European Review for Medical and Pharmacological Sciences 2018; 22: 7356-7363

Down-regulation of Treg by interference of enhances the killing effect of CIK on leukemia cell HL-60

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Abstract. – OBJECTIVE: Cytokine-induced killer cells (CIK) is a type of immune cell with antitumor activity induced by a variety of cytokines. Regulatory T cells (Treg) is a T cell subgroup featured as immunosuppressive function. Existing CIK cultivation system may inevitably induce Treg. Forkhead box protein 3 (Foxp3) is an essential transcription factor for Treg function. This study aimed to investigate the effects of CIK on the leukemia cell HL-60.

MATERIALS AND METHODS: This work silenced Foxp3 expression on the basis of CIK induction, aiming to investigate its killing effect on HL-60 cells. Peripheral blood mononuclear cells were separated and differentiated to CIK *in vitro*. $CD3^+CD56^+$ and $CD4^+CD25^+Foxp3^+$ Treg cells were detected by flow cytometry. CIK cells were co-cultured with HL-60 cells under the effector-target ratio at 20:1, 10:1, and 5:1, respectively. The killing activity of CIK on HL-60 cells was determined by CCK-8 assay.

RESULTS: The ratio of $CD3^+$, $CD3^+CD8^+$, and $CD3^+CD56^+$ cells gradually increased during CIK induction. Foxp3 interference significantly reduced Treg cell ratio on the 7^{th} day ($p < 0.05$). Treg cell ratio was significantly lower in Foxp3 interference group at 1.62% ± 0.07% compared with control ($p < 0.05$). The killing activity of CIK on HL-60 cells enhanced following the increase of effector-target ratio. Interference of Foxp3 significantly elevated the killing activity of CIK on HL-60 cells with effector-target ratio dependence ($p < 0.05$). CIK can effectively suppress HL-60 cell growth. Treg significantly inhibited the anti-tumor effect of CIK.

CONCLUSIONS: Interference of Foxp3 expression significantly declined Treg level and attenuated its suppression impact on CIK, thus enhancing the killing effect of CIK on HL-60 cells.

Key Words:
CIK, Treg, HL-60, Tumor immunotherapy.

Introduction

Leukemia is a type of malignant cloning proliferative disease originated from the hematopoietic stem (progenitor) cell dysplasia and abnormal proliferation. It is a common malignant disease in the blood system[1]. Acute promyelocytic leukemia (APL) is a specific dangerous type in acute myelocytic leukemia (AML). Its pathogenesis is associated with cytogenetics abnormity, accounting for about 10% of the adult AML[2]. Immune cell therapy refers to insert the competent immune cells cultured, amplified, and activated *in vitro* back to the patient, intending to kill tumor cells directly or through inducing immune effector cells. It has become the fourth kind of antitumor method after surgery, radiotherapy, and chemotherapy[3]. Cytokine-induced killer cells (CIK) is a kind of competent immune cells characterized as T lymphocytes antitumor activity[4] and NK cell-like non-major histocompatibility complex (MHC) restriction[5] induced by multiple cytokines and anti-CD3 monoclonal antibody. It can kill a variety of tumor cells, such as acute myelogenous leukemia, chronic lymphocytic leukemia, granulocytic leukemia, and B cell lymphoma[6-8]. It exhibits a broad prospect in the immunotherapy of hematological malignant tumor.

Regulatory T cells (Treg) is a kind of inhibitory T cell subgroup with immune regulating function. It can suppress various immune cells, including T cells, B cells, NK cells, and antigen presenting cells, thus has the function of alleviating immune response and inducing immune tolerance[9]. Interleukin 2 (IL-2) is an indispensable cytokine in the induction of CIK *in vitro*. However, the introduction of IL-2 will indirectly lead to the amplification of Treg cells, therefore attenuating the antitumor effect of CIK[10-12]. Although a number of studies[10-12] have confirmed the antitumor function of CIK, its effect is limited[7,13]. Furthermore, Treg generated during the process of CIK cultivation may produce an evident inhibitory effect on CIK, thus antagonizing the antitumor biological effect

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of CIK. Forkhead transcription factor 3 (Foxp3) is a type of head/winged helix transcriptional regulatory factor of foxhead family. It specifically expresses in $CD4^+CD25^+$ Treg cells, and is necessary to maintain the development and function of Treg cells[14]. In this work, we induced CIK *in vitro* and silenced Foxp3 expression upon viral interference, intending to explore its killing effect on APL cell line HL-60.

Materials and Methods

Main Reagents and Materials

Human APL cell line HL-60 was provided by the Chinese Academy of Medical Sciences (Beijing, China). Roswell Park Memorial Institute-1640 (RPMI-1640) medium and fetal bovine serum (FBS) were purchased from Gibco BRL. Co. Ltd. (Grand Island, NY, USA). Penicillin-streptomycin was purchased from Mediatech-Cellgro (Miami, FL, USA). Lipofectamine 2000 was purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA). Peripheral blood mononuclear cells (PBMC) separating medium Ficoll Paque PLUS was purchased from PE Gene Applied Biosystems (Foster City, CA, USA). ReverTra Ace quantitative PCR (qPCR) RT Kit and SYBR were purchased from Toyobo Life Science (Osaka, Japan). Rabbit anti β-actin, mouse anti Foxp3, and horseradish peroxidase (HRP) labeled secondary antibody were got from Abcam Biotechnology (Cambridge, MA, USA). Bicinchoninic acid (BCA) protein quantification kit was purchased from Bio-Tek Inc. (Winooski, VT, USA). Recombinant human interferon γ (IFN-γ) and IL-2 were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Mouse anti-human CD3 monoclonal antibody was got from BD Biosciences (San Jose, CA, USA). CD3-FITC, CD56-PE, CD4-FITC, CD25-PE, and Foxp3-APC were purchased from eBioscience (Santiago, CA, US). Cell Counting Kit-8 (CCK 8) Cell activity detection Kit was tog from Dojindo (Kumamoto, Japan).

CIK Induction

Peripheral blood was collected from healthy volunteers in heparin anticoagulant tube. PBMC was separated using density gradient centrifugation method. The peripheral blood was diluted by an equal volume of phosphate-buffered saline (PBS), and then added to the PBMC separating medium. After centrifuged at 300 ×g for 40 min, the white membrane layer was transferred to another centrifuge tube and diluted using more than three times of PBS. After centrifuged at 1000 r/min for 5 min, the cells were washed for one time. Next, the obtained PBMC was re-suspended using RPMI-1640 medium containing 10% FBS and 1% penicillin-streptomycin at 1×10^6/ml and inoculated in 24-well plate together with 100 ng/ml IFN-γ. After cultured at 37°C and 5% CO_2, the cells were treated by 100 ng/ml IL-2 and 50 ng/ml CD3McAb. CIK cells were collected on the 14th day for detection.

This study was approved by the Ethics Committee of Gaotang County People's Hospital of Shandong Province, LiaoCheng, Shandong, China.

CIK Phenotype Identification

CIK cells were collected on the 1st, 7th, and 14th day. The cells at 1×10^6/100 μl were added with 5 μl CD3-FITC and CD56-PE. After incubated at 4°C for 30 min, the cells were washed with PBS containing 2% FBS and tested by flow cytometry (FC 200 MCL, Beckman Coulter, Brea, CA, USA).

Foxp3 ShRNA Vector Construction and Cell Transfection

ShRNA eukaryotic expression plasmids PGPU6/GFP/Neo-Foxp3, PGPU6/GFP/Neo-Scramble, and PGPU6/GFP/Neo-Blank were designed and synthesized by Shanghai GenePharma Co. Ltd. (Shanghai, China). Foxp3 gene target sequence: forward, 5'-GTGAATAGGAAACCTGTGGTA-3'; reverse, 5'-TACCACAGGTTTCCTATTCAC-3'. Scramble sequence, forward, 5'-GCTGGAGACTTTCACAGGAAT-3'; reverse, 5'-ATTCCTGTGAAAGTCTCCA GC-3'. The cells were divided into four groups for CIK induction, including un-transfection group, PGPU6/GFP/Neo-Blank group, PGPU6/GFP/Neo-Scramble group, and PGPU6/GFP/Neo-Foxp3 group. The virus and Lipofectamine 2000 were diluted by optional minima essential medium (Opti-MEM) and incubated at room temperature for 5 min. Then they were mixed at room temperature for 30 min and added to the serum-free medium. The medium was changed to RPMI-1640 medium containing 10% FBS and 1% penicillin-streptomycin after 6 h.

$CD4^+CD25^+$ $Foxp3^+$ Treg Cell Detection

A total of 100 μl cell suspension was added with 5 μl FITC-CD4 and PE-CD25 flow cytom-

etry antibody at 4°C for 30 min. Next, the cells were washed by PBS containing 2% FBS for twice and fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 30 min. After treated by 0.1% Triton X-100 for 30 min, the cells were added by 5 µl APC-Foxp3 at room temperature for 20 min avoid of light. At last, the cells were re-suspended in 0.5 ml PBS containing 2% FBS and tested on flow cytometry to calculate the ratio of $CD4^+CD25^+$ $Foxp3^+$ Treg cells.

CCK8 Assay

HL-60 cell concentration was adjusted to 1×10^5/ml, while CIK concentration was adjusted to 2×10^6/ml, 1×10^6/ml, and 5×10^5/ml, leading to the effector-target ratio at 20:1, 5:1, and 1:1, respectively. The test group was added with 100 µl effector cells and target cells, single target group was added with 100 µl HL-60 cells and medium, single effector group was added with 100 µl CIK cells and medium with five replicates. The cells were incubated at 37°C and 5% CO_2 for 48 h. Next, a total of 20 µl CCK-8 solution was added to each well and incubated for 4 h. At last, the plate was tested at 490 nm on a microplate reader. Killing activity = {1-[(OD value in test group – OD value in effector group)/OD value in target group]} × 100%.

qRT-PCR

Total RNA was extracted using Trizol and reverse transcribed to complementary DNA (cDNA) using ReverTra Ace qPCR RT Kit. The reaction system contained 2 µg total RNA, 1 µl dNTP, 4 µl RT Buffer, 1 µl RT primer, 2 µl RT Enzyme, 1 µl RNase inhibitor, and ddH_2O. PCR amplification was composed of 40 cycles of 95°C for 15 s, 60°C for 30 s, and 74°C for 30 s. PCR reaction was performed on Bio-Rad CFX96 Real-Time PCR amplifier (Bio-Rad Laboratories, Hercules, CA, USA). The primer sequences were as follows. $Foxp3P_F$: 5'-GTGGCCCGGATGTGAGAAG-3', $Foxp3P_R$: 5'-GGAGCCCTTGTCGGATGATG-3'; β-$actinP_F$: 5'-GAACCCTAAG GCCAAC-3', β-$actinP_R$: 5'-TGTCACGCACGATTTCC-3'.

Western Blot

The protein was extracted and quantified by BCA method. The sample was diluted by 4× loading buffer and boiled for 5 min. A total of 50 µg sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. After blocked by 5% skim milk at room temperature for 60 min, the membrane was incubated in primary antibody at 4°C for 12 h (Foxp3 1:100, β-actin 1:800). After washed by PBST for three times, the membrane was incubated in HRP labeled secondary antibody (1:5000) at room temperature for 1 h. At last, the membrane was treated by ECL for 1-3 min and developed. Quantity One software (Quantity One software; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to analyze the protein band.

Statistical Analysis

SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. The measurement data was presented as mean ± standard deviation (SD). The Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data among groups. The Student's *t*-test was used to compare the differences between the two groups. $p < 0.05$ was depicted as statistical significance.

Results

CIK Phenotype Detection

Flow cytometry showed that all of $CD3^+$, $CD3^+CD8^+$, $CD3^+CD56^+$ cell number significantly increased following induction time extension. It revealed that was the lymphocytes with killing effect, including T cells and NK cells, suggesting successful CIK induction (Table I).

Foxp3 Interference Efficiency Detection

Compared with nontransfection group, green fluorescence protein (GFP) positive rate in CIK cells from blank vector group, scramble group,

Table I. Flow cytometry detection of CIK surface antigen expression (n = 3, %, mean ± SD).

Time point	$CD3^+$	$CD3^+CD8^+$	$CD3^+CD56^+$
1st day	41.62 ± 3.91*	11.56 ± 1.33*	1.46 ± 0.25*
7th day	96.51 ± 4.21#	59.75 ± 2.81#	18.60 ± 2.46#
14th day	98.13 ± 3.72#*	73.68 ± 3.23#*	39.22 ± 2.53#*

#$p < 0.05$, compared with 1st day, *$p < 0.05$, compared with 7th day.

or Foxp3 group was significantly higher (Figure 1A), suggesting high efficiency of virus infection. qRT-PCR results showed that shRNA targeting Foxp3 significantly declined Foxp3 mRNA expression in CIK cells (Figure 1B). Western blot demonstrated that shRNA targeting Foxp3 markedly inhibited Foxp3 protein expression in CIK cells (Figure 1C).

Interference of Foxp3 Reduced Treg Cell Ratio in Induction System

Flow cytometry revealed that no statistical difference of CIK cells was observed among blank group, scramble group, and Foxp3 interference group ($p > 0.05$). On the 1st day, the ratio of Treg cells was relatively low. In addition, no evident impact of virus infection was found on the expression of $CD4^+CD25^+Foxp3^+$ ($p > 0.05$). $CD4^+CD25^+Foxp3^+$Treg cell ratio significantly increased on the 7th day. Foxp3 interference markedly declined Treg cell ratio in the system ($p < 0.05$). Compared with the 7th day, Treg proportion on the 14th day slightly reduced, while its level in Foxp3 interference group was significantly lower (1.62% ± 0.07%) (Table II, Figure 2). The results demonstrated that Foxp3 interference showed no significant impact on CIK differentiation, whereas markedly suppressed Treg cell generation.

Interference of Foxp3 Enhanced the Killing Activity of CIK on HL-60 Cells

The killing activity of CIK on HL-60 cells enhanced following the increase of effector-target ratio under nontransfection condition. Blank or scramble vector transfection exhibited no significant impact on the killing activity of CIK on HL-60 cells ($p > 0.05$). Interference of Foxp3 significantly elevated the killing activity of CIK on HL-60 cells with effector-target ratio dependence ($p < 0.05$, Table III).

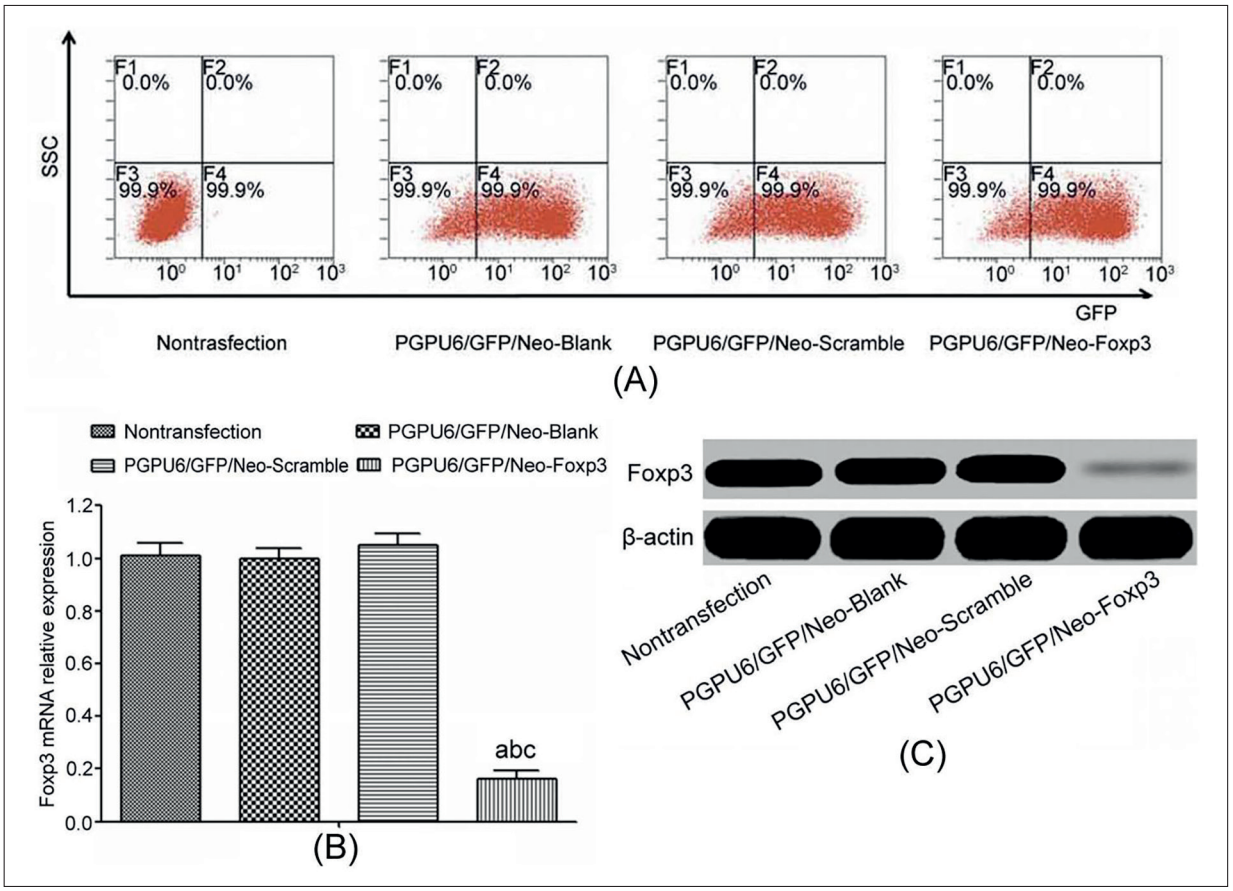

Figure 1. Foxp3 interference efficiency detection. ***(A)*** Flow cytometry detection GFP positive rate. ***(B)*** qRT-PCR detection of Foxp3 mRNA expression. ***(C)*** Western blot detection of Foxp3 protein expression. [a]$p < 0.05$, compared with nontransfection group. [b]$p < 0.05$, compared with PGPU6/GFP/Neo-Blank. [c]$p < 0.05$, compared with PGPU6/GFP/Neo-Scramble.

Table II. Flow cytometry detection of CIK and Treg cell surface antigen expression (n = 3, %, mean ± SD).

	Time point	Nontransfection	PGPU6/GFP/ Neo-Blank	PGPU6/GFP/ Neo-Scramble	PGPU6/GFP/ Neo-Foxp3
$CD3^+CD56^+$	1st day	1.81 ± 0.31	2.1 ± 0.42	2.2 ± 0.52	2.0 ± 0.35
	7th day	21.6 ± 3.02	19.7 ± 2.55	22.5 ± 2.94	20.6 ± 3.16
	14th day	37.8 ± 2.91	36.4 ± 3.43	39.2 ± 3.66	40.1 ± 4.08
$CD4^+CD25^+Foxp3^+$	1st day	2.12 ± 0.39	1.97 ± 0.42	2.08 ± 0.41	2.22 ± 0.51
	7th day	30.65 ± 1.85	31.53 ± 1.91	29.63 ± 2.03	15.38 ± 0.36[abc]
	14th day	21.23 ± 0.97	19.28 ± 1.05	20.56 ± 1.21	5.62 ± 0.07[abc]

[a]$p < 0.05$, compared with nontransfection group. [b]$p < 0.05$, compared with PGPU6/GFP/Neo-Blank. [c]$p < 0.05$, compared with PGPU6/GFP/Neo-Scramble.

Discussion

Following economy development, environmental pollution aggravation, life rhythm acceleration, and diet change, the incidence of leukemia shows the rising trend year by year, especially in the middle-aged and young adults and children under the age of 35[15]. It is estimated that the incidence of leukemia in China is 75.3/100000, including 44.4/100000 in male and 30.9/100000 in female[16]. Leukemia is featured as insensitive to chemotherapy and tiny residual lesion is easy to cause recurrence. There is still a lack of good prevention and treatment methods, leading to poor prognosis and high mortality[17]. Adoptive cellular immunotherapy (ACI) is a new immune method widely rised in recent years. It is a type of biological immunotherapy via inoculating immune cells with anti-tumor activity and direct or indirect mediating antitumor effect input the cancer patients to achieve the goal of cancer treatment[18]. CIK is a heterogeneous and activated *in vitro* lymphocyte group induced by multiple cytokines, such as IFN-γ, anti-CD3 monoclonal antibody, and IL-2. $CD3^+CD56^+$ cells are the major effector cells among the heterogeneous CIK cell group. Meanwhile, there is also a small amount of $CD3^+CD56^-$ T cells and $CD3^-CD56^+$ NK cells existed[19]. Compared with the anti-tumor immune cells previously reported, CIK is characterized

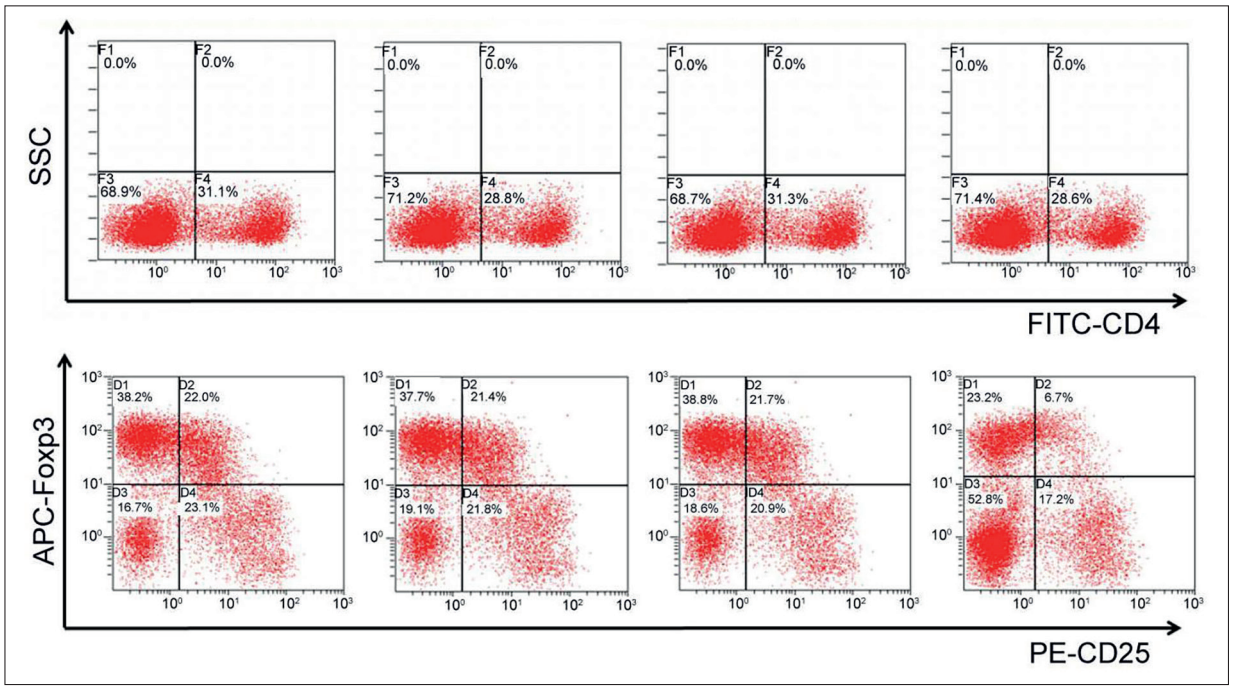

Figure 2. $CD4^+CD25^+Foxp3^+$ Treg cells on the 14th day.

Table III. The killing activity of CIK on HL-60 cells under different effector-target ratio (n = 5, %, mean ± SD).

Effector-target ratio	Nontransfection	PGPU6/GFP/ Neo-Blank	PGPU6/GFP/ Neo-Scramble	PGPU6/GFP/ Neo-Foxp3
5:1	16.58 ± 1.33	14.52 ± 1.26	15.36 ± 1.22	25.63 ± 1.34[abc]
10:1	31.56 ± 2.98*	29.59 ± 3.34*	30.85 ± 3.06*	46.69 ± 3.85[abc]*
20:1	42.96 ± 4.05[#]	40.62 ± 3.67[#]	41.67 ± 3.86[#]	66.87 ± 4.28[abc#]

[a]$p < 0.05$, compared with nontransfection group. [b]$p < 0.05$, compared with PGPU6/GFP/Neo-Blank. [c]$p < 0.05$, compared with PGPU6/GFP/Neo-Scramble. *$p < 0.05$, compared with effector-target ratio at 5:1. [#]$p < 0.05$, compared with effector-target ratio at 10:1.

as stronger capacity of *in vitro* proliferation and amplification, stronger migrating and invasive ability to tumor region, higher anti-tumor activity, and wider anti-tumor spectrum. It breaks the limitation of slow migration, lack of cell source, and low anti-tumor activity appeared in the lymphokine-activated killer cell (LAK), tumor infiltrating lymphocyte (TIL), anti-CD3 monoclonal antibody activated killer cell (CD3AK), and cytotoxic T lymphocyte (CTL)[20]. CIK can mediate killing effect on tumor cells via numerous pathways and mechanisms. It can directly kill target cells through the secretion of perforin and granular enzyme[21], express Fas Ligand to bind with Fas on tumor cell surface and trigger Fas/FasL to induce tumor cell apoptosis[4], and secrete a variety of antitumor factors to promote tumor cell apoptosis, such as TNF-α and IFN-γ[22].

With the deepening of the research on tumor immune mechanism, CIK is widely applied in clinical anti-tumor therapy. However, it is often difficult to achieve the desired clinical effect. The existence of Treg cells is one of the important causes leading to the CIK curative effect limitation. $CD4^+CD25^+$ T cells are a group of inhibitory T cell subgroup with immune regulating function. It can inhibit multiple immune cells to attenuate immune response and induce immune tolerance, including T cells, B cells, NK cells, and antigen presenting cells. The generation of Treg cells leads to tumor cell immune tolerance in addition to prevent the occurrence of autoimmune diseases[23], which is associated with down-regulating anti-tumor immunity, triggering immune escape, and promoting malignant tumor cell proliferation[24,25]. Treg cells can mediate the inhibitory effect on immune cells through cell direct contact and the release of immunosuppressive factors, resulting in the body loss of killing tumor cells ability. It was showed that $CD4^+CD25^+$ T cells can inhibit perforin and granular enzyme expressions in $CD4^+CD8^+$ effect T cells, suppress $CD4^+$ T cells to secrete Th1 cytokines, and make IL-2 mediated effect T cell in low proliferation and activity. Furthermore, it restrained the activation of $CD4^+CD8^+$ effect T cells via secreting TGF-β and IL-10, and suppressed the proliferation of NK cells and NKT cells with cytotoxicity[26,27]. Foxp3 is closely related to Treg cells expression and function. $CD4^+CD25^+$ Treg cells differentiation and development is regulated by the transcription factor Foxp3[14,28,29]. It was found that IL-2 not only can stimulate the proliferation and activation of effector T cell, and played a crucial role in the regulation of Treg cells development and function. Treg function stability not only depends on the role of IL-2[30], but also related to the IL-2 receptor configuration, affinity, and expression on Treg cell surface[31]. Animal studies revealed that Treg cells developed disorder in IL-2 or IL-2 receptor deleted mice, leading to an autoimmune disease characterized as $CD4^+$ cells activation enhancement and numerous autoantibodies generation[32,33]. *In vitro* studies also confirmed that IL-2 played a key role in promoting Treg development, proliferation, and amplification[34]. A variety of researches[10-12] demonstrated that *in vitro* culture system for CIK may inevitably lead to Treg cells generation and amplification, thus to produce an inhibitory effect on $CD3^+CD56^+$ cells, $CD3^+CD8^+$ T cells, and NK cells in CIK heterogeneous group. It attenuated the anti-tumor killing effect of CIK cells and form obstacles on tumor CIK immunotherapy. Tumor immunotherapy not only needs enough activated immune effector cells, also has to effectively break the immune tolerance, which plays an important role in enhancing the anti-tumor immune effect. Therefore, this research investigated the killing effect of Foxp3 in CIK on APL HL-60 cells.

Flow cytometry exhibited that $CD3^+$, $CD3^+CD8^+$, $CD3^+CD56^+$ cell number significantly increased following induction time extension, suggesting successful CIK induction. ShRNA

targeting Foxp3 significantly suppressed Foxp3 mRNA and protein expression in CIK cells. Compared with the non-transfection group, blank or scramble transfection group showed no evident impact on the killing effect of CIK on HL-60 cells. Foxp3 interference virus transfection significantly enhanced the killing activity of CIK on HL-60 cells with effector-target ratio dependence. Further detection revealed that interference of Foxp3 exhibited no significant impact on the differentiation of $CD3^+CD56^+$ cells but markedly inhibited Treg cells production, revealing that the enhancement of killing effect on HL-60 cells after Foxp3 interference depended on the low expression of Treg cells instead of increasing $CD3^+CD56^+$ effector cells. To sum up, we proposed that CIK is a kind of efficient tumor immunologic effector cells that can effectively restrain leukemia HL-60 cell growth. $CD4^+CD25^+Foxp3^+$ T cells markedly inhibited the anti-tumor effect of CIK. Interference of Foxp3 expression significantly declined Treg level and attenuated its suppression impact on CIK, thus enhancing the killing effect of CIK on HL-60 cells.

Conclusions

We showed that the CIK can effectively restrain leukemia HL-60 cell growth. $CD4^+CD25^+Foxp3^+$ T cells significantly inhibited the anti-tumor effect of CIK. Interference of Foxp3 expression significantly declined Treg level and attenuated its suppression impact on CIK, thus enhancing the killing effect of CIK on HL-60 cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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