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Original Article

Inhibition of autophagy in cytokine-induced killer cell promotes its cytotoxicity

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Abstract: Due to non-MHC-restricted and potent cytotoxicity against hematological malignancy, one group of heterogeneous effector T cells with diverse TCR specificities, cytokine-induced killer (CIK) cells are considered as a promising therapeutic approach of leukemia. Recently, it has been found that IL-2/IL-15-activated CIK cells were superior to that of generated with IL-2 protocol, however, the underlying mechanism is not fully elucidated. In this study, we found that in IL-2/IL-15-stimulated CIK cells, autophagy was dramatically down-regulated. We produced recombinant lentivirus containing shRNA specific against Beclin 1. Upon knockdown of Beclin 1 in CIK cells, the cytotoxic activity was considerably promoted. Re-expression of Beclin 1 in Beclin 1-knockdown CIK cells compromised their cytotoxic function, confirming that autophagy decreased the cytotoxic function of CIK cells. 3-MA treatment of CIK cells also enhanced their cytotoxic activity, potentially providing possibility to widespread application. Collectively, our study demonstrates autophagy negatively regulates the cytotoxicity of CIK cells on tumor cells, providing a novel approach for promoting the therapeutic efficacy of CIK cells against hematological malignancy.

Keywords: Cytokine-induced killer cells, cytotoxicity, autophagy

Introduction

During the past decades, adoptive cellular immunotherapy with the goal of eliminating tumor cells by stimulating immune response has received dramatic attention. Lymphokine-activated killer (LAK) cells and tumor-infiltrating lymphocytes (TIL) had potent in vitro cytotoxicity against susceptible tumor cells and harnessed established tumors in animal models. In clinical practice, LAK and TIL cells have shown modest efficacy against metastatic cancer. Cytokine-induced killer (CIK) cells are heterogeneous effector T cells with diverse TCR specificities, and have non-MHC-restricted cytotoxic activities against tumor cells. Therefore, CIK cells can eliminate tumor cells in a non-MHC-restricted manner and serve as an alternative cellular immunotherapy [1-3]. In recent years, the application of CIK cells has evolved from experimental observations into early clinical studies [4]. CIK cells have shown marked capability to proliferate and demonstrated superior over LAK cell in cytotoxic activities for cancer immunotherapy [5].

Macroautophagy (here we use autophagy) is a evolutionarily conserved autonomous digestion of intracellular components, involving the sequestration of cytoplasmic substances, such as macromolecules or organelles, in double-membrane autophagosomes, which fused with lysosomes, delivering their sequestered cargo to this compartment for enzymatic digestion [6]. Autophagy has long been recognized as a response to stress such as starvation to rapidly provide energy and anabolic building blocks to maintain energy homeostasis. Recently, autophagy has been shown as a vital cellular process involved in cellular homeostasis and survival mechanisms, cellular defense as part of both the innate and acquired immunity, anti-aging, as well as organisms development [7-10]. And mounting evidence suggests that autophagy can impact either the pathogenesis or the progression of human diseases [6, 11-13],

including cancer, neurodegenerative diseases, cardiovascular diseases, and inflammatory diseases. Because of its paramount importance, tight regulation of autophagy is required to prevent its unbalanced activation. To date, at least 35 autophagy-related (ATG) genes have been identified. Most of them contribute to autophagosome formation, and are conserved from yeast to human.

However, there are few reports addressing the role of autophagy underlying the mechanism of CIK cell on anti-tumor. Therefore, in the present study, we explored the autophagy of CIK cells, and further analysis the functions of autophagy on cytotoxic abilities. Our data demonstrated that autophagy inhibited cytotoxic capabilities of CIK, and autophagy deficiency would be a promising means to increase the therapeutic efficacy of CIK on tumor lysis.

Materials and methods

Reagents and cell lines

HL-60, KM3 and RPMI-8226 were obtained from ATCC (USA), and maintained in DMEM medium supplemented with 10% fetal calf serum (FCS), L-glutamine, and pen/strep antibiotics according to the manufacturer's instructions.

Generation of CIK cells

This study was approved by the Ethical Review Board of Chongqing Cancer Institute, and the blood was taken from all healthy volunteers after written consent of this study. CIK cells were generated from peripheral blood mononuclear cells (PBMC) with Ficoll separation as previously described [14]. Briefly, cells were resuspended with a density of 3×10^6 cells/mL in RPMI 1640, supplemented with 10% FCS, L-glutamine and Pen/Strep. 1000 U/mL IFN-g on was added on day 0, followed by 100 ng/mL anti-CD3 antibody and 500 U/mL IL-2 24 hours later. Thereafter, IL-2 alone or combined with 50 ng/mL IL-15 was added regularly.

Europium release assays

A non-radioactive cytotoxicity assay was used to assess the lytic activity of CIK [15]. Briefly, target cells were co-cultured with CIK cells in triplicates with E: T ratios of 100:1, 50:1, 40:1, 20:1, 10:1, and 5:1 in 96-well plates. After a period of 4 h, 20 mL supernatant was collected from each well and added to 200 μL europium solution (Perkin Elmer, USA). Fluorescence data was recorded using BD FACSCalibur (BD bioscience, USA). The percentage of specific cytolysis was calculated for each well as previously described [14].

Quantitative real-time PCR

Total RNA was prepared from CIK cells and was reverse-transcribed using qRT-PCR kit (Invitrogen). Quantitative real-time PCR was carried out on an ABI Prism 7900HT (Applied Biosystems). The program conditions as followed, 2-minute incubation at 50°C, then 95°C for 10 minutes, and followed with 95°C for 15 seconds and 60°C for 60 seconds for 40 cycles. We used b-actin as an internal control to normalize for differences in the amount of total RNA in each sample. The primer sequences for TLRs were obtained from InvivoGen (San Diego, CA), and the primer for b-actin were as follows: Forward: 5 TGGCGCTTTTGACTCAGGAT -3; Reverse: 5' GGGATGTTTGCTCCAACCAA -3'.

Autophagy analysis

Autophagy was analyzed by immunoblotting as described previously [16]. Briefly, in the immunoblotting analysis, cells were treated as indicated, and cell lysates were immunoblotted with anti-LC3 antibody to monitor the LC3-II conversion. Each experiment was performed at least 3 times.

Lentiviral vectors construction

pLVX-shRNA-zs Green was used to construct the recombinant lentivirus containing Beclin 1 or control shRNA sequences from Invivogen (San Diego, CA). Rat LC3 was cloned into pLVX-IRES-ZsGreen vector to construct the recombinant lentivirus. Lentivirus containing LC3 or Beclin 1 shRNA were produced by transfecting HEK293T cells with pMD.2G, psPAX2. An MOI of 20 was used to infect MSC, and the efficiency was determined at 48 hours under fluorescence microscopy.

Immunoblotting

Cells with proper treatment were collected and lysed in lysis buffer containing 1% Nonidet P-40, plus protease inhibitor cocktail (Roche,

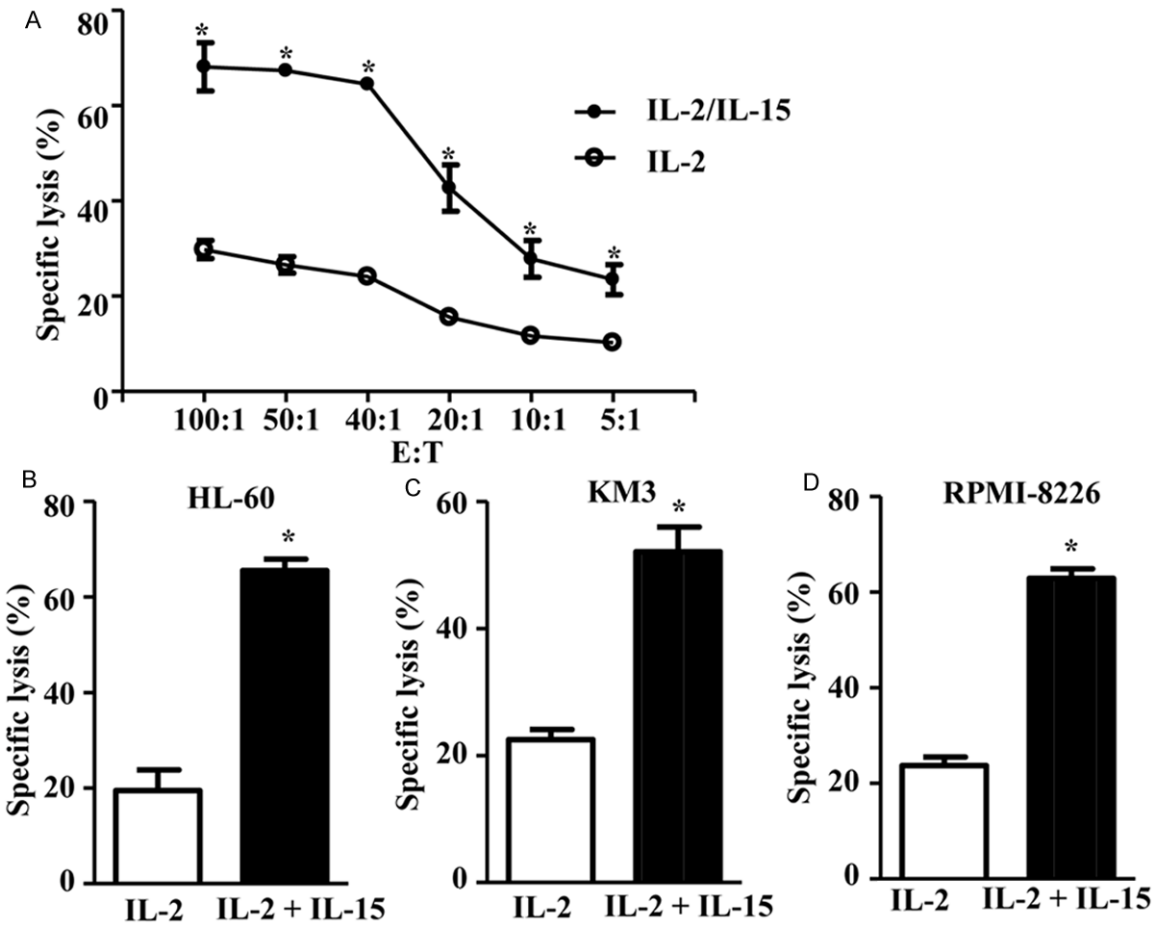

Figure 1. Cytotoxic capabilities of CIK cells on tumor cells. Cytoxicity of CIK cells generated with IL-2 alone or combined with IL-15 was assessed using europium release assay. (A) The day 10 CIK cells were co-cultured with HL-60 cells at (E):T ratios of 100:1, 50:1, 40:1, 20:1, 10:1 and 5:1 in 96-well plates. Four hours later, the specific lysis was analyzed as described in Materials and Methods. Data present here as Mean ± SD (n=5). (*$P < 0.05$) CIK cells were co-cultured with HL-60 (B), or KM13 (C), or RPMI-8226 (D) at E:T ratios of 40:1 for 4 hours, and the cytotoxicity was analyzed. Data present here as Mean ± SD (n=3). (*$P < 0.05$).

Indianapolis, IN). After brief vortexing and rotation, cell lysates were subjected to SDS-PAGE and transferred to PVDF membranes. These membranes were blocked with 5% fat-free milk in PBS for 30 min and incubated with indicated antibody in PBS with 0.5% fat-free milk for 1 h. The membranes were then washed in PBS and incubated for 1 h with HRP-conjugated secondary antibody. After subsequent washes, the protein bands were visualized with ECL plus immunoblotting detection reagents (Pierce).

Statistics

SPSS 20.0 was used to perform the statistics analyses. Significance was assessed using an independent two-tailed Student's *t* test or one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

In vitro cytotoxicity of CIK against leukemia cells

Both acute and chronic leukemia, especially myeloid leukemia are partially sensitive to CIK therapy [17, 18]. So in this study, we assessed the cytotoxicity of CIK cells against leukemia cell lines. It has been reported that IL-15-stimulated CIK cells were superior to IL-2-stimulated CIK cells in cytotoxicity against several leukemia cell lines [14]. Therefore, we would like to further explore the effect of IL-2/IL-15-stimulated CIK cells on other leukemia

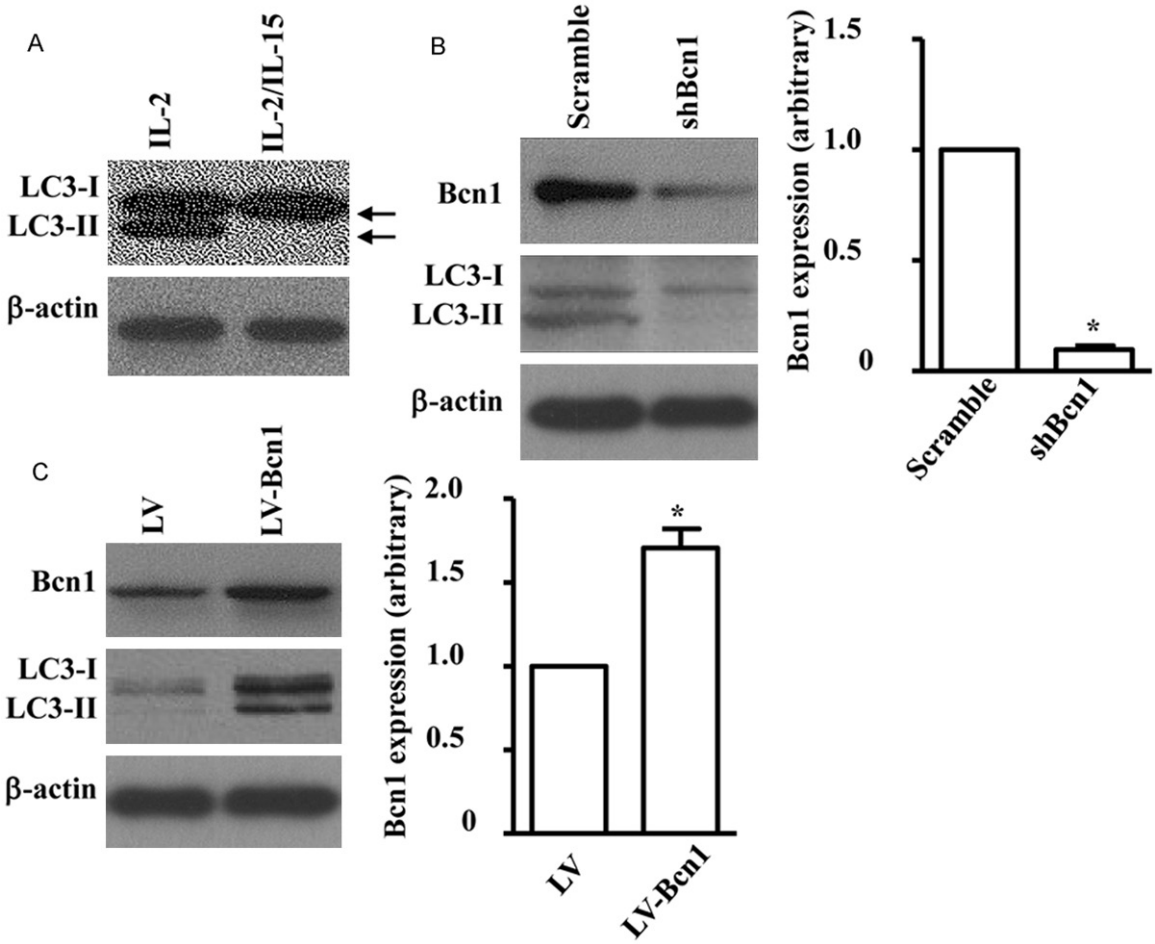

Figure 2. Autophagy was abrogated in CIK cells generated with IL-15 stimulation. (A) Cell lysate was prepared from day 10 CIK cells and then subjected to SDS-PAGE, then using immunoblot analysis with LC3 antibody. Day 10 CIK cells were infected with lentivirus containing shRNA specific to Beclin 1 (for knockdown, MOI=20) (B) or lentivirus containing Beclin1 sequence (for overexpression, MOI=20) (C) and cell lysates were prepared 48 hours later, which were subjected to SDS-PAGE and immunoblotting with Beclin 1 antibody. We showed a presentative image of immunoblotting from 3 independent experiments.

cell lines. Firstly, we determined to find the optimal effector to target (E:T) ratio using HL-60, a frequently used acute promyelocytic leukemia (APL) cell line. As shown in **Figure 1A**, E:T ratios of 100:1, 50:1, and 40:1 displayed similar cytotoxic activity, which were significantly stronger than ratios of 20:1, 10:1, and 5:1, therefore we used 40:1 ratio for thereafter study. To generalize our findings, we used two other cell lines, KM3 and RPMI-8226. KM3 cells are B-cell leukemia cells, while RPMI-8226 cells are multiple myeloma cells. At E:T ratio of 40:1, IL-2/IL-15-stimulated CIK cells showed impressively higher cytotoxic activity on HL-60 (**Figure 1B**), KM3 (**Figure 1C**), and RPMI-8826 (**Figure 1D**) cells than IL-2-stimulated CIK cells, suggesting the extensive cytotoxicity of IL-2/IL-15-stimulated CIK cells against multiple leukemia cell lines.

Autophagy was decreased in CIK cells generated by IL-2/IL-15-stimulation

Although IL-15 dramatically promoted the cytotoxic activity of CIK cells, the underlying mechanism is still not fully elucidated. Autophagy has been shown to be involved in T cell activation and other function, so we would like to explore the activity of autophagy during the generation of CIK cells. Firstly, we determined to check the status of autophagy in CIK cells generated by IL-2 or IL-2/IL-15 stimulation. As at day 10 after stimulation, CIK cells showed potent cytotoxic activity, we compared autophagy activity in Day

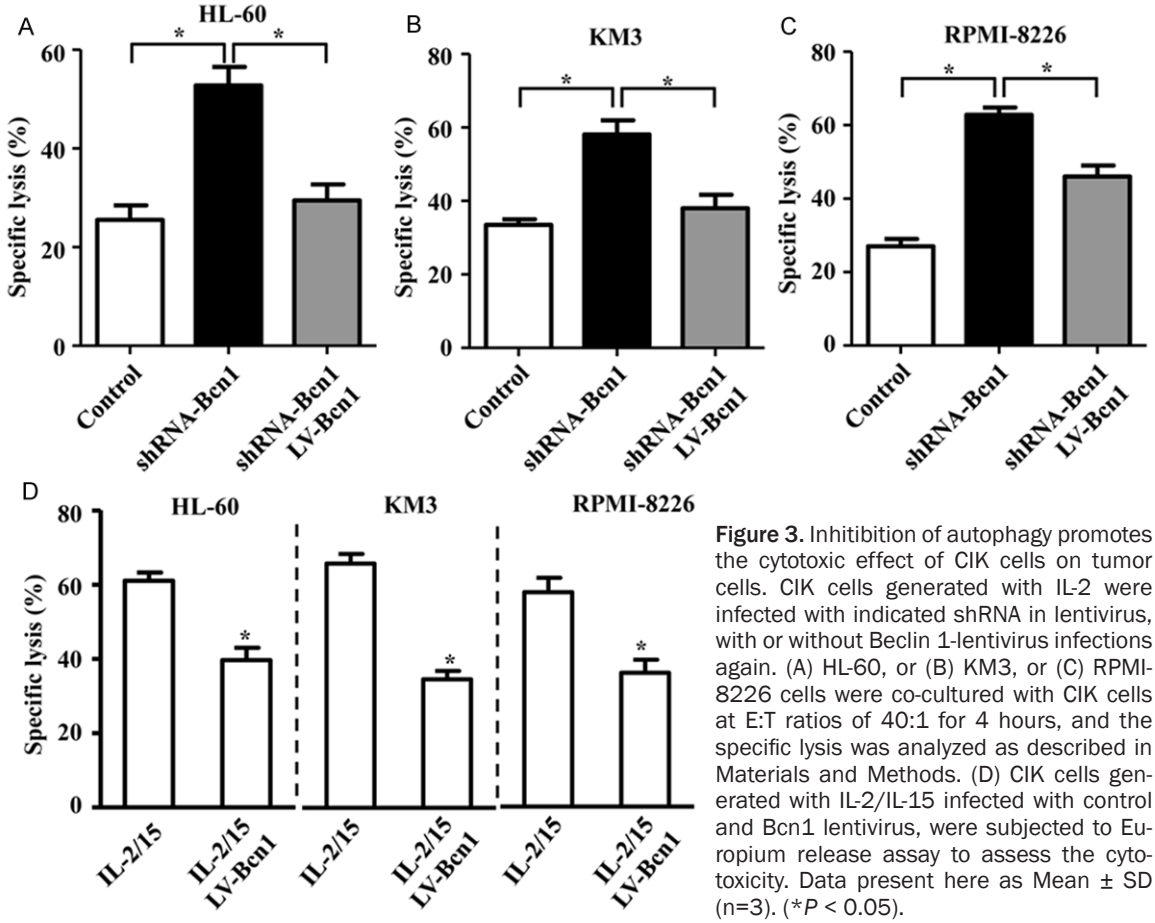

Figure 3. Inhitibition of autophagy promotes the cytotoxic effect of CIK cells on tumor cells. CIK cells generated with IL-2 were infected with indicated shRNA in lentivirus, with or without Beclin 1-lentivirus infections again. (A) HL-60, or (B) KM3, or (C) RPMI-8226 cells were co-cultured with CIK cells at E:T ratios of 40:1 for 4 hours, and the specific lysis was analyzed as described in Materials and Methods. (D) CIK cells generated with IL-2/IL-15 infected with control and Bcn1 lentivirus, were subjected to Europium release assay to assess the cytotoxicity. Data present here as Mean ± SD (n=3). (*$P < 0.05$).

10 IL-2- or IL-2/IL-15-stimulated CIK cells using immunoblotting of LC3. During the autophagy initiation, the recruitment of LC3-II, a phosphatidylethanolamine lapidated form of LC3-I protein, to the docking sites of autophagosome is usually thought as a hallmark of autopahgy. Our results showed that LC3-II expression was decreased significantly, indicating that autophagy was almost abrogated upon IL-15 stimulation (**Figure 2A**), suggesting the potential regulatory role of autophagy on cytotoxicity of CIK cells.

Inhibition of autophagy promotes cytotoxic activity of CIK cells

In order to further analyze the function of autophagy in CIK cells, we decided to knockdown Beclin 1 in CIK cells to determine the effect of autophagy on the cytotoxic activity of CIK cells. Beclin 1 is an essential regulator in initiating the autophagy. We used recombinant lentivirus system to perform the transfection. With a multiply of infection of 20, we infected the CIK cells with lentivirus containing specific shRNA to knockdown Beclin 1 expression, and the data demonstrated the Beclin 1 expression was decreased almost completely (by about 90%) (**Figure 2B**). For CIK cells infected with the recombinant Beclin 1-lentivirus, the expression of Beclin 1 was increased accordingly (**Figure 2C**). Next we co-cultured CIK-shRNA-Bcn1/CIK-control cells with leukemia cells, and the results demonstrated that knockdown of Beclin 1 promoted the cytotoxic activities considerably (**Figure 3A-C**). When the expression of Beclin 1 was rescued with Beclin 1 lentivirus, the cytotoxicity of CIK-shRNA-Bcn1 cells was decreased (**Figure 3A-C**). And upregulation of autophagy in IL-2/IL-15-stimulated CIK cells using Beclin 1 lentivirus decreased the cytotoxicity (**Figure 3D**), further confirming the essential roles of autophagy in regulating the cytotoxic activity of CIK cells.

3-MA treatment enhances cytotoxic activity of CIK cells

As the genetic modification of genes involved in autophagy is too burdensome and too artificial

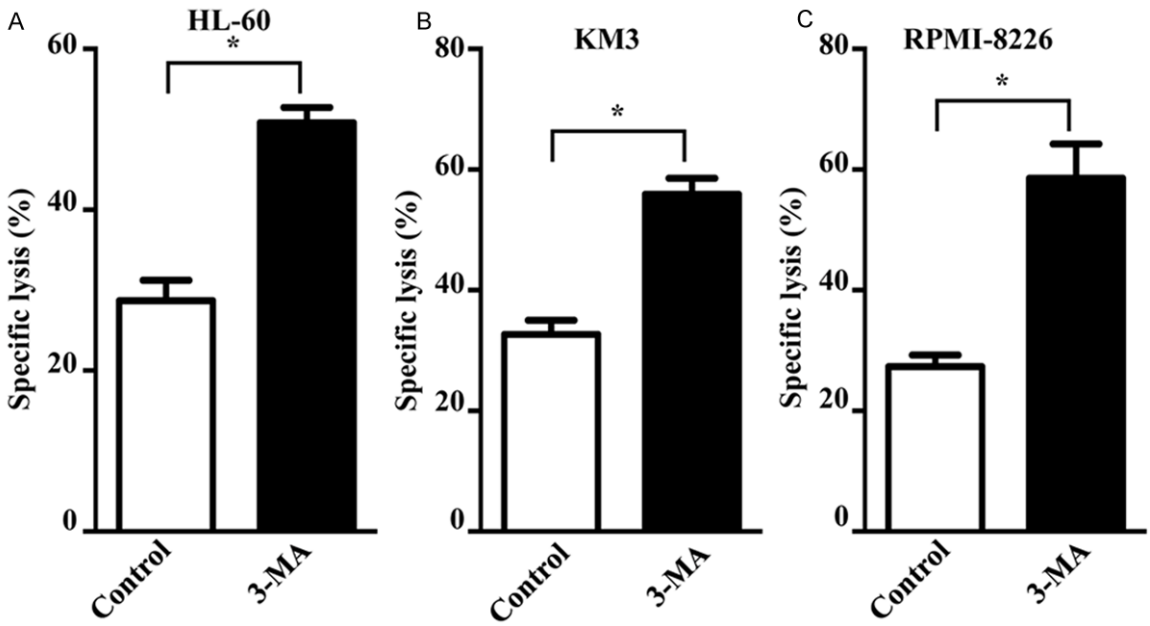

Figure 4. 3-MA treatment of CIK cells promotes the cytotoxic effect of CIK cells on tumor cells. CIK cells generated with IL-2 were treated with 3-MA (2 mM) for 12 hours. Europium release assay was used to assess the cytotoxicity of CIK cells. (A) HL-60, or (B) KM3, or (C) RPMI-8226 cells were co-cultured with CIK cells at E:T ratios of 40:1 for 4 hours, and the specific lysis was analyzed as described in Materials and Methods. Data present here as Mean ± SD (n=3). (*$P < 0.05$).

for its feasible application in vivo, we determined to utilize an alternative method to inhibit autophagy in CIK cell. 3-methyladenine (3-MA) is a cell permeable autophagic sequestration blocker, and inhibits autophagy by blocking autophagosome formation via the inhibition of type III Phosphatidylinositol 3-kinases. Before the detection of cytotoxic activity of CIK generated by IL-2/IL-15-stimulation, we treated the CIK cells with 3-MA (2 mM) for 12 hours. The cytotoxic activity of CIK cells treated with 3-MA showed considerable increase compared with CIK without treatment in all of cell lines (**Figure 4A-C**), suggesting the feasibility of pharmacological inhibitor in regulation of CIK cell cytotoxic activity, in turn making CIK cells as a more powerful tools on therapeutic application by autophagy manipulation.

Discussion

Autophagy is a lysosome-dependent degradation pathway involved in auto-digestion of cytoplasmic content. Originally thought as a nonselective process, autophagosomes are now also recognized to engulf substrates selectively, including damaged organelles and protein aggregates, but also bacteria, parasites and virions [19]. Currently, research on autophagy has also focused on adaptive immunity. It has been reported that autophagy is involved in T cell activation, proliferation, and so on. Therefore, in this study, it is no surprising to find that autophagy is an important regulator of cytotoxicity of CIK cells.

For leukemia patients, hematopoietic stem cell transplantation (HSCT) from a relative was considered as an important therapeutic option [20-22]. However, the therapeutic efficacy of HSCT was frequently compromised by relapse. Even for those therapeutically successful cases, graft-versus-host disease (GVHD) remains a big challenge due to the collapsed immunity of the recipient [23-25]. Transfer of only effector cells could be an alternative option.

CIK cells, along with LAK and TIL cells, have non-MHC-restricted potent cytotoxic activity, and potentially provide a promising immunotherapy of acute and chronic myeloid leukemia. Because of their superior cytotoxic activities over LAK and TIL cells, CIK cells have been considered to be a clinically feasible treatment of hematologic malignancy [2, 3, 5, 17]. Recently, Rettinger et al. [14] has reported that IL-15-

activated CIK cells was much superlative on cytotoxicity against diverse leukemia cells, compared to CIK cells generated by standard protocol. In this study, we found that autophagy was downregulated by IL-15-stimulation during the process of CIK generation. Knockdown of Beclin 1 expression in CIK cells would promote the cytotoxic function against tumor cells. Re-expression of Beclin 1 in Bcn1-knockdown CIK cells compromised the cytotoxicity largely, confirming the inhibitory role of autophagy on CIK functions. 3-MA is widely used as a pharmacological autophagy inhibitor, so we also tested the effects of 3-MA on CIK cells on promoting their cytotoxic ability. As expected, 3-MA considerably enhanced CIK cell cytotoxic activity on tumor cells, indicating his potential antitumor application.

Collectively, our studies demonstrated that autopahgy negatively regulated the cytotoxic function of CIK cells against tumor cells, and inhibition of autophagy promoted the therapeutic effects of CIK cells against hematological malignancy. Our study provided a novel strategy to promote the therapeutic efficacy of CIK cells against leukemia.

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Disclosure of conflict of interest

None.

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