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ConA mediates the activation of CTLL-2 and enhances its anti-tumor cell activity

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Abstract

CTLL-2 is a T-lymphocyte cell line with cytolytic activity that has been successfully developed by researchers in long-term culture, but it has lost its cytolytic ability to a large extent. ConA has been widely studied as a primary T-cell activator, but its effect on the activation and effector function of the T-lymphocyte cell line, CTLL-2 cells, has not been elucidated in literatures. In this paper, we explored the activation of CTLL-2 cells and changes in cell lysis activity against B16 melanoma cells under ConA treatment. The results showed that ConA induced CTLL-2 activation and enhanced CTLL-2-mediated cytotoxicity against B16 melanoma cells, indicating that ConA avails to restore the cytolytic activity of CTLL-2 cells.

Keywords

ConA, CTLL-2, B16, activation, cytotoxicity.

1. Introduction

CD69, a type II C-lectin membrane receptor, was described in early studies as a leukocyte activation marker that is rapidly induced and transiently expressed during lymphocyte activation, while its expression in resting lymphocytes is very low and essentially undetectable[1, 2]. CD25, the interleukin-2 receptor alpha-chain (IL-2Rα), is one of the component subunits of the interleukin 2 receptor [3]. CD25 is significantly up-regulated in expression at a later stage of T-cell activation and is regarded as a marker of T-cell activation in animals and humans [4]. Concanavalin A (ConA), the most widely studied plant lectin, is a tetravalent molecule with a well-defined structure and binding specificity[5, 6]. It is often used for T-cell activation in vitro, where T-cell activation is assessed by detecting the expression of CD25 and CD69[7].

CTLL-2 is a mouse T cell line with cytolytic activity that was successfully cloned by researchers in 1979. Because of its IL-2-dependent growth, CTLL-2 cells are often used for bioassays of IL-2 production and IL-2-related functional studies [8, 9]. CTLL-2 loses cytolytic activity to a large extent during the formation of cell lines after long-term culture [10]. It has been shown that specific gene mutations or agent treatments increase the cytolysis of CTLL-2 cells to a certain extent, suggesting that their cytolysis is dynamically moldable[11, 12]. ConA is commonly used to stimulate the activation of primary cells such as peripheral blood mononuclear cells (PBMCs) and Mouse splee cells [13, 14]，and its effects on the activation and effector function of cell lines such as CTLL-2 have not been reported. In this paper, we explored the changes in activation and effector function of CTLL-2 cells in response to ConA treatment by observing the expression of CD25 and CD69 and cytolysis in CTLL-2 cells, respectively.

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2. Material and method

2.1. Material

Concanavalin A (Con A) (C0412-5 MG) and mouse interleukin-2 (mIL-2) (I0523-20UG) purchased from Merck/Sigma-Aldrich were stored at -20°C, respectively.

2.2. Cells and cell culture

Mouse melanoma B16 cells and mouse T-lymphocyte line CTLL-2 cells were preserved and provided by the Department of Immunobiology, Jinan University. RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) was used for the culture of B16 cells and CTLL-2 cells, and an additional 0.5 ng/mL of mIL-2 was required for CTLL-2 growth.

2.3. LDH release assay

After being spreaded in 96-well plates at the appropriate density, the B16 cells (target cells) were allowed to attach to the wall and return to the growth state at 37℃ under 5% CO2 for about 8 h. CTLL-2 cells (effector cells), at different effector/target ratios, were added to the 96-well plates containing B16 cells as described above for co-culture. After co-culturing for 24, 2.5~10 μL of supernatant was aspirated into a new 96-well plate, followed by LDH release assay using Lactate Dehydrogenase (LDH) Cytotoxicity Assay Kit (CT0027, Beijing Leagene Biotechnology, China).

2.4. Live/dead staining

B16 cells were spread in 24-well plates at a density of approximately 3×10^4 per well and CTLL-2 cells were added for co-culturing after their attachment and recovery of growth status. After 24h of co-culture, supernatants and suspended CTLL-2 cells were discarded and adherent B16 cells were retained for subsequent staining to assess their cellular damage. Specifically, staining was performed at 37°C for 30 min by using the Live and Dead Cell Viability/Toxicity Assay Kit (KGAF001, KeyGEN BioTECH, China). Live/dead cells were observed by an inverted fluorescence microscope.

2.5. Flow cytometry analysis

A sufficient number of CTLL-2 cells (~5×10^5cells/well) were spread in a 24-well plate, followed by the addition of 5 ug/mL of ConA for incubation. Cells were collected after 24 h of incubation and stained with APC-conjugated CD25 (1:2000, ThermoFisher Scientific) and PE-conjugated CD69 (1:1000, ThermoFisher Scientific) antibodies, following detection by an Attune NxT flow cytometer (A24858, ThermoFisher Scientific, USA) and FlowJo VX software for analysing flow cytometry data.

2.6. Statistical analysis

Statistical differences of all data were derived from GraphPad Prism 9 software and analyzed by Student *t* test for unpaired data and one-way analysis of variance. All data were shown as mean ± standard deviation (SD).

3. Results

3.1. ConA upregulates CD69 expression in CTLL-2 cells

To explore the effect of ConA on CTLL-2 cell activation, CD25 and CD69, two surface molecules known to be up-regulated during activation, were used to assess CTLL-2 activation. CTLL-2 cells are IL-2-dependent, and their surfaces constitutively express the CD25 molecule [15]. There was no significant change in CD25 expression in CTLL-2 cells under ConA treatment, but the expression of CD69 was significantly up-regulated (Figure 1a). Consistent with this, the mean fluorescence intensity (MFI) of CD25 expression did not change significantly before and after

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ConA treatment, while CD69 expression was significantly higher after treatment than before (Figure 1b). The above results suggest that CTLL-2 can show a certain activation state under ConA stimulation.

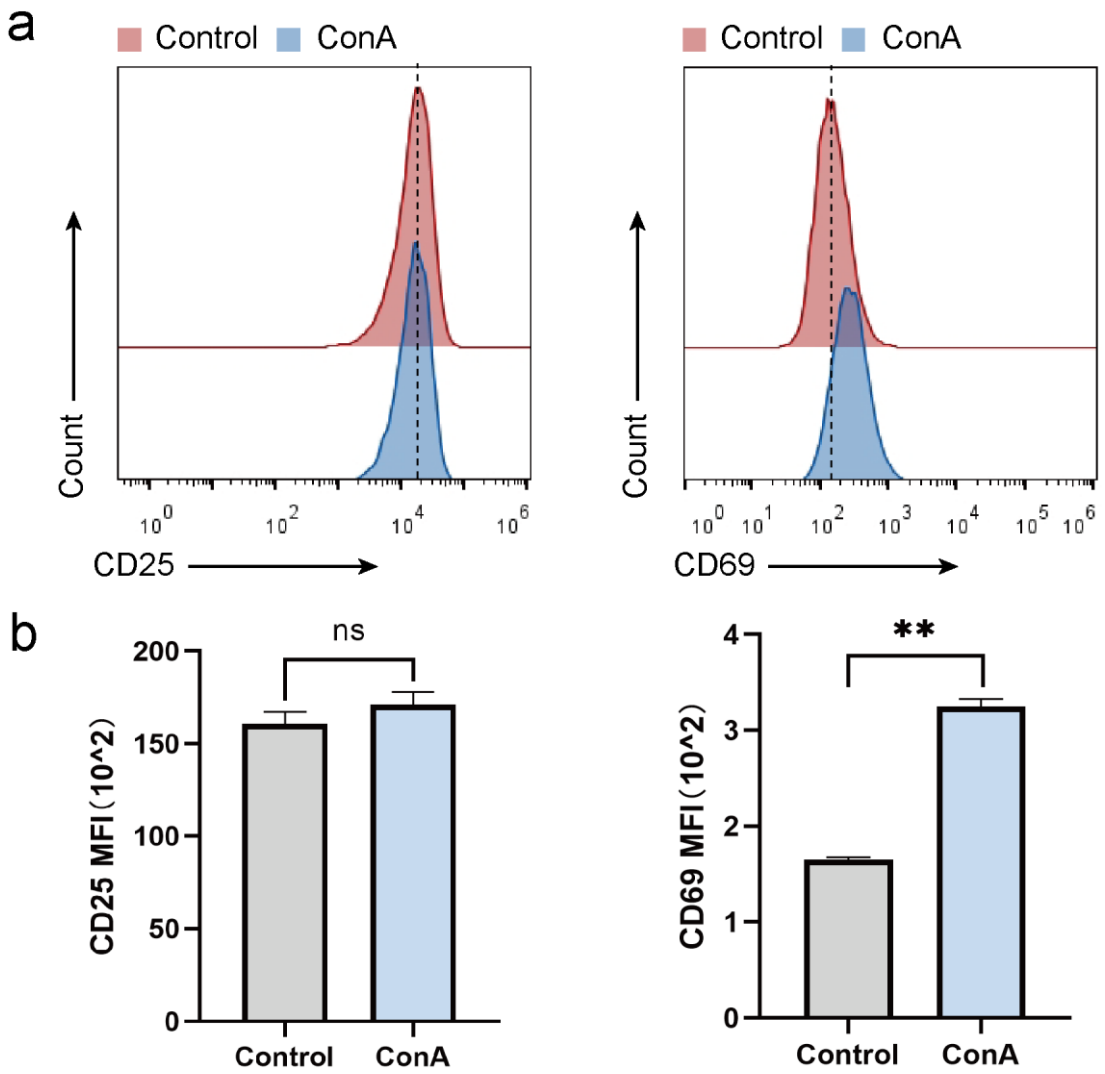

Figure 1. Flow cytometry analysis of lymphocyte activation markers CD25 and CD69. CTLL-2 was cultured in the presence and absence of 5 μg/mL ConA, and CD69 and CD25 expression was detected using flow cytometry after 24 h of incubation. (a) Overlaid histograms of CD25 and CD69 expression in CTLL-2 cells. (b) Effect of ConA on the mean fluorescence intensity of CD25 and CD69 in CTLL-2 cells. Data are expressed as means ± SD of three experiments. $^{**}P < 0.01$ vs. the control group; ns, not significant.

3.2. Determination of the appropriate target ratio

In order to determine cytotoxicity of CTLL-2 cells, we determined co-culture systems with different effector/target (E/T) ratios. Significant death of target B16 cells could be induced at an E/T of 5:1 compared to the control, and the highest number of B16 cell deaths was done when the E/T was 20:1 (Fig. 2a), and statistical data further visualised this difference (Fig. 2c).The results of the LDH release assay were consistent with the above results (Fig. 2b). To ensure that the lysis activity of CTLL-2 cells was evident, the E/T (20:1) that caused the most pronounced target cell death was selected for subsequent experiments.

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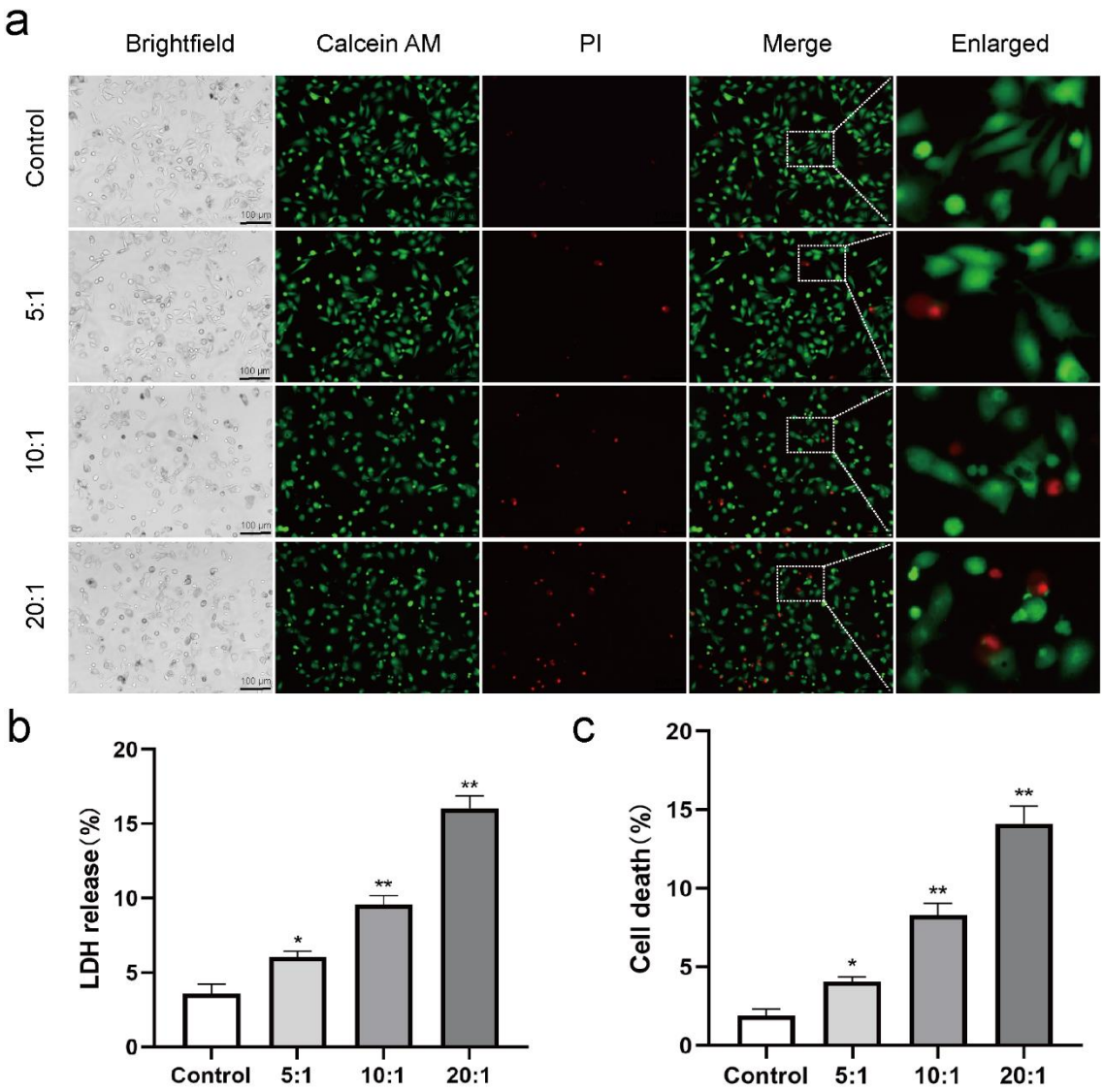

Figure 2. CTLL-2 cytolytic activity assay. Corresponding numbers of CTLL-2 cells were added to B16 cells for co-culture at different E/T (5:1, 10:1 and 20:1), and the assay of cell lysis activity was performed after 24 h of co-culture. Cytotoxicity was expressed as live-dead staining of B16 cells in the CTLL-2/B16 co-culture system (a) and LDH release from the co-culture system (b), respectively. (c) Statistical data of B16 cell death. Data are expressed as means ± SD of three experiments. *$P < 0.05$, **$P < 0.01$.

3.3. Regulation of CTLL-2 cytotoxicity against B16 cells by ConA

To further investigate effect of ConA on CTLL-2-mediated cytotoxicity, an E/T (20:1) CTLL-2/B16 co-culture system was adopted for test. ConA-treated CTLL-2 cells induced significantly higher numbers of target cell death (Fig. 3a) and increased LDH release (Fig. 3b) in B16 cells, and the statistics of the number of dead cells in B16 cells showed consistent results (Fig. 3c). The above data show that ConA significantly enhances the cytotoxic activity of CTLL-2 cells.

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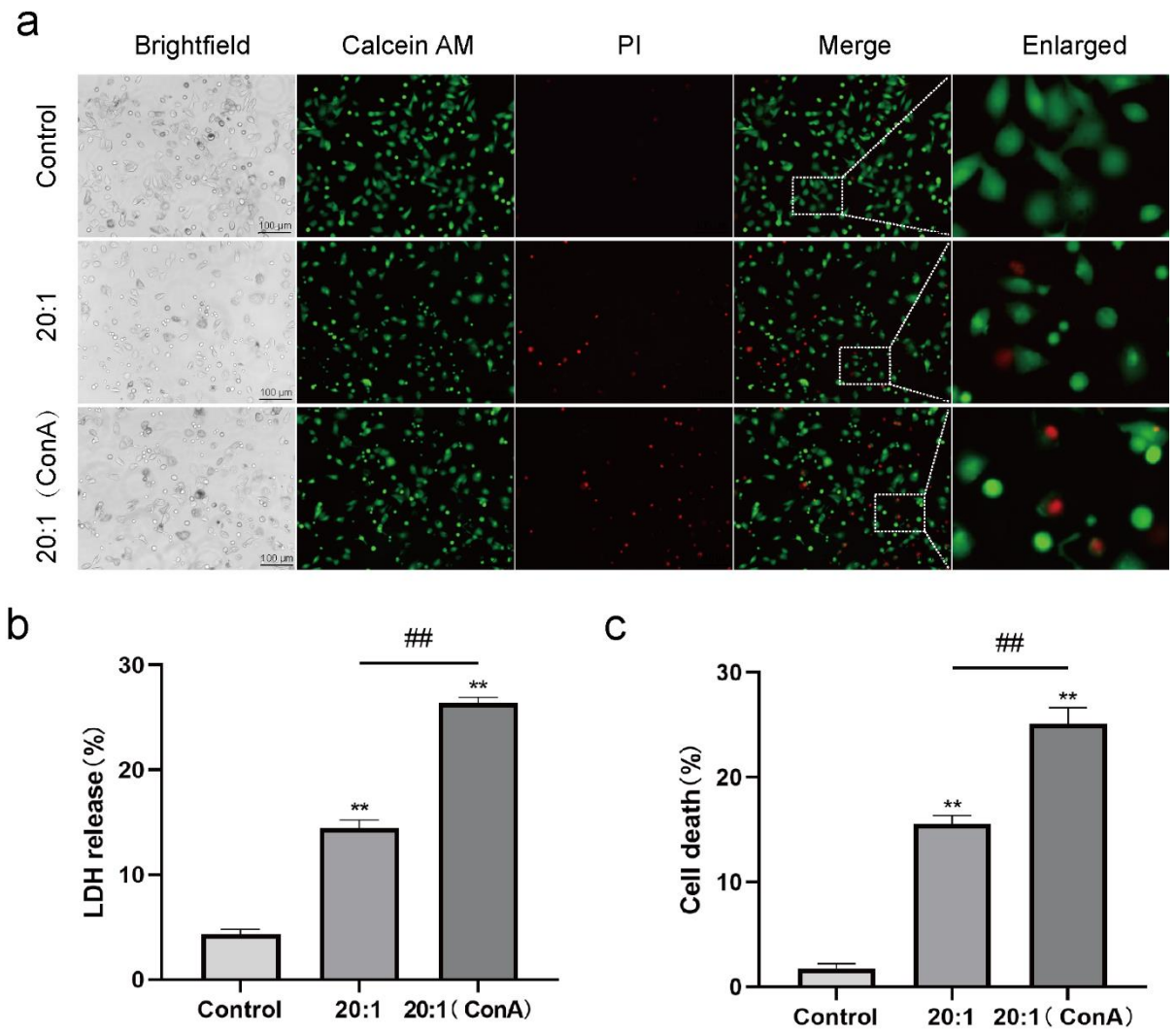

Figure 3. Effect of ConA on CTLL-2-mediated cytotoxicity. B16 cells were co-cultured with CTLL-2 cells treated and untreated with 5 μg/mL of ConA for 24 h. Cell death induced by CTLL-2 in target B16 cells (a) and LDH release in the co-culture system (b) were evaluated, respectively, and the deaths of B16 cells were subjected to statistically analysed (c). Data are expressed as means ± SD of three experiments.**P < 0.01 vs*. the control group，*##P ＜0.01*, the 20:1 (ConA) group *vs*. the 20:1 one.

4. Conclusion

Overall, ConA, as a primary T-cell activator, not only activates the T-lymphocyte cell line CTLL-2 cells *via* up-regulating the expression of CD69, but also enhances the cytolytic activity of CTLL-2 cells against B16 cells. The above results suggest that ConA might be utilized to restore the lost functions of CTLL-2 cells for long-term culture passage, to some extent.

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