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An additional CD28 costimulatory signal enhances proliferation and cytotoxicity of murine T cell-derived CIK cells

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Abstract

Objective: Cytokine induced killer (CIK) cells are *ex-vivo* expanded T cells endowed with both T and Natural Killer cell properties. The standard protocol for generation of CIK cells is to culture peripheral blood mononuclear cells (PBMC) in the presence of interferon- gamma (IFN-γ), monoclonal antibody (mAb) against CD3 and interleukin-2 (IL-2). However, this protocol lacks costimulatory signal (CD28), crucial for T cell activation. Herein, the proliferation and functional properties of murine thymocytes derived CIK cells generated with or without costimulatory activation provided by anti-CD28 mAb were examined.

Method: The proportion of CIK ($Thy1.2^+NK1.1^+$ and $CD8^+NK1.1^+$) cells in culture and the expression of cytotoxic granules (granzyme B and perforin) and proinflammatory cytokines (IFN-γ and tumor necrosis factor-alpha (TNF-α)) were determined by flow cytometry. Additionally, CIK cell cytotoxicity against YAC-1 murine lymphoma cells was measured by a propidium iodide-based assay.

Results: The addition of anti-CD28 to standard CIK culture conditions increased the number of $Thy1.2^+$ $NK1.1^+$ and $CD8^+$ $NK1.1^+$ (the major effector population) cells by almost 40% and 32%, respectively. Furthermore, the cytotoxic potential of CIK cells cultured with the addition of anti-CD28 mAb was also enhanced, with a corresponding increase in CIK cells expressing granzyme B, perforin, IFN-γ and TNF-α.

Conclusions: The addition of anti-CD28 mAb generated more effective murine T cell-derived CIK cells.

Keywords: CD28 monoclonal antibody; CD3 monoclonal antibody, Cytokine-induced killer cells; Murine thymocytes, Immunostimulation

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Introduction

Cytokine Induced Killer (CIK) cells, reported for the first time by Schmidt-Wolf *et al.*,[1] are *ex vivo* expanded T cells that display phenotypic and functional characteristics of both natural killer (NK) cells and cytotoxic T cells.[2] There are two main subsets of T cells in the human CIK cell culture, $CD3^+$ $CD56^+$ and $CD3^+$ $CD56^-$ cells (for murine CIK cell culture: $CD3^+$ $DX5^+$ / $CD3^+$ $NK1.1^+$ and $CD3^+$ $DX5^-$ / $CD3^+$ $NK1.1^-$).[3] Interestingly, the double positive cells are mainly $CD8^+$ T cells with a CD1d-independent, major histocompatibility complex (MHC)-unrestricted cytolytic activity against tumor targets.[4] Upon natural killer group 2 member D (NKG2D) recognition, CIK antitumor activity is mediated through the release of a variety of cytotoxic mediators including perforin, granzyme, interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α).[5] Besides anti-cancer activity, CIK cells also reduced graft-versus-host disease in an animal study.[6] At present, CIK cells have proven to be safe for adoptive transfer, and are widely used as immunotherapy for various cancers including lymphoma, metastatic renal cancer, hepatocellular carcinoma and relapsing hematological malignancies.[7] Almost all of the studies on CIK cells generated from human PBMC have focused on their antitumor effects.[8] However, other

applications of CIK cells and full elucidation of their differentiation process remain elusive. Moreover, murine CIK cells have been used for pre-clinical research, mostly adoptive immunotherapy for cancers.[9] Again, the expansion of murine CIK cells is problematic for preclinical studies as the cells could be expanded for a brief time; hence, has to be infused by 14 days of culture.[9] A variety of mouse models can provide clues to ascertain their usefulness in other diseases, so it would be very beneficial to generate murine T cell-derived CIK cells under optimal conditions.

Often the standard human CIK cell culture protocol is used to obtain murine CIK cells from various organs including the thymus, spleen and lymph nodes.[4,6,9] As it has already been proven that the major effector population of human CIK cells, i.e., $CD3^+$ $CD56^+$, is differentiated from a $CD3^+$ $CD56^-$ population, i.e., T cells,[10] the use of splenocytes or lymph nodes as the source of the $CD3^+$ $CD56^-$ subset results in the isolation of a mixed cell population. As the thymus contains more than 90% T cells, the use of murine thymocytes for CIK cell generation can eliminate the tedious and time-consuming T cell isolation process. Previously, Baker *et al.* also used thymocytes to generate CIK cells; however the yield of CIK cells from the thymus is relatively low, unlike that of the spleen.[9]

Moreover, the well-established protocol to obtain human CIK cells derived from peripheral blood mononuclear cells (PBMC) includes signaling through rIFN-γ, anti-CD3 mAb and rIL-2.[11] IFN-γ administered at the start of culture increases T cell cytotoxicity and facilitates the acquisition of a T-helper (T_H) 1 phenotype.[12] Next, rIL-2 further promotes proliferation, survival and differentiation of T cells.[13] Finally, anti-CD3 monoclonal antibody (mAb) supplied in the culture 24 hours (h) after the activation with rIFN-γ acts via T cell receptor (TCR) signaling and provides the first fundamental signal for cell activation.[14] In addition to TCR signaling, T cells require a second signal for full activation, and enhanced proliferation.[15] The additive signal provided by CD28 costimulation has been shown to enhance T cell proliferation.[16] The CD28 costimulatory signal in T and NKT cells is well known to act synergistically with the first TCR-derived signal.[17] Moreover, the CD28 costimulatory signal was shown to enhance the expression of chemokine receptors by CIK cells. Those CIK cells showed increased trafficking into the tumor site.[18] These observations demonstrated the importance of CD28 costimulatory signal in CIK cell activity. However, whether CD28 costimulatory signaling increases CIK cell proliferation and enhances cytotoxicity against tumor cells remains elusive. Also, whether enhanced cytotoxicity is due to increased number of CIK cell trafficking or their absolute potentiation of their cytotoxic ability is not clear.

Therefore, in the present study, we generated murine thymocytes derived CIK cells in the presence or absence of the CD28 costimulatory signal, and monitored the phenotype during the process of culture. Then, we measured the cytotoxicity of hence generated CIK cells against murine leukemic cells. Finally, we investigated for the production of cytotoxic granules and proinflammatory cytokines to determine the potential mechanisms enhancing cytotoxicity of CIK cells by the additional CD28 costimulatory signals. Interestingly, CD28 costimulatory signaling effectively enhanced CIK cell proliferation and potentiated their cytotoxic activity against tumor cells.

Methods

Animals

Four- to six-week-old male Balb/c mice were obtained from the National Laboratory Animal Center (Mahidol University, Bangkok, Thailand). All mice were housed at the Laboratory of the Department of Pharmacology, Siriraj Hospital and maintained on a 12-h light/dark cycle. All experimental procedures were in accordance with the guidelines of Mahidol University and the Office of the National Research Council of Thailand (NRCT) and approved by the Committee on Animal Care and Use of Siriraj Hospital (SiACUC). The animal protocol review number is SI-ACUP 013/2557.

Reagents

The following reagents were used in this study: Roswell Park Memorial Institute medium (RPMI 1640), fetal bovine serum (FBS) and 2-mercaptoethanol (Gibco Inc., Life Technologies, Carlsbad, CA, USA); mouse rIFN-γ (eBioscience, San Diego, CA, USA); anti-mouse CD3e mAb clone 145-2C11, anti-CD28 mAb clone 37.51 and mouse rIL-2 (BioLegend, San Diego, CA, USA); Phorbol 12-myristate 13-acetate (PMA) and ionomycin (Sigma-Aldrich, St. Louis, MO, USA); Brefeldin A solution (Catalogue no 347688, BD Biosciences, San jose, CA, USA), BD Cytofix/Cytoperm (BD Biosciences, San Jose, CA, USA); fluorescein isothiocynate (FITC)-labeled anti-mouse Thy1.2 mAb, phycoerythrin (PE)-labeled anti-mouse IFN-γ mAb, PE-labeled anti-mouse TNF-α mAb, PE-labeled anti-mouse granzyme-B mAb and PE-labeled anti-mouse perforin mAb (eBioscience); FITC-labeled anti-mouse CD8 mAb and PE-labeled anti-mouse NK1.1 mAb (BioLegend).

Cell line culture

The mouse T lymphoma cell line (YAC-1) was a kind gift from Dr. A. Wongkajornsilp (Mahidol University, Bangkok, Thailand). YAC-1 cells were cultured in RPMI 1640 supplemented with 10% FBS and antibiotics (100 μg/mL streptomycin and penicillin G 100 IU/mL) at 37°C in a humidified atmosphere with 5% CO_2.

Generation of CIK cells

CIK cells were generated as previously described[1] with slight modifications. Briefly, single cell suspensions were prepared from the thymus (3 mouse per experiment). The cells were cultured in RPMI 1640 containing 10% heat-inactivated FBS, 100 μg/mL streptomycin, 100 IU/mL penicillin G and 50 μM 2-mercaptoethanol in the presence of 1000 IU/mL mouse rIFN-γ at 37°C in a humidified atmosphere with 5% CO_2 for 24 h. Next, the cells were transferred to a dish pre-coated with 50 ng/mL anti-mouse CD3e mAb, and cultured with 300 IU/mL mouse rIL-2 in the presence or absence of 2 μg/mL anti-mouse CD28 mAb. The concentration of anti-CD28 mAb was chosen based on previous reports.[19] These cells were maintained at a density of 1×10^6 cells/mL, and medium (supplemented with 50 IU/mL mouse rIL-2) renewal was done every 2 to 3 days to ensure availability of nutrients for cell cell culture. After 3 weeks of culture, the cells were harvested and monitored by trypan

blue staining. Moreover, thymocytes at day 0 were used as control cells.

Detection of Immunophenotype by flow cytometry

The phenotypes of cells were determined by staining for both cell surface and intracellular markers on day 21. For cell surface markers, FITC-conjugated antibodies against CD8 or Thy1.2, and PE-conjugated antibody against NK1.1 were used. Briefly, one million cells (Control cells, or CIK cells) were washed once with phosphate buffered saline (PBS) and re-suspended in 100 µl PBS containing 1% FBS (FACS buffer). Then, the cell suspension was incubated with specific antibodies for 30 min in the dark on ice or at 4°C, washed twice with PBS and re-suspended in 100 µl FACS buffer. Analysis was performed using a flow cytometer (FACSCalibur, BD Biosciences). Data were analyzed using Flowing Software 2.5 (Cell Imaging Core, Turku Centre for Biotechnology, Turku, gFinland).

For intracellular cytokine (IFN-γ and TNF-α) staining, cells were stimulated for 24 h with 50 ng/mL PMA and 500 ng/mL ionomycin. Brefeldin A solution at final concentration of 10 µg/mL was added 4-6 hour prior to harvesting the cells. Subsequently, the harvested cells were monitored for cell death by trypan blue staining. The cells were fixed and permeabilized with BD Cytofix/Cytoperm according to the manufacturer's instructions, and then stained with PE-conjugated mAb against IFN-γ or TNF-α. For the intracellular cytolytic granule (granzyme B and perforin) stainings, the cells were only permeabilized using BD Cytofix/Cytoperm. These cells were then stained with PE-conjugated mAb against granzyme B or perforin. Cells were finally washed with PBS, resuspended in FACS buffer and immediately analyzed by flow cytometry. In parallel, thymocytes (day 0) stimulated with PMA/Ionomycin (24 hours) were similarly stained and analyzed (Control).

Assessment of cytotoxicity of CIK cells

The method for assessment of the cytotoxicity of CIK cells was adapted from Wongkajornsilp *et al.*, 2013. Briefly, the tumor-killing ability of control cells (thymocytes cultured in media only) and CIK (day 21) effector cells was assessed against YAC-1 target cells. YAC-1 cells (2×10^4 cells/well) prepared in serum-free RPMI were seeded (40 µL/well) in a 96-black well/clear bottom plate, and were co-cultured with the effector cells (40 µL/well) at the desired effector to target (E:T) ratios of 3.125:1, 6.25:1, 12.5:1 and 25:1 for 4 h at 37°C in a humidified atmosphere with 5% CO_2. Propidium iodide (PI) solution (20 µL/well of 10µg/mL PI in PBS) was added. After incubation for 1 h, fluorescence intensity was assessed at 482 nm excitation wavelength and 630 nm emission wavelength using the Spectra Max M5 microplate reader (Molecular Device, Sunnyvale, CA, USA). The % cytotoxicity was calculated using the following formula:

$$\% \text{ Cytotoxicity} = 100\ (Fl_x - Fl_0) / (Fl_{100} - Fl_0)$$

where, Fl_x represents the fluorescence intensity emitted from the wells containing the target cells co-cultured with the effector cells at various E:T ratios (3.125:1, 6.25:1, 12.5:1 and 25:1). Fl_0 represents the fluorescence intensity emitted from the wells containing the target cells only. Fl_{100} represents the fluorescence intensity emitted from the wells containing the target cells incubated with 20 µL/well of 0.04 N isopropanol.

Statistical analysis

The results are shown as the mean ± standard error of mean (SEM). Data were plotted using GraphPad Prism version 5.03 (GraphPad Software Inc., La Jolla, CA, USA). Two-way *ANOVA* with Bonferroni post-test was used to analyze the statistical significance of the difference between means for cell proliferation and cytotoxic assays. One-way *ANOVA* with Tukey's test was used to analyze statistical significance of the difference between means for phenotypic characterization and intracellular cytokine and cytolytic granule expression. $p < 0.05$ was considered statistically significant.

Results

CD28 costimulation effectively increases total cell numbers

We additionally administered anti-CD28 mAb in the well-established CIK cell culture conditions and analyzed the viability and proliferation of the cultured cells at the indicated times (**Figure 1**). As such, cultures at day 21 demonstrated viability more than 90% (**Table 1**) which is in agreement with that observed previously.[20] In comparison to the medium alone condition, the standard CIK cell culture conditions allowed

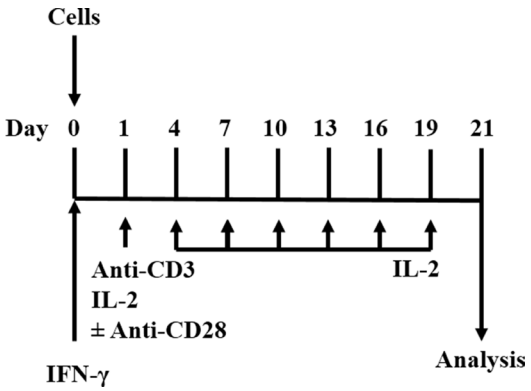

Figure 1. Schematic protocol for generation of thymocytes -derived murine CIK cells. Thymocytes maintained at a density of 1×10^6 cells/ml were stimulated with 1000 IU/ml IFN-γ for 24 h. Next, cells were transferred to 50 ng/ml anti-CD3 mAb-coated culture dishes, and subsequently stimulated with 300 IU/ml IL-2 ± 2 µg/ml anti-CD28 mAb. The culture was supplemented with 50 IU/ml IL-2 at an interval of 2–3 days. After 21 days, the CIK cells obtained were used for further assays.

Table 1. Cell viability of control thymocytes, CIK culture, and CIK culture + anti-CD28

Culture Conditions	Percentage of live cells (mean ± SD)	
	Unstimulated	PMA/I stimulated (24 hours)
Thymocytes (day 0)	99 ± 0.82	95.6 ± 2.83
CIK (day 21)	95 ± 2.45	93.6 ± 2.17
CIK + anti-CD28 (day 21)	97 ± 1.63	94.7 ± 2.05

SD, Standard Deviations; PMA, Phorbol 12-myristate 13-acetate; I, Ionomycin

cells to expand significantly at all time points tested (7, 14 and 21 days) after the start of the culture. Furthermore, cells cultured under the CIK cell conditions in which anti-CD28 mAb had been included showed markedly higher cell expansion than without the addition of anti-CD28 mAb (**Figure 2A**). Finally, by day 21, cultures reached a mean peak of 2-fold expansion in the group receiving both antibodies, and this expansion was significantly higher as compared with the group receiving anti-CD3 mAb alone (**Figure 2B**). Moreover, addition of anti-CD28 mAb at day 1 showed pronounced cell expansion when compared with those added at other time points (data not shown). Thus, we could affirm that earlier addition of anti-CD28 mAb to the well-established CIK cell culture condition enhances cell proliferation. Hence, further characterization of the cells was carried out using the cells obtained after 21 days of culture.

CD28 costimulation increases the percentage of CIK cells in culture

As murine CIK cells are positive for Thy1.2 and NK1.1, we identified the double positive population as CIK cells by flow cytometry. In the freshly isolated thymus, the majority of cells were T cells (99.86 ± 0.03% Thy1.2$^+$ NK1.1$^-$ cells) whereas double positive cells were rarely found (0.11 ± 0.02% Thy1.2$^+$ NK1.1$^+$). As expected, CIK cells expanded significantly under the CIK cell culture conditions (24.32 ± 1.85%). With the addition of anti-CD28 mAb, a higher proportion of CIK cells were obtained (40.60 ± 2.87%) (**Figure 3A**). Previous reports,[4] found that the majority of the CIK cell population (Thy1.2$^+$ NK1.1$^+$) is CD8$^+$. The addition of anti-CD28 mAb resulted in a higher proportion of CD8$^+$ NK1.1$^+$ cells (32.13 ± 0.53%) when compared with the CIK culture conditions without anti-CD28 mAb (16.70 ± 1.08%) (**Figure 3B**). Morphologically, the expanded CIK cells (day 21) growing in aggregates were larger in size and more irregular in shape when compared with control cells or fresh thymocytes (data not shown).

CD28 costimulation can potentiate cytotoxicity of CIK cells against tumor cells

As our previous results demonstrated that addition of anti-CD28 mAb to the standard CIK culture conditions enhances the proliferation of CIK cells, we next examined whether thus potentiated the CIK cell cytotoxicity against tumor cells. YAC-1 cells, a murine lymphoma cell line, were used as target cells to check CIK cell cytotoxicity. As expected, control cells had no cytolytic activity, but thymocytes grown in the CIK culture conditions showed significantly higher cytotoxicity against YAC-1 cells at all the effector-to-target (E:T) ratios tested (3.125:1, 6.25:1, 12.5:1 and 25:1). Notably, CD28 costimulation significantly augmented the tumor cytotoxicity of CIK cells (**Figure 4**).

CD28 costimulation increases the production of TH1 cytokines and cytolytic granules

CIK cells are known for their ability to abundantly produce the T_H1 cytokines IFN-γ and TNF-α.[21] Therefore, we examined whether the potentiation of CIK cell cytotoxicity against tumor cells could be attributed for the upregulation of cytotoxic granules and cytokines. Hence, the production of these cytokines by CIK cells was measured using flow cytometry. Thymocytes were expanded under the conditions described above, and harvested at day 21. The harvested cells were re-stimulated *in vitro* with PMA and ionomycin for 24 h. The viability of re-stimulated cells was more than 90% as checked by trypan blue dye staining (**Table 1**). Subsequent flow cytometry analysis revealed that the number of cells producing cytokines under standard CIK cell culture conditions was increased significantly when compared control cells (IFN-γ: 23.40 ± 1.40% vs. 4.36 ± 2.44%, respectively, and TNF-α: 25.81 ± 6.88% vs. 4.82 ± 2.59%, respectively). Notably, the addition of anti-CD28 to the CIK culture conditions tended to increase the number of cells producing both cytokines (IFN-γ: 26.09 ± 1.40%) and TNF-α (34.3 ± 5.88%) (**Figure 5A and B**).

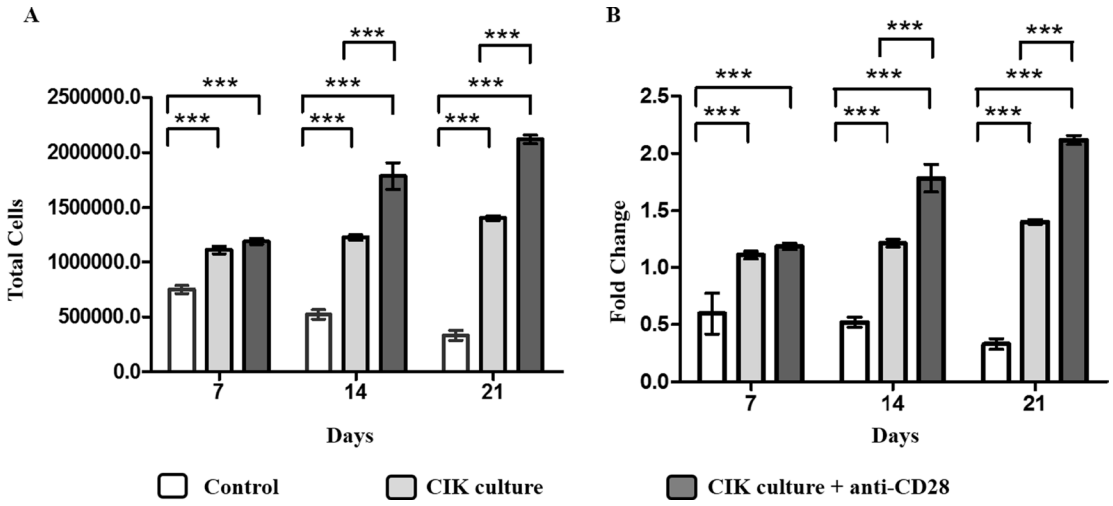

Figure 2. Increase in total cell numbers by the addition of anti-CD28 mAb to the CIK cell culture. A) Total cell numbers and B) fold change in total cells from thymocytes cultured in medium only (Control), standard CIK cell culture conditions (CIK culture), and the CIK cell culture conditions with the addition of anti-CD28 mAb (CIK culture + anti-CD28) on days 7, 14 and 21. Data shown are the mean absolute number of cells ± SEM from three separate experiments (*** $p < 0.001$).

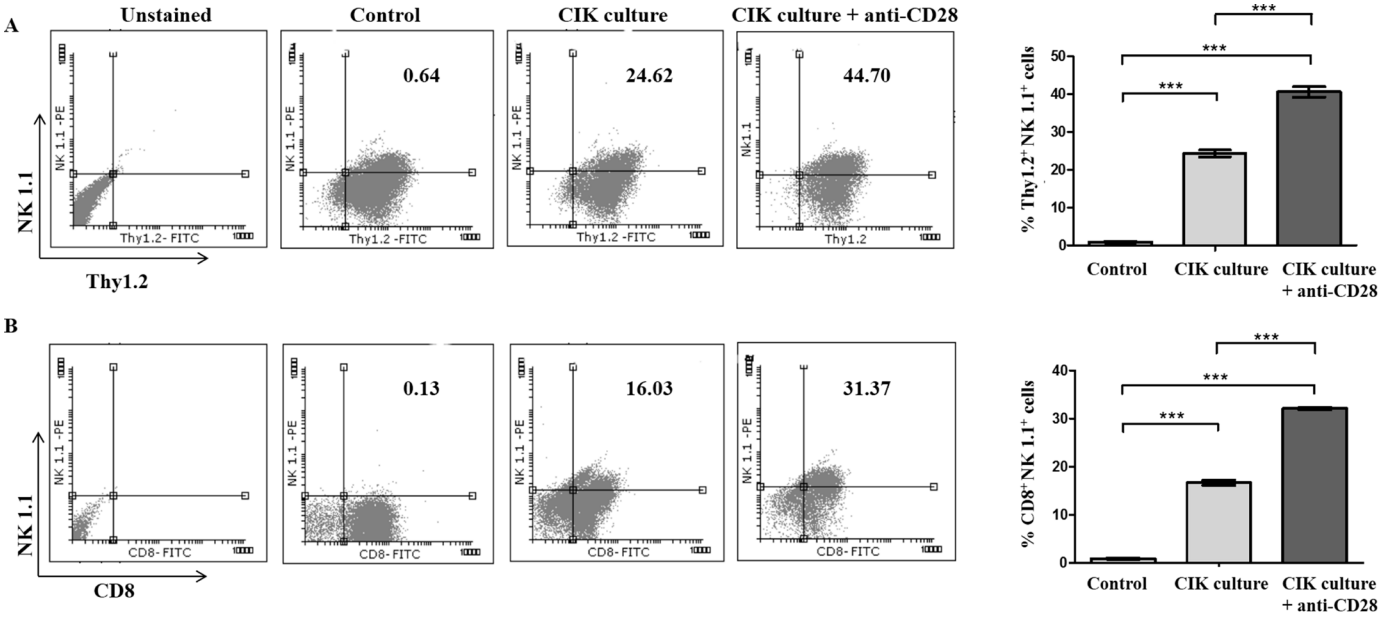

Figure 3. Increase in the major effector population of CIK cells by the addition of anti-CD28 mAb to the CIK cell culture. Flow cytometry analysis of thymocytes cultured in medium only (Control) and CIK cells (day 21) cultured under the standard CIK cell culture conditions (CIK culture), and CIK cell culture conditions with the addition of anti-CD28 mAb (CIK culture + anti-CD28) were stained with A) FITC-conjugated anti-Thy1.2 and PE-conjugated anti-NK1.1, and B) FITC-conjugated anti-CD8 and PE -conjugated anti-NK1.1. Unstained cells were used to define the negative populations. Dot plots showing the percentage of Thy1.2$^+$ NK1.1$^+$ and CD8$^+$ NK1.1$^+$ cells are representative of three experiments. The histogram shows the mean absolute percentages of Thy1.2$^+$ NK1.1$^+$ and CD8$^+$ NK1.1$^+$ cells ± SEM, respectively, from three independent experiments. Statistical significance was determined using one-way *ANOVA* to compare between multiple groups (*** $p < 0.001$).

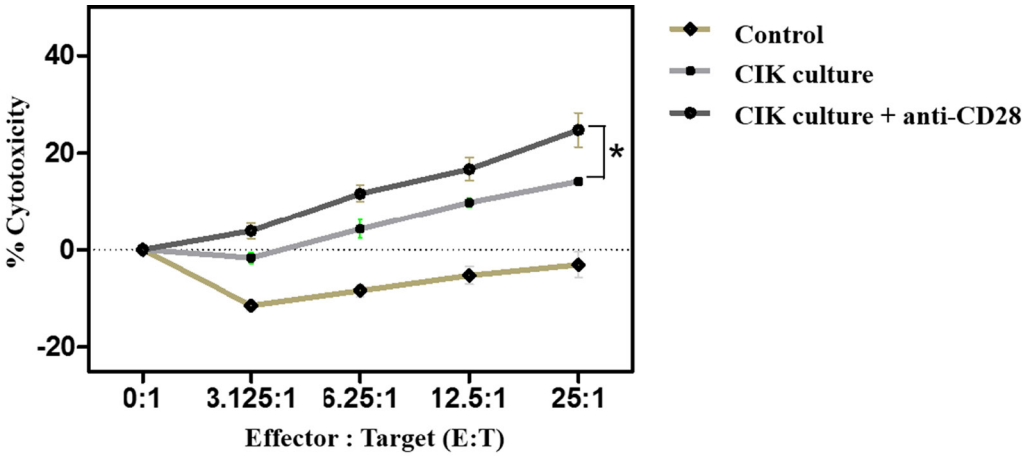

Figure 4. Enhancement of CIK cell cytotoxicity against YAC-1 cells by addition of anti-CD28 mAb to the CIK cell culture. Cytotoxicity of thymocytes cultured in medium only (Control), CIK cells (day 21) cultured under the standard conditions (CIK culture), and CIK cell culture conditions with the addition of anti-CD28 mAb (CIK culture + anti-CD28) were assessed against YAC-1 cells at different effector-to-target ratios, ranging from 3.125:1 to 25:1. Data are the mean ± SEM of three independent experiments (* $p < 0.05$).

Along with the production of T_H1 cytokines, CIK cells also release cytolytic granules.[22] We next addressed whether the addition of anti-CD28 mAb to the CIK culture condition enhances the production of cytolytic granules, as defined by the number of granzyme B$^+$ and perforin$^+$ cells by flow cytometry analysis. We confirmed that the CIK cells cultured under the standard CIK cell culture conditions had a significantly increased proportion of cells expressing cytolytic granules (granzyme B and perforin) when compared with thymocytes cultured in media only (granzyme B: 20.50 ± 1.99% vs. 2.02 ± 1.63%, respectively, and perforin: 18.88 ± 6.17 % vs. 3.72 ± 2.60%, respectively). Additionally, the inclusion of anti-CD28 mAb in the CIK culture conditions further enhanced the number of cells producing these granules (granzyme B: 24.38 ± 0.86% and perforin: 25.55 ± 8.64%) (**Figure 5C and D**).

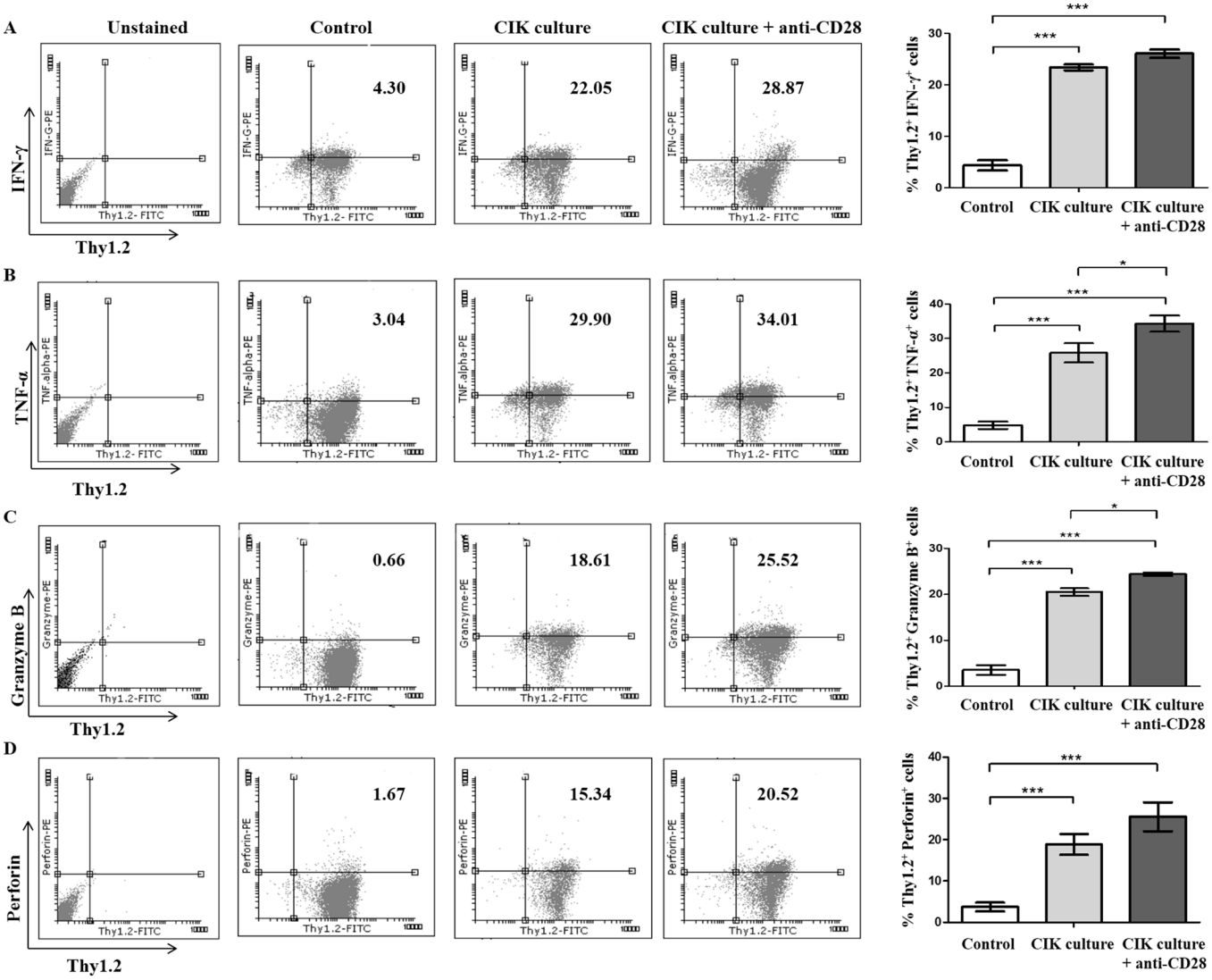

Figure 5. Increase in the major effector population of CIK cells producing T_H1 cytokines and cytolytic granules by the addition of anti-CD28 mAb to the CIK cell culture. Thymocytes cultured in medium only (Control) and CIK cells (day 21) cultured under the standard CIK cell culture conditions (CIK culture), and the CIK cell culture conditions with the addition of anti-CD28 mAb (CIK culture + anti-CD28) were stained for intracellular cytokines, A) FITC-conjugated anti-Thy1.2 and PE-conjugated anti-IFN-γ, B) FITC-conjugated anti-Thy1.2 and PE-conjugated anti-TNF-α and for intracellular cytolytic granules, C) FITC -conjugated anti-Thy1.2 and PE-conjugated anti-granzyme B and D) FITC-conjugated anti-Thy1.2 and PE-conjugated anti -perforin. Unstained cells were used to define the negative populations. Dot plots along with the percentage in A, B, C and D are representatives of three experiments for intracellular staining to determine the expression of the indicated cytokines and cytolytic granules. The histogram shows the mean absolute percentages of $Thy1.2^+$ IFN-γ^+, $Thy1.2^+$ TNF-α^+, $Thy1.2^+$ granzyme B^+ and $Thy1.2^+$ perforin$^+$ cells ± SEM, respectively, from three independent experiments. Statistical significance was determined using one-way *ANOVA* (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

Discussion

Over the past several years, CIK cells have come to be regarded as the primary candidate for adoptive immunotherapy. CIK cells were resistance to Fas-mediated apoptosis, and also express several anti-apoptotic genes such as CFLIP, Bcl-2, Bcl-xL, DAD-1 and surviving which make CIK cells a suitable candidate for immunotherapy.[23] Moreover, the dual T_H1 and NK properties that can kill abnormal cells such as cancerous cells have promoted these CIK cells as a unique immunotherapeutic approach.[12,24] There are numerous research groups who have proven the efficacy and safety of CIK cells to treat a variety of cancers through clinical studies.[2,7,24] Moreover, the functional properties of CIK cells, especially the production of IFN-γ, TNF-α, perforin and granzyme B, have been used to study the antiviral activity of human CIK cells against human immunodeficiency virus and Epstein–Barr virus.[25] At present, there are a wide variety of mouse-based disease models that mimic human pathologies.[1,6] These findings not only encourage investigating the antiviral activity of CIK cells in animal disease models but also provide a basis to broaden the application of CIK cell therapy to diseases other than cancer, such as asthma. Therefore, optimal generation of murine CIK cells and their use in preclinical studies will be highly beneficial when exploring other applications of CIK cells. Considering that the most

potent CIK population consists of $CD8^+$ NKT-like cells, which are believed to be differentiated from T cells,[11] we cultured T cells from mouse thymus to generate CIK cells.

The standard protocol to generate and expand human CIK cells is the incubation of PBMC in medium containing IFN-γ for 1 day, followed by stimulation with CD3 mAb and IL-2 for 21 days.[26] Immobilization with CD3 mAb is used to trigger T cell proliferation.[27] The addition of IFN-γ before IL-2 increases cellular cytotoxicity. Similarly, IL-2 is required as absence of this cytokine not only limits T cell expansion, but also results in the death of cells in culture.[1] Adopting the well-established protocol for culturing human CIK cells to obtain murine CIK cells defined as $Thy1.2^+$ $NK1.1^+$ cells, we could expand this rarely found population from the thymus as many as 24-fold. As this well-established CIK cell generation method lacks a costimulatory signal, we examined whether the incorporation of this signal could increase the expansion of CIK cells and enhance their functional properties. We found that addition of anti-CD28 mAb to the culture increases the CIK cell population up to 40-fold. Furthermore, it has also been reported that CIK cells are $CD8^+$ cells[9] and we confirmed that $CD8^+$ $NK1.1^+$ CIK cells were expanded up to16-fold in culture. Moreover, the addition of anti-CD28 mAb to CIK culture conditions increased this $CD8^+$ $NK1.1^+$ population even higher to 32-fold. Our results suggest that CD28 signaling acts synergistically with other stimuli. The reason behind enhanced proliferation of CIK cells mediated by CD28 signaling needs to be explored. Since, CD28 signaling was shown to enhance T cell survival by enhancing the anti-apoptotic factor Bcl-2;[28] whether this holds true for CIK cells needs to be justified. Moreover, addition of CD28 at times other than day 1 decreased the number of CIK cells, and the anti-proliferative effect of CD28 signaling at later time needs to be elucidated.

In addition to gaining higher numbers of CIK cells, it is also important to generate CIK cells that have potent cytotoxicity against tumor cells. To assess this we determined the *in vitro* cytotoxic activity of murine CIK cells towards YAC-1 cells. As expected, the additional CD28 costimulatory signal also enhanced the tumor killing activity of CIK cells.

The antitumor cytotoxicity of CIK cells is determined by their ability to produce T_H1 cytokines (IFN-γ and TNF-α) and cytolytic granules (perforin and granzyme B). Furthermore, cytolytic granules are reported to be important both *in vitro* and *in vivo* for the anticancer activity of CIK cells.[29,30] Therefore, we further tested whether enhanced antitumor cytotoxicity of CIK cells generated in presence of anti-CD28 mAb is due to an increased production of cytolytic mediator. Our results demonstrated that the additional CD28 costimulation augmented the production of all these mediators (IFN-γ, TNF-α, perforin and granzyme B) compared with those in absence of CD28 signaling. Thus, the inclusion of anti-CD28 mAb to the culture not only increased CIK differentiation and proliferation, but also potentiated the function of CIK cells.

Nevertheless, cytotoxicity of CIK cells has been improved by eliminating the suppressive factors like removing or downregulating regulatory T (Treg) cells.[31] Different strategies like addition of IL-15,[32] or IL-6[33] to the CIK cell culture were shown to inhibit Treg cells which subsequently enhanced the cytotoxicity. Moreover, the observation that CD28 costimulation suppresses induction of Treg cells from naïve precursors[34] might be responsible for the enhanced cytotoxicity of the CIK cells cultured in presence of anti-CD28 mAb; however, further studies is warranted.

In conclusion, the CD28 costimulatory signal enhances murine CIK cell differentiation and upregulation of cytotoxic mediator production, leading to greater cytotoxicity against tumor cells. It remains to be delineated how CD28 signaling enhanced the proliferation, and cytotoxicity of the CIK cells. Moreover, whether CIK cell can be used as an adoptive therapy for treatment of viral diseases and other human conditions using preclinical model is our future interest. Hopefully, CD28 costimulatory signal proved beneficial for generating murine CIK cells from pure T cells. This will in turn help not only for the development of more effective cancer immunotherapy, but also for exploring novel clinical applications of CIK cells in the future.

Competing interests

The authors declare that they have no competing interest.

Authors' contributions

KS and AW conceived the study. ST carried out the experiments and PP helped to analyze flow cytometry data. All authors have read and approved the final version of the manuscript.

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