APJAI
Asian Pacific Journal of
Allergy and Immunology

ORIGINAL ARTICLE

N-acetylcysteine potentiates the tumor cytotoxicity of cytokine-induced killer cells

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Abstract

Background: Cytokine-induced killer (CIK) cells are an *ex vivo* expanded heterogeneous population of natural killer (NK)-like T cells that can exert potent MHC-unrestricted antitumor activity. A number of pre-clinical and clinical studies have demonstrated that CIK cells can serve as a safe and potent immunotherapy of malignant tumors. N-acetylcysteine (NAC) has been demonstrated to enhance the T-cell functions by increasing their proliferation and cytokine production.

Objective: To investigate whether the incorporation of NAC to CIK cell culture could enhance the antitumor activity of CIK cells.

Methods: The phenotypes of human CIK cells, including $CD3^+CD56^+$, IFN-γ, granzyme B, and perforin, were determined by flow cytometry. The cytotoxic activity against the human erythroleukemic cell line (K562) and cholangiocarcinoma cell line (CL6) prelabeled with CFSE was investigated by flow cytometry. The mRNA expression levels of *IFNG*, *PRF1*, and *GZMB* were measured by real-time PCR.

Results: By adding NAC into CIK cell culture, the percentage of $CD3^+CD56^+$ cells along with the expression of Th1 cytokines and cytolytic granules increased significantly, resulting in an improvement of cytotoxicity against the cancer cell lines CL6 and K562.

Conclusions: The incorporation of NAC into CIK culture can markedly improve the cytotoxicity against cancer cells due to the significant increase in the major effector population of CIK cells expressing Th1 cytokines and cytolytic granules.

Key words: Cytokine-induced killer cells, N-acetylcysteine, Cancer immunotherapy, Cytotoxicity, Antitumor effect

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Introduction

Cytokine-induced killer (CIK) cells are a heterogeneous population of polyclonal T cells in which NK-like T cells are major effector cells. By culturing peripheral blood mononuclear cells (PBMCs) *ex vivo* in the presence of IFN-γ, anti-CD3 antibody, and interleukin (IL)-2 for a few weeks, CIK cells could be readily prepared, and were endowed with potent MHC-unrestricted antitumor cytotoxicity against both hematological and solid tumors.[1-3] Due to such a simple preparation protocol and their MHC-unrestricted tumor-killing activities, CIK cells have been widely applied in many clinical studies as a safe and effective cancer immunotherapy.[10-11]

N-Acetylcysteine (NAC), a precursor for glutathione synthesis, is a powerful antioxidant.[4] Recently, many possible effects of NAC on immune cell functions have been reported. Previous studies have shown that NAC can enhance the proliferation and cytokine production of T cells.[5] Furthermore, NAC is able to restore impaired T-cell function in cancer patients.[6] For adoptive T-cell therapy, by adding NAC to therapeutic T-cell expansion protocols, the adoptively transferred cells can retain the ability to resist DNA damage

and activation-induced cell death. These abilities can even last for 6 days after adoptive transfer without additional NAC supplementation.[7] In clinical practice, NAC is considered a safe medication for the treatment of acetaminophen overdose and for relieving productive cough. Hence, we aimed to investigate whether the incorporation of NAC into CIK cells culture could increase the proliferation and tumor-killing activities of CIK cells.

Materials and Methods

Cell lines

The human erythroleukemic cell line (K562) was kindly provided by Prof. Suradej Hongeng at Ramathibodi Hospital. The cholangiocarcinoma cell line (CL6) was a kindly given by Assoc.Prof. Adisak Wongkajornsilp at Siriraj Hospital. All the cell lines were cultured in a humidified incubator at 37°C and 5% CO_2. The K562 and CL6 cells were maintained in RPMI 1640 medium (Gibco, USA) and Dulbecco's modified eagle's medium (Gibco, USA), respectively. The growth medium was supplemented with 10% fetal bovine serum (FBS) (Biochrom, Germany), 100 ug/mL streptomycin, and 100 U/mL penicillin (Gibco, USA). These cells were chosen as target cells in the cytotoxicity assay and were passaged one day prior to the experiment to ensure log phase growth.

CIK cell generation

CIK cells were generated from PBMCs taken from healthy donors. The PBMCs were isolated from whole blood by Ficoll–Paque density centrifugation (IsoPrep, Robbins Scientific, CA). Cell viability was checked by the trypan blue exclusion test. The cells were seeded in a 10 cm dish at a density of 4 × 10^6 cells/mL and allowed to adhere for 1 h at 37°C and 5% CO_2 in RPMI 1640 (Gibco, USA) supplemented with 10% FBS (Biochrom, Germany), 100 ug/mL streptomycin, and 100 U/mL penicillin (Gibco, USA). The density of non-adhering PBMCs was adjusted to 1 × 10^6 cells/mL, and the cells were replated at 3 × 10^6 cells on a 6-well plate in the presence of 1,000 U/mL recombinant human IFN-γ (ImmunoTools, Germany) with or without 10 mM of NAC (Sigma-Aldrich, USA). After 24 h of incubation, 50 ng/mL anti-CD3 mAb (Biolegend, CA) and 300 IU/mL recombinant human IL-2 (Miltenyi Biotech, Germany) were added. Fresh medium and IL-2 were added every 3 days. The CIK cells were cultured for 14 days, and then harvested for the further experiments (**Figure 1**).

Detection of the CIK immunophenotypes by flow cytometry

The immunophenotypes of CIK cells were examined by determining the specific cell surface and intracellular protein markers by flow cytometry. The following fluorochrome conjugated antibodies were used: FITC-conjugated anti-human CD3 (clone UCHT-1, ImmunoTools, Germany), APC-conjugated anti-human CD56 (clone B-A19, ImmunoTools, Germany), PE-conjugated anti-human IFN-γ, (clone B27, ImmunoTools, Germany), APC-conjugated anti-human perforin (clone B-d48, Biolegend, CA), PE-conjugated anti-human granzyme B (clone REA226, Miltenyi Biotech, Germany), and APC-conjugated anti-human granzyme B (clone GB11, Biolegend, CA). For FAC staining of the intracellular proteins, the cells were stimulated by 50 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, USA) and 1 ug/mL ionomycin (Sigma-Aldrich, USA) for 1 h followed by 1 µl/ml GolgiPlug™ Protein Transport Inhibitor (BD Biosciences, USA) for 3 h. Fixation and permeabilization were performed using the BD Cytofix/Cytoperm™ Kit (BD Bioscience, USA) according to the manufacturer's instructions. Subsequently, the cells were labeled with fluorochrome conjugated antibodies. Data were obtained and analyzed using the CytoFLEX™ flow cytometer (Beckman Coulter, USA) and CytExpert software (Beckman Coulter, USA).

Quantitative real-time PCR analysis

RNA extraction was performed using the Real Genomics Total RNA Extraction Kit (RBC Bioscience, Taiwan) according to the manufacturer's instructions. The purity and quantity of the total RNA were measured on a NanoDrop system (Thermo Fisher Scientific, USA). cDNA synthesis from 1 ug of total RNA was carried out with the iScript™ Select cDNA Synthesis Kit (Bio-Rad, CA) according to the manufacturer's instructions. The gene-specific primers sequences were designed using the Primer-BLAST tool. All the genes, accession numbers, primer sequences, product sizes, and annealing temperatures used in this study are described in **Table 1**. The primers were ordered from Integrated DNA Technologies, USA. Real-time PCR was performed using the KAPA SYBR® FAST qPCR Master Mix Kit (Kapa Biosystems, USA) and the LightCycler® 480 Instrument II (Roche Applied Science, Germany). Each reaction mixture was 10 µL and also contained 10 ng of cDNA. The conditions were 95°C for 3 min, followed by 40 cycles of amplification at 95°C for 10 sec,

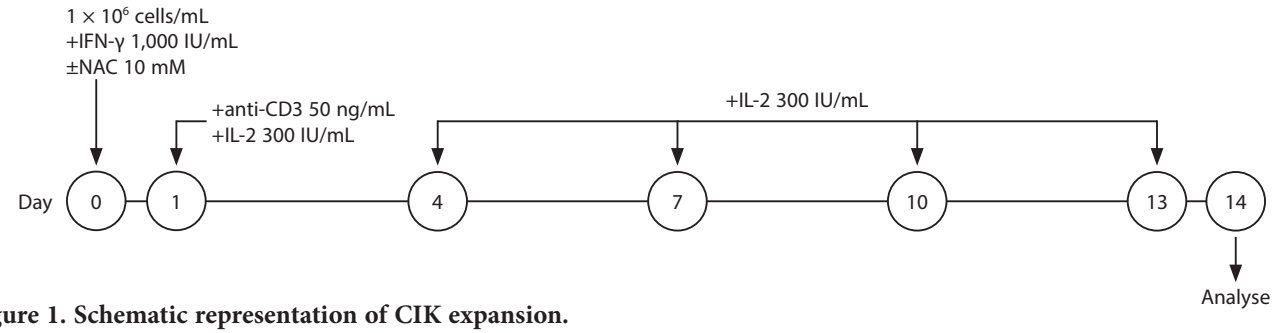

Figure 1. Schematic representation of CIK expansion.
CIK cells were expanded *in vitro* by the stimulation of PBMCs with 1,000 IU/ml of IFN-γ in the presence or absence of 10 mM NAC on day 0 of culture, followed by 50 ng/ml anti-CD3 mAb and 300 IU/ml IL-2 24 h later. IL-2 was added every 3 days. At day 14, the CIK cells were subjected to analysis.

Table 1. Primer sequences used for the real-time PCR analysis

Gene	Sequence (5'-->3')	Length (bp)	Annealing temp. (°C)	GenBank ID (accession)
GAPDH	Forward: AAATTCCATGGCACCGTCAAG Reverse: TGGTTCACACCCATGACGAA	248	57	NM_001256799.3
IFNG	Forward: CGTTTTGGGTTCTCTTGGCTG Reverse : TCTCCACACTCTTTTGGATGCT	242	57	NM_000619.2
PRF1	Forward: TCCTAAGCCCACCAGCAATG Reverse : TGGAGGCGTTGAAGTGGTG	215	57	NM_005041.5
GZMB	Forward: AGCCGACCCAGCAGTTTATC Reverse: GTGTGTGAGTGTTTTCCCAGG	229	57	NM_004131.5

57°C for 45 sec and 72°C for 1 sec. LightCycler® 480 software (Roche Applied Science, Switzerland) was used to determine the cycle threshold (Ct), with *GAPDH* considered a housekeeping gene for the normalization of each sample. Fold changes of the relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. Each reaction production was considered for its specificity by post-PCR melt curve analysis and agarose gel electrophoresis.

Flow cytometry assay of the cytotoxicity

Here, 1 × 10^6 K562 or CL6 in 1 ml phosphate buffer saline (PBS) was mixed with CellTrace™ CFSE (Invitrogen, CA) dissolved in DMSO at a final concentration of 1 μM. The cell/CFSE mixture was incubated at 37°C for 20 min and protected from light. The reaction was stopped by incubating the cell/CFSE mixture with 5 mL of culture medium containing 1% v/v of FBS for 5 min. The CFSE-labeled cells were collected by centrifugation and resuspended in fresh pre-warmed complete culture medium. The CFSE-labeled K562 or CL6 cells were used as the targets (T) while unlabeled CIK cells or NAC-treated CIK cells served as the effectors (E). The effector and target cells were mixed in complete culture medium at different E:T ratios of 100:1, 50:1, 25:1, 12.5:1, 6.25:1 in triplicate, with 15,000 target cells added per well. The target cells were incubated alone to measure spontaneous cell death. The mixed cells were incubated at 37°C for 4 or 24 h in a 96-well plate in a final volume of 200 μl per well in a humidified incubator at 37°C with 5% CO_2. As CL6 are adherent cells, for the co-cultured cells using these target cells, Trypsin–EDTA solution was used to detach the cells and the cells were then resuspended in PBS with 1% FBS and 0.025% (W/V) Trypsin–EDTA. For K562, the co-cultured cells were resuspended in PBS containing 1% of FBS. To identify dead cells, the co-cultured cells were stained with propidium iodide (PI) (Miltenyi Biotech, Germany) according to the manufacturer's instructions. The percentage of cells with $CFSE^+PI^+$ was determined as dead target cells. The percentage of cytotoxicity was calculated as cytotoxicity (%) = (dead target cells in the sample (%) – spontaneously dead target cells (%)) / (100 – spontaneously dead target cells (%)).[8]

Statistical analysis

Student's t-test was used for comparisons of the number of cells, percentage of protein markers in the flow cytometry analysis, and the gene expression level in the real-time PCR. Two-way ANOVA was used to analyze the significance of the differences between the means of the cytotoxicity experiments. The differences were considered as significant for $p < 0.05$(*), $p < 0.01$(**), $p < 0.001$(***), and $p < 0.0001$(****). The results are presented as the mean ± standard error of the mean (SEM).

Results

NAC did not affect the proliferation, but improved the differentiation of CIK cells

NAC has been reported to increase the proliferation of human T cells under certain conditions (0.4–3.1 mM) *in vitro* but to inhibit the proliferation at high concentrations (> 12.5 mM).[9] Hence, we found that 10 mM or less of NAC in the CIK culture did not alter the total number of CIK cells (**Figure 2A**). Thus, 10 mM NAC was tested for its ability to enhance CIK cell cytotoxicity. For the proliferation of control CIK cells and CIK cells treated with NAC, we counted cells at day 7 and 14, and there was no significant difference in fold change of the total cell number between these two groups (**Figure 2A**). Furthermore, the cell viability was checked by trypan blue exclusion test at day 0, 7 and 14. The viability of two groups of cells were more than 80%, and there was no significant difference in cell viability between groups (Data not shown).

Furthermore, as the major effector cells of CIK cells are $CD3^+CD56^+$ cells,[10] the proportion of the $CD3^+CD56^+$ subset after NAC treatment significantly increased from 28.44 ± 0.13 to 45.73 ± 1.26, as shown in **Figures 2B and 2C**.

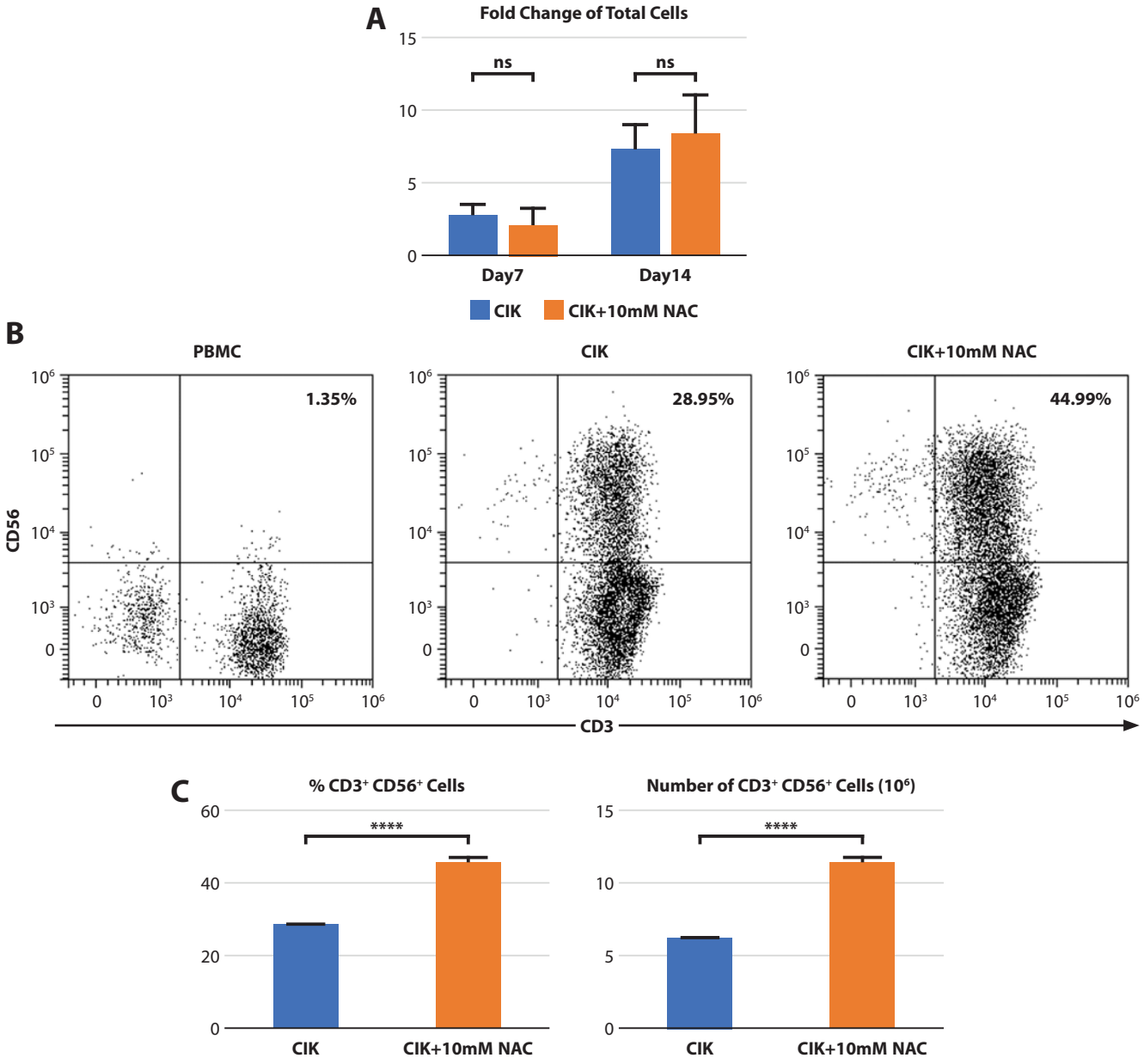

Figure 2. Quantification and specific surface marker analysis of CIK cells

A) Fold change of the total cell numbers between the control CIK cells and CIK cells treated with NAC. B) Percentages of the CIK cell specific surface markers $CD3^+CD56^+$ shown in dot plots. C) Histogram showing the percentages and the absolute number of $CD3^+CD56^+$ in CIK cultured with and without NAC. The data shown are representative of four independent experiments; $^{****}p < 0.0001$.

The expansion of CIK cells in the presence of NAC increased the expression of IFNG, PRF1, and GZMB

As the cytotoxic activity of CIK cells mainly depends on the production of IFN-γ and cytolytic effector proteins, including perforin and granzyme B, the acquisition of the cytotoxic phenotypes of CIK cells after treatment with NAC was examined by monitoring the relative gene expressions of IFN-γ, perforin, and granzyme B using real-time PCR. After NAC treatment, it was found that the levels of *IFNG*, *PRF1*, and *GZMB* expression were enhanced by approximately 9.6-, 4.4-, and 1.8-fold, respectively (**Figure 3A**). Thus, the expression levels of these genes encoding cytotoxic molecules in CIK cells treated with NAC were significantly increased, indicating an enhancement in the cytotoxicity of CIK cells with NAC treatment. In addition to gene expression, the productions of IFN-γ, perforin and granzyme B were measured by way of intracellular FACS staining (**Figure 3B**). The percentages of IFN-γ^+ and perforin$^+$ cells tended to increase upon NAC treatment. These FACS results show a relative similar tendency to those of the gene expression levels. Moreover, the absolute number of $CD3^+$ cells, in the bulk CIK cell culture, expressing IFN-γ, perforin and granzyme B was significantly increased in CIK cells pretreated with NAC in comparison to CIK control (**Figure 3C**).

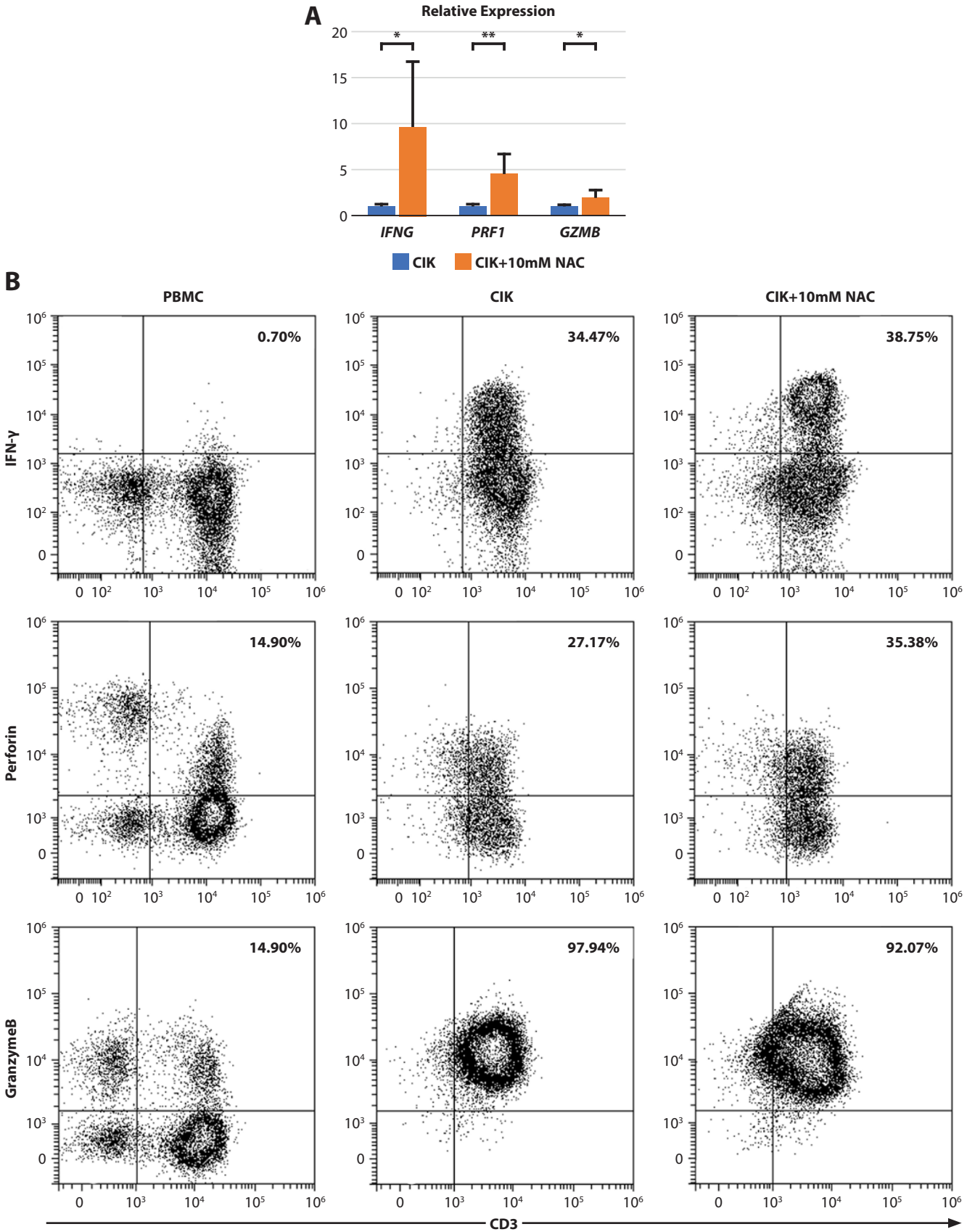

Figure 3. Expression of intracellular cytokines and cytolytic granules of CIK cells

A) Relative mRNA expression (fold change) values of *IFNG*, *PRF1*, and *GZMB* between the control CIK and NAC-activated CIK cells using *GAPDH* as the endogenous control. The relative fold changes were obtained using the $2^{-\Delta\Delta CT}$ method. B) FACS analysis of CD3 with IFN-γ, perforin, and granzyme B. The data shown are representative of four independent experiments. Statistical significance was indicated as $^{*}p < 0.05$ and $^{**}p < 0.01$.

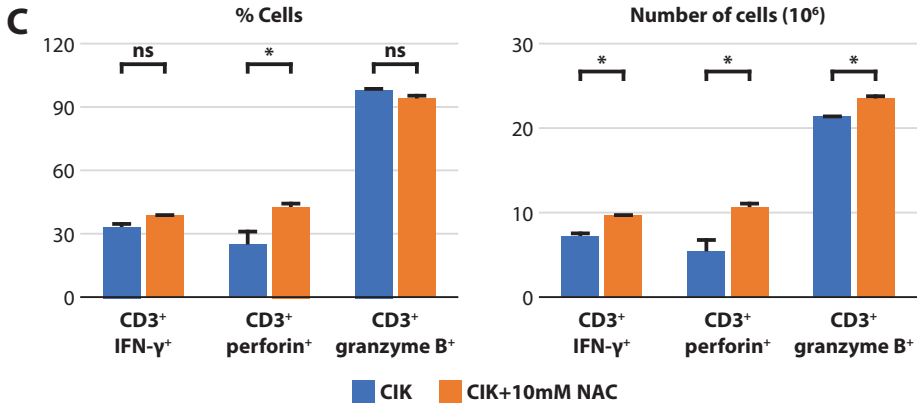

Figure 3. (Continued)
C) Histogram showing the percentages and the absolute number of $CD3^+IFN\text{-}\gamma^+$, $CD3^+perforin^+$ and $CD3^+granzyme\ B^+$ in CIK cultured with and without NAC. The data shown are representative of four independent experiments. Statistical significance was indicated as $^*p < 0.05$ and $^{**}p < 0.01$.

Culturing CIK cells with NAC enhanced their cytotoxicity against the cancer cell lines

To evaluate the tumor killing of CIK cells, the CFSE-labeled CL6 and K562 were used as target cells, as described in **Figure 4A**. The gating strategy was shown in a supplementary figure (**Figure S1**). The percentage cytotoxicity obtained from the co-culture between CIK and the target cells demonstrated that the CIK cells could kill CL6 and K562 cells in an E:T ratio- and time-dependent manner. As for the CIK cells pretreated with NAC, the effector cells showed a significantly higher percentage of cytotoxicity against both CL6 and K562 cells in comparison to the control. As shown in **Figure 4B**, for the cytotoxicity assay using CL6 cells as the target cells, the CIK cells and NAC-activated CIK cells effectively killed the target cells in an E:T ratio- and time-dependent manner. The maximum percentage cytotoxicity was shown in an E:T ratio of 25:1 in 24 h when NAC-activated CIK cells were used as effector cells. On the other hand, the ability of the control CIK cells to kill the K562 cells did not increase with increasing the E:T ratio. However, this ability could be enhanced by pretreating CIK cells with NAC, as shown in the result that the percentage cytotoxicity significantly increased approximately 2- and 3- fold at E:T ratios of 25:1 and 100:1, respectively, for 4 h co-culture, and approximately 4-fold at an E:T ratio of 100:1 for 24 h co-culture. Thus, the cytotoxicity assay results confirmed that the cytotoxic activity of CIK cells was improved by culturing CIK cells with NAC.

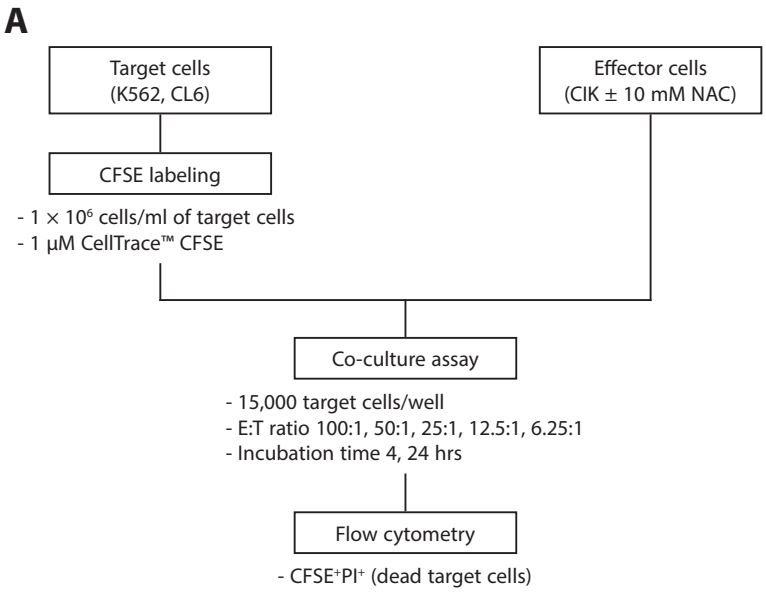

Figure 4. Cytotoxicity analysis of the CIK cells
A) Schematic representation of the cytotoxicity assay. The cytotoxicity of the control and NAC-treated CIK cells toward CL6 and K562, labeled with CFSE, was evaluated at various effector: target (E:T) ratios. The $CFSE^+PI^+$ dead target cells were detected by FACS analysis at 4 and 24 h co-culture.

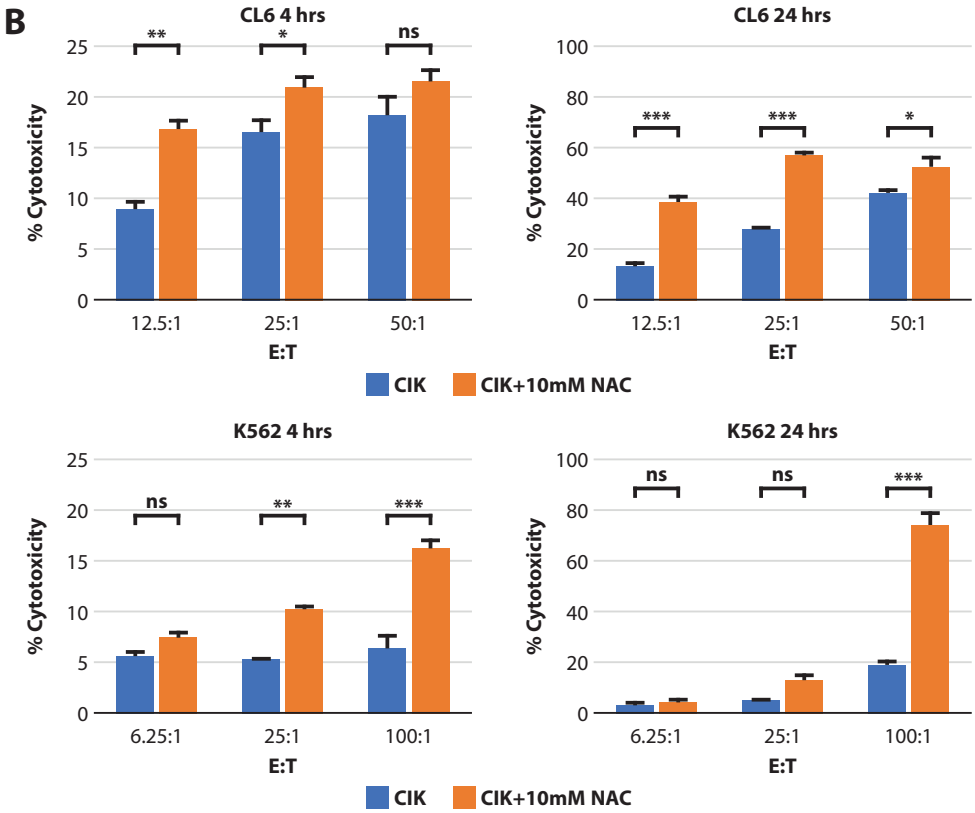

Figure 4. (Continued)
B) E:T-ratio-dependent cytotoxicity of the control and NAC-treated CIK cells against CL6 and K562 cells, at ratios of 12.5:1, 25:1, 50:1 and 6.25:1, 25:1, 100:1, respectively, at 4 and 24 h. The histogram represents the mean ± SEM. The data shown are representative of four independent experiments; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Discussion

Our study clearly demonstrated that the incorporation of NAC into CIK culture could markedly increase the cytotoxic phenotypes and tumor-killing activity of CIK cells. The *ex vivo* expanded CIK cultures in the presence of NAC has significant higher percentage of $CD3^+CD56^+$ when compare with CIK cultures alone (**Figure 2B-C**). Furthermore, NAC induced the expression level of *IFNG*, *PRF1*, *GZMB* as shown in the real-time PCR (**Figure 3A**). Similar result can be observed in the increasing of numbers of cells expressing cytolytic proteins, $CD3^+IFN\text{-}\gamma^+$, $CD3^+$perforin$^+$ and $CD3^+$granzyme B^+ in the bulk CIK cells pretreated with NAC, (**Figure 3C**). These subpopulations of CIK cells reflects cytotoxic activity of CIK cells. To affirm it, we investigated the antitumor activity of CIK cells against K562 and CL6 cell lines in the presence and absence of NAC. We found that the incorporation of NAC into CIK culture can markedly increase the tumor-killing activity of CIK cells as shown in **Figure 4B**. The percentage cytotoxicity against both CL6 and K562 significantly increased in comparison to the control.

The molecular pathway of NAC on CIK cytokine production is still unclear. Previous study demonstrated that NAC significantly up-regulated IFN-γ, IL-1β, IL-5, IL-12, and IL-12p40, and significantly down-regulated IL-10 production by PBMC after cellular stimulation.[12] Further study showed that treatment with NAC induced a significantly enhancement in NK-cell cytotoxicity activity.[13] The antioxidant activity exerted by NAC on thiol groups can also enhanced NK-cell-mediated cytotoxic binding and killing.[14,15] Furthermore, the promoting thiol expression and antioxidant gene by NAC can lead $CD8^+$ T cells persist longer in vivo and exerted superior tumor control.[16] Based on previous studies and our study, the mechanism underlying the CIK cytotoxic-enhancing activity by NAC is possibly due to the upregulating the key cytolytic proteins, IFN-γ, perforin and granzyme B.

Cytotoxicity activity of CIK cells against tumor cells include 2 signaling pathways, the binding of NK-cell receptors to their ligands, which are expressed on tumor cells and the induction of tumor cell apoptosis by Fas ligand via the Fas signaling pathway.[11,17] In term of the antitumor activity on different tumor targets, the molecular pathway that involves in tumor recognition is still not clearly identified. Previous studies demonstrated that cell surface adhesion molecules such as LFA-1 has a crucial role in binding and cytolytic activity of CIK cells,[18] and the cytotoxicity against BJAB were strongly inhibited by anti-LFA-1 mAb, while binding and cytotoxicity against KARPAS422 were slightly affect.[19] Therefore, even similar type of tumor cell line (BJAB and KARPAS422 which are human B lymphoma cell lines) can displayed different sensitivities to CIK-mediated cytotoxicity. Apart from LFA-1, CIK cells also express activating NK receptor, including DNAM-1, NKG2D and NKp30. Cell signaling through these receptors leads to activation of CIK and resulting in cytokine secretion

and cytotoxicity. By blocking of DNAM-1, lysis of MOLT4 cells (T-cell leukemia) was inhibited, whereas this blocking had no effect on RAJI cells (B-cell leukemia).[19]

Based on these previous findings, we can hypothesize that the possible mechanism underlines the different in susceptibility of K562 and CL6 to CIK cells and CIK cells pretreated with NAC (**Figure 4B**) may due to the expression of major ligand involving in target cell recognition by CIK cells. NAC should be further studied regarding its effect on the induction of CIK-mediated lysis of tumor targets through specific ligands. Given that NAC has long been used and proven to be a very safe medication, we propose that NAC should be added to CIK cell culture to generate potent CIK cells for the effective immunotherapy of cancers. Further studies are required to confirm the molecular mechanism by which NAC enhances the cytotoxic cytokine production and anti-tumor activity of CIK cells.

Acknowledgements

We are grateful to Prof. Suradej Hongeng at the Department of Pediatrics, Ramathibodi Hospital, Mahidol University for his helpful advice. We appreciatively thank Panwadee Pluangnooch and Chaipichit Phayangkhe for their isolation of PBMC. This research was supported by Siriraj Foundation (D-003658) and the National Science and Technology Development Agency (FDA-CO-2559-3325TH).

Availability of the Data and Materials

All the data supporting the findings of this study are available from the authors upon reasonable request.

Ethics Approval

The study protocol was approved by the Institutional Review Board of the Faculty of Medicine Siriraj Hospital (SIRB), Mahidol University (COA no. Si 606/2018).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Author Contributions

- KS designed the experiments.
- PE and CC performed the experiments.
- PE analyzed the data. PE and KS wrote the manuscript.
- All the authors reviewed and approved the final version of the manuscript.

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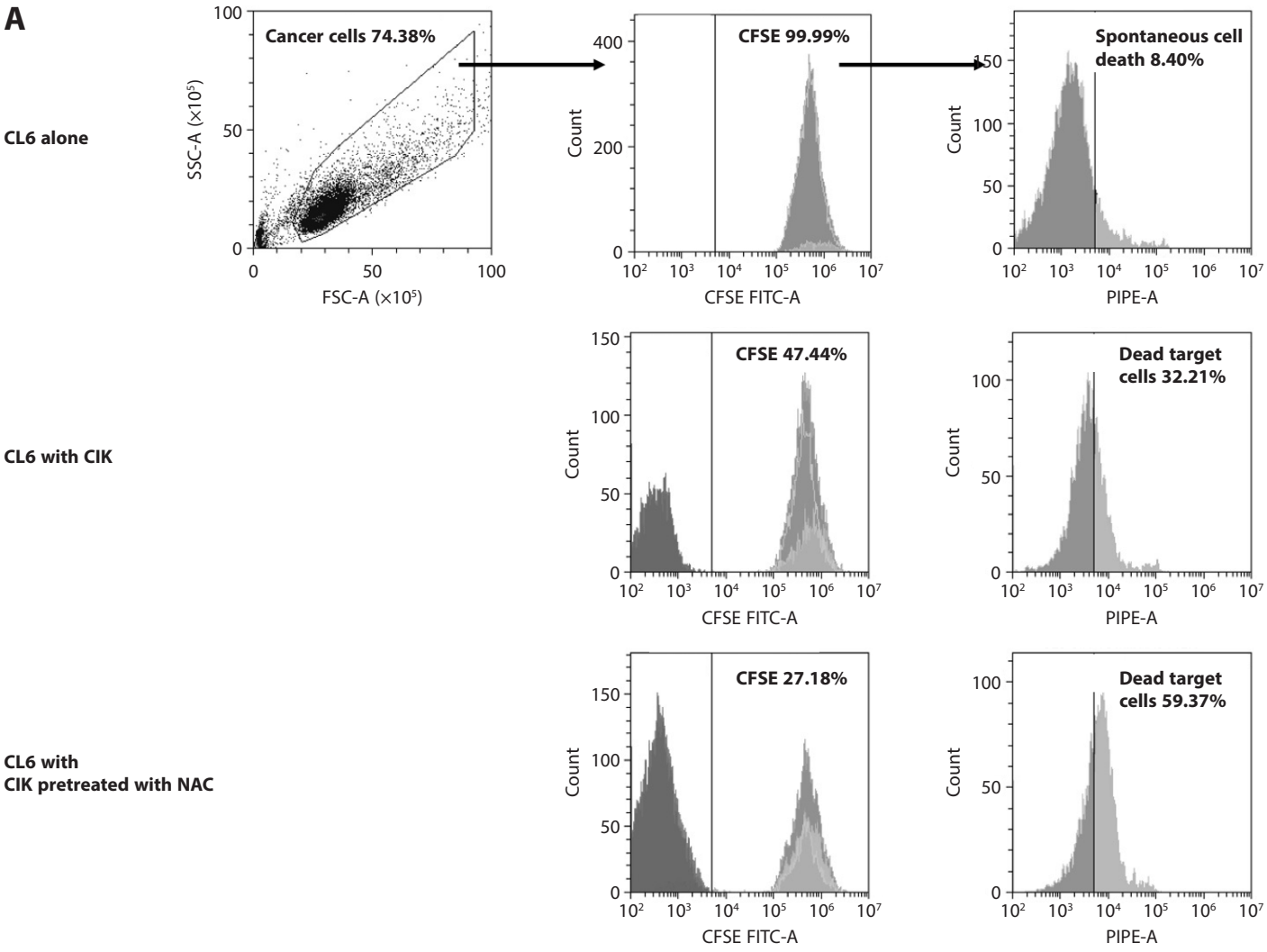
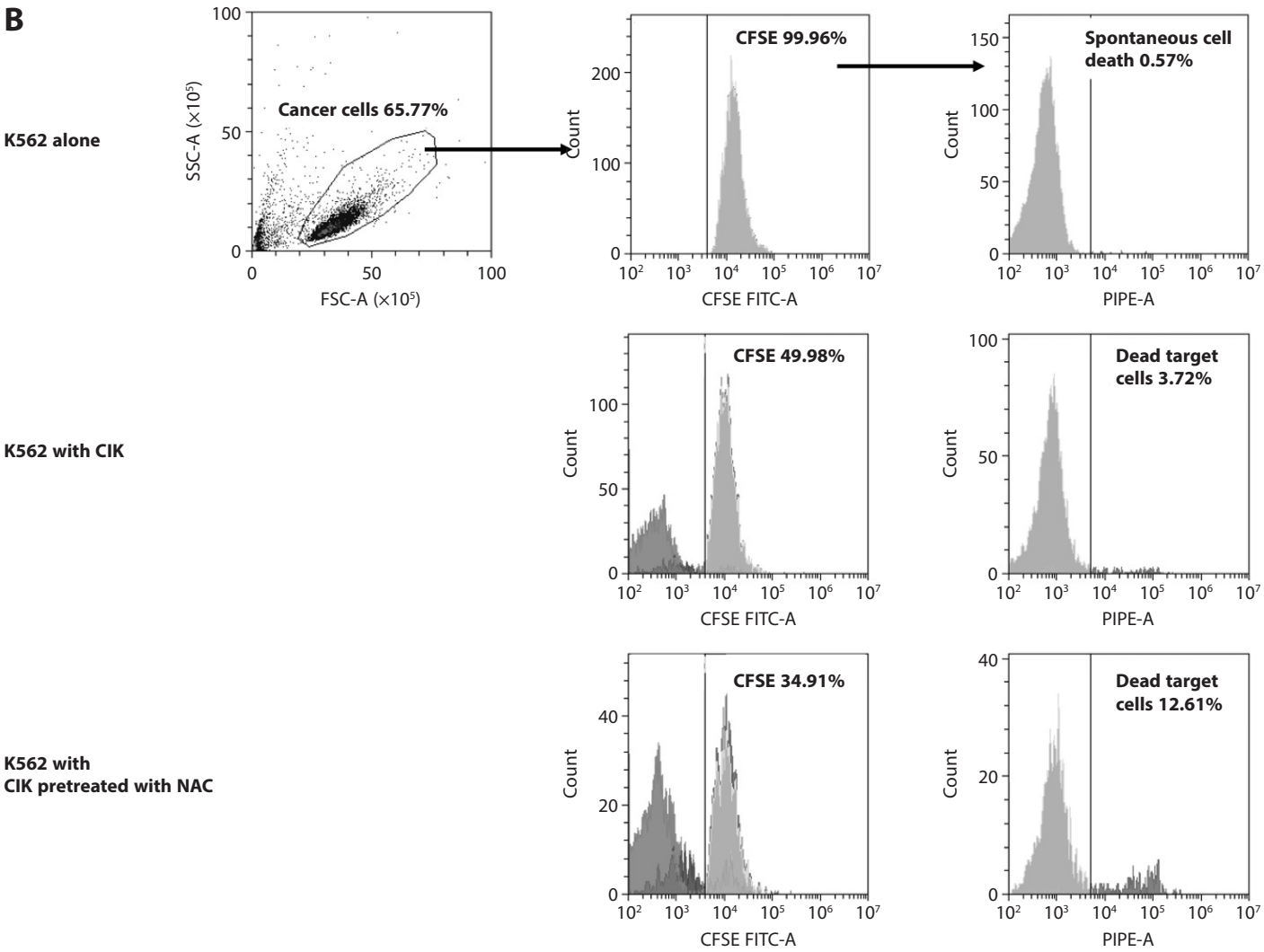
Supplemental material



Supplementary Figure 1. Gating strategy for the co-culture

In a first step CL6 (A) or K562 (B) were selected in a forward scatter channel plot (FSC) vs. sideward scatter channel plot (SSC). CL6 or K562 cells were displayed in histogram of CFSE. $CFSE^+$ were selected and then displayed in histogram of PI. The percentage of cells with $CFSE^+PI^+$ was determined as dead target cells. The percentage of spontaneous cell death of cancer cell culture alone was used as control.



Supplementary Figure 1. (Continued)