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Immunomodulatory Effects of Modified Colostrum, Whey, and Their Combination With Other Natural Products: Effects on Natural Killer Cells

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ABSTRACT

Objectives: Natural killer (NK) cells are important immune system effector cells providing innate defenses against intracellular infections, including viral infections, immune surveillance, and cancer immunoediting. The primary purpose of this study was to investigate whether modified ultra-filtrated colostrum (UC) and hydrolyzed whey (W) products or their combinations with other natural products with reported immunomodulatory properties will stimulate NK cell cytotoxic activity by activation of granzyme B and IFN-γ production.
Methods: The ability of study products to stimulate the cytotoxic activity of human-purified CD56$^+$ NK cells and the production of granzyme B and IFN-γ by activated NK cells was evaluated in the cytotoxic assay.
Results: All study products significantly increased NK-cell cytotoxic activity at an E: T ratio of 20:1. Treatment of cells with UC had a maximal cytotoxic effect at the minimal dose of 10 µg/ml, which exceeded the cytotoxic activity of IL-2. In contrast, the addition of egg yolk (CE) or CE + botanical blend (CEB) to UC resulted in a dose-dependent cytotoxic response with a maximal response at 1000 µg/ml. The maximal activity of blend products was comparable to UC activity. W exerted minimal stimulatory activity on NK cells. The magnitude of granzyme B and IFN-γ production was closely associated with the cytotoxic activity of NK cells stimulated with the study products.
Conclusions: All study product**s** demonstrated stimulatory activity on NK cells, with UC having a maximal effect on NK cell cytotoxicity. The study products can be used as dietary supplements to support NK cell activity in healthy individuals.

Introduction

In the past 2–3 decades, multiple natural products, including medicinal herbs and vitamins, have been investigated for their ability to modulate immune functions.[1,2] For example, berberine isolated from *Coptidis* rhizome suppressed the production of inflammatory cytokines and improved gut cell wall integrity, reducing the leakage of bacterial toxins into the systemic circulation.[3] Geniposide from *Gardenia jasminoides* prevents neutrophil migration to the colon mucosa and inflammatory cytokine production.[3] Ginsenosides modulated the activity of splenic T cells and restored circulating plasma cytokine levels.[4]

It has also been reported that multiple medicinal herbs (e.g., curcumin, garlic extract, and ginseng extract) and vitamins (group B vitamins, vitamin C, and D3) activate natural killer (NK) cells.[5]

Abbreviations: CE, Colostrum + egg yolk; CEB, Colostrum+ egg yolk+ botanical blend; IL, interleukin; INF, interféron; NK cells, Natural Killer cells; NKT cells, Natural Killer T cells; UC, ultra-filtered colostrum.

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NK cells are a subset of innate lymphoid cells capable of distinguishing normal cells from cells in distress (e.g., virus-infected and malignant cells).[6,7] Similar to phagocytic cells, they can be found in circulation and the tissues. NK cells play an essential role in immune surveillance and cancer immunoediting by identifying and eliminating cancer cells.[8] Unlike T cells, NK cells do not require antigenic priming and can control metastasis by "putting metastatic cells into a dormant state through the production of IFN-γ".[7] NK cells also shape anti-tumor immune responses by promoting the development of conventional dendritic cells at the tumor site and tumor-specific T cells.[7]

Additionally, NK cells are actively involved in anti-viral immune responses.[9] Acute viral infections with flavivirus and influenza virus can activate circulating NK cells and stimulate their migration to the site of infection. NK cells play a critical role in controlling tick-borne encephalitis virus, yellow fever virus, SARS-CoV-2, and influenza virus.

NK cells can be activated by intracellular and extracellular bacteria, including *Listeria monocytogenes, Francisella tularensis,* and *Chlamydia pneumoniae*.[10] Activated NK cells are capable of lysing bacteria-infected cells and producing cytokines (e.g., IFN-γ, GM-CSF, TNF-α, IL-6, IL-10, IL-17, and IL-22).[10] During bacterial infection, NK cells stimulate other immune cell anti-microbial activity (e.g., dendritic cells and macrophages). The proper function of NK cells is critical for supporting immune health, including anti-tumor and anti-viral immune responses.

We have previously reported that pretreatment of previously frozen PBMCs with bovine colostrum, the first meal of newborns in mammals, enhanced NK cells' ability.[11] Notably, the killing ability of NK cells depended upon colostrum composition, dose, and time of their exposure to colostrum. The PBMCs demonstrated the maximal cytotoxic activity 48 h post-colostrum treatment.[11] It has also been shown that ultra-filtered bovine colostrum (UC) and hydrolyzed whey (W) increased NK cell activity and the production of IL-2, IFN-γ, and TNF-α after oral administration in mice.[12–14] Additionally, the UC ingredient has been shown to have a reasonable safety profile as a dietary ingredient.[15]

Despite many studies on individual bioactive molecules isolated from natural products or extracts from a plant, studies of their combinations are limited. Nowadays, the vast majority of marketed dietary supplements are multi-component proprietary blends. Therefore, it is critical to investigate the immunomodulatory properties of multi-component natural products.

The study presented herein aims to evaluate the effect of UC and W alone or in combination with chicken egg yolk (CE) or egg yolk and proprietary botanical blend (CEB) on purified NK cell cytotoxic activity and granzyme B and IFN-γ production. The products used in this study were selected based on the reported immunomodulatory properties. UC stimulated proinflammatory cytokine production by LPS-activated PBMCs and suppressed their production when PBMCs are activated by phytohemagglutinin (PHA).[16] Fermented Whey (W) demonstrated modulatory effects on innate and adaptive immune responses, specifically anti-inflammatory properties.[17] All mushroom species used in this study also demonstrated immunomodulatory properties.[18] Mushroom beta-glucans activated the complement system and enhanced NK cell and macrophage activity.[19] Beta-glucans isolated from oats and barley enhanced the anti-cancer activities of immune cells by reducing PD-L1 expression and modulating dendritic cells, T cells, and NK cell activities.[20] Ultrafiltrates from plants with molecular weight 3-30 kDa enhance the cytotoxic activity of peripheral blood mononuclear cells (PBMCs).[21]

Materials and Methods

Compounds used in the study

The study products and their bioactive components, brand names, and product lots used in the study are listed in Table 1. All products were supplied by 4Life Research USA (Sandy, UT). Ultra-filtered colostrum (UC) was prepared by defatting whole bovine colostrum and ultra-filtering to concentrate the peptides and proteins with molecular sizes smaller than 10,000 Daltons. Chicken egg yolk extract was prepared by spray-drying the egg yolk. The bioactive components were extracted by water from the mycelia or fruiting body of the mushrooms.

The combined products of colostrum, egg yolk extract, and botanical blend were composed of ultrafiltered colostrum and egg yolk extract (>20%), soy phytosterols (>20%), inositol hexaphosphate (>15%), lemon peel powder (<5%), *Cordyceps sinensis* mycelia extract standardized to adenosine ≥0.28% and D-Mannitol ≥8.0% (>5%), *Grifola frondosa* fruiting body extract standardized to Proteoglycan 10.0% (<5%), *Lentinus edodes* fruiting body extract standardized to polysaccharides 10.0% (<5%), *Agaricus blazeii* fruiting body extract standardized to 40% polysaccharides (<5%), baker's yeast standardized to 3% polyphenols (<5%), aloe vera (*Aloe barbadensis*) leaf gel (<5%), oat seed extract standardized to 22% beta-glucan (<5%), and olive (*Olea europae*) leaf (<5%). Whey was hydrolyzed and enzymatically digested into bioactive peptides (>90% peptides and proteins).

Stock solutions of the study products (10 mg/mL) were prepared daily before the experiment. The products were diluted in PBS and sonicated in a 37°C water bath to dissolve them thoroughly. The product containing ultra-filtered and nano-filtered bovine colostrum, chicken egg yolk, and the botanical blend were centrifuged to remove undissolved particulates.

Reagents and cell line

Myelogenous leukemia cell line K562 was purchased from the Shanghai Institutes for Biological Sciences (SIBS, Shanghai, China). Passage 11 of K562 cells were used in these experiments. The following reagents were used in the study: anti-human CD3-FITC, anti-human CD56-APC, and 7-Aminoactinomycin D (7AAD) (Bi-

Table 1
Investigated products.

	Ingredient(s)	Brand name	Lot number
Ultra-filtered colostrum (UC)	Ultra-filtered bovine colostrum and maltodextrin	Transfer Factor Classic	B2025806
Colostrum + egg (CE)	Ultra-filtered and nano-filtered bovine colostrum, chicken egg yolk, maltodextrin, and silicon dioxide	Transfer Factor Tri-Factor	20050132
Colostrum + egg + botanical (CEB)	Ultra-filtered and nano-filtered bovine colostrum, chicken egg yolk, zinc methionine, inositol hexaphosphate, soy phytosterols, lemon peel powder, and the extracts of *Grifola frondosa*, *Lentinus edodes*, *Cordyceps sinensis*, *Agaricus blazeii*, baker's yeast, aloe gel, oat seed, and olive leaf	Transfer Factor Plus Tri-Factor	AA11302020-02
Fermented whey (W)	Enzyme-cleaved sweet whey	Whey Factor	2558321

olegend, San Diego, CA), Fixable Viability Dye eFluor™ 780 and carboxyfluorescein succinimidyl ester (CFSE), 123count eBeads™ Counting Beads (eBioScience – Fisher Scientific, San Diego, CA), fetal bovine serum (FBS) was Fisher Scientific (San Diego, CA), human CD56 MicroBeads, FcR-blocking antibodies (Miltenyi Biotec, San Diego, CA), IC fixation buffer (Invitrogen - Life Technologies), Quantikine® ELISA, Human Granzyme B Immunoassay, and Quantikine® ELISA, Human IFN-γ Immunoassay (R&D, Minneapolis, MN).

NK cell enrichment from PBMCs

Human peripheral blood mononuclear cells (PBMCs) were isolated from the blood donated by a healthy donor. The donor provided written informed consent before participation in the study. An equal volume of the whole blood was layered over Ficoll-Plaque™ Plus (GE Healthcare Life Sciences) and centrifuged at $400 \times g$ for 30 min. A translucent layer between Ficoll and serum was collected. Collected PBMCs were washed 2–3 times with PBS.

The supernatants were aspirated, and the cell pellet was resuspended in 80 µl of PBS + 3% FBS (running buffer) per 10^7 cells. Human CD56 microbeads were added at 20 µL microbeads per 10^7 cells. Cells were mixed by brief vertexing and incubated for 15 min at 4–6°C. Cells were washed by adding 2 ml running buffer per 10^7 cells and centrifuged at $300 \times g$ for 10 min. The supernatant was aspirated, and the cell pellets were resuspended in 500 µl of running buffer. The cell suspension was passed through a 70 µm nylon mesh filter before cell separation. $CD56^+$ cells were isolated using LS columns and a manual magnetic cell separator (Miltenyi).

The purity of isolated $CD56^+$ cell fraction was evaluated by flow cytometry. Enriched cells (1×10^6) were incubated with 20 µL of human FcR-blocking antibodies at 4°C for 15 min. Anti-human CD3-FITC, anti-human CD56-APC, and Live/dead- APC-eFluor780 were added to the sample. The sample was incubated at 4°C for 30 min in the dark. Cells were washed 3 times by adding 2 ml of Wash Buffer (PBS+2% FBS) and centrifugation at $400 \times g$ for 5 min. The cell pellet was resuspended in a 200 µL IC fixation buffer. Cells were incubated in the dark for 30 min at room temperature. Then, cells were washed twice by adding 2 mL wash buffer and centrifugation. Next, cells were resuspended in 250 µL PBS and analyzed by cytometry using Kaluza 2.2 software. Fifty thousand total cells were acquired. Prior to enrichment, PBMC contained 26.19% of $CD3^-CD56^+$ NK cells and 3.87% of $CD3^+$ $CD56^+$

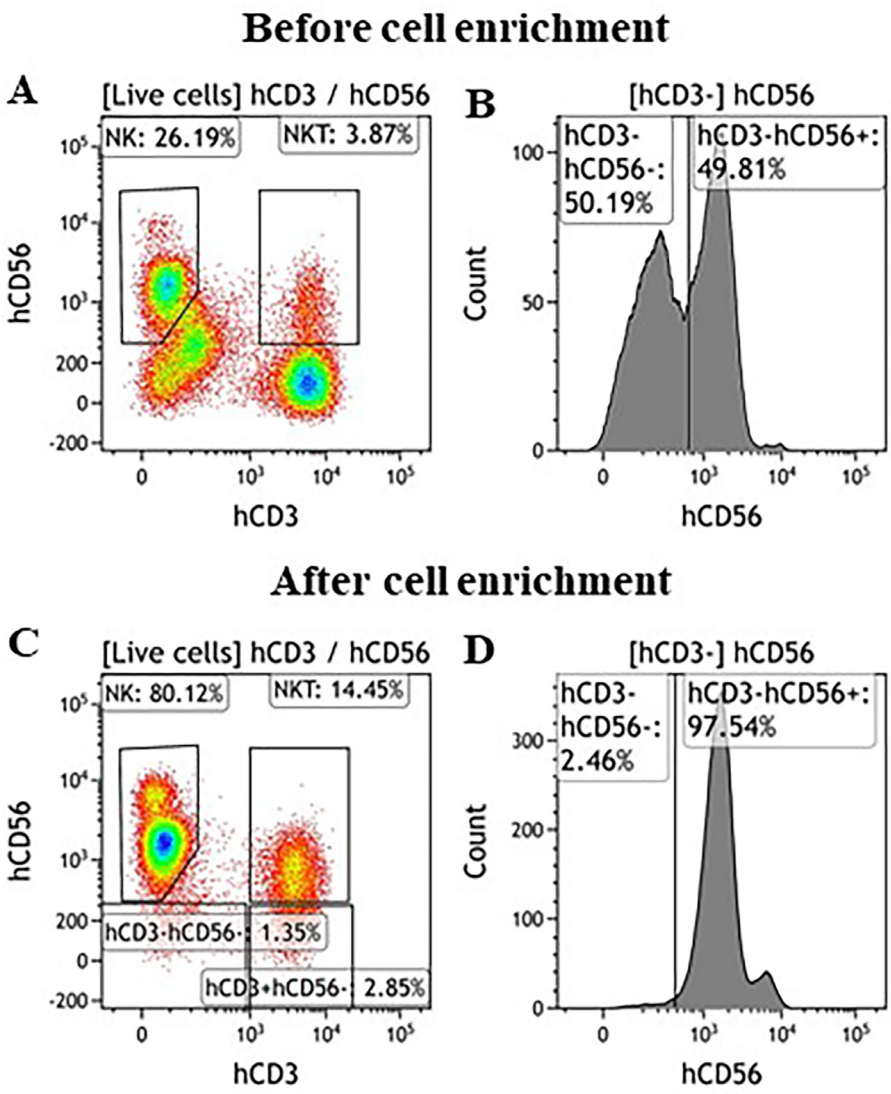

Figure 1. NK cell composition before and after enrichment with Miltenyi microbeads. A and B—percentages of $CD3^-CD56^+$ NK cells and $CD3^+$ $CD56^+$ NK T cells in PBMCs before enrichment; C and D—percentages of $CD3^-CD56^+$ NK cells and $CD3^+$ $CD56^+$ NK T cells after enrichment with $CD56^+$ microbeads.

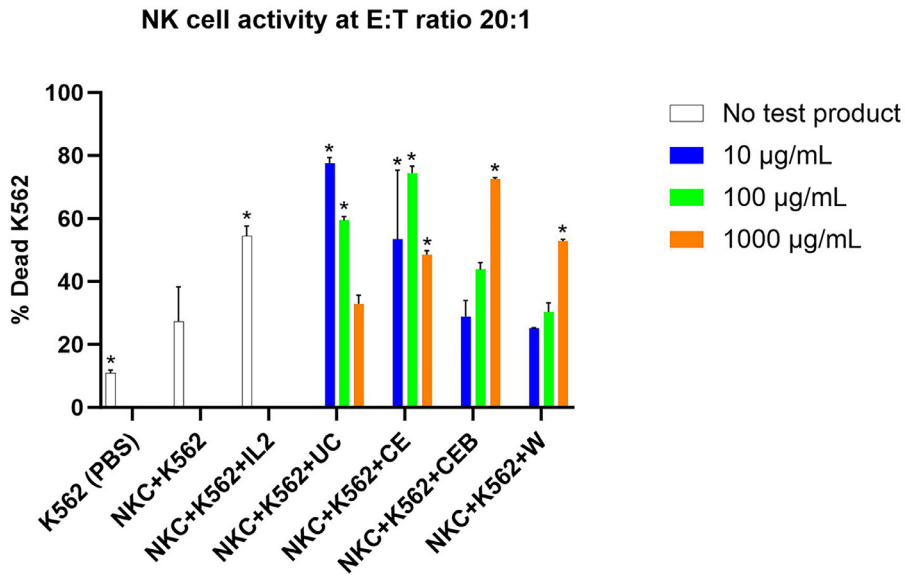

Figure 2. NK cell activity at an E:T ratio of 20:1. NK cells were cultured with K562 cells at an E:T ratio of 20:1. Plates were incubated for 48 h. The experiment was repeated twice with similar results. Data are presented as mean dead K562 cells + standard error of the mean (SEM) of duplicate testing. *Groups that are statistically different from control K562 + NK cells ($P < 0.05$).

NK T cells (Figure 1A and B). It has been determined that the enriched cell sample contained 80.12% of $CD56^+$ NK cells and 14.45% of $CD56^+CD3^+$ NK T cells (Figure 1C and D).

NK cell cytotoxicity assay

Freshly collected K562-target cells were labeled with 2.5 µM of CFSE. Cells were incubated for 5 min at 37°C in the dark, followed by adding 4–5 volumes of RPMI-1640 containing 20% of FBS to quench the labeling reaction. Then, cells were washed three times with complete media (RPMI-1640 plus 10% FBS). CFSE-labeled K562 target cells were co-incubated with the effector NK cells at 2:1 and 20:1 effector:target (E:T) ratios in a round-bottom 96-well plate in duplicated at 37°C, 5% CO_2 in a humidified incubator. The following controls were used in this study: K562 target cells only, K562 cells plus $CD56^+$ NK cells, and K562 cells plus $CD56^+$ NK cells plus IL-2 (50 U/mL). The study products were added to respective wells at 10, 100, and 1000 µg/mL. Plates were incubated for 48 h. Subsequently, 150 µL/well of supernatant was collected and stored at -80ºC for further analysis.

The remaining cells were washed twice with ice-cold PBS and stained 7AAD added at room temperature for 10 min in the dark. Cells were resuspended in 300 µL of PBS. Then 100 µL of 123 count eBeads™ were added to each sample. One tube containing only staining buffer and 100 µL of eBeads™ and one sample containing stained cells and no counting beads were used to calibrate the flow cytometer. Samples were analyzed by the flow cytometer using Kaluza software. Fifty thousand total cells were acquired.

The following equation was used for the calculation of absolute counts:

$$\frac{Absolute\,Count}{(cells/\mu L)} = \frac{(Cell\ Count \times eBead\ Volume)}{(eBead\ Count \times Cell\ Volume)} \times \frac{eBead}{Concentration}$$

Granzyme B and IFN-γ levels analysis

Cell supernatants collected in the NK cell cytotoxicity assay were removed from the freezer, thawed, and centrifuged to remove any cell debris. Granzyme B and IFN-γ were analyzed according to the manufacturer's recommendations. The optimal dilution of the samples was determined in the pilot experiment.

Statistical analysis

Data were analyzed in GraphPad Prism 9.2 software (La Jolla, CA) by two-way ANOVA. Dunnett's multiple comparison tests compared all treatment groups to the negative control, $K562^+$NK cells.

Results

NK cell cytotoxic activity

The addition of IL-2 to K562 cells + NK cells culture (positive control) at an effector:target (E:T) ratio of 20:1 increased NK cell cytotoxic activity 2.0-fold compared to the control (K562 cells + NK cells, Figure 1). All study products significantly increased NK-cell cytotoxic activity at an E: T ratio of 20:1 (Figure 2 and Figure 3). However, these products did not considerably affect NK-cell cytotoxic activity at an E: T ratio of 2:1 (data not shown).

Ultra-filtered colostrum (UC) increased NK-cell cytotoxicity 2.9-fold at a concentration of 10 µg/mL ($P < 0.0001$), 2.2-fold at 100 µg/mL ($P = 0.0003$), and 1.2-fold at 1000 µg/mL (difference was not statistically significant) compared to the control. UC's maximal stimulating effect exceeded the positive control effect (K562 cells + NK cells + IL-2) 1.4 times. The high dose of UC (1000 µg/mL) showed a stimulatory effect on NK-cell activity comparable to positive control. UC showed an inverse dose-response on NK-cell activity (Figure 2).

Ultra-filtered colostrum + egg (CE) was significant at all tested concentrations and had a bell-shaped dose-response relationship. The CE treatment increased NK-cell cytotoxic activity at 10 µg/mL 2.0-fold ($P = 0.0045$), 100 µg/mL 2.7-fold ($P < 0.0001$), and 1000 µg/mL 1.8-fold ($P = 0.032$) compared to the control (Figure 2).

Ultra-filtered colostrum + egg + botanicals (CEB) significantly increased NK cell cytotoxic activity at the highest dose (1000 µg/mL, $P < 0.0001$) (Figure 2). The stimulatory effect of CEB in smaller doses on NK cells was not statistically significant but presented a clear dose-response relationship (Figure 2). Similarly to CEB, only the highest dose of hydrolyzed whey (W, 1000 µg/mL) significantly increased the cytotoxic activity of NK cells ($P = 0.0059$). Treatment of cells with W at smaller doses also increased the activity of NK cells, but the differences were not statistically significant.

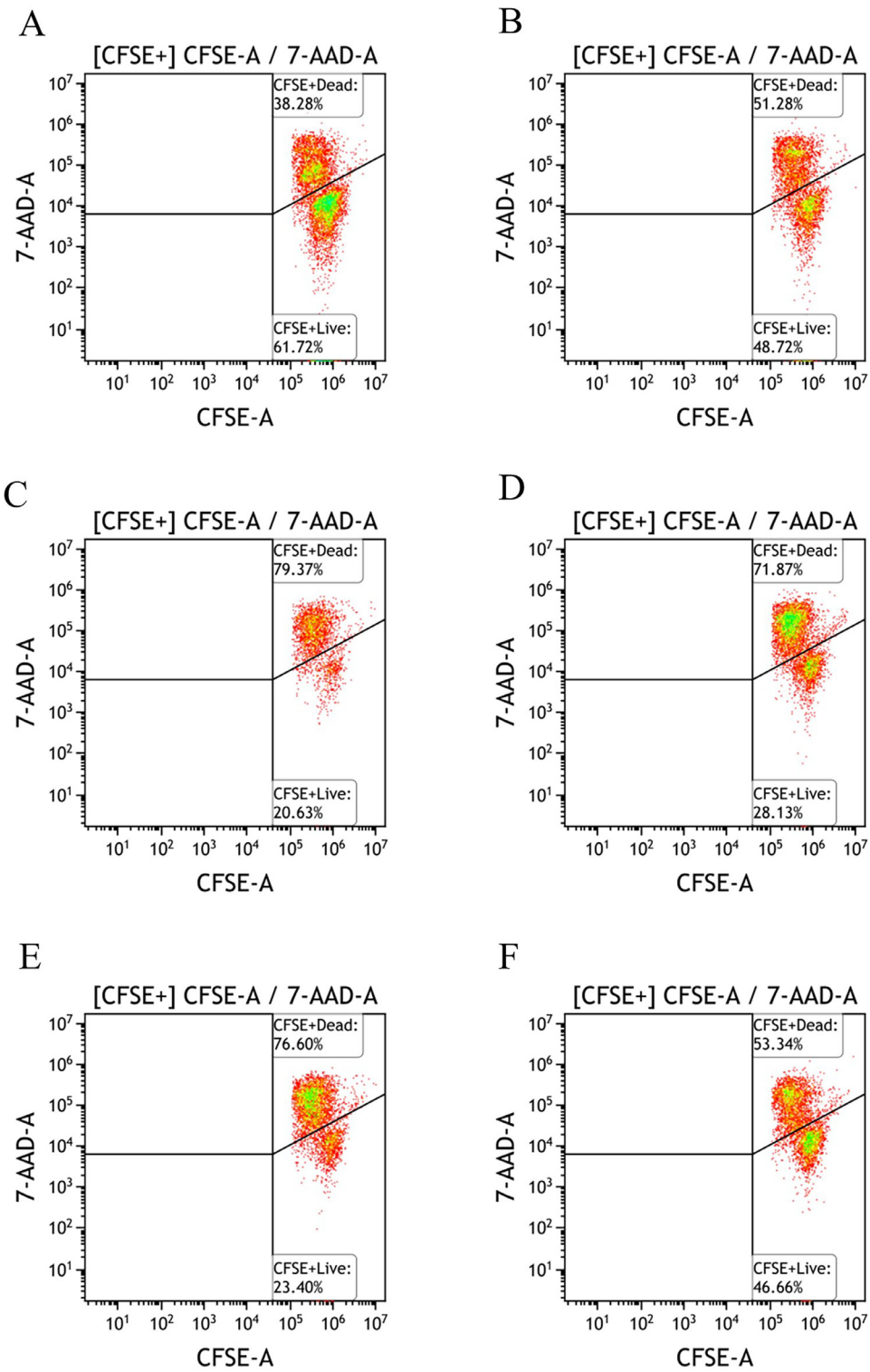

Figure 3. Representative images of NK cell cytotoxic activity. NK cells were cultured with K562 cells at an E:T ratio of 20:1. Plates were incubated for 48 h. A-NK cells + K563, B-NK cells + K563 + IL-2, C-NK cells + K563 + UC 10 μg/ml, D-NK cells + K563 + UC 1000 μg/ml, E-NK cells + K563 + CEB100 μg/ml, F- NK cells + K563 + W 1000 μg/ml.

Granzyme B and IFN-γ production by activated NK cells

The magnitude of granzyme B and IFN-γ production was closely associated with the cytotoxic activity of NK cells stimulated with the study products.

As presented in Figure 4, IFN-γ production was highest after treatment with low (10 μg/mL) and medium (100 μg/mL) doses of UC and similarly to NK-cell cytotoxic activity had an inverse dose-response pattern ($P < 0.0001$). Notably, the IFN-γ production was 10.8-fold higher following treatment with 10 μg/ml of UC and 7.4-fold following treatment with 100 μg/ml of UC compared to the positive control (K562 + NK cells + IL-2). CE increased IFN-γ release at all tested concentrations (CE 10 μg/mL $P = 0.0139$,100 μg/mL and 1000 μg/mL $P < 0.0001$), CEB ($P < 0.001$) and W ($P = 0.0070$) only increased IFN-γ secretion at the highest dose (1000 μg/mL).

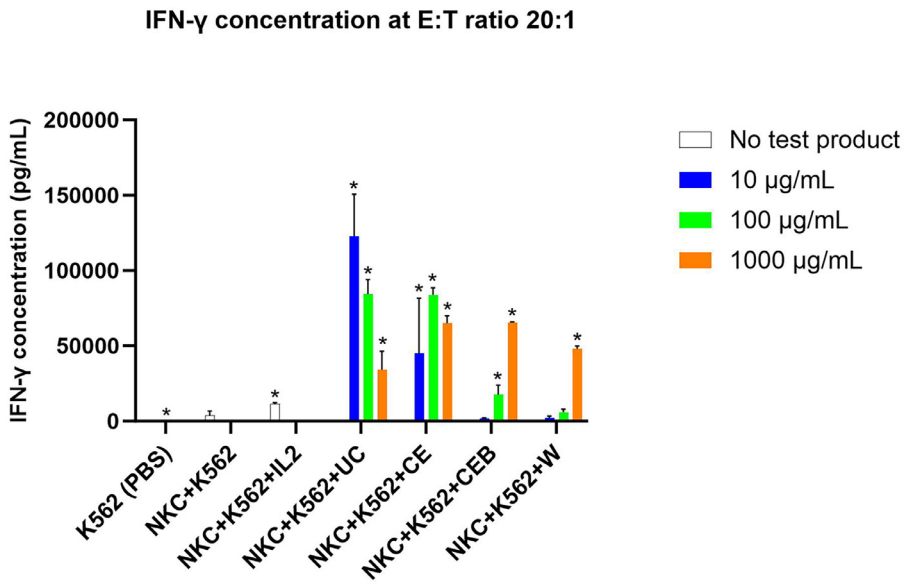

Figure 4. IFN-γ concentrations in supernatants. NK cells were cultured with K562 cells at an E:T ratio of 20:1. Plates were incubated for 48 h. Subsequently, 150 µL/well of supernatant was collected and stored at -80ºC for further analysis. The experiment was repeated twice with similar results. Data are presented as the mean ± standard error of the mean (SEM) of duplicate testing. *Groups statistically different from control K562 cells + NK cells ($P < 0.05$).

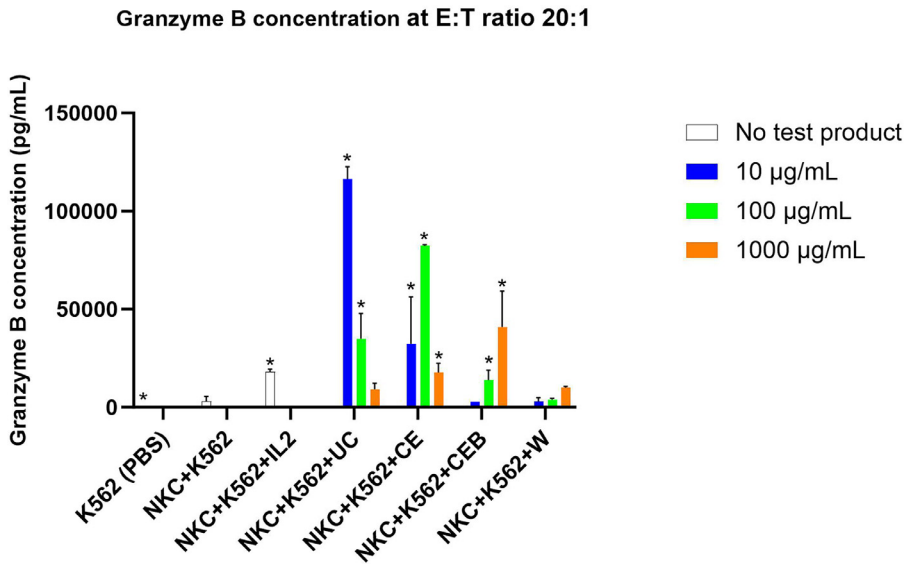

Figure 5. Granzyme B concentrations in the supernatants. NK cells were co-cultured with K562 cells at an E:T ratio of 20:1. Plates were incubated for 48 h. Subsequently, 150 µL/well of supernatant was collected and stored at -80ºC for further analysis. The experiment was repeated twice with similar results. Data are expressed as the mean ± standard error of the mean (SEM) of duplicate testing. *Groups statistically different from control K562 cells + NK cells ($P < 0.05$).

Similarly, granzyme B production increased following cell treatments with 10 µg/mL and 100 µg/mL of UC ($P < 0.0001$ and $P = 0.0046$, respectively) (Figure 5). The treatment with low (10 µg/mL, $P = 0.0105$) and medium (100mg/mL, $P < 0.0\,001$) doses of CE also increased granzyme B release. Finally, ultra-filtered colostrum + egg + botanicals only increased granzyme B production at a high concentration ($P = 0.0006$). Although there was a trend, granzyme B production was insignificant at any hydrolyzed whey concentration.

Discussion

NK cells are critical players in innate immune defenses. The primary functions of NK cells are the elimination of intracellular infections, immune surveillance, and cancer immunoediting.[8,22,23] Several viral infections (e.g., influenza virus and human immunodeficiency virus) could dysregulate NK cell functions, leading to a decreased cytotoxic activity of these cells.[24] Viral infections may lead to NK cell apoptosis.[24] Malignant cells produce inhibitory molecules suppressing the ability of NK cells to eliminate cancer cells via the reduction of their cytotoxic activities and capacity to produce inflammatory cytokines.[25] In addition, some studies suggest impaired NK cell functions may allow malignant cells to proliferate actively.[26]

Considering the importance of NK cell functions in supporting individuals' immune health, there is an urgent need to develop compounds supporting NK cell functions. Recently, NK cells have gained particular attention as immunotherapy for various malig-

nancies.[27] For example, NK cells can be genetically modified to express specific chimeric antigen receptors (CAR), recognizing, for instance, Her2 antigen in breast cancer cells.[27]

Our study has shown that NK cells treated with UC and its combinations with chicken egg yolk and botanical blend significantly activate human NK cell functions, as evidenced by an increased percentage of killed target cells. Interestingly, NK cell treatment with increasing doses of UC had an inverse effect on NK cell activity. This can be explained by the fact that colostrum stimulates IL-2 production and NK cells' cytotoxic activity at low concentrations and suppresses its production at high concentrations.[28] The addition of recombinant IL-2 restored the suppression of cytotoxic activity.

However, the addition of egg yolk and botanicals to UC reduced the production of IFN-γ and granzyme B, suggesting that other mechanisms of target cell killing may be involved. Potentially, CE or CEB-treated NK cells may exert their cytotoxic activity by inducing receptor-mediated apoptosis via the expression of the Fas ligand, a homotrimeric type II transmembrane protein, or TNF-related apoptosis-inducing ligand (TRAIL).[29] Another potential explanation may be the limited solubility of CE and CEB products.

In 2015, Gong and colleagues presented an assay developed for screening the tumoricidal activity of natural products.[30] Our study cannot be compared directly to the study by Gong et al. for several reasons. Still, both protocols can be used to screen natural products effectively for their ability to activate NK cells. The main goal of Gong's study was to identify natural compounds stimulating the tumoricidal activity of NK cells. Investigators used A549 lung adenocarcinoma cells as target cells and expanded in vitro NK cells.[30] Our study aimed to investigate the effects of several natural compounds on NK cell activity. We used leukemic cell line K562, commonly used as target cells, to evaluate the effects of various compounds on NK cells, not only the tumoricidal activity of NK cells. Like Gong's study, we used IFN-γ secreted by NK cells to assess their activation status.

The human NK cells in this study contained $CD56^+$ NK cells and $CD56^+CD3^+$ NKT cells. NKT cells are a small population of innate/adaptive cells expressing CD3 T cell receptors.[31] Glycolipids usually activate NKT cells from, for example, *Mycobacterium bovis* and *Borrelia burgdorferi* via glycolipid presentation through T cell receptors. Like classic NK cells, upon activation through the CD3 receptor, these cells produce inflammatory cytokines (TNF-α and IFN-γ). Additionally, NKT cells can produce anti-inflammatory cytokines like IL-4, IL-10, and transforming factor-beta.[31] It is unclear whether NKT cells contribute to cytotoxic activity in our study since UC preparation was defatted.

An analysis of 502 natural products for their effects on NK cytotoxic/tumoricidal activity revealed that only seven products exceeded the stimulatory action of IL-2 (positive control).[30] The stimulatory activity of UC and other tested products in the presented experiments surpassed positive control 1.1–1.4 times, suggesting that these products have a high potential to improve NK cell functions.

The number of obese people is growing exponentially in developed countries and worldwide. It is well-established that obese people are predisposed to viral infections and may more often develop cancer.[26] The number of NK cells, the ability to release cytotoxic granules, and the ability to produce inflammatory mediators like IFN-γ were reduced in severely obese people.[26] NK cell activity also declines with age. Although the number of cytotoxic NK cells seems to be higher in older adults,[32] a reduction in the killing capacity of infected cells by NK cells has been observed.[33] A study of healthy young and older adults revealed an age-associated decline in NK cell-killing capacity and the ability to secrete chemokines.[34] Dietary interventions with products like colostrum can potentially improve NK cell functions in such populations and decrease their susceptibility to bacterial and viral infections.

Conclusions

Ultra-filtered colostrum alone displayed the highest immune stimulatory activity on NK cells, while hydrolyzed whey had minimal activity on NK cell cytotoxic functions. The addition of egg yolk and botanicals did not further stimulate the cytotoxic activity of NK cells, potentially due to product solubility. Ultra-filtered colostrum and other study products can be used as dietary supplements in healthy individuals during flu and common cold seasons and in groups with decreased NK cell activities, such as older adults and those with obesity, to support their immune health and NK cell activity.

Declaration of competing interest

CS is an Editor-in-Chief, and EE is a member of the Editorial Board of the Current Therapeutic Research journal. All other authors declare no conflict of interest.

Author Contributions

DV, XH, CS, and EE—contributed to the experimental design; XY, YZ, MZ, CZ—conducted experiment; XH, DV, XY, YZ, MZ, CZ—formal data analysis and curation; DV, XH, CS, EE—contributed to the conception, design, and preparation of the manuscript. DV, XH, CS, XY, YZ, MZ, CZ, and EE—contributed substantially to drafting the manuscript and revising it critically for valuable intellectual content. All authors have read and agreed to the published version of the manuscript.

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