

Original Article

COMBINATIONS OF INTERLEUKIN 2/15/18 AND INTERLEUKIN 2/21 INCREASED EXPANSION AND CYTOTOXICITY OF UMBILICAL CORD BLOOD- DERIVED NATURAL KILLER CELLS AGAINST LEUKAEMIA

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ABSTRACT

The use of cytokines to expand natural killer (NK) cells offers both promising opportunities and notable challenges in the field of immunotherapy. The selection and optimisation of cytokines are critical to achieving efficient expansion of NK cells. Therefore, this study aimed to evaluate different cytokine combinations for the expansion and activation of cord blood-derived NK cells. Briefly, various interleukins (IL) combinations were tested: Group A (100 IU/mL IL-2 only), Group B: (100 IU/mL IL-2 and 100 IU/mL IL-15), Group C (100 IU/mL IL-2, 100 IU/mL IL-15 and 20 ng/mL IL-18) and Group D (100 IU/mL IL-2, 10 ng/mL IL-21). The NK cells were subsequently characterised by flow cytometry for CD56^{dim/bright} and CD16^{high/lo} subpopulations, as well as for their activating and inhibitory receptors. The cytotoxic effect of the different NK cell groups towards luciferase-expressing leukaemia cell line (Nalm6) and the secretion levels of tumour necrosis factor alpha (TNF- α) and interferon gamma (IFN- γ) were also assessed. NK cells treated with various combinations of cytokines demonstrated a higher proliferation rate and increased expression of CD56⁺CD16⁺ compared to the untreated (UT) group. Further subpopulation analysis revealed that groups B, C and D promoted the expansion of both cytokine-producing CD56^{bright} and cytotoxic CD56^{dim} NK cell subsets. Notably, only groups C and D exhibited significantly elevated levels of TNF- α and IFN- γ secretion. These groups also showed higher specific lysis (%) of Nalm6 cells ($p < 0.0001$). The combination of IL-2 with either IL-15 and IL-18 or IL-21 synergistically enhanced the expansion, activation, and cytotoxic function of NK cells, highlighting their potential application in immunotherapeutic strategies.

KEYWORDS: cord blood, natural killer cell, cancer immunotherapy, cytokine, interleukin

INTRODUCTION

In recent years, the critical role of the immune system in combating cancer has led to significant advancements in immunotherapy, including the adoptive transfer of immune cells, such as natural killer (NK) cells and chimeric antigen receptor (CAR)-modified T cells (1,2). The observed anti-tumour activity of NK cells in patients with acute myeloid leukaemia following allogeneic stem cell transplantation (allo-SCT) served as a pivotal trigger for the initiation of clinical trials exploring NK cells as a novel immunotherapeutic approach (3).

NK cells are a subset of lymphocytes that comprise approximately 10-15% of circulating peripheral blood lymphocytes. Unlike T and B cells, NK cells do not express any antigen-specific receptors. Instead, their mechanism of action is regulated by a finely tuned balance between activating and inhibitory receptors, such as the NK Group 2D (NKG2D), the human killer cell immunoglobulin-like receptors (KIRs), CD94-NKG2 heterodimers and natural cytotoxicity receptors (NCR) (4). These receptors recognise stress-induced ligands on target cells and initiate NK cell-mediated immune responses.

In the field of cancer research, NK cells cultured in interleukin-2 (IL-2) have demonstrated promising potential for use in immunotherapy (5). IL-2 is one of the earliest cytokines employed in this context due to its potent ability to stimulate NK cell proliferation, cytotoxicity, and cytokine production, particularly interferon gamma (IFN- γ). Various other modifications can be applied to NK cells before infusion, such as pre-treatment with specific cytokine(s), genetically enhanced expression of cytotoxic protein(s), adaptation from exposure to cancer cell lines, CAR or functionalization as a drug delivery vehicle (6). These modifications enable NK cells to exert a robust cytotoxic effect and effectively eliminate the circulating cancer cells.

Recent research has increasingly focused on cord blood-derived NK cells (CB-NK) due to their specific advantages over peripheral blood NK cells (PB-NK). Notably, CB-NK cells uniquely express CXCR4, a chemokine receptor that is responsible for leukocyte trafficking and immunoreactivity (7). Owing to their nascent origin, CB-NK cells exhibit a lower risk of graft-versus-host disease (GvHD) as compared to PB-NK, thereby supporting their application in allogeneic transplant settings. Additionally, frozen cord blood units deposited in public or private cord blood banks provide a readily available, off-the-shelf source for NK cell immunotherapy. Despite these advantages, several challenges remain in the development of NK-cell-based therapies, including optimal donor selection criteria, standardisation of cell culture protocols, and the scalability of manufacturing processes (8).

The ex vivo expansion and activation of NK cells commonly involve the use of specific cytokines that mimic physiological signals and enhance functional competence (9). While IL-2 is well-established in this context, the roles of other interleukins such as IL-15, IL-18 or IL-21 have yet to be thoroughly investigated. To our knowledge, there have been few experimentally-controlled studies of these cytokines in combination. IL-15 is a critical homeostatic cytokine for NK cell survival and maturation; it promotes sustained proliferation and supports memory-like NK phenotypes with less induction of regulatory T cells compared to IL-2 (5,10). IL-18, though not sufficient alone, acts synergistically with IL-12 or IL-15 to enhance IFN- γ secretion and prime NK cells for stronger cytotoxic responses, often mimicking inflammatory conditions (11,12). Meanwhile, IL-21 contributes to the differentiation and functional refinement of NK cells by increasing their cytolytic granule content and boosting antibody-dependent cellular cytotoxicity (ADCC), particularly in combination with other cytokines such as IL-15 (10,13). Together, these cytokines orchestrate a robust in vitro NK cell activation strategy that reflects both innate and adaptive-like properties, maximising their therapeutic potential. Optimising interleukin concentrations for NK cell expansion is challenging due to their dose-dependent effects

and potential for cellular exhaustion or unwanted differentiation. High levels of IL-2 may expand Tregs or induce apoptosis, while excessive IL-15 or IL-18 could cause overstimulation. IL-21 enhances NK cell function but may inhibit proliferation at high doses. Balancing these cytokines requires careful titration to maintain NK cell viability, expansion, and cytotoxicity.

This study aimed to isolate and characterise the NK cells isolated from cryopreserved mononuclear cells of umbilical cord blood. Thereafter, the cells were expanded in various combinations of cytokines and phenotypically assessed to determine the expression of activating and inhibitory receptors. Lastly, the cells underwent anti-tumour assessment at different doses against the leukaemia cell line. To our knowledge, this is the only study to compare several cytokine cocktails on CB-NK cells' expansion, activation, and anti-tumour efficacy.

MATERIALS AND METHODS

Ethics approval for cord blood samples

Ethical approval for the collection of human umbilical cord was obtained from the Medical Research and Ethical Committee (MREC, MOH) [NMRR-18-750-4121]. Twenty units (n = 20) of fresh cord blood were obtained from Hospital Tengku Ampuan Rahimah Klang (HTAR) with informed consent. The following shows different steps of generating NK cells from cord blood samples.

a) Isolation and cryopreservation of CB-MNCs

Cord blood mononuclear cells (CB-MNCs) were isolated via density gradient centrifugation using Ficoll-Paque™ PREMIUM (GE Healthcare Bio-Sciences AB, USA) to remove red blood cells and platelets. The resulting CB-MNCs were aliquoted into cryovials and stored under liquid nitrogen conditions. For subsequent use, the cryopreserved CB-MNCs were thawed and resuspended in a neutral buffer solution. NK cells were then purified from the CB-MNC population using the NK Cell Isolation Kit in combination with the MiniMACS™ Separator (Miltenyi Biotec, USA), following the manufacturer's instructions.

b) CB-NK cell expansion and activation

Purified CB-NK cells were cultured in 24-well plates at 37°C with 5% CO₂ containing NK MACS® Medium (Miltenyi Biotec, US) at a concentration of 5×10^5 cells/mL. The cultures were supplemented with 5% human serum (Sigma, US) and cytokine combination of either A: 100 IU/mL IL-2 (Miltenyi Biotec, US) only; B: 100 IU/mL IL-2 and 100 IU/mL IL-15 (Miltenyi Biotec, US); C: 100 IU/mL IL-2, 100 IU/mL IL-15 and 20 ng/mL IL-18 (MBL, Japan); D: 100 IU/mL IL-2 and 10 ng/mL IL-21 (Miltenyi Biotec, US). The concentration used for each cytokine was adapted based on previous studies (14). Cells were supplied with fresh medium supplemented with human serum and respective cytokine combinations every three days without the removal of pre-existing culture medium for 14 days.

c) CB-NK cell expansion and activation

Expanded CB-NK was harvested and stained with the directly-conjugated monoclonal antibodies (BD Biosciences, San Jose, CA; R&D Systems, Minnesota, US) as listed in supplementary Table 1. Flow cytometry was performed using a FACS Aria™ Fusion flow cytometer, analysing 5×10^5 viable cells, followed by data analysis using the BDFACS Diva software. 7-Aminoactinomycin D (7-AAD) was used to exclude non-viable cells. A group representing untreated NK cells (UT) was used as a comparator for both flow and cytokine secretion assays. The resulting subpopulations of CB-NK cells were categorised by either positively or negatively expressing CD56 and CD16 combinations (i.e. CD56⁺/CD16⁺). Double

positive CD56⁺/CD16⁺ subpopulation was further categorised by the level of CD56⁺ (bright or dim relatively) or CD16⁺ (high or low relatively) expressed to denote the functional subpopulation of CB-NK cells (15).

d) Cancer cell line

A target cell line was selected from the human B-cell precursor leukaemia cell lineage transduced with firefly luciferase: Nalm6-Fluc-Neo/eGFP-Puro (Imanix Life Sciences, Rochester, MN) was purchased for the cytokine secretion assay and bioluminescence cytotoxicity assay. The frozen cell line was thawed at 37°C and centrifuged at 200 × g for 3-5 minutes with 5 mL of complete growth media (RPMI-1640 medium containing 10 mM HEPES, 10% foetal bovine serum and 1% penicillin/streptomycin) (all from Gibco, Thermo Fisher Scientific Inc., USA). The cells were resuspended in a density of 1-3 × 10⁶ cells/mL and cultured at 37°C with 5% CO₂.

e) Cytokine secretion assay

CB-NK cells and Nalm6 cells were cocultured at a 1:1 ratio for 12 hours in a 37°C incubator with 5% CO₂. Supernatant was collected every hour and stored at -20°C until assay. The concentration of IFN-γ and tumour necrosis factor-α (TNF-α) was measured by ELISA using Human IFN-γ and TNF-α Mini TMB ELISA Development Kits (PeproTech, USA) and read by PerkinElmer EnVision Multimode Plate Reader according to the manufacturer's instructions (16).

f) Bioluminescence cytotoxicity assay

Luciferase-expressing Nalm6 cells were placed in 96-well round-bottom plates at a concentration of 3 × 10⁴ cells/100 μL in triplicate. Cells were given D-firefly luciferin potassium salt (150 μg/mL; Promega, US), and measured using the PerkinElmer EnVision Multimode Plate Reader for spontaneous apoptotic cell death through relative light units (RLU). Subsequently, CB-NK cells were added at a 1:1, 2:1, 5:1, 10:1 effector-to-target (E: T) ratio and incubated at 37°C for 12 hours. Data was collected every hour and recorded as test RLU. At the end of the experiment, cells were treated with Doxorubicin (Sigma, US) as a measure of maximal killing. Triplicate wells were averaged, and the percentage of specific lysis was calculated using the following equation (17,18):

$$\% \text{ Specific lysis} = \frac{(\text{spontaneous death RLU} - \text{test RLU})}{(\text{spontaneous death RLU} - \text{maximal killing RLU})} \times 100$$

Statistics

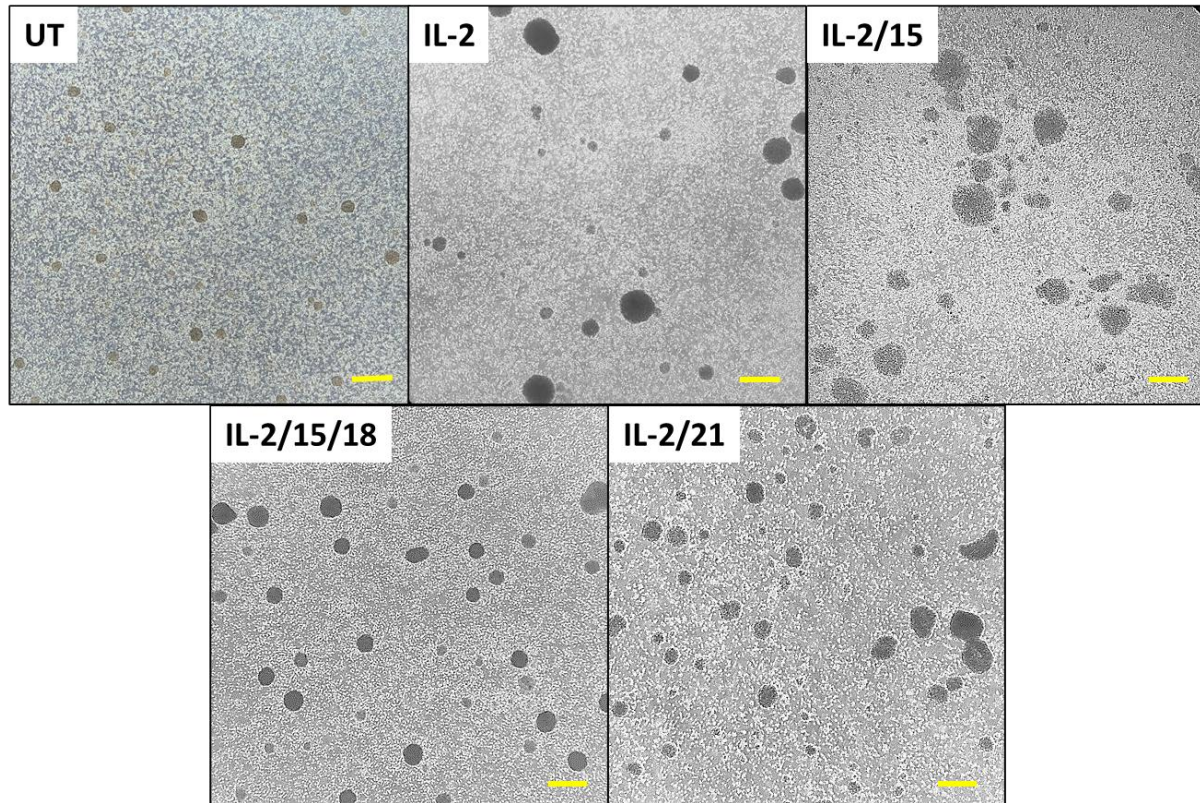
All graphs plotted and statistical analyses were performed via GraphPad software (GraphPad Prism v.8.4.3) (California, USA). Data was analysed using two-way analysis of variance (ANOVA). The significant differences between the groups were determined using Tukey's HSD post-hoc test. The data were presented as mean ± standard error mean (SEM) for technical replicates. Statistically significant differences were reported at (*) p<0.05, (**) 0.01, (***) 0.001 and (****) 0.0001.

RESULTS

a) NK cell expansion

To note, the following experiments were performed from CB-NK cells that were isolated from one unit of fresh cord blood with technical replicates. The average yield of purified NK cells was 1.9 × 10⁶, with a range of 0.8 × 10⁶ to 4.1 × 10⁶ cells. The mean fold-expansion of NK cells was 16.3 ± 6.6, 35.1 ± 5.9,

47.9 ± 9.7 and 23.8 ± 2.1 for cytokine combinations A, B, C and D, respectively. Supplementation of the culture with different growth factors, such as IL-15 and IL-18 or IL-21, resulted in the formation of larger NK cell aggregates, which is characteristic of actively proliferating immune cells (Figure 1).

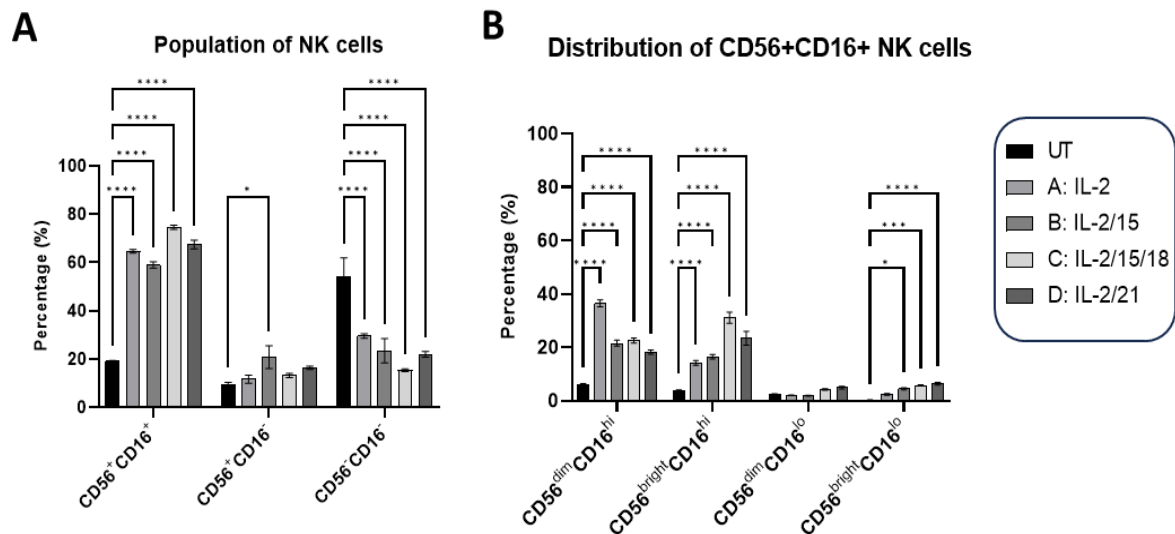


Images were captured at a magnification of 100 $\times$ with a scale bar (100 μ m).

Figure 1. Morphology of the NK cell population expanded after 14 days of culture. (UT) untreated; (A) IL-2 only; (B) IL-2 and IL-12; (C) IL-2, IL-15 and IL-18; or (D) IL-2 and IL-21.

b) Flow cytometric analysis of untreated versus expanded NK cells with various combinations of cytokines

To assess purity, the isolated cells were analysed by flow cytometry using anti-CD56, anti-CD3 or anti-CD19 for NK cells, T-cells and B-cells, respectively. NK cells were defined as CD56⁺/CD3⁻/CD19⁻ (data not shown). Overall, the purity of NK cells from each cytokine-treated group was higher compared to the UT group, as significantly shown in the CD56⁺CD16⁺ subpopulation in Figure 2(A). In addition, the population of CD56⁺CD16⁺ NK cells increased by ~3-fold ($p < 0.0001$) in all cytokine-treated groups (A-D) compared to the untreated (UT) group. Correspondingly, there was a significant reduction of non-NK cells, CD56⁻CD16⁻ ($p < 0.0001$) subpopulation in the treated groups. The CD56⁺CD16⁻ population remained relatively unchanged across all groups, except for group B, which showed a moderate increase ($p < 0.05$). Further analysis of the CD56⁺CD16⁺ subset was performed to identify the predominant population of 'functionally active' NK cells. As illustrated in Figure 2(B), cytokine treatment significantly increased CD56^{dim}CD16^{hi} ($p < 0.0001$) for all groups. A similar trend was observed for CD56^{bright}CD16^{hi} ($p < 0.0001$) for all groups. However, CD56^{bright}CD16^{lo} was significantly elevated in groups B ($p < 0.05$), C and D ($p < 0.0001$), but not in group A.

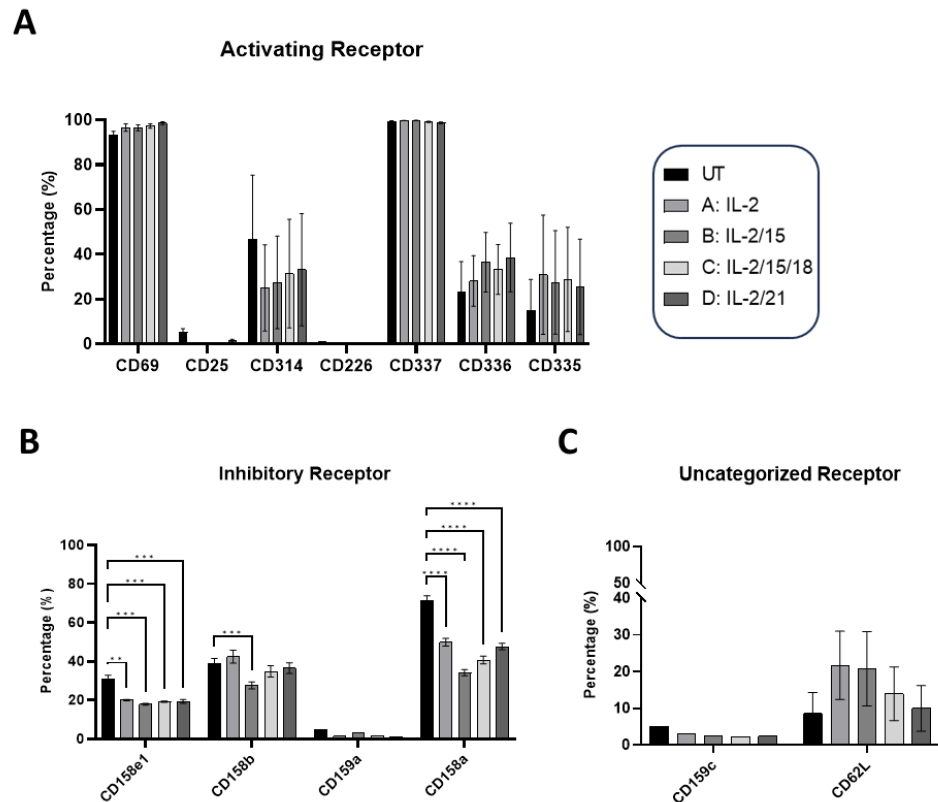


Asterisk denote significant differences (* $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$; Tukey's HSD critical value set at $\alpha = 0.05$).

Figure 2. (A) Distribution of NK cell subpopulation ($n = 5$, technical replicates) by CD56+/-CD16+/- expression and (B) further characterisation of CD56+CD16+ subpopulation by CD56bright/dimCD16hi/lo expression in different cytokine cocktails (A-D) and untreated control group (UT).

c) Effect of cytokine treatment on the expression of activating receptors as well as inhibitory receptors

As shown in Figure 3 (A), although there were no statistically significant differences, cytokine-treated NK cells exhibited notable changes of activating receptors, including CD69, CD25, CD314, CD226, CD337, CD336 and CD335. However, more pronounced alterations were observed in the expression of inhibitory markers, as presented in Figure 3 (B). Specifically, CD158e1 was downregulated by ~1.5 fold in groups A ($p < 0.05$), B, C and D ($p < 0.001$). No observable changes were detected for CD159a. In contrast, CD158a expression was markedly reduced in all cytokine-treated groups ($p < 0.0001$). The expression levels of CD159c and CD62L remained unchanged throughout the duration of the study (Figure 3C).

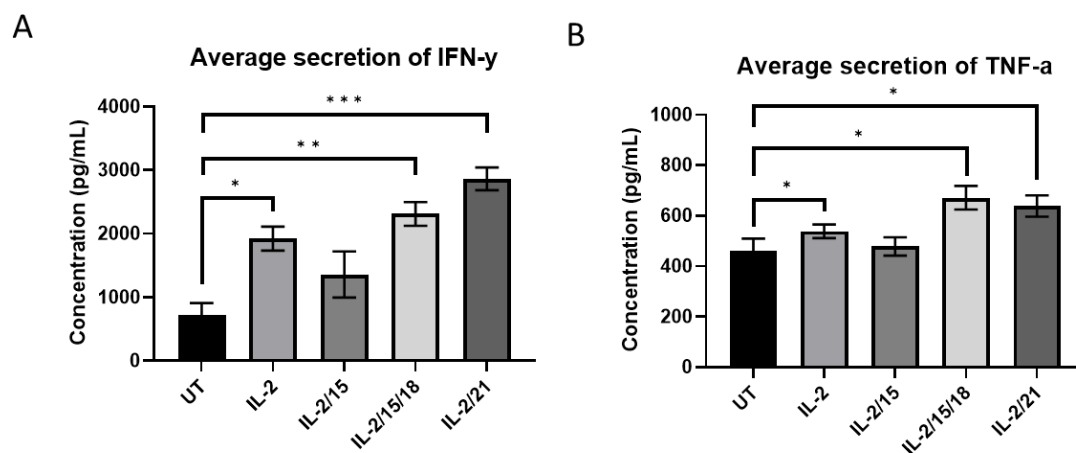


Asterisk denote significant differences (** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; Tukey's HSD critical value set at $\alpha = 0.05$).

Figure 3. Per cent of (A) activating receptors, (B) inhibitory receptors and (C) uncategorized receptors in CD56+CD16+ subpopulations of NK cells ($n = 2$, technical replicates) determined by flow cytometry.

d) Cytokine secretion assay

When cocultured with leukaemia cells for 12 hours, the CB-NK cells treated with combinations of IL-2, IL-15 and IL-18 (Group C) secreted the highest level of IFN- γ and TNF- α compared to UT or other cytokine combination groups, as illustrated in Figure 4.

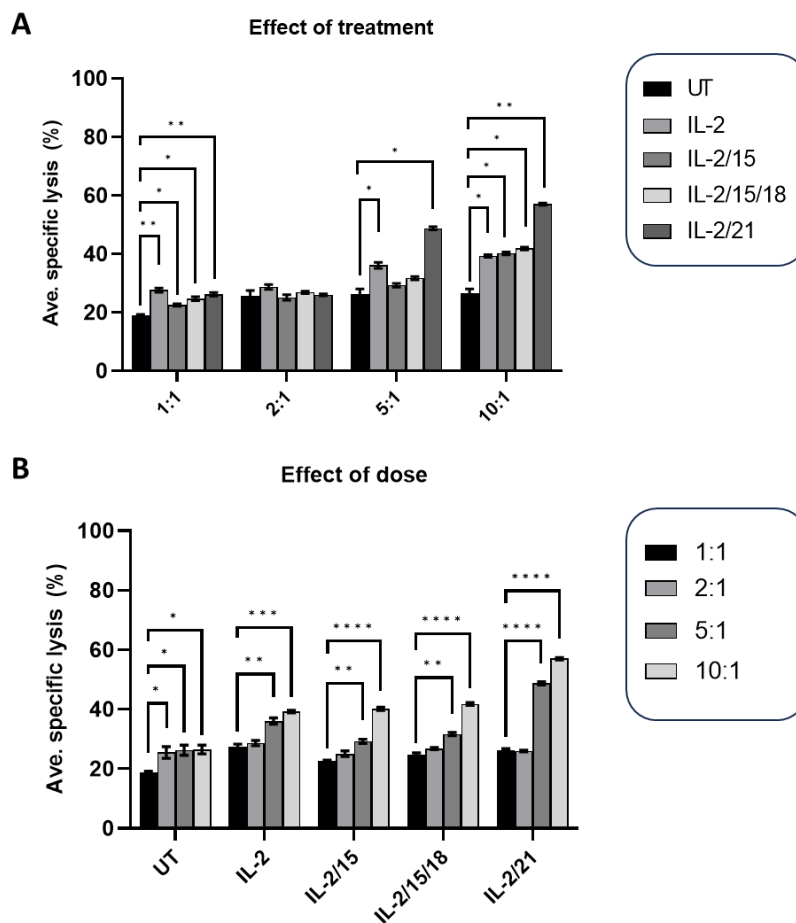


Asterisk denotes significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Tukey's HSD critical value set at $\alpha = 0.05$).

Figure 4. Averaged concentration (pg/mL) of secreted (A) TNF- α and (B) IFN- γ was plotted for all groups (n = 3, technical replicates). Note: Error bars represent SEM.

e) Bioluminescence cytotoxicity assay

As illustrated in Figure 5, the degree of NK cell-mediated cytotoxicity was directly proportional to the effector-to-target (E:T) ratio. Notably, NK cells treated with any of the cytokine combinations exhibited higher specific lysis (%) of Nalm6 leukaemia cells compared to the UT group. Among the treatment groups, NK cells treated with IL-2 and IL-21 (Group D) demonstrated the most potent cytotoxic activity in an E:T ratio-dependent manner, achieving over 59.6% specific lysis within 12 hours at an E:T ratio of 10:1.



Error bars represent SEM. Asterisk denotes significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Tukey's HSD critical value set at $\alpha = 0.05$).

Figure 5. Percentage of specific lysis (%) of Nalm6 cells after co-culture at different E:T ratios and various combinations of cytokine cocktails, to determine (A) the effect of different cytokines and (B) the effect of dose treatment at varying E:T ratios (n = 3).

DISCUSSION

NK cells have re-emerged as promising candidates for cancer immunotherapy after significant advancements in research and development. These innate lymphocytes possess the intrinsic ability to recognise and eliminate diseased or infected cells without the need for prior sensitisation. Naturally, the strength of its signals is weaker than that of cytotoxic CD8⁺ T-cells and is more susceptible to suppression by tumour-derived signals or the immunosuppressive tumour microenvironment. Interestingly, the ex vivo expansion and treatment of NK cells using various interleukins have yielded significant therapeutic benefits, many of which are currently under clinical investigations (19–23). However, it has been reported that these interleukins may elicit systemic adverse effects (e.g., cytokine storm or vascular leak damage); hence, their use should be confined strictly to ex vivo expansion protocols, with no direct in vivo administration (24). Nevertheless, concerns remain regarding potential transmission of residual pro-inflammatory cytokines, particularly IL-2, during NK cell transplantation. To mitigate this risk, the use of combinatorial interleukin strategies has been proposed, enabling a lower dependency on IL-2 while maintaining NK cell expansion and activation capabilities.

In the current study, when CB-NK cells were treated with IL-2 alone, these cells formed clusters starting on day 7 and increased by 16.3-fold in 14 days. When (C) IL-15 and IL-18, or (D) IL-21, were added with IL-2, the cell clusters were observed as early as day 4, and the NK cells grew more rapidly thereafter. By day 14, the expansion rate was 47.9-fold on average, inferring that the combination of IL-15 and IL-18 or IL-21 with IL-2 was more effective than IL-2 alone at promoting the growth of NK cells. Like previous studies, the incorporation of IL-15 manifests good expandability in the NK cells, similar to IL-2 alone. Specifically, these cytokines in their heterotrimeric receptors use cytokine-specific receptor α -chains, IL-2 receptor α (IL-2R α /CD25) for IL-2 and IL-15R α (CD215) for IL-15 (25–27).

According to the linear differentiation model, CD56^{bright} NK cells represent an immature phenotype that progresses into the more cytotoxic CD56^{dim} subset upon maturation. In their study, NK cells treated with IL-2 alone exhibited characteristics of highly differentiated cells, suggesting an enhancement of cytotoxic potential (28). In line with the present study's objective, the expansion and activation of NK cells towards the CD56^{dim}CD16^{hi} phenotype using alternative cytokine combinations was successfully achieved. The inclusion of additional interleukins alongside IL-2 or IL-15 was essential to achieve a functional balance, by preventing overstimulation or metabolic exhaustion of active NK cells, without compromising the anti-tumour activity. Previous reports identified IL-18 as an IFN- γ -inducing factor (IGIF), playing a pivotal role in promoting cytokine production (29). Furthermore, IL-18 is recognised as a proinflammatory cytokine that facilitates both type 1 and 2 immune responses. Conversely, IL-21, previously suggested to antagonise IL-15 activity, has also been implicated in the production of IL-10 and IFN- γ (30). However, in the context of NK cell treatment, groups C and D demonstrated superior cytokine secretion than the other groups and the UT group.

Paradoxically, cytokine treatment also led to an increase in the regulatory subpopulation of NK cells (CD56^{hi}CD16^{lo}), despite culture conditions being optimised to promote expansion of cytotoxic NK cells. We hypothesise that incorporating different interleukins besides IL-2 may create a more physiologically relevant microenvironment, supporting NK cells' growth and function. Similar to the in vivo setting, the presence of diverse growth factors and cytokines is necessary to maintain metabolic stability and immunological competence. This hypothesis is supported by enhanced secretion of IFN- γ and TNF- α by the cytokines in Groups C and D (Figure 4) that are instrumental in modulating NK cells' functions, initiating DC-NK cell crosstalk, and priming adaptive immunity (31). CD56^{dim} NK cells, while highly cytotoxic, are known to exhibit a high metabolic turnover in artificial culture settings. As a result, it has poorly affected the NK cells' biology and storage viability. Therefore, co-expanding regulatory CD56^{bright}

NK cells may help to maintain the viability and cytotoxic activity of CD56^{dim} NK cells, thus enhancing the overall therapeutic potential. This is further reflected in the greater specific lysis (%) of Nalm6 cells observed in Figure 5 (32).

In contrast to previous studies, our findings demonstrate that treatment of NK cells using various interleukins did not result in upregulation of activating receptors. Instead, cytokine treatment led to notable downregulation of inhibitory receptors, as shown in Figure 3(B), suggesting an initially baseline level of NK cell suppression. This observation may be attributed to the origin and developmental context of the tissue source. Specifically, CB-NK cells generally exhibit higher expression of inhibitory receptors compared to those derived from adult peripheral blood (33,34). This is likely due to the foetal immune system's requirement for stringent immunomodulation in utero to maintain tolerance between the foetal and maternal environment.

The two principal classes of inhibitory receptors expressed on human NK cells are the KIRs and the CD94-NKG2A heterodimer (35,36). These receptors transmit inhibitory signals and are critical for the education and functional tuning of NK cells. Previous studies have indicated that the responsiveness and efficacy of NK cells can be modulated based on the strength of these inhibitory signals (25,37). The relative increase in activating receptor expression and the reduction in inhibitory receptor levels are thought to enhance NK cell cytolytic function. This concept aligns with our current findings, in which cytokine-treated NK cells exhibited superior cytotoxic activity against cancer cells compared to their untreated counterpart.

Despite the lack of upregulation in activating receptor expression, NK cells demonstrated effective tumour cell lysis when co-cultured with Nalm6 target cells. This observation suggests that CB-NK cells may exist in a constitutively "primed" state, even in the absence of exogenous cytokine treatment. Among the activating receptors analysed, the UT group exhibited the highest expression of NKG2D/CD314, which declined following cytokine treatment. In the absence of external stimuli or target cell interaction, NKG2D could have functioned as the default 'surveillance-ready' receptor, maintaining immunological defence until a breach is detected. However, chronic exposure to pro-inflammatory cytokines such as TNF- α has been shown to downregulate NKG2D expression via the TNF- α /NF- κ B signalling axis. It is also plausible that the elevated baseline levels of NKG2D observed in the UT group may be attributed to the presence of specific NK cell subsets that inherently express high levels of this receptor (38). Nevertheless, the in vitro re-education of NK cells through cytokine treatment successfully rendered them cytotoxically active against tumour cells, independent of activating receptor upregulation, as demonstrated in Figure 5.

Our findings also revealed an elevation in the expression of NKp44 and NKp46 receptors on NK cells following cytokine treatment, although the increase was not statistically significant. Nevertheless, previous studies involving anti-NKp44 and anti-NKp46 treatment of NK cells have garnered considerable interest for their role in expanding high-purity NK cells, and thus warrant consideration in future investigations (9). Both of these receptors belong to the NCR family, which mediates non-major histocompatibility complex (non-MHC)-restricted recognition of tumour cells (40). NKp46 is constitutively expressed on both resting and activated NK cells, whereas NKp44 is an inducible receptor, serving as a hallmark of activated human NK cells. These activating receptors play a crucial role in the initiation of NK cell cytotoxic responses, particularly through ligand-receptor interactions that enable tumour cell recognition and lysis (41). Among the two, NKp44 is frequently cited as an activator status of NK cells due to its strong correlation with the interleukin-derived signalling pathway.

To assess the cytotoxicity performance, both UT and cytokine-treated NK cells were co-cultured with luciferase-transduced Nalm6 cells for 12 hours. The results demonstrated that the NK cell-mediated cytotoxicity, measured as the percentage of specific lysis of Nalm6 cells, was directly proportional to the effector-to-target (E:T) ratio. Among the treatment groups, NK cells treated with a combination of IL-2 and IL-21 exhibited the highest cytotoxic activity at an E:T ratio of 10:1, with an average increase of 59.6% in specific lysis compared to untreated NK cells. Furthermore, the cytotoxicity observed in group D was approximately 3-fold higher than that of UT NK cells, indicating a synergistic effect of IL-21 in enhancing NK cell-mediated cytotoxicity. This finding is consistent with previous reports demonstrating that IL-21 enhances NK cell cytotoxicity in a concentration-dependent manner, partly through the upregulation of IFN- γ and Granzyme B expression (42). Additional studies have confirmed the anti-tumour effects of IL-21 administration, producing significant tumour regression in murine models (43). Besides, IL-21 has also been shown to augment IL-2- or IL-15-induced NK cell activation, increase IFN- γ production and enhance NK-mediated response (44,45). Based on a Phase I clinical study conducted in 2007, IL-21 was well tolerated by patients with metastatic melanoma, supporting its potential clinical use in cancer immunotherapy (46).

One of the key limitations of this study was the suboptimal purity of the expanded NK cells, which remained below 80% across all treatment groups. Additionally, successful NK cell isolation was achieved from only one out of twenty units of cryopreserved CB-MNCs. Thus, the technical replicates employed may not be population-representative. To address this limitation, we recommend implementing strategies to enhance NK cell purity before downstream functional assays. One approach is to incorporate an additional purification step during the NK cell isolation process to eliminate contaminating cell populations. Alternatively, selectivity during the expansion phase could be improved by using antibodies targeting activating NK cell receptors. A recent study demonstrated that treatment with recombinant human IL-18 and IL-2, in combination with immobilised anti-NKp46 and/or anti-CD16 antibodies, yielded a population comprising approximately 95% CD3⁺CD56⁺ NK cells (47). These strategies should enhance both the quality and consistency of NK cell populations for future research and clinical applications.

CONCLUSION

In summary, the combination of IL-2, IL-15, and IL-18 (Group C), as well as IL-2 and IL-21 (Group D), resulted in the expansion of larger subpopulations of both CD56^{bright} and CD56^{dim}-expressing NK cells. While IL-2 alone was sufficient to activate NK cells, the addition of other cytokines significantly enhanced their proliferative capacity and functional specificity. Notably, only Groups C and D exhibited significant cytolytic activity against co-cultured Nalm6 leukaemia cells, underscoring the importance of incorporating interleukins beyond IL-2 to optimise NK cell efficacy. These findings suggest that further investigation into the role of CD56^{bright} subsets may be pivotal in sustaining cytotoxic NK cell function and improving long-term anti-tumour efficacy in immunotherapy.

Supplementary Materials: Supporting information related to this study can be accessed at: www.imrj.com/x. This includes Table S1: [List of phenotyping markers for NK cells].

Author Contributions: M.N.L. research design, data analysis, critical manuscript writing and reviewing; A.M.L.C.: data analysis and critical manuscript writing; P.S.P.: data analysis and critical manuscript writing; K.S.F.: critical manuscript writing and reviewing; N.A.F.: data analysis and reviewing; Q.H.L.: research design, data analysis, critical manuscript writing and reviewing; E.E.: data analysis and reviewing; Y.M.Y.: final approval

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Institutional Review Board Statement: This research was conducted in compliance with the Declaration of Helsinki and approved by the Medical Research and Ethical Committee (MREC) (JPP-IMR 18-043, approved on 2018-06-12). Twenty units of fresh cord blood were obtained from Hospital Tengku Ampuan Rahimah Klang (HTAR) with informed consent.

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Data Availability: Data will be made available upon reasonable request.

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Supplementary Table 1. List of phenotyping markers for NK cells

Surface markers	Specifications	Role
CD56	Anti-CD56-BUV395 (NCAM16.2)	Positive marker
CD16	Anti-CD16-PE-CG594 (3G8)	Positive marker
CD3	Anti-CD3-APC-H7 (SK7)	Negative marker
CD19	Anti-CD19-PE (HIB19)	Negative marker
CD25	Anti-CD25-APC (M-A251)	Activating receptor
CD69	Anti-CD69-APC (FN50)	Activating receptor
NKG2D	Anti-NKG2D/CD314-APC (1D11)	Activating receptor
NKp30	Anti-NKp30/CD337-Alexa Fluor® (p30-15)	Activating receptor
NKp44	Anti-NKp44/CD336-BB515 (p44-8)	Activating receptor
NKp46	Anti-NKp46/CD335-BB515 (9E2/NKp46)	Activating receptor
CD158a	Anti- KIR2DL1/CD158a-APC (HP-3E4)	Inhibitory receptor
CD158b	Anti- KIR2DL2/CD158b-BB515(CH-L)	Inhibitory receptor
CD158e1	Anti-NKB1/CD158e1-APC (DX9)	Inhibitory receptor

CD159a	Anti-NKG2A/CD159a-PE (131411)	Inhibitory receptor
CD159c	Anti-NKG2C/CD159c-BUV615 (134591)	Both activating & inhibitory
CD62L	Anti-CD62L-BB515 (SK11)	Both activating & inhibitory
