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Original

Optimization of IL-15–Enriched Cytokine-Induced Killer Cells Expansion: Phenotypic and Functional Characterization

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Abstract

Background: In adoptive cell therapy, cytokine-induced killer (CIK) cells have been used as a promising modality due to their potent antitumor activity and MHC-independent function. IL-15 has been proposed as a promising alternative to IL-2 because it enhances survival, maintains a memory-like phenotype, and improves cytotoxic function; however, its utility in optimizing CIK cells for the treatment of hepatocellular carcinoma (HCC) remains to be investigated.

Methods: CIK cells were generated from human peripheral blood mononuclear cells (hPBMCs) using a 12-day step-wise expansion protocol including treatment with IFN-γ, anti-CD3 antibody, IL-2, and IL-15 in culture medium containing human platelet lysate. The phenotypic characteristics of the cells were examined by flow cytometry, and the expression of $CD3^+$, $CD8^+$, and $CD56^+$ was assessed. The cytotoxic activity of CIK cells against HepG2 cells was evaluated by flow cytometry using a CFSE/PI assay at different effector-to-target ratios (E: T), and ELISA measured the secretion of IFN-γ and TNF-α.

Results: The expanded CIK cells exhibited a characteristic $CD3^+CD8^+$ and $CD3^+CD56^+$ phenotype. These cells demonstrated significant cytotoxic activity against HepG2 cells across different E: T ratio and showed enhanced production of IFN-γ and TNF-α, indicating robust antitumor activity.

Conclusion: The short-term IL-15-enriched expansion protocol generated stable CIK cells with an NK-like phenotype and significant cytotoxic activity against HCC cells. Increased secretion of inflammatory cytokines also indicated effective antitumor activation of these cells. These findings support further development and preclinical evaluation of IL-15-CIK-based cell therapy for the treatment of HCC.

Keywords:

Immunotherapy
Adoptive cell therapy
Cytokine-induced killer cells
Hepatocellular carcinoma

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1. INTRODUCTION

Immunotherapy has revolutionized the cancer treatment, serving as the "fifth pillar" of cancer therapeutics after surgery, radiotherapy, chemotherapy, and targeted therapy (1). Adoptive cell therapy (ACT) has emerged as one of the most promising immunotherapeutic strategies, involving the transfer of immune cells expanded and activated outside the body (*ex vivo*) into patients to eliminate cancer cells (2). Among various immune effector cell populations, cytokine-induced killer (CIK) cells have attracted considerable attention because of their rapid expansion, major histocompatibility complex (MHC)-unrestricted activity, strong antitumor activity with minimal toxicity, and reduced risk of graft-versus-host disease (GVHD) (3-5).

CIK cells are a heterogeneous population that mainly includes T cells ($CD3^+CD56^-$), NK cells ($CD3^-CD56^+$), and NK-like T cells ($CD3^+CD56^+$), derived from human peripheral blood mononuclear cells (hPBMCs) following *ex vivo* expansion with interferon-γ (IFN-γ), anti-CD3 antibodies (OKT3), and interleukin-2 (IL-2) (6, 7). In fact, the $CD3^+CD56^+$ subset represents the major effector population, exerting potent antitumor cytotoxicity in an MHC-unrestricted manner, making it useful against a broad range of cancers (8). The antitumor activity of CIK cells is mediated through the interaction of natural killer group 2 member D (NKG2D) with its ligands, such as MHC class I polypeptide-related sequence A and B (MICA and MICB) on the surface of tumor cells, as well as through the release of cytotoxic granules containing perforin and granzyme (9, 10). CIK cells also secrete cytokines, including IFN-γ and tumor necrosis factor-α (TNF-α), which can further enhance antitumor immunity through indirect immunomodulatory mechanisms in addition to their direct cytotoxic effects (11). The generation of CIK cells was first reported in 1991 by sequentially incubating hPBMCs with IFN-γ, anti-CD3 monoclonal antibody, and IL-2, often with IL-1, over a 14–21-day culture period (12). Although the classical approach is quite efficient, it has major limitations, including an increased risk of T-cell functional exhaustion during long-term culture and instability in their cytotoxic capacity (13, 14). To overcome these challenges, several corrective strategies have been investigated, among which the use of IL-15 has been proposed as a promising approach (15, 16). In contrast to IL-2, IL-15 increases survival and the maintenance of a memory-like phenotype in $CD8^+$ T cells and NK-like populations, while simultaneously reducing activation-induced cell death (17). Furthermore, IL-15 enhances cell's ability to recognize and eliminate tumor cells by increasing the expression of key activating receptors, including NKG2D (18). Although IL-15 offers clear advantages, evidence regarding the use of an IL-15-supplemented CIK cells protocol against hepatocellular carcinoma (HCC) remains scarce. Therefore, the present study aimed to design and optimize an IL-15-based protocol for generating CIK cells. In this study, the functional properties of CIK cells were evaluated by assessing their proliferation capacity, phenotypic characteristics, *in vitro* cytotoxic activity, and cytokine secretion pattern against the HepG2 cell line. By focusing on the specific role of IL-15, this study aims to provide an optimized strategy to generate CIK cells with improved function, paving the way for the development of more effective adaptive immunotherapy for HCC.

2. MTERIALS AND METHODS

2.1. Cell Culture

HepG2 cells were obtained from the Royan Institute (Tehran, Iran). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine. Cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Generation of CIK Cells

CIK cells were generated from hPBMCs isolated from a healthy volunteer. After obtaining informed consent, 30 mL of peripheral blood was collected into sodium heparin-containing Vacutainer tubes. hPBMCs were isolated from buffy coats by Ficoll–Hypaque density gradient centrifugation and washed three times with phosphate-buffered saline (PBS). The cells were then resuspended at a density of 3×10^6 cells/mL in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5% human platelet lysate (hPL) (Cell Tech Pharmed Co., Iran). To improve the expansion and effector functions of CIK cells, an optimized protocol supplemented with IL-15 was used. On day 0, cultures were stimulated with 1000 U/mL recombinant human interferon-γ (rhIFN-γ; PeproTech, Cranbury, NJ, USA) and 100 ng/mL immobilized anti-CD3 monoclonal antibody (OKT3; BioLegend, San Diego, CA, USA). On day 1, recombinant human interleukin-2 (rhIL-2; PeproTech, Cranbury, NJ, USA) and recombinant human interleukin-15 (rhIL-15; PeproTech, Cranbury, NJ, USA) were added at final concentrations of 500 U/mL and 50 ng/mL, respectively. Fresh medium and rhIL-15 were replenished every three days throughout the culture period, while the cell density was maintained at approximately 1×10^6 cells/mL. After 12 days of expansion, the viability of the CIK cell population exceeded 90% **(Figure 1)**.

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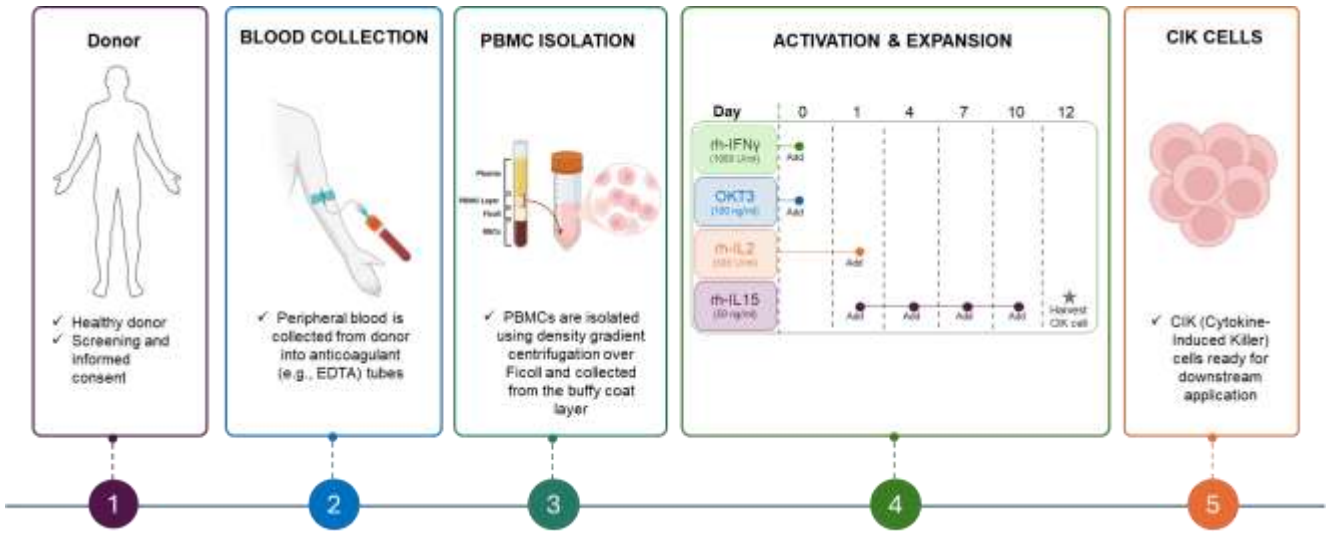

Figure 1. Timeline for generating the CIK cells. hPBMCs were isolated from healthy volunteer and incubated with IFN-γ, anti-CD3 antibody, IL-2, and IL-15 for 12 days, then stained with human monoclonal antibodies, including anti-CD3-PerCP, anti-CD56-PE, and anti-CD8-PE.

2.3. Phenotype analysis

The phenotype of the expanded CIK cells was analyzed by flow cytometry using the following fluorochrome-conjugated monoclonal antibodies: CD3-PerCP (Miltenyi Biotec, Bergisch Gladbach, Germany), CD8-PE (Cytomatin Gene, Iran), and CD56-PE (BD Biosciences, Heidelberg, Germany). Briefly, 1×10^5 CIK cells were washed once with PBS containing 1% bovine serum albumin (BSA) and resuspended in 100 µL of PBS/BSA buffer. The cells were incubated with the appropriate monoclonal antibodies for 20 min at 4 °C in the dark, washed twice with PBS, and finally resuspended in 100 µL of PBS.

Flow cytometric analysis was performed using a FACSCalibur flow cytometer (BD Biosciences, USA), and data were analyzed with FlowJo™ software. Live cells were identified and gated based on forward-scatter (FSC) and side-scatter (SSC) characteristics. The gated cells were then analyzed for CD3 expression. CD56 expression was subsequently assessed within the $CD3^+$ population, and cells co-expressing CD3 and CD56 were identified as the $CD3^+CD56^+$ CIK-cell population.

2.4. CFSE-Based Cytotoxicity Assay

The cytotoxic activity of CIK cells against HepG2 target cells was evaluated *in vitro* using a carboxyfluorescein succinimidyl ester (CFSE)/propidium iodide (PI) assay. HepG2 cells (1×10^7 cells/mL) were suspended in PBS and labeled with 1.25 µmol/L CFSE (Invitrogen, USA) for 5 min at 37°C. The labeling reaction was terminated by adding DMEM supplemented with 10% FBS, followed by three washes with complete medium. CFSE-labeled HepG2 cells were then seeded into 24-well plates at a density of 5×10^5 cells per well and co-cultured with CIK cells at effector-to-target (E: T) ratios of 30:1, 20:1, and 10:1. Wells containing CFSE-labeled HepG2 cells alone served as the control. After incubation for 12 h at 37°C in a humidified atmosphere containing 5% CO_2, PI was added to each well at a final concentration of 1 µg/mL and incubated for 5 min before analysis. Dead target cells were identified as CFSE/PI double-positive cells by flow cytometry. The percentage of specific target cell death (cytotoxicity) was measured using a FACSCalibur and Cell Quest software (BD Biosciences, San Jose, CA, USA) and then calculated as: Specific cytotoxicity (%) = [(experimental cell death (%) − spontaneous cell death (%)) / (100 − spontaneous cell death (%))] × 100.

Note: Experimental cell death refers to the percentage of target-cell death observed in the presence of CIK cells, whereas spontaneous cell death refers to the percentage of target-cell death observed in the target-cell-only control.

2.5. Cytokine production assay

HepG2 cells were co-cultured in the presence of CIK cells at different E: T ratios of 10:1, 20:1, and 30:1 in RPMI 1640 medium containing 10% FBS. After 12 hours of incubation, the supernatant was collected, and IFN-γ and TNF-α secretion was measured using ELISA kits (R&D Systems™; DIF50C for IFN-γ and DTA00D for TNF-α) according to the manufacturer's instructions. For comparison, CIK cells cultured under the same conditions in the absence of HepG2 cells were used as controls.

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2.6. Statistical Analysis

All experimental data were analyzed using ANOVA. Data are presented as mean values ± standard deviation (SD). All the experiments had at least three biological replicates. The $P < 0.05$ was considered statistically significant. All experimental data were analyzed using GraphPad Prism software (v.8.4.1; GraphPad Software Inc., San Diego, CA, USA).

3.RESULT

3.1. Phenotypes of generated CIK cells

CIK cells were successfully expanded from hPBMCs using an IL-15-supplemented protocol. The total cell number increased from 10×10^6 cells at the beginning of culture to 80×10^6 cells after 12 days, corresponding to an approximately 8-fold expansion. The viability of expanded cell populations throughout the culture period was >90%. These findings demonstrate that IL-15 supplementation effectively promotes cell survival and proliferation. The protocol for generation and expansion is shown in **Figure 1**.

Phenotypic analysis of the cultured cell population by flow cytometry showed a heterogeneous CIK cell population, predominantly composed of $CD3^+$ cells with significant $CD3^+CD8^+$ and $CD3^+CD56^+$ subsets. The enrichment of the $CD3^+CD56^+$ population highlights the development of NK-like cytotoxic characteristics in the presence of IL-15 (**Figures 2A and B**).

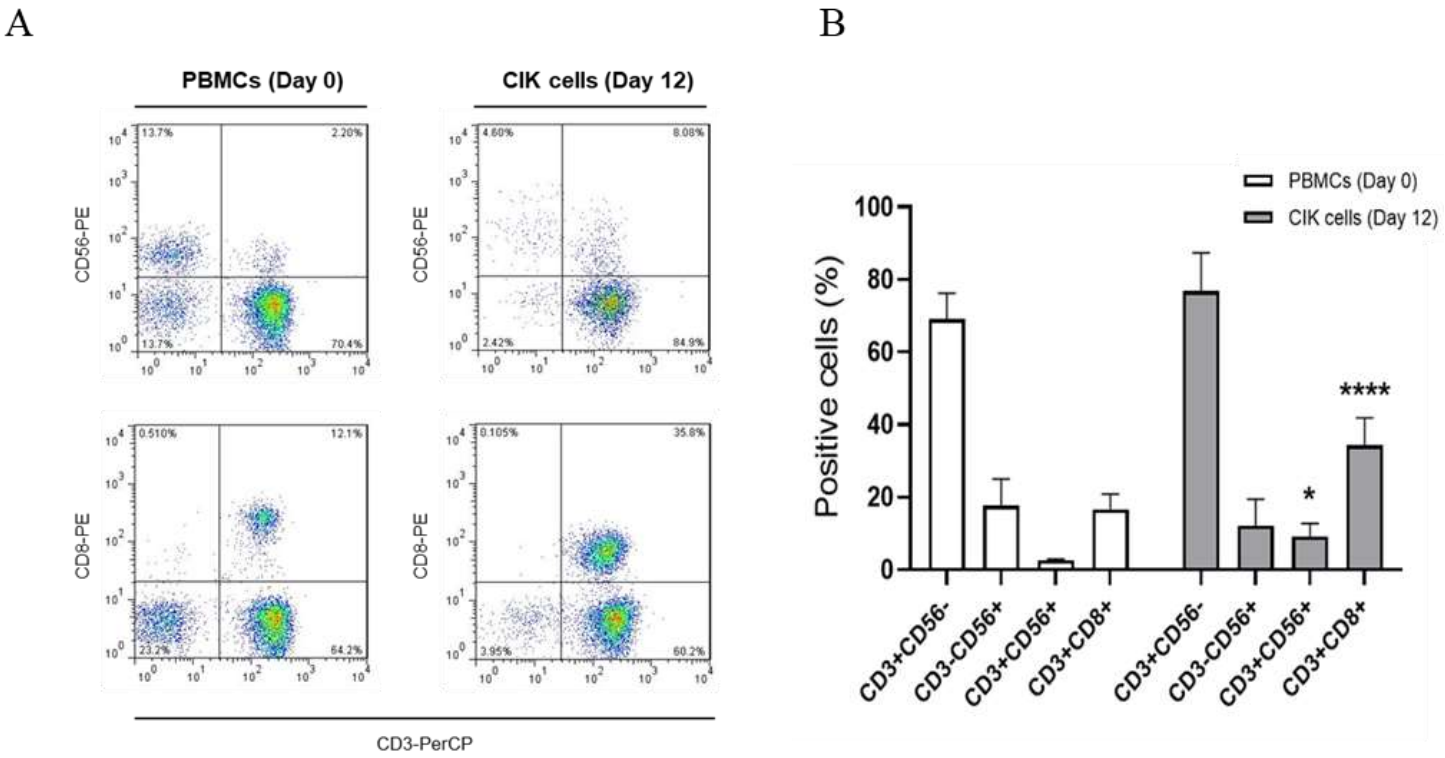

Figure 2. The phenotypic characterization of CIK cells. (A) Representative immunophenotypic analysis of hPBMCs and CIK cells. (B) Comparison of the phenotypic profiles of hPBMCs and CIK cells. The phenotypic comparison was performed using six independent samples, and the results are expressed as means ± SD. Data were analyzed using two-way ANOVA. *p<0.05, ****p<0.0001.

3.2. Cytotoxic activity of CIK cells on HepG2 cells

The antitumor activity of the generated CIK cells was evaluated using a CFSE/PI-based cytotoxicity assay. The expanded CIK cells demonstrated dose-dependent cytotoxicity against HepG2 cells. Specific lysis increased proportionally with higher E: T ratios, reaching a maximum of 22.3% at an E: T ratio of 30:1 after 12 hours of co-culture. These results confirm the antitumor activity of IL-15-enriched CIK cells against HCC cells (**Figure 3A and B**).

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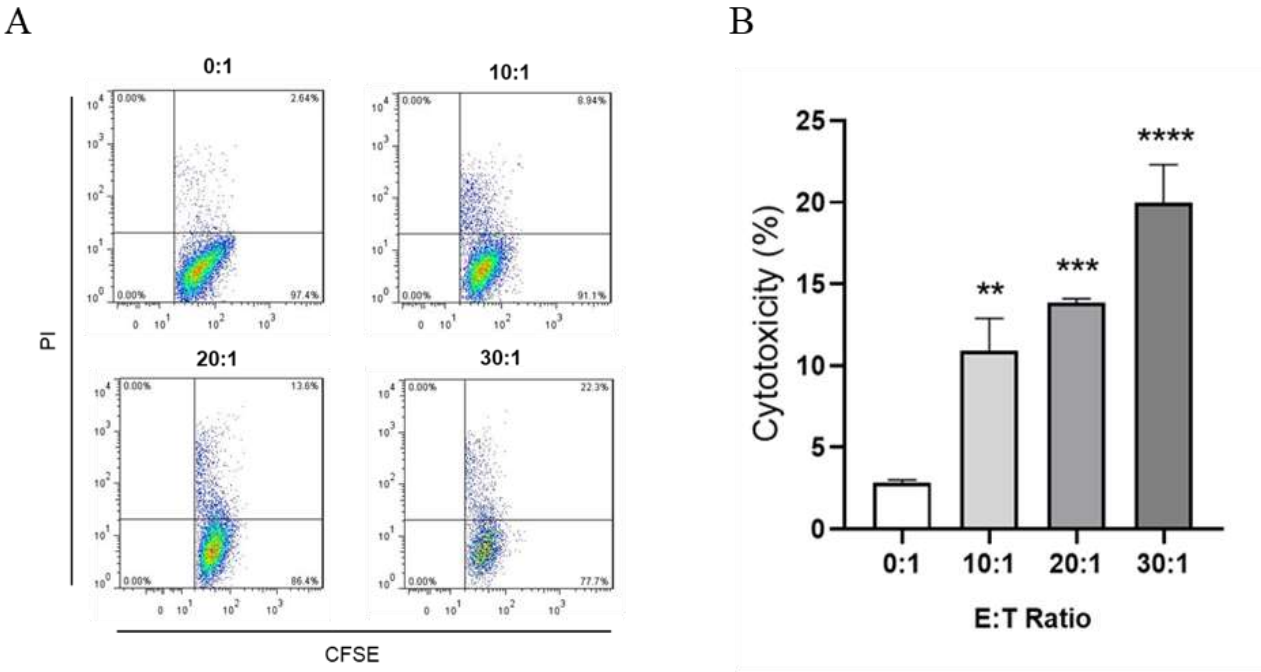

Figure 3. The cytotoxicity of CIK cells. (A) Cytotoxic activity (%) of CIK cells against HepG2 cells at different effector-to-target ratios, measured by flow cytometric assay. (B) To evaluate the cytotoxicity of CIK cells, CFSE-labeled HepG2 target cells were co-cultured with CIK effector cells at various E: T ratios for 12 h. After incubation, both floating and adherent cells were collected, washed with PBS, and stained with PI. Experiments were performed independently three times, and the results are expressed as mean ± SD. Data were analyzed using one-way ANOVA. **p<0.01, ***p<0.001, ****p<0.0001.

3.3. Cytokine production Profile

The cytokine secretion profile of IL-15-enriched CIK cells was analyzed by ELISA following 12 hours of co-culture with HepG2 cells. IFN-γ and TNF-α were the predominant cytokines secreted by the activated CIK cells. Compared with the control cells (CIK cells cultured alone), co-culture with HepG2 cells significantly increased the secretion of both IFN-γ and TNF-α. Importantly, cytokine secretion increased in a dose-dependent manner with increasing E: T ratios (10:1, 20:1, and 30:1) (**Figures 4A and B**).

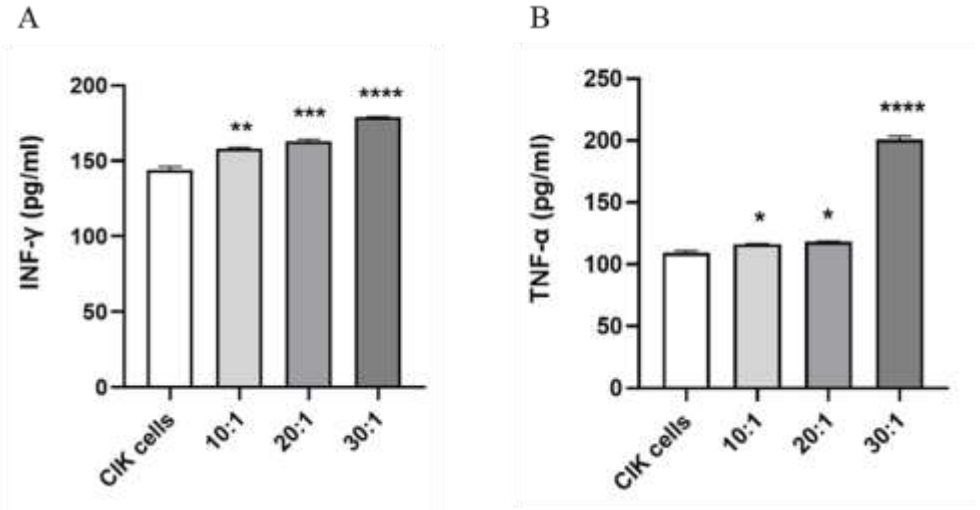

Figure 4. Cytokine production profiles of CIK cells. Cytokine release by CIK cells (IFN-γ (A) and TNF-α (B)) at different E: T ratios were quantified by ELISA. Experiments were performed independently three times, and the results are expressed as mean ± SD. Data were analyzed using one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

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4. DISCUSSION

In this study, CIK cells were successfully generated from hPBMCs using an optimized IL-15-enriched culture protocol, and their phenotypic and functional properties against HCC cells were investigated. The findings showed that the number of CIK cells were increased 8-fold over 12 days of *ex vivo* culture, while cell viability was maintained above 90%, demonstrating the ability of this protocol to support efficient expansion and maintain cell quality. These findings suggest that IL-15 provides an efficient cytokine microenvironment to support cell survival, proliferation, and functional maintenance. These results are in line with the growing evidence highlighting the pivotal role of IL-15 in promoting the stability, metabolic fitness, and functional integrity of cytotoxic lymphocytes, and reinforce its application in the design of next-generation immune cell-based therapeutic platforms (19, 20).

Phenotypic analysis revealed that the resulting cell population was heterogeneous but predominantly $CD3^+$, with a significant enrichment in the $CD3^+CD8^+$ and $CD3^+CD56^+$ subsets. The increase in the $CD3^+CD56^+$ subset is of great importance, as these cells have NK-like cytotoxic properties and can target tumor cells without MHC dependence (21, 22). Previous studies have also shown that IL-15 can enhance the cytotoxic phenotype of CIK cells and increase this subset more than IL-2 (15, 23). Therefore, IL-15 seems to play an important role in improving the function and efficiency of CIK cells.

Assessment of cytotoxic activity revealed that IL-15-enriched CIK cells exhibited a dose-dependent cytotoxic response against HepG2 cells, achieving 22.3% at an E: T ratio of 30:1 after 12 h of co-culture. Although this level of activity is considered moderate, it is biologically meaningful because it was achieved in a relatively short culture period and under conditions designed to maintain viability and clinical translatability. These findings are consistent with previous reports of IL-15-based CIK, which show that IL-15 can efficiently enhance antitumor activity and cytotoxic potential through innate immune pathways (18).

The antitumor activity of CIK cells has been attributed in previous studies to several MHC-unrestricted mechanisms, including recognition of NKG2D ligands and secretion of cytolytic granules such as perforin and granzymes (24). This feature is of particular importance in the context of HCC, as immune evasion in this tumor is often associated with reduced antigen presentation and impaired MHC class I-dependent recognition (25). Although these mechanisms were not directly investigated in the present study, they may contribute to the cytotoxic activity observed against HepG2 cells. Recent evidence in HCC immunotherapy also emphasizes that overcoming immune evasion mechanisms remains a major challenge; hence, the quasi-intrinsic cytotoxicity of CIK cells makes them a promising therapeutic option (26).

Beyond direct cytotoxic effects, CIK cells enriched with IL-15 secreted IFN-γ and TNF-α in an E: T ratio-dependent manner after co-culture with HepG2 cells. These cytokines are key pillars of antitumor activity, as they enhance tumor cell apoptosis, activate various components of the immune system, and intensify inflammatory signaling in the tumor microenvironment (27). The dose-dependent cytokine response pattern observed in this study suggests that IL-15 not only improves proliferation but also the functional quality of CIK cells. This finding is in line with the growing body of evidence highlighting the role of IL-15 in supporting survival, metabolic fitness, and sustained efficacy in adaptive cell therapy products (28).

In the context of recent literature, the present findings support the view that IL-15 is a key cytokine in the development of a new generation of immune cell therapy approaches. A 2025 review of the use of IL-2 and IL-15 in adaptive NK cells highlighted the importance of sustained cytokine support for survival, proliferation, and antitumor function, while also pointing out the limitations of IL-2, including toxicity and induction of terminal differentiation (29). Similarly, recent reviews of IL-15-based immunotherapy suggest that this cytokine remains an active focus of translational developments in cancer immunotherapy (30, 31). In this regard, the results of the present study extend this trend to the CIK cell setting in the context of HCC by demonstrating that a short-term IL-15-enriched protocol can generate cells with high survival, an NK-like phenotype, and measurable antitumor activity against HepG2.

An additional strength of this study is the relatively short 12-day culture duration, which may help limit exhaustion-associated phenotypes and preserve functional integrity. The use of human platelet lysate instead of FBS further improves clinical translatability by reducing xenogeneic exposure and aligning the protocol more closely with GMP-oriented manufacturing principles.

However, this study also has limitations. The functional evaluation was limited to *in vitro* assays using a single HCC cell line, and HCC heterogeneity may affect sensitivity to CIK-mediated killing. In addition, persistence, exhaustion marker expression, and *in vivo* efficacy were not assessed. Further studies using additional HCC models, mechanistic investigations, and appropriate *in vivo* models are necessary to validate these findings and to determine the potential therapeutic and clinical applicability of IL-15-enriched CIK cells. The potentially encouraging clinical data on adjuvant CIK cell therapy in HCC treatment may underscore the value of the next steps in translational research (32-34).

5. CONCLUSION

Overall, the IL-15-enriched culture protocol enabled the generation of viable and functionally active CIK cells,

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characterized by NK-like phenotypic features, dose-dependent cytotoxicity against HepG2 cells, and increased secretion of proinflammatory cytokines. These findings provide preliminary *in vitro* evidence supporting further investigation of IL-15-enriched CIK cells as a potential approach for HCC immunotherapy. However, additional studies using multiple HCC models, detailed mechanistic analyses, and *in vivo* validation are required to determine their therapeutic efficacy and clinical.

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Conflict of interest

The authors declare no conflict of interest.

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Ethical statement

This study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki and was approved by the Ethics Committee of Royan Research Institute (ethics code: IR.ACECR.ROYAN.REC.1399.045). Written informed consent was obtained from the participant (individual donor) before the study began.

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