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Cytokine-Induced Killer Cells as an Adoptive Cellular Immunotherapy Strategy for Hepatocellular Carcinoma

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Abstract: Background: Hepatocellular carcinoma (HCC) is the most common histologic type of primary liver cancer. HCC is the second highest mortality rate out of all major malignant carcinomas worldwide. Objectives: The aims of this study were to establish a rapid and easily handled culture method for sufficient expansion of viable and cytotoxic cytokine-induced killer (CIK) cells against HCC. Also, this study aimed to examine the morphologic, phenotypic, and functional characteristics of CIK cells. Method: Peripheral blood mononuclear cells (PBMCs) were cultured in a preliminary static culture to remove adherent cells. The suspended cells were cultured for 14 days with interferon-γ, human monoclonal anti-CD3 antibody and interleukin-2. Aliquots of induced PBMCs were harvested weekly to assess informative morphologic and phenotypic features of CIK cells. Mature CIK cells were subjected to functional assays that included the production of TNFα and the cytotoxic effect on HCC cell line, HepG2. Findings: CIK cells could be successfully expanded from all samples with a significant increase in T cells, natural killer cells, and natural killer T cells. TNFα concentration in the culture supernatant was significantly increased. The cytotoxic effect of CIK cells on HepG2 cells was nearly 60% at 40:1, effector: target ratio. Regression analysis was used to predict the CIK: HepG2 ratio required to achieve complete cytotoxicity. Conclusion: This study provides a detailed and simple strategy for culturing effective CIK cells. Mature CIK cells showed a high functional capacity against HCC; which will support the further ongoing practice of immunotherapy integration into different current cancer treatment protocols.

Keywords: Peripheral Blood Mononuclear Cells, CIK Cells, Natural Killer T Cells, Cytotoxicity, Liver Cancer

1. Introduction

Liver cancer is one of the very most commonly diagnosed malignant tumors. It is the second highest mortality rate worldwide; estimated to be responsible for 746,000 passings in 2012 [1]. The most common histologic type of primary liver cancer, hepatocellular carcinoma (HCC), is a malignant tumor emerging from hepatocytes. Globally, in the vicinity of 600,000 and one million new cases of HCC are diagnosed every year, with a survival rate ranged from 6 to 20 months [2]. HCC is an assertive malignancy with a poor prognosis, in which repeated hepatocyte damage sets up an endless loop of cell apoptosis and regeneration that in the long run outcomes in genomic instability and initiation of HCC [3].

HCC is affiliated with cirrhosis in 80–90% of cases. Hepatitis B virus infection is the main risk factor for HCC, globally, accounts for no less than half cases of HCC. Hepatitis C virus infection is the second most common risk factor, with a predicted 10–25% of all cases of HCC ascribed to it worldwide. Other significant risk factors include alcohol-induced cirrhosis, obesity, aflatoxin, fatty liver, iron overload, diabetes, and smoking [4]. Recently, non-alcoholic

fatty liver and non-alcoholic steatohepatitis, emerging from metabolic disorders such as insulin resistance syndrome, are included [5]. The liver is a tolerogenic organ with unique mechanisms of immune regulation. It has to anticipate peculiar immune responses to gut-derived antigens that steadily circulate through it [6].

Diagnosed cancers must have bypassed the body's antitumor immune responses to grow chronologically. Aside from liver tolerogenic nature, the loss of tumor-associated antigens, decreased major histocompatibility complex (MHC) antigen expression, inactivation of T cells by reduced T cell receptor signaling or IL-10, and transforming growth factor-β-mediated suppression, cause a scene of immune tolerance to tumors [7]. As the liver disease advances from cirrhosis to HCC, many immune cells' functions become impaired. T cells, both helper and cytotoxic types, diminish in numbers with constricted function and increased expression of inhibitory receptors. T helper 17 cells increase in number leading to increase tumor angiogenesis [8]. Cancer-associated fibroblasts inhibit natural killer (NK) cells function. Myeloid-derived suppressor cells suppress T cell stimulation, activate other immune-suppressive cells, and stimulate tumor angiogenesis [9].

Traditional treatments available for HCC patient are limited due to the advanced stage at which most patients are diagnosed. Surgical resection is a decent decision for most early-stage patients [10]. Sorafenib is a directed therapy, which is the standard first-line systemic drug for advanced HCC. However, patients with severe hepatic dysfunction or poor performance status do not derive any survival perk from this therapy [11]. Liver transplant is another perioperative intercession for advanced cases of HCC; nonetheless, there are limitations due to the deficient number of the matched donors and post-transplant allograft rejection [10]. HCC is extremely chemo-resistant as multi-drug resistance genes are reported to be highly expressed in HCC [12].

Unlike, the non-selectivity of traditional treatments, immunotherapy, hypothetically, could selectively target and devastate malignant cells with insignificant side effects [12]. It appears to work better in more immunogenic tumors like HCC. It is based on the redirection the patient's own immune system against cancer instead of targeting cancer itself (e.g. by chemotherapy). Adoptive cell therapy (ACT) is one of the major therapeutic options in cancer immunotherapy. It involves the transfer of an expansive number of *ex vivo*-cultured and functional immune cells into a tumor-bearing host [13]. It has employed numerous types of immune cells, including dendritic cells, cytotoxic T lymphocytes, lymphokine-activated killers, NK cells, and cytokine-induced killer (CIK) cells [14]. The ACT is a 'living' treatment in light of the fact that the managed cells can proliferate *in vivo* and keep up their antitumor effector functions. Such immunological stimulation may offset the strongly immune-suppressive microenvironment in the liver [15].

CIK cells, as the most frequently used ACT, are a heterogeneous cell population including natural killer T (NKT) cells ($CD3^+CD56^+$), T cells ($CD3^+CD56^-$), and NK cells ($CD3^-CD56^+$) cells. Normally, the most cytotoxic cells with double T/NK phenotype are uncommon but present (1% to 5%) in circulating blood [16]; yet, after *in vitro* expansion for 14 days, they may reach 20 to 30% of the total CIK cells [17]. Most of these NKT cells have been shown to be derived from the T cells and not from NK cells [18]. CIK cells have advantages of a higher proliferation rate, MHC-unrestricted activity, a strong activity against tumors with minimal toxicity and graft versus host disease [19], and exceptionally important, they are not hindered by immune-suppressive drugs [20]. Interferon-gamma (IFNγ) and tumor necrosis factor-alpha (TNFα) are the principle cytokines produced by CIKs [21].

Due to the number of advantages of CIK cells, they present a promising immunotherapy approach that could be used for HCC. Along these lines, the present study evaluates the potential of *in vitro* expansion of viable CIK cells from human peripheral blood mononuclear cells (PBMCs) and measures the proportion of the most effective subset $CD3^+CD56^+$ in the culture. In addition, the study examines TNFα secretion and the cytotoxicity of expanded CIK cells *in vitro* on HepG2 cell line.

2. Materials and Methods

2.1. Reagents

Roswell Park memorial institute medium (RPMI)-1640 with L-glutamine, RPMI-1640 without L-glutamine or phenol red, calcium-magnesium-free phosphate-buffered saline (PBS), penicillin-streptomycin-amphotericin B 100IU/100μg/0.25μg/mL, and Trypan blue 0.4% exclusion dye were all obtained from Lonza Verviers SPRL®, Belgium. Ficoll® paque plus was obtained from Sigma-Aldrich®, United Kingdom. Fetal bovine serum (FBS) was obtained from Biowest®, France. IFNγ and interleukin-2 (IL-2) were obtained from PeproTech®, United Kingdom. Low endotoxin, azide-free (LEAF™) purified anti-human CD3 antibody was obtained from Biolegend®, USA. Monoclonal FITC-conjugated anti-CD3 antibody, monoclonal PE-conjugated anti-CD56 antibody, and fluorescence-activated cell sorting buffer were obtained from BD Biosciences, USA. Cell counting kit-8 (CCK-8) and TNFα picoline ELISA kit were obtained from Boster Biological Technology®, USA. Heparin sodium was obtained from the Nile Co®, Egypt.

2.2. Generation of CIK Cells

2.2.1. Blood Collection

Peripheral blood (PB) samples from 10 healthy volunteers were collected by phlebotomy and emptied in sterile centrifuge tubes (50 mL) containing 5000 I.U. heparin sodium as anticoagulant followed by inversion several times, to ensure proper mixing, and labeling of the samples.

2.2.2. PBMCs Separation

PB samples were diluted 1:1 with PBS and mixed well, as dilution gives a better yield of mononuclear cells (MNCs)

[22]. MNCs were separated by density gradient using cooling swing out centrifuge (with no break) (Centurion®, United Kingdom) for 30 minutes and 1400 rotation/minute (rpm). Optimal centrifugation temperature (18-20°C) is required to keep density gradient medium at 1.077 g/mL [23]. The MNCs at the Blood-Ficoll® interface were aspirated and washed twice with 50 mL PBS, and then, washed once with RPMI-1640 medium and resuspended in 1 mL medium. Total MNCs (lymphocytes, monocytes, macrophages, and stem cells) were counted using hemocytometer by means of trypan blue exclusion dye.

2.2.3. Preliminary Static Culture of CIK Cells

Preliminary MNCs culture is required to remove adherent cells and obtain the suspension lymphocyte portion of MNCs *(Figure 1)*; which would later be differentiated into CIK cells. PBMCs were seeded at a density of 2×10^6 cells/mL and were allowed to adhere to tissue culture flask (Corning®, USA) containing 10 mL of RPMI-1640 medium for 2 hours in a humidified incubator (Shel Lab®, USA) at 37°C and 5% CO_2. After 2 hours, each flask contents were transferred to a previously labeled 50 mL centrifuge tube and centrifuged at 2000 rpm for 15 minutes to wash the suspended cells.

2.2.4. Final Culture of CIK Cells

For the generation of CIK cells, suspended MNCs were cultured at 37˚C and 5% CO_2 in RPMI-1640 medium containing 100 µL Pen/Strep/ Fungizone (10 µL/ 1 mL media) and IFNγ (1,000 U/mL) on day zero. 50 ng/mL human monoclonal anti-CD3 antibody and 300 U/mL IL-2 were added on day 1. Every 3 days, cells were counted, examined microscopically, and maintained at a density of 1×10^6 cells/mL with pre-warmed complete nutrient medium supplemented with IL-2. Fetal calf serum was not used in this culture protocol as it has many drawbacks and leads to serious misinterpretations in immunological studies [24, 25].

2.2.5. Suspension Culture Passage

When cultures were confluent, i.e. cells aggregate together and the medium appears unclear when the flask is swirled gently (~2.5×10^6 cells/mL), the culture was passaged. Aseptically half of the volume of cell suspension was removed and was placed into a new flask. Each flask was fed with 7 to 10 mL pre-warmed freshly prepared complete nutrient RPMI-1640 supplemented with IL-2.

2.3. Identification of CIK Cells

2.3.1. Morphologically

Microscopic examination of morphological changes, growth rate, count, and viability of MNCs during culture was carried out using an inverted microscope (Carl Zeiss®, Germany).

2.3.2. Phenotypically

CIK cells related surface phenotypes, CD3 and CD56, were identified by means of flow cytometric analysis on day 0, 7, and 14 of culture by FACS Calibur (Becton Dickinson, USA)

2.3.3. Functionally

The function of CIK cells was examined by investigation of TNFα secretion in vitro in the culture supernatant by ELISA assay and the cytotoxic effect of CIK cells in vitro on HCC cell line by using cell counting kit-8 (CCK-8) assay. CCK-8 allows a convenient assay using a water soluble tetrazolium salt, which produces a water soluble orange formazan dye upon bio-reduction by cellular dehydrogenases. The amount of formazan produced is directly proportional to the number of living cells.

2.4. Statistical Analysis

Analysis of data was performed with Statistical Package for Social Science (SPSS) version 19. The results of dependent variables were expressed as mean ± SD. The overall statistical significance of the difference between the groups' means was assessed by one-way analysis of variance (ANOVA) test followed by post hoc test to detect which means pairs are statistically significantly different. Regression between survival rate and CIK:HepG2 ratio was done using linear regression analysis. Correlation between measured variables was evaluated using Pearson's correlation coefficient. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Morphological Characterization of CIKs

During the entire culture period, the CIK cells' growth and maturation were observed under the inverted microscope. On day 4 of culture, microscopic examination of cultured MNCs showed that the cells began to grow double in number. Induced MNCs appeared as discrete single cells, round, and refractive to light. The onset of cluster-like formation of CIK cells could be observed on day 7 after first seeding of suspended MNCs. On day 10 of culture, most of the incubated cells began to grow and cluster together. On day 14, induced MNCs increased in number and form suspended large clusters which were a distinctive feature of mature CIK cells *(Figure 1)*.

Cell Count and Viability

It was found that the separated PBMCs count ranged from 30×10^6 to 34×10^6cell/mL and the lymphocytes account for 60.8 to 67.6% of total separated PBMCs. The viability range upon separation was 95–98%. ANOVA test results *(Table 1, Figure 2)* showed that the effect of culture duration on MNCs density *i.e.* growth rate, count, and viability was significant (P <0.001). The growth rate of induced MNCs *(Figure 2a)* was significantly increased on day 4 with culture time till reached maximum on day 7 then there was a significant decrease over days 10 and 14 (P <0.001), while the count of induced MNCs *(Figure 2b)* did not differ significantly between day zero and day 4 (P=0.291), but there was a very statistical significant increase in count on days 7, 10, and 14 (P <0.001). MNCs viability *(Figure 2c)* did not differ significantly on day 4 (P=0.275), 7 (P=0.323), and 10 (P=0.733), but it was significantly decreased from day 10 to day 14 (P=0.031).

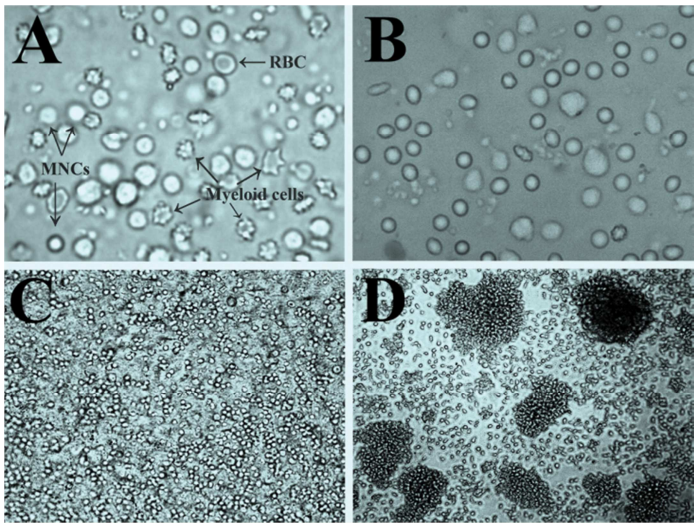

Figure 1. *Inverted microscopic images of PBMCs. (a) Before the preliminary static culture, showing round mononuclear cells along with round biconcave red blood cells (RBCs) contamination and irregularly shaped myeloid cells. (b) After the preliminary static culture, showing single discrete round cells with a clear center and refractive to light (c) After 7 days, the onset of cluster-like formation of CIK cells could be observed as the suspended cells began to clump together. (d) After 14 days, large suspended clusters of fully mature CIK cells were observed. (PBMCs images were obtained by an inverted microscope x100 (a and b) and x40 (c and d).*

Table 1. *Relation between density, number, and viability of induced MNCs and duration of culture. Data are presented as mean ± standard deviation. One-way ANOVA test was applied, followed by Tukey HSD post hoc test, (P <0.05), total sample size=20. †: Overall significance effect of culture duration on the dependent variable a: Significant increase versus the preceding time point. b: Significant decrease versus the preceding time point.*

Culture duration	MNCs density† (x 10^6 cells/mL)	MNCs number† (cells/flask x10^6)	Viability† (%)
Day 0	1	10	96.901 ± 1.50
Day 4	2.090 ± 0.100 [a]	20.900 ± 1	94.414 ± 0.80
Day 7	3.740 ± 0.124 [a]	74.800 ± 2.487 [a]	92.252 ± 0.93
Day 10	3.090 ± 0.139 [b]	123.600 ± 5.713 [a]	90.901 ± 1.84
Day 14	2.795 ± 0.190 [b]	223.600 ± 15.588 [a]	87.251 ± 2.38 [b]

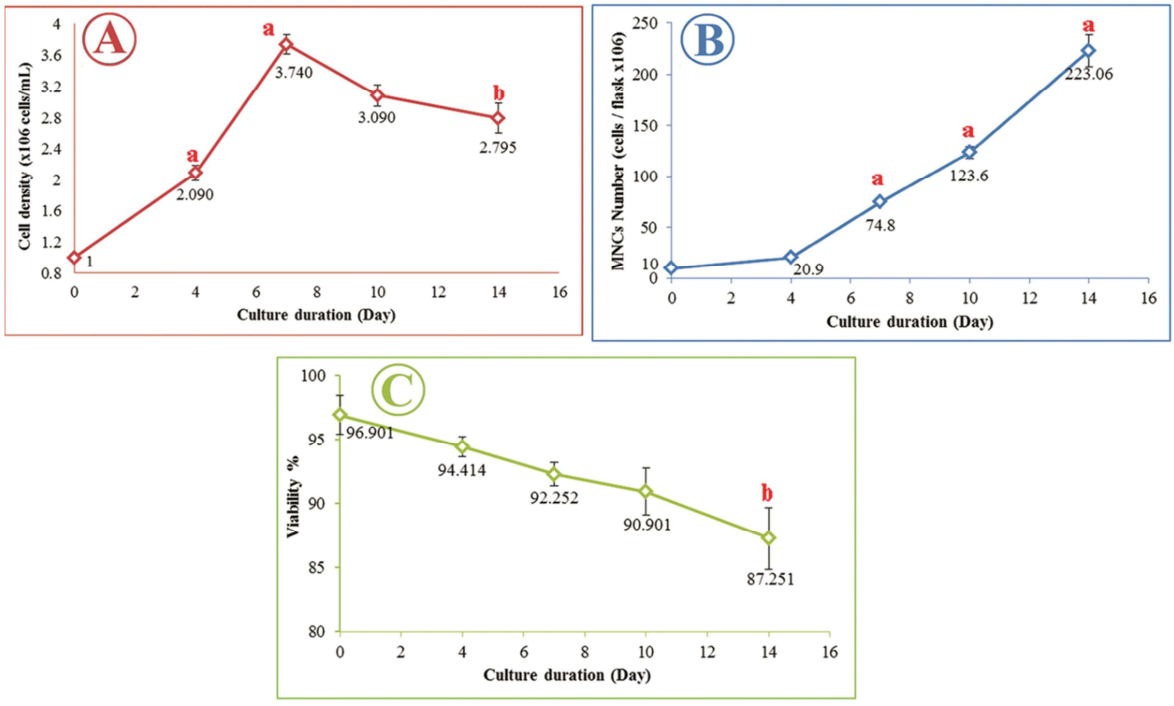

Figure 2. *Relation between density, number, and viability of induced MNCs and duration of culture. (a) Cell density, (b) Number of induced MNCs, (c) Viability. Data are presented as mean ± standard deviation. One-way ANOVA test was applied, followed by Tukey HSD post hoc test, (P <0.05), total sample size=20. a: Significant increase versus the preceding time point, b: Significant decrease versus the preceding time point.*

3.2. Phenotypical Characterization of CIKs

On day zero, MNCs were positive for CD3 (6.253 ± 3.357%) and CD56 (1.737 ± 0.274%). The percentages of T cells and NK cells were 5.867 ± 3.435 and 1.350 ± 0.226, respectively; while the percentage of NKT cells was 0.387 ± 0.091. The results showed that the expression of both CD3 and CD56 phenotypes were significantly affected by culture duration as determined by one-way ANOVA test (P <0.001). The expression of CD3 was significantly increased on day 7 (21.047 ± 2.912, P=0.042) and on day 14 (72.220 ± 8.698, P <0.001). The expression of CD56 showed more significant increase than CD3 on day 7 compared to day zero (12.123 ± 1.625, P=0.003) and on day 14 compared to day 7 (31.203 ± 3.500, P <0.001) *(Figure 3)*.

According to previous phenotypic results, percentages of T cells, NK cells, and NKT cells were estimated on day 7 and 14. One-way ANOVA analysis showed an overall statistically significant effect of culture duration on the expression of T cells, NKs, and NKT cells (P <0.001). Tukey HSD post hoc test *(Table 2 and Figure 3)* indicated that the percentage of T cells was insignificantly increased on day 7 (P=0.084), but it was significantly increased on day 14 (P <0.001). The percentage of NKs was significantly increased on day 7 (P <0.001), but it did not show a significant increase from day 7 to day 14 (P=0.523). The percentage of the most cytotoxic subset under investigation, $CD3^{+}CD56^{+}$, indicated that there was a significant increase in expression of NKT cells on day 7 (6.593 ± 0.843, P=0.029) and on day 14 (25.137 ± 3.656, P <0.001).

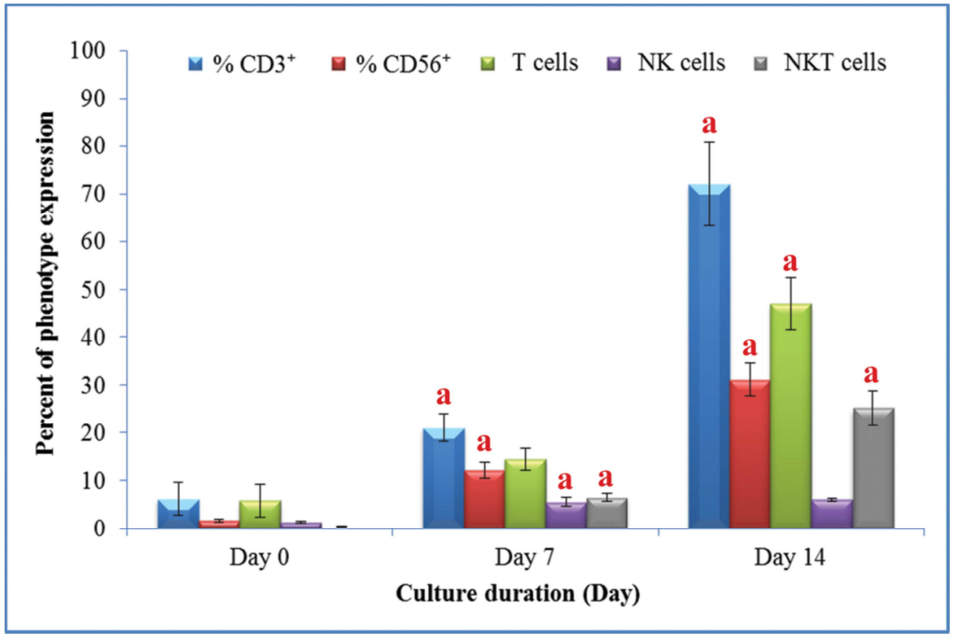

Figure 3. *Relation between the percent of phenotype expression on induced MNCs and culture duration. Data are presented as mean ± standard deviation. One-way ANOVA test was applied, followed by Tukey HSD post hoc test, (P <0.05), total sample size=9. a: Significant increase versus the preceding time point.*

Table 2. *The percentages of T cells, NK cells, and NKT cells on days 0, 7, and 14 of culture. Data are presented as mean ± standard deviation. One-way ANOVA test was applied, followed by Tukey HSD post hoc test, (P <0.05), total sample size=9. #: Overall significance effect of culture duration on the dependent variable a: Significant increase versus the preceding time point.*

Culture duration	% T cells †	% NK cells†	% NKT cells†
Day 0	5.867 ± 3.435	1.350 ± 0.226	0.387 ± 0.091
Day 7	14.453 ± 2.242	5.530 ± 0.934 [a]	6.593 ± 0.843 [a]
Day 14	47.083 ± 5.516 [a]	6.067 ± 0.247	25.137± 3.656 [a]

3.3. Functional Characterization of CIK Cells

3.3.1. Tumor Necrosis Factor Alpha Secretion

Tumor necrosis factor alpha was measured in culture supernatant at the beginning of the trial, day zero, and at the end of it, day 14. The result of TNFα concentration, on day zero, was *Nil*, *i.e.* undetectable. On day 14, the ELISA assay was performed on four different samples using a microplate reader at 450nm (Unicam®, United Kingdom). Pooled mean ± pooled standard deviation of TNFα concentration, calculated from 4 samples in octuplicate by the following equations, was 14.538 ± 6.672 pg/mL.

$$\text{Pooled mean} = \frac{(N_1M_1+N_2M_2+N_3M_3+N_4M_4)}{(N_1+N_2+N_3+N_4)} \quad (1)$$

Pooled standard deviation =

$$\sqrt{\frac{(N_1-1)S_1^2+(N_2-1)S_2^2+(N_3-1)S_3^2+(N_4-1)S_4^2}{(N_1+N_2+N_3+N_4-4)}} \quad (2)$$

Where N: number of test sample wells, M: mean of test sample wells, S: standard deviation from sample mean

3.3.2. Cytotoxicity Assay of CIK Cells

The cytotoxic effect of CIKs was measured on day 14 on mature CIK cells by using CCK-8 assay. It was investigated on hepatocellular carcinoma HepG2 cell line *in vitro*. HepG2 adherent cells were trypsinized for 10 minutes and were examined for detachment every 3–4 minutes by the inverted microscope. When the detached HepG2 hepatocytes were viewed as floating, compact, round with dense cytoplasm cells, and surrounded by spindle-shaped cells, 9 mL of RPMI-1640 + 1mL fetal bovine serum were added to neutralize the excess trypsin.

The survival rate is a part of survival analysis that represents the percentage of cells in a study or treatment group still alive for a given period of time after using a certain toxic agent. It was calculated by the following equation:

Percent survival rate of HepG2 cells =

$$\frac{A_{sample} - A_{CIK}}{A_{HepG2} - A_{blank}} \text{ X } 100 \quad (3)$$

Where Asample: sample wells, ACIK: CIK wells, AHepG2: HepG2 wells, Ablank: blank wells

The hypothesis test for the null hypothesis of zero slope between the two variables, X (CIK:HepG2 ratio) independent variable and Y (survival rate) dependent variable. One-way ANOVA analysis showed an overall statistically significant effect of CIK:HepG2 ratio on the percentage of HepG2 survival rate (P <0.001). Post hoc analysis using Tukey HSD test indicated that HepG2 survival rate at (5:1) ratio was insignificantly decreased from (1:1) ratio, yet it was significantly decreased at (10:1), (20:1), and (40:1) ratios (P <0.001). The minimum survival rate of HepG2 cells was at the CIK:HepG2 ratio of 40:1, which in turn means that the cytotoxic effect of CIK cells at (40:1) ratio was 58.889 ± 1.104 %.

3.4. Results of Regression Analysis

Regression analysis indicated that the survival rate is negatively correlated with the CIK:HepG2 ratio (r = –0.989) and that regression model could statistically significantly predict the outcome variable, the survival rate of HepG2 cells based on CIK:HepG2 ratio (P=0.001). A Significant regression equation was used to describe the statistical relationship between dependent variable (Y), survival rate, and independent variable (X), CIK:HepG2 ratio *(Figure 4)*, with a significant slope (P=0.001) and significant Y-intercept (P <0.001). Since the slope of the regression line was significantly different from zero, the null hypothesis of zero slope between the variables could be rejected and accept the alternative hypothesis that there is a significant relationship between the independent and dependent variables.

The regression equation was estimated [Y = – 1.559 X + 101.027]; where (–1.559) was the slope and (101.027) was the Y-intercept. From this equation, the CIK:HepG2 ratio required to achieve zero survival rate of HepG2 cells could be predicted. If Y is replaced with zero, then the X value would be 64.8. This means that at a CIK:HepG2 ratio of 65:1, the survival rate of HepG2 cells would be zero.

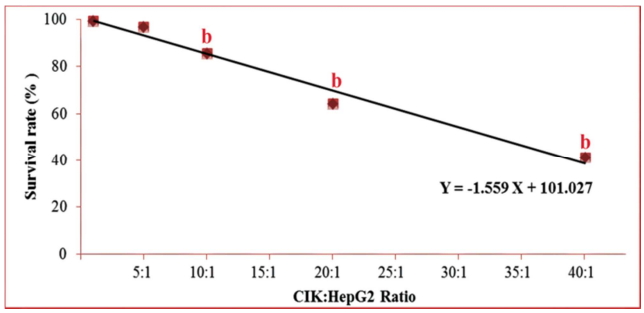

Figure 4. *Linear regression chart representing survival rate (%) of HepG2 cells against different ratios of CIK:HepG2 cells. One-way ANOVA test was applied, followed by Tukey HSD post hoc test, (P <0.05), total sample size=15. Linear regression analysis, (P <0.05), n=5. b: Significant decrease versus the increase in CIK:HepG2 ratio.*

3.5. Results of Correlation Analysis

Pearson's correlation analysis was applied to indicate the correlation between measured variables in the study *(Table 3)*. Duration of CIK cells culture showed a significant positive correlation with the number of induced MNCs (P=0.009), T cells expression (P=0.009), NK cells expression (P=0.025), and NKT cells expression (P=0.008) and a significant negative correlation with the viability of induced MNCs (P <0.001). Number of induced MNCs over the entire culture period showed a significant positive correlation with T cells expression (P=0.001), and NKT cells expression (P=0.001); while there was a significant negative correlation with the viability of induced MNCs (P=0.005). The growth rate of induced MNCs showed a significant positive correlation with NK cells expression (P=0.032). The viability of MNCs showed a negative correlation with T cells expression (P=0.009), NK cells expression (P=0.025), and NKT cells expression (P=0.008). T cells expression showed a positive significant correlation with the expression of NKT cells (P <0.001).

Table 3. *Results of Correlation Analysis. r: Pearson correlation coefficient, n=5. p: P value, p (2-tail) <0.05 was considered to be statistically significant. *: Significant correlation, +: Positive correlation, –: Negative correlation.*

		Culture duration	MNCs number	MNCs density	MNCs viability	T cells	NK cells	NKT cells
Culture	r	+1	+0.961*	0.690	–0.995*	+0.961*	+0.924*	+0.965*
duration	P		0.009	0.197	< 0.001	0.009	0.025	0.008
MNCs number	r	+0.961*	+1	0.517	–0.973*	+0.989*	0.802	+0.989*

		Culture duration	MNCs number	MNCs density	MNCs viability	T cells	NK cells	NKT cells
	P	0.009		0.372	0.005	0.001	0.103	0.001
MNCs density	r	0.690	0.517	+1	–0.666	0.474	+0.910*	0.489
	P	0.197	0.372		0.220	0.420	0.032	0.403
MNCs viability	r	–0.995*	–0.973*	–0.666	+1	–0.963*	–0.903*	–0.967*
	P	< 0.001	0.005	0.220		0.009	0.036	0.007
T cells	r	+0.961*	+0.989*	0.474	–0.963*	+1	0.788	+0.999*
	P	0.009	0.001	0.420	0.009		0.113	< 0.001
NK cells	r	+0.924*	0.802	+0.910*	–0.903*	0.788	+1	0.799
	P	0.025	0.103	0.032	0.036	0.113		0.105
NKT cells	r	+0.965*	+0.989*	0.489	-0.967*	+0.999*	0.799	+1
	P	0.008	0.001	0.403	0.007	< 0.001	0.105	

4. Discussion

Hepatocellular carcinoma is a disruptive cancer that occurs as an end result of chronic liver disease and cirrhosis [4]. Malignant liver cells often survive traditional treatment such as radiation and chemotherapy. Most importantly, small lesions and metastatic cells often remain and cause recurrence of disease [26]. Concomitant liver dysfunction with advanced tumor stages further impedes curative therapies. Thus, available treatments of this disease are highly complex, as they not only include the tumor biology and anatomic considerations within the liver but also the underlying function of the liver and the patient's functional status [10]. Immunotherapy is a new and promising treatment for a number of cancers. Cell-based immunotherapy is a set of therapeutic strategies based on manipulating and co-opting a patient's own immune cells, or donor cells and using immune cell functions to halt and reverse disease [27].

The present study evaluates the potential of *in vitro* expansion of viable CIK cells from human PBMCs and measures the proportion of the most effective subset $CD3^+CD56^+$ in the culture. In addition, the study examines TNFα secretion and the cytotoxicity of expanded CIK cells on HepG2 cell line. Different culturing protocols for CIK cells were reported; yet all these protocols share the main three growth factors pillars used in CIK culture, which are, IFNγ, IL-2, and monoclonal anti-CD3 antibody. The differences between them relied on different MNCs origin, nutrient growth medium, protein serum, growth factors' concentrations or different cytokines combinations.

In this study, the suspended subset of PBMCs, obtained from healthy volunteers, were cultured in RPMI-1640 medium without addition of protein serum to cultured cells, either in the form of fetal calf serum or as heat-inactivated plasma, as the foreign proteins may lead to sensitization of killer cells to react with them. This finding was in line with Kerbel and Blakeslee [24] and Reddy et al. [25], who reported that fetal calf serum should not be added to cell cultures as it causes many serious misinterpretations in immunological researches. Suspended cells were cultured for 14 days with IFNγ, monoclonal anti-CD3 antibody, and IL-2; which is T cell growth factor that promotes naïve T cells to differentiate into effector T cells, modulates differentiation of Th cells, promotes Treg cells development, augments cytolytic activity of NK, and regulates effector versus memory T cell generation [28].

Allogeneic protocols were reported by Iudicone et al. [29] and Guo et al. [30]. Autologous protocols were also reported by Niam et al. [31], Liu et al. [32], and Chan and Linn [33], who have obtained CIK cells from patients with myeloid leukemia, hepatocellular carcinoma, and polycythemia, respectively. According to Zhang et al. [34], the antitumor efficacy of autologous CIK cells derived from cancer patients was lower because of the immunosuppressive state of patients, compared with the allogeneic CIK cells obtained from adult healthy individuals. Protocols involved cord blood MNCs differentiation into CIK cells were also reported by Zhang et al. [35] and Durrieu et al. [36], who reported that CIK cells differentiated from cord blood differ in some receptors' expression and cytotoxic pathways, suggesting that the source of CIK cells may impact on therapeutic and cytotoxic efficacy.

The hypothesis of the protein serum effect on cultured CIK cells was followed by Guo et al. [30], Meng et al. [21], and Li et al. [37]. On the contrary, Niam et al. [31] used a complete nutrient medium of RPMI-1640 and 10% fetal calf serum, claiming that this was the optimal medium for CIK cell expansion, better than other serum-free MNCs culture media, despite that, the $CD3^+CD56^+$ effective subset reported comprised a median of 26.6%. Wei et al. [38] and Liu et al. [32] also used serum proteins, 10% fetal calf serum and 1% heat-inactivated plasma, respectively, during their CIK culturing protocols.

Other culturing protocols for CIK cells to enhance proliferation or selective tumor toxicity with different cytokines combinations were reported. Helms et al. [39] used IL-12 to shorten the *in vitro* expansion process for CIK cells and enhance their cytotoxic efficacy. Lin et al. [40] used IL-6 to decrease the proportion of Treg cells which inhibit cellular immunity against the tumor. Tao et al. [41] used IL-15 instead of IL-2 for *in vitro* CIK cells culture in order to enhance CIK cells–mediated cytotoxicity against leukemia. Rajbhandary et al. [42] used IL-21 to enhance the cytotoxic effect of CIK cells through increased expression of IFNγ, TNFα, granzyme B, and perforin. Ingersoll et al. [43] used IL-7 to expand cytotoxic CIK cells that improved survival in a xenograft mouse model of ovarian cancer. Iudicone et al. [29] used IL-15 on the 7^{th} day of culture instead of IL-2 to enhance CIK cells–mediated cytotoxicity against epithelial cancer cell lines.

During the entire culture period in the current study, the

inverted microscope was used to observe the growth and maturation of CIK cells. The onset of proliferation may be due to the direct effect of IL-2 that promoted naïve T cells to differentiate into effector cells [28] and the indirect effect of IFNγ mediated by monocytes activation, providing soluble proliferating factors (IL-12) and the initial mitogenic signal provided by the monoclonal anti-CD3 antibody on day 1 and sustained by the continuous presence of IL-2 every 3 days along the entire culture period [44]. The onset of cells maturation, cluster-like formation, could be observed on day 7, while fully matured suspended CIK clusters could be observed on day 14.

This goes with the results reported by Li et al. [45], who cultured MNCs for 14 days and found that the cells began to proliferate within 3 days and became fully matured as colonies in suspension within 14 days. On the other hand, these findings were not in line with Bonanno et al. [46], Niam et al. [31], and Wei et al. [38], who cultured human PBMCs for 21, 28, and 15 days, respectively, to reach the maturation stage. In addition, Chan and Linn [33] cultured human PBMCs for 26 days and they started counting and evaluating morphological and phenotypical changes of CIK cells on day 10 as they assumed that it is too early to assess cultured CIK cells before day 10.

In the present study, the count and viability of MNCs were reported every 3 days, before adjusting cell density. The results showed a significant increase in cells number with culture duration. This finding was not in line with Niam et al. [31], who reported that the number of CIK cells dropped to a median of 0.44 fold of starting number at the first counting at day 11 of culture, after which growth started from about day 14 and approached a plateau by day 28. The reported drop in number in his study was likely to be as a result of supplementing cell culture with more complete media and IL-2 at day 7, without adjustment to cell density. Thus, the crowded cell media and increased waste products led to cells death. When the cell density had been adjusted, from day 10 onward, the cells grew and showed obvious growth count from about day 14. The viability of induced PBMCs in this study was decreased on day 14 significantly with time to 87.251 ± 2.38%. These results go in compliance with Luo et al. [47], who cultured CIK cells from isolated PBMCs with cell viability (> 90%) and reported cell viability, about 2 weeks later, to be (> 85%).

This study aims at measuring the proportion of the most effective subset $CD3^+CD56^+$ in the culture. The results of CD3 and CD56 phenotypes showed a very significant increase in expression versus culture duration. This finding was supported by Mata-Molanes et al. [17], who reported that after 14 days of culture, the percentage of $CD3^+CD56^+$ subset reaches 20 to 30% of the total CIK cells. Guo et al. [30], who cultured CIK cells from healthy volunteers' blood donors for 14 days, reported $CD3^+CD56^+$ proportion on day 14 as 25.31 ± 7.42%. Li et al. [37], who cultured CIK cells from patients with early-stage melanoma blood for 14 days, reported $CD3^+CD56^+$ proportion on day 14 as 21.8 ± 8%. On the other hand, these results did not go in line with Bonanno et al. [46], who cultured PBMCs with different concentrations of anti-CD3 antibody (50, 250, and 250 ng/mL) reported that the proportion of $CD3^+CD56^+$ subsets on day 21 were 69.6%, 47.9%, and 29.3%, respectively.

The functional assays for CIK cells include the production of cytokines and the CIK cells' cytotoxic effect on HepG2 cell line *in vitro*. TNFα is one of the main cytokines produced by CIK cells and should be fit as a test for cytokines secreted by CIK cells [21]. TNFα can transmit apoptotic signals inside cells that can be killed by ligand binding. It is believed that TNFα usually gives the signal for cell survival; however, due to other signals, some tumor cells can undergo apoptosis [48].

In the present work, TNFα concentration, on day 14, was 14.538 ± 6.672 pg/mL. The present result was supported by Zhang et al. [34], who investigated the secretion of TNFα from expanded umbilical cord – CIK cells. The reported result was much less than that of the present study (6 ± 5.5 pg/mL); which may be due to different CIK cells origin [35] or the usage of fetal calf serum that affect immunological studies [24, 25].

The CIK cells' cytotoxic effect on cancerous cells showed variable degrees of efficacy in several tumors including malignant lymphoma either Hodgkin's disease or non-Hodgkin's lymphoma [49], hematological malignancies as acute myeloid and acute lymphocytic leukemia [50] and chronic lymphocytic leukemia [51]. Recent *in vitro* studies had further shown the potential activity of CIK cells differentiated from the blood of healthy or patient volunteers against breast cancer [30], pancreatic cancer [52], sarcomas [53], ovarian cancer [44], metastatic melanoma [54], glioblastoma or brain cancer [55], solid tumors [45], and gallbladder cancer [56]. In this study, the cytotoxic effect of CIK cells was investigated on HCC *in vitro*. The results showed a significant cytotoxic effect of CIK cells on HepG2 cells. We also could predict the CIK:HepG2 ratio required to achieve a complete cytotoxicity HepG2 cells, that is, 65:1.

5. Conclusion

Collectively, the present study has provided data to support the ongoing practice of generating CIK cells from human PB, which is an important and promising strategy for future work involving HCC immunotherapy. PB-derived MNCs can differentiate into CIK cells *in vitro* when cultured in complete nutrient media containing IFNγ, anti-CD3 antibody, and IL-2. The CIK culture showed different proportions of effector and cytotoxic subsets T cells, NK cells, and NKT cells. CIK cells showed high functional capacity as evidenced by secretion of cytokine TNFα and cytotoxicity against HCC cell line, HepG2. This study provides a simple and easily handled strategy for *in vitro* differentiation of human PB-derived MNCs into CIK cells. Further studies are also needed to address the *in vivo* anti-cancer immune response, toxicity of CIK cells to normal and cancerous cells, and the predictive value of many other factors related to malignant cells or tumor microenvironment.

Disclosure of Interest

The authors declare no conflict of interest. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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