

Autologous cytokine-induced killer cell therapy in clinical trial phase I is safe in patients with primary hepatocellular carcinoma

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Abstract

AIM: To investigate the influence of autologous cytokine-induced killer (CIK) cells on the phenotypes of CIK effector cells, peripheral T lymphocyte subsets and dendritic cell subsets in patients with primary hepatocellular carcinoma (HCC).

METHODS: Peripheral blood mononuclear cells (PBMC) were collected by a blood cell separator from 13 patients with HCC, then expanded by priming them with interferon-gamma (IFN- γ) followed by monoclonal antibody (mAb) against CD3 and interleukin-2 (IL-2) the next day. The phenotypic patterns of CIK cells were characterized by flow cytometry on d 0, 4, 7, 10, 13 and 15 of incubation, respectively. Then, 5 mL of venous blood was obtained from HCC patients before or 8-10 d after CIK cells were transfused into patients to assess the influence of CIK cells on the percentages of effector cells, and proportions of DC1 or DC2 in peripheral blood by flow cytometry.

RESULTS: After two weeks of *in vitro* incubation, the percentages of CD3⁺CD8⁺, CD3⁺CD56⁺, and CD25⁺ cells increased significantly from 33.5 \pm 10.1%, 7.7 \pm 2.8%, and 12.3 \pm 4.5% to 36.6 \pm 9.0% (P <0.05), 18.9 \pm 6.9% (P <0.01), and 16.4 \pm 5.9% (P <0.05), respectively. However, the percentages of CD3⁺CD4⁺ and NK cells had no significant difference. The percentages of CD3⁺ and CD3⁺CD8⁺ cells were kept at high levels during the whole incubation period, but those of CD25⁺, and CD3⁺CD56⁺ cells began to decrease on d 7 and 13, respectively. The proportions of type I dendritic cell (DC1) and type II dendritic cell (DC2) subsets increased from 0.59 \pm 0.23% and 0.26 \pm 0.12% before CIK cell therapy to 0.85 \pm 0.27% and 0.43 \pm 0.19% (all P <0.01) after CIK cell transfusion, respectively. The symptoms and characteristics of HCC patients were relieved without major side effects.

CONCLUSION: Our results indicated that autologous CIK cells can efficiently improve the immunological status in HCC patients, and may provide a potent approach for HCC patients as the adoptive immunotherapy.

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INTRODUCTION

Primary hepatocellular carcinoma (HCC) represents one of the most lethal neoplasms worldwide with a particularly high prevalence in China^[1]. Chronic viral hepatitis patients, especially hepatitis B or C patients, often fall victims to liver cirrhosis and subsequent HCC^[2,3]. The high percentage of chronicity may be due to the active combative mechanisms of the virus. In cirrhotic patients, the incidence of HCC annually has been reported to be between 2% and 7%. These findings indicate that prevention and early treatment of liver cancer, especially HCC, are an urgent and important issue.

HCC patients are often found to have functional deficiency in host adaptive immunity response and innate immunity response^[4]. Current therapeutic regimens including surgery, chemotherapy and radiotherapy for HCC often have very limited efficacy, and tumors tend to relapse or metastasize easily. Combination therapy becomes the most important means for treating HCC patients. Antitumor immunity is mainly dependent on cellular immune response. Therefore, cellular immunity dysfunction is one of the reasons why tumors are incurable, and easy to relapse or metastasize. Cytokine-induced killer (CIK) cells are shown to be a heterogeneous population, and the major population expresses both the T cell marker CD3 and the NK cell marker CD56, and is termed NKT cells. Cells with this phenotype are rare (1% to 5%) in natural peripheral blood mononuclear cells (PBMC)^[5]. CD3⁺CD56⁺ cells are able to expand nearly 1 000-fold when they are cultured with a cytokine cocktail comprising interferon- γ (IFN- γ), interleukin-2 (IL-2), mAbs against CD3, and interleukin-1 α (IL-1 α), and have a characteristic which is more effective in the treatment of tumors with a non-major histocompatibility complex (MHC)-restricted mechanism, and a most effective project^[6]. We have previously reported that CIK cells could suppress the growth of tumor cells *in vitro* when HCC cells were transplanted in mice^[7-10].

Dendritic cells (DCs) are specialized antigen-presenting cells (APC) in the immune system. They are critical for exerting T cell mediated immune responses, activating naïve T cells, and playing a critical role in innate immune response and adaptive immune response^[11]. DCs capture tumor-associated antigens (TAA) efficiently in peripheral tissues, transport these TAA from peripheral sites to primary and secondary lymphoid organs, express high levels of MHC I and MHC II molecules that present the processed TAA epitope specific T cells, express high levels of costimulatory CD80 and CD86 which are required to activate naïve and memory T cells, and synthesize important immunomodulatory mediators such as, IL-12, IFN- α , tumor necrosis factor (TNF)- α . DC contains at least two major distinct subsets, DC1 or DC2, which have mutually exclusive phenotypes and functions. DC1 is APC, and DC2 has been identified as the principal producer of IFN- α , a key cytokine involved in clearance of viral infections. They play a critical role in antiviral or antitumor immune response^[12,13]. We have recently reported that CIK cells could suppress the growth of HCC cells in animals or *ex vivo* effectively. In this study, we investigated the alterations of peripheral T lymphocyte subsets

and DC subsets in HCC patients for initial evaluation of autologous CIK therapy efficacy.

MATERIALS AND METHODS

Subjects

Thirteen patients (12 males and 1 female), mean age 46.8 ± 7.3 (range, 30-53) years, with confirmed diagnosis of HCC were recruited based on biochemical analysis and imaging examinations, such as ultrasonography, computed tomography and angiography. The general clinical data of HCC patients are summarized in Table 1. All cases were patients with hepatocirrhosis with more than 20 years of chronic HBV infection. After written informed consent was obtained from each patient, the patients with HCC began to receive CIK cell therapy protocol, which was approved by the Department of Health of Chinese PLA.

Reagents

Serum-free AIM-V medium was purchased from Invitrogen Corporation (GIBCO, USA). Recombinant human IL-2 (rhIL-2) was purchased from Beijing Red United Cross Pharmaceutical Co., LTD (China). Anti-CD3Ab was purchased from CIMAB (Cuba). Recombinant human IFN- γ (rh IFN- γ) was obtained from Shanghai Clonbiotech Co., LTD (China). Human albumin was obtained from Brief Introduction To Sino-Foreign Joint Venture, Harbin Sequel Bio-Engineering Medicine Co., LTD (China).

Isolation and culture of CIK cells

By using a blood cell separator (Spectra v 6.1, Cobe, USA), $(2-4) \times 10^9$ PBMC cells from each patient were obtained in a total volume of 50-60 mL. Cellular concentration was adjusted to 2×10^6 /mL in fresh serum-free AIM-V medium, and incubated at 37 °C in a humidified atmosphere of 50 mL/L CO₂ in the Lifecell tissue culture flask (Nexell Therapeutics Inc., USA). To generate CIK cells, 2 000 U/mL rhIFN- γ was added on the initial day. After 24 h of incubation, 50 ng/mL mAb against CD3, and 1 000 U/mL rhIL-2 were added. Fresh IL-2 and fresh AIM-V media were replenished every 3 d. On d 0, 4, 7, 10, 13, and 15, cell densities were determined, and the phenotypes were identified by flow cytometry (Becton Dickinson, USA), respectively. Cells were transfused back into HCC patients on days 10, 13, 15, respectively.

Surface marker analysis of CIK cells in cultured or peripheral blood

Incubated CIK cells were collected, washed, and stained with mouse against human CD3 and CD25 (mAbs) coupled to FITC respectively, and mAbs against CD4, CD8, CD16, CD19, and

CD56 coupled to PE (Becton Dickinson, San Jose, CA). Non-specific binding was determined using irrelevant mouse immunoglobulin isotypes IgG1-FITC, IgG2-FITC and IgG1 RD. Cells were incubated with Abs for 30 min at 4 °C. Excess Ab was removed and the stained cells were washed and analyzed or sorted by flow cytometry.

Analysis of DC subsets or CIK effector cells in peripheral blood

Five millilitre venous blood was obtained from each subject before and after CIK cell transfusion, respectively, to analyze DC subsets or effector cell phenotypes by flow cytometry. Briefly, blood cells were incubated with a lineage (lin) cocktail (anti-CD3, CD4, CD16, CD19, CD20, CD56) conjugated with FITC, PE-conjugated anti-CD11c or -CD123, PerCP-conjugated anti-HLA-DR for 30 min, then treated with FACS lysing solution for less than 10 min, washed by PBS, fixed with 2% paraformaldehyde for 20 min at 4 °C, and analyzed by flow cytometry^[14]. Other blood cells were used as described in cultured CIK cells to analyze the phenotypes of effector cells in peripheral blood.

Preparation and transfusion of CIK cell supernatants

Incubated CIK cells were transfused back into HCC patients via vein 3 times on days 10, 13 and 15, respectively. One-third of all CIK cells each time were collected by centrifugation for 20 min at 1 500 r/min, and washed twice in saline water (containing 5 g/L human albumin and IL-2 at 100 U/mL). About $(3-5) \times 10^9$ cells were resuspended in the same solution with 400-500 mL, then transfused back into patients intravenously.

Statistical analysis

The results were expressed as mean \pm SD. Results were analyzed by using SPSS software, and experiments were designed by self-pair. $P < 0.05$ was considered statistically significant.

RESULTS

Phenotypes of CIK cells in various culture time

The percentages of all effector cells varied over time *in vitro* incubation. The percentage of CD3⁺ T cells increased slowly, and kept a high level for a long time during the incubation period. The percentage of CD3⁺CD4⁺ decreased slightly, but that of CD3⁺CD8⁺ rose gradually. The percentage of CD3⁺CD56⁺ remarkably increased after incubation, and reached a maximum level on day 13, then gradually decreased during the further generations. The percentage of CD25⁺ increased rapidly after harvest, and reached a peak level on d 7, and then decreased rapidly (Figures 1 and 2).

Table 1 Clinical data of 13 hepatocellular carcinoma (HCC) patients

	Male/ Female	Age (yr)	HBV history	HCC stage	HBV viral load (copies DNA/mL)	ALT (U/L)	AFP (μ g/L)	TP (g/L)
1	M	32	10	Advanced	$<10^4$	34	>800	56
2	M	50	20	Advanced	5.02×10^6	40	31.9	65
3	F	52	12	Early	3.07×10^6	28	21.4	66
4	M	50	18	Advanced	1.69×10^5	150	60.6	63
5	M	49	15	Advanced	3.48×10^5	50	>1000	71
6	M	49	26	Advanced	2.02×10^6	35	121.9	73
7	M	45	10	Advanced	2.55×10^6	31	897.9	73
8	M	49	12	Advanced	3.44×10^5	98	<20	58
9	M	49	14	Advanced	1.66×10^5	78	139.6	84
10	M	51	15	Advanced	1.77×10^6	68	950	67
11	M	50	12	Advanced	$<10^4$	37	>1000	56
12	M	30	8	Early	1.26×10^7	50	>1210	68
13	M	53	14	Advanced	9.63×10^5	66	785.2	60

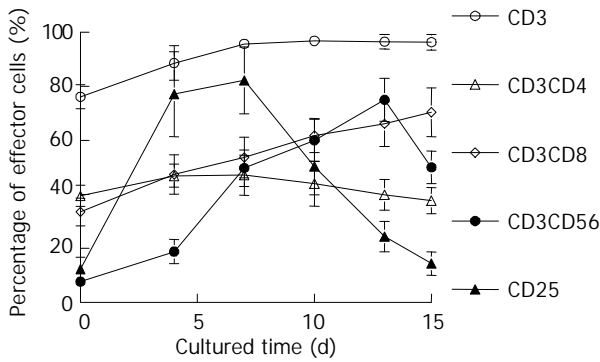


Figure 1 Dynamic analysis of CIK cellular phenotypes by flow cytometry.

Lymphocyte phenotype analysis before or after CIK cell transfusion

Before and 10 d after CIK cell transfusion, PBMCs were obtained from the same patient and analyzed for phenotypes by flow cytometry. Ten days after CIK cell transfusion, the proportions of CD3⁺CD8⁺, CD3⁺CD56⁺ and CD25⁺ in peripheral blood increased significantly from 33.5%, 7.7% and 12.3% to 36.6% ($P<0.05$), 18.9% ($P<0.01$) and 16.4% ($P<0.05$), respectively.

However, the proportions of CD3⁺CD4⁺ or NK cells declined slightly, and there were no significant differences before and after CIK cell transfusion (Figure 3). The longest observation time for patients was 108 d after CIK cell transfusion, others were 20-90 d, and the percentage of lymphocyte subpopulations was the same 8-10 d after CIK cell transfusion, and had no significant difference. Therefore, the proportions of effector cells in peripheral blood might last for more than 108 d. All the patients were under follow-up observation.

Analysis of DC subset proportion before or after CIK cell transfusion

The frequencies of DC1 and DC2 increased from 0.59% and 0.26% before CIK cell transfusion to 0.85% and 0.43% after CIK cell transfusion, respectively, and had a significant difference ($P<0.01$). Therefore, CIK cell treatment could enhance the proportions of DC1 and DC1 in peripheral blood in HCC patients (Figure 4).

HBV viral load analysis before and after CIK cell transfusion

Before CIK cell therapy, the average HBV viral load in HCC patients was 1.85×10^6 copies of DNA/mL. After CIK cell transfusion, the average viral load was decreased to 8.75×10^5 copies of DNA/mL in one month, 1.44×10^5 copies of DNA/mL in 2 mo, and 1.41×10^5 copies of DNA/mL in 3 mo (Figure 5).

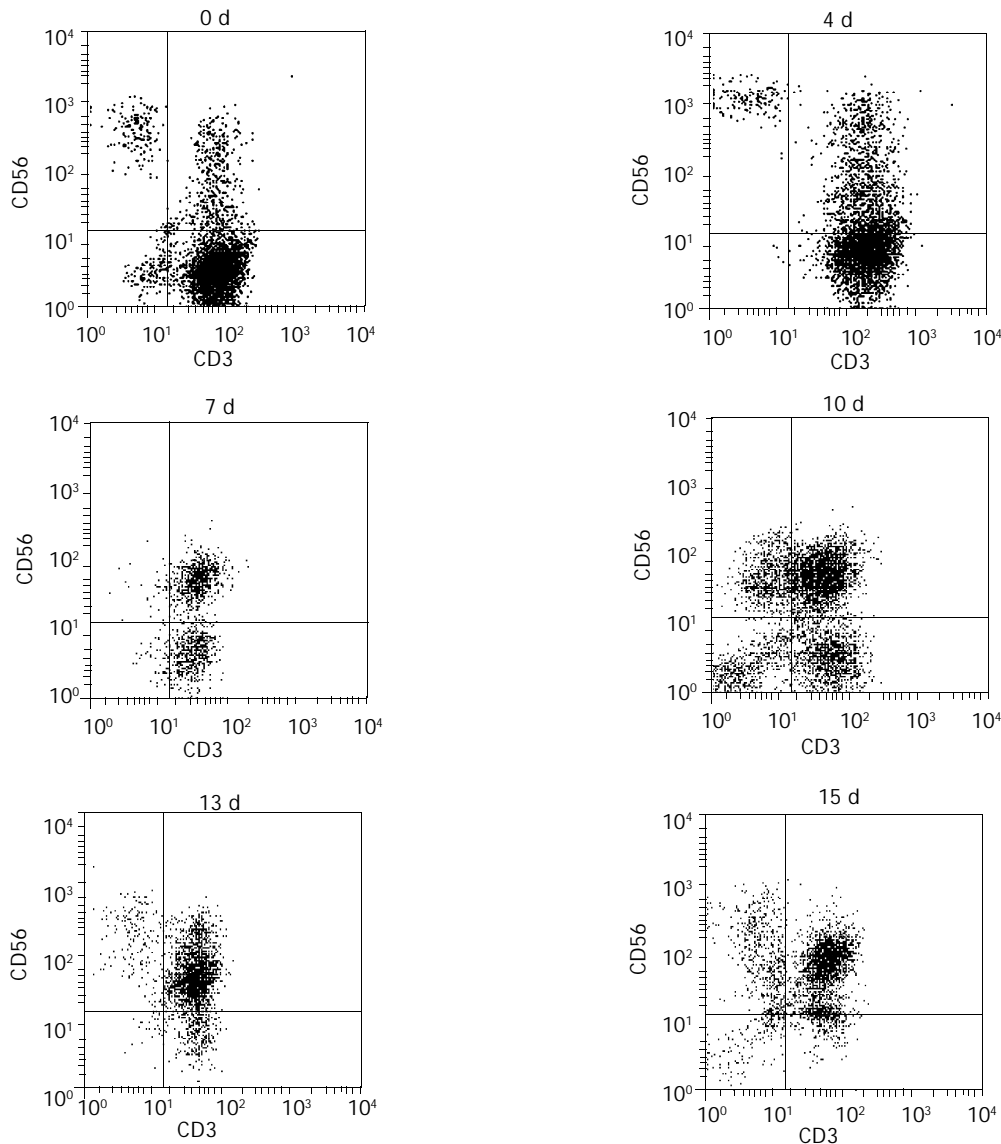


Figure 2 Phenotypic analysis of CD3⁺CD56⁺ cells in cultured cells by flow cytometry in various culture time.

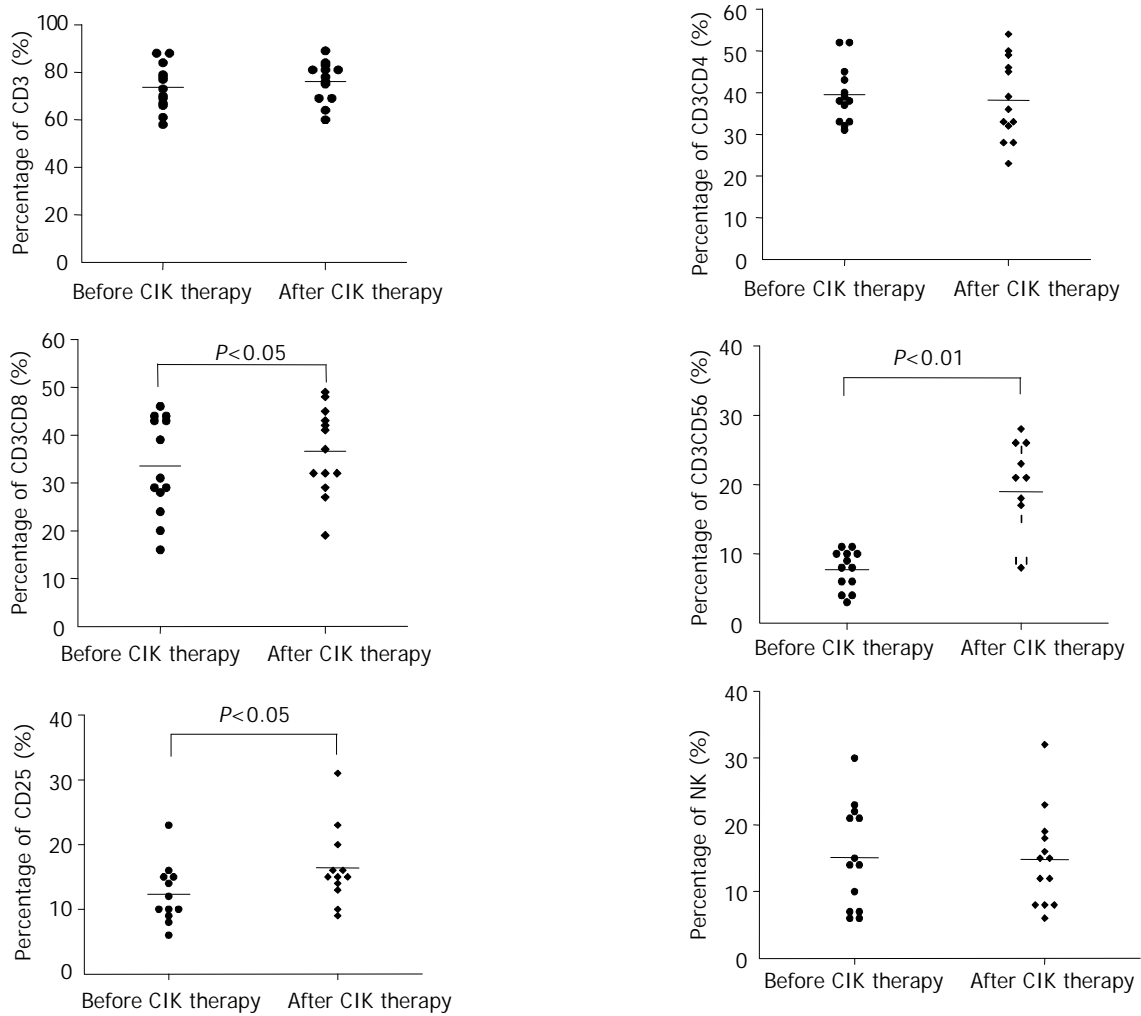


Figure 3 Percentages of lymphocyte subsets in peripheral blood of HCC patients before and after CIK cell therapy.

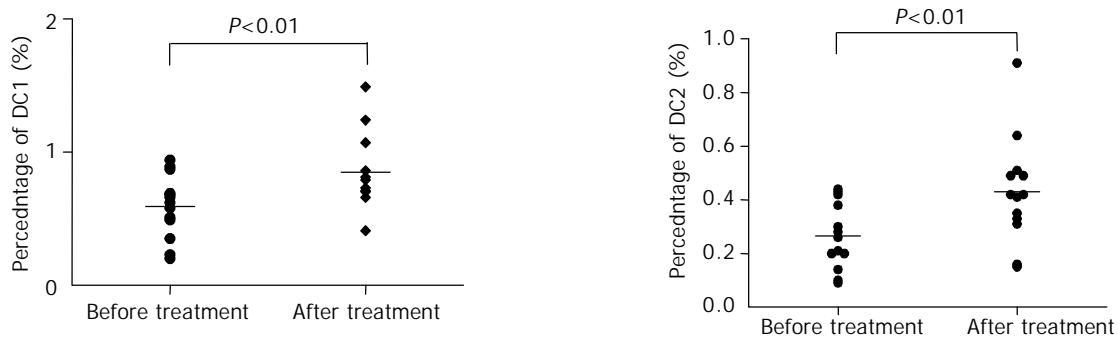


Figure 4 Proportions of DCs in HCC patient peripheral blood before and after CIK cell transfusion.

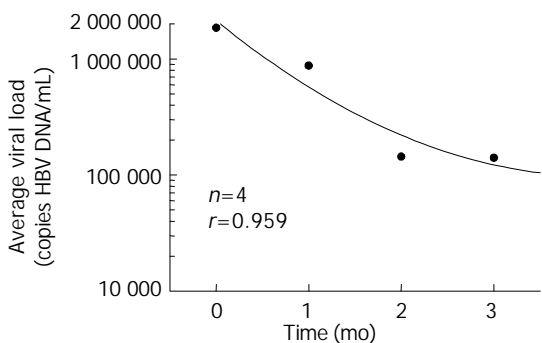


Figure 5 Average HBV viral load in HCC patient serum before and after CIK cell transfusion.

CIK cell therapy efficacy

After autologous CIK cell therapy, ameliorated symptoms, increased appetite, improved sleep and gained body weight were observed in most patients. The growth of tumors in all patients became slow down, the tumor volume was decreased in 3 patients. Most patients developed a fever 6 h after transfusion, and the body temperature was 37.5-40 °C, which could last for 6-8 h. Most fever patients recovered without any treatment. No side effects on liver and kidney were found. Therefore, the autologous CIK cell treatment was a safe and efficacious approach.

DISCUSSION

Heterogenous CIK cells could be generated from PBMC with

stimulation of multiple cytokines. The major marker of CIK cells was CD3⁺CD56⁺, termed NKT cells. It was thought that CIK cells which kill the tumor were non-MHC-restricted, but recently, NKT cells exerting efficient MHC- and non-MHC-restricted cytotoxicity against autologous tumor targets have been reported^[15]. One reason why tumors are difficult to cure is that tumor escapes the host immune monitor. The human immune system against the tumor is mainly dependent on the cellular immunity. The host immune response in HCC patients was significantly suppressed. The cell number and cytotoxicity are necessary for efficient immunotherapy of tumors. Autologous CIK cell immunotherapy is the most efficient approach of neoplasms. Our present study indicated that autologous CIK cell transfusion was safe and efficient to cure liver neoplasms.

In healthy condition, cells with phenotype of CD3⁺CD56⁺ were about 1-5% in peripheral blood^[5]. CD3⁺CD56⁺ cells could expand nearly 10-fold or even more than 100-fold under cytokine cocktail condition, and the cytotoxicity was greatly improved. The percentage of CD3⁺CD8⁺, CD3⁺CD56⁺ and CD25⁺ before CIK cell transfusion increased from 33.5%, 7.7%, 12.3% to 36.6%, 18.9%, and 16.4% after CIK cell transfusion in peripheral blood, respectively. The level of CD3⁺CD8⁺ could be kept as long as 100 d *in vivo*.

HBV chronic infection is the major pathogen of primary liver carcinoma. HCC patients had some immunity dysfunctions, including innate and adaptive immune responses^[4]. The study showed that the number of DCs was decreased or DCs displayed low function^[2,16,17]. DCs are considered unique APCs for their highly efficient capability of priming naïve T cells via direct cell-cell interactions and cytokine production, stimulating the propagation of naïve T cells, and playing a critical role in innate and adaptive immunity. They have been considered as one of the most potent regulators of the immunological mechanism^[18-20]. DC1 is of myeloid origin and could express CD11c, induce Th1 type of T cell differentiation and immunity. On the other hand, DC2 is of lymphoid origin and could express CD123, induce Th2 type of T cell differentiation and was involved in the induction of immunogenic tolerance^[21-23]. HBV patients exhibited a significant decrease in proportion of freshly isolated pDC1 to pDC2, and *ex vivo* generated DC1 function was impaired^[24]. DCs function was also suppressed in patients with HCC with hepatitis B and C virus infections^[25]. Decreased function of DCs might allow the development of tumor or the tumor itself might suppress the function of DCs^[26,27]. Our results showed that the proportions of DC1 and DC2 in the peripheral blood in HCC patients increased from 0.59% and 0.26% to 0.85% and 0.43% ($P < 0.01$) respectively after treatment with CIK cells. The reason was that some cytokines released by CIK cells, type I IFN for example, could promote the propagation or differentiation of DCs, enhancing the host immune response. Type I IFN can modulate DC activation/maturation and cytokine production in different ways depending on the experimental model and culture condition. The propagation of DC1 and DC2 could induce CTL against the tumor by producing more cytokines, and maintain the activity of CIK cells to kill the tumor efficiently^[28,29]. The studies showed that interaction between dendritic cells and CIK cells could lead to activation of both populations^[30]. This may provide a new approach in adoptive immunotherapy against tumors. Our data showed that HBV viral load decreased 3 mo after CIK cell therapy. The alpha-fetoprotein (AFP) in 6 cases significantly decreased. Serum alanine aminotransferase (sALT) decreased in 9 cases while enhanced in 2 cases. These results indicated that CIK cells played an essential role in anti-virus and anti-tumor treatment and improved the liver function. Our results also showed that CIK cell treatment efficacy was better when applied in combination with surgery or chemotherapy. For advanced HCC patients or those who were unfit for surgery or chemotherapy,

autologous CIK cell treatment could ameliorate symptoms, enhance quality of life and prolong the lives of patients.

In conclusion, autologous CIK therapy may greatly improve host immune responses. Therefore, CIK cells may have a major impact on immunotherapeutic protocols for patients with liver cancer.

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