

Effect of IL-12 on the proliferation and cytotoxicity of CIK cells to gastric adenocarcinoma cell BGC-823*

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Abstract Objective: The aim of the study was to evaluate the effect of interleukin-12 (IL-12) on the proliferation and cytotoxicity of cytokine-induced killer (CIK) cells *in vitro*. **Methods:** Three different combinations of cytokines, IL-2, IL-12 + IL-2, and IL-12, were used to proliferate CIK cells, adding IFN- γ , IL-1 and CD3 McAb in each one. Phenotype of the cells was analyzed by flow cytometry. The cellular proliferation and cytotoxic activity were determined by cytometry and MTT assay. **Results:** CIK cells generated by the three methods showed high reproductive activity, and no obviously difference in inducing CD3⁺CD56⁺ cells was found among the three groups. The group of IL-2 + IL-12 evidently enhanced both the proliferation and the cytotoxicity of the CIK cells compared with the other two groups ($P < 0.05$). **Conclusion:** IL-12 could be used to induce the CIK cells as well as IL-2. CIK cells induced by combining IL-12 with IL-2 had stronger proliferative ability and higher cytotoxicity to tumor cells *in vitro*, which could be used as a potential anti-tumor adoptive immunotherapy in clinic.

Key words Cytokine-induced killer (CIK) cell; interleukin-12 (IL-12); proliferative ability; cytotoxicity

Cytokine-induced killer (CIK) cells are a group of alloplasm cells, acquired from the mononuclear cell of human peripheral blood, cord blood or myeloid co-culture with some cytokine *in vitro*. CIK cells are called NK cells like T lymphocytes because of the characteristic CD3⁺CD56⁺ phenotype, have the anti-tumor activity of T lymphocytes and non-MHC-restricted in tumor cell killing. So they are considered to be the preferential project of anti-tumor adoptive immunotherapy [1–4].

Interleukin-12 (IL-12) is a heterodimeric cytokine having many biologic activities, generated from antigen presenting cells [5]. IL-12 with many anti-tumor bioactivities is a criticality regulatory molecule in immunological regulation, not only has direct inhibitory action to tumor, but also adjust organism immune system. So it has been the focus of attention and investigate at present [6–7]. This study used three different combinations of cytokine, and was designed to evaluate the effect of IL-12 on proliferation, cell phenotype and cytotoxicity of CIK cells.

Materials and methods

Reagents and instruments

Recombinant human IL-2 (Kingsley, Jiangsu, China); CD3 McAb (Tianguangshi, Beijing, China); recombinant human IFN- γ , recombinant human IL-1, and recombinant human IL-12 (PEPRO TECH, USA); rat anti-human CD3-Percp/CD4-FITC/CD8-PE, rat anti-human CD3-FITC/CD56-PE and goat anti-mouse IgG1 (BD, USA); leukomonocyte separating medium (Haoyang, Tianjin, China); RPMI 1640 (Gibco, USA); fetal calf serum (Sijiqing, Hangzhou, China); MTT (Solabio, China); health adult fresh peripheral blood (Harbin Red Cross Central Blood Station, China); flow cytometer (BD FACSCalibur).

Methods

Induction of CIK cells

Used three different combinations of cytokine: Group I (IL-2): IFN- γ , IL-1, IL-2 and CD3 McAb; Group II (IL-2 and IL-12): IFN- γ , IL-2, IL-12, IL-1 and CD3 McAb; Group III (IL-12): IFN- γ , IL-1, IL-12 and CD3 McAb. Peripheral blood mononuclear cells (PBMCs) were isolated from health adult fresh peripheral blood using Ficoll-Hypaque density gradient centrifugation. CIK cells with different combinations of cytokine were induced by the method of our laboratory. The cells were incubated in

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CO₂ incubator on humidity of 100% and at temperature of 37 °C.

Immunophenotype assay of CIK cells

Detected fluorescent antibody and corresponding negative control IgG1 20 µL were added into FCM test tubes. Collected the CIK cells on days 7, 14, and 21 after culture, washed 2 times using calcium and magnesium free PBS, added $1 \times 10^6/0.5$ mL into corresponding FCM tube and incubated away from light for 30 min at 4 °C, and waved every 10 min, washed 2 times using PBS, and then analyzed immediately with flow cytometry. The results were analyzed with CellQuest Pro. The percentage of CD3⁺CD56⁺ cells was CIK cells population.

Observation of the appearance of CIK cells colony and determination of proliferation activity

The changes in form of amplification colonies of every group cells were observed by light microscope and recorded the colonies condition of the three group cells on day 14. Every group cells were suspended to 1×10^6 /mL, 1 mL of the suspensions were incubated in 24-well culture plate, and added new culture medium and cytokine every 3 days and cells population were counted by trypan blue assay and calculated the average value at the same time. Growth curve was drawn according to the multiple of cell multiplication.

Cytotoxicity assay

Target cell was BGC-823 poorly differentiated gastric adenocarcinoma cell strain. The BGC-823 cells at logarithmic growth phase were suspended to 1×10^5 /mL, 5×10^4 /mL and 2.5×10^4 /mL and incubated in 96-well culture plate 100 µL every well in CO₂ incubator at 37 °C. Twenty-four hours later, three groups of cells were suspended to 1×10^6 /mL and incubated in 96-well culture plate. The ratios of effect to target were 10:1, 20:1 and 40:1, and every concentration had 4 wells. The blank of effector cells were the CIK cells that did not react with tumor cells. The blank of target cells were the BGC-823 that did not react with CIK cells. Forty-eight hours later, MTT were added into 96-well culture plate 20 µL every well and continue cultured for 4 h, removed supernatant, DMSO were added into 96-well culture plate 100 µL every well. Determined the absorbance value *A* of every well at 570 nm and calculated the ratio of cytotoxicity. The cytotoxicity ratio = $[1 - (A \text{ of experiment well} - A \text{ of effect cell control well}) / A \text{ of target cell control well}] \times 100\%$.

Statistical analysis

The data were handled with SPSS (V10.0) and expressed by mean ± SD. Statistical analysis was performed using the variance analysis and SNK-*q* test, with $P < 0.05$ considered significant.

Results

Morphology of cell multiplication

The mononuclear cells extracted were similar in size and refraction. On the third day after induced, lots of cells died, a little grown of colonies, but they were small (cell populations was about 10). The cells were grown and appeared pantomorphia. There was a lot of manipulus in nucleus – large granular lymphocyte (LGL) (Fig. 1a). On the sixth day, the cell colonies obviously increased and became bigger. We could see the white blotch colonies. The cell populations of the colonies were obviously increased. The cells were become accumulation, satiation and well refraction. A lot of cells emigrated surrounding the colonies (Fig. 1b).

Compared the three groups of CIK cells, they all had colonies. The cell colonies of group I (IL-2) and group III (IL-12) were less at earlier period. Some days later, the colonies were increased, and the colonies populations of every well of the two groups were 54.2 ± 8.3 and 48.8 ± 6.3 . The cell populations of every colony was about 50 to 80. There were a lot of tiny colonies in group II (IL-2 + IL-12) at earlier period. Some days later, the population of colonies was about 32.5 ± 9.7 which was obviously less than group I ($P < 0.05$). The cell populations of every colony were more than 150 (Fig. 2).

Cell proliferation activity

Monitoring the proliferation condition of CIK cells, we discovered that the three groups of CIK cells started growing on the third day, at the phase of quickly grow 14 days later, then at the platform phase after 21 days. The consequence of the cells amplification multiple on the 10th day using blood cell count meter was similar to that using microscope. The amplification multiple of group II was the highest (10.62 ± 0.33), that of group I was 6.14 ± 0.49 and that of group III was 5.66 ± 0.23 . There were significant difference in the three groups ($P < 0.05$). The effect of IL-12 combined with IL-2 was better than that of IL-12 or IL-2 alone. Fig. 3 showed the growth curves of CIK cells.

Immunophenotype of CIK cells

Induced 8 days later, the ratio of CD3⁺ cells in CIK cells was obviously increased, and CD3⁺CD56⁺ subgroup started amplification. Induced 2 weeks later, the proliferation of CD3⁺CD56⁺ cells achieved crest-time, and the proliferation ratio of IL-2 combined with IL-12 was a little higher. There were significant deviation among the three groups' phenotype at different induced times ($P < 0.05$), and the phenotype of the three groups had no obviously deviation at the same time. The ratio of CD3⁺ cells of group III was lower than that of the other two groups (Table 1 and Fig. 4).

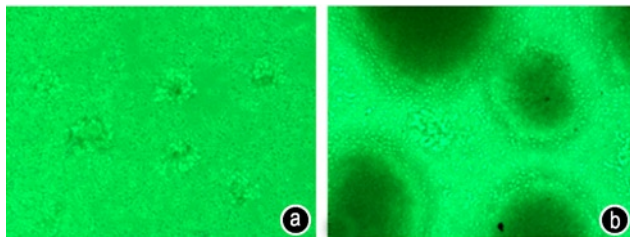


Fig. 1 Morphology of CIK cells in different days of inducement ($\times 200$). (a) the third day after induced; (b) the sixth day after induced

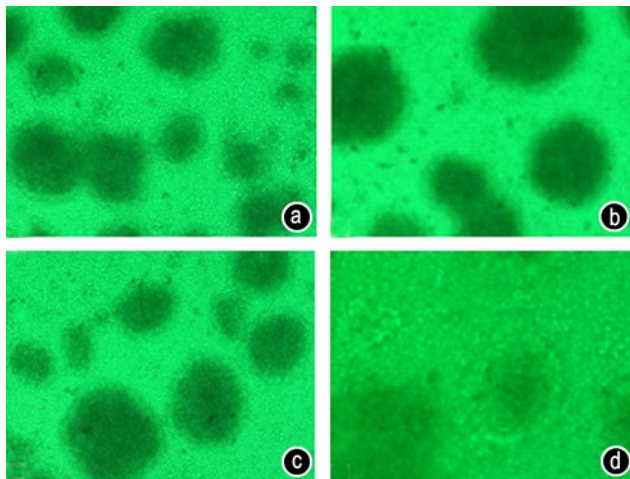


Fig. 2 Colonies of CIK cells cultured 10 days. (a) group I (IL-2), $\times 100$; (b) group II (IL-2 + IL-12), $\times 100$; (c) group III (IL-12), $\times 100$; (d) group II (IL-2 + IL-12), $\times 200$

Table 1 Percentage of CD3⁺CD56⁺ cells at different times ($\bar{x} \pm s$)

Groups	Percentage of CD3 ⁺ CD56 ⁺ cells (%)		
	7 d	14 d	21 d
Group I (IL-2)	5.71 \pm 1.09	11.27 \pm 0.06	19.90 \pm 0.07
Group II (IL-2 + IL-12)	7.14 \pm 1.64	14.79 \pm 0.21	20.89 \pm 0.50
Group III (IL-12)	5.90 \pm 0.40	12.02 \pm 0.35	20.19 \pm 0.16

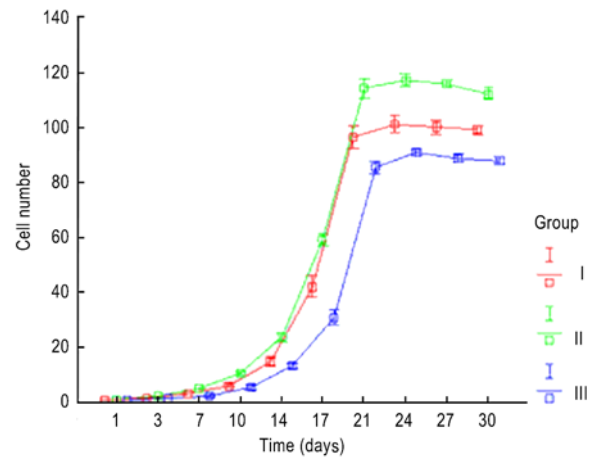


Fig. 3 Growth curves of CIK cells. I, group I (IL-2); II, group II (IL-2 + IL-12); III, group III (IL-12)

Cytotoxicity of CIK cells

The ratio of specificity cytotoxicity of CIK cells of group II to BGC-823 was obviously higher than that of group I and group III at different ratio of effect to target (E:T) ($P < 0.05$). There was no significant deviation between group I and group III ($P > 0.05$). And there was significant difference among different E:T of the three groups ($P < 0.05$; Table 2). We also discovered that cytotoxicity of CIK cells could maintain about 2 weeks and the strongest cytotoxicity was about at the 14th day.

Table 2 Cytotoxicity of CIK cells of the three groups ($\bar{x} \pm s$)

Groups	E:T		
	10:1 (%)	20:1 (%)	40:1 (%)
Group I (IL-2)	32.55 \pm 1.63*	41.88 \pm 2.22*	52.60 \pm 4.33*
Group II (IL-2 + IL-12)	43.44 \pm 2.12	62.85 \pm 5.36	80.19 \pm 1.86
Group III (IL-12)	30.44 \pm 2.83*	36.95 \pm 0.99*	48.94 \pm 3.25*

Compared with group II, * $P < 0.01$

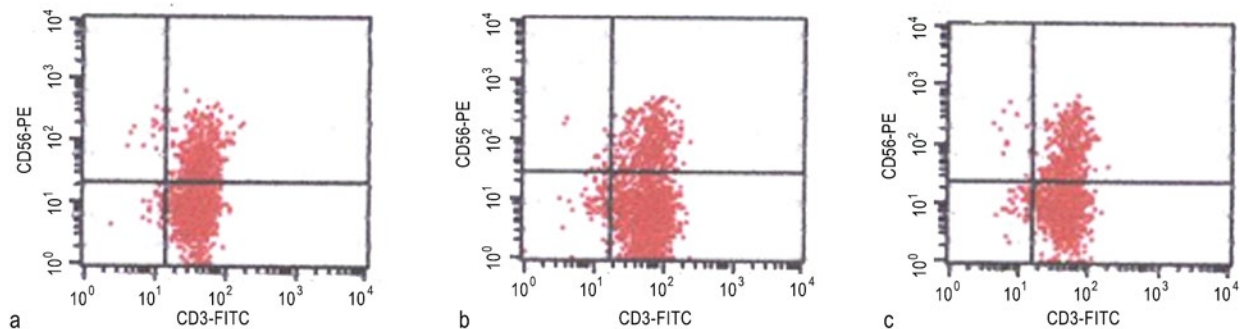


Fig. 4 The expression of CD3/CD56 of CIK cells of the three groups at day 21. (a) group I (IL-2); (b) group II (IL-2 + IL-12); (c) group III (IL-12)

Discussion

CIK cells are a group of alloplasm cell mass that acquired from mononuclear cell of peripheral blood or cord blood with some cytokine *in vitro*. The main components of CIK cells are CD3⁺CD56⁺ cells and CD3⁺CD8⁺ cells. CD3⁺CD56⁺ cell is primary effector cells as its highest proliferation and cytotoxicity. At present, the method of proliferation of CIK cells is the method reported by Schmidt-Woff *et al* in 1991 [2]. They use IL-1, IL-2, IFN- γ , anti-CD3 McAb and any other cytokines. Anti-CD3 McAb has cleavage stimulatory function that can help proliferation of CIK cell. If IFN- γ is added in culture system before IL-2 24 hours and combined with IL-1, the cytotoxicity of CIK cells can be increased. If IFN- γ and IL-2 are added at the same time or IFN- γ is added after IL-2, the cytotoxicity of CIK cells can be decreased. So the time of cytokines added is important to CIK cells.

IL-12 generated from activated macrophage and B cell is necessary factor in the differentiation of Th0 to Th1, can stimulate proliferation and cytotoxicity of immunocytes [7-9]. Our study indicated that the effect of IL-12 on CIK cells proliferation is a little lower than that of IL-2. The ability of IL-12 inducing CD3⁺CD56⁺ CIK cells is similar to that of IL-2, but the proliferation of CD3⁺ cells induced by IL-12 is less than that induced by IL-2. If induced by IL-12 combined with IL-2, the ratio of CD3⁺CD56⁺ CIK cells and total cellular score of CIK cells are obviously increased. So IL-12 has the ability of proliferation on CIK cell as IL-2, and IL-12 and IL-2 have synergistic action.

There has been a lot of studies of increasing cytotoxicity of CIK cells [10-13]. Direct inhibitory action to tumor of IL-12 has been indicated in many experimental animal models up to now [7, 14]. IL-12 directly acts on tumor cells through IL-12 receptor beta 1 to activate NF-kappaB and enhance IFN-gamma-mediated STAT1 phosphorylation [15]. IL-12 also induces IP-10 that can inhibit angiogenesis activity generated by tumor cell through IFN-gamma effect on IFN-gamma-acceptor, and finally eradicate tumor [16]. IL-12 has strong ability of inducing IFN- γ generation [17]. Studies have confirmed that tumor eradication induced by IL-12 depended on the effect of IFN-gamma produced by Th1 reaction on immunologic cells [18-19]. In addition, CIK cells have direct cytotoxicity on tumor cell, and also can generate a lot of inflammatory cell factors after activated, such as IFN- γ , IL-2, TNF- α and so on. These cytokines have direct repression effect on tumor cell and indirect effect through adjust immunologic system of organism. The findings of Helms *et al* demonstrate the potential to improve current CIK cell-based immunotherapy by increasing efficacy and shortening *ex vivo* expansion time. This holds promise for a highly efficacious cancer therapy utilizing synergistic effects of cytokine and cellular immunotherapy [20-23].

Our group studies that molecular cloning of IL-12 cDNA and expression in EL-4 cells, afterward, the antitumor immunity and vaccine effect induced by IL-12 synergized B7-1 gene transfected cells is detected in previously [24-27]. These explain that the cytotoxicity of CIK cells can be obviously enhanced by IL-12 combined with IL-2 in our studies.

Yang *et al* show that oncolytic adenovirus carrying hIL-12 (AdCN205-IL12) can produce high levels of hIL-12 in liver cancer cells, as compared with replication-defective adenovirus expressing hIL-12 (Ad-IL12) [28]. The data of Kim *et al* support that cisplatin delivery by IT-EP plus IL-12 gene delivery by IM-EP are more effective at inducing antitumor therapeutic responses through increased sensitivity of cisplatin-treated tumors to NK cell-mediated tumor killing. This combined approach may have some implications for treating melanoma in patients [29]. In addition, abundant of clinic studies of IL-12 activated CIK cells are reported by another laboratories [30-42].

It has been the hot spot of tumor immunotherapy that combined with cytokines can increase anti-tumor immunity of organism and decrease adverse reaction generated by large dose cytokine. Our study confirmed that combined with IL-2 and IL-12 can significantly increase the proliferation and cytotoxicity of CIK cells and the ratio of CD3⁺CD56⁺ cells in the course of inducing CIK cells. It is refer to clinical application that phenotype and vigor of CIK cells achieve dynamic balance from day 14 to 21 after induced by IL-2 combined with IL-12.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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