The Effects of IL-2 Concentration and Adding Manner on Proliferation and Function of CIKs

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1 **The effects of IL-2 concentration and adding manner on proliferation and**
2 **function of CIKs**

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10 **Abstract**

11 **Background:** Cytokine-induced killer cells (CIKs) adoptive cell transfer (ACT) is a common
12 malignant tumor treatment method. Interleukin-2 (IL-2) is one of essential cytokines for CIKs
13 culture. In most studies, only the effect of IL-2 concentration on function of CIKs was studied, but
14 the difference between multifarious adding manner of IL-2 was not explored.

15 **Methods:** This study established a novel sequential adding manner of IL-2. Different concentration

of IL-2 was added in different CIKs induction phase. Then, proliferation ability of CIKs was evaluated using cell proliferation curves, immune phenotype was analyzed by flow cytometry (FCM), IFN- γ secretion ability and cytotoxicity were detected by ELISA Kit and CCK-8 Kit respectively. Multiple comparison tests were conducted between each group to compare function of CIKs in 12 experimental groups.

Results: As the IL-2 concentration increased, the number of CIKs continued to increase in each group, but its function was not positively related with its number: CD3+ CD56+ subpopulation ratio, INF- γ secretion ability and cytotoxicity presented irregular changes. During quiescent phase and logarithmic growth phase, adding 300 and 1000 U/mL IL-2 respectively could obtain amounts of powerful CIKs (CD3+ CD56+ subpopulation ratio: 40.9%, INF- γ secretion ability: 542pg/mL, cytotoxicity: 40:1, 74.22).

Conclusions: Different concentration of IL-2 had a greater influence on biological function of CIKs in different growth phase, it was better to add IL-2 sequentially in quiescent phase and exponential growth phase of CIKs.

Key Words: cytokine-induced killer cells, interleukin-2, adoptive cell transfer, adoptive cell transfer

Background

Over 10,000 Chinese people were diagnosed with cancer averagely per day or around 7.5 people a minute in China in 2015 [1], malignant tumor has become one of the major diseases that takes seriously risks to Chinese health. Adoptive cell transfer (ACT) is a novel cancer treatment approach, which can stimulate or enhance a patient's immune function to kill cancer cell. Cytokine-induced killer cells (CIKs) is one of the most important ACT, it is a group of heterogeneous cells which obtained by co-culturing human peripheral blood mononuclear cells (PBMCs) with various cytokines (such as OKT3, IL-2, IFN- γ , etc.) in vitro. It is referred to as NK cell-like T lymphocytes, and CD3⁺ CD56⁺ subpopulation is its effector cell. In recent years, CIKs ACT has been widely used in various cancers and bring an inspiring clinical effect [2-7] because its strong antitumor activity and in MHC unrestricted manner [7]. As is well-known, clinical effect was directly affected by the number and function of immune cells.

Interleukin 2 (IL-2) is one of immune system signaling molecule, and initially named T cell growth factor (TCGF) [8, 9]. IL-2 plays an important role in promoting T cell proliferation and

function [10], also it was essential cytokines for CIKs growth. IL-2R on the surface of activated NK cells and T cells is the receptor of IL-2, formed by up to three subunits (α , β , and γ). The high-affinity interaction of IL-2 and IL-2R promote the activation of cell main signaling pathways and play a vital role in promoting the proliferation and function of NK cells [10-12]. Therefore, concentration and adding manner of IL-2 directly affect the quality of CIKs, and then affect its clinical efficacy. At present, in the cultivation of CIKs, IL-2 concentration and adding manner had no uniform standard. In this study, we performed a novel sequential addition: to add IL-2 with different concentration in different CIKs growth phase (quiescent phase and exponential growth phase). There are two primary aims: (1) to explore relationship between concentration, adding manner of IL-2 and CIKs function; (2) to establish a new culture system in order to obtain CIKs with the best proliferation capacity, activity and cytotoxicity.

Methods and methods

CIKs induction

CIKs ACT was approved by the Institutional Review Board of Lanzhou University Second Hospital. Informed consent was obtained from each patient. PBMCs were obtained by Ficoll-Hypaque density

centrifugation. Cells were resuspended at 1×10^6 CFU/mL in X-VIVO-20 media (LONZA) with 1000U/mL interferon- γ (IFN- γ , Peprotech) for the first 24 h. On day 1, 50ng/mL anti-CD3 monoclonal antibody (OKT3, Peprotech), 100U/mL interleukin-1 α (IL-1 α , Peprotech) and IL-2 (Peprotech) were added to the medium. Cells were cultured in 5% CO₂ at 37°C, and fresh medium and IL-2 were added every two days. The concentration of IL-2 was 300, 500 and 1000U/mL in first week; and 300, 500, 1000, 3000 and 6000U/mL in second week respectively. CIKs were harvested at day 16.

Proliferation of CIKs

Cell morphology was observed regularly, and the survival rate of CIKs was calculated by trypan-blue exclusion. Then, cell proliferation curves were drawn based on cell counting (proliferation fold = number of cells after proliferation / number of cells before proliferation).

Immune phenotype of CIKs

CIKs at day 16 from each group were harvested (1×10^6 CFU/mL, 100 μ L) and incubated for 30 min at 4 °C with anti-CD3-fluorescein isothiocyanate (FITC) and anti-CD56-Phycoerythrin (PE) (BD Bioscience). After washing twice, the immune phenotype of CIKs was characterized using flow

cytometry (FCM, BD FACSCalibur), and data analysis was performed with FlowJo V10.

IFN- γ secretion ability of CIKs

The expression levels of INF- γ were determined by ELISA (Proteintech): CIKs at day 16 from each group were harvested (1×10^6 CFU/mL). 100 μ L of sample was added into each well coated with antibody, and the plate was incubated at 37°C for 120 min; by washing 4 times, 100 μ L of detection antibody-biotinylated was added into each well, then the plate was incubated at 37°C for 60min; after washing, streptavidin-HRP solution (100 μ L per well) was added followed by incubation for 30mins; washing again, TMB substrate was added, and plates was sited in dark room for 15mins; stop solution was added and optical density (OD) value was detected at 450nm.

Cytotoxicity of CIKs

Cytotoxicity assay was performed by CCK-8 Kit (Yeasen Biotechnology). CIKs from each group were collected at different concentration and used as effector cells. Gastric cancer cells (MKN45) at exponential growth phase were used as target cells. The target cells were reacted with effector cells at an effector-target ratio (E:T) of 10:1, 20:1, 40:1, 80:1, 160:1. All samples were added to a 96-well plate and cultured in 5% CO₂ at 37°C. After 24h, 20 μ L CCK-8 solution was added in each well and

continued to be cultured for 4h. Then, OD value of each well was detected at 450nm. Cytotoxicity of CIKs was calculated using the formula: cytotoxicity = $[1 - (\text{OD experimental group} - \text{OD effector group}) / \text{OD control group}] \times 100\%$.

Statistic analysis

Statistical analysis was performed with GraphPad Prism 7.00. Proliferation curve data were analyzed by linear regression. Cytokine assay data used one-way ANOVAs. Cytotoxicity analysis was used two-way ANOVAs. Multiple comparisons test was carried out by dunnett's multiple comparisons test. $P < 0.05$ was considered as significant difference; $P < 0.01$ was considered as high significant difference; $P > 0.05$ was considered to be not a significant difference [2].

Result

Proliferation of CIKs

The viability of CIKs in each group was greater than 95%. In all groups, proliferation ability of CIKs enhanced with the concentration of IL-2 increased; high concentration IL-2 groups showed a better growth tendency, and its proliferation fold could reach 157.54 in day16 (Fig.1, Table 1).

Immune phenotype of CIKs

The results of FCM analysis showed that group A3 had the highest CD3+CD56+ subpopulation ratio (40.9%); and in multiple comparisons test, there was a high significantly difference between group A3 and other groups ($P<0.01$). Group A2 and A4 showed a higher ratio (30-40%); group A1, A5, B2 and B3 had middle ratio (20-30%); group B4, C3 and C4 had lower ratio (15-20%); group B5 and C5 had the lowest ratio (12.5, 10%) (Fig.2).

IFN- γ secretion ability of CIKs

The result of IFN- γ secretion ability showed in Fig.3: (1) group A3 had the highest IFN- γ production (542 pg/mL); (2) multiple comparison test showed that no significant difference was found between group A3 and A2, B2, B3 ($P>0.05$), and high significant difference between group A3 and the others ($P<0.01$).

Cytotoxicity of CIKs

In each group (Fig. 4), the lowest cytotoxicity appeared at E:T=10:1; after that, cytotoxicity increased as E:T raised, and it reached the highest at 40:1, but gradually decreased at 80:1 and 160:1. Then multiple comparison test was performed between the best E:T (40:1) and the others (10:1, 20:1, 80:1, 160:1) to compare the cytotoxicity of CIKs: (1) in all groups, the cytotoxicity of E:T = 40:1

was obviously better than 10:1 and 20:1 ($P<0.01$); (2) E:T = 40:1 showed a better cytotoxicity than 80:1 in group A3 and C4 ($P<0.05$), and it was more better in group C3 ($P<0.01$); also this superiority was not obvious in other groups ($P>0.05$); (3) the cytotoxicity of 40:1 was obviously better than 160:1 in group A4, B3 and B4 ($P<0.05$), this superiority was not significant in group B2 ($P>0.05$), and was very significant in other groups ($P<0.01$).

On the other hand, with a constant E:T (Fig. 5), group A3 showed a better cytotoxicity, then we compared group A3 with the others: (1) when E:T=10:1, there was a high significant difference between A3 and other groups ($P<0.01$); (2) at 20:1, no difference was found between group A3 and A1, A2, but high significant difference with the others ($P<0.01$); (3) at 40:1 and 80:1, no significant difference was found between the group A3 and A2, and high significant differences with the others ($P<0.01$); (4) at 160:1, no significant difference was found between group A3 and A1, A2, A4, however high significant differences with the others ($P<0.01$).

Discussion

IL-2 is a multicellular cytokine with multi-directional function, by interacting with its receptor IL-2R, it can promote cell proliferation and enhance cell activity. To date, there is no uniform

standard on concentration of IL-2 in CIKs culture. Li Y [13] and Meng Wang [14] respectively used 80ng/mL and 300 U/mL IL-2 to cultivate CIKs. The concentration of IL-2 in Niu L's [15] experiment was 400 U/mL, and the DC-CIKs ACT yielded good results in pancreatic cancer treatment. In other studies, its concentration was 500 or 1000 U/mL [16, 17]. Of course, recently, a large and growing body of studies has investigated effects of different concentration of IL-2 on CIKs. VitoloD [18] considered the concentration greater than 500 U/mL is beneficial to expansion of NK cells in vitro. Research by Xiong D [19] showed that when IL-2 concentration was less than 1000U/mL, there was no correlation between IL-2 and killing activity of NK cells; and high-concentration IL-2 (≥ 1000 U/mL) could further activate the killing activity of purified NK cells in vitro. Yet, some researchers [20] found 500U/mL was best. Other study [21] obtained the strongest proliferation ability of CIKs, when IL-2 was 1000U/mL. According to Zoll B [22], the appropriate IL-2 concentration in CIKs culture was 67-500U/mL. Nevertheless, other researchers hold a opposite view, they thought that different concentrations of IL-2 have a little effect on cytotoxicity of CIKs [23]. It is worth mentioning that although extensive researches have been carried out on this question, no study was found which adjusted IL-2 concentration according to

different growth phase of CIKs in domestic and foreign research.

In this study, we added IL-2 sequentially during CIKs culture, our research demonstrated that:

(1) IL-2 concentration was proportional to CIKs proliferation capacity, however, these CIKs did not have a powerful cytotoxicity; instead, its INF- γ secretion ability and cytotoxicity would decrease when IL-2 was over a certain concentration; (2) in quiescent phase of CIKs, low-concentration IL-2 (300U/mL) could enhance its CD3 + CD56 + subpopulation ratio, INF- γ secretion ability and cytotoxicity; (3) in exponential growth phase, low-concentration IL-2 (300, 500U/mL) had no advantages, also overdose (3000, 6000U/mL) was not suitable for CIKs growth; (4) with a consistent IL-2 concentration of CIKs in quiescent phase, CD3+ CD56+ subpopulation ratio and INF- γ secretion ability were reduced when IL-2 concentration of exponential growth phase was greater than 3000U/mL; CD3 + CD56 + subpopulation ratio, INF- γ secretion ability and cytotoxicity were lowest when IL-2 concentration of exponential growth phase was 6000 U/mL.

In short, our study indicated that CIKs need different dose IL-2 in different phase. Why did these phenomena occur? The first, the first week is quiescent phase of CIKs, cells are mostly at rest, T cell surface is not activated, it does not express or weakly express IL-2R. In quiescent phase,

multiple cytokines (OKT3, TNF α) are added to induce the expression of IL-2R, but this process takes time, so there is not enough IL-2R during this phase. At this time, the affinity between IL-2R β , IL-2R γ expressed by NK cells and IL-2 was lower, which means that IL-2 has a weak effect on T cells and NK cells. In a word, more IL-2 is not always better in quiescent phase of CIKs, vast amounts of IL-2 may cause cell intolerance, we recommend stimulating CIKs with low-concentration IL-2 (300U/mL), and increasing IL-2 concentration after NK and T cells are fully activated. Second, the second week is exponential growth phase of CIKs, CD69, CD25, CD71 are up-regulated after T cells undergo early, middle, and late activation, then CD25 and IL-2R β , IL-2R γ chain together constitute a high affinity IL-2R [24-26]. Meanwhile, after being fully activated, NK cells also express the high affinity receptor IL-2R α . Another reason is more IL-2 can induce target cells to increase IL-2R expression. That is to say, in exponential growth phase, the affinity between IL-2 and IL-2R could theoretically increase with the increase of IL-2 concentration, nevertheless, our study demonstrated that middle-concentration IL-2 (1000U/mL) showed a better experimental result than high-concentration (3000, 6000U/mL), the reason may be because excess IL-2 would induce enrichment of Treg cells [27, 28].

In this study, we established a sequential adding manner of IL-2 in CIKs culture (quiescent phase: 300U/mL, exponential growth phase: 1000U/mL), which can effectively promote proliferation ability, increase CD3⁺ CD56⁺ subpopulation ratio, enhance INF- γ secretion ability and cytotoxicity of CIKs. This sequential method can not only meet the basic needs of CIKs growth, but also can adjusting IL-2 concentration according to IL-2R expression of T cells in different phase, thereby reducing the experimental cost. It is beneficial to guide clinical treatment of CIKs ACT for malignant tumors.

Abbreviations

ACT: Adoptive cell transfer; CIKs: Cytokine-induced killer cells; FCM: Flow cytometry; IFN- γ : Interferon- γ ; IL-2: Interleukin-2; OD: Optical density.

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Author contributions

YL, YZ and TL contributed to the study conception and design. Material preparation, data collection and analysis were

performed by YLL and JLL. LZZ, JRZ, HXW and YYW prepared all the figures and tables. The first draft of the manuscript was written by YLL. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Consent for publication

This is not applicable for this study.

Ethics approval and consent to participate

The methodology for this study was approved by the Human Research Ethics committee of Lanzhou University Second Hospital (Ethics approval number: 2017A-044). Informed consent was obtained from all individual participants included in the study.

Competing interests

The authors declare that they have no competing interests.

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295 Tables and Figures

Table1 IL-2 concentration and CIKs numbers of 12 experimental groups

Group	Dose of IL-2 (U/mL)		Cell numbers in different time point ($\times 10^6/\text{ml}$, $\bar{X} \pm S$)				
	quiescent phase	exponential growth phase	day 4	day 7	day 10	day 13	day 16
A1	300	300	3.40 ± 0.01	8.63 ± 0.36	66.30 ± 1.67	125.77 ± 1.98	302.11 ± 2.92
A2	300	500	3.40 ± 0.02	9.15 ± 0.67	70.52 ± 1.38	128.74 ± 2.60	325.07 ± 3.84
A3	300	1000	3.42 ± 0.02	11.68 ± 1.26	72.50 ± 2.26	150.88 ± 1.50	384.37 ± 2.05
A4	300	3000	3.50 ± 0.02	12.58 ± 0.87	80.67 ± 1.13	158.10 ± 2.09	390.70 ± 1.29
A5	300	6000	3.48 ± 0.02	13.65 ± 0.89	85.13 ± 2.97	164.59 ± 2.22	402.66 ± 3.30
B2	500	500	3.52 ± 0.01	12.59 ± 0.76	92.68 ± 2.07	185.82 ± 1.35	415.31 ± 2.28
B3	500	1000	3.49 ± 0.02	14.72 ± 0.02	100.28 ± 2.74	191.14 ± 2.14	434.49 ± 2.19
B4	500	3000	3.53 ± 0.02	17.43 ± 0.48	104.94 ± 3.77	183.14 ± 2.13	443.03 ± 1.41
B5	500	6000	3.49 ± 0.03	18.44 ± 0.48	118.60 ± 1.51	208.99 ± 2.41	450.20 ± 1.66
C3	1000	1000	3.52 ± 0.03	16.75 ± 0.85	113.67 ± 3.40	205.11 ± 2.07	449.73 ± 1.79
C4	1000	3000	3.53 ± 0.03	20.37 ± 1.14	124.78 ± 1.37	212.18 ± 1.59	464.64 ± 2.57
C5	1000	6000	3.54 ± 0.01	23.39 ± 0.45	116.22 ± 1.73	220.58 ± 2.02	472.63 ± 1.55

Fig.1 The proliferation curves of CIKs in different experiment groups

Fig.2 The result of flow cytometry of CD3+CD56+ cell in CIKs on day 16

Fig.3 IFN- γ secretion ability of CIKs in different experiment groups

*: $P < 0.05$ was considered as significant difference; **: $P < 0.01$ was considered as high significant difference; ns: $P > 0.05$ was considered to be not a significant difference; ∇: Dunnett's multiple comparisons test was performed between group A3 and other groups.

Fig.4 The cytotoxicity of CIKs in different groups

Fig.5 The cytotoxicity of CIKs at different effector-target ratio

*: $P < 0.05$ was considered as significant difference; **: $P < 0.01$ was considered as high significant difference; ns:

$P > 0.05$ was considered to be not a significant difference; ∇: Dunnett's multiple comparisons test was performed between group A3 and other groups.

Figures

Figure 1

The proliferation curves of CIKs in different experiment groups

Figure 2

The result of flow cytometry of CD3+CD56+ cell in CIKs on day 16

Figure 3

IFN- γ secretion ability of CIKs in different experiment groups *: $P < 0.05$ was considered as significant difference; **: $P < 0.01$ was considered as high significant difference; ns: $P > 0.05$ was considered to be not a significant difference; ▽: Dunnett's multiple comparisons test was performed between group A3 and other groups.

Figure 4

The cytotoxicity of CIKs in different groups

Figure 5

The cytotoxicity of CIKs at different effector-target ratio *: $P < 0.05$ was considered as significant difference; **: $P < 0.01$ was considered as high significant difference; ns: $P > 0.05$ was considered to be not a significant difference; ☐: Dunnett's multiple comparisons test was performed between group A3 and other groups.