Emergence of peripheral CD3+CD56+ cytokine-induced killer cell in HIV-1-infected Chinese children

Geng-Feng Fu1, Xu Chen2, Hai-Yang Hu1, Hai-Tao Yang1, Xiao-Qin Xu1, Tao Qiu1, Lei Li1, Jin-Shui Xu1, Xi-Ping Huan1,2,* and Ya-Yi Hou3,4,*

1Jiangsu Provincial Center for Disease Prevention and Control, Nanjing, 210009, China
2School of Life Science, 3Immunology and Reproductive Biology Lab of Medical School and State Key Laboratory of Pharmaceutical Biotechnology and 4Jiangsu Key Laboratory of Molecular Medicine, Nanjing University, Nanjing 210093, China

Correspondence to: Y.-Y. Hou, Immunology and Reproductive Biology Lab of Medical School, Nanjing University, 22 Hankou Road, Nanjing, 210093, China; E-mail: yayihou@nju.edu.cn

*These authors are equally contributed to this paper.

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Abstract

Cytokine-induced killer (CIK) cells are immune effector cells characterized by co-expression of CD3 and CD56 molecules. We examined the quantities of CIK cells and the changes of these cell expressing NK cell receptors in HIV-1-positive children infected via mother-to-child transmission. The percentage of CIK cells was quantified and the changes in the surface cell receptor profiles in 18 HIV-1-infected children were examined. We found that CIK cell percentages were dramatically increased in HIV-1-infected children. Furthermore, the expressions of CD16, NKp30, NKp44, NKp46, NKp80 and CD244 on CIK cells were decreased, while the expressions of KIR3DL1 and NKG2D on CIK cells were increased in HIV-1-infected children. However, the expressions of KIR2D and NTB-A on CIK cells did not change in the HIV-1-infected children. CIK cells possessed the characteristics of promoting the maturation of dendritic cells and killing functions in HIV-1-infected children. Moreover, serum concentrations of IL-4 and IFN-γ were significantly increased in HIV-1-infected children compared with the HIV-negative controls. These changes likely occurred as a protective mechanism against transmission of maternal HIV-1 virus and thereby helped to limit viral spread, eliminate infected cells and help HIV-1-infected patients to slow the progression to AIDS.

Keywords: CD3+CD56+ cytokine-induced killer cell, HIV-1, mother-to-child transmission, receptor

Introduction

In 2009, an estimated 55 000 people were newly infected with HIV-1, of which ~1.3% were infants infected via mother-to-child transmission (MTCT). Unique genetic and immunological events distinguish antepartum from subsequent HIV-1 transmission because antepartum transmission occurs during the period where the child shares half of her/his MHC genes with the mother and where the mother tolerates the paternally derived fetal histocompatibility molecules (1). Antepartum HIV-1 transmission is related to the HLA class I alleles of the MHC because of their role in determining maternal–infant compatibility and modulating the CD8+ T-cell surveillance of virally infected cells (2–4).

Cytokine-induced killer (CIK) cells are immune effector cells characterized by co-expression of CD3 and CD56 molecules. CIK cells exhibit significant non-MHC-restricted lysis of target tumor or virus-infected cells (5–9). CIK cells demonstrate more potent cytolytic activity compared with CD3+CD56− cells (6,7). CIK cells are also called NK-like T cell because they bear the NK-specific marker CD56 and express NK cell receptors (10,11). FACS has shown that 8% of the lymphocytes were CD3+CD56+ cells in hemophagocytic syndrome (HPS) patients and that 31% of cytoplasmic CD3+ cells expressed NKp46, a specific marker of activated NK cells. This increase in CD3+CD56+ lymphocytes may have been secondary to activation of the immune system and proliferation of CD3+CD56+ T cells with NKp46 in the peripheral blood. These markers are useful for early diagnosis of HPS (12). NK cell receptors such as KIR3DL3, NKG2D, NKG7 and NK4 were expressed in unstimulated CIK cells, and NKG2E and NKG2C were up-regulated in CIK cells with target cells (13).

CIK cells are present in small populations in the peripheral blood of all individuals. Several previous reports have shown that CIK cells can be generated and expanded in vitro by incubating peripheral blood lymphocytes with anti-CD3 mAb, IL-2 and IFN-γ (14). In CD3+CD56+ cells
that were co-cultured with the EBV-transformed lymphoblastoid cell line, the concentration of IFN-γ released in the supernatant of co-cultures was higher than that of CIK cells cultured alone. CIK cells could be an adoptive immunotherapy for EBV-related malignancies, such as Hodgkin’s disease and nasopharyngeal carcinoma (15, 16). Furthermore, CIK cells can be adapted to other virus-related infections and reactivated in stem cell transplantation settings, e.g. cytomegalovirus (17).

The mechanisms involved in controlling the establishment of HIV-1 infection are not fully understood. In particular, the role of innate immunity in natural resistance in HIV-1-infected individuals has not been thoroughly evaluated. Innate immunity is very active at mucosal surfaces, which are the primary entry locations of HIV-1. Components of the innate immune system are known by NK cells, NK T cells and CD3+CD56+ cells, which have direct anti-HIV-1 activities through secretion of ILs, IFNs and chemokines (18,19). Activation of CD3+CD56+ cells may be important because of the ability to serve as an early source of regulatory cytokines, such as IFN-γ and IL-2 (20,21). In addition, CD3+CD56+ cells are very potent at destroying infected and transformed cells (22,23). Our previous study indicated that CD3dimCD56+ cells increased in individuals of HIV-1 infection with different routes of transmission (24). These changes may be involved in the prevention and control of HIV-1 infection.

The aim of this study was to evaluate the quantity and function of CD3+CD56+ CIK cells and the relationships between CIK cell percentage and NK cell receptor expression on CIK cells, secretion of some cytokines in plasma, CD4 T-cell counts and HIV-1 viral load (VL) in HIV-1-infected children.

Methods

Study subjects

This study enrolled 18 HIV-1-positive children who were not progressing into AIDS and not to be treated with anti-retrovirals, 13 of whom were boys and 5 were girls. Subject ages ranged from 2 to 12 years old (6.8 ± 3.1). Eighteen HIV-uninfected children, with the same sex distribution as the HIV-1-positive subjects, aged from 2 to 13 years old (6.8 ± 2.9) were included in the study as controls. All the HIV-1-positive diagnoses were confirmed at 18 months of age by western blot at the Jiangsu Provincial Center for Disease Prevention and Control. Based on epidemic investigations and laboratory confirmations in the China Information System for Disease Control and Prevention, study subjects were infected with HIV through vertical transmission from mother-to-child. The enrolled children had no recorded surgical or blood product transfusion histories. The HIV-1-negative control group children were recruited from Nanjing Children’s Hospital. Eligibility requirements for the control group included normal liver and kidney functions, no history of cancer or other diseases that may affect their immune system and negative maternal HIV-1 testing. The study was approved by the ethical committee of Jiangsu Provincial Center for Disease Prevention and Control.

Assay of CD3+CD56+ cell percentage and NK cell receptor expressions

Whole blood from study subjects was collected with anticoagulated EDTA for CD4 T-cell quantification within 6 h. Human lymphocyte separation medium (Sinopharm Chemical Reagent, Shanghai) was added 2:1 parts into the whole blood and fully mixed. The mixture was then centrifuged with 3000 r.p.m., 15 min at room temperature, and the PBMCs were separated for the receptor assay. The plasma was separated and frozen at −70°C for VL assay.

FACS flow cytometry analysis was performed to detect changes in CIK cell percentages and NK cell receptor profile expressions on CIK cells. First, the PBMCs were twice washed with Dulbecco’s PBS (1×) (Hyclone, Beijing). Next, mouse anti-CD45-allophycocyanin (APC) (eBioscience, CA, USA) was used to determine the gate of all white blood cells in the PBMC solution with FACS. The CIK cells were labeled with anti-CD56 PE-Cy5.5/CD3 Alex Flour 488 antibodies (Invitrogen, CA, USA) in the gate of CD45-positive cells. The following receptor antibodies were added, respectively, with the former antibodies to the PBMC solution: anti-CD16-PE and anti-NKp80-PE (RD System, MN, USA); anti-CD244-PE (eBioscience); anti-NKp44-PE, anti-KIR3DL1-PE and anti-NTB-A-PE (Becton Dickinson, CA, USA) and anti-KIR2DL1-PE, anti-NKp46-PE and anti-NKp30-PE (Becton Dickinson, CA, USA).

We conducted an assay of the receptors on CIK cells with the BD FACScalibur flow cytometer, and 10⁵ cells were acquired. The isotypic controls corresponding to the former antibodies were done before the former procedures.

Generation of CIK cells and dendritic cells

The PBMCs of study subjects were collected to separate the CIK cells and dendritic cells (DCs). DCs were generated as described previously (25). PBMCs were allowed to adhere in six-well plates at a density of 5 × 10⁶ cells ml⁻¹ for 1 h at RPMI-1640 with autologous heat-inactivated serum. Non-adherent cells were collected to generating CIK cells (see below). Adherent cells were cultured in 2 ml RPMI-1640 with 10% FCS and 500 U ml⁻¹ human granulocyte macrophage colony-stimulating factor (Peprotech, Rehovot, Israel) and 500 U ml⁻¹ human IL-4 (Pepro-Tech) per well for 7 days.

CIK cells were generated as described previously (26). In brief, PBMCs were prepared and grown in RPMI-1640 medium (Invitrogen, NY, USA), consisting of 10% FCS and 1000 U ml⁻¹ human recombinant IFN-γ (PeproTech). After 24-h incubation, 50 ng ml⁻¹ of anti-human CD3 (clone: OKT3, eBioscience, CA, USA) and 300 U ml⁻¹ IL-2 (PeproTech) were added. And, then cells were subcultured every 3 days in fresh complete medium and IL-2.

Co-culture of stimulated CIK cells with autologous DCs

CIK cells were harvested after incubated for 7 days as effector cells, and CIK cells were co-cultured for another 7 days with autologous 7 days of DCs as target cells with the effect/target ratio of 10:1. The target cells of each sample were 10⁵ cells.

Assay of subtype of DCs in the co-culture

The co-cultured cells were harvested after incubated for 7 days. Non-specific sites were blocked by incubation for 10 min on ice with fluorescence-activated cell sorter buffer (1× PBS,
2% heat-inactivated FBS and 0.1% sodium azide) containing 12% heat-inactivated FBS and 10 mg of total mouse IgG (Sigma–Aldrich, St Louis, MO, USA). The DCs were labeled with mouse anti-human monoclonal antibodies: PE-cyanine (Cy) 5.5-conjugated anti-HLA-DR; APC-conjugated anti-CD11c and PE-conjugated anti-CD123 (eBioscience). Then, the former cells were labeled with the following mouse anti-human monoclonal antibodies, respectively: FITC-conjugated anti-CD80, anti-CD83 and anti-CD86 (eBioscience). Dead cells were identified by trypan blue exclusion. Data acquisition of 105 events per sample was performed on a BD FACSCalibur device (BD Bioscience), and the analysis was done using CellQuest software (BD Bioscience).

**Analysis of killing function of CIK cells**

CIK cells were harvested after incubated for 7 days for the function analysis. The analysis was described as our previous study (27). In brief, K562 cells as the targets were collected, and the cells were labeled with a final concentration of 2 μM CFSE (Sigma–Aldrich) for 10 min. Then, the labeled cells were washed with PBS three times. The effect/target
ratio of 10:1 was set-up; the target cells of each sample were $10^5$ cells. Every sample was mixed to a final volume of 200 µl RPMI-1640 medium in 96-well flat-bottom plate and incubated for 4 h. Then, samples were put in an ice water bath and final concentration of 100 µg mL$^{-1}$ phosphatidylinositol (PI) (Sigma-Aldrich) was added in and incubated for 5 min in order to label the DNA of the dead cells. The dead target cells were analyzed within 60 min.

All samples were analyzed as described previously (27). Five thousand target events were collected. CFSE$^+$/PI$^+$ cells were considered as dead target cells. Percentage of specific target cell death (killing ratio) was analyzed by CellQuest software.

**Quantification of CD4 T cells**

In order to evaluate the relationship between CD4 T-cell counts and CIK cell percentages, CD4 T-cell quantification was performed in whole blood from study subjects. Whole blood was stained with mixed antibodies of ‘MultiTEST CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC Reagent’ (Becton Dickinson), and $10^5$ cells were acquired.

**VL assay of the HIV-1-infected children**

Plasma HIV-1 VL was quantified with AMPLICOR HIV-1 MONITOR (Roche, Basle, Switzerland) within 6 h of blood collection and plasma separation, in order to determine the effect of HIV-1 VLs on CIK cell percentages.

**Quantification of cytokines by ELISA**

Cytokines (IL-2, IL-4, IL-17, TNF-$\alpha$ and IFN-$\gamma$) released in plasma were measured using the specific eBioscience ELISA kits (eBioscience).

**Statistical analysis**

Results were expressed as mean ± standard deviation (SD). Statistical significance was assessed by non-parametric tests performed using Statistical Product and Service Solutions (SPSS, version 11.5). A cut-off of $P < 0.05$ was considered to be statistically significant. Correlation analysis was assessed by Stata 10.0, and a cut-off of $P < 0.05$ and a correlation coefficient between $-1$ and $1$, not including 0, was considered to be statistically significant.

**Results**

**CIK cell percentage**

The CIK cell percentage was notably increased 11.77-fold in total white blood cells in HIV-1-infected children. In order to quantify the percentage of CIK cells from PBMC in HIV-1-infected children, we labeled the CIK cells with anti-CD45, anti-CD3 and anti-CD56 antibodies. The CD3$^+$CD56$^+$ CIK cell population was detected by FACS. The percentage of CIK cells in HIV-1-infected children was dramatically increased, and there was a significant difference ($P < 0.001$) between HIV-1-infected and uninfected children (Fig. 1).
Expression of NK cell receptors on CIK cells

Expressions of KIR3DL1 and NKG2D receptors on CIK cells were increased by 3.23-fold and 1.39-fold, respectively. The percentages of KIR3DL1 ($P < 0.01$) and NKG2D ($P < 0.001$) receptors expressed were significantly increased in HIV-1-infected children compared with controls (Fig. 2).

The percentage of activated CD16, NKp30, NKp44, NKp46 and NKp80 receptors expressed on CIK cells were decreased by 6.70-, 41.04-, 4.74-, 14.38- and 2.99-fold, respectively. Moreover, the co-receptor of CD244 expressed on CIK cells was decreased by 1.30-fold. The percentage of these receptors expressed on CIK cells in HIV-1-infected children was dramatically decreased, and there was a significant difference in the receptor profiles between HIV-1-infected children and the controls (Fig. 3).

The expression of the KIR2D and NTB-A receptors on CIK cells was also examined. The percentages of these two receptors expressed on CIK cells had no significant difference between HIV-1-infected children and controls ($P > 0.05$, Fig. 4).

CIK cells promote maturation of DCs in HIV-1-infected children

CD80, CD83 and CD86 are important markers for the maturation of DCs in human peripheral blood. The percentages of CD80+, CD83+ and CD86+ DCs were significantly elevated in HIV-1-infected children ($**P < 0.01$). The values of CD80 on DCs co-cultured with CIK cells from HIV-1-negative children and from HIV-1-infected children were 17.63 ± 1.99 and 45.13 ± 4.41, respectively; the values of CD83 were 15.94 ± 2.71 and 34.93 ± 3.34 and the values of CD86
were 66.09 ± 4.57 and 81.23 ± 5.15, respectively. The expressing percentages of CD80, CD83 and CD86 on DCs co-cultured with CIK cells of HIV-1-infected children are obviously promoted compared with the co-cultured with CIK cells of HIV-1-negative controls (P < 0.01, Fig. 5).

Killing function of CIK cells
CIK cell killing function was assessed by K562 as the target cell. The percentage of CFSE$^+$/PI$^+$ cells in HIV-1-infected children was 16.42 ± 3.14. However, the percentage of CFSE$^+$/PI$^+$ cells in HIV-1-negative children was 10.88 ± 3.20. The difference between HIV-1-infected children and HIV-negative children was significant (P < 0.01) (Fig. 6) indicating that CIK cell killing function was promoted in HIV-1-infected children.

Quantification of CD4 T cells and VL in the HIV-1-infected children
To evaluate the effects of CD4 T-cell counts and VL on percentages of CIK cells in HIV-1-infected individuals, the number of CD4 T cells and VL in HIV-1-infected children were examined. The correlation between CD4 T-cell count and the percentage of CIK cell in the HIV-1-infected children was analyzed yielding a positive correlation coefficient of 0.961 (P < 0.001). The range of HIV-1 load in HIV-1-infected children was from 800 to 300,000 copies ml$^{-1}$. Usually, the VL and CD4 T-cell counts are reverse in HIV-1-infected person. As showed with the figure, the correlation between HIV-1 VL and the percentage of CIK cells in the HIV-1-infected children was negative. Therefore, CD4 T-cell counts and the percentage of CIK cell in the HIV-1-infected children was
a positive correlation as indicated and the correlation between HIV-1 VL and the percentage of CIK cells in the HIV-1-infected children was negative (Fig. 7).

**Secretion of cytokines in plasma**

IL-2, IL-4, IL-17, TNF-α and IFN-γ were analyzed in the plasma of HIV-1-infected children and HIV-negative children. The concentrations of IL-2 were 165.93 ± 78.82 and 127.66 ± 69.59 pg ml⁻¹ in HIV-negative children and HIV-1-infected children, respectively (P > 0.05). Concentrations of IL-4 were 4.97 ± 2.70 and 5.88 ± 1.99 pg ml⁻¹ in HIV-negative children and HIV-1-infected children, respectively (P < 0.05). Concentrations of TNF-α were 4.58 ± 3.24 and 4.13 ± 2.53 pg ml⁻¹ in HIV-negative children and HIV-1-infected children, respectively (P > 0.05). Concentrations of IFN-γ were 2.16 ± 0.03 and 2.18 ± 0.02 pg ml⁻¹ in HIV-negative children and HIV-1-infected children, respectively (P < 0.05) (Fig. 8).

**Discussion**

CIK cells are immune effector cells that co-express CD3 and CD56 molecules. CD3+CD56+ CIK cells are developed from CD3+CD56-T lymphocytes not from CD3-CD56+ NK cells in human blood. CD3+CD56+ CIK cells demonstrate a more potent cytolytic activity compared with CD3+CD56-T...
lymphocytes (28). Moreover, CD4 molecules are absent in most CIK cells because in humans, CIK cells mainly develop from CD4-CD8 T lymphocytes (29). Our results showed that the relationship between CD4 T-cell count and CIK cell percentage is a positive correlation. Therefore, further research is needed to determine how CD3+CD56+ CIK cell percentages in total white blood cells is significantly increased in HIV-1-infected children. A previous study by Koreck et al. (30) verified that the CD3+CD56+ cells were significantly decreased in the peripheral blood of patients with psoriasis. These results indicate that changes in CD3+CD56+ CIK cells are different in different diseases.

NK cell receptors such as NKG2D, expressed on non-MHC restricted, activated CD8+ T cells (31), were present on unstimulated CIK cells. Linn et al. (9) verified that up-regulation of NKG2C and NKG2E receptor expression on CIK cells correlated with cytotoxicity. Our results demonstrated that the expressions of NKG2D and KIR3DL1 on CIK cells were increased in HIV-1-infected children. Therefore, we presumed that these changes may be correlated with the increasing cytotoxicity of CIK cells. However, the expression of other activating receptors including NKp30, NKp44, NKp46, NKp80, CD16 and the cooperative receptor of CD244 on CIK cells were decreased significantly. These changes in CIK cells will be the important markers for early diagnosis of HIV-1 via MTCT. Kishi et al. (12) identified that the increase in CD3+CD56+ CIK cells and NK cells may reflect activation of immune reactions and proliferation of CD3+CD56+ T cells and activated NKp46+ NK cells in the peripheral blood; these are useful markers for early diagnosis of HPS.

Many studies have found a T_{n1}–T_{n2} shift in HIV-1-infected individuals, characterized by different secretions of relevant cytokines. During the course of HIV-1 infection, secretion of T_{n1} cytokines, such as IFN-γ, is generally decreased, whereas production of T_{n2} cytokines, IL-4, is increased. Bi-functional cytokines such as IFN-γ and IL-4 have been shown to have both inhibitory and stimulatory effects on HIV-1 (32). Moreover, cytokine profiles of asymptomatic (treatment naive) and symptomatic (undergoing treatment) HIV-1 patients was determined and found that IL-4 and IFN-γ were undetectable in most study subjects (33). Recent study showed that the mean levels of some cytokines were higher in pediatric patients than in adult patients (34), but IL-4 and IFN-γ were not detected in the study. Our results revealed that IFN-γ and IL-4 were significantly increased in plasma of HIV-1-infected children. As has been usually known, IL-4 is a cytokine that induces differentiation of naive helper T cells to T_{n2} cells. Upon activation by IL-4, T_{n2} cells subsequently produce additional IL-4. IFN-γ is produced predominantly by NK, NK T cells, CD4 and CD8 CTL. So it is needed further to determine the levels and origin of IFN-γ and IL-4 in plasma of HIV-1-infected children.

Interactions between DCs and CIK cells can lead to an increase in cell surface markers that are important for mediating cellular cytotoxicity (35). Our data showed that the expressions of CD80, CD83 and CD86 on the surface of DCs were increased significantly when CIK cells co-cultured with DCs, these three markers are the key signs for DCs maturation. Therefore, we presume that the percentage of CIK cells increase significantly in HIV-1-infected children, which can promote the maturation of DCs and affect the function of DCs further. Moreover, the expressions of NK cell receptors on CIK cells are changed dramatically; these changes are relative to the increasing killing function of CIK.
cells. However, we also must think much of the molecular mechanisms of these results in our future study.

In conclusion, our results suggested that the percentage of CD3+CD56+ CIK cells was increased and the expression of NK cell receptors on CIK cells. Moreover, CIK cells possessed the characteristics of promoting the maturation of DCs and killing functions in HIV-1-infected children. Additionally, cytokines in plasma were changed obviously in HIV-1-infected children. These changes likely occurred as a protective mechanism against transmission of maternal HIV-1 virus and thereby helped to limit viral spread, eliminate infected cells and help HIV-1-infected patients to slow the progression to AIDS.

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**References**


