

CD56^{dim} NK Cell is an Important Factor in T Cell Depletion of cART-Treated AIDS Patients

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Purpose: To investigate factors involved in T-cell depletion in combination antiretroviral therapy (cART)-treated human immunodeficiency virus 1 (HIV-1)-positive patients.

Patients and Methods: 29 HIV-1-positive patients were enrolled. The CD4+, CD8+ T cell subsets and CD56^{dim} NK cells were detected by flow cytometry. The concentrations of cytokines were measured by enzyme-linked immunosorbent assay. Extraction, amplification, and viral load quantification of specimens were performed using the Roche Cobas Ampliprep/Cobas TaqMan HIV-1 test.

Results: Compared with IR group, the total number of red blood cells (RBCs) and lymphocytes (LCs) in INR group was significantly reduced, and there was a significant positive correlation between the number of RBCs and that of LCs. The overall production rates of T cells-related cytokines were lower in INR group. However, the cell-surface expression of programmed death-1 (PD-1) on CD4+ T and CD8+ T cells were markedly elevated in INR group. Moreover, it was found that the proportion and the killing ability of CD56^{dim} NK cells significantly increased in INR patients, and significantly correlated with apoptosis of T lymphocytes.

Conclusion: A poor immune reconstitution in HIV-positive patients might result from multiple factors, including bone marrow suppression, high PD-1 expression on the surface of CD4+ T cells, and over-activation of T and NK cells. Besides, the activity of NK cells and RBCs count might be important auxiliary indicators for immune reconstitution and provided a reliable guidance for developing strategies to improve immune reconstitution.

Keywords: AIDS, T-cells, CD56^{dim} NK cells, antiretroviral therapy, immune reconstitution

Introduction

Acquired immunodeficiency syndrome (AIDS) is caused by infection with the human immunodeficiency virus (HIV-1), which is a member of the retroviral family of the lentivirus family. It mainly attacks lymphocytes expressing CD4 molecules, causing damage to the immune system. Although combined antiretroviral therapy (cART) can well control virus replication, certain patients may experience very little or no T-cell reconstitution.¹ According to the statistics about 20% of AIDS patients under cART cannot achieve satisfactory immune reconstitution.^{2,3} These patients are called immune non-responders (INRs). At present, there is some disagreement about the count standard of CD4+ T lymphocytes in INRs. One of them is that whose CD4+ T cells count is less than 200/ μ L after receiving cART treatment for two years.⁴⁻⁶ Severe changes in the immune function of the INR population, including decreased lymphocyte production, dysfunction of immune cells in tissues, changes in the proportion of regulatory T cells, and increased immune activation, immune senescence, and apoptosis.⁷

Although the specific mechanism leading to incomplete immune reconstitution is still unclear, a large number of studies have found that the possible cause is due to the decreased production and consumption of lymphocytes.^{4,8} In the

course of HIV-1 infection, many factors affect bone marrow hematopoietic function, leading to a decrease in lymphocyte production.⁹ Antiviral therapy can control the viral load but usually the virus replicates at a low level in the body, and persistent infection can cause cell apoptosis. These may cause the immune system to be disordered, lymphocytes are difficult to recover, and ultimately affect the immune system rebuilding.¹⁰

In the aspect of bone marrow hematopoiesis, the main reports currently focus on the destruction of hematopoietic cells by HIV-1. In vitro experiments, it was found that HIV-1 can infect hematopoietic progenitor cells (HPCs) to be latent foci of HIV-1, which is not easy to be cleared and is easily damaged.¹¹ In all stages of AIDS, patients often have anemia, lymphopenia, leukopenia, thrombocytopenia and other symptoms of bone marrow abnormalities.¹² The study found that the Tat protein of HIV-1 can cause a reduction in the expansion of bone marrow cells and a reduction in the proportion of the original subpopulation of hematopoietic stem cells by down-regulating the expression of a series of hematopoietic cytokines.¹³ Bone marrow suppression can reduce the production of lymphocytes to affect the immune reconstitution of AIDS patients.

After HIV-1 infection, the immune system will be fully activated to clear the virus. The incomplete clearance of HIV-1 leads to the continuous activation of the immune system, and activated lymphocytes is consumed. Activated immune cells produce a large number of cytokines, which on the one hand promotes the activation and differentiation of immune cells, and on the other hand regulates the replication of HIV-1. The results showed that the level of IL-10 in the elderly AIDS patients' serum increased, and the increased IL-10 could inhibit the replication of HIV-1.¹⁴ However, lymphocytes that excessively secrete cytokines can also cause the depletion of T cells through the activation-induced cell death (AICD) pathway. Chronic activation of T cells and related cytokines might be important factors to immune reconstitution of AIDS patients.¹⁵

As an immune checkpoint molecule, one role of programmed death 1 (PD-1) is to down-regulate T cells activity and prevent autoimmune tissue damage to maintain the homeostasis of the cell population.¹⁶ The expression of PD-1 molecules will slowly increase during early infection, and then gradually decrease after reaching a peak. During the progression of HIV-1 infection, the down-regulation of PD-1 has a positive effect on restoring the CD4+ T cells pool.¹⁷

Natural killer cells (NK cells), as innate immune lymphocytes, play an important role in eliminating virus-infected cells. According to the level of membrane marker CD56 molecules, NK cells can be further divided into CD56^{bright} and CD56^{dim} NK cells, which perform different functions.¹⁸ NK cells respond to viral infections without the need for clonal expansion, and can replace damaged CD8+ T cells during HIV-1 infection and kill the HLA down-regulated cells infected by virus.¹⁹ Compared with healthy controls, the proportion of NK cell CD56^{dim} subgroups in HIV-1 D subtype-infected people has decreased, which may not be conducive to virus clearance.²⁰ However, there is no clear description of the functional changes of the CD56^{dim} subgroup in the INR population and its correlation with T lymphocytes.

In this study, we investigated the difference of immune cells and related factors between the patients of INR and IR. The results may provide feasible index recommendations for early, convenient, and rapid assessment of the immune reconstitution status of AIDS patients.

Materials and Methods

Study Population and Ethical Approval

From October 2015 to April 2019, 29 HIV-1-positive patients with CD4+ T-cell count < 100 cells/ μ L that subsequently underwent cART in our hospital for 24 months were enrolled in this study. The study was approved by the Ethics Committee of the Fifth People's Hospital of Suzhou (China; Approval No. 2018003), and all the participants signed the written informed consent form prior to participating in the study. The study complies with the ethical guidelines of the 1975 Declaration of Helsinki.

Therapeutic Strategy

The antiviral regimen included tenofovir/zidovudine + lamivudine + efavirenz/nevirapine. After cART, the patients' plasma viral load dropped below the limit of detection (<50 HIV-1 RNA copies/mL). According to the number of CD4+ T cells after 24 months of treatment, 15 and 14 patients with CD4+ T-cell count < 200 and > 200 cells/ μ L were assigned to INR and IR

groups, respectively. The patients' general data at baseline included gender, age, route of transmission, duration of treatment, and CD4+ T-cell count.

Flow Cytometry

The CD4+ T-cell count in peripheral blood mononuclear cells (PBMCs) was determined by flow cytometry. In brief, the cells were labeled with fluorochrome-conjugated antibodies raised against CD3, CD4, and CD8 for 30 min at 4 °C. The cells were then fixed and permeabilized with the fixation/permeabilization solution from the Cytotfix/Cytoperm kit (BD Biosciences) as described by the manufacturer and labeled with fluorochrome-conjugated antibodies. We determined absolute and percentage values for T lymphocyte subsets CD3+, CD3+/CD4+T, CD3+/CD8+T, CD19+ B cells and CD3-/CD56+ NK cells in PBMCs using a flow cytometer (BD Biosciences, San Jose, CA, USA).¹⁷ Fluorescence was quantified using a BD Biosciences LSR Fortessa flow cytometer (BD Biosciences) and the FlowJo software package (FlowJo, LLC, Ashland, Oregon, USA).

COBAS AmpliPrep/COBAS TaqMan HIV-1 Test

The AmpliPrep/COBAS TaqMan HIV-1 test combines automated sample preparation for HIV-1 RNA purification using the AmpliPrep, and real-time polymerase chain reaction (PCR) amplification and detection using the COBAS TaqMan or the COBAS TaqMan analyzer. Extraction, amplification, and viral load quantification of specimens were performed using the Roche Cobas Ampliprep/Cobas TaqMan HIV-1 test (CAP/CTM) (Roche Molecular Systems, Branchburg, NJ). The TaqMan HIV-1 test was shown to have a linear dynamic range from 50 copies/mL to 1×10^7 copies/mL.²¹

Determination of Serum Levels of Cytokines

The concentrations of cytokines (IL-2, IL-4, IL-6, IL-10, IL-17, and IFN- γ) of the AIDS patients' serum were measured by enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA).

Statistical Analysis

Statistical analyses were performed by using SPSS 19.0 (IBM, Armonk, NY, USA) and GraphPad Prism 6.0 (GraphPad Software Inc. San Diego, CA, USA) software. The effect of treatment on erythrocytes and lymphocytes in INR and IR groups were analyzed by using paired *t*-tests. Comparisons between INR and IR groups were performed by using the unpaired *t*-test, and the correlation analysis using Spearman correlation coefficient.

Results

General Situation of the Research Object

The 29 HIV-1-positive patients were all male, and the HIV-1 infection route was homosexual sex. The patients' mean age in INR and IR groups before cART was 49 and 41 years old, respectively, and the mean CD4+ T-cell count of those groups was 64.69 and 88.60 cells/ μ L, respectively (Table 1). After 24 months of treatment, their average number of CD4+ T lymphocytes became 143.9 and 530.0 cells/ μ L, respectively.

The Numbers of RBCs and LCs are Correlated to the Outcome of Immune Reconstitution

The results showed that the numbers of RBCs and LCs in IR group were markedly elevated after cART compared with the initial treatment (Figure 1A and B). The mean number of RBCs and LCs was elevated by about 20% and 55.6%, respectively. However, the same trend was not observed in INR group (Figure 1C and D). After 2 years of treatment, the numbers of RBCs and LCs in IR group were significantly higher than those in INR group (Figure 1E and F). Through correlation analysis of the numbers of RBCs and LCs in patients who underwent cART, we found that both groups showed a positive correlation (Figure 1G and H). Our findings suggest that RBCs count is closely correlated to LCs count after a systemic treatment, which may be related to changes in the hematopoietic environment.

Table 1 Comparison of the General Information

Item	INR Group (n=15)	IR Group (n=14)
Gender		
Male	15	14
Female	0	0
Age (Median, years)	49 (27–61)	41 (29–64)
The route of transmission	Homosexual	Homosexual
Median duration of HAART (years)	3.1 (2.7–3.6)	2.9 (2.5–3.6)
Baseline level of CD4+T cells (cells/ μ L)	64.69 \pm 8.65	88.60 \pm 14.19
CD4+T cells level after treatment (cells/ μ L)	143.9 \pm 9.444	530.0 \pm 52.77

Note: Results are presented as mean \pm standard deviation of triplicate determinations.

Abbreviations: IR, immune responder; INR, immune non-responder.

Reduced Cytokine Secretion and Increased PD-1 Expression Suggest More Severe Immune Exhaustion in INR Patients

We further tested and compared the levels of T lymphocyte-related cytokines in the serum of INR and IR groups. The results showed that IL-2, IL-4, IL-10, IL-17 and IFN- γ in INR group were significantly lower than those in IR group (Figure 2A, $P < 0.05$). The ratio of CD4+ T cells and CD8+ T cells in INR group was lower than that in IR group, respectively (Figure 2B and C; $P < 0.05$). At the same time, the cell surface expression of PD-1 on CD4+ T and CD8+ T cells in INR group was significantly increased (Figure 2D and E; $P < 0.05$). The decreased secretion of cytokines and the high expression of inhibitory immune checkpoint PD-1 indicated that the patients in INR group had more severe immune failure. This is not conducive to the rebuilding of the immune system.

CD56^{dim} NK Cells in INR Patients May Inhibit Immune Reconstitution by Negatively Regulating PD-1+CD4+ T Lymphocyte Subsets

NK cells contribute to the resolution of adaptive immune responses via deletion of activated T cells, thus limit or exacerbate immune responses.²² In the present study, the results showed that there was no significant difference in the percentage of NK cells in PBMCs between the two groups (Figure 3A; $P > 0.05$). However, the proportion of CD56^{dim} NK cells was significantly higher in INR group than that in IR group (Figure 3B; $P < 0.05$). We further assessed the killing capability of CD56^{dim} NK cells by detecting the expression levels of CD107a, Granzyme B and Perforin in the cells. The results showed that these killing factors were surprisingly enhanced in INR group (Figure 3C-E; $P < 0.05$). These findings suggest that the increase in the number and function of CD56^{dim} NK cells may be directly related to the lower number of T lymphocytes in INR group. We further analyzed the correlation between CD56^{dim} NK cells with T lymphocytes, suggesting that CD56^{dim} NK cells are negative correlation with the proportion of CD4+T and PD-1+CD4+T lymphocyte subsets in INR group (Figure 3F and G). However, in IR group, CD56^{dim} NK cells showed no correlation with the proportion of CD4+T and PD-1+CD4+T lymphocyte subsets (Figure 3H and I). The above results suggest that the proportion of CD56^{dim} NK cells in the INRs could be responsible for reduction in PD-1+ T cells and might play roles in the immune depletion.

Discussion

In this study, the average proportion of CD56^{dim} NK cells in INR group was higher than that in IR group. We further detected the killing ability indicators (CD107a, perforin and granzyme B) of CD56^{dim} NK cells, and found that their expression levels all increased significantly. These results suggest that the reason why CD56^{dim} is not conducive to immune reconstitution in the INR population may be that despite a systemic therapy, virus components from HIV-1 latent infection continue to stimulate NK cells, resulting in an increased proportion of CD56^{dim} NK cell subsets and enhanced killing capability, which might destroy more T lymphocytes and lead to the depletion of CD4+ T lymphocytes. Zhang's

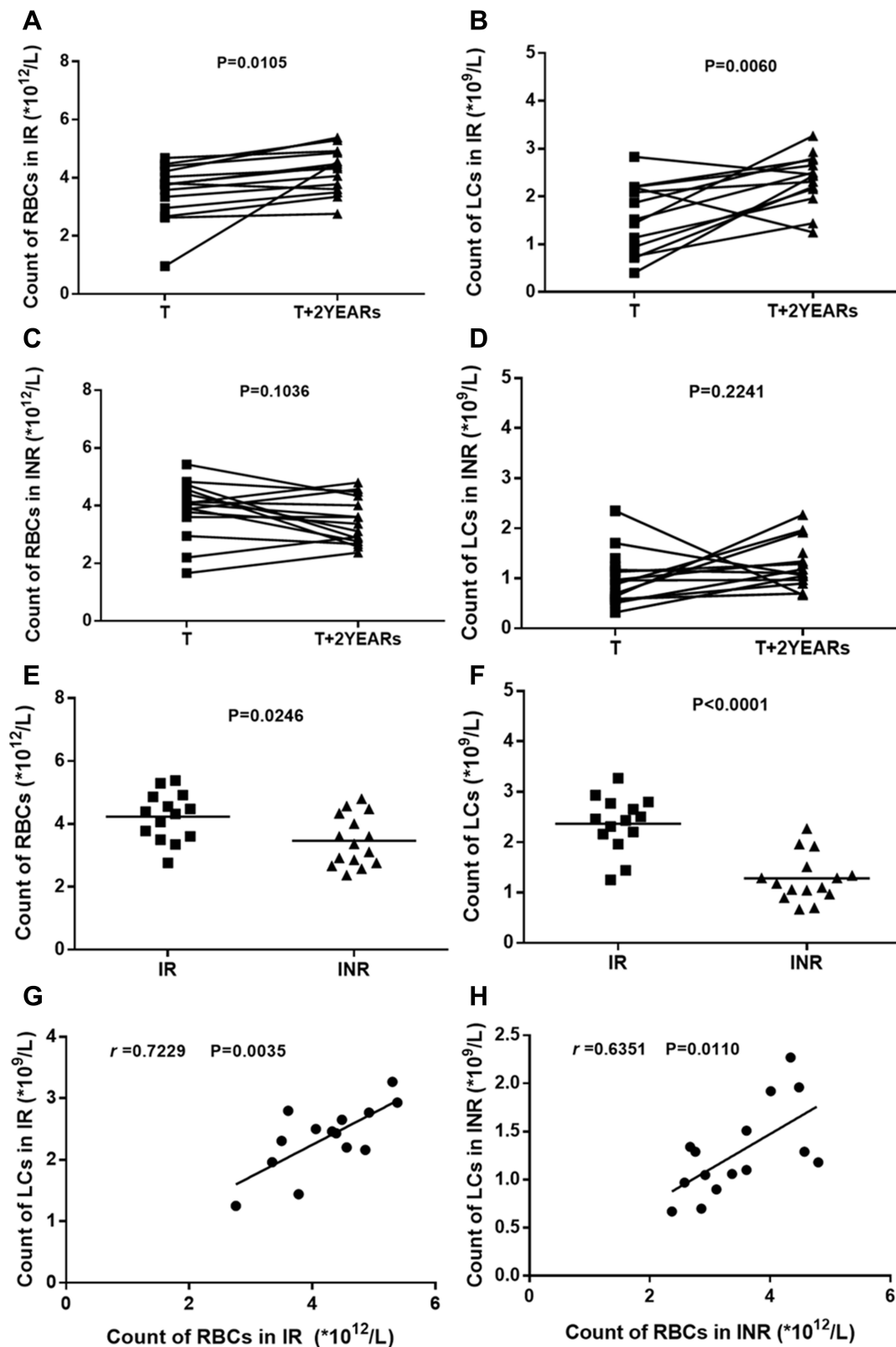


Figure 1 The numbers of RBCs and LCs are correlated to disease outcome. Detection of peripheral blood cells in INR and IR patients. Comparison of RBCs (A) and LCs (B) before and after 2 years' treatment in IR patients. Comparison of RBCs (C) and LCs (D) before and after 2 years' treatment in INR patients. Comparison of RBCs (E) and LCs (F) between IR and INR patients. The relationship between the counts of RBCs and LCs in IR (G) and INR (H) patients. All these counts were detected with FACS.

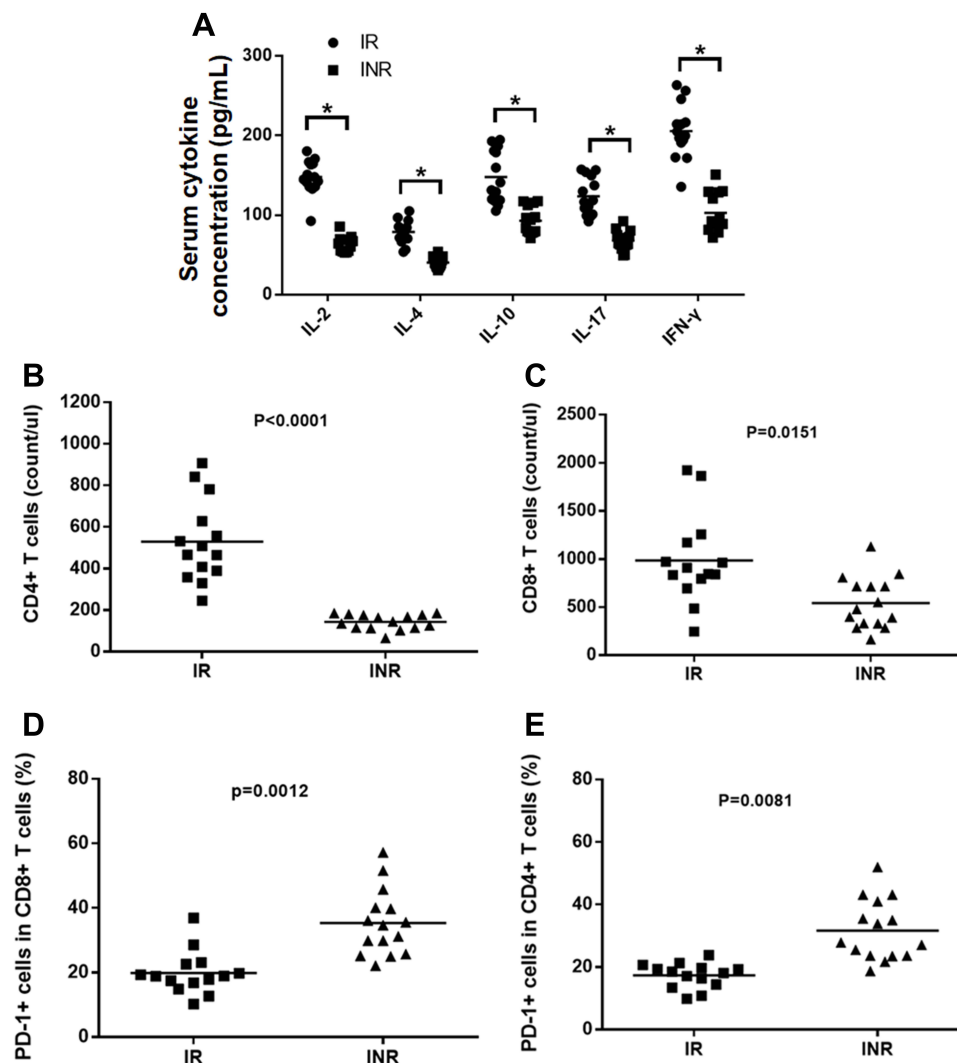


Figure 2 Reduced cytokine secretion and increased PD-1 expression suggest more severe immune exhaustion in INR patients. Detection of cytokines produced by T lymphocytes in peripheral blood: (A) Cytokine production by T cells. The count of CD4+ T cells (B) and CD8+ T cells (C) between IR and INR patients. Percentage of PD-1+ cells in CD4+ T cells (D) and CD8+ T cells (E) between IR and INR patients. * $P < 0.05$.

study also pointed out that the CD56^{dim} subgroup was found to be higher in INR patients than in IR patients.²³ These findings implied that the increased proportion of CD56^{dim} NK cells may not be conducive to immune reconstitution.

Transient PD-1 expression on cell surface is initiated upon T cell activation, while sustained PD-1 expression is a characteristic marker of T cell exhaustion.²⁴ In this study, we found that bone marrow suppression and PD-1 overexpression on the surface of T lymphocytes were more severe in INR group, which might lead to depletion of T lymphocytes. Interestingly, we found that the proportion of CD56^{dim} NK cells in AIDS patients was negatively correlated with the proportion of CD4+ and PD-1+CD4+ T cells. Since PD-1 expression on CD4+ T cells showed higher level in the late stage of HIV-1 infection,¹⁷ the increased and activated CD56^{dim} NK cells might play roles in the immune depletion, resulting in the proportion of CD4+ T cells and PD-1+CD4+ T cells decreased in INR patients.

We also found that a lower level of certain cytokines in INR patients than that in IR patients, for example, IL-2, IL-4, IL-10, IL-17 and IFN- γ , which have proven to be closely correlated to the activation and proliferation of T lymphocytes.^{25–27} The reduction of cytokine production from lymphocytes indicates that in the INR patient population, the cytokine production capacity of lymphocytes is significantly reduced, eventually leading to T lymphocyte depletion.

The cART can significantly improve the numbers of RBCs (>20%) and LCs (>85%), which can be a positive indicator of immune condition improvement. The number of RBCs and LCs in the peripheral blood sample can partially reflect the

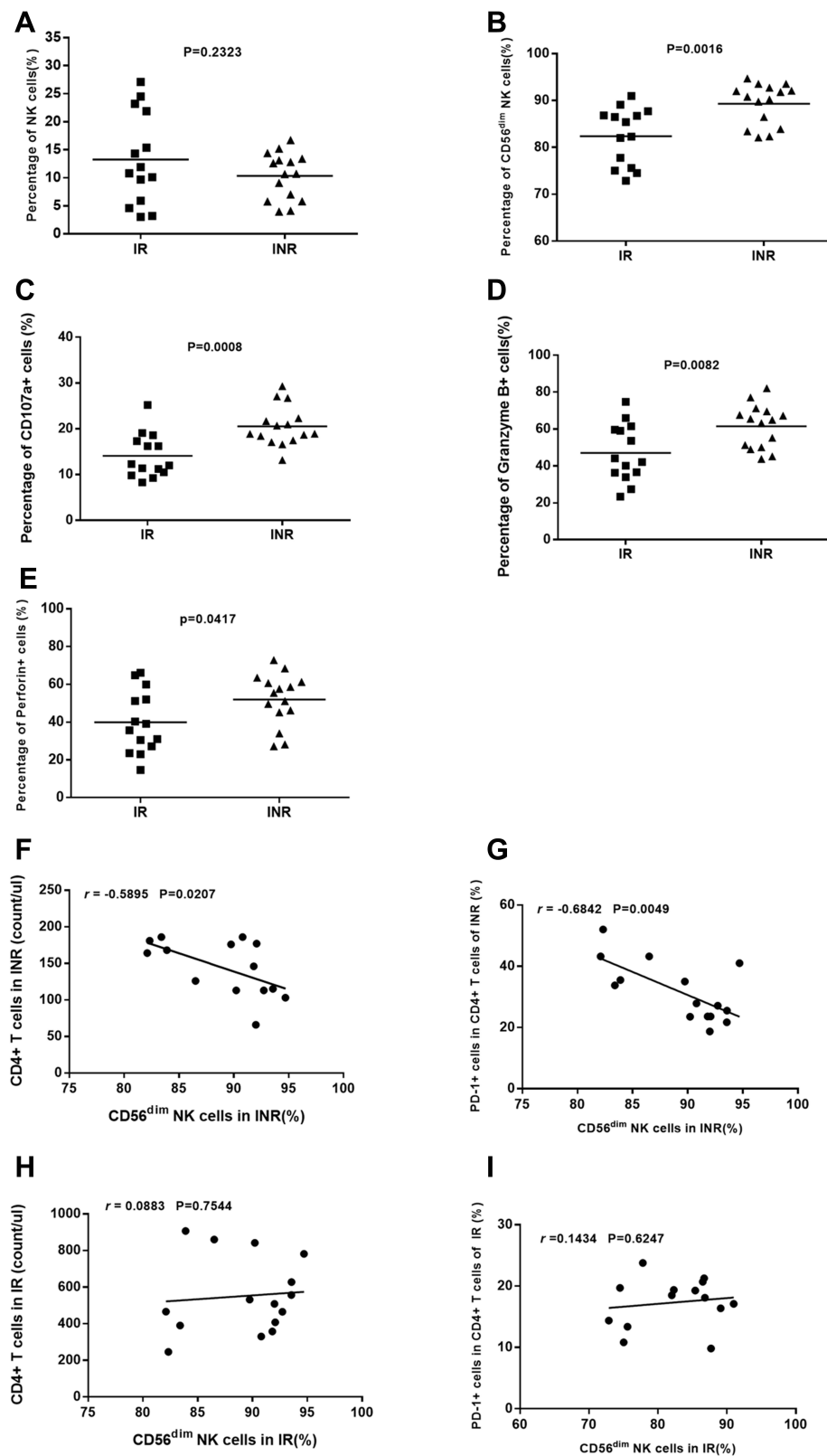


Figure 3 The proportion and the killing ability of CD56^{dim} NK cells significantly increased in INR patient, and significantly correlated with T lymphocytes exhaust. NK cells and CD56 subsets detected by using flow cytometry, and the killing capability of CD56^{dim} NK cells assessed by the related cellular molecules. Comparison of the percentages of NK cells (A), CD56^{dim} NK cells (B), CD107a+ cells in CD56^{dim} cells (C), Granzyme B+ cells in CD56^{dim} cells (D), and Perforin+ cells in CD56^{dim} cells (E) between IR and INR patients. Correlation between CD56^{dim} NK cells and CD4+ T lymphocytes (F), and between CD56^{dim} NK cells and PD-1+CD4+ T lymphocytes (G) in INR patients. Correlation between CD56^{dim} NK cells and CD4+ T lymphocytes (H), and between CD56^{dim} NK cells and PD-1+CD4+ T lymphocytes (I) in IR patients.

function of bone marrow.²⁸ There is a positive correlation between the number of RBCs and LCs after a systemic treatment. In the body, the cytokine levels are important to the hematopoietic environment.²⁹ Our results implied that the low levels of T cell-related cytokines in INR group might be insufficient to stimulate the bone marrow hematopoiesis.

Conclusion

This study revealed that the poor immune reconstitution might be caused by multiple factors, such as myelosuppression, T cell destruction enhanced by PD-1 pathway, decreased cytokine production by lymphocytes, and the increased killing efficiency of CD56^{dim} NK cells in INR patients. Among them, the red blood cell count may be an important auxiliary detection index for immune reconstitution, and the increased CD56^{dim} NK cell subset further enhanced the depletion of CD4⁺ T lymphocytes. We also found that not only the proportion of CD56^{dim} NK cells, but also their killing function are also enhanced in INR patients. These findings give us some clues to better understand the mechanisms of incomplete immune reconstitution in AIDS patients.

Abbreviations

cART, combination antiretroviral therapy; INR, immune non-responder; IR, immune responder; PD-1, programmed cell death protein 1; RBCs, red blood cells; LCs, lymphocytes; AICD, activation induced cell death.

Data Sharing Statement

The data that support the findings of this study are available from the study team but restrictions apply to availability of these data and so not publicly available. Data are however available from corresponding author upon reasonable request.

Ethics Approval and Informed Consent

Approval was obtained from the Fifth People's Hospital of Suzhou, China (Approval number: 2018003).

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Author Contributions

Feng Qian and Song Hu: conception, study design, methodology execution and acquisition of data; Yue-ping Zhu, Yin-ling Wang, Jin Liu, Jia-lu Qiao, Xi-ji Shu and Yong Gao: formal analysis and interpretation; Bin-lian Sun and Chuan-wu Zhu: supervision and funding acquisition. All authors made substantial contributions to draft the manuscript, revise or critically review the article; gave final approval of the version to be published; have agreed on the current journal; and agree to be accountable for all aspects of the work.

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Disclosure

The authors have no conflicts of interest to declare in this work.

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