Characterization Of Age-Related Immune Features After Autologous NK Cell Infusion

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Research Article

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Abstract

Objective: To analyze the characterization of age-related immune features after autologous NK cell infusion.

Background: Aging is a progressive loss of physiological function, accompanied with a functional decline of immune system, especially in T cell responses, which consequently leads to the increase of infections and cancers. However, understanding of immune ageing on body health is currently lacking.

Methods: In this study, we originally checked whether the administration of autologous NK cells would affect T cell senescence and exhaustion in healthy human beings with middle ages. Also, we detected whether NK cells infusion would affect senescence associated secretory phenotype (SASP)-related factors level.

Results: Results showed that senescent T cells including $CD28^-$, $CD57^+$, $CD28^-CD57^+$ and $CD28^-KLRG1^+$ subsets decreased significantly in both $CD4^+$ and $CD8^+$ T cells following once infusion of autologous NK cells. In addition, PD-1^+ and TIM-3^+ population within $CD4^+$ and $CD8^+$ T cells also dramatically declined after the infusion. Changes were continuously observed in senescent and exhausted T cell in 4 weeks after the intervention. Meanwhile, we found out that several key senescence associated secretory phenotype (SASP)-related factors including IL-6, IL-8, IL-1α, IL-17, MIP-1α, MIP-1β, MMP1 were significantly decreased after NK cell infusion. Moreover, gender did not influence the effects reducing extent caused by NK cell infusion, and whether the cells were frozen or fresh influenced several immune indexes.

Conclusion: Our findings imply that immune aging is a reversible process in healthy human beings and autologous NK cell administration can be introduced to alleviate the aging.

Introduction

Aging is characterized by a progressive loss of physiological function, and is a risk factor for several of the world's most prevalent diseases [1, 2]. According to the World Health Organization statistics, there will be more than 30% of percentage aged 60 years or older in China, American and several European countries by 2050 (https://www.who.int/ageing/en/), and related diseases will break out with age, including cancer, T2DM (type 2 diabetes), neurodegenerative disorders and cardiovascular disease.

Normally, aging is associated with a progressive decline in the function of immune system, among which natural killer (NK) cells and T cells are key components in innate and adaptive immunity, respectively [3-5]. NK cells, characterized by expressing CD16 and CD56, play critical roles as the first line of defense against virus-infection and cancer cells [6, 7]. Young individuals have high levels of functional NK cells. However, the NK-related activities decline with aging, leading to an increased incidence and severity of viral infections [8, 9]. In addition, Liu et al found that primary NK and CAR-NK cells have superior expansion capability and in vivo cytotoxicity after optimizing the cultural condition, which broaden cell therapy

application[10]. What's more, due to repeated antigenic stimulation throughout life, aging always accompanies with increasing accumulation of senescent and exhausted T cells, which in turn leads to impaired T cell-mediated responses [11-14]. This decline is largely responsible for the increased susceptibility to infection, reduced effectiveness of vaccination and higher incidences of diseases including cancer in the elderly [15-19].

Recent findings from several clinical studies showed that markers of T cell senescence (i.e. the loss of CD28 and/or gain of CD57 among CD4+/CD8+ T-cell)[15, 16] and T cell exhaustion (i.e. high expression of PD-1 among CD4+/CD8+ T-cell)[14] were usually higher in patients with HIV-infection, breast cancer or myeloid leukemia (AML) than in healthy controls. Of note, the population and/or numbers of senescent and exhausted T cells were reversed following anti-viral treatment and chemotherapy [20-23]. Importantly, these declining changes were mostly restricted to complete remission patients other than non-responders, implying that they are highly predictable and positively related to clinical outcome [23]. Natural killer cells play critical roles in immune clearance of aging-related senescent cells which may modulate T cell dysfunction. However, whether NK cells could boost immune system in sub-health population is still unknown.

Accumulation of senescent cells in aging may promote immune senescence by developing a senescence-associated secretory phenotype (SASP) and generating damage signals [24, 25]. Given the critical roles of NK cells in immune clearance of senescent cells and their declining activities with aging [8, 24, 26], here we firstly explored whether the administration of autologous NK cells would affect the peripheral population of senescent and exhausted T cells in healthy human beings with middle ages. Accordingly, the populations of $CD28^-$, $CD57^+$, $CD28^-CD57^+$ and $CD28^-KLRG1^+$ among both $CD4^+$ and $CD8^+$ T cells were assessed as senescent T cells, while the population of $PD1^+$ and $TIM3^+$ among both $CD4^+$ and $CD8^+$ T assessed as exhausted T cells here. Results showed that senescent T cells including $CD28^-$, $CD57^+$, $CD28^-CD57^+$ and $CD28^-KLRG1^+$ subsets decreased significantly in both $CD4^+$ and $CD8^+$ T cells following once infusion of autologous NK cells. In addition, $PD1^+$ and $TIM3^+$ population within $CD4^+$ and $CD8^+$ T cells also dramatically declined after the infusion. Decline was continuously observed in senescent and exhausted T cell in 4 weeks after the intervention. Meanwhile, chemokines, inflammatory cytokines, tumor necrosis factors and growth factors of serum were assayed. We found out that SASP-related factors including IL-6, IL-8, IL- 1α, IL-17, MIP-1α, MIP-1β, MMP1 were significantly decreased after NK cell infusion.

Materials And Methods

2.1 Subjects

This study (ClinicalTrials.gov identifier: ChiCTR-OOh-17011878) was approved by the Ethical Committee of Changzheng Hospital. Subjects were eligible for the study if they were 45-55 years old and disease-free. Subjects with a positive serology for human immunodeficiency virus (HIV) , hepatitis B virus (HBV),

hepatitis C virus (HCV), epstein-barr virus (EBV), cytomegalovirus (CMV), syphilis were also excluded, as were those with two or more abnormal testing results in live function tests including alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBil), indirect bilirubin (I-TBil), direct bilirubin (DBIL) and γ-GT. Also, Subjects with tumor marker alpha fetoprotein (AFP) and carcinoembryonic antigen (CEA) were excluded.

2.2 Study description

All subjects had physical examinations and medical questionnaires to assess the health status. Then, eligible subjects signed the informed consent form before entering the group and received a dose of autologous NK cells in two infusions in two days. Peripheral blood samples were obtained before cell infusion as baseline and at one- and four-weeks after cell infusion to evaluate the effects of NK cell administration on T cell senescence and exhaustion, as well as SASP.

2.3 In vitro natural killer cell amplification and cell infusion

Leukapheresis was carried out to collect PBMCs from subjects by Spectra Optia (TERUMO, USA). NK cells were PBMCs amplified *in vitro* using feeder cell free culture system with Natural killer cells culture kit (DAKEWE, China). In brief, cells were seeded into activator-coated flasks at 1~2X10^6 cells/ml and incubated in a 37℃-5% CO2 incubator (Thermo fisher, USA). Fresh NK medium was changed every 2-3 days until efficient amount of cells were obtained about 14 days later. Quality control was conducted by assessing samples taken during the whole culture period and the final cell product. The BacT/ALERT (bioMerieux, Durham, NC, USA) microbiological detection system was used for sterility, and the gel-clot technique using amoebocyte lysate from the horseshoe crab was used for endotoxin. Mycoplasma contamination tests were performed by PCR method using specific primers of mycoplasma (Yise Medical, China). Trypan staining was used to calculate cell number and cell viability of NK cells. NK cells was determined by expression of CD56 or CD16 and the absence of CD3, and quantified using flow cytometry with antibodies purchased from BD bioscience including anti-CD3 antibody (HIT3, FITC), anti-CD56 antibody (B159, APC), and anti-CD16 antibody (B73.1, APC) [27]. NK cells were resuspended in saline solution containing human serum albumin and intravenously injected into subjects at first drip rate of 20 drops/min following 40-60 drops/min in two equal lots in two days. At the end of every infusion, another 60-70 ml of saline solution was used to flush the pipeline of disposal transfusion set.

2.4 Immuno-phenotypic analysis of peripheral T cells

Peripheral blood mononuclear cells (PBMCs) were isolated using ficoll-paque (GE Healthcare, USA) for cellular phenotypic analysis of senescent and exhausted T cells by flow cytometry. For analysis of surface markers, cells were stained in PBS containing 2% fetal bovine serum (FBS, Thermo fisher, USA) with antibodies as indicated. Then flow cytometric analysis was carried out in BD LSRFortessa X20. The gating strategy to identify T cell subsets was applied as described previously [28]. Briefly, markers related to T cell senescence and exhaustion were also monitored using PD-1、TIM3、CD28 and CD57 antibodies. Antibodies used in this study were from BD Biosciences and eBioscience, including anti-CD4 (GK1.5,

1:100), anti-CD8 (53-6.7, 1:100), anti-CD25 (PC61, 1:100), anti- CD45RA (HI100, 1:100), anti-CXCR3 (G025H7, 1:100), anti-CCR4 (L291H4, 1:100), anti-CCR6 (G034E3, 1:100), anti-CCR7 (G043H7, 1:100), anti-CD127 (A019D5, 1:100), anti-CXCR5 (RF8B2, 1:100), anti-CD28 (CD28.2, 1:100), anti-CD57 (NK-1, 1:100), anti-KLRG1 (2F1, 1:100), anti-PD-1 (EH12.2H7, 1:100), anti-TIM3 (F38-2E2, 1:100).

2.5 Cytokine determination

Cytokines in blood plasma were detected by Luminex xMAP technology with multiplex assay kit (ProcartaPlex 8 Plex, Thermo Fisher, PPX-08), including MMP-1, MIP-1β, MIP-1α, IL-8, IL-1α, IL-6, IL-17A and IFN-γ.

2.6 Statistical analysis

Data in this study were represented as means ± SEM or means ± SD (the standard error of the mean). The statistical statistically significance was determined using a two-tailed paired Student t test. p value ≤ 0.05 were considered statistically significant.

Results

3.1 Baseline characteristics

During July 2017 to September 2018, 42 out of total 49 recruited subjects were with ages from 45 to 55 years old, for which these middle-age populations shown a significantly decreased percentage and activity of NK cells. During screening, 8 volunteers were excluded, among which 3 had incomplete detection index, 2 had two or more abnormal results in live function tests, 1 had abnormal immune index, 1 had positive infectious index and 1 had abnormal blood biochemical. Therefore, 34 volunteers were enrolled in the study, and received leukapheresis and subsequent NK cell administration. However, 2 subjects missed their sample collection after NK cell administration and had to exit from the study. Finally, 32 subjects successfully completed the study, among which 15 males and 17 females were included (Figure 1). Importantly, all the volunteers consulted and signed the informed consent form before participation. Meanwhile, 14 of 32 volunteers were re-injected cryopreserved NK cell,18 of 32 volunteers were re-injected fresh NK cell. The baseline characteristics of the subjects and information of NK cells used were listed in Supplementary Table 1.

3.2 Safety of autologous NK cells

After cell administration, all subjects had normal body temperature and blood pressure. And no one developed skin rashes, local infection and bleeding, fever, chills, difficult breathing, nausea and vomiting. Besides, one subject developed agrypnia in one week after cell infusion and recovered thereafter. One developed dizziness in one week after cell infusion and this phenomenon lasted for two weeks before recovering. 2 subjects developed fatigue, among which one developed mild fatigue and the other developed media fatigue, and both recovered in two weeks (Supplementary Table 2). Furthermore, we conducted routine blood test, hematological examination, urinary and virological examination at one

month later after cell infusion. No hepatotoxicity and nephrotoxicity were observed according to normal serum levels of ALT, AST, Urea, creatinine. Additionally, no abnormal C response protein (CRP), anti-thyroglobulin antibody (TGAb) and anti-thyroid peroxidase autoantibody (TPOAb) occurred, indicating no immune response and autoimmune effects were induced. Furthermore, no increased plasma levels of alpha fetoprotein (AFP) and carcinoembryonic antigen (CEA) were observed one month later, strongly confirming that autologous NK cell infusion is safe in terms of tumorigenicity.

3.3 Senescent CD4+ T cells decreased after NK cell infusion

Previous researches have proved that senescent cell accumulation accelerates aging-associated disorders and clearance of p16 positive cell delays this phenomenon [29]. NK cells play important roles in innate immunity for clearing senescent cells and defending against cancer [30, 31]. Thus, flow cytometry was carried out to detect populations of $CD4^+CD28^-$, $CD4^+CD57^+$, $CD4^+KLRG1^+$, $CD4^+CD28^-CD57^+$, $CD4^+CD28^-KLRG1^+$ as senescent $CD4^+$ T cells at baseline, one- and four-weeks after infusion. Results showed no significant changes in $CD4^+$ and $CD8^+$ populations at two time-points after cell infusion (Figure 2A-B). However, senescent $CD4^+$ T cells significantly decreased after NK cell infusion at one week and four weeks after infusion (Figure 2C). In addition, gender does not influence reducing extent caused by NK cell infusion (Figure 2D).

3.4 Senescent CD8+ T cells decreased after NK cell infusion

As we know, $CD8^+$ T cells are the main tumor killing cell group. $CD8^+$ cytotoxic T cell can attenuate tumor growth by expressing FasL and secreting granzyme B and IFN-γ. However, senescent T cell accumulation impaired T cell-mediated responses. So we check $CD8^+CD28^-$, $CD8^+CD57^+$, $CD4^+KLRG1^+$, $CD8^+CD28^-CD57^+$, $CD4^+CD28^-KLRG1^+$ percentage at baseline, one- and four-weeks after infusion. We found that senescent $CD8^+$ T cells significantly decreased after NK cell infusion at both one week and four weeks after NK cell infusion (Figure 2E). also, The effects induced by NK cell infusion are independent with gender (Figure 2F).

3.5 Exhausted T cells decreased after NK cell infusion

During chronic infections and cancer, which include persistent antigen exposure and inflammation, memory T cell differentiation exists [14]. It has been reported in human that T cell exhaustion happens during infections such as HIV and hepatitis C virus (HCV), as well as in cancer [17, 32, 33]. Importantly, exhausted T cells were characterized by high expression of PD-1, TIM-3, CTLA-4 and activation of their involved signaling pathways. The successful applications of anti-PD-1/PD-L1 antibodies in cancer immunotherapy recently have proved the significance and efficacy of treatments targeting T cell exhaustion [17]. Then we checked whether NK cell infusion affected the percentage of exhausted $CD4^+$ and $CD8^+$ T cells. Results showed that $CD4^+$PD-1^+T cell, $CD8^+$ PD-1^+ T cell, $CD4^+$TIM-3^+T cell and $CD8^+$TIM-3^+T cell were all significantly decreased after NK cell infusion at both one week and four weeks (Figure

3A). These results suggested that NK cell infusion might improve the function of T cells by alleviating exhausted status of T cells.

Moreover, The percentages of PD-1^{+} and TIM-3^{+} T cell decreased at one week and four weeks after NK cell infusion, which are independent of gender (Figure 3B).

3.6 Cell types influenced the effects of induced by NK cell infusion

In this study, we finally recruited 32 volunteers in all experiments. It should be pointed out that 14 of 32 volunteers were re-injected cryopreserved NK cell, and 18 of 32 volunteers were re-injected fresh NK cell. So we wanted to explore whether immune system effects influenced by NK cell infusion was dependent on infused cell types influence or not. We analyzed this two groups by two-tailed paired Student t test. The results suggested that fresh NK cell infusion group is better than frozen NK cell infusion group in promote immune system in sub-health population (Figure 4).

3.7 Key SASP-related factors reduced after NK cell infusion

Senescent cells accumulate with aging and lead to SASP-related factors including pro-inflammatory cytokines (IL-1α, IL-1β, IL-6), chemokines (IL-8, CXCL1), proteases (MMP-1,MMP-3,MMP-13). These SASP-related factors play critical roles to aging-related inflammation, diseases and morbidity [34-36]. Therefore, to check whether NK cell infusion decrease systematic levels of SASP-related factors, we measured cytokine levels in plasma collected before and after NK cell infusion. We found lower levels of key SASP components in plasma by NK cell infusion including IL-6, IL-8, IL- 1α, IL-17, MIP-1α, MIP-1β, MMP1, whereas a non-SASP-related factor, IFN-γ was not continuously significantly altered (Figure 5). These results indicated that NK cell infusion could attenuate SASP accumulation and improve CD4+ and CD8+ T cell activity.

Discussion

Here, we firstly uncovered that once administration of autologous NK cells in healthy human beings with middle ages could significantly decrease peripheral markers of T cell senescence and exhaustion as well as key SASP components, another critical factor highly related with aging. It's well documented that the immune system undergoes a progressive decline and deterioration with aging, which in turn results in an increase in incidence and severity of infections, impaired response to vaccines and development of cancer. Therefore, developing efficient measures to eliminate or at least alleviate the phenomenon of immune dysfunction may theoretically and practically restore or improve protective function of immune system.

Of note, autologous NK cell infusion was proved to be safe in people, because no adverse events were observed. Following autologous NK cell infusion, CD28^{-} , CD57^{+} and KLRG1^{+} subsets were significantly reduced within CD4^{+} and CD8^{+} T cells. Meanwhile, similar changes were observed in CD28^{-}CD57^{+} subsets and CD28^{-}KLRG1^{+} ones. Besides, T cell exhaustion was also alleviated significantly as indicated

by reduced levels of $CD4^+PD\text{-}1^+$, $CD4^+TIM\text{-}3^+$, $CD8^+PD\text{-}1^+$, $CD8^+TIM\text{-}3^+$. Although only once infusion was performed here, the effects were not transient but continuous for at least 4 weeks. And much longer-term effects should be evaluated in the future to further characterize the pharmacodynamics of NK Cell infusion.

Cellular senescence is one of the nine hallmarks of aging. Previous findings have reported that NK cells could eliminate senescent cells. Senescent cells share an important feature in SASP. We furtherly found out that NK infusion here also resulted in significant decrease of key SASP-related components including IL-6, IL-1α, IL-8, MIP-1α, MIP-1β, MMP1, but not IFN-γ which was a non-SASP-related factor [34, 35]. These findings strongly suggested that NK cell infusion alleviated cell senescence during aging. Senescent cells play critical roles in age-related immune dysfunction and inflammation, because they release dangerous signals to stimulate immune cells and usually these stimulations are chronic which in turn result in T cell senescence and exhaustion. Therefore, we strongly hypothesized NK cell infusion-related effects on senescent and exhausted T cells were probably at least partially due to their capacity to eliminate senescent cells in vivo. In the preliminary experiment, we also detected the influence of NK cells in T helper subsets, and the result revealed that NK cell may not affect the percentage of Th1, Th2, Th17 and Treg in $CD4^+$ T cell. Accordingly, more examinations have to be carried out in the future to address this issue.

Finally, we compared the gender effects on middle age, There are no significantly different trend in these two groups. These phenotypes suggest that the effects induced by NK cell infusion are independent with gender. However, we found that infused cell type, frozen or fresh, influenced the effects.

Conclusions

In conclusion, the present study originally uncovered the effect of NK cell infusion on T cell dysfunction and cellular senescence in healthy human beings with middle age. Our findings showed that once autologous NK cell administration was an efficient method to significantly alleviate T cell senescence and exhaustion, as well as key components of SASP. Our data importantly indicated that aging, as least for immune system, could be manipulated towards a younger direction by transfer of autologous NK cells. Further exploration should focus on the mechanism that autologous NK cell influence T cell senescence and exhaustion. For example, we would culture human T cell with serum sorted from volunteers before and after NK cell infusion. Also, we would sort senescent T cell and normal T cell from PBMC to explore their function. If possible, we would recruit an equal number of middle-age subjects to infuse normal saline, for that these experiment could explain whether the decreased senescent and exhausted T cell was result from NK cell infusion. Also, further investigation into these senescent and exhausted T cell populations, their origin, their function in immunologic pathologic conditions will greatly promote clinical use of NK immunotherapy.

Declarations

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Authors' contributions

Huanfeng Yang, Bin Li and Xueqi Wang were responsible for the conception of the article and project. Xiaofeng Tang, Biaolong Deng and Aiping Zang were responsible for analyzing data and writing the article. Xiaowen He, Ye Zhou, Daimeng Wang, Dan Li, Xueyu Dai, Xuhua Zhang, Ye Liu, Yonghua Xu, Jingjing Chen, Weijie Zheng and Luding Zhang helped us collect blood sample. Constance Gao helped us review the article. Huanfeng Yang, Bin Li and Xueqi Wang critically revised the entire manuscript and approved the final version.

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Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki in 1975 and the REMARK guidelines for biomarker studies. Signed informed consent was obtained from all participants. For the minors under the age of 18 years, informed consent has been obtained from a parent and/or legal guardian. This study was approved by the institutional research ethics committee of Shanghai Changzheng Hospital.

Consent for publication

Not applicable.

Competing interests

The authors have no competing interests.

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Figures

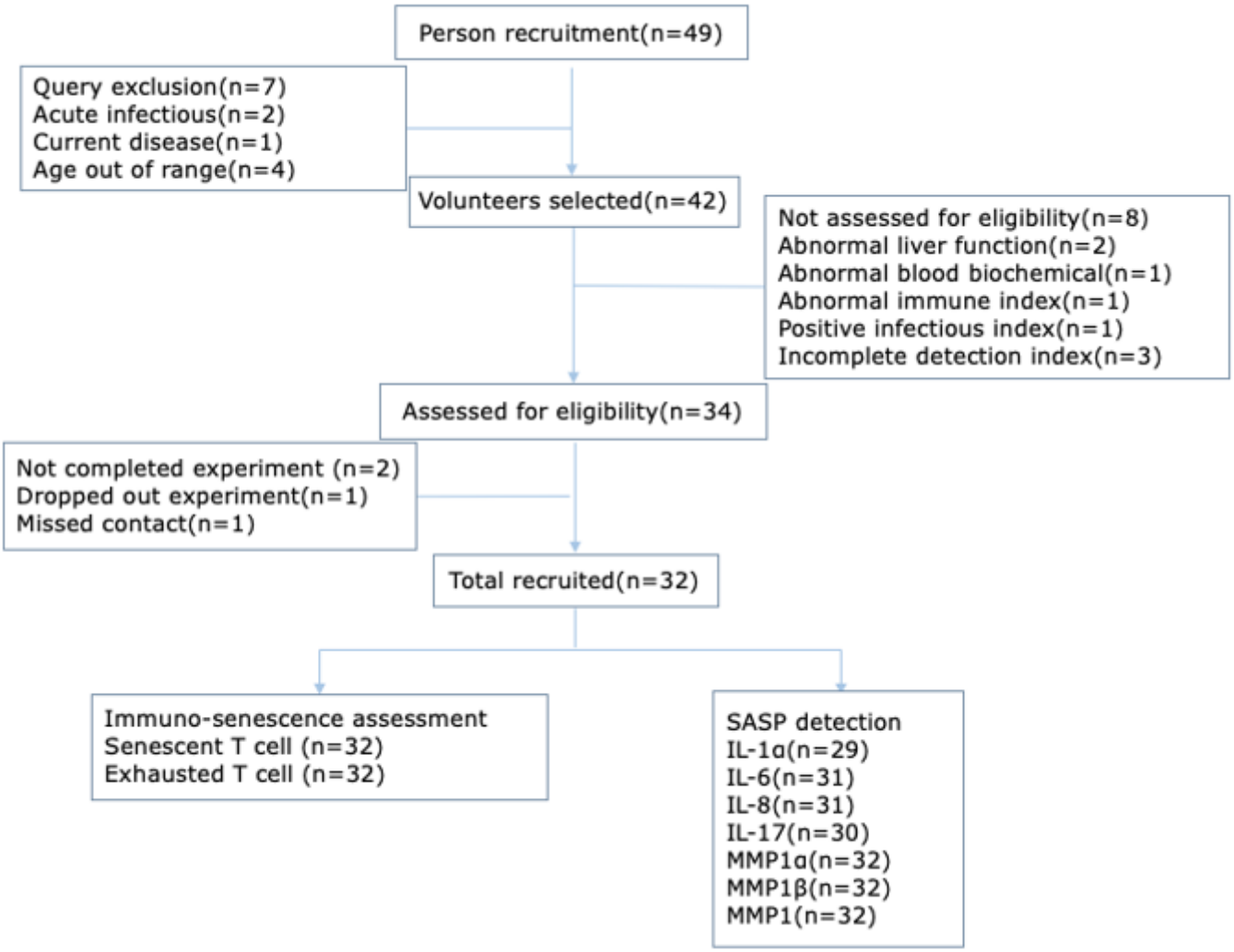

Figure 1

The study flow chart in line with the STROBE.

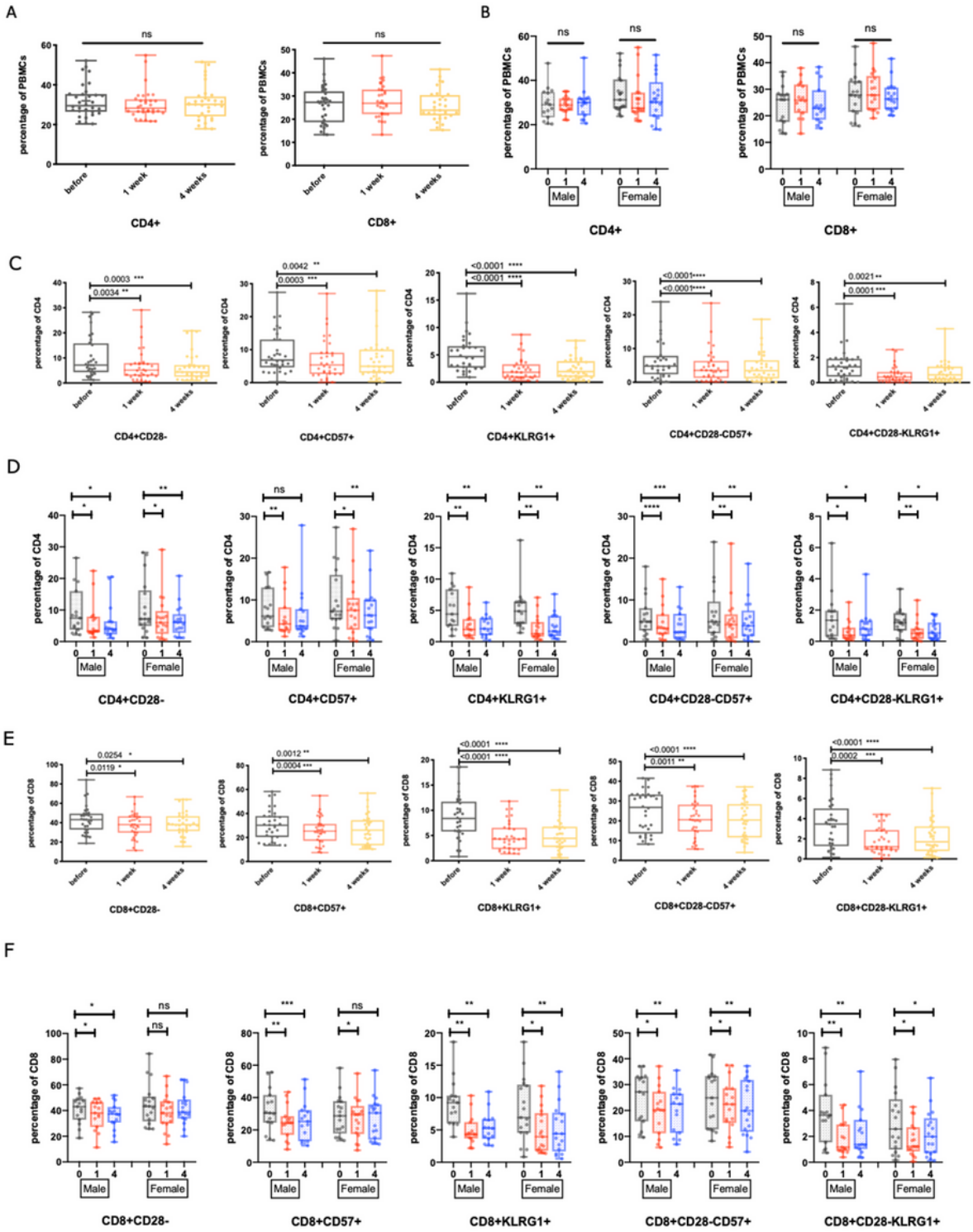

Figure 2

Senescent T cells decreased after NK cell infusion. (A). The percentages of total CD4+ and CD8+ T cell have no significant change on the one week and four weeks after NK cell infusion. (B). Gender doesn't influence the effects on total CD4+ and CD8+ T cell percentage. (C) The percentages of CD28-, CD57+, KLRG1+, CD28-CD57+ and CD28-KLRG1+ CD4+ T cells decreased at one week and four weeks after NK cell infusion. (D) Consistent with (Figure 3C), the percentages of CD28-, CD57+, CD28-CD57+ and CD28-

KLRG1+ CD4+ T cells decreased at one week and four weeks after NK cell infusion, which are independent with gender. (E) The percentages of CD28-, CD57+, KLRG1+, CD28-CD57+ and CD28-KLRG1+ cytotoxic T cells decreased at one week and four weeks after NK cell infusion. (F) Consistent with (Figure 4E), the percentages of CD28-, CD57+, CD28-CD57+ and CD28-KLRG1+ cytotoxic T cells decreased at one week and four weeks after NK cell infusion, which are independent with gender. Each marker has detected 32 volunteers, 15 volunteers are involved in Male group, and 17 volunteers are involved in Female group.. *P < 0.05; **P < 0.01; ***P < 0.001.

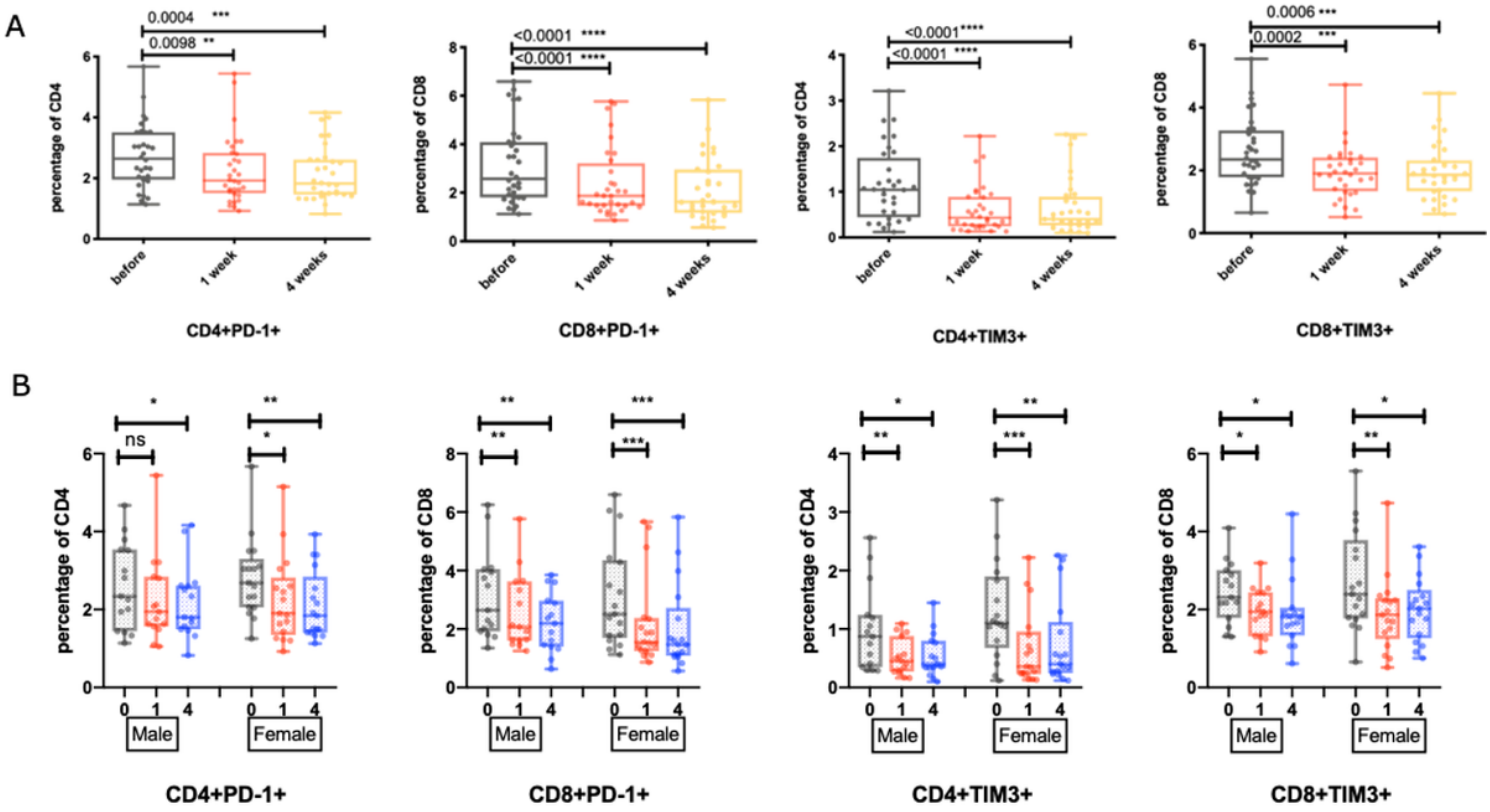

Figure 3

Exhausted T cells decreased after NK cell infusion. (A) The percentages of PD-1+ and TIM-3+ T cell decreased at one week and four weeks after NK cell infusion. (B) The percentages of PD-1+ and TIM-3+ T cell decreased at one week and four weeks after NK cell infusion, which are independent of gender. Each marker has detected 32 volunteers, 15 volunteers are involved in Male group, and 17 volunteers are involved in Female group. *P < 0.05; **P < 0.01; ***P < 0.001.

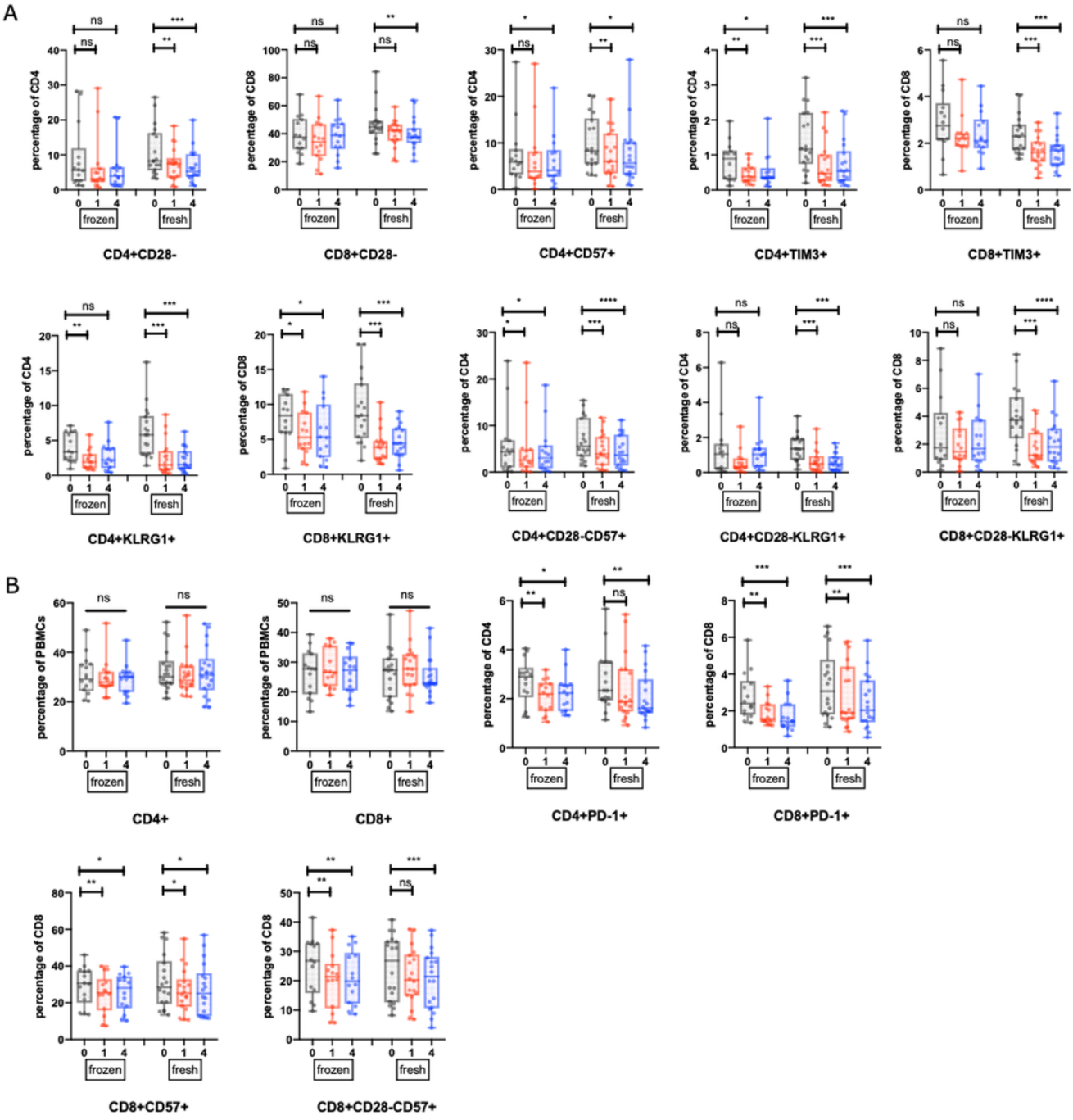

Figure 4

Cell types influence the effects of induced by NK cell infusion. (A) Marks shown that NK cell infusion group is better than frozen NK cell infusion group in promote immune system in sub-health population. (B) Marks have no significant difference between NK cell infusion group and frozen NK cell infusion group. Each marker has detected 32 volunteers, 14 volunteers are involved in frozen group, and 18 volunteers are involved in Fresh group. *P < 0.05; **P < 0.01; ***P < 0.001.

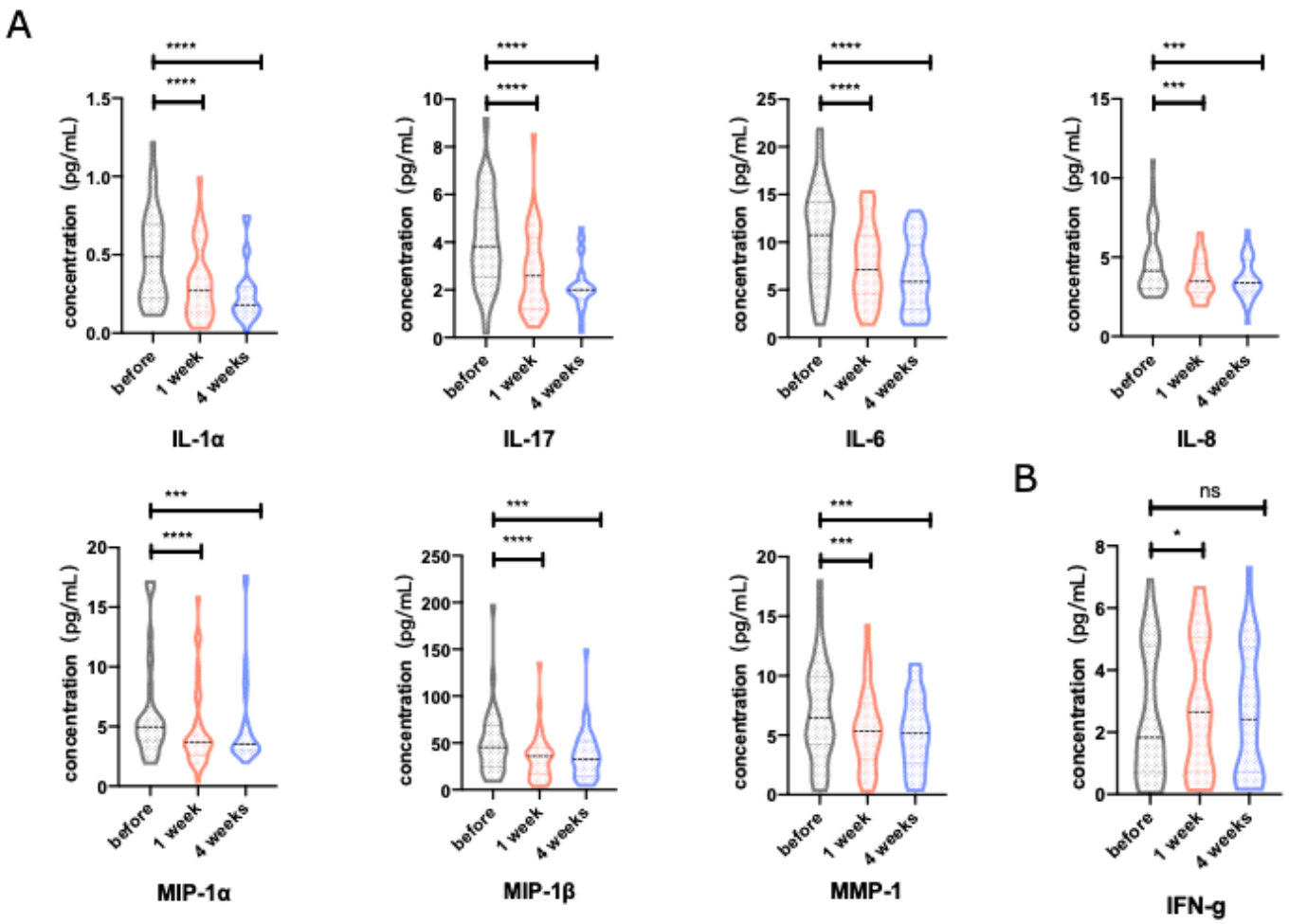

Figure 5

Key senescence associated secretory phenotype (SASP) components decreased after NK cell infusion.(A) IL-6, IL-8, IL-1α, IL-17, MIP-1α, MIP-1β, MMP1 decreased after NK cell infusion. (B) IFN-γ restored to the baseline four weeks after NK cell infusion while transient increase occurred at the first week. Data (IL-6 (n=31), IL-8 (n=31), IL-1α (n=29), IL-17 (n=30), MIP-1α(n=32), MIP-1β(n=32), MMP1 (n=32) and IFN-γ (n=32)) were analyzed by paired t test. *P < 0.05; **P < 0.01; ***P < 0.001.

Supplementary Files

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