Immune Normalization Strategy Against Suboptimal Health Status: Safe and Effective Mixed-Natural Killer Cell Therapy

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Research

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Abstract

**Background:** Immune normalization has emerged as a new paradigm in immunotherapy, which is proposed in cancer patients instead of the traditional immune enhancement therapy. The immune normalization strategy may also be implemented in cancer prevention of sub-healthy individuals.

**Methods:** We established *in vitro* cultured mixed-Natural Killer cells (NKM), which could be used to achieve immune normalization. We defined the sub-healthy individuals after analyzing the PD-1 ratio in PBMCs from 95 donors over 50 years of age.

**Results:** NKM were composed by approximately 20% NK cells (CD3<sup>-</sup>CD16<sup>+</sup> or CD56<sup>+</sup>), 30% NKT-like cells (CD3<sup>+</sup>CD16<sup>+</sup> or CD56<sup>+</sup>) and other T cells. The *in vitro* cytotoxicity of NKM was ten times higher than the peripheral blood mononuclear cells (PBMC). NKM cytotoxicity was negatively correlated with the ratio of regulatory T cells (CD3<sup>+</sup>CD4<sup>+</sup> T), and positively correlated with the ratio of NK cells, especially CD56<sup>bright</sup>CD16<sup>bright</sup> NK cells. We found the sub-healthy individuals displayed significantly higher ratio of CD3<sup>+</sup>PD-1<sup>+</sup> T cells in PBMC (Ratio > 4%) and higher ratio of CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>+</sup> T cells in CD3<sup>+</sup>CD8<sup>+</sup> T cells (Ratio > 10%) than the healthy controls. Then, we evaluated the potential clinical application of NKM therapy in one pancreatic cancer patient and four sub-healthy individuals.

**Conclusions:** NKM therapy showed good tolerance and no side effects were found. In sub-healthy individuals, the ratio of CD3<sup>+</sup>PD-1<sup>+</sup> T cells and CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>+</sup> T cells was significantly reduced after NKM treatment, which indicated that NKM therapy could potentially be used for cancer prevention and health care, thereby achieving the immune normalization.

**Trial registration:**

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**Introduction**

The immune system is an important barrier for the human body to resist various diseases. Its ability has rhythmic fluctuations[1, 2], and is affected by many factors, such as genetic factors and age[3, 4]. The reduction in the response capacity of immune cells will lead to various diseases, such as cancer, while the excessive activation of immune cells will also cause various diseases, such as autoimmune diseases. Thus, the balance of the immune system is an important factor in individual health[5]. Recently, the concept of “immune normalization” was brought up by Lieping Chen in cancer therapy[6], which also reminds us of abnormal immune changes in sub-healthy individuals. If we can normalize the individual
immunity by adjusting its immune system, a variety of diseases may be capable of being prevented. The development of cell therapy in the past decade has given us this hope.

Cell therapies, especially various immune cell therapies, have been used for cancer treatment, such as NK therapy[7] and CAR-T therapy[8]. Although the progress is rapid, due to the heterogeneity of cancer, further research is still required. Epidemiological data of colorectal cancer and lung cancer in recent decades tells us that cancer prevention and early detection are far more effective than their treatment in controlling cancer mortality[9, 10]. The application of immune cell therapy in disease prevention, especially cancer, may greatly reduce the occurrence or relapse of these diseases[11, 12]. Therefore, for the elder individuals who may have long-term immunocompromise, through cell immunotherapy to rebalance their immune system, we may eliminate mutant cells and invaders such as viruses and bacteria, thereby reducing the incidence of cancer.

Previously, the expression of immune checkpoint protein PD-1 on T cells was considered a sign of immune exhaustion[13, 14]. And PD-1 expression on PBMC (including CD4+ and CD8+ T cells) was significantly increased in the cancer[15-20], sarcoidosis[21] and chronic HCV infection[22]. In 2019, New England Journal of Medicine reported that Cortese, I. et al. found that in progressive multifocal leukoencephalopathy, PD-1 expression on PBMC was higher and could be significantly reduced after treatment with Pembrolizumab[23]. Furthermore, in severe COVID-19 patients, in addition to the decrease in peripheral blood T cells[24-28], the proportion of PD-1 expression in the remaining T cells was very high[27, 28]. These studies have shown that the proportion of PD-1 expression in PBMC may be an important sign of individual abnormality.

Here, we established an in vitro cultured mixed-NK (NKM) cell system using peripheral blood mononuclear cells (PBMC), and then studied the potential anti-tumor activity of these cells in vivo and in vitro. In order to be able to initially sort out the sub-healthy individuals, we analyzed the expression level of PD-1 in the NK and T cells of PBMC of 95 individuals over 50 years old, and developed a potential standard for this separation. Finally, we analyzed the efficacy and safety of NKM therapy in four sub-healthy individuals. Hence, we propose that our data represent the first-ever NKM cell therapy could be used as a safe tool for cancer prevention and health care.

**Methods And Materials**

**NKM Cell Culture**

The NKM cells were manufactured in an ISO Class 5 GMP laboratory (Lab of International Research Center for Regenerative Medicine, BOAO International Hospital, approved by Japanese Ministry of Health and Welfare). Primary PBMC were separated from 30-60ml whole blood. The T75 flask was pre-coated with OKM25 (FUKOKU, Japan). The PBMCs from individuals were cultured for 3-5 days in T75 and T225 flask with OKM100 medium (1750 JRU/ml of IL2 were included) (FUKOKU, Japan) containing 10% autologous inactivated plasma. Then the NKM cells were transferred to a CO₂ Bag which containing 1L
OKM200 medium (175 JRU/ml of IL2 were included) (FUKOKU, Japan) and 1% autologous inactivated plasma, cultured for 3-5 days. All NKM cells were incubated at 37°C in a humidified and 5% CO₂ incubator.

Flow Cytometer Analysis and Antibodies

To analyze the T cell subpopulations of PBMC and NKM cells, BD Tritest™ CD3 FITC/CD16+CD56 PE/CD45 PerCP and BD Tritest™ CD4 FITC/CD8 PE/CD3 PerCP (BD biosciences, Shanghai, China) were used for flow cytometer analysis. BD Pharmingen™ PE Mouse Anti-Human CD56 and BD Pharmingen™ FITC Mouse Anti-Human CD16 (BD biosciences, Shanghai, China) were used for the analyses of the NK cell subpopulations of PBMC and NKM cells. BD Pharmingen™ APC Mouse anti-Human CD279, BD Pharmingen™ APC Mouse Anti-Human CD152 and BD Pharmingen™ APC Mouse IgG1, κ Isotype Control (BD biosciences, Shanghai, China) were used for the flow cytometer analyses of the PD-1 and CTLA-4 cell subpopulations of PBMC and NKM cells. The flow cytometer analyses were performed according to the manufacturer’s instructions.

Cell Cytotoxicity Assay

Cell cytotoxicity of PBMC and NKM were assessed by measuring fluorescence intensity of green-fluorescent Calcein AM (#C3099; Thermo Fisher Scientific, Waltham, MA, USA) in intact cancer cells by using Terascan VPC instrument (Minerva Tech, Tokyo, Japan). Briefly, target cells (cancer cells: K562 cancer cells) were resuspend with DMEM medium containing 10% FBS, and mixed with 20μl Calcein AM at 37°C, 30min for staining. PBMC or NKM cells were co-cultured with pre-stained target cells at a certain E:T ratio for 2h or 4h of incubation time. The fluorescence intensity of living target cells is measured. Percent cytotoxicity of the assay was calculated by the following formula: % cytotoxicity = (1-[(average fluorescence of the sample wells - average fluorescence of the maximal release control wells)/(average fluorescence of the minimal release control wells - average fluorescence of the maximal release control wells)]) ×100[29].

Statistical Analysis

All of the experiments in our study were independently performed in triplicate. The data are presented as mean ± SD. All graphs were plotted and analyzed with GraphPad Prism 7.1 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). P-value > 0.05 was considered statistically not significant (ns), and the following denotations were used: **, p<0.001; and *, p<0.05.

Result

1. Generation and Characterization of NKM cells

In order to achieve safe and effective immunotherapy for patients, we established an in vitro culture system for the production of immune cells without any separation or any biological additives. The
autologous plasma was prepared and inactivated, then used in the cell culture process. The autologous immune cells are manufactured as shown in figure 1A and the method section, and we call these cells mixed-NK cells (abbreviated as NKM). After the culture is completed, we can obtain about 2.5 billion immune cells, including the NK cells and T cells.

We further analyzed the immune cell subsets of these NKM. Totally 369 NKM cells were analyzed for the distribution of NK cells and T cells. The results showed that the NKM were composed by approximately 20% NK cells (CD3-CD16+ or CD56+), approximately 30% NKT-like cells (CD3+CD16+ or CD56+), and other T cells. The T cells of NKM were composed by the approximately 30% regulatory T cells (Treg, CD3+CD4+) and 50% cytotoxic T cells (CD3+CD8+) (Figure 1B). In PBMC, the NK cells were mainly composed by the CD3+CD16+CD56neg NK cells and CD3+CD16+CD56dim NK cells (Supplementary Figure S1A). However, the NK cells from NKM were mainly composed by the CD3+CD16+CD56+ NK cells, of which the main subtype is CD3+CD16brightCD56bright NK cells (Figure 1C and Supplementary Figure S1B). Furthermore, we analyzed the NK subpopulations from donor-L at different stages of cell culture, including the primary culture stage (PBMC, T75 flask and T225 flask) and expansion stage (CO2Bag). The results showed that main NK subpopulations in PBMC were CD16bright NK cells (Figure 1D). The CD16bright NK cells transformed into CD16dim NK cells in primary culture stage (Figure 1E and 1F), and finally became CD16brightCD56bright NK cells after expanded proliferation (Figure 1G).

2. In vitro cytotoxicity and its correlations with the subpopulation of NKM cells

For the immune cells, the cytotoxicity on cancer cells is one of the most important functions. Thus, we conducted the in vitro cytotoxicity of NKM cells on K562 cancer cells, and compared them with PBMC and NK92 cells. In PBMC (from health donor), the average cytotoxicity is around 7.4% (E/T = 10:1, 2h incubation; n=9) and 16.8% (E/T = 10:1, 4h incubation; n=28). The average cytotoxicity of NKM cells (n=198) that we manufactured is around 65.6% (E/T = 10:1, 2h incubation), which is almost ten times higher than the PBMC (Figure 2A and Supplementary Table S1). Approximately 84% of NKM cells show more than 40% cytotoxicity. Even with more effector cells and more incubation times, the cytotoxicity of PBMC is still far inferior to NKM cells (Figure 2B and 2C). Then the cytotoxicity of NK92 cells was investigated with different E/T ratio (Supplementary Figure S2A), the result showed that the cytotoxicity of NKM cells (E/T = 10:1) is comparable to that of NK92 with an E/T ratio of 5:1 (2h incubation) (Supplementary Figure S2B). In summary, these results indicate that although the NKM cells have only half the cytotoxic capacity of pure NK cells (NK92), their cytotoxicity is much higher than that of PBMC.

As the NKM cells are a mixed cell population, we wonder which subsets of these cells are responsible for their in vitro cytotoxicity. Then we analyzed the subpopulation of the NKM cells, and performed correlation analysis with their in vitro cytotoxicity. We separated the NKM cells into NK cells (CD3-CD16+ or CD56+), NKT-like cells (CD3+CD16+ or CD56+), CD4T cells (Treg cells, CD3+CD4+), CD8T cells (cytotoxic T cells, CD3+CD8+). Strong positive correlation was observed between the cytotoxicity of NKM cells and the ratio of NK cells (r=0.58, p<0.0001), but a negative correlation with the ratio of Treg cells (r=-0.32,
p<0.0001) (Figure 3A and 3C). There is no correlation with the ratio of NKT-like cells and cytotoxic T cells (Figure 3B and 3D). Furthermore, we divide NK cells into three subpopulations: CD56⁺CD16⁻ NK cells, CD56⁺CD16⁺ NK cells and CD56⁻CD16⁺ NK cells. The cytotoxicity of NKM cells only showed a significant correlated with CD56⁺CD16⁺ NK cells (r=0.56, p<0.0001), while no correlation was found in CD56⁺CD16⁻ NK cells and CD56⁻CD16⁺ NK cells (Figure 3E-G). Thus, these data indicated that the in vitro cytotoxicity of NKM cells is mainly caused by the cytotoxicity of CD56⁺CD16⁺ NK cells, and is suppressed by Treg cells.

3. Potential therapeutic function of NKM cells in a pancreatic cancer patient (In vivo cytotoxicity)

After the characterization of NKM cells, the potential in vivo anti-tumor activity was also need to be investigated. Previous systematic reviews of immune cell therapy with NK cells indicated that allogeneic NK cells immunotherapy has better clinical efficacy than autogeneic therapy[30-32]. Thus, we recruited a patient with pancreatic cancer (T4N1M1c) whose cancer cells had metastasized and cannot sustain chemotherapy. Then we treated this patient with HLA-matched haploidentical NKM cell infusion (Donor: patient’s son). The treatment process was shown in the figure 4A. The patient received the intravenous injection of 20~30 ×10⁸ NKM cells each time. After six treatments, the patient showed well tolerated, and cancer metastatic foci of the renal pelvis and abdominal pelvic peritoneum were significantly reduced (Figure 4B and 4C). The patient's vital signs and physical condition were significantly improved. These results indicated that the NKM cells displayed high effective anti-tumor activity in the cancer patient, and suggested that the NKM could potentially be used for adjuvant cancer therapy.

4. Identify and recharacterize the sub-healthy individuals on the basis of PD-1 level on PBMCs

Previously, when analyzing the PBMC of many cancer patients, the researchers found that PD-1 expression is usually significantly induced in peripheral immune cells[18, 19, 33-35]. And we also found that in adolescents (10 to 20 years old), PD-1 expression of PBMC is very low (<2%) (Data not shown). Therefore, in order to identify sub-healthy individuals, we investigated PD-1 and CTLA-4 expression in NK and T cells of PBMC in 95 individuals over 50 years of age (Supplementary Table S2). The results showed that PD-1 was only expressed on T cells, including the Treg cells and cytotoxic T cells, but not NK cells, while CTLA-4 was not expressed in any PBMC (Figure 5A and 5B). High proportion of PD-1 expression in T cells may indicate that a large number of T cells were exhausted[13, 14], which in turn implies an abnormal immune system.

Further statistical analysis found that the proportion of CD3⁺PD-1⁺ cells in PBMC was usually less than 4%, and only 16.8% of individuals were higher than 4% (Figure 6A). The proportion of CD3⁺CD4⁺PD-1⁺ cells in CD3⁺CD4⁺ T cells was usually less than 10% and only 9.5% of individuals were higher than 10% (Figure 6B). The proportion of CD3⁺CD8⁺PD-1⁺ cells in CD3⁺CD8⁺ T cells was usually less than 10% and 37.9% of individuals were higher than 10% (Figure 6C). Here, in order to define sub-healthy individuals, we set the threshold of CD3⁺PD-1⁺/PBMC to >4% and the threshold of CD3⁺CD8⁺PD-1⁺/CD3⁺CD8⁺ to >10%.
In our cohort, about 4.2% of individuals showed these characteristics, so their immune system of these people may be abnormal, and we define these people as sub-healthy individuals.

5. Potential immune normalization of autologous NKM cells in sub-healthy individual

Since the NKM cells are mixed immune cells, including Treg cells that inhibit cytotoxicity and NK cells that produce cytotoxicity, we wonder that these NKM cells may display more effective functions in the immune normalization of sub-healthy individuals. Then we recruited four potential sub-healthy individuals to participate in this study. The treatment schedule was shown in the figure 7A. Approximately $20 \times 10^8$ autologous NKM cells were used for each treatment. The examination of PD-1 expression in T cells were performed before the treatment (pre-), after the 1st injection (mid-) and after the 3rd injection (post-). In Patient-M and Patient-Z, with a single injection of NKM cells, the PD-1 expression of PBMC, CD3+CD8+ and CD3+CD4+ could be significantly reduced to normal levels (Figure 7B and 7C). While in Patient-J, the effect of one injection of NKM cells is not obvious. After three injections of NKM cells, the PD-1 expression of PBMC, CD3+CD8+ and CD3+CD4+ eventually reduce to normal levels (Figure 7D). In Patient-C, three injections of NKM cells could only reduce the PD-1 expression of PBMC and CD3+CD8+, but not the PD-1 expression of CD3+CD4+ cells (Figure 7E). All sub-healthy individuals showed good tolerance and no side effects were found clinically. In summary, we found that with three autologous NKM cell therapies in general, we could successfully reduce the PD-1 expression in PBMC, and achieve the renormalization of the immune system in sub-healthy individuals.

Discussion

For decades, researches have struggled against various diseases, such as cancer, and have focused their efforts on "enhancing / improving" the immune system to eliminate the potential risks. The arising immune cell therapies have made these ideas a reality. Here, we presented a safe and effective NKM therapy for potential cancer adjuvant treatment and cancer prevention. We characterized the composition of our NKM cells and investigated the cytotoxicity in vitro and in vivo, and the results showed that they were significantly cytotoxic to K562 cells or hepatocellular carcinoma cells[36]. Although NKM therapy may not be as efficient as pure NK therapy, due to its ease of operation and safety (less immune-related side effects were found), we believed that NKM therapy is a sufficient treatment for cancer adjuvant therapy, especially in combination with surgery to improve the immune response and eliminate residual cancer cells.

Cancer epidemiology in Japan and the United States reminded us that cancer prevention is more efficient strategy for reducing the cancer incidence. Recently scientists have proposed that future cancer immunotherapy should be focus on the normalization of the immune system[6]. With the analyses of PD-1 expression in PBMC, we successfully defined the sub-healthy individuals, and further NKM therapy showed that the treatment could rapidly restore or rebalance the immune system of these individuals. We expanded NKM therapy to not only treat cancer patients, but also prevent the occurrence of cancer in patients through the normalization of peripheral immune system. Although the NKM therapy for sub-
healthy individuals still needs to be studied in more patients, we believe that the attempt of this study has allowed us to expand the application scenarios of immune cell therapy.

Finally, COVID-19 has spread rapidly around the world. In severe COVID-19 patients, the immune system collapsed severely, including the reduction of immune cells and functional T Cells exhaustion in the peripheral blood[28]. Just like our sub-healthy individuals, the PD-1 expression was significantly increased in CD4+ T and CD8+ T cells of PBMC in COVID-19 patients, and the increase was even more significant. Since our experiments in sub-healthy individuals successfully normalized their peripheral immune system, we believe that if we use NKM treatment for COVID-19 patients, we may be able to control the proportion of severe conversion, thereby reducing mortality.

In summary, the results presented in the current study demonstrate that newly established NKM therapy is a safe and efficient treatment for cancer adjuvant treatment and health care for sub-healthy individuals. The normalization of the peripheral immune system through NKM therapy could greatly expand its scope of application in the future.

Declarations

Ethics approval and consent to participate

This study was submitted and approved by the Medical Ethics Committee of BOAO International Hospital. All sample collection, healthy donor and patient consent, and healthy donor and patient recruitment followed Institutional Review Board protocols from BOAO International Hospital (QiongHai, P. R. China; approval number BIH-2018-1001 for adjuvant tumor therapy, BIH-2018-1002 for the health care of sub-healthy individuals). The study was conducted according to the principles of the Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and material

All data were added in the supplementary tables.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

H.W and X.L designed the study. Y.L, S.F and F.X performed the cell culture. Y.L, Hw.H, J.W and A.C performed the analyses of NKM cells. X.R and D.P recruited the patients and collected the clinic data. H.W, X.L and M.S analyzed the data and performed statistical analyses. H.W, M.S and X.L interpreted and discussed the data with all authors. H.W and M.S wrote the manuscript. All authors read and approved this manuscript.

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Figures
Figure 1

Characterization of NKM cells. A Manufacturing process of NKM. The whole process needs to be carried out in the GMP laboratory Class A ultra-clean workbench. B The composition of NKM. C The main NK cells in NKM were CD16brightCD56bright NK cells. The NKM from donor-N was analyzed by FACS. The main NKM cells were CD16+CD56+ cells (87.22%), while the majority was CD16brightCD56bright NK cells. D-G NK subpopulations of NKM cells in different culture stages, including primary PBMC (D), T75 flask (E), T225 flask (F), NKM product (G). The red circle in primary PBMC marked CD16bright NK cells, while the red circle in T75 and T225 flask marked CD16dim NK cells. The blue circle marked CD56brightCD16bright NK cells.
Figure 2

The NKM has very high in vitro cytotoxicity. A The cytotoxicity of PBMC and NKM. The cytotoxicity of 9 PBMCs (incubation for 2h) and 28 PBMCs (incubation for 4h) were obtained. The cytotoxicity of 198 NKMs (incubation for 2h) were obtained. B-C Labeled target cells (K562 cancer cells) were killed by the effector cells (PBMC or NKM). The number on the upper right showed the cytotoxicity of the effector cells. The ratio of effector cells and target cells was 10:1 or 20:1, and the incubation time was 2h or 4h.
The correlation of NKM cytotoxicity and cell subpopulations. A-D NK cells (CD3-CD16+/CD56+) positively correlated with NKM cytotoxicity, while Treg cells (CD3+CD4+ T) negatively correlated with NKM cytotoxicity. Totally 198 NKMs were used for the correlation analyses, and four subpopulations were analyzed, including NK cells (A), NKT-like cells (B), CD3+CD4+ T cells (C) and CD3+CD8+ T cells (D). E-G CD3-CD16+CD56+ NK cells correlated the NKM cytotoxicity. Totally 44 NKMs were used for the correlation analyses, and four subpopulations were analyzed, including CD56+CD16- NK cells (E), CD56+CD16+ NK cells (F) and CD56-CD16+ NK cells (G). The correlations and linear regressions were analyzed with GraphPad Prism 7.04.
Figure 4

A

Week 0  W2  W3  W4  W5  W6  W7
Medical  Blood  Examination  Collection

B

Before Treatment  After Treatment
Renal pelvis

C

Before Treatment  After Treatment
Abdominal pelvic peritoneum
Figure 4

The potential anti-tumor activity of NKM. A The schedule of NKM therapy for cancer patient. After physical examination, 20-30 ×10⁸ NKM cells were used for each injection, and six injections were performed in one course of treatment, with a time interval of one week. B-C The metastatic foci of the renal pelvis (B) and abdominal pelvis peritoneum (C) were significantly reduced after the NKM treatment.
Figure 5

A

Expression of PD-1/CTLA-4 in T cells

B

Expression of PD-1/CTLA-4 in NK cells
Figure 5

The expression of PD-1 or CTLA-4 in T cells and NK cells of PBMC. A PD-1 was expressed on peripheral T cells (including CD3+CD4+ T cells and CD3+CD8+ T cells), but CTLA-4 was not. B PD-1 and CTLA-4 were not expressed on peripheral NK cells.
Figure 6

Individual Distribution (A&B&C)
- CD3+PD-1+ / PBMC > 4% : 16.84%
- CD3+CD4+PD-1+ / CD3+CD4+ > 10% : 9.47%
- CD3+CD8+PD-1+ / CD3+CD8+ > 10% : 37.59%
- CD3+PD-1+ / PBMC > 4% & CD3+CD8+PD-1+ / CD3+CD8+ > 10% : 4.21%
Figure 6

Define sub-healthy individuals with PD-1 expression in PBMC. A The proportion of CD3+PD-1+ cells was less than 4% in the PBMC of healthy individual. PBMCs of 95 individuals (over 50 years old) were analyzed, in which 16.84% of the samples have a proportion of CD3+PD-1+ cells greater than 4%. B The proportion of CD3+CD4+PD-1+ cells was less than 10% in the CD3+CD4+ T cells of healthy individual. In these 95 individuals, 9.47% of the samples have a proportion of CD3+CD4+PD-1+ cells greater than 10%. C The proportion of CD3+CD8+PD-1+ cells was less than 10% in the CD3+CD8+ T cells of healthy individual. In these 95 individuals, 37.9% of the samples have a proportion of CD3+CD8+PD-1+ cells greater than 10%. In combination, we define that CD3+PD-1+ / PBMC > 4% and CD3+CD8+PD-1+ / CD3+CD8+ > 10% as sub-healthy individuals (4.21%).
Figure 7

A

Week 0  Week 2  Week 6  Week 10  Week 14
Medical Examination  Blood Collection
IV
IV
IV
IV
PBMCT Analysis

PBMC Analysis:
CD3+PD-1+ > 4%
CD3+CD28+PD-1+ > 10%

B

| Ratio (%) | p-value |
|----------|---------|
| pre-CD3' PD-1+ | 0.0008 |
| mHLA-CD3 PD-1+ | 0.0001 |
| post-CD3' PD-1+ | 0.0001 |

Patient Z

| Ratio (%) | p-value |
|----------|---------|
| pre-CD3' PD-1+ | 0.0001 |
| mHLA-CD3 PD-1+ | 0.0001 |
| post-CD3' PD-1+ | 0.0001 |

Patient M

| Ratio (%) | p-value |
|----------|---------|
| pre-CD3' PD-1+ | 0.0001 |
| mHLA-CD3 PD-1+ | 0.0001 |
| post-CD3' PD-1+ | 0.0001 |

| Ratio (%) | p-value |
|----------|---------|
| pre-CD3' PD-1+ | 0.0001 |
| mHLA-CD3 PD-1+ | 0.0001 |
| post-CD3' PD-1+ | 0.0001 |

Patient J

| Ratio (%) | p-value |
|----------|---------|
| pre-CD3' PD-1+ | ns |
| mHLA-CD3 PD-1+ | ns |
| post-CD3' PD-1+ | 0.0001 |

Patient C

| Ratio (%) | p-value |
|----------|---------|
| pre-CD3' PD-1+ | 0.0001 |
| mHLA-CD3 PD-1+ | 0.0001 |
| post-CD3' PD-1+ | 0.0001 |
Figure 7

Potential immune normalization of NKM therapy in sub-healthy individuals. A The schedule of NKM therapy for sub-healthy individuals. After physical examination, 20-30 ×10^8 NKM cells were used for each injection, and three injections were performed in one course of treatment, with a time interval of one month. The therapeutic effect detection during the NKM treatment was performed before the second injection. The final effect detection was performed one month after the end of NKM treatment. B-E Effective NKM therapy for immune normalization in four sub-healthy individuals. Patient Z (B), Patient M (C), Patient J (D) and Patient C (E) were recruited into the study. The proportions of CD3+PD-1⁺ / PBMC and CD3+CD8+PD-1⁺ / CD3+CD8⁺ were investigated before NKM treatment (pre-), during NKM treatment (mid-) and after NKM treatment (post-). The p-values were showed.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- CoverLetterforImmunityAgeing.docx
- SupTableS2.xlsx
- SupTableS1.xlsx
- SupFigureS2.pdf
- SupFigureS1.pdf