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ORIGINAL ARTICLE

Effect of Different Storage Conditions and Different Detection Time on the Results of Programmed Cell Death Protein 1 by Flow Cytometry

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SUMMARY

Background: **This study aimed to analyze the effect of different storage conditions and different detection time on the results of programmed cell death protein 1 (PD-1) in clinical peripheral blood samples by flow cytometry (FCM) after the completion of all procedure steps.**
Methods: **68 inpatients were randomly selected at the First Affiliated Hospital of Anhui Medical University in March 2025, and 68 peripheral blood specimens were collected. After all laboratory procedure steps were completed, cell suspension in PBS was prepared and stored both at 4℃ and at room temperature away from light, which were then divided into four different detection time groups: 0 hours, 4 hours, 8 hours, and 24 hours groups. The percentage results of seven cell populations, $CD45^+$ Lym cell population ($CD45^+$ Lym cells), $CD3^+$ T cell population ($CD3^+$ T cells), $CD3^+$ $CD4^+$ T cell population ($CD3^+$ $CD4^+$ T cells), $CD3^+$ $CD8^+$ T cell population ($CD3^+$ $CD8^+$ T cells), $CD3^+$$PD\text{-}1^+$ T cell population ($CD3^+$ $PD\text{-}1^+$ T cells), $CD3^+$ $CD4^+$ $PD\text{-}1^+$ T cell population ($CD3^+$ $CD4^+$ $PD\text{-}1^+$ T cells), and $CD3^+$ $CD8^+$ $PD\text{-}1^+$ T cell population ($CD3^+$ $CD8^+$ $PD\text{-}1^+$ T cells), were detected using FCM. Statistical software was used to compare and analyze the results.**
Results: **When stored at 4℃ away from light, compared with 0 hours group, the results of seven cell populations in the 4 hours and 8 hours groups did not change significantly and the results of the 24 hours group decreased significantly ($p < 0.05$). At room temperature away from light, the results of seven cell populations had no significant difference between the 0 hours and 4 hours groups. The results of seven cell populations in the 8 hours and 24 hours groups decreased significantly, with significant difference ($p < 0.05$).**
Conclusions: **The detection of PD-1 should not be carried out too long after all of the laboratory procedure steps have been completed. If samples are stored at 4℃ away from light, the detection time should be limited to 8 hours; if stored at room temperature away from light, the detection time should be limited to 4 hours. This is to ensure the accuracy and stability of the detection results.**
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KEYWORDS

storage condition, detection time, programmed cell death protein 1 (PD-1), flow cytometry (FCM)

INTRODUCTION

Programmed cell death protein 1 (PD-1), an important immunosuppressive molecule, belongs to the immunoglobulin superfamily and is a membrane protein com-

posed of 268 amino acids. It was originally cloned from apoptotic mouse T-cell hybridoma 2B4.11 [1-3]. Immune regulation targeting PD-1 is of great significance in combating tumors, infections, autoimmune diseases, and organ transplantation survival [4-7]. Its ligand PD-L1 can be used as a target, and the corresponding antibodies can be used the same. The combination of PD-1 and PD-L1 initiates programmed cell death of T cells, enabling tumor cells to obtain immune escape [8-9]. Flow cytometry (FCM) is a biotechnology that can detect the labeled fluorescence signals with high-speed by quantitative analysis and sort single cells or other biological particles in the suspension. It can be used to conduct continuous multi-parameter analysis of single cell flowing through optical or electronic detectors [10-11]. Since the 1970s, with the continuous improvement of FCM technology, its application has spread and has been used in the fields of basic and clinical medical research such as oncology, hematology, and immunology [12-13]. PD-1 expression on T cells (including $CD3^+$ T cells, $CD3^+$ $CD4^+$ T cells, and $CD3^+$ $CD8^+$ T cells) in peripheral blood can be measured by FCM with fluorescent PD-1 antibody. Clinical significance lies in the assessment of the immune status. However, flow cytometer is an advanced precision instrument and expensive to use. Frequent use would cause certain wear and tear to the instrument, and the maintenance cost is considerable as well. Generally, to minimize losses, the frequency of startup should be reduced and the probability of successful detection increased [14-15]. On the other hand, the fluorescent antibodies used for incubation are relatively sensitive to light and temperature. Strong light and high temperature conditions can easily cause a decrease in fluorescence intensity, affecting the detection results [16-17]. In view of this, after completing all the laboratory procedure steps of detecting PD-1 by FCM, it is of great benefit to explore the sample preservation conditions and detection time with the error controlled in the minimum range. This study aimed to explore the effect of different storage conditions and different detection time on the results of PD-1 by FCM to optimize storage conditions and detection time after comparing the differences in detection results. The study could provide reference value for the laboratory detection of PD-1 by FCM and ensure accuracy and stability of detection results.

MATERIALS AND METHODS

Sampling

68 inpatients were randomly selected at the First Affiliated Hospital of Anhui Medical University in March 2025, and 68 peripheral blood specimens were collected with no duplicate specimens. Among 68 inpatients, there were 38 males and 30 females, with an age range between 26 and 84 years; the average age was (60.24 ± 11.57) years.

Reagents and instruments

Blood collection tubes containing EDTA-K_2 anticoagulant were used as sampling tubes. Antibodies (CD45-PerCP, CD3-FITC, CD4-PE) and 10 x RBC lysis buffer were purchased from Beijing Tongsheng Times Biotechnology Co., Ltd. Antibodies (CD8-APC-Cy7, PD-1-APC) and flow sheath fluid were purchased from Tianjin Kuangbo Biotechnology Co., Ltd. Phosphate buffered saline (PBS) was purchased from Sangon Biotech (Shanghai) Co., Ltd. Beamcyte1026 flow cytometer was purchased from Changzhou Bidake Company, and 5810R refrigerated centrifuge was purchased from Eppendorf Company in Germany.

Detection methods

For PD-1 detection, 2.0 mL of venous blood were drawn and placed in the EDTA-K_2 anticoagulant tube. Then, 20.0 μL of CD45-PerCP/CD3-FITC/CD4-PE, 10.0 μL of CD8-APC-Cy7, 10.0 μL of PD-1-APC fluorescent antibodies, and 100.0 μL of blood sample were mixed in test tubes for incubation. After they were thoroughly mixed, the test tubes were kept at room temperature away from light for 15 minutes. Next, 2.0 mL of diluted 1 x RBC lysis buffer were added to the test tubes, mixed thoroughly, and left at room temperature away from light for 10 minutes. Then, the test tubes were centrifuged at 1,500 rpm/minute for 5 minutes, and the supernatants were discarded. Further, 2.0 mL of PBS were added in order to remove the residual RBC lysis buffer. The test tubes were centrifuged at 1,500 rpm/minute for 5 minutes, and the supernatants were discarded again. At last, 300.0 μL of PBS were added to the test tubes to form cell suspension and were prepared for detection.

Study groups

After completing all laboratory procedure steps, cell suspensions of 68 specimens in PBS were stored under two conditions: 4℃ and room temperature away from light, respectively. Meanwhile, they were divided into four different detection time groups: 0 hours group, 4 hours group, 8 hours group, and 24 hours group. The percentage results of seven cell populations were detected using FCM. The normal reference values, including $CD45^+$Lym cells (20.00 - 40.00%), $CD3^+$T cells (58.00 - 84.00%), $CD3^+CD4^+$T cells (27.00 - 51.00%), $CD3^+$ $CD8^+$T cells (15.00 - 44.00%), $CD3^+$-$PD\text{-}1^+$ cells (5.61 - 29.43%), $CD3^+CD4^+$-$PD\text{-}1^+$ cells (6.60 - 34.34%), and $CD3^+CD8^+$-$PD\text{-}1^+$ cells (4.21 - 44.89%), were listed.

Statistical analysis

SPSS 25.0 software was used for data analysis. Average age of patients and percentages of seven cell populations were expressed as mean ± standard deviation. The *t*-test was used for comparisons between two groups. p-value of less than 0.05 was considered significantly different.

Ethical approval

Ethical approval for this study was obtained from the Medical Ethics Committee of the First Affiliated Hospital of Anhui Medical University and was in accordance with the Declaration of Helsinki (reference number: PJ 2025-04-21), which ensured that all ethical considerations for the research were followed, in a manner that protected patient's confidentiality and privacy.

RESULTS

Clinical characteristics of inpatients

A total of 68 inpatients were randomly selected at the First Affiliated Hospital of Anhui Medical University in March 2025, and 68 peripheral blood samples were collected with no duplicate samples. Among them, the age range was between 26 and 84 years, and the average age was (60.24 ± 11.57) years.

Distribution of 68 inpatients regarding gender and different departments

Among 68 inpatients, there were 38 males and 30 females, with no significant difference ($p > 0.05$). In the distribution of the different departments, the main five departments were the Department of Medical Oncology, General Surgery, Respiratory and Critical Care Medicine, Tumor Radiotherapy, and Emergency Surgery, as shown in Table 1.

Comparison of the results of seven cell populations in different detection time groups at 4℃ away from light

The percentages of seven cell populations, which were, respectively, $CD45^+$Lym cells, $CD3^+$T cells, $CD3^+CD4^+$T cells, $CD3^+CD8^+$T cells, $CD3^+$PD-1^+T cells, $CD3^+CD4^+$PD-1^+T cells, and $CD3^+CD8^+$PD-1^+T cells, were detected and compared in different detection time groups at 4℃ away from light. The results showed that compared with 0 hours group, there was no significant difference in the results of seven cell populations in 4 hours and 8 hours groups ($p > 0.05$). The results of 24 hours group were evidently decreased, with significant difference ($p < 0.05$), and the data were shown in Tables 2 and 3.

Comparison of the results of seven cell populations in different detection time groups at room temperature away from light

The percentages of seven cell populations were detected and compared in different detection time groups at room temperature away from light. The results showed that compared with 0 hours group, there was no significant difference in the results of seven cell populations in the 4 hours group ($p > 0.05$). The results of 8 hours and 24 hours groups were evidently decreased, with significant differences ($p < 0.05$), and the data were shown in Tables 4 and 5.

DISCUSSION

PD-1 is an important immune checkpoint, which originally is a protective molecule in the human immune system. It acts like a brake to prevent inflammatory damage caused by excessive activation of T cells. Tumor cells take advantage of that characteristic and inhibit the immune system response by over-expression of immune checkpoint molecules. PD-1 can escape from immune surveillance and killing, thereby promoting the growth of tumor cells. It inhibits the activation of T cells and the production of cytokines by interacting with its ligand PD-L1, so that tumor cells can achieve immune escape and self-protection [18-19]. Kamphorst et al. selected patients with non-small cell lung cancer (NSCLC) in an advanced stage and detected the changes of Ki-67^+PD-1^+CD8^+ T cell levels in the peripheral blood of patients before and after treatment. The results showed that the level of Ki-67^+PD-1^+CD8^+ T cells in the peripheral blood of patients increased significantly following PD-1 targeted therapy and that T cell activation was not indiscriminate [20]. The antibody used in FCM was conjugated with fluorophore, which was susceptible to bright light and high temperatures. Under the condition of bright light irradiation, the conjugated double bond of the fluorophore broke, then a series of intermediate products were generated, which finally decomposed into colorless small molecular substances and resulted in the reduction of fluorescent antibody efficiency. High temperature could strengthen the dynamic force of fluorophore molecules, probably increasing the collision of fluorophore molecules and fluorescence quenching, which affected fluorescence intensity. Generally, when the temperature was 20℃, the fluorophore began to quench. As temperature increased, the fluorescence quenching became stronger, and the fluorescence could be quenched completely. At low temperature, the fluorescence intensity did not change significantly with the temperature and maintained a constant amount. Therefore, the low temperature condition had less influence on the fluorescent antibody, which was more conducive to the function of antibody [21-23]. Devine et al. had performed cell cycle analysis by FCM and mass cytometry (MCM) to analyze the cell cycle state of ex vivo samples from patients with hematologic malignancies. Before processing and analysis, clinical samples were usually stored for hours at room temperature or cryogenically frozen. No one had reported whether storage time and temperature affected the cell cycle state. They found that accurate cell cycle analysis required samples to be detected rapidly after collection, and that cryopreservation altered cell cycle fractions significantly. Their findings could provide reference value for the ideal approach of storage of samples [24]. Green et al. had carried out two experiments to explore the stability of fluorescent antibody conjugates. The results of their research showed that fluorescent antibody conjugates in liquid state should be stored at 4 to 5℃ or -20℃, of which the stability was better

Table 1. Distribution of 68 inpatients regarding gender and different departments.

	Number of inpatients	Number of all inpatients	Rate (%)
Gender			
Male	38	68	55.88
Female	30	68	44.12
Department			
Medical oncology	36	68	52.94
General surgery	24	68	35.29
Respiratory and critical care medicine	4	68	5.88
Tumor radiotherapy	3	68	4.41
Chemoradiotherapy center	1	68	1.47
Total	68	68	100.0

Table 2. Comparison of the results of four cell populations in different detection time groups at 4°C away from light.

Groups	n	$CD45^+$Lym (%)	$CD3^+$T (%)	$CD3^+CD4^+$T (%)	$CD3^+CD8^+$T (%)
0 hours	68	26.77 ± 12.70	64.73 ± 11.17	33.98 ± 10.92	26.29 ± 10.83
4 hours	68	25.86 ± 12.55	60.03 ± 10.99	31.69 ± 9.89	25.34 ± 10.21
8 hours	68	23.23 ± 10.34	56.11 ± 9.86	29.63 ± 8.62	22.72 ± 8.28
24 hours	68	15.61 ± 6.42 *	36.12 ± 7.18 *	18.64 ± 6.37 *	14.12 ± 4.33 *

Compared with 0-hours group, * $p < 0.05$.

Table 3. Comparison of the results of three cell populations in different detection time groups at 4°C away from light.

Groups	n	$CD3^+PD\text{-}1^+$T (%)	$CD3^+CD4^+PD\text{-}1^+$T (%)	$CD3^+CD8^+PD\text{-}1^+$T (%)
0 hours	68	6.44 ± 5.52	7.11 ± 6.99	7.79 ± 7.66
4 hours	68	6.15 ± 4.12	6.88 ± 5.45	7.27 ± 6.33
8 hours	68	5.35 ± 3.63	5.16 ± 4.42	6.64 ± 5.47
24 hours	68	2.49 ± 1.18 *	2.52 ± 1.22 *	2.63 ± 1.26 *

Compared with 0-hours group, * $p < 0.05$.

Table 4. Comparison of the results of four cell populations in different detection time groups at room temperature away from light.

Groups	n	$CD45^+$Lym (%)	$CD3^+$T (%)	$CD3^+CD4^+$T (%)	$CD3^+CD8^+$T (%)
0 hours	68	26.77 ± 12.70	64.73 ± 11.17	33.98 ± 10.92	26.29 ± 10.83
4 hours	68	20.57 ± 11.41	46.11 ± 7.87	23.53 ± 5.56	18.13 ± 4.15
8 hours	68	12.29 ± 4.91 *	33.02 ± 7.18 *	16.64 ± 6.37 *	13.31 ± 3.25 *
24 hours	68	10.13 ± 2.67 *	22.01 ± 4.32 *	10.48 ± 3.11 *	9.84 ± 1.93 *

Compared with 0-hours group, * $p < 0.05$.

Table 5. Comparison of the results of three cell populations in different detection time groups at room temperature away from light.

Groups	n	$CD3^+PD\text{-}1^+T$ (%)	$CD3^+CD4^+PD\text{-}1^+T$ (%)	$CD3^+CD8^+PD\text{-}1^+T$ (%)
0 hours	68	6.44 ± 5.52	7.11 ± 6.99	7.79 ± 7.66
4 hours	68	4.67 ± 2.23	4.88 ± 2.36	5.13 ± 2.54
8 hours	68	2.53 ± 1.02 *	2.77 ± 1.03 *	2.81 ± 1.06 *
24 hours	68	1.55 ± 0.51 *	1.62 ± 0.56 *	1.67 ± 0.61 *

Compared with 0-hours group, * $p < 0.05$.

than when stored at 25℃ [25]. Similar results were obtained in the analysis of this study; after completing all the steps of PD-1 detection in peripheral blood samples, the detection should not take too long, and it was better to store samples at 4℃ away from light. So, different storage conditions and different detection time had impacts on the results of PD-1 by FCM and we could limit the impacts.

CONCLUSION

In conclusion, PD-1 detection in peripheral blood should not be conducted too long after all laboratory procedure steps have been completed. If samples are stored at 4℃ away from light, the whole detection process should be completed within 8 hours; if stored at room temperature away from light, the whole detection process should be completed within 4 hours. This is to ensure accuracy and stability of the detection results.

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Data Availability Statement:
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of Interest:
The authors have no conflicts of interest to declare.

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