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Long term stability of immunophenotypic T cell subsets from whole blood of tacrolimus-based therapy kidney transplantation patients and healthy volunteers by flow cytometric analysis

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Abstract

Flow cytometric analysis has become popular in immunology to identify T cells subsets. Unfortunately, using this technique has been limited by requiring testing blood samples within 24 hours after collection. Therefore, preserving the stability of lymphocytes may be useful to prolong blood samples before analysis by flow cytometry. The aim of this study was to validate long term stability of T cell subsets (cell surface markers, CD3+, CD3+CD4+ and CD3+CD8+, and intracellular marker, CD4+IL-2) by flow cytometric technique using whole blood samples from tacrolimus-based therapy kidney transplantation patients and healthy volunteers. Whole blood samples were collected from 10 tacrolimus-based therapy kidney transplantation patients and 10 healthy volunteers, separated into 2 groups by stored at 4℃ and -20℃, and then analyzed by flow cytometry at day 1, day 3, day 7, day 15 and day 30 after collection. The percentage of T cell subsets were recorded at each time interval. The percentage of CD3+CD4+ subset in patient group was not significantly different up to 30 days after blood sample collection storage in both 4℃ (day 1 = 45.88 ± 4.78 VS day 30 = 41.32 ± 4.03) and -20℃ (day 1 = 42.76 ± 3.54 VS day 30 = 38.52 ± 2.97), $P > 0.05$. CD3+, CD3+CD8+, and CD4+IL-2 subset were not significantly different in both groups. Moreover, CD3+, CD3+CD4+, CD3+CD8+, and CD4+IL-2 subsets were significantly different between patient and healthy groups. These findings indicate that whole blood samples could preserve all cells for 30 days before flow cytometric analysis is done for both the patient and the healthy group.

Keywords: Flow cytometric analysis, Kidney transplantation patient, Long term stability of T cell, Whole blood sample, Tacrolimus

1. Introduction

Evaluation of T cell subsets by flow cytometer is useful in evaluating immune status in many immunodeficiency diseases such as human immunodeficiency virus (HIV) patients. It is routinely performed for monitoring infection stage and response to antiretroviral therapy. The accurate measurement of T cell subsets, both as absolute counts and percentage values, is critical not only in providing care to HIV infected patients but also in assessing new antiretroviral drugs and therapeutic vaccines [1].

Moreover, flow cytometric analysis to identify T cells subsets has become popular in other immunologic fields such as kidney transplantation [2]. Kidney transplantation is the most frequently performed solid transplantation in the world. It is the best treatment of end-stage renal disease (ESRD) because it enhances survival rate, decreases complications and improves the quality of life of transplant recipients [3]. All kidney transplantation patients must

receive immunosuppressive agents such as glucocorticoids, mycophenolate mofetil, cyclosporine and tacrolimus for suppressing the immune system activity and preventing the rejection of a transplanted organ [4]. Tacrolimus is an immunosuppressive agent in the calcineurin inhibitors group which inhibits T cells activation and reduces immune response to prevent graft rejection in solid organ transplantation patients [5-6].

Recent data showed tacrolimus concentration was well correlated with T cell subsets and T cell function and related to clinical outcome in solid organ transplantation patients [6-9]. T cells activation needs association of the T cell receptor CD3 complex and a second signal induced by stimulation of co-receptors such as CD2, CD4, CD8, or CD28 [10]. Cell-mediated immune responses include different T cells such as CD3, CD4 and CD8, and T cell-derived cytokines such as IL-2, IL-4, and IFN-ɣ. IL-2 plays a vital role in the immune protection against graft rejection in kidney transplantation patients [11-12]. Therefore, monitoring of T cell subsets may be another approach to follow the effectiveness of tacrolimus-treated kidney transplantation patients in the future.

There is limited information of time and temperature requirements of the sample holding before flow cytometric analysis of T cells subsets in these patients. This technique has been limited by the necessity to analyze samples shortly after blood collection [13]. Therefore, immunophenotyping in clinical laboratories is usually operated within 24 hours. Accordingly, the use of fixative products to preserve the stability of cells may use to allow a longer period before analysis [14].

Positive exploration of cytokine-producing cells in a mixed culture using flow cytometry has been achieved in previous studies [14-15]. Recently, the potential to measure cytokine production following whole blood activation has been described. Moreover, using multicolor staining techniques can detect several cytokines in conjunction with multiple surfaces and intracellular markers concurrently. This technique offers the ability to phenotype and positively identify cytokine-producing cells [16].

Assessment of cytokine production cells by flow cytometry can be performed following either whole blood activation or purified peripheral mononuclear cells (PBMCs) activation [17]. Whole blood activation, which eliminates cellular purification steps, is less labor-intensive and may represent a substantial improvement over PBMC culture [18]. The facilities to separate PBMCs are not available in general laboratories. Moreover, PBMCs requires large sample volume and may result in considerable cell loss during isolation. On the other hand, using whole blood requires less volume of blood than PBMC assays. This is useful particularly in blood volume-limited patients such as pediatric patients or field studies in endemic countries. There are several previous reports of whole blood samples being frozen successfully with good viability and recovery to screen for immunomodulators [15].

T cell subsets analysis by flow cytometry in many laboratory studies requires sample transportation from the field site to the laboratory. Presently, T cell subsets analysis techniques are not available in many clinical laboratories, necessitating transportation of specimens to specialized reference laboratories. Limitations of time and flow cytometer machines may delay analysis of the specimens. Investigation of storage time of T cell subsets before analysis can allow researchers to preserve samples longer especially in developing countries with limited access to flow cytometry machines. There was limited data for evaluating the effect of storage on T cell subsets of kidney transplantation patients especially for the long term [1].

Therefore, the aim of this study was to validate the stability of expression of 3 lymphoid surface markers from whole blood samples, total T cells (CD3+), T-helper (CD3+CD4+) cells and T-suppressor (CD3+CD8+) cells, and an intracellular marker, CD4+IL-2, which were stored at 4 ℃ and -20°C up to 30 days and then analyzed by flow cytometric technique using samples from tacrolimus-treated kidney transplantation patients and healthy volunteers.

2. Materials and methods

2.1 Subjects and study design

Ten tacrolimus-based therapy kidney transplantation patients and ten healthy volunteers were enrolled. This study was approved by the Khon Kaen University Ethics Committee in Human Research and the Institutional Review Board Number is IRB00001189 (Reference Number HE581019). Written informed consent was obtained from all participants.

Approximately 10 mL of whole blood was obtained from each subject and collected in EDTA (ethylenediaminetetraacetate) tubes. Each sample was divided into 5 mL tubes and stored at 4 ℃ and -20 ℃. All specimens were processed with the same procedure after blood sample collected and then analyzed by flow cytometer at day 1, day 3, day 7, day 15 and day 30.

2.2 Sample preparation

Zero-point one mL of each whole blood sample was moved from EDTA tubes into a 12X75 mm flow cytometry tube with cap and 0.005 mL TruStain FcX® (Biolegend Inc, CA, USA catalog No. 101319) was added

into each tube to block Fc receptors to reduce nonspecific immunofluorescent staining. And then, all samples were centrifuged and incubated for 10 minutes at room temperature.

2.3 Cell surface staining protocol

Each sample of conjugated fluorescent T cell surface antibodies was stained with a combination of PE anti-human human CD3® (Biolegend Inc, CA, USA catalog No. 300308), PerCP/Cy5.5 anti-human CD4® (Biolegend Inc, CA, USA catalog No. 300530) and Pacific Blue anti-human CD8® mAb (Biolegend Inc, CA, USA catalog No. 344718). The cells were incubated in the dark at 4℃ for 30 minutes and then washed in Cell Staining Buffer® (Biolegend Inc, CA, USA catalog No. 420201) according to the manufacturer's protocol.

2.4 Lysis of erythrocytes

The red blood cells were lysed using RBC Lysis Buffer® (Biolegend Inc, CA, USA catalog No. 420301) according to the manufacturer's protocol.

2.5 Intracellular staining protocol

For intracellular cytokine staining, cells were stimulated with Brefeldin A solution (Brefeldin A Solution®; Biolegend Inc, CA, USA catalog No. 420601) for 300 minutes for activation of cytokines. The cells were fixed in 1.0% paraformaldehyde (Fixation Buffer®; Biolegend Inc, CA, USA catalog No. 420801) and then washed in Cell Staining Buffer® (Biolegend Inc, CA, USA catalog No. 420201) prior to staining. For detected intracellular production cytokine, the fixed cells of each labeled antibody were re-suspended in Permeabilization Wash Buffer® (Biolegend Inc, CA, USA catalog No. 421002) according to the manufacturer's protocol. APC/Cy7 anti-human IL-2® (Biolegend Inc, CA, USA catalog No. 500342) was added for intracellular cytokines staining.

The cells were incubated in the dark at room temperature for 30 minutes and then washed in Cell Staining Buffer® (Biolegend Inc, CA, USA catalog No. 420201). Fixed and intracellular labeled cells were resuspended in 0.5% paraformaldehyde according to the manufacturer's protocol. Then all samples were stored in 2 groups at 4℃ and -20℃. Each storage sample group had the same cell preparation using the same protocol. After 24 hours (day 1), day 3, day 7, day 15 and day 30 all samples were analyzed by flow cytometer.

2.6 Flow cytometry analysis

For analysis, a 3-laser BD FACSCANTO® II Flow cytometer (BD Biosciences, NJ, USA) was configured for four-color analysis with appropriate electronic compensation for spectral overlap of the light emitted by different fluorochromes. The target cells (CD3+, CD3+ CD4+, CD3+ CD8+, and CD4+ IL-2) were gated for immunofluorescence analysis by FACSDiva Version 6.1.3 and shown in figure 1. Thresholds for positive results were set using isotype-matched control antibodies for the target antigens. Quality control of flow cytometry in this study is strict with the use of isotype controls before analysis and the laboratory has internal quality control (IQC) and external quality assessment (EQA) procedures.

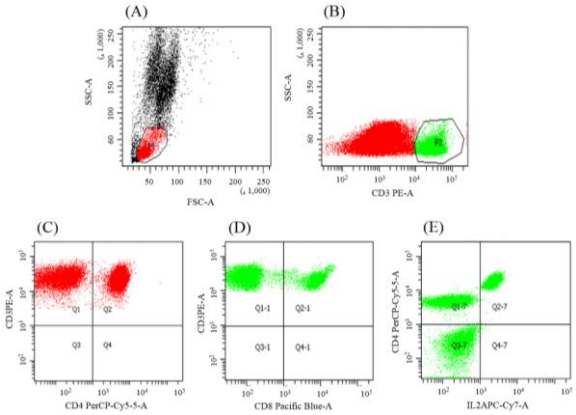

Figure 1. Representative plots showing the gating strategies leukocyte populations presented after red blood cell lysed whole blood by flow cytometry. (A) Dot plots using a light scattering feature from specimens and the respective gated population of lymphocytes (P1). (B) The respective gated population of CD3 T cells (P2). (C, D and E) Dot plots of subclass control and CD3 PE/CD4 PerCP-Cy5.5, CD3 PE/CD8 Pacific Blue and CD4 PerCP-Cy5.5/IL-2 APC/Cy7 with quadrant markers, respectively.

2.7 Statistical analysis

T cell populations (CD3+, CD3+CD4+ and CD3+CD8+) and T cell function (CD4+IL-2) are presented as percent mean ± SD. The percent mean values obtained for these parameters from 4 ℃ and -20 ℃ storage were compared for the difference using the independent t-test. To compare the T cell populations and T cell function of others on day 1, the paired t-tests were used for continuous variables in each group. The *p*-values (P) < 0.05 were considered to be statistically significant. All statistical analyses were carried out by STATA statistical software package version (StataCorp LP, College Station, Texas).

3. Results

This study was carried out to determine a protocol for T cell subsets by using whole blood which was stored at 4℃ and -20℃ from 1 to 30 days to assess the stability of T cell populations and T cell function compared with the standard method of carrying out all analysis on the first day. The percentage of T cells was preserved up to 30 days in samples collected in EDTA tubes. Figure 2-6 were shown a comparison between the percentages of T cell subsets in both group compared with day 1 (baseline).

For samples held at 4 ℃, the mean percentages of T cell subsets in healthy volunteers were not significantly different between day 1 and other days (P = 0.132, 0.186, 0.899 and 0.144 at day 3, 7, 15, and 30 compared with day 1, respectively). Moreover, the mean percentage of T cell subsets in the kidney transplantation patient group was also not significantly different between day 1 and the other days (P = 0.174, 0.135, 0.052 and 0.062 at day 3, day 7 day 15 and day 30 compared with day 1, respectively).

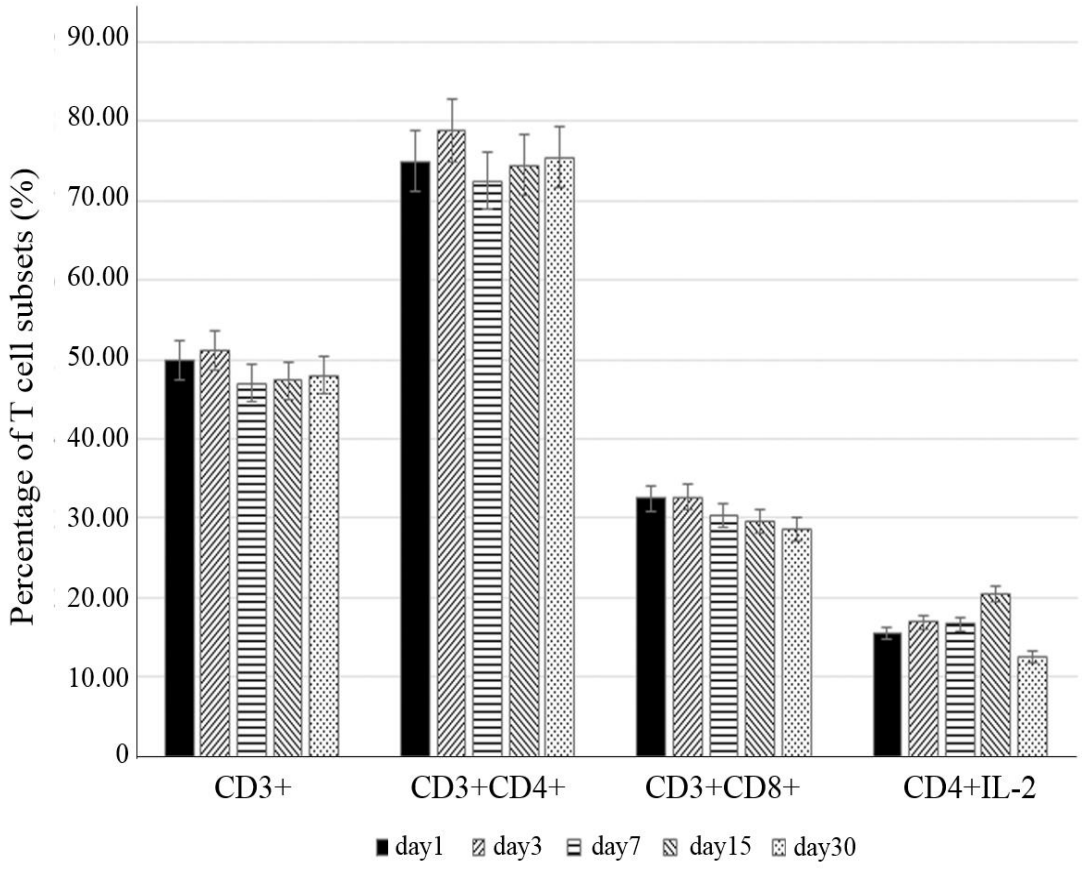

Figure 2. Bar charts averaging representative T cell populations (CD3+, CD3+CD4+, and CD3+CD8+) and T cell-derived cytokine (CD4+IL-2) present in healthy group at 4°C at day 1, day 3, day 7, day 15 and day 30 respectively.

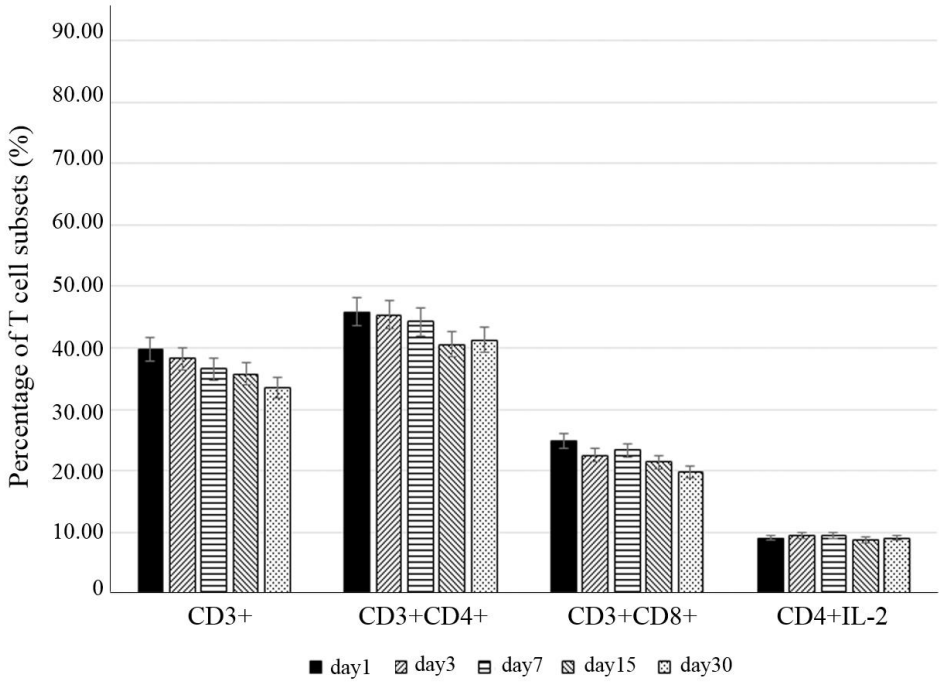

Figure 3. Bar charts averaging representative T cell populations (CD3+, CD3+CD4+, and CD3+CD8+) and T cell-derived cytokine (CD4+IL-2) present in patient group at 4℃ at day 1, day 3, day 7, day 15 and day 30 respectively.

For samples held at -20℃, the mean percentages of T cell subsets in healthy volunteers were not significantly different between day 1 and other days (P = 0.872, 0.942, 0.085 and 0.072 at day 3, 7, 15, and 30 compared with day 1, respectively). Moreover, the mean percentage of T cell subsets in the kidney transplantation patient group was also not significantly different between day 1 and the other days (P = 0.160, 0.104, 0.082 and 0.061 at day 3, day 5 day 15 and day 30 compared with day 1, respectively).

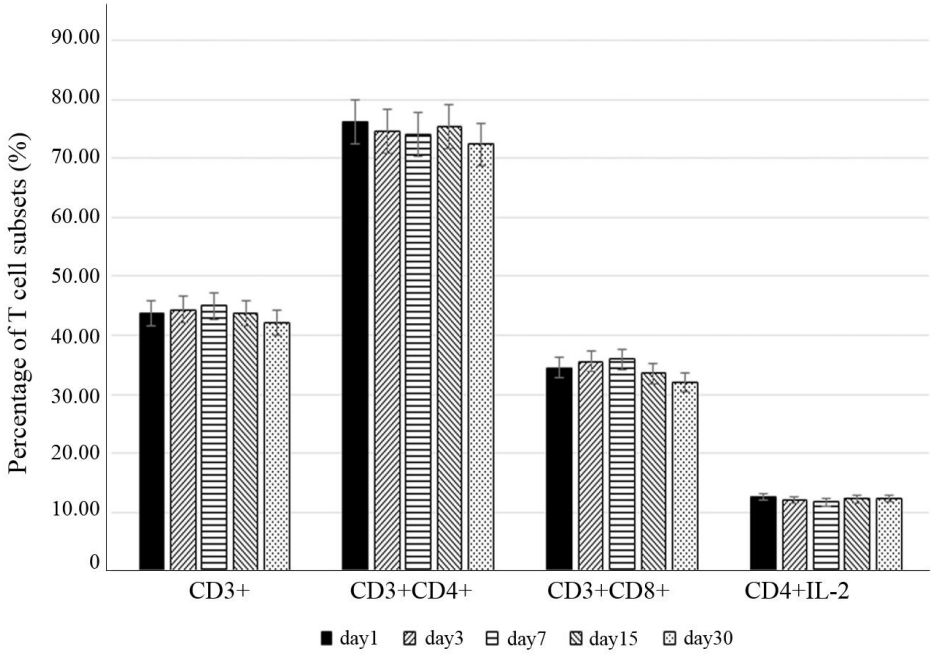

Figure 4. Bar charts averaging representative T cell populations (CD3+, CD3+CD4+, and CD3+CD8+) and T cell-derived cytokine (CD4+IL-2) present in healthy group at -20 ℃ at day 1, day 3, day 7, day 15 and day 30 respectively.

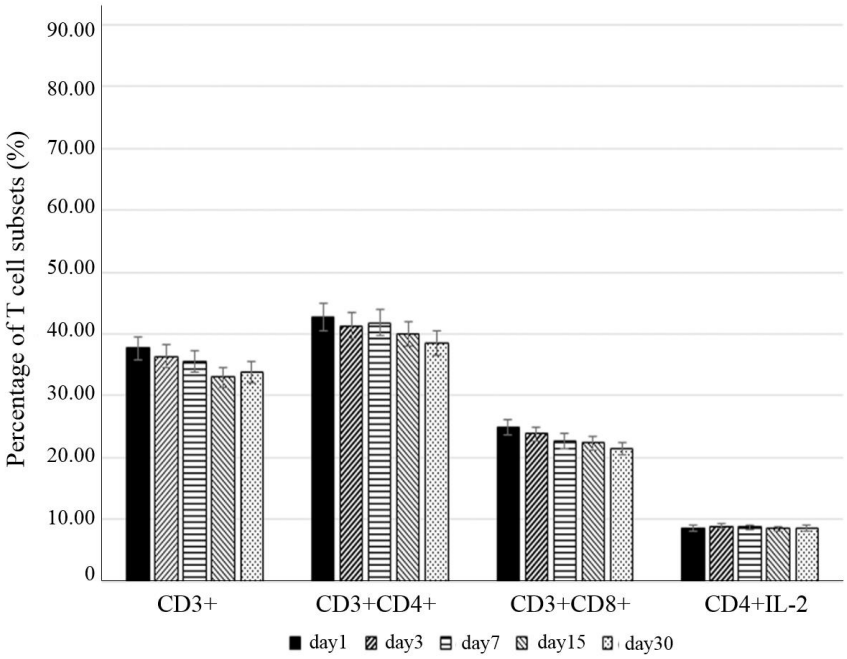

Figure 5. Bar charts averaging representative T cell populations (CD3+, CD3+CD4, and CD3+CD8+) and T cell-derived cytokine (CD4+IL-2) present in patient group at -20°C at day 1, day 3, day 7, day 15 and day 30 respectively.

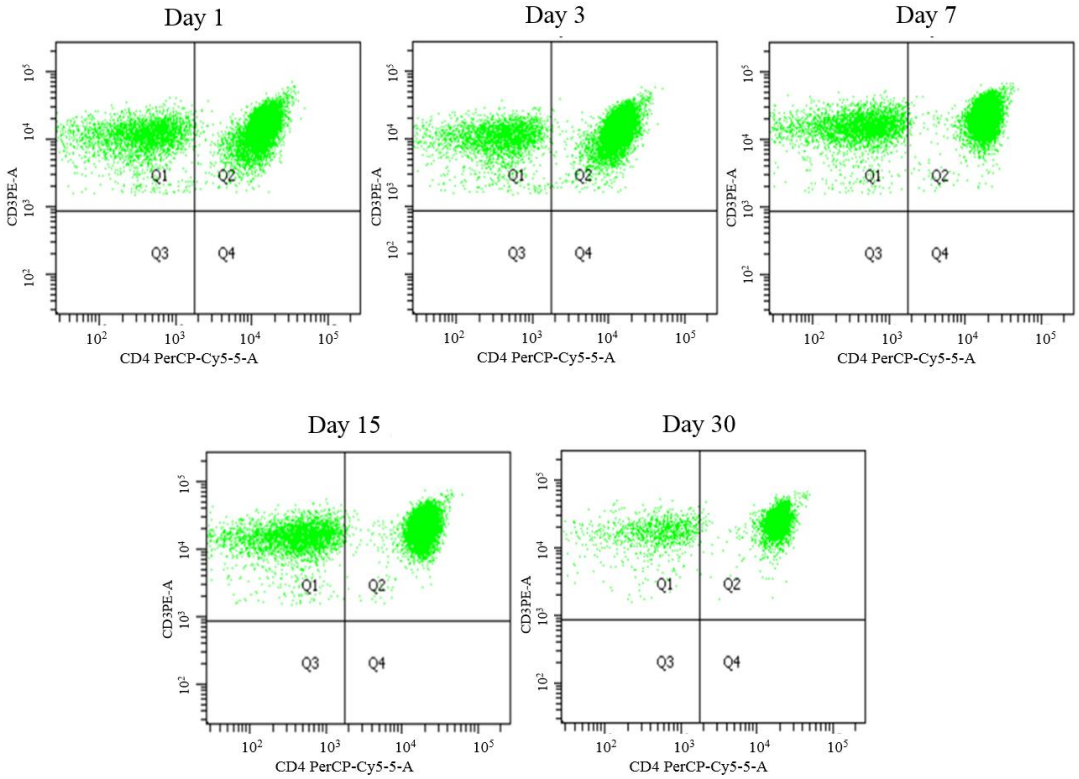

Figure 6. Standard flow cytometric analysis of T cell subsets of kidney transplant patients. Representative plots showing the gating strategies presented the respective gated population of CD3+CD4+T cells at Day 1 (baseline), 3, 7, 15 and 30 with quadrant markers respectively.

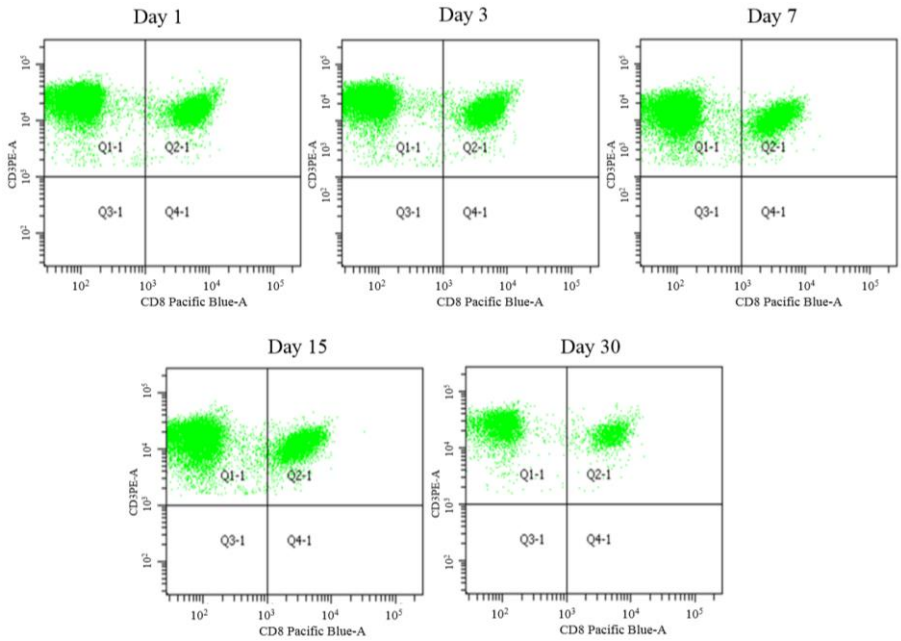

Figure 7. Standard flow cytometric analysis of T cell subsets of kidney transplant patients. Representative plots showing the gating strategies presented the respective gated population of CD3+CD8+T cells at Day 1 (baseline), 3, 7, 15 and 30 with quadrant markers respectively.

Moreover, the percentage of all T cell subsets was not significantly different between 4°C group and -20 ℃ group between both patient and healthy groups ($P = 0.41$, $P = 0.41$) as shown in fig 2 – 7. The percentage of all T cell subsets at 4 ℃ was significantly different between patient and healthy groups ($P < 0.005$) except CD4+IL-2 at day 30 as shown in figure 8.

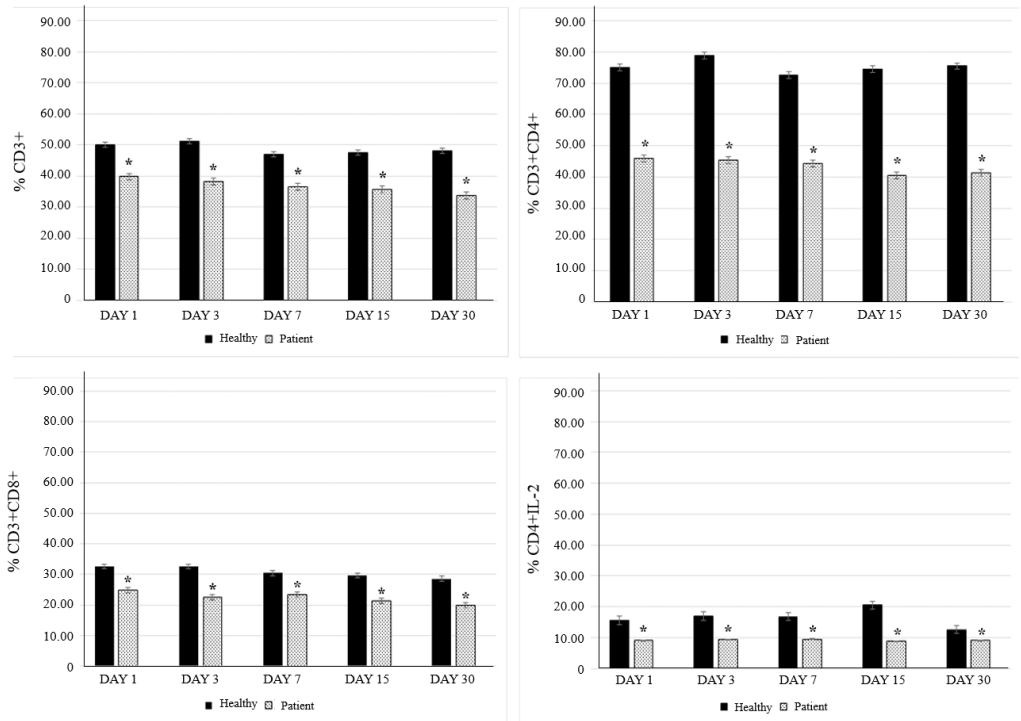

Figure 8. Bar charts averaging representative T cell populations ($CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$) and T cell-derived cytokine (CD4+IL-2) present in patient and healthy group at 4°C at day 1, day 3, day 7, day 15 and day 30 respectively. (*$P < 0.05$).

4. Discussion

Flow cytometry is widely used to determine immunophenotypic cell markers. In clinical and research laboratories, immunophenotypic markers such as T cells is usually performed using specimens on the same day after collection. Sometimes it is not possible to analyze samples immediately after staining for several reasons such as equipment failure and extended run times. Therefore, the post-sample processing stability should be considered.

Although the PBMCs assays are specific in T cell functional analyses (since there is no influence of other whole blood components such as neutrophils and plasma), it requires a large sample volume. Furthermore, massive cell loss occurs during isolation [10]. The alternative T cell assay by whole blood sample is expedient over PBMCs assays because it requires a small blood sample and does not need cell separation. Therefore, whole blood sample method is a simpler and more rapid sample analysis method.

This study evaluates the stability of immunophenotypic T cell subsets from whole blood samples of tacrolimus-based therapy kidney transplantation patients and healthy volunteers by flow cytometric analysis. Since kidney grafts are an extra cellular pathogen, cell-mediated immunity (especially T cells) play a vital part in immune response after kidney transplantation [12]. Effector T cells that develop from lymphoid organs infiltrate the graft and stimulate an inflammatory response. In T cell-mediated rejection, the graft is infiltrated by effector T cells, activated macrophages, B cells, and plasma cells and displays IFN-ɣ effects, increased chemokine expression, altered capillary permeability, and extracellular matrix, and decreased parenchymal function [5]. Apart from that, IL-2 is a key cytokine involved in the control of graft rejection. Therefore, T cell proliferation and IL-2 levels were used as the parameters to improve the assay from long term conditions [10].

Transportation of specimens and limitation of flow cytometer machines in many clinical laboratories may delay T lymphocytes analysis of the specimens. The Investigation of storage time of lymphocyte subsets before analysis could help researchers to have long-time preserve samples, especially in developing countries with limited flow cytometry machines. Most previous reports evaluating storage effects are from developed countries where all the laboratories are air temperature controlled [1]. Therefore, this study validated the stability of expression of 3 lymphoid surface markers, CD3+, CD3+CD4+ and CD3+CD8+, and an intracellular marker, CD4+IL-2, which were stored at 4 ℃ and -20 ℃ up to 30 days and then analyzed by flow cytometric technique using samples from tacrolimus-treated kidney transplantation patients and healthy volunteers.

The study in 2012 suggested that T cell subset immunophenotyping can be performed on aging specimens stored at room temperature or at 4 ℃ up to 72 hours, with results generated within published acceptable ranges. This allows off shift processing of specimens, which in their laboratory account for <10% of the total subset immunophenotyping volume [1]. Similarly, in 2011 this study demonstrated that most of the cell markers of both T lymphocytes and B lymphocytes were stable for one week at room temperature with the exception of the activation markers on T cells. Other studies have shown the stabilization of lymphocytes and immunologic markers for extended analysis using flow cytometry for up to 14 days by using Cyto-Chexv® blood preservative or TransFix®/EDTA Vacuum Blood Collection Tube [19-21]. The cost of blood preservative tubes is 7 USD/tube. This is very expensive when compared with using normal EDTA tubes (7USD/100tubes) which used in this study.

This study showed that a sample could be stored for 30 days at 4 ℃ and -20 ℃ and yet the analysis gave acceptable results without significant loss of cell surface and intracellular markers; the results were equivalent to the fresh specimen (day 1). These data confirmed that samples collected in the field or distant locations can be analyzed within one month and give accurate results up to 30 days if they are stained within 24 hours after blood collection. Moreover, the percentage of all T cell subsets in healthy group was significantly higher than in patient groups.

5. Conclusions

These findings indicated that storage of whole blood samples in EDTA tubes could hold lymphoid markers expression up to 30 days at both 4℃ and -20℃ storage. After sample collection and fixation in 24 hours, all T cell subsets were preserved up to 30 days before flow cytometric analysis without statistically significant changes in both patients and healthy volunteers. This study would be useful for other researchers who have limited access to flow cytometer machines, transportation of blood sample and amount of blood sample.

6. Acknowledgements

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