Scientific African 21 (2023) e01797

Contents lists available at ScienceDirect

Scientific African

journal homepage: www.elsevier.com/locate/sciaf

Blood collection tubes impact expression of activated CD4$^+$ and CD8$^+$ T cells in human whole blood assay☆

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ARTICLE INFO

Editor DR B Gyampoh

Keywords:
CD4$^+$ AND CD8$^+$ T cells
Activation status
Frequency
EDTA
Heparin

ABSTRACT

Background: T-lymphocyte subsets CD4 and CD8 play important role in host immune responses. However, little attention has been given to the impact of time lapse and the various anticoagulant blood collection tubes on the expression frequency and activation status of CD4$^+$ and CD8$^+$ T cells. To this end, we explore the impact of time (t<1 h and t=4 h) and collection tubes (EDTA and heparin) on the expression frequency and activation status of CD4$^+$ and CD8$^+$ T cells among healthy Ghanaian individuals.

Methods: A cohort of healthy individuals (n=9) is recruited, and blood samples obtained in Ghana for the frequency of CD4$^+$and CD8$^+$ T cells at various time points (<1 h and 4 h). The proportions of activation of these immune markers were profiled using immunophenotyping.

Results: Significant statistical differences in the activation frequency of CD69 expressing CD4$^+$T cells (t < 1 h and t=4 h; p=0.02) and CD69 expressing CD8$^+$ T cells from EDTA tubes at times (t < 1 h and t=4 h; p=0.05) was observed. No significant difference were observed with CD69 expressing cells in Heparin tubes. Notably, CD8$^+$ T cell activation frequency was observed to be consistently higher than that of CD4$^+$ T cell at the various study time points and in the collection tubes used. No marked alterations were observed witth the proportion of CD4$^+$ and CD8$^+$ T cells in the samples collected at the time points; <1 h and at 4 h.

Conclusion: The study shows that activation of CD4$^+$ and CD8$^+$ T cells in EDTA tubes differed significantly between both time points (t <1 h and t=4 h) but not in the heparin collection tubes. Therefore, it is important to take into account the elapsed time and the type of blood collection tubes when performing phenotypic characterization of activated immune markers.

Introduction

CD4$^+$ and CD8$^+$ T cells play key roles in adaptive immunity. CD4$^+$/CD8$^+$ ratio can serve as a marker for immune dysfunction, viral

☆ Authors Contribution: A.K., D.A.B., conceived and planned the experiment. A.K., and D.A.B., verified the analytical methods and carried out the experiments. A.K., P.O.P, D.A.B., A.S., A.L and K.N. discussed the results and contributed to the final manuscript.

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https://doi.org/10.1016/j.sciaf.2023.e01797
Received 19 January 2023; Received in revised form 1 May 2023; Accepted 6 July 2023
Available online 11 July 2023

reservoir size, aging, and inflammation [1]. $CD4^+$ T cells assist B cells in effective antibody production, enhancing macrophage activity as well as aid in the production of cytokines and chemokines by recruiting effector mediators to the infection site. $CD8^+$ T cells, on the other hand, are vital in eliciting accurate cytotoxic function and restraining intracellular infections.

The majority of these immune cells are transported through the bloodstream to effector sites. As a result, peripheral immune response analyses are crucial in examining disease response patterns, understanding treatment interventions and outcomes. Such analyses play a significant role in investigating disease pathogenesis, prognosis, diagnostics, and validating the clinical relevance of newly discovered biomarkers [2]. However, uncompromised blood samples are necessary for these analyses to isolate viable and functionally competent immune cells for further experimental analysis [3].

A quality cellular separation process must not only yield no contamination from red blood cells but also possess maximal functional capacity [4]. Factors such as age, ethnicity, smoking, alcohol intake and interval between blood draws have shown no statistical significance on T cell subsets expression pattern [5]. Previous studies have reported that the choice of collection tube, cell processing and preservation methods as well as instrumental variations and human subjectivity can influence immunophenotypic characterization of these immune cells [6–8]. The choice of blood collection system depends on the biomolecule(s) of interest and duration of storage [9,10]. The blood collecting tubes often include additives that mix with the blood when collected. These tubes have color-coded plastic caps and indicate which additives each tube contains. The tubes may contain additional substances that preserve the blood for processing in the medical laboratory. Using the wrong tube may therefore make the blood sample unusable. These substances are typically a thin film coating that is applied using an ultrasonic nozzle. The substances may include anticoagulants (EDTA, sodium citrate, sodium heparin) or a gel with intermediate density between blood cells and serum. Although there are numerous blood collection tubes used in biomedical laboratories, the ethylenediaminetetraacetic acid (EDTA) and heparin systems are the most commonly used. While EDTA is the preferred anticoagulant for hematological analysis, heparin is the most suitable choice, particularly over EDTA, for immunological studies due to its lower interference with most assays [11]. EDTA chelates calcium ions [9] and heparin deactivates thrombin blood clotting factor and factor Xa. [12]. Previous study by Bull et al. found no significant changes in cell viability, cell recovery and function of samples stored in EDTA, heparin and citrate [13].

While the choice of blood collection systems can impact immune markers of interest, it seems likely that lapsed time between sample collection and processing also affect the quality of immune cell activation and their expression frequency. For reliable immunophenotyping, the lapse time that is, the time between blood sample collection and immunoassay should be kept as short as possible as there is the potential for losses in the absolute cell numbers [11]. In resource-limited settings, most situations require that blood samples are transported from rural communities over long distances, using poor road networks to health/research centers prior to cell separation processes. Unlike at well-developed health facilities where analysis is performed within an hour (h) of collection, from our field studies, it takes a maximum of 4 h to transport and analysis the samples that are collected from these remote locations. Therefore, the effect of time lapse and type of collection system on the quality of immune activation remains to be studied.

To this end, we investigated the expression frequency and activation status of $CD4^+$ and $CD8^+$ T cells in EDTA and heparin collection tubes at different study time points ($t<$ 1 h and $t = 4$ h after sample collection).

Methods

Study population and ethics

This was a cross-sectional study. A total of nine (n=9) individuals from KNUST campus were recruited. Participants included healthy males and females (18–34 years). Participants suffering from chronic or infectious diseases were excluded from the study. Ethical approval was obtained from the Committee on Human Research Publication and Ethics of the School of Medicine and Dentistry of the Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana (CHRPE/AC/13417). Written informed consents were obtained from all study participants.

Blood sample collection

A quantitative volume of 10 mL of whole blood was collected from each study participant, 5 mL each was dispensed into EDTA and heparin tubes (BD Biosciences, USA).

Whole blood staining

To determine the expression pattern and activation status of T lymphocytes, whole blood staining was performed. A total of 200 μL of whole blood was pipetted from properly mixed blood in EDTA and heparin tubes into labelled wells in 96-well ELISA plates. The plates were centrifuged for 5 min at 1500 rpm at 4 ºC. After centrifugation, 180 μL of supernatant was removed from each well and discarded. A total of 5 μL of antibody cocktail (CD3-APC, CD4-Alexa488, CD8-PerCP-Cy5.5, and CD69-PE) was added to each of the wells. All reagents were obtained from eBioscience (Frankfurt, Germany) and ImmunoTools (Friesoythe, Germany). Plates were incubated for 30 min at 4 ºC in the dark, after which 100 μL of Red Blood Cell Lysing Solution (BD Biosciences, USA) was added to each well, mixed properly and incubated for 10 min at room temperature. The plates were thereafter centrifuged for 5 min at 1500 rpm after which 100 μL of the supernatant was removed and washing step repeated once. A fixing solution 100 μL was added to each well, incubated for 15 min at room temperature, after which the samples were centrifuged for 5 min at 1700 rpm. The plates were later flipped and blotted dry to remove supernatants. After further washing, cells were re-suspended in fixation and permeabilization buffer

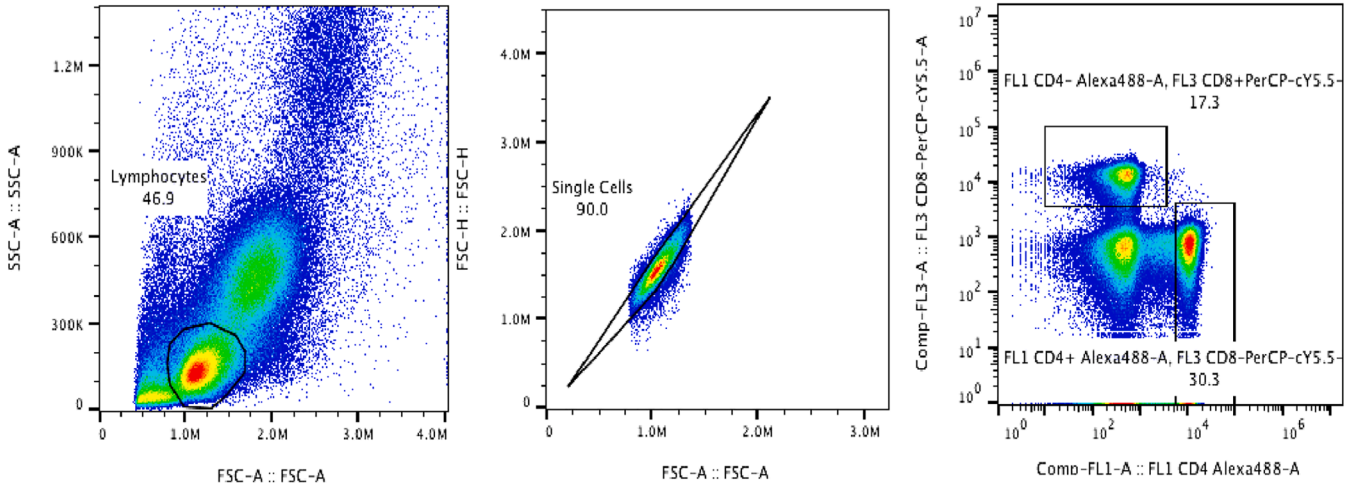

Fig. 1. CD4+ and CD8+ T-cells were gated on lymphocyte population single cells and analyzed for the% of cells expressing immune markers on the gated population. The figure shown here is a representative experiment of all the other analyses (A) Lymphocytes (B) Single cells (C) CD4 and CD8 T cells gates.

(eBioscience, USA).

Gating and flow cytometry measurements

The samples were measured with the Accuri C6 Flow Cytometer (Accuri Cytometers Inc, Ann Arbor, USA). Using the Accuri C6 software (BD Biosciences, USA), whole blood samples were acquired on medium fluidics flow rate at a threshold of 500,000 on the Forward-Side Scatter (FSC—H). ICS data were also acquired on medium/fast flow rate at a threshold of 500,000 on the Forward-Side Scatter (FSC—H). To correct spectral overlap, fluorescence compensation was performed using UltraComp ebeads (eBioscience, USA). FCS data was analyzed using the BD FASCDiva software. The figure below illustrates the gating strategy used in the FCS data analysis. Fig. 1.

Statistical analysis

Statistical analyses were performed using the software SPSS (IBM SPSS Statistics 22; Armonk, NY) and the PRISM 5 software (GraphPad Software, Inc., La Jolla, USA). Both Kolmogorov Smirnov and Shapiro-Wilk normality tests were applied to the datasets. Thereafter, paired/unpaired *t*-test or Mann-Whitney–U test were performed to compare two groups depending on whether the datasets were normally distributed or not. *P*-values of 0.05 or less were considered significant.

Results

Study demographics

Out of the 9 study participants, 5 (55.5%) were males, and 4 (44.4%) were females. The ages of the study participants ranged from 18 to 34 years (Table 1).

Proportion of $CD4^+$ and $CD8^+$ t cells in EDTA tube at time $<$ 1 h and t=4 h

Given that the ratio of CD4/CD8 in humans is an important immunological indicator of the immune status of a host, we investigated if the proportion of $CD4^+$ and $CD8^+$ T cells in samples stored in EDTA tubes was markedly altered at time $<$ 1 h and t=4 h. We observed no significant difference between $CD4^+$T cell collected in EDTA tubes at $t<1$ h and t=4 h; p=0.12 (Table 2). Similarly, we observed no difference in $CD8^+$ expressing T cell comparing $t<1$ h and t=4 h; p=0.75) (Table 2).

Proportion of $CD4^+T$ cells and $CD8^+T$ cells in heparin tubes at time ($t<1$ h) and time = 4 h

Having observed that EDTA tubes and time interval of 4 h had no effect on the proportion of $CD4^+$ and $CD8^+$ T cells expressed among the participants, we next investigated whether heparin collection tubes and the study time points ($t<1$ h and t=4 h) had any effect on expression frequency of $CD4^+$ and $CD8^+$ T cells. We observed that the percentage expression of $CD4^+T$ cells was not significant in heparin tubes at time $t<1$ h and t=4 h; p=0.18) (Table 2). The frequency of $CD8^+$T cells mirrored that of the $CD4^+T$ cells ($t<1$ h and t=4 h; p=0.66) (Table 2).

Activation status of $CD4^+T$ cells and $CD8^+T$ cells in edta tube at time $<$ 1 h and t=4 h

To determine the effect of EDTA collection tubes on the activation frequencies of $CD4^+T$ and $CD8^+$T cells at the two study time points, whole blood cells was stained using anti-human CD69 marker, an early activation marker of T cells and quantified with flow cytometry. We observed statistically significant differences in the activation frequency of CD69 expressing $CD4^+$T cells ($t<1$ h and t=4 h; p=0.02) and CD69 expressing $CD8^+$ T cells from EDTA tubes at times ($t<1$ h and t=4 h; p=0.05) (Table 3).

Activation status of $CD4^+T$ and $CD8^+T$ cells in heparin tubes at time $<$ 1 h and time = 4 h

To determine the activation of $CD4^+$ T and $CD8^+$ T cells in heparin-tubes, the expressing frequency of CD69 an early activation marker of T cells was assessed in whole blood staining at $t<1$ h and t=4 h. The levels of CD69 expressing $CD4^+$T cells (t<1 h and t=4h; p=0.86) and CD69 expressing $CD8^+T$ cells were not significantly altered at (t<1 h and t=4h; p=0.12) (Table 3).

Table 1
Age grouping of study participants.

Age groups (years)	Frequency	Percentage (%)
18–23	7	77.7
24–29	1	11.1
30- and above	1	11.1

Table 2
T cells expression at $t<1$h and $t=4$ h collected with EDTA and Heparin tubes.

Parameters	Time <1h	Time = 4h	*P*-value[⁂]
Expression of $CD4^+$ T cells at different time points in EDTA and Heparin tubes			
%$CD4^+$*T* cells [EDTA]	37.29± 1.90	42.71 ± 2.75	0.12
%$CD4^+$ T Cells [Heparin]	37.63 ± 1.63	40.83 ± 1.63	0.18
Expression of $CD8^+$ T cells at different time points in EDTA and Heparin tubes			
%$CD8^+$ T cells [EDTA]	25.83 ± 1.52	26.64 ± 2.06	0.75
%$CD8^+$ T Cells [Heparin]	26.42 ± 1.31	25.58 ± 1.38	0.66

[⁂] All tests for statistical significance were performed using student *t*-test after the datasets passed Shapiro Wilk test for normality. *P* values ≤ 0.05 were considered significant.

Table 3
T Cells activation at $t<1$h and $t=4$h collected with EDTA and Heparin tubes.

Parameters	Time <1h	Time = 4h	*P*-value
Activated $CD4^+$ T cells in EDTA and Heparin tubes			
%$CD4^+CD69^+$ T cells in EDTA tubes	0.38 ± 0.07	0.70 ± 0.10	0.02[‡]
%$CD4^+CD69^+$ T cells in Heparin tubes	0.60 ± 0.04	0.62 ± 0.11	0.86[⁂]
Activated CD8+ T cells in EDTA and Heparin tubes			
%$CD8^+CD69^+$ T cells in EDTA tubes	0.58 ± 0.07	0.91 ± 0.14	0.05[⁂]
%$CD8^+CD69^+$ T cells in Heparin tubes	1.00 ± 0.30	0.73 ± 0.40	0.12[⁂]

Statistical significance was based on either student *t*-test or Mann-Whitney U test after performing normality test using Shapiro Wilk test. *P* values ≤ 0.05 were considered significant. (NB [⁂] parametric test; [‡] non-parametric test).

Discussion

The activation of T cells is a highly intricate and complex process that entails biochemical mechanisms leading to cell differentiation and proliferation [14]. While anticoagulants have been found to have a minor effect on T cell recovery, viability, and function [15], the physical and phenotypic properties of T cells from different anticoagulants have a minimal impact on the expression of T cell subsets [16]. However, the time elapsed between sample collection and processing is believed to have an impact on the quality of immune cell activation and their frequency of expression but not widely explored especially in developing countries.

In this study, we evaluated CD4+ and CD8+ T cell activation status by measuring the expression of CD69, an early activation marker and a crucial co-stimulatory and signal-transmitting molecule for subsequent T-lymphocyte activation, proliferation, and gene induction [17,18]. Our results demonstrated statistically significant differences in CD4+ and CD8+ T cell activation levels in EDTA tubes at $t < 1$ h and $t=4$ h, but not in the heparin collection system. This difference may be attributed to the ability of EDTA to chelate Ca2+, which is essential for T cell activation, while heparin does not affect the levels of most ions in blood [19]. The study also found that the frequency of CD8+ T cell activation was consistently higher than that of CD4+ T cells at all time points and across all collection tubes containing different anticoagulants. Notably, the increase in CD8+ T cell activation was more pronounced in heparin tubes compared to EDTA tubes. These results are consistent with previous studies, which have shown that heparinized blood maintains more stable levels of bioactive TGF-β and better preserves IFN-γ secretion and cell viability over time compared to other collection tubes [15].

Studies have shown that CD8+ T cells express higher levels of TLR2 compared to CD4+ T cells when both are stimulated by TLR2 ligands and TCR agonists [19,20]. Furthermore, of the commonly used anticoagulants, heparin has been found to be more effective in maintaining the stability of immune markers compared to EDTA, as demonstrated in research by Coló Brunialti and colleagues. Their findings indicate that T cell activation was more efficiently achieved using heparinized blood than EDTA-treated blood [21]. A study examining the impact of different collection tubes (EDTA, heparin, citrate, and hirudin) on monocyte, neutrophil, and platelet function in whole blood found that the EDTA tube showed the least consistency and was the most unstable among the tubes tested [22]. This finding was supported by another study, which observed greater instability of physical and phenotypic parameters in samples collected in EDTA tubes compared to other collection tubes [23]. Here, we propose that the time intervals preceding sample processing play a critical role and must be considered when planning and executing immunology-based studies, particularly in developing countries, where access to well-equipped facilities for processing immune cells may not be readily available.

Further studies with larger sample sizes and appropriate fluorescence minus one (FMO) controls are necessary to address the major limitation of this study, which is the variability of data across time points and overlapping collection conditions. This is crucial, particularly in situations where batch effects are possible, or when sample processing may be delayed due to long distances between sample collection points and cell separation laboratories.

Conclusion

Based on the results of the study, it was observed that there was a significant difference in the activation of CD4+ and CD8+ T cells between EDTA and heparin tubes at different time points, with EDTA tubes showing instability over time. These findings suggest that

heparin is a more stable anticoagulant for immunological analysis. However, further investigations are required to assess the effect of prolonged time lapse on sample processing and immune markers. Taken together, the study provides valuable insights into the ideal blood collection tubes for sample processing, considering the impact on downstream functional immunological analysis.

Data availability

The data that support the findings of this study are available from the corresponding author, Alexander Kwarteng, upon reasonable request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to thank KCCR staff for their contribution to blood collections and sampling. We also thank the volunteers who took part in this study.

References

[1] W. Lu, V. Mehraj, K. Vyboh, W. Cao, T. Li, J.P. Routy, CD4:CD8 ratio as a frontier marker for clinical outcome, immune dysfunction and viral reservoir size in virologically suppressed HIV-positive patients, J Int AIDS Soc 18 (1) (2015) 20052.
[2] A. Posevitz-Fejfar, V. Posevitz, C.C. Gross, U. Bhatia, F. Kurth, V. Schutte, A. Bar-Or, S.G. Meuth, H. Wiendl, Effects of blood transportation on human peripheral mononuclear cell yield, phenotype and function: implications for immune cell biobanking, PLoS One 9 (12) (2014), e115920.
[3] R. Mallone, S.I. Mannering, B.M. Brooks-Worrell, I. Durinovic-Bello, C.M. Cilio, F.S. Wong, N.C. Schloot, T-cell workshop committee IoDS: isolation and preservation of peripheral blood mononuclear cells for analysis of islet antigen-reactive T cell responses: position statement of the T-cell workshop committee of the immunology of diabetes society, Clin. Exp. Immunol. 163 (1) (2011) 33–49.
[4] C. Nilsson, S. Aboud, K. Karlen, B. Hejdeman, W. Urassa, G. Biberfeld, Optimal blood mononuclear cell isolation procedures for gamma interferon enzyme-linked immunospot testing of healthy Swedish and Tanzanian subjects, Clin. Vaccine Immunol. 15 (4) (2008) 585–589.
[5] S.S. Uppal, S. Verma, P.S. Dhot, Normal values of CD4 and CD8 lymphocyte subsets in healthy indian adults and the effects of sex, age, ethnicity, and smoking, Cytometry B Clin. Cytom. 52 (1) (2003) 32–36.
[6] T.L. Williams, D. Andrzejewski, J.O. Lay, S.M. Musser, Experimental factors affecting the quality and reproducibility of MALDI TOF mass spectra obtained from whole bacteria cells, J. Am. Soc. Mass Spectrom. 14 (4) (2003) 342–351.
[7] M. de Almeida Santiago, E.F.B. de Paula Fonseca, C.F. da Silva Marques, E. Domingos da Silva, A.L. Bertho, A.C. Nogueira, Flow Cytometry as a Tool for Quality Control of Fluorescent Conjugates Used in Immunoassays, PLoS One 11 (12) (2016), e0167669.
[8] N. Aghaeepour, G. Finak, C.A.P.C. Flow, D. Consortium, H. Hoos, T.R. Mosmann, R. Brinkman, R. Gottardo, R.H. Scheuermann, Critical assessment of automated flow cytometry data analysis techniques, Nat. Methods 10 (3) (2013) 228–238.
[9] G. Juricic, A. Bakliza, A. Saracevic, L.M. Kopcinovic, S. Dobrijevic, S. Drmic, A.M. Simundic, Glucose is stable during prolonged storage in un-centrifuged Greiner tubes with liquid citrate buffer, but not in serum and NaF/KOx tubes, Clin. Chem. Lab. Med. 54 (3) (2016) 411–418.
[10] L. Fernandez, P. Jee, M.J. Klein, P. Fischer, S.L. Perkins, S.P. Brooks, A comparison of glucose concentration in paired specimens collected in serum separator and fluoride/potassium oxalate blood collection tubes under survey 'field' conditions, Clin. Biochem. 46 (4–5) (2013) 285–288.
[11] A.M. Diks, C. Bonroy, C. Teodosio, R.J. Groenland, B. De Mooij, E. De Maertelaere, J. Neirynck, J. Philippé, A. Orfao, J.J. Van Dongen, M.A. Berkowska, Impact of blood storage and sample handling on quality of high dimensional flow cytometric data in multicenter clinical research, J. Immunol. Methods 475 (2019), 112616. Dec 1.
[12] M.A. Smythe, J. Priziola, P.P. Dobesh, D. Wirth, A. Cuker, A.K. Wittkowsky, Guidance for the practical management of the heparin anticoagulants in the treatment of venous thromboembolism, J. Thromb. Thrombolysis 41 (1) (2016) 165–186.
[13] M. Bull, D. Lee, J. Stucky, Y.L. Chiu, A. Rubin, H. Horton, M.J. McElrath, Defining blood processing parameters for optimal detection of cryopreserved antigen-specific responses for HIV vaccine trials, J. Immunol. Methods 322 (1–2) (2007) 57–69.
[14] K. Hess, Y. Yang, S. Golech, A. Sharov, K.G. Becker, N-p Weng, Kinetic assessment of general gene expression changes during human naive CD4+ T cell activation, Int. Immunol. 16 (12) (2004) 1711–1721.
[15] M. Bull, D. Lee, J. Stucky, Y.L. Chiu, A. Rubin, H. Horton, M.J. McElrath, Defining blood processing parameters for optimal detection of cryopreserved antigen-specific responses for HIV vaccine trials, J. Immunol. Methods 322 (1) (2007) 57–69.
[16] J. Thornthwaite, P. Rosenthal, D. Vazquez, D. Seckinger, The effects of anticoagulant and temperature on the measurements of helper and suppressor cells, Diagn. Immunol. 2 (3) (1983) 167–174.
[17] S.F. Ziegler, F. Ramsdell, M.R. Alderson, The activation antigen CD69, Stem. Cells 12 (5) (1994) 456–465.
[18] A.G. Santis, M. López-Cabrera, J. Hamann, M. Strauss, F. Ánchez-Madrid, Structure of the gene coding for the human early lymphocyte activation antigen CD69: aC-type lectin receptor evolutionarily related with the gene families of natural killer cell-specific receptors, Eur. J. Immunol. 24 (7) (1994) 1692–1697.
[19] C. Higgins, The use of heparin in preparing samples for blood-gas analysis, Med. Lab. Obs. 39 (10) (2007) 16.
[20] S.M. Lee, Y.D. Joo, S.K. Seo, Expression and function of TLR2 on CD4 versus CD8 T cells, Immune Netw. 9 (4) (2009) 127–132.
[21] M.K. Coló Brunialti, E.G. Kallás, M. Freudenberg, C. Galanos, R. Salomao, Influence of EDTA and heparin on lipopolysaccharide binding and cell activation, evaluated at single-cell level in whole blood, Cytometry 50 (1) (2002) 14–18.
[22] C.S. Engstad, T.J. Gutteberg, B. Østerud, Modulation of blood cell activotion by four commonly used anticooguloints, Thromb. Haemost. 77 (4) (1997) 690–696.
[23] A.R. Ruiz, I.P. Fernandez, J.P. Baena, F. de La Torre, G.R. Ramírez, Influence of sample storage time and temperature on lymphocyte subset counts using a FACScount system, Haematologica 85 (5) (2000) 550–551.