2024, Scienceline Publication
World's Veterinary Journal
World Vet J, 14(2): 158-168.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2024.wvj20
PII: S232245682400020-14

Impact of Fixation of Camel Lymph Node Cells on Marker Expression Stability in Flow Cytometry

Mohammed Ali Al-Sukruwah[1], Hind Althagafi[2*], Najla K Al Abdulsalam[3], and Jamal Hussen[1*]

[1]*Department of Microbiology, College of Veterinary Medicine, King Faisal University, Al-Ahsa, Saudi Arabia*
[2]*Department of Biology, College of Science, Princess Nourah bint Abdulrahman University, P.O. Box 84428, Riyadh 11671, Saudi Arabia*
[3]*Department of Biological Sciences, College of Science, King Faisal University, P.O. Box 380, Al-Ahsa 31982, Saudi Arabia*

*Corresponding author's Email: hjalthagafi@pnu.edu.sa; jhussen@kfu.edu.sa

ORIGINAL ARTICLE
Received: March 20, 2024
Revised: April 22, 2024
Accepted: May 15, 2024
Published: June 25, 2024

ABSTRACT

Single cell immunophenotyping by flow cytometry has proven a useful and high sensitive method for the analysis of immune cell composition and phenotype in different lymphatic and non-lymphatic tissues. Fixation of stained cells is usually recommended when the cells need to be preserved for later analysis by flow cytometry to avoid changes in cell morphology and expression of the level of cellular antigens. In the present study, a stain-fix approach was used in combination with flow cytometry to investigate the impact of fixation of camel lymph node cell suspension (n = 5 camels) after labeling with monoclonal antibodies to some leukocyte antigens on their cellular composition and expression density of immune cell markers. The obtained results indicated that camel lymph node cell suspension stained with fluorochrome-conjugated mAbs to leukocyte antigens and fixed with paraformaldehyde (PFA) will keep stable values for their immune cell composition for at least six days when analyzed by flow cytometry. However, if cell subsets were to be identified, fixation may result in different values that were obtained when analyzing fresh stained unfixed cells. Especially the instability in the fluorescence intensity of CD14, CD172a, and MHCII will lead to significant changes in the frequency of monocyte subsets (classical versus intermediate or non-classical) and the identification of macrophage functional subtype (M1 versus M2). Similarly, the instability in CD44 expression may affect the identified phenotype of T cells with significantly lower frequency of activated T cells. In conclusion, flow cytometric data collected from stained and PFA-fixed cell suspension prepared from camel lymph nodes should be interpreted with care if the functional subtype of cells is to be identified based on surface molecule expression.

Keywords: Camel, Fixation, Flow cytometry, Immune cell, Lymph node

INTRODUCTION

Lymph nodes are secondary organs with specialized structures that filter the lymph throughout the body (Yang et al., 2019). Lymph nodes provide sites for cross-talk between antigen-specific adaptive immune cells and antigen-presenting cells for the development of adaptive immune responses (Liao and von der Weid, 2015; Rehfeld et al., 2017). The cellular composition of the node lymphatic tissue is dominated by lymphoid cells with lower numbers of neutrophils and monocytes and their derivatives, such as macrophages and dendritic cells. Alterations in the composition and phenotype of immune cells in the lymph node reflect important events of the immune response, including migration and homing of immune cells, antigen trapping, presentation, stimulation of adaptive immunity, and generation of effector cells (Lun et al., 2007). Therefore, the analysis of these changes provides an effective method to evaluate the immune response against infection and vaccination, or for the diagnosis of immunopathology (Koets et al., 2002; Caucheteux et al., 2013).

Single-cell immunophenotyping by flow cytometry has proven a useful and highly sensitive method for the analysis of immune cell composition and phenotype in different lymphatic (Aalbers et al., 2015) and non-lymphatic tissues (Dong et al., 2016; Hagberg et al., 2018; Tighe et al., 2019). Immunophenotyping steps usually include cell separation and the preparation of cell suspension followed by labeling cellular antigens with monoclonal antibodies conjugated with fluorescent dyes (Maecker et al., 2012). One of the big challenges during immunophenotyping studies, especially in veterinary medicine, where flow cytometers are still not available in every laboratory, is extending the time between cell staining and flow cytometric analysis without affecting cell composition or phenotype (Laurin et al., 2015). Fixation of stained cells represents a way to avoid protein denaturation and loss of antigenic structure leading to extending the time between sampling and analysis (Ng et al., 2012; Qin et al., 2021). In addition, the detection of intracellular epitopes, such as cytokines or signaling molecules, requires cell fixation and permeabilization to access the target epitope by antibodies (Paavilainen et al., 2010; Cheng et al., 2019). For the selection of a fixative agent, the fixative must not impact parameters related to cell structure, including cell size and granularity, as well as the reactivity of cellular epitopes with

To cite this paper: Al-Sukruwah MA, Althagafi H, Al-Abdulsalam NK, and Hussen J (2024). Impact of Fixation of Camel Lymph Node Cells on Marker Expression Stability in Flow Cytometry. *World Vet. J.*, 14(2): 158-168. DOI: https://dx.doi.org/10.54203/scil.2024.wvj20

the monoclonal antibodies. Cross-linking of cellular proteins and DNA using paraformaldehyde (PFA) is one of the most commonly used cell fixatives due to its ability to keep, to some extent, the cell structure, and the antigenic determinants (Cheng et al., 2019).

Fixation of human white blood cells induced a significant change in cell size and granularity as well as in the abundance of many cellular antigens (Pinto et al., 2005; Stewart et al., 2007). Similar effects of PFA on human blood leukocytes have been found in a study by Ng et al. (2012), with marked changes in cell count and reactivity to monoclonal antibodies (mAbs). Similarly, PFA fixation of pig peripheral blood mononuclear cells resulted in significant changes in the reactivity of mAbs to cell surface antigens (Schuberth et al., 1998).

In a fix-stain approach, a previous study analyzed the impact of PFA fixation of camel blood leukocytes on their reactivity to monoclonal antibodies to some cell surface antigens. The study results revealed that leukocytes fixed with PFA lost their subsequent binding to CD163 and WC1 monoclonal antibodies leading to the lack of identification of target cells. In addition, fixed cells showed reduced reactivity to CD14, CD172a, MHCII, CD11a, CD18, CD44, and CD45 monoclonal antibodies (Almohammed et al., 2022). In the present study, a stain-fix approach was used to investigate the impact of fixation of camel lymph node cell suspension after labeling with monoclonal antibodies to some leukocyte antigens on their cellular composition and expression density of immune cell markers.

MATERIALS AND METHODS

Ethical approval

The presrnt study was approved by the Ethics Committee of King Faisal University, Saudi Arabia (KFU-REC-2021- DEC -EA000326).

Animals and collection of lymph node samples

A total of five healthy camels (*Camelus dromedarius*) were selected from animals admitted for normal slaughtering at Al-Omran Slaughterhouse in Al-Ahsa Region in Saudi Arabia. The animals included in the study were examined for clinical signs of reproductive, gastrointestinal, or respiratory diseases by a veterinarian. In addition, the viscera were examined directly after euthanasia (via bleeding) to exclude animals with signs of abdominal, thoracic, or reproductive disorders. After collection, mesenteric lymph nodes were immediately placed on an ice pack in cold PBS containing 1 mM EDTA and transported to the laboratory within an hour.

Cell separation from lymph node samples

Lymph node cell suspension was prepared as previously described (Barut et al., 2022). After removing the fat and connective tissue from the capsule, the lymph nodes were placed in a sterile Petri dish filled with 10 mL of cold PBS-EDTA. The nodes were then cut into small pieces (2-3 cm) and minced using sterile scissors and forceps. The minced lymph nodes were suspended in PBS-EDTA and the cell supernatants were filtered through a cell strainer. The cells were washed in PBS-EDTA for 10 minutes at 4°C and 300 × g and the cell pellet were resuspended in PBS-EDTA and contaminating red blood cells were removed by hypotonic lysis. Finally, the cell pellet was resuspended in PBS-EDTA at 5.0 × 106 cell/mL. Cell viability (always more than 96%) was evaluated within one hour by flow cytometry after adding propidium iodide (2µg/mL) to the cells.

Antibody staining and flow cytometry

Lymph node cells were labeled with mAbs to camel leukocyte markers and analyzed on the flow cytometer (DiGiuseppe and Wood., 2019). For this, 1 x 10^6 cell/well were incubated in a 96-well plate with mAbs to CD45, CD44, CD172a, CD14, CD163, and MHCII. After incubation (15 minutes/ 4°C), cells were washed twice with PBS and were incubated with anti-mouse IgG1 and IgG2a (Invitrogen) conjugated with FITC and PE, respectively. Staining with isotype control antibodies was also done (Becton Dickinson Biosciences). Cells were then washed twice with PBS and analyzed on a Becton Dickinson Accuri C6 flow cytometer (Becton Dickinson Biosciences, San Jose, California, USA). Data from at least 100,000 cells were collected and analyzed with the flow cytometric software C-Flow (Becton Dickinson Biosciences, San Jose, California, USA).

Statistical analyses

Data analysis was performed using the flow cytometric software C-Flow (BD). The column statistic function of the Prism software (GraphPad) was used to calculate the means and standard error of the mean (SEM) of the analyzed parameters. Differences between means were tested with the t-test being considered significant with p-value < 0.05.

RESULTS AND DISCUSSION

Lymph nodes are filters of tissues and tissue fluids that trap antigens and provide sites for antigen presentation and activation of pathogen-specific immune responses (Liao and von der Weid, 2015). Their immune cell composition and phenotype may reflect pathologies in tissues they drain (Elmore, 2006; Ma et al., 2021). As there are limited data on the impact of fixation on the composition and phenotype of lymph node cells in the camel, the current study investigated the impact of fixation on the frequency of and marker expression on lymph node immune cells in camels. Antibody-labeled and paraformaldehyde-fixed lymph node cells were compared with unfixed cells by flow cytometry.

Impact of cell fixation on the cell composition of camel lymph node

Staining with the pan-leukocyte marker CD45 (Meza Guzman et al., 2024) and the myeloid marker signal regulatory protein α (SIRPα) identified total immune cells (CD45+ cells), myeloid cells (CD45+CD172a+), and lymphoid cells (CD45+CD172a-) within camel lymph node cells (Figure 1A-D). For all cell populations and all time points after fixation, no significant changes were observed between unfixed and fixed cells ($p > 0.05$) (Figure 1E-G).

Lymphocytes were identified as the major fraction of lymph node cells (86.6 %) based on their negative staining with CD14, while monocytes were identified as CD14+ cells with low granularity, as determined using the side scatter (SSC) parameter, with a mean percentage of (4.9 %) of total cells. Another minor population of CD14low cells with high SSC were identified as neutrophils with a mean percentage of (6.2 %) of total cells (Figure 2A). For all cell populations including monocytes, neutrophils, and lymphocytes, stability in their frequency was observed when comparing unfixed and fixed cells (Figure 2B). The percentages of these populations remained stable during the 6 days of measurement ($p > 0.05$). The obtained data indicated that the cellular composition of mAb-stained and PFA-fixed camel lymph node cells may be analyzed by flow cytometry with stable values for at least 6 days after fixation.

Impact of fixation on forward and side scatter properties of lymph node cell populations

The comparison between unfixed and fixed cells revealed a significant increase in the forward scatter (FSC) of fixed neutrophils (day 0) followed by normalization on day 1 and a subsequent decrease on days 3 and 6 after fixation ($p < 0.05$) (Figure 3A). For lymphocytes, a significant decrease in FSC was observed directly after fixation (day 0) and remained low on all other time points after fixation ($p < 0.05$) (Figure 3A). On the other hand, a marked increase in the SSC value was observed for monocytes and neutrophils directly after fixation and on all other time points ($p < 0.05$) (Figure 3B) when compared to unfixed cells. No Fixation-induced changes were found in the FSC value of monocytes or the SSC value of lymphocytes when comparing unfixed and fixed cells ($p> 0.05$) (Figure 3A-B).

Forward and side scatter values are important parameters that are commonly used for the identification of cells by flow cytometry (Givan, 2001; Nunez, 2001). While forward scatter is a measure that indicates cell size, side scatter reflects the structure complexity and granular content of a cell (Givan, 2001; Nunez, 2001). Increased FSC values are usually linked to stimulated phenotypes of monocytes and neutrophils (Hussen et al., 2016). Similarly, lymphocyte proliferation is associated with increased cell size with lymphoblasts being larger than lymphocytes (DiGiuseppe and Wood, 2019). In contrast, reduced cell size is an indicator of cell apoptosis and necrosis (Yurinskaya et al., 2017). On the other hand, reduced SSC, which indicates lower granularity, is usually linked to neutrophil degranulation upon stimulation (Hussen et al., 2016). In the present study, the fixation-induced changes in FSC and SSC properties of camel lymph node cells may interfere with the functional characterization of immune cells leading to false interpretation of stimulatory or inhibitory effects in functional studies or misinterpretation of cell viability studies.

Impact of cell fixation on cell markers expression on lymph node cells

The leukocyte antigens CD45 and CD44 are pan-leukocyte markers that are usually used for the identification of and gating on total immune cells for the subsequent analysis of cell subsets (Ratei et al., 2007; Gray et al., 2012). In addition, these molecules are used as markers for cell activation and migration of leukocytes (Gray et al., 2012; Senbanjo and Chellaiah, 2017). In the present study, fixed neutrophils and lymphocytes showed relative stability in the abundance (mean fluorescence intensity; MFI) of CD45 (Figure 4A) and CD44 (Figure 4B) directly after fixation followed by a continuous decrease in the MFI values starting on day 1 after fixation.

Only for monocytes, the expression stability by fixation remained until day 6 after fixation for CD45 and until day 3 after fixation for CD44. Although the observed fixation-induced change did not affect the percentage of cells stained positively with CD45 or CD44 antibodies, the reduced MFI values after 24 hours of fixation may lead to incorrect interpretation of the functional phenotype of the cells in terms of activation and migration status.

The myeloid antigens CD172a, CD14, and CD163 in addition to the class II major histocompatibility complex (MHCII) are cell markers usually used to identify monocytes, macrophages, and neutrophils (Broz and Krummel, 2015). Their detection is usually used for the identification of total myeloid cells (CD172a) (Hussen et al., 2016), for the classification of monocyte subsets (CD14 and MHCII) (Lyu et al., 2023), or for the characterization of the functional subtype of macrophages (MHCII and CD163) (Lyu et al., 2023; Feng et al., 2021). Except their CD163 expression, which indicated relative stability after fixation ($p > 0.05$), the monocyte phenotype was significantly affected by fixation in the present study. While CD14 and MHCII levels were significantly reduced on fixed monocytes, CD172a levels were significantly elevated after fixation ($p < 0.05$) (Figure 5A). In contrast, neutrophils did not show any significant fixation-induced changes in the abundance of any of the studied myeloid markers ($p > 0.05$) (Figure 5B).

Low CD14 expression has been linked to the phenotype of a non-classical subset of monocytes in camels and other species (Ziegler-Heitbrock, 2014). Similarly, heterogeneity in MHCII expression has been used for the classification of camel monocyte subsets with lower expression on classical than intermediate and non-classical monocytes (Hussen et al., 2020). The results indicated that fixation of camel lymph node cell suspension may lead to incorrect interpretation of data related to the heterogeneity of monocyte.

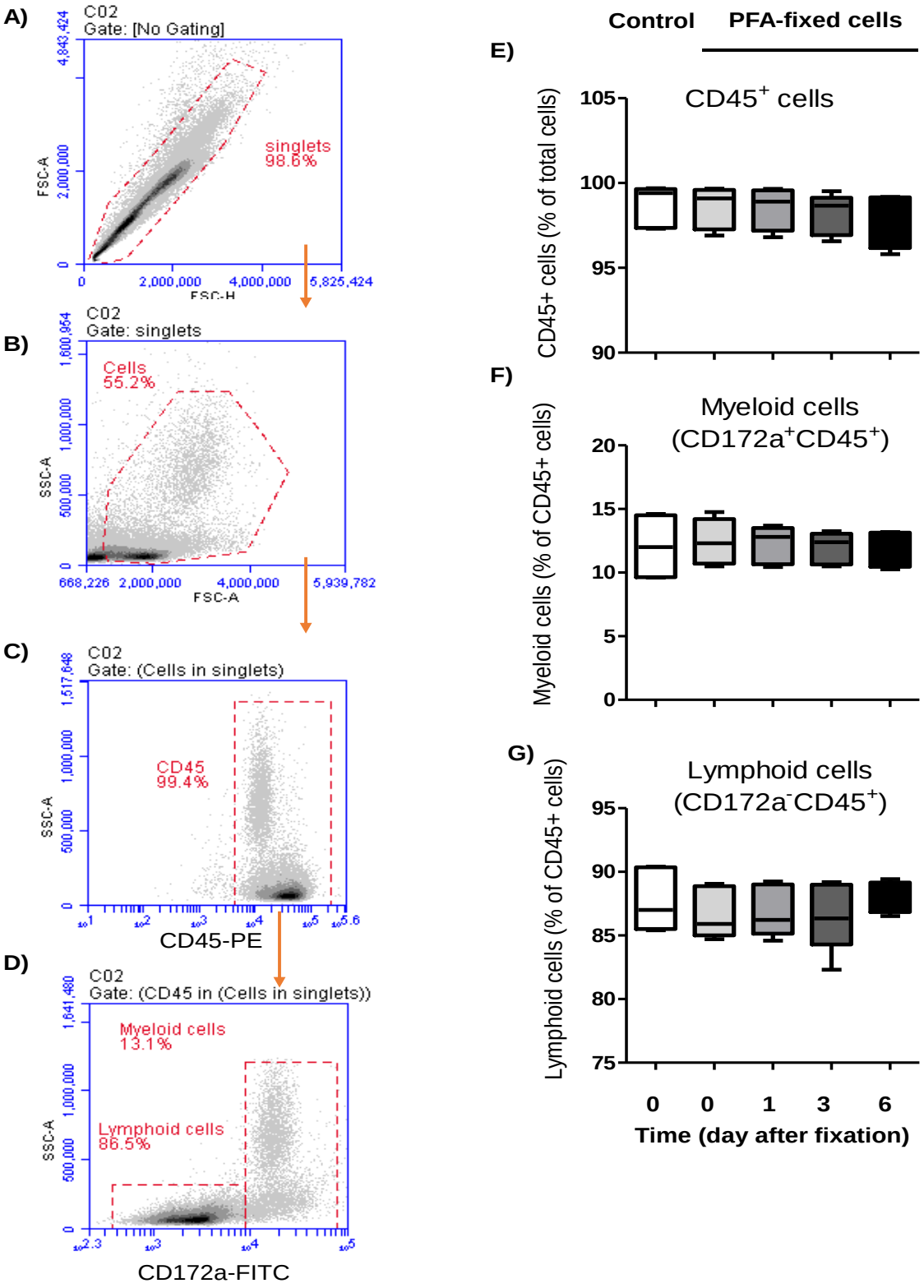

Figure 1. Gating strategy for the identification of camel lymph node cell populations using flow cytometry. After gating on single cells (**A**), lymph node cells were gated (**B**) based on their forward scatter (FSC) and side scatter (SSC) properties. Staining with CD45 (**C**) was used to identify total leukocytes and myeloid and lymphoid cells were identified based on their staining with CD172a (**D**). The percentage of total leukocytes (**E**), myeloid cells (**F**), and lymphoid cells (**G**) were calculated and presented as box plots.

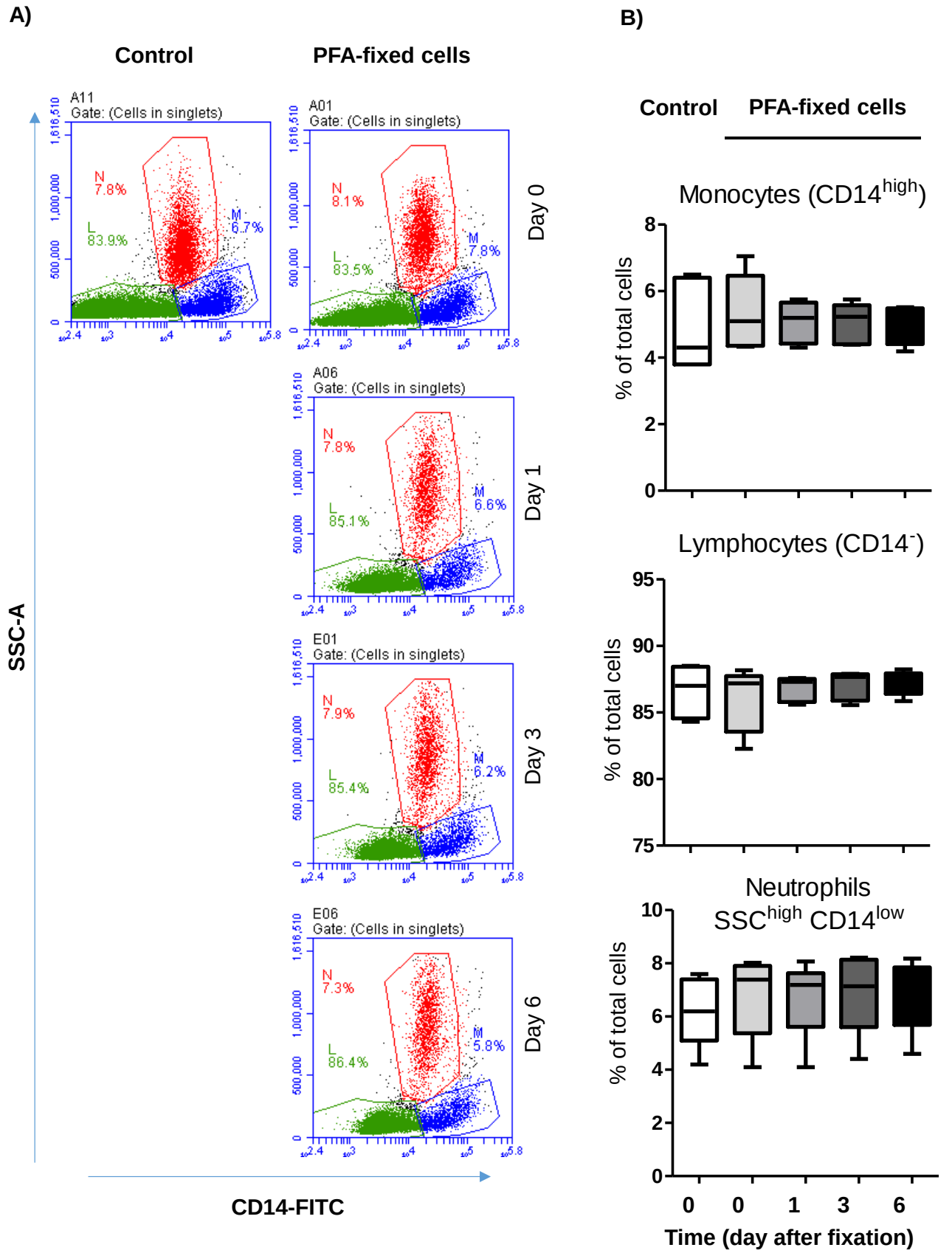

Figure 2. Identification of camel lymph node monocytes, neutrophils, lymphocytes based on their staining with monoclonal antibodies (mAbs) to CD14 (**A**) in a SSC/CD14 dot plot, and their percentages calculated for fixed and unfixed cells (**B**).

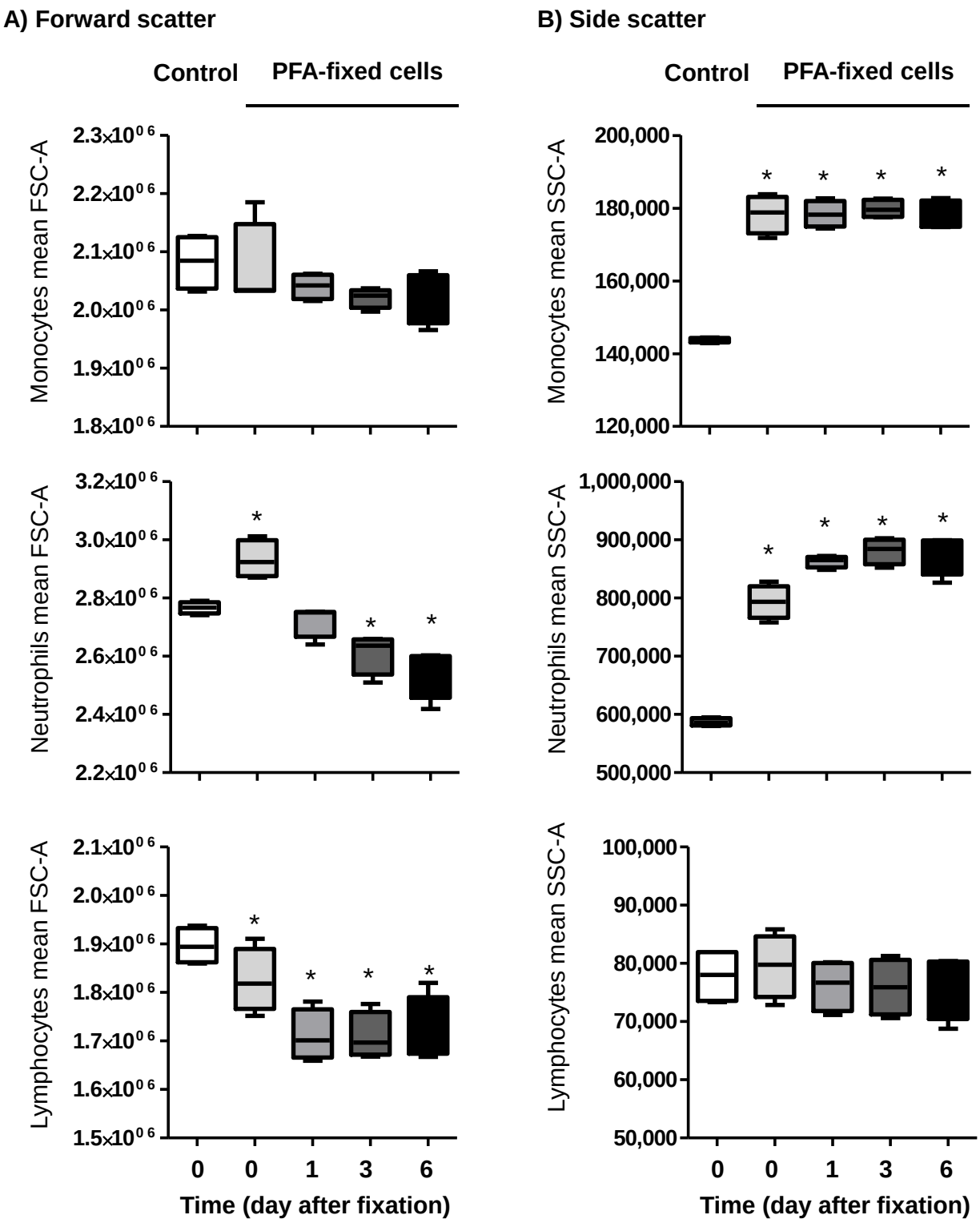

Figure 3. Changes in (**A**) forward scatter (FSC) and (**B**) side scatter (SSC) values for lymph node monocytes, neutrophils, and lymphocytes during 6 days after fixation. Lymph node cells were stained with mAbs to camel leukocyte antigens and analyzed by flow cytometry or were fixed with paraformaldehyde (PFA) and analyzed directly after fixation (d0) or on days 1, 3, and 6 after fixation. * indicated significant differences ($p < 0.05$).

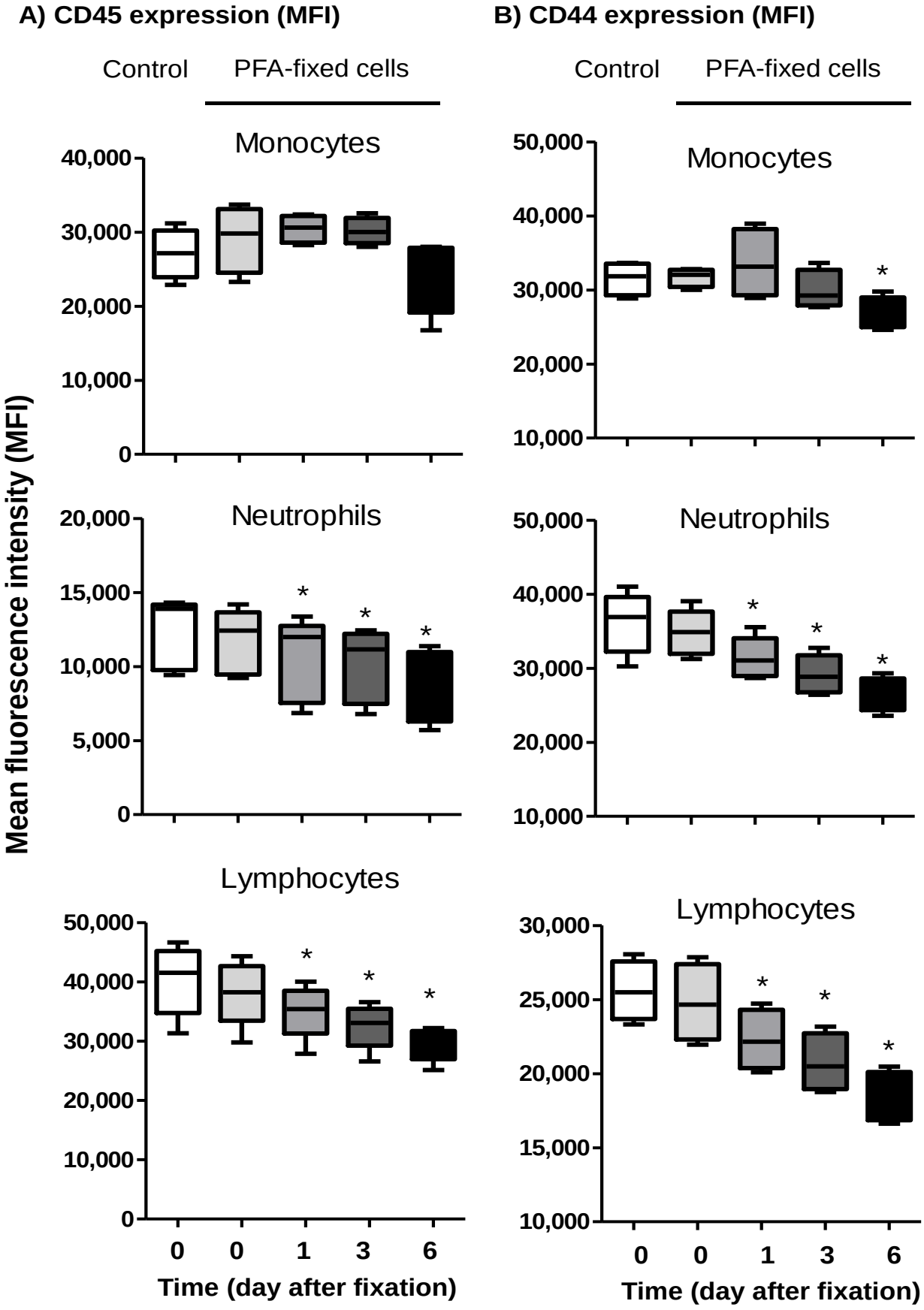

Figure 4. Changes in the abundance of CD45 (**A**) and CD44 (**B**) on lymph node monocytes, neutrophils, and lymphocytes during 6 days after fixation. Lymph node cells were stained with mAbs to CD45 and CD44 molecules and analyzed by flow cytometry or were fixed after staining with paraformaldehyde (PFA) and analyzed at day 0 (d0), d1, d3, and d6 after fixation. * indicated significant differences ($p < 0.05$).

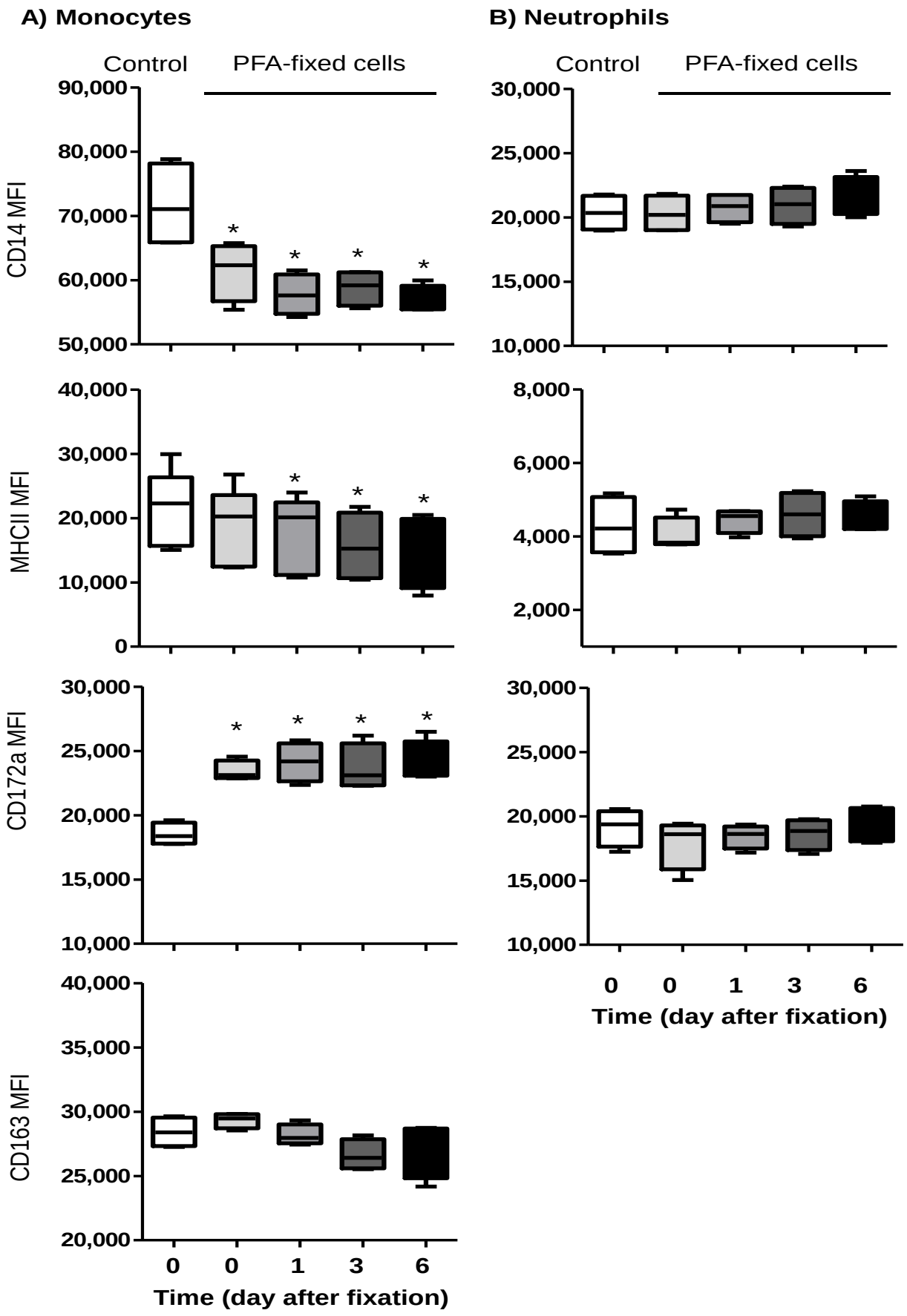

Figure 5. Fixation-induced changes in the expression levels of myeloid markers on lymph node monocytes (**A**) and neutrophils (**B**). Lymph node cells were stained with monoclonal antibodies(mAbs) to CD14, CD172a, MHCII, or CD163 molecules and analyzed by flow cytometry. Parallel set-ups were fixed after staining with paraformaldehyde (PFA) and analyzed at day 0 (d0), d1, d3, and d6 after fixation. * indicated significant differences ($p < 0.05$).

CONCLUSION

The obtained results of the study indicated that camel lymph node cell suspension stained with fluorochrome-conjugated mAbs to leukocyte antigens and fixed with PFA will keep stable values for the immune cell composition for at least six days after fixation. However, fixation may result in significant changes in the immunophenotype of monocytes, macrophages, and T cells.

DECLARATIONS

Acknowledgments

We thank Alomran Slauterhouse in Al-Ahsa, Saudi Arabia for providing the lymph node samples.

Authors' contributions

Mohammed Ali Al-Sukruwah did sample collection and manuscript revision. Hind Althagafi did sample analysis and original draft preparation. Najla Al Abdulsalam did study design and original draft preparation. Jamal Hussen designed the study, did flow cytometry and prepared original draft. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors have no competing interests to declare

Ethical considerations

The authors confirm that all authors have reviewed and submitted the manuscript to this journal for the first time.

Funding

This work was supported through the Annual Funding track (Project number: GRANT6,167) by the Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Al-Ahsa, Saudi Arabia.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author, [JH], upon reasonable request.

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