IMPACT OF CELL SEPARATION PROCEDURE ON THE IMMUNOPHENOTYPE OF DROMEDARY CAMEL PERIPHERAL BLOOD MONONUCLEAR CELLS AND TOTAL LEUKOCYTES

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ABSTRACT

The present study investigated the impact of the cell separation procedure and whole blood preservation on the viability and immunophenotype of camel peripheral blood mononuclear cells (PBMC) and total leukocytes. Mononuclear cells were separated by density gradient centrifugation, while total leukocytes were separated by hypotonic lysis of red blood cells. Cell apoptosis, necrosis, forward and side scatter properties, and cell staining patterns with monoclonal antibodies to selected immune cell markers were analyzed by flow cytometry. The results showed only low percentages of apoptotic and necrotic lymphocytes and monocytes within cells separated by both methods, indicating no impact of cell separation on viability. Similarly, comparable percentages of positively stained cells with monoclonal antibodies to the surface antigens CD45, CD44, MHC class I, CD11a, CD14, CD163, CD172a, CD4, BAQ44A, WC1, and MHC class II indicate no impact of the used cell separation methods on the immunophenotype of mononuclear cells. Subsequently, the next experiment was performed to see whether cryopreservation of camel whole blood followed by hypotonic lysis of red blood cells to separate total leukocytes would impact the viability or phenotype of PBMC. Although no impact of cryopreservation on total leukocyte count was observed, hypotonic lysis of cryopreserved blood for the separation of total leukocytes resulted in reduced viability of lymphocytes and monocytes with significant changes in their staining pattern with monoclonal antibodies. In conclusion, PBMC separated by density gradient centrifugation or total leukocytes separated by hypotonic lysis of fresh blood can be used for reliable identification of camel immune cell populations. However, using cryopreserved instead of fresh blood results in significant changes in cell viability and immunophenotype.

Keywords: Dromedary camel; lymphocytes; monocytes; flow cytometry; apoptosis; density gradient cell separation.

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INTRODUCTION

Peripheral blood represents an easily accessible sample source for the analysis of cellular immunity, with cells representative of both the innate and adaptive immune systems (Alexovic, Ulicna, Sabo, & Davalieva, 2024; Muller, Kroger, Schultze, & Aschenbrenner, 2024). Flow cytometric analysis of blood immune cell composition may be performed using whole blood samples, total leukocytes, or peripheral blood mononuclear cells (PBMC) (Dyikanov et al., 2024; Haider et al., 2022). In whole blood analysis, staining with antibodies to pan-leukocyte markers is required to exclude red blood cells from the analysis due to their dominance over white blood cells (Petriz, Bradford, & Ward, 2018). Alternatively, red blood cells are removed before or after staining with monoclonal antibodies (mAbs) to avoid their interference with the analysis (Connelly et al., 2022). Hypotonic lysis of red blood cells depends on short incubation of blood samples in distilled water followed by restoring the tonicity to keep the white blood cells intact (Li et al., 2018). Separation of blood mononuclear cells is another option and is performed by layering blood over the density solution followed by centrifugation at a specific speed and temperature (Fuss, Kanof, Smith, & Zola, 2009). In addition, it is not always applicable to analyze samples immediately after separation, and some samples therefore need to be preserved for later processing and analysis due to the limited availability of flow cytometry devices (Juhl, Christensen, Pedersen, Kastrup, & Ekblond, 2021). Therefore, several methods for the preservation of whole blood samples for flow cytometry were investigated in the literature (Braudeau et al., 2021; Rybakowska et al., 2021). The methods vary in terms of the required processing time, the need for specific preservative reagents, and the need for different preservation temperatures (Blackwell, Garcia, Keivanfar, & Bay, 2021; Braudeau et al., 2021; Serra et al., 2022). As recently reported, a similar immunophenotype of immune cells

was found when analyzing human blood stored at -80 ºC in a DMSO-based preservation medium and fresh blood samples (Serra et al., 2022).

After the identification of some mAbs with reactivity with the main cell surface markers of camel immune cells, several recent phenotyping and functional studies were undertaken for the characterization of the cellular immune compartment in camels. In most of these studies, immune staining was used in combination with flow cytometry for the characterization of cell populations and subpopulations in camel blood and tissues (J. Hussen, Althagafi, Al-Sukruwah, Falemban, & Abdul Manap, 2024; J. Hussen & Schuberth, 2020). For infection immunity studies in camels, monitoring the changes in immune cell composition and phenotype can be used for evaluating the immune response to infection or vaccination.

Because the impact of sample preparation on the flow cytometric analysis of viability and immunophenotype of camel blood mononuclear cells has not been investigated so far, the present study was undertaken to compare cell viability and reactivity with mAbs between mononuclear cells separated by density gradient centrifugation and total leukocytes separated by hypotonic lysis of red blood cells. In addition, the impact of whole blood preservation on PBMC immunophenotyping was investigated. We hypothesized that cell separation and cryopreservation differentially affect the viability and surface marker expression of dromedary camel immune cells. While such effects are well-documented in humans, dromedary camel leukocytes possess distinct structural properties, necessitating species-specific protocol validation.

MATERIALS AND METHODS

Animals and collection of blood samples: Blood samples (six for the first experiment and 12 for the second experiment) were collected from healthy dromedary camels (*Camelus dromedarius)* selected from the animals admitted at the Al-Omran Slaughterhouse in Al-Ahsa Region in Saudi Arabia. The animals included four males (two in each experiment) and 14 females with ages ranging from 5 to 9 years. Jugular vein blood was collected into EDTA-containing tubes and transported cooled within one hour to the laboratory.

Cryopreservation and thawing of whole blood samples: Blood cryopreservation was performed according to a previously described method (Serra et al., 2022). Briefly, blood was diluted in cryopreservation medium (Recovery™ Cell Culture Freezing Medium, Gibco by Life Technologies) in a 1 to 1 ratio (2 mL blood + 2 mL medium) in 15 mL tubes and kept at -80 ºC for 3 months. For sample recovery, the tubes were thawed by incubation in a water bath (37°C) for 5 min. Thawed blood samples were washed with PBS (1000 x g, 10 min) to remove the storage medium, followed by hypotonic lysis.

Hypotonic lysis of red blood cells and separation of total leukocytes: Leukocytes were separated from blood samples by removing red blood cells using hypotonic lysis (J. Hussen, 2021; Vuorte, Jansson, & Repo, 2001). This was performed by the addition of 5 mL distilled water for 20 seconds, followed by 5 mL of double-concentrated PBS to 2 mL blood (fresh or thawed samples for each animal). After washing by centrifugation (1000 x g, 10 min at 10ºC), the lysis step was repeated twice with centrifugation at 500 x g and 250 x g, for 10 min each, to remove the rest of red blood cells. Finally, the remaining leukocyte pellet was washed with PBS for 10 min at 100 x g and resuspended in PBS containing bovine serum albumin (0.5 %) and adjusted to 1 x 10^6 / mL.

Separation of peripheral blood mononuclear cells: Peripheral blood mononuclear cells (PBMC) were separated from fresh collected blood samples by density gradient centrifugation as previously described (J. Hussen, Al-Jabr, et al., 2023). This was done by diluting the blood sample (15 mL) in similar volume of cold PBS followed by carefully layering the mixture over 15 mL Lymphoprep™ (Containing Sodium Diatrizoate: 9.1% (w/v) and Polysaccharide: 5.7% (w/v) to achieve density of 1.077 g/mL; STEMCELL Technologies Inc. Vancouver, BC, Canada) in a sterile 50 mL falcon tube without mixing the blood with the Lymphoprep™. The tubes were then centrifuged immediately for 30 min at 1000 x g and 10ºC, without brake. Subsequently, the interphase was collected into a new tube, washed three times in PBS at 450, 200, and 125 x g (each round for 10 min), with brake. Finally, the cell pellet was suspended in PBS (2 x 10^6 cells/mL).

Analysis of cell viability: Viability of mononuclear cells separated by density gradient centrifugation or leukocytes separated by hypotonic lysis of blood was measured using the Annexin V-FITC Apoptosis Kit (Abcam; ab14085), according to the manufacturer's protocol. Briefly, cells were stained with Annexin V-FITC and propidium iodide (PI) diluted 1:100 in Kit buffer (100 µL /well), for 5 min at RT in the dark. The percentages of Annexin V+/PI- apoptotic cells, Annexin V+/ positive/PI+ necrotic cells, and Annexin V-/ PI- viable cells were calculated by flow cytometry (J. Hussen, Althagafi, Alalai, et al., 2024).

Staining with monoclonal antibodies: Separated PBMC or total leukocytes were labeled with mAbs to cell antigens followed by flow cytometric analysis (Table S1) (Jamal Hussen, Shawaf, Al-herz, Alturaifi, & Alluwaimi, 2018). The first staining step was performed by incubating the cells for 15 min at 4°C at a density of 1 x 10^6 cell/well in 96-well plates with mAbs to primary (unconjugated) mAbs against the cluster of differentiation (CD) CD45, CD44, CD11a, major histocompatibility (MHC) class-I, MHC class II, BAQ44A, WC1, CD4, CD172a, CD14, and CD163 (J. Hussen, 2021; J. Hussen, Alkuwayti, et al., 2023). After 15 min of incubation at 4 ºC, cells were washed twice with PBS/BSA buffer by centrifugation at 300 x g and 4ºC. Subsequently, the cell pellet was resuspended by shaking, and fluorochrome-labeled antibodies to mouse IgM, IgG1, and IgG2a (Invitrogen) diluted 1:100 with PBS/BSA buffer were added to the wells (10 µL /well). After a second incubation step for 15 min at 4ºC in the dark, a final wash was performed by adding 150 µL /well of PBS/BSA and centrifuging at 300 x g and 4ºC, the cells were analyzed using a flow cytometer (BD Accuri C6). A compensation matrix was generated using single-stained controls to correct for spectral overlap between detection channels. Furthermore, staining with appropriate isotype controls was used to ensure specific antibody binding. Single cells were identified by plotting FSC-A versus FSC-H to exclude doublets. Detailed antibody clone information and representative gating hierarchies are provided in Supplementary Table 1 and Supplementary Figures (Figure S1 and S2). Subsets of camel PBMC were identified based on their forward (FSC) and side scatter (SSC) properties. After gating on cell singlets in an FSC-H against FSC-A density plot, gates were set on lymphocytes and monocytes based on their FSC and SSC. Cell staining with the corresponding mAb was shown in a separate density plot after gating on the target cell population.

Statistical Analyses: Prism software (GraphPad) was used to calculate means and standard error of the mean (SEM) using the column statistic function. The Shapiro-Wilk test was used to test data normality. Because every animal contributed to both cell separation treatments (Hypotonic Lysis vs. Density Gradient Centrifugation in Experiment 1; Fresh vs. Cryopreserved in Experiment 2), paired Student's t-tests were utilized to evaluate differences between the two groups for all figures. To mitigate the increased risk of false-positive findings, p-values from the paired t-tests were adjusted using the Holm-Šídák method. An adjusted p-value less than 0.05 was considered indicative of statistical significance. Exact adjusted p-values, alongside 95% confidence intervals (CIs) and effect sizes (R squared) for all comparative analyses, are detailed in Supplementary Table 2.

RESULTS

Viability rates of dromedary camel mononuclear cells after density gradient centrifugation and hypotonic blood lysis:

Measurement of cell apoptosis and necrosis was performed using flow cytometry as shown in Figure 1A. Using hypotonic lysis (HL) of whole blood samples for separation of total leukocytes or density gradient centrifugation (DGC) for separation of PBMC resulted in similar ($p > 0.05$) percentages of viable cells within lymphocytes (97.3 for HL versus 96.7 for DGC) and monocytes (96.7 for HL versus 94.5 for DGC). For both lymphocytes and monocytes, there were no significant differences ($p > 0.05$) in the fraction of apoptotic cells or necrotic cells between the two procedures (Figure 1B).

Impact of cell separation procedure on forward and side scatter of camel lymphocytes and monocytes:

Cell side scatter (SSC) and forward scatter (FSC) are two important parameters that reflect shape change in cells being indicative of cell granularity and cell size, respectively. For both lymphocytes (100398 ± 2702 mean fluorescence intensity (MFI)) and monocytes (181976 ± 1689 MFI), hypotonic lysis of whole blood resulted in significantly ($p< 0.05$) higher SSC values compared to density gradient centrifugation (94163 ± 2916 MFI for lymphocytes and 175913 ± 1544 MFI for monocytes) (Figure 2A and B). In contrast, the FSC values were lower for cells separated by hypotonic lysis than by density gradient centrifugation. The difference was, however, significant only for lymphocytes (2025017 ± 19367 versus 2084535 ± 32974) ($p< 0.05$).

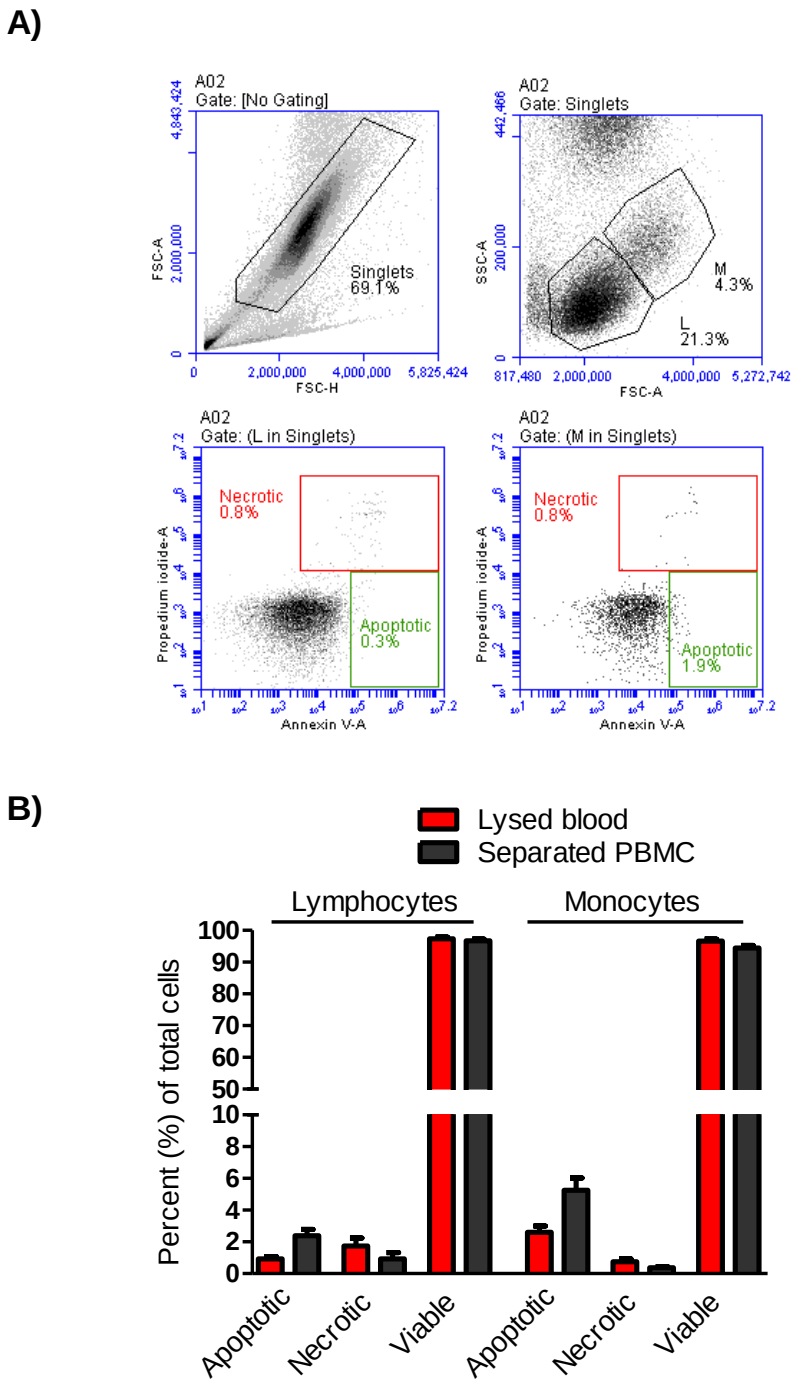

Figure 1. Analysis of apoptosis and necrosis. A) Total camel leukocytes (separated after hypotonic lysis of red blood cells) or camel PBMC (separated by density gradient centrifugation) were labeled with Annexin V and propidium iodide (PI). After gating on cell singlets in FSC-H against FSC-A density plot, gates were set on lymphocytes and monocytes based on their FSC and SSC properties. Apoptotic (Annexin+ / PI-) and necrotic cells (Annexin+ / PI+) were identified based on their staining for Annexin V and PI. B) The percentages of apoptotic, necrotic, and viable cells within lymphocytes and monocytes (mean ± sem; n = 6 camels; Paired t test with Holm-Šídák correction method).

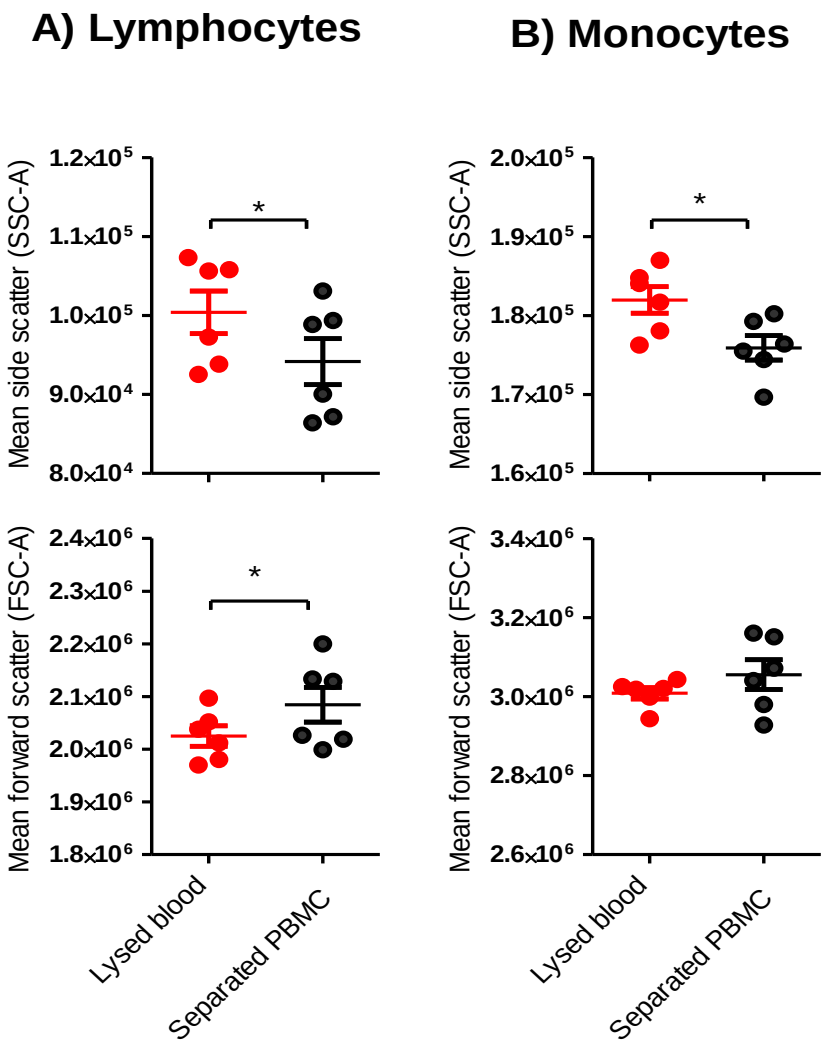

Figure 2. Forward and side scatter properties of monocytes and lymphocytes. Total camel leukocytes (separated after hypotonic lysis of red blood cells) or camel PBMC (separated by density gradient centrifugation) were analyzed by flow cytometry. The mean forward scatter (FSC) and side scatter (SSC) values of lymphocytes (A) and monocytes (B) were calculated and presented as scattered dot plots (mean ± sem; n = 6 camels). * Indicates a significant difference ($p < 0.05$).

Impact of cell separation procedure on the staining properties of camel mononuclear cells with monoclonal antibodies to selected cell surface antigens: For both cell separation procedures, most camel mononuclear cells (about 99%) stained positively with mAbs to the leukocyte marker antigens CD45, CD44, CD11a, and the major histocompatibility complex (MHC) class I, with no significant differences between cells separated by hypotonic lysis or density gradient centrifugation ($p > 0.05$) (Figure 3A). Similarly, staining with antibodies to CD14, CD163, and CD172a resulted in comparable fractions of positive cells ($p > 0.05$) between cells separated by hypotonic lysis or density gradient centrifugation (Figure 3A). In addition, the percentages of cells stained positively with antibodies to the lymphocyte marker antigens CD4, WC1, BAQ44A, MHCII, and CD11a (Supplementary Figure 2) revealed no significant differences in lymphocyte subset frequency between cells separated by hypotonic lysis and density gradient centrifugation ($p > 0.05$) (Figure 3B).

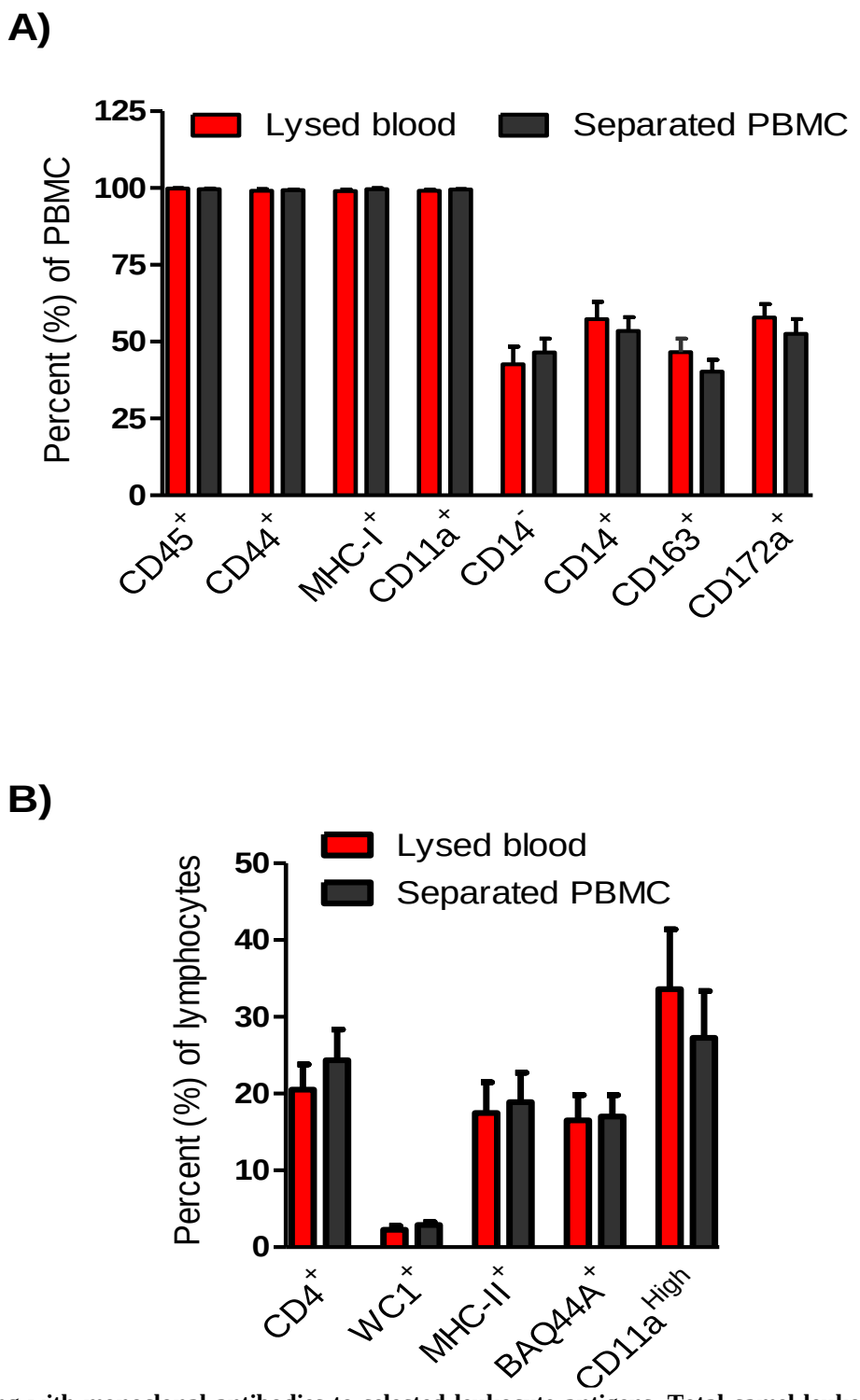

Figure 3. Staining with monoclonal antibodies to selected leukocyte antigens. Total camel leukocytes (separated after hypotonic lysis of red blood cells) or camel PBMC (separated by density gradient centrifugation) were stained with mAbs to cell surface markers and analyzed by flow cytometry. A) Percentages of PBMC with reactivity to mAbs to CD45, CD44, CD11a, MHC-I, CD14, CD172a, and CD163 (mean ± sem; n = 6 camels). B) Percentages of lymphocytes positive to CD4, WC1, BAQ44A, MHCII, and CD11a^{high} lymphocytes (mean ± sem; n = 6 camels; Paired t test with Holm-Šídák correction method).

Preservation of camel whole blood followed by hypotonic lysis induced changes in the viability of lymphocytes and monocytes: In this experiment, a comparison was made between fresh and preserved camel blood regarding cell count and the impact of hypotonic lysis on cell viability. While total leukocyte counts remained comparable between fresh and cryopreserved samples (Figure 4A), this metric does not reflect cellular vitality, which flow cytometric viability assays subsequently proved to be significantly compromised. For both lymphocytes and monocytes, cryopreservation of camel whole blood followed by leukocyte separation using hypotonic lysis of red blood cells resulted in a significant ($p < 0.05$) reduction in cell viability. For preserved blood samples, the percentage of apoptotic lymphocytes (10.6 ± 1.3 % of total cells) and monocytes (5.4 ± 0.9 % of total cells) were significantly higher than their percentages in fresh blood samples (0.9 ± 0.1 % of total cells for lymphocytes and 0.3 ± 0.05 % of total cells for monocytes). Similarly, in comparison to fresh blood samples, cryopreserved samples showed higher percentages of necrotic lymphocytes (8.0 ± 0.9 versus 1.9 ± 0.13) and monocytes (7.7 ± 1.1 versus 1.5 ±0.2) (Figure 4B).

Impact of whole blood preservation on the reactivity of camel mononuclear cells with monoclonal antibodies to selected cell surface antigens: In comparison to cells prepared from fresh blood, preservation of whole blood followed by separation of total leukocytes using hypotonic lysis of red blood cells resulted in significantly (adjusted $p < 0.05$) altered expression profiles across multiple markers. Specifically, preserved samples exhibited lower percentages of cells positively stained with mAbs to CD45 (89.8 ± 1.0 versus 99.1 ± 0.2 % of PBMC), CD44 (97.9 ± 1.0 versus 99.7 ± 0.2 % of PBMC), MHC class I (89.7 ± 1.6 versus 99.6 ±0.1 % of PBMC), and CD163 (13.6 ± 1.3 versus 19.3 ±1.3 % of PBMC) (Figure 5A). Conversely, no significant (adjusted $p > 0.05$) differences were observed between fresh collected and preserved blood samples regarding the percentage of cells reacted with antibodies to CD11a, CD14, and CD172a (Figure 5A).

In addition, significant differences in lymphocyte composition were observed between fresh and preserved blood samples. The percentages of lymphocytes stained positively with antibodies to the T helper cell marker CD4 (18.9 ± 1.5 versus 13.2 ± 1.4 % of lymphocytes) as well as the fraction of $CD11a^{high}$ lymphocytes (21.7 ± 2.1 versus 12.7 ± 1.4 % of lymphocytes) were significantly higher in fresh blood compared to preserved blood samples (Figure 5B). In contrast to this, preserved blood showed a higher percentage of cells positive for the γδ T cell marker WC1 (6.4 ± 0.6 versus 4.0 ± 0.5 % of lymphocytes), the B cell markers BAQ44A (18.9 ± 1.1 versus 14.1 ± 1.5 % of lymphocytes) and MHCII (33.3 ± 1.6 versus 26.0 ± 2.0 % of lymphocytes) (Figure 5B).

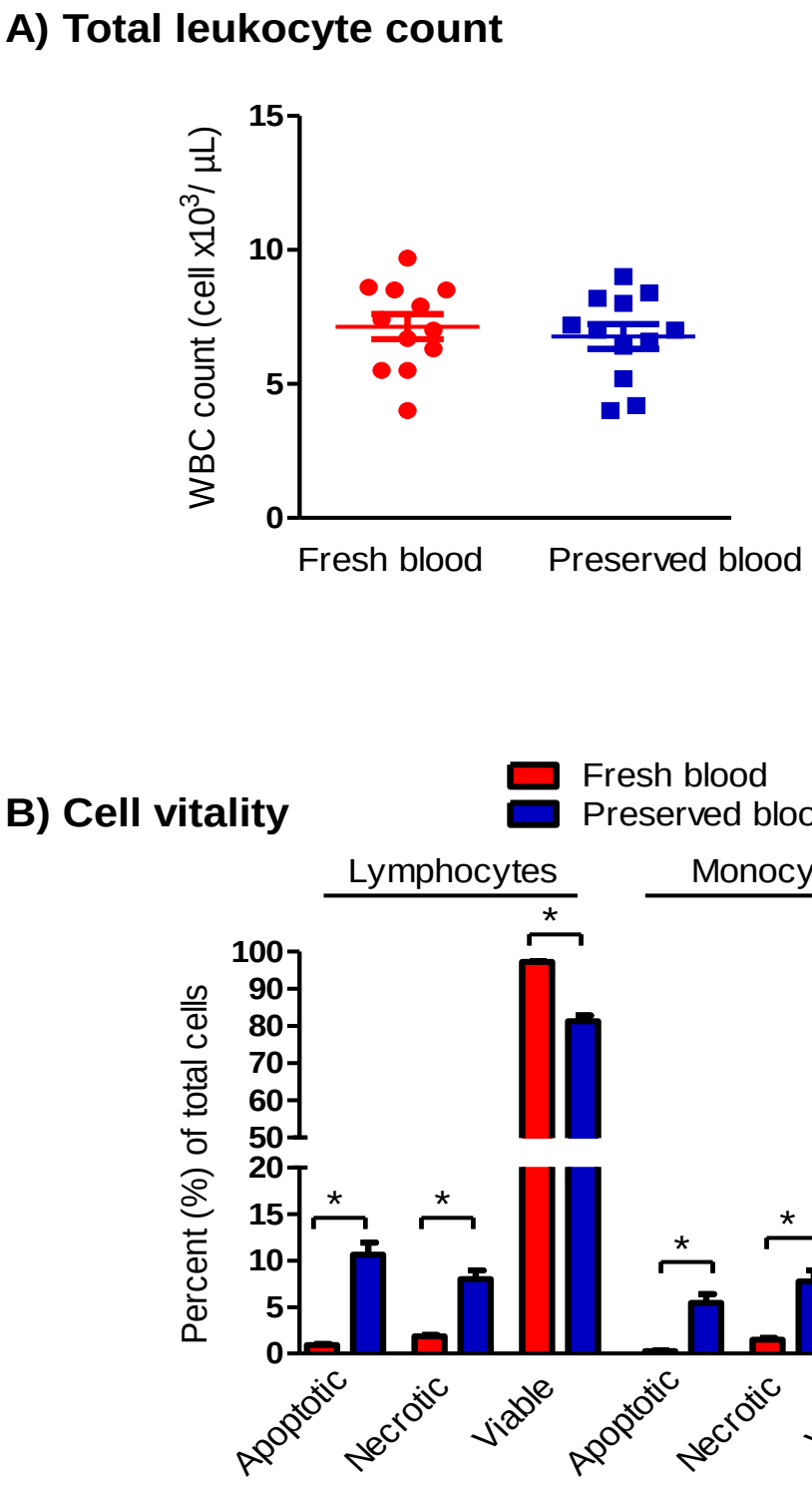

Figure 4. Impact of cryopreservation of whole blood on leukocyte count and cell viability of camel lymphocytes and monocytes. A) Total white blood cell count was determined by light microscopy using the Neubauer blood counter after addition of Turk solution to blood samples. B) Total leukocytes were separated from freshly collected or cryopreserved camel blood by hypotonic lysis of red blood cells. Separated cells were labeled with Annexin V and propidium iodide, and the percentages of apoptotic, necrotic, and viable cells within lymphocytes and monocytes were determined by flow cytometry and presented as mean ± sem (n = 12 camels; * indicates significant differences with p-value < 0.05; Paired t test with Holm-Šídák correction method).

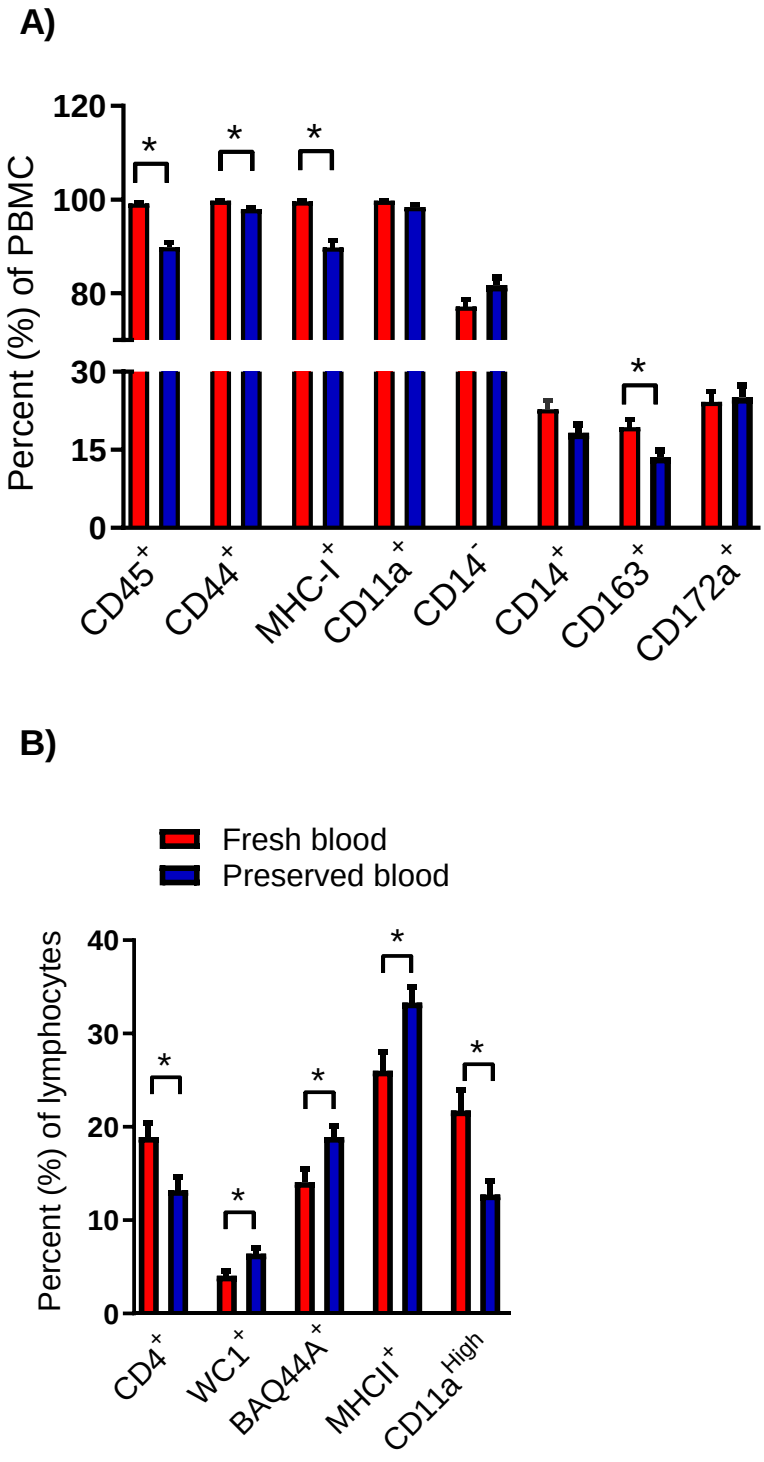

Figure 5. Staining camel leukocytes with monoclonal antibodies to selected leukocyte antigens. Total leukocytes were separated from freshly collected or cryopreserved camel blood by hypotonic lysis of red blood cells. Separated cells were labeled with mAbs to cell surface markers and analyzed by flow cytometry. A) Percentages of PBMC with reactivity to mAbs to CD45, CD44, CD11a, MHC-I, CD14, CD172a, and CD163 (mean ± sem; n = 12 camels). B) Percentages of lymphocytes positive to CD4, WC1, BAQ44A, MHCII, and CD11a^{high} lymphocytes (mean ± sem; n = 12 camels). All comparisons were made using Paired t test with Holm-Šídák correction method.

DISCUSSION

The analysis of immune cell dynamics represents one of the most important tools to explore the immune system in health and disease (Maecker, McCoy, & Nussenblatt, 2012; Robinson, Ostafe, Iyengar, Rajwa, & Fischer, 2023). The combination of immunofluorescence and flow cytometry enables the identification of changes in the composition and phenotype of peripheral blood mononuclear cells (PBMC) over the course of infection or inflammation (Perfetto, Chattopadhyay, & Roederer, 2004; Robinson et al., 2023). In comparison to human or many veterinary species, the characterization of camel immune cells is frequently limited by reagent availability. While human studies benefit from a vast, highly specific array of commercially available mAbs, camel immunophenotyping studies necessitate the use of cross-reactive antibodies directed against leukocyte antigens from other species.

To prepare the cells for flow cytometry, PBMC may be separated using density gradient centrifugation, analyzed in whole blood or in a white blood cell suspension prepared after removal of red blood cells. The present study investigated the impact of sample preparation on the viability and immunophenotype of camel PBMC.

The first experiment of the present study analyzed the impact of cell separation methods, density gradient centrifugation and hypotonic lysis of red blood cells, on the viability and reactivity of camel PBMC to antibodies against cell surface antigens. Cell viability analysis revealed minimal impact of both techniques on cell viability, with the major fractions of lymphocytes and monocytes being viable cells and only low percentages of apoptotic and necrotic cells. These results indicate that both techniques can be used for processing blood samples and the preparation of cell suspension for flow cytometry without affecting cell viability of PBMC.

Cell forward scatter (FSC) and side scatter (SSC), which indicate cell size and granularity, respectively, are important parameters that are employed in flow cytometry for the identification of cell populations based on their size and granularity (Bohmer, Bandala-Sanchez, & Harrison, 2011; McKinnon, 2018; Stanciu, Kwon, & Ehrhardt, 2016). In the present study, although the FSC and SSC of lymphocytes and monocytes were different between the two cell separation methods, this did not impact the identification of lymphocyte and monocyte cell populations based on their SSC and FSC characteristics. The significant differences observed in forward and side scatter properties following hypotonic lysis indicate morphological alterations, such as cellular swelling or degranulation (Cheon et al., 2025). While this study focused on viability and phenotypic stability, intact surface marker expression does not guarantee functional preservation. As cryopreservation is known to impact immune cell functions, future functional assays (e.g., proliferation, cytokine production) are necessary to fully validate the integrity of camel leukocytes.

Pan-leukocyte markers are cell surface molecules that are expressed on all immune cell populations and are, therefore, used for gating the whole leukocyte population in flow cytometry (Martini, Bernardi, Giordano, & Comazzi, 2020; van Dongen et al., 2012). For the dromedary camel, some mAbs that detect selected common leukocyte markers, including the protein tyrosine phosphatase CD45, the hyaluronic acid receptor CD44, the leukocyte function-associated antigen 1 (CD11a), and the major histocompatibility complex (MHC) class I are commercially available. On the other hand, lineage-specific markers such as the myeloid markers CD14, CD163, and CD172a and the lymphoid markers CD4 (T helper cells), BAQ44A (B2 cells), WC1 (γδ T cells), MHC class II molecules (B cells and monocytes) are used to identify myeloid and lymphoid cells and their functional subsets. In the present study, similar percentages of positively stained cells within PBMC indicate similar reactivity patterns of camel PBMC prepared by density gradient centrifugation or hypotonic lysis of whole blood with mAbs to the surface antigens CD45, CD44, MHC class I, CD11a, CD14, CD163, CD172a, CD4, BAQ44A, WC1, and MHC class II.

Collectively, the results of the first experiment indicate that both sample preparation procedures, the separation of PBMC by density gradient centrifugation and the separation of total leukocytes by hypotonic lysis of red blood cells, can be used for immunostaining of camel PBMC with comparable cell viability rate and immunophenotype.

Subsequently, the next experiment was performed to see whether cryopreservation of camel whole blood followed by hypotonic lysis of red blood cells to separate total leukocytes would impact the viability or phenotype of PBMC. The comparable leukocyte counts in freshly collected and cryopreserved blood samples indicate no impact of cryopreservation on leukocyte viability in camel. However, when cryopreserved blood was used for the separation of total leukocytes by hypotonic lysis, marked changes were observed in viability and immunophenotype of PBMC. Specifically, the higher proportion of apoptotic and necrotic PBMC, including both lymphocytes and monocytes, within cells prepared from cryopreserved blood indicates a negative effect of the combination of cryopreservation and hypotonic lysis on camel PBMC cell viability. In addition, the reduced fractions of cells positive for CD45, CD44, MHC class I, and CD163 within PBMC from preserved blood indicate significant changes in the immunophenotype of PBMC if leukocytes are separated from preserved blood. This effect is further supported by observed shifts in lymphocyte composition, characterized by reduced fractions of T helper cells and $CD11a^{high}$ lymphocytes, alongside increased proportions of B cells and WC1+ γδ T cells.

The profound loss of cell viability and alteration of immunophenotypic markers observed in cryopreserved camel leukocytes following hypotonic lysis can be attributed to synergistic osmotic and mechanical cellular stress (Zheng, Li, Shao, Li, & Song, 2024). One explanation for the observed changes in cell viability and phenotype in preserved camel blood could be a different tolerance of fresh and preserved cells to the hypotonic shock used to induce red blood lysis. Studies on human blood, where commercial lysis buffers with company-specific composition were used, showed better stability in immunophenotype after hypotonic lysis (Serra et al., 2022). Future studies may focus, therefore, on the comparison of different lysis buffers for their performance to prepare total leukocytes from preserved camel blood. Additionally, as the freezing rate of the samples was not controlled, which is a limitation of the current study, we cannot exclude a role of this factor in the obtained results. Furthermore, combined osmotic and hypothermic trauma has been found to be associated with the activation of stress-induced proteases leading to the cleavage of key surface diagnostic receptors. This may explain the significant phenotypic reductions in markers like CD45 observed via flow cytometry.

A limitation of the current study design is that the experimental design did not allow for the definitive uncoupling of cryopreservation-induced damage from lysis-induced damage. Specifically, there was no cryopreserved group processed without hypotonic lysis (due to the technical challenge of removing camel red blood cells without hypotonic lysis), nor was there a density gradient centrifugation arm for cryopreserved whole blood (due to the technical challenges of altered cellular buoyancy and hemolysis typically observed when centrifuging previously frozen whole blood over a density gradient). Additionally, there was a lack of baseline viability assessment immediately post-thaw but before lysis. Consequently, the observed reduction in viability and marker expression reflects the combined, synergistic damage of cryopreservation followed by acute hypotonic shock, rather than the isolated effects of either procedure alone. Additionally, it is important to note that cells separated by density gradient centrifugation include only mononuclear cells, whereas hypotonic lysis yields total leukocytes, including granulocytes. Although granulocytes were present in the lysed samples, our study focused on mononuclear populations (lymphocytes and monocytes) by gating on them based on forward and side scatter properties to allow direct comparison with the density gradient-isolated PBMCs. Future studies should incorporate granulocyte-specific functional markers to assess this distinct population.

Conclusions: In conclusion, PBMCs separated by density gradient centrifugation or total leukocytes separated by hypotonic lysis of fresh blood can be used for reliable identification of camel immune cell populations. On the other hand, using cryopreserved instead of fresh blood results in significant changes in cell viability and immunophenotype. As assessing apoptosis and cell percentages is insufficient to confirm complete functional preservation of immune cells, further studies are required to include functional assays such as cell proliferation, migration, and cytokine production.

Author Contributions: HA and JH designed the study, analyzed the samples, interpreted the data, critically revised the manuscript for important intellectual content, and approved the final version.

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Competing Interests: The authors have no relevant financial or non-financial interests to disclose

Data Availability: The datasets generated during the current study are available from the corresponding author on reasonable request.

Ethics approval: Animal sampling was conducted in accordance with the ARRIVE guidelines. Approval was granted by the Ethics Committee of King Faisal University, Saudi Arabia (KFU-REC-2024-JUN-ETHICS1843).

REFERENCES

Alexovic, M., Ulicna, C., Sabo, J., & Davalieva, K. (2024). Human peripheral blood mononuclear cells as a valuable source of disease-related biomarkers: Evidence from comparative proteomics studies. *Proteomics Clin. Appl., 18*(2), e2300072. doi:10.1002/prca.202300072

Blackwell, A. D., Garcia, A. R., Keivanfar, R. L., & Bay, S. (2021). A field method for cryopreservation of whole blood from a finger prick for later analysis with flow cytometry. *Am. J. Phys. Anthropol., 174*(4), 670-685. doi:10.1002/ajpa.24251

Bohmer, R. M., Bandala-Sanchez, E., & Harrison, L. C. (2011). Forward light scatter is a simple measure of T-cell activation and proliferation but is not universally suited for doublet discrimination. *Cytometry A., 79*(8), 646-652. doi:10.1002/cyto.a.21096

Braudeau, C., Salabert-Le Guen, N., Chevreuil, J., Rimbert, M., Martin, J. C., & Josien, R. (2021). An easy and reliable whole blood freezing method for flow cytometry immuno-phenotyping and functional analyses. *Cytometry B Clin. Cytom., 100*(6), 652-665. doi:10.1002/cyto.b.21994

Cheon, H., Kumar, S., Lee, I., Shin, S., Jang, H., Lee, Y. S., Seo, S. (2025). Multi-Channel Cellytics for Rapid and Cost-Effective Monitoring of Leukocyte Activation. *Biosensors (Basel), 15*(3). doi:10.3390/bios15030143

Connelly, A. N., Huijbregts, R. P. H., Pal, H. C., Kuznetsova, V., Davis, M. D., Ong, K. L., Hel, Z. (2022). Optimization of methods for the accurate characterization of whole blood neutrophils. *Sci. Rep., 12*(1), 3667. doi:10.1038/s41598-022-07455-2

Dyikanov, D., Zaitsev, A., Vasileva, T., Wang, I., Sokolov, A. A., Bolshakov, E. S., Goldberg, M. F. (2024). Comprehensive peripheral blood immunoprofiling reveals five immunotypes with immunotherapy response characteristics in patients with cancer. *Cancer Cell, 42*(5), 759-779 e712. doi:10.1016/j.ccell.2024.04.008

Fuss, I. J., Kanof, M. E., Smith, P. D., & Zola, H. (2009). Isolation of whole mononuclear cells from peripheral blood and cord blood. *Curr. Protoc. Immunol., Chapter 7*, 711-718. doi:10.1002/0471142735.im0701s85

Haider, P., Hoberstorfer, T., Salzmann, M., Fischer, M. B., Speidl, W. S., Wojta, J., & Hohensinner, P. J. (2022). Quantitative and Functional Assessment of the Influence of Routinely Used Cryopreservation Media on Mononuclear Leukocytes for Medical Research. *Int. J. Mol. Sci., 23*(3). doi:10.3390/ijms23031881

Hussen, J. (2021). Changes in Cell Vitality, Phenotype, and Function of Dromedary Camel Leukocytes After Whole Blood Exposure to Heat Stress in vitro. *Front. Vet. Sci., 8*, 647609. doi:10.3389/fvets.2021.647609

Hussen, J., Al-Jabr, O. A., Alkuwayti, M. A., Alrabiah, N. A., Falemban, B., Alouffi, A., Desquesnes, M. (2023). A Flow Cytometry Study of the Binding and Stimulation Potential of Inactivated Trypanosoma evansi toward Dromedary Camel Leukocytes. *Pathogens, 13*(1). doi:10.3390/pathogens13010021

Hussen, J., Alkuwayti, M. A., Falemban, B., Alhojaily, S. M., Adwani, S. A., Hassan, E. A. E., & Al-Mubarak, A. I. (2023). Impact of Selected Bacterial and Viral Toll-like Receptor Agonists on the Phenotype and Function of Camel Blood Neutrophils. *Vet. Sci., 10*(2). doi:10.3390/vetsci10020154

Hussen, J., Althagafi, H., Al-Sukruwah, M. A., Falemban, B., & Abdul Manap, A. S. (2024). Flow cytometric analysis of immune cell populations in the bronchial and mesenteric lymph nodes of the dromedary camel. *Front. Vet. Sci., 11*, 1365319. doi:10.3389/fvets.2024.1365319

Hussen, J., Althagafi, H., Alalai, M. A., Alrabiah, N. A., Al Abdulsalam, N. K., Falemban, B., Hebert, L. (2024). Surra-affected dromedary camels show reduced numbers of blood B-cells and in vitro evidence of Trypanosoma-induced B cell death. *Trop. Anim. Health Prod., 56*(7), 223. doi:10.1007/s11250-024-04078-9

Hussen, J., & Schuberth, H. J. (2020). Recent Advances in Camel Immunology. *Front. Immunol., 11*, 614150. doi:10.3389/fimmu.2020.614150

Hussen, J., Shawaf, T., Al-herz, A. I., Alturaifi, H. R., & Alluwaimi, A. M. (2018). Expression Patterns of Cell Adhesion Molecules on CD4+ T Cells and WC1+ T Cells in the Peripheral Blood of Dromedary Camels. *Pak. Vet. J., 38*(3). DOI: 10.29261/pakvetj/2018.055

Juhl, M., Christensen, J. P., Pedersen, A. E., Kastrup, J., & Ekblond, A. (2021). Cryopreservation of peripheral blood mononuclear cells for use in proliferation assays: First step towards potency assays. *J. Immunol. Methods, 488*, 112897. doi:10.1016/j.jim.2020.112897

Li, X., Tao, Y., Liu, J., Lu, W., Liu, H., & Dai, J. (2018). Optimized protocol of RBCs lysis for immunophenotypic analysis in the peripheral blood of tree shrew. *Acta. Biochim. Biophys. Sin. (Shanghai), 50*(4), 428-431. doi:10.1093/abbs/gmy008

Maecker, H. T., McCoy, J. P., & Nussenblatt, R. (2012). Standardizing immunophenotyping for the Human Immunology Project. *Nat. Rev. Immunol., 12*(3), 191-200. doi:10.1038/nri3158

Martini, V., Bernardi, S., Giordano, A., & Comazzi, S. (2020). Flow cytometry expression pattern of CD44 and CD18 markers on feline leukocytes. *J. Vet. Diagn. Invest., 32*(5), 706-709. doi:10.1177/1040638720945670

McKinnon, K. M. (2018). Flow Cytometry: An Overview. *Curr. Protoc. Immunol., 120*, 511-5111. doi:10.1002/cpim.40

Muller, S., Kroger, C., Schultze, J. L., & Aschenbrenner, A. C. (2024). Whole blood stimulation as a tool for studying the human immune system. *Eur. J. Immunol., 54*(2), e2350519. doi:10.1002/eji.202350519

Perfetto, S. P., Chattopadhyay, P. K., & Roederer, M. (2004). Seventeen-colour flow cytometry: unravelling the immune system. *Nat Rev Immunol, 4*(8), 648-655. doi:10.1038/nri1416

Petriz, J., Bradford, J. A., & Ward, M. D. (2018). No lyse no wash flow cytometry for maximizing minimal sample preparation. *Methods, 134-135*, 149-163. doi:10.1016/j.ymeth.2017.12.012

Robinson, J. P., Ostafe, R., Iyengar, S. N., Rajwa, B., & Fischer, R. (2023). Flow Cytometry: The Next Revolution. *Cells, 12*(14). doi:10.3390/cells12141875

Rybakowska, P., Burbano, C., Van Gassen, S., Varela, N., Aguilar-Quesada, R., Saeys, Y., Maranon, C. (2021). Stabilization of Human Whole Blood Samples for Multicenter and Retrospective Immunophenotyping Studies. *Cytometry A., 99*(5), 524-537. doi:10.1002/cyto.a.24241

Serra, V., Orru, V., Lai, S., Lobina, M., Steri, M., Cucca, F., & Fiorillo, E. (2022). Comparison of Whole Blood Cryopreservation Methods for Extensive Flow Cytometry Immunophenotyping. *Cells, 11*(9). doi:10.3390/cells11091527

Stanciu, C. E., Kwon, Y. J., & Ehrhardt, C. J. (2016). Forward-scatter and side-scatter dataset for epithelial cells from touch samples analyzed by flow cytometry. *Data Brief, 6*, 416-418. doi:10.1016/j.dib.2015.12.027

van Dongen, J. J., Lhermitte, L., Bottcher, S., Almeida, J., van der Velden, V. H., Flores-Montero, J., EuroFlow, C. (2012). EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. *Leukemia, 26*(9), 1908-1975. doi:10.1038/leu.2012.120

Vuorte, J., Jansson, S. E., & Repo, H. (2001). Evaluation of red blood cell lysing solutions in the study of neutrophil oxidative burst by the DCFH assay. *Cytometry, 43*(4), 290-296. PMID: 11260596. https://doi.org/10.1002/1097-0320(20010401)43:4<290::AID-CYTO1061>3.0.CO;2-X

Zheng, S., Li, Y., Shao, Y., Li, L., & Song, F. (2024). Osmotic Pressure and Its Biological Implications. *Int. J. Mol. Sci., 25*(6). doi:10.3390/ijms25063310