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Flow Cytometric Assessment of the Influence of Cryopreservation on Vitality, Recovery and Immunophenotype of Camel Peripheral Blood Mononuclear Cells

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ABSTRACT

Background: Immunophenotyping has been proven a valuable tool in diagnostic and research immunology laboratories to evaluate the immune status in health and disease. However, in some cases, cells cannot be analyzed immediately after separation and must be stored for later analysis. The present study was undertaken to investigate the impact of cryopreservation of camel PBMC on their vitality, recovery, shape, staining patterns with monoclonal antibodies and lymphocyte composition.

Methods: Flow cytometry was employed to analyze cell apoptosis, necrosis, shape change, staining patterns with mAbs and cellular composition of freshly separated and cryopreserved camel PBMC.

Result: The results showed similar lymphocytes and monocytes absolute numbers with comparable percentages of apoptotic and necrotic cells between fresh and preserved PBMC indicating no significant impact of cryopreservation on the recovery of camel PBMC. Although a cryopreservation-induced shape change was identified for camel lymphocytes, this change did not interfere with the identification of cryopreserved lymphocytes based on their SSC and FSC characteristics. In addition, the reactivity of camel PBMC with mAbs to the cell markers antigens CD45, CD44, CD11a, MHC-I, CD14, CD163 and CD172a remained unchanged for cryopreserved cells. However, mAbs to MHC-II and BAQ44A displayed modified binding to cryopreserved PBMC leading to significant changes in the positively stained B cells and increased MHC-II expression on monocytes. In conclusion, the results of the current study show that storing camel PBMC in cryopreservation media at -80 is an appropriate procedure for preserving the epitopes for all the used mAbs with modified binding to B cell markers.

Key words: Apoptosis, B cells, Camel, Flow cytometry, Monocytes, PBMC, T Cells.

INTRODUCTION

Peripheral blood mononuclear cells (PBMC), including cells of the innate and adaptive immune system, represent a valuable sample source for the evaluation of the immune system in health and disease (Pourahmad and Salimi, 2015; Alexovic *et al*., 2024). The major cell subpopulations that fall within the PBMC population include blood monocytes in addition to the lymphoid cells alpha beta ($\alpha\beta$) T cells, gamma delta (gd)T cells, B cells (Ambade *et al*., 2023; Shitikova *et al*., 2024). Immunophenotyping of PBMC using flow cytometry and fluorescent antibodies represents an essential procedure in diagnostic and research immunology laboratories (Heubeck *et al*., 2023; Preglej *et al*., 2023). The technique enables the simultaneous measurement of cell *via*bility, cell count, shape change and marker antigen expression (Rawat *et al*., 2019; Huang *et al*., 2024). In addition, several innate and adaptive functions, such as phagocytosis, proliferation and cytokine production could be analyzed using this single-cell analytic methodology (Ammann *et al*., 2020). However, due to the limited availability of flow cytometry devices because of their expensive prices, samples cannot always be analyzed immediately after separation and need therefore to be stored for later analysis in core facility laboratories (Juhl *et al*., 2021). In addition, in long-time clinical trials collecting and storing samples to be analyzed under the same conditions is usually advantageous to minimize variation in test results.

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Previous studies reported using several cell storage procedures for the preservation of PBMC for later analysis by flow cytometry (Hope *et al*., 2021; Browne *et al*., 2024). This includes storage of fixed unstained cells, fixed and stained cells, or unfixed and unstained cells (Pinto *et al*. 2005). For several species, cryopreservation of PBMC directly after their separation has been proven an effective procedure for storing cells and preserving their epitopes for later staining with mAbs and by flow cytometry (Li *et al*., 2021;

Efthymiou *et al*., 2022). In a recent study, (Hong *et al*. 2017) reported that storing human PBMC at -80°C in a DMSO-based cryoprotectant medium followed by thawing at 37°C is associated with high cell viability and recovery.

As no preservation methods have been investigated for storing camel PBMC for later analysis by flow cytometry, the present study was undertaken to comparatively analyze cell apoptosis, necrosis, shape change, staining patterns with monoclonal antibodies (mAbs) and cellular composition of freshly separated and cryopreserved camel PBMC.

MATERIALS AND METHODS

Collection of blood samples from camels

Blood samples were collected from nine dromedary camels (*Camelus dromedarius*. The camels were healthy camels selected from the animals admitted during February 2024 for normal slaughtering at Al-Omran Slaughterhouse (Al-Ahsa, Eastern Province, Saudi Arabia). The animals included five males and four females with ages ranging from 4 to 8 years (mean age 6.1±0.5). Blood (30 mL per animal) was collected from the jugular vein in EDTA-containing tubes. Collected blood samples were kept refregrated and transported within one hour to the Immunology Laboratory at King Faisal University for further processing.

Separation and cryopreservation of blood mononuclear cells

Separation of peripheral blood mononuclear cells (PBMC) was performed within one hour after blood collection. For this, 15 mL camel blood was diluted in 15 mL cold PBS and the mixture was carefully overlaid on 15 mL Lymphoprep™ (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 at 800 ×g for 30 min at 10°C. After centrifugation, the PBMC-containing interphase between the Lymphoprep™ and the plasma layers was collected into new sterile 50 mL tubes. Separated PBMC were washed in three rounds with cold PBS at 400, 200 and 100 ×g, each for 10 min to remove platelets. In the last centrifugation step, the cell suspension was divided into two parts in two 15 mL tubes. The washed cell pellet in one tube was suspended in cold RPMI 1640, adjusted to 2.1 × 10^6 cells/mL and further processed for flow cytometry (Hussen *et al*., 2024).

The pellet in the other tube was suspended in (2°C to 8°C) cooled cryopreservation medium (Recovery™ Cell Culture Freezing Medium, gibco by Life Technologies), adjusted to 2.1 × 10^6 cell/mL, dispensed in 1.5 mL cryo*vial*s and kept at -80°C until analysis. For cell recovery, the tubes were thawed by incubation in a water bath (37°C) for 5 min. Thawed cells were washed two times (300×g for 10 min each) with pre-warmed RPMI 1640 and finally suspended in RPMI medium.

Analysis of cell vitality

The percentage of apoptotic, necrotic and viable cells within fresh and preserved camel PBMC were measured by flow cytometry (Hussen *et al*., 2023b) using the Annexin V-FITC Apoptosis Kit according to the kit protocol (Abcam; ab14085). The washed cell pellet was stained in cell culture plates (96-wells) 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. Cells were classified upon excitation at 488 nm into Annexin V+/PI- apoptotic cells, Annexin V+/ positive/PI+ necrotic cells and Annexin V-/ PI- viable cells.

Analysis of shape change

Shape change in camel PBMC was measured by calculating the change in side scatter (SSC) area, indicating cell granularity and forward scatter (FSC) area, indicating cell size by flow cytometry (Hussen *et al*., 2023a).

Table 1: List of antibodies.

Antigen	Antibody clone	Target species	Labeling	Source	Isotype
CD45	LT12A	Llama	-	Kingfisher	Mouse IgG2a
CD44	LT41A	Llama	-	Kingfisher	Mouse IgG2a
CD11a	HUH73A	Goat	-	Kingfisher	Mouse IgG1
MHC-I	H58A	Cattle	-	Kingfisher	Mouse IgG2a
CD14	CAM36A	Camel	-	Kingfisher	Mouse IgG1
MHC-II	TH81A5	Pig	-	Kingfisher	Mouse IgG2a
CD172a	DH59b	Cattle		Kingfisher	Mouse IgG1
CD163	LND68A	Cattle	-	Kingfisher	Mouse IgG1
CD4	GC50A1	Cattle	-	VMRD,	Mouse IgM
WC1	BAQ128A	Cattle	-	VMRD,	Mouse IgG1
B cell	BAQ44A	Cattle	-	Kingfisher	Mouse IgM
Mouse IgM	poly	Mouse	APC	Thermofisher	Goat IgG
Mouse IgG1	poly	Mouse	FITC	Thermofisher	Goat IgG
Mouse IgG2a	poly	Mouse	PE	Thermofisher	Goat IgG

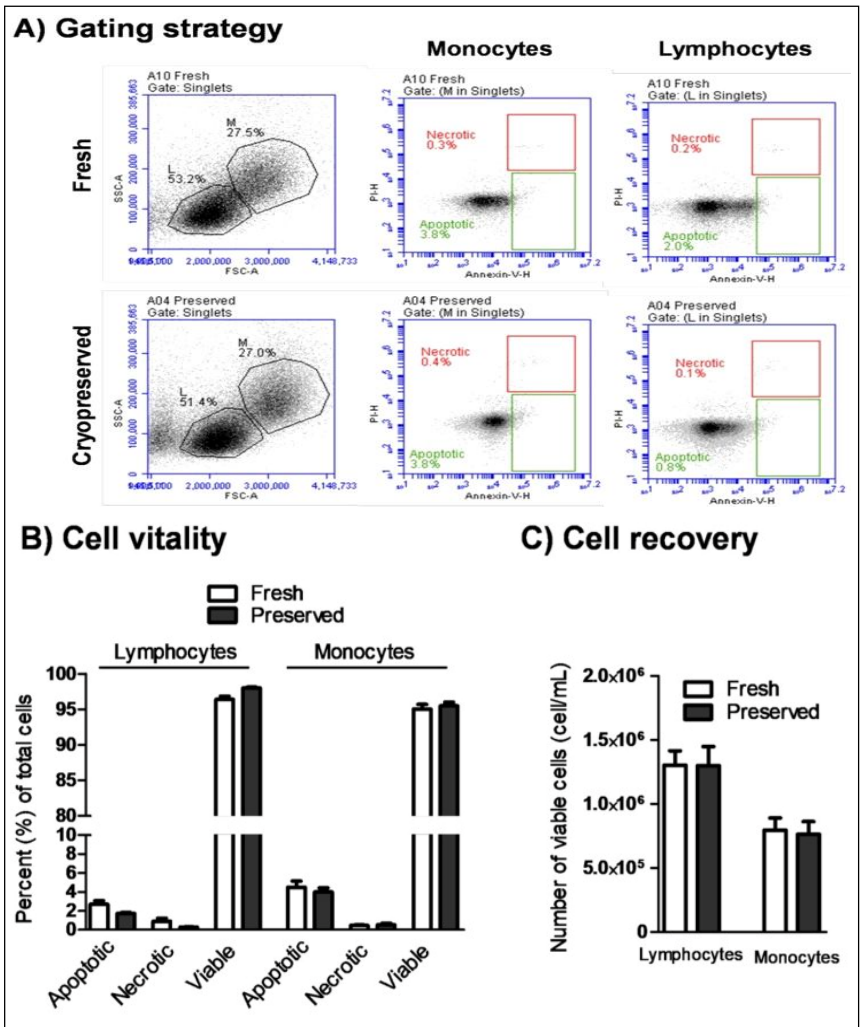
MHC: Major Histocompatibility Complex; WC1: Workshopcluster 1; APC: Allophycocyanin; FITC: Fluorescein isothiocyanate; PE: Phycoerythrin; poly: polyclonal.

Membrane immunofluorescence

Fresh separated and cryopreserved camel PBMC were labeled with monoclonal antibodies (Table 1) to camel leukocyte antigens and analyzed by flow cytometry (Hussen *et al*., 2018). Cells (1×10^6 cell/well) were incubated in the wells of a 96-well plate with mAbs to camel antigens: a cluster of differentiation (CD)45, CD44, CD11a, major histocompatibility (MHC) class-I, MHC-II, BAQ44A, workshop cluster (WC)1, CD4, CD172a, CD14 and CD163 (Hussen, 2021; Hussen *et al*., 2023b). After incubation (15 min; 4°C), cells were washed twice and were incubated with mouse secondary antibodies (IgM, IgG1, IgG2a; Invitrogen) labeled with different fluorochromes or with mouse isotype control antibodies (Becton Dickinson Biosciences). After washing, cells were 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

The software program C-Flow (BD) was used for the analysis of flow cytometric data. Surface molecule abundance was calculated using the mean fluorescence intensity of the corresponding cell type and molecule.



A) Fresh separated and cryopreserved camel PBMC were labeled in vitro with Annexin V and propidium iodide (PI). After excluding cell duplets, Annexin V against PI dot plots were used for the identification of apoptotic (Annexin+ / PI-) and necrotic (Annexin+ / PI+) cells within lymphocytes and monocytes (gates were done based on FSC and SSC properties. B) The percentages of apoptotic, necrotic and *via*ble cells within lymphocytes or monocytes were calculated and presented as mean ± sem. C) The absolute numbers of *via*ble lymphocytes and monocytes within fresh separated and cryopreserved PBMC (n = 9 animals) were counted per mL medium on the flow cytometer and cell counts were presented as mean ± standard error of mean (sem).

Fig 1: Analysis of cell vitality.

Means and standard error of the mean (SEM) were calculated using the column statistic function of the Prism software (GraphPad). Data normality was calculated using the Shapiro-Wilk test. For the comparison between fresh separated and cryopreserved cells, the Student's t-test (paired t-test) was performed; and the differences were considered significant if the p-value was less than 0.05. The correlation between molecule abundance on fresh and preserved cells, the linear regression function of GraphPad Prism was performed and the R Square was calculated.

RESULTS AND DISCUSSION

Impact of cryopreservation on cell vitality of camel mononuclear cells

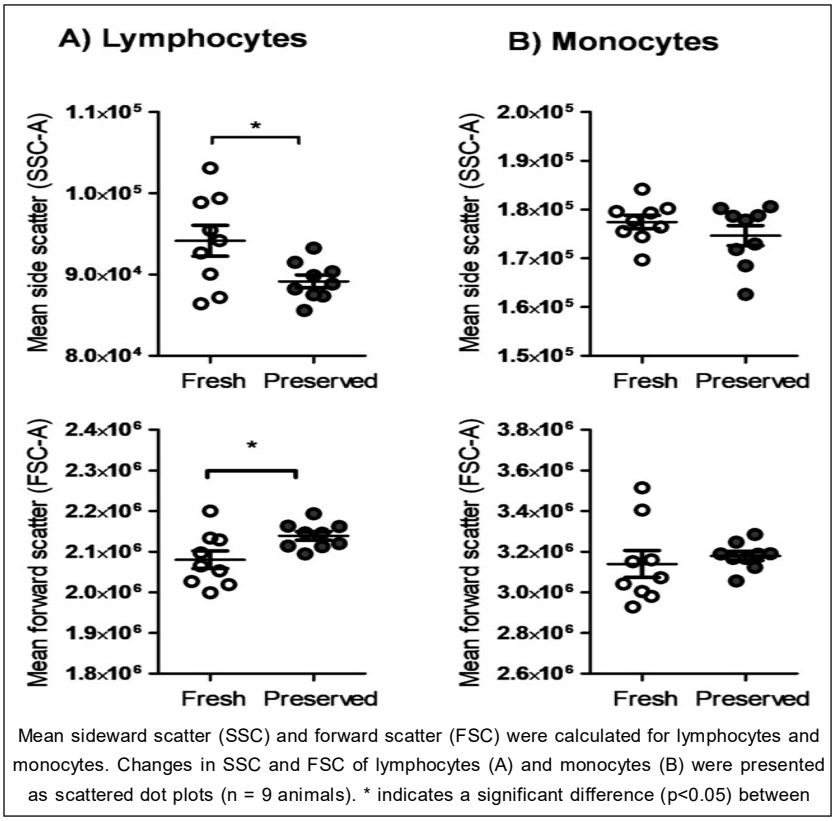
Cell apoptosis and necrosis of camel PBMC were analyzed using flow cytometry (Fig 1A). The comparison between fresh separated and cryopreserved cells revealed comparable percentages of apoptotic, necrotic and *via*ble cells within camel blood lymphocytes and monocytes (Fig 1B). The absolute numbers of *via*ble lymphocytes ($1.30\times10^6 \pm 0.11$ cell/mL) and monocytes ($0.79 \times 10^6 \pm 0.09$ cell/mL) within fresh PBMC showed no significant differences ($p > 0.05$) to the numbers of *via*ble lymphocytes ($1.30 \times 10^6 \pm 0.15$ cell/mL) and monocytes ($0.76 \times 10^6 \pm 0.09$ cell/mL) within cryopreserved PBMC (Fig 1C).

Shape change of cryopreserved camel mononuclear cells

Cryopreserved lymphocytes showed significantly ($p < 0.05$) reduced side scatter (SSC; $8.92 \times 10^4 \pm 0.08$) values, and increased ($p<0.05$) forward scatter (FSC; $21.39 \times 10^5 \pm 0.10$) values (Fig 2A) in comparison to the SSC ($9.41 \times 10^4 \pm 0.19$) and FSC ($20.80 \times 10^5 \pm 0.22$) values of freshly separated lymphocytes. For monocytes, however, both SSC and FSC parameters did not show significant ($p>0.05$) differences between fresh and cryopreserved cells (Fig 2B).

Impact of cryopreservation on the staining pattern of camel mononuclear cells with monoclonal antibodies (mAbs)

Monoclonal antibodies (mAbs) to the pan-leukocyte marker CD45, the cell adhesion molecules CD44 and CD11a as well as the major histocompatibility complex (MHC) class stained all camel PBMC positively with no significant differences in the percentage of stained cells between fresh and preserved PBMC (Fig 3 A, B). Similarly, the percentages of CD14-negative cells (lymphocytes), CD14-positive cells (monocytes), CD163-positive cells (monocyte subset) and CD172a-positive cells (myeloid cells) within PBMC did not differ significantly ($p>0.05$) between fresh and preserved cells (Fig 3 A, B).



Mean sideward scatter (SSC) and forward scatter (FSC) were calculated for lymphocytes and monocytes. Changes in SSC and FSC of lymphocytes (A) and monocytes (B) were presented as scattered dot plots (n = 9 animals). * indicates a significant difference ($p<0.05$) between

Fig 2: Cryopreservation-induced shape change in monocytes and lymphocytes. Fresh separated and cryopreserved PBMC were analyzed by flow cytometry.

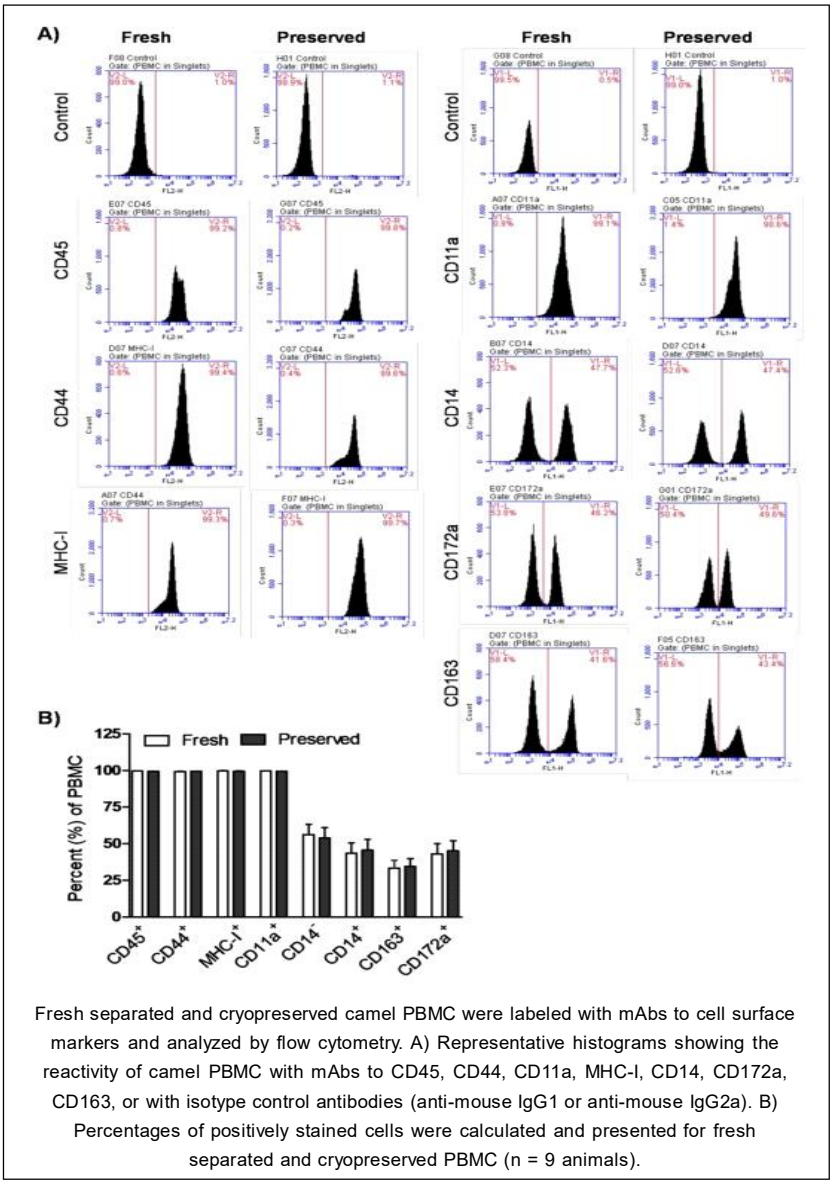
Impact of cryopreservation on camel lymphocyte composition

The analysis of lymphocyte composition (Fig 4A) revealed a higher ($p<0.05$) percentage of MHC-II-positive lymphocytes (B cells) within cryopreserved (26.9%±2.4) compared to fresh separated PBMC (18.6%±2.7) (Fig 4B). In contrast to this, the percentage of lymphocytes positively stained with BAQ44A mAb (B cell subset) was significantly lower ($p<0.05$) within preserved (13.3%±1.7) than fresh (17.4 %±2.3) PBMC (Fig 4B). On the other hand, the fractions of CD4-positive (T helper) cells, WC1-positive γδ T cells and CD11ahigh lymphocytes (activated lymphocytes) were comparable ($p>0.05$) between fresh and cryopreserved PBMC (Fig 4B).

Impact of cryopreservation on the expression of activation markers on camel monocytes

In comparison to freshly separated monocytes, cryopreserved monocytes showed higher ($p<0.05$) mean fluorescence intensity (MFI) of the cell activation marker MHC-II molecules (7939±1532 versus 2757±205 on fresh monocytes), while the abundance of CD163 did not differ significantly between fresh and preserved monocytes (Fig 5A). For the two monocyte activation markers, however, there was a positive correlation between the MFI values of fresh cells and those of preserved cells (R square = 0.7 for CD163 and 0.6 for MHC-II) (Fig 5B).

Immunophenotyping of peripheral blood mononuclear cells (PBMC) has been proven a valuable tool to evaluate



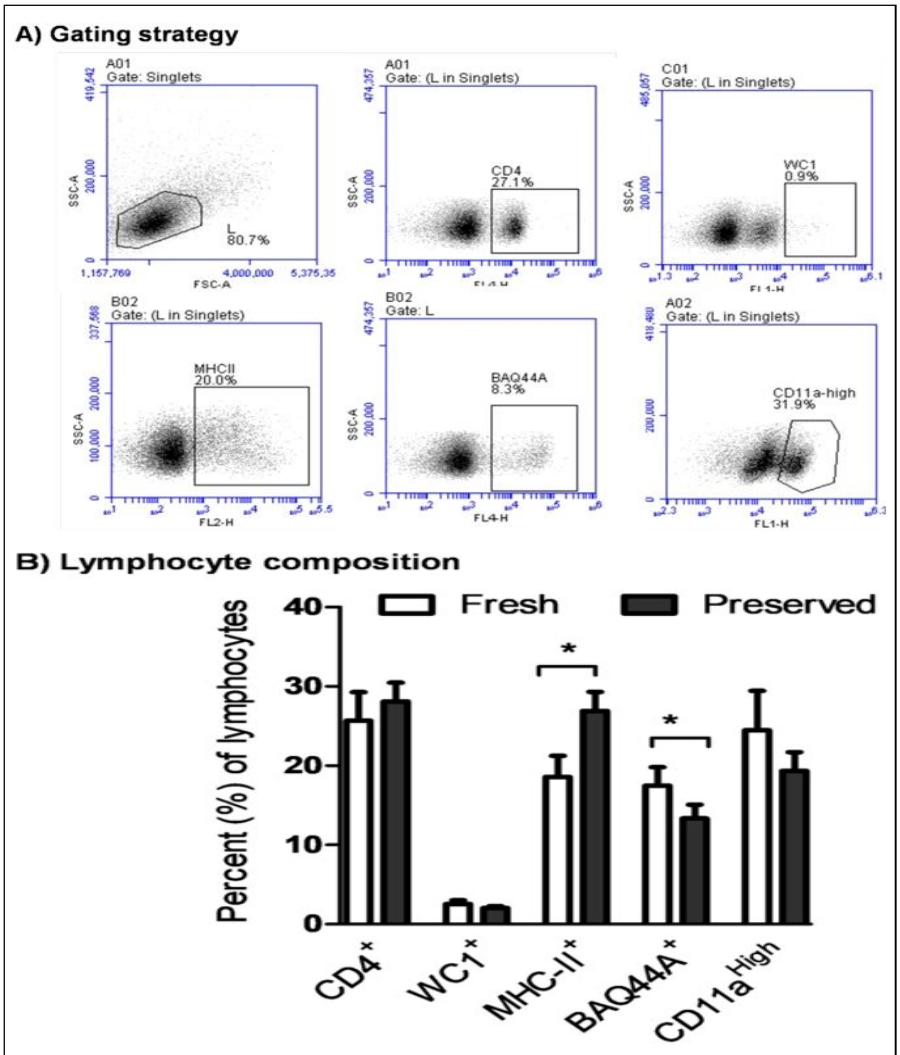
Fresh separated and cryopreserved camel PBMC were labeled with mAbs to cell surface markers and analyzed by flow cytometry. A) Representative histograms showing the reactivity of camel PBMC with mAbs to CD45, CD44, CD11a, MHC-I, CD14, CD172a, CD163, or with isotype control antibodies (anti-mouse IgG1 or anti-mouse IgG2a). B) Percentages of positively stained cells were calculated and presented for fresh separated and cryopreserved PBMC (n = 9 animals).

Fig 3: Staining pattern of PBMC with monoclonal antibodies (mAbs) to leukocyte markers.

the immune status in health and disease (Maecker *et al*., 2012). Single-cell analysis by flow cytometry is the most commonly used technique for immunophenotyping of PBMC (Perfetto *et al*., 2004). Due to the limited availability of flow cytometry devices in veterinary laboratories, cells are usually preserved to be analyzed in central or core facility laboratories. The present study aimed to investigate the influence of cryopreservation of camel PBMC on their vitality and immunophenotype.

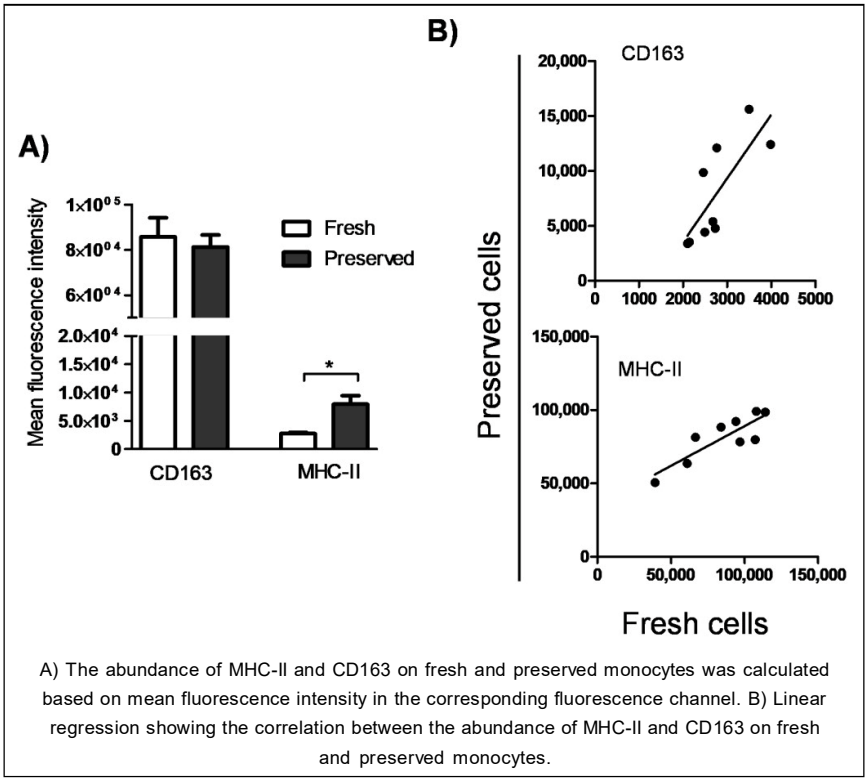
In the present study, the comparable percentages of dead, apoptotic and necrotic, cells within fresh and preserved camel lymphocytes and monocytes indicate that PBMC cryopreservation for 4 weeks did not impact their cell vitality. The similarity in absolute numbers of lymphocytes and monocytes between fresh and preserved PBMC also in support of this.

Cell light scatter parameters, including side scatter (SSC), which is indicative of cell granularity and forward scatter (FSC), which is indicative of cell size, are usually used for the identification of cell populations in flow cytometry (Stanciu *et al*., 2016). In the present study, although cryopreservation did not impact monocytes SSC or FSC values, the reduced SSC and increased FSC of preserved lymphocytes indicate a cryopreservation-induced shape change in this population. This change, however, did not interfere with the identification of



Fresh separated or cryopreserved camel PBMC were stained with mAbs to CD4, WC1, MHC-II, and BAQ44A and analyzed by flow cytometry. A) Representative dot plots showing the gating strategy for the identification of marker-positive cells. The percentage of positive cells within lymphocytes was calculated after setting a gate on lymphocytes. B) The percentage of CD4+ lymphocytes (T helper cells), WC1+ lymphocytes (γδ T cells), MHC-II+ lymphocytes (B cells), BAQ44A+ lymphocytes (B cell subset), CD11ahigh lymphocytes (activated lymphocytes) were calculated for fresh and preserved cells (n = 9 animals). * indicates significant differences ($p<0.05$).

Fig 4: Impact of cryopreservation of camel PBMC on lymphocyte composition.



A) The abundance of MHC-II and CD163 on fresh and preserved monocytes was calculated based on mean fluorescence intensity in the corresponding fluorescence channel. B) Linear regression showing the correlation between the abundance of MHC-II and CD163 on fresh and preserved monocytes.

Fig 5: Impact of cryopreservation on the abundance of monocyte activation marker expression. PBMC were stained with mAbs to MHC-II and CD163 and analyzed by flow cytometry.

cryopreserved lymphocytes based on their SSC and FSC characteristics.

The protein tyrosine phosphatase CD45 (Meza Guzman *et al*., 2024), the cell adhesion molecules CD44 (hyaluronic acid receptor) (Wu *et al*., 2024) and CD11a (Leukocyte function-associated antigen 1) (Lacouture *et al*., 2024) as well as the major histocompatibility complex (MHC) class I are common leukocyte markers expressed on all leukocyte populations (Kuzilkova *et al*., 2022), while the expression of the surface antigens CD14 (LPS receptor), CD163 (the receptor for hemoglobin-haptoglobin complexes) and CD172a (SIRPa) is limited to myeloid cells (mainly monocytes) within PBMC (Elnaggar *et al*., 2019). In the present study, the comparable percentages of marker-positive cells within fresh and preserved PBMC indicate no impact of cryopreservation on the staining pattern of camel PBMC with monoclonal antibodies to the indicated markers.

Blood lymphocytes population encompass several cell subsets, mainly including CD4+ alpha-beta (ab) helper T cells, CD8+ ab cytotoxic T cells, gamma delta γδ T cells, B cells and natural killer cells (NK cells), (Heubeck *et al*., 2023). In the present study, the commercially available monoclonal antibodies to camel CD4, WC1 (γδ T cell marker) (Gillespie *et al*., 2021), MHC-II and BAQ44A (B cell markers) (Stabel *et al*., 2022) were used to investigate the impact of cryopreservation on camel lymphocyte composition. The results indicate a significant increase in the proportion of B cells with a reduced BAQ44A+ B cell subset. This effect may lead to misinterpretation of results when B cells are comparatively analyzed in fresh and cryopreserved samples using the mentioned antibodies.

The expression pattern of the monocyte markers CD163 and MHC-II are commonly used for the characterization of monocyte subsets and the functional phenotypes of macrophages (Elnaggar *et al*., 2019). In the present study, the enhanced expression of MHC-II, which is a marker of inflammatory monocytes (Hussen *et al*., 2020), leads to the assumption that the cryopreservation or the thawing process either resulted in monocyte activation or higher accessibility of MHC-II epitopes on monocytes to the anti-MHC-II mAbs.

CONCLUSION

Comparative analysis of fresh-separated and cryopreserved camel PBMC by flow cytometry revealed no significant changes in cell viability four weeks after preservation. In addition, the reactivity of camel PBMC with mAbs to the cell markers antigens CD45, CD44, CD11a, MHC-I, CD14, CD163 and CD172a remained unchanged for cryopreserved cells. However, mAbs to MHC-II and BAQ44A displayed modified binding to cryopreserved PBMC leading to significant changes in the positively stained B cells and increased the abundance of MHC-II on cryopreserved monocytes. Collectively, the results of the current study show that storing camel PBMC

in cryopreservation media at -80°C is an appropriate procedure for preserving the epitopes for most of the used antibodies.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions.

Ethical approval

All animal procedures for experiments were approved by the Ethics Committee of King Faisal University, Saudi Arabia (KFU-REC-2024-JUN-ETHICS1843).

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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