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RESEARCH ARTICLE

Expression Patterns of Cell Adhesion Molecules on CD4$^+$ T Cells and WC1$^+$ T Cells in the Peripheral Blood of Dromedary Camels

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ABSTRACT

In human, mice and different veterinary species, T cells are subdivided into phenotypically different subpopulations based on their expressed T cell receptor and cell specific surface markers. As no data were available regarding the existence of T cell subpopulations in the peripheral blood of dromedary camels, the current study aimed at the identification of camel blood T cell subpopulations and the analysis of their adhesion molecules expression patterns. Using monoclonal antibodies cross-reactive with camel CD antigens, camel blood WC1-positive T cells and CD4-positive αβ T cells and their expression pattern of adhesion molecules were identified by flow cytometry. Under camel CD4-positive T cells, a major fraction (83%) with naïve phenotype (CD4$^+$ CD11a^{lo} CD44^{lo}) and minor fraction (13%) with effector phenotype (CD4$^+$ CD11a^{hi} CD44^{hi}) were identified. In addition, the analysis of age related effects on the proportion of lymphocyte subsets indicates that camels belong to the T cell high species.

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INTRODUCTION

The cellular immune system represents a complex network of specialized cells that interact together to ensure protection against invading pathogens. The cellular immune system of camel remains poorly studied when compared with other veterinary species (Hussen *et al.*, 2013; Duvel *et al.*, 2014; Gerner *et al.*, 2015).

Studies in human and different veterinary species revealed that peripheral blood T cells consist of different subpopulations, with distinct phenotypes and functions (Appay *et al.*, 2008; Herzig *et al.*, 2010; Duvel *et al.*, 2014). According to their expressed TCR, T cells can be subdivided into αβ T cells and γδ T cells, which react with different types of antigens. Whereas αβ T cells recognize peptide antigens presented on MHC molecules, γδ T cells respond to unconventional antigens such as lipid antigens in an MHC independent manner (Beetz *et al.*, 2008). Based on the expression of specific accessory molecules bovine αβ T cells, which are all known to express CD3 and CD2 molecules, can be subdivided into two main subsets; CD8$^+$ T cells and CD4$^+$ T cells (Duvel *et al.*, 2014). Little is known about the existence of subpopulations of T cells in the peripheral blood of camels.

After leaving the thymus, T cells circulate in the blood as naïve cells. Antigen stimulation induces proliferation and differentiation of naïve T cells into effector T cells (Berard and Tough, 2002). In the human and bovine systems, naïve and effector T cells can be distinguished based on the differential expression of numerous cell surface adhesion molecules like CD45, CD44, CD62L (L-selektin) and CD11a (LFA-1) that mediate lymphocyte homing and trafficking to sites of inflammation and lymphoid organs (Sallusto *et al.*, 2004; Blunt *et al.*, 2015; Maggioli *et al.*, 2015). The adhesion molecules CD44 and CD11a were used to describe human naïve (CD11a^{lo} CD44^{lo}) and effector (CD11a^{hi} CD44^{hi}) CD4+ T cells (Kohlmeier *et al.*, 2006). Whether functional subsets of camel T cells follow the same expression pattern of their human and bovine counterparts, it is unknown.

The current study aimed at the identification of T cell subsets in the peripheral blood of dromedary camel and the characterization of their expression pattern of cell surface adhesion molecules. In addition, age-related changes in the proportion of camel lymphocyte subsets were also analyzed.

MATERIALS AND METHODS

Animals and blood sampling: Blood samples were collected from fifteen apparently healthy dromedary camels (Camelus dromedaries) at Camel Research Center, King Faisal University, Al-Ahsa, Saudi Arabia. Blood samples were collected by venepuncture of the vena jugularis externa into EDTA vacutainer tubes (BD, Germany).

Separation of mononuclear cells (PBMC): Separation of camel PBMC was performed according to an established separation method (Hussen *et al*., 2017). Briefly, blood was layered on Ficoll-Isopaque (Sigma-Aldrich, Germany) and centrifuged for 30 min (3000 rpm; 10°C). The interphase containing mononuclear cells was washed 3 times in PBS and suspended in MIF buffer (PBS with bovine serum albumin (5 g/L) and NaN_3 (0.1 g/L). Purity of separated PBMC was evaluated by flow cytometry according to their scattering (FCS/SSC) properties and always exceeded 95%. Mean viability of separated cells, as determined by exclusion of propidium iodide (2 µg/ml, Calbiochem, Germany) was above 92%.

Monoclonal antibodies: The commercially available monoclonal antibodies (mAbs) used in this study are shown in Table 1.

Table 1: List of used primary monoclonal antibodies

Antigen	Antibody clone	Labelling	Source	Isotype
Mouse CD3	145-2C11	PE	Abcam	Hamster IgG
Human CD3	T3/2/16A9	Unlabeled	MyBiosource	Mouse IgG2a
Bovine CD4	CC8	FITC	Bio-Rad	Mouse IgG2a
Bovine CD4	GC50A1	Unlabeled	WSU	Mouse IgM
Bovine CD8	CC63	PE	Bio-Rad	Mouse IgG2a
Bovine WC1	CC15	FITC	WSU	Mouse IgG2a
Bovine WC1N2	BAQ4A	Unlabeled	WSU	Mouse IgG1
Bovine WC1	CC101	Unlabeled	Bio-Rad	Mouse IgG2a
Bovine WC1	BAQ128A	Unlabeled	WSU	Mouse IgG1
Bovine B cell	GC26A	Unlabeled	WSU	Mouse IgM
Activation marker	LH9A	Unlabeled	WSU	Mouse IgM
Activation marker	VPM30	Unlabeled	Abd Serotec	Mouse IgM
CD25	IL-A111	PE	Bio-Rad	Mouse IgG1
Bovine CD44	LT41A	Unlabeled	WSU	Mouse IgG2a
Mouse CD62L	MEL14	PerCP	Biolegend	Rat IgG2a
CD11a	G43-25B	PE	BD	Mouse IgG2a
CD11b	ICRF44	PE-Cy7	BD	Mouse IgG1
CD14	M5E2	PE	BD	Mouse IgG2a
CD18	6.7	FITC	BD	Mouse IgG1

Ig: Immunoglobulin.

Immunofluorescence and flow cytometry: Separated camel PBMC (4×10^5) were incubated with mAbs specific for different CD antigens (listed in Table 1) in MIF buffer. After incubation (30 min; 4°C), cells were washed twice and were incubated with mouse secondary antibodies IgG1, IgG2a, IgG2b (BD) and IgM (Beckmann Coulter) labelled with different fluorochromes. After washing, cells (100 000 events) were analyzed by flow cytometry (FACSCalibur, Becton Dickinson Biosciences, San Jose, California, USA). Flow cytometric data were analyzed using the FlowJo version 10 (FLOWJO LLC).

Statistical analyses: Statistical analysis was performed using the GraphPad Prism (v. 5) software. Results are expressed as means±S.E. of the mean (SEM). Student T test and one-factorial analysis of variance (ANOVA) were used for difference analysis between means and Bonferroni's correction was used to test normality distribution of data. Differences were considered statistically significant at a P-value of less than 0.05.

RESULTS

Immunophenotyping of camel blood lymphocytes: For immunophenotyping of camel T cell subsets, we tested different clones of monoclonal antibodies against T cell marker CD3, CD4, CD8 and TCR for their cross-reactivity with camel CD antigens (Table 1). As no cross-reactive antibodies could be found for camel CD3 or CD8, we only used monoclonal antibodies against CD4 and the bovine cell marker WC1 together with monoclonal antibodies to monocytes (CD14) and B cells (GC26A) to identify camel CD4-positive T cells, WC1-positive T cells and B cells (Fig. 1). With a mean of 26.6%±1.9 (mean±SEM) and a range between 18.4 and 42% B cells represented the major fraction of camel blood lymphocytes. On the other side WC1-positive T cells were only a minor fraction (7.4%±1.6; mean±SEM) under blood lymphocytes and ranged between 1 and 20% of total lymphocytes. The percentage of CD4+ T cells ranged between 14 and 33% of total lymphocytes with a mean of 24.6%±1.7 SEM. As no cross-reactive antibodies could be found for camel CD8, the percentage of CD4-nigative lymphocytes, which includes CD8+ αβ T cells and NK cells was calculated after exclusion of B cells and γδ T cells. CD4-nigative lymphocytes ranged between 31.4 and 60.8% with a mean±SEM of 47±2.6 (Fig. 1).

Adhesion molecules expression on camel blood T cell subsets: The expression pattern of adhesion molecules CD11a, CD11b, CD18 and CD62L on camel blood CD4-positive T cells and WC1-positive T cells was evaluated by flow cytometry. For the adhesion molecules CD11a and CD18, camel CD4-positive T cells and WC1-positive T cells showed comparative expression pattern. For CD11b and CD62L a higher mean fluorescence intensity was seen on camel WC1-positive lymphocytes than CD4-positive lymphocytes (Fig. 2).

CD11a surface expression defines naïve and effector camel CD4-positive T cells: Using monoclonal antibodies against the adhesion molecules CD11a and CD44, naïve (CD4+ $CD11a^{lo}$ $CD44^{lo}$) and effector (CD4+ $CD11a^{hi}$ $CD44^{hi}$) subsets of CD4-positive T cells were identified (Fig. 3). Camel effector CD4-positive T cells expressed significantly more CD44 than naïve CD4-positive T cells. Effector CD4-positive T cells represented a minor (17%±1.2) fraction of total CD4-positive T cells, whereas the majority of CD4-positive T cells showed naïve phenotype (82±1.2).

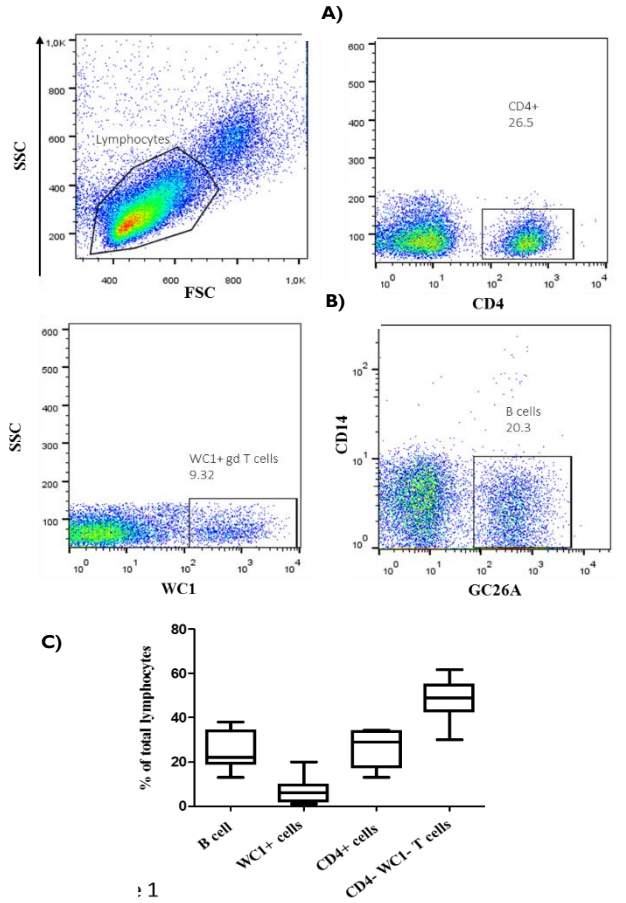

Fig. 1: Gating strategy for the identification of lymphocyte populations in peripheral blood of dromedary camel. Ficoll-separated camel PBMC were labelled with monoclonal antibodies specific for CD4, WC1 and B cell marker GC26A and were analysed by flow cytometry. A) In a SSC/FSC dot plot, a gate was set on lymphocytes according to their forward and side scatter characteristics. CD4-positive lymphocytes were gated in a SSC/CD4 plot. WC1-positive lymphocytes were shown in a SSC/WC1 plot. B cells were shown in a CD14/ GC26A plot to exclude CD14-positive monocytes. B) The percentages of gated lymphocyte populations were calculated and presented graphically (* = P<0.05).

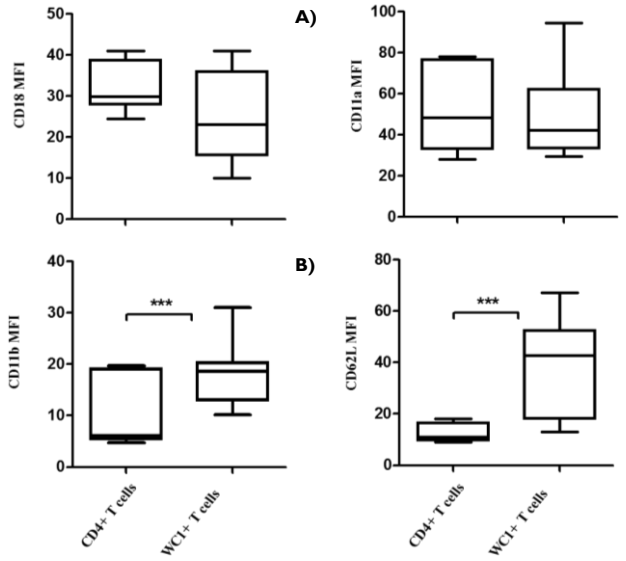

Fig. 2: Adhesion molecules expression on lymphocyte populations in peripheral blood of dromedary camel. Ficoll-separated camel PBMC were labelled with monoclonal antibodies specific for CD4, WC1, CD11a, CD11b, CD18 and CD62L and were analysed by flow cytometry. After gating on CD4-positive T cells or WC1-positive T cells the mean fluorescence expression for each adhesion molecule and subset were calculated and presented graphically as mean ± SEM (* = P<0.05).

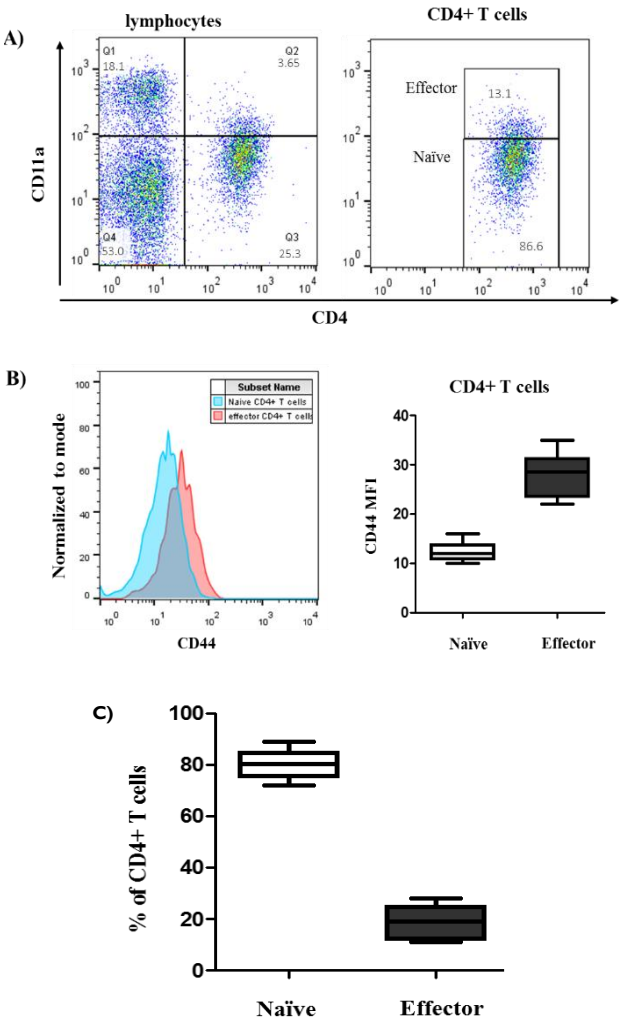

Fig. 3: Gating strategy for the identification of naive and effector CD4-positive T lymphocytes in the peripheral blood of dromedary camel. Ficoll-separated camel PBMC were labelled with monoclonal antibodies specific to CD4, CD11a and CD44 or with isotype control antibodies and were analysed by flow cytometry. A) After gating on lymphocytes (according to SSC/FSC characteristics) cells were plotted in a CD11a/CD4 plot to show the expression pattern of CD11a on CD4-positive and CD4-negative lymphocytes. A gate was set on CD4-positive cells to calculate the percentages of naïve (CD4+CD11a^{lo}) and effector (CD4+CD11a^{hi}) CD4-positive T cells. B) Mean fluorescence intensity of CD44 expression on naïve and effector CD4-positive T cells were shown as overlapping histogram and presented graphically. C) The percentages of naïve (CD4+CD11a^{lo}CD44^{lo}) and effector (CD4+ CD11a^{hi} CD44^{hi}) CD4-positive T cells of total CD4-positive T cells were calculated and presented graphically as mean ± SEM (* =P<0.05).

To see whether the percentage of effector CD4-positive T cells is changed in case of disease, we compared PBMC from a diseased animal with symptoms of respiratory infection (nasal discharge and fever) with PBMC from apparently healthy animal from the same age. As no monoclonal antibodies cross-reactive with camel IL-2 receptor, CD25, could be found, we labeled PBMC from the tow animals with monoclonal antibodies against the activation markers LHA9 (Mossad *et al.*, 2006) and VPM30, which were reported as activation markers of bovine and ovine lymphocytes. We found an elevated percentage of effector CD4+ T cells in blood of diseased animal, which correlated with enhanced expression of activation markers on blood lymphocytes. Lymphocytes from diseased animal showed higher expression of both activation markers than that from healthy animal (Fig. 4).

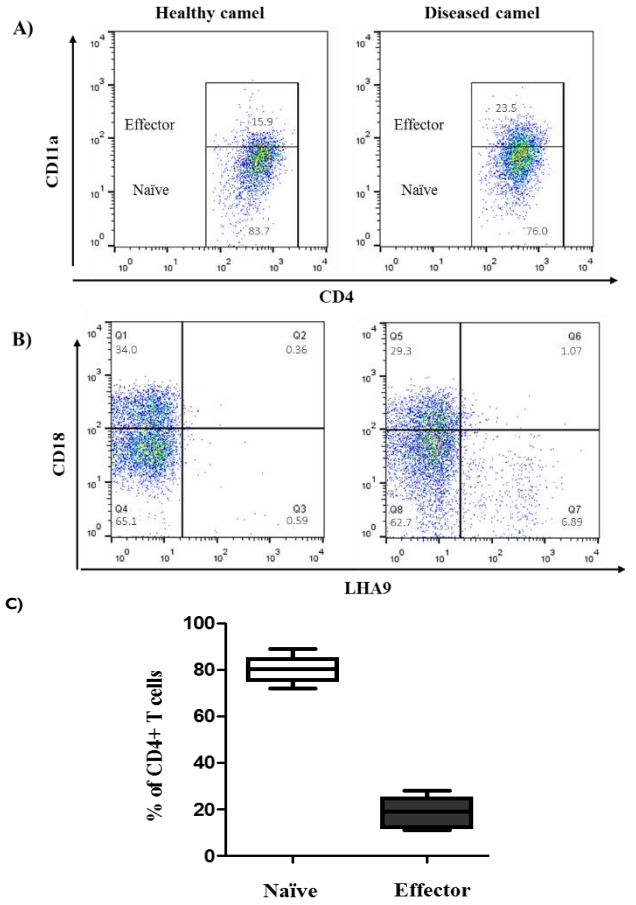

Fig. 4: Impact of age on the percentages of lymphocyte subsets in the peripheral blood of dromedary camels. Ficoll-separated camel PBMC were labelled with monoclonal antibodies specific to WC1, CD4, CD14, GC26A, CD11a and CD44 or with isotype control antibodies and were analysed by flow cytometry. After gating on distinct subset, the percentage of B cells (CD14- GC26A +), WC1-positive T cells, CD4-positive T cells, naïve ($CD4^+$ $CD11a^{lo}$ $CD44^{lo}$) and effector ($CD4^+$ $CD11a^{hi}$ $CD44^{hi}$) CD4-positive T cells were calculated and presented graphically for young (1-5 years old) and old (6-11 years old) camels as mean ± SEM (* = P<0.05).

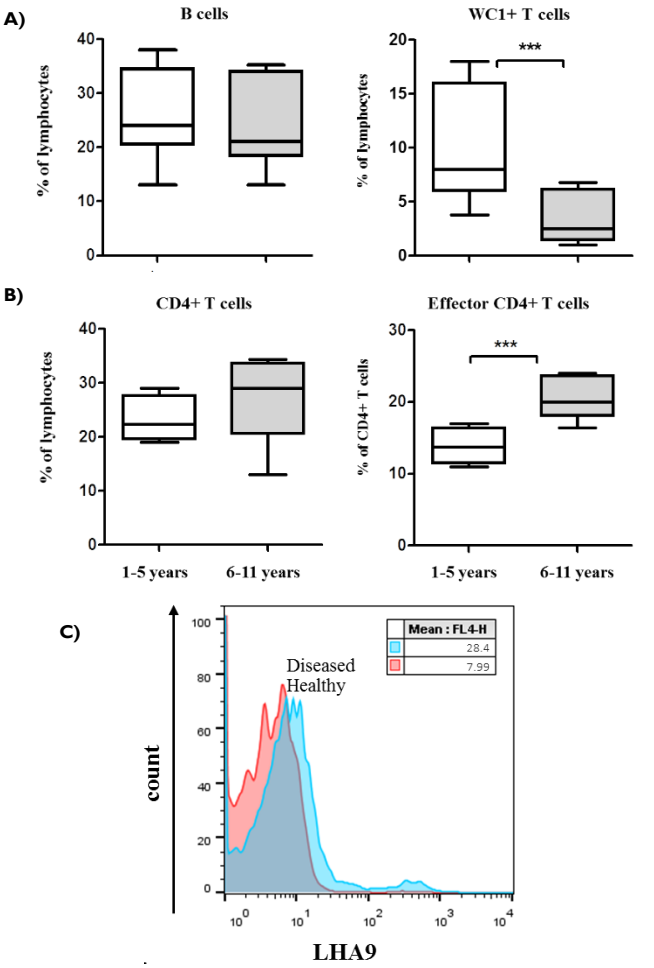

Fig. 5: Elevated proportion of effector CD4-positive T cells in blood of diseased animal accompanied by enhanced expression of activation marker on blood lymphocytes. Ficoll-separated camel PBMC of a healthy or diseased animal were labelled with monoclonal antibodies specific to CD4, CD11a, CD44, CD18 and LH9A or with isotype control antibodies and were analysed by flow cytometry. A) After gating on lymphocytes (according to SSC/FSC characteristics) cells were plotted in a CD11a/CD4 plot to show naïve ($CD11a^{lo}$ $CD44^{lo}$) and effector ($CD11a^{hi}$ $CD44^{hi}$) CD4-positive T cells. B) The expression of the activation marker LHA9 on gated lymphocytes was shown in a CD18/LHA9 plot. C) Mean fluorescence intensity of the activation marker LHA9 on lymphocytes of healthy and diseased animal was plotted as overlapping histograms.

Impact of age on adhesion molecules expression and cellular composition of camel lymphocytes: To evaluate the effect of age on the percentage of camel blood lymphocytes and adhesion molecules expression on their surface, animals were grouped according to their age as young (1-5 years old) and old (6-11 years old) camels. The percentage of blood B cells of total lymphocytes did not differ significantly between young and old camels. For WC1-positive lymphocytes, higher percentages were seen in blood from young camels than that from old camels. Although the percentage of CD4-positive T cells did not differ between young and old animals, old camels showed elevated percentages of effector CD4-positive T cells in comparison to young animals (Fig. 5).

DISCUSSION

Although some progress has been made in the analysis of camel humoral immune system (Muyldermans, 2013), little is known about cellular immunity in camels (Zidan *et al.*, 2000; Zidan *et al.*, 2000; Al-Mohammed Salem *et al.*, 2012). Our study aimed at the characterization of T lymphocyte subsets in the peripheral blood of dromedary camels and their adhesion molecules expression patterns.

Based on their expressed TCR and cell specific surface markers, T cells can be subdivided into phenotypically and functionally different subpopulations (Abeles *et al.*, 2012). In comparison to many other veterinary species, little is known about the existence of subpopulations of T cells in the peripheral blood of camels. Looking for monoclonal antibodies cross-reactive with camel CD antigens we firstly tested different clones of monoclonal antibodies against T cell marker CD3, CD4, CD8 and TCR from other species for their cross-reactivity with camel CD antigens (Table 1). As no cross-reactive antibodies could be found for camel CD3 or CD8, we only used monoclonal antibodies against CD4 and the bovine cell marker WC1 together with monoclonal antibodies to monocytes (CD14) and B cells (GC26A) to identify camel CD4-positive T cells, WC1-positive T cells and B cells.

To our knowledge, there are no reference values in the literature for the composition of camel blood

lymphocyte subsets. With a proportion of 26% B cells, 24% CD4-positive T cells and 7% WC1-positive T cells under total blood lymphocytes, our data suggest some similarities regarding lymphocyte composition between camel and bovine blood. Bovine lymphocytes, consist of 23% B cells , 20% CD4-positive T cells and 6% WC1-positive T cells (Oliveira and Hansen, 2008).

Adhesion molecules expression contributes to lymphocyte trafficking to inflamed tissues and secondary lymphoid organs through the interaction with ligands on endothelial cells of blood vessels and binding on extracellular matrix (Nourshargh and Alon, 2014). In our study the expression pattern of the adhesion molecules CD11a, CD11b, CD18 and CD62L on camel blood CD4-positive T cells and WC1-positive T cells was evaluated by flow cytometry. The higher expression of CD11b and CD62L on camel WC1-positive lymphocytes than CD4-positive lymphocytes seems to be similar to the expression pattern seen for bovine lymphocyte subsets (Waters *et al.*, 2003).

In human and bovine systems, naïve and effector T cells can be distinguished based on the differential expression of distinct cell surface adhesion molecules like CD44, CD62L and CD11a (Sallusto *et al.*, 2004; Foote *et al.*, 2005; Blunt *et al.*, 2015; Maggioli *et al.*, 2015). Analog to the human system (Kohlmeier *et al.*, 2006) and using monoclonal antibodies to the adhesion molecules CD44 and CD11a we could identify camel naïve ($CD11a^{lo}$ $CD44^{lo}$) and effector ($CD11a^{hi}$ $CD44^{hi}$) CD4-positive T cells with major naive (83%) and minor effector (17%) fraction of CD4-positive T cells. The activated phenotype of effector $CD4^+$ cells could be supported by elevated proportion of these cells together with upregulated expression of lymphocyte activation markers in a respiratory diseased animal.

Under different pathological (e.g. infection) (Duvel *et al.*, 2014) and physiological conditions (pregnancy) (Eger *et al.*, 2015), distribution of T cell subpopulations in blood can change significantly. Age-related changes in the composition of peripheral blood T cells were reported for different species (Romanyukha and Yashin, 2003; Elghetany and Lacombe, 2004). In young ruminants, γδ T cell compartment accounts for up to 60% of the total circulating mononuclear lymphocytes, which is in contrast to humans and mice, where γδ T cells represent only <5% of the total circulating lymphocytes (Guzman *et al.*, 2014). A large proportion of bovine γδ T cells express workshop cluster 1 (WC1), a transmembrane glycoprotein related to the scavenger receptor CD163 (Holm *et al.*, 2009). In our study the percentages of WC1-positive T cells were higher in young camels in comparison to older camels, which indicates that camels belong to the γδ T cell high species. In opposite to age related changes seen for γδ T cells, old camels showed elevated percentages of effector CD4-positive T cells in comparison to young animals.

Conclusions: This is the first study aimed at the characterization of lymphocyte subsets in camel peripheral blood. We identified camel $CD4^+$ T cells and $WC1^+$ T cells and analyzed their expression patterns of adhesion molecules. In addition, the functional heterogeneity of camel $CD4^+$ T cells was analyzed by identifying naïve and effector $CD4^+$ T cells. Furthermore, age related changes in the proportions of lymphocyte subsets were studied. A better understanding of the camel cellular immune system would help in the establishment of reference values to be involved in studies on special topics such as vaccination or infection immunity. In addition, characterization of camel T cells would help in the identification of disease biomarkers to be used in the diagnosis of camel diseases.

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Authors contribution: JH, AMA conceived and designed the study. JH and TS collected the samples and prepared it for flow cytometry. JH, MHA, AIA and HRA analyzed the labelled cells by flow cytometry. All authors contributed to data interpretation, manuscript preparation and revision of the final version.

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