

# IMPACT OF AGE AND PREGNANCY ON THE EXPRESSION LEVEL OF THE CORONAVIRUS (MERS-COV) RECEPTOR, DIPEPTIDYL PEPTIDASE 4 ON CAMEL BLOOD LEUKOCYTES

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## ABSTRACT

In dromedary camels, young animals have shown higher susceptibility to MERS-CoV infection than adult camels. The current study, analysed the impact of animal age and pregnancy on the expression density of DPP4 on the main populations of camel leukocytes using flow cytometry.

Adult camels showed significantly higher expression levels of DPP4 on their monocytes in comparison to newborn calves. Newborn calves and adult camels showed comparable expression levels of DPP4 on their neutrophils and lymphocytes. The results of the current study argue against a role of different DPP4 expression levels on blood leukocytes in the higher susceptibility of camel calves than to MERS-CoV. The comparable expression levels of DPP4 on monocytes, neutrophils, and lymphocytes from pregnant and non-pregnant female camels indicate no significant impact of pregnancy on DPP4 expression on blood leukocytes of camels.

**Key words:** Camel, corona virus, dipeptidyl peptidase 4, MERS-CoV), middle east

Dipeptidyl peptidase 4 (DPP4), which is a type II transmembrane glycoprotein involved in cleavage of dipeptides and degradation of incretins (Lambeir *et al*, 2003), has been identified as a functional receptor for the Middle East respiratory syndrome coronavirus (MERS-CoV) (Ohnuma *et al*, 2013; Raj *et al*, 2013; van Doremalen *et al*, 2014). Differential expression of DPP4 in the respiratory tracts has been shown to be responsible for higher susceptibility of humans than dromedary camels for MERS-CoV infection (Widagdo *et al*, 2016). In opposite to human, where DPP4 is mainly found on human T lymphocytes rather than monocytes (Pierson *et al*, 2008), dromedary camels have shown the highest expression of DPP4 on their blood monocytes (Al-Mubarak, 2018; Haverkamp *et al*, 2018).

Age-related changes of several innate and adaptive cellular immune responses have been described for different species (Romanyukha and Yashin, 2003; Elghetany and Lacombe, 2004), including dromedary camel (Gaashan *et al*, 2020). In dromedary camels (Hussen *et al*, 2020) as well as in several other animals species (Leung *et al*, 2000; Oliveira *et al*, 2012; Spadaro *et al*, 2019), pregnancy is

associated with modulations in several components of the immune system.

Expression pattern or density of DPP4 on blood leukocytes and its associated alterations by animal age or pregnancy has not been studied previously. The current study, therefore, comparatively analysed the expression of DPP4 on the main populations of peripheral blood leukocytes in newborn and adult camels and, during pregnant and non-pregnant states female camels.

## Materials and Methods

### Animals and blood sampling

For the comparison between newborn calves and adult camels regarding the expression of DPP4, blood samples were collected from 14 newborn camel calves (aged between 7 and 35 days) and 10 adult dromedary camels (*Camelus dromedarius*) aged between 7 and 9 years. For the evaluation of the impact of pregnancy on the expression level of DPP4 on blood leukocytes, blood samples were collected into vacutainer tubes containing EDTA from 12 pregnant and 9 non-pregnant apparently healthy she-camels. All camels were housed at the farm of

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the Camel Research Center, King Faisal University, Al-Ahsa, Saudi Arabia. All experimental procedures and management conditions used in this study were approved by the Ethics Committee at King Faisal University, Saudi Arabia (Permission number: KFU-REC/2020-09-25).

### **Isolation of leukocytes from camel blood**

Separation of camel leukocytes was performed after hypotonic lysis of blood erythrocytes as described previously (Hussen *et al*, 2017). Briefly, blood was suspended in distilled water for 20 sec and double concentrated PBS was added to restore tonicity. This was repeated (usually twice) until complete erythrolisis. Separated cells were finally suspended in MIF buffer (PBS containing bovine serum albumin (5 g/L) and NaN<sub>3</sub> (0.1 g/L)) at  $5 \times 10^6$  cells/ml. Cell purity of separated leukocytes was assessed by flowcytometry according to their FCS/SSC properties and always exceeded 90 %. The mean viability of separated cells was evaluated by dye exclusion (propidium iodide; 2 µg/ml, Calbiochem, Germany) and it was above 90%.

### **Immunofluorescence and flow cytometry**

After leukocyte separation, separated cells ( $1 \times 10^6$ ) were labeled with monoclonal antibodies against human DPP4 (goat IgG anti DPP4; R & D Systems) with cross-reactivity with camel DPP4 (Pierson *et al*, 2008; van Doremalen *et al*, 2014) diluted 1: 50 in PBS containing bovine serum albumin (5 g/l) and NaN<sub>3</sub> (0.1 g/l). After incubation at 4°C for 20 minutes, the cells were washed twice and further incubated with a secondary antibody against goat IgG labeled with fluorochrome (Alexa Fluor 488a-labelled rabbit F (ab')<sub>2</sub>-anti-goat IgG (H+L); Invitrogen). After incubation (20 minutes; 4°C), labelled cells were washed twice and analysed on the flow cytometer (Fig 1A). A Becton Dickinson FACS Calibur equipped with Cell Quest software (FACSCalibur; Becton Dickinson Biosciences, San Jose, California, USA) was used to collect the data. At least 100 000 cells were collected and analysed with the flowcytometric software FCS Express software Version 3 (De Novo Software, Thornton, Ontario).

### **Statistical Analyses**

Statistical analysis was performed with Prism (GraphPad). Results are presented as means  $\pm$  S.E. of the mean (SEM). Student *t* test was used for difference analysis between means. Differences were considered statistically significant at a p-value of less than 0.05.

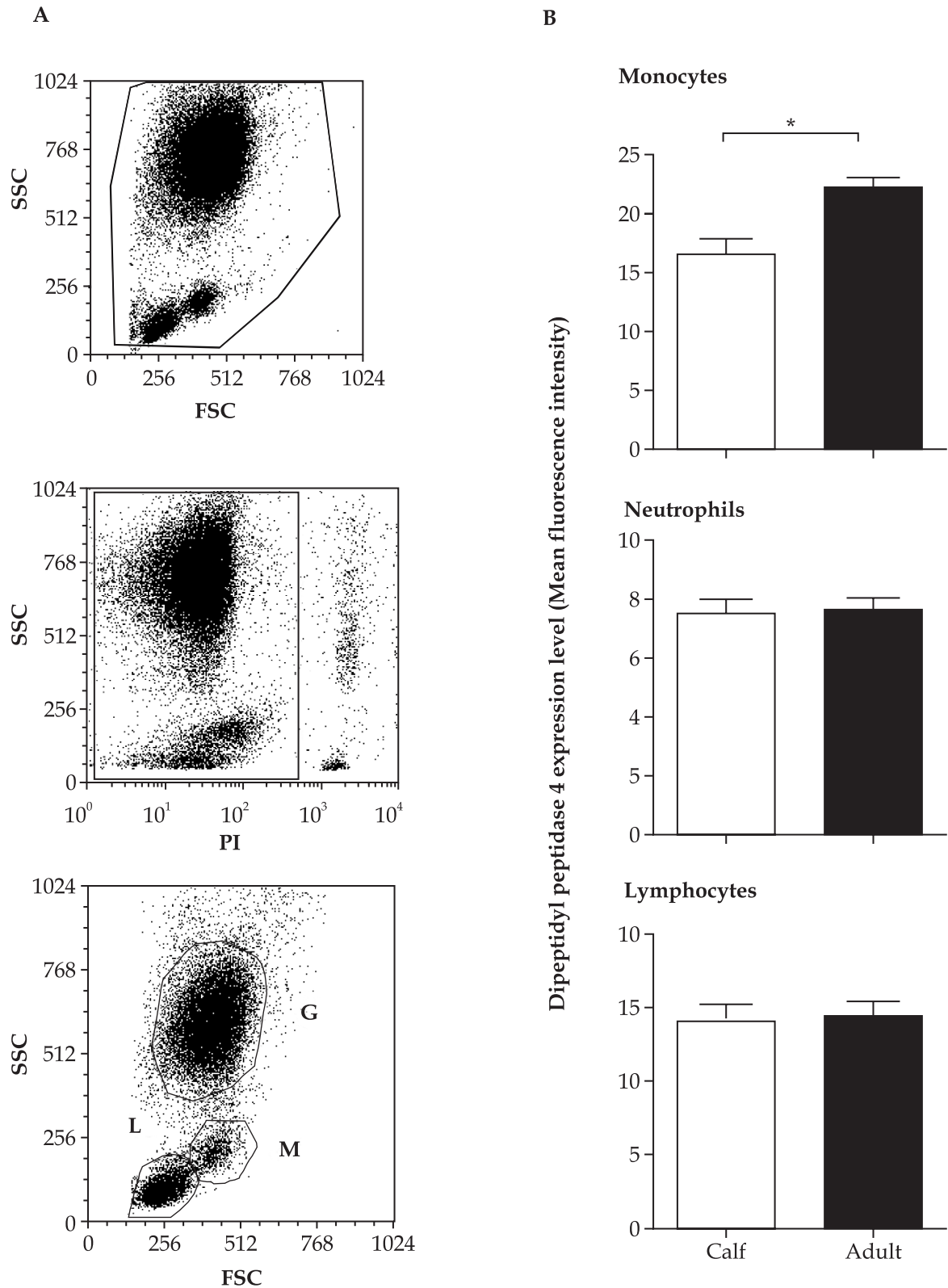
## **Results and Discussion**

In dromedary camels, young camels have shown higher susceptibility to MERS-CoV infection than adult camels (Khalafalla *et al*, 2015). Dipeptidyl peptidase 4 (DPP4) has been identified as a functional receptor for the MERS-CoV (Ohnuma *et al*, 2013; Raj *et al*, 2013; van Doremalen *et al*, 2014). The expression level of DPP4 on the surface of cells of respiratory tract has been found to be correlated positively with susceptibility to MERS-CoV infection in human (Cai *et al*, 2014; Meyerholz *et al*, 2016). In addition, the differential expression of DPP4 in the upper respiratory tracts has been shown to be responsible for higher susceptibility of humans than dromedary camels for MERS-CoV infection (Widagdo *et al*, 2016). Whether a different expression pattern or density of DPP4 on blood leukocytes contribute to this higher disease susceptibility in newborn camel calves than adult camels, it is unknown. The current study, therefore, analysed the impact of animal age on the expression density of DPP4 on the main populations of peripheral blood leukocytes of dromedary camels.

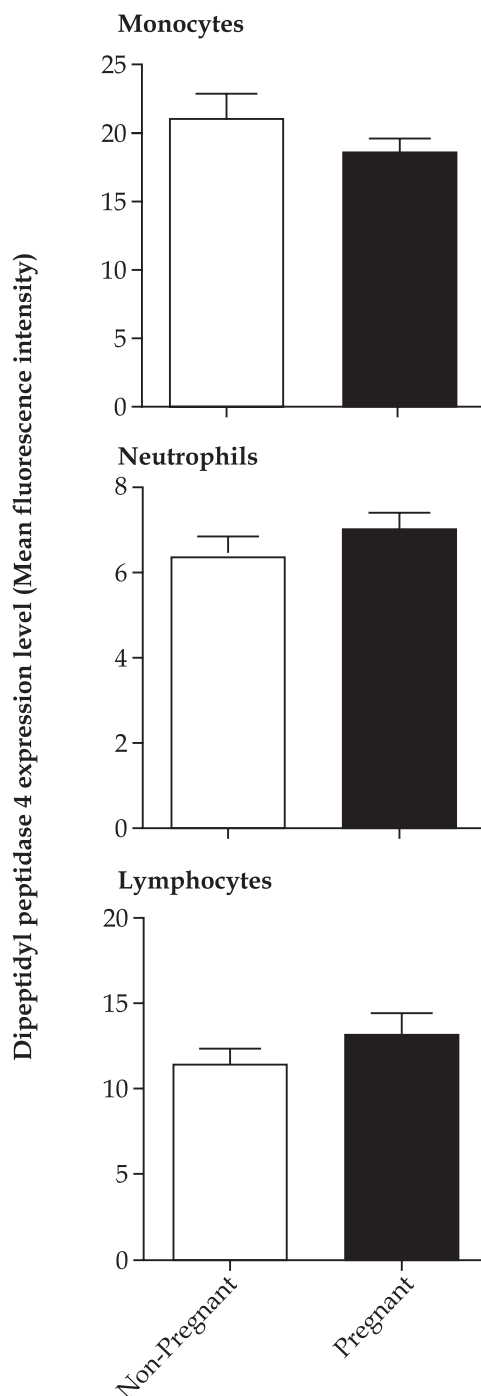
As shown in Fig 1B, monocytes from both newborn and adult camels expressed the highest levels of DPP4 when compared with neutrophils and lymphocytes (Fig 1B). The comparison between newborn calves and adult camels regarding the expression density of DPP4 on blood leukocytes revealed significantly higher expression levels on monocytes from adult camels than monocytes from calves (Fig 1B). For lymphocytes and neutrophils, however, newborn and adult camels showed comparable expression levels of DPP4 on their cells. Although the finding of the current study are in line with previous studies, showing highest DPP4 expression levels on camel monocytes (Al-Mubarak, 2018; Haverkamp *et al*, 2018), the results, however, argue against a role of different DPP4 expression levels on blood leukocytes in the higher susceptibility of camel calves than adults to MERS-CoV, as DPP4 is higher expressed on adult monocytes than calf monocytes. The evaluation of clinical importance of the higher abundance of DPP4 on adult monocytes needs further investigation.

### **The expression density of DPP4 on the surface of blood leukocytes from newborn and adult dromedary camels.**

Separated camel leukocytes were labeled with goat monoclonal antibodies to DPP4 and anti goat IgG secondary antibodies and labeled cells were analysed by flow cytometry. A) After gating on leukocytes,



**Fig 1. The expression density of DPP4 on the surface of blood leukocytes from newborn and adult dromedary camels.** Separated camel leukocytes were labeled with goat monoclonal antibodies to DPP4 and anti goat IgG secondary antibodies and labeled cells were analysed by flow cytometry. **A)** After gating on leukocytes, dead cells were excluded from the analysis by gating on propidium iodide negative cells. Based on their FSC/SSC properties, camel neutrophilic granulocytes (G), monocytes (M), and lymphocytes (L) were gated and the mean fluorescence intensity of DPP4 was calculated for each cell type. **B)** Data for cells from newborn and adult camels were presented as mean  $\pm$  SEM (\* =  $p < 0.05$ ).



**Fig 2. The impact of pregnancy on the expression density of DPP4 on the surface of camel blood leukocytes.** Separated camel leukocytes were labeled with goat monoclonal antibodies to DPP4 and anti goat IgG secondary antibodies and labeled cells were analysed by flow cytometry. After gating on leukocytes, dead cells were excluded from the analysis by gating on propidium iodide negative cells. Based on their FSC/SSC properties, camel neutrophilic granulocytes, monocytes, and lymphocytes were gated and the mean fluorescence intensity of DPP4 was calculated for each cell type. Data for cells from pregnant and non-pregnant animals were presented as mean  $\pm$  SEM (\* =  $p < 0.05$ ).

dead cells were excluded from the analysis by gating on propidium iodide negative cells. Based on their FSC/SSC properties, camel neutrophilic granulocytes (G), monocytes (M), and lymphocytes (L) were gated and the mean fluorescence intensity of DPP4 was calculated for each cell type. B) Data for cells from newborn and adult camels were presented as mean  $\pm$  SEM (\* =  $p < 0.05$ ).

Pregnancy is a physiologic process with several changes in the immunophenotype of camel blood leukocytes (Hussen *et al*, 2019). It is unknown, whether the expression density of DPP4 on blood leukocytes is affected by pregnancy in the dromedary she-camel. In the present study, the comparison between pregnant and non-pregnant female dromedary camels regarding the expression levels of DPP4 on their monocytes, neutrophils, and lymphocytes revealed no significant differences between the two groups (Fig 2). Although this indicates no significant effect of pregnancy on DPP4 expression on blood leukocytes, a direct pregnancy-associated change in DPP4 expression in the lung tissues can not be excluded.

### The impact of pregnancy on the expression density of DPP4 on the surface of camel blood leukocytes.

Separated camel leukocytes were labeled with goat monoclonal antibodies to DPP4 and anti goat IgG secondary antibodies and labeled cells were analysed by flow cytometry. After gating on leukocytes, dead cells were excluded from the analysis by gating on propidium iodide negative cells. Based on their FSC/SSC properties, camel neutrophilic granulocytes, monocytes, and lymphocytes were gated and the mean fluorescence intensity of DPP4 was calculated for each cell type. Data for cells from pregnant and non-pregnant animals were presented as mean  $\pm$  SEM (\* =  $p < 0.05$ ).

### Conclusions

The comparison between newborn calves and adult camels regarding the expression density of DPP4 on blood leukocytes revealed significantly higher expression levels on monocytes from adult camels than monocytes from calves. Newborn calves and adult camels showed comparable expression levels of DPP4 on their neutrophils and lymphocytes. The results of the current study argue against a role of different DPP4 expression levels on blood leukocytes in the higher susceptibility of camel calves to MERS-CoV. The comparable expression levels of DPP4 on monocytes, neutrophils, and lymphocytes

from pregnant and non-pregnant female camels indicates no significant impact of pregnancy on DPP4 expression on blood leukocytes of camels. Further work is needed to explore the impact of animal age and pregnancy on the local expression of DPP4 in the respiratory system of dromedary camel.

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