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UPREGULATION OF CORONAVIRUS (MERS-COV) RECEPTOR DIPEPTIDYL PEPTIDASE 4 ON CAMEL LEUKOCYTES AFTER BACTERIAL STIMULATION *IN VITRO*

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ABSTRACT

Middle East respiratory syndrome coronavirus (MERS-CoV) is an emerging zoonotic pathogen which causes high mortality rate in humans. Dromedary camels may play a central role in virus transmission to humans. Dipeptidyl peptidase-4 (DPP4), a transmembrane protein located on the cell surface of many epithelial and endothelial tissues was identified as the receptor for MERS-CoV. The current study investigated the possibility that bacterial stimulation of camel blood could affect the expression level of DPP4 on camel leukocyte subpopulation, which in turn may contribute to the higher susceptibility of camels with bacterial infection to MERS-CoV infection. DPP4 expression was evaluated by membrane immunofluorescence and flow cytometry. Stimulation of camel blood with the bacterial species *S. aureus* or *E. coli* resulted in the upregulation of DPPV on both monocytes and granulocytes, while *S. agalactiae* did not significantly modulate DPPV expression on either of the immune cells ($p > 0.05$). None of the bacterial species could induce a change in DPPV expression on lymphocytes from stimulated blood. Collectively, the present study showed an enhancing effect of bacterial stimulation on DPPV expression on camel monocytes and granulocytes.

Key words: Bacterial stimulation, coronavirus, dipeptidyl peptidase 4, leukocytes, MERS-CoV, Middle East respiratory syndrome

According to recent studies, dromedary camels play a pivotal role in Middle East Respiratory Syndrome Coronavirus (MERS-CoV) transmission to humans (Reusken *et al,* 2013; Adney *et al,* 2014; Reusken *et al,* 2014a; 2014b; Gossner *et al,* 2016; Haagmans *et al,* 2016; Harcourt *et al,* 2018; Te *et al,* 2022a). Members of the camelids family are currently discussed as a research model to study the pathogenesis of the MERS-CoV in human (Gultom *et al,* 2022; Te *et al,* 2022a; 2022b).

Dipeptidyl peptidase-4 (DPP4), which is also called CD26, is a transmembrane protein located on the cell surface of many epithelial and endothelial tissues in several organs such as kidneys, lungs, liver, and intestine (Lambeir *et al,* 2003; Klemann *et al,* 2016). Its expression levels and activity were found to be altered in several diseases, including inflammatory and infectious diseases and tumours (Hildebrandt *et al,* 2000; Dang and Morimoto, 2002; Klemann *et al,* 2016).

As the Middle East respiratory syndrome-coronavirus (MERS-CoV) emerged in 2012 (Zaki *et al,* 2012), DPP4 was identified as its receptor (Raj *et al,* 2013). After binding of the spike (S) protein of the virus on DPP4, virus entry is initiated by receptor mediated endocytosis (Lu *et al,* 2013; Wang *et al,* 2013). The expression level of CD26 on the surface of cells of respiratory tract has been found to be correlated positively with susceptibility to MERS-CoV infection (Cai *et al,* 2014; Meyerholz *et al,* 2016). CD26 Monoclonal Antibody was also able to inhibit MERS-CoV infection *in vitro* (Ohnuma *et al,* 2013)

In the current study, we investigated the upregulation of corona virus (MERS-CoV) receptor DPP4 on camel leukocyte subpopulation after bacterial stimulation *in vitro*.

Materials and Methods

Animals and blood sampling

Blood samples were collected from 3 male and 3 female dromedary camels (*Camelus dromedarius*) aged between 9 and 11 housed at the Camel Research Centre, King Faisal University, Al-Ahsa, Saudi Arabia. All animals were fed on hay and barley in addition to a mineral supplement. Water was available *ad libitum*. Blood was obtained by jugular venipuncture into

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vacutainer tubes containing EDTA (Becton Dickinson, Heidelberg, Germany). Collected blood samples were stimulated *ex vivo*.

Bacterial stimulation of camel blood samples

Whole camel blood was stimulated with *S. aureus*, *E. coli*, or *S. agalactiae*. Whole blood (1 ml) was diluted with 0.9 ml medium (RPMI-1640, Sigma-Aldrich, Deisenhofen, Germany) in sterile 12x75 mm glass tubes (BD Biosciences, San Jose, California, USA) (Hussen, 2021). Live bacterial suspension (0.1 ml; 10^7 bacteria/ml) was added to the diluted blood and the mixture was then incubated for 6 h at 37°C. A negative control tube containing 1 ml blood and 1 ml medium without bacteria was also included. After incubation, the tubes were then put on ice and immediately centrifuged at 4°C for 10 min at 1000xg. After removing the supernatant, the cell pellet was suspended in PBS.

Leukocytes separation from camel blood

Separation of blood leukocytes was performed after hypotonic lysis of blood erythrocytes (Hussen *et al,* 2017; Hussen *et al,* 2021). After centrifugation at 500 × g for 10 min (4°C) with break, the cell pellet was resuspended. The erythrolysis was repeated (usually twice) until the complete removal of red blood cells. Subsequently, the cells were suspended in 10 ml PBS and washed two times (250 × g and 100 × g for 10 min each) and finally adjusted to 5 x 10^6 cells/ml in MIF buffer (PBS containing bovine serum albumin (5 g/L) and NaN3 (0.1 g/L)).

Cell labeling with DPPV antibodies and flow cytometry

Separated camel blood leukocytes (4 x 10^5) were incubated with monoclonal goat IgG antibody (R & D Systems) specific for CD26 molecules (Pierson *et al,* 2008; van Doremalen *et al,* 2014) in PBS containing bovine serum albumin (5 g/l) and NaN3 (0.1 g/l). After 30 minutes incubation (4°C), cells were washed twice and incubated with a Alexa Fluor 488a-labelled rabbit F(ab')2-anti-goat IgG (H+L) secondary antibody. After incubation (30 minutes; 4°C), labelled cells were washed twice and analysed on the flow cytometer (Accuri C6, Becton Dickinson Biosciences, San Jose, California, USA). At least 100 000 cells were collected and analysed with the CFlow Software, Version 1.0.264.21 (BD Biosciences).

Statistical Analyses

Statistical analysis was performed with Prism (GraphPad). Results are presented as means ± S.E. of the mean (SEM). Differences between means were tested with one-factorial analysis of variance (ANOVA) and Bonferroni's correction for normally distributed data. Results were considered significant at a p-value of less than 0.05.

Results and Discussion

The present study investigated the impact of *in vitro* stimulation with different bacterial species on the expression level of DPP4 whose expression was evaluated by membrane immunofluorescence and flow cytometry using a goat polyclonal antibody to human DPP4, which is reactive with camel DPP4 (Widagdo *et al,* 2016). After the exclusion of cell doublets, gates were made on the different subsets of camel leukocytes (Fig 1A). DPP4 expression on cells from unstimulated blood and cells from blood stimulated with *Staphylococcus aureus* (*S. aureus*), *Streptococcus agalactiae* (*S. agalactiae*) and *Escherichia coli* (*E. coli*) was evaluated as the mean fluorescence intensity (Fig 1B).

The comparison between leukocytes populations regarding the basic expression level of DPPV confirms the higher expression level on monocytes compared to lymphocytes and granulocytes. However, unstimulated granulocytes and lymphocytes showed a comparable expression level of DPPV ($p<0.05$). Stimulation of whole camel blood with the bacterial species *S. aureus* or *E. coli* resulted in the upregulation of DPPV on both monocytes and granulocytes ($p<0.05$), while *S. agalactiae* did not modulate DPPV expression significantly on either of the immune cells ($p>0.05$). None neither of the bacterial species induced a change in DPPV expression on lymphocytes from stimulated blood, ($p>0.05$).

In a previous report, the expression level of DPP4 on monocytes increased significantly after stimulation with LPS (Al-Mubarak, 2018). In the humans, a similar enhancement of DPP4 expression was observed upon *in vitro* activation of human monocytes as well as on activated alveolar macrophages in human patients with chronic lung diseases (Zhong *et al,* 2013; Meyerholz *et al,* 2016).

The increased expression of DPPV on cells from the upper respiratory tract of human has been linked to their higher susceptibility to MERS-CoV infection compared to dromedary camels (Widagdo *et al,* 2016). The results from the present study indicated a possible role of bacterial infections in increasing the susceptibility of camels to MERS infection. Further studies are needed to confirm the findings

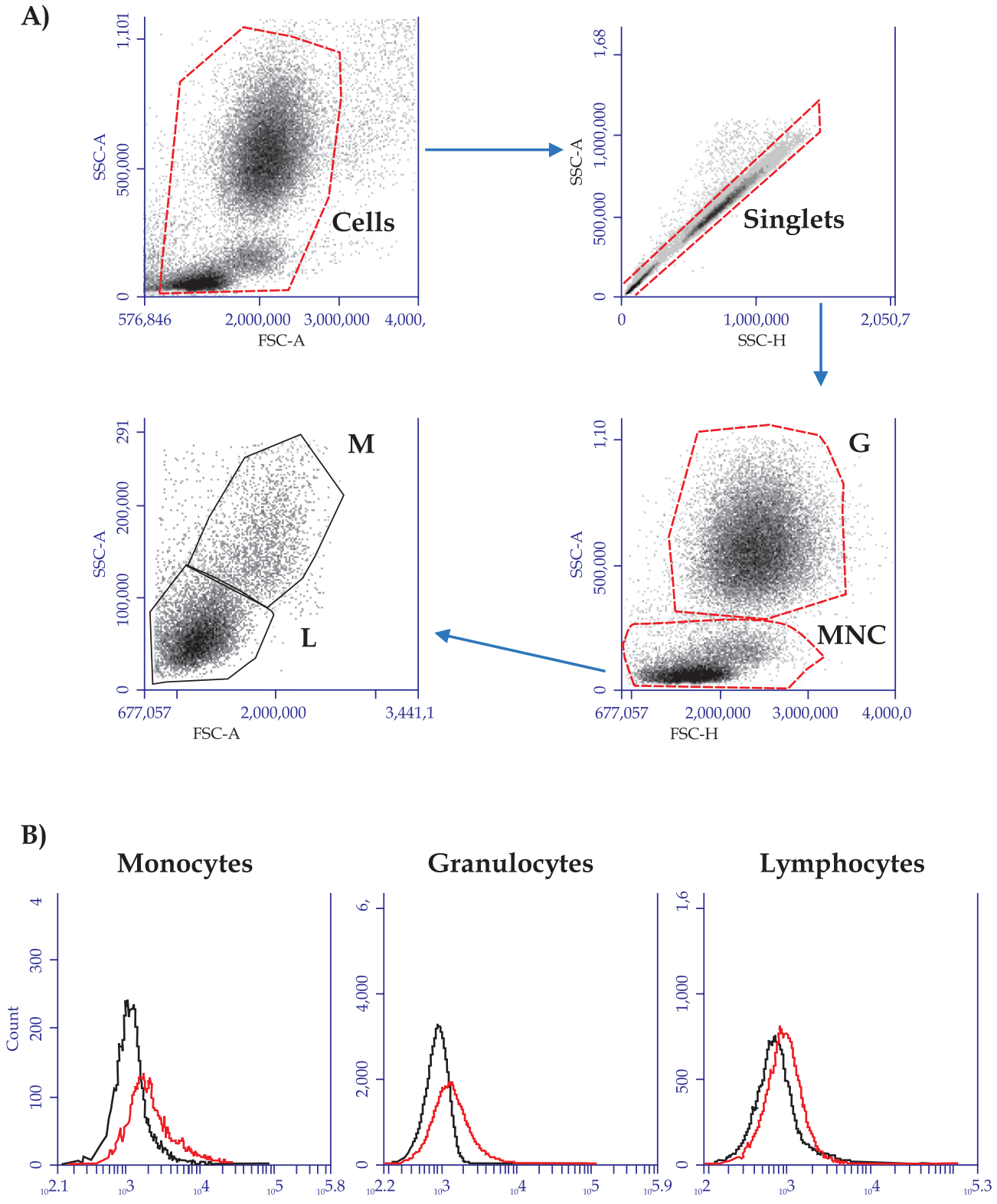

Fig 1. Flow cytometric analysis of DPP4 expression on the main leukocyte populations in stimulated and unstimulated peripheral blood of dromedary camel. Camel leukocytes were separated by hypotonic lysis of erythrocytes and the separated cells were labeled with polyclonal antibody to DPP4 and analysed by flow cytometry. A) After the exclusion of cell duplicates using a SSC-H/SSC-A dot plot, a SSC/FSC dot plot was used to identify granulocytes (G), monocytes (M) and lymphocytes (L) according to their forward and sideward scatter characteristics. B) After setting a gate on monocytes, granulocytes, and lymphocytes, the mean fluorescence intensity (MFI) of DPP4 for stimulated (red lined histogram) or unstimulated cells (black lined histogram) was presented in overlay histograms (data presented is for blood stimulated with *S. aureus*).

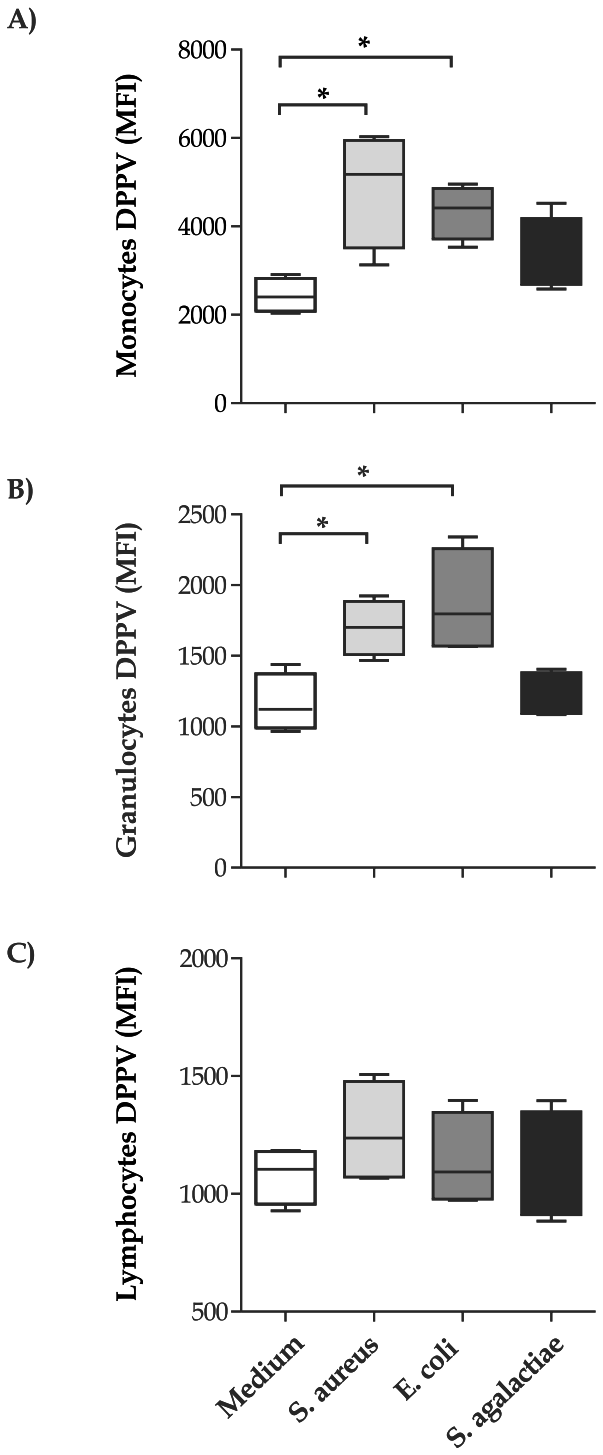

Fig 2. The effect of stimulation with live bacteria of camel blood on the expression level of DPP4 on the main leukocyte populations. Camel whole blood was stimulated for 4h with *S. aureus*, *E. coli*, or *S. agalactiae*. Separated leukocytes were labeled with polyclonal antibody to DPP4 and analysed by flow cytometry. After setting a gate on granulocytes, monocytes and lymphocytes, the mean fluorescence intensity (MFI) of DPP4 expression on granulocytes (G), monocytes (M) and lymphocytes (L) for stimulated and unstimulated leukocytes was calculated and presented graphically as mean +- SEM (* = $p<0.05$).

of the current study by the evaluation of disease susceptibility in healthy camels and camels with bacterial infections.

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Conflict of interest:

None.

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