DOI : 10.5958/2277-8934.2021.00009.6 *Vol 28 No 1, p 59-67*

IMMUNOMODULATORY EFFECT OF *Escherichia coli* LIPOPOLYSACCHARIDE ON PHENOTYPE AND FUNCTION OF BLOOD MONOCYTES IN CAMELS

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ABSTRACT

The objective of the current study was to investigate the effect of lipopolysaccharide (LPS) from *E. coli* on the phenotype and the function of the camel monocytes. Flow cytometry was used to analyse the expression of different myeloid markers and cell adhesion molecules on camel monocytes and to evaluate the ability of monocytes to engulf bacteria and to generate reactive oxygen species (ROS). In LPS-stimulated blood, monocytes showed shifting toward inflammatory macrophage-1 (M1) profile by enhancing the expression of high levels of MHCII molecules and reduced levels of CD163. Furthermore, LPS-stimulated monocytes upregulated the expression of the adhesion molecules CD62L and CD11b while downregulated the expression of CD18. Functionally, stimulation with LPS reduced the phagocytosis capability of monocytes but enhanced their ability to produce ROS. These results suggest a modulating effect of LPS on the phenotype, adhesion, and phagocytic functions of the camel blood monocytes and propose a possible new immune evasion mechanism.

Key words: Adhesion molecules, camel, innate immunity, monocytes, lipopolysaccharide, phagocytosis, ROS

Escherichia coli (*E. coli*) is a gram-negative bacterium, which causes several diseases in the dromedary camel. This includes mastitis and metritis in adult female camels and septicemia in the newborn camel calf leading to high mortality rates early in life (Aljumaah *et al,* 2011; Al-Ruwaili *et al,* 2012).

Monocytes are circulating immune cells with a key role in innate immunity to bacterial pathogens. In addition to their ability to ingest and kill bacteria, monocytes constitute the main source of tissue macrophages upon migration from the bloodstream to tissues (Soehnlein and Lindbom, 2010; Jakubzick *et al,* 2017; Pomeroy *et al,* 2017). The immunophenotype of blood monocytes is characteristic of their functional subtype. Depending on the type of the activating signal, monocytes undergo different phenotypic and functional changes. The expression of the monocytic markers CD172a, CD14, CD163, and MHCII are good indicators for the functional subtype of monocytes during their differentiation into macrophages (Schwartz and Svistelnik, 2012; Thawer *et al,* 2013; Hussen *et al,* 2014; Hussen and Schuberth, 2017). CD172a, which is known as the signal-regulatory protein alpha (SIRPa), is glycosylated cell surface receptor expressed on myeloid cells and functions as a regulatory receptor that inhibits cell signaling (Hussen *et al,* 2013). In camels, monocyte subsets I and II show higher abundance of CD14 than monocyte subset III (Hussen *et al,* 2020). Due to the low expression of CD14 and CD16, mouse monocytes are identified based on the expression of Ly6C and CD43 (Zawada *et al,* 2012).

Lipopolysaccharide is an important component of the gram-negative bacterial outer membrane and is considered a powerful activator of the innate immune response. The impact of LPS stimulation on the phenotype and function of camel monocytes has not been yet studied. The aim of the current study was to evaluate the immunomodulating effect, in terms of phenotype and function, of *E. coli*-lipopolysaccharide stimulation on camel blood monocytes *in vitro*.

Materials and Methods

Blood sampling

Blood samples were collected from 7 healthy dromedary camels (*Camelus dromedarius*) aged between 6 and 9 years by venipuncture of the vena jugularis externa into EDTA-containing vacutainer tubes (Becton Dickinson, Heidelberg, Germany).

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LPS whole blood stimulation

Whole blood stimulation was performed as described previously. Blood from healthy camels was stimulated with 1 µg/ml Lipopolysaccharide purified from *E. coli* O55:B5 (Sigma-Aldrich, Germany) at 37°C in 5% CO_2 or left without stimulation. After incubation for 4 h, blood samples were diluted with phosphate buffer saline (1:1) and centrifuged at 4°C for 10 min at 1000xg. After removing the supernatant, the cell pellet was resuspended in PBS.

Leukocytes separation

Separation of whole leukocytes from camel blood samples was performed with hypotonic lysis of blood erythrocytes (Hussen *et al,* 2013). Briefly, blood cells were suspended in distilled water for 20 sec. Later, double-concentrated PBS was added to restore tonicity. This step was repeated at least twice or until complete erythrolysis. The remaining cells were finally resuspended in MIF (Membrane Immunofluorescence) buffer composed of PBS containing 5 g/l of bovine serum albumin and 0.1 g/l of NaN_3 at a concentration of 5 x 10^6 cells/ml. The mean viability of the separated leukocytes was determined by the dye exclusion method using 2 µg/ml of propidium iodide (Calbiochem, Germany). The mean leukocyte viability in our experiments was above 95%.

Membrane immunofluorescence and flow cytometry

The expression of monocytic markers and cell adhesion molecules was analysed using membrane immunofluorescence test (Eger *et al,* 2015; Hussen *et al,* 2017). For blocking of FC receptor binding, separated camel blood leukocytes (4 x 10^5) were incubated with MIF buffer containing 5% autologous camel serum for 20 min at 4°C in 96 well round-bottom microtitre plates. After two times washing with MIF buffer (300 xg for 3 min at 4°C), cells were incubated with monoclonal antibodies (mAbs) specific for the monocytic markers CD172a, CD14, CD163, and MHCII and the cell adhesion molecules CD18, CD11a, CD11b, and CD62L cross-reactive with homologous camel molecules (0.2 µg of each mAb in 100 µl MIF buffer/well) (Hussen *et al,* 2017). After incubation for 15 min at 4°C, cells were washed with MIF buffer twice and incubated with mouse fluorochrome-labeled secondary antibodies (IgG1, IgG2a; 0.2 µg in 100 µl MIF buffer/well; Invitrogen) or with mouse isotype control antibodies (0.2 µg of each mAb in 100 µl MIF buffer/well; Becton Dickinson Biosciences, USA). After washing, the cells were analysed on a Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson Biosciences, California, USA). Data of 10^5 cells were collected and analysed with the flow cytometric software FlowJo (FLOWJO LLC). After the exclusion of dead cells (PI-negative cells), forward and sideward scatter were used to gate for monocytes. The median fluorescence intensity (MFI) for the selected CD marker was measured (Fig 1).

Phagocytosis Assay

Heat-killed *Staphylococcus aureus* (*S. aureus*) (Merck, Nottingham, UK) was labeled with fluorescein isothiocyanate (FITC) (Sigma-Aldrich, Missouri, USA). Leukocytes were separated from LPS-stimulated (4 h) or un-stimulated camel blood. Separated leukocytes were plated in 96-well plates at a density of 10^6 cells per well and incubated with the heat-killed FITC-labeled *S. aureus* (50 bacterial cells per leukocyte) for 30 minutes at 37°C in a 5% CO_2 incubator. Additionally, leukocytes, which were neither induced with LPS nor incubated with bacteria, were used as control. After incubation, propidium iodide (PI) (2 µg/ml final) was added to exclude dead cells and samples were analysed by flow cytometry. Phagocytic activity of monocytes was calculated as the percentage of cells expressing green fluorescence among all viable monocytes. The mean green fluorescence intensity (MFI) of phagocytosis-positive monocytes was measured as an indicator for the number of the phagocytosed bacteria by each monocyte.

Generation of ROS

The ROS-generation was measured as previously described (Hussen *et al,* 2016). LPS-stimulated or uon-stimulated camel leukocytes (1×10^6/well) were incubated without or with heat-killed non-opsonised (50 bacteria/cell) *S. aureus* (Pansorbin, Calbiochem, Merck, Nottingham, UK) for 20 min (37°C, 5% CO_2). For the detection of ROS, dihydrorhodamine (DHR123) (Mobitec, Goettingen, Germany) was added to the cells at a final concentration of 750 ng/ml. Later, the cells were washed with MIF buffer and the relative amount of the generated ROS was determined by the median green fluorescence intensity of gated monocytes.

Study ethics

This study obtained ethical approval from the Ethics Committee at King Faisal University, Saudi Arabia (Permission number: KFU-REC/2019-10-01).

Statistical Analyses

Statistical analysis was performed with the software Prism (GraphPad). Results were presented

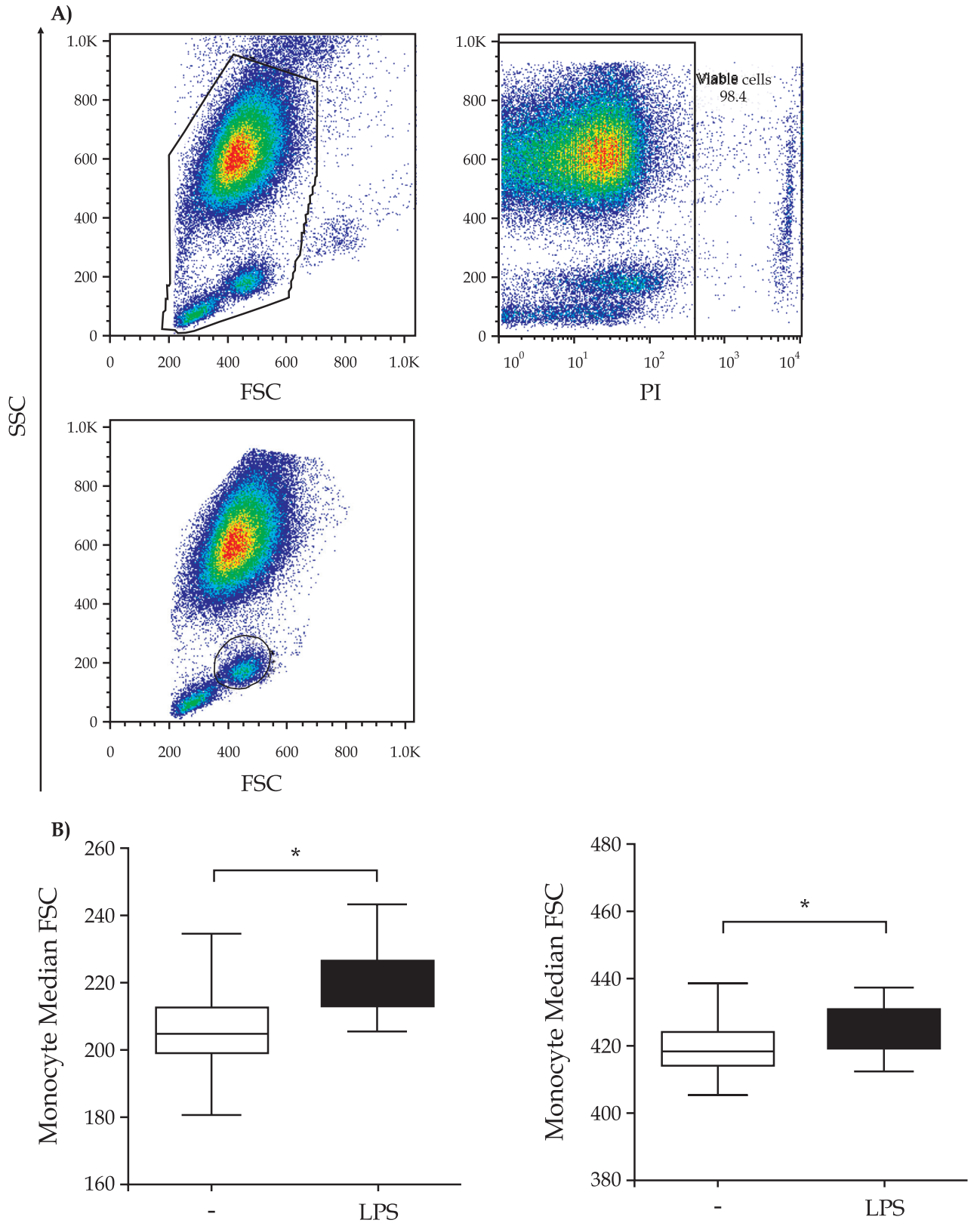

Fig 1. A) Gating strategy for flow cytometric analysis of the expression of myeloid markers and cell adhesion molecules on camel blood monocytes. After setting a gate on total leukocytes in an SSC/FSC dot plot, dead cells were excluded based on their positive staining with propidium iodide. In a SSC/FSC dot plot, monocytes were gated based on their forward and side scatter properties. The mean SSC and FSC of gated monocytes were measured and presented for unstimulated and stimulated cells (* = $p<0.05$).

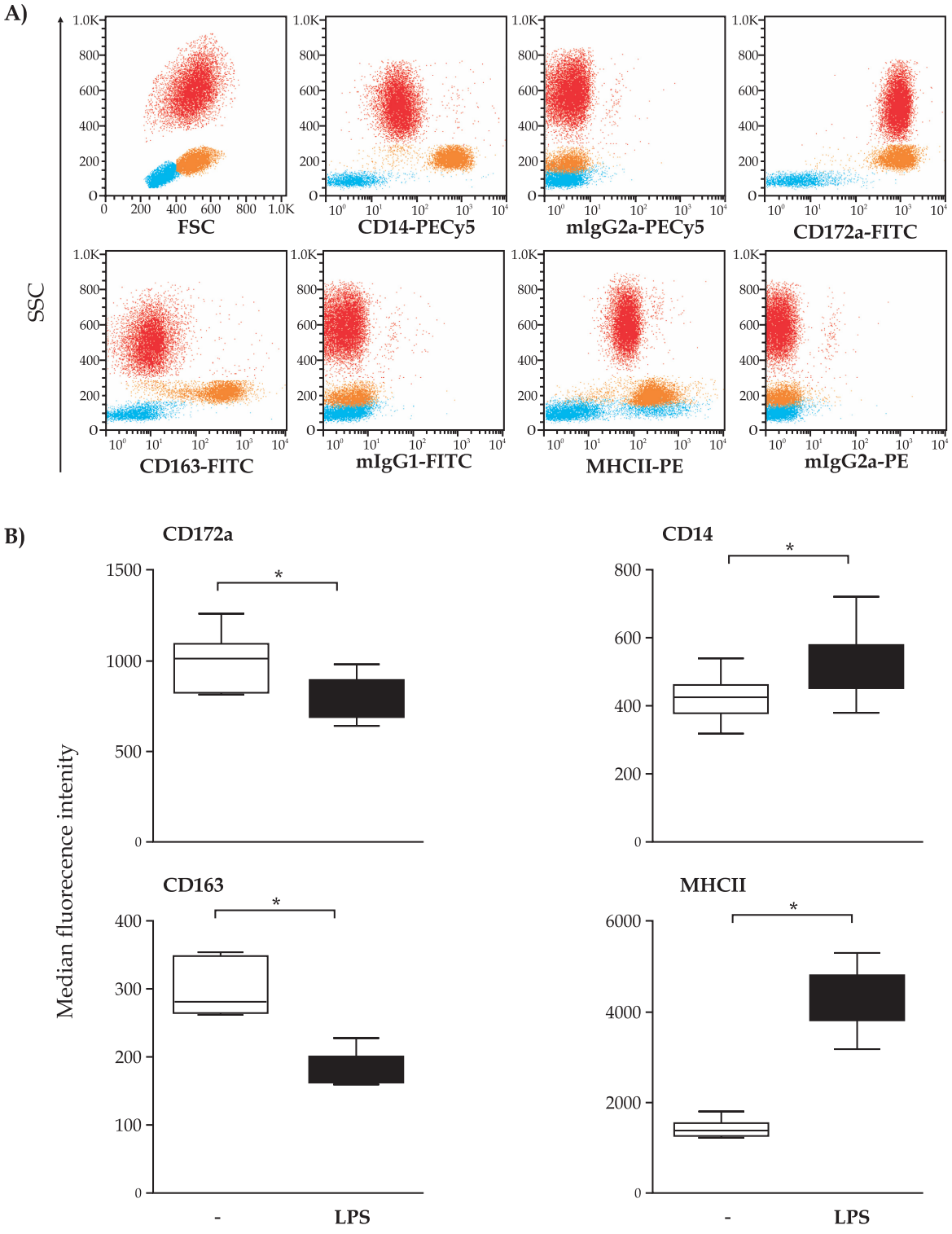

Fig 2. **A)** The staining pattern of camel leukocyte populations with monoclonal antibodies to CD172a, CD14, CD163, and MHCII. In an FSC against SSC dot plot, camel granulocytes, monocytes, and lymphocytes were identified based on their FSC and SSC characteristics. After setting gates on granulocytes (in red color), monocytes (in orange color), and lymphocytes (in blue colour), the staining patterns of different leukocyte populations with the used monoclonal antibodies were shown in separate dot plots. **B)** The impact of LPS-stimulation on the expression of the myeloid markers CD172a, CD14, CD163, and MHCII on camel blood monocytes. Camel's blood was stimulated with LPS for 4 h. After hypotonic lysis of erythrocytes, leukocytes were labeled with monoclonal antibodies to CD172a, CD14, CD163, and MHCII molecules. Labeled cells were analysed by flow cytometry. After setting a gate on monocytes, the main fluorescence intensities of labeled cells were calculated and presented as means ± SEM. (* = $p<0.05$).

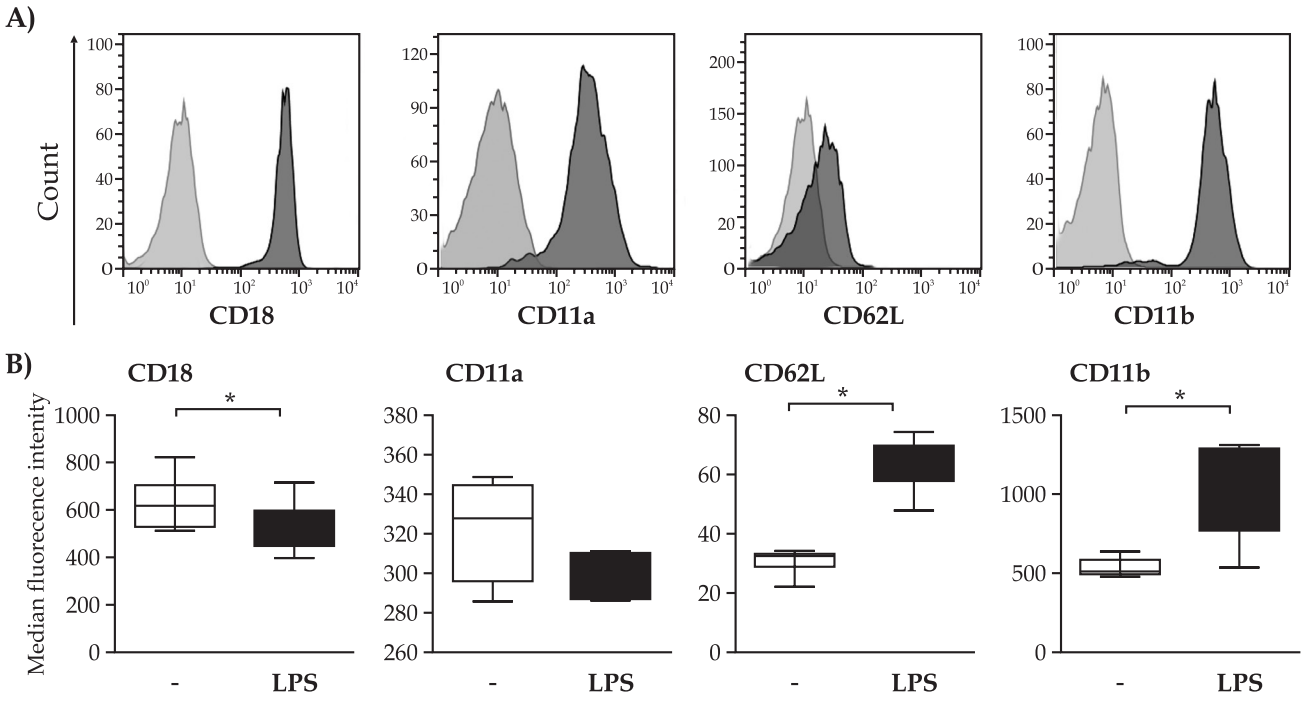

Fig 3. Influence of LPS-stimulation on adhesion molecules expression on blood monocytes. Camel blood was stimulated with LPS for 4 h. After hypotonic lysis of erythrocytes, separated leukocytes were labeled with monoclonal antibodies to CD18, CD11a, CD11b, and CD62L. Labeled cells were analysed by flow cytometry. A) Monocytes were gated based on their FSC and SSC properties. The staining of monocytes with monoclonal antibodies to CD18, CD11a, CD11b, and CD62L or with mouse isotype controls was shown as histograms. **B)** After setting a gate on monocytes, median fluorescence intensities of labeled cells for CD18, CD11a, CD11b, and CD62L were calculated and presented as means ± SEM. (* = $p<0.05$).

as means ± S.E. of the mean (SEM). The t-test (two groups) was used to test the difference between means. For the comparison between more than two groups (The impact of LPS on ROS production in monocytes with or without bacteria), the one-factorial analysis of variance (ANOVA) was used. A p-value of less than 0.05 was considered significant.

Results

LPS-stimulation modulates the expression of monocytic markers

Stimulation with LPS induced monocyte activation as measured by the increased median FSC and SSC (Fig 1).

In LPS-stimulated blood, monocytes changed the expression of different monocytic markers. The median fluorescence intensities (MFI) of the molecules CD172a (390 ± 16 versus 495 ± 22) and CD163 (112 ± 6 versus 182 ± 11) on monocytes were significantly reduced in LPS-stimulated blood in comparison to unstimulated blood. In contrary to this, LPS-stimulated blood showed higher MFI values for monocyte CD14 (345 ± 10 versus 285 ± 7) and MHCII molecules (4164 ± 117 versus 1455 ± 47) (Fig 2).

Effects of LPS-stimulation on the expression pattern of cell adhesion molecules on monocytes

LPS stimulation also modulated the expression of different cell adhesion molecules on blood monocytes. In comparison to unstimulated blood, the expression of CD11b (MFI 453 ± 58 versus 302 ± 15) and CD62L (MFI 69 ± 5 versus 25 ± 0.5) on monocytes was significantly increased in LPS-stimulated blood, while the expression of CD18 (MFI 372 ± 30 versus 481 ± 34) was significantly reduced. However, the expression of CD11a on monocytes did not change after stimulation with LPS (Fig 3).

Impact of LPS stimulation on phagocytosis capacity of monocytes

The capacity of the monocytes to phagocytose FITC-labelled *S. aureus ex vivo* was significantly affected by LPS-stimulation. In LPS-stimulated blood, the percentage of phagocytosis-positive monocytes was significantly lower than that in unstimulated blood (24 ± 2 versus 55 ± 9). The MFI of phagocytosis-positive monocytes, as an indicator for the number of bacteria ingested by each monocyte, was also lower in LPS-stimulated blood in comparison to unstimulated blood (598 ± 22 versus 780 ± 116) (Fig 4).

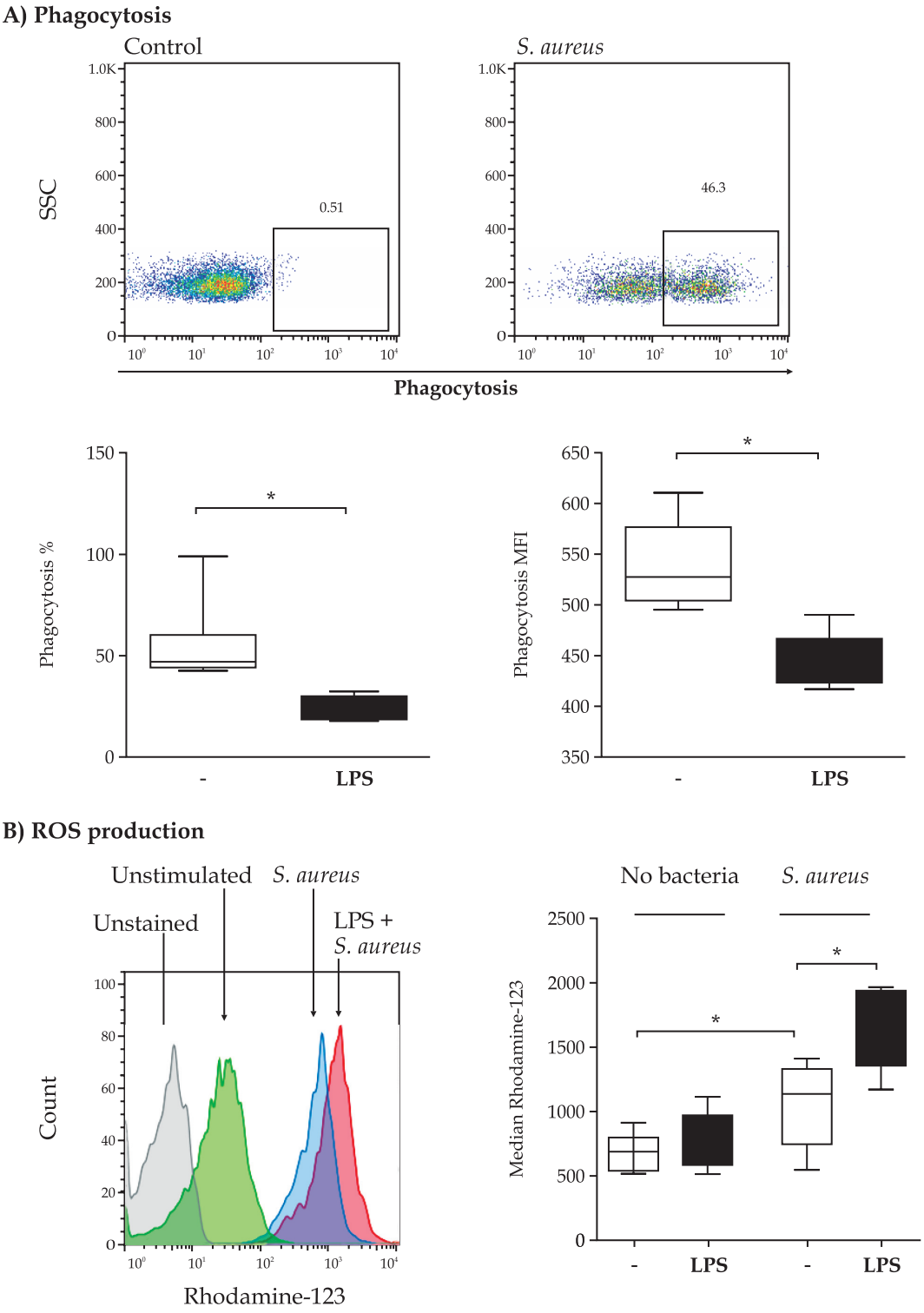

Fig 4. The impact of LPS stimulation on phagocytosis and ROS activity of the camel monocytes. Camel's blood was stimulated with LPS for 4 h or was left without stimulation (control). A) After red blood cell lysis, LPS-stimulated and un-stimulated leukocytes were incubated with FITC-labelled heat inactivated *S. aureus* and analysed by flow cytometry. After setting a gate on monocytes, phagocytosis-positive cells were defined based on their higher green fluorescence (representative results are shown in **A).** The percentage of phagocytosis and the median fluorescence intensities of green fluorescence-positive monocytes were calculated (means ±SEM). (* = $p<0.05$). **B)** LPS-stimulated and un-stimulated leukocytes were incubated with heat-inactivated *S. aureus* in the presence of the ROS-sensitive dye dihydrorhodamin 123 and labeled cells were analysed by flow cytometry (representative results are shown in B). After setting a gate on monocytes, ROS production was calculated as the median green fluorescence intensity of gated cells (means ± SEM). (* = $p<0.05$).

Impact of LPS stimulation on reactive oxygen generation in camel monocytes

Stimulation with *S. aureus* significantly induced ROS production in camel monocytes. In LPS-stimulated blood, monocytes produced significantly more ROS upon incubation with *S. aureus* when compared with monocytes from unstimulated (without LPS) blood (1654 ± 192 versus 1210 ± 67). LPS stimulation alone, however, did not induce a significant change in median ROS values of camel monocytes (Fig 4).

Discussion

Infections with the gram-negative bacterium *E. coli* are responsible for several illnesses in the dromedary camel including gastroenteritis and septicemia in camel calves and mastitis and metritis in adult she-camels (Aljumaah *et al,* 2011; Al-Ruwaili *et al,* 2012). Studies on the interaction of *E. coli* with the innate immune system of the dromedary camel are scarce. Monocytes play a key role in the antibacterial immune response through their ability to ingest and kill bacteria and to differentiate into different subtypes of tissue macrophages (Soehnlein and Lindbom, 2010; Jakubzick *et al,* 2017; Pomeroy *et al,* 2017). Depending on the type of the activating signal, monocytes undergo different phenotypic and functional changes.

To analyse the impact of the *E. coli* lipopolysaccharide (LPS) on the phenotype and the function of blood monocytes in dromedary camel, we used the whole blood stimulation model, which has the advantage of maintaining the microenvironment of immune cell interaction as it occurs *in vivo* (Gomes *et al,* 2010). LPS-stimulated camel blood monocytes showed polarisation toward the inflammatory macrophage (M1) subset as indicated by the upregulated expression of MHCII and downregulated expression of CD163 markers. The inflammatory nature of LPS-stimulated monocytes is also supported by the higher expression of the LPS-receptor CD14 and the lower expression of the signal-regulatory protein alpha (SIRPα), which functions as a regulatory receptor that inhibits cell signaling (Hussen *et al,* 2013).

Monocyte migration starts with their adhesion to endothelial cells of blood vessels, which is mediated by a set of cell adhesion molecules on monocytes and their ligands on endothelial cells (Imhof and Aurrand-Lions, 2004; Gerhardt and Ley, 2015). LPS stimulation of camel monocytes induced the upregulation of L-selectin, which is constitutively expressed on non-activated leukocytes and is rapidly shed upon chemotactic stimulation (Amulic *et al,* 2012). This indicates an inhibitory effect of LPS-stimulation on monocyte adhesion and likely transmigration. This is also supported by the LPS-induced downregulation of CD18, the beta chain of the cell adhesion molecule Mac-1 (CD11b/CD18), which mediates the subsequent firm adhesion of monocytes to the activated endothelium (Imhof and Aurrand-Lions, 2004; Gerhardt and Ley, 2015). However, the expression of CD11a was unchanged and the expression of CD11b was even enhanced on the LPS-stimulated monocytes in our study. These two molecules are essential for the adhesion of the migrating monocytes (Imhof and Aurrand-Lions, 2004; Hussen *et al,* 2013; Gerhardt and Ley, 2015; Hussen *et al,* 2016). CD11a requires to dimerize with CD18 to form the adhesion molecule LFA-1 (Roos and Law, 2001; van de Vijver *et al,* 2012). The lack of one of the heterodimer components renders this molecule nonfunctional. Similarly, the CD11b binds to CD18 to form the complement receptor 3 (CR3), which plays an important role in opsonisation and enhancing phagocytosis (Ley *et al,* 2007; Muller, 2013). Therefore, through the downregulation of CD18, LPS impairs leukocyte adhesion and phagocytosis.

Phagocytosis of bacterial pathogens and the subsequent killing of ingested bacteria are key anti-microbial effector mechanisms of monocytes during the first stages of the innate immune response (Hussen *et al,* 2013). Our data showed that LPS-stimulated monocytes have a reduced capacity to ingest *S. aureus*, but produced more ROS upon stimulation with the same bacteria. This indicates a negative effect of LPS on the antimicrobial capability and an enhancing effect on the pro-inflammatory function of monocytes.

In a previous report, we described three heterogenic subpopulations of monocytes in dromedary camels based on the expression profiles of MHCII and CD14 (Hussen *et al,* 2020). Subset one expresses high levels of CD14 and low levels of MHCII and is the most abundant monocytes. Subset two is a minor subset of monocytes, which expresses high levels of CD14 and MHCII and is considered the inflammatory monocytes with increased phagocytic activity. While subset three is another minor subpopulation of monocytes with low levels of CD14 and high levels of MHCII. LPS stimulation of camel monocytes in the current study seems to drive the monocyte population into a new subtype resembling subset two but with reduced phagocytic activity

resembling subset three. This might represent a new immune evasion mechanism by which *E. coli* escapes phagocytosis. Indeed, treatment of mouse bone marrow-derived macrophages with LPS was shown to induce tolerance and impaired *E. coli* phagocytosis (Kapellos *et al*, 2016).

Conclusions

The enhanced expression of MHCII molecules and the reduced levels of CD163 on LPS-stimulated camel monocytes indicate a shifting toward inflammatory macrophage-1 (M1) profile. LPS-stimulated monocytes increased the expression of the adhesion molecules CD62L and CD11b while decreased the expression of CD18. Functionally, stimulation with LPS reduced the phagocytosis capability of monocytes but enhanced their ability to produce ROS. Collectively, these results suggest a modulating effect of LPS on the phenotype, adhesion, and phagocytic functions of camel blood monocytes and propose a possible new immune evasion mechanism. Whether these effects contribute to the pathogenesis of *E. coli* infections in dromedary camels, needs further studies. Although the current study may contribute to the understanding of the response of camel monocytes to LPS, several questions are still open in this regards, including LPS-tolerance in camels and the characterization of functional subtypes of camel monocyte-derived macrophages.

Acknowledgements

The authors extend their appreciation to the Deputyship for Research & Innovation, Ministry of Education in Saudi Arabia for funding this research work through the project number IFT20010.

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