Bacterial Species-Specific Modulatory Effects on Phenotype and Function of Camel Blood Leukocytes

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Research article

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Abstract

Background: Recent studies have reported pathogen-species-specific modulating effects on the innate immune system. *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus agalactiae* are important pathogenic bacteria responsible for different infectious diseases in several animal species. In the present study, a whole blood culture and flow cytometry were used to investigate, whether stimulation with different bacterial species induces different immunomodulation patterns in camel leukocytes.

Results: Stimulation with either of the bacterial species resulted in the expansion of the camel CD14^{high}MHCII^{high} monocyte subset with a reduced fraction of CD14^{high}MHCII^{low} monocytes. For the CD14^{high}MHCII^{low} monocytes, however, only stimulation with *S. aureus* or *S. agalactiae* increased their fractions in blood. Although all bacterial species elicited the upregulation of cell surface MHC-II molecules on granulocytes, the increase was, however, highest on cells stimulated with *S. aureus*. The expression levels of the two adhesion molecules, CD11a and CD18, on neutrophils and monocytes were differently affected by bacterial stimulation. Functionally, *E. coli* failed to stimulate ROS production in monocytes, while induced a strong ROS production response in granulocytes. *S. agalactiae* elicited a weak ROS production in granulocytes when compared to the other two pathogens.

Conclusions: The different responsiveness of monocytes and granulocytes toward different bacterial species indicates different host-pathogen interaction mechanisms for the two cell populations. In addition, the phenotypic and functional differences between cells stimulated with *E. coli*, *S. aureus*, or *S. agalactiae* suggests pathogen-species-specific modulating effects of the bacterial pathogens on the camel innate myeloid cells.

1 Background

Pathogen-species-specific modulatory effects on the innate immune system have been described in several species and for different pathogens [1–7]. *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), and *Streptococcus agalactiae* (*S. agalactiae*) are major causative agents of different infectious diseases in the dromedary camel including metritis, mastitis, and neonatal infections [2, 8]. For the three different bacterial species, different host-pathogen interaction mechanisms and different disease outcomes have been reported [9, 10]. In animals with mastitis, infection with *E. coli* results mostly in severe acute inflammatory disease with clinical signs [1], whereas *S. aureus* or *S. agalactiae* are mainly responsible for mild subclinical infections of the udder [1, 3, 5, 11].

Based on the surface expression of CD14 and MHCII, camel monocytes have been recently classified into monocyte subset Mo-I (CD14^{high}MHCII^{low}), monocyte subset Mo-II (CD14^{high}MHCII^{high}), and monocyte subset Mo-III (CD14^{low}MHCII^{high}) [12]. Different phenotypic and functional differences have been identified between the three subsets of camel monocytes [12].

In addition to their essential role as first responders during the innate immune response, increasing evidence also suggests that granulocytes may contribute to adaptive immunity by processing and presenting antigens to T cells [13]. Upon antigen-specific stimulation, human neutrophilic granulocytes acquire MHC-II molecules and different co-stimulatory molecules rendering them to antigen-presenting cells able to stimulate CD4-positive T cell responses [14].

Studies on the pathogen-species-specific effect of bacterial pathogens on the innate immune system of camels are limited. The aim of the present study was, therefore, to analyze changes in phenotype and function of camel leukocytes in a whole blood culture with different bacterial species.

2 Materials And Methods

2.1 Blood collection

Blood samples were collected from six apparently healthy dromedary camels (*Camelus dromedarius*) housed at the Camel Research Center, King Faisal University, Al-Ahsa, Saudi Arabia. Blood was obtained by venipuncture of the *vena jugularis externa* into vacutainer tubes containing EDTA (Becton Dickinson, Heidelberg, Germany). The animals were only used for blood collection. Collected blood samples were stimulated ex vivo.

2.2 Whole blood stimulation with live bacteria

Whole blood stimulation was performed according to a previously established method [15]. The bacterial species used for in vitro stimulation included *S. aureus*, *E. coli*, *S. agalactiae*, which were isolated from milk samples collected from she-camels with mastitis (Bacteriology Unit, King Faisal University). Whole blood (1 ml) was diluted with 0.9 ml medium (RPMI-1640, Sigma-Aldrich, Deisenhofen, Germany) in sterile 12 × 75 mm glass tubes (BD Biosciences, San Jose, California, USA). 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 into icy water and immediately centrifuged at 4°C for 10 min at 1000xg. After removing the supernatant, the cell pellet was suspended in PBS.

2.3 Separation of blood leukocytes

Separation of whole camel leukocytes was done after hypotonic lysis of blood erythrocytes [16]. Briefly, unstimulated and stimulated blood samples suspended in PBS were centrifuged at 4°C for 10 min at 1000xg and the cell pellet was suspended in distilled water for 20 sec and double concentrated PBS was added to restore tonicity. This was repeated until complete erytholysis. Separated cells were finally suspended in MIF buffer (PBS containing bovine serum albumin (5 g/L) and NaN_3 (0.1 g/L)) at 5×10^6 cells/ml.

2.4 Analysis of cell viability

Cell viability of blood leukocytes was measured using the dye exclusion assay [17]. Stimulated and unstimulated leukocytes were incubated with the DNA-binding dye propidium iodide (PI; 2 µg/ml, Calbiochem, Germany) and were analyzed by flow cytometry (Fig. 1A-B). PI uptake versus exclusion was used to discriminate dead cells with permeable plasma membranes (PI-positive) from live cells with intact membranes (PI-negative).

2.5 Monoclonal antibodies

Monoclonal antibodies used in this study are listed in Table 1.

Table 1
List of antibodies

Antigen	Antibody clone	Label	Source	Isotype
CD14	TÜK4	-	WSU	mIgG1
MHCII	TH81A5	-	Kingfisher	mIgG2a
CD11a	G43-25B	PE	BD	mIgG2a
CD18	6.7	FITC	BD	mIgG1
mIgG2a	polyclonal	PE	Invitrogen	gIgG
mIgG1	polyclonal	FITC	Invitrogen	gIgG
Ig: Immunoglobulin; m: mouse; MHCII: Major Histocompatibility Complex class II, FITC: Fluorescein isothiocyanate, PE: Phycoerythrin, PerCP: Peridinin-Chlorophyll-Protein, BD: Becton Dickinson				

2.6 Membrane immunofluorescence and flow cytometry

The expression of different myeloid markers and cell adhesion molecules was analyzed using membrane immunofluorescence and flow cytometry [18]. Separated leukocytes (4×10^5) were incubated with unlabeled primary monoclonal antibodies (mAbs) specific for the cell markers CD14 and MHCII or with directly labeled monoclonal antibodies to the cell adhesion molecules CD11a and CD18 [16]. After incubation (15 min; 4 °C), cells were washed twice and cells labeled with anti-CD14 and anti-MHC-II were incubated with mouse secondary antibodies (IgG1, IgG2a; Invitrogen) labeled with different fluorochromes. Mouse isotype control antibodies (Becton Dickinson Biosciences) were also included. Washed cells were analyzed using the Accuric C6 flow cytometer (BD Biosciences). At least 100 000 total leukocytes were collected and analyzed with the CFlow Software, Version 1.0.264.21 (BD Biosciences) (Fig. 1A).

2.7 Generation of reactive oxygen species (ROS)

ROS generation was performed in 96-well round-bottom microtiter plates (Corning, NY, USA) [19]. Stimulated and non-stimulated leukocytes (1×10^6 /well) were incubated for 20 min (37 °C, 5% CO₂) with the ROS-sensitive dye dihydrorhodamine (DHR)-123, (750 ng/ml final, Mobitec, Goettingen, Germany). After incubation, cells were washed with MIF buffer, and the percentage of ROS-positive cells and the relative amount of generated ROS was determined by flow cytometry (Accuric C6 flow cytometer, BD Biosciences) after acquisition of 100 000 events.

3 Statistical Analyses

Statistical analysis was carried out using the software Prism (GraphPad software version 5). Results are expressed as mean $\pm$ S.E. of the mean (SEM). Differences between means were tested with one-factorial analysis of variance (ANOVA). Results were considered statistically significant at a p-value of less than 0.05.

4 Results

4.1 Leukocyte viability is not affected by in-vitro stimulation with live bacteria

The mean percentage of viable cells (PI-negative) among unstimulated cells (cells in medium control) was $99\% \pm 0.3$ for lymphocytes, $97\% \pm 0.6$ for monocytes, and $96\% \pm 0.6$ for neutrophils. In comparison to stimulation with other bacteria, stimulation with *E. coli* induced the lowest percentage of viable cells among neutrophils ($92\% \pm 1.6$) and monocytes ($93\% \pm 1.4$). However for all leukocyte populations, no significant changes ($p > 0.05$) were found in the fraction of viable cells after bacterial stimulation (Fig. 1A and B).

4.2 Bacterial stimulation differently induces ROS-production in camel leukocyte populations

In comparison to unstimulated granulocytes, all bacterial species induced a significant ($p < 0.05$) rise in the percentage of ROS-positive granulocytes (Fig. 3A) and the amount of ROS produced by granulocytes as measured by the mean fluorescence intensity (MFI) of the ROS-sensitive dye DHR-123 (Fig. 3B). The comparison between the three bacterial species revealed that stimulation with *S. aureus* ($39.1\% \pm 2.9$) or *E. coli* ($47.8\% \pm 6.0$) resulted in a significantly ($p < 0.05$) higher percentage of ROS-positive granulocytes in comparison to stimulation with *S. agalactiae* ($21.1\% \pm 4.1$). In addition, the increase in the amount of ROS produced by granulocytes (fold increase relative to medium control) was significantly higher after stimulation with *S. aureus* (4.9 fold $\pm$ 0.6) or *E. coli* (4.3 fold $\pm$ 0.6) than *S. agalactiae* (2.6 $\pm$ 0.4). In monocytes, only stimulation with *S. aureus* or *S. agalactiae* induced a significant ($p < 0.05$) increase in the

percentage of ROS-positive cells (Fig. 3C) and the amount of ROS produced by monocytes in comparison to unstimulated cells (Fig. 3D). In contrast to this, stimulation with *E. coli* did not significantly change the percentage of ROS-positive monocytes or the amount of ROS produced by monocytes ($p > 0.05$). For lymphocytes, none of the bacterial species induced significant changes ($p > 0.05$) in the percentage of ROS-positive cells or the MFI of DHR-123 (Fig. 3E, F).

4.3 Impact of bacterial stimulation on the expression of cell adhesion molecules on camel leukocytes

While the expression of the cell adhesion molecules CD11a and CD18 on blood lymphocytes was not affected significantly ($p > 0.05$) by bacterial stimulation (Fig. 4E, F), the expression level of the two molecules on neutrophils and monocytes was differently affected by bacterial stimulation. CD11a expression on granulocytes was significantly ($p < 0.05$) reduced after stimulation with *S. aureus* or *E. coli* (Fig. 4A), whereas only *E. coli* induced the reduction ($p < 0.05$) of CD11a expression on monocytes (Fig. 4C). Only for granulocytes, all bacterial species induced an increased ($p < 0.05$) expression of CD18 after stimulation (Fig. 4B).

4.4 Impact of bacterial stimulation on the composition of blood monocytes

In unstimulated blood, the monocyte population consisted of the CD14^{high}MHCII^{low} major monocyte subset (Mo-I; 82.4% $\pm$ 1.5 of total monocytes), the CD14^{high}MHCII^{high} inflammatory monocytes (Mo-II; 7.5% $\pm$ 1.2 of total monocytes) and the CD14^{low}MHCII^{high} monocyte subset (Mo-III; 4.3% $\pm$ 0.8 of total monocytes) (Fig. 5B and C). Stimulation with either of the three bacterial species induced a significant ($p < 0.05$) decrease in fraction of Mo-I (49.4% $\pm$ 3.4 for *S. aureus*, 61.5% $\pm$ 26 for *E. coli* and 45.7% $\pm$ 1.2 for *S. agalactiae*) with a significant 3 to 5 fold expansion ($p < 0.05$) of inflammatory Mo-II (4.8 fold $\pm$ 0.3 for *S. aureus*, 3.3 fold $\pm$ 0.6 for *E. coli* and 5.0 fold $\pm$ 0.8 for *S. agalactiae*) in comparison to unstimulated control (Fig. 5B and C). In addition, the fraction of Mo-III also increased ($p < 0.05$) after stimulation with *S. aureus* (9.0 $\pm$ 0.8% of total monocytes) and *S. agalactiae* (8.2 $\pm$ 0.8% of total monocytes) in comparison to unstimulated control (Fig. 5B and C).

4.5 Bacterial stimulation enhances MHC-II expression on blood granulocytes

Bacterial stimulation of whole blood activated significantly ($p < 0.05$) blood granulocytes as measured by increased forward scatter (FSC) values (Fig. 6A and B). The stimulation-induced increase in granulocytes FSC was comparable between all bacterial species ($p > 0.05$). In addition, stimulation with either of the bacterial species elicited the upregulation ($p < 0.05$) of cell surface MHC-II molecules on granulocytes

(Fig. 6C). The increase was, however, highest on cells stimulated with *S. aureus* (MFI 710 ± 154) in comparison to unstimulated blood (MFI 399 ± 61).

5 Discussion

The current study investigated the modulatory effects of whole blood stimulation with the bacterial species, *E. coli*, *S. aureus* or *S. agalactiae*, on the phenotype and function of camel leukocytes. The whole-blood culture used in the current study has been widely proven as an effective stimulation method, preserving the microenvironment of interaction between the pathogen and immune cells as it presents in vivo [20, 21].

In a recent report, three heterogenic subpopulations of monocytes have been described in dromedary camels based on the expression profiles of MHCII and CD14 [12]. Subset one (Mo-I) expresses high levels of CD14 and low levels of MHCII and is the most abundant monocytes. Subset two (Mo-II) 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 (Mo-III) is another minor subpopulation of monocytes with low levels of CD14 and high levels of MHCII. [12]. In the current study, stimulation with either of the bacterial species significantly reduced the fraction of Mo-I and induced an expansion in the inflammatory Mo-II. This effect seems in line with the reported expansion in camel Mo-II in she-camels with *E. coli*- or *S. aureus*-caused clinical endometritis (Hussen. et. al. in press). For human monocytes, an expansion of the intermediate CD14^{high} MHC-II^{high} monocytes was observed in several bacterial infectious diseases [22, 23]. This indicates functional similarity between camel Mo-II and human intermediate monocytes. For camel Mo-III, only stimulation with *S. aureus* or *S. agalactiae* but not with *E. coli* elicited Mo-III expansion. Whether this effect is unique to gram-positive bacteria, still to be investigated.

According to recent reports, granulocytes can acquire the function of antigen-presenting cells and contribute to adaptive immune responses by activating T cells in an MHC-II-dependent manner [14]. The stimulation-induced upregulation of MHC-II molecules on camel blood granulocytes although supports the reported role of granulocytes as antigen-presenting cells, seems, however, in contrast to the human system, where the upregulation of HLA-DR on granulocytes required antigen-specific stimuli, whereas exposure to innate Toll-like receptor ligands was not sufficient [13].

The generation of reactive oxygen species (ROS) is a key antimicrobial function of phagocytes that serves the effective killing of phagocytosed bacteria [24]. In the current study, *E. coli*, in contrast to *S. aureus* and *S. agalactiae*, failed to stimulate ROS production in camel monocytes. This seems contrary to cattle, where stimulation with *E. coli* elicited ROS generation in blood monocytes [15]. For granulocytes, although stimulation with any of the three pathogen species elicited ROS production, the magnitude of the response was significantly lower toward stimulation with *S. agalactiae* than *S. aureus* or *E. coli*. This

is also the case for the stimulation-induced change in CD11a expression on granulocytes, being only induced after stimulation with *S. aureus* or *E. coli*.

6 Conclusions

The different responsiveness of monocytes and granulocytes toward different bacterial species indicates different host-pathogen interaction mechanisms for the two innate cell populations. Whether this is due to different sets of pattern recognition receptors on camel monocytes and granulocytes, still to be investigated. In addition, the phenotypic and functional differences between cells stimulated with *E. coli*, *S. aureus*, or *S. agalactiae* argues for pathogen-species-specific effects of the bacterial pathogens on the camel innate myeloid cells. Further work is needed to understand the mechanisms responsible for the observed variation in the response of innate immune cells toward different bacterial species.

7 List Of Abbreviations

E. coli: *Escherichia coli*; *S. aureus*: *Staphylococcus aureus*; *S. agalactiae*: *Streptococcus agalactiae*; ROS: reactive oxygen species; MHC: major histocompatibility complex; CD: cluster of differentiation; MFI: mean fluorescence intensity. Mo-I: camel monocyte subset I; Mo-II: camel monocyte subset II; Mo-III: camel monocyte subset III.

8 Declarations

8.1 Ethics approval and consent to participate

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/2019-10-01).

8.2 Consent for publication

Not applicable

8.3 Availability of data and materials

The datasets used and/or analyzed during the current study are available on reasonable request.

8.4 Competing interests

The author declares that he has no competing interests.

8.5 Funding

The study was funded by the Deanship of Scientific Research at King Faisal University.

8.6 Authors Contributions

JH collected the samples, performed flow cytometry and prepared the manuscript.

8.7 Acknowledgements

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8.8 Conflict of interest:

None.

9 References

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Figures

Figure 1

Gating strategy for the identification of the main leukocyte populations in camel blood and the analysis of their viability. A) Separated camel leukocytes were labeled with propidium iodide and analyzed by flow cytometry. In a SSC/FSC dot plot, camel granulocytes (G) and mononuclear cells (MNC) were gated according to their forward and side scatter characteristics. In the MNC gate, monocytes (M) and lymphocytes (L) were identified based on their different forward and side scatter characteristics. In a separate FSC-A/FSC-H dot plot for each cell population, a gate was made on single cells (exclusion of duplets). In a SSC-A/FL-3 dot plot, viable cells were identified as PI-negative cells. B) For unstimulated cells, cells stimulated with *E. coli*, *S. aureus*, and *S. agalactiae*, the percentages of viable cells among granulocytes, monocytes, and lymphocytes were calculated and data were presented as mean ± SEM.

Figure 2

Flow cytometric analysis of ROS production in camel blood leukocytes. Camel leukocytes were separated from unstimulated and stimulated blood and separated cells were labeled with the ROS-sensitive dye DHR-123 and labeled cells were analyzed by flow cytometry A) In a SSC-A/FSC-A dot plot, a gate was set on leukocytes and duplets were excluded in a FSC-A/FSC-H dot plot. Camel granulocytes (G) and mononuclear cells (MNC) were gated according to their forward and side scatter characteristics. In the MNC gate, monocytes (M) and lymphocytes (L) were identified based on their different forward and side scatter characteristics. In a SSC-A/FL-1 dot plot, the percentage of DHR-positive cells and the mean green fluorescence intensity for each cell population were calculated.

Figure 3

Effect of bacterial species on ROS production in camel blood leukocytes. For unstimulated cells, cells stimulated with *E. coli*, *S. aureus*, and *S. agalactiae*, the percentages of DHR-positive cells and the mean green fluorescence intensity for each cell population were calculated. Data for monocytes (M) and lymphocytes (L) was presented as mean $\pm$ SEM. Different lowercase superscript letters indicate statistical significance ($P < 0.05$.)

Figure 4

Adhesion molecules expression on leukocyte populations in camel blood. Camel leukocytes were separated from unstimulated and stimulated blood and separated cells were labeled with monoclonal antibodies to CD11a and CD18 and labeled cells were analyzed by flow cytometry. For granulocytes (A-B), monocytes (C-D), and lymphocytes (E-F), the expression density (mean fluorescence intensity; MFI) of

CD11a and CD18 was calculated. Data for unstimulated and stimulated cells was presented as mean $\pm$ SEM. Differences between groups were considered significant if $P < 0.05$.

Figure 5

Impact of bacterial stimulation on the composition of camel monocytes. Camel leukocytes were separated from unstimulated and stimulated blood and were labeled with monoclonal antibodies to CD14 and MHCII molecules. Labeled cells were analyzed by flow cytometry. A) Gating strategy of camel monocyte subsets. After setting a gate on MNC, Camel monocytes were identified based on their forward

(FSC) and side scatter (SSC) properties. In a separate FSC-A/FSC-H dot plot, a gate was made on single cells (exclusion of duplets). B) Monocyte subsets Mo-I, Mo-II, and Mo-III were identified according to their CD14 and MHCII expression density (Mo-I: CD14^{high}MHCII^{low}, Mo-II: CD14^{high}MHCII^{high}, Mo-III: CD14^{low}MHCII^{high}). C) The percentage of each monocyte subset was calculated and presented for unstimulated and stimulated blood. Differences between groups were considered significant (*) if $P < 0.05$.

Figure 6

Stimulation-induced shape change and change in MHC-II expression in camel granulocytes. A) Shape change in granulocytes was measured as mean fluorescence intensity (MFI) of FSC-A of gated

granulocytes. B) Data for unstimulated cells and cells stimulated with different bacterial species were calculated and presented as means $\pm$ SEM (* = $p < 0.05$). C) MHC-II expression density on granulocytes from unstimulated blood and blood stimulated with different bacterial species was calculated and presented as means $\pm$ SEM (* = $p < 0.05$).