2023, Scienceline Publication
World's Veterinary Journal
World Vet J, 13(1): 214-221, March 25, 2023

ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2023.wvj23

NETosis and Calcium influx in Dromedary Camel Neutrophils after *In Vitro* Toll-like Receptor Stimulation

Khuzama Albahrani[1], Jumanah Alessa[1], Baraa Falemban[1], Mayyadah Abdullah Alkuwayti[2], and Jamal Hussen[1]*

[1]Department of Microbiology, College of Veterinary Medicine, King Faisal University, Al-Ahsa, Saudi Arabia
[2]*Department of Biological Sciences, College of Science, King Faisal University, Al Ahsa 31982, Saudi Arabia*

*Corresponding author's Email: jhussen@kfu.edu.sa

ORIGINAL ARTICLE
pii: S232245682300023-13
Received: 08 January 2023
Accepted: 27 February 2023

ABSTRACT

Neutrophilic granulocytes are vital immune cells of the early response to pathogens. They contribute to the antimicrobial response through phagocytosis, production of reactive oxygen species, cytokine production, degranulation, and NET-formation. Neutrophil extracellular traps (NETs), also known as NETosis, are a critical antibacterial effector mechanism of cells of myeloid effector cells, including neutrophils and macrophages. Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) that mediate pathogen sensing through the recognition of microbial structures known as pathogen-associated molecular patterns (PAMPs). The present study aimed to investigate the potential of several TLR ligands that mimic the sensing of bacterial and viral pathogens to stimulate NET-formation or Ca^{2+} influx in camel neutrophils. Neutrophils were purified from blood and were stimulated in vitro with ligands to TLR4, TLR2/1, TLR7/8, or TLR3. Net-formation was analyzed using the DNA-sensitive dye SYTOX™ Green and staining with antibodies to the neutrophil's granular enzyme myeloperoxidase. Real-time stimulation-induced Ca^{2+} influx was measured using the Ca^{2+-}sensitive dye Flou-4 and flow cytometry. Only the TLR4-ligand lipopolysaccharide (LPS) could induce NET-formation in camel neutrophils, while none of the investigated TLR agonists showed a Ca^{2+} influx-inducing effect in camel neutrophils. The current study represents the first report on the impact of direct activation of TLR on NET-formation and Ca^{2+} influx in camel neutrophils with a selective effect of LPS on NET-formation induction. Future studies may investigate the molecular mechanisms behind the different responsiveness of bovine and camel neutrophils to TLR stimulation.

Keywords: Camel, Ca^{2+} influx, Flow cytometry, Neutrophils, NETosis, Toll-like receptor

INTRODUCTION

Neutrophils are innate immune cells with a significant role in early defense against pathogens. They mainly contribute to antimicrobial response through the early detection of microbial structures and danger signals and the subsequent activation of other innate and adaptive immune cells essential for effectively eliminating pathogens (Soehnlein and Lindbom, 2010; Rosales et al., 2016). Neutrophils elicit their antimicrobial activity through several functions, including phagocytosis, production of reactive oxygen species, cytokine production, degranulation, and NET-formation (Akira and Takeda, 2004; Gordon, 2004; Tan et al., 2018).

Neutrophil extracellular traps (NETs), also known as NETosis, are a key antibacterial effector mechanism of cells of effector myeloid cells, including neutrophils and macrophages (Ciliberti et al., 2021). NETosis includes immobilizing intracellular DNA and nuclear chromatin to the extracellular space to build a network, where microbes are trapped and killed. The antimicrobial potential of NETs is mainly supported by many antimicrobial peptides released from their stores in neutrophil granules (Lippolis et al., 2006; Aulik et al., 2010; Remijsen et al., 2011). Although several models for NET-formation have been established for many veterinary species, including cattle, sheep, and goats (Worku et al., 2021), only a few studies investigated NET-formation in the dromedary camel (Hussen et al., 2022).

Toll-like receptors (TLR) are pattern recognition receptors (PRRs) expressed on and in different immune cells (Akira and Takeda, 2004; Beutler, 2004; Schmidt et al., 2004). They mediate the sense of pathogens through the recognition of microbial structures known as pathogen-associated molecular patterns (PAMPs, Ozinsky et al., 2000; Takeuchi and Akira, 2007; Radoshevich and Dussurget, 2016).

Together with a cluster of differentiation (CD)14, the LPS-binding protein (LBP), and MD-2, TLR4 is responsible for sensing gram-negative bacteria by the recognition of the PAMP lipopolysaccharide (LPS, Ohtsuka et al., 2001; Miyake, 2004; Johnzon et al., 2018). The interaction of the synthetic TLR-ligand Pam3CSK4 with TLR1/2 simulates innate sensing of Gram-positive bacteria (Mintz et al., 2013; Reid et al., 2021). Resiquimod (R848) and polyinosinic:

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polycytidylic acid (poly I:C) are synthetic agonists for the intracellular TLR8/TLR7 and TLR3, respectively, representing infection of viruses (Reid et al., 2021). The TLR-mediated release of NETs has been described after stimulation of neutrophils with different pathogens, including *Candida albicans* and *Staphylococcus aureus* (Pilsczek et al., 2010; Byrd et al., 2013; Block et al., 2022).

Changes in intracellular Ca^{2+} levels are a hallmark of several activation processes of neutrophils (Dixit and Simon, 2012). Under resting conditions, levels of neutrophils cytosolic Ca^{2+} are lower than in the extracellular compartment. After stimulation, neutrophils rapidly raise their intracellular Ca^{2+} levels through Ca^{2+} release from its cytosolic stores and/or Ca^{2+} influx from the extracellular milieu (Immler et al., 2018).

Several studies have investigated the impact of TLR activation on the phenotype and the function of neutrophils for humans and many other species (Byrd et al., 2013; Block et al., 2022). However, less is known about TLR activation in camel immune cells. The objective of the current study was to analyze the potential of several TLR ligands that mimic sensing of bacterial and viral pathogens to stimulate NET-formation or Ca^{2+} influx in camel neutrophils. The results of the present work would contribute to a better understanding of the interaction mechanisms of the camel immune response with different pathogen groups.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Ethics Committee of King Faisal University (approval no KFU-REC-2021- DEC - EA000326).

Animals and blood sampling

Blood samples were collected from five clinically healthy (based on clinical examination) dromedary camels (Camelus dromedarius) that were randomly selected from 35 camels reared on a camel farm in the eastern proven of Saudi Arabia. All camels were males from the Almajaheem breed with ages between 10 and 12 years old and body weights between 325 and 365 Kg. Blood sample collection was performed without anesthesia using venipuncture of the jugular vein into EDTA tubes (BD Biosciences, San Jose, California, USA), and collected blood was kept cooled until used for cell separation in the immunology laboratory at King Faisal University (usually after 1 hour).

Purification of camel neutrophils

Camel neutrophils were separated as previously described by Hussen et al. (2023a). Briefly, 5 mL camel blood was diluted with 5 mL phosphate buffered saline (PBS), and diluted blood was then layered (carefully without mixing them) on 5 mL of the lymphocyte separation medium Lymphoprep™ (Stemcell Technologies, Vancouver, Canada) in Corning® 15 mL centrifuge tubes. The blood was then centrifuged for 30 min at 800 × g. After removing the peripheral blood mononuclear cells (PBMCs) from the inter-phase, neutrophils were separated after erythrolysis. For erythrolysis, aquadest (5 mL) was used for 20 sec to lyse the RBCs and 5 mL of a 2x solution of PBS was then used to restore cell osmosity. The RBC-lysis was repeated until having a pure white cell pellet. Neutrophils were suspended at 1 × 107 cells/mL in HBSS buffer (Hank's balanced salt solution; MOLEQULE-ON, Auckland, New Zealand).

Toll-like receptor stimulation in camel neutrophils *in vitro*

The TLR stimulation was performed as previously described (Hussen et al., 2023b). The TLR ligands lipopolysaccharide (LPS), Pam3CSK4, R848, and Poly IC were purchased from Invivogen (San Diego, USA). Phorbol myristate acetate (PMA) was purchased from Calbiochem (Merck Millipore, Darmstadt, Germany). For the *in vitro* stimulation, 1 × 106 neutrophils were incubated in Roswell Park Memorial Institute (RPMI) medium for 4 hours at 37 °C with LPS (1 µg/mL), Pam3CSK4 (1 µg/mL), R848 (0.2 µg/mL), Poly IC (10 µg/mL), or phorbol 12-myristate 13-acetate (PMA; 10 ng/mL), or were left in medium without stimulation (negative control).

Measurement of neutrophil extracellular traps formation by SytoxGreen

Stimulated and non-stimulated neutrophils (5 x 105 cells per well of a 96-well cell culture plate) were incubated with one drop of the DNA-sensitive dye SytoxGreen (Invitrogen, Germany). After 15 min incubation at room temperature, the labeled cells were analyzed by flow cytometry (Accuri C6; BD Biosciences) by the acquisition of at least 30.000 neutrophils (Masuda et al., 2017).

Measurement of membrane myeloperoxidase

Membrane myeloperoxidase (MPO) was detected by labeling the cells with a mouse monoclonal (Clone 5B8) antibody against MPO conjugated with phycoerythrin (PE, Raskovalova et al., 2019). The antibody was purchased from BD Biosciences (San Jose California, USA). For cell labeling, 100 µL cell suspension (5 x 105 cells) was incubated as a

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pellet with 20 µL anti-MPO antibody for 15 min at 4°C followed by washing the cells with cold PBS supplemented with bovine serum albumin (MOLEQULE-ON, Auckland, New Zealand). Finally, the labeled cells were analyzed by flow cytometry (Accuri C6; BD Biosciences, San Jose, California, USA).

Real-time analysis of calcium influx

Purified camel neutrophils (1 x 107 cells / µL) were incubated for 30 min at 37°C with 1 µmol/l Fluo-4 AM (Molecular Probes, Eugene OR) in Ca^{2+}/Mg^{2+} HBSS (MOLEQULE-ON, Auckland, New Zealand). Cells were washed three times with HBSS (8 minutes, 300 xg) and finally suspended in HBSS. Baseline Fluo-4 fluorescence was measured for 20 sec before TLR agonists were added to the cells. The cellular response towards HBSS and ionomycin (Sigma-Aldrich, Germany, 250 nmol/L final) were used as negative and positive control stimulation, respectively (Hussen et al., 2016).

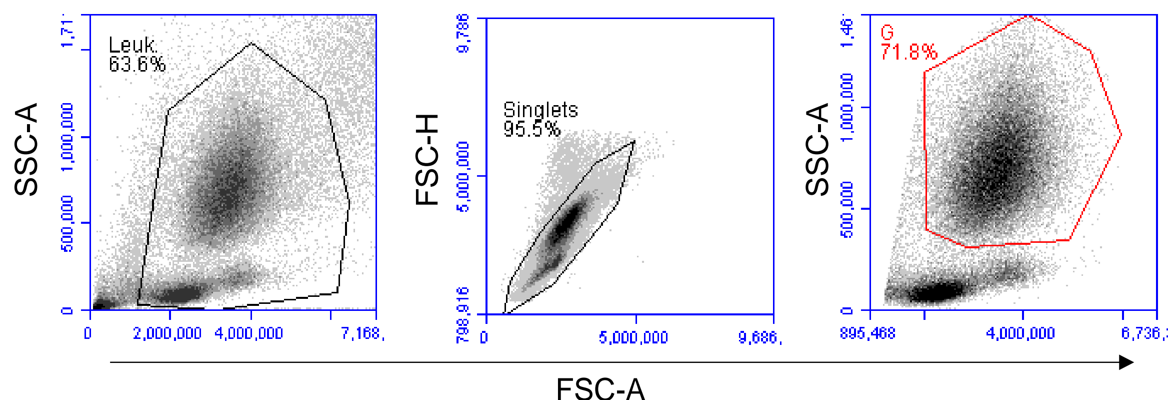
Statistical analysis

GraphPad Prism (San Diego, USA) was used for statistical analysis. Data normality was tested using Shapiro–Wilk test. The 1-factorial analysis of variance (ANOVA) test was used in combination with Bonferroni's multiple comparison tests to analyze the effect of different stimuli on NET-formation and Ca^{2+} influx of neutrophils. P-values less than 0.05 indicate significant differences between the means.

RESULTS AND DISCUSSION

In the current work, Ca-influx and NETosis responses were investigated in purified camel neutrophils upon in-vitro stimulation with different synthetic TLR-ligands. Neutrophil purification was performed using density gradient centrifugation over Ficol-Histopaque (Figure 1, Hussen et al., 2016). This method resulted in a pure neutrophil population (always more than 93%) with a vitality rate above 95% (propidium iodide-negative cells).

A) Blood leukocytes



B) Purified granulocytes

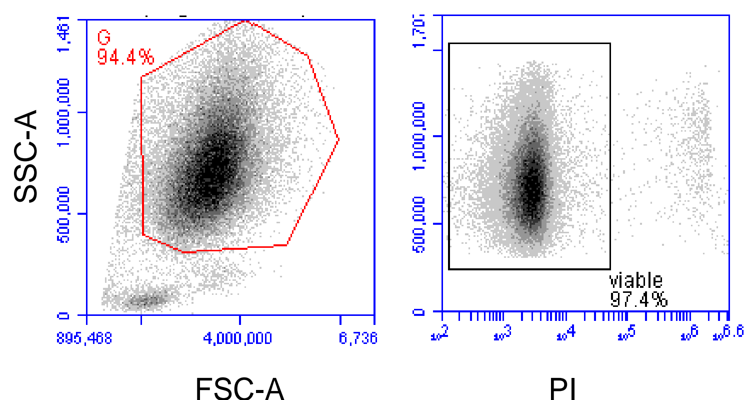


Figure 1. Purification of neutrophils from camel blood using density gradient centrifugation. **A**: After exclusion of cell debris (gate on leukocytes; Leuk) and cell duplicates (gate on singlets) in side scatter area against forward scatter area (SSC-A/FSC-A) and FSC-A/FSC-H dot plots, the fraction of neutrophilic granulocytes (G) was identified within leukocytes based on cell size (FSC-A) and granularity (SSC-A). **B**: Cell purity (percentage of neutrophils) and vitality (viability) of separated neutrophils were measured based on cell morphology (FSC-A/SSC-A) and staining with propidium iodide (PI), respectively.

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TLR-stimulation-induced NET-formation in camel neutrophils

Neutrophils NETosis (NET-formation) was measured based on the staining with the DNA binding dye SYTOX™ Green (Figure 2A-D). For control cells without stimulation, the percentage of neutrophils with enhanced SYTOX™ Green fluorescence was 2.0 % of total cells. Stimulation with PMA resulted in a 3-fold increase ($p < 0.05$) in the percentage of neutrophils with positive staining with SYTOX™ Green (7.01 %) as well as a 4-fold rise in the SYTOX™ Green mean fluorescence intensity (MFI: 1176 versus 4119 for non-stimulated cells) for the whole neutrophils population (Figure 2BC). For cells stimulated with TLR-ligands, only LPS stimulation resulted in a significant ($p < 0.05$) expansion in the SYTOX™ Green-positive cells (3.9 %) and enhanced MFI of total cells (MFI: 2555 versus 1176 for non-stimulated cells). The LPS-induced effect was, however, lower than that of PMA.

NET-formation was also confirmed by measuring the expression of the granular enzyme MPO on the surface of neutrophils (Figure 2E-H). For non-stimulated cells in medium control, the percentage of neutrophils with enhanced MPO staining was 3.6 % of total cells. Stimulation with PMA resulted in a 2-fold increase ($p < 0.05$) in the rate of neutrophils with positive staining with MPO (8.7 %) as well as a marked enhancement ($p < 0.05$) of the MPO mean fluorescence intensity (MFI: 950 versus 550 for non-stimulated cells) for the whole neutrophils population. With the exception of LPS, stimulation with TLR-ligands did not induce NET formation in neutrophils. In LPS-stimulated neutrophils, a marked ($p < 0.05$) expansion in the MPO-positive cells (6.07 %; Figure 2E) and an enhanced MPO MFI (MFI: 775) were observed (Figure 2F).

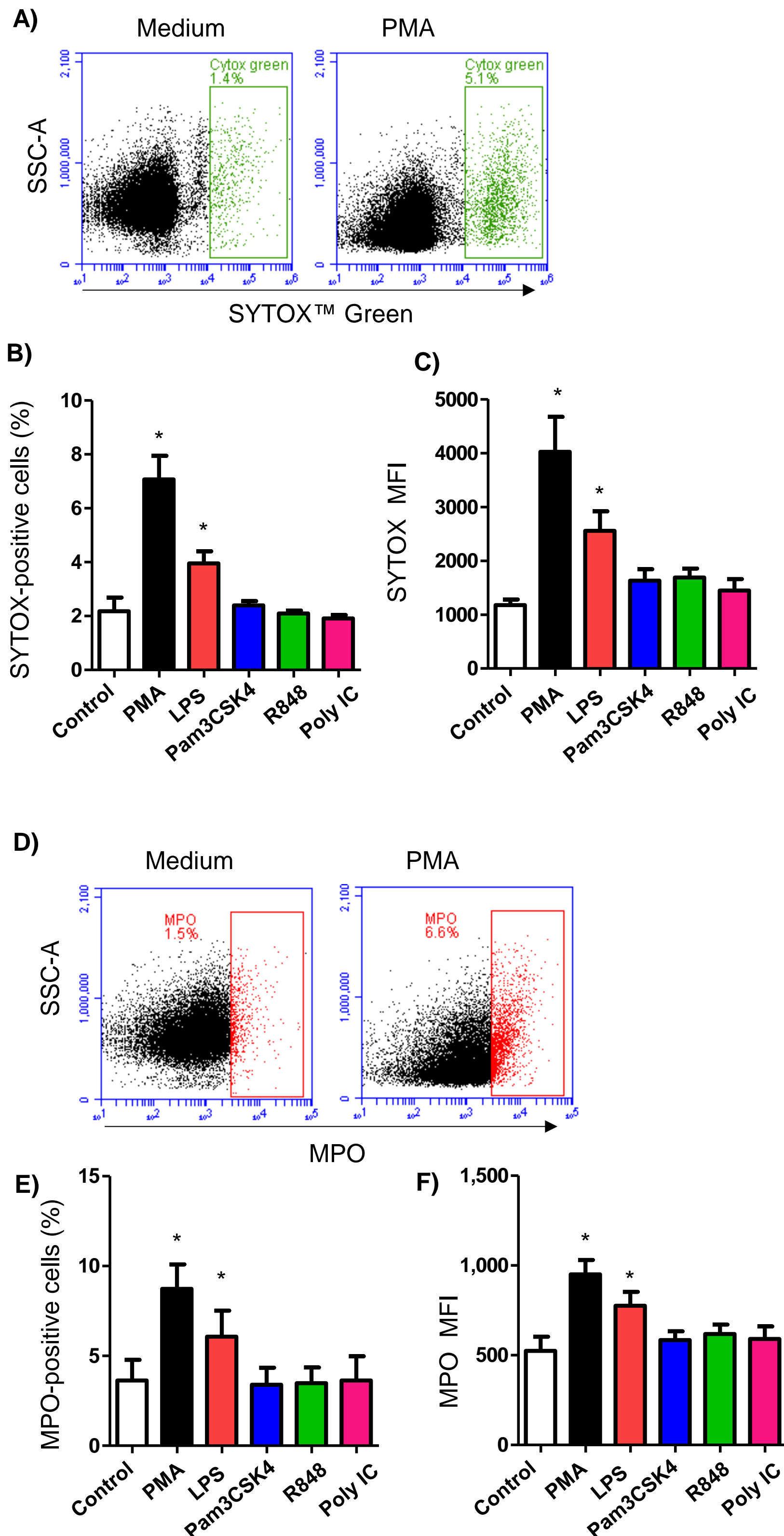

Figure 2. Neutrophils extracellular traps (NETs) in neutrophils. **A:** Purified neutrophils were stained with SYTOX™ Green and analyzed by flow cytometry. Cells with NET-formation were identified based on their positive staining with SYTOX™ Green. The percentage of SYTOX™ Green-positive cells (**B**) and the mean green fluorescence intensity (MFI) for all cells (**C**) were calculated and presented in graphs. **D:** Neutrophils were labeled with PE-conjugated monoclonal mouse antibodies to myeloperoxidase and labeled cells were analyzed using flow cytometry. Representative dot plots showing the percentage of MPO-positive neutrophils for non-stimulated and stimulated cells. The percentage of MPO-positive neutrophils (**E**), as well as MPO MFI for all neutrophils (**F**), were calculated and presented as mean and SEM (n = 5 camels; * $p < 0.05$ in comparison to control).

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Ca^{+2} influx in camel neutrophils after TLR stimulation

Stimulation-induced Ca^{+2} influx in purified camel neutrophils was analyzed using the Ca^{+2}-binding dey Fluo-4 (Figure 3A). Fluo-4 fluorescence was first explored for 20 seconds before adding a stimulant. For stimulation control, cells were stimulated with HBSS (negative control) and ionomycin (positive control). Stimulation with ionomycin resulted in a significant ($p < 0.05$) Ca^{+2} influx in neutrophils (71.3 ± 1.8 %) in comparison to non-stimulated (5.0 ± 1.9 %) cells (cells stimulated with HBSS, Figure 3B). In contrast to ionomycin, none of the TLR-ligands induced Ca^{+2} influx in purified camel neutrophils (Figure 3A, B).

A)

B)

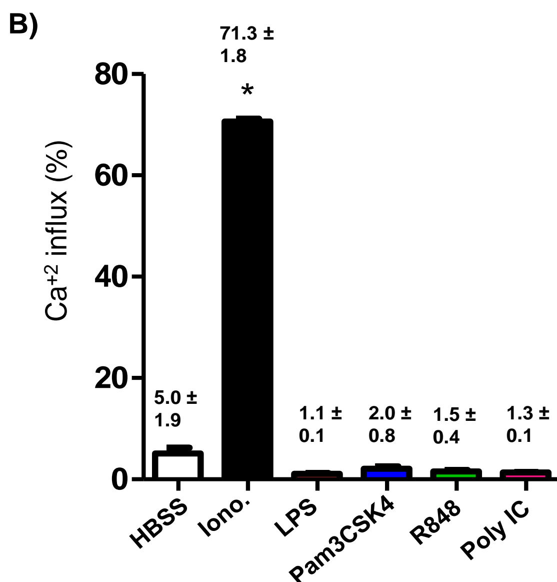

Figure 3. Purified camel neutrophils were stained with the calcium dey Fluo-4. **A:** For the measurement of real-time influx of calcium, Fluo-4 fluorescence height (FL1-H) was presented in relation to time. Neutrophils were defined based on their light scatter properties, and the influx of Ca^{2+} was measured as the fraction of cells that rose above the basic line after stimulation (arrows indicate the time point where stimuli were added). **B:** The fraction (%) of cells with the increased influx of Ca^{2+} in response to HBSS (negative control), ionomycin (Iono; positive control), or TLR ligands is presented graphically (n = 3 camels; * $p < 0.05$).

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Neutrophilic granulocytes are key immune cells in the early response to pathogens (Nathan, 2006; Mantovani et al., 2011; Kolaczkowska and Kubes, 2013; Malech et al., 2020; Burn et al., 2021). The interaction between neutrophils and pathogens is mediated through different receptors. TLRs are membrane and intracellular pattern recognition receptors interacting with PAMPs (Newton and Dixit, 2012; Zindel and Kubes, 2020). Although few recent studies analyzed the impact of some TLR-agonists on some functions of neutrophils in the dromedary camel (Hussen et al., 2023a; Hussen et al., 2023b), many questions still exist regarding the modulatory effect of TLR stimulation on neutrophils phenotype and function. Especially the potential of bacterial and viral TLR-agonists to induce NET-formation or Ca^{2+}influx in camel neutrophils has not been investigated so far. The present work investigated the effects of selected TLR agonists on NET-formation and Ca^{2+}influx in camel neutrophils.

Generation of neutrophils extracellular traps (NETs), also known as NETosis, is one of the effective mechanisms used by neutrophils for the extracellular killing of microbes (Brinkmann et al., 2004). During NETosis, neutrophils mobilize their DNA and nuclear proteins to build a network outside the cell. This network contains many antimicrobial peptides released from the neutrophil's granular stores. Microbes are trapped and killed inside this network (Brinkmann et al., 2004; Rada, 2019).

Receptor-mediated NET-formation has been recently described for human neutrophils. Receptors mediating NETosis in human neutrophils include TLRs, nod-like receptors, C-Type Lectin Receptors, FC receptors, and complement receptors (Chen et al., 2021). In the present study, only activation of TLR4 through the bacterial TLR4-ligand LPS showed the potential to induce NETosis in camel neutrophils. Studies in humans identified many TLRs that participate in NETosis by human neutrophils. This includes TLRs to viral, bacterial, fungal, and parasitic PAMPs (Chen et al., 2021). Although the results of the present work are in line with the reported TLR4-induced NETosis in human neutrophils, the lack of NET-formation after the stimulation of neutrophils with PAM3CSK4, R848, and Poly IC is in contrast to the human system, where TLR, TLR2, TLR7, and TLR8 were involved in the NETosis response to several pathogens in human neutrophils (Saitoh et al., 2012; Hiroki et al., 2019; Munoz-Caro et al., 2021).

The expression patterns of several members of the TLR group have been investigated for bovine and human neutrophils (Parker et al., 2005; Conejeros et al., 2015). Such studies are still lacking for camel neutrophils. In a recent study, activation of TLR-4, TLR-2/1, and TLR-7/8, but not of TLR-3, resulted in the activation of camel neutrophils with stimulation-induced shape change and modulation of activation markers expression (Hussen et al., 2023b).

A rise in cytosolic calcium levels represents an early step in the activation of neutrophils. It is associated with several functional activities, such as adhesion and migration to the site of infection, reactive oxygen species (ROS) production, and degranulation (Conejeros et al., 2015). To see whether TLR stimulation in camel neutrophils leads to Ca^{2+} influx, real-time flow cytometric measurement of changes in intracellular calcium concentrations in camel neutrophils was performed upon stimulation with TLR agonists. Present results showed that none of the used TLR ligands (LPS, Pam3CSK4, R838, or Poly IC) induced calcium influx in camel neutrophils. These results contradicted the reported increase of Ca^{2+} influx in bovine PMN exposed to Pam3CSK4 (Conejeros et al., 2015).

CONCLUSION

In conclusion, the present study evaluated the impact of selected TLR agonists representing PAMPs of bacterial and viral pathogens on NET-formation and Ca^{2+} influx in camel neutrophils. Only the TLR4-ligand LPS showed the potential to induce NET formation in camel neutrophils. None of the investigated TLR agonists showed a Ca^{2+} influx-inducing effect in camel neutrophils. The current study represents the first report on the impact of direct activation of TLR on NET-formation and Ca^{2+} influx in camel neutrophils. Further studies are required to investigate the molecular mechanisms behind the different responsiveness of bovine and camel neutrophils to TLR stimulation.

DECLARATIONS

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

Funding

This work was supported through the Annual Funding track by the Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia (Project number GRANT2,953).

Acknowledgments

None.

Authors' contributions

Khuzama Albahrani performed sample collection and preparation, manuscript revision. Jumanah Alessa did flow cytometry, manuscript revision. Baraa Falemban conducted data analysis, writing the original manuscript. Mayyadah

To cite this paper: Albahrani Kh, Alessa J, Falemban B, Alkuwayti MA, and Hussen J (2023). NETosis and Calcium influx in Dromedary Camel Neutrophils after *In Vitro* Toll-like Receptor Stimulation. *World Vet. J.,* 13 (1): 214-221. DOI: https://dx.doi.org/10.54203/scil.2023.wvj23

Abdullah Alkuwayti performed supervision, manuscript preparation and revision. Jamal Hussein carried out conceptualization, funding acquisition, data analysis, writing, and manuscript revision. All authors read and confirmed the final draft of the manuscript.

Competing interests

No conflict of interest to disclose.

Ethical consideration

The authors declare that the manuscript has not been published before and is not currently being considered for publication elsewhere. The originality of the final draft of the manuscript has been checked by all the authors.

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To cite this paper: Albahrani Kh, Alessa J, Falemban B, Alkuwayti MA, and Hussen J (2023). NETosis and Calcium influx in Dromedary Camel Neutrophils after *In Vitro* Toll-like Receptor Stimulation. *World Vet. J.,* 13 (1): 214-221. DOI: https://dx.doi.org/10.54203/scil.2023.wvj23