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The Modulation of *In Vitro* Differentiation of Monocyte-derived Macrophage by *Trypanosoma evansi* Antigens in the Dromedary Camel

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ABSTRACT

Studies on the camel immune response to *Trypanosoma (T.) evansi*, the causative agent of Surra, are very limited. In the present study, flow cytometry was employed to investigate the modulatory effects of different *T. evansi* antigens on the *in vitro* differentiation of camel blood monocytes into macrophages. For this, *in vitro*, separated camel monocytes were differentiated into monocyte-derived macrophages (MDM) in the presence or absence (control) of formalin-fixed (inactivated) *T. evansi* whole parasite (*T. evansi* group) or the purified Ro Tat 1.2 antigen (Ro Tat 1.2 group). The analysis of the antimicrobial functions of MDM (phagocytosis and reactive oxygen species (ROS) production) revealed reduced phagocytosis activity of camel MDM generated in the presence of *T. evansi* antigens. In addition, a lack of ROS-response was observed in camel MDM generated in the presence of *T. evansi* antigens after stimulation with PMA. These results indicated a compromising effect of *T. evansi* on the innate defense mechanisms in camels. Phenotypic analysis revealed the upregulation of major histocompatibility complex (MHC) class II molecules together with the lower abundance of the scavenger receptor for haptoglobin–hemoglobin complexes (CD163) on MDM generated in the presence of whole *T. evansi* parasites, indicating a polarizing effect of *T. evansi* on the differentiation of camel monocytes into an M1 phenotype. However, the reduced antimicrobial functions of these cells argue against their pro-inflammatory nature. Although both MDM generated in the presence of whole *T. evansi* antigens or their purified Ro Tat 1.2 proteins indicated similar expression levels of CD14 and MHCII molecules, the different abundance of the cell surface molecules CD172a, CD163, CD45, and CD44 indicated different phenotypes of the two MDMs. The results of the present study revealed compromising effects of *T. evansi* antigens on camel macrophages differentiated *in vitro* from blood monocytes. Whether these effects contribute to the *in vivo* pathogenesis of *T. evansi* in camels remains to be determined in future studies.

Keywords: Camel, Flow cytometry, Immunity, Macrophage, Monocyte, *Trypanosoma evansi*

INTRODUCTION

Surra is an arthropod-borne disease caused by *Trypanosoma* (*T.*) *evansi*, and a blood parasite derived from *T. brucei* (Diall et al., 2022; Birhanu et al., 2016) and affects several animal species, including dromedary and Bactrian camels with significant economic losses due to reduced fertility and high mortality rates (Birhanu et al., 2016). *T. evansi*, *T. brucei*, and *T. equiperdum* are currently classified under the subgenus *Trypanozoon* (Desquesnes et al., 2022). Several recent phylogenetic and immunological studies reported high genetic and antigenic similarities between *T. evansi*, *T. equiperdum*, and *T. brucei* (Lai et al., 2008; Carnes et al., 2015; Kocher et al., 2015) suggesting close relationship between the three parasites and leading to the assumption that *T. evansi* and *T. equiperdum* could be considered as subspecies of *T. brucei* (Desquesnes et al., 2022).

Upon leaving the bloodstream, blood monocytes can differentiate into different subsets of macrophages based on the tissue-specific modulatory effects of the microenvironment (Chen et al., 2023). Pro-inflammatory monocyte-derived macrophages (MDM), also known as classically-activated M1 macrophages, are usually differentiated under microbial stimuli like lipopolysaccharide or inflammatory cytokines like interferon-gamma (Muller et al., 2017). On the other hand, anti-inflammatory macrophages, also known as alternatively-activated M2 macrophages, are differentiated under the effect of helminthic antigens, nonsteroidal drugs, or the dominance of T helper 2 cytokines like interleukin (IL) 4 or IL13 (Chen et al., 2023). While M1 macrophages are inflammatory cells with enhanced antimicrobial functions like bacterial phagocytosis and killing capacity and production of inflammatory cytokines, M2 macrophages are anti-inflammatory cells with functions related to wound healing and resolution of inflammation (Eming et al., 2021). The killing of trypanosome is associated with the development of M1 macrophages that produce inflammatory mediators including tumor necrosis factor α (TNF-α), reactive oxygen species (ROS), and nitric oxide (NO; Stijlemans et al.,

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2007). Although these mediators were induced in a *T. evansi* mouse model, there was no correlation between their levels, the control of the parasitemia, and animal survival (Baral et al., 2007).

The development of effective *T. evansi* vaccines requires a better understanding of the pathological and immunological mechanisms involved in Surra. Studies on the immune response of camels to *T. evansi* are still limited to serological detection of specific antibodies in the serum of infected animals (Al-Harrasi et al., 2023; Habeeba et al., 2022), while the role of the cellular immune response has not been investigated so far. The main objective of the present study was, therefore, to investigate the modulatory effects of *T. evansi* antigens on the differentiation of monocytes into monocyte-derived macrophages in the dromedary camel.

MATERIALS AND METHODS

Ethical approval

Animals ethical approval was obtained from the Ethics Committee of King Faisal University, Saudi Arabia, with an approval number (KFU-REC-2021- DEC -EA000326).

The study was conducted in September 2023. Blood samples (5 mL) were collected from four (two male and two female) adult (9, 11, 11, 13 years old) healthy dromedary camels (Camelus dromedarius) reared at the farm of the Camel Research Center of King Faisal University, Al-Ahsa, Saudi Arabia. Blood samples were obtained by venipuncture of the jugular vein (vena jugularis externa) into vacutainer tubes containing EDTA (Becton Dickinson, Heidelberg, Germany).

Separation of mononuclear cells from camel blood

Camel peripheral blood mononuclear cells (PBMCs) were separated from buffy-coat blood by density gradient centrifugation over *Lymphoprep*™ (STEMCELL Technologies, Vancouver, Canada). For this, 5 mL of blood was diluted 1:2 with phosphate-buffered saline (PBS), and the mixture was layered on 5 mL of *Lymphoprep*™ in a 15 mL sterile falcon tube. After centrifugation at 4°C for 30 minutes at 800×g without a break, the PBMC-containing interphase was collected carefully using a 10 mL pipette. After three washes in cold PBS (400×g, 200×g, 100×g for 10 minutes at 4°C), the cells were analyzed for cell purity and vitality (Supplementary Figure 1), counted, and suspended in RPMI culture medium at 2×10^6 cell / mL (Duvel et al., 2014).

Monocyte isolation and *in vitro* differentiation into macrophages

Camel monocytes were separated by allowing camel PBMCs (1×10^6 cells / well) to adhere in 24-well culture plates (Nunc, NY, Rochester, USA) for 24 h at 37◦C and 5% CO_2 in serum-free RPMI 1640 culture media (MOLEQULE-ON, New Lynn, Auckland, New Zealand) followed by the removal of non-adherent lymphocytes by washing with fresh culture medium (Rao Muvva et al., 2019). A freeze-dried suspension of inactivated, purified, and fixed trypanosomes of the Variable Antigen Type (VAT) Rode Trypanozoon antigen type (Ro Tat) 1.2 (OIE-Reference Laboratory for Surra, Institute of Tropical Medicine, Antwerp, Belgium) was reconstituted with 1 mL RPMI medium (4×10^6 parasite / mL). Adherent monocytes were incubated for 4 days at 37°C and 5% CO_2 in culture medium containing whole *T. evansi* parasites (1×10^4 cells) or their purified Ro Tat 1.2 antigen (1 µg/mL) purchased from the OIE-Reference Laboratory for Surra (Institute of Tropical Medicine, Antwerp, Belgium). Day 4 macrophages were detached by incubation in cold PBS containing 5mmol/L EDTA on ice for 30 minutes, followed by several mixings of the well content using the pipette. Harvested cells were counted on the Accuri C6 flow cytometer (BD Biosciences) after the acquisition of 50 µl of the cell suspension (Eger et al., 2016).

Phagocytosis and reactive oxygen species assays

The bacterial phagocytosis by camel MDM was performed by incubating MDM with heat-killed *Staphylococcus aureus* (*S. aureus)* bacteria (Calbiochem, Nottingham, UK) labeled with a FITC labeling kit (Sigma-Aldrich, Missouri, USA) according to the manufacturer instructions (Silva et al., 2021). Camel MDM (1×10^3 cells in 100 µL RPMI medium) were incubated with *S. aureus-FITC* (20 bacteria/cell) for 30 minutes at 37°C and 5% CO2. After washing the plate with RPMI medium (300×g for 3 minutes), the cells were resuspended in 100 µL of PBS and analyzed by flow cytometry (Hussen et al., 2023).

Generation of ROS by MDM was measured in 96-well round-bottom microtiter plates (Corning, NY, USA) as previously described (Hussen et al., 2023). Camel MDMs (1×10^4/100 µL/well) were incubated in 50 µL RPMI culture medium alone or in medium containing 10 ng/mL phorbol-myristate-acetate (PMA; Invivogen Darmstadt, Germany) for 30 minutes (37°C, 5% CO2). After 15 minutes of incubation, dihydrorhodamine (DHR) 123 (Mobitec, Goettingen, Germany) was added to the cells at a final concentration of 750 ng/ml. The cells were washed in RPMI medium and ROS production was analyzed by flow cytometry.

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Phenotypic properties of monocytes-derived macrophages

The expression level of cell surface markers was evaluated by flow cytometry (Elnaggar et al., 2019) after cell labeling with monoclonal antibodies. For this, 1×10^3 MDM were incubated in 96 well plates with monoclonal antibodies to the cell surface molecules CD45, CD44, CD14, CD163, MHCII, and CD172a for 15 min at 4◦C on an ice pack. After two washings (3 min at 300×g) in PBS, secondary FITC-conjugated goat anti-mouse IgG1 and PE-conjugated goat anti-mouse IgG2a were added to the cells for 15 minutes at 4°C on ice in the dark. Finally, the cells were washed with PBS, resuspended in 100 µl PBS, and analyzed by flow cytometry.

Statistical analysis

Statistical analysis was performed using the Prism software (GraphPad). Means and standard deviation (SD) were calculated using the column statistic function Prism. Differences between means were tested with the One-way analysis of variance (ANOVA) and Bonferroni correction. P values less than 0.05 indicate that significant differences exist between the groups.

RESULTS AND DISCUSSION

The blood parasite *T. evansi*, the causative agent of Surra, is a World Organisation for Animal Health (WOAH) listed pathogen. However, surra is still a neglected disease in terms of research into improved prevention and control strategies (Desquesnes et al., 2022). Especially in camel *T. evansi* infection, studies on the immune response, the host-pathogen interaction mechanisms, and immune evasion strategies of the parasite are very limited (Birhanu et al., 2016; Diall et al., 2022). In the present study, flow cytometry was employed to investigate the modulatory effects of different *T. evansi* antigens on the *in vitro* differentiation of camel blood monocytes into macrophages. For this, *in vitro* differentiation of separated camel monocytes into monocyte-derived macrophages (MDM) was performed in the presence or absence of inactivated *T. evansi* whole parasite or the purified Ro Tat 1.2 antigen (Ro Tat 1.2 antigen is native Variable Surface Glycoproteins of bloodstream-form *T. evansi* trypanosomes).

For the analysis of the phagocytosis activity of MDM generated under *in vitro* stimulation with *T. evansi* antigens, day 4 macrophages were incubated with FITC-labeled *S. aureus* and their phagocytosis activity was analyzed by flow cytometry (Figure 1 A, B, C).

For MDM generated in medium control alone, the percentage of phagocytic cells was 15% of total cells with a mean fluorescence intensity (MFI, indicating the number of bacteria ingested by each cell) of 110182. The percentage of phagocytic cells within MDM generated in the presence of *T. evansi* (11.0% of total cells) or the purified Ro Tat 1.2 antigen (11.5% of total cells) was significantly lower than control MDM ($p < 0.05$). Similarly, the MFI values were significantly lower for *T. evansi* MDM (98718) and the purified Ro Tat 1.2 MDM (95171) compared to the control MDM (Figure 1 D and E, $p < 0.05$).

Stimulation of day-4 MDM generated without any trypanosomal antigens with the phorbol ester phorbol-12-myristate-13-acetate (PMA) induced a significant increase in the MFI signals of the ROS indicator Dehydrorohdamine-123 (DHR-123), indicating ROS generation in these cells ($p < 0.05$). In contrast, MDM generated in the presence of whole *T. evansi* parasites or their purified Ro Tat 1.2 antigen failed to respond to PMA stimulation with no increase in their ROS generation. For MDM without PMA stimulation, the baseline ROS amount was higher in MDM generated in the presence of the purified Ro Tat 1.2 antigen ($p < 0.05$, Figure 2).

Phagocytosis and the production of reactive oxygen species (ROS) are essential antimicrobial functions of macrophages (Fu and Harrison, 2021). Although it has not been proven in animals with Surra infection, the reduced phagocytosis activity of camel MDM generated in the presence of *T. evansi* antigens indicates a negative effect of the *T. evansi* parasite on the antimicrobial capacity of camel macrophages (Neaga et al., 2013). This is also supported by the lack of ROS-response in camel MDM generated in the presence of *T. evansi* antigens after stimulation with PMA. These results indicated a compromising effect of *T. evansi* on the innate defense mechanisms in camels, which may pave the way for secondary bacterial infections.

Upon leaving the bloodstream, monocytes can differentiate into different subsets of macrophages based on the local tissue-specific stimuli in their microenvironment (Hussen et al., 2014; Helft et al., 2015). The expression pattern of several cell surface molecules was analyzed to identify the functional phenotype of MDM generated under in vitro stimulation with T. evansi antigens. In comparison to control MDM generated in medium control alone, the presence of the whole *T. evansi* parasites during the *in vitro* differentiation of monocytes into macrophages resulted in MDMs with lower ($p < 0.05$) abundance of the cell surface molecules CD14, CD172a, CD163, CD45, and CD44, while the abundance of MHCII molecules was higher on *T. evansi* MDM ($p < 0.05$; Figure 5A-F). For MDM generated in the presence of the purified Ro Tat 1.2 antigen, only a reduced ($p < 0.05$) abundance of CD14 with enhanced ($p < 0.05$) MHCII expression was observed, compared to control MDM (Figure 3A-F).

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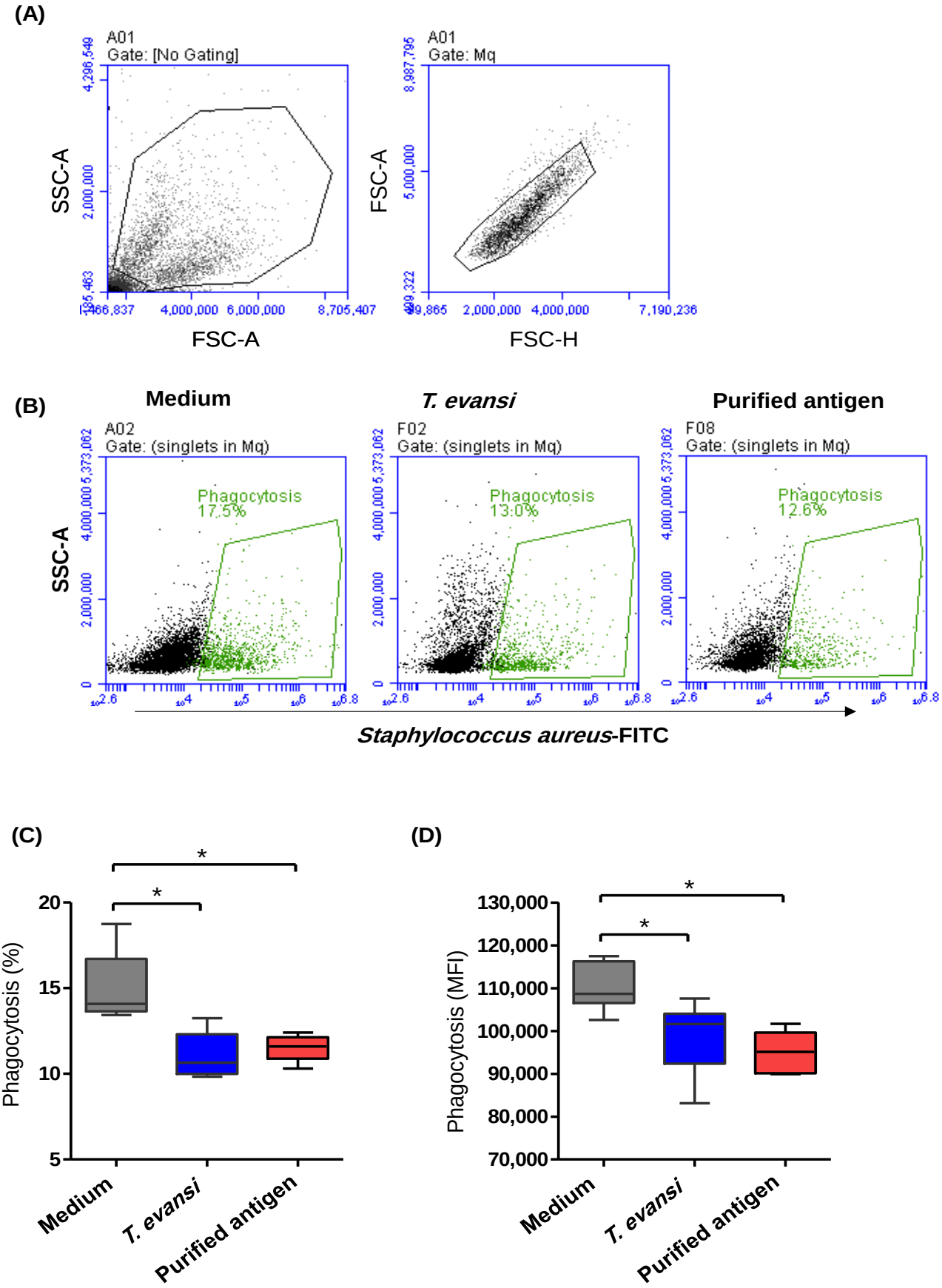

Figure 1. Phagocytosis of *Staphylococcus aureus* by camel monocyte-derived macrophages (MDM). Camel monocytes were allowed to differentiate into macrophages in the presence of whole inactivated *T. evansi* parasites or with purified Ro Tat 1.2 antigen or in culture medium alone for 4 days. **A:** Day-4 macrophages were gated based on their forward scatter (FSC) and side scatter (SSC) properties after the exclusion of cell duplets. **B:** The phagocytosis was analyzed by flow cytometry after incubation of MDM with FITC-labeled *Staphylococcus aureus*. The percentage of phagocytic cells (**C**) and the mean fluorescence intensity (MFI) of phagocytic cells (**D**) were calculated and presented as boxplots. * Indicates significant differences with a p-value less than 0.5.

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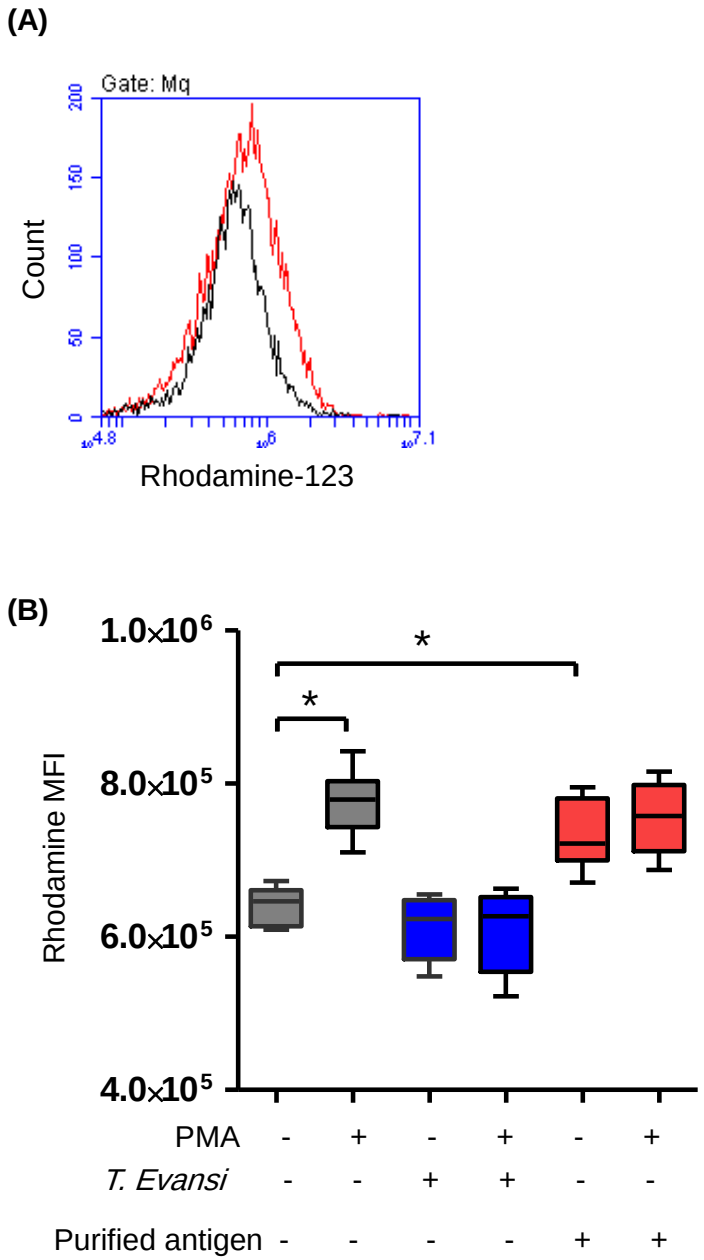

Figure 2. Reactive oxygen species (ROS) generation by monocyte-derived macrophages (MDM). Camel monocytes were allowed to differentiate into macrophages in the presence of whole inactivated *T. evansi* parasites or with purified Ro Tat 1.2 antigen or in culture medium alone for 4 days. **A:** Flow cytometric measurement of ROS production by day-4 macrophages stimulated with PMA (red line) or left without stimulation (black line) was performed after labeling the cells with dehydrorhodamine-123. **B:** Rhodamine-123 MFI values were calculated and presented for stimulated and non-stimulated cells as boxplots. * Indicates significant differences with a p value less than 0.5.

The major histocompatibility complex (MHC) class II molecules and the scavenger receptor for haptoglobin–hemoglobin complexes CD163 are recognized as markers of classically-activated inflammatory M1 MDM and alternatively-activated anti-inflammatory M2 MDM, respectively (Chavez-Galan et al., 2015; Hu et al., 2017). In the present study, the upregulation of MHCII molecules together with the lower abundance of CD163 molecules on MDM generated in the presence of whole *T. evansi* parasites indicates a polarizing effect of *T. evansi* on the differentiation of camel monocytes into an M1 inflammatory phenotype. The reduced antimicrobial functions of these cells, however, argues against their pro-inflammatory nature. The dominance of pro-inflammatory IFN-gamma-induced M1 macrophages has been found essential for the early control of parasitemia in *Trypanosoma*-infected animals (Magez et al., 2020). However, the reduced phagocytosis and ROS production capacities of camel MDM generated in the presence of *T. evansi* parasite may represent an immune escape mechanism that may contribute to the early establishment of the infection. Although both MDM generated in the presence of whole *T. evansi* antigens or their purified RoTat 1.2 proteins showed similar expression levels of CD14 and MHCII molecules, the different abundance of the cell surface molecules CD172a, CD163, CD45, and CD44 indicates different phenotypes of the two MDMs.

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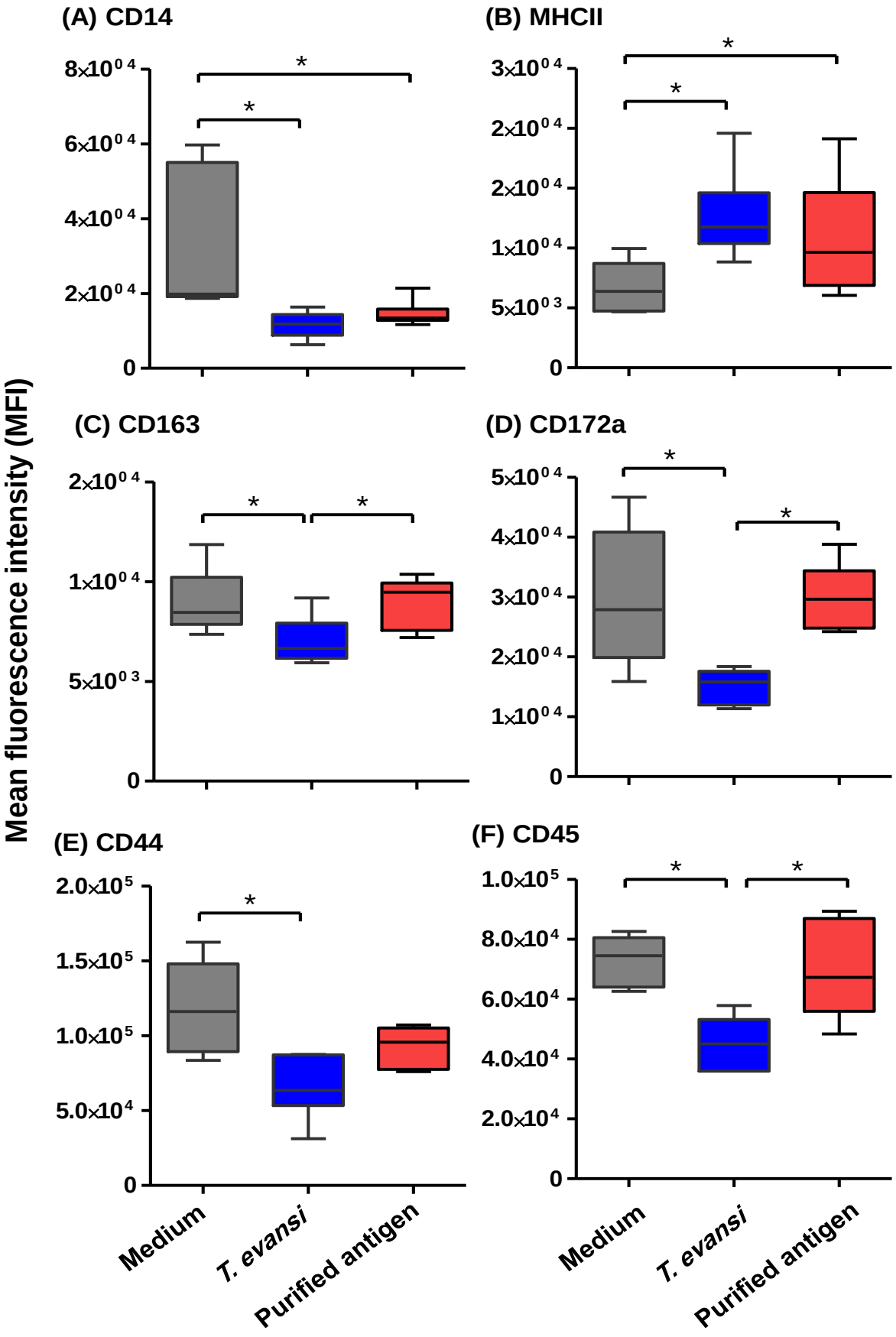

Figure 3. Phenotype of monocyte-derived macrophages (MDM) generated in the presence of whole inactivated *T. evansi* parasites or with purified Ro Tat 1.2 antigen or in culture medium alone. Day-4 macrophages were labeled with mAbs to several cell markers and analyzed by flow cytometry. MFI values indicating the expression levels of CD14 (**A**), MHCII (**B**), CD163 (**C**), CD172a (**D**), CD44 (**E**), and CD45 (**F**) were calculated and presented for the three setups of macrophages. * indicates significant differences with p value less than 0.5.

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CONCLUSION

The results of the present study indicated compromising effects of *T. evansi* antigens on the phenotype and function of camel macrophages differentiated *in vitro* from blood monocytes. The reduced phagocytosis activity of camel MDM generated in the presence of *T. evansi* antigens in addition to the lack of their ROS-response to stimulation with PMA indicates a compromising effect of *T. evansi* on the innate defense mechanisms in camels. Whether this could be related to the modulatory effect of other *T. evansi* antigens that are only present in the whole parasite but not in the purified RoTat 1.2 protein, it needs to be investigated in future studies. Future research may focus on the functional characterization of MDM generated from *T. evansi-infected* camels. For this, the analysis of key M1 cytokines like IFN-gamma or M2 cytokines like IL-10 may uncover the role of these cells during *T. evansi* infection.

DECLARATIONS

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Availability of data and material

The datasets generated during the current study are available from the corresponding author on reasonable request.

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Not applicable

Authors' contribution

Essa Ali Alhilal did sample collection and manuscript revision; Mayyadah Abdullah Alkuwayti did supervision and manuscript preparation; Noof Abdulrahman Alrabiah did manuscript preparation and revision. Omar Al-Jabr did manuscript preparation and revision; Jamal Hussen did Analysis, funding acquisition, and writing of the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

There is no competing interests to declare.

Ethical consideration

Ethical issues have been checked by all the authors.

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