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Research Article

Exploring Immune Evasion Strategies: CD200 And ATK/TLR2 Upregulation in Response to Chronic *T. evansi* Vesicular Fraction Stimulation

Nnanna Isaiah Ibeh[1]*, Onyiyechi Cynthia Okeke[1], Micheal Awo Okungbowa[2] and Isaiah Nnanna Ibeh[3]

[1]Department of Veterinary Anatomy, Faculty of Veterinary Medicine, University of Benin, Nigeria

[2]Department of Physiology, Igbinedion University, Edo State, Nigeria

[3]Department of Medical Laboratory Science, School of Basic Medical Sciences, University of Benin, Nigeria

***Corresponding Author:** Nnanna Isaiah Ibeh, Department of Veterinary Anatomy, Faculty of Veterinary Medicine, University of Benin, Nigeria.

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Abstract

Background: Trypanosoma evansi, the parasite responsible for surra, a disease affecting livestock and occasionally humans, has evolved strategies to evade the host immune system. A key mechanism involves extracellular vesicles (EVs) that contain proteins and molecules impacting host immune cells. Understanding these interactions is essential for developing effective treatments.

Aim: This study explores how *T. evansi*-derived EVs affect the expression of immune-related proteins CD200 and AKT/TLR2 in host cells, aiming to understand their role in immune evasion.

Method: We used RAW 264.7 and THP-1 cell lines, stimulating them with *T. evansi* EVs for 24 to 72 hours. Flow cytometry assessed CD200 expression. Quantitative real-time polymerase chain reaction (qRT-PCR) measured the expression of CD200 and AKT/TLR2, while enzyme-linked immunosorbent assays (ELISA) quantified pro-inflammatory cytokines IL-6 and TNF-α.

Results: Flow cytometry showed a significant upregulation of CD200 after 72 hours of stimulation with *T. evansi* EVs. ELISA revealed elevated IL-6 and TNF-α levels, suggesting an inflammatory response to the EVs. qRT-PCR indicated increased expression of TLR2 and AKT.

Conclusion: The findings suggest that *T. evansi* EVs may contribute to immune evasion by upregulating CD200, which can suppress host immune responses. The simultaneous increase in IL-6 and TNF-α implies that immune pathways are activated, indicating a complex host response to *T. evansi* infection. These insights into the parasite's immune evasion tactics could guide the development of new therapeutic strategies for surra and other parasitic diseases. Further research into CD200's role in immune modulation is warranted.

Keywords: *Trypanosoma evansi;* Extracellular Vesicles (EVs); Immune Evasion; CD200 Expression; Pro-inflammatory Cytokines; Host-Pathogen Interactions

Background of Study

Trypanosoma evansi is a protozoan parasite causing surra, a devastating disease in livestock and occasionally humans. Despite being closely related to *Trypanosoma brucei*, which causes sleeping sickness in humans, *T. evansi* has evolved unique mechanisms to evade the host immune response, allowing it to establish chronic infections [1,10]. Understanding these evasion strategies is crucial for the development of effective control measures and therapeutics.

One of the mechanisms employed by *T. evansi* involves the release of extracellular vesicles (EVs), which contain various proteins and molecules that modulate the host immune system. These EVs have been shown to play a significant role in immune evasion by suppressing the host's innate and adaptive immune responses [5]. However, the specific interactions between *T. evansi* EVs and host immune cells, as well as the underlying molecular mechanisms involved, remain largely unexplored [3].

Among the key players in immune regulation are CD200 and the ATK/TLR2 signalling pathway. CD200 is a cell surface glycoprotein that acts as a negative regulator of immune responses by interacting with its receptor, CD200R, on myeloid cells, leading to the suppression of pro-inflammatory cytokine production [2,15]. On the other hand, the ATK/TLR2 pathway plays a crucial role in recognizing and responding to microbial pathogens by activating innate immune responses. Understanding how *T. evansi* EVs modulate the expression of CD200 and ATK/TLR2 in host immune cells is essential for deciphering the parasite's immune evasion strategies [5,11].

In this study, we aim to investigate the effects of chronic *T. evansi* EV stimulation on the expression of CD200 and ATK/TLR2 in host immune cells . We hypothesize that *T. evansi* EVs modulate the expression of these molecules to suppress host immune responses, facilitating parasite survival and establishment of chronic infection [14]. To test this hypothesis, we will utilize *in vitro* and *in vivo* models to simulate chronic *T. evansi* infection and examine the expression levels of CD200 and ATK/TLR2 in immune cells exposed to *T. evansi* EVs [13].

By elucidating the mechanisms underlying *T. evansi* immune evasion strategies, this study will contribute to the development of novel therapeutic approaches targeting parasite-host interactions. Additionally, the findings may have broader implications for understanding immune evasion mechanisms employed by other parasitic infections, thus paving the way for the development of more effective control strategies against parasitic diseases.

Method

Experimental setup and cell culture

Cell preparation

RAW 264.7 and THP-1 cells were seeded in 6-well plates at a density of 1 × 10^6 cells per well and stimulated with *T. evansi* EVs at a concentration of 10 µg/mL for 24 hours. Control cells were left untreated. Following stimulation, cells were detached using trypsin-EDTA, washed with phosphate-buffered saline (PBS), and resuspended in staining buffer (PBS supplemented with 2% FBS) at a concentration of 1 × 10^6 cells/mL.

Antibody staining

Cells were incubated with anti-CD200 antibody (dilution 1:100) or appropriate isotype control antibody for 30 minutes at 4°C in the dark. After washing with staining buffer, cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature. Following fixation, cells were washed again and resuspended in staining buffer for flow cytometric analysis.

Flow cytometry

Stained cells were analyzed using a flow cytometer equipped with appropriate lasers and filters. A minimum of 10,000 events weree acquired for each sample. Data was collected in the form of forward scatter (FSC), side scatter (SSC), and fluorescence intensity. Analysis gates were set based on forward and side scatter properties to exclude debris and cell aggregates.

Extracellular vesicle (EV) isolation and stimulation

T. evansi EVs were isolated from culture supernatants using a combination of differential ultracentrifugation and size-exclusion chromatography. EVs were quantified using nanoparticle tracking analysis (NTA) and characterized by transmission electron microscopy (TEM) and western blotting. Subsequently, RAW 264.7 and THP-1 cells were exposed to *T. evansi* EVs at a concentration of 10 µg/mL for 24 hours to simulate chronic infection.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from treated cells using TRIzol reagent, and cDNA was synthesized using a reverse transcription kit. The expression levels of CD200 and ATK/TLR2 were quantified by qRT-PCR using specific primers and SYBR Green master mix. Gene expression was normalized to housekeeping genes, and fold changes were calculated using the comparative Ct method.

Enzyme-linked immunosorbent assay (ELISA)

The production of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), were measured in cell culture supernatants using commercially available ELISA kits. Cells were stimulated with *T. evansi* EVs, and supernatants were collected after 24 hours. ELISA assays were performed according to the manufacturer's instructions, and cytokine concentrations were determined by comparing optical densities to standard curves.

Statistical analysis

All experiments were performed in triplicate, and data was expressed as mean ± standard deviation (SD). Statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. P-values < 0.05 were considered statistically significant. Graphs and figures were generated using appropriate software to illustrate the results of the study.

Result

Flow Cytometric Analysis of CD200 Expression in Cell Suspension After Stimulation with Trypanosoma evansi Vesicular Fractions.

Flow cytometric analysis indicated that CD200 expression, as measured by mean fluorescence intensity (MFI), was significantly upregulated 72 hours after stimulation with Trypanosoma evansi vesicular fractions, compared to *ex vivo* levels. Statistical analysis confirmed a significant difference ($P < 0.0085$), indicating that the vesicular fractions from *T. evansi* stimulate increased CD200 expression over time (Figure 1). This finding suggests a potential impact of vesicular fraction stimulation on surface molecule expression, which may have implications for understanding the cellular responses to *T. evansi* infections.

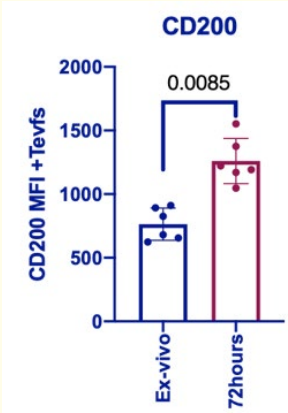

Figure 1: Flow cytometric analysis showing mean fluorescence intensity (MFI) of CD200 expression in cell suspension post-stimulation with Trypanosoma evansi vesicular fractions. The analysis was conducted *ex vivo* and 72 hours post-stimulation to compare the expression levels of CD200. A statistically significant increase in CD200 MFI was observed 72 hours post-stimulation compared to *ex vivo* ($P < 0.0085$). Data were assessed for normality using the Shapiro-Wilk and D'Agostino and Pearson tests, and the difference between groups was evaluated using a Student's t-test ($p < 0.05$ considered significant). The bars represent the mean ± standard deviation.

ELISA-Based Quantification of IL-6 and TNF-α Concentrations Following Stimulation with Trypanosoma evansi Vesicular Fractions.

Enzyme-linked immunosorbent assay (ELISA) was used to measure the concentrations of cytokines IL-6 and TNF-α in cell suspensions stimulated with *Trypanosoma evansi* vesicular fractions. The results showed that the levels of IL-6 were significantly higher 72 hours after stimulation compared to *ex vivo* ($P < 0.05$). Similarly, TNF-α concentrations were also significantly upregulated 72 hours post-stimulation, relative to the *ex vivo* measurements ($P < 0.05$) (Figure 2). These findings indicate an inflammatory response to *T. evansi* vesicular fractions over time, suggesting that prolonged exposure to these vesicles induces increased production of inflammatory cytokines. This response could provide insights into the host's immune reaction to *T. evansi* infections and help identify potential therapeutic targets.

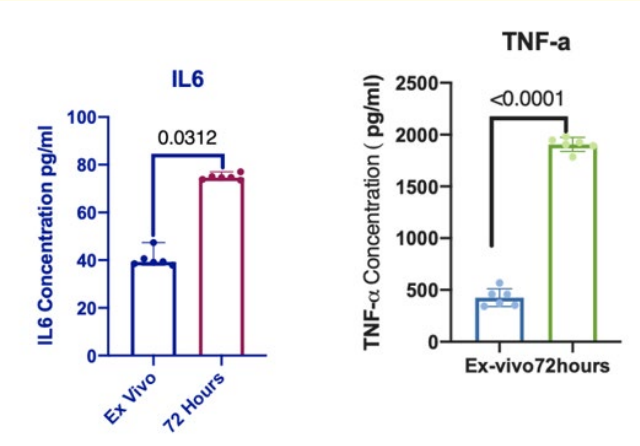

Figure 2: ELISA results showing concentrations of cytokines IL-6 and TNF-α in cell suspensions post-stimulation with *Trypanosoma evansi* vesicular fractions. Data were collected *ex vivo* and 72 hours after stimulation to assess the inflammatory response. A statistically significant increase in both IL-6 and TNF-α concentrations was observed 72 hours after stimulation compared to *ex vivo* ($P < 0.05$). The analysis involved a Student's t-test to determine the statistical significance of these differences. The bars represent the mean ± standard deviation.

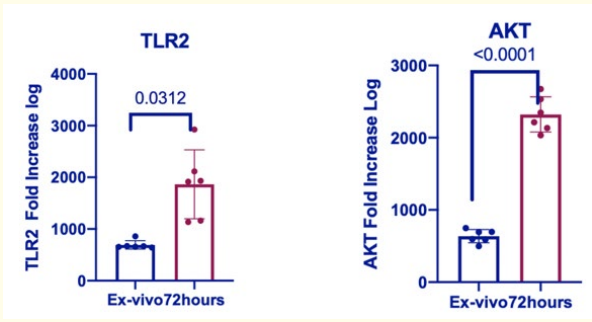

Figure 3: Quantitative PCR (qPCR) results showing the expression levels of Toll-like receptor 2 (TLR2) and the transcriptional protein AKT in cell suspensions post-stimulation with *Trypanosoma evansi* vesicular fractions. Data were collected *ex vivo* and 72 hours after stimulation to assess changes in gene expression. Both TLR2 and AKT were significantly upregulated 72 hours post-stimulation compared to *ex vivo* ($P < 0.05$). Statistical significance was determined using a Student's t-test. The bars represent the mean ± standard deviation.

Quantitative polymerase chain reaction (qPCR) was used to measure the expression levels of TLR2 and AKT in cell suspensions stimulated with Trypanosoma evansi vesicular fractions. Results indicated that TLR2 expression was significantly higher 72 hours after stimulation compared to *ex vivo* ($P < 0.05$). Similarly, AKT expression levels were also significantly elevated 72 hours post-stimulation, compared to *ex vivo* ($P < 0.05$) (Figure 3). This upregulation suggests that *T. evansi* vesicular fractions may trigger a robust response involving innate immune recognition and signaling pathways, potentially providing insights into the mechanisms of host-pathogen interactions, and suggesting possible targets for therapeutic intervention.

Discussion

The observed upregulation of CD200 following stimulation with *Trypanosoma evansi* vesicular fractions, along with the increase in cytokines IL-6 and TNF-α, and transcriptional protein AKT, suggests a complex interplay between host cells and parasite-derived components. CD200, also known as OX-2, is a membrane glycoprotein involved in immune regulation, often exerting an inhibitory effect on immune cells, particularly macrophages [8,9]. Its interaction with CD200 receptor (CD200R) on macrophages can modulate their activation state and subsequently affect inflammatory responses.

The significant increase in CD200 expression 72 hours after stimulation with *T. evansi* vesicular fractions suggests that these vesicles may influence immune responses through mechanisms involving CD200. Given the role of CD200 in dampening immune responses, its upregulation could represent a strategy by *T. evansi* to evade host defenses, allowing the parasite to persist within the host. This finding aligns with previous studies demonstrating that pathogens can utilize CD200-mediated pathways to reduce host immune responses, thereby aiding in their survival and proliferation [4].

The increase in cytokines IL-6 and TNF-α, along with the transcriptional protein AKT, indicates that while CD200 expression

may have a regulatory effect, the overall stimulation of immune pathways is evident. IL-6 and TNF-α are key cytokines involved in the inflammatory response, often serving as markers for immune system activation [14]. The increase in AKT, a crucial player in cell survival and metabolism, suggests that the vesicular fractions may also influence signaling pathways critical for macrophage activation and function (Jawalagatti., *et al.* 2023). This combination of immune activation with regulatory signals could reflect a balanced response to infection, with the host attempting to contain the parasite while avoiding excessive inflammation that could lead to tissue damage [1].

The upregulation of CD200, as noted in the context of *Trypanosoma evansi,* has significant implications for immune evasion and chronic infection. CD200 is known to act as an immune checkpoint molecule, generally suppressing immune cell activation when it binds to its receptor, CD200R, on myeloid cells. This interaction reduces the production of pro-inflammatory cytokines, potentially dampening the immune response. In the case of *T. evansi*, the upregulation of CD200 may be a deliberate strategy by the parasite to evade immune surveillance.

This mechanism could be related to the variant surface glycoprotein (VSG) gene, which is responsible for antigenic variation in trypanosomes like *T. brucei,* allowing them to escape immune detection. If the vesicular fractions of *T. evansi* contain VSGs, they could potentially impact host immune responses by modulating the expression of CD200, thereby reducing the activation of immune cells like macrophages. This reduction in macrophage activity could occur through the inhibition of CD40L, an important molecule on T cells that helps stimulate immune responses. By inactivating CD40L, *T. evansi* could effectively reduce the strength and efficiency of the adaptive immune response, allowing it to persist and establish chronic infection.

Additionally, the upregulation of TLR2 and AKT may also play a crucial role in the chronicity of *T. evansi* infection. TLR2 is part of the innate immune system, recognizing pathogen-associated molecular patterns (PAMPs) and initiating immune responses [6]. Its upregulation suggests an attempt by the host to recognize and respond to *T. evansi*, but the simultaneous upregulation of CD200 indicates a counteracting force by the parasite to suppress this response.

Similarly, the AKT signaling pathway is crucial for cell survival, metabolism, and immune regulation. If AKT is upregulated, it may indicate a shift toward cell survival and proliferation, potentially supporting the longevity and persistence of *T. evansi* within the host [2]. The combined upregulation of TLR2 and AKT, with the dampening effect of CD200 on adaptive immune responses, suggests a complex interplay where the host is trying to mount a defense, but the parasite's strategies effectively counterbalance this effort, leading to chronic infection [11].

These insights into how *T. evansi* manipulates immune pathways not only highlight the challenges in combating surra but also point to potential therapeutic targets. By focusing on CD200 and its interaction with VSGs, researchers may find new ways to block the immune evasion tactics of *T. evansi*. Similarly, understanding the roles of TLR2 and AKT in chronic infection could offer additional avenues for therapeutic intervention, helping to restore the balance in the host's immune response to combat parasitic infections more effectively like surra [14].

These findings have significant implications for understanding the host-pathogen interactions in *T. evansi* infections. The balance between immune activation and regulation may be a key factor in determining the outcome of such infections. The role of CD200 in this context warrants further investigation, particularly to determine if targeting CD200 or its receptor could offer therapeutic benefits in managing *T. evansi* infections or other similar parasitic diseases [14,15].

Given the potential regulatory role of CD200, future research should focus on the downstream effects of its interaction with CD200R on macrophages and other immune cells. Understanding these pathways could lead to novel strategies to modulate immune responses, potentially enhancing the host's ability to clear parasitic infections without causing excessive inflammation.

Conclusion

In this study, we investigated the impact of *Trypanosoma evansi* vesicular fractions on immune cell responses, with a focus on CD200 expression, inflammatory cytokines, and transcriptional proteins. Our results demonstrate that 72 hours post-stimulation, there is a significant upregulation of CD200, along with increased upregulation of IL-6, TNF-α, and AKT, indicating an immune

response to vesicular fractions. These findings suggest that *T. evansi* may use CD200-mediated mechanisms to modulate the host's immune response, potentially contributing to immune evasion and parasite persistence. Further research is required to elucidate the pathways involved and to explore therapeutic targets to improve the host's ability to combat *T. evansi* infection.

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Conflict of Interest

The authors declare no conflicts of interest related to the research, funding, or publication of this study. Transparency and ethical conduct have been maintained to uphold the integrity of the findings presented in this work.

Ethical Approval

This study has obtained ethical approval from the Institutional Review Board (IRB) with allocated number UB/VA200231 , ensuring that the research adheres to ethical principles and guidelines for the humane treatment of animals and ethical standards for experimental research involving human subjects.

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Limitations of the Study

This study has several limitations. First, the sample size was relatively small, potentially affecting the generalizability of the results. Additionally, the experiments were conducted *in vitro*, which may not fully replicate the complex *in vivo* environment. Furthermore, while we observed significant changes in cytokine and transcriptional protein levels, the exact signaling pathways involved remain unclear and require further investigation. Future studies should also consider exploring other immune cells and markers to provide a more comprehensive understanding of the immune response to *T. evansi*.

Authors Contribution

Each author played a significant role in the conception, design, execution, and analysis of the study. Nnanna Isaiah Ibeh experimental work, data analysis, Dr Onyiyechi Cynthia Okeke for manuscript writing, Professor M.A Okungbowa experimental work and script writing, and Professor I.N Ibeh for his contribution in the manuscript writing contributed. All authors critically reviewed and approved the final version of the manuscript.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used Gramerly/chingui A.I in order to correct sepellings and punctuations. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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