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Impact of *Trypanosoma evansi* Extracellular Vesicles on Immune Modulation: Insights into Macrophage Activation, Cytokine Dynamics, and Gene Expression

Nnanna Isaiah IBEH[1] (BMLs, MSc), Terry Nosa OMORODION[2] (BMLS, MSc, PhD) Micheal Awo OKUNGBOWA[2] (AMLS,MSc, PhD) and Isaiah Nnanna IBEH[3] (AMLS, MSc, PhD)

*[*1]Department of Veterinary Anatomy, Faculty of Veterinary Medicine, University of Benin, Nigeria.*
[2,3]Department of Medical Laboratory Science, School of Basic Medical Sciences, University of Benin, Benin City, Nigeria
** Corresponding Author: nnanma.ibeh@uniben.edu, +2348034834189,*
Authors email: terry.omorodion@uniben.edu, Michael.okungbowa@uniben.edu, uko.ibeh@uniben.edu

Abstract
The diverse host range and distribution of Trypanosoma evansi, a pathogenic trypanosome causing surra, in contrast to its ancestor, Trypanosoma brucei. The research focuses on the immune-modulatory potential of extracellular vesicles (EVs) derived from T. evansi, shedding light on their role in intercellular communication and host immune modulation. The investigation involves in vitro studies utilizing 12 mice, assessing F4/80 expression, flow cytometry analysis of CD86, CD206, and MHC II markers, and cytokine expression post-Tevfs stimulation. The sustained upregulation of F4/80 expression in macrophages post-stimulation indicates a prolonged engagement, suggesting a complex, time-dependent pattern. Flow cytometry reveals a significant upregulation of CD206, indicating a potential shift towards an anti-inflammatory M2-like macrophage phenotype. While trends are observed in CD86 and MHC II, statistical significance is not reached, implying alterations without statistical significance. Cytokine expression analysis indicates a significant upregulation of IL-10 and IL-4, suggesting an immunomodulatory effect favouring anti-inflammatory cytokine production. The noteworthy upregulation in Arginase-1, Toll-like receptor 9, and Inducible Nitric Oxide Synthase at 72 hours post-stimulation emphasizes the complexity of the immune response. The highly significant trend in Arginase-1 upregulation suggests a sustained impact, potentially influencing immune suppression and tissue repair. In conclusion, this research provides valuable insights into the intricate immune modulation orchestrated by T. evansi vesicular fractions. The observed patterns underscore the need for further investigations to unravel underlying mechanisms and explore potential therapeutic implications in the context of trypanosome infections. The study's focus on T. evansi-derived EVs opens avenues for translational research with potential real-world applications against trypanosome parasites.

Keywords: *Trypanosoma evansi, Extracellular vesicles, Immune response, Macrophage activation, Cytokine modulation, and Gene expression.*

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I. Background of Study

The distribution and host range of pathogenic trypanosomes vary widely, with Trypanosoma evansi exhibiting the broadest host range globally. In contrast, its ancestor, Trypanosoma brucei, had a more limited distribution(Benfoldi *et al.,* 2020). The divergence in distribution is attributed to *T. evansi'*s acquisition of new transmission modes through the loss of genetic material, allowing cyclical transmission in tsetse flies (Boushaki *et al.,* 2019;Selim *et al.,* 2022). This characteristic makes surra a multispecies and polymorphic disease, possibly constituting a complex of diseases induced by a group of parasites known as Trypanosoma evansi or a group of subspecies named *Trypanosoma brucei evansi* (Mirshekar *et al.,*2017).

In Africa and the Middle East, *T. evansi* causes diseases in camels, horses, and dogs, primarily in the northern regions of the Tsetse fly belt. *T. brucei brucei* infections in camels have been reported near the tsetse fly belt, while *T. congole*nse infections prove fatal to camels (Ramirez-Iglesias *et al*., 2017). Preventing their entry into the tsetse fly belt is crucial to eliminating *T. evansi* in the region. Surra prevalence in Africa is primarily influenced by camel infections, showing seasonal patterns due to vector activity and disease expression (Kamidi *et al*., 2017). Transhumant cattle herds migrating between the Tsetse belt and northern regions face challenges in distinguishing between *T. brucei and T. evansi* infections, with *T. evansi* infections uncommon in African cattle (Kamidi *et al*., 2017).

Extracellular vesicles (EVs) have become a focal point in biological research, acting as mediators of intercellular communication. In parasitology, the study of EVs derived from *Trypanosoma evansi* reveals their role in manipulating intercellular communication and modulating host immunity. This discovery prompts a reassessment of *T. evansi's* pathogenicity and mechanisms of persistence within its host (Wei *et al.,* 2021).

This research aims to provide a thorough analysis of the immune-modulatory potentials of *T. evansi*-derived extracellular vesicles. This includes examining the biochemical constituents, mechanisms, and potential limitations of these interventions addressing gaps in knowledge and seeking to pave the way for further investigations into the therapeutic potential of *T. evansi*-derived extracellular vesicles against Trypanosoma parasites in Balb/c mice. The need for alternative treatments due to the limitations of current medical technologies drives the investigation into *T.evansi* vesicular fractions. The study's potential economic and agricultural implications are significant, as positive outcomes could impact livestock populations in regions affected by *T. evansi*, contributing to agricultural economies.

In conclusion, this research addresses a complex and pressing issue in public health, offering potential solutions through the exploration of alternative treatments and a deeper understanding of the underlying biological mechanisms. The study's focus on *T. evansi*-derived extracellular vesicles highlights their potential as interventions against Trypanosoma parasites, providing a foundation for further translational research and real-world applications.

II. Materials and Method

Study Popultion and Design

In-vitro studies invlved 12 mice, acclimatized and culled in the department of Veterinary Medicine, University of Benin, Nigeria . During this period, the mice were housed in well-ventilated cages and provided with daily cage cleaning and a constant supply of food and water at a room temperature of 37°C. The mice will be fed with commercially prepared vital food to maintain uniform nutrition.

***T.evansi* Extraction**

In the extraction of live parasites and in-vitro culture mice were humanely culled, and blood and tissues were collected. The blood was centrifuged to obtain plasma, and tissues (liver, spleen, kidney, brain) were homogenized in PBS. The resulting homogenate underwent centrifugation to yield a supernatant, inspected under a microscope (400x) for T. evansi presence. Extracted parasites were transferred to a slide, covered, and examined under a microscope (1000x) to confirm identity (Maharana *et al I*., 2020).

Live Culture of T.evansi and vesicular fraction extraction.

For extracellular vesicle (EV) extraction , a *T. evansi* culture was grown, washed in PBS, and resuspended in a serum-free medium. After 16-24 hours, cellular debris was removed from the conditioned medium by centrifugation. The supernatant underwent ultracentrifugation to harvest EVs, following the method outlined by (Wei *et al*., 2021)

Determination of Protein Concentration of Vesicular Fractions derived from T.evansi

In the determination of protein concentration , samples were thawed, and EV suspensions were diluted in PBS. Standard protein solutions (0-2mg/dl) were prepared using BSA and Bradford reagent. Absorbance readings at 560nm were taken using a Spectrophotometer and a Nanodrop. Protein concentrations were calculated using standard curves and normalized to 35µg EV concentrations (Launrenzana *et al*., 2021)

Isolation of peritoneal Macrophages

Adult male mice aged 6-8 weeks were euthanized through CO2 inhalation following a protocol by Caserta & Pera (2021). The peritoneal cavity was opened, and fluid was collected using a syringe with a 25-gauge needle and ice-cold PBS, avoiding organ damage. After centrifugation at 400 x g for 10 minutes at 4°C, the pellet was resuspended in a 5% FBS 1640 RPMI complete medium. Cell counting utilized a hemacytometer and Trypan blue, adjusting the concentration as needed. Cells were seeded at 1 x 10^6 cells/mL per well in 6- or 12-well plates.

Cell Stimulation and Flow Cytometric Analysis.

In the subsequent in-vitro suspension and stimulation by extracellular vesicles (EVs),cells were plated with EVs at a concentration of 35µg/mL, incubated for specified times, including ex-vivo conditions. Suitable controls, both negative and positive (non-stimulated), were included, and cells were incubated until reaching 80-90% confluency at 37°C with 5% CO2. Cell concentration was calculated following the formula by (Caserta and Pera, 2021).

For Day 0/4 Staining, 1 ml of cell suspension was transferred to labeled FACs tubes under various conditions. Following a wash with 1 ml of FACs buffer, cells were centrifuged, resuspended, and incubated with FC block. Antibodies targeting surface markers were added, and controls were prepared simultaneously. After a 20-minute incubation in the dark and on ice, samples were washed, centrifuged, and acquired on the BD LSR Fortessa, capturing 10,000 events per sample. Subsequent analyses were performed using FlowJO (BD USA 10.8.1) according to the protocol by (Caserta and Ghezzi, 2021).

ELISA for Cytokine Evaluation

The procedure continued with 96-well ELISA plates subjected to overnight incubation at 4°C, coating each well with a capture antibody buffer. Standard solutions and undiluted samples were dispensed into duplicate wells, followed by incubation and washing steps. The working detector solution, containing detection antibody and avidin-HRP reagent, was added, and after another incubation and washing step, substrate solution was introduced. The resulting color change was measured at 450 nm using an ELISA plate reader, and cytokine concentrations were determined through standard curves (Caserta and Pera, 2021)

RT-QPRC fpr quantitative real time detection of transcriptional proteins

Quantitative RT-PCR, total RNA extraction utilized the RNeasy Plus Mini Kit from Qiagen. The RNA samples were treated with DNase I, and cDNA synthesis was performed using the High-Capacity cDNA Archive Kit from Applied Biosystems. Real-time quantitative amplification of specific genes involved PCR primers targeting various genes, and reactions were conducted in triplicates using Power Sybr Green Master Mix. An ABI 7500 Real-Time PCR System was used, and standard curves aided in normalizing expression levels to water, as detailed by (Caserta and Ghezzi, 2021)

Statistical Analysis:

GraphPad Prism 8.4.3 was used for the analysis of data acquired, and a combination of Parametric and non-parametric ANOVA (Paired One-way and Two-way analysis of variance) was adopted after data was passed through a normality test (Shapiro Wilk and D'Agostino & Pearson test) to determine their levels of distribution. P values are indicated as $P<0.05$ as significant and $P>0.05$ as non-significant. Post Hoc analysis was carried out using the Dunns multiple comparison test

III. Results;

Our findings elucidated that after stimulating cell suspension with Vesicular fractions of *T.evansi* Tevfs, a meticulous examination of F4/80 expression percentage was conducted throughout various incubation periods. Our observations delineated a sustained upregulation of F4/80 expression consistently observed from Ex vivo through 48 hours and 72 hours post-stimulation (38.50±3.72, 39.33±1.42 and 41.10±2.31). However, it is imperative to underscore that this observed trend did not attain statistical significance upon a comprehensive comparison of all incubation times in vitro (Figure 1.0).

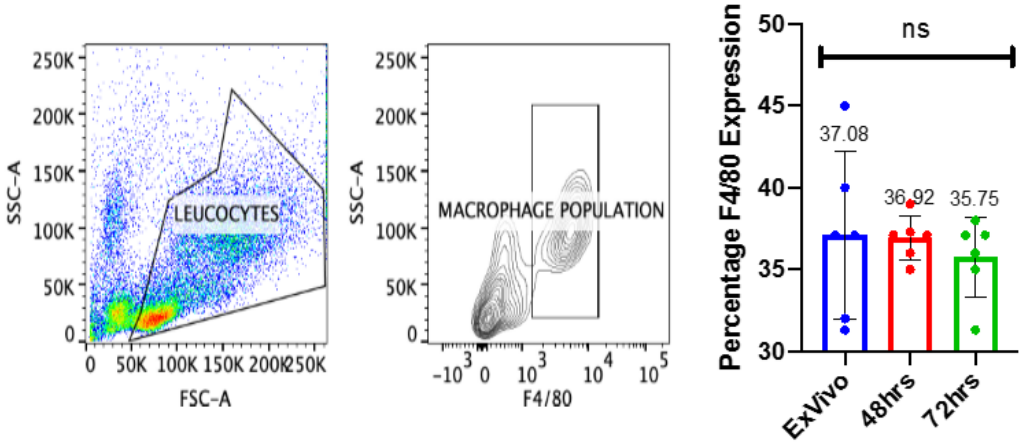

Figure 1.0 : **F4/80 Expression Post-Stimulation with 35mg/ml Mucuna pruriens Seed Crude Oil Extract (SCOEMP) at Different Incubation Times (Ex-vivo, 48 Hours, 72 Hours).** Data was passed through a shapiro wilk and D'Agostino & pearson to determine distribution and a Parametric ANOVA was used to analyse the variables and presented in Mean Standard Deviation. The scatter plot graph illustrates a comparison of F4/80 expression post-stimulation with 35 mg/ml Mucuna pruriens Seed Crude Oil Extract (SCOEMP) across

different incubation times (Ex-vivo, 48 hours, and 72 hours). The results of our investigation revealed that following the stimulation of cell suspension with SCOEMP, a comprehensive assessment of F4/80 expression percentage was meticulously conducted across various incubation durations. Non-significantly, we noted a consistent upregulation of F4/80 expression observed from Ex-vivo through 48 hours and 72 hours post-stimulation ($p>0.05$). n=6, $p<0.05$=Significant and $p>0.05$=Non-significant.

In Figure 2.0 and Table 1.0 following stimulation with vesicular fractions derived from *T.evansi*-Tevfs at the top concentration of 35µl/kg, diverse mean fluorescence expressions were discerned through flow cytometry utilising mice monoclonal antibody markers CD86, CD206, and MHC II. Notably, there was a significant upregulation of CD206 (mannose differentiation marker) upon Tevfs stimulation across various incubation times (ex vivo, 48 hours, and 72 hours) ($p < 0.05$). While certain trends were observed in the Tevfs stimulation on surface markers MHC II and CD86 II during the 48-hour and 72-hour incubation periods compared to the unstimulated control, these trends were statistically non-significant ($p > 0.05$).

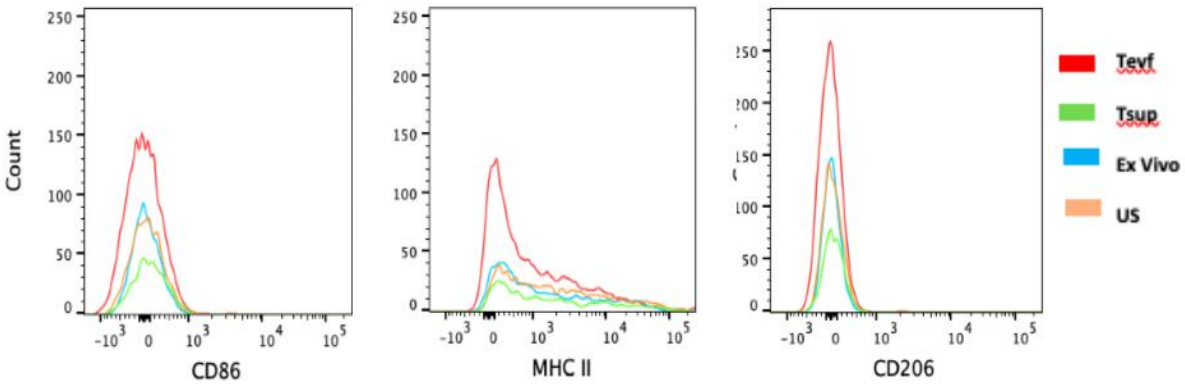

Figure 2.0: Comparative illustration of the Vesicular Fraction Stimulation across the Macrophage Surface Markers. The histogram Layout illustrates the normalized mean flourescence intensity of mouse monoclonal antibody CD86, MHC II and CD206 using a flow cytometry. Tevfs= Vesicular Fraction derived from T.evansi, T.sup,=T.evansi culture supernatant, ExVivo, US= unstimulated control, n=6

TABLE 1.0. Comparison of the macrophage differentiation markers CD86, MHC II and CD206 after stimulation with *Mucuna pruriens* seed crude oil extract-SCOEMP in-vitro

INCUBATION TIME	CD86	MHC II	CD206	POST HOC
EX VIVO	5221± 400.5^{a}	6874±780.2^{b}*	6842±317.3^{c}	B-A C-B* C-A*
48 HOURS	5011± 562.4^{a}	7444±849.7^{b}**	6659±403.7^{c}	B-A C-B ** C-A *
72 HOURS	5002±559.4^{a}	5682±719.0^{b}	7009±723.3^{c}**	B-A C-B C-A **

Key:
P value.
*= significant
**= Moderately significant
****= Highly significant
P<0.05= Significant
P>0.05= Nonsignificant, n=6

It was observed that upon Tevfs stimulation, in comparison to the unstimulated controls, there was a significant upregulation of IL-10 and IL-4 expression post-infection, as opposed to SCOEMP stimulation in vitro ($p<0.05$). molecular pathways, with Arginase-1 exhibiting a notably pronounced pattern over the 72-hour duration.

TABLE 2.0. Comparison between the Anti-inflammatory marker after stimulation with Vesicular fractions derived from *Trypanosoma evansi*-Tevfs and *Mucuna pruriens* seed-SCOEMP crude oil extract In-Vitro.

Anti-Inflammatory Cytokines pg/ml	PBS (Control)	Tevfs 35µg/ml	SCOEMP 35µg/ml	P.value	Post Hoc
IL-4	0^{a}	1906 ± 67.2^{b}**	753.0 ± 53.8^{c}	0.0001	C-B B-A*** C-A
IL-13	0^{a}	636.8 ± 91.7^{b}****	46.17 ± 15.79^{c}***	<0.0001	C-B*** B-A*** C-A***
IL-10	0^{a}	5084 ± 634.9^{b}**	238.7 ± 19.7^{c}	0.0001	C-B B-A** C-A

Key:
P value.
*= significant
**= Moderately significant
****= Highly significant
P<0.05= Significant
P>0.05= Nonsignificant

A noteworthy upregulation in the fold increase of the Arginase-1 gene (344.7±27.9 and 35.3±0.80, p<0.05), Toll-like receptor 9 (161.3±49.2 and 33.0±9.16, p<0.05), and Inducible Nitric Oxide Synthase (175.7±53.2 and 42.2±6.5, p<0.05) at 72 hours post-stimulation with Tevfs. Particularly, a highly significant trend was observed in the case of the Arginase-1 gene relative to the ex vivo timeline (p=<0.0001). These findings suggest a substantial modulation in the expression of genes associated with immune response and regulatory processes, emphasizing the impact of Tevfs stimulation on these

TABLE 3.0: Comparison of the Ex vivo and 72 hours incubation time to determine the level of expressions of pathogen-associated molecular patterns (TLR2 and TLR9) and macrophage activation markers across the Tevfs stimulation.

Transcriptional Proteins Log Fold Increase	**Ex Vivo 35µl**	**72Hours 35µl**	**P.value**
TLR2	45.07±4.5	145.1±4.5*	0.00312
TLR9	33.0±9.16	161.3±49.2**	<0.0018
Arginase-1	35.3±0.80	344.7±27.9****	<0.0001
iNOS	42.2±6.5	175.7±53.2**	<0.0015

Key:
P value.
*= significant
**= Moderately significant
****= Highly significant
P<0.05= Significant
P>0.05= Nonsignificant
TLR 2= Toll-Like Receptor 2
TLR 9 = Toll-Like Receptor 9
iNOS= Inducible Nitric Oxide Synthase
Tevfs=Vesicular fractions derived from *Trypanosoma evansi*

IV. DISCUSSION/CONCLUSION

Our investigation into the immune response triggered by vesicular fractions of *T. evansi* (Tevfs) stimulation revealed intricate dynamics in the expression of F4/80, CD86, CD206, and MHC II surface markers, as well as the modulation of cytokines and key genes associated with immune regulation. The comprehensive examination of these factors shed light on the nuanced interplay between the host's immune system and *T. evansi* vesicular fractions. The sustained upregulation of F4/80 expression observed throughout various incubation periods, from ex vivo to 72 hours post-stimulation, indicates a prolonged engagement of macrophages (Wei *et al.,* 2021). Despite the consistency of this trend, statistical analysis did not reveal significant differences among the incubation times. This suggests that the observed F4/80 upregulation may not follow a linear trajectory but rather a more complex, time-dependent pattern that warrants further investigation.The flow cytometry analysis, employing markers CD86, CD206, and MHC II, provided valuable insights into the immune response at the cellular level. A significant upregulation of CD206, a mannose differentiation marker associated with alternative macrophage activation, was noted upon Tevfs stimulation across various incubation times. This observation suggests a potential skewing towards an anti-inflammatory M2-like macrophage phenotype, emphasizing the intricate modulation of macrophage activation states by *T. evansi* (Guerreiro, 2018).

Although trends were observed in CD86 and MHC II surface markers during the 48-hour and 72-hour incubation periods, statistical analysis did not indicate significant differences compared to the unstimulated control. This implies that while there might be alterations in these markers, they do not reach statistical significance within the experimental parameters . The cytokine expression analysis revealed a distinctive upregulation of IL-10 and IL-4 post-Tevfs stimulation in contrast to SCOEMP stimulation. This indicates a potential immunomodulatory effect of *T. evansi* vesicular fractions, favoring anti-inflammatory cytokine production (Jawalagatti *et al*., 2023). The significant upregulation in the fold increase of Arginase-1, Toll-like receptor 9, and Inducible Nitric Oxide Synthase at 72 hours post-stimulation further emphasizes the complexity of the immune response.

Notably, the highly significant trend observed in the upregulation of the Arginase-1 gene relative to the ex vivo timeline suggests a time-dependent and sustained impact of Tevfs on this particular regulatory pathway. Arginase-1 is known for its involvement in immune suppression and tissue repair, indicating a potential mechanism through which *T. evansi* modulates the host immune response(Nguyen *et al*., 2023: Wei *et al*., 2021).

In conclusion, our findings highlight the intricate immune modulation orchestrated by *T. evansi* vesicular fractions on macrophage activation states, cytokine expression, and key regulatory genes. The observed patterns underscore the need for further investigations to decipher the underlying mechanisms and potential therapeutic implications of these immune interactions in the context of trypanosome infections.

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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) , 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.

AUTHOR 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 Nosa T Omorodion for manuscript writing, Professor M.A Okungbowa experimental work and script writing,and Professor I.N Ibeh for his contribution in the manscript writing contributed All authors critically reviewed and approved the final version of the manuscript.

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