Activation of macrophages by extracellular vesicles derived from red blood cells infected with *Babesia microti*

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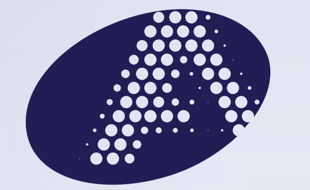

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Background

- Human babesiosis is an emerging tickborne disease in the United States caused by the intraerythrocytic protozoan parasite *Babesia microti* [1, 2].
- Despite an emergence of the disease in recent years, the pathogenesis and immune response to *B. microti* infection remain poorly understood.
- Studies in laboratory mice have shown a critical role for macrophages in the elimination of parasites and infected red blood cells [3, 4]. Importantly, the effector parasite molecules that activate macrophages are still unknown.
- Recent evidence identified a novel protein export mechanism in *B. microti* [5] that features a network of tubes of vesicles that extend from the parasite plasma membrane to the red blood cell (RBC) cytoplasm (Fig. 1B-C). Parasite-derived vesicles are eventually released to the extracellular environment [5].

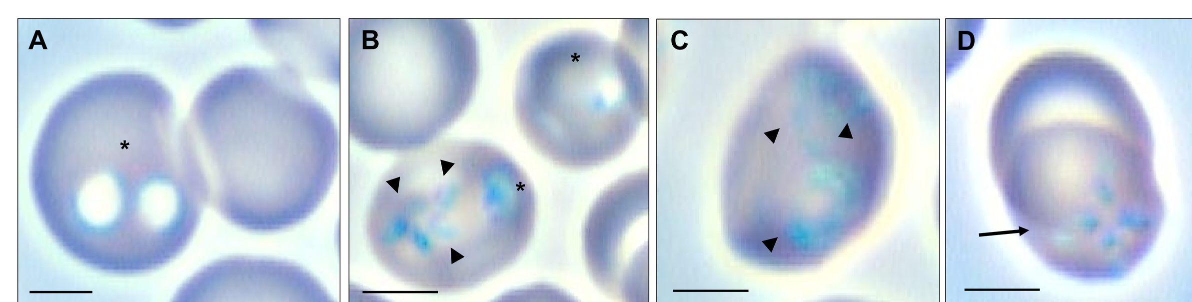


Fig. 1. Giemsa-stained blood smear from a *B. microti*-infected Syrian hamster showing ring forms of the parasite (**A** and **B**, asterisks), membranous extensions (**B** and **C**, arrowheads), and tetrad stages (**D**, arrow). Bar, 3 μm.

Hypothesis

- We postulate that, once released from *B. microti*-infected red blood cells (iRBCs), parasite-derived extracellular vesicles (EVs) participate in intercellular communication with neighboring cells. When EVs target macrophages as the recipient cells, changes in the modulation of cytokines with roles in the host innate immune response occur in response to EV-enclosed parasite antigens.

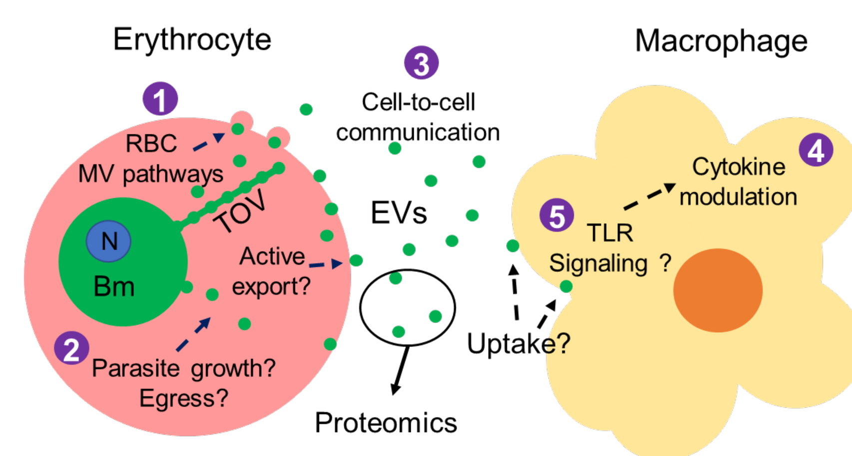


Fig. 2. Plausible biological roles of EVs in babesiosis. EVs harboring parasite antigens are released from iRBCs and cause phenotypic changes in neighboring macrophages. Bm, *B. microti*; N, nucleus; MV, microvesicle; TOV, tubes of vesicles [5].

Objectives

- Examine the expression of parasite antigens in enriched EV fractions collected from the supernatants of *in vitro* cultured iRBCs and plasma from infected hamsters.
- Evaluate the size distribution of EVs released from cultured RBCs during infection by Nanoparticle Tracking Analysis (NTA).
- Examine the uptake of EVs isolated from RBC culture supernatants and hamster plasma by mouse macrophages.
- Determine whether treatment of macrophages with EVs from Babesia iRBCs results in regulation of the transcription factor NF-κB and production of inflammatory cytokines.

Experimental Approach

Fig. 3. *In vitro* culture of *B. microti*-infected RBCs

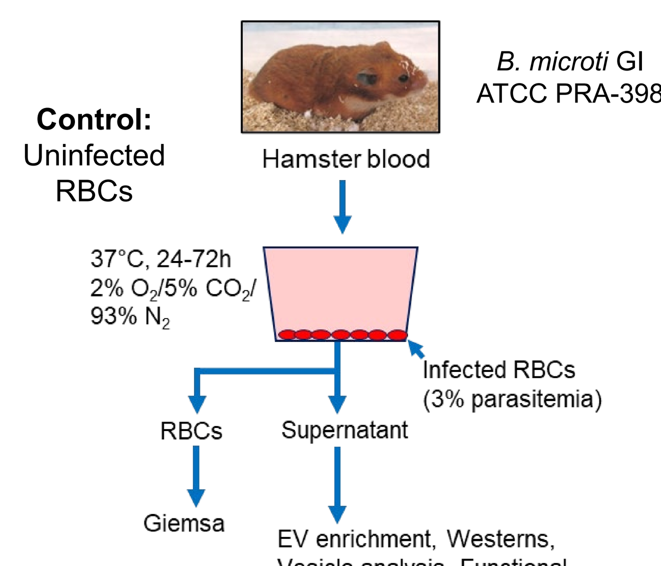
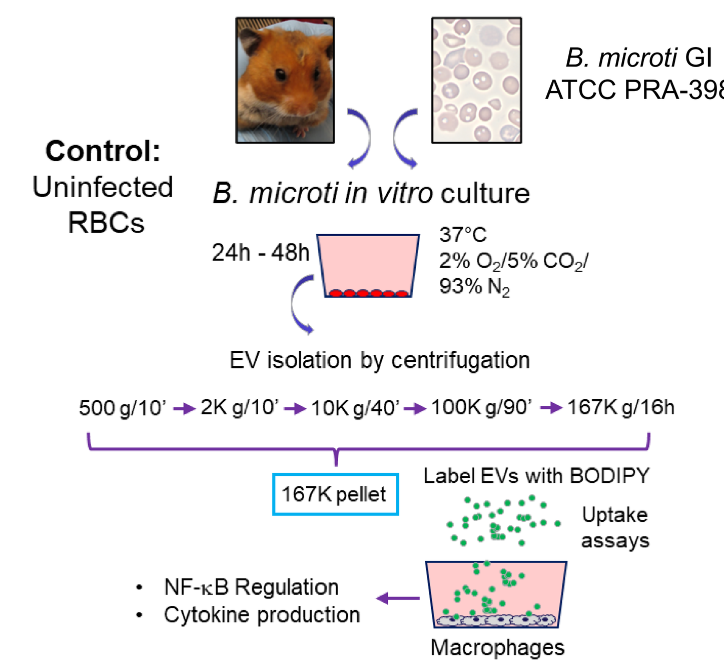


Fig. 4. Effects of EVs on macrophages



- Leukocyte-depleted blood was collected from *B. microti*-infected hamsters (14 days post-infection, 20% parasitemia), adjusted to 3% parasitemia with uninfected RBCs, and incubated in supplemented HL-1 medium according to Abraham, A., et al [6]. Parasitemia was monitored by microscopic examination (Fig. 3).
- EVs were enriched from RBC culture supernatants using commercial kits (SBI ExoMax) or sequential centrifugation and analyzed by Westerns, NTA, and functional assays (Figs. 3 and 4)
- EVs isolated from cultured RBCs were labeled with BODIPY (Fig. 4) and uptake assays were performed in macrophages (BEI Resources NR-9456).
- Macrophage responses to EV treatment were also determined by examining the activity of NF-κB and the production of cytokines in culture supernatants (Fig. 4).

Results

Fig. 5. Analysis of EVs isolated from iRBC supernatants by Westerns

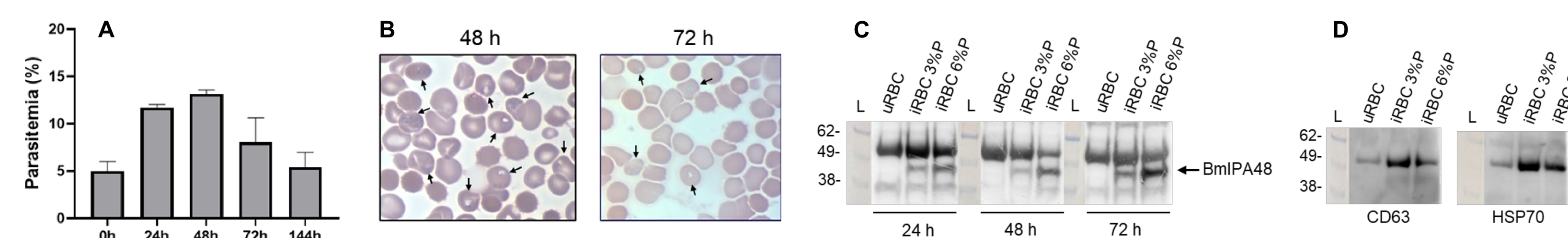


Fig. 5. Isolation of EVs from short term *in vitro* cultures of *B. microti*-infected hamster RBCs. **A,** parasitemia of iRBCs was determined by microscopy. Columns represent means+SEM of three replicates. **B,** representative image of intracellular stages of *B. microti* in infected RBCs (arrows). **C,** Western blot of *B. microti* antigen BmIPA48 (arrow) in EVs isolated after 24-72 h from iRBC cultures initiated with 3% and 6% parasitemia (P). uRBC, uninfected RBC. **D,** detection of host EV markers CD63 and HSP70 in EV preps. EVs were isolated from RBC supernatants using SBI ExoMax. L, protein ladder.

Fig. 6. Size distribution of EVs isolated from iRBC supernatants and plasma

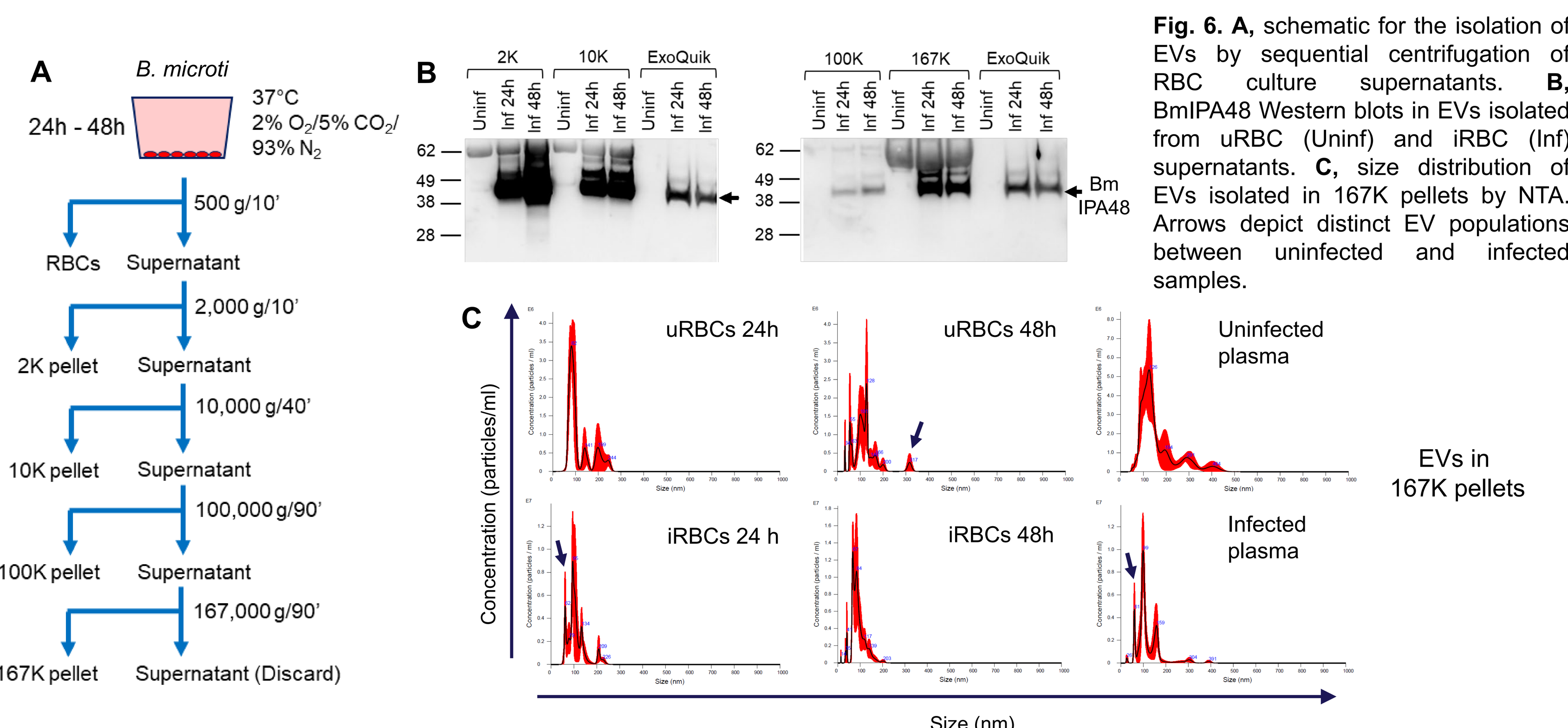


Fig. 6. A, schematic for the isolation of EVs by sequential centrifugation of RBC culture supernatants. **B,** BmIPA48 Western blots in EVs isolated from uRBC (Uninf) and iRBC (Inf) supernatants. **C,** size distribution of EVs isolated in 167K pellets by NTA. Arrows depict distinct EV populations between uninfected and infected samples.

Fig. 7. Uptake of BODIPY-labeled EVs by macrophages

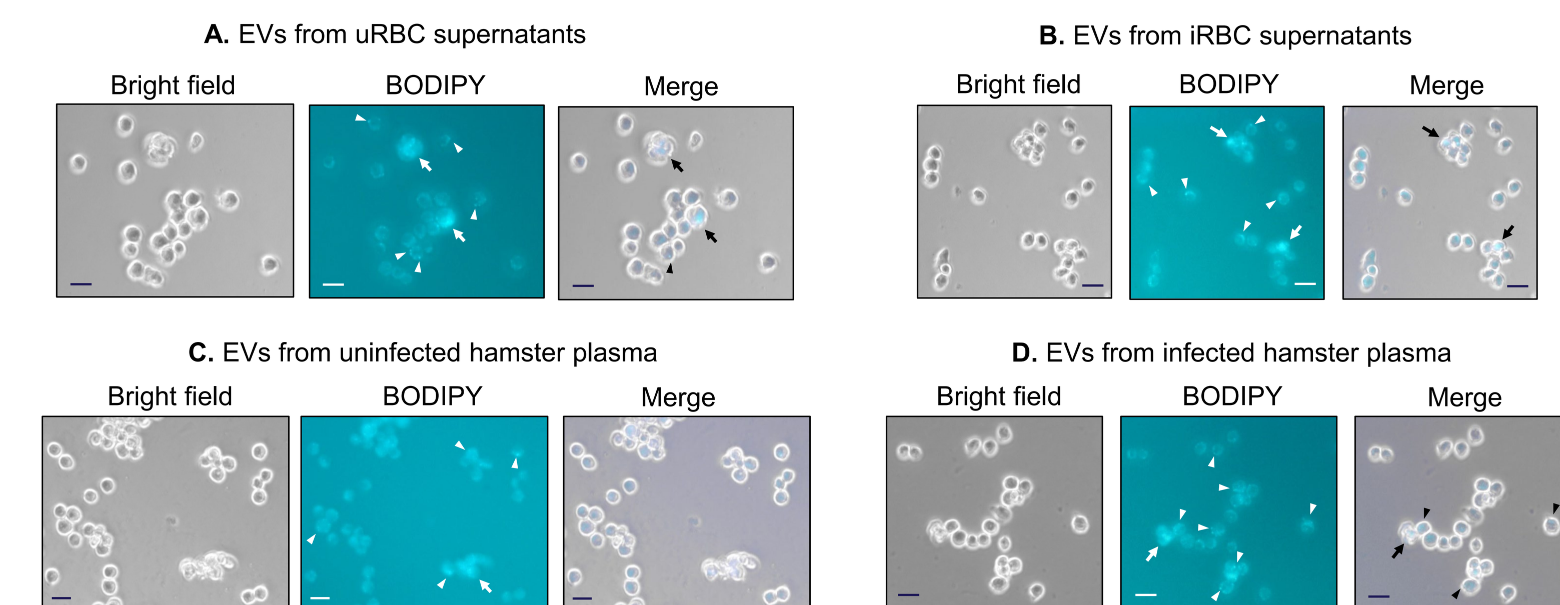


Fig. 7. Macrophage uptake of EVs isolated from uRBC supernatants **(A)**, iRBC supernatants **(B)**, uninfected hamster plasma **(C)**, and *B. microti*-infected hamster plasma **(D)**. EVs present in 167K fractions (Fig. 6A) were labeled with BODIPY dye and incubated with mouse macrophages (BEI Resources NR-9456) for 90 min. Arrows show internalization of BODIPY-labeled EVs. Arrowheads show localization of BODIPY-labeled EVs at the macrophage cell membranes. Bar, 15 μm.

Fig. 8. NF-κB and cytokine regulation in macrophages treated with EVs from iRBCs

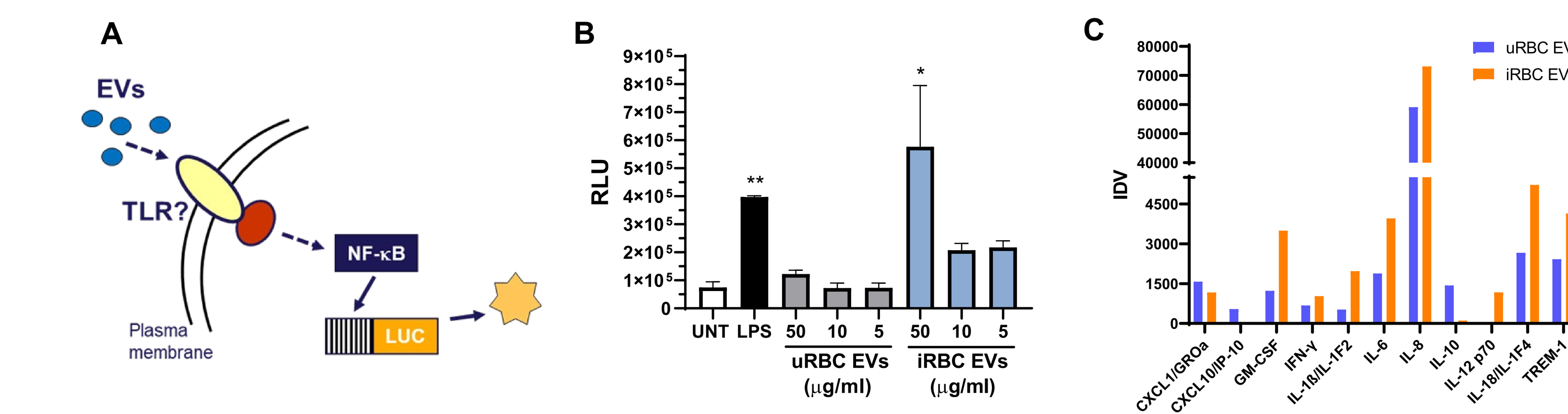


Fig. 8. Macrophage NF-κB activity and cytokine production in response to EVs from *Babesia* iRBCs. **A,** we hypothesize that EVs from iRBCs trigger macrophage Toll-like receptors (TLRs) resulting in downstream NF-κB activation. Experiments were performed in a macrophage line transfected with the luciferase gene under the control of an NF-κB responsive element (ATCC TIB-202-NFkB-LUC2). **B,** luciferase activity was measured in transgenic macrophages treated for 24 h with increasing concentrations of EVs from uRBC or iRBC supernatants. UNT, untreated; LPS-treated macrophages (5 μg/ml) were used as a positive control. Bars represent means±SEM of three experiments. **, *P*<0.01 compared to UNT; *, *P*<0.05 compared to 50 μg/ml of uRBC EVs. **C,** Production of cytokines after 24 h of EV treatment in macrophage supernatants as examined by protein arrays (R&D Systems). Densitometric analysis of protein spots showed changes in 11 cytokines in response to 50 μg/ml of iRBC EVs compared to uRBC EVs. Data correspond to one representative experiment of three performed.

Summary

- We present an *in vitro* model for the examination of macrophage responses to EVs derived Babesia-infected RBCs.
- Size distribution analysis of EVs from 167K fractions showed diverse vesicle populations among uninfected and infected samples. Distinct populations in infected culture supernatants and plasma were evident in the <100 nm size range.
- Uptake of EVs released into RBC culture supernatants and hamster plasma was observed in mouse macrophages *in vitro*.
- Macrophage NF-κB activity and dysregulation of cytokines were observed in response to iRBC-derived EVs.
- Future studies are required to identify additional host and parasite proteins present in isolated EV fractions, determine the biogenesis of EV release from iRBCs, elucidate the mechanisms of EV-mediated intercellular communication between iRBCs and macrophages, and decipher the signaling pathways involved in macrophage cytokine activation.

References

1. www.cdc.gov/parasites/babesiosis/resources/
2. Krause, P.J. 2019. Int. J. Parasitol. 49: 165-174.
3. Terkawi, M.A., et al. 2015. Infect. Immun. 83: 8-16.
4. Djokic, V., et al. 2018. Front. Microbiol. 9: 85.
5. Thekkiniath, J., et al. 2019. Life Sci. Alliance 2: 3.
6. Abraham, A., et al. 2018. J. Biol. Chem. 293: 19974-19981

Acknowledgments

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