Secretion of Extracellular Vesicles from *Babesia microti*-infected Erythrocytes: Biological Roles in Host Macrophage Activation

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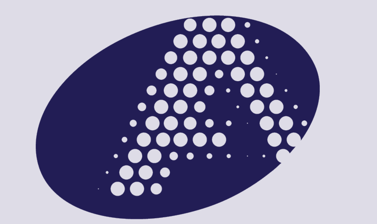


Poster 981

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].

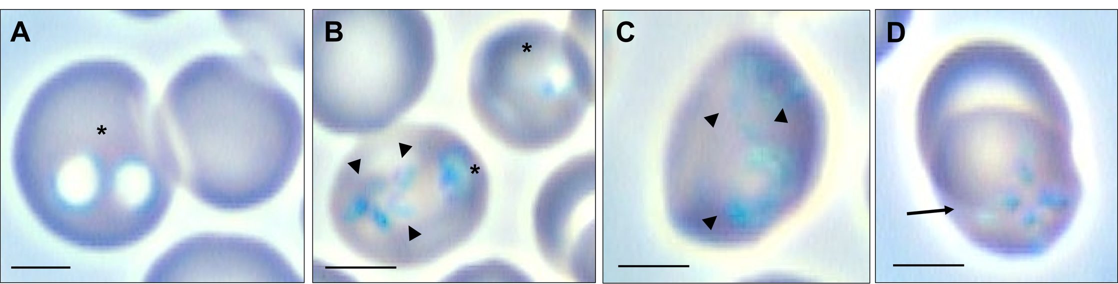


Fig. 1. Giemsa-stained blood smear from a *B. microti*-infected Syrian hamster showing ring forms of the parasite (**A** and **B**, asterisks), membranous extensions (**B** and **C**, arrowheads), and tetrad stages (**D**, arrow). Bar, 3 μm.

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 are likely to occur in response to EV-enclosed parasite antigens.

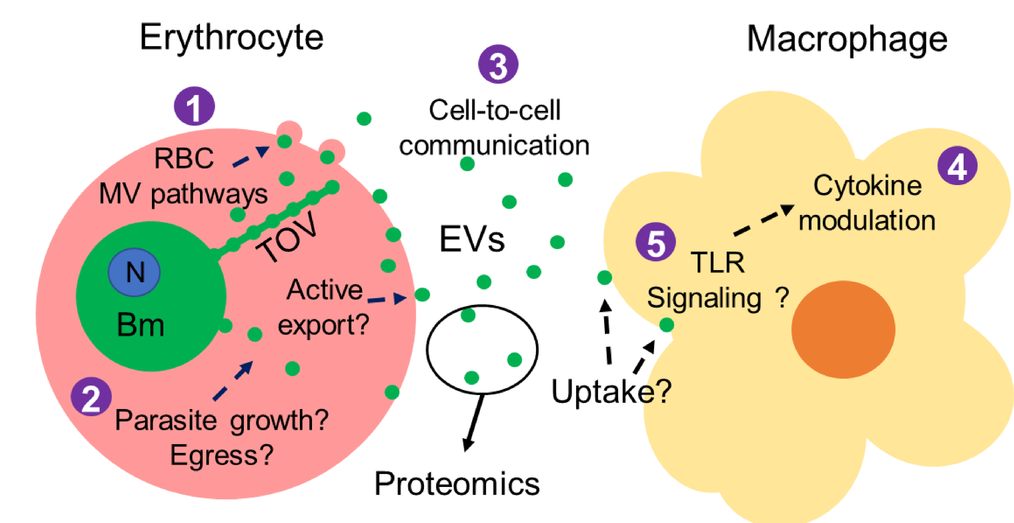


Fig. 2. Plausible biological roles of EVs in babesiosis. EVs harboring parasite antigens are released through RBC microvesicle (MV) pathways or actively exported from iRBCs and cause phenotypic changes in neighboring macrophages. Bm, *B. microti*; N, nucleus; MV, microvesicle; TOV, tubes of vesicles [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 cytokine profiles of mouse macrophages following exposure to *B. microti*-infected RBCs.
- Evaluate the contribution of parasite growth in the production of macrophage cytokines following co-incubation with iRBCs.

Experimental Approach

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

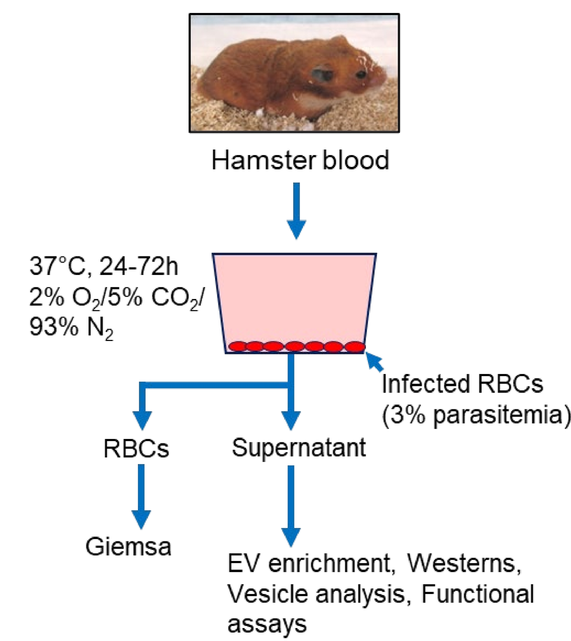
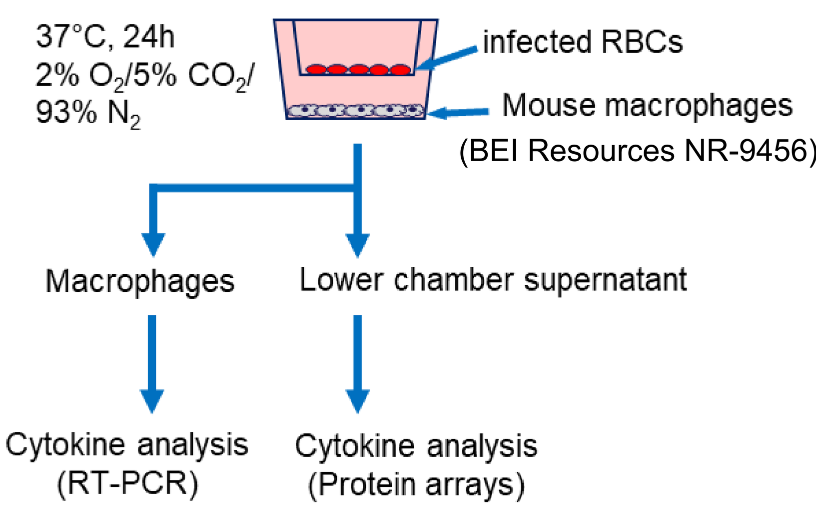


Fig. 4. Co-culture of *B. microti*-infected RBCs and macrophages



- Leukocyte-depleted blood was collected from *B. microti*-infected hamsters (14 days post-infection, 20% parasitemia) and adjusted to 3% parasitemia with uninfected RBCs.
- RBC cultures were incubated in supplemented HL-1 medium at 37°C, 2% O_2/5% CO_2/93% N_2 according to Abraham, A., et al [6].
- Parasitemia was checked daily by microscopic examination. EVs were enriched from RBC culture supernatants (Fig. 3) and analyzed by Westerns, NTA, and uptake assays in an immortalized murine macrophage line (BEI Resources NR-9456; www.beiresources.org).
- *B. microti*-infected RBCs were co-incubated with mouse macrophages (Fig. 4) and cytokine expression was examined by RT-PCR and protein arrays (R&D Systems).

Results

Fig. 5. Secretion of *B. microti* antigens into the supernatants of iRBCs

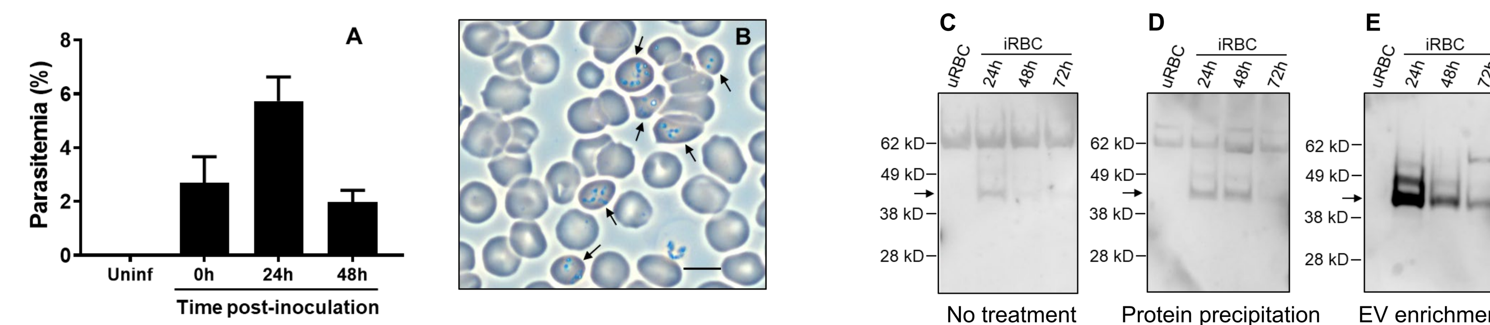


Fig. 5. Short term *in vitro* culture of *B. microti* in hamster RBCs. **A**, parasitemia was determined by microscopy. Columns represent means+SEM of four replicates. **B**, representative image of intracellular stages of *B. microti* in RBCs after 24h of culture. Bar, 5 μm. **C-E**, Western blots of *B. microti* antigen BmIPA48 in culture supernatants of iRBCs (arrows). **C**, no treatment of supernatants before Westerns; **D**, BmIPA48 detection following protein precipitation of culture supernatants; **E**, prior EV enrichment using a commercial kit (SBI ExoMax).

Fig. 6. Analysis of vesicle fractions isolated from iRBC supernatants and hamster plasma

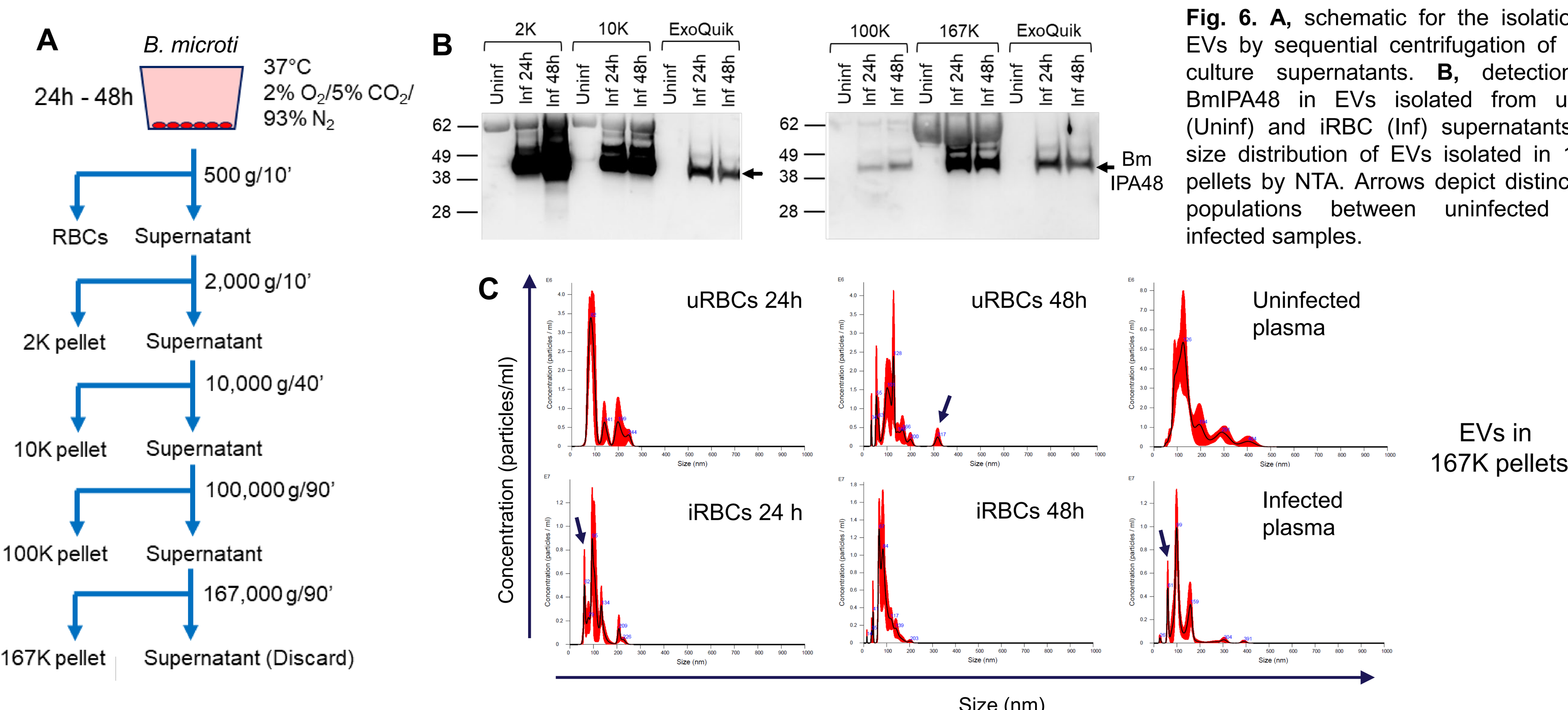


Fig. 6. A, schematic for the isolation of EVs by sequential centrifugation of RBC culture supernatants. **B**, detection of BmIPA48 in EVs isolated from uRBC (Uninf) and iRBC (Inf) supernatants. **C**, size distribution of EVs isolated in 167K pellets by NTA. Arrows depict distinct EV populations between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

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

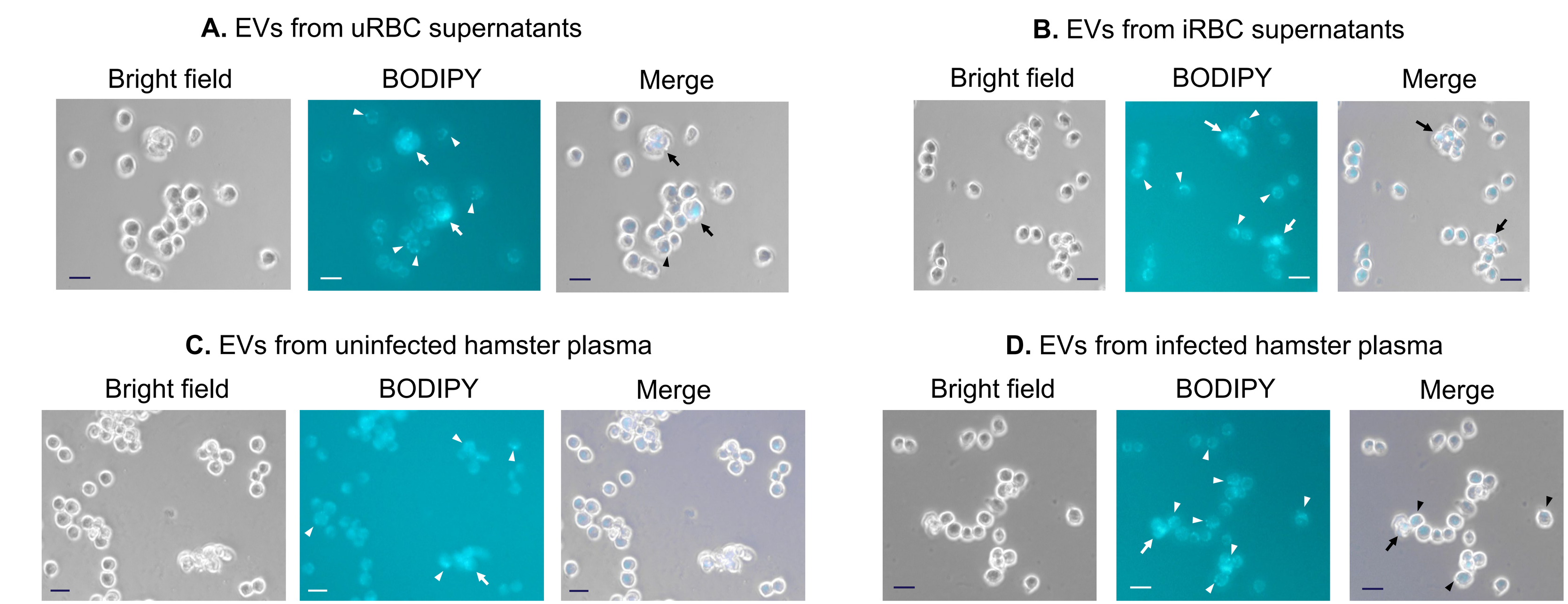


Fig. 7. Macrophage uptake of EVs isolated from uRBC supernatants **(A)**, iRBC supernatants **(B)**, uninfected hamster plasma **(C)**, and *B. microti*-infected hamster plasma **(D)**. EVs present in 167K fractions (Fig. 6A) were labeled with BODIPY dye and incubated with mouse macrophages (BEI NR-9456) 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. Cytokine activation in macrophages co-incubated with *B. microti*-infected RBCs

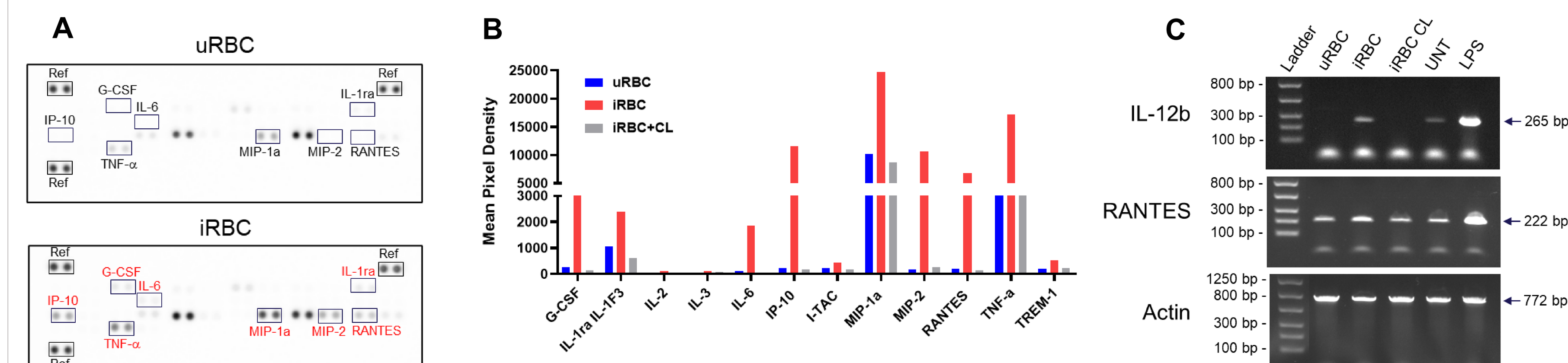


Fig. 8. Cytokine expression in mouse macrophages co-incubated with hamster uRBCs or iRBCs for 24 h (see Fig. 2). **A**, the production of 40 cytokines in macrophage supernatants was examined by protein arrays (R&D Systems). Cytokine increases in response to iRBC co-incubation are depicted in red. The addition of 170 μM of the antiparasitic drug clindamycin to the co-cultures abrogated this response. **B**, densitometric analysis of protein arrays showing alterations in 12 macrophage cytokines in response to iRBC co-incubation. **C**, RT-PCR analysis of IL-12b, RANTES, and actin in mouse macrophages co-incubated for 24h with uRBCs, iRBCs, or iRBCs with clindamycin (iRBC CL). Untreated (UNT) and LPS (5 μg/ml)-treated macrophages were used as negative and positive controls, respectively. Data correspond to one representative experiment of three performed.

Summary

- We established an *in vitro* culture model of *B. microti* iRBCs that allows the analysis of parasite antigen secretion over time and isolation and characterization of EVs from culture supernatants.
- 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 cytokines were upregulated in response to co-incubation with iRBCs. The requirement for parasite growth is critical in this response.
- Future studies will identify 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.

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