



UNIVERSITA' DEGLI STUDI DI PAVIA

PhD Program in Genetics, Molecular and Cellular Biology

Frontiers in cellular biology

“Cellular networks in normal, pathological and experimental conditions”

Article

Cell

**Extracellular Vesicles from *Trypanosoma brucei*
Mediate Virulence Factor Transfer and Cause Host
Anemia**

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Edoardo Errichiello

Trypanosoma brucei

3 subspecies:

T. b. gambiense



African sleeping sickness
(trypanosomiasis)

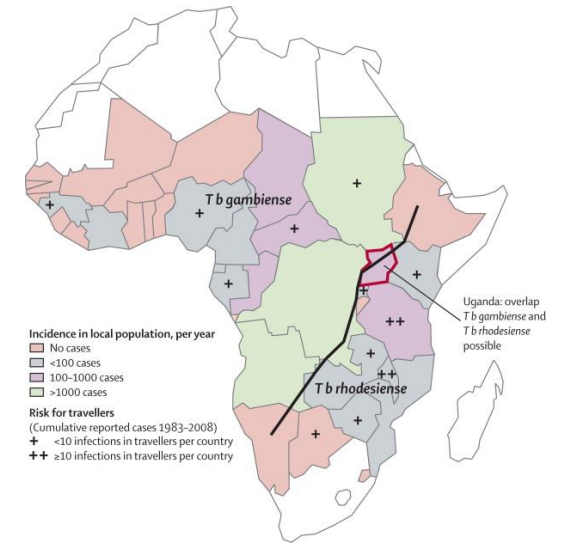
T. b. rhodesiense *

T. b. brucei

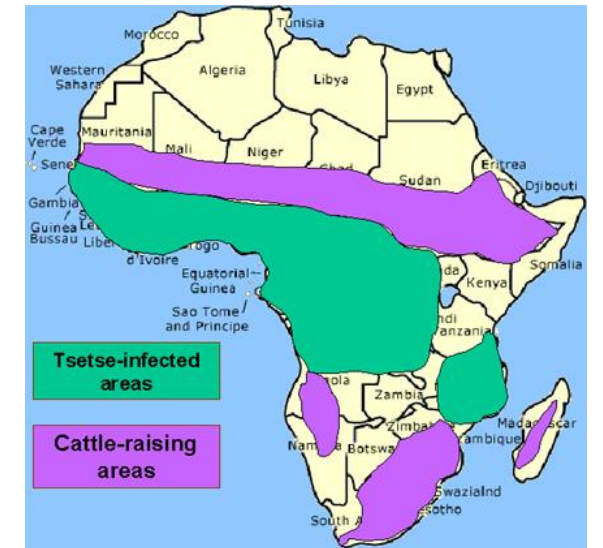
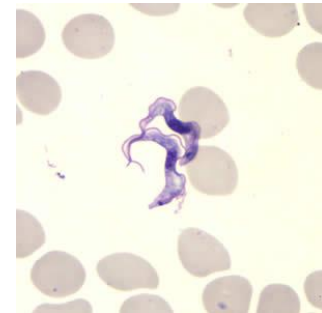
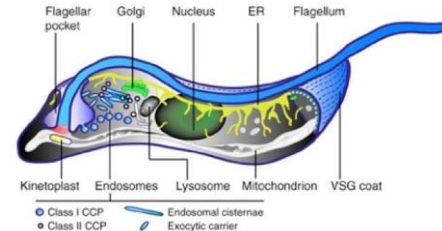
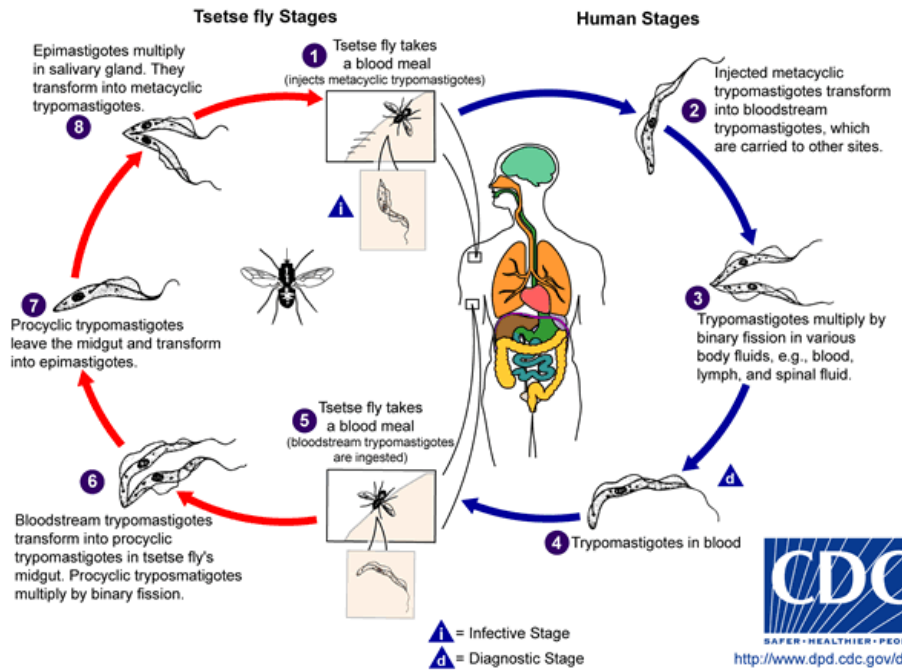


Nagana in animals (cattle)

* Produces the serum resistance-associated protein (SRA) necessary for human infectivity (circumvents the effects of circulating trypanosome lytic factor, TLF)

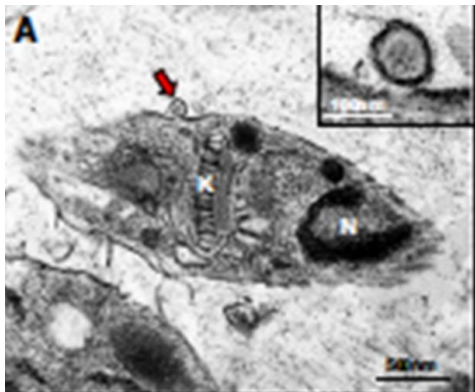


T. Brucei life cycle

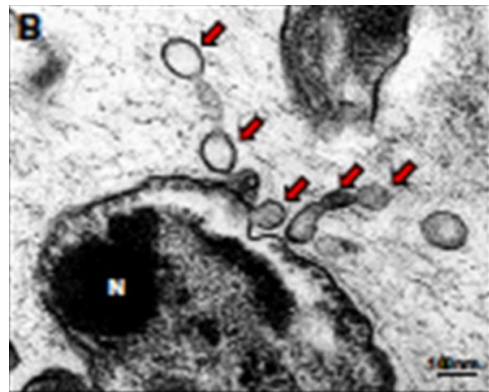


Previous evidences of EVs release in infectious diseases

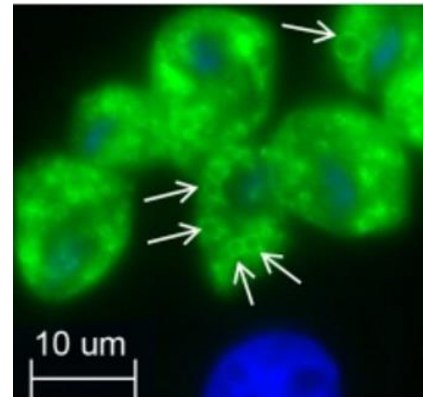
- *Trypanosoma cruzi* and *Leishmania spp.* have been shown to release EVs that interact with host cells and modulate immune responses (Marcilla et al., 2014)
- EVs derived from *Plasmodium falciparum*-infected erythrocytes promote parasite differentiation and regulate immune cells within the mammalian host (Mantel et al., 2013; Regev-Rudzki et al., 2013)
- The parasite *Trichomonas vaginalis* produces EVs that alter adherence to urogenital tract and modulate host immune response to infection (Twu et al., 2013)



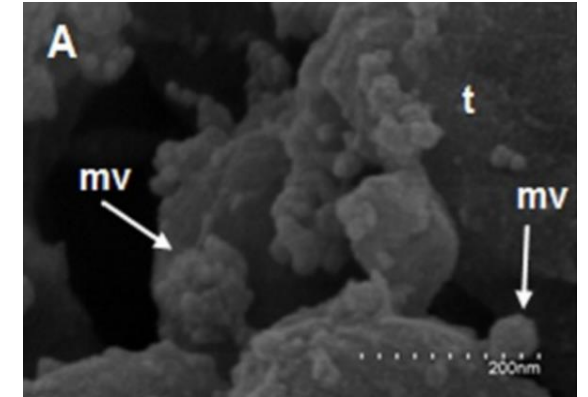
Atayde et al., 2015



Atayde et al., 2015



Twu et al., 2013



Marcilla et al., 2012

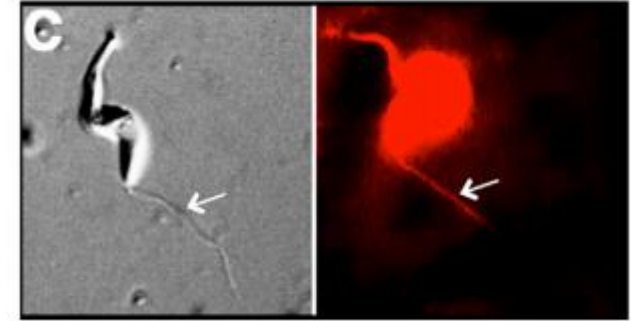
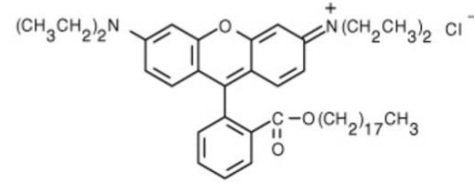
Flagellar membrane budding gives rise to nanotubes in *T. b. brucei*

Differential Interference Contrast (DIC, 40X)
video microscopy

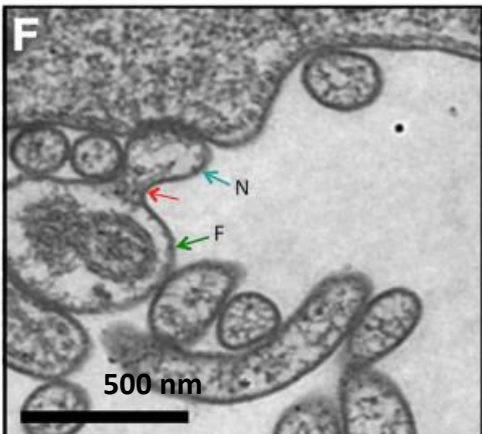


Highly dynamic filamentous structures extending from the posterior end of cells and connecting different trypanosomes

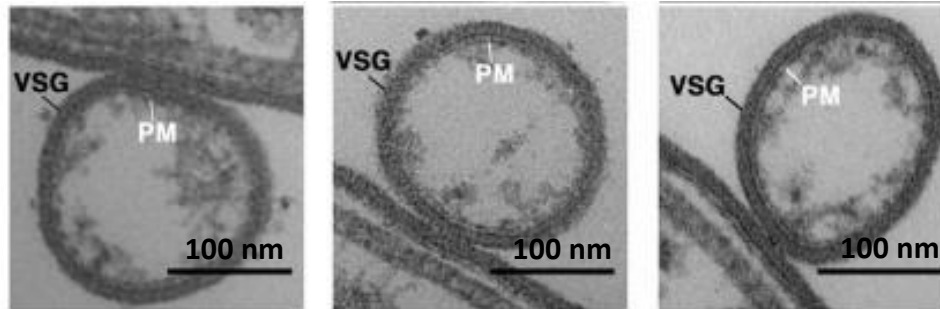
Labeling with the membrane binding dye octadecyl rhodamine B (R18)



Filaments are bounded by a lipid membrane (“membrane nanotubes”, >20 μm)



Transmission Electron Microscopy (TEM)



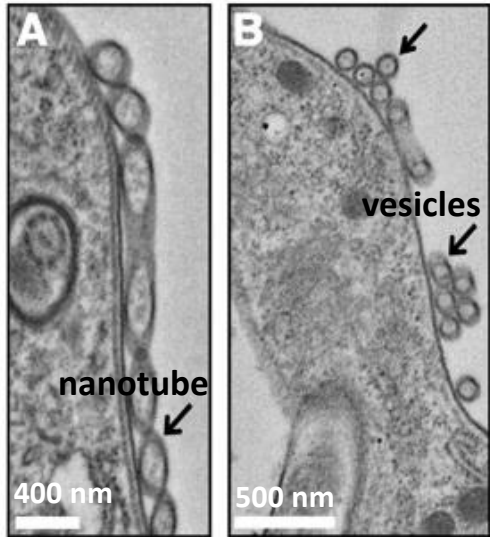
PM: plasma membrane
VSG: variant surface glycoproteins (surface coat)

Nanotubes develop from budding of the flagellar membrane, with similar characteristics

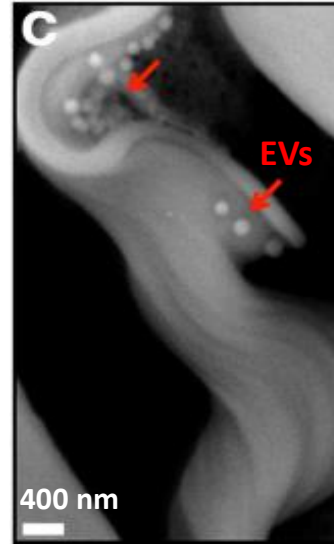
Flagellar membrane (green arrow, F) budding (red arrow) into nanotube (blue arrow, N)

Membrane nanotubes vesicularize into diffusible EVs

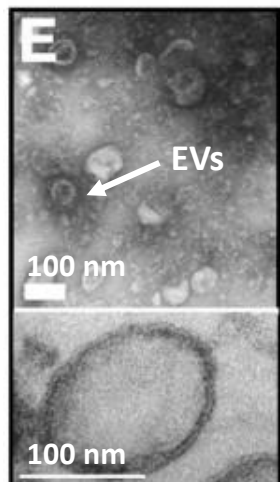
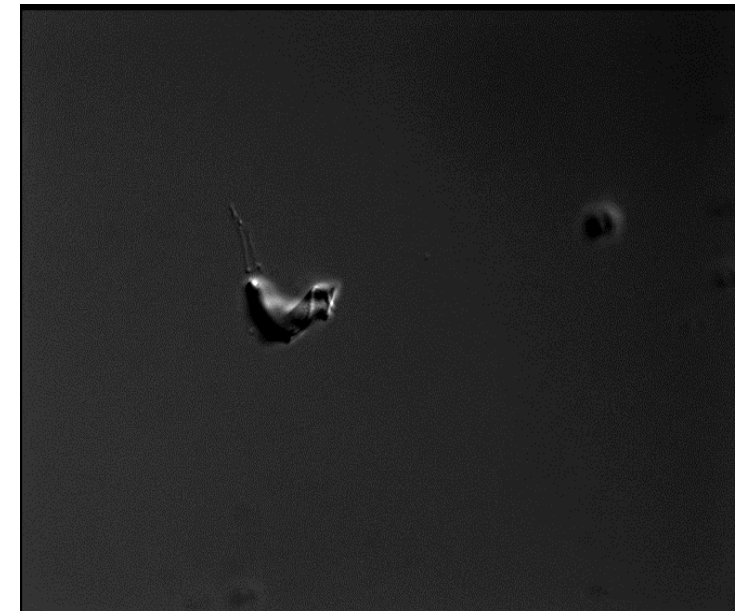
Transmission Electron Microscopy (TEM)



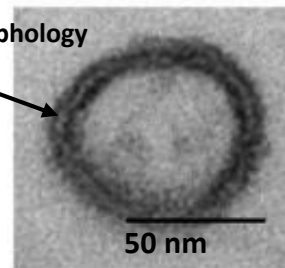
Scanning Electron Microscopy (SEM)



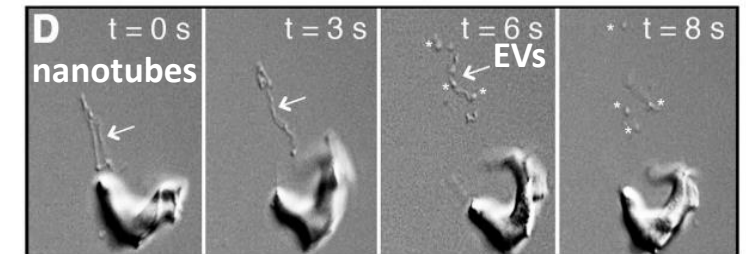
Differential Interference Contrast (DIC, 40X) video microscopy



membrane like morphology (10 nm)



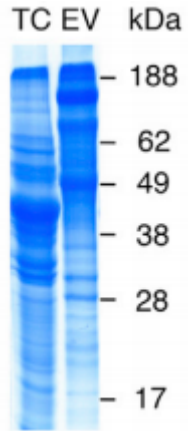
Purified EVs appear as unilamellar vesicles 70-80 nm in diameter with a 10-nm thick membrane



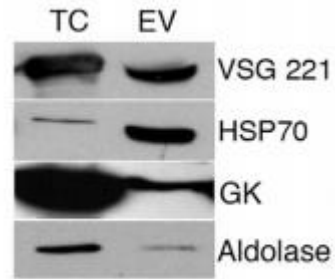
Nanotubes dissociation into free EVs

The EV proteome is enriched in flagellar membrane proteins and parasite virulence factors

SDS-PAGE protein profile

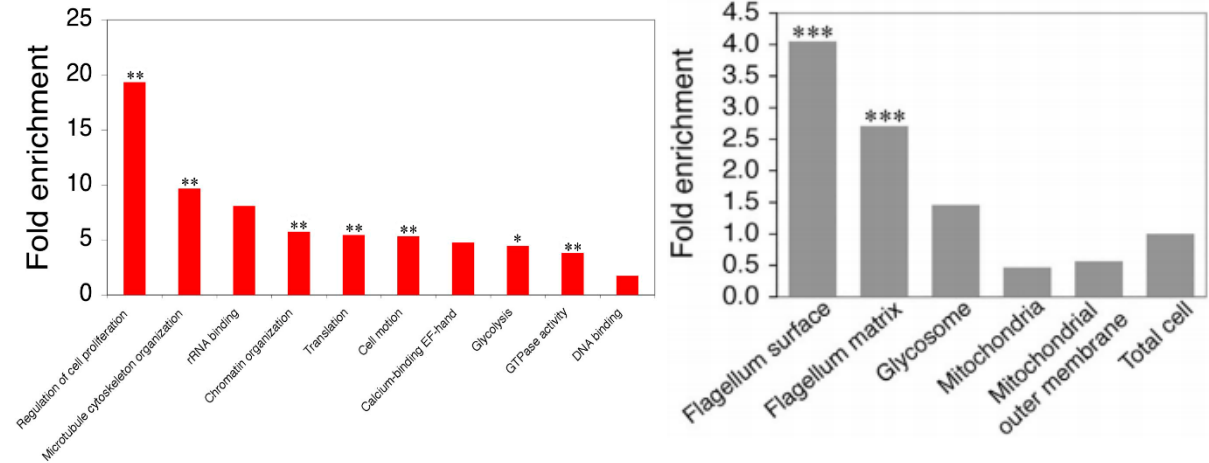


Western Blotting



VSG 221: Variant Surface Glycoprotein 221
GK: Glycerol Kinase

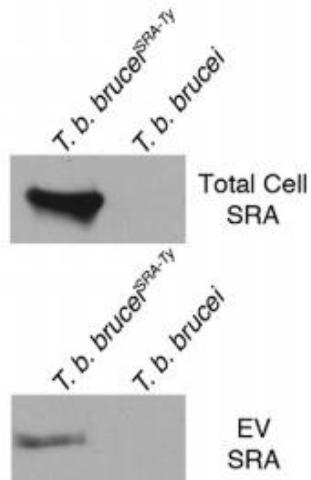
Proteomic analysis of EVs (156 proteins)



Purified EVs showed a different protein composition than total cell (TC)

EVs are enriched with flagellar matrix or membrane proteins (32%), but not with glycosome and mitochondrial proteins (2%)

Enrichment of EVs with flagellar proteins is consistent with a population of EVs being derived from nanotubes that form by budding of the flagellar membrane



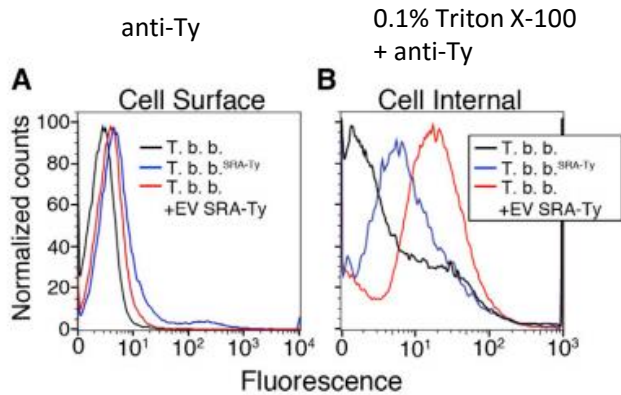
T. b. brucei^{SRA-Ty}: *T. b. brucei* line expressing a Ty-epitope tagged SRA (Ty-tag amino acid sequence: EVHTNQDPLD)

EVs contain the virulence factor SRA (Serum Resistance Associated protein)

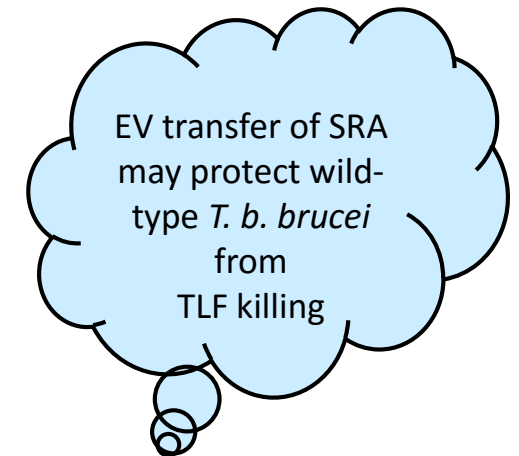
The flagellum may serve as part of a sorting pathway for delivery of biologically active molecules to neighboring cells

Can SRA be transferred to TLF (Trypanosome Lytic Factor) susceptible *T. b. brucei*?

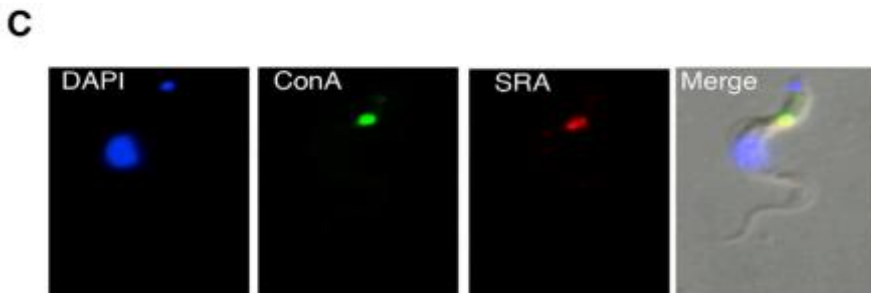
Flow cytometry of wild-type *T. b. brucei* incubated with EVs containing SRA-Ty



EVs from *T. b. brucei*^{SRA-Ty} are delivered to wild-type *T. b. brucei* where they accumulate in an intracellular location that becomes accessible to anti-Ty after cell permeabilization



Immunofluorescence microscopy of *T. b. brucei* treated with EVs containing SRA-Ty

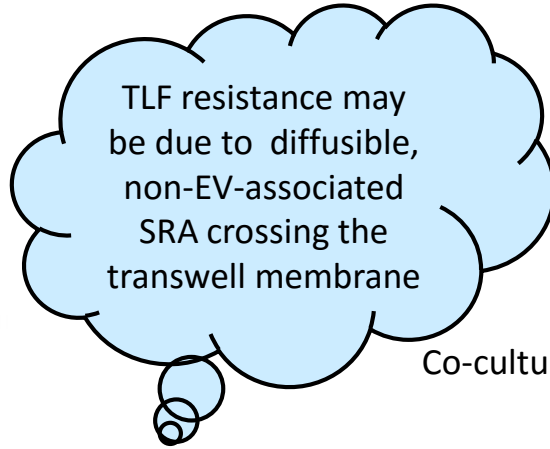
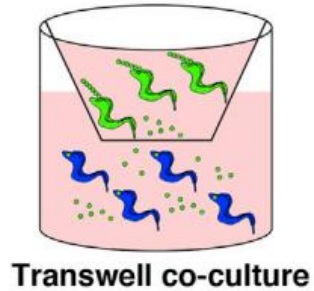


SRA-Ty delivered by EVs is internalized by wild-type *T. b. brucei* and co-localizes with concanavalin A (ConA), a marker for the endocytic pathway

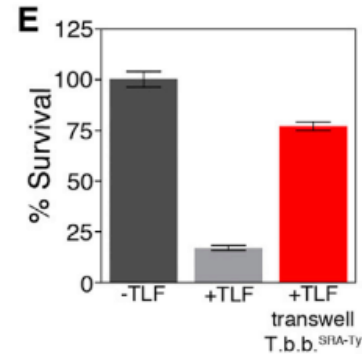
SRA from EVs localizes in the endolysosomal compartment of recipient Trypanosomes

T. b. rhodesiense EVs transfer SRA to *T. b. brucei* and confers TLF resistance

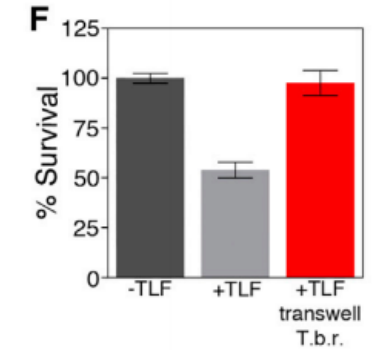
Transwell co-culture (0.2 μm filter) of wild-type *T. b. brucei* with *T. b. brucei*^{SRA-Ty} or *T. b. rhodesiense*



Co-culture of *T. b. brucei* with *T. b. brucei*^{SRA-Ty}

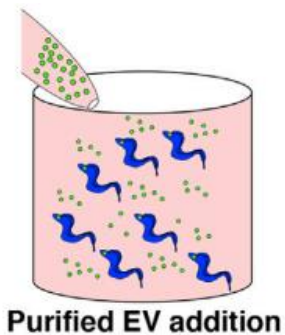


TLF overnight survival assays



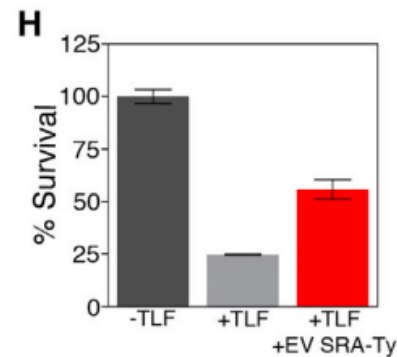
Co-culture of *T. b. brucei* with *T. b. rhodesiense*

Addition of EVs purified from *T. b. brucei*^{SRA-Ty} or *T. b. rhodesiense* directly to wild-type *T. b. brucei*

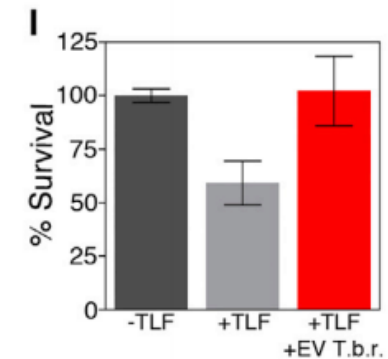


Purified EV addition

Addition of EVs purified from *T. b. brucei*^{SRA-Ty}



Addition of EVs purified from *T. b. rhodesiense*

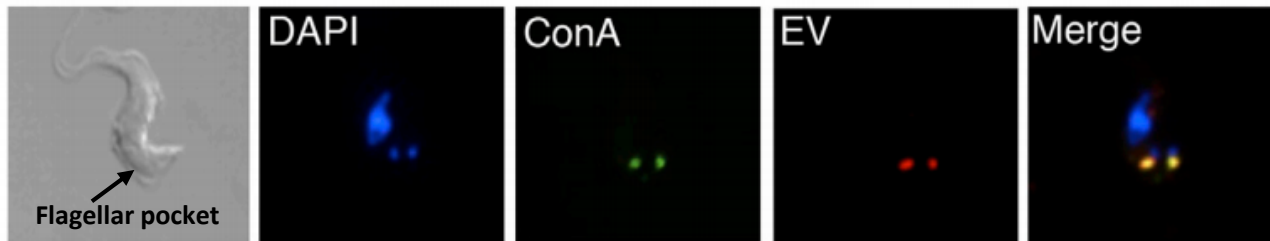


Functional SRA is transferred by EVs to co-cultured trypanosomes leading to TLF resistance

Trypanosome EVs are highly fusogenic and rapidly transfer proteins and lipids to recipient Trypanosomes

Fluorescence microscopy of *T. b. brucei* treated with Alexa-594-labeled EVs

3°C (inhibition of endocytosis)



37°C (permission of endocytosis)



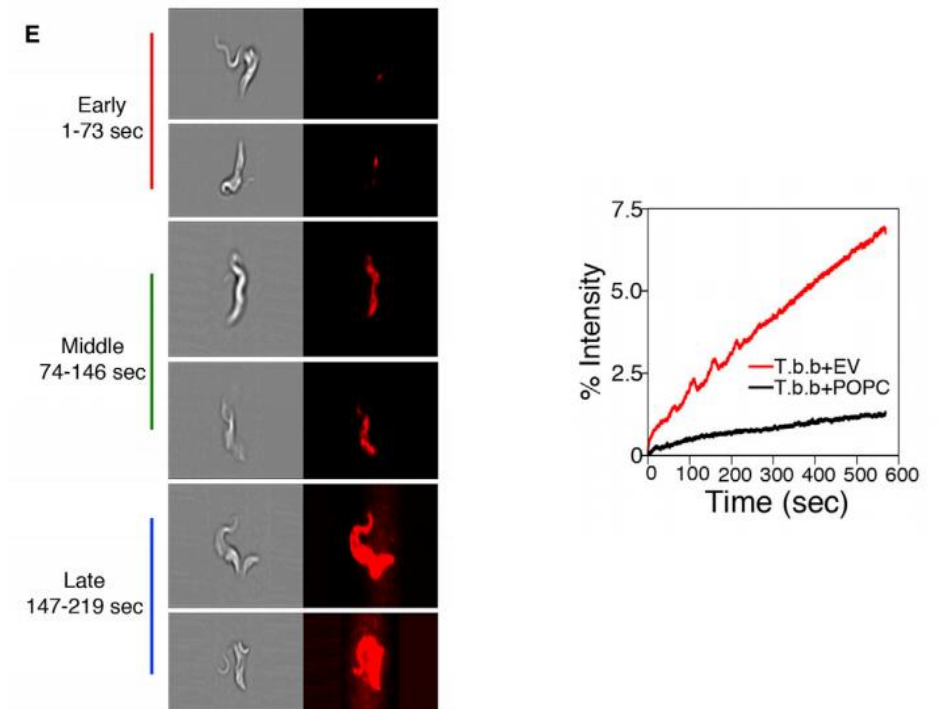
ConA: concanavalin A

EVs proteins interact with *T. b. brucei* at the flagellar pocket, and are endocytosed within endolysosomal vesicles

Membrane fusion assay by fluorescence dequenching of R18-labeled EVs incubated with *T. b. brucei*

R18: lipophilic fluorophore octadecyl rhodamine B

POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine



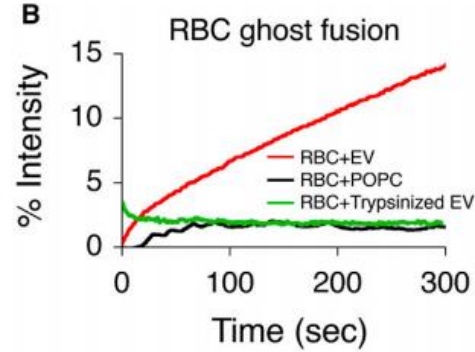
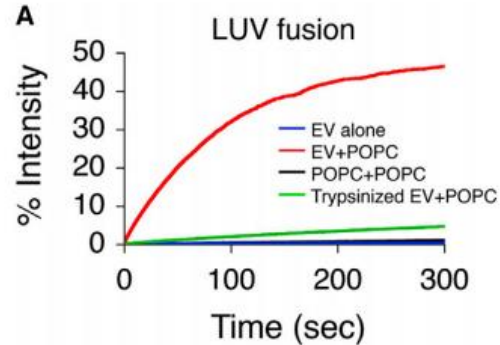
EVs fuse to the flagellar pocket membrane of *T. b. brucei* and rapidly equilibrate their lipids along the trypanosome membrane

Trypanosome EVs are fusogenic with artificial liposomes and human erythrocytes

Membrane fusion assay of R18-labeled EVs incubated with POPC-LUVs or ghost RBCs

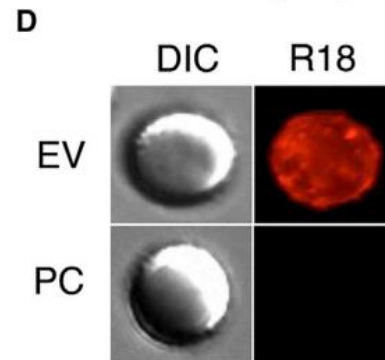
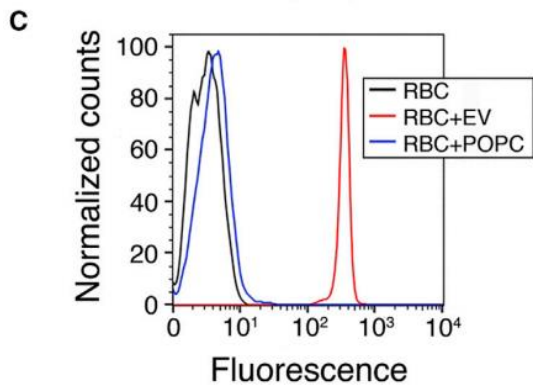
R18: lipophilic fluorophore octadecyl rhodamine B

POPC-LUV: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine-large unilamellar liposomes



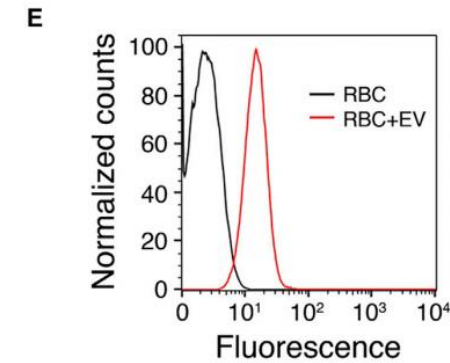
EVs trypsinization ablates fusion, indicating the presence of exposed EV protein(s) necessary for membrane fusion

Incubation of intact RBCs with R18-labeled EVs or POPC-LUVs



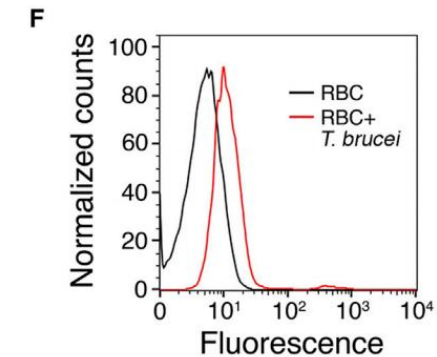
EVs fuse with intact erythrocytes and transfer R18

Incubation of Alexa-Fluor 488-labeled EV proteins with RBCs



Fusion also results in transfer of labeled EV proteins to RBCs

Transwell incubation of RBCs with R18-labeled *T.b. brucei*

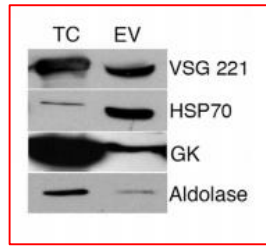
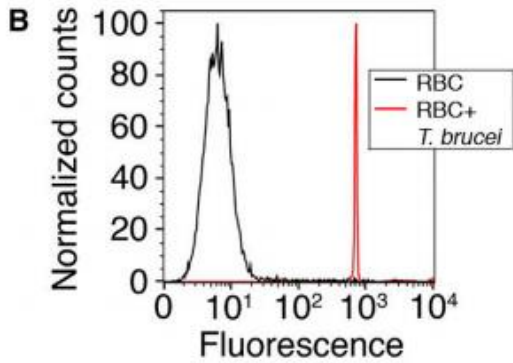


RBCs become labeled with R18 due to *T. b. brucei* EVs transfer

EVs transfer trypanosome lipids and proteins to host RBCs

T. b. brucei EV fusion modifies mammalian erythrocytes

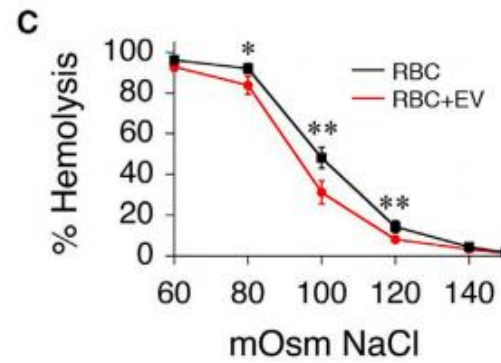
Erythrocytes transwell incubation with *T. b. brucei*, probed with anti-VSG 221



Does EVs fusion alter the physical properties of RBCs?

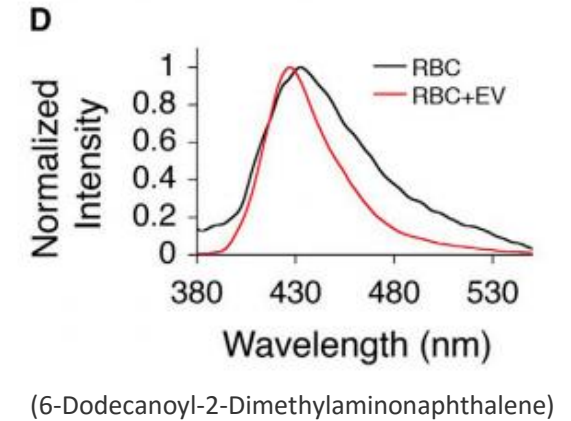
Fusogenic EVs may serve as vehicles for pathogen to host transfer of membrane proteins and lipids

Erythrocyte lysis test by quantifying hemoglobin concentration in supernatant



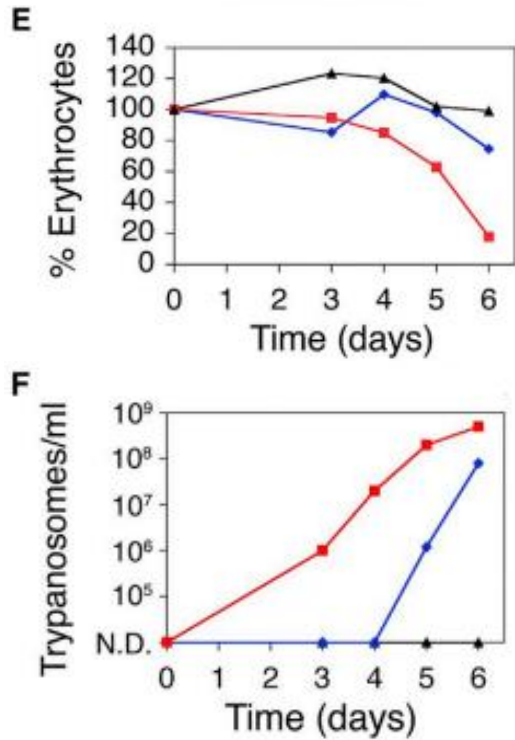
Human erythrocytes incubated with EVs are less sensitive to osmotic lysis, due to increased membrane rigidity

Laurdan emission spectra of erythrocytes



T. b. brucei EV fusion causes anemia in mice

Trypanosome acute infection

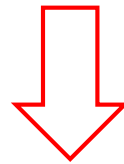
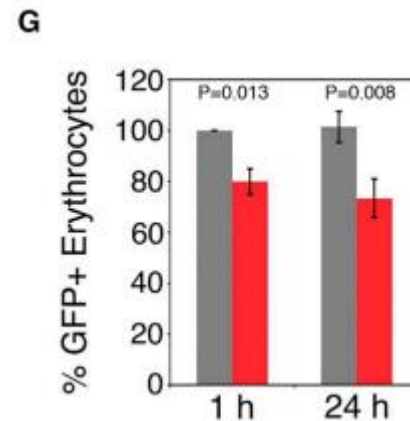


Heavily infected mouse
Moderately infected mouse
Mouse with undetectable parasitemia

During acute infection, the level of anemia correlates to parasitemia

Clearance of EV-altered RBCs

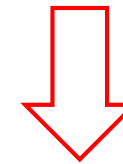
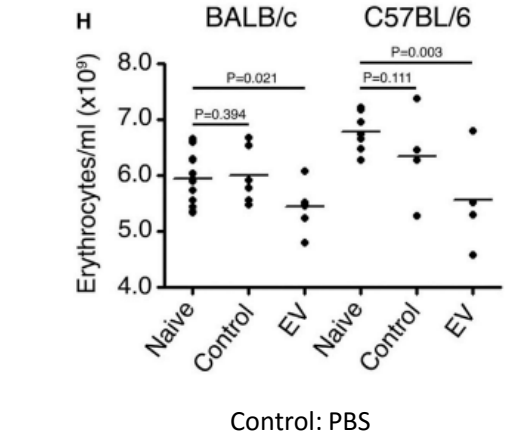
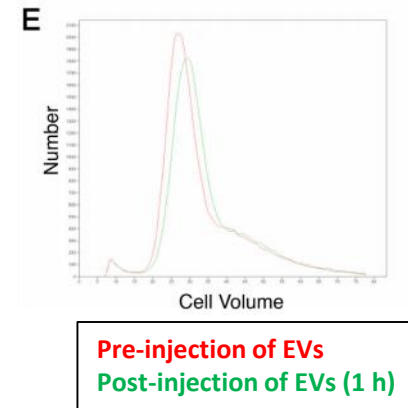
Murine GFP erythrocytes quantification after incubation with (red) or without (gray) purified EVs and injection into the tail vein of naive mice



Clearance occurs rapidly and the remaining EV-treated erythrocytes become stable in circulation after 24 hr

EVs fusion and RBCs loss

Purified EVs were intravenously injected into naive mice and erythrocytes quantified after 1 hr

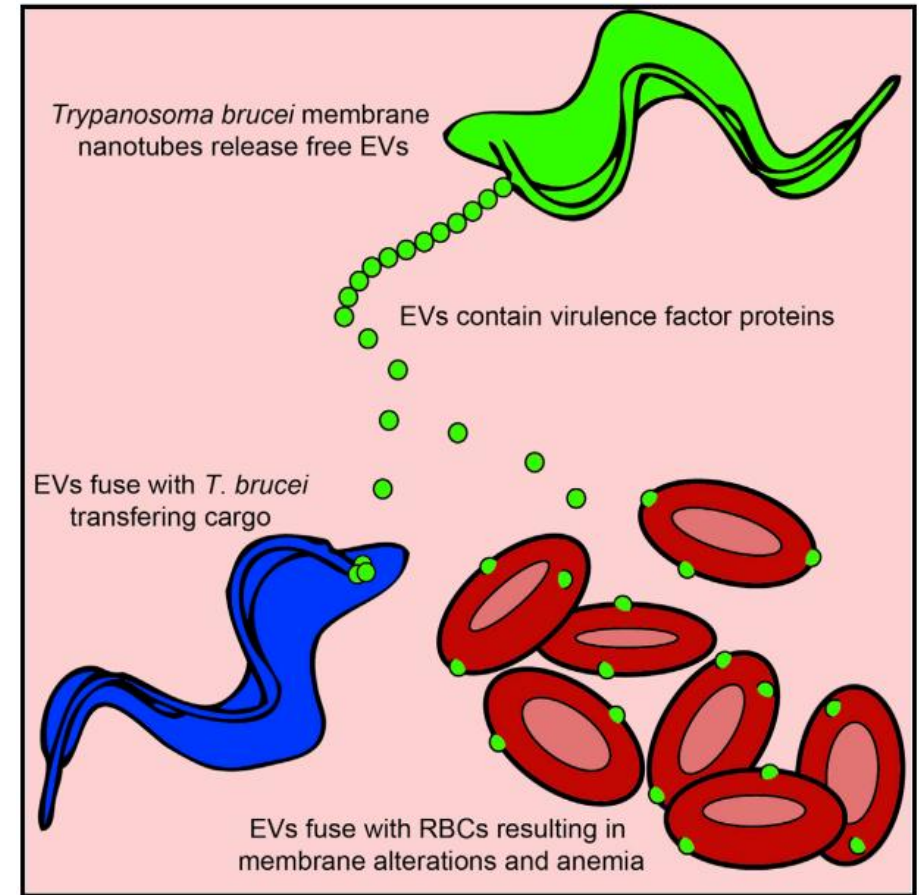


EVs injection causes an increase in erythrocyte volume due to lipid incorporation, and a decrease of erythrocytes

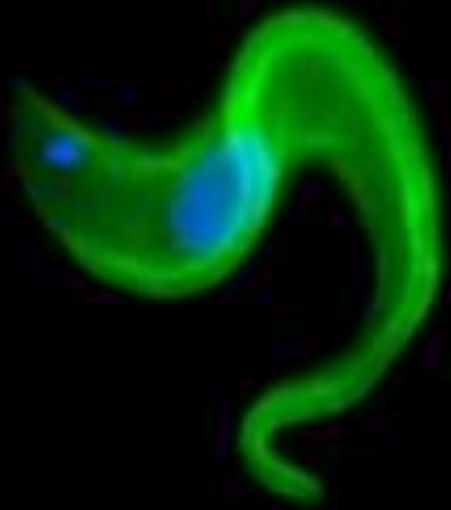
The highly fusogenic properties of trypanosome EVs directly alter the physical properties of erythrocytes and likely contribute to anemia

Conclusions and future perspectives

- Trypanosoma EVs contain several virulence factors necessary for human infectivity, including SRA
- Trypanosome-derived EVs are highly fusogenic with erythrocytes, resulting in physical changes to the cell membrane and rapid clearance
- In mouse model, these changes lead to erythrophagocytosis and are the cause of anemia during acute phase infection
- **This study opens the possibility of identifying inhibitors of EV fusion with host cells and may lead to development of drugs that will spare the host from disease-induced anemia, the major cause of morbidity**

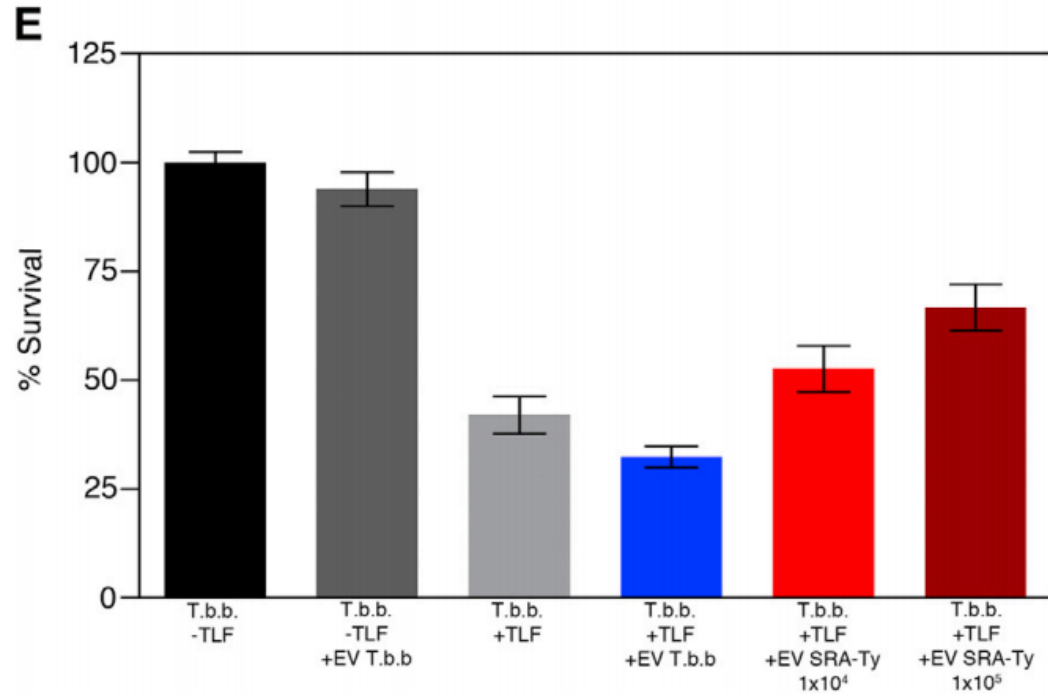


Thanks!



Supplemental Information

TLF overnight survival assays



Addition of EVs from *T. b. brucei*^{SRA-Ty} or *T. b. rhodesiense* but not EVs from wild-type *T. b. brucei* increased TLF resistance of recipient *T. b. brucei* in a dose-dependent manner