SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Nanotube and extracellular vesicle induction

Nanotubes were quantified by DIC of at least 200 trypanosomes at a magnification of 40× and expressed as percent of cells with associated nanotubes. Induction was utilized to acquire images in Figures 1A, 1C, 1D and 1E (complement active serum) and Figures 1F, 1G, 2A, 2B, 2C, 2D, S1C and S1D (RNAi). In all other experiments EVs were acquired from trypanosomes grown in heat inactivated serum or serum free conditions (for mass spectrometry).

Extracellular vesicle purification

T. brucei was grown in HMI-9 to a density of 1×10^6 cells/ml. Cells and cell debris were removed by centrifugation at 1200 × g for 10 min and the supernatant filtered (0.2 μm) and EVs collected by centrifugation at 100,000 × g for 70 min. EVs were washed with PBS and stored at 4°C in PBS (Bayer-Santos et al., 2013). Protein concentrations were determined by Bradford assay (Bio-Rad) and phospholipid concentration by the phospholipid assay kit (Sigma MAK122). EV yield was ~ 1 μg protein and 50-100 μg phospholipid per 1×10^6 cells corresponding to approximately 8.6×10^9 EVs.

Human erythrocytes

Blood was collected from healthy volunteers into citrate buffer by a licensed phlebotomist. Cells were used immediately or stored at 4°C for no longer than 1 week.

Microscopy

All DIC and immunofluorescence images were acquired with a Zeiss Axio Observer Z1 equipped with an AxioCam MRm controlled by AxioVision 4.6 software as previously described Sykes et al., 2015. For live cell imaging, trypanosomes from culture were aliquoted on slides at a density of 1×10^7 cells/ml and blood from infected mice was directly smeared on slides from tail clips. For fluorescence microscopy, trypanosomes were harvested at a density of 1×10^5 cells/ml, adjusted to 1×10^7 cells/ml and labeled with 40 μM R18 for 15 minutes at 37°C. Cells were then washed three times with sterile culture medium.

To visualize EV interaction with intact erythrocytes, 1×10^8 freshly collected human erythrocytes were incubated with 1.6 μg R18-labeled EV (by lipid) or an equivalent mass of R18-labeled POPC (Avanti Polar Lipids) large unilamellar vesicles (LUV) (see membrane fusion assay below for R18-labeling procedure) for 5 min at 37°C, washed phosphate buffered saline (PBS) and imaged. For immunofluorescence microscopy, erythrocytes were incubated with 1 μg EVs (by protein) for 5 min at 37°C, washed and blocked with 20% FBS. Cells were then incubated with antibodies against VSG 221 (1:5000) washed, incubated with Alexa-488 goat anti-rabbit IgG (Invitrogen) (1:1000) before blocking, washing and imaging.

SDS-PAGE and Western blotting

Antibodies used were against trypanosome VSG 221, aldolase, glycerol kinase (gifts from James Bangs, U. Buffalo; James Morris, Clemson University), human HSP70 (Abcam) and the yeast Ty epitope (1:5000).

Proteomic analysis

T. brucei EV proteins were digested with sequencing-grade trypsin (Promega) and resulting peptides were processed as described (Bayer-Santos et al., 2013). Peptides were loaded onto a C18 column (Thermo Scientific Acclaim® PepMap RSLC, 75 µm x 15 cm, nanoViper C18, 2 µm, 100 Å) connected to a quaternary RSLC nanoUHPLC system (Dionex Ultimate 3000, Thermo Fisher Scientific, San Jose, CA). The column was washed for 10 min with 2% acetonitrile (ACN)/0.1% formic acid (FA) (solvent A) at 300 nl/min flow rate. Peptides were eluted using the following gradient: 10-65 min, 5-40% solvent B (80% ACN/0.1% FA); 65-70 min, 40-95% solvent B; and 70-90 min, 95% solvent B. Eluting peptides were analyzed online by electrospray ionization on a Q Exactive Benchtop Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA). Full MS1 scan spectra were collected from 400-2000 *m/z* range at 70,000 resolution (at *m/z* 200). Top ten most intense parent ions were submitted to higher energy collision dissociation (HCD) with an isolation window of 4.0 *m/z* and 28% normalized-collision energy.

Raw LC-MS/MS files were submitted to database search against *T. brucei* TREU927 sequences (8776 sequences, downloaded from UniProt on 10/27/2014) using MaxQuant v1.5.0.25 (Cox and Mann, 2008). Searches were performed in two rounds using the following parameters: oxidation of methionine and acetylation of protein N-terminus as variable modifications, cysteine carbamidomethylation as a fixed modification, trypsin digestion with 2 missed cleavage sites allowed, 20 ppm for fragment mass tolerance, and 20 and 4.5 ppm peptide-mass tolerance for first and second search rounds, respectively. Identification false-discovery rate (FDR) was set at 1% for both peptide-spectrum matches (PSM) and protein level. Raw LC-MS/MS runs along with the MaxQuant

searching results were uploaded to PRIDE database (http://www.ebi.ac.uk/pride/) under accession number PXD002030.

EV proteome was compared to whole cell lysate (Butter et al., 2013), glycosomal (Guther et al., 2014), mitochondrial (Panigrahi et al., 2009), mitochondrial outer membrane (Niemann et al., 2013), and flagellum surface and matrix (Oberholzer et al., 2011) proteomes. Each subcellular proteome was tested for enrichment of EV proteins comparing to the whole cell lysate proteome and submitted to Fisher's exact test using the GraphPad software (http://graphpad.com/quickcalcs/contingency1/). The EV proteome was also analyzed for low abundance proteins using TriTryp DB. Total EV proteome was screened against life cycle stage specific ribosome profiling data (Jensen et al., 2014) and proteins with mRNA determined to have ≤ 125 RPKM of combined unique and non-unique reads were scored as "low abundance." The 125 RPKM cutoff was selected because it is ~ 10-fold lower than the abundant proteins HSP70 and enolase and ~ 50-fold lower than beta-tubulin. To confirm the presence of flagellar associated proteins all EV proteins were searched on TriTryp DB to identify microscopy data showing protein localization within proximity to the flagellum, flagellar pocket or cell surface of *T. brucei* (Bhogaraju et al., 2013; Esson et al., 2012; Gadelha et al., 2015; Hanrahan et al., 2009; Luo et al., 2006; Proto et al., 2011; Salmon et al., 2012; Zhou et al., 2015).

Scanning electron microscopy of purified EVs

EVs were fixed for TEM using the same method as BF *T. brucei.* After fixation EVs were washed and repelleted at 100,000 x g. Prior to resin embedding the EV pellet was embedded in 4% low melting point agarose and cut into 1 mm blocks.

SRA Transfer Assay

For flow cytometry *T. brucei* (8.5 x 10^6 /ml) was incubated with EVs at a concentration of 8.5 x 10^7 cell equivalents for 1 hr at 37°C. *T. brucei*, *T. brucei*$^{SRA\text{-}Ty}$ and EV treated *T. brucei* cells were chilled on ice for 30 min, washed with 1 x PBS and fixed with 0.05% paraformaldehyde (Sigma) in HMI9 media lacking serum proteins. An aliquot of fixed cells were washed and stored. Another aliquot was permeabilized with ice cold 0.1% Triton X-100 for 5 min. Cells were washed, blocked with 10% FBS and incubated with monoclonal anti-Ty (1:400) for 30 min following washes SRA was detected with a Alexa-488 anti-mouse IgG (1:1000) (Life Technologies). Cells were analyzed on a CyAn ADP Analyzer (Beckman Coulter). For microscopy *T. brucei* (1 x 10^7/ml) was incubated with EVs (1 x 10^8 cell equivalents) for 1 hr at 3°C or 30 min at 37°C. ConA was added to a final concentration of 5 μg/ml. Cells were chilled on ice for 30 min, washed and fixed with 0.05% paraformaldehyde in HMI9 media lacking serum. Cells were smeared onto microscope slides, air dried and permeabilized with methanol (-20°C) for 10 min. *T. brucei*, *T. brucei* SRA-Ty and EV treated *T. brucei* were then treated as above with monoclonal anti-Ty (1:1000) antibody and Alexa-488 anti-mouse IgG (1:1000).

Overnight Survival Assay

Co-cultured or transwell co-cultured trypanosomes (1× 10^5/ml) were treated with 1 ng TLF for 24 hours at 37°C. Trypanosomes (1 × 10^5/ml) were incubated with purified EVs

(1 x 10^4 - 1 x10^5 cell equivalents as described in figure) for 1 hr and then treated with 5 ng TLF for 24 hr at 37°C. After treatment surviving cells were counted by phase-contrast microscopy and compared against non-TLF treated cultures.

Saturation Binding

T. brucei cells were resuspended to a density of 5 x 10^6/ml at 3°C in serum-free HMI-9 medium containing 1% bovine serum albumin. Cells were incubated with increasing concentrations (by protein) of Alexa-594 labeled EVs for 15 min. Samples were washed with ice cold 1x PBS and analyzed by flow cytometry on a CyAn ADP Analyzer (Beckman Coulter) (DeJesus et al., 2013).

Binding Competition

T. brucei cells were resuspended to a density of 5 x 10^6/ml at 3°C in complete HMI-9. Cells were incubated with 2 μg (by protein) of Alexa-488 labeled EVs mixed with 2 μg, 10 μg , 20 μg or 50 μg of unlabeled competitor for 15 min. Samples were washed with ice cold 1x PBS and analyzed by flow cytometry on a CyAn ADP Analyzer (Beckman Coulter) (DeJesus et al., 2013).

EV and Microvesicle Purification

Procyclic *T. brucei* TREU667 were grown in SM medium containing 10% fetal bovine serum at 27°C. EVs were harvested from exponentially growing cells at 1 x 10^7/ml and prepared using the same protocol as BF EVs. Promastigote *Leishmania tarentolae* were grown in SDM-79 supplemented with 10% FBS at 27°C (Brun and Schonenberger, 1979). EVs were harvested from exponentially growing cells at 1.7 x 10^7/ml and prepared

using the BF EV protocol. This method has previously been used to purify *Leishmania* spp. EVs (Silverman et al., 2010). Human erythrocytes were washed in complete HMI-9 medium and incubated for 72 hr in 5% CO_2 at 37°C to allow for accumulation of micro vesicles (MVs) (Mantel et al., 2013). MVs were harvested by a series of differential centrifugation steps to remove cells and cell debris. Cells were spun at 1,200 x g for 10 min, supernatant was then spun twice at 4,000 x g for 10 minutes. The resulting supernatant was filtered though a 0.2 μm filter and subjected to a 100,000 x g spin for 70 minutes resulting in a pelleted fraction that was washed three times with 1 x PBS before use.

EV protein transfer

Purified EVs were labeled with an Alexa-594 labeling kit (Life Technologies) and $1x10^7$ cell equivalents were added to $1x10^8$ *T. brucei*. Cells were also treated with 5 μg/ml ConA-fluorescein isothiocyanate (Sigma) at 3°C serum-free HMI-9 medium containing 1% bovine serum albumin. After 15 minute cells were washed three times with ice cold 1x PBS and a subset was prepared for fluorescence microscopy. After washing cells were then warmed to 37°C for 15 min to allow for endocytosis to resume. Cells were then washed and prepared for fluorescence microscopy (Sykes et al., 2015).

Membrane fusion assay

Vesicle fusion was measured essentially as described by Vidal and Hoekstra (1995) (Vidal and Hoekstra, 1995). Purified EVs (160 μg lipid) were labeled with 80 μM R18 in 1 ml PBS at 4°C for 16 h. Unincorporated dye was removed by gel filtration (PD-10, GE Healthcare). Control POPC LUV were constructed by hydration of a thin lipid film with

PBS and extrusion through 0.1 μm polycarbonate filters. Assays were initiated in 1 ml of PBS with 1.6 μg R18-labeled EV lipid, or equivalent mass of control R18-labeled POPC LUV, to which 10 μg POPC LUV was added. The fluorescence of R18 was monitored in a Perkin Elmer Life Sciences LS-55 luminescent spectrofluorometer with 544 nm and 590 nm excitation and emission wavelengths respectively. 100 % de-quenching values were determined after addition of Triton X-100 to a final concentration of 0.01 %. For erythrocyte fusion assays, erythrocyte ghosts were prepared to eliminate spectral interference from hemoglobin. Human erythrocytes were osmotically lysed in 5 % carbonate buffer and washed into PBS. For EV fusion with *T. brucei* 1.6 μg R18-labeled EV lipid was added to 1x 10^6 BF *T. brucei*.

Purified EVs (160 μg lipid) were labeled with 80 μM R18 in 1 ml PBS at 4°C for 16 h. For erythrocyte fusion assays, erythrocyte ghosts were prepared to eliminate spectral interference from hemoglobin. Human erythrocytes were osmotically lysed in 5 % carbonate buffer and washed into PBS. For EV fusion with *T. brucei* 1.6 μg R18-labeled EV lipid was added to 1x 10^6 *T. brucei*.

For Amnis ImageStream analysis R18 labeled EVs were incubated with bloodstream form *T. brucei* cells (1x 10^6/ml) and immediately analyzed over the course of an ~5 minute run collecting 10,000 events.

Transwell co-culture

T. brucei (1× 10^5/ml) was co-cultured with *T. brucei* $^{SRA\text{-}Ty}$ or *T. b. rhodesiense* (1× 10^5/ml) in 0.2 μm transwell culture plates (Corning). Cells were co-cultured for 24 hours then split 1/10 and treated with TLF for an overnight survival assay. For erythrocyte co-

culture *T. brucei* (1×10^5/ml) was inoculated with mouse or human erythrocytes (1×10^8 cells/ml) in transwell plates.

For lipid exchange assays *T. brucei* cells were labeled with 40 μM R18, washed and then added to the transwell. Cells were grown for 24 to 48 hours and were split back every 24 hours to maintain log phase growth.

Co-culture selection

Wild-type *T. b. brucei* were grown together with a previously characterized cell line *T. b. brucei* $^{SRA\text{-}Ty}$ in 40 ml for 96 hours. Cells were selected with hygromycin at a concentration of 25μg/ml for 48 hours to ensure all Ty-SRA cells had died off (as determined by independent and mixed growth curve analysis). Surviving cells were then treated with TLF for an overnight survival assay.

Osmotic lysis assay

Freshly collected human erythrocytes were resuspended at 1×10^8 cells/ml in PBS, incubated with 200 μg EVs (by protein) for 30 min at 37°C. 5×10^5 erythrocytes were added to 150 μl of NaCl solutions, incubated for 30 min at 37°C, pelleted and the absorbance of the supernatant was read at 405 nm. Complete lysis was achieved by incubation in deionized H_2O.

Laurdan spectral analysis

Erythrocytes were washed, labeled with 2.5 μM Laurdan for 30 min at 37°C, washed three times with 37°C PBS and spectra recorded in quartz cuvettes with the Perkin Elmer Life Sciences LS-55 luminescent spectrofluorometer. Temperature was maintained at

37°C with a Perkin Elmer Life Sciences Biokinetics Accessory. Emission spectra were recorded from 360-600 nm with a 350 nm excitation wavelength. Spectra were background subtracted with an equal concentration of unlabeled erythrocytes in PBS.

Trypanosome infections and EV treatments

Trypanosome infections were initiated by intraperitoneal injection of 5×10^3 *T. b. brucei* Lister 427–221 parasites in retired breeder female BALB/c mice (Jackson Laboratory). Parasitemias were determined by the matching method (Herbert and Lumsden, 1976).

For *ex vivo* EV-treatment of erythrocytes, 2.5×10^8 erythrocytes from 10 week old mice expressing GFP in all cells from a ubiquitin promoter (C57BL/6-Tg(UBC-GFP)30Scha/J) (Jackson Laboratory) were collected in 1 ml PBS with 10 U/ml heparin and incubated for one hour at 37°C with or without 8×10^7 trypanosome EV equivalents / 2×10^7 RBC. Erythrocytes were injected into the tail vein of naive retired female breeder C57BL/6J mice. Whole blood collected from the tail vein was analyzed by flow cytometry (Beckman Coulter CyAn) and GFP-positive erythrocytes were quantified from the ratio of labeled to unlabeled erythrocytes in 1×10^6 cell counts.

For direct EV injections, wild-type BALB/c and C57BL/6J mice were injected intravenous (tail vein) with EV freshly prepared from 1×10^8 trypanosomes in a total volume of 150 μl PBS. Control mice were injected with an equal volume of PBS. At the indicated times, 1.5 μl of blood was collected from the tail vein and erythrocyte counts were determined via Coulter counting (Beckman).

SUPPLEMENTAL REFERENCES

Bayer-Santos, E., Aguilar-Bonavides, C., Rodrigues, S.P., Cordero, E.M., Marques, A.F., Varela-Ramirez, A., Choi, H., Yoshida, N., da Silveira, J.F., and Almeida, I.C. (2013). Proteomic analysis of Trypanosoma cruzi secretome: characterization of two populations of extracellular vesicles and soluble proteins. J Proteome Res *12*, 883-897.

Bhogaraju, S., Cajanek, L., Fort, C., Blisnick, T., Weber, K., Taschner, M., Mizuno, N., Lamla, S., Bastin, P., Nigg, E.A*., et al.* (2013). Molecular basis of tubulin transport within the cilium by IFT74 and IFT81. Science *341*, 1009-1012.

Brun, R., and Schonenberger (1979). Cultivation and in vitro cloning or procyclic culture forms of Trypanosoma brucei in a semi-defined medium. Short communication. Acta Trop *36*, 289-292.

Butter, F., Bucerius, F., Michel, M., Cicova, Z., Mann, M., and Janzen, C.J. (2013). Comparative proteomics of two life cycle stages of stable isotope-labeled Trypanosoma brucei reveals novel components of the parasite's host adaptation machinery. Mol Cell Proteomics *12*, 172-179.

Cox, J., and Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol *26*, 1367-1372.

DeJesus, E., Kieft, R., Albright, B., Stephens, N.A., and Hajduk, S.L. (2013). A single amino acid substitution in the group 1 Trypanosoma brucei gambiense haptoglobin-hemoglobin receptor abolishes TLF-1 binding. PLoS Pathog *9*, e1003317.

Esson, H.J., Morriswood, B., Yavuz, S., Vidilaseris, K., Dong, G., and Warren, G. (2012). Morphology of the trypanosome bilobe, a novel cytoskeletal structure. Eukaryot Cell *11*, 761-772.

Gadelha, C., Zhang, W., Chamberlain, J.W., Chait, B.T., Wickstead, B., and Field, M.C. (2015). Architecture of a Host-Parasite Interface: Complex Targeting Mechanisms Revealed Through Proteomics. Mol Cell Proteomics *14*, 1911-1926.

Guther, M.L., Urbaniak, M.D., Tavendale, A., Prescott, A., and Ferguson, M.A. (2014). High-confidence glycosome proteome for procyclic form Trypanosoma brucei by epitope-tag organelle enrichment and SILAC proteomics. J Proteome Res *13*, 2796-2806.

Hanrahan, O., Webb, H., O'Byrne, R., Brabazon, E., Treumann, A., Sunter, J.D., Carrington, M., and Voorheis, H.P. (2009). The glycosylphosphatidylinositol-PLC in Trypanosoma brucei forms a linear array on the exterior of the flagellar membrane before and after activation. PLoS Pathog *5*, e1000468.

Jensen, B.C., Ramasamy, G., Vasconcelos, E.J., Ingolia, N.T., Myler, P.J., and Parsons, M. (2014). Extensive stage-regulation of translation revealed by ribosome profiling of Trypanosoma brucei. BMC Genomics *15*, 911.

Luo, S., Fang, J., and Docampo, R. (2006). Molecular characterization of Trypanosoma brucei P-type H+-ATPases. J Biol Chem *281*, 21963-21973.

Mantel, P.Y., Hoang, A.N., Goldowitz, I., Potashnikova, D., Hamza, B., Vorobjev, I., Ghiran, I., Toner, M., Irimia, D., Ivanov, A.R.*, et al.* (2013). Malaria-infected erythrocyte-derived microvesicles mediate cellular communication within the parasite population and with the host immune system. Cell Host Microbe *13*, 521-534.

Niemann, M., Wiese, S., Mani, J., Chanfon, A., Jackson, C., Meisinger, C., Warscheid, B., and Schneider, A. (2013). Mitochondrial outer membrane proteome of Trypanosoma brucei reveals novel factors required to maintain mitochondrial morphology. Mol Cell Proteomics *12*, 515-528.

Oberholzer, M., Langousis, G., Nguyen, H.T., Saada, E.A., Shimogawa, M.M., Jonsson, Z.O., Nguyen, S.M., Wohlschlegel, J.A., and Hill, K.L. (2011). Independent analysis of the flagellum surface and matrix proteomes provides insight into flagellum signaling in mammalian-infectious Trypanosoma brucei. Mol Cell Proteomics *10*, M111 010538.

Panigrahi, A.K., Ogata, Y., Zikova, A., Anupama, A., Dalley, R.A., Acestor, N., Myler, P.J., and Stuart, K.D. (2009). A comprehensive analysis of Trypanosoma brucei mitochondrial proteome. Proteomics *9*, 434-450.

Proto, W.R., Castanys-Munoz, E., Black, A., Tetley, L., Moss, C.X., Juliano, L., Coombs, G.H., and Mottram, J.C. (2011). Trypanosoma brucei metacaspase 4 is a pseudopeptidase and a virulence factor. J Biol Chem *286*, 39914-39925.

Salmon, D., Vanwalleghem, G., Morias, Y., Denoeud, J., Krumbholz, C., Lhomme, F., Bachmaier, S., Kador, M., Gossmann, J., Dias, F.B.*, et al.* (2012). Adenylate cyclases of Trypanosoma brucei inhibit the innate immune response of the host. Science *337*, 463-466.

Silverman, J.M., Clos, J., de'Oliveira, C.C., Shirvani, O., Fang, Y., Wang, C., Foster, L.J., and Reiner, N.E. (2010). An exosome-based secretion pathway is responsible for protein export from Leishmania and communication with macrophages. J Cell Sci *123*, 842-852.

Vidal, M., and Hoekstra, D. (1995). In vitro fusion of reticulocyte endocytic vesicles with liposomes. J Biol Chem *270*, 17823-17829.

Zhou, Q., Hu, H., He, C.Y., and Li, Z. (2015). Assembly and maintenance of the flagellum attachment zone filament in Trypanosoma brucei. J Cell Sci *128*, 2361-2372.