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Supplementary Information for

African trypanosomes expressing multiple VSGs are rapidly eliminated by the host immune system

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Supplementary Information Text

Materials and Methods

Trypanosome cell lines and plasmid construction

T. brucei bloodstream form (BSF) parasites (strain Lister 427, antigenic type MiTat 1.2, clone 221a) (1) were cultured in HMI-11 as described in (2). PL1S and VSG13-expressing cell lines was described in (3) and (4), respectively. VSG8- and VSG11-expressing cell lines were described in (5). POT and PET cell lines were generated by transfecting pFAB9 and pFAB11 (6), respectively, in PL1S with an AMAXA nucleofector (Lonza), program X-001, using the previously optimized homemade Tb-BSF buffer (90 mM Na_2HPO_4, 5 mM KCl, 0.15 mM $CaCl_2$, 50 mM HEPES, pH 7.3) (7). pFAB9 contains two bidirectional promoters: a T7 promoter that drives transcription of the *BLE* gene to allow selection of positive clones with phleomycin and rDNA promoter followed by two Tet Operator sequences and a TDP1 gene with C-terminal TY1 tag and an aldolase 3'UTR. pFAB9 was linearized with *Not*I (New England Biolabs), for integration in of the rDNA spacer sequences and pFAB11 was linearized with *Sma*I (New England Biolabs). Overexpression *in vitro* was induced by adding 1 μg/ml of tetracycline to the medium. All cloning was performed using the In-Fusion® HD Cloning system (Clontech) following to the manufacturer's instructions.

Western blotting

Cells were lysed using Laemmli buffer, resuspended at 1×10^6 cells/μl and DNA digested with 200 U/ml Benzonase (Sigma). A SDS-PAGE was performed at 4ºC and proteins were transferred to a nitrocellulose membrane using an iBlot Dry Blotting System (Invitrogen) for 6 min and 30 sec. Membrane was blocked with 5% milk in PBS / 0.1% Tween for at least 1 hour and primary mouse anti-TY1 antibody (8) and rabbit anti-H2A (custom made) were incubated overnight at 4ºC in a 1:2000 and 1:5000 dilutions, respectively, in 3% milk in PBS / 0.1% Tween. Antibody against VSG9 was also incubated overnight at 4ºC in 1:20000 dilution, in 3% milk in PBS / 0.1% Tween. Membrane was washed three times with PBS / 0.1% Tween and incubated with anti-mouse horseradish peroxidase-linked secondary antibody (Amersham) at room temperature for 1 hr in a 1:20000 dilution in 3% milk in PBS / 0.1% Tween. Membrane was further washed three times with PBS / 0.1% Tween and developed with a Western Lightning Plus-ECL (PerkinElmer). Picture acquisition was made by a Chemidoc XRS+ (Bio-Rad).

Cell cycle profile

At each time point, 2 million cells were centrifuged for 10 min at 1300 g, 4ºC and washed once with ice-cold PBS. Cells were resuspended in PBS with 2 mM EDTA and slowly fixed with 2.5 ml of absolute ethanol. After fixing for at least one hour, cells were washed once and resuspended in 1 ml PBS / EDTA. Cells were incubated with 10 μg RNase A and 1 μg of propidium iodide for 30 min at 37ºC and further analyzed by flow cytometry for DNA content.

FAIRE and ChIP

Formaldehyde-assisted isolation of regulatory elements (FAIRE) and chromatin immunoprecipitation (ChIP) were performed as previously described (6). Quantitative PCR (qPCR) was performed using 1× SYBR Green PCR Master Mix (Applied Biosystems) using primers listed in *SI Appendix*, Table S1.

Transcript quantification

Parasites were harvested by centrifugation at 650 g for 10 min at 4°C and immediately resuspended in PureZOL (Bio-Rad) or TRIzol (Life Technologies). RNA was isolated following the manufacturer's instructions and RNA quantity and quality was assessed on a NanoDrop 2000 (Thermo Fisher Scientific). cDNA was generated from 200 ng of RNA using a Superscript cDNA Synthesis Kit (Life Technologies), according to manufacturer's protocol. Quantitative PCR (qPCR) was performed using 1× SYBR Green PCR Master Mix (Applied Biosystems) using primers presented in *SI Appendix*, Table S1. Negative controls lacking reverse transcriptase (RT-) were confirmed by qPCR. Amplification reactions were performed in duplicates. The $\Delta\Delta$Ct method was used to determine transcript levels relative to the normalizing gene.

Soluble-form VSG isolation and quantitative mass spectrometry

TDP1 was overexpressed for 48 hr in POT1 before isolation of soluble-form VSG (sVSG). Parental PL1S cell line was used as control. Isolation of sVSG was performed according to (9), except the eluate that was concentrated in Amicon Ultra-2 Ultracel-10 Membrane 10 kDa (Merck Millipore). sVSG samples at a concentration of 0.2 µg/µl in MilliQ water were reduced in 10 mM DTT (Sigma) at 56˚C for 1 hr and alkylated with 50 mM iodoacetamide for 30 min at 20˚C. Trypsin digestion (PROMEGA, Trypsin GOLD MS Grade) was performed overnight at 37˚C and 650 rpm. Samples were concentrated in a SpeedVac and then adjusted to 1% formic acid. Mass spectrometry data collection and process was performed as in (10). All mass spectrometry measurement files are deposited at the ProteomExchange consortium via PRIDE (PXD014803).

Generation VSG specific polyclonal IgM antisera

Specific polyclonal IgM antisera were generated against parasites expressing VSG9, VSG8 and POT2 mutants (with or without induction) as described in (11), with minor changes. Two mice were injected intra-peritoneally (i.p.) with 1×10^4 trypanosomes in HMI-11. Overexpression induction of parasites in culture was initiated 24 hr prior to mouse infection with 1 µg/ml tetracycline. Mice received doxycycline supplemented water (1 mg/ml of doxycycline hyclate, Sigma) 24 hours prior to infection and during the whole course of infection. On day 4 and 5 post-infection, mice were treated with 250 ng berenil/mouse injected i.p., to clear the parasites. On day 8 post injection, blood was collected via cardiac puncture, and serum was separated from whole blood by centrifugation.

VSG detection in live parasites by Fluorescence-activated cell sorting

1×10^6 cells/ml were harvested and washed with PBS. Cells were resuspended in 200 µl of cold HMI-11 with rabbit anti-VSG13 conjugated with Alexa Fluor 647 (diluted 1:5000) or with rabbit anti-VSG6 (diluted 1:10000, kind gift from Lucy Glover) and incubated for 30 min at 4ºC. After this period, cells were centrifuged, resuspended in 200 µl cold HMI-11 with Fc block (1:200, rat anti-CD16/CD32 ThermoFisher) and VSG8-specific polyclonal IgM antisera (diluted 1:50) and incubated for 30 min at 4ºC. In parallel, unstained cells were resuspended in 200 µl cold HMI-11 with Fc block (1:200, rat anti-CD16/CD32 ThermoFisher) and VSG9-specific polyclonal IgM antisera (diluted 1:100) and incubated for 30 min at 4ºC. Cells were again centrifuged, resuspended in 200 µl with donkey anti-rabbit IgG conjugated with Alexa Fluor 597 (1:500, Thermo Fisher) or with rat anti-mouse IgM conjugated with Alexa Fluor 488 (1:500, BioLegend). After washing, cells were resuspended in cold TDB, and immediately analyzed on a BD LSRFortessa™ X-20 (BD Biosciences) and FlowJo software.

Animal Experiments

Inbred C57BL/6J wild-type mice, 6-10 weeks old (Charles River, France) or RAG2-KO male mice, 6-10 weeks old (Instituto Gulbenkian de Ciência, Portugal) were housed in the specific pathogen-free facilities of the Instituto de Medicina Molecular – João Lobo Antunes. The animal facility and the experimental procedures complied with EU regulations and were approved by the Instituto de Medicina Molecular Animal Care and Ethics Committee (AWB_2016_07_LF_Tropism). Mice were infected by i.p. injection of 20 parasites of *T. brucei* POT1 and POT2 clones (with and without induction). Overexpression induction of parasites in culture was initiated 24 hours prior to mouse infection with 1 µg/ml tetracycline. Mice received doxycycline supplemented water (1 mg/ml of doxycycline hyclate, Sigma) 24 hours prior to infection and during the whole course of infection. Parasitemia was monitored throughout infection by collecting blood from the mouse tail. For parasite protein analysis, 25–100 µl of blood was collected from the mouse tail, in red blood cell lysis buffer (150 mM NH4Cl, 10 mM KHCO3, 1 mM EDTA.Na2, pH 7.4) and washed in 1× TDB and resuspended in Laemmli buffer. Mice were euthanized at the first signs of severe disease distress, with all efforts to minimize animal suffering.

Antibody repertoire

1×10^6 cells/ml were harvested and washed with PBS. Cells were resuspended in 200 µl of cold HMI-11 with either IgM polyclonal mouse antisera specific for VSG9 (1:50), or VSG8 (1:50), or mouse sera from animals infected with POT2 induced (1:50) or non-induced (1:50), or rabbit anti-VSG13 IgG conjugated with Alexa Fluor 647 (diluted 1:5000) (4), or VSG2 conjugated with APC (1:10000, kind gift from Nina Papavasiliou), or rabbit anti-VSG6 (1:10000, kind gift from Lucy Glover) for 30 min at 4ºC (4). After this period, cells incubated with mouse sera were centrifuged, resuspended in 200 µl cold HMI-11 with rat anti-mouse IgM conjugated with Alexa Fluor 488 (1:500, BioLegend) or donkey anti-rabbit IgG conjugated with Alexa Fluor 488 (1:500, Thermo Fisher) and incubated for 15 min at 4ºC. After washing, cells were resuspended in cold TDB, and immediately analyzed on a LSRFortessa™ X-20 (BD Biosciences) and FlowJo software.

Supplementary Figures

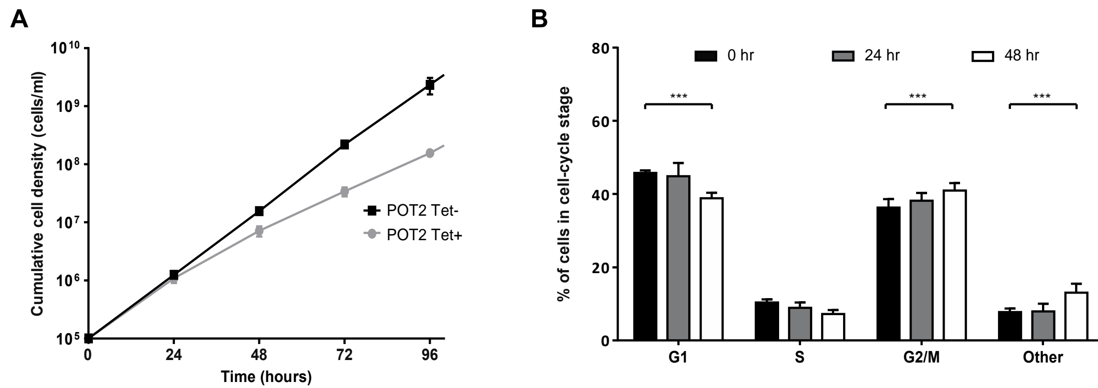

Fig. S1. **Inducible overexpression of TDP1 in bloodstream forms. (A)** Growth curve of POT2 before (Tet-, black curve) or after (Tet+, grey curve) induction of overexpression. Three independent experiments were analyzed and are represented as mean with SEM. **(B)** Cell cycle profile of POT2 after 24 and 48 hr of TDP1 overexpression. 'Other' represents cells with abnormal DNA content. Four independent experiments were analyzed and are represented as mean ± SEM. Statistical significance was determined by a two-way ANOVA with Dunnett's multiple comparison test. ***p-value < 0.001.

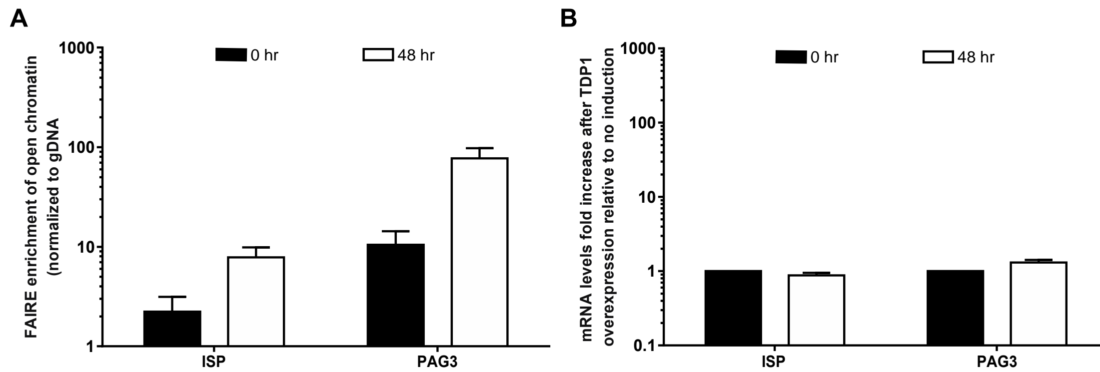

Fig. S2. **Low transcribed Pol II genes are not upregulated upon TDP1 overexpression. (A)** Chromatin conformation was measured by FAIRE at 48 hr after overexpression induction in POT1. DNA isolated by FAIRE was quantified by qPCR and normalized to gDNA copy number and ampicillin gene from DNA spike. Three independent experiments were analyzed and are represented as mean ± SEM. **(B)-fold**-increase of mRNA levels-fold after 48 hr of overexpression induction in POT1, measured by qPCR and normalized to non-induced cells. Three independent experiments were performed and are represented as mean ± SEM. **(A and B)** Statistical significance was determined by an unpaired t-test with Welch's correction.

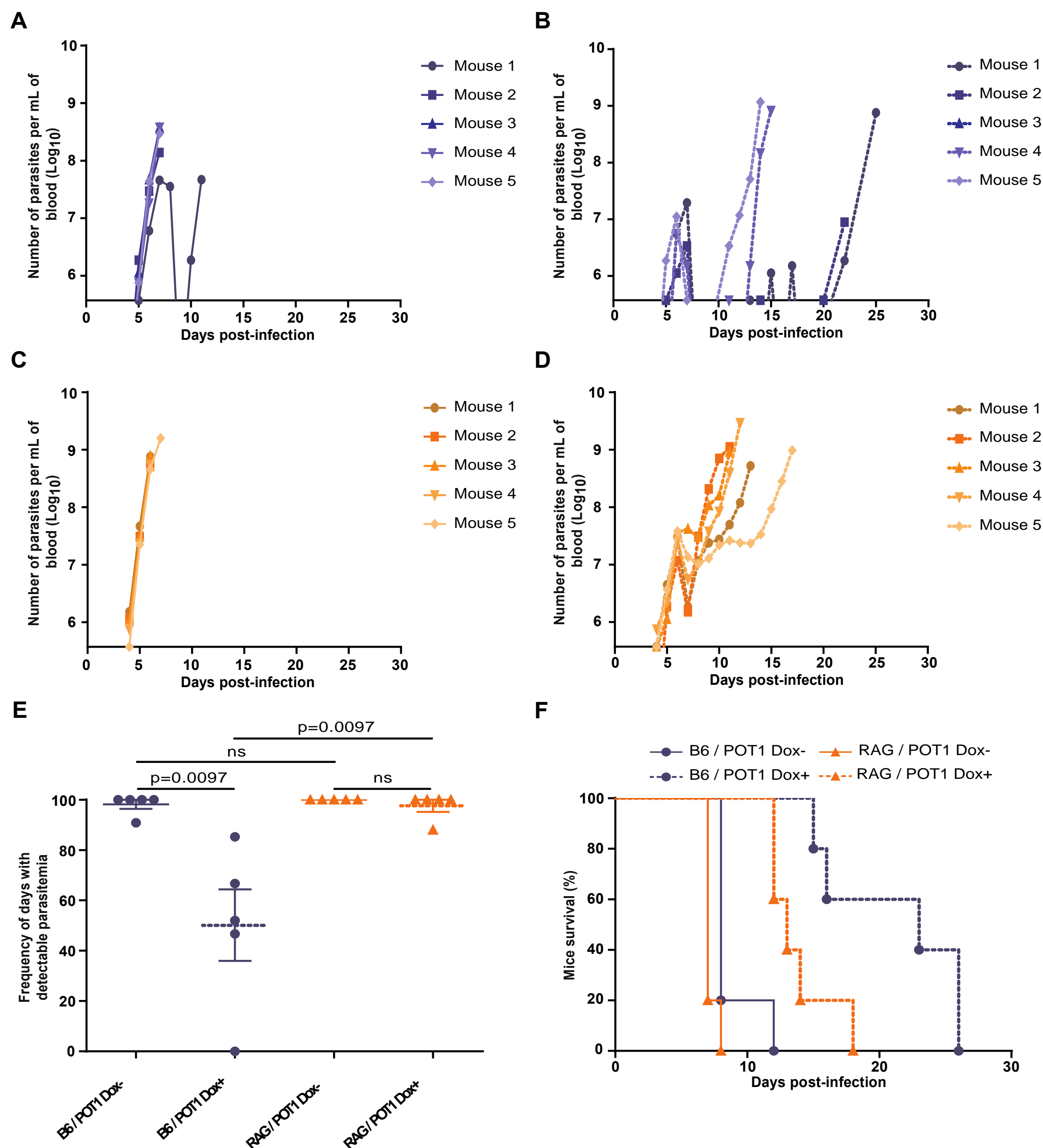

Fig. S3 **TDP1 overexpression increases mice survival upon infection and the immune system helps controlling parasitemia. (A-D)** Curves of parasitemia upon infection with POT1 parasites in C57BL6/J **(A-B)** or RAG2-KO mice **(C-D)** without or with induction of TDP1 overexpression with doxycycline (**A** and **C**; **B** and **D**, respectively). **(E)** Frequency of the number of days where parasitemia was detected relative to infection length for POT1 clones. Note that parasitemia started to be detected in C57BL/6J mice at day 5 post-infection and in RAG2 K.O. mice at day 4 post-infection. Statistical significance was determined by a two-tailed Mann-Whitney test (ns stands for not significant). **(F)** Survival curves of mice infection with POT1 clones. Statistical significance was determined by a Log-rank test obtaining the following p-values: B6/POT1 Tet- vs. Tet+: 0.0016; RAG/POT1 Tet- vs. Tet+: 0.0016; B6/POT1 Tet- vs. RAG/POT1 Tet-: 0.0158; B6/POT1 Tet+ vs. RAG/POT1 Tet+: 0.02.

Table S1. List of primers.

Primer name	Primer sequence (5'→3') forward / reverse
BSR_qPCR	CGGCTACAATCAACAGCATC / ACGATACAAGTCAGGTTGCC
VSG9_qPCR	ACTAAGCTCGTGGCGCAC / CGCGTAGTTGACGCATGAC
Luciferase_qPCR	ATGTCCGTTCGGTTGGCAG / CATACTGTTGAGCAATTCACG
VSG2_qPCR	AGCAGCCAAGAGGTAACAGC / CAACTGCAGCTTGCAAGGAA
VSG13_qPCR	ATAACGCATGGCCATCTTGAC / GTCGTTGCTGTGGATTGCTC
VSG6_qPCR	GCTATGAGACAGAAACTGAT / GGCTGCAACCCTGAGTGGTGC
18S_qPCR	ACGGAATGGCACCACAAGAC / GTCCGTTGACGGAATCAACC
mVSG639_qPCR	TCGCACTTTCAGCTCTGCTC / GCCGACCACTCGCTGTCC
GPEET2_qPCR	ACGGGACCAGAGGAAACTG / TAGAATGCGGCAACGAGAG
β-tubulin_qPCR	TTCAGGCTGGCCAATGCG / TACGGAGTCCATTGTACCTG
GAPDH_qPCR	AGATTGATGTCGTTGCTGTTGTG / ATGGCTTGCTCTTCGTAGTCG
Ampicillin_FAIRE DNA spike_qPCR	ATCGTTGGGAACCGGAGC / AGCGCAGAAGTGGTCCTG

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