Interspecies quorum sensing in co-infections can manipulate trypanosome transmission potential

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Quorum sensing (QS) is commonly used in microbial communities and some unicellular parasites to coordinate group behaviours. An example is Trypanosoma brucei, which causes human African trypanosomiasis, as well as the livestock disease, nagana. Trypanosomes are spread by tsetse flies, their transmission being enabled by cell-cycle arrested ‘stumpy forms’ that are generated in a density-dependent manner in mammalian blood. QS is mediated through a small (<500 Da), non-proteinaceous, stable but unidentified ‘stumpy induction factor’, whose signal response pathway has been identified. Although QS is characterized in T. brucei, co-infections with other trypanosome species (Trypanosoma congolense and Trypanosoma vivax) are common in animals, generating the potential for interspecies interactions. Here, we show that T. congolense exhibits density-dependent growth control in vivo and conserves QS regulatory genes, of which one can complement a T. brucei QS signal-blind mutant to restore stumpy formation. Thereafter, we demonstrate that T. congolense-conditioned culture medium promotes T. brucei stumpy formation in vitro, which is dependent on the integrity of the QS signalling pathway. Finally, we show that, in vivo, co-infection with T. congolense accelerates differentiation to stumpy forms in T. brucei, which is also QS dependent. These cross-species interactions have important implications for trypanosome virulence, transmission, competition and evolution in the field.

Trypanosoma brucei, Trypanosoma congolense and Trypanosoma vivax are African trypanosome species that can infect game animals and livestock with a co-infection frequency of up to 25% where analysed. All three species are spread by tsetse flies but undergo distinctive developmental pathways within the arthropod vector. Furthermore, only T. brucei is reported to undergo developmental transformation in the bloodstream of mammalian hosts in preparation for transmission, generating ‘stumpy forms’ that are G1 arrested and morphologically distinct from ‘slender forms’ that proliferate to establish each wave of parasitaemia. Other trypanosome species are described as monomorphic, or have ill-defined morphological configurations—a cytological indicator of cell-cycle position. Analysis of the first 14 days of infection showed that parasite number had a significant negative effect on the proportion of proliferating (2K1N, 2K2N) cells (general linear model, P = 0.001; Fig. 1a) such that parasites accumulated with a 1K1N configuration in individual infections when their numbers exceeded approximately 8 × 10⁷ ml⁻¹ (Supplementary Fig. 1). Although this was not associated with an accompanying morphological transition equivalent to T. brucei stumpy formation (Fig. 1b–d), Basic Local Alignment Search Tool (BLAST) and reciprocal BLAST analysis of the genome of T. congolense identified potential orthologues of a characterized set of 25 T. brucei genes required for stumpy formation (Supplementary Table 1), which is similar to the number in the T. vivax genome. Hence, T. congolense exhibited density-dependent cell-cycle arrest in vivo and encodes predicted orthologues of components of the T. brucei stumpy formation pathway.

To explore conservation of the signalling pathway responsible for stumpy formation between T. congolense and T. brucei, functional complementarity was examined (Fig. 2). Null mutants for Tb927.9.4080 (‘TbHYP2; previously identified as a component of the T. brucei quorum sensing (QS) response pathway’; Supplementary Table 2 and Supplementary Fig. 2) were initially generated in pleomorphic T. brucei EATRO 1125 by sequential allelic replacement (T. brucei AnTat1.1 90:13 ΔTbHYP2; Supplementary Fig. 3). As expected for a QS signalling pathway component, TbHYP2 null mutants lost the capacity for growth control in vivo (Supplementary Fig. 4). They also did not express the PAD1 marker for stumpy forms (Supplementary Fig. 4) and, when harvested and exposed to the developmental trigger cis-aconitate, they differentiated to the next life-cycle stage (procyclic forms) less efficiently than wild-type parasites, as assessed by expression of the procyclic surface protein EP procyclin and proliferation (Supplementary Fig. 4). Thereafter, the TbHYP2 null mutants were engineered (Fig. 2a) for doxycycline-inducible ectopic expression of the T. congolense orthologue of TbHYP2 (Tcl3000.0.19510, ‘TcHYP2’; E value: 5.9e-262, 45% identity, 58% similarity to TbHYP2; Supplementary Table 1). Figure 2b shows that upon inducible expression of TcHYP2, there was slowed progression of parasitaemia in vivo, which was potentially linked to either premature development to stumpy forms caused by overexpression of the QS signal pathway orthologue or a dominant negative consequence of TcHYP2 expression. Supporting the former, those parasites induced to express TcHYP2 exhibited PAD1 expression, while uninduced parasites remained PAD1 negative (Fig. 2c). Furthermore, when exposed to cis-aconitate the induced parasites expressed EP procyclin more effectively than the uninduced lines after 4 h and 24 h (Fig. 2d; P < 0.0001 and P = 0.0082, respectively). Combined, these assays showed that TcHYP2 can restore stumpy formation in a T. brucei TbHYP2 null mutant, providing evidence for functional complementarity between the genes.

Next, to explore interspecies cross talk in QS signals, the capacity for T. congolense to release a signal capable of inducing stumpy formation in T. brucei was tested. Initially, pleomorphic T. brucei (EATRO 1125 AnTat1.1 90:13; capable of stumpy formation),
monomorphic *T. brucei* (Lister 427 cells; incapable of stumpy formation) or culture-adapted *T. congolense* cells (IL3000) were incubated in the presence of 50 or 75% conditioned medium from *T. congolense* culture (TeCM; harvested from a *T. congolense* culture that had proliferated to $6 \times 10^8 \text{ml}^{-1}$ after three days without passage). These parasite species grow optimally in different
The presence of could still support active proliferation uninhibited by the accumulation of toxic metabolites. In contrast, culture media, but which was also capable of inducible RNA interference (RNAi) when exposed to TbHYP2 RNAi when exposed to TbHYP2 RNAi.

To explore whether the CAT-reporter response was mediated via the QS signalling pathway, the same analysis was carried out using a cell line containing the CAT–PAD 3′ UTR reporter construct, but which was also capable of inducible RNA interference (RNAi) of TbHYP2 (T. brucei AnTat1.1 90:13 CAT–PAD TbHYP2 RNAi). When exposed to TbCM (Fig. 3c) or TbCM (Fig. 3d) for 48 h and 72 h, CAT reporter expression was significantly reduced upon TbHYP2 RNAi compared with cells in which TbHYP2 RNAi was not induced (TbCM at 48 h, P = 0.0009; TbCM at 48 h, P < 0.0001).

Cell-cycle arrest in response to TbCM and TbCM was also reduced upon TbHYP2 RNAi (Supplementary Fig. 6). Confirming this was mediated through a QS response, a CAT reporter controlled by the 3′ UTR of the constitutively expressed T. brucei aldolase gene was not significantly affected by TbHYP2 RNAi when exposed to TbCM or a cell permeable mimic of the QS signal, 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (8-pCPT-cAMP; ref. 32; Supplementary Fig. 7). Hence, TbCM, as well as TbCM, can promote...
growth arrest and activation of stumpy reporter gene expression in pleomorphic *T. brucei* in vitro, which is mediated through the QS signalling pathway. In contrast, culture-adapted *T. congolense* did not show growth inhibition in *TbCM* containing QS signal activity sufficient to arrest *T. brucei* (Supplementary Fig. 5d,e). It remains to be established whether *T. congolense* are not responsive to the *T. brucei* QS signal or if this is a consequence of the culture-adapted *T. congolense* line used in the in vitro experiments, which has been subject to long-term passage. Unlike *TbCM*, 8-pCPT-cAMP could inhibit *T. congolense* growth in vitro, supporting the conservation of a QS signalling pathway between the species, although the effect was less pronounced than with *T. brucei* (Supplementary Fig. 5f,g).

Having demonstrated the potential for cross-talk in QS signals between *T. congolense* and *T. brucei* in vitro, we examined whether the same response was detectable in co-infections between these species in vivo. To enable unambiguous identification of *T. brucei* in the
Fig. 4 | Pleomorphic T. brucei introduced into an established T. congolense infection differentiate prematurely to stumpy forms in an effect mediated by QS signalling. a, Pleomorphic T. brucei EATRO 1125 encoding Ty1 epitope-tagged PFRA (T. brucei AnTat1.1 90:13 PFR–Ty1) were used to identify T. brucei in mixed infections by flagellar staining (green). Note that T. congolense cells show non-specific intracellular staining with the BB2 antibody that detects the Ty1 epitope. A PAD1-positive T. brucei stumpy cell (arrowheads) is shown surrounded by T. congolense cells on day 8 of the co-infection experiment. Scale bars, 10 μm. b, The proportion of each parasite species was determined by scoring more than 2,000 cells in the co-infections as PFR–Ty1 positive or negative, and applied to the total parasitaemia to calculate the effective parasitaemia of each species in the co-infections on days 6, 7 and 8. The effective T. brucei parasitaemia in the co-infections (purple, n = 3) remained lower than in the control ‘T. brucei only’ infections (blue, n = 3). Green: T. congolense in the co-infections (n = 3). Red: ‘T. congolense only’ infections (n = 3). e, PAD1-positive T. brucei on day 8 of the experiment (>500 cells scored, n = 3 for each condition tested). Despite lower T. brucei parasitaemia in the superinfections, there was a higher percentage of PAD1-positive cells compared with the single species T. brucei infections (unpaired t-test, **P < 0.01, two-sided). The error bars represent the mean ± s.d. d, A cell line with a Ty1 epitope-tagged PFRA protein and the capacity for doxycycline (Dox)-inducible TbHYP2 RNAi was generated. Effective RNAi targeting TbHYP2 was confirmed by northern blot using RNA collected after 48 h of culture ± Dox. Ethidium-bromide-stained ribosomal RNA acts as a loading control. e, The proportion of parasites of each species was calculated as in b (n = 3 for each condition tested). The effective T. brucei parasitaemia was lower in superinfections than in ‘T. brucei only’ infections, whether or not TbHYP2 RNAi was induced. The effective T. brucei parasitaemia was higher in co-infections where TbHYP2 RNAi was induced (+Dox) than in those without induction (–Dox), although the difference was not significant. f, PAD1-positive T. brucei on day 8 of the experiment (>500 cells scored, n = 3 for each condition tested). There were significantly more PAD1-positive T. brucei cells in the co-infections in which TbHYP2 RNAi was not induced (30–45%) than the co-infections where TbHYP2 RNAi was induced (1–3%) (**P < 0.0001, one-way analysis of variance with Tukey’s multiple comparisons test). The error bars represent the mean ± s.d. NS, not significant.

mixed infection, we generated a T. brucei pleomorphic line encoding a Ty1 epitope tagged PFRA protein (T. brucei AnTat1.1 90:13 PFR–Ty1)31. This allowed flagellar staining to distinguish T. brucei from T. congolense, with simultaneous co-labelling with PAD1 antibody and morphological analysis permitting quantitation of stumpy formation (Fig. 4a). Infections were initiated with T. congolense
followed, on day 4 post-infection, by a super-infection with T. brucei (Supplementary Fig. 8a). Control infections involving either T. congolense or T. brucei alone were analysed in parallel. The contribution of each species to the overall parasite load was then determined by scoring cell number and PFR labelling (Fig. 4b). T. brucei PAD1 expression (Fig. 4c) and cell-cycle status (Supplementary Fig. 9) were also measured. In the presence of a co-infection with T. congolense, T. brucei generated more PAD1-positive cells at a lower overall density of T. brucei than in T. brucei infections alone (Fig. 4c). Thus, 50% of T. brucei cells were PAD1-positive in the co-infection compared with <10% in the mono-infection (P = 0.0044), despite the T. brucei parasites comprising only a small proportion of the total parasite load, which was similar in the single- and mixed-species infections (1 × 10⁶ cells/ml⁻¹; Supplementary Fig. 8). Furthermore, although comprising only a minority of the overall parasitaemia in the co-infection (Fig. 4b), the T. brucei parasites assumed a stumpy morphology and exhibited an accumulation of QS signalling pathway.

To confirm that T. brucei null mutant infections (IL3000 strain) were used for both the infections and the in vitro experiments. This strain was derived from the ILC-49 strain that was isolated from a cow in the Trans Mara, Kenya†. The T. congolense IL3000 parasites used for the in vitro experiments were provided by A. MacLeod (University of Glasgow) in a blood straw. The T. congolense IL3000 parasites used for the in vitro experiments were supplied as culture-adapted bloodstream forms by L. Morrison (Roslin Institute, Edinburgh), who had received them from T. Baltz (University of Bordeaux). Six female MF1 mice were inoculated intraperitoneally with T. congolense IL3000. Parasitaemia was monitored daily from day 3 post-infection. Parasitaemia was estimated from a wet blood smear using the Herbert and Lumsden rapid matching method. Air-dried blood smears were fixed in ice-cold methanol and stored at −20°C before cell-cycle analysis.

Methods

Animal experiments. Animals were allocated to treatment groups at random from a group of female, age-matched adult MF1 mice that were at least 10 weeks old. No blinding was performed.

Infections to test functional complementation. Six female cyclophosphamide-treated MF1 mice were inoculated intraperitoneally with the T. b. rhodesiense T. b. rhodesiense null mutant cell line. In parallel, two mice were inoculated with the parental AnTat1.1 90:13 cell line. Parasitaemia was monitored daily from day 3 post-infection. Parasitaemia was estimated from a wet blood smear using the Herbert and Lumsden rapid matching method. Infections were monitored until parasites differentiated to stumpy forms or were terminated if the uncontrolled ascending parasitaemia was predicted to cause the death of the mice within the next 12 h. At the end of the experiment, the parasites were purified from whole blood by passage through a DE52 column (Whatman anion exchange cellulose, Z742600) at pH 7.8.

Infections to test functional complementation. Six female cyclophosphamide-treated MF1 mice were inoculated intraperitoneally with the T. b. rhodesiense T. b. rhodesiense overexpression T. b. rhodesiense null mutant cell line. One group (n = 3) was provided with doxycycline (200 μg/ml⁻¹ in 5% sucrose) in their drinking water from the time of inoculation to induce the T. b. rhodesiense overexpression. The other group (n = 3) received 5% sucrose only. In parallel, one mouse was infected with AnTat1.1 90:13 and one mouse was infected with the T. b. rhodesiense null mutant. Parasitaemia was monitored daily from day 3 post-infection. Parasitaemia was estimated from a wet blood smear using the Herbert and Lumsden rapid matching method. Infections were monitored until parasites differentiated to stumpy forms or were terminated if the uncontrolled ascending parasitaemia was predicted to become lethal within the next 12 h. At the end of the experiment, the parasites were purified from whole blood by passage through a DE52 column (Whatman anion exchange cellulose, Z742600) at pH 7.8.

Cell-cycle analysis. Methanol-fixed blood smears were rehydrated in phosphate-buffered saline (PBS) for 5 min. Slides were stained with 30 μl of 4′,6-diamidino-2-phenylindole (DAPI; 10 μg/ml⁻¹ in PBS) for 2 min in a humidity chamber and then washed for 5 min in PBS. Slides were then mounted with 40 μl Mowiol containing 2.5% 1,4-diazabicyclo(2.2.2)octane (DAABCO). N and K configurations were recorded by manual cell counting. For each sample and time point, 500 cells were counted, except when there was very low parasitaemia, in which case 200 cells were counted.

Generation of a T. b. rhodesiense null mutant with inducible T. b. rhodesiense overexpression. pENt6B-Y and pENt6P-Y vectors were used to generate the T. b. rhodesiense null mutant by sequential allelic replacement. Primers were designed to amplify regions of the
Southern blotting. A gene probe was produced to detect the presence of the \( \text{TbHYP2} \) gene and a 5'-UTR probe. The band was then visualized by autoradiography.

Western blotting. Protein samples were isolated on sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and blotted onto nitrocellulose membrane. Primary antibody dilutions were prepared in 5% milk and the membranes were incubated at room temperature. The membranes were washed twice with 50 mm Tris and 1.5 mm NaCl (pH 7.4) before the DNA was transferred to a nylon membrane overnight. The membranes were then ultraviolet cross-linked. Hybridization was carried out overnight at 40 °C with the DIG-labelled DNA probe in DIG Easy Hyb Buffer (Roche; 1106358001). The membrane was washed twice with 5 mm 2x saline sodium citrate and 0.1% sodium dodecyl sulfate at room temperature, and then twice for 15 min with 0.5x saline sodium citrate and 1% sodium dodecyl sulfate at 68 °C. The membrane was blocked with 1x Maleic acid buffer containing 1% DIG, before the addition of 2μL anti-DIG (Roche; 11093274910) and incubation for 30 min. Final detection used the chemiluminescent substrate CDP-Star (Roche; 11685627001) diluted 1:100 in detection buffer (100 mm Tris HCl and 100 mm NaCl pH 9.5).

Flow cytometry. Between 2x10^6 and 5x10^6 cells were washed twice in PBS before fixing in 500μl of 2% formaldehyde and 0.05% glutaraldehyde for more than 1 h at 4 °C. The cells were then washed three times in PBS and resuspended in 2% BSA in PBS for 30 min. Cells were then resuspended in primary antibody diluted in 2% BSA in PBS (aDAP1 was diluted 1:200 and eDAP1 procyclin (Cedar Lane Laboratories) was diluted 1:500) and incubated overnight at 4 °C. The cells were then fixed twice in PBS and resuspended in secondary antibody diluted in 2% BSA in PBS (a rabbit CY5 and a mouse fluorescein isothiocyanate were each diluted 1:1000). The cells were washed twice in PBS and resuspended in 500μl PBS containing 0.02 μg/ml DAPI. Samples were then processed on an LSR II flow cytometer (BD Biosciences). Positive controls and secondary antibody-only controls were included. Analyses were performed using FlowJo software version 10.1 (https://www.flowjo.com).

Conditioned medium generation. For the generation of conditioned medium, cultures of \( T. brucei \) AnTat1.1 90:13 were established at 1x10^5 cells ml^{-1} in HMI-9. Cells were propagated and harvested from 10^6 to 1x10^7 cells ml^{-1} in TSBFS3 [1]. and incubated for three days at 34 °C (and 5% CO₂). Conditioned medium was collected at a range of densities from 5x10^6 to 1x10^7 cells ml^{-1} and prepared as for TCM by filtering the conditioned supernatant through a 0.22μm filter. Conditioned medium was stored for a maximum of five days at 4 °C before use. Alongside flushes for conditioned medium generation, flasks containing either HMI-9 or TBSFS3 without parasites were prepared, and these control media were treated in the same way as the conditioned medium and used as negative controls in all the conditioned medium experiments.

CAT reporter experiments. A plasmidic cell line with a CAT reporter under the control of the \( \text{PADI} \) 3' UTR was used to report on stumpy formation. Additionally, a plasmidic cell line with inducible \( \text{TbHYP2} \) RNAi (ref. 1) was transfected with the \( \text{PADI} \) 3' UTR construct or a control construct with CAT reporter expression controlled by the 3' UTR of the constitutively expressed \( \alpha \)-tubulin gene.

The CAT reporter \( T. brucei \) cells were washed once with HMI-9 and resuspended at a density of 2x10^6 cells ml^{-1} in a mixture of 75% conditioned medium or control medium and 25% HMI-9. Cultures were incubated for three days at 37 °C (and 5% CO₂) without passage. Each day, the cell number was estimated using a Beckman Coulter Z2 Coulter Particle count and size analyser (or haemocytometer if there were a number of dead cells) and checked for enzymatically linked immunosorbent assay (ELISA) samples were collected. For experiments involving \( \text{TbHYP2} \) RNAi, induction of the RNAi with doxycycline was initiated on day one before the addition of conditioned medium and maintained throughout the experiment. CAT ELISA samples were prepared by collecting 5ml of culture, washing the cells three times with PBS and resuspending them in 1mL \( \text{TbHYP2} \) lysate buffer (Roche) for 25 min at room temperature. The lysis reaction was centrifuged to pellet debris and the supernatant was snap frozen in liquid nitrogen and stored at −80 °C.

Samples were analysed by CAT ELISA (Roche; 11636727001) to determine their CAT concentration according to the manufacturer's instructions. Each sample was loaded into two wells of a 96 well plate. The CAT concentration of the samples was estimated by comparing the absorbance at 405 nm with that of a CAT standard curve (provided by the manufacturer). The standard curve included a range of CAT concentrations of 0.0625–2ng ml⁻¹, as well as a blank of 0 ng ml⁻¹. Absorbance was measured using a BioTek ELx808 Absorbance Microplate reader with Gen5 data analysis software (BioTek). Reads of duplicate wells at 405 nm were averaged and converted to CAT concentrations using the standard curve. The CAT concentration per cell was calculated using the number of cells in each 5ml sample collected during the experiment.

Superinfection experiment. Six female MF1 mice were inoculated intraperitoneally with \( T. congolense \) IL3000 on day 0; each mouse received approximately 2.4x10^6 cells. On day 4, three \( T. congolense \)-infected and three previously uninfected mice were inoculated intraperitoneally with \( T. brucei \) AnTat1.1 90:13 with a TY- tagged PFR; each mouse received approximately 1.2x10^6 cells. Stocks used for infection were mixed before division between the mice to ensure that single species infections and co-infections were initiated with the same \( T. brucei \) inoculum.

Parasitaemia was monitored daily from day 2 post-infection. The total parasitaemia was estimated from a wet blood smear using the Herbert and Lumsden rapid matching method. Blood (10μl) was collected on each day of infection and the cells were washed in 200μl cold phosphate-buffered saline (PBS) and resuspended in 125μl cold PBS before 125μl 8% parafomaldehyde in PBS was added. Cells were fixed on ice for 10 min and then resuspended in 130μl 0.1 M glycine in PBS and kept at 4 °C overnight. The samples were then resuspended in PBS and used for immunofluorescence. The proportion of the co-infection parasitaemia contributed by each species was estimated by counting the number of PFR-TY1-positive cells (>2,000 cells scored). At the end of the experiment, parasites were purified from whole blood by passage through a DE52 column (Whatman anion exchange column, Z742600) at pH 7.8. Purified parasites were fixed with parafomaldehyde for immunofluorescence and these samples were used to determine the proportion of \( T. brucei \) that were PAD1 positive in the infections (>500 cells scored), as well as the KN configuration of these cells.

Superinfection experiment with \( \text{TbHYP2} \) RNAi induction. Six female MF1 mice were inoculated intraperitoneally with \( T. congolense \) IL3000 on day 0; each mouse received approximately 2.4x10^6 cells. On day 4, all six \( T. congolense \)-infected and six previously uninfected mice were inoculated intraperitoneally with \( T. brucei \) AnTat1.1 90:13 with a TY- tagged PFR and infected with inducible RNAi targeting \( \text{TbHYP2} \); each mouse received approximately 1.2x10^6 cells. Stocks used for infection were mixed before division between the mice to ensure that all infections were initiated with the same \( T. brucei \) inoculum.

On day 1 of the experiment, doxycycline (200μg ml⁻¹ in 5% sucrose) was provided in the drinking water of three of the \( T. congolense \)-infected and three of the uninfected mice. The remaining mice received 5% sucrose only. Parasitaemia was monitored and samples were collected as for the initial superinfection experiment.
Immunofluorescence. Paraformaldehyde-fixed cells were adhered to polysine slides (VWR; 631-0107). 20 µl 0.1% triton in PBS was applied for each well for 2 min. This was then aspirated and the wells were washed with a large drop of PBS. The wells were blocked with 2% BSA in PBS for 45 min at 37°C in a humidity chamber before the application of 20 µl primary antibody. They were then incubated with primary antibody (diluted in 2% BSA in PBS, 1:100 dilution) and BB2 1:200 for 45 min at 37°C in a humidity chamber. Positive control wells and secondary antibody-only wells were included for each experiment. The wells were each washed five times by repeatedly applying and aspirating PBS. They were incubated with 20 µl secondary antibody (diluted in 2% BSA in PBS, α-rabbit Alexa flour 488 1:500 and α-mouse Alexa flour 568 1:500) for 45 min at 37°C in a humidity chamber. 20 µl of a DAPI working dilution (10 µg·ml⁻¹) was then applied to each well for 1 min, followed by five washes with PBS. Slides were mounted with a cover slip by the application of Mowiol containing 2.5% DABCO and analysed on a Zeiss Axioskop 2 plus or Zeiss Axio Imager Z2. QCapture Suite Plus Software (version 3.1.3.10, https://www.qimaging.com) was used for the image capture. Images of BB2, PAD1 and DAPI staining were overlaid in ImageJ 64 (ref. 39) and cell counts were performed using the Cell Counter plugin.

Bioinformatic analysis. The BLASTp tool on TritypDB (ref. 40) was used for the identification of orthologues.

Statistical analysis. Most statistical analyses were carried out in GraphPad Prism version 6 (GraphPad Software, www.graphpad.com). A general linear model approach was used to analyse T. congolense parasitaemias for cell-cycle arrest using Minitab17 (www.minitab.com/). This model tested the significance of the effect of parasitaemias for cell-cycle arrest using Minitab17 (www.minitab.com/). This model tested the significance of the effect of parasitaemias for cell-cycle arrest using Minitab17 (www.minitab.com/). This model tested the significance of the effect of parasitaemias for cell-cycle arrest using Minitab17 (www.minitab.com/). This model tested the significance of the effect of parasitaemias for cell-cycle arrest using Minitab17 (www.minitab.com/). This model tested the significance of the effect of parasitaemias for cell-cycle arrest using Minitab17 (www.minitab.com/).

Data availability. The data that support the findings of this study are available either within the manuscript or from the corresponding author on reasonable request.

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References
1. Brown, S. P. & Buckling, A. A social life for discerning microbes. Cell 135, 600–603 (2008).
2. Leggett, H. C., Brown, S. P. & Reece, S. E. War and peace: social interactions in infections. Philos. Trans. R. Soc. Lond. B Biol. Sci. 369, 20130365 (2014).
3. Vassella, E., Reuner, B., Yutzy, B. & Boshart, M. Differentiation of African trypanosomes is controlled by a density sensing mechanism which signals cell cycle arrest via the CAMP pathway. J. Cell Sci. 110, 2661–2671 (1997).
4. Auty, H. et al. Trypanosome diversity in wildlife species from the Serengeti and Luangwa Valley ecosystems. PLoS Negl. Trop. Dis. 6, e1832 (2012).
5. Cox, A. P. et al. Constraints to estimating the prevalence of trypanosome infections in East African zebu cattle. Parasit. Vectors. 3, 80 (2010).
6. Takeet, M. I. et al. Molecular survey of pathogenic trypanosomes in naturally infected Nigerian cattle. Res. Vet. Sci. 94, 555–561 (2013).
7. Pinchbeck, G. L. et al. Trypanosoma in the Gambia: prevalence in working horses and donkeys detected by whole genome amplification and PCR, and evidence for interactions between trypanosome species. BMC Vet. Res. 4, 7 (2008).
8. Rotureau, B. & Van Den Abbeele, J. Through the dark continent: African trypanosome development in the tsetse fly. Front. Cell. Infect. Microbiol. 3, 53 (2013).
9. Vickers, K. Morphology and mitochondrial activity in sleeping sickness trypanosomes. Nature 208, 762–766 (1965).
10. Vickers, K. The fine structure of Trypanosoma congolense in its bloodstream phase. J. Protozool. 16, 54–69 (1969).
11. Gardiner, P. R. & Wilson, A. J. Trypanosoma (Duttoniella) vivax. Parasitol. Today 3, 49–55 (1987).
12. Mony, B. M. et al. Genome-wide dissection of the quorum sensing signalling pathway in Trypanosoma brucei. Nature 505, 681–685 (2014).
13. Dean, S. D., Marchetti, R., Kirk, K. C. & Matthews, K. A surface transporter family conveys the trypanosome differentiation signal. Nature 459, 213–217 (2009).
14. Coustou, V., Guegan, F., Plazolles, N. & Baltz, T. Complete in vitro life cycle of Trypanosoma congolense: development of genetic tools. PLoS Negl. Trop. Dis. 4, e618 (2010).
15. Hirumi, H. & Hirumi, K. Continuous cultivation of Trypanosoma brucei blood stream forms in a medium containing a low concentration of serum protein without feeder cell layers. J. Parasitol. 75, 985–989 (1989).
16. MacGregor, P. & Matthews, K. R. Identification of the regulatory elements controlling the transmission stage-specific gene expression of PADI in Trypanosoma brucei. Nucleic Acids Res. 40, 7705–7717 (2012).
17. Laxman, S., Riechers, A., Sadilek, M., Schwede, F. & Beavo, J. A. Hydrolysis products of CAMP analogs cause transformation of Trypanosoma brucei from slender to stumpy-like forms. Proc. Natl Acad. Sci. USA 103, 19194–19199 (2006).
18. Bastin, P., Bagherzadeh, Z., Matthews, K. R. & Gull, K. A novel epitope tag system to study protein targeting and organellar biogenesis in Trypanosoma brucei. Mol. Biochem. Parasitol. 72, 235–239 (1996).
19. Esparwapa, S. M., Estrela, S. & Brown, S. P. Within-host dynamics of multi-species infections: facilitation, competition and virulence. PLoS ONE 7, e38730 (2012).
20. Balner, O., Sears, S. C., Schotzau, A. & Brun, R. Intraspecific competition between co-infecting parasite strains enhances host survival in African trypanosomes. Ecology 90, 3367–3378 (2009).
21. Bruce, M. C. et al. Cross-species interactions between malaria parasites in humans. Science 287, 845–848 (2000).
22. Portugal, S. et al. Host-mediated regulation of superinfection in malaria. Nat. Med. 17, 732–737 (2011).
23. MacGregor, P., Szoor, B., Savill, N. J. & Matthews, K. R. Trypanosomal immune evasion, chronicity and transmission: an elegant balancing act. Nat. Rev. Microbiol. 10, 431–438 (2012).
24. Duggle, S. P., Griffin, A. S., Campbell, G. S. & West, S. A. Cooperation and conflict in quorum-sensing bacterial populations. Nature 450, 411–414 (2007).
25. Feyre, E. M., Wissmann, B. V., Welburn, S. C. & Lutumba, P. The burden of human African trypanosomiasis. PLoS Negl. Trop. Dis. 2, e333 (2008).
26. MacLean, L. et al. Severity of human African trypanosomiasis in East Africa is associated with geographic location, parasite genotype, and host inflammatory cytokine response profile. Infect. Immun. 72, 7040–7044 (2004).
27. MacLean, L. M., Odiit, M., Chisi, J. E., Kennedy, P. G. & Sternberg, J. M. Focus-specific clinical profiles in human African trypanosomiasis caused by Trypanosoma brucei rhodesiense. PLoS Negl. Trop. Dis. 4, e906 (2010).
28. Wellde, B. et al. Trypanosoma congolense. I. Clinical observations of experimentally infected cattle. Exp. Parasitol. 36, 6–19 (1974).
29. Herbert, W. J. & Lumsden, W. H. Trypanosoma brucei: a rapid “matching” method for estimating the host’s parasitism. Exp. Parasitol. 40, 427–431 (1976).
30. Kelly, S. et al. Functional genomics in Trypanosoma brucei: a collection of vectors for the expression of tagged proteins from endogenous and ectopic gene loci. Mol. Biochem. Parasitol. 154, 103–109 (2007).
31. Raskind, W. S. ImageJ (NIH, Bethesda, Maryland, 2015), http://imagej.nih.gov/ij/.
32. Adleit, M. et al. TriTrypDB: a functional genomic resource for the Trypanosomatidae. Nucleic Acids Res. 38, D457–D462 (2010).

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**Author contributions**

K.R.M. conceived and supervised the study. E.S. and K.R.M. devised the experiments. E.S. and J.Y. planned and carried out the experiments. E.S., K.R.M. and A.I. collated, analysed and interpreted the data. K.R.M. and E.S. wrote the manuscript.

**Competing interests**

The authors declare no competing financial interests.

**Additional information**

Supplementary information is available for this paper at doi:10.1038/s41564-017-0014-5. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to K.R.M.

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Experimental design

1. Sample size

Describe how sample size was determined.

For the analysis of phenotypes 3-5 animals per treatment were routinely used for analysis. Our previous analyses (e.g. Mony, B.M., et al., Genome-wide dissection of the quorum sensing signalling pathway in Trypanosoma brucei. Nature, 2014. 505(7485): p. 681-5) indicate that this sample size is sufficient to detect differences between cell lines and treatment groups (for example where gene silencing is activated by provision of doxycycline). Using that data as an exemplar, we tested 5 genes for effects with and without doxycycline mediated gene-silencing in vivo. Using cell cycle status as the measured parameter, the effect size ranged from 0.637 to 1.804. Those values were then used to calculate the power for different sample sizes. This showed that a sample size of 3-5 per group (+ or - DOX), or total of 6 to 10 allowed us to achieve 80% power for all test genes except one.

In the current manuscript, the visual analytical assays applied (manual scoring by microscope) to the different treatments and groups (cell cycle scoring, analysis of PAD1 staining, scoring of flagellar labelling for parasite species, morphological analysis) required analyses to be limited to 3 animals per group. Data were examined before analysis to ensure normality and that no transformations were required. P values of less than 0.05 were considered statistically significant.

2. Data exclusions

Describe any data exclusions.

No data were excluded from the analyses.

3. Replication

Describe whether the experimental findings were reliably reproduced.

All figures include information on the replicate number for each experiment. Experiments were also validated in additional pilot experiments or independent replicates.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

Animals were allocated at random into treatment groups from a group of female, age matched MF1 mice.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

No blinding was done.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or the Methods section if additional space is needed).

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.).
- A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly.
- A statement indicating how many times each experiment was replicated.
- The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section).
- A description of any assumptions or corrections, such as an adjustment for multiple comparisons.
- The test results (e.g. p values) given as exact values whenever possible and with confidence intervals noted.
- A summary of the descriptive statistics, including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range).
- Clearly defined error bars.

See the web collection on statistics for biologists for further resources and guidance.

7. Software

Describe the software used to analyze the data in this study. Most statistical analyses were carried out in GraphPad Prism version 6 (GraphPad Software, La Jolla, California, USA, www.graphpad.com). A General Linear Model was used to analyse T. congolense parasitaemias for cell cycle arrest using Minitab®. This model tested the significance of the effect of parasitaemia on % 2K1N, 2K2N cells and incorporated mouse as a random factor, which was not significant (p=0.55).

For all studies, we encourage code deposition in a community repository (e.g. GitHub). Authors must make computer code available to editors and reviewers upon request. The Nature Methods guidance for providing algorithms and software for publication may be useful for any submission.

Materials and reagents

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

The materials and datasets generated during and/or analysed during the current study are available either within the manuscript or from the corresponding author on reasonable request.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

2.5x10^6 cells were washed twice in PBS prior to fixing in 500μl 2% formaldehyde/0.05% glutaraldehyde >1h at 4°C. Cells were then washed 3x in PBS and resuspended in 2%BSA:PBS for 30 min. Cells were then resuspended in primary antibody diluted in 2%BSA:PBS (αPAD1 was diluated 1:200, αEP procyclin (Cedar Lane laboratories) was diluated 1:500) and were incubated overnight at 4°C. The cells were washed twice in PBS and were resuspended in secondary antibody diluted in 2%BSA:PBS (α-rabbit CY5 and α-mouse FITC were each diluated 1:1000). The cells were washed twice in PBS and were resuspended in 500μl PBS containing 0.02μg/ml DAPI. Samples were then processed on an LSRII flow cytometer (BD Biosciences). Positive controls and secondary antibody only controls were included. Analysis was performed using FlowJo software (Tree Star).
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used. T. congolense parasites of the IL3000 strain were used both for infections and in vitro experiments. This strain was derived from the ILC-49 strain that was isolated from a cow in the Trans Mara, Kenya. The T. congolense IL3000 parasites used for in vivo experiments were provided by Dr Annette MacLeod (University of Glasgow) in a blood straw. The T. congolense IL3000 parasites used for in vitro experiments were supplied as culture-adapted bloodstream forms by Dr Liam Morrison (Roslin Institute, Edinburgh), who had received them from Professor Théo Baltz (University of Bordeaux).
   b. Describe the method of cell line authentication used. Morphological distinction from T brucei in vitro and in vivo. In vivo competition experiments used labelled PFR staining to unambiguously distinguish T brucei form T. congolense.
   c. Report whether the cell lines were tested for mycoplasma contamination. No
   d. If any of the cell lines used in the paper are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use. No

Animals and human research participants

Policy information about studies involving animals: when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals
    Provide details on animals and/or animal-derived materials used in the study.
    Female, age matched MF1 mice were used

Policy information about studies involving human research participants

12. Description of human research participants
    Describe the covariate-relevant population characteristics of the human research participants.
    Not applicable