Supplemental information

The discarded flagellum of *Trypanosoma cruzi* provides very early targets for protective $CD8^+$ T cells

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Supplemental figures and legends

Figure S1

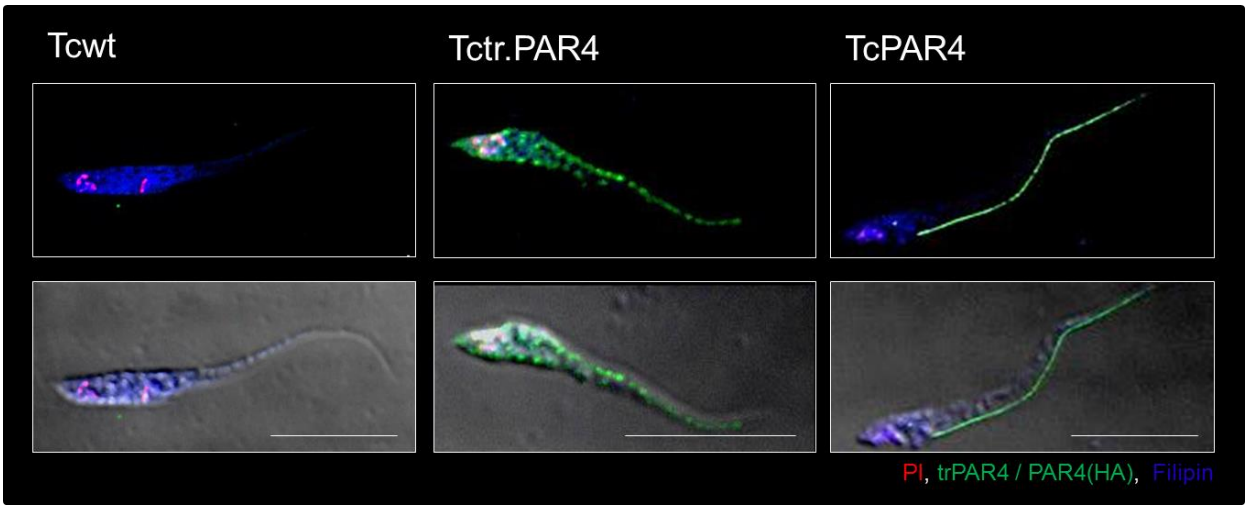


Figure S1 related to Figure 1: Deleting flagellar localization signal retains PAR4 in *T. cruzi* cytoplasm.

Fluorescence microscopy (top panel) and DIC overlay (bottom panel) showing localization of transgenically expressed tr.PAR4 or PAR4 in the epimastigote stage of Tctr.PAR4 or TcPAR4 strains respectively. Tcwt served as the control. PAR4/tr.PAR4, DNA or plasma membrane marked by anti-HA, PI and filipin respectively. Scale bar = 10µ

Figure S2

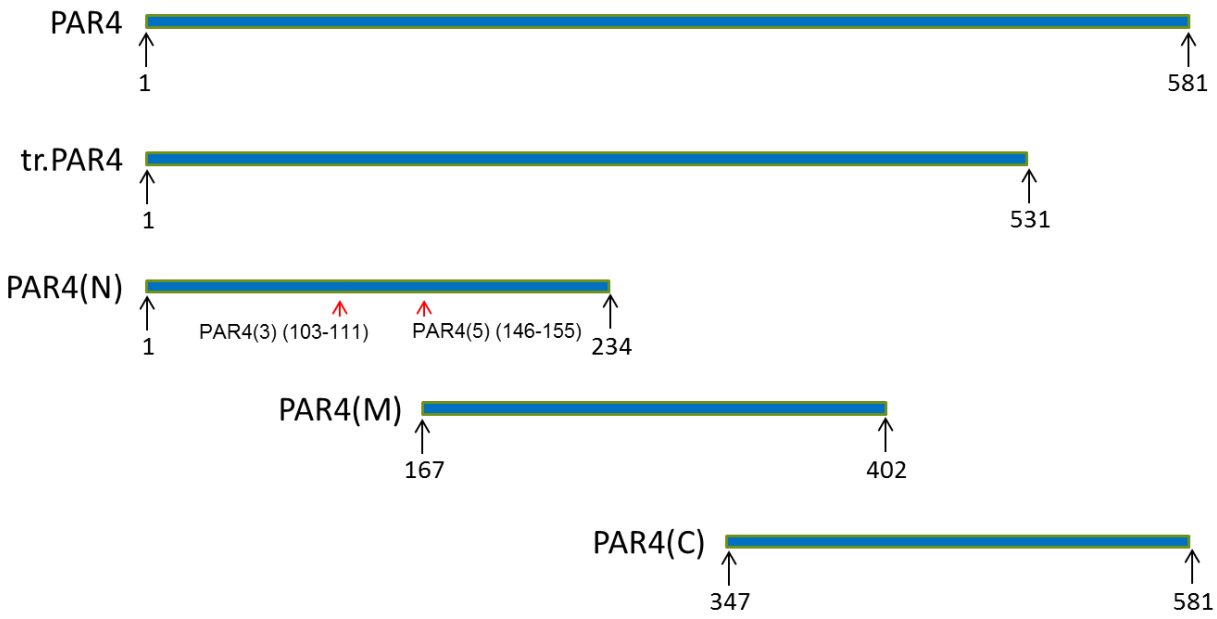


Figure S2 related to Figure 1: Schematic representation of the relative sizes and positions of transgenically expressed proteins.

Relative lengths and positions of the full or truncated gene products expressed in transgenic *T. cruzi*, bacteria or mouse fibroblast cells. The numbers beneath the arrows indicate amino acid/ approximate positions of $CD8^+$ T cell epitopes.

Figure S3

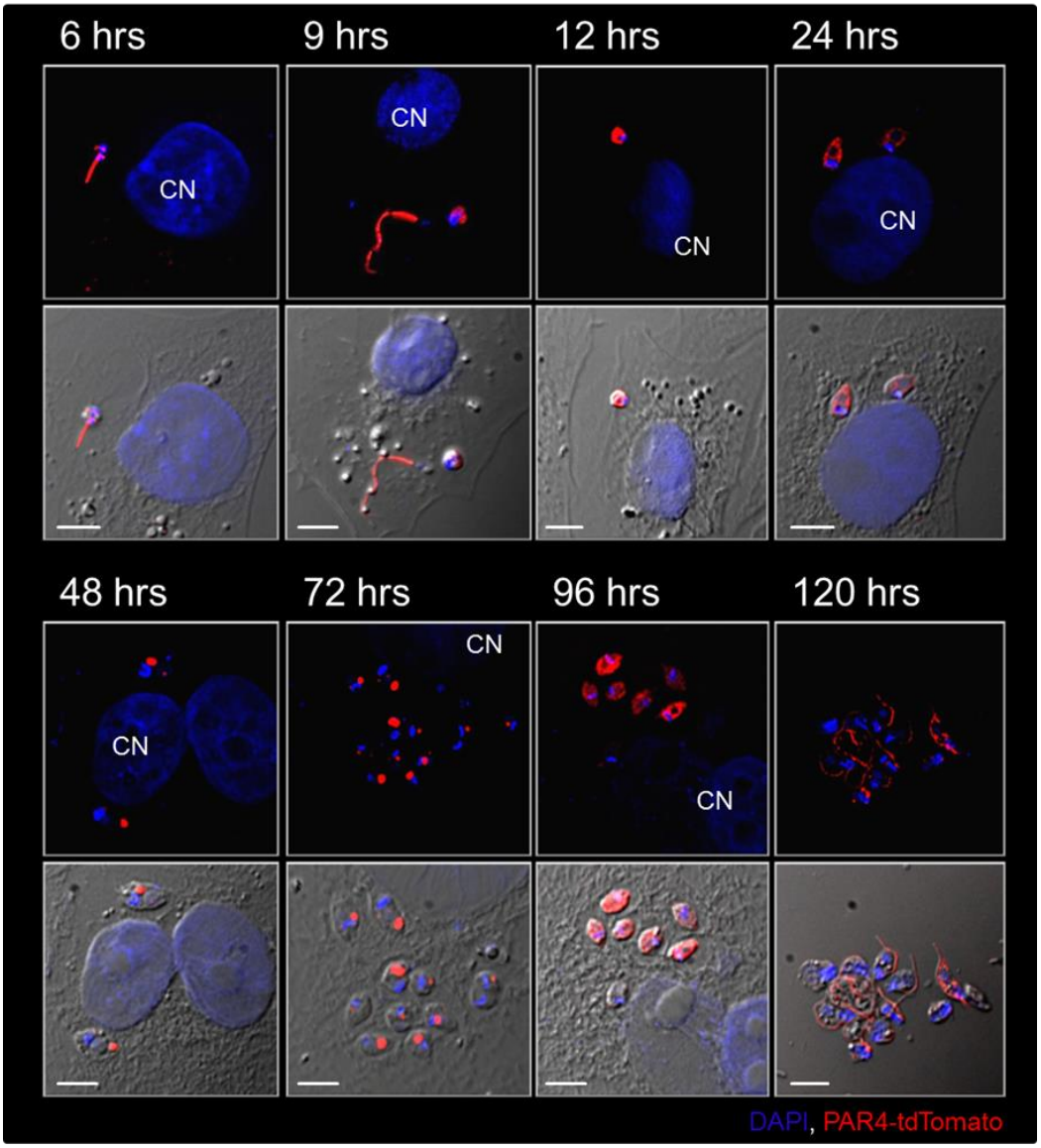
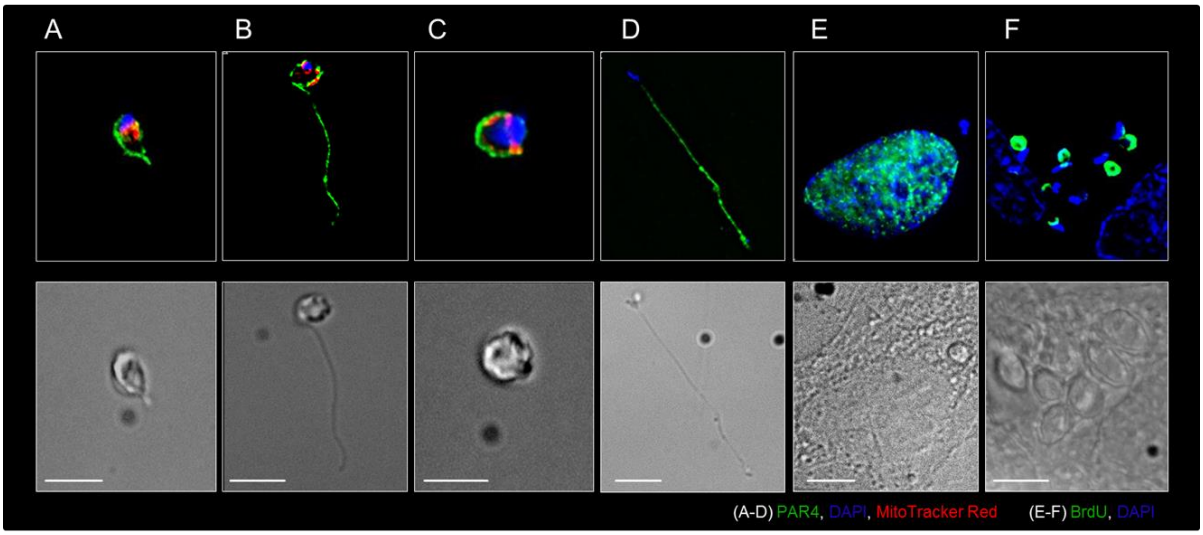


Figure S3 related to Figure 2: Localization of PAR4 in intracellular life cycle stages of TcPAR4

Representative fluorescence microscopy (top panel) and DIC overlay (bottom panel) indicating the localization of PAR4 in TcPAR4 (tdTomato) through the indicated time points, post-infection of vero cells. CN indicates host cell nucleus. Scale bar= 10µ

Figure S4



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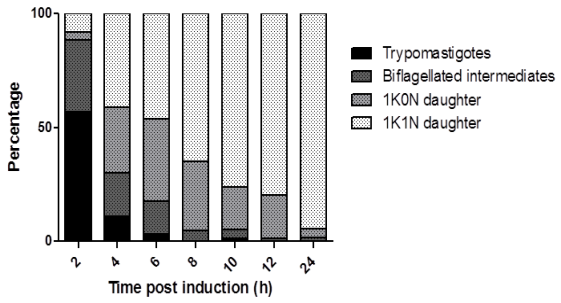

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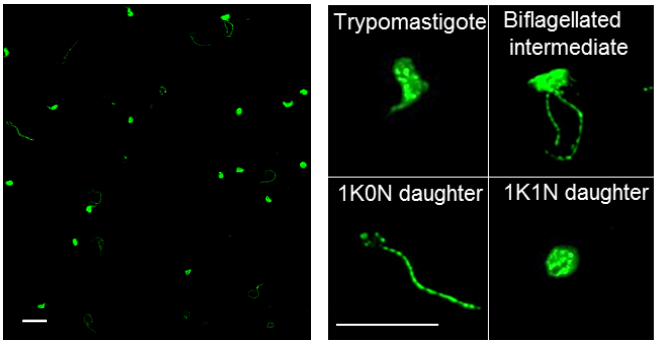

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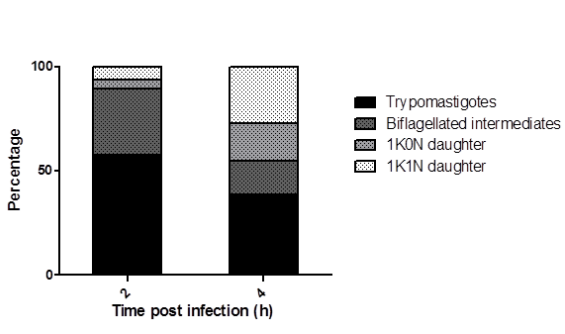

Figure S4 related to Figure 2: Asymmetric division in *T. cruzi* amastigogenesis produces anucleate daughter cells with functional mitochondria.

(A-D) Fluorescence microscopy (top panel) and DIC imaging (bottom panel) showing newly generated 1K1N (A) and 1K0N (B) daughter amastigotes (0-4 hrs), each with active mitochondria. Mitochondria are maintained long term (>16 hrs) in the 1K1N (C), but not 1K0N (D) daughter. (E-F) Absence (E) or presence (F) of BrdU incorporation in freshly formed (first generation, $\leq$12 hrs post infection) or later stage (further generations, $\geq$24 hrs) amastigotes, in host cells. (G) Relative percentages of trypomastigotes, biflagellated intermediates, 1K1N and 1K0N stages observed at various time points during axenic amastigogenesis (n $\geq$150 for each timepoint). (H) Mixture of daughter cell types 8 hrs post-induction of axenic conversion of TctdTom (left) and magnified image of each morphological type (right) (pseudocolored green for clarity). (I) Relative percentages of trypomastigotes, biflagellated intermediates, 1K1N and 1K0N stages observed during intracellular amastigogenesis at the initial time points post co-incubation (infection) of TctdTom and vero cells (n $\geq$50 for each timepoint). Right panel shows Intracellular parasites at various stages of amastigogenesis in human foreskin fibroblast cells infected with TctdTom *T. cruzi* (6 hrs of co-incubation). PAR4, DNA, DNA replication or functional mitochondria indicated by anti-PAR4 (green), DAPI (blue), BrdU (green) incorporation in DNA or MitoTracker red localization, respectively. Scale bar = 10µ.

Figure S5

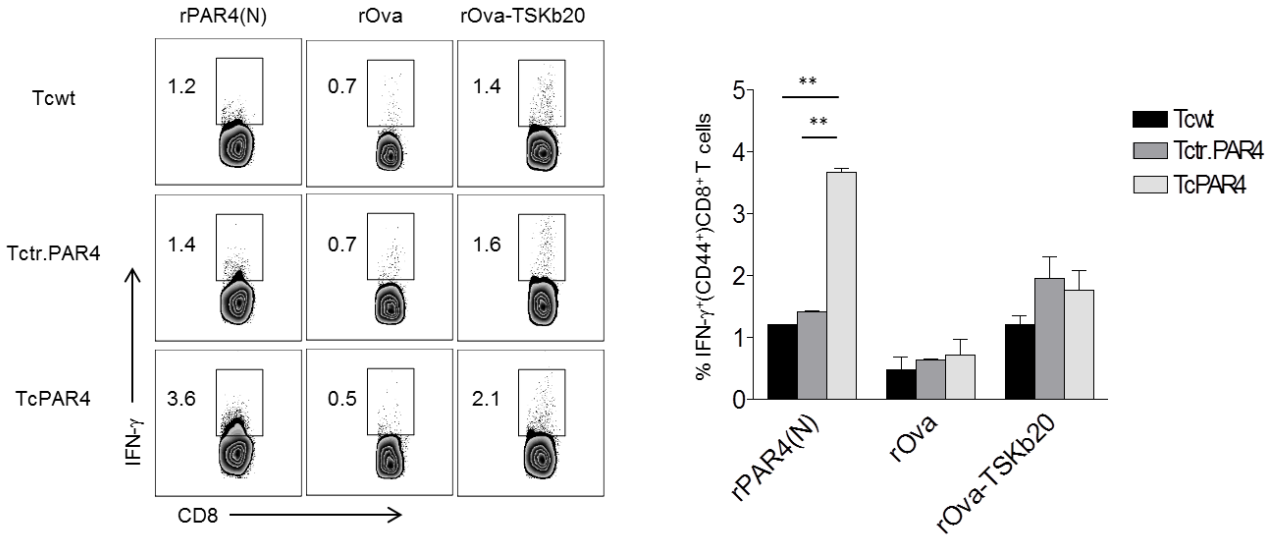

Figure S5 related to Figure 4: PAR4 localization to *T. cruzi* flagellum is critical to enhanced PAR4-specific response in TcPAR4 infection

Intracellular IFN-γ staining of splenocytes from Tcwt, Tctr.PAR4 or TcPAR4 infected (180 dpi) mice, restimulated with rPAR4(N), rOva or rOva-TSKb20 for 16hrs. Histograms are gated on $CD8^+$ $CD44^+$ lymphocytes, with the inset numbers indicating the percentage of IFN-γ producing $CD8^+$ T cells. The bar graph presents mean $\pm$ s.e.m from one of three separate experiments with 3 mice/ group. ** indicates $p \leq 0.01$ by student t-test.

Figure S6

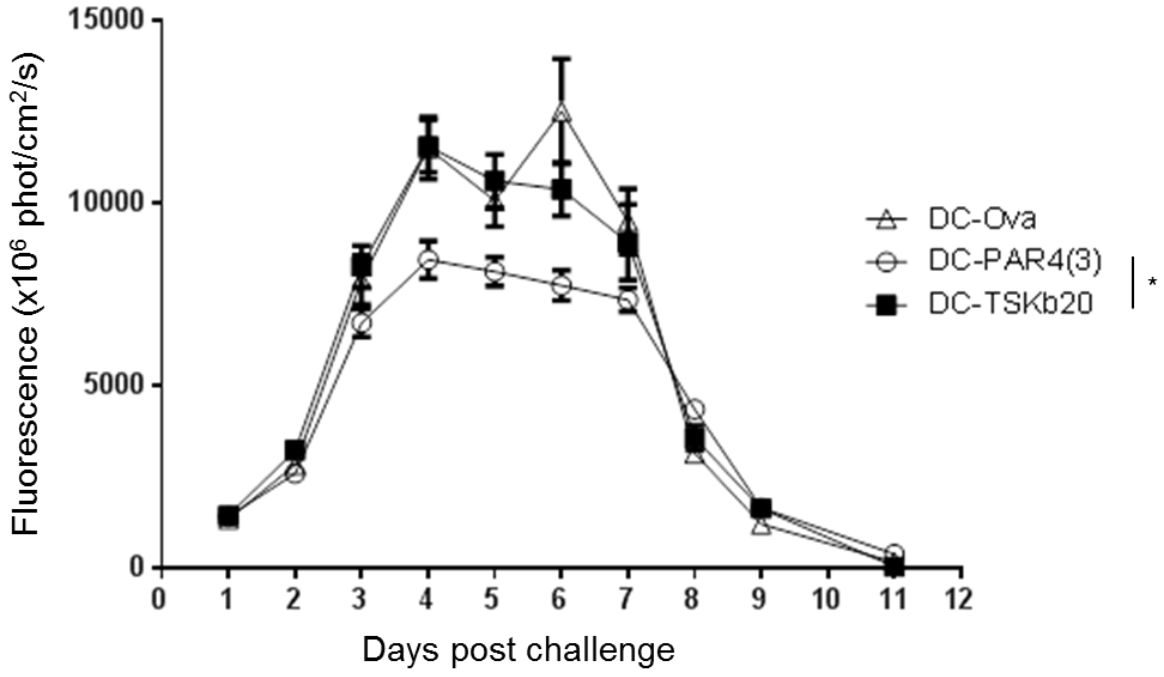

Figure S6 related to Figure 6: Better control of *T. cruzi* challenge on immunization against PAR4(3) compared to with TSKB20

Control of TctdTom challenge based on mean fluorescent signal in the feet (N=6) of mice vaccinated against PAR4(3) (DC-PAR4(3)), TSKb20 (DC-TSKb20) or SIINFEKL (DC-Ova) at the indicated time points. ** indicates $p \leq 0.01$ by student t-test.

Figure S7

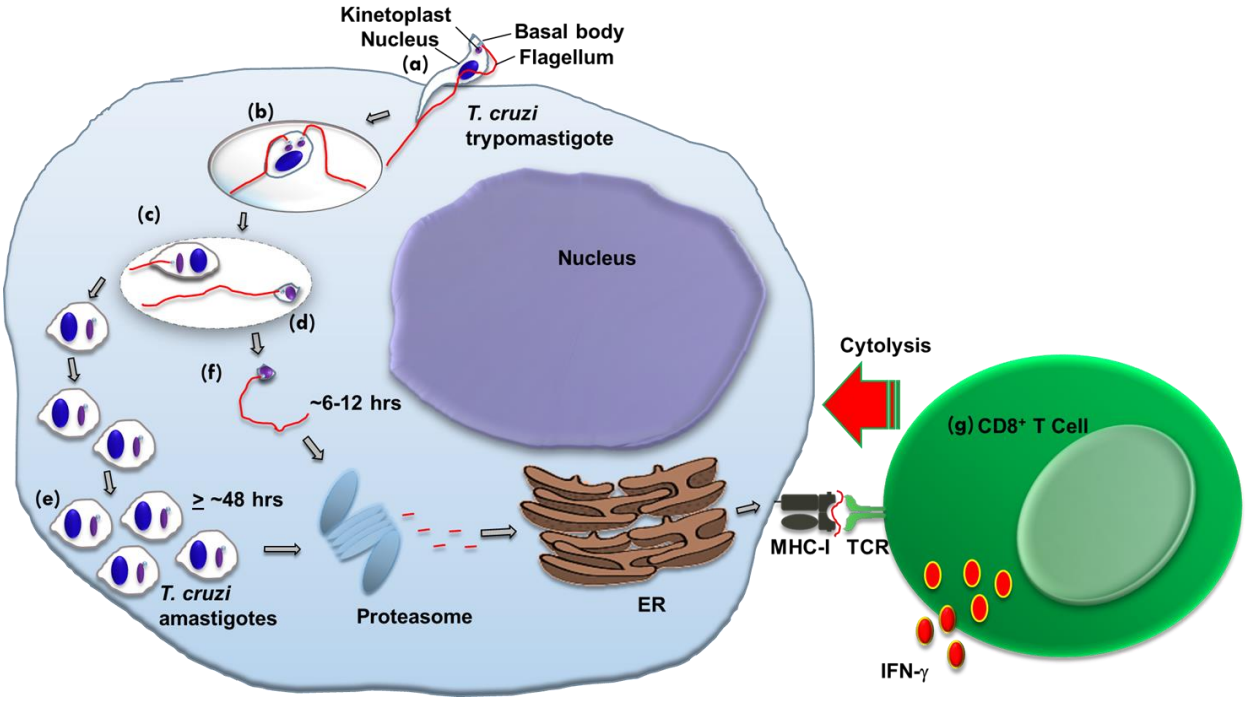

Figure S7 related to figure 7: Proposed model of amastigogenesis and antigen presentation in *T. cruzi* –infected cells.

Upon entry of the host cell, the *T. cruzi* trypomastigote (a) initiates a cell division by replicating the kinetoplast, basal body and flagellum to form an intermediate, biflagellated stage inside the lysosomal vacuole (b). Cytokinesis of the biflagellated stage results in a 1K1N daughter amastigote, (c) with a kinetoplast, basal body, flagellar stub and nucleus, and a 1K0N daughter cell (d) possessing a kinetoplast, basal body and an extensive flagellum but no nucleus. Following release from the vacuole, the 1K1N amastigote undergoes multiple rounds of replication (e), increasing the parasite burden that will ultimately occupy most of the host cell cytoplasm in 4-5 days (not shown). The 1K0N daughter rapidly undergoes degradation in host cell cytosol (f) and the constituent proteins are available for proteasomal degradation within ~6-12 hrs post-infection. Proteins from the replicating amastigotes, including trans-sialdiase proteins, also accumulate in the host cytosol and enter the class I MHC antigen presentation

pathway within 48 hrs post-infection. The proteasome degraded peptides are imported into the endoplasmic reticulum and selected peptides are presented with the class I MHC molecules on the host cell surface. Previously primed $CD8^+$ T cells with appropriate T cell receptors (g) will recognize the host cell as being infected and will respond with the production of cytokines or the release of granules resulting in cytolysis of the infected host cell. MHC-associated peptides that are presented earlier in the infection process (e.g. those from the flagellum) invite earlier detection and effector function of appropriately primed *T. cruzi*-specific T cells.

Supplemental methods

ELISPOT assay for IFN-gamma

T cells specific to rPAR4, rPAR4(N), PAR4(3), PAR4(5), TSKB20, SIINFEKL or rOVA were determined in TcPAR4 or Tcwt infected, or naïve control mice by ELISPOT assay as described previously (Eickhoff et al., 2011). In short, splenocytes from the mice (1×10^6 per well) were incubated for 18 hrs with the corresponding antigen at 1ug/ well for proteins or 5uM for the peptides, in nitrocellulose bottom ELISPOT plates (Millipore) coated with anti-mouse IFN-γ (BD Biosciences). After overnight stimulation and washing off the splenocytes, wells were incubated with biotinylated-anti-mouse IFN-γ (BD Biosciences), streptavidin-HRP (BD Biosciences) and AEC substrate (Sigma). Images of developed ELISPOT plates were captured using a CTL Analyzer and spots counted using Immunospot Software v3.2 (CTL). PMA/ionomycin (iono) combination served as control. Data are represented as the number of IFN-γ spot forming cells per million spleen cells.

Intracellular cytokine staining for IFN-γ

To determine intracellular IFN-γ production, $1.5x10^6$ spleen cells from TcPAR4, Tctr.PAR4 or Tcwt infected, or naïve mice were restimulated with recombinant proteins (at 1µg/ well): *T. cruzi* PAR4 (rPAR4), ovalbumin (rOVA), ovalbumin with its SIINFEKL epitope replaced by the immuno-dominant ANYKFTLV (Martin et al., 2006) from *T. cruzi* (rOVA-TSKb20), or peptides (at 5µM): PAR4 (3), PAR4(5), TSKb20 (5 µM) or SIINFEKL; or transgenic MC57G murine (C57BL/6) fibroblasts expressing: PAR4 protein's amino terminal (MC-PAR4(N)), middle portion (MC-PAR4(M)) or carboxy terminal (MC-PAR4(C)) (Figure S2), ovalbumin (MC-Ova) or ovalbumin with its SIINFEKL epitope replaced by the immune-dominant ANYKFTLV (Martin et al., 2006) from *T. cruzi* (MC-OVA-TSKb20), and processed for intracellular cytokine staining (ICS). The splenocytes were washed in PAB (2% BSA, 0.02% azide in PBS) and stained for surface expression of CD4, CD44 and CD8 using anti-CD4 PE, CD44 FITC and anti-CD8

eFluor450 (BD Pharmingen). All cells for ICS were fixed and permeabilized using Cytofix/Cytoperm (BD Pharmingen) on ice for 15 min and washed in PermWash (BD Pharmingen). The cells were then stained with anti- IFN-γ APC (BD Pharmingen) for 30 min on ice. Cells were washed and fixed in 2% formaldehyde for 20 min at 4°C, then washed and resuspended in PAB for flow cytometric analysis using a CyAn flow cytometer (Beckman Coulter) and analyzed with FlowJo software (Tree Star).

ELISA

To determine the relative concentrations of PAR4 in the trypomastigote stage from TcPAR or Tcwt, ELISA was performed as described before (Kurup and Tarleton, 2013). In short, polyclonal hyper-immune sera raised in Balb/c mice against recombinant *T. cruzi* PAR4(N) expressed in bacteria (primed and boosted 21 days later, to collect sera on day 28) was used to probe whole cell lysates (50µg/ well) from various *T. cruzi* strains. The reaction was developed using anti-mouse HRPase antibody (Jackson ImmunoResearch) conjugate and TMB microwell peroxidase substrate system (KPL) as per the manufacturer's protocols.

In vitro cytotoxicity assay

In vitro cytotoxicity of splenocyte effector cells derived from TcPAR4 or Tcwt infected mice on target MC-PAR4(N) or MC-Ova were assayed as described before (Cao et al., 2010). Briefly, effector cells were harvested, counted, washed, and resuspended to 1×10^6 cells/ml in media. Effectors and CFSE-labeled target cells were mixed at a range of E:T, in sterile 96-well flat bottom plates at 1×10^5 targets/well. Cultures were incubated for 5 h at 37°C under 5% CO_2. 7-AAD was then added to samples, incubated for 30 min in the dark, washed and cytolysis assessed by flow cytometry. Maximum cytolysis control was achieved by treating target cells with Cytofix/Cytoperm (BD Pharmingen) and minimum cytolysis control represented untreated target cells. Percentage of specific lysis was determined using the equation: [(% cytolysis $_{sample}$ - %cytolysis $_{min}$)/ (%cytolysis $_{max}$ - %cytolysis $_{min}$)] × 100%., where %cytolysis= 1-% live cells.

Supplemental references

Cao, L.F., Krymskaya, L., Tran, V., Mi, S., Jensen, M.C., Blanchard, S., and Kalos, M. (2010). Development and application of a multiplexable flow cytometry-based assay to quantify cell-mediated cytolysis. Cytometry Part A : the journal of the International Society for Analytical Cytology 77, 534-545.

Eickhoff, C.S., Vasconcelos, J.R., Sullivan, N.L., Blazevic, A., Bruna-Romero, O., Rodrigues, M.M., and Hoft, D.F. (2011). Co-administration of a plasmid DNA encoding IL-15 improves long-term protection of a genetic vaccine against Trypanosoma cruzi. PLoS Negl Trop Dis 5, e983.

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