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Perpetual expression of PAMPs necessary for optimal immune control and clearance of a persistent pathogen

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Abstract

Pathogen-associated molecular patterns (PAMPs) are known to be fundamental in instigating immune responses but their role in influencing these responses beyond their initiation is less well understood. Here, using the protozoan parasite *Trypanosoma cruzi*, which is deficient in strong PAMPs, we demonstrate a requirement for the continuous expression of PAMPs for optimal anti-pathogen immunity. Although co-inoculating with, temporarily anchoring of and transgenic expression of exogenous PAMPs all result in enhanced early adaptive immune responses, only the continuous expression of bacterial PAMPs on transgenic *T. cruzi* sustains these responses, resulting in enhanced pathogen clearance. These findings demonstrate that PAMPs function to potentiate adaptive immune responses well beyond their initiation and may determine the efficiency of control of pathogens capable of long-term persistence.

Introduction

Pathogen associated molecular patterns (PAMPs) are known to be critical in initiating innate immune responses and to inducing and directing subsequent adaptive immunity[1–4]. PAMPs are effective indicators of the presence of particular pathogens in part because they are unique to classes of pathogens and because they are often required for pathogen survival and thus cannot be altered, suppressed or easily hidden by pathogens. Thus, most of the studies that established the role of PAMPs in innate and adaptive immune responses are based on negating the ability of the host to respond to these molecular patterns[3,5] rather than by blocking PAMP expression. This however, has limited our ability to address the relevance of PAMPs to adaptive immunity after its onset.

In this work, we make use of the protozoan pathogen *Trypanosoma cruzi* to evaluate the role of PAMPs in influencing adaptive immunity at and beyond its initiation. *T. cruzi* is the etiological agent of Chagas disease, the highest impact human parasitic disease in the Americas. Previous studies have shown that *T. cruzi* trypomastigotes stimulate a very weak

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host cell response during invasion[6] and elicit significantly delayed adaptive immune responses[7], strongly suggesting the relative absence of potent PAMPs on live, invading parasites. Although several endogenous PAMPs have been identified in *T. cruzi*[8,9], their failure to impact the strength of the adaptive immune responses[10] as well as the ability of certified PAMPs to potentiate immune responses when co-inoculated with *T. cruzi*[7] have argued against the ready availability of these putative PAMPs on live *T. cruzi*[11] and their relevance to anti-*T. cruzi* immunity. This apparent absence of strong endogenous PAMPs, as well as the genetic pliability of *T. cruzi*, makes this pathogen an ideal template for expression of bona fide PAMPs from other pathogens, and thus for the study of the role of PAMPs beyond the initial phases of immune recognition.

In this study, we generate transgenic *T. cruzi* expressing potent exogenous protein PAMPs and show that this expression not only induces superior innate immune responses, but also drives more rapid and persistently stronger adaptive immunity in mice. These studies demonstrate that constitutive expression of PAMPs is instrumental in maintaining strong adaptive immunity beyond its onset in an infection, leading to better pathogen control and in some cases, complete parasitological cure. Additionally, these findings provide new understanding of how the lack of molecular patterns on the part of some eukaryotic pathogens may fail to incite early immune responses and thus provide an opportunity for establishment of persistent infections.

Results

PAMP transgenic *Trypanosoma cruzi* enhances innate immunity

We chose to express protein PAMPs in *T. cruzi*, since PAMP expression can be stably generated by transgenesis of a single gene, in contrast to polysaccharides or nucleic acid PAMPs which would require transfer of entire biosynthetic pathways into *T. cruzi*. Genes encoding the *Salmonella typhimurium* flagellin (*fliC*), the ligand for both TLR5[12] and Neuronal Apoptosis Inhibitory Protein (NAIP)5/ IL-1β-converting enzyme protease-activating factor (Ipaf)[13] and *Neisseria meningitidis* porin (*porB*), a ligand for TLR1/2[14] were amplified by PCR and cloned into the pTREX plasmid[15] with a *T. cruzi* secretory signal peptide from gp72[16] at their 5' end (Supplementary Fig. S1a). PAMP-transgenic *T. cruzi* expressing FliC (TcgFliC) or PorB (TcgPorB) were engineered by transfecting these constructs into wild-type (WT), Brazil strain *T. cruzi* (Tcwt). The signal peptide ensured secretion (Supplementary Fig. S1b) of the protein PAMPs expressed by the PAMP-transgenic *T. cruzi* in epimastigote, trypomastigote and amastigote life stages (Supplementary Fig. S1c).

Stimulation of TLRs 1/2 or 5 ultimately activates the transcription factors NFkB/ AP-1, to promote immunity, primarily by the production of inflammatory cytokines[3,17]. NFkB/ AP-1 reporter cell lines exhibited substantially increased NFkB/ AP-1 activation by TcgFliC or TcgPorB live trypomastigotes (Fig. 1a) or epimastigote lysates (Supplementary Fig. S2) relative to Tcwt parasites. FliC, is also an NAIP5/ipaf ligand that induces IL-1β production in antigen presenting cells (APCs)[13]. FliC-expressing *T. cruzi* potentiated strong caspase1 activation (Fig. 1b) and modestly enhanced production of IL1β in TLR5-deficient

macrophages (Fig. 1c and 1d) demonstrating that *T. cruzi*-expressed FliC exhibits both of the PAMP properties of *Salmonella* flagellin.

The innate immune response-inducing activity of PAMP-transgenic *T. cruzi* trypomastigotes was also evident in IL-12*yet40* reporter mice in which cells expressing IL12/23 p40 subunit also express yellow fluorescent protein (YFP)[18]. Peritoneal exudate macrophages exposed in vitro to PAMP-transgenic *T. cruzi*, produced IL-12 at an increased frequency relative to those exposed to WT *T. cruzi* (Fig. 2a). Additionally, TcgFliC and TcgPorB infections of IL-12*yet40* reporter mice resulted in a more rapid and increased infiltration of the IL-12 producing $CD11c^+$ $CD8\alpha^+$ classical dendritic cells (cDCs) into the draining lymph nodes (Fig. 2b). TcgFliC infection also altered the lineage bias of the inflammatory cells infiltrating or prevailing at the site of infection, with increased numbers of blood-derived monocytes ($CD45^+CD11b^+CD11c^-Gr\text{-}1^{int}$), macrophages ($CD45^+CD11b^+CD11c^-F4/80^+$), inflammatory DCs ($CD45^+CD11b^+CD11c^{hi}Gr\text{-}1^{int}$) and other non-classical ($CD45^+CD11b^+CD11c^+CD8\alpha^-$) DCs, as compared to WT *T. cruzi* infection (Fig. 3a and 3b). Classical ($CD45^+CD11b^-CD11c^+CD8\alpha^+$) DCs remained undetectable at the site of infection in either case. TcgFliC infection was also associated with enhanced recruitment of IL-12 producing monocytes (Fig. 3a) to the site of infection. The innate immune enhancing effect of PAMP transgenesis was also evident systemically with considerably higher serum levels of IL-12 and TNFα, as compared to Tcwt infection (Fig. 3c).

IFNγ produced by naïve $CD8^+$ T cells, in a TCR independent, IL-12 mediated manner early in the infection appears to serve vital functions in the initial immune responses to a number of pathogens[19,20]. To measure IFNγ induced early in response to *T. cruzi* infection, we used the IFNγ reporter (Yeti) mice, wherein cells expressing IFNγ concurrently express enhanced-yellow fluorescent protein (eYFP)[21]. At time points when *T. cruzi* specific $CD8^+$ T cells are not yet detectable[7] we observed significantly higher proportions of $IFN\gamma^+CD8^+$ T cells in the draining lymph nodes with TcgFliC infection, compared to that with Tcwt (Fig. 3d). Taken together, these results indicate that the expression of bacterial PAMPs in *T. cruzi* markedly enhances innate immune activation both in vitro and in vivo.

PAMP transgenic *Trypanosoma cruzi* enhances adaptive immunity

A robust *T. cruzi* specific $CD8^+$ T cell response is crucial for the control of *T. cruzi* infection in mice[22–24] and we have previously shown that the $CD8^+$ T cell response to *T. cruzi* in C57BL/6 mice is dominated by cells specific for peptides encoded by the trans-sialidase gene family[25]. Hence, we can use $TSKb20^+CD8^+$ T cells as a surrogate for the total $CD8^+$ T cell response mounted against *T. cruzi*, and track this response using the$TSKb20/K^b$ tetramers[25]. Mice infected with PAMP- transgenic *T. cruzi* mounted a more rapid (Fig. 4a) and substantially stronger $TSKb20^+$ $CD8^+$T cell response that was also maintained at higher levels throughout the infection (Fig. 4b and 4c) relative to WT infected mice. This potentiation of T cell responses by PAMP-transgenic *T. cruzi* was also evident in the IFNγ and TNFα production by antigen-experienced $CD8^+$ (Fig. 4d) and $CD4^+$ (Fig. 4e) T cells. Infection with PAMP transgenic *T. cruzi* also elicited higher serum levels of *T. cruzi* specific antibodies compared to infection with Tcwt parasites (Fig. 4f).

To reaffirm that the enhanced adaptive immune responses observed with PAMP-transgenesis in *T. cruzi* were indeed dependent on signaling through pattern recognition receptors (PRRs) targeted by the transgenic PAMPs, we infected MyD88$^{-/-}$ mice, that are deficient in the primary adaptor for multiple TLRs and are unresponsive to TLR5, TLR1/2[3] or (IL-1β from) NAIP5/ipaf stimulation[26]. As expected, the *T. cruzi*-infected MyD88$^{-/-}$ mice showed a delayed generation of TSKb20-specific T cells relative to wild-type mice with the pattern of responses being similar irrespective of the expression or not of the bacterial PAMPs (Fig. 4g). This result indicates that the enhanced adaptive immune response to *T. cruzi* conferred by PAMP transgenesis is a result of increased triggering of host PRRs and the consequential effects downstream of MyD88 signaling.

Since many pathogens express multiple PAMPs that cooperate to potentiate adaptive immune responses[27,28] we attempted to co-express both FliC and PorB in *T. cruzi*. Although C57Bl/6 mice infected with TcgFliC-PorB generated a more rapid and stronger TSKb20^{+}CD8^{+} T cell response as compared to Tcwt, this response was not improved upon that generated by either PAMPs transgenically expressed alone (Supplementary Fig. S3a). This outcome may be a result of technical limitation of protein over-expression in this system since attempts to overexpress multiple other genes in *T. cruzi* also resulted in reduced TcgFliC potency and reduced expression of the second protein (Paraflagellar Rod Protein 4, PAR4) (Supplementary Fig. S3b and S3c). This result also suggests that the impact of PAMPs on immune responses may be determined by their relative expression levels in a pathogen.

Continuous expression of FliC sustains enhanced adaptive immunity

A canonical concept in immunology is that strong innate immunity invokes more potent adaptive immune responses[3,5]. This concept is supported by many studies demonstrating that co-delivery of TLR-ligands with antigens or vaccines significantly boosts adaptive immune responses[29]. However, to our knowledge, no studies have directly investigated the impact on adaptive immunity- of a transient presence of PAMPS at the initiation of infection, to a continuous expression of PAMPs throughout the course of infection. Given that PAMP-transgenesis in *T. cruzi* not only initiated a more rapid TSKb20^{+}CD8^{+} T cell response in mice but also resulted in a response that was maintained at unusually high levels, we suspected that PAMPs may have a continuous instructive role in maintaining strong adaptive immune responses. To determine the consequences of transient versus continuous expression of PAMPs on adaptive immune responses to *T. cruzi*, we tethered various PAMPs to *T. cruzi* using GPI-anchors. Initial experiments showed that molecules linked in this fashion were readily incorporated into the surface of trypomastigotes of *T. cruzi* and had a half-life of ~ 12 hrs (Supplementary Fig. S4a). The signaling potency of FliC delivered by the GPI tether (Tc-GPI-FliC) or by endogenous expression (TcgFliC) was equivalent as indicated by their similar abilities to induce NFkb/AP-1 activation in reporter cells (Supplementary Fig. S4b), or IL-12 production in peritoneal exudate macrophages (Supplementary Fig. S4c). Additionally, Tc-GPI-FliC, TcgFliC or native FliC co-inoculated with *T. cruzi* all potentiated similar innate immune responses *to T.* cruzi in vivo (Fig. 5a), and resulted in nearly identical early TSKb20^{+}CD8^{+} T cells responses (Fig. 5b). However only in the infection with TcgFliC, was the TSKb20-specific response maintained above the

level of the Tcwt infection into the chronic stage (Fig. 5b). The delivery of other individual or combinations of PAMPs with *T. cruzi* infection by GPI-anchors or by co-inoculation enhanced innate immune responses in mice – some much more strongly than TcgFliC (Supplementary Fig. S4d). But only infection with the PAMP-transgenic *T. cruzi* resulted in the long-term maintenance of enhanced adaptive responses (Supplementary Fig. S4e). Repeated injection of flagellin, which is known to induce IL-12 production and activate Th1 responses[30,31], failed to maintain the enhanced $CD8^+$ T cell responses provided by transgenic expression of flagellin (Supplementary Fig. S4f). Thus, continued expression of PAMPs acts to maintain stronger adaptive immune responses, exceeding those elicited by transient PRR engagement at the initiation of infection or thereafter.

FliC transgenesis boosts immune control of *Trypanosoma cruzi*

To investigate the impact of the PAMP-induced enhancement of innate and adaptive immune responses on the parasite control during the course of *T. cruzi* infection, we first monitored the phenotype of *T. cruzi*-specific $CD8^+$ T cells in these mice. Drug-induced cure of *T. cruzi* infection results in a gradual shift in the $TSKb20^+CD8^+$ T cells from a predominant T-effector phenotype ($CD127^{lo}$) to a majority T-central memory (T_{CM})-like ($CD127^{hi}$) phenotype[22], accompanied by a decrease in the frequency of $CD8^+$ T cells expressing KLRG1, a marker for repeated antigenic stimulation[22,32]. At 296 dpi, TcgFliC-infected mice exhibited higher proportions of T_{CM} among the $TSKb20^+CD8^+$ T cells and decreased numbers of $KLRG1^+(CD44^+)CD8^+$ T cells in comparison to mice infected with wild-type *T. cruzi* (Fig. 6a), suggesting more effective control of the infection with TcgFliC This supposition was confirmed using qPCR to measure *T. cruzi* DNA in muscle tissue from these mice (Supplementary Fig. S5). At 400 dpi, *T. cruzi* DNA was undetectable in mice infected with TcgFliC, but was consistently detected in tissues from Tc-GPI-FliC, Tc-GPI +FliC and Tcwt infected mice (Fig. 6b). In contrast, neither changes in the phenotypes of *T. cruzi*-specific $CD8^+$ T cells, nor a decrease in tissue parasite loads were evident in the case of TcgPorB infected mice, despite their enhanced innate and adaptive immune reponses (Supplementary Fig. S6)

We have previously used immunosuppression to reveal otherwise undetectable infection and as a definitive measure of drug-induced cure in *T. cruzi* infection[22]. One of three TcgFliC-infected mice immunosuppressed with cyclophosphamide exhibited no detectable parasites after immunosuppression, indicating clearance of the infection. The enhanced control of the TcgFliC infection relative to wild-type *T. cruzi* infection was not due to a decrease in virulence of the FliC-transgenic parasites, as $IFN\gamma^{-/-}$ mice infected with TcgFliC or Tcwt showed similar peripheral blood parasite loads and mortality patterns (Supplementary Fig. S7), nor to immune responses directed at FliC presented as an antigen (Supplementary Fig. S8). Taken together, these data indicate that FliC transgenesis, but not co-inoculation or temporary surface-anchoring potentiates anti-*T. cruzi* adaptive immune responses and facilitates control and clearance of *T. cruzi* infection.

Discussion

One of the key paradigms in immunology is that innate immune mechanisms detect microbial infections through their characteristic PAMPs and trigger the specific antimicrobial host defense responses appropriate to that infection[29,33,34]. Once initiated, these pathogen-specific (adaptive) immune responses bring about control of the infection and often, a long-term specific immunological memory. However, we have very limited knowledge of the role of PAMPs in influencing the adaptive immunity beyond its initiation, in large part because, by their very nature, PAMPS are crucial for pathogen survival and thus cannot be turned off during an infection. In this study we provide unequivocal evidence that the expression of classical bacterial PAMPS in the protozoan pathogen *T. cruzi* results in substantially enhanced innate and adaptive immune responses and more efficient pathogen control. These data add to the wealth of information indicating that *T. cruzi* has an extremely quiet entry into hosts[6] and a considerably delayed induction of anti-parasitic immune responses[7].

Though PAMPs are highly conserved structures that are extremely difficult for pathogens to alter or sacrifice, there is some plasticity in PAMP display. For example, host detection of LPS in *Porphyromonas gingivalis* and *Escherichia coli* is modulated by differential acylation of lipid-A[35,36], while *Yersinia pestis* synthesizes LPS-lipid A that is a poor TLR4 ligand[37] and *Pseudomonas aeruginosa* down-regulates its flagellin expression when in airway passages[38]. Although it is unlikely that any pathogen will be able to make all its PAMPs entirely invisible to the immune system, we believe that potential PAMPs could be rendered immunologically inconsequential by concealing or modifying, without significantly impacting pathogen biology. Multiple PAMPs (e.g. GPI anchors, DNA, GIPL-ceramide), have been attributed to *T. cruzi*[8,9] but these molecules seem to be relatively insignificant to the downstream immune responses generated, with comparable adaptive immunity in mice that are either responsive or (genetically) unresponsive to these ligands[10]. Perhaps this is not surprising since these putative PAMPs would be “hidden” from their respective TLRs in live, intact *T. cruzi*. However, when strong bacterial PAMPs are transgenically expressed and released by *T. cruzi*, significantly improved innate and adaptive immune responses are generated. So we propose that the failure of *T. cruzi* to display potent PAMPs may indeed be another example of innate immune evasion employed by pathogens.

The observed evasion of innate immune responses may not only be important in delaying the adaptive immune response – thus allowing for firm establishment of the infection, but also may promote the persistence of *T. cruzi*. In the presence of a bacterial PAMP, *T. cruzi* infection is better controlled and even completely cleared in some cases. It has to be noted that complete clearance of *T. cruzi* infection is normally extremely rare. Given the increased level of *T. cruzi*-specific $CD8^+$ T cells with a Tcm phenotype and the nearly undetectable tissue parasite load in mice infected with PAMP transgenic *T. cruzi*, we predict that most of these mice would eventually cure these infections if allowed sufficient time. Importantly, this enhanced control of *T. cruzi* infection derived from expression of bacterial PAMPs is not associated with any evidence of increased immunopathology..

The relative absence of PAMPs in *T. cruzi* provided a unique opportunity to study the importance of PAMP expression beyond the early induction of adaptive immune responses. Though there is a wealth of literature demonstrating how the strength of innate immunity determines the potency of adaptive immune responses[33,34], few studies have focused on the impact of innate immune responses on adaptive immunity once an infection is established. When potent bacterial PAMPs were either co-inoculated with, temporarily surface-anchored on, or constitutively expressed- by the invading *T. cruzi*, the resulting adaptive immune responses were not only accelerated, but also peaked early at levels that were above that seen in mice infected with WT *T. cruzi*. However, it was only when *T. cruzi* perpetually expressed the PAMPs that the stronger adaptive immune responses were maintained throughout the course of the infection, eventually leading to a better control of the pathogen and sterile clearance in some cases. It is likely that the locally enhanced inflammatory milieu, coupled with the improved antigen processing and presentation by more highly activated APCs resulting from continuing PAMP exposure[33,39] potentiated the quality, quantity and longevity of T and B cells[40–43]. Although transgenic expression of FliC and PorB induced similar boosting of immune responses, it was only in the case of TcgFliC that this response corresponded with better parasite control. FliC is distinctive in its ability to induce IL-1β through the intracellular NAIP5/ipaf receptor stimulation and given that *T. cruzi* spends the majority of its time in vertebrates within the cytoplasm of host cells that are likely to express the NAIP5/ipaf receptor[44], it is possible that NAIP5/ipaf-IL-1β activation contributes to enhanced recognition and control of TcgFliC infected cells. IL-1β levels have also been shown to correlate with $CD8^+$ T cell abundance in adipose tissue[45] which incidentally is a major depot for *T. cruzi* persistence chronically. A possible confounder in the interpretation of our results is that FliC expressed by TcgFliC may act as a target antigen for adaptive immune responses, contributing to the control of FliC-expressing parasites. However the absence of detectable FliC specific T cells in TcgFliC-infected mice argues against this possibility.

Heterologous expression of bacterial PAMPs in *T. cruzi*, despite prompting stronger adaptive immune responses, could only marginally (though significantly) accelerate the initial induction of these responses. Hence the deficiency of effective PAMPs in *T. cruzi* may be only one of the several factors that contribute to the marked delay in initiation of anti-*T. cruzi* immune responses. An additional important trigger for induction of adaptive responses is the exposure of damage associated molecular patterns (DAMPs)[46]. Revelation of DAMPs from either host or *T. cruzi* would not be expected until 4–5 days post-infection, with the initial round of exit of *T. cruzi* from infected host cells.

This study provides significant new insights into the *T. cruzi*-host interface and identifies some of the contributing factors for the ability of *T. cruzi* to persist indefinitely in most hosts, despite the generation of potent immune responses. The contribution of inadequate PAMP expression in the persistence of other pathogens, in particular eukaryotic pathogens that lack many of the classical PAMPs, is worthy of further exploration. Our findings also advance the idea that innate immune responses may have an extended, instructive role on the adaptive immunity, thus playing an even more significant part in the effective control of pathogens than was previously appreciated. The inability of classical adjuvants to generate a

long-lasting enhancement of T cell responses has been a hurdle in the development of T cell based vaccines[29]. Expression of heterologous PAMPs would be expected to enhance the effectiveness of live vaccines and periodic or continuous exposure to PAMPs might have therapeutic benefit in persistent infections wherein endogenous PAMPs are inadequate.

Methods

Mice and infections

C57BL/6, B6.IFNγ–knockout (IFNγ$^{-/-}$), MyD88 Knockout (MyD88$^{-/-}$), B6.IL-12*yet40* reporter (IL-12*yet40*) and IFNγ reporter (Yeti) mice were purchased from The Jackson Laboratory or bred and maintained in our animal facility under specific pathogen-free conditions to use females of 6–8 weeks for the experiments. *T. cruzi* epimastigotes were transfected[16] with pTREX plasmid[15] containing the coding sequence of *Salmonella typhimurium* flagellin (FliC), *Neisseria meningitidis* (FAM18 strain) porin (PorB) or *T. cruzi* paraflagellar rod protein 4 (PAR4) genes, with or without fusion to an upstream N-terminal portion of the *T. cruzi* gp72 gene or influenza haemagglutinin (HA)-tag, to generate transgenic *T. cruzi*. All infections were initiated by inoculating vero cell culture passaged trypomastigote stage *T. cruzi*, intra-peritoneally (i.p) (10^4 parasites) or subcutaneously in the ear (s.c) (5×10^4 parasites) or the foot pad (f.p) (10^4 parasites). Native FliC was inoculated i.p in mice at 10μg/animal[31] at 10 day intervals. All animal protocols were approved by the University of Georgia Institutional Animal Care and Use Committee.

Reporter cell assays

The ability of various *T. cruzi* strains to induce NFkB/ AP-1 activation by TLR stimulation was assayed using THP1-Blue-CD14 reporter cells (Invivogen), following the manufacturer's protocol. 10^4 live *T. cruzi* trypomastigotes were incubated with 2×10^6 reporter cells for 9 hours at 37°C/5% CO_2, and the nuclear translocation of activated NFkB/ AP-1 was determined by colorimetrically quantifying the secreted embryonic alkaline phosphatase (SEAP). To determine the IL-12 production induced in cells by *T. cruzi*, 10^5 peritoneal exudate macrophages from IL-12yet40 reporter mice were incubated with 10^3 *T. cruzi* trypomastigotes for 18 hrs at 37°C/5% CO_2. The proportion of YFP$^+$ macrophages was determined by flow cytometry. LPS or media served as controls.

Determination of caspase 1 activity

Active caspases were detected with FLICA Apoptosis Detection kit (Immunochemistry Technologies) following the manufacturer's protocol. After 12 hours incubation of 2×10^4 TcgFliC or Tcwt with 2×10^5 RAW blue (TLR5−) mouse macrophages (Invivogen), the latter were incubated with a fluorescent inhibitor peptide specific to caspase 1 (FAM-YVAD-FMK) for 60 min at 37°C/5% CO_2. Inhibitors were removed by rinsing; the cells were fixed and then analyzed with a florescence plate reader

Western blot and ELISA

To determine the presence of FliC, TcgFliC lysate[47] or culture (12h) supernatant, were probed with anti-FliC Mab (1:500) (Biolegend) by western blot. Sera collected from C57BL/6 mice infected with various *T. cruzi* strains, 30 dpi were assayed for anti-*T. cruzi*

antibodies by ELISA. To determine the relative concentrations of haemagglutinin (HA) tagged protein (PAR4-HA) in the trypomastigote stage from various strains of transgenic *T. cruzi*, serial dilutions of whole cell lysates were assayed with anti-HA antibody (1:1000) (Roche). A purified HA-tagged protein (*T. cruzi* PAR2) expressed in *E. coli* was used as the standard.

Intracellular cytokine staining

To measure IL-1β production, 2×10^5 RAW blue ($TLR5^-$) mouse macrophages (Invivogen) were incubated with 2×10^4 TcgFliC trypomastigotes for 18hrs. *E. coli* lipopolysaccharide (LPS) +ATP or media served as controls. The induced IL-1β in macrophages were determined by staining using the Cytofix/Cytoperm intracellular staining kit (BD Pharmingen) following the manufacturer's protocol. Similarly, to determine intracellular IFNγ and TNFα production, 1.5×10^6 spleen cells from TcgFliC, TcgPorB or Tcwt infected, or naïve mice were restimulated with *T. cruzi* peptide TSKb20 (5 μM), *T. cruzi* whole cell lysate (10 μg) or FliC and processed for intracellular cytokine staining (ICS). Recombinant Ovalbumin protein (ova) or recombinant Ovalbumin protein with TSKb20 peptide substituted for SIINFEKL(Ova-TS20) served as a controls. 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 (1:100), CD44 FITC (1:400) and anti-CD8 eFluor450 (1:800) (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-IL-1β PE (1:100) (R&D systems), anti-IFNγ APC (1:400) or anti-TNFα PECy7 (1:400) (both 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.

Phenotyping cells by flow cytometry

T cell phenotypes were determined[22] by staining with tetramer-phycoerythrin (TSKb20-PE; ANYKFTLV peptide on $H2K^b$ NIH Tetramer Core Facility) and the following: anti-CD62L APC (1:100), anti-CD44 FITC (1:400), anti-CD8 efluor-450 (1:800), anti-CD127 PEcy7 (1:100) and anti-KLRG1 APCcy7 (1:100) (all from eBioscience). Anti-CD4 PECy5 (1:200) (Invitrogen) and anti-B220 PECy5 (1:200) (Invitrogen) staining were used for a dump channel.

To determine the phenotypes of cells infiltrating the site of infection, the tissue (ear) was enzymatically digested to dissociate the cells . In the case of draining lymph nodes, the cells were dissociated by gently crushing between the ground edges of glass slides. After FcR (CD16/ 32) block, cell surface markers were used to differentiate several cell lineages[48]. DCs, infiltrating monocytes or resident macrophage subsets were differentiated using the following mAb conjugations: CD11c APC (1:100), CD8α efluor450, CD11b APC/ eFluor780 (1:800), Gr-1(Ly6C/Ly6G) PerCP/Cy5.5 (1:200), F4/80 PECy7 (1:200) and CD45 PE (1:200) (BD Pharmingen, ebioscience, or BioLegend). DCs were defined with CD11c, with further differentiation into $CD11b^-CD8\alpha^+$ cDCs and $CD11b^+$ ($F4/80^-$) DCs. Monocytes and iDCs were defined as $CD11b^+$ $CD11c^-Gr\text{-}1^{int}$ and $CD11b^+$ $CD11c^+$ $Gr\text{-}1^{int}$ respectively[49]. Macrophages were identified as $F4/80^+$ $CD11b^+$ $CD11c^-$. IL-12 producing

DCs were defined as $CD11c^+$ $CD8\alpha^+$ ($CD11b^-$) YFP^+ in IL-12*yet40* reporter mice as described before[18]. Data is represented as the percentage of each cell type over all the cells derived by enzymatic digestion, representing the total cellularity at the site.

IFNγ producing CD8 T cells in the draining lymph nodes of yeti mice were defined as $CD4^-B220^-CD8^+YFP^+$ as described before[50]. At least 5×10^5 (blood) or 5×10^6 (peripheral tissue/ lymph node) cells were acquired per sample using a CyAn flow cytometer (Beckman Coulter) and analyzed with FlowJo software (Tree Star).

Serum cytokine assay

Blood collected from C57Bl/6 mice inoculated with TcgFliC or Tcwt and sera separated to assay for various cytokines using the Q-Plex™ Mouse Cytokine Screen ELISA (Quansys Biosciences) or for IL-12 alone using eBioscience mouse IL-12p70 flowCytomix following the manufacturer's protocol. Luminescence intensity of each sample was measured and the concentration of each cytokine was determined using the Q-View™ software (Quansys Biosciences) in the former or by flow-cytometry for the latter.

Temporary anchoring of PAMPs on *Trypanosoma cruzi*

FSL-biotin GPI anchor with a single biotin F-moiety (FSL-CONJ(1Biotin)-SC2-L1, KODE Biotech Materials Ltd) was used to coat *T. cruzi* trypomastigotes as per the manufacturer's protocol. 1×10^6 trypomastigotes were incubated with 2μg of FSL-biotin in 100 μl serum free RPMI 1640 media. After washing to remove the excess FSL-biotin, the parasites were incubated on ice with streptavidin (Sigma), at 5 times the molar concentration of FSL-biotin (to give Tc-GPI). Excess streptavidin was removed by washing and various biotinylated ligands: FliC-biotin, Pam3CSK4-biotin (Pam3Cys-Ser-(Lys)4-biotin, invivogen), ODN-biotin (oligodeoxynucleotide- biotin, invivogen), all at 3× molar concentration of FSL-biotin or Pam3CSK4-biotin and ODN-biotin together, each at 1.5× molar concentration of FSL-biotin were incubated with the Tc-GPI for 30 min on ice to yield Tc-GPI-FliC, Tc-GPI-Pam3CSK4, Tc-GPI-ODN or Tc-GPI-ODN-Pam3CSK4 respectively. FliC was biotinylated using EZ-link sulfo-NHS-LC Biotinylation kit (Thermo scientific) following the manufacturer's protocol. The various PAMP anchored *T. cruzi* strains were washed twice with RPMI1640 to remove excess PAMPs, counted, re-suspended in complete RPMI1640 and used for in vitro or in vivo assays. Tc-GPI was used as the control. PAMPs when co-inoculated with *T. cruzi*, were used at approximately the same quantities (in w/v) as was used to label *T. cruzi* above.

Real time PCR

The skeletal muscle tissue from mice were analyzed by real-time PCR for the presence of *T. cruzi* (DNA) using the following primer pairs- TCSat30: GGCGGATCGTTTTCGAG and TCSat179: AAGCGGATAGTTCAGGG.[51]

Assessment of infectivity and clearance of *Trypanosoma cruzi*

To assess the infectivity of different strains of *T. cruzi*, $IFN\gamma^{-/-}$ mice were inoculated with 10^4 trypomastigotes of TcgFliC or Tcwt strains. Blood was collected from the tail vein at 21dpi to quantify the number of parasites using a compound light microscope and expressed

as the number of live trypomastigotes per 100 (40×) fields. Survival was monitored daily. Complete clearance of *T. cruzi* from infected mice was assessed by real time PCR amplification of *T. cruzi* DNA in skeletal muscle obtained following immunosuppression with cyclophosphamide (200mg/kg; 4× at 2 day intervals)[22].

Statistical analysis

Data are presented as the mean plus/minus the standard error of mean (s.e.m). Statistical analyses compared the groups with a two-tailed student t-test. Only p values of less than 0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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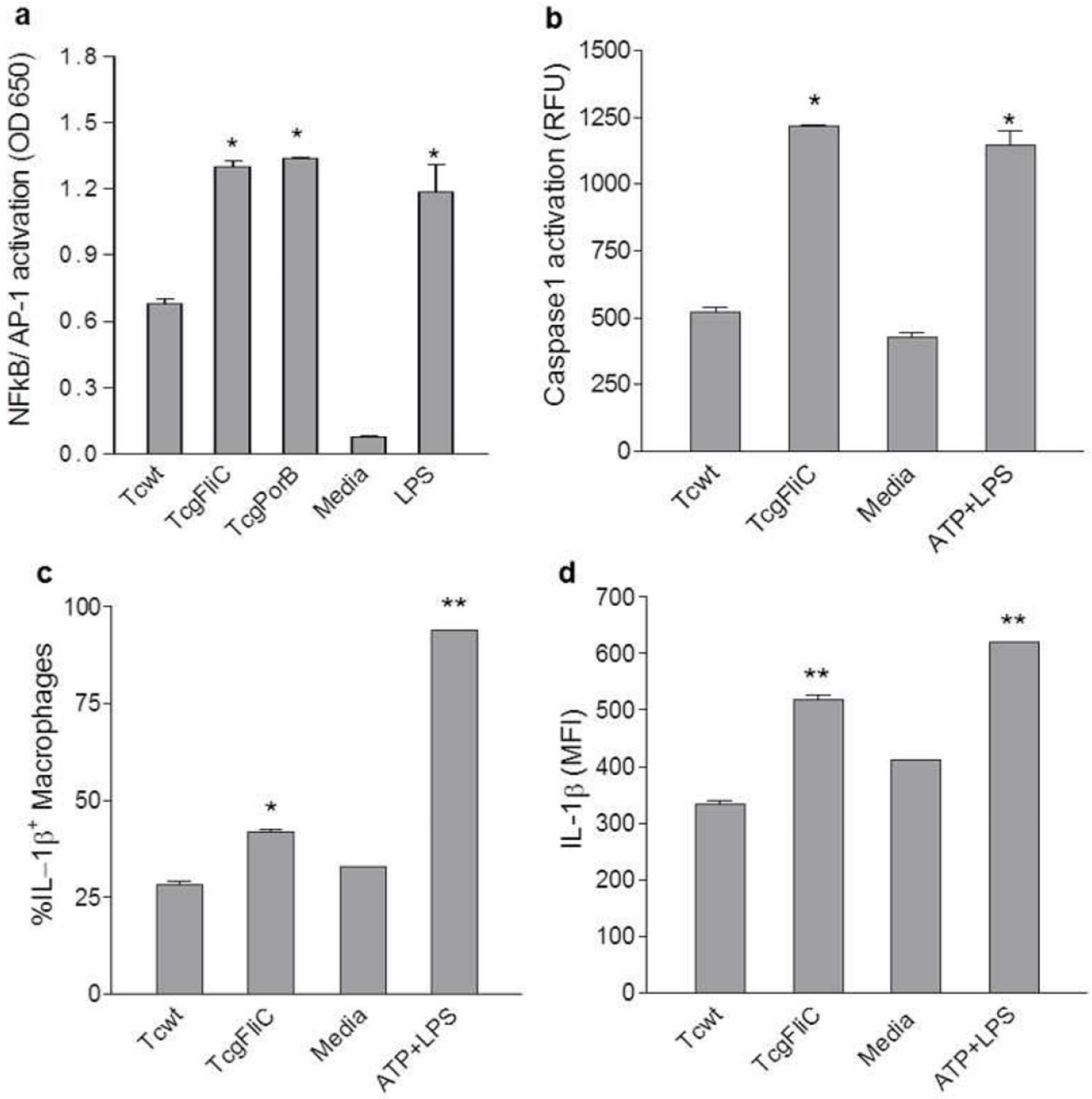

Figure 1. PAMP transgenic *T. cruzi* enhances cellular innate immune responses

(a) NFkB/ AP-1 activation in reporter cells incubated with Tcwt, TcgFliC or TcgPorB trypomastigotes for 12 hrs. Media or *E. coli* derived LPS were used as negative and positive controls, respectively. (b) Caspase1 activation in (TLR5^{-}) macrophages incubated with Tcwt or TcgFliC trypomastigotes for 12 hrs. Media or ATP with *E. coli* derived LPS served as controls. (c) The proportion of (TLR5^{-}) macrophages producing IL-1β on incubation with Tcwt or TcgFliC trypomastigotes for 18 hrs. (d) IL-1β mean fluorescence intensity (MFI) per cell by TLR5^{-} macrophages incubated with Tcwt or TcgFliC trypomastigotes for 18 hrs.

Media or ATP with *E. coli* derived LPS served as controls. All data show mean ± s.e.m and are representative of at least 3 separate experiments. * or ** indicates $p \leq 0.05$ or ≤ 0.01 respectively, as determined by student t-test comparing the indicated groups to Tcwt.

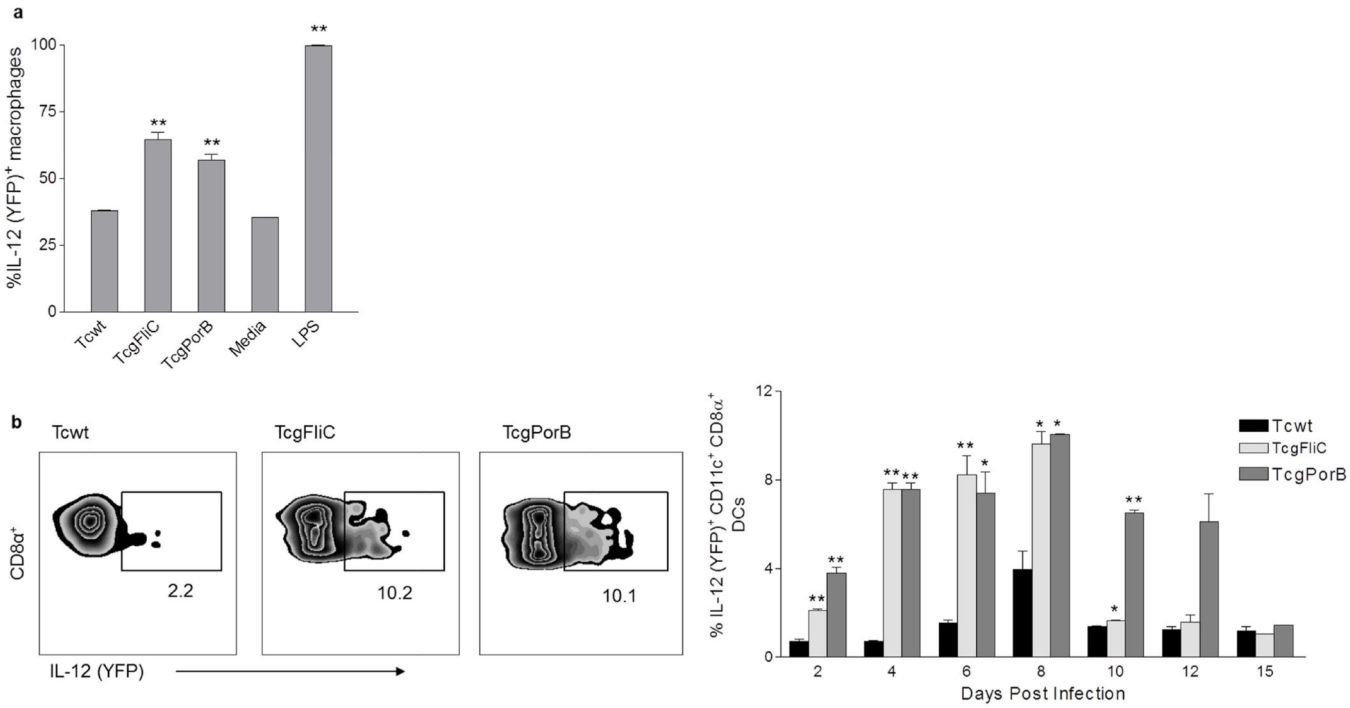

Figure 2. PAMP transgenic *T. cruzi* induces higher frequencies of IL-12 producing APCs

(a) The proportion of peritoneal exudate macrophages with induced IL-12 (YFP) on incubation with Tcwt, TcgFliC or TcgPorB trypomastigotes for 18 hrs. Media or *E. coli* derived LPS served as controls. Data presented as mean ± s.e.m and are representative of 3 separate experiments. (b) IL-12 producing ($CD11c^+$ $CD8\alpha^+$) cDC recruitment into the draining lymph nodes on Tcwt, TcgFliC or TcgPorB infection of IL-12*yet40* mice. Flow plots show representative data from 6 dpi, with the numbers inset indicating the percentage of IL-12 producing cDCs. Right panel shows the kinetics of IL-12^+ ($CD11c^+$ $CD8\alpha^+$) cDC recruitment. Data are represented as mean± s.e.m from one of 3 separate experiments, with at least 3 mice/ group. * or ** indicates $p \leq 0.05$ or ≤ 0.01 respectively, as determined by student t-test comparing the indicated groups to Tcwt (a), at the corresponding time points (b).

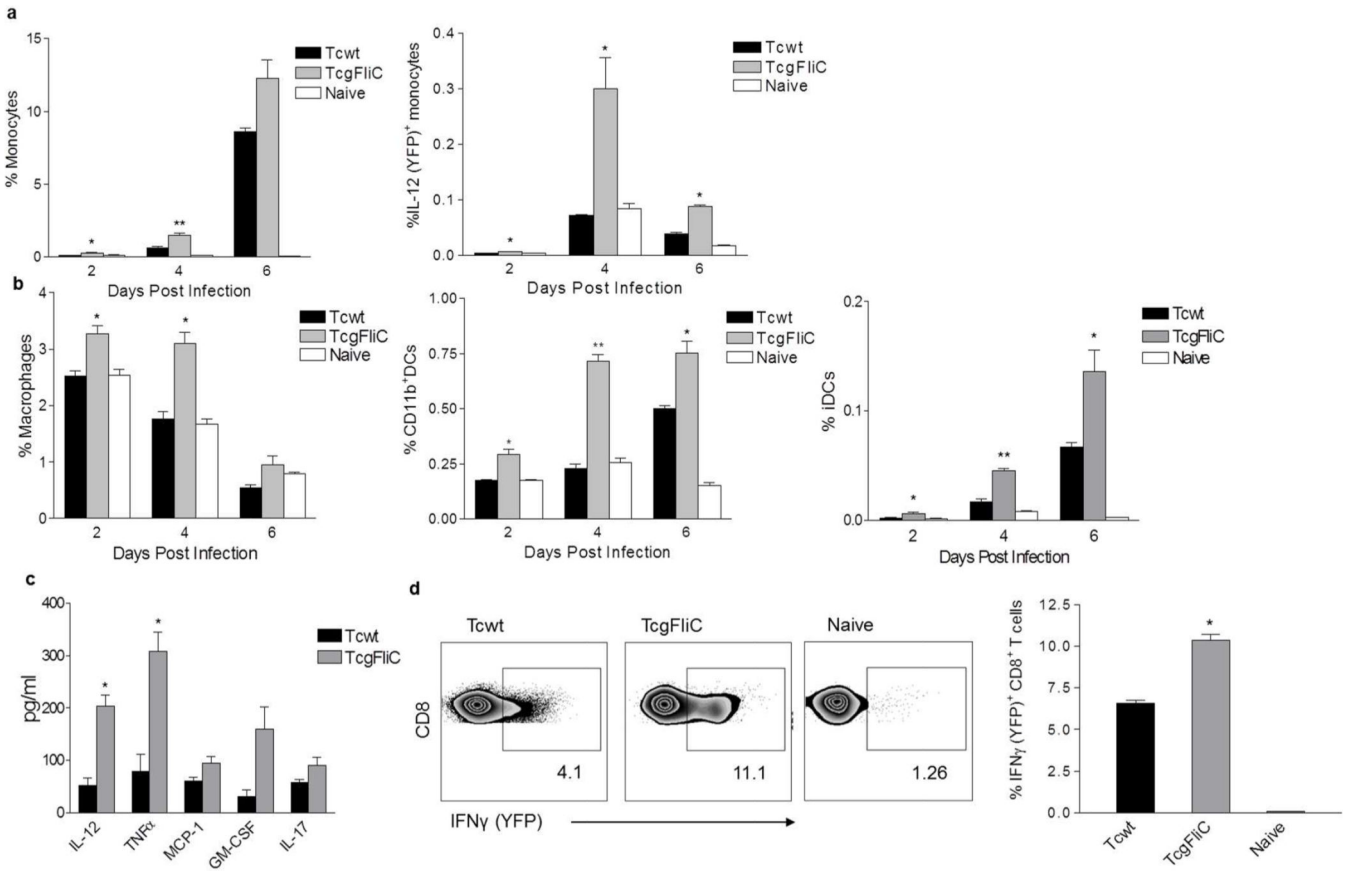

Figure 3. PAMP transgenesis in *T. cruzi* enhances the systemic innate immune responses

(a) The proportion of blood derived monocytes and their IL-12 producing subset at the site (s.c) of Tcwt or TcgFliC infection in IL-12*yet40* mice at various time points post infection. Naïve mice were inoculated with media alone. (b) The proportion of resident macrophages, $CD11b^+$ DCs or iDCs at the site of Tcwt or TcgFliC infection in IL-12*yet40* mice at various time points post infection. Naïve mice were inoculated with media alone. (c) Serum levels of various cytokines in C57Bl/6 mice infected with Tcwt or TcgFliC, 4 dpi. (d) Percentage of $CD8^+$ T cells producing IFNγ in the draining lymph nodes with Tcwt or TcgFliC infected (f.p) Yeti mice, 6dpi. Representative flow plots with the numbers inset indicating the percentage of IFNγ producing $CD8^+$ T cells. All data represented as mean ± s.e.m from one of 3 separate experiments with 3–6 mice/group. * or ** indicates $p \leq 0.05$ or ≤ 0.01 respectively, as determined by student t-test comparing the indicated groups to Tcwt (c and d), at the corresponding time points (a and b).

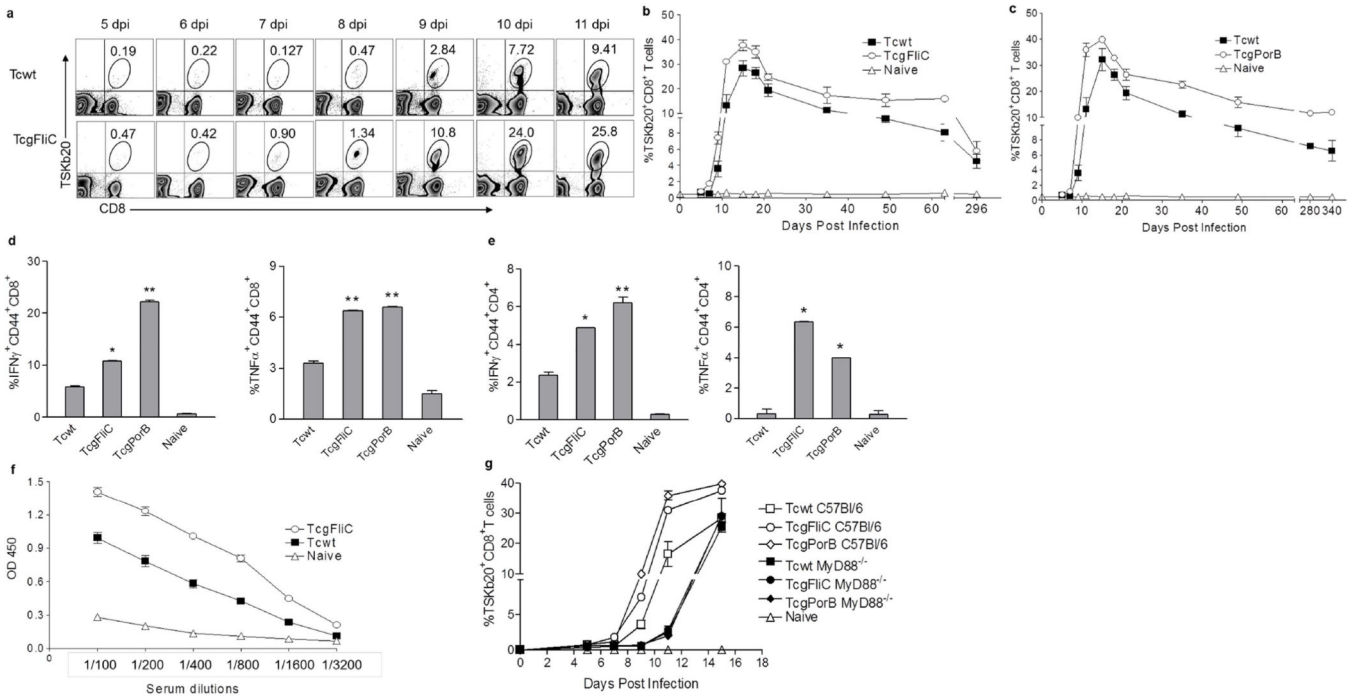

Figure 4. PAMP transgenesis in *T. cruzi* enhances the adaptive immune responses generated

(a) Flow plots show early time points post-infection in C57BL/6 mice with Tcwt or TcgFliC, with numbers inset indicating percentage of $TSKb20^{+}CD8^{+}$ T cells. (b and c) The kinetics of $TSKb20^{+}CD8^{+}$ T cell frequencies in circulation in TcgFliC (b) and TcgPorB (c) compared to Tcwt infection of C57BL/6 mice. Data represented as mean ± s.e.m from one of 6 separate experiments, with at least 6 mice/ group. (d and e) The percentage of $CD8^{+}(CD44^{+})$ (d) or $CD4^{+}(CD44^{+})$ (e) T cells producing IFNγ (left) or TNFα (right) in response to TSKb20 peptide or *T. cruzi* whole cell lysate re-stimulation respectively in Tcwt, TcgFliC or TcgPorB infected C57BL/6 mice, 180dpi. Data represented as mean ± s.e.m from one of 3 separate experiments, with 3 mice/ group. (f) Anti-*T. cruzi* antibody titers in sera of mice infected with Tcwt or TcgFliC in C57Bl/6 mice, 30dpi. Data shown as mean ± s.e.m, representing 2 separate experiments, with 3 mice/ group. (g) The kinetics of $TSKb20^{+}CD8^{+}$ T cell frequency in circulation on Tcwt, TcgFliC or TcgPorB infection of $MyD88^{-/-}$ or C57Bl/6 mice. Data represented as mean ± s.e.m from one of 3 separate experiments, with 3–6 mice/ group. *$p \leq 0.05$ or **$p \leq 0.01$, as determined by student t-test comparing the indicated groups to Tcwt.

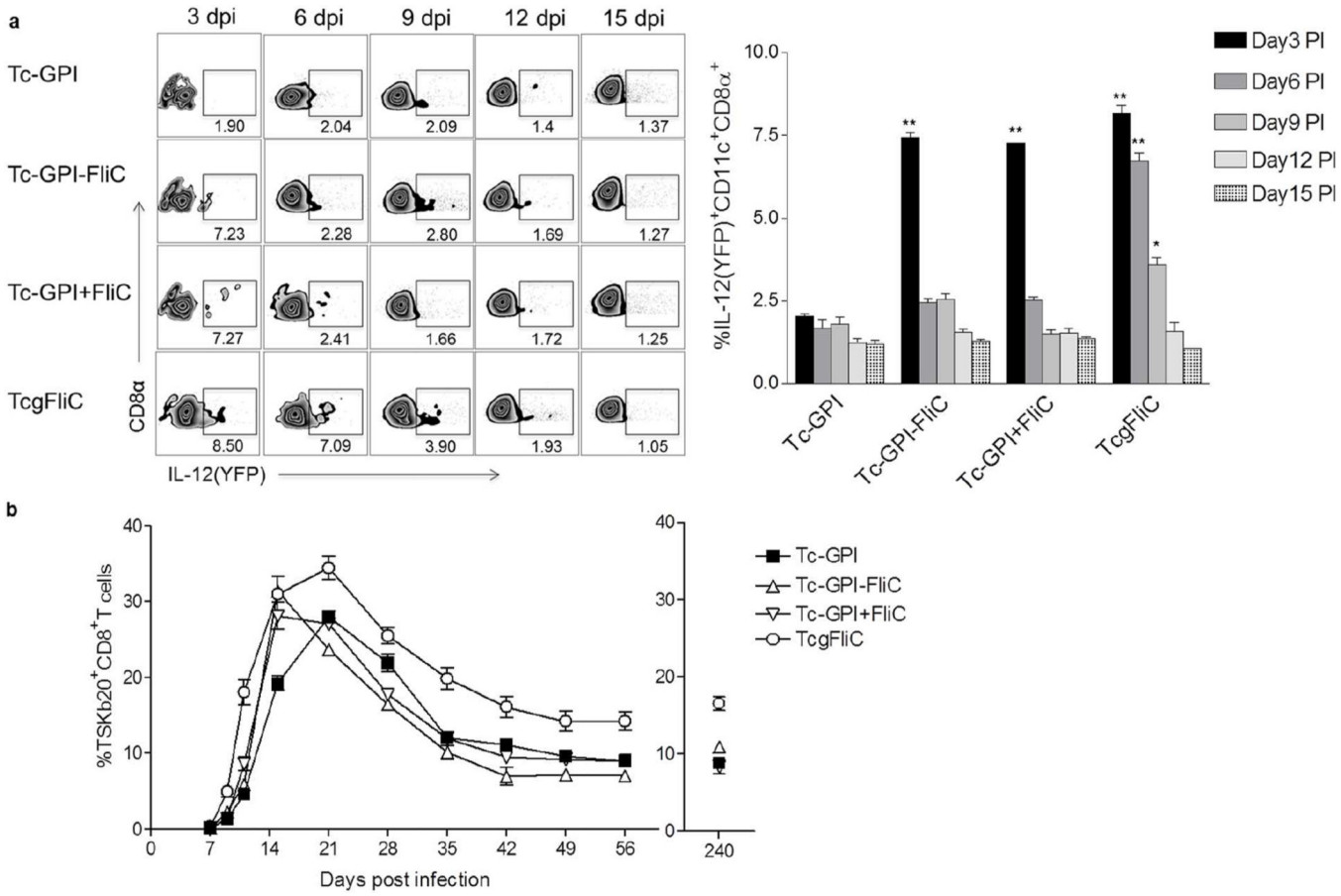

Figure 5. Continuous expression of FliC is required to sustain the enhanced adaptive immunity

(a) The proportions of IL-12 producing cDCs recruited into the draining lymph nodes on various days post infection with *T. cruzi* having FliC temporarily surface-anchored (Tc-GPI-FliC), co-inoculated with (Tc-GPI+FliC), or constitutively expressed in (TcgFliC), compared to the background strain (Tc-GPI) in IL-12*yet40* reporter mice. The flow panel shows representative flow plots, with the numbers inset indicating the %IL-12$^+$ cDCs. Data are represented as mean ± s.e.m from one of 3 separate experiments, with at least 3 mice/ group/time point. *p≤ 0.05 or **p≤ 0.01, as determined by student t-test comparing the indicated groups to Tcwt-GPI at the corresponding time-ponts. (b) TSKb20$^+$CD8$^+$ T cell frequency in circulation in Tc-GPI, Tc-GPI-FliC, Tc-GPI+FliC or TcgFliC infection of C57BL/6 mice. Data represented as mean ± s.e.m combining two experiments, with 3–6 mice/ group.

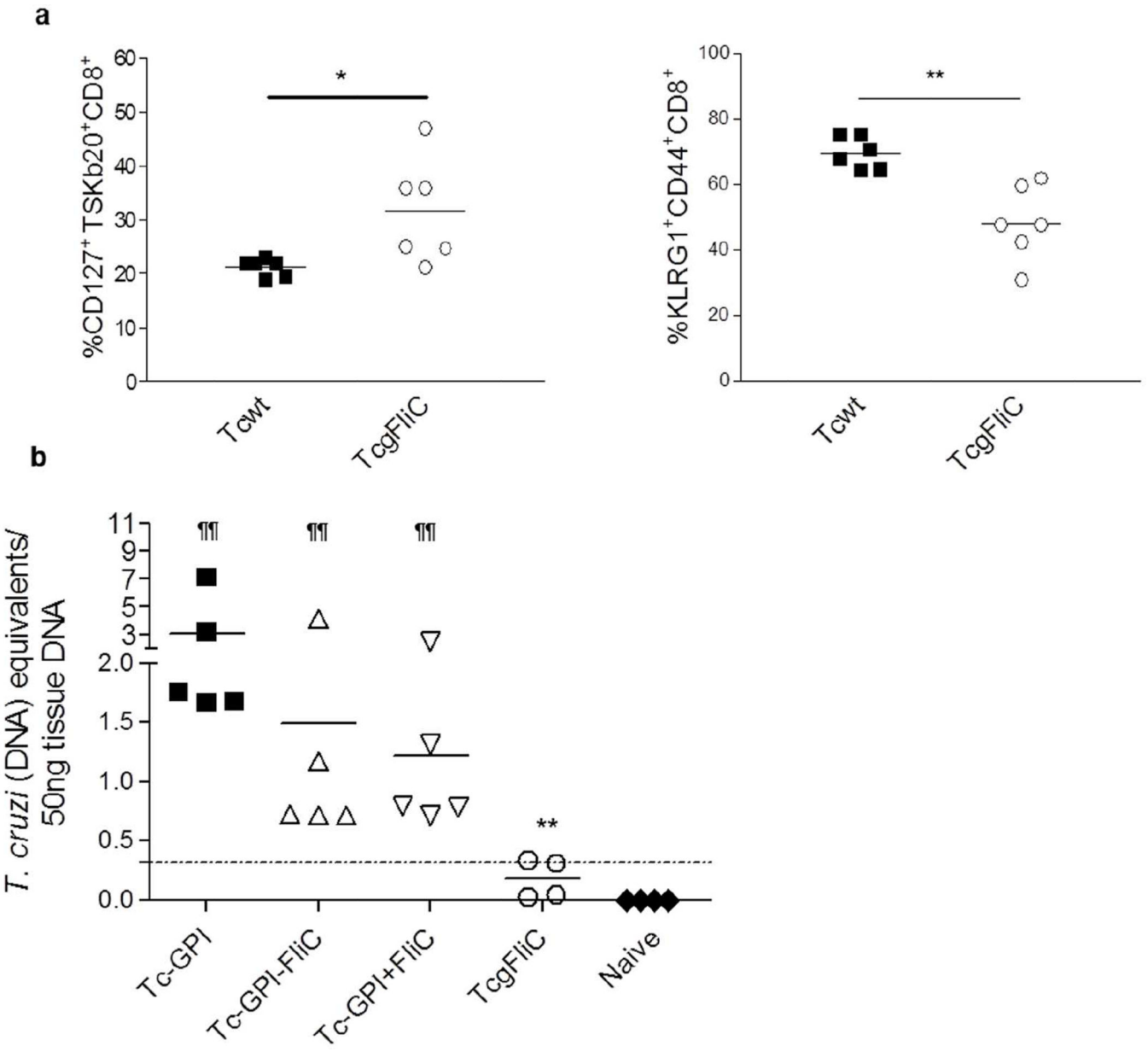

Figure 6. FliC transgenesis enhances control of *T. cruzi* infection in mice

(a) Frequency of $CD8^+$ T cells in circulation having Tcm like phenotype ($CD127^{hi}$ $TSKb20^+$ $CD8^+$ (left) and $KLRG1^{lo}$ $CD44^+$ $CD8^+$ (right)), in Tcwt or TcgFliC infected C57BL/6 mice, 296 dpi. Data represents 3 separate experiments with 3–6 mice/ group. *p≤ 0.05 or **p≤ 0.01, as determined by student t-test.(b) *T. cruzi* DNA in skeletal muscle of C57BL/6 mice inoculated with Tcwt, Tc-GPI-FliC, Tc-GPI+FliC or TcgFliC as determined by quantitative real-time PCR, 400 dpi. Horizontal bars represent the mean. Naïve mice served as control. The dotted line represents the threshold of detection for the assay. Data are representative of 3 separate experiments initiated with at least 5 mice/ group. ¶¶ or ** indicates p≤ 0.01 comparing the indicated groups to naïve or Tcwt infected mice, respectively, as determined by student t-test.