Incomplete recruitment of protective T cells is associated with *Trypanosoma cruzi* persistence in the mouse colon

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Abstract

Trypanosoma cruzi is the etiological agent of Chagas disease. Following T cell mediated suppression of the acute phase infection, this intracellular eukaryotic pathogen persists long-term in a limited sub-set of tissues at extremely low-levels. The reasons for this tissue-specific chronicity are not understood. Using a dual bioluminescent:fluorescent reporter strain and highly sensitive tissue imaging that allows experimental infections to be monitored at single-cell resolution, we have undertaken a systematic analysis of the immunological micro-environments of rare parasitized cells in the mouse colon, a key site of persistence. We demonstrate that incomplete recruitment of T cells to a subset of colonic infection foci permits the occurrence of repeated cycles of intracellular parasite replication and differentiation to motile trypomastigotes at a frequency sufficient to perpetuate chronic infections. The life-long persistence of parasites in this tissue site continues despite the presence, at a systemic level, of a highly effective T cell response. Overcoming this low-level dynamic host:parasite equilibrium represents a major challenge for vaccine development.

KEY WORDS: *Trypanosoma cruzi*, Chagas disease, chronic persistence, murine imaging, colon, T cell recruitment

INTRODUCTION

The insect-transmitted protozoan parasite *Trypanosoma cruzi* is the causative agent of Chagas disease, and infects 5-7 million people in Latin America (1). Despite decades of effort, only limited progress has been made in developing a vaccine, and doubts remain about the feasibility of vaccination as a method for disease control (2,3). In humans, *T. cruzi* infection passes through an acute stage that lasts 2-8 weeks, during which parasitaemia is readily detectable, although symptoms are generally mild and non-specific. With the induction of the adaptive immune response, in which $CD8^+$ IFN-γ^+ T cells play a key role (4,5), there is a significant reduction in the parasite burden. However, sterile clearance is not achieved and parasites persist as a chronic life-long infection. One-third of those infected with *T. cruzi* eventually develop Chagasic pathology, although symptoms can take decades to become apparent. Cardiomyopathy is the most common clinical outcome (6-8), followed by digestive tract dysfunction and megasyndromes, which are reported in about 10% of infected individuals, often in parallel with cardiac disease.

Although the innate immune system is able to detect the parasite (9,10), there is a delay in the subsequent induction of an adaptive response relative to other pathogens (5,11). This, together with a rapid rate of parasite division (12) and broad cell type tropism, allows *T. cruzi* to disseminate widely during the acute stage, with most organs and tissues becoming highly infected (13). The $CD8^+$ T cell response, which predominantly targets a sub-set of immunodominant epitopes in members of the hypervariable *trans*-sialidase surface antigen family (14,15), is critical for controlling the acute stage infection in mice, in combination with antibody-mediated responses. The parasite burden is reduced by 2-3 orders of magnitude as the

disease transitions to a chronic dynamic equilibrium (13). Understanding why the immune system then fails to eliminate the remaining parasites is a central question in Chagas disease research. This information is crucial to underpin rational vaccine design and immunotherapeutic interventions.

Because of the complexity and long-term nature of Chagas disease in humans, mice have been important experimental models for research on interactions between parasite and host. They display a similar infection profile to humans, exhibit chronic cardiac pathology, and are widely used in drug and vaccine development (16). Bioluminescence imaging studies have revealed that the GI tract is a major parasite reservoir during chronic infections and that the degree of containment to this region is determined by both host and parasite genetics (13,17). Parasites are also frequently detectable in the skin, and in some mouse models, such as C3H/HeN, skeletal muscle can be an important site of persistence (4,18). In the colon, the most frequently infected cells are myocytes located in the gut wall. However, the extent of infection is low, and in many cases, this entire organ contains only a few hundred parasites, concentrated in a small number of host cells (18). After transition to the chronic stage, *T. cruzi* also exhibits a reduced proliferation rate, although the cycle of replication, host cell lysis and re-infection appears to continue (12). Evidence for a form of dormancy in *T. cruzi* has been reported (19), however whether this is analogous to dormant/quiescent life-cycle stages observed in other parasites, such as *Toxoplasma gondii* bradyzoites and *Plasmodium vivax* hypnozoites (20), remains to be established.

Multiple studies have shown that experimental *T. cruzi* vaccines have protective efficacy and can reduce both parasitaemia and disease severity (21-26). However, evidence for complete parasite elimination after challenge, is lacking. In contrast, drug-cured infections can confer long-lasting protection against re-challenge with a homologous parasite strain (3), although sterile protection was only achieved in ~50% of animals. Re-challenge with a heterologous strain did not result in sterile protection, although there was >99% reduction in the peak acute stage parasite burden. All drug-cured animals that displayed re-infection transitioned to the canonical chronic stage equilibrium and organ distribution, without passing through an elevated acute stage parasitaemia. Once established in permissive sites, such as the GI tract, parasites appear to survive the systemic *T. cruzi*-specific IFN-γ^+ T cell response generated by the primary challenge. In the absence of information on the immunological micro-environment of these persistent parasites, the reasons for this are unclear. Resolving this question will have a major strategic impact on the development of an effective vaccine.

Progress in this area has been limited by technical difficulties in locating and analysing the rare infection foci in permissive tissue sites, such as the colon. Here, we describe the application of a *T. cruzi* bioluminescent:fluorescent dual reporter strain and enhanced tissue processing and imaging procedures that have allowed us to show that incomplete homing of leukocytes, including T cells, to foci of intracellular infection is associated with the ability of the parasite to persist in the colon.

Results

Cellular immunity suppresses the colonic parasite load during chronic *T. cruzi* infection. Myocytes in the colonic gut wall are an important site of *T. cruzi* persistence in murine models of chronic Chagas disease. However, infected host cells are extremely rare and unevenly distributed (18), and their immunological micro-environment has not been systematically investigated. To assess the role of the immune response in controlling infection at a cellular level in this tissue compartment, we infected C3H/HeN mice with *T. cruzi* CL Luc::mNeon, a parasite line that constitutively expresses a bioluminescent:fluorescent fusion protein (27). This reporter strain can be used in combination with *ex vivo* imaging and confocal microscopy of colonic wall whole mounts to detect infection foci at single cell resolution (Materials and Methods). When infections had reached the chronic stage (>100 days post-infection), one cohort of mice was immunosuppressed with cyclophosphamide, an alkylating agent that is generally suppressive of leukocyte populations, including both innate cells and T cells (28), and which has been widely used to drive the reactivation of low-level *T. cruzi* infections in experimental settings (29-32). Cyclophosphamide itself has no growth promoting effect on intracellular amastigotes (Fig. S1). Treatment led to a major reduction in peripheral blood mononuclear cells (PBMCs) within 5-10 days (Fig. 1a and b; Fig. S2). In parallel, other groups of mice were subjected to antibody-mediated depletion of the circulating $CD4^+$ or $CD8^+$ T cell populations (Materials and Methods). This was achieved, with high specificity, in a similar time-scale (Fig. 1c; Fig. S2). Circulating anti-*T. cruzi* serum antibody levels were not significantly altered by

150 cyclophosphamide treatment, or by depletion of the $CD4^+$ or $CD8^+$ T cell subtypes
151 (Fig. 1d).
152
153 Examination of mouse tissue and organs by *ex vivo* bioluminescence imaging
154 (13,17) revealed that cyclophosphamide-induced immunosuppression had resulted
155 in a widespread increase in the intensity of infection (Fig. 2 and 3), including in the
156 skin, skeletal muscle, GI tract and heart. With $CD8^+$ depletion, skeletal muscle and
157 the skin were the only tissue sites where we observed a significant enhancement in
158 the level of infection, with increases of greater than one order of magnitude in some
159 instances. However, there was a wide variation in the parasite burden between mice
160 (Fig. 2 and 3), reflecting the dynamic nature of chronic disease infections (13). In the
161 internal organs, including the GI tract, there was no significant increase in infection
162 as a result of $CD8^+$ T cell depletion, at least within the time-frame of the experiment.
163 $CD4^+$ T cell depletion did not promote a relapse in any of the organs or tissue sites
164 examined (Fig. 2 and 3).
165
166 To further investigate the effect of perturbing the immune system, we undertook
167 detailed confocal microscopy analysis of external gut walls after removal of the
168 mucosal layer (Materials and Methods). This technique allows systematic
169 assessment of the full length and thickness of the longitudinal and circular smooth
170 muscle layers of the colon at a single cell level (18). Consistent with the *ex vivo*
171 imaging data (Fig. 3), this revealed that cyclophosphamide treatment had resulted in
172 a significant increase in the number of parasite infected cells (Fig. 4). Furthermore, in
173 the absence of PBMCs (Fig. 1a), it is implicit from the resulting parasite
174 dissemination, that the circulating serum antibodies are unable to maintain

suppression of the infection at this and other sites during the chronic stage (Fig. 2 and 3). However, specific depletion of either the $CD4^+$ or the $CD8^+$ T cell repertoires (Fig. 1c), by themselves, did not have a significant effect on the number of infected cells in the colonic gut wall (Fig. 4d).

Parasites persisting in the colon can induce effective localised T cell recruitment. At any one time, the majority of the parasite population that persists in the colon is found in a small number of infected cells that typically contain several hundred replicating amastigotes, or occasionally, differentiated non-dividing trypomastigotes (12). The remainder of the population is more widely distributed, with considerably lower numbers of parasites per infected cell. To better understand the process of long-term parasite survival, we investigated the cellular micro-environment of persistent infection foci. When infections had advanced to the chronic stage, peeled colonic wall whole mounts were examined by confocal microscopy (Materials and Methods), and compared to those of naïve, age-matched control mice. In the tissue from non-infected mice, using DAPI staining to highlight nuclei, an average of 55 host cells were identified in 200 μm diameter circles positioned around randomly selected nuclei within the whole mounted gut wall (Fig. 5a). Most cells had elongated nuclei typical of smooth muscle myocytes. In the infected group, parasitized cells were identified by green fluorescence (Materials and Methods). Scanning revealed that total cellularity in the immediate locality of infection foci was similar in most cases to that in non-infected colonic tissue; the cellularity of 95% of infection foci was within 3 x S.D. of the background mean, compared with 98% around randomly selected cells from naïve control regions (Fig. 5a and b). However, on occasions there was evidence of highly localised cellular infiltration, with 3.4% of

infection foci surrounded by a local cellularity that was >4 x S.D. above the background mean. Within these intense infiltrates, host cells with more rounded nuclei, typical of lymphocytes, predominated. In contrast to the majority of parasitized cells that had not triggered a detectable localised response (Fig. 5c), amastigotes in these inflammatory infiltrates frequently displayed an irregular morphology suggestive of immune-mediated damage, as judged by the diffuse pattern of green fluorescence (compare Fig. 5c and d).

We investigated the nature of these cellular infiltrates, by staining colonic gut wall whole mounts from chronically infected mice with specific immune cell markers (Materials and Methods). This revealed, as expected, that leukocytes (identified by anti-CD45 antibodies) constituted close to 100% of the infiltrate population (Fig. 6a). A major proportion of the recruited cells were also positive when stained with anti-CD3 antibodies, specific markers for the T-cell receptor complex (Fig. 6b and c), with both $CD4^+$ and $CD8^+$ T cells represented within this population (Fig. 6d). To assess the local density of stained immune cells, we examined 200 μm diameter circular tissue sections centred on each infection focus using Z-stack confocal microscopy. A series of imaged sections starting 5 μm above and 5 μm below the centre of the parasite nest (a total volume of 314 μm^3) were generated, and the number of stained cells in the infection micro-environment determined in 3-dimensions (Fig. S3). In sections of colonic smooth muscle from non-infected mice, leukocytes were dispersed and rare, with an average of ~1 $CD45^+$ cell per 314 μm^3, although they were more numerous in the sub-mucosal tissue (Fig. S4). Using a cut-off value of 3 x S.D. above the respective background level, 40 - 45% of infection foci displayed evidence of leukocyte infiltration (Fig. 6e). Therefore, despite being a site of parasite

persistence, dynamic homing of leukocytes, including T cells, to parasitized cells in the murine colon is a characteristic of chronic stage infection, although at any one point in time, not all parasite nests will have triggered this type of recruitment response. Given the 'snapshot' nature of imaging, our data therefore suggest that in the majority of cases, a likely outcome of colonic cell invasion will be infiltration of leukocytes prior to completion of the *T. cruzi* intracellular cycle, and the presumptive destruction of the parasites (Fig. 5d).

Incomplete homing of protective T cells allows a subset of intracellular colonic infections to complete their replication cycle. Evidence indicates that *T. cruzi* rarely occupies individual colonic myocytes for extended periods (>2 weeks) (12), suggesting that parasites are either efficiently eliminated by the immune response, or that they complete a cycle of replication, trypomastigogenesis and host cell lysis within this period. In addition, there is considerable variation in the level of infection within individual colonic cells at any one time, with parasite numbers that can range from 1 to >1000 (12). We therefore investigated whether the immune response induced against infected cells increased in line with the intra-cellular parasite burden. When the levels of infiltrating leukocytes in the local environment of infected cells were compared with the number of intracellular *T. cruzi* parasites, we found no apparent correlation (Fig. 7a, b and c). This was the case irrespective of whether anti-CD45, anti-CD4 or anti-CD8 antibodies were used to assess the nature of the cellular infiltrate. It is implicit therefore, that the elapsed duration of an individual intracellular infection, as inferred from the extent of parasite proliferation, is not a determinant of the likelihood of detection and leukocyte homing to that site.

Of 237 infected colonic cells detected in 13 animals, only 4 (~1.7%) contained parasites that had clearly differentiated into flagellated trypomastigotes, the life-cycle stage that disseminates the infection by re-invasion of other host cells, or via transmission to the blood-sucking triatomine insect vector. Of these, three contained large numbers of parasites (>1,000), while the fourth contained 128. In each case, the leukocyte densities in the local micro-environment were within a range similar to host cells where the infection was less mature, as judged by the number of intracellular amastigotes and their lack of differentiation into trypomastigotes. In the example shown (Fig. 8a and b), Z-stack imaging was used to serially section a large nest containing >1,000 parasites, and shows mature trypomastigotes in the act of egress, despite the recruitment of a small number of $CD45^+$ leukocytes, including $CD8^+$ T cells (Fig. 8c and d). Therefore, for a small proportion of infected cells, including in some cases large and mature parasite pseudocysts, the host immune system is either not triggered locally by an infection, is too slow to respond, or is in some way suppressed. As a result, in the colon, it is possible for the entire cycle of parasite proliferation, differentiation and egress to occur in the absence of effective intervention by a cellular immune response, at a level sufficient to allow prolongation of the chronic infection.

Discussion

Despite the generation of a vigorous and specific $CD8^+$ T cell response (4,14,33,34), *T. cruzi* infections in mice are rarely cleared to sterility, even in vaccinated animals. Instead, parasites persist predominantly in a small number of reservoir tissue sites, typically for the life-time of the host (10). One possibility is that intermittent dissemination from these locations to less permissive organs, which include the heart, could promote repeated episodes of infection, resulting in localised inflammatory responses that contribute to disease pathology in a cumulative manner (35). Understanding why the immune system efficiently suppresses, but fails to eliminate *T. cruzi* infections, is one of the key challenges in Chagas disease research. Here, using tissue processing techniques that allow the immunological micro-environment of infection sites in the colon to be assessed at single cell resolution, we demonstrate that $CD45^+$ leukocytes, including both $CD4^+$ and $CD8^+$ T cells, are frequently recruited to chronic infection foci within a reservoir tissue. However, for a small sub-set of infected cells, effector cell recruitment is either absent, or too slow to prevent completion of the intracellular cycle of parasite proliferation and differentiation to the motile trypomastigote stage (Fig. 8). Thus, chronic *T. cruzi* infections in the colon are not characterised by a generalised tissue-specific latency, but by a dynamic equilibrium between host and pathogen.

T cell recruitment during *T. cruzi* infection is driven by secretion of chemokines from infected cells. For example, the CXCR3 ligands CXCL9 and CXCL10 have been implicated in cardiac infiltration (36). IFN-γ and TNF-α expression by antigen specific $CD8^+$ T cells (4), and subsequent iNOS expression (37-39), potentially from recruited innate monocytes/macrophages or from somatic cells of the infected tissue, then increases the local concentration of reactive nitrogen species. In Chagas disease, the resulting inflammatory environment tightly controls the number of infected cells, but can also act as the key driver of chronic immunopathology (7,14,40,41). An important observation from our study is that the likelihood of T cell recruitment in the colon is not linked with the maturity of individual *T. cruzi* nests, as judged by the intracellular parasite load (Fig. 7). As a result, in some parasitized cells, differentiation to the flagellated trypomastigote form can occur without inducing infiltration of leukocytes in sufficient time to block a productive infection (Fig. 8).

The reasons why protective T cells are not recruited to a small sub-set of infection foci are unclear. Hypothesised mechanisms to account for *T. cruzi* immune evasion include a general absence of pathogen associated molecular patterns (PAMPs) (42), cytokine-mediated inhibition of effector responses (10), insufficiently strong chemoattractant signalling in low parasite load settings (40), the extensive antigenic diversity expressed by the large families of *trans*-sialidase and mucin genes (14,43,44), and stress-induced cell-cycle arrest and dormancy (19). However, none of these obviously correspond with our observation that there is an apparent lack of association between the extent or longevity of an individual cellular infection and the magnitude of localised leukocyte recruitment (Fig. 7). Some highly infected host cells appear to be invisible to the immune system, whereas other much smaller nests

trigger massive cellular infiltration. One explanation could be that a slow-down in the intracellular amastigote replication rate during chronic stage infections (12) contributes to reduced immune detection. In circumstances where the infected cell is in an area of the colon that is otherwise parasite-free, this may be sufficient to permit completion of the initial replication cycle. However, after trypomastigote egress and host cell lysis, the resulting tissue disruption and production of damage associated molecular patterns (DAMPs) could act to enhance leukocyte recruitment into the area, leading to the destruction of parasites that have re-invaded host cells in the vicinity of the initial infection. In contrast, trypomastigotes which migrate further from this DAMP-enriched locality may be able to establish a productive infection in the absence of rapid immune detection. Despite a diverse and complex antigenic repertoire, induction of the T cell response in draining lymph nodes is known to be highly focussed (14), and once T cell recruitment has been triggered, parasite destruction can be initiated (Fig. 5d). Widespread parasite dormancy was not evident in the colon (12), and does not appear to be necessary for immune evasion in this tissue site.

Success or failure of the immune system in eliminating these rare chronic infection foci may be a largely stochastic process resulting from the dynamic interplay between the host and pathogen at a single cell or tissue micro-domain level. If parasites were able to universally suppress innate detection pathways, with concomitant reduction in localised chemokine output, this would have a negative impact on host survival, and thus long-term *T. cruzi* transmission. Conversely, if nests were always detected by the immune system before completion of the replication cycle, the parasite would risk host-wide elimination. The ability of *T. cruzi*

352 to persist in some organs/tissues, may therefore be dependent on the propensity, or
353 otherwise, of these tissues to amplify the chemokine signals triggered by low-level
354 infection, with a possible role for closely adjacent re-infections in the amplification
355 process. In mice, there are strain-specific differences in the extent of such tissue-
356 restriction during chronic infections. This could have parallels in humans, and
357 account for the heterogeneous profile of disease progression.

364

365 *T. cruzi* infection induces a high titre polyclonal B cell/antibody response during the
366 acute stage of infection, which although delayed and initially unfocussed (45), does
367 contribute to parasite control and can protect against virulent infections. In the
368 chronic stage, a role for the humoral response in suppressing the dissemination of
369 persistent parasites is unresolved (10), and a key role for B cells has not been
370 identified. Here, we show that in the absence of PBMCs, circulating antibodies,
371 which in the short-term are not profoundly affected by cyclophosphamide treatment
372 (46) (Fig. 1d), are unable to compensate for T cell depletion and maintain tissue-
373 specific repression of the parasite burden (Fig. 2 and 4d). If the humoral response
374 does have a significant protective role during the chronic stage, for example,
375 involving opsonisation of the parasite through FcR-antibody binding, then this
376 function could be lost on depletion of key cellular effectors. In addition, our results do
377 not exclude the possibility that parasite-specific antibodies could act to limit
378 infections at a systemic level, over a longer duration, perhaps by controlling
379 trypomastigote numbers or restricting their spatial dissemination.

380

381 The central role of $CD8^+$ T cells in suppressing *T. cruzi* infections is well established,
382 and in various parasite:mouse strain combinations, depletion of circulating $CD8^+$ T

383 cells leads to partial recrudescence in specific organs (4,5,15,34). In the
384 experimental model outlined here, this relapse took place in skeletal muscle and skin
385 (Fig. 2 and 3), although had the period of $CD8^+$ depletion been extended, we cannot
386 exclude the possibility that relapse, as inferred from the bioluminescence signal,
387 would also have been identified at other sites. When we examined the effect of $CD8^+$
388 T cell depletion at a cellular level in the colon, where tissue processing procedures
389 allow systematic analysis, we found no significant increase in the number of infected
390 cells, in contrast to the major rebound observed with cyclophosphamide-mediated
391 reduction of the entire PBMC population (Fig. 1-4). Whether this was a result of less
392 efficient depletion of $CD8^+$ T cells at this site, or that the protective role is better
393 covered by $CD4^+$ T cells or innate populations, will be an important question to
394 address. A non-redundant function for $CD4^+$ T cells is less well established in murine
395 models of Chagas disease (47-49), although in humans with untreated HIV co-
396 infections, parasites become easily detectable in the bloodstream and can result in
397 CNS pathology (50). Since depletion of either $CD4^+$ or $CD8^+$ T cells by themselves
398 did not promote the level of systemic relapse observed with cyclophosphamide
399 treatment over the time period analysed (Fig. 2 and 3), our results therefore suggest
400 that either both lymphocyte sub-types are able to contribute to suppression of
401 chronic stage infections in the colon, or that innate monocytes/macrophages are able
402 to provide a covering role during this time-period. The further development of tissue
403 processing and imaging procedures applicable to other organs and tissues, to allow
404 systematic analysis of chronic infections at single cell resolution, will be an important
405 step in extending these observations more widely.
406

If our findings in experimental mice are translatable to humans, this will have important implications for anti-*T. cruzi* vaccine development. Vaccines protect by presenting non-tolerised antigens in the correct immunological context, to expand small numbers of antigen-specific naïve T and B cells, which then generate a sub-population of memory cells. The expanded memory populations then allow more rapid deployment of adaptive effectors on future contact with the pathogen. However, *T. cruzi* is able to persist indefinitely in hosts that already have expansive systemic populations of effective T cells. Unless vaccines can prevent parasites from accessing permissive sites after the initial infection, or they are able to enhance successful homing of adaptive effector cells, it will be difficult to achieve sterilising immunity. Drug-cured infections can confer complete protection against re-challenge with a homologous strain, but with heterologous strains, despite the prevention of an acute stage peak, the infection proceeds directly to a status that is analogous to the chronic stage in terms of parasite burden and tissue distribution (3). Therefore, it is likely that successful anti-*T. cruzi* vaccines will require an ability to eliminate parasites at the initial site of infection during the first intracellular replication cycle. This will be a considerable challenge.

Materials and Methods

Mice and parasites. All experiments were performed using female C3H/HeN mice, purchased from Charles River (UK). They were maintained in individually ventilated cages, under specific pathogen-free conditions, with a 12-hour light/dark cycle, and provided with food and water *ad libitum.* Research was carried out under UK Home Office project licenses PPL 70/8207 and P9AEE04E4, with approval of the LSHTM Animal Welfare and Ethical Review Board, and in accordance with the UK Animals (Scientific Procedures) Act 1986 (ASPA). The *T. cruzi* line CL Luc::mNeon, a derivative of the CL Brener strain (discrete typing unit TcVI), was used in all experiments. It had been genetically modified to express a bioluminescent:fluorescent fusion protein containing red-shifted luciferase and mNeonGreen fluorescent domains (27,51). For infections, C3H/HeN mice, aged 6-8 weeks, were inoculated i.p. with $1x10^3$ bloodstream trypomastigotes obtained from immunodeficient CB17-SCID mice, as described previously (30). Mice were then monitored by *in vivo* bioluminescence imaging (17) which indicated that they had transitioned to the chronic stage by 50-60 days post-infection. Experiments were performed when mice had been infected for at least 100 days.

455

456 **Suppression of the murine immune response.** General immunosuppression was
457 achieved by injecting mice i.p. with cyclophosphamide (200 mg/kg) at 4-day
458 intervals, up to a maximum of 3 injections, in accordance with animal welfare
459 (17,30). Circulating $CD8^+$ T cells were depleted by i.p. injection of 400 μg of the YTS
460 169.4 monoclonal anti-CD8α (2BScientific), diluted in PBS, at 4-day intervals, up to a
461 maximum of 4 times (Fig. 1C). The same regimen was applied for depletion of $CD4^+$
462 T cells, using the GK1.5 monoclonal antibody (2BScientific).

463

464 **Tissue processing and imaging.** When mice were sacrificed, organs and tissues
465 were removed and transferred to a Petri dish in a standardized arrangement, soaked
466 in 0.3 mg/ml d-luciferin in PBS, and examined by *ex vivo* bioluminescence imaging
467 using the IVIS Spectrum system (Caliper Life Science) and the LivingImage 4.7.2
468 software (52). The skin was removed from the carcass, and following subcutaneous
469 adipose tissue removal, was placed fur down, soaked in 0.3 mg/ml d-luciferin and
470 imaged under the same conditions as the internal organs. The carcass was placed
471 dorsal side up, soaked in 0.3 mg/ml d-luciferin, and imaged as above.

472

473 Colonic muscularis walls were isolated by peeling away the mucosa, whole mounted
474 as described previously (18), and then exhaustively searched for parasites (green
475 fluorescence) with a Zeiss LSM880 confocal microscope. Small tissue sections (~5
476 mm^2) around parasite nests were excised from the whole mount by scalpel, washed
477 twice in PBS and incubated for 2 days in 1:300 primary antibody diluted in PBS / 5%
478 fetal calf serum / 1% Triton-X100 at 4°C. Following 2 further washes in PBS,
479 secondary antibody diluted 1:500 in the same blocking/permeabilising solution was

added to the tissue sections, and incubated for 3 hours at room temperature. Sections were then mounted in Vectashield, containing the DNA stain DAPI, and imaged by confocal microscopy. Colonic muscularis walls from naïve aged-matched mice were similarly prepared as controls, with and without the primary antibody.

For accurate determination of intracellular parasite and surrounding host cell numbers, tissue samples were imaged in 3-dimensions (Z-stacking), with the appropriate scan zoom setting (18). The Image Browser overlay function was used to add scale bars, and images were exported as .TIF files to generate figures. Primary antibodies used were as follows: anti-luciferase (G7451, Promega), CD45 (Tonbo Biosciences, 30-F11), CD3 (Abcam, ab11089), CD4 (Abcam, ab25475), CD8 (Abcam, ab25478). The secondary antibodies were Invitrogen A-11055, Invitrogen A-21434, Invitrogen A-11007.

Flow cytometry. At each time-point, mice were placed in a "hot box" and left at 38^{o}C for 10 minutes. They were then placed in a restrainer and the lateral tail vein punctured using a 0.5M EDTA (pH 7.4) soaked 21G needle. A single drop of blood was transferred to a 2 ml tube and 10µl 0.5M EDTA added to prevent clotting. Each sample was then mixed with 400 µl ice-cold PBS and placed onto 300 µl Histopaque 1083 (Sigma-Aldrich), and spun at 400 *g* for 30 minutes in a microcentrifuge. The monocytic layer was aspirated using a pipette, mixed with 1 ml ice-cold PBS, pelleted and resuspended in 200 µl flow cytometry buffer (PBS, 5% fetal bovine serum, 0.05% sodium azide), and 1 µl of the cocktail of conjugated antibodies added (1:200 dilution in each case). After 1 hour incubation in the dark, cells were pelleted and re-suspended in 2% paraformaldehyde in PBS, followed by a further 45 minutes

505 incubation in the dark. The stained fixed cells were then pelleted, re-suspended in
506 filtered flow cytometry buffer and transferred to standard flow cytometry tubes.
507 Samples were analysed using a BD Bioscience LSRII flow cytometer, with plots
508 created and analysed in FlowJo V.10.6.1. The following antibodies were used: CD45
509 (ThermoFisher, 30-F11, Super Bright 600), CD3 (ThermoFisher, 17A2, FITC), CD4
510 (ThermoFisher, RM4-5, eFluor 450), and CD8 (ThermoFisher, SK1, Alexa Fluor
511 780).
512
513 **α-*T. cruzi* antibody ELISA.** 96-well plates were coated with sonicated *T. cruzi* CL
514 Luc::mNeon trypomastigote lysate; 100 μl (0.5 μg) per well diluted in 15 mM Na_2CO_3,
515 34.8 mM $NaHCO_3$. The plates were incubated at 4°C overnight to allow antigen
516 binding, washed 3x with PBS / 0.05% Tween 20, and then blocked with PBS / 2%
517 milk powder. Diluted murine serum samples, collected from each Histopaque
518 separation, were further diluted to 1:1600. These were aliquoted in triplicate (100 μl
519 per well) and incubated for 1 hour at 37°C. Horse radish peroxidase (HRP)
520 conjugated anti-mouse IgG secondary antibody (Abcam, ab99774) was then added
521 (1:5000; 100 μl per well), and the plates incubated for a further 1 hour. After the
522 addition of HRP substrate (80 μl per well) (Stabilised TMB, Life Technologies), the
523 plates were incubated at room temperature in the dark for 5 minutes and read using
524 a FLUOstar Omega plate reader (BMG LABTECH), after the addition of 40 μl 1M
525 HCl.
526
527 **Testing for the effect of cyclophosphamide on *T. cruzi* growth.** A 96-well plate
528 was seeded with 5000 MA104 cells/well, and 18 hours later these were infected with
529 culture derived *T. cruzi* CL Luc::mNeon trypomastigotes at an MOI of 5:1. Invasion

was allowed to occur overnight, the wells were washed thoroughly with serum-free medium, and intracellular amastigotes allowed to proliferate for 24 hours. Cyclophosphamide was then added up to a concentration of 200 μM. 3 days later, the intensity of green fluorescence was recorded on a FLUOstar Omega plate reader, and the impact on parasite growth assessed.

Statistics. Analyses were performed in GraphPad PRISM v8.0. S.D. Fold change in bioluminescence intensity was compared using a one-way ANOVA with Dunnett's pairwise comparisons. Background cellularity and $CD45^+$, $CD4^+$ and $CD8^+$ cut-offs were set as mean + 3 x S.D. Data sets were compared using a 2-sample t-test with Welch correction. If data were not normally distributed, as assessed using a Shapiro-Wilk test, a Mann-Whitney rank sum test was used.

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738 **FIG 1** Suppression of cellular immunity in mice chronically infected with *T. cruzi.* (a)
739 C3H/HeN mice chronically infected (>100 days) with *T. cruzi* CL Luc::mNeon (n=6)
740 were immunosuppressed by i.p. inoculation with cyclophosphamide (200 mg/kg) at
741 4-day intervals, up to a maximum of 3 injections (Materials and Methods). The %
742 events recorded as peripheral blood mononuclear cells (PBMCs) at different time
743 points after the initiation of treatment for individual mice are shown. Also included in
744 the day 1 values are additional data points (n=24) from immunocompetent
745 chronically infected mice. (b) Flow cytometry plots showing the loss of detectable
746 events in the PBMC gate (black oval) over the course of cyclophosphamide
747 treatment (see also Fig. S2). PBMCs were identified based on the spectral forward

748 (FFC, Y-axis) and side (SSC, X-axis) scatter. (c) Effective depletion of T cell subsets
749 by treatment of mice with specific anti-CD4 or anti-CD8 antibodies (Materials and
750 Methods). The graphs show the $CD4^+$ and $CD8^+$ flow cytometry events of individual
751 mice as a % of the total $CD3^+$ population over the treatment periods. (d) ELISA mean
752 absorbance readings (using anti-mouse IgG secondary antibody) for serum from
753 chronically infected mice that had been treated with cyclophosphamide, or treated
754 with anti-CD4 or anti-CD8 antibodies. Microtitre plates containing *T. cruzi*
755 trypomastigote lysates were prepared as described (Materials and Methods).
756 Dashed red lines identify the mean, $\pm$1 x S.D. and $\pm$2 x S.D. values, determined from
757 immunocompetent chronic stage controls (n=28). One of the anti-CD8 antibody
758 treated mice died between day 5 and 9, and was excluded from subsequent
759 analysis.
760
761 **FIG 2** Fold change in radiance (p/s/cm^2/sr) established by *ex vivo* bioluminescence
762 imaging of internal tissues and organs from C3H/HeN mice chronically infected with
763 *T. cruzi* (control), and after treatment with cyclophosphamide, anti-CD4 or anti-CD8
764 antibodies, as indicated (Materials and Methods). Infection intensities were
765 determined using LivingImage software to draw individual regions of interest around
766 each organ and tissue sample (17). Data from infected mice were normalised to
767 account for variations in background radiances of different tissue types by using
768 matching tissues from uninfected controls to establish the fold change. The maximal
769 value from the uninfected organs was used. The dashed line indicates the detection
770 threshold, equal to the mean +2 SDs of the bioluminescence background derived
771 from the fold change between empty regions of interest in tissue from age-matched
772 uninfected mice and empty regions from chronically infected animals. Control data

773 points also include values from additional immunocompetent chronically infected
774 mice (n=17) (18). Means are compared with a one-way ANOVA with post hoc
775 Dunnett's pairwise comparisons test; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.
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777
778 **FIG 3** Tissue-specific impact of T cell depletion on parasite burden. C3H/HeN mice
779 chronically infected with *T. cruzi* CL Luc::mNeon were treated with
780 cyclophosphamide, anti-CD4 or anti-CD8 antibodies as outlined in the legend to Fig.
781 1. 16 days post-treatment initiation, organs and tissues were examined by *ex vivo*
782 imaging (52) (Materials and Methods). (a) Representative bioluminescence images
783 of internal organs from treated mice arranged as shown in the inset (left). (b) Dorsal
784 bioluminescence images following removal of internal organs, fur, skin and major
785 adipose depots (Material and Methods). (c) *Ex vivo* bioluminescence imaging of skin
786 (adipose tissue removed). Radiance ($p/s/cm^2/sr$) is on a linear-scale pseudo-colour
787 heat map. The heat map image of skeletal bioluminescence after treatment with anti-
788 CD8 antibodies is shown at an increased minimum and maximum radiance ($1x10^4$ –
789 $1x10^6$) to avoid saturation of the image. The complete radiance data set is shown in
790 Fig. 2.
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793 **FIG 4** Control of parasites in the colon of chronically infected mice is lost on
794 suppression of cellular immunity. (a) Colon sections from C3H/HeN mice chronically
795 infected with *T. cruzi* CL-Luc::mNeon were pinned luminal side up and examined by
796 e*x vivo* bioluminescence imaging. Radiance ($p/s/cm^2/sr$) is on a linear-scale pseudo-
797 colour heat map. Upper inset, colonic sections from non-treated infected mice; lower

inset, section from mice immunosuppressed by cyclophosphamide treatment (Materials and Methods). (b) Schematic highlighting the distinct layers of the GI tract. The dashed red line and arrow indicate the position above which tissue can be peeled off to leave the external colonic wall layers (18). (c) External gut wall whole mounts were examined in their entirety at a 3-dimensional level by confocal microscopy. Examples of parasite infected cells in immunocompetent mice and their locations, detected by green fluorescence (mNeon). DAPI staining (blue) identifies host cell nuclei. Scale bars=20 μm. (d) The total number of parasitized cells counted in each whole mounted colonic gut wall for the control and the immune-depleted groups. Each dot represents a single mouse, with the colons examined 12-22 days post treatment initiation (see Fig. 1d). **** = p≤0.0001. Differences between control values and those obtained from mice that had been treated with anti-CD4 and anti-CD8 antibodies were non-significant.

FIG 5 Defining the localised cellularity of *T. cruzi* infected host cells in the colonic gut wall. (a) Images of whole mounted colonic gut wall from C3H/HeN mice chronically infected with *T. cruzi* CL-Luc::mNeon (Materials and Methods). When infection foci were identified, 200 μm diameter circles were drawn centred on each parasite cluster or 'nest'. Circles were placed by centring on randomly selected cells in the case of non-infected age-matched controls (top left panel). DAPI-stained nuclei (blue) that fell within this disc (highlighted by white dots) were counted as a measure of cellularity. Intracellular parasites can be identified by green fluorescence. These are indicated by white arrows in the lower images. (b) Background cellularity around randomly selected cells (n=48) on whole mounted colonic gut walls from

naïve age-matched C3H/HeN mice was established as above. With tissue from chronically infected mice, localised cellularity was calculated using circles centred on parasite foci (green) (n=247). Individual values are indicated by blue (non-infected) and green (infected) dots. The dashed lines indicate 3 x S.D. and 4 x S.D. above the background mean. (c) An infected myocyte where the local cellularity is equivalent to the background level and the intracellular amastigotes (green) are structurally intact. (d) Zoomed-in image of an intense cellular infiltrate (nuclei, blue) in which the *T. cruzi* parasites (green) display an irregular and diffuse morphology. Parasite DNA is identifiable as small discrete DAPI-stained spheres throughout this inflammatory focus (examples indicated by white arrows). Scale bars=20 μm.

FIG 6 T cells are major constituents of the leukocyte population recruited to chronic stage infection foci. (a) Confocal images of colonic gut wall sections from chronically infected mice (Materials and Methods). Rare infection foci were identified by mNeonGreen fluorescence (parasites) after exhaustive searching of whole mounted gut walls. Staining with anti-CD45 (orange) reveals that hematopoietic cells constitute the vast majority of the infiltrate population. Host cell nuclei were identified by DAPI staining (blue). (b) Anti-CD3 staining of cellular infiltrates shows that T cells constitute a majority of the population. Blue, host cell nuclei; red, CD3 staining; green, parasite fluorescence. (c) Serial Z-stack imaging (Materials and Methods) through the same cellular infiltrate as in b, showing selected sections through the infiltrate. (d) Histological sections containing cellular infiltrates and associated infection foci (parasites, green; indicated by white arrows in right-hand image) stained with either anti-CD4 (purple) or anti-CD8 (yellow) antibodies. Scale bars=20

μm. (e) Whole mounts containing infection foci were stained with anti-CD45, anti-CD4, or anti-CD8 antibodies and the number of positive host cells in the immediate vicinity (314 μm^3 volume) was determined by serial Z-stack confocal imaging. Each dot corresponds to a single infection focus. The horizontal dashed line is 3 x above the S.D. of the mean background level in non-infected tissue. In the case of anti-CD45 staining, none of the 50 tissue regions examined from non-infected mice contained CD45+ve positive cell numbers above this value. 41%, 45% and 42% of infection foci identified by CD45, CD4 and CD8 staining, respectively, were above this cut-off.

FIG 7 Lack of correlation between intracellular parasite load and localised T cell infiltration during chronic infections. (a) Comparison of the parasite numbers in infected colonic gut wall cells with the local leukocyte cell density. Infection foci were identified in whole mounts of colonic tissue, which were then stained with anti-CD45 antibody (Materials and Methods). The parasite and cell numbers in a tissue volume of 314 μm^3 were determined using serial Z-stack imaging, with leukocytes identified by orange staining and parasites by green fluorescence. The horizontal dashed line is 3 x above the S.D. of the mean background level in non-infected tissue. Each dot identifies a single infection focus, with tissue samples derived from 6 mice (71 infection foci). The confocal images show representative infection foci used to generate the data, and illustrate the varying extents of leukocyte infiltration. (b) Similar analysis of infection foci using anti-CD4 staining (purple). Tissue samples were derived from 3 mice (54 infection foci). (c) Analysis of infection foci using anti-CD8 staining (yellow). Tissue derived from 4 mice (116 infection foci).

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875 **FIG 8** Incomplete recruitment of leukocytes to parasite nests allows progression of
876 *T. cruzi* through the full intracellular infection cycle. (a) An intense bioluminescent
877 focus in a chronic stage distal colon viewed by *ex vivo* imaging (Materials and
878 Methods). Radiance (p/s/cm^2/sr) is on a linear-scale pseudocolour heatmap. (b)
879 Confocal imaging of the corresponding parasite nest showing representative serial Z-
880 stack images taken along the depth of the infected cell. The Z-axis position relative
881 to the centre of the nest is indicated above each of the images. Parasite numbers
882 (>1000) were established from green fluorescence and the characteristic DAPI
883 staining of the parasite kinetoplast DNA (the mitochondrial genome) (18) (blue).
884 Infiltrating leukocytes (orange) were identified by staining with anti-CD45 antibodies
885 (Materials and Methods). Scale bar=20 μm. (c) Enlarged images of a small cluster of
886 infiltrating CD45$^+$ (orange) and CD8$^+$ (yellow) cells in close vicinity to the nest. White
887 arrows indicate leukocytes corresponding to CD8$^+$ T cells. (d) Egress of
888 differentiated trypomastigotes into the extracellular environment. Data from the
889 infected cell captured in these images was not included in Fig. 7 since the parasite
890 burden was too great to determine numbers with precision.

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