Animal Models for Exploring Chagas Disease Pathogenesis and Supporting Drug Discovery

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SUMMARY

Infections with the parasitic protozoan *Trypanosoma cruzi* cause Chagas disease, which results in serious cardiac and/or digestive pathology in 30-40% of individuals. However, symptomatic disease can take decades to become apparent, and there is a broad spectrum of possible outcomes. The complex and long-term nature of this infection places a major constraint on the scope for experimental studies in humans. Accordingly, predictive animal models have been a mainstay of Chagas disease research. The resulting data have made major contributions to our understanding of parasite biology, immune responses and disease pathogenesis, and have provided a platform that informs and facilitates the global drug discovery effort. Here, we provide an overview of available animal models, and illustrate how they have had a key impact across the field.

INTRODUCTION

Chagas disease results from infection with the parasitic protozoan *Trypanosoma cruzi* and is a significant public health problem across much of Latin America, with an estimated 6-7 million people infected (1). In addition, the disease has begun to take on an increasingly global perspective, with an additional 0.5 million infected individuals now resident outside endemic regions, particularly in the US and Europe (2, 3). In the immediate future, there is little prospect of a useable vaccine against *T. cruzi* infection, the current drugs have limited efficacy, and the mechanisms of disease pathogenesis are poorly understood. Research progress in these areas has been limited by the long-term nature of the infection, the range and complicated features of disease pathology, and the wide genetic diversity of the parasite.

T. cruzi is a member of the Kinetoplastida (family Trypanosomatidae), a group of flagellated protozoa that also includes *Trypanosoma brucei* subsp. and *Leishmania* spp., parasites that cause African trypanosomiasis and leishmaniasis, respectively. The *T. cruzi* species has been sub-divided into six major genetic lineages, or Discrete Typing Units (DTUs TcI-TcVI) (4, 5), with a seventh, the bat-derived TcBat, that has more recently been described (6). TcI and TcII are thought to represent long-established ancestral lineages, with TcV and TcVI proposed to be hybrids derived from TcII and TcIII. There have been reports of associations between DTUs and traits such as host-preference, drug-susceptibility and disease pathology. However, definitive evidence that provides a molecular basis for the proposed associations remains elusive. A recent review (5) is recommended for a comprehensive update on *T. cruzi* population biology.

T. cruzi is an obligate intracellular parasite, has an extremely wide host range, and is able to infect most, if not all mammals (Table 1). In endemic regions, transmission is primarily vector-mediated via hematophagous triatomine bugs (7). They deposit faeces contaminated with non-replicating metacyclic trypomastigote forms of the parasite onto the skin whilst taking a blood meal. Infection occurs when parasites are then rubbed or scratched into the wound in response to irritation, or transferred to sites where they can cross mucous membranes, such as the eye. Other important routes of infection include congenital transmission, the oral route through consumption of parasite contaminated food or drink, and medical interventions such as blood transfusion or organ transplantation (8 - 11). Upon infection, the flagellated metacyclic trypomastigotes invade host cells, escape from the parasitophorous vacuole and differentiate into the ovoid amastigote intracellular stage that is characterised by a considerably reduced flagellum length (Fig. 1). Amastigotes then replicate in the cytoplasm by binary fission in an asynchronous manner (12). In some host cell types, such as myocytes, parasite numbers can rise to beyond 1000, although a few hundred is more typical (13). This is followed by differentiation into non-replicative bloodstream form trypomastigotes, rupture of the host cell and release of these flagellated infectious forms into the bloodstream or interstitial spaces (Fig. 1). Parasite propagation is then enabled by infection of other host cells or by uptake in a triatomine blood meal, where the parasite undergoes a further round of differentiation into the insect-form epimastigote stage, that is flagellated and replicative.

Human infections with *T. cruzi* are usually life-long (14). The initial acute stage is characterised by a patent parasitemia, and typically the infection manifests as a mild, non-specific, febrile-like illness, although in many cases it can be asymptomatic. As a

result, the majority of those infected are unaware of their status. On occasions, the acute stage disease can be more serious, especially in children, leading to encephalopathy or myocarditis, sometimes with fatal consequences (15, 16). The acute stage usually resolves within 6-8 weeks. This is accompanied by immune-mediated reduction of the parasite burden and entry into an asymptomatic disease phase, that in 60-70% of those infected, lasts for their life-time. The remainder of infected individuals develop progressive cardiac and/or digestive pathology over a period of years, with a broad spectrum of outcomes (17, 18). Chronic *T. cruzi* infection is a leading cause of cardiomyopathy in many areas of Latin America and constitutes the main public health burden associated with the disease (19, 20). The digestive pathology, which includes megaoesophagus and megacolon, can also result in severe morbidity, with surgery and palliative care being the only options to alleviate symptoms (21, 22).

The nitroheterocyclic agents benznidazole and nifurtimox are the drugs currently used to treat *T. cruzi* infections (23 - 25). However, both have important limitations, particularly against infections in adults that have progressed to the chronic stage. Cure rates are sub-optimal (typically 50-80%), and toxicity, combined with long administration periods (2-3 months), has a negative impact on the ability of patients to complete the course of treatment (26 - 28). It is also difficult to monitor treatment efficacy. Highly sensitive PCR methods to detect residual infection are confounded by the intermittent nature of bloodstream parasites, which are often confined to rare infection foci in deep tissue, and there is a lack of robust biomarkers for sterile cure (25). These factors have acted as a constraint on clinical trials, since they necessitate long follow-up periods to confirm curative outcomes and the subsequent impact on

preventing or alleviating symptoms. In addition, features of parasite biology, such as a possible role for dormancy or quiescence, could also have a role in recrudescence (29 - 31). Nevertheless, drug development consortia, which bring together big pharma with the not-for-profit and academic sectors, have had successes in advancing lead anti-parasitic compounds into pre-clinical testing (32, 33). As we describe in this review, predictive animal models have had a crucial role in driving progress in this and other areas of Chagas disease research.

T. CRUZI: A HIGHLY PROMISCUOUS PARASITE

Naturally-acquired infections with *T. cruzi* have been reported in at least 150 mammalian species (34), and the consensus is that most, if not all mammals, are susceptible (Table 1). Infected animals are ubiquitous throughout South and Central America, Mexico and the Southern USA (35 - 40). In the latter area, infections amongst wild life have been detected from the East Coast to the West (41), and infection rates of 10 - 50% are commonly reported (42 - 45). The potential for infected pets and domestic livestock to act as a parasite reservoir (Table 1) is frequently highlighted as a potential threat to public health. Surveys in countries such as Brazil, Argentina, Colombia and the USA (46 - 49) show that dogs often harbour parasites and develop symptoms of Chagas disease similar to those in humans. For example, infected working dogs (US border control) can display serious Chagasic cardiac pathology (50, 51). Although spill-over of *T. cruzi* infection from domestic animals to humans is widely considered an issue of concern, evidence suggests that geographical and epidemiological context are critical factors that determine the extent of this risk (40, 52 - 54). In the Southern USA for example, although *T. cruzi* infections of triatome vectors

are widely reported, autochthonous human cases are rare. House construction methods and other structural barriers reduce the opportunities for home colonisation [55].

In addition to mammals, infections of other vertebrate groups have occasionally been reported (Table 1). Short term infection in chickens following parasite inoculation has been observed, but these are transient, and this has led to the hypothesis that birds are largely refractory to prolonged natural infection, possibly as a result of higher ambient body temperature (41^{o}C) and/or innate immune responses (56, 57). However, more recently, examination of an American barn owl (a road-kill victim in Mexico) has revealed *T. cruzi* infection in multiple organs and tissues (58). More research is therefore required to establish the species range and epidemiological significance of avian infections. There have also been reports of both naturally acquired and experimental infections in lizards (59, 60), although how widespread *T. cruzi* infections are within natural populations remains to be determined. Finally, experimental infections of zebra fish larvae have led to the suggestion that this host could serve as a model to investigate *T. cruzi* motility *in vivo* (61). The transparent nature of larvae has allowed parasite dissemination to a variety of organs and tissues to be imaged over an extended period (7 days post-infection). However, wider utilisation of the zebra fish model will be restricted by the apparent inability of the parasite to transition through its intracellular life-cycle in this background (Fig. 1).

Because of the transient and often asymptomatic/non-specific nature of symptoms in humans, there are few data on the tissue distribution of parasites during the acute phase of the disease. In experimental mammalian infections, *T. cruzi* is pan-tropic

during the acute stage, and unusually amongst eukaryotic protozoan parasites, seems capable of invading and proliferating in any nucleated cell. In mice, for example, although the initial distribution profile can be influenced by the parasite strain or inoculation route (62 - 65), infections then disseminate rapidly such that parasites become readily detectable in the bloodstream and widely distributed in organs and tissues (66 - 69). This can be clearly demonstrated by *in vivo* and *ex vivo* bioluminescence imaging (see below for further details), which has revealed that acute stage infections are characterised by a high parasite burden in the skin, and in all major internal organs (Fig. 2). When infections transition to the chronic stage, immune-mediated mechanisms reduce parasite numbers by up to 1,000-fold (70 - 72). They become restricted predominantly to a smaller number of sites, commonly the GI tract, skin and skeletal muscle, depending on the mouse:parasite combination (65, 70, 73). Persistence in the chronic stage reflects tissue-specific immune tolerance rather than tropism on the part of the parasite (65). In hamsters, which are amenable to *in vivo* and *ex vivo* imaging, parasites are also broadly disseminated during the acute stage, with the skin being a major site of infection (74).

OVERVIEW OF ANIMAL MODELS USED IN CHAGAS DISEASE RESEARCH

Non-human primates

In Chagas disease research, mice, dogs, rats, hamsters and non-human primates (NHPs) are the most widely used animal models (Table 1). Of these, NHPs represent a small fraction of the total, reflecting access, cost factors, ethical issues, and their inappropriateness for high-throughput studies. Experimental *T. cruzi* infections of capuchin monkeys (*Cebus apella*) were described more than 30 years ago (75, 76),

however since then, reports of direct inoculation of NHPs with parasites have been rare. With the exception of vaccine protection studies (77), most recent experimental work has utilised baboons (*Papio hamadryas*) or cynomolgus macaques (*Macaca fascicularis*) that acquired natural infections from wild triatomine bugs within enclosures at primate research centres (78 - 81). Analysis of both experimental and naturally acquired infections indicates that disease progression and immune responses in these models have many similarities to the situation in humans. Macaques with naturally acquired infections have also been used in drug testing studies, such as those that led to the identification of an orally administered benzoxaborole as a candidate anti-*T. cruzi* drug (82). Although their close evolutionary relationship to humans offers advantages across a number of research areas, including congenital transmission (83), for the reasons outlined above, it is unlikely that NHP use in Chagas disease research will expand significantly in the foreseeable future.

Mice and other rodents

Mice have a long history as an animal model in experimental Chagas disease research (84 - 86), and are by far the most commonly used species. They offer many advantages. These include the availability of in-bred strains and transgenic models that display a wide range of susceptibility to infection (Table 2), and a spectrum of disease pathology that overlaps with that in humans (87 - 91). In addition, mice are cost-effective, easy to maintain, amenable to high-throughput experimentation, and there is a plethora of well-validated experimental reagents. Accordingly, murine models have played the central role in dissecting the immune response, exploring the mechanisms that underpin disease pathogenesis, and as integral components of the

drug development pipeline. The infection profile in mice mirrors that in humans, with an initial acute stage where parasites are readily detectable in the bloodstream (peaking 10 - 30 days post-infection, depending on the model), followed by transition to the life-long chronic phase in which bloodstream parasites are detectable only intermittently. With some mouse:parasite strain combinations, death is a common outcome (92, 93), from either acute myocarditis, cardiomyopathy or encephalopathy, pathologies that can also be fatal in human infections. Therefore, mice display a spectrum of disease severity that often overlaps with that observed in humans, making them valuable experimental models for studying Chagas disease pathology.

A major drawback of most animal models, including mice, is that during the chronic stage of Chagas disease, it has been difficult to monitor the progression of infection, even using PCR-based methodologies. This is due to the transient low level parasitemia and the sequestration of rare infection foci within deep tissue sites. These technical limitations have been partially overcome by the development of *in vivo* bioluminescence imaging techniques applicable to murine infections (63, 65, 70, 94 - 96). Using *T. cruzi* genetically modified to express a codon-optimised, red-shifted luciferase (emission max. 617 nm) (97, 98), it has been possible to image infections throughout the chronic stage, in real-time, using non-invasive approaches (65, 70). Two factors are responsible for the enhanced imaging properties of red-shifted luciferase; first, there is a reduced propensity of light towards the red-end of the visible spectrum to undergo scatter, resulting in improved tissue penetration; second, red light is less subject to absorbance within tissue, where haemoglobin is the principal chromophore. One limitation of bioluminescence imaging is that it is not readily applicable to visualising infections at a cellular level, for example, by using fixed tissue

sections or tissue clearing approaches. This arises because enzyme-mediated oxidation of the luciferin substrate, which results in light emission, is ATP-dependent. This limitation has now been overcome by linking fluorescent reporter genes in-frame with the gene encoding red-shifted luciferase, such that parasites express a fusion protein that is both bioluminescent and fluorescent (Fig. 3A) (99). Because fluorescence is an intrinsic property of the molecule, this has enabled infections to be imaged at single-cell resolution in tissue sections after bioluminescence-guided sampling (Fig. 2B, Fig. 3B, C, D) (73, 100).

In addition to mice, other rodent species, particularly hamsters and rats, have been used as models to explore aspects of disease pathogenesis, although fewer studies have been reported. This may reflect the reduced availability of commercially available reagents, or the decreased experimental flexibility achievable with these larger rodents. Nevertheless, hamsters are amenable to bioluminescence imaging (74), and have proven useful for studying cardiac disease (101 - 103). Intriguingly, Syrian hamsters (*Mesocricetus auratus*) infected with the *T. cruzi* CL Brener strain (DTU TcVI) do not develop reproducible chronic cardiac pathology, but do display hindlimb muscle hypertonia and a gait dysfunction similar to spastic diplegia (74). Rats have similarly been utilised across the spectrum of Chagas disease research, including as models for congenital transmission (104, 105) and immune responses to infection (106 - 108).

Canine models

Canine experimental models have also been widely used, particularly in the area of chemotherapy (109, 110). Studies have included attempts to optimise dosing

regimens with the front-line drug benznidazole (111, 112), the testing of novel lead compounds (113, 114), and assessment of combination therapy (115). Animals with both experimental and naturally acquired infections have been utilised in these experiments. In the case of vaccine research, canines have been the subject of several studies (116 - 119), although evidence for sterile protection remains elusive. The well-reported susceptibility of dogs to *T. cruzi* infection and the associated pathology has resulted in them becoming useful models for studies on basic immunology (120 - 123), disease pathogenesis (124, 125) and symptom alleviation strategies (126). In Chagas disease research, other species are less commonly utilised in an experimental context, at least in a systematic manner.

THE ROLE OF ANIMAL MODELS IN DISSECTING THE IMMUNE RESPONSE TO *T. CRUZI* INFECTION

Chagas disease pathology is predominantly immune-driven, with a chronic inflammatory aetiology leading to tissue damage, cardiac fibrosis, and neuronal destruction. The type of immune response depends on the phase of infection and the life cycle stage of the parasite. During the initial infection, neutrophils and macrophages are recruited to the bite-site, as the first responders. Parasites at this stage are metacyclic trypomastigotes (Fig. 1), pre-adapted for migration from the insect hindgut into the mammalian host. They are complement-resistant due to the expression of numerous complement regulatory proteins (for review, see ref #127). During the first few days, monocytes, macrophages and neutrophils constitute the greatest number of infected cells (128). Highly motile non-replicative extracellular bloodstream trypomastigotes, generated during the first infection cycle (Fig. 1), then

play the key role in systemic parasite dissemination, provoking an antibody response from B cells. At this stage, the parasite also engenders a polyclonal, antigen-independent B-cell response through the release of mitogenic factors (128 - 131). In addition, trypomastigotes are exposed to other humoral immune factors, such as complement components, against which they employ multiple resistance mechanisms (127). The replicating intracellular amastigotes (Fig. 1) generate a T cell response in which CD8+ cytotoxic T cells are critical for parasite control. However, this response occurs more slowly against *T. cruzi* than against other microbial infections (128). As with many pathogens that have evolved to cause chronic infections, *T. cruzi* deploys a variety of mechanisms to evade or downregulate the immune response (71). In addition, with the widely disseminated nature of the infection, organ-specific and tissue-resident immune mechanisms then come into play.

Immunity and pathology are inextricably linked during *T. cruzi* infection, whether cardiac, digestive or both (see below). However, in humans, the decades-long progression from infection to overt clinical disease complicates detailed understanding of the interplay between host and parasite. Therefore, experimental animals that display faster progression to symptomatic disease, and which offer options to standardise or vary multiple parameters, play a vital role in studies focussed on immune-pathology. Mice are by far the most commonly used animal in this area of research. Their small size makes them the model of choice for *in vivo* and *ex vivo* imaging (Fig. 2 and 3). It also allows experiments to be scaled up, statistically powered, and rapidly repeated, as required. Limitations in these areas have been a criticism of some studies in which larger mammals are used as the infection model. The murine immune system has been widely studied and is genetically tractable, with many

immunological null mutants and conditional mutants available to researchers (Table 2). The number of tools and reagents available to interrogate the immune response, such as monoclonal antibodies, is more comprehensive than for any other animal. Mice can also exhibit both digestive and cardiac Chagas disease (for example, 100, 132), although models that develop late-stage GI tract megasyndromes have yet to be established. Here, we illustrate examples, amongst many, which highlight how mouse models have been key to dissecting the nature of the immune response to *T. cruzi* infection.

The role of innate immunity

Infected myeloid cells are thought to traffic *T. cruzi* from the parasite contaminated bite-site, around the host. Deletion of the signalling lymphocytic activation molecule 1 (Slamf1) receptor on macrophages reduces both the number of amastigote nests, and the number of parasites per nest, in the hearts of acutely infected mice. Consequently Slamf1-/- mice do not normally succumb to a lethal challenge infection with the virulent TcY (DTU TcII) strain, whereas wild type mice die (133). Absence of Slamf1 renders macrophages and dendritic cells less able to support parasite replication, and it has been inferred that this results in fewer parasites being trafficked to the heart, explaining the resistance to acute myocarditis (133). Further research has identified strain-specific differences in infection outcomes in the Slamf1 null background; some parasite strains behave similarly to TcY, with reduced virulence, whereas infections with other strains, such as TcVFRA (DTU TcVI), result in decreased macrophage NADPH oxidase activity and a higher parasite burden (134). These studies demonstrate the extent to which *T. cruzi* genetic variation is an important factor that adds complexity to Chagas disease research, and highlight how the flexibility of murine

models allows these types of differential response to infection to be captured. In terms of wider *T. cruzi* tissue dissemination, the relative contributions of bloodstream trypomastigotes versus the trafficking of infected myeloid cells has yet to be addressed in experimental models, particularly during the chronic phase. At this stage of the infection, bloodstream trypomastigotes are rare, but dynamic spatio-temporal changes in the location of infection foci is a characteristic feature (70). The recent development of dual reporter parasites (Fig. 3) will allow the application of new approaches to this question (99).

Acquired immunity

Murine models have been central to analysis of acquired immunity against *T. cruzi* infection, and have made major contributions to identifying the roles, and assessing the interplay, of B cells, CD4+ Th1 cells, Th17 cells, and most crucially, CD8+ cytotoxic T cells. For example, B cells function in the maintenance of the antigen specific CD8+ T cell response during murine infections, and B cell depletion results in a contraction of the CD8+ T cell population (135). The remaining *T. cruzi*-specific CD8+ T cells exhibit decreased effector function and a higher degree of apoptosis. However, the CD8+ T cell response in B cell-depleted mice can be rescued by administration of IL-17A. Consistent with this, IL-17 receptor null mutant mice display a similar phenotype to B cell depleted-mice. Earlier work had demonstrated that *T. cruzi* infection triggers the development of an IL-17 secreting B cell population, where parasite trans-sialidase mediated sialylation of CD45 (a surface protein common to all white blood cells) activates signalling via the protein kinases Src and Btk, to promote IL-17 production (136). As vindication of the translatability of findings on the complex interplay of this arm of the acquired immune response in mouse models, this pathway also functions

401 in human B cells *in vitro*. This study highlights the co-ordination required between B
402 and cytotoxic T lymphocytes, the two main adaptive effector cell types involved in long-
403 term control of *T. cruzi* infections.
404
405 Studies in mice have also revealed a multitude of mechanisms which have evolved in
406 *T. cruzi* that delay or disrupt the generation of a functional parasite antigen-specific
407 response. One of these is the secretion of proteins that act as B cell mitogens in a T
408 cell-independent, non-antigen specific manner. Three parasite proteins have been
409 shown to have this property in murine infection models; proline racemase, malate
410 dehydrogenase and trans-sialidase (129 - 131). This immune evasion strategy delays
411 the generation of an effective, high-titre antibody response, allowing trypomastigotes
412 to disseminate through the host during the acute phase of infection.
413
414 Murine infection models have been crucial in establishing the major role of CD8+
415 cytotoxic T cells in bringing the parasite burden under control during the acute stage
416 of infection, and continually suppressing their numbers during the chronic phase. Their
417 importance reflects the cytosolic niche of *T. cruzi* across a wide range of nucleated
418 cell types – only T cells which can recognise antigen presented in the context of MHC
419 class I can eliminate pathogens of this type. The CD8+ T cell antigen specificity in
420 mice is remarkably skewed towards immunodominant members of the parasite trans-
421 sialidase gene superfamily (137). Generation of this dominant T-cell response is
422 independent of CD4+ T cell help for priming. However, the magnitude of the CD8+
423 response is significantly decreased in the absence of CD4+ T cell help, and the
424 parasite burden cannot then be controlled (138). Given the extensive variability of the
425 trans-sialidase superfamily (139), both within and between strains, this may explain

the reduced level of immune protection against heterologous challenge in drug-cured infection models (140), compared with the levels that are achievable with a homologous strain challenge.

One of the strengths of mice as an experimental model is the availability of a vast range of transgenic strains which can be exploited to more precisely characterise immunological phenomena (Table 2). For example, *T. cruzi* infections of mice with genetically engineered tolerance to the two most immunodominant trans-sialidase epitopes demonstrated that this skewed T cell response does not impede the effectiveness of the immune response. The tolerized mice behaved similarly in their ability to control infections and to generate *T. cruzi*-specific memory T cells (141). This immunodominance phenomenon has been demonstrated in T cells from human patients, albeit to a lesser extent (137).

Although CD8+ T cells play the major effector role in systemic control of *T. cruzi* infection, the possibility that other tissue-specific determinants are also involved can be explored using mouse models. For example, myocytes display lower levels of MHC I expression than many other tissues and increasing MHC I levels, by muscle-specific inducible expression, can enhance CD8+ T cell mediated control of infection. However, this effect is short-lived and the parasite-specific T cells then develop an exhausted phenotype, with parasite replication then increasing rapidly (142). CD8+ T cell-depletion in mice also results in expansion of the parasite burden in skeletal muscle, while the level in the gut remains constrained, suggesting that other immune effectors, or cell populations, are involved in control of parasites in the GI tissue niche (13). Tissue-specific elements in the immune control of parasite numbers, such as

451 decreased MHC I expression, may explain the life-long persistence of the parasite and
452 its survival in specific reservoir niches. Addressing these issues in humans, is
453 experimentally complex, and animal models have therefore been the main focus of
454 research in this area.
455
456 In humans, the reactivation of acute symptoms of Chagas disease in
457 immunosuppressed patients with low CD4+ T cell counts, particularly those with
458 HIV/AIDS, demonstrates that this T cell type plays an important role in controlling the
459 infection (143). A type I pro-inflammatory response is required to initiate control of
460 infection, and CD4+ Th1 cells play a significant role in controlling both systemic and
461 mucosal infection (144, 145). In murine footpad infection models, during the initial
462 stages of infection, the IFN-γ required for parasite control is produced primarily through
463 infiltrating CD4+ Th1 cells, rather than NK cells (128). In this model, CD4+ T cells
464 contribute more to the initial control of the nascent infection than CD8+ T cells. Mice
465 lacking CD4+ T cells are less able to control the initial parasite growth than mice
466 lacking CD8+ cells. However, both T cell populations are required for long-term
467 control, as has been demonstrated by infections carried out in mice lacking expression
468 of MHC class I, MHC class II, or both (146). Furthermore, CD4+ T cell help is
469 necessary for the antigen specific CD8+ T cell population to reach its maximal level
470 (138).
471
472 As indicated above, IL-17A plays an important role in orchestrating protection against
473 *T. cruzi* infection. IL-17 is secreted by many immune cell types, including B cells and
474 γδ T cells. Adoptive transfer experiments using trans-sialidase-specific *in vitro* derived
475 Th1 and Th17 cells, into $RAG^{-/-}$ immunodeficient mice, showed that a combination of

Th17 and CD8+ T cells provides greater protection against lethal challenge than the transfer of Th1 and CD8+ T cells. CD8+ cells alone do not provide significant control of infection. These experiments indicate a significant role for Th17 cells in driving the protective cytotoxic CD8+ T cell response, and that complete protection could be generated in the absence of Th1 T cell help (147). In contrast to B cells, the role of Th17 cells in CD8+ T cell help was independent of IL-17A production, and was instead dependent on IL-21, a cytokine not produced in significant quantities by Th1 cells. (147).

The role of non-murine models in exploring the immune response

Other animal models have been utilised less extensively, and at a less detailed level, to explore the immune response to *T. cruzi* infection. In the case of canines, as with other non-murine models, the reduced availability of tools and reagents, the limitations of current genetic modification procedures, and the lack of defined in-bred strains have rendered them less suitable. The examples below illustrate some of the difficulties associated with the use of canines as experimental models. A positive correlation was found between levels of IgG1 antibodies and myocarditis in dogs, in both the acute and chronic phase (125, 148). In contrast, a separate study (149) found a negative correlation between IgG1 levels and cardiomegaly in chronic phase infections. These studies employed experimentally infected mongrel dogs (148), naturally infected stray dogs (125) and experimentally infected beagles (149). Therefore, compared to experiments with mouse models, in canine studies, the control of variables such as host and parasite genetics, environment and co-infections are intrinsic issues that are more difficult to control, and can confound comparisons between different studies. However, dogs are important in the context of Chagas disease - they act as a reservoir

of infection and live in close proximity to humans. Further research on how they respond to infections is therefore epidemiologically relevant and justified.

Immune responses have been also been studied in naturally infected macaques, where the infecting parasite strains are wild isolates, rather than well-characterised laboratory strains. To add complexity, naturally infected animals can often be infected with multiple *T. cruzi* strains, and possibly other pathogens. Nevertheless, such studies have value. In one example, 15 chronically infected macaques were divided into three groups based on pathology - asymptomatic, mild or moderate Chagasic cardiomyopathy (150). There were some differences between the three populations, such as higher levels of circulating granzyme A+ NK cells and CD8+ T cells in the symptomatic versus asymptomatic populations. The asymptomatic population displayed a higher level of circulating and splenic monocytes, NK, and NKT cells than the non-infected control animals. In contrast, those with symptomatic cardiomyopathy (mild or moderate) exhibited a high degree of pro-inflammatory responses composed of activated CD8+ T cells and B cells, with increased levels of TNF-α and IFN-γ. These findings echo some of the results obtained from human Chagas patients at various stages of the disease, suggesting that immune driven pathogenesis in the macaque has similarities with the process in humans.

In another study, in which macaques had been naturally infected with parasites from two different DTUs (TcI and TcIV), T effector memory cell ($CD28^{-}$ $CD95^{+}$) levels were higher than in uninfected controls. In addition, the infected cohorts had higher proportions of fully differentiated memory ($CD45RA^{-}$ $CD27^{-}$ $CD28^{-}$) CD8+ T cells. This was also the case for the CD4+ memory T cell population (81). However, there was

no correlation between the immune response, disease status, and the parasite DTU. Although there was a correlation between antibody levels and parasitemia, this did not extend to cellular immunity or disease status. NHPs offer advantages for studying the immune response, based on their closer evolutionary link to humans, but mice seem likely to continue being the predominant animal models for exploring this aspect of Chagas disease. The research flexibility that they offer allows more expansive and tightly controlled studies to be undertaken, with an enhanced scope for downstream confirmational experiments.

WHY DOES CHRONIC *T. CRUZI* INFECTION RESULT IN HEART DISEASE?

Chronic cardiac abnormalities are the most common manifestation of *T. cruzi* infection (17-20), although there is a wide range of disease outcomes, and it can take decades for symptoms to become apparent. Human experimental research in this area is largely limited to observational studies and a small number of clinical trials (27, 151-153). In humans, cardiac complications range from minor myocardial issues, through to arrhythmias, sudden organ failure and death (19-20). Identifying the triggers of pathology and the determinants of disease progression and severity have been key research questions. Earlier studies had suggested that progressive Chagasic heart disease is an autoimmune-driven process that could continue, even in the absence of parasites, through involvement of autoreactive antibodies and T cells (154 - 158). It is widely considered that this hypothesis had a negative impact on both anti-parasitic drug development and vaccine design strategies. However, a critical role for autoimmunity has now been largely discounted, with animal experimentation having been crucial in reaching this conclusion. Studies using neonatal heart transplants in

551 mice demonstrated that on-going cardiac infection is a pre-requisite for tissue damage
552 (159). Autoimmune phenomena that do develop in murine infections subside when
553 parasites are cleared by drug treatment (19). Curative benznidazole treatment during
554 the acute stage of murine infections prevents the development of cardiac pathology,
555 whereas if treatment is postponed, the beneficial effects are reduced, since irreversible
556 damage has occurred in the interim (Fig. 4) (132). Early treatment of infected mice
557 with other experimental trypanocidal agents (160), or with vaccine-based therapy,
558 produced a similar trend in terms of an ability to block chronic disease progression
559 (161). In dogs, comparable outcomes were achieved following benznidazole
560 treatment, even when sterile cure was not achieved (162, 163). Collectively, these
561 studies have important implications for public health policy - they suggest that early
562 treatment of *T. cruzi* infections will be crucial to maximise the likelihood of preventing
563 progressive cardiac pathology. The translational value of this inference was confirmed
564 by the results of the BENEFIT clinical trial, which revealed that benznidazole treatment
565 of patients with advanced Chagas cardiomyopathy is delivered too late to alleviate the
566 disease (27).
567
568 During chronic stage *T. cruzi* infections, the parasite load in mice is extremely low,
569 although *in vivo* imaging studies show that the remaining infection foci are
570 metabolically active and highly dynamic in space and time (65, 70). The gut and skin,
571 and in some strains, skeletal muscle, appear to provide immunologically permissive
572 niches that permit long-term maintenance of the infection (Fig. 5). Our hypothesis is
573 that these tissues act as reservoirs with potential for intermittent trafficking of
574 parasites, or parasite-infected cells, to other sites such as the heart (164). Detectable
575 cardiac infections in mice are intermittent, and their frequency is dependent on the

576 mouse:parasite strain combination (65, 132). For example, in BALB/c mice chronically
577 infected with the *T. cruzi* CL Brener strain, analysis reveals that <10% of animals have
578 detectable cardiac infection at any one time, whereas in C3H/HeN mice infected with
579 *T. cruzi* JR (DTU TcI) parasites, cardiac infections exceed 80% (Fig. 5A) (65).
580 Significant cardiac fibrosis can be detected in both these models, although it is more
581 severe in the latter. A possible explanation for these observations is that episodic re-
582 infections of the heart are seeded from more long-term permissive tissues sites, at a
583 low frequency in the BALB/c mouse, and a higher frequency in the C3H/HeN mouse.
584 As this is a process that occurs for the life-time of the infected individual, the resulting
585 cardiac-localised inflammatory immune responses, including interferon-γ-induced
586 nitric oxide, would then continuously eliminate the parasites, but give rise to collateral
587 tissue damage (164). The cumulative nature of cardiac pathology can be explained by
588 the fact that heart muscle has a low regenerative capacity (165), and the tissue repair
589 involves fibro-fatty replacement to compensate for the loss of cardiomyocytes.
590
591 In humans, assessment of parasite tissue distribution during the chronic asymptomatic
592 stage is a major technical challenge, and most investigative research has focussed on
593 material derived from autopsies, or from patients undergoing heart transplants.
594 However, such “end-point” analysis of parasite location may not be representative of
595 earlier phases of disease development, such as those occurring in on-going
596 asymptomatic infections, the period during which immune-mediated tissue damage is
597 accumulating. The frequent inability to detect infection foci in post-mortem histological
598 sections from patients with chronic heart disease (166-170), which was originally taken
599 as evidence for an autoimmune aetiology (171), can now be viewed as a consequence
600 of intermittent transient cardiac infections. This type of mechanism could provide an

explanation for the variable nature of symptomatic pathology, whereby timing and severity are influenced by the impact of host and parasite genetics and a range of environmental factors. Animal models, particularly murine, provide an accessible platform that will allow these issues to be experimentally addressed.

ANIMAL MODELS FOR DIGESTIVE CHAGAS DISEASE (DCD)

While there have been encouraging advances in understanding the pathogenesis of cardiac Chagas disease, progress on DCD has been more limited. Anti-parasitic chemotherapy is not recommended for *T. cruzi*-positive individuals who display digestive symptoms, but retain normal cardiac function (14,172). Reasons include the lack of systematic clinical trials to assess treatment efficacy in this context, and the absence of pre-clinical animal studies to justify treatment and inform study design. Therapeutic options for DCD are instead focussed on palliative measures, such as dietary changes and surgery to remove the affected bowel region (14, 173 - 175), with considerable risk of mortality when surgical intervention is used against late-stage disease (176). To date, there have been no clinical trials to investigate the effectiveness of the front-line drug benznidazole, or other therapeutic agents, specifically against DCD.

Human DCD clinically presents as dilation and dysfunction of the gastrointestinal (GI) tract, which causes symptoms such as abdominal pain, achalasia, constipation and faecaloma (173, 174). In severe cases, progressive organ dilatation can develop into megasyndromes, typically affecting the colon or the oesophagus. Dilation stems from the degeneration of enteric neurons, a process that leads to dysperistalsis and smooth

muscle hypertrophy. Early theories of DCD pathogenesis, based on human autopsy studies, suggested that pathology stemmed from irreversible neuron losses that occurred during the initial acute stage of infection, which only became apparent years later, after being 'unmasked' by subsequent age-related denervation (177 - 179). This hypothesis was supported by an inability to regularly detect gut-resident parasites in chronic disease patients. However, studies have since repeatedly detected *T. cruzi* DNA, antigen and inflammatory infiltrates in post-mortem and biopsy samples from human DCD cases (180 - 184), suggesting that chronic parasite persistence could have a role in pathogenesis. By inference, anti-parasitic drug treatment may have potential benefits in terms of limiting disease progression. However, as set out above for cardiac disease, interpretation of data from advanced and terminal stages of the disease has many caveats in terms of the underlying mechanisms of pathogenesis, and how these relate to the temporal or spatial distribution of infection.

Given the long-term, multifactorial nature of DCD, studying the cause-and-effect relationships between infection, host response and tissue pathology can only be achieved, realistically, using experimental animal models. There is no well-established model of advanced digestive megasyndromes, although enlargement of parts of the GI tract has been reported in some observational studies of experimentally infected mice (185), rats (186), rabbits (187), hamsters (188) and macaques (189). Nevertheless, seminal studies conducted in Brazil in the 1960s showed the possibility of reproducing histopathological aspects of human DCD in rats and mice (190 - 193). The first major mechanistic insight, namely that much of the collateral nervous tissue damage was dependent on the production of reactive nitrogen species, as part of the anti-parasitic inflammatory response, was first suggested from studies using a nitric

651 oxide synthase (NOS) inhibitor in a Wistar rat model (194), findings that were
652 subsequently confirmed by studies using transgenic mice (195).
653
654 The most commonly reported animal model of DCD is based on *T. cruzi* Y strain
655 infection of outbred Swiss mice, which exhibit significant acute denervation and
656 delayed intestinal transit (196 - 200). One drawback of this model is related to the high
657 virulence of the TcY strain - in order to study the chronic phase, sub-curative
658 benznidazole treatment is typically given to elongate the life-span of infected mice.
659 Nevertheless, the data show that in chronic *T. cruzi* infection, there is decreased
660 intestinal motility and an impaired neuronal cholinergic response, providing further
661 insight into enteric nervous system dysfunction in experimental DCD. Other murine
662 models have been used to investigate key questions related to disease progression;
663 for example, why do only a relatively small subset of infected people go on to develop
664 DCD? In this context, it is intriguing that deletion of the nucleotide-binding
665 oligomerization domain-containing protein 2 (NOD2) gene, which encodes an
666 intracellular pathogen sensing protein, results in chronic dysperistalsis in normally
667 DCD-resistant C57BL/6 mice infected with the *T. cruzi* RN25 (DTU TcII) strain (201).
668 Experiments such as this further highlight the potential of transgenic mouse models to
669 identify host determinants of clinical outcomes.
670
671 As outlined above, bioluminescence imaging and PCR studies of chronically infected
672 mice have demonstrated that the GI tract serves as a major long-term reservoir site of
673 *T. cruzi* infection, particularly the stomach and large intestine (13, 70, 100, 202 - 206),
674 and that this is reproducible across diverse mouse:parasite strain combinations. These
675 observations have underpinned the development of a robust model for DCD that

involves C3H/HeN mice infected with the *T. cruzi* JR strain (100, 206). This combination recapitulates several of the key clinical manifestations, including a significantly delayed GI transit linked to localised persistence of the parasite, and associated lesions in enteric neurons within the wall of the large intestine (Fig. 6). Interestingly, the extent of this transit delay varies considerably between different mouse:parasite strain combinations, indicating that both host and pathogen genetics are determinants of pathology, which may be reflective of disease diversity in humans.

With the development of these mouse models, and as our understanding of DCD continues to evolve, it has become possible to experimentally interrogate whether curative drug treatment can prevent the onset of DCD, or mitigate its severity. Additionally, the models provide a framework to explore the underlying mechanisms of neuronal denervation. Curative benznidazole treatment, initiated towards the end of the acute stage was found to reverse DCD, restoring normal GI transit time, in association with a significant recovery of myenteric neuron density in the colon (206). Non-curative treatment, which initially reduced the parasite burden close to background levels led to transient alleviation of DCD, but this was then followed by gradual relapse of the residual infection and an eventual return of symptoms. Analyses of host gene expression in colon tissue following curative treatment showed that the observed functional recovery and regeneration of the enteric nervous system were linked to resolution of chronic inflammation, and a transition to a tissue proliferative repair response by neuronal tissue. This included the upregulation of multiple enteric nervous system related genes (206). Therefore, animal studies indicate that acute denervation does not fully account for the development of chronic disease symptoms, challenging the previous consensus on the nature of DCD aetiology (177, 178).

Furthermore, they provide an experimental rationale for timely therapeutic intervention, targeted at the parasite, as a means of preventing or slowing the development of symptomatic DCD.

The potential of organoids

It is clear that comprehensive approaches combining advanced molecular techniques and robust animal models are essential to unravel the complex interplay between different stages of *T. cruzi* infection and enteric neuropathy. Recent reports have also uncovered new levels of interconnection between the gut immune and nervous systems (207 - 209), which were previously unrecognised. As alluded to above, current insights into the complex neuro-immune interactions triggered by *T. cruzi,* and their effects on health and infection, are mostly based on animal models. Although animal models are essential to advance pre-clinical *in vivo* studies, there could be differences between human and rodent biological systems that are not sufficiently captured by experimental models. To bridge this gap, the development of advanced *ex vivo* technologies derived from human cells will be a key step. These technologies should closely mimic tissue-like physiological processes, be more accessible, reproducible and scalable to allow the effective study of biological processes.

The development of 3D tissue cultures known as organoids, derived from stem cells, has significantly transformed biomedical research by offering an alternative to animal models. The creation of 'mini-guts' from pluripotent stem cells, which were among the first organoids capable of forming a gut epithelial layer (210), marked a significant advancement in the field. Currently, human intestinal organoids can be developed from human embryonic and induced pluripotent stem cells which replicate the intricate

architecture of the human intestine (211, 212). The field has further advanced by integrating a functional enteric nervous system into the human intestinal organoids (213, 214). Such technologies will have enormous potential in the field of *T. cruzi* infection biology. For example, in the case of drug development, only a small number of agents that are effective in 2D human cell culture, prove to be curative when moved to *in vivo* testing, highlighting the need for a platform that bridges *in vitro* cell culture and animal models. Currently, most researchers employ human intestinal organoids in their most basic form, consisting solely of the gut epithelium (215 - 218). More recently, this approach has been applied to modelling *T. cruzi* infection using mouse gut epithelial organoids (219). However, the simplicity of these systems restricts their ability to explore more complex questions in infection biology. To effectively utilise gut organoid technology for studying neuro-immune cross-talk, such as that involved in interactions with *T. cruzi,* it will be crucial to develop innovative approaches that encompass both the immune and enteric nervous systems. The field has moved towards developing this integrated approach (220 - 223), particularly in the context of enteric bacterial, viral and parasitic GI tract pathogens, including helminths and *Cryptosporidia* (224 - 226). Applications of these next-generation human intestinal organoids will overcome current limitations, provide a new system for drug testing, and compliment data generated through animal experimentation.

THE ROLE OF ANIMAL MODELS IN DRUG DEVELOPMENT

The limited effectiveness and toxicity of the current front-line drugs, benznidazole and nifurtimox (25, 227, 228), have been major drivers of the global effort to produce improved therapeutics for *T. cruzi* infections. Mice have been the most frequently used

751 animal model in the drug development field, where iterative approaches and high-
752 throughput experimental capacity are crucial. The translational value of drug efficacy
753 data obtained using murine models is supported by a number of observations. In both
754 humans and mice, benznidazole and nifurtimox treatment can result in sterile cure (26
755 - 28, 111, 229), whereas the azole drug posaconazole fails to produce regular curative
756 outcomes (26, 230). Also, murine infection profiles and the nature of disease pathology
757 display many similarities to that observed in humans (65, 70). Technical advances,
758 such as the development of highly sensitive *in vivo* bioluminescence imaging
759 procedures (98), have further enhanced the utility of murine models in drug testing.
760 With the availability of bioluminescent strains that encompass the wide spectrum of
761 parasite genetic and phenotypic diversity (231), there is now the means to ensure that
762 lead compounds have *in vivo* activity that covers the *T. cruzi* species, prior to being
763 progressed into clinical trials.
764
765 With *T. cruzi* infections, a pressing issue associated with drug efficacy testing, in both
766 humans and animal models, is the confirmation of sterile cure. As mentioned above,
767 the extremely low parasite burden during chronic stage infections, the intermittent
768 nature of parasitemia, and the sequestration of rare infection foci in deep tissue sites
769 are problematic issues, that can lead to false-cure diagnoses. In clinical trials, this has
770 necessitated long-term follow-up using PCR-based diagnostic approaches, which
771 have cut-off points that vary between different studies (26 - 28, 232). Similar
772 challenges are also associated with animal models. For example, microscopic
773 detection of bloodstream parasites is infrequent during the chronic stage (233), and
774 PCR methodologies, even at a tissue level, are subject to variable read-outs (111,
775 230).

Highly sensitive *in vivo* bioluminescence imaging has been a valuable addition to the drug testing pipeline (229, 230). It provides a capacity for real time non-invasive monitoring of infection and is a scalable procedure. The process can also be adapted to optimise the detection of parasites that survive treatment (Fig. 7). To achieve this, three weeks following the cessation of treatment, mice are typically immunosuppressed using cyclophosphamide to promote the outgrowth of parasite persisters. At the final stage, organs and tissues can be removed and examined by *ex vivo* imaging (Fig. 7B), a procedure where the limit of detection is <12 parasites (73). Two recent reports serve to illustrate where bioluminescence imaging has played an integral role in progressing promising treatments towards clinical testing. First, a new class of cyanotriazole compounds that selectively inhibit the parasite topoisomerase II was shown to rapidly cure *T. cruzi* infections (32). Second, a series of pyrrolopyrimidines that inhibit *T. cruzi* cytochrome b were found to cure infections when administered in combination with low sub-efficacious doses of benznidazole (33). End-point *ex vivo* imaging can also add value to the process of compound assessment. Using parasites that express bioluminescent:fluorescent fusion proteins, infections can be imaged at single cell resolution (73, 99). With non-curative treatments, this allows residual parasites to be localised to specific organs or tissue sites, to determine if non-equitable drug distribution could be a factor in recrudescence. The immunosuppression step is omitted in this case, to avoid perturbing parasite distribution. At an experimental level, other approaches such as incorporation of the thymidine analogue EdU into parasite DNA, can also be used to assess the replicative status *T. cruzi* amastigotes that have survived *in vivo* drug treatment (234).

In addition to *in vivo* testing of compound efficacy, mice are often the model of choice for initial assessment of pharmacokinetic properties, toxicity, compound distribution and half-life (235 - 238). However, differential compound metabolism between mice and humans could be a significant factor in reducing the cross-species translational value of drug efficacy data. A recent innovative approach to circumventing this problem has involved the generation of transgenic mice in which several of the genes encoding members of the drug-metabolising cytochrome P450 superfamily (239) have been replaced with their human equivalents. These “humanised” mice should increase the predictive power of *in vivo* pre-clinical testing and enhance the relevance of pharmacokinetic studies.

Other animal models are less commonly used in the drug development pipeline. As mentioned above, macaques that were naturally infected with *T. cruzi* played an important role in characterising the curative potential of a class of benzoxaboroles (82), and canine models have often been used to explore drug efficacy, with treatment of both naturally acquired and experimental infections (109 - 115). However, given the logistics and financial costs associated with research using larger mammals, and the reduced experimental flexibility, it is difficult to foresee a situation where they replace mice as the default model for routine *in vivo* testing of compound efficacy. Nevertheless, these other animal models have potential for contributing significantly to our understanding of disease pathogenesis (51, 101, 120, 121, 123 - 126) and the role of anti-parasitic drug treatment in blocking the progression of chronic disease pathology (114, 120).

CONCLUDING REMARKS

The most important questions in Chagas disease research are: (i) What are the drivers of pathology, and can we intervene to block disease progression? (ii) Can we develop new anti-parasitic drugs with improved efficacy and reduced toxicity? Predictive animal models have had a central role in the fundamental research required to address these issues, and are integral components of the drug development pipeline. These studies have provided new insights into the triggers of both cardiac and digestive pathology, enabled the immune response to be explored at exquisite detail, and helped advance new therapeutics into the pre-clinical phase. Although the extensive host range of the *T. cruzi* parasite provides a wide variety of experimental options, mice remain by far the most commonly used animal models. Flexibility and convenience are major factors, together with on-going improvements in imaging technology, and the availability of in-bred strains and transgenic lines.

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Biographical Profiles

Archie Khan is a Pharmacist by training and received her PhD in Neuroscience from UCL School of Pharmacy, London, where she studied the mechanisms of action of phytocannabinoids and cognitive enhancers in the brain. She then worked as a Research Fellow at the London School of Hygiene and Tropical Medicine, investigating chronic parasitic infections in the context of the enteric nervous system, particularly *Trypanosoma cruzi* in digestive Chagas disease. Recently, she was awarded a Marie Curie EU Fellowship and is now based at INSERM in Nantes, France, where she undertakes research into neuro-immune interactions in the gut during health and infection, using human intestinal and colonic organoids. She has been exploring her interest in the intersection of neuroscience and parasitology for over 7 years.

Martin Taylor gained his PhD in Molecular Parasitology from the University of London. He then held a post-doctoral fellowship in the laboratory of Professor Piet Borst at the Netherlands Cancer Institute in Amsterdam, where he researched antigenic variation in the African trypanosome, *Trypanosoma brucei.* He returned to the UK in 1995 to take up a post-doctoral position in the laboratory of Prof. John Kelly at the London School of Hygiene and Tropical Medicine. He is currently Associate Professor of Molecular Biology at LSHTM. His research interests range from the basic biochemistry and molecular biology of the kinetoplastid parasites (trypanosomes and *Leishmania*), to the development of refined and predictive pre-clinical models of Chagas disease, human African trypanosomiasis and leishmaniasis to accelerate drug and vaccine development. He also uses these models to research the pathogenesis and immunology of these diseases.

Amanda Fortes Francisco obtained a BSc in Nutrition and an MSc and PhD in Biological Sciences in Brazil. In 2012, she was awarded a scholarship from the Brazilian National Council for Scientific and Technological Development to undertake post-doctoral research on *Trypanosoma cruzi* in the Kelly lab at LSHTM. Since 2014, she has been funded by the Drugs for Neglected Diseases initiative (DNDi), a not-for-profit research organization that is developing new treatments for neglected diseases. Currently, her main projects as an Assistant Professor are related to drug discovery and Chagas disease pathogenesis using *in vivo* bioluminescent imaging systems. To provide context, for more than 50 years, only two drugs have been available to treat Chagas disease, and both have major limitations. Amanda had a key role in the *in vivo* testing and regimen optimisation of two new drug candidates that were described last year in *Science* and *Science Translational Medicine*.

Shiromani (Shani) Jayawardhana is a Scientific Officer at the London School of Hygiene and Tropical Medicine, in the Department of Infection Biology. She was awarded a BSc in Biomedical Sciences at Kings College, London and has been working with John Kelly on Chagas disease pathogenesis and drug discovery since 2014. Her major focus has been to investigate infection recrudescence and *Trypanosoma cruzi* replication following non-curative drug treatment in experimental mouse models. Her expertise in bioluminescence and fluorescence imaging technology also enables her to participates in a range of other projects studying Chagas disease pathogenesis. Shani is in the final stages of writing her PhD thesis.

Richard Atherton is a Research Assistant and PhD candidate in the Department of Infection Biology at the London School of Hygiene and Tropical Medicine (LSHTM).

Richard graduated from the University of Leeds with a BSc in Microbiology before completing an MSc in Infectious Disease Control at the LSHTM. His major interests are host-pathogen interactions and the biological mechanisms that underpin pathogenesis. Building on previous experience working in virology, his research has shifted to protozoan pathogens, where he is focused on dissecting the cell and molecular biology of the zoonotic parasite *Trypanosoma cruzi.* Specifically, he is investigating the processes of intracellular parasite differentiation and egress from infected host cells.

Francisco (Paco) Olmo, received his PhD in Clinical Medicine and Public Health from the University of Granada in 2015. Following a post-doctoral Marie Skłodowska-Curie Fellowship at the London School of Hygiene and Tropical Medicine, he was promoted to Assistant Professor in the Department of Infection Biology in 2018. Currently, he is Tenured Associate Professor in the Department of Parasitology at the University of Granada. Francisco has over 15 years of experience researching the complex biology of the kinetoplastids, a group of flagellated protozoan parasites that cause human disease (including *Trypanosoma cruzi* and *Leishmania spp*.). He has participated in the development of several molecular and cellular tools and experimental models that have important applications in the drug discovery field. Additionally, these technologies are enabling the community to study host-parasite interactions at a molecular level.

Michael Lewis is an Associate Professor of Host-Pathogen Interactions at the University of Warwick Medical School, a post he has held since 2023. He graduated with a BSc in Genetics and an MSc in Medical Molecular Microbiology from the University of Nottingham. Michael was then awarded a PhD from the London School

of Hygiene and Tropical Medicine (2008) for work focussed on *Trypanosoma cruzi* biology and genetics. Following post-doctoral studies at LSHTM, he established himself as an independent investigator through receipt of an EU Marie Curie International Fellowship and a New Investigator Award from the UK Medical Research Council. His research is centred on the pathogenesis of infectious diseases caused by protozoan parasites, principally *T. cruzi* and Chagas disease.

John Kelly is Professor of Molecular Biology at the London School of Hygiene and Tropical Medicine (LSHTM). After graduating from Glasgow University with a Biochemistry BSc and PhD, he undertook post-doctoral research in London at the National Institute for Medical Research and the Imperial Cancer Research Fund, organisations that subsequently merged to form the Crick Institute. Post-doctoral research covered ribosome function and interferon-regulated gene expression. In 1986, he was recruited by LSHTM to undertake research on trypanosome molecular biology, where his group pioneered genetic modification of *Trypanosoma cruzi*, the causative agent of Chagas disease. They have since developed a range of genetic tools and applied these to investigating drug mode-of-action, parasite biochemistry and molecular genetics. With the development of highly-sensitive *in vivo* imaging procedures and murine models of Chagas disease, recent research has focussed on disease pathogenesis and drug discovery through collaborations with DND*i*, GSK, Novartis and other academic partners.

Figure 1. Mammalian stages of the *Trypanosoma cruzi* life-cycle. Flagellated metacyclic trypomastigotes are transmitted by triatomine bugs and infect mammalian cells where they differentiate into replicative amastigotes that divide by binary fission. After 4-7 days, the parasites transform into the non-replicative bloodstream form trypomastigote stage, escape from the host cell, and continue to propagate the infection. In the example shown, MA104 cells (an African Green monkey foetal kidney cell line) were infected with *T. cruzi* CL Brener parasites (DTU TcVI) expressing mScarlet fluorescence (red). mScarlet is co-expressed with luciferase as part of a bioluminescence:fluorescence fusion protein reporter encoded by an engineered gene stably integrated into a ribosomal RNA expression site (99). DNA (host and parasite) is stained with Hoechst (blue). White scale bars = 10 μM. The metacyclic trypomastigote (left) has been enlarged for visual effect.

Figure 2. Using bioluminescent/fluorescent imaging to monitor murine acute stage *Trypanosoma cruzi* infections. BALB/c mice were infected with the *T. cruzi* (CL Brener strain) that had been genetically modified to express a fusion protein that is both bioluminescent and fluorescent (see Fig. 3) (99). (A) *Ex vivo* bioluminescence imaging reveals the widespread dissemination of *T. cruzi* in mouse tissue and organs at the peak of the acute stage of infection (day 14). (B) Fluorescence imaging of various tissue sections obtained from acutely infected mice (the cardiac muscle and bladder images are from reference 234). With the exception of skeletal muscle, host cell DNA is pseudo-coloured red (DAPI), and parasites are green (or yellow when imaged on a red background). In the skeletal muscle image, DNA is stained blue (also

DAPI), with actin stained in red (mouse mAb; Thermo Fisher MA5-12542), giving the typical striated pattern. Scale bars = 20 μm

Figure 3. Single cell resolution imaging of a chronic *Trypanosoma cruzi* infection focus in the mouse colon. (A) Organisation of the bioluminescent:fluorescent fusion gene, following integration into a *T. cruzi* ribosomal locus (99). The red-shifted (R-S) luciferase, linker (black), and mNeonGreen (mNeonG) sequences are indicated. (B) *Ex vivo* bioluminescence image of an external colonic wall layer from a chronically infected CH3/HeN mouse imaged using the IVIS Spectrum system (Caliper Life Science). (C) Detection of fluorescent parasites in the tissue using a Zeiss LSM880 confocal microscope (Ex506 nm, Em517 nm) after bioluminescence-guided imaging. (D) Serial z-stack sections of the tissue encompassing the infected cell can be used to generate a 3-D image, with parasite numbers then determined on the basis of DNA staining (DAPI, blue). White arrows indicate each parasite (adapted from 73).

Figure 4. The development of cardiac fibrosis in *Trypanosoma cruzi* infected mice can be blocked when curative benznidazole treatment is initiated in the acute stage, but not the chronic stage. BALB/c mice infected with *T. cruzi* (CL Brener strain) were subject to curative benznidazole treatment (20 days, 100 mg/kg). (A) Quantification of collagen content (Masson's trichome stain) in cardiac sections as a marker of fibrosis. Data are from mice where treatment was initiated 14, 22, 66 or 100 days post-infection (132), and the cardiac tissue then harvested 169 days post-infection. (B) Micrographs highlighting the extent of cardiac fibrosis (collagen

deposition – blue) in control mice, and infected mice where benznidazole treatment was initiated 22 or 110 days post-infection.

Figure 5. Tissue distribution of *Trypanosoma cruzi* during chronic murine infections revealed by *ex vivo* bioluminescence imaging. BALB/c mice were infected with *T. cruzi* CL Brener (DTU TcVI), and C3H/HeN mice were infected with *T. cruzi* JR (DTU TcI), as indicated. (A,B) When infections had progressed to the chronic stage (>100 days), tissues and organs were arranged as shown and examined by *ex vivo* bioluminescence imaging (73). (C) *Ex vivo* imaging of skin, fur side down and with adipose tissue removed. Upper image; BALB/c, Tc CL Brener. Lower image; C3H/HeN, Tc JR. Parasite strains express a red-shifted luciferase gene that was integrated into a *T. cruzi* ribosomal locus (70).

Figure 6. Visualising the impact of *T. cruzi* infection on enteric neurons. (A) Compressed z-stack fluorescence image of a colon tissue whole-mount from a C3H/HeN mouse 42 days post-infection (DPI) with a green fluorescent *T. cruzi* JR reporter strain. Expanded panels (right) show 4 μm sliced single z-stack images of parasites (green) in close proximity to nerves (red; anti- Tuj1) in the region highlighted by the white arrow. Host cell DNA, grey. (B) Compressed z-stack whole-mount immunofluorescence images of colon tissue neuronal cell bodies in the myenteric nerve plexus of a C3H/HeN mouse 336 DPI with *T. cruzi* JR (anti-Hu; magenta). The diffuse cell morphology (lower image) illustrates the progressive deterioration of the enteric nervous system during chronic stage DCD.

Figure 7. Investigating Chagas disease drug efficacy using bioluminescence imaging. (A) BALB/c mice chronically infected with *T. cruzi* CL Brener were treated orally with 30 mg/kg benznidazole (BNZ) for either 5 or 20 days. Ventral and dorsal *in vivo* images of 2 mice per drug regimen are shown. 117 days post-infection (DPI), both mice that were treated for 5 days were designated as non-cured, and euthanised (yellow dots). Mice given with the longer dosing regimen, were further treated with cyclophosphamide (injected with 200 mg/kg on 136, 140 and 144 DPI) to promote the outgrowth of any remaining parasites. (B) *Ex vivo* images of organs and tissues from the mice treated with benznidazole for 20 days (176 DPI). Mice that are bioluminescence negative by both *in vivo* and *ex vivo* imaging are designated as cured.

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The authors declare that they have no financial or other competing interests that are relevant to this study.

Table 1. The wide host range of *Trypanosoma cruzi*[1]

Host species	Reported type of infection	Common research uses	Ref.
Macaque	Natural (primate research centre)	Drugs, vaccines, immunopathology	78, 82
Spider/howler monkeys	Natural (field acquired)	-	240
Chimpanzee	Natural (primate research centre)	-	241
Tamarin	Natural (field acquired and zoo)	-	242, 243
Mouse	Various, including experimental	Most widely used experimental model	100, 244
Rat	Various, including experimental	General experimental model	245, 246
Hamster	Experimental	General experimental model	74, 102
Bat	Natural (field acquired)	-	6, 247
Opossum	Natural (field acquired)	-	42, 248
Raccoon	Natural (field acquired)	-	249, 250
Horse	Natural (domesticated environment)	-	46, 251
Cattle	Natural (domesticated environment)	-	252, 253
Sheep	Natural (domesticated environment)	-	46, 254
Pigs	Natural (domesticated environment)	-	255
Dog	Various, including experimental	Drugs, vaccines, immunopathology	115, 256
Fox	Natural (field acquired)	-	257, 258
Cat	Various	-	259, 260
Armadillo	Natural (field acquired)	-	248, 261
Lama	Natural (domesticated environment)	-	262
Sloth	Natural (field acquired)	-	263
Lizard	Natural (field acquired and experimental)	-	59, 60
Owl	Natural (field acquired)	-	58
Zebrafish	Experimental	-	61

Footnotes:

[1]This table is illustrative of the wide range of species where infection with T. cruzi has been reported, not a comprehensive list.

Table 2. Examples of transgenic null mutant mouse strains used to explore the impact of *Trypanosoma cruzi* infection

Target gene	Cell types involved	Phenotype during infection with *T. cruzi*	Ref.
Slamf1	myeloid cells - macrophages, dendritic	Survive lethal challenge, strain-specific reduced parasite replication in macrophages and dendritic cells, reduced parasite infiltration into heart, reduced NADPH oxidase 2 activity in infected macrophages	133, 134
TLR-2	myeloid cells - macrophages, dendritic	No significant effect on parasite burden, proinflammatory cytokine production unaffected	264
TLR-9	myeloid cells - macrophages, dendritic	Enhanced parasitemia, impaired IFN-γ response	265
TLR-2/ TLR-9	myeloid cells - macrophages, dendritic	Enhanced parasitemia over TLR9 null alone, increased mortality in acute infection. Mortality not as great as with the MYD88 null mutant, impaired IFN-γ response	265
MYD-88	myeloid cells - macrophages, dendritic	Enhanced parasitemia, increased mortality in acute infection, decreased production of IFN-γ, lL-12., TNF-α and reactive nitrogen species	264
IFN-γ	NK, $CD4^+$ T, $CD8^+$ T cells	Enhanced parasitemia, increased and rapid mortality in acute infection,	266
IL-1R	macrophages, monocytes, dendritic cell subsets	Enhanced parasitemia, prevention of bradycardia, no effect on other arrhythmias	267
IL-4	Th2 T cells,	Decreased parasitemia, lower mortality, reduced anti-trypanosome IgG1	266
IL-6	widely expressed	Enhanced parasitemia, increased mortality, reduced splenocyte recall response, no impact on IFN-γ secretion	268
IL-10	monocytes, Th2 T cells, Tregs	Enhanced parasitemia, increased morbidity, decrease in $CD8^+$ T cell response and IFN-γ production,	269
IL-12	myeloid cells	Enhanced parasitemia, increased and rapid mortality in acute stage infection, impaired IFN-γ response	266, 270
IL-17A	Th17, $CD8^+$ T, γδT, NK cells, neutrophils	Enhanced parasitemia, increased mortality, increased tissue damage, decreased activation of effector functions in immune cells	271

IL-17RA	widely expressed	Increased mortality without enhanced parasitemia, reduced neutrophil recruitment, increased inflammatory tissue damage,	272
IL-18	widely expressed	No significant difference to wild type (Tulahuen or Y strain). Mice infected with Colombian strain showed reduced myocarditis in chronic stage	270, 273
CCR-5	widely expressed	Increased parasitemia and cardiac parasitism in the acute stage, and reduced survival. Directs migration of macrophages and T cells to the heart. Not essential for maintaining inflammation in the heart during chronic infection	274
$P47^{phox}$	phagocytic cells	Increased mortality, increased iNOS expression, increased pro-inflammatory cytokine production, impaired activation of $CD8^+$ T cells	275
Inducible NO synthase	widely expressed	No impact on the extent of infection, but enhanced expression of TNF-α, IL-1, and MIP-1α	276
MicroRNA-155	hematopoietic cells	Increased mortality, increased cardiac parasite burden, decreased pro-inflammatory cytokine production. Decreased $CD8^+$, NK and NK-T cell populations.	277
5-lipoxygenase	widely expressed, but not by T cells	Transiently increases parasitemia, and enhances survival rate. Reduced cardiac inflammation, collagen deposition, and migration of CD4+, CD8+, and IFN-γ-producer cells	278
STAT-1	most abundant in lymphoid tissues	Increased parasitemia and parasite tissue load, reduced survival rate. Higher systemic levels of IFN-γ, IL-10 and IL-17	279

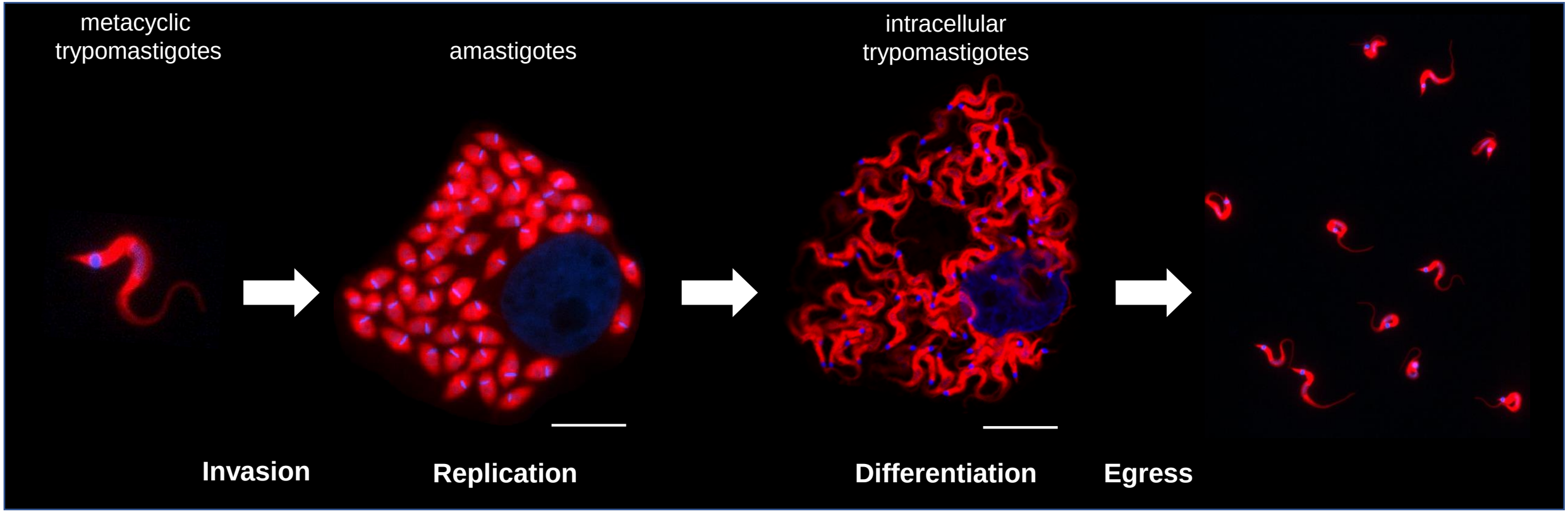

Figure 1. Mammalian stages of the *Trypanosoma cruzi* life-cycle. Flagellated metacyclic trypomastigotes are transmitted by triatomine bugs and infect mammalian cells where they differentiate into replicative amastigotes that divide by binary fission. After 4-7 days, the parasites transform into the non-replicative bloodstream form trypomastigote stage, escape from the host cell, and continue to propagate the infection. In the example shown, MA104 cells (an African Green monkey foetal kidney cell line) were infected with *T. cruzi* CL Brener parasites (DTU TcVI) expressing mScarlet fluorescence (red). mScarlet is co-expressed with luciferase as part of a bioluminescence:fluorescence fusion protein reporter encoded by an engineered gene stably integrated into a ribosomal RNA expression site (99). DNA (host and parasite) is stained with Hoechst (blue). White scale bars = 10 μM. The metacyclic trypomastigote (left) has been enlarged for visual effect.

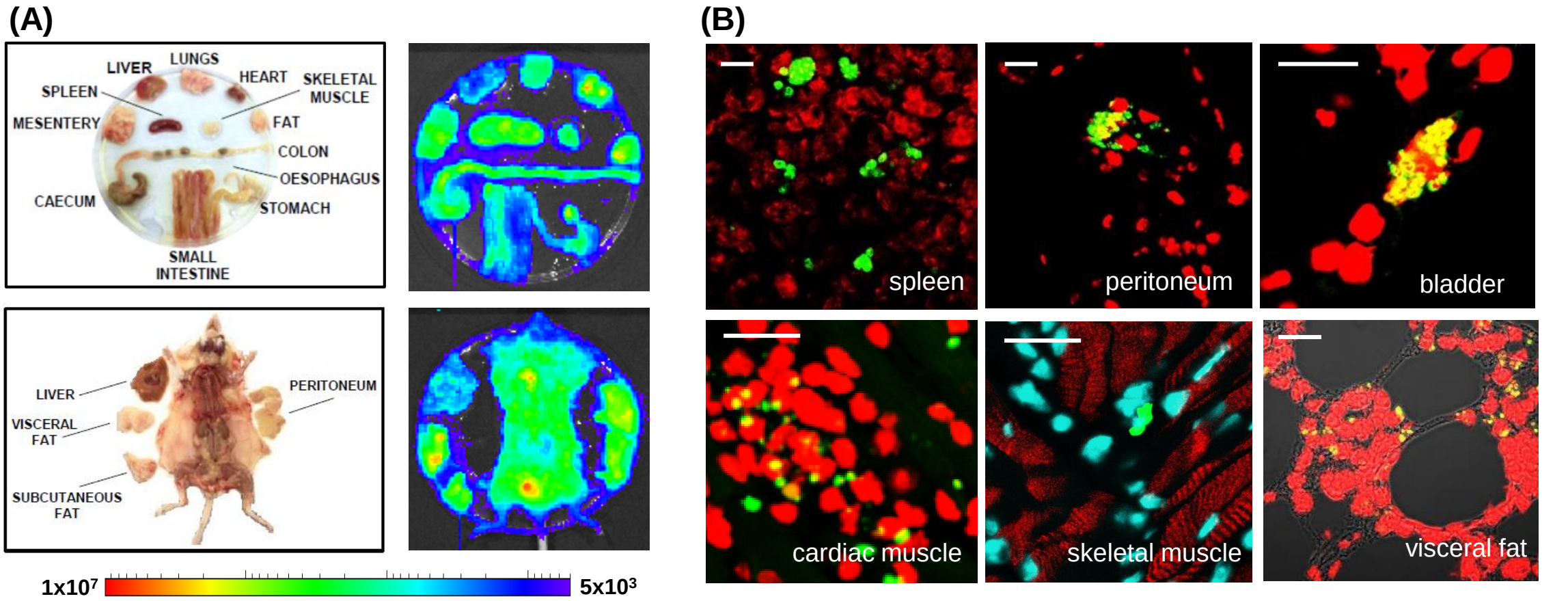

Figure 2. Using bioluminescent/fluorescent imaging to monitor murine acute stage *Trypanosoma cruzi* infections. BALB/c mice were infected with the *T. cruzi* (CL Brener strain) that had been genetically modified to express a fusion protein that is both bioluminescent and fluorescent (see Fig. 3) (99). (A) *Ex vivo* bioluminescence imaging reveals the widespread dissemination of *T. cruzi* in mouse tissue and organs at the peak of the acute stage of infection (day 14). (B) Fluorescence imaging of various tissue sections obtained from acutely infected mice. With the exception of skeletal muscle, host cell DNA is pseudo-coloured red (DAPI), and parasites are green (or yellow when imaged on a red background). In the skeletal muscle image, DNA is stained blue (also DAPI), with actin stained in red (mouse mAb; Thermo Fisher MA5-12542), giving the typical striated pattern. Scale bars = 20 μm

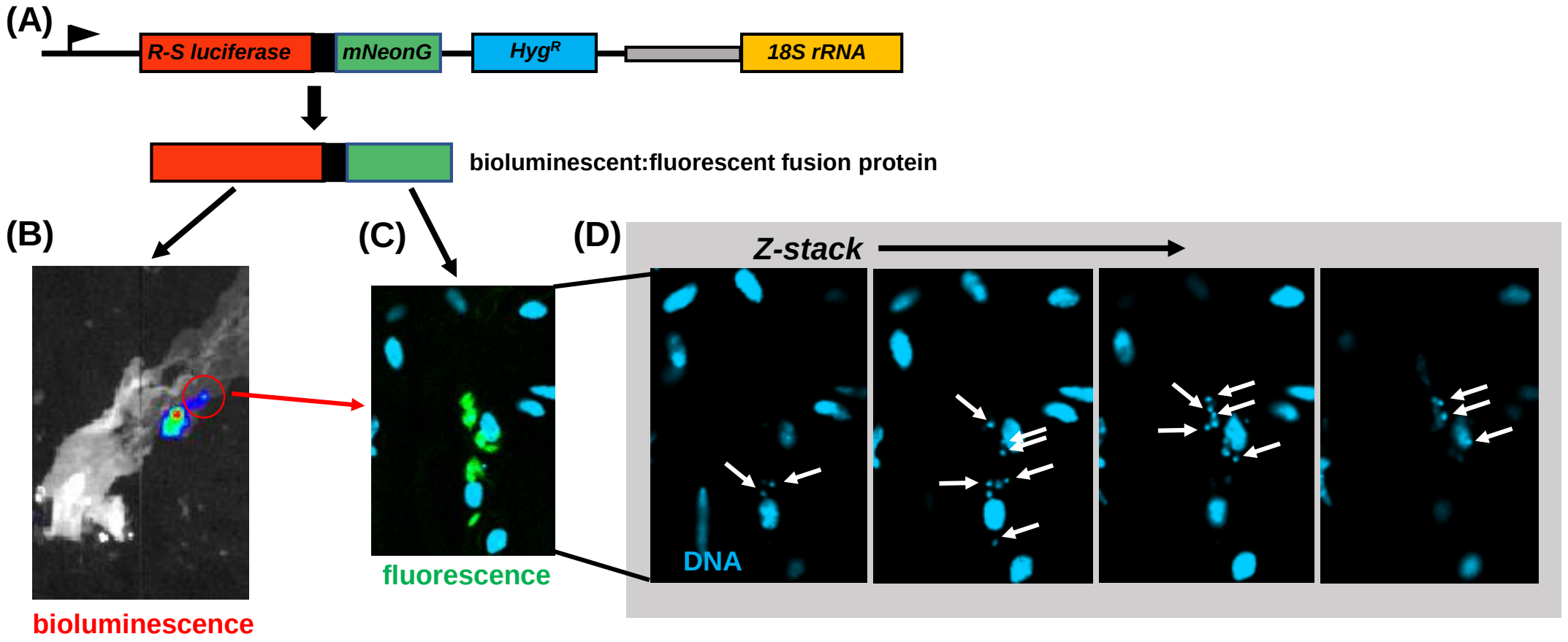

Figure 3. Single cell resolution imaging of a chronic *Trypanosoma cruzi* infection focus in the mouse colon. (A) Organisation of the bioluminescent:fluorescent fusion gene, following integration into a *T. cruzi* ribosomal locus (99). The red-shifted (R-S) luciferase, linker (black), and mNeonGreen (mNeonG) sequences are indicated. (B) *Ex vivo* bioluminescence image of an external colonic wall layer from a chronically infected CH3/HeN mouse imaged using the IVIS Spectrum system (Caliper Life Science). (C) Detection of fluorescent parasites in the tissue using a Zeiss LSM880 confocal microscope (Ex506 nm, Em517 nm) after bioluminescence-guided imaging. (D) Serial z-stack sections of the tissue encompassing the infected cell can be used to generate a 3-D image, with parasite numbers then determined on the basis of DNA staining (DAPI, blue). White arrows indicate each parasite (adapted from 73).

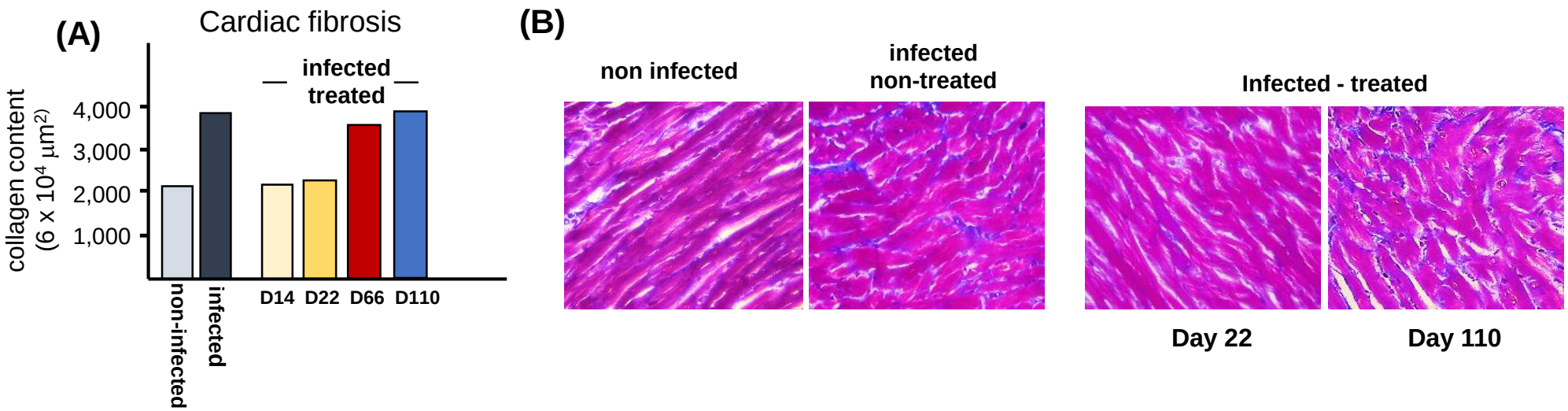

Figure 4. The development of cardiac fibrosis in *Trypanosoma cruzi* infected mice can be blocked when curative benznidazole treatment is initiated in the acute stage, but not the chronic stage. BALB/c mice infected with *T. cruzi* (CL Brener strain) were subject to curative benznidazole treatment (20 days, 100 mg/kg). (A) Quantification of collagen content (Masson's trichome stain) in cardiac sections as a marker of fibrosis. Data are from mice where treatment was initiated 14, 22, 66 or 100 days post-infection (132), and the cardiac tissue then harvested 169 days post-infection. (B) Micrographs highlighting the extent of cardiac fibrosis (collagen deposition – blue) in control mice, and infected mice where benznidazole treatment was initiated 22 or 110 days post-infection.

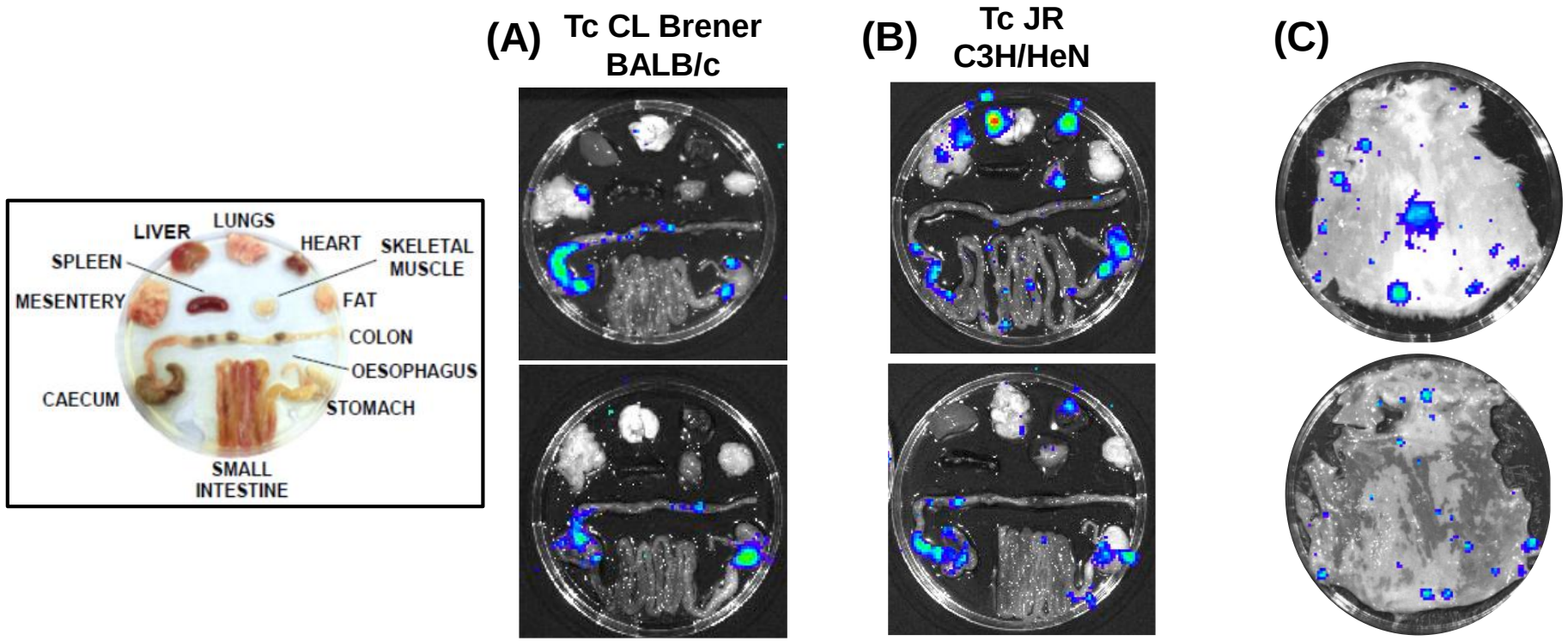

Figure 5. Tissue distribution of *Trypanosoma cruzi* during chronic murine infections revealed by *ex vivo* bioluminescence imaging. BALB/c mice were infected with *T. cruzi* CL Brener (DTU VI), and C3H/HeN mice were infected with *T. cruzi* JR (DTU I), as indicated. (A,B) When infections had progressed to the chronic stage (>100 days), tissues and organs were arranged as shown and examined by *ex vivo* bioluminescence imaging (73). (C) *Ex vivo* imaging of skin, fur side down and with adipose tissue removed. Upper image; BALB/c, Tc CL Brener. Lower image; C3H/HeN, Tc JR. Parasite strains express a red-shifted luciferase gene that was integrated into a *T. cruzi* ribosomal locus (70).

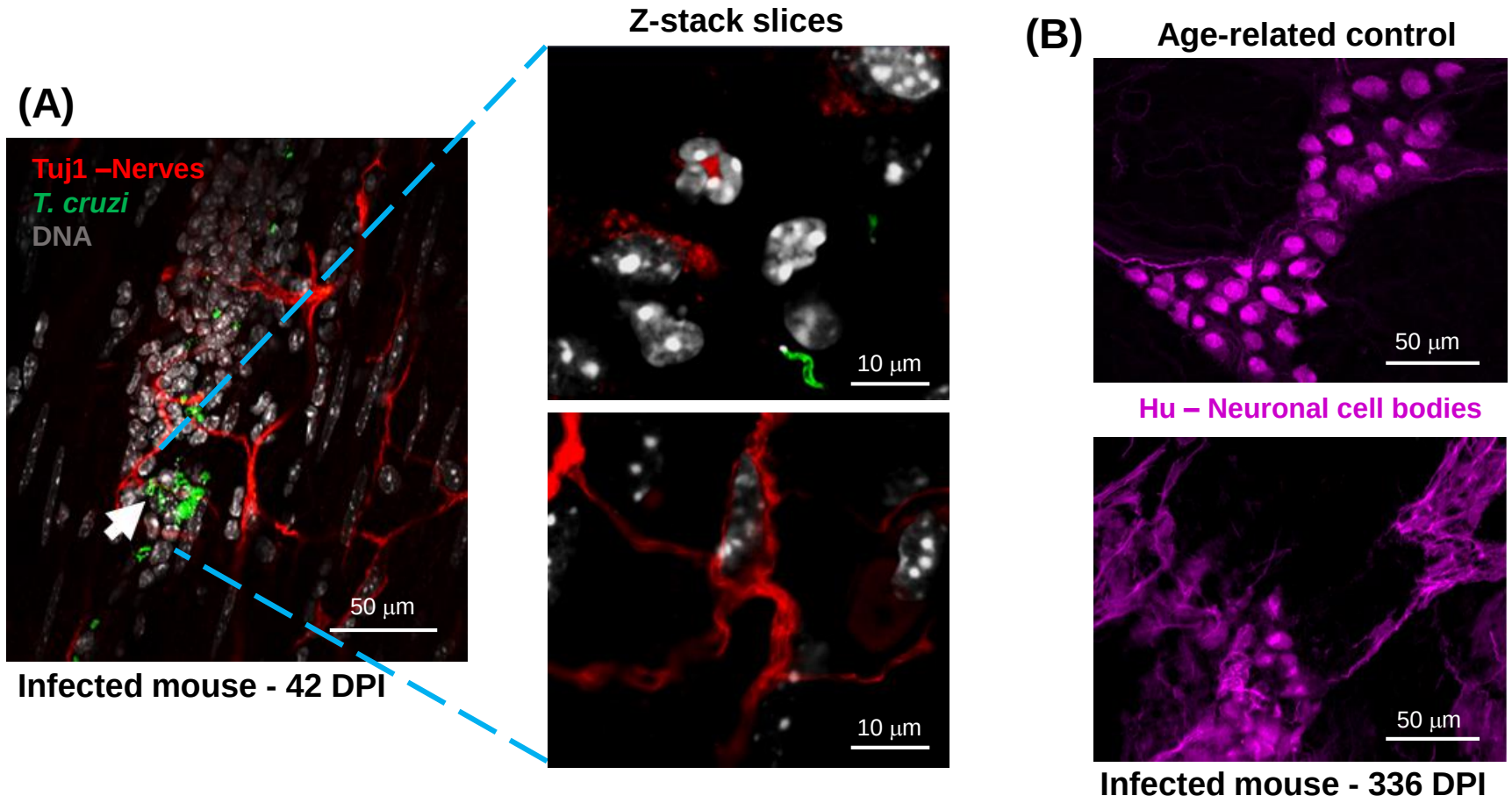

Figure 6. Visualising the impact of *T. cruzi* infection on enteric neurons. (A) Compressed z-stack fluorescence image of a colon tissue whole-mount from a C3H/HeN mouse 42 days post-infection (DPI) with a green fluorescent *T. cruzi* JR reporter strain. Expanded panels (right) show 4 μm sliced single z-stack images of parasites (green) in close proximity to nerves (red; anti- Tuj1) in the region highlighted by the white arrow. Host cell DNA, grey. (B) Compressed z-stack whole-mount immunofluorescence images of colon tissue neuronal cell bodies in the myenteric nerve plexus of a C3H/HeN mouse 336 DPI with *T. cruzi* JR (anti-Hu; magenta). The diffuse cell morphology (lower image) illustrates the progressive deterioration of the enteric nervous system during chronic stage DCD.

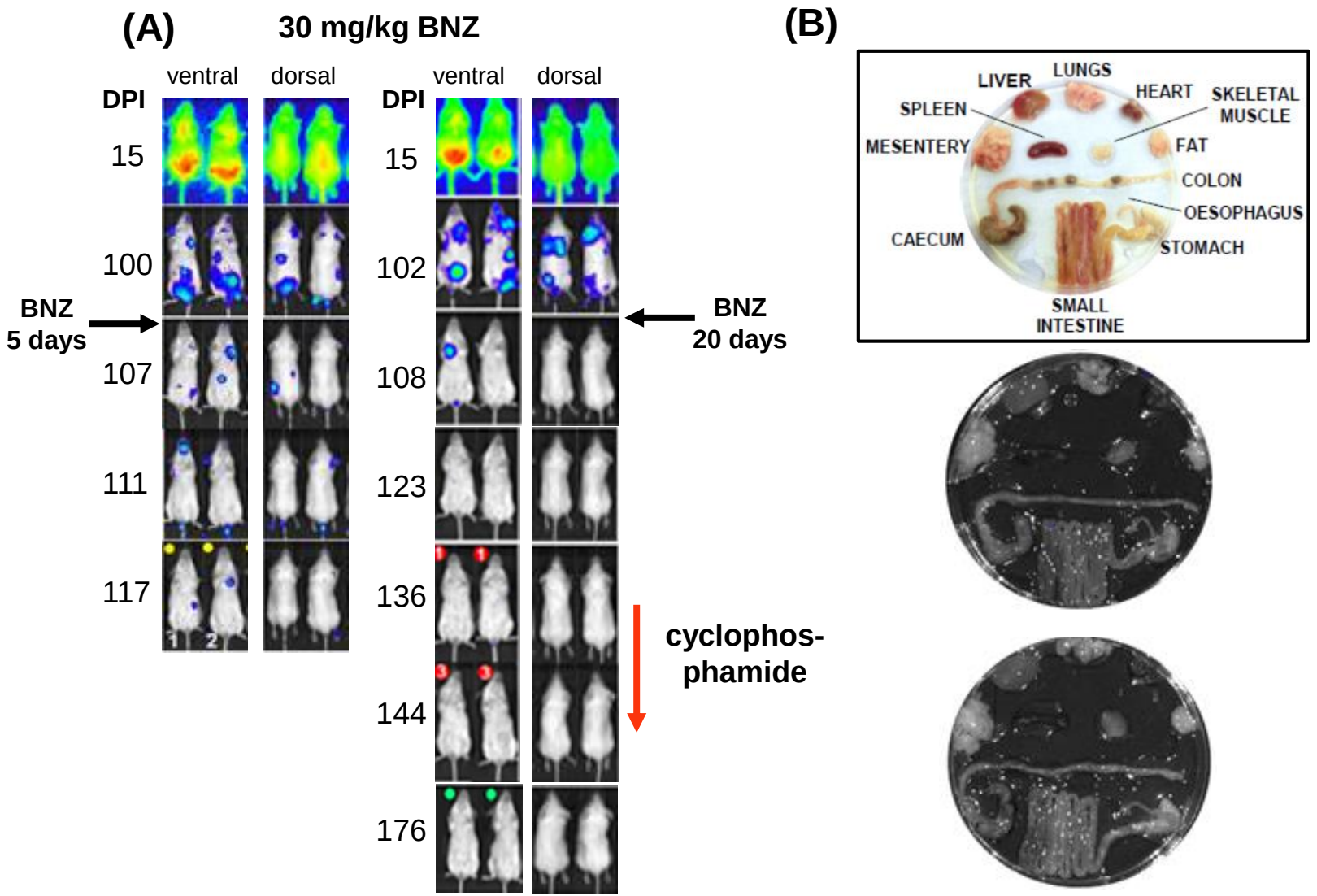

Figure 7. Investigating Chagas disease drug efficacy using bioluminescence imaging. (A) BALB/c mice chronically infected with *T. cruzi* CL Brener were treated orally with 30 mg/kg benznidazole (BNZ) for either 5 or 20 days. Ventral and dorsal *in vivo* images of 2 mice per drug regimen are shown. 117 days post-infection (DPI), both mice that were treated for 5 days were designated as non-cured, and euthanised (yellow dots). Mice given with the longer dosing regimen, were further treated with cyclophosphamide (injected with 200 mg/kg on 136, 140 and 144 DPI) to promote the outgrowth of any remaining parasites. (B) *Ex vivo* images of organs and tissues from the mice treated with benznidazole for 20 days (176 DPI). Mice that are bioluminescence negative by both *in vivo* and *ex vivo* imaging are designated as cured.