

## Using Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes as a Model to Study *Trypanosoma cruzi* Infection

Adriana Bozzi,<sup>1,6</sup> Nazish Sayed,<sup>1,2,3</sup> Elena Matsa,<sup>1,2,3</sup> Gabriele Sass,<sup>4,5</sup> Evgenios Neofytou,<sup>1,2,3</sup> Karl V. Clemons,<sup>4,5</sup> Rodrigo Correa-Oliveira,<sup>6</sup> David A. Stevens,<sup>4,5</sup> and Joseph C. Wu<sup>1,2,3,\*</sup>

<sup>1</sup>Stanford Cardiovascular Institute, 265 Campus Drive, Rm G1120B, Stanford, CA 94305, USA

<sup>2</sup>Department of Medicine, Division of Cardiovascular Medicine, Stanford University, School of Medicine, Stanford, CA 94305, USA

<sup>3</sup>Department of Radiology, Stanford University, School of Medicine, Stanford, CA 94305, USA

<sup>4</sup>Division of Infectious Diseases and Geographic Medicine, Stanford University, School of Medicine, Stanford, CA 94305, USA

<sup>5</sup>California Institute for Medical Research, San Jose, CA 95128, USA

<sup>6</sup>Instituto René Rachou, FIOCRUZ, Belo Horizonte, Brazil

\*Correspondence: [joewu@stanford.edu](mailto:joewu@stanford.edu)

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### SUMMARY

Chagas disease (ChD) is one of the most neglected tropical diseases, with cardiomyopathy being the main cause of death in *Trypanosoma cruzi*-infected patients. As the parasite actively replicates in cardiomyocytes (CMs), the heart remains a key target organ in the pathogenesis of ChD. Here we modeled ChD using human induced pluripotent stem cell-derived CMs (iPSC-CMs) to understand the complex interplay between the parasite and host cells. We showed that iPSC-CMs can get infected with the *T. cruzi* Y strain and that all parasite cycle stages can be identified in our model system. Importantly, characterization of *T. cruzi*-infected iPSC-CMs showed significant changes in their gene expression profile, cell contractility, and distribution of key cardiac markers. Moreover, these infected iPSC-CMs exhibited a pro-inflammatory profile as indicated by significantly elevated cytokine levels and cell-trafficking regulators. We believe our iPSC-CM model is a valuable platform to explore new treatment strategies for ChD.

### INTRODUCTION

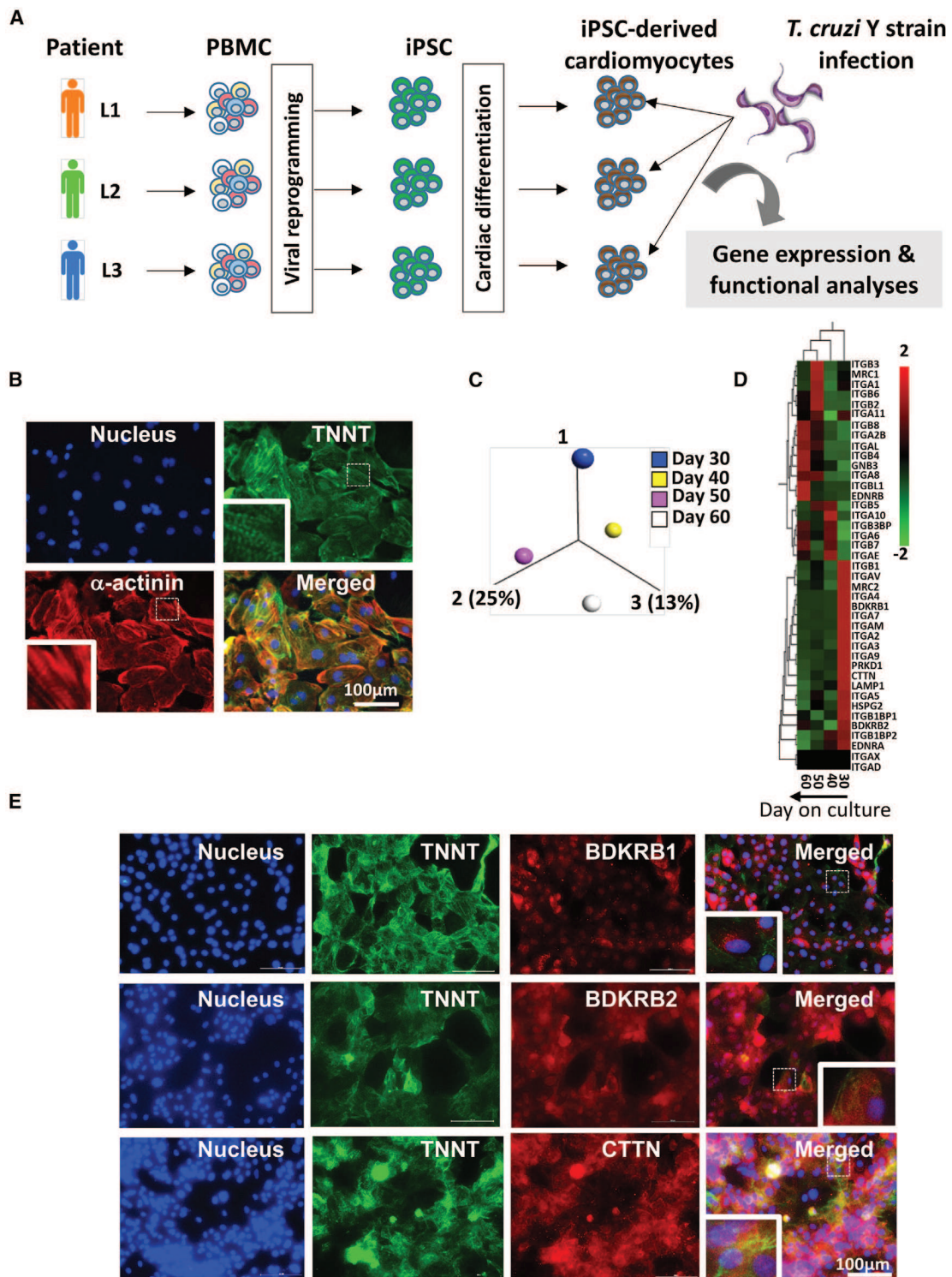
Chagas disease (ChD) is a parasitic, systemic, and chronic disease caused by the protozoan *Trypanosoma cruzi*, with at least 6 million people estimated to be infected worldwide. *T. cruzi* parasites are mainly transmitted to humans by the infected feces of blood-sucking triatomine bugs often known as the “kissing bug”. Despite intensive efforts to control this disease over the years, ChD has recently experienced a resurgence in endemic countries and emerged in non-endemic areas, such as the United States (due to factors such as immigration, blood transfusion, and organ transplantation) (Bern et al., 2011). Currently available therapeutic options for ChD are limited, with benznidazole (BNZ) or nifurtimox being recommended for treatment of acute phase and early in the chronic infection (de Andrade et al., 1996).

Characterized by a progressive inflammatory reaction, ChD presents an acute phase, generally asymptomatic, followed by a lifelong chronic phase, with distinct clinical forms known as indeterminate, cardiac (CARD), and digestive (Brener, 1980). The most common and severe manifestation of ChD is the CARD form that causes congestive heart failure, arrhythmias, and conduction abnormalities, often leading to stroke and sudden death. This type of dilated cardiomyopathy (DCM) is characterized by chronic inflammation, fibrosis, cardiac hypertrophy, and thromboembolic

events. During its obligate intracellular stage, *T. cruzi* trypomastigotes can actively replicate in many different cell types such as macrophages, smooth and skeletal muscles, cardiomyocytes (CMs), and endothelial cells. Many studies investigating the immune response following *T. cruzi* infection and pathogenesis in the chronic phase of ChD have shown that the inflammatory response in the myocardium is an important aspect in the pathogenesis of ChD (Gomes et al., 2003; Sousa et al., 2014; Souza et al., 2004).

CARD is clearly the result of an inflammatory process. Therapeutic measures are aimed at treating the consequences of disease such as cardiac failure. This scenario necessitates attention to efficient diagnosis, treatment, and clinical management (Andrade et al., 2014). To this end, it is essential to improve our understanding of the progression of parasitic infection and standardization of models designed for ChD drug discovery. Rodents are the most commonly used models to study ChD, but fundamental differences between mice/rats and humans make murine models less than optimal for the following reasons: (1) the murine resting heart rate is approximately 6- to 10-fold faster than the human rate and (2) murine and human CMs also differ in fundamental aspects such as cardiac development, electrical properties, and ion channel contributions. As an alternative, in this study we developed a new model to study ChD using human induced pluripotent cell-derived CMs (iPSC-CMs).





**Figure 1. Human iPSC-CMs and Receptors to *Trypanosoma cruzi* Invasion**

(A) Overview of study design. PBMCs from three healthy individuals were reprogrammed to iPSCs using a Sendai virus vector expressing Oct4, Sox2, Klf4, and c-Myc. iPSC clones were differentiated into iPSC-CMs, infected with *T. cruzi* Y strain, and submitted for gene expression and functional analysis.

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## RESULTS

### Receptors to *T. cruzi* Invasion Are Expressed on iPSC-CMs

Peripheral blood mononuclear cells (PBMCs) from three healthy subjects were reprogrammed to iPSCs using the non-integrating Sendai virus method (Figure 1A). We achieved up to 90% efficiency in differentiating iPSC-CMs, as assessed by  $\alpha$ -actin and troponin T expression and spontaneously contractility of iPSC-CMs (Figure 1B). Next, these iPSC-CMs were tested for their capacity to be infected by *T. cruzi* trypomastigotes. The gene expression of host receptors used by *T. cruzi* parasites to invade cells was analyzed in uninfected iPSC-CMs cultured on days 30, 40, 50, and 60. Principal-component analysis (PCA) (Figure 1C) and heatmap (Figure 1D) showed a differential gene expression pattern depending on the days being analyzed. The majority of receptors showed a high gene expression on day 30. We confirmed by immunofluorescence assay the expression of bradykinin receptor-1, bradykinin receptor-2, and cortactin in more than 80% of iPSC-CMs (Figure 1E), suggesting that parasitic infection of iPSC-CMs was possible starting on day 30.

### Index of *T. cruzi* Infection in iPSC-CMs

The potential of *T. cruzi* (Tc) Y strain to invade iPSC-CMs was verified after 3 and 24 h interaction, in ratios of 1:5 and 1:10 (iPSC-CMs:parasites), respectively. The infection index grew with time interaction and ratio analyzed. A ratio of 1:10 enabled infection of 100% of iPSC-CMs by 24 h, therefore the higher ratio was not required (Figure 2A). Almost 50% of iPSC-CMs had the amastigote *T. cruzi* forms in their cytoplasm in a low ratio (1:5) after interaction (3 h). Therefore, all further infections were performed with 3 h interaction and at 1:5 ratio.

### Time Course of *T. cruzi* Infection in iPSC-CMs

To explore *T. cruzi* infection in iPSC-CMs, we next analyzed the time course of infection. The number of amastigote form parasites increased exponentially and trypomastigote form parasites were observed in supernatant at 96 h of infection (Figure 2B). Importantly, the number of iPSC-CMs decreased significantly with around 91% rendered nonviable when infected with *T. cruzi* as compared with only 11% in the absence of infection (Figure 2C). Moreover, this decrease was further exacerbated following 72 and 96 h of infection (Figure 2D), and the morphology of

the iPSC-CMs started to change to a vacuolated cytoplasm. At the same time-points, significant levels ( $p < 0.05$ ) of reactive oxygen species (ROS) and reactive nitrogen species (RNS) were quantified in the supernatant of iPSC-CMs cultured with Tc Y strain (Figure 2E), suggesting that *T. cruzi* infection led to oxidative stress and apoptosis in iPSC-CMs.

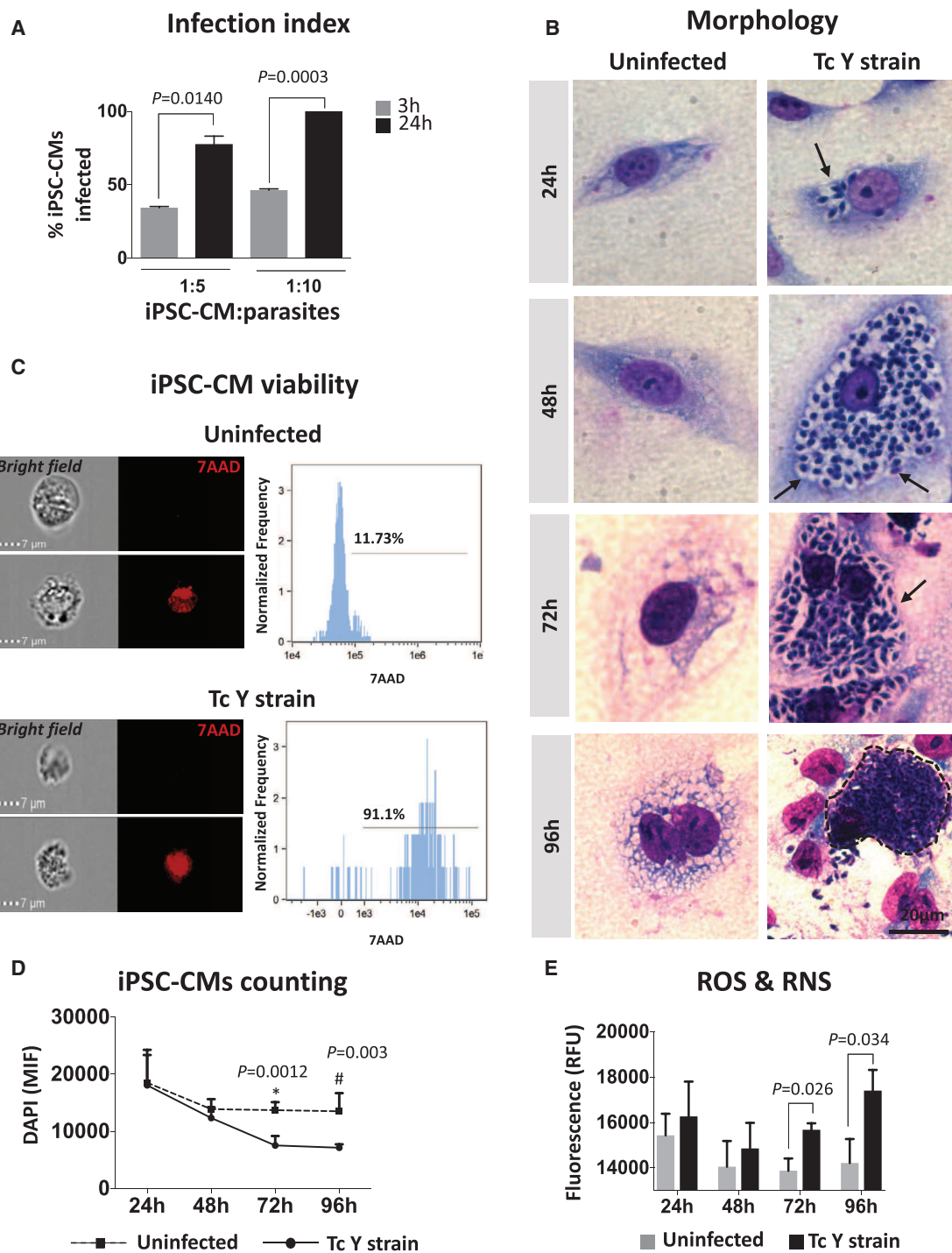
### iPSC-CM Contractility following *T. cruzi* Infection

All human iPSC-CM lines cultured with Tc Y strain were measured for their beat rate, peak height, and beat duration following 24 and 48 h of infection (Figures S1A–S1C). After 48 h of infection, iPSC-CMs were beating at nearly 200 beats per minute and experienced a significant increase in their peak height that was 3.27 times greater than that detected in the controls (Figures S1A and S1B). Although the differences in the beat duration were not significant, they were 11.83 times lower in non-infected iPSC-CMs (Figure S1C). These results were similar in all iPSC-CM lines. Next, we investigated the effects of *T. cruzi* infection on genes that encode calcium-handling proteins. We analyzed the gene expression level of *CACNA1C* (voltage-dependent L-type calcium channel), *RYR2* (ryanodine receptor 2), *CASQ2* (calsequestrin 2), *TRDN* (triadin), and *ATP2A2* (sarco(endo)plasmic reticulum calcium-ATPase 2) (Figure S2A). The majority of  $Ca^{2+}$  handling genes analyzed were down-regulated in iPSC-CMs following *T. cruzi* infection. Moreover, we found low expression of *PKP2* gene (Figure S2B) that codes for the desmosomal protein plakophilin-2 and plays an important role in cell-cell adhesion and transcription of genes that control intracellular calcium cycling. Taken together, these data suggest that our iPSC-CM model reproduced an arrhythmic stress during *T. cruzi* infection that was characterized by tachycardia.

### Sarcomeric Proteins and Connexin 43 Distribution in iPSC-CMs

To investigate the influence of *T. cruzi* infection on sarcomere structure and cell junction formation in iPSC-CMs, we tracked the distribution of  $\alpha$ -actinin, troponin T, and connexin 43 during the course of infection (Figure S1D). While the non-infected iPSC-CMs had organized myofibrils with  $\alpha$ -actinin and troponin T staining at the Z line of sarcomeres at all times-points (Figure S1D), the infected samples exhibited sarcomeric disarray as early as 24 h post-infection, which increased throughout the course of infection (48–96 h) (Figure S1D). The sarcomere localization of troponin T was similar to control cells

(B) Immunofluorescence images of iPSC-CMs confirming the presence of sarcomeric proteins such as  $\alpha$ -actinin and troponin T. (C and D) Variability in gene expression of receptors for *T. cruzi* invasion on iPSC-CMs cultured on days 30, 40, 50, and 60. (C) PCA with computation of closest neighboring samples; (D) heatmap showing hierarchical clustering between iPSC-CMs cultured on different days. (E) Immunofluorescence images of iPSC-CMs showing the expression of receptors to *T. cruzi* such as bradykinin receptor 1 (BDKR1), bradykinin receptor 2 (BDKR2), and cortactin (CTTN). The expression of TNNT was used as a control. Scale bars, 100  $\mu$ m.



**Figure 2. Infection of Human iPSC-CMs with *Trypanosoma cruzi***

(A) iPSC-CMs were cultured with *T. cruzi* (Tc) Y strain at the ratio 1:5 or 1:10 (iPSC-CMs:parasites) for 3 or 24 h. The percentage of iPSC-CMs with amastigote forms inside the cytoplasm was plotted as the average of three different counting areas. Significant statistical differences ( $p < 0.05$ ) are indicated between samples.

(B) iPSC-CMs uninfected and infected with Tc Y strain in a ratio of 1:5 were stained with Giemsa after 24, 48, 72, and 96 h. The arrows point to amastigote *T. cruzi* forms in iPSC-CMs' cytoplasm, and the dotted line surrounds a nest of trypanomastigote *T. cruzi* outside ruptured iPSC-CMs. Scale bar, 20  $\mu\text{m}$ .

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until 48 h after *T. cruzi* infection. Following 72 and 96 h of infection, troponin T lost periodicity in the Z line of cardiac myofibrils (Figure S1D, Tc Y strain). By analyzing the connexin 43 distribution, we observed a loss of cell-cell junction at 24 h post-infection, which became more obvious at later timepoints (48–96 h) (Figure S1D, Tc Y strain).

### Cytokines and Chemokines Secreted by iPSC-CMs following *T. cruzi* Infection

To investigate iPSC-CMs in relation to the inflammation in ChD, we next analyzed the secretion of cytokines and chemokines by iPSC-CMs following *T. cruzi* infection. Although CMs do not typically produce cytokines and chemokines, significant levels were found after 48 h of *T. cruzi* infection (Figure 3). Interestingly, these results showed a predominance of inflammatory activators (Figures 3A and 3B) compared with regulators (Figure 3C).

### iPSC-CM Gene Expression Affected by *T. cruzi* Infection

We next performed RNA sequencing (RNA-seq) to analyze the iPSC-CM gene expression as described previously (Zhao et al., 2017). Uninfected and Tc Y strain-infected samples were grouped separately according to PCA analysis, which showed variability in gene expression before and after *T. cruzi* infection (Figure S3A). Heatmap analysis found approximately 11,000 genes that were differentially expressed ( $p < 0.05$ ) following *T. cruzi* infection (Figure S3B). As ChD is characterized by an inflammatory process that results in progressive fibrosis affecting ventricular contractility and structure, we focused on the expression of genes that are related to cardiac remodeling, inflammation, and contractility. We found 78 genes that were significantly down- or up-regulated in iPSC-CMs infected with *T. cruzi*, and we compared these results with previously published gene expression analyses of other cardiomyopathies induced by Coxsackievirus (COX) (Sharma et al., 2014), familial DCM (Sun et al., 2012), and doxorubicin (DOX) (1 and 10 mM) (Burridge et al., 2016) (Figure 4A). All experiments were performed in a comparable manner including having similar differentiation protocols and seeding densities of iPSC-CMs. Similarly, close attention was paid to make sure that identical amounts of iPSC-CMs were seeded and exposed

to *T. cruzi* when compared with uninfected controls. In contrast to COX, DCM, and DOX, iPSC-CMs infected with *T. cruzi* for 24 h showed inflammatory activity with a high expression of genes for chemoattractant and adhesion proteins (CXCL3, CXCL6, CXCL12, CXCL14, CXCL17, and CLEC4D) and pro-inflammatory proteins (IL6ST, IL-9R, TNFAIP1, IRAK1, MRC2, and HMGB1) (Figure 4B). Furthermore, genes expressing extracellular matrix proteins such as collagen type I and IV (COL1A1, COL1A2, and COL4A4), laminin (LAMA1), and metalloproteinase (MMP9 and MMP24) were significantly expressed in iPSC-CMs infected with *T. cruzi*. Interestingly, the majority of genes for sarcomeric proteins (ACTA2, MYH11, MYL2, MYBPC3, TNNC1, TNNT2, and TMOD1) and cytoskeleton (DMD and VCL) were downregulated in our iPSC-CM model following *T. cruzi* infection (Figure 4C), which correlated well with the vacuolated morphology of infected iPSC-CMs.

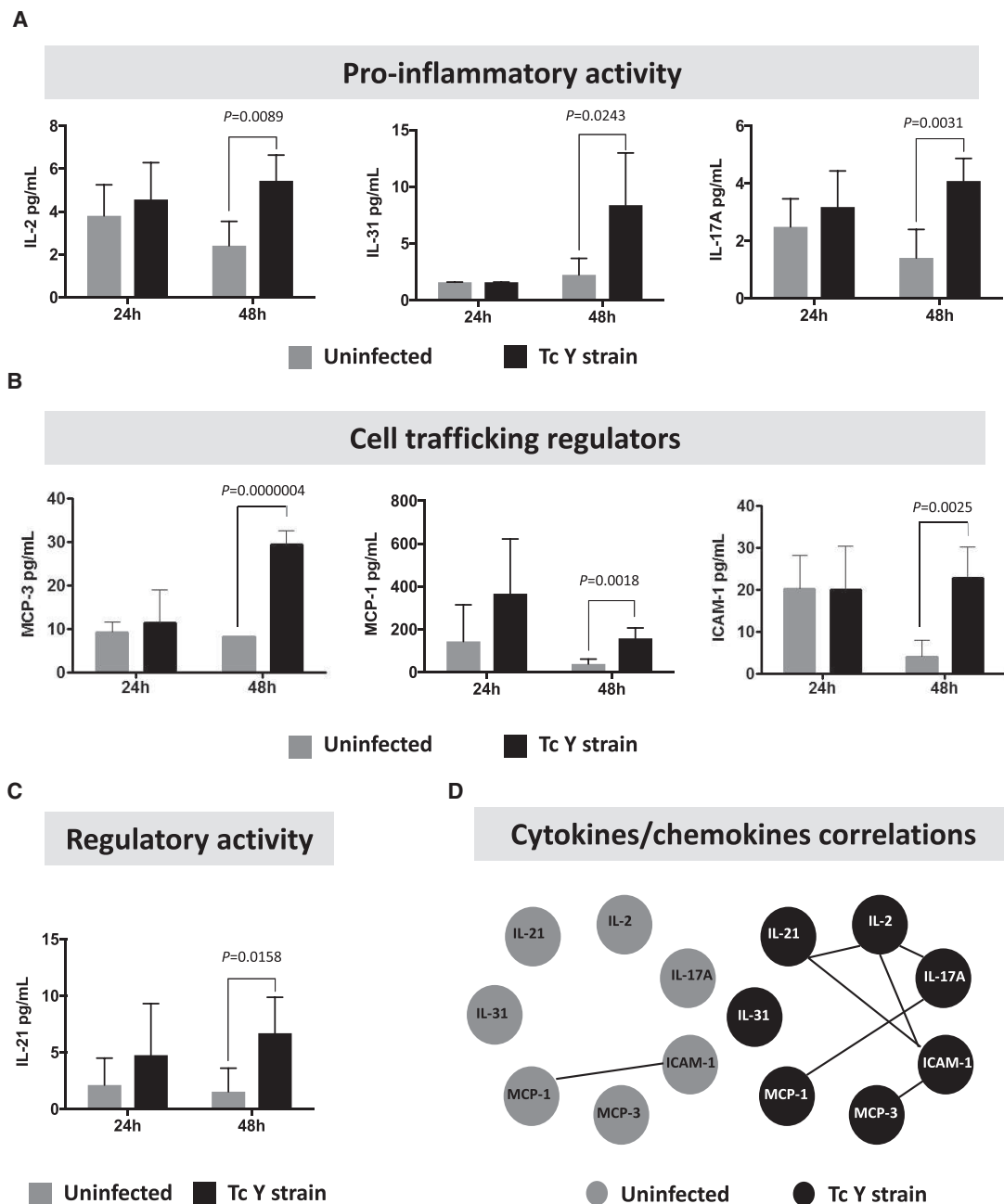
## DISCUSSION

This study was designed to develop an *in vitro* platform using human cardiac cells to study ChD that remains a public health problem in affected regions. As ~30%–40% of all Chagasic patients exhibit a severe clinical phenotype including heart failure, there is a compelling need to understand the mechanisms involved in ChD using human cardiac cells. Even though ChD has been studied previously using human PBMCs (Gomes et al., 2014; Sousa et al., 2014) or murine models (Garzoni et al., 2003; Pereira et al., 1993), it has been difficult to model ChD in human cardiac cells due to lack of available human samples. Our group has successfully reprogrammed adult cells into iPSCs (Churko et al., 2013) and differentiated them into CMs (Burridge et al., 2014), thereby providing an ideal *in vitro* platform for modeling human cardiac diseases (Liu et al., 2018; Sayed et al., 2016; Sharma et al., 2017), drug screening (Liang et al., 2013; Matsa et al., 2016), and precision medicine (Sayed et al., 2019; Sayed and Wu, 2017). Until now, this platform has not been used to model cardiac diseases that are triggered by parasitic infection such as *T. cruzi*. Here, we generated an iPSC-CM model that can be infected by Tc Y strain to recapitulate all phases of the parasite's life cycle including: (1) infective stage, (2) trypomastigotes

(C) iPSC-CM viability assay: images (bright and 7-AAD fluorescence fields) and histograms representatives of iPSC-CMs uninfected and infected with Tc Y strain stained with 7-AAD.

(D) Number of iPSC-CMs over the time course of *T. cruzi* infection. iPSC-CMs uninfected (dotted line) and infected (Tc Y strain) were stained with DAPI and the nucleus counted by size. The graph shows the average of triplicates of the iPSC-CMs after exclusion of trypanomastigote nuclei. Significant statistical differences ( $p < 0.05$ ) are indicated among samples.

(E) Total production of free radicals. Reactive oxygen species and reactive nitrogen species (ROS/RNS) were measured in the supernatants of iPSC-CMs uninfected and infected with Tc Y strain (1:5, 3 h interaction). The average of biological triplicates is expressed in columns graphs. Significant statistical differences are indicated among samples ( $p < 0.05$ ).



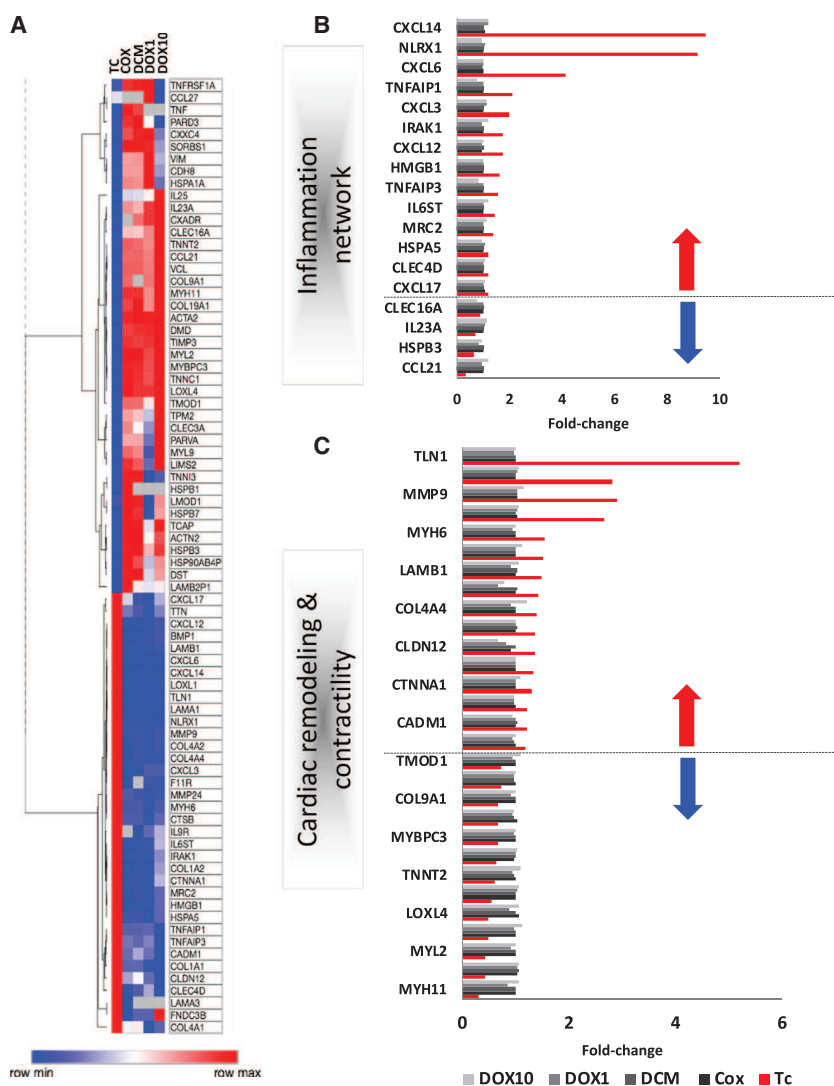
**Figure 3. Regulators of Inflammation Delivered by iPSC-CMs Infected with *T. cruzi***

(A–C) Sixty-three human molecules were measured in the supernatants of iPSC-CMs uninfected and infected with *T. cruzi* (Tc Y strain) (1:5, 3 h interaction). Graphs show most significantly affected (A) pro-inflammatory cytokines, (B) cell trafficking regulators, and (C) regulatory cytokines produced by iPSC-CMs following 24 and 48 h *T. cruzi* infection. Significant statistical differences are indicated among samples ( $p < 0.05$ ).

(D) Significant positive correlation between cytokines and chemokines produced by iPSC-CMs at 48 h *T. cruzi* infection ( $p < 0.05$ ; Spearman and Pearson correlation). These results are averages of three different iPSC-CM lines, with each line being tested in duplicates.

transforming into amastigotes, (3) multiplying amastigotes, (4) amastigotes transforming into trypomastigotes, and (5) trypomastigotes bursting out of host cells.

ChD has an acute and a chronic phase, in which the acute phase is the first contact between the parasite and the host that is usually asymptomatic and characterized



#### Figure 4. Comparing Gene Expression Among Different Cardiomyopathies

(A) Significant genes expressed in iPSC-CMs infected with *T. cruzi* (Tc) (1:5, 3 h interaction) for 24 h were compared with gene expression of iPSC-CMs infected with Coxsackievirus (COX), iPSC-CMs from dilated cardiomyopathy (DCM) patients, or iPSC-CMs treated with doxorubicin 1  $\mu$ M (DOX1) and 10  $\mu$ M (DOX10).

(B and C) Differentially expressed genes in iPSC-CMs infected with *T. cruzi* compared with COX, DCM, DOX1, and DOX10. Graphs show genes related to the network of (B) inflammation, (C) cardiac remodeling and contractility. Red arrows indicate upregulated genes and blue arrows indicate downregulated genes.

by elevated parasitic load. In contrast, the chronic phase is characterized by subpatent parasitemia and disease progression (Menezes et al., 2011). In our study, we infected iPSC-CMs with *T. cruzi* that reflected both the acute phase (24 and 48 h) as well as the chronic phase (72 and 96 h), in which an exponential high parasitic load was observed (Figure 2B). This successful demonstration of human iPSC-CM infection with *T. cruzi* makes our model a valid *in vitro* platform to study disease mechanisms of ChD as well as understand host-parasite cell interactions. Furthermore, it provides a unique platform for screening new drugs in the management of ChD. To that end, our data showed that BNZ, the first line of drug for ChD, significantly reduced the percentage of iPSC-CMs infected with *T. cruzi* (Figure S4).

Clinically, ROS/RNS are elevated during the acute phase of *T. cruzi* infection; however, we detected ROS/RNS in iPSC-CMs during the chronic phase of our iPSC-CM model. This could be due to the different strains of *T. cruzi*, as ROS/RNS production depends on the pathogen's virulence (Andrade et al., 1999). We used the Y strain of *T. cruzi*, which is known to be less infective to myoblasts when compared with other strains, and thereby responsible for a low level of parasitemia (Andrade et al., 2010). In addition, *T. cruzi* has a very efficient antioxidant machinery that can regulate the immediate ROS production thereby allowing the parasite to evade the early host response.

Interestingly, the time course of *T. cruzi* infection mirrored some aspects of clinical manifestations observed in patients with ChD (Medeiros et al., 2017). The



co-existence of inflammatory infiltration and fibrosis in ChD results in an abnormal electrocardiogram characterized by arrhythmias, conduction disturbances, and repolarization changes (Spencer et al., 2011). Our iPSC-CMs showed a high beat rate, high peak height, and low beat duration matching arrhythmias in patients with ChD (Figures S1A–S1C). This resulting tachycardia may be due to altered distributions of sarcomeric proteins,  $\alpha$ -actinin (contractility protein), troponin T (regulatory protein), and connexin 43 (gap junction protein), which happened at 72 h post-infection (Figure S1D). Moreover, it could also be due to the low expression of calcium-handling genes such as *RYR2*, *ANK2*, *CACNA1C*, *TRDN*, and *CASQ2* (Figure S2). Indeed, decreased *CASQ2* levels have been linked to arrhythmias triggered by  $\beta$ -adrenergic stimulation (Tavi et al., 2005). Similarly, disruptions in  $\alpha$ -actinin distribution in mouse embryonic CMs has been observed following 72 h of *T. cruzi* infection (Melo et al., 2006). Notably, in our model the *T. cruzi* infection interfered with sarcomere organization and intercellular junctions, which could explain the conduction disturbances seen in both acute and chronic patients with ChD.

Previous studies in ChD have analyzed gene expression in murine heart models (Henaio-Martinez and Parra-Henaio, 2015), but little is known about the variability in genome responses over the time course of the *T. cruzi* infection. Given that ChD is characterized by intense inflammatory infiltrates (Rocha et al., 2007), we analyzed the genes that can affect inflammation, cardiac remodeling, and contractility to assess the variations in gene expression signatures in ChD-infected iPSC-CMs. ChD is known to show a robust immune response orchestrated by innate immune cells with high levels of interleukin-12 (IL-12), interferon gamma, and tumor necrosis factor alpha (Andrade et al., 2014). Indeed, our infected iPSC-CMs showed a similar response with an increase in IL-2, IL-17A, MCP-1, and ICAM-1. However, the normal heart does not constitutively express inflammatory cytokines, suggesting that physical and/or chemical stresses due to *T. cruzi* infection might be responsible for the immune response from the CMs. This is consistent with findings in existing literature analyzing cytokine levels in PBMCs of patients with ChD (Gomes et al., 2014; Guedes et al., 2012). Furthermore, the increase in pro-inflammatory markers in iPSC-CMs correlated with the advent of iPSC-CM dysfunction, suggesting that these cytokine levels were high enough to trigger CM dysfunction. Finally, the comparison of gene expression between *T. cruzi* and other cardiomyopathies (Figure 4) revealed genes that could be used as potential targets to manage ChD, including *CXCL6*, *CXCL12*, *CXCL14*, and *CXCL17*, as these chemokines are known to contribute to the regulation of immune cell migration (Lu et al., 2016).

In summary, our study generates the first compelling data to demonstrate the feasibility of using human iPSCs to model ChD. We showed that human iPSC-CMs can be infected by the *T. cruzi* strain and can recapitulate the key aspects of cardiac dysfunction observed during ChD cardiomyopathy. Importantly, our study highlights a list of relevant genes that might play an important role in ChD pathogenesis, making them potential targets for pharmacological intervention. However, questions concerning the specificity of the drug effects still remain to be answered. We believe our study may pave the way to using the iPSC technology as an ideal platform to screen for novel anti-parasitic drugs against ChD cardiomyopathy.

## EXPERIMENTAL PROCEDURES

An expanded section is available in the [Supplemental Experimental Procedures](#). Human iPSCs were derived from fibroblasts or PBMCs obtained from patients using approved IRB protocols 17576 and 29904.

### Differentiation of iPSC-CMs

iPSCs were differentiated into iPSC-CMs on Matrigel-coated plates using our established CM differentiation protocol (Burrige et al., 2014). In brief, iPSCs were treated with CHIR99021, a *Wnt* activator for 2 days followed by IWR treatment in RPMI medium plus B27 supplement without insulin. From day 7 onward, cells were placed in RPMI plus B27 medium until beating was observed. At this point, cells were glucose starved for 3–4 days to purify iPSC-CMs and further maintained RPMI plus B27 medium.

### Invasion Assay with *T. cruzi*

Beating iPSC-CMs were plated in 24-well plates at  $2 \times 10^5$  cells/well in RPMI plus B27 with insulin for the *T. cruzi* infection. To optimize the infection, we analyzed two target effector ratios, 1:5 and 1:10 (iPSC-CMs:parasites) at two time points (3 and 24 h) in a volume of 500  $\mu$ L per well. An infection time-response curve was used to define the optimal time of infection for use in further analysis with biological assays. An uninfected group was subjected to the same conditions and medium, except for the presence of the parasite. This group represented the vehicle/mock group, and all the data from the *T. cruzi* infection group were compared with the uninfected group.

### Statistical Analysis

PCA and hierarchical clustering heatmap analysis were performed for gene expression with Omics Explorer version 3.2 software (Qlucore, NY, USA). MANOVA test with  $p < 0.05$  significance was used for comparisons. Statistical analyses for functional data were performed using Graph Pad Prism v7.0 software. Data are presented as mean  $\pm$  SEM. Comparisons were conducted by Student's t test, using the Bonferroni-Dunn method or one-way ANOVA. Correlation analyses were done using Pearson or Spearman's correlation coefficient. Results were considered significant when  $p < 0.05$ .





## ACCESSION NUMBERS

The GEO accession number for RNA-seq in this paper is GEO: GSE129676.

## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.stemcr.2019.04.017>.

## AUTHOR CONTRIBUTIONS

A.B., conception and design, data collection, data analysis, and manuscript writing; N.S., conception and design, data analysis, and manuscript writing; E.M., data collection and data analysis; G.S., data collection and data analysis; E.N., data analysis; K.V.C., conception and design; R.C.O., conception, design, financial support, and manuscript writing; D.A.S., conception, design, financial support, and manuscript writing; J.C.W., conception and design, financial support, manuscript writing, and final approval of manuscript.

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