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Gap Junctions and Chagas Disease

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Abstract

Gap junction channels provide intercellular communication between cells. In the heart, these channels coordinate impulse propagation along the conduction system and through the contractile musculature, thereby providing synchronous and optimal cardiac output. As in other arrhythmogenic cardiac diseases, chagasic cardiomyopathy is associated with decreased expression of the gap junction protein connexin43 (Cx43) and its gene. Our studies of cardiac myocytes infected with *Trypanosoma cruzi* have revealed that synchronous contraction is greatly impaired and gap junction immunoreactivity is lost in infected cells. Such changes are not seen for molecules forming tight junctions, another component of the intercalated disc in cardiac myocytes. Transcriptomic studies of hearts from mouse models of Chagas disease and from acutely infected cardiac myocytes *in vitro* indicate profound remodelling of gene expression patterns involving heart rhythm determinant genes, suggesting underlying mechanisms of the functional pathology. One curious feature of the altered expression of Cx43 and its gene expression is that it is limited in both extent and location, suggesting that the more global deterioration in cardiac function may result in part from spread of damage signals from more seriously compromised cells to healthier ones.

3.1. INTRODUCTION

Gap junction channels are composed of the connexin family of transmembrane proteins that assemble as end-to-end alignments of hexameric connexin subunits (Fig. 3.1). These structures form intercellular conduits that are permeable both to current-carrying ions (primarily K⁺) and to second messenger molecules with molecular mass (Mr) < 1 kDa such as Ca²⁺, IP₃ and cyclic AMP. The connexin gene family in mammals includes more than 20 isoforms encoded by separate genes (Söhl and Willecke, 2004), and such isoforms are named according to the molecular weight (in kDa) of the protein predicted from its cDNA (Goodenough et al., 1996); genes encoding connexins follow a different nomenclature, where they are divided into several subfamilies and identified according to the order of their discovery. Gap junction channels are critical in the heart, where they mediate synchronized rhythmic contractions and maintain cardiac homeostasis by allowing the free diffusion of metabolites between cardiac myocytes. Connexin43 (Cx43, encoded by the *Gjal* gene in

rodents and by *GJA1* in humans) is the most abundant gap junction protein in ventricular myocytes, being localized at intercalated discs in normal myocardium (see Duffy et al., 2006 for review). In addition, Cx40 (encoded by *Gja5*) and connexin45 (Cx45, *Gjc1*) are found more prominently in atrium in the working myocardium and in the conduction system, and connexin37 (Cx37, *Gja4*) is a major component of gap junctions between endothelial cells in the vessel wall.

Gap junction function and distribution within and between cells are affected by phosphorylation state of the connexins that form them and by other factors including intracellular pH and protein–protein interactions (see Hervé et al., 2004; Spray et al., 2001 for reviews). In diverse cardiac disease states, such as myocardial infarction and ischaemia, significant remodelling of the distribution of Cx43 occurs in ventricles, resulting in disorganization of normal microconduction pathways and arrhythmias (Severs, 2001); similarly, altered Cx40 distribution has been associated with atrial fibrillation (see Chaldoupi et al., 2009 for review).

Studies of alterations in cardiac myocytes during *in vitro* infection with *Trypanosoma cruzi* indicate that the parasite is capable of impairing host cell functioning through alterations in cell–cell communication (de Carvalho et al., 1992). Such an effect is expected to be of particular importance in the heart, where maintenance of synchronous contractions requires functional gap junctions (see Duffy et al., 2006; Severs et al., 2006 for reviews). Because gap junctional communication is important in normal cardiac conduction, and because chagasic cardiomyopathy resulting from infection with *T. cruzi* is associated with arrhythmias, a major focus of our laboratories has been the examination of the expression and distribution of Cx43 in widely used *in vivo* and *in vitro* models of infection.

Chagas disease has both acute and chronic stages, and in both, there may be cardiac involvement. While myocarditis may be observed during acute infection, chronic infection may result in arrhythmias, congestive heart failure or thromboembolic events (see Tanowitz et al., 1992, 2009 for review). Dilated cardiomyopathy usually occurs many years after the initial infection. There is little tissue parasitism in the chronic stage, but conduction pathways are damaged with resultant disturbed heart rhythm. Mechanisms postulated by various authors to explain the development of chronic chagasic heart disease include autoimmunity, microvascular disturbances and autonomic nervous system derangements (see Marin-Neto et al., 2007 for review). Clearly, there is evidence for each: inflammation is present in the absence of appreciable parasite burden; verapamil is therapeutically useful because it alleviates vasospasms; and there is destruction of autonomic ganglia in chronic disease. However, mechanisms responsible for the arrhythmogenic nature of the disease have received little attention.

3.2. LOSS OF GAP JUNCTIONS AND COUPLING IN RODENT CHAGASIC CARDIOMYOPATHY

In early studies, our laboratory groups examined spontaneous beating rate of cultures of *T. cruzi*-infected mouse and rat cardiac myocytes, finding opposite effects in the two murine species (Aprigliano et al., 1993; Bergdolt et al., 1994; de Carvalho et al., 1992; see de Carvalho et al., 1994 for review): in infected mouse cells, beat rate was higher, whereas in infected rat myocytes, beat rate was slower and less rhythmic. Studies also revealed that variability in interbeat interval was much higher in infected neonatal rat cardiac myocytes (de Carvalho et al., 1992); mouse myocytes exhibited decreased chronotropic response to norepinephrine (Aprigliano et al., 1993), whereas rat heart cells showed the opposite effect (Bergdolt et al., 1994). The extent to which these differences reflect parasite strain is unknown, but the strikingly different effects that parasite strain can cause are illustrated by a

recent gene profiling experiment performed on L₆E₉ myoblasts infected with four different *T. cruzi* species that identified distinct transcriptomic fingerprints caused by each parasite (Adesse et al., 2010).

Studies in both rat (de Carvalho et al., 1992; see Fig. 3.2) and mouse cardiac myocytes following infection with Y or Tulahuan strains of *T. cruzi* (Adesse et al., 2008; de Carvalho et al., 1992) demonstrated that Cx43 immunofluorescence in infected cell pairs was substantially lower at 72 h after infection; coupling between non-parasitized cells in infected dishes was not affected, indicating that factors secreted into the medium are unlikely to be responsible for the decrease in Cx43 abundance by infected myocytes. Junctional conductance and dye transfer were also measured electrophysiologically at 72 h after infection in the rat myocytes (de Carvalho et al., 1992), and those results indicated a strong decrease in coupling, but only in cases where both cells were infected.

We also have examined expression of the major cardiac gap junction protein Cx43 employing immunoblots and RNA measurement. In both rat and mouse, Western blots of Cx43 protein and Northern blots of *Gja1* mRNA indicated only slightly decreased levels in rat astrocytes and leptomeningeal cells at up to 72 h after infection in culture (Campos de Carvalho et al., 1998). This only slight decrease in Cx43 mRNA at later time points in culture is consistent with recent microarray studies described below. However, immunofluorescence has uniformly detected strikingly less Cx43 in junctional regions of infected rat cells. Such a dramatic decrease in Cx43 immunostaining without major change in total Cx43 protein might indicate that an antigenic site became “cryptic” as a consequence of parasitic infection (as was reported to occur between CNS glial cells in kainate-induced excitotoxicity; Hossain et al., 1994). However, similar results were obtained using other Cx43 antibodies, and we suggested initially that the infection altered Cx43 distribution within the cell, presumably as a result of altered trafficking and retention in non-junctional compartments (de Carvalho et al., 1992, 1994).

Another peculiarity with regard to Cx43 immunostaining arose in initial studies using an antibody (181A) raised by Dr. Elliot Hertzberg against an epitope on the carboxyl terminus of Cx43. This antibody seemed to stain the intracellular parasite, raising the possibility that *T. cruzi* might either express Cx43 or hijack it intracellularly. However, the use of antibodies directed to different residues of the cytoplasmic Cx43 C-terminal tail demonstrated that a *T. cruzi* surface protein actually cross-reacts with certain Cx43 antibodies. As illustrated in Fig. 3.3, immunogold analysis using the 181A antibody (residues 346–360) displayed typical localization at cell–cell contacts in non-infected cardiac myocytes (Fig. 3.3A, arrows). However, in *T. cruzi*-infected cells, membrane Cx43 immunolocalization was absent and there was consistent staining of the amastigotes (Fig. 3.3B, arrowheads). Confocal microscopy showed that using a commercially available anti-Cx43 antibody (Sigma), recognizing an adjacent region (residues 363–382) of the C-tail, there was no staining of the intracellular parasites (Fig. 3.3C). Highly infected cardiac cells lose Cx43 immunoreactivity for both Sigma and 181A antibodies (Fig. 3.3C and D, stars), but non-parasitized cells displayed normal Cx43 plaques (Fig. 3.3C and D, arrows). Thus, staining of *T. cruzi* by 181A antiserum is likely due to the recognition of a homologous protein, present in all three life forms of the parasite (Fig. 3.3F), showing a different molecular weight from Cx43 as compared to mouse heart lysates used as positive controls. Staining of a 120-kDa band was observed in immunoblots using the 181A antibody (Fig. 3.3F) but not with the Sigma antibody (Fig. 3.3E).

Our previous observations (Bergdolt et al., 1994; Garzoni et al., 2003) and those of others (Rodriguez et al., 1995) demonstrated that intracellular calcium concentrations in the host cell were increased during initial times of infection with trypomastigotes and that these

calcium waves could propagate in a cluster of neighbouring cells (Garzoni et al., 2003). To re-examine the issue of whether *T. cruzi* invasion could modulate gap junctional communication, we examined Cx43 expression/distribution in mouse cardiac myocytes during initial infection with the Y strain. In cultured mouse myocytes, the infection with the Y strain of *T. cruzi* had an interesting effect on Cx43 expression. At the first hour of infection, which corresponds to initial steps of this interaction, Cx43 protein levels were substantially increased, whereas Cx43 transcripts were unaltered (Fig. 3.4). These observations suggest an effect of parasite invasion on connexin trafficking: As *T. cruzi* depends on lysosome recruitment for the formation of the parasitophorous vacuole (Andrade and Andrews, 2004), Cx43 removal from the plasma membrane could be impaired. Further analysis at 24–72 hours post-infection (hpi) revealed a 61% decrease in protein levels (Fig. 3.4A) and a 20% decrease in mRNA levels at the final time point (Fig. 3.4), when cells are highly parasitized, with abundant intracellular amastigotes and no Cx43 staining (Fig. 3.3). The changes in Cx43 levels detected in mouse cardiac myocytes infected with the Y strain are in contrast with previous observations on rat glial cells infected with Tulahuan strain, which showed no significant alteration in levels of either Cx43 or its phosphorylation state at 72 hpi (Campos de Carvalho et al., 1998). These discrepancies could be explained by the differences in parasitaemia (which was considerably lower in the glial study) or in parasite strain pathogenicity *in vivo* (Caetano et al., 2010) or *in vitro*, as recently demonstrated in our study that used oligonucleotide microarrays to compare the infection of rat myoblasts with four reference strains of *T. cruzi* (Adesse et al., 2010).

Thus, there seems to exist a direct relationship between host cell parasitism and Cx43 downregulation *in vitro*, as demonstrated in Figs. 3.3 and 3.4, in which highly infected cells display decreased Cx43 protein and mRNA levels. This hypothesis is further supported by a recent study that showed recovery of infected myocytes after treatment with amiodarone, a potent anti-arrhythmic that is commonly prescribed for chagasic patients with severe cardiac compromise (Dubner et al., 2008). Interestingly, this compound is also an inhibitor of sterol biosynthesis in fungi (Courchesne, 2002; Courchesne et al., 2009) and trypanosomatids (Benaïm et al., 2006; Serrano-Martín et al., 2009). When used in micromolar concentrations (1.6–6.5 µg/ml) in infected myocyte cultures, amiodarone induced profound morphological alterations to amastigotes that led to clearance of the parasitism and host cell recovery. Among the changes observed was a marked recovery of Cx43 immunoreactivity and spontaneous contractility rate (Adesse et al., 2011). These observations, combined with that of minimal change in Cx43 transcripts during *in vitro* infection (Fig. 3.4), reinforce the idea that the Cx43 protein downregulation that follows *in vitro* infection results from impaired protein synthesis, rather than altered Cx43 gene expression. Amiodarone is a promising compound, as it has a strong bioavailability, is highly lipophilic (Van Herendael and Dorian, 2010) and selectively eradicates *T. cruzi* infection in doses much lower than what is recommended by the American Heart Association for daily dosage in cases of atrial fibrillation (200–400 mg/day) with no side effects (Schweizer et al., 2011).

The murine models of chagasic infection have brought important contributions to the understanding of the arrhythmogenic impact of Cx43 remodelling. During acute infection with *T. cruzi* (11 days post-infection with the Y strain or 30 days post-infection with the Brazil strain), Cx43 protein levels are decreased in atria and ventricles, consistent with what was observed in the *in vitro* infection (Adesse et al., 2008). Such a reduction in overall Cx43 abundance in the infected heart presumably reflects inhomogeneous distribution, a condition that is a prominent feature of ventricular conduction disorders (see Severs, 2001 for review). The reduction in the expression of Cx43 levels in the infected heart may be induced by the parasite *per se*. However, the effects of soluble factors present in the serum and the inflammatory response on Cx43 expression cannot be entirely ruled out. When serum from chagasic cardiomyopathic patients was added to cultured cardiomyocytes or isolated rabbit

hearts, there was a substantial impairment of dye spread through gap junctions, atrioventricular conduction block and changes in heart rate (Costa et al., 2000). This observation seems to contradict the results described above, in which we observed substantial impairment in the coupling and Cx43 expression of infected cells but not in non-parasitized cells in infected dishes (Adesse et al., 2008, de Carvalho et al., 1992). The difference between these data could be in part explained by the high concentrations of serum proinflammatory cytokines and chemokines found during chronic infection in which parasite load is much reduced. It has been shown that growth factors, such as transforming growth factor- β (TGF- β), can regulate gap junction intercellular communication (Chandross et al., 1995; see Chanson et al., 2005 for review). TGF- β is required for the invasion of host cells and is produced early upon infection, and constantly throughout the acute and chronic phases (see Araújo-Jorge et al., 2008 for review). Recently, it was demonstrated that the addition of 2 ng/mL TGF- β in cardiomyocytes *in vitro* downregulated Cx43 protein expression in non-infected myocytes, resulting in reduced organization of gap junctions similar to the pattern observed in infected cultures. These results were further reinforced when the TGF- β receptor type 1 (ALK-5) was inhibited by SB-431542, which completely reversed the effect of TGF- β and *T. cruzi* infection on Cx43 expression. The authors suggested that TGF- β produced in infected culture could affect both infected and non-infected cells and affect the pattern of Cx43. In addition, because TGF- β regulates a diverse array of cellular processes, including tissue development and repair (see Ramos-Mondragón et al., 2008, Yarnold and Brotons, 2010 for reviews), the high levels of TGF- β and consequent disorganized expression of Cx43 could both act in synergy to promote dysrhythmias in chagasic patients (Waghabi et al., 2009).

Confocal microscopy experiments revealed that acute infection (30 days post-infection with the Brazil strain) induces connexin remodelling with lateralization of Cx43 plaques, that is, delocalization from the intercalated discs (Fig. 3.5A–B). Such remodelling is commonly observed in cardiac diseases such as hypertrophic cardiomyopathy (Seidel et al., 2010), myocardial infarction (Wang et al., 2010) and heart failure (Akar et al., 2004) and contributes to impairment of impulse propagation.

In a murine model of chronic *T. cruzi* infection (Y strain), we observed structural damage to the myofibrils, mitochondria and sarcoplasmic reticulum with intercalated disc discontinuity, as shown in the electron micrographs in Fig. 3.5C and D. Interestingly, using oligonucleotide microarrays, we have previously described that both in *in vitro* and in *in vivo* models of infection, there are marked changes in the expression of genes related to contractile proteins as well as to the intercalated disc (Adesse et al., 2010; Goldenberg et al., 2009; Mukherjee et al., 2008). An important recent report indicated that in human chagasic cardiomyopathic hearts, Cx43 distribution is altered in areas of fibrosis and this observation was most prominent in patients with cardiomegaly (Waghabi et al., 2009). These findings highlight an evolving concept that many types of cardiomyopathy target expression or involve mutations in molecular components of the intercalated disc (see Saffitz et al., 2007 for review and Celes et al., 2007 for changes in sepsis). Thus, as pointed out in recent commentary (Spray and Tanowitz, 2007), cardiomyopathies, including chronic chagasic cardiomyopathy, may be considered to be “junctionopathies”. Taken together, these data may explain in part the dysrhythmias and conduction abnormalities that attend this infection (see Section 3.4).

3.3. NOT ALL JUNCTIONAL PROTEINS ARE AFFECTED BY *TRYPANOSOMA CRUZI* INFECTION

As emphasized above, our studies have consistently demonstrated that *T. cruzi* infection decreases Cx43 expression at appositional membranes in cultured cardiac myocytes. In

order to investigate effects of *T. cruzi* infection on other protein families, we used Mardin-Darby canine kidney (MDCK) cells to evaluate Cx43 and the tight junction protein zona occludens-1 (ZO-1) expression after *T. cruzi* infection (48 h). Immunocytochemistry with antibodies specific for Cx43 (Fig. 3.6A) demonstrated that gap junctional staining was significantly reduced in most of the infected cells, although uninfected neighbours could display normal Cx43 abundance and distribution (Fig. 3.6A). Although a number of connexins (including Cx43) are associated with ZO proteins (Giepmans and Moolenaar, 1998; Sorgen et al., 2004; Toyofuku et al., 1998), the infection did not affect the ZO-1 tight junction proteins in MDCK cells (Fig. 3.6B). These results demonstrate that *T. cruzi* infection disrupts specifically Cx43 gap junction protein and spares several proteins that form tight junctions (F. Fortes, A.C. Campos de Carvalho and R. Goldenberg, unpublished observations).

3.4. MICROARRAY EXPERIMENTS HAVE REVEALED PROFOUND CHANGES IN GENE EXPRESSION IN THE CHAGASIC MOUSE, BOTH IN ACUTE AND IN CHRONIC DISEASE PHASES

We have published several papers characterizing the impact of chagasic cardiomyopathy and acute infection of neonatal rodent myocytes on gene expression by host tissue or cells (Adesse et al., 2010; Goldenberg et al., 2009; Manque et al., 2011; Mukherjee et al., 2003; 2008; Soares et al., 2010). Our most extensive data set involved hybridization on Duke oligonucleotide arrays of RNA extracted from four biological replicas of hearts from control and C57Bl6 mice infected with the Colombian strain of *T. cruzi* for 8 months (i.e. chronic stage) (Soares et al., 2010). For this chapter, we further analyzed those data with respect to heart rhythm determinant (HRD) genes selected using our prominent gene expression analysis (PGA; Iacobas et al., 2010a). In this data set, Cx43 (*Gja1*) was slightly but not significantly reduced (1.8-fold reduction, $p = 0.07$), whereas Cx37, the major gap junction protein in endothelial cells, was strongly upregulated (3.3-fold, $p = 0.02$). Of the cadherins (Cdh) for which there were adequate data (Cdh4, 5 and 13), only Cdh13 was regulated (-1.7 -fold, $p = 0.02$). Other HRD genes downregulated include those encoding the inwardly rectifying potassium channel J8 (-1.6 , $p < 0.04$) and Pklac (-1.7 , $p = 0.01$). Upregulated HRD genes included Lamin A (1.7-fold, $p = 0.04$), Myh7 (2.7-fold, $p = 0.006$) and TGF β 2 (1.9-fold, $p = 0.005$). Some other genes encoding proteins that have been associated with Cx43 were not significantly affected, including Cx40 and Cx45, catenins, CAR and plakophilin. Another metric by which to examine the impact of disease on experimental intervention is to use coordination analysis to determine the degree to which network interlinkage is altered. Results of such a network analysis are shown in Fig. 3.7, where we have compared the extent to which HRD genes are ($p < 0.05$) synergistically (Pearson coefficients > 0.9) or antagonistically (Pearson coefficients < -0.9) coordinately expressed among themselves (Fig. 3.7A–D) and with connexins. Inspection of the networks in normal heart reveals that there are many synergistic interlinkages and very few antagonistic connections (Fig. 3.7A and B); in the infected heart, synergistic connections are reduced, whereas antagonistic ones are greatly increased (Fig. 3.7C and D). This network remodelling is especially evident when the interlinkages are focused on connexin genes in control and infected hearts (Fig. 3.7E). With respect to individual gap junction genes, Cx43 (*Gja1*) is synergistically expressed with five genes (*Gja4*, *Gjc1*, *Cav3*, *Ctmd1*, *Scr5a*), *Gja4* with eight (*Gja1*, *Cdh5*, *Ctnna2*, *Dsg2*, *Epas1*, *Pcdh18*, *Scn5a* and *Tjap1*), *Gja5* with six (*Atplal*, *Dsc2*, *Pcd1*, *Pcdhga12*, *Tgfb2* and *Tjap1*) and *Gjc1* with four (*Gja1*, *Ctnnd1*, *Cxad5*, *Pcdh7*). By contrast, these interlinkages are radically different in *T. cruzi*-infected heart. *Gja1* is synergistically expressed with six genes (*Gja5*, *Ank3*, *Pdch18*, *Pcdhga12*, *Ryr2* and *Tjp1*) and antagonistically with three genes (*Gjc1*, *Cdh5* and *Lmma1*). *Gja4* becomes synergistically expressed with only two genes (*Adrbk1* and *Ctnnal1*) and antagonistically

with eight (*Abcc9*, *Casq2*, *Cdh12*, *Csrp3*, *Cxddr*, *Gaa*, *Kcnj8*, *Pbp2*). *Gja5* becomes synergistically expressed with three genes (*Gja1*, *Tjp1* and *Ttn*) and antagonistically with only two (*Gjc2* and *Pcdh1*). *Gjc1* becomes synergistically expressed with five genes (*Cay3*, *Cdh5*, *tmnd1*, *Hand2* and *Pcdh1*) and antagonistically with six (*Gja1*, *Gja5*, *Atpla1*, *Epas1*, *Tjap1* and *Tjp1*). These pairwise interconnections among HRD genes are illustrated in Fig. 3.7A–B, showing the remarkable extent of alteration in response to parasitic infection. Although the resulting altered topology is specific for Chagas disease, profound remodelling of the HRD genomic fabric was also reported in other arrhythmogenic conditions such as chronic constant and intermittent hypoxia (Jacobas et al., 2010b).

3.5. CONCLUSIONS

Chagas disease is the result of infection with *T. cruzi*. Acute infection is accompanied by an acute myocarditis with myonecrosis, inflammation and intracellular parasites. Despite the small number of cells that are infected, nevertheless, there is often functional deterioration, manifested both as arrhythmias and as compromised cardiac output. Infection of cultured cardiac myocytes can be viewed as an *in vitro* correlate of acute infection of the heart. These infected cultured cells display altered chronotropy and synchronous activity even though few cells are infected. This suggests that there are signals sent out from infected cell to non-infected cells that may alter the physiological responses of cells within the whole culture dish. This is likely similar to what occurs in the heart with alterations in gap junctions as a result of infection. Chronic chagasic heart disease is associated with profound conduction disturbances associated with fibrosis, lipid accumulation and cellular and tissue level hypertrophy. We now appreciate that even during the chronic phase of the disease, there is a persistence of the parasite with a low-level continuous infection that is associated with fibrosis and vasculopathy. In part, this manifestation of dysfunction as a consequence of only a small number of cells being affected may reflect the anatomy of the tissue that is targeted. The heart is composed of specialized conduction and contraction myocytes, and optimized output depends upon the progressive synchronized activation of the contractile myocardium. Thus, reducing gap junction expression in only a small number of cells could provide focal slowing of conduction or focal compromise of chamber contraction.

Infection of cardiac myocytes and more globally, infection of the animal, leads to functional uncoupling of cardiac myocytes, as a consequence of reduced expression of Cx43 and its gene. A variety of methods have been used to evaluate the changes in gap junction expression in the chagasic heart. These methods include functional studies in which dye coupling, junctional conductance or conduction synchrony were evaluated, by immunostaining and Western blotting and measurements of gene expression, either through Northern blots or, more recently, from microarray analysis. The findings from these studies include the observation that the cardiac gap junction protein and the channels that it forms are a target of infection. In a population of acutely infected cardiac myocytes, gap junction abundance and immunoreactivity with certain antibodies are severely compromised, as are functional coupling and synchronous contraction. In adjacent non-infected cells, gap junction expression and function are less affected so that there is a mosaic of cells that are either connected or disconnected to their neighbours depending on presence and extent of parasitaemia. In chronic chagasic cardiomyopathy, the number of parasitized cells is low, but circulating factors such as IL-1 β and TGF- β are elevated in the chronically inflamed myocardium, resulting in not only reduced expression of Cx43 but also structural remodelling due to fibrosis.

In summary, the available data suggest that the effect on gap junctions of small numbers of infected cells in both acute and chronic disease has a critical role in the underlying pathophysiological processes which result in clinical chagasic cardiomyopathy.

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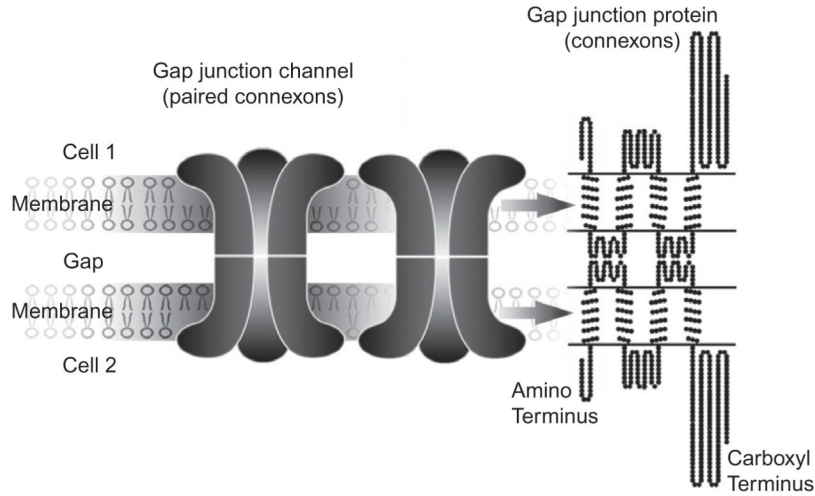


FIGURE 3.1. Schematic representation of gap junction and connexin structures

Two cells are coupled through connexon hemichannels, each composed of six subunits of connexin (Cx). Gap junction channels connect two cells' cytoplasms, allowing free exchange of small metabolites such as Ca^{2+} , IP3 and cAMP, which in heart tissue are important for maintaining synchronous contraction. Gating properties of gap junctions can be regulated by Cx structure, which consists of four primarily α -helical transmembrane domains, cytoplasmic amino and carboxyl termini and a cytoplasmic loop, all of which contain some regions of α -helix, and extracellular loops that are primarily β -structure. A number of proteins that bind to cardiac connexins are known, and more are certain to be discovered, linking the connexin into an intercellular signalling complex, the Nexus. Binding sites may either correspond to structured regions within the connexin molecules or be unstructured, leading to presumably low-affinity and dynamic interactions.

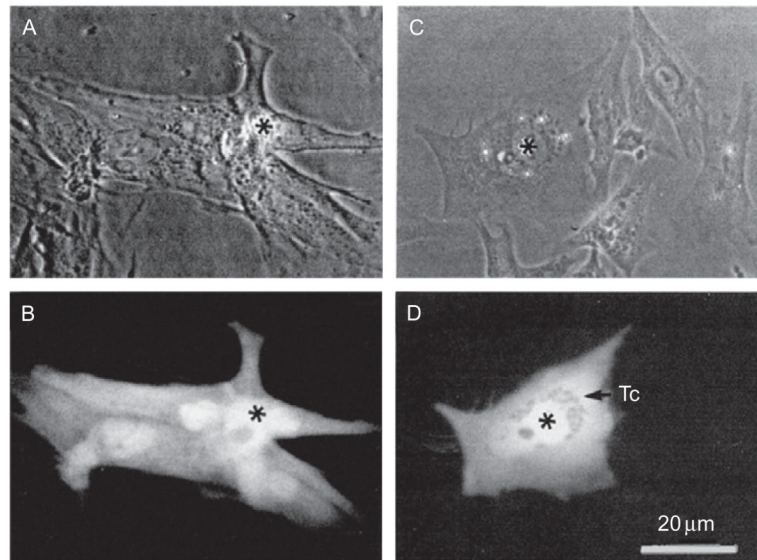


FIGURE 3.2. Trypanosoma cruzi infection impairs cell–cell coupling

The micrographs depict the pioneer experiment that tried to understand the basis of arrhythmogenesis in *T. cruzi* infection. Cultured cardiac myocytes were injected with the dye lucifer yellow (LY) that spreads to adjacent cells through gap junctions. Non-infected cells (A,B) were capable of transmitting LY to up to six cells, whereas when the injection was done in a highly infected cell (C,D), dye spread was abolished. Asterisks indicate the cells that were injected. Bars = 20 μm. From de Carvalho et al. (1992).

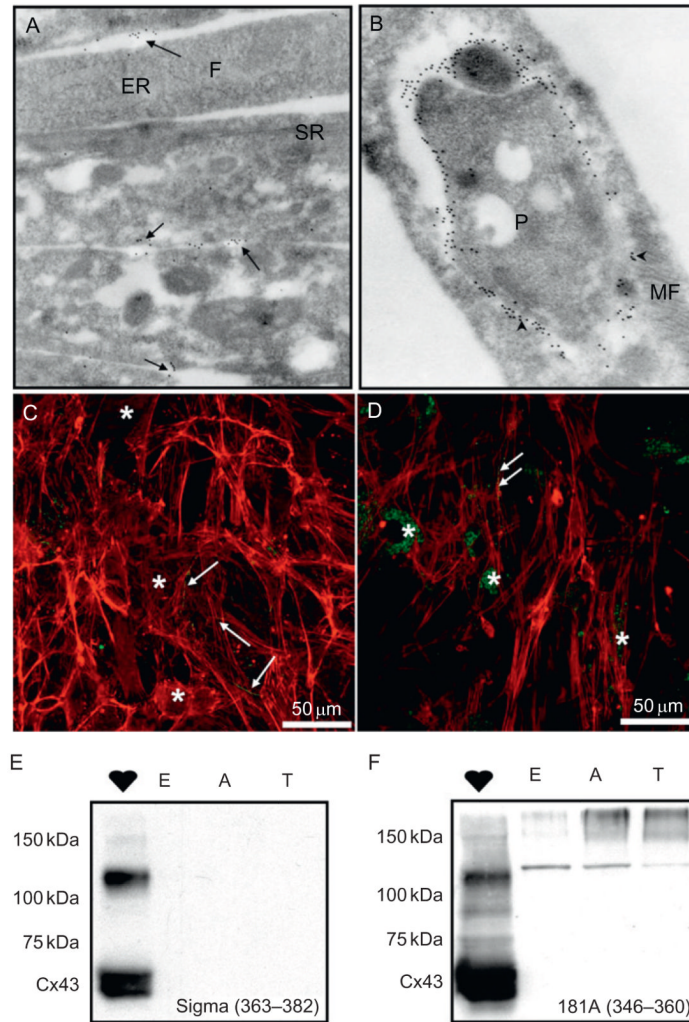


FIGURE 3.3. Does *Trypanosoma cruzi* express connexin43?

The use of specific antibodies directed to different residues of connexin43 C-terminal tail demonstrated that Cx43 shares a homologous residue with a *T. cruzi* surface protein. (A and B) Immunogold analysis using the 181A antibody (residues 346–360) displayed typical localization at cell–cell contacts in non-infected cardiac myocytes (A, arrows). However, in *T. cruzi* infected cells, membrane Cx43 immunolocalization was absent and there was a consistent staining of the amastigotes (B, arrowheads). Confocal microscopy using a commercially available anti-Cx43 antibody (Sigma), recognizing residues 363–382 of the C-tail, reveals no staining of the intracellular parasites (C). Highly infected cardiac cells lost Cx43 immunoreactivity for both Sigma and 181A antibodies (C and D, stars), but non-parasitized cells displayed normal Cx43 plaques (C and D, arrows). Staining of *T. cruzi* by 181A antiserum is likely due to the recognition of a homologous protein, present in all three evolutive forms of the parasite (F), showing a different molecular weight from Cx43 as compared to mouse heart lysates used as positive controls. Staining of a 120-kDa band was observed in immunoblots using the 181A antibody (F) but not with the Sigma antibody (E). (E, epimastigote; A, amastigote; T, trypomastigote; SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; F, fibroblast; P, parasite; MF, myofibril.)

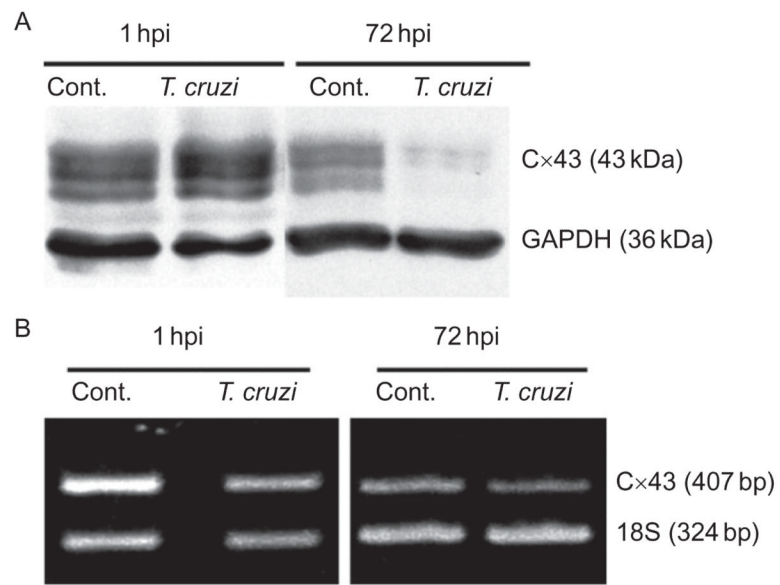


FIGURE 3.4. Connexin43 protein and mRNA expression during in vitro infection with *T. cruzi*
 Mouse cardiac myocytes were cultivated and infected with the Y strain of *T. cruzi*. Protein analysis showed that infection induces a bidirectional effect on Cx43, starting with a significant increase at 1 hour post-infection (hpi), followed by a normalization in protein levels until 72 hpi, when there is a drop of 61% in protein levels. Semiquantitative RT-PCR showed no alteration on Cx43 mRNA at 1 hpi, but a significant decrease in Cx43 transcripts at 72 h.

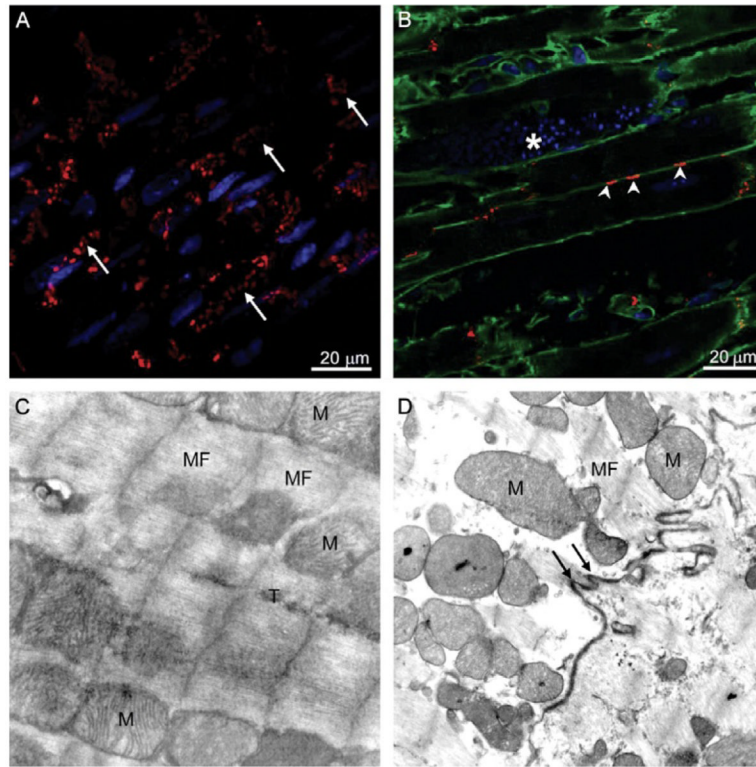


FIGURE 3.5. Cardiac Chagas disease affects connexins and intercalated discs morphology
 Hearts from acutely infected mice (30 days post-infection with the Brazil strain) were harvested and processed for immunohistochemistry for Cx43 (red) and F-actin (green) (A–B). Non-infected animals (A) displayed abundant Cx43 staining (red) in cell–cell contacts, mainly in the intercalated discs (arrows). Acutely infected myocardium (B) presented amastigotes pseudo-cysts (*), as revealed by DAPI staining in blue and lateralization of Cx43 in neighbour cells (arrowheads). Transmission electron microscopy revealed that during chronic Chagas disease (180 days post-infection with the Y strain), there are foci of severe damage to myocytes (D) in which cells are hypertrophied and display mitochondria swelling and disarray of contractile elements as compared to age-matched uninfected mouse hearts (C). The arrows point to a region where myofibrils anchor to intercalated discs, indicating substantial cellular disorganization. Original magnification: 8000 \times (C) and 10,000 \times (D). M, mitochondria; MF, myofibril; T, T-tubule.

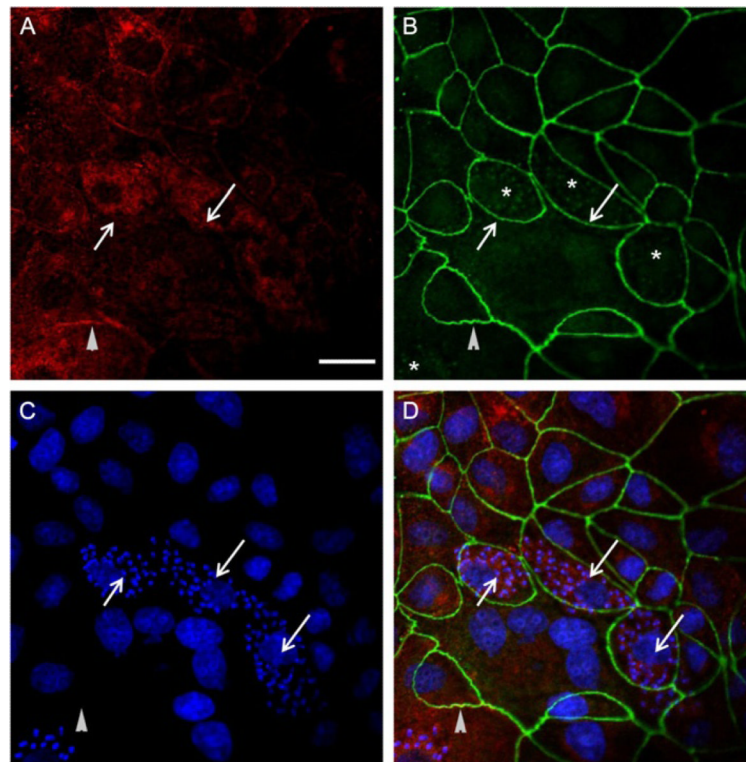


FIGURE 3.6. Trypanosoma cruzi infection affects Cx43 but not other junctional proteins
 MDCK2 cells were cultured and infected with *T. cruzi* (Brazil strain) for 72 h. Immunofluorescence for Cx43 (A, in red) and Zona Occludens-1 (B, in green) showed that despite the drastic decrease in Cx43 immunoreactivity in most of the highly infected cells (*), ZO-1 distribution was maintained intact (arrows mark regions where Cx43 was lost but ZO-1 was still present, arrowhead where Cx43 was still present in nonparasitized cells). With nucleic acid staining with TOPRO3, is possible to visualize host cell nuclei and also kinetoplastid DNA from intracellular amastigotes (small spots in (C)). In (D) the merged image is displayed. Bars = 20 μ m.

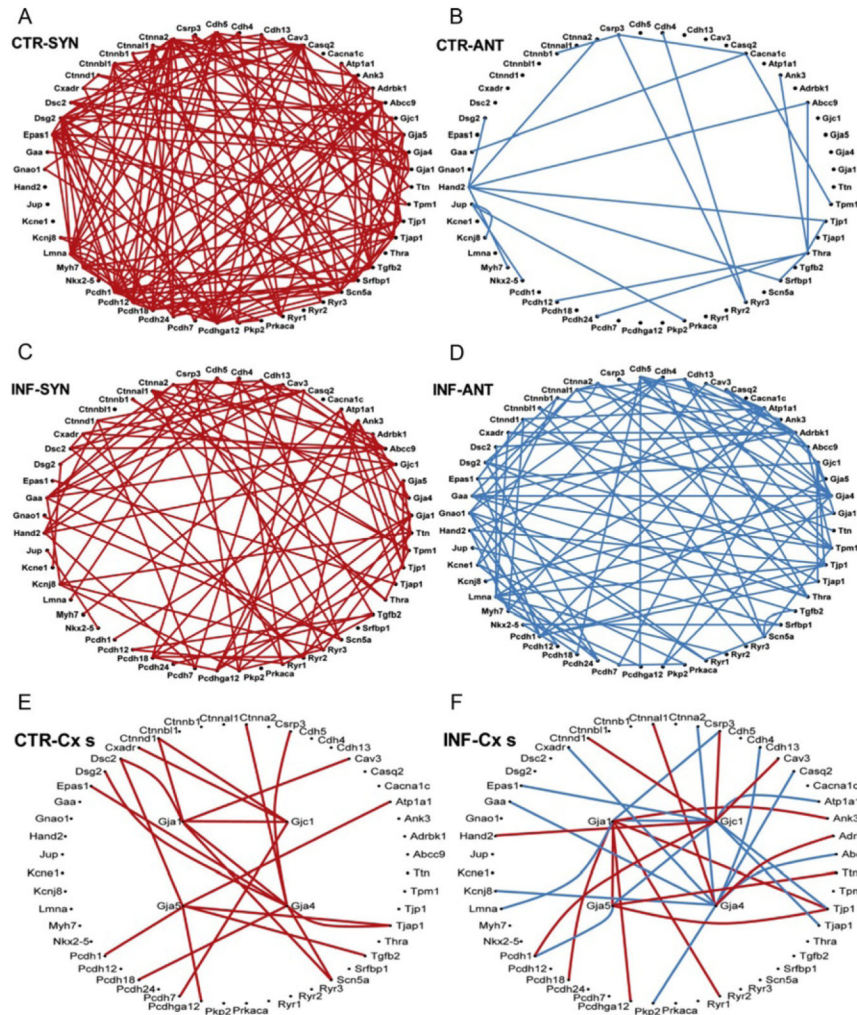


FIGURE 3.7. Remodelling of heart rhythm determinant (HRD) gene interlinkages in the chagasic heart and impact on connexin-dependent HRD networks
 (A) Synergistic coordinations (red lines) are very common between HRD gene pairs in normal heart. (B) Antagonistic coordinations (blue lines) between HRD gene pairs are rare in normal heart. (C and D) In infected heart, synergistic coordinations decrease and antagonistic coordinations greatly increase, indicating profound network remodelling (E and F). Expression coordinations of connexin genes with HRD genes are substantially altered by *T. cruzi* infection, with different HRD genes showing synergistic coordination (red lines) and the appearance of numerous antagonistic coordinations.