# Junction communication in the immune system: modulation of the GAP junctions by infection with *Toxoplasma gondii*

# Comunicação juncional no sistema imunológico: modulação das junções GAP em infecção por *Toxoplasma gondii*

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**ABSTRACT**
*Toxoplasma gondii* is a protozoan parasite responsible for toxoplasmosis, and may be causing any problems in different systems. Some of these complications are associated with the change of intercellular communication mediated by Junctions Communicators that allows direct communication between tissues. However, there are still systems that are not fully consistent with the junctional communication, including the innate immune system, represented by Macrophages. Thus, we used J774-G8 macrophage cell line culture infected by the RH strain of Toxoplasma in its tachyzoite form. The results revealed that in J774-G8 cells The Cx43 and Phalloidin proteins interact in the plasma membrane of the J774-G8 lineage, and they undergo a sensitive reduction in the membrane after 72 hours of infection with the parasite *Toxoplasma gondii*. The evaluation of the Cx43 protein expression by immunoelectrophoretic transfer has been shown to be altered (elevated) in J774-G8 macrophage cells infected with the parasite *Toxoplasma gondii* 24 and 48 hours compared to uninfected cells.

**Keywords:** Gap junction, Macrophages, *Toxoplasma gondii.*

**RESUMO**
*Toxoplasma gondii* é um protozoário responsável pela toxoplasmose, podendo causar alterações em diferentes sistemas. Algumas dessas complicações estão associadas à alteração da comunicação intercelular mediada por Junctions Comunicantes que permite a comunicação direta entre os tecidos. No entanto, ainda existem sistemas que não são totalmente consistentes com a comunicação juncional, incluindo o sistema imunológico inato, representado pelos macrófagos. Assim, usamos a cultura da linha de células de macrófagos J774-G8 infectados com a cepa RH de Toxoplasma em sua forma de taquizoíta. Os resultados revelaram que nas células J774-G8 as proteínas Cx43 e Faloidina interagem na membrana plasmática da linhagem J774-G8, sofrendo uma sensível redução na membrana após 72 horas de infecção pelo parasita *Toxoplasma gondii.* A avaliação da expressão da proteína Cx43 por transferência imunoeletroforética demonstrou estar alterada (elevada) em células de macrófagos J774-G8 infectadas com o parasita *Toxoplasma gondii* 24 e 48 horas em comparação com células não infectadas.

**Palavras-chave:** Junção Comunicante, Macrófagos, *Toxoplasma gondii.*

## 1 INTRODUCTION

Gap junction channels are composed by the connexin family of transmembrane multigenic proteins that assemble as end-to-end alignments of hexameric connexin subunits (KOVAL et al., 2014).

Such proteins can be divided into nine structural domains: four transmembrane domains presenting α-helix structure; A C-terminal portion; An N-terminal portion; Two extracellular loops; An intracellular or cytoplasmic loop between the transmembrane regions 2 and 3, characterized for representing the region of less similarity and less conservation between the connexins, there by forming intercellular conduits for current-carrying ions and molecules Mr < 1000 Da such as Ca2þ, IP3 and Cyclic AMP (HERVE et al., 2004; KUMAR & GILULA, 1996; NEIJSSEN et al., 2005).

Physiologically junctional channels play an important role in the development of multicellular organisms. Particularly, in the immune system, the expression of Cx43 can modulate the formation of lymphoid cells (MONTECINO-RODRIGUEZ et al., 2000).

The hemi channels formed by Cx43 are highly expressed in several cells, such as microglia, astrocytes and endothelium, and they are highly sensitive to DAMPs **(**Damage Associated Molecular Pattern) and PAMPs (Pathogen Associated Molecular Patterns) (CONTRERAS et al., 2004; HANSSON and SKIÖLDEBRAND, 2015).

When the channels become active under conditions of mechanical or ischemic stress and allow the release of molecules such as ATP, glutamate, or $NAD^+$, causing different physiological responses (LILLY et al., 2015). Changes in the amount and distribution of the communicating junctions may be associated with severe cardiac conditions (REAUME et al., 1995).

Nogueira et al. (2016) demonstrated that parasite infection by *Toxoplasma gondii* (*T. gondii*) induces changes in the junctional complex of retinal pigment epithelial cells, altering the adhesion junctions.

*T. gondii* is the parasitary agent of toxoplasmosis. This protozoan has the characteristic of being obligatory intracellular (LÜDER et al., 2001). It is capable of infecting and replicating in any nucleated cell of homothermal animals, including man, and some heterothermal animals (FEITOSA, 2017).

The present parasite is a pathogen with highly successful infection, having infected about 30% of the global human population (HIDE, 2016). Studies that involve the interaction of macrophages with *T. gondii* have shown that the parasite also has the capacity to inhibit macrophage activation, and reduces the coupling and the connexin

expression between astrocytes and the leptomeningeal cells parasitized, leading to the belief that this may be a general cellular response to infection with intracellular parasites (DE CARVALHO et al, 1998; DOS SANTOS et al., 2011).

This parasite has several mechanisms of escape and promotes changes in the parasitized cell to ensure the maintenance of its survival, as demonstrated by Seabra et al. (2002), in which mouse macrophages activated with IFN-γ and LPS infected with *T. gondii* tachyzoites have NO production inhibited due to the reduction of iNOS expression in the infection process. It was demonstrated the presence of mechanisms of escape of the microbicidal action of activated macrophages involving the exposure of Phosphatidylserine (FS), mimicking apoptosis and facilitating *T. gondii* infection (DOS SANTOS et al., 2011).

De Carvalho et al. (1998) demonstrated that both *T. gondii* and *Trypanosoma cruzi* (*T. cruzi*) influence the communicating junctions in a similar way, suggesting that the decrease in junctional communication could be a result of secondary changes in cellular metabolism due to infection.

The parasitic infection changes not only the production of cytokines, but also the pattern of intercellular communication activity by gap junctions, demonstrating the modification of the structural pattern of the junctional communication organization, as well as its functional profile in the studies with the protozoan *T. cruzi* (COSTA et al., 2000).

When infected, *in vitro* and *in vivo* by *T. cruzi* and *T. gondii*, pia mater and arachnoid cells (the meningeal cells) also showed reduction in the cellular coupling and in the marking for Cx43 and Cx26, however the total levels of Cx43 protein did't are significantly changed, suggesting that the infection interrupts the trafficking and assembly of the protein in functional channels in the plasma membrane (DE CARVALHO et al, 1998).

Therefore, the objective of this work is to evaluate the modulation of expression and positioning of gap junctions formed by Connexin 43 (Cx43) in macrophage cell line (J774-G8 cells) before and after infection with the *T. gondii*.

## 2 MATERIALS AND METHODS

### 2.1 CELL CULTURE OF MACROPHAGE LINEAGE J774-G8

The lineage of macrophage cell j774-g8 the cells were plated, with an initial density of $1x10^{-6}$ cells/ml in plastic bottles of 25cm² (corning / usa) in glass laminate (number 1) (fisherbrand / fisher scientific). the culture were kept in medium dmem (dulbecco's modified eagle's medium) supplemented with 10% of fetal bovine serum (v/v) (gibco-life technologies, rockville, md), penicillin 1000 ui/ml and streptomycin 100 ui/ml (sigma chemical company, st louis, mo). the cells were kept at 37°c in humid atmosphere, 5% of $co_2$ (culture $co_2$ incubator, model ccl-170b-8, sigapore).

### 2.2 OBTAINING TACHYZOITE

Tachyzoite form of *T. gondii* (RH Strain) were provided by the Technology Laboratory in Cell Culture (LTCC) by UEZO, Brazil-RJ. These parasites were obtained through the number of the Ethics Committee of the Universidade Estadual do Norte Fluminense Darcy Ribeiro (ID 124396).

Tachyzoites of *T. gondii* (RH strain) were inoculated intraperitoneally into BALB/c mice. After 2-3 days, tachyzoites were harvested using intraperitoneal wash with phosphate buffered saline (PBS; pH 7.3) and then were counted.

### 2.3 CELL PARASITE INTERACTION

The cells were plated in plastic bottles of 25 cm² (CORNING / USA) or in glass laminate organized on the 24 well plates. After 48 hours, the culture has reached the confluence. The tachyzoite of *T. gondii* was resuspended in medium DMEM (Dulbecco's modified eagle's medium) and placed in contact with the cell in the proportion of 10 parasites for each cell. This was kept for 1 hour in the greenhouse in humid atmosphere to 5% $CO_2$ (Culture $CO_2$ Incubator, model CCL-170B-8, Sigapore). Then, the culture medium was discarted and replaced for DMEM (Dulbecco's modified eagle's medium) supplemented with 10% fetal bovine serum (GIBCO-Life Technologies, Rockville, MD), penicillin 1000 UI/mL and streptomycin 100 UI/ml (Sigma Chemical Company, St Louis, MO), were kept to 37°C in humid atmosphere to 5% $CO_2$ (Culture $CO_2$ Incubator, model CCL-170B-8, Sigapore) in the period of 24, 48 and 72 hours.

### 2.4 DETERMINATION OF PROTEIN CONCENTRATION

The protein concentration was determined by the Bradford's method, 1976. The

optical density readings was perfomed at wavelength of 595 nm in spectrophometer (SHIMADZER, model Uv-2450)

## 2.5 WESTERN BLOT

The cells were taken and serape in a solution of sodium bicarbonate, PH: 8,3 and centrifuged 10 minutes (New technique centrifuge, model NT 810, São Paulo). It was added tween 20 (1%) (Sigma) and was homogenated and resuspended in sodium bicarbonate solution 1 mM with the protease inhibitor cocktail for storage -20°C (Protease inhibitor: PMSF – 50 mM in ethanol; Leupeptin – 5 mg/mL; EDTA - 200 mM; Aprotinin – 10 mg/mL; E-64 – 1 mM; Pepstatin – 1 mg/mL; Antipain – 10 mM; 0-fenantrolin – 200 mM) (Sigma). The separation of the proteins was by the technique of gel electrophoresis polyacrylamide denaturant (SDS-PAGE). It was added 23 mg sample-solubilized protein in the concentrated gel of protein solubilized in sample buffer in the concentrated gel to 10% on plates with 1,5mm of thickness. The electrophoretic was performed at a constant voltage of 150 mv to 2 hours (System BIO-RAD).

After it, the gel was placed in contact with the membrane in buffer and the transfer under a constant current for the membrane was incubated in buffer TBS milk 5% and tween 20- 0,5% after 5 washes with TBS containing tween 20 a 0,5% (TBS-T).

The membrane was incubated with antibody anti-connexin 43 (GJA1 antibody (ab11370), Abcam) diluted in TBS-T containing skimmed milk powder 3%. The nitrocellulose was washed with TBS-T, as already described, and was incubated with secondary antibody antirabbit linked to horseradish peroxidase for chemiluminescence, for 2 hours. After a new wash with TBS-T, the nitrocellulose was incubated with the ECL solution (Peroxidase substrate for chemiluminescence) added to Peroxide for 10 minutes for development in the ChemiDoc MP imaging system BIO-RAD, and evaluated by the Image lab 5.1 program.

## 2.6 IMMUNOFLUORESCENCE

The cells were washed with PBS and fixed with 4% Paraformaldehyde (Vetec) during 1 hour and taken with PBS-TRITON X-100$^{R}$ (Sigma) at one concentration of 1% and incubated with bovine serum albumin (Sigma) without immunoglobulin diluted in PBS, 2% a 30 minutes. Primary anti-Cx43 antibody (GJA1 antibody (ab11370), Abcam) was incubated overnight 4°C, after which cells were washed and incubated with secondary polyclonal goat anti-rabbit Alexa Fluor 488 antibody (Goat antiRabbit IgG

(H+L) Cross-Adsorbed Secondary Antiboby, Alexa Fluor 488 (A-11008), Invitrogen) for 2 h at 37°C (Culture $CO_2$ Incubator, model CCL-170B-8, Sigapore). F-actin filaments were stained with Alexa Fluor 546 Phalloidin (A22283) (Life Technology) for 2h at 37ºC and DNA was stained with DAPI (4',6 diamidino-2-phenylindole). The markings were observed at an epufluorence microscope AXIOVERT 40 Plus Carl Zeiss. The experiments were submitted to observation simple or section at Z plan (dimensional reconstruction) using the confocal microscope LSM710 QUASAR (Carl Zeiss, Oberkochen, Germany)

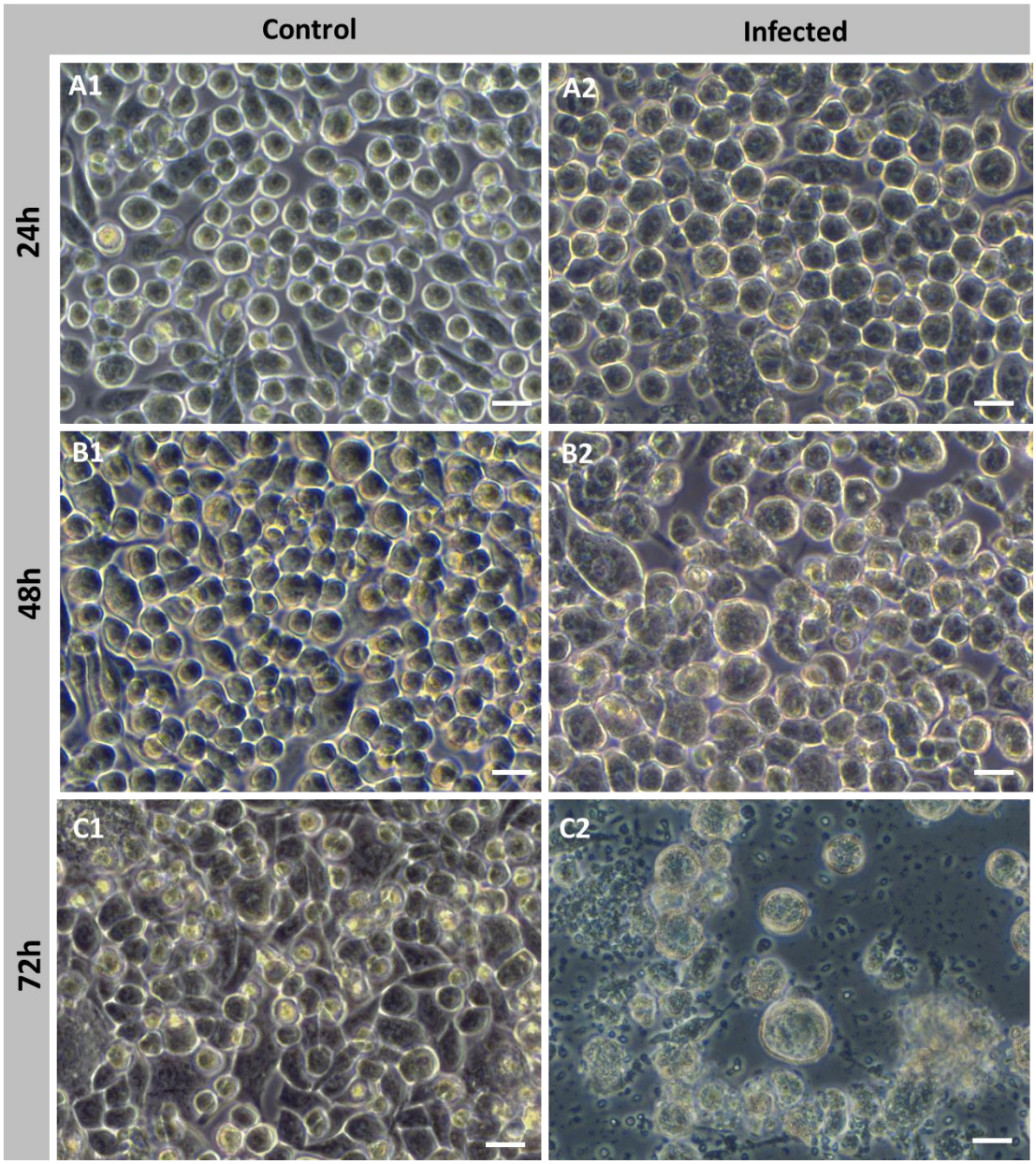
### 2.7 STATISTICAL ANALYSIS

To compare the analysis of variance was used one-way Anova with pos test of turkey. All statistics were made through the software Graphpad Prism v6.0. The values of significance were less than 0,001 ($p < 0,001$).

## 3 RESULTS

The results in figure 1 shown the morphological aspects of J774-G8 cells in the control (Figure 1 A1, B1 and C1) and infected (Figure 1 A2, B2 and C2) groups after the parasite-cell interaction for 24, 48 and 72 hours on phase contrast micrographs. Comparing the control and treated groups, is possible to observe that the progression of the infection cause morphological changes in the cells. The micrographs demonstrate the reduction in the number of cells caused by the detachment and cell lysis, in addition to the change in the cellular form (B2 and C2) and the increase in the quantity of free parasites in the culture (C2).

Figure 1: Micrographs in phase contrast using J774-G8 cells control (number 1) and infected with *T. gondii* (number 2) in the 24, 48 and 72 hours. Observe the reduction in the number of cells and morphological changes with the progression of the infection time with the protozoa, as well as the increase in the number of parasites. calibration bar 20µm. Increases of 400 X (40x objective).



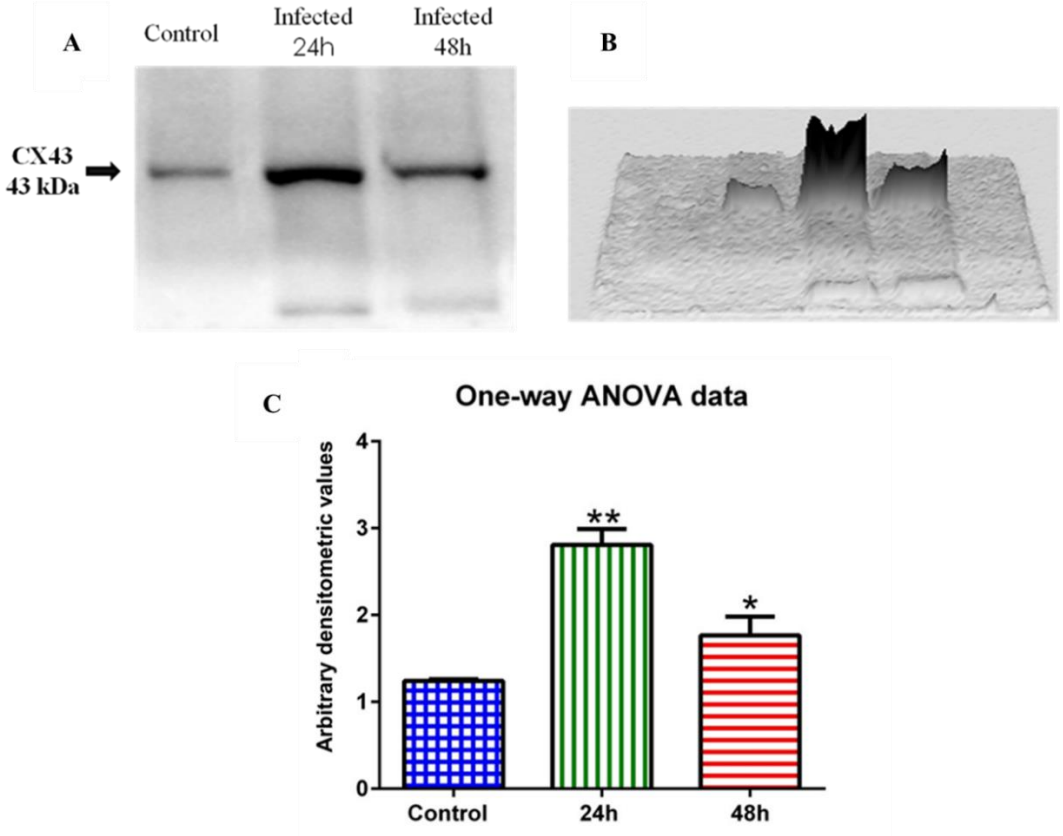

The expression of connexin 43 (Cx43) protein levels was analyzed by immunoelectrotransference assays of control and infected J774-G8 cells at 24 and 48 hours. We observed that Cx43 was expressed not only in control cells, but also in infected cells at all analyzed incubation times in this experiment (Figure 2A).

However, the band representing the interaction with the parasite in the 24 hour incubation period showed a higher expression of Cx43 than in control cells (uninfected), but also in the 3-dimensional image of the membrane marked with the antibody (Figure 2B).

The same findings were revealed at 48 hours of infection, with Cx43 expression at higher values than those found in control cells, however at lower values than those found at 24 hours of infection (Figures 2A and B).

The densitometric data confirmed the visible findings represented on the membrane marked with the antibody, highlighting the significant difference between the densitometric values.

Figure 2: In A and B, respectively, two and three dimensional images of one of the representative Western Blot experiments of cultures of J774-G8 (uninfected) or infected J774-G8 macrophage cell line (24 and 48 hours). The 43 kDa label is a result of the use of the polyclonal antibody for Cx43. Equal amounts of protein were loaded. The anti-Cx43 antibody detected the presence of the Cx43 protein in the control J774-G8 cells and in the infected J774-G8 with incubation of 24 and 48 hours. Note the increase in expression of Cx43 in relation to the control in infected J774-G8 cells in 24 and 48 hours, however, it is possible to notice the decrease in expression of Cx43 when compared to the 24 hour. In C we have the densitometric graphic representative of the bands observed in the Western blot image of J774-G8 cell cultures. This experiment was perfomed three times. Significance: $p < 0.0001$ (One Way Anova).




To assess the positioning of the Cx43 proteins in macrophages J774-G8, immunofluorescence experiments were realized (Figure 3). We performed the simultaneously labeling of the cells with the anti-connexin 43 and phalloidin antibodies, which bind to F-actin filaments, as well as incubation with the DAPI dye, which marks the cell nucleus.

When analyzing the experiment confocal microscopy system, we can observe the cell nucleus marking with DAPI (Figure 3A). The results of figure 3B indicated the location of Cx43 essentially on the plasma membrane of uninfected J774-G8 cells.

In parallel, the cells were immunostained with Faloidin (anti-F-actin), demonstrating that the actin filaments are homogeneously distributed by J774-G8 cells, being observed mainly in the plasma membrane framework and the organization of the cytoskeleton (Figure 3C).

Figure 3D shows the colocalization between the two proteins, Cx43 and Phalloidin (yellow dots resulting from the colorimetric interaction of green and red of the two antibodies), indicating that connexin 43 is interacting with the cytoskeleton in a possible anchoring process, being decisive for the formation of functional junction channels.

In the immunofluorescence experiments of the cell cultures infected with the parasite *T. gondii* were possible to observe the changes caused by the parasite-cell interaction in the junction complex and in the cell structure, in 72 hours of infection.

In figure 4A, it is possible to observe the cell nucleus marking by DAPI in blue and the nucleus of the protozoan *T. gondii* indicated by the arrow. In Figure 4B, we can highlight the absence of the staining for Cx43, as well as the staining for phalloidin (Figure 4C) showing a significant decrease in cells that shows a possible disorganization of the cellular cytoskeleton, which may suggest the connection between the cytoskeleton and connexin 43 in the J774-G8 macrophage lineage.

In Figure 4D, consequently, it was not possible to observe the colocalization between the Cx43 and Phaloidin proteins as demonstrated in the control, indicating that the cytoskeleton interacts with connexin 43.

Figure 3: Immunofluorescence demonstrated in confocal microscopy (LSM710, Zeiss, Germany), indicating the tag for protein connexin 43 (Cx43) and Phalloidin (marker actin filaments) in J774-G8 macrophage cell line. The labeled for Phalloidin and Cx43 can be observed on the plasma membrane of cells (B and C). We observed that the nucleus of the cells was labeled with DAPI nuclear stain. In micrograph D is demonstrated interposing images, showing a possible colocalization between Cx43 and F-actin (Arrows in points highlighted - yellow). Calibration bar: 50μm.

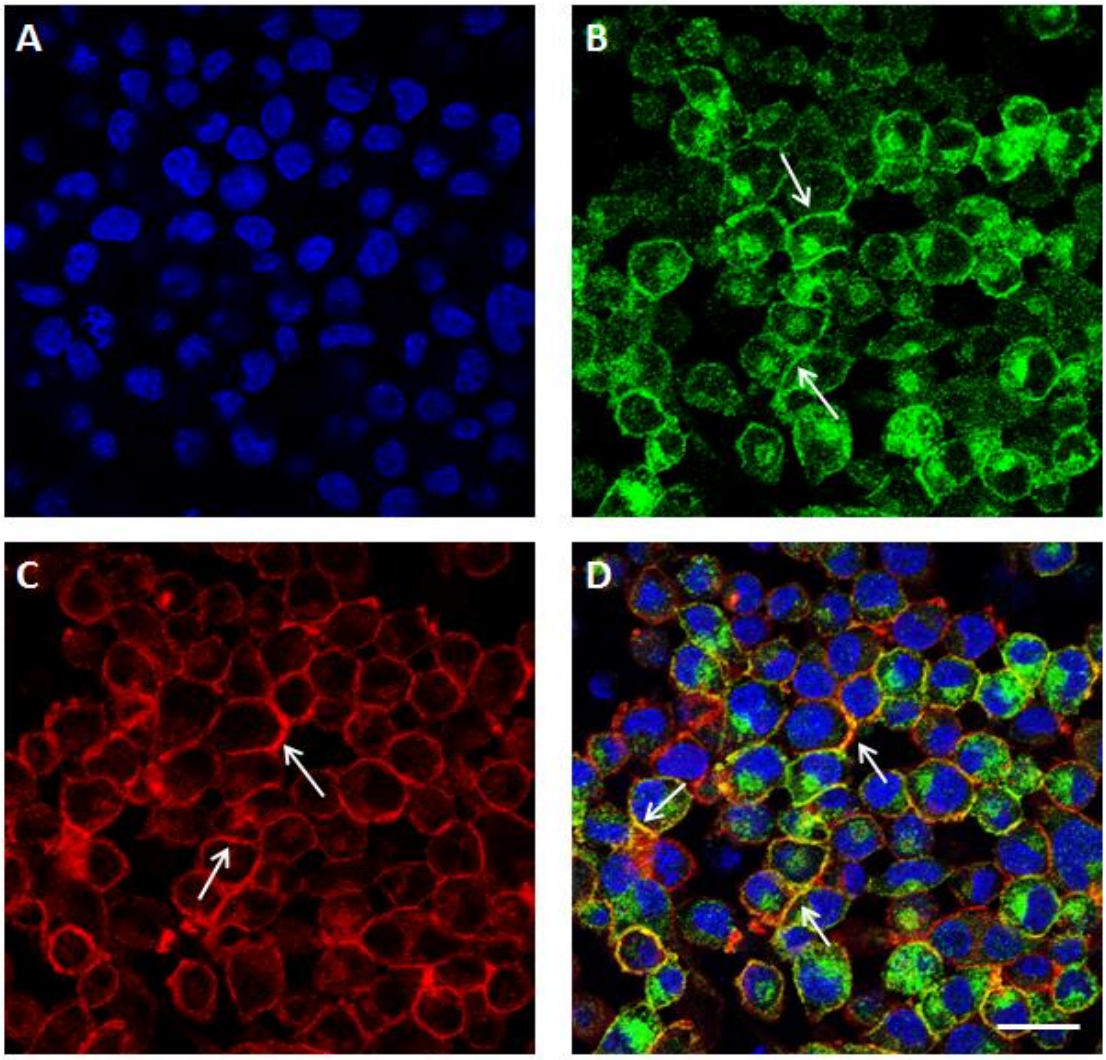
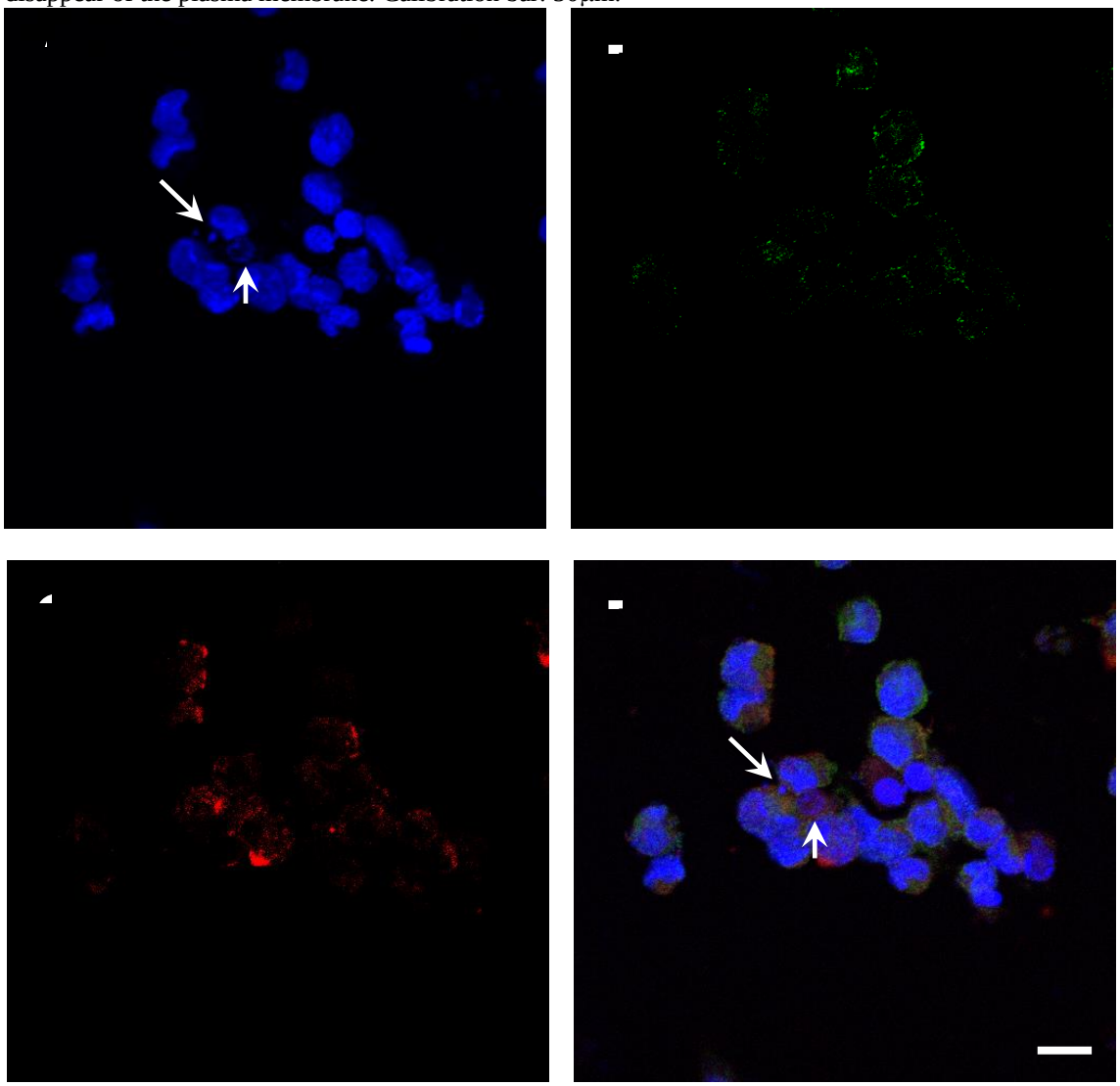


Figure 4: Immunofluorescence demonstrated in confocal microscopy (LSM710, Zeiss, Germany), indicating the tag for protein connexin 43 (Cx43) and Phalloidin (marker actin filaments) in J774-G8 macrophage cell line infected with *Toxoplasma gondii* by 72 hours. In A and D arrows demonstrated infection with parasite. In this experiment, Cx43 and F-actin labeling were disrupted of the plasma membrane of the J774-G8 cells after infection (B, C). In D the colocalization between Cx43 and F-actin disappear of the plasma membrane. Calibration bar: 50μm.



## 4 DISCUSSION

Some of the complications associated with toxoplasmosis may be connected with modulation of intercellular communication, with that we sought to study not only the changes related to cell growth aspects associated with macrophages cell lineage J774-G8 infected with *T. gondii* as well as the structural behavior of the junction communicating. Thus, it would be possible to analyze a possible junctional communication profile in a microenvironment of acute infection generated by *T. gondii*.

It is known that the progression of the parasite cell interaction in the acute model of *in vitro* infection is marked by the decrease in the number of cells due to the maintenance of the parasite's survival (Figure 1).

In order to evaluate whether infection with *T. gondii* would alter the spatial organization of Cx43 protein in J774-G8 cells, we performed immunofluorescence experiments using simultaneous labeling with the anti-Cx43 and Phalloidin antibodies (anti-F Actin).

The results of Figure 3 indicated the location of Cx43 essentially on the plasma membrane of J774-G8 cells, as well as with literature (Fortes et al. 2004). At the same time, the cells were immunolabelled with phalloidin (anti-F-actin), demonstrating that the actin filaments are homogeneously distributed in the J774-G8 cells, being observed mainly in the plasma membrane (Figure 3). These data are in complete agreement with Benchimol (1985), in which macrophage cells actin filaments and microtubules are scattered in the cytoplasm of the cells and with high concentration near the cell surface (plasma membrane) and are involved in the mobility of pseudopodia, as well as in vesicular and organelle trafficking (ALLISON et al., 1971).

We show the co-localization of the Cx43 and F-actin proteins in migratory cells of the immune system, indicating that Cx43 is interacting with the cytoskeleton in a possible anchoring process, which is decisive for the formation of functional junctional channels (Figure 3).

The connexons are transported from the Golgi complex to the plasma membrane of the cell through microtubules and need this structure to be inserted into the plasma membrane (MUSIL & GOODENOUGH, 1993; DHEIN, 1998, GAIETTA et al., 2002; SEGRETAIN & FALK, 2004). However, to date no work has made this association in cells that have a dynamic behavior, such as macrophage cells.

The structural and molecular organization model of the cellular junctions of Giepmans (2004), involved with cell adhesion and communication formed in particular by Cx43 highlight α-catenin and α-actinin involved in the formation of the cellular cytoskeleton and the insertion of connexin in the plasma membrane, having the β-catenin present in the protein complex which is associated with the E-cadherin adhesion protein which is co-located and co-immunoprecipitated with the Cx43. According to Fujimoto et al. (1997), β-catenins are co-localized with Cx43 at the beginning of junctional plaque formation in the cellular plasma membrane, being essential for membrane connexin insertion and insertion.

Immunofluorescence patches performed on cultures of cells infected with the *T. gondii* parasite indicated a marked decrease in the labeling for the Cx43 protein (Figure 4B), associated with the marked decrease in the labeling for phalloidin (Figure 4C). These results are in agreement with De Carvalho et al. (1998), which demonstrated structural changes in cardiac myocytes during *T. gondii* infection *in vitro*, and in this same study it was observed that the parasite is able to impair the functioning of the host cell through changes in cell-cell communication.

In addition to observing the decrease in the expression of Cx43 on the plasma membrane of J774-G8 macrophages, it was observed that infection with the parasites can alter the cellular organization, as highlighted by Seabra et al. (2004) in relation to the inhibition of the production of NO with *T. gondii* infection, being able to depolymerize the F-actin proteins in the infection process, and disorganize the cellular cytoskeleton.

Thus, we can indicate that the cytoskeleton interacts intimately with Cx43 in this model, because the Cx43 protein is not present in the membrane of the infected cells of the J774-G8 line, and that they present disorganization in the cytoskeleton, with absence of F-actin (Phalloidin). This hypothesis can be elucidated in Figure 3, which demonstrates the intimate relationship between cytoskeletal proteins in the maintenance and insertion of junctional membrane proteins in the plasma membrane (GIEPMANS, 2004).

To study the possible changes related to cellular communication in the macrophage cell infection model with *T. gondii*, analyzes of the protein expression levels of Cx43 were performed. Expression levels of Cx43 remained in the control cells (Figure 2A and 2B), which reinforced the immunofluorescence result (Figure 3A).

However, expression levels of Cx43 after 24 hours of infection were significantly higher than those expressed by uninfected cells, which was evidenced by the densitometry represented by the statistical analysis of the graph of figure 2C.

The infectious-inflammatory process triggers the production of various cytokines and interleukins that are capable of activating various cells of the immune system, in particular macrophages (VEGA & DE MAIO et al., 2003; CHANSON et al, 2005). This process of activation may lead to an increase in the communication levels between cells, especially if the information needs to be passed on, as in the presentation of antigens to the cells responsible for immunological memory (NEIJSSEN & NEEFJES, 2007). This communication can also take place through communicating junctions, as presented not only by our findings in this work, but also by other authors (EUGENÍN et al., 2003).

However, even in the cytokine release process, it is observed the alteration of the cellular framework in cases of parasitic infection, with the disorganization of the cytoskeleton demonstrated by our data and which is corroborated by several authors (SEABRA et al., 2004).

In this way, it is possible to infer from the Western blot that in the first 24 hours of infection with *T. gondii*, there is signaling for the macrophage to try to improve its cellular communication profile, increasing the production of Cx43 which could allow the formation of effective communicating junctions in the acute infection process. However, production levels decrease in 48 hours of infection (Figure 2), virtually disappearing within 72 hours post-infection, as demonstrated in immunofluorescence (Figure 4), which accompanies the disappearance of the cytoskeleton organization.

However, it is possible that within 24 hours of infection no longer occurs to functional coupling through the communicating junctions formed by the Cx43 in macrophages, since the changes in the cytoskeleton, linked to the depolymerization of F-actin, become evident after 2 hours of infection with *T. gondii*, *in vitro* experiments (SEABRA et al., 2004)

With this, it is possible to infer that the junctional communication in macrophages can play an important role in the inflammatory microenvironment, since it can be modulated by factors that activate the innate immune response, as well as its altered expression in several moments of the parasitic infection, the which may lead us to a prominent role in the infectious-inflammatory environment.

## 5 CONCLUSION

Considering the results obtained, we can conclude that the protein Cx43 and Phalloidin interact in the J774-G8 macrophage plasma membrane uninfected with the parasite *T. gondii*, but suffer a significant reduction in the membrane after 72 hours of infection due to the disorganization of the cellular cytoskeleton, but the protein expression, by immunoelectrophoretic transfer of Cx43 was shown to be increased in 24 and 48 hour macrophage J774-G8 lineage cells with the parasite *Toxoplasma gondii*, when compared to uninfected cells, suggesting increased protein expression for cell signaling during the infection process.

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