Effect of *Toxoplasma gondii* infection on the junctional complex of retinal pigment epithelial cells

ALANDERSON R. NOGUEIRA[1], FERNANDA LEVE[3], JOSÉ MORGADO-DIAZ[3], ROBERTO CARLOS TEDESCO[2,4] *and* MIRIAN CLAUDIA S. PEREIRA[1]*

[1] *Laboratório de Ultraestrutura Celular, Instituto Oswaldo Cruz, Fiocruz, Av. Brasil 4365, Manguinhos, Rio de Janeiro, RJ, 21040-900, Brazil*
[2] *Laboratório de Biologia Estrutural, Instituto Oswaldo Cruz, Fiocruz, Av. Brasil 4365, Manguinhos, Rio de Janeiro, RJ, 21040-900, Brazil*
[3] *Divisão de Biologia Celular, Coordenação de Pesquisa, Instituto Nacional de Câncer, Rua André Cavalcanti 37, 5° Andar, Rio de Janeiro, RJ 20230-051, Brazil*
[4] *Disciplina de Anatomia Descritiva e Topográfica da Universidade Federal de São Paulo – UNIFESP, Rua Botucatu 740, São Paulo-SP 04023-900, Brazil*

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SUMMARY

Ocular toxoplasmosis is the most frequent cause of uveitis, leading to partial or total loss of vision, with the retina the main affected structure. The cells of the retinal pigment epithelium (RPE) play an important role in the physiology of the retina and formation of the blood–retinal barrier. Several pathogens induce barrier dysfunction by altering tight junction (TJ) integrity. Here, we analysed the effect of infection by *Toxoplasma gondii* on TJ integrity in ARPE-19 cells. Loss of TJ integrity was demonstrated in *T. gondii*-infected ARPE-19 cells, causing increase in paracellular permeability and disturbance of the barrier function of the RPE. Confocal microscopy also revealed alteration in the TJ protein occludin induced by *T. gondii* infection. Disruption of junctional complex was also evidenced by scanning and transmission electron microscopy. Cell–cell contact loss was noticed in the early stages of infection by *T. gondii* with the visualization of small to moderate intercellular spaces. Large gaps were mostly observed with the progression of the infection. Thus, our data suggest that the alterations induced by *T. gondii* in the structural organization of the RPE may contribute to retinal injury evidenced by ocular toxoplasmosis.

Key words: *Toxoplasma gondii*, retinal pigment epithelial cells, junctional complex.

INTRODUCTION

Toxoplasma gondii, an intracellular obligate parasite in the phylum Apicomplexa, has widespread geographic distribution. Epidemiological surveys indicate that up to 30% of the world's population may be infected (reviewed by Robert-Gangneux and Dardé, 2012), although the disease is typically asymptomatic in immunocompetent individuals. However, immunocompromised individuals may develop encephalitis (Contini, 2008) and severe ocular lesions with loss of visual acuity (Rothova *et al*. 1993; Commodaro *et al*. 2009).

Ocular toxoplasmosis is the most common ocular disease among adolescents, with the retina being the most often affected structure. *Toxoplasma gondii* is the principal pathogen responsible for intra-ocular inflammation in infected individuals, with uveitis occurring in 85% of cases (Talabani *et al*. 2010). It is possible to find cysts in the retina with destruction of the ocular architecture (Tedesco *et al*. 2004) and necrotic lesions in the choroid (retinochoroiditis) (Vallochi *et al*. 2002) as a result of the inflammation caused by mononuclear cells at the site of infection.

The eye is an immune privileged site and is protected against damage caused by inflammation and microorganisms (Stein-Streilein, 2008, 2013). Retinal pigment epithelial cells (RPE) have a crucial role in retinal physiology and induction and maintenance of the immune privilege (Wenkel and Streilein, 1998; Sugita, 2009). RPE cells constitute the outer blood–retinal barrier, an interface between the retina and choroidal circulation. One of the functions of the RPE cells is to repel the immune effector cells, mainly lymphocytes that can penetrate the retina and cause damage to its tissue. Failure of the blood–retinal barrier leads to breakdown of the immune privilege and the development of intra-ocular inflammation (Willermain *et al*. 2002; Cunha-Vaz *et al*. 2011).

The integrity of the inner and outer blood–retinal barrier is essential for the maintenance of the retinal morphology and function. The outer blood–retinal barrier is provided through restriction of RPE paracellular permeability (Rizzolo, 1997). The RPE selective permeability is determined by a junctional complex composed of tight junctions (TJs),

* Corresponding author: Laboratório de Ultraestrutura Celular, Instituto Oswaldo Cruz, Fiocruz, Av. Brasil 4365, Manguinhos, Rio de Janeiro, RJ 21040-900, Brazil. E-mail: mirian@ioc.fiocruz.br

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GAP junctions and adherent junctions (Bissell and Radisky, 2001). The TJs are dynamic, complex structures, consisting of transmembrane proteins, including claudins, TJ-associated MARVEL protein family (occludin, tricellulin and MarvelD3) and junctional adhesion molecules, that regulate membrane polarity, cell morphology, cell growth and paracellular diffusion (Rizzolo *et al*. 2011; Lu *et al*. 2014; Rizzolo, 2014). Claudins and occludin are the principal proteins regulating epithelial permeability and active barrier functionality. However, the stability of TJs and their role in signalling pathways involved in barrier function are also regulated by the association of the zonula occludens with the actin cytoskeleton (Shin *et al*. 2006; Lu *et al*. 2014). Thus, any alteration in the complex junction organization may affect the blood–retinal barrier (Ban and Rizzolo, 1997). The aim of this study was to investigate the effects of infection by *T. gondii* on the integrity of TJs and its consequences on the barrier function in RPE cells.

MATERIAL AND METHODS

Parasites

The tachyzoite forms of *T. gondii*, RH strain, were maintained by successive infection of Swiss Webster mice (18–20 g). Ten female mice were intraperitoneally infected with 10^6 tachyzoites/animal. Three days post infection (3 dpi), the peritoneal exudate was collected and centrifuged for 7 min at 200 ***g*** to remove macrophages and cellular debris. The supernatant was then centrifuged for 10 min at 1000 ***g*** and the sediment containing the tachyzoites was resuspended in 10 mL of Dubelcco's modified Eagle medium (DMEM, Sigma Chemical Co., MO, USA) without serum. Total parasites per mL were quantified using a Neubauer chamber. All procedures with animals had been approved by the Committee of Ethics for Use of Animals of the Oswaldo Cruz Foundation.

Cell culture and parasite infection

The spontaneously immortalized human RPE lineage (ARPE-19, ATTC n° CRL-2302™) was acquired from the Banco de Células do Rio de Janeiro (Centro de Recursos Biológicos) (http://www.bcrj.hucff.ufrj.br). Cells were maintained in DMEM-F12 media supplemented with 10% of bovine fetal serum (SFB; Sigma), 1 mM *L*-glutamine plus 1000 U mL^{-1} penicillin, and kept at 37 °C in 5% CO_2 humidified atmosphere. The ARPE-19 cells were dissociated with 0·01% trypsin and 0·01% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS), pH 7·2, and isolated cells were seeded either at 2×10^5 cells mL^{-1} on 24-well culture plates containing glass coverslips, or 5×10^4 cells mL^{-1} on polycarbonate filter (Transwell, 0·4 μm pore size, 6·5 mm diameter – Costar, Cambridge, MA, USA). The choice of this cellular model was based on its physiological similarities to the RPE *in vivo*.

ARPE-19 cells were cultivated for 15 days prior to *T. gondii* infection. Afterwards, the cultures were infected with tachyzoite forms of *T. gondii* (RH strain) at a ratio of 5:1 parasites/host cell. Infection was interrupted after 1, 2 and 4 h of interaction.

Indirect immunofluorescence

ARPE-19 cultures, controls and *T. gondii*-infected cells, were fixed for 20 min at 4 °C with 4% paraformaldehyde in PBS, pH 7·2. After washing, the cells were permeabilized with PBS containing 0·5% Triton X-100 (3 × 10 min), washed with PBS and PBS containing 4% bovine serum albumin (BSA) (3 × 10 min). Cells were next incubated overnight at 4 °C with anti-occludin antibody (1:50; Zymed Laboratories), then washed and incubated for 1 h at 37 °C with the anti-rabbit IgG antibody conjugated with TRITC dye (1:400; Sigma). The samples were mounted with 2·5% 1,4-diazabicyclo [2·2·2]octane (DABCO) and observed using confocal laser scanning microscope BX51 Olympus and LSM 510 META (Zeiss).

Electron microscopy analysis

Uninfected and *T. gondii*-infected cells grown on polycarbonate filters (Transwell) were fixed for 1 h at 4 °C with 2·5% glutaraldehyde in sodium cacodylate buffer 0·1 M, pH 7·2 containing 3·5% sucrose. After fixation, the cultures were washed with sodium cacodylate buffer 0·1 M, pH 7·2 containing 3·5% sucrose and post-fixed for 1 h at 4 °C with 1% OsO_4 in similar buffer. Afterwards, the samples were dehydrated through a crescent series of acetone and embedded in PolyBed 812 resin. Ultra-thin sections were stained with 5% uranyl acetate and 2% lead citrate. The samples were analysed using a JEOL transmission electron microscope (JEM/1011). For scanning electron microscopy (SEM), the cultures were fixed as described above, dehydrated in acetone and critical point dried. The samples were gold sputter-coated (20 nm) and observed using the JEOL scanning electron microscope (JSM 6390LV) instrument.

Transepithelial electrical resistance (TEER)

ARPE-19 cells were seeded and cultivated for 15 days in Transwell polycarbonate filters (0·4 μm pore size, 0·33 cm^2 surface area) (Corning Life Sciences, Lowell, MA). After confluence, the cultures were infected with tachyzoite forms of *T. gondii* (RH strain) at a ratio of 5:1 parasites/host

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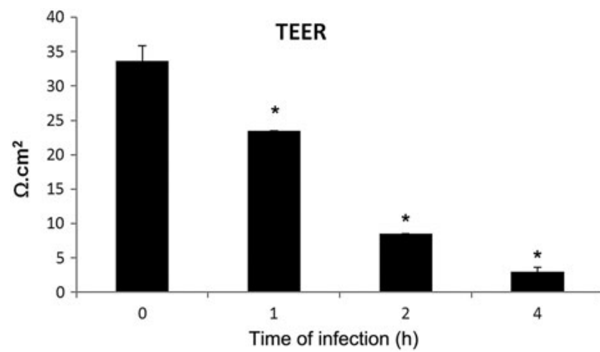

Fig. 1. *Toxoplasma gondii* infection affects the function of outer blood–retinal barrier *in vitro*. TEER measurement was performed to evaluate tight junction functionality in *T. gondii*-infected ARPE-19 cultures compared with control. A significant reduction after 2 and 4 h in TEER was observed in ARPE-19 cells infected by *T. gondii*. (*) Statistically significant, ANOVA, post-test Bonferroni, $P<0{\cdot}01$.

cell. As above, the kinetics of infection was interrupted after 1, 2 and 4 h of interaction, and TEER values were determined using a Millicel-ERS system (Millipore Corporation, Billerica, MA). All TEER values were normalized for the area of the filter (0·6 cm^2) and obtained after background subtraction (i.e., filter and bath solution).

Statistical analysis

The Student's *t*-test was used to determine the significance of differences between mean values of the percentage of infection in three independent experimental assays. For TEER measurement, each experiment was repeated at least three times using different batches of ARPE-19 cell cultures and parasites. Each time point in an experimental set contained triplicate cultures. Results of *T. gondii*-infected cultures were compared with the corresponding control by one-way ANOVA followed by Bonferroni's post-test, using GraphPad Prism version 4.0 for Windows (Graph-Pad Software, San Diego, CA). A P-value $\leqslant 0{\cdot}05$ was considered significant.

RESULTS

The ARPE-19 lineage was used to evaluate the barrier function of RPE after *T. gondii* infection. Confluent ARPE-19 cultures presented a morphologic profile characteristic of this cell type (Fig. 2), with juxtaposed cells, eccentric nuclei and well-established intercellular contacts.

The functionality of the TJ complex, which is responsible for size- and charge-selective transport properties, was assessed by TEER measurement. Uninfected cells achieved a maximal TEER value of approximately 32 $\Omega\ cm^{-2}$. *Toxoplasma gondii* infection disrupted this value, with *T. gondii*-infected cells demonstrating a significant decrease of TEER as compared with control cells. After 1 h of infection, a decrease in TEER ($P\leqslant 0{\cdot}05$) was observed (Fig. 1). However, a maximal reduction was evidenced within a time course of 4 h ($P\leqslant 0{\cdot}01$), achieving a TEER value of 3 $\Omega\ cm^{-2}$, which correspond to 10-fold decrease compared with control cells.

TJ integrity was also analysed by indirect immunofluorescence detection of occludin in uninfected and *T. gondii*-infected ARPE-19 cells. Confocal microscopy of ARPE-19 cells showed continuous junctional staining along cell borders, demonstrating a well-developed TJ structure (Fig. 2). Loss of continuity occludin staining within intercellular junction was observed in early stage of infection (1 h). A clear disruption of TJs was seen by the prominent intercellular spacing and absence of occludin staining after 4 h of parasite–host cell interaction (Fig. 2). Intracellular tachyzoites were also labelled with anti-occludin antibody, which may be related to unspecific cross-reaction with parasite proteins.

Ultrastructural analysis of ARPE-19 cells revealed the presence of microvilli at the cell surface and large numbers of mitochondria and endoplasmic reticulum processes (Fig. 3). The junctional complex was visualized at intercellular regions (Fig. 3), allowing cohesion between bordering cells in the monolayer to be observed. Infection with *T. gondii* affected the junctional complex, leading to the loss of intercellular contact. After 2 h of infection, it was possible to observe an intact junctional domains flanked by slight intercellular spaces (Fig. 3). The lack of association between neighbouring cells was a gradual process, which became more pronounced with a reduction of junctional integrity at a later stage of infection. At 4 h post-infection, we observed alterations in the RPE junctional complex revealed by the formation of large lacunae between adjacent cells, with excessive areas of reduced cell–cell contact (Fig. 3).

To ensure that the observed effects were predominant in infected cultures, we evaluated the temporal analysis of *T. gondii*–ARPE-19 cells interaction by SEM. SEM analysis of uninfected ARPE-19 cultures revealed a uniform monolayer with juxtaposed cells with plentiful surface microvilli, showing well-preserved and defined cell–cell contacts (Fig. 4). However, as observed by MET, *T. gondii* infection altered the organization of ARPE-19 cell culture. Disturbance in the cell–cell contacts was evident with the progress of infection. Small to moderate intercellular spaces were observed after 2 h of interaction (Fig. 4), and this effect became more evident after 4 h of infection. In this time course, large intercellular lacunae and also rounded cells were visualized in the infected cultures, associated with the disruption of contacts between adjacent cells (Fig. 4).

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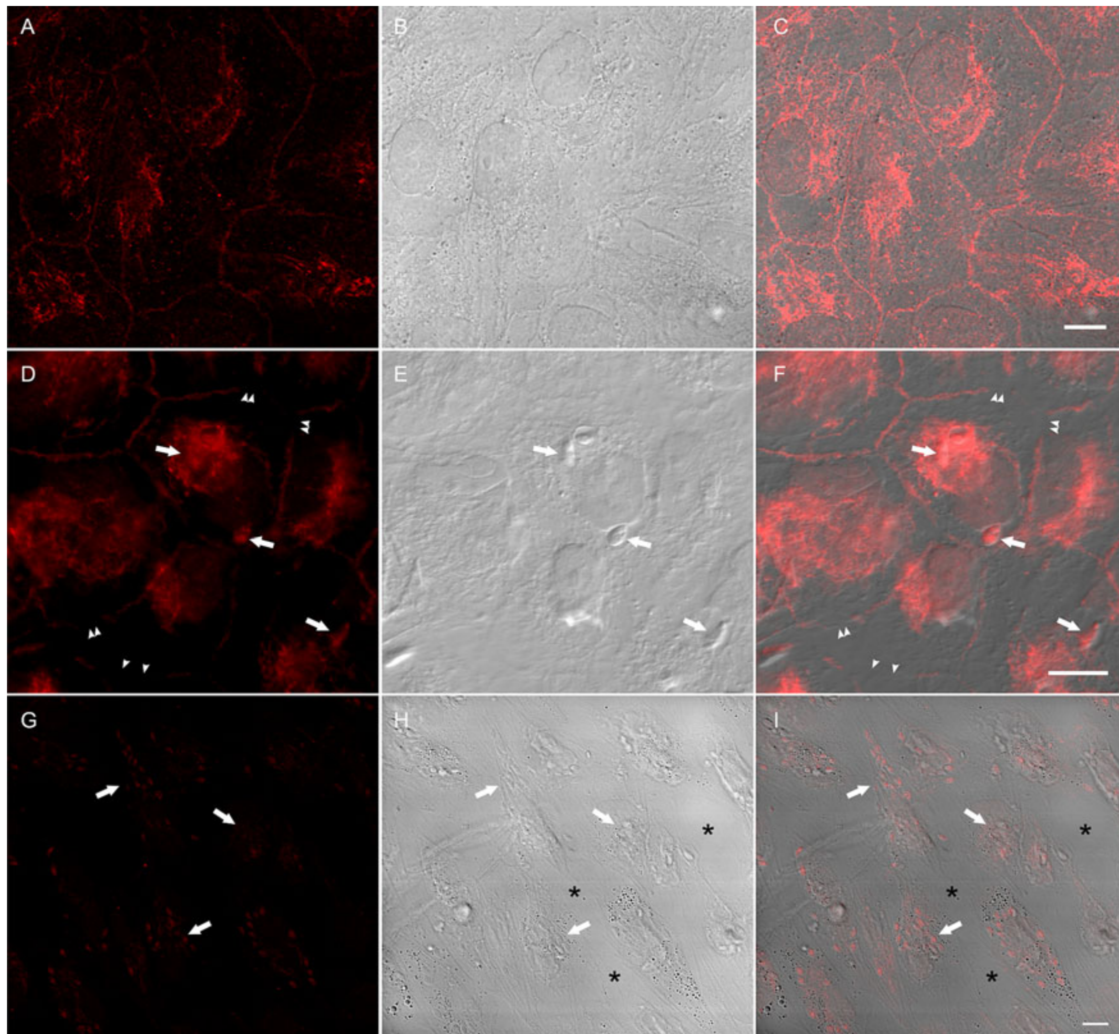

Fig. 2. Immunofluorescence images of tight junction distribution in uninfected and *T. gondii*-infected RPE cells. (A) ARPE-19 cells display a continuous positive staining for occludin in cell–cell contact. Alterations in tight junction distribution were revealed by loss of occludin labelling (arrowhead) after 1 h of *T. gondii* infection (B). A severe disruption of cell–cell contacts (*) was seen after 4 h of infection (C), showing no occludin labelling. An unspecific labelling was visualized in the intracellular parasites (arrows) which may be related with antibody cross-reaction. DIC (B, E and H) and merge images (C, F and I). Bar = 10 μm.

DISCUSSION

Retinochoroiditis is an important ophthalmologic manifestation of toxoplasmosis, resulting in devastating consequences in loss of visual acuity (Stanford *et al*. 2005). A hallmark of *T. gondii* infection is the parasite's ability to overtake biological barriers during acute infection and reactivation of chronic disease. Circulating *T. gondii* tachyzoites access the retinal microenvironment by crossing the retinal endothelium through a mechanism mediated either by paracellular transmigration, mediated by the interaction of adhesion molecules of the parasite (MIC2) and target cell (ICAM-1), or by hitchhiking via leucocyte migration through vascular endothelium (Furtado *et al*. 2012). Evidences have also demonstrated that *T. gondii* invade through both the retina and choroidal vasculature (Remington and Desmonts, 1976; Tedesco *et al*. 2004). An *ex vivo* assay carried out in a human eyecup demonstrated the migration of tachyzoites through retina layers (Furtado *et al*. 2013). Intraretinal parasites were visualized at the nerve fibre layer and ganglion cell layer as well as outer layers, including outer plexiform layer and outer nuclear layer. Evidence of tachyzoites and cysts has been observed in the RPE cells of patients with retinochoroiditis, suggesting that RPE may be a vulnerable infection site for *T. gondii* (Nicholson and Wolchok, 1976). However, the mechanism involved in overcoming the barrier of RPE cells is not well understood. Therefore, in the present study, we analysed the changes in retinal barrier function during *T. gondii* infection in order to understand the loss of immune privilege in ocular toxoplasmosis.

The outer blood–retinal barrier is characterized by low permeability and is responsible for maintenance

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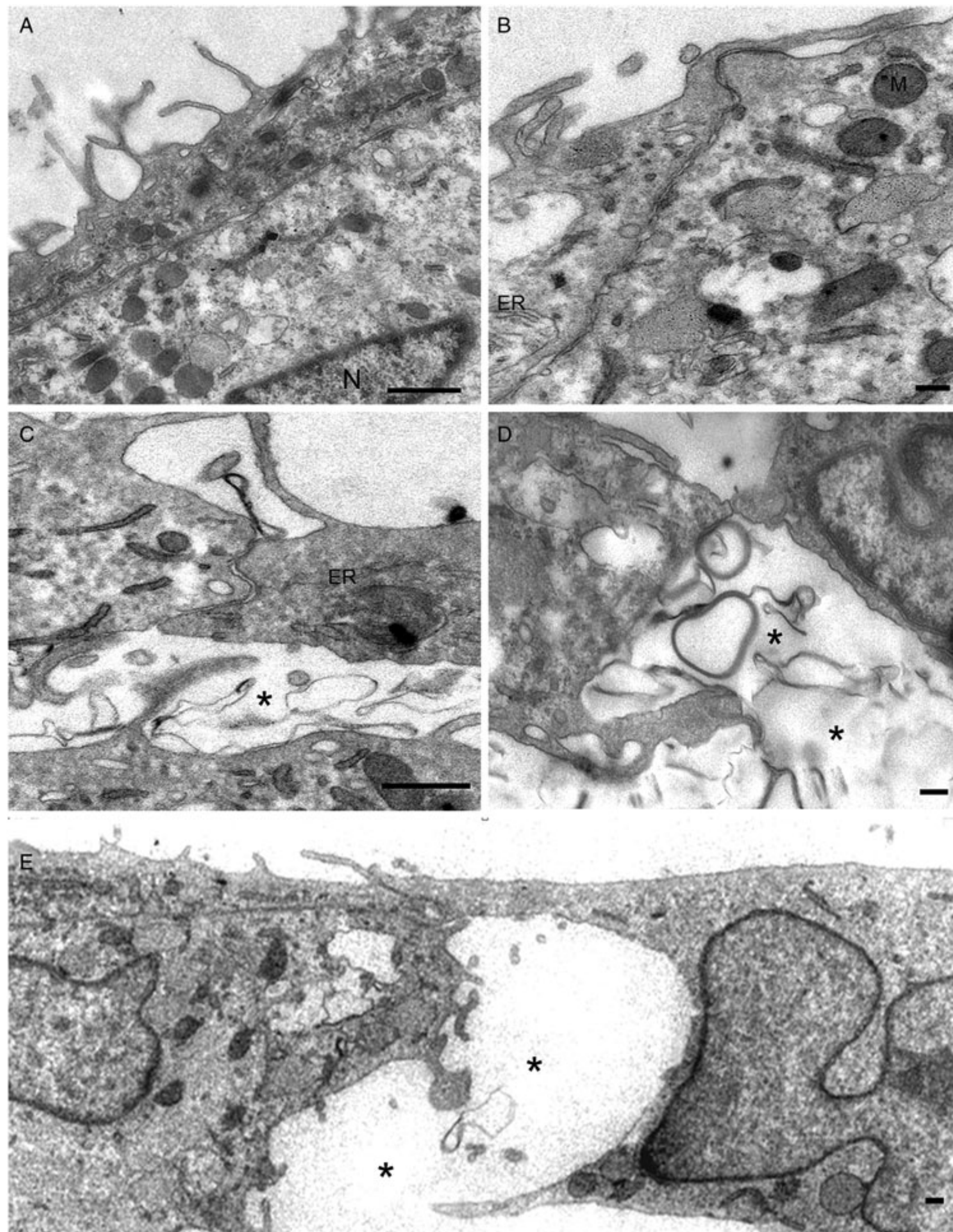

Fig. 3. Ultrastructural analysis of RPE intercellular junction integrity after *T. gondii* infection. Transmission electron micrograph revealed an intact tight junction complex in control ARPE-19 monolayer (A and B). Microvilli on the surface of the cell, melanin granules and intracellular organelles, such as mitochondria (M), endoplasmic reticulum (ER) and nucleus (N) were also observed (A and B). Junction breakdown (*) was observed after 2 h of *T. gondii* infection (C) and became more evident at later time point (4 h) (D and E), showing large gaps in the region of intercellular contact. Bar = 1 μm (A). Bar = 0·2 μm (B–E).

of the immunologically privileged site of the human eye by protecting neural retinal cells from damage (Campbell and Humphries, 2012). Intercellular contacts are essential to preserve the selective barrier function of the RPE (revised by Rizzolo *et al*. 2011), without which pathogen infection may result in a breach of this functional barrier (Moyer *et al*. 2009). Our findings demonstrate that *T. gondii* infection disturbs the RPE barrier by affecting the junctional complex organization and retinal permeability. This effect was clearly demonstrated by a reduction in TEER that results in a 10-fold decrease of the barrier permeability after 4 h of infection. Disruption of cell–cell contact was also evidenced in *T. gondii*-infected ARPE-19 cells. Alterations in the spatial distribution of TJs were evidenced by lack of occludin labelling in *T. gondii*-infected ARPE-19 cells. Additionally, infection by *T. gondii* induced the formation of large gaps between the RPE cells visualized by electron microscopy (TEM and SEM), corroborating the cell permeability data. Taken together, these results suggest that TJ dysfunction makes the retinal epithelium permissive to the influx of

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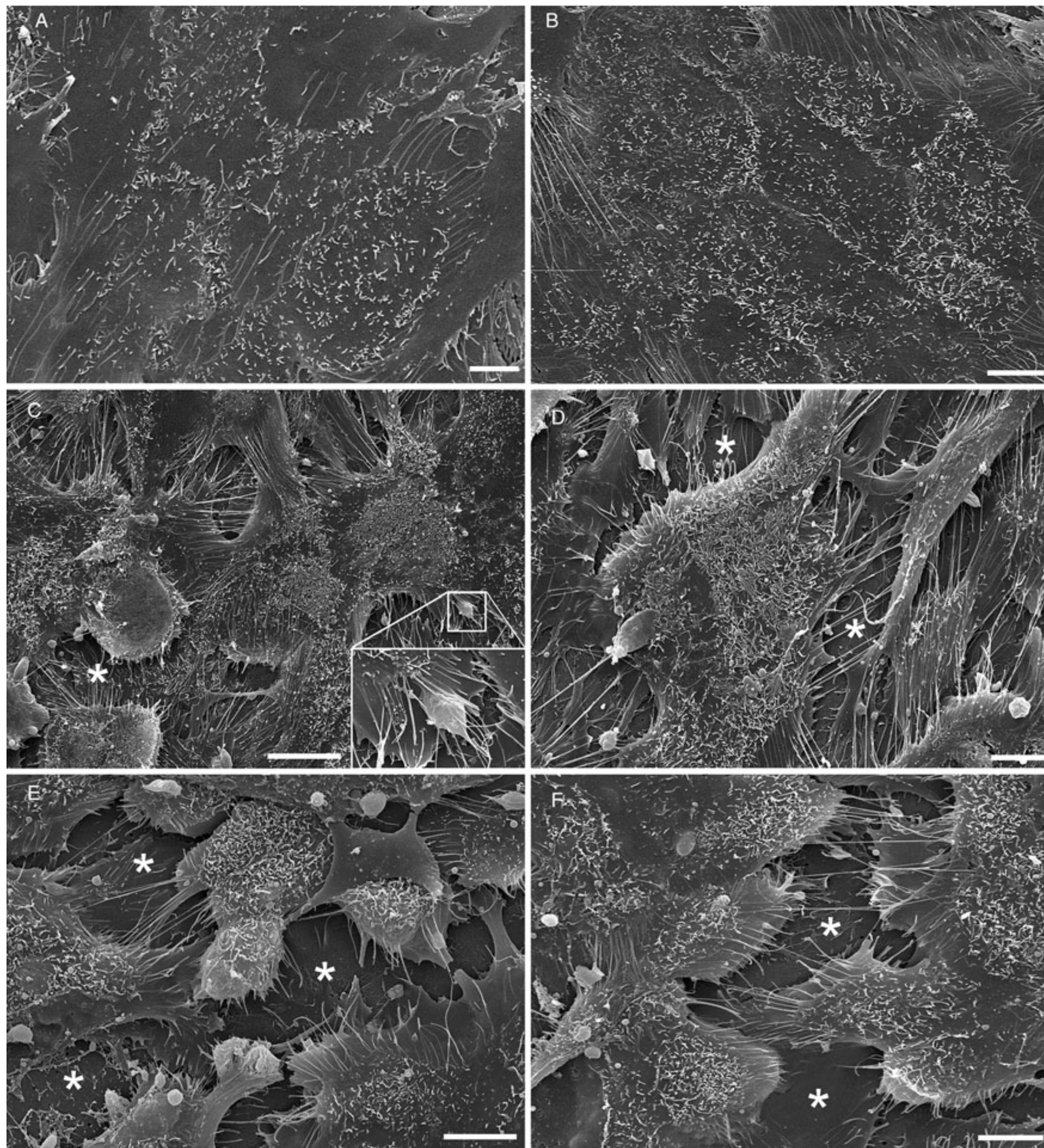

Fig. 4. SEM analysis of non-infected and *T. gondii*-infected RPE cells. Highly confluent monolayer of control ARPE-19 cells showing microvilli on the apical surface and intact cell–cell contacts (A and B). After 2 h of infection, a disruption of junctional complex was observed in *T. gondii*-infected cultures (C, D). This event was intensified after 4 h of infection where intercellular gaps (*) were clearly visualized (E and F). Note the presence of parasites in the invasion process (insert C). Bar = 10 μm (A, D, E and F); 20 μm (B and C).

inflammatory cells and may contribute to the severity of the ocular infection. The alteration in the TJ may be directly or indirectly induced by the parasite during acute phase or reactivation of toxoplasmosis, since the innate immunity response triggered by *T. gondii* infection leads to the production of cytokines or chemokines which may also contribute to paracellular permeability. Breakdown of cell–cell junctional integrity has been associated with inflammatory mediators and the generation of reactive oxygen species (ROS) induced by pathogens (Mayhan, 2001; Rao, 2008; Hamada *et al*. 2013). Also, it has been demonstrated that high level of ROS production activates matrix metalloproteinases (MMPs) as MMP-1, -2 and -9 (Haorah *et al*. 2007), which can degrade protein constituents of TJ (occludin, claudin and ZO-1, -2 and -3) through phosphorylation of their tyrosine residues (Bauer *et al*. 2010; Feng *et al*. 2011). Phosphorylation of occludin and the presence of intraepithelial lymphocytes ($\gamma^{\delta+}$ iIELs) in the intestinal mucosa, an important barrier that must be crossed for parasite dissemination in mammalian host, have been reported to regulate the epithelial barrier integrity in response to *T. gondii* and Salmonella challenge (Dalton *et al*. 2006). In congenital transmission of *T. gondii*, MMP-2 and -9 seem to participate both in extracellular matrix (ECM) degradation and placental

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barrier dysfunction, leading to an efficient tachyzoite transmission to the fetus (Wang and Lai, 2013).

Indeed, it has been shown that RPE cells have dual functionality: (i) they can prevent the inflammatory response by secreting immunosuppressive factors that maintain the immune privilege of the eye and retinal barrier; or (ii) by contrast can play an important role in triggering an intense inflammatory response upon infection by pathogens, abrogating barrier function (Holtkamp *et al*. 2001). Thus, it appears possible that inflammatory mediators and/or ROS secreted by RPE cells in response to infection with *T. gondii* may be one of the mechanisms underlying the disruption of TJs and thus to blame for the disruption of the retinal barrier. One striking fact upon this subject is the migration of RPE cells to deeper layer of retina (Tedesco *et al*. 2004).

Several lines of evidence have also highlighted the effect of viral and bacterial infection in blood–retinal barrier permeability (Zhang *et al*. 2005; Ramadan *et al*. 2006; Moyer *et al*. 2009). Destruction of barrier function was reported in RPE cells infection by HIV-1. Changes in claudin are modulated by HIV-1 transactivator Tat protein (HIV-1 Tat) and disturb the TJ organization, mediating HIV invasion in the ocular tissue. This effect seems to be regulated by activation of NF-κB and oxidative stress (Bai *et al*. 2008). In addition, endophthalmitis caused by *Bacillus cereus* induces a rapid inflammatory response and loss of retinal function (Moyer *et al*. 2009). Toxins and/or other mediator factors secreted by *B. cereus* may be involved in TJ disruption and retinal damage (Moyer *et al*. 2009), leading to visual impairment.

In summary, our findings demonstrate that *T. gondii* infection induces changes in TJ complex of RPE cells, a likely culprit in breakdown of the blood–retinal barrier. Improved understanding of the mechanism of this process should advance methods to prevent or treat ocular toxoplasmosis.

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