Besnoitia besnoiti and *Toxoplasma gondii*: two apicomplexan strategies to manipulate the host cell centrosome and Golgi apparatus

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SUMMARY

Besnoitia besnoiti and *Toxoplasma gondii* are two closely related parasites that interact with the host cell microtubule cytoskeleton during host cell invasion. Here we studied the relationship between the ability of these parasites to invade and to recruit the host cell centrosome and the Golgi apparatus. We observed that *T. gondii* recruits the host cell centrosome towards the parasitophorous vacuole (PV), whereas *B. besnoiti* does not. Notably, both parasites recruit the host Golgi apparatus to the PV but its organization is affected in different ways. We also investigated the impact of depleting and over-expressing the host centrosomal protein TBCCD1, involved in centrosome positioning and Golgi apparatus integrity, on the ability of these parasites to invade and replicate. *Toxoplasma gondii* replication rate decreases in cells over-expressing TBCCD1 but not in TBCCD1-depleted cells; while for *B. besnoiti* no differences were found. However, *B. besnoiti* promotes a reorganization of the Golgi ribbon previously fragmented by TBCCD1 depletion. These results suggest that successful establishment of PVs in the host cell requires modulation of the Golgi apparatus which probably involves modifications in microtubule cytoskeleton organization and dynamics. These differences in how *T. gondii* and *B. besnoiti* interact with their host cells may indicate different evolutionary paths.

Key words: *Besnoitia besnoiti*, *Toxoplasma gondii*, microtubule cytoskeleleton, Golgi apparatus, centrosome, parasite-host interaction.

INTRODUCTION

Obligate intracellular parasites have co-evolved with hosts to be able to invade their cells and flourish. To be successful they need to establish specific molecular parasite-host cell interactions, and then manipulate the host cell structures, mechanisms and pathways in order to replicate and grow. These events are strictly required for the completion of their life cycles and are the basis of their pathogenesis. Therefore, the understanding of the molecular mechanisms underlying host cell invasion is a crucial step for defining therapeutic strategies.

Apicomplexan parasites are a unique and diverse group of protozoa characterized by having a structure called the apical complex, which is involved in host-cell invasion. *Besnoitia besnoiti* and *Toxoplasma gondii* are both apicomplexan parasites, belonging to the family Sarcocystidae, and are phylogenetically closely related (Ellis *et al*. 2000; Marcelino *et al*. 2011). *Besnoitia besnoiti* has received little attention until recently. Members of the Bovidae are the only known intermediate hosts and its definitive host remains unknown. Cattle that survive the acute phase of infection (characterized by nasal and ocular discharges, anorexia and generalized weakness) retain low body condition and have chronic scleroderma, pronounced thickening of the limbs and difficult and painful locomotion (Pols, 1960). Bovine besnoitiosis causes significant economic losses in the cattle industry of Africa and Middle East, having been considered by EFSA an emerging disease in the EU

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due to its increased prevalence and geographic expansion (EFSA, 2010). *Toxoplasma gondii* infects warm-blooded vertebrates, including humans, and can be found in most regions of the world. Infection can result in a wide spectrum of clinical signs depending on the host animal species. Congenital infection is common, resulting in one of the major causes of abortion in sheep (Innes, 2010). In humans, most infections generate few or no symptoms, but acute infections are a concern, particularly during pregnancy and in immunocompromised individuals.

Toxoplasma gondii is one of the most studied apicomplexan parasites and, therefore, is a good comparative model for studies on *B. besnoiti*. The molecular mechanisms involved in host-cell invasion by *T. gondii* parasites have been extensively studied, and some similarities were found with other apicomplexan parasites, as for example *Plasmodium*. However, nothing is known about the host cell invasion strategies used by *B. besnoiti*.

We have previously shown that the microtubule cytoskeleton of the host cell has an active role during the first steps of *B. besnoiti* host cell invasion, because the host cell microtubule cytoskeleton is rearranged in order to surround the parasite during the first steps of cell entrance (Reis *et al.* 2006). Also, for *T. gondii*-infected host cells, the microtubule cytoskeleton surrounds the parasitophorous vacuole (PV) soon after invasion (Walker *et al.* 2008; Sweeney *et al.* 2010) which is accompanied by the detachment of the centrosome, the major microtubule-organizing centre (MTOC) of animal cells, from the nuclear envelope, and further association with the PV (Coppens *et al.* 2006; Walker *et al.* 2008; Wang *et al.* 2010). Interestingly, it is now known that the alteration of centrosome positioning is involved in the repositioning of the pericentrosomal Golgi ribbon (Bornens, 2012), and that the Golgi apparatus and centrosome are functionally linked (Sütterlin and Colanzi, 2010). For example, Golgi proteins like GM130, Cdc42 and Tuba regulate centrosome morphology and function (Kodani and Sütterlin, 2008; Kodani *et al.* 2009); and a centrosomal protein, like AKAP450, is an important linker between the centrosome and the Golgi apparatus (Rivero *et al.* 2009). Additionally, the correct morphology and positioning of the Golgi apparatus is achieved by the organized cooperation of microtubules growing from both the centrosome and the Golgi apparatus (Hurtado *et al.* 2011). Also, it has been demonstrated that the Golgi apparatus nucleates microtubules in different epithelial cell lines (Efimov *et al.* 2007; Rivero *et al.* 2009). In fact, the nucleus-centrosome-Golgi defines an intrinsic polarity axis (Bornens, 2008; Dupin *et al.* 2009; Luxton and Gundersen, 2011) that has been shown to be essential for several cellular functions, and that can be remodelled during cell-stage transitions. For example, the centrosome is repositioned and microtubule arrays reoriented during cell migration (Yvon *et al.* 2002; Luxton and Gundersen, 2011).

To gain new insights about the cellular mechanisms underlying *B. besnoiti* host cell invasion we decided to go further, studying the role of the host microtubule cytoskeleton, centrosome and the intrinsic cell polarity axis in *B. besnoiti* host invasion. For this we have profited from the fact that we recently described the human TBCC-domain containing 1 (TBCCD1), a protein that localizes at the centrosome and is involved in centrosome–nucleus connection (Gonçalves *et al.* 2010*a*). The knockdown of TBCCD1 causes the dissociation of the centrosome from the nucleus and disorganization and spreading of the Golgi apparatus throughout the cytoplasm, suggesting that TBCCD1 is a key regulator of centrosome positioning and consequently of internal cell organization (Gonçalves *et al.* 2010*a*). Therefore, we have investigated the impact of over-expressing or depleting TBCCD1 from host cells in the ability of *B. besnoiti* to recruit the centrosome and invade and replicate in these cells. We extended these studies to *T. gondii* to investigate the differences and similarities between the two apicomplexan parasites and if, during host invasion, the cellular mechanisms are conserved.

Here we show that both *B. besnoiti* and *T. gondii* parasitophorous vacuoles (PVs) cross-talk with the host microtubule cytoskeleton, but each parasite promotes distinct arrangements of these polymers independently of the host cell type. Moreover, only *T. gondii* consistently displaces the centrosome from the host cell nucleus to the PV membrane which is accompanied by Golgi apparatus disorganization. In fact, although both parasites seem to preferentially establish the PV close to the host cell Golgi apparatus, *B. besnoiti* causes Golgi compaction even under conditions where this structure was dispersed prior to invasion, e.g. TBCCD1 depletion. Results from invasion and replication assays for both parasites in a host background of TBCCD1 over-expression or depletion, point to the idea that successful establishment of PVs in the host cell requires modulation of Golgi apparatus. The mechanisms underlying this modulation seem to be different for *T. gondii* and *B. besnoiti*, but probably rely on the microtubule cytoskeleton exploitation by both parasites. The differences found between these two Apicomplexa parasites are most likely a result of two distinct evolutionary mechanisms that might reflect the parasites' distinct tissue tropism and pathogeny.

MATERIALS AND METHODS

Cell culture and parasite culture

Vero cells were grown in Dulbecco's modified Eagle's medium (DMEM) with Glutamax (Invitrogen), whereas

hTERT-RPE-1, hTERT-RPE-1-centrin–GFP and hTERT-RPE-1 overexpressing TBCCD1-GFP were grown in DMEM/F12 with Glutamax (Invitrogen), both supplemented with 10% foetal bovine serum (Invitrogen) and non-essential amino acids (Invitrogen). These cell lines were cultured in a 5% CO_2 humidified atmosphere at 37 °C as exponentially growing sub-confluent monolayers.

Besnoitia besnoiti Bb1Evora03 strain (Cortes *et al.* 2006) and *T. gondii* (ME49 strain SAG1-Luciferase-BAG1-GFP, a kind gift from Andrea Crisanti, Imperial College London, UK) tachyzoites were grown in Vero cells and maintained in DMEM with Glutamax (Invitrogen), supplemented with 10% foetal bovine serum and non-essential amino acids (Invitrogen). Tachyzoites were harvested from the supernatant by centrifugation at 770 *g* for 10 min.

Generation of viral particles and stable cell lines

To produce viral particles carrying the TBCCD1-GFP transgene, HEK293T cells were co-transfected with the plasmids pHR-SIN-TBCCD1-GFP, pCMVR8.9 and pMDG using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The viral particles present in the culture medium were collected at 48 and 72 h post-transfection and used to infect hTERT-RPE-1 cells. GFP-positive cells were selected by cell sorting using a FacsAria Multicolor cell sorter. Single cells were isolated in 96-well plates, from which individual clonal cell lines were derived.

Immunofluorescence

For immunofluorescence, the cells were fixed with cold methanol (10 min at −20 °C), blocked with 3% bovine serum albumin (20 min), incubated 1 h with the primary antibodies (anti α-tubulin, Sigma, clone DM1A; anti γ-tubulin, Sigma, clone GTU88; anti-Golgin 97, Molecular Probes, clone CDF4; rat anti-α-tubulin, AbD Serotec, clone YL1/2; rabbit anti-*B. besnoiti* (Marcelino *et al.* 2011); cat anti-*T. gondii* (kindly supplied by Helga Waap, LNIV, INIAV, Portugal); washed and incubated 1 h with secondary antibodies (anti-mouse Alexa 488, Molecular Probes; anti-mouse Alexa 594, Molecular Probes; anti-rabbit Alexa 488, Molecular Probes; anti-rabbit Alexa 594, Molecular Probes; anti-rat Alexa 546, Molecular Probes; anti-cat FITC F-4262 SIGMA). DNA was stained with DAPI ($1 \mu g \mu L^{-1}$; Sigma) in PBS for 2 min. The preparations were washed in PBS and mounted in MOWIOL 4-88 (Calbiochem) mounting medium supplemented with 2·5% (w/v) DABCO (Sigma). Cells were examined with a fluorescence microscope (Leica DMRA2) and image acquisition was performed with a cooled CCD camera and MetaMorph Imaging Software (Universal Imaging Corporation). The image processing was carried out with ImageJ Software.

Transfection of hTERT-RPE-1 and hTERT-RPE-1 centrin-GFP cell lines with small interference RNAs

Cells (2×10^4 cells seeded in 12-well plates) were transfected using Oligofectamine (Invitrogen) with 100 nM of a mixture of four siRNAs from Dharmacon (ON-TARGETplus Duplex; Lafayette, CO, USA) and Ambion (Silencer Select siRNAs; Austin, TX, USA) that silences the expression of human TBCCD1 gene. After either 54 or 66 h of transfection (for the 18 or 6 h post-invasion time points, respectively), purified tachyzoites of *B. besnoiti* or *T. gondii* were added to the transfected cells, both at a multiplicity of infection of 10 parasites cell^{-1}. The experiment was stopped at 72 h of transfection, and coverslips where then processed by immunofluorescence.

Statistical analyses

Statistical analyses were performed using student's *t*-test and one way ANOVA. The software programs SPSS Statistics version 19 and Microsoft Excel 2007 were used.

Invasion and replication assays

For the replication assay, $1·5 \times 10^4$ cells of hTERT-RPE-1, hTERT-RPE-1 over-expressing TBCCD1-GFP and hTERT-RPE-1 transfected with siRNA for TBCCD1, were invaded with $1·5 \times 10^5$ parasites. 18 h after invasion the cells were processed for immunofluorescence with the polyclonal antibodies against *B. besnoiti* and *T. gondii*. The parasite number in each vacuole was counted.

For the invasion assay, $1·5 \times 10^4$ cells of hTERT-RPE-1, hTERT-RPE-1 over-expressing TBCCD1-GFP and hTERT-RPE-1 transfected with siRNA for TBCCD1, were invaded with $1·5 \times 10^5$ parasites. After 1 h, the cells were washed twice with PBS 1× to eliminate non-invaded parasites. After 17 h the cells were processed for immunofluorescence with the polyclonal antibodies against *B. besnoiti* and *T. gondii*. The number of invaded cells *vs* the number of non-invaded cells was counted.

Wound healing assay

hTERT-RPE-1 cells were invaded with *B. besnoiti* and *T. gondii* (10 parasites cell^{-1}). After 18 h of parasite invasion, confluent cells were wounded with a 0·1–10 μL pipette tip, and live image frames from 120, 360 and 500 min of recovery were analysed.

RESULTS

Host microtubule cytoskeleton is differentially rearranged around the PV of B. besnoiti *and* T. gondii *during parasite invasion and replication*

We have previously shown that during the first steps of *B. besnoiti* host cell invasion, the microtubule cytoskeleton of the host cell is playing an active role. From the first minutes of invasion, the host cell microtubules start to interact with the entering parasite to finally surround the entire PV (Reis *et al.* 2006). Similarly, it was also described for *T. gondii* that infected host cells form circular, basket-like structures of microtubules that surround the PV, as soon as 30 min after invasion (Walker *et al.* 2008). To further investigate the involvement of the host microtubule cytoskeleton during *B. besnoiti* host cell invasion in comparison to what was observed for *T. gondii* invasion, a time-course study of the rearrangements of the microtubules of host cells in response to *B. besnoiti* and *T. gondii* invasion was performed by immunofluorescence microscopy (IF). For this, we used RPE-1 and Vero cells, and collected samples of invaded cells at different time points. These invaded cells were then processed for IF using a monoclonal antibody against *α*-tubulin (Fig. 1A and B), whereas parasites inside the host cells were stained with a polyclonal sera against *B. besnoiti* or *T. gondii*. We observed host cell microtubules around the *B. besnoiti* PV after 15 min of invasion (Fig. 1A), suggesting an interaction established early after invasion. As the invasion proceeds and parasites start to replicate, the micro-tubules progressively present a more complex arrangement around the PV (Fig. 1A). In fact, as the number of parasites increases inside the PV, the microtubules create an alveolus-like structure surrounding each parasite, which finally originates a microtubule rosette with an organization resembling that of the parasites inside the vacuole. These structures are clearly seen at 24 h and 30 h after invasion (see zoomed detail of 24 h and 30 h in Fig. 1). The alterations of the host microtubule cytoskeleton during *B. besnoiti* invasion are slightly different from those observed when host cells are invaded by *T. gondii* (Fig. 1B). In fact, and according to what was already described in the literature (Coppens *et al.* 2006; Walker *et al.* 2008), micro-tubules originate a basket-like structure of micro-tubules around the PV, but even for longer times of invasion and higher number of parasites inside the PV (see zoomed detail for 24 and 30 h in Fig. 1B), this microtubule structure does not show the charac-teristic alveolar arrangement of the microtubules surrounding individual *B. besnoiti* inside the PV (Fig. 1A). This suggests that throughout invasion, the PV of both apicomplexan parasites interacts with the host microtubule cytoskeleton, but each of them promote distinct arrangements of these polymers.

It is also important to mention that microtubule rearrangements underlying *B. besnoiti* and *T. gondii* host cell invasion, and parasite replication, are not a cell-type specific process because they were also observed in Vero cells (supplementary Fig. S1).

Host cell centrosome is recruited by T. gondii *but not by* B. besnoiti

The fact that *B. besnoiti* and *T. gondii* host cell invasion and establishment of the PV trigger a response of the host cell microtubules surrounding the PV, led us to investigate if the PV membrane has the ability to nucleate/organize microtubules, being therefore involved in the remodelling of the host microtubule cytoskeleton. Previous studies reported that *T. gondii* infection increases the number of *γ*-tubulin staining foci within the host cell (Walker *et al.* 2008). These foci were described to mainly localize at the PV membrane and were consistent with the normal size of the host MTOC. Due to the differences observed between *T. gondii* and *B. besnoiti* in rearranging the host microtubule cytoskeleton, we investigated if *B. besnoiti* host cell invasion also originates *γ*-tubulin foci in the host cell, able to nucleate microtubules. For this we invaded RPE-1 cells constitutively expressing GFP-centrin, a cen-triolar marker, with *B. besnoiti* (Fig. 2A1 and A2) and *T. gondii* (Fig. 2B1 and B2) for 18 h, and then we depolymerized host cell microtubules by treating cells with nocodazole (30 μM). After this period, nocodazole was removed by washing the cells with fresh medium and microtubules were allowed to recover for 5 and 15 min in order to localize microtubule nucleation sites. Then, these cells were processed for IF using a monoclonal antibody against *γ*-tubulin in order to localize all the sites with ability to nucleate microtubules; and with a monoclonal antibody against *α*-tubulin to label the recovering microtubule cytoskeleton. Either, in the case of *T. gondii* or *B. besnoiti* invaded cells, we were not able to observe the described additional foci of *γ*-tubulin nucleating microtubules at the PV mem-brane (Walker *et al.* 2008), or in their vicinity (Fig. 2).

The fact that, under our conditions, we did not observe that the PV membrane has the ability to recruit *γ*-tubulin and nucleate/polymerize microtu-bules, but *B. besnoiti* and *T. gondii* invasion provokes host microtubule rearrangements during the first steps of host cell invasion (Reis *et al.* 2006; Walker *et al.* 2008; Sweeney *et al.* 2010) and during PV establishment (current work), led us to study the impact of both parasites in the centrosome. In fact, it was conceivable that the manipulation of the centro-some by the parasites could explain the observed microtubule rearrangements during host invasion. Supporting this hypothesis were the data showing that *T. gondii* invasion promotes the detachment of the centrosome from the nuclear envelope, which
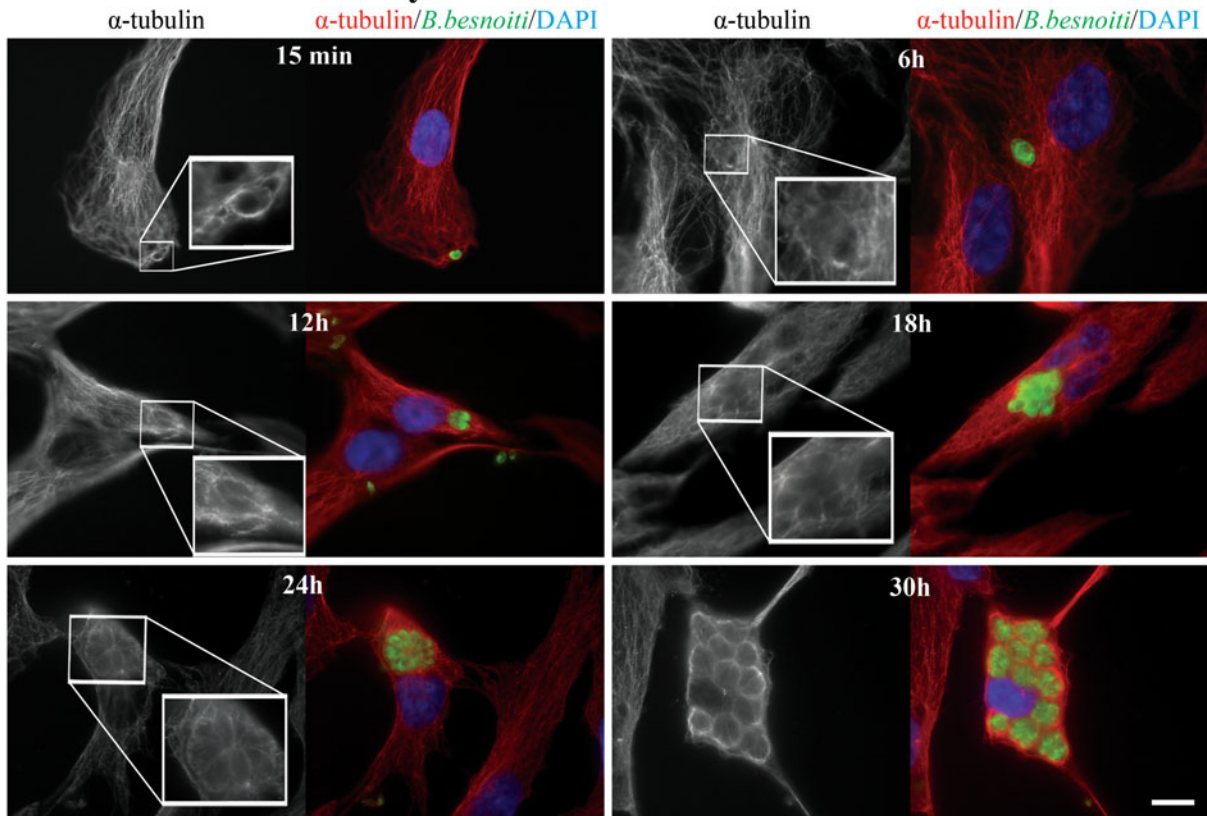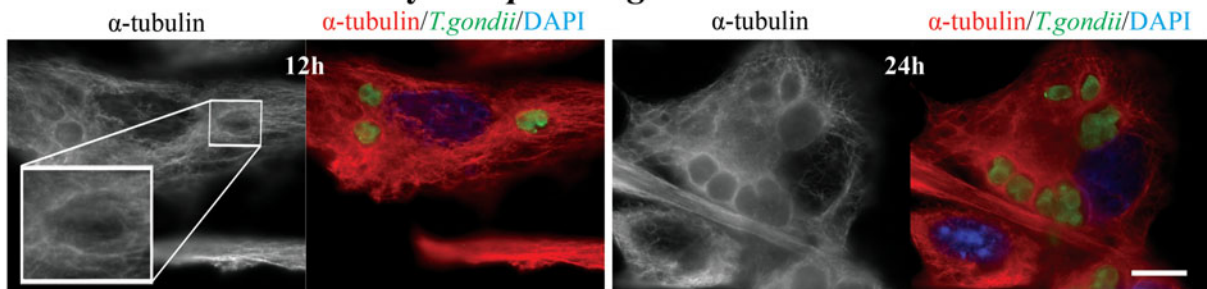
Fig. 1. Host microtubule cytoskeleton rearrangement around the parasitophorous vacuole of *B. besnoiti* or *T. gondii*. Indirect immunolocalization of host cell α-tubulin during invasion by *B. besnoiti* and *T. gondii*. Different time points from 15 min to 30 h of invasion are shown. On the left side, in grey, α-tubulin shows the structure of host cell microtubules, and its organization surrounding the parasitophorous vacuoles (PV). Zoomed areas correspond to detailed views of the close interaction between host cell microtubules and PVs. On the right side α-tubulin is shown in red, parasites in green (polyclonal antibodies against *B. besnoiti* and *T. gondii* were used), and blue for DNA staining with DAPI. (A) *B. besnoiti* invading RPE-1 cells. (B) *T. gondii* invading RPE-1 cells. Scale bars = 7 μm.

further localizes close to the PV (Coppens *et al.* 2006; Walker *et al.* 2008; Wang *et al.* 2010).

To study the impact of *B. besnoiti* on the host cell centrosome we used RPE-1 cells invaded for 6 and 18 h and processed the cells by IF using an antibody against γ-tubulin (to allow the identification of the microtubule nucleating centres, including centrosomes), and the polyclonal antibodies against the parasites. The referred invasion time points were selected due to the fact that they correspond to the stage in which microtubules were found to surround a well-established PV, but replication of the parasite has not occurred yet (6 h, see Fig. 1A); and a more

advanced stage of microtubule reorganization, where the microtubule rosette-like structures start to be evident in *B. besnoiti*-invaded cells (18 h, see Fig. 1A). The results obtained are shown in Fig. 3 and a graphical view is presented in supplementary Fig. S2. In comparison with control, non-invaded RPE-1 cells, we found that RPE-1 cells invaded with *B. besnoiti* for 6 h do not present significant alterations, in respect to γ-tubulin staining or altered localization of the centrosomes. In fact, in a series of three independent experiments we observed that the number of invaded cells with mislocated centrosomes is similar (about $32 \pm 2.6\%$, $n = 230$) to the
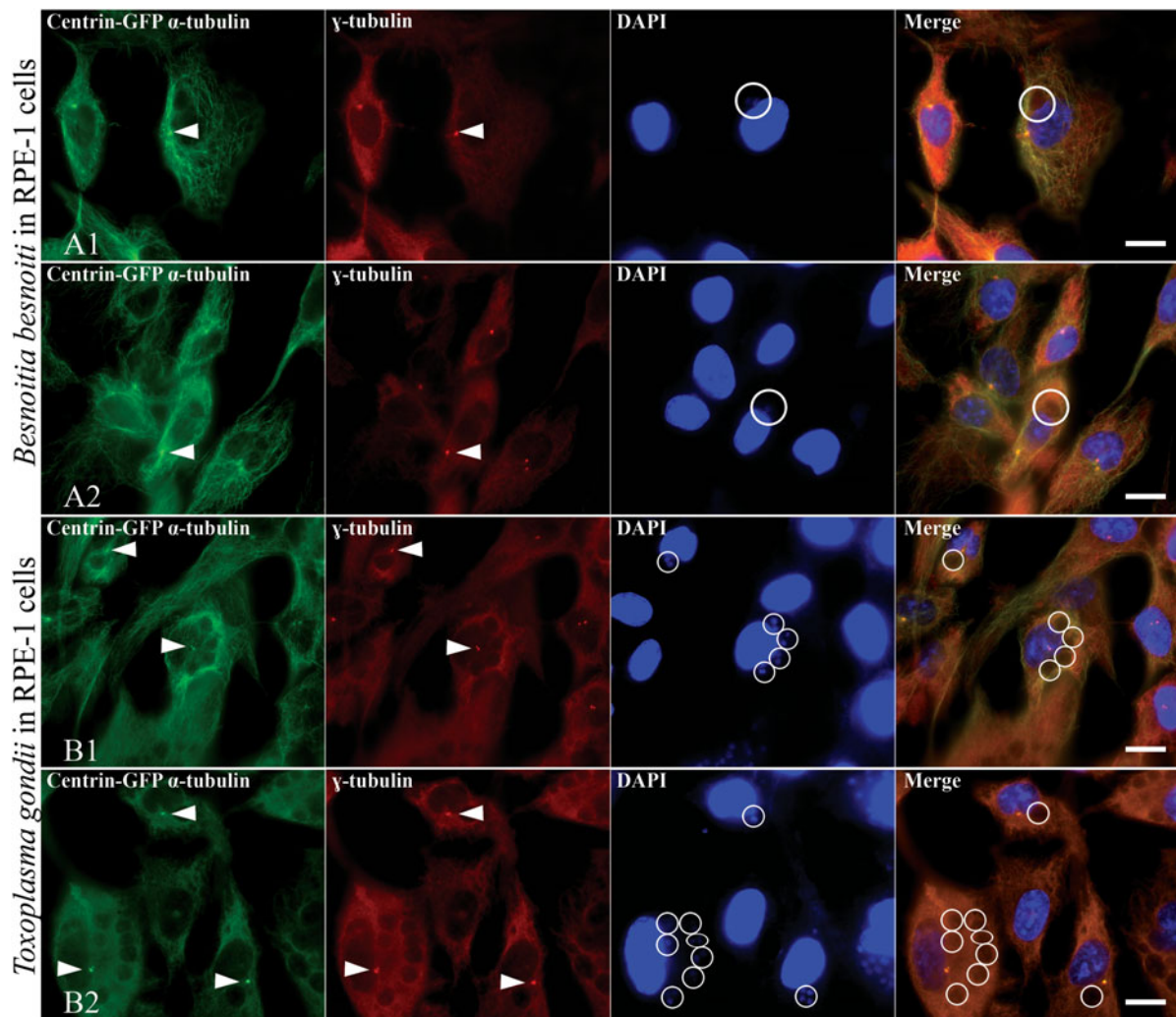
Fig. 2. Investigating the presence of γ-tubulin staining foci additional to the host centrosome in RPE-1 cells invaded by *B. besnoiti* or *T. gondii*. Indirect immunolocalization of *B. besnoiti* and *T. gondii* in RPE-1 cells at 18 h post-invasion and after nocodazole treatment (15 min recovery). (A1 and A2) RPE-1 cells invaded with *B. besnoiti*. (B1 and B2) RPE-1 cells invaded with *T. gondii*. Antibodies against γ-tubulin (red) and α-tubulin (green) were used. RPE-1 cells constitutively express centrin-GFP (green). DNA was stained with DAPI (blue). White circles represent the limits of each parasitophorous vacuole, which can be confirmed by the DAPI staining of the parasites' nuclei. White arrowheads point to the host cell centrosome, which can be seen either by centrin-GFP (green), or in γ-tubulin staining (red). No other γ-tubulin foci were detected neither in *B. besnoiti* nor in *T. gondii* invaded cells. Scale bars = 7 μm.

non-invaded RPE-1 cells ($30 \pm 7.7\%$, $n = 354$). At a later stage (18 h post-invasion) there was only a slight increase of mislocated centrosomes (from $30 \pm 7.7\%$, $n = 354$, in non-invaded RPE-1 cells, to $40 \pm 1.2\%$, $n = 452$, in RPE-1 invaded by *B. besnoiti*), and this mislocation represents a slight increase in the average nucleus-centrosome distance, from $2 \pm 1.2\,\mu m$ ($n = 307$) to $2.3 \pm 1.7\,\mu m$ ($n = 167$), which is not statistically significant (student's *t* test; measurement exemplified in Fig. 3B). Again γ-tubulin staining was observed only at the centrosomes (Fig. 3). Therefore, and contrary to *T. gondii* (Coppens *et al*. 2006; Walker *et al*. 2008; Wang *et al*. 2010), host cell invasion by *B. besnoiti* does not seem to require any significant alteration of the localization of the host cell centrosome. In this context, and to withdraw any hypothesis that the results obtained for *B. besnoiti*

invasion were a consequence of our experimental design or manipulation, we also investigated if in our host cell invasion experimental conditions *T. gondii* was able to recruit the centrosome towards the PV. These experiments confirmed that RPE-1 cells at 18 h post-invasion with *T. gondii* present a clear increase of mislocated centrosomes (far from the nucleus – $70 \pm 2.8\%$, $n = 727$), without affecting centrosomal γ-tubulin staining, in comparison to non-invaded RPE-1 cells (control cells; $30 \pm 7.7\%$, $n = 354$). Moreover, the distance of nucleus–centrosome increases from $2 \pm 1.2\,\mu m$ ($n = 307$) in non-invaded RPE-1 cells to $5 \pm 3.2\,\mu m$ ($n = 405$) in invaded cells, a statistically significant difference (student's *t* test, $P < 0.005$), in agreement with the results described by others (Coppens *et al*. 2006; Walker *et al*. 2008; Wang *et al*. 2010). Analysis of
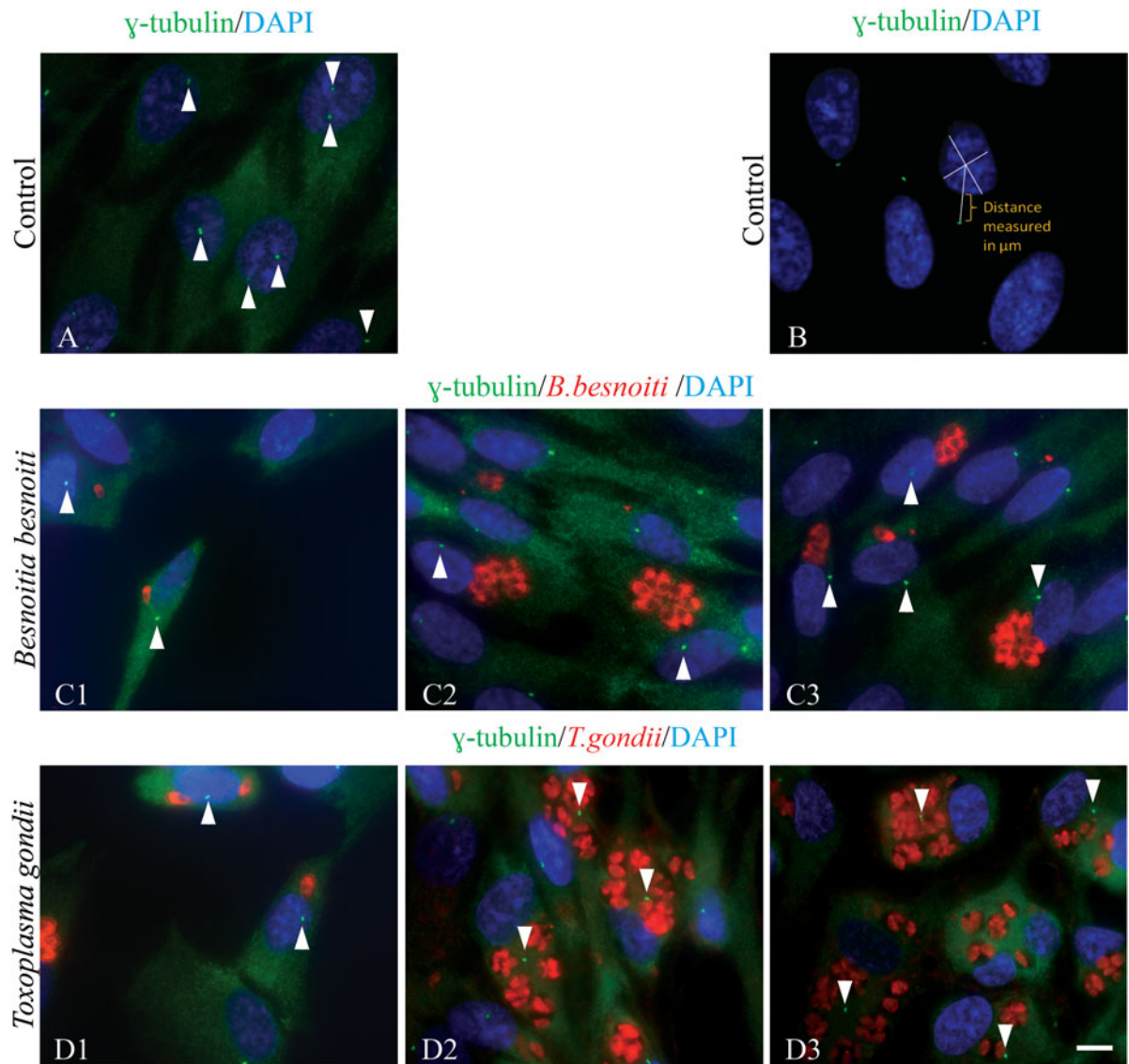
Fig. 3. Host centrosome–nucleus position in RPE-1 host cells invaded with *T. gondii* or *B. besnoiti*. Indirect immunolocalization of *T. gondii* and *B. besnoiti* invading RPE-1 cells. Antibodies against γ-tubulin, *B. besnoiti* (polyclonal antibody) and *T. gondii* (polyclonal antibody) were used. DNA was stained with DAPI. The position of the centrosomes can be seen in green (γ-tubulin staining), in relation to the position of parasites in each parasitophorous vacuole (red) and host cell nucleus (blue). Arrowheads indicate the host centrosome positioning. (A) RPE-1 cells, non-invaded control. (B) IF imaging showing how the distance between the host cell nucleus and centrosome was measured using ImageJ software (centrosome-green; nucleus-blue). (C1) *B. besnoiti*, 6 h post-invasion. (C2 and C3) *B. besnoiti*, 18 h post-invasion – the position of the centrosome is maintained close to the host cell nucleus. (D1) *T. gondii*, 6 h post-invasion. (D2 and D3) *T. gondii*, 18 h post-invasion – note the displacement of the centrosomes away from the nuclei and closer to the parasitophorous vacuoles. Scale bar=7 μm.

the data obtained for 6 h after *T. gondii* host cell invasion shows that the recruitment of the centrosome by this parasite has not been established yet (Fig. S2A). This suggests that the phenomenon is more relevant during PV development and parasite replication than in the first steps of invasion. Additionally, the results obtained validate our experimental system for *B. besnoiti*, showing that, despite the fact that both parasites recruit host cell microtubules to the PV, only *T. gondii* consistently displaces the centrosome from the host cell nucleus to the PV membrane.

Golgi recruitment in RPE-1 cells invaded by B. besnoiti *and* T. gondii

It is now well established that centrosome/ microtubules are involved in the organization and positioning of the pericentrosomal Golgi ribbon (Bornens, 2012), and that the Golgi apparatus and centrosome are functionally linked (Sütterlin and Colanzi, 2010). Also, it has already been demonstrated that Golgi membranes are able to nucleate microtubules (Efimov *et al*. 2007; Rivero *et al*. 2009). The fact that only *T. gondii* recruits the centrosome
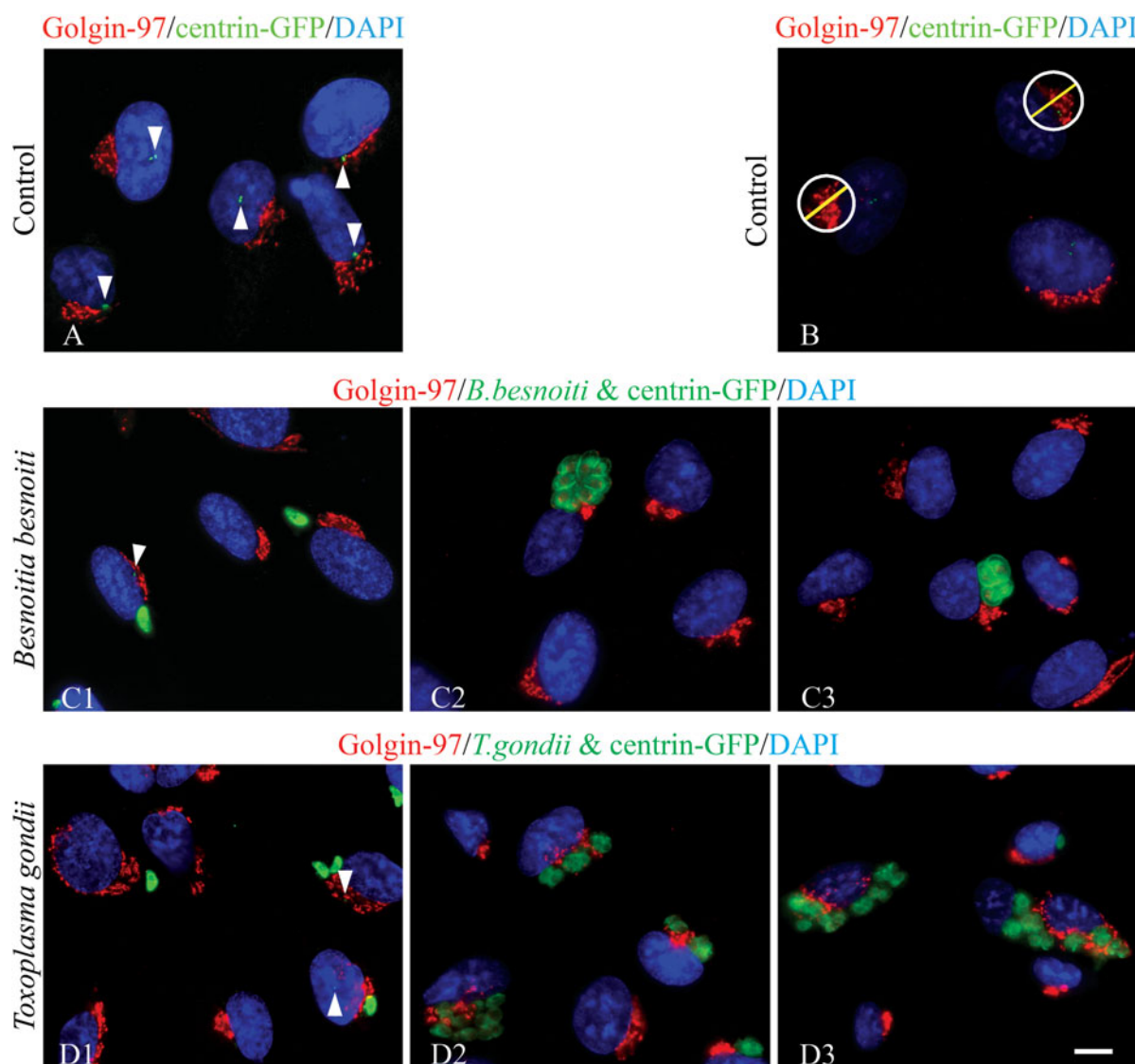
Fig. 4. *Besnoitia besnoiti* and *T*. *gondii* recruit Golgi apparatus in invaded RPE-1 cells. Indirect immunolocalization of the Golgi complex in *B*. *besnoiti*- and *T*. *gondii*-invaded RPE-1 cells. Antibodies used were *B*. *besnoiti* polyclonal antibody (green), *T*. *gondii* polyclonal antibody (green) and anti-Golgin 97 (red). DNA was stained with DAPI (blue). (A) RPE-1 cells constitutively expressing centrin-GFP, non-invaded control. (B) IF imaging showing how the measurement of Golgi diameter was performed in non-invaded RPE-1 host cells using ImageJ software (Golgi-red; centrosome-green; nucleus-blue). (C1) *B*. *besnoiti*, 6 h post-invasion. (C2 and C3) *B*. *besnoiti*, 18 h post-invasion. (D1) *T*. *gondii*, 6 h post-invasion. (D2 and D3) *T*. *gondii*, 18 h post-invasion. The relative position of the Golgi apparatus to the centrosome can be seen in A, C1 and D1, through centrin-GFP (green – white arrowheads). In red, Golgi apparatus of the invaded host cells is consistently close and around the parasitophorous vacuole of both parasites. During *T*. *gondii* invasion the Golgi ribbon is completely fragmented. Scale bar=7 μm.

to the PV, but both parasites cause the reorganization of the host microtubule cytoskeleton, leads us to hypothesize that this could also be related to different strategies for Golgi apparatus recruitment during host infection. To test this hypothesis, RPE-1 and RPE-1 cells constitutively expressing GFP-centrin were invaded for 6 h (Fig. 4C1 and D1) and 18 h (Fig. 4C2, C3, D2 and D3) by *B*. *besnoiti* and *T*. *gondii*. The cells were processed for IF to assess the position and integrity of the Golgi apparatus in relation to the PV. For this we have used a monoclonal antibody against Golgin-97, a Golgi marker, and polyclonal antibodies against *B*. *besnoiti*

and *T*. *gondii*. The position of the Golgi apparatus in relation to the centrosome was determined through GFP-centrin (Fig. 4C1 and D1).

Notably, and contrary to what was observed for the centrosome recruitment, both parasites seem to preferentially establish the PV close to the host cell Golgi apparatus, which was consistently found close/around the PV in invaded RPE-1 cells (Fig. 4). However, a more detailed observation revealed that both parasites affect Golgi apparatus organization differently. The establishment of the PV by *B*. *besnoiti* parasites close to the Golgi apparatus, in the majority of the cases, does not seem to cause

fragmentation/disorganization, but instead to induce Golgi apparatus compaction (Fig. 4C1–C3). On the contrary, *T. gondii* tends to cause fragmentation and dispersion of the Golgi apparatus (Fig. 4D1–D3) which is in agreement with the data in the literature (Coppens *et al*. 2006; Walker *et al*. 2008). Indeed, in two independent experiments, at 18 h post-invasion by *T. gondii*, $57 \cdot 1 \pm 2 \cdot 5\%$ ($n = 298$) of the invaded cells present a fragmented Golgi apparatus, while only $15 \cdot 9 \pm 4 \cdot 1\%$ ($n = 302$) of cells invaded with *B. besnoiti* show a dispersed Golgi (value close to RPE-1 non-invaded control cells – $14 \cdot 8 \pm 2 \cdot 4\%$, $n = 366$) (Fig. S3B). To better analyse and quantify these observations we measured the Golgi apparatus diameter, as shown in Fig. 4B. We observed that at 18 h of invasion the Golgi diameter for cells invaded by *T. gondii* ($11 \cdot 3 \pm 4 \cdot 9 \, \mu$m, $n = 168$) was not significantly different from that of non-invaded cells ($9 \cdot 8 \pm 4 \cdot 6 \, \mu$m, $n = 264$), while in cells invaded by *B. besnoiti* the Golgi diameter ($7 \cdot 4 \pm 3 \cdot 2 \, \mu$m, $n = 162$) shows a statistically significant decrease in comparison to the value found in control cells ($9 \cdot 8 \pm 4 \cdot 6 \, \mu$m; one way ANOVA, $P < 0 \cdot 05$) (Fig. S3D).

It is also important to note that while there is no obvious recruitment of the centrosome by any of the parasites at 6 h of invasion (Fig. 3), in what refers to the Golgi apparatus, at 6 h of invasion it is already detected in the proximity of the *T. gondii* and *B. besnoiti* PVs (Fig. 4C1 and D1). Also, in the case of *T. gondii*, $33 \cdot 6 \pm 1 \cdot 6\%$ ($n = 283$) of invaded cells already showed a fragmented Golgi apparatus (Fig. S3A).

The impact of B. besnoiti *and* T. gondii *invasion on host cell centrosome and Golgi apparatus recruitment in cells depleted of the centrosomal protein TBCCD1*

Because both *T. gondii* and *B. besnoiti* parasites recruit the Golgi apparatus during host cell invasion, but only *T. gondii* associates this with the simultaneous recruitment of the centrosome, we decided to investigate if in this case both events were linked. If this was so, then *B. besnoiti* should have a different mechanism to recruit the Golgi apparatus that does not require the recruitment of the centrosome. For this we studied the ability of *T. gondii* and *B. besnoiti* to invade and replicate in RPE-1 cells in the background of the depletion of the centrosomal protein TBCC-domain containing 1 (TBCCD1). We have recently reported that the knockdown of TBCCD1, a protein related to tubulin cofactor C involved in the tubulin folding pathway, in RPE-1 cells, causes the displacement of the centrosome from the nucleus and disorganization of the Golgi apparatus (Gonçalves *et al*. 2010*a*). However, the major microtubule nucleating activity of the centrosome is not affected by TBCCD1 silencing. Therefore, we also investigated the ability of both parasites to recruit the centrosome and Golgi apparatus in a host cell that already has mispositioned centrosomes and disorganized Golgi apparatus.

Despite the misplacement of the centrosome caused by the TBCCD1 siRNA treatment, the PV of *B. besnoiti* is in close association to the nucleus, and continues to be surrounded by host microtubules (Fig. S4) in a similar way to that observed in invaded RPE-1 WT cells (Fig. 1A). Additionally, no obvious association to the mislocated centrosome was observed (Fig. 5B1–B3). In the case of *T. gondii* the recruitment of the centrosome is no longer obvious, as it was in invaded RPE-1 WT cells (Fig. 5C1–C3). Notably, the mispositioning of the centrosome in these cells does not affect the efficiency of invasion (data not shown), nor replication (Fig. 6A and B) of *B. besnoiti* and *T. gondii*, as shown by the results of a series of two independent assays.

Concerning the Golgi apparatus organization and recruitment by the two parasites, we observed that in TBCCD1 siRNA-treated RPE-1 cells invaded for 6 h (Fig. 7C and D) and 18 h (Fig. 7E–H) by *B. besnoiti* and *T. gondii*, the PVs are in close association with Golgi elements that sometimes completely surround the PV. Unexpectedly, in *B. besnoiti*-invaded cells, after 18 h of invasion, there was an accentuated decrease in the percentage of cells presenting Golgi apparatus fragmentation/dispersion ($36 \cdot 4 \pm 1 \cdot 9\%$, $n = 168$) in comparison to control cells ($78 \cdot 1 \pm 4\%$, $n = 220$). These scores were obtained from two independent experiments (see Fig. 7B for the measurement approach). This is an early phenomenon, as it can already be detected at 6 h post-invasion. Although in these cells the Golgi diameter is greatly increased since it is dispersed throughout the cytoplasm, at 18 h of invasion we can find a statistically significant difference (one way ANOVA, $P < 0 \cdot 05$) between Golgi diameter in siRNA non-invaded cells ($29 \cdot 9 \pm 7 \cdot 4 \, \mu$m, $n = 138$) and siRNA cells invaded by *B. besnoiti* ($19 \cdot 4 \pm 7 \cdot 3 \, \mu$m, $n = 109$). No difference is found in terms of Golgi diameter for TBCCD1 siRNA treated cells invaded by *T. gondii*, neither for 6 h of invasion ($29 \pm 6 \cdot 8 \, \mu$m, $n = 163$) nor for 18 h of invasion ($28 \cdot 8 \pm 7 \cdot 5 \, \mu$m, $n = 117$). These results (summarized in Fig. S3) show that, contrary to what is observed in *T. gondii* invaded cells, *B. besnoiti* invasion causes the compaction of the Golgi apparatus, which is already observable in RPE-1 WT cells (Fig. 4), and becomes more obvious in TBCCD1 siRNA cells. Consequently, *B. besnoiti* invasion seems to rescue Golgi from the disorganization and dispersion provoked by TBCCD1 depletion.

Host cell centrosome recruitment in RPE-1 cells over-expressing TBCCD1 by B. besnoiti *and* T. gondii

Taking into account the role of TBCCD1 in nucleus-centrosome connection, and the results observed during *B. besnoiti* and *T. gondii* invasion of RPE-1
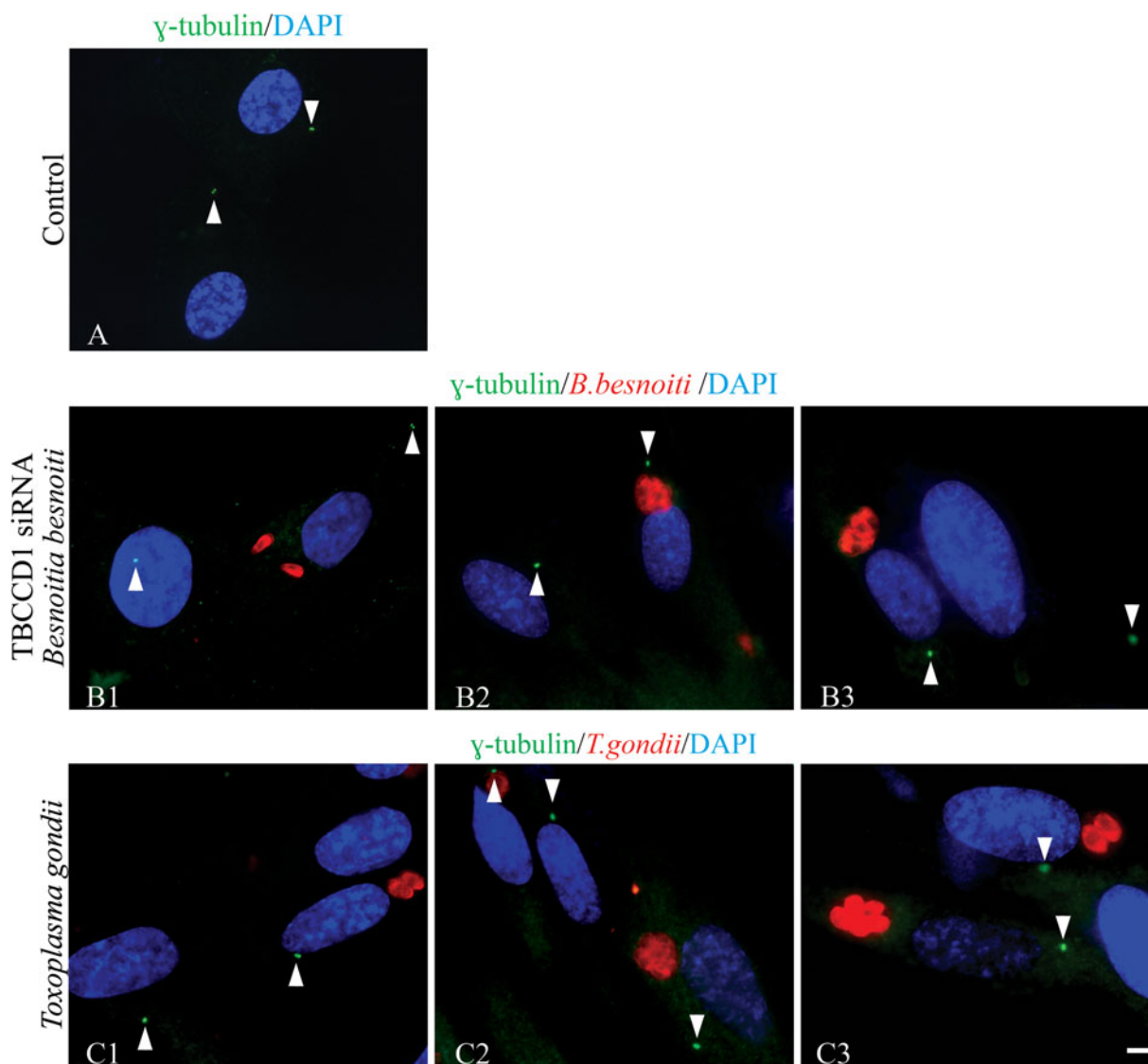
Fig. 5. Mislocated host centrosomes caused by TBCCD1 knockdown are not recruited neither by *B. besnoiti* nor by *T. gondii*. Indirect immunolocalization of *T. gondii* and *B. besnoiti* invading TBCCD1 siRNA RPE-1 cells. Antibodies were used against γ-tubulin, *B. besnoiti* (polyclonal antibody), and *T. gondii* (polyclonal antibody). DNA was stained with DAPI. The position of the centrosomes can be seen in green (γ-tubulin staining – white arrowheads), in relation to the position of each parasitophorous vacuole (red) and host cell nucleus (blue). (A) TBCCD1 siRNA RPE-1 cells, non-invaded control. (B1) *B. besnoiti*, 6 h post-invasion. (B2 and B3) *B. besnoiti*, 18 h post-invasion. (C1) *T. gondii*, 6 h post-invasion. (C2 and C3) *T. gondii*, 18 h post-invasion. In RPE-1 cells depleted of TBCCD1 the parasitophorous vacuoles of both parasites are not associated with host cell centrosomes. Scale bar=7 μm.

cells with decreased TBCCD1 levels, we addressed the capability of the two parasites to recruit the centrosome in RPE-1 over-expressing TBCCD1 at 6 h (Fig. 8B1 and C1) and 18 h (Fig. 8B2, B3, C2 and C3) of host cell invasion. RPE-1 cells over-expressing TBCCD1 do not present an obvious phenotype related to centrosome positioning (Fig. 8A). In the case of *B. besnoiti*, in what concerns host centrosome recruitment, the invasion of RPE-1 cells in the background of TBCCD1 over-expression does not significantly differ from that of WT RPE-1 invasion (Fig. 8B1–B3). In fact, in three independent experiments (Fig. S2), there is only a small increase of mislocated centrosomes, at 18 h of host cell invasion, from $23 \pm 12\%$ ($n = 445$) in non-invaded RPE-1 cells

over-expressing TBCCD1, to $39 \pm 12\%$ ($n = 350$) when these cells are invaded by *B. besnoiti*. As for the distance between the nucleus and the mislocated centrosomes at 18 h of invasion, the difference is not statistically significant (student's t test), from $1 \cdot 4 \pm 0 \cdot 8 \, \mu$m ($n = 199$) in non-invaded cells to $1 \cdot 7 \pm 1 \, \mu$m ($n = 129$) in invaded cells. As expected from these results, *B. besnoiti* shows similar rates of invasion (data not shown) and replication (Fig. 6C) in WT and over-expressing TBCCD1 RPE-1 cell lines.

Interestingly, we observed that after 18 h of invasion, the distance between the nucleus and mislocated centrosomes in *T. gondii*-invaded RPE-1 cells over-expressing TBCCD1 ($1 \cdot 6 \pm 1 \cdot 2 \, \mu$m, $n = 167$) is reduced in comparison to that measured
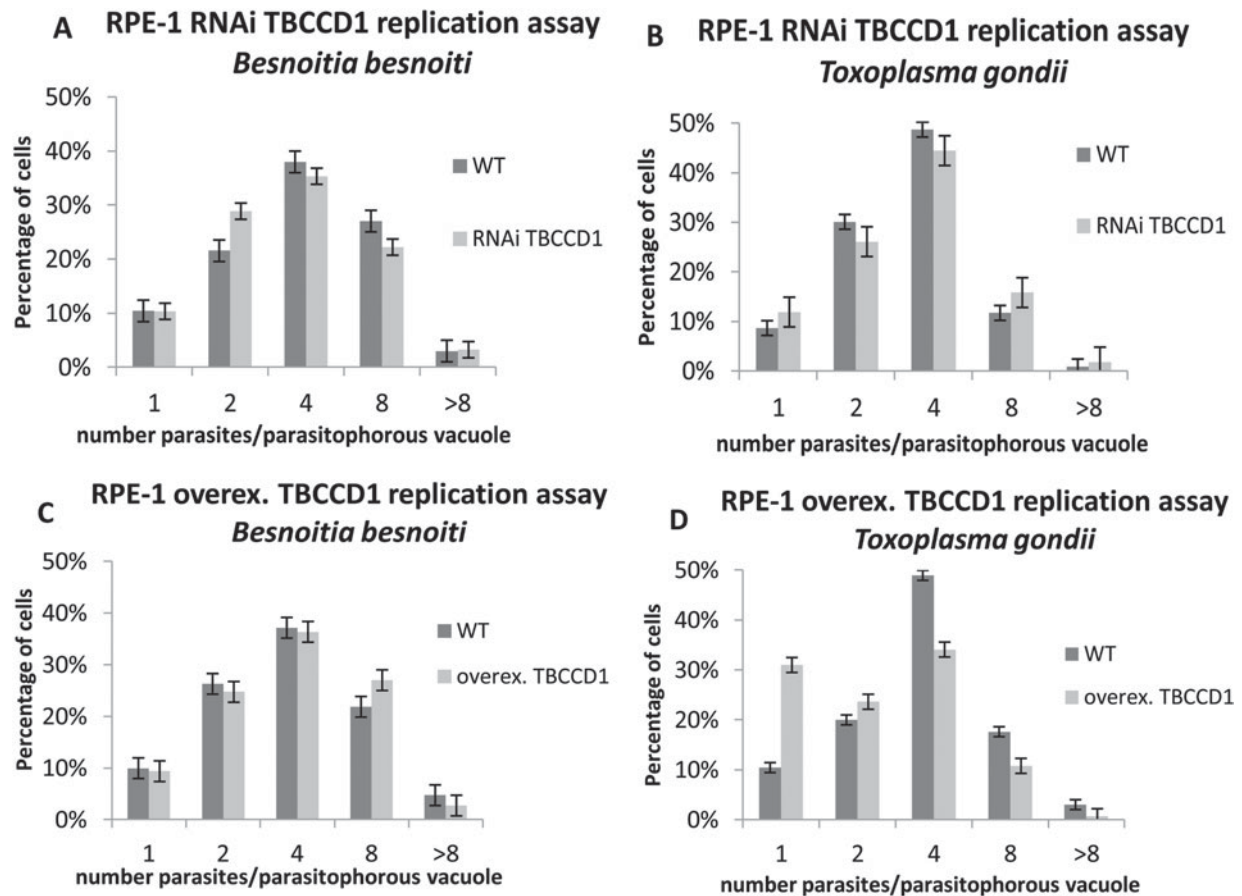
Fig. 6. (A) Graphic representing *B. besnoiti* replication assays in cells RPE-1 RNAi for TBCCD1. (B) Graphic representing *T. gondii* replication assays in cells RPE-1 RNAi for TBCCD1. (C) Graphic representing *B. besnoiti* replication assays in cells RPE-1 over-expressing TBCCD1. (D) Graphic representing *T. gondii* replication assays in cells RPE-1 over-expressing TBCCD1. The mean percentage of cells ($\pm$ S.D.) in two independent experiments is shown ($527 < n < 794$).

in WT-invaded RPE-1 cells ($5 \pm 3 \cdot 2\,\mu m$, $n = 405$), and the value is very similar to that found in RPE-1 WT non-invaded cells ($2 \cdot 1 \pm 1 \cdot 3\,\mu m$, $n = 307$). Additionally, the proportion of *T. gondii*-invaded RPE-1 cells over-expressing TBCCD1 presenting mislocation of the centrosome decreases to $40 \pm 8 \cdot 2\%$ ($n = 518$), in comparison to the percentage values found in RPE-1 WT cells invaded for the same period of time ($70 \pm 2 \cdot 8\%$, $n = 727$). These results (summarized in Fig. S2) show that *T. gondii* parasites have a greater difficulty to recruit the host cell centrosome in RPE-1 cells over-expressing TBCCD1 than in RPE-1 WT cells. This suggests that normally *T. gondii* directly/indirectly manipulates the host cell system of factors involved in centrosome–nucleus connection, as for example TBCCD1. The question, still, is why *T. gondii* invasion/infection requires the recruitment of the host centrosome towards the PV. Therefore, we investigated the ability of *T. gondii* to invade and replicate in RPE-1 cells over-expressing TBCCD1. The results of the replication assay, and the number of parasites in each PV, are presented in Fig. 6D. Notably, *T. gondii* replication is delayed in RPE-1 cells over-expressing TBCCD1 when compared with WT RPE-1 cells. This observation

indicates that *T. gondii* replication requires an efficient recruitment of the host centrosome, and that it is able to manipulate the molecular mechanisms involved in the nucleus–centrosome connection. In the case of the invasion assay, no significant differences were found between *T. gondii* invasion of RPE-1 WT cells and RPE-1 cells over-expressing TBCCD1 (data not shown). Altogether the results show again that *B. besnoiti* and *T. gondii* parasites control different host molecular pathways during the establishment of invasion.

Golgi recruitment and organization in RPE-1 cells over-expressing TBCCD1 invaded by B. besnoiti *and* T. gondii

The overall appearance of Golgi apparatus in RPE-1 cells over-expressing TBCCD1 (Fig. 9A) is similar to that of RPE-1 WT cells (Fig. 4A). However, since we observed that *T. gondii* has an increased difficulty in recruiting the centrosome in these cells, it was interesting to investigate if this would also reflect any differences in the relationship between the Golgi apparatus organization and localization, and the PV, in comparison to RPE-1 WT-invaded cells.
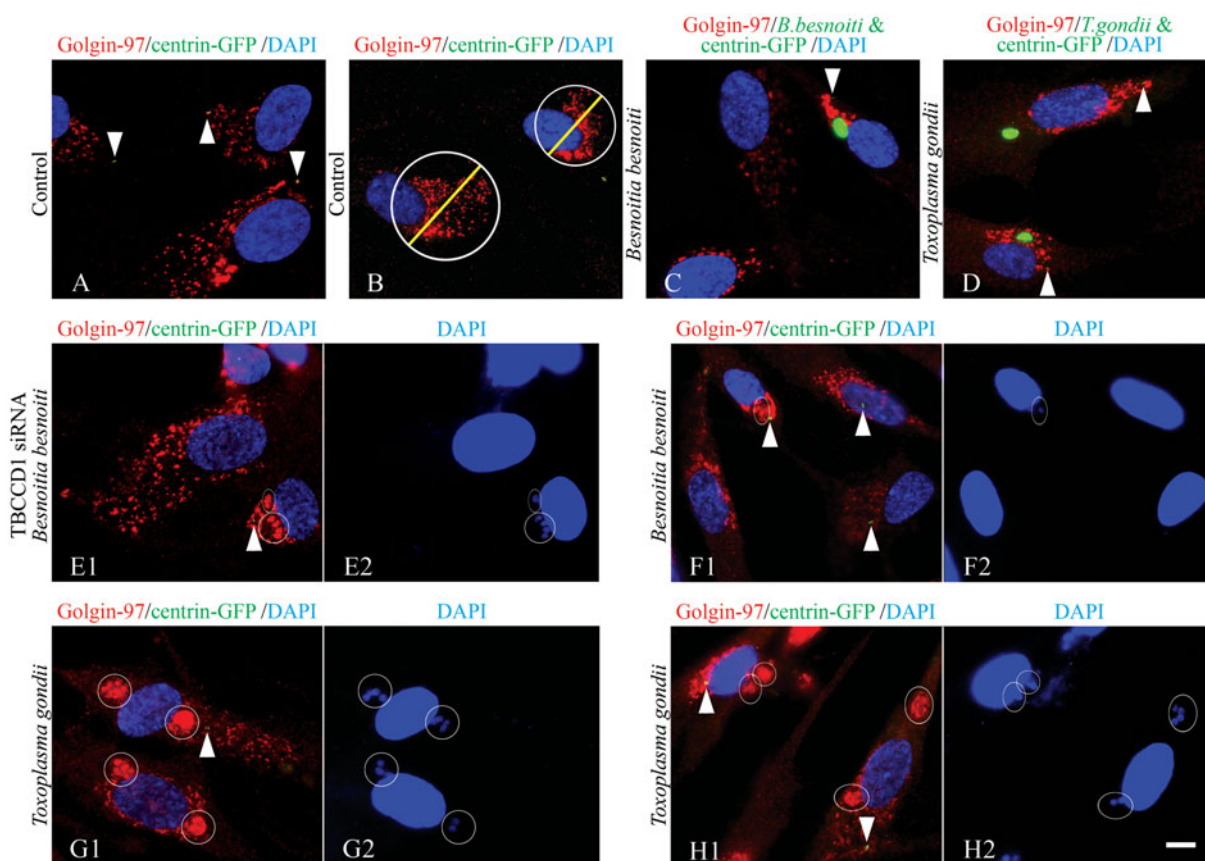
Fig. 7. Golgi apparatus organization in RPE-1 cells depleted of TBCCD1 and invaded by *B. besnoiti* or *T. gondii*. Indirect immunolocalization of host cell Golgi complex in *B. besnoiti* and *T. gondii* invaded TBCCD1 siRNA RPE-1 cells constitutively expressing centrin-GFP. Antibodies used were anti-Golgin 97 (red), *B. besnoiti* polyclonal antibody (in image C-green); and *T. gondii* polyclonal antibody (in image D-green). DNA was stained with DAPI (blue). The relative position of the Golgi apparatus to the centrosome can be determined by centrin-GFP labelling (green – white arrowheads). In images E1, F1, G1 and H1, white circles represent the limits of each parasitophorous vacuole, which can be confirmed by the DAPI staining of parasites nuclei on the right image (E2, F2, G2 and H2), and by the red staining of the parasite Golgi apparatus with anti-Golgin 97. (A) TBCCD1 siRNA RPE-1 cells constitutively expressing GFP-centrin, non-invaded control. In these cells the Golgi ribbon is fragmented and dispersed throughout the cytoplasm. (B) IF imaging showing how the measurement of Golgi diameter was performed in non-invaded RPE-1 host cells treated with siRNA TBCCD1 using ImageJ software (Golgi-red; centrosome-green; nucleus-blue). (C) *B. besnoiti*, 6 h post-invasion. Golgi apparatus is close to the parasitophorous vacuole, and shows less fragmentation than in the non-invaded control cells. (D) *T. gondii*, 6 h post-invasion. Golgi apparatus is fragmented and close to *T. gondii* parasitophorous vacuoles. (E and F) *B. besnoiti*, 18 h post-invasion. Golgi apparatus of the invaded host cells is consistently more compact (when compared with the control cells, in (A)) and close to the parasitophorous vacuoles. (G and H) *T. gondii*, 18 h post-invasion. Note that Golgi apparatus is completely fragmented throughout the host cell cytoplasm. Scale bar=7 μm.

Thus, RPE-1 cells over-expressing TBCCD1 were invaded with *T. gondii* and *B. besnoiti*, and the invasion was stopped at 6 h (Fig. 9B1 and C1) and 18 h (Fig. 9B2, B3, C2 and C3). Comparing the results shown in Figs 4 and 9 it is clear that over-expressing TBCCD1 does not cause any difference in Golgi recruitment by *T. gondii* or by *B. besnoiti*. In a series of two independent experiments (Fig. S3A, B), we observed that at 18 h of invasion by *T. gondii*, RPE-1 cells over-expressing TBCCD1 present $43 \cdot 9 \pm 4 \cdot 1\%$ ($n = 253$) of the invaded cells with a fragmented Golgi apparatus, in comparison to the $57 \cdot 1 \pm 2 \cdot 5\%$ ($n = 298$) of the invaded cells with a fragmented Golgi apparatus in WT RPE-1 cells, and the non-invaded RPE-1 cells over-expressing

TBCCD1 ($15 \pm 2 \cdot 5\%$, $n = 261$). The observed small decrease in the percentage of *T. gondii*-invaded RPE-1 cells over-expressing TBCCD1 that contain a fragmented Golgi apparatus, may be related with a more stable pericentrosomal region provided by higher levels of TBCCD1. In the case of RPE-1 cells over-expressing TBCCD1 invaded by *B. besnoiti*, a percentage of $14 \cdot 5 \pm 3 \cdot 7\%$ ($n = 257$), similar to that in non-invaded RPE-1 cells over-expressing TBCCD1 ($15 \cdot 2 \pm 2 \cdot 5\%$, $n = 261$), and to that in invaded RPE-1 WT cells ($15 \cdot 9 \pm 4 \cdot 1\%$, $n = 302$), was found.

The Golgi diameter parameter was also evaluated in the background of TBCCD1 over-expression during invasion by both parasites (Fig. S3C, D).
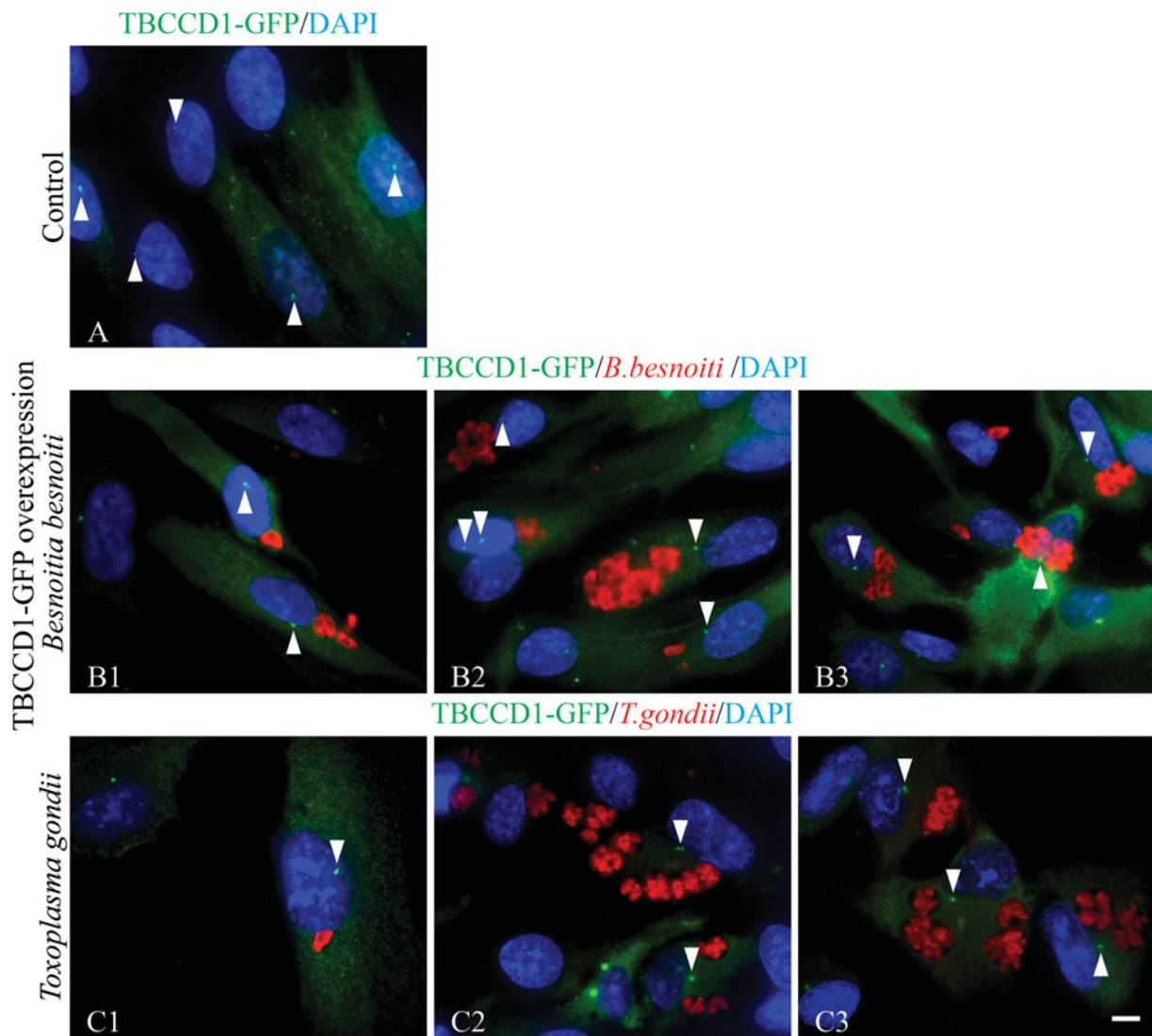
Fig. 8. Host centrosome–nucleus position in RPE-1 host cells over-expressing TBCCD1-GFP invaded with *T. gondii* or *B. besnoiti*. Indirect immunolocalization of *T. gondii* and *B. besnoiti* invading RPE-1 cells over-expressing TBCCD1-GFP. Antibodies against *B. besnoiti* (polyclonal antibody) and *T. gondii* (polyclonal antibody) were used. DNA was stained with DAPI. The position of the centrosomes is identified by TBCCD1-GFP (green – white arrowheads), in relation to the position of each parasitophorous vacuole (red) and host cell nucleus (blue). (A) RPE-1 cells, non-invaded control. (B1) *B. besnoiti*, 6 h post-invasion. (B2 and B3) *B. besnoiti*, 18 h post-invasion. (C1) *T. gondii*, 6 h post-invasion. (C2 and C3) *T. gondii*, 18 h post-invasion. Scale bar=7 μm.

In RPE-1 over-expressing TBCCD1 cells, in two independent experiments, at 18 h, cells invaded by *B. besnoiti* have a statistically significant decrease in Golgi apparatus diameter ($6 \cdot 9 \pm 3 \, \mu$m, $n = 149$), when compared with non-invaded cells over-expressing TBCCD1 ($10 \cdot 3 \pm 4 \, \mu$m, $n = 168$; one-way ANOVA, $P < 0 \cdot 05$). In the case of cells invaded by *T. gondii*, the Golgi apparatus diameter does not differ ($10 \pm 4 \, \mu$m, $n = 153$) from that of non-invaded cells ($10 \cdot 3 \pm 4 \, \mu$m, $n = 168$).

The impact of B. besnoiti *and* T. gondii *invasion in RPE-1 cells migration*

Considering the differences described above in the recruitment of the host cell centrosome by the PVs of the two parasites, and the known involvement of the centrosome in cell motility, we compared the effects of *T. gondii* and *B. besnoiti* in the ability of cells to migrate, by performing wound-healing assays.

For this purpose RPE-1 WT cells were grown in glass coverslips and then invaded for 18 h by either *T. gondii* or *B. besnoiti*. Confluent RPE-1 WT cells were then wounded with a micropipette tip, and the wound closing was followed. At minute 0, the proportion of invaded cells in the monolayer was similar in both experiments for the two parasites: $17 \cdot 85 \pm 4 \cdot 5\%$ in the experiments with *B. besnoiti*, and $16 \cdot 55 \pm 2\%$ in the experiments with *T. gondii*. Images were then captured at 120, 360 and 500 min of recovery and showed that cells invaded with *T. gondii*, in contrast to those invaded with
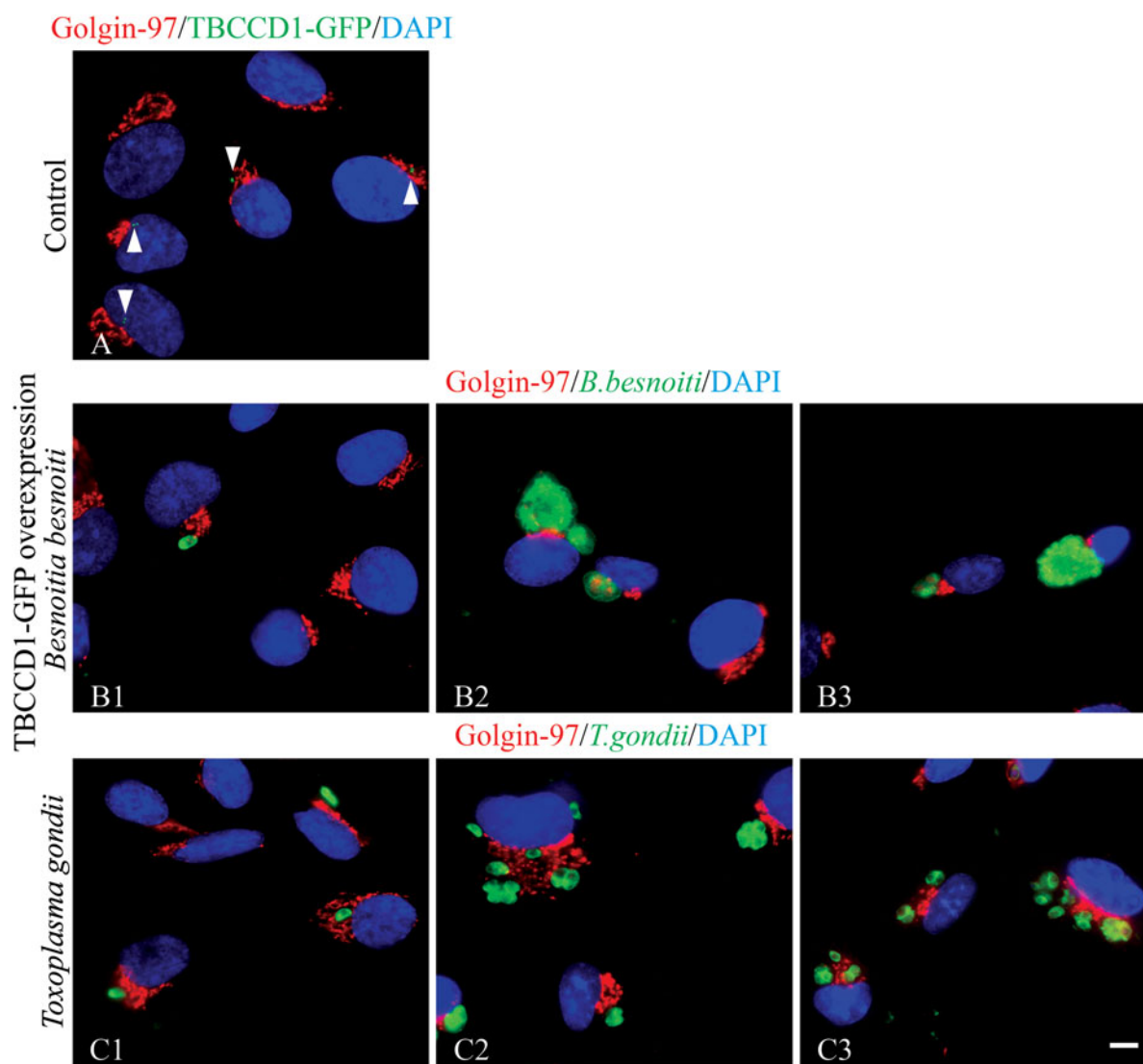
Fig. 9. Golgi apparatus organization in RPE-1 cells over-expressing TBCCD1-GFP and invaded by *B. besnoiti* or *T. gondii*. Indirect immunolocalization of the Golgi complex in *B. besnoiti* and *T. gondii* invaded RPE-1 cells over-expressing TBCCD1-GFP. Antibodies used were *B. besnoiti* polyclonal antibody (green), *T. gondii* polyclonal antibody (green), and anti-Golgin 97 (red). DNA was stained with DAPI (blue). (A) RPE-1 cells over-expressing TBCCD1-GFP (green – white arrowheads), non-invaded control. (B1) *B. besnoiti*, 6 h post-invasion. (B2 and B3) *B. besnoiti*, 18 h post-invasion. (C1) *T. gondii*, 6 h post-invasion. (C2 and C3) *T. gondii*, 18 h post-invasion. In red, Golgi apparatus of the invaded host cells is consistently close and around the parasitophorous vacuole of both parasites. In *B. besnoiti* invaded cells, the Golgi ribbon seems to be more compact, whereas in *T. gondii* invasion is fragmented, when compared with non-invaded cells. Scale bar=7 μm.

B. besnoiti, present a significant delay in wound closing in comparison to control cells. The difference is visible at 500 min when control cells almost closed the wound, which in the case of cells invaded by *T. gondii* is still visible (Fig. 10).

To try to establish a relationship between the delay of wound closing in invaded cells and the ability of *T. gondii* to recruit the centrosome to the vicinity of the PV, we studied the orientation of the centrosome and Golgi apparatus in invaded and non-invaded cells at the leading edge of a closing wound. In fact, it has been described in several different cell types that the centrosome reorients towards the leading edge when cells are stimulated to migrate (Gomes *et al*. 2005;

Etienne-Manneville, 2008; Schmoranzer *et al*. 2009; Vinogradova *et al*. 2009). Therefore, we observed by IF invaded and non-invaded RPE-1 cells in the periphery of the wound edge stained with an antibody against γ-tubulin (Fig. 11A1, B1, B2, C1 and C2) and Golgin-97 (Fig. 11A2, B3, B4, C3 and C4) and we found no obvious difference between the orientation of the centrosome in invaded and non-invaded cells. In two independent assays, we measured the angle between the direction of the wound and the axis connecting the centrosome and nuclear centre, in infected and non-infected WT RPE-1 cells (as exemplified in Fig. 12C) and, surprisingly, we observed that the mean angle of the centrosome
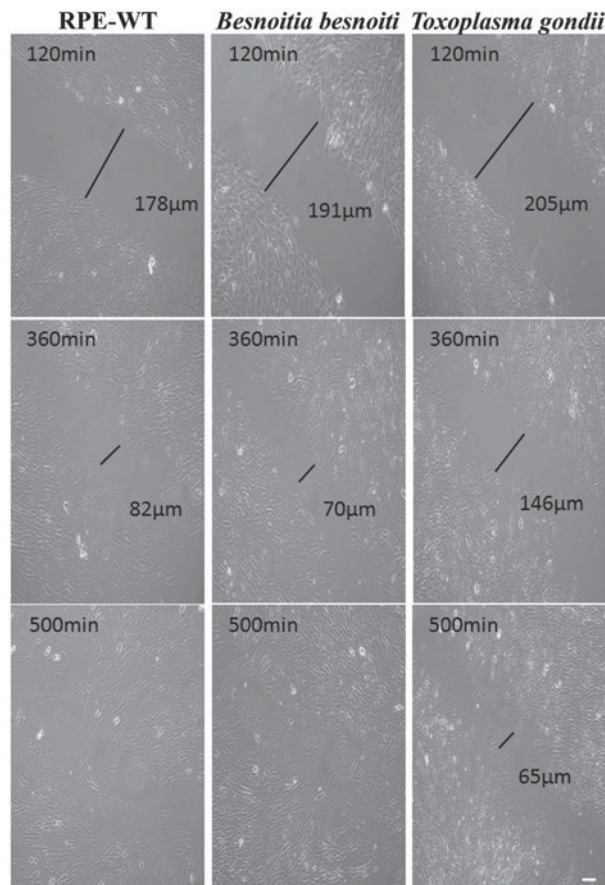
Fig. 10. Wound-healing assay in RPE-1 WT cells invaded by *T. gondii* and *B. besnoiti*. Non-invaded and invaded RPE-1 were grown to confluence, wounded and imaged for 600 min. Frames from 120, 360 and 500 min are shown. In this picture we can see that cells invaded with *T. gondii* show a significant delay in wound closing in comparison to control cells. This delay is not observed in cells invaded with *B. besnoiti*. Scale bar=50 μm.

towards the wound is quite similar in WT RPE-1 non-invaded cells ($68 \pm 1 \cdot 5$, $n = 587$), invaded by *B. besnoiti* ($76 \cdot 7 \pm 2$, $n = 101$) and invaded by *T. gondii* ($70 \cdot 5 \pm 2 \cdot 5$, $n = 183$), Fig. 12A. Concerning Golgi apparatus positioning, we counted the number of cells in the leading edge with a Golgi apparatus located within the 90° angle facing the wound (as shown in Fig. 12D; Hurtado *et al.* 2011). The results are summarized in Fig. 12B and show that migrating cells invaded by *T. gondii* present a similar localization of Golgi apparatus relative to the leading-edge in comparison to those non-invaded (which is consistent with the observations in Fig. 11C3 and C4). This suggests that the delay in wound heal closing of invaded cells cannot be ascribed to problems of centrosome or Golgi apparatus positioning but must be related to other factors. In the case of RPE-1 cells invaded by *B. besnoiti* we found the lowest value for Golgi reorientation ($62 \pm 3 \cdot 9\%$, $n = 101$; Fig. 12B), andnon-invaded RPE-1 WT cells had $74 \pm 3 \cdot 3\%$ ($n = 587$) of cells with an oriented Golgi towards the leading edge. Interestingly, the images presented in

Fig. 11C3 and C4 show that in migrating cells invaded by *T. gondii* the Golgi apparatus is less disorganized (non-fragmented), similar to the non-invaded cells (compare Fig. 11A2).

DISCUSSION

Previous work by our group demonstrated that *B. besnoiti* invasion induces a host cell microtubule rearrangement, as microtubules start to surround the parasite upon the first minutes of invasion and originate a cone-shaped microtubule network (Reis *et al.* 2006). In the present study we show that this rearrangement of host microtubules occurs, not only in the initial steps of host cell invasion, but also during PV establishment and parasite replication, supporting the idea that invasion requires a clear cross-talk between the host microtubule cytoskeleton and the *B. besnoiti* PV. This interaction was also observed during *T. gondii* invasion and is in accordance with what has already been described by other authors (Sehgal *et al.* 2005; Coppens *et al.* 2006; Walker *et al.* 2008; Sweeney *et al.* 2010). In fact, it is now known that host microtubules affect *T. gondii* invasion by hastening the time to parasites' initiation of host cell invasion (Sweeney *et al.* 2010). Altogether these data support the idea that *B. besnoiti* and *T. gondii* require the establishment of an interaction with the host cell microtubule cytoskeleton from the initial to advanced steps of infection. However, this requirement does not seem to be a universal feature for the apicomplexan parasites because it was reported that the closely related *Neospora caninum* does not cause any alterations in the microtubule host cell cytoskeleton organization (Coppens *et al.* 2006).

Differences between distinct members of the phylum Apicomplexa in terms of how they interact with and modulate mechanisms and pathways of the host cell, can also be extended to the interaction of these parasites with the host centrosome. In general, the centrosome has a central cellular localization in close connection with the nucleus. In the majority of cell types the Golgi apparatus also adopts an organization around the centrosome, microtubules being involved in its organization and, in turn, the Golgi apparatus is also involved in microtubule nucleation (Efimov *et al.* 2007; Rivero *et al.* 2009; Vinogradova *et al.* 2009). This internal organization of the cytoplasm creates an intrinsic polarity axis that is required for different cell activities such as formation of immune synapses, wound healing, cell migration, cell growth and differentiation (Yvon *et al.* 2002; De Anda *et al.* 2005) and cilia assembly (Gonçalves *et al.* 2010*b*). Having this in mind, we hypothesized that *B. besnoiti* and *T. gondii* may differentially explore this intrinsic polarity axis during host invasion, PV establishment and parasite replication. To test this hypothesis we have also investigated the position and state of organization
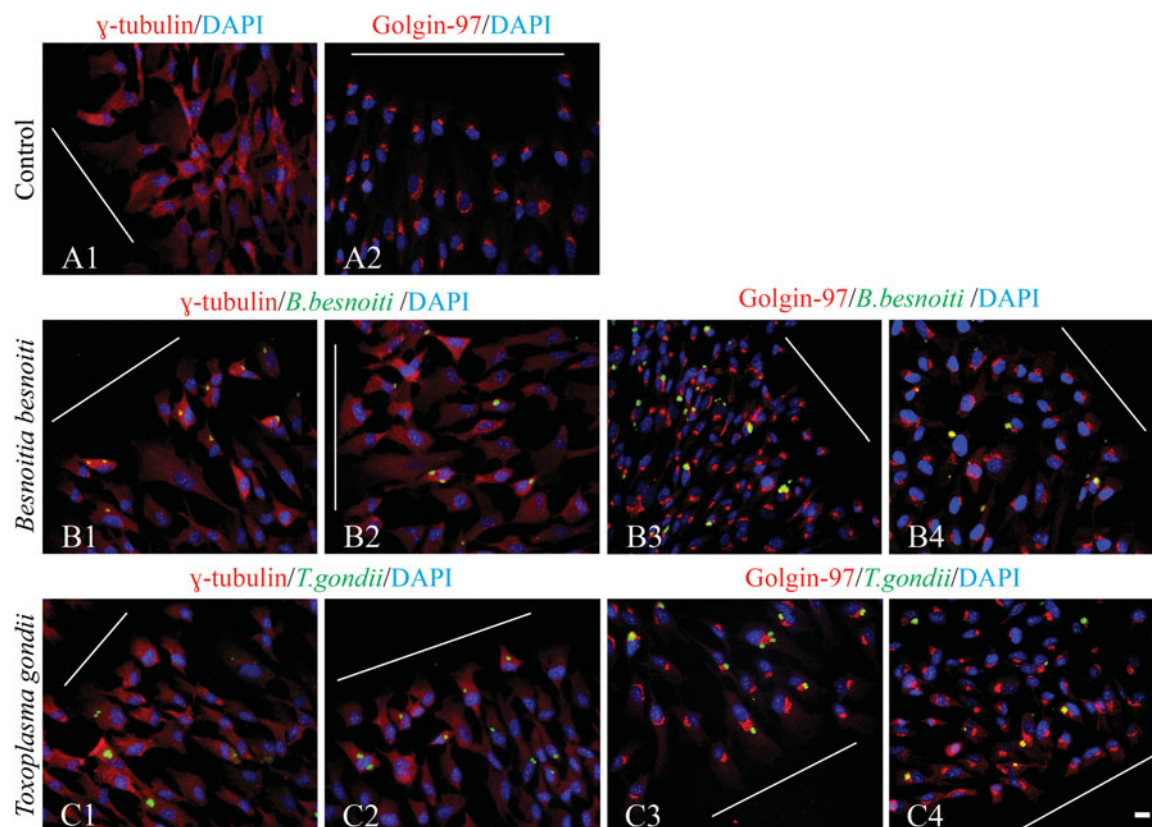
Fig. 11. Indirect immunolocalization of centrosome and Golgi complex in *B. besnoiti* or *T. gondii* invaded RPE-1 cells, during wound closure migration. Antibodies used were *B. besnoiti* polyclonal antibody (green), *T. gondii* polyclonal antibody (green), anti-Golgin 97 (red) and anti-γ-tubulin (red). DNA was stained with DAPI (blue). White lines indicate the position of the wound edge. In A1, B1, B2, C1 and C2, the orientation of the host cell centrosomes towards the wound edge can be seen in red. (A1) RPE-1 cells, non-invaded control. (B1 and B2) *B. besnoiti*, 18 h post-invasion. (C1 and C2) *T. gondii*, 18 h post-invasion. In A2, B3, B4, C3 and C4 the orientation of the host cell Golgi complex can be visualized (red). (A2) RPE-1 cells, non-invaded control. (B3 and B4) *B. besnoiti*, 18 h post-invasion. (C3 and C4) *T. gondii*, 18 h post-invasion. Scale bar=20 μm.

of Golgi apparatus in cells invaded with either *B. besnoiti* or *T. gondii*. In accordance with the literature (Coppens *et al*. 2006; Walker *et al*. 2008), we observed that *T. gondii* invasion consistently causes the fragmentation of the host cell Golgi apparatus, and the association of this cellular compartment with *T. gondii* PV. Interestingly, *B. besnoiti*, although it does not recruit the centrosome, is also engaged in the localization of the host cell Golgi complex close to the PV. But this relocalization is not accompanied by Golgi apparatus fragmentation. It is possible that both parasites strictly require the localization of Golgi apparatus close to the PV to achieve a successful invasion. In the case of the Golgi fragmentation observed during *T. gondii* invasion, this may be a second effect directly caused by centrosome recruitment. In fact, loss of microtubules by depolymerizing agents causes the movement of the centrosome away from the nucleus, accompanied by Golgi disorganization (Salpingidou *et al*. 2007). Also, centrosomes delocalized to positions far from the centre of the cell due to depletion of the centrosomal protein TBCCD1 cause a dramatic Golgi apparatus disorganization (Gonçalves *et al*.

2010*a*). However, it seems that Golgi structure integrity and positioning are not critical for protein modification or global secretion (Thyberg and Moskalewski, 1999; Miller *et al*. 2009; Yadav *et al*. 2009; Hurtado *et al*. 2011), thus the fragmented Golgi in *T. gondii*-invaded cells could maintain its global functions. In fact, it has been shown that in the presence of a disassembled host cell Golgi, parasites are able to grow and replicate normally (Shaw *et al*. 2000).

To try to explain the differences found between *B. besnoiti* and *T. gondii* in recruiting the host centrosome, we have studied the ability of both parasites to replicate in cells where the positioning of the centrosome and the organization of Golgi apparatus had been manipulated through changes in the expression levels of TBCCD1, a putative regulator of nucleus–centrosome connection (Gonçalves *et al*. 2010*a*). *Toxoplasma gondii* presents an increased difficulty in the recruitment of the centrosome in host cells over-expressing TBCCD1, accompanied by a delay in replication. However, this delay is not observed in cells treated with TBCCD1 targeting siRNAs, where the Golgi apparatus is already
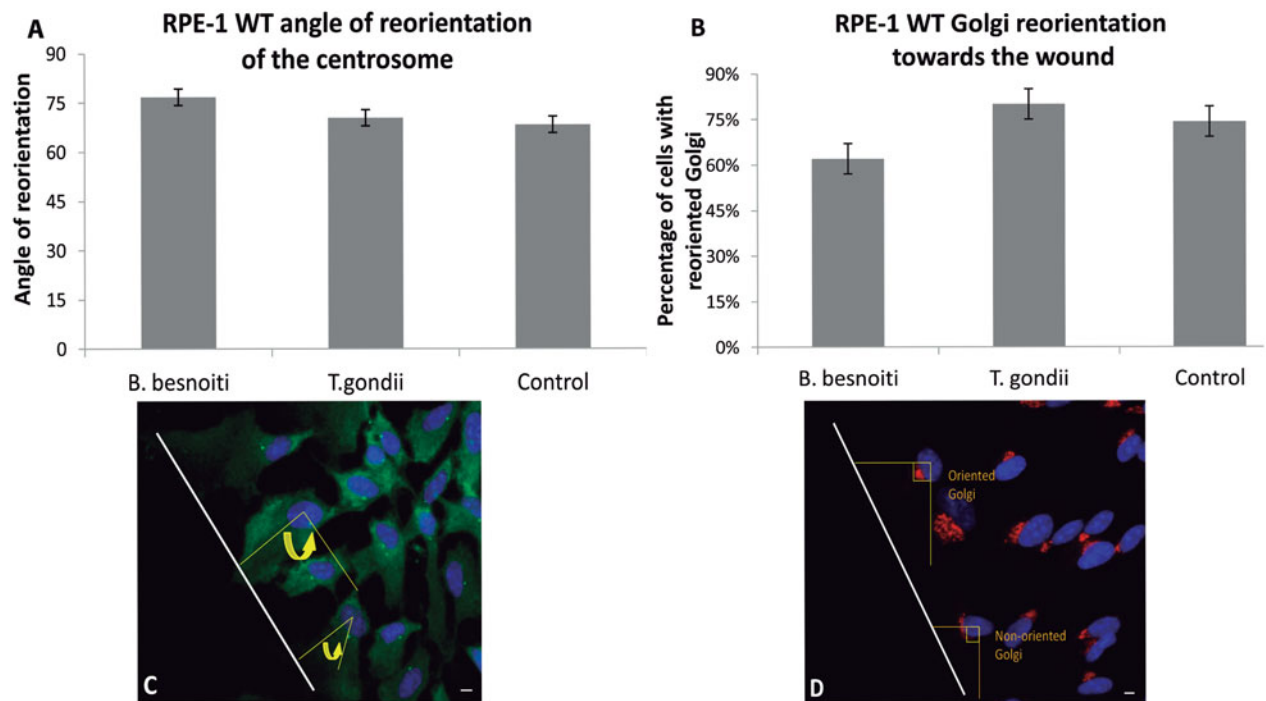
Fig. 12. (A) Graphic representing the angle of centrosome reorientation towards the wound leading edge, in non-invaded RPE-1 cells, and in invaded RPE-1 cells by *B. besnoiti* or *T. gondii*. (B) Graphic representing the percentage of cells with a Golgi complex reoriented towards the wound leading edge, in non-invaded RPE-1 cells, and in RPE-1 cells invaded by *B. besnoiti* and *T. gondii*. (C) Schematic IF of how the measurement of the angle of the centrosome in relation to the leading edge (white line) was performed using ImageJ software (centrosome – green; nucleus – blue). (D) Schematic IF of how the reorientation of the Golgi towards the leading edge (white line) was determined, with an example of a cell with an oriented Golgi, and another with a non-oriented Golgi. ImageJ software was used (Golgi – red; nucleus – blue). The mean angle of reorientation of the centrosome (± s.d.) and the mean percentage of cells with a reoriented Golgi (± s.d.) in two independent experiments are shown ($101 < n < 587$). Scale bars = 3 μm.

disorganized. Over-expression or depletion of TBCCD1 does not affect *B. besnoiti* replication. Notably, in RPE-1 TBCCD1 depleted cells invaded by *B. besnoiti* there is a reorganization of the Golgi ribbon previously fragmented due to TBCCD1 depletion. Together, these results strongly suggest that ultimately, during host invasion, both parasites require the manipulation of Golgi apparatus organization which, in *T. gondii*, is achieved through Golgi disorganization and in *B. besnoiti* by Golgi apparatus condensation observed in wild type RPE-1 cells and more prominently in TBCCD1 RPE-1 siRNA treated cells. This would explain why, in TBCCD1-depleted cells, where Golgi is already fragmented, *T. gondii* presents normal rates of replication. We suggest that *T. gondii* achieves this disorganization by a mechanism that involves manipulation of the host centrosome that is more relevant during PV development and parasite replication. In the case of *B. besnoiti* it seems that this parasite developed the ability to reorganize the Golgi apparatus even if it is completely disorganized, as in the case of cells depleted of TBCCD1.

It is known that perturbing Golgi apparatus positioning, by disconnecting it from the centro-some, has a more dramatic effect on directional cell migration than disrupting the Golgi apparatus itself (Hurtado *et al*. 2011). Indeed, it was described for *T. gondii* that the parasite is able to manipulate the migratory response of dendritic cells and macro-phages, which present a hypermigratory phenotype that may facilitate parasite dissemination (Lambert *et al*. 2006, 2010; Weidner *et al*. 2013). Also, it was reported that *T. gondii* suppresses both centrosome reorientation and migratory response in host fibro-blasts (Wang *et al*. 2010). In our wound-healing assays, we observed a delay in closing the wound by cells invaded with *T. gondii*, but most of the cells in the edge of the wound showed an oriented centro-some and Golgi towards the leading edge, supporting the fact that the delay does not seem to be due to an altered orientation of these organelles. The discrep-ancy between ours and the above-mentioned pub-lished results may be explained by the use of different cell lines in the two studies. In fact, although several studies reported centrosome reorientation towards the leading edge in migrating cells (Yvon *et al*. 2002), this is not true for all cell types, as positioning of the centrosome and cytoplasmic organization are highly dependent on geometrical constraints imposed by both the substratum/cellular matrix and cell–cell interactions (Pouthas *et al*. 2008; Dupin *et al*. 2009).

Consequently, the relevance of centrosome reorientation for cell migration is still open to debate (Yvon et al. 2002).

In conclusion, we show that *B. besnoiti* and *T. gondii* require the manipulation of the organization of Golgi apparatus, which probably creates better conditions for their replication inside the cell. However, the mechanism (or mechanisms) underlying this modulation is distinct for both parasites but probably linked to the interaction with the host cell microtubule cytoskeleton, including those microtubules nucleating from the Golgi.

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