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Development and characterization of monoclonal antibodies against *Besnoitia besnoiti* tachyzoites

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Research Article

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Abstract

This is the first report on the development and characterization of eight monoclonal antibodies (MABs) generated against whole- and membrane-enriched tachyzoite extracts of the apicomplexan parasite *Besnoitia besnoiti*. Confocal laser scanning immunofluorescence microscopy was used to localize respective epitopes in *B. besnoiti* tachyzoites along the lytic cycle. A pattern compatible with dense granule staining was observed with MABs 2.A.12, 2.F.3 and 2.G.4, which could be confirmed by immunogold electron microscopy for MABs 2.A.12 and 2.F.3. In particular, MABs 2.F.3 and 2.G.4 were secreted during early invasion, proliferation and egress phases. MABs 3.10.8 and 5.5.11 labelled the tachyzoite surface, whilst MABs 1.17.8, 8.9.2 and 2.G.A recognized the apical tip, which is reminiscent for microneme localization. Besides, the epitopes recognized by the latter two (MABs 8.9.2 and 2.G.A) exhibited a redistribution from the anterior part across the parasite surface towards the posterior end during invasion. Most MABs developed were genus-specific. Indeed, the MABs cross-reacted neither with *T. gondii* nor with *N. caninum* tachyzoites. In summary, we have generated MABs that will be useful to study the key processes in the lytic cycle of the parasite and with additional promising diagnostic value. However, the molecular identity of the antigens recognized remains to be elucidated.

Introduction

Besnoitia besnoiti is a cyst-forming apicomplexan parasite (Marotel, 1912) responsible for bovine besnoitiosis, a re-emerging disease in Europe characterized by both local and systemic clinical signs (Álvarez-García *et al*., 2013; Cortes *et al*., 2014). *Besnoitia besnoiti*, together with the closely related parasites *Toxoplasma gondii* and *Neospora caninum*, belongs to subfamily Toxoplasmatinae (Tenter *et al*., 2002). Members of this subfamily are characterized by the presence of two sequential asexual parasite stages that develop in the intermediate hosts. First, rapidly dividing tachyzoites are responsible for the acute stage of the disease, and subsequently tachyzoites undergo differentiation to bradyzoites that form tissue cyst during the chronic stage (Álvarez-García *et al*., 2014; Gutiérrez-Expósito *et al*., 2017).

Morphologically, *B. besnoiti* tachyzoites closely resemble the tachyzoite stage of *N. caninum* and *T. gondii*. The *B. besnoiti* invasive stages possess the typical apicomplexan secretory organelles of apicomplexan parasites such as rhoptries, micronemes and dense granules, which may most likely, similar to *T. gondii* and *N. caninum*, play an important role in various aspects of the host–parasite relationship (Dubey *et al*., 2003; Langenmayer *et al*., 2015).

To date, only a few *Besnoitia* proteins have been studied. Protein disulfide isomerase (*Bb*PDI), a protein that acts as a chaperone in the endoplasmatic reticulum, is thought to be secreted by *B. besnoiti* micronemes during host cell invasion (Naguleswaran *et al*., 2005; Marcelino *et al*., 2011). On the other hand, calcium-dependent protein kinase 1 (*Bb*CDPK1), which is expressed exclusively in plants, fungi algae and apicomplexan parasites, represents a promising target for a new generation of drugs named bumped kinase inhibitors, and is expected to play a role in tachyzoite invasion and proliferation (Jiménez-Meléndez *et al*., 2017). Moreover, a few proteomic studies have been carried out in order to investigate relevant biological processes as well as the host immune response associated with *B. besnoiti* infection that identified several proteins involved in conservative pathways among Toxoplasmatinae parasites (Fernández-García *et al*., 2013; García-Lunar *et al*., 2013*b*). However, the contribution of these studies has been hampered by the absence of *B. besnoiti* genome sequence which became available only very recently (Schares *et al*., 2017). Surface antigens and proteins from secretory organelles, which have been described to be highly immunogenic in *N. caninum* and *T. gondii*, and which are therefore considered to be valuable and specific diagnostic

targets (Schares et al., 2000), could not be identified in *B. besnoiti* proteome (García-Lunar et al., 2013*b*).

We have shown earlier that at least 25 *B. besnoiti* antigens cross-reacted with specific anti-*N. caninum* antibodies, and these may be responsible for false-positive reactors (García-Lunar et al., 2013*b*; García-Lunar et al., 2015). Thus, the development of monoclonal antibodies (MABs) was regarded as a suitable approach to develop novel markers for diagnostic purposes and for studies on *B. besnoitia* cell biology. Eight MABs directed against different compartments of *B. besnoiti* tachyzoite antigens were identified and respective epitopes were localized during the lytic cycle in tachyzoites by immunofluorescence and immunogold transmission electron microscopy (TEM). In addition, the genus, species and stage specificity was investigated in the closely related protozoans *B. tarandi*, *N. caninum*, *T. gondii* and *Sarcocystis* spp. as well as in the bradyzoite stage of *B. besnoiti*.

Materials and methods

Parasites

Tachyzoites from the BbSpain-1 isolate of *B. besnoiti* (Fernández-García et al., 2009*b*), *B. tarandi* (Dubey et al., 2004), the Nc-1 isolate of *N. caninum* (Dubey et al., 1988) and TgME49 isolate of *T. gondii* (Lunde and Jacobs, 1963) were grown in Marc-145 cell monolayers with DMEM supplemented with 5% fetal calf serum. Tachyzoites were separated from host cells as previously described (Fernández-García et al., 2009*a*), centrifuged at $1350 \times \boldsymbol{g}$ for 10 min, and stored at −80 °C until use for Western blot assays (Fernández-García et al., 2009*b*).

Besnoitia besnoiti bradyzoites were released by a trypsin treatment of a skin biopsy sample from a chronically naturally infected cow, following a previously described method (Fernández-García et al., 2009*a*). *Sarcocystis* spp. cystozoites were purified from naturally infected bovine hearts as previously described by More et al. (2008) and were pelleted by centrifugation and stored at −80 °C until use for Western blot experiments.

Production of hybridomas

MABs were developed against a whole *B. besnoiti* tachyzoite extract and an enriched membrane extract that were prepared following previous procedures (Wouda et al., 1998; Schares et al., 2013). Briefly, whole *B. besnoiti* tachyzoite extract was obtained from detergent lysate of purified *B. besnoiti* tachyzoites that were pelleted through a 20% sucrose cushion in PBS for 1 h at $13\,000 \times \boldsymbol{g}$ at 4 °C (Wouda et al., 1998). Next, tachyzoite pellets were suspended in PBS containing 1% (vol/vol) Triton X-100. Enriched membrane extract was prepared by surface biotinylation and subsequent immunoprecipitation (Schares et al., 2013).

For the production of MABs against intact whole purified *B. besnoiti* tachyzoites, BALB/C mice were immunized with 50 μg of the respective extract four times with 15 days intervals. Three days prior to the fusion, mice were boosted by an intraperitoneal injection of 50 μg of whole *B. besnoiti* tachyzoite extract. After euthanasia, splenocytes were fused with P3 × 63 Ag8653 myeloma cells. The supernatants of the hybridomas were screened for antibodies by ELISA. Positive hybridomas were cloned by limiting dilution and recloned at least four times.

MABs against the enriched membrane extract of *B. besnoiti* tachyzoites were obtained following a previously reported procedure (Schares et al., 1999*a*,*b*; Aguado-Martínez et al., 2010). Briefly, mice were immunized by an intraperitoneal injection of 50 μg of *B. besnoiti* membrane-enriched extract on day 0. Three days prior the fusion, mice were boosted by a subcutaneous injection of 50 μg of the respective extract. Then, mice were euthanized and splenocytes were fused with SP2/0 myeloma cells. The supernatants of the resulting hybridomas were screened for antibodies against *B. besnoiti* tachyzoites by Western blot and positive hybridomas were cloned by limiting dilution and recloned at least twice.

Isotyping of MABs was performed with a commercially available kit (IsoQuick™ Kit for Mouse Monoclonal Isotyping, Sigma-Aldrich, Madrid, Spain).

SDS-PAGE and Western blot

SDS-PAGE was carried out using 15% polyacrylamide gels (Fernández-García et al., 2009*a*; Schares et al., 2010; García-Lunar et al., 2013*a*; Schares et al., 2013). A total of 4×10^7 tachyzoites of *B. besnoiti* and *B. tarandi*, 2×10^7 tachyzoites of *N. caninum* and *T. gondii* and 10^7 *Sarcocystis* spp cystozoites were used as antigens. Both reducing and non-reducing conditions were employed for each antigen. For reducing conditions, dithiothreitol or β-mercapto-ethanol was added to the five times concentrated loading buffer. Parasite antigens and marker proteins separated in SDS-PAGE were electrophoretically transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore, Germany). After blocking with PBS-TG [PBS, 0.05% (v/v) Tween 20, 2% (v/v) fish gelatine liquid (Serva, Heidelberg, Germany)], the antigen-coated membrane was cut into strips and stored frozen at −20 °C until used (Schares et al., 2010). To detect antibodies against parasite antigens, the incubation of the strips with serum was performed as previously described by Schares et al. (1999*a*) with few modifications. Strips were probed with undiluted hybridoma supernatants and reactions visualized using peroxidase conjugates [anti-mouse IgG (H + L) or anti-mouse IgG (Fc), MinX Hu,Bo,Ho, Dianova, Hamburg, Germany] and 4-chloro-1-naphthol as a substrate.

Protein localization dynamics throughout the lytic cycle by immunofluorescence

Protein localization dynamics alongside the lytic cycle of *B. besnoiti* tachyzoites were studied using infected Marc-145 cells cultured on coverslips, and six out of the eight MABs, namely MAB 2.F.3, 2G.4, 2.G.A, 8.9.2, 5.5.11 and 3.10.8. For this, 5×10^4 Marc-145 cells were placed on sterile 13 mm coverslips in 24-well plates and cultured overnight at 37 °C/5% CO_2. Subsequently, *B. besnoiti* BbSpain-1 tachyzoites were collected by scrapping an infected culture flask, passing the suspension through a 25-gauge needle, and the number of viable parasites was determined in a Neubauer chamber by trypan blue exclusion. Subsequently, the Marc-145 monolayers were exposed to *B. besnoiti* tachyzoites during 30 min, 1, 2, 6, 24, 48 and 72 h. After these time points, extracellular/non-adherent parasites were removed by three PBS washes, and the specimens were fixed in either 2% paraformaldehyde in PBS (2% PFA-PBS) or in 2% paraformaldehyde-0.05% glutaraldehyde in PBS (2% PFA + 0.05%GA-PBS) for 10–30 min at room temperature (RT). After fixation, the infected monolayers were permeabilized in PBS containing 0.2% Triton X-100 (Merck Chemicals) for 20 min at RT, and blocking of unspecific binding sites was carried out using PBS/0.1% Triton X-100/3% bovine serum albumin (BSA; Roche) for 15 min at RT. Then, cultures were labelled with a polyclonal rabbit antiserum against *B. besnoiti* tachyzoites (Gutiérrez-Expósito et al., 2012) (1:1000 dilution) to delineate the tachyzoite surface, and with non-diluted hybridoma, supernatants (MABs 2.F.3, 2G.4, 2.G.A, 8.9.2, 5.5.11 and 3.10.8) for 30 min at RT. Following three washes with PBS, coverslips were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:1000)

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and Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:1000) (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at RT. Parasite and host cell nuclei were stained with 40, 6-diamidino-2-phenylindole (DAPI), and finally, coverslips were mounted on glass slides with ProLong® Gold antifade reagent (Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA) and sealed with clear nail polisher. Single stacks of immunofluorescence staining were captured with a Leica TCS-SP5 confocal laser-scanning microscope (Leica Microsystems) in the Spanish National Centre for Biotechnology (CNB, Madrid). Image processing was performed using the LAS AF (Leica Microsystems) and the ImageJ software (NCBI, http://rsb.info.nih.gov/ij/).

Transmission electron microscopy

Besnoitia besnoiti tachyzoite-infected Vero cells were grown in T25 tissue culture flasks. After a 72 h culture period, the medium was removed and cells were washed with 100 mM sodium cacodylate buffer, pH 7.2, and were fixed in cacodylate buffer containing 3% paraformaldehyde and 0.05% glutaraldehyde for 1 h at 20 °C. The cell layer was removed with a cell scraper, washed in cacodylate buffer, centrifuged, and the pellet was resuspended in cacodylate buffer. Cells were then dehydrated by sequential washing in progressively increasing concentrations of pre-cooled (−20 °C) ethanol (30–50–70–90–100%), and finally embedded in LR-White resin as previously described (Hemphill *et al.*, 2004; Risco-Castillo *et al.*, 2007). Infiltration of the resin was carried out over 3 days at −20 °C, with one resin change/day. Finally, the specimen blocks were polymerized at 60 °C over night. Sections of 80 nm thickness were prepared using a Reichert and Jung ultramicrotome, placed onto formvar-carbon-coated nickel grids (Plano GmbH, Wetzlar, Germany), and air-dried.

For immunogold labelling, sections were on-grid-labelled in a moist chamber with MAB culture supernatants that were used undiluted for 1 h at 20 °C, followed by three washes in PBS, 10 min each. Goat anti-rabbit conjugated to 10 nm diameter gold particles (Aurion, Wageningen, The Netherlands) was applied at a dilution of 1:5 in PBS-0.3% BSA as secondary antibody. After another three washes, 10 min each, grids were air-dried and contrasted with uranyle acetate and lead citrate (Hemphill *et al.*, 2004). Specimens were viewed on a CM12 TEM operating at 80 kV.

Results and discussion

MABs labelled the surface, the apical tip and the granular content of B. besnoiti tachyzoites

We have developed eight MABs that clearly label different subcellular compartments of *B. besnoiti* tachyzoites (Fig. 1). The characterization of these antibodies is timely, since tools for cell biological studies on *B. besnoiti* are essentially missing. Only five MABs against *B. besnoiti* tachyzoites have been developed in the past. Njagi *et al.* (2004) developed three MABs that were shown to label the apical cone and one MAB that stained cytoplasmic granules of the tachyzoites. Based on immunofluorescence results and the *in vitro* neutralizing properties of these MABs, it was suspected that these antibodies recognized proteins localized in secretory organelles, but the precise subcellular location was not confirmed. Similarly, Shkap *et al.* (1995) characterized one MAB that showed different recognition patterns, labelling a 70 kDa polypeptide in the soluble cytosol and the membrane fraction of *B. besnoiti* tachyzoites. Thus, the MABs we have generated can be added to this limited panel of biological reagents on *B. besnoiti* cellular biology and, potentially, also diagnosis.

In the present study, MABs 2.A.12, 2.F.3 and 2.G.4 labelled cytoplasmic granular content compatible with what has been observed for dense granule proteins (Leineweber *et al.*, 2017), MABs 2.G.A, 1.17.8 and 8.9.2 labelled the apical tip of the tachyzoites, which resembles microneme staining (Li *et al.*, 2015), whereas MABs 3.10.8 and 5.5.11 appeared to bind to structures on the tachyzoite surface (Schares *et al.*, 1999*b*). Interestingly, the later ones were produced using an enriched membrane antigen extract of tachyzoites, prepared by surface biotinylation and subsequent immunoprecipitation (Schares *et al.*, 2013). In the past, Schares *et al.* (1999*b*) obtained four MABs directed against biotinylated *N. caninum* tachyzoites and, similarly, three of them labelled the outer membrane surface of the parasite. TEM analysis was carried out with all MABs. However, only MABs 2.A.12 and 2.F.3 gave conclusive results, confirming dense granule staining as shown in Fig. 2.

Epitope co-localization dynamics showed distinctive pattern for each MAB along the lytic cycle

The lytic cycle in Toxoplasmatinae parasites is a conserved process, and involves key events that occur sequentially, during which specialized organelles secrete their contents in an orchestrated manner. First, apical micronemes release their contents during the early attachment–invasion process, mediating attachment and also re-orientation of tachyzoites. At the onset, and also during the invasion process, rhoptry proteins are released, and the dense granules discharge their contents shortly prior, or upon completion of host cell invasion, and they are involved in parasitophorous vacuole (PV) formation and maturation, and eventually parasite egress to ensure parasite dissemination and survival (Black and Boothroyd, 2000; Blader *et al.*, 2015). Initially, two MABs representative of each subcellular localization were selected for the studies of localization dynamics during the lytic cycle of *B. besnoiti* tachyzoites: 2.F.3, 2G.4 (dense granules), 2.G.A, 8.9.2 (micronemes), and 5.5.11 and 3.10.8 (tachyzoite surface). Moreover, different fixatives should be employed to characterize proteins in closely related parasites in order to obtain a concise picture of their localization, particularly for those proteins located in subcellular compartments. Thus, we selected two fixation methods for crosslinking proteins, one comprised only of paraformaldehyde, and the other one employing combined paraformaldehyde/glutaraldehyde fixation. In addition, a precipitation fixation protocol using methanol was also employed, but this resulted in either absence of labelling or lower staining intensity (data not shown). In general, paraformaldehyde fixation was more efficient in retaining the reservoirs of intracellular epitopes, whilst combined paraformaldehyde/glutaraldehyde fixation resulted in improved preservation of proteins after secretion. This is in agreement with the previous studies (Sohn *et al.*, 2011; Pastor-Fernández *et al.*, 2016*a*, 2016*b*). Membrane staining, most likely on the surface of tachyzoites, was visualized regardless the fixation protocol.

Immunofluorescence staining with MABs 2.F.3 and 2.G.4 confirmed the punctated, granular-dense granules-like staining (Fig. 3). Secretion of respective proteins recognized by these antibodies was observed with both fixatives, and with MAB 2.F.3 most pronounced after 1–2 h post infection (hpi), extending up to PV maturation at 24 hpi. Interestingly protein secretion in evacuoles was visible in samples fixed with paraformaldehyde and glutaraldehyde at 6 hpi. In *T. gondii*, numerous studies have demonstrated that the parasite modifies the PV and its host cell by secreting numerous rhoptry and dense granule proteins. In particular, Dunn *et al.* (2008) reported that the dense granule protein TgGRA7 was present in strand-like structures in the host cytosol together with other dense granule proteins. Similar findings were reported for the *Neospora* rhoptry protein NcROP2

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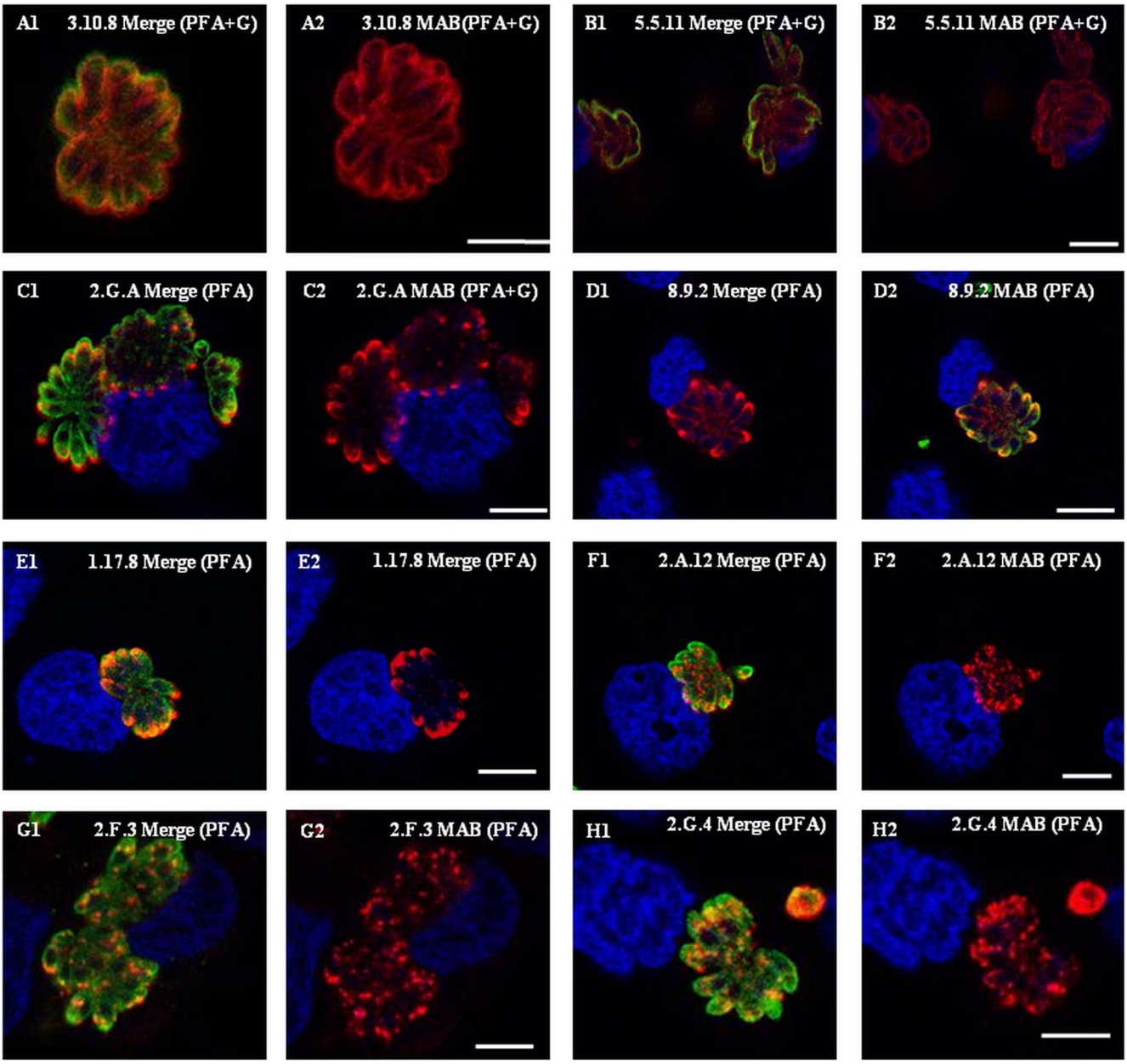

Fig. 1. Confocal laser scanning microscopy of MABs labelling the surface (A and B), the apical tip (C–E) and granular content (F–H) of *Besnoitia besnoiti* tachyzoites. Infected cultures were fixed with either PFA or PFA + GA and double labelled with anti-*B. besnoiti* polyclonal antibody (green) and mouse MABs (red). Nuclei were stained with DAPI (blue). All the images show a single-stack 1 μm slice. Scale bars represent 5 μm.

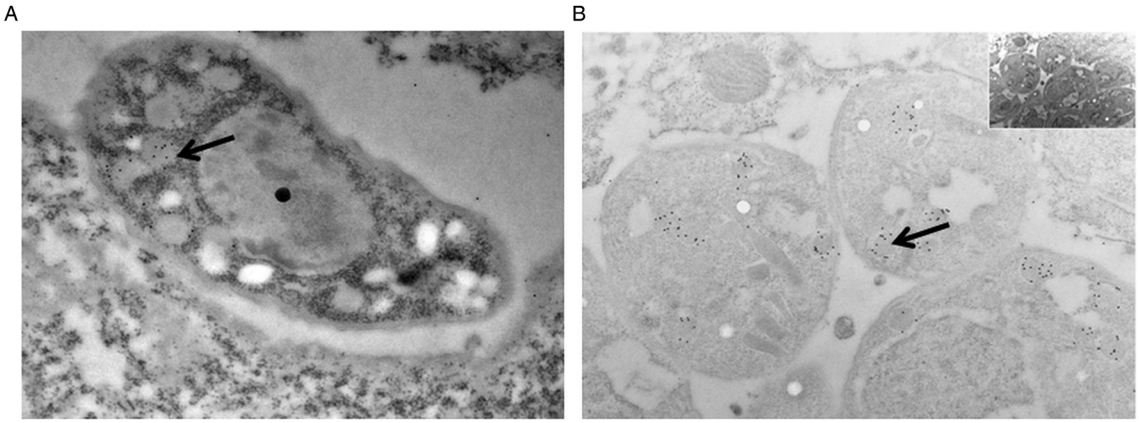

Fig. 2. Immunogold-TEM of *Besnoitia besnoiti* tachyzoites stained with MABs 2.A.12 (A) and 2.F.3 (B) and anti-mouse-10 nm gold particles. Arrows pint towards gold particles indicating dense granule staining. Bars = 1.8 μm.

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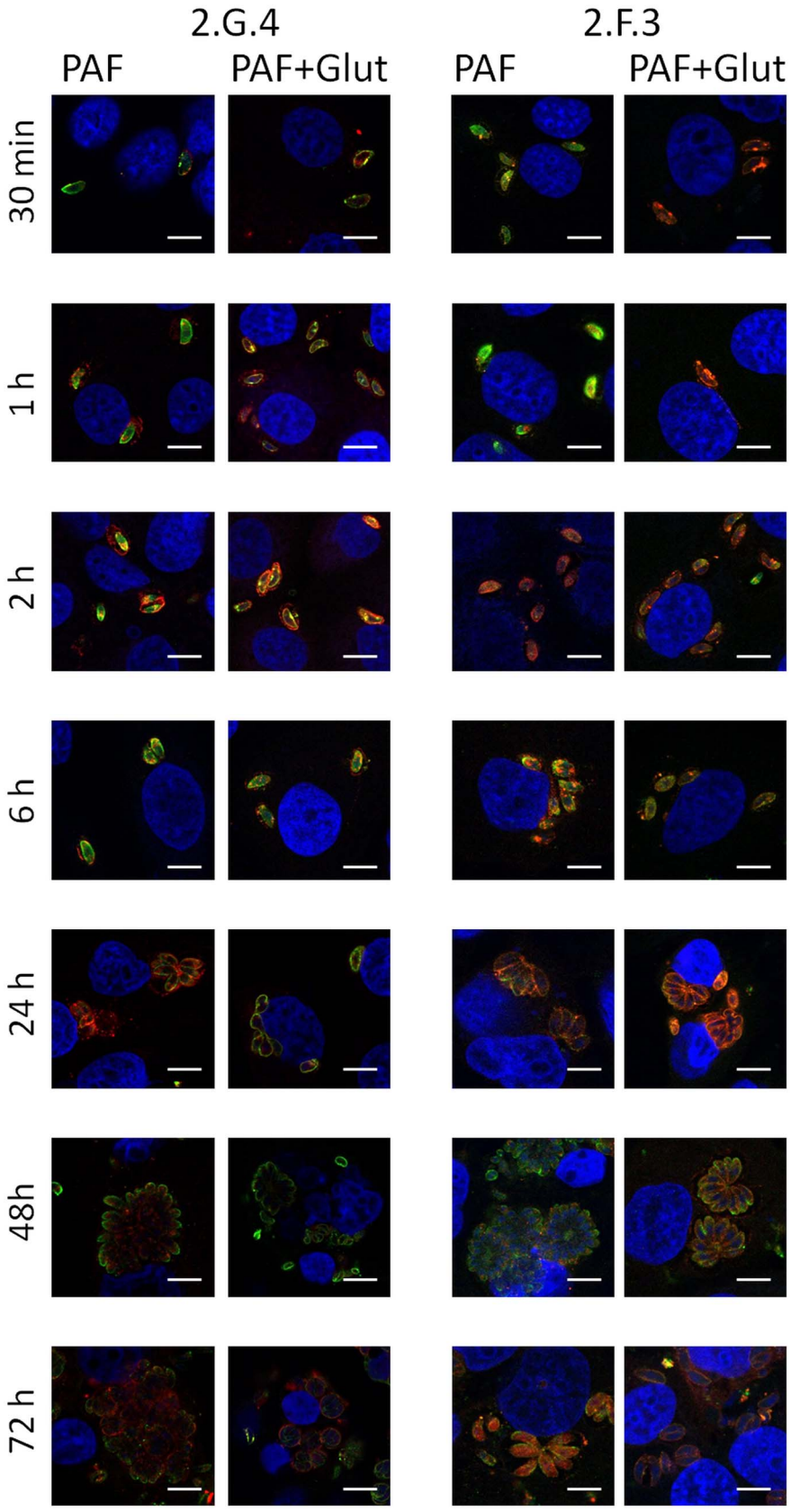

Fig. 3. Confocal laser scanning microscopy at different stages of the lytic cycle of *Besnoitia besnoiti* tachyzoites, stained with MABs displaying a granular staining pattern compatible with dense granule localization (2.F.3 and 2.G.4). Infected cultures were fixed with paraformaldehyde (PFA) or paraformaldehyde combined with glutaraldehyde (PFA + GA), and infected cells were double labelled with a polyclonal antibody against *B. besnoiti* tachyzoites (green) and monoclonal antibodies (red). Nuclei were stained with DAPI (blue). All the images show a single 1 μm slice. Scale bars represent 5 μm.

(Pastor-Fernández *et al.*, 2016*b*). Accordingly, the proteins labelled with our MABs are likely to participate in invasion, PV formation and/or PV maturation. However, *B. besnoiti* Sp-1 tachyzoites are able to survive in the extracellular environment for extended periods of time, and thus exhibit a more asynchronous invasion process compared with *Neospora* or *Toxoplasma* (Frey *et al.*, 2016). From 24 hpi onwards, the granular staining pattern observed with MAB 2.F.3 changed to a more pronounced staining of the membrane, and protein secretion was again detected during parasite egress at 72 hpi. The labelling pattern of MAB 2.G.4 was similar to the one displayed by 2.F.3, but a few remarkable differences were noted (Fig. 3). Protein secretion into evacuoles was detected earlier at 1 hpi, in close proximity to the host cell nucleus, and secretion extended up to 6 hpi in parallel with a punctated

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Fig. 4. Confocal laser scanning microscopy at different stages of the lytic cycle of *Besnoitia besnoiti* tachyzoites, stained with MABs that exhibit a staining pattern compatible with microneme localization (2.G.A and 8.9.2). Infected cultures were fixed with paraformaldehyde (PFA) or paraformaldehyde combined with glutaraldehyde (PFA + GA), and were double labelled with a polyclonal antibody against *B. besnoiti* tachyzoites (green) and monoclonal antibodies (red). Nuclei were stained with DAPI (blue). All the images show a single 1 μm slice. Scale bars represent 5 μm.

staining. Once the PV was formed, a slight labelling seemed to be associated with the membrane of the tachyzoites, and a granular staining was again observed during egress. Similar findings regarding dense granules staining were observed in closely related parasites. In *Neospora*, NcNTPase and NcGRA7 protein secretion was also evident during early invasion, PV maturation and egress. However, NcNTPase was scattered throughout the tachyzoite cytoplasm, whereas NcGRA7 labelling was observed closer to the parasite surface, suggestive of differential protein trafficking (Pastor-Fernández *et al.*, 2016*a*).

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