

## Identification of an immunogenic protein of *Giardia lamblia* using monoclonal antibodies generated from infected mice

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*The humoral immune response plays an important role in the clearance of Giardia lamblia. However, our knowledge about the specific antigens of G. lamblia that induce a protective immune response is limited. The purpose of this study was to identify and characterise the immunogenic proteins of G. lamblia in a mouse model. We generated monoclonal antibodies (moAbs) specific to G. lamblia (1B10, 2C9.D11, 3C10.E5, 3D10, 5G8.B5, 5F4, 4C7, 3C5 and 3C6) by fusing splenocytes derived from infected mice. Most of these moAbs recognised a band of  $\pm 71$  kDa (5G8 protein) and this protein was also recognised by serum from the infected mice. We found that the moAbs recognised conformational epitopes of the 5G8 protein and that this antigen is expressed on the cell surface and inside trophozoites. Additionally, antibodies specific to the 5G8 protein induced strong agglutination (> 70-90%) of trophozoites. We have thus identified a highly immunogenic antigen of G. lamblia that is recognised by the immune system of infected mice. In summary, this study describes the identification and partial characterisation of an immunogenic protein of G. lamblia. Additionally, we generated a panel of moAbs specific for this protein that will be useful for the biochemical and immunological characterisation of this immunologically interesting Giardia molecule.*

Key words: *Giardia lamblia* - immunogenic antigen - monoclonal antibody

*Giardia lamblia* is the causative agent of giardiasis in humans and several animal species, colonising the small intestinal lumen (Adam 2001). This parasite is widely distributed worldwide (Lengerich et al. 1994). The signs and symptoms of giardiasis include diarrhoea, abdominal pain, flatulence, weight loss and malabsorption of nutrients. In most cases, the infection remains asymptomatic (Adam 2001, Eckmann & Gillin 2001). The immunological mechanisms involved in clearance of *Giardia* infections are not fully elucidated. In immunocompetent patients, the infection is self-limiting, which indicates the presence of effective host defence mechanisms against this intestinal parasite (Faubert 2000, Eckmann 2003). The humoral and cellular immune responses play an important role in the control of *Giardia* infections (Faubert 2000, Singer & Nash 2000, Roxstrom-Lindquist et al. 2006). However, our knowledge about the immunogenic antigens of *G. lamblia* is limited. Several antigenic proteins of *G. lamblia* have been described, including variable surface proteins (VSPs), cyst wall proteins (CWPs), giardins, tubulins, arginine deaminase, ornithine carbamoyl transferase and enolase, among others (Luján et

al. 1995, Langford et al. 2002, Abdul-Wahid & Faubert 2004, Davids et al. 2006). Several of these molecules are recognised by the systemic and secretory antibody responses of infected humans and mice (Palm et al. 2003, Abdul-Wahid & Faubert 2004, Téllez et al. 2005, Davids et al. 2006). The proteins  $\alpha$ -1 giardin and CWP-2 provide marked protection against *G. lamblia* infection (Larocque et al. 2003, Jenikova et al. 2011).

The purpose of this study was to identify and characterise the immunogenic proteins of *G. lamblia* in a mouse model by generating monoclonal antibodies (moAbs) specific to *G. lamblia* by using splenocytes derived from infected mice. Those moAbs will help to further characterise the *Giardia* antigens that are recognised during infection.

### MATERIALS AND METHODS

**Mice** - C3H/HeJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). This strain of mouse is susceptible to infection with the *G. lamblia* clone GS/M-83-H7 (Byrd et al. 1994).

***G. lamblia* culture** - *G. lamblia* trophozoites [clone GS/M-83-H7 (ATCC 50581)] were obtained from the American Type Culture Collection. Axenic *G. lamblia* cultures were maintained in the TYI-S-33 medium, which was supplemented with newborn calf serum (NBCS) and antibiotics at 37°C.

***G. lamblia* protein extract** - *G. lamblia* soluble extract proteins were obtained using the method described by Gottstein et al. (1990), with slight modifications.

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Confluent cultures of *G. lamblia* trophozoites were harvested during log-phase growth and were incubated on ice for 15 min. The harvested trophozoites were washed three times with sterile phosphate buffered saline (PBS). Then, the trophozoites were resuspended in 1.5 mL of PBS and frozen (liquid nitrogen) and thawed [room temperature (RT)] three times in the presence of a protease inhibitor cocktail [23 mM 4-(2-aminoethyl) benzenesulphonyl fluoride, 0.3 mM pepstatin A, 0.3 mM E-64, 2 mM bestatin and 100 mM sodium ethylenediamine tetraacetic acid (Sigma, St. Louis, MO, USA)].

The *G. lamblia* lysate was sonicated for 2 min (30 cycles) (Brandon Sonifier 250, Shelton, CT, USA). Cell debris was removed by centrifugation (10,000 g for 30 min). The protein concentration of the soluble antigen preparation was determined by the Bradford method (Bio-Rad, Hercules, CA, USA).

**Generation of *Giardia* specific B-cell hybridomas** - B-cell hybridomas were generated from splenocytes of *G. lamblia*-infected C3H/HeJ mice. Eight-14-week-old male C3H/HeJ mice were infected and re-infected with  $5 \times 10^6$  *G. lamblia* trophozoites (GS/M-83-H7) using a sterile animal-feeding needle for peroral inoculation. The *G. lamblia* inoculum was prepared by washing in vitro-cultivated trophozoites three times with ice-cold sterile PBS and resuspending them in 200  $\mu$ L of sterile PBS. Primary infection occurred on day 0, while the second and third challenges took place on days 42 and 84, respectively. The infected mice were bled from the tail vein every week and the serum was recovered and stored at  $-80^\circ\text{C}$ . Infected mice were sacrificed by cervical dislocation five days after the last infection. Splenocytes were fused with the myeloma cell line P3 x 63.Ag8 using polyethylene glycol 1500 (Roche, Indianapolis, IN). moAbs specific to *G. lamblia* proteins were selected on the basis of their antigen recognition and were subcloned by limiting dilution (Velazquez et al. 2005).

**Western blotting assays** - Soluble *G. lamblia* proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12%) under modified reducing conditions. *G. lamblia* protein extract (500  $\mu$ g) was mixed with an equal volume of modified 2X SDS-PAGE sample buffer (0.2% SDS, 0.2% 2-mercaptoethanol). These modifications in the SDS-PAGE sample buffer were performed after preliminary experiments indicated that the antigen-recognition of the moAb 5G8.B5 was sensitive to the standard concentration of SDS and 2-mercaptoethanol (Velazquez et al. 2005). The proteins were electrotransferred to a nitrocellulose membrane for 20 min using a semi-dry blotting system (Owl HEP-1, Thermo Scientific, Rockford, IL, USA) with a 120-mAmp current. The nitrocellulose membranes were blocked with PBS containing 5% fat-free dry milk and 1% bovine serum albumin (BSA) for 1 h at RT. The blocked membranes were then incubated for 1 h at RT either with mouse serum from pre-infected mice, infected and re-infected mice (diluted 1:25) or with several moAbs [anti-VSP H7 ascites serum (moAb G10/4), anti-5G8 protein (moAb 5G8.B5) and control antibodies (isotype control antibody) (IgG2b, from Sigma, St. Louis MO, USA)

and 40F (IgG2a)]. All moAbs were tested at 5  $\mu$ g/mL. After five washes with PBS, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (diluted 1:7500 with PBS-0.1% BSA) (Sigma, St. Louis MO, USA) for 1 h at RT. The membranes were washed and developed using a SuperSignal West Pico Chemoluminescent Substrate kit (Thermo Scientific, Rockford, IL, USA).

**Flow cytometric analysis of *G. lamblia* trophozoites** - For cell surface staining, trophozoites were washed twice with ice-cold PBS. Then, the cells were incubated with the moAb 5G8.B5 (IgG2b) or the isotype control antibody (IgG2b) at 5  $\mu$ g/mL in D5F-0.05%  $\text{NaN}_3$  for 1 h at  $4^\circ\text{C}$ . After three washes with cold PBS, the trophozoites were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (diluted 1:200 with D5F-0.05%  $\text{NaN}_3$ ) (Sigma, St. Louis, MO, USA) for 1 h at  $4^\circ\text{C}$ . Then, the trophozoites were washed three times with cold PBS and fixed for 15 min at RT with 1% paraformaldehyde (PFA) (Sigma, St. Louis MO, USA) in Dulbecco's Modified Eagle's Medium (Sigma, St. Louis MO, USA) supplemented with 5% heat-inactivated foetal bovine serum (FBS) containing 0.05%  $\text{NaN}_3$  (D5F-0.05%  $\text{NaN}_3$ ). At least 10,000 trophozoites were acquired using fluorescence-activated cell sorting (FACS) (Canto II FACS, Becton Dickinson, CA, USA). For intracellular staining of the trophozoites, the *Giardia* cells were fixed for 15 min with 1% PFA at RT. After two washes with ice-cold PBS, the trophozoites were permeabilised for 5 min at RT with 0.1% triton X-100 (Sigma, St. Louis MO, USA). The permeabilised cells were then stained as described above. The fixation procedure did not alter the antigenicity of the *Giardia* proteins.

**Confocal microscopy analysis** - *G. lamblia* trophozoites were obtained from confluent cultures and washed three times with ice-cold sterile PBS. The trophozoites were fixed for 15 min in 3.7% formaldehyde. The dry glass slides were placed in PBS containing 1% milk and 5% FBS (GIBCO) for 1 h. After three washes with ice-cold PBS, the slides were stained (cell-surface staining or intracellular staining) in a humidity chamber for 1 h at 5  $\mu$ g/mL, using the moAb 5G8.B5 (IgG2b) or the isotype control antibody (IgG2b). After two washes with cold PBS, the slides were incubated for 1 h with FITC-conjugated goat anti-mouse IgG (diluted 1:100) (Sigma, St. Louis, MO, USA). The stained slides were preserved with mounting media (Vectashield) and analysed by confocal microscopy using a TCP-SP2 Confocal Laser Scanning microscope (Leica Microsystems, Heidelberg, Germany). The samples were observed through a 100X plan apochromatic oil immersion lens (NA: 1.32) and analysed with Leica Confocal Software (Leica LCS 2.0 Build 0871).

**Immunoabsorption assay** - Each well of a six-well plate (Corning, USA) was treated with 1 mL of moAb 5G8.B5 or control antibody (25  $\mu$ g/mL) in sterile PBS for 1 h at  $37^\circ\text{C}$ . Then, the wells were blocked with sterile PBS containing 1% BSA for 1 h at  $37^\circ\text{C}$ . After five washes with sterile cold PBS,  $2 \times 10^6$  *G. lamblia* trophozoites (pre-incubated at  $4^\circ\text{C}$ ) suspended in 1 mL of

PBS-1% BSA were added to each well and incubated for 20 min at 4°C. After five washes with sterile cold PBS, 2 mL of TYI-S-33 medium (supplemented with 10% of NBCS) was added to the wells and incubated for 10 min at 4°C. The adherent cells were harvested and cultivated in TYI-S-33 medium supplemented with 10% NBCS.

**Agglutination assay** - Two million *G. lamblia* trophozoites were incubated at 4°C for 1 h with several concentrations of the moAb 5G8.B5 or the isotype control antibody (0, 0.6, 1.2, 2.5, 5 or 10 µg/mL) in ice-cold PBS in each well of a six-well plate (Corning, USA). Agglutination of the trophozoites was assessed by microscopy (Rivero et al. 2010).

**Statistical analysis** - The data were analysed by the Kruskal-Wallis and Tukey methods (Statistical Package for the Social Sciences 2002). Statistical significance was accepted when  $p < 0.05$ .

## RESULTS

**Generation of B-cell hybridomas specific for *G. lamblia* proteins** - To identify and characterise the immunogenic proteins from *G. lamblia*, we generated B-cell hybridomas specific for *G. lamblia* (GS/M-83-H7) antigens by fusing splenocytes from infected mice with the myeloma cell line P3 X 63.Ag8. We generated nine moAbs that were specific for *Giardia* proteins (1B10, 2C9.D11, 3C10.E5, 3D10, 5G8.B5, 5F4, 4C7, 3C5 and 3C6) (Fig. 1). All of the moAbs were of the IgG2b isotype (except for the moAb 1B10, which was IgG2a) and recognised a band with a relative molecular mass of  $\pm 71$  kDa. This immunogenic band was also recognised by serum from infected and re-infected mice (Fig. 2). To further characterise this immunogenic protein, we selected the B-cell hybridoma 5G8.B5 for its ability to produce large amounts of moAbs. To evaluate the antigen recognition by the moAb 5G8.B5, western blotting

analysis was performed. However, when the moAb recognition was evaluated, considerable difficulty was encountered in optimising the western blotting conditions. Eventually, results were obtained by reducing the SDS and 2-mercaptoethanol concentrations in the buffer prior to SDS-PAGE (see Materials and Methods). These observations indicated that antigen recognition by the 5G8.B5 antibody was sensitive to the SDS and 2-mercaptoethanol conditions, suggesting that this moAb recognised a conformational epitope of the  $\pm 71$  kDa protein.

**5G8 protein expression in *G. lamblia* trophozoites** - The relative level and distribution of expression of the 5G8 protein in *G. lamblia* trophozoites (GS/M-83-H7) was explored by performing flow cytometry analysis. Almost all permeabilised GS/M-83-H7 trophozoites ( $\approx 94\%$ ) expressed the 5G8 protein (Fig. 3B), while only a small percentage ( $\approx 2-5\%$ ) of those trophozoites expressed high levels ( $10^4-10^5$  FI units) of the 5G8 protein on the cell surface (Fig. 3A). Confocal microscopy analysis confirmed that the 5G8 protein was found both at the cell surface (Fig. 4A) and inside the trophozoites (Fig. 4B). The 5G8 protein was detected as an intense and diffuse mark in all parts of trophozoites, including the ventral disc and flagella (Fig. 4A). To further characterise this immunogenic protein, we enriched the trophozoite population expressing the 5G8 protein on the cell surface by a panning technique using the moAb 5G8.B5. Approximately 50-70% of the enriched cell population [5G8 (+) trophozoites] expressed the 5G8 protein on the cell surface.

**5G8 protein is different from the VSP H7** - VSPs are immunogenic proteins that cover the cell surface of *G. lamblia*. Only one VSP is expressed on the surface of *G. lamblia* trophozoites at any point in time (Touz et al. 2008, Rivero et al. 2010). The *G. lamblia* clone GS/M-83-H7 expresses the immunogenic VSP H7. The predicted molecular weight of this protein is  $\pm 56$  kDa, but the mi-

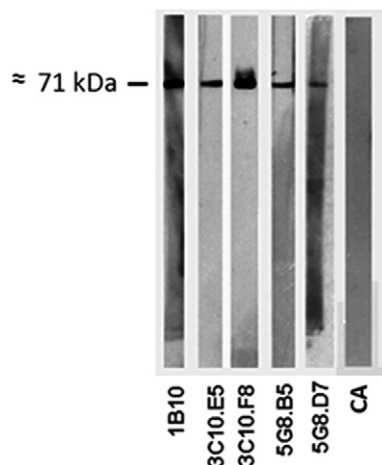


Fig. 1: antigen recognition by monoclonal antibodies (moAbs) specific for *Giardia lamblia* proteins. Western Blot analysis of *G. lamblia* specific moAbs (1B10, 3C10.E5, 3C10.F8, 5G8.B5 and 5G8.D7). Antigen recognition of some the moAbs generated. The most of them recognised a band of  $\pm 71$  kDa. CA: control antibody.

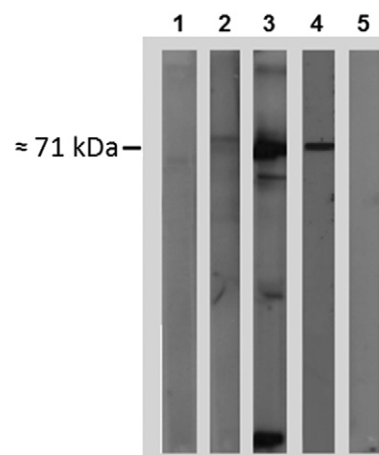


Fig. 2: the 71 kDa *Giardia lamblia* protein is recognised by sera from infected and re-infected mice. 1: pre-infected serum; 2: serum from infected mice (5th week post-infection); 3: serum from re-infected mice (2nd week post re-infection); 4: 5G8.B5 monoclonal antibody (moAb); 5: isotype control antibody (IgG2b).



gration of this protein in as SDS-PAGE gel is considerably higher ( $\pm 72$  kDa). To determine whether the *Giardia* antigens VSP H7 and 5G8 corresponded to the same protein molecule, we evaluated the expression of these antigens on the cell surface of *G. lamblia* trophozoites [GS/M-83-H7 and 5G8 (+)] by FACS analysis, using moAbs specific to the VSP H7 (G10/4 moAb) and 5G8 (5G8.B5 moAb) proteins. The antigen recognition by both moAbs was clearly different (Fig. 5), indicating that the 5G8 protein and the VSP H7 protein are not the same molecule. Additionally, confocal microscopy data showed differences in the antibody recognition pattern between the 5G8 and VSP H7 proteins (unpublished observations).

**Agglutination assay** - The agglutinating capacity of the moAb 5G8.B5 was evaluated in GS/M-83-H7 and 5G8 (+) trophozoite cultures. The moAb 5G8.B5 induced a strong agglutination of 5G8 (+) trophozoites at the different concentrations tested (Table). Large clumps of agglutinated

cells were observed at antibody concentrations of 2.5 and 5.0  $\mu\text{g}/\text{mL}$  (Fig. 6). In contrast, the moAb 5G8.B5 showed a lower agglutination capacity for GS/M-83-H7 trophozoites (Fig. 6, Table). The isotype-matched control antibody did not show an agglutinating capacity on *Giardia* cells at any of the tested antibody concentrations.

## DISCUSSION

The immunological mechanisms involved in the control and clearance of *Giardia* are not completely understood. The humoral and cellular immune responses play an important role in *Giardia* infection (Faubert 2000, Singer & Nash 2000, Roxstrom-Lindquist et al. 2006). The development of a vaccine against this infection has been hampered by a lack of knowledge about the *Giardia* proteins that induce a protective response during infection. In the present study, we generated moAbs specific for *G. lamblia* antigens from splenocytes derived from infected mice. These moAbs have allowed us to partially

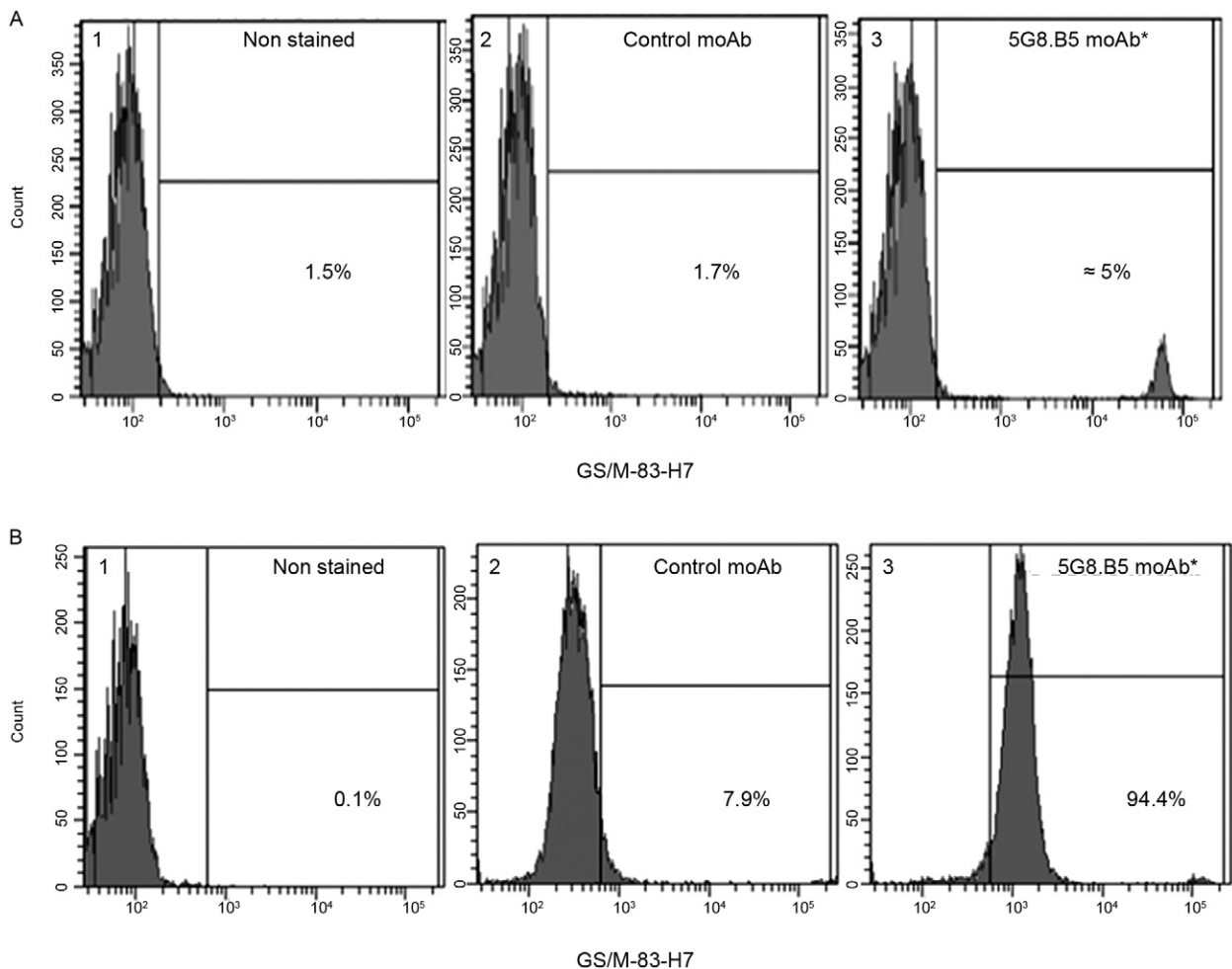


Fig. 3: 5G8 protein expression in *Giardia lamblia* trophozoites. Fluorescence-activated cell sorting analysis was performed using the 5G8.B5 monoclonal antibody (moAb) and control antibodies (isotypes IgG2b and IgG2a). A: cell surface staining of *G. lamblia* trophozoites (GS/M-83-H7) {1: non-stained control cells; 2: control moAb [40F (IgG2a)]; 3: 5G8.B5 moAb}; B: inside staining of *G. lamblia* trophozoites (GS/M-83-H7) {1: non-stained control cells; 2: control moAb [40F (IgG2a)]; 3: 5G8.B5 moAb}. Statistical significance was accepted when  $p < 0.05$  from control antibody (marked with asterisks).

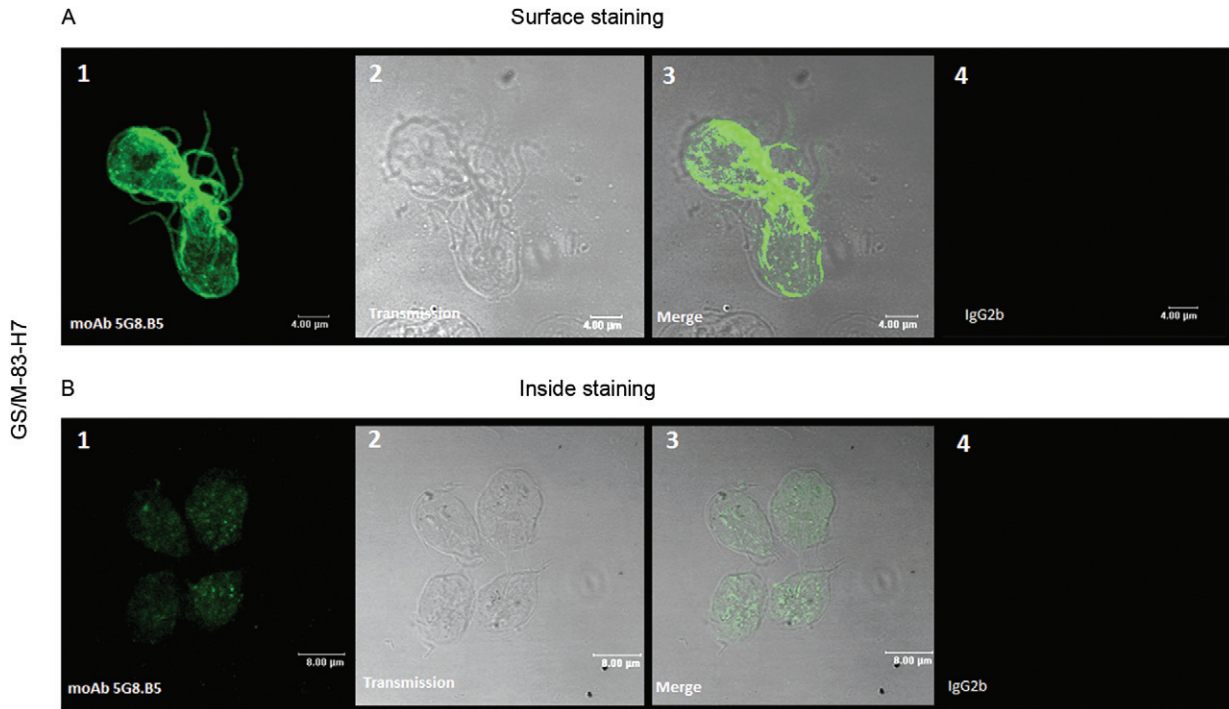


Fig. 4: confocal microscopy analysis of *Giardia lamblia* trophozoites. Surface (A) and inner (B) staining: 1: 5G8.B5 monoclonal antibody (moAb); 2: transmission microscopy image; 3: merge of 5G8.B5 moAb and transmission microscopy image; 4: isotype control antibody (IgG2b).

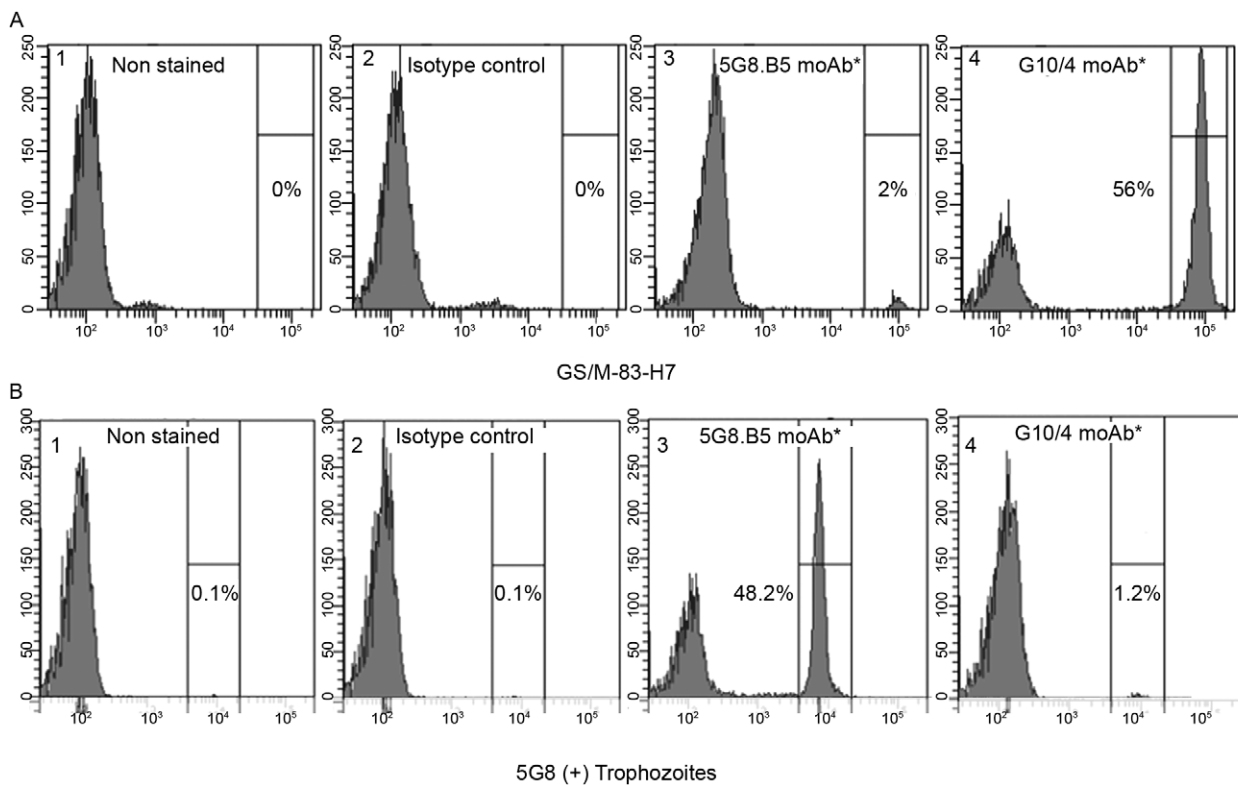


Fig. 5: *Giardia lamblia* 5G8 protein is different from variant surface protein (VSP) H7. Fluorescence-activated cell sorting analysis was performed using the monoclonal antibodies (moAbs): G10/4 ( $\alpha$ -VSP H7), 5G8.B5 ( $\alpha$ -5G8 protein) and isotype control antibody (IgG2b). Cell surface staining of *G. lamblia* GS/M-83-H7 (A) and 5G8 (+) trophozoites (B). 1: non-stained control cells; 2: isotype control antibody; 3: 5G8.B5 moAb; 4: G10/4 moAb. Statistical significance was accepted when  $p < 0.05$  from control antibody (marked with asterisks).

characterise, at the biochemical and immunological levels, a highly immunogenic protein recognised by the immune system of infected mice. The antigen recognition of most of the moAbs was directed toward a protein band of  $\pm 71$  kDa (5G8 protein). Primary and secondary infections with *G. lamblia* are associated with differences in the antibody recognition pattern. However, the antibody responses [faecal (IgA) and serum (IgG)] against the 71 kDa protein were maintained during the primary and secondary infections, demonstrating the immunogenic properties of this antigen (Velazquez et al. 2005). In vitro agglutination of *G. lamblia* trophozoites was induced by the moAb 5G8.B5, suggesting that in the infected host the 5G8 *Giardia* protein may induce specific antibodies that could promote *Giardia* agglutination, thus preventing the colonisation of the parasite. Further studies are needed to characterise this immunogenic protein at the molecular level and evaluate its protective role during

*Giardia* infection. The *G. lamblia* genome database (*Giardia* DB - *Giardia* Genomic Resources - giardiadb.org/giardiadb/), together with digestion and mass spectrometry analysis of the antigenic band, may help to identify this *G. lamblia* antigen (Luque Garcia et al. 2008).

FACS analysis revealed that the 5G8 protein was expressed inside of *Giardia* trophozoites (> 90% of total cell population) and only a small percentage ( $\approx 2\text{-}5\%$ ) of those trophozoites expressed high levels of the 5G8 protein on the cell surface. The pattern of expression of this antigen indicates that the 5G8 protein may be transported by the vesicular traffic system toward the plasma membrane (Luján & Touz 2003). Current studies in our laboratory are examining the precise subcellular localisation of the 5G8 protein in the trophozoite and its trafficking to the plasma membrane of the parasite.

*G. lamblia* undergoes antigenic variation, a process resulting in the variation of the proteins that completely cover the trophozoite cell surface. The *G. lamblia* strain GS/M-83-H7 constitutively expresses the VSP H7, which has a relative molecular mass of  $\pm 56$  kDa, but the migration of this protein in SDS-PAGE is considerably higher ( $\pm 72$  kDa) and can vary with the preparation of the acrylamide gel (Nash & Mowatt 1992, Luján & Touz 2003). VSP H7 is expressed on the surface of trophozoites and is immunogenic. The flow cytometry analysis showed a different antibody recognition pattern between the moAbs specific for the proteins 5G8 and VSP H7, indicating that these *Giardia* molecules are different proteins. In addition, the expected behaviour for a VSP such as VSP H7, which is capable of changing spontaneously in vitro with high frequency (Nash et al. 1990), was observed for VSP H7 protein, but not for the 5G8 antigen. It has been estimated that the frequency of change (in vitro) for an individual VSP in *G. lamblia* is approximately once every six-13 generations (48-72 h) (Nash et al. 1990). In contrast, the expression of the 5G8 protein on the surface of *G. lamblia* trophozoites did not change during in vitro culture for at least three weeks (unpublished observations). This result strongly suggests that the 5G8 protein is not a VSP.

In summary, this paper describes the identification and partial characterisation of an immunogenic protein of *G. lamblia*. Additionally, we generated a panel of moAbs specific for this protein, which will be useful for the biochemical and immunological characterisation of this immunologically interesting *Giardia* molecule.

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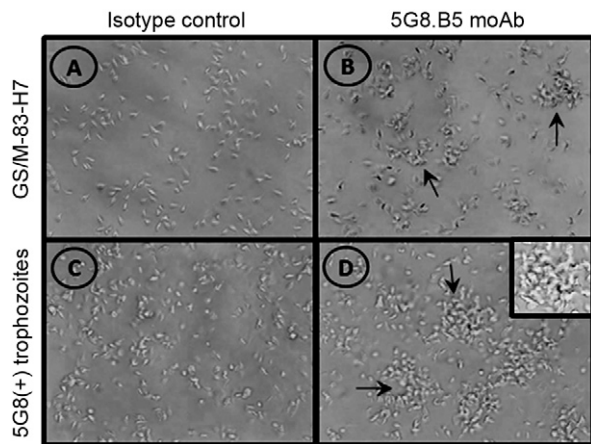


Fig. 6: *Giardia lamblia* agglutination by the monoclonal antibody (moAb) 5G8.B5. The agglutinating capacity of the 5G8.B5 moAb was evaluated on GS/M-83-H7 and 5G8 (+) trophozoite populations. Control isotype (A, C) and 5G8.B5 (B, D) at different concentrations (0, 0.6, 1.2, 2.5, 5 and 10  $\mu\text{g}/\text{mL}$ ).

TABLE  
Monoclonal antibody 5G8.B5 induces  
*Giardia lamblia* trophozoites agglutination<sup>a</sup>

Antibody ( $\mu\text{g}/\text{mL}$ )	Isotype control antibody	moAb 5G8.B5
0	-	-
0.6	+/-	+
1.2	+/-	++
2.5	+/-	+++
5	+/-	+++
10	+/-	++

a: -: < 1%; +/-: 1-10%; +: 11-30% (low); ++: 31-60% (medium); +++: 61-90% (high).

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