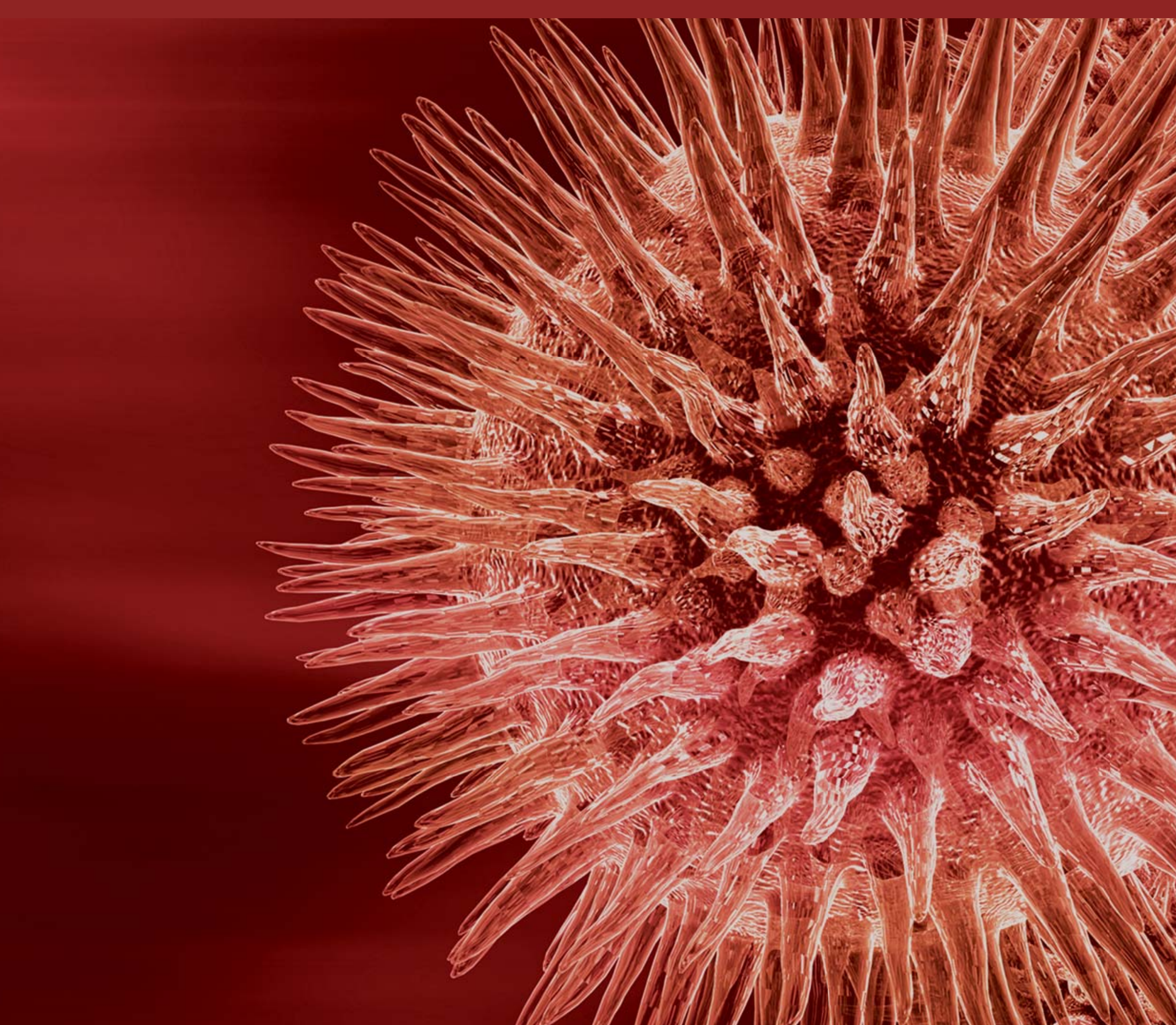
Journal of Biomedicine and Biotechnology

Immunology and Cell Biology of Parasitic Diseases 2011

Guest Editors: Luis I. Terrazas, Abhay R. Satoskar, and Jorge Morales-Montor



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Editorial

Immunology and Cell Biology of Parasitic Diseases 2011

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Infections caused by parasitic pathogens are still a global health problem. Parasitic infections are considered to be one of the leading causes of high morbidity and mortality in underdeveloped countries, particularly in children and the elderly. They affect the physical and intellectual capabilities of these populations, resulting in high treatment and rehabilitation expenses. Moreover, in developed countries, many parasitic infections are now a reemerging health problem, mainly due to massive migration of infected people from endemic countries, who carry the infection to novel and naïve fields. The goal of this special issue was to invite contributions from leading scientists in the field of parasitology with particular emphasis on immunobiology of parasitic diseases. In this special issue, we will discuss recent advances made in understanding the cellular and molecular basis of parasitic infections as well as immunological mechanisms for control of these diseases. In this light, we have new proposals as to how *Trypanosoma cruzi* can invade host cells as well as new methods to study cellular research in helminth infections. These original research papers and reviews are focused on different parasitic diseases emphasizing the need for high-impact research on public-health-related infectious diseases, including helminths (schistosomiasis, cysticercosis, trichinellosis, among others) and protozoan (toxoplasmosis, trypanosomiasis, amebiasis, and malarial) infections. We also have a couple of reviews to emphasize the current exciting research topic, how helminthic infections modulate immune responses to allergens and autoimmune diseases. Another hot topic of research is the search for cells that are key sources of Th2 responses during helminth infections and we have a nice review about the latest developments in this field. And of course, the role of regulatory T cells in modulating parasitic infections cannot be missed.

This special issue was mainly supported by attendees of our IV Mexican Immunoparasitology meeting which has been held regularly every two years and was created to facilitate meetings for the exchange of ideas between Mexican scientists and overseas guest scientists interested in the field of the immunoparasitology. Though each time it has become increasingly difficult to find the right places for parasitological as well as in immunological meetings for discussion, this is an important area that requires exchange of knowledge. Similar to last year, approximately 35% of the papers submitted to this special issue were rejected or withdrawn after a rigorous review by our referees, thus trying to maintain the quality of JBB.

We start with an original contribution by X. Du et al., demonstrating that deficiency in IFN-γ leads to decreased egg burden in mice infected with *S. japonicum*, through an enhanced activation of T cells during acute infection. Their data suggests that IFN-γ is not always a positive regulator of immune responses and that, in certain situations, the disruption of IFN-γ signaling may upregulate the cytotoxic T-cell-mediated immune responses to this parasite. M. Esquivel-Velázquez et al. commented on how to improve the immunodiagnosis of neurocysticercosis (NCC), a disease of the central nervous system that is considered a public health problem in endemic areas. The authors highlight that immunodiagnosis of NCC is a useful tool that can provide important information on whether a patient is infected or not, but there are many drawbacks as not all infected patients can be detected by this technique. Thus, several important

methods for developing an immunodiagnostic test for NCC are listed and discussed in their contribution. The identification of a set of specific and representative antigens of *T. solium* and a thorough compilation of the many forms of antibody response of humans to the diverse forms of *T. solium* disease are also stressed as necessary. Following the *T. solium* paper, we have an original paper by A. Ochoa-Sánchez et al. that use a hamster model for taeniasis where they identified specific antigens from this tapeworm that could be used for differential diagnosis. With regards to another important helminth parasite, *Trichinella spiralis*, R. Hernández-Bello et al. evaluated the *in vitro* effects of sex steroids on the molting process of *Trichinella spiralis*. The molting process is the initial and a very crucial step in the development of the muscular larvae to adult worm. They demonstrate that progesterone is the only steroid that causes an effect by decreasing the molting rate. These authors also demonstrate that caveolin expression is downregulated by progesterone and estradiol. By using flow cytometry, a protein that is recognized by a commercial antiprogesterone receptor antibody was detected. The authors conclude that their findings may have strong implications in the host-parasite coevolution. The observation of sex-associated susceptibility to this infection could point to the possibility of using antihormone therapy to inhibit parasite development. Another very interesting contribution, which may have important application on parasitology, is the one of K. Nava-Castro et al., who designed a new method to disaggregate and analyze single isolated cells from helminths using flow cytometry. These authors mention that traditional methods to analyze molecules in parasites, particularly in complex parasites, such as worms, require homogenization of the whole helminth parasite, preventing evaluation of individual cells or specific cell types in a given parasite tissue or organ. The authors remark that the extremely high interaction between helminths and host cells (particularly immune cells) is an important point to be considered. It is really hard to obtain fresh parasites without host cell contamination. Then, it becomes crucial to determine which of the analyzed proteins are exclusively of parasitic origin, and not a consequence of host cell contamination. In their contribution, they describe a method to isolate and obtain purified helminth cells. On another front, the last few years have helped recognize helminths as very important parasites which can significantly modulate the immune response of their hosts. A series of studies have taken advantage of these properties and now there are several investigations on the immune modulatory capacities of helminthic infections. Thus, y. Osada and t. Kanazawa discuss both sides (benefit and harm) of the possible use of schistosome antigens to treat patients suffering from concomitant diseases. They argue the pros and cons of the hygiene hypothesis and also comment on the importance of controlling schistosomiasis. In a similar theory regarding helminths as potential regulators of the immune response, E. Daniłowicz-Luebert et al. have provided a comprehensive review on how different helminthic infections can modulate specific and allergy-related immune responses. They give us a growing list of parasites which have been reported to modulate beneficially allergic and autoimmune responses. Finally, with respect to helminths, S. Leon-Cabrera and A. Flisser present a review on the role of basophils as a key cell type in inducing Th2-type responses in helminth infections. They compare several recently published papers which support the theory that basophils are Th2-inducers versus papers where this role is mainly attributed to helminth-modulated dendritic cells. Thus this review focuses on the origin of the Th2 responses during helminth infections as its central theme.

Protozoa are the most deadly parasites in the world, and in this special issue, we have several original papers describing their novel methods of infection, new genetic mechanisms of regulation as well as the role of recently discovered transcription factors in the development of these parasites. This section starts with a review on the role of interleukin-10 in malaria by M. Niikura et al. Interleukin- (IL)-10, an anti-inflammatory cytokine, is known to inhibit protective immune responses against malarial parasites and is involved in exacerbating parasitemia during *Plasmodium* infection. In contrast, IL-10 is regarded as necessary for suppressing severe pathology during *Plasmodium* infection. Thus, the authors summarize the role of IL-10 during murine malarial infection, focusing especially on coinfections with lethal and nonlethal strains of malaria parasites. R. Tuteja et al. discuss emerging data on the functions of transcription factors in malaria. *Plasmodium falciparum* is responsible for causing the most lethal form of malaria in humans. Although it shares some common features with eukaryotic transcription, it is assumed that mechanisms of transcriptional control in *P. falciparum* somehow differ from those of other eukaryotes. They argue that transcription factors are slowly emerging to have more defined roles in the regulation of gene expression in parasites. Coming back to immunity to malaria, S. Pathak et al. suggest that a protein from *Plasmodium* named PfP0 induces a deviant humoral response which may contribute to immune evasion mechanisms of the parasite. S. Muñiz-Hernández et al. demonstrate extensively the contribution of the residual body in the spatial organization of *Toxoplasma gondii* within the parasitophorous vacuole. They characterize the structure and function of the residual body in intracellular tachyzoites. They conclude that the absence of the network and presence of atypical residual bodies in ΔGRA2-HXGPRT knockout mutant parasites affect the intravacuolar organization of tachyzoites and their exteriorization. *Trypanosoma cruzi* is a flagellated protozoan and is the causative agent of Chagas disease which is prevalent in several regions in America. D. Pérez-Morales et al. analyzed the response of *T. cruzi* to temperature-induced stress. They discovered 24 proteins which showed changes in amounts of particular forms of protein in response to temperature stress, suggesting a potential role for them in heat shock response. In another study on *T. cruzi* by G. Ballesteros-Rodea et al. described the flagellar motility of this parasite, a phenomenon that had been poorly analyzed. Here the authors conclude that such movements are not random and are of capital importance for the survival of this parasite. Another extended protozoa infections is amoebiasis. With respect to this disease, C. Bañuelos et al. used genetic manipulation of the MVB protein trafficking pathway of

Entamoeba histolytica to overexpress 166 amino acids of its N-terminal Bro1 domain in trophozoites which resulted in diminished phagocytosis rates.

Finally, TP. Velavan and O. Ojurongbe performed a state-of-the-art review on the role of T regulatory cells in the outcome of both helminth and protozoan infections. Tregs have been observed in a variety of experimental as well as natural parasitic infections, and their role sometimes appears to favor parasite colonization but in many others these cells appear to help the host in order to maintain a regulated immune response avoiding hyperresponsiveness to disease. Thus, a fine balance in this T-cell subset is of great importance to maintain health and prevent disease development.

We hope that this collection of articles in the second special issue of immunobiology of parasitic diseases is of great interest to our readers and we also hope that you enjoy reading the contributions of the authors.

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Erratum

Erratum to "Flagellar Motility of *Trypanosoma cruzi* Epimastigotes"

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The authors would like to make the following changes.

(1) In the Results Section *3.3. Vectorial Analysis of Parasites Trajectories*, third paragraph, the sentence: this behavior was clearly observed when speed and rotational angle were analyzed together (Figure 7) should be replaced by the following sentence: this behavior was clearly observed in the parasite trajectories, like the one pictured in Figure 7.

(2) The legend of Figure 7, Figure 7: *Analysis of parasite trajectory*. The trajectory of free-swimming epimastigotes was determined using the speed and rotational angle results form a 10-second recording. The histogram is representative of the average behavior of 20 randomly selected epimastigotes, should be replaced by Figure 7: *Analysis of a single parasite trajectory*. The trajectory of a typical free-swimming epimastigote was recorded for 10 seconds.

(3) Figure 7 that is in the original paper should be replaced by the following figure.

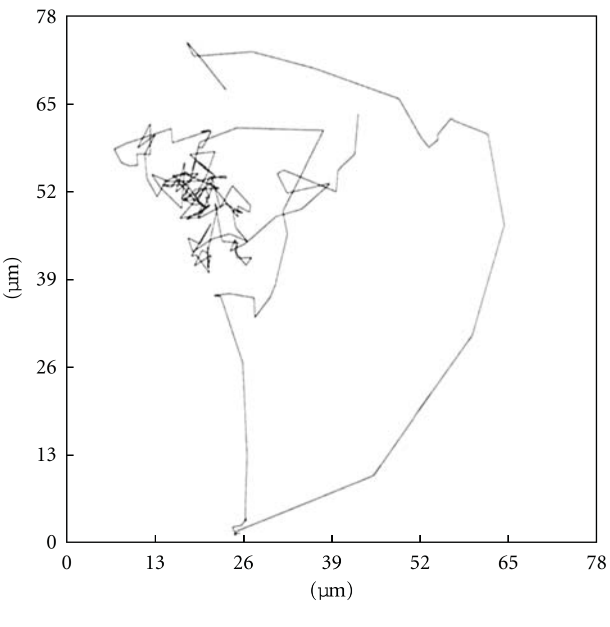

Figure 7

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Research Article

EhADH112 Is a Bro1 Domain-Containing Protein Involved in the *Entamoeba histolytica* Multivesicular Bodies Pathway

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EhADH112 is an *Entamoeba histolytica* Bro1 domain-containing protein, structurally related to mammalian ALIX and yeast BRO1, both involved in the Endosomal Sorting Complexes Required for Transport (ESCRT)-mediated multivesicular bodies (MVB) biogenesis. Here, we investigated an alternative role for EhADH112 in the MVB protein trafficking pathway by overexpressing 166 amino acids of its N-terminal Bro1 domain in trophozoites. Trophozoites displayed diminished phagocytosis rates and accumulated exogenous Bro1 at cytoplasmic vesicles which aggregated into aberrant complexes at late stages of phagocytosis, probably preventing EhADH112 function. Additionally, the existence of a putative *E. histolytica* ESCRT-III subunit (EhVps32) presumably interacting with EhADH112, led us to perform pull-down experiments with GST-EhVps32 and [^{35}S]-labeled EhADH112 or EhADH112 derivatives, confirming EhVps32 binding to EhADH112 through its Bro1 domain. Our overall results define EhADH112 as a novel member of ESCRT-accessory proteins transiently present at cellular surface and endosomal compartments, probably contributing to MVB formation during phagocytosis.

1. Introduction

Entamoeba histolytica, the causative agent of human amoebiasis provokes the second worldwide highest rates of morbidity and mortality due to protozoa [1]. *E. histolytica* trophozoites obtain host nutrients from a very active uptake and efficient engulfment of bacteria, red blood cells (RBC), and cell debris [2], which makes them to be considered as professional phagocytes. Since *E. histolytica* phagocytosis-deficient mutants have a diminished virulence *in vitro* and *in vivo* [3, 4], and nonvirulent *E. histolytica* strains exhibit reduced rates of phagocytosis [5], this cellular event has been defined as a key virulence factor.

EhCPADH, an *E. histolytica* protein complex formed by the EhADH112 adhesin and the EhCP112 cysteine protease, has been widely involved in adherence to, phagocytosis, and destruction of target cells [6]. Bioinformatics analysis revealed that EhADH112 is structurally related to mammalian ALIX [7], an evolutionarily conserved, ubiquitously expressed and multifunction scaffold protein, originally identified by its association with proapoptotic signaling partners [8, 9]. Additional evidence has established that ALIX modulates other cellular mechanisms, including receptor downregulation [10, 11], endosomal protein sorting [12–14], integrin-mediated cell adhesion and extracellular matrix assembly [15], actin-based cytoskeleton remodeling [16, 17], and membrane invagination and abscission in cytokinesis and retroviral budding [18].

ALIX is an abundant cytoplasmic protein with a multimodular architecture containing an N-terminal "banana"-

shaped Bro1 domain [19], a middle "V"-shaped domain [20] and a C-terminal proline-rich region [21]. This tripartite domain organization occurs in the majority of ALIX orthologues and provides them multiple protein-binding sites for specific roles in several cellular processes, and the possibility of linking proteins into distinct networks, thus acting as scaffold proteins [22].

Much of what is known about ALIX has stemmed from the characterization of its closest orthologue, yeast BRO1, a crucial component of the Endosomal Sorting Complexes Required for Transport (ESCRT) pathway [23, 24]. The ESCRT machinery comprises a set of protein complexes (ESCRT-0,-I, -II, -III, and -associated proteins) most of them constituted by the so-called Vacuolar protein sorting (Vps) factors. The assembly of the ESCRT apparatus at the endosomal surface is required to selectively transport ubiquitinated receptors and other cargo proteins into late endosomes known as multivesicular bodies (MVB), towards final degradation into the vacuole or lysosome [25, 26]. In this process, human ALIX or yeast BRO1 promotes endosomal membrane scission for intraluminal vesicles formation of MVB, drived by the direct association of their N-terminal Bro1 domains to the human CHMP4 or yeast Vps32 (also named Snf7) ESCRT-III subunits, respectively [19, 20].

Despite significant advances in the understanding of EhADH112 functions related to parasite virulence [6], its structural relationship with ALIX and BRO1 proteins [7] and recent evidence regarding the existence of most ESCRT components [27] and MVB-like organelles in *E. histolytica* [28], the potential role of EhADH112 in ESCRT-dependent protein sorting and trafficking along the MVB pathway had not yet been explored. The Bro1 domain occurs in a wide group of eukaryotic proteins that serve as scaffold for linking different cellular networks, including MVB formation dependent on ESCRT. In fact, a well-known hallmark for Bro1 domain functionality is its ability to bind to ESCRT-III subunits. Here, we initiated the characterization of the N-terminal residues comprising the small EhADH112 Bro1 domain originally defined by the Pfam protein domain database [29], but later extended by crystallographic experiments [19]. The tertiary structure modeling of EhADH112, presented in this study, predicted the spatial conformation needed for putative interaction with ESCRT-III subunits via the Bro1 domain. To explore if EhADH112 could be involved in MVB formation during phagocytosis, we generated a trophozoite population (ANeoBro1) overexpressing the first 166 amino acids of the EhADH112 Bro1 domain. ANeoBro1 trophozoites dramatically diminished their rates of phagocytosis, possibly due to an impairment of EhADH112 functions, seemingly produced by exogenous Bro1 accumulation in cytoplasmic vesicles and aberrant complexes, and its absence in plasma membrane and phagosomes, where EhADH112 exerts its role for target cell adherence and phagocytosis. Electron immunolocation of EhADH112 in structures resembling MVB, together with its finding in both soluble and insoluble subcellular fractions, suggested its participation in MVB formation. Moreover, EhADH112 *in vitro* binding to a protein homologous to Vps32 (EhVps32), strengthened our hypothesis regarding the EhADH112 contribution to ESCRT-mediated protein sorting along the MVB pathway by virtue of its Bro1 domain. Altogether, our results define EhADH112 as a novel member of Bro1 domain-containing proteins present at cellular surface and endosomal compartments with a potential role in the MVB pathway.

2. Materials and Methods

2.1. Tertiary (3D) Protein Modeling. The EhADH112 primary sequence was submitted to the Phyre Server (http://www.sbg.bio.ic.ac.uk/~phyre/) and validated by the Swiss Model Database. EhADH112 3D modeling was performed with human ALIX (2oev) and yeast BRO1 (1z1b) crystallized sequences as templates. Results were documented and analyzed through the DeepView-Swiss-Pdb Viewer software.

2.2. E. histolytica Cultures. Trophozoites of clone A (strain HM1: IMSS) were axenically cultured in TYI-S-33 medium at 37°C [30]. Medium for transfected trophozoites (ANeo, ANeoADH112 and ANeoBro1 populations) was supplemented with 40 μg/mL geneticin (G418) (Life Technologies, Gaithesburg, MD). Trophozoites were harvested in logarithmic growth phase for all experiments and cell viability was monitored by microscopy using Trypan blue dye exclusion tests.

2.3. PCR Amplification of the Bro1-FLAG Encoding Fragment. A 498 bp fragment from the 5′ end of the *EhAdh112* gene, corresponding to the first 166 amino acids of the EhADH112-Bro1 domain was PCR-amplified, using the sense (1–24 *EhAdh112* nt) 5′-GGGGTACCATGAATAGACAATTCATTCCTGAA-3′ and the antisense (477–497 *EhAdh112* nt) 5′-CGGGATCCTTACTTATCGTCGTCATCCTTGTAATCACTACCTGCTGCACATAGTTGG-3′ oligonucleotides, 10 mM dNTPs, 100 ng of *E. histolytica* genomic DNA as template and 2.5 U of *Taq* DNA polymerase (Gibco). PCR was carried out for 30 cycles comprising 1 min at 94°C, 30 sec at 59°C, and 40 sec at 72°C. The sense oligonucleotide contained a *Kpn*I restriction site, whereas the antisense oligonucleotide contained the FLAG (DYKDDDDK) tag-encoding sequence (underlined) [31] and a *Bam*H1 restriction site. Oligonucleotides used in this work specifically recognize sequences present in the *EhAdh112* gene but not those present in the previously reported *EhAdh112*-like genes [7].

2.4. Generation of ANeoBro1 Trophozoites by Transfection with the pNeoBro1FLAG Plasmid. The PCR-amplified product *(Bro1FLAG)* was cloned into the *Bam*H1 and *Kpn*I sites of the pExEhNeo (pNeo) plasmid, which contains *E. histolytica*-specific transcription signals and the G418 resistance (Neo^R) conferring gene as selectable marker [32], producing the pNeo*Bro1FLAG* construct. *Escherichia coli* DH5α bacteria were transformed with the pNeo*Bro1FLAG* or pNeo plasmids. Both plasmids were purified using the QIAGEN Maxi kit (Chatsworth, CA) and automatically sequenced. Plasmids

(200 μg) were transfected by electroporation as previously described [32] into exponentially growing trophozoites of clone A, generating the ANeo and ANeoBro1 trophozoite populations.

2.5. RT-PCR Experiments. cDNAs were synthesized using 1 μg of DNase-treated total RNA, 10 mM dNTPs, 200 U AMV reverse transcriptase (Gibco) and 0.5 μg of oligo dT (Gibco) in a final volume of 10 μL, for 1 h at 42°C. PCR amplifications were carried out using 4 μL (~330 ng) of cDNA, 1 U of *Taq* polymerase, 2 mM dNTPs, and 100 ng of the sense (1–24 nt) and the antisense (477–497 nt) oligonucleotides from the *EhAdh112* gene. Additionally, the sense (1–17 nt) 5′-ATGATTGAACAAGATGG-3′ and the antisense (780–794 nt) 5′-TTAGAAGAACTCGTC-3′ primers were used to amplify a 794 bp fragment of the Neo^R gene. Each PCR was performed as described above. Amplified products were separated by 1% agarose gel electrophoresis, ethidium bromide-stained and visualized in a Gel Doc 1000 apparatus (BioRad).

2.6. Immunofluorescence Assays. Trophozoites grown on coverslips were fixed with 4% paraformaldehyde (PFA) (Sigma) at 37°C for 1 h, permeabilized with 0.5% Triton X-100 in PBS (PBS-Triton) for 30 min and incubated with 1% bovine serum albumin (BSA) for 40 min at 37°C. Trophozoites were incubated with mouse monoclonal antibodies against EhADH112 (mαEhADH112) (1 : 10) or rabbit polyclonal anti-FLAG (pαFLAG) (USBiological) antibodies (1 : 500), overnight (ON) at 4°C, followed by incubation with fluorescein-isothiocyanate (FITC)-labeled anti-mouse or anti-rabbit secondary antibodies (1 : 100) (Zymed), respectively, for 1 h at 37°C.

For colocalization experiments, PFA-fixed trophozoites were incubated ON at 4°C with mαEhADH112 and pαFLAG antibodies, followed (1 h at 37°C) by FITC-labeled anti-mouse IgM and tetramethyl-rhodamine-isothiocyanate-(TRITC)-labeled anti-rabbit IgG secondary antibodies. For some experiments, trophozoites were first incubated with fresh RBC (1 : 40) for different times at 37°C, fixed with PFA, contrasted with 3 mM diaminobenzidine (DAB) and treated for immunofluorescence assays as above. All preparations were preserved using the antifade reagent Vectashield (Vector) and examined through a Nikon inverted microscope attached to a laser confocal scanning system (Leica).

2.7. Adherence and Phagocytosis Kinetics. Trophozoites were incubated for 5, 10, and 15 min with freshly obtained human RBC (10^8 cells/mL) (1 : 100 ratio) at 4°C for adherence, or at 37°C for phagocytosis experiments [33]. RBC were contrasted with DAB and counted at random from three independent experiments to determine the number of RBC adhered to or ingested by 100 trophozoites.

2.8. Transmission Electron Microscopy (TEM) Assays. Fast-freeze fixation followed by cryosubstitution was used for ultrastructural location of EhADH112 and the FLAG-tagged Bro1 recombinant polypeptide. Transfected trophozoites were pelleted and placed into the hole of a 7 mm diameter antiadhesive plastic ring positioned on a foam rubber support and frozen on a copper mirror precooled to liquid nitrogen temperature using a Reichert KF 80 unit. Freeze substitution was achieved with a Reichert CS autosystem in acetone containing 4% osmium tetroxide for 48 h at −80°C. Afterwards, samples were brought to room temperature at a rate of 4°C/h and embedded in epoxy resins. Ultrathin sections were obtained and stained with uranyl acetate and lead citrate. For immunogold labeling, thin sections were placed on formvar-coated nickel grids and incubated for 15 min in 0.1 M ammonium chloride. Then, sections were washed twice with PBS (5 min each), blocked with 1% BSA for 15 min, and incubated with polyclonal rabbit anti-EhADH112 (pαEhADH112) or monoclonal mouse anti-FLAG (mαFLAG) (Sigma) antibodies at 1 : 50 or 1 : 20 dilutions, respectively, for 60 min. Then, sections were washed and incubated for 1 h with goat anti-rabbit or anti-mouse IgG (1 : 20) antibodies conjugated to 15 nm colloidal gold (BBI International, Cardiff, UK). Finally, sections were washed and treated as above to be observed through a Zeiss EM 910 electron microscope. For phagocytosis experiments, trophozoites were first incubated with fresh RBC (1 : 40 ratio) at 37°C for 15 min and treated for TEM as described.

2.9. Isolation of E. histolytica Membranes. *E. histolytica* cellular fractions were obtained as described by Aley et al. [34]. Briefly, trophozoites from wild-type clone A (40×10^6) were harvested, washed twice with 19 mM potassium phosphate buffer, pH 7.2, and 0.27 M NaCl (PD solution) and pooled. Cell pellet was resuspended to 2×10^7 cells/mL PD solution containing 10 mM $MgCl_2$ and rapidly mixed with an equal volume of 1 mg/mL concanavalin A in the same buffer. After 5 min, cells were spun at 50 ×g for 1 min to remove the excess of concanavalin A. The supernatant was discarded and cell pellet was resuspended in 12 mL of 10 mM Tris-HCl buffer, pH 7.5, containing 2 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM $MgCl_2$. After 10 min swelling in hypotonic buffer, cells were homogenized by 18–20 strokes of a glass Dounce homogenizer with a tight-fitting pestle (Wheaton Scientific Div.). Cell lysis and membrane sheets formation were verified by phase-contrast microscopy. The homogenate was layered over a two-step gradient consisting of 8 mL of 0.5 M mannitol over 4 mL of 0.58 M sucrose, both in Tris buffer, and spun at 250 ×g for 30 min. Material remaining at the top of the 0.5 M mannitol (SN1) was centrifuged at 40 000 ×g for 1 h to separate soluble molecules (SN2) from small membrane fragments and vesicles (P2). Large plasma membrane fragments and other heavy debris formed a tight pellet at the bottom of the gradient (P1). This pellet was resuspended in 1 mL Tris buffer containing 1 M α-methyl mannoside and left on ice for 40 min with occasional mixing. Plasma membranes free of concanavalin A were diluted into three volumes of Tris buffer, homogenized by 80 strokes with a glass Dounce homogenizer, layered on a 20% sucrose Tris gradient and spun for 30 min at 250 ×g. Vesiculated plasma membranes floating above the initial sucrose layer (SN3) were collected and then concentrated by centrifugation at 40 000 ×g for 1 h. The pellet (P4), enriched

in plasma membranes, was resuspended in Tris buffer. All steps were performed at 4°C. Samples (50 μg) were analyzed by SDS-PAGE (10%) and transferred onto nitrocellulose membranes for Western blot assays using rabbit polyclonal antibodies against the last C-terminal 243 amino acids of EhADH112 (pαEhADH243) and peroxidase-labeled anti-rabbit IgG secondary antibodies, at 1 : 300 and 1 : 10 000 dilutions, respectively. As a control, mouse monoclonal anti-*E. histolytica* actin and peroxidase-labeled anti-IgG corresponding secondary antibodies were used at 1 : 1 500 and 1 : 10 000, respectively.

2.10. In Vitro Binding Assays. A plasmid encoding glutathione S-transferase (GST)-*EhVps32* was constructed by inserting a PCR fragment containing the EhVps32 coding sequence into the *Bam*H1 site of pGEX-5X-1 (Pharmacia). Plasmids used for *in vitro* synthesis of *EhAdh112* and *EhAdh112*-truncated derivatives were constructed by inserting PCR-amplified or restriction fragments containing the corresponding EhADH112 coding sequences in the polylinker of pGBKT7 (Clontech). GST-*EhVps32* and GST alone were expressed in *E. coli* BL21 bacteria. Cultures (50 mL) were induced at 30°C after addition of 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and incubated for additional 2.5 h before proceeding with the GST fusion protein purification [35]. [^{35}S]-EhADH112 and [^{35}S]-EhADH112 truncated derivatives were synthesized *in vitro* using the Promega TNT coupled transcription-translation system in the presence of [^{35}S] methionine (1 000 Ci/mmol). Labeling reaction (2 μl) was added to glutathione beads loaded with GST-EhVps32 or GST proteins and incubated at 4°C for 1 h in 500 mL of a 10 mM Tris HCl, pH 8.0, 1 mM EDTA and 150 mM NaCl solution (STE) with 1% (v/v) Triton X-100. After five washes with STE/1% Triton X-100, beads were boiled in sample buffer and proteins were separated by 10% polyacrylamide SDS-PAGE. Bound proteins were detected by autoradiography or Coomassie blue staining of gels.

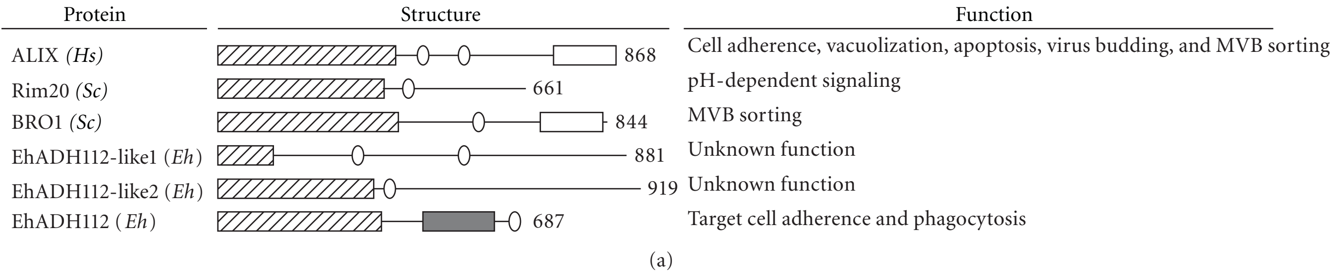
3. Results and Discussion

3.1. Tertiary Structure Modeling of EhADH112 Predicts Conformational Similarities to ALIX. Bro1 domain-containing proteins are highly conserved among eukaryotes and exhibit distinct functions in several cellular processes (Figure 1(a)), depending on the partners they interact with [22]. EhADH112 displays 40% homology and 20% identity to human ALIX [7] and its primary sequence aligns with the portion of ALIX corresponding to the N-terminal Bro1 and the middle “V” domains. More divergent relatives of ALIX only contain the Bro1 domain but otherwise, bear little resemblance to the remaining structure [36, 37]. Although EhADH112 lacks the proline-rich C-terminal tract that harbors the majority of protein-binding sites linking ALIX to various functions [7, 22], instead, this protein has a target cell adherence domain, which binds to a 97 kDa protein in epithelial cells [38]. Besides EhADH112, the *E. histolytica* genome predicts two *EhAdh112*-related genes encoding the EhADH112-like1 (898 amino acids) and EhADH112-like2 (919 amino acids) proteins [7], both displaying a putative Bro1 domain at their N-terminus (Figure 1(a)). However, the existence of these proteins has not yet been confirmed in trophozoites.

Our current predictions for EhADH112 tertiary structure resulted in protein overlapping to ALIX (Figure 1(b)). Models determined the boomerang or “banana”-shaped spatial conformation for the N-terminal EhADH112 Bro1 domain, mostly made of α-helices forming a solenoid, with helices 6 through 11 arranged in a tetratricopeptide repeat-like structure [19], and a central core arranged in two extended three-helix bundles forming elongated arms that fold back into a “V” (Figure 1(b)). Arms conformation suggests that the “V” domain may act as a hinge, changing in response to ligand binding, as described for ESCRT and viral proteins interaction with ALIX [18]. Apparently, EhADH112 conserves the two hydrophobic patches [19] required for ALIX or BRO1 endosomal membrane targeting and association, via direct binding to ESCRT-III CHMP4 or Vps32 subunits, respectively (Figures 1(b) and 1(c)), or Src-tyrosine kinase docking. Since the conserved interaction of ALIX or BRO1 with ESCRT-III is necessary for membrane inward budding and MVB biogenesis during protein sorting and trafficking [39], it is possible that EhADH112 could associate to putative *E. histolytica* ESCRT-III components via its Bro1 domain.

3.2. Generation of Trophozoites Overexpressing the Bro1 Recombinant Polypeptide. To initiate the characterization of the EhADH112 Bro1 domain, trophozoites of clone A (HM1:IMSS) were transfected with the pNeo*Bro1FLAG* plasmid (Figure 1(d)) driving the expression of the recombinant Bro1 polypeptide and generating the ANeoBro1 population. The presence and expression of plasmids in trophozoites was confirmed by RT-PCR amplification of the *Neo*^{R} gene. *Neo*^{R} was amplified from ANeo (transfected with empty pNeo) and ANeoBro1 trophozoites (Figure 1(e), lane 3), whereas a transcript corresponding to the *Bro1FLAG* fragment, was only detected in the ANeoBro1 population (Figure 1(e), lane 4), as expected. No amplification was detected in the absence of reverse-transcriptase in reaction mixtures or using total RNA from wild type clone A trophozoites (Figure 1(e), lanes 1 and 2).

3.3. ANeoBro1 Trophozoites Localize the Exogenous Bro1 Recombinant Polypeptide in Cytoplasmic Compartments. As part of the EhCPADH complex, an *E. histolytica* surface heterodimer involved in target cell adherence, phagocytosis, and destruction, EhADH112 is located at trophozoite plasma membrane and cytoplasmic vacuoles [6]. To determine the location of the Bro1 polypeptide overexpressed by ANeoBro1 trophozoites and to distinguish it from the Bro1 domain present in endogenous EhADH112, immunofluorescence experiments were carried out using polyclonal antibodies against the FLAG tag (pαFLAG) and monoclonal antibodies (mαEhADH112) against the EhADH112 carboxy terminus adherence epitope (444–601 amino acids). ANeo



(a)

(b)

(c)

(d)

(e)

Figure 1: Structural features of EhADH112 and generation of ANeoBro1 trophozoites. (a) Structural features of representative Bro1 domain-containing proteins and EhADH112- and EhADH112-like proteins: Bro1 domain (squares containing diagonal lines), coiled coil regions (ellipses), proline-rich tracts (white squares), and adherence region (gray square). *Hs: Homo sapiens; Sc: Saccharomyces cerevisiae; Eh: Entamoeba histolytica*. Numbers: amino acid number of each protein. (b-c) Spatial conformation of EhADH112 and its Bro1 domain. (b) Ribbon representation for predicted EhADH112 tertiary and human ALIX crystallized structures and (c) Bro1 domains from EhADH112, ALIX and yeast BRO1 proteins. N: amino terminus. C: carboxy terminus. TPR: tetratricopeptide repeat. (d) Schematic depiction of the pNeo*Bro1FLAG* plasmid. K: *Kpn*I restriction site. B: *Bam*H1 restriction site. (e) Transcripts obtained by RT-PCR assays using oligonucleotides for the Neo^R gene or *Bro1FLAG* sequence and cDNAs synthesized from nontransfected clones A (lane 2), ANeo (lane 3), and ANeoBro1 (lane 4). Lane 1 corresponds to the reaction mixture without reverse transcriptase, using total RNA from ANeoBro1.

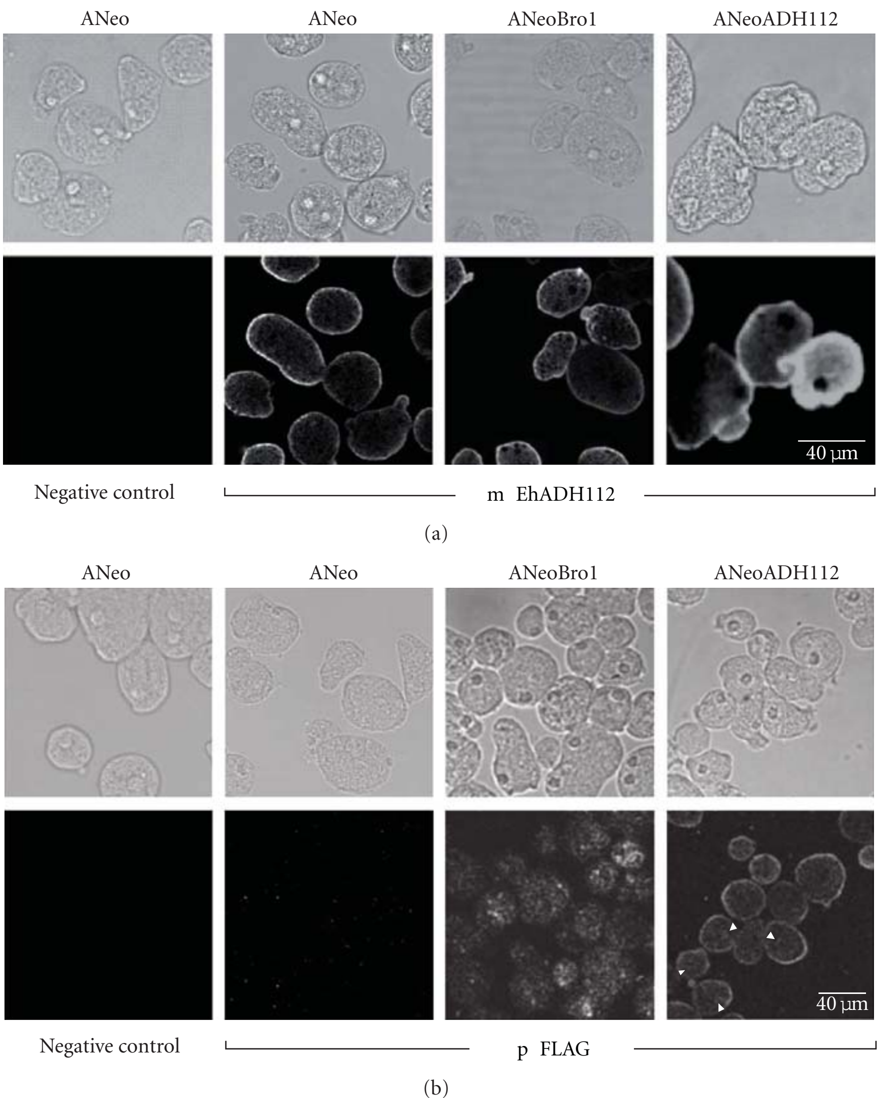


Figure 2: Cellular location of EhADH112 and the Bro1 recombinant polypeptide in transfected trophozoites. Confocal microscopy images of permeabilized ANeo, ANeoBro1, or ANeoADH112 trophozoites incubated with (a) mαEhADH112 or (b) pαFLAG antibodies. Negative controls correspond to trophozoite preparations only incubated with secondary antibodies. *Top*: phase contrast images. *Bottom*: corresponding confocal sections. Arrowheads: small cytoplasmic vesicles.

trophozoites, as well as a trophozoite population named ANeoADH112 that overexpresses the EhADH112 full length protein fused to a FLAG tag (EhADH112-FLAG), were used here as additional controls [7].

By confocal microscopy, mαEhADH112 antibodies revealed the presence of EhADH112 at the plasma membrane of permeabilized ANeo, ANeoBro1, and ANeoADH112 trophozoites (Figure 2(a)), although fluorescence was higher in ANeoADH112 population, since these trophozoites express both, endogenous EhADH112 and exogenous EhADH112-FLAG.

As expected, pαFLAG antibodies gave no reaction with ANeo trophozoites (Figure 2(b)), but traced FLAG-tagged Bro1 as punctuated structures and patches of different sizes in the cytoplasm of ANeoBro1 trophozoites. Interestingly, no signals were detected at plasma membrane (Figure 2(b)), suggesting that the EhADH112 carboxy end, absent in the exogenous Bro1 recombinant polypeptide, could be participating in EhADH112 targeting to the trophozoite surface. Since EhADH112-FLAG appeared at the plasma membrane (Figure 2(b)) and some cytoplasmic vacuoles (Figure 2(b), arrowheads) of ANeoADH112 trophozoites, in a similar pattern to the one exhibited by endogenous EhADH112 in all parasite populations analyzed (Figure 2(a)), we rule out that the FLAG tag could be causing the defective targeting of recombinant Bro1 to the membrane. Negative results were likewise obtained with nontransfected or transfected trophozoites treated only with secondary antibodies. Here, we show results obtained by assaying ANeo trophozoites (Figures 2(a) and 2(b)).

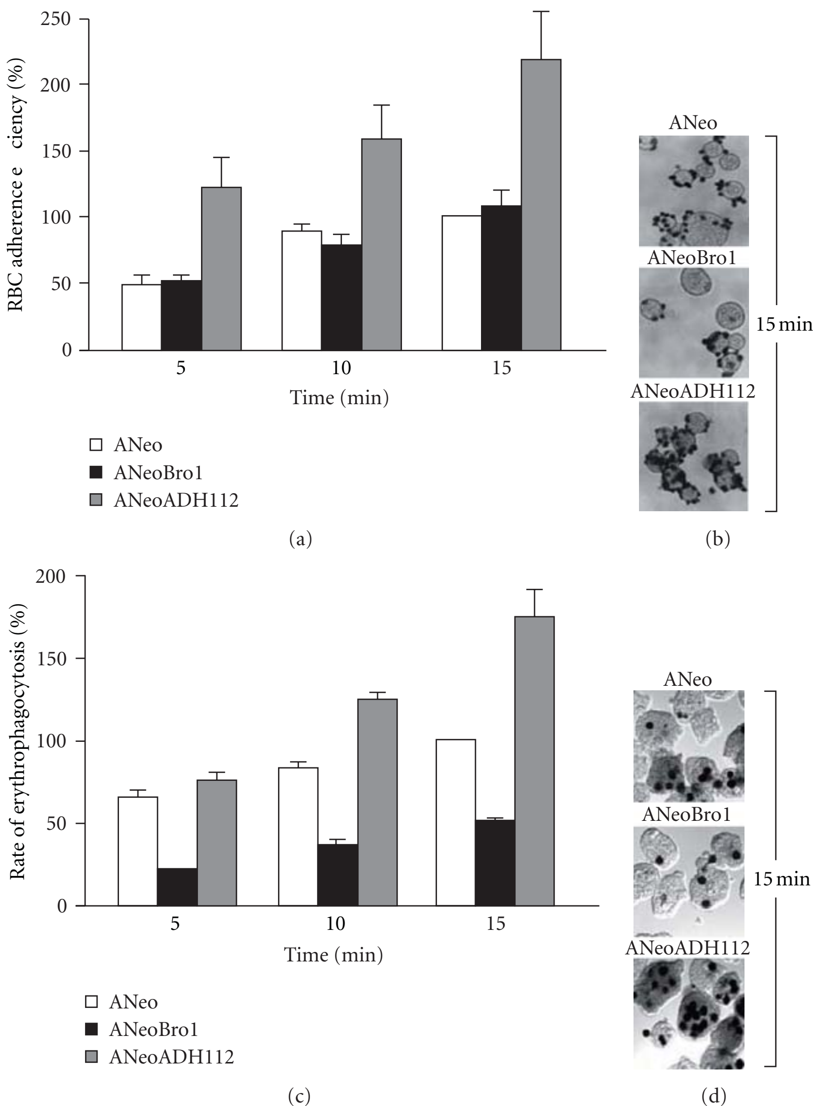


Figure 3: RBC adherence efficiency and rates of erythrophagocytosis exhibited by ANeo, ANeoBro1, and ANeoADH112 trophozoites. (a-b) Trophozoites were incubated with RBC (1 : 100) at 4°C for adherence and (c-d) at 37°C for phagocytosis, for different times. RBC were contrasted with DAB and counted in 100 randomly selected trophozoites under the light microscope to obtain the mean of RBC adhered or ingested by trophozoite. Bars represent the mean ± standard error of at least three independent experiments performed by duplicate.

From these experiments, we can conclude that the Bro1 recombinant polypeptide expressed by ANeoBro1 trophozoites is located at a different site than EhADH112, and that transfection procedures or expression of FLAG-tagged proteins did not affect the location of endogenous EhADH112 in trophozoites.

3.4. Expression of the Exogenous Bro1 Recombinant Polypeptide by ANeoBro1 Trophozoites Diminishes Erythrophagocytosis but Not Target Cell Adherence. EhADH112 has been previously characterized by the properties conferred by its C-terminus for target cell primary contact, internalization, and phagocytosis [6, 38]. To further understand the Bro1 domain contributions to EhADH112 functions in trophozoites, we carried out RBC adherence and erythrophagocytosis assays. Adherence efficiency and erythrophagocytosis rates exhibited by ANeo trophozoites at 15 min were taken as 100% in all experiments. Again, as an additional control we used ANeoADH112 trophozoites.

After 15 min of RBC incubation at 4°C, ANeo and ANeoBro1 trophozoites adhered a mean of 5.7 ± 3 and 6.6 ± 2 RBC per trophozoite, respectively (Figures 3(a) and 3(b)), meanwhile at 10 and 5 min they adhered 4.78 ± 1 and 4.56 ± 2, and 2.73 ± 2 and 2.8 ± 2 RBC per trophozoite, respectively. In contrast, ANeoADH112 showed an increased avidity to attach RBC, by adhering above twice more RBC than ANeo and ANeoBro1 at 15, 10, and 5 min (12.5 ± 2, 8.7 ± 2 and 7 ± 2 RBC per trophozoite, respectively) (Figures 3(a) and 3(b)). These results indicate that exogenous expression of the Bro1 recombinant polypeptide did not affect the adherence function of trophozoites and suggest

that the EhADH112 N-terminus may not be promoting target cell binding.

Interestingly, noteworthy differences were found in assays determining phagocytosis activity of transfected trophozoites. ANeo trophozoites ingested 14.3 ± 2 RBC per parasite at 15 min, 11.6 ± 2 RBC at 10 min and 9.4 ± 2 RBC at 5 min (Figure 3(c)), whereas ANeoBro1 trophozoites only ingested 7.1 ± 2, 5 ± 2 and 2.8 ± 2 RBC per trophozoite at 15, 10 and 5 min, respectively (Figures 3(c) and 3(d)). As previously reported [7], ANeoADH112 trophozoites exhibited augmented phagocytosis rates, by ingesting 76% more RBC than ANeo trophozoites at 15 min of erythrophagocytosis (Figures 3(c) and 3(d)), and 40% and 10% more, at 10 and 5 min, respectively (Figure 3(c)). These latter experiments indicate that overexpression of Bro1 results in a dominant negative effect on phagocytosis. We hypothesized that this phenomenon may be due to recruitment and association of proteins probably involved in target cell internalization and phagocytosis by the truncated EhADH112 protein instead of the endogenous one, thus producing a competition for protein binding sites and reducing trophozoites rates of ingestion, despite efficient primary cell contact. Otherwise, the Bro1 recombinant polypeptide *per se*, could be producing a conformational change in EhADH112 or preventing EhADH112 accessibility for the interaction with its counterparts, therefore causing a functional impairment.

Transfection procedures did not modify EhADH112 location and function, since ANeo trophozoites displayed similar results to the ones presented by nontransfected clone A trophozoites (data not shown).

3.5. The Bro1 Recombinant Polypeptide Exhibits a Different Cellular Location to Endogenous EhADH112 during Phagocytosis. Previous work using TEM and immunofluorescence experiments determined that the EhADH112 C-terminus mediates target cell adherence but also contributes to phagocytosis activity of *E. histolytica* trophozoites [6, 38]. The diminished phagocytosis rates exhibited by ANeoBro1 trophozoites led us to precise the location of EhADH112 and the overexpressed Bro1 polypeptide after 5 min of erythrophagocytosis.

Through confocal microscopy, and in agreement to preceding findings, EhADH112 was detected at plasma membrane, target cell contact sites, membrane extensions, and phagosomes of nontransfected clone A trophozoites (data not shown). A similar location was determined for EhADH112 in ANeo trophozoites (data not shown), which adhere to and phagocyte RBC in the same way than wild-type trophozoites do. Regarding ANeoBro1 trophozoites (Figure 4(a)), we observed EhADH112 at the trophozoite plasma membrane, cytoplasmic vacuoles and phagosomes (Figure 4(a), top). Besides, small phagosome-neighbouring vesicles that may correspond to endosomes or lysosomes were detected (Figure 4(a), asterisks). Otherwise, the Bro1 recombinant polypeptide was found in cytoplasmic punctuated and vesicular structures and, significantly, it was accumulated in vacuolar compartments (Figure 4(a), arrows) that did not overlap to RBC location (Figure 4(a)). Images also evidenced that exogenous Bro1 did not reach trophozoites plasma membrane at early stages of phagocytosis and was absent in RBC-containing phagosomes. The different location of EhADH112 and recombinant Bro1 in trophozoites under basal culture conditions, even during phagocytosis, together with the impairment of ANeoBro1 erythrophagocytosis rates and the presence of aggregates containing exogenous Bro1, suggest that this polypeptide could be interfering with the function of trophozoite proteins present at the plasma membrane, probably participating in membrane remodeling and RBC internalization into phagosomes. Control experiments, omitting primary antibodies gave no signals in trophozoites (Figure 4(a), bottom).

3.6. Exogenous Bro1 Accumulates in Aberrant Compartments at Late Stages of Phagocytosis. It has been previously shown that endogenous EhADH112 protein changes its location within trophozoites during RBC phagocytosis. After target cell contact, this adhesin is translocated from the trophozoite plasma membrane to the phagocytic vacuoles. As the ingestion process advances, EhADH112 is found in RBC and after 30 min, it comes back to the plasma membrane [6]. To elucidate whether exogenous Bro1 localizes at some point to the plasma membrane or to phagosomes and to better understand its fate along the erythrophagocytosis process, we followed this recombinant polypeptide in ANeoBro1 trophozoites at different times of RBC ingestion. Immediately after RBC interaction (0 min), exogenous Bro1 appeared in randomly distributed cytoplasmic vesicles and patches of distinct sizes and morphologies (Figure 4(b)). As RBC ingestion progressed (5 min), the exogenous Bro1 recombinant polypeptide accumulated in a ring-like structure surrounding RBC-containing compartments probably corresponding to phagosomes or phagolysosomes (Figure 4(b)). This Bro1-enriched large structure achieved a closer proximity to RBC at 10 min of phagocytosis, although no RBC overlapping was observed (Figure 4(b)). At late phagocytosis stages (15 and 20 min), the Bro1 recombinant polypeptide appeared in huge tubular structures (Figure 4(b)). In yeast, deletion or inactivation of several Vps factors, which assemble into the ESCRT machinery during protein sorting and trafficking through endosomal compartments, induces the formation of enlarged vacuolated and tubulated organelles that fail to mature into MVB [40, 41]. Hence, the large vacuoles observed in ANeoBro1 trophozoites may correspond to aberrant endosomal compartments where the exogenous Bro1 polypeptide is stuck, affecting the dynamics of RBC internalization from the membrane to the phagosomes. Work in progress in our laboratory, using specific biochemical markers, will allow us to determine the identity of these structures.

Since immunoelectron microscopy is a key technique to place macromolecular functions within a cellular context, we addressed the ultrastructural location of exogenous Bro1 in ANeoBro1 trophozoites under basal culture conditions and at 15 min of erythrophagocytosis. For these experiments we used mouse monoclonal antibodies against the FLAG tag (mαFLAG), which resulted to be more sensitive for specific

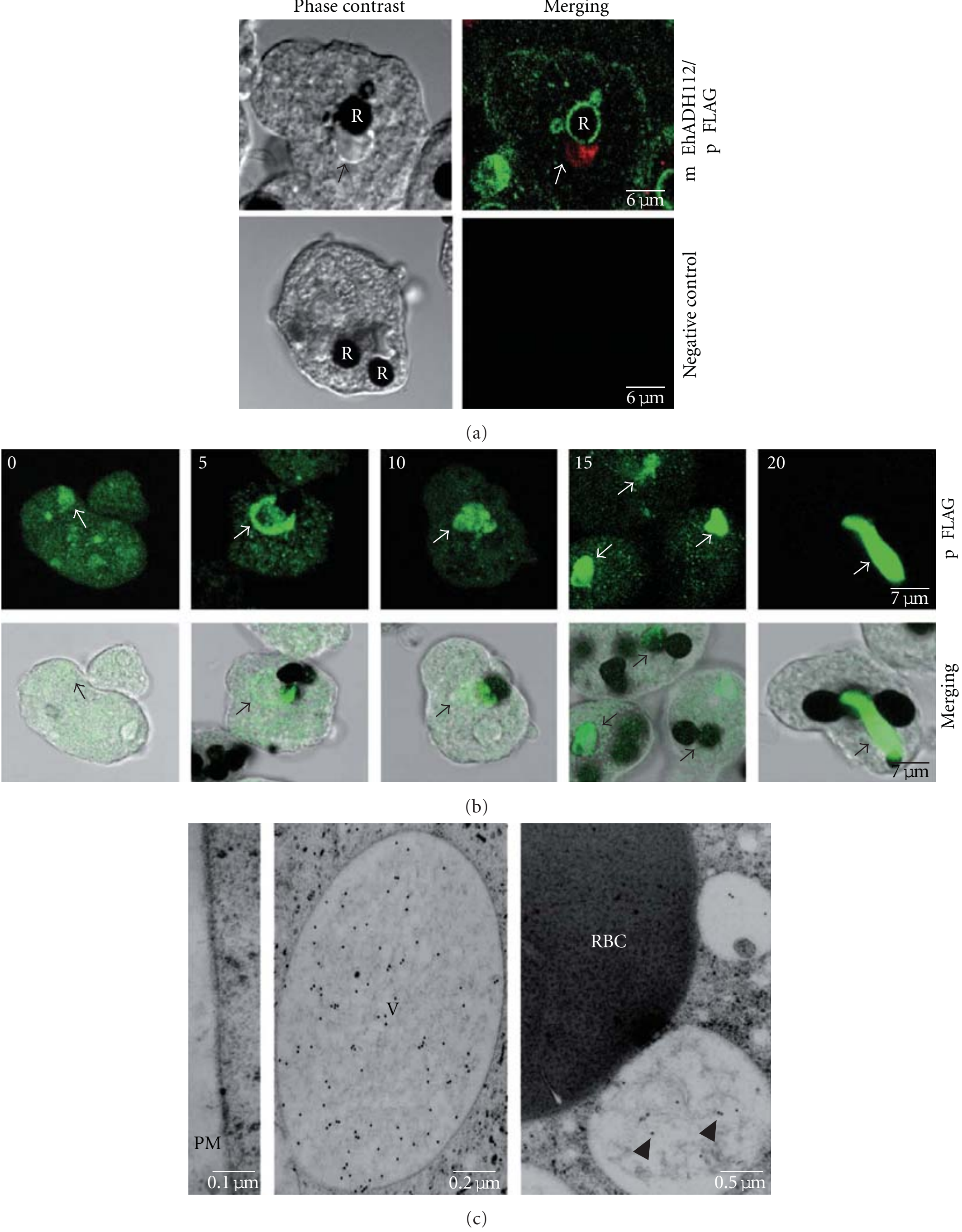


Figure 4: Differential location of endogenous EhADH112 and the Bro1 recombinant polypeptide in ANeoBro1 trophozoites during phagocytosis. (a) Cellular immunolocation of endogenous EhADH112 and exogenous Bro1 in ANeoBro1 trophozoites after RBC ingestion. Trophozoites were incubated with RBC for 5 min and permeabilized. *Top*: after RBC contrasting with DAB, EhADH112 was detected by mαEhADH112 antibodies and FITC-labeled anti-mouse secondary antibodies. The FLAG-tagged Bro1 recombinant polypeptide was detected by pαFLAG antibodies and TRITC-labeled anti-rabbit secondary antibodies. *Bottom*: trophozoites incubated with FITC-labeled anti-mouse IgM and TRITC-labeled anti-rabbit IgG secondary antibodies. Preparations were examined through laser confocal microscopy. R: RBC. Arrows: recombinant Bro1 containing vacuoles. Asterisks: EhADH112 present in endosomal or lysosomal-like compartments. (b) Immunolocation of exogenous Bro1 in ANeoBro1 trophozoites at different times of erythrophagocytosis. Trophozoites were incubated with fresh RBC and treated as described above. The Bro1 recombinant polypeptide was detected by pαFLAG and FITC-labeled anti-rabbit secondary antibodies. Preparations were examined through a laser confocal microscope. *Top*: confocal sections. *Bottom*: merging of phase contrast images and laser confocal corresponding sections. Arrows: vesicles and large vacuoles containing exogenous Bro1. (c) Ultrastructural location of exogenous Bro1 in ANeoBro1 trophozoites after RBC ingestion. Ultrathin sections of ANeoBro1 trophozoites were processed for immunogold labeling and TEM as described above. The Bro1 recombinant polypeptide was detected by mαFLAG antibodies and gold-labeled secondary antibodies. *Left*: plasma membrane (PM). *Middle*: a huge vacuole. *Right*: vacuoles in the proximity of RBC after 15 min phagocytosis. V: vacuole. Arrowheads: Bro1 gold-labeled particles.

recognition of the FLAG-tagged Bro1 recombinant polypeptide than the pαFLAG ones.

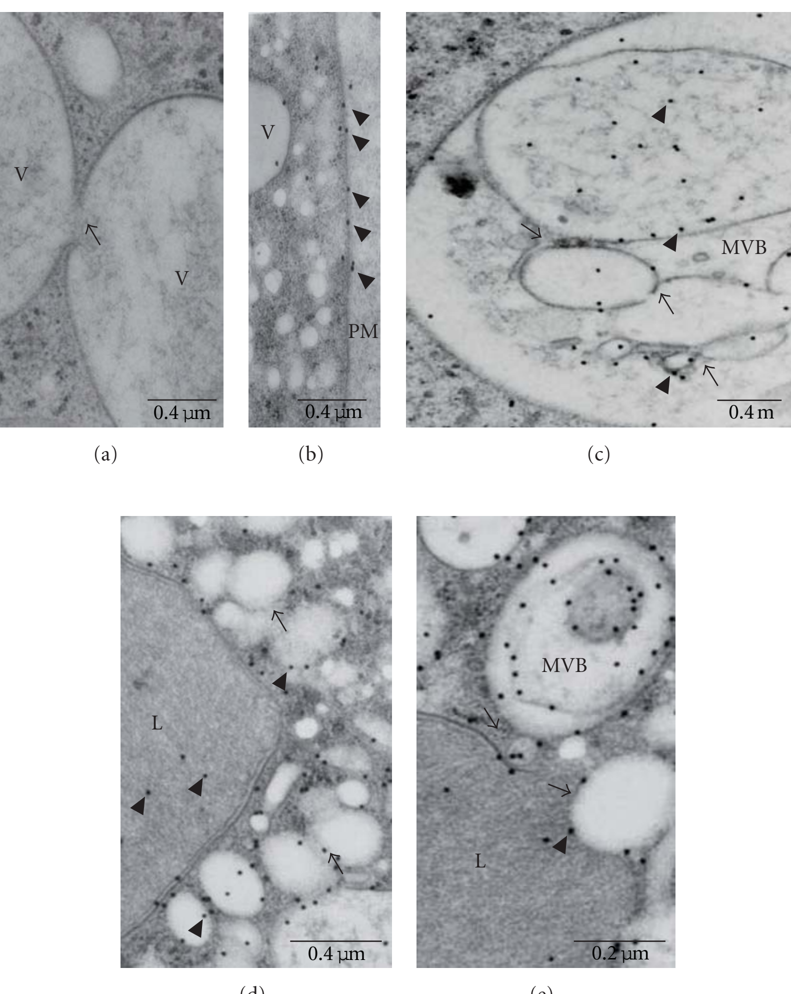
In agreement with the immunofluorescence results showed here (Figures 2, 4(a) and 4(b)), mαFLAG antibodies did not reveal any signal at the plasma membrane of ANeo-Bro1 trophozoites in culture (data not shown), and neither at 15 min of RBC ingestion (Figure 4(c), left panel). Numerous gold particles associated to fibrillar material were observed within huge vesicles (Figure 4(c), middle panel) that might correspond to the large vacuoles detected in Figures 4(a) and 4(b). Moreover, mαFLAG antibodies recognized some vesicles close to RBC (Figure 4(c), right panel), confirming that recombinant Bro1 remains in vacuolar compartments of different sizes, but not in RBC or phagosomes (Figure 4(c), right panel). Taken together, our immunolocation results revealed that under culture conditions, exogenous Bro1 is profusely distributed in different-sized cytoplasmic vesicles of ANeoBro1 trophozoites. Similarly, at initial steps of target cell contact, the Bro1 recombinant polypeptide remains in the cytoplasm of ANeoBro1 trophozoites, which preserve their ability to attach to target cells, but significantly decreased their phagocytosis rates. Location of exogenous Bro1 in small structures that gradually come together to transform into exaggerated vacuolar compartments at late stages of phagocytosis suggests that at these sites, recombinant Bro1 could also be retaining proteins involved in RBC internalization, targeting, and phagocytosis, some of them even affecting the function of EhADH112, or that this huge protein complexes may block the recycling of proteins back to the trophozoite membrane, where tentatively they would contribute to the membrane remodeling and protein assembly processes required for phagosome formation.

3.7. EhADH112 Localizes in Structures Resembling MVB in ANeoBro1 Trophozoites. According to our immunofluorescence assays, the location of endogenous EhADH112 at the plasma membrane of ANeoBro1 trophozoites was not affected by the overexpression of recombinant Bro1 under basal conditions. In fact, this resulted in RBC adherence efficiencies similar to that displayed by wild-type and ANeo trophozoites. Since EhADH112 was also found in cytoplasmic vacuoles that could differ or not from that in which recombinant Bro1 accumulated, we assessed the ultrastructural location of EhADH112 in ANeoBro1 trophozoites by immunogold labeling experiments, using polyclonal rabbit antibodies against EhADH112 (pαEhADH112) (Figure 5), which gave a better reactivity on frozen ultrathin sections than mαEhADH112 ones. Of note, pαEhADH112 antibodies do not recognize the EhADH112-like protein sequences previously reported by our group, since Western blot assays specifically detect the band corresponding to the predicted molecular weight of EhADH112 (data not shown).

Through TEM, pαEhADH112 antibodies revealed EhADH112 at the plasma membrane of both, ANeo (data not shown) and ANeoBro1 trophozoites (Figure 5(b)). Gold labeling was also observed at external and internal faces of vesicle membranes and inside vesicles, frequently associated to fibrillar and membranous material (Figures 5(c)–5(e)). Interestingly, EhADH112 was abundant within large vacuoles containing several tubular and vesicular structures. Huge organelles, containing intraluminal vesicles of different sizes and shapes, the majority of them immunolabeled, appeared in many trophozoites (Figure 5(c)). By their morphology, these compartments may correspond to MVB, which showed a high similarity to the ones described in mammals by Denzer et al. [42]. We also detected numerous whitish EhADH112-carrying vesicles outside electrodense structures with lysosome appearance, which also exhibited EhADH112 signals (Figure 5(d)). These lysosome-like structures presented a delimiting double membrane labeled with EhADH112 (Figures 5(d) and 5(e)). Strikingly, several vacuolar structures, one contained inside the other, which suppose membrane inward budding of vesicles (Figure 5(e), arrows), bordered lysosome-like organelles and carried EhADH112. Recurrent and profuse docking of EhADH112 to the membrane of different-sized vesicles, suggest its possible participation in protein sorting along endosomal compartments and vesicle fusion processes (Figures 5(d) and 5(e)).

3.8. EhADH112 Appeared on RBC and Phagosomes during Phagocytosis. At the beginning of erythrophagocytosis, trophozoite membrane proteins make contact with RBC. Immediately, not yet well-understood signaling processes occur, allowing the recruitment of molecules with different roles in the uptake and digestion of target cells. In this paper, our immunofluorescence experiments evidenced that overexpression of the Bro1 recombinant polypeptide in ANeoBro1 trophozoites did not affect EhADH112 location at target cell contact sites and phagosomes (Figure 4(a)). Further, TEM ultrastructural observations, located EhADH112 near to and at invaginating membranes surrounding RBC in trophozoites of ANeo (data not shown) and ANeoBro1 populations (Figure 4(c)). Inside trophozoites, in the vicinity of delimiting membranes surrounding RBC, several small whitish vesicles were seen (Figure 6(b)), sometimes as if they were being released from huge vesicles. By their appearance [42], these vesicles may correspond to MVB (Figure 6(b), asterisks). As phagocytosis advanced, more abundant gold particles were found on RBC (Figures 6(b) and 6(c)). Furthermore, around phagosomes containing RBC, we observed whitish vesicles in arrangements apparently organized, some of them labeled with gold particles (Figure 6(c)).

Mammalian ALIX and yeast BRO1 are cytoplasmic proteins that associate with endosomal compartments to function in concert with components of the ESCRT machinery during MVB formation [41, 43]. In the endocytic pathway, MVB are formed from early endosomes and then, they fuse to late endosomes or lysosomes [12, 44]. Here, our findings strengthen the role of EhADH112 not only as an adhesin at the trophozoite surface, but also as a protein whose similarity to other Bro1 domain-containing proteins such as ALIX, could assign it an alternative function in endosome, phagosome, and MVB formation. The presence of gold labeling at the surface of small vesicles at the proximity of larger vacuoles could mean that EhADH112 is being



(a) (b) (c)

(d) (e)

Figure 5: Ultrastructural location of EhADH112 in ANeoBro1 trophozoites. Ultrathin sections of ANeo or ANeoBro1 trophozoites were first prepared by cryosubstitution and incubated with pαEhADH112 and gold-labeled secondary antibodies, and then contrasted and analyzed by TEM. (a) ANeoBro1 trophozoites only incubated with gold-labeled secondary antibodies. (b–e) EhADH112-immunogold labeling in (b) plasma membrane (PM), (c) a structure resembling a MVB, (d) a lysosome-like organelle, (e) MVB and vesicles fusing to a lysosome. V: vacuole. MVB: multivesicular bodies. Arrows: vesicular fusion areas. Arrowheads: EhADH112 gold-labeled molecules.

targeted to these sites to perform a putative function in vesicle formation or that it is *per se* a conveyor protein which carries other molecules involved in this process. Moreover, the possibility of EhADH112 participation in vesicle biogenesis implies that it could be translocated from the plasma membrane to the delimiting membranes of internal vesicles. Hence, EhADH112 could be a soluble or insoluble membrane-associated protein, depending on its function and the protein or proteins it binds to.

3.9. EhADH112 Is Mostly Present in Membrane Subcellular Fractions. To investigate whether native EhADH112 remains as a soluble or insoluble membrane-associated protein in wild-type trophozoites, we carried out the procedure described by Aley et al. [34] followed by Western blot assays, using rabbit polyclonal antibodies against the last 243 amino acids of EhADH112 (pαEhADH243), which detect the carboxy-terminus of the adhesin. pαEhDH243 antibodies recognized the expected 78 kDa band corresponding to the EhADH112 molecular weight in crude extracts (TP) obtained after disruption of trophozoites incubated with concanavalin A in the presence of protease inhibitors (Figure 7(a), left panel, lane 1). After centrifugation at 250 $\times$g for 30 min on a mannitol/sucrose gradient, EhADH112 appeared in the supernatant (SN1), which contains vesicles, small membrane fragments, and soluble proteins (Figure 7(a), left panel, lane 2). We also detected a weak band in the pellet (P1), which contains large fragments of plasma membranes and cell debris (Figure 7(a), left panel, lane 3). As a control, we used mouse monoclonal antibodies against actin (mαactin), which reacted with the corresponding 43 kDa protein in all fractions (Figure 7(a), left panel, lanes 1 to 3).

Then, we ultracentrifuged (40 000 $\times$g) the SN1 fraction to obtain the supernatant (SN2), where soluble components remain, and the pellet (P2), containing internal vesicles and

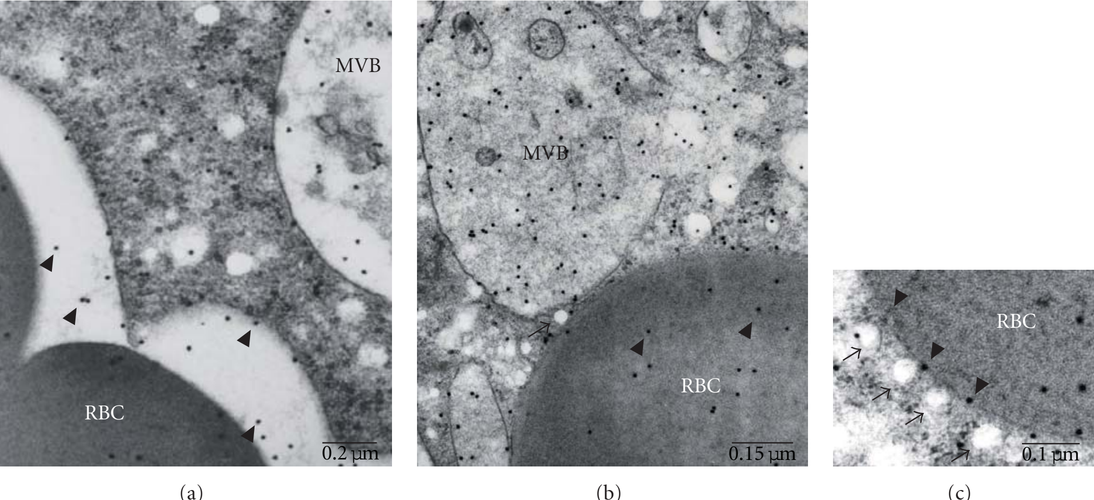


Figure 6: Ultrastructural location of EhADH112 in ANeoBro1 trophozoites after RBC ingestion. After 15 min of interaction with RBC, ANeoBro1 trophozoites were processed for immunogold labeling using pαEhADH112 and secondary antibodies, and contrasted and analyzed by TEM as described above. (a) Section from a trophozoite containing a RBC into phagosomal compartments, displaying a cytoplasmic MVB. (b) MVB and RBC neighboring vesicles; (c) RBC surrounded by small vesicles. MVB: multivesicular bodies. RBC: red blood cells. Arrowheads: EhADH112 gold-labeled molecules. Asterisk: disruption of a larger vacuole and release of vesicles.

small membrane fragments. EhADH112 was only found in P2 (Figure 7(a), middle panel, lane 2), suggesting that it could be mostly a membrane-associated protein. Actin was also absent in the SN2 fraction and strongly detected in P2 (Figure 7(a), middle panel, lane 2).

Next, we processed the P1 fraction, that according to Aley et al. [34], contains vesiculated and nonvesiculated plasma membrane fragments. The P1 fraction (Figure 7(a), left panel, lane 3) was homogenized with α-methyl mannoside and centrifuged at 250 ×g on a sucrose cushion to separate vesiculated (P4) from nonvesiculated membranes and debris (P3). P3 did not react with pαEhADH243 antibodies (Figure 7(a), right panel, lane 1), indicating that EhADH112 is poorly present in nonvesiculated membranes. Then, we concentrated the supernatant (SN3) by ultracentrifugation at 40 000 ×g to obtain vesiculated membrane fragments (P4). pαEhADH243 antibodies revealed the presence of EhADH112 in P4 (Figure 7(a), right panel, lane 2), thus, confirming that this protein is associated to vesiculated plasma membrane fragments. Meanwhile, actin was localized in P3 and P4, attached to nonvesiculated and vesiculated plasma membranes (Figure 7(a), right panel, lanes 1 and 2).

These data showed the presence of EhADH112 in membrane vesicles, supporting results obtained by microscopy experiments. They also evidence that EhADH112 could be associated to plasma membrane during the active vesicular traffic exhibited by trophozoites, probably inside small vesicles that eventually fuse to plasma membrane.

Most Bro1 domain-containing proteins are cytosolic, and it has been noticed that ALIX is transiently recruited to the plasma membrane to promote membrane fission during budding and release of viral particles, in association to late-acting ESCRT proteins [45]. EhADH112 was originally described as a component of the heterodimeric EhCPADH complex, present at trophozoite plasma membrane and cytoplasmic vacuoles under culture conditions. Interestingly, during erythrophagocytosis, this complex is found at different locations. First, the protein is found at the trophozoite plasma membrane, particularly, at target cell contact sites. Then, this protein is detected together to internalized erythrocytes, in phagosomes and phagolysosomes. At late stages, the EhCPADH complex is detected again at the trophozoite plasma membrane, thus suggesting protein recycling or even, a putative participation of EhADH112 in membrane remodeling. Additional evidence has consistently confirmed the presence of EhADH112 in both, trophozoite plasma membrane and cytoplasmic vesicles. According to our current subcellular fractioning experiments, EhADH112 is mostly present in membrane fractions. However, as it was shown in Figure 7(a), EhADH112 is also found in fractions containing soluble proteins. Therefore, we cannot discard the possibility that EhADH112 exists in soluble form under particular conditions not yet explored. Since the precise protein location is largely determined by its function in the cell, it must be considered that Bro1 domain containing proteins such as ALIX are ubiquitous in order to serve as scaffold proteins connecting several biological processes. Therefore, additional functions should be investigated for EhADH112 to better understand its subcellular location in trophozoites. Particularly, the presence of EhADH112 in different membrane compartments, strongly supports the hypothesis that EhADH112 could perform a role in the endocytic pathway, although it remains to be established if the previously reported *E. histolytica* ESCRT machinery is indeed participating in this process.

3.10. EhVps32 Binds to the N-Terminus of EhADH112. One conserved feature of the majority of Bro1 domain-containing

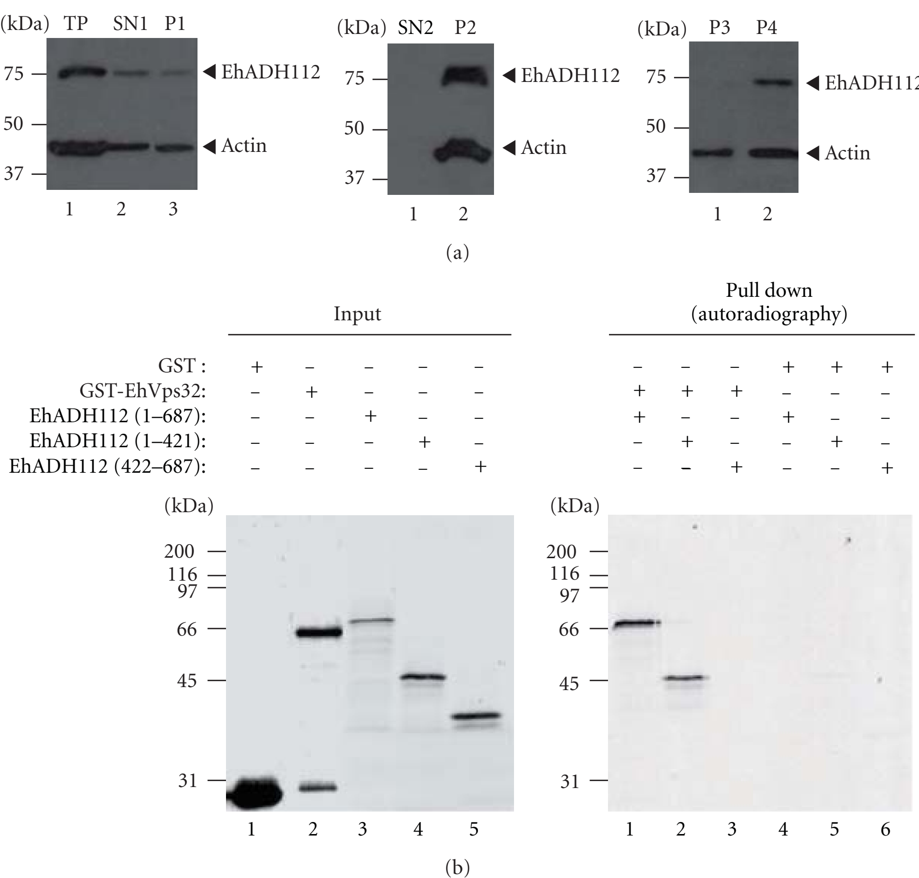


Figure 7: EhADH112 major presence in membrane subcellular fractions and EhADH112 interaction with EhVps32. (a) Location of EhADH112 in wild-type trophozoites subcellular fractions. Proteins (50 μg) from different fractions of trophozoite extracts were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. Western blot assays were performed using pαEhADH243 and mαactin antibodies and corresponding peroxidase-labeled anti-rabbit or anti-mouse IgG secondary antibodies. Membranes were revealed by chemiluminescence. *Left*: SN1 and P1 fractions resulting after total proteins (TP) centrifugation at 250 ×g for 30 min in a mannitol/sucrose gradient. *Middle*: SN2 and P2 fractions resulting after SN1 ultracentrifugation at 40 000 ×g for 60 min. *Right*: P3 and P4 fractions obtained after ultracentrifugation of solubilized P1 at 40 000 ×g for 60 min. (b) Binding of EhADH112 to EhVps32 through the Bro1 domain. GST or GST-EhVps32 proteins were immobilized on glutathione-Sepharose beads and incubated with *in vitro* synthesized [^{35}S]-EhADH112 or [^{35}S]-EhADH112 derivatives. *Left*: purified GST and GST-EhVps32 fusion proteins or [^{35}S]-EhADH112 and [^{35}S]-truncated derivatives (autoradiography, lanes 3, 4 and 5) used for binding experiments (8% of the total reaction mixture). *Right*: pulled-down proteins electrophoresed on 10% SDS-polyacrylamide and detected by autoradiography.

proteins is their ability to bind the ESCRT-III component Vps32 or CHMP4. This interaction allows BRO1 or ALIX targeting to endosomes during MVB formation and virus budding [13, 19]. Here, we investigated whether EhADH112 interacts with an *E. histolytica* protein homologous to yeast Vps32. First, we found in the *E. histolytica* genome database a protein sequence (EhVps32) with an *e*-value of 2.5 *e*-12, displaying 48% homology and 25% identity to yeast Vps32. According to multiple sequence analysis and Pfam database predictions, EhVps32 contains a Snf7 domain, present in all members of the Snf7 family. Additionally, the predicted EhVps32 secondary structure using the Jpred program, suggested that EhVps32 conserves the characteristic five α-helices present in the Snf7 family protein. To confirm the predicted interaction between EhADH112 and EhVps32 proteins, pull down experiments were performed. Thus, we expressed a GST-EhVps32 fusion protein in bacteria. GST alone or purified GST-EhVps32 were immobilized on glutathione-sepharose beads (Figure 7(b), left panel, lanes 1 and 2, resp.) and incubated with [^{35}S]-labeled EhADH112 (Figure 7(b), left panel, lane 3) or [^{35}S]-EhADH112 (1-421 amino acids) (Figure 7(b), left panel, lane 4) and [^{35}S]-EhADH112 (422–687 amino acids) derivatives (Figure 7(b), left panel, lane 5), previously synthesized by a coupled transcription-translation system, as described in Section 2.10. GST-EhVps32 beads retained EhADH112 (Figure 7(b), right panel, lane 1), and the EhADH112 (1–421) derivative (Figure 7(b), right panel, lane 2), but not the EhADH112 (422–687 amino acids) polypeptide (Figure 7(b), right panel, lane 3). As expected, GST alone was unable to bind EhADH112 and EhADH112 derivatives (Figure 7(b), right panel, lanes 4 to 6). Proteins present in

pull-down reaction mixtures were Coomassie blue stained as an additional control (data not shown). Together, these results strongly suggest that EhADH112 binds EhVps32 through the Bro1 domain, as it has been reported for other Bro1 domain-containing proteins [19, 20].

Although we do not know yet the identity of other proteins associated to or transported by EhADH112 in live trophozoites, based on these results, we hypothesized that EhADH112 may interact with ESCRT proteins *in vivo*, as it has been reported for ALIX and BRO1 [19, 39, 46, 47]. Interestingly, EhADH112 seems to be a novel member of a subfamily of Bro1 domain-containing proteins present at cellular surface [48] that alternatively could regulate the assembly of proteins at endosomal membranes for MVB biogenesis. Translocation of EhADH112 from the plasma membrane to internal vesicles, endosomes, or phagosomes and back to the surface, could also be related to a scaffold function, as it has been described for its homologues in yeast and mammals [22]. However, protein partnerships in different cellular networks should be addressed further.

4. Conclusions

In this work, we reported for the first time the functional characterization of the N-terminus of EhADH112, an *E. histolytica* Bro1 domain-containing protein involved in parasite virulence. A dramatic decrease of phagocytosis rates displayed by trophozoites overexpressing an EhADH112 Bro1 recombinant polypeptide, together with an exaggerated accumulation of this protein in aberrant compartments, suggested that the Bro1 domain recruits proteins participating in phagocytosis. Moreover, EhADH112 localization at trophozoite plasma membrane, MVB and phagosomes and in both soluble and insoluble subcellular fractions, provided additional support for an alternative role for this protein in the endosomal MVB pathway. This function is conserved among Bro1 domain-containing proteins, which interact with ESCRT components to associate to endosomes. Here, we also showed the *in vitro* association of EhADH112 with an *E. histolytica* protein homologous to the ESCRT-III Vps32 subunit, as a putative hallmark for EhADH112 Bro1 domain function in endosomal protein sorting. Additional efforts should be made to better understand the role of EhADH112 in ESCRT-mediated MVB biogenesis and other functions also assigned to Bro1 domain-containing proteins.

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Review Article

Are Basophils Important Mediators for Helminth-Induced Th2 Immune Responses? A Debate

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Helminth parasites induce Th2 immune responses. Immunological mechanisms leading to Th2 induction are mainly dependent on IL-4. However, early source of IL-4 has not been precisely identified. Noticeably, basophils seem to be important mediators for inducing and maintaining the Th2 response probably because they secrete IL-4 and exert functions similar to APCs. Nevertheless, recent experimental evidence points that DCs could be also significant participants during this process. The involvement of basophils during memory responses is also discussed.

1. Introduction

Intestinal helminth infections still represent a public health problem in many developing tropical and subtropical countries affecting the health of human beings and of livestock [1, 2]. $CD4^+$ T cells are the main cellular mediators in host helminth interactions. In response to different antigens, these cells differentiate in four types of T-helper cells Th1, Th2, Th17, and regulatory T cells. Helminths and their antigens induce Th2 immune responses, and protection against these parasites seems to be dependent on this polarization.

Th2-cells secrete type 2 cytokines such as interleukin-4 (IL-4), IL-5, IL-9, IL-13, but non-T cells including basophils, mast cells, B cells, and eosinophils can also produce them. The initial priming for Th2 differentiation is dependent on the IL-4 receptor α chain (IL-4R) and transcription factors STAT6 and GATA3, as well as processing and presenting antigens from antigen presenting cells (APCs) and upregulation of costimulatory molecules [3]. A type 2 immune response is characterized by activation and expansion of $CD4^+$ Th2-cells, mucosal epithelial cells, eosinophils, basophils, production of immunoglobulin E (IgE) and mast cell, and goblet cell hyperplasia [4]. Moreover, basophils and mast cells are activated by IgE-immune complexes through crosslinked-high-affinity Fc receptors (FcRs) for IgE located on the cell surface. Then, these cells are able to degranulate and release cytokines, chemokines, proteases, serotonin, histamine, and heparin, resulting in smooth muscle hypercontractibility, increased permeability, and inflammatory cell recruitment that, accompanied by mucus production, will facilitate clearance of parasites (Figure 1).

Basic aspects about activation of Th1- and Th17-type immune responses are well characterized. Nevertheless, the immunological mechanisms leading towards induction of Th2 immune responses remain to be elucidated. Early production of IL-4 is essential for Th2 differentiation [3]. Dendritic cells (DCs) are efficient APCs that express costimulatory molecules CD40 and CD86 and produce cytokines (IL-12, IL-13, and IL-6) necessary for the activation and differentiation of $CD4^+$ T cells during Th1 or Th17 responses [5]. However, DCs are not able to produce IL-4.

Recently, it has been documented that basophils are involved in development and amplification of type 2 immune responses during helminth infections, because they are capable of producing and secreting IL-4 in response to helminth antigens and by crosslinking of antigen-specific IgE complexes. Furthermore, it has been suggested the possible role of basophils as APCs, since they constitutively express MHC-class-II, costimulatory molecules such as CD40, CD80, and CD86, and the lymph-node-homing receptor CD62L [6–8].

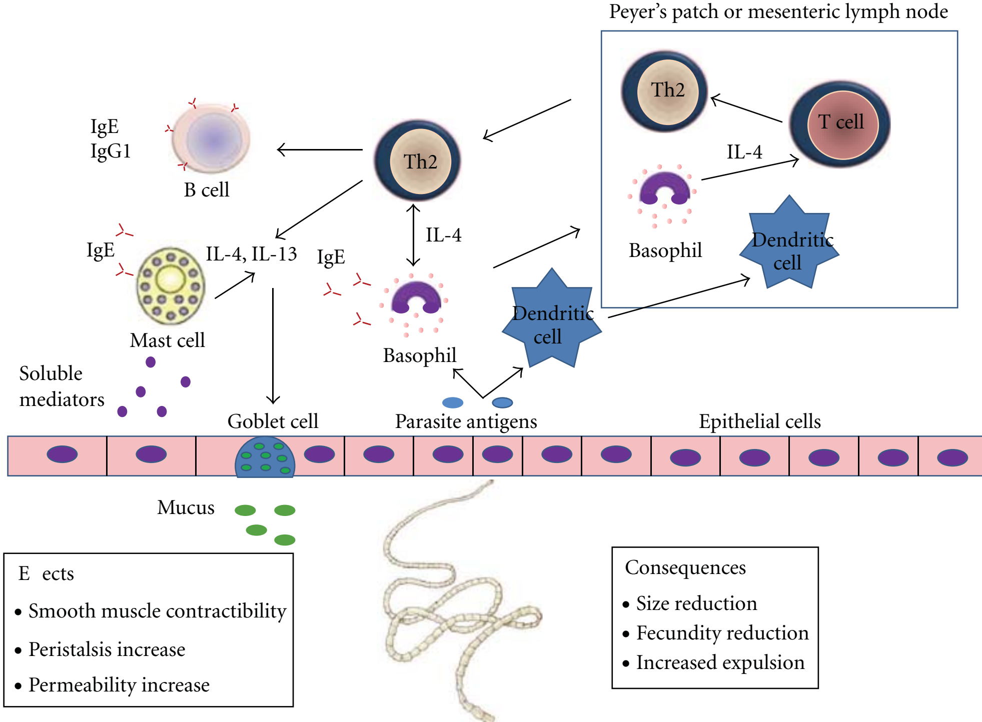

Figure 1: Components of type 2 immune response effective against gastrointestinal (GI) helminth parasites. In the primary response, APCs process and present antigens via MHC-class-II, upregulate costimulatory molecules, and, in an IL- 4 milieu, prime naïve T cells to become Th2-cells. Th2-cells and other reactive cells secrete IL-4 and IL-13, promoting B class switching to IgE and IgG1. IgE immune complexes activate basophils and mast cells by crosslinking of FcR. Activated mast cells and basophils secrete soluble mediators inducing changes in smooth muscle contractility, peristalsis, and intestinal permeability increase. IL-4 also induces goblet cell proliferation and mucus production. All of these elements induce a hostile environment for the parasite, provoking their expulsion or their reduction in size and fecundity.

This information indicates that this cell type is a potential early source of IL-4 that could promote differentiation of $CD4^+$ Th2-cells or even present antigens to $CD4^+$ T cells. Additionally, recent data have revealed a function of basophils not only in the initiation and maintenance of type 2 responses, but also in protective immunity and memory responses. Nonetheless, the potential enrolment of basophils in the initiation of Th2 immunity is under study, and results obtained from different research groups have become controversial, which highlights the importance of investigating the interactions between helminths and this cell type. The main goal of this paper is to provide an overview of recent findings in this regard.

2. Cell Types Involved in Initiating and Maintaining Type 2 Immune Responses

DCs control differentiation of naïve T cells into Th1 and Th17 effectors cells through cytokine production like IL-12, IL-6, and IL-23. After stimulation with Toll-like receptors' ligands, DCs initiate signal cascades resulting in presentation of peptides by MHC-class-II to T cells with upregulation of costimulatory molecules CD40, CD80, and CD86 [9]. Th1 response is promoted by IL-12 secretion from DCs [10] whereas Th17 by IL-1, IL-6, or IL-23 from this same APC [11].

The role of DCs in the induction of Th2 responses has also been studied [12], and it is well known that some Th2-type helminth antigens are able to modify DCs towards a phenotype that may induce Th2 differentiation [13–15]. However, Th2-associated DCs signals have not been identified yet. In spite of the fact that DC express MHC-class-II and costimulatory molecules, very little is known about how DCs could sense Th2-type antigens, the nature of DCs subsets, whether they are sufficient to initiate Th2 responses, and if it is necessary establishing cooperation with innate immune cells.

Basophils are odd polymorphonuclear granulocytes, mainly found in the blood and peripheral tissues representing less than 1% of total leukocytes in blood and spleen. These cells mature in the bone-marrow before entering the blood stream, express FcRs and are capable of secreting Th2 cytokines such as IL-4 and thymic stromal lymphoprotein (TSLP), both important molecules for Th2 induction [16, 17]. Basophils can be activated through an IgE-dependent or IgE-independent process secreting, in consequence, important amounts of IL-4 [18, 19] as well as mediating degranulation and releasing preformed mediators [16].

Basophils are rapidly recruited into the bone-marrow, small intestine, blood stream, and other tissues during helminth infections and allergic inflammation [20, 21].

Echinococcus multilocularis extracts as well as the glycoprotein IPSE/α-1 expressed in *Schistosoma mansoni* eggs induce release of IL-4 and IL-13 from basophils in the presence of IgE [22]. This demonstrates that basophils are able to respond to parasitic antigens and could be important in generating Th2 responses during helminth infections. Constitutively and under stimulation of allergens and helminth antigens, basophils express MHC-class-II and costimulatory molecules. Moreover, these cells rapidly produce IL-4 upon activation, and they are capable of forming conjugates with $CD4^+$ T cells, suggesting a role for basophils in Th2 differentiation of naïve $CD4^+$ T cells.

In the last decade, the role of basophils and DCs has been studied in experimental models where both cell populations were depleted mainly by different methods: treatment with the monoclonal antibody anti-FceRI (MAR-1) and administration of diphtheria toxin (DT) to animals that express the diphtheria toxin receptor under the CD11c promoter (CD11c-DTR), respectively. In 2010, a new genetically basophil-deficient mouse strain was developed [23]. Results induced discussion about the role of DCs and basophils in the initiation of Th2 responses. Studies from different groups regarding this topic are discussed what in follows.

3. Basophils as Initiators of Type 2 Immune Responses

Several reports have suggested that, in some conditions, DCs may not be crucial for promotion of the Th2-cell development. In the OVA-papain subcutaneous immunization model, a cysteine protease that mimics the activity of enzymes secreted by Th2 response-related helminth parasites promoted upregulation of MHC-class-II and costimulatory molecules expression in basophils, suggesting that these cells could exert functions similar to APCs [6]. Interestingly, transfer of OVA-loaded basophils into MHC-class-II-deficient mice was sufficient for Th2 polarization, while MAR-1-mediated basophil depletion abolished this response, suggesting that antigen presentation by basophils was enough for triggering Th2 responses [6].

Splenic basophils from mice infected with *Strongyloides venezuelensis* were MHC-class-II positive. Furthermore, this cell population expressed CD80 and CD86, as well as CD62L, additionally showing secretion of IL-4 [7], in agreement with Sokol and colleagues [6]. In absence of DCs, basophils were able to induce development of antigen-specific Th2-cells *in vitro*, suggesting that these cells produce sufficient IL-4 for induction of naïve $CD4^+$ T cells into Th2-cells. When basophils were incubated in the presence of antigen and IgE complexes, an enhanced Th2 response was observed, supporting that these cells could capture IgE complexes and increase their own ability as APC [7].

Parasite expulsion and protective immunity in *Trichuris muris* infection is dependent on $CD4^+$ Th2-cells [24]. During this infection in mice, basophils were identified as a cell population that expressed IL-4, MHC class II, and CD62L [8], suggesting their potential to enter into lymphoid tissues, act as APC, produce IL-4, and lead T-cell polarization towards Th2-cells. *In vivo* depletion of basophils with MAR-1 impaired immunity to this parasite, supporting that basophils could facilitate development of protective Th2 immunity. In *T. muris*-infected mice, exhibiting expression of MHC-class-II restricted to $CD11c^+$ DC populations, animals showed minimal induction of Th2 cytokines in response to the infection. Interestingly, production of Th2 cytokines was restored when a neutralizing monoclonal antibody to IFN-γ was used [8]. These results suggest that DCs can induce protective Th2 responses in an environment where Th1 cytokines have been previously blocked, whereas basophils and other cell populations with ability to produce IL-4 could facilitate Th2 differentiation by reciprocal blocking Th1 responses.

Taken together, all these experiments using mice where basophils have been abrogated with specific antibodies (Table 1), support the idea that these cells could interact with T cells in order to promote Th2 differentiation through antigen presentation and cytokine production. Therefore, the role of DCs in the initiation of Th2 responses is questioned.

However, a recent study suggests that Th2-cell development cannot be so simple, requiring cooperation between DCs and basophils. Using the CD11c-DTR model, it has been demonstrated that DCs are required for the induction of antigen-specific Th2 responses after subcutaneous immunization with OVA-papain [25], in contrast to previous results by Sokol and coworkers [6]. Nevertheless, DCs isolated from these immunized mice failed to induce IL-4 production of OVA-specific T cells *in vitro*. When a combination of DCs and basophils was cocultured in presence of T cells, IL-4 production was clearly increased. Also, lymph-node DCs and dermal DCs released reactive oxygen species (ROS) after immunization with OVA-papain. Release of ROS induced the production of oxidized lipids that triggered induction of TSLP in epithelial cells [25]. TSLP has a key role in the induction of Th2 responses [26, 27]. In this way, the authors demonstrated that TSLP inhibit the ability of DCs to stimulate Th1 responses by suppressing IL-12 production in DCs and inducing production of CCL7. Remarkably, this chemokine could mediate the recruitment of IL-4 secreting basophils into the lymph-node promoting Th2 differentiation.

Other results support the notion of cooperation between DCs and basophils. For example, depletion of DCs in the CD11c-DTR model resulted in impaired production of Th2 cytokines from $CD4^+$ T cells in response to *Schistosoma mansoni* infection or after *S. mansoni* egg injection [28]. On the contrary, depletion of basophils with MAR-1 did not alter levels of cytokines or Th2 induction in this experimental system. However, induction of the Th2 response was not completely ablated in CD11c-depleted animals suggesting that some DCs could remain or the involvement of other APCs. An additional study suggests that basophils are recruited into draining lymphatic nodes (dLNs) after *S. mansoni* egg injection [8]. In mice in which MHC-class-II expression was restricted to $CD11c^+$ DC, *S. mansoni* egg injection increases $CD4^+$ T cells in dLNs. Adoptive transfer of primary wild-type basophils augmented the proliferation

Table 1: Experimental evidence related to the role of basophils and DC in induction of Th2 immunity.

Reference	Methods employed	Conclusions
[6]	OVA-papain immunization model Basophil depletion mediated by MAR-1 DC manipulation in CD11c-DTR model	Basophils express MHC-class-II and costimulatory molecules DCs are not required for the development of papain-induced Th2 responses DCs are not able to induce $CD4^+$ Th2-cells *in vitro* except if basophils are included in the culture
[8]	*T. muris*-infected mice and injection of *S. mansoni* eggs Basophil depletion mediated by MAR-1 DC manipulation in CD11c-DTR model	Basophils express MHC-class-II and IL-4 Depletion of basophils *in vivo* impairs immunity to *T. muris* Minimal induction of Th2 cytokines in mice in which MHC-class-II expression was restricted to $CD11c^+$ cells. Th2 cytokines production was restored with a neutralizing monoclonal antibody to interferon-γ
[7]	*S. venezuelensis*-infected mice	Basophils secrete IL-4 and express CD80, CD86, CD62L, and MHC class II Basophils induce antigen-specific Th2-cells *in vitro* in the absence of DC Enhanced Th2 responses result when basophils are cultured with antigen-specific IgE
[48]	*N. brasiliensis* infection Basophil depletion mediated by anti-Thy-1.2 mAb	In the absence of IL-4 and/or IL-13-producing T cells, basophils contribute to efficient worm expulsion
[28]	*S. mansoni* infection and *S. mansoni* egg injection Basophil depletion mediated by MAR-1 DC manipulation in CD11c-DTR model	Depletion of DCs results in impaired $CD4^+$ T cell production of Th2 cytokines; depletion of basophils has no effect
[29]	*N. brasiliensis* infection Basophil depletion mediated by MAR-1	Depletion of basophils does not diminish the development of IL-4 producing $CD4^+$ T cells Basophils recruitment into the dLN depends on IL-3
[23]	OVA-papain immunization model and *N. brasiliensis* infection Basophil-deficient mice Mcpt8Cre and DC-ablated mice	Basophils are not required for *in vivo* priming of Th2-cells in *N. brasiliensis*-infected mice or after immunization with OVA-papain, with constitutive deletion of basophils DC-ablated mice are impaired in mounting a Th2-cell response against OVA-papain despite normal basophil recruitment

of dLN $CD4^+$ T cells in response to injection of parasite eggs [8], supporting that after exposure to helminth antigens basophils are recruited into dLN and they could cooperate together with DCs in the proliferation and expansion of $CD4^+$ T cells.

Nevertheless, recent studies have questioned the use of DT for depleting DCs, claiming side effects of DT administration. Furthermore, the use of MAR-1 for depleting basophils has been related with activation of mast cells and induction of anaphylaxis. Then, in 2010, Ohnmacht and coworkers [23] reported a new transgenic mouse strain (Mcpt8Cre) with constitutive deletion of basophils. After immunization with OVA-papain, Mcpt8Cre mice developed a normal Th2-cell response suggesting that in papain-induced Th2-cell differentiation, basophils play a minor role. In addition, mice with constitutive deletion of DCs are impaired in mounting a Th2-cell response against OVA-papain despite normal basophil recruitment, confirming that papain-induced Th2-cell differentiation depends on DC and not on basophils (Table 1). Similar results were obtained with *N. brasiliensis*-infected mice where basophils seem not be required for *in vivo* priming of Th2-cells [23, 29]. Interestingly, excretion/secretion (E/S) products from *N. brasiliensis* and *Acanthocheilonema viteae*, as well as *S. mansoni* soluble egg antigen and *Echinococcus granulosus* antigen B [30], have been related with inhibition of DCs maturation and DC production of IL-10, favoring a Th2 response [31]. Recently, it has been demonstrated that excreted-secreted antigens of the cestode *Taenia crassiceps* (TcES) affect murine and human DC activities [32, 33]. DCs pulsed with TcES abrogated their capacity to respond to proinflammatory stimuli such as LPS, decreasing expression of maturation and costimulatory molecules. TcES-exposed murine bone-marrow-derived DCs failed to release proinflammatory cytokines, while they preserved IL-10 production [32]. Similarly, TcES enhances production of IL-10 by human DCs, but not IL-12, IL-1β, TNF, and IL-6 [33]. Additionally, when TcES-exposed DCs are used as APCs, they suppress IFN-γ production and increase IL-4 levels in $CD4^+$ T cells, supporting the idea that helminth-derived products may modulate DCs in order

to acquire a special phenotype with capacity to bias Th2 responses. In spite that *T. crassiceps* shares antigenic resemblance with *Taenia solium*, the cestode parasite of humans [34], studies are necessary to analyze the role of basophils and DCs in the induction of Th2 responses because there are no published reports, except those performed in experimental infections with *T. solium* in hamsters. The intestinal mucosa shows an increase in IFN-γ expression detected by *in situ* hybridization during the first week of infection while IL-13 is seen between days 2 and 16 after infection and IL-4 since the second week when parasite expulsion occurs. These findings demonstrate that coexpression of Th1 and Th2 cytokines takes place in this experimental infection [35]. It would be interesting to determine whether *T. solium*-excreted/-secreted antigens have the ability to interact with DCs and basophils, in order to elucidate important early steps in the activation of the host immune response resulting in the expression of Th1 or Th2 cytokines.

Some authors propose that DCs alone can drive Th2-cell differentiation and basophils could help to sustain and amplify the type 2 response [36]. However, the multiple studies described, demonstrate the importance of basophils and DCs in the induction of Th2 responses, suggesting that there are several pathways in which these cells can act independently or in cooperation. The vision of the type 2 immune response are like a complex network with duplicated essential functions which indicates that basophil-dependent response, DC-dependent response, and DC/basophil-dependent response could simultaneously occur *in vivo*. Therefore, it is necessary to reassess the role of each one depending on the infection setting.

4. Participation of Basophils in Memory Response against Helminths

The high prevalence of helminths in endemic populations suggests that the complete elimination of parasites is seldom generated [37]. However, some evidence about the rapid development of resistance to reinfection in individuals with long-term exposure [38] and the decrease of infection intensity in adults of endemic areas [39] indicates that immunity is acquired with cumulative exposure. The long exposure or persistence of antigens keeps the immunological memory due to long-term survival by antigen-specific memory T cells and B cells [40]. Then, antibody production by memory B cells is a key process for maintaining the acquired immune response. Noticeably, helminth infections are characterized by IgE production, and high levels of this antibody class are associated with resistance to reinfection [41, 42].

Basophils are capable of binding antigen-specific IgE antibodies through FcR and undergo activation [16]. This fact is important during secondary infections with parasites. Interestingly, experimental evidence shows that mechanisms required for parasite expulsion during primary and secondary infections are different. During primary mice infection with *N. brasiliensis,* a protective immune response dependent on IL-13, IL-4, and $CD4^+$ T cells develops, which is able to stimulate goblet cell proliferation, mucin secretion, and physiological changes in the gut; eosinophils, IgE, and accumulation of basophils are not necessary for expulsion in a primary infection [23, 43, 44]. During secondary infections, worm expulsion occurs in mast-cell-deficient c-$Kit^{W\text{-}sh}$ mice, even in the absence of $CD4^+$ T cells. However, when these mice were depleted of basophils, worm expulsion was impaired [45]. These data indicate that, in a primary infection, $CD4^+$ T cells stimulate a non-$CD4^+$ T-cell group involved in the memory response which could participate in the expulsion of worms after a secondary infection. In agreement with these results, Mcpt8-basophils-deficient mice showed an efficient *N. brasiliensis* worm expulsion during the primary infection, while this ability was impaired during the secondary infection [23]. These studies suggest that basophils play a role in the protective immunity against helminths.

Yoshimoto and colleagues [7] reported that basophils might efficiently take up low doses of antigen in an IgE-dependent way. Moreover, when these cells are pulsed with antigen-IgE complexes, they are capable of improving their APCs ability. In secondary infections, basophils could bind IgE and become sensitized to antigens that have been previously in contact with the host. Sensitized basophils can synthesize and release IL-4 and IL-13 [46], both key molecules for B-cell differentiation and IgE production [47]. This information highlights the possibility of an amplification loop between basophil activation and immunoglobulin production. Also, basophils could be directly activated by some allergens and parasites [6, 46] expanding their own capacity for introducing excretory or secretory proteins from helminths and promoting Th2 differentiation.

Basophils have been shown to be able to enhance immunological memory responses. In mice immunized with allophycocyanin, basophils exert the ability to *in vitro* bind this protein on their cell surface, becoming activated and releasing IL-4 and IL-6 after 6 weeks of immunization. This activation is FcR dependent, as no release of IL-4 and IL-6 was detectable on FcR-deficient mice [49]. Therefore, it has been proposed that after a primary immunization, antigen-specific IgE is captured by FcR on basophils and, after a second one, activation occurs releasing major cytokines for B-cell stimulation and in consequence immunoglobulin production [49]. Recent studies have shown that IgD-cross-linking leads to basophil activation and expression of B-cell-activating factor (BAFF) and IL-4, stimulating production of T-independent antibodies from B cells [50]. Also, KU812 cells, a human basophilic line, are capable of producing IL-4 and IL-13, and induce synthesis of IgE and IgG4 in human normal B cells [51]. During memory response, secretion of these cytokines could be important since depletion of basophils leads to decrease of production of IgG1 and IgG2 as well as increase of susceptibility to *Streptococcus pneumonia*-induced sepsis [49]. These results indicate that basophils may not only contribute to support the Th2 response through IL-4 production but also by directly activating and promoting IgE production from B cells.

In a primary infection, the immune response takes longer to develop, probably because of the higher number of parasite antigens that need to be recognized in order to induce the host immune response [52]. In addition, in

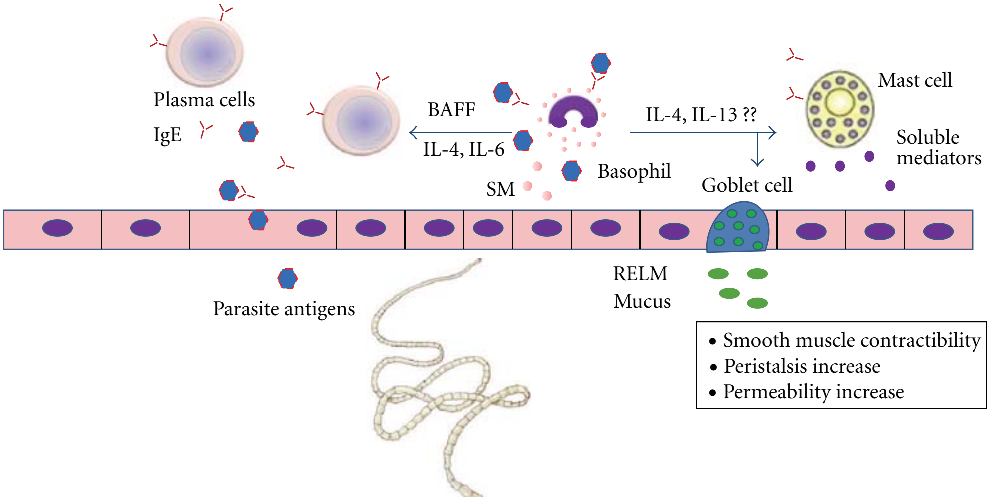

Figure 2: Possible role of basophils in secondary immune responses against GI helminths. Basophils could be directly activated by parasite antigens or take up antigen through parasite-specific IgE antibodies developed in previous infections. IL-4, IL-13, IL-6, and BAFF, released by activated basophils, stimulate antibody production from B cells and may enhance Th2-associated immune responses. It remains to be determined if these mechanisms take place *in vivo*. SM: soluble mediators.

Heligmosomoides polygyrus-infected mice most of the IgG1 and IgE antibodies are polyclonal or have low-affinity specificities; comparatively, after multiple infections affinity of specific IgG and IgA antibodies matured preventing adult worm development [53]. It has been suggested that parasites possess the ability to promote nonspecific-antibody production, but this topic is currently under study. In spite of the fact that development and maintenance of long-lived IgE-producing plasma cells remains unclear, both induction of specific IgE-producing plasma cells during a primary infection and development of high-affinity antibodies during repetitive infections could explain the rapid basophil activation during secondary infections. Remarkably, it has been shown that, after immunization, basophils, additional to B cells, are the only population able to capture on their surface intact antigens present in the plasma through a mechanism mediated by antigen-specific IgE antibodies [54].

All this information suggests that during secondary challenges specific-IgE antibodies and parasite-derived products could activate basophils. Production of IL-4, IL-6, and BAFF by activated basophils may stimulate T-independent antibody production from B cells resulting in an amplification loop. Local release of IL-4 and IL-13 could stimulate other innate cells like goblet and mast cells, leading to an effective and more rapid protective response (Figure 2).

Oral immunization with recombinant functional *Taenia solium* calreticulin (rTsCRT) reduces tapeworm burden in the experimental model of intestinal taeniasis in hamsters [55]. Calreticulin has been identified in several parasites [56, 57], and it has been demonstrated that CRT-specific IgE antibodies develop in many infections [58, 59]. In addition, basophils isolated from individuals living in a hookworm endemic area are able to release histamine in the presence of CRT [60], suggesting the existence of basophils precharged with CRT-specific IgE antibodies. CRT is also recognized by IgE antibodies of *Heligmosomoides polygyrus*-infected mice and induces degranulation of an IgE-sensitized basophil cell line [58]. Therefore, it could be interesting to analyze if TsCRT immunization is able to induce antigen-specific IgE production and, if these antibodies are able to sensitize basophils. Sensitized basophils can synthesize and release IL-4 and IL-13 promoting Th2 differentiation and helping to support a protective immune response. Elucidation of these mechanisms will allow the development or more efficient vaccines against helminths.

5. Concluding Remarks

Helminths induce Th2 immune responses. Immunological mechanisms leading to Th2 induction are mainly dependent on IL-4. However, early source of IL-4 has not been precisely identified. Noticeably, basophils seem to be important mediators for inducing and maintaining the Th2 response probably because they secrete IL-4 and exert functions similar to APCs. Nevertheless, recent experimental evidence points that DCs could be also significant participants during this process, suggesting that Th2 immune responses could be occurring through multiple nonexcluding pathways, which have not been completely elucidated yet. Also, it is necessary to analyze these findings under an overall view since the nature of the immune response depends on the characteristics of individual products as well as the particular interactions of each helminth with its host. Finally, although the role of basophils in the induction of memory responses against helminths needs further investigation, it opens an interesting research field focused on developing vaccines based on antigens that promote Th2 responses, long-lived plasma cells, and specific antibody production.

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Research Article

Plasmodium Riboprotein PfP0 Induces a Deviant Humoral Immune Response in Balb/*c* Mice

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Passive immunization with antibodies to recombinant *Plasmodium falciparum* P0 riboprotein (rPfP0, 61–316 amino acids) provides protection against malaria. Carboxy-terminal 16 amino acids of the protein (PfP0C0) are conserved and show 69% identity to human and mouse P0. Antibodies to this domain are found in 10–15% of systemic lupus erythematosus patients. We probed the nature of humoral response to PfP0C0 by repeatedly immunizing mice with rPfP0. We failed to raise stable anti-PfP0C0 hybridomas from any of the 21 mice. The average serum anti-PfP0C0 titer remained low ($5.1 \pm 1.3 \times 10^4$). Pathological changes were observed in the mice after seven boosts. Adsorption with dinitrophenyl hapten revealed that the anti-PfP0C0 response was largely polyreactive. This polyreactivity was distributed across all isotypes. Similar polyreactive responses to PfP0 and PfP0C0 were observed in sera from malaria patients. Our data suggests that PfP0 induces a deviant humoral response, and this may contribute to immune evasion mechanisms of the parasite.

1. Introduction

Ribosomal phosphoprotein P0 is a highly conserved neutral protein found in the 60S ribosomal subunit of eukaryotes [1]. P0, along with the related acidic ribosomal phosphoproteins P1 and P2, forms a pentameric protein complex $(P1)_2$-P0-$(P2)_2$ that has a role in the assembly of the GTPase-binding site in the large subunit of ribosomes [2–4]. P0 is vital to cell survival as knocking it out is lethal in *Saccharomyces cerevisiae* and *Plasmodium berghei* [5, 6]. It has been postulated to have multiple other functions including apurinic-apyrimidinic endonuclease activity in *Drosophila melanogaster* [7], regulation of gene expression in *Drosophila*, and apoptosis and carcinogenesis in humans [7–10]. P0 has been shown to be present on the surface of *Plasmodium spp.*, *Toxoplasma gondii*, *Saccharomyces cerevisiae* [11] as well as on the surface of neuronal, hepatic, and other cell lines [12, 13]. Human P proteins have been studied extensively because of their association with systemic lupus erythematosus (SLE), an autoimmune disorder. Approximately, 10 to 15% of patients suffering from SLE possess autoantibodies against the conserved 16 carboxy-terminal amino acids [14]. Clustal analysis reveals that this region of the protein is highly conserved across diverse species [15]. Human and mouse P0, for instance, differ only in six amino acids and are identical in the lupus domain (Figure 1). We have previously shown that 87% of adult residents in high-transmission malaria areas of eastern India possessed antibodies against *Plasmodium falciparum* P0 (PfP0) [16]. Similarly, 60% of adults residing in Kenya showed substantial T-cell responses to PfP0 protein [17]. Polyclonal and monoclonal antibodies against PfP0 have been shown to block parasite invasion of red blood cells

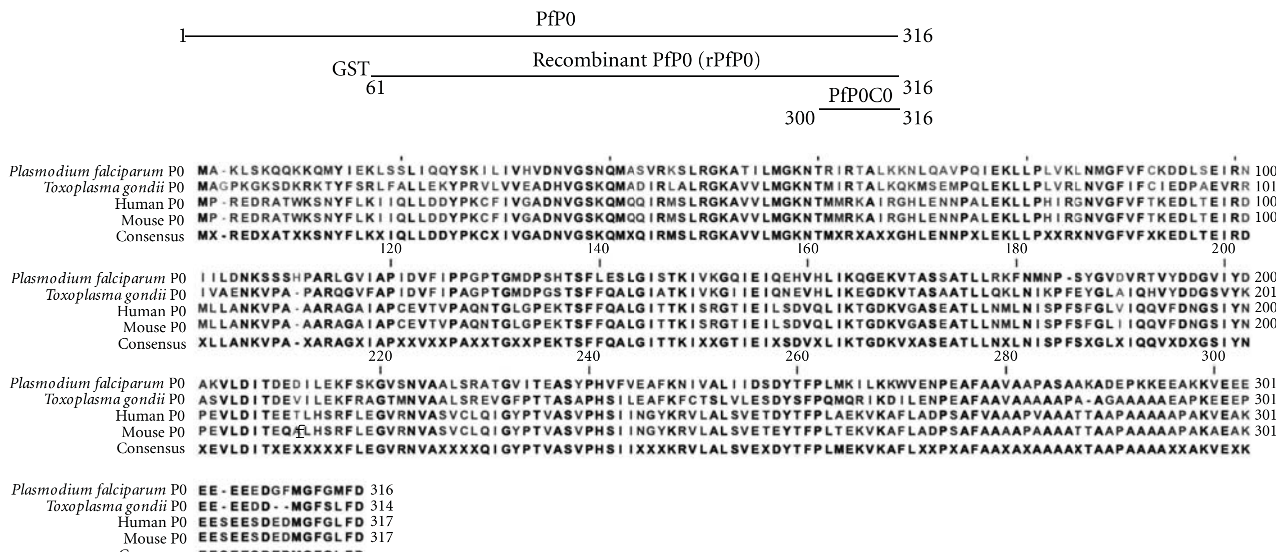


Figure 1: Schematic representation of PfP0, recombinant PfP0, and PfP0C0 and P0 multiple sequence alignment.

in vitro and *in vivo* [15, 18, 19]. When we attempted to raise monoclonal antibodies (mAbs) against the major fragment of PfP0, recombinant PfP0 (rPfP0, 61−316 amino acids), we found that the first mouse, receiving 7 injections (4 weekly, 3 monthly), gave rise to unstable hybridomas reacting to the amino-terminus of the protein. The second mouse receiving 9 injections of the protein (4 weekly, 5 monthly), gave rise to several independent mAb clones, most of them reacting exclusively to the extreme carboxy-terminal, PfP0C0 (300−316 amino acids, Figure 1) [19]. The serum from this mouse reacted exclusively with rPfP0 and PfP0C0, but did not recognize other overlapping peptides derived from the protein [20]. PfP0C0 shows 69% identity to carboxy-terminal of human P0. This predominance of antibodies towards the lupus domain could have been a result of the age of the mouse (8 months), because of breakdown of immune tolerance following repeated immunizations, or both. Alternatively, it was possible that it was an idiosyncratic response of that mouse.

PfP0 is a potential vaccine candidate since anti-PfP0 antibodies were shown to protect against malarial infection in the murine model [18, 19]. Because of its conserved nature and the homology of the carboxy-terminal domain to the human protein, it is also likely to behave like an autoantigen. It was important to ascertain the quality and quantity of humoral response induced by the protein after repeated immunizations. We therefore undertook this systematic study wherein we attempted to raise mAbs against PfP0C0 after repeated immunizations with rPfP0. Selection processes in the central and peripheral levels govern the survival of B cells capable of responding to a particular immunogen, whereas peripheral antigen-driven selection processes determine the type and extent of humoral response. We reasoned that if splenic B cells are a reflection of the whole B cell response and that B cell specificity does not bias hybrid formation, then the frequency of hybridomas formed should reflect immunogenicity of different epitopes of PfP0. We also investigated the nature of the serum anti-PfP0C0 response.

We failed to raise a single anti-PfP0C0 hybridoma from any of the 21 mice used in these subsequent experiments, suggesting that the first success in raising hybridomas against the PfP0C0 domain was probably due to an unusual response observed in that one single mouse (of combined 23 mice used in the two studies). We observed connective tissue fibrosis of the spleen from the fourth month of the immunization schedule, and this increased progressively with further immunizations. Postmortem examination revealed pathological changes in the liver, heart, kidneys, and lungs of the mice. The average serum anti-PfP0C0 titre remained low ($5.1 \pm 1.3 \times 10^4$) even after 10 boosters. Dinitrophenyl (DNP) adsorption studies revealed that the humoral response was largely polyreactive. This polyreactivity was not confined to any particular immunoglobulin isotype, but was distributed across all isotypes. Sequence analysis of the seven hybridomas obtained in the initial study revealed that they were derived from a single clone. We analysed sera from uncomplicated malaria patients to determine the extent of polyreactivity to the protein. Our analysis revealed a similar polyreactive response to PfP0 and the PfP0C0 epitope in these sera. Our data suggests that PfP0 induces a deviant humoral response in mice. Induction of deviant, low-titre, polyreactive responses is likely a method of evading the host immune system.

2. Materials and Methods

2.1. Mice. Six-week-old female Balb/*c* mice were obtained from the local animal breeding facility at the Tata Institute of Fundamental Research, Mumbai, India. Mice were bred and maintained under specific pathogen-free conditions. All experimental research was conducted according to recommendations of the CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals).

2.2. Immunizations and Establishment of Hybridomas. Immunogens and peptides: the expression and purification of

recombinant PfP0 (amino acids 61−316) as a GST fusion protein was done as described earlier [21]. The recombinant *E. coli* cells containing *PfP0* gene fragment were grown for 2 h at 37°C, induced with IPTG, harvested, and lysed. The cell lysates were centrifuged, and insoluble particulate fraction containing the fusion protein was resolved on SDS-polyacrylamide preparative gel. A strip of the gel corresponding to MW of the recombinant protein was excised, electroeluted, dialysed against PBS, and concentrated. The identity of the protein was confirmed by Western blot analysis as described [22]. Ant brain homogenate, *P. falciparum* phosphofructokinase, *P. falciparum* enolase were prepared as described [23–25]. P0 protein from *Toxoplasma gondii* (TgP0) was cloned in pGEX4T1 and expressed as a GST fusion protein. The recombinant, full-length GST−TgP0 was extracted from induced cell pellets using sarkosyl [26], purified from the lysate using Glutathione sepharose beads (GE Healthcare), washed thrice with PBS containing 150 mM NaCl, and eluted using 100 mM glutathione. The identity of the protein was confirmed using SDS-PAGE and Western blotting (data not shown) Bovine serum albumin coupled to PfP0C0 (BSA-PfP0C0) was prepared as described earlier [19].

The synthetic peptides MoP0C0 (EESEESDEDMGFGLF-D), PfP0C0 (EEEEEEDGFMGFGMFD) were obtained from Mimotopes, Canberra, Australia. Bovine insulin, ssDNA, and dsDNA were obtained from Sigma (St Louis, MO, USA).

Immunization and hybridoma establishment was done as described by Rajeshwari et al., 2004 [19]. Briefly, 50 μgs of protein emulsified in Freund's adjuvant was administered intraperitoneally in mice. The animals received four weekly injections followed by monthly injections. Except for the first immunization where Freund's complete adjuvant was used, the rest of the immunizations employed Freund's incomplete adjuvant. 4 days before fusion, the mice were boosted with 250 μg of the immunogen in saline. Mice were sacrificed after each monthly injection, their serum was collected, their spleens were harvested, and the splenocytes were fused with the mouse myeloma Sp2/0 cells. Antibody-secreting clones were selected by ELISA [27]. Immunogen-recognizing hybridomas were subcloned to monoclonality by limiting dilution. For rPfP0 immunizations, mice were given up to seven monthly immunizations; three mice were sacrificed after each monthly immunization.

2.3. Human Serum Samples. Serum samples were collected from patients of uncomplicated malaria as well as immune adults living in high endemicity area in Orissa, India. Presence or absence of *P. falciparum* infection was confirmed by microscopic examination of Giemsa-stained thick and thin blood smears or by using commercial kits. The samples were collected after informed consent and after obtaining the requisite clearances from the Ethics Committee, SCB Medical College and Hospital, Cuttack, India.

2.4. ELISA. Antibody response was determined by ELISA [19]. Briefly, Maxisorp plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with whole antigens (5 μg/mL) or synthetic peptides (200 ng/mL) in PBS, pH 7.2. The plates were blocked with 1% BSA before adding hybridoma supernatants or appropriate dilutions of the serum, developed using rabbit anti-mouse Ig conjugated to horseradish peroxidase (Boehringer Mannheim, Mannheim, Germany) and ABTS (Boehringer Mannheim), and the absorbance (optical density; OD) was measured at 405 nm with EL808 Ultra Microplate reader (Biotek Instruments Inc, Winooski, VT, USA).

To detect polyreactivity, DNP was conjugated to CNBr-activated sepharose beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK) as per manufacturer's instructions. Briefly, 2,4-DNP-ε-lysine (Sigma) was dissolved in 0.1 M NaHCO3 pH 8.3 containing 0.5 M NaCl, added to sepharose beads swollen in 1 mM HCl, and incubated on an end-over-end mixer for 1 h at room temperature. After washing away the excess ligand, the active sites were blocked using 0.1 M Tris-HCl buffer, pH 8.0. The beads were washed with three cycles of 0.1 M acetic acid/sodium acetate, pH 4.0 containing 0.5 M NaCl followed by a wash with 0.1 M Tris-HCl, pH 8 containing 0.5 M NaCl. Aliquots of serum dilutions were incubated or not with DNP-sepharose beads for 1.5 hour on a nutator and centrifuged. The supernatant was diluted further for use in ELISA. Percent polyreactivity was calculated as under:

% polyreactivity

$$= \frac{\text{OD of unadsorbed serum} - \text{OD of adsorbed serum}}{\text{OD of unadsorbed serum}} \times 100. \quad (1)$$

2.5. Determination of RNA Sequence of Anti-PfP0C0 mAb Clones. Anti-PfP0C0 antibody producing hybridoma clones (1A4, 1B3, 2C11, 1E5F4, 1F6, 2G1, and 2H1) were lysed in Trizol (Invitrogen, Carlsbad, CA, USA), and RNA was prepared according to manufacturer's protocol. cDNA was prepared from the RNA using 3′ primer (heavy or light chain constant primer) and MMLV reverse transcriptase (NEB). PCR amplifications of light and heavy chains were performed using primers described in Table 1. Conditions used to amplify PCR fragments were as follows: DNA melting at 91°C for 1 min, primer annealing 52°C for 2 min, and polymerase extension at 72°C for 1.5 min for 30 cycles were carried out. The PCR products were TA cloned in pGEM-T vector system (Promega, Madison, WI, USA), and ligation mixes were used to transform XL-1 Blue *E. coli* bacteria. Positive clones were sequenced using M13F (5′d [GTAAAACGACGGCCAG]3′) and M13R (5′d [CAGGAAACAGCTATGAC]3′) primers.

2.6. Statistical Analysis. Paired Wilcoxan signed-rank test was used to compare and evaluate the statistical significance of the unadsorbed and adsorbed OD of mouse sera. Nonparametric Mann-Whitney test was used to evaluate the statistical significance of the human serum data. GraphPad Instat software was used for analysis.

3. Results and Discussion

3.1. Recombinant PfP0 Immunizations Failed to Yield Stable Anti-PfP0C0 mAb Producing Hybridomas. We were

Table 1: Primers used to amplify antibody variable regions.

Heavy chain 3′ primer	
IgG1	5′ AGG CTT ACT AGT ACA ATC CCT GGG CAC AAT 3′
Heavy chain 5′ primers	
HC1	5′ AGG TCC AGC TGC TCG AGT CTG 3′
HC2	5′ AGG TCC AGC TGC TCG AGT CAG 3′
HC3	5′ AGG TCC AGC TTC TCG AGT CTG 3′
HC4	5′ AGG TCC AGC TTC TCG AGT CAG 3′
HC5	5′ AGG TCC AAC TGC TCG AGT CTG 3′
HC6	5′ AGG TCC AAC TGC TCG AGT CAG 3′
HC7	5′ AGG TCC AAC TTC TCG AGT CTG 3′
HC8	5′ AGG TCC AAC TTC TCG AGT CAG 3′
Kappa light chain 3′ primer	
MKC	5′ GCG CCG TCT AGA ATT AAC ACT CAT TCC TGT TGA A 3′
Kappa light chain 5′ primers	
LC1	5′ CCA GTT CCG AGC TCG TTG TGA CTC AGG AAT CT 3′
LC2	5′ CCA GTT CCG AGC TCG TGT TGA CGC AGC CGC CC 3′
LC3	5′ CCA GTT CCG AGC TCG TGC TCA CCC AGT CTC CA 3′
LC4	5′ CCA GTT CCG AGC TCC AGA TGA CCC AGT CTC CA 3′
LC5	5′ CCA GAT GTG AGC TCG TGA TGA CCC AGA CTC CA 3′
LC6	5′ CCA GAT GTG AGC TCG TCA TGA CCC AGT CTC CA 3′
LC7	5′ CCA GTT CCG AGC TCG TGA TGA CAC AGT CTC CA 3′

interested in raising mAbs against the recombinant *P. falciparum* ribosomal protein P0 (PfP0) as polyclonal antibodies to this protein had inhibited parasite invasion of red blood cells [15, 19]. In a previous study, we were successful in raising stable mAbs against PfP0 after multiple immunizations only in one mouse, and most of these were against PfP0C0 [19]. Since the extreme C-terminal domain of human P0 protein is homologous to PfP0 carboxy-terminus (PfP0C0; 300−316 amino acids) and is the major P-protein autoantigen involved in lupus [14], we were interested in probing the nature of the humoral response to PfP0C0 and wanted to determine the frequency with which we would obtain mAbs against this domain of PfP0 protein. The full-length protein shows extremely poor expression, hence we used the major fragment of the protein (rPfP0, 61−316 amino acids) for immunizations.

We immunized 6-week-old Balb/*c* mice with four weekly injections of rPfP0 in Freund's complete adjuvant, followed by monthly boosters. Spleens from 3 mice each were harvested after each monthly injection (5th through 11th immunization) for hybridoma preparation. Table 2, shows the results for 5th, 7th, 9th, and 11th immunization. Similar results were obtained for the remaining time points. As is seen from Table 2, we failed to obtain any stable clones against PfP0C0, despite repeated immunizations or the age of the mice. Screening of about 1800 clones yielded only 22 stable clones. Most clones were either polyreactive or stopped antibody production within a few days and failed to recognize the carboxy-terminal epitope. Thus, neither age of the animal nor repeated immunizations with a potential autoantigen could be the reason for the success of raising hybridomas in the initial study.

Our failure to raise stable hybridomas against PfP0C0 cannot be attributed to faulty technique, since we succeeded in obtaining multiple stable clones against a variety of proteins (Table 2). These include ant brain homogenate, *Drosophila* translin which bears 52% homology with the mouse protein [28], parasite proteins such as *P. falciparum* fructokinase-β, and potential autoantigenic proteins such as *P. falciparum* enolase [29, 30] and TgP0. The identity of PfP0 and mouse P0 C-terminus over the extreme 16 amino acids is 69% (Figure 1). This homology of PfP0 to mouse P0 alone cannot be the only reason for lack of hybridomas, since both *P. falciparum* enolase and TgP0 show considerable homologies with mouse enolase and mouse P0 (75% and 62.5%, resp.), and these were amenable to generation of a large number of stable hybridomas. Secondary antigenic challenges result in the generation of long-lived plasma cells capable of secreting high-affinity antibodies to the immunizing antigen [31]. It is tempting to speculate that PfP0 is an unusual antigen in that it failed to induce a robust B cell response, since repeated immunizations yielded very few stable hybridomas, and none of them were against the immunogenic carboxy-terminus of the antigen.

3.2. Earlier mAbs Raised against PfP0C0 Arose from a Single B Cell Clone. Since in our previous studies we obtained several mAbs exclusively against PfP0C0 from one mouse, we analyzed the isotype and sequences of the seven mabs (1A4, 1B3, 2C11, 1E5F4, 1F6, 2G1, and 2H1) from this earlier study to establish their relatedness, if any. Isotype analysis showed that all the mAbs belonged to IgG1 isotype. We performed RT-PCRs and amplified products of the heavy and the light chains of these seven mAb clones and sequenced them

Table 2: Serum titre and hybridomas obtained following repeated immunizations with various proteins.

Immunization number	Number of mice screened	Number of clones screened	Stable clones (specificity)	Serum titre ($\times 10^4$)	Splenic fibrosis
Recombinant PfP0					
4	16	ND	−	2.5 ± 0.6	−
5	2	120	0	ND	−
7	3	282	0	2.4 ± 0.1	+
9	3	478	9 (Mouse P0, polyreactive)	5.3 ± 1.3	++
11	2	571	13 (non-PfP0C0, GST)	4.0 ± 1.0	+++
Ant-brain homogenate					
4	1	700	33	5	−
Drosophila Translin					
3	1	290	22	>10	−
Plasmodium falciparum Phosphofructokinase					
3	1	190	7	>50	−
Plasmodium falciparum enolase					
5	1	290	10	>50	−
Toxoplasma gondii P0					
5	1	300	>70	>17	−

ND: not done; −: negative, +: mild, ++: moderate, +++: severe.

Table 3: Results of histopathological investigations of mice following repeated recombinant PfP0 administrations.

Organ	Severity	Changes observed
Liver	+++	Severe swelling and derangement of cords, infiltration of mononuclear cells around the portal triad
Kidneys	+++	Tubular necrosis and diffuse necrotic changes along with infiltration of inflammatory cells around the necrotic area
Lungs	+++	Edema, congestion, MNC infiltration, diffuse interstitial and patchy pneumonia
Heart	+	Mild congestion and swelling of fibres
Spleen	+++	Loss of lymphoid cells, replaced by connective tissue
Brain	−	No abnormality detected

−: negative, +: mild, +++: severe.

(Figure 2). It was observed that the CDR2 and CDR3 regions were identical for the heavy chain in all seven clones. Two single base changes in CDR1 region of 1A4 and 1E5F4 and 3 base changes in 2C11 gave rise to some amino acid changes in the CDR1 region of 3 out of 7 clones. Regarding the light chain, 1A4 and 2C11 showed certain base changes, while all the other five clones were identical. Although the CDR3 region of 1A4 was decidedly different, the CDR1 and 2 regions were near identical. The remaining regions of the IgG1 chains of these clones were identical (data not shown). It is therefore apparent that the clones obtained in the earlier study originated from a single B cell clone. This could be due to an unusual expansion of the clone, possibly due to breakdown of tolerance, and was definitely an aberrant incidence since subsequent analysis failed to reproduce our earlier result of obtaining mAb clones against PfP0C0 (Table 2).

3.3. Recombinant PfP0 Immunizations Resulted in Pathological Changes in the Mice. During the course of spleen harvesting for hybridoma generation, we noticed mild connective tissue fibrosis of the spleen from the 7th immunization (Table 2). The animals started to become sick following the booster immunization. This fibrosis worsened progressively, and by 11th immunization the spleen became difficult to harvest (Figure 3). Marked splenic fibrosis had not been observed in the earlier study. Since the aim of that study was to obtain mAbs to PfP0C0, postmortem pathological examination had also not been undertaken. Postmortem analysis of the mice revealed pathological changes in all the major organs such as the heart, lungs, liver, and kidneys (Table 3). Interestingly, no abnormality was detected in the brain. These findings suggested that repeated immunizations with rPfP0 resulted in a humoral autoimmune response, possibly because of the homology between the parasite P0 and mouse P0. The brain is well separated from blood by the blood-brain barrier. Antibodies do not normally cross this barrier [32]. The pathological changes observed in all the major organs excluding the brain suggested autoantibodies as a possible cause of the damage. We therefore investigated the serum Ig response to PfP0C0.

3.4. Recombinant PfP0 Induces a Low-Titre, Largely Polyreactive Serum Anti-PfP0C0 Response. Sera collected from the mice after each monthly immunization with rPfP0 were tested for their anti-PfP0C0 response. The serum titre remained low even after the 11th administration of the protein

	CDR1	CDR2	CDR3
1A4_HC	SGFTFTSYAMS	ISSGGS	QWE
1B3_HC	SGFTFSSYAMS	ISSGGS	QWE
2C11_HC	LDYSFSSYAMS	ISSGGS	QWE
1E5F4_HC	LDSSFSSYAMS	ISSGGS	QWE
1F6_HC	SGFTFSSYAMS	ISSGGS	QWE
2G1_HC	SGFTFSSYAMS	ISSGGS	QWE
2H1_HC	SGFTFSSYAMS	ISSGGS	QWE

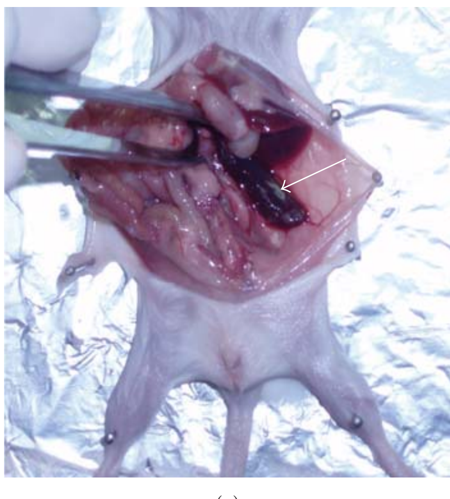
	CDR1	CDR2	CDR3
1A4_HC	TCTGGATTCA-CTTTCACTAGTTATGCCATGTCT	ATTAGTAGTGGTGGTAGT	CAGTGGGAG
1B3_HC	TCTGGATTCA-CTTTCAGTAGTTATGCCATGTCT	ATTAGTAGTGGTGGTAGT	CAGTGGGAG
2C11_HC	TCTGGATTATTCTTTCAGTAGTTATGCCATGTCT	ATTAGTAGTGGTGGTAGT	CAGTGGGAG
1E5F4_HC	TCTGGATTCATCTTTCAGTAGTTATGCCATGTCT	ATTAGTAGTGGTGGTAGT	CAGTGGGAG
1F6_HC	TCTGGATTCA-CTTTCAGTAGTTATGCCATGTCT	ATTAGTAGTGGTGGTAGT	CAGTGGGAG
2G1_HC	TCTGGATTCA-CTTTCAGTAGTTATGCCATGTCT	ATTAGTAGTGGTGGTAGT	CAGTGGGAG
2H1_HC	TCTGGATTCA-CTTTCAGTAGTTATGCCATGTCT	ATTAGTAGTGGTGGTAGT	CAGTGGGAG

	CDR1	CDR2	CDR3
1A4_LC	KSSQSLLNSGNQKNYL	GASTRESG	QNDHSYPYTF
1B3_LC	KSSQSLLNSRNQKNYL	FASTRESG	QQHYNTPLTF
2C11_LC	KSSQTLLNSRNQKNYL	FASTRESG	QQHYNTPLTF
1E5F4_LC	KSSQSLLNSRNQKNYL	FASTRESG	QQHYNTPLTF
1F6_LC	KSSQSLLNSRNQKNYL	FASTRESG	QQHYNTPLTF
2G1_LC	KSSQSLLNSRNQKNYL	FASTRESG	QQHYNTPLTF
2H1_LC	KSSQSLLNSRNQKNYL	FASTRESG	QQHYNTPLTF

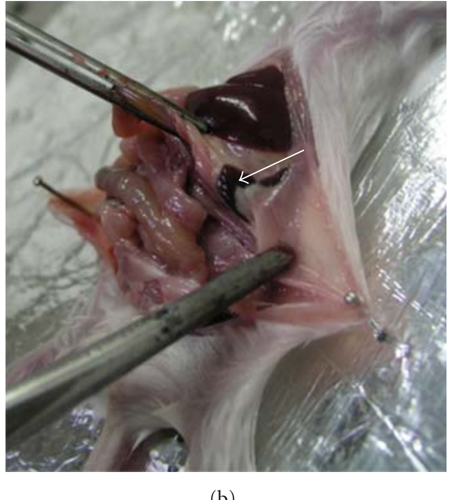
	CDR1
1A4_LC	AAGTCCAGTCAGAGTCTGTTAAACAGTGGAAATCAAAAGAACTACTTG
1B3_LC	AAGTCCAGTCAGAGCCTTTTAAATAGTAGAAATCAAAAGAACTATTTG
2C11_LC	AAGTCCAGTCAGACCCTTTTAAATAGTAGAAATCAAAAGAACTATTTG
1E5F4_LC	AAGTCCAGTCAGAGCCTTTTAAATAGTAGAAATCAAAAGAACTATTTG
1F6_LC	AAGTCCAGTCAGAGCCTTTTAAATAGTAGAAATCAAAAGAACTATTTG
2G1_LC	AAGTCCAGTCAGAGCCTTTTAAATAGTAGAAATCAAAAGAACTATTTG
2H1_LC	AAGTCCAGTCAGAGCCTTTTAAATAGTAGAAATCAAAAGAACTATTTG

	CDR2	CDR3
1A4_LC	GGGGCATCCACTAGGGAATCTGGG	CAGAATGATCATAGTTATCCGTACACGTTC
1B3_LC	TTTGCATCCACTAGGGAATCTGGG	CAGCAACATTATAACACTCCGCTCACGTTC
2C11_LC	TTTGCATCCACCAGGGAATCTGGG	CAGCAACATTATAACACTCCGCTCACGTTC
1E5F4_LC	TTTGCATCCACTAGGGAATCCGGG	CAGCAACATTATAACACTCCGCTCACGTTC
1F6_LC	TTTGCATCCACTAGGGAATCTGGG	CAGCAACATTATAACACTCCGCTCACGTTC
2G1_LC	TTTGCATCCACTAGGGAATCTGGG	CAGCAACATTATAACACTCCGCTCACGTTC
2H1_LC	TTTGCATCCACTAGGGAATCTGGG	CAGCAACATTATAACACTCCGCTCACGTTC

Figure 2: Sequence determination of anti-PfP0C0 mAbs obtained in the first study. Seven mAbs obtained in our earlier study [19], namely, 1A4, 1B3, 2C11, 1E5F4, 1F6, 2G1 and 2H1 were sequenced to determine their relatedness. Except for some minor variations (in red), all seven mAbs were nearly identical, suggesting that they had arisen from a single B cell clone.



(a)



(b)

Figure 3: Splenic fibrosis observed after repeated immunizations with recombinant PfP0. (a) Spleen of control mice. (b) Spleen of a mouse following repeated immunization with recombinant PfP0 (four weekly and seven monthly). Arrows point to the spleen. Note the reduced size of the spleen and fibrosis around it. Histopathological analysis revealed a loss of lymphoid cells and extensive replacement of splenic tissue with connective tissue.

(Table 2). By contrast, high-titre responses were obtained after just 4 or 5 immunizations against a variety of other proteins, including those that displayed a high degree of homology to mouse proteins such as *P. falciparum* enolase, *Drosophila* translin, and TgP0. The low titre of the sera, coupled with the unstable polyreactive hybridomas obtained, suggested the possibility that the induced anti-PfP0C0 response was polyreactive. Polyreactive antibodies are low affinity antibodies that can bind a range of biological molecules of both self- and non-self-origin [33, 34]. They are normal serum constituents and are a significant part of the initial immune response. DNP treatment is commonly used to remove polyreactive antibodies in animals not exposed to the hapten [35, 36]. We treated the sera with DNP-sepharose beads and determined the anti-PfP0C0 titre of adsorbed and unadsorbed sera in ELISA. Treatment with DNP led to a statistically significant reduction in the anti-PfP0C0 reactivity of the sera, indicating that PfP0 immunization induced a largely polyreactive anti-PfP0C0 response. Figure 4(a) shows a typical response of DNP adsorbed and unadsorbed serum sample. The extent of polyreactivity remained high (median value 32%) even after repeated boosters (Figure 4(b)). This polyreactive response was not confined to a particular isotype, but was found across all isotypes, since DNP treatment resulted in statistically significant reduction in anti-PfP0C0 response in all isotypes (Figure 4(c)). We also tested the sera for reactivity against dsDNA, ssDNA, and insulin. Figure 4(d) shows the result of a single serum sample. Similar results were obtained for other samples (data not shown).

3.5. Malarial Infection Also Results in a Low-Titre, Largely Polyreactive Serum Anti-PfP0 and Anti-PfP0C0 Response. We wished to determine if serum from patients of uncomplicated malaria also showed a similar polyreactive response to PfP0. The protein is highly conserved across *P. falciparum*. Amongst the 13 strains reported in PlasmoDB [37], the DNA sequence is identical except for a single synonymous mutation in one strain (Figure 5). PfP0 is also abundantly expressed on the surface of merozoites [18]. Although we normally see elevated antibody responses to PfP0 in malaria immune adults as compared to patients [18], we had not assessed the polyreactive component earlier. We tested sera from immune adults as also patients of uncomplicated malaria for anti-PfP0 and anti-PfP0C0 antibodies. In agreement with our earlier results, immune adults, but not patients, showed an elevated response to PfP0 (Figure 6(a), $P < 0.01$). Analysis of the response for polyreactivity to PfP0 revealed that the patient's sera were highly polyreactive (Figure 6(b); median value 44.8%). By contrast, sera from immune adults exhibited minimal polyreactivity to the protein ($P = 0.001$; median value 13.9%). Similar results were obtained for PfP0C0 (data not shown).

Polyreactive autoantibodies have been reported in *Plasmodium-chabaudi*-infected mice [38]. The autoantibodies found in clinically protected persons resident in malaria hyperendemic areas are similar to those present in disorders such as SLE, rheumatoid arthritis, Sjogren's syndrome, polymyositis, scleroderma, and Hashimoto's thyroiditis. These can bind to double- and single-stranded DNA, erythrocytes, immunoglobulins, ribonucleoproteins, and enolase. However, anti-thyroglobulin antibodies, autoreactive to B cells and found in normal persons, are not enhanced in such a population, indicating that it is not a matter of random non-specific polyclonal B cell activation against conserved antigens [18, 25, 39, 40]. It is unlikely that homology of PfP0 to the host protein is responsible for the observed polyreactive response since repeated immunizations with Pf enolase or bovine serum albumin coupled to PfP0C0 (BSA-PfP0C0) failed to induce a polyreactive response. Interestingly, although specific, BSA-PfP0C0 administration did not induce a high titre anti-PfP0C0 response (titre $\sim 2\times 10^4$,

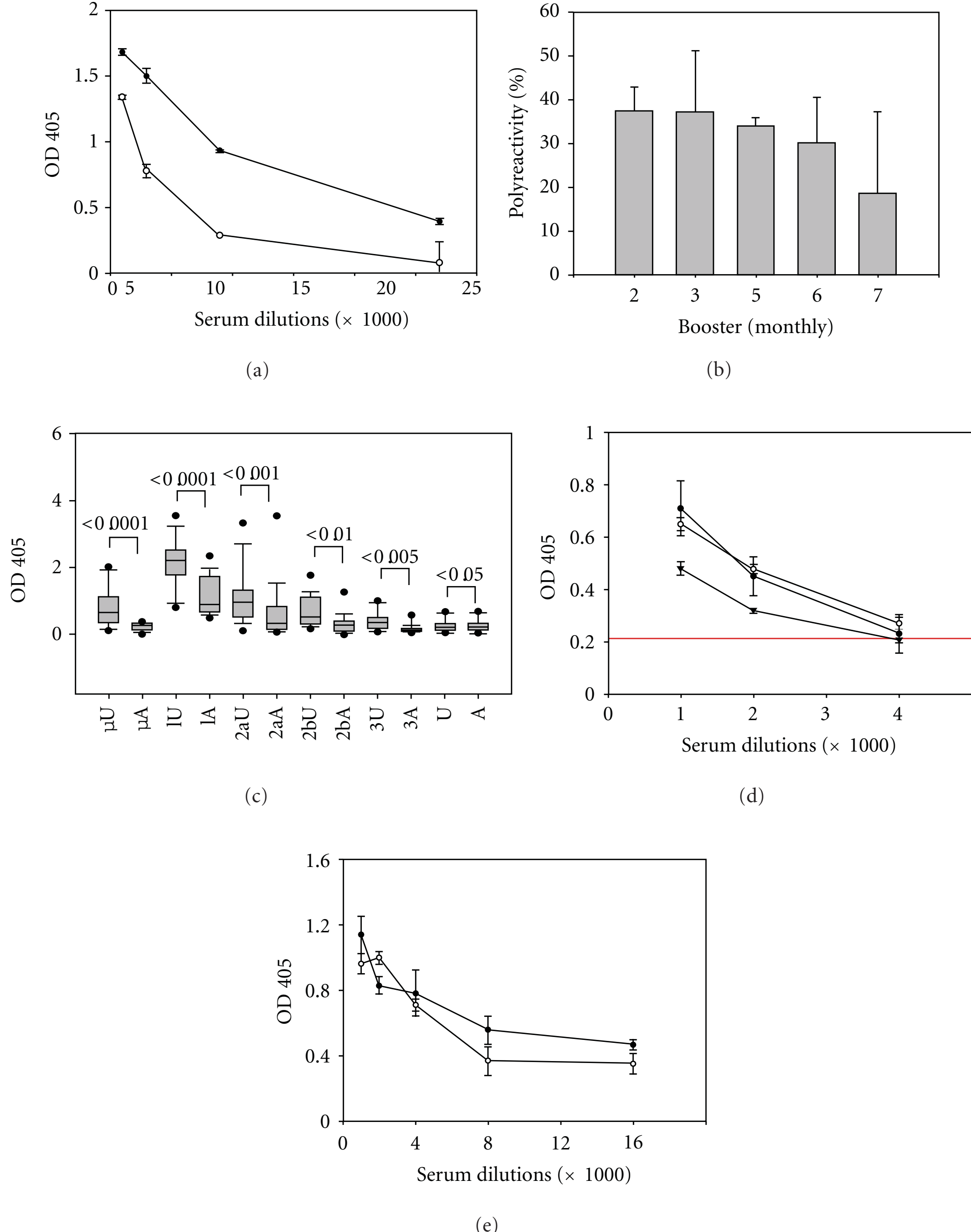


Figure 4: Polyreactive serum anti-PfP0C0 response induced following repeated immunizations with rPfP0 but not BSA-PfP0C0. (a) Sera of mice administered recombinant PfP0 were treated or not with DNP-sepharose beads. Anti-PfP0C0 titre before and after treatment was determined in an ELISA. A representative example of anti-PfP0C0 response of DNP adsorbed (empty circles) and unadsorbed (filled circles) sera. Error bars represent the SEM of three readings. (b) Mice were immunized by four weekly and up to seven monthly injections of rPfP0. Aliquots of the sera were treated with DNP-sepharose beads to adsorb out polyreactive antibodies. The bar graphs depict the percent polyreactivity in sera of mice after multiple immunizations as indicated. Error bars represent the SEM of 3 sera. (c) Box plots showing the 25th and 75th percentiles, together with the median, with whiskers showing the minimum and maximum difference and filled circles representing the outliers, in the ELISA OD responses of DNP unadsorbed (U) and adsorbed (A) sera of all 21 mice immunized with recombinant PfP0; μ (IgM), γ1 (IgG1) γ2a (IgG2a), γ2b (IgG2b), γ3 (IgG3), and α (IgA). Statistically significant values obtained by Paired Wilcoxan signed-rank test are indicated. (d) Autoreactivity of a representative serum sample against bovine insulin (closed circle), dsDNA (open circle), and ssDNA (closed triangle) of mice immunized with rPfP0. Red line represents the response of control unimmunized sera at the lowest dilution (1 : 1000). Error bars represent the SEM of three readings. Similar results were obtained with other sera. (e) Mice were administered BSA-PfP0C0, and the sera were treated or not with DNP-sepharose beads. Anti-PfP0C0 titre before and after treatment was determined in ELISA. A representative example of anti-PfP0C0 response of DNP adsorbed (empty circles) and unadsorbed (filled circles) sera. Error bars represent the SEM of three readings.

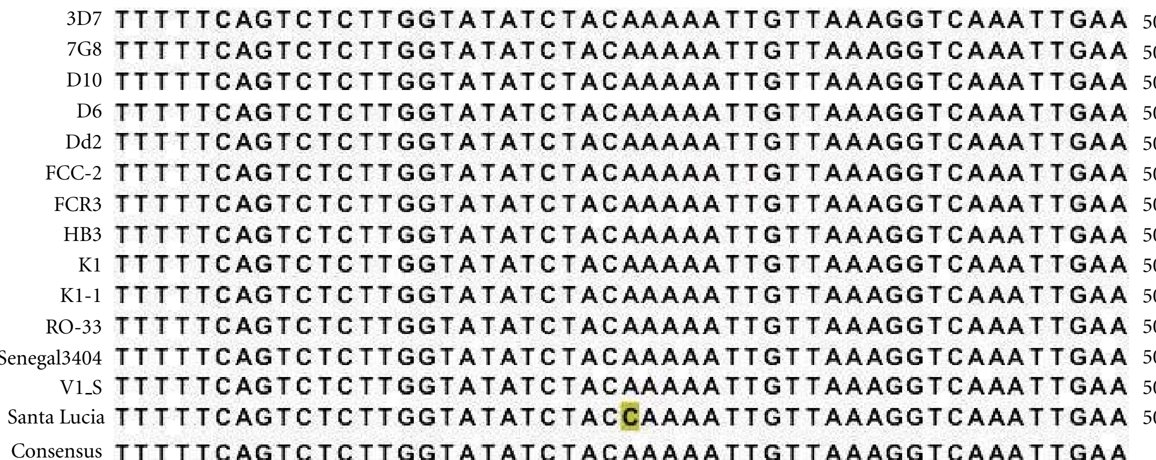


Figure 5: DNA sequence alignment of P0 across *P. falciparum* strains showing one synonymous mutation (marked in yellow) in the Santa Lucia strain. The rest of the sequences were identical across strains.

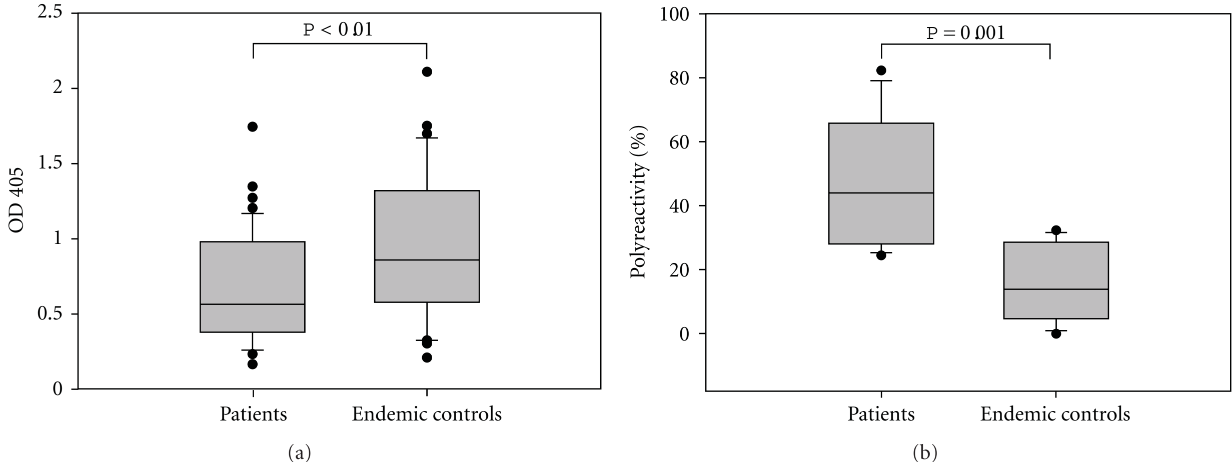


Figure 6: Polyreactive serum anti-PfP0C0 response observed in uncomplicated malaria patients, but not in immune adults living in high endemicity areas. (a) Box plots showing the 25th and 75th percentiles, together with the median, with whiskers showing the minimum and maximum difference and filled circles representing the outliers in anti-PfP0 responses of sera from patients of uncomplicated malaria ($n = 49$) and immune adults ($n = 30$) as determined in ELISA. (b) Box plots showing the 25th and 75th percentiles, together with the median, with whiskers showing the minimum and maximum difference and filled circles representing the outliers of % polyreactivity in anti-PfP0 responses of sera from patients and immune adults. The sera were treated or not with DNP-sepharose beads, and anti-PfP0 titre before and after treatment was determined in ELISA to determine the % polyreactivity.

Figure 4(e)). However, this response was autoreactive, since the sera were reactive to mouse P0C0, albeit at a tenfold lower concentration (titre $\sim 2 \times 10^3$, data not shown). Thus, the potentially autoreactive epitope coupled to a carrier protein failed to induce a polyreactive response, but the recombinant protein containing 61–316 amino acids induced a low-titre, polyreactive response. This suggests that the capacity to induce polyreactivity probably lies in the 61−300 region of the protein. It is also possible that the conformation of the protein is an important factor in the induction of polyreactivity or that rPfP0 acts like a B cell mitogen. Further investigations are needed to determine if a particular region and/or the conformational structure of the protein is responsible for induction of a polyreactive response.

Normal human serum contains natural autoantibodies that recognize self-antigens [41]. Analysis of human monoclonal autoantibodies derived from normal peripheral blood B cells has shown that natural autoantibodies are polyreactive and express germline immunoglobulin variable region genes with little or no somatic mutation [42, 43], although polyreactivity has also been observed in affinity-matured antibodies [44]. The capacity to adopt multiple conformational states in equilibrium allows polyreactive antibodies to bind multiple, structurally unrelated antigens [45]. Polyreactive antibodies have been reported to be induced in murine model of malaria [38]. We show that immunization with a parasite-derived protein can also induce a polyreactive response. Interestingly, we find people suffering from malaria also exhibit a polyreactive response to PfP0. This polyreactive response was confined to patients, since sera from immune adults showed a comparatively non-polyreactive response to the protein. We have earlier shown

that PfP0 is expressed on the surface of merozoites [18]. Thus, it could be that exposure to low levels of the protein (as would happen in the case of immune endemic adults) results in specific response to the protein, and this specific response protects against malarial disease. Exposure to high doses of the protein (e.g., during active malarial disease or during immunization protocols), on the other hand, possibly results in a polyreactive response. Further investigations are needed to see if low doses of PfP0 with or without a strong adjuvant result in a specific anti-PfP0 response.

Antibodies have a crucial role in the control of malarial infection. Opsonization by antibodies to parasite proteins expressed on the surface of merozoites or infected erythrocytes facilitates their removal and eventual destruction by macrophages [46]. The malarial parasite has been shown to subvert the host immune system in multiple ways including antigenic variation and antigenic polymorphisms, interference with dendritic cell maturation, and induction of apoptosis in memory B and T cells [47–50]. Here, we show that a conserved, parasite-derived protein induces a deviant humoral response in the host. A propensity to express or secrete conserved antigens that induce a weak polyreactive humoral response could be one more mechanism of enhancing parasite survival.

Abbreviations

PfP0: *Plasmodium falciparum* P0
rPfP0: Recombinant PfP0
PfP0C0: Carboxy-terminal 16 amino acids of PfP0
SLE: Systemic lupus erythematosus
mAbs: Monoclonal antibodies
DNP: Dinitrophenyl
TgP0: *Toxoplasma gondii* P0
BSA-PfP0C0: Bovine serum albumin coupled to PfP0C0.

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Research Article

Proteomic Analysis of *Trypanosoma cruzi* Epimastigotes Subjected to Heat Shock

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Trypanosoma cruzi is exposed to sudden temperature changes during its life cycle. Adaptation to these variations is crucial for parasite survival, reproduction, and transmission. Some of these conditions may change the pattern of genetic expression of proteins involved in homeostasis in the course of stress treatment. In the present study, the proteome of *T. cruzi* epimastigotes subjected to heat shock and epimastigotes grow normally was compared by two-dimensional gel electrophoresis followed by mass spectrometry for protein identification. Twenty-four spots differing in abundance were identified. Of the twenty-four changed spots, nineteen showed a greater intensity and five a lower intensity relative to the control. Several functional categories of the identified proteins were determined: metabolism, cell defense, hypothetical proteins, protein fate, protein synthesis, cellular transport, and cell cycle. Proteins involved in the interaction with the cellular environment were also identified, and the implications of these changes are discussed.

1. Introduction

Chagas disease is endemic and is recognized as a major health problem in many Latin American countries, where it is estimated that approximately 10–12 million people are affected by this condition, causing 15,000 deaths per year [1, 2]. This disease is caused by the hemoflagellated parasite *Trypanosoma cruzi*, which has a complex life cycle, alternating its different developmental stages (trypomastigotes, epimastigotes, bloodstream trypomastigotes, and amastigotes) between two different hosts: the insect vector (Hemiptera, family Reduviidae, subfamily Triatominae) and the reservoir (many vertebrates, including man) [3]. Because of the digenetic life cycle of this parasite, it is exposed to growth conditions that differ significantly between the invertebrate and vertebrate hosts, as the physiological characteristics of these two hosts are clearly heterogeneous [4]. These differences are observed in the temperature, pH, nutrient availability, redox potential, and so forth, in which each represent a cellular stress to the parasite [5, 6]. In addition, once the parasite is inside the insect or vertebrate, it is exposed to fluctuations that occur due to the switch from the extracellular to the intracellular environment and the host immune response [6]. In mammalian cells, the parasite is exposed to low pH values, as the parasitophorous vacuole is acidified over time [7], as well as free radicals, which are part of the defense mechanism that the host cell develops [8]. In the digestive tube of the insect vector, the presence of digestive enzymes and a hemolytic factor produce a potentially hostile environment for the parasite [9]. Some of these alterations may lead to cell death, but, like many other parasites, *T. cruzi* has developed mechanisms that allow it to survive and multiply under fluctuating environmental conditions. These adaptations have produced alterations in gene expression modulated by environmental factors [6]. In *T. cruzi*, one of the stresses experienced during the passage

from one host to another is the exposure to sudden changes in temperature, while, inside the insect, the parasites are subjected to an average temperature of 26°C, but, once they enter the mammalian host, they face temperatures of 37°C or more, experiencing a heat shock [10]. Nonlethal heat shock is produced when there is a sudden increase in the environmental temperature; it causes specific changes in the pattern of gene expression and in cellular functions of the organisms, generating a cellular stress response [11].

In trypanosomes, all protein-encoding genes are organized into large polycistronic transcription units (several genes are transcribed by a single mRNA); therefore, gene regulation is controlled by the stability and/or translation of specific mRNAs. Additionally, posttranslational modifications play an important role in modulating protein function [12]. Thus, gene expression in *T. cruzi* is regulated primarily at the posttranscriptional level, and, like other organisms, there has been a poor correlation between mRNA levels and protein expression. Therefore, proteomic analysis of *T. cruzi* is important for the study of changes in global gene expression in specific physiological conditions in this parasite.

Characterizing the gene expression pattern of *T. cruzi* under stress conditions is essential because cellular stress is part of the parasite life cycle. It is important to identify which proteins are modified during this particular phenomenon in order to have a broader understanding of the biology of this pathogen. At this time, there are no safe chemotherapeutic agents or effective preventive vaccines against the parasite. To identify potential therapeutic candidates, methodologies can be used to study a large number of proteins that may be involved in parasite survival after the passage of one host to another.

In this study, a general proteomic analysis of *T. cruzi* parasites exposed to heat shock was performed. The protein pattern of epimastigotes under normal growth conditions was compared to the pattern after heat shock exposure, and, for the first time, twenty-four proteins with a different abundance between the two conditions were identified. Several groups of proteins with important roles were identified, including those localized on the surface.

2. Materials and Methods

2.1. Parasites. T. cruzi epimastigotes of the strain Ninoa (MHOM/MX/1994/Ninoa) [13, 14] were routinely maintained at 28°C in liver infusion tryptose (LIT) media supplemented with 10% fetal calf serum and 25 μg/mL hemin [13].

2.2. Heat Shock Assays. Cultures were grown in 25 cm^2 culture dishes at a concentration of 3×10^6 cells/mL in a final volume of 10 mL fresh LIT medium. After three days of incubation at 28°C, which corresponded to the exponential growth phase, and when the culture had reached a density of $25 \times 10^6 \pm 5 \times 10^6$ cells/mL, the culture was divided into two equal parts. Five mL of the culture was maintained at 28°C for use as a control, and the other 5 mL was subjected to a sudden change in temperature by incubation at 37 or 42°C for 3 h. For each condition, after the incubation period, cultures were harvested by centrifugation at 2,500 ×g for 10 min at 4°C and washed three times with PBS. Finally, the wet weight of the cell button was determined before proceeding with the protein extraction. Before protein extraction, samples of parasites were fixed and Giemsa-stained.

2.3. Protein Extraction. The pellet of parasites was resuspended in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.0008 g/mL tris (hydroxymethyl-aminomethane)) with protease inhibitors (12 mM EDTA, 1 mM PMSF, 0.001 mM pepstatin and 0.1 mM leupeptin) in a ratio of 3 mL lysis buffer/g of wet weight. The pellets were stirred vigorously for 3 min, incubated on ice for 10 min and centrifuged at 12,800 ×g for 15 min, and the supernatant was then recovered (protein extract). Protein extracts were kept at −70°C until use, and the protein integrity was assessed by 12% SDS-polyacrylamide gel electrophoresis (PAGE). Protein concentrations were determined using the 2D-Quant kit (Amersham Biosciences), according to manufacturer's instructions.

2.4. Two-Dimensional Gel Electrophoresis (2-DE). For isoelectric focusing (IEF), aliquots of 300 μg of protein in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% IPG buffer (pH range 3–10), 8 mg dithiothreitol (DTT), trace bromophenol blue) from control or heat-shock-treated epimastigotes lysates were applied to 17 cm immobilized pH gradient (IPG) gel strips (Bio-Rad) with a nonlinear (NL) pH range of 3–10, by in-gel sample rehydration, overnight. The proteins were then focused in a PROTEAN IEF Cell (Bio-Rad) using the following protocol: 250 V, 20 min, 20°C; 10 000 V, 2.5 h, 20°C; 40 000 V/h, 6–8 h, 20°C. Before the second dimension, proteins were reduced (10 mg/mL DTT) and alkylated (25 mg/mL iodoacetamide) in equilibration buffer (6 M urea, 2% SDS, 0.05 M Tris-HCl pH 8.8, 30% glycerol, trace bromophenol blue) [15]. Proteins were separated in a second dimension on 10% SDS-PAGE gels using a vertical PROTEAN II XL system (Bio-Rad) and standard Tris/glycine/SDS buffer with the following program: 16 mA, 30 min; 24 mA, 6–8 h.

2.5. Gel Staining and Image Analysis. Gels were stained with silver nitrate, according to the following protocol: fixation for 12 h in 50% methanol/10% ethanol; incubation for 1 h in 5% methanol/1% ethanol; three washes of 5 min in deionized water; incubation for 1.5 min in 0.02% sodium thiosulfate; three washes of 30 s in deionized water; staining with 0.2% silver nitrate for 40 min; three washes of 60 s in deionized water; visualization with developing solution (6% sodium carbonate, 0.05% formaldehyde, 0.002% sodium thiosulfate); finally, 6% acetic acid was added [15]. Observed protein pIs were calculated using the chart of Immobiline DryStrip pH 3–10 NL 24 cm, pH as function of distance at 20°C and 8 M urea (GE Healthcare). The molecular mass was calculated based on the protein migration distance

(Rf) calibrated against Silver Stain SDS-PAGE Molecular Weight Standard Mixtures Wide Molecular Weight Standard (Sigma Aldrich). Silver-stained gel images were digitalized using a Fluor-S MultiImager (Bio-Rad). Image analysis was performed using PDQuest 2D analysis software (Bio-Rad). After background subtraction and noise filtering, spots were detected and quantified. The spots were measured by taking the optical density (OD), which is calculated by dividing the incident light intensity by the transmitted light intensity and taking the log (base 10) of the ratio. An OD value was assigned to each pixel, and linear interpolation of each OD was used to express quantification. Spots that showed a change of at least 1-fold in intensity between control and heat-shock-treated samples (mean OD value of control/mean OD value of heat shock treated) from three gel replicates were selected for identification.

2.6. Mass Spectrometry. Protein extracts from heat-shock-treated *T. cruzi* of the strain Ninoa were subjected to 2D-PAGE and were silver stained using the silver SNAP stain for mass spectrometry kit (Pierce), following the manufacturer's instructions. Selected spots were excised manually from the gel and identified by peptide mass fingerprinting (PMF). Briefly, proteins were subjected to in-gel digestion with trypsin (20 ng/μL) at 37°C overnight. After treatment with ammonium bicarbonate, the peptides were extracted with 50% acetonitrile/5% formic acid and concentrated under a vacuum. The peptides generated were incorporated into a metal plate using the acid α-cyano-4-hydroxycinnamico as a matrix. The peptide mixtures were then ionized in a Voyager DE-PRO matrix-assisted laser desorption/ionization/time-of-flight (MALDI-TOF) mass spectrometry system (Applied Biosystems, USA) equipped with a nitrogen laser at 337 nm, and ion/mass (m/z) or peptide fingerprints were obtained. Mass spectra were acquired in reflector mode and externally calibrated with Sequazyme Mass Standards Kit (Applied Biosystems). Proteins were identified by database searching with the peptide fingerprints using the Protein Prospector database (http://prospector.ucsf.edu) and MS-Fit UniProt.2006.03.21 (http://www.pir.uniprot.org/), based on the following search parameters: the *Trypanosoma* species of origin, pI ± 0.1% and MW ± 2% with tolerance of 75 ppm, one tryptic miss-cleavage allowed, variable modifications of methionine (oxidation), cysteine (carbamidomethylation) and pyroglutamate formation at N-terminal glutamine of peptides.

3. Results and Discussion

In *T. cruzi*, the heat shock response has been correlated to heat shock proteins (HSPs) expression [4, 10, 16–18], but the identity of other proteins involved in this process has not been reported. However, due to the physiological changes in cells subjected to heat shock, it is expected that a diverse set of proteins with a wide variety of functions involved in optimizing cellular metabolism will be expressed and that the heat shock response will not be limited to the production of chaperones [19–21]. In this study, we identified, for the first time, spots showing a different abundance in *T. cruzi* epimastigotes subjected to heat shock compared to control parasites. This means that these spots showed a change in the amount of this particular form of the protein in response to temperature stress, suggesting a potential role in the heat shock response. In 2-DE gel, we can measure the amount of a particular form of a protein in a specific biological situation and experimental setting. The particular form of a protein we observe may result, as has been mentioned by Jungblut et al. in 2008, from transcripts from indistinguishable genes, indistinguishable parts of genes, or posttranslational modifications [22].

Studies on the nonlethal heat shock response in *T. cruzi* epimastigotes have been performed at different growth temperatures ranging from 37 to 43°C [10]. The types of responses observed at different temperatures differ and depend on the strain. In previous work, the Maracay strain exposed to 42°C for 4 h was observed to acquire a round shape [4]. The Silvio X-10/4 strain incubated at the same temperature for 3 h did not show this transformation [10]. de Carvalho et al. [16] determined that the CL strain does not have a classic stress response at 37°C. In another study using *T. cruzi* epimastigotes, a large increase in the expression level of HSP70, one of the key molecules induced in the heat shock response in many organisms, occurred only after a 3 h incubation at 42°C and was not observed after the same incubation time at 37 or 40°C [10]. In Mexico, it has been reported that the vast majority of isolates analyzed belong to the discrete typing unit (DTU) genetic group I, including the Ninoa strain, which we used in this work and for which some of the relevant biological characteristics have been described [13, 14, 23]. As this was the first study performing heat shock assays on the Ninoa strain, the first aim was to determine which temperature was adequate for the production of heat shock. For this, the effect of two nonlethal heat shock temperatures were tested on the mobility of *T. cruzi* epimastigotes, bearing in mind that movement is an indirect parameter of the effect of temperature on parasite homeostasis. Mobility inhibition curves of the *T. cruzi* strain Ninoa incubated at 37 or 42°C were generated. As shown in Figure 1(a), the experimental temperature of 37°C did not have any effect on the mobility of parasites, which remained virtually unchanged compared to the control temperature (28°C). In contrast, when the incubation temperature was elevated to 42°C, a decrease in the parasites' mobility was observed, which was time dependent. Also, parasites at 42°C showed slight morphological changes at 3 h of incubation, some of them began to acquire a round shape (Figure 1(b)). Based on the previous curve and the literature [10, 16], parasites were incubated at 42°C for 3 h to induce heat shock, which led to a mobility rate of about 75% and some morphological changes.

Protein extracts from control and heat-shock-treated parasites were subjected to 2-DE gel electrophoresis. Figure 2 shows 2-DE gels representative of three independent experiments from *T. cruzi* epimastigotes of the strain Ninoa under control (3 h at 28°C) (Figure 2(a)) and heat shock (3 h at 42°C) (Figure 2(b)) conditions, stained with silver nitrate. An average of 506 ± 73 spots were found in gels from

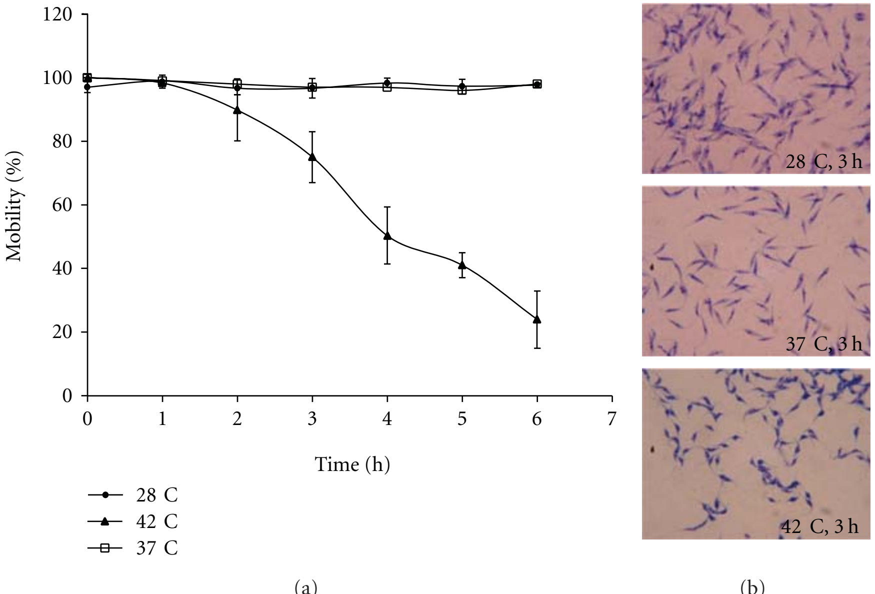

Figure 1: *T. cruzi* epimastigotes mobility and morphology after heat shock. (a) *T. cruzi* of the strain Ninoa in log phase growth were incubated at 28, 37, or 42°C for 1 to 6 h. Aliquots of each culture were taken each hour, and the number of mobile and immobile parasites was determined in a hemocytometer by observation in a microscope. The data are representative of three independent experiments and are shown as the mean ± SD. (b) Epimastigotes Giemsa-stained in log phase growth incubated at 28, 37, or 42°C for 3 h are shown.

parasites incubated at 28°C, whereas gels obtained from heat-shock-treated parasites had an average of 521 ± 31 spots. The number of proteins detected was comparable to and even higher than those previously reported by Parodi-Talice et al. in 2004, where an average of 350 spots was found using protein extracts from epimastigotes at the same pH range, run on a 10% polyacrylamide gel, and stained with silver nitrate [24]. An analysis of the distribution of the protein isoelectric point (pI) or the molecular weight (MW) for both conditions was performed. Extracts from parasites growing at either 28°C or 42°C showed a normal distribution of proteins based on established pI or MW ranges (data not shown). In fact, these two conditions had almost identical values for both distributions; most of the proteins had a pI of 5.1–7.0 and a molecular weight of 31–70 kDa, as was previously reported by Parodi-Talice et al. [24]. Additionally, as previously was reported by the same authors, most of the proteins were localized in the acidic region and, consequently, the resolution of the separation of proteins was lower in this region of the gel than in the basic area (Figure 2). In different studies about the *T. cruzi* proteome over pH range of 3–10, there is a constant finding that the acidic region shows a low resolution and that the proteins with molecular weights above 90 kDa are subrepresented [24, 25]. Perhaps the intrinsic characteristics of parasite proteins are involved in this particular distribution.

A master gel from control and heat-shock-treated parasites is shown in Figure 2(c) and Figure 2(d), respectively, which contains all of the spots found in the gels of three independent experiments.

Then, for the selection of proteins identified by MS, we only considered those points that had a differential abundance between control and experimental conditions (OD relation), were consistently found in three independent experiments, and were in sufficient concentrations for identification by PMF. Ultimately, we obtained peptide mass fingerprints for twenty-four spots. The spots that were analyzed by MS are indicated on the proteome of 42°C (Figure 2(b)), each with a number corresponding to Table 1, where they are listed and their main characteristics are described. Nineteen of the twenty-four spots corresponded to particular forms of proteins in which the value of the average intensity of three independent experiments was higher compared to the control spot, whereas, in the remaining five identified spots, the intensity was lower in the heat-stress experiments with respect to the control spots (Table 1). These spots represent a particular form of the identified protein.

Other parameters included in Table 1 are the MOWSE score, which is a conventional measured validation of peptides identified by MS that reflects the match between the alignment of the theoretical and experimentally determined masses, and, finally, the absolute probability that this match is a random event. Therefore, the protein with the highest score is the most likely to be the protein [26]. The percentage of coverage refers to fragments of the candidate protein sequence that aligned with the ion signals of peptides generated by the mass spectrometer. The accession numbers for each protein correspond to UniProt protein entries. Finally, the abundance (in number of folds) of the particular form of the protein in heat shock compared with the same particular form of the protein in control conditions was calculated.

The twenty-four particular forms of identified proteins showed a variability of functions and belonged to some of the functional groups of proteins that have been described to

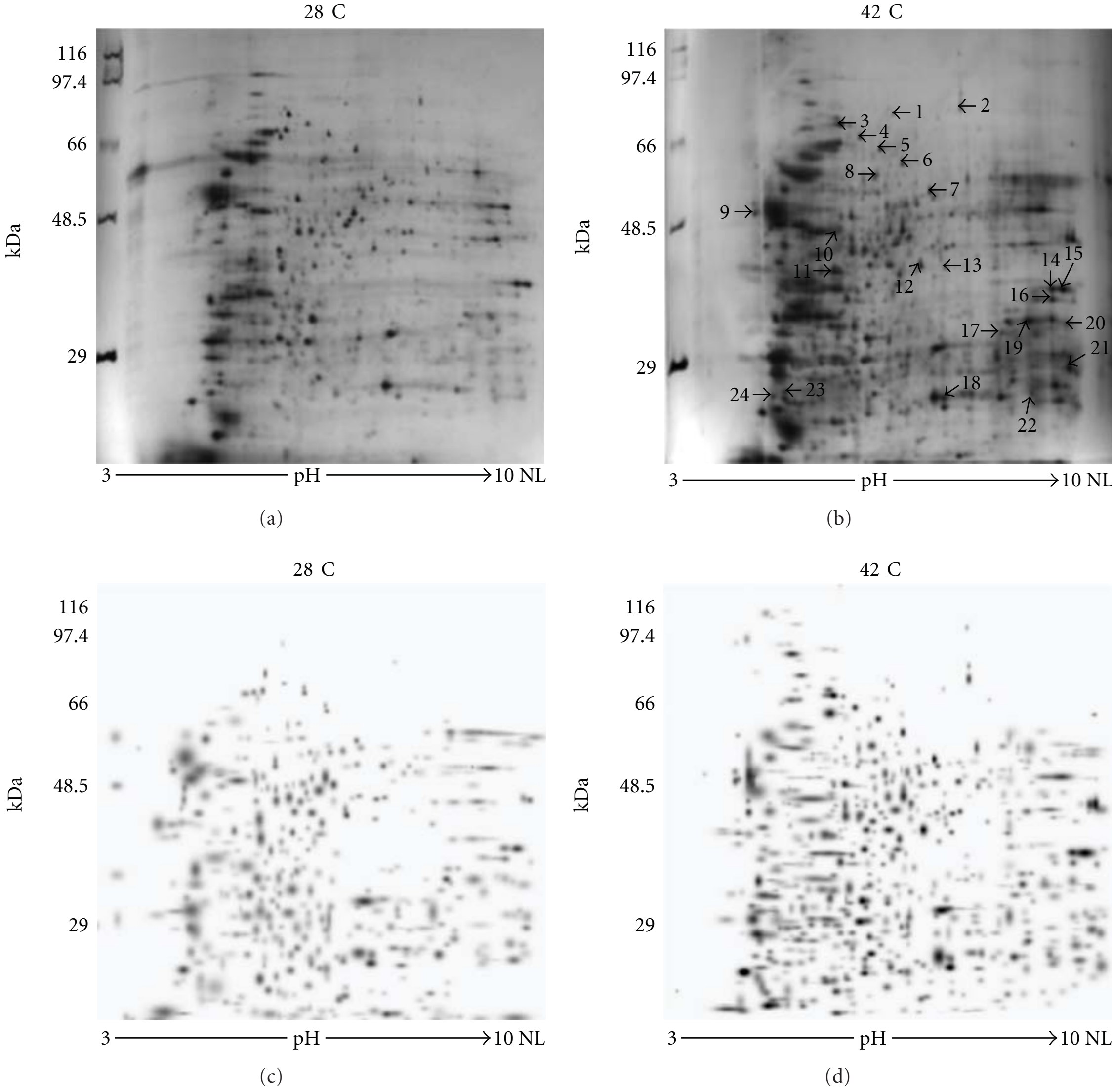

Figure 2: 2-DE polyacrylamide gels of control and heat-treated parasite extracts. (a) and (b) Silver-stained 2-DE gels of soluble proteins (300 μg per gel) from *T. cruzi* epimastigotes at normal growth (28°C, 3 h) (a) or heat shock (42°C, 3 h) temperatures (b). The proteins were firstly separated in a pH range of 3–10 NL and then on 10% SDS-PAGE. An average of 506 $\pm$ 73 spots were found in gels from parasites incubated at 28°C, and gels obtained from heat-shocked parasites had an average of 521 $\pm$ 31 spots. The molecular standards are indicated in kDa. The gels are representative of three independent experiments. In 2-DE gel from 42°C, the spots indicated by numbers were identified by MS (Table 1). (c) and (d) Master gels of control (c) or heat shock conditions (d). Three gel images obtained from different experiments from each condition (28°C or 42°C) were aligned and matched each other to generate an average master gel using the PDQuest software.

be induced by heat shock in many of the organisms analyzed by proteomic methods. The twenty-four particular forms of proteins identified were grouped into functional categories according to the Functional Catalogue 2004 [27]. Eight functional categories were assigned, and the percentages of particular forms of proteins identified that corresponded to each category were determined: metabolism (25%), cell defense (16.66%), interaction with the cellular environment (16.66%), hypothetical proteins (16.66%), protein fate (12.5%), cellular transport (4.16%), protein synthesis (4.16%), and cell cycle (4.16%). Six of the particular forms of proteins had a MOWSE score lower than 67; therefore, they are not discussed hereafter.

Particular forms of proteins involved in cell metabolism represented the largest group, which included particular forms of proteins involved in energy generation such as ATPase and ATP synthase. Another enzyme was found to be involved in the energy-generating process by means of the catabolism of aromatic amino acids, the hydroxyacyl-alpha-L-aromatic dehydrogenase [28]. Homologous proteins increase in abundance from other organisms like bacteria, and plants under thermal stress have been identified in previous proteomic analyses similar to this study [19–21]. Within the same functional category of proteins, a lower intensity of the NAD/FAD-dependent dehydrogenase, involved in oxidative phosphorylation, was observed after

Table 1: Particular form of proteins with a different abundance from *T. cruzi* epimastigotes subjected to heat shock identified by MALDI-TOF.

Particular form of a protein identity	Spot	Accession number	Theoretical pI/MW (kDa)	Experimental pI/MW (kDa)	Sequence coverage (%)	MOWSE score	Abundance (folds relative to control)
Metabolism							
Transitional endoplasmic reticulum ATPase, putative	3	Q4DWB5	5.4/86.1	5.4/77	50.5	$7.47\ e+6$	2.057
ATP synthase, putative	7	Q4E1T6	6.0/53.7	5.9/57.6	22.8	101	1.876
Glutamate dehydrogenase, putative*	13	Q4CR18	6.0/43.2	6.1/43.4	24.5	16.8	1.317
Calcium translocating P-type ATPase*	16	Q86QH6	8.3/37.3	8.2/37.9	28.2	62.1	1.197
NAD-/FAD-dependent dehydrogenase, putative	11	Q4CVH0	5.3/43.0	5.3/43.4	21.4	72.6	1.072
Aromatic L-α-hydroxy acid dehydrogenase	17	Q9NJT2	6.8/33.7	6.9/34.1	25.3	204	−1.228
Cell defense							
Heat shock protein 70 (HSP70), putative	6	Q4DTM9	5.8/70.9	5.8/63.4	29.7	5611	1.776
Thiol transferase Tc52	10	Q7Z0C8	5.4/48.9	5.5/48.5	50.5	473601	1.544
Tryparedoxin peroxidase, putative	22	Q4CX87	7.6/25.5	7.8/25.6	35.0	124	1.125
Chaperone DNAJ protein, putative	19	Q4E244	7.6/35.3	7.6/34.9	41.7	2349	1.065
Interaction with the cellular environment							
Surface glycoprotein Tc-85/16*	8	Q2VYD0	5.5/66.8	5.6/61.5	27.6	1.81	1.821
Mucin-associated surface protein (MASP), putative	9	Q4CYQ5	4.4/50.0	4.2/53	29.3	692	1.403
Surface protease gp63, putative	5	Q4DTZ3	5.7/78.6	5.6/66	46.1	$3.25\ e+9$	1.218
Mucin-associated surface protein (MASP), putative*	23	Q4DQ68	4.8/26.5	4.8/26.6	45.3	29.4	−1.514
Hypothetical protein							
Hypothetical protein	1	Q4D9E4	5.6/88.3	5.7/82.3	18.9	1160	1.577
Hypothetical protein	15	Q4CYM6	8.4/39.2	8.5/39.4	41.2	1197	1.569
Hypothetical protein	14	Q4CTH8	8.2/39.5	8.2/39.4	24.3	5713	1.537
Hypothetical protein	20	Q4E5C7	8.7/34.8	8.7/34.6	44.3	3723	−1.114
Protein fate							
Ubiquitin hydrolase, putative	4	Q4E680	5.6/81.1	5.5/71.4	38.6	$1.23\ e+7$	1.390
Ubiquitin-conjugating enzyme E2, putative	24	Q4DVH2	4.5/25.7	4.5/26	34.8	96.6	−1.293
Proteasome regulatory non-ATPase subunit, putative*	18	Q4CPL0	6.0/25.2	6.1/25.7	41.7	7.13	−1.226
Protein synthesis							
Cysteinyl-tRNA synthetase, putative*	2	Q4CQ34	6.1/89.2	6.2/85.1	13	28.9	1.396
Cellular transport							
Rab6 GTPase-activating protein, putative	12	Q4DHN9	5.8/44.2	5.8/44.3	25.7	4739	1.342
Cell cycle							
Retrotransposon hot-spot (RHS) protein, putative	21	Q4D6R8	8.6/29.0	8.7/28.8	36.1	1456	1.206

* These proteins were not discussed because their MOWSE score is lower than 67.

heat shock. In wheat, a decrease in the expression of ATP synthase was observed after heat shock, and it was suggested that this enzyme might affect energy-dependent processes involved in the resistance to thermal shock [19]. In *T. cruzi*, a decline in energy supply is also a consequence of nutritional stresses of trypomastigotes [29].

There have been few proteomic studies aimed at studying the cellular stress response in pathogenic organisms [30]; however, it has been suggested that the physiology and other biological properties, including pathogenicity, are influenced by environmental parameters [31]. Other particular forms of proteins identified in this study were those involved in the interaction with the cellular environment, including a set of particular forms of proteins found on the parasite surface. Interestingly, among the particular forms of surface proteins that showed an increase in intensity as a result of stress treatment, a mucin-associated surface protein (MASP) was found, which is a *T. cruzi*-specific protein with until lately unknown function [32], but, recently, a MASP has been associated with invasion process ([33] and unpublished data from our laboratory). An increase of abundance of the surface metalloproteinase GP63 was also observed, which has been implicated in virulence, host cell infection, and the release of parasite surface proteins [32]. The attachment to the insect midgut has been described as one of the crucial steps that allow *T. cruzi* epimastigotes to continue their life cycle. For this process, the parasites express different proteins, including the GP82 and GP90 surface glycoproteins, whose appearance on the parasite surface is accompanied by the morphological differentiation from epimastigotes into metacyclic forms [34]. Perhaps the heat shock response triggers a protein expression pattern very similar to that shown during differentiation to trypomastigotes, the infective stage, as suggested by Alcina et al. [35]. For bacteria, it has been suggested that the synthesis of virulence factors is influenced by the availability of nutrients, pH, growth phase, and temperature [30].

Particular forms of proteins involved in cell defense represented 16.6% of the particular forms of proteins identified. HSP70 and the chaperone DnaJ protein (HSP40) increased their intensity after heat shock. The increase in HSP70 (1.7-fold) was similar to what was previously reported in a similar heat stress experiment by Olson et al. [10], where a 2.5-fold increase was observed. This also supports that a true heat shock response was induced in our model. Two other identified proteins, thiol transferase Tc-52 and tryparedoxin peroxidase, are known to be involved in responding to oxidative stress [36, 37]. It has been reported that, after a heat-shock response, oxidative stress is generated, which could occur for several reasons. After heat stress, many proteins are denatured, including antioxidant proteins, which leads to an accumulation of reactive oxygen species, characteristic of aerobic metabolism. It has also been shown that the reactivity of superoxide increases with temperature [36, 38].

Additionally, a small percentage of the particular forms of proteins identified were proteins implicated in protein fate, such as modification and destination. In eukaryotic cells, most of the proteins in the nucleus and cytoplasm are degraded in the 26S proteasome rather than in lysosomes, which is a multisubunit complex located in the cytoplasm and nuclear compartments. The proteasome proteolytic pathway controls a wide variety of cellular functions like the quality control of proteins, which includes the degradation of mutated or damaged proteins [39]. Ubiquitin-conjugating enzyme E2 performs the second step in the ubiquitination reaction that targets a protein for degradation via the proteasome. For *T. cruzi*, an essential role of the proteasome in the transformation to different parasite stages has been reported [40]. An increase in abundance of proteasome proteins was observed in parasites subjected to metacyclogenesis and exposed to Benznidazole [41, 42].

Particular form of protein involved in cellular transport the Rab6 GTPase- (guanosine-triphosphatase-) activating protein, which is involved in the regulation of membrane transport in *T. brucei* [43] was identified in this work. A protein retrotransposon hot spot was also found, which is associated with polymorphic subtelomeric regions [44] and whose role in heat stress could not be determined.

It has been proposed that, although some stress response mechanisms are highly conserved across species, others, like those involved in the optimization of metabolism and cell function or regulation, vary from organism to organism [21]. For this reason, we expected to find proteins with a variety of functions involved in stress resistance. In addition, in the case of *T. cruzi*, which has a large number of proteins with unknown functions, this is more relevant because of the potential that these proteins could be involved in the biology of the parasite. Some of the particular forms of proteins identified in our study have already been found in proteomic studies in which the parasite was subjected to different stresses: nutritional stress, stress by exposure to Benznidazole and metacyclogenesis [41, 42]. Thus, we suggest a convergence of resistance mechanisms to other stresses, including the parasite transformation from one stage to another. Finally, because of the increase in abundance of some surface proteins, it will be interesting to study the infectivity of parasites undergoing heat stress and to determine whether a relationship exists between this response and the infection process.

4. Conclusions

In summary, in this study, the *T. cruzi* epimastigotes proteome subjected to heat shock was described, and particular forms of different proteins with different abundance after heat shock were identified by PMF. To our knowledge, this is the first time a bulk of proteins different from HSPs were found to be differentially abundant during heat shock in *T. cruzi*.

Taken together, these findings offer new insights into the basic physiological processes involved in the *T. cruzi* response to heat shock; this response is vital for parasite survival.

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Research Article

Flagellar Motility of *Trypanosoma cruzi* Epimastigotes

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The hemoflagellate *Trypanosoma cruzi* is the causative agent of American trypanosomiasis. Despite the importance of motility in the parasite life cycle, little is known about *T. cruzi* motility, and there is no quantitative description of its flagellar beating. Using video microscopy and quantitative vectorial analysis of epimastigote trajectories, we find a forward parasite motility defined by tip-to-base symmetrical flagellar beats. This motion is occasionally interrupted by base-to-tip highly asymmetric beats, which represent the ciliary beat of trypanosomatid flagella. The switch between flagellar and ciliary beating facilitates the parasite's reorientation, which produces a large variability of movement and trajectories that results in different distance ranges traveled by the cells. An analysis of the distance, speed, and rotational angle indicates that epimastigote movement is not completely random, and the phenomenon is highly dependent on the parasite behavior and is characterized by directed and tumbling parasite motion as well as their combination, resulting in the alternation of rectilinear and intricate motility paths.

1. Introduction

Trypanosoma cruzi is a flagellated protozoan that is the causative agent of Chagas disease, a debilitating and incurable fatal illness that affects 20 million people in Latin America [1]. This parasite presents a complex, biphasic life cycle, moving between invertebrate and vertebrate hosts in which three alternate developmental forms (epimastigotes, metacyclic and bloodstream trypomastigotes, and amastigotes) can be identified by their morphological features. Amastigotes are characterized by the presence of a short flagellum, while epimastigotes and trypomastigotes have a long flagellum that emerges from the flagellar pocket, an organelle that together with the cytostome is involved in the endocytic and exocytic pathways of the parasite [2–5].

Like other flagellated cells, the flagellum of trypanosomatids propels the parasite through the action of its mechanochemical oscillator, which generates motile forces. Cilia and flagella are organelles that have been highly conserved over the course of evolution and are encountered in a variety of organisms from protists to mammals. Therefore, the trypanosomatid flagellum shows the characteristic pattern of nine pairs of peripheral doublets and one central pair of axoneme microtubules that is conserved among eukaryotes. The peripheral microtubules bear inner and outer dynein arms, radial spokes, and nexin links, while the central-pair microtubules show specific projections. The doublets are tethered to the cell via the basal body and are attached to each other by nexin links. The dyneins are the molecular motors that cause adjacent microtubule doublets to slide past one another. The action of the dyneins is coordinated through a dynein-regulating complex, and the signals are transmitted across the radial spokes, which together produce the waveforms of cilia and flagella [6–8]. This sliding is translated into flagellar bending.

The flagella of trypanosomatids also present another structure that is unique to kinetoplastids, euglenoids, and dinoflagellates, which is named the paraflagellar rod (PFR) (reviewed in [9–11]). The PFR is a lattice-like structure that runs parallel to the axoneme within the flagellar membrane.

It has proximal, intermediate, and distal domains; its diameter is similar to that of the axoneme; it is anchored to the axoneme via connections to doublets 4 to 7 [12–14].

Because the flagellum is composed of diverse structures, and is highly dynamic, intense, and highly coordinated, conformational changes of the axonemal microtubules, associated structures and PFR are required to perform all of the functions in which the flagellum is involved. Reverse and forward genetic tools have been used to evaluate many aspects of flagellar function and movement. The main flagellar components have been silenced, disrupting flagellar motility. Thus, it has been shown in trypanosomes that the flagellum plays key roles in cell morphogenesis [15], attachment to the insect host epithelium [16], mitochondrial DNA segregation [17], and cell division [18]. In addition, flagellar motility has shown to be essential for *T. brucei* survival in the mammalian bloodstream [19].

Although the genetic approaches have allowed for a considerable increase in the knowledge of different aspects of flagellar function and movement, little is known about parasite motility modes and flagellar beat. Quantitative analyses of the flagellar beat in *T. brucei* [20, 21] and *L. major* [22] have been reported; however, there exists no description of flagellar beating in *T. cruzi*.

Recent structural analysis of the PFR during epimastigote flagellar beating in *T. cruzi* suggests that the three portions (proximal, intermediate, and distal) of the PFR are rearranged, undergoing dynamic remodeling during flagellar beating [5]. Nevertheless, a quantitative understanding of epimastigotes beating is still lacking, and very little is known about the motility of *T. cruzi* epimastigotes.

In the present study, we analyzed the motility and flagellar beating of *Trypanosoma cruzi* epimastigotes. Images of free-swimming epimastigotes were captured by video microscopy, and a quantitative analysis of the distance traveled by individual epimastigotes was performed. The time trajectory of each parasite was decomposed into distance, speed, and rotational angle to facilitate a more detailed quantitative vectorial analysis, and a population-wide analysis was also performed.

2. Materials and Methods

2.1. Parasites. Epimastigotes from the *T. cruzi* CL-Brenner strain (a well-characterized strain and a reference strain for the genome project) were cultured in liver infusion tryptose medium (LIT) containing 10% fetal bovine serum (FBS) and 0.1 mg/mL hemin at 28°C. For all experiments, the parasites were grown in homogeneous conditions and were sampled for use in experiments while in the logarithmic growth stage at 48 hours. The stock cultures were grown every 4 days using 1×10^6 parasites as an initial inoculum to guarantee the homogeneity and active motility of the parasites.

2.2. Video Microscopy and Motility Assays. The parasites were labeled with carboxy-fluorescein succinimidyl ester (CFSE) green fluorescent stain (Molecular Probes, Eugene, OR) as follows. Epimastigotes (5×10^6) in log-phase growth were washed with 1X PBS and stained in 1 mL of PBS with 0.5 mM of CFSE at room temperature for 20 min in the dark. After three washes with 1X PBS, 10 μL drops of epimastigotes (5×10^6/mL of 1X PBS) were placed onto coverslips, and the parasites were allowed to move freely. The motility videos of the stained parasites were taken through an inverted confocal laser microscope (Leica SP5, DM 16000, Mo) with a 488 nm fluorescence excitation filter and an HCXPLAPO lambda blue 63x 1.4 NA oil objective. The images were captured and analyzed using the LAS AF software (*Leica Application Suite Advanced Fluorescence Lite*/1.7.0 build 1240 Leica Microsystems). The images were captured in the time series mode at a rate of 10 frames/sec and recorded at 10 sec/sample.

2.3. Image Processing and Statistical Treatment of Measurements. For motility assays, the movement of the labeled cells was measured and analyzed using the Image-Pro plus V 6.0 Media Cybernetics program. For trajectory analysis, we returned the datasets x_i and y_i, which represent the parasite horizontal and vertical coordinates, respectively, at time t_i, after the start of recording. The first step of the trajectory analysis was the calculation of the distance traveled between two consecutive times, t_i and t_{i+1}. For this calculation, we used the following well-known formula from vector analysis, which we implemented in *MATLAB 7.1*:

$$d_i = \sqrt{(x_{i+1} - x_i)^2 + (y_{i+1} - y_i)^2}. \tag{1}$$

Because all time intervals between consecutive points are 1 second long, each distance d_i is numerically equal to the corresponding velocity v_i. Once all instantaneous velocities v_i for a given parasite were obtained, the average individual parasite velocity $\overline{v}$ and the corresponding velocity histograms were calculated. Next, the angles between two consecutive displacements $[t_{i-1}, t_i]$ and $[t_i, t_{i+1}]$ were calculated for each parasite using the following equation (also implemented in a custom *MATLAB 7.1* routine):

$$\theta_i = s_i \cos^{-1}\left(\frac{(x_{i+1} - x_i)(x_i - x_{i-1}) + (y_{i+1} - y_i)(y_i - y_{i-1})}{d_{i-1} d_i}\right), \tag{2}$$

where d_{i-1} and d_i are the distances traveled by the parasite during displacements $[t_{i-1}, t_i]$ and $[t_i, t_{i+1}]$, respectively, and s_i is given by

$$s_i = \frac{(x_i - x_{i-1})(y_{i+1} - y_i) - (y_i - y_{i-1})(x_{i+1} - x_i)}{|(x_i - x_{i-1})(y_{i+1} - y_i) - (y_i - y_{i-1})(x_{i+1} - x_i)|}. \tag{3}$$

From the theorems of vector analysis, it can be proven that $s_i = 1$ for a counter-clockwise rotation, while $s_i = -1$ otherwise. Once all angles θ_i were calculated for a parasite trajectory, we computed the corresponding histogram and repeated the procedure for all of the parasites studied. Because all histograms were approximately symmetrical, for each trajectory, we further calculated the average of the absolute value of the angle θ_i as a measure of the intricacy of the corresponding trajectory.

3. Results

3.1. Motility Analysis of Free-Swimming Epimastigotes. The motion of free-swimming epimastigotes was analyzed using video microscopy, and the videos were processed with the Image-Pro plus V 6.0 Media Cybernetics program to study the cells motility traces. Figure 1 illustrates the cell motility and flagellar beating of *T. cruzi* epimastigotes. The parasites were highly motile, and showed great variability of movements and trajectories, which was characterized by alternating periods of translational cell movement, tumble and shutdown (Figures 1(a), 1(b), and 1(c)) that resulted in different distance ranges traveled by the cells (Figure 1(d)). The main waveform was a tractile beat that initiated at the tip of the flagellum and propagated toward the base, resulting in forward cell propulsion. In the free-swimming epimastigotes in which cell motion was not impeded, the wave amplitude did not change detectably along the entire free flagellum (Figure 2), as observed in *Leishmania major* [22]. This tractile beat was occasionally interrupted for short periods of base-to-tip wave beats that led to the reorientation of the anterior flagellar end and then the parasite body with no evident backward motility (Figure 1(e) light blue region, Figure 3 and Video 1). These data suggest that *T. cruzi* shows a forward cell propulsion with a tip-to-base flagellar wave propagation, as has been reported for Leishmania and African trypanosomes [20, 22].

3.2. Free-Swimming Epimastigotes Travel Different Distance Ranges. Quantitative analysis of cell motility traces of the fluorescent epimastigote population showed that the average velocities of the parasites ranged from 1 μm/10 sec to 400 μm/10 sec (Figure 4). The highest percentage of parasites (30%) traveled at the maximum velocity (400 μm/10 sec), while the next highest percentage of epimastigotes (21%) traveled at the minimum velocity (1 μm/10 sec). The remaining epimastigotes traveled different distances at different speeds between these extremes (20% at 21–40 μm/10 sec, 14% at 41–60 μm/10 sec, 7% at 61–90 μm/10 sec, and 8% at 81–100 μm/10 sec) at variable levels without displaying a particular pattern. Even though the differences in the average velocities may have resulted from differences between cells in motion and cells attached to the microscope slide, all of the parasites had a vigorous motion, and the amplitude and frequency of the flagellar wave was constant.

3.3. Vectorial Analysis of Parasites Trajectories. The time trajectories of free-swimming epimastigotes through the liquid medium were decomposed into distance, speed, and rotational angle to facilitate a more detailed quantitative comparison. Vector analysis was then applied to characterize the speed and rotational angles of all individual trajectories of each parasite using a custom *MATLAB 7.1* routine, and the average behavior of the epimastigote population was determined.

The speed data showed that the speed of the parasites could be described by a Maxwell-Boltzmann distribution, suggesting that epimastigotes undergo Brownian-like motion. The wide parasite-to-parasite variability in average speed ($v_i = 6.37 \pm 0.7$) suggests that, in addition to Brownian motion, flagellar beating also contributes to the motility of epimastigotes (Figure 5).

As shown in Figure 6, the rotational angle analysis indicated that the angular change was approximately symmetrical over the entire range from -180 to 180 degrees in the free-swimming parasites. This finding indicated that there is no preference in the direction toward which parasites rotate, and it suggests a high degree of stochasticity. As the rotational angle histograms were not flat but rather showed a different bias for each parasite, the movement of parasites was not completely random, and the phenomenon was highly dependent on parasite behavior. The smaller angles were produced during directional drift through directed motion, whereas the larger angles were produced during the spin and tumble stage when the flagellum does not show coherent directed motion. The rotational angle histogram for free-swimming parasite motion showed a peak centered at small angles, indicative of almost rectilinear paths, with few abrupt changes in direction. This behavior was clearly observed when speed and rotational angle were analyzed together (Figure 7). The same symmetrical behavior was observed in all the randomly chosen parasites analyzed, and the average angular change was $\theta_i = 85 \pm 15$, even for parasites that had traveled different distances.

Taken together, these results indicate that epimastigotes from the same population taken from the same cell culture and exposed to identical environmental conditions do not follow a single motility mode.

4. Discussion

Even though that the motility process of trypanosomatids is important for survival, completion of the life cycle, and establishment of parasite infection, the molecular mechanisms involved remain poorly understood.

The recent publication of complete genome sequences for *T. cruzi*, *T. brucei*, and *L. major* [23–25], the studies of the trypanosome flagellar proteome [19, 20], and the feasibility of genetic manipulability of these organisms have shed light on the key roles of some molecules that participate in parasite motility and flagellar function. However, little is currently known about the general characteristics of motility and the flagellum beating processes, and an in-depth quantitative description against which hypotheses could be tested is lacking. For the "Tritryps," quantitative analyses of the flagellar beating and free-swimming motility properties have only been reported in *T. brucei* [20, 21] and *L. major* [22].

Using video microscopy and vectorial analysis of parasite trajectories, we have found that the motility of free-swimming epimastigotes of *T. cruzi* is characterized by (a) a large variability of movements and trajectories with alternating periods of translational movement, tumble, and shutdown of the parasites; (b) forward motility resulting from tip-to-base flagellar wave propagation; (c) a symmetrical rotational angle that indicates a high degree of stochasticity;

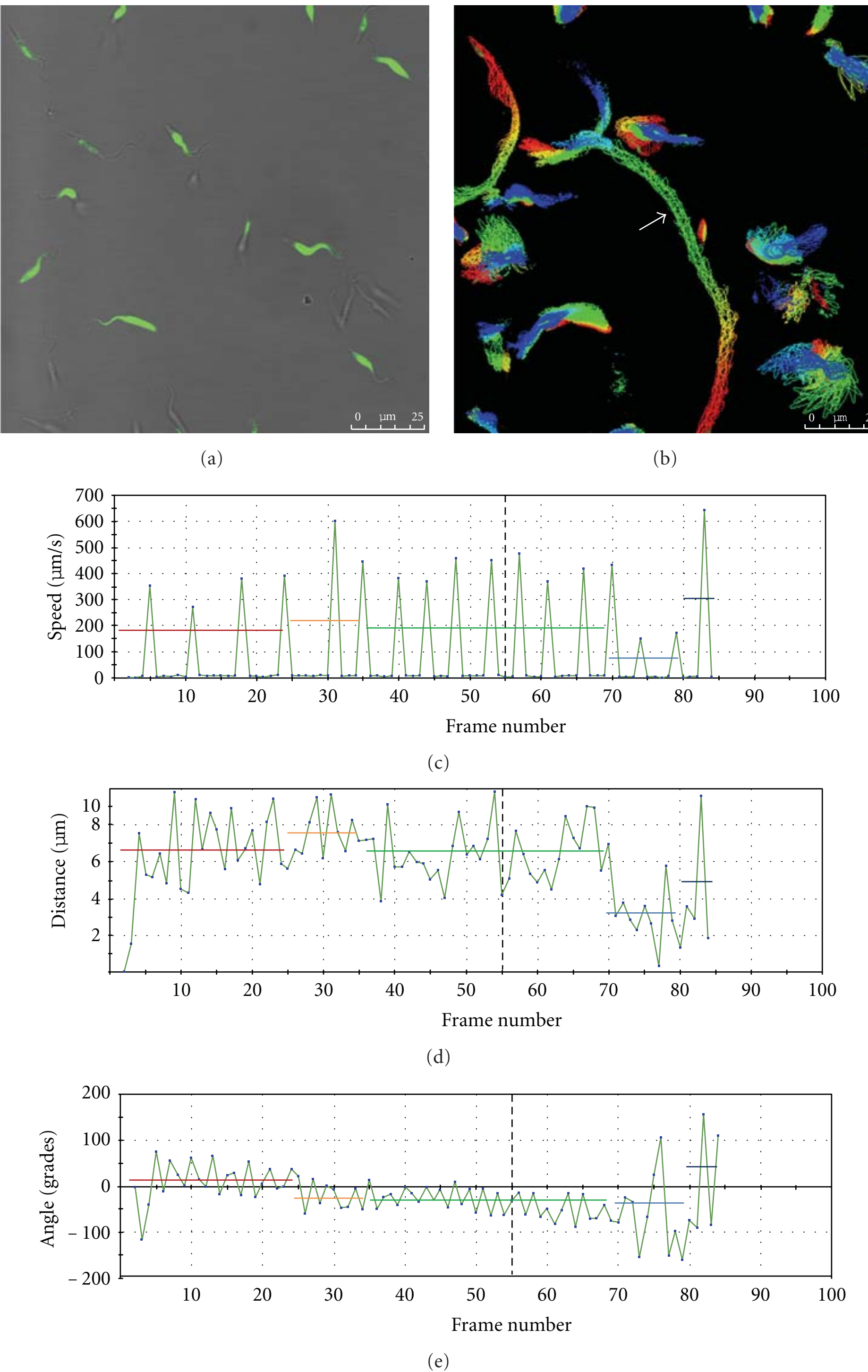


Figure 1: *Trypanosoma cruzi motility analysis by time-lapse microscopy.* (a) Direct observation of CFSE green fluorescent-labeled free-swimming epimastigotes analyzed by confocal microscopy. (b) Motility traces of epimastigotes. The positions of each individual cell are plotted at 10 frames/sec and recorded for 10 sec/sample. The starting position of each epimastigote is in red. The video clips used to generate these motility traces are available as supplementary material available at doi: 10.1155/2012/520380 (Video 1). The arrow indicates the parasite trajectory analyzed in panels (c, d, and e). The graphics represent the distance (c), angles (d), and speed (e) through consecutive frames of the selected parasite (arrow) of panel b. The color bars indicate the average value of each segment analyzed using the same color scale as the motility traces.

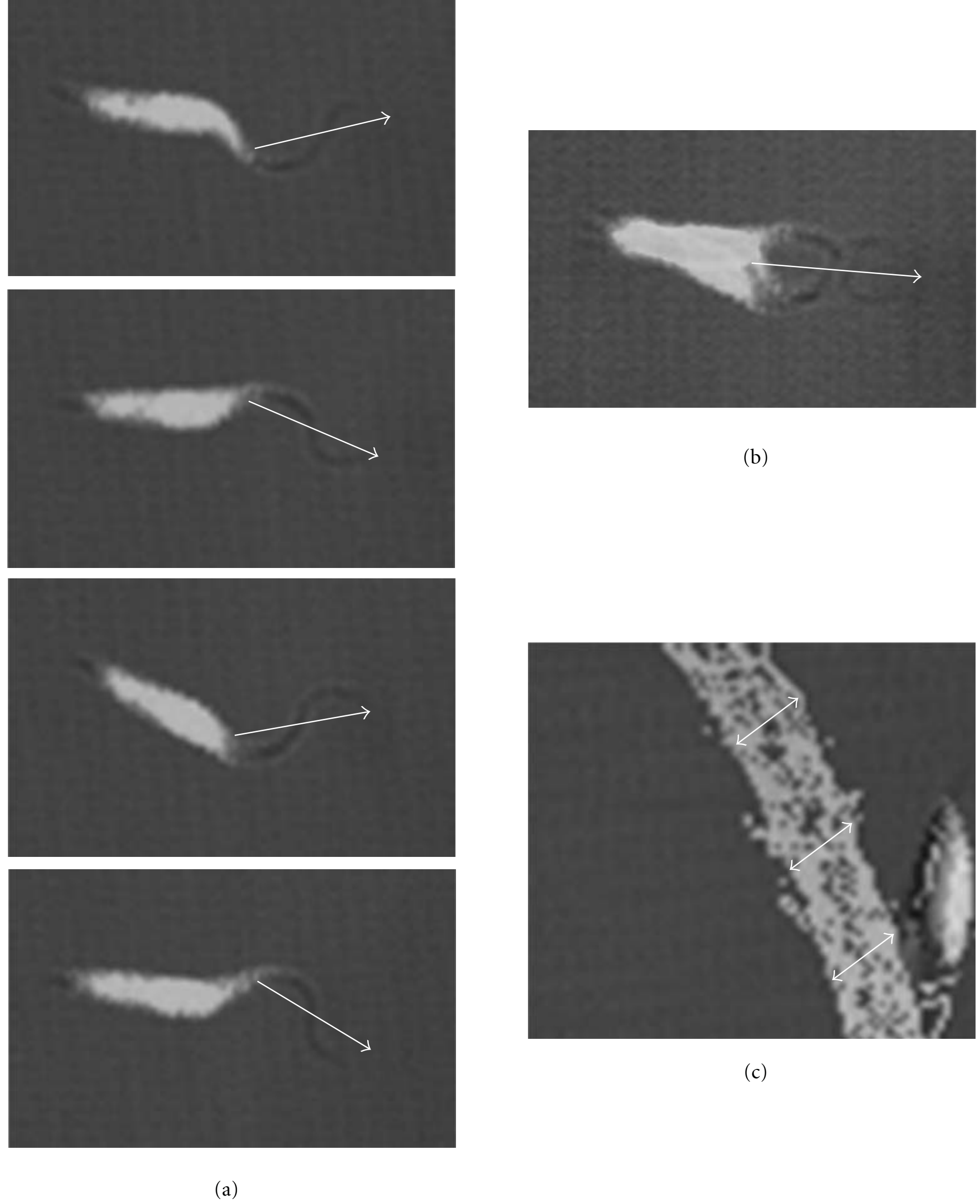


Figure 2: *Analysis of the symmetrical flagellar beating of* T. cruzi. (a) Parasite position and direction of motion (arrows) of four consecutive frames of an epimastigote with forward cell propulsion obtained from the green region of Figure 1. The amplitude and frequency of the flagellar wave were constant and symmetrical. (b) Fixed and superimposed images from panel a. (c) Plots of the movements and displacement of the epimastigote of panel (a). The arrows indicate the symmetry of parasite and flagellar wave contours during the cell displacement.

(d) flagellar beating that directs the epimastigote motility; (e) sporadic asymmetric base-to-tip beats that represent a ciliary beat of trypanosomatid flagella and produce a directional drift for the cell.

As for other trypanosomatids, the epimastigotes of *T. cruzi* swim very actively in any direction in the culture medium while leading with the anterior end. The parasite motility analysis presented in this work indicates that the cell traces have patterns similar to those previously reported for *T. brucei* [26, 27] and that the beating initiated from the flagellar tip is similar to that reported for *Leishmania* [22] and *T. brucei* [20]. This property of trypanosomatid flagella is distinctive compared with most other organisms in which beating is initiated from the proximal end of the flagella. In these organisms, it was believed that bend formation and sustained regular oscillation depended upon a localized resistance to interdoublet sliding, which is usually conferred by structures at the flagellar base, known as the basal body. Similarly, in trypanosomes, it has been proposed that they may have some tip initiating or capping structure to provide resistance to doublet sliding in a manner similar to the basal body. This attachment structure has been found in *Crithidia deanei*, *Herpetomonas megaseliae*, *Trypanosoma brucei*, and *Leishmania major* [28]; therefore, this structure may also

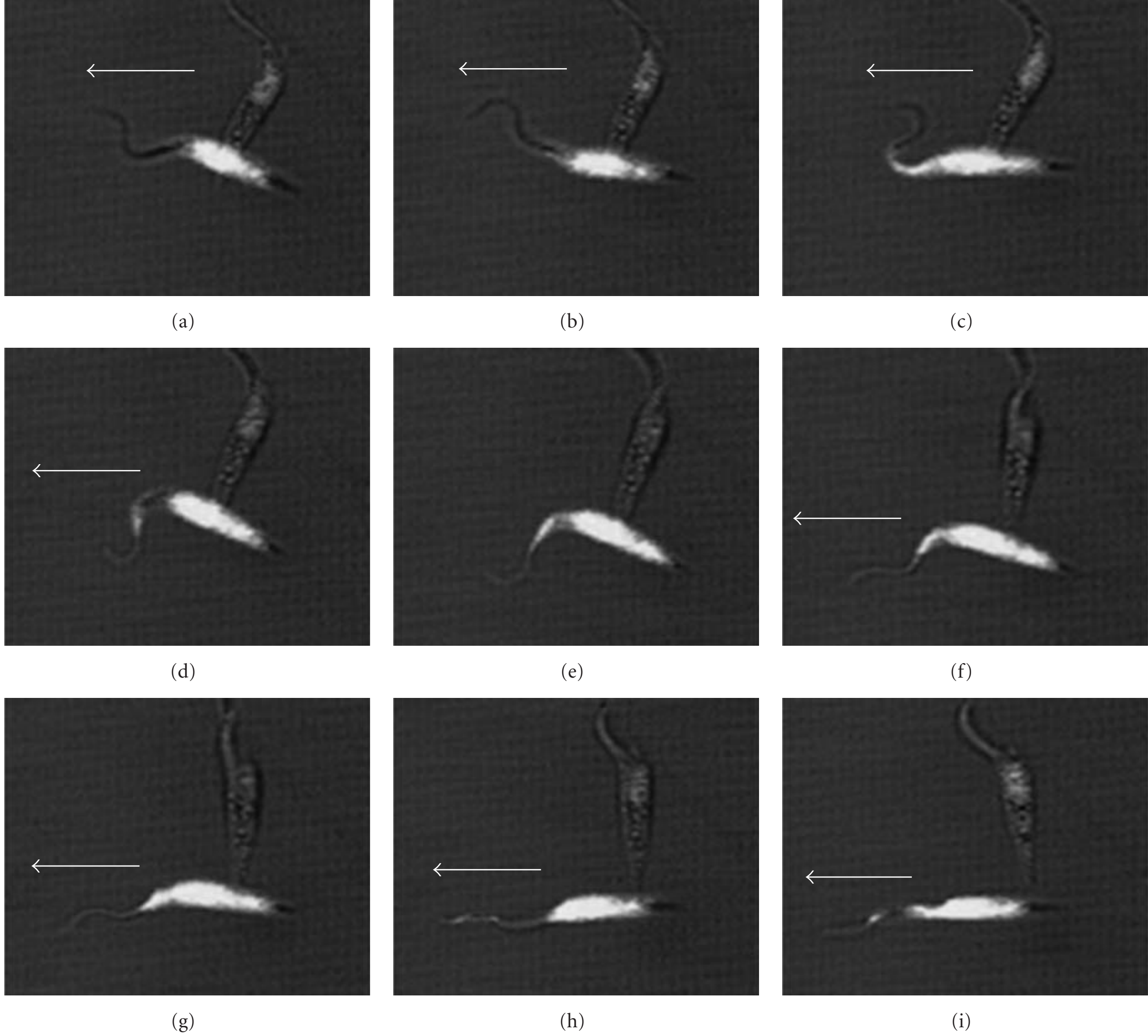


Figure 3: *Illustration of the ciliary beat of* T. cruzi. The forward parasite motility resulting from tip-to-base flagellar wave propagation (panels a–d) was interrupted by a sudden pause in which almost no movement of the parasite was observed (Ø). A base-to-tip wave propagation was observed before the parasite reinitiated the tip-to-base flagellar beat that resulted in a very little translational motion and clearly reoriented the parasite re-orientation and restarted the forward motion of the cell (panels f–i). These images correspond to those from an epimastigote obtained from the light blue region of Figure 1.

be present in *T. cruzi*, although future experiments will be needed to demonstrate this characteristic.

The beat initiation from points other than the flagellum tip, as reported for *L. major* and *T. brucei*, was difficult to evaluate in the *T. cruzi* epimastigotes because high-speed video microscopy was not used in this work. However, it was possible to detect that in addition to the tip-to-base flagellar wave, the epimastigotes also showed another type of beat that was propagated in the opposite direction (base-to-tip, which is characteristic of ciliary beating) at a much smaller frequency and in a highly asymmetric mode. This ciliary beat started spontaneously and interrupted the initiation of the main tip-to-base beat, producing a sudden pause in which almost no movement was observed. Then, a short interval of base-to-tip waves was noted before the parasite reinitiated the tip-to-base flagellar beat that resulted in a very little translational motion and clearly produced a change in epimastigote orientation and the resumption of forward cell motion. This switch between flagellar and ciliary beating has been reported in *T. brucei* [20], *L. major*, *C. deanei*, *C. fasciculata* [22], and *C. oncopelti* [29]. These results indicate that these organisms are able to sustain at least two kinds of beat types and suggest that they are likely able to maintain other types of beats when are in different microenvironments during their life cycle.

The variability of movements and trajectories of *T. cruzi* epimastigotes is consistent with the tumbling, intermediate, and persistent motility modes observed in *T. brucei*. These motility modes are the result of the cell elongation that correlates with cell stiffness, which has been shown to affect

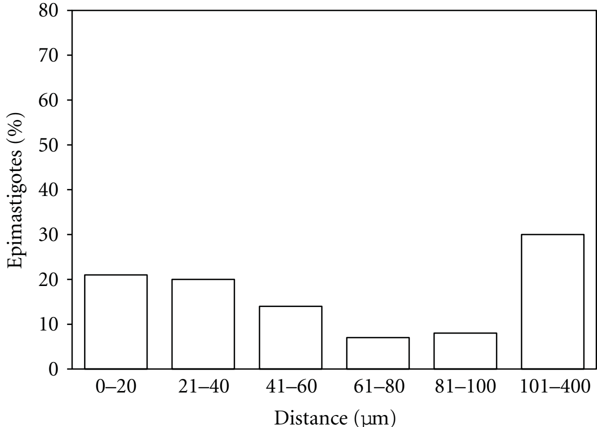


Figure 4: *Quantitative motility analysis of free-swimming epimastigotes.* The analysis of motility traces of fluorescent epimastigotes showed that the average velocities of the parasites ranged from 1 μm/10 second to 400 μm/10 sec. The histograms show the range of distances that each cell traversed per 10 sec. For each parasite trajectory, 100 randomly chosen cells were analyzed.

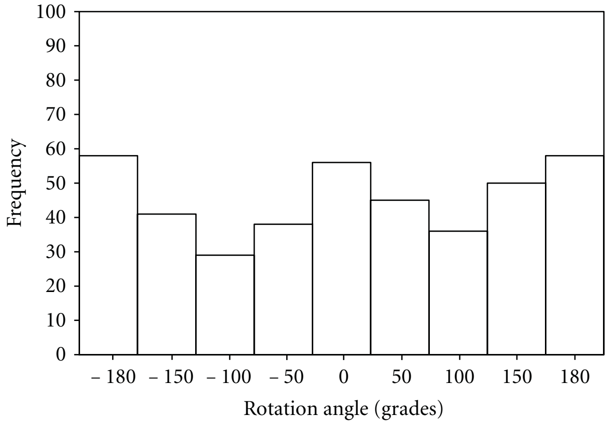


Figure 6: *Analysis of flagellar rotational angle.* The angles between two consecutive displacements were calculated for each parasite. The histogram is representative of the average behavior of 20 randomly selected epimastigotes.

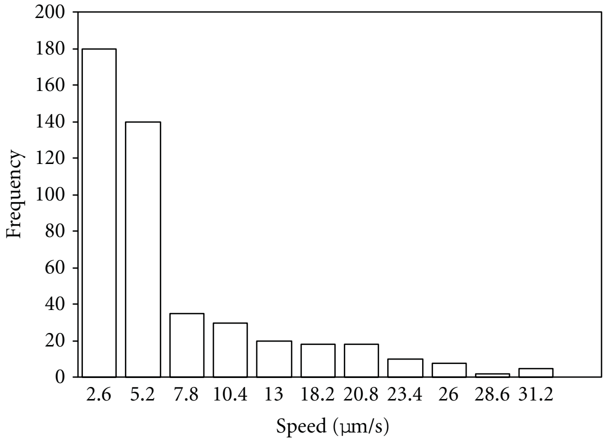


Figure 5: *Analysis of cell speed.* The speeds at which cells traveled through consecutive points during 1-sec time intervals were quantified in CFSE green fluorescent-labeled epimastigotes. The histogram is representative of the average behavior of 20 randomly selected epimastigotes.

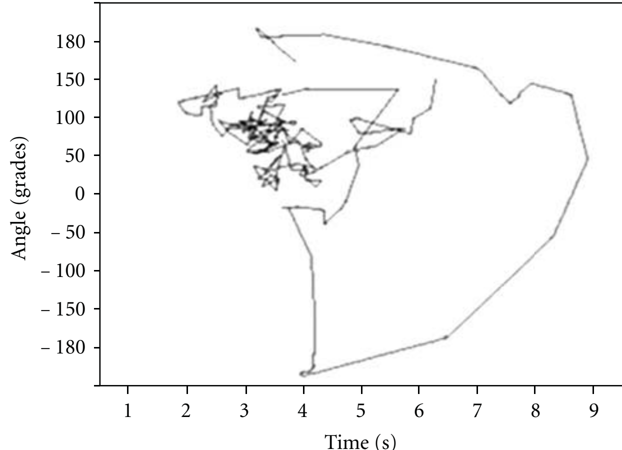
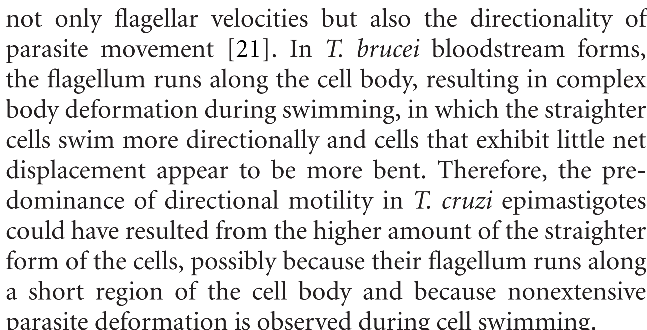


Figure 7: *Analysis of parasite trajectory.* The trajectory of free-swimming epimastigotes was determined using the speed and rotational angle results form a 10-second recording. The histogram is representative of the average behavior of 20 randomly selected epimastigotes.


not only flagellar velocities but also the directionality of parasite movement [21]. In *T. brucei* bloodstream forms, the flagellum runs along the cell body, resulting in complex body deformation during swimming, in which the straighter cells swim more directionally and cells that exhibit little net displacement appear to be more bent. Therefore, the predominance of directional motility in *T. cruzi* epimastigotes could have resulted from the higher amount of the straighter form of the cells, possibly because their flagellum runs along a short region of the cell body and because nonextensive parasite deformation is observed during cell swimming.

A recent analysis of the structural organization of the PFR of *T. cruzi* epimastigotes in flagella in straight versus bent states [5] suggests that the PFR changes according to the movement of the axoneme. The PFR is composed of discrete filaments structured in lattice-like arrays with three distinct domains (proximal, intermediate, and distal), and it has been proposed that the three domains may work together. According to this proposal, when the proximal domain is compressed, the distal or opposite domain is stretched in an alternating pattern during flagellar beating. The intermediate domain would follow this dynamic of movement, and in a bent state, the filaments would get closer [5]. Therefore, the different motility modes observed during the swimming of epimastigotes could be an indication that the intermediate domain is able to change its position between the proximal

and distal filaments, allowing the switch between flagellar and ciliary beating. The movement of this domain would alternate the periods of translational cell movement, tumble, and shutdown of the parasite and result in the consequent reorientation of the epimastigotes. The mechanism by which the different modes of motility are coordinated is still unknown, but the minor components of the PFR [30] could participate as motor, anchoring, or connector proteins in the different modes of movement. However, future biochemical and genetic studies will be necessary to determine the type, function, and possible participation in the flagellar beat of these proteins.

The symmetry and equal amplitude waves through the flagellum observed in the directional motility of *T. cruzi* epimastigotes closely resemble that reported for promastigotes of *L. major*, which also has a free flagellum that is only attached along a short region of the cell body [22, 31]. Quantitative analysis of the rotational angle and study of separated parasite displacement and flagellar beating, using the corresponding fixed and superimposed images, revealed that, as in *Leishmania*, *T. cruzi* did not show changes in amplitude as the wave travelled from tip to base. In contrast, in *T. brucei*, the amplitude was reduced toward the proximal end, possibly due to the attachment of its flagellum along most of the cell body [20]. To test this hypothesis, future experiments comparing the flagellar beating of epimastigotes versus bloodstream and metacyclic trypomastigotes of *T. cruzi* will be useful.

The trypanosomatid flagellum is completely different from rotary-motor based bacterial flagella [32] and more complex than most other microtubule-based eukaryotic flagella [33, 34]. *T. cruzi*, like the other two "Tritryps" whose genome sequences have been determined, can be genetically manipulated for functional studies, is easily grown and transformed in culture, and its motility is an important part of its life cycle. For these reasons, the "Tritryps" are becoming very attractive models for the analysis of flagellar function and the study of genes involved in human genetic disorders linked to flagellar motility defects. Therefore, the quantitative description of motility and flagellar beating of *T. cruzi* reported in this work provides a useful platform for future genetic experiments to test parasite motility and flagellar function hypotheses.

In summary, our quantitative motility analysis results offer insights into flagellar beating of the American trypanosome and provide new detail on an important, yet poorly understood, motility mode of *T. cruzi*.

Acknowledgments

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Review Article

Regulatory T Cells and Parasites

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Human host encounters a wide array of parasites; however, the crucial aspect is the failure of the host immune system to clear these parasites despite antigen recognition. In the recent past, a new immunological concept has emerged, which provides a framework to better understand several aspects of host susceptibility to parasitic infection. It is widely believed that parasites are able to modulate the magnitude of effector responses by inducing regulatory T cell (Tregs) population and several studies have investigated whether this cell population plays a role in balancing protective immunity and pathogenesis during parasite infection. This review discusses the several mechanism of Treg-mediated immunosuppression in the human host and focuses on the functional role of Tregs and regulatory gene polymorphisms in infectious diseases.

1. Immunomodulation by Parasites

In this paper, we specify parasites as eukaryotic pathogens that largely include protozoa and helminths and survive off their host partly or completely for their life cycle. They employ various strategies to evade against an effective host innate immune system. Innate immunity rarely eliminates parasites but can successfully inhibit growth while they recruit antigen-specific T and B cells to differentiate into effector cells that thwart the infection [1]. For an effective parasite survival, evasion of adaptive immunity remains the key [2]. In this scenario, parasites strike a balance with the host immune system to increase their survival rate. This balance is accomplished by complex alteration of the innate and acquired immune response of the host where regulatory T (Tregs) cells play an important role [3].

2. Regulatory T Cells

Understanding the complex cellular and molecular mechanism that regulates the host immune response to parasitic infections still remains a key issue in immunology. The crippling effect of host immunity on onset of an infection is due to the fact that parasites induce Tregs that in turn suppress antiparasite effector cells [4]. The Tregs are a subset of T cells that function to control immune responses. The primary role of Tregs is active suppression of several pathological and physiological immune responses in the host, thereby contributing to the maintenance of immune homeostasis [5–7]. Although Tregs are defined as T cells with suppressive activity on immune responses, it had been documented that regulatory T cell populations remain diverse [8]; a few of them are induced in response to infectious challenge and the others are considered as natural regulators [9]. Parasites can ably manipulate natural Tregs by amending the T cell immune response at the infection site to an extent that could lessen the infection burden, thereby prevailing in the host for a longer time frame [10]. The well-characterized Tregs are $CD4^+CD25^+$ population and represent about 10% of peripheral $CD4^+$ T cells both in mice and humans [11]. Tregs are considered as negative regulators of T cell immune response and these natural Tregs originate during thymic development and appear first in the fetal circulation [12]. The suppressor activity is enriched in naturally occurring Tregs such as $CD4^+CD25^+$ that plays a vital role in the initiation and orchestration of immune responses [13, 14]. The $CD4^+CD25^+$ population reveals a high expression of Foxp3 transcription factor which is vital for differentiation and function of naturally occurring Treg cells [15] and for programming the suppressor T cell function [16, 17]. $Foxp3^+$

Tregs play an essential role in controlling the voracity of the response as they generally strike a balance that limits potentially harmful immune-mediated pathology to the host while still allowing sufficient immune pressure against the pathogen [18].

3. Mechanism of Suppression

T-cell receptors remain the key to trigger suppressive function in both naturally occurring and induced Tregs [19]. The regulation of T cells is either by contact-dependent regulation or by soluble factors such as immunosuppressive cytokines. To date, no precise mechanism has been clearly postulated to explain the suppressor function exhibited by Tregs.

3.1. Contact-Dependent Mechanism. Many different hypotheses have demonstrated how Tregs are regulated based on the contact-dependent suppressive mechanism. However, two specific mechanisms are reviewed here. One mechanism is the interaction of T effector ligand CD80 and CD86 with cytotoxic-T-lymphocyte-associated protein (CTLA-4). This interaction triggers the transmission of immunosuppressive signals on T effector cells thus inhibiting effector T-cell function [20] (Figure 1(a)). CTLA-4 is expressed at high levels on $CD4^+CD25^+$ Tregs, and there is substantial evidence that CTLA-4 expressed by natural Tregs has a key role in Treg-mediated suppression both *in vivo* and *in vitro* [6, 21, 22]. In another model, the costimulatory molecules CD80 and CD86 expressed in antigen-presenting cells (APCs) interact with CTLA-4 leading to consequential signalling and activation of IDO (indoleamine 2,3 dioxygenase) in dendritic cells (DCs), an enzyme responsible for immune tolerance on effector T cells [23] (Figure 1(b)). IDO catalyzes the conversion of tryptophan to kynurenine that provides immunosuppressive effects in the local environment of DCs by cytotoxicity or by *de novo* generation of Tregs [8]. Studies have reported decreased activation of T cells and T cell deletion in association with reduced tryptophan concentration in cultures [23, 24]. Studies have also demonstrated that human adaptive Tregs preferentially expressed granzyme B and are capable of killing allogenic tumour cells in a perforin-dependent manner [25]. In line with these studies, it is demonstrated that both subtypes $CD4^+CD25^+$ Tregs exhibit perforin-dependent cytotoxicity against a variety of autologous target cells including $CD4^+$, $CD8^+$, $CD14^+$ monocytes and dendritic cells [26].

3.2. Immunosuppressive Cytokine Mediated. In contrast to contact dependent suppressive mechanism, reports indicate that cytokines such as IL-10 and transforming growth factor (TGF-ß) are needed for *in vitro* mediating suppression [27, 28]. Several *in vivo* studies have indicated the role of immune suppressive cytokines in suppression. In this model, Treg-dependent inhibition of tumor-specific CD8 T cell-mediated cytotoxicity requires expression of the TGF-ß receptor by CD8 cells thereby demonstrating a specific role of TGF-ß signaling in the inhibition of cytotoxicity independent of cellular proliferation [29] (Figure 1(c)). The IL-10 cytokine hampers the antigen presenting ability by downregulating the MHC class II and costimulatory molecules on DCs thereby preventing the maturation and activation of dendritic cells both in humans and in mice [30]. TGF-ß also downregulates the MHC class II expression and costimulatory molecules on DCs [31].

In addition, in mouse models, a recent study had demonstrated that helminth parasites have evolved a novel mechanism to directly expand $Foxp3^+$ Tregs which may be a key part of the parasite's strategy to survive in the host for a longer time [32]. On infection with intestinal helminth *H. polygyrus,* an expansion of the Foxp3-expressing $CD4^+$ T cells was observed. The *H. polygyrus* excretory-secretory antigen (HES) induced Tregs and was demonstrated to suppress *in vitro* effector cell proliferation. The hypothesis proposed that HES ligated the transforming growth factor TGF-ß receptor and promoted Smad2/3 phosphorylation. The Foxp3 induction by HES was lost in dominant-negative TGF-ßRII cells and was eliminated by the TGF-ß signaling inhibitor [32].

4. Tregs and Tropical Diseases

Tregs can reduce injurious host inflammatory and immune responses through mechanisms of cell-to-cell contact, inhibitory cytokines, and cytokine deprivation. This prevents an overexuberant immune response with bystander tissue damage during the host response to infections [33]. However, Tregs may also blunt Th2 responses such as IL-5-dependent eosinophil activation required to kill parasites. The interplay and balance among host Th1, Th2 and Tregs responses is crucial in the defense against a parasitic infection [34]. Some of the earliest studies emphasized that natural Tregs help control the extent of immune-mediated pathology. During malarial infection increased numbers of $CD4^+CD25^+Foxp3^+$ T cells have been found in both human and murine malaria infection [35, 36]. Evidences of the role of Tregs as suppressors of T-cell responses in malaria was initially demonstrated in murine models, where Tregs are known to be associated with increased or delayed parasite growth [37, 38]. Higher Tregs numbers are associated with increased parasite load and development of human infection caused by *P. falciparum* [39, 40]. Given these associations between severe disease and exacerbated immune pathology, a number of studies have explored the role of $CD4^+CD25^{hi}$ $Foxp3^+CD127^{-/lo}$ Tregs in determining the outcome of malaria infection. In a study conducted among Gambian children with severe, uncomplicated clinical malaria and with healthy (controls), Tregs were unable to control the inflammation during acute and severe *P. falciparum* infections, suggesting that this component may be rapidly overwhelmed by virulent infections [41]. Tregs may be beneficial to the host in the later part of the infection—when parasitemia is being cleared—by downregulating the inflammatory response and thereby preventing immune-mediated pathology [41]. On the other hand, if Tregs mediate their suppressive effects too early, this could hamper the responses required for initial control of parasitemia,

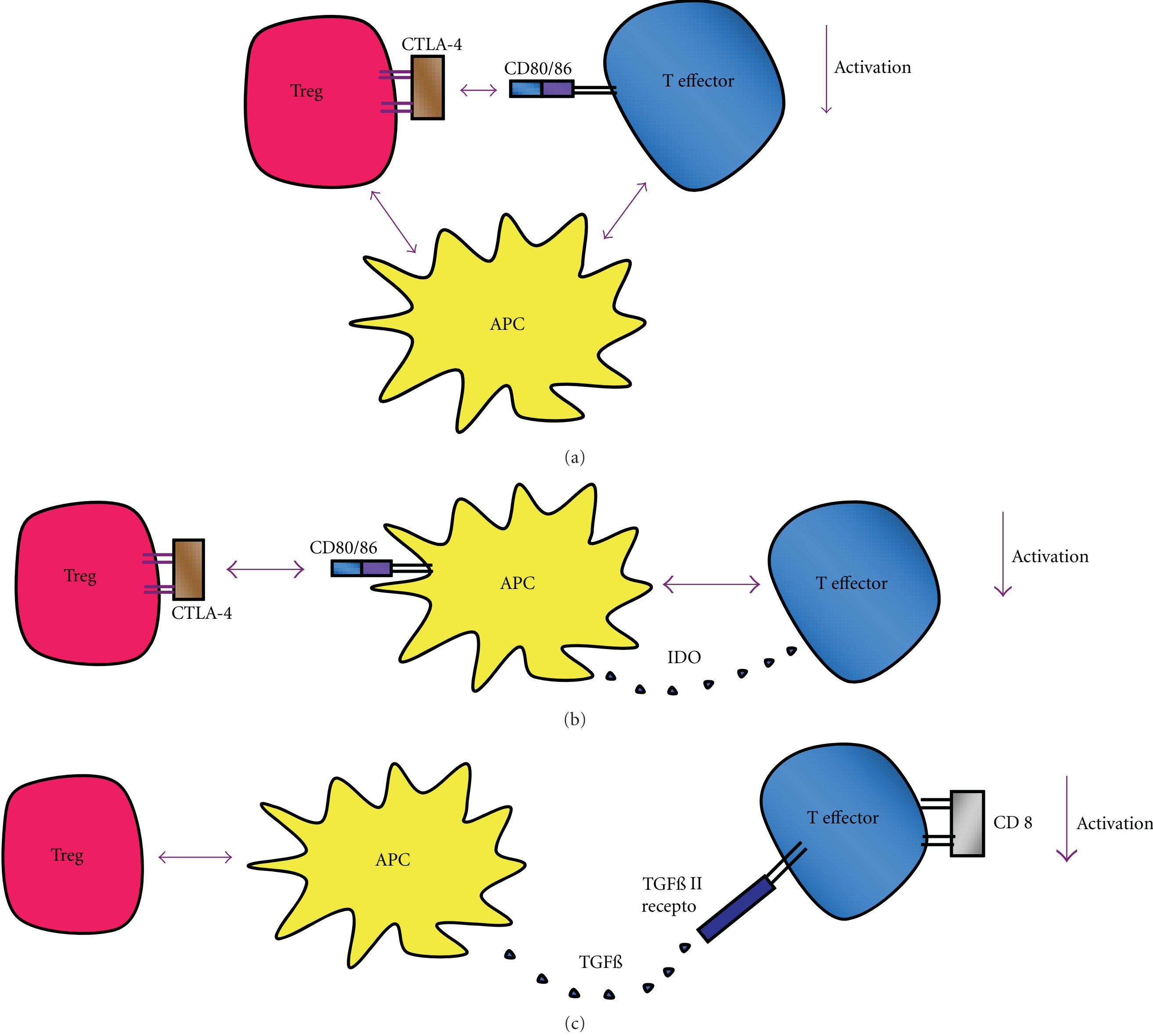

Figure 1: Mechanism(s) of suppression: illustrates various molecular and cellular mechanisms to explain how Tregs can suppress host immune responses. (a) In contact dependent mechanism the costimulatory molecules, namely, CD80/86, interact with CTLA-4 to trigger immunosuppressive signals on T effector cells leading to subtle effector cell function. (b) In contact dependent mechanism, CD80/86 expressed in APC interact with CTLA-4 leading to consequential signalling and activation of IDO leading to immunosuppressive effects. (c) The crosstalk of TGFß expressed in APC to TGFß II receptor leads to immunosuppressive cytokine-mediated suppressor function.

permitting unbridled parasite growth, which may also lead to severe disease. It was also demonstrated that patients with acute *P. vivax* infection presented a significant augmentation of circulating Tregs producing anti-inflammatory (IL-10 and TGF-ß) as well as pro-inflammatory (IFN-γ, IL-17) cytokines, which were further positively correlated with circulating parasites [42]. Malaria-specific induction of Tregs has been observed in a variety of experimental malaria infections in mice [36, 43], but their role in preventing severe malarial pathology is still unclear.

Tregs are widely believed to be involved in silencing the immune response during chronic stages of any filarial infection. Although patients with chronic Onchocerciasis (river blindness) posses higher worms burden, they reveal little/no signs of dermatitis. Studies have argued that Tr1 (a subset of $CD4^+$ T cells) induce a substantial increase in IL-10, IL-5, and IFN-γ levels conferring an immunosuppressive effect [44] in chronic onchocerciasis individuals, whereas in animal models studies had demonstrated that a subtle immune response is mediated by female worms [45, 46] and is dependent on TGF-β and IL-10, two cytokines closely implicated in the activity and induction of Tregs [47–49]. In *Litomosoides sigmodontis* model, the infective L3 stage induces the proliferation of $CD4^+Foxp3^+$ Tregs,

which translates to an increased percentage of $CD4^+$ T cell population at the site of infection expressing $Foxp3^+$ cells within 7 days after infection [49]. The $CD4^+$ regulatory T cell population has also been found significantly higher in several other filarial infections, including *Brugia malayi* [50], the gastrointestinal nematode *Heligmosomoides polygyrus* [51, 52], and the gut/muscle-dwelling nematode *Trichinella spiralis* [53].

Chagas, a tropical disease caused by *Trypanosoma cruzi* is known to cause cardiomyopathy, an inflammatory response in the heart [54]. The occurrence of larger proportion of $Foxp3^+$ population has been demonstrated to control the inflammatory responses in the heart during chagas disease [55]. Therefore, $Foxp3^+$ cells may be involved in a possible mechanism to prevent exacerbation of the inflammatory responses [54, 55]. A similar pattern of Tregs role was established with African trypanosomiasis in a mouse models where naturally occurring $Foxp3^+$ Tregs induce IL-10 production with subtle IFN-gamma response by $CD4^+$ and $CD8^+$ effector T cells. In addition, Tregs also downregulate classical activation of macrophages resulting in decreased activity of TNF-alpha [56]. The Treg activity is believed to decrease the tissue damage in the host cells suggesting a cardinal role for naturally occurring Tregs in the development of a tolerant phenotype during African trypanosomiasis [57].

The immune response against *T. gondii*, an intracellular parasite and the etiological agent of toxoplasmosis, has been largely characterized and demonstrated that cellular immunity plays a vital role in controlling infections [58]. Of which, Tregs were demonstrated to modulate the protective immune response against *T. gondii*, thereby driving a powerful Th1 immune response [59, 60]. Also, it is believed that the absence of Tregs in toxoplasmosis may induce an uncontrolled inflammatory response [60].

Foxp3-expressing Tregs have been implicated in parasite-driven inhibition of host immunity during chronic infection [32]. One of the major effects of chronic helminth infections is induction of T-cell hypo responsiveness [61]. The mechanisms involved have been thought to be multiple and the involvement of natural and inducible Tregs in down regulating effector T cell responses upon chronic infection has been proposed [62]. The cytokines IL-10 and TGF-ß have been associated with down regulation, indicating that regulatory populations are activated during infection. The importance of IL-10 as a crucial mediator of regulation in parasite infections has been well recognized both in humans and murine [63–65]. In human filariasis, heavily infected individuals have high IL-10 levels and IL-10 messenger RNA production which was inversely correlated with T cell proliferation [34, 66]. Similarly infection in experimental *Schistosoma mansoni* was shown to be associated with immunoregulatory mechanisms, including Treg that may help control morbidity and dampen resistance to reinfection. Treg responses control both Th1 and Th2 responses in an IL-10 independent manner [67, 68] and are associated with regulation of granuloma formation in chronic infections [69]. In a study conducted among patients infected with *S. mansoni* in Kenya, it was revealed that few patients had higher proportions of $CD3^+/CD4^+/CD25^{high}$ Tregs that subsequently decreased after treatment with praziquantel. The study concluded that not all *Schistosoma mansoni*-infected individuals develop high percentages of circulating Tregs. The effective treatment decreases the proportion of Tregs and their phenotypes, possibly because of the removal of constant exposure to antigens from intravascular, egg-producing adult worms [70]. In a NOD mice model, treatment with *S. mansoni* egg antigen (SEA) was shown to upregulate TGF-ß on T cells and Th2 cells resulting in the expansion of $Foxp3^+$ that remain crucial in determining the SEA-mediated diabetes outcome [71]. Also, hsp60 peptide (SJMHE1) of *S. japonicum* induces $CD4^+CD25^+Foxp3^+$ Tregs both *in vivo* and *in vitro* resulting in subsequent release of IL-10 and TGF-ß [72].

Nematode infections have been shown to induce regulatory cell expansion in both mice and humans [73]. In a study conducted in Lima, Peru, among human T lymphocyte virus (HTLV-1) patients with or without *Strongyloides* infection, increased proportions of $CD4^+CD25^+Foxp3^+$ Tregs were observed in patients with *S. stercoralis* and HTLV-1 coinfection in comparison to normal controls [33]. Furthermore, those with increased proportions of $CD25^+FoxP3^+$ cells had decreased antigen driven production of IL-5 and lower eosinophil counts. This reduced response is inversely correlated with the proportion of CD4 cells, which are $CD4^+CD25^+FoxP3^+$, suggesting a role for these cells in blunting antigen-driven protective responses.

Visceral Leishmaniasis (VL) represents a parasitic disease that has been shown not to induce expansion of natural Tregs. In a study conducted among patients presenting with symptoms of Kala-azar, frequencies of $Foxp3^+$ cells in patient with VL before and after treatment did not increase, neither were they elevated when compared to endemic controls. It was therefore concluded that active VL is not associated with increased frequencies of peripheral Foxp3 Treg or accumulation at the site of infection [74]. While active VL does not induce expansion of Treg, it has been shown in animal models that Treg is directly responsible for its reactivation [75]. During the primary infection, *L. major* can disseminate to other tissues within the body which may persist after parasite reduction and healing [76, 77]. These persistent parasites are associated with the establishment of strong immunity against reinfection, a state that is referred to as concomitant immunity [75, 78, 79]. Tregs have been found abundantly at these reinfection sites while it reduces at the site of initial infection confirming its importance in reactivation of VL infection. Significant increase in IL-10 production by dermal and LN $CD4^+$ T cells has been shown during the reactivation process confirming a role of IL-10 mediated *Leishmaniasis* both in susceptible and resistant individuals [80, 81]. In macaque (*Macaca mulatta*) model, *L. braziliensis* strain that produces self-healing dermal lesions was used to characterize the systemic and local cell-mediated immune responses that led to controlled growth of granulomas in the infected host. Resolution of infection was observed to be dependent on concomitant recruitment of interleukin- (IL-)-10-producing $CD4^+CD25^+$ regulatory T (Treg) cells that suppress the effector T-cell-mediated inflammatory response [82].

5. Regulatory Gene Polymorphisms

Parasites exert a selection pressure on their hosts and are accountable for driving diversity within gene families and immune gene polymorphisms in a host population. The pathogen driven selection on immune genes can potentially alter the primed sequence and can direct to substantial changes in gene expression [1]. A number of loci were known to be associated to Treg activity. Genes such as *IL-2*, *IL-4*, *IL-10*, *IL-13*, *STAT-4*, *STAT-6*, *GITR*, *TLRs*, and *Foxp3* are established as key players in regulating Tregs [1]. The investigation of human polymorphisms in loci associated with Treg activity may underlie both susceptibility to infection and level of Treg expression. Many of these polymorphisms evolved and are maintained in a human population exposed to infectious diseases. Genotype associations may predict likely susceptibility and allow identification of subjects at the risk of developing the disease and may be subjected to therapeutic treatment.

Investigation of human polymorphisms in immune relevant genes has been used to determine the level of Treg expression and thereby the extent of susceptibility to parasitic infection [10]. The single-nucleotide polymorphisms (SNPs) in the promoter region of the genes such as *STAT6*, *Foxp3*, and *TNFRSF18* were well characterized for their functional role [4, 10, 83]. One gene of interest that plays a key role in the function of Tregs is the *IL-10* gene locus. In populations exposed to *Leishmania braziliensis* in Bahia, Brazil, genetic analysis of the *IL-10*-819C/T SNP polymorphism, located in the *IL-10* promoter, showed that the C allele increased the risk of lesions. The *IL-10*-819 C/C genotype was associated with higher levels of *IL-10* than C/T and T/T genotypes demonstrating a vital role for *IL-10* in skin lesions in humans infected with *L. braziliensis* [84]. Also, *IL-10* promoter polymorphism was recently shown to influence nonspecific total IgE levels, but not schistosomiasis-specific immunity [85]. In chagas disease low *IL-10* expression was associated with cardiac function and it was demonstrated that the polymorphic allele, which correlates with lower expression of *IL-10*, was associated with the development of chagas disease cardiomyopathy. *IL-10* gene polymorphism and *IL-10* expression are important in determining susceptibility to chagasic cardiomyopathy [86]. Also, in malaria infection it was shown that common IL-10 promoter haplotypes condition susceptibility to severe malaria anemia and functional changes in circulating *IL-10*, TNF-alpha, and *IL-12* levels in children with falciparum malaria [87]. In a study among subjects infected with urinary schistosomiasis in Mali, an association was found between *STAT6* (rs324013) gene polymorphism and infection level in subjects under 20 years while the same study did not observe any association with *IL-4* and *IL4R* polymorphism [88]. *IL-13* promoter polymorphism has also been associated with urinary schistosomiasis [89].

6. Conclusion

In summary, the field of infectious disease immunology is at an exciting intersection with new concepts in regulation of immune responsiveness. Despite extensive studies, there is still much that remains unclear about the mechanism and activities of Treg. A more comprehensive understanding of the mechanisms and gene-expression pathways that underlie the Treg activities will be essential if new therapeutic strategies are to be developed. The ability of the Treg cells to control many facets of the immune response makes them an interesting model to study possible immune-modulatory intervention.

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Research Article

The Hamster Model for Identification of Specific Antigens of *Taenia solium* Tapeworms

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Humans acquire taeniasis by ingesting pork meat infected with *Taenia solium* cysticerci, which are the only definitive hosts of the adult stage (tapeworm) and responsible for transmitting the human and porcine cysticercosis. Hence, detection of human tapeworm carriers is a key element in the development of viable strategies to control the disease. This paper presents the identification of specific antigens using sera from hamsters infected with *T. solium* tapeworms analyzed by western blot assay with crude extracts (CEs) and excretion-secretion antigens (E/S Ag) obtained from *T. solium* cysticerci and tapeworms and extracts from other helminthes as controls. The hamster sera infected with *T. solium* tapeworms recognized specific bands of 72, 48, 36, and 24 kDa, in percentages of 81, 81, 90, and 88%, respectively, using the *T. solium* tapeworms E/S Ag. The antigens recognized by these hamster sera could be candidates to improve diagnosis of human *T. solium* taeniasis.

1. Introduction

Taenia solium is a cestode that causes taeniasis when the adult worm (tapeworm) is lodged in the human intestine and cysticercosis when the larval stage (cysticercus) establishes in the central nervous system, skeletal muscle, and other organs of both pigs and humans [1]. Taeniasis causes only mild symptoms in the human carrier, which accounts for the disease remaining frequently undiagnosed, whereas cysticercosis of the central nervous system (neurocysticercosis, NCC) causes clinical manifestations that range from mild symptoms to death [2–4].

A study performed in hospitalized patients at the National Institute of Neurology and Neurosurgery in Mexico City during the period from 1995 to 2009 revealed that the annual frequency for hospitalizations due to NCC remained constant, with approximately 2.4% of neurology consultations attributable to NCC, whereas mortality from this disease has diminished, which can be explained by an opportune diagnosis [5]. Seroprevalence studies show that up to 12% of the populations in endemic areas carry anticysticerci antibodies [6].

The mean prevalence of *T. solium* taeniasis in endemic countries is 4%, though in some countries a prevalence of up to 7% has been reported. Some studies suggest that the disease is more common among women and individuals between 30 and 40 years of age [7, 8]. Epidemiological studies in Central and Latin America show that in the last thirty years, Colombia, Honduras, and Guatemala are the countries with the highest annual frequency of taeniasis, whereas Costa Rica has the lowest number of cases [9]. On the other hand, in four communities in Guatemala (Quesada, El Tule, El Jocote, and Santa Gertrudis) in the period from 1991 to 1994, 92 positive cases were detected among 3,399 studied samples (2.7%), and 98% of the recovered parasites were *T. solium*. A 1% frequency was detected in children aged 6 months to 4 years [8, 10].

Identification of the *Taenia* species (*T. saginata*, *T. asiatica*, and *T. solium*) is fundamental in the control and prevention of taeniasis/cysticercosis transmission in endemic areas. The methods used for these studies have been based on microscopic detection of eggs in feces [11] and on the detection of tapeworm coproantigens [12]. Diagnosis of

tapeworms through coproparasitoscopic analysis lacks sensitivity and specificity since the eggs of different taenias are morphologically indistinguishable, and although the detection of coproantigens is specific for the genus, it does not distinguish between species. However, at the experimental level, techniques based on DNA identification are capable of distinguishing between species of taenias [13].

Taeniasis in dogs has been diagnosed using ELISA test to detect antibodies that recognize components in the tapeworms excretion/secretion antigens (E/S Ag) [14, 15]. Recently, two serological methods (ELISA and IET) have been used for detection of *T. solium* taeniasis. These methods use two antigens obtained from adult stage (tapeworms) E/S Ag of *T. solium*: ES33 and ES38 and their recombinants (rES33 and rES38) which give sensitivity and specificity above 97% and 91%, respectively [16, 17].

Epidemiological studies show that human with *T. solium* taeniasis is the risk factor in the transmission of cysticercosis in pigs and humans [6, 18]. However, little effort has been made to identify new specific antigens in order to develop a low-cost, feasible method, which is both sensitive and specific for detecting tapeworm carriers. Thus, the present study was carried out in order to identify specific antigens from *T. solium* tapeworm, using the *T. solium* taeniasis-hamster model.

2. Materials and Methods

2.1. Biological Material. Taenia solium cysticerci and tapeworms were obtained by dissecting them from skeletal muscles from naturally infected pigs and from small intestine of experimental infected hamster. Animals were processed according to the Official Mexican Norms: NOM-009-ZOO-1994 for sanitary processing of meats and NOM-033-ZOO-1995 for humanitarian sacrifice of domestic and wild animals. Cysticerci were washed in cold sterile phosphate-buffered saline (PBS), pH 7.4. Viability of cysticerci in each lot was determined by incubation of 20 cysticerci at 37°C in RPMI 1640 medium (Sigma) complemented with pig bile at 25% for 24 h [19]. Cysticerci were considered viable, when the scolex of the larva evaginates and displays contractile movements. The number of evaginated parasites was counted and a mean percentage of viability was established.

2.2. Development Taenia solium Tapeworms. Golden hamsters (*Mesocricetus auratus*) were treated orally with praziquantel (PQZ 3 mg/animal). A week later, the animals were infected orally with eight cysticerci [20]. Briefly, animals were immunosuppressed by intraperitoneal administration of methylprednisolone (2 mg/animal) every 15 days. Five weeks later, the animals were killed by intraperitoneal administration of sodium pentobarbital (200 mg/kg). *Taenia solium* tapeworms were recovered from the small intestine, washed with antibiotic complemented PBS (penicillin 1×10^6 U/L and streptomycin 2 g/L, Sigma), and cultivated in RPMI medium to obtain the E/S Ag. Blood samples were obtained from the hamsters before infection to be used as a negative control serum (preimmune serum) and shortly before killing. Blood samples were incubated at room temperature for 30 min and centrifuged at 735 *g* for 10 min to obtain serum. The recovered sera were stored at −20°C until needed.

2.3. Antigens Preparation

Cysticerci E/S Ag. Cysticerci were incubated for 6 hours at room temperature in Petri dishes containing RPMI medium with antibiotic and EDTA (1 mM). The culture medium was recovered and centrifuged at 9000 *g* for 20 min, the supernatant was filtered through 0.45 *μ*m membranes (Millipore), dialyzed against PBS, and concentrated in an AMICON unit using 3000 PM membranes (Millipore). Protease inhibitors (TLCK, PMSF, and EDTA) were added and the antigens were stored at −20°C [21].

Tapeworm E/S Ag. Tapeworms were incubated at 37°C in Petri dishes containing RPMI medium with antibiotic (penicillin 1×10^6 U/L and streptomycin 2 g/L, Sigma). The medium was exchanged every 8 hours on the first day of incubation and every 12 hours during the following 3 to 4 days. Viability of tapeworm was monitored daily through microscopic observations of morphology and mobility. The media recovered from the incubation were pooled and centrifuged at 9000 *g* for 20 min. The supernatant was dialyzed against PBS, concentrated, protease inhibitors (TLCK, PMSF, and EDTA) added and the samples were frozen at −20°C until used [17].

Crude Extracts (CE). The parasites (*Taenia solium, Taenia saginata, Taenia taeniaeformis, Hymenolepis diminuta*, and *Ascaris lumbricoides*) were homogenized individually by a polytron (Brinkmann Instruments, Inc) at maximal power for 3 min in PBS, pH 7.4, using a ratio of 1 g parasite per 5 ml of extraction solution, complemented with protease inhibitors. The suspension was centrifuged at 19,870 *g* for 30 min and the supernatant was dialyzed against PBS at 4°C, overnight. The resulting mixture was ultracentrifuged at 100,000 *g* for 30 min; the supernatant was distributed in aliquots and frozen at −20°C.

Recombinant Antigens. We isolated clones that code for the P29 and antigen B (AgB or paramyosin) antigens by screening an expression library of *T. solium* adult stage constructed in *λ*ZAPII vector with a hyperimmune rabbit serum anti-CE of *T. solium* tapeworm. Recombinant AgB preparation was carried out according to a previously established protocol [22], and the *T. solium* P29 antigen (TsP29) was produced using the pRSETB vector and the recombinant antigen purified by metal affinity chromatography [Jiménez et al., unpublished results]. The concentration of purified proteins was determined by the Lowry method and diluted at 1 mg/ml in PBS [23].

Production of Hyperimmune Sera. Hyperimmune sera against *Taenia solium* (cysticerci and tapeworm crude extracts, E/S Ag, AgB, and P29 antigens) were prepared in eight-week-old female hamsters. Prior to immunization,

Table 1: Percentages and recovery of *Taenia solium* tapeworm in immunosuppressed hamsters.

Cysticerci lot	Hamsters used/infected	Infection (%)	Tapeworms recovered (size)	Recovery (%)
1	13/9	69.23	37 (10–30 cm)	36
2	7/3	42.85	10 (20–30 cm)	18
3	10/9	90	37 (10–30 cm)	46
4	18/15	83.33	52 (15–40 cm)	36
Total	**48/36**	**74.35**	**134**	**34**

blood samples were taken to obtain a preimmune serum to serve as negative control in western blot assays. Hamsters were immunized subcutaneously with 50 μg of each antigen/animal, every 15 days. After the final immunization, sera were obtained, antibody titers were determined by ELISA and stored at −20°C. Hyperimmune sera against CE of known helminthes were obtained from rabbits and prepared according to the same immunization scheme.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The electrophoretic patterns of the CE and E/S Ag of *T. solium* cysticerci and tapeworms were determined employing 50 and 15 μg, respectively, in a 10% SDS-PAGE adding 2-mercaptoethanol as a reducing agent [24]. Electrophoresis was carried out in a MiniProtean II (Bio-Rad) camera at 100 V. The crude extract gels were stained with Coomassie brilliant blue and the E/S Ag gels were silver-stained.

Western Blot Assay. We carried out preparative 10% SDS-PAGE using 2 μg of protein for the crude extracts or E/S Ag and 200 ng of pure antigens for every linear millimeter of gel as well as a prestained molecular weight marker (Bench-Mark Prestained protein ladder, Invitrogen). The antigens were transferred onto nitrocellulose membranes (Hybond-C, Amersham Biosciences) using a Bio-Rad Mini Transblot equipment (100 V/1 h) in cold. The membrane was washed with PBS, cut into 2 mm wide strips, and stored at −20°C.

For the identification of specific tapeworm antigens, the membranes with the different extracts were confronted with the sera from hamsters infected with *T. solium* tapeworms and preimmune sera (1 : 100), as well as with the hyperimmune hamster and rabbit sera (1 : 1000) diluted in PBS-Tween 0.3% and 5% fat-free milk. The membranes were incubated for 1 h at room temperature under constant agitation and subsequently washed three times for 5 min with PBS-Tween 0.3%. A second peroxidase-conjugated hamster or rabbit anti-IgG antibody (Zymed) at a 1 : 2000 dilution was added and incubated for 1 h under constant stirring at room temperature. The membrane was washed as previously, and the antibodies bound to the membranes were developed using diaminobenzidine (5 μg/ml) and 0.3% H_2O_2. To discard cross-reactions to other helminthes, membranes containing extracts from *T. saginata, T. taeniaeformis, H. diminuta,* and *A. lumbricoides* were tested against the same sera. Bands obtained in membranes by western blot were analyzed with the 1D Image Analysis Software (*Kodak Digital Science).*

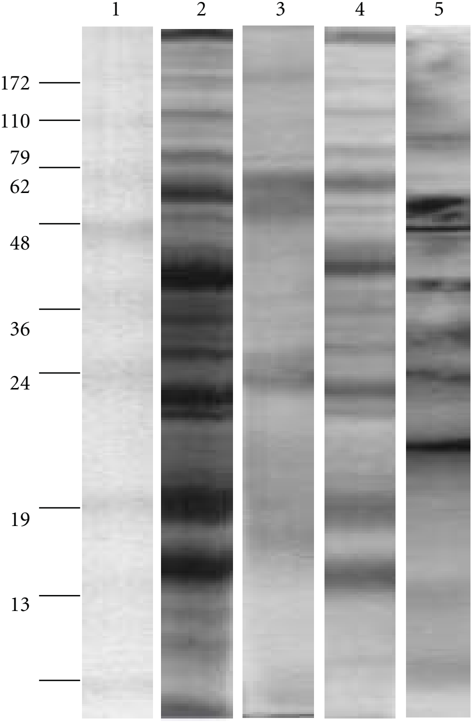

Figure 1: 10% SDS-PAGE. Lane 1: molecular weights (Bench-Mark prestained protein ladder, Invitrogen), and *Taenia solium*. Lanes 2: tapeworm crude extract (CE), Lane 3: tapeworm excretion/secretion antigens (E/S Ag), Lane 4: cysticerci CE, Lane 5: cysticerci E/S Ag. Lanes 2 and 4 stained with Coomassie blue, lanes 3 and 5 with silver stain.

3. Results

We obtained several lots of cysticerci from naturally infected pigs acquired from different regions in Mexico. Lots presented 90, 85, 66, and 90% of scolex evagination and a mean viability percentage of 83%. Table 1 shows that, at the time of death (5 weeks), the number and length of recovered tapeworms vary in each lot, with a mean number of three tapeworms recovered from each hamster and a mean length of 25 cm. Recovered tapeworms and cysticerci were incubated in RPMI medium supplemented with antibiotics to obtain the E/S Ag.

The composition of CE from *T. solium* cysticerci and tapeworms observed in 10% SDS-PAGE stained with Coomassie blue shows very similar complex patterns (Figure 1, lanes 2 and 4), with bands between 13 to 200 kDa.

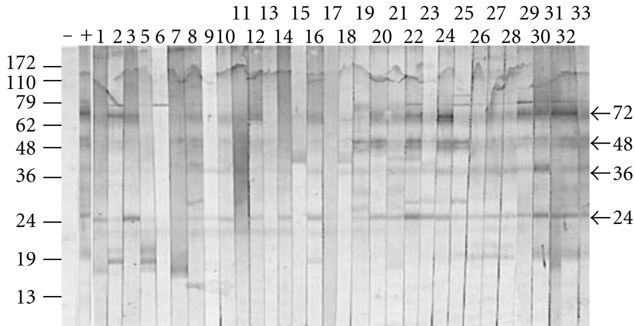

Figure 2: Western blot with tapeworm E/S Ag of *Taenia solium* was incubated with hamster sera infected with *T. solium* tapeworms (1–33). Preimmune (lane −) and hyperimmune hamster sera antitapeworm E/S Ag of *T. solium* (lane +) were used as negative and positive controls. Peroxidase-conjugated antihamster IgG antibody was used as second antibody.

Table 2: Recognition percentage of the different bands in tapeworm E/S Ag of *Taenia solium* by 33 hamster sera infected with *T. solium* tapeworms.

Band (kDa)	Serum number	Recognition (%)
85	4 (23,24,25,26)	9
80	C+, 15 (2,6,8,19,22,24–33)	45
77	C+, 23 (2,5,6,8,10,12,16,20,23–26,27,29–33)	69
72	C+, 27 (2–5,7,8,10–12,15,16,18–33)	81
52	C+, 16 (2–8,19–25,29,32)	48
48	C+, 27 (2–5,7,8,10–14,16,19–33)	81
43	4 (15,18,21,22)	12
36	C+, 30 (1–5,7–14,16,18-33)	90
30	C+, 5 (19,21,24, 32,33)	15
27	C+, 4 (22,23,24,25)	12
25	C+, 1 (19)	3
24	C+, 29 (1–5,7–12,14,16,17,19–33)	88
22	C+, 22 (1–14,16–20,26,27,31)	66
19	C+, 4 (2,5,27,28)	12
17	C+, 10 (2,5,16,25–28,30,32,33)	30
15	4 (1,2,7,29)	12

In contrast, the silver-stained patterns from *T. solium* tapeworms (lane 3) and cysticerci (lane 5) E/S Ag are different among themselves and different from patterns obtained for the CE of the tapeworms (lane 2) and cysticerci (lane 4). In the case of the tapeworm E/S Ag, eight bands ranging from 20 to 170 kDa were observed, the 62–78, 48, 36, and 20–24 kDa bands being distinct. For the cysticerci's E/S Ag the recognizing were in the region of 30–110 kDa, with five distinct bands of 64, 50, 40, 30, and 28 kDa and a doublet in the 90 kDa region (lane 5).

When the 33 hamster sera infected with *T. solium* tapeworms (lanes 1–33) were tested against tapeworm *T. solium* E/S Ag, sixteen bands of different molecular weight were easily distinguished between the 13 to 172 kDa. However, four bands in the regions of 72, 48, 36, and 24 kDa were recognized in 81, 81, 90, and 88%, respectively. It is worth mentioning that the preimmune sera used as negative controls (lane −) reacted mildly with a band in the 20 kDa region, and this band was discarded from further analysis. The positive control (lane +), a hyperimmune antitapeworm E/S Ag hamster serum, reacted intensely with bands between 20 and 172 kDa, as well as with the 72, 48, 36, 30, 24, and 20 kDa bands, recognized by the sera from hamster infected with *T. solium* tapeworms (Figure 2, Table 2).

The results of western blot with CE of *T. solium* tapeworm and the hamster sera infected with *T. solium* tapeworms show that hamster sera recognized bands in the range of 40 to 160 kDa, with 40 kDa band being one of the most recognized (27%). Four bands (160, 100, 90, and 70 kDa) were recognized by 3 to 12% of the sera. The negative control (lane −), preimmune serum, did not show any reaction, whereas the positive control (lane +), a hyperimmune

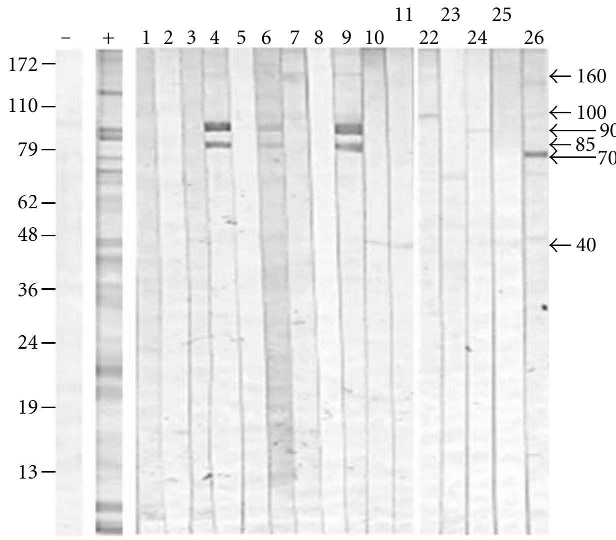

Figure 3: Western blot with *Taenia solium* tapeworm CE was tested with hamster sera infected with *T. solium* tapeworms (1–33). Preimmune (lane −) and hyperimmune hamster sera against *T. solium* tapeworm CE (lane +) were used as negative and positive controls. WB shows representative samples of the assay. Number of lanes is corresponding to serum number of the assay.

Table 3: Recognition percentages of the different bands in CE of the *T. solium* tapeworms by 33 hamster sera infected with *T. solium* tapeworms.

Band (KDa)	Serum number	Recognition (%)
160	C+, 8, 10, 26	9
100	C+, 8, 16, 20, 22	12
90	C+, 5, 7, 10, 24	12
85	C+, 5, 7, 10	9
70	C+, 26	3
40	C+,11,12,13,15,18,19, 24,25,26	27

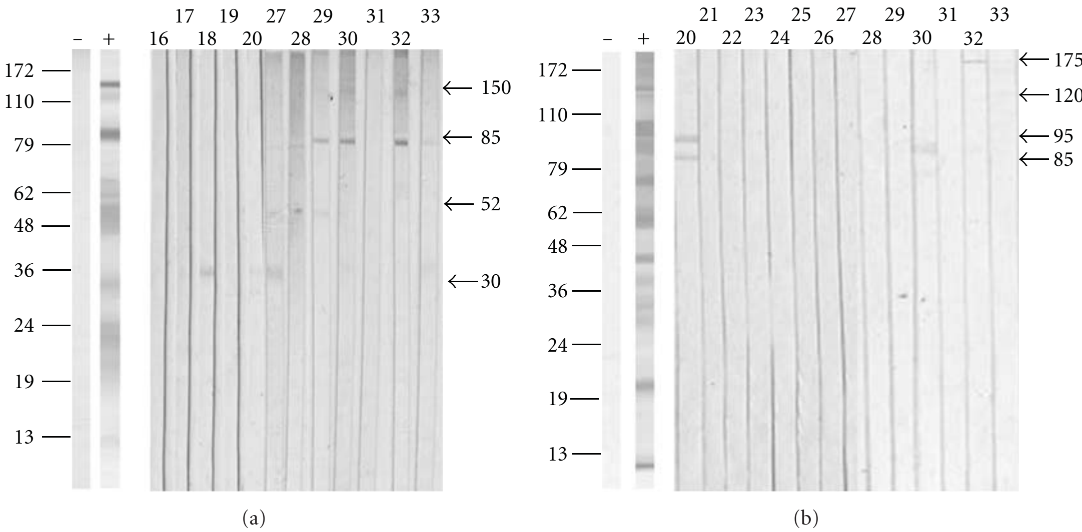

Figure 4: (a) Western blot with *Taenia solium* cysticerci E/S Ag was tested with 33 hamster sera infected with *T. solium* tapeworms (1–33). Preimmune (lane −) and hyperimmune hamster sera anti-*T. solium* cysticercus E/S Ag (lane +) was used as negative and positive controls. WB shows representative samples of the assay. Number of lanes is corresponding to serum number of the assay. (b) Western blot with *Taenia solium* cysticercus CE was tested with 33 hamster sera infected with *T. solium* tapeworms (1–33). Preimmune (lane −) and hyperimmune hamster sera anticysticerci CE (lane +) were used as negative and positive controls. WB shows representative samples of the assay. Number of lanes is corresponding to serum number of the assay.

hamster serum antitapeworm CE, recognized several bands in the range of 10 to 170 kDa (Figure 3, Table 3).

On the other hand, in the hamster sera infected with *T. solium* tapeworms in the E/S Ag of *T. solium* cysticerci, only four bands of 150, 85, 52, and 30 kDa were recognized in 6, 21, 9, and 27%, respectively. It should be noted that these bands and others in the 19 to 200 kDa range were also recognized by the hyperimmune hamster serum anticysticercus E/S Ag used as a positive control (lane +). Conversely, the preimmune serum did not recognize any bands on the membrane (lane −) (Figure 4(a), Table 4(a)).

When cysticerci CE antigens were used in membrane, only three hamster sera infected with *T. solium* tapeworms reacted with the 175, 120, 95, and 85 kDa bands, which represent recognition of 3, 3, 6, and 6%, respectively. Preimmune (lane −) hamster serum did not react with any component of the CE cysticerci. The positive control (lane +) recognized membrane bands in the range of 10 to 200 kDa, showing a strong response with the 95, 85, 70, 60, 45, 30, 20, and 13 kDa bands (Figure 4(b), Table 4(b)).

Since hamster sera infected with *T. solium* tapeworms frequently recognized bands in the 85, 90, and 29 kDa regions and because AgB and P29 antigens are located in these regions, and have been used for diagnosis of cysticercosis and echinococcosis [25, 26], we performed a western blot using the 33 hamster sera infected with *T. solium* tapeworms with these proteins of *T. solium*. Ninety-six percent of the infected hamster's sera recognized 85 and 95 kDa bands at different intensities and showed slight reaction to some bands below this range (Figure 5(a)), while the same sera showed a 28% recognition of the 29 kDa band (Figure 5(b)). The hyperimmune anti-AgB and anti-P29 sera, used as positive controls, specifically recognize the 85, and 95 (antigen B) and the 29 kDa (P29 antigen) bands, as well as other lower weight bands presumably representing degradation products of these antigens.

The results of the western blot assay with CE of other helminthes show that two hamster sera infected with *T. solium* tapeworms recognized a band of approximately 100 kDa in the CE of *T. saginata* (Figure 6(a)). In the case of CE from *T. taeniaeformis,* we observed that three sera reacted with a band of approximately 100 kDa showing 9% recognition (Figure 6(b)). In the case of *H. diminuta,*

Table 4: (a) Recognition percentage of *T. solium* cysticerci E/S Ag by 33 hamster sera infected with *T. solium* tapeworms. (b) Recognition percentage of the 33 hamster sera infected with *T. solium* tapeworms on antigens of *T. solium* cysticerci CE.

(a)

Band (kDa)	Serum number	Recognition (%)
150	C+, 30,32	6
85	C+, 5, 27–30,32,33	21
52	C+, 27,28,29	9
30	C+, 1,3,12,14,18,20,26,30,33	27

(b)

Band (kDa)	Serum Number	Recognition (%)
175	C+, 32	3
120	C+, 33	3
95	C+, 20,30	6
85	C+, 20,30	6

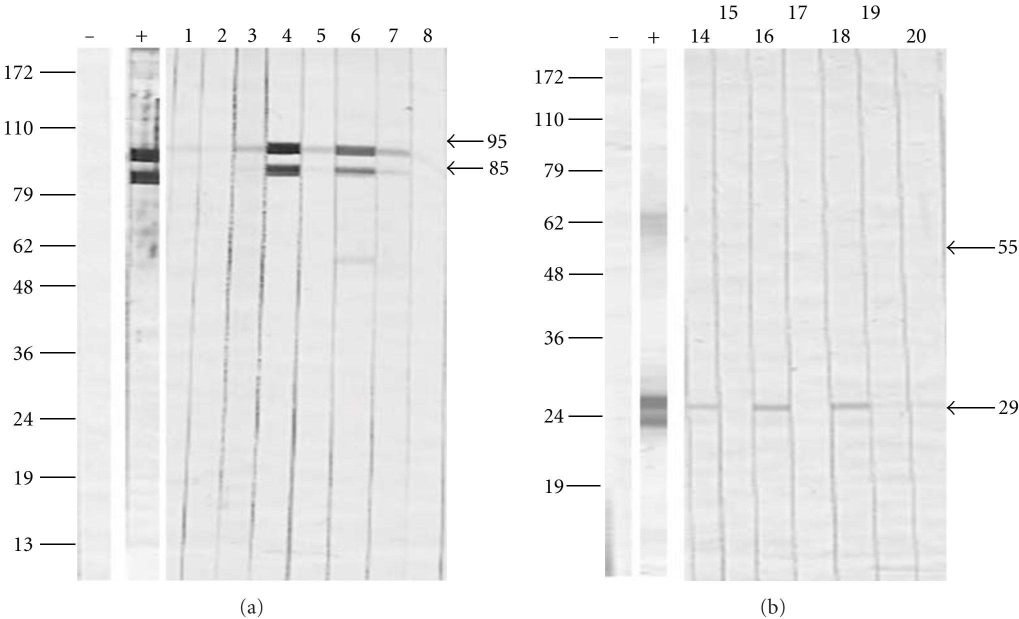

Figure 5: Western blot with AgB (a) and P29 (b) recombinants from *Taenia solium* was incubated with 33 hamster sera infected with *T. solium* tapeworms. Preimmune hamster normal sera (lane −) were used as negative controls. Hyperimmune anti-AgB and anti-P29 from *T. solium* sera from hamster were used as positive controls (lanes +). WB shows representative samples of the assay. Number of lanes is corresponding to serum number of the assay.

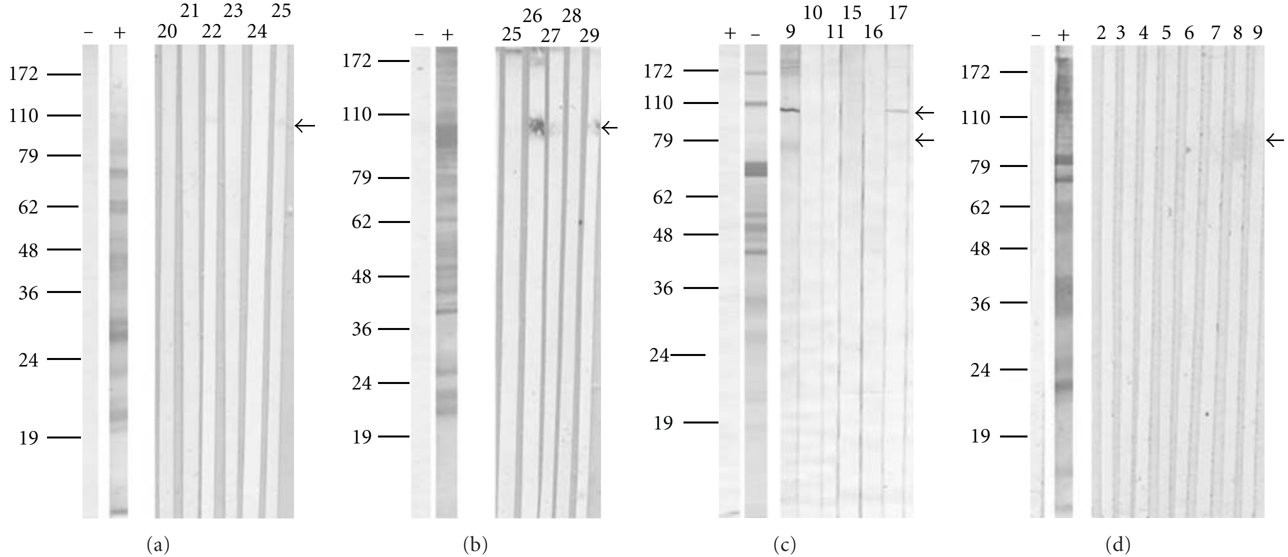

Figure 6: Western blot shows cross-reaction with other helminthes. Crude extracts (CE) from (a) *Taenia saginata*, (b) *Taenia taeniaeformis*, (c) *Hymenolepis diminuta*, and (d) *Ascaris lumbricoides* were confronted with 33 hamster sera infected with *T. solium* tapeworms. Hyperimmune sera prepared in rabbits against each of the CE were used as positive controls (lanes +) and preimmune sera from rabbit were used as negative controls (lanes −). WB shows representative samples of the assay. Number of lanes is corresponding to serum number of the assay.

the sera reacted primarily with bands in the 100, 81, and 50 kDa regions (Figure 6(c)). Additionally, only one serum recognized bands in the 80 and 100 kDa region in the CE of *Ascaris lumbricoide*s (Figure 6(d)). The preimmune hamster (data not shown) and rabbit sera used as negative controls (lane −) showed no reaction in the membranes with CE from these helminthes; in contrast, hyperimmune rabbit sera (lane +) against CE of helminthes recognized bands in the 20 and 172 kDa range, when tested against CE of *T. saginata*, *T. taeniaeformis*, *H. diminuta,* and *A. lumbricoides.*

4. Discussion

In view of the continuing threat to public health represented by NCC, numerous studies have been conducted to develop better and cheaper diagnostic methods [27]. Currently,

there are different serological methods available that employ ELISA and western blot using *T. solium* cysticercus glycoprotein isolated with *Lens culinaris* or its recombinants. These methods have 98% sensitivity and 100% specificity in serum [28, 29]. In contrast, diagnosis of taeniasis has not received the same attention. There are several reasons for this: taeniasis remains frequently unrecognized by the host since the symptoms are usually mild and unspecific, and the high risk involved handling the tapeworm eggs [1, 30]. To eliminate the risk of exposure to human feces contaminated with *T. solium* eggs, we used the taeniasis model in golden hamsters. This model is safe since the tapeworms that develop in the hamster's intestine do not reach sexual maturity and do not produce infective eggs [20, 31]. Table 1 shows that in immunosuppressed animals infected with eight cysticerci, the rate of established parasites is 34.89%, with variable permanence at 5 weeks of infection, a rate which is in accord with previous publications [31, 32].

The 10% SDS-PAGE assays show that all fractions present great complexity. For the CE, we observed a similar pattern for both the cysticercus and tapeworms showing bands in the range of 13 to 200 kDa. Conversely, we observed great differences in the banding patterns of E/S Ag between the cysticercus and the tapeworm. In the E/S Ag from the tapeworm, 12 bands in the 13 to 110 kDa were distinguished, and these results are similar to those reported by Wilkins and coworkers [17], who identified approximately 16 bands. For the cysticercus E/S Ag, we found 20 bands in the 13 to 172 kDa range, with distinct bands of 64, 54, 46, and 30 kDa. These data are also consistent with previous findings of 13 to 19 bands described in the cysticercus E/S Ag [33, 34].

The western blot assays on membranes containing *T. solium* tapeworm E/S Ag confronted by the 33 hamster sera infected with *T. solium* tapeworms identified four antigens of 72, 48, 36, and 24 kDa with a recognition of 81, 81, 90, and 88%, respectively. Wilkins and coworkers [17] showed that sera from *T. solium* tapeworm-infected patients confronted by adult stage E/S Ag detected two bands of 32.7 and 37.8 kDa. The 37.8 kDa band matches with the 36 kDa band identified in this paper by hamster sera infected with *T. solium* tapeworms and presumably corresponds to the same antigen.

To determine the specificity of the identified bands, we performed a western blot assay by confronting the hamster sera infected with *T. solium* tapeworms with cysticerci E/S Ag and with the CE from both cysticerci and tapeworms of *T. solium*, which showed that four bands were recognized, 150, 85, 52, and 30 kDa, with the 30 kDa bands showing the highest percentage of recognition (27%). This band does not match with the bands identified by the hamster sera infected with *T. solium* tapeworms in the tapeworm E/S Ag. Additionally, the hamster sera infected with *T. solium* tapeworms frequently recognized two other bands at varying intensities and corresponding to a 95–85 kDa band in the case of cysticerci CE and a 29 kDa band in the case of E/S Ag of cysticerci and tapeworm. These bands match with two well-known diagnostic antigens of cysticercosis and echinococcosis, AgB and P29 [25, 26]. However, these antigens are not recommended for the specific diagnosis of *T. solium*, since they are both present in other helminthes [26, 35].

In this paper, we observed that hamster sera infected with *T. solium* tapeworms reacted mildly with 100 kDa in the CE from *T. saginata*, *T. taeniaeformis, H. diminuta,* and *A. lumbricoides*. However, there are reports of the lack of cross-reactions using *T. solium* tapeworms E/S Ag and human sera with other parasitic infections, including *T. saginata* [17, 27].

It is worth mentioning that tapeworm-infected hamsters, whether immunosuppressed or not, produce antibodies against *T. solium* from the first week of infection and titers increasing as the infection is prolonged to finally decrease after the worm is expelled [32, 36]. This suggests that these bands (72, 42, 36, and 24 kDa) could be used to detect serum antibodies to human tapeworms as well as to produce antibodies that detect them in samples of patients with taeniasis. In addition, the recombinant forms of these bands could improve the suggested method for diagnosis of taeniasis [17].

Humans are the only definitive host of *T. solium* tapeworm and, thus, responsible for causing both human and swine cysticercosis [6, 18]. It is also known that humans can be easily treated for this parasitosis by antihelminthic drugs, such as praziquantel and albendazole [12, 37, 38]. Therefore, further studies should be undertaken with sera from human patients with taeniasis or in endemic areas to determine if these antigens could be candidates for the specific detection of *T. solium* taeniasis. In developing countries, where taeniasis-cysticercosis is an endemic disease and continues to be a significant public health problem, it is essential to have reliable and cheap diagnostic tools to screen populations, as a primary measure for control and eradication programs [5, 38].

5. Conclusions

We identified four specific antigens in the *T. solium* tapeworm E/S Ag that are useful in the detection of tapeworms in hamsters, which could be potentially useful in the diagnosis of human *T. solium* taeniasis.

Acknowledgments

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Review Article

Modulation of Specific and Allergy-Related Immune Responses by Helminths

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Helminths are master regulators of host immune responses utilising complex mechanisms to dampen host protective Th2-type responses and favour long-term persistence. Such evasion mechanisms ensure mutual survival of both the parasite and the host. In this paper, we present recent findings on the cells that are targeted by helminths and the molecules and mechanisms that are induced during infection. We discuss the impact of these factors on the host response as well as their effect in preventing the development of aberrant allergic inflammation. We also examine recent findings on helminth-derived molecules that can be used as tools to pinpoint the underlying mechanisms of immune regulation or to determine new anti-inflammatory therapeutics.

1. Introduction: Features of Helminth-Induced Immune Responses

Helminths have developed a unique evolutionary dialogue with their hosts' immune system due to their longevity within the host, their complex life cycles, and multicellular nature. These pathogens induce very different immune responses in comparison to bacteria, fungi, viruses, or protozoa. Cells of the innate and adaptive immune system are important for the initiation of type 2 immunity, which characterises the response to helminth infection, as well as allergic reactions. The key players in T helper (Th) 2-type immunity are $CD4^+$ Th2 cells and involve the cytokines interleukin (IL-)4, IL-5, IL-9, IL-10, and IL-13 and immunoglobulin (Ig)E. $CD4^+$ Th2 cells also express some of the cytokines mentioned above as well as the chemokine ligand CCL11 and the chemokine receptor CCR3 [1–3]. These factors lead to recruitment and infiltration of eosinophils, basophils and mast cells, and expansion of alternatively activated macrophages [4]. Notably, Th2-type immune responses are composed of three major features: inflammation, wound repair, and, most importantly, resistance to helminths.

Parasites have developed various strategies to modulate the immune system and ultimately suppress host protective Th2-type immune responses for example, by induction of innate and adaptive regulatory cells, anti-inflammatory cytokines and specific inhibitory antibody isotypes (reviewed by Anthony et al. [2]).

Hence, helminth parasites are master regulators of immune responses in order to ensure life-long persistence in the host. One strategy of immune regulation that has evolved is the secretion of a wide range of immunoregulatory molecules, which are able to target various host cells and alter them to induce a highly directed host response known as a "modified Th2-type response." This response is designed to limit a possibly detrimental Th2 immune response, thus restraining the extreme symptoms that are often observed in allergy or in aspects of helminth diseases such as fibrosis in *Schistosoma mansoni* [3]. In immunological terms, the modified Th2 response is defined by the development of specific antibody isotypes, including induction of IgG4 accompanied by a decrease in IgE, as well as IL-4 and IL-5, while IL-10 levels from different regulatory cell sources increase [5]. These mechanisms can lead to attenuation of pathology and clinical symptoms, tolerance, and ultimately persistence of the worm, which is associated with a hyporesponsive immune system. Asymptomatic infection assures long-term survival of the parasite within the host and therefore sustains parasite feeding, completion of the life cycle, and successful reproduction [3, 5].

Many studies of animal and human helminth infections have shown their potential for downregulating the immune system. Moreover, relevant epidemiological studies have observed that helminth-infected populations exhibit lower levels of immunopathological diseases such as Th1-related autoimmune diseases or abberrant Th2-related diseases for example, asthma or allergic rhinitis. These observations indicate an inverse global distribution of allergy/autoimmune diseases and helminth infections, the first being an expanding problem of developed and industrialized countries, while the latter being a feature in developing countries [6–9]. Such findings support the hypothesis that the immune system has coevolved to operate in the presence of immunomodulatory helminth infections, while in the absence of exposure to helminths, the immunoregulatory components that would normally prevent allergy and autoimmune disease become weakened [5, 10].

Here, we focus on recent advances in cellular mechanisms that are employed and modulated during helminth infections as well as on reports from field research and studies on animal models. These studies have identified helminths and helminth-derived products that play a role either in induction of Th2 responses and immune modulation in parasitic infections (reviewed in Table 1) or in downregulation of bystander Th2-type immunopathologies like allergic asthma, allergic inflammation and airway hyperreactivity, food allergy, eczema, atopic dermatitis (Th1/Th2), or anaphylaxis (reviewed in Table 2).

2. Epidemiological Evidence of Helminth-Induced Immunoregulation

2.1. Immunoregulation during Helminth Infection in Humans. It has long been known that helminths induce a specific immune phenotype in the majority of persons that allows for establishment of infection while simultaneously preventing or reducing signs of disease in the host [11]. Antigen-specific cellular hyporesponsiveness was described for filarial infections more than 30 years ago by Ottesen et al. studying a population in the Cook Islands endemic for lymphatic filariasis. In this study lymphocytes from adults infected with the filarial species *Wuchereria bancrofti* showed significantly lower levels of proliferation in response to filarial antigen compared with endemic controls who were negative for all signs of infection or disease but constantly exposed to infection and, therefore, putatively consistently exposed to the antigens [12]. Later, another study from the same group made the distinction between microfilariae (mf) positive asymptomatically infected persons and mf negative patients showing clinical symptoms of filariasis (e.g., elephantiasis or hydrocoele) [13]. The resulting data suggested that the outcome of disease depends on the host response together with a mechanism of immune modulation induced by the parasite (reviewed by Ottesen [14]). Epidemiological studies of helminth-infected persons recognise distinct clinical outcomes that depend on immune regulation induced by the parasite in conjunction with the genetic background of the host (reviewed by Maizels and Yazdanbakhsh [3]). Resistant individuals are constantly exposed to the parasite but show no signs of infection or disease; this group develops an appropriate response, defined by equal proportions of Th1, Th2, and T regulatory (Treg) cells with a balance of IgG4 and IgE levels. A second group develops a hyporesponsive phenotype characterised by asymptomatic infection, which tolerates the presence of fecund adult worms. This group has high levels of regulatory cells and IL-10, leading to a modified Th2 response. Finally, a small proportion of patients develops a hyperresponsive phenotype (characterised by an immunopathological response) [3, 11, 14]. In *S. mansoni*, *W. bancrofti*, and *Brugia malayi* infections, the main pathological response is a result of overreactive T cell responses that cause inflammation and injure the host. This group exhibits increased IgE responses, and the Treg compartment is greatly diminished. In *W. bancrofti* and *B. malayi* infections, this can result in elephantiasis, whereby the lymphatic tissue becomes dilated and hypertrophic. Parasite death leads to the release of antigenic material that causes lymphatic obstruction in the vessels and chronic inflammation [14]. A second, rare result of these infections is tropical pulmonary eosinophilia characterised by chronic lung obstruction, peripheral blood eosinophilia, and extremely elevated levels of IgE, greater than in elephantiasis [15]. In *Onchocerca volvulus* infection the rare chronic hyperreactive form, known as Sowda, is also defined by high levels of IgE [16]. This is accompanied by strong Th2 responses, including IL-4, IL-5, and IL-13. Thus a fine balance of different aspects of immunity is required to develop a response beneficial to the host.

Therefore, immune modulation acts in favour of persistent infection and continuous transmission while simultaneously enabling the host to tolerate infection by diminishing clinical symptoms. Coinfection studies of helminths with other diseases provide supporting evidence that helminths have great ability to modulate immune responses, some aspects of which may then affect bystander infections. In fact, protective inflammatory immune responses that typically develop in bacterial or intracellular parasitic infections can be downregulated when a coinfecting helminth is present, resulting in increased susceptibility in the host to the former infection. For example, individuals coinfected with latent tuberculosis (TB) and *W. bancrofti* showed lower levels of TB-protective proinflammatory cytokines in vitro than TB patients without filarial infection. This effect was reversed after treatment with microfilaricidal drugs [47]. Research focusing on individuals coinfected with multiple filarial species and *Plasmodium falciparum* demonstrated a modulation of the antimalarial immune response by helminths that increased anti-inflammatory responses [48]. After stimulation with malaria antigen, filarial-infected individuals demonstrated significantly lower levels of interferon (IFN)-γ, tumour necrosis factor (TNF)-α and IL-17A, and higher levels of IL-10 from $CD4^+$ T cells compared with filarial-uninfected individuals. Similarly, a study examining the effect of helminth infections on responses to oral cholera vaccine demonstrated that *Ascaris lumbricoides* infection was associated with lower cholera antigen-specific IL-2 cytokine responses [49]. These epidemiological studies demonstrate the ability of helminths to modulate immune responses to themselves as well as to concurring and unrelated infections.

Table 1: Helminth-derived immunomodulatory and Th2-inducing molecules. CXCL: chemokine ligand; DC: dendritic cell; Ig: immunoglobulin; GI: gastrointestinal; IFN-γ: interferon gamma; IL: interleukin; LPS: lipopolysaccharide; MAP: mitogen activated kinase; MHC: major histocompatibility complex; MIF: macrophage migration inhibitory factor; PBMC: peripheral blood mononuclear cell; STAT: signal transducer and activator of transcription; TGF: transforming growth factor; Th: T helper cell; TLR: Toll-like receptor; Treg: T regulatory cell.

Helminth species	Name	Molecule	Mechanisms of immune modulation during infection	Reference
Echinococcus granulosus	AgB	Antigen B	Reduces expression of costimulatory molecules on human DCs and induces Th2	[17]
Fasciola hepatica	FheCL1	Cysteine protease	Prevents release of macrophage inflammatory mediators by degrading TLR3	[18]
Schistosoma mansoni	Lyso-PS	Lyso-phosphatidylserine	Lyso-PS treated DCs induce IL-10$^+$ Treg through TLR2 and promote Th2 polarization	[19, 20]
	DsRNA	Double-stranded RNA	Triggers TLR3 to activate STAT1 and induces expression of type 1 IFNs in DCs	[21, 22]
	Omega-1	Ribonuclease	Reduces expression of costimulatory molecules and IL-12 in DCs and induces IL-4 and Foxp3 in CD4$^+$ cells	[23–25]
	IPSE/alpha-1	Glycoprotein	Induces IgE-dependent IL-4 production from basophils in vivo	[26]
	SmCKBP	Chemokine-binding protein	Suppresses neutrophil recruitment by inhibiting the mammalian chemokine CXCL8	[27]
Ascaris suum	PAS-1	200 kDa protein	Suppresses proinflammatory cytokines and neutrophil influx after exposure to LPS	[28]
Necator americanus	*Na*-ASP-2	High homology to C-C chemokines	Secreted by infective larvae, recruits neutrophils in vitro and in vivo	[29]
	Na-NES	Metalloprotease	Cleaves the eosinophil chemoattractant CCL11 and prevents its action in vitro and in vivo	[30]
Nippostrongylus brasiliensis	Acetylhydrolase	Acetylhydrolase	Inactivates mammalian platelet-activating factor, thus potentially regulating GI inflammation	[31]
Trichinella spiralis	*Ts*-MIF	MIF homologue	Inhibits migration of human PBMCs, similar to human MIF	[32]
Trichuris muris	43 kDa IFN-γ homologue	IFN-γ homologue	Binds to IFN-γ receptor in mice	[33]
Acanthocheilonema viteae	AvCystatin /Av17	Cysteine protease inhibitor	Downregulates T cell responses. Interferes with macrophage MAP kinase signaling pathways to induce IL-10	[34, 35]
	ES-62	Phosphorylcholine-containing glycoprotein	Reduces CD4$^+$ T cell proliferation and IL-2 production, inhibits IL-4 and IL-13 production Inhibits IL-12 production from macrophages after exposure to LPS in a TLR4-dependent manner Induces an anti-inflammatory phenotype in DCs	[36–39]
Brugia malayi	*Bm*-ALT-1, *Bm*-ALT-2	Abundant larval transcript proteins	Expression of *Bm*-ALT in recombinant *Leishmania mexicana* parasites diminishes IFN-γ-mediated killing and induces GATA-3 and SOCS-1 in these macrophages	[40]
	Bm-CPI-2	Cysteine protease inhibitor	Blocks antigen presentation via MHC class II by interfering with asparaginyl endopeptidase	[41, 42]
	Bm-TGH-2	Homologue of TGF-β	Binds to mammalian TGF-β receptors thus potentially influencing Treg differentiation	[43]
Litomosoides sigmodontis	*Ls*-cystatin	Cysteine protease inhibitor	Reduces antigen-specific proliferation of spleen cells	[44]
Onchocerca volvulus	Onchocystatin	Cysteine protease inhibitor	Suppresses antigen-specific proliferation of PBMCs, induces IL-10 from stimulated PBMCs Reduces expression of MHC class II molecules and CD86 on human monocytes	[45, 46]

Table 2: Effects of helminth infections and helminth-derived products/molecules on improvement of symptoms in allergy-related experimental animal models.

	Allergy model	Suppressive mechanism	Reference
Defined helminth molecules; (from helminth species)			
smCKBP; (*S. mansoni*)	Hapten-induced contact hypersensitivity	NA[3]	[27]
Sm22.6, PIII and Sm29; (*S. mansoni*)	OVA-induced AI[1]	$CD4^+Foxp3^+$ Treg	[65]
As-MIF; (*A. simplex*)	OVA-induced AI	IL-10, TGF-β, and Treg dependent	[66]
PAS-1; (*A. suum*)	APAS-3-induced AI	Possibly IL-10	[67]
	OVA-induced AHR[2]	$CD4^+CD25^+$ T cell-, $CD8^+$ T cell-dependent and IL-10/TGF-β-, IFN-γ-mediated	[68, 69]
Nippocystatin (NbCys); (*N. brasiliensis*)	OVA-induced AI	Cathepsin B and cathepsin L-dependent mechanism	[70]
AvCystatin (Av17); (*A. viteae*)	OVA-induced AHR	IL-10, macrophages, partially $CD4^+CD25^+$ T cells	[71]
ES-62; (*A. viteae*)	Oxazolone- (Oxa-) induced skin immediate hypersensitivity OVA-induced AHR	Mast cell mediated, TLR4-dependent	[72]
Helminth infection and undefined products			
S. mansoni infection; males and females + eggs laid in infection; males (no eggs)	Pen V-induced systemic anaphylaxis	IL-10-producing B cells	[73]
S. mansoni infection; males (no eggs)	OVA-induced AHR	B cell- and IL-10-dependent suppression of pulmonary eosinophil infiltration	[74]
S. mansoni infection; eggs	OVA-induced AI	Egg-treatment: $CD4^+CD25^+Foxp3^+$Treg	[75]
S. japonicum egg antigen (SEA)	OVA-induced AI	$CD4^+CD25^+$ Treg, possibly IL-10	[76]
A. suum adult worm extract (ASC)	OVA-induced AHR	NA	[77]
Heligmosomoides polygyrus infection	OVA-induced AHR	IL-10	[78]
	OVA-induced AHR HDM-induced AI	IL-10-independent, $CD4^+CD25^+$ T cells IL-10-independent Breg cells	[79, 80]
	Peanut extract-induced food allergy	IL-10	[81]
H. polygyrus excretory-secretory product (HES)	OVA-induced AI	HES-induced Treg cells	[82]
N. brasiliensis infection	OVA-induced AHR	IL-10 from unknown cell source	[83]
N. brasiliensis excretory-secretory product (NES)	OVA-induced AI	NA (IL-10 independent)	[84]
Strongyloides stercoralis infection	OVA-induced AI	NA	[85]
Strongyloides venezuelensis infection	OVA-induced AI	NA	[86]
T. spiralis infection	OVA-induced AI	Correlated with increased IL-10, TGF-β, $CD4^+CD25^+$ Treg	[87]
L. sigmodontis infection	OVA-induced AHR	TGF-β, $CD4^+CD25^+$ Treg	[88]

[1] (AI): airway inflammation, associated with inflammatory cell influx into the lungs and local Th2 cytokine production.
[2] (AHR): airway hyperreactivity, mainly described as lung bronchiole and smooth-muscle tissue remodelling leading to airflow obstruction, resulting in altered airway function. Chronic airway inflammation may lead to AHR, however, different immunopathological pathways govern the regulation of AI and AHR (discussed elsewhere [89–93]).
[3] NA data not available.

2.2. Inverse Correlation between Helminth Infections and Allergic Reactions. Eradication of helminth infections in industrialised countries in the past 30 years has had a great impact on the prevalence of diseases associated with inappropriate immune responses. The observed increase in the appearance of allergy-related diseases might be a result of this and altered hygienic measures in everyday life. One possible explanation is the hygiene hypothesis [50, 51], which includes a number of factors like improved public health, use of antibiotics and vaccines that in consequence reduce the occurrence of viral, bacterial or helminth infections early in life, contributing to higher numbers of individuals with allergic, and/or autoimmune disorders.

There are numerous cohort studies determining allergic disease status and immune responses to parasite-specific antigens or environmental allergens of helminth-infected

individuals in parasite-endemic areas (reviewed by others [6, 8]). However, when interpreting this data it is important to take into consideration the time of first infection, duration and intensity of infection, helminth species, as well as host genetic and environmental factors.

Infections with trematodes, whipworms and hookworms were described to be negatively correlated with the allergen skin prick test [52]. Results from a study of Gabonese school children tested for skin reaction to house dust mites (HDMs) and other allergens, for *Schistosoma* eggs in urine and microfilariae of *Loa loa* and *Mansonella perstans* in blood, showed lower prevalence of a positive skin test to HDMs in children with urinary schistosomiasis. Moreover, schistosome antigen-specific concentrations of IL-10 were significantly higher in infected children and concentrations of IL-10 were negatively associated with a positive skin test result [53].

Several population studies done in endemic areas of *Schistosoma* infections in Brazil [54, 55] and of *Ascaris*, *Trichuris*, and *Ancylostoma* in Ecuador [56] revealed a strong inverse association between helminth infections and immediate skin test reactivity to common environmental aeroallergens. Araujo et al. [54] and Cooper et al. [56] reported a negative association between very high levels of IgE antibodies and allergen skin test reactivity. It is possible that anti-helminth-IgE competes for the same high-affinity IgE receptors targeted by allergen-specific IgE [54]. Although other studies from Africa reported on a reverse association between hookworm infections and sensitization to HDM *Dermatophagoides pteronissinus* (Der p 1), they did not show a significant association between skin test sensitivity to HDMs, allergen-specific IgEs and between total serum IgE levels and helminth-specific IgEs [57, 58].

The contribution of helminth infections to the inverse correlation with allergy-related diseases was demonstrated in studies with anthelminthic treatment. Results from a randomized, controlled trial on the effect of repeated treatment with anthelminthics on a population of Gabonese school children showed a significant increase in the rate of developing skin sensitivity to HDMs. This was in part mediated by reductions of *Ascaris* and/or *Trichuris* infections, directly pointing to the importance of a presence of live worms to suppress allergic responses during infection and also preventing allergy later in life [59]. Lynch et al. also reported an effect of anthelminthic treatment on the allergic reactivity of children in the tropics in Venezuela. The authors showed that the effective elimination of intestinal helminth infection resulted in significantly decreased total serum IgE levels. However, immediate-hypersensitivity skin test reactivity and serum levels of specific IgE antibody against common allergens were significantly increased in treated children [60]. Also other studies reported that anthelminthic treatments increase allergen skin reactivity to environmental allergens [61, 62]. Rodrigues et al. reported that Brazilian children with heavy infection in early childhood with *Trichuris trichiura* had a lower prevalence of allergen skin test reactivity to a wide spectrum of common environmental allergens (HDMs, *Blomia tropicalis*, fungi, animal, and cockroach allergens) later in childhood, even when the helminth infection was no longer present [63]. Additionally, a population study in Taiwan showed that schoolchildren bearing pinworm *Enterobius* were diagnosed with lower prevalence of asthma and allergic rhinitis; moreover, these children had reported an infection of pinworms early on in life [64]. Thus, early exposures and infections with helminths may have a protective effect and suppress allergic inflammation later during life.

Additionally, host genetic factors may play a role in prevalence of helminth infections and allergies. Notably variants of genes involved in Th2 immune responses like *IL13* or *STAT6* were associated with increased risk of asthma and allergy [94–97]. Studies among African-Americans shed a light on the limited understanding we have on asthma genetics. Polymorphisms in candidate genes associated with asthma showed higher frequency among certain ethnic backgrounds that developed strong Th2 immune responses [6, 98, 99]. Thus, genes, which in the evolution of the immune system were initially responsible for induction of Th2-type immune responses and for parasite expulsion, now cause problems with allergy-related diseases when living in "sterile" and modern societies.

2.3. Helminth Infections that Promote Allergic Reactions. In contrast to population studies reporting a negative correlation between allergy and helminth infections as discussed above, various studies from South America, Europe, and Asia on *A. lumbricoides* infections and allergy reported increased rather than decreased prevalence of asthma [100–102]. For example, two cross-sectional surveys among schoolchildren in former East Germany revealed that children with low doses of helminthic antigens (low worm burden) but *Ascaris*-IgE seropositivity had higher levels of total IgE and higher prevalence of allergic rhinitis and asthma to inhalant allergens [101]. It was proposed that heavy parasitic infections might generate immune suppressive mechanisms, whereas mild worm exposure and low-level contact with helminths may enhance reactivity to environmental allergens or even potentiate the Th2 immune response that in some cases may promote allergic inflammation. As *Ascaris* infection has been well-documented as a risk factor for asthma (described elsewhere [103–106]), there are two main hypotheses why *Ascaris* promotes allergic reactions. The first relates to the specific pulmonary phase in the *Ascaris* life cycle that causes inflammation and eosinophilia, which results in IgE-mediated asthma with high total- and specific-IgE titres against larvae and adult worms [107]. The second hypothesis of increased allergy in *Ascaris* infection is explained by cross-reactivity of *Ascaris*-specific molecules with environmental allergens like HDMs or the storage mites *B. tropicalis* that induce allergen-specific IgEs leading to strong Th2-type responses and therefore contributing to overall pathogenesis [108]. Moreover, *Ascaris*-tropomyosin, a pan allergen, induced wheal and flare in skin prick tests and histamine release from basophils of asthmatics and nonasthmatics. These results suggest that *Ascaris*-tropomyosin in some allergic individuals potentiates the pathogenesis of asthma and other allergic diseases [109–111].

Interestingly, the promotion of atopy can also be observed in parasitic infections where the human is not the definitive host and, therefore, chronic infection is

not established, for example, *Toxocara* spp. [52, 112]. In addition, the nematode *Anisakis simplex*, regularly infecting marine mammals as definitive hosts but accidentally also leading to gastrointestinal (GI) infections in humans, who ingest raw fish, was described to cause allergic reactions. Allergy to *A. simplex* is being recognised as an occupational disease with a wide spectrum of allergic reactions (rhinitis, asthma urticaria, allergic contact dermatitis, and anaphylactic shock) [113–115]. As both parasites cause accidental and infrequent infections, in case of infection the immune system may be exposed to Th2-inducing events rather than to immunomodulatory mechanisms that occur in chronic infections. These may contribute to the immune pathology and lead to allergic reactions.

It is important to keep in mind that variations in epidemiological studies may have many confounding factors like types of parasitic infections, intensity and timing of the infection, other coinfections, first exposure to allergen as compared to parasitic infection, types of allergens, nutritional status of the patients, medication received, and genetic background.

3. Host Cells Targeted by Helminth Infections

Current research has focused particularly on identifying the cells targeted by parasitic immune modulation, and the exact helminth-derived molecules responsible for this. Immune modulation by the parasite occurs through production of specific parasite-derived molecules that target mammalian host immune cells and signaling pathways. During infection this is strictly dependent on live parasites as shown by the recovery of cellular hyporesponsiveness in patients treated with anthelminthic chemotherapy [116]. In addition, a study by Da'dara and Harn demonstrated that killing of schistosome parasites could reinstate immune responses to a bystander vaccine targeting human immunodeficiency virus-1, which were greatly diminished in the presence of the parasite [117]. Similarly, another study demonstrated that prior elimination of intestinal helminths in patients vaccinated with bacille Calmette-Guerin resulted in greater protective responses to the vaccine compared with patients who did not receive anthelminthic chemotherapy [118]. During active infection, live worms are thus normally essential to ensure constant release of immunomodulatory molecules. Both GI and tissue-dwelling helminths can establish long-term chronic infections and have many immunomodulatory molecules in common, which, despite the different anatomical locations target similar host cells employing comparable regulatory mechanisms. Thus, experimental models together with human studies have helped to elucidate the mechanisms and cell targets underlying parasite-induced immune modulation. In this section, we discuss some of the most common and relevant examples.

3.1. Dendritic Cells. As professional antigen presenting cells (APCs) required in priming the adaptive immune response, dendritic cells (DCs) are an essential driving force of Th2 induction in helminth infections and in allergy-related diseases. The field of research on DCs targeted by parasite-induced immune modulation is as vast as that on macrophages, and there is definitive evidence of a specific DC phenotype that develops in helminth infection, demonstrating modulation by the parasite to deviate host inflammatory responses. Several groups could show that in helminth infection, DCs mature only selectively or show impaired functions in terms of TLR responsiveness [119–122]. As DCs are the main messenger cells to communicate with T cells and initiate an immune response, interference with their functions represents a key mechanism for helminths to induce an environment conducive to their survival [123].

The downregulation of proinflammatory cytokines appears to be a frequent feature in helminth-mediated modulation of the Th2-type response [124]. Murine DCs stimulated with lipids from *S. mansoni* eggs matured to induce specifically Tregs that produced IL-10, reducing Th1 responses whilst producing a modulated Th2 response [19]. However, as reviewed by Carvalho et al. [124] DCs that have been previously exposed to helminth products can also effectively prime Th2 cells. *S. mansoni* soluble egg antigen (SEA) is a particularly strong inducer of Th2 responses, and importantly the glycoprotein omega-1 was identified as the main Th2-inducing component in SEA [23]. Human DCs exposed to *B. malayi* mf showed higher levels of apoptosis and decreased production of IL-12 and IL-10 [125]. In fact when human monocytes that were being differentiated to DCs in vitro were stimulated with *B. malayi* mf antigen, they produced significantly decreased levels of IL-12p40, IL-12p70, and IL-10 in response to bacterial adjuvant [121]. Massacand et al. also demonstrated how DCs exposed to excretory-secretory (ES) products from both *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus* had reduced expression of the proinflammatory cytokine IL-12/23p40 [126]. Similarly, Balic et al. reported that bone-marrow-derived DCs exposed to *N. brasiliensis* ES (NES) products actively matured and could induce a Th2 response when transferred into naïve mice [119]. High levels of IL-4, IL-5, and IL-10 were detected after reexposure to NES. In vitro, these DCs produced high levels of IL-6 and IL-12p40 but full production of IL-12p70 was blocked. Finally it was shown that murine lipopolysaccharide- (LPS-) or CpG-matured DCs exposed to ES products from *Taenia crassiceps* showed diminished responses to stimulation through TLRs 4 and 9. These DCs significantly decreased levels of proinflammatory cytokines, including IL-12p40 and IL-12p70, as well as TNF-α and IL-15. IL-10 was, however not affected in this study [127]. Thus, downregulation of proinflammatory cytokines seems to be a frequent mechanism in immune modulation by helminths.

Combined with suppression of proinflammatory cytokines, a key aspect in modulation of DCs is the downregulation of costimulatory molecules, leading to induction of a Th2 response [124]. Dowling et al. illustrated that stimulation of murine bone-marrow-derived DCs with *A. lumbricoides* pseudocoelomic body fluid (ABF) influenced DC maturation by inducing expression of IL-6, IL-12p40, and macrophage inflammatory protein 2. However, DC maturation was only partial as levels of costimulatory molecules CD80, CD86, CD40, OX40L, and major histocompatibility

complex (MHC) class II were not affected. Furthermore, when DCs were cultured in the presence of ovalbumin (OVA) with ABF and then transferred into naïve mice, restimulation resulted in significantly increased levels of IL-4, IL-5, and IL-10 compared to DCs cultured without ABF [128]. Coculturing of ES products but not adult worm antigen from *H. polygyrus* with LPS-treated DCs induced a semimature phenotype with moderate expression of CD40, CD86, and MHC class II [120]. In fact when DCs were treated with ES and OVA and subsequently cultured with OVA-specific T cells, the T cells developed a regulatory phenotype, expressing CD25 and high levels of IL-10 [120]. Thus, targeted DCs could suppress both Th1 and Th2 responses. Furthermore, a novel subset of DCs expressing low levels of CD11c have also recently been identified in mice chronically infected with *H. polygyrus* [129]. In this infection setting, DCs acted as efficient APCs and induced high numbers of $CD4^+$ T cells expressing Foxp3 but only low numbers of $CD4^+$ effector T cells. This population of DCs was suggested to be one of the main target cells involved in inducing an immunoregulatory environment in *H. polygyrus* infection, allowing the establishment of chronic infection [129]. Nevertheless, DCs are clearly essential for efficient priming of the Th2 response, as depletion of $CD11c^+$ cells during development of the adaptive $CD4^+$ T cell response in *S. mansoni* infection drastically impaired the Th2 response [130].

3.2. Alternatively Activated Macrophages. Macrophages that are activated by the Th2-type cytokines IL-4 and IL-13 develop an alternatively activated phenotype and have a well-described role in helminth infections (reviewed by Hoerauf et al. [131]). Alternatively activated macrophages (AAMs) are recruited in large numbers to the sites of helminth infection where they can proliferate, as recently shown [4]. AAMs are characterised in mice by expression of specific markers, including arginase-1, resistin-like molecule (RELM)-α (also known as found in inflammatory zone, FIZZ1), Ym-1, Ym-2, acidic mammalian chitinase (AMCase), and mannose receptor C type (MRC)-1 [132]. AAMs are important in tissue homeostasis, downregulation of the adaptive immune system, acting as effector cells against parasites, and to reduce or heal any ensuing damage caused by infection [133]. In particular, AAM-derived arginase-1 is important for wound healing [134]. AAMs recruited during *B. malayi* infection have been demonstrated to drive $CD4^+$ Th2 responses, deviating the immune system from inducing a proinflammatory Th1 response that could be detrimental to parasite survival [135]. Monocytes from *B. malayi* asymptomatically infected patients have also been described to show an alternatively activated phenotype, expressing the AAM-specific markers arginase-1, MRC-1, resistin, and CCL18 as well as the downregulatory cytokines transforming growth factor (TGF)-β and IL-10 [136] (similar to what has been described for mouse AAMs [137]). However, translation of characterised markers into the human system in general should be done with caution, as reports indicate arginase-1 may not be a reliable marker for AAMs in humans as it is found in other cell types [138]. Furthermore, human monocytes do not express arginase-1 after stimulation with IL-4 and IL-13, unlike mouse macrophages (reviewed by Raes et al. [139]). This indicates that the translation from murine to human data can be difficult, and reliable markers for alternative activation in human monocytes should be further investigated.

The importance of AAMs is clear from numerous mouse models. Macrophages from *B. malayi* infected mice were shown to be fully capable of processing and presenting antigen and providing costimulation for T cells, however the resulting antigen-specific T cell population remained suboptimal with cell proliferation but not Th2 cell cytokine production being impaired [140]. These macrophages were IL-4-dependent and required direct contact with T cells to induce hyporesponsiveness [141]. In a mouse model of *S. mansoni* infection, arginase-expressing macrophages played an essential role in host protection by suppressing expression of the classical inflammatory cytokines IL-12 and IL-23 [142]. Removal of these cells resulted in an accumulation of parasite eggs in the intestines and intestinal haemorrhage, thus preventing the eggs from being excreted. Slightly different effector functions of AAMs come into play in GI helminth infections. Large numbers of AAMs were shown to accumulate in *N. brasiliensis* infection in the gut, characterised by expression of arginase-1, RELM-α and Ym-1, in an IL-4- and IL-13-dependent manner [143]. Macrophage depletion resulted in impaired worm expulsion from the small intestine and prevented smooth muscle hypercontractility.

RELM-α in particular has a clear immunomodulatory role during infection. RELM-α was shown to be essential in regulating lung inflammation, granuloma size and fibrosis in mice challenged with *S. mansoni* eggs [144]. Additionally, RELM-α deficient mice could not control Th2 cell differentiation or cytokine production. Moreover, Pesce et al. illustrated that challenge of RELM-α deficient mice with *S. mansoni* eggs resulted in elevated eosinophil levels and IgE titres. In the same study RELM-α deficient mice challenged with *N. brasiliensis* infection produced increased Th2 responses, manifested by elevated lung pathology and expulsion of adult worms. Importantly, addition of exogenous RELM-α reversed the pathological Th2 responses in this infection [145]. This represents RELM-α as a key host-derived molecule induced by helminth infection to allow prolonged parasite survival. These studies point at a host protective role for AAMs in specific tissue dwelling and GI helminth infections.

3.3. Regulatory T Cells. Among the cell types targeted during helminth infections, $CD4^+$ Tregs are particularly implicated in controlling innate and adaptive immunity. Natural $CD4^+$ Tregs develop in the thymus and express the transcription factor Foxp3, the IL-2 receptor α chain (CD25), CTLA-4, and a range of immunosuppressive proteins including IL-10 and TGF-β [146]. Additionally, other adaptive $CD25^+$ Treg populations exist in the periphery that develop from non-regulatory T cell subsets, with their expression of IL-10 and TGF-β distinguishing them from activated $CD4^+CD25^+$ nonregulatory T cells. Tregs are important in reducing

pathology in the host via suppression of both Th1 and Th2 responses, thus preventing disease symptoms as demonstrated by evidence of Treg involvement in both Th2-mediated allergies and Th1-mediated autoimmunity (reviewed by Else [147]). In a murine model of schistosomiasis $CD4^+CD25^+$ T cells expressing high quantities of IL-10 were shown to play a significant role in reducing immunopathology, especially in the chronic stage of infection [148]. McKee and Pearce illustrated that in *S. mansoni*-infected mice $CD4^+CD25^+Foxp3^+$ cells produced significant levels of IL-10 that were required to prevent DC-derived IL-12, thereby suppressing Th1 responses [149]. In fact, isolated schistosome eggs were demonstrated to be capable of modulating Th2 responses, without the need for the former life cycle stages that develop during infection. Schistosome eggs injected into mice were shown to induce a population of $Foxp3^+$ cells that expanded to control the $CD4^+$ T cell response, preventing inflammatory Th1 responses while modulating Th2 responses to prevent Th2-mediated immunopathology [150]. Both natural and adaptive Tregs have been described in filarial-infected persons, with the adaptive Treg population producing high levels of IL-10 [151]. Our group has shown that in *H. polygyrus* infection, $Foxp3^+$ Tregs are a requirement for limiting immunopathology and represent a potential source of IL-10 [152, 153]. Thus, while Treg depletion did not affect worm burdens, the Th2 response was greatly accelerated and augmented, with high levels of IL-4 and IL-13 being produced. The uncontrolled Th2 response led to increased immunopathology in the intestine, demonstrating that Tregs were essential in regulating this response [153]. $Foxp3^+$ Tregs were also induced after stimulation with *H. polygyrus* ES products, reducing proliferation of effector cells by activating the TGF-β signaling pathway [82]. During infection, Tregs may therefore be seen as important effector cells required to prevent or reduce pathology in the host by modulating the ensuing Th2 response, thereby simultaneously allowing establishment of chronic infection.

3.4. B Cells. Host protection as well as regulation by antibodies and B cells is being recognised as an essential component in Th2 responses in helminth infections [154]. In *S. mansoni*-infected mice, where the dominant isotypes are IgG1 and IgE [154], blockade of B cell production resulted in high levels of the proinflammatory cytokines IFN-γ and IL-12 but low levels of the Th2 cytokines IL-4 and IL-10 in acute infection [155]. Moreover mice deficient in B cells could not downregulate granuloma formation in the chronic stage of infection, and this mechanism was mediated by FcγR, indicating a role for antibodies in down modulation of pathology [156]. In fact, antibody isotypes are demonstrated to have an important role in determining the outcome of helminth infection in the host. The cytokines IL-4 and IL-13 act on B cells to induce both IgG1 and IgE in mice and IgG4 and IgE in humans. High levels of IgG4 but low levels of IgE are found in the blood of hyporesponsive, asymptomatic persons infected with *B. malayi*, *W. bancrofti,* and *O. volvulus* [16, 157]. IgG4 correlates with high levels of IL-10 and the presence of adult worms in hyporesponsive persons. In Bancroftian filariasis, high levels of IgG4 [158] but low levels of IgE [159] were found in mf positive individuals compared to patients with clinical disease (elephantiasis or tropical pulmonary eosinophilia). IgE is known to activate degranulation of mast cells (MCs), basophils and eosinophils and induce antibody-dependent cell-mediated cytoxicity (ADCC) [154, 160]. IgG1 in mice and IgG4 in humans compete with IgE for binding sites and therefore may inhibit such processes. In fact, IgG4 has downstream suppressive effects, such as inhibiting complement activation [161]. Thus, inhibitory IgG4 may prevent immunopathological responses in helminth asymptomatically infected individuals and can simultaneously provide an indication of the clinical outcome in infected persons.

Regulatory B cells have been first described in autoimmune diseases, where the main mediator of suppression is IL-10 [154]. Helminth infections can also induce specific B cell phenotypes with regulatory properties as shown in infection with *S. mansoni* and *H. polygyrus* [162]. In *S. mansoni* infection, a particular subset of B cells has been described that are $CD1d^{high}$ and express high levels of IL-10 (defined as $CD19^+IL\text{-}10^+CD1d^{high}CD5^+CD21^{high}CD23^+IgD^+IgM^{high}$ cells). Transfer of these cells into OVA-sensitized mice reduced OVA-induced allergic airway inflammation via induction of $Foxp3^+$ Tregs; the effect was TGF-β independent as showed in anti-TGF-β mAb treatment experiments [163]. Furthermore, another group demonstrated that transfer of IL-10-producing B cells from IL-4 deficient mice infected with *S. mansoni* provided complete resistance to experimentally induced anaphylaxis when applied to naïve mice. Thus, absence of IL-4 was essential to prevent exacerbation of this allergic response [73]. In another study, the same group proved that the protective response of *S. mansoni* infection was entirely dependent on B cells as depletion of IgM^+ B cells resulted in mice becoming completely susceptible to anaphylaxis [74]. Interestingly, adoptive transfer of a population of $CD19^+CD23^{high}$ B cells from *H. polygyrus*-infected mice could reduce the effects of allergic airway inflammation of OVA-sensitized mice, however, in an IL-10 independent manner. These mesenteric lymph node B cells reduced secretion of IL-5 and infiltration of eosinophils into the airways, suppressing allergen-induced pathology [80]. Thus, B cells from helminth infections exhibit regulatory capacity in unrelated diseases and are of therapeutic interest in allergies, therefore their phenotype and functional aspects should be further investigated.

4. Immunomodulatory Effects of Helminth-Derived Molecules and Their Role in Modulation of Allergy-Related Diseases

There are numerous studies examining the effects of different helminth infections (e.g., *S. mansoni* [73–75], *H. polygyrus* [78, 81, 164], *N. brasiliensis* [83], *Trichinella spiralis* [87], and *Litomosoides sigmodontis* [88]) on allergy-related diseases in experimental animal models, where helminths show the capacity to suppress abberrant Th2 immune responses. In addition, ES products either from *H. polygyrus* [82] or

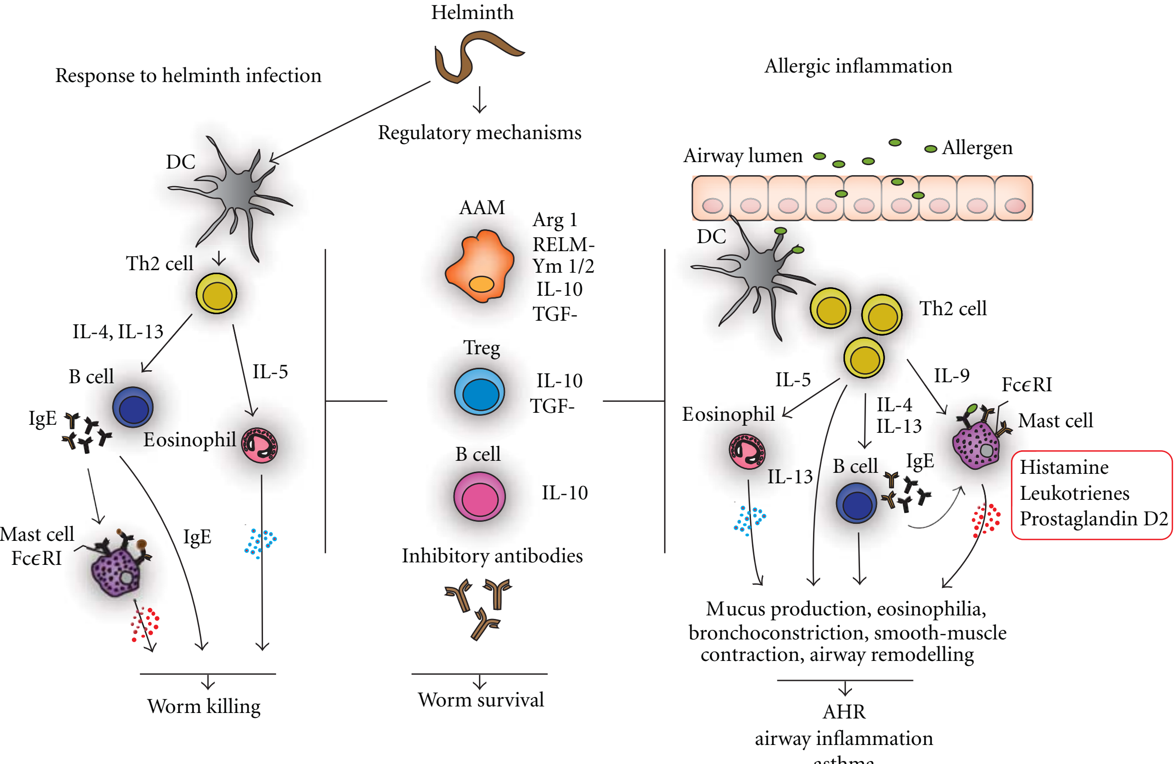

Figure 1: Regulatory and effector mechanisms of Th2-type responses. *Th2 immune response in helminth infection*: helminth infection induces a protective Th2 immune response. Professional antigen presenting cells such as dendritic cells (DCs) process helminth antigens and display them to $CD4^+$ T cells that differentiate into polarised T helper 2 cells. Th2 cells produce cytokines such as IL-4, -5, and -13 that activate and attract macrophages, eosinophils, and other innate immune cells as well as B cells. IL-4 and -13 induce differentiation of antigen-specific B cells and production of large amounts of antibodies (characteristically IgE). Antibodies opsonise the helminths leading to killing via eosinophils or neutrophils, as well as macrophages by antibody-dependent cellular toxicity (ADCC). IgEs bind to Fcε-receptors (FcεRI) on mast cells (MCs). Sensitized MCs secrete large amounts of histamine and other mediators and facilitate the attraction and accumulation of further immune cells, which result in larvae killing. *Regulatory mechanisms in helminth infection:* Helminths induce immunoregulation via modulation of immune cells leading to alternatively activated macrophages (AAMs), regulatory T cells (Treg), and B cells. AAMs in mice express among others arginase-1 (Arg 1), resistin-like molecule-α (RELM-α), Ym-1, Ym-2, IL-10, and TGF-β and contribute to wound healing. Treg produce IL-10 and transforming growth factor-β (TGF-β), whereas B cells can elicit regulatory mechanisms via IL-10. These cellular changes lead to modified Th2 immune responses and larvae survival as well as blocking of unrelated inflammation such as allergic immune responses. *Allergic inflammation:* DCs present allergen-derived peptides to T cells. Stimulation of allergen-specific T cells results in differentiation of $CD4^+$ T cells into Th2 cells that produce Th2 cytokines. IL-4 directs T cell differentiation towards a Th2-related phenotype. IL-5 regulates the recruitment and differentiation of eosinophils and stimulates them to release mediators. Additionally, IL-4 and -13 induce Ig-class switching to IgE by B cells. IgEs bind to high affinity FcεRI on MCs. Sensitized, IL-9-activated, and maturated MCs degranulate and release preformed mediators, including histamine, leukotrienes, and prostaglandins. This allergic inflammatory cascade leads to increased mucus production, eosinophilia, bronchoconstriction, smooth-muscle contraction, and remodelling in the lungs, which result in allergic-airway hyperreactivity (AHR), airway inflammation or asthma. The allergic inflammation may be suppressed by a spill-over effect of immunomodulatory mechanisms of helminth infections.

N. brasiliensis [84] were described to have similar beneficial immunoregulatory effects. Furthermore, helminths act through various cell types and can interfere with allergy symptoms in animal models via distinct mechanisms including Tregs [79, 82, 88], B cells [73, 80, 163], and induction of regulatory cytokines like IL-10 [78, 81, 83, 87] or TGF-β [87, 88, 163], as discussed in the previous section and reviewed in Table 2.

These and other studies led to the discovery of specific immunomodulatory helminth-derived molecules and products that induce a microenvironment beneficial to the parasite, while at the same time preventing immune-related pathology associated with vigorous Th2 responses (Figure 1). However, as mentioned earlier, persistence of the parasite is not obligatory to reduce allergic symptoms in later life [63]. Moreover, these functionally and structurally diverse molecules are expressed throughout the parasite life cycle and interact directly with host cells. A summary of helminth-derived immunomodulatory molecules and products is given in Table 1. We focus here on molecules that are relevant for

human disease, established animal models of host-parasite interactions, and defined molecules that downregulate unrelated Th2 inflammation.

4.1. Homologues of Mammalian Cytokines and Chemokines. The existence of parasite-derived homologues of host mammalian anti-inflammatory cytokines reveals an apparent evolutionary struggle between the parasite and the host, whereby the parasite has evolved mechanisms to establish chronic infection. For example, two TGF-β homologues found in *Brugia* species, *Bm-tgh-1* and *Bm-tgh-2*, have been well characterised, the second of which is thought to have an immunomodulatory role. TGH-2 is secreted by adult worms and in its recombinant form was shown to bind the human TGF-β receptor, thus potentially influencing Treg development [43, 165]. *S. mansoni* male worms express a member of the TGF-β receptor family known as SmRK-1 [166] for which mammalian TGF-β may be a ligand involved in worm development [167]. *H. polygyrus* ES contains remarkable TGF-β-like activity, inducing Foxp3 expression in naïve T cells and modulating immune functions, thereby maintaining worm burdens to induce chronic infection [82].

The beneficial role of homologues of mammalian chemokines such as macrophage migration inhibitory factor (MIF) is less apparent. MIF homologues have been described in multiple helminth species (reviewed elsewhere [168, 169]). Mammalian MIF has numerous functions, in particular as a proinflammatory cytokine [169]. Helminth MIF has direct chemotactic effects on human monocytes but appears to be associated with anti-inflammatory, modified Th2-type responses [170]. In a murine model of asthma, a recombinant MIF-like protein from *A. simplex* (*As*-MIF) was described to have a protective effect and to suppress the Th2-type response, reduce production of IL-4, -5 and -13 in the bronchoalveolar lavage fluid (BAL), inhibit eosinophilia, goblet cell hyperplasia, and ameliorate lung hyperreactivity. The effect of *As*-MIF was possibly mediated by IL-10 and TGF-β production in the BAL as well as via Treg induction in the lungs of *As*-MIF-treated animals when compared to asthmatic controls [66].

Another helminth-derived molecule with structural similarities to mammalian chemokines is *Ancylostoma*-secreted protein-2 (*Na*-ASP-2) secreted by the infective larvae of *Necator americanus* hookworm. Recombinant *Na*-ASP-2 was shown to recruit high numbers of neutrophils when injected into an air pouch (a sterile inflammatory setting) in mice. The influx of neutrophils was suggested to create permeability in host vessels, thus facilitating larval migration [29]. Hence, cytokine homologues seem to be exploited by the parasite for efficient immune evasion; however more work is needed to assess which of those molecules participate in suppression of unrelated inflammation.

4.2. Protease Inhibitors. The cystatins and serpins are the best-characterised protease inhibitors of helminths that have immunomodulatory potential. Mammalian cysteine proteases are essential for efficient processing and presentation of antigen on MHC class II to induce an appropriate adaptive T cell response. Mammalian cystatins play a vital role in regulating these pathways; however, helminth cystatins from *Acanthocheilonema viteae*, *B. malayi*, *N. brasiliensis*, and *O. volvulus* have been shown to interfere with this process to dampen antigen-dependent immune reactions (reviewed by Klotz et al. [171]). *Bm*-CPI-2, a cystatin from *B. malayi*, was illustrated to interfere with antigen processing, which led to a reduced number of epitopes presented to T cells in vitro [41]. Studies from our own group have demonstrated that onchocystatin (rOv17) from *O. Volvulus* reduced antigen-driven proliferation of peripheral blood mononuclear cells in a monocyte-dependent manner [45]. Recombinant AvCystatin (rAv17) from *A. viteae* has potent immunomodulatory roles illustrated by its ability to reduce antigen-specific and unspecific T cell responses [34]. AvCystatin is recognised by macrophages and upon uptake induces phosphorylation of the mitogen-activated protein kinase signaling pathways ERK1/2 and p38 in macrophages. This led to tyrosine kinase-dependent IL-10 production in macrophages [34, 35]. Furthermore, we could show in an OVA-induced airway hyperreactivity mouse model that AvCystatin-treated asthmatic mice exhibited amelioration of the disease. AvCystatin administered intraperitoneally (i.p.) during the sensitization phase as well as before challenge with OVA, suppressed recruitment of eosinophils into the lungs, OVA-specific and total IgE levels, and reduced allergen-specific IL-4 production. IL-10 is a key element in AvCystatin-induced immunomodulation, as blocking of IL-10 with an anti-IL-10R mAb reversed the beneficial effect on cell recruitment and production of IgE [71]. Moreover, depletion of macrophages with clodronate liposomes before airway allergen challenge diminished the antiallergic effect of AvCystatin [71].

The rodent filarial species *L. sigmodontis* secretes a cystatin at various stages of the life cycle, which after injection via microosmotic pumps into the peritoneal cavity of *L. sigmodontis*-infected mice greatly decreased nitric oxide production and proliferation of antigen-specific spleen cells [44].

Similarly, recombinant cystatin from the ES product of the GI nematode *N. brasiliensis* (named nippocystatin, NbCys) was shown to inhibit cathepsins L and B, and suppressed antigen processing by APCs [172]. Furthermore, OVA-sensitized and NbCys-treated mice showed decreased OVA-specific spleen cell proliferation, reduced OVA-specific IgE levels and cytokine production due to inhibition of cathepsin B and L-dependent antigen processing [70].

Similar to cystatins, serpins (serine protease inhibitors) have important roles in mammalian biological processes including regulation of complement activation, inflammatory pathways, and cell interactions. *Bm*-SPN-2 is a serpin expressed by *B. malayi* microfilariae, which could inhibit proteases of human neutrophils, thereby interfering with and potentially circumventing the most abundant leukocyte to encounter mf in the bloodstream [173]. However, another study by Stanley and Stein contests the enzymatic activity of *Bm*-SPN-2 [174]. Thus, further studies should clarify the role of serpins as immunomodulatory molecules. Taken together, protease inhibitors such as cystatins are a class of

molecules found in numerous helminths that have important immunomodulatory functions.

4.3. ES-62. A secreted 62 kDa glycoprotein from *A. viteae* known as ES-62 is one of the best-characterised helminth immunomodulators. ES-62 has been shown to exhibit a plethora of well-documented anti-inflammatory properties [175] and contains phosphorylcholine moieties, which are largely responsible for immunomodulation (reviewed by Harnett et al. [176]). OVA-specific $CD4^+$ T cells exhibited lower levels of proliferation and IL-2 production after challenge with ES-62, as well as inhibition of IL-4 and IL-13 [36]. It was also shown that ES-62 acts on macrophages to inhibit production of IL-12 if the cells were subsequently exposed to LPS and IFN-γ [37, 38]. ES-62 could also be shown to act on bone-marrow-derived precursors of DCs to inhibit a proinflammatory response induced by LPS [39] and B cells by modulating T and B cell interactions [177]. ES-62 is also active against FcεRI-mediated MC responses in vitro. It directly inhibited FcεRI-induced release of allergy mediators from human MCs by selectively blocking key signal transduction events. ES-62 also interfered with MCs in vivo in a mouse model of immediate-type hypersensitivity to oxazolone, thereby diminishing ear swelling and MC-dependent hypersensitivity, as well as ex vivo MC degranulation in mice that were previously injected with the molecule. Moreover, ES-62 ameliorated OVA-induced murine airway inflammation, airway hyperresponsiveness, lung pathology, and eosinophilia by suppression of MC function [72]. Thus, ES-62 is likely to be a key mediator of filarial-induced immunoregulation, and its properties might be used to dampen overwhelming unrelated inflammation in the future.

4.4. Schistosome-Derived Antigens. Schistosome soluble egg antigen (SEA) and ES products released by the egg stage of the parasite contain potent Th2-inducing and immunomodulatory activity. SEA from *S. mansoni* was shown to be an extremely strong inducer of Th2 responses without the need for live infection or the addition of adjuvant [178]. Recently it was shown that omega-1, a hepatotoxic ribonuclease, is one of the key players in this response [23, 24]. Omega-1 is a glycoprotein, which was demonstrated to polarise human monocyte-derived and $CD11c^+$ murine DCs in a direction supporting Th2 responses even in the presence of LPS. In fact, SEA depleted of omega-1 was not able to sufficiently induce a Th2 response in vitro [24]. However, the Th2-suppressive actions of schistosome-derived antigens clearly highlight the ability of this helminth to modulate host immune responses. ES- or omega-1-treated DCs display the typical modulated phenotype that is critical for induction of a Th2 response, including reduced expression of costimulatory molecules and a lowered efficiency in participating in DC-T cell conjugates [23]. In fact, this study demonstrated that omega-1 was shown to alter the morphology of DCs, possibly preventing T cell activation. IPSE/alpha-1 is another glycoprotein present in SEA and abundant in ES, which could induce IL-4 production from human basophils in an IgE-dependent but antigen-independent mechanism [179]. *S. mansoni* also expresses glycans that have been shown to exhibit immunomodulatory functions. Okano et al. demonstrated that glycan-containing SEA could induce high levels of IL-5, IgE production, and eosinophilia when administered intranasally into mice [178]. The same group was subsequently able to show that *S. mansoni* SEA contained the carbohydrate lacto-N-fucopentaose III (LNFPIII), which induced high levels of IL-4, IL-5, and IL-10 by nasal lymphocytes bypassing the need for an adjuvant [180]. DCs are also targeted by lyso-phosphatidylserine (Lyso-PS) present in SEA, to dampen the Th2 response. Lyso-PS was shown to act on DCs via the TLR2 pathway, endowing them with the ability to induce IL-10-producing Treg cells [19].

Whole SEA, as well as SEA-derived molecules, has been described to interfere with animal models of allergy-related diseases. Yang et al. used *S. japonicum* SEA to test its effect in experimentally induced asthma in mice. SEA increased IL-10 production and expression of Foxp3 on $CD4^+CD25^+$ T cells, both with immunosuppressive activity, while at the same time decreasing the expression of Th2 (IL-4 and IL-5) cytokines, suppressed antigen-induced airway inflammation, recruitment of inflammatory cells into the lungs, and development of asthma [76]. *S. mansoni* egg-secreted chemokine-binding protein (smCKBP) was reported to significantly diminish ear swelling of hapten-sensitized mice in a contact hypersensitivity model. Isolation of the inflammatory infiltrate from hapten-treated ears of smCKBP-treated mice showed reduction of neutrophilia when compared to cells isolated from the ears of control animals. Smith et al. showed that smCKBP has specific in vivo activity, suppressing immediate or local inflammation [27]. Additionally, three *S. mansoni* antigens Sm22.6 (soluble protein associated with the *S. mansoni* tegument), PIII (a multivalent antigen of *S. mansoni* adult worms), and Sm29 (a membrane bound glycoprotein on the *S. mansoni* adult worm tegument) were evaluated in the OVA-induced airway inflammation mouse model. All three antigens showed a beneficial immunomodulatory effect in this allergy model characterised by suppression of airway inflammation, reduced eosinophilia in the lungs, decreased OVA-specific IgE levels, and lower Th2-specific cytokine production in BAL when compared to asthmatic animals. Sm22.6 significantly induced higher levels of IL-10, while both Sm22.6 and PIII lead to increased expression of $CD4^+Foxp3^+$ T cells suggesting that Treg cells might be involved in the modulation of this aberrant Th2 inflammation [65].

4.5. Molecules of Ascaris suum . Although *Ascaris* infections are described to support allergic inflammation (Section 2.3), products from *A. suum* have also been described to contain immunomodulatory activity, in particular the 200 kDa protein PAS-1. Administration of PAS-1 to mice that had been injected with LPS into sterile air pouches resulted in a significant decrease in neutrophil migration as well as suppressing proinflammatory cytokines IL-6, TNF-α, and IL-1β [28]. In particular, significant effects of PAS-1 have been demonstrated in allergy. Itami et al. [67] used a murine model of asthma induced by another *A. suum*-molecule, APAS-3 that promotes IgE production [181], to test the

suppressive effect of PAS-1 [182]. In this experimental model of asthma induced by APAS-3, PAS-1-treated mice showed decreased eosinophil migration into the lungs, lower IL-4, IL-5, CCL11 and RANTES production in the BAL, as well as reduced airway hyperreactivity. Moreover, IL-10 production was considerably increased after PAS-1 treatment when compared to APAS-3-immunized controls. Thus, *A. suum* has allergenic components as well as suppressive products that downregulate allergic responses [67]. More recently, a study from the same group showed suppressive effect of PAS-1 also in OVA-induced airway inflammation. In line with previous findings, PAS-1 reduced eosinophilia, decreased production of Th2 associated cytokines in the BAL and OVA-specific IgE and IgG1 levels in serum. Moreover, the effect was observed in IL-12- but not in IL-10- and IFN-γ-deficient animals, thus IL-10 and IFN-γ played a role in the immunomodulatory effect of PAS-1 [68]. Furthermore, the authors reported that the immunomodulatory effect of PAS-1 was mediated by $CD4^+CD25^+$ and $CD8^+$ T cells as shown by adoptive cell-transfer into OVA-sensitized and challenged mice. Recipients of PAS-1-primed $CD4^+CD25^+$ T cells had increased levels of IL-10/TGF-β whereas mice with transferred PAS-1-primed $CD8^+$ T cells showed prominent IFN-γ production [69]. Those three cytokines were described to play important roles in the suppression of allergic diseases and reported to be strongly induced during successful allergen-specific immunotherapy in humans as reviewed by Holgate and Polosa [183].

5. Conclusion

Helminths have had millions of years to evolve regulatory mechanisms that circumvent the mammalian immune response and allow for the longevity characteristically observed during infection. Thus, understanding of this coevolutionary nature is important in order to comprehend the iimmunomodulatory potential of helminth-derived molecules. This will better allow the development of therapeutical targets in symptomatic disease in helminth infections and help to break transmission of infection in endemic countries. Furthermore, the genetic basis underlying differences in responses of different individuals to helminth infections should be examined to develop a full understanding of these parasites, and current studies are beginning to address this. In particular, relevant experimental models for filarial disease are required to study both asymptomatic and pathological responses.

The connection between helminth infections and allergy is of particular interest. The epidemiological data reported in this paper together with experimental studies clearly support the view of the immunomodulatory potential of helminth-derived products to interfere with and even prevent aberrant allergic inflammation. These interactions are summarised in Figure 1.

Research should focus on studying the immunomodulatory effects of helminth-derived products on allergy and strive to develop new therapeutic strategies by identifying the mechanisms and pathways utilised by such molecules in mediating their immunomodulatory effects.

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Research Article

New Method to Disaggregate and Analyze Single Isolated Helminthes Cells Using Flow Cytometry: Proof of Concept

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In parasitology, particularly in helminthes studies, several methods have been used to look for the expression of specific molecules, such as RT-PCR, western blot, 2D-electrophoresis, and microscopy, among others. However, these methods require homogenization of the whole helminth parasite, preventing evaluation of individual cells or specific cell types in a given parasite tissue or organ. Also, the extremely high interaction between helminthes and host cells (particularly immune cells) is an important point to be considered. It is really hard to obtain fresh parasites without host cell contamination. Then, it becomes crucial to determine that the analyzed proteins are exclusively from parasitic origin, and not a consequence of host cell contamination. Flow cytometry is a fluorescence-based technique used to evaluate the expression of extra-and intracellular proteins in different type cells, including protozoan parasites. It also allows the isolation and recovery of single-cell populations. Here, we describe a method to isolate and obtain purified helminthes cells.

1. Introduction

Flow cytometry is a technique that uses the principles of light scattering, light excitation, and fluorescence, to analyze size, complexity, molecular or cellular characteristics of single cells or particles by suspending them in a stream of fluid [1]. This technique is widely used to analyze the expression of both extracellular and intracellular proteins, secreted molecules and DNA-content, among others [2]. It is also used in basic and clinical research (immunology, hematology, molecular biology) and recently in other research areas such as microbiology, algae, and plant biology. Among the main advantages of flow cytometry are (1) the multiparametric analysis of mixed populations by identifying specific markers on these cells and (2) the separation of particular subpopulations by electrical or mechanical means to divert cells with measured characteristics within the range specified by the user [1].

Helminthes present a formidable challenge to the mammalian defense mechanisms. They are large, meaning that their size exceeds that one of the host defense cells by several orders of magnitude. Furthermore, the parasite and the host interphase is outside the cells, partially because most helminthes have a relatively impermeable cuticle composed of proteins that can be structurally arranged so they become "hard". It is unclear how the mammalian defense mechanisms could incapacitate these macroscopic animals. Furthermore, it is worth noting that tissue dwelling nematodes poses a completely different problem than the better studied gastrointestinal nematodes [3]. In the latter instances, live parasites can be successfully eliminated from within the mammal by such effectors as mucin of increased viscosity and enhanced peristaltic activity. Such mechanisms are not available in the case of tissue dwelling nematodes, which have to be killed and perhaps broken down and digested before they can be successfully eliminated. Thus, many host cells are

literally covering all helminth body, as well as inside the parasite. This characteristic of helminthes makes it really difficult to isolate and characaterize antigens, or proteins that belong exclusively to the parasite, without host interference or contamination. Thus, finding a method that is capable to render cells of only parasite origin is a challenge for researchers.

For the past years, in our laboratory, we have been trying out to describe the role of sex steroids on different helminthes parasites. Also, the specific receptors of the parasite able to respond to these molecules have been partially characterized [4, 5]. Previously, have been detected mRNA and protein, respectively, for steroid hormone receptors by using real-time reverse-transcriptase polymerase chain reaction (RT-PCR) and fluorescent microscopy methods in several parasites. However, as previously mentioned, due to the extremely high interaction between helminthes and immune or other host cell types, which may eventually lead to host cell invasion into several parasitic tissues, it is critical to determine that the detected and analyzed protein(s) are exclusively found in the helminth parasitic cells, and not as a consequence of host immune (or any other type) of cell contamination. Thus, it was crucial to develop a method that could sort out very specifically, and without any possible error, the cross-contamination between host and parasite cells. To date, the available methods to dissect single parasite cells are quite expensive and complicated (microscopic laser dissection, for instance). Here we describe a method to dissect different helminthes cells, and by using flow cytometry, we were able to determine that there was no host cell contamination and provide information that demonstrate that, these cells are from only helminthes cell population based on both proportions and size of cells, as well as microscopic characterization.

2. Materials and Methods

2.1. Ethics Statement. Animal care and experimentation practices at the Instituto de Investigaciones Biomédicas are frequently evaluated by the Institute's Animal Care and Use Committee, according to the official Mexican regulations (NOM-062-ZOO-1999). Mexican regulations are in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH and The Weatherall Report) of the USA, to ensure compliance with established international regulations and guidelines. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Instituto de Investigaciones Biomédicas (Permit Number: 2011–16). Mouse, rats, and pigs sacrifice to obtain parasites was performed under anesthesia, and all efforts were made to minimize animal suffering.

2.2. Harvesting and Preparing T. crassiceps and T. solium cysticerci, and Trichinella spiralis Larvae for Experimentation. *Taenia crassiceps* cysticerci for each experimental session were obtained from intraperitoneally infected female mice and placed in tubes containing sterile PBS (1x) supplemented with 100 U/mL of antibiotics-fungizone (Gibco, Grand Island). The tubes were centrifuged for 10 min at 1,500 rpm at 4°C and the supernatants were discarded. The packed cysticerci were incubated in DMEM serum-free medium (Sigma, St. Louis, Missouri). They were then centrifuged 3 times for 10 min at 1500 rpm for washing. After the final wash, the numbers of viable (complete, translucent and motile cystic structures) cysticerci were counted under a binocular microscope. Ten viable nonbudding cysticerci of approximately 2 mm in diameter were selected and dispensed in 24-well culture plates (Falcon, BD Labware, Franklin Lakes, New Jersey) in 1 mL DMEM serum-free medium (Gibco BRL) and maintained at 37°C under 5% CO_2 until used. *Taenia solium* cysticerci were dissected from the muscle of infected pigs in independent experiments. The fibrous capsule surrounding each parasite was carefully separated under a dissection microscope. Once dissected, cysticerci were placed in tubes containing sterile PBS (1x) supplemented with 100 U/mL of antibiotics-fungizone (Gibco, Grand Island, NY). The tubes were centrifuged for 10 min., at 800 g at 4°C, and the supernatant was discarded. The packed cysticerci were incubated in DMEM serum-free medium (Gibco, BRL, Rockville, MD). They were then centrifuged 3 times for 10 min at 800 g for washing. After the final wash, the numbers of viable (complete and translucent cystic structures) cysticerci were counted using a binocular microscope and placed in 24-well culture plates (Falcon, BD Labware, Franklin Lakes, New Jersey) in 1 mL DMEM serum-free medium (Gibco BRL) and maintained at 37°C under 5% CO_2 until used. *Trichinella spiralis* (ISS 406) was maintained in the laboratory by serial passage infections in BALB/c mice and/or Sprague-Dawley rats. The infective-stage muscular larvae (ML) were recovered from experimentally infected mice at 30 days p.i. by a standard pepsin-hydrochloric acid digestion method. Larvae were washed several times with PBS, and 4,000 ML were used to infect Sprague-Dawley rats. Adult parasites were recovered from 1 to 6 days p.i. from the small intestine. The NBL (24–72 h old) were obtained by incubating 4-, 5-, and 6-day-old adult parasites in RPMI medium (Gibco BRL) supplemented with 10% FBS (Hyclone) and antibiotics (Gentamicin 50 mg/L and Amphotericin B, Sigma, 2 .5 mg/L).

2.3. T. crassiceps, T. solium, and T. spiralis Cell Isolation. *T. crassiceps*, *T. solium* and *T. spiralis* cells were extracted by tissue disruption according to the following protocols. *T. crassiceps* parasites were macerated using a nylon mesh "sandwich" (150 mm. Small Parts) and a syringe plunger in 1 mL of RPMI media. Meshes were washed with media, and cell suspension was centrifuged at 300 ×g for 5 min, and cells in pellet were recovered in PBS. *T. solium* parasites were disaggregated initially by passing them through a 3 mL syringe to recover internal cells. Disrupted cysticerci were macerated as described for *T. crassiceps* cells. Cells recovered in RPMI media, centrifuged at 300 ×g for 5 min and pellets were recovered in PBS. *T. spiralis* larvae were disaggregated using a micropestle (Eppendorf, USA) until no more clumps were visible. Cells were centrifuged at 300 ×g for 5 min and cells in pellet recovered in PBS. Parasite cell viability was >80% for all three parasites.

2.4. Analysis of Host-Derived Cells in T. crassiceps and T. solium cysticerci, and T. spiralis Larvae Cells by Flow Cytometry. *T. crassiceps* and *T. spiralis* larvae cells were stained with the following antibodies for 10 min at 4°C: anti-mouse CD3-FITC, anti-mouse CD4 FITC, anti-mouse CD8-PE-Cy5, anti-mouse CD19-PE, anti-mouse Mac-1, and anti-mouse Mac-3 (all from BD Biosciences), and washed with 500 μL of staining buffer (PBS pH.7.4, 2% Fetal Bovine Serum, 0 .02% NaN_2). Cells were fixed in 2% paraformaldehyde solution and stored until analysis protected from light. *T. solium* cysticerci-derived cells were stained with anti-human MHC-I-biotin antibody, washed once, and stained with APC-coupled Streptavidin (BD Biosciences). All samples were analyzed by flow cytometry using an FACS Calibur (BD, Biosciences) and data analyzed using the FlowJo© software.

2.5. Specific Determination of T. crassiceps and T. solium cysticerci, and T. spiralis Larvae Cells by Flow Cytometry. Helmith-derived cells were fixed in 2% paraformaldehyde solution for 10 min at 37°C and centrifuged at 300 ×g for 5 min. Afterwards, they were incubated in absolute methanol for 30 min at 4°C and centrifuged at 300 ×g for 5 minutes and then washed twice with 500 μL of staining buffer (PBS pH.7.4, 2% Fetal Bovine Serum, 0 .02% NaN_2) and resuspended in same buffer (100 μL/test). *T. crassiceps* and *T. solium* cells were incubated in presence of mouse antiparamyosin (1 μg/test) (kindly provided by Dr. Pedro Ostoa, Instituto de Investigaciones Biomédicas, UNAM). *T. spiralis* cells were incubated in the presence of mouse anticaveolin (1 μg/test) (kindly provided by Dr. Guadalupe Ortega-Pierres, CINVESTAV, IPN.) at room temperature for 20 min, and subsequently washed with 1 mL of staining buffer. Immediately after, cells were centrifuged at 300 ×g for 5 min. Cell pellets were resuspended separately in presence of the secondary antibody FITC or Alexa488-conjugated goat anti-mouse (Zymed) and incubated at 4°C for 30 min in the dark. After this second incubation, cells were washed twice in staining buffer and centrifuged at 300 ×g for 5 min. Cell pellets were resuspended in 500 μL of staining buffer in absence of light and analyzed by flow cytometry using an FACS Calibur (BD, Biosciences). Data was analyzed with FlowJo software.

2.6. Nuclei Staining with Diamidino-Phenylindole (DAPI) of Isolated Cells. For DAPI fluorescent staining, *in vitro* cultivated primary cells were collected, fixed in paraformaldehyde (Sigma) for 10 min, and permeabilized with 0 .2% Triton X-100 (Merck) for 5 min. DAPI at a concentration of 0 .5 μg/mL was added to the fixed cells on the slide, followed by an incubation for 20 min in the dark. Slides were then rinsed in PBS, Fluoprep (bioMerieux) was added, and a cover slip applied. The stained biological substrates were visualized using an optical microscope. For light microscopy applications, we used a Nikon Eclipse E600 microscope equipped with a Nikon DXM1200 F CCD (Nikon Corp.; Tokyo, Japan). Immunofluorescence staining experiments were carried out using a Nikon Eclipse 80 i microscope and a Nikon DXM1200 C CCD (Nikon Corp.). For DAPI visualization, a 350 LP filter was used (Nikon, Corp.). Image processing and analysis was carried out using Adobe Photoshop CS3 (Adobe Systems Inc., San Jose, CA, USA) and Image Pro Plus 6 .2 (Media Cybernetics, Bethesda, MD, USA).

3. Results

3.1. Taenia solium, Taenia crassiceps, and Trichinella spiralis Single Cells. In Figure 1, it is clear that we were able to obtain isolated single cells of the cestodes (a) *Taenia solium*, (b) *Taenia crassiceps*, and the nematode (c) *Trichinella spiralis*. The composition shown in the first photograph of every row shows the complete parasites, before of the isolation process. In the second picture of every row, the first step, by disrupting the parasites and, showing clumps of parasites, and, debris of pieces of the same. Finally, in the third picture of every row, the isolated single cells of the three parasites are depicted. It is interesting to note that, *Taenia crassiceps* (b) cells are multinucleated, and, due to the size (10 μm) it cannot be a complete parasite. The same can be said also for *Taenia solium* (a) and *Trichinella spiralis* (c). Cell viability was >80% for all three parasites after disaggregation (data not shown).

3.2. DAPI Stained Normal Isolated Parasite Cells. Often, one may wish to monitor the presence or expression of several different molecules in cell culture. One component that is almost always monitored is the cell nucleus, which is stained with DAPI, a molecular probe characterized by $\lambda_{ex} = 358$ nm and $\lambda_{em} = 461$ nm. DAPI binds to the inner groove of DNA present in cell nuclei and results in a blue emission that can be seen in the fluorescent microscope. Staining of DAPI in these parasite-isolated cells not only allows us to visualize the cell nuclei, but also allows an easy quantification of the number of cells in a given field of view. A representative example of the images processing steps and subsequent, phase contrast, counting of cell nuclei stained with DAPI, and the overlay using both techniques are shown in Figure 2, where (a) is the composition to show *T. solium* cells, (b) *T. crassiceps* cells, and (c) *T. spiralis* cells.

3.3. Size and Complexity of T. crassiceps, T. solium, and T. spiralis Are Different from the Host Cells. Flow cytometry analysis firstly showed that (b) *T. solium*, (c) *T. crassiceps* and (d) *T. spiralis* cells were different in size and complexity from mouse spleen cells (a). In fact, parasite cells were approximately 3-fold smaller and exhibited less complexity (Figures 3(b), 3(c), and 3(d)) than the mouse spleen cells (Figure 3(a)).

In addition, parasite cells showed no expression of the membrane markers CD3, CD4, CD8 which are typically present in some types of mammalian leukocytes or MHC I, marker of all types of mammalian cells (Figure 4).

3.4. Expression of Paramyosin and Caveolin-1 Is Only on Parasites and Not Host Cells. On the other hand, in Figure 5, the FACS analysis showed that *T. solium* isolated cells

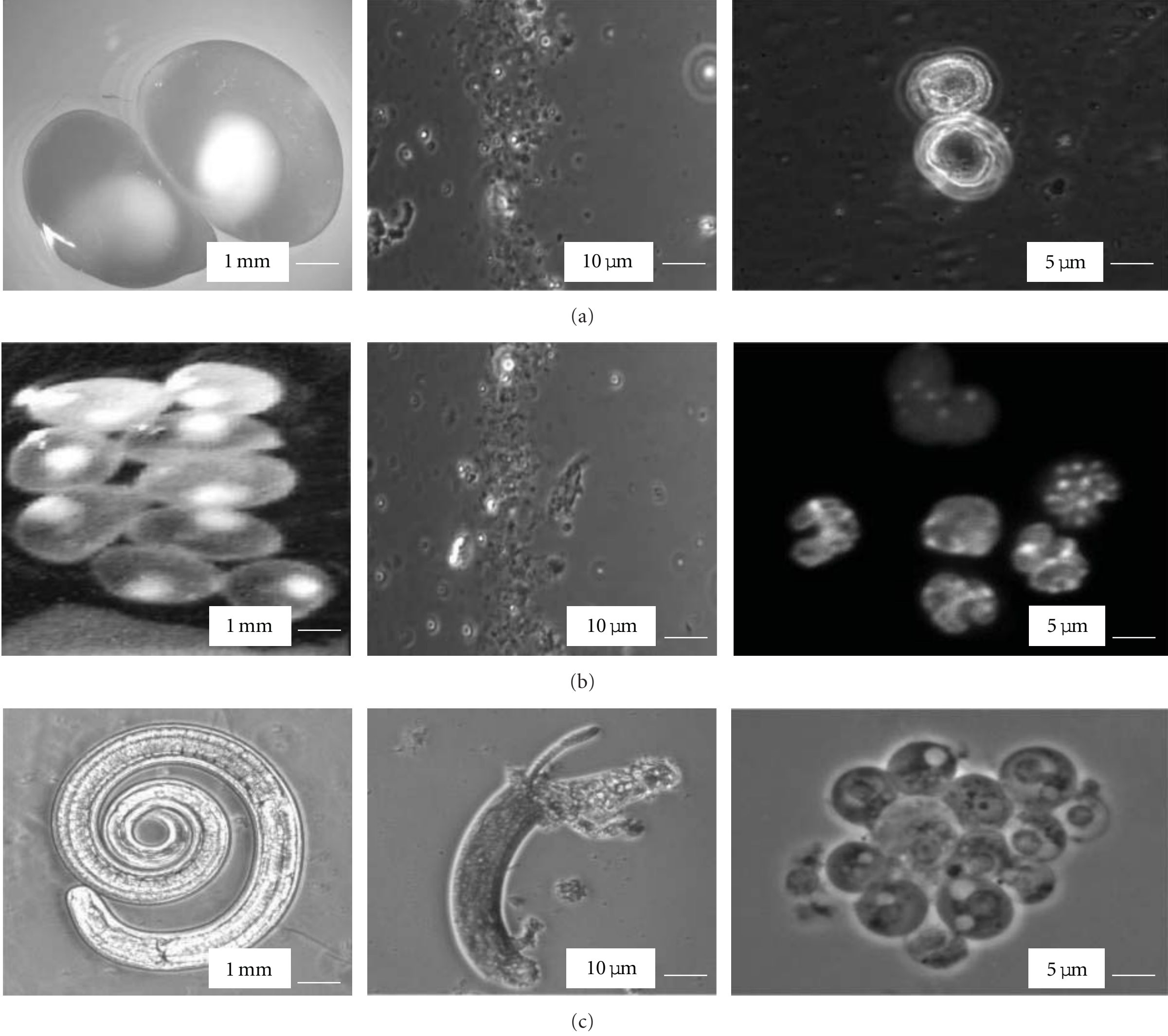

Figure 1: Imaging of the process of getting single cells from *T. solium*, *T. crassiceps*, and *T. spiralis* after mechanical disaggregation. (a) *T. solium* cysticerci (first picture), pieces of *T. solium* after disgregation (middle picture), and *T. solium* isolated cells (third picture of panel (a)). In (b) *T. crassiceps* (first picture), pieces of *T. crassiceps* after disaggregation (middle picture), and *T. crassiceps* isolated cells (third picture of panel (b)) and (c) *Trichinella spiralis* (first picture), pieces of *T. spiralis* after disaggregation (middle picture) and *T. spiralis* isolated cells (third picture of panel (c)). Pictures were taken using an inverted microscope (Olympus, MO21, Tokyo) at 10x and 100x magnification.

expressed calreticulin (a) and *T. crassiceps* isolated cells expressed paramyosin (Ag-B) (b), an exclusive component of the cytoskeleton of cestodes, nematodes, and insects, while the host cells did not showed expression of this molecule (not shown). In the case of *T. spiralis* isolated single cells, there was a clear expression of caveolin-1 (cloned, sequenced, and expressed exclusively of this parasite), while the host cells analyzed to look for the expression of this protein have a negative dying (not shown).

4. Discussion

Several methods are currently employed to evaluate expression of specific molecules in helminthes, including real-time reverse-transcriptase polymerase chain reaction (real-time RT-PCR), western blot, double-dimension gels, and several microscopic assays. However, all these methods require homogenization of the whole helminth parasite cells, thereby preventing evaluation of individual cells or specific cell types in a given parasite tissue or area and cannot differentiate from parasite cells and host cell contamination cells. The problem with analyzing helminths proteins is that helminth tissues are usually highly contaminated by host immune cells, thus preventing researchers to determine that the molecule they are trying to characterize truly belong to their parasites. Our method of isolation of helminth cells is highly specific and is able to determine and sort specifically the parasite cells from the host cells. In addition, parasite cells can be sorted out and recovered alive to perform experiments in these isolated cells, like, *in vitro* culture, transfection, and possibly regeneration of complete parasites derived from single cells.

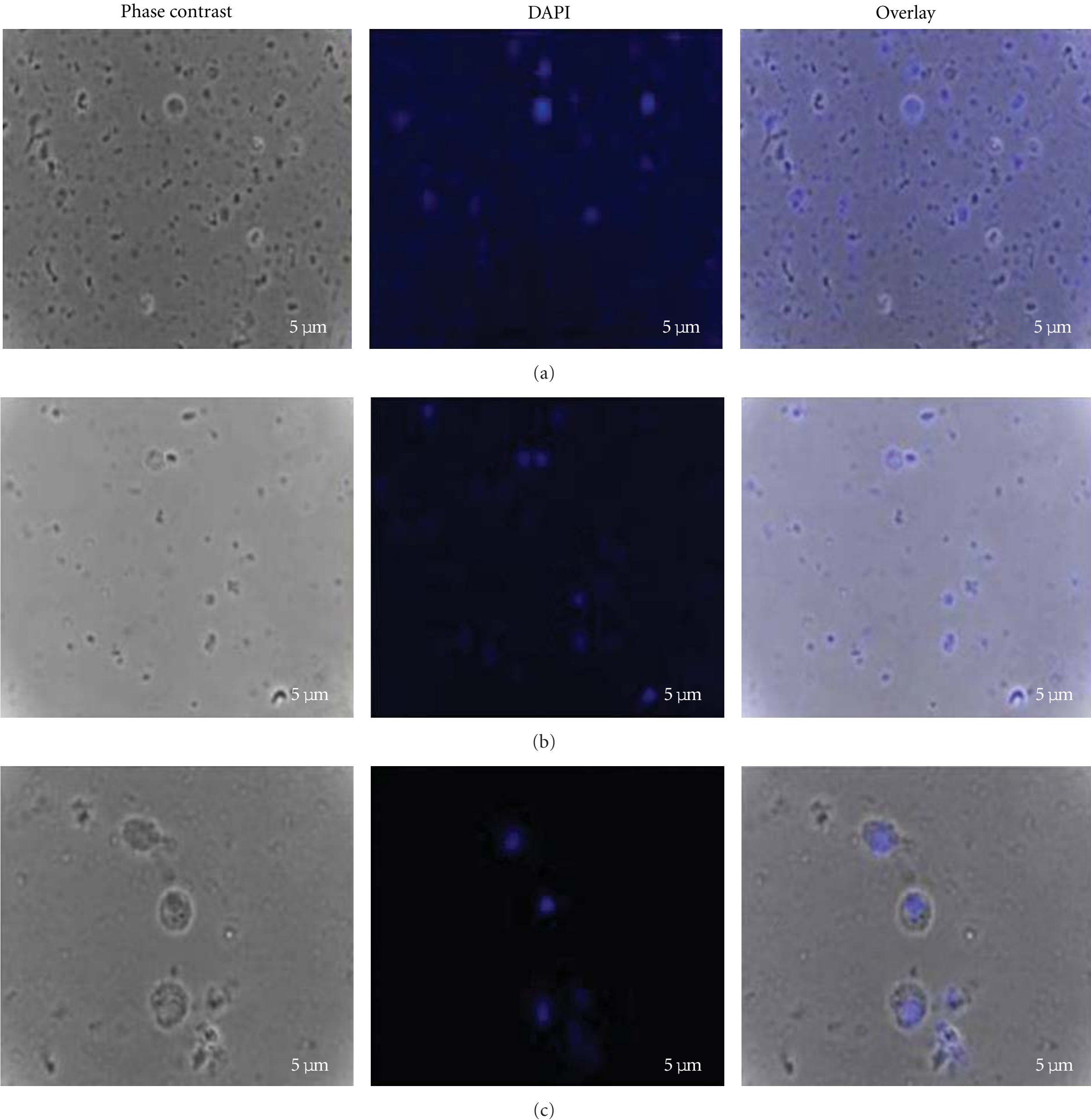

Figure 2: Disruption of parasite tissue and staining of primary cells. After isolation, primary cells were fixed and nuclei were stained with DAPI. Composed figure depicts stained cells with DAPI (produces blue color) of *Taenia solium*, *Taenia crassiceps*, and *Trichinella spiralis* cells.

The disaggregation, and separation for further flow cytometry analysis shown in here, is an important tool for determining protein expression of cell surface molecules and has also been shown to be useful for identifying expression of intracellular proteins in permeabilized cells [6]. Therefore, our method can be a potentially useful tool in the field of new protein helminth parasite discovery biology by providing a technique for analyzing all type of proteins from helminth parasite origin within intact parasite cells, with no host cell contamination [7]. This type of analysis has not been previously reported and may pose increased difficulties due to nonspecific binding of antibodies to intracellular proteins.

In addition to providing information on cells expressing specific proteins, once that we have the disaggregated parasites, the cells specifically detected as parasites cells, by size and granularity and by using a single specific protein marker (as paramyosin and caveolin-1 in our case), are gated to use to determine the problem protein. This gives an indication of the relative amount of the searched protein expressed by an individual cell, thus providing a means to quantify that protein on each cell. Another advantage of this technique is that expression of proteins of interest can be correlated with the degree of activation, maturation, or differentiation of given parasite cell types. Finally, if complex mixtures of parasite helminthes cells are present (as is the case), flow

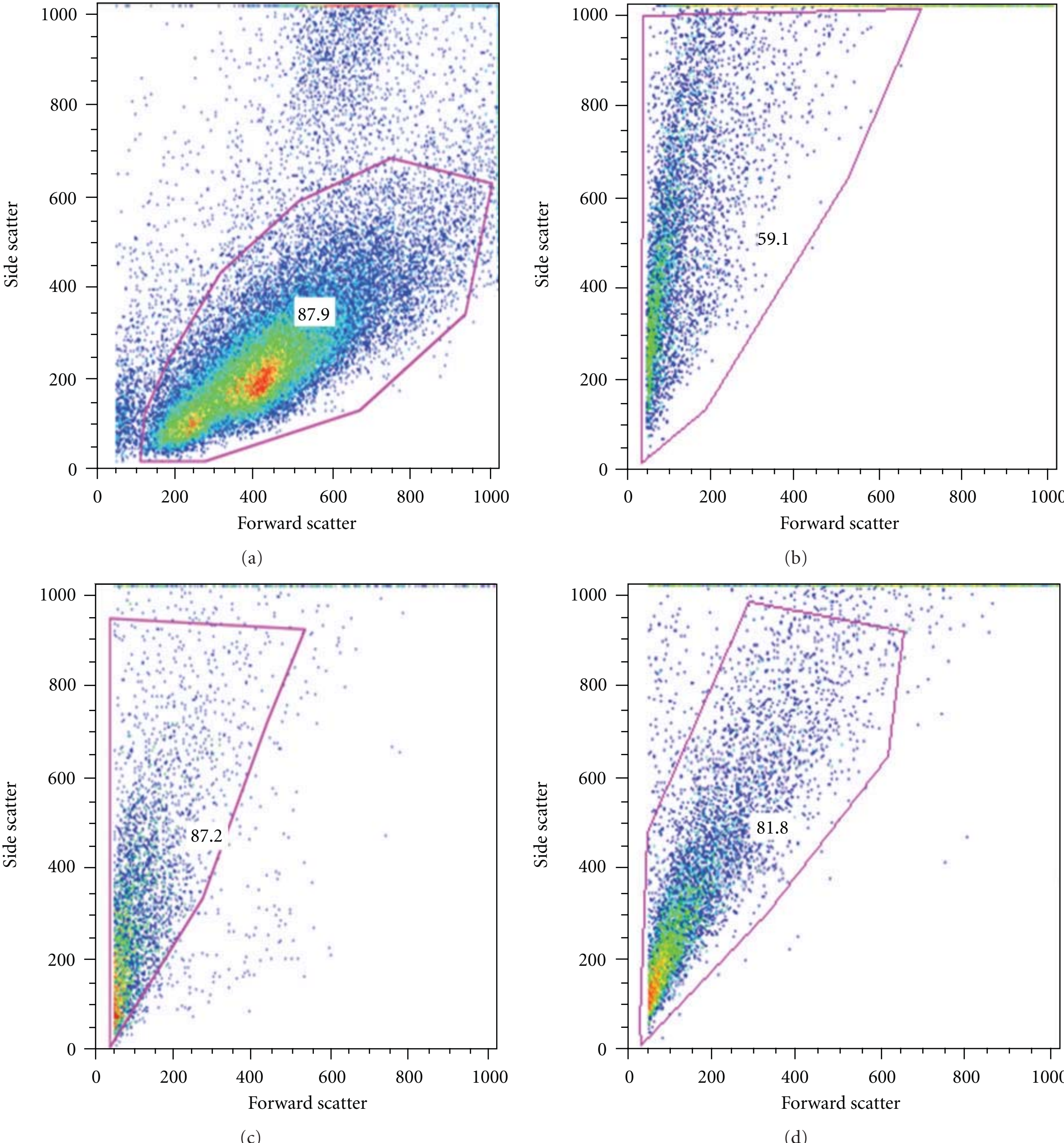

Figure 3: Forward/Side scatter of splenocytes and parasites isolated cells. (a) Spleen cells, (b) *T. solium* cells, (c) *T. crassiceps* cells and (d) *T. spiralis* cells were disaggregated by tissue disruption, washed twice with FC buffer, and fixed using Lyse/Fix buffer (BD Biosciences).

cytometry can be used to sort subpopulations of cells and, therefore, identify proteins expressed by specific parasite cell types. This method is, however, limited in its inability to specify the location within the cell (nucleus, cytoplasm, mitochondria) of intracellular proteins that are identified.

Specifically in the field of parasite hormone receptors, flow cytometry can be used to identify intracellular expression of steroid hormone receptors. We have been able to demonstrate and characterize expression of estrogen hormone receptors, from *T. crassiceps* [4] Using real-time reverse-transcriptase polymerase chain reaction (RT-PCR) and fluorescent microscopy methods, we were able to determine that it was present, respectively, for this receptor in the whole parasite [8]. This method could, therefore, be useful to answer a variety of scientific questions related to hormone receptors biology in the complex-host parasite interaction, including determining the responsiveness of specific cell types of parasites to steroid hormone treatment, for instance. This method would, therefore, be a very useful tool for rapid, high throughput measurement of not only hormone receptors, or receptors in general, but at the protein level in single, intact helminthes isolated cells.

On the other hand, it was critical to determine that the obtained cell populations were exclusively found in the

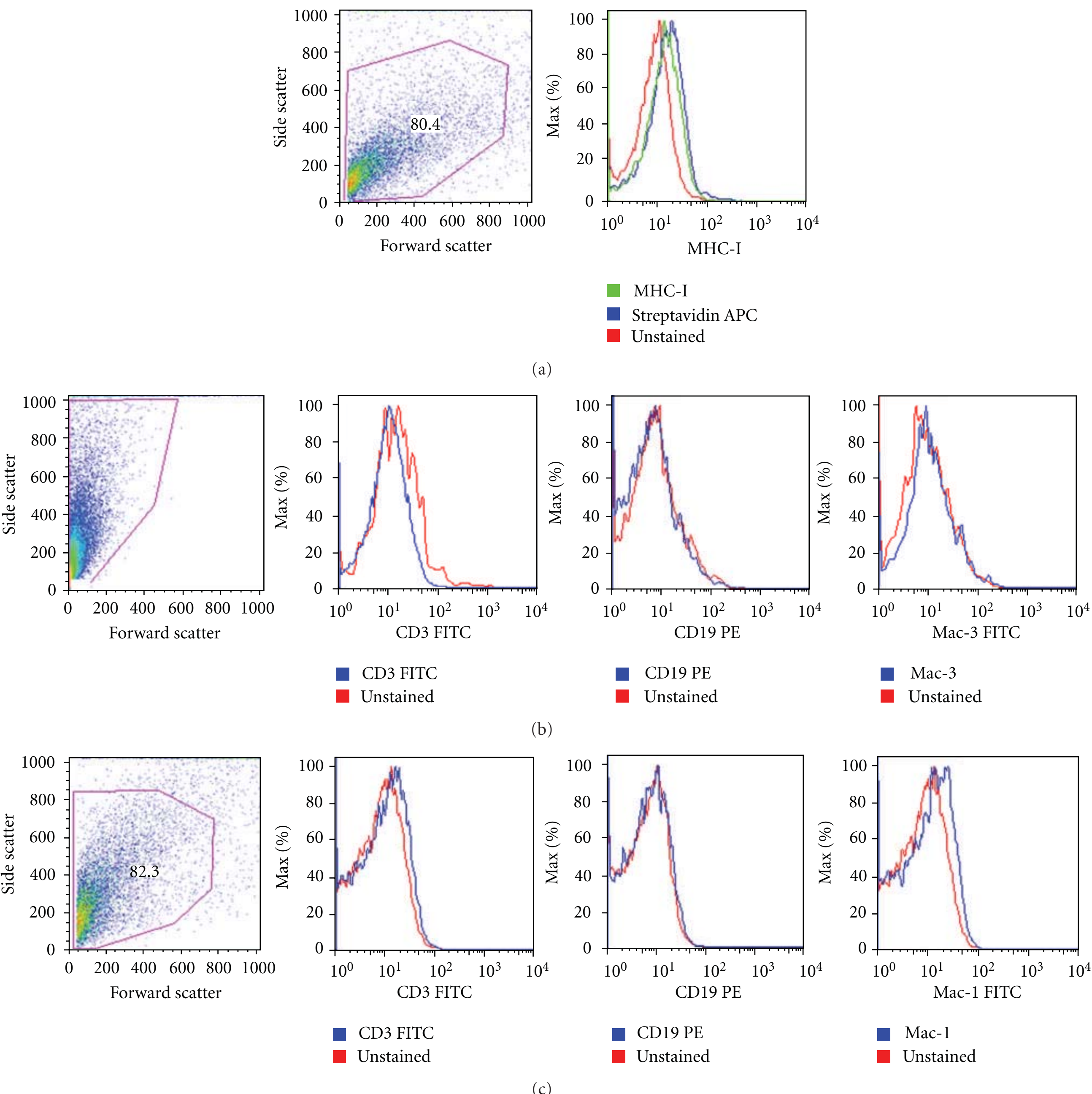

FIGURE 4: Non-host-contaminated parasite isolated cells. Cells from (a) *T. solium* cells, (b) *T. crassiceps* cells, and (c) *T. spiralis* cells were disaggregated by tissue disruption and stained with anti-hMHC-I, anti-mCD3, anti-mCD19, anti-mCD11 b, and anti-mMac-3 antibodies.

T. crassiceps and *T. solium* cysticercus, and *T. spiralis* larvae, and not a consequence of host immune, or other type of cell contamination, because, again, as shown elsewhere, there is extremely high interaction between parasites and host cells, which may eventually lead to host cell invasion into several parasitic tissues [9]. For this reason, an alternative use of flow cytometry was employed to differentiate proteins from *T. crassiceps*, *T. solium*, and *T. spiralis* and their murine host by identifying exclusive molecules of the parasite, which are neither synthesized nor expressed by the host. This is the case of paramyosin, a muscle protein found only in invertebrates, such as *Drosophila melanogaster*, *Caenorhabditis elegans* [10, 11], *Taenia solium* [12, 13] and *T. saginata* [14], and caveolin-1 a protein that is implicated in *T. spiralis* differentiation [15]. The flow cytometry studies showed that presence of the analyzed cells protein belonged specifically to the parasites, because paramyosin was only detected in *T. crassiceps* and *T. solium* cells, and caveolin-1 was only expressed in *T. spiralis*. In contrast, the αanti-paramyosin or αanti-caveolin-1 antibody did not recognize cells extracted from mouse, but they were positive for CD3, CD4, CD8, CD19, and macrophage antibodies, contrary to parasite cells. These results demonstrate

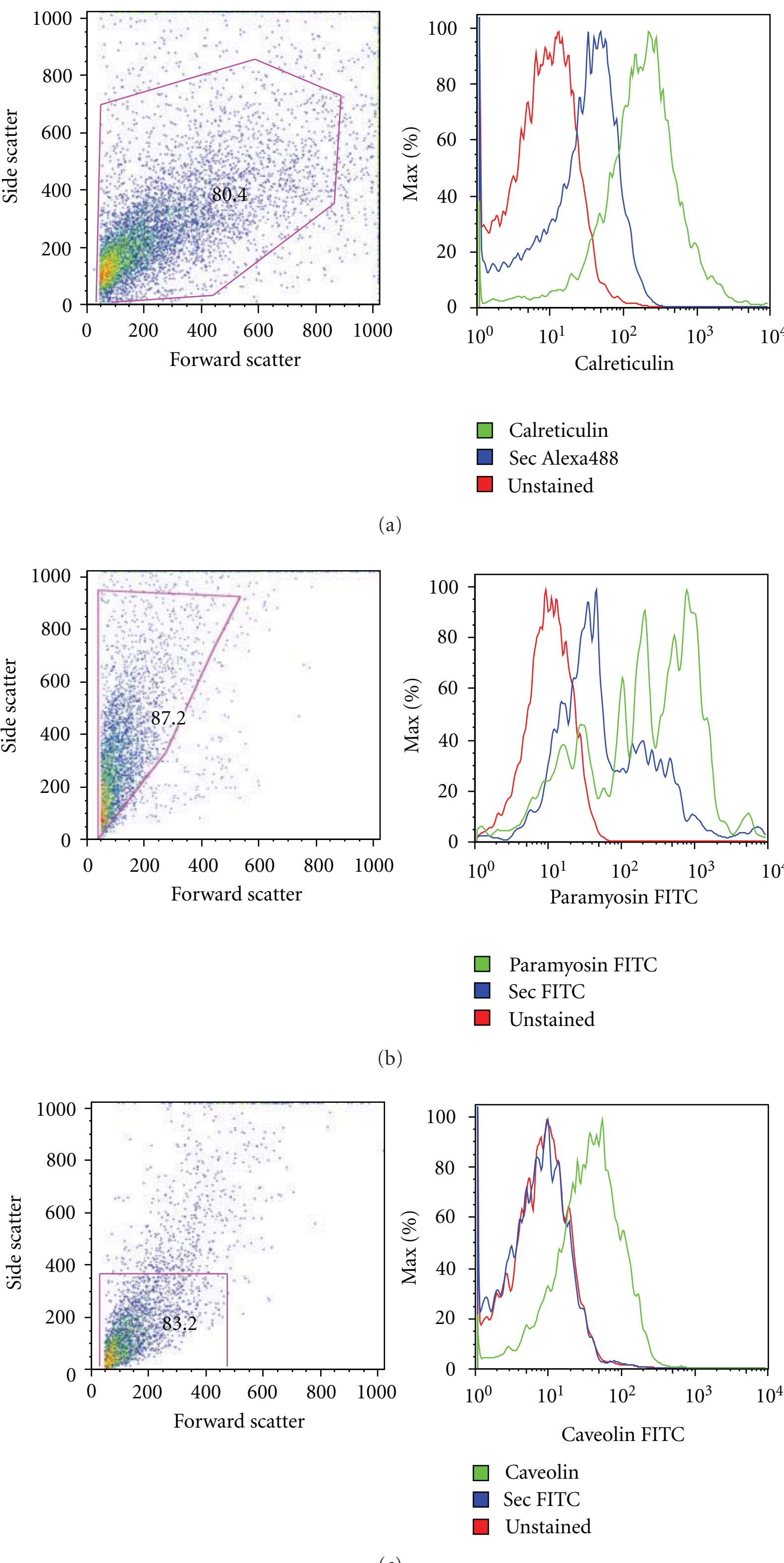

Figure 5: Expression of parasite-specific proteins on (a) *T. solium,* (b) *T. crassiceps,* and (c) *T. spiralis*. Anticalreticulin and antiparamyosin antibodies were obtained by mice immunization with cloned and expressed proteins from *T. solium* and *T. crassiceps*. Anticaveolin antibodies were obtained by mice immunization with a cloned and expressed protein from *T. spiralis*.

that the analyzed parasite cells are in fact from the three parasite's origin and not from other sources, and simultaneously accentuate the potential use of flow cytometry for differential identification of molecules from organisms with extremely close contact, such as helminthes parasites and their hosts.

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Research Article

Contribution of the Residual Body in the Spatial Organization of *Toxoplasma gondii* Tachyzoites within the Parasitophorous Vacuole

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Toxoplasma gondii proliferates and organizes within a parasitophorous vacuole in rosettes around a residual body and is surrounded by a membranous nanotubular network whose function remains unclear. Here, we characterized structure and function of the residual body in intracellular tachyzoites of the RH strain. Our data showed the residual body as a body limited by a membrane formed during proliferation of tachyzoites probably through the secretion of components and a pinching event of the membrane at the posterior end. It contributes in the intravacuolar parasite organization by the membrane connection between the tachyzoites posterior end and the residual body membrane to give place to the rosette conformation. Radial distribution of parasites in rosettes favors an efficient exteriorization. Absence of the network and presence of atypical residual bodies in a ΔGRA2-HXGPRT knock-out mutant affected the intravacuolar organization of tachyzoites and their exteriorization.

1. Introduction

Toxoplasma gondii is an obligate intracellular parasite that actively invades host cells through a sequential secretion of proteins from Apicomplexa-specific secretory organelles, namely, micronemes and rhoptries [1] as well as by the participation of the parasite motility based on its subpellicular cytoskeleton [2]. The highly replicative and invasive form of *Toxoplasma*, the tachyzoite, proliferates within an intracellular compartment named the parasitophorous vacuole (PV). The PV delimiting membrane (PVM) is formed at the time of invasion from both the host cell membrane components and parasite-secreted products [3, 4]. Once installed within the host cell, the PV is rapidly encaged by host cell intermediate filaments and microtubules [5], whilst the PVM associates with host cell mitochondria and endoplasmic reticulum [6–8]. Studies showed the formation of host-microtubules-based invaginations of the PVM—named Host Sequestering Tubulo-structures or HOSTs [5]—that serve as conduits for nutrient acquisition from the host cytoplasm to the PV lumen. Apart from rhoptry proteins [9], the PVM is also decorated with several proteins secreted from a third type of Apicomplexa-specific secretory organelles, the dense granules, which contain the GRA proteins [1].

Observation of infected cells by electron microscopy showed that a membranous nanotubular network (MNN) of 40–60 nm in diameter assembles at the invaginated posterior end of the parasite during the first hour following invasion and further extends into the PV space in order to connect with the PVM [10]. Immunoelectron microscopy analysis showed that the MNN has a stable association with several GRA proteins including GRA2 [10], GRA4 [11], GRA6 [11], and GRA9 [12] and showed that GRA2 contributes to the formation of a multiprotein complex within the MNN [13]. Transmission electron microscopy (TEM) analysis in thin sections of embedded infected host cells with GRA2 *Toxoplasma* knock-out mutant showed that deletion of the corresponding gene leads to complete disappearance of the MNN without altering parasite *in vitro* proliferation [14, 15].

Once tachyzoites have established metabolic connections with the host cell by means of the MNN, the HOSTS, and the PVM, they begin to divide asexually mainly by endodiogeny, a process that is characterized by the synchronous assembling of two daughter parasites within each mother cell. Once two sets of intracellular organelles have been assembled within the mother cell, daughter cells emerge from the mother, leaving remnants of the mother cell at their posterior end [16]. These apparent remnants have been referred to as the residual body (RB) of division [17]. After the third division, tachyzoites organize in rosettes around the RB. To date there is not data about the fine structure of the RB or its function.

Analysis of the intravacuolar arrangement of tachyzoites during endodiogeny has been successfully achieved by transmission electron microscopy (TEM) [15, 18, 19]. By using a method proposed by Tanaka for scanning electron microscopy (SEM) in which apical plasma membrane is removed thus preserving the integrity and spatial distribution of intracellular compartments and organelles [15, 18, 19], it was possible to know the relationship between the intravacuolar organization of proliferating tachyzoites and the MMN [15, 18, 19].

In the present study we characterized the intravacuolar organization of tachyzoites of the RH strain of *T. gondii* in rosettes during proliferation in an attempt to better characterize origin, structure, and function of the RB. We additionally determined the contribution of GRA2 protein in the intravacuolar organization of tachyzoites by studying GRA2 knock-out mutant-infected cells.

2. Materials and Methods

All reagents were purchased from SIGMA (St Louis, Mo). Specific reagents for electron microscopy were from Polysciences (Warrington, Pa) unless otherwise indicated.

2.1. Animals. *BALB/c* mice used for parasite infections were maintained in an animal facility with regulated environmental conditions in terms of temperature, humidity, and filtered air. Animals were maintained according to the country official norm NOM-062-ZOO-1999 (http://www.sagarpa.gob.mx/Dgg/NOM/062zoo.pdf) for the production, care, and use of laboratory animals (México).

2.2. Cell Culture and Preparation of Parasites. Madin-Darby Canine Kidney epithelial cells (MDCK, ATCC-CCL 34) were used as host cells for both parasite invasion and proliferation. MDCK cells were maintained in Dulbecco Minimum Essential Medium (DMEM) (GIBCO, USA), supplemented with 10% fetal calf serum (FCS, Equitech-Bio, USA), under a 5% CO_2 atmosphere, at 37°C.

Parasites of the RH strain (wild type) were maintained by intraperitoneal passages in female Balb/c mice [20]. After cervical dislocation, tachyzoites were harvested from intraperitoneal exudates, washed in phosphate-buffered saline (PBS, 138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.1 mM KH_2PO_4, and pH 7.4), and filtered through 3 μm pore polycarbonate membranes (Millipore, Bedford, Mass) [20].

The ΔGRA2-HXGPRT knock-out mutant [15] constructed in the RH strain background and was maintained in MDCK cells. Prior to each experiment, cells were lysed and parasites were harvested, rinsed in PBS, counted, and suspended in appropriate medium.

2.3. Infection of MDCK Cells with Tachyzoites. MDCK host cells were grown on sterile coverslips in DMEM supplemented with 10% FCS for 24 h to reach between 80–90% confluency. Cells were exposed to parasites at the ratio of 5 : 1 parasites per host cell, incubated for 2 h and washed with PBS to discard extracellular parasites. Infected MDCK cells were maintained in DMEM with 10% fetal calf serum under a 5% CO_2 atmosphere at 37°C and at desired times.

2.4. Identification of Components of RB by Immunofluorescence. MDCK cells infected for 24 h were fixed in 3.7% paraformaldehyde for 20 min, permeabilized for 10 min in 0.1% Triton X-100, blocked in 0.5% BSA, and incubated for 2 h with the following primary antibodies diluted in PBS: monoclonal antibody (mAb) TG05.54 anti-SAG1 [21], mAb TG17.43 anti-GRA1, mAb TG17.179 anti-GRA2 [22], and rabbit serum anti-GRA6 [11], each at the dilution of 1 : 500, or mAb T5.2A3 anti-ROP1, mAb T34A5 anti-ROP2, each at the dilution of 1 : 25 [23] (the mAbs anti-ROP proteins were provided by J. F. Dubremetz, CNRS UMR 5539, Université Montpellier II, France, and the rabbit serum anti-GRA6 was obtained from L. D. Sibley, Department of Molecular Microbiology, Washington School of Medicine, Saint-Louis, MO). Cells were rinsed in PBS, incubated for 1 h with goat anti-mouse IgG (H+L) or with goat anti-rabbit IgG (H+L), both coupled to Alexa Fluor 488 (Molecular Probes, USA). To detect nuclei in tachyzoites organized in rosettes, cells were incubated for 1 h with 10 μg/mL of the fluorescent stain 4′6-diamidino-2-phenylindole specific for double-stranded DNA (DAPI, Sigma-Aldrich Co., Mexico).

Coverslips were mounted on glass slides in Vectashield Mounting Medium (Vector Laboratories, UK) and analyzed with an AxioScope II fluorescence microscope coupled to an AxioCam II RC digital camera (Carl Zeiss). Fluorescent images were acquired and processed using the AxioVision software 4.5.

2.5. Fine Structure of the RB by TEM and SEM-Tanaka. For TEM, MDCK cells infected for 24 h were fixed for 1 h in 2.5%

glutaraldehyde. Cell monolayers were scraped off, rinsed in PBS and fixed for 1 h in 1% OsO_4 at 4°C, rinsed, gradually dehydrated in ethanol, and finally embedded in Spurr's resin [20]. Thin sections were obtained with an Ultracut E ultramicrotome (Reichert Jung, Austria) and stained with uranyl acetate and lead citrate. Copper grids with the sections were examined in a JEOL 1400 transmission electron microscope at 80 keV (JEOL LTD, Japan). Digital images were obtained and processed with Adobe Photoshop software (USA).

For SEM using the Tanaka method, MDCK cells were in fected for 1, 6, 12, and 24 h and then processed according to Travier et al. [15]. Briefly, at selected times, infected mono layers were fixed with 2% glutaraldehyde and 1% OsO_4 in PBS, ethanol dehydrated, critical point dried in CO_2 atmosphere in a Samdry-780A apparatus (Tousimis Research, USA), and gold coated in a Denton Vacuum Desk II (INXS. Inc, Florida) [20]. Coverslips containing the infected monolayers were attached to SEM aluminum holders, and the apical plasma membrane of host cells was removed by an adhesive tape. Both the treated coverslips and the adhesive tapes recovered from the rod were gold coated and analyzed using a SEM JEOL 65LV (JEOL, LTD, Japan). Digital images were recorded, and photocompositions were realized with the Adobe Photoshop software.

2.6. Ionomycin-Induced Egress Assay. Coverslips with MDCK cells infected for 24 h were mounted within observation chambers and then were exposed to 0.1 μM ionomycin (in 0.001% DMSO in PBS) to induce parasite egress [24]. Exteriorization was recorded under time lapse mode in a phase contrast microscope using an AxioCam RC digital camera (Carl Zeiss) and the AxioVision software. Sequential images were processed using the Adobe Photoshop software.

2.7. Three-Dimensional Reconstruction of the Rosette. Three-dimensional model of a rosette was built using AutoCAD software version 2007, and it was based on the morphological properties of intravacuolar tachyzoites micrographed by SEM and on the spatial distribution of the tachyzoites nuclei stained with the DAPI dye. Two orthogonal views were required to design the pictorial 3D rosette. The front isometric view of the tachyzoite, providing the width and height dimensions, was divided in 26 longitudinal sections of 190 nm, each with a total length of 5.14 μm. The top isometric view provided the width and depth dimensions of each cross-section. Both parameters were taken in consideration to create the geometry of the parasite, consisting of 26 planar section curves. Sweeping the planar sections along a defined spine designed to be the main geometry axis, allowed the creation of a complex multisection solid. Each 3D tachyzoite was adapted in specific position, around a 3D RB model, according to the interparasite distance observed in SEM micrographs of rosettes in order to construct the respective 3D digital model.

2.8. Statistical Analysis. Variance analysis of data was achieved by using the Student's t-test.

3. Results

3.1. Tachyzoites Organize around an RB to Form Intravacuolar Rosettes. By detaching the plasma membrane of infected cells, the spatial distribution of the intravacuolar tachyzoites was exposed further showing their relationship with the MNN and the RB under the high resolution of an SEM (Figure 1(b)). After 24 hours of proliferation, most of the tachyzoites were organized within the vacuole in rosettes around an RB located in the center of the structure (Figures 1(a) and 1(b), arrow). Tachyzoites were surrounded by the MNN and tightly associated through their posterior end to the RB (Figure 1(b)). During the detaching process, most of MNN and vacuolar components remained associated to the rosette while detached apical membrane remained free of parasites or of any MNN component (Figure 1(c)).

3.2. The Residual Body Is Related to the Rosette Organization. In SEM images, the RB was clearly identified as a round structure located in the center of the rosette with diameter of 1.43 μm ± 1.0 (measured in 11 rosettes analyzed) that was linked to the posterior ends of parasites in proliferation (Figure 2(A), rectangle). A magnification of the interaction zone between the RB and the posterior end of the tachyzoites showed a close association between both membrane areas (Figure 2(B)). In order to further examine the fine structure of the RB and its relationship with the daughter tachyzoites in the rosette, thin sections of infected MDCK cells were analyzed by TEM. A membrane was found limiting the periphery of the RB (Figure 2(C), insets (a), (b)). At the interior of the RB were identified several organelles characteristics of tachyzoites such as dense granules, rhoptries, nuclear fragments, mitochondria, and Golgi between others, suggesting their origin from components that were trapped in the RB during the division of the tachyzoites (Figure 2(C)). The structural analysis of the interaction zone showed a membrane continuity between the membrane of the posterior end of the tachyzoites and the RB membrane, with the presence of an apparent communication between the cytoplasm of both the RB and the tachyzoites (Figures 2(C)–2(E), white arrows). In regions of the RB membrane not involved in the intermembrane interaction, we detected a typical three-membrane pellicle (Figure 2(C); inset (b)). According to the magnification shown in Figure 2(E), the polar posterior ring (indicated by double arrows) appears to contribute to stabilizing the intermembrane junction. Polar posterior ring of tachyzoites can be clearly identified by a submembrane electron dense zone at the posterior end of the tachyzoites.

Identification of some proteins present in the RB was made by immunofluorescence with antibodies against proteins from secretory organelles such as dense granules and rhoptries. Dense granule proteins GRA1, GRA2, and GRA6 that are normally secreted in the PV [1] were detected in the RB (Figure 3, arrows). Interestingly, GRA5, a protein that has been described associated with the PVM [22, 25], was also found in the RB. During the focusing of the tachyzoites in the rosettes by phase contrast microscopy, the definition of the RB was lost showing an apparent absence of the structure. When the RB is focused, then the rosette appeared blurry.

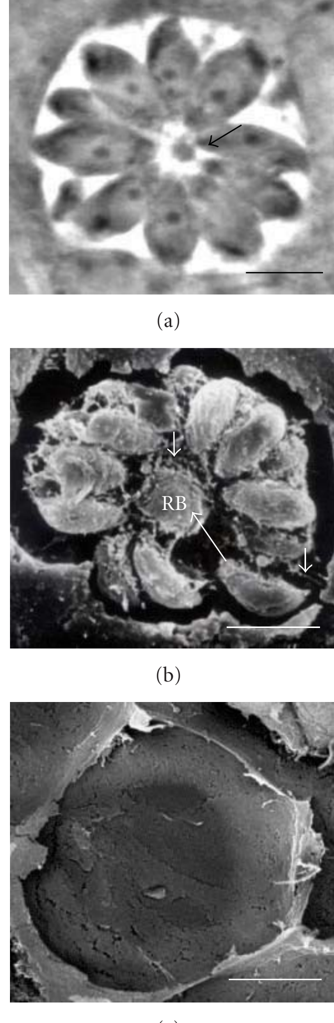

Figure 1: Intravacuolar organization of tachyzoites in MDCK cells. (a) Phase contrast micrograph of a rosette of intravacuolar tachyzoites and (b) SEM-Tanaka micrographs of a rosette (B). Long arrows indicate the RB; arrowheads show intravacuolar network extensions connecting parasites to the PVM; short arrows indicate the position of the apical end in tachyzoites; asterisks show the empty space observed around parasites, at the periphery of the PV. The counterpart of the PV was devoid of any material from the MNN or of tachyzoites (c). Bar = 5 μm.

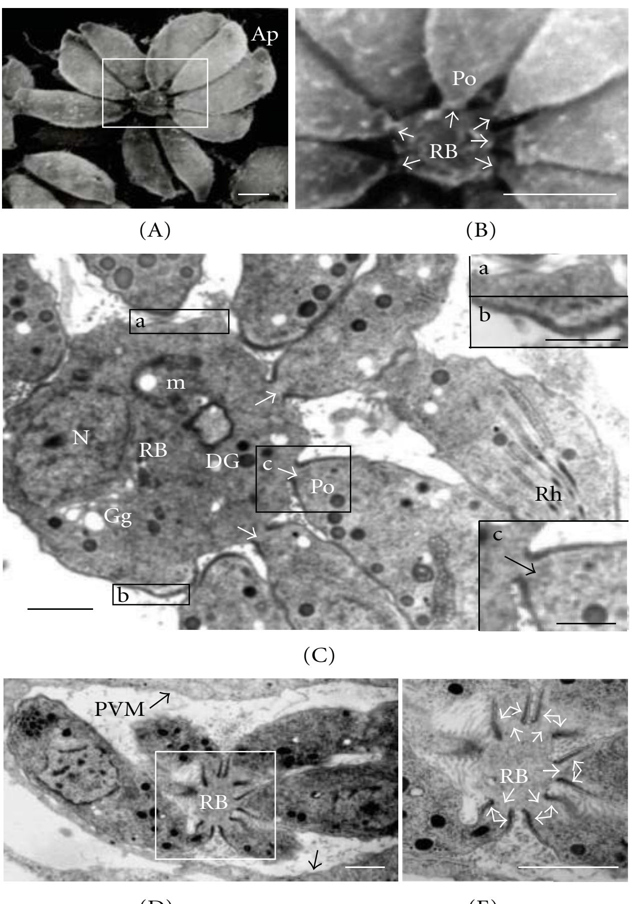

Figure 2: The residual body is a structure derived from a mother cell that contributes to the intravacuolar organization of parasites in rosettes. (A) Intravacuolar "rosette" micrographed after SEM-Tanaka processing. (B) Magnification of the area squared in (A) showing a link between the RB and the posterior ends of the proliferating tachyzoites (arrows). (C–E) micrographs obtained by thin sectioning. Arrows in (C–E) indicate membrane fusion between the RB limiting membrane and the tachyzoites membrane at the posterior end and the continuity between tachyzoites and the RB cytoplasm. (E) High magnification of the area squared in (D). Asterisks in (E) indicate accumulation of MNN at the periphery of the RB, and double arrows indicate parasites' posterior polar ring. Insets in (C) represent the different types of membranes that limit the RB: a unit membrane (a) or a pellicle-like composed of three layers (b). Inset (c) in (C) indicates membrane fusion between the RB and the posterior end of a parasite. Ap: apical end of the tachyzoite; DG: dense granule; Gg: Golgi; m: mitochondria; N: nucleus; PVM: parasitophorous vacuole membrane; Po: posterior end of the tachyzoite; RB: residual body; Rh: rhoptries. Bars = 1 μm; Bars in insets b and c = 500 nm.

That is why we decided to focus on the rosette rather than the RB.

Antibodies against SAG1, the parasite major surface protein [21], labelled the plasma membrane of proliferating parasites but not the RB membrane (Figure 3, arrow in SAG1); probably the availability of the RB membrane was limited by the binding of the tachyzoites. Proteins from rhoptries ROP1 and ROP2 were detected only in the apical end of parasites but not in the RB indicating the specificity of the staining (Figure 3). DNA staining with DAPI showed the presence of the nuclei of tachyzoites and only a slight RB labeling (Figure 3). In the particular case of DAPI, we had to focus on the RB because the signal we were looking for was precisely within the RB, that is why in the image of phase contrast microscopy the RB appeared as a clear and well-defined structure.

3.3. Formation of the RB and MNN during Intravacuolar Proliferation. In order to characterize the formation of the RB during endodiogeny, infected MDCK cells were cultured

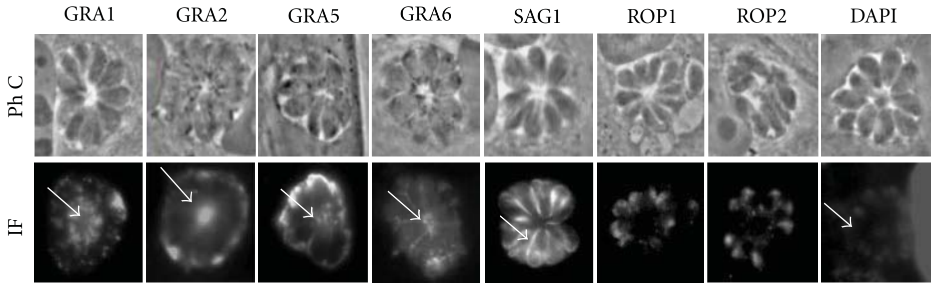

FIGURE 3: Localization of GRA proteins in the RB. MDCK cells infected with RH tachyzoites were incubated with antibodies directed against the dense granule proteins GRA1, GRA2, GRA5, and GRA6, rhoptry proteins (ROP1, 2), or membrane protein SAG1. GRA proteins marked the RB (arrows), while the stain with nuclear marker, DAPI, showed a weak staining of RB. Immunofluorescence micrographs (IF) are shown with their respective phase contrast microscopy images (PhC).

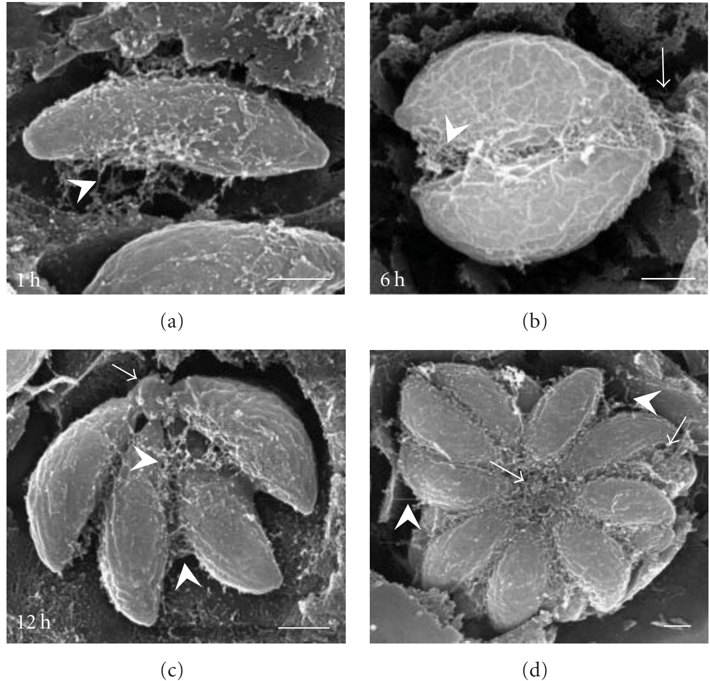

FIGURE 4: SEM-Tanaka sequence of intravacuolar proliferation of tachyzoites. Arrowheads indicate extensions of the MNN, which connect the incurved face of a single parasite to the PVM (1 h), the incurved faces of parasites after the first and second divisions (6, 12 h), or which connect the rosette to the PVM (24 h). Arrows point out the RB (6–24 h). Asterisks indicate the apparent empty space at the PV periphery (1–24 h). Timing indicated in lower left corners represents the replication time after invasion. Bars = 1 μm.

for 1, 6, 12, and 24 h to obtain PVs containing 1, 2, 4, 8, and 16 parasites, and they were processed for SEM as described above. After 1 h of invasion, the MNN was detected mainly concentrated on the incurved face of recently invaded parasites (Figure 4(a), forming a web that kept the first parasite attached to the PVM (arrowhead). Parasites that resulted from the first division at 6 h after invasion (Figure 4(b)) remained connected with the PVM via extensions of the MNN located at the parasite posterior ends (arrow) as well as on their incurved face (arrowhead). In addition, we detected a residual body that kept the two parasites united by their posterior ends (asterisk). At twelve hours after invasion, the RB acquired a spherical shape while the network that surrounded the parasites favoured interparasitic cohesion (Figure 4(c)). At 24 h of proliferation of parasites, the RB was found in the centroid of the rosette with the presence of several parasitic interconnections (Figure 4(d), arrow). There were also connections between parasites and the PVM (arrowheads).

3.4. The Residual Body Contributes to the Intravacuolar Organization of the Parasites. To study whether the RB contributes to the efficient use of intravacuolar space by the proliferating parasites, tachyzoites organized in rosettes were stained with the fluorescent dye for nuclei, DAPI, and serial optical sections obtained in a confocal microscope (Figure 5(a)). Serial images showed that the nuclei and thus parasites are arranged in two adjacent planes which contain

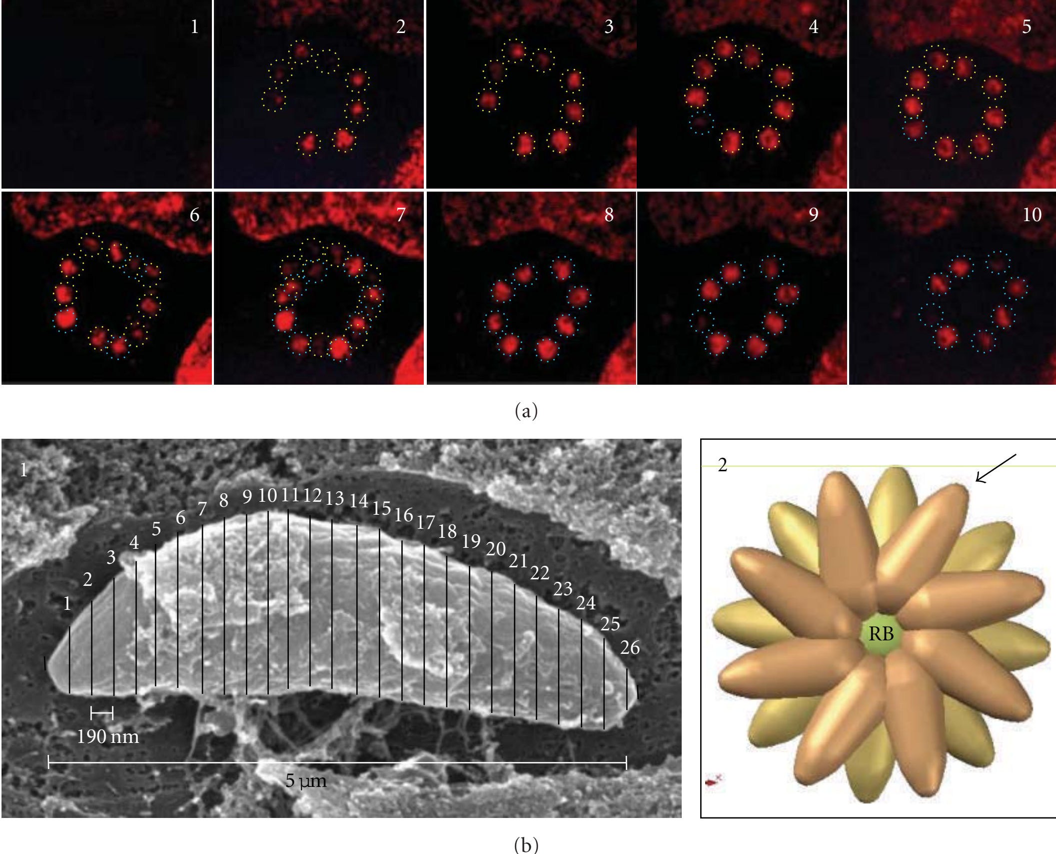

Figure 5: Proliferating tachyzoites arranged in rosettes are localized in two planes within the PV. (a) Confocal serial sections of proliferating tachyzoites arranged in rosette and stained with DAPI to show nuclear distribution. Confocal images 3 and 8 show the spatial arrangement of tachyzoites in two planes each containing 8 parasites. The interaction between both planes is showed in confocal image 7. (b) SEM-Tanaka micrograph of tachyzoite after 1 h of invasion that was used as a template to build the 3D digital model of the rosette; it was sectioned in 26 portions of 190 nm each and was used to build a digital model that was in turn used to design a 3D digital model of a rosette (C). Arrow, apical extreme of parasite, RB: residual body. Bar = 5 μm.

each 8 parasites (Figure 5(a), insets 3 and 8 resp.). Parasites in both planes showed an interspersed distribution; however, in a certain optical section (inset 7) all nuclei were visible although with clear differences in their respective confocal planes (see inset 7, Figure 5(a)). By imaging tachyzoites and rosettes by SEM and their nuclei by confocal microscopy, we could develop a three-dimensional digital model of the rosette (Figures 1 and 5(b), and inset 1). The exact location of the parasites in the two planes was deduced from the images of the nuclei obtained by confocal microscopy (Figure 5(a)), resulting in the three-dimensional arrangement of the rosette shown in Figure 5(b) (inset 2). According to the 3D model of the rosette, the parasites are interspersed at different levels in order to optimize the available space. Each parasite is pointing outward in an organization in the form of wagon wheel in order to define possible externalization individual routes.

3.5. The Absence of the MNN Alters the Cohesion between the Parasites and the Intravacuolar Arrangement. The ΔGRA2-HXGPRT strain (ΔGRA2) is an RH mutant knocked out for expression of GRA2 that has been previously characterized to lack of the typical MNN [14, 15]. We used the ΔGRA2 strain in order to determine if the lack of expression of GRA2 protein could modify the intravacuolar organization of the tachyzoites and the structure of the RB. Firstly, the absence of protein GRA2 did not alter the invasive capacity finding that approximately 40% of the cells were infected with both the RH strain as the ΔGRA2 strain (data not shown). To follow the intravacuolar development of the tachyzoites, cells were invaded for 1, 6, 12 and 24 h and processed for SEM as described in Figure 4. In all the intravacuolar development stages, tachyzoites were covered by an amorphous material (Figure 6). Typical RBs were not detected, and the MNN was observed as an abundant amorphous material covering the parasites with only few fibers interconnecting parasites and attaching them to the PVM (Figures 7(a) and 7(b), arrowhead). An interesting observation was to find ΔGRA2 strain at 24 h organized in clusters of 2 to 8 parasites in the same PV but not in the typical rosette arrangement (Figures 6(e) and 6(f), and 7). In most cases, a clear lack of interparasite cohesion was evidenced by the parasites separation even in the cluster distribution (E). Parasites were found attached to the RB through fibrous tubules leaving spacing between the body and the posterior end (Figures 6(e) and 6(f)). It

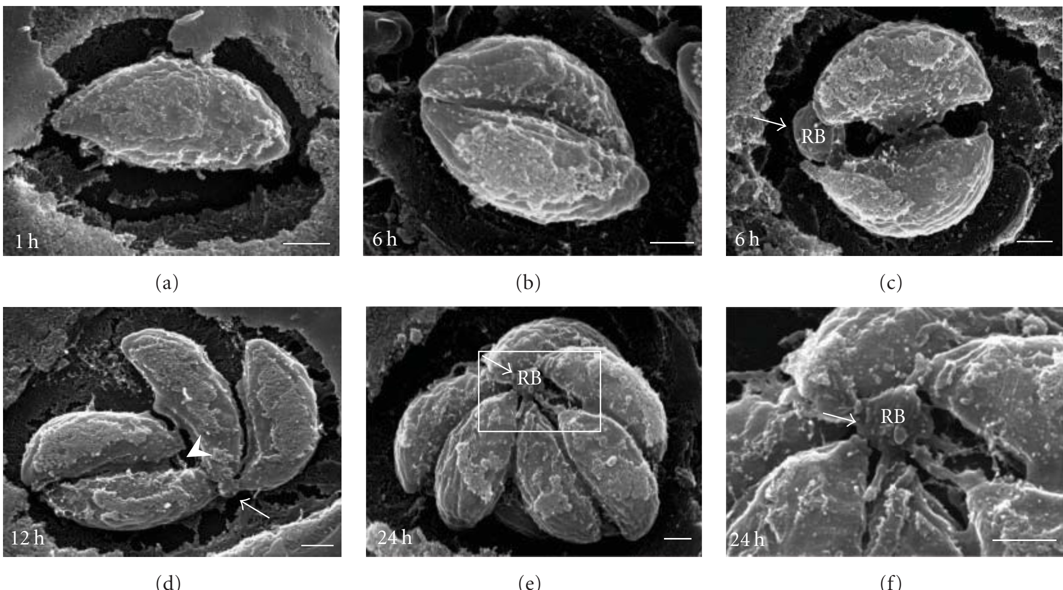

Figure 6: The intravacuolar MNN plays a structural role in keeping parasites tightly organized as "rosettes" during endodiogeny. (a–f) SEM-Tanaka micrographs of MDCK cells, infected by ΔGRA2 mutant for 1, 6, 12, and 24 h, show loss of the MNN and alteration of interparasite cohesion within the PV. Arrows in (c–f) indicate residual amorphous and fibrous material at the parasites posterior end instead of the typical RB. Arrowheads in (d) show membranous fibers linking parasites together and to the PVM. Asterisks in (a–f) indicate an apparent empty space at the PV's periphery. Bar = 1 μm.

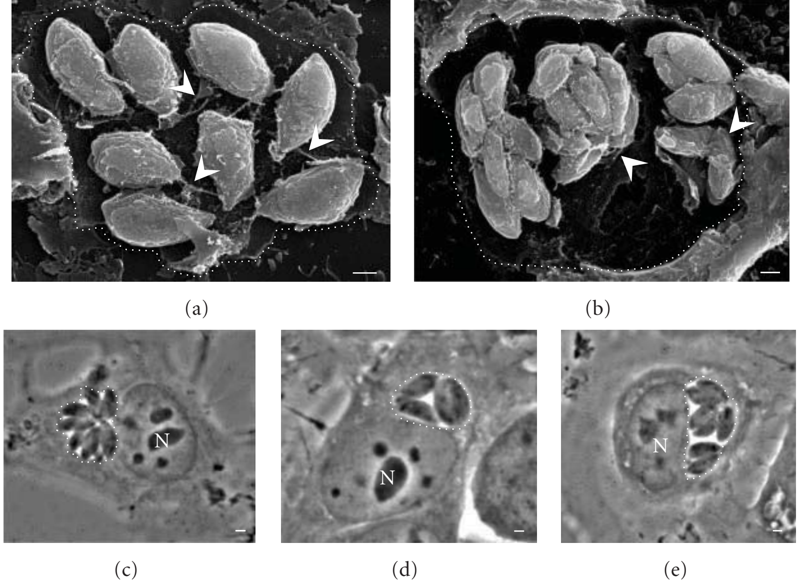

Figure 7: Absence of the GRA2 protein results in asynchrony of proliferation with loss of MNN, RB, and intravacuolar parasite cohesion. SEM-Tanaka micrographs of MDCK cells, infected by ΔGRA2 mutants for 24 h, show loss of interparasite cohesion with presence of isolated parasite aggregates within the same PV (a, b). Arrowheads indicate membranous fibers cross-linking parasites and parasites connected to the PVM. Phase contrast micrographs of live MDCK cells infected with (c) RH *Toxoplasma* strain or (d, e) ΔGRA2 parasites. Dotted lines were drawn to delimit the PVs. Bars = 1 μm.

is possible that the structural modifications of the RB and the type of interaction with the tachyzoites altered somehow the intravacuolar organization, therefore rosettes were not formed.

3.6. The RB Promotes an Orderly and Efficient Externalization of the Parasites. To determine the involvement of RB in the externalization of the tachyzoites from infected cells, MDCK cells infected with RH or ΔGRA2 strains were exposed to calcium ionophore ionomycin to induce the externalization, and this was recorded in real time by time-lapse video microscopy. Parasites of the RH strain left the PV and the host cell after the ionomycin stimulus by propelling themselves in a synchronous and in a centrifugal way along

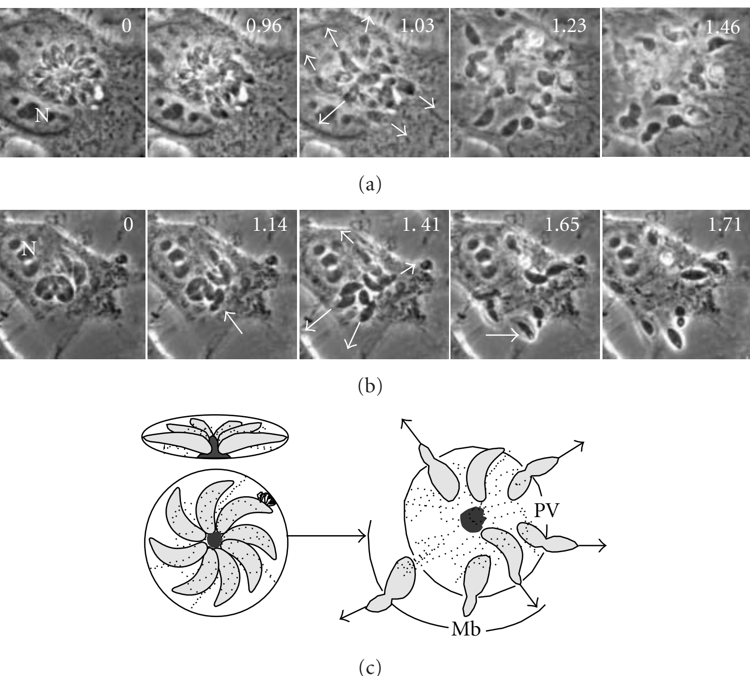

Figure 8: During exteriorization of tachyzoites, the residual body remains inside the PV. (a) Egress of RH tachyzoites arranged as "rosettes" from infected MDCK cells was induced by 0.1 μm ionomycin and recorded by time lapse videomicroscopy. Externalizing parasites are indicated by the arrows. Arrowheads show the constriction of the tachyzoites passing through the host plasma membrane. The asterisk indicates remnants of the RB after the egress of parasites from a rosette. (b) The exteriorization of tachyzoites of ΔGRA2 strain was slightly slower than the RH strain. Several ΔGRA2 tachyzoites remained trapped in the cytoplasm or the nucleus (asterisks). Numbers in the upper right corners indicate the timing in seconds. N: nucleus of the host cell. Bars = 5 μm. (c) represents the individual exteriorization routes followed by tachyzoites organized in rosettes.

individual routes to reach the extracellular medium as fast as 1.3 ± 0.5 seconds (Figure 8(a), inset 1.03′′, arrows). The trigger for the output started with a vibratory movement of the tachyzoites followed by twirling and sliding movements that were oriented to transverse the PVM followed by the plasma membrane. During externalization, there were two constrictions of the parasites, the first when they traversed the vacuolar membrane and the second when they passed through the cell membrane. The RB after the exteriorization remained inside the host cell (Figure 8(a), 1.23′′, asterisk). The externalization of the ΔGRA2 strain was more erratic although very similar to the RH strain (1.6 ± 0.7 seconds). Although many parasites left the cell, several of them could not do it being trapped in the nucleus or the cytoplasm. Apparently, differences in egress time were not observed; however, one event important to remark is the fact that some parasites of DGRA2 mutant even if leave of parasitophorous vacuole are unable to leave their cells staying into of cytoplasm.

4. Discussion

During the development of *Toxoplasma* within the PV, proteins secreted from dense granules contribute to the formation of new membranes, including those that form the PV and the MNN, but their function is poorly known in part due to the few experimental approaches available to isolate them and to gain access to the PV [1, 26].

Several technical procedures have been used to examine the intravacuolar arrangements of *Toxoplasma* including TEM analysis on thin sections and integration of serial optical sections obtained by confocal microscopy, although most of them have limitations in terms of image interpretation, resolution, or technical difficulty. One possibility to examine the inner structure of a cell and its organelles is by using the technique previously developed by Tanaka [18] and recently used in the study of *Toxoplasma* [15, 19]. In this method, the plasma membrane of cells previously processed for SEM is mechanically detached, exposing the spatial distribution of the intracellular organelles.

By applying the SEM technique in infected cells, we were able to study the arrangement of the intravacuolar tachyzoites in rosettes (Figure 1). According to our results, the rosette may represent a type of organization adopted by parasites to optimize the cytoplasmic space available for proliferation in cells with different phenotypes such as neurons, epithelial cells, muscle cells.

During endodiogeny, the favored type of tachyzoite division [27], the mother parasite forms two new dome-shaped conoids, each with an associated inner membrane complex and a set of microtubules and secretory organelles. Most of the mother cell cytoplasm and organelles are incorporated into the two daughter cells [28]. Although directly linked to the endodiogeny process, the RB is a structure that has been reported but poorly characterized; even more, it has been

suggested that the RB is degraded into the VP during posterior endodiogeny cycles [17]. The RB has also been considered as a product of stress condition, and it has been assumed that it is generated by treatments that affect an adequate assembly of the daughter cells. The RB is more easily observed when artificially enlarged as the result of ectopic protein expression or treatment with several drugs that affect the cytoskeleton [29, 30]. Presence of large RB (higher to 5 μm) containing mitochondria and dense granules observed after exposure to actin-modifying drugs delayed or inhibited the parasite egress [31]. In parasites overexpressing myosin, the RB observed was lacking organelles or DNA [29].

In our study and under normal culture conditions and in absence of drugs, tachyzoites of RH strain proliferate around an RB to form intravacuolar rosettes. The RB is an interesting structure that has been considered as a waste material without a defined function [16, 29, 31, 32]. Here, we showed that it is a natural structure located in the centroid of the rosette. It was detected from the first cell division in PV containing two tachyzoites and appeared simultaneously to the formation of the MNN.

We consider that the RB may contain remnants released from the rear during cell division, but finding the parasites stably bound to it in the rosettes is very possible that the residual body fulfills a role as an organizer system in the rosette formation.

Although variations in the size of the RB were detected, there is no evidence to suggest that large or small residual bodies represent a defect in the process of endodiogeny.

The MNN has been considered as a tubule connection system between parasites and the PVM for exchange of nutrients and/or molecules between the host cell and the parasite [1]. The MNN consisted in tubules that keep connections between each daughter tachyzoites keeping them in close proximity to the PV [19]. Data obtained with RH and ΔGRA2 strains indicate that the MNN is an important structure involved in the maintenance of internal parasitic cohesion within the PV and that, somehow favors replicative cycle synchronization.

Absence of the GRA2 protein in the ΔGRA2 strain resulted in a complete loss of the MNN [14, 15] with the presence of atypical RBs. ΔGRA2 parasites organized in "clusters" instead of rosettes. *GRA2* disruption resulted in the loss of parasite division synchrony, as observed by phase contrast microscopy in live cells and by SEM (Figures 6 and 7). These data suggest that the RB as well as the MNN favor the parasitic cohesion during the intravacuolar division and the parasitic arrangement in rosettes: the structural complex of MNN-RB and its extensions to the PVM would anchor the recently internalized parasites to the PVM to immobilize them as an initial necessary step to allow the synchronized proliferation of the parasites.

According to the SEM analysis in numerous samples, we proposed that formation of RB during organization of the rosette could involve the following steps (Figure 9); (I) a first parasite starts the endodiogeny process; (II) at the same time, MNN components are secreted through the posterior end of the parasite, followed by an apparent pinching event at the posterior end with a trapping of the pellicle and cytoplasm components into a nascent RB that remains linked to the membrane of the posterior end of the parasite; (III–V) during the next replication cycles, the RB increases its size and the amount of stored material keeping all the time the daughter tachyzoites attached through their posterior end to the RB membrane. Maintenance of the interparasite space attached to the RB membrane determines the distribution of the parasites in a rosette organization (VI).

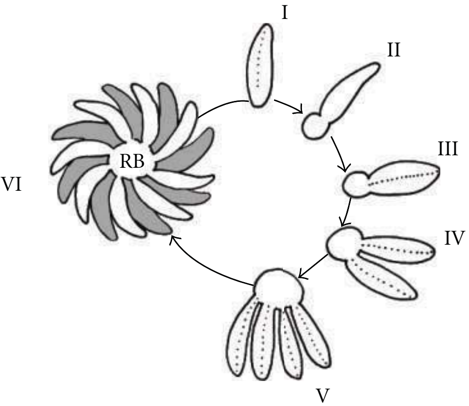

Figure 9: Formation of RB through of the endodiogeny process. (I) The first parasite into to PV; (II) during the early stages of the first division of the endodiogeny process, components are secreted and released toward the posterior end to form a first RB; (III) as tachyzoites proliferate, they release components to the posterior end and enrich the RB; (IV–VI) every new daughter remains attached to the RB starting the radial organization of the rosette.

To date, there are no reports about the presence of RBs in infected animal tissues with *Toxoplasma*. Most reports about the intravacuolar organization of the parasite within infected animal tissues correspond to the presence of tissue cysts in animal models of toxoplasmosis. Of course, the study of the presence of RB's in the infected animal could be interesting and validate that our observations done *in vitro* are also occurring *in vivo*.

Although we studied the proliferation of tachyzoites in epithelial cells *in vitro*, under physiological conditions, tachyzoites or bradyzoites also come into contact with epithelial cells as enterocytes or endothelial cells from the vascular tissue, so it is possible that *in vivo Toxoplasma* can be organized in rosettes with a central RB.

5. Conclusions

Our study showed that (1) the RB is a spheroid structure occurring naturally during endodiogeny of RH strain (2) is possible to observe this structure as soon as the first parasite division takes place, and it is formed simultaneously to the organization of the MNN; (3) it is limited by a membrane and it is probably formed from the first division by a pinching event of the posterior end membrane and through secretion of parasite's components; (4) during endodiogeny, daughter, tachyzoites remain attached to the RB membrane showing a continuity between the RB and tachyzoites cytoplasm; (5) while the MNN determines interparasite cohesion, the RB

defines the spatial position in the rosette organization, acting like an organizing center during proliferating as a strategy to allow successful coordinated parasite division; (6) the RB may contain cytoplasmic organelles, such as mitochondria, nuclear fragments, and dense granules; (7) during exteriorization, the RB could determine the adequate parasite orientation with the aim to favor an efficient egress through individual routes of exteriorization; (8) the ability of exteriorization of the parasites attached to the RB indicates they are mature enough to display all the events involved in exteriorization, such as motility, conoid extrusion, and ropthry secretion, and the RB does not represent an obstacle for such dynamic secretory processes; (9) interdigitated distribution in tachyzoites around the RB could optimize the intravacuolar space during proliferation; (10) lack of GRA2 protein produced atypical amorphous MNN and RB and absence of rosettes.

Acknowledgments

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Review Article

Role of Interleukin-10 in Malaria: Focusing on Coinfection with Lethal and Nonlethal Murine Malaria Parasites

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Interleukin- (IL-) 10, anti-inflammatory cytokine, is known to inhibit the protective immune responses against malaria parasites and to be involved in exacerbating parasitemia during *Plasmodium* infection. In contrast, IL-10 is regarded as necessary for suppressing severe pathology during *Plasmodium* infection. Here, we summarize the role of IL-10 during murine malaria infection, focusing especially on coinfection with lethal and nonlethal strains of malaria parasites. Recent studies have demonstrated that the major sources of IL-10 are subpopulations of $CD4^+$ T cells in humans and mice infected with *Plasmodium*. We also discuss the influence of innate immunity on the induction of $CD4^+$ T cells during murine malaria coinfection.

1. Introduction

Malaria, caused by protozoan parasites of the genus *Plasmodium*, is the major parasitic disease in tropical and subtropical regions, including parts of the Americas, Asia, and Africa. With more than 200–300 million clinical cases globally and approximately 1 million deaths per year, malaria represents the most important infectious disease worldwide. Four species of *Plasmodium* infect humans: *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*.

Human malarial parasites develop through two stages in humans: a liver stage and a blood stage. The asexual blood stage of the parasite is the cause of malarial pathologies. Therefore, it is important to prevent the replication of this stage of parasite. Particularly, *P. falciparum* causes severe pathologies such as cerebral malaria, severe anemia, and respiratory injury in the blood stage. It is necessary to understand the mechanism of protective immunity against the blood stage of the parasite during malaria infection. Nevertheless, it is difficult to investigate the human *in vivo* immune response against malaria parasite for many reasons. Consequently, murine malaria models with *P. berghei*, *P. yoelii*, and *P. chabaudi* have been used to elucidate the immune interaction in hosts and to demonstrate many factors associated with malarial defense mechanisms [1].

2. Parasite Killing: The Role of Proinflammatory Cytokines during Human and Murine Malaria Infection

Interferon- (IFN-) γ and Interleukin- (IL-) 12 play a crucial role in the clearance of intracellular pathogens [2–5]. Low levels of IFN-γ and IL-12 production have been observed in young African children with severe anemia during infection with *P. falciparum* [6]. The IFN-γ-mediated responses have been shown to be involved in protection against infection with *P. falciparum* [7]. In murine malaria, IFN-γ produced by $CD4^+$ T cells has been shown to play a pivotal role in protective immunity against *P. chabaudi* (*Pc*) AS [8], nonlethal *P. berghei* (*Pb*) XAT [9], and *P. yoelii* (*Py*) 17XNL [10] infection. Actually, IFN-γ-depleted mice infected with murine malaria parasites show high levels of parasitemia and eventually die. IL-12 is a necessary factor for clearance of nonlethal *Pc* AS [11], *Pb* XAT [12], and *Py* 17XNL [13], suggesting that IL-12 plays an important role in protective immunity via IFN-γ production in murine malaria. Production of IFN-γ and IL-12 is suppressed by anti-inflammatory cytokines such as IL-10. It is possible that enhancement of IL-10 production contributes to suppression of parasite killing, considering that IL-10 plays a detrimental role during *P. falciparum* infection.

3. Source and Biological Effect of IL-10

IL-10, an anti-inflammatory cytokine, plays an important role in regulating immune responses in hosts, as does TGF-β. The major source of IL-10 is known to be T cell subsets including Th1 cells, Th2 cells, Tr1 cells ($CD25^+Foxp3^-$), and regulatory T (Treg) cells ($CD25^+Foxp3^+$). In antigen-primed T cells, Th2 cells were originally believed to be the major source of IL-10. Stimulation of Th1 cells with IL-27 upregulates IL-10 production and enhances IFN-γ expression slightly [14, 15]. Tr1 were identified as a subset of $CD4^+$ cells that produce high levels of IL-10, low levels of IL-2, but not IL-4. They develop from naïve T cells under the influence of IL-27 [15–17]. IL-10 is also produced by naturally occurring Treg cells [18]. TGF-β induces the expression of IL-10 [19]. Moreover, IL-2, an important activator of suppressive activity by Treg cells, enhances IL-10 production [20, 21]. Today, it is known that the source of IL-10 is not only T cell subsets but also almost all leukocytes [22–25].

Apparently, monocytes/macrophages are the main target cells of inhibitory IL-10 effects [26]: IL-10 inhibits the release of proinflammatory mediators from monocytes/macrophages, and thereby inhibits the LPS- and IFN-γ-induced secretion of TNF-α, IL-1β, IL-6, IL-8, G-CSF, and GM-CSF [27, 28]. Furthermore, IL-10 inhibits the antigen presentation of monocytes/macrophages. Moreover, the IL-10-induced inhibition of IL-12 synthesis in antigen-presenting cells results in reduced IFN-γ production in T cells [29]. Actually, IL-10 inhibits both the proliferation and the cytokine synthesis of $CD4^+$ T cells, including the production of IL-2 and IFN-γ by Th1 and of IL-4 and IL-5 by Th2 [30, 31].

4. Detrimental Effect of IL-10 on the Outcome of Human and Murine Malaria Infection

High levels of IL-10 and TNF in plasma have been characteristic of young African children with malarial anemia and high levels of parasitemia [32–39]. In common IL-10 promoter variants, the -1082A/-819T/-592A (ATA) haplotype has been associated with increased susceptibility to severe anemia [39]. Their IL-10 : IL-12 ratio was higher than that in the non-ATA haplotype. On the other hand, the -1082G/-819C/-592C (GCC) haplotype has been associated with protection against severe anemia [39]. The IL-10 : IL-12 ratio in the GCC haplotype was lower than that in the ATA haplotype. These findings suggest that a high IL-10 : IL-12 ratio is associated with the downregulation of IFN-γ production and that it causes development of severe anemia during *P. falciparum* infection.

Lethal *Py* 17XL-infected mice show higher levels of IL-10 and TGF-β production than nonlethal *Py* 17XNL-infected mice early in infection [40, 41]. High levels of IL-10 and TGF-β are associated with inhibition of proinflammatory response, resulting in high levels of parasitemia, severe anemia by which RBCs ruptured, causing parasite replication and the death of infected mice. Depletion or deficiency of IL-10 [40, 42], or the blockade of IL-10 receptor [41] regulates parasitemia during lethal *Py* 17XL infection and prolongs survival of infected mice. Couper et al. [42] reported that the major source of IL-10 in lethal *Py* 17XL-infected mice is $CD4^+$ Tr1 cells, just as it is in toxoplasmosis [43] and cutaneous leishmaniasis [44] (Figure 1).

In nonlethal *Py* 17XNL-infected mice, the production of IL-10 and TGF-β is induced in the late phase of infection [41]. The population of $CD4^+$ Tr1 cells has been shown to be the major source of IL-10 in nonlethal *Py* 17XNL as well as lethal *Py* 17XL infection. Moreover, IL-10-deficient mice show marked suppression of the replication of parasites compared with that in wild-type mice [42] (Figure 1). These findings suggest that enhanced-IL-10 production suppresses inflammatory response against malaria parasites, resulting in high levels of parasitemia and anemia by replication of parasites in infected mice. Results show that IL-10 plays a detrimental role during human and murine malaria infection.

5. Role of Anti-Inflammatory Cytokines during Murine Malaria Infection

Reportedly, a low IL-10/TNF ratio is associated with severe malarial anemia [36–38]. These results suggest that low levels of IL-10 production are associated with enhancement of TNF production, followed by increased IFN-γ production. The enhancement of TNF production might be associated with the aggravation of disease severity, such as severe anemia, by which phagocytosis of uninfected RBC occurs [45], or dyserythropoiesis [46]. Moreover, results obtained using mouse models have suggested that IL-10 plays a protective role in the host during murine malaria infection. Although IL-10-deficient mice show lower levels of parasitemia than wild-type mice do during murine malaria infection, they indicate severe diseases such as hepatic pathology [42, 47, 48] and cerebral pathology [49, 50]. Actually, inflammation, which is involved in parasite killing, is upregulated in IL-10-deficient mice, but excessive inflammation, such as the increase of IFN-γ production, also presents the risk of developing hepatic pathology and/or cerebral pathology. Therefore, it seems that IL-10 might be necessary for suppression of hepatic pathology and cerebral pathology in the host during infection.

B6 mice infected with *Pb* NK65 display hepatic pathology and die within 2 weeks. The development of severe hepatic pathology is involved in IL-12 [11], IFN-γ, and $CD8^+$ T cells [9]. The IL-12 production is induced through a MyD88-dependent pathway in DCs or macrophage and engenders hepatic pathology in a perforin/granzyme-dependent manner during *Pb* NK65 infection [51]. Coinfection with nonlethal *Pb* XAT or *Py* 17XNL prevents the development of hepatic pathology caused by *Pb* NK65 infection and prolongs survival of mice [47]. In fact, IL-10 KO mice coinfected with nonlethal *Pb* XAT or *Py* 17XNL showed severe hepatic pathology, suggesting that IL-10 is involved in suppression of disease severity during coinfection [47] (Figure 2(b)). During lethal *Py* 17XL or nonlethal *Py* 17XNL infection, IL-10, which is derived from $CD4^+$ Tr1 cells, is also necessary for the prevention of hepatic pathology [42]. Nevertheless, it remains unclear whether IFN-γ and $CD8^+$ T cells are

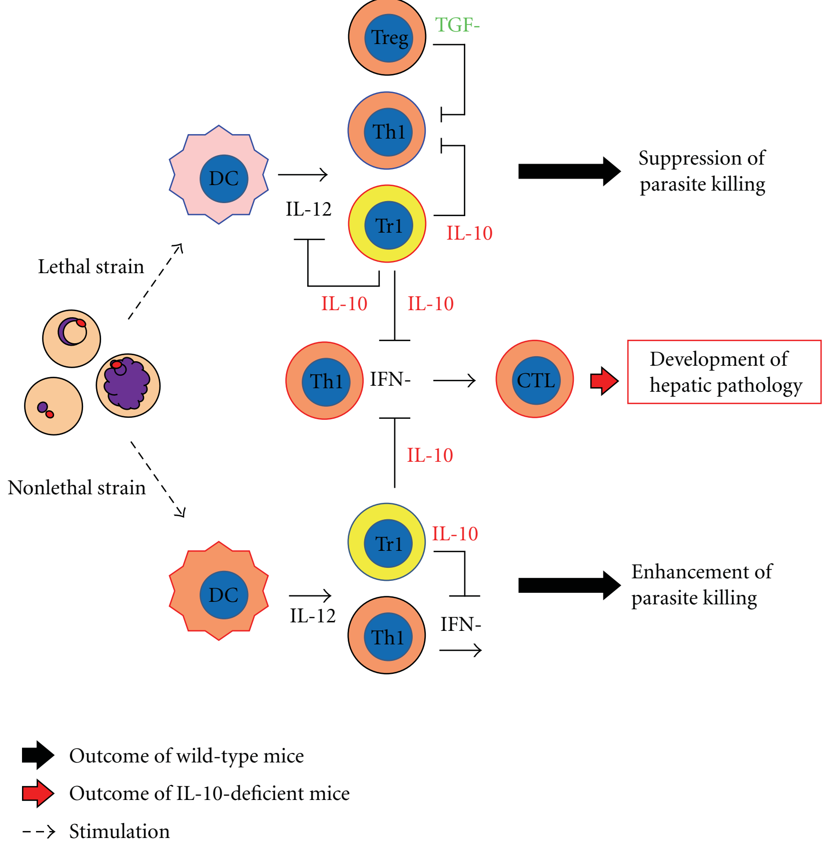

Figure 1: Scheme of immune responses during lethal *P. yoelii* 17XL and nonlethal *P. yoelii* 17XL infection: DC: dendritic cell; Th: helper T cell (CD4$^+$ T cells); Tr1, IL-10-producing CD4$^+$ T cells; Treg, regulatory T cells; CTL, cytotoxic T cells (CD8$^+$ T cells). In a lethal *P. yoelii* 17XL infection, Tr1 are induced. IL-10 inhibits proinflammatory cytokine production for parasite killing, producing high levels of parasitemia and the death of mice. Tr1 are also induced in nonlethal *Py* 17XNL as well as lethal *Py* 17XL infection. However, parasite killing occurs during nonlethal *Py* 17XNL infection. When IL-10 is ineffective (depletion or deficiency of IL-10 or the blockade of IL-10 receptor) in mice infected with nonlethal *Py* 17XNL or lethal *Py* 17XL, excessive inflammation is induced in association with the development of hepatic pathology.

associated with development of hepatic pathology in mice infected with *Py* 17XL or *Py* 17XNL (Figure 1).

Mice infected with *Pb* ANKA show similar features to human cerebral malaria (CM) regarding neurologic signs and histopathological findings, considering that *Pb* ANKA infection in mice might be an experimental model of CM (ECM) [52, 53]. Proinflammatory cytokines, such as IFN-γ and lymphotoxin-α, are known to accelerate ECM development [54] (Figure 2(c)). In contrast, anti-inflammatory cytokines, such as IL-10, prevent ECM development [55, 56]. However, it remains unclear how IL-10 suppresses ECM development, because high levels of IL-10 production were observed in spleen [57] and plasma [58] of mice singly infected with *Pb* ANKA.

The ECM development is suppressed by the simultaneous presence of murine AIDS during *Pb* ANKA infection [55]. Results demonstrated that murine AIDS-mediated protection of ECM is dependent on IL-10, which is produced by splenic CD4$^+$ T cells, with the use of anti-IL-10 mAb. It is particularly interesting that coinfection with parasites such as nonlethal *Pb* XAT [50] or Filaria [59] has also been shown to prevent ECM development. The suppressive effect of coinfection with nonlethal *Pb* XAT or Filaria on ECM during *Pb* ANKA infection was abrogated in IL-10 KO mice [50, 59], suggesting that IL-10 plays a crucial role in the suppression of ECM during coinfection with other parasites (Figure 2(b)).

In contrast to coinfection with nonlethal *Pb* XAT, the suppressive effect of coinfection with nonlethal *Py* 17XNL on ECM during *Pb* ANKA infection is independent of IL-10 [50, 60]. A recent study demonstrated that Treg cells, which are expanded by IL-2/anti-IL-2 complexes, suppress the recruitment of pathogenic CD4$^+$ and CD8$^+$ T cells to brains and protect mice from developing ECM during *Pb* ANKA [61]. The IL-2/anti-IL-2 complexes enhanced the levels of Foxp3 and CTLA-4 expression and increased the levels of IL-10 production from Treg cells during *Pb* ANKA infection. However, the suppression of ECM by Treg cells was dependent on CTLA-4 but not on IL-10 [61] (Figure 2(c)). The suppressive effect of coinfection with nonlethal *Py* 17XNL on ECM during *Pb* ANKA infection was not reversible by depleting antibodies against CD25$^+$-bearing CD4$^+$ T cells or CTLA4$^+$-bearing CD4$^+$ T cells [60]. A key factor that has a suppressive effect on ECM by coinfection with nonlethal *Py* 17XNL has not yet been discovered.

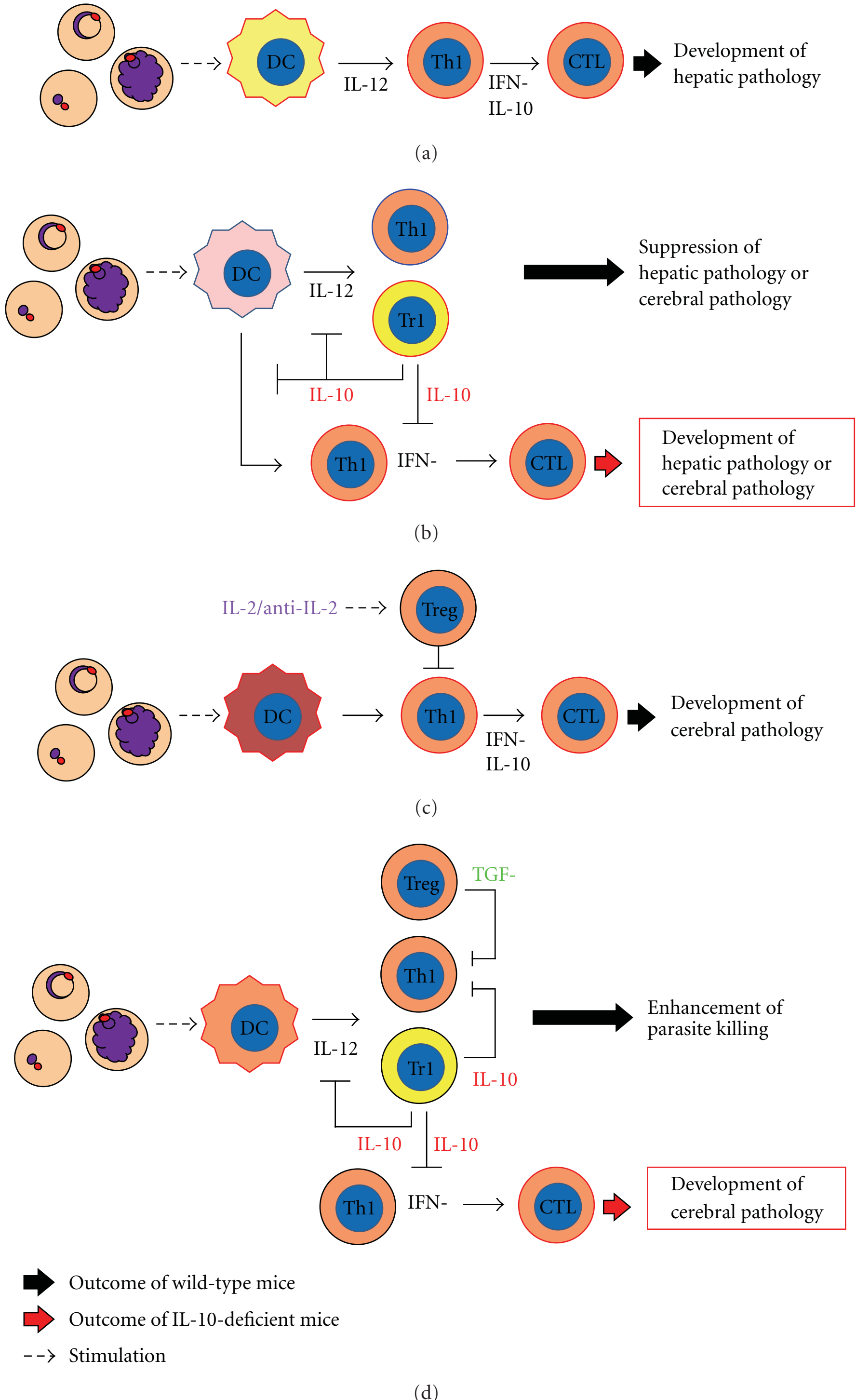

Figure 2: Role of anti-inflammatory responses during murine malaria infection. (a) Immune responses during lethal *P. berghei* NK65 infection. (b) Possible mechanism by which development of disease severity such as hepatic or cerebral pathology is suppressed by coinfection with nonlethal murine malaria parasites. (c) Immune responses during lethal *P. berghei* ANKA infection. (d) Immune responses during *P. chabaudi* AS infection. (a) and (c) A type of DC activated by lethal *P. berghei* NK65 or *P. berghei* ANKA might induce pathological Th1 and CTL. The pathological Th1 and CTL are involved in excessive inflammation and the development of severe pathology, such as hepatic pathology (*P. berghei* NK65) or experimental cerebral malaria (ECM) (*P. berghei* ANKA). (b) A type of DC activated by nonlethal malaria parasites before activation by lethal parasites might expand Tr1, but not pathological Th1, and might subsequently suppress severe disease. IL-10, which might be produced by Tr1, inhibits proinflammatory cytokine production and expansion of pathological Th1 during coinfection. Therefore, coinfected IL-10 deficient mice develop severe pathology, such as hepatic pathology or ECM. (d) In *P. chabaudi* AS infection, IL-10 from Tr1 and Treg are associated with suppression of proinflammatory cytokine production and expansion of pathological Th1. A deficiency of IL-10 or TGF-β contributes to development of ECM.

Development of severe disease such as hepatic pathology and cerebral pathology generally involves excessive inflammation in murine malaria parasites. Little is known about the differences between the developmental mechanisms of hepatic pathology and cerebral pathology during *Pb* NK65 and *Pb* ANKA infection, respectively. However, IL-10 can downregulate excessive inflammation during *Pb* NK65 or *Pb* ANKA infection. It is associated with the suppression of hepatic pathology and cerebral pathology. Results show that IL-10 plays a protective role in the host during *P. falciparum* infection.

6. A Different Type of DC Induced by Lethal and Nonlethal Murine Malaria Infection

How are different subsets of $CD4^+$ T cells, such as pathological $CD4^+$ T cells [62, 63], IL-10-producing $CD4^+$ T cells [42], and Treg cells [64], induced between lethal and nonlethal murine malaria infection? The development of CM is inhibited completely by the simultaneous presence of nonlethal *Py* 17XNL [60], lethal *Pb* K173 [57], and nonlethal *Pb* XAT [50]. However, protection from CM was not induced in mice when they were infected with *Py* 17XNL on day 4 after *Pb* ANKA infection [60]. Similarly, coinfection with *Pb* XAT on day 1 or day 3 after infection with *Pb* ANKA failed to protect mice from cerebral malaria (Niikura et al. unpublished data). In simultaneous infection with *Pb* ANKA and *Pb* K173, suppression of ECM was associated with the induction of cytokines such as IFN-γ, IL-10, and IL-12 on day 1 after infection [57]. These findings suggest that the presence of other parasites might modulate some key factors/cells that are involved in innate immunity in early infection with *Pb* ANKA. Actually, DCs are important for initiating immune responses against malaria parasites. It is possible that immune responses induced by DCs produce protective and pathological effects, respectively, when mice are infected with nonlethal and lethal parasites. Therefore, DCs might contribute to the determination of the virulence of malaria parasites. In coinfection, a type of DC activated by nonlethal malaria parasites before activation by lethal parasites might fail to expand pathological $CD4^+$ T cells and subsequently fail to suppress severe disease.

Wykes et al. [13] showed that although DCs from mice infected with nonlethal *Py* 17XNL were fully functional, DCs from mice infected with lethal *Py* YM were unable to produce IL-12 or present antigens to T cells. Apparently, lethal malaria causes a failure of DC function, resulting in the suppression of Th1 immune responses (Figure 1). Similar to lethal *Py* 17XL infected mice, it is possible that mice infected with lethal *Py* YM induce IL-10-producing $CD4^+$ T cells. Although IL-10 might inhibit the DC function, such as antigen presentation and release of proinflammatory cytokines, little is known about whether IL-10 associates with a different type of DC induced between lethal and nonlethal murine malaria infection. Toll-like receptors (TLRs) play an important role in the innate immune system against pathogens [65]. Therefore, TLRs might be associated with disease severity during malaria infection. During lethal *Py* 17XL infection, TLR9 signaling in DCs is known to be crucial for the activation of Treg cells that suppress Th1 immune responses, causing high levels of parasitemia [64]. In contrast, MyD88, but not TLR signaling, has been shown to be necessary for elimination of parasites in mice infected with nonlethal *Py* 17XNL [66]. Accordingly, a different type of DC induced between lethal and nonlethal murine malaria infection might induce different subsets of $CD4^+$ T cells, such as IL-10-producing $CD4^+$ T cells or Treg cells.

7. Is IL-10 Necessary for Host Protection against Murine Malaria Parasites?

Although IL-10-deficient mice suppressed an increase of parasitemia during coinfection with lethal and nonlethal parasites, mice were unable to eliminate parasites completely and eventually died [47, 50]. These results suggest that the lethal strains of malaria parasites may modulate the induction of adaptive immunity independent of IL-10. Millington et al. [67] demonstrated that *Plasmodium* infection inhibits the induction of adaptive immunity to heterologous antigens by modulating DC function. According to their paper, hemozoin (HZ), rather than infected RBC membranes, was a key factor involved in the suppression of murine DC function. On the other hand, it has been shown that repeated stimulation through TLR9, which is the receptor for HZ, engenders tolerance to signaling through TLR4 [68].

In fact, HZ activates DCs through the TLR9-MyD88 pathway [69]. A recent study has demonstrated that parasite protein-DNA complex, but not HZ, plays a crucial role in TLR9-mediated activation of DCs during infection [70]. Stimulation through TLR9 might be associated with development of severe hepatic pathology, because MyD88 pathway, which is activated by TLR9 stimulation, is known to be involved in severe hepatic pathology caused by *Pb* NK65 [51]. Coban et al. [71] and Griffith et al. [72] reported that the TLRs-MyD88 signaling pathway might play a critical role in ECM during lethal *Pb* ANKA infection. It has been shown that ECM is prevented by oral treatment with E6446, which is a synthetic antagonist of nucleic acid-sensing TLRs [73]. In contrast, it is demonstrated that murine cerebral malaria development is independent of Toll-like receptor signaling [74, 75]. It remains controversial whether TLRs-MyD88 signaling pathway is associated with ECM development.

In summary, IL-10 is necessary for suppression of hepatic pathology or ECM in the host although IL-10 entails a risk of downregulation of protective immunity against malaria parasites. $CD4^+$ T cells of different kinds, such as pathological $CD4^+$ T cells, IL-10-producing $CD4^+$ T cells, or Treg cells, are induced during different kinds of *Plasmodium* spp infection. To induce a more effective immune response in host defense against *Plasmodium* spp, it is necessary to elucidate the interaction of innate and acquired immune cells such as DCs, $\alpha\beta$ T cells, and $\gamma\delta$ T cells.

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Research Article

Sex Steroids Effects on the Molting Process of the Helminth Human Parasite *Trichinella spiralis*

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We evaluated the *in vitro* effects of estradiol, progesterone, and testosterone on the molting process, which is the initial and crucial step in the development of the muscular larvae (ML or L1) to adult worm. Testosterone had no significative effect on the molting rate of the parasite, however, progesterone decreased the molting rate about a 50% in a concentration- and time-independent pattern, while estradiol had a slight effect (10%). The gene expression of caveolin-1, a specific gene used as a marker of parasite development, showed that progesterone and estradiol downregulated its expression, while protein expression was unaffected. By using flow citometry, a possible protein that is recognized by a commercial antiprogesterone receptor antibody was detected. These findings may have strong implications in the host-parasite coevolution, in the sex-associated susceptibility to this infection and could point out to possibilities to use antihormones to inhibit parasite development.

1. Introduction

The sex steroids hormones, 17β-estradiol (E_2), progesterone (P_4), and testosterone (T_4), act upon the reproductive system of mammalians by binding to specific sex steroid receptors, which determine changes in reproductive physiology and behaviour [1, 2]. Recently, it has been shown that sex steroids participate not only in reproductive physiology, but also in a number of different functions, which include immune modulation, brain activity, bone metabolism, and lung and heart physiology. Moreover, also strong direct effects of sex steroids on parasites to modulate different parasite functions have been reported [3–7].

Recent information reveals that sex hormones can affect the course of worm infection [8–12], as in the case of the cestodes *Taenia crassiceps* and *Taenia solium* [13–15]. In line with this statement, it is known that the frequency of *T. solium* pig cysticercosis is increased during pregnancy, when there is a significant increase in progesterone levels [13, 16]. It has also been demonstrated that the castration in naturally infected male boars induces an increase in the prevalence of cysticercosis, which highlights the possible role of host androgens to restrict parasite establishment and estrogens to facilitate it [13]. Furthermore, another helminth, *T. crassiceps* (a close relative of *T. solium*) has shown to be affected by *in vitro* sex steroid treatment. Specifically, 17β-estradiol increases the reproduction of *T. crassiceps* parasites, while testosterone or dihydrotestosterone decreases it [17]. *In vivo,* when castrated mice are treated with 17β-estradiol, the number of parasites as well as their infective capacity

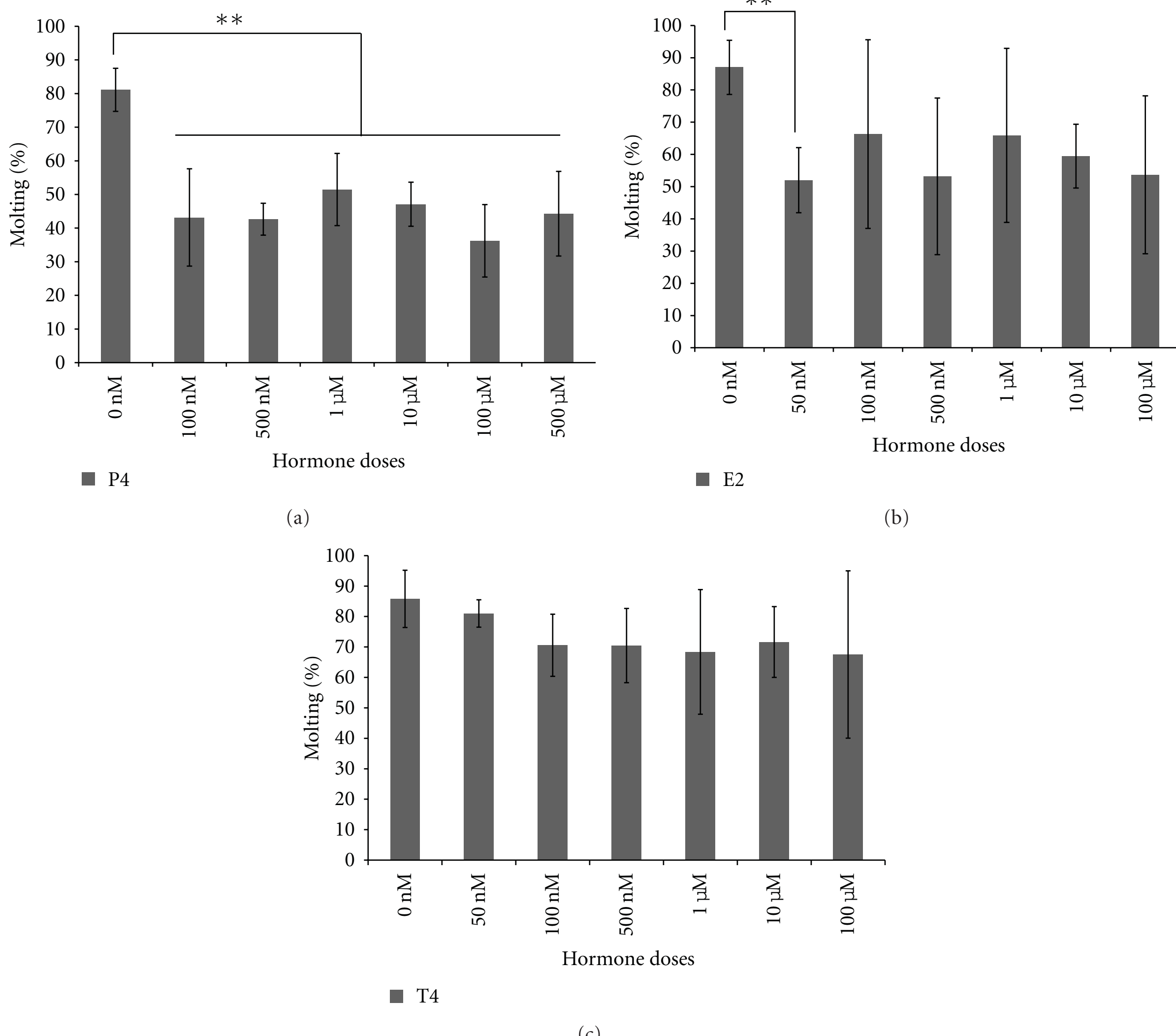

Figure 1: Dose-dependent curves of sex steroid hormones on *in vitro* molting of muscle larve of *T. spiralis*. One hundred muscle larve (ML) of *T. spiralis* were incubated for 36 hours with different concentrations of progesterone (P4), estradiol (E2), or testosterone (T4). Progesterone inhibited the molting rate of the muscle larva of *T. spiralis* in a concentration-independent pattern (a). Estradiol inhibit only at 50 nM concentration (b), while testosterone has no any effect on molting (c). Each point represents the mean (SD) of quintuplicate determinations of the number of larvae in molting process.

increases up to 200% [7, 18] meanwhile progesterone has the opposite effect in castrated mice of both sexes: a decrease in the parasite loads of almost 100% [19].

T. spiralis is an intracellular parasitic nematode of mammalian striated muscles. The life cycle of this parasite is completed within a single host and the parasite resides in two distinct intracellular habitats. The infective stage of *T. spiralis* is part of a nurse cell-larva complex found in striated muscle of prey eaten by carnivores. Digestive enzymes in the stomach release the larva from the muscle tissue and the parasitic L1 migrate to small intestinal sites at the base of villi where they reside in a syncytium of epithelial cells.

Consistent with the level of coevolution evident from parasite adaptation to the host is the assumption that *T. spiralis* can exploit the hormonal microenvironments within the host [8]. This suggests a system of transregulation (term coined by us) in which the parasite exploits host hormones and growth factors to facilitate infection and potentially increase growth and reproduction rates; this process has been described in at least eight parasitic infections that are caused by both protozoan and metazoans [10]. Furthermore, endocrine factors, related to sex and age, are well recognized as modulators of the immune response [11, 12] and by having a direct effect over the parasite. Thus, sex steroid hormones play key roles in the susceptibility to trichinellosis at two levels: (a) protective immune response or (b) direct effect on parasite development [11, 15]. Steroid hormone effects are not only restricted to cestode parasites, but also to nematodes such as *Ancyclostoma dudodenale*, whose number of larval and adult stages is increased by sex steroid hormones in several organs of mice [20]. Moreover, adult and muscle larvae of *T. spiralis* are increased in ovarectomized female

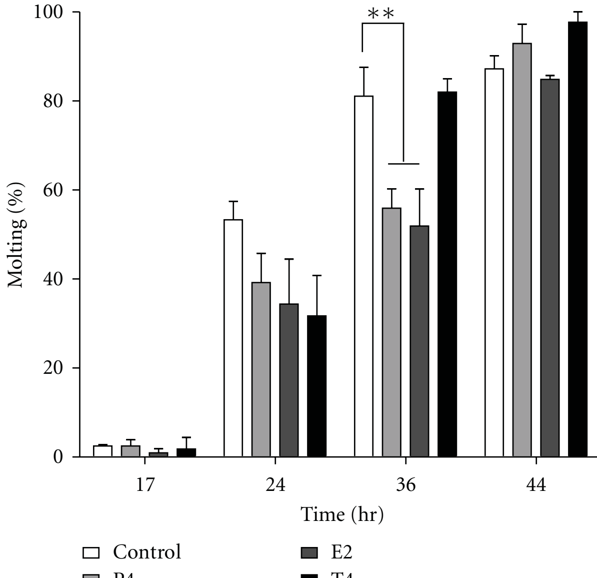

Figure 2: Time curves of *T. spiralis* molting process after exposure to progesterone (P4), estradiol (E2), and/or testosterone (T4). The parasites were cultured for 44 hours. Progesterone and estradiol had 35–50% of inhibitory effect on molting rate compared with control and testosterone. The maximum inhibitory effect was at 36 hr of culture. Each point represents the mean (SD) of 5 assays counting the number of molting larvae and the mean was obtained. $^{**}P <$ 0.05. The hormone concentration was as follows: P4, 100 mM; E2, 50 nM, and T4, 50 nM.

rats [10], suggesting that estrogens and progesterone are restrictive factors for parasite establishment, while androgens should play a permissive role to the infection. In the case of the nematode *T. spiralis,* males are generally more susceptible than females to the infection, since in mice the males present more parasite burden at both intestinal and muscle level than females do [21–23]. This finding may represent an interesting approach in trichinellosis by *T. spiralis*, because if we know that sex steroids can specifically down-regulate genes involved in the fecundity and oviposition of the parasite, we can propose the use of sex steroid analogous to modulate this effect. Taking into consideration this information, the aim of the present study was to explore the role of sex steroids on *T. spiralis* development, evaluating its *in vitro* effects on the molting process, which is the key in the maintenance of the infectious cycle in the host. The *in vitro* effect of progesterone, estradiol, and testosterone on *T. spiralis* was studied through pharmacological and molecular (RT-PCR, immunohistochemistry and flow cytometry) approaches, in order to figure out the mechanism of sex steroids actions in the parasite.

2. Materials and Methods

2.1. Obtention of Parasites. T. spiralis was maintained in the laboratory by serial passage infections in Wistar rats. The infective-stage ML were recovered as described in [24]. Briefly, the carcass of experimentally infected mice at 30 days of infection was digested by a standard pepsin-hydrochloric acid digestion method to obtain LM stage.

2.2. Sex Steroids Dose-Response Time Curves. Culture grade estradiol, progesterone, and testosterone were obtained from Sigma (Sigma-Aldrich, USA). For *in vitro* tests, estradiol and progesterone water soluble (Sigma-Aldrich, USA) were dissolved in RPMI 1640 free serum culture medium (Gibco) at desired stock concentration and sterilized by passage through a 0.2 mm millipore filter. Testosterone was dissolved in absolute ethanol to the desired stock concentration and sterilized by passage through a 0.2 mm millipore filter. For cultures, the experimental design was as follows: 100 muscular larvae were cultured in 24-well plates and 6 wells per condition were used. Groups designed were as follows: control (only RPMI); control vehicle (EtOH-RPMI); estradiol; progesterone; testosterone. For time-response curves, parasites from all treatments were cultured during 48 hr, with medium change at 24 hr, using optimal physiological concentration for estradiol (50 nM), progesterone (100 nM), and testosterone (50 nM). Culture wells contained 2 mL of RPMI medium and were incubated at 37°C and 5% CO_2. Steroids were prepared to add 100 μL to 5 mL of the medium in each well to final concentration. From concentration-response curves of each steroid, we selected physiological concentration for estradiol, progesterone, and testosterone, as well as, increased concentration of each in pharmacological concentration (above 10 μM concentration). Survival and molting were determined under light microscopy using Axiovert Ziess Microscope and 25x Neo Plan objective.

2.3. Morphologic Analysis of T. spiralis Treated with Sex Steroids. T. spiralis ML cultured with or without hormones were observed at different hours under light microscopy using Axiovert Ziess Microscope and 25x Neo Plan objective. The microphotographies generated were modified and contrasted using a software image (Adobe Photoshop CS3, US).

2.4. RNA Extraction of Cultured Parasites in Presence of Sex Steroids. Total RNA was isolated from *T. spiralis* of each *in vitro* treatment (positive expression control) using Trizol reagent (Invitrogen, Carlsbad, Calif.). In brief, parasites were homogenized in Trizol reagent (1 mL/0.1 g tissue), and 0.2 mL of chloroform was added per mL of Trizol. The aqueous phase was recovered after 10 min of centrifugation at 14,000 g. RNA was precipitated with isopropyl alcohol, washed with 75% ethanol, and redissolved in RNAse-free water. Total RNA concentration was determined by absorbance at 260 nm and its purity was verified after electrophoresis on 1.0% denaturing agarose gel in presence of 2.2 M formaldehyde. Total RNA was stored at −70°C

2.5. Immunohistochemistry of Caveolin-1 (CAV-1) in the Parasite. T. spiralis ML obtained at 36 hr of culture with or without the sex steroids were embedded and frozen in tissue freezing medium (Triangle Biomedical Science, USA) at −70°C. Four-micrometre-thick sections of parasites were fixed with 4% paraformaldehyde for 30 min, permeabilised

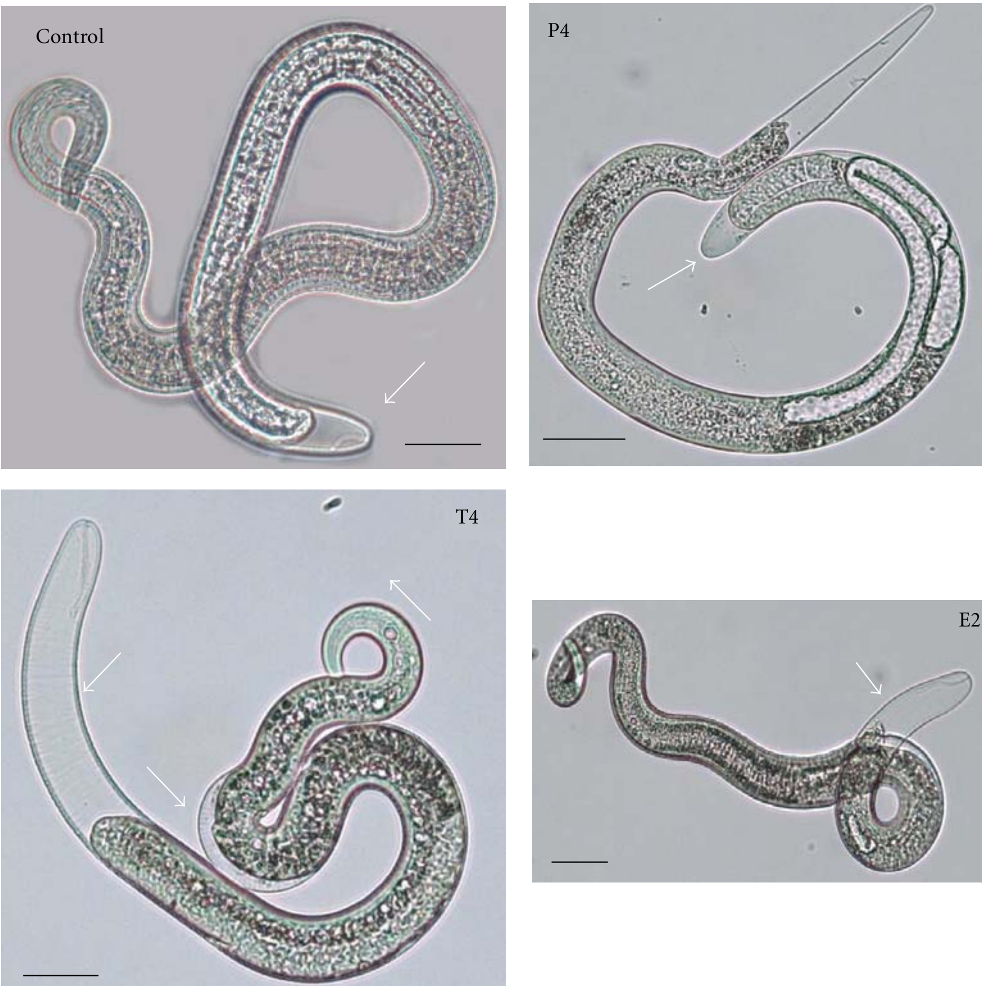

Figure 3: Micrographies of ML parasites cultures in presence of sexual steroids. Parasites cultured at 36 hr with progesterone (P4, 100 mM), estradiol (E2, 50 nM), or testosterone (T4, 50 nM) or in absence of sex steroid (control) were observed in Axiovert microscopy using 25x objective. In all cases, the old cuticle starts to detach (clear zone) at the apical or basal of the parasite (arrows).

in 1% PBS-SDS and blocked with RPMI medium containing 0.5% BSA and 5% FBS, as described in [25]. Cross-sections were incubated with the previously obtained polyclonal anti-Ts-Cav-1 antibody and then incubated with the secondary antibody, fluorescein-isothiocyanate-(FITC-) conjugated goat anti-mouse antibody (Sigma-Aldrich) [25]. Control experiments were performed by incubation secondary antibody. The background fluorescence in samples was reduced by contrast with 0.025% Evans Blue. Samples were analysed in a Leica TCSSP5 confocal laser-scanning microscope (Leica Microsystems, Germany). The images were constructed using Leica Confocal software.

2.6. Flow Cytometry to Detect Progesterone (PR) Receptor in Parasites Cells. *T. spiralis* cells were extracted by tissue disruption using a micropestle (Eppendorf, USA) until no more clumps were visible. Cells were centrifuged at 300 g for 5 min and recovered in PBS. Cells were stained with the following antibodies for 10 min at 4°C: anti-mouse CD3-FITC, and anti-mouse CD19-PE, and anti-mouse Mac-1 (all from BD Biosciences). For intracellular staining, cells were initially fixed with 2% paraformaldehyde solution for 10 min at 37°C, permeabilized with methanol 100%, washed twice with 500 μL of staining buffer (PBS pH. 7.4, 2% Fetal Bovine Serum, 0.02% NaN_2), and stained with anti-caveolin-1 and anti-PR (Santa Cruz Biotechnology, USA). For primary antibodies recognition, cells were stained with FITC coupled anti-mouse IgG and PE-coupled anti-rabbit IgG for 10 minutes at room temperature, washed, and stored until the analysis is protected from light. Ovary-derived cells were stained with anti-PR as described for *T. spiralis* cells. All samples were analyzed by flow cytometry using an FACS Calibur (BD, Biosciences, USA) and data analyzed using the FlowJo software.

2.7. Experimental Design and Statistical Analysis. Hormone dose-response time curves were estimated in 4 independent experiments. The response variable used in statistical analyses was the total number of the molted parasites that showed motility in all wells of each hormone concentration

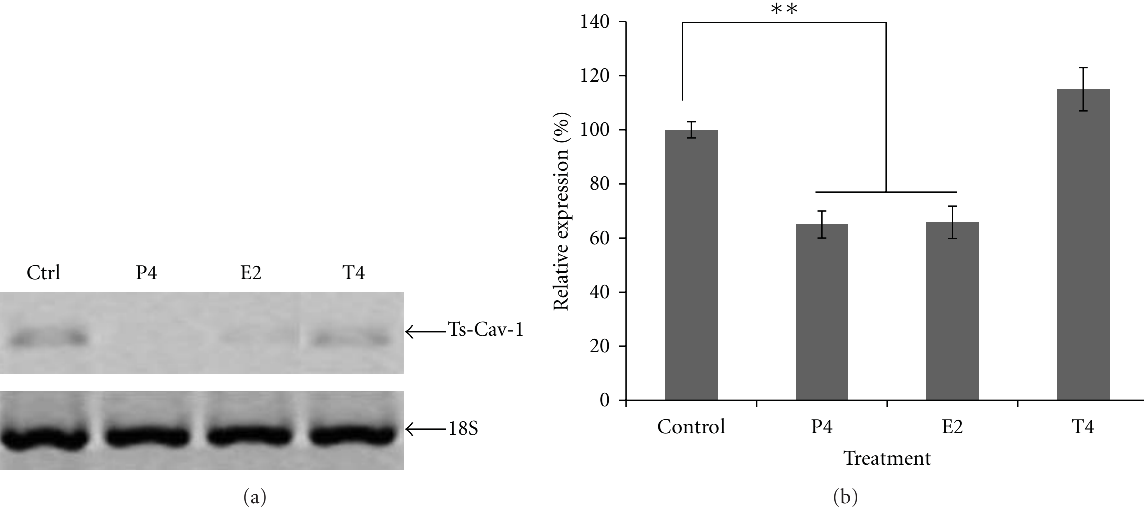

FIGURE 4: Ts-Cav-1 gene expression in *T. spiralis* ML cultured in presence of sex steroids. A single band of 306 bp, corresponding to the caveolin-1 of *T. spiralis,* was detected in all parasites cultured at 36 hr (a). Progesterone and estradiol reduced the expression level of this gene at least 40%, while testosterone has no effect on the expression of this gene (b). Densitometric analysis is shown (b). 18 S was used as constitutive expression gene. Data are represented as mean ± SD. $^{**}P < 0.05$.

and time of exposure, for every experiment. Hormones, their concentrations and times of exposure were the independent variables. The data for the 4 replicates of each treatment were expressed as an average. Data were analyzed using one-way variance analysis (ANOVA). If ANOVA showed significant differences among treatments, a Tukey Test was applied for test significance. Differences were considered significant when $P < 0.05$.

2.8. Ethics Statement. Animal care and experimentation practices at the Instituto de Investigaciones Biomédicas are frequently evaluated by the Institute Animal Care and Use Committee, and by governmental (official Mexican regulations (NOM-062-ZOO-1999)), in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health of the USA, to ensure compliance with established international regulations and guidelines. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Instituto de Investigaciones Biomédicas. Rats were sacrificed to obtain the L1 using sevorane as anesthesia, and all efforts were made to minimize suffering.

3. Results

When *T. spiralis* parasites were *in vitro* exposed to progesterone, a decrease in the molting process rate about 35–50% was observed in all treated parasites compared to control groups (Figure 1(a)). However, this molting-inhibiting effect mediated by progesterone was independent of the tested concentrations (Figure 1(a)). For estradiol, an inhibitory effect was observed for the lowest concentration, but just an inhibitory tendency was observed in all cases independent of the culture concentration tested, without any significant differences (Figure 1(b)). In the case of testosterone, there was no apparent effect on the molting of *T. spiralis in vitro* (Figure 1(c)).

Concomitantly, the molting-inhibiting effect of progesterone (100 nM) and estradiol (50 nM) was maintained through all 40 hours of *in vitro* culture, reaching its highest response on 36 h in culture, in relation to untreated parasites (Figure 2). Consistently, when parasites were exposed to testosterone (50 nM), no differences on the percent of molting were observed neither during the first hours of culture nor at the end of the process (Figure 2).

It is important to mention that viability of the molted parasites was verified daily by means of worm motility in the culture plate, which was constant through all days of *in vitro* culture. Injured parasites were recognized by a progressive internal disorganization: development of clear areas inside of the parasite and increasing number of internal vesicules (Figure 3). Differentiated worms in absence of hormones had a normal development, reaching their tipical shape characteristic of adult at 40 hours in culture and have a normal internal organization and the molting process is ongoing well. Once again, in the presence of estradiol and progesterone, the molting rate is decreased, while in the testosterone-treated parasites, the molt rate is slightly accelerated (Figure 3).

In order to look for molecular effects of sex steroids, we sort out the expression of caveolin-1 (Cav-1), which is a marker protein for oocyte development in the female worm of *T. spiralis*. A single band corresponding to the expected molecular weight of the amplified fragment of Cav-1 (approximately 306 bp) was detected from *T. spiralis* control and treated with sex steroids (Figure 4). Relative expression of the mRNA of the Cav-1 gene in the parasite revealed that estradiol and progesterone decrease the expression by 40%, while treatment with testosterone increased the same by 10% (Figure 4).

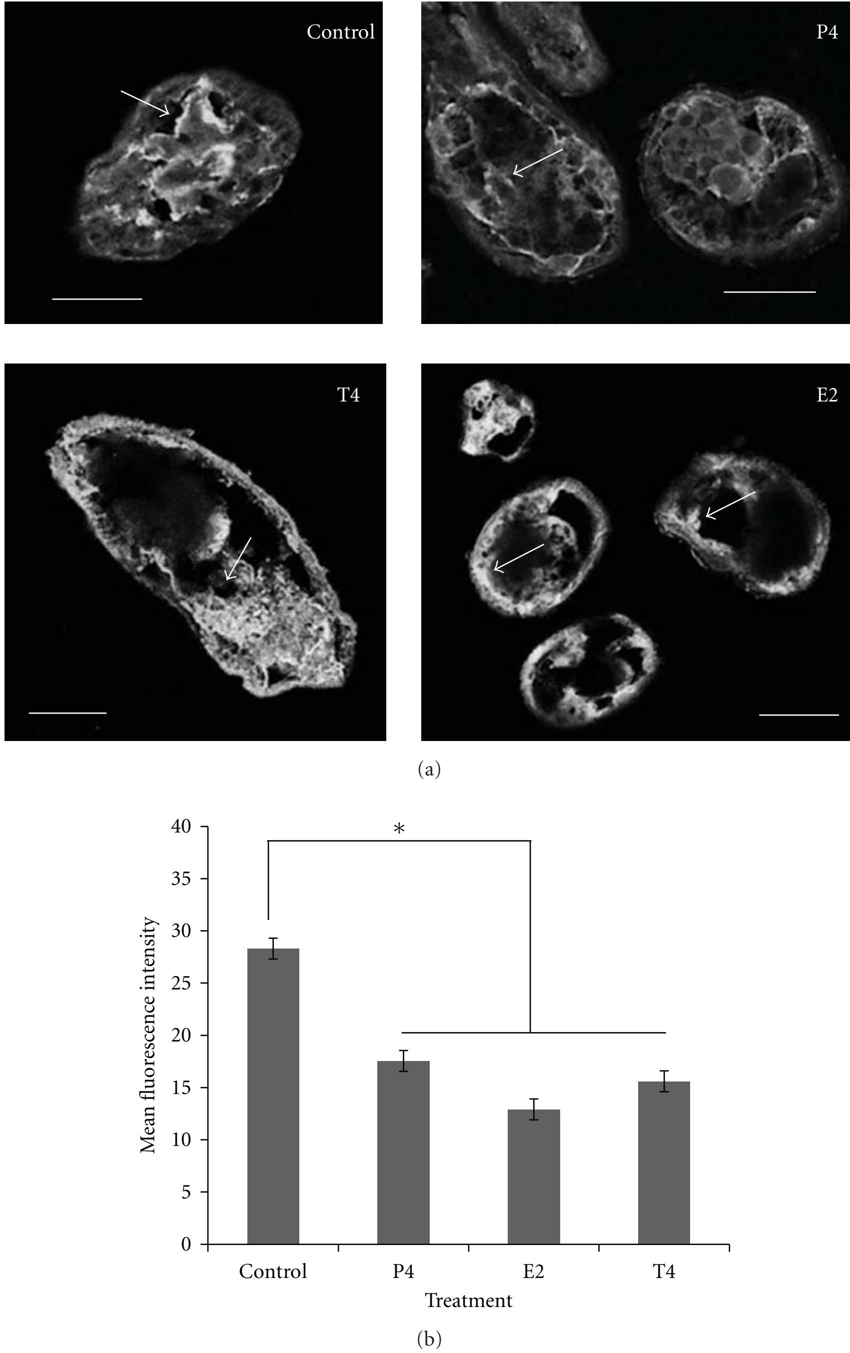

FIGURE 5: Indirect immunofluorescence of Ts-Cav-1 protein expression in muscle larvae (ML) cultured. Four-micrometre sections from parasites cultured without or with sex steroids (P4, E2 or T4) were treated with anti-Ts-Cav-1 antibody and fluorescein isothiocyanate-conjugated goat anti-mouse antibody and observed under confocal laser microscopy. In all cases of parasite treated there was no changes on Ts-Cav-1 protein expression. Bars = 25 μm.

Interestingly, when we look for the expression pattern of Cav-1 by immunohistochemistry, there were no apparent differences between the untreated parasites and the ones exposed to sex steroids (Figure 5(a)). When we quantified this pattern of expression, this pattern was confirmed, since quantification of cells expressing Cav-1 was no different when we compared between treatments (Figure 5(b)).

In order to determine if the effect of progesterone was mediated by a putative progesterone receptor in the parasite, we perform flow cytometry using commercial anti-PR antibodies. As shown in Figure 6(a) (upper row), *T. spiralis* cells do not express immune cell markers such as CD3, CD19, or Mac-1, which are typically present in some types of mammalian leukocytes, which suggest that there

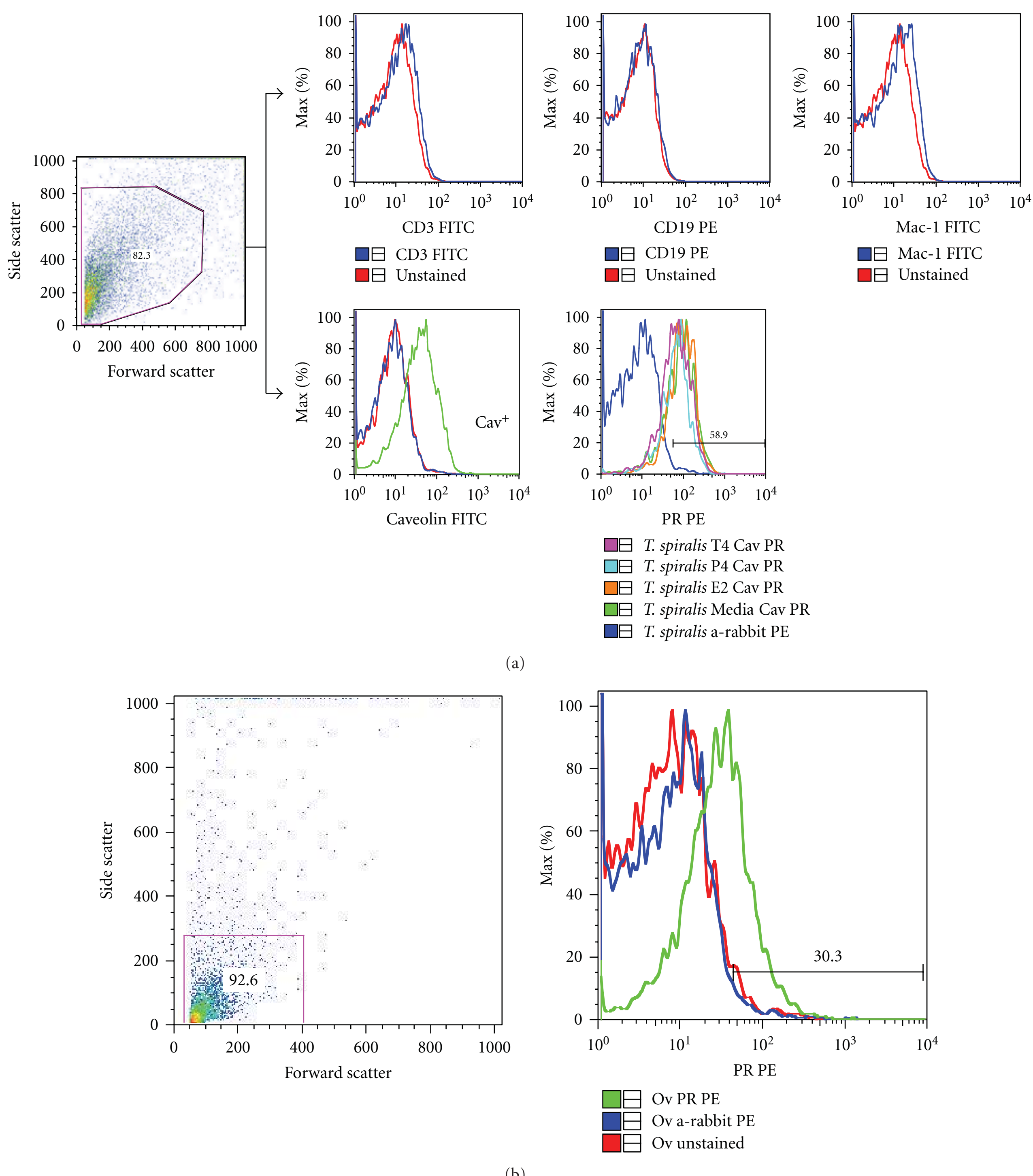

Figure 6: Progesterone receptor (PR) and host markers expression on *T. spiralis* cells. (a) *T. spiralis* cells were stained with anti-mCD3, anti-mCD19, and anti-mMac-1 (upper row); cultured in presence of media (green line), E2 (orange line), P4 (blue line), and T4 (pink line) and stained with anti-Cav-1 of *T. spiralis* and anti-PR specific antibodies (middle row). (b) Ovary-derived cells were stained with anti-PR antibody as a positive control for PR.

is no contamination with host cells. We then analyzed the expression of the classic progesterone receptor (PR) on parasitic cells that expressed caveolin-1 (a protein cloned, sequenced, and expressed exclusively by this parasite), in response to the steroid hormones estradiol, progesterone, and testosterone. As shown in Figure 6(a) (middle row), cav^+ cells showed a specific binding of anti-PR antibody, which suggested the presence of a PR-like receptor of parasitic

origin. PR expression was also detected in ovary, as an expression control tissue (Figure 6(b)).

4. Discussion

Estrogens and androgens play an important role at different system levels for maintenance of homeostasis in vertebrates. They are implicated not only on the reproductive behavior and physiology, but also act as modulators of immune system, brain activity, and lung and heart physiology. In the last years, increasing information reveals that sex hormones can affect the course of parasite infection [8, 9, 11, 12] by modulating the Th1 or Th2 response to lead a susceptibility/resistance to infections. In that way, sex differences on parasitemia can reflect the suppressive effects of testosterone and the increasing effects of estrogens in the immune system [26], principally on Th2 immune responses in females leading to a higher production of interleukins (i.e., IL-4, -5, -6 and -10) [27]. Therefore, males of several species are more susceptible to infections caused by different parasites as well as the prevalence and intensity of parasitic infection than females do [28, 29]. For example, male mice are less resistant to protozoan like *Plasmodium berghei* [30] or *Trypanosoma cruzi* [31] or nematodes like *Strongyloides sp*. [32] than females, while the administration of potent estrogenic compounds as estradiol increased the resistence to the parasite [31–33]. Otherwise, progesterone protects mice from *T. crassiceps* infections [19].

Particularly on *T. spiralis* infections, it has been described a resistance/susceptibility associated to gender's host; in which males are more susceptible than females [22, 23]. Interestingly, in pregnant rats, where progesterone levels is increased, the resistance to *T. spiralis* infections throught parasite loads in muscle are increased compared to parasite loads in muscle observed in virgin rats. This effect was mediated by the ability of sera to mediate death in newborn larvae (NBL) of *T. spiralis* in antibody-dependent cell cytotoxicity assays [34]. Also, progesterone can activate effector peritoneal cells to eliminate NBL in a rapid and antibody-independent manner [35]. However, the intervention of the host's immune response in dealing with the parasite and the possibility of additional direct effects of sexual steroid hormones on the parasite's physiology should not be hastily discarded. Here we describe the effects of progesterone, estradiol, and testosterone upon the molting process of *T. spiralis* larvae. First of all, it was clear that progesterone has a direct inhibitory effect on the percent of worms that are going through the molting process *in vitro*. In fact, progesterone exerts a marked molting-inhibitory effect in a concentration-independent pattern, maintained entire time in culture, making that all parasites differentiate at 36 hours of *in vitro* culture.

T. spiralis parasites not only showed molting, but they also presented a constant motility in the culture plate, which suggests that neither estradiol nor progesterone affected parasite viability and therefore they were alive during the culture process. Moreover, progesterone induced a decrease in the rate of the molting process of the worm in the cultured parasites, with respect to untreated parasites. Estradiol and progesterone inhibited the expression of Ts-Cav-1 in the LM, one gene that is implicated in the maturing and development of the NBL [25]. This observation result is important because it suggests that the LM of *T. spiralis* could modify the expression of specific genes through steroid receptors similar to those found in other invertebrates [36, 37]. However, no differences on expression of Ts-Cav-1 protein were observed. One explanation is because the Ts-Cav-1 protein is a molecule that is accumulated in the plasma membrane of the parasite's cells, principally oocytes. We thought that this protein generated in all treatments during the first hours of being cultured, was cumulated in the membrane, and for 36 hr of culture, we do not see any difference in the accumulated protein, although existed a down-regulation in the gene expression at this time on estradiol or progesterone cultures existed. Interestingly, neither percent molting nor molting rate depends on testosterone concentrations. These findings, could explain in part the decreased parasite loads observed in females on infection with *T. spiralis* and even in pregnant females: sex steroids present in the pregnant female environment avoid the normal rate of molting process on the LM to adult female which directly impacts in the potential adult female that releases NBL. Our results contribute to the knowledge of the mechanisms by which the host microenvironment affects the parasite and parasite protein regulation and expression. As it can be seen, direct effects of sex steroids upon helminth parasites (cestodes, nematodes, and trematodes) are not unusual. In fact, previous results suggest that these pathogens are not only directly affected by hormones, but they have also developed several strategies to exploit the host's endocrine microenvironment [4, 38], which include degradation of host proteins as an alternative source of aminoacids [39], development of parasitic-sex steroid receptors [40, 41], and cross-activation of signal transduction pathways [42, 43].

5. Conclusions

Here, we describe different effects of sex steroids that probably have different action mechanisms directly upon *T. spiralis*. Progesterone effects could explain, at least partially, the low parasite burdens observed on experimental trichinellosis infection in pregnant and in nonpregnant rats [34]. To our knowledge, this is the first report where antitrichinella effects are described for sex steroids and open a promissory field in the design of new strategies that include the antihormone therapy in the control of trichinellosis caused by *T. spiralis*.

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Review Article

Schistosome: Its Benefit and Harm in Patients Suffering from Concomitant Diseases

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Schistosomiasis is an important tropical disease affecting approximately 200 million people worldwide. Because of its chronicity and robust immunomodulatory activity, the effects of schistosomes on other diseases, such as allergies, autoimmunity, and infectious diseases, have been studied extensively in both epidemiological and experimental settings. In this paper, we summarize the beneficial and harmful effects of schistosomes. The importance of controlling schistosomiasis is also discussed.

1. Introduction

Schistosoma spp., blood flukes, are parasitic helminths found mainly in developing countries with a tropical or subtropical climate and affect 200 million people worldwide [1]. *Schistosoma mansoni*, *japonicum,* and *mekongi* harbor in veins of the portal system and lay eggs in the blood vessels. The deposition of numerous eggs in the intestines and liver results in intestinal and hepatic granulomatous lesions, fibrosis, portal hypertension, and hepatosplenomegaly. In contrast, *Schistosoma haematobium* mainly harbors in the venous plexus of the bladder and/or rectal venous plexus. This worm usually causes bloody urine but is also considered to have an etiological relationship with bladder cancer [2]. Because of the extensive distribution of schistosomes and morbidity due to egg deposition, researchers have been interested in the influences of schistosome infections on concomitant diseases [3]. As mentioned, *S. haematobium* is an important carcinogenic factor of bladder cancer although the worm itself does not have mutagenic activity [4]. This parasite is also suggested to be a risk factor for the transmission of HIV [5]. These effects are attributable to pathological lesions caused by the parasites. On the other hand, most helminthic parasites including schistosomes are known to induce robust Th2-polarization. Especially in schistosome infections, egg deposition in the host tissues was reported to be the major stimulus of Th2 responses [6], and egg proteins (e.g., omega-1, IPSE, and peroxiredoxin) are involved in the Th2-biasing activity [7–11]. In addition, schistosome eggs have immunomodulatory potential inducing the alternative activation of macrophages [12] and regulatory T-cell expansion [13]. Because of its robust systemic Th2-inducing and immunomodulatory ability, this worm has been studied most extensively for its bystander effects on various immunological phenomena *in vivo*. In this paper, we summarize the effects of a concurrent infection of schistosomes on immunological disorders and parasitic/microbial infections.

2. Effects of Schistosomes on Immunological Disorders or Infectious Diseases in Mice and Humans

2.1. Allergy. Effects of helminths (including schistosomes) on allergic diseases have been studied extensively in both experimental and epidemiological settings. In experimental asthma or airway hypersensitivity models, schistosome infections and antigen administrations have been consistently shown to protect the animals from the diseases (Table 1). In most studies on the antiasthmatic effects of schistosomes, cellular infiltration (including eosinophils) into bronchoalveolar lavage fluid (BALF) was diminished and simultaneously IL-4, IL-5, and IgE levels were reduced. In contrast, an increase in Treg cells and augmentation of IL-10 production

were observed. Fallon and Mangan [14] designated this kind of Th2 response (Treg dominant and IL-10 dominant) a "modified Th2 response" as opposed to the conventional "allergic Th2 response." They demonstrated that in infected mice, IL-10-producing CD1d^{high} B cells induce Treg cells and consequently ameliorate allergic airway inflammation [15]. In the study, IL-10 was indispensable to the effects of schistosomes. The authors also showed the importance of B cells, and IL-10 in the suppression of systemic anaphylaxis by schistosomes [16]. Smits et al. [17] found that in adoptive transfer experiments, spleen cells (especially B cells and CD4^{+} T cells) from chronically infected mice could confer protection against pulmonary infiltration by white blood cells, especially eosinophils. In addition, administration of anti-IL-10R antibody cancelled out the effects of the cell transfer. These studies seem to support the critical importance of B cells, Treg cells and IL-10 in schistosome-induced protection against airway allergic inflammation. In studies by another group, however, IL-10 signaling was not essential for the antiallergic immunomodulation by schistosomes [18, 19]. The reason for this discrepancy is unclear, but as the authors point out, other immunomodulatory factors may be able to compensate the absence of IL-10 [19].

Antiallergic effects of schistosomes have also been demonstrated in humans. In a study in Brazil, both asthmatic symptoms and skin reactivity to indoor allergens were reduced in *S. mansoni*-infected asthmatics compared to non-infected individuals [42]. In another study, higher expression levels of HLA-DR, IL-10R (in monocytes), CTLA-4 and CD40L (in CD4^{+} T cells) were observed in *S. mansoni*-infected asthmatics [43]. According to that paper, the main sources of IL-10 were monocytes and Treg cells. A meta-analysis of current parasite infections and atopy [44] revealed that schsitosomiasis was protective against allergic skin sensitization as well as some other helminths (*Ascaris lumbricoides*, *Trichuris trichiura*, hookworm). The same research group also reported a meta-analysis of interrelationships between helminths and asthma [45]. Hookworm infections were shown to be protective, but no beneficial effect was found for schistosome infections, probably due to the insufficient number of studies (only two). Collectively, anti-asthmatic effects of schistosomes have been confirmed in animal models and suppressive effects on allergic skin reactivity have been confirmed in humans. However, more epidemiological (especially intervention) studies are necessary to conclude whether schistosome infections have beneficial or detrimental effects on asthmatic patients.

2.2. Autoimmunity. Autoimmune disorders had been considered Th1-mediated diseases for a long time. As the Th2-biasing ability of parasitic helminths and consequent downregulation of Th1 responses have been well known, antiautoimmune effects of such parasites had been attributed to the downregulation of Th1 responses in infected animals. However, some major autoimmune diseases are now considered to be dependent on Th17, a newly found pathogenic T-cell subset that mainly produces IL-17 [46]. With this finding, the antiautoimmune properties of helminths have been revisited. In recent years, downmodulation of Th17 responses by parasitic helminths has been reported [24, 36, 47, 48]. If the suppressive activity on both Th1 and Th17 is common to parasitic helminths, helminths may become ideal sources of drug screening for treatment of autoimmune disorders. That is because both T-cell subsets are involved in the pathogenesis of some autoimmune diseases [49]. Moreover, the unstable nature of Th17 [49] reinforces this idea.

As summarized in Table 1, schistosomes suppress various autoimmunity models in rodents. An upregulation of IL-4 and downregulation of IFN-γ responses are almost commonly observed. In addition, responses of IL-17 and/or TNF-α, both of which play pathological roles in autoimmune arthritis [24, 26] and hapten-induced colitis [36, 40], were also downregulated by schistosome infections. Moreover, our study in mice with collagen-induced arthritis (CIA) revealed that the disease-associated local augmentation of bone-destructive cytokines (i.e., IL-6 and RANKL) was abrogated in infected animals [24]. These results indicate that schistosomes have suppressive effects on both Th1/Th17 and inflammatory cytokines. In addition, schistosomes suppress other pathogenic mediators such as autoreactive antibodies. Schistosomes decreased levels of anti-insulin IgG [30], anti-TSHR IgG2a [41], and anticollagen IgG [24, 25]. This effect was also observed in humans, as Mutapi et al. recently reported that *S. haematobium* infection intensity was inversely related to autoreactive antinuclear antibody (ANA) levels [50]. The authors also found that antihelminthic treatment increased ANA levels. However, Rahima et al. reported the presence of antinuclear antibodies in *S. mansoni*-infected mice and that sera from patients with systemic lupus erythematosus (SLE) reacted with cercarial antigens [51], suggesting that schistosomes trigger some kinds of autoimmunity. In conclusion, large-scale cross-sectional studies may be necessary to reveal the interrelationships between schistosomiasis and autoimmunity.

It is reasonable to hypothesize that the downmodulation of proinflammatory cytokines and pathogenic antibodies is involved in the antiautoimmune activity of schistosomes, at least partially. As regulatory cytokines are known to downregulate proinflammatory cytokines and pathogenic antibodies, it is important to determine the "essential" regulatory cytokines in each disease models for elucidation of suppressive mechanisms. For this purpose, it is necessary to perform experiments of cytokine neutralization with specific antibodies or experiments using gene-targeted animals. In Th1/Th17-dependent autoimmunity, IL-4, the key cytokine of Th2 responses, may be responsible for the alleviation of the disease symptoms. For instance, by using gene-targeted mice, STAT6 (a key signaling molecule in the response to IL-4 and IL-13) was shown to be indispensable to the *S. mansoni* egg-induced suppression of experimental autoimmune encephalomyelitis (EAE) [28] and of TNBS-induced colitis [35]. In contrast, IL-4 and IL-13 were dispensable to the suppression of DSS-induced colitis (Th2 cytokine dominant and macrophage-mediated colitis) by male worms of *S. mansoni* [39]. In the same study, authors demonstrated that IL-10 and TGF-β were also dispensable to the anticolitic effects of schistosome, by using specific antibodies against IL-10R and TGF-β. IL-10 was not a crucial regulatory cytokine

Table 1: Suppressive effects of schistosome on experimental allergy and autoimmunity in rodents.

Category of animal models	Diseases	Schistosome	Treatment	Proposed mechanisms	Refs
Allergy	Asthma/Airway hypersensitivity or inflammation	Sm	Infection (male)	IL-5 ↓, IL-10↑	[20]
			Infection, Egg i.p.	IL-4 ↓, IL-5 ↓, IgE ↓, Treg↑, Independent of IL-10	[18]
			Infection (chronic)	B cells and $CD4^+$ T cells, Dependent on IL-10	[17]
			Infection and Adoptive transfer	IL-10-producing $CD1d^{high}$ B cells → Treg↑	[15]
			Sm22.6, PIII	IL-4 ↓, IL-5 ↓, IgE ↓, Treg ↑, independent of IL-10	[19]
		Sj	Infection (male, mixed)	IL-4 ↓, IL-5 ↓, IgE ↓, IL-10 ↑	[21]
			Infection and adoptive transfer	DC → IL-4 ↓, IL-5 ↓, IL-10 ↑	[22]
		Sj	SEA, Eggs (i.p., p.o.)	Treg↑	[23]
	Systemic anaphylaxis	Sm	Infection	IL-10-producing B cell	[16]
Autoimmunity	Collagen-induced arthritis (CIA)	Sm	Infection	IL-17 ↓, TNF-α ↓, IL-6 ↓, RANKL ↓, anticollagen IgG ↓	[24]
		Sj	Infection	IL-4 ↑, anticollagen IgG ↓	[25]
	Adjuvant-induced arthritis (AIA)	Sj	Sj16 i.p.	TNF-α ↓, IL-1β ↓, NO ↓, IL-10 ↑	[26]
	Experimental autoimmune encephalomyelitis (EAE)	Sm	Infection	IL-12p40 ↓, IFN-γ ↓, TNF-α ↓, IL-4 ↑	[27]
			Egg i.p.	IFN-γ ↓, IL-4 ↑, TGF-β ↑, IL-10 ↑, dependent on STAT6	[28]
		Sj	SEA i.p.	IFN-γ ↓, IL-4 ↑	[29]
	Type 1 diabetes in NOD mice	Sm	Infection, Egg i.p.	Inhibition of Ab class switch (anti-insulin IgG ↓)	[30]
			SEA, SWA i.p.	NKT ↑	[31]
			SEA i.p.	Treg ↑	[32]
	Streptozotocin-induced diabetes (multiple low dose)	Sm	Infection		[33]
	TNBS-induced colitis	Sm	Infection	IL-2 ↑, IL-4 ↑	[34]
			Egg i.p.	IFN-γ ↓, IL-4 ↑, dependent on STAT6	[35]
			SWA i.p.	IFN-γ ↓, IL-17 ↓, TGF-β ↑, IL-10 ↑	[36]
		Sj	Egg i.p.	IFN-γ ↓, IL-4 ↑, IL-10 ↑, Treg ↑	[37]
			Egg i.p.	IFN-γ ↓, IL-4 ↑, TLR4 ↓	[38]
	DSS-induced colitis	Sm	Infection (male)	Dependent on macrophages, independent of Treg, IL-10, IL-4, IL-13 and TGF-β	[39]
			Infection	TNF-α ↓	[40]
	Grave's hyperthyroidism	Sm	Infection	Anti-TSHR IgG2a ↓	[41]

↓: down-regulation, ↑: up-regulation, Sm: *S. mansoni*, Sj: *S. japonicum*.

also in other helminthic infections, that is, piroxicam-induced colitis was suppressed by *Heligmosomoides polygyrus* [48], and EAE was suppressed by *Fasciola hepatica* [47], both in IL-10-deficient mice. In the latter study, however, TGF-β was shown to be responsible for anti-EAE effects of the worms [47]. Taken together, the involvements of IL-4, IL-10, and TGF-β in antiautoimmune effects of helminths depend on the disease models and helminth species. Further investigations using various autoimmunity models and gene-targeted animals would be necessary for comprehensive elucidation of the suppressive mechanisms by regulatory cytokines in schistosome infections.

Regarding the participation of regulatory cell populations in schistosome-induced antiautoimmune effects, Treg cells, macrophages, and other types of cells (e.g., NKT cells) have been suggested. Although Treg cell population is known to expand by schistosome infection or SEA administration [13, 32], their involvement seems to depend on the disease

models. For instance, Cooke et al. have been studying antidiabetic effects of *S. mansoni* using a spontaneous T1D model (NOD mouse) [30], and they demonstrated that Treg cells were essential in the suppression of T1D by cell transfer experiments [32]. The authors (Zaccone et al.) showed that splenocytes from nontreated NOD mice successfully transmitted diabetes into NOD/SCID recipients, whereas splenocytes from SEA-treated NOD mice had a reduced capacity to transmit diabetes. They also showed that SEA had various immunomodulatory effects on dendritic cells (DCs), macrophages, and T cells of NOD mice [12, 32]: for example, increased expressions of TGF-β, galectins, PD-L1, and so forth. In contrast to this T1D model, Smith et al. showed that depletion of Treg cells did not influence the suppressive effect of schistosomes on DSS-induced colitis [39]. They demonstrated that macrophages (but NOT alternatively activated macrophages) played an essential role in the amelioration of the colitis.

In some of the studies in Table 1, the injection of eggs or SEA was not effective [27, 39, 40]. Likewise, in our study of CIA, SEA injection was not effective [52]. Taken together with findings that mice infected with male worms become resistant to DSS-induced colitis [39], egg-derived substances are not sufficient to explain all of the immunomodulatory activities of schistosomes. Indeed, injection of soluble worm antigens (SWAs) could prevent T1D in NOD mice [31] and TNBS-induced colitis [36]. Therefore, differential effects of worms and eggs should be further elucidated for a precise understanding of the immunomodulatory mechanisms of schistosomes. In addition, further studies on differences between single-sex infections and mixed-sex infections may be necessary.

2.3. Parasitic Infections. In tropical developing countries, infections with multiple microbes/parasites are common. Consequently, the immune responses and/or pathological lesions caused by one pathogen may affect the outcome of other infections. Therefore, influences of parasitic infections on concomitant diseases have been studied. In this section, we focus on the effects of schistosomes on other parasitic infections. Malaria is the world's deadliest and most widely distributed parasitic disease. Consequently, there are more than a few experimental studies on the interrelationships between schistosomiasis and malaria (Table 2). The influence of schistosome infections depends on the species of the malarial parasites and mouse strains used in the experiments. For instance, in CBA/Ca mice, *S. mansoni* protected against *P. chabaudi* infection, worsened *P. yoelii* infection, and had no effect on *P. berghei* infection [53]. Even when the same parasite, *P. chabaudi,* was used, schistosomes exacerbated the parasitemia and mortality in C57BL/6 mice but ameliorated the outcome in A/J mice [54, 55]. Although these complicated outcomes should be taken into consideration, in general, schistosome infections seem to exacerbate rodent malaria, that is, increase of parasitemia, mortality, and hepatosplenomegaly [56]. Detrimental effects of schistosome infections on malarial outcome are also reported in humans [57, 58]. In Kenyan school children, even a light *S. mansoni* infection was shown to exacerbate hepatosplenomegaly of malarial patients [57]. In a study in Senegal, heavy *S. mansoni* infections significantly increased the incidence of malarial attacks [58]. However, the modification of antibody responses by concomitant schistosomiasis is controversial [59, 60]. The mechanism responsible for the exacerbation (or amelioration) of malaria is clear in neither mice nor humans. Helmby et al. [54] suggested diminished production of TNF-α in schistosome-infected mice to have contributed to the increase in parasitemia of *P. chabaudi*. Yoshida et al. extensively analyzed possible mechanisms of protective effects of schistosomes against *P. chabaudi* in A/J mice [55]. They observed an enhanced Th1 response to *P. chabaudi* in schistosome-infected mice and that an anti-IFN-γ antibody abrogated the schistosome-induced protective effect against *P. chabaudi.* As monoinfection with *S. mansoni* induced a Th2-dominant response, upregulation of IFN-γ seemed to derive from the mixed infections of both parasites. They also observed the upregulation of iNOS gene expression in spleen from mice with mixed infections, suggesting an increase of splenic nitric oxide production to have contributed to the protection from *P. chabaudi.* Although the mechanism of the exacerbation of rodent malaria by schistosome is yet to be sufficiently analyzed, Treg cells have been shown to play an important role in the exacerbation of *P. yoelii* infection by preceding *H. polygyrus* infection [61]. Likewise, the induction/expansion of Treg cells by schistosome might be involved in the increased susceptibility to rodent malaria. The Th1 immune response protects against malarial parasites, but it also plays pathological roles in malaria, especially cerebral malaria [62]. Thus, schistosome as a representative Th2-biasing helminth is expected to suppress brain pathology. Indeed, in a model of cerebral malaria using mice infected with *P. berghei* ANKA, *S. mansoni* reduced the incidence of cerebral malaria and delayed death [63]. Moreover, the administration of IPSE/alpha-1, a Th2-inducing schistosomal egg protein [9, 10], also delayed death. Bucher et al. reported that brain pathology was reduced in schistosome-infected mice although they did not observe any beneficial effect on mortality [64].

Influence of schistosomes on other protozoan infections has also been investigated (Table 2). Regarding *Leishmania major*, reports vary, with Yoshida et al. finding no effects of schistosome on the outcome of *L. major* infection [67], and La Flamme et al. reporting an exacerbation of experimental leishmaniasis [68]. The reason for the discrepancy is not clear but might be intensity of the *S. mansoni* infection (20 cercariae in the former versus 70 cercariae in the latter). In the case of *L. donovani*, schistosome-preinfected mice failed to control growth of the protozoa in the liver [70]. In the coinfected mice, hepatic egg granulomas were shown to provide a favorable microenvironment for the growth of the amastigotes. Likewise, *S. mansoni* exacerbated *Trypanosoma cruzi* infection [71] and *Toxoplasma gondii* infection [72]. In general, schistosome infections seem to be detrimental to animals infected with protozoan parasites.

In marked contrast to that for protozoan parasites, protective immunity against intestinal helminths is usually Th2 dependent. Therefore, the Th2-dominant environment

Table 2: Effects of schistosome on other parasitic infections in rodents.

Category	Parasites	Schistosome	Mouse strain	Effects	Refs.
Malaria	*Plasmodium berghei*	Sm	CBA/Ca	No effect	[53]
	Plasmodium berghei ANKA	Sm	Swiss albino	Parasitemia ↑, mortality ↑	[65]
	Plasmodium berghei NK65	Sm	BALB/c	Parasitemia ↑, mortality ↑	[66]
	Plasmodium chabaudi	Sm	CBA/Ca	Parasitemia ↓	[53]
	Plasmodium chabaudi AS	Sm	A/J	Mortality ↓	[55]
			C57BL/6	Parasitemia ↑, mortality ↑	
		Sm	C57BL/6	Parasitemia ↑	[54]
	Plasmodium yoelii	Sm	CBA/Ca	Parasitemia ↑	[53]
		Sm	BALB/c	Parasitemia ↑, mortality ↑	[56]
Cerebral Malaria	*Plasmodium berghei* ANKA	Sm	ICR HSD	Cerebral malaria ↓	[63]
		Sm	C57BL/6	Brain pathology ↓, no effect on mortality	[64]
Other protozoan infections	*Leishmania major*	Sm	BALB/c	No effect	[67]
			C57BL/6	No effect	
		Sm	C57BL/6	Parasitemia ↑, lesion resolution delayed	[68]
	Leishmania mexicana	Sm	Outbred	Incubation period shortened	[69]
	Leishmania donovani	Sm	C57BL/6	Parasitemia ↑	[70]
	Trypanosoma cruzi	Sm	Albino	Parasitemia ↑, mortality ↑	[71]
	Toxoplasma gondii	Sm	Albino	Mortality ↑	[72]
Helminthic infections	*Hymenolepis diminuta*	Sm	NMRI	Expulsion ↑	[73]
	Strongyloides venezuelensis	Sm	C57BL/6	Migration ↓	[67]
		Sj	C57BL/6	Migration ↓, expulsion ↑	[69]
	Trichuris muris	Sm	AKR	Expulsion ↑	[74]

↓: downregulation, ↑: upregulation; Sm: *S. mansoni*, Sj: *S. japonicum*.

produced by schistosomes can be expected to protect against intestinal helminths. Indeed, as summarized in Table 2, schistosome infections protected mice from intestinal helminths. Likewise, in a human study in Brazil [75], *S. mansoni* egg counts were inversely correlated with *A. lumbricoides* and *T. trichiura*. (One exception was *Ancylostoma,* which was positively correlated with schistosome infections). Although protection against intestinal helminths is commonly observed in experimental settings, the mechanisms differ in each case. For instance, protection against a lung-migratory parasite, *Strongyloides venezuelensis*, mainly involved eosinophil-mediated killing of larvae in the lungs [76]. In addition, intestinal mucosal mastocytosis induced by *S. japonicum* infection rendered mice resistant to harboring of adult worms [76]. Antigen cross-reactivity between schistosomes and *S. venezuelensis* [67, 76] may have also contributed to the protection. In contrast, in the case of *Hymenolepis diminuta* and *Trichuris muris* (nonmigratory intestinal helminths), the protection seems to be dependent on accelerated expulsion from the intestines [73, 74]. Overall, schistosome infections appear to be beneficial to animals infected with intestinal helminths.

2.4. Bacterial Infections and Vaccinations. Prolonged bacteremia in schistosomiasis patients was first reported more than 50 year ago, and the relationship between enterobacteria infections and schistosomiasis has been long studied [3]. The prolonged enterobacterial infection is referred to "prolonged septicemic enterobacteriosis." The schistosome-induced exacerbation of enterobacterial infections has also been observed in experimental settings [77, 78]. As most studies were conducted before the "molecular immunology age," the mechanisms of exacerbation of bacterial infections have not been sufficiently elucidated. However, the mechanisms are likely similar to those responsible for the schistosome-induced increase in susceptibility to protozoan parasites, that is, a reduction in Th1-dependent protective immunity as a consequence of augmented Th2 responses. Indeed, a Th1-inducing protozoan parasite, *L. donovani,* did not affect the growth of *Salmonella paratyphi* A in infected hamsters [78]. Moreover, impairment of the bactericidal function of macrophages from schistosome-infected mice was reported [79]. In addition to the immunomodulation by schistosomes, there are direct schistosome-bacteria interactions providing worm bodies as foci for bacterial multiplication [3, 80, 81]. Another considerable influence of schistosomes on bacterial infections is a reduction in vaccine efficacy. The *Mycobacterium bovis* BCG-induced protective response against *Mycobacterium tuberculosis* in mice was reduced by *S. mansoni* infection [82]. The authors also reported increased susceptibility to intravenous BCG

inoculations and lung pathology in *S. mansoni*-infected mice [83]. In these studies, decrease in IFN-γ and nitric oxide in response to PPD were observed.

2.5. Viral Infections and Vaccinations. As hepatotropic viruses, that is, hepatitis B virus (HBV) and hepatitis C virus (HCV), cause liver cirrhosis during chronic infections, the synergistic exacerbation of hepatic pathology is expectable outcome of concurrent infections of HBV/HCV and schistosomes. Because of a lack of suitable animal models for infections of these hepatotropic viruses, major findings in schistosome-HBV/HCV coinfections have been obtained from epidemiological studies. Regarding HBV, schistosomiasis (especially the severe hepatosplenic form) was correlated with a higher frequency of HBV infection [3, 84, 85]. This observation could be explained by an increased susceptibility to HBV caused by schistosome infections. On the other hand, there are reports of no relationship between schistosomiasis and HBV [86, 87]. In addition, experiments with animals do not support increased susceptibility to HBV in schistosomiasis. Some evidence comes from an experiment using woodchucks infected with both schistosomes and woodchuck hepatitis virus (WHV) [88]. As HBV and WHV belong to the same family (family Hepadnaviridae), a concurrent infection by schistosomes and WHV in woodchucks is a good model of concurrent infections of schistosomes and HBV in humans. The authors reported no impact of the schistosome infection on WHV serum markers. Another paper on HBV transgenic mice [89] reported an inhibition of HBV replication during schistosome infection. In that study, the antiviral effects of schistosomes were attributed to IFN-γ and nitric oxide. Overall, it seems premature to conclude the presence of certain positive or negative effects of schistosomes on HBV infection. Regarding HBV vaccines (both serum derived and recombinant), they were immunogenic in schistosomiasis patients although reduced responses to vaccination were observed in hepatosplenic schistosomiasis [90–92].

In contrast to the controversial effects on HBV infections, detrimental effects of schistosomes on HCV infections have been clearly demonstrated, that is, schistosomes weaken anti-HCV immune responses and worsen liver disease. According to studies in Egypt, patients with coinfections were characterized by a more advanced liver pathology, greater viral burden, higher levels of anti-HCV antibodies, and progression to chronic hepatitis [93–95]. Moreover, schistosomiasis was shown to be inversely correlated with HCV-specific $CD4^+$ T cells, $CD8^+$ T cells, and/or Th1 cytokine responses [95–100]. In addition to the modulatory effects of schistosomes on HCV-specific immune responses, SEA of *S. mansoni* [101] and *S. haematobium* [102] were shown to enhance *in vitro* viral replication in a hepatoblastoma cell line (HepG2) and peripheral blood mononuclear cells (PBMCs), respectively. Likewise, in other viral infections, schistosomes were shown to suppress specific CTL and cytokine responses and to prevent viral clearance [103–107]. It is interesting that the granulomas in *S. mansoni*-infected mice provide a microenvironment suitable for viral expansion [107], as in the case of a hepatic infection with the protozoan parasite *L. donovani* [70].

3. Concluding Remarks

Based on the experimental and epidemiological findings reviewed here, it can be concluded that schistosome infections are generally beneficial to patients with intestinal helminth infections and detrimental to patients with bacterial, viral, or protozoan infections. In most tropical or subtropical countries where schistosomiasis is endemic, more serious infectious diseases (e.g., HIV/AIDS, tuberculosis, and malaria) are also endemic. Therefore, control of schistosomiasis (especially infections of *S. mansoni* and *S. haematobium*) has been given low priority compared to control of such infectious diseases. However, if the detrimental bystander effects of schistosomes on concomitant bacterial, viral, or protozoan infections are properly considered, the importance of controlling schistosomiasis should be more emphasized.

In 2002, J. F. Bach summarized epidemiological trends of allergic and autoimmune diseases during recent several decades in developed countries [108]. According to the paper, the prevalence of the immunological disorders such as asthma, T1D, multiple sclerosis (MS), and Crohn's disease (CD) was increasing, whereas the prevalence of infectious diseases such as rheumatic fever, hepatitis A, tuberculosis, mumps, and measles was decreasing. These phenomena may be explained by the "hygiene hypothesis" in which microbial and helminthic infections prevent immunological disorders. Along with this hypothesis, schistosome infections are expected to prevent or alleviate symptoms of immunological disorders. According to the experimental studies until now, antiallergic and antiautoimmune effects of schistosomes are also plausible in humans. However, schistosomes could not be directly used for therapeutic treatment because of their pathogenicity. Instead, purified immunomodulatory products or recombinant proteins could be tested for clinical use. Indeed, considerable numbers of helminths' products have been shown to protect against experimental immunological disorders [109]. The immunomodulators exert their effects via Toll-like receptors (TLRs) and/or C-type lectin receptors (CLRs) [109]. Taken together with the finding that systemic administration of TLR agonists could prevent experimental autoimmunity and allergy [110], appropriately synthesized TLR agonists may be able to mimic or replace the prophylactic or therapeutic effects of schistosomes.

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Review Article

Emerging Functions of Transcription Factors in Malaria Parasite

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Transcription is a process by which the genetic information stored in DNA is converted into mRNA by enzymes known as RNA polymerase. Bacteria use only one RNA polymerase to transcribe all of its genes while eukaryotes contain three RNA polymerases to transcribe the variety of eukaryotic genes. RNA polymerase also requires other factors/proteins to produce the transcript. These factors generally termed as transcription factors (TFs) are either associated directly with RNA polymerase or add in building the actual transcription apparatus. TFs are the most common tools that our cells use to control gene expression. *Plasmodium falciparum* is responsible for causing the most lethal form of malaria in humans. It shows most of its characteristics common to eukaryotic transcription but it is assumed that mechanisms of transcriptional control in *P. falciparum* somehow differ from those of other eukaryotes. In this article we describe the studies on the main TFs such as myb protein, high mobility group protein and ApiA2 family proteins from malaria parasite. These studies show that these TFs are slowly emerging to have defined roles in the regulation of gene expression in the parasite.

1. Introduction

Transcription is the synthesis of an RNA molecule complementary to the DNA template by the action of several enzymes. Transcription, whether prokaryotic or eukaryotic, has three main sequential events such as initiation, elongation, and termination [1, 2]. Initiation is the most important step and involves the binding of RNA polymerase to double-stranded DNA. Elongation is the covalent addition of nucleotides to the 3′ end of the polynucleotide chain, and termination involves the recognition of the transcription termination sequence and the release of RNA polymerase [1, 2]. Transcription is the first checkpoint in the gene expression. The sequence of DNA that is transcribed into an RNA molecule is called a "transcription unit" and it usually encodes at least one gene. Transcription is considerably more complex in eukaryotic cells as compared to bacteria. In bacteria, all the genes are transcribed by a single RNA polymerase, but the eukaryotic cells contain multiple different RNA polymerases that transcribe various classes of genes [3]. This enhanced complexity of eukaryotic transcription most probably facilitates the sophisticated regulation of gene expression needed to direct the activities of the many different cell types of multicellular organisms [4]. Three distinct nuclear RNA polymerases are responsible for transcribing different classes of genes in eukaryotic cells. The ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) are transcribed by RNA polymerases I and III, and the protein-coding genes are transcribed by RNA polymerase II to yield mRNAs [3, 5].

For efficient transcription, RNA polymerase requires other proteins, commonly known as transcription factors (TFs), to produce the transcript. TFs or sequence-specific DNA-binding factor is a protein that specifically binds to DNA sequence and controls the stream of genetic information from DNA to mRNA [4]. TFs along with other proteins in a complex control the transcription by promoting (activator), or blocking (repressor) the recruitment of RNA polymerase to specific genes (Figure 1). The main functions of TFs are to bind to RNA polymerase, to bind another TF, and to bind to cis-acting DNA sequences. TFs usually work in groups or complexes forming multiple interactions which allow for varying degrees of control over rates of transcription. In eukaryotes genes are generally in an "off" state, thus TFs mainly work to turn "on" the gene expression.

While in bacteria the genes are expressed constitutively until a TF turns it "off." The eukaryotic genes generally contain a promoter region upstream from the gene and/or enhancer region upstream or downstream from the gene with some motifs that are specifically recognized by different types of TFs [3]. One distinct quality of TFs is that they have DNA-binding domains (DBDs) that give them the ability to bind to specific promoter or enhancer sequences. The binding of TFs triggers the other TFs to bind and this creates a complex that ultimately facilitates binding by RNA polymerase, thus initiating the process of transcription [3, 4].

The basal transcription complex and RNA polymerase II bind to the core promoter of protein encoding gene which is normally within about 50 bases upstream of the transcription initiation site [3]. Further transcriptional regulation is controlled by upstream control elements (UCEs), generally present within about 200 bases upstream of the initiation site (Figure 1). Sometimes TATA box, present in the core promoter for Pol II, the highly conserved DNA recognition sequence for the TATA-box-binding protein, TBP, helps in the assembly of transcription complex at the promoter (Figure 1). General transcription factors (GTFs) are an important class of TFs essential for transcription (Figure 1). Generally GTFs do not bind to DNA, but they form part of the large transcription preinitiation complex which interacts with RNA polymerase directly (Figure 1). The most common GTFs are transcription factors IIA (TFIIA), TFIIB, TFIIE, TFIIF, and TFIIH [3, 6].

Malaria caused by the mosquito-transmitted parasite *Plasmodium falciparum* is the most serious and widespread parasitic disease of humans. Malaria is the cause of enormous number of deaths every year in the tropical and subtropical areas of the world. Among four species of *Plasmodium*, *P. falciparum* causes the most fatal form of malaria [7, 8]. Each year, approximately two hundred and twenty-five million people become infected with malaria and around 781,000 die as a result according to the World Health Organization's 2010 World Malaria Report [9]. The malaria parasite has 14 chromosomes, more than 7000 genes and a four-stage life cycle as it passes from humans to mosquitoes and back again [7, 10]. It is very efficient at evading the human immune response. *P. falciparum* has a complex life cycle that involves defined morphological stages accompanied by the stage-specific gene expression in both the human and mosquito host, but the mechanisms of transcriptional control in this parasite are not well known [11]. *P. falciparum* contains characteristics which are common to eukaryotic transcription [12]. But it also has exclusive patterns of gene expression and an AT-rich genome. The genome analysis reveals a relative paucity of transcription-associated proteins and specific cis-regulatory motifs [11]. These observations have led to reflect a reduced role for the TFs in transcriptional control in the parasite.

It has been suggested that protein levels during the life cycle of malaria parasite are controlled through posttranscriptional mechanisms, thus it is possible that posttranscriptional regulation may play a major role in the control of gene expression in *P. falciparum*. The comparisons of mRNA and protein levels across seven major developmental stages of the *P. falciparum* life cycle were conducted [13]. Even though reasonably high correlations were observed between the transcriptome and proteome of each stage, a considerable fraction of genes was found to display a delay between the peak abundance of mRNA and protein [13]. The quantitative protein expression profiling during the schizont-stage of the *P. falciparum* development revealed that extensive posttranscriptional regulation and posttranslational modifications occur in malaria parasites. These observations further support that the posttranscriptional gene regulation events are widespread and of presumably great biological significance during the intraerythrocytic development of *P. falciparum* [14]. In a recent interesting study, it has been reported that PfCLKs (cyclin-dependent kinase-like kinase) play crucial role in malaria parasites erythrocytic replication, presumably by participating in gene regulation through the posttranscriptional modification of mRNA [15]. The rate of mRNA decay is also an essential aspect of post-transcriptional regulation in all organisms. The half-life of each mRNA is precisely related to its physiologic role and thus plays an important role in determining levels of gene expression. By using genome-wide approach to describe mRNA decay in *P. falciparum*, it was observed that the rate of mRNA decay increases severely during the asexual intraerythrocytic developmental cycle [16].

As global efforts to eradicate malaria have been unsuccessful, there is a vital requirement to decipher the biology of *Plasmodium* and in particular the mechanisms of gene regulation that manage its developmental cycle, so as to propose novel strategies to fight malaria. There are only few TFs well-characterized from *P. falciparum*. These are Myb1 protein, high mobility group box (HMGB) proteins, and the Apetala2 (AP2) domain-containing proteins [17]. The studies on these factors will be described in the following sections.

2. Myb Protein

Myb is an abbreviation derived from "myeloblastosis," an old name for leukemia. This family of proteins was first characterized in the avian myeloblastosis virus (AMV). Myb proteins, highly conserved in eukaryotes, belong to tryptophan cluster family and regulate gene expression by binding to DNA [18]. Their characteristic Myb DBD of approximately 50 residues contains three tandem repeats (R1, R2, and R3) with three frequently spaced tryptophan residues [18, 19]. The structure of the Myb domain is similar to the helix-turn-helix motif of prokaryotic transcriptional repressors and eukaryotic homeodomains. Myb proteins bind DNA in a sequence-specific manner and regulate the expression of genes involved in differentiation and growth control [18].

Myb protein was recognized in the *P. falciparum* genome by aligning about 200 nonredundant eukaryotic Myb proteins and generating a consensus sequence analogous to the characteristic DNA-binding domain [20]. This consensus was used as query for the *Plasmodium* database which resulted in the annotation of a 414 amino acid long open reading frame PfMyb1 [20]. Initially, only one Myb domain

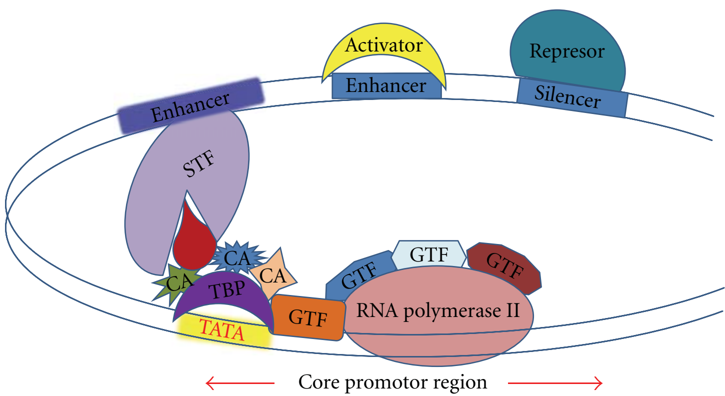

Figure 1: Representation of general transcription machinery: CA: coactivator, GTF: general transcription factor, STF: specific transcription factor, and TBP: TATA-binding protein.

(R2) was identified in PfMyb1 but the alignment of complete sequence of PfMyb1 (PlasmoDB number PF13_0088) with the DBD of three proteins, DdMybH, DdMyb2, and DdMyb3 of *Dictyostelium discoideum* resulted in the recognition of three Myb domains situated in the C-terminus of the protein as in DdMyb2 and DdMyb3 of *D. discoideum*, whereas in most of the Myb proteins DBD is located in the N-terminus [20]. However, in place of tryptophan, PfMyb1 contains imperfect repeats with a tyrosine or a phenylalanine. Moreover, a critical cysteine residue, which is conserved as the tryptophan residues was also found in R1 and R2, and it most likely plays a role in redox regulation [20]. This detailed computational analysis of PfMyb1 confirmed that it is a genuine Myb protein conserved in all the *Plasmodium* species [20]. It was further reported that PfMyb1 is expressed throughout all the erythrocytic developmental stages of the parasite (rings, early and late trophozoites, as well as early and late schizonts) [20]. The expression was analyzed in two different clones of *P. falciparum*, 3D7 and the gametocyte-less F12 derived from 3D7, and the difference in the mRNA profile resides in a lower expression of the *Pfmyb1* transcript in the ring stage of F12 compared to 3D7, followed by a quick increase in early F12 trophozoites. Myb-DNA-binding activity was observed with a prototype (*mim-1*) and two putative *Plasmodium* Myb regulatory elements, pfmap1 (MAP kinase) and pfcrk1 (cdc2-related protein kinase) genes. These genes were originally reported to be expressed preferentially during erythrocytic asexual and sexual stages, respectively [21, 22]. This interaction was confirmed to be specific since it was inhibited by specific competitors and anti-PfMyb1 antibody in band-shift assays. During erythrocytic development, the band-shift profiles were clearly different in the 3D7 and the gametocyte-less F12 clones, in contrast to the transcript level [20]. In a follow-up study, the same group used long double-stranded RNA (dsRNA) to reduce the cognate messenger and encoded protein and reported that the parasite cultures treated with dsRNA of PfMyb1 showed growth inhibition [23]. As a result of this dsRNA inhibition, the parasite mortality occurred during trophozoite to schizont stages of the development suggesting that PfMyb1 is essential for parasite growth [23]. They have also shown that PfMyb1 binds to a number of promoters such as the promoter of phosphoglycerate kinase, calcium-dependent kinase, TATA-binding protein, proliferating cell nuclear antigen, phosphatase, histones, and cyclin-dependent kinase within the parasite nuclei, and therefore directly regulates the key genes involved in cell cycle regulation and progression [23].

3. High-Mobility-Group Box Protein

In eukaryotes, the high-mobility-group (HMG) box nuclear factors are highly conserved throughout evolution. HMG box domain is composed of around 80 amino acids folded in three α-helices arranged in an L shape, and this domain is involved in DNA binding [24]. HMG box proteins can bind to non-B-type DNA structures such as cruciform and distorted AT-rich DNA sequences in a nonsequence-specific fashion [25]. This binding triggers DNA bending and assists the binding of nucleoprotein complexes that in turn repress or activate transcription [24]. HMG box domains are also involved in a variety of protein-protein interactions. HMG box proteins actively participate in chromatin remodeling by increasing nucleosome sliding and accessibility of the chromatin [25].

A *P. falciparum* gene encoding a typical HMG box protein was reported [26]. The gene for PfHMG consisted of one putative DNA-binding domain contained within a single exon. The amino acid sequence revealed that PfHMG lacks the acidic C-terminal domain, which is present in the HMG of higher eukaryotes and interacts with basic proteins such as histones [24, 26]. This domain is also absent in the HMG of yeast and *Babesia bovis* [26]. The northern blot analysis of PfHMG RNA expression showed that HMG is expressed in all the stages of the asexual erythrocytic life cycle, with the highest level of transcript at early schizont stages [26].

In another study, four putative *P. falciparum* HMG box proteins including one previously reported were predicted by sequence homology [26, 27]. PfHMGB1 was annotated within chromosome 12, PfHMGB2 and PfHMGB3 on chromosomes 8 and 12, and PfHMGB4 within chromosome 13, respectively [27]. PfHMGB1 (PlasmoDB number PFL0145c)

and PfHMGB2 (PlasmoDB number MAL8P1.72) are small proteins under 100 amino acids long and contain one characteristic HMG box domain similar to B-Box of mammalian HMGB1 [27, 28]. PfHMGB4 (PlasmoDB number MAL13P1.290) encodes a 160 amino acids long protein, but PfHMGB3 is a larger protein (2,284 amino acid), with two HMG box domains and several additional putative functional motifs, including one Myb domain [27]. The sequence analysis showed that the PfHMGB1 contains 45, 23, and 18%, while PfHMGB2 shares 42, 21, and 17% homology with *Saccharomyces cerevisiae*, human, and mouse HMG box proteins, respectively [28]. The in vitro studies performed with both the recombinant proteins showed that they were able to interact with distorted DNA structures and bend linear DNA. These proteins were expressed in both asexual- and gametocyte-stage cells, and PfHMGB1 is preferentially expressed in asexual erythrocytic stages and PfHMGB2 in gametocytes. The subcellular localization study revealed that both factors were present in the nucleus, but PfHMGB2 was also detected in the cytoplasm of gametocytes [27]. On the basis of differences in their levels of expression, subcellular localizations, and capabilities for binding and bending DNA, these factors most likely have role in transcriptional regulation of *Plasmodium* development [27]. In an interesting study it was reported that PfHMGB1 and PfHMGB2 are effective inducers of proinflammatory cytokines such as TNFα from mouse peritoneal macrophages [28]. These observations imply that secreted PfHMGB1 and PfHMGB2 are most likely responsible for producing host inflammatory immune responses associated with malaria infection [28].

The role of HMGB2 protein in regulation of sexual stage gene expression was evaluated by disrupting the *Plasmodium yoelii* gene encoding HMGB2. It is in vivo function in the vertebrate host the mouse and the mosquito *Anopheles stephensi* was studied [29]. It has been reported that the parasites lacking PyHMGB2 develop into gametocytes but have severe impairment of oocyst formation [29]. It was also shown that PyHMGB2 is not required for asexual growth, but it is involved in controlling the genes which are important for oocyst development in the mosquito. These results suggest that the protein expression in sexual stages is transcriptionally and translationally regulated, where PyHMGB2 acts as an important regulator of sexual stage development [29].

4. ApiA2 Family

Activator protein-2 (AP-2) or Apetala2 family of transcription factors constitutes a family of closely related and evolutionarily conserved proteins that bind to the DNA consensus sequence GCCNNNGGC and stimulate target gene transcription. Four different isoforms of AP-2 have been identified in mammals, termed AP-2 α, β, γ, and δ [30, 31]. These proteins share a characteristic helix-span-helix motif at the carboxyl terminus, which, together with a central basic region, mediates dimerization and DNA binding. The amino terminus contains a proline/glutamine-rich domain, which is responsible for transcriptional activation. The general functions of the family appear to be the cell-type-specific stimulation of proliferation and the suppression of terminal differentiation during embryonic development. The proteins are able to form hetero- as well as homodimers [30, 31]. The AP-2 factors are primarily localized in the nucleus, where they bind to the target sequences and regulate the target gene transcription.

Using a comparative genomic analysis, it has been shown that the apicomplexans possess this AP2 family of proteins which is commonly known as ApiAP2. About 20–27 members of this ApiAP2 family are present in different genomes, and *P. falciparum* ApiAP2 gene family has 27 members, which are largely conserved across *Plasmodium* species [17]. All of these are expressed throughout the four stages of the intraerythrocytic development cycle [31, 32]. Each of these proteins contain one to four copies of the AP2 DNA-binding domain and similar to plants, these domains in ApiAP2 proteins are also approximately 60 amino acids long and are found in both single- and tandem-domain arrangements [31, 32].

By using protein-binding microarrays, the DNA-binding specificities of two ApiAP2 proteins representing different classes of AP2 domain architectures from *P. falciparum* were demonstrated [33]. The gene with PlasmoDB number *PF14_0633* encodes an 813-aa protein, which shows high level of expression during the ring stage of development [34]. It contains a single 60 amino acids AP2 domain and an adjoining AT-hook DNA-binding domain [33]. *PF14_0633* has orthologues in all the sequenced *Plasmodium* genomes and all the other sequenced apicomplexan genomes. The other ApiAP2 gene with PlasmoDB number *PFF0200c* shows high level of expression in late-stage parasites and encodes a 1,979 amino acid protein containing two AP2 domains in tandem. These two AP2 domains are linked with each other by a conserved 17 amino acid sequence [33]. It was reported that in *Plasmodium* spp., the orthologous tandem AP2 domains of PFF0200c share ~95% amino acid sequence identity, but the individual AP2 domains of PFF0200c share only 35% identity with each other [33]. These AP2 domains specifically bind with unique DNA sequence motifs that are found in the upstream regions of different sets of genes that are coregulated during asexual development. Interestingly, despite the sequence deviation between ApiAP2 proteins from distantly related Apicomplexan species (*P. falciparum* and *Cryptosporidium parvum*), the DNA-binding specificities of orthologous pairs of AP2 domains are highly conserved, that is, TGCATGCA, although their downstream targets may vary. This demonstrated an interaction between *Plasmodium* transcription factors and their putative target sequences [33].

Using PEXEL/VTS search, it was reported that AP2 proteins do not have motifs for apicoplast targeting, mitochondrial transit, endoplasmic reticulum trafficking, transmembrane domains, or host cell surface targeting but the classical lysine- and arginine-rich nuclear localization signals were identified concluding that this protein family consisted of TFs [33]. The *Plasmodium*-based global yeast two-hybrid study suggested that ApiAP2 proteins interact with each other and with chromatin-remodeling factors, *Plasmodium* histone acetyltransferase GCN5 [35]. The binding to

chromatin-remodeling factors may help in the recruitment of these complexes to specific chromosomal locations and facilitate interaction with the core transcription machinery. The crystal structure of the DNA-bound dimer of the AP2 domain of PF14_0633 exhibits many of the canonical features of similar DNA-binding domains [36]. The structure of PF14_0633 shows that it dimerizes through a three dimensional domain-swapping mechanism in which the α-helix of one protomer is packed against the β-sheet of its dimer mate. It was further reported that the dimerization of the AP2 domain of PF14_0633 aligns Cys76 residues of each monomer with one another with enough proximity to permit the disulfide bond formation. It was interesting to note that the Cys76 residue is conserved in all the orthologues of PF14_0633 in *Plasmodium spp.*, however it is not conserved in other related apicomplexan species [36]. This DNA-induced dimerization of the AP2 domain of PF14_0633 facilitates the conformational rearrangement of the rest of the protein or its interaction partners and this concurrently loops out intervening DNA among pairs of binding sites enriched in the upstream regions of a set of sporozoite-specific genes [36].

In a recent comprehensive study, the global DNA-binding specificities for the entire *P. falciparum* ApiAP2 family of DNA-binding proteins was biochemically and computationally characterized [37]. Their results revealed that the majority of proteins bind diverse DNA sequence motifs and occur in functionally related sets of genes. In a number of proteins, multiple AP2 domains within the same ApiAP2 protein were reported to bind distinct DNA sequences. In addition to high affinity primary motif interactions, the interactions with secondary motifs were also observed [37]. By mapping these sequences throughout the parasite genome, the results of this study provide a basis for developing a regulatory network underlying parasite development [37]. The overall studies on ApiAP2 family of proteins in *P. falciparum* suggest that these proteins are main components of gene regulation in the parasite. Although further work is needed in order to determine how ApiAP2 proteins function as transcriptional regulators. But the DNA-binding sequence specificity of these proteins, their conservation across Apicomplexa, and the extremely consistent expression patterns of their predicted downstream targets suggest their vital function in regulating parasite development.

During its asexual life cycle, the *P. falciparum* develops into several distinct morphological forms occupies various compartments in its human host and often faces drug treatment. The microarray-based transcriptomic studies of these stages reported remarkable changes in the steady-state mRNA levels of several genes, suggesting that differential gene expression is essential for development. It is well established now that gene regulation in *P. falciparum* consists of a bulk transcriptional event characteristic of the majority of genes from which differential expression of a minority of genes is selected by a combination of pretranscriptional and posttranscriptional mechanisms. Therefore, the modulation of expression of the targeted genes is the outcome of the blend of these diverse interactions.

Some of these important TFs have also been characterized from other protozoan parasites. For example, a member of the HMGB was identified in *Entamoeba histolytica* [38] and some Myb family members were characterized from *Trichomonas vaginalis* (reviewed in [39]). The information compiled in this paper suggests that *P. falciparum* indeed contains few TFs which are responsible for the gene regulation in various stages of its development. Relatively, little is known about how the parasite uses these few TFs to globally regulate the transcription in order to produce the proteins essential for its development and pathogenesis. Future work is undoubtedly required in order to solve this mystery. It has been suggested that combinatorial gene regulation might be the general mode of transcriptional regulation in *P. falciparum* or it can be assumed that the effect of various TFs on gene expression is additive. Therefore a very useful anti-plasmodial approach should be to target and inactivate one or more of these TFs with drugs. This strategy will directly or indirectly affect the gene regulation and consequently the function of several downstream genes and crucial biological processes. Due to the effect on numerous genes this approach will be very helpful, and it will be relatively difficult for the parasite to develop resistance to this line of drugs.

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Review Article

Immunodiagnosis of Neurocysticercosis: Ways to Focus on the Challenge

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Neurocysticercosis (NCC) is a disease of the central nervous system that is considered a public health problem in endemic areas. The definitive diagnosis of this disease is made using a combination of tools that include imaging of the brain and immunodiagnostic tests, but the facilities for performing them are usually not available in endemic areas. The immunodiagnosis of NCC is a useful tool that can provide important information on whether a patient is infected or not, but it presents many drawbacks as not all infected patients can be detected. These tests rely on purified or semipurified antigens that are sometimes difficult to prepare. Recent efforts have focused on the production of recombinant or synthetic antigens for the immunodiagnosis of NCC and interesting studies propose the use of new elements as nanobodies for diagnostic purposes. However, an immunodiagnostic test that can be considered as "gold standard" has not been developed so far. The complex nature of cysticercotic disease and the simplicity of common immunological assumptions involved explain the low scores and reproducibility of immunotests in the diagnosis of NCC. Here, the most important efforts for developing an immunodiagnostic test of NCC are listed and discussed. A more punctilious strategy based on the design of panels of confirmed positive and negative samples, the use of blind tests, and a worldwide effort is proposed in order to develop an immunodiagnostic test that can provide comparable results. The identification of a set of specific and representative antigens of *T. solium* and a thorough compilation of the many forms of antibody response of humans to the many forms of *T. solium* disease are also stressed as necessary.

1. Introduction

Neurocysticercosis (NCC) is a disease caused by the metacestode or larval form of the tapeworm *Taenia solium* when it lodges in the central nervous system (CNS) and is endemic of the Andean area of South America, Brazil, Central America and Mexico; China, the Indian subcontinent, and South-East Asia; sub-Saharan Africa [1–3]. It is considered a public health problem as it is the main cause of late-onset epilepsy [4] and it is also the most important parasitic disease of the nervous system [3, 5, 6]. Cysticerci may also locate elsewhere in skeletal muscles, heart, eyes, diaphragm, tongue, and subcutaneous tissues, causing a condition simply referred to as cysticercosis.

NCC is a disease difficult to diagnose based on the clinical picture as it presents a variety of nonspecific symptoms and in 50% of the cases none [7]. The symptoms differ according to the location of the cysts in the brain (parenchymal or ventricular) and the number and the state of the parasites (vesicular, degenerating, or calcified) [5, 8–12]. The severe forms of NCC seriously impair the patients' health and may lead to death. Medical diagnosis of NCC is impossible on clinical data alone. The definitive diagnose is made using a combination of methods including images of the cysts in the brain (by computed tomography or magnetic resonance imaging) and immunological methods (detection of specific antibodies or antigens). As this is a disease frequently associated to poverty [3, 13, 14], the availability and high

costs of neuroimages or sophisticated immunological assays in endemic areas limit the diagnostic capacity [15]. An effective immunodiagnosis of NCC would be the most practical way to facilitate medical diagnosis for millions of poor people in endemic countries and it would also supply sero-epidemiological studies with a low-cost indicator of prevalence of infection. In addition, a positive immune test would raise the clinical suspicion of early nonsymptomatic NCC which, if confirmed, would allow offering early treatment. Interesting advances in immunodiagnostic assays for NCC have been made during the past few years, involving the use of synthetic or recombinant antigens [16–29] and some efforts have been done to detect specific antigens or antibodies in noninvasive ways for the patient [30–33]. Most reports initially claim very high specificity/sensitivity scores, sometimes even as high as 100/100%. Enthusiasm soon calms as the methods are applied by different laboratories, in larger numbers of cases and in various epidemiological scenarios of the disease [31, 34–37].

Immunodiagnosis of NCC can be done by two ways: by identifying antibodies against cysticercal antigens, or by identifying parasite's antigens directly. Here is a review of the recent studies made in the area of immunodiagnosis of NCC and the methods used in each case are discussed.

2. Biological Factors Involved in Neurocysticercosis

Host factors as age, gender, or race are involved in the severity of NCC (Figure 1). Age has an effect upon the number of cysticercal lesions and on the state of the cysticerci in the brain (vesicular, colloidal): while vesicular cysticerci increase with aging, colloidal cysticerci diminish without representing an increment on severity of NCC [38]. Sexual dimorphism has been reported in many parasitic infections as malaria, schistosomiasis, tripanosomiasis, toxoplasmosis, and cysticercosis [39]. The effect of sex hormones upon the immune system is evident, for example, in *T. solium* cysticercosis, the prevalence of naturally infected pigs almost doubles in castrated or pregnant pigs [40]. In general, females generate more robust humoral and cell-mediated immune responses than males, but males present a stronger inflammatory response to infectious organisms [41]. In NCC, females present increased levels of IL5/Il6/IL10 in cerebrospinal fluid (CSF) [42] as well as a higher leukocyte counts than men [38] and more frequently present severe NCC [43] which may have a relation with the location of the cysts in the brain and with higher inflammatory profiles in female [44]. Furthermore, females present higher immunoglobulin levels than men to different antigenic challenges [45] and in seroepidemiological surveys females show the highest anticysticerci response [46].

The genetic differences between different populations also play a role in infectious diseases [47–49], especially on terms of susceptibility to disease as in the case of malaria [50]. In NCC, some antigens from the HLA complex have been involved in the relative risk for developing parenchymal NCC [51], but studies of this type are scarce. A study identifying genes responsible for the pleomorphic presentation of the disease has not been done, but the effect of the genetic background upon the development of this particular disease is clear between subjects from different endemic areas, as is the case of India in which single cysticercotic granuloma (SCG) is the most frequent presentation of NCC while in Latin America it is not [35, 52].

Furthermore, genetic variability of the parasite itself has been described. Genetic variability of cysticerci has been found at different levels, from the global level (which identified two genotypes: Asia and Africa/Latin America) [53–57]) to the regional and the community level [58].

With all these factors involved, it is hard to find a single immunodiagnostic test that can detect all true NCC cases and that can work worldwide.

3. Problems, Advances, and Perspectives in the Immunodiagnosis of Neurocysticercosis

Historically, tests developed to diagnose NCC either by detecting specific antibodies or antigens have shown that not all cases could be detected (false negatives) and many other cases are detected as positive (false positives) when they are not. The first case is related to the number, state, and location of the cysticerci in the brain and involves patients with degenerating, dead, or single cysticerci. The second case involves patients with parasitic diseases closely related to cysticercosis (as echinocococcis or hymenolepiasis), patients exposed to the parasite but that did not become infected, patients with cysticercosis outside the central nervous system and patients who once were infected but resolved the infection without consequences. Additionally, almost half of NCC cases are asymptomatic [12] and the symptomatic cases present a variety of unspecific symptoms as chronic epilepsy and headaches as the most common [8].

Immunodiagnostic tests for NCC initially claim to have very good sensitivity/specificity scores, but as they are being tested by other groups, in larger number of cases and in areas with different degrees of endemicity the scores are lower [31, 37, 52]. Also the scores lower because many immunodiagnostic tests rely on purified or semipurified antigens and the procedure of purification is complex and frequently require technical expertise. This question, coupled with differences in the selection of the NCC patients and the control groups, provokes large variations within and between tests and low reproducibility between laboratories. The need to find new antigens for immunodiagnosis of NCC which can improve the diagnostic capacity of actual tests persists. These new antigens have to be tested by various laboratories to prove that the sensitivity/specificity maintains between tests putting special attention on the selection of NCC patients and controls so the results can be compared. Some efforts have been done in recent years to try to make a more uniform immunodiagnostic test [20, 24] and to make comparable the results from different tests between laboratories [16, 26, 59, 60].

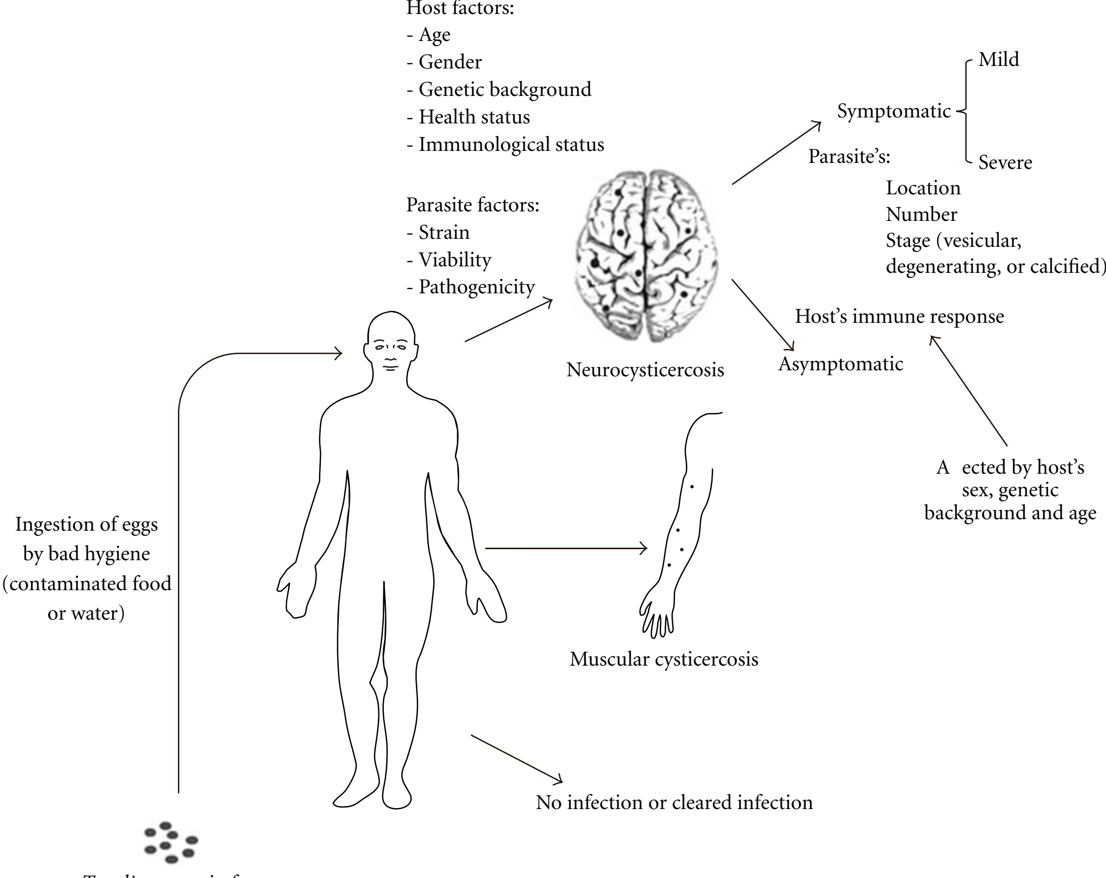

Figure 1: Factors involved in NCC. The development of NCC depends on many factors from either the host or the parasite. The factors affecting the immune response of the host are particularly important for the immunodiagnosis of NCC as they may affect the results between individuals.

4. Detection of Antigens or Antibodies for the Immunodiagnosis of NCC

Immunodiagnostic tests can be divided in two major groups: the ones that use an antigenic mixture or single antigens to try to find antibodies against them; and the ones that use specific antibodies to find specific antigens in the samples.

Looking for antibodies against cysticercal antigens can be done using a variety of samples from the human body: serum, CSF, urine, saliva, and so forth, but a major disadvantage of this approach is that false positives can result as antibodies do not necessarily indicate an active infection with viable metacestodes but a resolved one or exposure to the parasite [61]. Another disadvantage is that cross-reactivity may occur with other parasitic diseases, most commonly *Echinococcus granulosus* [25, 62–67], although cross-reactivity has also been reported with other diseases as hymenolepiasis, fasciolasis [62], toxoplasmosis [67, 68], malaria [67], amoebiasis [67], syphilis [68], hepatitis B [68], toxocariasis [62], cerebral tuberculosis [37, 67], mononucleosis [68], among others [25, 37, 65], which may be due to the selection of bad characterized samples and not necessarily to the fact that patients are infected with something else than *T. solium*. However, the search for antibodies in samples has the advantage that mixtures of antigens can be used (as parasite extracts or semipurified antigens) while the search for antigens needs to have specific antibodies against the desired antigens but can indicate an active infection [15, 69]. When looking for antibodies against parasite antigens, the most frequently used samples are serum or CSF and the most common immunoglobulin is IgG as it is the predominant antibody detected in NCC, although IgA, IgE, and IgM can also be detected but have little value in diagnosis [13, 70], though they can be used for follow-up, as NCC patients show undetectable levels of IgG4, IgM, and IgA antibodies in saliva after drug treatment, while antibodies in serum persist longer regardless of the subtype [33].

5. Antigens Used for Inmunodiagnosis of NC

Multiple antigens have been used for the immunodiagnosis of cysticercosis, among them are low molecular mass (LMM) antigens [31], excretory/secretory (ES) [30, 31, 71–74], crude soluble extract (CSE) [31, 68], total saline extract [59], antigen B [75, 76], lentil lectin glycoproteins (LLGPs) [52],

vesicular fluid (VF) [68, 77], membrane and scolex extracts [68], somatic antigens [74], recombinant proteins [16–18, 20, 21, 25–28], and synthetic peptides [19, 24, 29]. The source of these antigens has been commonly *Taenia solium* (the parasite responsible for NCC) but related species as *Taenia crassiceps* [5, 24, 68, 77–79], *Taenia saginata* [59, 80], or *Taenia taeniformis* [81] have also been used as antigen sources; and among the multiple methods used to date for the immunodiagnosis of NCC complement fixation, agglutination, radioimmunoassays, ELISA and Western Blot (WB) can be counted [34, 77, 80, 82, 83]. Some of these methods are too old, but have the advantage of being cheap, so they could be used with new antigens and yield different results.

Most commonly for the immunodiagnosis of NCC, serum or CSF samples are used with some advantages and disadvantages for each one. For instance, it is proposed that the detection of antigens or antibodies in CSF is better than in serum because there is a release of parasite antigens directly to it or local production of antibodies, but it is more difficult and dangerous to obtain than serum and requires special facilities. However, WB with LLGPs (LLGP-WB, considered by many to be the most reliable method for serologically detecting NCC) both samples have no significant differences in performance, although antigen detection by ELISA is better in CSF than in serum, but this test is less sensitive than LLGP-WB [69].

LLGP-WB [84] has a sensitivity of >90% and a specificity of 100% [2]. This assay involves the separation of 7 glycoprotein antigens (50, 42–39, 24, 21, 18, 14, and 13 kDa) by SDS-PAGE and its recognition in an immunoblot by serum of CSF antibodies. However, in recent studies mainly in Indian patients where almost two thirds of the NCC patients have an SCG [35, 52], LLGPs have shown to be less sensitive than for multiple cysticerci. In these cases, sensitivity has been reported to range between 50 and 80% and specificity between 94 and 100% [22, 35, 85, 86]. Also this test has been shown to be less sensitive in children than in adults and the pattern of protein recognition also differed in these age groups (children tend to recognize the higher molecular mass proteins while adults tend to recognize LMM with serum or CSF antibodies) [87] probably due to the time of infection and the antigenic stimuli. The most common bands identified by serum antibodies are, along with the 18 and 14 kDa, the 29 kDa for SCG and the 31 kDa for multiple lesions [35]. In Latin American NCC patients, where multiple lesions are more common than SCG, the most common recognized bands were 39–42 kDa and 21 kDa [60]. So there seems to be differences not only in the amount of antibodies produced between SCG and multiple lesions [88, 89], but also in the antigens recognized by these antibodies. To try to improve the diagnosis of SCG patients, LLGPs have been unfolded and reduced trying unmask epitopes that may detect antibodies in these SGC patients and resulted that the unfolding of the diagnostic proteins with urea exhibited the maximum antibody binding and in this conformation 46% of the patients with SGC that were serologically negative became positive [35].

Problems like this one, or the difficulty to differentiate between NCC and cysticercosis elsewhere have lead to the search of new antigens and methods to immunodiagnose NCC. Antigenic extracts from *Taenia solium* cysticerci have been reevaluated [31, 68, 90], as well as antigens from other related species as *Taenia taeniformis* [81], *Taenia saginata* [59, 80] or *Taenia crassiceps* [5, 24, 68, 77–79], which have been useful for identifying new protein candidates for the immunodiagnosis of NCC and also have shown drawbacks as the propensity to cross-reactions which has lead to tests with low specificity. However, the use of related species as the ones mentioned above provide an alternative source of parasitic antigens as there are difficulties to obtain *Taenia solium* cysticerci from a natural infected source, even in areas of high endemicity [78, 91]. However, the use of parasitic extracts present many drawbacks that need to be considered if intended for immunodiagnosis as the variation between isolates and differences in the methods of extraction by different laboratories, which leads to poor reproducibility and invalid comparisons between results.

6. Advances in the Methods for Inmunodiagnosis of NC

New methods for the immunodiagnostic of NCC have also been developed involving the reevaluation of common methods with new antigens (e.g., ELISA or WB), or the use of different samples as urine or saliva [30, 31, 33], as they have the advantage that they are very simple to obtain and do not cause any harm to the patient. They are also convenient to assay in endemic areas as facilities to obtain CSF or serum samples are not frequently present [92]. However with these samples not very good results have been obtained in terms of sensitivity or specificity, especially with patients with SCG, and the methods need to be validated with well-defined positive and negative NCC samples and with samples from other parasitic diseases. In the case of urine, sensitivity has been reported to be of 92% which decreased to 62.5% for SCG patients when antigens were detected with monoclonal antibody-ELISA [32]. Results were not very good for antibody detection either by ELISA or LLGP-WB in urine, with reported sensitivities ranging from 44 to 76% and specificities from 33 to 66%, despite the antigen used (ES, LMM, or CSE) [30, 31]. Saliva has been tested for the detection of antibodies against CSE or antigen B (composed by 2 immunologically identical polypeptides prepared by collagen-binding method [75, 76]) with no much better results for any of the immunoglobulins tested (IgG, IgG1, IgG4, IgM, and IgA), although it was shown that IgG4 in saliva could be a useful tool for patients' follow-up after treatment [33]. Nonetheless, these and other studies lack the appropriate controls (well-defined positive and negative samples, and well-defined samples of patients with other infections to assess cross-reactions). Another method that has emerged for the diagnosis of NCC, although it is not immunologically very interesting, is the amplification by PCR of *T. solium* DNA present in the CSF of NCC patients, with a high reported sensitivity of 96.7% [93], though

control samples from patients with other parasitic infections were not included. Other methods for the immunodiagnosis of NCC have also been tried in an attempt to substitute the LLGP-WB assay as its performance is expensive and requires technical expertise. Among these methods is the dot blot which is easier to perform and has shown to have sensibility and specificity similar to ELISA [64, 67, 90, 94].

In addition, there have been efforts to try to correlate the result in an immunodiagnostic test with the location and state of the cysticerci in the brain. An example is the detection of the antigen HP10 in CSF, which correlates with the location of the cyticerci in the brain: when located in the subarachnoideal space or the ventricles, HP10 could be detected, but when located in the parenchyma HP10 could not be detected; and when cysts were damaged, HP10 levels were reduced significantly [15]. Also high antigen levels in CSF suggest the presence of subarachnoid NCC [69]. Many studies have reported tests that can differentiate live from dead cysticerci by the detection of excretory/secretory (ES) antigens in CSF or serum [71–74], or by the detection of antibodies against a 10 kDa protein from the vesicular fluid of *Taenia solium* [17, 25]. These studies support the idea that infected hosts produce antibodies of different specificities as the cysticercus develops, degenerates, and dies because the antigens released by the parasite in each state are different [72].

Many of the most recent efforts in the field of immunodiagnosis of NCC have centered in the production of recombinant proteins or synthetic peptides that could provide a reliable source of antigens without depending on obtaining cysts from naturally infected hosts. The advantage of these antigens is that they would make comparable results between and within laboratories as there is no need to purify cysticercal antigens. However, synthesized or recombinant proteins do not have the same glycosilation pattern as those obtained directly from the parasite (recombinant proteins) or do not have any glycosilation at all (chemically synthesized proteins). This often affects the sensibility of the produced protein, which is frequently assessed with sera that were positive to the native protein, but rarely with different patients' sera to assess if the produced protein could detect more cases than the native one [16, 19–21]. A sensitivity of 95% means that the produced protein can detect 95% of the cases that the native protein detected, though that does not represent a real improvement in immunodiagnosis of NCC unless it can also detect cases that the native protein could not.

The principal protein targets to produce or synthesize are the components of the LLGP-WB that are recognized by NCC patients, and several of them have been already synthesized and tested preliminarily. For instance, from the members of the 8 kDa family, Ts18var1 has been produced in insect cells [16] as well as TsRs1, Ts18var1, and Ts18 Var3 [20]; the 14 and 18 kDa proteins produced by recombination [27]; Ts14, Ts18var1, TSRS1, and TSRS2var1 by chemical synthesis [29], and full-length Ts18 and Ts14 by chemical ligation [19], Ag1V1/Ag2 by recombination [26] as well as Ts8B1, Ts8B2, Ts8B3 [18], Ts14 [27] and a 10 kDa protein [17, 25]; GP50, which is not a member of the 8 kDa family but it is part of the LLGPs, was produced by recombination in bacteria and in a baculovirus expression system [16, 21]. Other proteins outside those from LLGPs that have also been produced or synthesized include T24 (integral membrane protein that does not bind to lentil lectin) produced in a droshophila cell line [22]; HP6-Tsag (oncospheral adhesion protein of *Taenia saginata*) in bacteria and baculovirus systems with similar specificities between the systems (93–95%), but higher sensitivity for the inactive cases by the baculovirus protein (48–64%) [95]; peptide NC-1 selected by phage-display [23]; peptides KETc12, 410, and 413 synthesized from a cDNA library of *T. crassiceps* [24], and recombinant TS24 and Es33 [28]. The methods of production are varied, as well as the results and the ways to evaluate the produced protein, some giving very good sensitivities but in other cases, the native protein is much better than the produced one.

Finally a very interesting approach for the diagnosis of cysticercosis in pigs has been developed. This approach involves the production of nanobodies (camelid-derived single-domain antibody fragments) by recombination after immunizing dromedaries with cisticercal antigens its evaluation for serodiagnosing cysticercosis in pigs. The selected nanobodies had the advantage that did not cross-react with other closely-related parasitic diseases as *Taenia hydatigena, Taenia saginata, Taenia crassiceps,* or *Trichinella spiralis*, although cross-reactivity with other parasites as *Echinococcus granulosus* was not assessed. Nanobodies are heavy-chain-only antibodies that recognize antigens as firmly as normal antibodies do but are about one tenth their size [96]. This characteristic allows them to often recognize epitopes that are not readily accessible to conventional antibodies [97]. Nanobodies have been used also in the diagnosis of trypanosomiasis [98] or malaria [99], but mostly there are being directed to treat more efficiently autoimmune diseases or cancer than commercial conventional antibody therapies [100]. Nanobodies have beneficial production and stability properties [97] which, along with their antigen-recognition characteristics, make them a promising tool for the diagnosis and treatment of many diseases in which NCC may be included, and this approach should be translated to the NCC field as it may provide an assay with higher sensitivity, especially for diagnosing SCG patients which are often negative in conventional tests, by recognizing epitopes that are not recognized by conventional antibodies.

Despite all these efforts and alternatives, a definitive immunodiagnostic test for NCC has not been achieved. The need to account with a more reproducible and sensitive immunodiagnostic test than the actual ones remains, and some studies have focused on this issue by synthesizing antigens from LLGPs used in WB and other antigens, but still there is the need to find new antigens that can detect those cases that are negative in LLGP-WB like SCG cases. Different protein expression systems have been tried to overcome the problem of requiring a natural source of antigens and the difficulties of purifying cysticercal antigens, but the produced proteins lack the natural glycosilation pattern and show diminished sensitivity than the native proteins. New approaches to diagnose NCC should be explored, as

the use of nanobodies that could result very interesting in detecting difficult cases. Developing an immunodiagnostic test that could detect 100% of the true NCC cases and exclude 100% of the true negative cases has been difficult, especially considering biological factors that are involved and the many forms of cysticercosis as differences in the genetics of the host and of the parasite, and there is still much to do to improve the current tests. The biological factors, especially those related to genetic differences of the host or the parasite, that affect NCC may explain why a particular immunodiagnostic test first reports very high sensitivity and specificity scores that then lower as it is being applied in different regions and by different laboratories; and these factors could also make impossible the development of a single immunodiagnostic test, but local tests that can detect cases in a certain endemic area can be developed.

Special attention should be paid in the cases selected to evaluate new tests so results can be compared with other tests, especially in the cases selected as controls (healthy individuals and with other parasitic diseases) as these are responsible for the specificity reported and an adequate selection can assure that results can be compared between tests. Also, attention should be put in including different subsets of NCC clinical types, as these differences affect the amount of antibodies or antigens that can be detected. Extraparenchymal forms of NCC are associated with higher circulating antigen levels and more reactive antibody bands in LLGP-WB than intraparenchymal forms [69, 101–104]. These differences can affect the estimation of the performance of newly developed tests, so attention should be put in the number of cases of each sub-type that are included.

A plausible approach to identify antigens that can be useful for the serodiagnosis of NCC is the separation in 2 dimensions of cysticercal antigens and their recognition by hosts' immunoglobulins. This method can give us useful information about the differences in recognition of the parasite's antigens by different hosts (immunological diversity) and about the antigens that are recognized by many or all infected hosts (if there are any) to select antigens for use in an immunodiagnostic test to detect true positive cases.

Finally, the following proposals for improving the actual immunodiagnostic tests are made.

6.1. Proposals for Improvement. (1) *T. solium* disease is present in many countries around the world and many research groups are working to develop an immunodiagnostic test that can detect all NCC individuals although so far that goal has not been accomplished. Cooperation is necessary to concert a worldwide effort to carefully design a research plan concordant with the complexities of *T. solium* disease, and to develop and test in the short term with a minimal number of options from which to select the most proficient immunodiagnosis of NCC the possibility to be put to immediate production and general use while further research for improvement continues.

(2) Clearing the problem of antigen cross-reactivity and species representation is necessary to succeed in developing an immunodiagnostic test for NCC. Purification of antigen(s) or epitopes critically certified to be exclusive of *T. solium* and present in all members of a representative sample of parasite specimens of an endemic site is mandatory. Some likely candidates have been proposed [105], although further research is necessary to determine if they fulfill the conditions mentioned above. A way of avoiding the high costs and demanding technical skills involved in the purification of natural antigens is the use of those present in phage display peptide libraries [23] or the production of recombinant or chemically synthesized antigens [106]. Antigens present in only *T. solium* but not in other *Taenia* species would constitute the candidate antigen preparation (CAP).

(3) It is also necessary to study and characterize the presumed wide spectrum of humans' antibody production in *T. solium* disease in order to calibrate the candidate antigen preparation that would include all infected individuals. Western Blots using CAP in reaction with representative samples of all subsets of infected individuals (regardless of whether the parasite had established or not) if possible, or at least of confirmed cysticercosis and NCC samples, would provide the images necessary to construct all immunological profiles of the infected individuals. Computer-assisted image analysis of WB and cluster analysis could address this problem. The set of CAP that reacts with all or most infected individuals in which the parasite was established would constitute the definitive antigen preparation (DAP).

(4) Rather than attempting to develop ways to distinguish each of the different subsets of NCC disease, efforts in immunodiagnosis could focus on improving diagnosis of NCC (to include all NCC and NCC + cysticercosis samples and exclude cysticercosis, taeniosis, and infected but not established samples), while for the prevalence of *T. solium* disease, in whatever its form, it should only clearly distinguish members of the infected (established or not) from the not infected.

Three are the classes of *T. solium* disease that matter the most and perhaps require different strategies: the contact case, the NCC case (whether it is only NCC or NCC + cysticercosis elsewhere), and the tapeworm carrier. For this purpose, it is indispensable to construct representative and certified negative and positive control panels of the samples CSF, serum, and feces from each geographic area upon their reaction with DAP. Certification of the members of cysticercosis elsewhere and of the noninfected individuals is complicated by its need of whole-body scans in search of cysticerci located elsewhere of CNS. Additional negative control samples from a culturally and historically certified community or geographic area without *T. solium* disease and low in infectious diseases in general would be useful to establish the cut-off values for immunotesting with DAP.

(5) Once the problem of antigen specificity and representation is solved, there should be no major problem to Immunodiagnose NCC in the CSF of a symptomatic neurological patient nor of an intestinal tapeworm in the feces, preferably by antigen detection (this is to distinguish cysticercosis located elsewhere and live from dead cysticerci in the CNS because antibodies could persist after the death of the parasite for unknown periods of time).

(6) However there would remain serious problems to tackle for serology, the most accessible sample useful for the detection of early nonsymptomatic NCC cases in the general population and for epidemiological studies of *T. solium* disease prevalence. The major problem for serology in unambiguously detecting asymptomatic NCC cases is the potential location elsewhere of the parasite (cysticercosis elsewhere or taeniosis) that produces false-positive results or the low reactivity of patients with few live cysticerci or with dead cysticerci (NCC or elsewhere) that produces false-negative results. Adding to positive serology a marker of CNS damage [107, 108] as a sign of CNS involvement could help in discriminating NCC from other forms of *T. solium* disease.

The development of an effective and definitive immunodiagnostic test for NCC is possible, but a series of considerations and evaluations need to be addressed first as stated above, and a worldwide effort is required to develop a test that could be effective everywhere. Nonetheless, the effort is necessary and the result would be very useful to help eradicate this disease.

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Research Article

Upregulated Expression of Cytotoxicity-Related Genes in IFN-γ Knockout Mice with *Schistosoma japonicum* Infection

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It is well accepted that IFN-γ is important to the development of acquired resistance against murine schistosomiasis. However, the *in vivo* role of this immunoregulatory cytokine in helminth infection needs to be further investigated. In this study, parasite burden and host immune response were observed in IFN-γ knockout mice (IFNg KO) infected with *Schistosoma japonicum* for 6 weeks. The results suggested that deficiency in IFN-γ led to decreased egg burden in mice, with low schistosome-specific IgG antibody response and enhanced activation of T cells during acute infection. Microarray and qRT-PCR data analyses showed significant upregulation of some cytotoxicity-related genes, including those from the granzyme family, tumor necrosis factor, Fas Ligand, and chemokines, in the spleen cells of IFNg KO mice. Furthermore, $CD8^+$ cells instead of NK cells of IFNg KO mice exhibited increased transcription of cytotoxic genes compared with WT mice. Additionally, *Schistosoma japonicum*-specific egg antigen immunization also could activate $CD8^+$ T cells to upregulate the expression of cytotoxic genes in IFNg KO mice. Our data suggest that IFN-γ is not always a positive regulator of immune responses. In certain situations, the disruption of IFN-γ signaling may up-regulate the cytotoxic T-cell-mediated immune responses to the parasite.

1. Introduction

Interferon gamma (IFN-γ) is a cytokine with multiple immunoregulatory functions that mediates the host defense against various pathogen infections. The broad effects of IFN-γ include activation of macrophages and antiviral immunity, enhancement of antigen presentation, induction of MHC-peptide complexes, orchestration of lymphocyte-endothelial interactions, regulation of T cell polarization toward Th1, cellular proliferation, and stimulation of apoptosis [1]. The importance of these diverse IFN-γ-mediated functions is also highlighted by the examination of schistosome infections, which are classically a type of multicellular parasitic infections.

It is well accepted that T-cell-mediated immunity, mainly that mediated by $CD4^+$ T cells, is important to the development of acquired resistance against schistosomes. Following infection by normal cercariae, a predominant Th1 immune reaction is observed in the early phase, which then shifts to an egg-induced Th2-biased profile. Many immunization studies, especially using a variety of animal models vaccinated with attenuated cercariae [2–6] suggest that Th1 cytokines, including IFN-γ and IL-2, and the activated macrophages may be beneficial in preventing schistosomiasis. Also, some immuno-epidemiological studies on reinfection following drug treatment have shown that people living in endemic areas acquire some form of protective immunity after years of exposure to *Schistosoma mansoni*, *Schistosoma haematobium*, or *Schistosoma japonicum* [7–9]. Th1 response (particularly IFN-γ production) to schistosomulum antigen is hypothesized to be the key to schistosomiasis resistance in these subjects [10, 11]. Thus, an important strategy for vaccine design and development of an immune response against schistosomes involves induction of inherent IFN-γ production, which will facilitate the mounting of a Th1 response, especially at the early stage of infection [12].

It has been theoretically speculated that increased worm burdens and/or higher worm fecundity would be present in *Schistosoma japonicum*-infected IFN-γ knockout mice (IFNg KO mice). However, in our studies, a very interesting

phenomenon showed that the absence of IFN-γ made little difference in the worm burdens, while lower egg burdens were observed in IFNg KO mice. To explore some other possible immunological events in the absence of IFN-γ signaling in *Schistosoma japonicum* infection, the characteristics of the host immune responses were investigated in infected IFNg KO mice with lower egg burdens.

2. Materials and Methods

2.1. Experimental Mice and Parasites. Six- to eight-week-old female IFN-γ knockout (IFNg KO) mice and the wild-type (WT) control C57BL/6J (B6) mice were purchased from Model Animal Research Center of Nanjing University (Nanjing, China). All mice were maintained and bred under specific pathogen-free conditions at Nanjing Medical University. All experiments were undertaken with the approval of Nanjing Medical University Animal Ethics Committee. *Schistosoma japonicum* (*S. japonicum*, a Chinese mainland strain) cercariae were maintained in *Oncomelania hupensis* snails as the intermediate host, which were purchased from Jiangsu Institute of Parasitic Disease (Wuxi, China).

2.2. Infection with S. japonicum and Assessment of Parasite Burden. IFNg KO mice and WT mice were percutaneously infected with 40 $\pm$ 2 *S. japonicum* cercariae through their shaved abdomen. There were ten mice in each group. At 6 weeks after-infection, all mice were sacrificed to measure the parasitological parameters. After perfusion of the thoracic aorta, the recovery of worms was calculated by perfusate sedimentation plus residual worms from the intestinal mesenteric vessels. Weighed liver samples were digested in 5% KOH at 37°C overnight to count the released eggs under a microscope. The released eggs were microscopically counted. Parasite burden was measured by the total number of worms recovered, released eggs in the liver, eggs per pair counted, and eggs per gram liver sample counted. Two independent experiments were carried out.

2.3. Detection of Schistosome-Specific IgG Antibodies in Serum by Enzyme-Linked Immunosorbent Assay (ELISA). Serum was prepared from the peripheral blood sample at day 0, 3 weeks, and 6 weeks after *S. japonicum* infection. The levels of *S. japonicum* soluble adult worm preparation-(SWAP-) and egg antigen-(SEA-) specific IgG antibodies in sera were measured using an indirect ELISA. The concentrations of coated SWAP and SEA were 6 μg/mL and 5 μg/mL, respectively. All serum samples were diluted to 1 : 100 in phosphate buffered saline (PBS). Secondary goat anti-mouse IgG labeled with horseradish peroxidase (HRP) (ABD, USA) was diluted to 1 : 1500 and used to detect the primary antibody. Each sample was assayed in triplicate. On each plate, positive and negative tests were provided as quality controls. OD (optical density) values were read at 450 nm (Clinibio 128C, ASYS Hitch GmbH, Austria).

2.4. Isolation of Splenocytes, Calculation of the Percentage of $CD8^+$ and NK Cell by Flow Cytometry, and Purification of $CD8^+$ and NK Cell Subsets by Magnetic-Activated Cell Sorting (MACS). Spleens were aseptically removed when uninfected mice and 6-week *S. japonicum*-infected IFNg KO and WT mice were sacrificed. Spleen cells were prepared by gently forcing spleen tissue through a fine nylon net into incomplete RPMI 1640 containing 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. After removal of erythrocytes, the cells were resuspended and diluted to a final concentration of 1.0×10^7 cells/mL. The viability of splenocytes was >95%, as assessed by trypan blue dye exclusion.

Each one of the 1.0×10^6 splenocytes was, respectively, put into a tube and labeled with FITC anti-mouse CD19 to stand for "B cells," labeled with APC anti-mouse CD3 and FITC anti-mouse CD4 to mark "$CD3^+CD4^+$ cells", and labeled with APC anti-mouse CD3, PE anti-mouse NK1.1 and FITC anti-mouse CD8a to count the percentage of $CD3^-NK1.1^+$ or $CD3^+CD8^+$ cells in the splenocytes by flow cytometry.

For isolation of mouse $CD8^+$ and NK cells from a mouse spleen cell suspension, the splenocytes were readjusted to a concentration of 1×10^8 cells into 400 μL buffer, incubated with 100 μL mouse anti-CD8a cell microbeads or mouse anti-NK cell (CD49b, DX5) microbeads (Miltenyi Biotec GmbH, Germany) for 15 min at 4–8°C, and washed with buffer one time. NK^+ and $CD8a^+$ cells were separated using magnetic activated cell sorting (MACS; Mini Macs, Miltenyi Biotec), by applying the cell suspension to a plastic column equipped with an external magnet. The sorted NK and $CD8^+$ cell suspensions were, respectively, incubated with PE-conjugated rat anti-mouse CD49b (DX5) monoclonal antibody or FITC-conjugated rat anti-mouse CD8a monoclonal antibody to confirm the purity by flow cytometry (Miltenyi Biotec GmbH, Germany). Purified NK and $CD8^+$ cells were used for the microarray analysis.

2.5. Measurement of Type 1/Type 2 Cytokine Levels in the Splenocyte Culture Supernatants. Isolated splenocytes harvested from uninfected mice and 6-week *S. japonicum*-infected IFNg KO and WT mice were cultivated without or with 10 μg/mL SEA and 1 μg/mL ConA for 72 hours. Next, the supernatants were collected for the Th1/Th2 cytokine assay. Cytokine levels were examined using the Bio-Plex mouse Th1/Th2 cytokine assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for interleukin (IL)-12p70, IFN-γ, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, IL-4, IL-5, and IL-10 according to the recommended procedure and protocols of the manufacturer. Parameters were read on the Bio-Plex suspension array system, and the data were analyzed using Bio-Plex ManagerTM software with 5 PL curvetting.

2.6. Microarray Analyses of Splenocytes, Purified $CD8^+$, and NK Cells from S. japonicum-Infected Mice. The isolated splenocytes, $CD8^+$, and NK cells harvested from IFNg KO and WT mice at 6 weeks after-infection were subjected to gene expression profile analyses. First, total RNA was extracted using the TRIzol reagent (Invitrogen Life Technologies) and purified using the RNeasy kit (QIAGEN).

An equal amount of total RNA from five to six mice per group was mixed and cDNA was generated using the One-Cycle Target Labeling and Control Reagents (Affymetrix). The cRNA was made with the GeneChip IVT Labeling Kit (Affymetrix). Biotin-labeled, fragmented (200 nt or less) cRNA was hybridized for 16 hours at 45°C to Affymetrix Mouse 430 2.0 arrays (Affymetrix) by the Microarray Facility. The arrays were washed and stained and were subsequently read using a GeneChip Scanner 3000. The fluorescence signal was excited at 570 nm, and data were collected on a confocal scanner at 3 μm resolution. Data sorting and analysis were acquired by GeneSpring GX7.0 software (Agilent). After the normalization and filtering procedure, the system identified the differentially expressed genes that had differences of 2-fold or greater. These genes were placed into pathways based on the KEGG and GENEMAP databases. Significant pathways with differentially expressed genes were identified ($P < 0.05$) by use of the Fisher's Exact Test and Chi-square (χ^2) test. The -LgP is given to assess the significance of a particular pathway category.

2.7. Quantitative Real-Time PCR (qRT-PCR). Total RNA was extracted from the isolated splenocytes harvested from IFNg KO and WT mice at 6 weeks after-infection using TRIzol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. The total RNA was transcribed to cDNA using a commercially available reverse transcription kit (Epicentre, USA). The cDNA was employed as a template in the following real-time PCR. Primers specific for β-actin, granzyme A (*gzma*), granzyme B (*gzmb*), granzyme K (*gzmk*), perforin 1 (*prf1*), Fas Ligand (*fasl*), chemokine (C-C motif) ligand 5 (*ccl5*), killer cell lectin-like receptor, subfamily K, member 1 (*klrk1*), and tumor necrosis factor (ligand) superfamily, member 9 (*tnfsf9*) are shown in Table 1. The PCR reaction was carried out in a 10 μL reaction mixture containing 2 μL of cDNA, 2× Master Mix (eENZYME, USA), and 0.625 μL of forward primer and reverse primer, respectively (Invitrogen, CA). The qRT-PCR was performed using an ABI 7900 Real-time PCR system with the following program: 95°C for 10 min, 40 cycles at 95°C for 15 sec, and at 68°C for 1 min. To create the PCR melting curve, the amplified product was submitted to incubation at 95°C for 2 min; 60°C for 20 sec; 99°C for 10 sec. β-actin was used as an internal control. The relative transcription levels of individual target genes were normalized using the internal control. The identity and purity of the PCR product were confirmed by melting curve analysis. All data were analyzed using PE Applied Systems Sequence Detector 1.3 software. The threshold cycle number was used to quantify the target gene transcription level for each sample using the comparative threshold cycle method. The results represent the expression level of the target gene relative to the expression level of β-actin.

2.8. Immunization of Mice with SEA and Cytotoxicity Assay of Purified CD8⁺ and NK Cells. A volume of 100 μL PBS containing 50 μg of soluble egg antigen (SEA) was, respectively, injected at 3 different subcutaneous sites on the back of IFNg KO and WT mice. Booster immunizations were conducted at 1 and 2 weeks after the initial vaccination. The animals were sacrificed at 7 days after the last immunization. NK cells and $CD8^+$ cells separated from the splenocytes were, respectively, submitted to cytotoxicity and qRT-PCR assays.

Table 1: qRT-PCR primers and products.

Gene symbol	Primer sequence (5′ → 3′)	Product (bp)
β-actin	F: 5′CCTCTATGCCAACACAGTGC3′ R: 5′GTACTCCTGCTTGCTGATCC3′	211
gzma	F: 5′TGTGAAACCAGGAACCAGATG3′ R: 5′GGTGATGCCTCGCAAAATA3′	256
gzmb	F: 5′TGCTCTGATTACCCATCGTCC3′ R: 5′GCCAGTCTTTGCAGTCCTTTATT3′	89
gzmk	F: 5′CCCACTGCTACTCTTGGTTTC3′ R: 5′GGCATTTGGTCCCATCTCTA3′	252
prf1	F: 5′CCCACTCCAAGGTAGCCAAT R: 5′GCTGTAAGGACCGAGATGCG	265
fasl	F: 5′GGTTCTGGTGGCTCTGGTT3′ R: 5′ACTTTAAGGCTTTGGTTGGTG3′	105
ccl5	F: 5′ACCACTCCCTGCTGCTTTG3′ R: 5′CACACTTGGCGGTTCCTTC3′	131
klrk1	F: 5′GCAGTTCTTGCCTCACTTCC3′ R: 5′AGCTCCTCCTCGTCTTCTTC3′	97
tnfsf9	F: 5′TGTTCCTATCTTCACCCGC3′ R: 5′GACTGTCTACCACCAACTCCTT3′	290

The gene transcription levels for some cytotoxic molecules, including *gzma*, *gzmb*, *gzmk*, *prf1*, *fasl,* and *tnfsf9*, were detected in purified $CD8^+$ cells by qRT-PCR, as described above. For the cytotoxicity assay of NK cells, 1×10^6/mL of YAC-1 cells labeled with ^{3}H-TdR 10 μCi were cultured in an incubator (37°C, 5% CO_2) for 2 hours, shaking every 30 min. Next, YAC-1 cells were washed with the RPMI-1640 in triplicate and adjusted to 1×10^5/mL. A volume of 100 μL of YAC-1 cells and 100 μL of purified NK cells of 1×10^7/mL were added to 96-well plates (effector-target ratio = 100 : 1). Additionally, 100 μL of YAC-1 cells and 100 μL of RPMI-1640 were added to wells as the blank control. A total of 100 μL of YAC-1 cells and 100 μL of 1% Triton X-100 were added to wells as the maximum release control. All plates were cultured in 5% CO_2, 37°C for 4 hours. A Liquid Scintillation Counting System collected the data. Cytotoxicity of NK cells is calculated by the following formula:

$$\frac{{}^3\text{H-TdR release value of Experiment} - {}^3\text{H-TdR release value of Blank well}}{{}^3\text{H-TdR release value of Maximum well} - {}^3\text{H-TdR release value of Blank well}} \times 100\%. \quad (1)$$

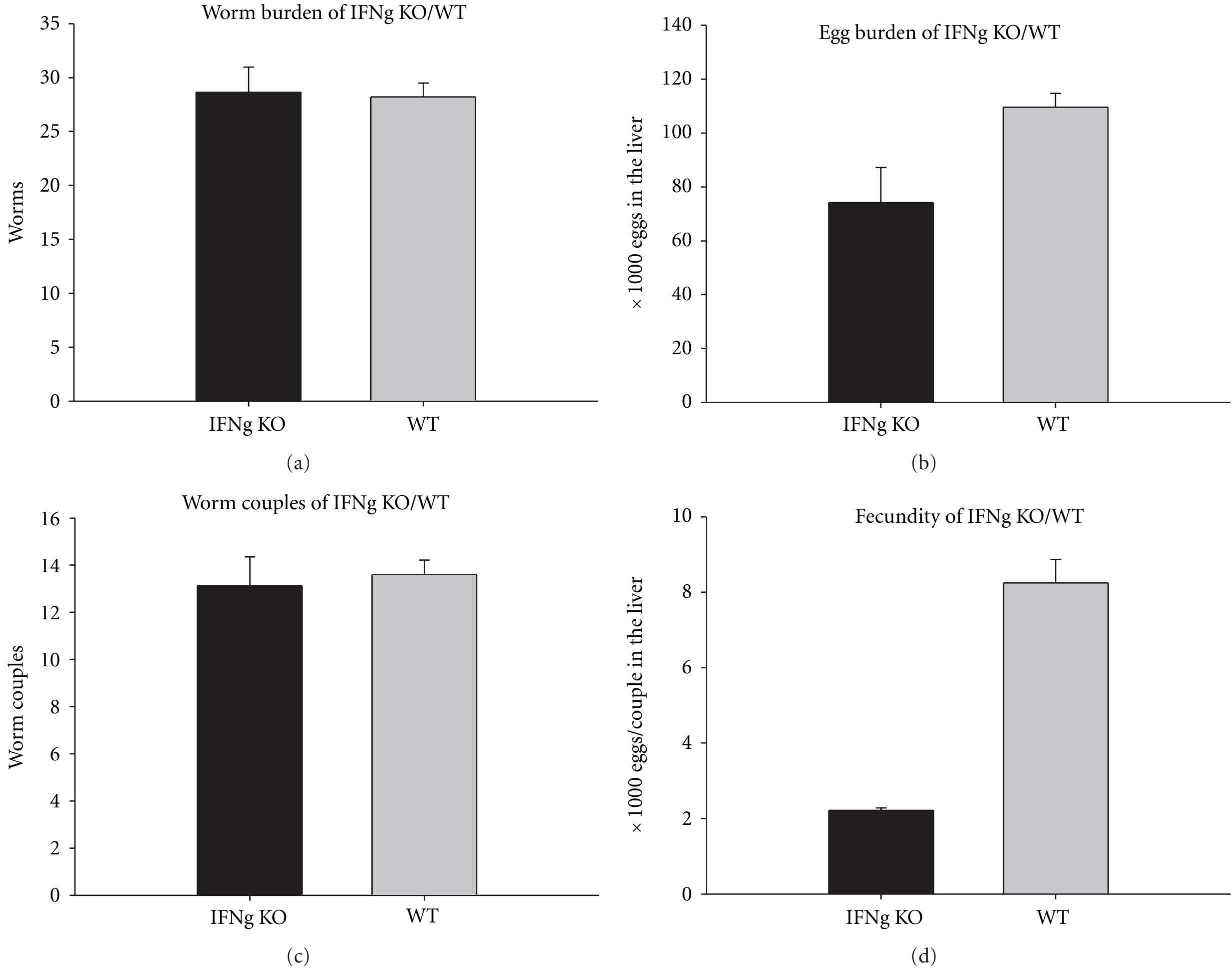


Figure 1: Parasite burden of IFNg KO mice and WT mice ($n = 10$, resp.) at 6 weeks after-infection with *Schistosoma japonicum* (compared with WT mice, $^{**}P < 0.01$). (a) Total worms were recovered by portal perfusion at 6 weeks after-infection. (b) Eggs deposited in the liver were counted after digestion of the liver with 5% KOH. (c) Worm pairs were recovered by portal perfusion at 6 weeks after-infection. (d) Eggs deposited per worm couple in the liver. Data are representative of two independent experiments with the similar results.

2.9. Statistical Analysis. The data are presented as mean ± SEM. Significance was tested using unpaired t-test, ANOVA, or the Mann-Whitney test where appropriate, or in the case of microarray data, Fisher's Exact Test, Chi-square (χ^2) test. All statistics were analyzed with SPSS 16.0 software. Significant values were indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$.

3. Results

3.1. Deficiency of IFN-γ Signaling Led to Decreased Egg Burden. To investigate the outcome of infection with *S. japonicum* in the absence of IFN-γ, parasite burden was evaluated at six weeks after the 40 cercariae challenge. Two independent animal experiments showed that the total egg number in the liver of IFNg KO mice was significantly lower than that in WT mice ($P < 0.01$), although there was little difference in worm recovery between these two groups, as in one of these experiments shown in Figures 1(a)–1(c). The number of eggs per pair of worms is a significant index of the fecundity of *Schistosoma japonicum*, which can exclude the difference of pairs and be objective to assess the pathological damage of liver by the deposit of eggs. As shown in Figure 1(d), the number of eggs per pair in IFNg KO mice was much lower than that in WT mice, indicating that the absence of IFN-γ might have a deleterious effect on the fecundity of worms.

3.2. IFN-γ-Deficient Mice Displayed a Low Schistosome-Specific IgG Antibody Response. To study the humoral response in the acute infection, schistosome-specific IgG levels in sera were determined by ELISA. With the progress of *S. japonicum* infection, SWAP-specific IgG antibody levels in mice sera continued to rise. Although there was no difference in worm numbers between IFNg KO and WT mice, SWAP-specific IgG antibodies of IFNg KO mice at 3 and 6 weeks after-infection were significantly lower than those of WT mice (Figure 2(a)). *S. japonicum* worms begin

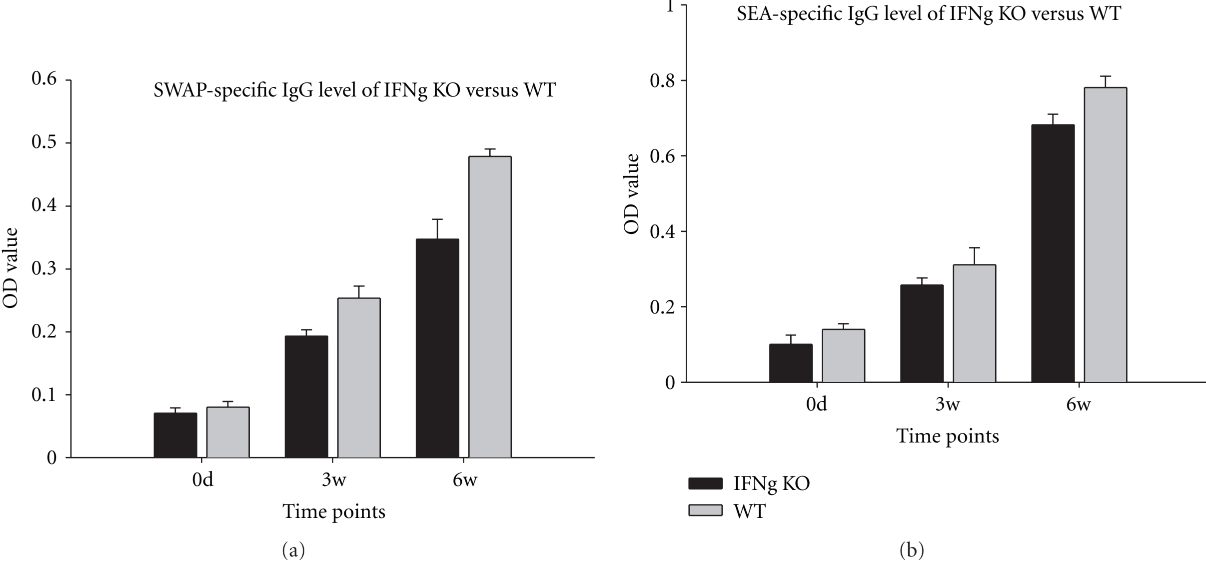


Figure 2: Dynamics of SWAP- and SEA-specific IgG antibody levels in IFNg KO and WT mice ($n = 10$, resp.) according to ELISA of sera harvested at day 0, 3 weeks, and 6 weeks after-infection (compared with WT mice, $^*P < 0.05$, $^{**}P < 0.01$). Data are representative of two independent experiments with the similar results.

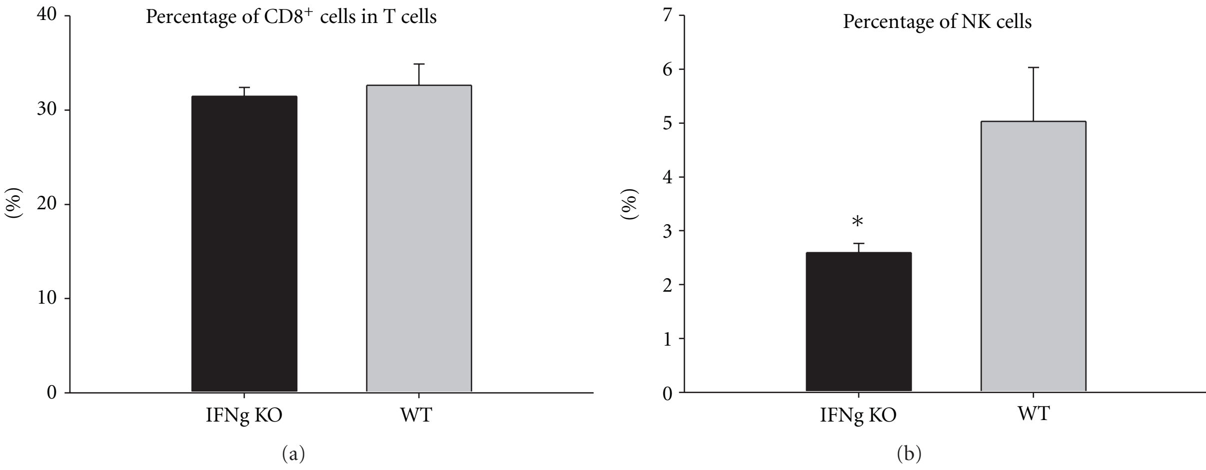


Figure 3: Percentage of $CD8^+$ cells among T cells and NK cells among spleen cells as determined by FACS at 6 weeks after-infection with *Schistosoma japonicum* (compared with WT mice, $^*P < 0.05$).

to lay eggs at approximately 3 weeks after infection. Thus, SEA-specific IgG antibody level in sera was significantly elevated at 6 weeks after-infection. Comparably, SEA-specific IgG antibody level in sera from IFNg KO mice at 6 weeks after-infection was also lower than that from WT mice (Figure 2(b)).

3.3. The Percentage of T-Cell Subsets of IFNg KO Mice Were Comparable to WT Mice, While There Were Fewer NK Cells in the Splenocytes of IFNg KO Mice. Before infection, no significant difference was observed in the total number of cells in the spleens of the IFNg KO mice compared to WT mice, nor were there any alterations of splenic cell populations with respect to CD3, CD4, CD8, CD19, and NK1.1 surface markers. At 6 weeks after *S. japonicum* infection, there were no significant differences in the percentage of $CD3^+$, $CD4^+$, $CD8^+$, and B cells among the spleen cells between IFNg KO and WT mice. Also, little differences were observed in the percentage of $CD4^+$ and $CD8^+$cells among T cells between these two mice groups. However, the percentage of NK cells in IFNg KO mice was significantly lower than that in WT mice ($P < 0.05$). Figure 3 showed the percentages of $CD8^+$ cells among $CD3^+$ T cells and NK cells among the spleen cells at 6 weeks after *S. japonicum* infection.

3.4. Deficiency of IFN-γ Enhanced the Activation of T Cells during Acute Infection with S. japonicum. To assess the effects of IFN-γ deficiency on the cellular immune response,

Th1/Th2 cytokines, IFN-γ, IL-12, TNF-α, IL-2, IL-10, IL-4, IL-5, and GM-CSF in the splenocyte culture supernatant were measured. All cytokine levels before infection were very low and close to baseline (data not shown). At 6 weeks after *S. japonicum* infection, cytokine expression of both IFNg KO and WT mice without any stimulation also stayed at low levels (Figure 4). With ConA stimulation, IL-12, TNF-α, IL-5, IL-10, and GM-CSF of IFNg KO mice were significantly higher than those of WT mice ($P < 0.05$). Furthermore, with specific stimulation of SEA, IL-5 and GM-CSF levels in IFNg KO mice were higher than those of WT mice ($P < 0.05$). More importantly, levels of IL-10 in sera from IFNg KO mice were much lower than those in WT mice, which might contribute to immune activation in IFNg KO mice.

3.5. Microarray and qRT-PCR Analyses of Splenocytes Showed That Some Genes Related to Cytotoxicity Were Significantly Upregulated in IFNg KO Mice. Based on the above-described parasitological and immunological differences between *S. japonicum*-infected IFNg KO and WT mice, a gene expression profiling approach was used to compare the functional gene expression changes in the spleen cells. All differentially expressed genes with 2-fold or greater changes were placed into pathways based on the KEGG and GENEMAP databases. The value of "-LgP" stands for the significance of a specific pathway in IFNg KO mice compared with that in WT mice (Figure 5). Pathway analysis of splenocytes (Figure 5(a)) showed that several immune-related pathways, including cytokine-cytokine receptor interaction, hematopoietic cell lineage, leukocyte transendothelial migration, Toll-like receptor signaling pathway, cell adhesion molecules (CAMs), complement and coagulation cascades, natural killer cell-mediated cytotoxicity, MAPK signaling pathway, antigen processing and presentation, PPAR signaling pathway, and apoptosis, were significantly enhanced in IFNg KO mice. The differentially expressed genes in the pathways of cytokine-cytokine receptor interaction and natural killer cell mediated cytotoxicity were listed in Table 2. For some genes, transcription levels of proinflammatory factors chemokines and their receptors (such as *ccl2, ccl5, cxcl2, ccr5,* and *cxcr6*) and cytotoxicity-related molecules (such as *gzma, gzmb, gzmk, prf1, fasl, klrc1*, *klrd1*, *klrg1,* and *klrk1*) were significantly upregulated in IFNg KO mice.

Notably, some genes belonging to the signaling pathway of natural killer cell-mediated cytotoxicity should be mentioned. Several genes related to cytotoxic effects, including *gzma, gzmb, gzmk, prf1,* and *fasl,* and some genes related to activating and recruiting killer cells were also examined by qRT-PCR detection. Relative transcription levels of *gzma, gzmb, gzmk, fasl, ccl5,* and *klrk1* were significantly higher in IFNg KO mice than those in WT mice, which were consistent with the microarray data (Figure 6).

3.6. Microarray Data for Purified CD8$^+$ Cells and NK Cells Revealed That the CD8$^+$ Cell Subset Might Play More Important Role in the Cytotoxic Effect in S. japonicum Infection. To investigate the cytotoxic genes expression associated with CD8$^+$ cells and/or NK cells in IFNg KO mice infected with *S. japonicum*, we purified CD8$^+$ cells and NK cells from splenocytes of IFNg KO and WT mice by MACS for further microarray analysis. The purity of CD8$^+$ cells and NK cells are about 99% and 80%, respectively. The differentially expressed genes between IFNg KO and WT mice with 2-fold or greater changes were also placed into pathways based on the KEGG and GENEMAP databases. Most of the increased immune-related pathways in purified CD8$^+$ and NK cells (Figures 5(b) and 5(c)) were seen in spleen cells, such as cytokine-cytokine receptor interaction, hematopoietic cell lineage, leukocyte transendothelial migration, Toll-like receptor signaling pathway, cell adhesion molecules (CAMs), complement and coagulation cascades, natural killer cell-mediated cytotoxicity, antigen processing, and presentation. Furthermore, it was found that natural killer cell-mediated cytotoxicity exhibited more significance of enhancement in purified CD8$^+$ cells than in NK cells. As listed in Table 2, those genes associated with cytotoxicity, including the granzyme family members *gzma, gzmb, gzmk* and *prf1*, *fasl,* and *tnf*, strongly enhanced the transcriptional levels in CD8$^+$ cells of IFNg KO mice compared with those of the WT mice. Unlike CD8$^+$ cells, there was little difference in those transcripts in NK cells. In addition, NK cells might not be excluded from function as regulators of immune response to *S. japonicum* infection through upregulated transcription of some cytokines, chemokines and CD molecules, such as *ccl2*, *ccl4*, *il18*, *il18r1*, *il6*, *cd14*, and *cd28,* in IFNg KO mice.

3.7. Schistosoma japonicum-Specific Egg Antigen Could Activate CD8$^+$ T Cells in IFNg KO Mice. To ascertain whether the specific antigen may directly induce the cytotoxic activity of CD8$^+$ cells or NK cells, rather than complicated factors in the infectious course, IFNg KO and WT mice were immunized with *Schistosoma japonicum*-specific egg antigen (SEA). Next, CD8$^+$ cells and NK cells were sorted from the splenocytes. As Figure 7 illustrates, expression of *gzma*, *gzmb*, *gzmk*, *prf1*, *fasl,* and *tnfsf9* in purified CD8$^+$cells was measured by qRT-PCR. Although only gzmb and tnfsf9 were significantly higher in IFNg KO mice relative to WT mice, other genes showed a trend of enhanced expression in IFNg KO mice. Thus, *Schistosoma japonicum* SEA might activate the cytotoxic ability of CD8$^+$ cells in IFNg KO mice. Meanwhile, to assess the cytotoxicity of NK cells, purified NK cells stimulated by *Schistosoma japonicum* SEA were cocultured with YAC-1 cells, which are specific target cells for activated NK cells. As Figure 8 illustrates, cytotoxicity of NK cells from IFNg KO mice was decreased, although there was no significant difference between these two groups.

4. Discussion

It has been well documented that IFN-γ plays significant protective roles in the host response to *Leishmania*, *Toxoplasma gondii* [13], *Plasmodium* [14], *Candida albicans* [15], and other intracellular pathogens. As for many extracellular metazoan parasites, such as schistosomes, most studies support the hypothesis that the Th1 response, especially IFN-γ secretion, can activate macrophages and/or other effectors

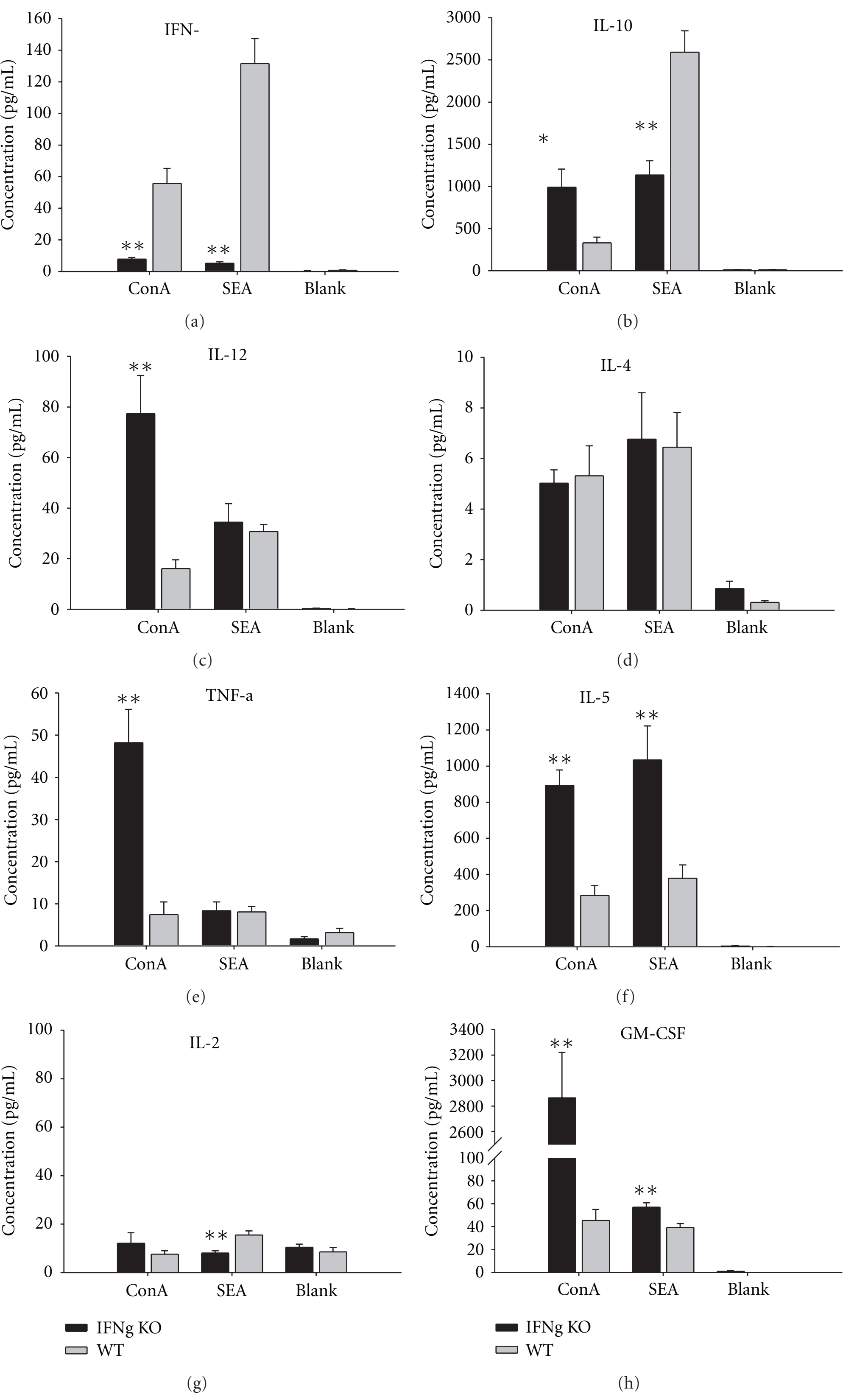
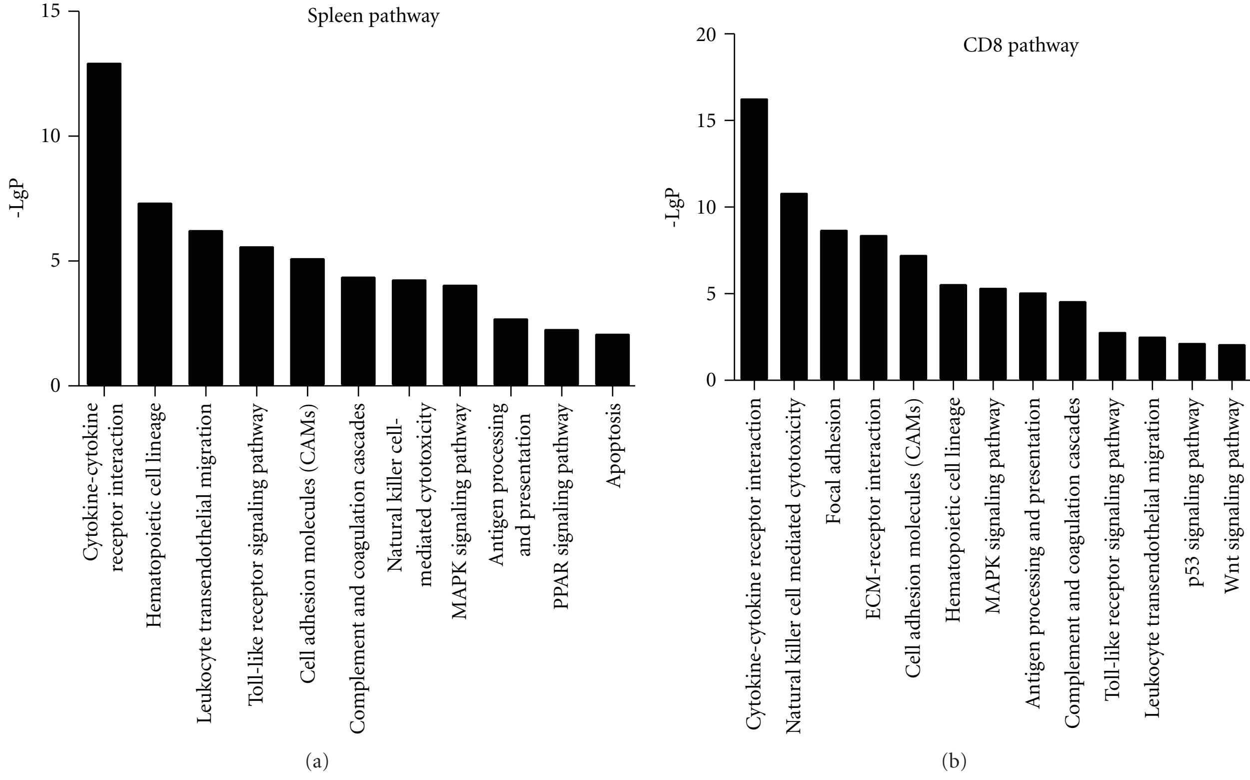


Figure 4: Type 1/Type 2 cytokine levels in the supernatant of splenocyte cultures of IFNg KO and WT mice ($n = 10$, resp.) at 6 weeks after *Schistosoma japonicum* infection by Bio-Plex detection (compared with WT mice, $^{*}P < 0.05$, $^{**}P < 0.01$). Data are representative of two independent experiments with the similar results.



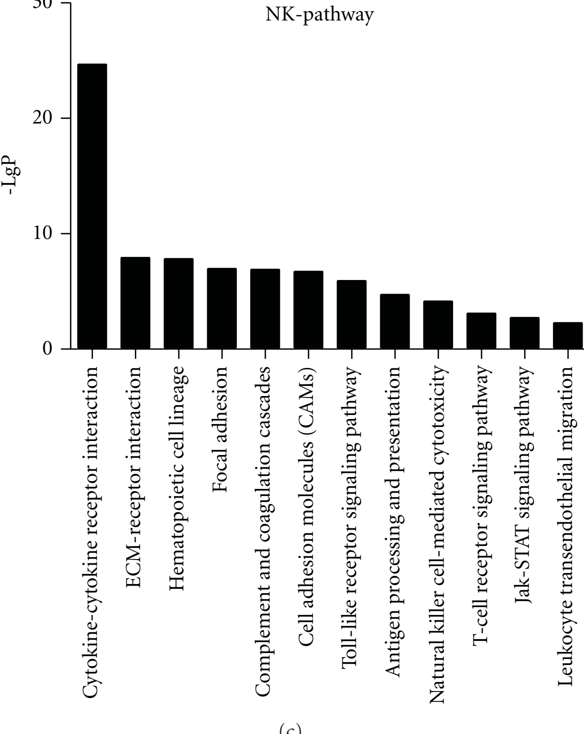


Figure 5: Significantly upregulated pathways with differentially expressed genes in splenocytes, purified $CD8^{+}$ cells, and NK cells in 6-week *Schistosoma japonicum*-infected IFNg KO mice compared with WT mice based on KEGG and GENEMAP databases. The value of "-LgP" stands for the significance of a particular pathway category.

Table 2: Signal intensities of some characteristic genes in spleen cells, purified CD8$^+$ and NK cells from 6-week *Schistosoma japonicum*-infected mice.

Gene Symbol	Probe Set ID	Spleen cells			Purified CD8$^+$ cells			Purified NK cells		
		SI* of IFNg KO	SI of WT	Change**	SI of IFNg KO	SI of WT	Change**	SI of IFNg KO	SI of WT	Change**
ccl2	1420380_at	241.3	48.3	Increase	80.5	54.1	No change	219.8	43	Increase
ccl4	1421578_at	674.3	293.7	No change	2028.4	874.2	Increase	6176.4	2540.6	Increase
ccl5	1418126_at	19335.4	6000.8	Increase	32722	27917.8	No change	25966.1	29725.3	No change
ccl9	1417936_at	3048.6	2043.1	Increase	939.3	493.4	Increase	14887.6	10318.9	Increase
ccr2	1421188_at	414.5	141.6	No change	586.5	211.5	Increase	4102.4	1825.7	Increase
ccr3	1422957_at	141.5	97	No change	247	29.4	Increase	575.3	130.2	Increase
ccr5	1424727_at	594.2	257.3	Increase	1422.7	1105	Increase	1757.1	1424.8	Increase
cxcl2	1449984_at	3436.5	1584.8	Increase	2948.7	690.5	Increase	5645.8	1727.6	Increase
cxcr6	1422812_at	2547.3	438.4	Increase	6390	1782.7	Increase	8213.3	2695.1	Increase
csf1r	1419873_s_at	3946.4	2641.7	Increase	4913.4	1210.7	Increase	4166.8	2346.6	Increase
il1b	1449399_a_at	2322.7	1027.5	Increase	1919.8	1355.5	Increase	1587.8	1663	No change
il18	1417932_at	1280	941	No change	1722.4	658.9	Increase	1498.4	840.9	Increase
il18r1	1421628_at	2336.8	279.4	Increase	5965.7	2332.4	Increase	10221.2	4810	Increase
il18rap	1456545_at	1326.6	392.7	Increase	3042	1722	Increase	5229.3	4758.1	No change
Il6	1450297_at	115	44.9	No change	20.7	33.6	No change	5888.1	2484.2	Increase
tnf	1419607_at	291.9	195	No change	472.2	253.8	Increase	906.7	551.2	Increase
gzma	1417898_a_at	13097.3	2514.9	Increase	22373.6	11803.6	Increase	32005.9	35300.9	No Change
gzmb	1419060_at	6568.9	1127.1	Increase	17907	6863.3	Increase	14257.1	14818.8	No Change
gzmk	1422280_at	2049.2	400.9	Increase	10278.1	2464.3	Increase	3299.7	1849.7	Increase
prf1	1451862_a_at	677.4	262.8	Increase	3098.3	2575.4	Increase	2369.2	2338.4	No Change
fasl	1449235_at	514.6	232.2	Increase	1198.3	497	Increase	2026	1903.7	No Change
klrc1	1425005_at	1237.6	272.5	Increase	2809.8	1154.7	Increase	9477.8	6187.2	Increase
klrd1	1460245_at	2968.6	1158.5	Increase	12620.7	11113.5	Increase	14710.6	10420.8	Increase
klrg1	1420788_at	2706.3	315.4	Increase	4224.1	1961.4	Increase	6138.2	4830.9	Increase
klrk1	1450495_a_at	1109.5	459.2	Increase	6708.1	2665	Increase	10344.4	8147.4	Increase
cd14	1417268_at	2277.3	1158.6	Increase	2004.7	515	Increase	2112.1	870.8	Increase
cd28	1417597_at	1154.5	742.5	No change	5199.3	3430.5	Increase	2448.6	1127.7	Increase
cd3e	1422105_at	2558.9	1382.3	Increase	5797.7	5959.3	No change	3458.3	3198.7	No change
cd8a	1444078_at	1131	634.3	Increase	12587.6	13751.8	No change	1425.6	2022	Decrease
cd8b1	1426170_a_at	3568.2	1429.2	Increase	27357.8	25575	No change	4259.4	3833.2	No change

* SI stands for "signal intensity." ** Indicates statistically significant difference between IFNg KO and WT groups.

(cells/molecules), which might participate in eliminating larvae in the early stages of infection. Contrary to expectations, our studies showed that IFN-γ-deficient mice infected with *Schistosoma japonicum* were found to have a significantly decreased egg burden in the liver compared to WT mice, while no obvious difference in worm burdens between these two groups. In the early stage of *Schistosoma japonicum* infection, the deficiency of IFN-γ concomitant with an impaired antibody response had no significant impact on the schistosomula. It is possible that the disruption of IFN-γ signaling altered some immunological or physiological internal environmental of the host, either as direct effect or as compensatory consequence, so the worm fecundity might be affected or some eggs might be destroyed.

Killer cell-mediated cytotoxicity was addressed in IFN-γ knockout mice infected with *Schistosoma japonicum*. Microarray data of splenocytes showed that some transcripts of granzymes, perforin, FasL, and TNF family members that are normally involved in cytotoxicity were significantly upregulated in the absence of IFN-γ during the acute infection. These molecules are mainly induced by two major cytotoxic lymphocyte subsets, natural killer (NK) cells and CD8$^+$ T cells. Although the effector functions of NK cells and CD8$^+$ T cells are carried out in similar way, their activation modes and action stages are different [16]. NK cells both produce IFN-γ and respond to IFN-γ. In our studies of *Schistosoma japonicum* infection, the number of NK cells from IFNg KO mice was significantly lower than that in wild-type mice, and the transcripts of some cytotoxicity-related genes in splenic NK cells from IFNg KO mice could not be increased. In contrast, purified CD8$^+$ T cells from *Schistosoma japonicum*-infected IFNg KO mice or

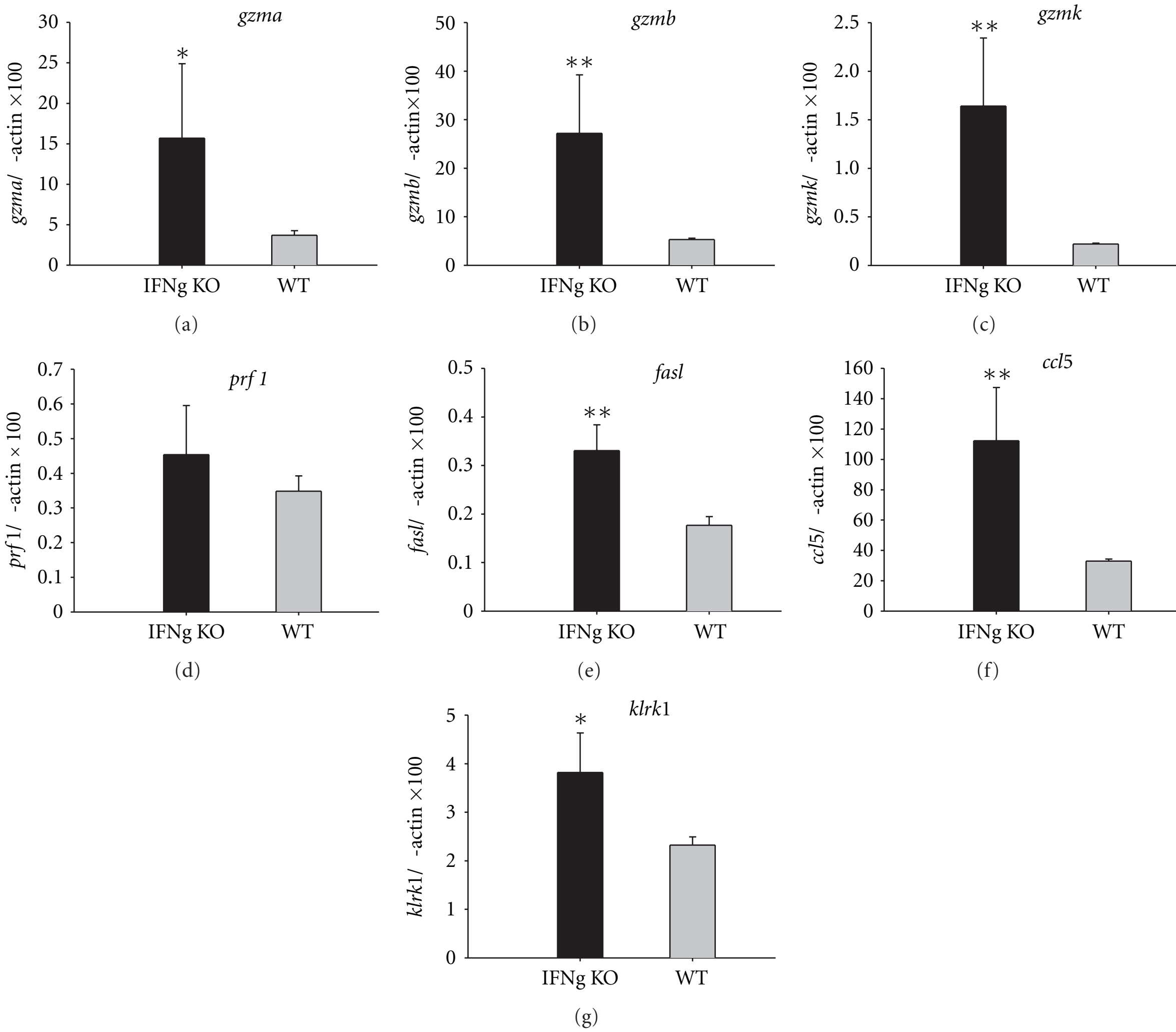


Figure 6: Relative transcription levels of *gzma*, *gzmb*, *gzmk*, *prf1*, *fasl*, *ccl5,* and *klrk1* in splenocytes of *Schistosoma japonicum*-infected IFNg KO and WT mice by real-time PCR. Data were analyzed using the Mann-Whitney test for statistical support (compared with WT mice, $^{*}P < 0.05$, $^{**}P < 0.01$).

SEA-immunized IFNg KO mice showed higher transcription of these cytotoxic molecules in IFNg KO mice compared to WT mice, which was consistent with upregulated expression of cytotoxic genes in infected spleen cells. Potentially, activation of $CD8^+$ T cells might play more important role in the cytotoxic effect during *Schistosoma japonicum* infection. Induction of $CD8^+$ T cell activation and expression of cytotoxin transcripts requires at least two independent stimuli, activation of the TCR and costimulation via a cytokine milieu [17]. Firstly, most $CD8^+$ T cells express TCR that can recognize a specific MHC I-bound antigenic peptide, which is commonly derived from an intracellular pathogen via antigen processing. However, peptides may also be derived from exogenous antigens that intersect class I presentation pathway after endocytosis by APCs. It is generally accepted that dendritic cell and its derived cytokines are the most efficient at cross-presenting exogenous antigens [18]. Cross-priming of $CD8^+$ T cells could not be excluded in *Schistosoma japonicum* infection, which needs to be clearly elucidated in the future. Secondly, the cytokine milieu, including IL-6/IL-12 and some γc-dependent cytokines, could regulate the expression of granzymes and perforin [19]. We found that deficiency of IFN-γ could influence a wide range of cytokines and other inflammatory molecules, which might activate the immune response in *Schistosoma japonicum* infection. The levels of some of the Th1 and Th2 type cytokines, especially IL-12, in spleen cell culture supernatant from IFNg KO mice were significantly higher than those from WT mice with ConA stimulation. Microarray data of splenocytes from mice infected with *Schistosoma japonicum* showed upregulated gene expression of some proinflammatory factors, chemokines, cytokines, and cell adhesion molecules in IFNg KO mice. These factors might contribute to the recruitment of activated lymphocytes and other immune cells and lead to intensive inflammatory environment. It is suggested that deficiency of IFN-γ signaling may enhance the cellular immune capacity of some certain lymphocytes in response to schistosome antigens.

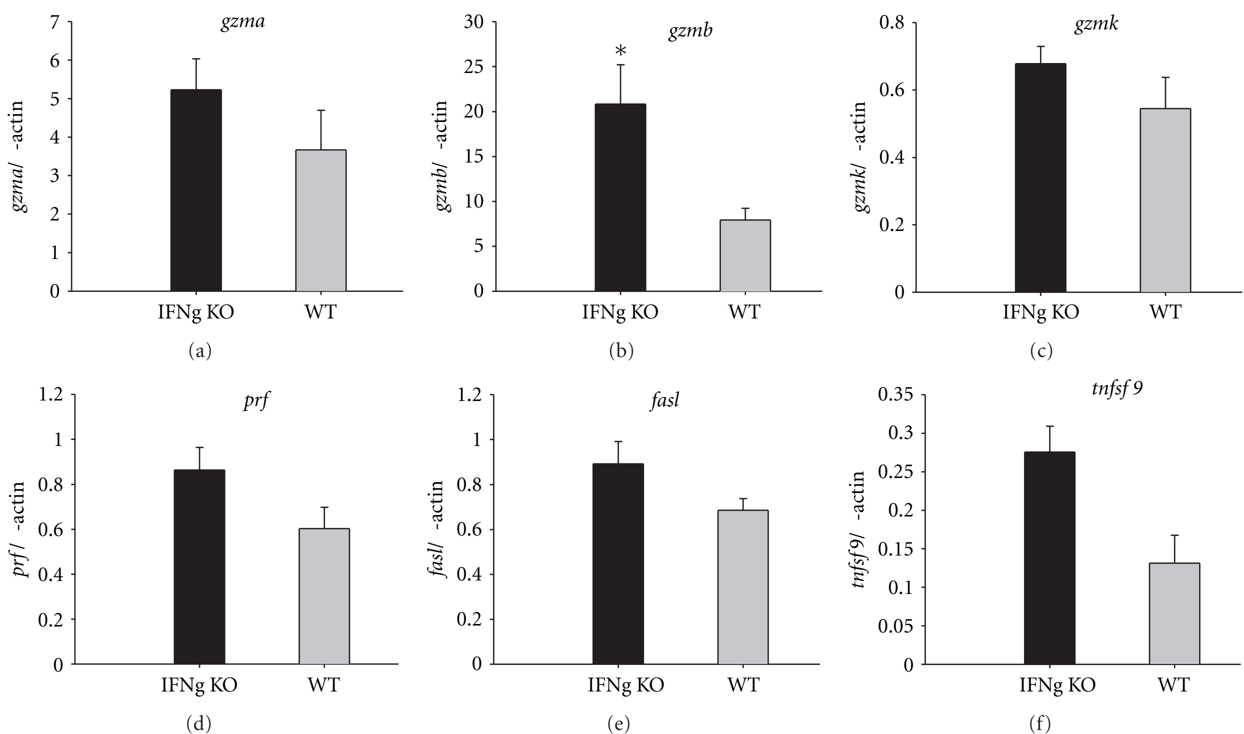


Figure 7: Expression of *gzma, gzmb, gzmk, prf1, fasl* and *tnfsf9* in purified $CD8^+$ cells from SEA-immunized mice, as measured by qRT-PCR (compared with WT mice, $^*P < 0.05$, $^{**}P < 0.01$).

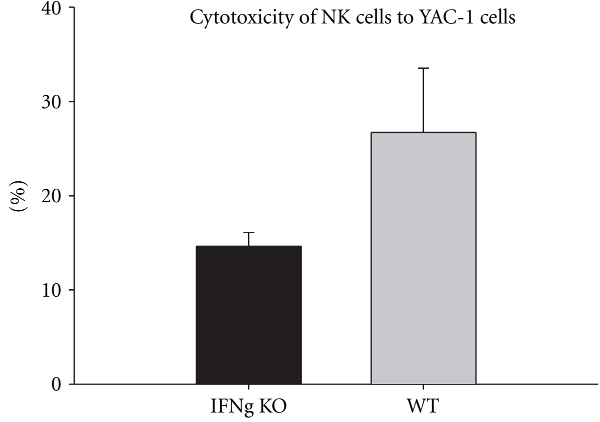


Figure 8: Cytotoxicity of NK cells exposed to YAC-1 cells from IFNg KO and WT mice immunized with SEA.

In our model of *Schistosoma japonium* infection, it was implied that IFN-γ negatively regulated the $CD8^+$T cell response. Dalton et al. reported that T-cell cytolytic activity was enhanced in IFNg KO mice with *Mycobacterium bovis* infection [20]. Another study suggested that CD8 T cells from IFN-γ gene knockout donors induce more severe lethal graft-versus-host disease (GVHD) compared to CD8 T cells from wild-type (WT) donors in fully MHC-mismatched strain combinations [21]. Thus, it is important to note that, in some specific circumstances, IFN-γ might play a negative role depending on the concentrations of IFN-γ, microenvironments, different infectious agents, or different phases of the immune response. Besides well-known protective effects, the negative modulation of IFN-γ has been gaining increasing attention. IFN-γ negatively regulates activation and migration of dendritic cells and NK cells [22–24]. Also, IFN-γ promotes the development and differentiation of regulatory T cells and apoptosis of activated $CD4^+/CD8^+$ cells [25]. These studies using IFNg KO mouse model help to define the *in vivo* role of this immunoregulatory cytokine.

Finally, some literature supports the hypothesis that $CD8^+$ T cells may participate in the protective immunity against schistosomes. In a study of a *Schistosoma mansoni* vaccine candidate molecule, Sm28GST, it was reported that immunization with Sm28GST could induce antigen-specific CTL effects, leading to a decreased parasite burden and alleviated liver pathology. Transfer of Sm28GST-specific $CD8^+$ cells also conferred protection, and this protective effect of Sm28GST was significantly decreased after treatment with anti-$CD8^+$ cell antibody [26, 27]. Nevertheless, we do recognize there is no direct evidence regarding how extracellular parasites activated $CD8^+$ T cells or whether some cytotoxic granules could destroy schistosome eggs. In our present work, the fact that there was no difference in worm numbers suggests that these $CD8^+$ T cells might not have any deleterious effect on primary infections in IFNg KO mice despite the up-regulation of a range of enzymes involved in expression of cytotoxity. Further studies of the mechanism through which IFN-γ interacts with $CD8^+$ T cells may contribute to a better understanding of the immunity during *Schistosoma japonicum* infection.

5. Conclusion

This study shows that IFN-γ knockout has no obvious effect on worm burden and results in reduced egg burden in *S. japonicum* infection of mice. In IFN-γ knockout mice, many cytotoxicity-associated genes are upregulated during the infection. These results indicate that IFN-γ is not always a positive regulator of immune responses and it might play multiple roles in *S. japonicum* infection.

Authors' Contributions

X. Du, D. Zhang, M. Ji, and G. Wu participated in the design of the study, drafted the paper. X. Du, J. Wu, M. Zhang, Y. Gao and M. Hou performed majority of the experiments. All authors read and approved the final paper.

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