

# Disruption of antigenic variation is crucial for effective parasite vaccine

Fernando D Rivero<sup>1</sup>, Alicia Saura<sup>1</sup>, Cesar G Prucca<sup>1</sup>, Pedro G Carranza<sup>1</sup>, Alessandro Torri<sup>1</sup> & Hugo D Lujan<sup>1</sup>

*Giardia lamblia* is a human intestinal pathogen. Like many protozoan microorganisms, *Giardia* undergoes antigenic variation, a mechanism assumed to allow parasites to evade the host's immune response, producing chronic and/or recurrent infections. Recently, we found that the mechanism controlling variant-specific surface protein (VSP) switching in *Giardia* involves components of the RNA interference machinery and that disruption of this pathway generates trophozoites simultaneously expressing many VSPs. Here we use these altered trophozoites to determine the role of antigenic variation in a gerbil model of giardiasis. Our results show that either primary infection with trophozoites simultaneously expressing many VSPs or immunization with purified VSPs from the transgenic cells protects gerbils from subsequent *Giardia* infections. These results constitute, to our knowledge, the first experimental evidence that antigenic variation is essential for parasite survival within hosts and that artificial disruption of this mechanism might be useful in generating vaccines against major pathogens that show similar behavior.

A particular characteristic of parasitic organisms is their capacity to adapt to changes in the environment<sup>1,2</sup>. However, during an infection, the survival of pathogenic organisms depends not only on their aptitude to colonize a host but also on their ability to counteract the host's defense mechanisms<sup>3</sup>. Surface antigenic variation is considered a major process that allows parasite evasion of the immune system, leading to chronic infection despite continuous immune pressure<sup>4</sup>. Antigenic variation has been demonstrated in many microorganisms, including *Giardia*, that use various mechanisms to switch the expression of their variable surface antigens<sup>4</sup>.

*G. lamblia* is one of the most common causes of intestinal disease worldwide. In developed countries, it is a frequent cause of diarrhea in children in day care centers, institutionalized individuals, backpackers, travelers and homosexuals. In developing countries, about 200 million people have symptomatic giardiasis, with some 500,000 new cases reported annually<sup>5,6</sup>. Phylogenetic studies identified seven *G. lamblia* assemblages (A to G)<sup>7</sup>. Assemblages A (represented by the WB isolate) and B (represented by the GS/M isolate) have been shown to infect humans<sup>8</sup>.

*Giardia* has a simple life cycle consisting of infective cysts and vegetative trophozoites. Infection is transmitted by ingestion of cysts, which are passed in the feces<sup>9</sup>. Trophozoites are responsible for the clinical manifestations associated with the disease, which vary from asymptomatic infections to acute or chronic diarrhea<sup>10</sup>. Symptoms in humans typically occur 2 weeks after infection and last 2–5 d<sup>11</sup>. Giardiasis in humans and in gerbil models is self-limiting if the immune system of the host is fully developed, but chronic infections can occur in the absence of any apparent immunodeficiency<sup>11</sup>.

The biological role of antigenic variation is speculative, although the most accepted theory is evasion of the host immune defenses<sup>11</sup>. However, there are no experimental data directly supporting this

hypothesis, because disruption of antigenic variation has not been accomplished in any parasitic organism until recently.

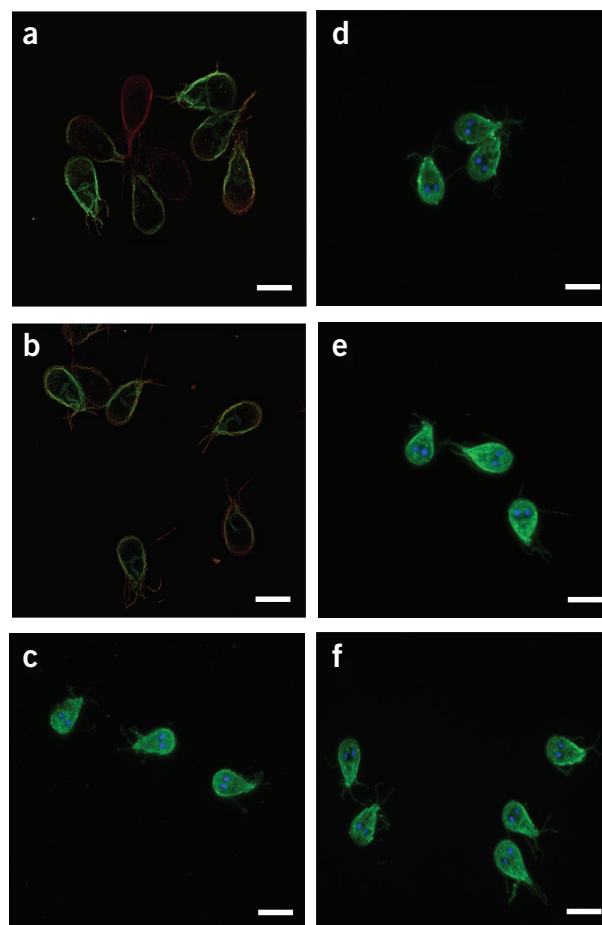
Antigenic variation in *G. lamblia* involves VSPs, which are cysteine-rich integral membrane proteins that possess a variable extracellular amino-terminal region, a conserved carboxy-terminal domain that includes a unique transmembrane region and a short, five-amino acid cytoplasmic tail<sup>12</sup>. VSPs form a thick coat that represents the host-parasite interface<sup>13</sup>. Only one VSP, from a repertoire of more than 190 homologous genes<sup>14</sup>, is expressed on the surface of a given trophozoite at any time<sup>11</sup>, but switching expression to an antigenically distinct VSP occurs even in culture<sup>15</sup>. Recently, we proposed that the control of surface antigen expression in *G. lamblia* involves a mechanism similar to RNAi<sup>6</sup>. Many, if not all, VSP genes (*vsps*) are simultaneously transcribed, but a system comprising an RNA-dependent RNA polymerase (RdRP) generates antisense RNA targeting all but one *vsp* transcript<sup>16</sup>. The duplexes formed between the sense and antisense *vsp* RNAs are subsequently cleaved to 22- to 25-nucleotide interfering RNAs by a Dicer/Argonaute system. We showed the direct involvement of RdRP and Dicer by experiments in which these enzymes were knocked down, leading to a change from single to multiple VSP expression in individual *G. lamblia* trophozoites<sup>16</sup>.

Infections with *G. lamblia* are characterized by a strong immune response, particularly to VSPs<sup>11</sup>. Because a humoral immune response in *G. lamblia*-infected hosts coincides with the elimination of the original VSP, a functional role of antibodies in the selection of phenotypic variants during the course of infection has been proposed<sup>15,17</sup>. Moreover, monoclonal antibodies (mAbs) specific for the different VSPs have shown cytotoxicity against the antigen-expressing trophozoites<sup>18,19</sup>. Because *Giardia*'s mechanism of protection may depend on switching expression among immunologically distinct VSPs, the host should be able to prevent infection by simultaneously developing

<sup>1</sup>Laboratory of Biochemistry and Molecular Biology, School of Medicine, Catholic University of Cordoba, Cordoba, Argentina. Correspondence should be addressed to H.D.L. (hlujan@ucc.edu.ar).

Received 30 October 2009; accepted 18 March 2010; published online 25 April 2010; doi:10.1038/nm.2141

**Figure 1** Expression of VSPs in wild-type *G. lamblia* clones and in *G. lamblia* with deregulated antigenic variation. (a,b) Representative direct immunofluorescence confocal images of trophozoites in which Dicer (a) or RdRP (b) have been knocked down. Unfixed cells expressing VSP9B10 were labeled with tetramethylrhodamine isothiocyanate (TRITC)-conjugated mAb 9B10 (red), and trophozoites expressing VSP1267 were labeled with FITC-conjugated mAb 5C1 (green). Many trophozoites expressing VSP9B10 on the surface also express VSP1267 (yellow). (c) Immunofluorescence image of a fixed uncloned population of the WB isolate that was stained with the CRGKA-specific mAb 12F1 (green) and counterstained with DAPI (blue). (d–f) Representative images of indirect immunofluorescence assays with mAbs specific for VSPs (green) counterstained with DAPI (blue), showing clonal populations of *Giardia lamblia* WB9B10 (d), WB1267 (e) and GS/M-H7 (f). These cells are only labeled by the specific mAb. Scale bars, 20  $\mu$ m.



specific immune responses to all variable surface molecules. Our idea is that deregulating antigenic variation could be useful in generating effective vaccines.

To analyze this hypothesis, we performed experiments in the gerbil model of giardiasis, which better resembles human infections<sup>20,21</sup> than do most mouse models and in which parasites are known to undergo antigenic variation<sup>22,23</sup>.

## RESULTS

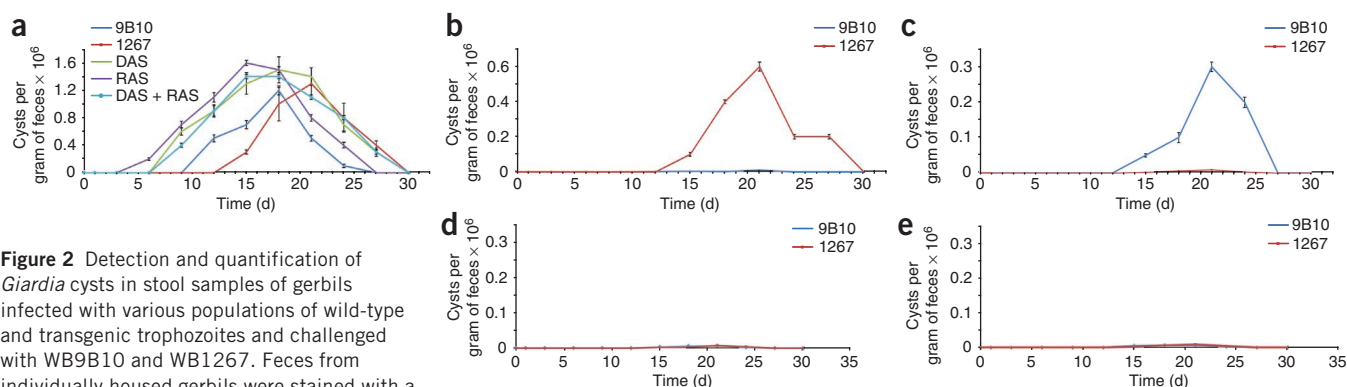
### Detection of VSPs in wild-type and transgenic *G. lamblia*

To study the role of VSPs during experimental infections, we generated *G. lamblia* trophozoites simultaneously expressing many VSPs by knocking down the expression of Dicer or of RdRP (DAS or RAS trophozoites, respectively). We used the *G. lamblia* WB isolate because well-characterized mAbs against many VSPs of this isolate are available<sup>16</sup>. Additionally, we obtained trophozoite populations expressing only one VSP on their surface by limiting dilution of cells screened with mAbs to specific VSPs. *G. lamblia* WB trophozoites in which Dicer (Fig. 1a) or RdRP (Fig. 1b) had been knocked down expressed many VSPs, as indicated by confocal direct immunofluorescence assays showing co-localization of more than one VSP on the surface of individual cells. Addition of percentages of cells expressing various VSPs, as judged by reactivity to a panel of mAbs directed to diverse VSPs, further supported that these cells simultaneously express many VSPs (Supplementary Table 1). All trophozoites from uncloned cultures can be labeled with mAbs specific for the cytoplasmic tail common to all VSPs (Fig. 1c). In culture, trophozoites spontaneously switch

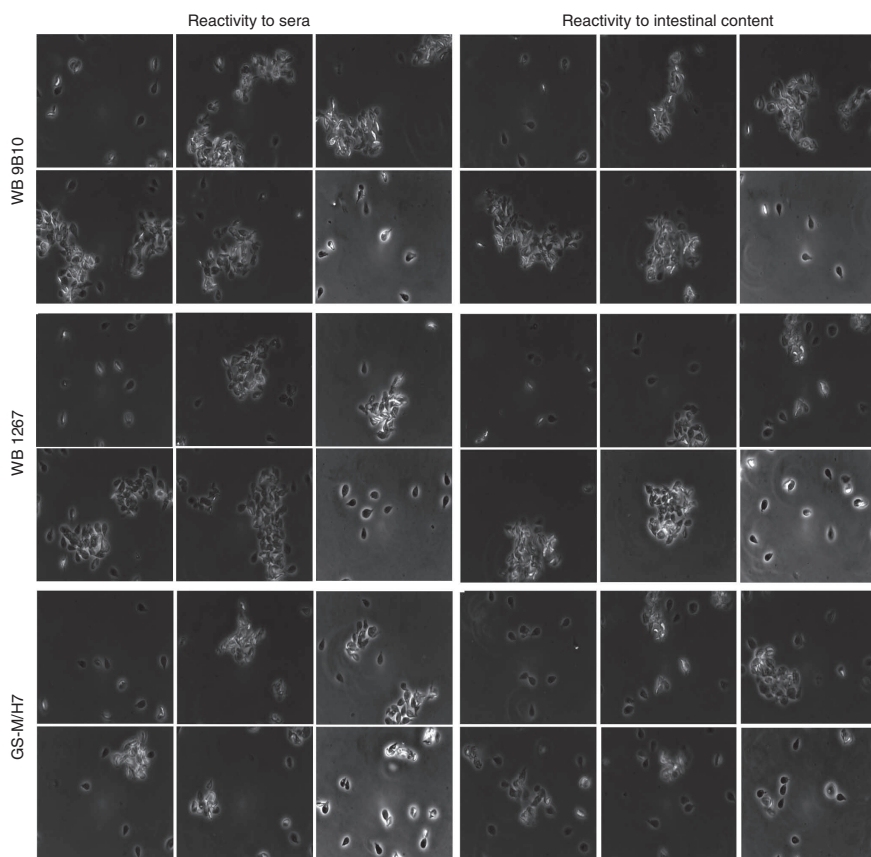
their VSP, thus we maintained populations of cells expressing a specific VSP for only 12 h in culture after selection to assure the homogeneity of the populations. We determined unique VSP expression in trophozoites by indirect immunofluorescence assays with a panel of VSP-specific mAbs (Fig. 1d–f and Supplementary Table 1).

### Protection from *G. lamblia* infection in the gerbil model

We initially used these *G. lamblia* populations to infect specific pathogen-free, *Giardia*-naive gerbils. We initiated infection by orogastric



**Figure 2** Detection and quantification of *Giardia* cysts in stool samples of gerbils infected with various populations of wild-type and transgenic trophozoites and challenged with WB9B10 and WB1267. Feces from individually housed gerbils were stained with a FITC-labeled mAb 7D2 specific for CWP2 and counted daily over 1 month. (a–e) Graphs depicting the number of cysts released per gram of feces under different conditions. (a) Gerbils initially infected with clonal populations of trophozoites WB9B10, WB1267 or transgenic trophozoites in which Dicer (DAS) or RdRP (RAS) were knocked down, as well as with a 1:1 mixture of both. (b–e) Gerbils previously infected with *G. lamblia* WB9B10 (b), WB1267 (c), DAS (d) and RAS (e) were challenged 2 months after primary infection with clonal populations of *Giardia* WB9B10 and WB1267. Figures represent the mean value  $\pm$  s.d. of five independent experiments.



**Figure 3** Serum and intestinal contents of gerbils infected with transgenic trophozoites expressing the full repertoire of VSPs are able to agglutinate different *Giardia* clones *in vitro*. Representative images of phase-contrast microscopy of clones WB9B10, WB1267 and GS/M H7 challenged with serum (left) or intestinal content (right). Each group of six images shows trophozoites incubated with serum or intestinal content from uninfected gerbils (top left), serum or intestinal content of gerbils infected with the corresponding clone (top middle), VSP-specific mAb to the corresponding clone (top right), serum or intestinal content of gerbils infected with DAS trophozoites (bottom left), serum or intestinal content of gerbils infected with RAS trophozoites (bottom middle) and serum or intestinal content of gerbils infected with a heterologous clone (bottom right). Sera and intestinal contents from infected or immunized gerbils agglutinate the trophozoites similar to the specific mAbs to VSPs, as compared to the controls. Scale bar, 25  $\mu$ m.

and found similar results (**Supplementary Tables 2 and 3**). Immunofluorescence assays performed on stool from selected gerbils with a mAb directed to *Giardia* CWP2 clearly showed release of cysts in infected gerbils and an absence of cysts in the protected gerbils (**Supplementary Fig. 1**). We also inoculated gerbils previously infected with *G. lamblia*

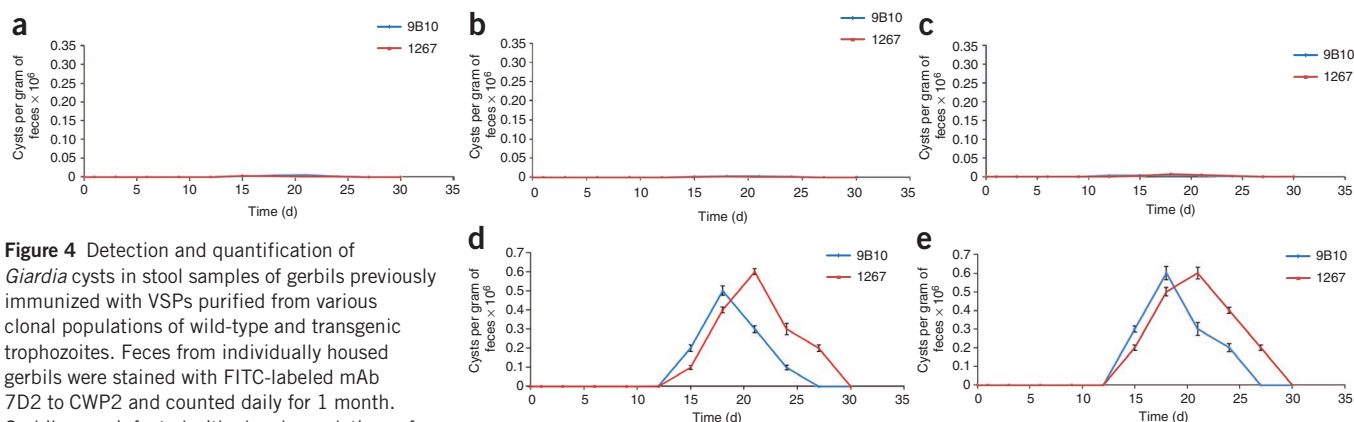
strains DAS, RAS or DAS + RAS with cysts obtained from the original infections (to which the VSPs of the released trophozoites were unknown). Similarly to those gerbils inoculated with particular trophozoites populations, these gerbils were refractory to infection (**Supplementary Table 4**). Additionally, the signs of disease observed during the primary infection were not apparent during the challenge infection. Taken together, these results strongly suggest that an immune response to multiple VSPs is necessary to prevent the establishment of new infections.

We collected serum and intestinal content from the infected and control gerbils and incubated them *in vitro* with trophozoites expressing one or several VSPs. Serum or intestinal content of uninfected gerbils had no effect on parasite morphology, viability or motility (**Fig. 3**). Conversely, when we incubated clonal trophozoite populations expressing a unique VSP with mAbs directed against these antigens or with the serum or intestinal content of gerbils infected with that same clone, detachment and agglutination of nearly all cells took place (**Fig. 3**), indicating that antibodies against that surface antigen were present. Incubation with serum or intestinal content of gerbils infected with another clone did not show any significant effect (**Fig. 3**). When we exposed any clonal population to the serum or intestinal content of gerbils infected with DAS, RAS or DAS + RAS *G. lamblia*, strong agglutination of the trophozoites occurred (**Fig. 3**). These results indicate that the DAS or RAS *G. lamblia*-infected gerbils were able to develop a strong immune response to VSPs on trophozoites and that other antigens that may be on the surface seem irrelevant to conferring protection.

We also tested serum and intestinal content from gerbils immunized with VSPs of isolate WB against trophozoites of isolate GS/M<sup>25</sup>. Serum and intestinal content of gerbils infected with DAS and RAS trophozoites

inoculation of trophozoites and evaluated the release of cysts, which clearly indicates gerbil infection, by identification of *Giardia* in stool samples by immunofluorescence assays with a mAb specific for *Giardia* cyst wall protein-2 (CWP2)<sup>24</sup>. We quantified the number of cysts per gram of feces to determine the infectivity and virulence of each population. All populations were able to establish infections (**Fig. 2a**). The appearance of cysts in stool and the number of cysts varied slightly among the various populations. During the second week of infection, infected gerbils showed episodes of diarrhea and weight loss (~5–6% compared to uninfected gerbils). However, all infected gerbils were able to self-cure by day 30 after infection (**Fig. 2a**). To avoid the possibility that some gerbils might be chronically infected with a small number of trophozoites, we treated half of the gerbils with metronidazole to cure any undetectable infection.

To determine whether the primary infection with trophozoites expressing either a particular VSP (VSP9B10, VSP1267 or VSPA6) or the entire repertoire of VSPs (DAS, RAS or DAS + RAS) conferred protection from subsequent infections, we challenged the gerbils with clonal trophozoite populations expressing a specific VSP at 2 months after cure of the primary infection. The results of cyst elimination in gerbils previously infected with clones WB9B10 or WB1267 (**Fig. 2b,c**), or from gerbils previously infected with DAS or RAS trophozoites (**Fig. 2d,e**), clearly indicate that most gerbils infected with trophozoites expressing a single VSP were refractory to a secondary infection with cells expressing the same VSP but were easily re-infected with trophozoites expressing another VSP (**Fig. 2b,c**). In contrast, most gerbils that were originally infected with trophozoites simultaneously expressing many VSPs were protected from subsequent infection with clonal populations (**Fig. 2d,e**). We performed the same challenge experiments at 2, 4 and 12 months from the original infection



**Figure 4** Detection and quantification of *Giardia* cysts in stool samples of gerbils previously immunized with VSPs purified from various clonal populations of wild-type and transgenic trophozoites. Feces from individually housed gerbils were stained with FITC-labeled mAb 7D2 to CWP2 and counted daily for 1 month. Gerbils were infected with clonal populations of trophozoites of WB9B10 or WB1267. Immunizations were carried out with VSPs purified from DAS and RAS transgenic trophozoites (and a mixture of both). Graphs depict the number of cysts released per gram of feces under various conditions. (a–c) Gerbils previously immunized with VSPs purified from DAS (a), RAS (b) or the mixture of both (c) were challenged with clones WB9B10 or WB1267. (d,e) Control gerbils (d) or those immunized with the intracellular protein BiP (e) were challenged with clones WB9B10 or WB1267. Figures represent the mean value  $\pm$  s.d. of five independent experiments.

showed partial agglutination of the GS/M cells (30–40%), indicating that VSPs from both isolates share common epitopes (Fig. 3).

### Immunization of gerbils with purified VSP

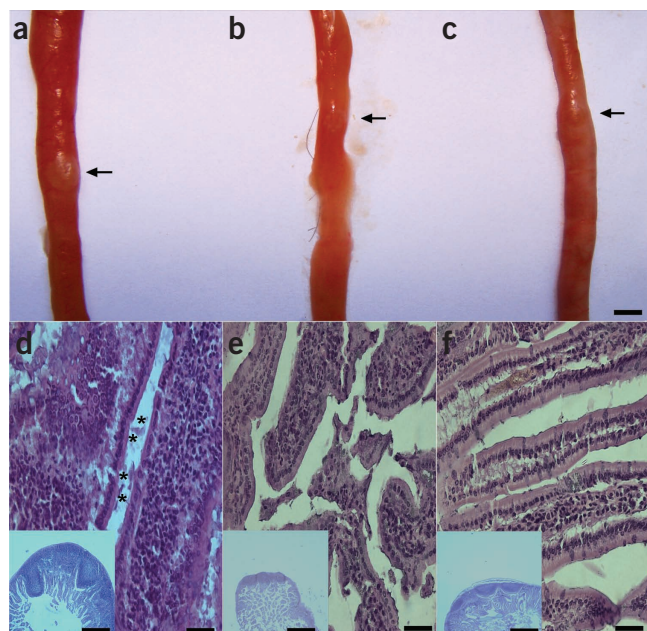
Because vaccination with trophozoites is not practical, we first tested whether purified VSPs can resist their passage through the harsh conditions of the upper gastrointestinal tract by incubating trophozoites with high trypsin concentrations (Supplementary Fig. 2) or at various pHs (Supplementary Fig. 3). In both cases, the stability of the VSPs was evident, as judged by their reactivity to specific mAbs. Therefore, we purified the entire repertoire of VSPs from altered trophozoites with a mAb that reacts with the five-amino acid cytoplasmic tail present in all VSPs<sup>26</sup> and used the mixture as immunogen (Supplementary Fig. 4). We used an intracellular antigen of *Giardia*, GRP78/BiP, a molecular chaperone of the endoplasmic reticulum<sup>27</sup>, as a control. We then immunized gerbils with these preparations, without adjuvant, by orogastric administration of three doses, 3 d apart each. In all cases, vaccination did not trigger any signs of the

disease, indicating that VSPs alone are not toxic (data not shown). After 2 months, we inoculated the gerbils with parasites expressing particular VSPs. Similarly to the results observed during primary infections, oral immunization with the entire repertoire of VSPs generated a strong immune response that prevented infection of these gerbils (Fig. 4). In contrast, control gerbils inoculated with vehicle only or with GPR78/BiP were readily infected by clonal populations (Fig. 4). Individual purified VSPs used as immunogen conferred protection only against trophozoites expressing the same VSP, as shown in the infection experiments (data not shown). The protection generated by VSP preparations lasted up to 1 year (Supplementary Tables 5–7), and the serum and intestinal content of immunized gerbils showed similar results to those obtained by infecting gerbils (data not shown).

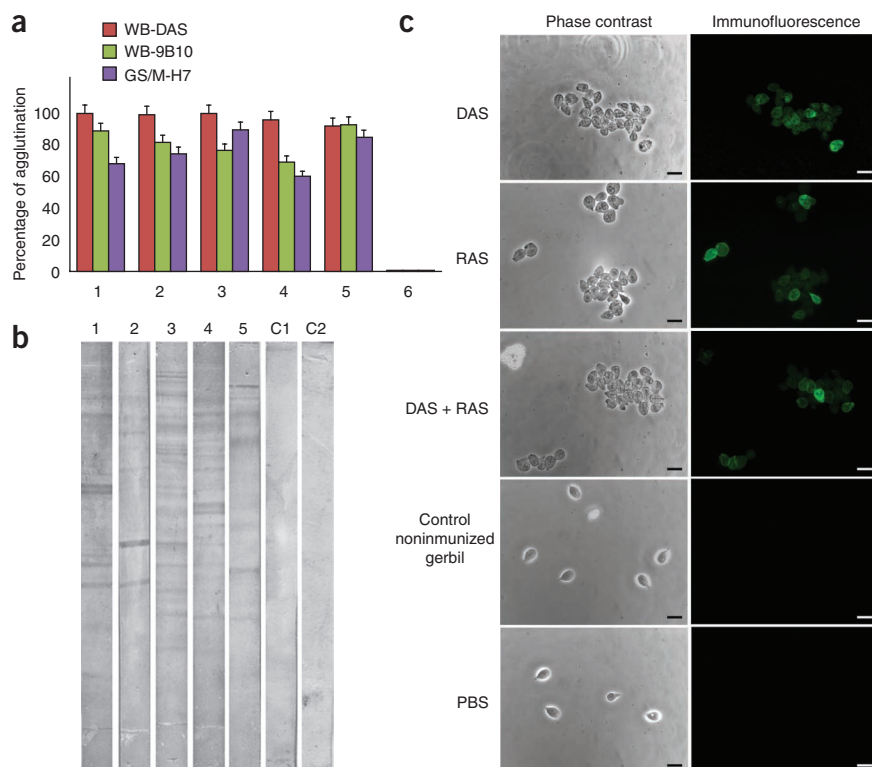
Notably, although infection by *Giardia* has been reported to cause little or no inflammation<sup>5,10,11</sup>, the intestine of infected gerbils showed an increase in the size of Peyer's patches and more neutrophil, mast cell and lymphocyte infiltrations of the gut epithelium compared with uninfected gerbils (Fig. 5). The macroscopic and microscopic alterations of the upper small intestine observed in the infected gerbils were not evident in the immunized gerbils (Fig. 5).

### Protection against heterologous isolates

In addition to testing the serum and intestinal content of gerbils immunized with WB-derived VSPs against trophozoites of the GS/M isolate (Fig. 3), we challenged some of those gerbils with GS/M trophozoites 12 months after immunization. Two out of ten gerbils



**Figure 5** Intestinal morphology of the gerbil's upper small intestine during infection and challenge. (a–c) Macroscopic examination of upper small intestines from experimental gerbils. (a) Intestine of a gerbil during primary infection with trophozoites expressing the entire repertoire of the VSPs (DAS) 15 d after inoculation. Arrows point to an increase in size of the Peyer's patches compared to an uninfected gerbil used as control (b). (c) Intestine of a gerbil immunized with purified VSPs obtained from DAS trophozoites. Scale bar, 0.5 cm. (d–f) Microscopic examination of upper small intestines from experimental gerbils. (d) Infected gerbil showing a clear enlargement of Peyer's patches and moderate inflammatory infiltrate in mucosa and submucosa. Asterisks depict some *Giardia* trophozoites at the intestinal lumen. (e) Control uninfected and unvaccinated gerbil. (f) Vaccinated gerbil showing histologically normal intestinal mucosa. Scale bars, 100  $\mu$ m. Insets show a general morphology of the upper small intestine (scale bars, 200  $\mu$ m).



**Figure 6** VSP-specific antibodies react to heterologous isolates. (a) Sera from infected humans agglutinate cells and recognize the surface of the trophozoites. Sera from five infected humans (1 to 5) and a newborn *Giardia*-naive individual (6) were directly labeled with FITC and used to test their reactivity to VSPs on transgenic and wild-type trophozoites by indirect immunofluorescence. Results are expressed as percentage of agglutination. Error bars represent s.d. (b) Sera of infected humans detect many *Giardia* DAS proteins. Sera from five infected humans (1 to 5), a newborn *Giardia*-naive individual (C1) and secondary antibody only (C2), were tested for their reactivity to VSPs on DAS trophozoites by western blotting. A differential banding pattern is observed for each infected individual. (c) Sera from gerbils immunized with purified VSP agglutinate the cells of a new human *G. lamblia* isolate and recognize the surface of the trophozoites. Sera from randomly selected DAS-, RAS- and DAS + RAS-immunized gerbils and a *Giardia*-naive gerbil used as control were directly labeled with FITC and used to test their reactivity by indirect immunofluorescence to VSPs on a new *G. lamblia* isolated axenized from cysts collected from an acutely infected human. The sera from immunized gerbils agglutinate the trophozoites and label the surface of the parasites in contrast to an unimmunized gerbil control. Scale bars, 20  $\mu$ m.

became infected. The number of cysts released in the feces of these infected gerbils was lower, and we did not observe any signs of the disease when compared to infections with trophozoites of the GS/M isolate expressing VSP H7 (GS/M-H7) in nonimmunized gerbils in nonimmunized gerbils (data not shown).

To determine whether immunized gerbils were protected against challenge infection with novel isolates, we orally administered viable cysts obtained from humans with acute giardiasis to the gerbils. Five different isolates were used. In all cases, no VSP-immunized gerbil became infected ( $n = 5$  per group). We obtained similar results when we used trophozoites obtained by excystation of cysts isolated from humans (axenized trophozoites). The human sera agglutinated and labeled the surface of transgenic trophozoites (Fig. 6a and Supplementary Fig. 5). Western blot experiments of DAS trophozoite lysates incubated with serum from these subjects (in contrast to serum from a newborn individual used as control) showed many bands, indicating that these subjects had been exposed to multiple VSPs (Fig. 6b). Moreover, serum from VSP-immunized gerbils agglutinated and labeled the surface of trophozoites axenized from these human isolates (Fig. 6c). Experiments using these gerbils' intestinal content showed identical results (data not shown), indicating the presence of VSP-specific antibodies (most likely secretory IgA).

## DISCUSSION

*G. lamblia* is one of the most prevalent parasites in the world, common to humans and domestic and farm gerbils<sup>7</sup>. At present, controlling the disease is limited to chemotherapy<sup>28</sup>, but only a few drugs are available, and problems such as side effects, drug resistance<sup>29</sup> and a high rate of post-curative re-infection make this impractical in endemic areas. Therefore, vaccines against *G. lamblia* are considered a promising approach for controlling this parasite<sup>30</sup>.

The host-parasite interactions that direct the outcome of *Giardia* infection remain poorly understood<sup>10</sup>. Hosts are able to develop a strong

immunological response to both intracellular<sup>30,31</sup> and surface<sup>11,30</sup> antigens, and antigenic variation has been suggested as the cause of prolonged infections<sup>11,15</sup>. Indeed, what the host detects very early during infection is the surface coat formed by VSPs<sup>13</sup>. Additionally, VSPs have been implicated in enhanced resistance of the parasite to intestinal proteases<sup>32</sup>, as well as in host selection due to variable intestinal flora<sup>33</sup>.

To determine the precise role of antigenic variants during the course of infection, we generated trophozoite populations simultaneously expressing many VSPs. In a series of experiments designed to overcome the deficiencies of previous models (gerbils free of previous *Giardia* or related parasites infections, identical flora in all gerbils, gerbils that reproduce the symptoms of human infections), we used these transgenic parasites for experimental infections in gerbils. To our knowledge, this represents the first time that organisms in which the mechanism of antigenic variation has been disrupted have been used to determine not only the course of infection but also the host's response to the parasite.

This study shows that gerbils can successfully be protected against challenge infections with *G. lamblia*. Our results also show that antigenic variation is essential for parasites to evade the host immune response, because either trophozoites simultaneously expressing many VSPs or VSPs purified from the transgenic cells were able to confer strong protection. Additionally, induction of a considerable response against *G. lamblia* with the entire repertoire of VSPs was associated with a concomitant reduction of the signs of infection, suggesting that vaccination causes protection from infection rather than parasite clearance after infection. In this regard, macroscopic and microscopic examination of the upper small intestine of infected gerbils showed a high degree of inflammation when compared to vaccinated and control gerbils.

Although we did our work in a gerbil model, the results are consistent with earlier studies in humans<sup>25,34</sup> that showed that some individuals may be chronically infected and others re-infected with or without symptoms, although serum and intestinal *Giardia*-specific

antibodies (particularly secretory IgA) were detected<sup>25,34</sup>. These results showed that the characteristics of infections in adult humans are highly variable. Thus, we needed to use laboratory animals in which results are consistent and reproducible to analyze the course of *G. lamblia* infections<sup>35–41</sup>.

Early studies were performed in the *G. muris* mouse model<sup>30,35,41</sup>. But *G. muris* cannot infect humans, and, until recently, it was not known whether it also undergoes antigenic variation<sup>42</sup>. For these reasons, although the immune response in this model could be determined, the influence of antigenic variation during the course of the mouse infection was not considered<sup>35</sup>. Subsequently, an adult mouse model of *G. lamblia* infection was established<sup>39</sup>. Many mouse strains and several *G. lamblia* isolates as well as clones derived from them were used to determine that only the clone expressing VSPH7 from the GS/M isolate was able to infect adult mice<sup>39</sup>. It is unclear why only this clone could sustain an infection. What seems evident from those studies and from the recently reported genome of GS/M is that this strain notably varies from isolate WB<sup>43</sup>. However, over the course of many years, GS/M-H7 was widely used to study the immunology of giardiasis. In all of these studies, the role of the VSPs and the cellular and humoral response was clearly demonstrated<sup>30,31,41</sup>, indicating that B cell-dependent mechanisms are most likely responsible for surface antigen switching<sup>11,37,38</sup>.

We here showed that the serum or intestinal content of infected or immunized gerbils was able to agglutinate trophozoites similarly to mAbs against VSPs, or sera from mice or humans<sup>44–48</sup>. These results support the hypothesis that antibodies are generated to *Giardia*'s VSPs and may be responsible for the clearance of the infection.

Studies performed with *G. muris* trophozoites showed that sera from susceptible mice were able to immobilize and lyse trophozoites *in vitro*<sup>46</sup>. Immobilization was suggested as a key part of a defense system against *Giardia*, as in the lumen of the small intestine it would result in the inability of the parasite to attach to the epithelium. Lysed trophozoites would then release intracellular molecules, but an immune response against intracellular antigens seems ineffective in clearing the infection, because antibodies cannot penetrate the surface barrier represented by the VSPs<sup>13</sup>. Thus, VSPs should be considered the principal target of a protective immune response against *Giardia*. In our study, antibodies generated to VSPs of the WB isolate could also agglutinate some trophozoites of GS/M, indicating that VSPs from both assemblages infecting humans share common epitopes, as was reported to occur between VSPs from *G. lamblia* and *G. muris*<sup>42</sup>.

For veterinary use, a commercial vaccine is available<sup>49</sup>. The vaccine consists of killed trophozoites and has been reported to reduce shedding of cysts and to prevent clinical signs of the disease<sup>49,50</sup>. However, studies of the efficacy of this vaccine have been highly criticized<sup>51,52</sup>, suggesting that a vaccine preparation consisting of inactivated parasites of unknown origin is not effective enough to protect domestic animals. To be effective, a vaccine may need to target *Giardia* of diverse assemblages. VSPs administered orally are highly immunogenic, do not induce mucosal tolerance and are resistant to proteolytic digestion and low pH. Further studies using VSPs that provide insight into the mechanisms involved in resisting antigen destruction in the gut and avoiding mucosal tolerance may be relevant to efforts to develop oral vaccines against other pathogens<sup>53</sup>.

Vaccination with the entire repertoire of VSPs demonstrates the role of antigenic variants in evading the host's immune system and confirms the key role of antigenic variation as an adaptive mechanism developed by parasites to cause chronic and recurrent infections. Because many pathogens undergo antigenic variation, avirulent

strains of parasites in which the mechanism of antigenic variation can be deregulated to allow the expression of multiple variants, as recently demonstrated for *Plasmodium falciparum*<sup>54</sup>, might constitute a helpful tool for the development of vaccines. The demonstration of this proof of principle in *Giardia* might induce a rethinking of the current strategies regarding immunoprophylaxis for parasites that have antigenic variation.

## METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemedicine/>.

*Note: Supplementary information is available on the Nature Medicine website.*

## ACKNOWLEDGMENTS

We thank T.E. Nash (National Institute of Allergy and Infectious Diseases, US National Institutes of Health) for providing VSP-specific mAbs and *Giardia* strains and M.E. Rivarola and the Pathology Service of the School of Medicine, Catholic University of Cordoba, for technical assistance. This work was supported by grants from the Agencia Nacional para la Promoción de la Ciencia y la Tecnología, Consejo Nacional de Investigaciones Científicas y Técnicas, Catholic University of Cordoba, the Howard Hughes Medical Institute and the EU CONTENT Project. H.D.L. is a Howard Hughes Medical Institute International Research Scholar and a Member of the Scientific Investigator's Career of the Consejo Nacional de Investigaciones Científicas y Técnicas.

## AUTHOR CONTRIBUTIONS

F.D.R. performed most of the gerbil and *in vitro* experiments; C.G.P. generated transgenic trophozoites and performed confocal and epifluorescence assays; A.S. and P.G.C. collaborated in gerbil experiments and developed monoclonal antibodies. A.T. collected and evaluated human samples. All authors analyzed the data. H.D.L. supervised the project and wrote the manuscript.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at <http://www.nature.com/naturemedicine/>.

Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions/>.

- Barrett, L.G., Thrall, P.H., Burdon, J.J. & Linde, C.C. Life history determines genetic structure and evolutionary potential of host-parasite interactions. *Trends Ecol. Evol.* **23**, 678–685 (2008).
- Vonlaufen, N., Kanzok, S.M., Wek, R.C. & Sullivan, W.J. Jr. Stress response pathways in protozoan parasites. *Cell. Microbiol.* **10**, 2387–2399 (2008).
- Zambrano-Villa, S., Rosales-Borjas, D., Carrero, J.C. & Ortiz-Ortiz, L. How protozoan parasites evade the immune response. *Trends Parasitol.* **18**, 272–278 (2002).
- Deitsch, K.W., Lukehart, S.A. & Stringer, J.R. Common strategies for antigenic variation by bacterial, fungal and protozoan pathogens. *Nat. Rev. Microbiol.* **7**, 493–503 (2009).
- Adam, R.D. Biology of *Giardia lamblia*. *Clin. Microbiol. Rev.* **14**, 447–475 (2001).
- Savioli, L., Smith, H. & Thompson, A. *Giardia* and *Cryptosporidium* join the 'Neglected Diseases Initiative'. *Trends Parasitol.* **22**, 203–208 (2006).
- Monis, P.T., Caccio, S.M. & Thompson, R.C. Variation in *Giardia*: towards a taxonomic revision of the genus. *Trends Parasitol.* **25**, 93–100 (2009).
- Thompson, R.C., Hopkins, R.M. & Homan, W.L. Nomenclature and genetic groupings of *Giardia* infecting mammals. *Parasitol. Today* **16**, 210–213 (2000).
- Luján, H.D., Mowatt, M.R. & Nash, T.E. Mechanisms of *Giardia lamblia* differentiation into cysts. *Microbiol. Mol. Biol. Rev.* **61**, 294–304 (1997).
- Buret, A.G. Pathophysiology of enteric infections with *Giardia duodenalis*. *Parasite* **15**, 261–265 (2008).
- Nash, T.E. Antigenic variation in *Giardia lamblia* and the host's immune response. *Phil. Trans. R. Soc. Lond. B* **352**, 1369–1375 (1997).
- Nash, T. Surface antigen variability and variation in *Giardia lamblia*. *Parasitol. Today* **8**, 229–234 (1992).
- Pimenta, P.F., da Silva, P.P. & Nash, T. Variant surface antigens of *Giardia lamblia* are associated with the presence of a thick cell coat: thin section and label fracture immunocytochemistry survey. *Infect. Immun.* **59**, 3989–3996 (1991).
- Morrison, H.G. *et al.* Genomic minimalism in the early diverging intestinal parasite *Giardia lamblia*. *Science* **317**, 1921–1926 (2007).
- Nash, T.E. Surface antigenic variation in *Giardia lamblia*. *Mol. Microbiol.* **45**, 585–590 (2002).
- Prucca, C.G. *et al.* Antigenic variation in *Giardia lamblia* is regulated by RNA interference. *Nature* **456**, 750–754 (2008).
- Gottstein, B. & Nash, T.E. Antigenic variation in *Giardia lamblia*: infection of congenitally athymic nude and scid mice. *Parasite Immunol.* **13**, 649–659 (1991).

18. Nash, T.E. & Aggarwal, A. Cytotoxicity of monoclonal antibodies to a subset of *Giardia* isolates. *J. Immunol.* **136**, 2628–2632 (1986).
19. Hemphill, A., Stager, S., Gottstein, B. & Muller, N. Electron microscopical investigation of surface alterations on *Giardia lamblia* trophozoites after exposure to a cytotoxic monoclonal antibody. *Parasitol. Res.* **82**, 206–210 (1996).
20. Deselliers, L.P., Tan, D.T., Scott, R.B. & Olson, M.E. Effects of *Giardia lamblia* infection on gastrointestinal transit and contractility in Mongolian gerbils. *Dig. Dis. Sci.* **42**, 2411–2419 (1997).
21. Buret, A., Hardin, J.A., Olson, M.E. & Gall, D.G. Pathophysiology of small intestinal malabsorption in gerbils infected with *Giardia lamblia*. *Gastroenterology* **103**, 506–513 (1992).
22. Aggarwal, A. & Nash, T.E. Comparison of two antigenically distinct *Giardia lamblia* isolates in gerbils. *Am. J. Trop. Med. Hyg.* **36**, 325–332 (1987).
23. Moss, D.M., Mathews, H.M., Visvesvara, G.S., Dickerson, J.W. & Walker, E.M. Antigenic variation of *Giardia lamblia* in the feces of Mongolian gerbils. *J. Clin. Microbiol.* **28**, 254–257 (1990).
24. Luján, H.D., Mowatt, M.R., Conrad, J.T., Bowers, B. & Nash, T.E. Identification of a novel *Giardia lamblia* cyst wall protein with leucine-rich repeats. Implications for secretory granule formation and protein assembly into the cyst wall. *J. Biol. Chem.* **270**, 29307–29313 (1995).
25. Nash, T.E., Herrington, D.A., Losonsky, G.A. & Levine, M.M. Experimental human infections with *Giardia lamblia*. *J. Infect. Dis.* **156**, 974–984 (1987).
26. Mowatt, M.R., Aggarwal, A. & Nash, T.E. Carboxy-terminal sequence conservation among variant-specific surface proteins of *Giardia lamblia*. *Mol. Biochem. Parasitol.* **49**, 215–227 (1991).
27. Luján, H.D., Mowatt, M.R., Conrad, J.T. & Nash, T.E. Increased expression of the molecular chaperone BiP/GRP78 during the differentiation of a primitive eukaryote. *Biol. Cell* **86**, 11–18 (1996).
28. Gardner, T.B. & Hill, D.R. Treatment of giardiasis. *Clin. Microbiol. Rev.* **14**, 114–128 (2001).
29. Upcroft, J.A., Upcroft, P. & Boreham, P.F. Drug resistance in *Giardia intestinalis*. *Int. J. Parasitol.* **20**, 489–496 (1990).
30. Faubert, G. Immune response to *Giardia duodenalis*. *Clin. Microbiol. Rev.* **13**, 35–54, (2000).
31. Müller, N. & von Allmen, N. Recent insights into the mucosal reactions associated with *Giardia lamblia* infections. *Int. J. Parasitol.* **35**, 1339–1347 (2005).
32. Nash, T.E., Merritt, J.W. Jr. & Conrad, J.T. Isolate and epitope variability in susceptibility of *Giardia lamblia* to intestinal proteases. *Infect. Immun.* **59**, 1334–1340 (1991).
33. Singer, S.M. & Nash, T.E. The role of normal flora in *Giardia lamblia* infections in mice. *J. Infect. Dis.* **181**, 1510–1512 (2000).
34. Nash, T.E., Herrington, D.A., Levine, M.M., Conrad, J.T. & Merritt, J.W. Jr. Antigenic variation of *Giardia lamblia* in experimental human infections. *J. Immunol.* **144**, 4362–4369 (1990).
35. Anders, R.F., Roberts-Thomson, I.C. & Mitchell, G.F. Giardiasis in mice: analysis of humoral and cellular immune responses to *Giardia muris*. *Parasite Immunol.* **4**, 47–57 (1982).
36. Aggarwal, A. & Nash, T.E. Antigenic variation of *Giardia lamblia* in vivo. *Infect. Immun.* **56**, 1420–1423 (1988).
37. Gottstein, B., Deplazes, P. & Tanner, I. *In vitro* synthesized immunoglobulin A from nu/nu and reconstituted nu/nu mice against a dominant surface antigen of *Giardia lamblia*. *Parasitol. Res.* **79**, 644–648 (1993).
38. Stäger, S., Gottstein, B., Sager, H., Jungi, T.W. & Muller, N. Influence of antibodies in mother's milk on antigenic variation of *Giardia lamblia* in the murine mother-offspring model of infection. *Infect. Immun.* **66**, 1287–1292 (1998).
39. Byrd, L.G., Conrad, J.T. & Nash, T.E. *Giardia lamblia* infections in adult mice. *Infect. Immun.* **62**, 3583–3585 (1994).
40. Bouza, M., Maciques, I., Torres, D. & Nunez, F.A. *Giardia lamblia* in Mongolian gerbils: characteristics of infection using different human isolates. *Exp. Parasitol.* **96**, 43–46 (2000).
41. Eckmann, L. Mucosal defences against *Giardia*. *Parasite Immunol.* **25**, 259–270 (2003).
42. Ropolo, A.S., Saura, A., Carranza, P.G. & Lujan, H.D. Identification of variant-specific surface proteins in *Giardia muris* trophozoites. *Infect. Immun.* **73**, 5208–5211 (2005).
43. Franzén, O. *et al.* Draft genome sequencing of *Giardia intestinalis* assemblage B isolate GS: is human giardiasis caused by two different species? *PLoS Pathog.* **5**, e1000560 (2009).
44. Dunn, L.A., Upcroft, J.A., Fowler, E.V., Mathews, B.S. & Upcroft, P. Orally administered *Giardia duodenalis* extracts enhance an antigen-specific antibody response. *Infect. Immun.* **69**, 6503–6510 (2001).
45. Heyworth, M.F. Relative susceptibility of *Giardia muris* trophozoites to killing by mouse antibodies of different isotypes. *J. Parasitol.* **78**, 73–76 (1992).
46. Belosevic, M. & Faubert, G.M. Lysis and immobilization of *Giardia muris* trophozoites *in vitro* by immune serum from susceptible and resistant mice. *Parasite Immunol.* **9**, 11–19 (1987).
47. Butscher, W.G. & Faubert, G.M. The therapeutic action of monoclonal antibodies against a surface glycoprotein of *Giardia muris*. *Immunology* **64**, 175–180 (1988).
48. Hill, D.R., Burge, J.J. & Pearson, R.D. Susceptibility of *Giardia lamblia* trophozoites to the lethal effect of human serum. *J. Immunol.* **132**, 2046–2052 (1984).
49. Olson, M.E., Ceri, H. & Morck, D.W. *Giardia* vaccination. *Parasitol. Today* **16**, 213–217 (2000).
50. Olson, M.E., Morck, D.W. & Ceri, H. Preliminary data on the efficacy of a *Giardia* vaccine in puppies. *Can. Vet. J.* **38**, 777–779 (1997).
51. Anderson, K.A. *et al.* Impact of *Giardia* vaccination on asymptomatic *Giardia* infections in dogs at a research facility. *Can. Vet. J.* **45**, 924–930 (2004).
52. Uehlinger, F.D. *et al.* Efficacy of vaccination in preventing giardiasis in calves. *Vet. Parasitol.* **146**, 182–188 (2007).
53. Mann, J.F., Acevedo, R., Campo, J.D., Perez, O. & Ferro, V.A. Delivery systems: a vaccine strategy for overcoming mucosal tolerance? *Expert Rev. Vaccines* **8**, 103–112 (2009).
54. Tonkin, C.J. *et al.* Sir2 paralogs cooperate to regulate virulence genes and antigenic variation in *Plasmodium falciparum*. *PLoS Biol.* **7**, e84 (2009).

## ONLINE METHODS

**Parasites.** *G. lamblia* WB<sup>12</sup> and GS/M<sup>12</sup> as well as human isolates were cultured in TYI-S-33 medium<sup>24</sup>. Clones expressing various surface antigens were obtained by limiting dilution in 96-well culture plates and selected by immunofluorescence with specific mAbs<sup>24</sup>. Reactive clones were then expanded overnight in culture medium and tested for homogeneity before use. WB clones 1267 (mAb 5C1), 9B10 (mAb 9B10), A6 (mAb 6E7) and GS/M-H7 (mAb G10/4) were used in control experiments and in challenge infections<sup>12,22,55,56</sup>.

**Silencing of *Giardia* Dicer and RNA-dependent RNA polymerase.** Complementary DNA sequences of the genes encoding the *Giardia* enzymes RdRP and Dicer were cloned into plasmid pTubHA.pac, transfected into *Giardia* by electroporation and selected as previously reported<sup>16</sup>.

**Immunofluorescence microscopy.** Confocal and immunofluorescence assays were performed as previously reported<sup>16</sup>. The percentage of cells expressing particular VSPs was calculated by counting 500 cells in triplicate experiments or by flow cytometry<sup>16</sup>.

**Polyacrylamide gel electrophoresis and western blotting.** Trophozoite protein extracts were subjected to PAGE (SDS-PAGE) and western blotted as previously reported<sup>24</sup>.

**Gerbils.** Specific pathogen-free, 6-week-old outbred male *Meriones unguiculatus* (Animal Facility, Catholic University of Cordoba) were used and housed in air-conditioned (18–22 °C, 40–50% humidity) racks with a 12-h light-dark cycle. Gerbils were given autoclaved food and sterile water supplemented with a mixture of filter-sterilized vitamin solution *ad libitum*. Before infection, gerbils were tested for negativity for serum antibodies against *Giardia* antigens by ELISA. After infections, some control groups of gerbils were orally treated with 20 mg metronidazole (Sigma) for 3 d, 10 d before challenge, to eliminate the presence of any *Giardia* in their intestines<sup>22</sup>. All protocols were approved by the Institutional Review Board of the Catholic University of Cordoba.

**Infections.** Infections were induced in gerbils by orogastric inoculation of  $2 \times 10^5$  trophozoites or  $1 \times 10^2$  cysts resuspended in 0.5 ml of 2× PBS. Some control gerbils received 0.5 ml PBS by the same route. We used freshly collected cysts from infected gerbils or human subjects to avoid rapid loss of viability and infectivity<sup>57</sup>. Fecal collection from infected gerbils was performed daily from day 0 to day 30. Cysts or trophozoites were identified visually by light microscopy or by immunofluorescence assays with cyst-specific 7D2 mAb<sup>24</sup> or antibody to trophozoite-specific antigens (GPR78/BiP; 9C9 mAb<sup>27</sup>). Periodically, randomly selected gerbils were killed and the small intestines were isolated, slit open longitudinally and suspended in culture medium at 4 °C for 30 min. The supernatants were collected and examined for *Giardia* trophozoites by light and fluorescence microscopy or placed in culture medium for up to 6 d<sup>39</sup>. *Giardia* cysts excreted by gerbils were quantified by collecting stool pellets from individually housed gerbils over a 24-h period. The stool samples were weighed, resuspended in 2 ml of PBS and filtered. The filtrate was centrifuged at 250g for 10 min. After three washes, the pellet was suspended in 2 ml PBS, and cysts were stained and counted in a hemocytometer. Gerbils were considered uninfected if no cysts were found in the feces or no trophozoites were detected after 6 d in culture.

**Purification of variant-specific surface proteins from DAS and RAS transgenic trophozoites.** The entire repertoire of VSPs expressed in the transgenic trophozoites was purified by immunoaffinity with the 12F1 mAb<sup>16</sup> generated against the conserved five-amino acid tail of VSPs, as previously reported<sup>58</sup>. Purified VSPs were resuspended in PBS containing 0.01% Tween 20, quantified and used to orally immunize gerbils<sup>44</sup>. Individual VSPs were purified by the same methodology with specific mAbs.

**Purification of *Giardia* GRP78/BiP.** Trophozoites of the *G. lamblia* isolate WB clone 9B10 were transfected with the pTubHA.pac plasmid containing the full-length cDNA of BiP/GRP78 (ref. 27) and three copies of the hemagglutinin epitope tag. Transgenic trophozoites were lysed with PBS-0.01% Tween 20, and BiP was isolated using a hemagglutinin-specific antibody (Sigma).

**Oral immunizations.** Gerbils were immunized by three successive oral administrations of 200 µg of parasite proteins suspended in sterile PBS containing 0.01% Tween 20. The same amount of antigen was administered to gerbils when BiP or purified VSPs were used as immunogen.

**Blood samples.** Blood samples from gerbils were collected after the first day of infection or immunization to detect the presence of circulating gerbil antibodies to *Giardia*. Blood from human subjects was obtained immediately after finding *Giardia* cysts in their stool samples or from control, uninfected newborn individuals. All protocols were approved by the Institutional Review Board of the Catholic University of Cordoba, and appropriate informed consent was obtained for all human subjects.

**Intestinal contents.** Secretions of small intestines of infected, uninfected and immunized gerbils were collected essentially as reported for mice<sup>45</sup>. The supernatants were filter-sterilized and maintained at –70 °C until use.

**Agglutination assays.** Test were performed in 96-well plates with flat-bottom wells. Approximately  $5 \times 10^5$  trophozoites per well were incubated at 4 °C for 1 h with various dilutions of gerbil intestinal secretions, gerbil sera or VSP-specific mAbs (all heat inactivated) in TYI-S-33 medium without adult bovine serum. After mixing by repeated pipetting, agglutination of trophozoites was assessed by microscopy. Binding of mouse or human antibodies to the surface of the parasites was detected with TRITC-conjugated goat antibodies to mouse or human immunoglobulins. Although it was previously reported that there is cross-reaction of goat antibody to hamster IgG with gerbil immunoglobulins<sup>36</sup>, we were unable to detect gerbil antibodies with hamster-specific immunoglobulin reagents from multiple commercial sources; therefore, gerbil sera and intestinal contents were directly labeled with FITC as previously reported<sup>16</sup>.

55. Carranza, P.G. *et al.* Simultaneous expression of different variant-specific surface proteins in single *Giardia lamblia* trophozoites during encystation. *Infect. Immun.* **70**, 5265–5268 (2002).

56. Adam, R.D. *et al.* Antigenic variation of a cysteine-rich protein in *Giardia lamblia*. *J. Exp. Med.* **167**, 109–118 (1988).

57. Deng, M.Y. & Cliver, D.O. Degradation of *Giardia lamblia* cysts in mixed human and swine wastes. *Appl. Environ. Microbiol.* **58**, 2368–2374 (1992).

58. Luján, H.D., Mowatt, M.R. & Wu, J.J. Lu, Y., Lees, A., Chance M.R. & Nash T.E. Purification of a variant-specific surface protein of *Giardia lamblia* and characterization of its metal-binding properties. *J. Biol. Chem.* **270**, 13807–13813 (1995).