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Cytokines, Antibodies, and Histopathological Profiles during *Giardia* Infection and Variant-Specific Surface Protein-Based Vaccination

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ABSTRACT Giardiasis is one of the most common human intestinal diseases worldwide. Several experimental animal models have been used to evaluate *Giardia* infections, with gerbils (*Meriones unguiculatus*) being the most valuable model due to their high susceptibility to *Giardia* infection, abundant shedding of cysts, and pathophysiological alterations and signs of disease similar to those observed in humans. Here, we report cytokine and antibody profiles both during the course of *Giardia* infection in gerbils and after immunization with a novel oral vaccine comprising a mixture of purified variant-specific surface proteins (VSPs). Transcript levels of representative cytokines of different immune profiles as well as macro- and microtissue alterations were assessed in Peyer's patches, mesenteric lymph nodes, and spleens. During infection, cytokine responses showed a biphasic profile: an early induction of Th1 (gamma interferon [IFN-γ], interleukin-1β [IL-1β], IL-6, and tumor necrosis factor [TNF]), Th17 (IL-17), and Th2 (IL-4) cytokines, together with intestinal alterations typical of inflammation, followed by a shift toward a predominant Th2 (IL-5) response, likely associated with a counterregulatory mechanism. Conversely, immunization with an oral vaccine comprising the entire repertoire of VSPs specifically showed high levels of IL-17, IL-6, IL-4, and IL-5, without obvious signs of inflammation. Both immunized and infected animals developed local (intestinal secretory IgA [S-IgA]) and systemic (serum IgG) humoral immune responses against VSPs; however, only infected animals showed evident signs of giardiasis. This is the first comprehensive report of cytokine expression and anti-*Giardia* antibody production during infection and VSP vaccination in gerbils, a reliable model of the human disease.

KEYWORDS giardiasis, animal models, cytokines, immunization

Giardia lamblia infection is one of the most common intestinal diseases worldwide (1). *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. Trophozoites are responsible for the clinical manifestations associated with the disease, which vary from asymptomatic infections to acute or chronic diarrhea. Symptoms in humans typically occur 2 weeks after infection and are usually mild, such as cramps and mild chronic diarrhea, even though severe complications associated with malabsorption are also frequent (2). The mechanisms by which *G. lamblia* causes disease are poorly understood. The parasite is suspected to be noninvasive, and little or no mucosal inflammation has been reported during acute infection in humans (3). Disease severity may depend on multiple parasite- and host-related factors (4). In some

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cases, particular variant-specific surface proteins (VSPs) have been suggested to be involved in pathogenicity, but antigenic variation is, undoubtedly, responsible for the chronicity of *Giardia* infections (5). Nevertheless, the stimulus causing VSP switching remains elusive.

Although *Giardia* species inhabit the intestinal tracts of almost all classes of vertebrates, *G. lamblia* is the only recognized species found in humans and most other mammals (1). Several experimental models have been used to evaluate the clinical signs and the pathology of giardiasis as well as the course of antigenic variation during *Giardia* infections (6). Mice are not infected with *Giardia* assemblage A (represented by the human WB isolate) but can be naturally infected with *Giardia muris*. In contrast to most human isolates of *G. lamblia*, *G. muris* and most isolates from the other assemblages do not cause infection in humans and cannot proliferate in culture. Moreover, the murine model of giardiasis does not reproduce all the symptoms of the disease found in humans (7). Conversely, *Meriones unguiculatus* rodents (gerbils) are one of the most useful animal models since they present high susceptibility to infections by oral inoculation of either cysts or trophozoites, abundant cyst elimination in the feces of infected animals, and pathophysiological alterations similar to those observed in humans (6, 8, 9). In this animal model, infections cause disease symptoms, including diarrhea and loss of weight (10, 11), making it a suitable model for the study of the course of *Giardia* infections. However, the immune response of these rodents to *Giardia* infections is poorly known due to the lack of genetically modified animals and gerbil-specific immunological tools (8, 9). *Giardia* undergoes surface antigenic variation, a mechanism by which parasitic microorganisms can evade the host's immune response (12). Antigenic variation in *Giardia* involves variant-specific surface proteins (VSPs). VSPs are cysteine-rich integral membrane proteins that form a dense coat on the parasite. Of a repertoire of over 200 homolog genes encoded in the parasite genome, only one VSP is expressed on the surface of every single trophozoite at any given moment (12); however, a switch in expression to an antigenically distinct VSP has been reported to occur spontaneously (13, 14).

In a previous work, we reported that a mechanism similar to RNA interference (RNAi) is involved in the control of the expression of surface antigens and that knocking down the expression of key enzymes of the RNAi pathway produced a change from expression of single to multiple VSPs in individual *Giardia* trophozoites (15). Then, we hypothesized that trophozoites expressing the complete VSP repertoire on their surfaces would confer protection against future infections. Therefore, we performed experiments using altered parasites of the *Giardia* WB isolate in the gerbil model of giardiasis. Our results showed that the animals initially infected with cells expressing all of the VSPs encoded in their genome are largely protected from infection by *Giardia* clones expressing a unique VSP on their surface or by cysts obtained from infected individuals. Additionally, immunization with the entire repertoire of VSPs purified from these transgenic cells also provided protection against subsequent infections (5). Similar results to those observed in gerbils were obtained in young and adult dogs and cats (16). However, the gerbil immunological basis of *Giardia* infection compared to VSP-based vaccine protection has still not been studied.

The aims of this work were to determine cytokine profiles, histopathological and macromorphological alterations of relevant tissues (intestine, spleens [SPL], Peyer's patches [PPs], and mesenteric lymph nodes [MLNs]), and production of anti-VSP-specific antibodies after gerbil infection or vaccination by using new reagents and techniques developed for this animal model.

RESULTS AND DISCUSSION

To describe the pathology by which *G. lamblia* causes disease in our model, the course of the infection in gerbils inoculated with WB1267 trophozoites (parasites from the WB isolate expressing the VSP1267 on their surface) was followed by quantifying trophozoite load in the small intestine and cysts in stool samples. Trophozoites reached their maximum number at 7 days postinfection (dpi) (Fig. 1A), followed by a rapid

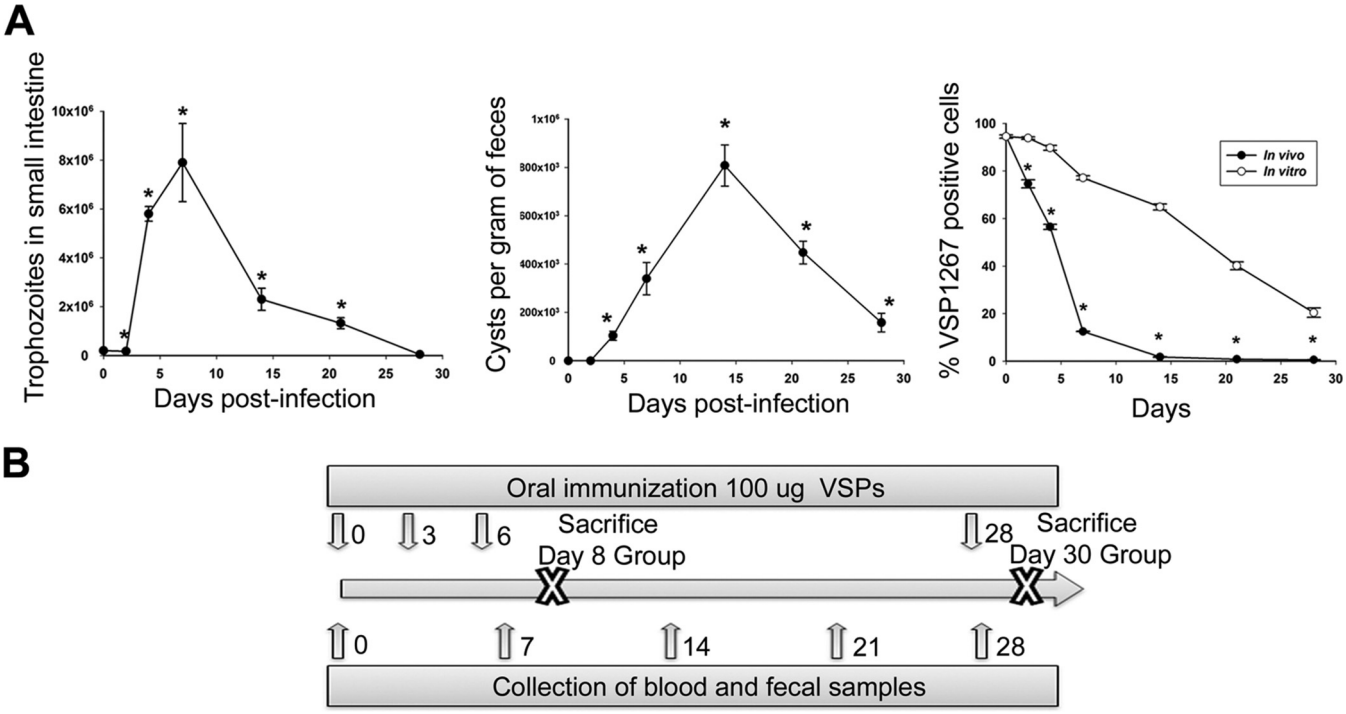


FIG 1 Infection and immunization. (A) Gerbil infection kinetics. Animals were infected intragastrically with 2×10^5 *G. lamblia* trophozoites resuspended in PBS–5 mM cysteine. Cysts were quantified by immunofluorescence using an anti-CWP2-specific MAb. Trophozoites were counted from small intestine at the indicated time points. Clonal expression of VSP1267 was evaluated by immunofluorescence assays with the MAb 5C1. Each point represents the mean value ± standard error of the mean of the results obtained in three independent experiments ($n = 12$). *, $P < 0.01$ compared to results in uninfected animals or to *in vitro* culture. (B) Immunization scheme. Immunization was performed with VSPs as described in Materials and Methods.

decrease but persistence up to 28 dpi. The rapid decrease in the number of parasites is very consistent among all gerbil infections reported to date (5, 9, 17) and varies only slightly from decreases reported in mice, dogs, cats, and humans (16, 18, 19), suggesting that the course of *Giardia* infection is similar in all host species.

The expression of VSP1267 evaluated in trophozoites recovered from the infected animals showed a marked decrease of the original VSP level at as early as 2 dpi (Fig. 1A), indicating early antigenic switching after infection establishment. The occurrence of *in vivo* switching during the first stages of infection and prior to the formation of specific antibodies has been previously described (20). However, the reason for this fast switching is not clear. Selection of a VSP, rather than switching, has been suggested for VSPH7 of the assemblage B isolate GS-M in the mouse model of infection (21); however, the large number of trophozoites recovered at 4 dpi indicated that VSP switching is more likely to occur than selection of a different VSP in surviving trophozoites. Several stimuli other than antibodies have been proposed to induce antigenic variation (22, 23). Thus, it is likely that environmental changes (culture versus intestinal conditions) could trigger VSP switching. It is also possible that mechanisms of the innate immune system, such as oxidative stress (reactive nitric and oxygen species) and/or soluble intestinal factors (22, 23) might favor this accelerated change in VSP expression over *in vitro* switching (Fig. 1A). In contrast to trophozoite load, the kinetics observed in cyst elimination in stool samples showed a peak between 14 and 21 dpi (Fig. 1A), following a pattern previously described for *Giardia* infections in gerbils (5, 9).

The visual analysis of PPs showed a progressive size increase from 2 dpi, with PPs reaching their largest size at 21 dpi (Fig. 2A). The histopathological analysis of infected animals also showed a clear weight gain of MLNs from 2 dpi, with a peak at 4 dpi; after that point, MLN weight began to decrease until it reached normal levels at 21 dpi (Fig. 2B). Histological sections of MLNs from infected animals (Fig. 2C) showed reactive lymphadenitis with lymphoid hyperplasia and abundant eosinophilic areas enriched in

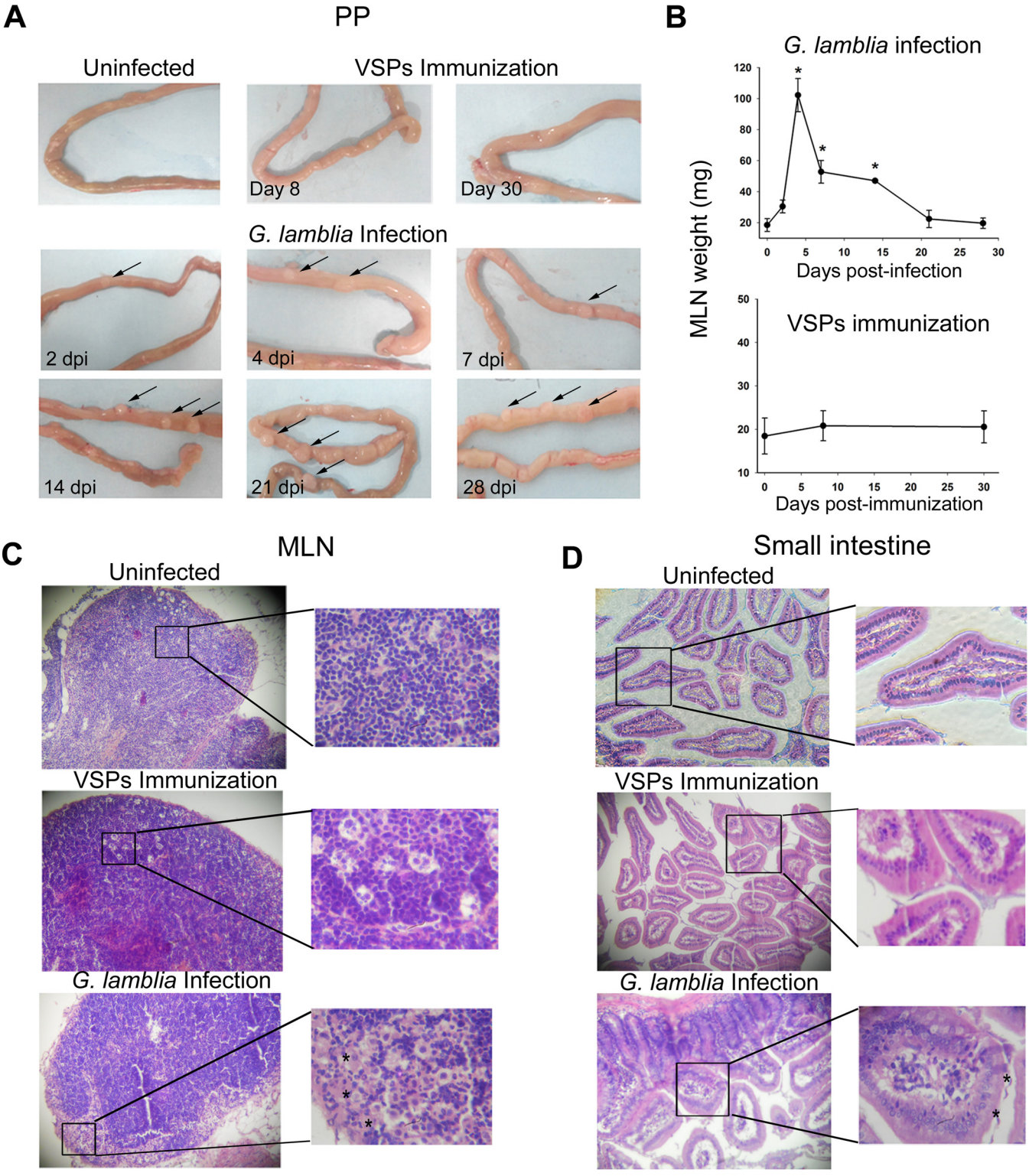
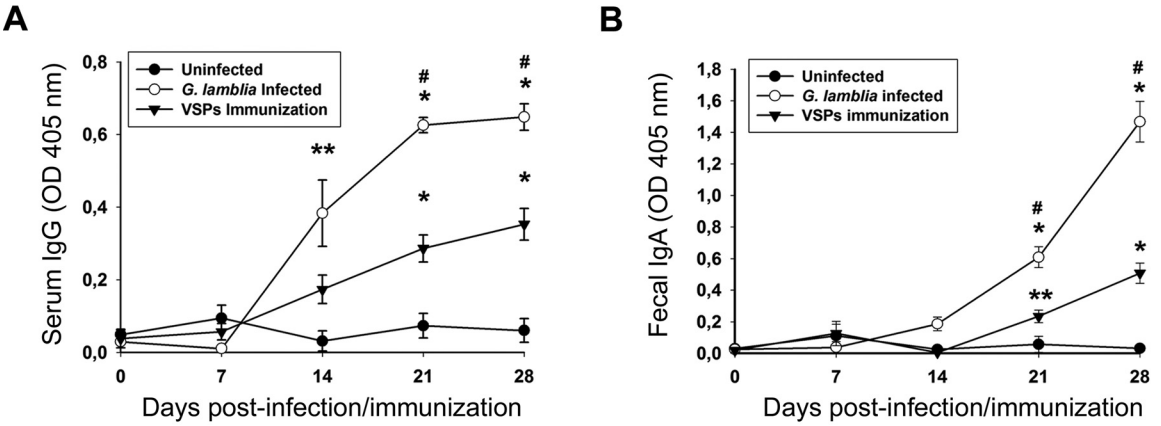


FIG 2 Histopathological analysis. (A) Macroscopic examination of upper small intestines. Arrows indicate an increase in size of the Peyer's patches in the infected versus uninfected gerbils used as controls. (B) Mesenteric lymph node size. The MLN size from experimental gerbils was determined by weighing at the indicated day postinfection. Each point represents the mean value ± standard error of the mean of the results obtained in three independent experiments ($n = 12$). *, $P < 0.05$ compared to results in uninfected animals (0 dpi). (C and D) Light microscopy images. Representative images (magnification, ×20) from MLN and upper small intestine from uninfected, VSP-immunized, or infected gerbils are shown. Slides were stained by hematoxylin-eosin. Asterisks indicate some *Giardia* trophozoites in the intestinal lumen from infected gerbils (7 dpi) and eosinophilic areas enriched with histiocytes in an MLN section (4 dpi).

histiocytes. Both lymphoid hyperplasia and histiocyte accumulation occurred at the expense of the three ganglion compartments (cortex, paracortex, and medulla); however, histiocytosis was more marked at the medullar sinus (Fig. 2C). Histopathological analysis of the small intestine revealed marked changes in the intestinal mucosa during *Giardia* infection, including a generalized inflammatory process with pronounced alterations of the gut architecture, such as flattening of villi, crypts, and goblet cell hyperplasia, inflammatory infiltrate in mucosa and submucosa, and increases in the number of intraepithelial leukocytes and in mucus secretion (Fig. 2D). These intestinal alterations have also been observed by previous reports in different models of giardiasis including in gerbils (11, 17). No morphological alterations were observed at this level in vaccinated or uninfected animals. All infected gerbils were able to develop a systemic humoral immune response, as evidenced by specific IgG, and an intestinal secretory response (secretory IgA [S-IgA]) (Fig. 3). Notably, the development of the humoral response correlated with the decrease in cyst number (Fig. 3D and E), indicating that these systemic and local humoral immune responses could be related to the control of giardiasis. Not only innate defense mechanisms but also both acquired humoral and cell-mediated immune responses protect against *Giardia* infections (7). In fact, several studies have demonstrated that a variety of immunological factors participate in the control of primary infection by *G. lamblia*, including $CD4^+$ T cells and their cytokines as well as B cells and their antibodies (24, 25). Previous research has suggested that humoral immunity is important for parasite elimination since hypogammaglobulinemic individuals (7) and mice genetically deficient in functional B cells or specifically lacking IgA-expressing B cells tend to present prolonged infections (26). Accordingly, antibodies produced in the intestinal mucosa may directly act in giardiasis control by binding to surface antigens and, thereby, interfering with their adherence to the intestinal mucosa as well as promoting their immobilization and agglutination (5). In this study, experimental infection by *G. lamblia* stimulated an intestinal secretory-specific immune response. The increase in the sizes of both PPs and MLNs would indicate an active response of these immune compartments.

The evaluation of the vaccine outcome revealed that immunization with VSPs was also able to induce a marked humoral response, showing significant levels of serum IgG and fecal S-IgA against both the whole parasite and individual VSPs (Fig. 3A to C). The persistence of *Giardia* at the site of infection provoking a constant stimulation of the immune system, the presence of other antigens different from VSPs, and the adjuvant effect of the inflammatory reaction present during the course of infection could be the reasons for the higher antibody levels present in the natural infection than after immunization with the multi-VSP vaccine. In previous works we have shown that both infection with the homologous VSP-expressing *Giardia* clone (i.e., primary VSP1267 infection and secondary VSP1267 infection) and immunization with VSP and infection with *G. lamblia* expressing either VSP1267 or VSP9B10 are protective; therefore, although the vaccine humoral responses were lower than responses seen in natural infection, these antibody levels would be sufficient to provide protection. Remarkably, no histopathological changes were observed in the vaccinated animals (Fig. 2A to C), indicating that the inflammatory process may be causing the signs of the disease since oral vaccination with purified VSPs did not induce signs of giardiasis.

With respect to the mechanisms involved in immune response induction in humans or experimental hosts infected with *G. lamblia*, previous studies have shown that the parasite and/or its components induce a mixed Th1/Th2 response (24, 27, 28), and, more recently, reports have described a key role of a Th17 response in the control of *Giardia* infections. Interleukin-17 (IL-17) appears to be essential for the control of these infections in diverse hosts such as mice, cattle, and humans (29–32). In the present work, cytokines that characterize Th1 proinflammatory events (gamma interferon [IFN-γ], tumor necrosis factor alpha [TNF-α], IL-1β, and IL-6), Th2 mediator induction (IL-4 and IL-5), Th17 (IL-17), and immune regulation (transforming growth factor β [TGF-β] and IL-10) were chosen to characterize the immune response induced by parasite infection and VSP vaccination. Cytokine mRNA levels were quantified in the



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	Serum IgG			Fecal IgA		
	Uninfected	*G. lamblia* 1267 Infection	VSPs Immunization	Uninfected	*G. lamblia* 1267 Infection	VSPs Immunization
anti-VSP1267	0,083 +/- 0,054	0,629 +/- 0,247	0,423 +/- 0,121	0,060 +/- 0,043	0,843 +/- 0,272	0,515 +/- 0,138
anti-VSP9B10	0,127 +/- 0,057	0,182 +/- 0,166	0,469 +/- 0,132	0,084 +/- 0,055	0,172 +/- 0,09	0,610 +/- 0,108

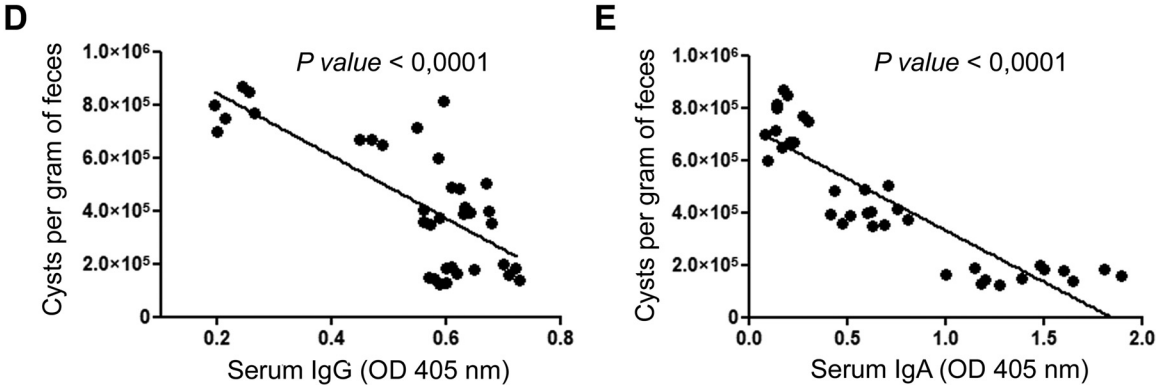

FIG 3 Systemic and intestinal humoral immune responses. Blood and stool samples were collected at 0, 7, 14, 21, and 28 days after infection or immunization. Serum IgG (A) and secretory IgA (B) levels were determined by ELISA using a polyclonal mouse anti-gerbil IgG and IgA developed in our laboratory (see Materials and Methods). Plates were sensitized with *G. lamblia* WB1267 soluble extract. Each point represents the mean value ± standard error of the mean of the results obtained in three independent experiments ($n = 12$). *, $P < 0.01$ compared to results in uninfected animals; **, $P < 0.05$ compared to results in uninfected animals; #, $P < 0.01$ for results in infected animals versus those in animals immunized with VSPs (Tukey's test between the groups). (C) Levels of specific serum IgG and fecal IgA anti-VSP1267 or anti-VSP9B10. Samples of blood and stool from 28 dpi or immunizations were tested. Plates were sensitized with immunopurified VSP1267 or VSP9B10, respectively. Values are the means ± standard errors of the means ($n = 12$). (D and E) Linear correlation between cyst numbers and either serum IgG or fecal IgA response. Data from 14, 21, and 28 dpi were included in the analysis.

SPL, PPs, and MLNs from gerbils at 14 and 28 dpi or at 8 and 30 days after immunization (Fig. 4A). Cytokine profiles showed marked differences during the course of both processes, and only *P* values of <0.05 (Fig. 4, asterisks) were considered in the analysis.

The infection analysis showed an induction of a mixed Th1/Th2/Th17 response at 14 dpi, including high levels of the specific proinflammatory IL-1β. At 28 dpi, IL-5 mRNA levels were significantly increased in all tissues. In PPs, the IL-5 increase was accompanied by a rise in IL-4 and a decrease in IFN-γ transcripts, whereas in MLNs only TGF-β transcript levels were increased. No significant differences were observed in the other studied cytokines. The increase in IL-4 and IL-5 mRNA levels and a decrease in IFN-γ

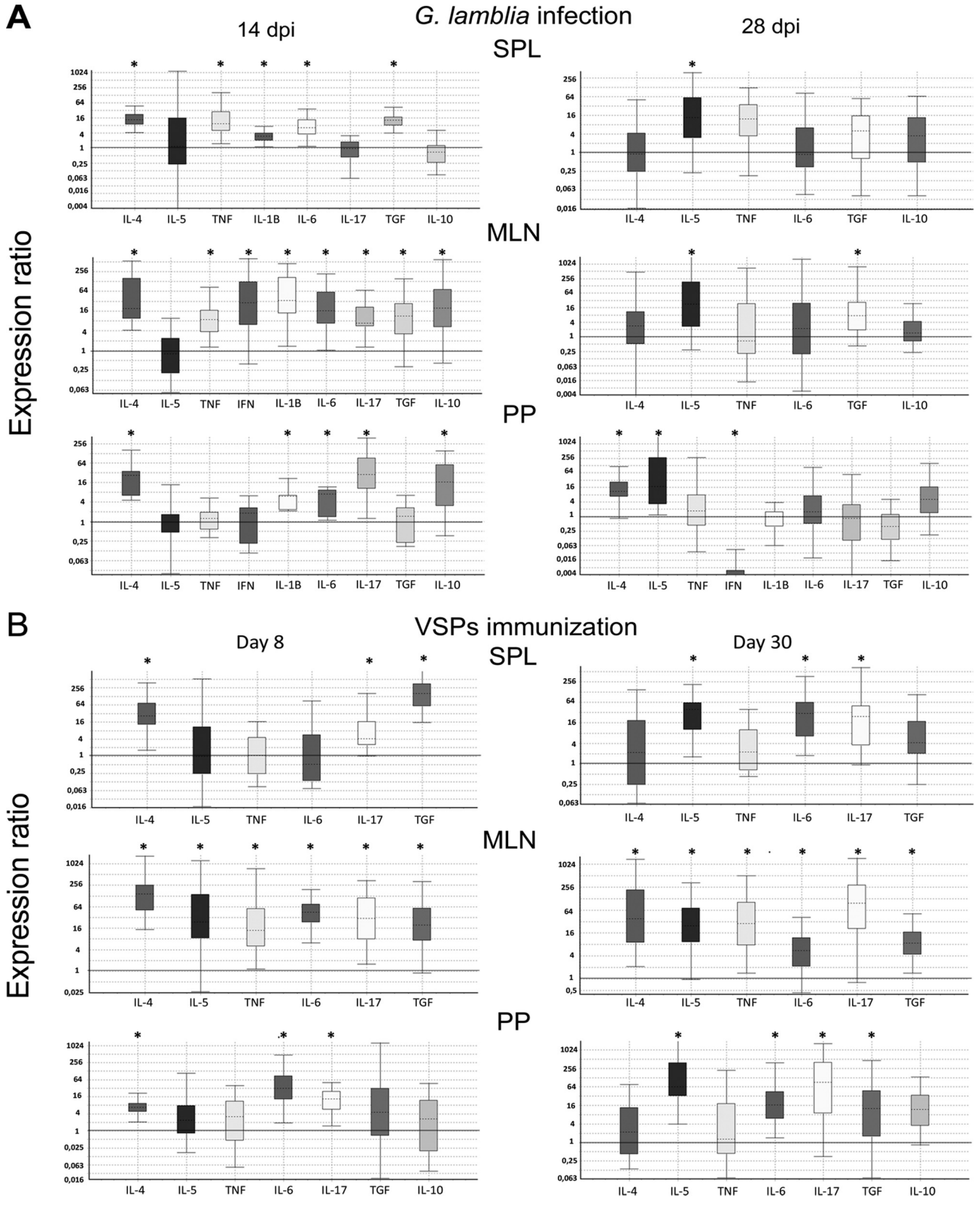

FIG 4 Transcript expression of different cytokines in infected or VSP-immunized gerbils. At 14 and 28 days postinfection (A) or at 8 and 30 days postimmunization (B), the animals were sacrificed, and cells were obtained from PPs, MLNs, and SPL. After total RNA extraction (TRIzol) and cDNA synthesis, cytokine transcript levels were determined via quantitative PCR. Data represent the increase rate compared to the levels in uninfected animals. All data were normalized to the corresponding reference gene using the ΔC_T method and analyzed by REST software. Only those cytokines with detectable transcript levels are shown in the graphs. A P value of <0.05 (*) was considered significant. Data are representative of three independent experiments with four animals per group.

expression observed at 28 dpi in PPs (Fig. 4A) suggest the development of a classic Th2 response. The higher levels of IL-4 produced by PP cells might have inhibited the development of the Th1 T cell subset (33), causing inhibition of IFN-γ production. This switch from an initial Th1/proinflammatory response to a marked Th2 response in the late infection likely is a regulatory mechanism induced by the parasite to evade the deleterious effects associated with the effector molecules produced during this type of immune response, namely, nitric oxide, among others (34–36). The differences observed in cytokines among the three tissues are likely due to the fact that *Giardia* trophozoites do not invade the mucosa and to immune response compartmentalization of the peripheral lymphoid organs, especially in PPs and MLNs.

We have previously shown that a vaccine based on a cocktail of *Giardia* immunogenic VSPs was able to induce a protective immune response in gerbils (5) and domestic animals (16). In this work, we also evaluated the immune response generated by immunization with this protein mix. To assess the possible toxicity of repeated doses, which could induce a local inflammation, and to further assay the vaccine's early immunostimulatory activity, histopathological changes and cytokine levels were measured 2 days after the application of the last vaccine dose (day 8). Furthermore, to characterize the type of response generated by this protective vaccine, a boost with an additional oral administration of the VSP mix was performed 28 days following the immunization protocol, and 2 days later (day 30), different analyses were performed. In agreement with previous findings, immunization with VSPs did not induce any obvious inflammation, nor did it produce signs of the disease (5). PPs and MLNs did not show increased size, and no significant histopathological alterations in the small intestine of vaccinated gerbils were observed (Fig. 2A to C).

With respect to the cytokine profile developed by the vaccine, the early response was characterized by a marked increase in IL-4, IL-6, IL-17, and TG-β, mainly in MLNs and PPs and, to a lesser extent, in the SPL (Fig. 4B). In MLNs, increased levels of IL-5 and TNF-α were also observed. The responses at day 30 maintained high levels of IL-6, IL-17, and TGF-β whereas IL-4 remained high only in MLNs and SPL. Importantly, at this immunization stage, in addition to the high levels of IL-17, a strong increase in IL-5 was observed (Fig. 4B).

The possible protective role of Th1-, Th2- or Th17-type immunity in *Giardia* infection was not clear. Evidence from different experimental models has been controversial. In humans, for example, it has been shown that *Giardia* can stimulate the production of IFN-γ from human $CD4^+$ T cells *in vitro* although these cells do not trigger cytotoxicity or migration (37). A recent study has shown that increased levels of IFN-γ, IL-4, and IL-5 in fecal samples from infected individuals are associated with a prolonged course of *G. lamblia* infection, suggesting that these cytokines may favor parasite persistence (28). Moreover, increased levels of IFN-γ, IL-4, and IL-5 were also associated with increased length of *G. lamblia* infections (24). In particular, the participation of IL-6 in the control of *Giardia* infection in mice has been suggested to be important (38, 39). IL-6 participates in both T and B cell responses and has been involved in the promotion of T cell differentiation toward Th17 cells (40). In our study, increased levels of IL-6 transcripts were observed early during the infection (14 dpi) as well as after immunization (days 8 and 30) (Fig. 4). In MLNs, this increase was accompanied by an increase in TGF-β, a cytokine also involved in Th17 development. In fact, very high levels of IL-17 were found in the immunized animals, particularly at day 30, when 80- and 100-fold increases in IL-17 in PPs and MLNs, respectively, were reached (Fig. 4B). Moreover, a significant increase in IL-17 levels during *Giardia* infection, particularly at 14 dpi, was observed in PPs and MLNs (Fig. 4A). Accordingly, although several studies have described an important role of IL-17 in different models of giardiasis in recent years (41), this is the first report that describes this immune response in gerbil infections. In addition, a Th17 response to immunization with VSPs, consisting of an increase in IL-6, TGF-β, and IL-17, shows the importance of these proteins as relevant protective immunogens of *Giardia*.

The influence of Th2-mediated responses on mucosal IgA immunoregulation is also well known. For instance, IL-5 promotes switching to the IgA isotype (7), an antibody

that is important in eradicating both *G. muris* and *G. lamblia* infection (26). In this case, the high levels of IL-4 and IL-5 present in PP cells would be in agreement with the S-IgA detected in fecal samples. The pronounced Th2-type profile observed in PPs at 14 and 28 dpi was also observed in gerbils (9), which showed the specific increase of serum IgG1 and IgG2, as well as in *G. muris*-infected mice (42). Moreover, it has been demonstrated that TGF-β induces an IgA class switch on B lymphocytes (43), suggesting that the increase in mRNA levels of this cytokine observed both during infection and after immunization with VSPs could contribute to the production of intestinal S-IgA. The protective role of this cytokine has also been reported in humans. For instance, Taherkhani et al. (44) determined the association between TGF-β1 polymorphism and susceptibility to giardiasis, showing that individuals with symptomatic disease have lower levels of S-IgA than asymptomatic and control groups. In addition, the frequency of the TGF-β1 polymorphisms was significantly higher in symptomatic patients than in asymptomatic ones. TGF-β has also been identified as a critical cytokine for commitment to Th17 development, upregulating IL-23R expression (45). Reports have shown that *Giardia* antigens trigger a Th2-mediated response (44), which might not provide protection and may also even be harmful. Therefore, the relative success of *Giardia* in completing its life cycle during a primary infection might also be due to the capacity of the parasite to trigger a Th2-type response.

A strong Th1 response is also important in reducing *Giardia* trophozoite load and fecal cyst counts (34, 35). It appears that the association of *Giardia* with macrophages elicits mainly an oxidative response, with nitric oxide having an important role (36). In the context of a Th1 response, a protective role has also been described for TNF-α. Accordingly, a significant increase in this cytokine was observed in MLNs and SPL at 14 dpi, as well as in MLNs of VSP-immunized animals at 8 and 30 dpi (Fig. 4).

In conclusion, the VSP-based vaccine was able to induce high levels of IL-17, IL-6, IL-4, and IL-5, stimulating the production of intestinal S-IgA and serum IgG, without obvious signs of inflammation.

Here, we also describe a very extensive cytokine profile in the gerbil model of giardiasis for the first time. In recent years, the induction of a Th17 immune response in mouse models has been strongly associated with protection against giardiasis. Here, in a more valuable model we have demonstrated that a strong IL-17 response can be detected during infection and VSP-based vaccination. Although the gerbil model presents high similarities to human giardiasis, the species has been little explored due to the previous lack of immunological tools. The results presented in this study greatly contribute to the understanding of the immune response in this suitable experimental model of giardiasis and provide conclusive explanations of the involvement of the immune system during both symptomatic infections and protective vaccination.

MATERIALS AND METHODS

Ethics. All procedures performed on animals were conducted according to protocols approved by the Institutional Committee for Care and Use of Experimental Animals. These protocols adhere to the U.S. Public Health Service (PHS) guidelines for animal research. No animals were harmed during the collection of blood and fecal samples.

Parasites. *Giardia lamblia* WB (ATCC 50803) was cultured in TYI-S-33 medium. Clones expressing VSP1267 were obtained by limiting dilution in 96-well culture plates and selected by immunofluorescence with a specific monoclonal antibody (MAb), 5C1. Reactive clones were then expanded overnight in culture medium and tested for homogeneity before use.

Vaccine preparation. The generation of transgenic trophozoites expressing the whole repertoire of VSPs has been previously reported (15). Briefly, the complementary sequences of the gene encoding *Giardia* Dicer were cloned into plasmid pTubHA.pac. Antisense fragments were amplified by PCR from genomic DNA (gDNA) of clone WB/9B10 and then cloned into the vector. *Giardia* was transfected by electroporation, and transgenic trophozoites were selected with puromycin. Silencing of Dicer was verified by quantitative reverse transcription-PCR (qRT-PCR), and expression of multiple VSPs was determined by immunofluorescence assays using a panel of MAbs directed to different VSPs (5, 15). The entire repertoire of VSPs expressed in transgenic trophozoites was then purified by immuno-affinity using MAb 12F1 generated against the conserved 5-amino-acid tail of VSPs (5, 15). Animals were immunized by three oral administrations of 100 μg of VSPs every 3 days (5). The animals from the day 30 group received a booster dose 2 days before sacrifice.

Gerbils. Specific-pathogen-free, 6-week-old outbred gerbil (*Meriones unguiculatus*) males and females (Animal Facility, Catholic University of Cordoba) were used; they were housed in air-conditioned (18 to 22°C, 40 to 50% humidity) racks with a 12-h light-dark cycle. Gerbils were fed *ad libitum* with autoclaved food and sterile water supplemented with a mixture of filter-sterilized vitamin solution. Before infection, gerbils were tested for the presence of serum antibodies against *Giardia* antigens by enzyme-linked immunosorbent assay (ELISA) and for *Giardia* cysts in stool samples by light and immunofluorescence microscopy using cyst-specific MAb 7D6. In both infection and immunization experiments, four animals per group were used, and three independent assays were performed.

Infections. Gerbil infection was induced by orogastric inoculation of 2×10^5 trophozoites resuspended in 0.5 ml of 1× phosphate-buffered saline (PBS) (5). Control gerbils received 0.5 ml of PBS by the same route. Feces were collected weekly from week 0 to week 4. Cysts were identified visually by light microscopy and immunofluorescence assays with MAb 7D6. *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 250 × *g* for 10 min. After three washes, the pellet was suspended in 2 ml of PBS, and cysts were stained and counted in a hemacytometer (5). Gerbils were considered uninfected if no cysts were found in the feces.

Trophozoite recovery. At the indicated times postinfection (Fig. 1A), the gerbils were euthanized, and the first portion of small intestine (10 cm) was removed and placed in TSY-S-33 medium on ice. The intestine pieces were minced after 30 min on ice, and parasites were counted with a hemacytometer. The percentage of trophozoites expressing VSP1267 was determined by immunofluorescence assays using the specific MAb 5C1.

Blood samples. Blood samples from gerbils were collected weekly to detect the presence of circulating gerbil antibodies to *Giardia* antigens.

Fecal extracts. Fecal samples were obtained from each animal on the same day of blood sample collection. Samples of feces (0.1 g) were placed in polystyrene microtubes containing 0.2 ml of PBS, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail tablets (Complete, EDTA-free; Roche Diagnostics). After incubation at 4°C for 1 h, the tubes were centrifuged (10,000 × *g*, 4°C, 10 min) to remove insoluble materials. The supernatant (fecal extract) was collected and stored at −70°C until use (46).

Total protein extraction from *G. lamblia* trophozoites. Trophozoites were washed twice with PBS and resuspended in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (Complete, EDTA-free; Roche Diagnostics). After incubation at 4°C for 1 h, samples were centrifuged at 10,000 × *g* at 4°C for 10 min, and the supernatant was collected. Total protein concentration was determined by the bicinchoninic assay (BCA) method (Pierce BCA protein assay kit; Thermo Fisher Scientific).

Immunoenzymatic assay (ELISA). An ELISA was performed to determine the levels of IgG and IgA antibodies in serum and fecal samples, respectively, from infected and immunized gerbils. Mouse anti-gerbil IgG and IgA antibodies were developed in our laboratory (see below). Microtiter plates (96-well; Greiner Bio-One, Germany) were coated with *G. lamblia* WB1267 soluble extract (5 μg/ml) in carbonate buffer (50 mM, pH 9) and incubated overnight at 4°C. Plates were washed twice with PBS and blocked with PBS plus 10% bovine serum albumin (PBS-BSA) at room temperature (RT) for 1 h. Serum samples were diluted 1:2 in PBS-BSA and incubated at RT for 1 h. Positive- and negative-control sera were included in each plate for all assays. After plates were washed, the anti-gerbil IgG and anti-gerbil IgA polyclonal antibodies were added (1:500 in PBS–1% BSA) and incubated at RT for 1 h. Finally, an alkaline phosphatase-labeled secondary antibody was used (1:2,000 in PBS–1% BSA). After 1 h at RT, plates were washed, and the reaction product was revealed with 1 mg/ml *p*-nitrophenyl phosphate (Sigma Chemical Co., USA) diluted in glycine buffer (0.1 M, pH 10.4). The reaction was stopped with 50 μl of 3 M NaOH. Optical density (OD) was measured using a microplate reader at 405 nm (Multiskan; Thermo Scientific). For determination of anti-VSP1267 and anti-VSP9B10 humoral responses, the ELISA plates were coated with the corresponding immunopurified proteins using the specific MAbs 5C1 to VSP1267 and anti-9B10 to VSP9B10.

Production of mouse anti-gerbil IgG and IgA polyclonal antibodies. Mouse anti-gerbil IgG and IgA antibodies were developed by immunization of BALB/c mice with gamma and alpha heavy chains, respectively. First, total Igs were purified from normal gerbil serum by affinity chromatography using a thiophilic adsorption kit (Pierce). After SDS-PAGE, the two specific heavy chains were successfully traced using commercial antibodies, anti-human alpha chain and gamma chain (Sigma-Aldrich) (which cross-reacted with gerbil Igs), and purified from the polyacrylamide gel. The immunization protocol consisted of four weekly doses with 50 μg of gerbil alpha or gamma chain; the former dose was emulsified with complete Freud adjuvant, and the latter was emulsified with incomplete Freud adjuvant. Ten days after the last immunization, mice were bled, and the polyclonal serum was stored for later use.

RNA extraction and cDNA synthesis. Total RNA was extracted from gerbil spleens (SPL), Peyer's patches (PPs), and mesenteric lymph nodes (MLNs) using TRIzol reagent (Thermo Fisher Scientific), according to manufacturer's instructions. After DNase I (Roche) treatment (2 h at 37°C), total RNA was spectrophotometrically quantified, and several PCRs were performed to check for the presence of genomic DNA. When no DNA was detected, cDNA synthesis was performed using Superscript IV reverse transcriptase (Invitrogen), according to the manufacturer's instructions (20-μl final volume), and stored at −70°C for subsequent quantitative PCRs (qPCRs).

Real-time RT-PCR. cDNA (8 μl) was used in a final volume of 10 μl; a triplicate for each gene was performed. Each PCR cycle consisted of an initial incubation at 95°C for 15 min, a denaturation step (94°C, 1 min), an annealing step (58°C, 30 s), and an elongation step (72°C, 30 s). There were a total of 40 cycles and a final incubation at 72°C for 10 min (additional extension step). The melting curve was performed

TABLE 1 Oligonucleotide sequences used in qPCR analysis

Primer target	Direction[a]	Primer sequence	Product size (bp)	Reference
IL-5	Fw	5′-ATTCTAACTCTCGCCTGGGTCTGG-3′	315	47
	Rv	5′-GAACTGCCGTGCTCTCCGTCTC-3′		
IL-4	Fw	5′-CAGGGTGCTCCGCAAATTT-3′	67	48
	Rv	5′-GACCCCGGAGTTGTTCTTCA-3′		
IL-6	Fw	5′-AGGATCCAGGTCAAATAGTCTTTCC-3′	77	48
	Rv	5′-TTCCGTCTGTGACTCCAGTTTCT-3′		
GAPDH	Fw	5′-CATGGCCTTCCGAGTTCCT-3′	60	47
	Rv	5′-TTCTGCAGTCGGCATGTCA-3′		
IL-17	Fw	5′-AGCTCCAGAGGCCCTCGGAC-3′	236	49
	Rv	5′-AGGACCAGGATCTCTTGCTG-3′		
TNF-α	Fw	5′-GCTCCCCCAGAAGTCGGCG-3′	254	49
	Rv	5′-CTTGGTGGTTGGGTACGACA-3′		
IL-1β	Fw	5′-GGCAGGTGGTATCGATVATC-3′	494	49
	Rv	5′-CACCTTGGATTTGACTTCTA-3′		
IL-10	Fw	5′-CATGGGTCTTGGGAAGAGAA-3′	196	50
	Rv	5′-CCATTCCCAAAGGAATTGAA-3′		
TGFβ	Fw	5′-GCTACCACGCCAACTTCTGT-3′	197	50
	Rv	5′-GTTGGACAACTGCTCCACCT-3′		
IFNγ	Fw	5′-CCATGAACGCTACACACTGCATC-3′	224	50
	Rv	5′-GAAGTAGAAAGAGACAATCTGG-3′		

[a]Fw, forward; Rv, reverse.

from 50°C to 90°C, with readings taken every 1°C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene. All data were normalized to the corresponding reference gene transcription level using the comparative ΔC_T (where C_T is threshold cycle) method and analyzed using REST 2009 software (Relative Expression Software Tool, version 2.0.13; Qiagen). The primers used in the reaction mixtures are described in Table 1 (47–50).

Histological studies. At the indicated times postinfection or postimmunization (Fig. 2), gerbils were euthanized, and the MLNs and the first portion of the small intestine (10 cm) were removed and fixed by immersion in 4% paraformaldehyde. Fixed tissues were dehydrated, embedded in paraffin, cut, and stained with hematoxylin-eosin for morphological analysis.

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