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α 1-giardin based live heterologous vaccine protects against *Giardia lamblia* infection in a murine model

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Abstract

Giardia lamblia is a leading protozoan cause of diarrheal disease worldwide, yet preventive medical strategies are not available. A crude veterinary vaccine has been licensed for cats and dogs, but no defined human vaccine is available. We tested the vaccine potential of three conserved antigens previously identified in human and murine giardiasis, α 1-giardin, α -enolase, and ornithine carbamoyl transferase, in a murine model of *G. lamblia* infection. Live recombinant attenuated *Salmonella enterica* Serovar Typhimurium vaccine strains were constructed that stably expressed each antigen, maintained colonization capacity, and sustained total attenuation in the host. Oral administration of the vaccine strains induced antigen-specific serum IgG, particularly IgG_{2A}, and mucosal IgA for α 1-giardin and α -enolase, but not for ornithine carbamoyl transferase. Immunization with the α 1-giardin vaccine induced significant protection against subsequent *G. lamblia* challenge, which was further enhanced by boosting with cholera toxin or sublingual α 1-giardin administration. The α -enolase vaccine afforded no protection. Analysis of α 1-giardin from divergent assemblage A and B isolates of *G. lamblia* revealed >97% amino acid sequence conservation and immunological cross-reactivity, further supporting the potential utility of this antigen in vaccine development. Together, these results indicate that α 1-giardin is a suitable candidate antigen for a vaccine against giardiasis.

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Conflicts of interest

The authors declare no conflicts of interest.

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Keywords

Giardiasis; live oral vaccines; animal models

1. Introduction

Giardia lamblia is one of the most common protozoan pathogens of the human intestine and a leading cause of diarrheal disease worldwide [1]. *Giardia* infections are also frequent in domestic cats, dogs, and ruminants, and zoonotic transmission has been proven [2]. *Giardia* is highly contagious, since ingestion of as few as ten cysts can cause infection [3], making the pathogen a threat to the safety of public water supplies. *Giardia* exists in two forms, the infectious cyst, which is resistant to many common disinfectants, and the trophozoite, which colonizes the small intestinal lumen and causes disease. Although the clinical symptoms of giardiasis, including diarrhea, abdominal pain, malabsorption and weight loss, can be severe, the infection is usually not accompanied by significant mucosal inflammation and is normally self-limiting in non-endemic regions [4,5]. Chronic infections occur in endemic regions, which may be related to re-infection with different strains, but the exact reasons are poorly understood.

Despite the clinical importance of *G. lamblia*, no preventive medical strategies for humans have been developed to date. A crude veterinary *Giardia* vaccine (GiardiaVax®), composed of total lysate of *G. lamblia* trophozoites, attenuates giardiasis symptoms and prevents cyst shedding in cats and dogs [6] but not in calves [7]. Immune responses to cysts antigens develop during the course of infection [8], and several live and DNA vaccines against the encystation-specific antigen, cyst wall protein 2, reduce cyst shedding and transmission of *Giardia* [9–11], suggesting that it is possible to protect entire animal populations at risk of infection (herd immunity). These transmission-blocking vaccines did not reduce trophozoite numbers in the small intestine, which limits their potential clinical utility, as vaccines that only protect against cysts but not trophozoites may not prevent escape of very small number of cysts, which could allow development of full-blown disease in the host, given the extremely high infectivity of the parasite [3].

Key to the development of defined vaccines against *Giardia* is the molecular identification of candidate antigens and evaluation of their protective efficacy. A number of studies over the last twenty-five years have reported on *Giardia* antigens recognized by antibodies from infected patients or experimentally infected animals. At least twenty antigens ranging from 14–125 kDa have been characterized in crude extracts of trophozoites or cysts [8,12], but only a few of these were identified at the molecular level. Among the best-characterized *Giardia* antigens are members of the family of variable surface proteins (VSPs), which constitute a major fraction of surface proteins in trophozoites and whose exact functions remain poorly understood. Some 200 different VSP genes are encoded in the *G. lamblia* genome, of which only one is normally expressed per trophozoite [13]. In a population of trophozoites, different VSPs are expressed simultaneously, and switches in the expression of specific VSPs occur rapidly during the course of *Giardia* infection [14,15]. It may be possible to develop pharmacological or genetic interventions that allow expression of multiple VSPs on single trophozoites, which could be useful for a vaccine based on

attenuated *Giardia* [16,17]. However, different giardial strains have distinct VSP genetic repertoires [18,19], and no strategies are available to identify defined virulence mutants of the parasite. Thus, the high VSP variability and lack of antigenic conservation and cross-reactivity suggest that VSPs are not promising targets for the development of defined vaccines against *Giardia*.

More recent studies have combined high-resolution two-dimensional electrophoresis and mass spectrometry to identify at least sixteen immunoreactive proteins, including VSPs, α -giardins, arginine deiminase (ADI), ornithine carbamoyl transferase (OCT), and α -enolase, recognized by convalescent sera or breast milk from humans with giardiasis [20,21]. Eleven of twelve antigens identified in a murine giardiasis model [22] were also recognized by antibodies from previously infected humans [20,21]. This extensive overlap suggests that the antigen specificity of the murine and human immune responses to *G. lamblia* are remarkably similar and provides important validation for the use of murine giardiasis models for understanding and preventing the human infection. The conserved antigens identified in human and murine giardiasis are mainly proteins with structural or metabolic functions, and, unlike VSPs, are not associated with antigenic variation, making them potentially useful antigen targets for defined vaccines.

Successful vaccines against *Giardia* and other important enteric pathogens must activate mucosal defenses in the intestinal tract. Numerous live vectors and adjuvant-based approaches have been employed to achieve effective vaccination by mucosal routes, with varying degrees of success [23,24]. Live vaccine vectors rely on antigen synthesis and delivery by attenuated microbes. Because the microbes are selected for their natural ability to interact intimately with the mucosal as well as systemic immune system, they generally induce robust and long-lasting specific immunity [25–27]. Furthermore, a great deal is known about the pathogenicity, physiology, and genetics of *Salmonella* serotypes, and *Salmonella* can be genetically manipulated with ease. For these reasons, *Salmonella*-based vaccine systems are among the most advanced and promising technologies for inducing immunological protection against enteric pathogens.

In this study, we report the construction of new *Salmonella*-based vaccines against giardiasis. Four of the most promising conserved antigen candidates were tested: α 1-giardin, ADI, OCT, and α -enolase, based on their frequency of recognition by different human and murine immune sera [20–22]. Three of these antigens were successfully expressed in attenuated *Salmonella* vectors and examined for their ability to induce specific immune response and protection against *G. lamblia* in a preclinical murine giardiasis model. We show here that vaccination with one of them, α 1-giardin, provides marked protection against *G. lamblia* infection, making it a suitable candidate antigen for a vaccine against giardiasis.

2. Materials and methods

2.1. *Giardia* isolates

The following *G. lamblia* isolates were used in this study: strain WB, clone 6 (WB/C6, ATCC 50803), GS/M-83-H7 (GS/M, ATCC 50581) [28], Portland-1 (ATCC 30888), BR-7 (ATCC PRA-41), UNO/04/87/1 (UNO, ATCC 50184), BRIS/83/HEPU/106 (106) [29], and

BRIS/87/HEPU/713 (713) [30]. The 106 and 713 isolates were kind gifts of Dr. J. Upcroft (Queensland Institute of Medical Research). All other isolates were obtained from the American Type Culture Collection. Isolates were grown axenically at 37°C in trophozoite growth medium (Diamond TYI-S-33 medium, pH 7.1, containing 10% adult bovine serum and 0.5 mg/ml bovine bile).

2.2. Bacterial vaccine strains

S. enterica serovar Typhimurium strain χ^{9373} [*pmi*-2426 (*gmd-fcl*)-26 $P_{fur81}::TT\ araC$ $P_{BAD.fur}$ $P_{crp527}::TT\ araC$ $P_{BAD.crp}$ *asdA21::TT\ araC* $P_{BAD.c2}$ *araE25* *araBAD23* *relA198::araC* $P_{BAD.lacI}$ TT] was used for antigen expression and delivery [31].

Attenuation of this strain is accomplished in the absence of mannose and arabinose by incomplete LPS synthesis (*pmi*) and lack of ferric uptake regulator (*fur*) and cAMP receptor protein (*crp*). Furthermore, deletion of the *asd* gene causes bacterial lysis in the absence of exogenous diaminopimelic acid, a nutrient essential for cell wall synthesis but not available in the mammalian host. This mutation is also used to establish a balanced-lethal vector-host system by use of expression vectors with the wild-type *asd* gene as the only selective marker. For cloning purposes, *E. coli* χ^{6212} [$F^- \lambda^- \Phi 80$ (*lacZYA-argF*) *endA1* *recA1* *hsdR17* *deoR* *thi-1* *glnV44* *gyrA96* *relA1* *asdA4*] carrying pYA232 plasmid was used as an intermediate host [32]. This *E. coli* DH5-derived strain is negative for *asd* and allows expression of constructs under the control of the P_{trc} and related promoters. Bacterial strains were grown aerobically at 37°C on LB agar or in LB broth supplemented with 0.1% glucose and 0.1% mannose (LBGM), with or without tetracycline (15 µg/ml) or diaminopimelic acid (50 µg/ml) as appropriate.

2.3. Construction and stability testing of expression plasmids

Plasmid pYA3342 was used for cloning and cytoplasmic expression of selected antigens [33]. This plasmid expresses the *asd* gene, which can complement the deleted gene in the vaccine strain, thus constituting a balanced-lethal host/vector system that allows stable plasmid maintenance in the mammalian host without antibiotic selection. Full-length genes of α 1-giardin, α -enolase, OCT, and ADI were amplified by PCR from genomic DNA of *G. lamblia* WB/C6, using the following primers (restriction sites used for cloning are underlined): α 1-giardin, 5'-CAT GCC ATG GGC CCG AAG GTC ACC GAC ATT GCG-3' (sense), 5'-CTG AGG ATC CCT ACT TCA CGC GCC AGA GGG TG-3' (anti-sense); ADI, 5'-CAT GCC ATG GGC ACT GAC TTC TCC AAG GAT AAA G-3' (sense), 5'-CTG AGG ATC CTC ACT TGA TAT CGA CGC AGA TG-3' (anti-sense); OCT, 5'-CAT GCC ATG GGC CCG TTC AAG CAG ACC CGC CAC-3' (sense), 5'-CTG AGG ATC CTC ACT CCA TCT TGC AGT CAT G-3' (anti-sense); α -enolase, 5'-CAT GCC ATG GAG GCT CCG TCT ACG ATC-3' (sense), 5'-CTG ACT GCA GTC ACT TCC AGG CCT CGA AAC C-3' (anti-sense). The ATG translation start site was provided within the upstream *Nco* I restriction site used for cloning, and glycine was inserted as a second amino acid into the antigen sequences when needed (Fig. 1). PCR products were purified, digested with the respective restriction enzymes, and inserted into pYA3342. The plasmids were transformed by heat shock into competent *E. coli* cells χ^{6212} (pYA232). Recombinant clones were examined by restriction analysis and sequencing of the plasmid insert. Plasmids carrying the correct inserts were isolated and transformed into *S. enterica* serovar

Typhimurium χ^{9373} by electroporation. Controls were transformed with pYA3342 without antigen gene insert ("Vector"). Transformants containing Asd⁺ plasmids were selected on LBGM agar without diaminopimelic acid. Plasmid stability was tested by consecutive subculturing of the *Salmonella* strains (1:1,000 dilution) twice a day in LBGM without arabinose, in the absence or presence of diaminopimelic acid, for 5 days encompassing >70 generations. Afterwards, bacteria were plated onto LBGM, and colonies were tested by PCR for the presence of intact antigen inserts. Vaccine strains were further examined for LPS integrity by polyacrylamide gel electrophoresis of cell wall extracts followed by silver staining [34] and were found to produce intact LPS.

2.4. Antigen expression in vaccines

Antigen synthesis in recombinant *Salmonella* vaccine strains was evaluated by sodium dodecyl sulfate-PAGE and immunoblot analysis using polyclonal mouse antisera generated against recombinant forms of the respective antigens. *Salmonella* in exponential growth phase were lysed in SDS sample buffer (2% SDS, 2 mM β -mercaptoethanol, 4% glycerol, 0.04 M Tris-HCl, pH 6.8, 0.01% w/v Bromophenol blue) by boiling at 95°C for 5 min. Recombinant antigens were produced in *E. coli* and purified by affinity chromatography [22]. Bacterial lysates and purified antigens were size-separated on a 4–20% SDS-PAGE gel and electrotransferred to a PVDF membrane. After overnight blocking at 4°C in PBST containing 5% dried nonfat milk and 1% goat serum, membranes were stained for 1 h at room temperature with optimal dilutions of mouse antisera raised against the recombinant forms of the respective *Giardia* antigens in PBST containing 1% dried milk and 1% goat serum [35], washed with PBST and incubated for 1 h at room temperature with HRP-labeled goat anti-mouse IgG (1:10,000 dilution, Southern Biotech, Birmingham, AL). After washing filters (3 × 30 min in PBST), bound HRP was detected by enhanced chemiluminescence (ECL Plus, Amersham, Piscataway, NJ).

2.5. Mouse immunizations and *Giardia* challenges

Salmonella were grown in LB broth with 0.1% mannose and 0.1% arabinose to late-log phase (OD₆₀₀ of 0.8) and washed twice before immunization. Female BALB/c mice (8-weeks old, The Jackson Laboratory) were fasted for 3 h and given 10⁹ CFU of *Salmonella* vaccine strain or vector control by oral gavage in 200 μ l of a buffer (PBS with 0.01% gelatin). A second immunization was given two weeks later. Buffer alone was used as a control. In some experiments, immune boosting was performed using one of two strategies: 1. Oral co-administration of 10 μ g cholera toxin together with the live vaccine vectors in both immunizations [11]; 2. Sublingual administration of 15 μ g purified recombinant antigen followed immediately by oral gavage of live vaccine vector for the first immunization, and replacement of the second live vector immunization with sublingual administration of 15 μ g antigen [36]. Six weeks after the second immunization and/or boosting, mice were infected with *G. lamblia* GS/M trophozoites by oral gavage (10⁷/mouse in 0.2 ml of TYI-S-33 medium). Infections were initiated with trophozoites, rather than cysts, because protection must be directed against the disease-causing trophozoite form of the parasite, rather than the transient cyst form, to be clinically protective, and trophozoite inoculation leads to more robust infection than cyst inoculation and thus represents a more stringent test of vaccination-induced protection. Furthermore, although mice can be infected

with *G. lamblia* cysts [37], this strategy has rarely been exploited in the published literature, presumably because it is difficult to routinely obtain large enough numbers of *G. lamblia* cysts of sufficient quality and purity.

At different times after infection, the small intestine was removed, opened longitudinally, placed in 10 ml of ice-cold PBS for 10 min, and shaken vigorously to detach trophozoites, which were counted in a hemacytometer under a phase-contrast microscope. To evaluate bacterial colonization of the *Salmonella* vaccine strains, Peyer's patches, mesenteric lymph nodes, and spleen were collected and homogenized. Homogenates were plated onto MacConkey agar supplemented with 1% glucose and 0.1% mannose, and incubated overnight at 37°C. Identity of colonies was confirmed by PCR using primers for the *Salmonella invA* gene [38] and primers for the respective *Giardia* antigen.

2.6. Analysis of vaccine immunogenicity

Blood was obtained by tail vein bleeding, diluted 1:10 with PBS containing 2 mM EDTA, and centrifuged at $2,000 \times g$ for 10 min at 4°C. Supernatants were stored at -80°C. For collection of luminal contents, the small intestine was opened longitudinally, and placed into 2 ml PBS with protease inhibitors (Mini Complete EDTA Free, Roche, Indianapolis, IN) for 10 min at room temperature with gentle agitation. After centrifugation at $8,000 \times g$ for 15 min at 4°C, supernatants were collected and stored at -80°C. Specific antibody levels were determined by ELISA using purified recombinant *Giardia* antigens produced in *E. coli* [22]. Antigens were suspended in 50 mM bicarbonate buffer (pH 9.6) and added to 96-well plates at 100 ng protein/well. After overnight incubation at 4°C, plates were blocked for 2 h with PBS, 5% non-fat dried milk, 0.5% Tween 20, and washed with PBS, 0.5% Tween 20. Plates were incubated for 2.5 h at room temperature with serial dilutions of the serum samples in PBS, 1% BSA, 0.5% Tween 20, washed, and further incubated with HRP-conjugated anti-mouse IgG or IgA antibodies, followed by development with tetramethyl benzidine/hydrogen peroxide. The reactions were stopped with 1.2 M sulphuric acid, and plates were read at 450 nm.

Levels of antibodies against *Salmonella* were assayed by whole-cell ELISA. *Salmonella* strain χ^{9373} was cultured overnight, washed twice in PBS, and added to microtiter plates at 10^7 bacteria/well in 50 μ l. Plates were dried overnight at room temperature, and bacteria were fixed for 5 min with 0.15% glutaraldehyde in 0.15 M phosphate buffer (pH 7.0), followed by a 30 min incubation with 0.15 M glycine in 0.15 M phosphate buffer to block unreacted aldehyde groups. Plates were subsequently handled as described for the *Giardia* antigen ELISA.

2.7. Sequence analysis and cross-reactivity studies of α 1-giardin

Genomic DNA was extracted from diverse *G. lamblia* isolates using lysis buffer (0.1 M NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, 1% SDS, and 100 μ g/ml proteinase K), followed by phenol-chloroform extraction. The genes for α 1-giardin and triose phosphate isomerase (TPI) were amplified by PCR using Phusion polymerase (New England Biolabs, Ipswich, MA) and the following primers: α 1-giardin, 5'-CAT GCC ATG GGC CCG AAG GTC ACC GAC ATT GCG-3' (sense) and 5'-CTG AGG ATC CCT ACT TCA CGC GCC AGA GGG

TG-3' (anti-sense); TPI, 5'-ATC GGY GGT AAY TTY AAR YGA-3' (sense) and 5'-CAC TGG CCA AGY TTY TCR CA-3' (anti-sense) [39]. Amplification was conducted for 30 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 2 min, followed by final extension at 72°C for 10 min. PCR products were separated by agarose electrophoresis, gel-purified (Zymoclean Gel DNA Recovery Kit, Zymo Research, Orange, CA), and sequenced from both directions. Sequences were aligned using the ClustalW2 multiple sequence alignment program [40]. Sequences of α 1-giardin are available under the following GenBank accession numbers: AY781324.1 (WB/C6), HQ171976 (106), HQ171977 (713), HQ171980 (Portland-1), HQ171979 (BR-7), HQ171978 (GS/M), and HQ171981 (UNO).

To test immunological cross-reactivity of α 1-giardins, cell lysates were prepared from different *G. lamblia* isolates by lysing log-phase grown cells in 0.1 M NaCl, 50 mM Tris (pH 7.2), 1% NP-40, and protease inhibitors (Mini Complete EDTA Free). Samples (30 μ g/lane) were separated by SDS-PAGE and subjected to immunoblot analysis as described above.

2.8. Data analysis

Trophozoite counts were $\log_{10}$ transformed, and means and SEM were calculated from the log values. Samples without detectable trophozoites were assigned a log value equivalent to half of the detection limit of the assay (10^4 trophozoites/intestine). For ELISA, means and SEM were calculated from the data of individual wells. Differences between groups were compared by Mann-Whitney rank sum test or t-test as appropriate, with $p < 0.05$ considered as significant and $p < 0.01$ as highly significant.

3. Results

3.1. Construction and *in vitro* characterization of recombinant vaccines against *Giardia*

Eleven of the twelve *Giardia* antigens previously identified in murine studies were conserved across diverse *Giardia* isolates and were also recognized by humans [20–22]. Therefore, we considered them good candidates for broadly active vaccines against giardiasis. To begin to test their utility as vaccination targets, we selected four antigens for vaccine development, α 1-giardin, α -enolase, OCT, and ADI, since they were the most frequently detected antigens in several human and murine studies [20–22]. As vaccine strategy, we employed attenuated live *Salmonella* vectors, because they can be given by the preferred oral vaccination route, are highly immunogenic, and are safe to administer [25,26]. These vaccine vectors employ a strategy of regulated delayed attenuation such that they are fully virulent and invasive at the time of oral inoculation, thereby allowing strong engagement of the mucosal immune system, but become progressively attenuated due to exhaustion of arabinose, which is needed for maintaining the virulent state, but is not available in the murine host [41]. At the same time, arabinose exhaustion leads to induction of bacterial expression of the vaccine antigen inside the host.

Full-length genes encoding each of the target antigens were obtained by PCR from the genome strain, *G. lamblia* WB/C6, and inserted into the *Salmonella* expression plasmid, pYA3342, which carries an *asd* marker but no antibiotic resistance gene (Fig. 1). The plasmid was introduced into *S. enterica* serovar Typhimurium vaccine strain χ^{9373} , which

has multiple genomic deletions that attenuate virulence and lacks its endogenous *asd* gene [31]. Trans-complementation of the *asd* gene by the plasmid allows bacteria to grow without diaminopimelic acid, an essential nutrient not available in the mammalian host. The genes encoding three of the antigens, α 1-giardin, α -enolase, and OCT, were separately introduced into *Salmonella*. Stability testing showed that all three plasmids were maintained with intact inserts in the attenuated *Salmonella* strain χ^{9373} for at least five days of continuous culture encompassing >70 generations in the presence and absence of diaminopimelic acid. Of note, these tests were done without arabinose, which suppresses antigen expression in this system [42], so we could evaluate the impact of antigen on bacterial growth under conditions of maximal antigen expression. In contrast, we could not obtain stable ADI-carrying *Salmonella* clones in our experiments, since stability testing of the initial transfectants invariably showed loss of the ADI gene insert after several days in culture. These observations suggested that full-length ADI may be toxic to *Salmonella*, which precluded further analysis of this antigen. Antigen expression is tightly regulated by arabinose in the *Salmonella* vectors, so it is possible that continued bacterial growth with arabinose to suppress antigen production may allow maintenance of an intact ADI-carrying plasmid.

Expression from all three stable vaccine vectors led to synthesis of the respective antigens in immunoreactive forms, as shown by immunoblot analysis of extracts of each of the three *Salmonella* χ^{9373} strains with polyclonal mouse antibodies generated against the respective recombinant antigen (Fig. 2). Moreover, expression levels were equivalent (within a <2-fold range) between the three antigens, as determined by comparison with known amounts of the recombinant forms (Fig. 2). Together, these data show that the new recombinant *Salmonella* vaccine vectors were stable *in vitro* and expressed α 1-giardin, α -enolase, and OCT in immunoreactive forms.

3.2. Immunogenicity of recombinant vaccines

We next tested the ability of the three new vaccine vectors to induce antigen-specific immune responses in mice. Murine models of *G. lamblia* infection had been used successfully for prior immunological and vaccine studies [22,43,44]. Adult BALB/c mice were given 10^9 CFU of each vaccine twice by oral gavage two weeks apart, and were examined after six weeks by ELISA for development of IgG antibodies against the immunizing *Giardia* antigen. The vaccine strains expressing α 1-giardin and α -enolase elicited high levels of specific IgG in the serum (Fig. 3A), indicating that these two vaccines were strongly immunogenic *in vivo*. In contrast, the OCT-expressing vaccine did not induce specific IgG antibodies (Fig. 3A). All three vaccine strains stimulated strong antibody responses to *Salmonella* antigens, which were similar to the specific antibody levels observed after administration of a control vector without antigen insert (Fig. 3A). These data suggest that the failure to induce OCT-specific IgG was not related to the lack of immune engagement of the OCT-expressing *Salmonella* vaccine strain.

Based on their immunogenicity, we subsequently focused on α 1-giardin and α -enolase. Some studies have indicated that secretory IgA is required in host defense against *Giardia* [45], while other reports have suggested that IgA is not important for clearance of *Giardia* [43]. To determine whether secretory IgA is induced upon immunization, we assayed the

effect of oral vaccine administration on antigen-specific IgA production in mucosal secretions. Mice were again immunized orally twice two weeks apart, and specific IgA levels in intestinal luminal washes were tested after six weeks by ELISA. Both α 1-giardin and α -enolase vaccines induced modest levels of specific secretory IgA (Fig. 3B). Furthermore, for one of the immunogenic vaccines, α 1-giardin, we determined the levels of antigen-specific IgG subtypes in the serum as indirect indicators of the type of T cell responses stimulated by vaccination. We found significantly increased levels of α 1-giardin specific IgG_{2A}, but not IgG₁ (Fig. 3C), suggesting activation of T cell responses related to IFN- γ but not IL-4 by the α 1-giardin vaccine. Thus, oral administration of attenuated recombinant *Salmonella* vaccine strains as a delivery vehicle for *Giardia* antigens elicited systemic IgG against α -enolase, and IgG_{2A} against α 1-giardin, and mucosal IgA responses to both antigens.

3.3. Stability and attenuation of *Salmonella* vaccine strains *in vivo*

A successful live vaccine needs to be stable *in vivo* to allow effective initial colonization, continued antigen production, and immune stimulation, and must remain fully attenuated to be safe. To evaluate colonization and stability of the new vaccine strains, we infected BALB/c mice with a single oral dose of the *Salmonella* strains carrying α 1-giardin, α -enolase, or no antigen-encoding insert (Vector). After seven days, we determined colonization of Peyer's patches, mesenteric lymph nodes, and spleen by CFU assay. Bacterial numbers in Peyer's patches and mesenteric lymph nodes were not significantly different for the two antigen-expressing and the empty vector strains (Fig. 4). Furthermore, splenic CFU were similar for the α -enolase expressing and the empty vector strains, but no colonization of the spleen could be detected for the α 1-giardin expressing *Salmonella* strain (Fig. 4). Analysis of the colonies in the CFU assays by PCR for the antigen inserts revealed that 100% (10/10) of tested *Salmonella* colonies from mice given the α 1-giardin vector carried the full-length gene. For the α -enolase vector, 90% (9/10) had maintained the full-length gene. Together, these results show that all the recombinant *Salmonella* vaccine vectors were fully capable of colonizing the intestinal mucosa initially, which either reflects the minimal impact of antigen synthesis on bacterial fitness, or the delayed antigen expression phenotype of the vectors, requiring several cell divisions for exhaustion of arabinose and maximal expression [42]. On the other hand, maximal bacterial synthesis of α 1-giardin appears to interfere with systemic survival of the vaccine bacteria.

To examine the attenuation of the vaccine strains, mice were given two oral doses of 10^9 CFU two weeks apart, and bacterial CFU were enumerated six weeks later in homogenates of Peyer's patches, mesenteric lymph nodes, and spleen. No *Salmonella* were recovered from any of the organs for either of the two antigen-expressing or the empty vector strains, indicating that the vaccines had been cleared from the host by six weeks. Thus, antigen expression did not alter attenuation of the recombinant *Salmonella* strains.

3.4. Protective capacity of α 1-giardin expressing vaccine

To test whether the two immunogenic vaccines induced immune protection against infection, mice were immunized orally twice two weeks apart and were challenged orally six weeks later with *G. lamblia* GS/M trophozoites. Four days after infection, trophozoite

numbers in the small intestine were determined. Administration of the α 1-giardin vaccine conferred significant protection, with a >80% reduction in trophozoite load relative to buffer and vector controls (Fig. 5A). In contrast, the α -enolase vaccine had no significant protective effect under the same conditions (Fig. 5A).

To explore whether the protective capacity of the α 1-giardin vaccine could be further improved, we employed two boosting strategies. In one approach, we co-administered the common mucosal adjuvant, cholera toxin, together with the live vaccine in each of the two immunizations. In the other approach, we gave a modest amount (15 μ g) of recombinant purified α 1-giardin by the sublingual route followed immediately by oral gavage of the live vaccine vector, and replaced the second gavage of the live α 1-giardin vaccine vector with sublingual α 1-giardin administration [36]. Both approaches led to enhanced protection against subsequent oral *G. lamblia* GS/M challenge, with a significant >5-fold reduction in trophozoite load relative to mice given the α 1-giardin vaccine alone, leading to an overall decrease of >95% (~2 log) in trophozoite load relative to buffer or vector controls (Fig. 5B). Furthermore, a time course analysis demonstrated that the protective effects of the boosting strategies were sustained throughout the acute infection challenge (Fig. 5C). Cholera toxin given alone with the live control vector did not elicit protection against subsequent *G. lamblia* challenge (Fig. 5C). Taken together, these results show that expression of the *Giardia* antigen, α 1-giardin, in a recombinant *Salmonella* vaccine strain provides marked protection against giardiasis, which is further enhanced by different immune boosting strategies.

3.5. Phylogenetic conservation and immunological cross-reactivity of α 1-giardin

Because the α 1-giardin vaccine induced protection against *Giardia*, it is a good candidate for further vaccine development. For a vaccine to be broadly active against diverse *G. lamblia* strains, the target antigen should be highly conserved. We therefore evaluated sequence conservation of α 1-giardin among diverse isolates of *G. lamblia*. Seven different isolates were used, representing geographic diversity in origin and both major genetic assemblages, A and B, that include all known *G. lamblia* isolates with disease-causing potential in humans. The genes for α 1-giardin and TPI, a commonly used standard for comparing diverse *Giardia* isolates [46], were amplified from genomic DNA by PCR, and the products were directly sequenced. Analysis of α 1-giardin sequence revealed >89% identity at the nucleotide level and >97% at the amino acid level for all seven *Giardia* isolates (Table 1). Stretches of up to 80 amino acids (of a total protein length of 295 amino acids) were 100% identical in all isolates. Sequence conservation of α 1-giardin was higher than that of TPI (average amino acid identity of 99.1% for α 1-giardin vs. 95.3% for TPI).

Beyond sequence conservation, a useful antigen should exhibit epitope conservation in the form of immunological cross-reactivity between different *Giardia* isolates. To explore antigen cross-reactivity, we examined cell extracts from different *Giardia* isolates by immunoblotting with sera from mice immunized with the α 1-giardin expressing *Salmonella* vaccine strain. The immune sera recognized native α 1-giardin in all five *G. lamblia* isolates we tested, whereas staining of the immunoblots with sera from mice given empty vector *Salmonella* strains showed multiple bands against *Salmonella* but no *Giardia*-specific bands

(Fig. 6). Detection of α 1-giardin was stronger in *G. lamblia* isolates belonging to Assemblage A compared to Assemblage B. For example, densitometric analysis of immunoblots revealed that the α 1-giardin band of the Assemblage A isolate, WB/C6, was ~16-fold more intense than that of the Assemblage B isolate, GS/M. This may be related to the fact that the α 1-giardin gene in the vaccine strain was obtained from WB/C6. However, despite the apparently lower levels of antibodies against α 1-giardin of *G. lamblia* GS/M, immunization provided marked protection against this *G. lamblia* strain (Fig. 5), indicating either that lower antibody levels are sufficient for immune protection or that protection does not depend on induction of specific antibodies. Taken together, these results demonstrate that α 1-giardin is structurally conserved and immunologically cross-reactive between diverse *G. lamblia* isolates, strongly supporting the utility of α 1-giardin as a conserved antigen for an effective vaccine against giardiasis.

4. Discussion

Vaccinations against debilitating and deadly infectious diseases are among the greatest achievements of modern medicine. Successful vaccines are often attenuated forms of the virulent pathogens, because they closely mimic the critical interactions with the host immune system, yet do not cause overt disease. Attenuation depends on isolation of spontaneous mutants of the respective pathogen, or the ability to engineer targeted mutations by genetic means. Neither of these strategies has been applied to the protozoan parasite, *Giardia* (or to most other parasites). Thus, mutant *Giardia* that colonize the host and engage the immune system, but can not cause diarrheal disease, have not been reported, partly because suitable animal models of giardiasis-associated diarrhea are lacking and the underlying pathophysiology is incompletely understood [47]. Because it is doubtful that an attenuated form of *Giardia* can be developed as an effective vaccine, it is necessary to employ critical *Giardia* antigens in cell-free or heterologous live defined vaccine systems, analogous to what has been proposed for other enteric protozoan pathogens [48]. The current study shows that this strategy can be successful against giardiasis, since expression of a particular conserved antigen, α 1-giardin, in recombinant attenuated *Salmonella* conferred protection of the host against subsequent *Giardia* challenge. Although protection has so far not been absolute in a murine model, the results are encouraging, as they, together with those with variable *Giardia* surface antigens [17] and induction of herd immunity [9–11], indicate that effective defined vaccine strategies against giardiasis are feasible.

An important issue in vaccine design is the question of how much protection is needed to prevent clinical symptoms after infectious challenge. In the present study, we obtained up to 100-fold reduction in *Giardia* numbers after immunization and boosting, leaving <1% of parasite burden in the host at peak infection compared to naïve animals. Although numerically impressive, the murine model system of giardiasis can not be used to determine whether this reduction would be sufficient to attenuate symptomatic disease. Mice are generally powerful models for immunological studies of many microbial infections, as also demonstrated by the almost complete overlap in conserved *Giardia* antigens detected in murine and human infections [22]. Yet, overt clinical symptoms do not occur after *Giardia* infection of mice, making them unsuitable for establishing dose/response relationships for parasite numbers and disease symptoms. Unfortunately, other animal models, particularly

gerbils, have their own shortcomings, including the lack of molecular and immunological tools, and well-developed live vaccine systems. Thus, it will ultimately be necessary to conduct human studies to determine the exact relationship between reduced infectious burden and attenuated clinical symptoms in immunized individuals [3].

The protective antigen, α 1-giardin, is a member of the α -giardin protein family, which consists of 21 members in *G. lamblia* [49]. These proteins display distant homology to vertebrate annexins, which are characterized by their calcium- and phospholipid-binding properties, but have no immediate mammalian analogs [49]. Giardins were originally identified as structural proteins in the attachment disc of the parasite [50], but the different family members were later found to have more diverse localizations [49]. At least two α -giardins, α 1 and α 2, as well as δ -giardin, are localized partly at the trophozoite plasma membrane, both on the cytoplasmic and extracellular side, and may play a role in parasite attachment to the intestinal epithelium [49,51]. Freshly excysted parasites have especially high levels of surface-exposed α 1-giardin [52]. Blockade of surface-localized α 1-giardin by secretory IgA antibodies, which are induced by immunization, may interfere with epithelial attachment of the parasite and thus favor removal in the bulk flow of the intestinal lumen. Similarly, antibodies against δ -giardin can inhibit parasite attachment in vitro [51], although it is not known whether this antigen confers protection in vivo, precluding direct comparison with our α 1-giardin observations. In any case, the functions of α 1- and other giardins are poorly understood at present, making it difficult to propose a specific mechanism by which immune responses against them can contribute to clearance of the parasite.

Irrespective of the mechanism of α 1-giardin dependent immune protection, the antigen has desirable features for a vaccine candidate. It is one of the most common conserved antigens recognized by humans with giardiasis, but has no homolog in mammals [20,21]. Antibodies against α 1-giardin develop very early during human infections [20]. It is expressed throughout the giardial life cycle, especially in excysting cells which initiate infection and trophozoites which cause disease [49,52], and is shed in the feces [52]. Importantly, α 1-giardin exhibits marked sequence conservation and immunological cross-reactivity among diverse *Giardia* isolates. Only a single amino acid variation was observed in the previously identified immunogenic C-terminal region (amino acids 160–200) of α 1-giardin [52]. Interestingly, the same region shows epitope similarity with α 7.1-giardin, another conserved and antigenic α -giardin [52], raising the possibility that the protection afforded by immunization with α 1-giardin may be partly mediated by cross-reactivity with α 7.1-giardin.

In contrast to α 1-giardin, immunization with an α -enolase expressing candidate vaccine did not protect against infection. This enzyme catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway and has other, non-glycolytic functions in many microbes [53,54]. It is normally localized in the cytoplasm of *G. lamblia* trophozoites, making it a seemingly unlikely immune target. However, α -enolase is released from trophozoites upon contact with intestinal epithelial cells without evidence of cell death [35], which presumably makes it accessible to host immune recognition. Such a secreted antigen, no longer physically associated with trophozoites, could act as an infection indicator to the host and perhaps as an immune trigger for enhancing intestinal motility or altering other host conditions that indirectly limit the ability of *Giardia* to colonize the host [55]. Although

attractive in principle, the lack of protection upon immunization with the α -enolase vaccine argues against the relevance of this potential defense mechanism. Released α -enolase may also have direct functions in *Giardia* colonization, as it has for other pathogens [56], although immunization against α -enolase either did not disrupt these functions, or their disruption did not affect parasite colonization.

Our live attenuated *Salmonella* α 1-giardin vaccine conferred protection and elicited specific mucosal IgA and systemic IgG_{2A}, but not IgG₁, antibodies. An earlier report had employed an attenuated *Salmonella* vector expressing a particular *Giardia* VSP antigen to show induction of mucosal IgA and serum IgG₁, but not IgG_{2A}, although protection against infection was not assessed [27]. Another study used a different attenuated *Salmonella* vector system expressing *Giardia* cyst wall protein 2 to demonstrate antigen-specific induction of IFN- γ and IL-4 producing T cells and specific serum IgG_{2A}, but not IgG₁, which was associated with protection against cysts shedding [11]. Together, these findings suggest that induction of Th1-type immune responses and systemic IgG_{2A} may be indicative of effective immune protection against *Giardia*.

The *Salmonella*-based vaccine employed here expresses antigen in the bacterial cytoplasm once expression is induced due to exhaustion of arabinose [33]. Although effective for α 1-giardin, these localization and expression characteristics may not be optimal for other *Giardia* antigens. For example, the lack of a specific antibody response to OCT, whose total expression levels in *Salmonella* were similar *in vitro* to those of α 1-giardin and α -enolase, may be related to unfavorable bacterial antigen localization or insufficient immune access *in vivo*, or perhaps a need for higher antigen expression levels. Other antigens may be toxic in the *Salmonella* vaccine vector, as exemplified by ADI, for which we could not obtain stably expressing clones under conditions of maximal antigen production. Modifications of the antigen or different expression strategies may permit reliable antigen production and immunogenicity, and ultimate evaluation of the protective potential of these antigens. Furthermore, the observed increase in protection achieved by immune boosting strategies, such as cholera toxin co-administration or sublingual antigen administration, strongly suggests that the protective capacity of α 1-giardin and perhaps other antigens can be enhanced by improvement of vector design and antigen delivery. Such optimized *Salmonella* vaccine vector design and delivery may also facilitate the construction of multi-subunit vaccines against giardiasis, analogous to what has been achieved for other important human pathogens [57–60]. Finally, it should be noted that even if attenuated *Salmonella*-based vaccines should prove not to be ideal for vaccination against giardiasis, insights into protective antigens gained with these systems will nonetheless be valuable, as they should be applicable to other vaccination strategies.

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Abbreviations

ADI	Arginine deiminase
OCT	Ornithine carbamoyl transferase
VSP	variable surface protein

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HIGHLIGHTS

- *Giardia lamblia* is a leading protozoan cause of diarrheal disease worldwide.
- Preventive medical strategies are not available against giardiasis.
- Immunization of mice with a live $\alpha 1$ -giardin vaccine protected against infection.
- $\alpha 1$ -giardin is highly conserved between divergent *Giardia* isolates.

Fig. 1.

Map of antigen-encoding plasmids. Expression plasmids employ the Asd^+ plasmid pYA3342. Antigen synthesis is controlled by the P_{trc} promoter. Full-length genes of the depicted antigens were inserted into the multiple cloning site (MCS) of the plasmid using the indicated restriction sites. A transcriptional terminator, 5ST1T2, is provided on the plasmid.

Fig. 2.

G. lamblia antigen expression in *S. enterica* serovar Typhimurium strain χ^{9373} . Full-length genes of $\alpha 1$ -giardin ($\alpha 1G$), α -enolase (ENO), and OCT were inserted into the vaccine vector pYA3342, which was transformed into the attenuated χ^{9373} *Salmonella* strain. Vector without insert was used as a control. Stable clones were identified and examined for antigen expression by immunoblotting of total bacterial extracts from 6×10^7 bacteria/lane, using polyclonal mouse antisera generated against recombinant forms of the respective antigens. Known amounts of the indicated GST- or His-tagged recombinant proteins were loaded for comparison. Densitometric estimation of protein amounts indicated that 1.1, 0.9, and 0.7 μg of $\alpha 1$ -giardin, α -enolase, and OCT, respectively, were produced in 6×10^7 bacteria of the respective vaccine strains. The immunoblots for OCT showed a non-specific band (*) that was also present in vector controls.

Fig. 3.

Antibody responses induced by immunization with *Salmonella* vaccine strains. BALB/c mice were orally immunized twice two weeks apart with *Salmonella* vaccine strains synthesizing α1-giardin (α1G), OCT, or α-enolase (ENO), or carrying plasmid without antigen insert (Vector). Buffer without bacteria was administered as a control (Buffer). Antibody levels were assayed six weeks after the second immunization. (A) Specific IgG antibodies against purified recombinant forms of the respective antigens, and against whole *Salmonella*, were determined by ELISA in serum samples (diluted 1:50). Data are mean ± SEM (n 3). *p<0.05 (t-test) relative to “Buffer” controls at the same time point. Anti-*Salmonella* IgG levels were slightly lower in mice vaccinated with the α1-giardin expressing

vector in the shown experiment, but in other experiments such a decrease was not observed. (B) Luminal washes of the small intestine were collected and diluted (1:2), and IgA antibodies against recombinant forms of the antigens were determined by ELISA, and are shown normalized against total IgA levels in the washes (which had means of 39, 71, 43, and 62 $\mu\text{g/ml}$ for vaccination with Buffer, Vector, $\alpha 1$ -giardin, and α -enolase, respectively). Data are mean $\pm$ SEM of 5–6 mice. * $p < 0.05$ (t-test) relative to “Buffer” controls. (C) The indicated IgG subclass antibodies against $\alpha 1$ -giardin were determined by ELISA in serum (diluted 1:90) of $\alpha 1$ -giardin immunized and vector control mice. Data are mean $\pm$ SEM of 4 mice each. * $p < 0.05$ (t-test) relative to “Vector” for each isotype.

Fig. 4.

Initial colonization of murine tissues after vaccine administration. BALB/c mice were given a single oral dose (10^9 CFU) of the *Salmonella* vaccine strains synthesizing α 1-giardin (α 1G) or α -enolase (ENO), or carrying plasmid without antigen gene insert (Vector). After seven days, bacterial numbers were determined by CFU assay in homogenates of Peyer's patches (PP), mesenteric lymph nodes (MLN), and spleen. Each data point represents a single animal, with short horizontal lines showing geometric means of each data set. The long bold horizontal line indicates the detection limit of the assay. Statistical analysis of the data revealed no significant differences for PP and MLN, while CFU of the α 1G vaccine were significantly different ($p < 0.05$ by t-test) from the spleen CFU in the Vector and ENO groups.

Fig. 5.

Protection against *G. lamblia* infection by oral immunization with $\alpha 1$ -giardin expressing *Salmonella* vaccine. BALB/c mice were orally immunized twice two weeks apart with *Salmonella* vaccine strains producing $\alpha 1$ -giardin ($\alpha 1\text{G}$) or α -enolase (ENO), or carrying plasmid without antigen insert (Vector). Buffer without bacteria was administered as a control (Buffer). In addition, in panels B and C, several mouse groups were given one of two booster treatments: Oral co-administration of 10 μg cholera toxin (CT) with the live vaccine vectors in both immunizations (labeled “ $\alpha 1\text{G} + \text{CT}$ ”), or sublingual administration of 15 μg purified recombinant antigen (i.e., $\alpha 1$ -giardin; ag) followed by oral gavage of live vaccine vector for the first immunization and replacement of the second live vector immunization with sublingual administration of 15 μg antigen alone (labeled “ $\alpha 1\text{G} + \text{ag}$ ”). In all groups, six weeks after the first immunization, mice were challenged with 10^7 *G. lamblia* GS/M trophozoites, and trophozoite numbers in the small intestine were determined four days later (A, B) or at the indicated times (C). Values are mean $\pm$ SEM of 10–14 mice (A), 6–8 mice (B), or 3–5 mice (C) in each group. Each panel represents an independent set of experiments conducted at separate times by different investigators. * $p < 0.05$ (Mann-Whitney test) relative to “Buffer” controls at the same time after infection. # $p < 0.05$ relative to “ $\alpha 1\text{G}$ ” alone in panel B. The dashed line in panel C represents the detection limit of the assay.

Fig. 6.

Immunological cross-reactivity of $\alpha 1$ -giardins of diverse *G. lamblia* isolates. Cell lysates from the indicated *G. lamblia* isolates, and from *Salmonella* vaccine strains as controls, were examined by immunoblotting with sera from mice immunized with the *Salmonella* vaccine strain synthesizing $\alpha 1$ -giardin ($\alpha 1G$) or carrying plasmid without antigen gene insert (Vector). Two different exposure times (t_{expos}) were used to reveal different band intensities on the blots. The asterisk denotes a non-specific band also found in the Vector controls.

Table 1

Sequence conservation of α 1-giardin

Assemblage	α 1-giardin									
	<i>G. lamblia</i> isolates	% nucleotide identity	Amino acids (position)						TPI	
			81	83	93	130	174		% nucleotide identity	
A	WB/C6	100	V	L	V	V	A		100	
	106	100	V	L	V	V	A		99	
	713	100	V	L	V	V	A		99	
	Portland-1	100	V	L	V	V	A		100	
	BR-7	99	V	L	A	V	A		99	
B	GS/M	90	I	L	A	A	A		79	
	UNO	89	I	F	A	A	V		79	

The genes for α 1-giardin and TPI were amplified by PCR from the DNA of seven genetically and geographically diverse *G. lamblia* isolates, and the resulting products were sequenced. Sequences were aligned and percentage of sequence identity at the nucleotide level was determined relative to the reference strain WB/C6. The α 1-giardin sequences were further compared at the amino acid level, with the amino acid positions shown for which differences were observed relative to the WB/C6 sequence.