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MICROBIAL IMMUNITY AND VACCINES

Identification of Conserved Candidate Vaccine Antigens in the Surface Proteome of *Giardia lamblia*

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ABSTRACT *Giardia lamblia*, one of the most common protozoal infections of the human intestine, is an important worldwide cause of diarrheal disease, malabsorption, malnutrition, delayed cognitive development in children, and protracted postinfectious syndromes. Despite its medical importance, no human vaccine is available against giardiasis. A crude veterinary vaccine has been developed, and experimental vaccines based on expression of multiple variant-specific surface proteins have been reported, but poorly defined vaccine components and excessive antigen variability are problematic for pharmaceutical vaccine production. To expand the repertoire of antigen candidates for vaccines, we reasoned that surface proteins may provide an enriched source of such antigens since key host effectors, such as secretory IgA, can directly bind to such antigens in the intestinal lumen and interfere with epithelial attachment. Here, we have applied a proteomics approach to identify 23 novel surface antigens of *G. lamblia* that show >90% amino acid sequence identity between the two human-pathogenic genetic assemblages (A and B) of the parasite. Surface localization of a representative subset of these proteins was confirmed by immunostaining. Four selected proteins, uridine phosphorylase-like protein-1, protein 21.1 (GL50803_27925), α1-giardin, and α11-giardin, were subsequently produced in recombinant form and shown to be immunogenic in mice and *G. lamblia*-infected humans and confer protection against *G. lamblia* infection upon intranasal immunization in rodent models of giardiasis. These results demonstrate that identification of conserved surface antigens provides a powerful approach for overcoming a key rate-limiting step in the design and construction of an effective vaccine against giardiasis.

KEYWORDS giardiasis, immunization, surface antigens, vaccines

Giardia lamblia (synonym, *G. intestinalis*) causes one of the most common protozoal infections of the human intestine and is an important cause of diarrheal disease, with hundreds of millions of annual cases worldwide (1). In developing countries, prevalences as high as 40% have been reported (2). In the United States, *G. lamblia* is one of the two most common causes of outbreaks of parasitic disease, with prevalence rates of 1 to 7% (1, 3). Infections are more frequent and severe in young children, particularly in day care centers (3, 4), and among travelers, hikers, and military personnel in the field (5).

The parasite exists in two forms, the infectious cyst, which is resistant to many common disinfectants, and the disease-causing trophozoite form, which colonizes the proximal small intestine (6, 7). Cysts are highly contagious as ingestion of as few as 10 can cause infection (8, 9). Symptomatic giardiasis is characterized by watery diarrhea, epigastric pain, nausea,

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and vomiting, which may lead to malabsorption and malnutrition (10). Persistent infection of children in developing countries can lead to stunting and cognitive impairment (11). While rarely fatal in developed countries, infection can cause protracted postinfectious syndromes (12–14). Furthermore, although treatment with antimicrobials such as metronidazole is often successful, failures can occur in up to 40% of cases (15), and resistance to all major antigiardial drugs has been reported (16, 17).

Giardiasis is self-limiting in >85% of cases in areas of nonendemicity, indicating that effective immune defenses exist. Symptoms of giardiasis are much less severe in regions of endemicity than of nonendemicity, suggesting gradual build-up of immunity (18). Secretory IgA (19, 20), intestinal hypermotility (21, 22), and CD4 T cells (23) are important in the host defense, but other effectors have been proposed (24).

A human vaccine against giardiasis is not available. A crude veterinary vaccine (GiardiaVax), composed of total cell lysates of a mixture of sheep, dog, and human isolates, reduces symptoms and duration of cyst output in cats and dogs (25). Interestingly, the vaccine has also been used as an immunotherapeutic agent in dogs with chronic giardiasis that had failed standard drug treatment (26), raising the intriguing possibility that a *G. lamblia* vaccine may be effective postexposure. However, poorly defined antigen preparations are not desirable in human vaccines as they are difficult to standardize and carry an increased risk of adverse effects. Attenuated *G. lamblia* has never been reported; such forms would probably not establish infection in the host and would therefore fail to activate mucosal immunity.

Multiple *G. lamblia* proteins are recognized by immune sera, but only a few have been identified at the molecular level or proven to be protective (18, 27, 28). Among the best-characterized antigens of *G. lamblia* are variant-specific surface proteins (VSPs) (29), whose functions remain poorly understood (30). Some 200 different VSP genes are present in the *G. lamblia* genome (31, 32), but only one is expressed per trophozoite (33). Switches in VSP expression are common (34), confounding development of VSP-specific immune protection. VSP expression is regulated by RNA interference, and its silencing allows concurrent expression of multiple VSPs in individual trophozoites and has been proposed as a basis for a vaccine (35–37). However, the human-pathogenic assemblage A and B isolates have completely different VSP repertoires with >400 antigens altogether (31, 32, 38), posing significant technical challenges for the pharmaceutical production of a reliable vaccine.

Several conserved, non-VSP antigens have been identified, including giardins, arginine deiminase, and ornithine carbamoyl transferase (39). These proteins, unlike VSPs, do not undergo antigenic variation. Several of these are cytoplasmic but are actively and selectively secreted upon contact with epithelial cells, thus explaining their immunogenicity (40, 41). To expand the repertoire of antigen candidates for vaccine development, we reasoned that surface proteins may provide an enriched source of such antigens since at least one of the key host effectors against the parasite, secretory IgA, can directly bind to such antigens in the intestinal lumen and thereby interfere with normal attachment to the epithelium (19). Here, we report on a proteomics approach to identify novel conserved surface antigens of *G. lamblia* as candidates for vaccine development and demonstrate the utility of the identified protein as candidate vaccine antigens.

RESULTS

Immunological cross-protection between *G. lamblia* strains of the two human-pathogenic genetic assemblages. Successful vaccine antigens should ideally confer protection against different strains of the two genetic assemblages (A and B) of human-pathogenic *G. lamblia*. Because of marked genetic diversity between the assemblages, particularly among the immunodominant VSPs (32, 42), it is not clear how much immunologically relevant overlap exists in the host response to infection with divergent *G. lamblia* strains. To address this question, we utilized a giardiasis model in gerbils, which are highly susceptible to a wide range of *G. lamblia* strains (43), for a controlled cross-protection trial with a primary infection followed by pharmacological clearance and secondary challenge. Gerbils were inoculated with the assemblage A

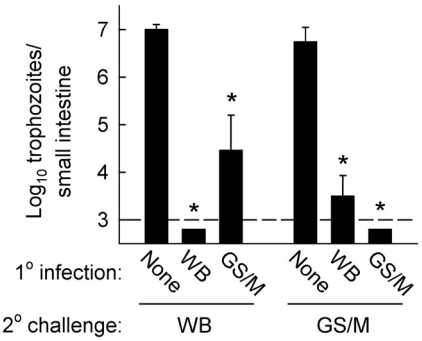

FIG 1 Immunological cross-protection between assemblage A and B strains of *G. lamblia* in gerbil model of giardiasis. Gerbils were orally infected (primary [1°] infection) with *G. lamblia* WB (assemblage A) or *G. lamblia* GS/M (assemblage B) or left uninfected (None). After 4 weeks, all animals were treated with metronidazole (20 mg/kg, twice a day for 3 days) to clear any residual infection. After a 1-week break to allow metronidazole elimination, gerbils were orally rechallenged (secondary [2°] infection) with the indicated *G. lamblia* strains. Ten days later, parasites were enumerated in the small intestine (means + SE, $n = 4$ gerbils/group; *, $P < 0.05$ for results versus those of control gerbils without primary infection, Kruskal-Wallis test with Dunn's *post hoc* test; the dashed line represents the detection limit of the assay).

strain, WB, or the assemblage B strain, GS/M, or left uninfected. After 4 weeks, an adequate time for induction of strong adaptive immune responses (44), all animals were treated with metronidazole to eradicate any residual infection and subsequently challenged with either of the two *G. lamblia* strains. Primary infection with either *G. lamblia* strain led to complete protection upon secondary challenge with the same strain (Fig. 1). Importantly, secondary challenge with the respective other, assemblage-mismatched strain also conferred marked protection, as indicated by >100-fold reduction in trophozoite load in the small intestine at the peak of infection upon secondary challenge (Fig. 1). Although cross-protection was slightly less complete than protection against assemblage-matched strains, these data strongly suggest that immunologically relevant cross-protection exists between divergent *G. lamblia* strains and thus provide the rationale for identifying conserved antigens that mediate this protection.

Proteomic analysis of *G. lamblia* surface proteins. Our previous studies and those of others had used whole trophozoite extracts of *G. lamblia* to identify proteins recognized by immune serum as candidate vaccine antigens (20, 39). This strategy could have biased antigen identification toward more abundant proteins while potentially underestimating less abundant surface proteins. Because surface proteins may be important targets of protective IgA responses (19), we pursued an alternative approach focused on identifying conserved cell surface proteins. Trophozoites of the two divergent *G. lamblia* strains, WB (assemblage A) and GS/M (assemblage B), were labeled with a reactive but non-membrane-permeant biotin (*N*-hydroxysuccinimide [NHS]-biotin) (Fig. 2). Incubation of biotin-labeled cells with a fluorescently labeled streptavidin revealed strong staining at the trophozoite surface and flagella, which was not observed in controls not labeled with biotin (Fig. 2). Furthermore, immunoblot analysis of total cell lysates with anti-biotin showed a range of biotin-labeled proteins that were not seen in control lysates (Fig. 2), demonstrating the specificity of the biotin labeling.

Next, we immunoprecipitated biotin-labeled proteins from total cell lysates of *G. lamblia* WB by streptavidin affinity chromatography, digested the precipitates *in situ* with trypsin, and identified the resulting peptides by sequential liquid chromatography and tandem mass spectrometry (Fig. 2). Comparison of the peptides with simulated digests of all gene products predicted from the *G. lamblia* WB genome sequence led to the identification of 86 proteins. Of these, 15/86 (17%) were annotated as VSPs, which are known to be abundant at the trophozoite surface (33), thus validating our experimental strategy for identifying surface proteins. Bioinformatics analysis showed that 24 (28%) of the 86 identified proteins, including the VSP proteins, have well-defined transmembrane regions, with (22/24) or without (2/24) signal peptides. An additional 21 (25%) of the proteins have motifs for palmitoylation, which can mediate an alternative

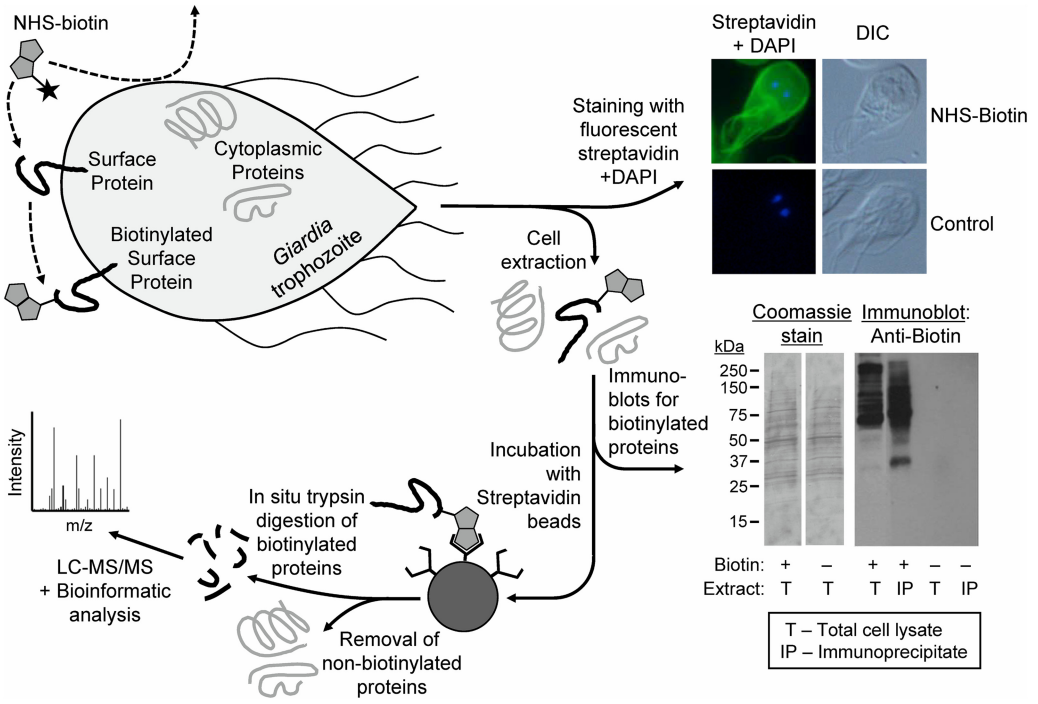

FIG 2 Biotin labeling of surface proteins in *G. lamblia* trophozoites. Intact trophozoites were reacted with non-membrane-permeant NHS-biotin for 2 h at 4°C. After biotinylation and washing steps, biotin-labeled and unlabeled control cells were assessed by immunofluorescence with fluorescent streptavidin (green) and counterstained with DAPI (blue). Differential interference contrast microscopy (DIC) was used to visualize the cells for comparison. In parallel, total cell lysates were prepared and analyzed directly or subjected to immunoprecipitation with streptavidin-agarose beads. Precipitated proteins were separated by SDS-PAGE. Total protein was visualized by Coomassie staining, and biotinylated proteins were detected by immunoblotting with an anti-biotin antibody (bottom right). Immunoprecipitates were subsequently digested with trypsin, and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

mode of membrane binding in *G. lamblia* (45), whereas glycosylphosphatidylinositol (GPI) anchoring of membrane proteins is rare in the parasite (46). Thus, >50% of the identified proteins have features that are commonly associated with membrane proteins.

The same strategy for surface protein identification was then applied to the GS/M strain of *G. lamblia*. Mass spectrometry of tryptic digests and comparison of the peptides with simulated digests of the predicted gene products of the *G. lamblia* GS/M genome led to the identification of 51 proteins. As for the WB strain, a significant number (6/51, 12%) of identified proteins were annotated as VSPs. Comparison of the identified proteins in WB and GS/M revealed that 27 were found in both strains. None of these were annotated as VSPs, which underlines that the VSP repertoires of the two *G. lamblia* strains are largely nonoverlapping (31, 32, 42). Of the 27 common proteins, 23 showed greater than 90% amino acid sequence identity between WB and GS/M (Table 1), indicating that the vast majority of the identified non-VSP surface proteins are conserved between the two divergent assemblage A and B strains.

Confirmation of surface protein localization. To confirm the surface localization of the identified proteins by immunofluorescence staining, *G. lamblia* WB trophozoites were allowed to attach to glass slides and were fixed with paraformaldehyde but left nonpermeabilized for selective staining of surface targets accessible to antibodies from the outside. As a control, fixed cells were permeabilized with Triton X-100 to permit antibody access to all proteins regardless of location. For staining, we used previously validated antibodies (47, 48) or generated new mouse polyclonal antibodies against the recombinant proteins. Immunoblotting of total *G. lamblia* lysates and staining with the new antibodies revealed the expected single bands, confirming their specificity (Fig. 3A). Staining and immunofluorescent imaging of the fixed trophozoites revealed that all five putative surface proteins for which we had antibodies, i.e., β-giardin, α1-giardin, α11-giardin, uridine

TABLE 1 Conserved *G. lamblia* surface proteins identified by mass spectrometry

Group and gene product[a]	WB locus[b]	GS/M locus[b]	% identity for WB vs GS/M	% identity vs human	Comment(s)[c]
Proteins with <20% identity to human proteins					
β-Giardin	4812	2741	100	4.8	Secretome
γ-Giardin	17230	4532	99.4	13.9	
Protein disulfide isomerase 5	8064	3370	99.1	17.5	TM regions
Pyruvate-flavodoxin oxidoreductase	114609	4147	96.3	5.6	Palmitoyl motifs
Carbamate kinase	16453	2343	95.9	7.2	Secretome
Aminoacyl-histidine dipeptidase	15832	2286	95.7	14.0	Palmitoyl motifs
Pyruvate, phosphate dikinase	9909	4299	95.6	4.2	Palmitoyl motifs
Pyruvate-flavodoxin oxidoreductase	17063	1047	95.4	5.6	Palmitoyl motifs
Uridine phosphorylase-like protein (UPL)-1	9779	1523	94.8	17.2	Palmitoyl motifs
Protein 21.1	27925	3278	94.5	8.2	Secretome
α1-Giardin	11654	1672	92.2	11.1	Secretome
α11-Giardin	17153	2473	90.9	15.2	Secretome
Proteins with ≥20% identity to human proteins					
Cytosolic HSP70	88765	2353	98.3	62.3	Palmitoyl motifs, secretome
Bip	17121	3283	98.2	55.4	TM regions, secretome
Peroxiredoxin 1	14521	730	98.0	50.2	Palmitoyl motifs, secretome
Ribosomal protein L4	17547	4247	97.8	35.4	
Elongation factor 2	17570	1765	97.7	52.5	Palmitoyl motifs, secretome
Dynamin	14373	1962	97.7	36.7	
Ribosomal protein L5	17395	1856	97.3	52.4	
Peroxiredoxin 1	16076	730	97.0	50.2	Palmitoyl motifs, secretome
Ribosomal protein L3	16525	348	96.8	46.3	
Ribosomal protein S8	5845	260	95.9	40.6	Secretome
Elongation factor 1-γ	12102	1508	90.3	23.9	Palmitoyl motifs, secretome

[a]Live *G. lamblia* trophozoites were labeled with non-membrane-permeant NHS-biotin, cell extracts were prepared, and biotin-labeled proteins were immunoprecipitated by streptavidin affinity chromatography. Precipitates were digested *in situ* with trypsin, and the resulting peptides were analyzed by high-pressure liquid chromatography coupled with tandem mass spectroscopy. Peptides were compared to simulated digests of all gene products predicted from the *G. lamblia* genome sequences. The 23 listed gene products were identified in both *G. lamblia* strains (WB and GS/M) and had >90% amino acid identity between the strains. Comparison with all human proteins was done for the GS/M gene sequences and used to separate the *G. lamblia* proteins into two groups depending on the degree of identity. In each group, the proteins are sorted in order of decreasing identity between the two *G. lamblia* strains.

[b]Locus tags for genes of strains WB and GS/M are preceded by GL50803 (*G. lamblia* ATCC 50803) and GL50581 (*G. lamblia* ATCC 50581), respectively.

[c]Key features of the identified proteins were explored by sequence analysis for transmembrane (TM) regions and palmitoylation motifs. Proteins also identified in prior studies of the *G. lamblia* secretome (41, 63) are indicated.

phosphorylase-like protein 1 (UPL-1), and protein 21.1, were detectable without permeabilization (Fig. 3B). As a control, a known intracellular protein, calmodulin kinase (CAMK) (47), could not be stained under the same conditions, demonstrating the selectivity of our approach (Fig. 3B). Staining patterns varied from what appeared to be specific or preferential to the adhesion disc (β-giardin, protein 21.1) to localized (α1- and α11-giardin) or generalized (UPL-1) surface staining. By comparison, in permeabilized cells, staining for all five proteins was generally strong and widespread (Fig. 3B), suggesting that the proteins were not exclusively present at the surface.

To localize proteins for which we did not have specific antibodies, we opted for expressing them in *G. lamblia* WB as fusion proteins with a hemagglutinin (HA) epitope tag and staining with an anti-HA antibody. All five targeted proteins (pyruvate-flavodoxin oxidoreductase, pyruvate-phosphate dikinase, dynamin, peroxiredoxin-1, and ribosomal protein S8) could be stained without permeabilization, while a known intracellular control protein, NEK kinase, showed no staining (Fig. 3C). Staining patterns and intensity, again, ranged from fairly selective, such as the strong flagellar and head staining of pyruvate-phosphate dikinase, to more generalized and weaker (e.g., dynamin) effects. After permeabilization, all five proteins and the NEK kinase control showed strong staining across the cells (Fig. 3C), suggesting that these proteins were not limited to the surface. Together, the staining data confirm that all 10 of the tested surface proteins identified by selective biotin labeling and mass spectrometry were expressed at the trophozoite surface and accessible to external antibodies.

Immunogenicity and protective capacity of selected conserved surface antigens. Candidate antigens that have limited similarity to human proteins are generally preferable for vaccines since the risk of adverse immunological cross-reactions upon

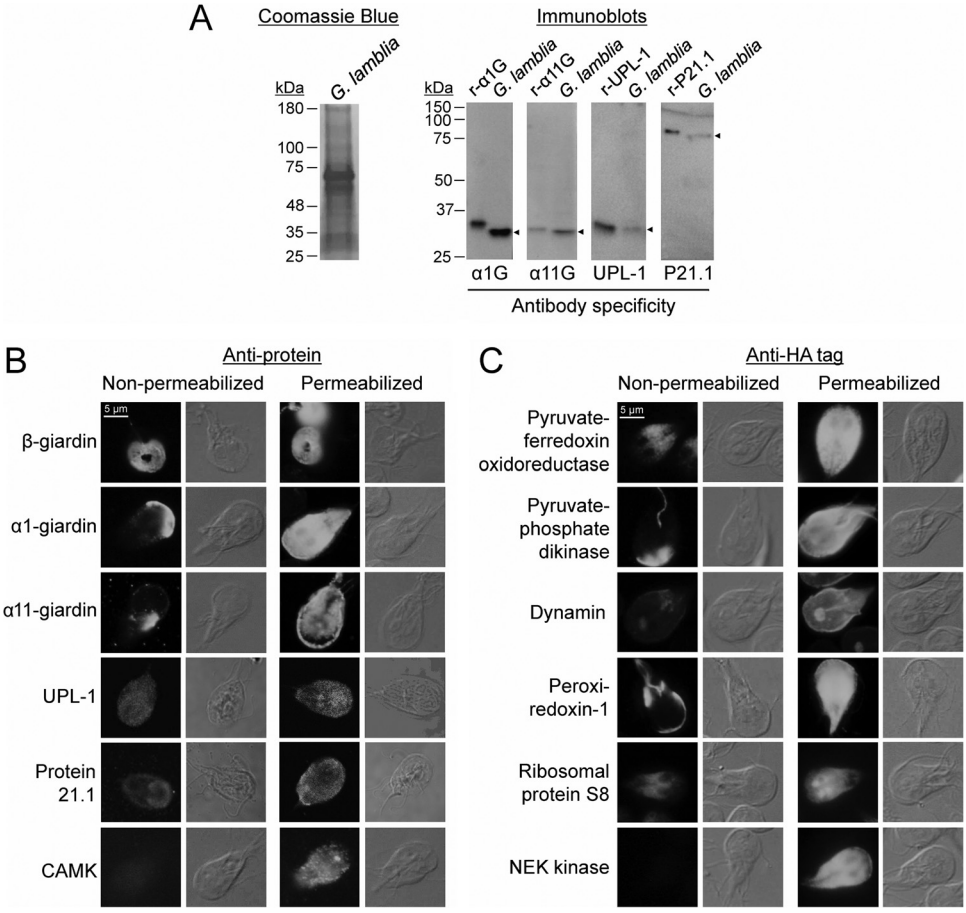

FIG 3 Confirmation of surface localization of protein candidates. (A) Polyclonal mouse antibodies were generated against the indicated antigens (α1G, α1-giardin; α11G, α11-giardin; UPL, uridine phosphorylase-like protein; P21.1, protein 21.1) and tested for specificity by immunoblotting with total cell lysates of *G. lamblia* WB trophozoites. His-tagged recombinant forms (r-) of the respective antigens were used as controls (the epitope tag adds ~2.2 kDa to the mass of the native proteins). (B and C) Live *G. lamblia* WB trophozoites were allowed to attach to glass coverslips and fixed with paraformaldehyde. Cells were permeabilized with Triton X-100 or left nonpermeabilized. The indicated proteins were then detected by immunofluorescence staining with specific antibodies (Anti-protein) or by staining of transgenic trophozoites expressing epitope (HA)-tagged forms of the proteins with an anti-HA tag, as indicated. Calcium/calmodulin-dependent protein kinase (CAMK) and NEK kinase were used as controls for proteins with known cytoplasmic localization.

immunization should be reduced. In evaluating the similarity of the identified *G. lamblia* surface proteins to human proteins, we selected a subset of 12 (of 23) that had $<$20% similarity (and E values of $>10^{-30}$) relative to any predicted human protein (Table 1). Out of these, we produced four representative examples, α1-giardin, α11-giardin, UPL-1, and protein 21.1, as recombinant His-tagged proteins in *Escherichia coli* and purified them by nickel column chromatography to $>$80% purity in soluble form (Fig. 4A). A fifth protein, β-giardin, was also expressed in *E. coli* but could not be readily purified in soluble form.

To test the immunogenicity of the candidate antigens, we administered individual purified antigens together with the prototype mucosal adjuvant cholera toxin to mice by intranasal administration. Immunization induced high titers of serum IgG for all four antigens (Fig. 4B), whereas significant mucosal secretory IgA was elicited for only two of them, α11-giardin and UPL-1 (Fig. 4C). As a further evaluation of the immunogenicity of the selected antigens, we tested antibody titers in patients with laboratory-confirmed giardiasis (49). Healthy and presumed-unexposed individuals were used as controls. Infection induced significant increases in serum IgG titers against three of the four antigens (α1-giardin, α11-giardin, and protein 21.1) (Fig. 4D) and in serum IgA titers against all four antigens

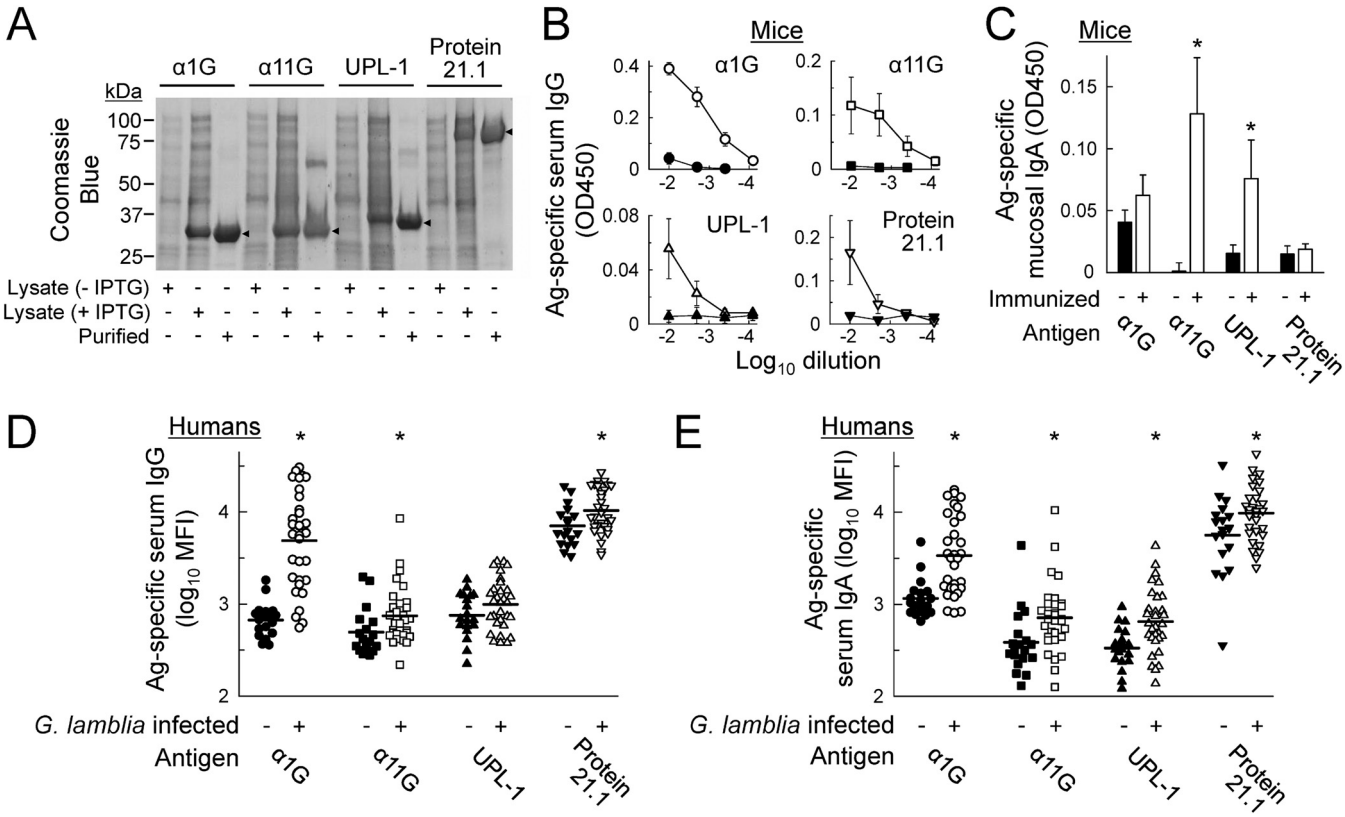

FIG 4 Immunogenicity of candidate *G. lamblia* antigens. (A) The indicated proteins (α1G, α1-giardin; α11G, α11-giardin; UPL, uridine phosphorylase-like protein) were produced in recombinant form in *E. coli*, purified by nickel affinity chromatography, and visualized by SDS-PAGE and Coomassie blue staining. (B) C57BL/6 mice were immunized by three intranasal administrations 2 weeks apart with recombinant proteins and cholera toxin as an adjuvant. Cholera toxin alone was used as a control. Mice were bled, and serum IgG levels against the respective proteins were determined by ELISA (means $\pm$ SE, $n \geq 3$ mice/group; open symbols, immunized mice; closed symbols, mock-immunized controls). (C) Mucosal washes were prepared from the small intestine of immunized and control mice and used to test antigen-specific IgA levels (means $\pm$ SE, $n \geq 3$ mice/group; *, $P < 0.05$ by *t* test). (D and E) Sera from *G. lamblia*-infected patients (+) and age-matched, presumed-unexposed controls (−) were tested for IgG and IgA titers against the indicated antigens by multiplex beads immunoassays. Each data point represents one individual. *, $P < 0.05$ by Mann-Whitney U test. Ag, antigen.

(Fig. 4E). Together, these data show that the four selected antigens are immunogenic after mucosal immunization or natural infection in mice and humans.

To assess protective capacity, antigen-immunized mice were challenged 2 weeks after the last of three immunizations by oral gavage with *G. lamblia* GS/M trophozoites, and parasite load in the small intestine was determined after 5 days, the time of peak parasite burden in control mice (19). Immunization with all four antigens conferred significant protection, as evidenced by a 50- to 500-fold reduction in trophozoite numbers compared to those in mock-immunized mice (Fig. 5A). Furthermore, immunization with one of the antigens, α1-giardin, shortened the duration of infection since most immunized mice had eradicated infection by day 20, while none of the nonimmunized controls were cleared at that time (Fig. 5B). Despite strong induction of antigen-specific IgG after immunization (Fig. 4B), protection was not significantly correlated with IgG titers for any of the four antigens (Fig. 5C), suggesting that IgG is not likely to act as an antigiardial effector in this system. Similarly, mucosal IgA, which had been shown to play a role in clearance of *G. lamblia* infection (19, 20), was not important for protection since immunization of IgA-deficient mice with two of the antigens, α1-giardin or UPL-1, conferred significant protection that was similar to that in wild-type mice (Fig. 5D).

Finally, immunization with α1-giardin also induced significant protection in gerbils against subsequent *G. lamblia* infection, indicating that protection was not host specific (Fig. 5E). Together, these data show that the newly identified surface antigens are promising candidates for a protective vaccine against giardiasis.

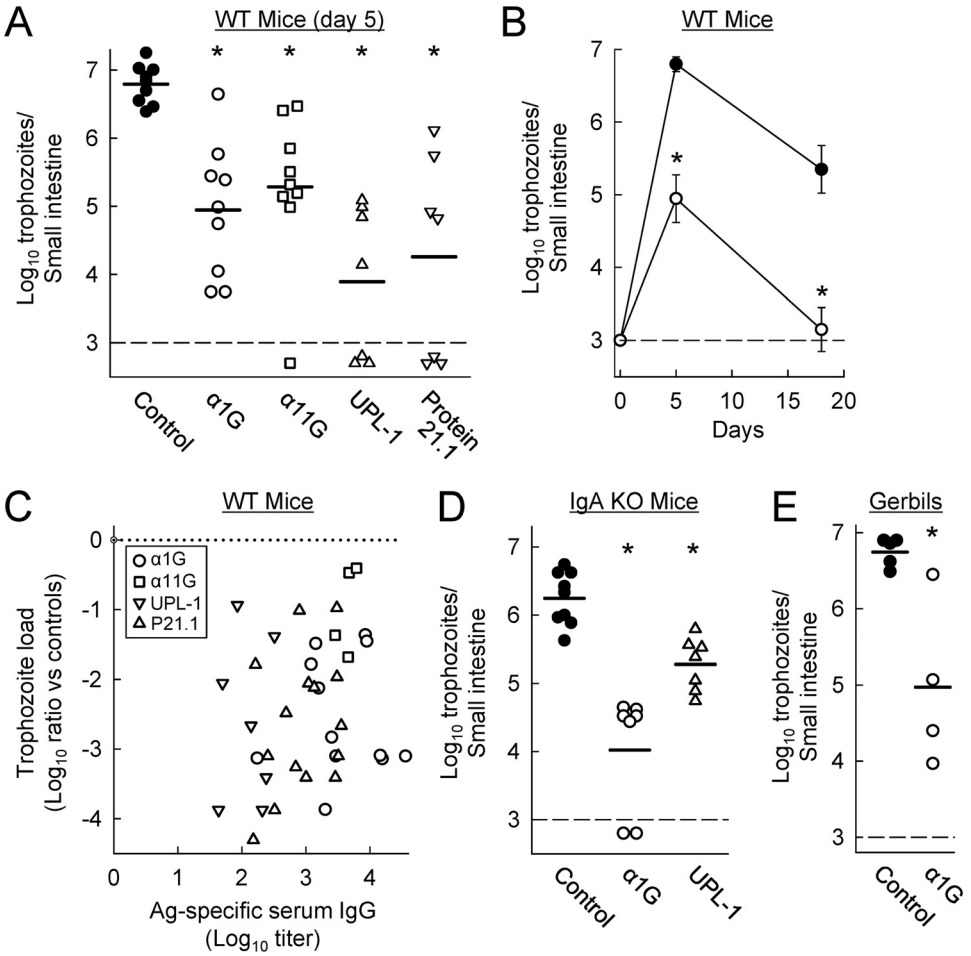

FIG 5 Immunization with candidate antigens confers protection against *G. lamblia* infection. (A to D) C57BL/6 wild-type (WT) and IgA-deficient (IgA knockout [KO]) mice were intranasally immunized with the indicated antigens (α1G, α1-giardin; α11G, α11-giardin; UPL, uridine phosphorylase-like protein) or given cholera toxin alone as a control. Two weeks after the last immunization, plasma was obtained to determine antigen-specific IgG titers; mice were orally inoculated with *G. lamblia* GS/M trophozoites, and live trophozoites were enumerated in the small intestine after 5 days (A, C, and D) or after 5 and 18 days (B). Symbols in panels A, C, and D depict individual mice, with geometric means shown as horizontal lines in panels A and D. Data in panel B are means ± SE ($n = 6$ to 8 mice/group). The dashed lines in panels A, B, and D represent the detection limits of the assay. Panel C shows the correlations of antigen-specific IgG titers and reduction of trophozoite numbers relative to those of the controls for individual mice and the indicated antigens (the dotted line represents the trophozoite load in controls). (E) In a separate experiment, gerbils were intranasally immunized with α1-giardin and cholera toxin or given cholera toxin alone as a control and subsequently challenged by oral administration of *G. lamblia* GS/M trophozoites. After 10 days, live trophozoites were enumerated in the small intestine (each symbol represents one animal; geometric means are shown as horizontal lines). Significance relative to values for the controls is indicated (*, $P < 0.05$; Kruskal-Wallis test with Dunn's *post hoc* test for panels A and D and a Mann-Whitney U test for panels B and E).

DISCUSSION

Modern vaccine development depends on identification of suitable antigens that can elicit protective immunity. An experimental subunit vaccine has been reported for *G. lamblia* using the encystation-specific cyst wall protein, CWP2 (50). The vaccine reduces cyst shedding and transmission of *G. lamblia* in mice but has no effect on the disease-causing trophozoites (50, 51). Another antigen, α1-giardin, has been shown to reduce trophozoite load upon immunization with a live recombinant *Salmonella*-based vaccine vector (52) and after DNA vaccination (53). Independent identification and testing of this antigen as a purified protein in the present study confirmed and expanded these findings. We also identified a second member of the α-giardin family, α11-giardin (54), as a protective, conserved antigen against giardiasis and discovered that two other conserved giardins, β-giardin and γ-giardin, are expressed at the

trophozoite surface. The family of giardins has >20 members (54) with differential localizations in the cytoplasm, attachment disc, or at the plasma membrane (54–57). Several are found in the attachment disc and may contribute to parasite attachment (58), but the functions of most giardins are poorly understood. Our findings indicate that select members of this gene family, including α1, α11, β, and γ, are attractive candidate antigens for an effective vaccine against giardiasis.

The surface proteome analysis revealed two unexpected antigens, UPL-1 and protein 21.1, that were not previously known to be surface expressed. The functions of UPL-1 are not known, but it has 95 to 97% amino acid identity with uridine phosphorylase, which is an important enzyme for nucleotide synthesis in the cytoplasm of *G. lamblia* (59, 60), and has been shown in another parasite, *Toxoplasma*, to be an immunodominant protein (61). Expression of its closely related gene product, UPL-1, at the cell surface was therefore unexpected and suggests possible extracellular functions. Similar to the case for UPL-1, little is known about another protein identified in the *G. lamblia* surface proteome, protein 21.1 (open reading frame [ORF] GL50803_27925). It is characterized by multiple ankyrin repeats that mediate protein-protein interactions in many biologic systems, but the functions of ankyrin repeat-containing proteins are highly diverse, providing little clues as to the potential role of protein 21.1 in *G. lamblia*. Its preferential localization in the attachment disc might suggest a role in parasite attachment, but this remains to be explored. Nonetheless, the unexpected identification of these two protective proteins demonstrates that unbiased identification of conserved surface proteins, many of which were previously found to be immunogenic (20, 39) and confirmed here, can reveal promising vaccine candidates even without any actual or predicted knowledge of their cellular functions.

VSPs, which are among the most abundant and immunogenic surface proteins in *G. lamblia* (33), were identified in our proteomics approach, but subsequent bioinformatics criteria for strong conservation between the two human-pathogenic *G. lamblia* assemblages removed them from further analysis. Their exclusion is a consequence of the known diversity and lack of overlap between the VSP gene repertoires of different *G. lamblia* strains and assemblages (30–32, 42) and documents the selective nature of our experimental strategy. Although we did not pursue VSPs in this study, it may be possible to exploit VSPs as vaccine antigens if trophozoites are genetically manipulated to express most or all possible variants on the surface (36, 37). Vaccination with live strains of such broad-spectrum VSP-expressing vaccine strains of *G. lamblia* would require attenuation and infection with cysts from both assemblages, which poses multiple technical and biological challenges, not the least being that attenuated *G. lamblia* parasites have never been reported in and may not engage the mucosal immune system. The alternative, a "supervalent" vaccine with >400 purified VSP antigens for cell-free vaccination is feasible in principle (37) but would be practically prohibitive to manufacture and may not reliably induce immunity for all VSP antigens or for *G. lamblia* strains with different VSP repertoires. In comparison to vaccines based on numerous VSPs, our proteomics and selection strategy allow identification of well-defined single conserved antigens that would be expected to be relatively easy to manufacture for a pharmaceutically tractable subunit vaccine.

The proteins identified by our surface labeling strategy were highly enriched for features typically associated with surface proteins, such as predicted transmembrane regions or palmitoylation motifs. Furthermore, surface localization was confirmed for a select number of the identified proteins, albeit none of them was exclusively membrane associated. However, a number of the identified proteins had no known features of classical membrane proteins. It is possible that other, poorly defined mechanisms of membrane association may account for some of these proteins. They may also not be actual membrane proteins but contaminations in the immunoprecipitations or labeled with biotin independent of surface localization. For example, the endoplasmic reticulum in *G. lamblia* has recently been shown to have connections to the surface (62), so the biotin label may travel along this pathway and thereby generate background labeling. Consistent with this possibility, several of the identified proteins without

transmembrane regions or palmitoylation sites are translational factors associated with the endoplasmic reticulum. Alternatively, biotin labeling as done in this study may label some secreted proteins since a number of the identified proteins overlap those found in studies of the secretome of *G. lamblia* (41, 63).

All four tested antigens reduced infection by >98% of peak parasite burden and completely prevented infection in several mice, and one antigen also shortened the infection duration; yet it is unclear how much reduction in infectious load would be required to attenuate or prevent potential clinical symptoms. This question cannot be readily answered in murine models of giardiasis because no meaningful clinical disease develops in these preclinical models. In any case, complete protection may not be necessary to achieve significant public health benefits for a vaccine, as documented for vaccines against rotavirus and other enteric infectious agents (64, 65).

Adjuvants and suitable administration routes are critical for effective induction of mucosal immunity, particularly effectors that are active against *G. lamblia* (66). Our studies show that IgG and mucosal IgA are not likely to be important in this context since no correlation was found between IgG titers and protection, and immunizations were effective even in the absence of IgA. Other effectors, such as intestinal hypermotility (21, 22) or specific antimicrobial peptides (67), could be involved and may need to be targeted by effective adjuvants. For the present study, we used the prototype adjuvant cholera toxin and intranasal immunization with excellent efficacy, but it is likely that further vaccine development will require optimization of these parameters. Cholera toxin and related adjuvant toxins have been tested as adjuvants in humans but are not approved due to toxicity concerns (68, 69). Mutant forms of the toxin have been developed and may eventually be suitable for human use (70). Other adjuvants, particularly alum, are used routinely in human vaccines, but their effectiveness is currently not optimal (66). It may also be possible to express defined antigens in live attenuated vectors that can be given orally and induce mucosal immunity (52), so further advances in live vector design may allow the best conserved antigens to be delivered with such technologies. Key to all of these vaccine strategies is knowledge of the most protective antigens, so the current systematic strategy to reveal an array of conserved antigen candidates provides a powerful new approach for overcoming a key rate-limiting step in the design and construction of an effective vaccine against giardiasis.

MATERIALS AND METHODS

***G. lamblia* isolates and cultivation.** We used *G. lamblia* isolates WB clone C6 (ATCC 50803; human, assemblage A) (71) and GS/M clone H7 (GS/M; ATCC 50581; human, assemblage B) (9). Parasites were grown in modified TYI-S33 medium to no more than 80% confluence (72).

Biotin labeling of surface proteins. Unattached parasites were decanted and attached, intact cells were resuspended in ice-cold phosphate-buffered saline containing 0.2 M sucrose, pH 7.4 (PBS-S). Parasites were placed on ice for 5 min, concentrated by centrifugation for 5 min at 600 × *g* and 4°C, and cells were combined into new tubes. Cells were washed two times in PBS-S (50 ml/tube) and then a third time with PBS-S containing a protease inhibitor cocktail (Complete Mini-EDTA; Roche) plus 1 μM E-64 (PBS-S+; Sigma). Cells were counted and resuspended in cold PBS-S+ at a density of 1×10^8 cells/ml, placed into 1.5-ml microcentrifuge tubes, and labeled with 2 mM EZ-Link NHS-PEG4-biotin (Thermo Scientific) for 2 h with continuous rotation under refrigeration. Unlabeled controls were processed under identical conditions but without added NHS-PEG4-biotin. Following the labeling reaction, viability of trophozoites was assessed with fluorescein diacetate (25 μg/ml) and propidium iodide (6 μg/ml), and excess biotin was quenched with 0.5 M Tris-HCl (pH 8.0) to a final concentration of 50 mM. Parasites were washed three times with ice-cold PBS-S in new 50-ml tubes.

Following the biotinylation and washing steps, biotin-labeled and unlabeled control cells were assessed by immunofluorescence assay for localization of the biotin label. Ten microliters of cells was allowed to attach to prewarmed eight-well slides at 37°C for 10 min, which were then fixed for 10 min at 37°C with 2% paraformaldehyde in PBS. Cells were rinsed in PBS, permeabilized in 0.5% Triton X-100 for 10 min, blocked (10% goat serum, 1% glycerol, 0.1% bovine serum albumin [BSA], 0.1% fish gelatin, and 0.04% sodium azide in PBS) for at least 30 min at room temperature, and probed with streptavidin-Alexa 488 (1:800 diluted in blocking buffer; Molecular Probes) for 30 min. Cells were washed four time in PBS, postfixed in 4% paraformaldehyde (PFA) for 5 min, rinsed with PBS, and mounted in Prolong Gold with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes). Biotin conjugates associated with whole cells were imaged on an E800 Nikon (New York City, NY) microscope with an EXFO (Vanier, Canada) X-cite fluorescent 120-W metal halide illuminator and imaged with a DMX 1200F Nikon fluorescence sensitive digital camera.

Purification of biotinylated proteins. Washed biotinylated and control cells were concentrated by centrifugation and transferred to new 1.5-ml microcentrifuge tubes. Biotinylated membrane proteins were processes and purified essentially as described previously (73) with some modifications. Briefly, cell pellets were directly lysed, and membrane proteins were solubilized in 1 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 2% SDS, 0.1% Igepal CA-630, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 μM E-64, Complete Mini protease inhibitor cocktail), and insoluble material was removed by centrifugation at 13,000 $\times$ *g* for 5 min at 4°C. Lysis buffer was used to preequilibrate streptavidin-agarose by batch method under refrigeration and continuous rotation for 10 min. Approximately 1 mg of cellular protein (estimated at $\sim$5 μg of membrane protein per 5 $\times$ 10^7 cells) was added per 150 μl of equilibrated streptavidin-agarose slurry. Biotinylated proteins were incubated with streptavidin-agarose for 2.5 h under refrigeration and with continuous rotation. Subsequent washing to remove unbound proteins was done as described previously (73).

Mass spectrometry. Trypsin-digested peptides were analyzed by high-pressure liquid chromatography coupled with tandem mass spectroscopy (HPLC-MS/MS) using nano-spray ionization as described previously (74) with the following modifications. The nanospray ionization experiments were performed using a QSTAR-Elite hybrid mass spectrometer (AB Sciex) interfaced with nano-scale reversed-phase HPLC (Tempo) using a 10-cm glass capillary (100-μm inside diameter), packed with 5-μm C_{18} Zorbax beads (Agilent Technologies, Santa Clara, CA). Peptides were eluted from the C_{18} column into the mass spectrometer using a linear gradient (5 to 60%) of acetonitrile (ACN) at a flow rate of 400 μl/min for 1 h. The buffers used to create the ACN gradient were buffer A (98% H_2O, 2% ACN, 0.2% formic acid, and 0.005% trifluoroacetic acid [TFA]) and buffer B (100% ACN, 0.2% formic acid, and 0.005% TFA).

MS/MS data were acquired in a data-dependent manner in which the MS1 data were acquired at *m/z* of 400 to 1,800 and the MS2 data were acquired from *m/z* of 50 to 2,000. Peptide identifications were made using the paragon algorithm executed in Protein Pilot, version 2.0 (AB Sciex). A comprehensive library of *G. lamblia* protein sequences was obtained from GiardiaDB (https://giardiadb.org/giardiadb/). Proteins were identified by at least two peptides with a confidence of >95% for the peptide identification. The MS was set to disregard ions from certain high-abundance nongiardial proteins. The following programs were used to evaluate putative membrane association: Giardia DB, release 36 (www.giardiadb.org/giardiadb/); SignalP Server, version 3.0 (http://www.cbs.dtu.dk/services/SignalP/); TMHMM Server, version 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), and CSS-Palm for palmitoylation site prediction (http://csspalm.biocuckoo.org/online.php).

Cloning of epitope-tagged proteins and immunofluorescence assays. Using genomic DNA from *G. lamblia* WB/C6, full-length cDNAs and the corresponding promoter of the respective target genes were amplified by PCR and cloned into a vector that introduces a C-terminal triple hemagglutinin (HA) tag and allows for stable expression of the target gene under its own promoter (75). Trophozoites were transfected with each vector and selected for stable expression by puromycin selection as described previously (76, 77).

For staining, live trophozoites were allowed to attach to poly-L-lysine-coated coverslips for 10 min at 37°C, washed in warm PBS, fixed with 4% paraformaldehyde for 30 min, and left untreated or permeabilized with 0.5% Triton X-100 for 10 min. Fixed cells were blocked for 30 min in 5% goat serum, 1% glycerol, 0.1% bovine serum albumin, 0.1% fish gelatin, and 0.04% sodium azide and stained for 1 h at room temperature with primary antibodies against the target proteins. Antibodies against calmodulin (47) and β-giardin (48) were validated previously, while those against α1-giardin, α11-giardin, protein 21.1, and UPL-1 were generated by immunizing mice with recombinant forms of these proteins (see below) and validating them by immunoblotting with whole-cell extracts of *G. lamblia* WB. Antibodies against the HA tag were obtained commercially (Sigma). After the primary staining step, slides were washed and further incubated for 1 h with Alexa 488-labeled secondary antibody against the Ig isotype of the primary antibody. After a rinse with water, coverslips were mounted with Prolong Gold with DAPI (Molecular Probes). Staining was examined using a Nikon Eclipse E800 microscope equipped with an X-Cite 120 fluorescence lamp.

Production of recombinant proteins. Sequences of candidate antigens were obtained from the published genome sequence of *G. lamblia* GS/M (ATCC 5058), and cDNAs were generated by total gene synthesis with codon optimization for *E. coli* (GenScript Biotech). Genes were inserted into NdeI-XhoI cloning sites of the bacterial expression vector pET-15b (Millipore Sigma), and vectors were transformed into T7 Express lysY/Iq Competent *E. coli* (NEB) per the manufacturer's protocol. Cells were grown at 37°C in LB medium with carbenicillin until they reached an optical density at 600 nm (OD_{600}) of 0.4 to 0.6, and protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 to 3 h. Recombinant proteins were purified from bacterial lysates using HisPur Ni-nitrilotriacetic acid (NTA) resin (Thermo Scientific), concentrated using Amicon Ultra centrifugal filters (EMD Millipore), and stored at a concentration of >1 mg/ml at 4°C and in a buffer of 300 mM NaCl, 25 mM HEPES, 100 mM arginine, 20 mM imidazole, 10% glycerol, 0.1% Tween 20, and protease inhibitors. Purity of the soluble recombinant proteins was analyzed by SDS-PAGE. Endotoxin contamination in the antigen preparations was determined with a ToxinSensor Chromogenic LAL Endotoxin assay kit (GenScript) and found to range between 0.79 and 0.99 endotoxin units/ml (equivalent to 79 to 99 pg/ml, or approximately 0.4 to 0.5 pg per dose upon intranasal administration in mice). Preliminary tests showed that endotoxin had no impact on *G. lamblia* infectivity in mice since three intranasal administrations of a 5,000-fold-higher dose (2.5 ng of lipopolysaccharide [LPS] from *E. coli* O127:B8) than the amounts found in the antigen preparations over 4 weeks yielded similar numbers of trophozoites in the small intestine compared to numbers in mice not given LPS (7.35 $\pm$ 0.10 versus 7.63 $\pm$ 0.18, $n = 5$ and 6, respectively; *P*, not significant by

Mann-Whitney U test). Furthermore, PCR analysis with universal 16 rRNA primers did not detect any contaminating bacterial DNA in the antigen preparations.

Human serum samples and multiplex beads immunoassays. Serum samples from 31 Norwegian adult patients with laboratory-confirmed giardiasis were collected (49). Most participants acquired infection by traveling in areas where *G. lamblia* is endemic, including Africa ($n = 11$), Europe ($n = 7$), Asia ($n = 6$), and the Americas ($n = 4$), and three were of uncertain origin. Control sera were obtained from 19 presumed-unexposed individuals. Patients and controls matched for age (means and standard deviations [SD] of 42.3 ± 20.4 and 39.6 ± 16.8 years for infected patients and controls, respectively) and sex (45% and 37% female, respectively). The study was approved by the Regional Committee for Medical Research Ethics of Western Norway.

For titer determinations, recombinant antigens or glutathione *S*-transferase (GST) as a control protein was separately coupled by sodium *N*-hydroxysulfosuccinimide and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide HCl cross-linking chemistry to 4-μm Cyto-Plex polystyrene beads with different levels of internal fluorescence (Thermo Fisher Scientific, Waltham, MA). Bead density was determined by microscopic counting. Each well of a MultiScreen HTS HV filter plate was prewetted with 200 μl of assay buffer (PBS containing 1% BSA and 0.05% Tween 20), and 10^4 beads of each protein-labeled type were added. Diluted human serum (1:50 in assay buffer) was added (100 μl/well), and plates were incubated for 1 h at room temperature and washed with assay buffer; Alexa Fluor 488-labeled AffiniPure goat anti-human IgA or Alexa Fluor 555-labeled goat anti-human IgG(H+L) antibody (Jackson Immunoresearch, Ely, UK) was added to the wells (1:400 or 1:500 dilution, respectively, in 200 μl assay buffer), and samples were incubated for an additional hour at room temperature. Wells incubated with Alexa Fluor 488-labeled mouse anti-His antibody (1:100 in 200 μl; Novus Biologicals, Abingdon, UK) were used as positive controls since all recombinant antigens carried the His epitope tag. GST-tagged beads were used as negative controls. After incubation, beads were washed twice in fluorescence-activated cell sorting (FACS) buffer (PBS containing 1% BSA), resuspended in 160 μl/well of FACS buffer, transferred into a round-bottom microplate, and analyzed in an LSRFortessa flow cytometer (BD Biosciences, San Jose, CA). IgA and IgG responses were quantified by determining the median fluorescence intensity (MFI) for each protein-coupled bead population using FlowJo software, version 10.4.2 (FlowJo LLC, Ashland, OR).

Antigen immunizations. Adult (6- to 8-week-old) female C57BL/6 mice (The Jackson Laboratory), IgA-deficient mice on a C57BL/6 background (19), and adult (10-week-old) female Mongolian gerbils (*Meriones unguiculatus*; Charles River Laboratories) were used for these studies. For intranasal immunizations, animals were placed under anesthesia with ketamine and xylazine, and a small volume (5 μl in mice; 10 μl in gerbils) of a mixture of antigen (50 to 100 μg) and cholera toxin (10 μg) was instilled into one of the nares. Cholera toxin alone was used as a control. Animals were left recumbent for at least 1 min and then allowed to recover in their cages. Immunizations were done three times at least 2 weeks apart. Mice were bled via the tail vein to obtain plasma, or the small intestine was removed and rinsed with PBS and protease inhibitors to obtain mucosal washes. Antibody levels against the immunizing antigens were determined by enzyme-linked immunosorbent assay (ELISA) in plasma or mucosal washes (20).

***G. lamblia* infections in rodents.** For infection challenges we used wild-type (C57BL/6) mice, IgA-deficient mice on a C57BL/6 background (19), and Mongolian gerbils. Infection challenges were done at least 2 weeks after the last immunization. Mice were treated for 2 days before and throughout the infection with an antibiotic cocktail (1.4 mg/ml neomycin, 1 mg/ml ampicillin, and 1 mg/ml vancomycin in the drinking water), while gerbils were not pretreated. *G. lamblia* trophozoites (WB/C6 or GS/M) were grown to mid-logarithmic phase and administered in growth medium by oral gavage to gerbils (10^7 trophozoites in a 400-μl volume) or mice (10^6 trophozoites in a 200-μl volume). At different times after infection, the small intestine was removed, opened longitudinally, placed into 2 to 5 ml of PBS, and cooled on ice for 10 min. After vigorous shaking of samples, live trophozoites were counted in a hemocytometer. Euthanasia of mice and gerbils was performed with CO_2 inhalation followed by removal of a major organ. All animal studies were reviewed and approved by the University of California San Diego Institutional Animal Care and Use Committee.

Statistical analysis. An unpaired *t* test, Mann-Whitney U test, or Kruskal-Wallis test with Bonferroni correction and the appropriate *post hoc* tests were used to compare results between animal groups as appropriate. Data are presented as means ± standard errors (SE) or as individual data points and geometric means. For the human samples, differences in specific antibody responses between individuals in the exposed and presumed-unexposed group were analyzed by a Mann-Whitney U test using SPSS Statistics, version 24. Differences with *P* values of <0.05 were considered statistically significant for all experiments.

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