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Mucosal vaccination in a murine gnotobiotic model of *Giardia lamblia* infection

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ABSTRACT *Giardia lamblia* is an important protozoan cause of diarrheal disease worldwide, delayed development and cognitive impairment in children in low- and middle-income countries, and protracted post-infectious syndromes in developed regions. *G. lamblia* resides in the lumen and at the epithelial surface of the proximal small intestine but is not mucosa invasive. The protozoan parasite is genetically diverse with significant genome differences across strains and assemblages. Animal models, particularly murine models, have been instrumental in defining mechanisms of host defense against *G. lamblia*, but mice cannot be readily infected with most human pathogenic strains. Antibiotic pretreatment can increase susceptibility, suggesting that the normal microbiota plays a role in controlling *G. lamblia* infection in mice, but the broader implications on susceptibility to diverse strains are not known. Here, we have used gnotobiotic mice to demonstrate that robust intestinal infection can be achieved for a broad set of human-pathogenic strains of the genetic assemblages A and B. Furthermore, gnotobiotic mice were able to eradicate infection with a similar kinetics to conventional mice after trophozoite challenge. Germ-free mice could also be effectively immunized by the mucosal route with a protective antigen, α1-giardin, in a manner dependent on CD4 T cells. These results indicate that the gnotobiotic mouse model is powerful for investigating acquired host defenses in giardiasis, as the mice are broadly susceptible to diverse *G. lamblia* strains yet display no apparent defects in mucosal immunity needed for controlling and eradicating this lumen-dwelling pathogen.

KEYWORDS giardiasis, germ-free mice, small intestine, immunization, α1-giardin

Giardia lamblia (syn. *Giardia intestinalis* and *Giardia duodenalis*) is a major cause of diarrheal disease worldwide with hundreds of millions of annual cases (1). The parasite is highly contagious as ingestion of as few as 10 cysts can cause infection (2). Symptomatic giardiasis is characterized by diarrhea, epigastric pain, nausea, vomiting, malabsorption, and malnutrition. In low- and middle-income countries, giardiasis is a leading cause of delayed development and cognitive impairment in children (3). While rarely fatal in high-income countries, infection can disable patients for extended periods and cause protracted post-infectious syndromes despite apparently successful treatment (4).

The parasite exists in two forms, the infectious cyst, which is resistant to many common disinfectants, and the disease-causing trophozoite form, which resides in the lumen and at the epithelial surface of the proximal small intestine (SI) (1). Giardiasis is self-limiting in >85% of cases in non-endemic areas, indicating that effective immune defenses exist. Symptoms of giardiasis are much less severe in endemic than non-endemic regions, suggesting gradual build-up of immunity (5). Immunization against giardiasis is possible (6–10), but a human vaccine has not been developed. Treatment with antimicrobials (e.g. metronidazole) is often successful, but failures occur in up to

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20% of cases, and resistance to all major antigiardial drugs has been demonstrated (11, 12).

G. lamblia is genetically diverse. Genome studies of different human and veterinary isolates have shown overall sequence similarities of <80% nucleotide identity in protein-coding regions, a difference that would justify separate species designations (13), yet for historical reasons, a single species name has been retained in most of the literature, albeit alternative proposals have been made (14). Instead, different isolates have been divided into eight genetic assemblages, A–H, based on genetic similarities (15). Across assemblages, different genes and gene families show varying degrees of conservation. Some genes, such as β-giardin, show only minimal variation between different isolates (16), while other genes, such as those belonging to the family of variant-specific surface proteins, are largely unique to different assemblages (13, 17). The functional implications of such gene variability on host-microbial interactions are not well understood. Assemblage A and B strains infect a range of mammalian hosts, including humans, and can be zoonotic, while strains of the other assemblages are primarily found in specific hosts among domestic animals and wildlife. Human infections with assemblage A and B strains occur worldwide (18), making it important that any new therapeutic and preventive interventions are effective against strains of both assemblages.

Animal models have played an important role in understanding giardial pathogenesis. For example, gerbil models of giardiasis have been used for exploring antigenic variation in *G. lamblia* and mechanisms of small intestinal malabsorption after infection (19–21). Mouse models have been instrumental for demonstrating the role of various immuno-regulatory cytokines, including IL-6 (22, 23), IL-17 (24, 25), and TNF-α (26), as well as of different effector cells, including CD4 T cells (27), B cells (28), and mast cells (29), in host defense against the parasite. Despite their proven utility, mice are not readily infectible with *G. lamblia*, since only limited strains, including GS/M, have been found to infect adult mice with an intact intestinal microbiota (30, 31). Suckling mice with an immature microbiota and treatment of adult mice with antibiotics can render mice more susceptible to infection, suggesting that the normal microbiota plays a role in determining or modifying susceptibility to *G. lamblia* infection in mice (32–35), but the broader impact on the infectivity of divergent strains is not known. Based on reports of the feasibility of *G. lamblia* infections in germ-free mice (36, 37), we set out in this study to systematically explore the role of the microbiota in determining murine infectivity of diverse strains of *G. lamblia* in gnotobiotic murine models and the host defenses that are operative in those models.

RESULTS

Germ-free mice are highly susceptible to infection with diverse *G. lamblia* strains

Prior studies had shown that adult mice can be infected with the *G. lamblia* assemblage B strains, GS/M strain (30), particularly when pretreated with antibiotics (32). Another assemblage B strain, H3, also requires antibiotic conditioning for persistent infection of mice (34). Consistent with these prior findings, we also observed that adult C57BL/6 mice given a cocktail of antibiotics (ampicillin, neomycin, and vancomycin) in the drinking water could be orally infected GS/M (Fig. 1A). In contrast, mice not treated with antibiotics were not infectible with GS/M and even antibiotics did not permit infection with two assemblage A strains, WB/C6 or 713, in our animal facility (Fig. 1A). We confirmed that antibiotic administration was effective since the number of fecal bacteria was significantly reduced by ~1 log for aerobes and >5 logs for anaerobes but did not completely eradicate bacterial colonization (Fig. 1B).

The observed differences in infectivity may reflect biologic differences among the *G. lamblia* strains or could be related to residual intestinal microbes that were not eradicated despite the use of a cocktail of three different antibiotics targeting a broad spectrum of bacteria. To distinguish between these possibilities, we infected germ-free adult

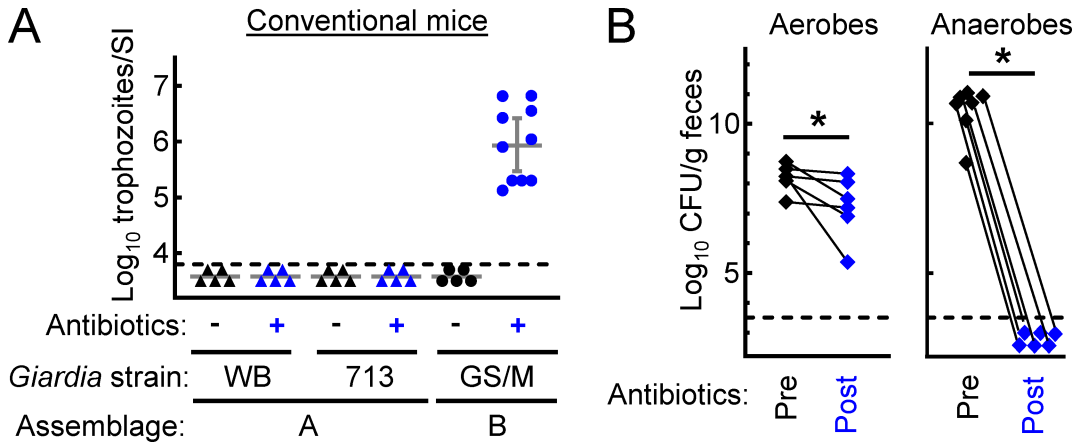

FIG 1 Infectivity of diverse *G. lamblia* strains in conventional mice. (A) Conventional adult C57 mice were given a cocktail of antibiotics (neomycin, ampicillin, and vancomycin) in the drinking water (+) or regular drinking water (–) and were orally infected with the indicated strains of *G. lamblia*. After 5 days, trophozoite numbers in the SI were determined. Data points (triangles for assemblage A strains, circles for assemblage B strain) represent data from individual mice, and gray lines show geometric means and 95% confidence intervals. The dashed line denotes the sensitivity of the assay. (B) Impact of antibiotic treatment on fecal aerobic and anaerobic bacteria before (Pre) and after (Post) antibiotic treatment for 2 weeks, as determined by CFU assay. Each data point represents an individual animal. Lines connect the CFU counts in the same animal before and after treatment. The dashed black line shows the detection limit of the assay, *P < 0.05 (Kruskal-Wallis test with Dunn's post hoc test).

C57BL/6 mice with a range of *G. lamblia* strains of both human-pathogenic genetic assemblages, A and B. Robust intestinal infection was observed for all five strains tested, albeit with differences in peak trophozoite numbers after 5 days (Fig. 2A). Furthermore, consistent with other studies (34, 36), *G. lamblia* GS/M-infected gnotobiotic mice excreted cysts in the stool at levels similar to antibiotic-treated conventional mice (Fig. 2B), indicating that giardial encystation was not impacted by the lack of a normal microbiota. These results show that germ-free mice constitute a facile model of *G.*

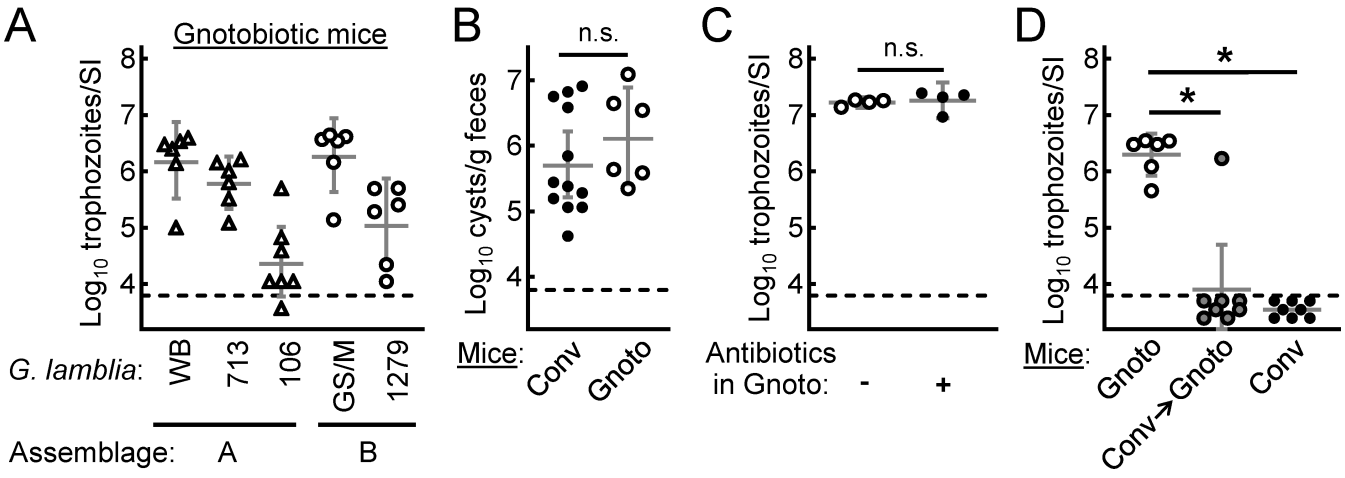

FIG 2 Infectivity of diverse *G. lamblia* strains in gnotobiotic mice. (A) Germ-free mice were orally infected with indicated strains of *G. lamblia* (making them gnotobiotic with respect to giardiasis). Trophozoites in the SI were enumerated after 5 days. (B) Fecal cyst excretion was determined in conventional mice given an antibiotic cocktail (Conv + Abx) and gnotobiotic mice (Gnoto) at 5 days after infection with *G. lamblia* GS/M. (C) Gnotobiotic mice were given a cocktail of antibiotics (neomycin, ampicillin, and vancomycin) in the drinking water (+) or regular drinking water (–) and infected with *G. lamblia* GS/M. After 5 days, trophozoite numbers were determined in the small intestine. (D) Germ-free mice were co-housed with conventional mice (Conv) for 2 weeks to allow association with a conventional fecal microbiota (Conv → Gnoto) and remained cage mates throughout the experiment. Gnotobiotic mice were used as controls. All three groups were orally infected with *G. lamblia* WB, and trophozoites in the small intestine were enumerated after 5 days. In all graphs, symbols represent individual mice, gray lines depict geometric means and 95% confidence intervals, and dashed black lines show the detection limit of the assays. *P < 0.05 (Student t-test in panels B and C or Kruskal-Wallis test with Dunn's post hoc test in panel D); n.s., not significant.

lamblia infection with a range of divergent strains and normal giardial growth and differentiation.

Microbial colonization of germ-free mice interferes with susceptibility to *G. lamblia*

To better understand how antibiotics and germ-free status facilitate *G. lamblia* infection, we treated germ-free mice with the same antibiotics cocktail used for conventional mice and infected them with *G. lamblia*. No significant difference was observed in the trophozoite load on day 5 compared with untreated germ-free mice (Fig. 2C), suggesting that the antibiotics had no effect on the parasites or host in the absence of a microbiota. To directly establish the role of the microbiota in interfering with *G. lamblia* infection, we co-housed germ-free adult mice with conventional mice for 2 weeks to facilitate microbial colonization with a normal fecal microbiota and challenged them with *G. lamblia* without antibiotic conditioning. The conventionalized ex-germ-free mice could no longer be infected with the WB strain, making them similar to conventional controls not treated with antibiotics (Fig. 2D). Together, these results show that the normal microbiota of mice is critical in determining murine susceptibility to a broad range of *G. lamblia* strains.

Gnotobiotic mice control and eradicate *G. lamblia* infection

Germ-free mice are known to have defects in mucosal immune development (38–41), which may explain their broad susceptibility to *G. lamblia*. To determine whether relevant immune defects exist after infection, we performed a time course analysis of intestinal trophozoite loads after oral infection of germ-free mice. Peak trophozoite levels were observed by 4–7 days after infection with both *G. lamblia* strains, GS/M and WB, followed by modest decreases at day 14 and levels at or below the detection limit by day 28 (Fig. 3). This time course was similar to that after infection of antibiotic-treated conventional mice with GS/M (Fig. 3). Although the practical detection limits do not allow definite conclusions about complete parasite eradication, the time course data strongly suggest that the lack of microbiota did not compromise adaptive murine immune defense

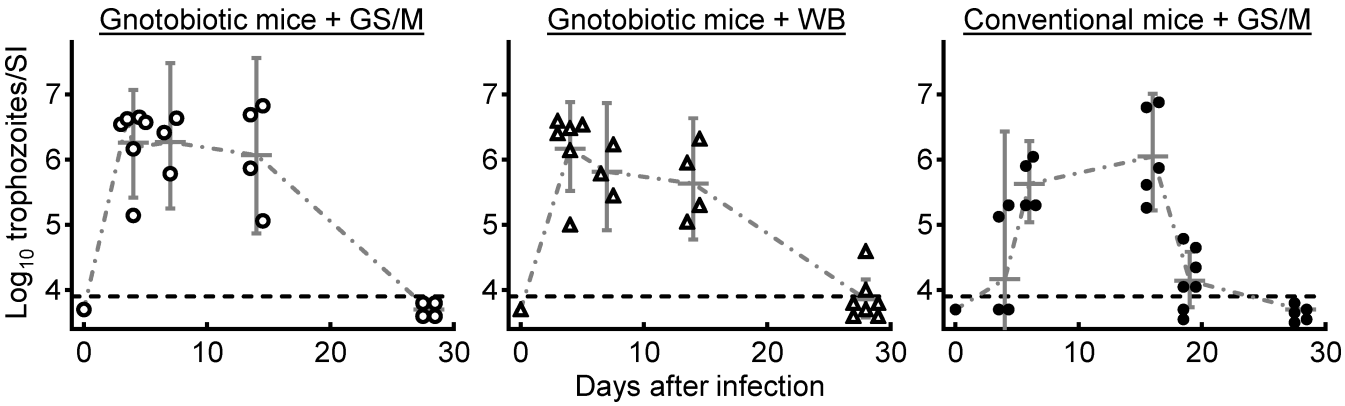

FIG 3 Time course of *G. lamblia* clearance in gnotobiotic mice. Gnotobiotic and antibiotics-treated conventional mice were orally infected with *G. lamblia* strains GS/M (assemblage B) or WB (assemblage A). At the indicated times after infection, trophozoite numbers were determined in the SI. Data points represent individual mice, horizontal and vertical gray lines represent geometric means and 95% confidence intervals, and dashed dotted gray lines connect the means of the different time points. The dashed black line depicts the detection limit of the assay. We confirmed that the cohort of mice for these studies had no *Giardia* before infection and these data are included in the graphs as day 0. For graphical clarity, the data are shown as below sensitivity (since the log scale would not allow indication of true zero). Statistical analysis of the early (day 4–7), middle (day 14–19), and late periods (day 28) after infection showed no significant differences for GS/M infection between gnotobiotic vs. conventional mice in the middle and late periods, while the early period showed a significant albeit modest difference [geometric means and 95% confidence intervals of $\log_{10}$ trophozoite numbers of 6.3 (5.9–6.7) vs 5.0 (4.2–5.8); n = 8–9 mice/group, $P < 0.05$, by Mann-Whitney test], which suggested a slightly delayed early colonization with *G. lamblia* GS/M in conventional compared with gnotobiotic mice in these experiments, although other experiments did not support this suggestion (see Fig. 1 and 2).

against *G. lamblia* GS/M or WB infection, greatly enhancing the utility of the gnotobiotic *G. lamblia* infection model.

Effective mucosal immunization of gnotobiotic mice against *G. lamblia*

Given the observation of spontaneous clearance of *G. lamblia* infection in gnotobiotic mice, we next explored whether germ-free mice could be immunized for protection against infection. Prior studies had revealed several conserved antigens in the surface proteome of *G. lamblia* (42). Of these, α1-giardin had proven to be a robustly protective antigen (7, 42), while β-giardin, which is the most highly conserved antigen between the two divergent *G. lamblia* strains GS/M and WB (42), had not been evaluated for protective capacity. Therefore, we expressed recombinant His-tagged β-giardin in *Escherichia coli* and purified it by nickel affinity chromatography (Fig. 4A). Both β-giardin and the previously produced α1-giardin displayed >90% purity by Coomassie Blue-stained SDS-PAGE. Germ-free mice and conventional controls were immunized thrice by intranasal administration of the antigens together with the mucosal adjuvant and cholera toxin and challenged with *G. lamblia* (Fig. 4B). Immunization with α1-giardin conferred marked protection against infection with *G. lamblia* GS/M in both gnotobiotic and conventional mice, as indicated by a 50- to 100-fold reduction in trophozoite load on day 5 (Fig. 4C). In contrast, β-giardin induced no significant protection in either group of mice (Fig. 4C).

Because germ-free mice can be effectively immunized (Fig. 4C) and be infected with divergent *G. lamblia* strains of both human pathogenic lineages (Fig. 2A), they constitute an excellent model for testing cross-protection of vaccine antigen candidates against diverse strains of *G. lamblia*. The recombinant α1-giardin used for the present studies was based on the genome sequence of the assemblage B strain GS/M (42), so we asked whether this form of α1-giardin can confer cross-protection against the assemblage A strain, WB. Immunization of germ-free mice with GS/M-derived α1-giardin induced significant protection against *G. lamblia* WB infection, albeit the ~1 log reduction in peak trophozoite load was less than the ~2 log reduction seen for *G. lamblia* GS/M

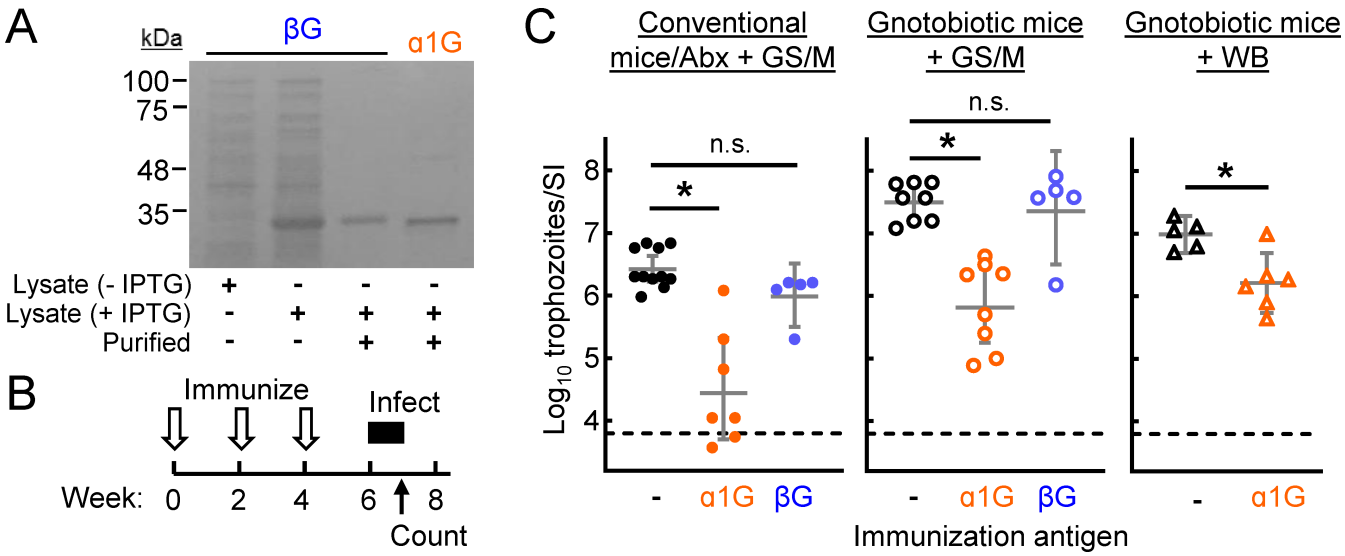

FIG 4 Mucosal immunization of gnotobiotic mice. (A) Recombinant His-tagged forms of β-giardin (βG) and α1-giardin (α1G) were produced in *E. coli* after induction with isopropyl-β-d-thiogalactopyranoside (IPTG), purified by nickel affinity chromatography, and visualized by SDS-PAGE and Coomassie Blue staining. (B) Immunization and infection schedule. (C) Conventional mice given antibiotics (Abx) and gnotobiotic mice were intranasally immunized three times over 4 weeks with α1G or βG or left non-immunized (–). Two weeks after the last immunization, mice were orally challenged with *G. lamblia* strains GS/M (assemblage B) or WB (assemblage A). After 5 days, trophozoites were enumerated in the SI. Each data point represents an individual animal, gray lines show geometric means and 95% confidence intervals, and the dashed black line shows the detection limit of the assay. *$P < 0.05$ vs non-immunized controls (Kruskal-Wallis test with Dunn's post hoc test in left and middle panels or Student's *t*-test in right panel); n.s., not significant.

(Fig. 4C). Taken together, these findings indicate that germ-free mice can be effectively immunized against *G. lamblia* infection and be used as a model for testing cross-protection of candidate vaccine antigens against diverse strains of the parasite.

CD4 T cells are critical for mucosal immunization of gnotobiotic mice against *G. lamblia*

To explore potential immune effectors of immunization-induced protection of gnotobiotic mice against *G. lamblia*, we first determined antibody titers against the immunizing antigen, since B cells and their products have been shown to contribute to immune defense against the parasite (28). In conventional mice, immunization induced a strong plasma IgG response to α1-giardin and a weak IgG response to β-giardin, while neither antigen elicited specific plasma IgG in germ-free mice (Fig. 5A). Immunization, particularly of gnotobiotic mice, appeared to modestly increase the total plasma IgG level, which may be related to the use of the immune-stimulatory adjuvant cholera toxin, but the increases were not accompanied by increased levels of antigen-specific antibodies (Fig. 5A). Similarly, no antigen-specific secretory IgA antibodies were observed after immunization in conventional or gnotobiotic mice (Fig. 5B). Although we have not comprehensively excluded any possible involvement of B cells, our findings did not suggest an important role for B cell effectors in immunization-induced protection in gnotobiotic mice.

In the apparent absence of B cell involvement, we next investigated the role of T cells, particularly CD4 T cells, in the immunization-induced protection of gnotobiotic mice. Prior work had shown that these cells are important in immunity to *G. lamblia* (43), although development of specific CD4 T cell subsets may be compromised in germ-free mice (44). Germ-free mice and conventional controls were immunized intranasally with the protective antigen α1-giardin, and CD4 T cells were subsequently ablated by systemic injection of the cytotoxic rat monoclonal anti-CD4 antibody, GK1.5 (45). CD4 T cell-depleted mice and controls treated with normal rat IgG were challenged with *G. lamblia* GS/M, and trophozoite load was determined after 5 days. CD4 T cell depletion completely abrogated immune protection in both gnotobiotic and conventional mice (Fig. 5C). These results show that CD4 T cells are critical for immune protection in mucosally immunized gnotobiotic mice, underlining that these mice can mount an adaptive immune response that is protective against the lumen-dwelling mucosal pathogen, *G. lamblia*.

DISCUSSION

Limited infectivity in murine models of *G. lamblia* has been a challenge for studies of the infectious pathogenesis of this unique lumen-dwelling protozoan pathogen. Prior work had identified only limited strains, including GS/M and H3, of human-pathogenic *G. lamblia* that could infect mice (30, 31, 34). Our studies confirm that several strains other than GS/M are not infectious in conventional mice with an intact microbiota. Antibiotic pretreatment can render mice more susceptible to infection as shown in prior work (32), including in studies with the H3 strain (34), and confirmed here, hinting at the importance of the microbiota for limiting infectivity. The current work directly demonstrates this gate-keeping role of the microbiota, since germ-free mice could be infected with all of the tested divergent strains of *G. lamblia*. These findings indicate that the observed lack of murine infectivity with *G. lamblia* is not an intrinsic property of most strains but relates to the existing murine microbiota exerting a form of intestinal colonization resistance to *G. lamblia* as an external microbial invader. This resistance can be partly overcome by reducing the microbiota through oral treatment with broad-spectrum antibiotics but only in a selective fashion for a subset of *G. lamblia* strains. The mechanisms of colonization resistance remain to be determined but are not likely to involve defects in host development since resistance could be rapidly conferred by colonizing adult germ-free mice with a conventional microbiota. Competition for scarce nutrient resources in the lumen (36, 46), bacterially produced bacteriocins that inactivate *G.*

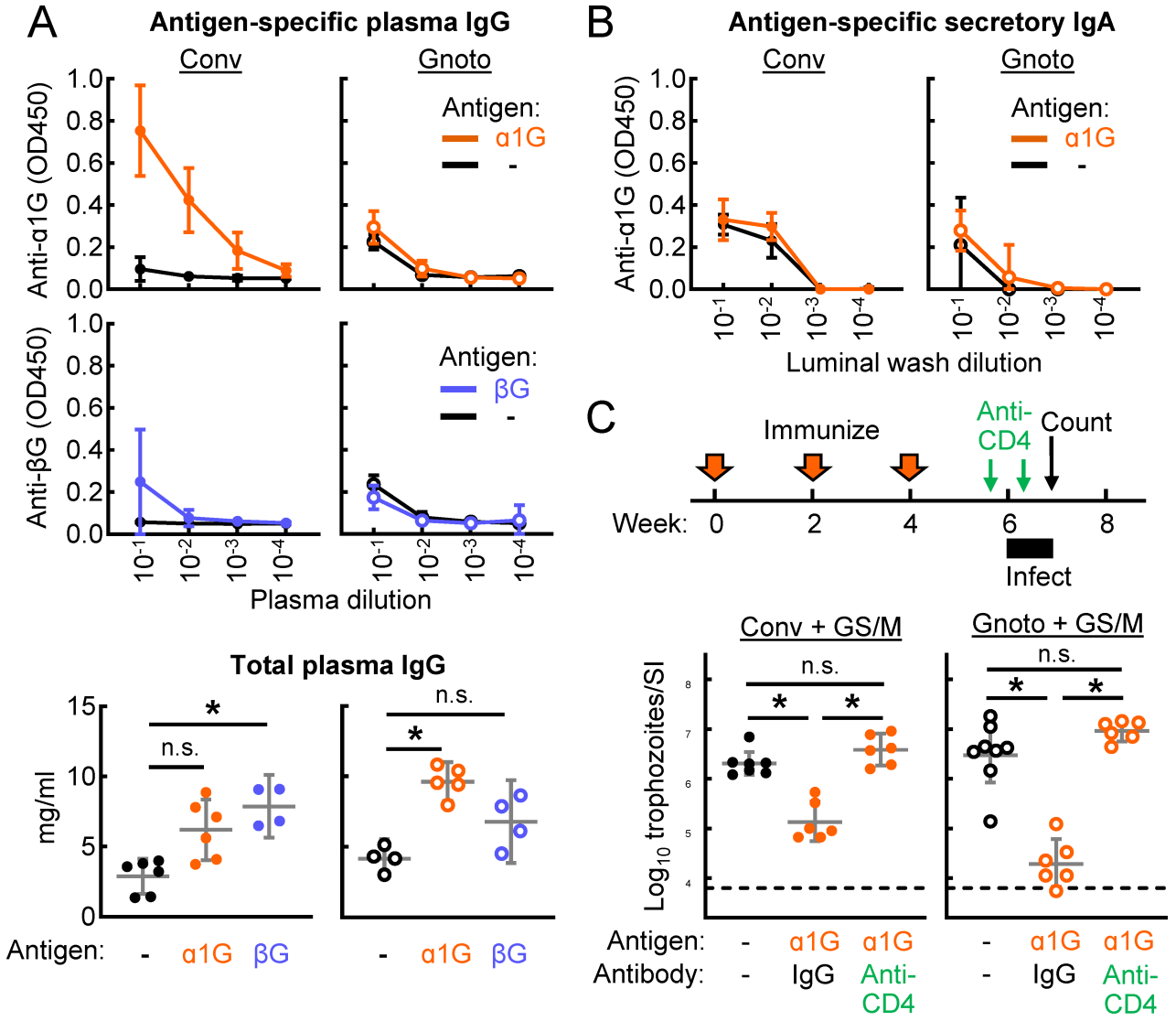

FIG 5 Immune effectors of immunization-induced protection against *G. lamblia* in gnotobiotic mice. Conventional mice treated with antibiotics (Conv) and gnotobiotic mice (Gnoto) were immunized intranasally three times with the antigens, α1-giardin (α1G) or β-giardin (βG), or left non-immunized (–). (A) Plasma was obtained from immunized mice and non-immunized controls from the same initial cohort before *G. lamblia* challenge and tested by enzyme-linked immunosorbent assay for IgG antibodies against the immunizing antigens (top panels). Plasma samples were assayed for total IgG by ELISA (bottom panels). (B) Mucosal washes were obtained from the small intestine of mice immunized with α1G and non-immunized control mice (–) and tested for specific IgA antibodies against the immunizing antigen. Specific antibody levels are shown as means and 95% confidence intervals of the absorbance readings at 450 nm (OD450, n = 3–4 mice/group), while total IgG levels are shown for individual animals, with gray lines depicting means and 95% confidence intervals. (C) Two weeks after the last immunization, mice were orally inoculated with *G. lamblia* GS/M trophozoites, as indicated in the experimental schedule. One day before and 1 day after infection, mice were injected with the cytotoxic anti-CD4 antibody GK1.5 or rat IgG as a control. After 5 days, trophozoites were enumerated in the small intestine (SI). Each data point represents an individual animal, gray lines represent geometric means and 95% confidence intervals, and the dashed black line shows the detection limit of the assay. *P < 0.05 (Kruskal-Wallis test with Dunn's post hoc test); n.s., not significant.

lamblia trophozoites (47), or stimulation of host immune defenses (48) could potentially all contribute, alone or in combination, to the colonization resistance against *G. lamblia* (49). By comparison, murine infections with the closely related protozoan *Giardia muris* do not require antibiotic conditioning (28), suggesting that this murine parasite may have adapted to the normal microbiota of mice, so that potential resistance mechanisms such as nutrient competition or bacteriocin production have little impact on its growth and survival in the small intestine.

Previous studies had indicated that germ-free mice have selective intestinal immune defects, including reduction of intestinal dendritic cell subsets (50), attenuated mucosal IgA responses (51), and diminished Th17 development and IL-17 secretion (27). We also observed a decreased antibody response to mucosal immunization, confirming that germ-free mice have an attenuated immune response. Both IgA and IL-17 can contribute to host defense against *Giardia* (24, 25, 28), making it plausible that germ-free mice could be compromised in their anti-giardial defense. However, our findings demonstrate that gnotobiotic mice could clear *G. lamblia* infection without delay and could be effectively protected against infection by mucosal immunization. It remains possible that the mice had low levels of infection below the detection limit of our assays which might contribute to spread to other individuals. Nonetheless, trophozoite clearance by >99.9% strongly implies that the immune defects in germ-free mice have apparently little impact on the adaptive immune response that is relevant for controlling and eradicating intestinal infection with *G. lamblia* GS/M or WB, although another strain, H3, has been reported to cause long-term colonization of mice (34). CD4 T cells, which are required for giardial clearance of conventional mice (43), were also critical in mediating immunization-induced immune protection in our gnotobiotic mouse studies. The proficient intestinal immune response to *G. lamblia* under gnotobiotic conditions may indicate either that development and maturation of the necessary immune cells and competencies are constitutive and independent of ongoing microbial colonization or stimulation or that they can be rapidly activated from a dormant state by mucosal immunization or giardial infection.

Previous work had identified several conserved antigens that elicit protection against *G. lamblia* infection in mice upon mucosal immunization (10, 42, 52). Unexpectedly, we found in the present work that immunization with another vaccine antigen candidate, β-giardin, did not protect against giardiasis in either gnotobiotic or conventional mice. This protein, like several of the protective antigens, is found on the surface of trophozoites (42), so inaccessible cellular localization is not likely to account for the lack of protection. Any unrecognized sequence variations are also not likely to explain the negative findings, since β-giardin is more conserved across genetic assemblages that any other surface protein identified in proteomics studies (42), which is a major reason why it is used as a target gene for diagnostic and genotyping purposes (16). The most plausible explanation may be the apparent poor immunogenicity of the antigen in our models, since specific IgG titers were at least 100-fold lower compared with immunization with the protective antigen α1-giardin under the same conditions. Purification of recombinant β-giardin presented unique challenges since it required denaturing conditions and renaturing before immunization. Although this process was sufficient to render the protein water soluble, it may have not been enough to result in a native protein that was adequately immunogenic. Contrary to our present immunization studies, however, prior work with human reconvalescent sera after giardiasis had identified β-giardin as an immunogenic antigen (53). Similarly, *G. lamblia* infection in mice elicits specific antibodies against β-giardin (54). Thus, native β-giardin can be immunogenic, so it remains possible that a fully immunogenic form of recombinant β-giardin may still confer protection against giardiasis.

Gnotobiotic murine models of *G. lamblia* infections have utility in different areas of giardiasis research. One of them, as shown herein, is vaccine development, as the models allow infection with diverse and divergent *G. lamblia* strains encountered in human infections. Vaccine antigen candidates can be tested for efficacy against a representative and diverse range of such strains, greatly increasing the chances that such antigens will ultimately prove to be broadly effective in clinical studies. Similarly, development of new drugs against giardiasis could benefit from *in vivo* testing against different *G. lamblia* isolates, including those resistant to existing antigiardial drugs (11). Another research area that can benefit from gnotobiotic giardiasis models is the exploration of the triangular interactions between *Giardia*, commensal microbiota, and the host. For example, associating germ-free mice with defined commensals and other bacteria

can facilitate the testing of specific hypotheses such as the importance of bacteriocin production (47) or nutrient consumption for giardial colonization and host metabolism (34, 36). Conversely, the impact of *Giardia* on bacterial microecology, such as biofilm formation (55), or on host functions, including morphologic changes or mucin expression (37, 56, 57), could be examined under simplified conditions with defined bacterial species. Colonization of germ-free mice can be done with giardiasis-associated, dysbiotic microbiota to determine the impact on intestinal physiology (36, 37, 58). Finally, the gnotobiotic giardiasis models offer new opportunities for exploring mucosal immune development and function, since infection is limited strictly to the intestinal lumen and epithelial surface and induces an effective intestinal adaptive immune response in the absence of a microbiota and without overt mucosal inflammation (59).

MATERIALS AND METHODS

G. lamblia isolates and cultivation

The following *G. lamblia* strains were used: assemblage A strains WB (ATCC 50803) (60), BRIS/83/HEPU/106 (106) (7, 61) and BRIS/83/HEPU/713 (713) (7, 61) and assemblage B strains GS/M clone H7 (GS/M; ATCC 50581) (2) and BRIS/91/HEPU/1279 (1279) (62). Parasites were grown in modified TYI-S-33 medium with 100 µg/mL each of penicillin and streptomycin (63) to no more than 80% confluence.

Mice

Adult (6–12 weeks) male and female germ-free C57BL/6NTac mice were obtained from Taconic Biosciences and bred in the UC San Diego Gnotobiotic Facility as previously described (64). Briefly, breeding colonies of germ-free mice were routinely housed in flexible film isolators (64). For *G. lamblia* infections, mice were transferred to individual ventilated cages (Sentry SPP Mouse, Allentown) and handled in a Class II laminar flow cabinet with sterile gloves and surgical attire. Germ-free status of the animals was routinely confirmed by plating fecal homogenates on tryptic soy blood agar and Columbia blood agar and incubating the plates for up to 3 days at 37°C under both aerobic (room air) and anaerobic (AnaeroPack System, Mitsubishi) conditions (64). Microbial culture techniques have proven to be highly sensitive and relevant for detection of contaminations, practically superior to PCR determination of bacterial DNA in stool (whose thresholds are unreliable), and more sensitive than microscopy-based methods (65, 66). Conventional male and female (6–12 weeks) C57BL/6 mice (Jackson Laboratory) were used as controls. For the association of germ-free mice with a conventional microbiota, adult (8–12 weeks) germ-free mice were co-housed with conventional mice at a 1:1 ratio (four mice/cage) for 2 weeks before *G. lamblia* infections. In preliminary studies, we confirmed that microbial colonization of germ-free mice reached a plateau within 7 days of initial microbial exposure, as determined by CFU assays of fecal pellets [geometric means and ranges of log10 CFU/g feces after 7 days of exposure: 10.0 (range 9.8–10.2) under aerobic and 10.8 (10.6–11.1) under anaerobic culture conditions, compared with 8.2 (7.4–8.7) and 10.2 (8.7–11.0), respectively, in conventional control mice; $n \geq 3$ mice]. Establishment of microbial colonization was also confirmed upon autopsy by the disappearance of the mega-cecum characteristic for germ-free mice. All animal studies were reviewed and approved by the University of California San Diego Institutional Animal Care and Use Committee.

G. lamblia infections and evaluations

Conventional mice were treated for 1 week before and throughout the infection with an antibiotic cocktail of 1.4 mg/mL neomycin, 1 mg/mL ampicillin, and 1 mg/mL vancomycin in the drinking water. Water containing the antibiotic cocktail was replaced every 3–4 days. Efficacy of the antibiotic treatment was assessed by collecting fecal pellets from individual mice and plating serial dilutions of the fecal homogenates onto tryptic

soy blood agar and Columbia blood agar. Plates were incubated for 24–48 h at 37°C under aerobic (room air) and anaerobic (AnaeroPack System) conditions, and CFU were determined and related to stool weight. The cocktail of antibiotics (100 µg/mL each) had no impact on the *in vitro* growth of *G. lamblia* strains GS/M or WB (doubling times over 48 h: GS/M controls, 11.7 ± 2.4 h, GS/M + cocktail, 11.4 ± 2.0 h; WB controls, 9.7 ± 1.4 h, WB + cocktail, 9.4 ± 1.1 h; means ± SD, $n = 3$ separate experiments). For *Giardia* infections, *G. lamblia* trophozoites were grown to mid-logarithmic phase and administered in TYI-S-33 growth medium with penicillin and streptomycin by oral gavage to mice (10^6 trophozoites in a 200 µL volume). Trophozoite preparations were tested for bacterial contamination by plating samples onto tryptic soy blood agar, followed by incubation under aerobic conditions. At different times after infection, mice were euthanized by controlled CO_2 inhalation and the small intestine was removed, opened longitudinally, placed into 5 mL PBS, and cooled on ice for 10 min. After vigorous shaking of samples, live trophozoites were counted in a hemocytometer.

For evaluation of cyst excretion, fecal pellets were collected from individual mice, weighed, and fixed in 10% formalin for 24 h at 4°C to preserve and inactivate the cysts. Subsequently, samples were homogenized, washed twice with PBS by centrifugation and resuspension, and stained for 30 mins at room temperature with a 1:100 dilution of FITC-labeled polyclonal goat anti-*Giardia* antibody (LSBio) in PBS with 1% BSA. After three washes with PBS, samples were resuspended in 200 µL PBS and cysts were manually counted in a hemocytometer using a fluorescence microscope.

For depletion of CD4-positive cells, mice were injected intraperitoneally twice, 1 day before and 1 day after *G. lamblia* infection, with 200 µg/mouse of the monoclonal rat IgG2b anti-CD4 antibody, GK1.5 (BioXcell) (45), or with purified rat IgG (BioLegend) as a control.

Production of recombinant proteins

Sequences of α1-giardin and β-giardin were obtained from the published genome sequence of *G. lamblia* GS/M (13), and cDNAs were generated by total gene synthesis with codon optimization for *Escherichia coli* (GenScript Biotech). Genes were inserted into the *Nde*I-*Xho*I cloning sites of the bacterial expression vector pET-15b (Millipore Sigma), and vectors were transformed into T7 Express lysY/Iq Competent *Escherichia coli* following the manufacturer's protocol (New England BioLabs). Cells were grown at 37°C in Luria-Bertani broth with carbenicillin until they reached an optical density at 600 nm (OD600) of 0.4–0.6. Protein expression was induced by the addition of 1 mM isopropyl-β-d-thiogalactopyranoside for 2–3 h. Recombinant proteins were purified from bacterial lysates by nickel affinity chromatography (HisPur Ni-NTA Resin, Thermo Scientific). Non-denaturing conditions were used for α1-giardin purification as described before (42), whereas β-giardin purification was not successful under these conditions and instead required denaturing conditions and subsequent renaturing using sequential dialysis with 0.3 M guanidine hydrochloride in PBS followed by dialysis with 0.1 M arginine, 50 mM NaCl, 10 mM phosphate, and pH 7.0 in PBS. Recombinant proteins were stored at 4°C at a concentration of >1 mg/mL 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 recombinant proteins was analyzed by SDS-PAGE.

Antigen immunizations

For intranasal immunizations, mice were placed under anesthesia with ketamine and xylazine, and a small volume (6 µL) of a mixture of antigen (30–40 µg) and cholera toxin (2–4 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 2 weeks apart. Infection challenges were done 2 weeks after the last immunization. To assess antibody levels, mice were bled before infection via the tail vein into ethylenediaminetetraacetic acid containing tubes and

plasma was prepared by centrifugation. Antibody levels against the immunizing antigens were determined by ELISA as described before (54).

Statistical analysis

An unpaired *t*-test, Mann-Whitney U test, or Kruskal-Wallis test with Bonferroni correction and the appropriate post hoc tests, as appropriate and indicated in the figure legends, were used to compare results between animal groups using GraphPad Prism. Results from male and female mice were not significantly different and were combined. Data of all infections are shown from individual mice (i.e., biological replicates), with geometric means and 95% confidence intervals included. Antibody data are shown as means ± 95% confidence intervals of the results of individual mice. Differences with P values of < 0.05 were considered statistically significant.

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