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Authors

Zhou, Jiarong
Miyamoto, Yukiko
Ihara, Sozaburo
et al.

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BRIEF REPORT

Codelivery of Antigens and Adjuvant in Polymeric Nanoparticles Coated With Native Parasite Membranes Induces Protective Mucosal Immunity Against *Giardia lamblia*

Jiarong Zhou,[1] Yukiko Miyamoto,[2] Sozaburo Ihara,[2,3] Ashley V. Kroll,[1] Noelle Nieskens,[2] Vivien N. Tran,[2] Elaine M. Hanson,[2] Ronnie H. Fang,[1] Liangfang Zhang,[1] and Lars Eckmann[2]

[1]Department of NanoEngineering and Moores Cancer Center, University of California San Diego, La Jolla, California, USA, [2]Department of Medicine, University of California San Diego, La Jolla, California, USA, and [3]Division of Gastroenterology, Institute for Adult Diseases, Asahi Life Foundation, Tokyo, Japan

The protozoan pathogen *Giardia lamblia* is an important worldwide cause of diarrheal disease and malabsorption. Infection is managed with antimicrobials, although drug resistance and treatment failures are a clinical challenge. Prior infection provides significant protection, yet a human vaccine has not been realized. Individual antigens can elicit partial protection in experimental models, but protection is weaker than after prior infection. Here, we developed a multivalent nanovaccine by coating membranes derived from the parasite onto uniform and stable polymeric nanoparticles loaded with a mucosal adjuvant. Intranasal immunization with the nanovaccine induced adaptive immunity and effectively protected mice from *G. lamblia* infection.

Keywords. protozoa; giardiasis; intestinal mucosa; nanovaccine; biomimetic nanoparticle; multivalent vaccine.

Giardia lamblia is a major worldwide cause of diarrheal disease, characterized by epigastric pain, malabsorption, and malnutrition [1]. Infection occurs mostly by the fecal-oral route, particularly through consumption of contaminated water [2]. Giardiasis in children is a leading cause of delayed development and cognitive impairment, and the infection can cause protracted postinfectious syndromes in adults [3–5]. Established infection is managed with antimicrobial drugs, particularly metronidazole [4], but drug resistance and treatment failures are a clinical challenge [6]. A crude veterinary vaccine was developed and shown to be effective at reducing symptoms in cats and dogs [7], but commercial production has been discontinued. A human vaccine has not been realized. Immunization with α1-giardin, a highly conserved, membrane-associated *G. lamblia* antigen, induces protection in mice, although protection is weaker than after prior infection, suggesting that a single antigen may be insufficient for maximal protection [8]. An experimental vaccine utilizing multiple variant surface proteins (VSPs) from a transgenic *G. lamblia* strain has shown promise in domestic animals [9], but the VSP repertoires of the genetically diverse human-pathogenic assemblages are largely nonoverlapping, making the production of a tractable vaccine challenging.

Biomimetic nanoparticles have recently emerged as attractive systems to enhance the potency and breadth of conventional therapies [10]. Advantages of nanoparticles for vaccine applications include the ability to protect encapsulated payloads from degradation, target specific immune subsets, and drain efficiently into lymphatic tissues [11]. In particular, cell membrane coating technology has enabled a facile approach for the design of multivalent nanovaccines to treat diseases such as cancer and bacterial infections [12–14]. By stripping the outer membrane layer from cells and coating it onto synthetic nanomaterials, this approach to nanoparticle functionalization faithfully preserves native surface antigens and bypasses the burdensome task of antigen identification and validation [10, 11]. Given the compositional and structural similarity of *G. lamblia* to mammalian cell membranes, we leveraged this technology to engineer a parasite membrane-coated nanovaccine for mucosal delivery to protect against *G. lamblia* infection.

METHODS

G. lamblia Strains

G. lamblia WB clone C6 (assemblage A, American Type Culture Collection [ATCC] 50803) and GS/M clone H7 (GS/M; assemblage B, ATCC 50581) were grown in modified TYI-S-33 medium to <80% confluence before membrane harvest and murine infections.

Nanoparticle Fabrication and Characterization

For nanoparticle synthesis, poly(lactic-*co*-glycolic) acid (PLGA) dissolved in acetone was added into water, after which the organic solvent was evaporated under vacuum. Parasite membranes were prepared by hypotonic lysis and mechanical disruption. Nanoparticles were mixed with parasite membranes at a 1:1 membrane protein to polymer ratio and sonicated in a bath sonicator for 4 minutes at room temperature. Polyethylene glycol (PEG)-coated nanoparticles were used as controls. CpG-loaded nanoparticles were fabricated as reported [12]. Size, zeta potential, and morphology of the nanoparticles were determined by dynamic light scattering and transmission

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Correspondence: Lars Eckmann, MD, University of California San Diego, Department of Medicine, 9500 Gilman Drive, La Jolla, CA 92093-0063 (leckmann@ucsd.edu).

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electron microscopy (TEM). Technical details are provided in the Supplementary Materials.

Mouse Immunizations and Challenges

Adult BALB/cJ mice were intranasally immunized with nanoparticles or α1-giardin [8] and a mucosal adjuvant 3 times 2 weeks apart. Two weeks after the last immunization, plasma antibody titers were determined by enzyme-linked immunosorbent assay (ELISA) using lysates from *G. lamblia* GS/M trophozoites as antigen. For challenges, trophozoites grown to mid-log phase were administered by oral gavage (10^6 in 200 µL). After 5 days, the small intestine was removed, opened, cooled on ice, and agitated. Live trophozoites were counted in a hemocytometer. Fecal cyst output was determined in stool homogenates by staining with fluorescein isothiocyanate-labeled polyclonal goat anti-*Giardia* antibody and enumeration by fluorescence microscopy. Results from male and female mice were not significantly different and were combined. For technical details, see Supplementary Materials. Animal studies were reviewed and approved by the University of California San Diego Institutional Animal Care and Use Committee.

RESULTS

Membranes were isolated from the GS/M strain of *G. lamblia* by hypotonic and mechanical lysis of trophozoites grown to mid-log phase, followed by differential centrifugation (Figure 1A). High-energy sonication of the isolated membranes with bare PLGA nanoparticle cores led to the formation of *G. lamblia* (GL) membrane-coated nanoparticles (GL/GSM-NP for designation of parasite and strain used during fabrication) by self-assembly. The coating process is guided by electrostatic interactions and a preferential drive towards lower energy states. Characterization by dynamic light scattering revealed that the nanoparticles had an approximately 40 nm size increase after coating due to the addition of the parasite membrane layer (Figure 1B). Furthermore, the surface zeta potential of GL/GSM-NP was similar to that of purified membranes and markedly less negative than that of uncoated PLGA cores, indicating that the surface of GL/GSM-NP was covered by parasite membranes. Visualization with TEM confirmed that nanoparticles were coated with a thin layer of membrane (Figure 1C). Immunoblot analysis of a representative membrane-associated protein, α1-giardin [8], showed the protein in purified parasite membranes and GL/GSM-NP, but not in control nanoparticles coated with red blood cell membranes (Figure 1D). Finally, we determined that GL/GSM-NP could be lyophilized for long-term storage (>1 month) without significant change in size upon reconstitution (Figure 1B). Overall, these results demonstrate that stable and uniformly sized nanoparticles could be readily and reliably fabricated to display membrane-associated proteins found on the surface of *G. lamblia* trophozoites.

We next sought to investigate the protective potential of GL/GSM-NP. BALB/c mice were intranasally immunized with 60

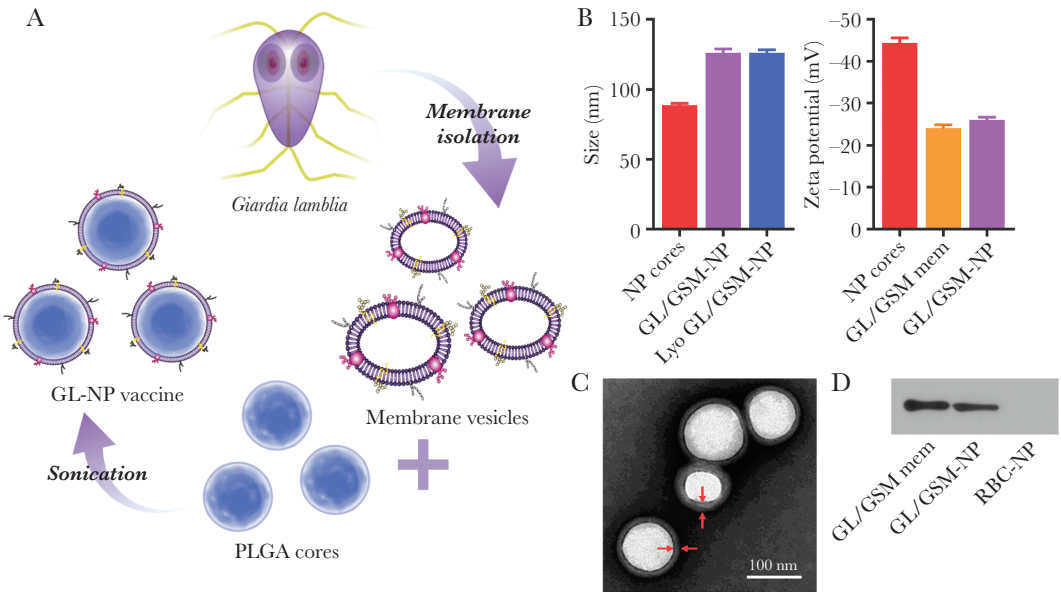

Figure 1. Construction and characterization of a *Giardia lamblia* membrane-coated nanovaccine. *A*, Log-phase *G. lamblia* trophozoites are disrupted by mechanical lysis under hypotonic conditions, and membranes are collected by ultracentrifugation. Isolated membranes are mixed with polymeric nanoparticle cores made from poly(lactic-*co*-glycolic) acid (PLGA), and upon high-energy sonication, membranes self-assemble onto the cores to yield *G. lamblia* membrane-coated nanoparticles (GL-NP). The nanoparticle cores can be preloaded with adjuvants before membrane coating. *B*, Size (left) of bare PLGA cores (NP cores), nanoparticles coated with membranes from *G. lamblia strain* GS/M (GL/GSM-NP), and GL/GSM-NP reconstituted after lyophilization (Lyo GL/GSM-NP) and surface zeta potential (right) of nanoparticle cores, isolated membranes before coating (GL/GSM mem), and GL/GSM-NP were determined by dynamic light scattering. Data are shown as mean + SD, $n = 3$. *C*, Representative TEM image of GL/GSM-NP negatively stained with uranyl acetate. The membrane layer is outlined by red arrows. *D*, Immunoblot analysis of the membrane-associated protein α1-giardin in GL/GSM mem, GL/GSM-NP, and control nanoparticles coated with human red blood cell membranes (RBC-NP).

μg of GL/GSM-NP together with 5 μg of the mucosal adjuvant cholera toxin (CTX), which is commonly used as a gold standard for preclinical studies. An inert PEG-coated nanoparticle (PEG-NP) was included as a control. After 3 immunizations, plasma immunoglobulin G (IgG) antibodies against GS/M trophozoite lysates were markedly elevated in mice immunized with GL/GSM-NP, while mice given PEG-NP had only background antibody levels (Figure 2A). Subsequent oral challenge with GS/M trophozoites showed significant protection (>3 logs) of GL/GSM-NP immunized mice at the peak of infection compared to naive mice or mice given the PEG-NP controls (Figure 2B).

G. lamblia displays significant genetic heterogeneity, as indicated by distinct genetic assemblages. Human-pathogenic strains fall into assemblages A and B, so we explored whether a combination nanovaccine could be made that encompasses strains from both assemblages. We isolated membranes from both the GS/M strain (assemblage B) and the WB strain (assemblage A) and fabricated nanoparticles with an equal mixture of the two (GL/Pool-NP). Immunization with GL/Pool-NP together with CTX induced high levels of *G. lamblia*-specific IgG antibodies (Figure 2A). Oral challenge of immunized mice with live trophozoites revealed marked protection that was even greater than after immunization with GL/GSM-NP and CTX (Figure 2B). Most (6 of 7) of the GL/Pool-NP immunized mice had no detectable parasites, suggesting that the mixture of different *G. lamblia* strains could confer near complete protection against infection. By comparison, immunization with

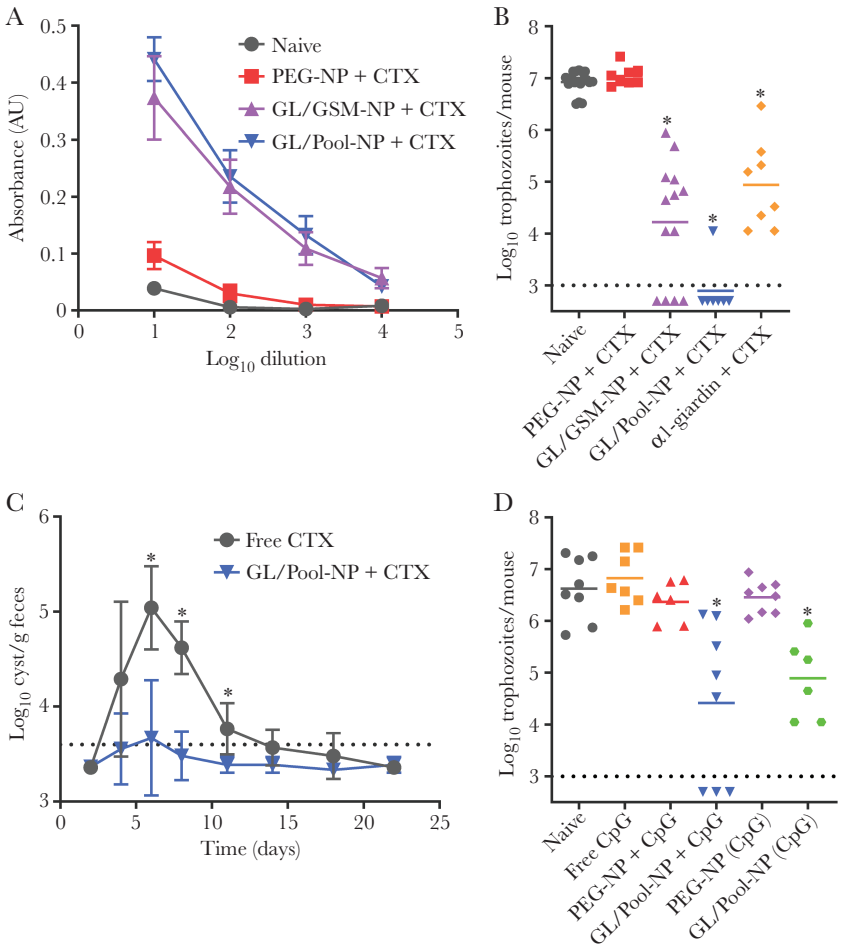

Figure 2. Immunogenicity and protective capacity of *Giardia lamblia* membrane-coated nanovaccines. *A–C*, Adult BALB/c mice were given 3 intranasal immunizations 2 weeks apart of the indicated nanoparticle (NP) formulations (PEG-NP, control NPs coated with polyethylene glycol; GL/GSM-NP, NPs coated with membranes from *G. lamblia* GS/M only; GL/Pool-NP, NPs coated with a 1:1 mixture of membranes isolated from *G. lamblia* strains GS/M and WB) or purified α1-giardin together with cholera toxin (+ CTX). *A*, Two weeks after the last immunization, plasma IgG titers against *G. lamblia* GS/M lysates were determined by ELISA. Data are shown as mean ± SD, $n \geq 6$ for each group. *B*, The indicated groups of mice were orally challenged with *G. lamblia* GS/M trophozoites, and live trophozoites in the small intestine were enumerated after 5 days. Each data point represents an individual animal. Horizontal bars show means. *$P < .01$ versus naive controls (one-way ANOVA). *C*, Immunized and control animals were orally challenged with *G. lamblia* GS/M trophozoites, and fecal cyst excretion was monitored at the indicated time points by immunostaining and counting. Data are shown as mean ± SD, $n \geq 5$ for each group. *$P < .01$ (Student's unpaired *t*-test). *D*, Mice were intranasally immunized with CpG only (free CpG) or various NP formulations together with free CpG (+ CpG), or the indicated nanoparticles with preloaded CpG [NP(CpG)]. Mice were challenged with *G. lamblia* GS/M trophozoites, and live trophozoites in the small intestine were enumerated after 5 days. Each data point represents an individual animal. Horizontal bars show means. *$P < .01$ versus naive controls (one-way ANOVA).

α1-giardin and CTX also conferred protection against GS/M challenge but to a significantly lesser degree (mean $\log_{10}$ counts of 4.95 for α1-giardin vs < 3 for GL/Pool-NP; $P < .0001$; Figure 2B), suggesting that multiple antigens present in the nanoparticle membrane coating act in synergistic fashion. Furthermore, time course analysis of fecal cyst shedding showed reduced peak levels on day 6 in GL/Pool-NP immunized mice compared to CTX controls, and clearance by 11 days compared to day 22 in the controls (Figure 2C). These results indicate that peak infection, infection duration, and cumulative infectious load were all markedly decreased in immunized animals.

The murine infection model relies on antibiotics conditioning to achieve consistent high-level infection. To determine whether this regimen affected nanovaccine efficacy, we conducted immunization studies without antibiotics treatment. As expected, infection in CTX-treated control animals was lower than in mice pretreated with antibiotics, confirming that the microbiota can influence *G. lamblia* susceptibility. Nonetheless, GL/Pool-NP immunization of mice not treated with antibiotics conferred complete protection against GS/M challenge (Supplementary Figure 1), indicating that antibiotics did not impact vaccine efficacy.

Given the strong protective effect of the nanovaccine, we next investigated whether the model adjuvant, CTX, which showed excellent activity but is not suitable for human use, could be replaced with one that has greater clinical potential. A major advantage of nanovaccines is that adjuvants and antigens can be incorporated together to achieve colocalized delivery to antigen-presenting cells, thus promoting strong immune activation against the appropriate antigenic targets [11]. In initial tests, we found that coadministration of GL/Pool-NP and CpG oligodeoxynucleotides, which are Toll-like receptor 9 agonists that have shown clinical promise as adjuvants, induced high *G. lamblia*-specific plasma IgG levels (Supplementary Figure 2), underlining the utility of CpG as a mucosal adjuvant. Subsequently, we fabricated a nanoformulation that encapsulates a murine-active CpG inside a *G. lamblia* membrane-coated nanoparticle, GL/Pool-NP(CpG). Encapsulation of CpG into the core protects the agonist from degradation without compromising adjuvanticity. Intranasal administration of GL/Pool-NP(CpG) without additional free adjuvant induced *G. lamblia*-specific plasma IgG, albeit at somewhat lower levels than after coadministration of GL/Pool-NP and CpG (Supplementary Figure 2). This difference in IgG induction efficiency may be related to the lower CpG dose after nanoencapsulation compared to CpG coadministration (0.45 μg vs 5 μg, respectively), because current strategies for loading of CpG into the hydrophobic PLGA core remain to be optimized. Nonetheless, despite an approximately 10-fold difference in adjuvant dose, immunization with the consolidated GL/Pool-NP(CpG) formulation achieved marked protection (approximately 2 log) comparable to coadministration of GL/Pool-NP and free CpG (Figure 2D). These results demonstrate the excellent potential of a unified nanoparticulate delivery system that incorporates both antigen and adjuvant for effective mucosal vaccination against *G. lamblia*.

DISCUSSION

We have developed a multivalent parasite membrane-coated nanovaccine to protect against *G. lamblia* infection. By presenting parasite membrane antigens to the immune system in an immunostimulatory context, the nanovaccine was able to elicit strong *G. lamblia*-specific immune responses. Membrane coating enables antigenic material to be preserved in its native form, leading to improved antigenicity compared to heat-denatured or recombinant antigens, which should lead to higher avidity antibodies that are more protective against infection. Parasite membrane-coated nanovaccines are inherently multiantigenic, thereby eliciting broader immune responses and better protection than can be achieved with single antigen vaccines [11], consistent with the greater protection afforded by the new nanovaccine compared to the single antigen α1-giardin. The specific underlying immune mechanisms remain to be established but it is likely that vaccine-induced generation of a broad set of T cells with specificity against different antigens is involved. Importantly, our platform does not require time-consuming and costly antigen identification or genetic construction of mutant parasites, allowing it to be readily applied to many clinical strains of the parasite, either alone or in combination. This approach can significantly accelerate vaccine development and allow the production of nanovaccines custom-tailored to different and potentially evolving epidemiologic situations.

Our studies have limitations in their focus on quantitative evaluation of parasitic burden, while the potential of the nanovaccine to protect against clinical disease could not be assessed. Standard murine models are unsuitable to address these questions, because mice do not develop the typical manifestations of human giardiasis, although it has been reported that combination of *G. lamblia* infection with other conditions such as protein malnutrition or microbial dysbiosis, or other animal models such as gerbils, are associated with symptoms that mimic specific aspects of human infections [15]. Future studies will have to address the protective capacity of the nanovaccines in these models.

The membrane-coated nanovaccine has a polymeric nanoparticle core that contributes several important functionalities, such as providing the final formulation with improved stability, consistency, and antigen display [11]. Nanoparticle sizes can also be fine-tuned to optimize uptake by antigen-presenting cells and promote efficient lymphatic drainage. Importantly, different adjuvants can be encapsulated inside the nanoparticles, thus strengthening immune activation due to antigen-adjuvant colocalization [11]. The efficacy of existing and future mucosal adjuvants will have to be systematically established, but the

nanovaccine platform could incorporate different adjuvants. Our nanoformulation is a nonliving biocompatible system that can be easily fabricated, exhibits excellent colloidal stability, and can be lyophilized for long-term storage. While cell membrane coating nanotechnology has been leveraged for a variety of biomedical applications [10], our nanoformulation demonstrates that eukaryotic parasite membranes can be used in this application, paving the road for the development of novel vaccine formulations against other important eukaryotic pathogens beyond *G. lamblia*.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Escobedo AA, Almirall P, Robertson LJ, et al. Giardiasis: the ever-present threat of a neglected disease. Infect Disord Drug Targets **2010**; 10:329–48.
2. Huang DB, White AC. An updated review on *Cryptosporidium* and *Giardia*. Gastroenterol Clin North Am **2006**; 35:291–314, viii.
3. Berkman DS, Lescano AG, Gilman RH, Lopez SL, Black MM. Effects of stunting, diarrhoeal disease, and parasitic infection during infancy on cognition in late childhood: a follow-up study. Lancet **2002**; 359:564–71.
4. Gardner TB, Hill DR. Treatment of giardiasis. Clin Microbiol Rev **2001**; 14:114–28.
5. Rogawski ET, Bartelt LA, Platts-Mills JA, et al. Determinants and impact of *Giardia* infection in the first 2 years of life in the MAL-ED birth cohort. J Pediatric Infect Dis Soc **2017**; 6:153–60.
6. Arguello-Garcia R, Leitsch D, Skinner-Adams T, Ortega-Pierres MG. Drug resistance in *Giardia*: mechanisms and alternative treatments for giardiasis. Adv Parasitol **2020**; 107:201–82.
7. Olson ME, Ceri H, Morck DW. *Giardia* vaccination. Parasitol Today **2000**; 16:213–7.
8. Jenikova G, Hruz P, Andersson MK, et al. α1-Giardin based live heterologous vaccine protects against *Giardia lamblia* infection in a murine model. Vaccine **2011**; 29:9529–37.
9. Serradell MC, Saura A, Rupil LL, et al. Vaccination of domestic animals with a novel oral vaccine prevents *Giardia* infections, alleviates signs of giardiasis and reduces transmission to humans. NPJ Vaccines **2016**; 1:16018.
10. Fang RH, Kroll AV, Gao W, Zhang L. Cell membrane coating nanotechnology. Adv Mater **2018**; 30:1706759.
11. Zhou J, Kroll AV, Holay M, Fang RH, Zhang L. Biomimetic nanotechnology toward personalized vaccines. Adv Mater **2020**; 32:1901255.
12. Kroll AV, Fang RH, Jiang Y, et al. Nanoparticulate delivery of cancer cell membrane elicits multiantigenic antitumor immunity. Adv Mater **2017**; 29:1703969.
13. Wei X, Gao J, Wang F, et al. In situ capture of bacterial toxins for antivirulence vaccination. Adv Mater **2017**; 29:1701644.
14. Wei X, Ran D, Campeau A, et al. Multiantigenic nanotoxoids for antivirulence vaccination against antibiotic-resistant gram-negative bacteria. Nano Lett **2019**; 19:4760–9.
15. Bartelt LA, Roche J, Kolling G, et al. Persistent *G. lamblia* impairs growth in a murine malnutrition model. J Clin Invest **2013**; 123:2672–84.