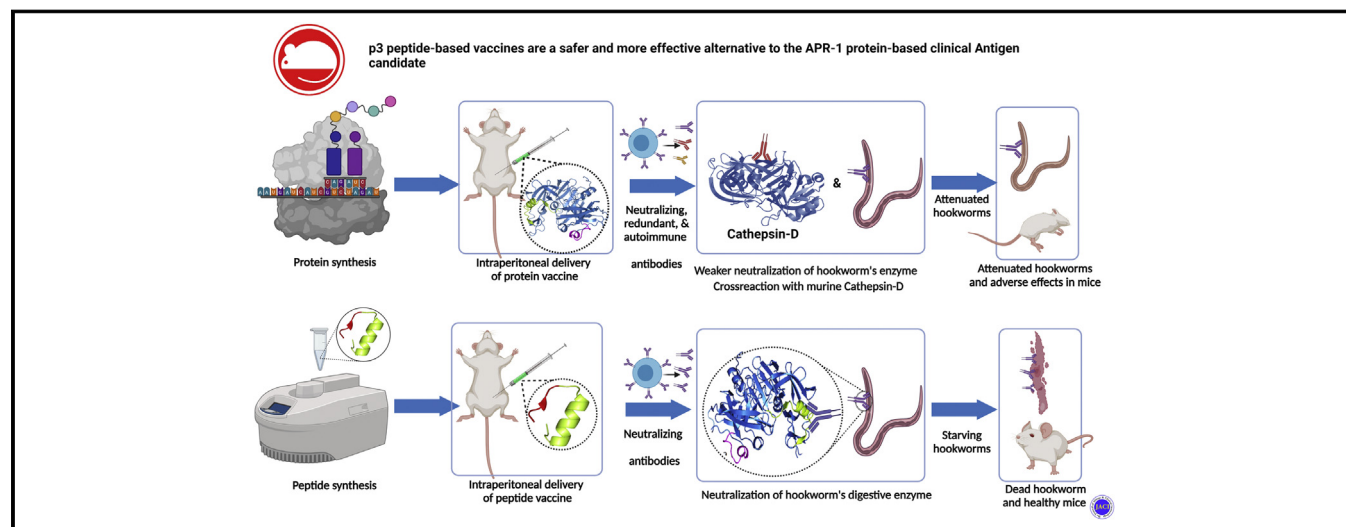


Development of a peptide vaccine against hookworm infection: Immunogenicity, efficacy, and immune correlates of protection



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GRAPHICAL ABSTRACT



Background: Approximately 400 million individuals are infected with hookworms globally. Protective vaccines are needed to prevent reinfections, which often occur after drug treatment in endemic areas. Ideal vaccines are highly efficacious and well tolerated, and do not present risks to patient safety. Peptide vaccines can generate specific, highly protective responses because they focus on minimal antigenic target(s) with a specific immunoprotective mechanism. *Necator americanus* aspartyl protease 1 (*Na*-APR-1) is one of the most promising hookworm vaccine antigens. The neutralizing epitope p3 (TSLIAGPKAQVEAIQKYIGAEL), together with universal the T_H epitope P25 (KLIPNASLIENCTKAEL), has been used previously to produce peptide vaccines and was found to protect BALB/c mice against rodent hookworm infections, resulting in worm burden reductions of up to 98%. However, because of

extensive digestion in the gastrointestinal tract, large oral vaccination doses were necessary to achieve this level of efficacy. **Objective:** We sought to overcome the limitations of oral vaccine delivery and to investigate protective efficacy and immune correlates of protection. Herein, we examined 5 different peptide vaccines following intraperitoneal injection, to compare their efficacy with that of the clinical protein antigen APR-1. **Methods:** BALB/c mice were immunized with p3-P25-based antigen that was adjuvanted with (1) lipid core peptide, (2) polymethyl methacrylate, (3) linear polyglutamine, and (4) branched polyglutamine (BL₁₀), or with (5) CpG/aluminum hydroxide adjuvant (alum)-adjuvanted control and protein-based (6) CpG/alum-adjuvanted *Na*-APR-1. The mice sera, saliva, and feces were sampled for immune response evaluation. The immunized mice were further challenged via hookworm

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Disclosure of potential conflict of interest: M. Skwarczynski and I. Toth are coinventors in a patent application titled "Self-assembling, self-adjuvanting system for delivery of vaccines" filed by The University of Queensland (application no. AU 2020900058). The rest of the authors declare that they have no relevant conflicts of interest.

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larvae infection, and protection was evaluated by conducting intestinal hookworm counts.

Results: BL₁₀ and lipid core peptide generated the highest serum anti-*Na*-APR-1 IgG and fecal anti-APR-1 IgG titers, but only BL₁₀ generated significant fecal anti-*Na*-APR-1 IgA titers. Upon challenge, immunization with CpG/alum-adjuvanted p3-P25, BL₁₀, and lipid core peptide provided the highest worm burden reductions of 75%, 77%, and 59%, respectively, whereas the group immunized with *Na*-APR-1 had only modest worm reduction of 26%. The relationships between serum anti-*Na*-APR-1 IgG, fecal anti-*Na*-APR-1 IgA and IgG, and worm burden reduction were established with R^2 values greater than or equal to 0.9, and the crucial role of both anti-*Na*-APR-1 IgG and IgA responses was identified.

Conclusions: We demonstrated for the first time that p3-based vaccine candidates are safer and can deliver higher protection against hookworm infection compared with the clinical vaccine candidate, *Na*-APR-1. (J Allergy Clin Immunol 2022;150:157-69.)

Key words: Hookworm, infection challenge, intraperitoneal immunization, nanovaccine, peptide vaccine, aspartyl protease

Hookworm parasites plague tropical regions, infecting about half a billion individuals across the globe.^{1,2} The most prevalent species are *Necator americanus* (*N americanus*) and *Ancylostoma duodenale* (*A duodenale*), followed by *Ancylostoma ceylanicum* (*A ceylanicum*).³⁻⁵ Adult worms feed on host blood and establish infection and patency in the small intestine, which can endure for years. Heavy infection/burden in the young, women of reproductive age, or those with compromised nutritional status results in substantial blood loss,^{6,7} leading to debilitating iron-deficiency anemia. Wide-scale infections hamper the productivity and development of affected communities.⁸ Anthelmintic drugs provide a partial solution; however, reinfection can occur immediately after worm expulsion. There is also evidence of reduced cure rates with the mainstay anthelmintic drugs, prompting fear of emerging drug resistance as has been documented for nematode parasites of livestock.⁹⁻¹⁴ Consequently, anthelmintic vaccines are preferable, because they provide the host with long-lasting protection against infection.

Hookworms digest host hemoglobin and serum proteins as their source of nourishment. Aspartyl protease-1 (APR-1) initiates the digestion process in the hookworm gut by cleaving intact host hemoglobin.^{15,16} Neutralization of APR-1 deprives the worms of nourishment and leads to worm burden reduction. Immunization of dogs and hamsters with recombinant APR-1 as a vaccine antigen resulted in 33% and 44% hookworm burden reductions, respectively.^{17,18} Owing to these promising outcomes, *Na*-APR-1 is 1 of 2 leading vaccine antigens being examined in clinical trials.^{19,20} Individuals chronically infected with hookworms have been found to generate antibody titers against non-neutralizing epitopes in the *Na*-APR-1 sequence, for example, S₁₀₇L epitope (SRKCPFYDIACML). A neutralizing epitope (A₂₉₁Y and its extended version p3) was mapped and identified, and antibodies that bound to A₂₉₁Y (AGPKAQVEAIQKY) or p3 (TSLIAGPKAQVEAIQKYIGAEEL) were found to neutralize *Na*-APR-1 enzymatic activity *in vitro*.^{15,16,21,22} Antibodies generated against p3 better recognized *Na*-APR-1 and neutralized the enzyme more efficiently *in vitro* compared with those generated

Abbreviations used

alum:	Aluminum hydroxide adjuvant
APR:	Aspartyl protease
BL ₁₀ :	Branched polyoleucine
CpG-ODN:	Oligodeoxynucleotide adjuvant
LCP:	Lipid core peptide
IgG1:	Subclass 1 of IgG
IgG2a:	Subclass 2a of IgG
LL ₁₀ :	Linear polyoleucine
PBST:	PBS containing 0.05% tween 80
PDI:	Polydispersity index
PMA:	Polymethyl acrylate

against A₂₉₁Y following intraperitoneal immunization of BALB/c mice.²² Furthermore, conjugation of p3 to hydrophobic lipid moieties, such as lipid core peptide (LCP), resulted in increased immunogenicity and enhanced recognition of the parent protein (*Na*-APR-1) by generated antibodies.²² However, an infection challenge was not conducted to evaluate the overall efficacy of these peptide vaccines.²² Furthermore, T_H-cell epitope (P25) (KLIPNASLIENCTKAEL) P25 was included to support uniform humoral response,²³ and it was used in previous peptide-based hookworm vaccine research studies.²⁴⁻²⁷ P25 was derived from the fusion protein of the Morbillivirus canine distemper virus and well proven for its ability to improve humoral immune responses in BALB/c mice.^{23,28}

Our group recently developed additional p3-based vaccines by conjugation of the peptide antigen to polyoleucine and LCP.^{24,25} The vaccines, designed for oral administration, generated modest anti-*Na*-APR-1 and anti-p3 IgG titers, yet oral immunization with both polyoleucine and LCP p3-conjugates resulted in high worm burden reductions (up to 94%).^{24,25} However, orally dosed peptides and proteins are often largely digested in the stomach and intestines by digestive enzymes.^{29,30} Thus, as expected, the oral vaccines had to be administered in high doses (6 × 100 µg per mouse). When the number of doses was reduced (4 × 100 µg per mouse), vaccine efficacy was reduced to 55% to 80% worm burden reductions.²⁶ The efficacy in this study was predominantly attributed to serum anti-*Na*-APR-1 IgG titers, because fecal anti-*Na*-APR-1 IgA titers were low to undetectable. Thus, it is highly likely that injection-based delivery of these vaccines could significantly reduce dose quantity and immunization frequency.

Although oral bioavailability/delivery may be preferable, injectable vaccines still have benefits and are suitable as a comparative control. The immunogenicity of the used adjuvanting moieties is difficult to evaluate following oral administration due to the added complexity of several factors, including oral bioavailability/delivery efficacy and digestion/proteolysis of the delivered antigen. These factors obscure conclusions on the true immunogenicity of peptide vaccines. Therefore, the role and protective mechanisms offered by systemic immunity need to be investigated. And notably, the protein subunit hookworm vaccines that are currently in clinical trials—aluminum hydroxide adjuvant (alum)/monophosphoryl lipid A-adjuvanted glutathione transferase-1 and *Na*-APR-1—are injection-based vaccines.^{19,20} Therefore, injection of novel vaccine candidates provides the best means for comparison between these and the clinically tested *Na*-APR-1 vaccine antigen.

In this study, we aimed to investigate the immunogenicity and protection offered by peptide-based vaccine candidates in

comparison to *Na*-APR-1 protein-based vaccine administered via intraperitoneal injection. To our knowledge, this is the first study comparing the anti-hookworm efficacy of a peptide-based vaccine with clinically tested *Na*-APR-1 protein-based vaccine. Thus, this enables direct comparison of protective efficacy and investigation of the dominant immune responses that correlate with protection, that is, worm and egg burden reductions. To this end, we synthesized the vaccine candidates, evaluated the physicochemical properties, then immunized BALB/c mice ($n = 8$ per group) twice with either peptide vaccine candidates or CpG/alum-adjuvanted *Na*-APR-1 protein as a positive control. Immunized mice were infected with the rodent hookworm, *Nippostrongylus brasiliensis*, and protection was evaluated by intestinal worm counts and fecal egg counts in all groups. Serum, saliva, and fecal samples were evaluated for antigen-specific immune responses to determine the predominant protective immune response(s) that correlated most precisely with worm/egg burden reductions.

METHODS

Protected L-amino acids, 4-methylbenzhydrylamine rink amide resin, 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, and 4-pentynoic acid were purchased from Novabiochem (Läufelfingen, Switzerland) and Mimotopes (Melbourne, Australia). Piperidine, *N*, *N*, dimethylformamide, trifluoroacetic acid, *N,N* diisopropylethylamine, chromatography-grade acetonitrile, methanol, and dichloromethane were purchased from Merck (Darmstadt, Germany). Linear azide-terminated polymethylacrylate (2400 g/mol) and triisopropylsilane were obtained from Sigma-Aldrich (Castle Hill, Australia). Snake skin dialysis membrane, 10,000 *Da* molecular weight cutoff, was obtained from Thermo Fischer (Scoresby, Australia). 4-Pentynoic acid was purchased from Novachem Pty Ltd (Victoria, Australia). Dde-protected C16 lipoamino acid and 2-[(4, 4-dimethyl-2,6-dioxocyclohex-1-ylidene) ethylamino]-d,l-hexadecanoic acid were synthesized as described previously.³¹ Flat-bottom, high binding affinity ELISA 96-well plates were purchased from Sarstedt (Nümbrecht, Germany). Goat anti-mouse IgG-horseradish peroxidase (HRP), goat anti-mouse subclass 2a of IgG (IgG2a)-HRP, and goat anti-mouse subclass 1 of IgG (IgG1)-HRP antibodies were purchased from Bio-Rad (Gladesville, Australia), while goat anti-mouse IgA-HRP antibody was purchased from Life Technologies (Scoresby, Australia).

Equipment

Shimadzu reverse-phase HPLC instruments (Shimadzu Corp, Kyoto, Japan) equipped with Vydac C4 columns were used to analyze and purify the vaccine compounds. An ESI-MS electrospray ionization mass spectrometer (Sciex API-3000, Sciex, Vaughan, Ontario, Canada) was used for identification measurements. Secondary structural conformations of the vaccine compounds were evaluated using a Fourier transform infrared spectrophotometer (Spectrum, PerkinElmer, Buckinghamshire, UK) equipped with attenuated total reflectance, and a Zetasizer Nano (Malvern Instruments, Worcestershire, UK) was used to measure the particle size distribution of self-assembled vaccines via dynamic laser scattering. A BMG CLARIOStar fluorescence plate reader (BMG LABTECH, Cary, NC) was used to assess *in vitro* neutralization/proteolysis of wild-type *Na*-APR-1 enzymes. Electron micrographs of self-assembled vaccine compounds were captured with a JEM-1010 transmission electron microscope (HT7700 Exalens, Hitachi Ltd, Jeol Ltd, Tokyo, Japan). OriginPro 2020 (OriginLab Corporation, Northampton, Mass) and Graphpad Prism v8.3 (GraphPad Software, Inc, San Diego, Calif) were used for mathematical and statistical presentation of the data.

Vaccine synthesis

We synthesized the peptide vaccine compounds using fluorenylmethyloxycarbonyl solid-phase peptide synthesis, as described previously.²⁶ The

peptide-polymer conjugate, p3, was synthesized by conjugation of fluorenylmethyloxycarbonyl-synthesized peptide to polymethyl acrylate polymer using copper-catalyzed alkyne azide cycloaddition, as described previously.²⁶

Particle size distribution

Peptide vaccines were dissolved in PBS at a concentration of 0.2 mg/mL. Self-assembled particle size distribution was measured using dynamic light scattering at 25°C and a measurement angle of 173°.

Electron microscopy

Vaccine particle imaging was captured via transmission electron microscope at 100 kV. The images were analyzed using AnalySIS software (Megaview III, Münster, Germany).

Immunization of mice

Fifty-six female BALB/c mice (6–8-week old) (Animal Resources Centre, Perth, Australia), divided into 7 groups of 8 mice each, were used for this study. Seven groups of mice were intraperitoneally injected with the following formulations: (1) PBS, (2) CpG/alum-adjuvanted p3-P25, (3) LCP peptide vaccine, (4) polymethyl acrylate (PMA) peptide vaccine, (5) branched polyoleucine (BL₁₀) peptide vaccine, (6) linear polyoleucine (LL₁₀) peptide vaccine, or (7) CpG/alum-adjuvanted APR-1 enzyme. Naive preimmunization samples (sera, saliva, and feces) were collected from all mice 2 days before immunization. The mice were then immunized by intraperitoneal injection (50 μ L) of the peptide vaccines (25 μ g) in PBS. PBS alone was administered to the negative control group, whereas the 2 positive control groups were given either oligodeoxynucleotide adjuvant (CpG-ODN) 1826/alum-adjuvanted p3-P25 or CpG-ODN 1826/alum-adjuvanted *Na*-APR-1 protein. The CpG/alum mixture was prepared by mixing 10 μ g of CpG-ODN 1826 (Invivogen, San Diego, Calif) and 0.25 mL of 2% Alhydrogel (Invivogen) with 0.25 mL antigen solution in PBS (p3-P25 or *Na*-APR-1, adjusted to 25 μ g per immunization) by pipetting up and down for 5 minutes. The mice were immunized twice with a 2-week interval. Infection challenge was conducted 2 weeks after the second immunization. Postimmunization samples were collected 2 days before challenge and at euthanasia, 1 week postchallenge. Collected sera was diluted 10-fold with PBS; saliva samples were stored undiluted, and fecal samples were homogenized in PBS (0.1 g/mL) containing 0.05% tween 20 and 0.05% sodium azide, followed by centrifugation and transfer of the supernatant.

Immunogenicity assays

ELISA was used to evaluate the immunogenicity of peptide vaccines compared with negative and positive controls. ELISA 96-well plates (high binding affinity with flat-bottom clear wells) were coated with 0.1 mL of antigen solution (either p3 or *Na*-APR-1 protein, 1 μ g/mL) in carbonate buffer (pH 9.6). The plates were incubated for 1.5 hours at 37°C, then washed with deionized water, followed by PBST wash buffer (PBS containing 0.05% tween 80), and dried. The plates were blocked using 5% skim milk in PBS solution, then incubated and washed, as above. A volume of 100 μ L of 0.5% skim milk in PBST was added to all wells, except those in the first column, which were instead filled with 135 μ L. A volume of 15 μ L of each serum sample (initial dilution 1:100) was added to each first column well and mixed thoroughly, followed by 3-fold serial dilutions along the rows. For fecal samples, 100 μ L of 0.5% skim milk in PBST and 50 μ L of fecal extract were added to the first row, followed by 3-fold serial dilutions. The plates were then incubated and washed as described previously. Secondary antibody solution was prepared in 0.5% skim milk in PBST at a 1:4000 dilution for goat anti-mouse IgG-HRP, goat anti-mouse IgG1-HRP, goat anti-mouse IgG2a-HRP, and goat anti-mouse IgA-HRP. A volume of 100 μ L was added to each well depending on the measured antibody isotype or subclass in the assay. The plates were incubated and then washed as described previously. Peroxidase substrate solution, *o*-phenylenediamine dihydrochloride, was applied for 15 minutes, then the reaction was stopped by adding 100 μ L of 1 N sulfuric acid. The absorbance was

measured via a SpectraMax 250 microplate reader (Molecular Devices, Hampton, Va) at 450 nm. The titer was described as the lowest dilution that produced an absorbance above the mean absorbance of the control wells (containing naive mouse serum that was sampled preimmunization) plus 3 SDs.

Enzyme neutralization assay

Our neutralization assay protocol was based on the method reported by Pearson et al,^{21,32,33} but slightly modified to evaluate immune sera instead of purified antibodies. A volume of 50 μ L of recombinant *Na*-APR-1 wild-type enzyme (0.2 μ g/mL) solution in acetate buffer (50 mM sodium acetate, pH 3.5), expressed as previously reported,³²⁻³⁴ was added to white opaque fluorescence 96-well plates, either in the presence or absence of immune mouse serum (0.5 μ L). Sera from 4 mice within a group were pooled to result in 2 representative serum samples per group. Binding was allowed to occur for 30 minutes at 37°C. The total reaction volume was adjusted to 100 μ L with acetate buffer (pH 3.5). A prefluorescence substrate, MoCAc-GKPILFFRLK hemoglobin-substitute, was added immediately before fluorescence intensity measurements to give a final concentration of 1 μ M. The measurements were conducted using a fluorescence plate reader at 5-minute intervals for a total duration of 2 hours (37°C, excitation wavelength of 330 nm, emission wavelength of 390 nm, with suitable optic filters). The photomultiplier tube gain was set to 75%, and focal height was set to autoadjust for maximum signal output. To evaluate the relative inhibition of proteolysis kinetics from one immunized group to another, the increase in relative fluorescence units was integrated over 30 minutes from the onset of measurements.

Infection challenge

The lifecycle of *N brasiliensis* was maintained in Sprague Dawley rats, with *N brasiliensis*-third-stage larvae recovered from rat feces. Two weeks after the second immunization, a larval suspension was prepared and BALB/c mice were infected by injecting 750 larvae into the scruff, as recommended for establishing infection in BALB/c mice by rat-adapted larvae.³⁵ Mice were euthanized 7 days postinfection. Following euthanasia, the intestines were isolated, and feces collected from the colon and large intestines, as described previously.^{24,25,35}

To determine fecal egg counts, fecal samples were weighed and dispersed in 4 to 10 mL of saturated salt solution, and then 300 μ L volumes were loaded into the 2 chambers of the counting slide and counted under the microscope. Egg count per gram of feces was determined using Equation 1, where V_T is the total volume, in milliliters, of concentrated salt solution used and W_t is the weight of feces in grams.

$$\text{egg/g} = \frac{(\text{Count}_1 + \text{Count}_2) \times 1000 \times V_T}{600 \times W_t} \quad (\text{Equation 1})$$

To determine intestinal worm count, the intestines—from cecum to pylorus—were cut open longitudinally and placed in a petri dish that contained 5 mL of PBS. The dishes were then incubated at 37°C for 1 hour. After incubation, the petri dishes were placed under a dissecting microscope and the adult worms were counted.

To analyze immune response-efficacy relationships, group worm count data were fitted as a function of group serum log₁₀ anti-*Na*-APR-1 IgG titers [$f(\text{Serum log IgG titers})$], fecal log₁₀ anti-*Na*-APR-1 IgA titers [$f(\text{fecal log IgA titers})$], and fecal anti-*Na*-APR-1 IgG titers [$f(\text{fecal log IgG titers})$] for all groups using Equation 2. In Equation 2, worm count is the measured mean worm count (y-axis values; primary protective efficacy end point), $\text{Count}_{\min}$ and $\text{Count}_{\max}$ are the minimum and maximum count values at the lower and upper plateaus, respectively, P is the power exponent, logAb titer is the log₁₀ antibody titer value (x-axis values), and x_0 is the fit constant at the midpoint of log₁₀ antibody titer values.

$$\text{worm count} = \text{Count}_{\min} + \frac{\text{Count}_{\max} - \text{Count}_{\min}}{1 + 10^{P(\log x_0 - \log \text{Ab titer})}} \quad (\text{Equation 2})$$

Mice condition was monitored on daily basis during animal studies for mobility, piloerection, and body condition, at prechallenge and postchallenge

stages. Mice that exhibited very poor clinical condition were given a score of 4 and had to be euthanized; these mice were emaciated and had clearly visible segmented vertebrae and skeletal structure, with low mobility and poor grooming. Mice with poor clinical condition were given a score of 3; these mice were thin and had moderately visible vertebrae, with moderate mobility, and lacked grooming. Underconditioned mice were given a score of 2, showed no signs of weight loss or skeletal structure, and had slightly reduced mobility and grooming. Finally, well-conditioned mice were warranted a score of 1; these mice appeared well-fed and well-groomed and exhibited high mobility.

Statistical and correlation analyses

One-way ANOVA with Dunnett multiple comparisons test was used for “parametric” statistical assay evaluation, whereas the Mann-Whitney U test was used as a “nonparametric” method to evaluate the significance of the infection challenge results (worm and egg burdens), as described previously.^{24,25} Significance was set at P less than .05 (*), P less than .01 (**), P less than .001 (***), and P less than .0001 (****).

In correlation analysis, the simplest function was generally chosen to fit the results; that is, if data points allow for a linear fit, then a linear function is chosen; however, if nonlinear pattern of data emerges, then exponential or sigmoidal functions are used. It is also worth mentioning that the sigmoidal function was preferably chosen to fit efficacy data, due to its close mechanistic relationship to neutralization mechanism, which lead to its frequent use in fitting efficacy results in the literature, that is, antibody log₁₀ concentration/titers versus neutralization efficacy.^{26,27,36,37} Dose-response sigmoidal functions have been used in several studies to fit neutralization profiles at different concentrations of purified monoclonal, or serum polyclonal, antibodies against several pathogens.³⁷⁻⁴⁰ Therefore, because of its close mechanistic relationship with efficacy, it was used in this study to evaluate efficacy among different mice groups; this was to extract the underlying meaningful efficacy information that would help as guidance in improving future vaccine designs against hookworm parasites. The correlation constants are derived and mentioned as mean $\pm$ SD, in addition to correlation coefficient (R^2), which generally describes goodness of the fit.

Ethics statement

Animal experiments were approved by the James Cook University Animal Ethics Committee (ethics no. A2571). Animals were housed at James Cook University, and allowed free access to pelleted food and water, in accordance with the Queensland Animal Care and Protection Act.

RESULTS

Synthesis and characterization of vaccines

Peptide vaccines p3-P25, LCP, BL₁₀, and LL₁₀ were synthesized using fluorenylmethyloxycarbonyl-based solid-phase peptide synthesis, whereas PMA was synthesized by conjugating p3-P25 to PMA via copper(I)-catalyzed azide-alkyne cycloaddition (click) reaction (Fig 1), all as described previously.²⁶ The extent of PMA conjugation after click reaction (93%) was examined by elemental microanalysis.

The peptide vaccines were designed to self-assemble in aqueous media to form nanoparticles that can potentiate their immunogenicity. Nanoparticle size was measured using dynamic light scattering and transmission electron microscope. PMA (230 $\pm$ 5 nm, Polydispersity index [PDI] = 0.16 $\pm$ 0.10) and LL₁₀ (22 $\pm$ 1 nm, PDI = 0.36 $\pm$ 0.10) formed uniform particles with relatively small PDI values, whereas LCP (9 $\pm$ 1 nm, PDI = 0.86 $\pm$ 0.10) and BL₁₀ (14 $\pm$ 1 nm, PDI = 0.79 $\pm$ 0.20) showed a higher tendency to aggregate (Fig 2). Vaccine peptide secondary structure was characterized as described previously.²⁶ All peptides adopted a similar conformation: predominantly α -helix

and random coil with a minor β -sheet content (see Fig E1 in this article's Online Repository at www.jacionline.org).

Immunogenicity of the vaccines

Mice (8 per group) were immunized twice ($2 \times 25 \mu\text{g}$ vaccine doses), with a 2-week interval between immunizations. Serum, feces, and saliva were collected 2 days before each immunization for antigen-specific immunogenicity evaluations. Vaccine immunogenicity and immune response quality were evaluated via ELISA using either p3 or *Na*-APR-1 as an antigen (Fig 3; see Figs E2-E4 in this article's Online Repository at www.jacionline.org). Immune sera, fecal extracts, and saliva were evaluated for antigen-specific antibody titers. Sera from *Na*-APR-1-, LCP-, LL₁₀-, and BL₁₀-immunized mice generated significantly higher anti-*Na*-APR-1 IgG titers compared with controls (Fig 3, A). The other vaccines, such as PMA and p3-P25, failed to induce the production of significantly elevated anti-*Na*-APR-1 IgG titers in the sera compared with the negative control, although some mice within these groups did show detectable IgG levels. Fecal extracts of *Na*-APR-1-, LCP-, and BL₁₀-immunized mice generated significantly elevated fecal anti-*Na*-APR-1 IgG titers compared with the PBS group (Fig 3, B). Moreover, fecal extracts from BL₁₀-immunized mice had significantly elevated anti-*Na*-APR-1 IgA titers (Fig 3, C). No other group generated significantly elevated anti-*Na*-APR-1 IgG or IgA titers from fecal samples. Fecal extract from the LL₁₀ group had significantly elevated fecal anti-p3 IgG titers, similar to those generated by BL₁₀; however, the LL₁₀ group's fecal anti-*Na*-APR-1 IgG titers were very low and not significantly different from that of the PBS control group (Figs 3 and E3). Serum anti-p3 IgG titers for p3-P25 LCP, BL₁₀, and LL₁₀ were much higher (10^4 - 10^5 , ca. 1000-fold) (Fig E2, D and E) than anti-*Na*-APR-1 IgG serum titers (10^1 - 10^2) (Figs 3, A, and E2, B). Immunization over time shows that serum anti-p3 IgG titer levels are increasing after the second immunization; however, these increased only lightly postchallenge (Fig E2, A-D). Moreover, fecal anti-*Na*-APR-1 IgG titers correlated with serum anti-*Na*-APR-1 IgG titers (Fig E2, F); the results suggest that the levels of APR-1-specific fecal IgG started increasing steadily after serum titers reached threshold titer level of ca. $10^{1.1}$.

Salivary anti-p3 IgG and IgA titers were also evaluated (Fig E4). The BL₁₀ group had significantly elevated anti-p3 IgG titers, and the LCP group had significantly elevated anti-p3 IgA titers compared with control groups, but salivary antibody titers from the other groups, including the positive controls, were not significantly different. Serum anti-*Na*-APR-1 IgE and serum anti-human cathepsin-D IgG titers were evaluated for all groups (see Fig E5 in this article's Online Repository at www.jacionline.org). The mice did not generate any detectable anti-*Na*-APR-1 IgE titers; however, *Na*-APR-1-immunized mice generated low, albeit significantly elevated, titers of anti-human cathepsin-D IgG ($10^{0.9}$; $P < .01$).

In vitro Na-APR-1 enzyme neutralization

Immunized mouse serum samples were mixed to form 2 pools for each group. These pooled samples were then characterized for their *Na*-APR-1 neutralization capacity (inhibition of enzymatic activity) *in vitro* using a synthetic prefluorescence substrate. The percent increase in fluorescence was integrated over 30 minutes and the area under the curve presented for each group

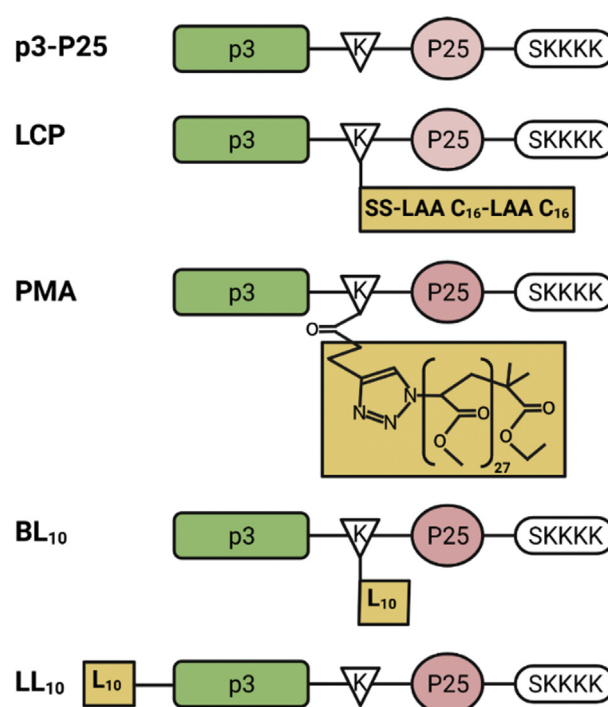


FIG 1. Peptide-based vaccines against hookworm infection. Peptide p3 (green round-edge rectangle) is an APR-1-derived neutralizing B-cell epitope (TSLIAGPKAQVEAIQKYIGAEI), P25 (pink circle) is a T_H-cell epitope (KLIPNASLIENCTKAEI), SKKKK (white oval) is a solubilizing moiety, K (white triangle) is a branching lysine, and 10-mer polyolefin (L₁₀), PMA, and LCP, where S is serine (S) and -LAA-C16 is 2-amino-D,L-hexadecanoic acid, are adjuvanting moieties (orange rectangles).

(Fig 4). All peptide vaccine groups (LCP, BL₁₀, LL₁₀, PMA, and p3-P25) had a significantly reduced area under the curve compared with the PBS group ($P < .0001$), indicating significant neutralization. The highest neutralization values were observed in BL₁₀, and the positive control, p3-P25/alum/CpG, groups ($P > .05$). Sera from the PMA and LL₁₀ groups had weaker neutralization capacity compared with sera from the p3-P25/alum/CpG group ($P < .001$). Surprisingly, sera from the *Na*-APR-1-positive control group did not induce significant inhibition of *Na*-APR-1 enzymatic activity, despite the fact that purified anti-APR-1 antibodies generated by APR-1 adjuvanted with complete Freund's adjuvant were previously reported to exhibit potent neutralization.³²

Infection challenge

All mice were infected with 750 *N brasiliensis* larvae suspended in PBS via injection into the scruff 2 weeks after the second immunization, then euthanized 7 days later to evaluate parasite burden. Adult worms were counted in the intestines, and eggs were counted in the feces (Fig 5). Immunization with CpG/alum-adjuvanted p3-P25, LCP, and BL₁₀ resulted in significant worm burden reductions of 75% ($P < .01$), 56% ($P < .05$), and 77% ($P < .01$) in mice intestines, respectively, compared with the PBS control group (P according to 1-way ANOVA with Dennett's multiple comparisons test). Notably, all groups had significant worm burden reductions according to the commonly used nonparametric Mann-Whitney *U* test. Surprisingly, the lowest

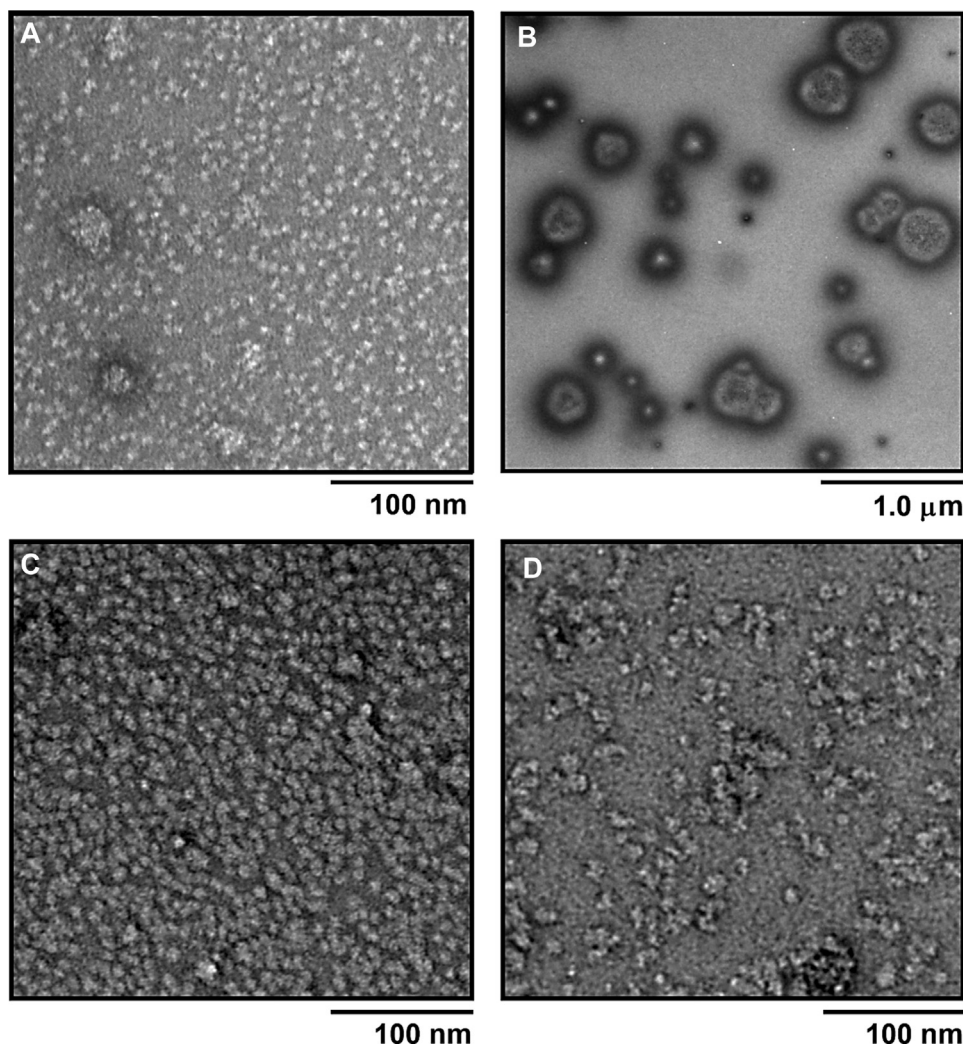


FIG 2. Electron micrographs of the peptide vaccines: (A) LCP, (B) PMA, (C) BL₁₀, and (D) LL₁₀.

mean reduction of 26% was observed for *Na*-APR-1 protein-immunized positive control mice; this reduction was not significant using parametric statistics ($P > .05$). Egg count was significantly reduced in PMA, LCP, and BL₁₀ groups by 62% ($P < .05$), 87% ($P < .01$), and 64% ($P < .05$), respectively, according to parametric statistical comparisons. Again, all groups except LL₁₀ had significant egg burden reductions according to the nonparametric Mann-Whitney U test, with the lowest mean reductions: 30% and 51%, observed in the LL₁₀ and APR-1-immunized groups, respectively ($P > .05$, 1-way ANOVA). Two mice from the *Na*-APR-1-immunized group were euthanized early due to very poor clinical conditions, and while 2 additional mice maintained tolerable conditions, their condition was steadily declining before euthanasia. *Na*-APR-1-immunized mouse sera were analyzed for potential autoimmune (anti-cathepsin-D IgG) or allergic (anti-*Na*-APR-1 IgE) responses (see Fig E5). Anti-*Na*-APR-1 IgE and anti-cathepsin-D IgG were not significant (low and undetectable) in all peptide vaccine groups; however, 3 mice from the *Na*-APR-1-immunized group had detectable anti-human cathepsin-D IgG antibodies.

CpG/alum-adjuncted *Na*-APR-1-immunized mice only exhibited poor to very poor clinical conditions/scores at

postchallenge stage. Two mice were emaciated (ca. 20% loss in body weight) and in a very poor clinical condition (score of 4); thus, they were euthanized on day 4 postchallenge. Two other mice from the same group were thin (ca. 7% loss in body weight) and were in poor clinical condition (score 3), by day 4 postchallenge. Therefore, they were kept for evaluation on euthanasia day 7 postchallenge. The mice in the other groups were well-conditioned (score 1) at prechallenge or postchallenge stage.

Relationships between immune responses and protection

Relationships were established between humoral immune responses and mean worm count, which was used as the primary end point of protection. Mean worm count/reduction values were plotted against mean serum anti-*Na*-APR-1 IgG, fecal anti-*Na*-APR-1 IgG, or mean fecal anti-*Na*-APR-1 IgA (Fig 6; Fig E6, G and H). However, anti-p3 antibody titers were used in the relationship instead of anti-*Na*-APR-1 titers for *Na*-APR-1-immunized mice (Fig 6) to allow logical comparison among the groups and to exclude additional neutralizing and immunogenic epitopes within the APR-1 sequence. A sigmoidal relationship

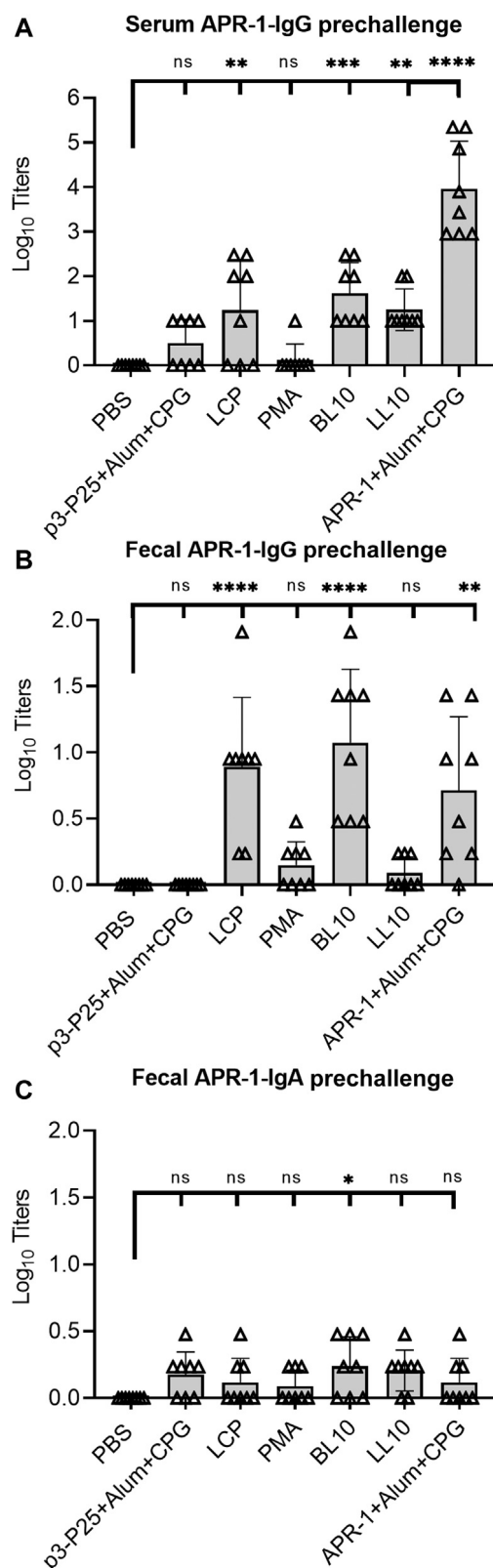


FIG 3. Immunogenicity of the vaccines. Serum anti-*Na*-APR-1 IgG titers (A), and fecal anti-*Na*-APR-1 IgG (B) and IgA (C) titers. One-way ANOVA with Dunnett's multiple comparisons test was used to statistically compare vaccinated groups to the PBS group. Significance was set at $P < .05$ (*), $P < .01$ (**), $P < .001$ (***), and $P < .0001$ (****).

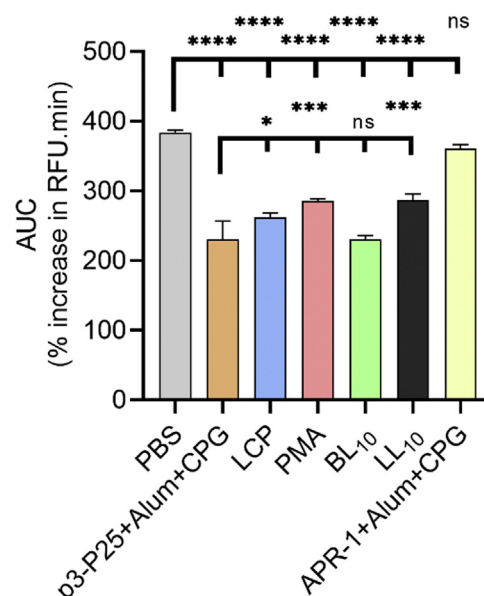


FIG 4. *In vitro* *Na*-APR-1 enzyme neutralization by pooled immune sera. The lower the integration area (area under the curve, AUC), the higher the neutralization. RFU, Relative fluorescence units. One-way ANOVA with Dunnett's multiple comparisons test was used to statistically compare vaccinated groups to the PBS group. Significance was set at: not significant $P > .05$ (ns), $P < .05$ (*), $P < .001$ (***), and $P < .0001$ (****).

between immune response and protection was found between mean serum *Na*-APR-1 IgG titers and worm reduction ($R^2 = 0.98$); however, the p3-P25 and PMA groups were omitted because they deviated from the pattern (Fig 6, A). Moreover, a strong sigmoidal relationship was found between fecal anti-*Na*-APR-1 IgA and worm reduction, which represented all peptide vaccines except LL₁₀ ($R^2 = 0.98$) (Fig 6, B). Fitting all groups, including LL₁₀-immunized mice, resulted in a weaker correlation ($R^2 = 0.7$).

In-depth analysis of immune correlates of protection

To more deeply analyze the protective humoral immune response(s), mice were regrouped on the basis of individual worm burden reductions compared with the PBS control group, because this signified the primary end point of protection. This approach aimed to isolate the unique and significant immune response(s) that distinguished the mice that were most effectively protected. The mice were sorted into 3 groups; high (>75% hookworm burden reduction), moderate (>50% hookworm burden reduction), or low (<50% hookworm burden reduction) (Fig 7, A). The new groupings remained consistent when egg burden reductions were considered, instead (Fig 7, B). The low protection group was not significantly different in worm or egg burden reduction compared with the PBS control group, whereas the moderate and high protection groups had significantly lower burdens of parasite eggs and adult worms ($P < .01$ to $P < .0001$).

We simultaneously analyzed the immune responses associated with different levels of protection in the mice, including serum anti-p3 IgG, serum anti-*Na*-APR-1 IgG titers (Fig 7, C), fecal

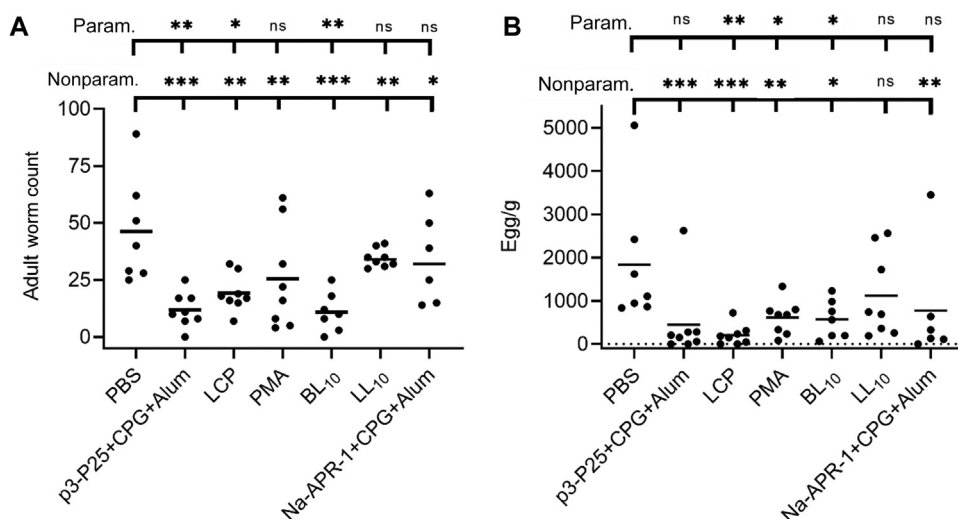


FIG 5. Infection challenge of vaccinated mice presented as (A) intestinal adult worm count, and (B) egg count per gram of feces. *Nonparam*, Nonparametric; *ns*, nonsignificant; *Param*, parametric. Horizontal solid lines show the mean value of each group. One-way ANOVA with Dunnett's multiple comparisons test was used for parametric statistical evaluation, whereas the Mann-Whitney *U* test was used for nonparametric statistical evaluation compared with the negative control group. Significance was set at $P < .05$ (*), $P < .01$ (**), and $P < .001$ (***).

anti-*Na*-APR-1 IgG, fecal anti-*Na*-APR-1 IgA (Fig 7, D), and salivary anti-p3 IgG and IgA (see Figs E6 and E7 in this article's Online Repository at www.jacionline.org). Highly protected mice had significantly higher serum anti-p3 ($P < .01$) and anti-*Na*-APR-1 IgG ($P < .05$) titers compared with mice in the low protection group (Fig 7, C). In addition, some of the highly protected mice had undetectable serum anti-*Na*-APR-1 IgG titers (Fig 7, C). Fecal anti-*Na*-APR-1 IgG and IgA significantly increased from low to moderate to highly protected mice ($P < .05$), and the increase in fecal anti-*Na*-APR-1 IgA titers in the high protection group was highly significant ($P < .001$). In contrast to serum antibodies, all the highly protected mice had detectable fecal anti-*Na*-APR-1 IgG or IgA titers (Fig 7, C and D).

DISCUSSION

The protective efficacy of a peptide vaccine depends on the strength, quality, and type of immune response(s) produced. In addition to systemic humoral immunity, measured as serum antigen-specific antibody titers, a vaccine's ability to induce mucosal humoral responses in the saliva and feces affects overall efficacy.⁴¹ Interestingly, mucosal immune responses can be developed even after parenteral vaccine administration.⁴¹⁻⁴⁴

Here, we immunized mice intraperitoneally with full-length *Na*-APR-1 protein adjuvanted with CpG/alum and several p3-peptide-based vaccine candidates. Although mice immunized with a protein-based formulation produced high IgG titers against *Na*-APR-1, only a small proportion of the produced antibodies recognized p3 peptide (Fig 8). Thus, immunization with full-length protein did not produce adequate levels of systemic and mucosal neutralizing anti-p3 antibodies and did not protect mice against hookworm challenge (Fig 5). Yet, the 26% worm burden reduction in the BALB/c-*N brasiliensis* infection challenge model was similar to the 33% reduction observed in the dog-*Ancylostoma caninum* infection-challenge model¹⁷

following immunization with *Na*-APR-1. This supports the reliability of our infection model. We recently established that serum anti-*Na*-APR-1 IgG is a significant immune correlate of protection, based on an oral immunization study.²⁶ Here, the same peptide vaccines were administered intraperitoneally, but at a greatly reduced dose ($2 \times 25 \mu\text{g}$ vs $4 \times 100 \mu\text{g}$), with significant anti-*Na*-APR-1 serum IgG and fecal IgA titers. Immunization with LCP, p3-P25, and BL₁₀ resulted in superior protection (59%, 75%, and 77%, respectively), significantly outperforming the clinical vaccine candidate based on *Na*-APR-1 protein. It should be noted that the current challenge was performed using *N brasiliensis* parasites and *Na*-APR-1 protein.⁴⁵ *N brasiliensis*-APR-1⁴⁵ is 80% identical (86% similar) to the *Na*-APR-1 sequence (see Fig E8 in this article's Online Repository at www.jacionline.org); thus, they likely have most epitopes in common, including the neutralizing epitope, p3, which is completely conserved between the APR-1 proteins of the 2 species.

All peptide-based vaccines were self-assembled into small nanoparticles ranging in size from 10 nm to 22 nm, except for the PMA conjugate, which assembled into 230-nm particles. BL₁₀ and LCP nanoparticles (10-15 nm) induced higher anti-p3 IgG titers than did LL₁₀ (22 nm), and much higher titers than did PMA (230 nm), suggesting that smaller sized nanovaccines were more immunogenic. The most immunogenic peptide nanovaccines, LCP and BL₁₀, also triggered the highest *Na*-APR-1-specific serum IgG and fecal IgA titers (Figs 3 and 8), which were associated with 60% to 80% worm burden reductions (Figs 5-7). The *Na*-APR-1 vaccine was not only poorly protective (26% worm burden reduction), but most mice in the *Na*-APR-1-immunized group also exhibited poor clinical scores (4 of 8) and 2 had to be euthanized on day 4 postinfection, potentially due to the generation of autoantibodies against murine cathepsin-D (Figs 5 and E5). Therefore, the peptide vaccine candidates examined were not only more effective but also much safer.

Although the peptide vaccines were administered to mice by injection, most generated not only systemic but also mucosal

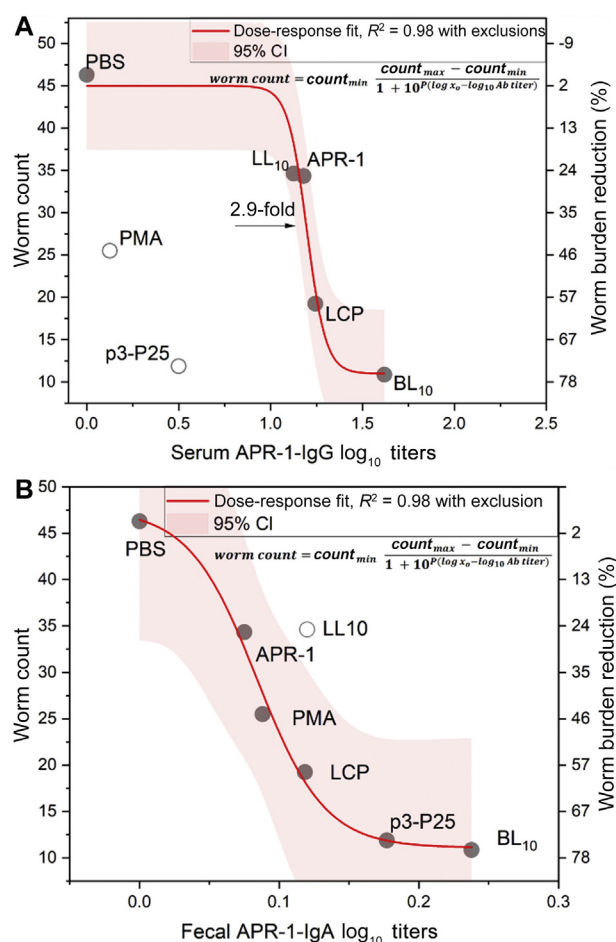


FIG 6. The relationship between mean adult worm count and mean serum anti-*Na*-APR-1 IgG (A), or mean fecal anti-*Na*-APR-1 IgA (B) from each group. The sigmoidal fit between worm count and serum anti-*Na*-APR-1 IgG (red curve, Fig 6, A) was $R^2 = 0.98$; PMA and p3-P25 sera were excluded because they had very low, nonsignificant serum anti-*Na*-APR-1 IgG titers that did not explain their efficacy. The sigmoidal fit between worm count and fecal anti-*Na*-APR-1 IgA (Fig 6, B) was $R^2 = 0.98$; LL₁₀ was excluded because it had nonsignificant serum anti-*Na*-APR-1 IgG and fecal anti-*Na*-APR-1 IgG titers, unlike the other groups. Open symbols show the mean titer values that were excluded to generate the curve, whereas closed symbols show the included mean titer values. Best-fit coefficients, constants, and power exponents are provided. Fit constants for sigmoidal fit between worm count and serum anti-*Na*-APR-1 IgG titers (mean $\pm$ SD) were “worm” Count_{max} = 46 ± 3.1 , “worm” Count_{min} = 11.0 ± 1.7 , the center point ($\approx$ IC50) log x_0 = 1.20 ± 0.010 , and for the “decay exponent/slope” $P = -8.366 \pm 2.12$, whereas fitting constants for sigmoidal fit between worm count and fecal anti-*Na*-APR-1 IgA titers (mean $\pm$ SD) were “worm” Count_{max} = 47 ± 2.85 , “worm” Count_{min} = 11.09 ± 1.77 , the center point ($\approx$ IC50) log x_0 = 0.0840 ± 0.005 , and for the “decay exponent/slope” was steeper with $P = -17.890 \pm 5.495$.

humoral T_H2 responses, as demonstrated through saliva and fecal extracts, with significant anti-*Na*-APR-1 and anti-p3 IgG and IgA titers from the feces, as well as serum anti-*Na*-APR-1 and anti-p3 IgG titers in sera (Figs 3 and E2-E4). Furthermore, the positive controls that were adjuvanted with CpG-ODN 1826/alum mixture produced mainly T_H2 responses, as demonstrated by the production of excessive IgG1 over IgG2a (Fig E2, D, and E2, E). In addition, anti-p3 IgG titer levels closely matched anti-p3 IgG1 subclass titers for all mice groups (Figs E2, B, and E2, D), which supported a dominant T_H1 immune response. Although CpG-

ODN is reported to trigger T_H1-biased responses,⁴⁶ in this study, CpG-ODN was not able to switch the humoral responses toward T_H1 in the presence of alum, as evidenced by undetectable IgG2a titers in most mice (Fig E3): this included *Na*-APR-1-immunized mice. However, it was reported previously that CpG-ODN has mucosal adjuvanting potential,⁴⁶ which may explain the mucosal humoral responses of the positive control groups (Fig 3).

Immune correlates of protection are defined as immune responses that are responsible for, and statistically related to, protection, whereas absolute immune correlates of protection are defined as the threshold level of immune response(s) that, if surpassed, significantly correlates with protection.⁴⁷ Thus, to determine immune correlates of protection against hookworm infection, we established relationships between promising immune responses and protection (Figs 6, 7, E6, and E7). Three main effector immune mechanisms of protection are considered for helminth parasites, including hookworms: all are antibody-mediated.^{27,48-50} They are neutralizing antibody responses against essential parasite protein(s); and antibody-mediated cellular responses mediated by either IgG2 (with macrophages and/or natural killer cells; tolerable response) or IgE (with macrophages, basophils, and/or mast cells; allergic response). In contrast, cellular responses, that is, cytotoxic T-cell responses, cannot protect against extracellular hookworm infections because they require antigen presentation by host cell MHC-I molecules, which is not the case in helminth infection. By regrouping mice on the basis of response efficacy (Fig 7), highly protected mice (>75% worm reduction) showed significantly high anti-*Na*-APR-1 IgG titers ($P < .05$) in the serum and feces, and very highly significant anti-*Na*-APR-1 IgA in the feces ($P < .01$) compared with weakly protected mice (<50% worm reduction). This suggests that the mucosal humoral response, especially fecal anti-*Na*-APR-1 IgA, also plays an important protective role, and possibly provides even the most important protective mechanism. However, salivary anti-p3 IgG and IgA were not significantly different between low and highly protected groups ($P > .05$, Fig E7). This can be explained by localized targeting of mucosal responses (fecal anti-*Na*-APR-1 IgG and IgA) at infected intestinal sites, because this would be more crucial for protection, providing focused responses around adult hookworms. Our approach has highlighted the most promising protective immune responses among vaccinated groups, thus allowing for the establishment of potentially novel mucosal immune-correlate(s) of protection for BALB/c-*N. brasiliensis* infection.

The immune response-protection relationship between serum anti-*Na*-APR-1 IgG and mean worm count (Fig 6, A) excluded PMA and p3-P25. These were serologically nonimmunogenic compounds with *Na*-APR-1 titers less than $10^{0.6}$, a value that was previously shown to be the limit for detectable worm reduction.^{26,27} In other words, the sera of p3-p25 and PMA mice deviated from the relationship, that is, mean anti-*Na*-APR-1 IgG titers less than $10^{0.6}$; thus, their efficacy is potentially related to their ability to induce mucosal immunity (Fig 8). Moreover, a strong relationship between fecal anti-*Na*-APR-1 IgA and mean worm count (Fig 7, D) was established with or without the LL₁₀ group. Fecal samples from LL₁₀-immunized mice, unlike the other groups, had very low to undetectable anti-*Na*-APR-1 IgG titers. Therefore, serum anti-*Na*-APR-1 IgG and fecal anti-*Na*-APR-1 IgA were most likely responsible for this group’s modest worm reduction. Fecal anti-*Na*-APR-1 IgA appears to be effective at very low titers, based on the plotted dose-response curve (Fig 6,

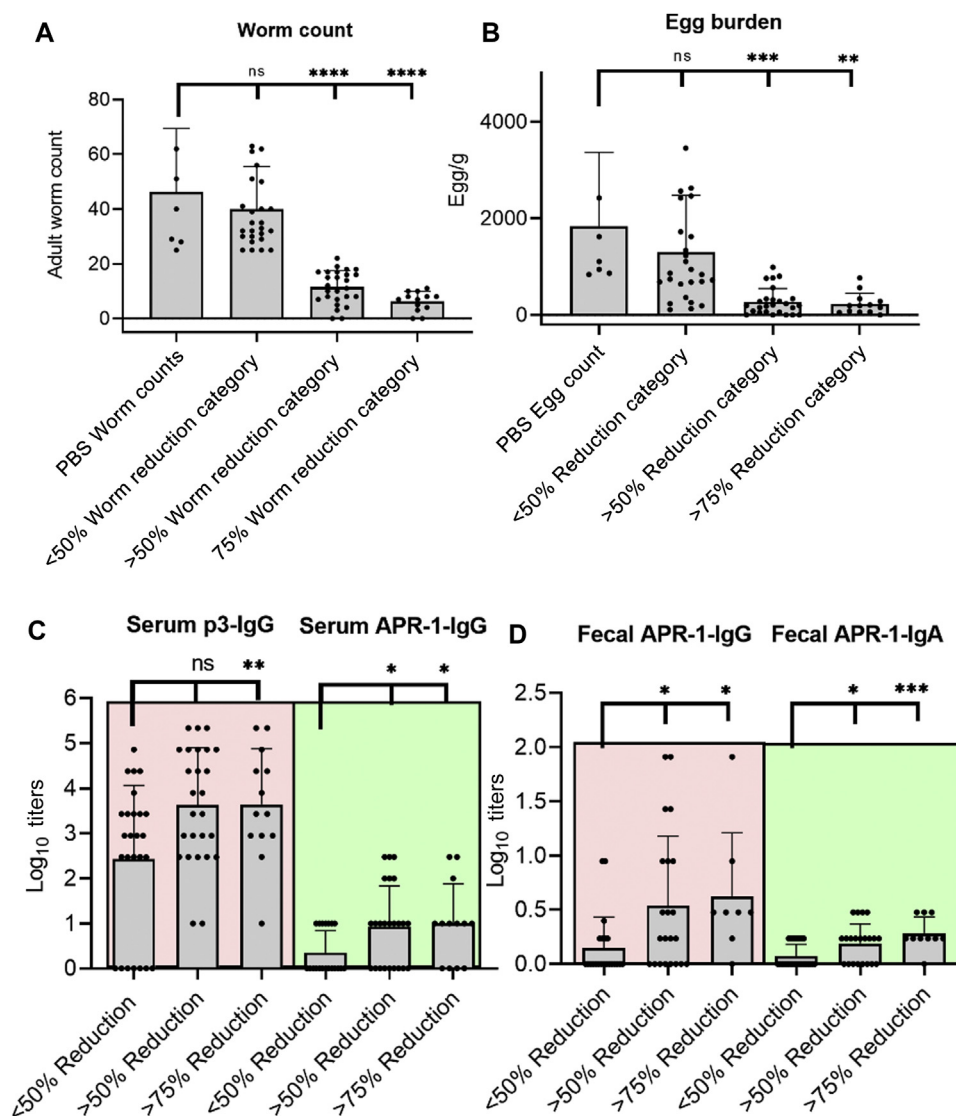


FIG 7. The contribution of various antibodies to protective efficacy for individual mice. Immunized mice were regrouped according to worm burden reduction, “high” (>75%), “medium” (>50%), and “low” (<50%) (A), to determine immune responses that distinguished highly protected mice. Egg burden reductions (B) of the regrouped mice corresponded with worm count categorization. The significant contribution of immune responses to protection (C and D), including serum anti-p3 IgG titers or serum anti-*Na*-APR-1 IgG (serum *Na*-APR-1-IgG) titers (Fig 7, C) and fecal anti-*Na*-APR-1 IgG (fecal *Na*-APR-1-IgG) or IgA (fecal *Na*-APR-1-IgA) (Fig 7, D) among regrouped mice. ns, Nonsignificant. One-way ANOVA with Dunnett’s multiple comparisons test was used for statistical evaluation. Significance was set at $P < .05$ (*), $P < .01$ (**), and $P < .001$ (***).

B), where 50% efficacy corresponds to very low titers of $10^{0.1}$, whereas 75% efficacy corresponds to titers of $10^{0.18}$. Moreover, IgA was found to be protective even in the absence of adequately protective serum or fecal IgG titers, as observed in the PMA and p3-P25 groups. The efficacy of fecal antigen-specific IgA against hookworms found here is in agreement with the previously reported association of high levels of protection against infection in hamsters.⁵¹ Similar associations between parasite-specific fecal IgA titers and parasite burdens were also observed in immunized mice for other intestinal parasites, such as *Heligmosomoides polygyrus* and *Eimeria maxima*.⁴⁰ Fecal IgA were found to be highly protective against hookworms and could potentially offer protection against other gastrointestinal helminth infections.

Therefore, future studies should consider evaluating pathogen-/antigen-specific mAb responses to identify potential correlates of protection, thus facilitating the design of highly protective vaccines. In addition, the duration between final immunization and infection challenge can be increased in future studies. This could be useful to evaluate whether the vaccine candidate could maintain a long-lived mucosal immune response because it is often reported that the local gastrointestinal environment imposes switching to regulatory responses. Other relationships between immune responses and protection (egg burden reduction or worm burden reduction) are provided in this article’s Online Repository (Fig E6, A-H). Anti-p3 antibodies in the serum and feces correlated well with worm burden reduction in APR-1–

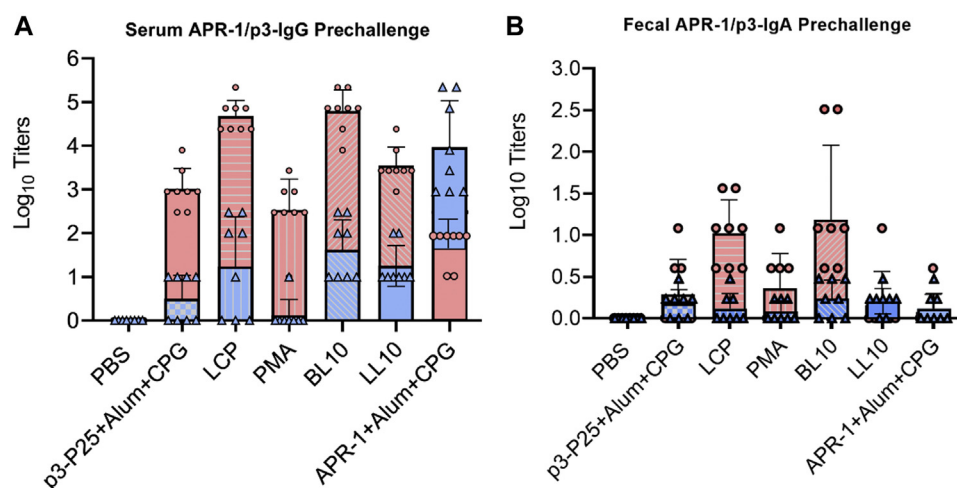


FIG 8. Differences between serum anti-p3 and anti-*Na*-APR-1 IgG log₁₀ titers (A), and fecal anti-p3 and anti-*Na*-APR-1 IgA log₁₀ titers (B) between all immunized groups. Average titers per anti-p3 group are represented by red bars, individual mice are represented by red circles, anti-*Na*-APR-1 group average titers are represented by blue bars, and individual mice are represented by blue triangles.

immunized mice among the other groups. This suggests that the p3 epitope is the main protective neutralizing epitope within the APR-1 sequence. In other words, data points in the immune response-efficacy relationships from the “anti-p3 antibody titers” of the APR-1-immunized group (Fig 8) did not deviate from the pattern of the other p3-based vaccine groups.

In summary, LCP and BL₁₀ proved to be highly immunogenic protective vaccines compared with the current clinical vaccine candidate, APR-1, and other peptide vaccine candidates, in the BALB/*c-N brasiliensis* challenge model,^{19,20} even with just 2 modest intraperitoneal doses (2 × 25 μg). These promising peptide vaccine candidates self-assembled into small nanoparticles 10 to 15 nm in diameter. They induced strong humoral systemic and mucosal antigen-specific IgG and IgA responses that resulted in worm burden reductions of 58% to 77% and fecal egg burden reductions of 69% to 89% in the BALB/*c-N brasiliensis* challenge infection model. Vaccine efficacy was predominantly attributed to the fecal IgA response (ca. 80%, Fig E9), followed by fecal/serum IgG responses (ca. 20%, Fig E9); thus, special attention should be given to mucosal humoral responses in future studies. Furthermore, the recognition quality of antibodies generated by p3-based peptide vaccines can be further improved in future research, thus closing the gap between anti-p3 titers and anti-APR-1 titers.

Hookworm vaccines need not achieve sterilizing protection, that is, 100% worm reduction, because 80% worm burden reduction in humans was modeled to have a highly positive impact in hookworm-epidemic areas.^{52,53} Moreover, hookworm-related morbidity is directly linked to infection intensity, and controlled, low-burden experimental human hookworm infection is well tolerated, and has even been shown to carry some health benefits in humans.⁵⁴⁻⁵⁶ Thus, our vaccine candidates are highly promising and should progress to further preclinical studies on larger animals (eg, dogs) and finally into clinical investigations.

CONCLUSIONS

In this study, we synthesized 5 peptide vaccine candidates against hookworms, immunized BALB/*c* mice twice, intraperitoneally, and conducted an infection challenge using rodent

hookworms (*N brasiliensis*) to evaluate protective efficacy of the vaccine candidates compared with *Na*-APR-1, the clinical vaccine antigen. We found that the clinical vaccine candidate, APR-1 protein antigen, was not as effective as p3-peptide in the BALB/*c-N brasiliensis* infection-challenge model. Alum/CpG-adjuvanted *Na*-APR-1 antigen produced the highest anti-*Na*-APR-1 IgG titers; however, the produced antibodies failed to neutralize the enzymatic activity of *Na*-APR-1. In contrast, p3 antigen focused immune responses on the neutralizing epitope within APR-1. Furthermore, the mucosal anti-APR-1 IgA response has been suggested as a powerful immune correlate of protection, whereas serum IgG responses were also protective but only to a limited extent. Several strong relationships between immune responses and efficacy were established, including determination of the contribution of combined immune responses to overall protective efficacy. Worm burdens were greatly reduced in mice after *N brasiliensis* larval challenge following double intraperitoneal immunization with small nanovaccine doses. The generated serological IgG responses were predominantly of the IgG1 subclass, even with protein and peptide controls using CpG/alum. Our findings support further investigations on peptide-based vaccines against hookworms, and subsequent clinical trials. We propose that p3-based peptide vaccines provide a safer and more effective alternative to protein subunit vaccines, including the APR-1-based clinical vaccine candidate.

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Clinical implications: We propose that p3-based peptide vaccines provide a safer and more effective alternative to the APR-1-based clinical vaccine candidate.

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SECONDARY STRUCTURE BY FOURIER TRANSFORM INFRARED (FTIR)

The compounds were dissolved in PBS 2 mg/mL, placed on the attenuated total reflection attachment, and their FTIR spectra were measured in triplicate from 500 to 4000/cm at 25°C, with a 4/cm resolution. The spectra were processed and deconvoluted using the method described by Yang et al.^{E1} To obtain their secondary structure contents, Originpro 2020 software was used. Briefly, the method involves derivatizing the spectrum followed by specific secondary structure peak assignments in the second derivative of the spectra; integration area of a specific structure's assigned peak(s) over total peak area yields the content of this specific secondary structure.

A shift in serum efficacy from oral to injection-based delivery was also observed where 50% worm reduction efficacy corresponded to $10^{1.25}$ (intraperitoneal) instead of $10^{0.75}$ (oral), and 75% worm reduction efficacy corresponded to $10^{1.4}$ (intraperitoneal) instead of $10^{0.9}$ (oral)^{E2,E3}; thus, this shift suggests a change in effective concentration by about $10^{0.5}$, approximately 3-fold, in favor of the oral immunization route (Fig E6, A and G). This suggests that oral immunization can produce a more effective response at lower serum titers, potentially due to the localization of lymph nodes near afflicted intestinal sites where adult worms reside; thus, antibody generation is more concentrated around the worms in the intestines. Furthermore, mean fecal anti-APR-1 IgG titers were plotted against mean worm reduction values (Fig E6, G). A linear relationship fitted most of the mice groups' data points, fitted $R^2 = 0.91$, with the exclusion of the 2 groups nonresponding PMA and p3-P25. These 2 groups had very low to undetectable titers in most mice for serum as well as fecal anti-APR-1 IgG; thus, fecal anti-APR-1 IgA titers of these groups are most likely responsible for their protective efficacy.

IMMUNOLOGIC RESPONSES AGAINST HOOKWORMS

The promising immune response-protection relationships were combined with the aim of determining the potential contribution of each immune response to overall protection, expressed as worm burden reduction. Because serum IgG and fecal IgG behaved in a similar manner and both of their relationships with worm burden reduction had the same excluded peptide vaccines, that is, PMA and p3-P25, therefore, either fecal or serum IgG was combined with fecal IgA responses to determine the contribution of each immune response to overall protective efficacy (Fig E9).

By including the omitted data point(s), from previous immune response-protection relationships, in a multivariate fit, it should help determine the contribution of each immune response. In other words, group worm count data can be fitted as a function of 2 or more immune responses, without exclusions/omissions of any data points; thus, the deviation in the immune response-protection relationship from one response can be compensated by the other immune response(s). To this end, group worm count data were fitted as a function of both [f (fecal log IgA titers)] and [f (fecal log IgG titers)] using Equation 3, where A and B are confectations that determine the contribution of fecal anti-APR-1 IgA and anti-APR-1 IgG, respectively. Combining the relationships established for fecal, or serum, IgG and fecal IgA anti-APR-1 titers demonstrated that fecal anti-APR-1 IgA contributed to 80% to 85% of worm reduction of candidate compounds, whereas serum IgG or fecal IgG is responsible only for 15% to 20% of worm reduction (Fig E9, A and B); fitting combined relationships gave $R^2 = 0.96$ including all tested vaccines. Moreover, oral delivery immune responses as well as intraperitoneal delivery immune responses (fecal IgG and IgA) were plotted in 3-dimensional plot against worm count and fitted using log normal surface fit; fitting gave $R^2 = 0.9$ (Fig E9, C).^{E3} In the 3-dimensional plot, it is observed that at low fecal IgA titers, the worm count reductions were subtle with the increase in fecal IgG titers; however, when fecal IgA titers increased, the worm count reductions were considerably steeper with the increase in fecal IgG titers (Fig E9, C).

$$\begin{aligned} \text{Predicted worm count} &= A \times f_{(\text{fecal log IgA titers})} \\ &+ B \times f_{(\text{fecal log IgG titers})} \end{aligned} \quad (\text{Equation 3})$$

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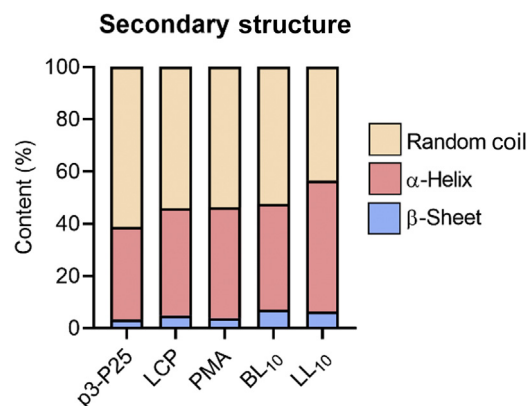


FIG E1. Secondary structures of peptide vaccines evaluated by ATR-FTIR. *ATR*, Attenuated total reflection.

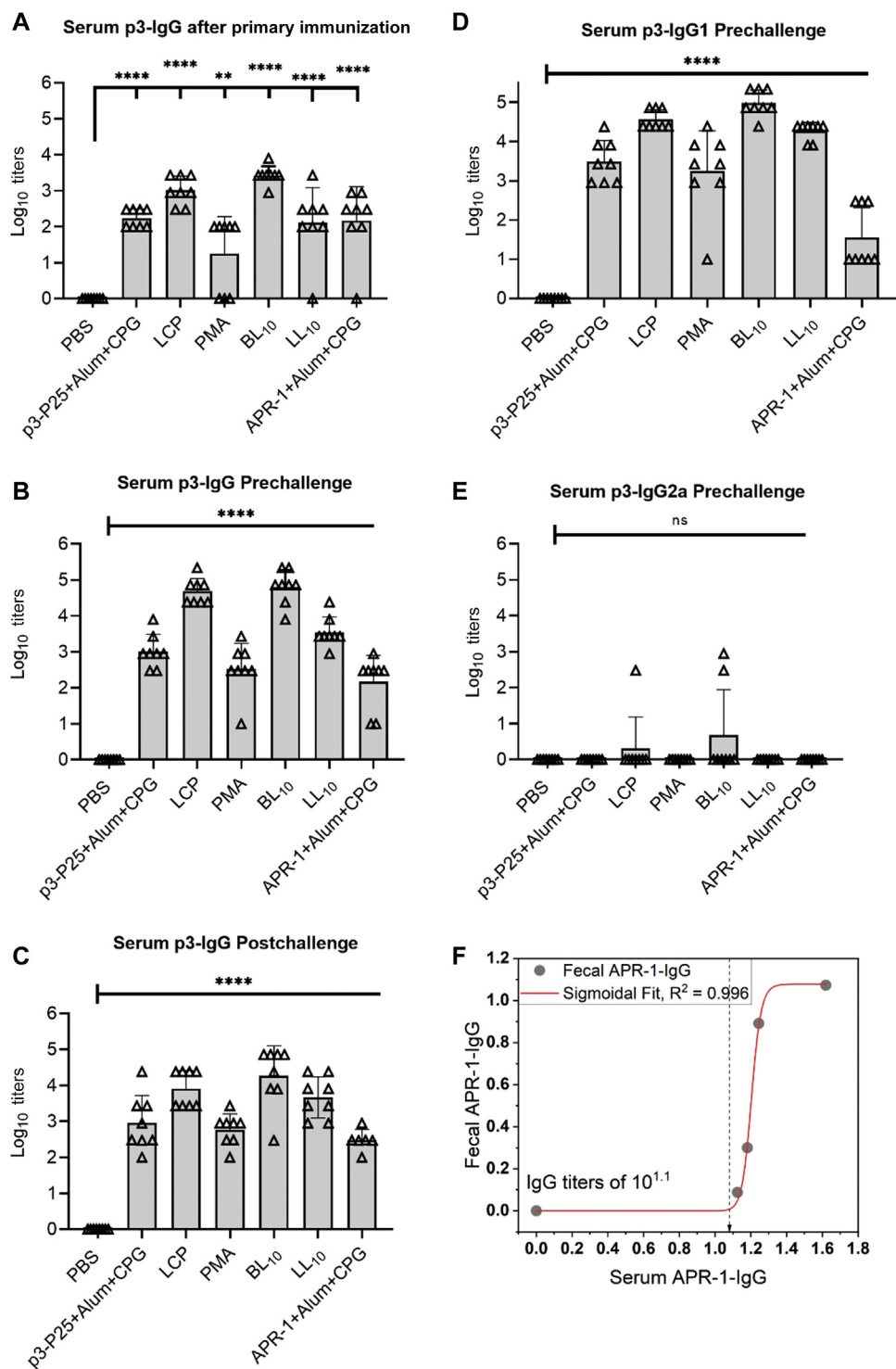


FIG E2. Generation of antigen-specific serum IgG over the immunization course; after primary immunization (A), prechallenge (B), and postchallenge (C), in addition to antigen-specific IgG subclasses, IgG1 (D), and IgG2a (E), and the relationship between serum anti-*Na*-APR-1 IgG log₁₀ titers and fecal anti-*Na*-APR-1 IgG log₁₀ titers (F) fitting to sigmoidal function gave $R^2 > 0.99$. *ns*, Nonsignificant.

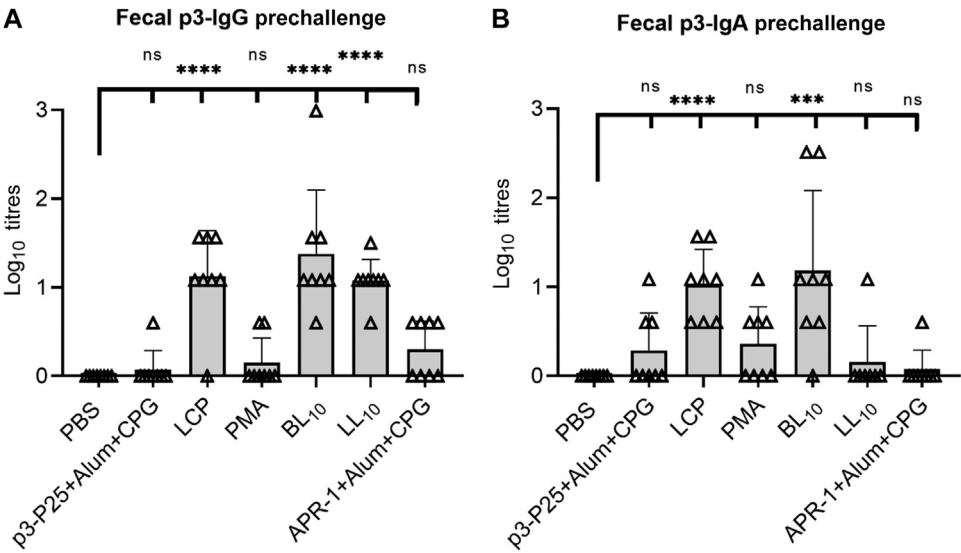


FIG E3. Fecal anti-p3 IgG (A) and IgA (B) titers. *ns*, Nonsignificant.

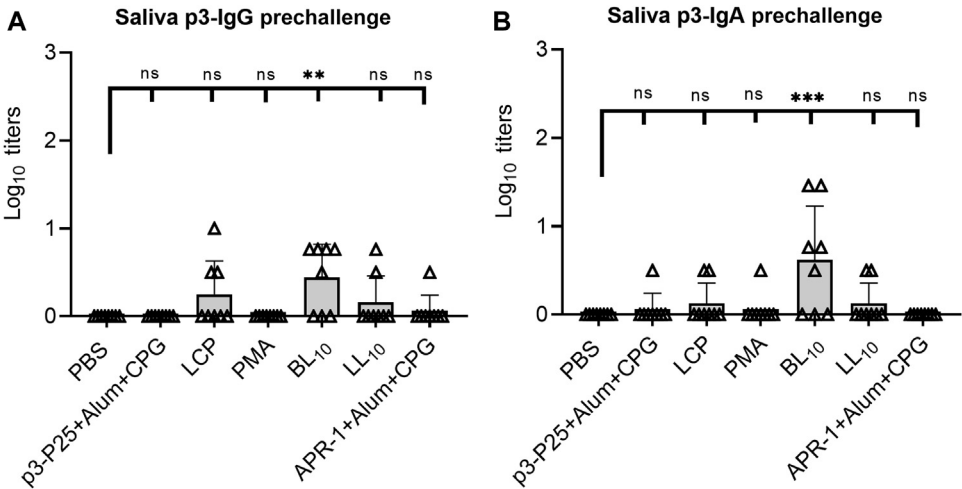


FIG E4. Salivary anti-p3 IgG (A) and IgA (B) titers. *ns*, Nonsignificant.

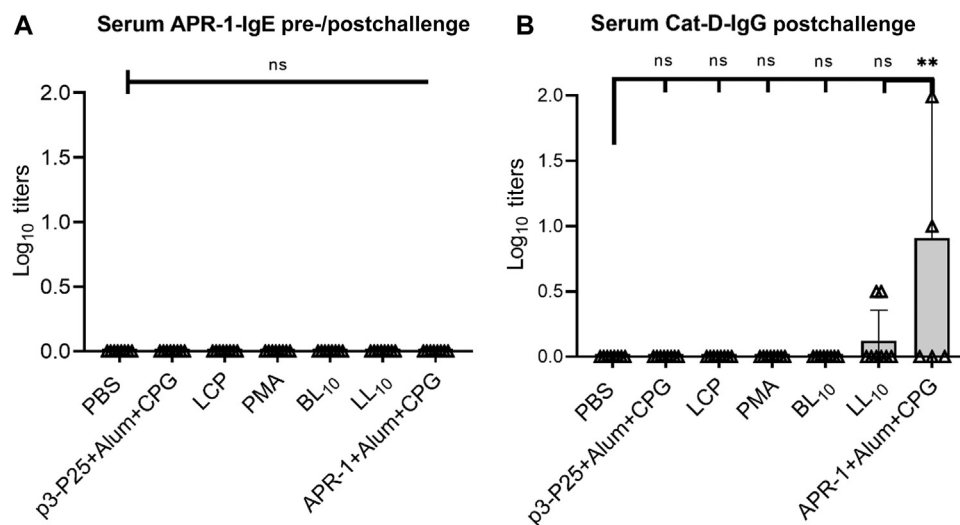


FIG E5. Serum APR-1-IgE titers as indicator of allergic response (**A**), and serum anti-human cathepsin-D IgG titers as indicator of autoimmune response (**B**). Mice groups did not generate detectable IgE titers against native protein in all groups' sera; however, peptide nanovaccines did not generate significant APR-1-specific anti-human cathepsin-D IgG titers, in the serum, whereas APR-1-immunized group generated low IgG titers that recognized human cathepsin-D. *Cat-D*, Cathepsin-D; *ns*, nonsignificant.

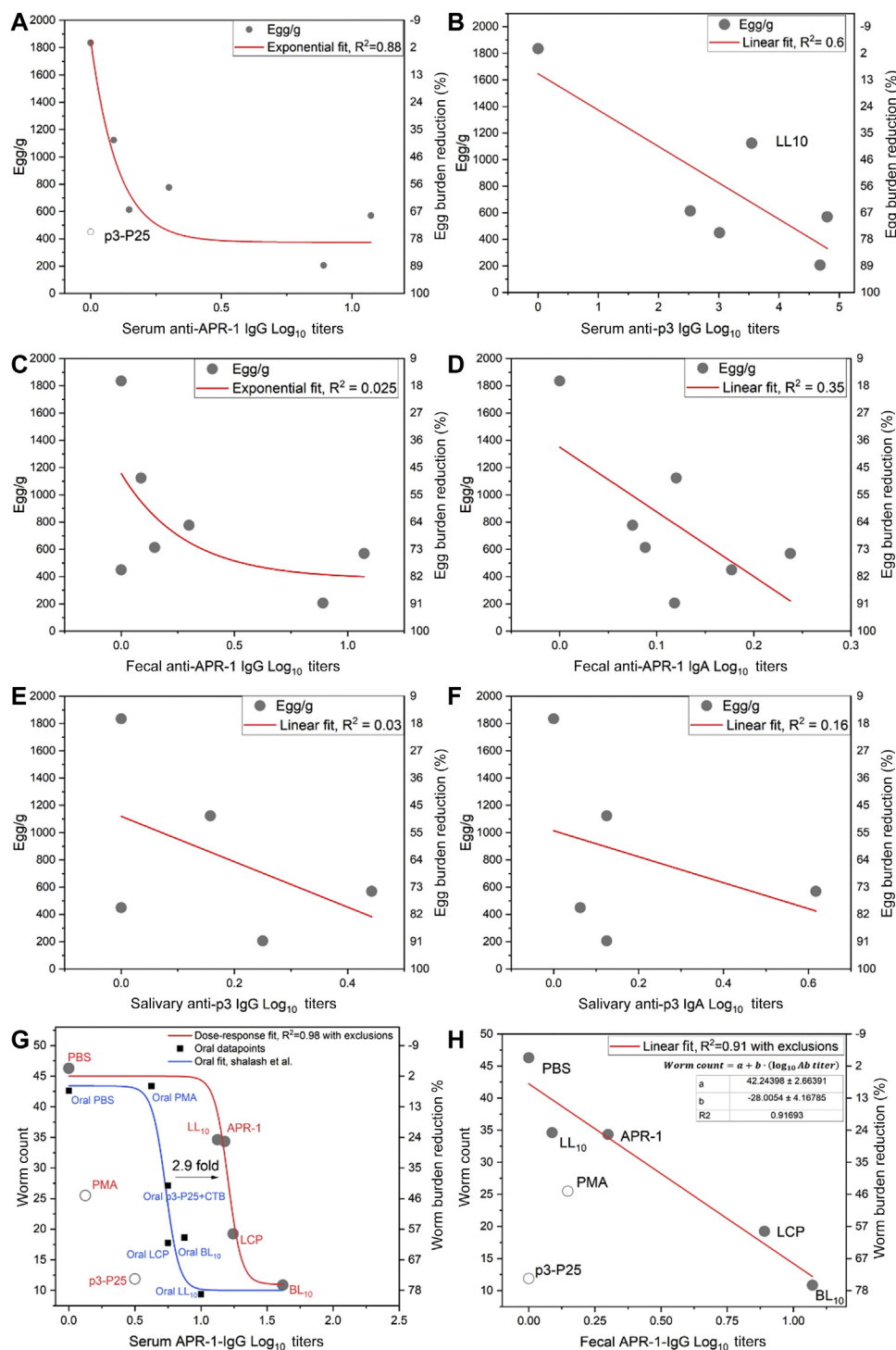


FIG E6. The relationship between egg burden and (A) serum anti-APR-1 IgG titers (exponential fit gave $R^2 = 0.88$), (B) serum anti-p3 IgG titers (linear fit gave $R^2 = 0.6$), (C) fecal anti-APR-1 IgG titers (exponential fit gave very poor R^2 of 0.025), (D) fecal anti-APR-1 IgA (linear fit gave low R^2 value of 0.35), (E) salivary anti-p3 IgG titers (linear fit gave very poor R^2 of 0.03), or (F) salivary anti-p3 IgA titers (linear fit gave very low R^2 of 0.16). Finally, the blue curve (Fig E6, G) represents oral serum titers-efficacy fitting results from Shalash et al^{E3} for the same peptide vaccines; higher antibody titers (ca. 3-fold) are required for the injection route to achieve the same level of worm reduction efficacy as those observed with oral vaccine delivery (Fig E6, G). Linear fit between worm count and fecal anti-APR-1 IgG (H) gave $R^2 = 0.91$; PMA and p3-P25 were excluded because they did not have significant serum or fecal anti-APR-1 IgG titers.

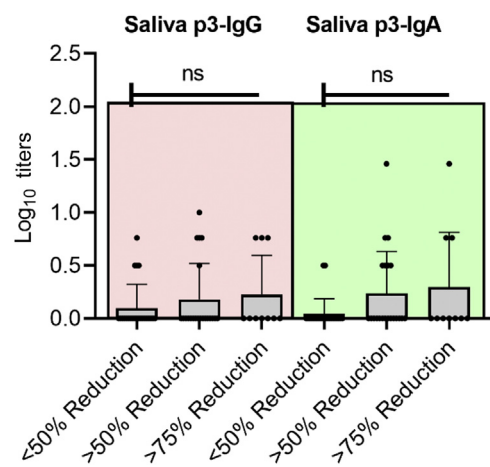


FIG E7. The lack of contribution of salivary anti-p3 IgG and IgA titers to protection, investigated via categorization/reshuffling procedure. *ns*, Nonsignificant.

CLUSTAL 2.1 multiple sequence alignment

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Nb-APR-1      AGLEGGQQTQEHYSHTARAPGGDMPRLLLLLLALFAVGYGAIHHRRAYHSRRDVQSIPLTR
Na-APR-1      -----MARLVFLVLCTAAASVHRRLFHQARRHVTSSVLSR
Mouse         -----MKTPGVLLLLGLLASSFAIIRIPLRKFTSIRRTMTE
Human         -----MQPSSLLPLALCLLAAPASALVRIPLHKFTSIRRTMSE
               .  :: * *  :.  :           :           :..

Nb-APR-1      QRTLRLRLLLSGSWEDYQKQRNHYQKLLAKYAANKAGKLQSTNEIDELLRNYMDAQYFG
Na-APR-1      QPTLRERLIASGSWEDYQKQRYHYRKKILAKYAANKASKLQSANIDEELLRNYMDAQYFG
Mouse         VGGSVEDLILKGPITKYSMQ-----SSPKTTEPVSELLKNYLDAYYFG
Human         VGGSVEDLIAKGPVSKYSQA-----VPAVTEGPIPEVLKNYMDAQYFG
               * *: .*. .*.           :   : *:*:*:*:*:*:*

Nb-APR-1      TIQIGTPAQNFTVIFDGTSSNLWVPSRKCPFYDIACMLHHRYSYGASSTYKEDGRKMAIQ
Na-APR-1      VIQIGTPAQNFTVIFDGTSSNLWVPSRKCPFYDIACMLHHRYSYGASSTYKEDGRKMAIQ
Mouse         DIGIGTPPQCFTVVFDTGSSNLWVPSIHCKILDIAWVHHKYNYSKSSSTVYKNGTSFDIH
Human         EIGIGTPPQCFTVVFDTGSSNLWVPSIHCKILDIAWVHHKYNYSKSSSTVYKNGTSFDIH
               * **** * ********* : * : **** :*:*:* * **** :* : *

Nb-APR-1      YGTGSMKGFISKDTVCVAG-----ICAESQPF AEATSEPGLTFIAAKFDGILGM
Na-APR-1      YGTGSMKGFISKDIVCIAG-----ICAEEQPF AEATSEPGLTFIAAKFDGILGM
Mouse         YGSGSLSGYLSQDTSVPCKSDQSKARG--IKVEKQIFGEATKQPGIVFAAKFDGILGM
Human         YGSGSLSGYLSQDTSVPCKSASSASALGGVKVERQVFGEATKQPGITFIAAKFDGILGM
               **:*.:.*:.*:*.:.           : * * * .*:.*:*.:.*****

Nb-APR-1      AFPEIAVLGVQPVFHTFIEQKKVSPVFAFWLNRNPDSDLGGEITLGGMDARRYVDPITW
Na-APR-1      AFPEIAVLGVTPVFHTFIEQKKVSPVFAFWLNRNPESEIGGEITFGGVDTRRYVEPITW
Mouse         GYPHISVNNVLPVFDNLMMQKLVQKIFSYLNRDPEGQPGGELMLGGTDSKYHGLSY
Human         AYPRISVNNVLPVFDNLMMQKLVQKIFSYLNRDPAQPGGELMLGGTDSKYHGLSY
               .:*.*:.* * ****.:.*:.* * . :*:.*:*.:. :*: * :* * :* :*:

Nb-APR-1      TPVTRRGYWQFKMDTVKGGSSVACPNQCQAIADTGTSLIAGPKAQVEAIQKYIGAEP LM
Na-APR-1      TPVTRRGYWQFKMDMVQGGSSSIACPNQCQAIADTGTSLIAGPKAQVEAIQKYIGAEP LM
Mouse         LNVTRKAYWQVHMDQLEVGNETLCKGGCEAIVDTGTSLLVGPVEEVKELQKAIGAVPLI
Human         LNVTRKAYWQVHLDQVEVASGLTLCKEGCEAIVDTGTSLLVGPVDEVELQKAIGAVPLI
               ***:.***.:*: :. . * * :*:.*:*.:.*:.* :*. :* * * * :

Nb-APR-1      KGEYMIPCDKVPSPLELTFVIEGKAFTLKGEDYVLSVKTGGKTIKLSGFMGMDPFRIGE
Na-APR-1      KGEYMIPCDKVPSPDVFSIIDGKTFTLKGEDYVLTVAAGKSICLSGFMGMDPFRIGE
Mouse         QGEYMIPCEKVSSLPTVYLKGGKKNYELHPDKYILKVSQGGKTIKLSGFMGMDIPPPSGP
Human         QGEYMIPCEKVSTLPAITLKLGGKGYKLSPEYTLKVSQAGKTIKLSGFMGMDIPPPSGP
               :*****:*.*:.* : : * * : * : * * .*:.*:*.:.*****:* *

Nb-APR-1      LWILGDVFIGRYYTVFDIGEARVGAQAKAENGIPVPPAVREVRYELFNTNSAEEDTFMNV
Na-APR-1      LWILGDVFIGRYYTVFDVGQARVGAQAKSEDFGFPVGTPTVTRFRQLQEDSDSDEDDVFTF --
Mouse         LWILGDVFIGRYYTVFDRDNNRVGFANAVVL-----
Human         LWILGDVFIGRYYTVFDRDNNRVGFAEARL-----
               ***** ***** : *****

Nb-APR-1
Na-APR-1
Mouse
Human

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FIG E8. Protein sequences alignment of mouse cathepsin-D, human cathepsin-D, *Nb*-APR-1, and *Na*-APR-1; yellow highlighted sequence showing lack of similarity of p3 epitope (within APR-1 proteins) to corresponding sequences in mouse or human cathepsin-D sequences. Mouse cathepsin-D (UniProt P18242) sequence is 89% similar (80% identical) to human cathepsin (UniProt P07339), and only 46% identical to hookworm *Na*-APR-1 enzyme sequence (UniProt Q9N9H3) using CLUSTALW, whereas hookworm *Na*-APR-1 enzyme sequence (UniProt Q9N9H3) is 86% similar (80% identical) to *Nb*-APR-1 sequence.^{E4} *Nb*, *N. brasiliensis*.

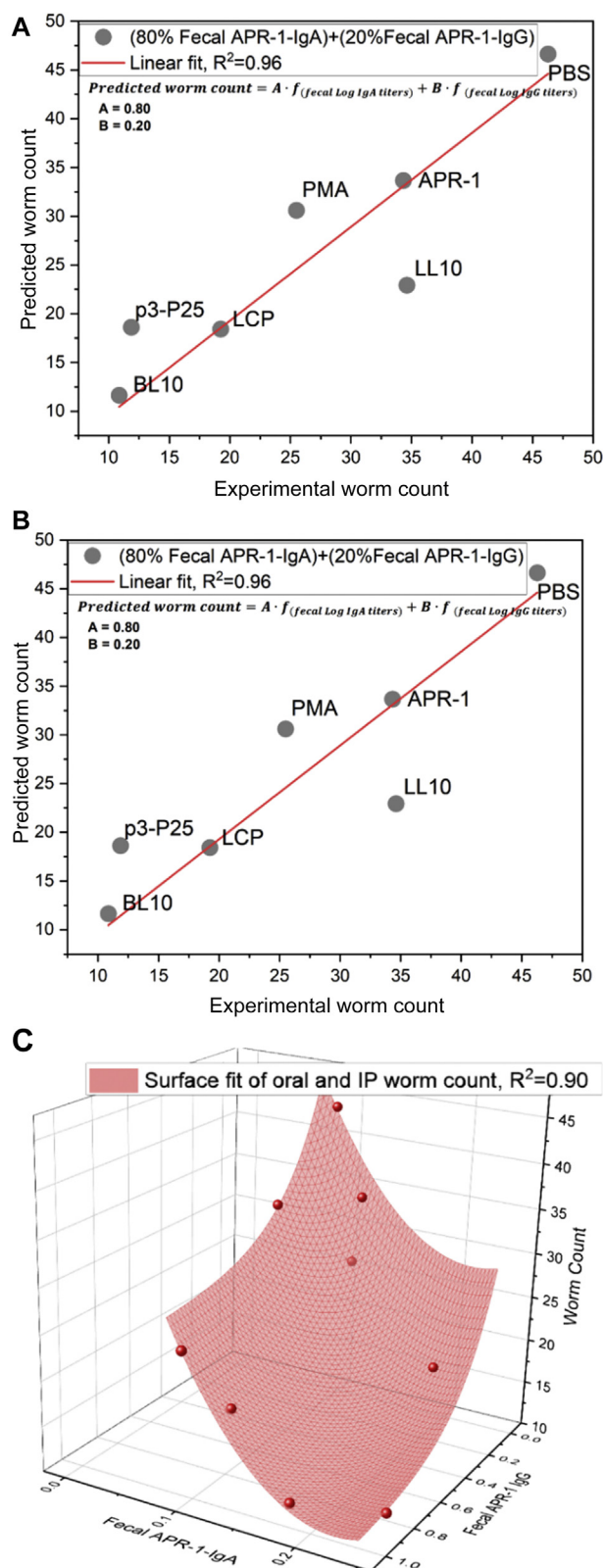


FIG E9. Combined immune correlates of protection. The relationship between predicted and experimental worm count using combination of the 2 established relationships; fecal APR-1 IgG and IgA vs worm counts (A), or serum APR-1 IgG and fecal APR-1-IgA vs worm counts (B) for prediction of corresponding adult worm count; multivariate fit gave $R^2 = 0.96$. Three-dimensional surface fit between adult worm count (y-axis) and fecal anti-APR-1 IgG (z-axis) and IgA (x-axis) from both injection and oral immunization routes of the employed peptide vaccines, surface log-normal fit gave $R^2 = 0.90$ (C).