

Safety and immunogenicity of novel group A streptococcal peptide vaccines targeting cryptic epitopes (J8-K4S2 and P*17-K4S2): a first-in-human, double-blinded, phase 1 randomised controlled trial

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Abstract

The rapidity and aggressiveness of many group A streptococcal (*Streptococcus pyogenes*) clinical syndromes highlight the urgent need for a vaccine. Two related combination cryptic epitope peptide vaccines, J8-CRM+K4S2-CRM ('J8-K4S2') and p*17-CRM+K4S2-CRM ('p*17-K4S2'), are novel vaccine candidates under investigation. In this single-centred, two-stage, unblinded sentinel safety dosing, then randomized, double-blinded, placebo-controlled trial, 30 volunteers were recruited (NCT04882514). Ten volunteers (Stage 1) were given test doses of J8-K4S2 and then p*17-K4S2 in a staggered, unblinded fashion at weeks 0, 3 and 6 to confirm immediate safety of the products. This was followed by twenty volunteers (Stage 2) who were randomized to receive either of the trial vaccines, J8-K4S2 or p*17-K4S2, or comparator (RabAvert) vaccine following the same vaccination schedule. The primary outcome was vaccine safety; the secondary outcome was immunogenicity. Both vaccine candidates were well tolerated and elicited robust immune responses. The most common adverse events noted were transient injection site pain, injection site erythema, fatigue and headache. These were similar for those receiving either the novel vaccines or the rabies vaccine comparator. There were no high-grade attributable adverse events and no severe adverse events overall.

Immunogenicity assessment by ELISA demonstrated that both vaccine candidates consistently induced strong and sustained antibody responses against their respective target antigens, J8 and p*17, with lower responses to K4S2 commensurate with the lower dose of K4S2 present in the vaccines. Both vaccines induced antibody responses to the surface of different strains of live *S. pyogenes*. All 20 participants who received the candidate vaccines achieved at least a 4-fold rise in J8- or p*17-specific antibody titers from baseline, demonstrating 100% seroconversion, while 90% also seroconverted to K4S2. Notably, these elevated antibody responses persisted for at least six months post-vaccination, remaining significantly above pre-immunisation levels, highlighting the durability of the immune response induced by both vaccines.

The safety and immunogenicity profiles for J8-K4S2 and p*17-K4S2 were favourable and support ongoing development of these promising vaccine candidates.

Introduction

Streptococcus pyogenes (group A *Streptococcus*; Strep A) infections result in over 650,000 deaths annually and have been the inciting pathogen in a surge of outbreaks worldwide post-COVID19¹. Clinical syndromes range from mild pharyngitis to more severe presentations of necrotizing fasciitis, septicemia and in rare cases streptococcal toxic shock syndrome. Additionally, through molecular mimicry, untreated *S. pyogenes* infections, even mild, can lead to rheumatic heart disease (RHD) and post-streptococcal glomerulonephritis². There is a clear and urgent need worldwide for a vaccine against this pathogen. The vaccine, however, must balance efficacy while avoiding concerns of autoimmunity.

One of *S. pyogenes*'s major virulence factors is the M-protein, which defines its serotype or *emm* types. While each strain of *S. pyogenes* expresses only one type of M-protein, there are over 275 known distinct

emm types³. Cross-strain immunity is limited, providing an individual minimal cross-protection against newer strain encounters. There are also substantial geographical variations in circulating *emm* types, further creating challenges for vaccine development⁴. One vaccine strategy has been to incorporate the epitopes of M-proteins from multiple *emm* type strains, though there are inherent limitations to this approach due to the large number of non-cross-reactive M-proteins⁵⁻⁷; another has been to define peptides from the highly conserved regions from the M-protein⁸⁻¹¹. Still further strategies consider employing non-M-protein targets, such as other *S. pyogenes* virulence factors¹²⁻¹⁵.

A concern with all *S. pyogenes* vaccine candidates is whether they may initiate acute rheumatic fever (ARF), an autoimmune sequela thought to derive from molecular mimicry, where antibodies target the heart (RHD) and/or brain (rheumatic chorea)¹⁶. T-cells are also thought to contribute to pathogenicity via cross-reactivity with heart proteins, such as cardiac myosin and tropomyosin. While the precise identity of autoimmune epitopes on *S. pyogenes* has not been defined, the B-repeat section of the M-protein has been suggested to be a possible region^{17,18}. With these considerations for vaccine design, minimal epitopes were defined from the C-repeat region of the M-protein to reduce the risk of host cross-reactivity. The main focus was on the highly conserved C3 repeat region as previous studies demonstrated that host (mouse and human) antibodies to epitope 'p145' in this region could opsonize multiple strains of *S. pyogenes*^{9,10,19-21}. Furthermore, others have shown that antibodies to the M-protein demonstrate greater opsonophagocytic killing compared to non-M-protein antibodies^{12-14,22}. Two M-protein epitopes from p145, 'J8'²³ and 'p*17'²⁴ were identified. J8 is a 12-amino acid (aa) peptide flanked by amino acids from a yeast protein (GCN4) for structural stability²³; and p*17 is a 20-mer variant peptide designed through amino acid substitutions²⁴. When these peptides were conjugated to diphtheria toxoid (DT) or cross-reacting material 197 (CRM₁₉₇) and tested in the Lewis rat model of valvulitis, they did not induce rheumatic fever following vaccination^{25,26}. Importantly, J8 and p*17 vaccines induced antibodies in mice and rats that recognized *S. pyogenes* irrespective of *emm* type^{24,27,28}. Vaccination with these vaccine candidates protected mice from both upper respiratory tract infection (unpublished) and invasive disease following skin challenge^{24,27,29-31}. Additionally, J8-DT, when administered with Alum and delivered intramuscularly, was shown to be safe and immunogenic in a single-dose pilot clinical study³².

The conserved region of the M-protein is immunologically cryptic, and as such humans require years of repeated exposure to develop antibodies to it¹⁰. Similarly, naïve mice do not develop antibodies following infection, but do generate antibodies following vaccination that can then be boosted naturally following streptococcal exposure, leading to enhanced protection from subsequent challenge infections³³. The characteristic of being highly conserved, and able to safely prime for the induction of antibodies that can be boosted by infection, makes this region a highly desirable vaccine candidate.

While vaccines based on J8 and p*17 are strongly protective in animal models against all *S. pyogenes emm* types tested, the most highly virulent strains (such as *covr/s* mutants) upregulate other virulence factors, including the interleukin-8 (IL-8) protease, *S. pyogenes* cell-envelope protease (SpyCEP), which reduces neutrophil chemotaxis to the site of infection^{27,29}, impairing the level of vaccine-induced immunity. It was

observed that immunity to these virulent strains can be restored by incorporating the epitope ('S2') derived from a catalytically inactive truncated fragment of SpyCEP²⁹ into the M-protein epitope-based vaccine. S2 is a 20-aa synthetic peptide that can induce SpyCEP-neutralizing antibodies of equivalent potency to those induced by recombinant SpyCEP^{27,29,34,35}. S2 was further modified with four N-terminal lysine residues to improve solubility (therefore name K4S2)³⁰. To enhance immunogenicity for an outbred population, each peptide (J8, p*17, K4S2) was individually chemically conjugated to CRM₁₉₇. The conjugated peptides were then mixed before adsorption to aluminum hydroxide (Alum), creating the two related novel *S. pyogenes* vaccines – J8-CRM+K4S2-CRM ('J8-K4S2') and p*17-CRM+K4S2-CRM ('p*17-K4S2')³⁶. Both vaccine candidates had been evaluated for safety in a general toxicology study in Sprague-Dawley rats. No evidence of toxicity was observed, and all vaccinated animals developed robust and sustained immune responses³¹. The development of these vaccine candidates was spurred on by results from a previously undertaken pilot study with the related predecessor vaccine J8-DT. The vaccine was evaluated for safety in humans with eight healthy individuals receiving a single intramuscular dose. There were no adverse events and participants generated J8-specific antibodies within 28 days post-vaccination³². Collectively, these findings supported the safety of both vaccines and their suitability for evaluation in a Phase 1 clinical trial in healthy adults³¹.

Here, we present the results of a phase 1, two-staged, first-in-human study consisting of initial unblinded sentinel safety dosing followed by a double-blind, randomized, placebo-controlled study to assess safety, tolerability, and immunogenicity of both vaccine candidates. To our knowledge, these vaccines represent the first cryptic epitope-based antibody-inducing vaccines to have completed a Phase I trial.

Results

Participants:

Participants were enrolled in two stages (sentinel safety dosing and randomized administration; Fig. 1). Demographics and baseline characteristics of the participants involved in the trial are outlined in Table 1. Of note, there was a random predominance of female volunteers. The mean ages for the sentinel safety doses were 24 and 29.2 years of age for the J8-K4S2 and p*17-K4S2, respectively (range 19–34 years of age); the mean age for the RCT stage was 30.9, 32.0, and 32.4 years of age, respectively for each of the arms, with an overall range of 19–44 years of age. Participant comorbidities are noted (Table 1). The CONSORT figure (Fig. 1) outlines enrollment.

Table 1
Demographics of participants for the test doses (Stage 1) and the RCT (Stage 2)^{††}.

	STAGE 1		STAGE 2		
	J8-K4S2	p*17-K4S2	Control	J8-K4S2	p*17-K4S2
	n = 5	n = 6	n = 10	n = 5	n = 5
Sex					
Male	1 (20%)	2 (33%)	3 (30%)	1 (20%)	0 (0%)
Female	4 (80%)	4 (66%)	7 (70%)	4 (80%)	5 (100%)
Age					
mean	24	29.2	30.9	32	32.4
range	19–32	22–34	19–43	21–44	29–40
Comorbidities					
HEENT	0	1 (20%)	2 (20%)	2 (40%)	3 (60%)
Neuro/Psych	2 (40%)	1 (20%)	7 (70%)	2 (40%)	2 (40%)
Resp	0	1 (20%)	0	0	1 (20%)
Cardio	0	1 [†]	0	0	0
GI	0	0	1 (10%)	0	1 (20%)
MSK	0	0	9 (90%)	0	0
Metabolic	0	0	2 (20%)	0	1 (20%)
Skin	0	2 (40%)	2 (20%)	1 (20%)	0
Blood	0	0	3 (30%)	2 (40%)	1 (20%)
Allergies	0	0	5 (50%)	3 (60%)	2 (40%)
Other	2 (40%)	0	8 (80%)	4 (80%)	5 (100%)
†- One individual enrolled in Stage 1 received the first dose but subsequently removed when their slight mitral valve prolapse was deemed to have met exclusion criteria. All scheduled safety follow-ups were completed and was included in the safety population but excluded from the immunogenicity analyses. See Supplementary data file.					
††- Study compliance was excellent; however, we did have one participant in our RabAvert control group unable to complete their last follow-up visit secondary to a family emergency.					

Safety: The vaccines demonstrated a favourable safety and reactogenicity profile.

Stage 1: Sentinel safety doses were administered to five volunteers for each of the two vaccine combinations in a staggered fashion to ensure ample consideration of any potential adverse events.

Participant demographics and adverse event (s) (AEs) for this stage are noted (Table 1 and **Supplementary Table 1,2**). There were no Grade 3 or 4 vaccine-attributable AEs for the sentinels and the trial proceeded. Of note, there was one participant in the J8-K4S2 test arm who developed transient nephrotic range proteinuria noted on urinalysis collected pre-dose on the day of first dosing (resulted after dosing visit) that recurred on the day of third dose (resulted after dosing) though they had a normal point-of-care (POC) creatinine prior to each dose and urinalyses preceding that were deemed clinically non-significant (1.0 g/L or negative on dipstick urinalyses). Nine weeks after their last dose, they developed peripheral edema and persisting nephrotic range proteinuria that was later diagnosed as minimal change disease (MCD) via renal biopsy. Detailed review by the trial's data safety monitoring board (DSMB) determined this event was non-attributable (see **Supplemental data** for further details). Symptoms and proteinuria resolved by three months.

Stage 2

In the **placebo-controlled RCT stage**, all participants reported at least one AE over the course of the trial. The total number of AEs reported by study arm were 160 (control), 92 (J8-KS42), 76 (p*17-K4S2) for a total of 328 AEs (Fig. 2A). Of those, all but nine were categorized as mild (Grade 1); nine were Grade 2 AEs and all were headaches requiring over-the-counter medications to treat them. Of the AEs deemed vaccine attributable ("definitely", "probably" and "potentially related"), there were a total of 73 AEs (36 in control; 28 in J8-K4S2; and 9 in p*17-K4S2) (Fig. 2B). Five of those AEs were Grade 2 severity and again, all were headaches requiring treatment with medications – three were reported from the control arm; two were reported from the J8-K4S2 arm.

AE's specific to reactogenicity are graphed separately (Fig. 3A). The most common AE reported was injection site pain, reported at least once by 13 of 20 participants (65%). Overall AEs reported, whether attributable or not, are tabulated (Fig. 3B).

With specific attention to cardiac and renal-related AEs, echocardiograms were performed with each visit and any abnormalities reviewed by two independent cardiologists. Reported abnormalities were not clinically significant and within the bounds of normal variation. There were no echocardiographic features of rheumatic carditis. There was one case of a "trivial anterior pericardial effusion" noted in a participant in the J8-K4S2 arm on the same day as receiving their third dose (D42) that resolved by repeat echocardiogram two weeks later (D56). None of the findings met protocol stopping criteria and none required intervention.

There were no vaccine-attributable renal AEs.

Immunogenicity: Vaccination induced robust and durable IgG responses.

IgG response over the course of vaccination.

Immunogenicity was evaluated by measuring vaccine antigen-specific IgG responses to J8, p*17, and K4S2 peptides, as well as responses to the carrier protein (CRM₁₉₇). IgG levels were measured at baseline (D0) and after each vaccination (D21, D42, D56) and at 6 months (D224) after the last vaccination, to characterize the kinetics of the antibody response. Following vaccination, all participants who received J8-

K4S2 or P*17-K4S2 vaccine mounted an antibody response to vaccine antigens with a boosting effect after each vaccination and a decline by 6 months post-vaccination (**Supplementary Figs. 1, 2**). For descriptive immunogenicity analyses participants from Stages 1 and 2 are presented together. A marked increase in vaccine antigen-specific IgG titers relative to pre-vaccination (D0) levels was noted (Fig. 4). For the J8-K4S2 cohort, the J8-specific geometric mean IgG titer (GMT) increased by > 20-fold after one vaccination (D21) to > 254-fold ($p < 0.005$) after the final vaccination (D56). Furthermore, even at the 6-month follow-up (D224), titers were significantly higher (48-fold, $p < 0.05$) than pre-vaccination levels (Fig. 4A). Likewise, for the P*17-K4S2 cohort, titers increased by > 15-fold following one vaccination, rising to > 101-fold ($p < 0.001$) after the third vaccination. These titers remained > 21-fold elevated up to six months post-vaccination (Fig. 4C). Using a serologic response definition of > 4-fold increase in titer relative to baseline⁶, all 20 vaccinated participants met criteria for seroconversion for the M-protein component of both vaccines. Total IgG responses were robust and predominantly IgG1, consistent with a Th1-skewed immune response (**Supplementary Fig. 3**). Furthermore, vaccine antigen -specific IgG responses were comparable across Stage 1 and Stage 2 despite a > 9-month gap between cohorts, demonstrating the stability of the vaccine formulation when stored at 4°C (**Supplementary Fig. 4**).

Both vaccine candidates elicited antibody responses to the SpyCEP epitope, K4S2. Consistent with the low dose of K4S2 in the vaccine, these responses were modest. IgG responses increased over time from a mean (for all 20 participants) of 1.2-fold to 5.7-fold between D21 and D56 with an overall slight decline by D224 (Fig. 4B, D). Responses to carrier protein CRM followed an increasing pattern of antibody response consistent with vaccine boosting (**Supplementary Fig. 5**).

Vaccine vs RabAvert control responses.

In exploratory pooled stage comparisons, the *S. pyogenes* vaccine responses between *S. pyogenes* vaccinated and RabAvert® (control) vaccinated cohorts were compared by performing area under the curve (AUC) analysis, which takes into account an entire series of dilutions (1 in 80 to 1 in 5120) for each participant at each time point (**Supplementary Fig. 1, 2**). The RabAvert control group ($n = 10$) was analysed at all time points against all antigens (J8, p*17 K4S2 and CRM) in parallel with *S. pyogenes* vaccine recipients, under blinded conditions (**Supplementary Fig. 6**).

Participants receiving each active vaccine in Stages 1 and 2 are presented together by vaccine candidate for descriptive immunogenicity analyses. Because the RabAvert comparator was administered only in randomized Stage 2, comparisons between the active-vaccine cohorts and the RabAvert cohort were exploratory and should not be interpreted as randomized treatment comparisons. At baseline (D0), all 30 participants had negligible J8- and p*17-specific IgG (geomean AUC 4.1 and 4.5, respectively; Fig. 5A, B), consistent with the cryptic nature of these M-protein-derived antigens. In contrast, all participants had higher pre-existing antibodies to K4S2 (geomean AUC 43.2; Fig. 5C). Following immunisation, recipients of J8-K4S2 or p*17-K4S2 ($n = 10$ per cohort) showed a progressive increase over time in antigen-specific IgG compared with RabAvert control. In the J8-K4S2 cohort, J8-specific geomean AUC increased 65-fold, 145-fold and > 322-fold after the first, second and final third dose, respectively. Responses after the second and third doses were significantly higher ($p < 0.005 - 0.001$), than those observed in the RabAvert group

(**Supplementary Fig. 7A**). By D56, 100% of participants had mounted a J8-specific response. At 6 months post-vaccination (D224), antibody levels declined but remained 146-fold higher than the RabAvert control ($p < 0.01$; **Supplementary Fig. 7A**). Analysis of AUC demonstrated significant increases from D0 to D56 and from D0 to D224 (both $p < 0.05$; Fig. 5A(i-ii)). No appreciable increase in J8-specific IgG was observed in the RabAvert group over the study period (Fig. 5A(i-ii)).

Similarly, in the p*17-K4S2 cohort, p*17-specific IgG increased relative to the RabAvert control, with geomean AUC fold-changes ranging from 49-fold at D21 to 133-fold at D56, which was significantly higher ($p < 0.01 - 0.005$), than the fold-change AUC at D0 (**Supplementary Fig. 7B**), with 100% of participants mounting a p*17-specific response. Six months after the final vaccination, antibody levels remained 66-fold higher than the RabAvert control and significantly higher than D0 ($p < 0.01$; **Supplementary Fig. 7B**). Analysis of AUC demonstrated significant increases in p*17-specific IgG from D0 to D56 and from D0 to D224 (both $p < 0.01$; Fig. 5B(i-ii)). Furthermore, p*17-specific IgG responses were significantly greater in the p*17-K4S2 cohort than in the RabAvert control group at both D56 ($p < 0.001$, Fig. 5B(i)) and D224 ($p < 0.01$, Fig. 5B(ii)). p*17-specific IgG levels in the RabAvert group remained negligible at all measured time points (Fig. 5B).

K4S2-specific responses from participants who received either the J8-K4S2 or p*17-K4S2 vaccine, are presented together for descriptive immunogenicity analyses (Fig. 5C). Responses to K4S2 were modest in both vaccine arms, consistent with the low K4S2 dose in the vaccines. Nevertheless, K4S2-specific IgG levels increased significantly from D0 to D56 ($p < 0.01$) which were also significantly higher than the RabAvert control at the corresponding time point ($p < 0.01$; Fig. 5C(i)). An increasing trend at D224 was also noted, but it was not significant (Fig. 5C(ii)). For both vaccine cohorts, AUC fold-change in K4S2-specific responses compared to RabAvert demonstrated a significant increase ($p < 0.05-0.01$), beginning after the second vaccination (**Supplementary Fig. 7C, D**). These elevated responses were sustained in the J8-K4S2 cohort through to the 6-month follow-up timepoint (**Supplementary Fig. 7C**). The RabAvert group exhibited no detectable non-specific antibody responses to any of the vaccine antigens and maintained baseline levels throughout the course of the study (**Supplementary Fig. 6**).

CRM-specific responses in the vaccinated and RabAvert control cohort demonstrated high baseline levels at D0 (geomean AUC 4511.4), consistent with cross-reactivity between CRM and diphtheria toxoid (DT) and likely reflecting DT vaccination in the population (Fig. 5D). Nevertheless, a clear increase in CRM-specific IgG was observed after the final immunisation, with all participants registering a 4-fold or greater increase in AUC than the corresponding time point in the control group ($p < 0.001$). By D224, levels had declined but remained significantly higher (2.3 -fold, $p < 0.001$) than in the RabAvert control group (Fig. 5D(i)). The RabAvert group maintained the baseline level until at least the 6-month time point, when the last samples were collected (Fig. 5D, **Supplementary Fig. 6(iv)**).

Vaccination elicited broadly reactive antibodies against two distinct emm-type strains of *S. pyogenes*

To characterise vaccine-induced antibody responses to live *S. pyogenes*, IgG binding to the bacterial surface was assessed by flow cytometry using clinically relevant and distinct *emm*-types; M1 and M75 strains. Total IgG was purified from seven matched sera collected before vaccination (D0) and after the third vaccination

(D56), from a randomly selected subset of participants comprising three individuals from the J8-K4S2 cohort and four individuals from the p*17-K4S2 cohort (Fig. 6A). Purified IgG was evaluated for binding to live *S. pyogenes* bacteria. Representative histograms from one participant from each vaccine group against each bacterial strain are shown in Fig. 6B-E. Flow cytometry analysis demonstrated significantly greater binding of D56 IgG compared with matched D0 IgG for both M1 and M75 strains (Fig. 6B-E).

Notably, pre-vaccination IgG exhibited significant binding to both strains, reflecting naturally acquired antibodies arising from prior exposure to *S. pyogenes*. Binding of D0 IgG to *S. pyogenes* strains was significantly greater than that observed for the negative control (secondary antibody only) (Tx value > 3000). However, post-vaccination (D56) IgG from the seven participants tested showed a marked increase in binding to both *S. pyogenes* strains relative to matched pre-vaccination samples, demonstrating vaccine-induced enhancement of antibody recognition of the bacterial surface.

Consistent with these findings, analysis of Super-Enhanced Dmax (SE Dmax) % positive values³⁷, which estimate the proportion of events that are truly positive relative to the reference population, showed significantly higher binding of D56 IgG than that of matched D0 IgG across all participants tested against both M1 and M75 strains (Fig. 6F). Collectively, these data demonstrate that vaccination induced robust antibody responses capable of recognising the surface of clinically relevant *S. pyogenes* strains representing distinct *emm*-types.

Discussion

The worldwide prevalence of *S. pyogenes* and its ability to cause severe rapidly progressive disease manifestations warrant ongoing efforts for development of a safe and effective vaccine. These Phase 1 trials employed two alum adjuvanted, dual conjugated peptides targeting two of *S. pyogenes*'s major virulence factors, the M-protein and SpyCEP. Preclinically, vaccine candidates J8-K4S2 and p*17-K4S2 showed no clinical toxicity and induced robust immune responses in Sprague–Dawley rats³¹ with no evidence of cross-reactive cardiac or neuronal antibodies in an autoimmune Lewis rat valvulitis model^{25,26}. Consistent with these findings, the current human study demonstrated a well-tolerated safety profile alongside excellent immunogenicity.

Reactogenicity, following administration of J8-K4S2 and p*17-K4S2 was comparable to that observed in the RabAvert vaccine control group. The frequency and severity of AEs were also consistent with those reported in other phase 1 trials of alum-adjuvanted vaccines, where the most common AEs are local injection site reactions, followed by malaise, low-grade fever and headaches, with > 80% in most cases experiencing at least one of those symptoms with dosing^{38–40}. For an additional cross-check of our control, the AEs reported from the original trials with RabAvert were confirmed for similar reactions to our cohort⁴¹.

Vaccination with each of the J8-K4S2 and p*17-K4S2 vaccines elicited a robust response for both M-protein peptide-specific IgG responses, where they produced peak IgG GMT levels > 254 and > 101, respectively, after three doses and were durable to at least 6 months. At 6 months post-vaccination, J8- and p*17-specific IgG levels remained > 146-fold and > 66-fold above baseline, respectively. Titers increased from

baseline for each of the components with pre-vaccination J8- and p*17-specific IgG levels being low or undetectable, and all participants mounting antigen-specific responses. The low pre-existing titers were predictable, even in an adult population with years of pre-existing exposure to *S. pyogenes*. As shown previously, humans only develop significant levels of antibodies to the C3 region of the M-protein after many years of intense exposure^{10,20} and naïve mice do not make antibodies to this region following sequential infections⁴².

In contrast to the M-protein peptide-specific responses, the pre-existing levels of antibody responses to K4S2 were elevated in about half of participants, demonstrating that this region is not cryptic. While all participants were screened for both anti-streptolysin-O titers (ASOT) and anti-DNaseB at baseline with threshold cutoffs before enrolment to exclude recent acute infection, it is highly likely that population-level exposure to *S. pyogenes* led to a level of immunity against the SpyCEP component. The K4S2-CRM component still elicited seroconversion in > 90% of participants. This was lower than anticipated, although not unexpected given the baseline levels of K4S2 specific IgG in participants (as noted) and the low K4S2 dose evaluated in these studies. Future iterations of the vaccine candidate may need to contain higher doses of this SpyCEP epitope. When we compare the results of M-protein-specific titers from a prior 30-valent M-protein vaccine⁷, the GMTs for our study's participants were in a comparable range.

The carrier protein, CRM₁₉₇, elicited a robust IgG response with titers increasing from baseline and persisting at higher levels after 6 months post-vaccination. There had been concern that the pre-existing CRM-specific response might lead to 'carrier-induced epitope suppression' (CIES) whereby pre-existing immunity to a carrier protein can attenuate responses to conjugated antigens^{43–46}. However, this was not observed, as both J8-K4S2 and p*17-K4S2 vaccines led to not only a significant primary response but also to significant and sequential boosting following the second and third doses of the vaccines.

The antibody levels for these vaccines that are required for protection in humans are not known. However, in pre-clinical studies we showed that naïve immunodeficient SCID mice that received memory B-cells from J8-DT-immunized mice and then were given a streptococcal infection were protected. These mice had no detectable antibody at the time of challenge but were able to rapidly generate modest titers of between 1000 and 10,000 – sufficient to suppress the infection by day 5⁴⁷. Those titers are similar to what we have observed in this study. The total IgG responses were predominantly IgG1, a cytophilic subclass associated with Fc receptor engagement and bacterial clearance, suggesting the induction of functional antibacterial antibody responses.

One of the key findings of this study was that vaccine-induced antibodies could recognise the surface of live *S. pyogenes* strains representing distinct *emm* types. Vaccination with either J8-K4S2 or p*17-K4S2 significantly enhanced IgG binding to both M1 and M75 strains, demonstrating that the immune responses elicited by these vaccines extend beyond recognition of the immunising peptides and are capable of targeting native antigens expressed on the bacterial surface.

Notably, measurable antibody binding to live *S. pyogenes* was detected in pre-vaccination samples, likely reflecting naturally acquired immunity generated through repeated exposure to *S. pyogenes* throughout life.

Previous epidemiological, immunological and human challenge studies have demonstrated that recurrent streptococcal exposures shape the antibody repertoire, resulting in the accumulation of both strain-specific and cross-reactive antibodies in adults^{4,48}. Importantly, despite the presence of pre-existing antibodies, vaccination significantly increased IgG binding to both M1 and M75 strains, indicating an enhancement of the existing immune repertoire. These findings have important implications for the deployment of a future *S. pyogenes* vaccine. Unlike preclinical models, vaccination in real-world settings will occur in populations with heterogeneous histories of streptococcal exposure and varying levels of pre-existing immunity. The observation that both J8-K4S2 and p*17-K4S2 are highly immunogenic therefore provides encouraging evidence that these vaccines can reinforce naturally acquired immune mechanisms and broaden functional immunity against clinically relevant strains. Given the extensive *emm*-type diversity of *S. pyogenes*, the induction of antibodies capable of recognising epidemiologically distinct strains represents an important step towards the development of a broadly protective vaccine.

This is the first Phase I vaccine study, to our knowledge, targeting cryptic or subdominant B-cell epitopes. As these epitopes are cryptic within the M-protein, only an epitope-based strategy can elicit a specific antibody response to them. Although they are cryptic in naïve animals, exposure to live *S. pyogenes* can boost vaccine-induced antibodies and enhance protective efficacy, showing that the epitopes are not cryptic to memory B-cells³³. If this finding is reproduced in human vaccinees, it is possible that intermittent community exposure to *S. pyogenes* will lead to durable immunity post vaccination without need for subsequent boosters.

Further characterisation of the functional activity of these epitope-induced antibodies will be important. However, preclinical studies in mice have shown that epitope-induced antibodies bind various *S. pyogenes* strains and mediate high-level bactericidal killing^{27,28,30}. Furthermore, murine studies also show that prior exposure to one strain of *S. pyogenes* does not hinder vaccine-induced immunity against heterologous strains³³.

This study demonstrates that both J8-K4S2 and p*17-K4S2 are promising candidates to take forward for further studies. While J8 and p*17 are overlapping epitopes in the C3-repeat region of the M-protein, they have many differences. J8 contains only 12 (sequential) amino acids derived from the M-protein that partially overlap the 20 amino acids from the p*17 epitope. Furthermore, p*17 has two non-native amino acid substitutions (within the overlap region) designed to promote structural stability of the peptide^{10,20,21,23,24}. Detailed analyses of the minimal epitopes recognized by each of the volunteers will enable a clearer understanding of whether one or both of these vaccine antigens should be included in the vaccine.

A limitation of the study was the reduced amount of the K4S2-CRM vaccine per dose (6.25µg) and p*17-CRM vaccine dose (25µg) than initially intended. While all vaccinees responded to each component of the vaccine, future studies may incorporate higher doses. The small study size also limits the ability to perform any subgroup analysis, including any sex and gender considerations. Future phase 2 studies will be powered for more in-depth immunogenicity analyses.

In summary, J8-K4S2 and p*17-K4S2 demonstrated excellent safety and immunogenicity as first-in-human *S. pyogenes* vaccine candidates. The data support further development of these vaccine products.

Methods

Trial vaccine

Two synthetic peptide conjugate vaccines were manufactured under Good Manufacturing Practices (GMP) at Auspep Clinical Peptides, Australia. The first vaccine candidate contained the peptides J8 and K4S2; the second vaccine candidate, peptides p*17 and K4S2. Each peptide was chemically conjugated to the carrier protein CRM₁₉₇ to enhance its immunogenicity (i.e., J8-CRM₁₉₇, p*17-CRM₁₉₇, and K4S2-CRM₁₉₇) and was then adjuvanted with Alhydrogel ('Alum'). The vaccine J8-CRM+K4S2-CRM/Alum contained approximately 50 µg J8-CRM₁₉₇, 6.25 µg K4S2-CRM₁₉₇ and Alhydrogel® (~ 250 µg elemental aluminium) per dose. The vaccine p*17-CRM+K4S2-CRM/Alum contained 25 µg p*17-CRM₁₉₇, 6.25 µg K4S2-CRM₁₉₇ and Alhydrogel® (~ 250 µg elemental aluminium) per dose. RabAvert® vaccine was used as the comparator. Products were stored as a sterile liquid suspension of single-dose aliquots of 1.15mL ± 5%, maintained at 5°C ± 3°C in a temperature-monitored refrigerator. Doses of 1.0 mL were administered intramuscularly (IM) on study day 0, day 21 ± 7 and day 42 ± 7.

Trial design

The trial protocol has previously been published³⁶. In brief, the study was divided into two stages. Stage 1 was an open label sentinel safety test dosing stage for each of the two vaccine candidates (J8-K4S2 or p*17-K4S2), with 10 healthy participants. Stage 2 involved randomized and blinded administration of the two vaccine candidates and a comparator rabies vaccine (RabAvert) to 20 healthy participants (Fig. 1). Trial endpoints were safety (primary) and immunogenicity (secondary)³⁶. The inclusion and exclusion criteria are shown in the Supplementary Data (**Extended data – Protocol**).

The two-stage design was to maximize safety. The test doses for each product were given in a staggered manner with at least two days between each participant to enable prompt identification of any immediate adverse events. Any Severe Adverse Event (SAE), grade 4 AE, clinically significant valve abnormality on echocardiography, or episode of acute rheumatic fever (ARF) would have halted the trial, at least temporarily, for a Data Safety and Monitoring Board (DSMB) review.

Trial procedures

The study was approved by the University of Alberta Research Ethics Review Board (REB# Pro00089919) as well as the Health Products and Food Branch of Health Canada and was carried out in accordance with the Declaration of Helsinki and the principles of Good Clinical Practices. The trial was registered with ClinicalTrials.gov: NCT04882514 (May 6, 2021).

For Stage 1, five sentinel participants received a 3-dose series of the first vaccine candidate (J8-K4S2), followed by another five participants who received the second vaccine candidate (p*17-K4S2) (Fig. 1). These doses were given in a staggered fashion with a minimum of two days in between to monitor for any immediate reactions/vaccine concerns. On the day of dosing, each participant was required to have normal creatinine (on point-of-care testing), negative pregnancy test (where applicable), normal ECG and physical exam, as well as a normal urinalysis within the previous 48 hours, and no clinically significant abnormalities noted on their last echocardiogram before they were approved by the study physician to receive the dose. Participants were also required to remain in clinic for monitoring 30 min post-dosing. Safety laboratory tests were performed at baseline, with each dosing visit (week 0, 3, 6) and at week 6 and 26 (6-month post-last dose). After all participants had received their respective 3-dose series and the week 6 follow-up data collected, data were compiled, analyzed and presented to the DSMB for assessment and approval to proceed as planned to Stage 2. Participants from Stage 1 received their week 26 follow-up (6-month post-last dose) thereafter per planned scheduling.

Stage 2 proceeded with 20 participants enrolled and receiving a 3-dose series of either of the two vaccine candidates or the comparator rabies vaccine. Both participants and study staff were blinded to the assignment. Safety assessments and pre-dosing requirements were as above in Stage 1.

Assessment of safety and tolerability

At each study visit, participants were assessed for clinical, laboratory, ECG and echocardiographic abnormalities. Adverse events were documented using standard toxicity tables, adapted from the National Institute for Allergy and Infectious Diseases (NIAID) Division of Microbiology and Infectious Diseases (DMID) toxicity tables⁴⁹. Unsolicited adverse events were also collected throughout the study up to and including the final 6-month follow-up visit (week 26). REDCap, version 15.0.37 was used for all data collection.

Assessment of immunogenicity

Immunogenicity was assessed as vaccine antigen-specific IgG responses to the target peptides (J8, p*17, and K4S2) and IgG responses to the carrier protein (CRM₁₉₇), captured as anti-CRM antibodies. Blood samples were collected from each participant prior to study vaccination (screening), prior to first vaccination (D0) and 2–3 weeks after each vaccination (D21 ± 7; D42 ± 7; D56 ± 7), and at the 6-month follow-up visit (D224 ± 7). Immunogenicity was measured by ELISA and reported as peptide-specific serum IgG antibody titers. ELISA plates (Nunc Maxisorp) were coated with vaccine peptides (J8, p*17, K4S2) and blocked with 5% skim milk in PBS with 0.05% Tween-20. Participant serum was 2-fold diluted, starting at a dilution of 1 in 80 and incubated in coated wells to allow peptide-specific antibodies to bind to the immobilised peptides. Bound antibodies were detected with goat anti-human IgG conjugated to horseradish peroxidase. Goat anti-mouse IgG1 conjugated to horseradish peroxidase was used for IgG subtyping analysis. Pooled human serum from Fiji, a region with endemic *S. pyogenes*, served as the positive control. For negative controls, antigen-coated wells were processed with all reagents except the test serum. To

minimise background noise, all samples were also tested on non-antigen-coated plates, and these values were subtracted from antigen-coated plate readings to clean the data. Data were captured as the optical density (OD) at absorbance 450nm. Titers were defined as the reciprocal of the highest dilution of antiserum with an absorbance greater than 0.112. This value was derived based on the baseline (at D0) OD readings in all 30 participants reflective of background binding to the ELISA plate. The fold change between titer at pre-vaccination and post each and final vaccination (2 weeks and 6 months) were determined. Seroconversion was defined as a minimum four-fold ($\geq 4\times$) rise in vaccine antigen-specific IgG antibody titers relative to baseline (pre-vaccination) values, measured at the designated post-third dose timepoint. For comparisons between vaccine recipients and the RabAvert control group, antigen-specific IgG responses were measured by ELISA using serial serum dilutions ranging from 1:80 to 1:5120. Antibody responses were measured by calculating the area under the curve (AUC) from the resulting titration curves, providing an integrated measure of antigen-specific IgG binding across the dilution range. Higher AUC values indicate stronger antigen-specific antibody responses.

Flow Cytometric Assessment of Vaccine-Induced Antibody Binding to *S. pyogenes*

To evaluate vaccine-induced antibody recognition of the *S. pyogenes* surface, matched plasma samples collected prior to immunisation (D0) and two weeks after the third vaccination (D56) were analyzed from a subset of participants vaccinated with either J8 + K4S2 (n = 3) or p*17 + K4S2 (n = 4). Total IgG was purified from plasma using Protein G affinity chromatography according to the manufacturer's instructions⁴⁸. Purified IgG preparations were subsequently volume-adjusted to match the original plasma volume.

Purified total IgG was diluted 1:40 and incubated overnight with live *S. pyogenes* M1 and M75 strains, in triplicate, normalised to OD₆₀₀ 0.5, with shaking at 100 rpm. Bacteria were then washed and stained with PE-conjugated anti-human IgG Fab antibody (Thermo Fisher Scientific; 1:250 dilution) to detect surface-bound human IgG. Samples were analysed on a BD LSR Fortessa flow cytometer, and fluorescence intensity distributions were compared between matched D0 and D56 samples. Differences in fluorescence intensity distributions were quantified using the Probability Binning algorithm in FlowJo^{13,37} (BD Biosciences). The resulting T(x) statistic was used to assess differences between populations, with values > 4 considered significant ($P < 0.01$)^{37,50}. D56 samples were compared with matched D0 samples and secondary antibody-only controls; representative T(x) values are shown above selected overlaid histograms. The proportion of antibody-positive events was quantified using the Super-Enhanced Dmax (SE Dmax) algorithm in FlowJo, which estimates the percentage of positive events relative to the matched D0 reference population. SE Dmax values were calculated for each participant against the M1 and M75 strains, and individual participant values were plotted as violin plots for each strain.

Statistical analysis

Sample size was calculated using data from a pilot study of the J8-DT vaccine in eight healthy volunteers, employing the J8-specific antibody titer was taken as the continuous immunogenicity design endpoint³². The mean (SD) titers in the pilot were 1.6 (0.29) $\mu\text{g/mL}$ and 4.5 (2.4) $\mu\text{g/mL}$ at baseline and 28 days post-vaccination, respectively. With the assumption that the immunogenicity of both the J8-K4S2 or p*17-K4S2

vaccine candidates would be at least as strong and using a t-test with $\alpha = 0.05$ and power = 0.8, eight subjects per group would be needed to demonstrate an increase in titers after vaccination compared to the commercial vaccine (RabAvert), by standard sample size calculations for normally distributed data.

Primary outcome. Descriptive statistics were used to calculate the frequencies of AEs in each study arm. Given the small patient numbers, comparative statistics were not computed.

Secondary outcome. Immunogenicity was assessed by comparing post-vaccination responses with pre-vaccination baseline responses within each vaccine cohort, and through exploratory comparisons between active-vaccine and RabAvert control recipients. Participants receiving the same vaccine candidate in Stages 1 and 2 were pooled for within-cohort analyses. Longitudinal responses were analysed using paired non-parametric methods (Wilcoxon matched-pairs signed-rank test or Friedman test, as appropriate), while comparisons between independent groups were performed using the Mann-Whitney U test or Kruskal-Wallis test with pairwise Wilcoxon rank-sum comparisons. As RabAvert was administered only in Stage 2, comparisons between pooled active-vaccine cohorts and the RabAvert cohort were exploratory and should not be interpreted as randomized treatment comparisons. Flow cytometry data were analysed using FlowJo software and presented as T(x) or SE Dmax, as described in the methods and relevant figure legends.

Declarations

Written informed consent was obtained from all participants prior to their enrollment in the study.

Data availability

The data that support the findings of this study are available from the corresponding author upon request. Source data are provided with this paper.

Competing interests

Michael F Good and Manisha Pandey are co-inventors on the patents related to the vaccine candidates in trial. All remaining authors declare no competing interests.

Author contributions

Conceptualization: M.F.G., M.P., M.T.H., D.L.T, M.H., G.T., V.M-S.; **Clinical Trial Lead:** M.T.H., V.M-S.; **Clinical investigators:** W.S., C.B., A.T-C., C.O., B.T.; **Study coordination:** K.K., I.O., A.S.; **Scientific Lead:** M.F.G., M.P.; **Vaccine manufacture oversight, QC, regulatory:** M.P., S.R., M.B.; **Funding acquisition:** M.F.G., M.P., M.T.H., V.M-S., D.L.T, M.H; A.S., J.O.; **Methodology and experiments:** M.P., S.R., V.O., C.S., A.C., Z.X., C.W., J.D.; **Data interpretation:** M.F.G., M.P., V.M-S., V.O., C.S., M.Y., K.K., S.J. and G.T. **Product development and commercial support:** E.K, C.D.; **Original draft:** V.M-S., M.P., M.F.G.; **Manuscript review:** All authors. All authors read and approved the final version of the manuscript for submission.

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Figures

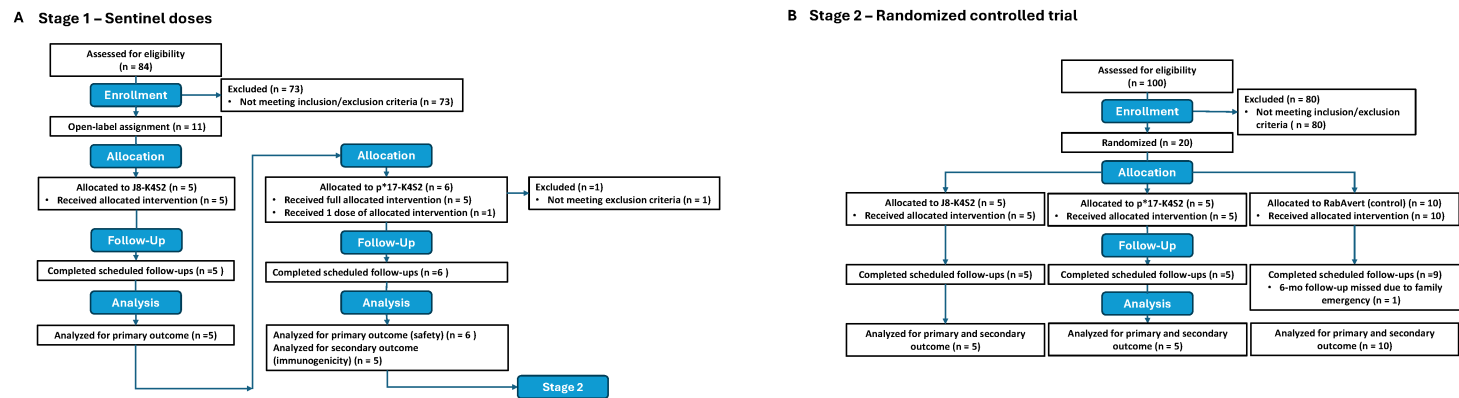


Figure 1

CONSORT Flow diagram of enrolled participants for Stage 1(A) and Stage 2 (B).

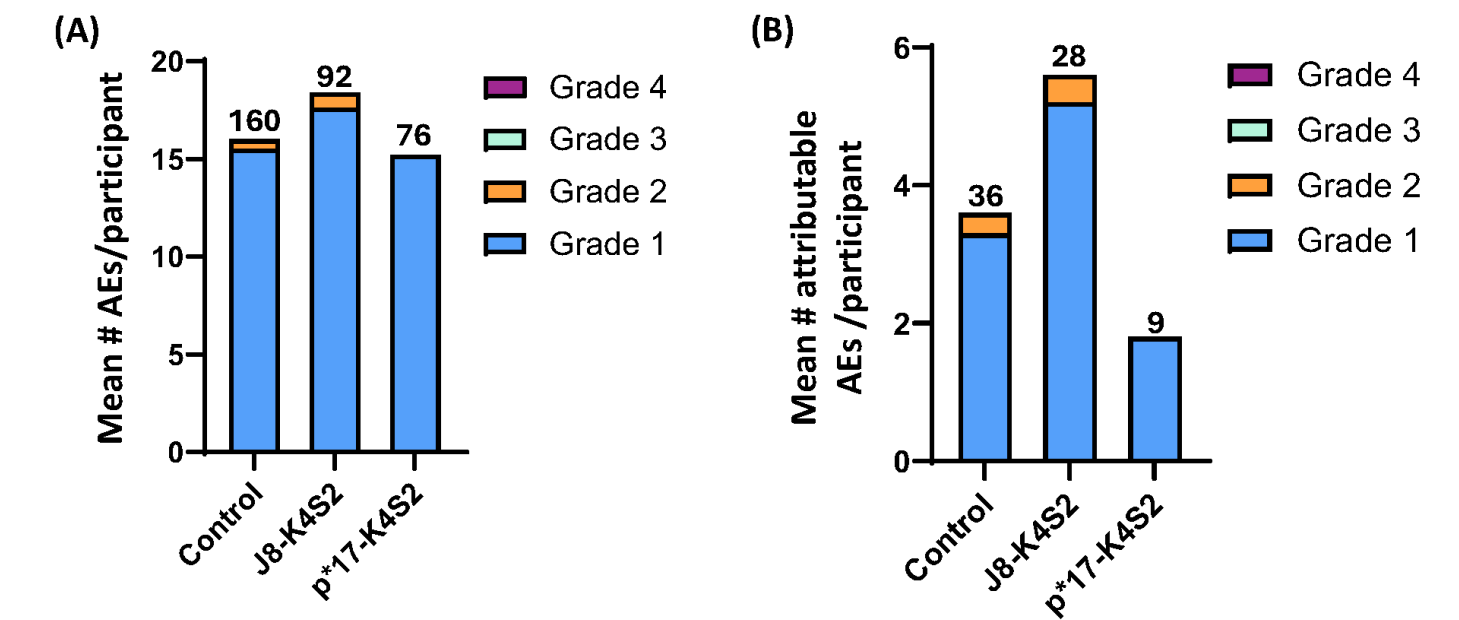


Figure 2

Adverse events (AEs) by study arm– averaged by number of participants in the arm. (A) Overall AEs; (B) Attributable AEs (definite, probably, possibly). All of the attributable Grade 2 AEs were headaches requiring over the counter medication by participants. Individual participants contribute multiple events over the course of the study. The number above the bar refers to the number absolute number of AEs.

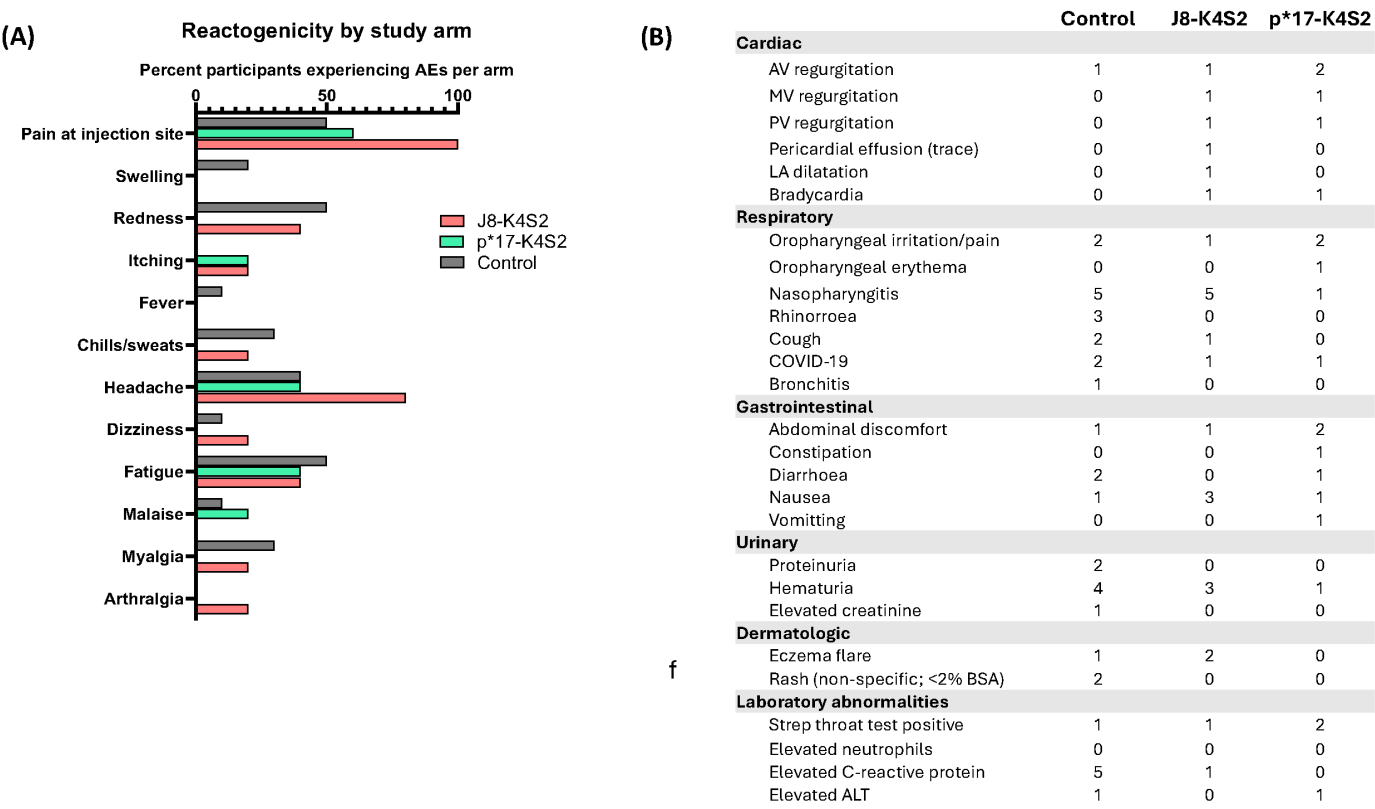


Figure 3

Adverse events (AEs) by system and study arm.(A) Symptoms of reactogenicity – described as a percent of the total participants in each arm; (B) Additional AEs by system – individual events noted. Please see text for additional details.

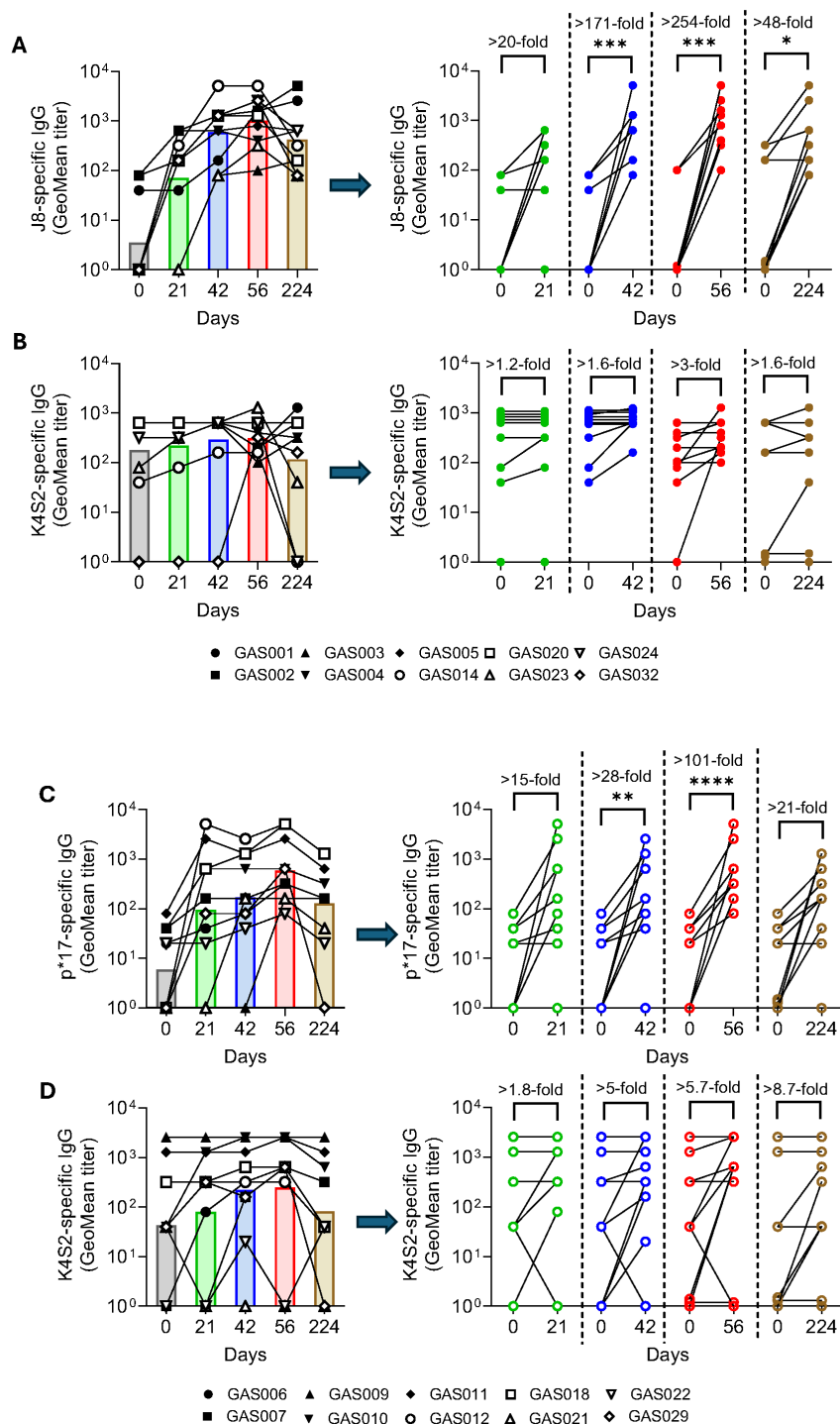


Figure 4

Vaccine antigen-specific serum IgG responses in trial participants. Participants received intramuscular vaccinations with either J8-CRM + K4S2-CRM/Alum or p*17-CRM + K4S2-CRM/Alum in Stages 1 and 2 (n=5 each) on D0, D21, and D42. Antigen-specific IgG responses were measured by ELISA, and the mean titer for each participant was calculated from duplicate measurements using an OD cut-off of 0.112. Antigen-specific mean titer for the J8-K4S2 cohort for (A) J8 and (B) K4S2, and for the P*17-K4S2 cohort for

(C) p*17 and (D) K4S2 are shown. Individual participant titers across each time point are plotted as connected lines, with each participant denoted by a distinct symbol. Data were background-corrected by subtracting OD values obtained from plates without antigen. For descriptive immunogenicity analyses participants from Stages 1 and 2 are presented together by vaccine candidate and the geometric mean titer (GMT) (n=10) is shown as bars. Post-vaccination responses (D21,42, 56 and D224) were compared with D0 and fold-change in GeoMean titers are shown. Statistical analyses for repeated measurements were performed using Friedman's test with Dunn's multiple comparisons correction. Statistical significance is indicated as * $p \leq 0.05$, ** $p \leq 0.01$ *** $p \leq 0.005$, and **** $p \leq 0.001$.

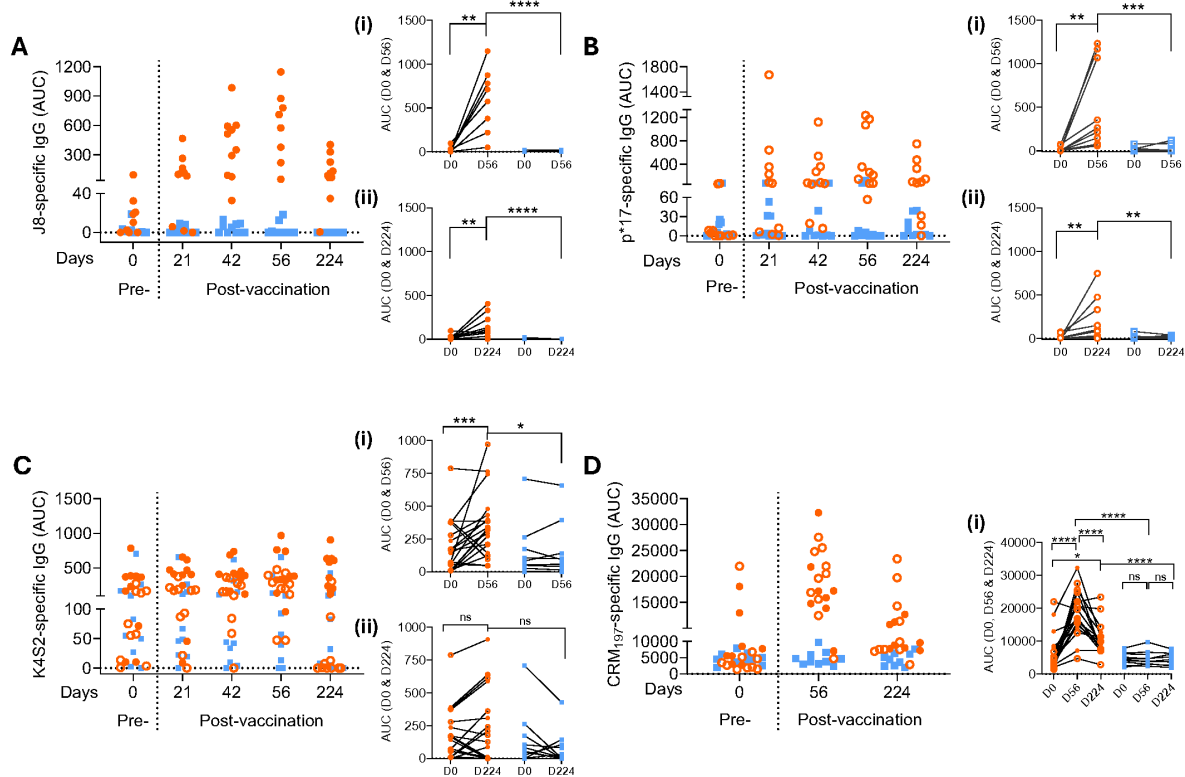


Figure 5

Vaccine antigen-specific serum IgG responses in vaccinated and control cohorts. Participants received intramuscular vaccinations with either J8-CRM + K4S2-CRM/Alum, p*17-CRM + K4S2-CRM/Alum in Stages 1 and 2 (n=5 per stage), or RabAvert (control; Stage 2 only, n=10) on days 0, 21, and 42. Serum samples were collected before vaccination (D0), three weeks (± 7 days) after each dose (D21, D42, and D56), and six months after the final vaccination (D224). Antigen-specific IgG responses were measured by ELISA. Optical density (OD) was measured at 450 nm, and area under the curve (AUC) was calculated from titration curves ranging from dilutions of 1:80-1:5120. AUC values are shown for (Ai-ii) J8-specific, (Bi-ii) p*17-specific, (Ci-ii) K4S2-specific, and (Di) CRM197-specific serum IgG responses. Data were background-corrected by subtracting OD values obtained from plates without antigen. For descriptive immunogenicity analyses, participants receiving each active vaccine in Stages 1 and 2 are presented together by vaccine candidate (J8-K4S2, n=8; and p*17-K4S2, n=10). For analyses of K4S2- and CRM-specific immune responses, all participants who received J8-K4S2 or p*17-K4S2, were analyzed as a single cohort (n=18). Exploratory

comparisons of within-group changes from baseline (D0) to post-vaccination time points (D56 or D224) were performed using a two-tailed Wilcoxon matched-pairs signed-rank test. Comparisons between the active vaccine recipients and the RabAvert control group at days 56 and 224 were performed using a two-tailed Mann-Whitney U test. Statistical significance is indicated as ns (not significant), * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$, and **** $p \leq 0.001$.

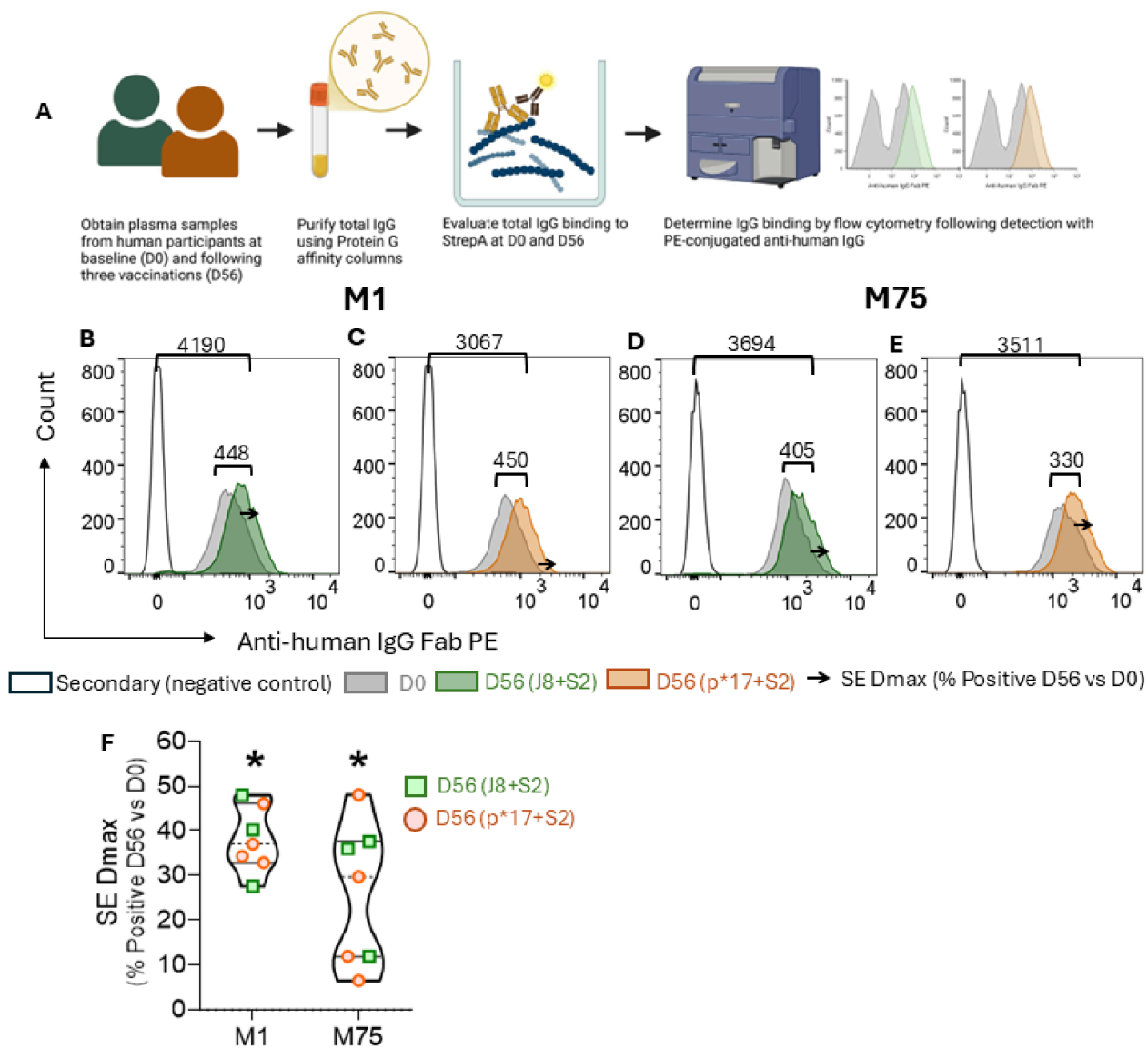


Figure 6

Vaccine-induced total IgG binding to Strep A M1 and M75 strains assessed by flow cytometry. (A) Schematic of flow cytometry-based assessment of IgG binding to StrepA. Matched participant sera samples collected on D0 (pre-vaccination) and D56 (post-third vaccination) from J8-K4S2 (n = 3) and p*17-K4S2 (n = 4) vaccine recipients were purified for total IgG using Protein G columns and volume-matched back to the original serum volume. Purified total IgG from D0 and D56 samples was incubated overnight with M1 and M75 StrepA at a 1:40 dilution with end-over-end mixing. The following day, samples were stained with anti-

human IgG Fab-PE (Thermo Fisher, 1:250) and antibody binding was assessed by flow cytometry. (B-E) Histograms shown are representative of two participants from each vaccine group for M1 (B and C) and M75 (D and E) StrepA. The secondary antibody-only negative control is represented by the black outline, baseline (D0) total IgG binding by the grey histogram, and D56 total IgG binding by green (J8+S2 recipients) and orange (p17+S2 recipients) histograms. The T(x) values, displayed at the top of each histogram, were determined using the probability binning algorithm in FlowJo by comparing D56 total IgG with matched D0 total IgG or with the secondary-only negative control. A T(x) value of >4 was considered significant ($P < 0.01$). The Super-Enhanced Dmax (SE Dmax) algorithm was applied to determine the percentage of truly positive events, indicated by the arrow on each histogram. (F) Violin plot of SE Dmax % positive values for all participants tested, comparing D56 total IgG with matched D0 total IgG for M1 and M75 StrepA strains. Green squares represent D56 J8+K4S2 participants and orange circles represent D56 p*17+K4S2 participants. SEDmax % positive values were calculated relative to the matched D0 sample (defined as 0). Technical triplicates were averaged for each participant, and participant-level values were analysed using a one-sample t-test against a hypothetical value of 0. Statistical significance is indicated as * $p < 0.05$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [WCHRIDCCSAPGASVaccineTrial27Jun2025final.pdf](#)
- [Suppdataawprotocolupdated.pdf](#)