nature communications

Article

https://doi.org/10.1038/s41467-024-54665-5

Streptococcus pyogenes pharyngitis elicits diverse antibody responses to key vaccine antigens influenced by the imprint of past infections

Received: 25 April 2024

Accepted: 19 November 2024

Published online: 03 December 2024

Check for updates

Joshua Osowicki[1,2,3,17] ✉, Hannah R. Frost[1,17], Kristy I. Azzopardi[1], Alana L. Whitcombe[4,5], Reuben McGregor[4,5], Lauren H. Carlton[4,5], Ciara Baker[1], Loraine Fabri[1,6,7], Manisha Pandey[8], Michael F. Good[8], Jonathan R. Carapetis[9,10,11], Mark J. Walker[12,13,14], Pierre R. Smeesters[1,2,6,7], Paul V. Licciardi[2,15], Nicole J. Moreland[4,5,18], Danika L. Hill[16,18] ✉ & Andrew C. Steer[1,2,3,18]

Knowledge gaps regarding human immunity to *Streptococcus pyogenes* have impeded vaccine development. To address these gaps and evaluate vaccine candidates, we established a human challenge model of *S. pyogenes* pharyngitis. Here, we analyse antibody responses in serum and saliva against 19 antigens to identify characteristics distinguishing 19 participants who developed pharyngitis and 6 who did not. We show that pharyngitis elicits serum IgG responses to key vaccine antigens and a muted mucosal IgA response, whereas IgG responses are minimal and IgA responses more pronounced in participants without pharyngitis. Serum IgG responses to pharyngitis in adult participants resemble those in children and are inversely correlated with the magnitude of pre-existing responses. While a straightforward correlate of protection is not evident, baseline antibody signatures distinguish clinical and immunological outcomes following experimental challenge. This highlights the influence of a complex humoral imprint from previous exposure, relevant for interpreting immunogenicity in forthcoming vaccine trials.

Streptococcus pyogenes is a highly adapted human-restricted pathogen with an immense global burden of communicable and non-communicable diseases across a diverse clinical spectrum spanning acute infections and post-infectious syndromes with chronic disease outcomes[1]. *S. pyogenes* ranks in the global top ten infection-related causes of death, with more than 500,000 deaths every year attributable to rheumatic heart disease[2] and acute invasive infections[3]. In recognition of the clear unmet need, the World Health Organisation Product Development for Vaccines Advisory Committee lists *S. pyogenes* as a global priority pathogen for new vaccine research and development[4]. While recent efforts have successfully reinvigorated the field[5,6], the modern *S. pyogenes* vaccine development ecosystem remains fragile[7].

Critically, there is no established human immune correlate of protection to predict the efficacy of *S. pyogenes* vaccines[8]. The epidemiology of *S. pyogenes* infections strongly suggests that partial immune protection accumulates with repeated exposure through childhood[8–10]. However, unlike vaccine-preventable bacterial diseases caused by *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Neisseria meningitidis*, there are no primary or acquired immunodeficiency syndromes classically associated with susceptibility to *S. pyogenes* infection from which the basis for naturally acquired

A full list of affiliations appears at the end of the paper. ✉e-mail: joshua.osowicki@rch.org.au; danika.hill@monash.edu

protection may be inferred. The contribution of M protein serotype- or *emm* genotype-specific humoral immunity, initially established by streptococcal research pioneer Rebecca Lancefield[11], remains uncertain[8], with mixed findings from longitudinal cohort studies[12–15], historical human challenge trials[16–19], animal models[20] and in vitro assays[21].

In the early 20th century, hundreds of thousands of children and adults were vaccinated for the prevention of *S. pyogenes* clinical syndromes, all considered then as scarlet fever[22]. The last time the efficacy of an *S. pyogenes* vaccine was evaluated in humans was in 1970s challenge trials in which parenteral and mucosal monovalent M protein vaccines were protective against homologous pharyngeal challenge, although a correlate of protection could not be demonstrated[17,18]. Since a contentious 1979 United States Food and Drug Administration ruling that slowed *S. pyogenes* vaccine research was revoked in 2006[23,24], many M protein and non-M protein antigens have proven to be protective in a variety of animal models[25]. Human immunogenicity for most of these antigens has been demonstrated in natural cohorts[12,15], pooled human immunoglobulin products[26,27], and a small number of early-phase vaccine trials[28–32]. Still, by 2016, in the face of scientific, commercial, and regulatory barriers, *S. pyogenes* vaccines were considered impeded vaccines[33].

As part of resurgent global *S. pyogenes* vaccine development efforts[5,6], the establishment of new human infection models has been prioritised as a platform for early efficacy evaluation to accelerate vaccine development[34] and to explore immune responses. The CHIVAS-M75 trial established the world's only modern *S. pyogenes* human infection model in healthy adult volunteers[35]. We have previously described a distinct systemic and mucosal cellular and cytokine signature of experimental human pharyngitis in CHIVAS-M75 participants[36].

Here, interrogated longitudinal systemic, mucosal, functional, and binding antibodies to *S. pyogenes* in serum and saliva collected from 25 participants in the CHIVAS-M75 trial, before and after challenge, 19 of whom developed acute symptomatic pharyngitis and 6 who did not. These data will lay the foundations for immune assessments in forthcoming trials to evaluate promising vaccine candidates[7].

Results

Antibodies induced by challenge have modest activity in functional in vitro bacterial adhesion and opsonophagocytic assays

We first investigated mucosal and systemic functional responses against the *emm*75 *S. pyogenes* challenge strain[37] using an established serum opsonophagocytic killing assay[38] and an adapted bacterial adhesion assay[39] (Fig. 1). Saliva collected before and 1 week after the challenge affected bacterial adhesion to a pharyngeal cell line (Detroit 562), however, there were no consistent changes related to the challenge or between those who did and did not develop pharyngitis (Fig. 1B). We detected no serum opsonophagocytic activity against the challenge strain for 23 of 25 participants in samples from before and 1 month after challenge (Fig. 1C). Opsonophagocytic activity was observed at baseline in just two participants, one who subsequently developed pharyngitis (SN010) and one who did not (SN013). These were the only participants with increased opsonophagocytic killing after 1 month. Both had sustained pharyngeal colonisation with *S. pyogenes*, anti-streptolysin O seroconversion[35], and increased inhibition by post-challenge saliva in the adhesion assay. In summary, neither functional assay correlated with clinical outcome.

Pharyngeal challenge induces heterogenous serological responses against key vaccine antigens

To evaluate humoral immune responses, we quantified IgG in serum and IgA in saliva against a panel of 17 antigens comprising vaccine candidates and known targets of *S. pyogenes* immunity (Fig. 2A and Table S1). IgG and IgA responses were highly variable across antigens for all participants at pre- and post-challenge time-points (Fig. 2B), including inconsistent responses against protein antigens and related derivative peptides, such as the M75 protein and its derivative antigens: the N-terminal M75-HVR peptide and C-repeat peptides p145, J8 and P*17 (Supplementary Figs. 3, 4). When analysed as fold-change relative to pre-challenge titres, participants who developed pharyngitis had increased post-challenge serum IgG responses at 1 month against several key vaccine antigens (SpyCEP, SLO, ScpA, GAC) and a trend towards lower post-challenge saliva IgA responses at one week, especially for GAC and M75-HVR (Fig. 2C, F). Serum IgG and saliva IgA kinetics were different—serum IgG responses generally continued to increase between the 1 week and 1 month time-points, whereas IgA changes were most evident at the 1-week visit and returned towards baseline at 1 month. (Supplementary Fig. 1). Conversely, among the smaller group of 6 participants who did not develop pharyngitis, there were trends towards lower serum IgG and higher saliva IgA post-challenge responses. These trends were antigen-specific, as total IgA concentration in saliva was not affected by challenge or pharyngitis (Supplementary Fig. 2). Saliva IgG and serum IgA responses were below the limit of detection in our assays.

We sought to determine the frequency of responses to each antigen and if this differed by pharyngitis outcome. A response was defined as an increase of 25% or greater above pre-challenge titres, a threshold previously applied for IgG responses to other bacterial pathogens[40,41], and used here to capture the breadth of antigens recognised by the modest antibody response induced by a single infection. Serum IgG responses were more common among participants with pharyngitis, whereas antigen-specific saliva IgA responses occurred with similar or higher frequency among participants without pharyngitis (Fig. 2D). Consistent with the fold-change findings, a response was observed most frequently against four vaccine candidate antigens (SpyCEP, SLO, ScpA and GAC). The breadth of the serum IgG response was higher among participants with pharyngitis, with a median response to four antigens, compared to one antigen among participants without pharyngitis (Fig. 2E). When analysing saliva, participants with pharyngitis responded to a median of two antigens, while there was a trend toward a greater breadth of IgA responses among participants without pharyngitis, including one participant with a saliva IgA response to 11 different antigens. Overall, the most robust post-challenge differences among participants after pharyngitis were decreased saliva IgA for GAC and M75-HVR and increased serum IgG for SpyCEP, SLO, ScpA and GAC (Fig. 2F, all other antigens Supplementary Figs. 3–5). The opposing trends observed for IgG and IgA responses led us to consider if these responses were somehow related. However, there was no correlation observed between the fold-change of saliva IgA responses and serum IgG responses at either 1 week or 1 month (Fig. 2G).

Experimental *S. pyogenes* pharyngitis induces a humoral immune response maintained for at least 3 months

For the four antigens associated with the most frequent serum IgG responses (SpyCEP, SLO, ScpA and GAC), we proceeded to use a multiplex bead-based assay to determine the absolute concentration of antigen-specific serum IgG[42,43]. Responses to SpnA and DnaseB were also analysed because they can be used clinically (with SLO) as evidence of recent *S. pyogenes* infection[44]. For all six antigens, participants with a 25% increase in IgG concentration (termed responders) all developed pharyngitis, except for a single participant who did not develop pharyngitis and was a responder to ScpA (Fig. 3B). The increase in IgG to all antigens in responders occurred as early as the 1-week outpatient visit and was maintained out to 3 months indicating that when antibodies were induced, they were durable for several months. At each time-point, the absolute IgG concentration for responders was equivalent to non-responders, except for IgG against

https://doi.org/10.1038/s41467-024-54665-5

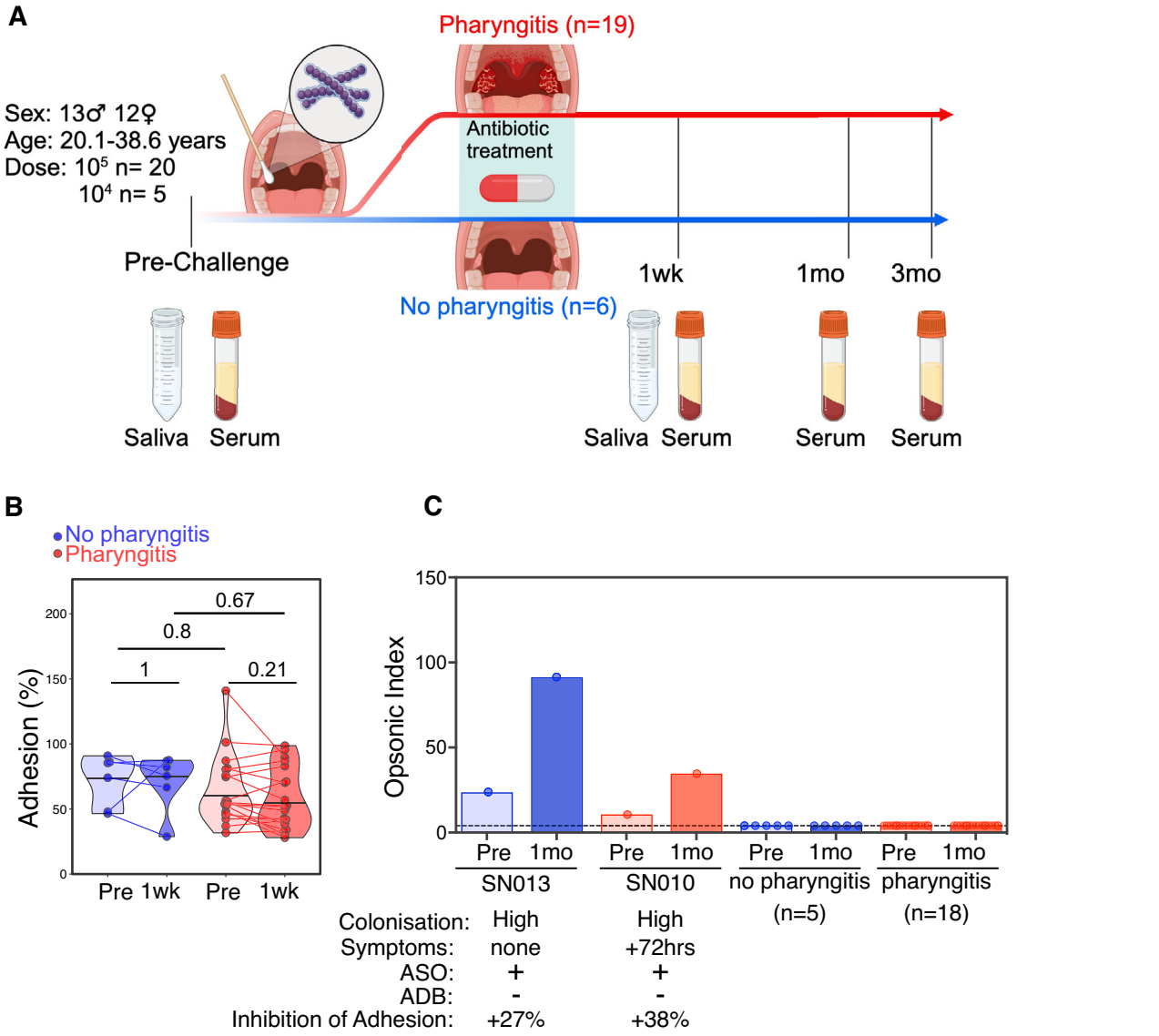


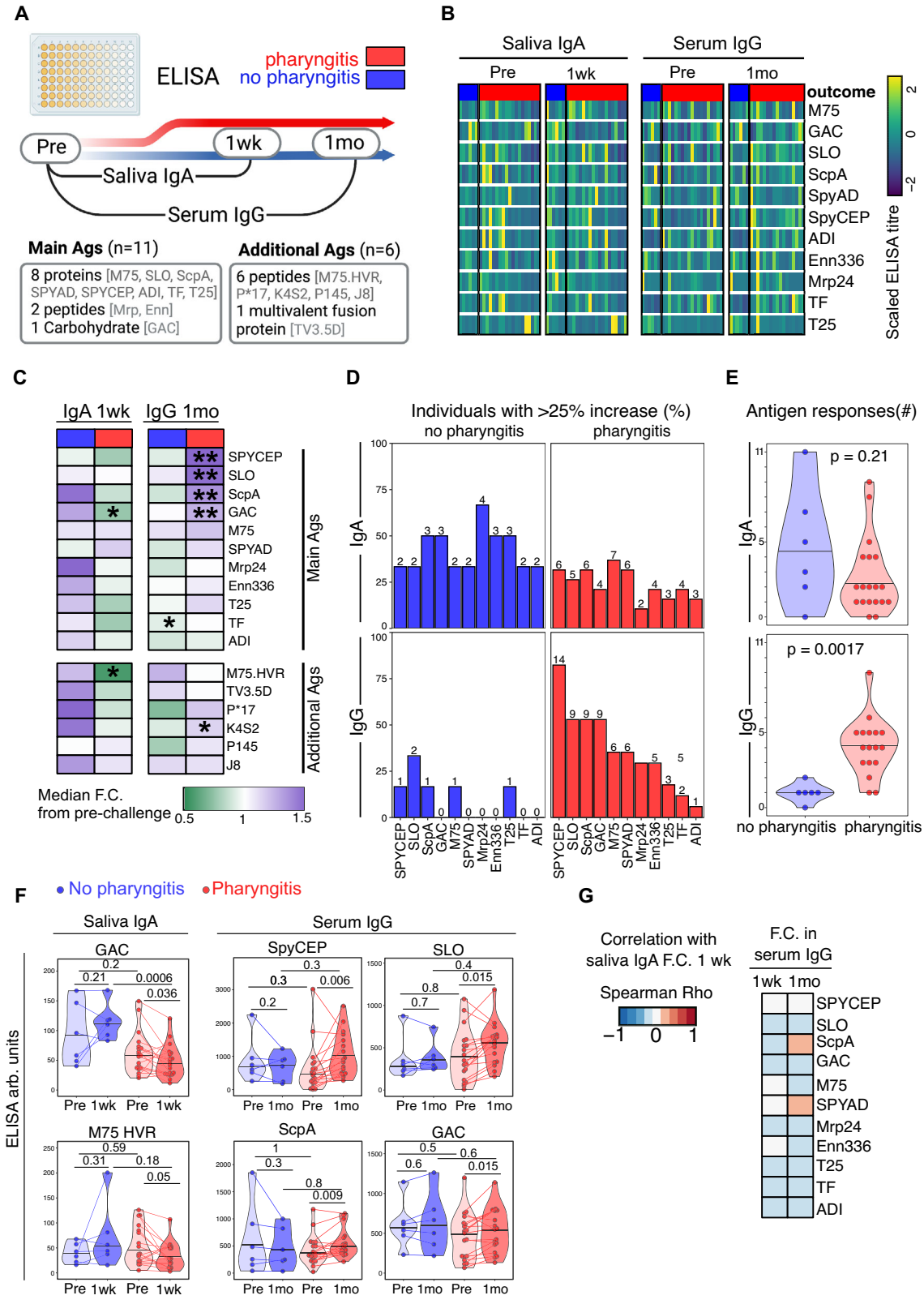
Fig. 1 | Experimental human *S. pyogenes* pharyngitis is not associated with new serum opsonophagocytic responses or inhibition of adherence by saliva. **A** Schematic overview of challenge study timelines and samples analysed in this study. Created in BioRender. Hill, D. (2023) BioRender.com/g84v832. **B** Bacterial adherence to Detroit 562 cells in the presence of saliva collected pre-challenge and 1 week after pharyngitis diagnosis ($n = 19$) or discharge without pharyngitis ($n = 6$). Bacterial burden determined by CFU of recovered bacteria and % adherence determined relative to bacteria-only controls. Responses for each group are shown as median + range, with within-group comparisons performed using two-sided paired Wilcoxon signed-rank tests and between-group comparisons using two-sided Mann–Whitney tests, p values are shown following false discovery rate (FDR)-adjustment for multiple comparisons. **C** Serum opsonophagocytic killing assay results from pre-challenge and one-month post-challenge samples ($n = 25$), highlighting results from two participants with a detectable opsonophagocytic response (SN013, SN010; mean and standard error of three technical replicates shown), and grouping the remainder of non-responders by clinical pharyngitis outcome (blue = non-pharyngitis, $n = 5$; red = pharyngitis, $n = 18$). Opsonic index determined by the linear regression of the two dilutions closest to 50% killing. Text descriptions below describe clinical and infection parameters for SN013 and SN010.

SpnA, which had a higher median concentration for non-responders at all time-points. There was a trend towards higher pre-challenge titres in non-responders, most evident for SpyCEP, SpnA and SLO.

Antibody responses to experimental human *S. pyogenes* pharyngitis in adults resemble responses to natural infection in children

Current *S. pyogenes* initiatives are focused on advancing a paediatric product. One concern regarding human challenge research is that immune responses in healthy adult participants may not be generalisable to children[34]. We compared serum IgG responses from the multiplex bead-based assay for the CHIVAS-M75 adult participants with previously published data for healthy children ($n = 15$) and children 1 month following microbiologically-confirmed *S. pyogenes* pharyngitis ($n = 22$) from New Zealand (aged 5 to 14 years)[45]. IgG concentrations were equivalent among pre-challenge adult samples and healthy children for all antigens except ScpA (Fig. 4). Post-challenge adult samples had equivalent IgG concentrations for all six antigens to convalescent serum from children, with a trend towards elevated responses in the paediatric samples for SpnA only. These results provide some reassurance that pre-existing serum antibody concentrations and the magnitude of post-challenge responses in adult human challenge participants resemble those observed before and after pharyngitis in children.

https://doi.org/10.1038/s41467-024-54665-5



Pre-existing humoral immunity inversely correlates with the post-challenge response

We sought to explore the higher pre-challenge IgG responses in non-responders. For five out of six antigens measured with the multiplex bead-based assay, there was a significant inverse correlation between pre-challenge IgG concentration and antibody fold-change by the 1-month outpatient visit, with a similar trend observed for GAC. Participants who did not develop pharyngitis were enriched among samples with higher pre-challenge serum IgG levels and minimal or negative post-challenge fold-change (Fig. 5A). This inverse correlation was consistent for the four antigens measured by both multiplex bead-based assay and ELISA. Pre-challenge saliva IgA was inversely correlated with the fold-change in IgA by the 1-week outpatient visit for ScpA and M75 (Fig. 5B), although participants did not cluster distinctly

Fig. 2 | Experimental pharyngeal challenge with *Streptococcus pyogenes* induces mucosal and systemic antibody responses against major vaccine antigens. **A** Schematic overview of the time-points and antigens studied to detect IgG from serum and IgA saliva. Antigens were grouped into two categories; Main included leading vaccine candidates and key virulence factors, and Additional included peptides or domains of proteins included among Main Ags (M protein, SpyCEP, T antigen). Created in BioRender. Hill, D. (2023) BioRender.com/m87o308. **B** Heatmap showing the relative abundance of IgA and IgG responses pre and post-challenge at indicated time-points in pharyngitis and non-pharyngitis participants for 11 main antigens. ELISA titres are shown with responses scaled across IgA and IgG separately. Pharyngitis outcome indicated as coloured bar (red = pharyngitis, blue = no pharyngitis). **C** Heatmap showing the median fold-change observed relative to baseline at 1 week for IgA, and 1 month for IgG with associated *p* values from two-sided paired Wilcoxon signed-rank test after FDR-adjustment for 11 main antigens and six additional peptide or vaccine antigens. (*$P_{adjusted} < 0.05$, **$P_{adjusted} < 0.01$). **D** The percentage of pharyngitis and non-pharyngitis participants that showed a 25% increase in antibodies post-challenge, with 1 week for IgA and 1 month for IgG. Numbers indicate how many participants responded to each antigen. **E** A tally of the number of antigens to which participants showed a 25% increase in IgA and IgG (shown in **D**), with participants split by pharyngitis outcome. *P* value determined from two-sided Mann–Whitney *U*-test. **F** Representative examples of antigens that showed significant change after challenge (as shown in **C**) expressed in ELISA arbitrary units (arb. units). Within-group comparisons were performed using a two-sided paired Wilcoxon signed-rank test, and groups were compared using a two-sided Mann–Whitney test, all FDR-adjusted for multiple comparisons. **G** Spearman correlation of fold-change in saliva IgA at 1 week with fold-change in serum IgG at 1 week and 1 month post-challenge. All comparisons were $p > 0.1$.

based on outcome. In contrast to the IgG finding, participants without pharyngitis were enriched among samples with lower pre-challenge IgA and positive post-challenge fold-change. Correlation coefficients for pre- versus post-challenge IgG and IgA responses, measured by ELISA and multiplex bead-based assay, were negative for the majority of antigens tested. These data suggest that pre-existing antibodies may affect mucosal and systemic antibody responses to *S. pyogenes* infection, either by direct interference or as a surrogate for immune memory.

Unbiased clustering reveals distinct serological signatures associated with experimental human pharyngitis and the humoral response to challenge

To investigate whether clinical outcome was associated with distinct serological features, we used non-metric multidimensional scaling incorporating serum IgG data for 19 antigens (Table S1) from pre-challenge and the 1-month outpatient visit to generate an unbiased view of each participant's serological signature. We analysed the IgG data to assess the correlation between each individual's responses to all other individuals across the 19 antigens. Then, we used multi-dimensional scaling to simplify these complex relationships into two dimensions. Participants clustered according to pharyngitis outcome, suggestive of a pre-challenge serological state associated with protection from pharyngitis (left panel Fig. 6A, B). For most participants, pre- and post-challenge points were clustered closely together (middle panel Fig. 6A), consistent with previous *S. pyogenes* exposures eliciting a distinct individual serological imprint. Where there was a change in multidimensional space coordinates for participants in response to challenge, this movement was predominantly left-to-right along Dimension 1 (middle panel Fig. 6A) and was almost exclusively limited to the pharyngitis group (right panel Fig.6B). This suggests that, after challenge, some participants acquired a serological signature closer to the pre-challenge state of participants who did not develop pharyngitis. Dimension 1 was correlated with high serum IgG responses against GAC, SpyCEP, ScpA, K4S2, DnaseB, and SpnA, and low IgG responses to several antigens, including J8, ADI and TF (Fig. 6C). In keeping with this, participants with a left-to-right post-challenge shift along Dimension 1 were predominantly those we previously identified as IgG responders against two or more of SpyCEP, GAC, SLO and ScpA (Fig. 6A middle and right), indicating that responses to these antigens were major drivers of the clustering. Together, these data suggest that while individual human *S. pyogenes* serological signatures are highly variable, there are common features that may be associated with protection against pharyngitis.

Baseline antibody levels are associated with clinical outcome and severity of experimental human *S. pyogenes* pharyngitis

We correlated 37 baseline antibody variables to eight clinical features to investigate how pre-existing humoral immunity related to pharyngitis outcome and severity (symptoms, signs, time to diagnosis, intensity of bacterial colonisation) (Fig. 7A)[35]. Ten variables significantly correlated with one or more clinical features (Fig. 7B). While there were variable correlations between different antigens and clinical variables, for most antigens, the directionality of correlation was strongly concordant. Broadly, baseline serum IgG against three antigens often described as cryptic (ADI, TF[46] and J8[47]) correlated with severity of infections, whereas baseline antibodies against 7 other antigens were inversely correlated with severity: serum IgG against K4S2, DnaseB, SPyAD, SpyCEP, SpnA, T25 and saliva IgA against p145.

We then used sparse partial least squares discriminant analysis (sPLS-DA) to discern which among the 37 immune variables most effectively distinguished between participants who did and did not develop pharyngitis. Analysing these variables in a multidimensional space, the sPLS-DA model pinpointed 5 variables that most significantly explained the immunological distinctions related to pharyngitis outcomes. Furthermore, the model revealed a single dimension along which participants were distinctly clustered according to their pharyngitis status (Fig. 7C). The five pre-challenge variables that contributed to this first dimension and were associated with not developing pharyngitis were low serum IgG to TF and ADI, high serum IgG to SpnA and T25, and high saliva IgA to p145 (Fig. 7D), which were all robustly different between pharyngitis and non-pharyngitis groups (Fig. 7E). Together, these data suggest that the clinical outcome following *S. pyogenes* challenge is influenced by pre-existing humoral immunity and that systemic responses to some antigens may discriminate individuals with increased susceptibility to symptomatic pharyngitis.

Discussion

This study of mucosal and systemic antibody responses in the CHIVAS-M75 human challenge trial advances our understanding of immunity against *S. pyogenes* and underlines the potential for the human model to accelerate vaccine development. Pharyngeal challenge induced heterogeneous and highly individualised patterns of saliva IgA and serum IgG responses, with durable responses against conserved vaccine antigens (e.g. SpyCEP, ScpA, SLO and GAC). Serum IgG responses in healthy adult challenge participants were comparable to responses in children following natural pharyngitis. While a straightforward correlate of protection was not evident, baseline antibody signatures, reflecting the imprint of past infections, were identified that distinguished clinical outcomes following experimental challenge. Pre-existing antibodies inversely correlated with the magnitude of IgG induced by challenge, exemplifying the complex interactions between a highly adapted ubiquitous pathogen and its only natural host, with important ramifications for understanding vaccine immunogenicity.

Our study highlights the limitations of currently available functional serology assays, as the serum opsonophagocytic killing and salivary bacterial adhesion assays provided limited insight into the

https://doi.org/10.1038/s41467-024-54665-5

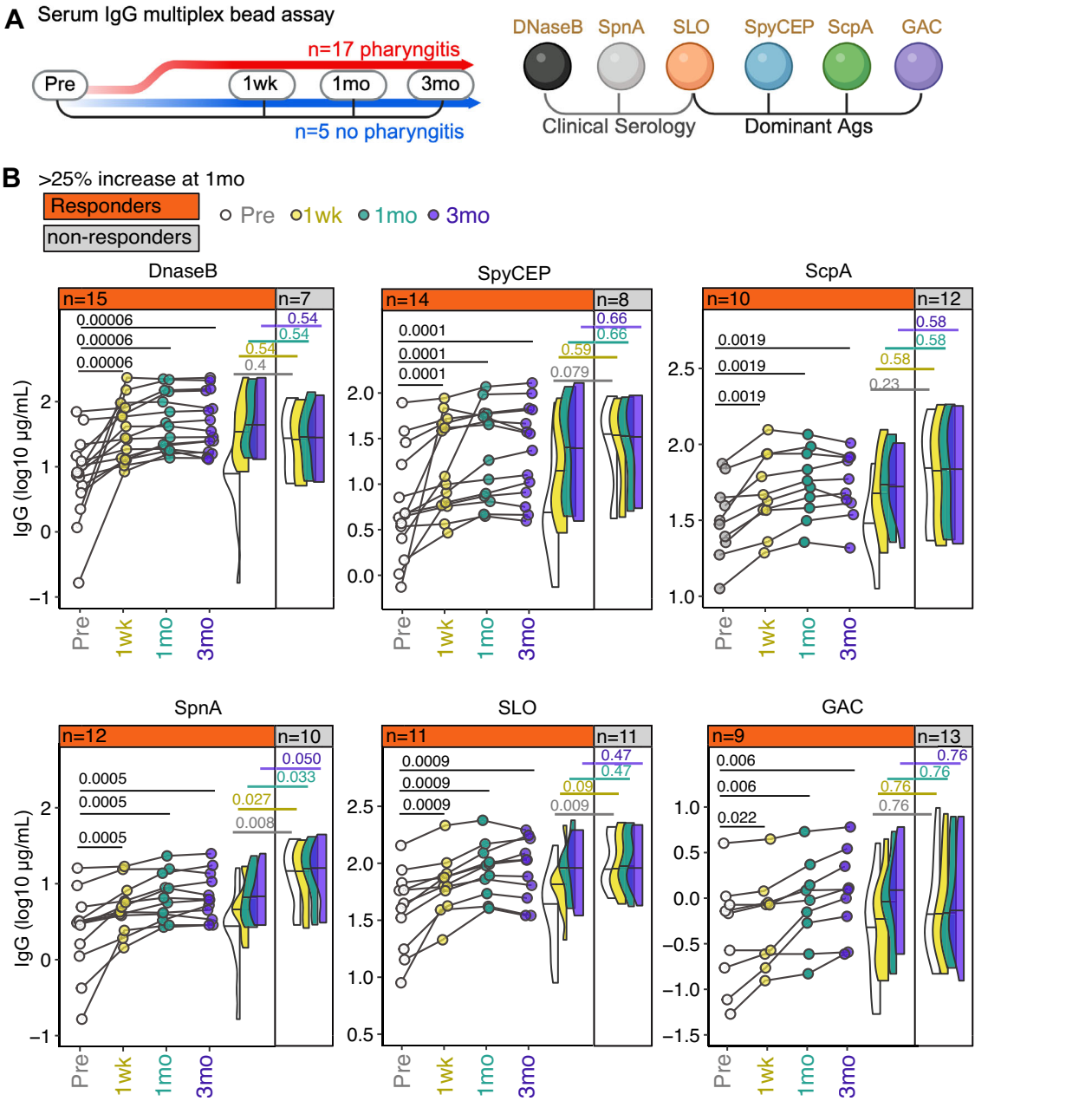


Fig. 3 | Antibody responses induced by experimental pharyngeal challenge with *Streptococcus pyogenes* are maintained for at least 3 months. A Schematic overview of time-points and antigens measured by a multiplex bead-based assay for IgG antibodies in sera. Created in BioRender. Hill, D. (2023) BioRender.com/m17l045. **B** IgG responses for 6 antigens at indicated time-points with the cohort split into those who showed a 25% increase in IgG at 1-month post-challenge (responders) and those that did not (non-responders). For Responders, each dot represents an individual, and lines connect paired samples. The violin plots summarise the distribution (median+range) of responses across time-points for each group, with colour corresponding to time-point. The analysis included 22 participants with samples available from all four time-points. *P* values were determined for within-group paired sample comparisons using Friedman's test followed by a two-sided Wilcoxon signed-rank test with FDR-adjustments, and for between-group comparisons using Kruskal–Wallis and Dunn's multiple comparison testing.

basis of susceptibility and protection. It is possible that the pre-screening and exclusion of individuals with high serum *emm*75-specific IgG from the CHIVAS-M75 trial[19,35] may have contributed to the lack of opsonophagocytic killing observed. However, the M75 protein of the *emm*75 challenge strain was not the target of opsonisation by pooled human immunoglobulin (in the same assay) in a recent study[21]. These findings are consistent with 1970s human studies in which induction of a type-specific serum bactericidal response by M protein vaccines was not a reliable correlate of protection, which was observed against symptomatic pharyngitis (parenteral and mucosal vaccines) and colonisation (mucosal vaccine only)[16–18]. Expanding the scope of the modern human challenge model to include other *emm*-type strains will enable further evaluation of the opsonophagocytic killing assay as a correlate of protection. For multi-component vaccines including secreted virulence factors (e.g. SpyCEP and SLO), additional assays may be required to capture the diversity of functional antibody responses[48,49].

Acute symptomatic *S. pyogenes* pharyngitis is characterised by a combined humoral and cellular immune response to contain and clear the focal infection[36]. The human model enabled simultaneous sequential assessment of IgA in saliva and IgG in the blood. Antibody responses in these compartments did not correlate in magnitude or

https://doi.org/10.1038/s41467-024-54665-5

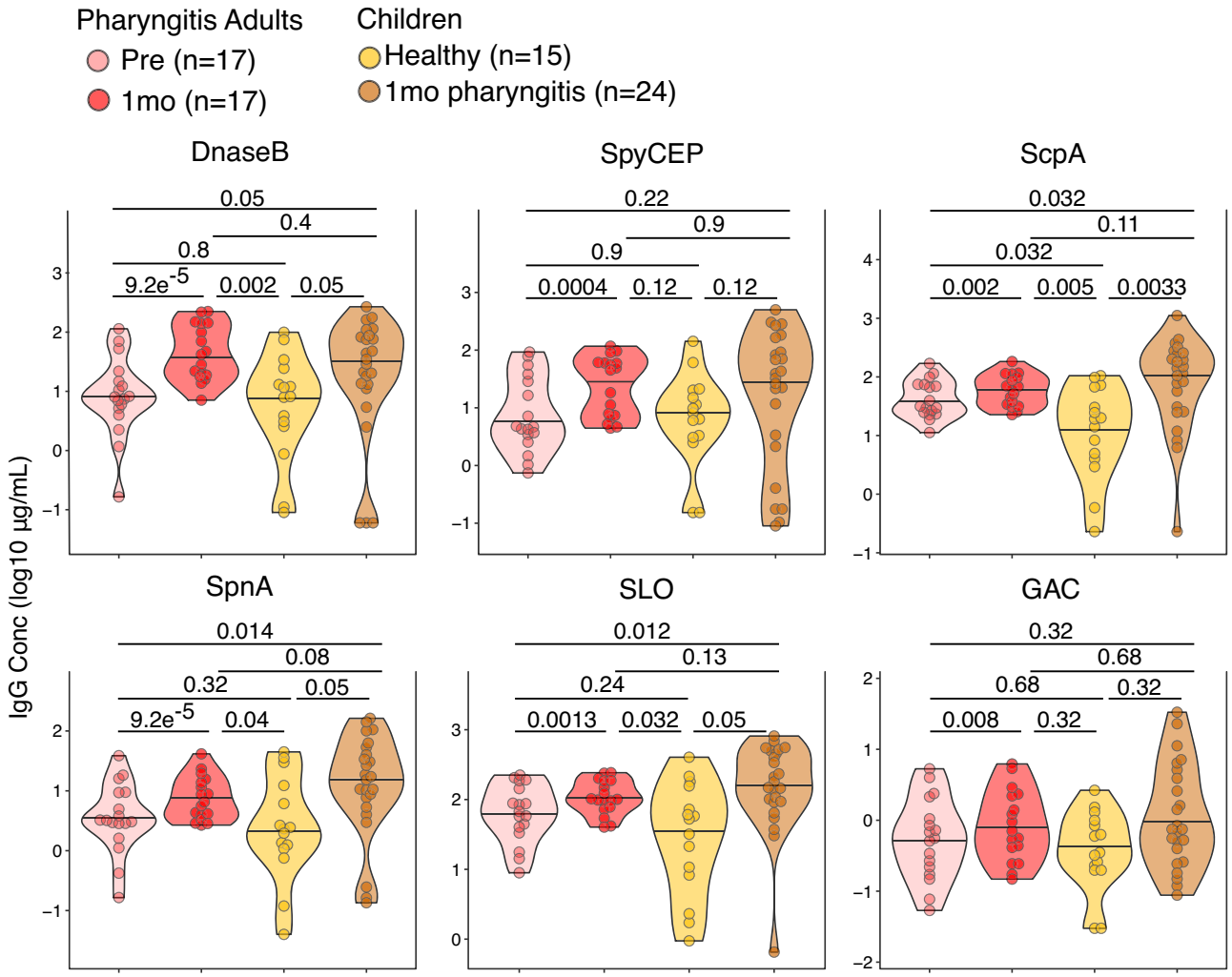


Fig. 4 | Antibody responses to experimental human *Streptococcus pyogenes* pharyngitis in healthy adults are similar to natural responses in children. Serum IgG concentrations measured by multiplex bead-based assay to 6 antigens among challenge participants pre- and 1-month post-challenge was compared to healthy children and children >1 month after acute pharyngitis (aged between 5 and 14 years)[45]. *P* values were determined using Dunn's multiple comparison testing or Wilcoxon signed-rank test for paired data, both two-sided.

antigen-specificity, strongly suggesting they are produced and regulated independently. Overall, individual antibody responses were highly variable, as in previous longitudinal cohort studies of children[12,15,50]. Whilst every tested antigen was immunogenic in at least one participant, serum IgG responses were predominantly raised against conserved antigens with a role in evading human immunity (e.g. SpyCEP, SLO and ScpA), while responses to the M protein, conventionally considered as immunodominant, were modest and infrequent. In saliva, IgA responses were most pronounced against surface-bound antigens contributing to adhesion and invasion (e.g. M- and M-like-proteins) and were more common among the six participants who did not develop pharyngitis. Where serum IgG responses were produced, they were maintained for at least 3 months, which suggests that *S. pyogenes* pharyngeal infection may induce long-lived antibody-secreting cells[51], as serum antibodies would have waned substantially based on IgG serum half-life alone[52]. Overall, the post-challenge antibody responses described here add to recent longitudinal studies[12,15,50] undermining the historical conception of asymptomatic colonisation (carriage) with *S. pyogenes* as immunologically inert.

The mucosal and systemic pre-challenge antibody profiles of CHIVAS-M75 participants fit with repeated *S. pyogenes* exposures throughout early life, driving the development of humoral immune memory. The inverse correlations we observed between baseline antibody levels and post-challenge responses resemble antibody feedback-mediated suppression of B cell responses, the basis for anti-D prophylaxis during pregnancy for Rhesus-negative women[53] and recently reported to influence antibody responses to SARS-CoV-2 and malaria vaccines[54–56]. This phenomenon is distinct from the original antigenic sin[57], which would have boosted rather than suppressed antibody responses from pre-existing B cell memory, particularly against the highly conserved antigens. Although it remains to be seen whether antibody feedback will affect *S. pyogenes* vaccines, it is reassuring that increased antigen dose can overcome feedback in mice[58], suggesting that vaccine design may overcome suppression.

The limitations of the human *S. pyogenes* pharyngitis model have been discussed in depth previously[19,35,37] and broadly relate to generalisability[34], including age of participants, direct inoculation, and use of a single strain, among others. This included pre-screening to exclude individuals with high type-specific anti-*emm75* serum IgG. Reassuringly, we showed that pre- and post-challenge antibody levels among healthy and convalescent adults resembled those of healthy and convalescent children, the initial target group for vaccine development. The challenge strain, *emm75*, ranks among the top 10 most frequently isolated across all settings[59]. The confirmed inoculum from

https://doi.org/10.1038/s41467-024-54665-5

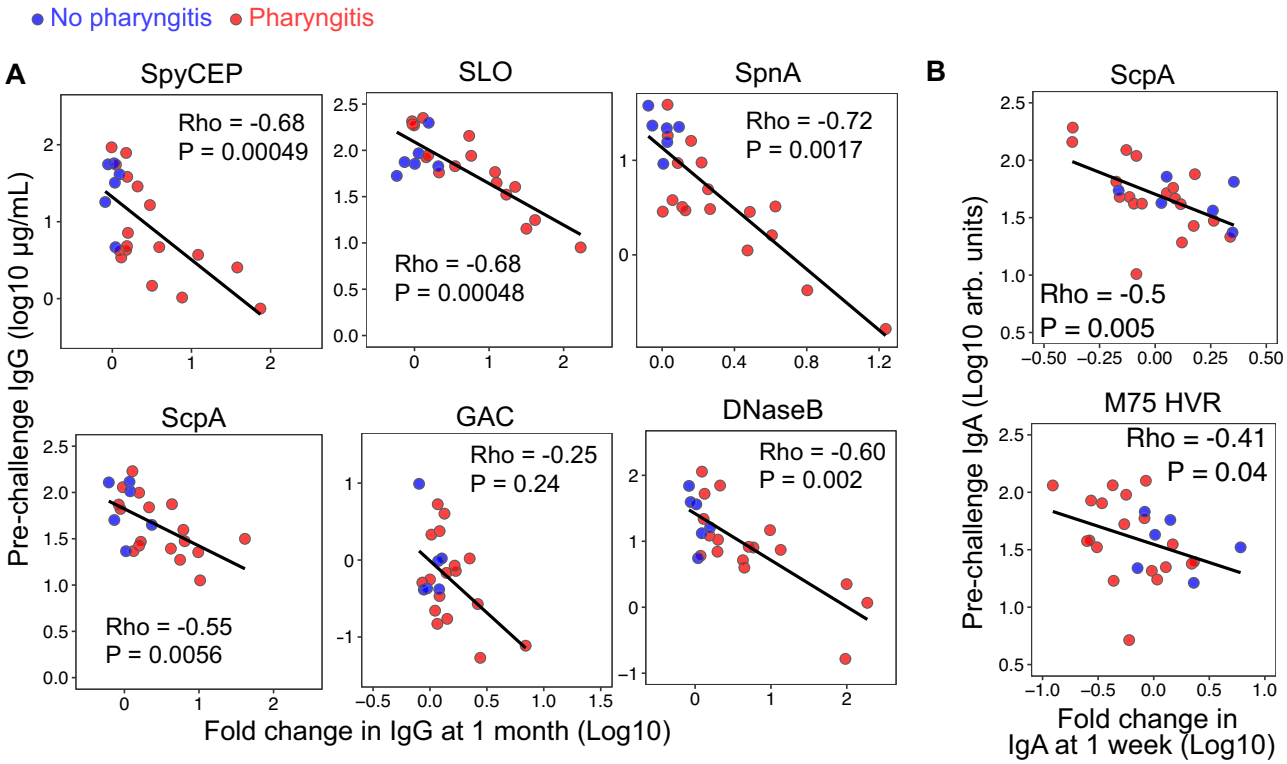


Fig. 5 | Pre-challenge baseline titres negatively correlate with post-challenge antibody responses. A Correlation between IgG responses to 6 antigens measured by multiplex bead-based assay in samples from pre-challenge with those at 1-month post-infection (pharyngitis $n = 17$, no pharyngitis $n = 6$). **B** Correlation between anti-ScpA and anti-M75 IgA responses measured by ELISA from pre-challenge and 1-week post-challenge (pharyngitis $n = 19$, no pharyngitis $n = 6$). Coefficient and p value were determined using Spearman's method (two-sided) with a linear regression line included to indicate linear trends. Each dot represents an individual participant with the pharyngitis group shown in red and non-pharyngitis in blue.

CHIVAS-M75 is at least 20 times lower than the dose delivered in 1970s human trials, which successfully demonstrated vaccine-induced protection[17,18,35] and up to 10,000 times lower than in animal models used in pre-clinical vaccine evaluation[25]. This argues against concerns the human model may be too stringent to support the assessment of natural and vaccine-induced protective immunity. The major limitation of the immunological analyses is that the sample size, including just six participants without pharyngitis, precluded definitive conclusions regarding antigen-specific antibody results related to protection or susceptibility to pharyngitis. More fundamentally, whether our findings may contribute to defining a correlate of natural protection in future challenge and/or longitudinal surveillance studies is an open question, and its highly possible that correlates of protection from vaccination will be different.

Human challenge trials are poised to evaluate the efficacy of *S. pyogenes* vaccines for the first time in almost 50 years. Our findings highlight the power of the experimental human *S. pyogenes* pharyngitis model as a platform for investigating systemic and mucosal immunity. Clinical and immunological responses to *S. pyogenes* exposure are influenced by an individual's imprint of past infections, underscoring the importance of assessing pre-existing immunity in planned vaccine trials in adults and children. This work will facilitate future studies that link responses to vaccination and infection with subsequent clinical outcomes, inform the interpretation of early-phase vaccine trial results, and converge on correlates of protection to support vaccine development.

Methods

Experimental pharyngitis model

Details of the CHIVAS-M75 study protocol, *emm*75 *S. pyogenes* challenge strain, and trial results have been described previously[19,35,37]. The study, collection of samples, and related immunology research was approved by the Alfred Hospital Human Research Ethics Committee (500/17). Written informed consent was obtained from all participants. In the CHIVAS-M75 trial, 19 of 25 participants were diagnosed with pharyngitis, comprising 18/20 that received a dose of 10^5 colony-forming units (CFU)/ml, and 1/5 that were given a lower dose (10^4 CFU/ml). The clinical picture of acute pharyngitis was supported by microbiological (qPCR and culture), biochemical (C-reactive protein, cytokines and chemokines) and clinical streptococcal serology (anti-streptolysin O, anti-DnaseB) results. Considering all available data, one participant (SN057) who was not contemporaneously diagnosed with pharyngitis[35] clearly did have pharyngitis, and they have been categorised as such in this study. All participants with pharyngitis had *S. pyogenes* detected by throat swab qPCR and culture at multiple time-points[60]. *S. pyogenes* colonisation was detected in only one of the 6 participants (participant SN013) who did not develop pharyngitis[35].

CHIVAS-M75 samples

Serum and saliva were collected from participants at screening, the evening prior to the challenge, 8 to 12 h after the challenge, daily during the remainder of the inpatient period, then at 1-week, 1-month, 3-month and 6-month outpatient visits. Blood was collected in serum separator tubes (BD Vacutainer SST Gold 8.5 mL) and allowed to clot. Within 2 h of collection, tubes were centrifuged at 1500 × *g* for 15 min at 20 °C, and then aliquots were stored at −80 °C. Saliva samples were collected after a 30-min fast from food and drink beginning with an initial brief mouth rinse with 50–100 mL of tap water, repeated after 25 min. After 30 min, participants began depositing fluid into a 50 mL Falcon tube standing upright in a cup of ice, to a total of 5–10 mL over the next 30 min. The fluid was then centrifuged at 2600×*g* for 15 min at 4 °C, then phenylmethylsulfonyl fluoride (Sigma) was added to a final

https://doi.org/10.1038/s41467-024-54665-5

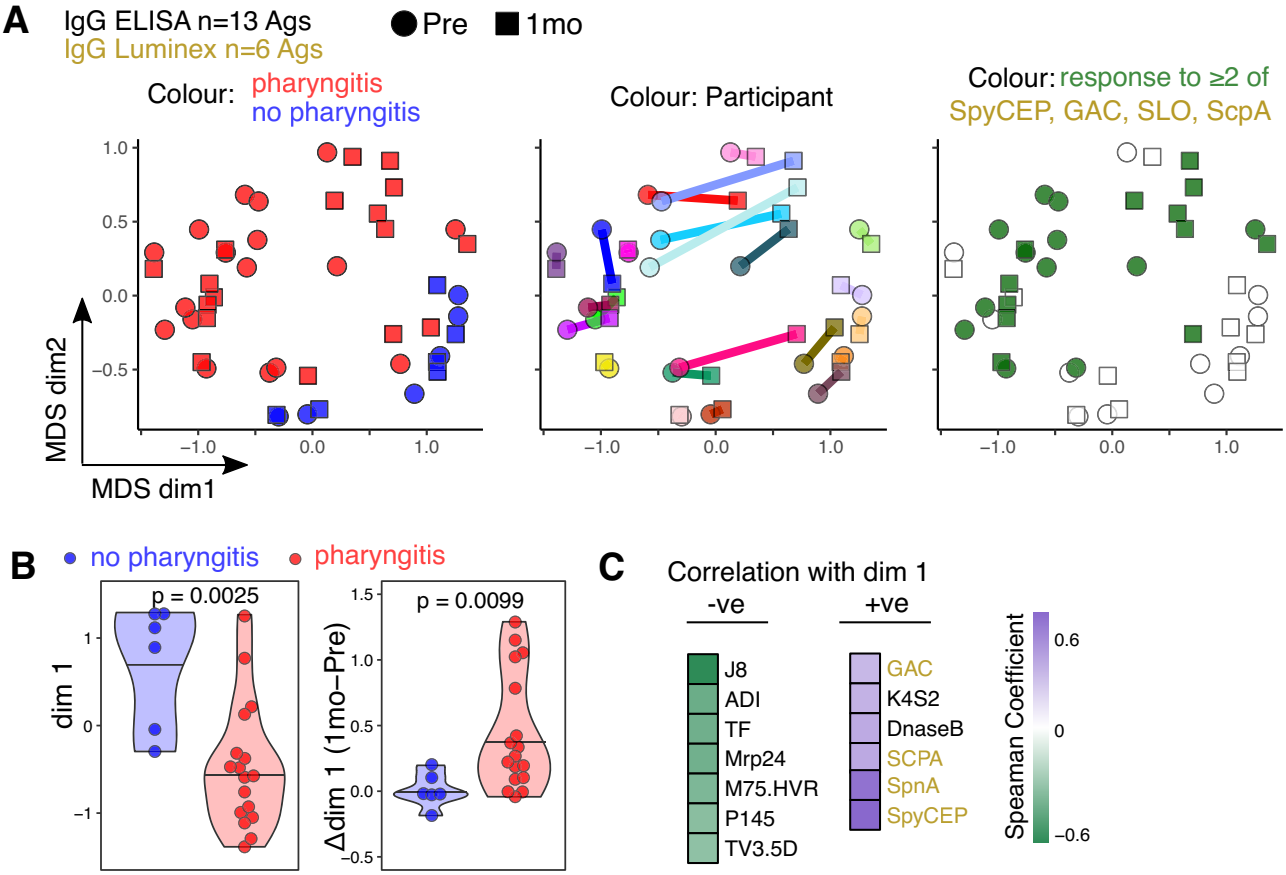


Fig. 6 | Serological signatures differ by clinical outcome following experimental pharyngeal human challenge with *S. pyogenes* and are shaped by infection. A Non-metric multidimensional scaling (MDS) was performed using IgG responses to 19 antigens for 23 individuals. Each point represents a sample, with a shape denoting time-point and coloured to indicate pharyngitis outcome (left), paired samples (centre), or responses to 2 or more of the dominant antigens SpyCEP, SLO, ScpA or GAC. **B** Violin plots of first MDS dimension values pre-challenge (left), or at 1 month with pre-challenge values subtracted (right), between pharyngitis and non-pharyngitis groups. *P* value determined by two-sided Mann–Whitney *U*-test. **C** Two-sided Spearman correlation analysis between MDS dimension 1 and IgG responses, with only antigens with significant correlation shown ($p < 0.01$ after FDR-adjustment). Antigens with results from ELISA assays are shown in black font, and multiplex bead-based assays are shown in gold font.

concentration of 500 nM, and aliquots were stored at −80 °C until analysis.

Antigens

A panel of 17 *S. pyogenes* vaccine candidate antigens, comprising recombinant proteins, synthetic peptides, and purified carbohydrate (Table S1) was investigated. Synthetic peptides from the M75 protein hypervariable region (M75-HVR), M-related protein (Mrp) 24 and Enn336 protein, were produced by GenScript with 95% purity, and collaborators at Griffith University provided p145, J8, P*17 and K4S2[61]. Recombinant proteins produced in BL21(DE3) pLysS *E. coli* representing the full-length M75 protein[21], T25 pilus, and TeeVax-3[62] were provided by collaborators at the University of Auckland. University of Queensland collaborators provided recombinant proteins representing arginine deiminase (ADI), trigger factor (TF), and C5a peptidase (ScpA)[63]. Recombinant proteins representing *S. pyogenes* Adhesion and Division protein (SpyAD), Cell Envelope Protease (SpyCEP), and Streptolysin O (SLO), plus purified native Group A Carbohydrate (GAC)[64], came from GSK Vaccines Institute for Global Health.

Enzyme-linked immunosorbent assays (ELISA)

ELISAs were done for all 17 antigens using serum and saliva from all 25 CHIVAS-M75 participants. In this study, data from ELISAs performed using samples from two time-points were analysed for serum IgG (pre-challenge and 1 month, or 3-month for 1 participant who failed to attend a 1-month follow-up visit) and saliva IgA (pre-challenge and 1-week follow-up visit). All antigens were resuspended to 5 μg/mL in 0.1 M coating carbonate buffer (0.03 M Na_2CO_3, 0.07 M $NaHCO_3$, pH 9.6), except full-length M75 protein and GAC, which were used at 1 μg/mL and coated at 50 μL per well onto 96-well medium-binding ELISA plates (Greiner Bio-One) overnight at room temperature. Plates were then blocked with 200 μL of 2% w/v bovine serum albumin (BSA, Sigma) in phosphate-buffered saline (PBS) for 1 or 2 h at 37 °C for IgG and IgA, respectively, followed by five washes in wash buffer (PBS with 0.05% v/v Tween20, PBS-T). Serial dilutions were then performed using diluent (PBS with 0.5% w/v BSA and 0.05% v/v Tween20) for sera (1:100, 1:300, 1:900, 1:2700) or saliva (1:50, 1:100, 1:200, 1:400) and then 50 μL added per well in duplicate. A set of healthy adult sera and saliva with high reactivity against the antigen panel were included as standardisation controls on each plate in duplicate; sera at 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, and saliva at 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200 dilutions. Plates were then incubated for 1 h at 37 °C (orbital shaking for saliva) and then washed five times with PBS-T. Negative controls included both antigen-coated wells without serum and blocked wells with positive control serum, all of which had an absorbance at OD_{450nm} of ~0.05 across all plates. Horseradish peroxidase-labelled goat anti-human IgG (Southern Biotech Goat anti-human HRP-IgG cat#2040-05 lot#B3919-XD29C, diluted 1:5000) or IgA (Southern Biotech Goat anti-human HRP-IgA cat#2050-05 lot#G0919-QC50D, diluted 1:2000) secondary antibodies were added

https://doi.org/10.1038/s41467-024-54665-5

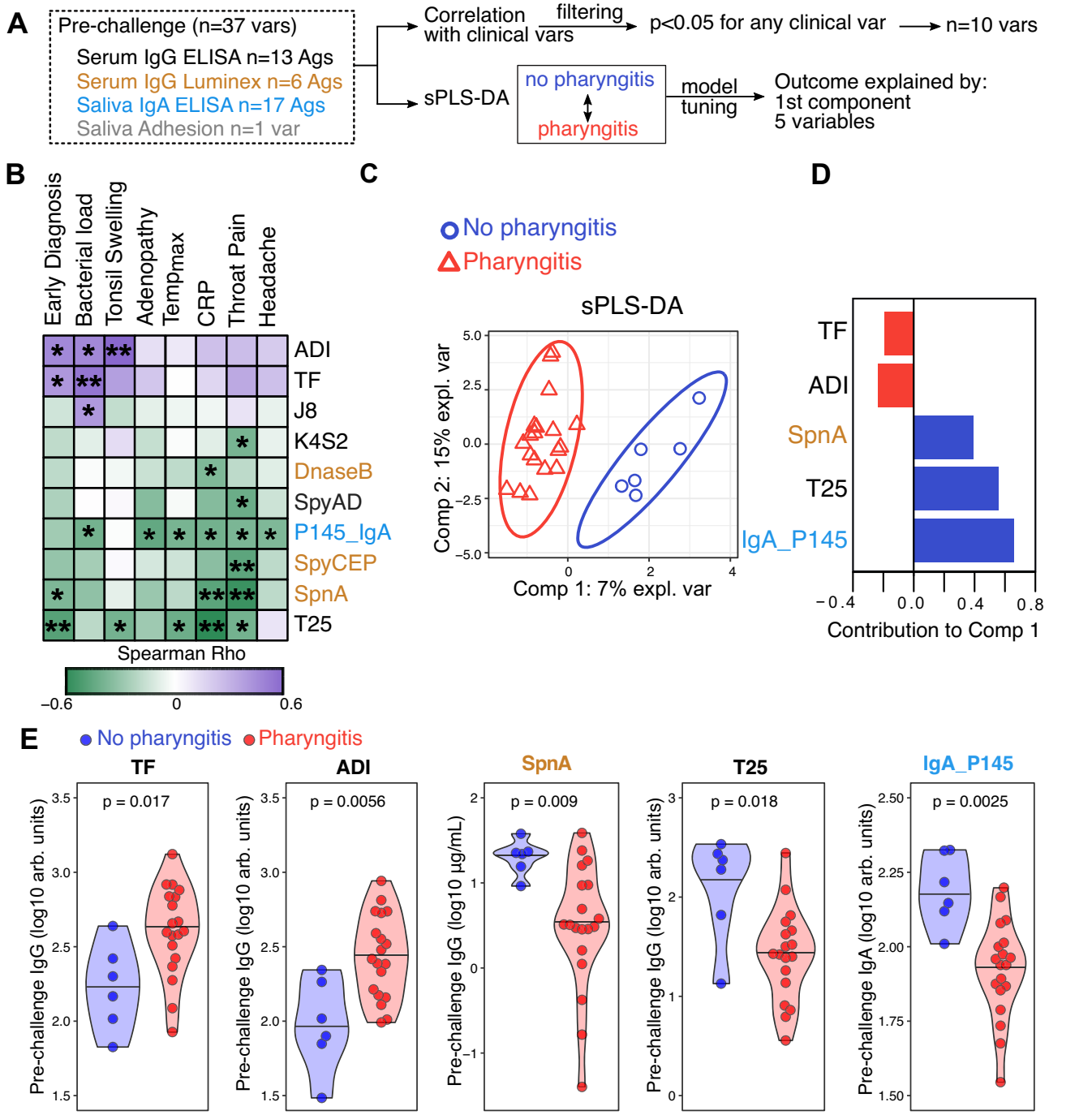


Fig. 7 | Baseline antibody titres are associated with clinical pharyngitis outcome following experimental human challenge with *Streptococcus pyogenes*. **A** Schematic overview of methods and variables included in correlation analysis and sparse partial least squares discriminant analysis (sPLS-DA). **B** Heatmap of correlation coefficients between various clinical variables and pre-challenge antibody levels. Antigens shown are those that showed significant two-sided Spearman correlation for one or more clinical variables. Asterisks denote unadjusted *p* values *<0.05, **<0.01. **C** Samples distributed across two dimensions from sPLS-DA. **D** Contributions of top 5 variables to sPLS-DA dimension 1, with variables in red higher in the pharyngitis group and variables in blue higher in the non-pharyngitis group. **E** Pre-challenge antibody responses against the top five variables from sPLS-DA, differentiating between participants who subsequently developed pharyngitis (red) and those who did not (blue). *P* value determined using the two-sided Mann–Whitney test. Antigens with results from ELISA assays are shown in black (IgG) and blue (IgA) text, and multiplex bead-based assays (IgG) are shown in gold text.

at 50 µL/well for 1 h at 37 °C, followed by five washes with PBS-T and three washes with reverse osmosis water. Tetramethylbenzidine substrate (Life Technologies) was added at 50 µL/well and developed at room temperature for 1 min (SLO, GAC, full-length M75 protein) or 5 min (all other antigens). Reactions were stopped with 1 M phosphoric acid, and then absorbance was measured at OD_{450nm} using a MultiSkan Go instrument with SkanIt Software version 4.1 (Thermo Fisher Scientific). Serum IgG end-point titres for each antigen and each sample are shown in Supplementary Fig. 5. Results were discarded, and the assay was repeated if the R^2 of the reference curve was ≤0.993 or the standard deviation between replicates was high (>10%). Across all antigens and both isotypes, the coefficient of variation between

duplicate samples ranged between 2 and 5%, indicating strong intra-assay concordance. Human salivary IgA was quantified using the Human IgA SimpleStep ELISA® Kit (Abcam) according to the manufacturer's instructions, with 1:5000 and 1:10,000 dilutions tested.

Multiplex bead assays

Antigen-specific IgG antibody responses were measured using a previously described multiplex bead-based assay (Luminex)[42,43]. Briefly, beads coupled with *S. pyogenes* antigens were incubated with sera diluted 1:8000 in assay buffer (PBS pH 7.4, containing 1% IgG-free BSA). Following washing, bound IgG was detected with phycoerythrin labelled anti-human IgG (Jackson ImmunoResearch Labs cat#709-116-098 lot#144968). Median fluorescence intensity signals for each sample were measured with a MagPix instrument and the xPonent software version 4.2 (Luminex Corporation). Standard curves were generated from pooled human immunoglobulin (Privigen; CSL Behring), which was buffer exchanged into PBS, and fitted using five-parameter logistic regression with Belysa software (Merck). Antigen-specific IgG concentrations in µg/mL were quantified by interpolation.

Paediatric comparator data are from a previously published study of *S. pyogenes* infections from Auckland, New Zealand[43,45]. Available data were shown for children (5–14 years old) with *S. pyogenes* pharyngitis ($n = 24$) and healthy controls ($n = 15$) who were deemed low risk for developing serious *S. pyogenes* disease, including acute rheumatic fever. Convalescent sera were obtained ~4 weeks after microbiologically-confirmed *S. pyogenes* positive pharyngitis and were included in the comparator analysis if a serological response to at least one antigen in the multiplex bead-based assay was observed when compared to matched healthy children. For both datasets, only data for the 6 relevant antigens were analysed for this study.

Bacterial isolates and growth

M75 *Streptococcus pyogenes* bacteria (611024, accession number CP033621)[37] were grown stationary at 37 °C with 5% CO_2 in Todd-Hewitt broth (Oxoid) with 1% (w/v) yeast extract (THY; Bacto) or on solid THY agar with 0.005% 2,3,5-tetraphenyltetrazolium chloride (TTC; Sigma).

Opsonophagocytic killing assays (OPKA)

OPKAs were carried out as previously described[21,38,65]. Briefly, HL-60 cells were differentiated for 3 or 4 days using 0.8% dimethylformamide at 37 °C with 5% CO_2 and diluted in opsonization buffer (10% v/v heat-inactivated foetal bovine serum (HyClone)), 0.1% w/v gelatin (Sigma) in Hanks' balanced salt solution with Ca/Mg) to 1×10^7 cells/ml. Frozen *emm*75 bacterial stocks were thawed, washed and diluted in opsonization buffer to ~120,000 CFU/ml and pre-incubated for 30 min at room temperature with 20 µl heat-inactivated participant sera. Baby rabbit complement and differentiated HL-60 cells were then added and incubated for 60 min at 37 °C with 5% CO_2. Surviving bacteria were recovered, then enumerated by serial dilution and plating 10 µl drips on TTC-THY agar and incubating overnight at 37 °C with 5% CO_2[21]. Bacteria were enumerated using a ProtoCOL3 automated colony counter (Synbiosis). The dilution of sera resulting in 50% killing was calculated as the opsonic index using the Opsotiter software version 3 (license obtained from the Pathogen Reference Laboratory at the University of Alabama at Birmingham)[66]. Positive and negative control sera was included in every assay.

Bacterial adhesion assays

The adhesion assay using Detroit 562 (D562) human pharyngeal cells was performed as described previously[37,39], with the addition of a pre-incubation step with saliva described below. D562 cells were maintained in an assay medium (Dulbecco's Modified Eagle Medium with 10% foetal bovine serum, and 1x Pen-Strep (Sigma)). M75 *S. pyogenes* was grown to mid-exponential phase (OD_{600nm} ~ 0.5), washed, and resuspended in 1 ml of assay media. Input inoculum per test was ~10^6 CFU/well to achieve a multiplicity of infection of five *S. pyogenes* CFU to one D562 cell. Bacteria were pre-incubated for 30 min at 37 °C with participant saliva (40 µl) collected pre-challenge and 1 week following discharge, then added to D562 cells plated in Triplicate wells were averaged, and assays performed at least twice per sample. µL of assay medium per well of 24-well tissue culture plates (Nunc) in triplicate. Plates were then centrifuged for 5 min at 200×*g* before incubation for 1 h at 37 °C in 5% CO_2. After the incubation, non-adherent bacteria were removed by washing three times with 1 mL PBS, then total cell-associated bacteria (adherent plus invasive) were recovered by adding 200 µl 0.25% trypsin, then lysis with 0.025% Triton-X (Sigma) in distilled water. Bacteria were then enumerated by serial dilution and plating 10 µl drips on TTC-THY agar and incubating overnight at 37 °C with 5% CO_2[67]. M75 *S. pyogenes* adhesion (%) was determined as CFU in saliva sample wells relative to control wells (without saliva).

Statistical analysis

Analysis of raw data and data extrapolation was performed using Prism versions 8 to 9 (GraphPad). Statistical analysis was conducted in R version 3.8[68], using packages ggplot2, pheatmap, viridis, tidyverse, reshape2, ggpubr, RColorBrewer, gghalves, vegan, ggbeeswarm, mixOmics, Polychrome and uwot. For serum IgG ELISAs, a four-parameter logistic curve was generated from the standard curve for each antigen for each plate, and a titre for each serum sample was calculated relative to that curve and expressed as arbitrary units (arb. units). For antigen-specific saliva IgA ELISAs, sample arb. units values were calculated relative to the reference control saliva at the 1:50 dilution. For total IgA, values were extrapolated from a log-transformed five-parametric logistical standard curve, generated using known amounts of IgA. *P* values were determined using two-sided non-parametric methods (Mann–Whitney tests for unpaired and Wilcoxon signed-rank test for paired two group comparisons). Participants were deemed to have a response where the arb. units post-challenge was 25% greater than the pre-challenge value. This cutoff was used to determine (1) the proportion of individuals who responded to each antigen; and (2) the number of antigens to which each participant responded. A comparison of different response thresholds is shown in Supplementary Fig. 6. Where repeated two group comparisons were performed across the panel of antigens, *p* values were adjusted for multiple corrections using the false discovery rate (FDR) approach[69]. For three or more groups, paired sample comparisons were performed using Friedman's test, followed by the Wilcoxon signed-rank test with FDR-adjustments, and between-group comparisons using Kruskall–Wallis and Dunn's multiple comparison testing.

Multidimensional scaling was performed using the vegan package in R using ELISA or multiplex bead-based assay data for 18 antigens that were each z-score transformed. A matrix of Spearman correlation coefficients, comparing each participant's antibody responses to every other participant, was generated and used to calculate a distance matrix for each person and each variable. This was used to perform non-metric multidimensional scaling to reduce the dimensionality of the data to 2[70]. The coordinates for dimension 1 from the multi-dimensional scaling analysis were compared between groups, as well as subtracting pre-challenge from 1-month coordinates.

Sparse partial least squares discriminant analysis was performed using the mixOmics package in R. ELISA variables were used except where multiplex bead-based assay data was available. Correlation was determined by Spearman correlation coefficient for clinical variables on discrete numerical scales[35]. The initial model allowed for ten components, model performance evaluated down to two components, then tuned using three Mfold validation and 100 iterations which returned five immune variables and one dimension as optimal to explain the difference between pharyngitis and non-pharyngitis participants.

Reporting summary
Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability
The ELISA and multiplex bead-based assay data generated in this study and the human challenge study clinical outcome data have been deposited in the Zenodo repository under accession code 13327362 [https://zenodo.org/records/13347362].

Code availability
The R code used to generate all analyses in this study has been deposited in the Zenodo repository under accession code 13327362 [https://zenodo.org/records/13347362].

References

1. Brouwer, S. et al. Pathogenesis, epidemiology and control of Group A Streptococcus infection. *Nat. Rev. Microbiol.* **21**, 431–447 (2023).
2. Roth, G. A. et al. Global burden of cardiovascular diseases and risk factors, 1990-2019: update from the GBD 2019 study. *J. Am. Coll. Cardiol.* **76**, 2982–3021 (2020).
3. Ralph, A. P. & Carapetis, J. R. Group a streptococcal diseases and their global burden. *Curr. Top. Microbiol. Immunol.* **368**, 1–27 (2013).
4. Immunization VaB, Vaccine Prioritization & Platforms Team. Strategic Priority 7: Regional engagement strategy to identify IA2030 global priority endemic pathogens. Product Development for Vaccines Advisory Committee (PDVAC) Meeting, World Health Organization. (https://www.who.int/news-room/events/detail/2023/12/12/default-calendar/2023-meeting-who-product-development-for-vaccines-advisory-committee-meeting-pdvac).
5. Osowicki, J. et al. WHO/IVI global stakeholder consultation on group A Streptococcus vaccine development: report from a meeting held on 12-13 December 2016. *Vaccine* **36**, 3397–3405 (2018).
6. Vekemans, J. et al. The path to group A Streptococcus vaccines: World Health Organization Research and development technology roadmap and preferred product characteristics. *Clin. Infect. Dis.* **69**, 877–883 (2019).
7. Walkinshaw, D. R. et al. The *Streptococcus pyogenes* vaccine landscape. *NPJ Vaccines* **8**, 16 (2023).
8. Frost, H., Excler, J. L., Sriskandan, S. & Fulurija, A. Correlates of immunity to group A Streptococcus: a pathway to vaccine development. *NPJ Vaccines* **8**, 1 (2023).
9. Lorenz, N. et al. Serological profiling of group A Streptococcus infections in acute rheumatic fever. *Clin. Infect. Dis.* **73**, 2322–2325 (2021).
10. Tsoi, S. K., Smeesters, P. R., Frost, H. R., Licciardi, P. & Steer, A. C. Correlates of protection for M protein-based vaccines against group A Streptococcus. *J. Immunol. Res.* **2015**, 167089 (2015).
11. Lancefield, R. C. Current knowledge of type-specific M antigens of group A streptococci. *J. Immunol.* **89**, 307–313,(1962).
12. Hysmith, N. D. et al. Prospective longitudinal analysis of immune responses in pediatric subjects after pharyngeal acquisition of group A Streptococci. *J. Pediatr. Infect. Dis. Soc.* **6**, 187–196 (2017).
13. Guirguis, N., Fraser, D. W., Facklam, R. R., El Kholy, A. & Wannamaker, L. W. Type-specific immunity and pharyngeal acquisition of group A Streptococcus. *Am. J. Epidemiol.* **116**, 933–939 (1982).
14. Wannamaker, L. W., Denny, F. W., Perry, W. D., Siegel, A. C. & Rammelkamp, C. H. Jr. Studies on immunity to streptococcal infections in man. *AMA Am. J. Dis. Child* **86**, 347–348 (1953).
15. Salie, M. T. et al. Serum immune responses to group A Streptococcal antigens following pharyngeal acquisitions among children in Cape Town, South Africa. *mSphere* **8**, e00113–e00123 (2023).
16. D'Alessandri, R. et al. Protective studies with group A streptococcal M protein vaccine. III. Challenge of volunteers after systemic or intranasal immunization with type 3 or type 12 group A Streptococcus. *J. Infect. Dis.* **138**, 712–718 (1978).
17. Fox, E. N., Waldman, R. H., Wittner, M. K., Mauceri, A. A. & Dorfman, A. Protective study with a group A streptococcal M protein vaccine. Infectivity challenge of human volunteers. *J. Clin. Invest.* **52**, 1885–1892 (1973).
18. Polly, S. M., Waldman, R. H., High, P., Wittner, M. K. & Dorfman, A. Protective studies with a group A streptococcal M protein vaccine. II. Challenge of volunteers after local immunization in the upper respiratory tract. *J. Infect. Dis.* **131**, 217–224 (1975).
19. Osowicki, J. et al. Controlled human infection for vaccination against Streptococcus pyogenes (CHIVAS): establishing a group A Streptococcus pharyngitis human infection study. *Vaccine* **37**, 3485–3494 (2019).
20. Rivera-Hernandez, T. et al. Vaccine-induced Th1-type response protects against invasive group A Streptococcus infection in the absence of opsonizing antibodies. mBio **11**, e00122-20 (2020).
21. McGregor, R. et al. Naturally acquired functional antibody responses to group A Streptococcus differ between major strain types. *mSphere* **8**, e0017923 (2023).
22. Gill FA. A review of past attempts and present concepts of producing streptococcal immunity in humans. *Q Bull. Northwest Univ. Med. Sch.* **34**, 326–339 (1960).
23. Revocation of status of specific products; Group A streptococcus. Direct final rule. *Fed. Regist.* **70**, 72197–72199 (2005).
24. Asturias, E. J. et al. Safety of *Streptococcus pyogenes* vaccines: anticipating and overcoming challenges for clinical trials and post-marketing monitoring. *Clin. Infect. Dis.* **77**, 917–924 (2023).
25. Harbison-Price, N. et al. in *Streptococcus pyogenes: Basic Biology to Clinical Manifestations* (eds Ferretti J. J., Stevens D. L. & Fischetti V. A.) Ch. 31 (The University of Oklahoma Health Sciences Center, 2022).
26. Reglinski, M., Gierula, M., Lynskey, N. N., Edwards, R. J. & Sriskandan, S. Identification of the *Streptococcus pyogenes* surface antigens recognised by pooled human immunoglobulin. *Sci. Rep.* **5**, 15825 (2015).
27. Bensi, G. et al. Multi high-throughput approach for highly selective identification of vaccine candidates: the Group A Streptococcus case. *Mol. Cell. Proteom.* **11**, M111.015693 (2012).
28. Sekuloski, S. et al. Evaluation of safety and immunogenicity of a group A streptococcus vaccine candidate (MJ8VAX) in a randomized clinical trial. *PLoS ONE* **13**, e0198658 (2018).
29. Pastural, E. et al. Safety and immunogenicity of a 30-valent M protein-based group a streptococcal vaccine in healthy adult volunteers: a randomized, controlled phase I study. *Vaccine* **38**, 1384–1392 (2020).
30. McNeil, S. A. et al. A double-blind, randomized phase II trial of the safety and immunogenicity of 26-valent group A streptococcus vaccine in healthy adults. *Int. Congr. Ser.* **1289**, 303–306 (2006).
31. McNeil, S. A. et al. Safety and immunogenicity of 26-valent group A streptococcus vaccine in healthy adult volunteers. *Clin. Infect. Dis.* **41**, 1114–1122 (2005).
32. Kotloff, K. L. et al. Safety and immunogenicity of a recombinant multivalent group A streptococcal vaccine in healthy adults: Phase 1 trial. *J. Am. Med. Assoc.* **292**, 709–715 (2004).
33. Steer, A. C. et al. Status of research and development of vaccines for *Streptococcus pyogenes*. *Vaccine* **34**, 2953–2958 (2016).
34. Abo, Y. N. et al. Strategic and scientific contributions of human challenge trials for vaccine development: facts versus fantasy. *Lancet Infect. Dis.* **23**, e533–e546 (2023).

35. Osowicki, J. et al. A controlled human infection model of *Streptococcus pyogenes* pharyngitis (CHIVAS-M75): an observational, dose-finding study. *Lancet Microbe* **2**, e291–e299 (2021).
36. Anderson, J. et al. Immune signature of acute pharyngitis in a *Streptococcus pyogenes* human challenge trial. *Nat. Commun.* **13**, 769 (2022).
37. Osowicki, J. et al. A controlled human infection model of group A Streptococcus pharyngitis: which strain and why? mSphere https://doi.org/10.1128/mSphere.00647-18 (2019)
38. McGregor, R., Jones, S., Jeremy, R. M., Goldblatt, D. & Moreland, N. J. An opsonophagocytic killing assay for the evaluation of group A Streptococcus vaccine antisera. *Methods Mol. Biol.* **2136**, 323–335 (2020).
39. Khemlani, A. H. J., Proft, T. & Loh, J. M. S. Assays to analyze adhesion of group A Streptococcus to host cells. *Methods Mol. Biol.* **2136**, 271–278 (2020).
40. Zhu, H. et al. Molecular correlates of vaccine-induced protection against typhoid fever. *J. Clin. Invest.* https://doi.org/10.1172/JCI169676 (2023).
41. Knuutila, A. et al. Simultaneous determination of antibodies to pertussis toxin and adenylate cyclase toxin improves serological diagnosis of pertussis. *Diagnostics* **11**, 180 (2021).
42. Whitcombe, A. L. et al. An eight-plex immunoassay for group A streptococcus serology and vaccine development. *J. Immunol. Methods* **500**, 113194 (2022).
43. Whitcombe, A. L. et al. Increased breadth of group A Streptococcus antibody responses in children with acute rheumatic fever compared to precursor pharyngitis and skin infections. *J. Infect. Dis.* **226**, 167–176 (2022).
44. Hanson-Manful, P. et al. The novel group A Streptococcus antigen SpnA combined with bead-based immunoassay technology improves streptococcal serology for the diagnosis of acute rheumatic fever. *J. Infect.* **76**, 361–368 (2018).
45. Bennett, J. et al. Risk factors for group A streptococcal pharyngitis and skin infections: a case control study. *Lancet Reg. Health West Pac.* **26**, 100507 (2022).
46. Henningham, A. et al. Conserved anchorless surface proteins as group A streptococcal vaccine candidates. *J. Mol. Med.* **90**, 1197–1207 (2012).
47. Ozberk, V., Pandey, M. & Good, M. F. Contribution of cryptic epitopes in designing a group A streptococcal vaccine. *Hum. Vaccin. Immunother.* **14**, 2034–2052 (2018).
48. Carducci, M. et al. Development and characterization of a hemolysis inhibition assay to determine functionality of anti-Streptolysin O antibodies in human sera. *J. Immunol. Methods* **526**, 113618 (2024).
49. Turner, C. E., Kurupati, P., Wiles, S., Edwards, R. J. & Sriskandan, S. Impact of immunization against SpyCEP during invasive disease with two streptococcal species: *Streptococcus pyogenes* and *Streptococcus equi*. *Vaccine* **27**, 4923–4929 (2009).
50. Keeley, A. J. et al. *Streptococcus pyogenes* colonization in children aged 24–59 months in the Gambia: impact of live attenuated influenza vaccine and associated serological responses. *J. Infect. Dis.* **228**, 957–965 (2023).
51. Corcoran, L. M. & Tarlinton, D. M. Regulation of germinal center responses, memory B cells and plasma cell formation-an update. *Curr. Opin. Immunol.* **39**, 59–67 (2016).
52. Mankarious, S. et al. The half-lives of IgG subclasses and specific antibodies in patients with primary immunodeficiency who are receiving intravenously administered immunoglobulin. *J. Lab Clin. Med.* **112**, 634–640 (1988).
53. Bowman, J. M. The prevention of Rh immunization. *Transfus. Med. Rev.* **2**, 129–150 (1988).
54. Schaefer-Babajew, D. et al. Antibody feedback regulates immune memory after SARS-CoV-2 mRNA vaccination. *Nature* **613**, 735–742 (2023).
55. Inoue, T. et al. Antibody feedback contributes to facilitating the development of Omicron-reactive memory B cells in SARS-CoV-2 mRNA vaccinees. *J. Exp. Med.* https://doi.org/10.1084/jem.20221786 (2020).
56. McNamara, H. A. et al. Antibody feedback limits the expansion of B cell responses to malaria vaccination but drives diversification of the humoral response. *Cell Host Microbe* **28**, 572–585.e7 (2020).
57. Koutsakos, M. & Ellebedy, A. H. Immunological imprinting: understanding COVID-19. *Immunity* **56**, 909–913 (2023).
58. Tas, J. M. J. et al. Antibodies from primary humoral responses modulate the recruitment of naive B cells during secondary responses. *Immunity* **55**, 1856–1871.e6 (2022).
59. Smeesters, P. R. et al. Global *Streptococcus pyogenes* strain diversity, disease associations, and implications for vaccine development: a systematic review. *Lancet Microbe* **5**, e181–e193 (2024).
60. Fabri, L. V. et al. An emm-type specific qPCR to track bacterial load during experimental human *Streptococcus pyogenes* pharyngitis. *BMC Infect. Dis.* **21**, 463 (2021).
61. Pandey, M. et al. Combinatorial synthetic peptide vaccine strategy protects against hypervirulent CovR/S mutant streptococci. *J. Immunol.* **196**, 3364–3374 (2016).
62. Loh, J. M. S. et al. A multivalent T-antigen-based vaccine for group A Streptococcus. *Sci. Rep.* **11**, 4353 (2021).
63. Rivera-Hernandez, T. et al. An experimental group A Streptococcus vaccine that reduces pharyngitis and tonsillitis in a nonhuman primate model. *mBio* **10**, e00693-19 (2019).
64. Di Benedetto, R. et al. Rational design of a glycoconjugate vaccine against group A Streptococcus. *Int. J. Mol. Sci.* **21**, 8558 (2020).
65. Jones, S. et al. Development of an opsonophagocytic killing assay for group a streptococcus. *Vaccine* **36**, 3756–3763 (2018).
66. Burton, R. L. & Nahm, M. H. Development and validation of a four-fold multiplexed opsonization assay (MOPA4) for pneumococcal antibodies. *Clin. Vaccin. Immunol.* **13**, 1004–1009 (2006).
67. Frost, H. R. et al. Validation of an automated colony counting system for group A Streptococcus. *BMC Res. Notes* **9**, 72 (2016).
68. R Core Team. R: a language and environment for statistical computing. (R Foundation for Statistical Computing, 2020).
69. Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B* **57**, 289–300 (1995).
70. Hill, D. L. et al. Immune system development varies according to age, location, and anemia in African children. *Sci. Transl. Med.* https://doi.org/10.1126/scitranslmed.aaw9522 (2020).

Acknowledgements

We thank Prof. Thomas Proft and Dr Jacelyn Loh (University of Auckland) for providing T25 and TeeVax-3 antigens. We thank Dr Danilo Gomes Moriel (GSK Vaccines Institute for Global Health) for supplying GAC, SLO, SpyCEP and SpyAD antigens, including for the development of the Luminex multiplex bead-based assay. GSK Vaccines Institute for Global Health is an affiliate of GlaxoSmithKline Biologicals SA. We are grateful to Dr Julie Bennett and Prof. Michael Baker at the University of Otago, Wellington, New Zealand, and all contributors to the study from which comparator IgG data was obtained for streptococcal pharyngitis in children. The CHIVAS-M75 study was funded by the Australian National Health and Medical Research Council (1099183). This study was partially funded by the Maurice Wilkins Centre for Molecular Biodiscovery (3716490, N.J.M.), the University of Auckland School of Medicine Foundation (3714694, N.J.M.), Australian National Health and Medical Research Council Early Career or Investigator Fellowships (J.O, D.L.H, M.J.W, A.C.S) and the Michelson Prize from the Human Immunome Project and Michelson Medical Research Foundation (D.L.H.) J.O. is also supported by a Melbourne Children's Campus Clinician–Scientist fellowship and A.C.S. by a Viertel Senior Medical Research Fellowship. J.O.,

H.R.F., K.I.A., D.L.H. and A.C.S. are Human Infection Challenge Network for Vaccine Development members.

Author contributions

Conceptualization: A.C.S., J.O., H.R.F., N.J.M. and D.L.H. Methodology and experiments: H.R.F., K.I.A., A.L.W., R.M., L.H.C., C.B., L.F. and P.V.L. Data analysis: D.L.H., H.R.F., R.M., L.H.C., A.L.W. and N.J.M., Patient cohorts: A.C.S. and J.O. Original draft: D.L.H., J.O., and H.R.F. Manuscript review: D.L.H, J.O., H.R.F., A.C.S., N.J.M., P.R.S., P.V.L., M.J.W., M.F.G. and M.P. Funding acquisition: A.C.S., J.R.C., J.O., M.F.G., M.J.W., M.P., P.R.S., D.L.H., A.C.S., D.L.H. and N.J.M. jointly led this research.

Competing interests

A.C.S. and J.R.C. are co-chairs of the Australian Strep A Vaccine Initiative (ASAVI), and A.C.S. co-chairs the Strep A Vaccine Global Consortium (SAVAC). N.J.M. is co-leader of Rapua te mea ngaro ka tau, a New Zealand-based *S. pyogenes* vaccine initiative. M.F.G., M.P., P.R.S. and M.J.W. are inventors of patents related to *S. pyogenes* vaccines. The remaining authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-024-54665-5.

Correspondence and requests for materials should be addressed to Joshua Osowicki or Danika L. Hill.

Peer review information *Nature Communications* thanks Ryan Thwaites, Elena Mitsi and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. A peer review file is available.

[1]Tropical Diseases Research Group, Murdoch Children's Research Institute, Melbourne, VIC, Australia. [2]Department of Paediatrics, University of Melbourne, Melbourne, VIC, Australia. [3]Department of Infectious Diseases, Royal Children's Hospital Melbourne, Melbourne, VIC, Australia. [4]Maurice Wilkins Centre for Molecular Biodiscovery, University of Auckland, Auckland, New Zealand. [5]School of Medical Sciences, Faculty of Medical and Health Sciences, The University of Auckland, Auckland, New Zealand. [6]Microbiology Laboratory, European Plotkin Institute of Vaccinology, Université libre de Bruxelles, Brussels, Belgium. [7]Department of Paediatrics, Brussels University Hospital, Academic Children Hospital Queen Fabiola, Université libre de Bruxelles, Brussels, Belgium. [8]The Institute for Glycomics, Griffith University, Gold Coast, QLD, Australia. [9]Wesfarmers Centre for Vaccines and Infectious Diseases, The Kids Research Institute Australia, University of Western Australia, Perth, WA, Australia. [10]Faculty of Health and Medical Sciences, University of Western Australia, Perth, WA, Australia. [11]Department of Paediatric Infectious Diseases, Perth Children's Hospital, Perth, WA, Australia. [12]School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, QLD, Australia. [13]Australian Infectious Diseases Research Centre, The University of Queensland, Brisbane, QLD, Australia. [14]Institute for Molecular Bioscience, The University of Queensland, Brisbane, QLD, Australia. [15]Vaccine Immunology Research Group, Murdoch Children's Research Institute, Melbourne, VIC, Australia. [16]Department of Immunology, School of Translational Medicine, Monash University, Melbourne, VIC, Australia. [17]These authors contributed equally: Joshua Osowicki, Hannah R. Frost. [18]These authors jointly supervised this work: Nicole J. Moreland, Danika L. Hill, Andrew C. Steer. ✉e-mail: joshua.osowicki@rch.org.au; danika.hill@monash.edu