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Expanding strain coverage of a group A *Streptococcus* pilus–expressing *Lactococcus lactis* mucosal vaccine

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Abstract

Group A Streptococcus (GAS) is a human pathogenic bacterium that can trigger a wide range of diseases, including the autoimmune diseases acute rheumatic fever and rheumatic heart disease, causing major morbidity and mortality in many low- and middle-income countries. Primary intervention programs have had limited success thus far, and a licensed vaccine has yet to be developed. The pilus of GAS is known to be involved in host cell adhesion, biofilm formation and immune evasion. We have a mucosal vaccine in development that expresses the pilus of GAS on the surface of the nonpathogenic bacterium *Lactococcus lactis*. To expand strain coverage, we combined seven *L. lactis* constructs, each expressing a different GAS pilus variant, and investigated the systemic and mucosal immune responses following immunization. Mice immunized with this combination showed specific immunoglobin G and immunoglobin A responses to the GAS pilus proteins of vaccine strains, at levels comparable to mice immunized with a single construct. Cross-reactivity to pilus proteins of nonvaccine strains was also evident. Furthermore, protective efficacy against a homologous strain of GAS in a murine nasopharyngeal colonization model was observed. Overall, this study provides further evidence for using pilus-expressing lactic acid bacteria as a vaccine to prevent upper respiratory tract GAS infections.

INTRODUCTION

Streptococcus pyogenes or Group A Streptococcus (GAS) is a pathogenic bacterium that causes a plethora of diseases in humans, from minor skin and throat infections, such as impetigo and pharyngitis, to severe invasive infections, such as streptococcal toxic shock syndrome and necrotizing fasciitis. From a global perspective, there are an estimated 600 million cases of GAS pharyngitis per year, and over 162 million children suffer from impetigo at any one time.[1,2] Invasive GAS infections are rare but are associated with high mortality.[1] GAS infections can also result in poststreptococcal autoimmune diseases such as acute rheumatic fever, which can further develop into rheumatic heart disease.[3,4] A review in 2017 reported 33.4 million cases and 319 400 deaths attributed to rheumatic heart disease worldwide.[5] A need for a safe and effective vaccine against the life-threatening conditions caused by GAS is therefore paramount.

Pili are long, flexible hairlike filaments that protrude from the bacterial surface.[6] First identified in 2005,[6] GAS pili have since been shown to be involved in host cell adhesion, biofilm formation and immune evasion.[7–10] The major component of the pilus structure is the backbone pilin, also known as the T-antigen, which polymerizes to form fibers up to 10 μm long.[6] The T-antigen has been used as a serological marker for GAS infection in addition to M-typing.[11] However, serological

typing has been largely replaced by genotyping of the *emm* gene, which encodes the M protein, and the *tee* gene, which encodes the T-antigen. There are also one or two ancillary pilins (AP1 and AP2) attached to either end of the pilus.[12] AP1 is the main adhesin of the pilus, though, in some pili, the T-antigen has also been shown to have adhesive properties.[10,13–15] AP2 serves as an adapter protein for sortase-mediated cell wall anchorage.[12,16] The proteins involved in pilus formation are encoded in the variable genomic region known as the fibronectin- and collagen-binding T-antigen (FCT) region, of which nine different types (FCT-1 to FCT-9) have been identified.[16–18] FCT-3 and FCT-4 are by far the most prevalent FCT regions found in different GAS strains.[18] FCT-7, 8 and 9 are all derivatives of FCT-4 lacking either the gene for AP1 (FCT-7 and 9) or carrying a different regulator gene (*rofA* is replaced by *nra* in FCT-8).[18] Sequence analysis of the major and minor pilus subunits has revealed 21 T-antigen variants that shared < 95% identity.[19] The most diversity was seen among FCT-3- and FCT-4-type strains.[16,19] AP1 variants have been less well studied but generally grouped into the same phylogenetic clusters as the T-antigen.[16] AP2 variants were far more conserved and grouped into only five clusters.[16] The diversity in the pilus genes is far more limited compared with other GAS vaccine targets such as the hypervariable M-protein.[20] This makes the pilus an attractive vaccine target as it is also well exposed on the bacterial surface and plays a major role in adhesion during the initial stages of GAS colonization in the upper respiratory tract or skin.[7]

We have previously shown that the food-grade bacterium *Lactococcus lactis* expressing the FCT-4–type pilus could be used as a mucosal vaccine.[21] Immunization of rabbits with heat-killed recombinant *L. lactis* bacteria *via* oral gavage elicited pilus-specific antibody responses that inhibited bacterial adhesion and promoted the opsonophagocytic killing of different GAS strains tested.[21] Furthermore, intranasal immunization of mice with *L. lactis* expressing the FCT-4–type pilus improved clearance rates of GAS following nasopharyngeal challenge.[21] However, immunization with a single pilus type was not expected to provide adequate vaccine coverage because of its antigenic diversity.

In this study, we hypothesize that a combination of seven *L. lactis* strains, each expressing a different GAS pilus type, will generate a vaccine with extended strain coverage. We have named this vaccine GASPEL (GAS pili expressed on *L. lactis*). We show improved strain coverage in GASPEL-immunized mice, and that GAS clearance rates following nasopharyngeal challenge remain as effective as immunization with a single construct.

RESULTS

Expression of GAS pili on the surface of *L. lactis*

The pilus operons from GAS M6T6 (FCT-1 type), GAS M49T18.2 (FCT-3 type) and GAS M4T4 (FCT-5 type) serotypes were cloned and expressed in *L. lactis* MG1363 under the control of the constitutive lactococcal P23 promoter as previously described.[21] We have designated these *L. lactis* constructs as PilM6T6, PilM49T18.2 and PilM4T4, respectively (Figure 1a). Including our four previously reported constructs, PilM1T1 (GAS M1T1, FCT-2 type),[22] PilM89T11 (GAS M89T11, FCT-4 type),[21] PilM28T28.1 (GAS M28T28.1, FCT-4 type)[21] and PilM2T2 (GAS M2T2, FCT-6 type),[10] a total of seven constructs were assessed in this study (Figure 1a). This represented at least one pilus operon from each of the six main FCT types (FCT-1 to FCT-6), plus an additional FCT-4–type pilus to enhance coverage of this region of higher diversity. Of note is that cloning the PilM2T2 and PilM4T4 operons excluded two to three predicted sortases that were considered redundant (Figure 1a). Pilus expression was confirmed by flow cytometry using rabbit polyclonal antibodies to the respective T-antigens (Figure 1b).

Intranasal immunization with single *L. lactis* pilus constructs induces systemic antibody responses in mice

After confirming pilus expression on our *L. lactis* constructs, we sought to analyze the immune response to individual pilus constructs after immunization. Groups of mice ($n = 5$) were immunized intranasally with 10 µL of 10^8 colony-forming units (CFU) of each construct on 3 consecutive days. This dosing schedule was performed a further two times, 2 weeks apart (Figure 2a). The sera from each group of mice were analyzed by ELISA for their antibody response to the respective pilus subunits, the T-antigen and AP1 (Figure 2b). AP2 was excluded from analysis as antibodies to this subunit are less likely to play a significant biological role in immunity because of its known "hidden" location at the base of the pilus structure.[12] All constructs generated specific serum immunoglobin (Ig) G to their cognate T-antigen above the limit of detection (LOD). The response to cognate AP1 was not as pronounced, with most groups showing reactivity close to or below the LOD. Only PilM89T11- and PilM2T2-immunized mice consistently produced AP1-specific serum IgG well above the LOD (Figure 2b). T-antigen serum IgA responses were above the LOD for all groups except for PilM28T28.1-immunized mice (Figure 2b). Serum IgA responses to AP1 proteins were all below the LOD (Figure 2b).

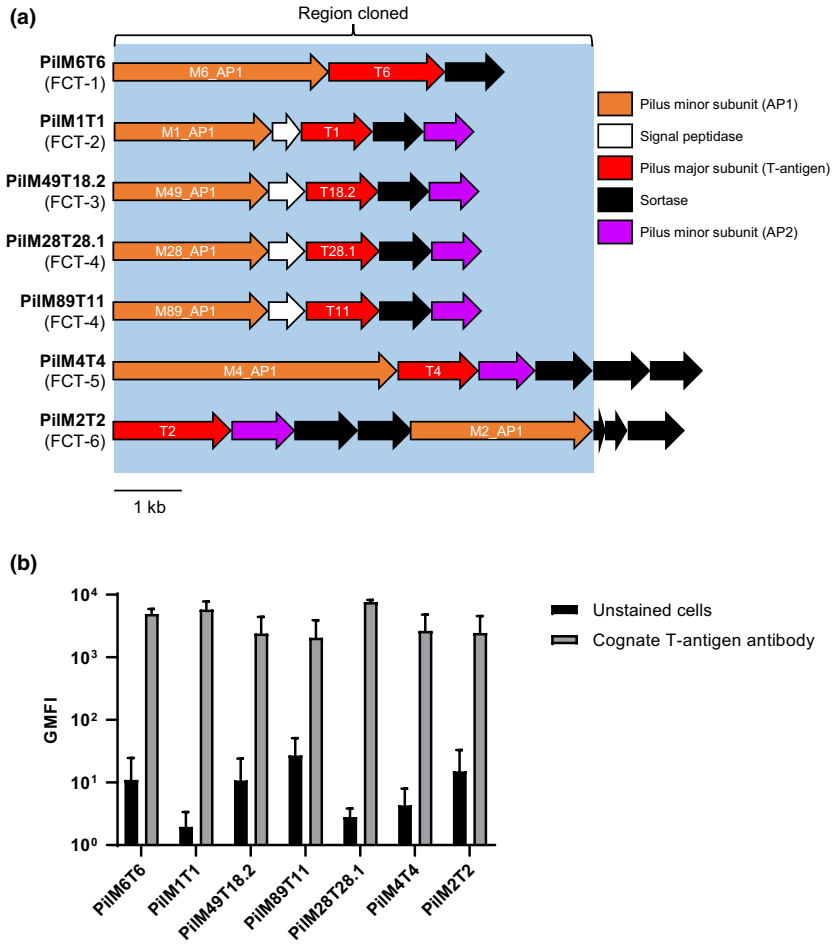

Figure 1. Cloning and expression of the GAS pilus on *Lactococcus lactis*. Pilus operons from GAS M6T6 (PilM6T6), GAS M1T1 (PilM1T1), GAS M49T18.2 (PilM49T18.2), GAS M89T11 (PilM89T11), GAS M28T28.1 (PilM28T28.1), GAS M4T4 (PilM4T4) and GAS M2T2 (PilM2T2) were cloned and expressed in *L. lactis*. **(a)** Schematic representation of the gene organization and respective gene sizes in each pilus operon cloned. The shaded area designates the genes included in the constructs used in this study. **(b)** Pilus expression was analyzed by flow cytometry using rabbit polyclonal antibodies specific to each respective T-antigen. Anti-rabbit IgG-Alexa Fluor 647 was used for detection. The graph represents the GMFI from 10 000 events on a log scale (n = 3). GAS, group A Streptococcus; GMFI, geometric mean fluorescent intensity; FCT, fibronectin- and collagen-binding T-antigen.

Intranasal immunization with GASPEL induces systemic antibody responses comparable to single-construct immunization

We next investigated the effect of combining all seven *L. lactis* pilus constructs into a single vaccine (GASPEL). Mice (n = 5) were immunized intranasally with an equal CFU ratio of each *L. lactis* construct (a combined total of 10^8 CFU), following the same immunization regimen (Figure 2a). All T-antigen serum IgG responses remained above the LOD after GASPEL immunization (Figure 3a) but were significantly lower than mice immunized with individual PilM49T18.2 ($P < 0.05$), PilM89T11 ($P < 0.05$) or PilM4T4 ($P < 0.001$) pilus constructs (Figure 3a). GASPEL immunization improved the T28.1 response compared with immunization with PilM28T28.1 alone ($P < 0.05$). Serum IgG responses to AP1 were similar between GASPEL-immunized and single-construct–immunized mice, with the exception of M2_AP1, which showed a significant drop ($P < 0.0001$). GASPEL antisera were also able to bind directly to *L. lactis* expressing fully assembled pili as analyzed by flow cytometry (Supplementary figure 1). However, high background staining was also observed with preimmune and empty vector sera so specificity cannot be confirmed (Supplementary figure 1).

Serum IgA responses to AP1 proteins remained low after GASPEL immunization (Figure 3b). Significantly lower responses to T4 (7.5-fold, $P \leq 0.05$), T6 (~4-fold, $P \leq 0.05$), T18.2 (~2-fold, $P \leq 0.05$) and T11 (3-fold, $P \leq 0.01$) were observed in GASPEL-immunized mice compared with

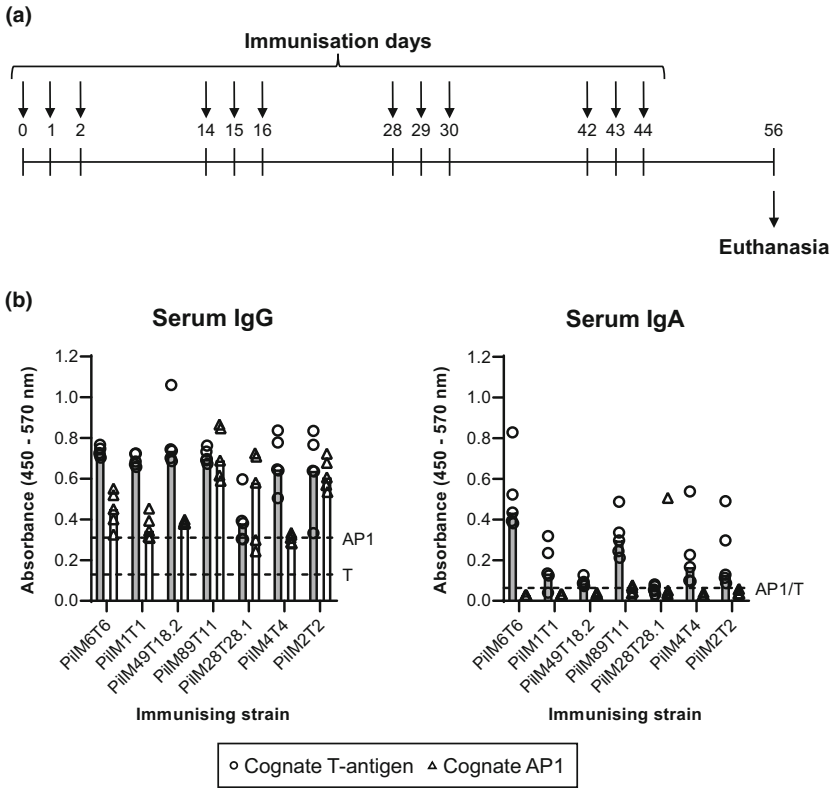

Figure 2. Intranasal immunization with individual pilus constructs induces pilus-specific systemic antibody responses. FVB/n mice ($n = 5$ per group) were immunized intranasally with 10^8 CFUs of live recombinant *Lactococcus lactis* PilM6T6, PilM1T1, PilM49T18.2, PilM89T11, PilM28T28.1, PilM4T4 or PilM2T2. **(a)** Immunization schedule. **(b)** Serum antibodies from each mouse to cognate T-antigen (circles/gray bars) and AP1 proteins (triangles/white bars) were measured by ELISA at a 1:100 serum dilution. Symbols indicate data from individual mice from a single experiment; bar indicates the group median. The horizontal dashed line indicates the LOD. AP, ancillary pilin; CFU, colony-forming units; Ig, immunoglobulin; LOD, limit of detection.

single-construct immunization (Figure 3b). An increased response to T28.1 (~5.5-fold, $P \leq 0.0001$) was, however, observed in GASPEL-immunized mice (Figure 3b).

Immunization with GASPEL protects against nasopharyngeal colonization in mice

As shown in our previous study, GAS M89T11, which expresses the FCT-4–type pilus, is one of the best colonizing strains in mice.[21] Mice immunized with PilM89 showed a higher clearance rate when infected with GAS M89T11 than the control mice.[21] To confirm that protective efficacy is still maintained when immunizing with GASPEL, FVB/n mice ($n = 19$ or 20) were immunized intranasally with GASPEL and then subsequently challenged with GAS M89T11. The nasal shedding of bacteria from infected mice was monitored by gently pressing the nose of each mouse ten times on an agar plate for bacterial culture and enumeration. The burden of GAS (as measured by daily CFU counts shed from each mouse) trended lower in GASPEL-immunized mice, but this was not significant (Figure 4a). It was also noted that several mice in both groups appeared to stop shedding the bacteria but then started again at a later timepoint, suggesting potential reinfection (Figure 4a). When examining the loss of carriage excluding reinfection, a significant reduction ($P = 0.0085$) in carriage rate in GASPEL-immunized mice was observed (Figure 4b). For this analysis, mice were scored as having cleared the infection if negative cultures were returned for at least 2 consecutive days.

Significantly higher ($P \leq 0.0001$) serum IgG and IgA titers were detected against T11 and M89_AP1 in GASPEL-immunized mice than in the empty vector control group (Figure 5a). T11- and M89_AP1–specific mucosal antibody responses could also be detected in postchallenge bronchoalveolar lavage (BAL) and nasal samples and were significantly higher ($P \leq 0.001$) than control (Figure 5b). Within the GASPEL-immunized group, significant positive correlations were observed between serum and nasal IgA with T11 (Supplementary figure 2). Significant positive correlations were also

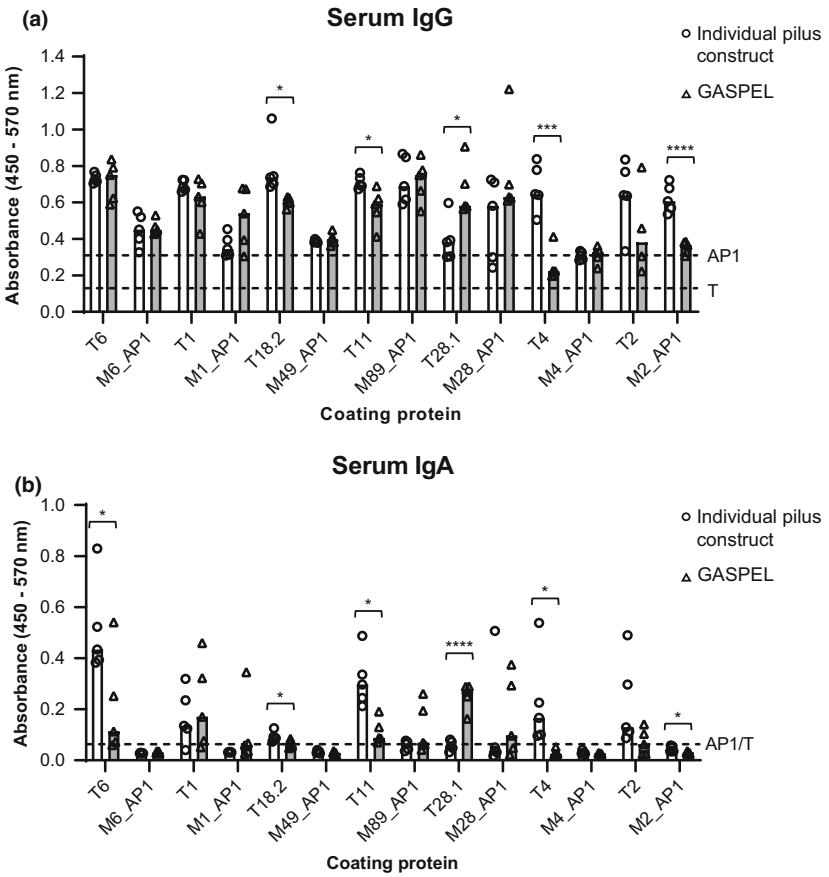

Figure 3. Comparison of serum antibody responses from individual construct and GASPEL-immunized mice. Mice ($n = 5$) were immunized intranasally with a total of 10^8 CFU of GASPEL (an equal mix of seven *Lactococcus lactis* pilus constructs). Serum **(a)** IgG or **(b)** IgA antibody responses from GASPEL-immunized mice (triangles/gray bars) were compared with those from mice previously immunized with each individual construct (circles/white bars). For GASPEL-immunized mice, serum from each mouse was tested against all T-antigen or AP1 proteins as indicated. For mice immunized with each individual construct, serum was tested against their cognate T-antigen or AP1 protein only. The serum was tested at a 1:100 dilution. Symbols indicate data from individual mice; bar indicates the group median. $*P \leq 0.05$, $***P \leq 0.001$, $****P \leq 0.0001$, unpaired *t*-test. The horizontal dashed line indicates the LOD. AP, ancillary pilin; CFU, colony-forming unit; GAS, group A Streptococcus; GASPEL, GAS pili expressed on *L. lactis*; LOD, limit of detection.

observed between serum IgA and both nasal and BAL IgA with M89_AP1 (Supplementary figure 2). While there were no significant correlations between antibody levels and carriage rates, several significant inverse correlations were observed between antibodies (BAL AP1 IgA, BAL T11 IgA, serum AP1 IgG) and average CFU counts (Supplementary figure 2).

GASPEL antibodies cross-react with nonvaccine T-antigens

To analyze whether GASPEL immunization could provide broad coverage to the vaccine and nonvaccine strains, pooled prechallenge sera and mucosal samples were tested against a panel of 21 recombinant T-antigens (Figure 6). Antigen-specific serum IgG responses toward T-antigens in the GASPEL vaccine were all above the LOD except for T2, which was not detected (Figure 6a). Cross-reactivity was seen toward T18.1 and T28.2, as might be expected from sequence phylogeny (Figure 6c). Cross-reactivity to T3.1, T3.2 and T13 was also observed at levels comparable to detected vaccine antigens. Cross-reactivity above the LOD was also observed to T5, T7, T8 and T9 when compared with the control group (Figure 6a).

In the absence of sufficient BAL and nasal samples to test these against the panel of 21 T-antigens, pooled fecal samples were used as a surrogate for the mucosal response. Similar to the serum IgG response, fecal IgA responses to all T antigens present in the GASPEL vaccine were observed, except for T2. Furthermore, cross-reactivity was observed to all other T-antigens except for T8 and T9 (Figure 6b).

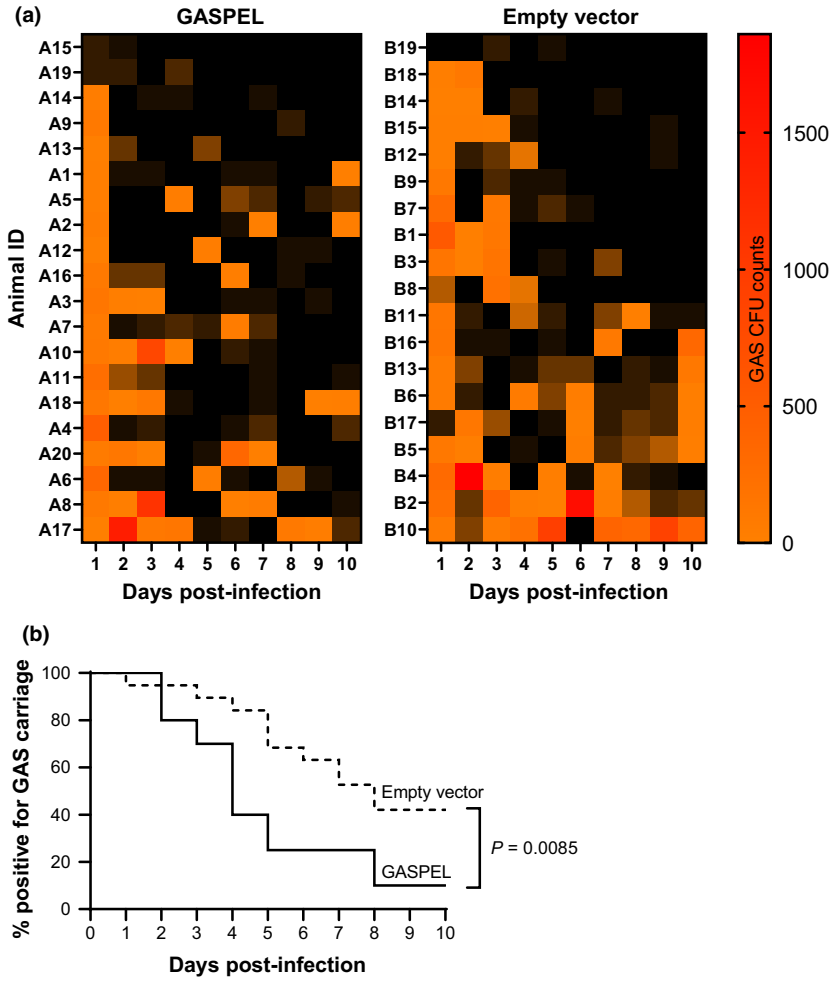

Figure 4. Intranasal immunization of mice with GASPEL protects against GAS M89T11 nasopharyngeal challenge. FVB/n mice were immunized intranasally with *Lactococcus lactis* empty vector (n = 19) or GASPEL (n = 20). Mice were subsequently challenged intranasally with 10^8 CFU GAS M89T11. Mice were monitored for nasal shedding of bacteria daily. **(a)** Total CFU counts recovered from the nose of each mouse per day. Each row represents counts from an individual mouse. There were no significant differences in CFU counts between empty vector and GASPEL-immunized mice by two-way ANOVA. **(b)** The percentage of mice positive for GAS carriage excluding reinfection. Loss of carriage is determined as returning no bacteria growth for 2 consecutive days. Data are combined from two independent experiments. Statistical analysis was performed using the log-rank test. CFU, colony-forming unit; GAS, group A Streptococcus; GASPEL, GAS pili expressed on *L. lactis*.

DISCUSSION

Previous investigation using *L. lactis* expressing the GAS pilus from M89T11 demonstrated that mucosal vaccination could provide protective efficacy against nasopharyngeal challenge in mice.[21] In the present study, we further explored the potential of this pilus-based vaccine by extending vaccine coverage using combined *L. lactis* constructs that express pili from multiple FCT regions. We show that a mix of seven *L. lactis* constructs in our GASPEL vaccine could still generate pilus-specific serum antibodies despite the GASPEL vaccine containing one-seventh the dose of each respective construct, suggesting that a lower dose may function as effectively for some constructs, or that sufficient similarity between pili in GASPEL boosts responses to individual pilus proteins. There were a few exceptions (T18.2, T11, T4, M2_AP1) where IgG and IgA levels were comparatively lower in GASPEL-immunized mice, and hence altering the ratio or dose of these constructs in the vaccine may be beneficial. It was noted that the antibody responses between studies with GASPEL immunization could vary for some antigens, for example, a decrease in T2 and an increase in T4 response was observed in challenge study samples *versus* the immunization study samples. This may be explained by batch variation of live vaccine

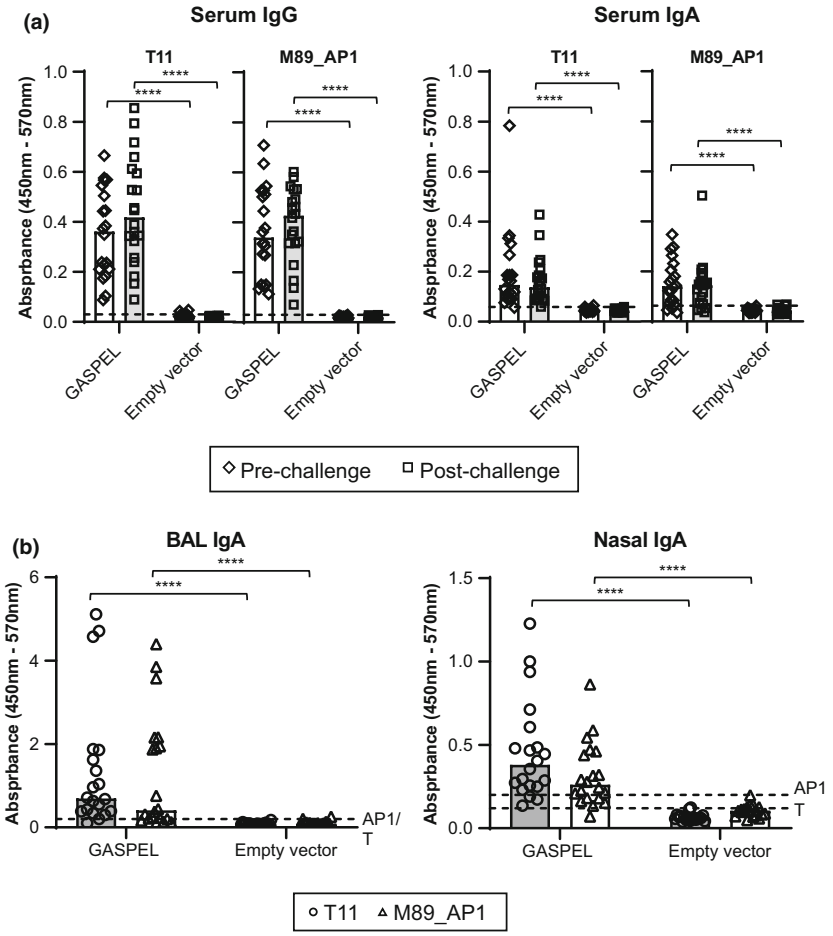

Figure 5. Postchallenge analysis of systemic and mucosal immune responses. FVB/n mice were immunized intranasally with *Lactococcus lactis* empty vector ($n = 19$) or GASPEL ($n = 20$). Mice were subsequently challenged intranasally with 10^8 CFU GAS M89T11. **(a)** IgG and IgA responses in the sera were measured by ELISA at a 1:100 dilution against T11 or M89_AP1 as indicated. Serum was collected before the challenge (7 days after the final immunization; diamonds/white bars) and after the challenge (on day 10 after the challenge; squares/gray bars). **(b)** IgA responses in the BAL and nasal samples collected 10 days after the challenge were measured against T11 (circles/gray bars) and M89_AP1 (triangles/white bars) by ELISA. Bars represent the median values. Data are combined from two independent experiments. The horizontal dashed line indicates the LOD. ****$P \leq 0.0001$, Mann–Whitney *U*-test. AP, ancillary pilin; BAL, bronchoalveolar lavage; CFU, colony-forming unit; GAS, group A Streptococcus; GASPEL, GAS pili expressed on *L. lactis*; Ig, immunoglobulin; LOD, limit of detection.

components and would need to be addressed in future studies.

Interestingly, we noted an increase in the T28.1 response when PilM28T28.1 was combined in the GASPEL vaccine, suggesting the presence of cross-reactive epitopes to this T-antigen. Gaining a better understanding of cross-protective epitopes would be beneficial to ensure the best combination of pili for broad strain coverage. We expected a higher immune response to T-antigens as these subunits are multimerized to form the pilus backbone, while there is only a single AP1 on the pilus tip. This was observed in the majority of mice, with AP1 responses being close to or below the LOD. Importantly, GASPEL-immunized mice were able to reduce the carriage rate of GAS in the nasopharynx after the intranasal challenge compared with the empty vector control, with comparable differences to those reported previously for immunization with PilM89T11 alone.[21] However, apparent reinfection was also observed in some animals, possibly because of them being housed in groups of up to five per cage. This suggests that protection was not sufficient in these closed-housing conditions.

Given that GAS is a respiratory pathogen, a robust mucosal immune response in the respiratory tract is likely to be beneficial in preventing early infection and disrupting onward transmission. We explored IgA responses in BAL and nasal secretions after GASPEL vaccination and GAS challenge. All were elevated compared with empty vector–immunized mice, but there was clear heterogeneity in

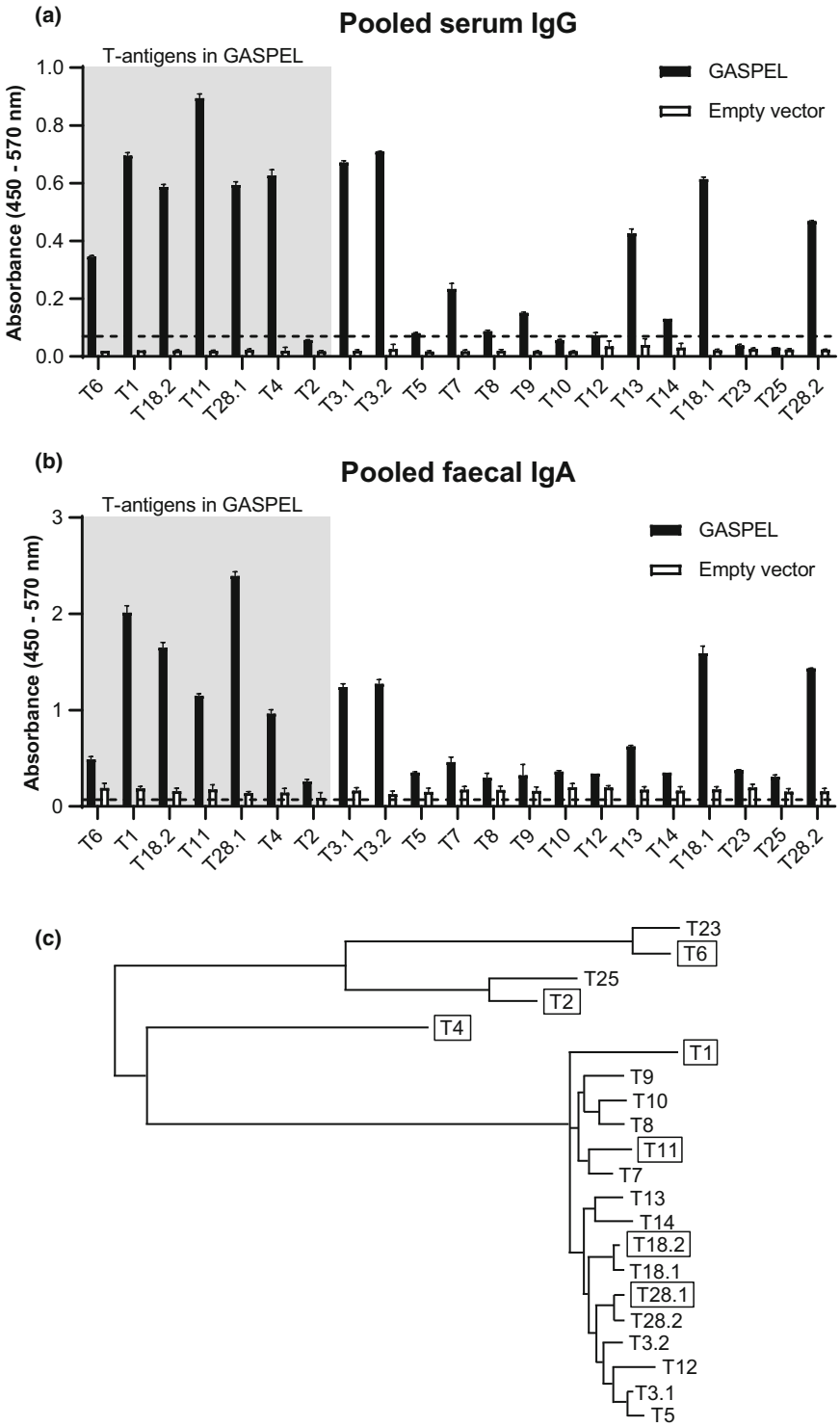

Figure 6. Cross-reactivity of serum and fecal samples to recombinant T-antigens from various GAS strains. GASPEL-immunized (black bars) or *Lactococcus lactis* empty vector–immunized (white bars) postimmunization, prechallenge samples were pooled within each group to provide enough sample volume to test against a panel of the major 21 T-antigens by ELISA. **(a)** Serum IgG was tested at a 1:100 dilution. **(b)** Fecal IgA was tested at 100 mg mL^{-1}. **(c)** Phylogenetic tree generated from amino acid sequences of 21 full-length T-antigens on GenomeNet. T-antigens present in the GASPEL vaccine are outlined. Data are presented as the mean with standard deviation of two technical replicates. The horizontal dashed line indicates the LOD. GAS, group A Streptococcus; GASPEL, GAS pili expressed on *L. lactis*; Ig, immunoglobulin; LOD, limit of detection.

response between individual animals. High BAL anti-T11 or anti-M89_AP1 IgA absorbances correlated with reduced CFU shed during the 10-day monitoring period. As preinfection anti-M89_AP1 serum IgG and IgA correlated strongly with BAL IgA, measuring these responses may prove to be valuable predictors of protective efficacy.

While we have not confirmed whether these antibodies function by opsonophagocytosis or neutralization, previous studies suggest that both mechanisms are likely to play a role. Antibodies against AP1 proteins and some T-antigens have been shown to block adhesion to human cell lines,[10,21] while antibodies against the T-antigen have been shown to be opsonophagocytotic.[21,23] Further antibody analysis will be required to confirm this for GASPEL.

Our primary aim for GASPEL was to expand strain coverage. Sequence analysis of *tee* genes has previously revealed 21 major *tee* types with high stability over time and geographical location.[19] We observed cross-reactive IgG antibodies to the 10 out of 14 T-antigens not included in GASPEL. IgG absorbances were at similar levels to vaccine T-antigens for T3.1, T3.2, T13, T18.1 and T28.2. Based on phylogenetic analysis, cross-reactivity to T18.1 and T28.2 were expected as these T-antigens share greater than 95% sequence identity to T18.2 and T28.1, respectively. Cross-reactive epitopes have also been previously described between T18, T3 and T13.[24] For the remainder of the T-antigens, serum IgG level was low and unlikely to provide sufficient protection against these strains. Additional *L. lactis* pilus constructs may be required to ensure a broadly efficacious vaccine. The diversity of AP1 has been less extensively studied; however, based on the same phylogenetic clustering of *ap1* genes and *tee* genes observed,[16] we would expect a similar extent of cross-reactivity. Performing an experimental investigation of cross-reactive epitopes would be ideal for reducing the number of constructs required to achieve global coverage.

The use of *L. lactis* as a vector for mucosal vaccine delivery has been studied by others (reviewed in[25]). *L. lactis* offers several advantages, including its high safety profile as a food-grade bacterium, inexpensive and simple production, pain-free oral or nasal delivery and inherent adjuvancy.[25] However, its use is not without challenges. Genetic modifications employed to express vaccine antigens will require greater regulatory approval, and vector-specific immune responses could prove to be a double-edged sword. However, there is a strong rationale for generating mucosal immunity against respiratory pathogens such as GAS. Preventing early colonization of GAS at the site of infection would be highly beneficial to avoid downstream infection and disrupt transmission.

We show here that intranasal delivery of GASPEL can generate both mucosal and systemic immune responses; however, the response was low for many of the constructs, particularly against the AP1 antigen. While further investigation is clearly needed to optimize the dose and explore the extent of GASPEL coverage and efficacy, we show that including additional *L. lactis* pilus constructs can expand strain coverage while maintaining protective efficacy against respiratory challenge.

METHODS

Animal ethics

All animal experiments were performed in the Vernon Jansen Unit (University of Auckland, New Zealand) in accordance with relevant guidelines and regulations approved by the University of Auckland Animal Ethics Committee (reference number 8448).

Bacteria and cell culture conditions

GAS strains for infection were cultured in brain heart infusion medium (Becton Dickinson, Sparks, USA) at 37°C under static conditions. *L. lactis* MG1363 harboring expression plasmid for GAS pilus operon was cultured in M17 medium (Becton Dickinson) supplemented with 0.5% glucose (Sigma-Aldrich, St Louis, USA) and 200 μg mL^{-1} kanamycin (Sigma-Aldrich). BL21(DE3)pLysS *Escherichia coli* (Novagen, Darmstadt, Germany) harboring expression vector for pilus recombinant proteins was cultured in Luria-Bertani broth at 37°C and 200 rpm with 30 μg mL^{-1} chloramphenicol (Sigma-Aldrich) and 50 μg mL^{-1} ampicillin (Sigma-Aldrich).

Cloning and protein expression

The complete pilus operon was amplified from genomic DNA by PCR, cloned into the pLZ12-Km2 P23R vector and electroporated into *L. lactis* MG1363 as described previously.[21] To generate recombinant proteins of individual pilus proteins, the regions between the predicted N-terminal signal peptide sequence and the C-terminal sortase signal were amplified by PCR, cloned into the expression vector pET32a3c or pROEXhtb and transformed into *E. coli* BL21(DE3)pLysS. Proteins were expressed and purified as previously described.[23] GAS strains and primers used in this study are listed in Supplementary table 1.

Mouse immunization

Lactococcus lactis constructs were grown to mid-exponential phase, resuspended in phosphate-buffered saline (PBS)/10% glycerol (Sigma-Aldrich) and then frozen at −80°C in aliquots. When required, aliquots were thawed at room temperature, washed and resuspended in PBS. Bacteria were enumerated by plating to confirm the inoculation dose. Five- to seven-week-old male or female FVB/n mice ($n = 5$) were immunized under isoflurane anesthesia intranasally with 10^8 CFU of live *L. lactis* in a volume of 10 μL. This was administered on 3 consecutive days, 2 weeks apart, for a total of 12 doses (days 0, 1, 2, 14, 15, 16, 28, 29, 30, 42,

43 and 44). Preimmune samples were collected on day 0, and immune samples were collected on day 56. BALs were performed with 1 mL PBS. Nasal washes were performed with 0.5 mL PBS, and fecal samples were resuspended at 100 mg mL^{-1} in PBS containing 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, MO, USA) as previously described.[26]

ELISA

MaxiSorp plates (Nunc, Denmark) were coated with 1 μg mL^{-1} recombinant protein in PBS overnight at 4°C prior to incubation with biological samples. Detection was performed using goat anti-mouse IgG-HRP (Thermo Fisher Scientific, Waltham, USA) or goat anti-mouse IgA-HRP (Abcam, Hangzhou, China) and 3,3,5,5-tetramethylbenzidine (Thermo Fisher Scientific). Absorbance at 450 and 570 nm was determined on an EnSpire multilabel plate reader (Perkin Elmer, Waltham, USA). Absorbances were measured from a 1:100 (serum) or undiluted (BAL, nasal and fecal) sample. Limits of detection (LODs) were calculated as the average absorbance +3 × the standard deviation of negative control (buffer-only) wells.

Flow cytometry

Each *L. lactis* strain in this study was analyzed for pilus expression as previously described[22] using T-antigen–specific rabbit polyclonal antibodies (10 μg mL^{-1}) and anti-rabbit IgG-Alexa Fluor 647 (Abcam). T-antigen–specific rabbit antisera were generated by immunizing New Zealand White rabbits with recombinant T-antigen emulsified in incomplete Freund's adjuvant (Sigma-Aldrich) as previously described.[21] Polyclonal antibodies were affinity purified from antisera and validated by ELISA. Flow cytometry analysis was performed on an LSRII Flow Cytometer and analyzed using FlowJo v10 software.

Murine nasopharyngeal challenge

Five- to six-week-old male FVB/n mice (n = 19 or 20) were immunized under isoflurane anesthesia intranasally with 10^8 CFU live GASPEL constructs or live *L. lactis* with empty vector in a 10-μL volume on 3 consecutive days. Additional boosters were given 2 weeks apart for a total of 12 doses. Ten days after the final immunization, mice were given a nasopharyngeal challenge as previously described.[21] Mice were infected with 10^8 CFU/5 μL/mouse of exponential phase GAS M89T11 and monitored daily for nasal shedding. Monitoring was performed by gently pressing the nares of each mouse onto the surface of a brain heart infusion/kanamycin agar plate ten times, spreading the bacteria on the agar surface and culturing the plates overnight at 37°C. Mice were scored as having cleared the infection if no colonies were cultured for at least 2 consecutive days.

Phylogenetic analysis of T-antigens

The 21 T-antigen amino acid sequences were downloaded from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). Alignment and phylogenetic reconstructions were performed using the function "build" of ETE3 version 3.1.1[27] as implemented on GenomeNet (https://www.genome.jp/tools/ete/). A maximum likelihood phylogenetic tree was inferred using PhyML version 20160115 run with model and parameters: pinv e -f m --alpha e --bootstrap −2 -o tlr --nclasses 4.[28] Branch supports were the χ^2-based parametric values returned by the approximate likelihood ratio test.

Statistical analysis

ELISA data are presented as median and individual biological replicates. Data were tested for normal distribution, and the differences between the two groups were evaluated by an unpaired t-test. A paired t-test was used when comparing the pre- and postinfection samples from the same animal. GAS carriage rates were evaluated using the log-rank test. Analyses were performed using GraphPad Prism version 9.0.2 (GraphPad Software, LLC).

DATA AVAILABILITY

The data generated during and/or analyzed during this study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

Adrina Hema J-Khemlani: Data curation; formal analysis; investigation; methodology; validation; visualization; writing – original draft; writing – review and editing. **Devaki Pilapitiya:** Formal analysis; investigation; writing – review and editing. **Catherine Jia-Yun Tsai:** Formal analysis; investigation; writing – review and editing. **Thomas Proft:** Conceptualization; funding acquisition; methodology; project administration; supervision; visualization; writing – review and editing. **Jacelyn Mei San Loh:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; supervision; visualization; writing – original draft; writing – review and editing.

CONFLICT OF INTEREST

The authors have no relevant competing interests to disclose.

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SUPPORTING INFORMATION

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