Evaluation of novel Streptococcus pyogenes vaccine candidates incorporating multiple conserved sequences from the C-repeat region of the M-protein

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Abbreviations. CD, Circular Dichroism, CRR, C repeat region

ABSTRACT

A major challenge for *Streptococcus pyogenes* vaccine development is the identification of epitopes that confer protection from infection by multiple *S. pyogenes* M-types. Here we have identified and characterised the distribution of common variant sequences from individual repeat units of the C-repeat region (CRR) of M-proteins representing 77 different M-types. Three polyvalent fusion vaccine candidates (SV1, SV2 and SV3) incorporating the most common variants were subsequently expressed and purified, and demonstrated to be alpha-helical by Circular Dichroism (CD), a secondary conformational characteristic of the CRR in the M-protein. Antibodies raised against each of these constructs recognise M-proteins that vary in their CRR, and bind to the surface of multiple *S. pyogenes* isolates. Antibodies raised against SV1, containing five variant sequences, also kill heterologous *S. pyogenes* isolates in *in vitro* bactericidal assays. Further structural characterisation of this construct demonstrated the conformation of SV1 was stable at different pHs, and thermal unfolding of SV1 a reversible process. Our findings demonstrate that linkage of multiple variant sequences into a single recombinant construct overcomes the need to embed the variant sequences in foreign helix promoting flanking sequences for conformational stability, and demonstrates the viability of the polyvalent candidates as global *S. pyogenes* vaccine candidates.

INTRODUCTION

Streptococcus pyogenes (group A Streptococcus, GAS) is a human pathogen that colonises the throat or skin, resulting in diseases that vary in presentation and clinical severity. *S. pyogenes* infection is the leading cause of bacterial pharyngitis, and is also responsible for cellulitis, kidney disease (post-streptococcal glomerulonephritis (PSGN), rheumatic fever (RF) and rheumatic heart disease (RHD). Further *S. pyogenes* is also the causative agent of severe invasive diseases including necrotising fasciitis and toxic shock syndrome (1). *S. pyogenes* associated diseases are estimated to kill half a million people each year (2). The majority of these deaths are attributable to RHD, and mainly occur in developing nations. As RF and RHD are autoimmune sequelae of *S. pyogenes* infection in which immune molecules and cells raised against epitopes of bacterial proteins attack host protein and tissues (3), antibiotic treatment is an unviable option for the treatment of primary episodes of RF/RHD. A preventive vaccine is therefore the ideal approach for prevention of RF/RHD and other *S. pyogenes* associated diseases.

The M-protein, the major virulence factor of *S. pyogenes* is the favoured target of vaccine development (4,5). This protein is the most abundant protein on the bacterial surface and contributes to immune evasions, adherence and intracellular invasion (6). Structurally, the protein forms a coiled-coil dimer that extends from the bacterial surface to beyond the peptidoglycan layer (7,8). The hypervariable amino-terminus is followed by A, B and C-repeat regions that increase in amino acid conservation between variant M-proteins. Diversity in the hypervariable region is used to categorise *S. pyogenes* into M-types based on discriminatory serological responses to this region, or emm-types, based on nucleotide sequence variation in the corresponding region of the *emm* gene (9). To date more than 150 emm-types have been described. *S. pyogenes* expressing M1, M5 and M6 are classically associated RHD, M49 and M57 with PSGN, and M1, M3 and M18 with severe invasive disease (1). Associations between M-type and disease are not as strong in many populations where streptococcal infection is endemic (10-12). Rather, large numbers of M-types circulate (13,14), none of which dominates disease associations.

The presence of epitopes within the central region of the M-protein responsible for immunologically cross-reactive immune responses which could contribute to RHD (15) preclude the use of full length M-proteins as a vaccine candidate. While the hyper-variable amino-terminal region induces antibodies that are bactericidal, these antibodies are also M-type specific, and only effective in killing homologues of *S. pyogenes* isolates. Nevertheless, the utility of incorporating multiple N-terminal epitopes from multiple M-proteins into a single formulation has been evaluated (5,16,17). An alternative approach is to target of the less immunogenic, but more conserved C-Repeat Region (CRR) of the M-protein (4,18-22), which may provide protection against multiple M-types. Epitope mapping of the CRR has previously identified a short amino acid sequence, that maintains helicity when flanked by yeast GCN4 sequences. When conjugated to Diphtheria Toxoid (DT) or other carrier molecules, this chimeric peptide induces murine antibody responses that kill *S. pyogenes* in *in vitro* assays and protect mice from intraperitoneal and intranasal challenge (4,20,23-26). This full chimeric 29mer peptide is called J14, and the internal sequence homologous to M-protein sequence is known as $J14_i$.

The $J14_i$ target sequence within the CRR is only found in the third CRR block (CRRB3) of Class II M-proteins, the CRRB closest to the bacterial surface. A variant sequence ($J14.1_i$) is present in Class I M-proteins (27). Additional variants of $J14_i$ are found in CRRB1 and CRRB2, which are further from the bacterial surface (11,28-32). While it is well recognized that a single CRRB is sufficient for the biological role of the M-protein (33), it is not clear whether antibodies to the CRRB3 are adequate to protect against infection with *S. pyogenes* strains in all biological conditions. For instance highly mucoid strains may not be able to project the surface proximal CRRB sufficiently far enough beyond the capsule for recognition by antibody. Furthermore, vaccine pressures may select for replacement or mutation of this CRRB resulting in variants that do not react with J14 antibodies (34-36).

Here we have evaluated a panel of polyvalent recombinant vaccine constructs incorporating common $J14_i$-variants found in CRRB1, CRRB2 and CRRB3 from multiple M-types. By linking the variants in a manner that maintains heptad periodicity across the entire protein, we have eliminated the need for heterologous GCN4 sequence. Antibodies raised against these novel constructs bind to a wide range of M-proteins, and

the surface of heterologous *S. pyogenes* strains. Further we also show that antibodies raised against SV1, a construct containing five common $J14_i$-variant sequences kill *S. pyogenes* in *in vitro* bactericidal assays.

EXPERIMENTAL PROCEDURES

Bacterial strains. *S. pyogenes* isolates used in this study are listed in Table 1. *S. pyogenes* M1, 88-30 and ES61 possess the *emm1*, *emm97* and *emm65* genes. The corresponding M-proteins expressed by these genes differ in amino acid sequence in their CRRs. Unless otherwise specified *S. pyogenes* were grown in Todd Hewitt Broth (THB), and on Todd Hewitt agar supplemented with 2% horse blood.

Peptide synthesis and protein expression. Peptides used in this study are listed in Table 2. All peptides were synthesised using Fmoc chemistry, and purified to greater than 90% using HPLC prior to use in ELISA. The J14-variants contain $J14_i$-variant amino acid sequence flanked by GCN4 amino acid sequence. p145 peptides contain $J14_i$-variant sequence and an additional six amino sequences native to the M-protein, with no GCN4 sequence. Reverse translation of the amino acid sequence of SV1, SV2 and SV3, taking into account codon bias in *Escherichia coli,* was used to design nucleic acid sequences encoding each of these vaccine candidates. These nucleotide sequences were synthesised by DNA2.0 (USA), cloned into the expression vector pET41 (Novagen, Inc), and subsequently transformed into *E. coli* C43 (Lucigen Inc, USA). All recombinant proteins were expressed by the addition of 1mM IPTG to growth media, and purified through nickel affinity chromatography.

Immunisation. Prior to immunisation, J14 was conjugated to DT through a C-terminal cysteine using 6'-maleimido-caproyl *n*-hydroxy succinimide (MCS). Recombinant vaccine constructs were not conjugated to DT as previous studies using recombinant proteins that include J14 have shown they are immunogenic in the absence of additional carrier molecules (37). Female B10.Br mice were immunised subcutaneously at the tail base with 25 μg of J14-DT or recombinant protein emulsified in Complete Freunds Adjuvant (CFA). Control mice were immunised with phosphate buffered saline (PBS) and CFA. Mice received booster injections consisting of 5 μg of antigen dissolved in PBS at days 21 and 28 and blood was collected at day 35 via tail bleed.

ELISA. ELISAs were performed as previously described (19,25). Ninety-six well ELISA plates were coated with peptide or protein (5.0 $\mu g.ml^{-1}$) dissolved in carbonate coating buffer and blocked with 5% skim milk/PBS/Tween20. Primary sera were diluted 1:2 down the plates, which were then incubated at 37ºC for 90 min. The plates were washed in PBS-Tween, and secondary goat anti-mouse IgG conjugated to Horse Radish Peroxidase added. After a further 90 min incubation, the plates were washed, incubated with SigmaFAST OPD tablets (Sigma, USA) and absorbance (450_{nm}) measured using a Benchmark (BioRad, USA) microplate reader. The antibody titre endpoint was determined as the lowest dilution in which the absorbance was three standard deviations higher than absorbance observed in sera from PBS immunised mice. Titres for each group are expressed as the arithmetic mean ± standard error. Statistical comparisons between groups were performed using the Kruskal-Wallis test with Dunns post-test used to compare individual groups.

To compare binding of J14-DT sera to the $J14_i$-variant sequences, sera diluted 1:1,000 was added to the wells of ELISA plates containing p145-variant peptides and ELISA completed as described above. The mean absorbance associated with each peptide was determined after subtraction of mean absorbance measured for PBS-control sera. Competition ELISAs were also performed to assess the relative affinity of J14 antisera to peptides containing $J14_i$-variants. For these experiments ELISA plates were blocked with 5% skim milk/PBS Tween as described above. One hundred microlitres of antisera diluted 1:5,000 was then added to all wells of the plate. Free peptide ($10\mu g.ml^{-1}$) was added to the first row, and subsequently diluted 1:2 down the plate to a final concentration of $0.02\mu g.ml^{-1}$. After incubation for 30 minutes, the peptide/antibody mixtures were transferred to the wells of a second set of ELISA plates that had been pre-coated with J14. The competition ELISA was then completed as per the standard protocol and percent inhibition calculated by comparing the optical density of wells containing antisera pre-incubated with peptide to wells on the same plate containing antisera that had been incubated in the absence of peptide. A non-specific peptide and normal mouse sera were used as controls in these experiments.

Immunofluorescence microscopy and FACS. Immunofluorescence microscopy (IFA) and FACS were used to qualitatively assess binding of antibodies to the surface of *S. pyogenes* (38). For IFA *S. pyogenes* was

grown overnight, fixed to a polylysine slide (Biolabs Scientific, New Zealand) with 3% paraformaldehyde and blocked with 5% skim milk/PBS/Tween. To prevent non-specific Fc-IgG binding by streptococcal surface proteins slides were also blocked with non-specific human polyclonal IgG (AbD Serotec, UK). After the secondary blocking step, the slides were incubated with antisera diluted 1:200, washed, and incubated with secondary goat anti-mouse-IgG-FITC labelled antibody (Invitrogen, USA) diluted 1:200 in 0.5% skim milk/PBS/Tween. Prolong Gold (Life Technologies) and a coverslip was added to each sample, and slides viewed under a Leica Confocal microscope at a wavelength of 500-650nm.

FACS was also employed to investigate the binding of antisera to the surface of logarithmically growing *S. pyogenes*. Bacteria were first grown to mid-log phase, harvested, and washed in PBS. Non-specific binding of antibodies to the surface of the bacteria was inhibited by incubations with human IgG diluted 1:150 in 0.3% (w/v) BSA/PBS/Tween. Cells were then incubated with primary antibody diluted 1:50 in 0.3% (w/v) BSA/PBS/Tween. After primary incubation, the bacteria were washed and incubated with secondary antibody (goat anti-mouse FITC conjugated IgG). Subsequently, the cells were washed, and fixed with 4% paraformaldehyde/PBS and examined in a FACSort flow cytometer (Becton Dickson, USA) with Cellquest software. Unpaired t-tests were used to compare histograms of vaccine serum with PBS serum with a confidence interval of 95%.

Bactericidal assays. Bactericidal assays were performed as a measure of the functionality of anti-vaccine antibodies (38,39). Overnight cultures of *S. pyogenes* were diluted 10^{-5} in PBS, and mixed with 50µl of heat inactivated murine sera containing antibodies of interest and 200µl of fresh human blood (a source of complement and neutrophils). After incubation for 3 hours duplicate 50µl samples was mixed with 20 ml of molten agar (less than 50°C) containing 2% defibrinated horse blood, and added to petri-dish. After overnight incubation the colony forming units (CFU) were determined for each plate. Bactericidal activity is reported as the percent killing of *S. pyogenes* in samples incubated in the presence of immune sera compared to bacteria incubated in the presence of heat inactivated normal mouse sera. All human blood used in bactericidal assays were pre-screened for the absence of streptococcal antibodies.

Structural characterisation of proteins. The secondary structure of proteins and peptides was assessed by Circular Dichroism (CD) spectroscopy (40). Samples were dissolved in 10 mM sodium phosphate buffer (pH 7.0) with 20 mM NaCl and varying concentrations of 2,2,2-trifluoroethanol (TFE) to a final concentration of 0.03 $mg.ml^{-1}$. Spectra were acquired from 190 to 260 nm as the average of 3 repeats at 20°C with a spectral bandwidth of 1 nm and a scanning speed of 10 nm/min using a JASCO J-715 CD spectrometer equipped with a Peltier temperature control. Acquired data were corrected for buffer baseline, normalised to reported mean residue ellipticity [Θ] and smoothed using the program ACDP. The secondary structure content for each sample was subsequently predicted with spectrum deconvolution using a linear combination of three Fasman prototype spectra (α-helix, β-strand and random coil) as implemented in the program ACDP (41).

The conformational stability of SV1 at varying pH was assessed by dissolving the protein in 20 mM NaCl, 10% TFE and 10 mM pH buffer; sodium acetate was used to buffer at pH 4 and pH 9, sodium phosphate from pH 5 to 8, and glycine at pH 10. Samples were prepared 2 hrs before measurement and assessed at 20°C. To investigate the thermal stability, samples were dissolved in 20 mM NaCl, 10% TFE and 10 mM sodium phosphate (pH 7.0), and the CD at 222 nm was monitored while changing the temperature at a rate of 1 K min^{-1}. The temperature was increased from 20°C to 80°C and then immediately decreased at the same rate to 20°C. Full spectra were acquired as described above before starting the heating ramp (20°C), at 80°C, and again after the cooling ramp (20°C).

RESULTS

Distribution of J14-like sequences in multiple M-types. To identify common variants of $J14_i$, the amino acid sequences in the C-repeat region of 164 M-proteins representing 77 different M-types and five non-typable M-types was examined. One hundred and thirty three of these M-proteins were retrieved from the Genbank database and the remaining sequences obtained by nucleotide sequencing and translation of *emm*-genes from 31 *S. pyogenes* isolates in the laboratory (9). The emm-types of these proteins were previously reported (11), or determined through comparison to the emm-protein database at the Centre for Disease Control (http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm). $J14_i$ was present in CRRB3 of 52% of proteins (Table 3). The remaining M-proteins all possessed $J14.1_i$ in CRRB3. While J14 was not present in

the CRRB1 or CRRB2, J14.1$_i$ was found in both repeat blocks. Together, J14$_i$ and J14.1$_i$ accounted for 53% of all J14$_i$-variants present in all CRRBs. Another sixteen J14$_i$-variant sequences were distributed throughout CRRB1 and CRRB2 blocks in different M-proteins. However five J14$_i$-variants (J14$_i$, J14.1$_i$, J14.2$_i$, J14.4$_i$ and J14.29$_i$) accounted for greater than 90% of all J14$_i$-variants.

As the presence of multiple proteins of the same M-type may skew the percentage distribution of individual J14$_i$-variants, the distribution of J14$_i$-variants using individual representatives of each M-type was calculated. Using this analysis the same five J14i-variants were still the most abundant, and still accounted for greater than 90% of all variants. Of the thirty M-types represented more than once in the complete dataset, sixteen displayed intra-M-type variation in the CRR. In seven instances this variation occurred through apparent deletion or duplication of CRRBs, resulting in differing numbers of repeat units. In three other cases, proteins of the same M-type possessed the same number of CRRBs, but contained variant J14$_i$ sequences. Five M-types possessed CRRBs that differed both in the number of CRR blocks, and J14$_i$-types.

The affinity of J14-DT antibodies is greater for J14 than J14$_i$-variants. Anti J14-DT antibodies bind to p145, a 20mer helical peptide that includes J14$_i$ and an additional six amino acids from the M-protein (20,25). To examine the affinity of J14 antisera for J14$_i$-variant sequences, a panel of twelve p145-like peptides were used in ELISA with J14-DT antisera diluted 1:1,000 (Figure 1A). When compared to mean absorbance observed using J14, the mean absorbance for all p145-variant peptides, including p145, was significantly lower. In turn, the mean absorbance observed with p145 was at least two-fold higher than that observed with other p145-variants. While J14-DT antisera bound to p145, p145.2, p145.3 and p145.5, the absorbance observed with another eight peptides was negligibly higher than negative control PBS sera, suggesting that anti-J14$_i$ antibodies only recognise a subset of J14$_i$-variants.

As the attachment of the p145-variant peptides to ELISA plates may occur with different efficiencies, or alter the conformation of peptides and reduced recognition by J14 antibodies, competition ELISA using free p145 variants and bound J14 were next conducted. As expected the pre-incubation of antisera with increasing

concentrations of free J14 resulted in a reduction in binding bound J14, with fifty percent inhibition occurring at 0.1 $\mu g.ml^{-1}$ free J14. Preincubation of sera with p145, p145.2 and p145.3 also reduced binding to bound J14. No decrease in absorbance was observed with an unrelated control peptide or other p145 variant peptides (Figure 1B).

Novel vaccine candidates are immunogenic. Based on the frequency of $J14_i$-variants, and the limited capacity of J14 antibodies to bind to $J14_i$-variant sequences, three novel *S. pyogenes* vaccine candidates were designed, expressed and purified (Figure 2). SV1 includes the five most common $J14_i$-variants ($J14_i$, $J14.1_i$, $J14.2_i$, $J14.4_i$ and $J14.29_i$). As previous work by Dale *et al*. (16) demonstrated that peptide sequences present on the terminal ends of polyvalent vaccine constructs are less immunogenic than epitopes embedded within the constructs, copies of $J14_i$ and $J14.1_i$ were incorporated in both the central and distal positions in SV1. SV1 also lacks the GCN4 flanking regions present in J14. SV2 is comprised of single copies of $J14_i$ and $J14_i.1$ flanked by GCN4 amino acid sequence. SV3 includes multiple copies of $J14_i$ and $J14_i.1$, but no GCN4 flanking sequence. In all instances the amino acid sequences were incorporated into the fusion constructs in a manner such that the heptad periodicity required to maintain the propensity for coiled coil formation, as seen in the M-protein, is maintained across the full length of the recombinant constructs.

To examine the immunogenicity of vaccine constructs, B10.Br mice were subcutaneously immunised with each of the recombinant proteins or J14-DT. All the antigens were immunogenic, with mean titres against the immunising antigen ranging from 153,600 ± 21,000 for the SV1 group to 1,280,000 ± 174900 for the J14-DT immunised group (Figure 3). When ELISA were conducted against J14, the mean titres observed for the J14-DT and SV2 groups were at least ten-fold higher than mean titres observed for SV1 and SV3 groups, suggesting that some of the immune responses against J14 targets GCN4 amino acod sequence. As the true target of antibodies raised against the vaccine candidates is the M-protein, ELISA were next conducted against the M1 protein, an M-protein possessing two $J14.2_i$ repeat sequences, and J14. In contrast to the earlier findings the greatest titres were observed with the SV1 (273,100 ± 34,000), and lowest titres observed for the J14-DT (141,100 ± 37,300). However there was no statistical difference between the mean titres of any group. ELISAs and Western blot performed against two other purified M-proteins, M65 and

Comment [d1]: Did you not do elisas again the individual J14 variant peptides (I vaguely remember Michelle doing this). Think it would be good to include especially to confirm that abs to all variants, as the strains tested in the binding/functional assays only contain three of the 5 SV1 variants, 2 of which are found in the other recombinant proteins (J14 and J14.1).

Comment [d2]: MG-I agree, especially as you use ELISA/inhibition assay against these range of peptides to show that J14 abs do/don't cross reaction, so to compare apples with apples, would also be good to show they DO with all or SV1 contruct in particular…

Comment [d3]: Add this info

M124, with differing CRRs also demonstrated the affinity of SV1, SV2 and SV3 antibodies for heterologous M-proteins was as great as that of J14-DT antisera (data not shown). Isotyping profiles of sera raised against all antigens were also similar (IgG1>IgG2b>IgG2a>IgG3) (Figure 4). Together these results demonstrate that recombinant antigens lacking GCN4 sequences induced significant antibody responses that recognise heterologous M-proteins.

Vaccine antibodies bind to the surface of *S. pyogenes*. To examine the binding of antibody to the bacterial surface, IFA and FACS were conducted with multiple *S. pyogenes* isolates possessing different CRRs. The fluorescence observed through IFA demonstrated that J14-DT, SV1 and SV3 antibodies bound to the surface of *S. pyogenes* M1, ES61 and 88-30 with relatively equal intensity (Figure 5). However, the fluorescence observed with SV2-antisera was qualitatively weaker than that observed with other sera. No fluorescence was observed using PBS control sera. When assessed by FACS using bacteria grown to log-phase only SV1 and SV2 antibodies were shown to bind all three *S. pyogenes* at levels statistically greater PBS sera (Figure 6). J14-DT antisera bound to *S. pyogenes* M1, but not ES61 or 88-30 in these experiments. SV3 antibodies only bound to *S. pyogenes* 88-30.

Antibodies raised against SV1 are bactericidal. To examine the functionality of antibodies raised against each of the recombinant vaccine antigens *in vitro* bactericidal assays were performed with *S. pyogenes* 88-30 and *S. pyogenes* ES61. In these assays heat inactivated immune and control sera were incubated with *S. pyogenes* and human blood that was pre-screened to confirm the absence of natural anti-streptococcal antibodies. All experiments were performed independently in triplicate and results presented as the reduction in CFU recovered when compared to incubation of bacteria with PBS immune sera (Table 4). Only antibodies raised against SV1 were found to bactericidal towards both *S. pyogenes* 88-30 and ES61. The mean percent reduction in recovery of *S. pyogenes* 88-30 was 27 ± 14%, 28 ± 28% and 38 ± 17% for SV1, SV2 and SV3 respectively. The mean percent reduction in CFU in growth of *S. pyogenes* ES6 in the presence of SV1 antibodies was 78 ± 17%. No reduction in growth of *S. pyogenes* ES61was observed when incubated with SV2 or SV3 antibodies.

Structural properties of vaccine candidates. Maintenance of the helical structure of amino acid sequence within $J14_i$, matching the conformation of the M-protein is considered to be an important factor in the efficacy of vaccines from the conserved region of the M-protein (20). The propensity for recombinant vaccine antigens and J14 to form alpha-helical secondary structure was therefore assessed using CD spectroscopy (Figure 7). After deconvolution, SV3 was the only construct with greater than 50% alpha-helical structure in aqueous solution. When the spectra were examined in increasing concentrations of TFE, all proteins were predicted to assume alpha-helical structure at 10% TFE, irrespective of the presence of GCN4 flanking regions. In contrast, only 50% of J14 was predicted to form alpha-helical structure in 40% TFE.

Antibodies raised against SV1 were the only ones to kill both strains of *S. pyogenes* evaluated in this study, suggesting it to be the most promising candidate. To evaluate the stability of the secondary structure of SV1 CD analysis was performed on this construct at different temperatures and pHs. When exposing SV1 to increasing temperatures, the alpha-helical secondary structure was increasingly lost (Figure 8A). At 80°C the CD spectrum resembled that of an unfolded protein. The spectra at 20°C before and after the heating/cooling are almost identical and clearly show the existence of alpha-helical structure. Thus, the thermal unfolding of SV1 was a reversible process, since the CD at the same temperature in the cooling and heating phases agreed at all temperatures. Exposing SV1 to environments with varying pH values did not result in any significant changes of the alpha-helical structure as judged by the mean residue ellipticity at 222 nm (Figure 8B).

DISCUSSION

Group A streptococcal infection is a leading cause of bacterial related morbidity in the developing world. Increases in the prevalence of invasive disease have also resulted in a re-evaluation of the medical importance of this pathogen in developed nations. Early studies using whole M-protein resulting in the development of RF-like symptoms in vaccinees (42), and later reports describing the contribution of epitopes within the M-protein to the pathogenesis of RF and RHD (1) have prohibited the use of full length M-proteins as *S. pyogenes* vaccine candidates. Subsequent studies therefore targeted the immunogenic, but hypervariable amino-terminal region of multiple M-proteins (5,16,17), or the more conserved but less

immunogenic C-terminal region (4,18-22). Our approach to developing a broad spectrum vaccine capable of preventing infection from the majority of *S. pyogenes* M-types has been to construct polyvalent recombinant proteins that incorporate common sequences from the CRR of variant M-proteins, thereby offering protection against a wide range of *S. pyogenes* isolates. In choosing amino acid sequences from the CRR for incorporation into vaccine constructs, we examined the distribution of $J14_i$-variants in single representatives of individual M-proteins, removing biases that may occur through the over-representation of particular emm-types in epidemiological studies (28-30). Unlike the amino-terminal sequences, of which more than one hundred have been described, only eighteen $J14_i$-variants were found amongst the 77 different M-types we analysed, demonstrating the practicality of this approach in terms of strain coverage. Intra-M-protein differences in the possession of $J14_i$-variant sequences also provide added support for a polyvalent approach to *S. pyogenes* vaccine design.

Previously we reported that fusion of J14 with the H12 domain of SfbI, another *S. pyogenes* surface protein that has been proposed as a *S. pyogenes* vaccine candidate, resulted in loss of the alpha-helical secondary structure (37). By incorporating a flexible spacer sequence, and incorporating two J14 sequences in tandem, the alpha-helical J14 structure could be restored. In this study all polyvalent constructs were designed to ensure that both heptad periodicity required for coiled coil structure as observed in the parental M-protein, and alpha helicity were maintained along the full length of the proteins. The secondary conformation of all proteins, including those without GCN4 flanking sequences, was subsequently confirmed through circular dichroism. In comparison to J14 and SV2, SV1 and SV3, lack GCN4 amino acid sequence. A greater proportion of the latter's amino acid sequences therefore correspond to M-protein amino acid sequence. Our finding that while J14 and SV2 induced much greater anti-J14 titres, whole M-protein titres were similar for all groups, even in the absence of carrier molecule, suggest that a significant proportion of J14-DT and SV2 antibodies are directed against GCN4 sequence or epitopes that span the GCN4-$J14_i$ junction. The similar M-protein titres observed for all groups also demonstrate that a carrier molecule such as DT is not required for induction of significant immune responses by SV1, SV2 and SV3 in murine models. Together our data demonstrate that the rationale design of a vaccine undertaken here has resulted in constructs that do not require non-M-protein flanking sequence for conformational integrity, or carrier molecule for the induction of significant immune responses.

IFA and FACS both demonstrated that vaccine sera recognised M-protein on the surface of *S. pyogenes*. However the binding of sera to the surface of *S. pyogenes* strains in IFA experiments was qualitatively different to the binding observed in the FACs experiments. IFA was conducted on *S. pyogenes* in stationary phase growth, whereas the FACS was conducted on bacteria in log-phase growth. *S. pyogenes* in logarithmic growth phase are considered to have more capsule on their surface that bacteria in stationary phase (43,44). Thus the CRR is may be more accessible to immune molecules in stationary phase than in logarithmic growth. The failure of SV3 and J14-DT antibodies to bind to M1 GAS when grown to log-phase would suggest this to be the case for this isolate. However the ability for all antibodies to bind to 88-30, irrespective of growth phase, may also suggest that the total amount of capsule present on the surface differs between isolates, and may impact antibody binding capacity.

Comment [d4]: MG - Why wouldn't it also be the case for SV2

SV1 is the most promising vaccine of the three candidates assessed. SV1 possesses the five most common $J14_i$-variants, and is the only candidate to include $J14_i$-variants from CRRB1. In all 101 M-type/$J14_i$-variant combinations examined in the current study, 71% possess two or three of the variant epitopes present in SV1 in their CRRBs. Another 21% possess two copies of $J14.1_i$, but only contain 2 CRRBs in their full length sequence, demonstrating the advantage that inclusion of multiple sequences has in providing greater strain coverage. SV1 does not require foreign flanking amino acid sequence for maintenance of secondary structure, or require conjugation to carrier molecule for induction of anti-M-protein immune responses. SV1 antibodies also bind to the surface of *S. pyogenes* when grown to stationary or log-phase and kill heterologous *S. pyogenes* isolates in *in vitro* assays. Additionally, the structural studies indicates that SV1 will maintain the conformation required for induction of appropriate immune responses when admixed in formulations with varying chemical properties. The immunological and chemical properties suggest it to be a promising global *S. pyogenes* vaccine candidate.

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FIGURE LEGENDS

Figure 1. Specificity of J14-DT antibodies as determined by ELISA. (A) Mean absorbance measured using J14-DT antisera diluted 1:1000 and p145-variant peptides variant peptides. (B) Competitive ELISA of J14, p145-variants and J14-DT antisera. Data is presented as percentage binding of samples when the mean absorbance of samples pre-incubated with peptide was compared to the homologous samples that was not pre-incubated with peptide. pNS is an unrelated control peptide.

Figure 2. Schematic diagram of novel recombinant vaccine candidates. The recombinant constructs consist of linked in a fusion construct such that the heptad sequence required for coiled coil structure, is maintained throughout the length of the protein. SV2 also includes amino acid sequence derived from the GCN4 protein.

Figure 3. Serum antibody titres of B10.Br mice immunised with SV1, SV2, SV3 or J14-DT. The immunising antigen is shown in the x-axis. ELISAs were performed against (A) immunising antigen, (B) peptide J14 or (C) M1. Titres are shown in log scale. Horizontal bars represent the arithmetic mean titre for each group.

Figure 4. Mean isotype titres in response to immunisation of mice with vaccine candidates. ELISAs were performed against (A) the immunising antigen, (B) J14 or, (C) recombinant M1 protein. The antigen used to generate the antisera is shown on the X-axis,

Figure 5. Immunofluorescence microscopy of *S. pyogenes* M1, 88-30 and ES61. Stationary phase bacteria were incubated with antibodies raised against PBS, SV1, SV2 and SV3, and J14-DT, respectively and viewed using a x100 objective.

Figure 6. FACS analysis of binding of vaccine antisera to the surface of S. pyogenes M1, 88-30 and ES61 GAS. Bacteria were grown to mid-log phase, incubated with primary antisera, and FITC-labelled

secondary antisera. Binding of antigen specific antisera to the bacterial surface was determined by comparing histograms of vaccine serum with PBS serum using unpaired t-tests.

Figure 7. Circular dichroic spectra of proteins and J14. Samples were titrated in increasing concentrations of TFE, and CD were acquired and secondary structure contents assessed by fitting with Fasman prototype spectra. All protein vaccine constructs, irrespective of GCN4 content, were found to form 100% alpha helical structure in 10% TFE. In contrast the alpha-helical content of J14 was only 50% and required significntly larger amounts of TFE (40%).

Figure 8. Structural stability of SV1 as monitored by Circular Dichroism. (A) CD of SV1 at 222 nm in the heating phase (red curve) and the subsequent cooling phase (blue curve). (B) Full CD spectra of SV1 acquired at 20°C before heating (black curve), at 80°C (red curve), and after cooling at 20 (blue curve). (C) The alpha-helical structure of SV1 at varying pH. CD spectra were acquired at different pH and the CD at 222 nm for each pH was evaluated as difference to the CD observed at pH=7. In the pH range from 4 to 10, less than 0.1% change of the CD was observed when compared to the CD at neutral pH. Data shown are the result of three independent measurements.

Table 1. *S. pyogenes* strains used in this study. The emm-type and $J14_i$-variant sequences is also indicated.

GAS strain	Emm-type	CRRB1	CRRB2	CRRB3
M1	Emm1	J14.2	J14.2	$J14_i$
88-30	Emm97	$J14.2_i$	$J14.57_i$	$J14_i$
ES61	Emm65	$J14.36_i$	$J14.1_i$	$J14.1_i$

Table 2. Peptides used in this study

peptide	amino acid sequence
J14	KQAEDKVKASREAKKQVEKALEQLED KVK
p145	LRRDLDASREAKKQVEKALE
p145.1	LRRDLDASREAKKKVEADLA
P145.2	LRRDLDARSEAKKQVEKDLA
P145.3	LRRDLDTSREAKKQVEKDLA
P145.4	LRRDLDASREAKKQLEAEHQ
P145.5	TARDLEAVRQAKAQVEAALK
P145.12	LSRDLEASRAAKKELEAKHQ
P145.32	LSRDLEASRAAKKDELEAEH
P145.36	LSRDLEASRAAKKELEANHQ
P145.40	LSRDLEASREANKKVTSELT
P145.52	LSRDLEASREAAKKDLEAEH

Table 3. Distribution of $J14_i$-variants sequences in individual CRRBs of variant M-proteins.

peptide	amino acid sequence	% distribution of $J14_i$-variants			
		CRRB1	**CRRB2**	**CRRB3**	**All**
$J14_i$	ASREAKKQVEKALE	0.0	0.0	46.3	16.3
$J14.1_i$	ASREAKKKVEADLA	3.8	43.8	53.8	35.7
$J14.2_i$	ASREAKKQVEKDLA	27.3	34.0	0.0	20.0
$J14.3_i$	TSREAKKQVEKDLA	0.0	0.6	0.0	0.2
$J14.12_i$	ASRAAKKELEAKHQ	6.1	0.0	0.0	1.8
$J14.4_i$	ASREAKKQLEAEHQ	21.2	8.0	0.0	9.0
$J14.5_i$	AVRQAKAQVEAALK	1.5	0.6	0.0	0.7
$J14.6_i$	ASREAKKQLEAEQQ	0.0	0.6	0.0	0.2
$J14.8_i$	ASRAAKKELEAEHQ	7.6	1.9	0.0	2.9
$J14.9_i$	ASREAKRQVEKDLA	0.0	1.2	0.0	0.4
$J14.29_i$	ASRAAKKDLEAEHQ	15.9	4.9	0.0	6.4
$J14.35_i$	ASRAAKKDLEAEHR	1.5	0.0	0.0	0.4
$J14.36_i$	ASRAAKKELEANHQ	4.5	0.0	0.0	1.3
$J14.41_i$	ASRAAKKKVEADLA	0.0	1.2	0.0	0.4
$J14.53_i$	GSRAAKKELEAKHQ	7.6	0.0	0.0	2.2
$J14.55_i$	ASRAAKKELETNHQ	1.5	0.0	0.0	0.4
$J14.57_i$	ASREAKKQVEKGLA	0.0	3.1	0.0	1.1
$J14.58_i$	ASRAAKKDLEAKHQ	1.5	0.0	0.0	0.4

Table 4. Bactericidal activity of *S. pyogenes* vaccine antisera

Sera	88-30	ES61
SV1	27 ± 15	78 ± 17
SV2	28 ± 28	0 ± 0
SV3	38 ± 17	0 ± 0