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Conserved anchorless surface proteins as group A
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Conserved Anchorless Surface Proteins as Group A Streptococcal Vaccine Candidates

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1 **Abstract**

2 *Streptococcus pyogenes* (group A *Streptococcus*; GAS) causes ~700 million human
3 infections each year, resulting in over 500,000 deaths. The development of a commercial
4 GAS vaccine is hampered by the occurrence of many unique GAS serotypes, antigenic
5 variation within the same serotype, differences in serotype geographical distribution, and
6 the production of antibodies cross-reactive with human tissue that may lead to
7 autoimmune disease. Several independent studies have documented a number of GAS
8 cell wall-associated or secreted metabolic enzymes that contain neither N-terminal leader
9 sequences nor C-terminal cell wall anchors. Here, we applied a proteomic analysis of
10 serotype MIT1 GAS cell wall extracts for the purpose of vaccine development. This
11 approach catalogued several anchorless proteins and identified two protective vaccine
12 candidates, arginine deiminase and trigger factor. These surface-exposed enzymes are
13 expressed across multiple GAS serotypes exhibiting $\geq 99\%$ amino acid sequence identity.
14 Vaccine safety concerns are alleviated by the observation that these vaccine candidates
15 lack human homologs, while sera from human populations suffering repeated GAS
16 infections and high levels of autoimmune complications do not recognize these enzymes.
17 Our study demonstrates anchorless cell surface antigens as promising vaccine candidates
18 for the prevention of GAS disease.

19

20 **Keywords**

21 *Streptococcus pyogenes*, arginine deiminase, trigger factor, vaccine.

1 **Introduction**

2 The Gram-positive bacterium group A *Streptococcus* (GAS, *S. pyogenes*) is a major
3 human pathogen causing the ubiquitous infections of pharyngitis and impetigo and, less
4 commonly, life-threatening invasive diseases including streptococcal toxic shock-like
5 syndrome and necrotizing fasciitis [1]. Repeated GAS infections may trigger a suite of
6 non-suppurative immune sequelae including acute rheumatic fever, rheumatic heart
7 disease and acute post-streptococcal glomerulonephritis [1]. Globally, GAS causes ~700
8 million human infections each year, resulting in over 500,000 deaths [2].

9

10 A safe and efficacious commercial vaccine to prevent GAS infection has yet to be
11 developed, and this critical public health goal is fraught with pitfalls posed by the
12 pathogen's unique phenotypic features. In contrast to *Streptococcus pneumoniae*, where
13 commercial vaccines have been developed using conjugated capsular polysaccharides of
14 the most common serotypes, the CPS of all GAS strains is a homopolymer of hyaluronic
15 acid, a common sugar in human connective tissues, and therefore immunologically inert.
16 The antigenically diverse surface-anchored GAS M proteins are capable of eliciting
17 protective immunity; however, comparison of the encoding (*emm*) genes indicates
18 sequence diversity in the N-terminal hypervariable region and adjacent 'A' repeat
19 domains generate over 100 distinct GAS *emm* types [3]. Regions of the M protein
20 dimeric coiled-coil structure are implicated in eliciting cross-reactive antibodies against
21 human cardiac and basal ganglia tissue that are key to the immuno-pathogenesis of
22 rheumatic fever, thereby raising important concerns regarding vaccine safety.
23 Nonetheless, immune sera raised against 30 defined amino-terminal M protein fragments

1 in the 30-valent experimental M protein vaccine did not cross-reacted with human brain,
2 kidney or heart tissue in indirect immunofluorescence assays. These authors also
3 demonstrated considerable cross-protection of the 30-valent vaccine against non-vaccine
4 M serotypes [4].

5
6 Recently, a series of independent studies have documented a class of GAS cell
7 wall-associated or secreted metabolic enzymes that contain neither N-terminal leader
8 sequences nor C-terminal cell wall anchors [5-9]. Perhaps because these molecules do not
9 fit common categories of bacterial vaccine antigen, e.g. carbohydrate capsule, surface-
10 anchored protein, or secreted toxin, their potential as immunogens has not been pursued.
11 Here, we adopt a post-proteomic approach to reveal the vaccine efficacy of selected
12 highly-conserved and non-human-crossreactive anchorless cell wall-associated enzymes
13 against lethal GAS infection.

14 **Materials and Methods**

15 **GAS strains used in this study.** The *S. pyogenes* strains utilised in this study have been
16 described previously: 5448 (*emm1*) [8], 5448AP (*emm1*) [10], HSC5 (*emm14*) [11],
17 NS88.2 (*emm98.1*) and NS192 (*emm100*) [12, 13], pM1 (*emm1*) [14], 2036 (*emm6*) [15],
18 DSM2071 (*emm23*) [16], NV-2728 (*emm12*) (Novartis Vaccines, Siena, Italy), pL1
19 (*emm54*) (M. Batzloff, unpublished), and 20174 (*emm3*) (M. Kotb, unpublished).

20
21
22 **Protein identification by 2D electrophoresis.** To identify the major cell wall-associated
23 proteins of invasive M1T1 clonal 5448 *S. pyogenes* isolate [8] grown in the presence of

1 the cysteine protease inhibitor E64, a two-dimensional (2D) sodium dodecyl sulfate-
2 polyacrylamide gel electrophoresis (PAGE) proteomic analysis of a mutanolysin cell wall
3 extract was performed as previously described [17].

4
5 **Construction of expression plasmids and recombinant protein purification.** The
6 genes *sagP* (Genbank accession number AF468045), *ropA* (AAM80241) and *fba*
7 (AAK36400) encode ADI, TF and FBA respectively. Primers were designed (Table 1) to
8 amplify the genes from GAS strain 5448 for recombinant gene expression and the
9 resultant recombinant proteins purified as described previously for *ropA* [18]. The
10 production of recombinant M1 protein is described elsewhere [19].

11
12 **Preparation of pepsin M protein extracts.** Extracts were prepared as previously
13 described [20].

14
15 **ELISA.** ELISA was performed as previously described [15], and the titre designated the
16 highest serum dilution with an absorbance (450nm) cut-off greater than 0.2.

17
18 **Immunization and intraperitoneal challenge of mice with GAS.** Three immunization
19 and intraperitoneal challenge experiments were performed. For the first experiment, four
20 to six weeks old (Animal Resource Centre, Australia) female BALB/c mice ($n = 20$ for
21 test antigens; $n = 30$ for controls) were subcutaneously immunized with 10 μ g
22 recombinant protein adjuvanted with CFA in the primary immunization. Two mice
23 immunized with recombinant ADI died of incidental causes prior to challenge. Mice were

1 challenged via the intraperitoneal route on day 56 with approximately 8×10^6 CFU of
2 GAS M1 serotype strain pM1 [14]. Results of independent replicates ($n = 10$) were
3 combined for each test antigen. Mice were monitored twice a day for ten days after
4 challenge and euthanized when they exhibited defined humane endpoints approved by
5 University of Wollongong and Queensland Institute of Medical Research Animal Ethics
6 Committees. For the second experiment, five weeks old (Charles River, Italy) female CDI
7 mice ($n = 48$ for ADI, ADI and TF combination, and Alum negative control; $n = 42$ for
8 TF; $n = 24$ for M protein positive control) were intraperitoneally immunized with 10 μ g
9 recombinant protein combined with Alum on days 0, 21 and 35. Groups of mice were
10 challenged via the intraperitoneal route on day 55 with 1.35×10^6 CFU of GAS serotype
11 M1 strain pM1. Results of three independent replicates were combined and presented for
12 each test antigen. For the third experiment, five weeks old (Charles River, Italy) female
13 CDI mice ($n = 48$ for ADI and TF combination, and Alum negative control; $n = 24$ for M
14 protein positive control) were intraperitoneally immunised with 10 μ g recombinant
15 protein combined with Alum on days 0, 21 and 35. Groups of mice were challenged via
16 the intraperitoneal route on day 55 with 1.2×10^2 CFU of GAS serotype M12 strain M12-
17 2728. Results of three independent replicates were combined and presented for each test
18 antigen. In experiments two and three, mice were monitored on a daily basis for at least
19 one week after treatment and euthanized when they exhibited defined humane endpoints
20 that had been pre-established for the study in agreement with Novartis Animal Welfare
21 Policies. Experimental protocols described in this report complied with the Australian
22 Code of Practice for the Care and Use of Animals for Scientific Purposes (National
23 Health and Medical Research Council, Australia).

Intraperitoneal immunization and subcutaneous challenge of mice with a hypervirulent *covS* mutant GAS strain. Four to six weeks old female C57BL/J6 mice (Animal Resource Centre, Australia) ($n = 40$ for ADI and TF; $n = 21$ for ADI and TF combination; $n = 80$ for M1 protein positive control; $n = 79$ for PBS negative control) were immunized on days 0, 21 and 28 with 10 μ g recombinant protein adjuvanted with CFA in the primary immunization. One PBS negative control mouse died of incidental causes prior to challenge. Mice were challenged via the subcutaneous route on day 56 with approximately 2×10^8 CFU of hypervirulent *covS* mutant GAS strain 5448AP of serotype 1 [10]. Results of independent replicates ($n = 10$) were combined for each test antigen. Mice were monitored twice a day for ten days after challenge and euthanized when they exhibited defined humane endpoints approved by the University of Wollongong Animal Ethics Committee.

Opsonophagocytosis killing assay. GAS strain 5448 was grown to stationary phase and the opsonophagocytosis killing assay performed with the specific polyclonal mouse serum raised against M1 protein, ADI or TF (adjuvanted with CFA), as previously described [19]. Two biological replicates were undertaken using quadruplicate samples.

Detection of cell wall-associated proteins in cell wall extracts. SDS-PAGE and western blotting was performed as described previously [21]. The primary serum was polyclonal mouse anti-serum directed against each recombinant protein (adjuvanted with

CFA), whilst the secondary anti-serum was goat anti-mouse IgG-HRP conjugate (Chemicon). Antibodies were diluted 1:2,000.

Detection of proteins on GAS cell surface using flow cytometry. Flow cytometry was utilized to determine whether candidate antigens were localized on the surface of GAS isolates 5448 (M1T1) [8], pM1 (M1) [15] and pL1 (M54) using pooled polyclonal mouse anti-sera ($n = 10$) directed against each recombinant protein (adjuvanted with CFA); and M12-2728 (M12) using pooled polyclonal mouse anti-sera ($n = 10$) directed against each recombinant protein (adjuvanted with Alum). Incubations were performed for either 1 h on ice or overnight at 4°C. A 1 mL volume of logarithmic-phase bacteria ($OD_{600} = 0.6$), were washed twice in filtered PBS and non-specific binding sites and Ig-binding proteins blocked with 3% (w/v) BSA/PBST followed by normal human IgG diluted 1:150 in 0.3% (w/v) BSA/PBST, respectively. Cells were washed and incubated with primary antibody diluted 1:50 in 0.3% (w/v) BSA/PBST. Anti-M Protein or anti-pep M extract serum and serum from mice immunized with PBS were utilized as the positive and negative controls, respectively. Bacteria were washed and incubated with the secondary antibody, goat anti-mouse FITC conjugated IgG (Zymed, USA) diluted 1:150 in 0.3% (w/v) BSA/PBST. Finally, cells were washed and fixed with 4% (w/v) paraformaldehyde/PBS and either analyzed in a FACSort flow cytometer (Becton Dickson, USA) with CellQuest software (version 3.1f) or a BD Accuri C6 flow cytometer (BD Accuri Cytometers, USA) with BD CFlow software. Cell-surface-associated FITC-fluorescence (excitation: 488 nm; emission: 520 nm) was analysed using a uniform population of gated cells. Histograms displaying FL-1 (FITC) fluorescence were prepared using FlowJo software

(version 7.2.5). Specific antibody binding was calculated by subtracting the average geometric mean of the no primary antibody control from the test sample. Each antigen was tested in triplicate.

Testing the reactivity of human endemic serum with recombinant proteins. The reactivity of recombinant protein antigens with pooled serum was tested by ELISA as described previously [22]. Human serum was obtained from two populations (i) school-aged Aboriginal children ($n = 30$) living in remote communities in the Northern Territory, Australia; (ii) Indian children and young adults (9 years old - 22 years old) living in Chandigarh, India, pooled into the following categories: individuals without disease symptoms ($n = 10$); patients with pharyngitis ($n = 8$); patients with acute rheumatic fever ($n = 9$); patients with rheumatic heart disease ($n = 10$). Test serum was diluted two-fold starting with a 1:100 dilution and the secondary antibody, goat anti-human IgG-HRP (Bio-Rad), was diluted 1:2,000. Recombinant M1 protein was incubated with a 1:200 dilution of Aboriginal serum or Indian rheumatic heart disease serum on plates as a control to normalise data. Two experimental biological replicates were performed.

Production of polyclonal rabbit serum. Rabbit polyclonal serum was raised against ADI, TF or M1 protein according to previously stated methods [22].

Cross-reactivity of candidate antigen polyclonal sera with human heart extract. Human heart tissue (5 g) was homogenised in 5 mL of PBS containing complete protease inhibitor cocktail (Roche) using a PolyTron PT2100. Debris was removed by

centrifugation at 12,000 x *g* for 30 min at 4°C. The reactivity between human heart proteins and test serum from rabbits immunised with individual recombinant antigens was tested by ELISA. 96-well plates were coated with 20 µg/mL of the human heart extract in 0.1M carbonate coating buffer (pH 9.6) and blocked with 1% (w/v) gelatin in PBS. The test serum was diluted two-fold starting with a 1:10 dilution in 1% (w/v) gelatin. Naive rabbit serum was utilized as a negative control. The secondary antibody, goat anti-rabbit IgG-HRP (Dianova), was diluted 1: 3,000 in 1% (w/v) gelatine. Two biological replicates were undertaken using duplicate samples.

Transmission immunogold electron microscopy. GAS cells were grown to stationary phase, then immuno-gold labeled and visualized as previously described [21].

Detection of proteins on the cell surface using immunofluorescence microscopy.

Confocal immunofluorescence microscopy was performed as previously described [23] using a 100X/1.0 FLUOTAR PL oil immersion objective lens with a Leica TCS SP confocal microscope mounted on a Leica DM IRBE inverted microscope (Leica Microsystems, Germany) configured for the detection of FITC.

Statistical analyses. Differences in survival of mice immunized with recombinant protein antigens compared to sham-immunized mice were determined by the log-rank test. Differences were considered statistically significant at $P < 0.05$. Statistical analysis of human serum ELISA and human heart extract cross-reactivity ELISA were performed using a one-way analysis of variance. To determine the shift in cell surface fluorescence,

1 the geometric mean of the flow cytometry histograms resulting from the surface labelling
2 of the test serum were compared to the geometric mean of the sham-immunized serum
3 histogram using an unpaired *t*-test with a confidence interval of 95%. All statistical tests
4 were performed using GraphPad™ Prism software version 4.2.

6 **Results**

7 **Proteomic Analysis of GAS Cell Wall Extracts**

8 Proteomic analysis of GAS cell wall extract was undertaken using isolate 5448, a
9 representative of the globally disseminated MIT1 clonal serotype [8]. The cell wall
10 extract was separated by two-dimensional gel electrophoresis and the landmark proteins
11 identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.
12 In total, 31 distinct cell-wall associated proteins were identified (Fig. 1a; Supplementary
13 Table 1). Among these were metabolic enzymes that share significant amino acid
14 sequence identity with human homologs (e.g. GAS α -enolase shares 49% identity with
15 human α -enolase), which raises concern for autoimmune consequences [24]. For further
16 characterization we chose three candidates that are not represented in the human
17 proteome and do not share significant amino acid sequence identity with other human
18 proteins as determined by BLAST-P interrogation (Supplementary Table 1): arginine
19 deiminase (ADI) [25, 26] and trigger factor (TF) [27, 28], which are potential GAS
20 virulence determinants, and fructose bisphosphate aldolase (FBA), which affords vaccine
21 protection against *Streptococcus pneumoniae* [29]. All three enzymes lack N-terminal
22 signal sequences and C-terminal cell wall anchors [7]. The genes encoding these three
23 candidate antigens are highly conserved, sharing $\geq 99\%$ amino acid sequence identity.

Each enzyme is detected in cell wall fractions across a range of disparate GAS M serotypes (Fig. 1b).

Surface Localization of Candidate Antigens

To investigate the presentation of the three vaccine candidates on the GAS cell surface, immunofluorescence microscopy, flow cytometry and immuno-electron microscopy were undertaken utilizing antigen-specific polyclonal anti-serum. Anti-serum raised against the well-characterized surface-bound M protein was used as the positive control, while serum from mice immunized with PBS or animal matched pre-immune serum was used as the negative controls for this series of experiments. Polyclonal anti-serum specific for M1 protein, ADI, TF and FBA clearly bound to the GAS cell surface, whereas pre-immune serum (Fig. 1d) or sham immunized serum did not react (Fig. 1e). When tested on GAS isolates 5448 (M1), pM1 (M1) and pL1 (M54) using flow cytometry, in comparison to sham-immunized serum, polyclonal sera raised against full length M protein or pepsin M extract, ADI or TF were observed to produce a significant shift in fluorescence ($P < 0.05$; Fig. 1c and Fig. 3a). Following surface binding to the M12-2728 (M12) GAS isolate, anti-TF and anti-M protein sera produced a significant shift in fluorescence compared to PBS sham sera in flow cytometry ($P < 0.05$; Fig. 3a). Anti-ADI serum also resulted in a shift, which was not statistically significant ($P > 0.05$; Fig. 3a).

Anchorless Proteins Elicit Protective Immunity

Conjecture exists in the scientific literature regarding the biological relevance and certitude of surface expression of anchorless proteins in GAS [5, 30]. To validate our findings and to test protective vaccine efficacy of the selected anchorless surface proteins, full-length M1 protein, ADI, TF and FBA were adjuvanted with Complete Freund's Adjuvant (CFA) and used to subcutaneously immunize BALB/c mice and the survival recorded following lethal intraperitoneal M1 GAS challenge. Immunization of mice with anchorless proteins adjuvanted with CFA resulted in high titer serum IgG antibodies (Fig. 2a), demonstrating that robust antibody responses are engendered following vaccination. Following lethal intraperitoneal M1 GAS challenge, BALB/c mice immunized with full-length M1 protein, ADI and TF were observed to have significantly increased survival ($P < 0.05$), compared to sham-immunized mice (Fig. 2c). As experimental immunization with FBA ($P > 0.05$) failed to protect against lethal intraperitoneal M1 GAS challenge (Fig. 2c), the protective efficacy of FBA was not further evaluated. Next, we explored the capacity of ADI and TF to elicit protective immunity using Alum, an adjuvant approved for human use. Immunization of CD1 mice with anchorless proteins adjuvanted with Alum were again observed to have significantly increased survival ($P < 0.05$), compared to sham-immunized mice (Fig. 2d).

Invasive GAS infections are known to be caused by both wild type and hypervirulent *covRS* forms with differing transcriptional profiles [31]. Thus, an alternate GAS challenge model was explored in which CFA-adjuvanted full-length M1 protein, ADI, TF or a combination of ADI and TF were used to immunize C57BL/6 mice prior to subcutaneous challenge with the hypervirulent *covS* mutant M1 strain, 5448AP [10].

Immunization with ADI alone resulted in a trend toward increased survival ($P > 0.05$), whilst immunization with M1 protein or the 1:1 combination of ADI and TF engendered protective immunity against subcutaneous challenge with 5448AP ($P < 0.05$) (Fig. 3b). The ability of ADI and TF to confer protective immunity against heterologous GAS challenge was investigated. Intraperitoneal immunization of CD1 mice with M12 protein or a 1:1 cocktail of ADI and TF adjuvanted with Alum resulted in significant protection against intraperitoneal challenge with the M12 GAS strain M12-2728 ($P < 0.05$) (Fig. 3c). The observed protection of mice may be due to a concert of factors including antibody-mediated opsonophagocytic killing of GAS (Fig. 2b), antibody-mediated neutralization of the enzymatic activity of ADI or TF, or non-antibody mediated mechanisms.

Reactivity of Candidate Antigens with Human Sera and Cross-Reactivity with Human Heart Tissue

The Aboriginal population of the Northern Territory of Australia suffers some of the highest reported rates of GAS disease and autoimmune sequelae [32]. We examined whether members of this population have pre-existing immunity against ADI and TF, hypothesizing that an immune response against these protective antigens may be lacking in a human population that suffers repeated GAS infections [33]. The Aboriginal immune response was determined using a pool of serum ($n = 30$) obtained from Aboriginal children living in remote communities of the Northern Territory suffering endemic GAS infection. In stark comparison to M1 protein ($P < 0.001$), there were minimal serum antibody responses directed against ADI and TF (Fig. 4a). The human immune response

mounted against the anchorless proteins was also investigated using four pools of serum isolated from individuals living in Chandigarh, India, classified into the following clinical disease categories: individuals living in endemic area showing no symptoms ($n = 10$), pharyngitis ($n = 8$), acute rheumatic fever ($n = 9$), and rheumatic heart disease ($n = 10$). The serum antibody response detected against ADI and TF for each of these four pools of sera was significantly less than the response engendered by M1 protein ($P < 0.01$) (Fig. 4b - 4e). Finally, polyclonal M1 protein antiserum strongly cross-reacted with human heart extract, in contrast to anti-ADI and anti-TF polyclonal sera (Fig. 4f).

Discussion

GAS is a major human pathogen causing ~700 million human infections each year, resulting in over 500,000 deaths [2]. Although several surface proteins and virulence factors have been previously assessed as vaccine candidates [34], there is no commercial vaccine available to prevent GAS infection. Following a proteomic analysis of an M1T1 cell wall extract, we selected three anchorless proteins; ADI, FBA and TF; for further characterization as putative GAS vaccine candidates.

Anchorless proteins are highly conserved and are localized on the surface of GAS.

Several previously characterized GAS vaccine candidates; M protein, SfbI, SOF and FbaA [34] are not conserved between GAS serotypes nor expressed by all serotypes of GAS. The genes encoding each of the anchorless vaccine antigens of this study were ubiquitous amongst sequenced GAS genomes with $\geq 99\%$ conservation amongst serotypes (Table 2). In addition, the anchorless vaccine antigens were detected in

mutanolysin derived cell wall extracts of eight representative clinical GAS isolates (Fig. 1b). Whilst the anchorless proteins lack signal sequence(s) for export and the traditional LPXTG motif known to anchor Gram-positive surface proteins to the cell wall, they were detected on the GAS cell surface using immunofluorescent microscopy (Fig. 1e), flow cytometry (Fig. 1c and Fig. 3a) and immuno-EM (Fig. 1d). At this stage their mechanism of export is unknown. Anchorless GAS cell surface-associated proteins of cytoplasmic origin is by no means a new phenomenon. The glycolytic surface exposed plasminogen-binding virulence factors of GAS, α -enolase [35] and GAPDH [36] are established examples of two traditionally cytoplasmic proteins lacking a LPXTG motif located on the surface of GAS possessing a dual biochemical and virulence-associated functionality. In addition to ADI, TF and FBA, both α -enolase and GAPDH were amongst the 31 proteins identified in the proteomic analysis of GAS cell wall extracts (Fig. 1a),

Anchorless proteins protect against lethal GAS challenge. When adjuvanted with CFA or the human-approved adjuvant Alum, ADI and TF elicited a protective immune response against intraperitoneal challenge with a homologous M1 isolate (Fig. 2c and Fig. 2d). Anti-ADI and anti-M1 protein sera generated following immunization of mice resulted in the killing of M1 GAS in whole human blood (Fig. 2b). Opsonophagocytic killing of GAS is hypothesised to play a role, at least in part, in the efficacy of protective GAS antigens by facilitating bacterial clearance in the infected host. When administered in a two-antigen cocktail combination ADI and TF were observed to act synergistically, conferring significant protection against lethal subcutaneous M1 GAS challenge ($P < 0.05$; Fig. 3b) and against intraperitoneal challenge with a heterologous M12 isolate ($P <$

0.05; Fig 3c). It is possible that vaccine preparations containing a number of protective GAS antigens (or fragments thereof) may result in complete protection. The addition of ubiquitously expressed antigens such as ADI or TF to multi-antigen GAS vaccine preparations may help overcome issues of serotype dependent protection in cases where the expression of the individual antigens is limited to a subset of serotypes.

Anchorless proteins do not react with human sera or heart tissue. During early immunization studies of human subjects with GAS vaccine preparations, the administration of partially purified M3 protein was reported to result in three cases of acute rheumatic fever [37]. It has been subsequently established that the host immune response to M protein can produce antibodies that react with host tissue such as cardiac myosin [38]. As a consequence, there has been a heightened concern that host antibodies generated against GAS vaccine antigens may cross-react with human proteins and tissues potentially leading to auto-immune disease. To address the issue of cross-reactivity, we tested the capacity of human serum collected from individuals living in an area of endemic GAS disease to react with the anchorless antigens. The lack of an immune response against ADI and TF in both these populations, which have high rates of autoimmune sequelae [32], suggest that these proteins are not involved in triggering autoimmune disease. Moreover, the lack of an immune response against these potentially protective antigens, despite repeated infection, suggests that GAS may have evolved a mechanism to shield these antigens from the normal host immune response. When incubated directly with human heart extract, polyclonal anti-ADI and anti-TF serum antibodies did not significantly react ($P > 0.05$; Fig. 4c), in contrast to anti-M1 protein

1 serum. These results suggest that unlike full length M protein, the B-cell epitopes
2 contained within ADI and TF do not cross-react with human cardiac proteins.

3
4 In conclusion, two anchorless cell surface proteins of GAS, ADI and TF, were
5 identified in this study and found to confer protection against lethal GAS challenge in a
6 variety of mouse models. These protective vaccine candidates are highly-conserved, are
7 expressed across multiple GAS serotypes, and lack significant amino acid sequence
8 identity with any known human protein. Furthermore, sera from human populations
9 suffering repeated GAS infections and high levels of autoimmune complications did not
10 recognize these enzymes, and polyclonal antiserum raised against ADI and TF did not
11 cross-react with human heart extract. This novel class of antigen warrants further
12 investigation as vaccine candidates for the prevention of GAS disease, a major cause of
13 morbidity and mortality worldwide.

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4

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35

1 **Table 1.** Oligonucleotide primers used in this study for construction of expression
 2 plasmids.
 3

Primer	Direction	bp	Sequence (5' to 3')
ADI fwd	Forward	22	CACCATGACTGCTCAAACACCA
ADI rev	Reverse	24	TTAAATATCTTCACGTTCAAATGG
TF fwd	Forward	22	CACCATGTCTACATCATTTGAA
TF rev	Reverse	21	TTACTTAACGCTTGCTGTGCT
FBA fwd	Forward	22	CACCATGGCAATCGTTTCAGCA
FBA rev	Reverse	27	TTATGCTTTGTAGCTGATCCGAAAAC

4

Table 2. Amino acid identities of ADI, TF and FBA amongst sequenced GAS proteomes determined using BLAST-P searches available at NCBI (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi).

GAS strain	Serotype	Amino acid sequence identity (%)		
		ADI	TF	FBA
M1	<i>emm1</i>	99	99	100
M49 591	<i>emm49</i>	99	100	ND
MGAS10394	<i>emm6</i>	99	100	99
MGAS315	<i>emm3</i>	100	100	99
MGAS8232	<i>emm18</i>	99	99	100
SSI-1	<i>emm3</i>	100	100	99
MGAS10270	<i>emm2</i>	99	99	100
MGAS10750	<i>emm4</i>	99	99	100
MGAS2096	<i>emm12</i>	99	99	99
MGAS5005	<i>emm1</i>	99	99	100
MGAS6180	<i>emm28</i>	99	99	100
Manfredo	<i>emm5</i>	99	99	99
NZ131	<i>emm49</i>	99	99	100
MGAS9429	<i>emm12</i>	99	99	99

Abbreviation: ND; not determined.

Figure 1 Identification, serotype distribution and surface localization of novel GAS antigens. **(a)** Representative two-dimensional gel electrophoresis profile of GAS strain 5448 mutanolysin cell wall extract. Protein spots identified by peptide mass mapping following trypsin digest and MALDI-TOF MS are denoted by numbered arrows. Arrows numbered 18 correspond to ADI, arrow number 13 corresponds to TF, arrow number 10 corresponds to FBA. Molecular mass markers are indicated in kilo-Daltons (kDa). **(b)** Western blot detection of cell surface-associated proteins (ADI, TF and FBA) in cell wall extracts of eight representative GAS isolates (*emm* type provided here in brackets); from left to right, 5448 (*emm1*), NS88.2 (*emm98.1*), NS192 (*emm100*), DSM2071 (*emm23*), HSC5 (*emm14*), 20174 (*emm3*), pM1 (*emm1*), 2036 (*emm6*). Multiple blots were used to compile image. The size of each protein is indicated in kilo-Daltons (kDa). **(c)** Quantification of antigens on the surface of GAS M1 isolate 5448 using flow cytometry. Histogram for test antigens and M1 protein control indicated with solid line, histogram for sham-immunized serum denoted by shading. ADI, TF and M1 protein were observed to have a significant difference in shift ($P < 0.05$) in comparison to the sham-immunized serum, which is indicated with an asterisk (*). Samples were analysed in triplicate. **(d)** Detection of anchorless surface proteins on the surface of GAS strain 5448 using immuno-electron microscopy. Specific polyclonal rabbit anti-serum (upper panel) shows accumulation of gold particles (indicated by arrows) at the cell surface in comparison to the corresponding pre-immune serum (lower panel). Scale bars represent 200 nm. **(e)** Detection of M1 protein, ADI, TF and FBA via immunofluorescence on the surface of GAS strain pM1 using anti-sera from BALB/c mice ($n = 10$) immunized with the cell surface-associated antigens and stained with goat anti mouse IgG-FITC. Fluorescence

image (upper panel) and transmission image (lower panel). Scale bars indicate 5 μ m. For each experiment (a-e), two independent replicates were prepared and representative data is presented.

Figure 2 ADI, TF and FBA antigens produce high titre antibodies and protect against lethal intraperitoneal GAS challenge. **(a)** Serum-specific IgG antibody titers in BALB/c mice ($n = 10$) following subcutaneous immunization of antigen on day 0, 21 and 28. Mice serum was tested for specific reactivity on days 0, 20, 27 and 41. The absorbance cut-off for the determination of titer was 0.2. Red bars represent the arithmetic mean, two independent experimental repeats were performed. Serum obtained from sham-immunized mice did not produce measurable titres against ADI, TF, FBA or M1 protein. **(b)** Percent survival of the serotype M1 GAS strain 5448 following opsonophagocytosis killing assay. GAS were pre-incubated with specific polyclonal mouse serum raised against M1 protein, ADI or TF (serum from PBS sham immunized mice was included as a negative control) and subsequently incubated with normal heparinised human blood. Quadruplicate data from two repeat experiments is presented. Percent survival of GAS was calculated in comparison to the number of GAS (cfu/mL) surviving in the PBS sham immunised serum control. * indicates a significant difference ($P < 0.01$) between the test antiserum and the PBS sham immunised control serum. **(c)** Kaplan-Meier survival curves for intraperitoneal challenge of immunized BALB/c mice with a lethal dose of pM1 GAS, 8×10^6 colony forming units (CFU) ($n = 18$ for ADI; $n = 20$ for TF and FBA). M1 protein ($n = 30$) was selected as a positive control, whilst PBS was the negative control ($n = 30$). Antigens were co-administered with CFA in the primary immunization. Two

1 independent replicates were performed for ADI, TF and FBA, three independent
 2 replicates were performed for the controls, M1 and PBS, each repeat contained $n = 10$
 3 mice for each antigen or control. **(d)** Kaplan-Meier survival curves for intraperitoneal
 4 challenge of CD1 mice with 1.35×10^6 CFU of pM1 GAS ($n = 48$ for ADI, $n = 42$ for
 5 TF). M1 protein ($n = 24$) was used as a positive control, whilst PBS was the negative
 6 control ($n = 46$). Antigens were co-administered with Alum. Three independent replicates
 7 were performed, all data is presented.

8

9 **Figure 3 (a)** Quantification of antigens on the surface of GAS isolates pM1, pL1 and
 10 M12-2728 using flow cytometry. Histogram for test antigens and positive control
 11 indicated with solid line, histogram for sham-immunized serum denoted by shading. Sera
 12 raised against M protein, pepsin M protein extracts, ADI and TF produced a shift in cell
 13 surface FITC fluorescence compared to PBS sham serum. A significant shift in
 14 fluorescence ($P < 0.05$) is indicated with an asterisk (*). Samples were analyzed in
 15 triplicate. **(b)** Kaplan-Meier survival curves for subcutaneous challenge of C57BL/J6
 16 mice (co-immunized with Freund's complete adjuvant) with a lethal dose of 5448AP
 17 GAS, 2×10^8 CFU ($n = 40$ for ADI, $n = 40$ for TF, $n = 21$ for ADI + TF cocktail). M1
 18 protein ($n = 80$) was selected as a positive control, whilst PBS was the negative control (n
 19 $= 79$). At least two independent repeats were performed, all data is presented. **(c)** Kaplan-
 20 Meier survival curves for intraperitoneal challenge of CD1 mice (co-immunized with
 21 Alum) with 1.2×10^2 CFU of M12-2728 GAS ($n = 48$ for ADI + TF cocktail). M12
 22 protein ($n = 24$) was selected as a positive control, whilst Alum was the negative control
 23 ($n = 48$). Three independent replicates were performed, all data is presented.

1

2 **Figure 4** Reactivity of antigens with human serum and cross-reactivity with human heart
3 extract. The human IgG response against M1 protein, ADI and TF was determined using
4 **(a)** a pool of serum ($n = 30$) from Aboriginal children living in remote communities of
5 the Northern Territory (Australia) suffering endemic GAS infection, **(b)** a pool of serum
6 ($n = 10$) from individuals with no disease symptoms in Chandigarh (India), **(c)** a pool of
7 serum ($n = 8$) from patients with pharyngitis in Chandigarh (India), **(d)** a pool of serum (n
8 $= 9$) from patients with acute rheumatic fever in Chandigarh (India), and **(e)** a pool of
9 serum ($n = 10$) from patients with rheumatic heart disease in Chandigarh (India). **(f)**
10 Reactivity of rabbit polyclonal antisera raised against M1 protein, ADI or TF with human
11 heart extract. * Response against M1 protein is significantly higher than test antigens (P
12 < 0.01). Error bars represent the standard error of the mean. For each component of figure
13 (a-f) duplicate data from two independent replicate experiments is presented.

Figure 1

Figure 2

Figure 3

Figure 4

Supplementary Table 1. Peptide mass fingerprinting identification of cell wall-associated proteins of *S. pyogenes* isolate 5448, and percent identity of cell wall-associated proteins with human homologs.

Spot	Protein ^a	Function or pathway	Accession no. ^b	Molecular mass (kDa) ^c	pI ^c	Peptide matches ^d	Coverage (%) ^e	Human homolog ^f	
								Accession no. ^{b, g}	Identity (%) ^h
1	Enolase*	Glycolysis; Virulence factor	P69950	47.2	4.74	24	68.9	P09104	49
2	GAPDH	Glycolysis; Virulence factor	Q5XDW3	35.8	5.34	19	57.0	P04406	44
3	Chaperone protein DnaK (HSP70)	Chaperone	Q5XAD6	64.8	4.62	22	47.4	P38646	51
4	Putative carbamate kinase	Amino acid biosynthesis; arginine metabolism; pyrimidine nucleotide biosynthesis	Q8K6Q9	33.2	4.71	17	57.9	n/a	0
5	Putative alkyl hydroperoxidase	Peroxidase activity	Q7CEM3	20.5	4.65	11	58.1	P32119 ^o	39
6	General stress protein, Gls24 family	Stress protein	Q5XBW8	17.5	4.65	11	92.6	n/a	0
7	Putative thioredoxin	Electron transport	Q5XA57	13.6	5.06	8	45.8	B4DZQ3 ^o	41
8	LSU ribosomal protein L12P	Protein biosynthesis	Q5XCB4	12.9	4.42	9	77.0	n/a	0
9	Triosephosphate isomerase	Involved in several metabolic pathways	P69888	26.5	4.57	16	69.3	P60174	40
10	Fructose-bisphosphate aldolase	Glycolysis; sixth step	Q5XA12	31.0	4.91	16	52.1	n/a	0
11	Ribosome recycling factor	Protein synthesis	P68904	20.6	5.68	11	64.3	Q96E11	28
12	Putative phosphotransferase system (PTS), enzyme II component B	Protein-N(PI)-phosphohistidine-sugar phosphotransferase activity	Q7CN95	17.9	6.13	14	78.5	n/a	0
13	Trigger factor	Protein folding and transport	Q5XA08	50.6	4.47	20	46.9	Q02790 ⁺	23
14	60 kDa chaperonin (GroEL)	Chaperone	Q8K5M5	56.9	4.75	28	62.4	P10809	47
15	Putative dipeptidase	Proteolysis and peptidolysis	Q99ZU1	51.3	4.87	17	46.1	Q96KP4 ^u	43
16	Ornithine carbamoyltransferase	Arginine degradation; arginine deiminase pathway; second step	P65609	37.8	5.19	22	70.5	P00480	40
17	6-phosphofructokinase	Key control step of glycolysis	Q8P0S6	35.7	5.34	22	57.6	Q01813	37
18	Arginine deiminase	Virulence factor	Q5XAY2	46.2	4.99	26	68.5	n/a	0
19	Purine nucleoside phosphorylase	Purine-nucleoside phosphorylase activity	Q5XCL3	28.8	4.98	12	39.0	P00491 ^u	45

20	Superoxide dismutase (Mn)	Virulence factor	Q8P0D4	22.5	4.87	9	57.0	P04179	50
21	Putative transketolase	Calcium ion binding; transketolase activity	Q8K670	77.5	4.98	16	29.5	P29401 ^Ω	33
22	10 kDa chaperonin (GroES)	Chaperone	P63771	10.3	4.79	8	86.5	P61604	32
23	30S ribosomal protein S6	Transcription	P66600	11.1	4.97	6	74.0	B9ZVW4 ^Ω	25
24	Phosphoenolpyruvate-protein phosphotransferase	Carbohydrate active-transport system	Q5XBI4	63.1	4.60	20	33.4	n/a	0
25	Putative NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	Metabolic enzyme	Q99Z67	50.4	5.06	23	50.1	P47895 ^Ω	32
26	Putative 2-dehydropantoate 2-reductase	Pantothenate biosynthesis	P65667	33.8	4.84	16	52.1	n/a	0
27	Protein phosphatase 2C	Phosphoprotein phosphatase activity	Q5XAP6	27.0	4.60	17	76.8	Q8N819	23
28	Adenylate kinase	Essential for maintenance and cell growth	P69882	23.7	4.83	18	84.0	P54819	39
29	Transcription elongation factor GreA	Transcription elongation	Q5XDQ7	19.7	5.08	12	77.8	n/a	0
30	M protein type 1*	Virulence factor	Q10372	54.2	6.53	14	33.3	Q15149 [†]	21
31	Chaperone protein DnaK (HSP70) fragment	Chaperone	Q8K624	64.8	4.61	9	15.0	P38646	51

^a GAS proteins implicated in human autoimmune complications are denoted by an asterisk (*).

^b Swiss-Prot/TrEMBL accession number.

^c Theoretical values obtained from Swiss-Prot/TrEMBL database.

^d Number of tryptic peptides detected by MALDI-TOF MS that could be matched to the protein.

^e Percentage of protein sequence covered by the matched peptides.

^f Top match identified by BLAST-P searches of the *Homo sapiens* database (using an E threshold of 1) at the Expasy/SIB BLAST network service (<http://au.expasy.org/tools/blast/>).

^g n/a (not applicable) indicates a human homolog of similar function was not identified.

^h GAS proteins without a known human homolog have zero percent identity.

[†] Human homolog identified using BLAST-P search does not have the same function as GAS protein.

^Ω GAS or human protein has unknown function.