

# Conserved anchorless surface proteins as group A streptococcal vaccine candidates

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**Abstract** *Streptococcus pyogenes* (group A *Streptococcus* (GAS)) causes ~700 million human infections each year, resulting in over 500,000 deaths. The development of a commercial GAS vaccine is hampered by the occurrence

of many unique GAS serotypes, antigenic variation within the same serotype, differences in serotype geographical distribution, and the production of antibodies cross-reactive with human tissue that may lead to autoimmune

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disease. Several independent studies have documented a number of GAS cell wall-associated or secreted metabolic enzymes that contain neither N-terminal leader sequences nor C-terminal cell wall anchors. Here, we applied a proteomic analysis of serotype MIT1 GAS cell wall extracts for the purpose of vaccine development. This approach catalogued several anchorless proteins and identified two protective vaccine candidates, arginine deiminase and trigger factor. These surface-exposed enzymes are expressed across multiple GAS serotypes exhibiting  $\geq 99\%$  amino acid sequence identity. Vaccine safety concerns are alleviated by the observation that these vaccine candidates lack human homologs, while sera from human populations suffering repeated GAS infections and high levels of autoimmune complications do not recognize these enzymes. Our study demonstrates anchorless cell surface antigens as promising vaccine candidates for the prevention of GAS disease.

**Keywords** *Streptococcus pyogenes* · Arginine deiminase · Trigger factor · Vaccine

## Introduction

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

A safe and efficacious commercial vaccine to prevent GAS infection has yet to be developed, and this critical public health goal is fraught with pitfalls posed by the pathogen's unique phenotypic features. In contrast to *Streptococcus pneumoniae*, where commercial vaccines have been developed using conjugated capsular polysaccharides (CPS) of the most common serotypes, the CPS of all GAS strains is a homopolymer of hyaluronic acid, a common sugar in human connective tissues, and therefore immunologically inert. The antigenically diverse surface-anchored GAS M proteins are capable of eliciting protective immunity; however, comparison of the encoding (*emm*) genes indicates sequence diversity in the N-terminal hypervariable region, and adjacent 'A' repeat domains generate over 100 distinct GAS *emm* types [3]. Regions of the M protein dimeric coiled-coil structure are implicated in eliciting cross-reactive antibodies against human cardiac and basal

ganglia tissue that are key to the immuno-pathogenesis of rheumatic fever, thereby raising important concerns regarding vaccine safety. Nonetheless, immune sera raised against 30 defined amino-terminal M protein fragments in the 30-valent experimental M protein vaccine did not cross-react with human brain, kidney, or heart tissue in indirect immunofluorescence assays. These authors also demonstrated considerable cross-protection of the 30-valent vaccine against non-vaccine M serotypes [4].

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

## Materials and methods

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

**Protein identification by two-dimensional electrophoresis** To identify the major cell wall-associated proteins of invasive MIT1 clonal 5448 *S. pyogenes* isolate [8] grown in the presence of the cysteine protease inhibitor E64, a two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) proteomic analysis of a mutanolysin cell wall extract was performed as previously described [17].

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

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

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	TTATGCTTTGTTAGCTGATCCGAAAAC

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

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

*Immunization and intraperitoneal challenge of mice with GAS* Three immunization and intraperitoneal challenge experiments were performed. For the first experiment, 4- to 6-week-old (Animal Resource Centre, Australia) female BALB/c mice ( $n=20$  for test antigens;  $n=30$  for controls) were subcutaneously immunized with 10  $\mu\text{g}$  recombinant protein adjuvanted with complete Freund's adjuvant (CFA) in the primary immunization. Two mice immunized with recombinant ADI died of incidental causes prior to challenge. Mice were challenged via the intraperitoneal route on day 56 with approximately  $8 \times 10^6$  CFU of GAS M1 serotype strain pM1 [14]. Results of independent replicates ( $n=10$ ) were combined for each test antigen. Mice were monitored twice a day for 10 days after challenge and euthanized when they exhibited defined humane endpoints approved by University of Wollongong and Queensland Institute of Medical Research Animal Ethics Committees. For the second experiment, 5-week-old (Charles River, Italy) female CDI mice ( $n=48$  for ADI, ADI and TF combination, and Alum negative control;  $n=42$  for TF;  $n=24$  for M protein positive control) were intraperitoneally immunized with 10  $\mu\text{g}$  recombinant protein combined with Alum on days 0, 21, and 35. Groups of mice were challenged via the intraperitoneal route on day 55 with  $1.35 \times 10^6$  CFU of GAS serotype M1 strain pM1. Results of three independent replicates were combined and presented for each test antigen. For the third experiment, 5-week-old (Charles River, Italy) female CDI mice ( $n=48$  for ADI and TF combination, and Alum negative control;  $n=24$  for M protein positive control) were intraperitoneally immunized with 10  $\mu\text{g}$  recombinant protein combined with Alum on days 0, 21, and 35. Groups of mice were challenged via the intraperitoneal route on day 55 with  $1.2 \times 10^2$  CFU of GAS serotype M12 strain M12-2728. Results of three independent replicates were combined and presented for each test antigen. In experiments 2 and 3, mice were monitored on a daily basis for at least 1 week after treatment and euthanized

when they exhibited defined humane endpoints that had been pre-established for the study in agreement with Novartis Animal Welfare Policies. Experimental protocols described in this report complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, Australia).

*Intraperitoneal immunization and subcutaneous challenge of mice with a hypervirulent covS mutant GAS strain* Four- to six-week-old female C57BL/6 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  $\mu\text{g}$  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  $2 \times 10^8$  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 10 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), while the secondary anti-serum was goat anti-mouse IgG-horseradish peroxidase (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) [14], 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 ( $OD_{600}=0.6$ ) was washed twice in filtered phosphate buffered saline (PBS) and non-specific binding sites and Ig-binding proteins blocked with 3% (w/v) bovine serum albumin (BSA)/PBS with Tween 20 (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 fluorescein isothiocyanate (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 FACS sort 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 analyzed 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 (1) school-aged Aboriginal children ( $n=30$ ) living in remote communities in the Northern Territory, Australia; (2) Indian children and young adults (9–22 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 twofold 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 normalize 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].

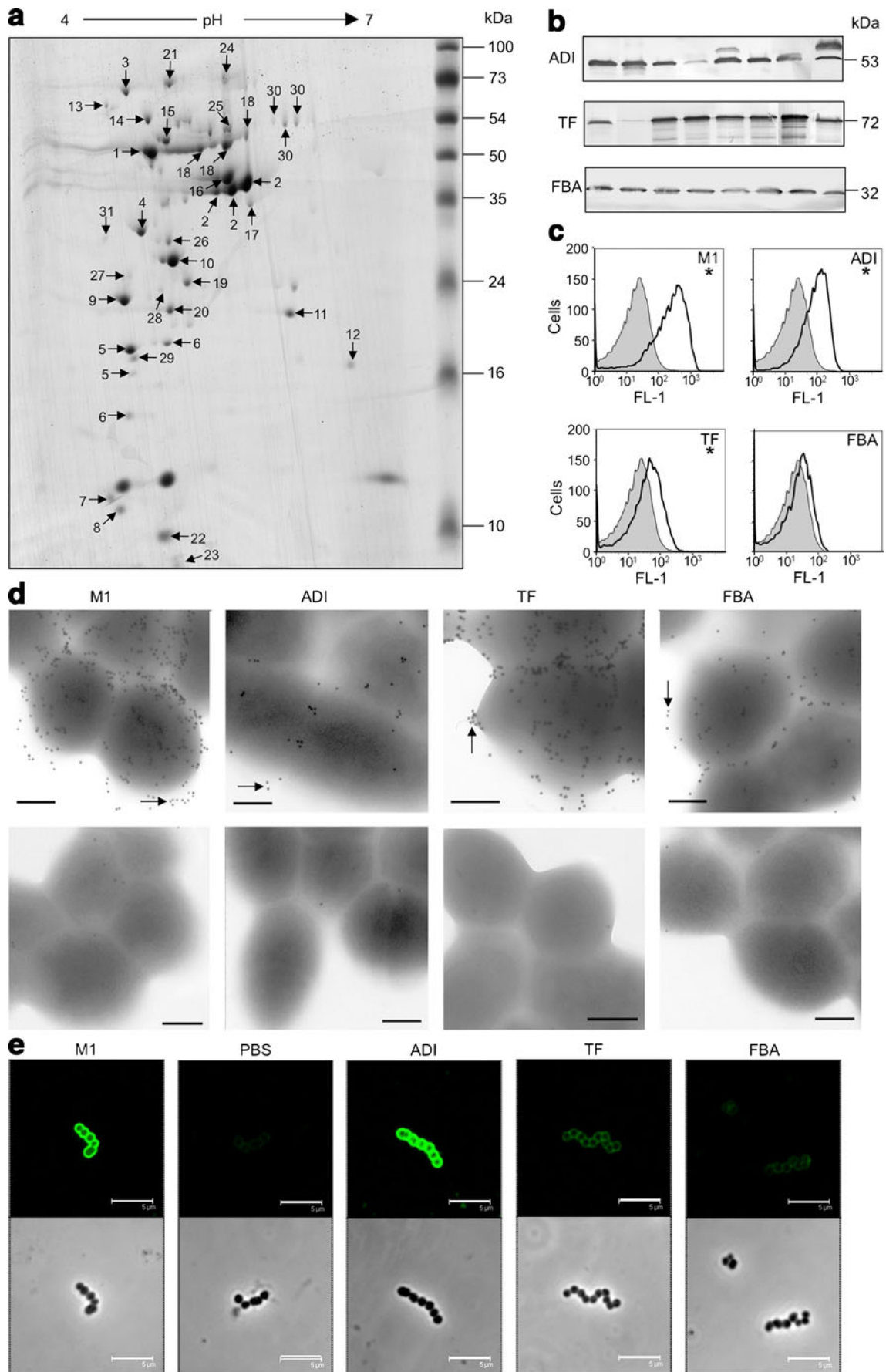
**Fig. 1** Identification, serotype distribution, and surface localization of novel GAS antigens. **a** Representative two-dimensional gel electrophoresis profile of GAS strain 5448 mutanolysin cell wall extract. Protein spots identified by peptide mass mapping following trypsin digest and MALDI-TOF 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 analyzed 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  $\mu$ m. For each experiment (a–e), two independent replicates were prepared, and representative data are presented

**Cross-reactivity of candidate antigen polyclonal sera with human heart extract** Human heart tissue (5 g) was homogenized in 5 mL of PBS containing complete protease inhibitor cocktail (Roche) using a PolyTron PT2100. Debris was removed by centrifugation at 12,000 $\times$ g for 30 min at 4°C. The reactivity between human heart proteins and test serum from rabbits immunized with individual recombinant antigens was tested by ELISA. Ninety-six-well plates were coated with 20  $\mu$ g/mL of the human heart extract in 0.1 M carbonate coating buffer (pH 9.6) and blocked with 1% (w/v) gelatin in PBS. The test serum was diluted twofold 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 and 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 with 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, the geometric mean of the flow cytometry histograms resulting from the surface labelling of the test serum were compared with the geometric mean of the sham-immunized serum histogram using an unpaired  $t$  test with a confidence interval of 95%. All statistical tests were performed using GraphPad™ Prism software version 4.2.

## Results

### Proteomic analysis of GAS cell wall extracts

Proteomic analysis of GAS cell wall extract was undertaken using isolate 5448, a representative of the globally disseminated M1T1 clonal serotype [8]. The cell wall extract was separated by two-dimensional gel electrophoresis and the landmark proteins identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. In total, 31 distinct cell-wall-associated proteins were identified (Fig. 1a; Supplementary Table 1). Among these were metabolic enzymes that share significant amino acid sequence identity with human homologs (e.g., GAS  $\alpha$ -enolase shares 49% identity with human  $\alpha$ -enolase), which raises concern for autoimmune consequences [24]. For further characterization, we chose three candidates that are not represented in the human proteome and do not share significant amino acid sequence identity with other human proteins as determined by BLAST-P interrogation (Supplementary Table 1): ADI [25, 26] and TF [27, 28], which are potential GAS virulence determinants, and FBA, which affords vaccine protection against *S. pneumoniae* [29]. All three enzymes lack N-terminal signal sequences and C-terminal cell wall anchors [7]. The genes encoding these three 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

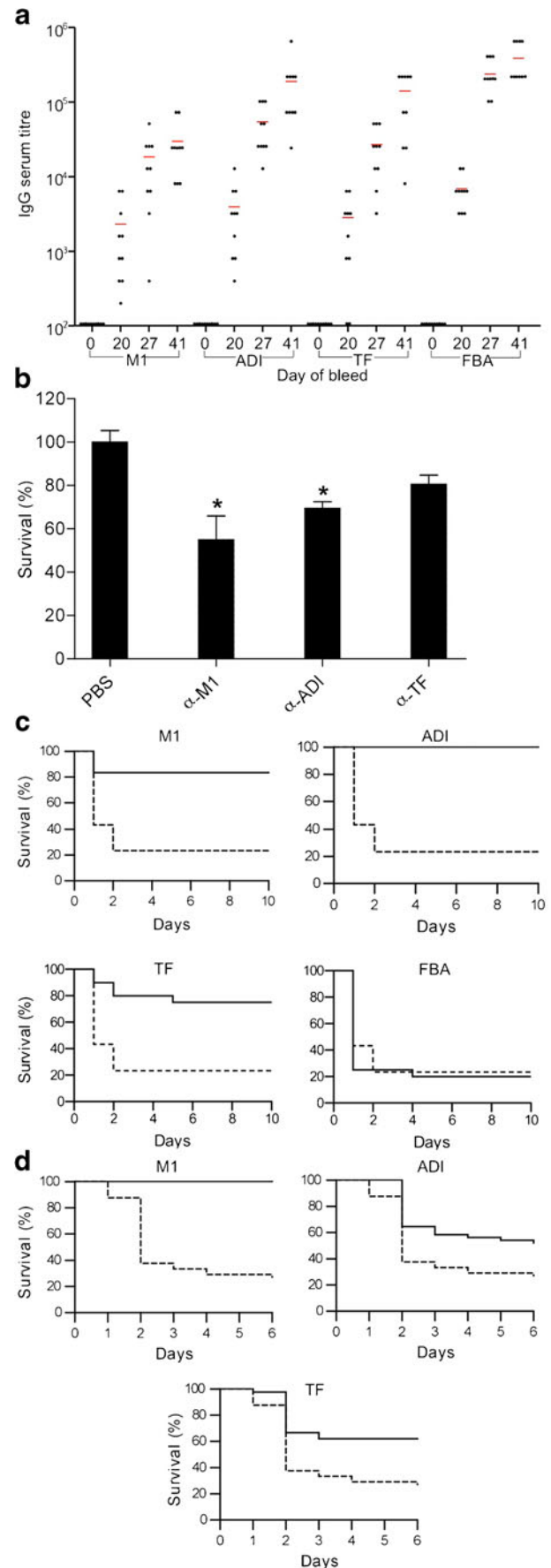
were undertaken utilizing antigen-specific polyclonal anti-serum. Anti-serum raised against the well-characterized surface-bound M protein was used as the positive control, while serum from mice immunized with PBS, or animal matched pre-immune serum was used as the negative controls for this series of 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 ( $P < 0.05$ ; Figs. 1c and 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 with 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 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 with 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 with 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

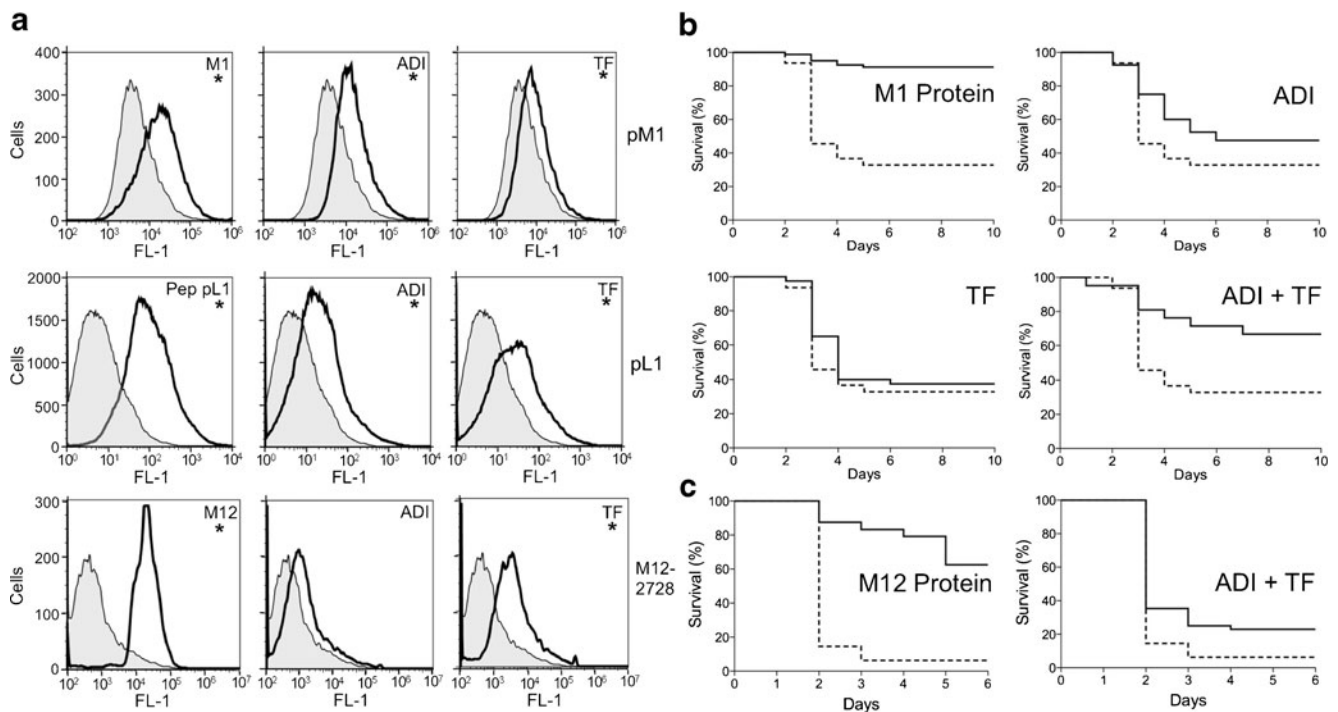
**Fig. 2** ADI, TF, and FBA antigens produce high-titer antibodies and protect against lethal intraperitoneal GAS challenge. **a** Serum-specific IgG antibody titers in BALB/c mice ( $n=10$ ) following subcutaneous immunization of antigen on days 0, 21, and 28. Mice serum was tested for specific reactivity 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 titers 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 heparinized human blood. Quadruplicate data from two repeat experiments are presented. Percent survival of GAS was calculated in comparison to the number of GAS (colony-forming units (CFU) per milliliter) surviving in the PBS sham-immunized serum control. Asterisk indicates a significant difference ( $P<0.01$ ) between the test antiserum and the PBS sham-immunized control serum. **c** Kaplan–Meier survival curves for intraperitoneal challenge of immunized BALB/c mice with a lethal dose of pM1 GAS,  $8 \times 10^6$  CFU ( $n=18$  for ADI;  $n=20$  for TF and FBA). M1 protein ( $n=30$ ) was selected as a positive control, while PBS was the negative control ( $n=30$ ). Antigens were co-administered with CFA in the primary immunization. Two independent replicates were performed for ADI, TF, and FBA; three independent replicates were performed for the controls, M1 and PBS; each repeat contained  $n=10$  mice for each antigen or control. **d** Kaplan–Meier survival curves for intraperitoneal challenge of CD1 mice with  $1.35 \times 10^6$  CFU of pM1 GAS ( $n=48$  for ADI,  $n=42$  for TF). M1 protein ( $n=24$ ) was used as a positive control, while PBS was the negative control ( $n=46$ ). Antigens were co-administered with Alum. Three independent replicates were performed; all data are presented



TF were used to immunize C57BL/J6 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 ( $P>0.05$ ), while 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



**Fig. 3** **a** Quantification of antigens on the surface of GAS isolates pM1, pL1, and M12-2728 using flow cytometry. Histogram for test antigens and positive control indicated with *solid line*; histogram for sham-immunized serum denoted by *shading*. Sera raised against M protein, pepsin M protein extracts, ADI, and TF produced a shift in cell surface FITC fluorescence compared with PBS sham serum. A significant shift in fluorescence ( $P < 0.05$ ) is indicated with an *asterisk*. Samples were analyzed in triplicate. **b** Kaplan–Meier survival curves for subcutaneous challenge of C57BL/6 mice (co-immunized with CFA) with a lethal dose of 5448AP GAS,  $2 \times 10^8$  CFU ( $n = 40$  for

ADI,  $n = 40$  for TF,  $n = 21$  for ADI+TF cocktail). M1 protein ( $n = 80$ ) was selected as a positive control, while PBS was the negative control ( $n = 79$ ). At least two independent repeats were performed; all data are presented. **c** Kaplan–Meier survival curves for intraperitoneal challenge of CD1 mice (co-immunized with Alum) with  $1.2 \times 10^2$  CFU of M12-2728 GAS ( $n = 48$  for ADI+TF cocktail). M12 protein ( $n = 24$ ) was selected as a positive control, while Alum was the negative control ( $n = 48$ ). Three independent replicates were performed; all data are presented

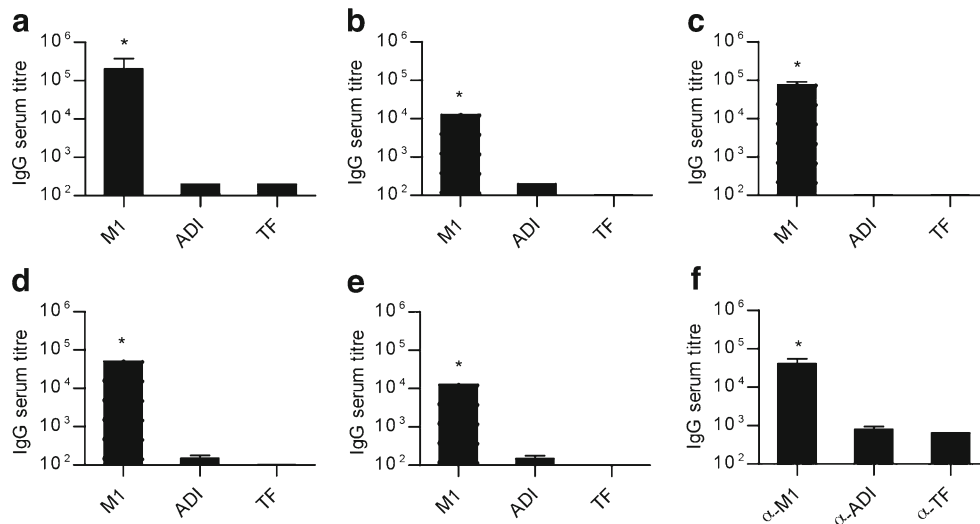
whether members of this population have pre-existing immunity against ADI and TF, hypothesizing that an immune response against these protective antigens may be lacking in a human population that suffers repeated GAS infections [33]. The Aboriginal immune response was determined using a pool of serum ( $n = 30$ ) 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–e). 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 or expressed by all serotypes of GAS. The genes encoding each of the anchorless vaccine antigens of this study were ubiquitous among sequenced GAS genomes with  $\geq 99\%$  conservation among serotypes. In addition, the anchorless vaccine antigens were detected in mutanolysin-derived cell wall extracts of eight representative clinical GAS isolates (Fig. 1b). While





**Fig. 4** Reactivity of antigens with human serum and cross-reactivity with human heart extract. The human IgG response against M1 protein, ADI, and TF was determined using **a** a pool of serum ( $n=30$ ) from Aboriginal children living in remote communities of the Northern Territory (Australia) suffering endemic GAS infection, **b** a pool of serum ( $n=10$ ) from individuals with no disease symptoms in Chandigarh (India), **c** a pool of serum ( $n=8$ ) from patients with pharyngitis in Chandigarh (India), **d** a pool of serum ( $n=9$ ) from patients with acute

rheumatic fever in Chandigarh (India), and **e** a pool of serum ( $n=10$ ) from patients with rheumatic heart disease in Chandigarh (India). **f** Reactivity of rabbit polyclonal antisera raised against M1 protein, ADI, or TF with human heart extract. *Asterisk*: response against M1 protein is significantly higher than test antigens ( $P<0.01$ ). Error bars represent the standard error of the mean. For each component of figure (**a–f**), duplicate data from two independent replicate experiments are presented

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 (Figs. 1c and 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,  $\alpha$ -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  $\alpha$ -enolase and GAPDH were among 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, d). 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 hypothesized 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, the

combination ADI and TF was 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. 4f), in contrast to anti-M1 protein serum. These results suggest that, unlike full-length M protein, the B cell epitopes contained within ADI and TF do not cross-react with human cardiac proteins.

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

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