Recombinant Group A Carbohydrate backbone embedded into Outer Membrane Vesicles is a potent vaccine candidate targeting Group A Streptococcus from *Streptococcus pyogenes* and *Streptococcus dysgalactiae* subsp. *equisimilis*

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Running title: First recombinant carbohydrate-based vaccine candidate for Group A Streptococcus

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Background: Group A Streptococcus (GAS) are responsible for a wide range of human-exclusive infections, annually killing more than 500,000 people. Antibiotic resistance incidence of invasive GAS tripled in the past decade and emphasises the need to develop a universal GAS vaccine. We have produced, for the first time, a recombinant polyrhamnose backbone (pRha), a validated universal GAS vaccine candidate. *E. coli* outer membrane vesicles (OMVs) carrying pRha were investigated for their immunogenicity and efficacy in an animal model.

Methods: OMVs decorated with pRha were administered to C57BL/6J mouse and rabbit models. Flow cytometry, ELISA, Luminex, immunofluorescence microscopy and serum bactericidal assay assays were conducted to investigate the ability of pRha-specific antibodies to recognise and kill clinical (hypervirulent) GAS strains.

Results: Our results suggest that pRha-OMVs induce specific antibodies which recognise Group A Carbohydrate (GAC) from *S pyogenes* and *S. dysgalactiae* subsp.

equisimilis. Increased IgG levels correlate with increased bactericidal killing of the hypervirulent GAS M89 strain. Elevated IL-17a from pRha-OMV-immunised splenocytes indicates possible stimulation of long-term memory immune cells.

Conclusion: We are the first to report efficacy and potency of this unique, exogenously produced polysaccharide, pRha, in the induction of humoral-mediated immune responses to GAS.

Key words: Group A Streptococcus, Group A Carbohydrate, Rhamnose, Outer membrane vesicles, M protein, Vaccine

Topic: *Streptococcus pyogenes*, immunoglobulins, polysaccharides, opsonophagocytosis, acute rheumatic fever, M protein, invasive Group A Streptococcus, hyaluronic acid

Introduction

Streptococcus pyogenes, frequently described as Group A Streptococcus (GAS), is a beta-haemolytic Gram-positive human exclusive bacterium causing a wide range of illnesses in both healthy and immunocompromised individuals. Breaching of immune barriers by GAS leads to acute suppurative effects in the skin and pharyngeal epithelia, causing localised skin infections, tonsillitis and enlarged lymph nodes in the throat [1]. Prolonged prevalence of GAS in the epithelial cells has proven to lead to life-threatening complications such as streptococcal toxic shock syndrome, necrotising fasciitis and, importantly, post-streptococcal autoimmune sequelae [2]. One of these major autoimmune sequelae is rheumatic heart disease, which kills >300,000 patients worldwide [1, 3]. Incidence of antibiotic resistance has tripled in the past decade, and the common penicillin-based antibiotics now only eradicate ~65% of all tonsilitis cases caused by GAS infections [4, 5].

52 Identification of GAS is based on the detection of its main surface antigen, the Group A
53 Carbohydrate [GAC]. This consists of a polyrhamnose (pRha) backbone (→3)α-Rha(1→2)α-
54 Rha(1→) with an immunodominant N-acetylglucosamine (GlcNAc) side chain present on
55 every α-1,2-linked rhamnose [6-8]. Recent studies have reported the presence of negatively
56 charged glycerol-phosphate onto approximately every fourth GlcNAc sidechain [9]. Earlier
57 research proposed GAC as a potential antigen due to i) its abundant presence in the cell wall
58 contributing ~50% of GAS by weight, ii) its conservation across all >200 GAS serotypes and
59 sequenced GAS genomes [10], and iii) the absence of cross-reactivity between antibodies
60 raised against endogenously-produced pRha and human cardiac antigen [11, 12].

61 GAC antibodies were frequently found at high-titres in serum samples, directly correlating with
62 the presence of GAS in the throat of children [13], and anti-GAC antibody titres persist in
63 patients with rheumatic valvular disease [14]. Moreover, a strong body of evidence shows that
64 injecting purified or synthetic components of GAC conjugated to a carrier protein elicits an
65 immune response that confers protection against multiple GAS serotypes [13, 15]. However,
66 the anti-GAC antibodies raised from immunisation have safety concerns: N-GlcNAc-specific
67 antibodies against the sidechain cross-react with human tissues [16, 17]. Importantly, this was
68 not seen in antibodies raised against the pRha backbone alone [11, 12] suggesting that pRha
69 could be a safe and effective vaccine candidate.

70 Outer membrane vesicle (OMV)-based vaccine candidates produced in *E. coli* have been in
71 development for more than ten years and have shown efficacy against several pathogens [18,
72 19]. Here, we show that recombinantly produced pRha-OMVs carry the correct pRha structure,
73 as revealed by linkage analysis, identical to the natively-produced pRha in a GAS mutant
74 strain that is deficient in the GlcNAc side chain [11, 12]. We show that *E. coli* pRha-OMV are
75 immunogenic in a mouse model; moreover, the stimulated IgG antibodies are able to opsonise
76 GAS isolates and *S. dysgalactiae* subsp. *equisimilis* (SDSE) and promote the killing of
77 hypervirulent GAS strains. Increased IL-17a indicated the possible stimulation of cellular-
78 mediated immune response in pRha-OMV vaccinated animals. We thus present an alternative

route to recombinantly produce a universal GAS OMV-based vaccine candidate, removing the requirement to isolate the pRha chemically or enzymatically from GAS cells.

Materials and Methods

Bacterial strains and growth conditions

E. coli and GAS isolates were used from stored glycerol stocks (25%) and streaked out onto appropriate media (LB and Todd–Hewitt Broth [THY], respectively) to obtain single colonies. A single *E. coli* colony was cultured in 10 mL LB medium supplemented with 150 µg/mL erythromycin, grown at 37˚C, 200 rpm overnight and used to inoculate 1 L medium, grown for 18 h. GAS isolates were grown at 37 °C, 5% CO_2 in THY. Both *E. coli* and GAS isolates were used as overnight cultures for Western blot analysis or used at 1:100 to $O.D_{600}$ 0.4 for serum bactericidal assay, flow cytometry and ELISA assays.

Ethical approval

The experiments involving mice were approved by the University of Dundee welfare and ethical use of animals committee and conducted in accordance with the UK Home Office approved project licence [PPL PEA2606D2]. The experiments involving rabbits were conducted in association with Davids Biotechnologie GmbH, Germany.

Purification of pRha-OMVs

E. coli pRha-OMVs and negative control (empty OMVs without pRha) were isolated from CS2775 cells transformed with plasmids pHD0136 and pHD0139 [9]. Cells were harvested by centrifugation (4,500 RPM, 30 min, 4ºC). The supernatant was filtered (0.45µm) and subjected to ultracentrifugation (Type 45Ti, 45,000 RPM, 4 hrs, 4ºC). The pellets were weighed, resuspended in PBS, and protein content determined using the Bradford method. The OMVs were analysed for their average size distribution in the Zetaview nanoparticle tracking analysis instrument, and LPS levels assessed using the Limulus Amoebocyte Lysate (LAL) assay (Thermo Fisher, UK).

104 **Immunisation**

105 Mouse Model: Five- to six-week-old female C57BL/6J mice were acquired from Charles River
106 Laboratories, UK, and were acclimatised for 10 days prior to immunisation. Mice (n=6) were
107 immunised subcutaneously with pRha-OMV (10 µg diluted in 100 µl of endotoxin free
108 PBS/mouse/injection) or *E. coli* OMV (n=6) on day 0, immediately following tail bleed for pre-
109 immune sera. Identical booster injections were administered on day 21 and day 49. Animals
110 were euthanised by CO_2 asphyxiation and bled by cardiac puncture on either day 70 or day
111 84. For adjuvant studies, Imject Alum (Sigma) was used with 10 µg of pRha-OMV (1:1 ratio);
112 adjuvant with PBS only (1:1 ratio) served as a negative control. PBS/Alum vaccinated mice
113 (n=3) were used for baseline measurements.

114 Rabbit Model: For New Zealand White rabbit immunisation, the animal (n=1) was bled for pre-
115 immune serum prior to immunising with the purified OMV pRha (150 µg diluted in 150 µl of
116 endotoxin-free PBS/injection) on day 0. Booster injections were administered on days 14, 28,
117 42 and 56, at 100 µg in 100 µl of endotoxin-free PBS/injection. The animal was culled by CO_2
118 asphyxiation and bled by cardiac puncture. Final bleed serum was taken on day 63 and
119 affinity-purified (0.53 mg/ml) by Davids Biotechnologie GmbH.

120 **Flow cytometry**

121 Antibody binding to GAS and *E. coli* cells was analysed using the procedure adapted from
122 [20]. Bacterial cells (2×10^6 CFU) were resuspended in nonspecific human IgG (Sigma, UK)
123 for one hour on ice, then washed with PBS and diluted 1:100 with immunised mice serum
124 (PBS or pRha-OMV) overnight at 4°C. Cells were washed twice with PBS and stained with
125 1:250 dilution of Alexa fluor 488-conjugated goat anti-mouse IgG (Thermo Fisher, UK),
126 incubated for 20 minutes at 4°C in the dark. Cells were washed twice (PBS) and fixed with
127 500 µl of 4% PFA and analysed by flow cytometry (BD Bioscience). A total of 10,000 events
128 were acquired and data was analysed using FlowJo software version 10.6.2.

129 **ELISA**

130 For antigen-antibody interaction, plates were coated with 50 µl/well of 1 µg/ml pRha-
131 OMV/negative control OMVs or *S. pyogenes* cells adapted from [21, 22]. Briefly, bacterial cells
132 at OD_{600} of 0.4, from the overnight culture, were resuspended (1:100) in PBS. Mouse serum
133 from immunised animals was added, 50 µl/well at 1:1,000. Bound antibodies were probed
134 using 50 µl/well of 1:1,000 of anti-mouse IgG HRP (Sigma–Aldrich) and 75 µl/well of
135 tetramethylbenzidine substrate (Sigma–Aldrich). The reaction was stopped using 75 µl/well of
136 1 M H_2SO_4, and absorbance read at 450 nm.

137 **Luminex**

138 Immunoglobulins in mouse sera were quantified using a Milliplex Multiplex assay (Merck
139 Millipore, UK). Immunoglobulin beads (IgA, IgG1, IgG2a, IgG2b) from Mouse Antibody
140 Isotyping 7-Plex ProcartaPlex were used as analytes and the samples were prepared
141 according to manufacturer's instructions (Thermo Fisher, UK).

142 **Immunoblot analyses**

143 Antisera from pRha-OMV vaccinated animals were tested for binding to *E. coli* cells or GAS
144 by Western blot analysis using 12.5% acrylamide PAGE gels poured according to
145 manufacturer's instructions (Thermo Fisher, UK). Cells of *E. coli* negative control and *E. coli*
146 pRha-OMV were used directly from overnight cultures. Overnight-grown GAS cells were
147 washed with PBS and incubated with 6 µl of PlyC (0.7 mg/ml) for one hour (37°C, 300 RPM)
148 and centrifuged for 14,000 RPM, 5 mins. The resulting pellets were resuspended in 2x SDS-
149 PAGE loading dye and subjected to Western blot analysis according to manufacturer's
150 instructions (Invitrogen, UK). The PVDF membranes were blocked with 5% non-fat dried milk
151 in Tris-Buffered Saline, 0.1% Tween® 20. Immunised mouse sera were used at 1:1000 dilution
152 to probe the blots (overnight, 4°C, 20 RPM) followed by goat anti-mouse IgG HRP at 1:1000
153 dilution for two hours at 4°C, 20 RPM. Rabbit anti-GAC antibodies (Abcam, UK) with goat anti-
154 rabbit IgG HRP (Abcam, UK) (1:2,500) were used as a positive control. Antibody binding was

155 visualised using Clarity Western ECL Substrate (Biorad, UK) and viewed under the Gel Doc
156 imaging system (GeneSys software, Syngene).

157 **Isolation and *ex vivo* re-stimulation of splenocytes**

158 Isolation of mouse spleens and re-stimulation with antigens was conducted according to
159 published procedures [23]. Briefly, spleens were rinsed and flushed, using 21G needles, with
160 5 ml of RPMI 1640 media (supplemented with 100 U/ml Pen-Strep (Sigma, UK), 200 mM L-
161 glutamine (Gibco, UK) and 10% heat-inactivated foetal bovine serum (Sigma, UK)). The
162 dispersed cells were centrifuged (300 x g, 10 min) and red blood cells were lysed in lysis buffer
163 (Sigma, UK). Cells were washed and centrifuged again by the addition of 10 ml of PBS (300
164 x g, 10 min). Pellets were resuspended in complete RPMI 1640 media and adjusted to $7.5x10^6$
165 cells/ml; 1.5 x 10^6 cells [200 µl] were added to each well of a 96 well plate followed by 50 µl of
166 1 µg/ml pRha-OMV and incubated for three days. Cells were pelleted (400 x g, 10 min) and
167 supernatants subjected to ELISA analysis for IL-17a (Thermo Fisher, UK).

168 **Microscopic analysis**

169 GAS M89 cells were grown as mentioned above. Briefly, overnight culture of M89 strains were
170 washed twice with PBS (10,000 RPM, 5 minutes). Washed cells were stained overnight at 4°C
171 with pre-rabbit or pRha-OMV post immune sera at 1:100 dilution. Prior to adding secondary
172 antibody (goat anti-rabbit FITC in 1:50) the cells were washed twice with PBS. The FITC-
173 stained cells were mounted and viewed using a DeltaVision microscope; images were
174 deconvoluted and analysed using softWoRx imaging system.

175 **Serum Bactericidal assay (SBA)**

176 SBA assays were carried out according to [24, 25]. Briefly, in a 96 well plate 25 µl of HBSS
177 medium containing $CaCl_2$ and $MgCl_2$ (Gibco) were added as buffer, followed by 25 µl of rabbit
178 antiserum (1:100 dilution): either pre-immune or pRha-OMV immune serum. The antibodies
179 were incubated with 12.5 µl of M89 (2×10^5) cells in the presence of activated or inactivated
180 baby rabbit complement (37°C, 3h, 5% (v/v) CO_2), then 12.5 µl of mixed cells was spotted at

181 the start of the incubation (T0) and at the end of incubation (T3) onto sheep blood agar plates
182 (Oxoid, UK) and incubated overnight (37˚C, 5% (v/v) CO_2). Colonies were counted from
183 duplicate wells and analysed for the percentage of bacterial survival.

184 **Statistical Analysis**

185 Data was analysed using GraphPad Prism Version 8 (La Jolla, CA, USA). One-way analysis
186 of variance [ANOVA] with Bonferroni's correction, two-tailed T-test followed by Dunn's post-
187 test were used to test the statistical significance. FlowJo analysis software (FlowJo, USA) was
188 used to plot the geometric mean fluorescence intensity (gMFI) of flow cytometric data.

189 **Results**

190 **Recombinantly produced polyrhamnose-OMVs carry the antigen on Lipid A**

191 The polyrhamnose (pRha) carbohydrate antigen with the immunospecific repeat unit of two
192 rhamnose sugars, linked via the alpha1,2-1,3 repeating unit, is found in GAS, GCS and *S.*
193 *mutans,* and also in newly emerging isolates from *S. dysgalactiae* subsp. *equisimilis* [11, 26-
194 28]. We recombinantly expressed the gene cluster from *S. mutans sccA-G* and *S. pyogenes*
195 in *E. coli* [9, 29]. Our analysis revealed that the pRha thus produced is deposited on the outer
196 membrane Lipid A, transferred onto it in the inner membrane via the O-antigen ligase WaaL
197 [Fig 1A & Supplementary figure 1A] [30, 31]. Subsequently, we isolated OMVs that were either
198 decorated with the pRha (pRha-OMV) [Fig 1B] or lacking the pRha (empty OMVs) and
199 analysed them using Group A Carbohydrate specific antibody. Only the pRha-OMVs were
200 detected by the antibodies, confirming the decoration of the OMVs with pRha [Fig 1B]. The
201 purified OMV's were quantified and tested for LPS content using the LAL kit, confirming the
202 endotoxin levels at 0.7 EU/ml [Supplementary figure 1B]. The OMV average particle sizes
203 were in agreement with previously reported *E. coli* OMVs, with a diameter range of 50–250
204 nm [Supplementary figure 1C] [32].

205 OMVs have been shown to induce IgG antibodies in animals [33] as well as in humans [34].
206 We immunised mice with either pRha-OMV or control OMVs and measured the levels of pRha-

207 OMV-specific IgG by exposing the antibodies to *E. coli* control strain that produces a higher
208 level of polyrhamnose [Fig 1C, D]. The pRha-OMV-specific IgG levels increased in pRha-OMV
209 immunised animals compared to those in control animals (immunised with empty OMV),
210 demonstrating increased binding of the anti-pRha-OMV antibodies to the polyrhamnose
211 compared to anti-OMV titres [Fig 1C, D].

212 **pRha-OMV stimulated antibodies selectively bind to the pRha epitope**

213 Final bleed sera from each group were pooled and analysed by flow cytometry for their ability
214 to recognise the pRha antigen expressed on *E. coli* cells compared with control cells lacking
215 pRha. Histograms for antibody deposition and extraction of gMFI from the flow cytometry data
216 generated using pRha-OMV immunised animals clearly revealed increased IgG antibody
217 deposition onto *E. coli* pRha-bound OMVs, compared to control anti-OMV IgG [Fig 2A, B],
218 indicating that selective paratopes are expressed in the sera of the pRha-OMV vaccinated
219 animals. Importantly, the gMFI for pRha-OMV was significantly higher than that of the negative
220 control (OMV) on exposure to pRha positive *E. coli* cells suggesting strong immunogenicity of
221 the pRha [Fig 2B]. This agrees with the observation that OMVs alone stimulated production of
222 antibodies that recognise *E. coli* cells without pRha, and, to a lesser degree, *E. coli* decorated
223 with pRha [Fig 2B]. Interestingly, the levels of cross-reactivity were similar for both species
224 (~50 gMFI units), supporting our interpretation that pRha-OMVs stimulate production mainly
225 of antibodies against the pRha but also, to a lower extent, against the OMVs.

226 Using ELISA, serum from the pRha-OMV-vaccinated animal showed significantly higher
227 binding affinity for the pRha-bound *E. coli* than for OMV or PBS immunised animals [Fig 2C].
228 These data indicate that the pRha antigen on the *E. coli* cell was recognised robustly by the
229 pRha-OMV-immunised animals. Control, OMV-immunised, animal sera showed background
230 staining possibly as a result of anti-OMV activity in these animals. Although, the antibody
231 response from the OMV vaccinated animals was lower towards the pRha producing *E. coli*
232 cells [Fig 2C], the anti-OMV titre in these animals was significantly higher in negative control
233 cells alone compared to anti-pRha-OMV titre groups [Fig 2D]. Immunoblot analysis of pRha-

234 OMV immunised serum showed a strong signal in *E. coli* pRha-OMV lysate confirming the
235 presence of GAC [Supplementary Fig 1D].

236 **Validation of IgG subtypes in pRha-OMV immunised mice**

237 Previous research has shown a correlation between frequent exposure to polysaccharides
238 and increased IgG subtypes in humans and animals [35-37]. Hence, we examined the effect
239 of pRha-OMV on the IgG subtypes in mice (IgG1, IgG2a, IgG2b and IgG3) using Luminex
240 analysis [Fig 3]. The Ig subtypes were analysed in pooled sera of the PBS and pRha-OMV
241 immunised animals on day 49 (after the administration of two boosters) and on day 70 (after
242 three boosters) [Fig 3]. Our results show a pattern of IgG subtype responses, revealing
243 increased levels of total IgG2b [Fig 3C], IgG3 [Fig 3D] and IgG2a [Fig 3B], with lower levels of
244 IgG1 [Fig 3A], from both PBS and anti-pRha OMV sera. However, the anti-pRha OMV IgG
245 subtypes were significantly higher compared to PBS immunoglobulin levels and sustained
246 until the end of the immunisation schedule (day 70).

247 **pRha-OMV antibodies recognise clinical GAS isolates**

248 The efficacy of the pRha-OMV antibodies was further investigated by testing their ability to
249 recognise GAS isolates using flow cytometry and ELISA analyses. To assess the opsonisation
250 of a wide range of clinical GAS serotypes, flow cytometry was performed using antibody titres
251 from all groups. The gMFI obtained from the flow cytometry data showed significantly
252 increased antibody deposition in sera of animals immunised with pRha-OMV compared to that
253 in control immunised animals [Fig 4A]. However, not all GAS serotypes were recognised by
254 the pRha-OMV IgG. Addition of aluminium adjuvant to the pRha-OMVs increased antibody
255 deposition in mouse sera by recognising most of the GAS serotypes, except for M28 and $M1_{UK}$
256 [Fig 4B]. The degree of IgG binding to the GAS isolates varied highly across all tested
257 serotypes; the pRha-OMV with aluminium performed better than pRha-OMV serum alone [Fig
258 4A, B]. ELISA assays confirmed a similar response: pRha-OMV sera recognising GAS

259 serotypes, including the dominant clade M1T1 ($M1_{UK}$), a new *emm*1 *S. pyogenes* lineage [Fig
260 4C] [38].

261 Clinical GAS isolates, along with wild type M1 isolates M5448 (M1 WT) and a related genetic
262 mutant delta *gacI* (delta M1WT expressing only the pRha backbone) [11] were probed with a
263 commercially available Group A Carbohydrate antibody and compared to pRha-OMV and
264 negative control (OMV alone or PBS-immunised) mouse sera. GAS isolates show an intense
265 band at ~25 kDa on Western blots probed with sera from pRha-OMV mice, confirming the
266 specificity of the pRha antibodies. The band was absent in the control OMV sera. The positive
267 control (GAC antibody) displayed a stronger signal representing the presence of complete
268 GAC components (including GlcNAc/glycerol-phosphate sidechain) [Figure 4D].

269 **pRha-OMV sera stimulate IL-17a production in murine splenocytes**

270 We investigated the effect of the pRha-OMV immunogen on IL-17a production in splenocytes.
271 IL-17a, a key cytokine mediator, contributes to activation of T cells by mediating protective
272 innate immunity against GAS [39]. Increased IL-17a levels were detected in pRha-OMV
273 vaccinated splenocytes restimulated with pRha-OMV antigen, compared with splenocytes
274 from the PBS group [Fig 5A]. The data suggest that pRha-OMV induces a humoral mediated
275 immune response, and also a cytokine-mediated cellular immune response [39, 40],
276 potentially via carbohydrate-specific T helper cells [41].

277 **pRha-OMV immunised rabbit serum recognises GAS from *S. dysgalactiae* subsp.**
278 ***equisimilis* and kills the hypervirulent M89 GAS bacteria**

279 Having established that pRha-OMV antibodies raised in C57BL/6J mouse models are efficient
280 in opsonising GAS pathogens, we investigated the effect of pRha-OMV immunised rabbit
281 antibodies on the binding and killing of GAS strains. Increased rabbit pRha-OMV IgG antibody
282 deposition was observed on newly emerged *S. dysgalactiae* subsp. *equisimilis* (SDSE)
283 isolates, which have replaced their Group G Carbohydrate gene cluster with a functional
284 Group A Carbohydrate cluster (SDSE_*gac*) [27]. The anti-pRha-OMV sera recognised and

285 binds to SDSE_*gac* isolates Stg485 and Stg652 [Fig 5B]. Microscopic examination of the M89
286 strain treated with pRha-OMV rabbit serum displayed uniform cocci (long chains) revealing
287 the classical structure of GAS [Fig 5C]. Furthermore, rabbit pRha-OMV antibody, but not rabbit
288 pre-immune serum, was able to kill the hypervirulent strain M89 [Fig 5D]. Overall, these data
289 confirm the ability of recombinantly produced pRha to induce type-specific functional
290 antibodies that aid in promoting the recognition and killing of the M89 hypervirulent strain.

291 **Discussion and concluding remarks**

292 Several recent studies have addressed the production of universal GAS vaccine candidates
293 targeting the GAS polyrhamnose backbone of the group A carbohydrate. These approaches
294 focused on chemical extraction of natively produced pRha from GAS cells or chemical
295 conjugation to a carrier protein [12, 42]. We explored an alternative production route, and, for
296 the first time, we have succeeded in producing recombinant pRha embedded into *E. coli* OMVs
297 and evaluated them as vaccine candidates against GAS. The data demonstrate that pRha-
298 OMVs were effectively presented to the mouse immune system and triggered a strong immune
299 response specific for the pRha carbohydrate when supplemented with an adjuvant. This effect
300 agrees with the findings of a study investigating *E. coli* OMV decorated with β-(1→6)–linked
301 poly-N-acetyl-D-glucosamine (PNAG) [43]. PNAG is a conserved polysaccharide antigenic
302 component found in bacteria, fungi and protozoan cells. Immunisation studies on PNAG-OMV
303 provoked polysaccharide specific IgG antibodies.

304 The prevalence of IgG subtypes has been widely studied within the GAS infection models.
305 One of the most abundant subclasses of human IgG found against the GAS M proteins is IgG3
306 [44]. Notably, antibody isotype class switching from IgM to mouse IgG3 constant regions was
307 proven to be driven by the frequent exposure to polysaccharide antigens [45, 46]. A strong
308 IgG1 response was observed in humans exposed to polysaccharides from pneumococcal
309 pathogens [35] and increased IgG2a and IgG2b levels were detected in mice exposed to M
310 protein-based vaccines from *Streptococcus pyogenes* [47].

311 Previous studies have shown that immunisation of rabbits with the pRha backbone extracted
312 from native GAS promotes opsonophagocytic killing of multiple GAS serotypes and also
313 protects the animals against systemic GAS challenges [11]. Here, we observed that anti-pRha-
314 OMV IgG promoted bactericidal killing of the hypervirulent M89 strain mediated by baby rabbit
315 complement. Strikingly, recognition of SDSE_*gac* isolates by pRha-OMV antibodies
316 broadened the potential protection of a pRha-based vaccine to target newly emerging SDSE
317 isolates.

318 A major finding of this study is the increased expression of IL-17a stimulated by the pRha-
319 OMV antigen in mouse splenocytes. Carbohydrates are known to trigger a T cell independent
320 immune response and hence conjugating them with a carrier protein elicits T cell dependent
321 immune response. Recognition of polysaccharides by $CD4^+$ T cells were shown to induce
322 activation and differentiation which can be analysed by measuring the IL-17A cytokine [48].
323 IL-17-mediated protective immunity via neutrophil recruitment has been shown against GAS
324 [39], *Klebsiella pneumoniae* [49] and *Streptococcus pneumoniae* [50]. However, we cannot
325 rule out the possibility of a role for endogenous *E. coli* antigens in inducing IL-17a, which
326 needs further exploration.

327 In summary, we have demonstrated that the recombinantly produced pRha in *E. coli* OMVs
328 induces robust IgG antibodies in mice and stimulates IgG binding to all tested GAS serotypes.
329 Importantly, the antibodies efficiently kill the hypervirulent strain M89 implying possible IgG-
330 mediated protection in *in vivo* models. The antigenic properties shared between GAS and *E.*
331 *coli* cells requires further research, and possible avenues include producing detoxified LPS
332 recombinant pRha-OMV and studying its immunogenicity in animal models. Future exploration
333 of recombinantly-produced pRha-OMVs as a universal GAS vaccine candidate, as well as the
334 conjugation of recombinant pRha to a carrier protein, remains to be investigated.

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341 References

342 1. Cunningham, M.W., *Pathogenesis of group A streptococcal infections.* Clin Microbiol Rev,
343 2000. **13**(3): p. 470-511.
344 2. Ferretti JJ, S.D., Fischetti VA,, *Streptococcus pyogenes : Basic Biology to Clinical*
345 *Manifestations.* 2016.
346 3. Carapetis, J.R., et al., *The global burden of group A streptococcal diseases.* The Lancet
347 Infectious Diseases, 2005. **5**(11): p. 685-694.
348 4. Passàli, D., et al., *Group A streptococcus and its antibiotic resistance.* Acta
349 otorhinolaryngologica Italica : organo ufficiale della Societa italiana di otorinolaringologia e
350 chirurgia cervico-facciale, 2007. **27**(1): p. 27-32.
351 5. Musser, J.M., et al., *Reduced In Vitro Susceptibility of Streptococcus pyogenes to β-Lactam*
352 *Antibiotics Associated with Mutations in the pbp2x Gene Is Geographically Widespread.* J Clin
353 Microbiol, 2020. **58**(4).
354 6. Lancefield, R.C., *A serological differentiation of human and other groups of hemolytic*
355 *streptococci.* The Journal of experimental medicine, 1933. **57**(4): p. 571-595.
356 7. Mc, C.M., *Further studies on the chemical basis for serological specificity of Group A*
357 *streptococcal carbohydrate.* J Exp Med, 1958. **108**(3): p. 311-23.
358 8. Coligan, J.E., T.J. Kindt, and R.M. Krause, *Structure of the streptococcal groups A, A-variant*
359 *and C carbohydrates.* Immunochemistry, 1978. **15**(10-11): p. 755-60.
360 9. Zorzoli, A., et al., *Group A, B, C, and G Streptococcus Lancefield antigen biosynthesis is*
361 *initiated by a conserved α-d-GlcNAc-β-1,4-l-rhamnosyltransferase.* The Journal of biological
362 chemistry, 2019. **294**(42): p. 15237-15256.
363 10. Davies, M.R., et al., *Author Correction: Atlas of group A streptococcal vaccine candidates*
364 *compiled using large-scale comparative genomics.* Nature Genetics, 2019. **51**(8): p. 1295-
365 1295.
366 11. van Sorge, N.M., et al., *The classical lancefield antigen of group a Streptococcus is a virulence*
367 *determinant with implications for vaccine design.* Cell Host Microbe, 2014. **15**(6): p. 729-740.
368 12. Gao, N.J., et al., *Site-Specific Conjugation of Cell Wall Polyrhamnose to Protein SpyAD*
369 *Envisioning a Safe Universal Group A Streptococcal Vaccine.* Infectious Microbes & Diseases,
370 2021. **Latest Articles**.
371 13. Sabharwal, H., et al., *Group A streptococcus (GAS) carbohydrate as an immunogen for*
372 *protection against GAS infection.* J Infect Dis, 2006. **193**(1): p. 129-35.
373 14. Dudding, B.A. and E.M. Ayoub, *Persistence of streptococcal group A antibody in patients with*
374 *rheumatic valvular disease.* J Exp Med, 1968. **128**(5): p. 1081-98.
375 15. Kabanova, A., et al., *Evaluation of a Group A Streptococcus synthetic oligosaccharide as*
376 *vaccine candidate.* Vaccine, 2010. **29**(1): p. 104-14.
377 16. Kaplan, M.H. and M. Meyeserian, *An immunological cross-reaction between group-A*
378 *streptococcal cells and human heart tissue.* Lancet, 1962. **1**(7232): p. 706-10.
379 17. Kirvan, C.A., et al., *Mimicry and autoantibody-mediated neuronal cell signaling in Sydenham*
380 *chorea.* Nat Med, 2003. **9**(7): p. 914-20.
381 18. Gerritzen, M.J.H., et al., *Spontaneously released Neisseria meningitidis outer membrane*
382 *vesicles as vaccine platform: production and purification.* Vaccine, 2019. **37**(47): p. 6978-
383 6986.

384 19. van der Pol, L., M. Stork, and P. van der Ley, *Outer membrane vesicles as platform vaccine*
385 *technology.* Biotechnology journal, 2015. **10**(11): p. 1689-1706.
386 20. Rivera-Hernandez, T., et al., *An Experimental Group A Streptococcus Vaccine That Reduces*
387 *Pharyngitis and Tonsillitis in a Nonhuman Primate Model.* mBio, 2019. **10**(2): p. e00693-19.
388 21. Reglinski, M., et al., *A recombinant conjugated pneumococcal vaccine that protects against*
389 *murine infections with a similar efficacy to Prevnar-13.* NPJ vaccines, 2018. **3**: p. 53-53.
390 22. Fisher, M., et al., *Shr is a broad-spectrum surface receptor that contributes to adherence and*
391 *virulence in group A streptococcus.* Infection and immunity, 2008. **76**(11): p. 5006-5015.
392 23. Moffitt, K.L., R. Malley, and Y.J. Lu, *Identification of protective pneumococcal T(H)17 antigens*
393 *from the soluble fraction of a killed whole cell vaccine.* PLoS One, 2012. **7**(8): p. e43445.
394 24. Palumbo, E., et al., *Antigen identification starting from the genome: a "Reverse Vaccinology"*
395 *approach applied to MenB.* Methods Mol Biol, 2012. **799**: p. 361-403.
396 25. McGregor, R., et al., *An Opsonophagocytic Killing Assay for the Evaluation of Group A*
397 *Streptococcus Vaccine Antisera.* Methods Mol Biol, 2020. **2136**: p. 323-335.
398 26. Chochua, S., et al., *Emergent Invasive Group A Streptococcus dysgalactiae subsp. equisimilis,*
399 *United States, 2015-2018.* Emerging infectious diseases, 2019. **25**(8): p. 1543-1547.
400 27. King, H., et al., *Molecular basis for recognition of the Group A Carbohydrate backbone by the*
401 *PlyC streptococcal bacteriophage endolysin.* Biochemical Journal, 2021. **478**(12): p. 2385-
402 2397.
403 28. Tanaka, D., et al., *Genetic features of clinical isolates of Streptococcus dysgalactiae subsp.*
404 *equisimilis possessing Lancefield's group A antigen.* Journal of clinical microbiology, 2008.
405 **46**(4): p. 1526-1529.
406 29. Shibata, Y., et al., *Expression and characterization of streptococcal rgp genes required for*
407 *rhamnan synthesis in Escherichia coli.* Infect Immun, 2002. **70**(6): p. 2891-8.
408 30. Ruan, X., et al., *The WaaL O-antigen lipopolysaccharide ligase has features in common with*
409 *metal ion-independent inverting glycosyltransferases.* Glycobiology, 2012. **22**(2): p. 288-99.
410 31. Han, W., et al., *Defining function of lipopolysaccharide O-antigen ligase WaaL using*
411 *chemoenzymatically synthesized substrates.* J Biol Chem, 2012. **287**(8): p. 5357-65.
412 32. Shin, H.-S., et al., *Detection of Gram-negative bacterial outer membrane vesicles using DNA*
413 *aptamers.* Scientific Reports, 2019. **9**(1): p. 13167.
414 33. Rappazzo, C.G., et al., *Recombinant M2e outer membrane vesicle vaccines protect against*
415 *lethal influenza A challenge in BALB/c mice.* Vaccine, 2016. **34**(10): p. 1252-1258.
416 34. Wedege, E., et al., *Functional and specific antibody responses in adult volunteers in new*
417 *zealand who were given one of two different meningococcal serogroup B outer membrane*
418 *vesicle vaccines.* Clinical and vaccine immunology : CVI, 2007. **14**(7): p. 830-838.
419 35. Parkkali, T., et al., *IgG subclasses and avidity of antibodies to polysaccharide antigens in*
420 *allogeneic BMT recipients after vaccination with pneumococcal polysaccharide and*
421 *Haemophilus influenzae type b conjugate vaccines.* Bone Marrow Transplant, 1999. **24**(6): p.
422 671-8.
423 36. Cooper, L.J., et al., *Variable domain-identical antibodies exhibit IgG subclass-related*
424 *differences in affinity and kinetic constants as determined by surface plasmon resonance.*
425 Mol Immunol, 1994. **31**(8): p. 577-84.
426 37. Hovenden, M., et al., *IgG subclass and heavy chain domains contribute to binding and*
427 *protection by mAbs to the poly γ-D-glutamic acid capsular antigen of Bacillus anthracis.* PLoS
428 Pathog, 2013. **9**(4): p. e1003306.
429 38. Lynskey, N.N., et al., *Emergence of dominant toxigenic M1T1 Streptococcus pyogenes clone*
430 *during increased scarlet fever activity in England: a population-based molecular*
431 *epidemiological study.* The Lancet. Infectious diseases, 2019. **19**(11): p. 1209-1218.
432 39. Dileepan, T., et al., *Robust antigen specific th17 T cell response to group A Streptococcus is*
433 *dependent on IL-6 and intranasal route of infection.* PLoS Pathog, 2011. **7**(9): p. e1002252.

434 40. Narita, K., K. Asano, and A. Nakane, *IL-17A plays an important role in protection induced by*
435 *vaccination with fibronectin-binding domain of fibronectin-binding protein A against*
436 *Staphylococcus aureus infection.* Medical Microbiology and Immunology, 2017. **206**(3): p.
437 225-234.
438 41. Avci, F.Y., et al., *A mechanism for glycoconjugate vaccine activation of the adaptive immune*
439 *system and its implications for vaccine design.* Nature medicine, 2011. **17**(12): p. 1602-1609.
440 42. Di Benedetto, R., et al., *Rational Design of a Glycoconjugate Vaccine against Group A*
441 *Streptococcus.* International Journal of Molecular Sciences, 2020. **21**(22): p. 8558.
442 43. Stevenson, T.C., et al., *Immunization with outer membrane vesicles displaying conserved*
443 *surface polysaccharide antigen elicits broadly antimicrobial antibodies.* Proceedings of the
444 National Academy of Sciences, 2018. **115**(14): p. E3106-E3115.
445 44. Falconer, A.E., et al., *Distinct IgG1 and IgG3 subclass responses to two streptococcal protein*
446 *antigens in man: analysis of antibodies to streptolysin O and M protein using standardized*
447 *subclass-specific enzyme-linked immunosorbent assays.* Immunology, 1993. **79**(1): p. 89-94.
448 45. Harmer, N.J. and R. Chahwan, *Isotype switching: Mouse IgG3 constant region drives*
449 *increased affinity for polysaccharide antigens.* Virulence, 2016. **7**(6): p. 623-626.
450 46. Dillon, M.J., et al., *Contribution of murine IgG Fc regions to antibody binding to the capsule of*
451 *Burkholderia pseudomallei.* Virulence, 2016. **7**(6): p. 691-701.
452 47. Mills, J.-L.S., et al., *M-protein based vaccine induces immunogenicity and protection from*
453 *Streptococcus pyogenes when delivered on a high-density microarray patch (HD-MAP).* npj
454 Vaccines, 2020. **5**(1): p. 74.
455 48. Nishat, S. and P.R. Andreana, *Entirely Carbohydrate-Based Vaccines: An Emerging Field for*
456 *Specific and Selective Immune Responses.* Vaccines, 2016. **4**(2): p. 19.
457 49. Murakami, T., et al., *Two Types of Interleukin 17A-Producing γδ T Cells in Protection Against*
458 *Pulmonary Infection with Klebsiella pneumoniae.* J Infect Dis, 2016. **214**(11): p. 1752-1761.
459 50. Wang, Y., et al., *Cross-protective mucosal immunity mediated by memory Th17 cells against*
460 *Streptococcus pneumoniae lung infection.* Mucosal Immunology, 2017. **10**(1): p. 250-259.

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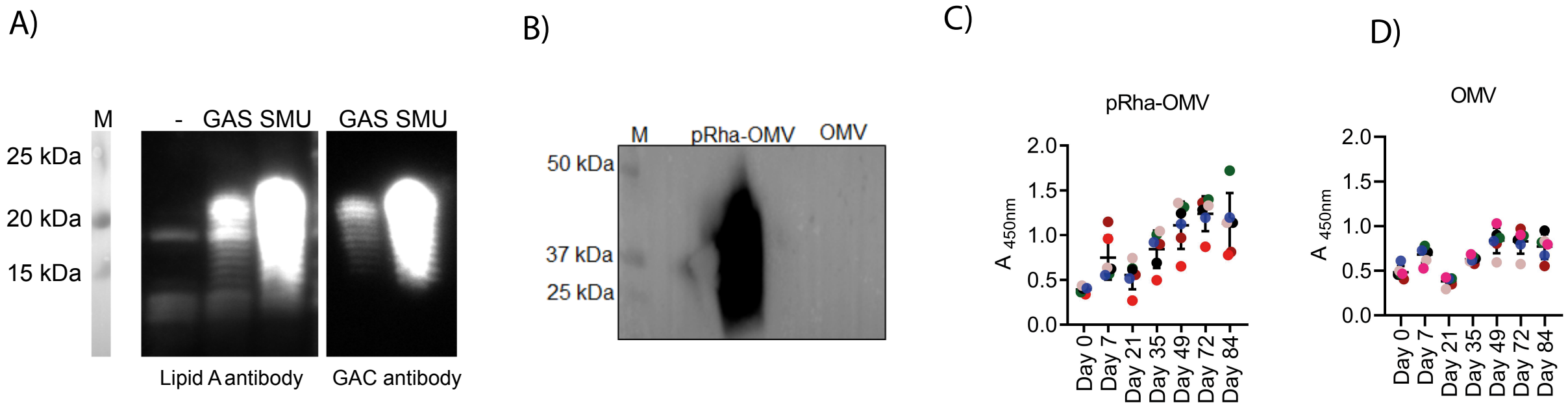

Figure 1: Purification and Immunisation of recombinantly produced pRha-OMV: A) Western blot of pRha produced in *E. coli* cells from the GAS and *Streptococcus* mutans (SMU) gene clusters and probed with Lipid A antibody and GAC specific antibody. B) Representation of immunoblot analysis of recombinantly produced *E. coli* producing pRha-OMV and *E. coli* OMV were stained for 1:1000 Rabbit anti-GAC antibody followed by goat anti-IgG HRP. Molecular mass markers are given in kilodaltons. C) pRha-OMV specific IgG antibodies were measured by coating the 96-well plate with MEGA cells (*E. coli* cells expressing longer chain of GAS pRha) and analysed using sandwich ELISA. The anti-pRha-OMV sera (C) or OMV vaccinated mice sera (D) collected at each stage of immunisation were tested against MEGA. Bound antibodies were detected using pre-titrated, biotin-conjugated antibody. Data shown are mean ± S.E.M. from technical replicates.

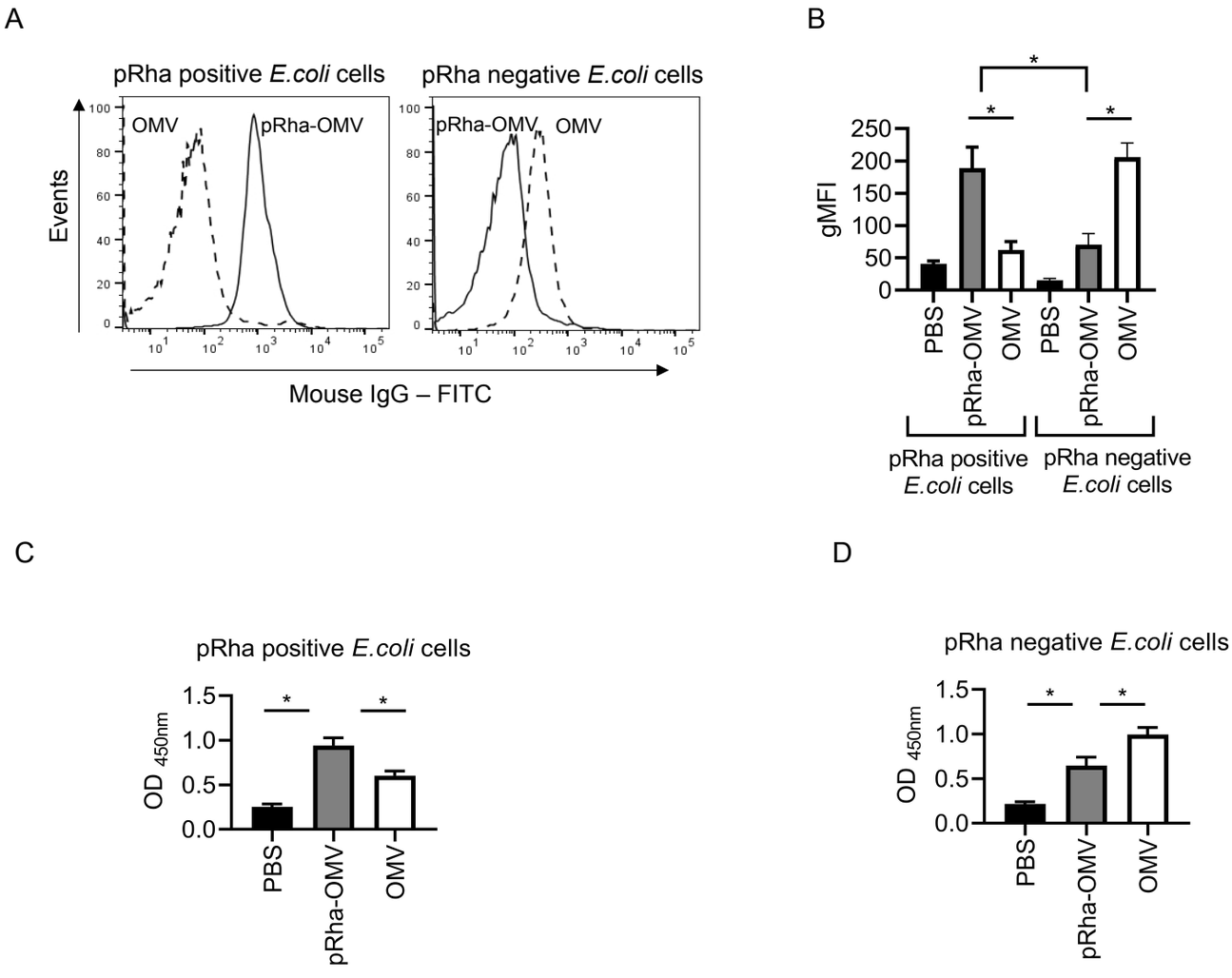

Figure 2: pRha-OMV induced IgG antibodies selectively binds to pRha positive *E. coli* cells: A) Representative histograms for antibody deposition on pRha positive *E. coli* cells or pRha negative *E. coli* cells stained either using pooled 1:100 antiserum from pRha-OMV immunised sera (open black line) or 1:100 anti-serum from OMV alone immunised sera (dashed line). The stained cells were probed with anti-mouse IgG FITC and read in flow cytometer analysis. (B) IgG deposition of pRha-OMV immunised or OMV alone immunised or PBS animal sera analysed using geometric mean fluorescence intensity (gFMI) by treating either with the pRha positive *E. coli* cells or pRha negative *E. coli* cells. C) Plates were coated with pRha positive *E. coli* cells or pRha negative *E. coli* cells (D) and probed with a 1:1000 dilution of murine sera recovered from pRha-OMV immunised or OMV immunised or PBS mice. Bound antibodies were detected using a 1:1000 dilution of HRP-conjugated goat anti-mouse IgG. Flow data shown are collected from at least 10000 events. Statistical analyses were conducted using ANOVA followed by Bonferroni post hoc-test *P<0.05. Data shown are mean±S.E.M. of three independent experiments.

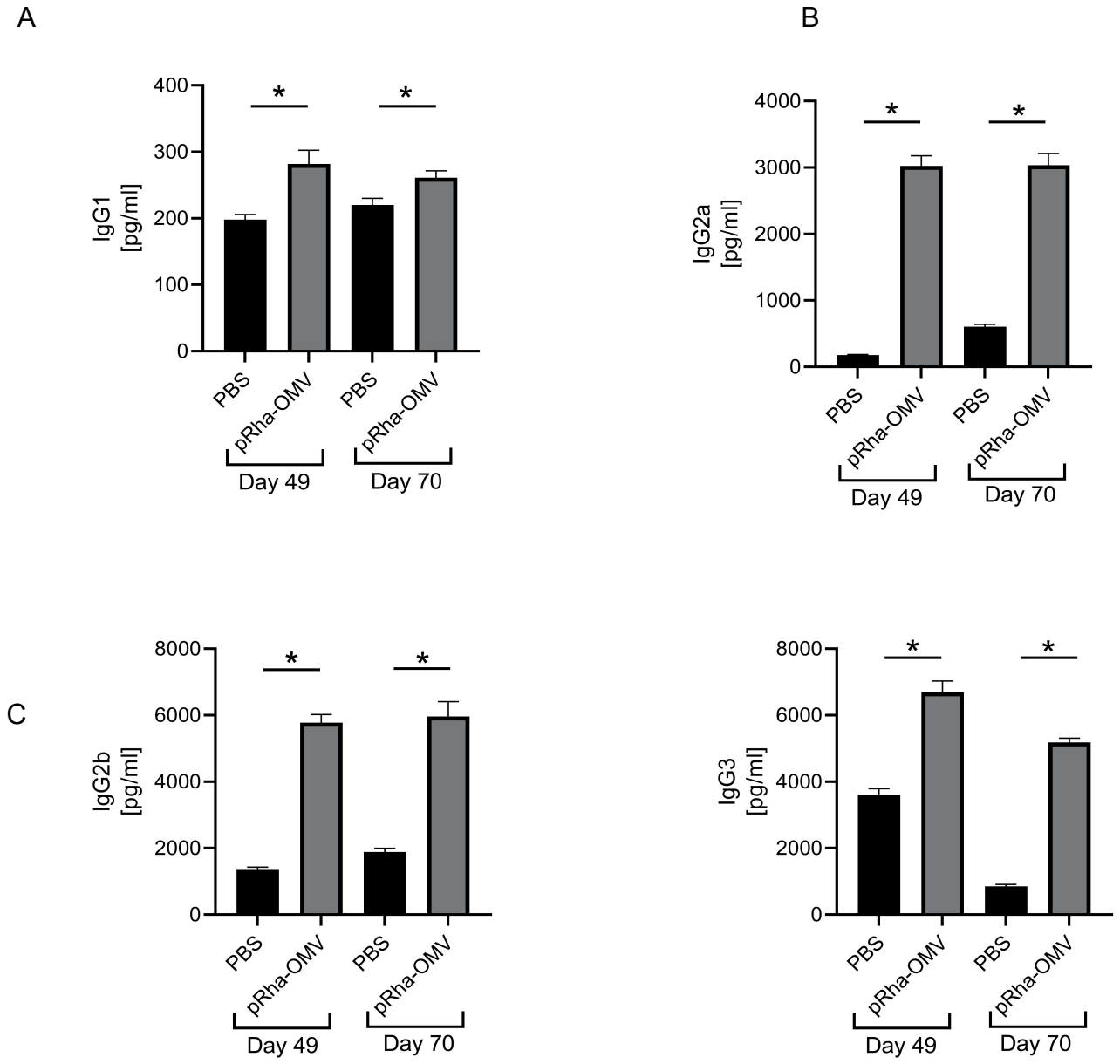

Figure 3: Quantification of Immunoglobulin subtypes against pRha-OMV in mice sera using Luminex analysis: A) IgG1 B) IgG2a C) IgG2b and D) IgG3 immunoglobulins were compared between day 49 (after two boosters) and day 70 (after three boosters) on the pooled pRha-OMV immunised mice sera or with PBS sera and analysed using Luminex. *$p < 0.05$ unpaired two-tailed T test (vs PBS). Data shown are mean±S.E.M. from technical replicates.

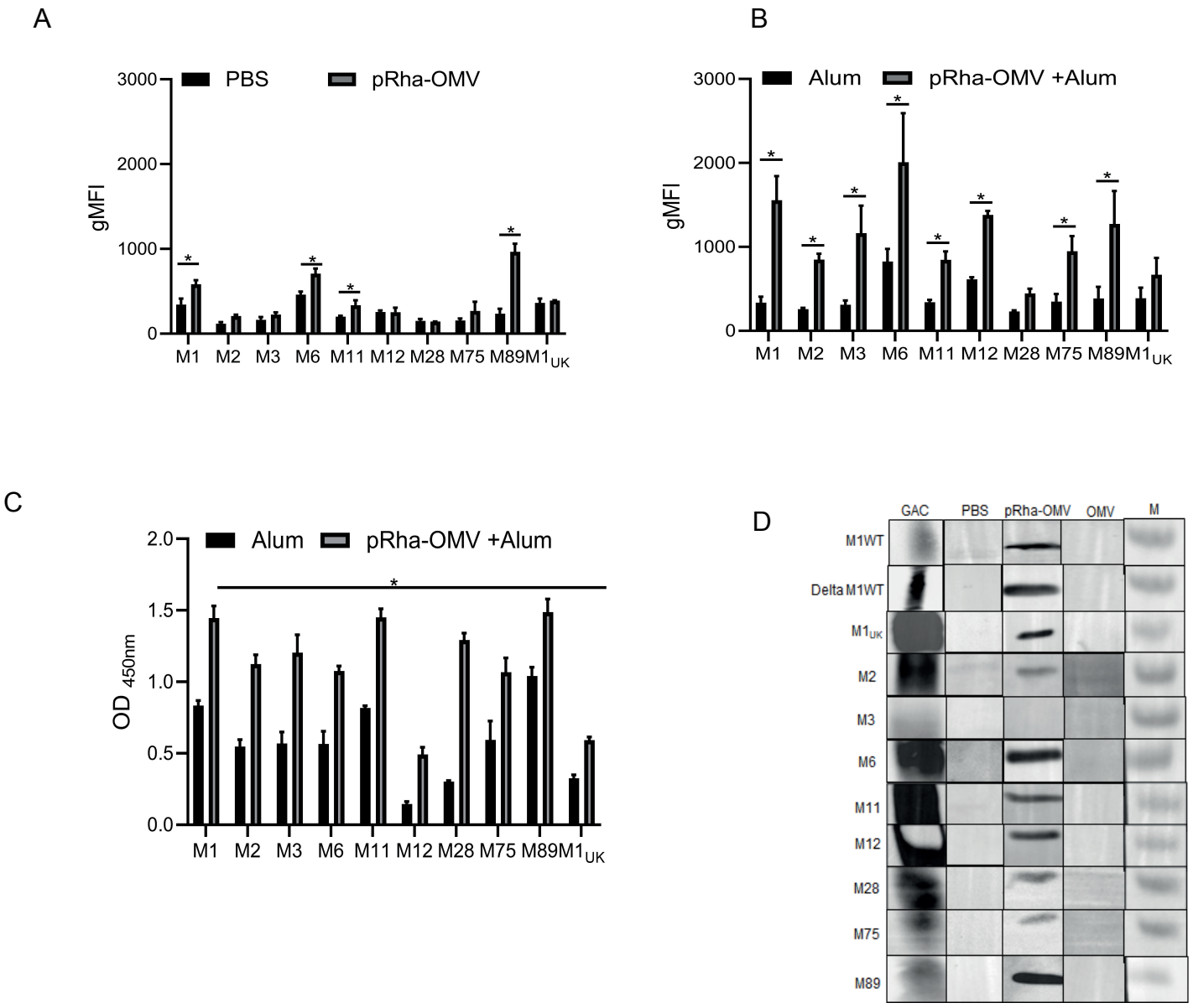

Figure 4: pRha-OMV vaccinated antibody deposition on GAS clinical isolates: A) Graph showing geometric mean fluorescence intensity (gMFI) extracted from flow cytometry analysis of clinical GAS isolates stained with either PBS or pRha-OMV pooled mice sera (1:100) followed by anti-mouse IgG alexafluro 488 channel B) Graph showing gMFI extracted from flow cytometry analysis of clinical GAS isolates stained with either alum or pRha-OMV with alum pooled mice sera (1:100) followed by anti-mouse IgG alexafluro 488 channel C) Whole cell ELISA analysis conducted to measure the antiserum from pRha-OMV with alum (1:1000) or antiserum from alum (1:1000) binding to clinical GAS strains D) Immunoblot analysis of clinical GAS isolates stained using pooled mice sera from the animals immunised with either PBS or pRha-OMV or OMV were used to probe the GAS lysates at 1:1000 dilution. The GAS lysates were further probed with anti-mouse IgG HRP (1:1000). Anti-Group A Carbohydrate antibody (GAC) was used as a positive control (1:1000). Statistical analyses were conducted using ANOVA followed by Bonferroni post hoc-test *P<0.05 (PBS vs pRha-OMV or Alum vs pRha-OMV+Alum). Data shown are mean±S.E.M. of three independent

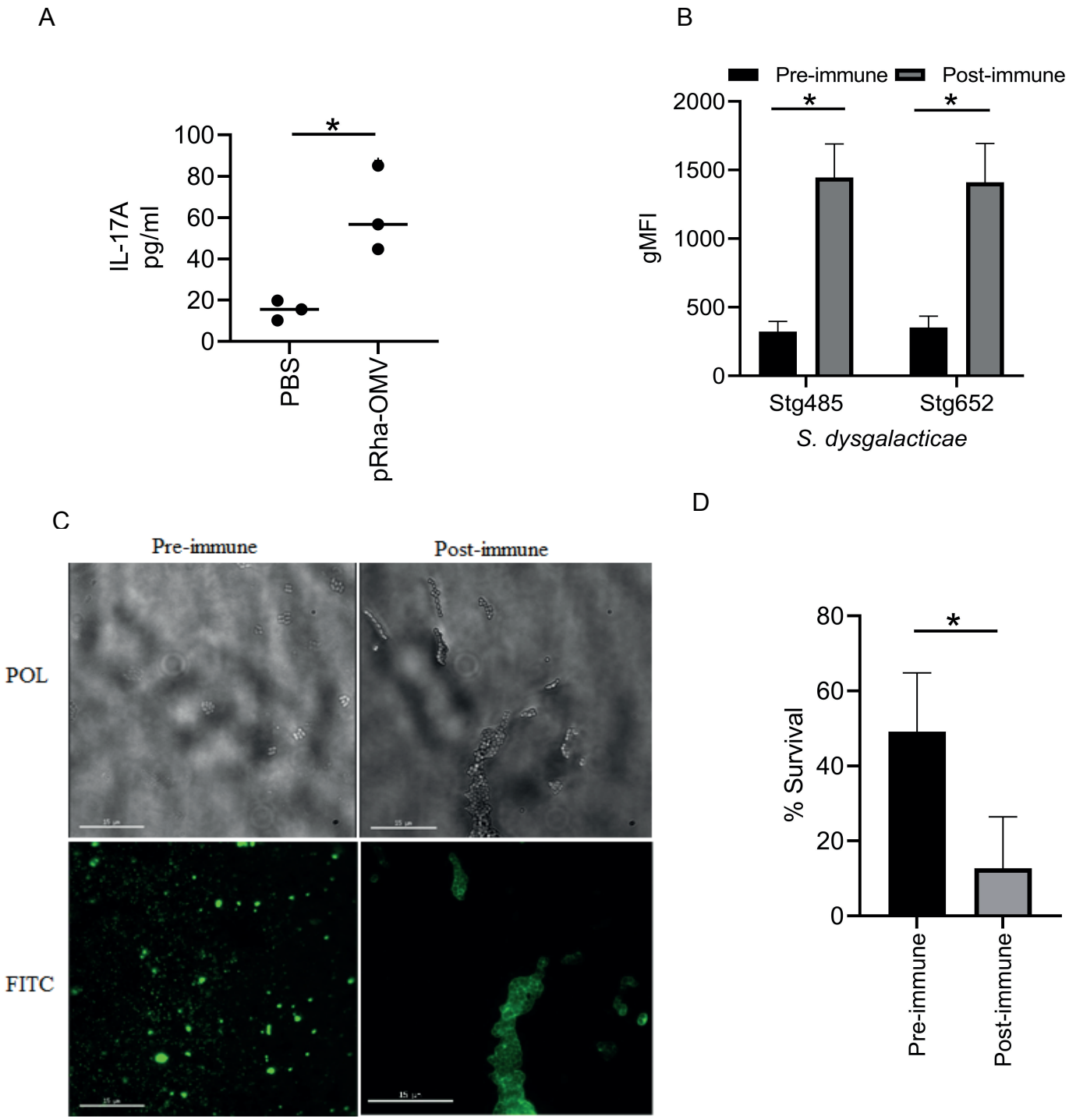

Figure 5: Rabbit immunised pRha-OMV IgG promotes killing of the hypervirulent strain M89: A) Graph showing the cytokine mediator IL-17a measured in the supernatants of individual mice splenocytes (PBS or pRha-OMV) restimulated with recombinant OMV pRha antigen (10 µg/ml). Results displayed as mean ± SEM from technical replicates B) Antibody deposition measured using a flow cytometry assay on *Streptococcus dysgalacticae* containing GAC and lacking Group G Carbohydrate (GGC) (Stg485 and Stg652) stained in 1:1000 rabbit antiserum from pre-rabbit sera and pRha-OMV immunised sera. C) Representative images of immunofluorescent staining of hypervirulent strains M89 stained with either with pre-immune rabbit sera or pRha-OMV immunised rabbit sera (1:1000) followed by goat anti-Rabbit IgG FITC (1:50) channel. Polarised (POL) and FITC channel were used to document the images using deltavision microscopy D) Percentage of survival of M89 hypervirulent GAS strains were analysed using antiserum from the pRha-OMV immunised or pre-immune rabbit in the presence of 5% baby rabbit serum. *$p < 0.05$ unpaired two-tailed T test (PBS vs pRha-OMV or pre-immune sera vs post-immune sera). Data shown are mean±S.E.M. of three independent experiments for B, D.