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1 **A novel, multiple-antigen pneumococcal vaccine protects against**
2 **lethal *Streptococcus pneumoniae* challenge**

3

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14 **Running Head:** A new multi-antigen pneumococcal vaccine

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24 **Abstract word count:** 188

25 **Conflict of interest statement:** WC was supported by a MRC CASE Studentship in
26 collaboration with ImmunoBiology Ltd; JSB and DG have received fees for consultancy work
27 from ImmunoBiology Ltd; CE, AM, PC and CB are employees of ImmunoBiology Ltd

28

29 **Funding statement:** WC was supported by an MRC CASE Studentship; GE and ER-S are
30 supported by MRC grants MR/R001871/1 and R/N02687X/1 respectively. This work was
31 undertaken at UCLH/UCL who received a proportion of funding from the Department of
32 Health's NIHR Biomedical Research Centre's funding scheme.

33

34 **Abstract 211 words**

35 Current vaccination against *Streptococcus pneumoniae* uses vaccines based on capsular
36 polysaccharides from selected serotypes, and has led to non-vaccine serotype replacement
37 disease. We have investigated an alternative serotype-independent approach, using
38 multiple-antigen vaccines (MAV) prepared from *S. pneumoniae* TIGR4 lysates enriched for
39 surface proteins by a chromatography step after culture under conditions that induce
40 expression of heat shock proteins (Hsp, thought to be immune adjuvants). Proteomics and
41 immunoblots demonstrated that compared to standard bacterial lysates, MAV was enriched
42 with Hsps and contained several recognised protective protein antigens, including
43 pneumococcal surface protein A (PspA) and pneumolysin (Ply). Vaccination of rodents with
44 MAV induced robust antibody responses to multiple serotypes, including non-pneumococcal
45 conjugate vaccine serotypes. Homologous and heterologous strains of *S. pneumoniae* were
46 opsonised after incubation in sera from vaccinated rodents. In mouse models, active
47 vaccination with MAV significantly protected against pneumonia, whilst passive transfer of
48 rabbit serum from MAV vaccinated rabbits significantly protected against sepsis caused by
49 both homologous and heterologous *S. pneumoniae* strains. Direct comparison of MAV
50 preparations made with or without the heat-shock step showed no clear differences in
51 protein antigen content and antigenicity, suggesting that the chromatography step rather
52 than Hsp induction improved MAV antigenicity. Overall, these data suggest that the MAV
53 approach may provide serotype-independent protection against *S. pneumoniae*.

54

55 **Keywords:** *Streptococcus pneumoniae*, vaccine, protein antigens, multiple-antigen vaccine

56 Introduction

57 *Streptococcus pneumoniae* is a common cause of community-acquired pneumonia (CAP),
58 septicaemia and meningitis (1), as well as of non-invasive diseases such as acute otitis
59 media (AOM) and bronchitis (2). Over 90 different serotypes of *S. pneumoniae* have been
60 identified, determined by the characteristics of the capsular polysaccharide (CPS) (3). There
61 are currently two vaccines available to prevent *S. pneumoniae* infections – the
62 pneumococcal polysaccharide vaccine (PPV) and the pneumococcal conjugate vaccine
63 (PCV). Each consists of capsular polysaccharide antigen from a limited panel of *S.*
64 *pneumoniae* serotypes. In the UK, PPV remains the first choice for adult vaccination (4) and
65 PCV is routinely included in childhood immunisation schedules worldwide as it has greater
66 efficacy in infants than the PPV. Unfortunately, in developing countries the high cost of PCV
67 restricts its availability, and in addition serotype coverage is reduced as PCV was designed
68 to include the most prevalent serotypes in North America (5). Furthermore, serotype
69 replacement in response to PCV vaccination alters the ecology of *S. pneumoniae*, reducing
70 the efficacy of polysaccharide vaccines over time (6). A vaccine based on protein antigens
71 may provide a low cost alternative approach capable of inducing cross-serotype protection
72 (7, 8).

73

74 One vaccine approach dependent on protein antigens is a whole cell approach, a cost-
75 effective method of immunising with a large number of potential protein antigens to
76 potentially induce serotype-independent protective immunity. In addition, a whole cell
77 approach could target both humoral and cellular host immunity (9, 10), potentially enabling
78 clearance of both disease and colonisation. Several groups have therefore studied a whole
79 cell vaccine approach against *S. pneumoniae*, including progression to early phase clinical
80 trials (11–13). An alternative to maintaining protein antigens as part of whole *S. pneumoniae*
81 bacterium is using a bacterial lysate as a vaccine, which could result in a more stable
82 preparation that is better suited to vaccine delivery than a whole bacterium. However, the

83 antigenicity of whole cell lysates maybe weak and require enhancing (14). One method of
84 enhancing immunogenicity is altering the preparation of the lysate to ensure increased
85 representation of immunoprotective proteins. This can be partially achieved using anion
86 exchange chromatography with a pH 8.0 buffer to preferentially capture several well known
87 *S. pneumoniae* antigens which all have a pI of 7.5 or lower, including PiuA, PiaA, PsaA,
88 RrgA, RrgB, ClpP, PspA and Ply. In addition, growth under stress conditions such as high
89 temperatures to induce heat shock proteins (Hsps) could increase antigenicity (15) as Hsps
90 facilitate the cross-presentation of peptides (16, 17) and act as natural adjuvants by
91 stimulating macrophages and dendritic cells to cause cytokine secretion (18–20). As a result,
92 Hsps have been studied as vaccines that protect against cancer as well as microbial
93 pathogens (21), with a number of bacterial Hsps showing promise as vaccine candidates
94 (22–24), including in a models of lethal lung infection (25–27). For example, mice
95 intranasally immunised with the *S. pneumoniae* Hsp DnaJ (Hsp40) or Hsp caseinolytic
96 protease P (ClpP) were protected from *S. pneumoniae* infection, including against systemic
97 challenge with a panel of heterologous strains (28). Hence Hsps are potential vaccine
98 antigens with advantageous immunomodulatory properties that could be used as a
99 component of a broadly protective *S. pneumoniae* vaccine.

100

101 Here we present data on a multiple-antigen approach to a novel *S. pneumoniae* vaccine
102 based on bacterial lysates that combines the advantages of a whole cell approach with the
103 potential additional benefit of increased Hsps and surface antigens content in the vaccine
104 preparation.

105 **Materials and Methods**

106 **Bacterial strains and growth conditions**

107 *S. pneumoniae* was grown in either Todd-Hewitt medium (THY; Oxoid, UK) containing 5%
108 yeast extract or the defined Hoeprichs' media, and on 5% blood Columbia agar (Oxoid)
109 plates containing 5% defibrinated horse blood (TCS Biosciences, UK) at 37° C 5% CO₂.
110 Growth in medium was assessed using optical density (OD) at 580-600 nm, with bacterial
111 stocks grown to mid-log phase (OD_{580nm} 0.4-0.5) before storage in 10% glycerol at -80° C.
112 Bacterial counts were determined as previously described (29–31) by plating 10-fold serial
113 dilutions of aliquots on 5% blood Columbia agar plates after overnight incubation at 37 °C in
114 5% CO₂. The TIGR4 strain-derived multiple-antigen vaccine (MAV) was made from *S.*
115 *pneumoniae* TIGR4 (American Type Culture Collection ATCC® BA-334™), and MAV
116 batches IPS004, IPS005 and IPS014 from TIGR4 B7.1 (PlyD6) which expresses an
117 inactivated pneumolysin toxin made as previously described (32). Additional *S. pneumoniae*
118 strains used in this manuscript were: D39 (serotype 2); 0100093 (serotype 3); 23F, a gift
119 from Prof B Spratt (Imperial College London); 18C, from the ATCC (ATCC® BAA-1662™),
120 EF3030 (serotype 19F), a gift from Prof D Briles (University of Alabama); Strain 1777/39
121 (19A), a gift from Prof J Paton (University of Adelaide).

122

123 **MAV, Heat-killed lysate and heat-killed whole cell preparation**

124 MAV were made from *S. pneumoniae* TIGR4 cultured in Hoeprichs' media (made in-house)
125 in 1 L shake flasks at 37°C to an of OD₆₀₀ of 1.2 before heat shocked at 42°C for 30 minutes.
126 This step was omitted for the non-heat shocked preparation MAV^{IPS005}. Bacteria were then
127 centrifuged twice with wash buffer (40 mM Tris, 150 mM NaCl, 1 mM MgCl₂, pH 8.0),
128 incubated with lysis buffer (40 mM Tris, 20 mM NaCl, 1 mM MgCl₂, and 0.5% w/v sodium
129 deoxycholate (NaDOC), pH 8.0) for 1 h at 4°C, before homogenisation (EmulsiFlex C5 high
130 pressure homogeniser, Avestin, Germany), and incubation with 0.1% w/v octaethylene glycol
131 monododecyl ether (C₁₂E₈) for 4 h at 4 °C. Sample supernatants were harvested using a 5

132 mL Capto Q column (GE Healthcare, UK). Protein was eluted and collected as 5 mL
133 fractions at 400 mM and 500 mM concentrations of NaCl. IPS004 and IPS014 MAV batches
134 were made as described above using the TIGR4 B7.1 (PlyD6) with heat shock at 37°C for 30
135 minutes, and lysis of IPS004 in NaDOC (0.5%) and C₁₂E₈ (0.1%) and of IPS014 in NaDOC
136 (0.5%) and Triton-X 100 (1%). For heat-killed TIGR4 lysates (HKL) and heat-killed whole cell
137 (HKWC) preparations TIGR4 were also cultured in Hoeprichs' media in 1 L shake flasks at
138 37°C to an of OD₆₀₀ of 1.2, followed by killing by incubation at 65 °C for 45 minutes which
139 was confirmed by culture on Columbia agar plates. For the HKL, lysis using NaDOC and
140 high pressure homogenisation was as described above. C₁₂E₈ was added to both the HKL
141 and HKWC before filtration.

142

143 **Vaccine characterisation**

144 Vaccine samples were analysed with SDS-PAGE using a 4-12% NuPage gel (Invitrogen,
145 USA), MES (Invitrogen) running buffer, and staining for protein with InstantBlue (Expedeon,
146 UK). For Western blot analysis, gels were subsequently blotted onto polyvinylidene fluoride
147 (PVDF) membranes and probed with the appropriate antibodies diluted in 5% milk / PBS:
148 anti-Hsp60 (GroEL; SPS-875; StressGen, USA) at 1:2000; anti-pneumolysin (ab49568;
149 Abcam, UK) at 1:2000; anti-Hsp70 (made in-house) at 1:500; anti-PspA (sc17483, Santa
150 Cruz, USA) at 1:1000. Protein concentrations were determined using bicinchoninic
151 acid protein assays (Pierce, USA). For haemolysis assays vaccine preparations were serial
152 diluted in phosphate-buffered saline (PBS) and an equal volume of 2% defibrinated horse
153 blood added and incubated at 37°C for 30 minutes followed by centrifugation at 1000 g for 1
154 minute, and measuring the absorbance of the supernatants at 490 nm.

155

156 **Capillary gel electrophoresis**

157 Analysis of samples by capillary gel electrophoresis (CGE) was conducted by deltaDOT,
158 London BioScience Innovation Centre, using the high performance capillary electrophoresis

159 (HPCE) platform, PEREGRINE. Peaks were manually selected and raw data was expressed
160 as peak area corrected for migration time and then expressed as a percentage of the total
161 corrected peak area or AUC (inclusive of triton peak (peak 1) and inter-peak regions (A –
162 G)), normalised to an external protein standard. This compensates for day-to-day variation
163 and allows comparison between runs on different ‘sections’ of capillary. Peak 1 is the
164 detergent triton peak.

165

166 ***In vitro* assays**

167 *S. pneumoniae* whole cell ELISAs were performed using bacterial cultures at an OD₅₈₀ of
168 0.4-5, alkaline phosphatase (AP) conjugated secondary antibodies, and the substrate para-
169 nitrophenylphosphate (pNPP) (Sigma) as previously described (33, 34). Absorbance was
170 read at 450 nm, subtracting readings at 630 nm (Versamax). The ELISA titre represents the
171 theoretical sample dilution that would result in an OD₄₅₀₋₆₃₀ of 0.1. For detection of anti-Ply
172 and anti-PspA antibodies, the appropriate antigen was diluted in carbonate buffer to a
173 final concentration of 1 µg/mL and 100 µL was transferred to each well of a 96 well Maxisorp
174 ELISA plate, and incubated overnight at 4 °C. Plates were then washed x 3 with ELISA wash
175 buffer (1% v/v Tween-20 / PBS), blocked for 1 h with block buffer (1 % w/v BSA / PBS) at 37
176 °C then washed as previous. Serum samples were diluted to a starting dilution of 1/100 to
177 1/300. Doubling dilutions of the pooled sera samples were assayed in duplicate. Plates were
178 incubated at 37 °C for 1 h, washed and goat anti-mouse IgG HRP diluted to 1/20,000 in
179 added before incubation at 37 °C for 1 h, before washing. TMB substrate was added before
180 incubation at room temperature in the dark. Plates were read OD_{450 nm} and end point titres
181 were calculated using the linear part of each titration curve. IgG surface-binding was
182 assessed using previously described flow cytometry assays (35–37) and species appropriate
183 secondary antibodies: anti-human IgG secondary antibody (1:200) conjugated to PE (Sigma-
184 Aldrich); goat anti-mouse IgG, conjugated to FITC (Bio-Rad, USA). FACS analysis of
185 bacterial cells was performed on the FACSVerser flow cytometer (Becton Dickinson, USA),

186 and FACSuite (Becton Dickinson) and FlowJo (Becton Dickinson) software. FACs surface
187 binding comparisons of MAV^{IPS004} and MAV^{IPS005} were conducted by ImmBio: 100 μ L of
188 bacterial suspension was placed in each 5 mL FACS tube and incubated overnight at 4°C.
189 Cells were washed with PBS/0.1% Tween 20 (PBS-T). Pre-adsorbed sera samples were
190 diluted serially (two-fold) starting from 1 in 25 to 1 in 800 in PBS / 1% BSA. Cells and sera
191 were incubated together for 2 hours at room temperature and then washed with PBS-T. Goat
192 anti-mouse IgG detection antibody in PBS / 1% BSA was added and incubated for 2 hours.
193 Cells were then fixed with formalin for 30 minutes at room temperature. Following washing in
194 PBS-T, samples were resuspended in PBS / 1% FCS. The mean fluorescence intensity
195 (MFI) was read by flow cytometry requiring 100,000 events for each sample. The mean
196 fluorescence intensity (MFI) multiplied by the number of cells in quadrant 1 is shown for each
197 serotype. This represents the degree of antibody binding to each serotype. Multiplexed-
198 electroluminescence assays were conducted as previously described (38–40) using a Meso
199 Scale Discovery (MSD, MD USA) platform assay (41) and 5 μ g/ml of *S. pneumoniae*
200 proteins and 10 μ g/ml of capsular polysaccharide. After incubation of each antigen-coated
201 plate with blocking agent, washing, and incubation with diluted test sera for 45 min at room
202 temperature, plates were washed and MSD Sulfo-Tag-labelled goat anti-mouse IgG
203 secondary antibody added for reading using a MSD SECTOR Imager 2400 or 6000.

204

205 **Quantitative comparison of protein content using Tandem Mass Tags (TMT)** 206 **and mass spectrometry (MS)**

207 TMT labelling procedure followed manufacturer's recommendation (Thermo Fisher). In brief
208 protein lysates from two replicates of HKL and MAV were reduced with tris(2-carboxyethyl)
209 phosphine and alkylated with iodoacetic acid before an overnight acetone precipitation.
210 Protein pellets were digested overnight at 37°C in 200 mM TEAB solution containing 2.5 μ g
211 trypsin (Promega) with the resulting peptides labelled with different isobaric tags (Tandem
212 Mass Tags, TMTs 126 – 128). Labelled peptides were mixed and injected on to an XBridge

213 C18 column, (5 μ m, 4.6 mm id and 25 cm long; Waters) for the first dimension high pH RP-
214 HPLC separation under a linear gradient consisting of mobile phase A (10 mM ammonium
215 formate, pH 10.0) and up to 70% B (90% acetonitrile in mobile phase A) for 2 hours at flow
216 rate of 0.5 mL/min, using a Jasco system consisting an autosampler, semi-micro HPLC
217 pumps and UV detector. Eluted fractions were collected and concatenated into eighteen
218 tubes and vacuum dried.

219

220 Nano-LC and MS/MS was performed using a U3000 direct nano system coupled with nano-
221 electrospray and LTQ-Orbitrap Discovery mass spectrometer (Thermo). The twelve HPLC
222 fractions containing the mixture of fourplex labelled peptides were resuspended in 0.1%
223 formic acid and each was separated on a PepMap C18 reversed phase nano column (3 μ m,
224 100 \AA , 50 cm length; Thermo) under a column flow rate of 0.3 μ l/min using linear gradient of
225 5 – 25% for 180 min, 25 – 32% for 20 min and 32 – 90% for 10 min of 95% acetonitrile and
226 0.1% formic acid. MS scan and MS/MS fragmentation were carried out in Orbitrap and LTQ
227 respectively using 2 cycles of top 3 data-dependent acquisition with dynamic exclusion mode
228 enabled and total cycle time at approximately 30 milliseconds. The first cycle used collision-
229 induced dissociation (CID) fragmentation generating spectra for peptide sequencing, and the
230 second High energy CID (HCD) generating spectra both for peptide sequencing and relative
231 quantitation via report ions.

232

233 Mass spectra processing, database searching and quantitation were performed using
234 Thermo Proteome Discoverer 1.4 with built-in Sequest against UniProt *S. pneumoniae*
235 FASTA database, (release (2014.04.03)). Spectra from the 12 fractions were added together
236 as one sample during searching. Initial mass tolerances by MS were set to 10 ppm. Up to
237 two missed tryptic cleavages were considered. Methionine oxidation was set as dynamic
238 modification whereas carboxymethylation on cysteine and TMT6plex labels on N-terminal
239 amino acid and lysine side chain were set as static modifications. Peptides at rank 1 with
240 high confidence are considered to be unambiguously sequenced. Quantification was based

241 on the relative abundances of TMT tag as the reporter ions for each peptide in the HCD
242 spectra with all TMT channels present. Ratios were calculated from relative abundances of
243 each labelled peptide in the sample based on reporter ion intensities and for every protein
244 identified, each was assigned a series of quantification ratio relative to each group.

245

246 ***In vivo* methods**

247 All *in vivo* experiments using mice were performed according to UK national guidelines for
248 animal use and care. Experiments performed at UCL were approved by the UCL Biological
249 Services Ethical Committee and the UK Home Office (Project Licence PPL70/6510).
250 Experiments used 6 week old outbred female CD1 mice obtained from Charles River
251 Laboratories. Mice were vaccinated with 75 µg of protein in 100 µL PBS using either
252 intraperitoneal injection at day 0, 14 and 28 or subcutaneous (SC) vaccination on day 0 and
253 21. Tail bleeds (5µL per mouse) were collected on day 42 and mice challenged with *S.*
254 *pneumoniae* on day 49. For the pneumonia model mice were inoculated with 5×10^6 CFU of
255 *S. pneumoniae* in PBS intranasally (IN) under isoflurane (4%; MiniRad) anaesthesia. After
256 either 24 or 48 h, the mice were euthanized with pentobarbitone, and blood, sera,
257 bronchoalveolar lavage fluid (BALF), lung, and spleen collected as previously described (9,
258 36, 42, 43). Lungs and spleens were macerated through a 0.2 µm filter. For the colonisation
259 model, mice were anaesthetised with aerosolised isoflurane (4%) and inoculated with 5×10^6
260 CFU *S. pneumoniae* suspended in 10µl of PBS. At designated time points post-infection the
261 mice were culled and the nasal washes obtained by retrograde washing of the nares with
262 500 µL PBS via the trachea. To assess survival, mice were vaccinated by intraperitoneal
263 inoculation with 75 µg of the MAV^{IPS014} vaccine together with the adjuvant system (Sigma,
264 S6322) on day 1, 10 and 22 before intranasal challenge with 1×10^7 CFU TIGR4 *S.*
265 *pneumoniae* on day 50. Disease development was monitored over 6 days and mice culled
266 when exhibiting signs of severe disease (42). For the passive transfer model mice were
267 injected intraperitoneally (IP) with 200 µl of serum harvested from rabbits vaccinated by SC

268 injection with 375 μ g MAV on days 0, 21 and 35, or with 2 x 200 μ L Prevenar 13 (day 0 and
269 21) at Envigo, UK. Mice were challenged 6 hours later by IP inoculation of 1×10^4 CFU of *S.*
270 *pneumoniae* and culled at 24 h to obtain blood samples for plating. For the sepsis model,
271 sera from vaccinated mice or rabbits were transferred to mice via intravenous (IV) injections
272 to the lateral tail vein. After 4 h, the mice were inoculated IV with 5×10^5 CFU of *S.*
273 *pneumoniae*, and culled 4 h later to collect blood. For the pre-opsonisation clearance model
274 *S. pneumoniae* were opsonised by incubation in 100% of rabbit immune serum for 1 h at 37
275 $^{\circ}$ C, then 5×10^5 CFU *S. pneumoniae* were inoculated IV into mice which were then culled 4
276 h later to obtain blood for CFU quantification by plating. To calculate target organ CFU
277 aliquots of blood, lung and spleen tissues were plated at appropriate dilutions on 5% blood
278 Columbia agar plates containing 5mg/mL gentamicin (Sigma). Additional experiments to
279 raise antisera with different vaccine preparations were performed at a commercial
280 organisation, Churchill Applied Biotechnology Ltd, according to institutional guidelines under
281 their UK Home Office Project Licence. For these experiments, six groups of female CD-1
282 mice (n=10) were immunised subcutaneously with the 0.75 μ g of MAV on day 0 and day 21.
283 Mice were culled on day 35 and terminally bled, and sera prepared for the investigation of
284 antibody responses.

285

286 **Flow cytometry phenotypic screening of inflammatory cell populations**

287 Lungs of vaccinated mice were harvested 24 hours after infection and single cell
288 suspensions prepared by homogenating the tissues and filtering them with 100 μ m cell
289 strainers. Red blood cells were lysed with RBC lysis buffer (Biolegend, 420301) and washed
290 cells were resuspended at a concentration of 10^6 cells/ml in blocking buffer (PBS-1% BSA
291 containing anti-Fc receptor antibodies (Biolegend, TruStain FcXTM)). The cells were seeded
292 in round-bottom 96-well plates (100 μ l/well) and incubated for 30 min on ice. Cells were
293 washed and stained with a mixture of antibodies diluted 1:100 for 30 minutes in ice. The
294 antibodies used were: anti-mouse CD19 Brilliant Violet 480 (BD Bioscience, 566167), anti-

295 mouse CD11c PE-Cy7 (Biolegend, 117317), anti-mouse Ly-6G PerCP-Cy5.5 (Biolegend,
296 127615), anti-mouse F4/80 Brilliant Violet 421 (Biolegend, 123131), anti-mouse CD3 PE
297 (Biolegend, 100205), anti-mouse CD4 APC and anti-mouse CD8 APC-Cy7 (Biolegend,
298 100713). The cells were washed three times with PBS and stained with Zombie Green
299 Fixable viability kit (Biolegend, 423111) (1:500) for 15 min according to the manufacturer's
300 instructions. After two extra washes with PBS-1% BSA cells were fixed with
301 paraformaldehyde (PFA) and fluorescence assessed using a XX (BD Bioscience, UK). The
302 samples were analysed on a FACS Verse flow cytometer (BD Bioscience).
303 Neutrophil/monocyte population and the lymphocyte populations were initially identified
304 using forward and side scatter dimensions and the immune sub-populations were defined as
305 follows: macrophages CD11c+ F4/80+ Ly-6G-, neutrophils Ly-6G+ CD11c- F4/80-, B cells
306 CD19+ CD3-, and T cells CD3+ CD19-. T cells population were further subdivided using
307 CD4 and CD8 markers. Lung homogenate cytokines levels (IL-1, IL-6, IL-10, TNF- α) were
308 determined by using a Luminex magnetic bead array assay (R&D systems) according to
309 manufacturer protocols.

310

311 **Statistical methods**

312 Statistical analyses were conducted using Prism 7 (Graph Pad, USA). Parametric data are
313 presented as means, and error bars represent standard deviation. Comparisons between
314 multiple groups were conducted using analysis of variance and the Holm-Sidak or Dunnett's
315 post-test to compare between experimental groups. Non-parametric data were analysed
316 using the Mann-Whitney U test. For the disease development model, data were analysed
317 using the log-rank (Mantel-Cox) test.

318 **Results**

319 **Formulation of a *S. pneumoniae* MAV**

320 A multiple-antigen Hsp-enriched preparation based on a whole cell preparation was
321 formulated using the ImmBio platform technology as previously described (32). Heat shock
322 was used to enrich for Hsps and anion exchange chromatography to enrich for negatively
323 charged *S. pneumoniae* antigens (e.g. PspA and Ply) (Fig. 1A). Immunoblots determined
324 which elution fractions contained the highest concentration of Hsp60 and Hsp70 protein, and
325 demonstrated a marked increase in the expression of both Hsp60 and 70 content in the MAV
326 compared to bacterial heat-killed lysate (HKL) (Fig. 1B and C). A pooled human IgG
327 preparation known to recognise multiple *S. pneumoniae* protein antigens (9) was used to
328 probe MAV and HKL to determine whether there were differences in their non-Hsp protein
329 content. This demonstrated variations in number, intensity and molecular weights of bands
330 identified after incubation in sera from MAV or HKL vaccinated animals (Fig. 1D). Ply activity
331 in the MAV, HKL and a heat-killed whole cell (HKWC) preparation formulated with the wild-
332 type TIGR4 strain was assessed using a haemolysis assay. HKWC and HKL did not cause
333 lysis of red blood cells, probably due to degradation of Ply during the heat-killing step,
334 whereas MAV caused red cell lysis, suggesting the MAV preparation still contained active
335 Ply (Fig. 1E).

336

337 **Proteomic analysis of MAV preparations**

338 MS/MS sequencing was used to identify proteins in the MAV and HKL preparations, with
339 TMT labelling to assess relative protein quantitation. A total of 627 proteins were identified
340 and compared between MAV and HKL preparations. Of these, 57 were increased >2-fold in
341 MAV compared to HKL (Supplementary Table 1), including several Hsps and important
342 known surface antigens such as PavB and several lipoproteins, including PsaA, PiaA, and
343 the Th17 antigens SP_0148 and SP_2108 (44) (Table 1). Conversely, 152 proteins were
344 decreased by more than 2-fold by the vaccine preparation process, including multiple

345 proteins required for basic metabolic functions (e.g. ribosomal proteins) and capsule
346 synthesis, but also the virulence factors and protective antigens Ply, PspA, and PspC
347 (Supplementary Table 2). Previously we have published data obtained using deltaDOT®
348 capillary gel electrophoresis (CGE) demonstrating consistent protein content between
349 different batches of MAV preparations (32).

350

351 **Vaccination with MAV induces functional antibodies**

352 To assess immunogenicity of MAV, mice were immunised by subcutaneous injection with
353 either MAV, HKL, HKWC or the negative control buffer using a two dose schedule 21 days
354 apart. Whole cell ELISAs demonstrated that pooled serum obtained one week after the
355 second vaccination with MAV contained markedly higher antibody responses to the *S.*
356 *pneumoniae* TIGR4 strain than those detected in serum from HKL-vaccinated mice. No
357 statistically significant anti-TIGR4 response was identified in sera from mice vaccinated with
358 HKWC (Fig. 2A). The serum antibody response to MAV was dominated by IgG with no
359 significant IgM response compared to buffer vaccinated mice (Fig. 2B). Significantly
360 increased levels of anti-TIGR4 IgG were also detected in the BALF of mice immunised with
361 MAV, but not in nasal washes (Fig. 2C). Whether serum IgG induced by the MAV, HKL and
362 HKWC preparations can recognise and bind to the surface of live *S. pneumoniae* was
363 assessed using a flow cytometry assay that correlates with protection (36). Compatible with
364 the ELISA data, when *S. pneumoniae* TIGR4 were incubated in serum from mice vaccinated
365 with MAV there were higher levels of IgG binding than after incubation in serum from mice
366 vaccinated with HKL or HKWC. Incubation in sera from HKWC vaccinated mice also resulted
367 in less IgG binding to *S. pneumoniae* in comparison to sera from HKL-vaccinated mice (Fig.
368 3A, B, C). To investigate IgG binding to heterologous strains, the IgG binding assays were
369 repeated using *S. pneumoniae* serotypes 18C, 23F, 3, and 19F (EF3030) strains. Sera from
370 MAV vaccinated mice significantly increased serum IgG binding to the 18C, 23F, 3 and
371 EF3030 (19F) strains compared to in a buffer-vaccinated control serum (Fig. 3D, E).

372

373 Sera from MAV vaccinated mice bind to multiple protein antigens

374 Immunoblots against *S. pneumoniae* lysates from TIGR4, D39 and 19A strains
375 demonstrated that antibodies from MAV and HKL vaccinated serum recognised a number of
376 proteins which were largely conserved between the three strains. The antigens recognised
377 after probing with sera from MAV and HKL vaccinated mice overlapped, although a band at
378 approximately 75kDa (potentially consisting of multiple proteins) was recognised by serum
379 from MAV vaccinated mice but not recognised by serum from HKL vaccinated mice (Fig.
380 4A). An MSD multiplex assay that measures IgG levels to a panel of known *S. pneumoniae*
381 surface and immunogenic proteins was used to identify some of the protein antigens
382 recognised by sera from vaccinated mice (38). IgG in serum from MAV-vaccinated mouse
383 recognised all the antigens included in the MSD panel proteins, including PspC (CbpA),
384 PspA, PsaA, PiaA, PiuA, and the pilus proteins RrgA and RrgB, all of which have previously
385 been shown to be protective vaccine candidates in mice (45–48) (Fig. 4B). In contrast, IgG in
386 serum from HKL-vaccinated mice recognised fewer proteins with no responses to PspC,
387 LytC, PcsB, PiaA, PiuA, family 1 PspA, SP_0609, SP_2027, Spr0057 (StrH) and StkP.

388

389 Comparison of MAV preparations made with and without Hsp induction

390 To assess the role of Hsp induction for the immunogenicity of the MAV preparations, MAV
391 preparations were made with (MAV^{IPS004}) and without (MAV^{IPS005}) the Hsp induction step.
392 Comparing the two MAV preparations using immunoblots showed no clear differences in
393 expression of the Hsps Hsp70 and Hsp60, nor in the expression of the immunogenic
394 proteins PspA and Ply (Fig. 5A). The capillary gel electrophoresis profiles of both
395 preparations suggested only minor overall differences in their protein constituents of (Fig.
396 5B). Both preparations were used to generate antisera using vaccination experiments in
397 mice conducted at Churchill Applied Biotechnology Ltd. Sera recovered from mice
398 vaccinated with MAV^{IPS004} and MAV^{IPS005} treated mice showed no major statistically

399 significant differences in flow cytometry assays of IgG binding to live *S. pneumoniae* or
400 whole cell ELISA titres against the TIGR4 *S. pneumoniae* strain (Fig. 5C). Specific protein
401 antigen ELISAs demonstrated increased antibody titres to Ply in serum from mice vaccinated
402 with the heat shock MAV^{IPS004} compared to those vaccinated with MAV^{IPS005} (Fig. 5D), and
403 reduced titres to PspA (Fig. 5E). When measured using the MSD multiplex assay (38) there
404 were no differences between the two vaccine preparations in responses to other antigens
405 (PiuA, PsaA, RrgA and RrgB, data not shown). These data indicate that the heat shock step
406 in MAV preparation had limited effects on heat shock content or overall antigenicity.

407

408 **Protective efficacy of vaccination of mice with MAV TIGR4 preparations**

409 Mouse models were used to determine if vaccination with Hsp induced MAVs resulted in
410 protective immunity against *S. pneumoniae*. 24 h after challenge using the pneumonia
411 model, mice vaccinated with MAV had at least one log₁₀ fewer bacterial CFU in both the
412 blood and lungs compared to buffer-vaccinated controls (Fig. 6A and B). In contrast, MAV
413 vaccination did not reduce the density of bacterial CFU in nasal washes obtained 2 weeks
414 after inoculation in a model of *S. pneumoniae* nasopharyngeal colonisation with TIGR4 (Fig.
415 6C). In order to eliminate potential adverse effects caused by an active Ply, new MAV
416 preparations termed MAV^{IPS004} and MAV^{IPS014}, denoting different batches, were prepared
417 using a mutated TIGR4 strain expressing a detoxified Ply (49). MAV^{IPS004} and MAV^{IPS014} both
418 contained similar levels of detoxified Ply as measured by ELISA assay that stimulated an
419 antibody response that recognised native Ply (32), and absence of haemolysis in the red
420 blood cell assay. Rabbit sera obtained from rabbits vaccinated subcutaneously on days 0,
421 21, 35 with 375 µg of MAV^{IPS004}, *S. pneumoniae* vaccine Prevenar as a positive control, or
422 buffer were used for passive immunisation of mice followed by intraperitoneal challenge of 1
423 x 10⁴ CFU TIGR4 *S. pneumoniae* after 6 h. When culled 24 h post-challenge, blood CFU
424 were recovered in over 65% of the mice given sera from buffer-vaccinated rabbits whilst
425 there were no CFU detected in mice given serum obtained from MAV^{IPS004} vaccinated rabbits
426 (Fig. 6D). Two mice (16%) developed septicaemia after passive administration of sera from

427 rabbits given Prevenar. In an alternative sepsis model, mice were challenged by intravenous
428 inoculation with 5×10^5 CFU of *S. pneumoniae* TIGR4 or ATCC[®] BAA-1662 (serotype 18C
429 *S. pneumoniae*) strains after pre-incubation of the bacteria for 1 h in 100% rabbit sera
430 obtained from animals immunised with MAV^{IPS014}, Prevenar or buffer controls. Mice were
431 culled at 4 h to assess rate of bacterial clearance from the blood by quantifying CFU. Those
432 challenged with the TIGR4 strain were almost completely protected against infection if the
433 bacteria were incubated in sera from either MAV^{IPS014} or Prevenar vaccinated rabbits pre-
434 challenge (Fig. 6E). Pre-incubation of the 18C strain prior to intravenous challenge in sera
435 from either MAV^{IPS014} or Prevenar vaccinated rabbits pre-challenge reduced bacterial CFU
436 recovered from the blood by over one log₁₀ compared to pre-incubation in sera from buffer
437 vaccinated rabbits (Fig. 6F). To support these data vaccination with MAV^{IPS014} was compared
438 to a buffer control in a protection study, which demonstrated that mice given a three dose
439 vaccination schedule with MAV^{IPS014} were protected against the development of fatal
440 infection after pneumonia challenge with TIGR4 (Fig. 7A). The effects of vaccination on the
441 inflammatory response to pneumonia challenge was assessed using flow cytometry of lung
442 and BALF cell populations 24 h after intranasal infection with TIGR4. Despite clear
443 reductions in lung and blood CFU in vaccinated mice, there were no differences in the
444 proportions of neutrophils in BALF between MAV- or buffer-vaccinated mice, indicating MAV-
445 vaccination resulted in an increased BALF neutrophil response for the level of bacterial
446 infection (Fig. 7B, C). In addition, in lung homogenates MAV vaccinated mice had reduced
447 proportions of neutrophils and macrophage lineage cells and a corresponding increase in T
448 cells (Fig. 7D). The increase in T cell proportion within lung homogenates in vaccinated
449 mice did not alter the CD4 / CD8 proportions compared to the data for control mice (data not
450 shown). Lung homogenate cytokine levels were variable between mice but showed
451 increased IL1, IL6, TNFalpha, and IL10 responses in vaccinated mice compared to controls,
452 again suggesting vaccinated mice were able to mount a more sustained inflammatory
453 response than controls (Fig. 7 E-H). BALF and blood cytokine levels were in general too low
454 and variable for consistent patterns to be identified.

455

456 Overall, these results indicate that the MAV is able to protect against *S. pneumoniae*
457 infection with the homologous or a heterologous strain at a similar level of protection to that
458 provided by Prevenar, and associated with significant changes in the inflammatory response
459 to pneumonic infection.

460 **Discussion**

461 Although the existing childhood conjugated capsular polysaccharide *S. pneumoniae*
462 vaccines are highly effective, the lack of both protection against non-vaccine serotypes and
463 convincing evidence of serotype replacement in vaccinated populations, as well as the high
464 cost of these vaccines, has stimulated continued interest in alternative vaccine approaches
465 (50, 51). Vaccines based on protein antigens could overcome these disadvantages, allowing
466 production of relative cheap vaccines that target cross-protective antigens (52–55).
467 Presentation of a large number of protein antigens derived from a whole cell approach has
468 the additional potential advantages of inducing immune responses to multiple antigens,
469 thereby potentially avoiding vaccine escape mutants developing and inducing stronger
470 cross-protective responses (56, 57).

471

472 The data presented here shows that a novel *S. pneumoniae* multiple protein antigen vaccine
473 approach induces antibody that recognises homologous and heterologous strains, and
474 protects against invasive pneumonia and sepsis. The MAV approach uses a whole cell
475 lysate that has been manipulated to increase expression of Hsps and the anion exchange
476 column and running buffer are optimised for the capture of known *S. pneumoniae* antigens
477 as well as Hsp proteins. Proteomic analysis confirmed that the MAV approach alters relative
478 levels of *S. pneumoniae* proteins within the preparation compared to a simple bacterial
479 lysate, with altered expression of a total of 209 proteins of the 627 proteins analysed. The *S.*
480 *pneumoniae* TIGR4 strain actually contains approximately 2000 genes (58), so the number
481 of proteins with altered content in the MAV may in fact be substantially larger. Both
482 immunoblotting and TMT-MS confirmed that the MAV had increased Hsp content compared
483 to a simple lysate. In addition, there was increased expression of multiple surface proteins
484 including known protective antigens (mainly lipoproteins), although there were also reduced
485 amounts of other protein antigens that are known to induce protective immunity in mice.

486

487 Importantly, whole cell ELISAs, MSD for IgG responses to individual antigens, or a flow
488 cytometry assay of IgG binding to *S. pneumoniae* all demonstrated enhanced antibody
489 responses in mice vaccinated with the MAV compared to the HKL, demonstrating the benefit
490 of the MAV approach in making a potentially more effective vaccine. Unlike HKL, MAV
491 induced antibody responses to all the protein antigens tested using the MSD system
492 suggesting that the MAV approach may induce antibody responses to a very high proportion
493 of the proteins in the preparation. Furthermore, although iTRAQ demonstrated reduced
494 content in the MAV preparation compared to HKL for the important antigens PspC and Ply,
495 vaccination with MAV still induced stronger IgG responses to these antigens when tested
496 using MSD than HKL. These data again suggest the MAV approach enhances
497 immunogenicity. The improved serological responses to MAV compared to HKL might be
498 predicted to be due to the increased Hsps content leading to formation of Hsp-peptide
499 complexes and thereby increasing antigen presenting ability (27, 32). However, direct
500 comparison of MAV preparations with or without a heat shock step as vaccines in mice did
501 not show any major differences in protein content (including Hsp 60 and 70) or in
502 immunogenicity apart from a reduction in antibody responses to PspA. These data suggest
503 that the anion exchange chromatography step alone enhances immunogenicity of the MAV
504 preparations, and seems to increase heat shock protein content independent of the heat
505 shock step. In addition, the lower temperature used for making MAV preparations compared
506 to that required for making HKL preparations could have allowed some proteins to retain
507 stronger immunogenicity.

508

509 Data from a pneumonia and two separate sepsis mouse models demonstrated active or
510 passive vaccination with MAV improved protection against *S. pneumoniae*. Time course
511 experiments carried demonstrated that vaccination with the MAV preparation delayed and
512 protected against lethal infection. The total T cell populations in the lung were also increased
513 in MAV-vaccinated mice. This effect in T cell proportions probably reflects the accumulation
514 of antigen specific cells during pneumonia in infected tissue, but further experiments would

515 be needed to confirm this. Overall the data demonstrate vaccination with MAV had
516 significant effects on the inflammatory response during *S. pneumoniae* pneumonia that is
517 likely to contribute to protective efficacy, with improved neutrophil recruitment into the
518 alveolar space and increased numbers of T cells within the lung parenchyma.

519

520 MAV also induced IgG that bound to heterologous strains in the flow cytometry assay, an
521 assay which correlates with improved phagocytosis *in vitro* and in protection in mouse
522 models (35–37). Indeed, in a mouse model of sepsis passive vaccination with sera from
523 MAV-vaccinated rabbits gave a similar level of protection against both the homologous
524 TIGR4 and heterologous 19C strains as passive vaccination with sera from Prevenar
525 vaccinated rabbits. Whether vaccination with MAV can induce stronger protection than
526 vaccination with HKL or HKWC in the mouse models has not been tested; due to the
527 relatively low sensitivity of the mouse models this is likely to require inappropriately large
528 numbers of mice per test group. Lack of sensitivity is also why vaccination followed by
529 disease challenge experiments would be unlikely to show any significant differences
530 between MAV preparations with and without heat shock steps, and why these experiments
531 were not performed. The level of protection varied between models, with complete
532 prevention of septicaemia in some models e.g. IV TIGR4 infection, versus reductions in
533 bacterial CFU in the blood only (e.g. 18C serotype sepsis model). The latter is likely to slow
534 the progression of disease but not prevent fatal infection. Vaccination with MAV failed to
535 protect against colonisation, but this is not that surprising given the lack of detectable
536 antibody in nasal washes and previous data showing that anti-protein antibody is often
537 ineffective at reducing nasopharyngeal *S. pneumoniae* CFU (in some cases, even when
538 Th17 mediated immunity has also been induced) (9, 10, 61). Prevention of nasopharyngeal
539 colonisation will probably require vaccines that induce strong cell-mediated immune
540 mechanisms, which may require vaccination in combination with specific adjuvants (24, 43,
541 58). This would be an important area for further investigation. Future experiments should

542 also assess whether MAV vaccination modulates pulmonary inflammatory responses to *S.*
543 *pneumoniae* pneumonia challenge.

544

545 The MAV approach described here can induce cross-protective immunogenicity of protein
546 antigens which stimulate antibody and perhaps Th17 cell responses (44, 63) without
547 requiring the identification of specific protective antigens nor production of recombinant
548 proteins for inclusion in subunit vaccines. MAV requires limited downstream processing and
549 rapidly produces a high yield of vaccine product, considerably reducing vaccine costs and
550 making the vaccine more likely to be affordable in low and middle income countries. The
551 MAV approach therefore offers a promising opportunity for a novel next generation *S.*
552 *pneumoniae* vaccine, and has recently completed a phase I trial in 36 subjects (Clinical Trial
553 Registry Number: NCT0257635; 59).

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- 786

787 **Figures**

788

789 **Figure 1** Formulation of a multiple antigen *S. pneumoniae* TIGR4-derived vaccine
790 preparation (MAV). **A** Ion exchange (IEX) chromatogram showing the purification of the
791 MAV. Light green line indicates NaCl elution concentration, the brown line is the resulting
792 conductivity in the system; the blue line is the UV trace showing concentration of eluted
793 proteins (mAu); the dark green line is the pressure in the system; fractions collected are
794 numbered in red; total volume is recorded on the x-axis (mL). **B** Detection of Hsp60 and
795 Hsp70 by Western blot in selected IEC fractions; BCA assay protein concentrations for these
796 fractions are shown in the table. **C** A comparison of the heat shock protein content (Hsp60
797 and Hsp70) as measured by immunoblotting of heat-killed lysate (HKL) and MAV. Bar chart
798 shows pixel intensity quantification (ImageQuant TL; GE Lifesciences) for Hsp60 and Hsp70
799 bands. **D** Immunoblots of 5 µg of total protein of either MAV or HKL probed with pooled
800 human IgG at 1:20 000 (Pentaglobin; Paviour Pharmaceuticals, New Delhi). **E** Comparison
801 of the haemolytic activity against horse red blood cells in serial 2 fold dilutions of MAV from
802 neat to 1:64 (filled circles) and HKL (squares), and HKWC (crosses) preparations, with a
803 saponin positive control (empty circles).

804

805 **Figure 2** MAV is immunogenic in a mouse model of subcutaneous vaccination. CD1
806 mice were vaccinated subcutaneously with 75 µg on day 0 and day 21, and culled at 28
807 days to obtain serum. **A** Whole cell IgG ELISA against *S. pneumoniae* TIGR4 for pooled
808 sera harvested from tail vein bleeds (10 µL per mouse, n = 6) **B** Whole cell IgG and IgM
809 ELISAs against *S. pneumoniae* TIGR4 for pooled sera from MAV vaccinated mice (n = 5)
810 and **C** against *S. pneumoniae* TIGR4 for pooled BALF and nasal washes from MAV
811 vaccinated mice. Data are presented as mean and 95% CI. *P* values calculated using the
812 Mann Whitney t test with * = $p < 0.05$; ** = $p < 0.01$.

813

814 **Figure 3** Binding of immune mouse sera to the surface of *S. pneumoniae* strains. **A**
815 IgG surface binding assays of *S. pneumoniae* TIGR4 incubated in sera from vaccinated mice
816 is shown by geometric mean fluorescence index (MFI). Error bars represent SD of technical
817 replicates. Significance is calculated with the Holm-Sidak test, with $* = p < 0.05$. **B**
818 Representative flow cytometry histograms showing IgG positive *S. pneumoniae* TIGR4
819 populations. White histogram – buffer negative control serum; black histogram – serum from
820 MAV vaccinated mice; dark grey – serum from HKL vaccinated mice; light grey – serum from
821 HKWC vaccinated mice. **C** IgG binding to TIGR4 in immune serum diluted to 25, 12.5, 6.25
822 and 3.125%. Data points are means of technical replicates; error bars represent standard
823 deviations. Significance values are calculated between each dilution curve using a two-way
824 ANOVA and compared to the buffer negative control. **** = $p < 0.001$. **D** Mean fluorescent
825 IgG surface binding to *S. pneumoniae* 18C, 23F, ST3 and 19F strains incubated in sera from
826 MAV or buffer vaccinated mice. Error bars represent standard deviations for technical
827 replicates. Significance is calculated with the Holm-Sidak test, with $* = p < 0.05$. **E**
828 Representative histograms showing a shift in IgG positive populations against different
829 strains of *S. pneumoniae*: white histogram – IgG binding in buffer vaccinated serum; shaded
830 histogram – binding in MAV vaccinated serum.

831

832 **Figure 4** Identification of protein antigens recognised by sera from vaccinated mice. **A**
833 Immunoblots of *S. pneumoniae* TIGR4, D39 or 19A strains whole cell lysates probed with
834 serum diluted 1:1000 from mice vaccinated with either HKL or MAV. **B** Identification of
835 protein antigens recognised by sera from vaccinated mice using MSD. Values are
836 normalised to a negative control from buffer-vaccinated mice sera. Mean values are shown
837 with error bars representing standard deviations for sera from mice vaccinated with MAV (n
838 = 3, black columns); HKL vaccinated serum ($n = 1$, grey columns).

839

840

841

842 **Figure 5**

843 A comparison of heat shocked MAV vs non-heat shocked MAV. **A** MAV preparations were
844 made with (MAV^{IPS004}) and without (MAV^{IPS005}) the Hsp induction step. Protein bands were
845 compared using a Coomassie gel (top). 3 µg (lanes 7 & 9) and 5 µg (lanes 8 & 10) of each
846 MAV was loaded respectively; immunoblots of the MAV preparations were also probed for
847 the presence of the key *S. pneumoniae* protein antigens (PlyD6, PspA) and Hsps (Hsp70,
848 Hsp60). **B** Capillary gel electrophoresis (CGE) analysis was conducted to determine the
849 protein constituents of each preparation. Each peak is denoted by a number, and inter-peak
850 regions marked by a letter. Quantification of peaks is shown in the bar chart on top. CGE
851 traces are shown below. **C** Both MAV^{IPS004} and MAV^{IPS005} were used to generate antisera
852 using vaccination experiments in mice. Sera recovered from mice vaccinated with either
853 preparations were analysed using flow cytometry assays of IgG binding to live *S.*
854 *pneumoniae* (serotypes 1, 2 (D39), 4 (TIGR4), 6B, 8, 19A, 22F and 23F) and results are
855 represented as mean fluorescence intensity (MFI) in the appropriate gate (Q1) **D** ELISAs
856 detecting anti-Ply and **E** anti-PspA responses were conducted in duplicate. Sera from the
857 experiments described above were diluted as shown on the x-axis, and OD 450 nm was
858 measured for each MAV, and a buffer control. Abbreviations: MAV, multi-antigen vaccine;
859 HS, heat shocked; NHS, non-heat shocked; MK, molecular weight marker; AUC, area under
860 curve

861

862 **Figure 6** Vaccination with MAV preparations protects mice against *S. pneumoniae*
863 challenge. **A** Lung and **B** blood CFU 24 hours after challenge by intranasal inoculation with 1×10^7
864 CFU *S. pneumoniae* TIGR4 strain of mice vaccinated twice subcutaneously with 75 µg
865 of MAV or a negative control buffer (n = 10 per group). **C** Nasal wash two weeks after
866 nasopharyngeal colonisation with 5×10^6 CFU *S. pneumoniae* TIGR4 of mice vaccinated
867 twice subcutaneously with 75 µg of MAV or a negative control buffer (n = 8 per group). **D**
868 Blood CFU 6 hours after challenge by intraperitoneal inoculation with 1×10^4 CFU *S.*
869 *pneumoniae* TIGR4 strain of mice passively vaccinated with 200 µl of sera from rabbits

870 obtained from animals vaccinated three times with 375 μg of MAV^{IPS004}, twice with 0.2 mL of
871 Prevenar, or a negative control buffer ($n = 12$ per group). **E and F** Blood CFU 4 hours in
872 mice after challenge by intravenous inoculation with 5×10^5 CFU of the *S. pneumoniae* **E**
873 TIGR4 or **F** ATCC[®] BAA-1662 (18C) strains that have been incubated pre-inoculation in
874 sera obtained from rabbits vaccinated with MAV^{IPS014}, Prevenar, or a negative control buffer
875 ($n = 5$ to 10). For all panels each symbol represents data from a single mouse, and
876 horizontal bars represent median values. Statistical significances were calculated using a
877 Mann Whitney t test (panels A to D) or Dunnett's multiple comparisons test (panels E and F).
878 Significance abbreviations: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

879

880 **Figure 7** Vaccination with MAV preparations increase survival of mice and alters the
881 inflammatory response after TIGR4 *S. pneumoniae* pneumonia challenge. **A** Percentage
882 survival of mice over 6 days after challenge by intranasal inoculation with 1×10^7 CFU *S.*
883 *pneumoniae* TIGR4 strain of mice vaccinated three times (day 1, 10, 22) intraperitoneally
884 with 75 μg of MAV^{IPS014} or a negative control buffer ($n = 15$ per group). Significance has
885 been calculated using the log-rank (Mantel-Cox) test. **B to D** Target organ CFU (**B**),
886 inflammatory cell populations in BALF (**C**) and lung (**D**) 24 h after challenge with 1×10^7 CFU
887 *S. pneumoniae* TIGR4 strain in MAV^{IPS014} vaccinated and control mice. Inflammatory cell
888 data are shown as a percentage of total cells recovered from lungs of MAV- and buffer-
889 vaccinated mice; CFU data show lung, blood or BALF CFU recovered 24 h after challenge
890 with each symbol representing data from a single mouse and horizontal bars represent
891 median values. **E to H** Lung homogenate cytokine levels (pg / ml) 24 h after challenge with
892 1×10^7 CFU *S. pneumoniae* TIGR4 strain in MAV^{IPS014} vaccinated and control mice. For
893 panels (**B**) to (**H**) statistical significances were calculated using a Mann Whitney t test.
894 Significance abbreviations: * = $p < 0.05$; ** = $p < 0.01$.

Figure 1

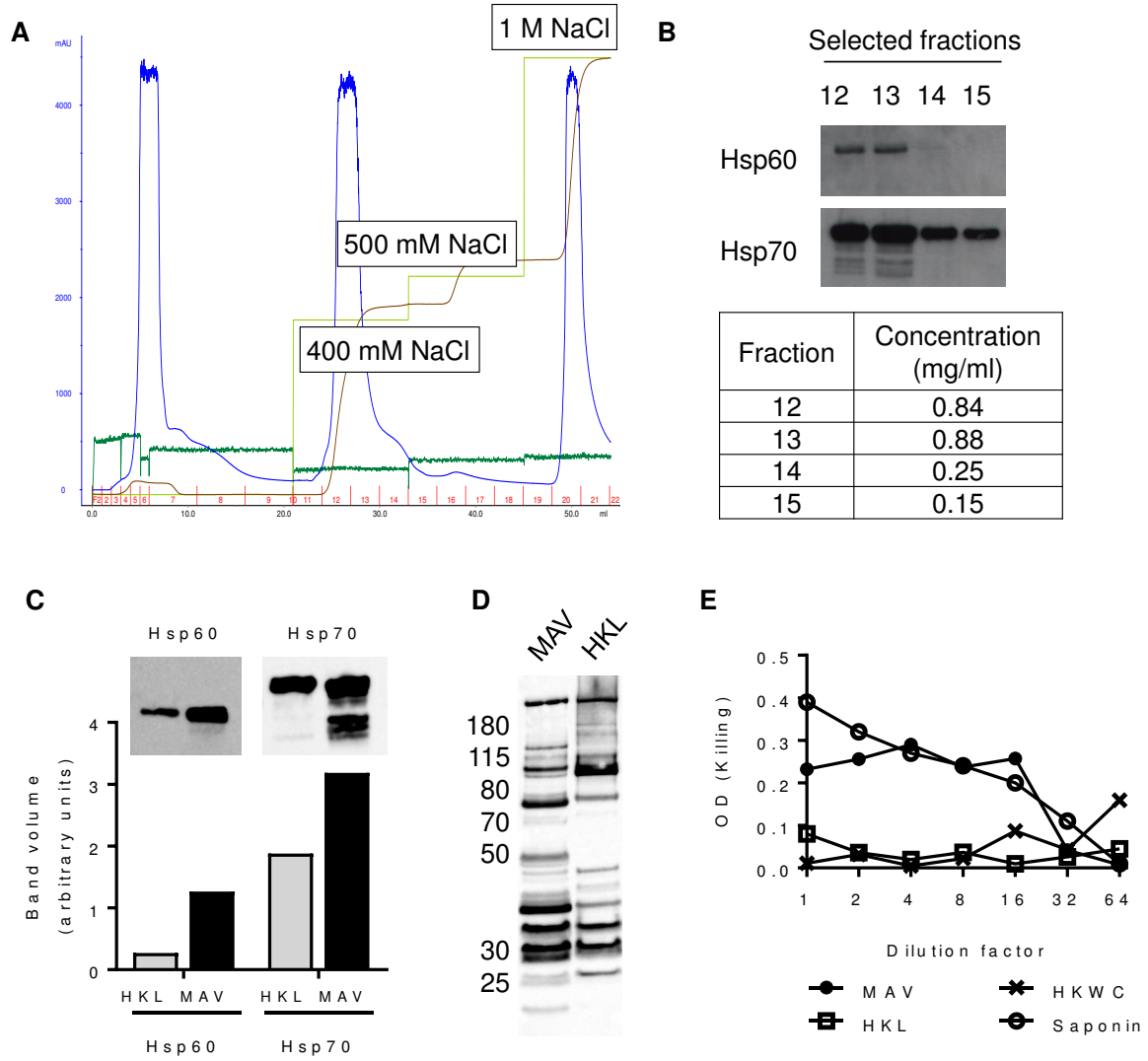


Figure 2

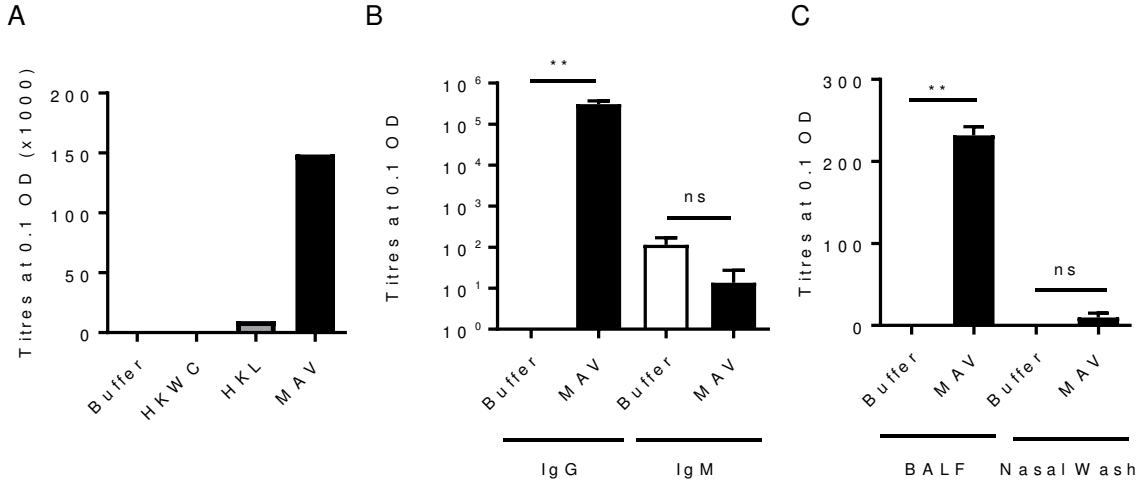


Figure 3

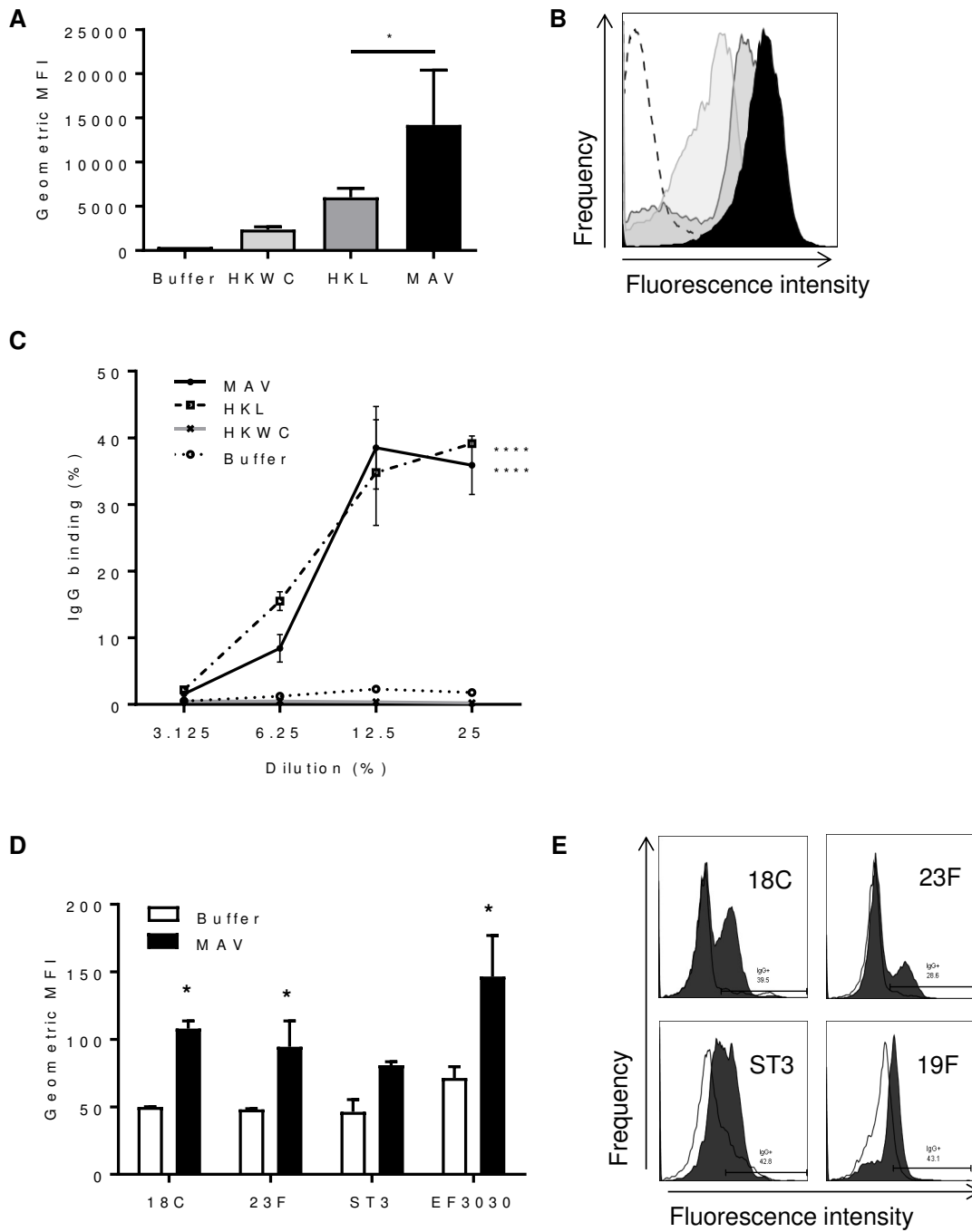


Figure 4

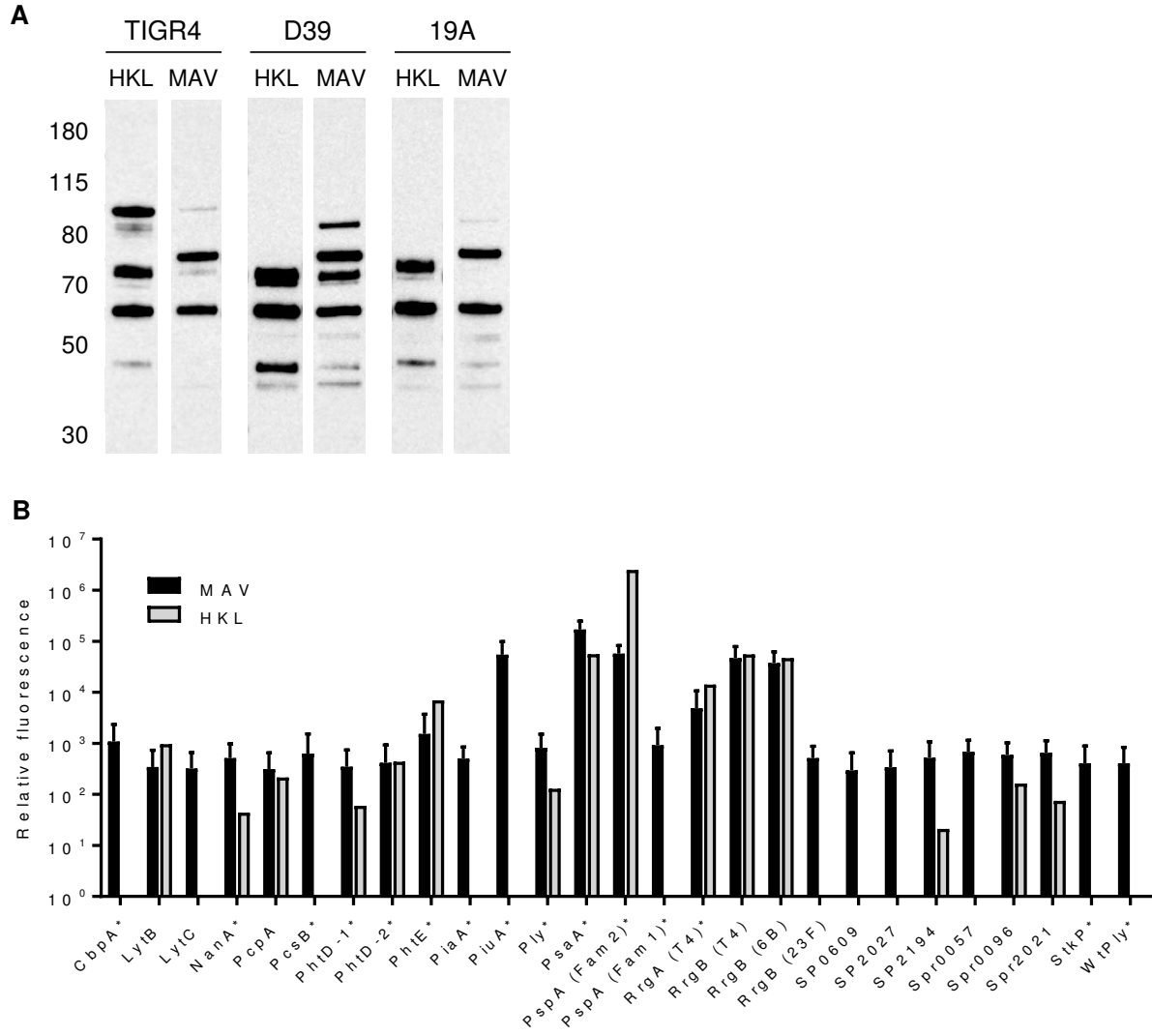


Figure 5

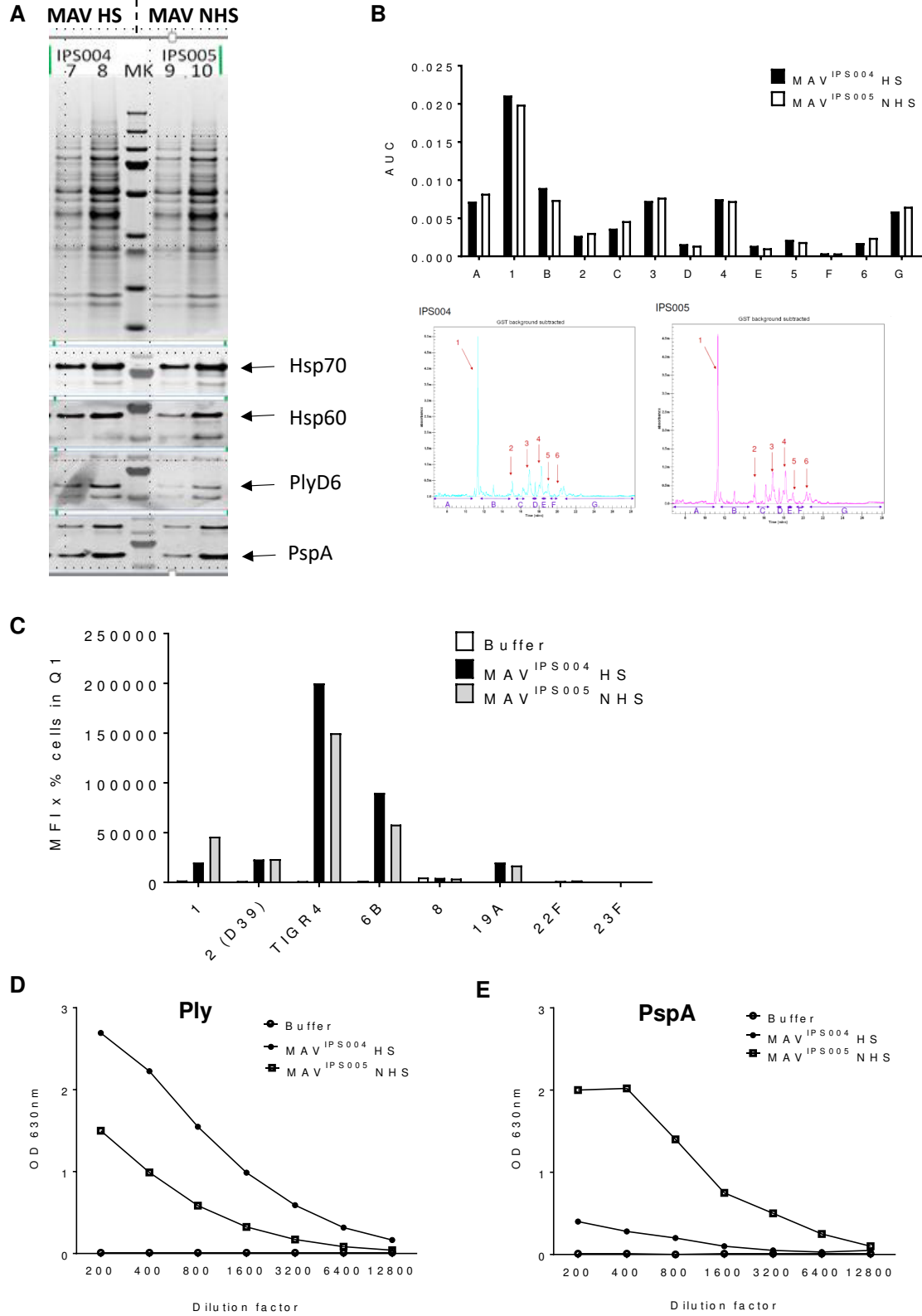


Figure 6

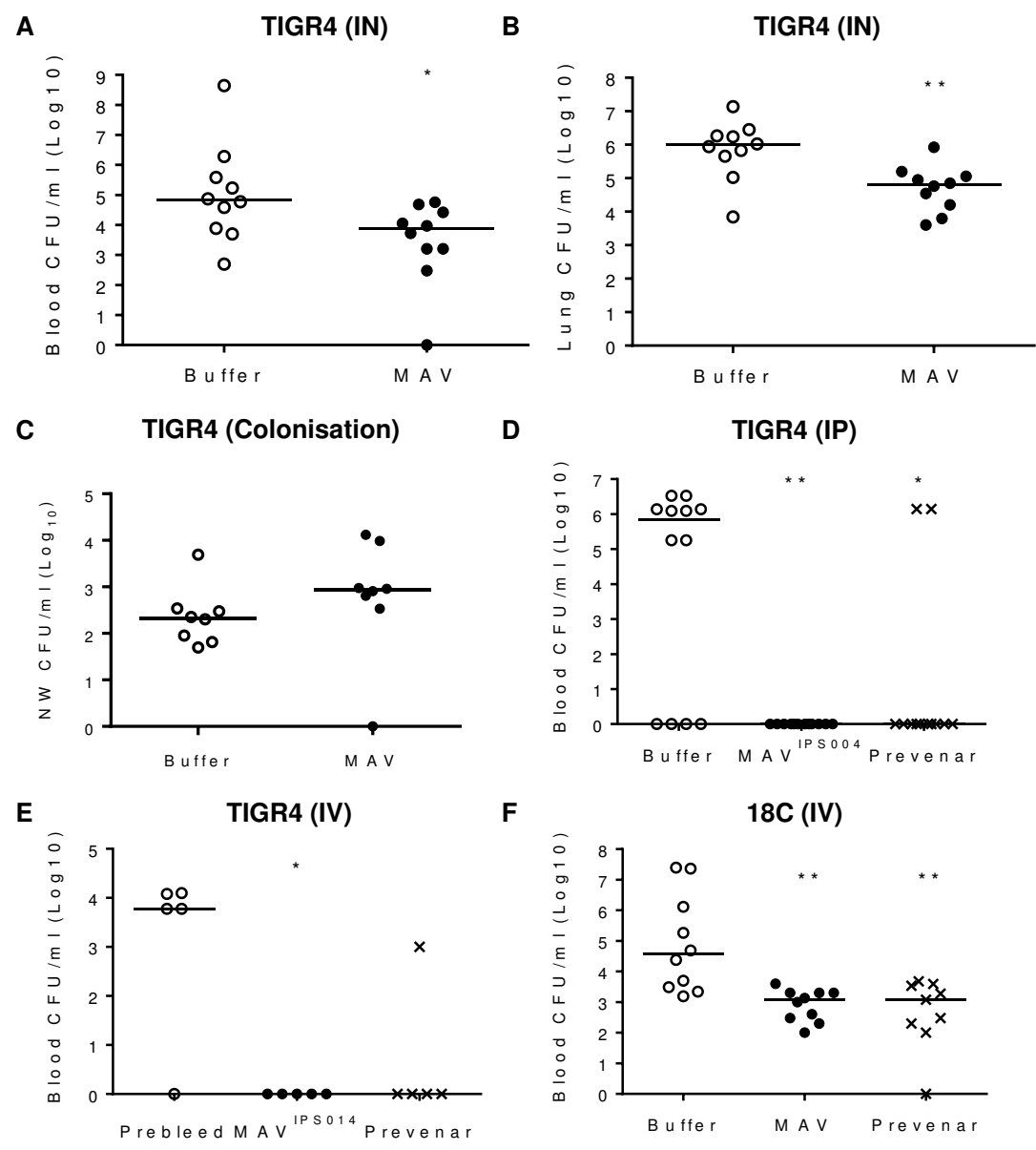
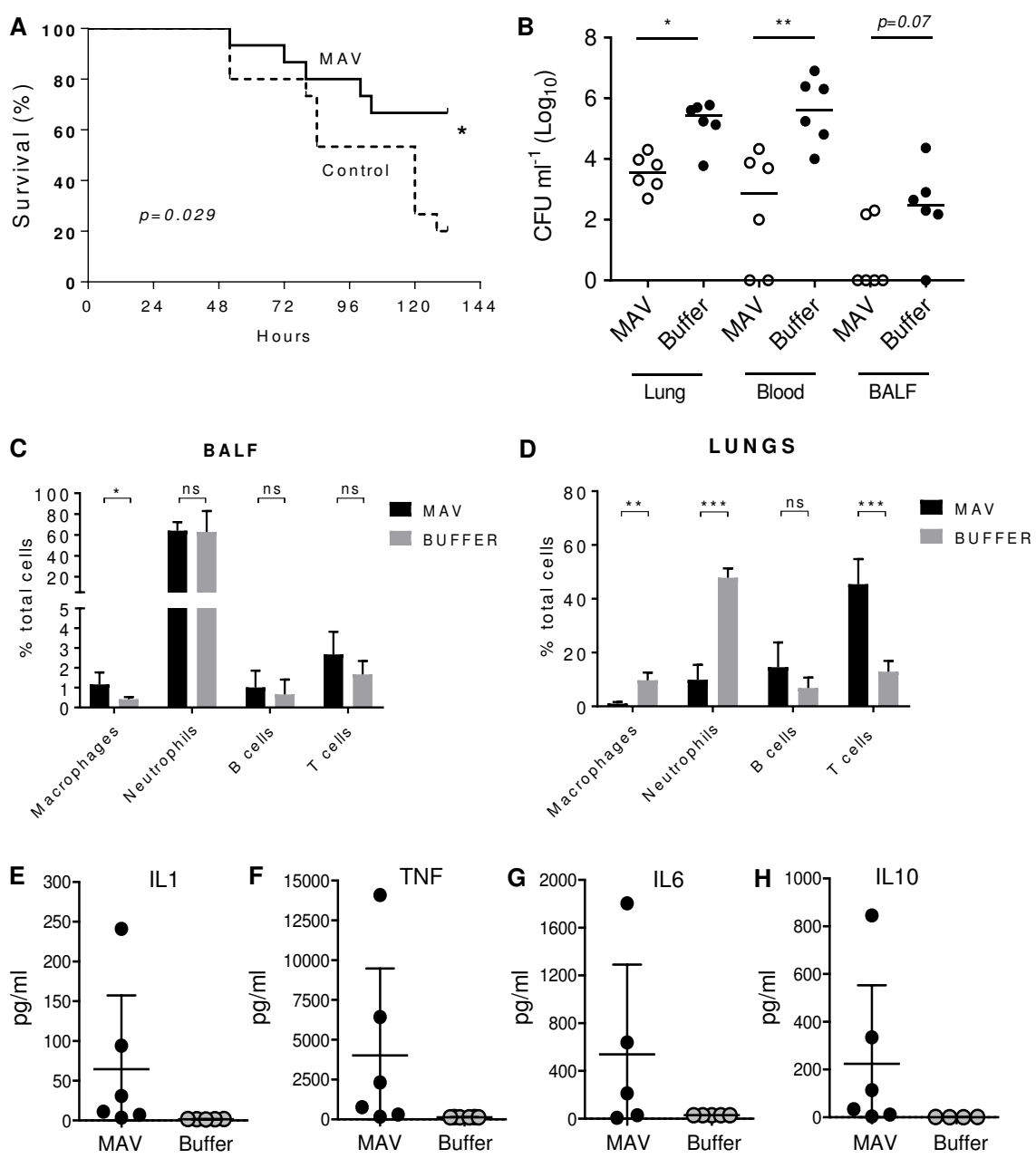


Figure 7



1 **Table 1 Selected proteins that TMT-MS/MS analysis show have increased**
 2 **expression in the MAV TIGR4.1 compared to the TIGR4 HKL preparation.**

SP #	Protein	Fold change	References
Heat shock proteins			
SP_0338	Putative ATP-dependent Clp protease, ATP-binding	2.67	(60)
SP_0517	Chaperone protein (DnaK)	4.33	(61, 62)
SP_0516	Protein GrpE (GrpE)	3.03	(63, 64)
SP_0519	Chaperone protein (DnaJ)	2.37	(65, 66)
SP_1906	60 kDa chaperonin (GroL)	2.95	(67)
SP_1907	10 kDa chaperonin (GroS)	3.56	(68)
Known immunogens			
SP_0082	Cell wall surface anchor family protein (PavB)	5.33	(69)
SP_0148	ABC transporter, substrate-binding protein	3.10	(70, 71)
SP_0149	ABC transporter substrate binding protein (MetQ)	2.52	(70, 72)
SP_0629	L,D-carboxypeptidase (DacB)	3.94	(73)
SP_0845	Nucleoside ABC transporter protein	2.37	(74)
SP_1032	Iron-compound ABC transporter (PiaA)	2.22	(47, 75)
SP_1650	Manganese ABC transporter lipoprotein (PsaA)	4.03	(76, 77)
SP_2093	Putative uncharacterized membrane protein	2.78	(78)
SP_2108	Maltose/maltodextrin-binding protein (MalX)	3.19	(71)

3
 4 Only proteins with an increased fold change of 2 and above are shown