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

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Running Head: A new multi-antigen pneumococcal vaccine

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

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

Introduction

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

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

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

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

Materials and Methods

Bacterial strains and growth conditions

S. pneumoniae was grown in either Todd-Hewitt medium (THY; Oxoid, UK) containing 5% yeast extract or the defined Hoeprichs' media, and on 5% blood Columbia agar (Oxoid) plates containing 5% defibrinated horse blood (TCS Biosciences, UK) at 37° C 5% CO_2. Growth in medium was assessed using optical density (OD) at 580-600 nm, with bacterial stocks grown to mid-log phase (OD_{580nm} 0.4-0.5) before storage in 10% glycerol at -80° C. Bacterial counts were determined as previously described (29–31) by plating 10-fold serial dilutions of aliquots on 5% blood Columbia agar plates after overnight incubation at 37 °C in 5% CO_2. The TIGR4 strain-derived multiple-antigen vaccine (MAV) was made from *S. pneumoniae* TIGR4 (American Type Culture Collection ATCC® BA-334™), and MAV batches IPS004, IPS005 and IPS014 from TIGR4 B7.1 (PlyD6) which expresses an inactivated pneumolysin toxin made as previously described (32). Additional *S. pneumoniae* strains used in this manuscript were: D39 (serotype 2); 0100093 (serotype 3); 23F, a gift from Prof B Spratt (Imperial College London); 18C, from the ATCC (ATCC® BAA-1662™), EF3030 (serotype 19F), a gift from Prof D Briles (University of Alabama); Strain 1777/39 (19A), a gift from Prof J Paton (University of Adelaide).

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

MAV were made from *S. pneumoniae* TIGR4 cultured in Hoeprichs' media (made in-house) in 1 L shake flasks at 37°C to an of OD_{600} of 1.2 before heat shocked at 42°C for 30 minutes. This step was omitted for the non-heat shocked preparation MAV^{IPS005}. Bacteria were then centrifuged twice with wash buffer (40 mM Tris, 150 mM NaCl, 1 mM $MgCl_2$, pH 8.0), incubated with lysis buffer (40 mM Tris, 20 mM NaCl, 1 mM $MgCl_2$, and 0.5% w/v sodium deoxycholate (NaDOC), pH 8.0) for 1 h at 4°C, before homogenisation (EmulsiFlex C5 high pressure homogeniser, Avestin, Germany), and incubation with 0.1% w/v octaethylene glycol monododecyl ether ($C_{12}E_8$) for 4 h at 4 °C. Sample supernatants were harvested using a 5

mL Capto Q column (GE Healthcare, UK). Protein was eluted and collected as 5 mL fractions at 400 mM and 500 mM concentrations of NaCl. IPS004 and IPS014 MAV batches were made as described above using the TIGR4 B7.1 (PlyD6) with heat shock at 37°C for 30 minutes, and lysis of IPS004 in NaDOC (0.5%) and $C_{12}E_8$ (0.1%) and of IPS014 in NaDOC (0.5%) and Triton-X 100 (1%). For heat-killed TIGR4 lysates (HKL) and heat-killed whole cell (HKWC) preparations TIGR4 were also cultured in Hoeprichs' media in 1 L shake flasks at 37°C to an of OD_{600} of 1.2, followed by killing by incubation at 65 °C for 45 minutes which was confirmed by culture on Columbia agar plates. For the HKL, lysis using NaDOC and high pressure homogenisation was as described above. $C_{12}E_8$ was added to both the HKL and HKWC before filtration.

Vaccine characterisation

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

Capillary gel electrophoresis

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

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

In vitro assays

S. pneumoniae whole cell ELISAs were performed using bacterial cultures at an OD_{580} of 0.4-5, alkaline phosphatase (AP) conjugated secondary antibodies, and the substrate para-nitrophenylphosphate (pNPP) (Sigma) as previously described (33, 34). Absorbance was read at 450 nm, subtracting readings at 630 nm (Versamax). The ELISA titre represents the theoretical sample dilution that would result in an $OD_{450-630}$ of 0.1. For detection of anti-Ply and anti-PspA antibodies, the appropriate antigen was was diluted in carbonate buffer to a final concentration of 1 µg/mL and 100 µL was transferred to each well of a 96 well Maxisorp ELISA plate, and incubated overnight at 4 °C. Plates were then washed x 3 with ELISA wash buffer (1% v/v Tween-20 / PBS), blocked for 1 h with block buffer (1 % w/v BSA / PBS) at 37 °C then washed as previous. Serum samples were diluted to a starting dilution of 1/100 to 1/300. Doubling dilutions of the pooled sera samples were assayed in duplicate. Plates were incubated at 37 °C for 1 h, washed and goat anti-mouse IgG HRP diluted to 1/20,000 in added before incubation at 37 °C for 1 h, before washing. TMB substrate was added before incubation at room temperature in the dark. Plates were read OD450 nm and end point titres were calculated using the linear part of each titration curve. IgG surface-binding was assessed using previously described flow cytometry assays (35–37) and species appropriate secondary antibodies: anti-human IgG secondary antibody (1:200) conjugated to PE (Sigma-Aldrich); goat anti-mouse IgG, conjugated to FITC (Bio-Rad, USA). FACS analysis of bacterial cells was performed on the FACSVerse flow cytometer (Becton Dickinson, USA),

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

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

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

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

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

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

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

In vivo methods

All *in vivo* experiments using mice were performed according to UK national guidelines for animal use and care. Experiments performed at UCL were approved by the UCL Biological Services Ethical Committee and the UK Home Office (Project Licence PPL70/6510). Experiments used 6 week old outbred female CD1 mice obtained from Charles River Laboratories. Mice were vaccinated with 75 µg of protein in 100 µL PBS using either intraperitoneal injection at day 0, 14 and 28 or subcutaneous (SC) vaccination on day 0 and 21. Tail bleeds (5µL per mouse) were collected on day 42 and mice challenged with *S. pneumoniae* on day 49. For the pneumonia model mice were inoculated with 5 x 10^6 CFU of *S. pneumoniae* in PBS intranasally (IN) under isoflurane (4%; MiniRad) anaesthesia. After either 24 or 48 h, the mice were euthanized with pentobarbitone, and blood, sera, bronchoalveolar lavage fluid (BALF), lung, and spleen collected as previously described (9, 36, 42, 43). Lungs and spleens were macerated through a 0.2 µm filter. For the colonisation model, mice were anaesthetised with aerosolised isoflurane (4%) and inoculated with 5 x 10^6 CFU *S. pneumoniae* suspended in 10µl of PBS. At designated time points post-infection the mice were culled and the nasal washes obtained by retrograde washing of the nares with 500 µL PBS via the trachea. To assess survival, mice were vaccinated by intraperitoneal inoculation with 75 µg of the MAV^{IPS014} vaccine together with the adjuvant system (Sigma, S6322) on day 1, 10 and 22 before intranasal challenge with 1x10^7 CFU TIGR4 *S. pneumoniae* on day 50. Disease development was monitored over 6 days and mice culled when exhibiting signs of severe disease (42). For the passive transfer model mice were injected intraperitoneally (IP) with 200 µl of serum harvested from rabbits vaccinated by SC

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

Flow cytometry phenyotypic screening of inflammatory cell populations

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

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

Statistical methods

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

Results

Formulation of a *S. pneumoniae* MAV

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

Proteomic analysis of MAV preparations

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

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

Vaccination with MAV induces functional antibodies

To assess immunogenicity of MAV, mice were immunised by subcutaneous injection with either MAV, HKL, HKWC or the negative control buffer using a two dose schedule 21 days apart. Whole cell ELISAs demonstrated that pooled serum obtained one week after the second vaccinaton with MAV contained markedly higher antibody responses to the *S. pneumoniae* TIGR4 strain than those detected in serum from HKL-vaccinated mice. No statistically significant anti-TIGR4 response was identified in sera from mice vaccinated with HKWC (Fig. 2A). The serum antibody response to MAV was dominated by IgG with no significant IgM response compared to buffer vaccinated mice (Fig. 2B). Significantly increased levels of anti-TIGR4 IgG were also detected in the BALF of mice immunised with MAV, but not in nasal washes (Fig. 2C). Whether serum IgG induced by the MAV, HKL and HKWC preparations can recognise and bind to the surface of live *S. pneumoniae* was assessed using a flow cytometry assay that correlates with protection (36). Compatible with the ELISA data, when *S. pneumoniae* TIGR4 were incubated in serum from mice vaccinated with MAV there were higher levels of IgG binding than after incubation in serum from mice vaccinated with HKL or HKWC. Incubation in sera from HKWC vaccinated mice also resulted in less IgG binding to *S. pneumoniae* in comparison to sera from HKL-vaccinated mice (Fig. 3A, B, C). To investigate IgG binding to heterologous strains, the IgG binding assays were repeated using *S. pneumoniae* serotypes 18C, 23F, 3, and 19F (EF3030) strains. Sera from MAV vaccinated mice significantly increased serum IgG binding to the 18C, 23F, 3 and 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 $\log_{10}$ 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 10^4 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

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

Discussion

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

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

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

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

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

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

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

References

1. **Said MA**, **Johnson HL**, **Nonyane BAS**, **Deloria-Knoll M**, **O'Brien KL**. 2013. Estimating the burden of pneumococcal pneumonia among adults: a systematic review and meta-analysis of diagnostic techniques. PLoS One **8**:e60273.
2. **Monasta L**, **Ronfani L**, **Marchetti F**, **Montico M**, **Brumatti L**, **Bavcar A**, **Grasso D**, **Barbiero C**, **Tamburlini G**. 2012. Burden of disease caused by otitis media: Systematic review and global estimates. PLoS One **7**.
3. **Sørensen UB**, **Henrichsen J**, **Chen HC**, **Szu SC**, **Sorensen UBS**, **Henrichsen J**, **Chen HC**, **Szu SC**. 1990. Covalent linkage between the capsular polysaccharide and the cell wall peptidoglycan of *Streptococcus pneumoniae* revealed by immunochemical methods. Microb Pathog **8**:325–334.
4. **Maruyama T**, **Taguchi O**, **Niederman MS**, **Morser J**, **Kobayashi H**, **Kobayashi T**, **D'Alessandro-Gabazza C**, **Nakayama S**, **Nishikubo K**, **Noguchi T**, **Takei Y**, **Gabazza EC**. 2010. Efficacy of 23-valent pneumococcal vaccine in preventing pneumonia and improving survival in nursing home residents: double blind, randomised and placebo controlled trial. BMJ **340**:c1004.
5. **Webster J**, **Theodoratou E**, **Nair H**, **Seong AC**, **Zgaga L**, **Huda T**, **Johnson HL**, **Madhi S**, **Rubens C**, **Zhang JSF**, **El Arifeen S**, **Krause R**, **Jacobs TA**, **Brooks AW**, **Campbell H**, **Rudan I**. 2011. An evaluation of emerging vaccines for childhood pneumococcal pneumonia. BMC Public Health **11 Suppl 3**:S26.
6. **Hanage WP**, **Finkelstein JA**, **Huang SS**, **Pelton SI**, **Stevenson AE**, **Kleinman K**, **Hinrichsen VL**, **Fraser C**. 2010. Evidence that pneumococcal serotype replacement in Massachusetts following conjugate vaccination is now complete. Epidemics **2**:80–4.
7. **Pichichero ME**, **Khan MN**, **Xu Q**. 2016. Next generation protein based *Streptococcus pneumoniae* vaccines. Hum Vaccin Immunother **12**:194–205.
8. **Daniels CC**, **Rogers PD**, **Shelton CM**. 2016. A Review of pneumococcal vaccines: current polysaccharide vaccine recommendations and future protein antigens. J

Pediatr Pharmacol Ther **21**:27–35.

9. **Wilson R**, **Cohen JM**, **Reglinski M**, **Jose RJ**, **Chan WY**, **Marshall H**, **de Vogel C**, **Gordon S**, **Goldblatt D**, **Petersen FC**, **Baxendale H**, **Brown JS**. 2017. Naturally acquired human immunity to pneumococcus is dependent on antibody to protein antigens. PLoS Pathog **13**:e1006137.

10. **Wilson R**, **Cohen JM**, **José RJ**, **de Vogel C**, **Baxendale H**, **Brown JS**. 2015. Protection against *Streptococcus pneumoniae* lung infection after nasopharyngeal colonization requires both humoral and cellular immune responses. Mucosal Immunol **8**:627–39.

11. **Hvalbye BK**, **Aaberge IS**, **Løvik M**, **Haneberg B**. 1999. Intranasal immunization with heat-inactivated *Streptococcus pneumoniae* protects mice against systemic pneumococcal infection. Infect Immun **67**:4320–5.

12. **Malley R**, **Lipsitch M**, **Stack A**, **Saladino R**, **Fleisher G**, **Pelton S**, **Thompson C**, **Briles D**, **Anderson P**. 2001. Intranasal immunization with killed unencapsulated whole cells prevents colonization and invasive disease by capsulated pneumococci. Infect Immun **69**:4870–3.

13. **Xu X**, **Meng J**, **Wang Y**, **Zheng J**, **Wu K**, **Zhang X**, **Yin Y**, **Zhang Q**. 2014. Serotype-independent protection against pneumococcal infections elicited by intranasal immunization with ethanol-killed pneumococcal strain, SPY1. J Microbiol **52**:315–323.

14. **Cohen JM**, **Chimalapati S**, **de Vogel C**, **van Belkum A**, **Baxendale HE**, **Brown JS**. 2012. Contributions of capsule, lipoproteins and duration of colonisation towards the protective immunity of prior *Streptococcus pneumoniae* nasopharyngeal colonisation. Vaccine **30**:4453–4459.

15. **Zügel U**, **Kaufmann SH**. 1999. Role of heat shock proteins in protection from and pathogenesis of infectious diseases. Clin Microbiol Rev **12**:19–39.

16. **De Nagel DC**, **Pierce SK**. 1991. Heat shock proteins implicated in antigen processing and presentation. Semin Immunol **3**:65–71.

17. **Ortmann B**, **Androlewicz MJ**, **Cresswell P**. 1994. MHC class I/beta 2-microglobulin

609 complexes associate with TAP transporters before peptide binding. Nature **368**:864–
610 7.
611 18. **Asea A**, **Kraeft SK**, **Kurt-Jones EA**, **Stevenson MA**, **Chen LB**, **Finberg RW**, **Koo**
612 **GC**, **Calderwood SK**. 2000. HSP70 stimulates cytokine production through a CD14-
613 dependant pathway, demonstrating its dual role as a chaperone and cytokine. Nat
614 Med **6**:435–42.
615 19. **Huang Q-Q**, **Sobkoviak R**, **Jockheck-Clark AR**, **Shi B**, **Mandelin AM**, **Tak PP**,
616 **Haines GK**, **Nicchitta C V.**, **Pope RM**. 2009. Heat shock protein 96 is elevated in
617 rheumatoid arthritis and activates macrophages primarily via TLR2 signaling. J
618 Immunol **182**:4965–4973.
619 20. **Warger T**, **Hilf N**, **Rechtsteiner G**, **Haselmayer P**, **Carrick DM**, **Jonuleit H**, **von**
620 **Landenberg P**, **Rammensee H-G**, **Nicchitta C V**, **Radsak MP**, **Schild H**. 2006.
621 Interaction of TLR2 and TLR4 ligands with the N-terminal domain of Gp96 amplifies
622 innate and adaptive immune responses. J Biol Chem **281**:22545–53.
623 21. **Jonasch E**, **Wood C**, **Tamboli P**, **Pagliaro LC**, **Tu SM**, **Kim J**, **Srivastava P**, **Perez**
624 **C**, **Isakov L**, **Tannir N**. 2008. Vaccination of metastatic renal cell carcinoma patients
625 with autologous tumour-derived vitespen vaccine: clinical findings. Br J Cancer
626 **98**:1336–41.
627 22. **Floto RA**, **MacAry PA**, **Boname JM**, **Mien TS**, **Kampmann B**, **Hair JR**, **Huey OS**,
628 **Houben ENG**, **Pieters J**, **Day C**, **Oehlmann W**, **Singh M**, **Smith KGC**, **Lehner PJ**.
629 2006. Dendritic cell stimulation by mycobacterial Hsp70 is mediated through CCR5.
630 Science (80-) **314**:454–8.
631 23. **Friedland JS**, **Shattock R**, **Remick DG**, **Griffin GE**. 1993. Mycobacterial 65-kD heat
632 shock protein induces release of proinflammatory cytokines from human monocytic
633 cells. Clin Exp Immunol **91**:58–62.
634 24. **Kang HK**, **Lee H-Y**, **Lee Y-N**, **Jo EJ**, **Kim JI**, **Aosai F**, **Yano A**, **Kwak J-Y**, **Bae Y-S**.
635 2004. *Toxoplasma gondii*-derived heat shock protein 70 stimulates the maturation of
636 human monocyte-derived dendritic cells. Biochem Biophys Res Commun **322**:899–

904.

25. **Lima KM**, **Santos SA**, **Lima VMF**, **Coelho-Castelo AAM**, **Rodrigues JM**, **Silva CL**. 2003. Single dose of a vaccine based on DNA encoding mycobacterial hsp65 protein plus TDM-loaded PLGA microspheres protects mice against a virulent strain of *Mycobacterium tuberculosis*. Gene Ther **10**:678–85.

26. **Bonato VLD**, **Gonçalves EDC**, **Soares EG**, **Santos RR**, **Sartori A**, **Coelho-Castelo AAM**, **Silva CL**. 2004. Immune regulatory effect of pHSP65 DNA therapy in pulmonary tuberculosis: Activation of CD8+ cells, interferon-γ recovery and reduction of lung injury. Immunology **113**:130–138.

27. **Chionh YT**, **Arulmuruganar A**, **Venditti E**, **Ng GZ**, **Han JX**, **Entwisle C**, **Ang CS**, **Colaco CA**, **McNulty S**, **Sutton P**. 2014. Heat shock protein complex vaccination induces protection against *Helicobacter pylori* without exogenous adjuvant. Vaccine **32**:2350–2358.

28. **Cao J**, **Li D**, **Gong Y**, **Yin N**, **Chen T**, **Wong CK**, **Xu W**, **Luo J**, **Zhang X**, **Lam CWK**, **Yin Y**. 2009. Caseinolytic protease: a protein vaccine which could elicit serotype-independent protection against invasive pneumococcal infection. Clin Exp Immunol **156**:52–60.

29. **Cohen JM**, **Khandavilli S**, **Camberlein E**, **Hyams C**, **Baxendale HE**, **Brown JS**. 2011. Protective contributions against invasive Streptococcus pneumoniae pneumonia of antibody and Th17-cell responses to nasopharyngeal colonisation. PLoS One **6**.

30. **Yuste J**, **Sen A**, **Truedsson L**, **Jönsson G**, **Tay LS**, **Hyams C**, **Baxendale HE**, **Goldblatt F**, **Botto M**, **Brown JS**. 2008. Impaired opsonization with C3b and phagocytosis of Streptococcus pneumoniae in sera from subjects with defects in the classical complement pathway. Infect Immun **76**:3761–3770.

31. **Hyams C**, **Trzcinski K**, **Camberlein E**, **Weinberger. DM**, **Chimalapati S**, **Noursadeghi M**, **Lipsitch M**, **Brown JS**. 2013. Streptococcus pneumoniae capsular serotype invasiveness correlates with the degree of factor H binding and opsonization

with C3b/iC3b. Infect Immun **81**:354–363.

32. **Cecchini P**, **Entwisle C**, **Joachim M**, **Pang Y**, **Dalton KA**, **Hill S**, **McIlgorm A**, **Chan W-Y**, **Brown JS**, **Colaco CA**, **Bailey CR**, **Clarke SW**. 2015. Next generation vaccines: development of a novel *Streptococcus pneumoniae* multivalent protein vaccine. Bioprocess J **14**:18–33.

33. **Hyams C**, **Camberlein E**, **Cohen JM**, **Bax K**, **Brown JS**. 2010. The *Streptococcus pneumoniae* capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. Infect Immun **78**:704–715.

34. **Chimalapati S**, **Cohen J**, **Camberlein E**, **Durmort C**, **Baxendale H**, **de Vogel C**, **van Belkum A**, **Brown JS**. 2011. Infection with conditionally virulent *Streptococcus pneumoniae* Δpab strains induces antibody to conserved protein antigens but does not protect against systemic infection with heterologous strains. Infect Immun **79**:4965–4976.

35. **Jomaa M**, **Yuste J**, **Paton JC**, **Jones C**, **Dougan G**, **Brown JS**. 2005. Antibodies to the iron uptake ABC transporter lipoproteins PiaA and PiuA promote opsonophagocytosis of *Streptococcus pneumoniae*. Infect Immun **73**:6852–6859.

36. **Cohen JM**, **Wilson R**, **Shah P**, **Baxendale HE**, **Brown JS**. 2013. Lack of cross-protection against invasive pneumonia caused by heterologous strains following murine *Streptococcus pneumoniae* nasopharyngeal colonisation despite whole cell ELISAs showing significant cross-reactive IgG. Vaccine **31**:2328–2332.

37. **Chimalapati S**, **Cohen JM**, **Camberlein E**, **MacDonald N**, **Durmort C**, **Vernet T**, **Hermans PWM**, **Mitchell T**, **Brown JS**. 2012. Effects of deletion of the *Streptococcus pneumoniae* lipoprotein diacylglyceryl transferase gene *lgt* on ABC transporter function and on growth *in vivo*. PLoS One **7**:e41393.

38. **Turner P**, **Turner C**, **Green N**, **Ashton L**, **Lwe E**, **Jankhot A**, **Day NP**, **White NJ**, **Nosten F**, **Goldblatt D**. 2013. Serum antibody responses to pneumococcal colonization in the first 2 years of life: results from an SE Asian longitudinal cohort study. Clin Microbiol Infect **19**:E551-8.

693 39. **Kantsø B**, **Green N**, **Goldblatt D**, **Benfield T**. 2015. Antibody response is more likely
694 to pneumococcal proteins than to polysaccharide after HIV-associated invasive
695 pneumococcal disease. J Infect Dis **212**:1093–1099.
696 40. **Ferreira DM**, **Neill DR**, **Bangert M**, **Gritzfeld JF**, **Green N**, **Wright AKA**, **Pennington**
697 **SH**, **Moreno LB**, **Moreno AT**, **Miyaji EN**, **Wright AD**, **Collins AM**, **Goldblatt D**,
698 **Kadioglu A**, **Gordon SB**. 2013. Controlled human infection and rechallenge with
699 *Streptococcus pneumoniae* reveals the protective efficacy of carriage in healthy
700 adults. Am J Respir Crit Care Med **187**:855–864.
701 41. **Goldblatt D**, **Ashton L**, **Zhang Y**, **Antonello J**, **Marchese RD**. 2011. Comparison of
702 a new multiplex binding assay versus the enzyme-linked immunosorbent assay for
703 measurement of serotype-specific pneumococcal capsular polysaccharide IgG. Clin
704 vaccine Immunol **18**:1744–51.
705 42. **Brown JS**, **Hussell T**, **Gilliland SM**, **Holden DW**, **Paton JC**, **Ehrenstein MR**,
706 **Walport MJ**, **Botto M**. 2002. The classical pathway is the dominant complement
707 pathway required for innate immunity to *Streptococcus pneumoniae* infection in mice.
708 Proc Natl Acad Sci U S A **99**:16969–16974.
709 43. **Yuste J**, **Botto M**, **Bottoms SE**, **Brown JS**. 2007. Serum amyloid P aids
710 complement-mediated immunity to *Streptococcus pneumoniae*. PLoS Pathog **3**:1208–
711 19.
712 44. **Moffitt KL**, **Gierahn TM**, **Lu YJ**, **Gouveia P**, **Alderson M**, **Flechtner JB**, **Higgins**
713 **DE**, **Malley R**. 2011. TH17-based vaccine design for prevention of streptococcus
714 pneumoniae colonization. Cell Host Microbe **9**:158–165.
715 45. **Ahmed MS**, **Derbyshire S**, **Flanagan B**, **Loh C**, **Mccormick M**, **Barocchi M**,
716 **Masignani V**, **Finn A**, **Zhang Q**. 2014. Immune responses to pneumococcal pilus
717 RrgA and RrgB antigens and their relationship with pneumococcal carriage in
718 humans. J Infect **68**:562–571.
719 46. **Xu J**, **Dai W**, **Chen B**, **Fan X**. 2015. Mucosal immunization with PsaA protein, using
720 chitosan as a delivery system, increases protection against acute otitis media and

721 invasive infection by *Streptococcus pneumoniae*. Scand J Immunol **81**:177–85.
722 47. **Xin W**, **Li Y**, **Mo H**, **Roland KL**, **Curtiss R**. 2009. PspA family fusion proteins
723 delivered by attenuated *Salmonella enterica* serovar Typhimurium extend and
724 enhance protection against *Streptococcus pneumoniae*. Infect Immun **77**:4518–28.
725 48. **Jomaa M**, **Terry S**, **Hale C**, **Jones C**, **Dougan G**, **Brown J**. 2006. Immunization with
726 the iron uptake ABC transporter proteins PiaA and PiuA prevents respiratory infection
727 with *Streptococcus pneumoniae*. Vaccine **24**:5133–9.
728 49. **Kirkham L-AS**, **Kerr AR**, **Douce GR**, **Paterson GK**, **Dilts DA**, **Liu D-F**, **Mitchell TJ**.
729 2006. Construction and immunological characterization of a novel nontoxic protective
730 pneumolysin mutant for use in future pneumococcal vaccines. Infect Immun **74**:586–
731 93.
732 50. **Moffitt K**, **Malley R**. 2016. Rationale and prospects for novel pneumococcal vaccines.
733 Hum Vaccin Immunother **12**:383–92.
734 51. **Chan W-Y**, **Cohen J**, **Brown J**. 2016. The new first-line defense: the potential of
735 nasopharyngeal colonization in vaccine strategies. Vaccine Dev Ther **Volume 6**:47–
736 57.
737 52. **Khan MN**, **Pichichero ME**. 2012. Vaccine candidates PhtD and PhtE of
738 *Streptococcus pneumoniae* are adhesins that elicit functional antibodies in humans.
739 Vaccine **30**:2900–2907.
740 53. **Glover DT**, **Hollingshead SK**, **Briles DE**. 2008. <i>Streptococcus pneumoniae<i>
741 surface protein PcpA elicits protection against lung infection and fatal sepsis. Infect
742 Immun **76**:2767–2776.
743 54. **Salha D**, **Szeto J**, **Myers L**, **Claus C**, **Sheung A**, **Tang M**, **Ljutic B**, **Hanwell D**,
744 **Ogilvie K**, **Ming M**, **Messham B**, **van den Dobbelsteen G**, **Hopfer R**, **Ochs MM**,
745 **Gallichan S**. 2012. Neutralizing antibodies elicited by a novel detoxified pneumolysin
746 derivative, PlyD1, provide protection against both pneumococcal infection and lung
747 injury. Infect Immun **80**:2212–20.
748 55. **Tostes RO**, **Rodrigues TC**, **Da Silva JB**, **Schanoski AS**, **Oliveira MLS**, **Miyaji EN**.

2017. Protection elicited by nasal immunization with recombinant Pneumococcal surface protein A (rPspA) adjuvanted with whole-cell pertussis vaccine (wP) against co-colonization of mice with *Streptococcus pneumoniae*. PLoS One **12**:e0170157.

56. **Malley R**, **Anderson PW**. 2012. Inaugural Article: Serotype-independent pneumococcal experimental vaccines that induce cellular as well as humoral immunity. Proc Natl Acad Sci **109**:3623–3627.

57. **Babb R**, **Chen A**, **Hirst TR**, **Kara EE**, **McColl SR**, **Ogunniyi AD**, **Paton JC**, **Alsharifi M**. 2016. Intranasal vaccination with gamma-irradiated *Streptococcus pneumoniae* whole-cell vaccine provides serotype-independent protection mediated by B cells and innate IL-17 responses. Clin Sci (Lond) **130**:697–710.

58. **Tettelin H**, **Nelson KE**, **Paulsen IT**, **Eisen JA**, **Read TD**, **Peterson S**, **Heidelberg J**, **DeBoy RT**, **Haft DH**, **Dodson RJ**, **Durkin AS**, **Gwinn M**, **Kolonay JF**, **Nelson WC**, **Peterson JD**, **Umayam LA**, **White O**, **Salzberg SL**, **Lewis MR**, **Radune D**, **Holtzapple E**, **Khouri H**, **Wolf AM**, **Utterback TR**, **Hansen CL**, **McDonald LA**, **Feldblyum T V**, **Angiuoli S**, **Dickinson T**, **Hickey EK**, **Holt IE**, **Loftus BJ**, **Yang F**, **Smith HO**, **Venter JC**, **Dougherty BA**, **Morrison DA**, **Hollingshead SK**, **Fraser CM**. 2001. Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. Science (80-) **293**:498–506.

59. **Hughes CE**, **Harvey RM**, **Plumptre CD**, **Paton JC**. 2014. Development of primary invasive pneumococcal disease caused by serotype 1 pneumococci is driven by early increased type I interferon response in the lung. Infect Immun **82**:3919–26.

60. **Lu Y-J**, **Zhang F**, **Sayeed S**, **Thompson CM**, **Szu S**, **Anderson PW**, **Malley R**. 2012. A bivalent vaccine to protect against <i>Streptococcus pneumoniae and Salmonella typhi. Vaccine **30**:3405–12.

61. **Plumptre CD**, **Ogunniyi AD**, **Paton JC**. 2013. Vaccination against Streptococcus pneumoniae using truncated derivatives of polyhistidine triad protein D. PLoS One **8**:e78916.

62. **Zhang Z**, **Clarke TB**, **Weiser JN**. 2009. Cellular effectors mediating Th17-dependent

clearance of pneumococcal colonization in mice. J Clin Invest **119**:1899–1909.

63. **Schmid P**, **Selak S**, **Keller M**, **Luhan B**, **Magyarics Z**, **Seidel S**, **Schlick P**, **Reinisch C**, **Lingnau K**, **Nagy E**, **Grubeck-Loebenstein B**. 2011. Th17/Th1 biased immunity to the pneumococcal proteins PcsB, StkP and PsaA in adults of different age. Vaccine **29**:3982–9.

64. **Entwisle C**, **Hill S**, **Pang Y**, **Joachim M**, **McIlgorm A**, **Colaco C**, **Goldblatt D**, **De Gorguette D'Argoeuves P**, **Bailey C**. 2017. Safety and immunogenicity of a novel multiple antigen pneumococcal vaccine in adults: A Phase 1 randomised clinical trial. Vaccine **35**:7181–7186.

Figures

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

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

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

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

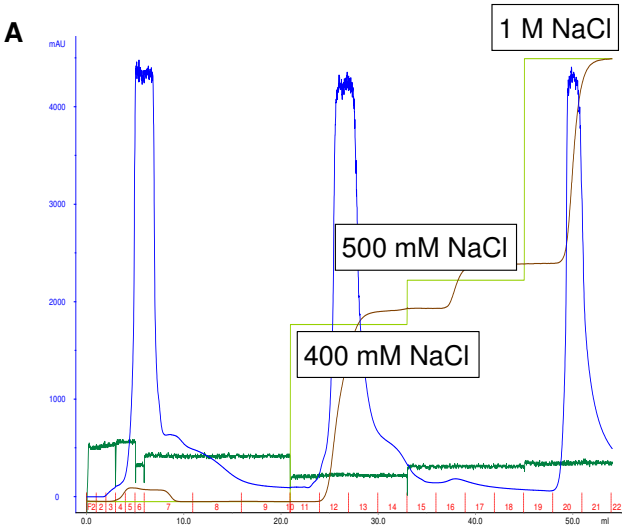
842 **Figure 5**

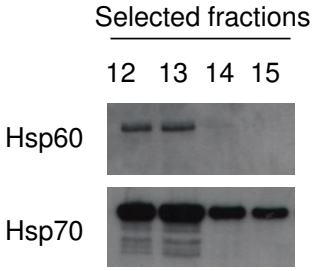
843 A comparison of heat shocked MAV vs non-heat shocked MAV. **A** MAV preparations were
844 made with (MAV^{IPS004}) and without (MAV^{IPS005}) the Hsp induction step. Protein bands were
845 compared using a Coomassie gel (top). 3 µg (lanes 7 & 9) and 5 ug (lanes 8 & 10) of each
846 MAV was loaded 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 barchart 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* (serotpyes 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 shcok; 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
864 x 10^7 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 x 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 x 10^4 CFU *S.*
869 *pneumoniae* TIGR4 strain of mice passively vaccinated with 200 µl of sera from rabbits

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

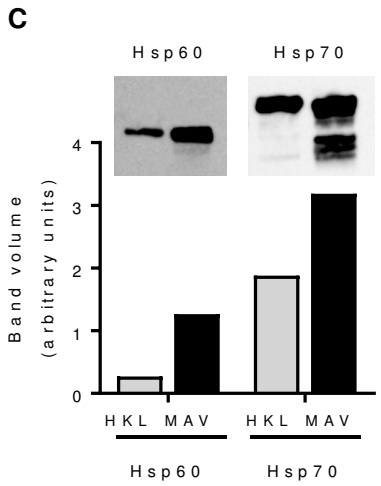
Figure 7 Vaccination with MAV preparations increase survival of mice and alters the inflammatory response after TIGR4 *S. pneumoniae* pneumonia challenge. **A** Percentage survival of mice over 6 days after challenge by intranasal inoculation with 1 x 10^7 CFU *S. pneumoniae* TIGR4 strain of mice vaccinated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

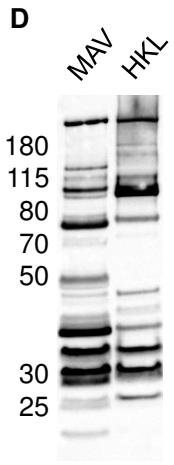
Figure 1





Fraction	Concentration (mg/ml)
12	0.84
13	0.88
14	0.25
15	0.15





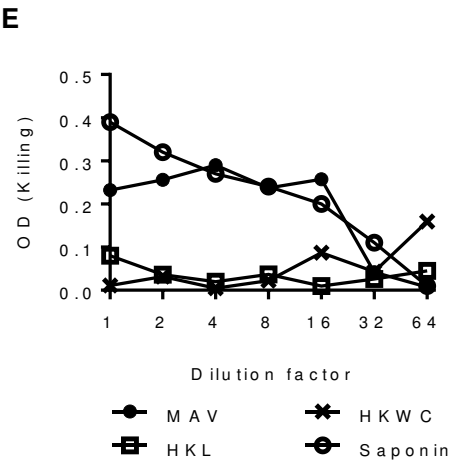
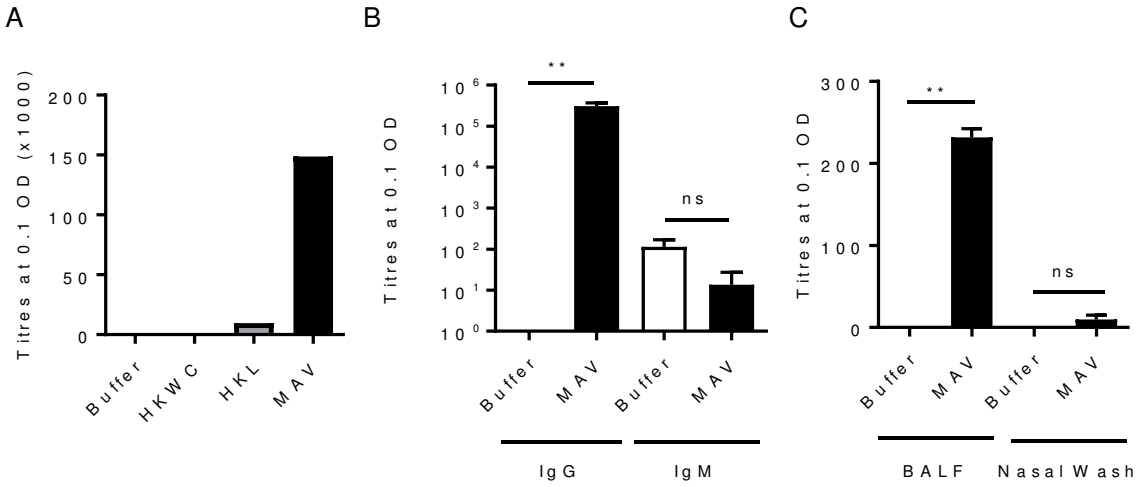


Figure 2



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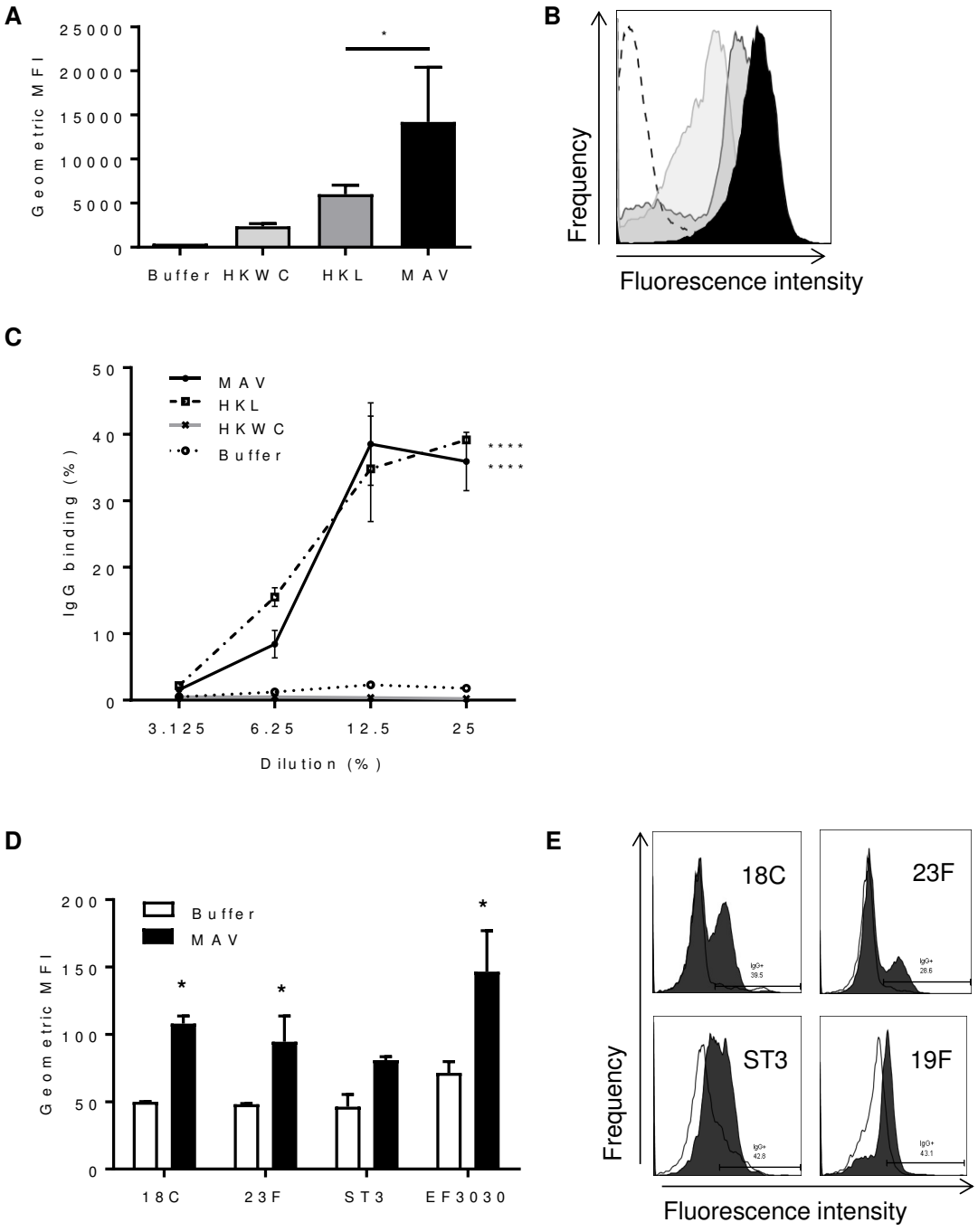


Figure 4

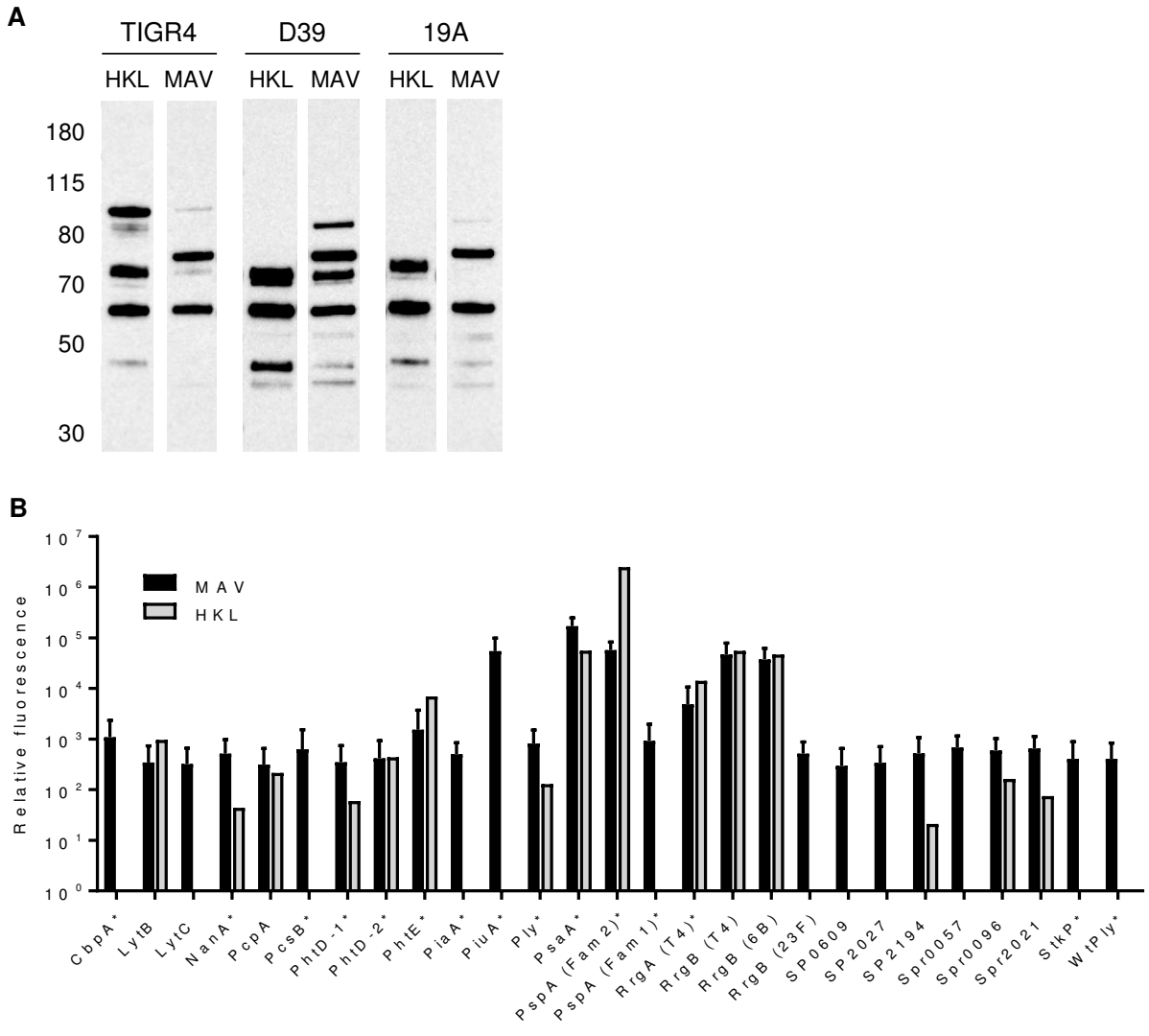
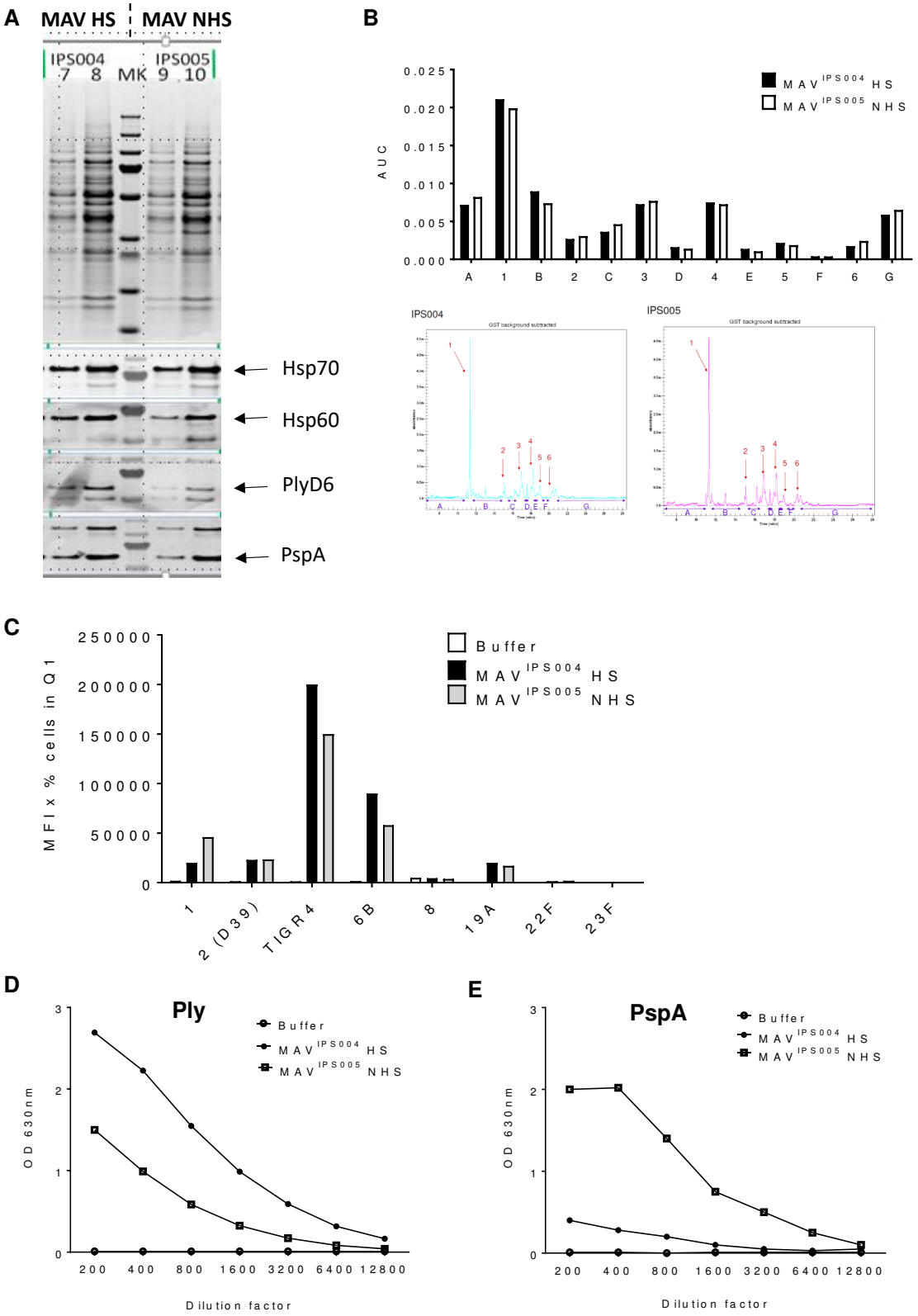


Figure 5



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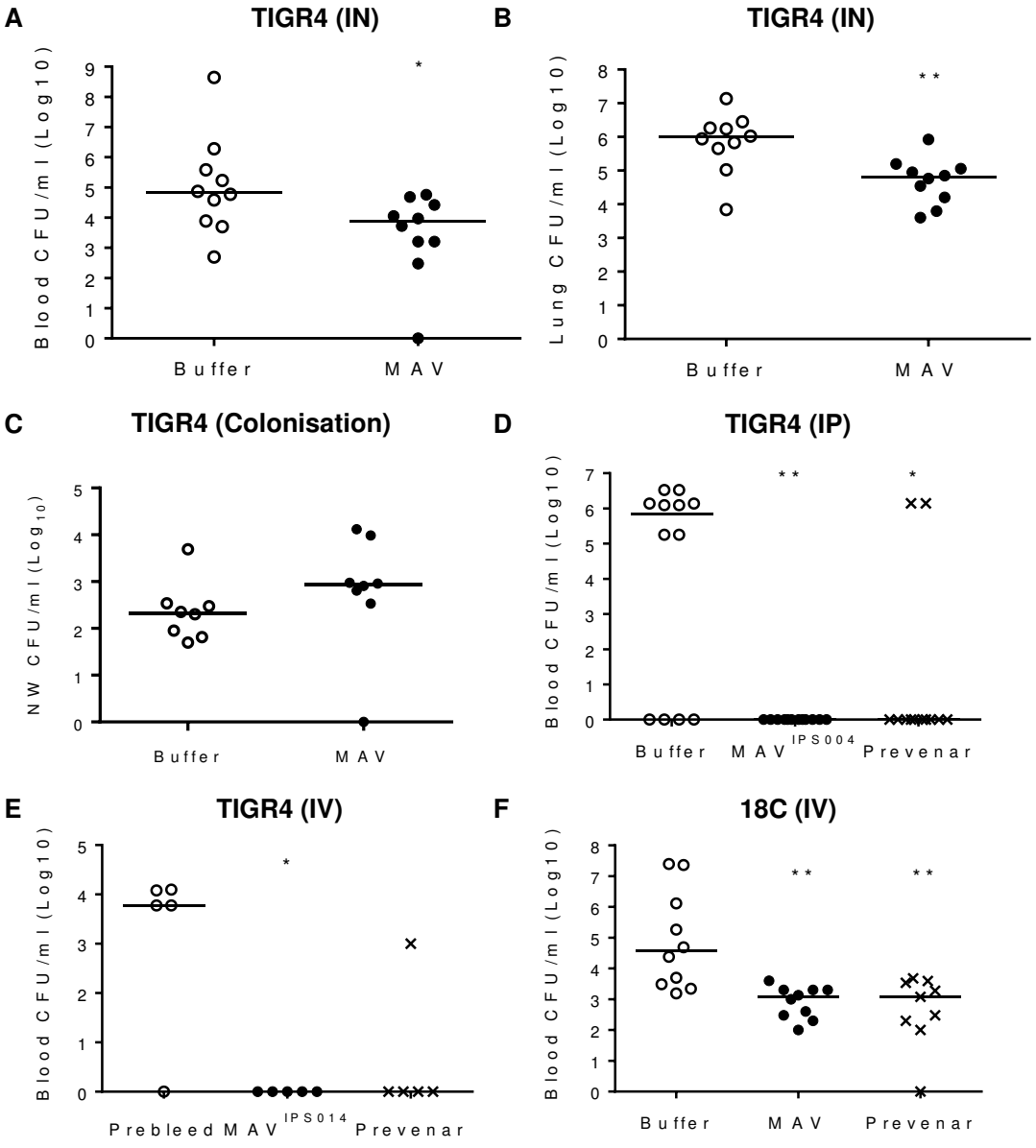

Figure 7

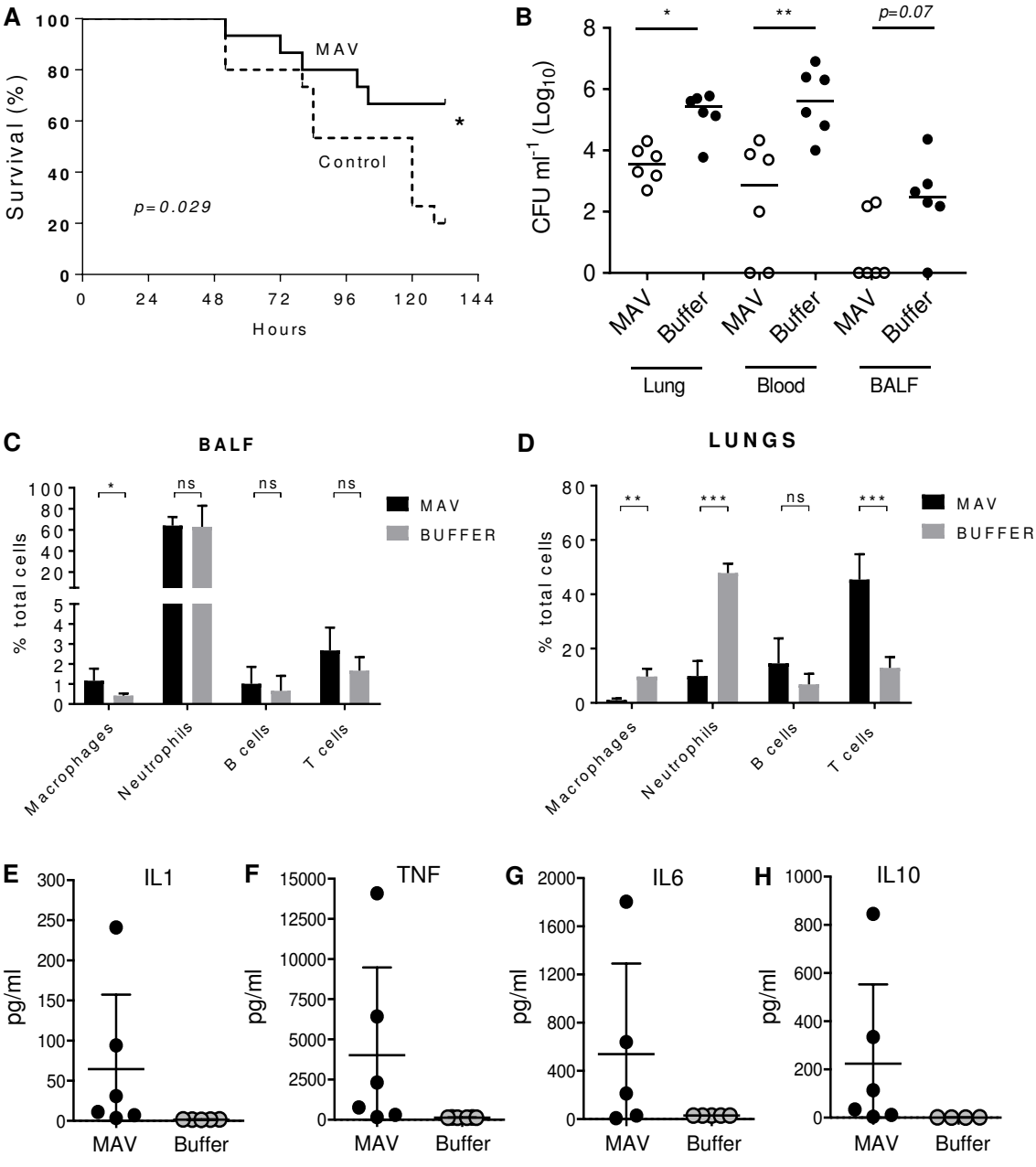

Table 1 Selected proteins that TMT-MS/MS analysis show have increased expression in the MAV TIGR4.1 compared to the TIGR4 HKL preparation.

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)

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