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Vaccination with the *Staphylococcus aureus* secreted proteins EapH1 and EapH2 impacts both *S. aureus* carriage and invasive disease

Elizaveta Elshina [a,1,2], Elizabeth R. Allen [a,1], Amy Flaxman [a,1], Pauline M. van Diemen [a,3], Anita Milicic [a], Christine S. Rollier [b], Yuko Yamaguchi [a], David H. Wyllie [a,*]

[a] Jenner Institute, Centre for Cellular and Molecular Physiology, University of Oxford, United Kingdom
[b] Oxford Vaccine Group, Department of Paediatrics, University of Oxford, and NIHR Oxford Biomedical Research Centre, Centre for Clinical Vaccinology and Tropical Medicine, University of Oxford, United Kingdom

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ABSTRACT

Introduction: There is a need for an efficacious vaccine reducing infections due to *Staphylococcus aureus*, a common cause of community and hospital infection. Infecting organisms originate from *S. aureus* populations colonising the nares and bowel. Antimicrobials are widely used to transiently reduce *S. aureus* colonisation prior to surgery, a practice which is selecting for resistant *S. aureus* isolates. *S. aureus* secretes multiple proteins, including the protease inhibitors extracellular adhesion protein homologue 1 and 2 (EapH1 and EapH2).
Methods: Mice were vaccinated intramuscularly or intranasally with Adenovirus serotype 5 and Modified Vaccinia Ankara viral vectors expressing EapH1 and EapH2 proteins, or with control viruses. Using murine *S. aureus* colonisation models, we monitored *S. aureus* colonisation by sequential stool sampling. Monitoring of *S. aureus* invasive disease after intravenous challenge was performed using bacterial load and abscess numbers in the kidney.
Results: Intramuscular vaccination with Adenovirus serotype 5 and Modified Vaccinia Ankara viral vectors expressing EapH1 and EapH2 proteins significantly reduces bacterial recovery in the murine renal abscess model of infection, but the magnitude of the effect is small. A single intranasal vaccination with an adenoviral vaccine expressing these proteins reduced *S. aureus* gastrointestinal (GI) tract colonisation.
Conclusion: Vaccination against EapH1 / EapH2 proteins may offer an antibiotic independent way to reduce *S. aureus* colonisation, as well as contributing to protection against *S. aureus* invasive disease.

1. Introduction

Staphylococcus aureus is able to asymptomatically colonise the human skin, nares [1] and bowel [2], and to cause a plethora of diseases ranging from superficial skin infections [3] to severe and life threatening endocarditis, pneumonia, and sepsis [1]. *S. aureus* hospital and community acquired infections are a major health concern and economic burden, aggravated by the increasing incidence of antibiotic resistance and the limited supply of new therapeutics [4]. There is no licensed vaccine against *S. aureus*; two major Phase III clinical trials aiming to reduce the incidence of severe infection post-surgery, or during haemodialysis, have been unsuccessful [5,6].

Persistent *S. aureus* carriage is an important phenomenon, present in 20–30% of the population, which represents the source of human infection [7]. It is a state characterised by ongoing exposure to and acquisition of new *S. aureus* strains [8], but a low loss rate. In contrast, the rest of the population, described as intermittent or non-carriers, carry *S. aureus* following exposure but lose carriage rapidly [9].

S. aureus transcription is highly dynamic, with multiple virulence proteins produced in response to external stimuli, including neutrophils [10]. These proteins modify critical aspects of the innate and adaptive immune response [11]. Neutralising *S. aureus* subversion proteins has been proposed as one route to an effective *S. aureus* vaccine [12]. Substantial support for this hypothesis comes from studies of vaccination against surface protein A, an immunoglobulin binding protein, which affects both the development of *S. aureus* invasive disease [13–16] and bacterial colonisation [17].

EapH proteins are secreted inhibitors of neutrophil serine proteases, including neutrophil elastase, whose mechanism of

* Corresponding author.
E-mail address: david.wyllie@ndm.ox.ac.uk (D.H. Wyllie).
[1] These authors contributed equally to this work.
[2] Present address: Department of Pathology, University of Cambridge.
[3] Present address: Virology, APHA-Weybridge, New Haw, Addlestone.

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action, defined by co-crystallisation and mutagenesis studies, involves non-covalent binding to mammalian serine proteases [18]. EapH1 and EapH2 operate by different mechanisms, despite their primary amino acid homology [19]. Consequences of EapH-mediated serine protease inhibition include the protection from degradation of important S. *aureus* immune modulatory proteins, resulting in preserved formyl-peptide receptor antagonism by *S. aureus* Chemotaxis inhibitory protein (CHIPS), formyl peptide receptor-like 1 inhibitor (FLIPr) and FLIPr-like proteins, and preserved alternative pathway inhibition by Staphylococcal complement inhibitor (SCN) proteins [20].

Eap itself is a much larger cell-surface protein containing four to six repetitive MHC class II analogue protein (Map) domains connected with short linkers [21]. It is secreted, but a proportion of the protein binds back to the *S. aureus* cell wall [22] via interaction with a neutral phosphatase [23], and the protein can be detected in the pseudocapsule that encloses staphylococcal abscess communities [24]. It has a wide range of biological activities, with different domains having specialised functions [25]. Proteins binding to Eap domains include collagen [26], vitronectin [22], fibrinogen [27], plasmin [22], ICAM-1 [27], C4 [28], and, like EapH proteins, neutrophil serine proteases [18]. Functionally, Eap alters bacterial agglutination, tissue adherence, inhibition of neutrophil recruitment [22,27], bacterial persistence within host tissues [24], inhibition of classical and lectin complement pathways at the level of C3 proconvertase formation and thence impairing phagocytosis and killing by neutrophils [28] and impairment of neutrophil serine protease activity [18,20].

Here, we investigated the potential of EapH1 and EapH2 as vaccines, electing to use viral vectored regimes to administer these [29] We used a well-studied regime, in which adenoviral priming is followed eight weeks later by a modified Vaccinia Ankara (MVA) vaccine expressing the same antigen. This regime induces both potent antibody and T cell responses, and both T cell and antibody mediated protection have been observed in other disease models [30–33]; regime selection was motivated by the role of both antibody [34] and T cell based protection in the control of *S. aureus* carriage and disease in animal models [35].

2. Materials and Methods

2.1. Bioinformatic analyses

Gene presence/absence in *S. aureus* genomes was determined using tblastn (NCBI Blast suite v. 2.4.0), requiring significant homology over ≥95% of the gene length. Genomes analysed are described in Supplementary Data, Table S1.

2.2. Bacterial strains and growth

S. aureus strains used were Newman (obtained from Prof T Foster, Trinity College Dublin), USA300 JE2 (FPR3757) (obtained from ATCC), while we isolated the SaF_1 strain [36]. For infection of mice, *S. aureus* was grown in tryptic soy broth (TSB, Oxoid, UK) overnight at 37 °C, 130 rpm, and an aliquot recultured at 37 °C for 2.5 h. Bacteria were resuspended in 10 mL Phosphate Buffered Saline (PBS, Sigma Aldrich, UK) at approximately 10^8 cells/mL. The actual bacterial concentrations were determined by dilution plating.

2.3. Recombinant protein production

Synthetic DNA encoding C-terminal Map domain with N-terminal His-tag and 3C protease site (ThermoFisher) (Supplementary Data, Table S2) were inserted into the pOPIN-F vector. Protein expression used BL21 (DE3) *E. coli* with purification on nickel columns. On average 2 to 10 mg of protein was obtained from 50 mL culture.

2.4. Antigens and viral vectors

Vaccine 'Eap' contained the C-terminal Map domain (domain 5) of *S. aureus* Eap protein (a. a. 481–584 of WP_001549158.1). The vaccine 'EapH1_2' contained Map domains of EapH1 (a. a. 24-141 of WP_001549607.1) and EapH2 (a. a. 24-144 of WP_000769689.1); vaccine 'Eap_EapH1_2' was composed of all three antigens (Supplementary Data, Tables S2 and S3). DNA sequences encoding vaccine constructs were synthesised including a 3′ V5 epitope tag and IMX313 oligomerization domain [37], and were subcloned into a mammalian expression vector (patent WO2014053861A2). Adenovirus and MVA production was as previously described [38,39]. The expression of antigens from the viral vectored vaccines was confirmed by infecting HeLa cells and expression of proteins was assayed using a sandwich ELISA using monoclonal anti-V5 epitope capture antibody and anti IMX 313 rabbit polyclonal antibody (kind gift of Dr F Hill, Imaxio SA, Lyon, France) for primary detection. We conducted these assays both on supernatants and cellular lysates obtained 24 h after infection with 1 to 100 MOI of virus. Cells were lysed with PBS containing 1% Tween 20 and Complete protease Inhibitor Cocktail (Roche).

2.5. Ethics

All mouse procedures were conducted in accordance to the Animal (Scientific Procedures) Act 1986 (Project licences 30/2825 & 30/3385) and were approved by the University of Oxford Animal Care and Ethical Review Committee.

2.6. Animals

2.6.1. Intravenous challenge model

To determine the protective effect of the vaccines we used a murine intravenous challenge model [24]. For the three IV challenge experiments in this study, a total of 111 female BALB/c mice were used, obtained from Envigo (formerly Harlan Laboratories) age 6 weeks. We have previously determined that this assay has 90% power to detect a 0.85 log decrease with $\alpha = 0.05$ and a group size of 8 [40]. Mice used in experiments #1 and #2 were colonised with *S. aureus* in the breeding facility. In experiment #3 mice from a different barrier were used which were not *S. aureus* colonised; these were experimentally colonised with *S. aureus* USA300 JE2 (FPR3757) prior to vaccination, as described previously [36]. Mice were housed randomly in individually filtered cages of 3, 4 or 6 and received normal diet and water *ad libitum.* In all three experiments, mice received experimental or control vaccines via an intramuscular injection with 10^9 IU AdHu5, followed 8–10 weeks later by a boost vaccination comprising 10^7 PFU MVA. Two weeks after the boost vaccination, mice were challenged with $\sim 10^7$ CFU *S. aureus* Newman and monitored as described previously [41,42]. Three days post infection mice were sacrificed to harvest both kidneys; one was used for MRI analysis and one for quantitative culture, as described [36,43,44]. A summary of experimental design for experiments 1 to 3 is shown in Supplementary Data, Table S4.

2.6.2. Intranasal vaccination and experimental colonisation of mice

Eighty female CD1 mice aged 6 weeks were anaesthetised with isoflurane and received a single 100 μl intranasal dose of PBS containing 10^9 IU AdHu5 or 10^7 PFU MVA expressing EapH1_2 or no antigen. This administration was performed dropwise into both nostrils and appeared well tolerated. A co-caging strategy was

used, such that equal numbers of mice from each vaccination group were included in each cage [36]. Food and water was available *ad libitum*. To assess efficacy of vaccines in reducing *S. aureus* GI carriage, mice were experimentally colonised with USA300 26 days after vaccination. For experimental colonization of mice, *S. aureus* USA300 was prepared as described in 'Bacterial strains and growth' at PBS at 5×10^9 cfu/ml. Cage environments, but not the mice themselves, were contaminated with ~5 mL of an aerosolised *S. aureus* inoculum. Bedding was changed after seven days. Follow-up involved faecal sampling from individual mice and quantitative culture, as described previously [36].

2.6.3. Intranasal infection model

Anaesthetised mice were challenged with 10^7 CFU *S. aureus* USA300 bacterial suspension in 0.05 mL PBS administered dropwise into each nostril. Mice were monitored at 4 timepoints in the 24 h following intranasal challenge for signs of illness. 24 h post infection mice were sacrificed, lungs harvested, the right lung homogenised in PBS, and *S. aureus* quantification performed as above.

2.6.4. Measuring murine antibody responses

The antibody response to vaccination in mice was assessed by LIPS assay as previously described [40,45]. ELISA with recombinant His-tagged Eap was used to determine anti-Eap antibody response, because high levels of background signal were observed in LIPS assays. Regression analysis was use to estimate the end-point titre value equivalent to three times the background.

2.6.5. IFN-γ Enzyme-Linked ImmunoSpot (ELISpot) assay

Individual mouse peripheral blood samples were treated with ACK lysis buffer to remove RBCs and resuspended in MEM α-modification media supplemented with 10% heat-inactivated FCS (Gibco BRL). PBMCs were stimulated with 5 μg/mL of peptides spanning EapH1 and EapH2 portions of the immunogen in the presence of a mouse splenocyte suspension (5×10^6 cells/mL) from the same mouse strain overnight, and IFN-γ spot forming cells (SFC) quantified [37].

3. Results

3.1. Conservation of EapH proteins across S. aureus lineages

We assessed presence and conservation of genes encoding EapH1 and EapH2 in two sets of *S. aureus* genomes: 104 clinical *S. aureus* representative of all major lineages [8], and 170 closed *S. aureus* genomes available in GenBank, using tblastn (see Methods). In common with other recognised core *S. aureus* genes, such as IsdA and IsdB, we detected EapH2 in all strains examined, and EapH1 in all but one (Supplementary Fig. S1). Genetic diversity relative to the query sequences used (WP_001549607.1, WP_000769689.1 respectively) was less (median 97%) for EapH1 than for EapH2 (median 99%). Both genes are very highly conserved relative to Eap (median 83%) which as expected [7] was detected in all isolates examined.

3.2. Eap and EapH protein production from viral vectors

We investigated combinations of EapH1, EapH2 and Eap proteins as vaccine antigens. Three constructs, designated Eap, EapH1_2 and EapH1_2_Eap, were designed to contain different combinations of Map domains from the Eap, EapH1 or EapH2 proteins (Fig. 1A and B). These constructs were expressed from both adenovirus human serotype 5 (AdHu5) and modified vaccinia Ankara (MVA) vectors using expression cassette containing a eukaryotic secretion sequence, and a 3′ V5 tag (for antigen detection) together with an IMX313 multimerising sequence, included because it has been shown to increase immunogenicity of some proteins in the context of viral vectored vaccines [46]. Cytosolic presence and secretion of antigens from viral vectors *in vitro* was confirmed by EIA (Fig. 1C and D). Adenovirus and MVA, which did not contain the antigen expression cassette, were used as negative controls.

3.3. Serological response to vaccination

Balb/c mice were vaccinated intramuscularly in a prime boost regime with adenoviral and MVA vectors expressing Eap, EapH1_2,

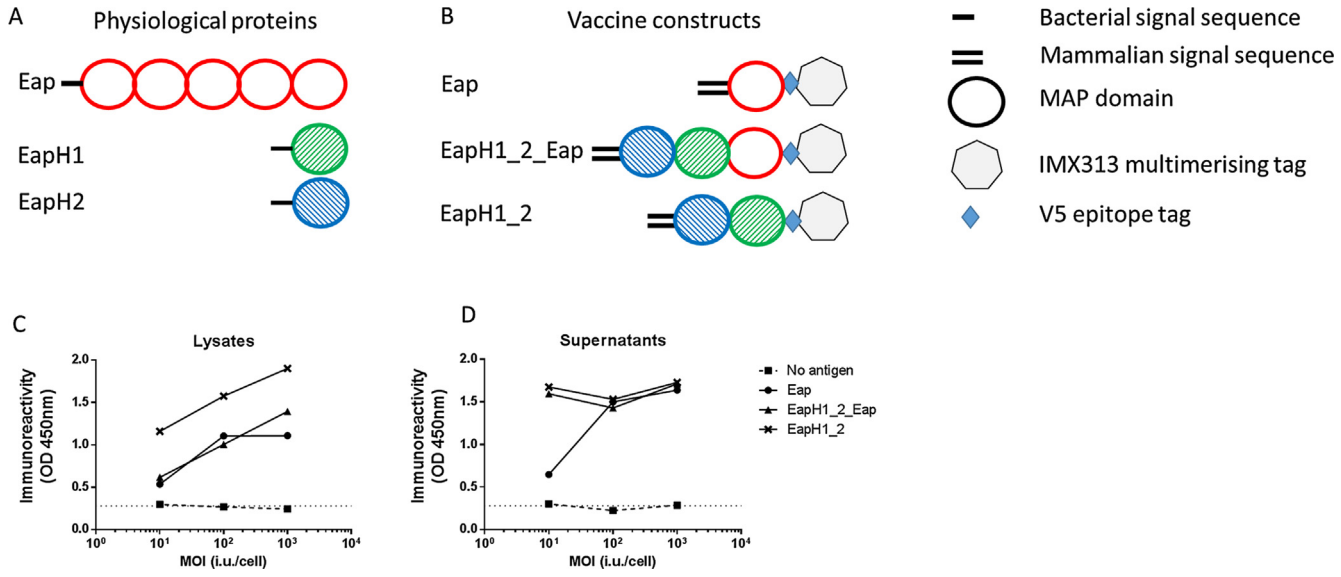

Fig. 1. Production of recombinant Eap, EapH1 and EapH2 proteins from viral vectors. Domains from the physiological Eap, EapH1, and EapH2 proteins (A) were expressed from AdHu5 and MVA vectors behind a mammalian signal sequence, and fused to V5 epitope tag and IMX313 multimerising domain (B). V5 and IMX313 tagged proteins were detected from both supernatant (C) and cell lysates (D) following infection of HeLa cells with adenoviruses expressing these constructs. Absorbance in a semi-quantitative enzyme immunoassay is shown in (C) and (D). Mean readings from triplicate infections are shown.

EapH1_2_Eap, or no antigen (Fig. 2A). Control animals were vaccinated with vectors without Eap proteins and did not have detectable serological responses to EapH1 and EapH2 (Fig. 2B and C), but had weak IgG responses to Eap (median endpoint titre 1/200, Fig. 2D), suggestive of their exposure to Eap or a cross-reacting protein. This is compatible with our observations that a ST-15 *S. aureus* strain colonises in the Balb/c mice studied in this experiment, and with reports of the generation of anti-Eap antibodies during *S. aureus* carriage [36,47].

As shown by serological analysis two weeks after boost, all components of all the vaccines were immunogenic, producing a strong antibody response against the respective *map* domains of EapH1, EapH2 and Eap proteins (Fig. 2B–D). Eap vaccination significantly increased anti-Eap immune responses (Fig. 2D). Additionally, increased immune responses against Eap were observed in 2/5 of animals immunised with EapH1_2 alone (Fig. 2D), suggesting that cross reactivity of antibodies generated to EapH1_2 with Eap may occur in a subset of animals.

3.4. EapH1 and EapH2 reduce abscess numbers and bacterial recovery in i.v. Challenge models

Following vaccination, mice were challenged intravenously (i.v.) with *S. aureus* strain Newman. All mice survived the challenge until day 3, when a post mortem was performed. Abscess formation in the right kidney of each mouse was analysed using post-mortem MRI [43]; numbers were significantly lower in the EapH1_2 vaccinated group in comparison to the control group ($p = 0.04$) (Fig. 2F). Median bacterial counts in the EapH1_2 vaccine group were 0.5 $\log_{10}$ CFU/g lower than that in the control group (median 7.0 vs. 6.5 CFU/g; $p = 0.31$, Mann-Whitney test) (Fig. 2E). Since power calculations using historical data from this infection model [40] indicated the experiment was only powered to detect a 0.9 $\log_{10}$ decrease in bacterial numbers, we performed two additional experiments to increase study power.

Experiment 2 exactly replicated the first one, in that Balb/c mice were naturally colonised with *S. aureus* (Fig. 2). Experiment 3 used Balb/c mice from the same supplier, but from a different breeding barrier in which natural colonisation did not occur; these mice were experimentally colonised with *S. aureus* aged 6 weeks (Fig. 4, see Methods). Following Adenovirus/MVA vaccination with EapH1_2, a reduction in both bacterial recovery and abscess formation was seen across the three experiments (Fig. 3A and B, respectively). Heterogeneity in vaccine impact on bacterial recovery between experiments was not significant in linear modelling (heterogeneity $p > 0.1$). Thus, these results indicated that the intramuscular vaccination with EapH1/H2 expressing viruses causes a small protective effect.

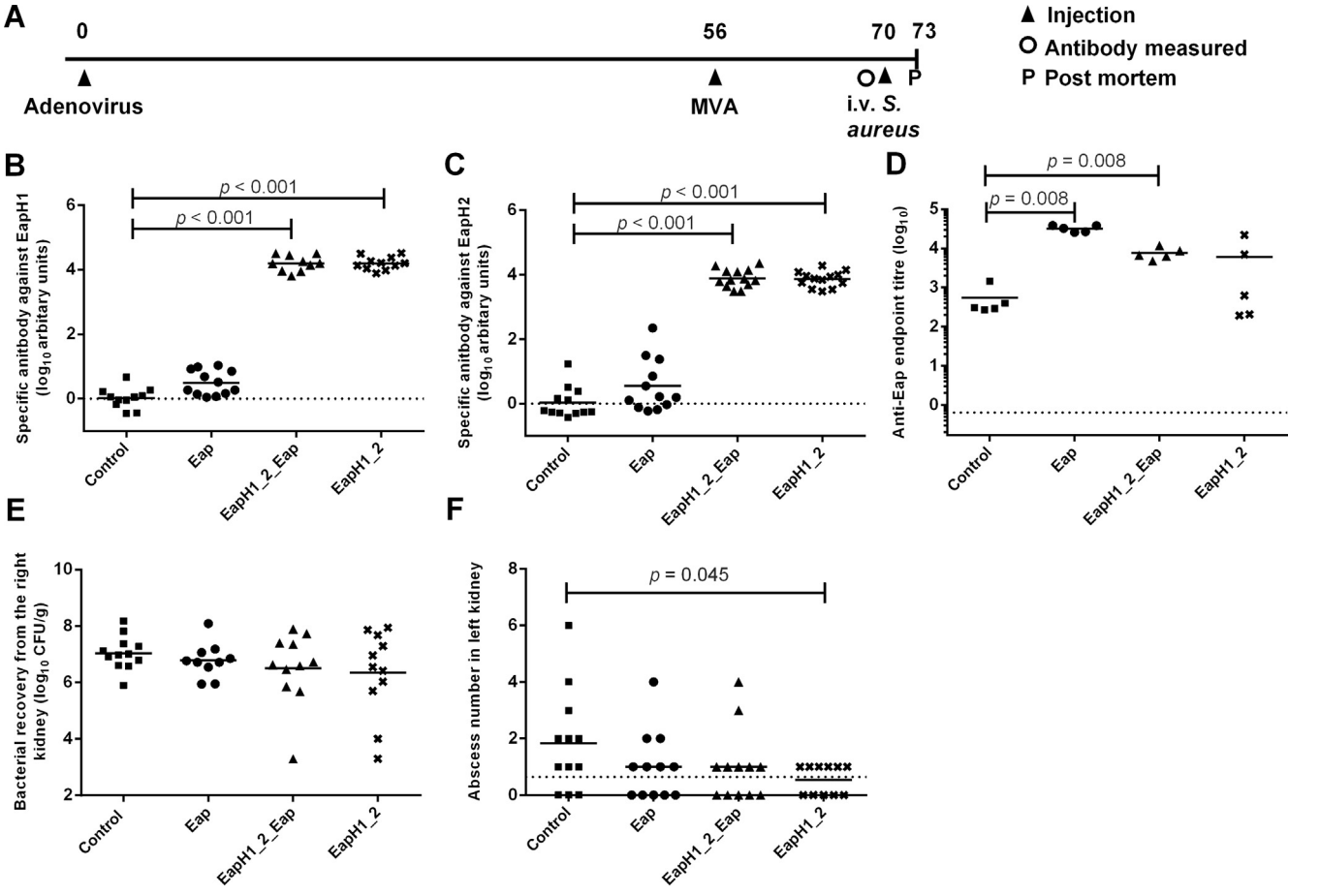

Fig. 2. Immunogenicity and protection induced by EapH vaccination. Balb/c mice were vaccinated with viral vectors expressing Eap, EapH1_2, EapH1_2_Eap. A control vector ('control') was prepared and titred identically to the antigen containing vectors; it differed from the test vectors only in the presence of the antigen in the expression cassette. Experimental timescale is shown in (A); numbers above the horizontal line indicate days after adenoviral injection. Specific antibody against (B) EapH1 and (C) EapH2 was determined by LIPS one day before challenge. Points represent individual animals' results and horizontal bars are medians. Anti-Eap endpoint antibody titres were determined on a random subset of five animals from each vaccination group by ELISA (D). 3 days following IV challenge, bacterial recovery from the right kidney was determined (E) and the number of abscesses in the left kidney was determined by post-mortem MRI (F). Statistical significance determined was by Mann-Whitney tests.

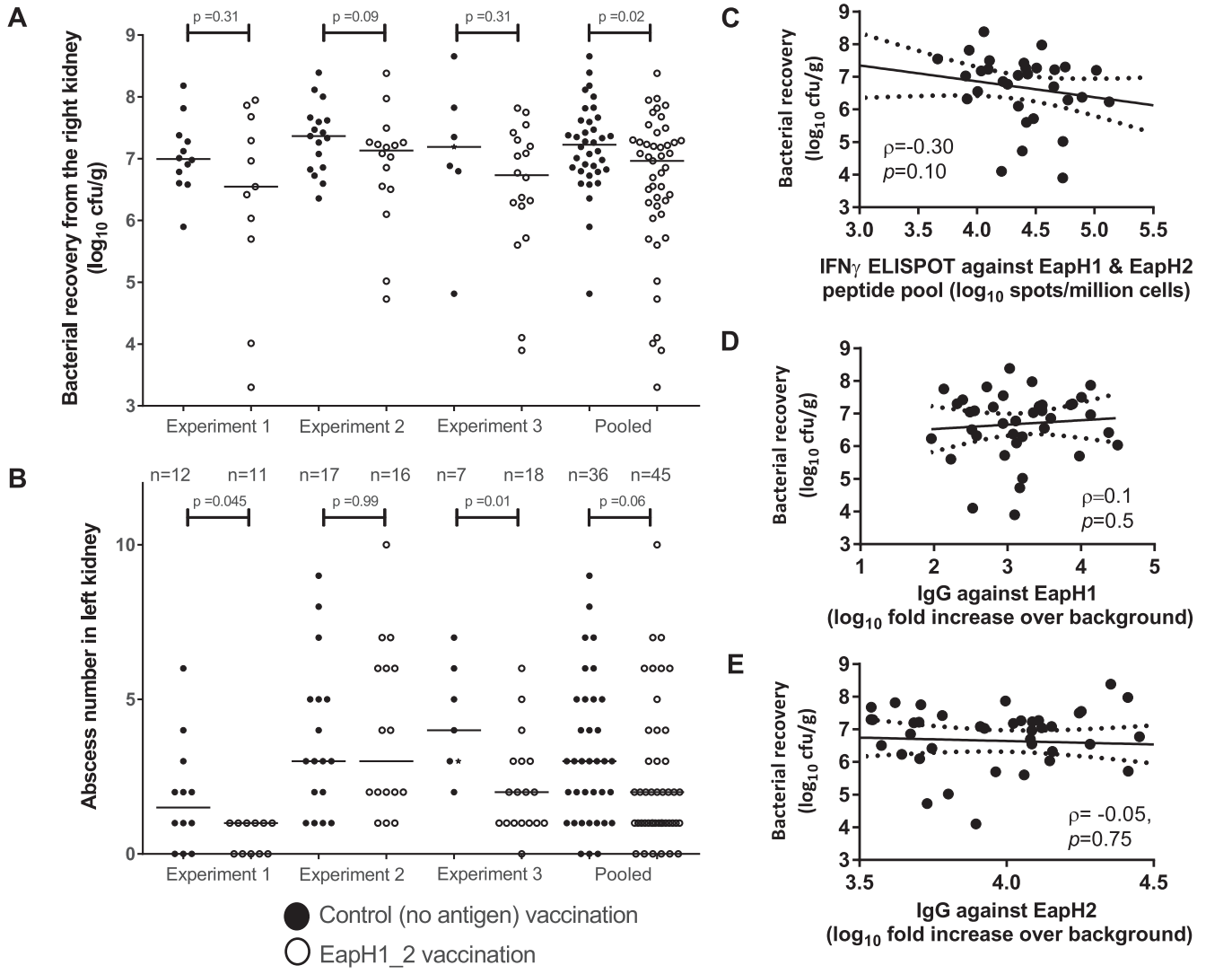

Fig. 3. Protection following EapH1_2 vaccination. Summary of protection data seen with EapH1_2 vaccination in three experiments (#1, #2, #3). Balb/c mice were vaccinated with viral vectors expressing Eap, EapH1_2, EapH1_2_Eap. A control vector ('control') was prepared and titred identically to the antigen containing vectors; it differed from the test vectors only in the presence of the antigen in the expression cassette. Experiment #1 is shown in Fig. 2; #2 was identical in design to #1, using naturally colonised Balb/c mice. #3 was identical in design but used experimentally colonised Balb/c mice (see methods). Bacterial recovery from right kidney (A) and abscess number determined by MRI for left kidney are shown in (B), with significance determined by Mann-Whitney tests. In experiment 3, only 5 mice were available in the control group. Three mice in experiment 2 and one mouse in experiment 3 reached humane endpoint before the end of the experiment and are plotted with a bacterial recovery of 10^9 cfu/ml and abscess number of 10, shown as stars. Associations between renal bacterial load and peripheral blood anti-EapH1_2 IFN-γ ELISPOT (experiments 2 & 3) (C), anti-EapH1 antibody (experiments 1–3) (D) and anti-EapH2 antibody (experiments 1–3) (E) on the day before challenge in animals vaccinated with EapH1_2. For (C)–(E) linear regression lines with 95% confidence intervals are displayed with Spearman's ρ and p values.

3.5. Protection and immunological responses to vaccine antigens

To investigate correlates of protection, in the latter two experiments, IFN-γ secreting PBMCs were quantified using blood samples obtained one day before i.v. challenge. These experiments showed induction of a geometric mean of 24,000 IFN-γ secreting EapH1/H2 responsive PBMCs per million cells by the Adenovirus/MVA regime. However, neither blood ELISpot nor serological responses against EapH1 or EapH2 were significantly associated with protection (ρ = −0.3, 0.1, −0.05; p = 0.1, 0.5, 0.75 respectively; Fig. 3C–E).

3.6. Intranasal vaccination with EapH1_2 accelerates loss of S. aureus carriage

Intramuscular vaccination (experiments #1–3, above), in which *S. aureus* carriage levels in stools were monitored, did not consistently impact faecal carriage; a significant effect of vaccination on faecal carriage was seen in only one of the three experiments (experiment #1, in which a significantly higher proportion of mice were *S. aureus* negative in the EapH1_2 vaccinated group than the control group, p = 0.006) (Supplementary Data, Fig. S2).

We postulated that intranasal vaccination might generate specific mucosal immune responses controlling carriage. To test this, we studied outbred CD1 mice. We chose this strain because we wanted to study an outbred mouse strain, and found the CD1 strain not to be colonised with *S. aureus*, but to be colonisable by *S. aureus* (Fig. 4). We also had difficulty in sourcing Balb/c mice, a strain we had studied in earlier experiments, which lacked *S. aureus* colonisation. Mice were vaccinated intranasally with a single dose of either adenovirus Hu5 expressing EapH1_2, a control adenovirus expressing no antigen, MVA expressing EapH1_2, or MVA expressing no antigen (Fig. 4A). Vaccination induced systemic antibody responses against EapH1 and EapH2 (Fig. 4B and C). 26 days

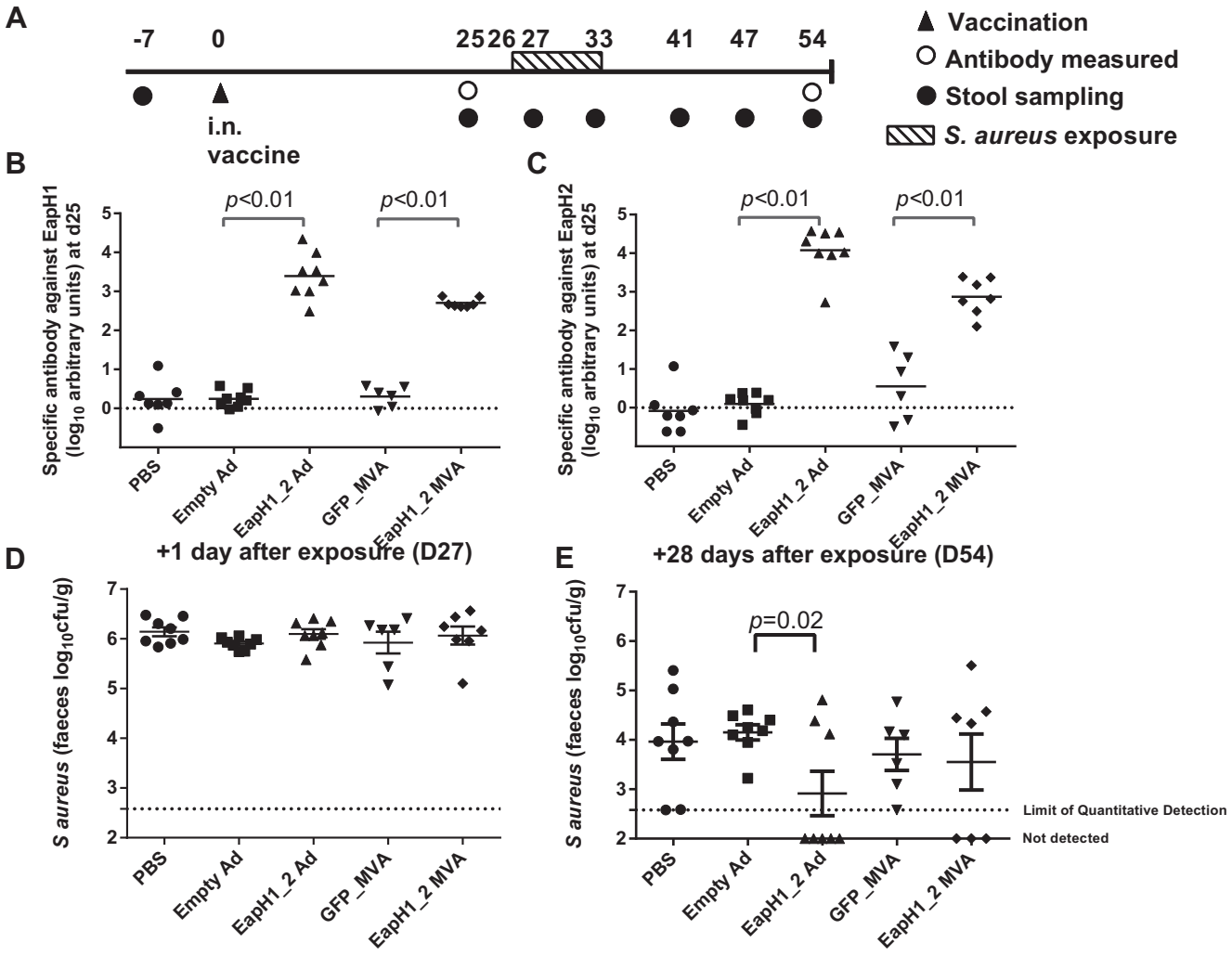

Fig. 4. Intranasal vaccination with vectors expressing EapH1_2. Experimental timescale (A). CD1 mice were vaccinated intranasally with a single dose of a viral vector (AdHu5 or MVA) expressing EapH1 and EapH2, or no antigen. Serological responses to vaccination were measured and significance determined by Dunn's multiple comparison tests (B and C). 26 days after vaccination, mice were exposed to *S. aureus* by environmental contamination, and GI carriage of *S. aureus* was monitored. Carriage levels at 1 day (D) and 28 days (E) after *S. aureus* exposure, with significance determined by Mann-Whitney tests. Data from one experiment is shown. Each dot represents the result from one animal and the bar represents the median observation. The limits of detection of direct and enrichment cultures are shown.

later, mice were exposed to *S. aureus* USA300 by environmental contamination. Stool cultures one day later were positive in all mice (Fig. 4D). One week later the contaminated bedding was changed for fresh bedding, and carriage levels of *S. aureus* in stools were monitored. At the end of follow-up (day 54 after vaccination, 28 days after experimental colonisation), a significantly higher proportion of mice were *S. aureus* negative in the adenovirus-EapH1_2 group than the control groups (Fisher exact test, $p = 0.01$); median counts were also significantly lower (Mann-Whitney test, $p = 0.02$) (Fig. 4E).

Similar results were seen in two additional, independent experiments: stool bacterial load declined over time in all groups in the CD1 mouse population studied (Supp. Figure S3 F). Control adenovirus (without EapH1_2 expression) and PBS vaccinated mice did not differ significantly in stool counts at any time point. By 28 days after vaccination, the EapH1_2 expressing adenovirus vaccinated animals had significantly lower bacterial counts than those in the PBS or control adenovirus immunised groups ($p = 0.01$ for both comparisons).

Thus, mucosal EapH1_2 vaccination does not prevent the acquisition of carriage, but rather accelerates loss of carriage in murine models of colonisation.

3.7. Intranasal vaccination with EapH1_2 and S. aureus pneumonia

We tested whether the immune response elicited by i.n. vaccination reduced bacterial counts in the lung following intranasal challenge with *S. aureus* in two independent experiments. We administered i.n. vaccines as described above; 28 days later, we challenged mice with *S. aureus* via a nasal route (see Methods). 24 h after infection, bacterial counts in the right lung did not differ significantly between animals administered i.n. control adenovirus and those receiving i.n. adenovirus expressing EapH1 H2 (Supp. Figure S4).

4. Discussion

Here we investigate the impact of vaccination with the highly conserved neutrophil serine protease inhibitors EapH1 and EapH2 proteins [18,20]. Viral vectored vaccines expressing an EapH1 and EapH2 fusion protein reduce bacterial counts in a murine i.v. challenge model of *S. aureus* disease. This model focuses on the early stages of *Staphylococcus aureus* bacteraemia; while clinical relevance has been questioned following humans trials in which

activity in this model did not translate into efficacy [5,6], it remains in use, with an emphasis on the selection of vaccines increasing the biological effect of those vaccines tested formerly [48].

Although the effect of EapH antigens in the intravenous challenge model is modest relative to that reported from multi-antigen vaccines [48], EapH1 and EapH2 may have value as components of a multivalent *S. aureus* vaccine aiming to reduce invasive disease. Given the modest effect size, the lack of a positive correlation with either peripheral blood gamma-interferon secreting T cell numbers, or antibody responses, may simply reflect limited protective efficacy, and hence limited power in the association study. Alternatively, protective efficacy may reside in T cell [35] or antibody [49] subpopulations which were not monitored in the studies performed.

We performed the initial experiments in mice which are already colonised with *S. aureus*, a colonisation status recently recognised to be widespread [47]; we considered these mice relevant because the human population is ubiquitously exposed to *S. aureus* antigens. Serological analysis indicated that Eap was one antigen to which the mice initially studied were exposed; anti-EapH1 and EapH2 antibody responses were not detected. Previous work indicates that Eap vaccination can impact bacterial load [24], but we did not observe this; it is possible that the natural anti-Eap immune response was partially protective and could not be enhanced using the Eap domain vaccines we tested.

S. aureus carriage is an important phenomenon which represents the source of human infection [7]. In humans, nasal carriage is most heavily studied, although it is very commonly associated with bowel carriage [50,51]. Modelling indicates all humans can acquire *S. aureus*, detectable by nasal swabs [8]. In some individuals, termed persistent carriers, the loss rate following acquisition is very low, whereas in others the loss rate is much higher, and nasal cultures are intermittently or rarely positive [8]. In this paper we quantified GI tract carriage in mice. We elected to do so because GI tract carriage of *S. aureus* represents a highly prevalent reservoir of virulent, antimicrobial resistant organisms in humans [51,52], and for practical reasons: in previous work we found stool sampling to be much easier, more sensitive, and less invasive than nasal sampling in mice [36]. We used the antimicrobial resistant USA300 MRSA strain, the control of whose spread is an important public health priority [46,53–57]. Akin to the situation in humans, we observed that all mice acquired colonisation following experimental exposure [36]. Subsequently, lower concentrations of bowel *S. aureus* were observed in the group receiving intranasal vaccination with EapH1 and EapH2, suggesting this intervention increases the rate of loss of *S. aureus* bowel carriage in mice.

More detailed mechanistic studies will be needed to identify the mechanism behind the reduction in bacterial counts in stool in adenovirus-EapH1-H2 vaccinated animals. It is possible that mucosal adenovirus vaccination specifically generates effective mucosal immunity. Induction of IgA via B cell interactions with Peyer's patch dendritic cells [58] is one possibility. By contrast, detailed mechanistic studies using HIV gag antigens showed that *i.m.* vaccination with adenoviruses generates substantial mucosal cellular responses [59,60], although it remains possible that the prolonged antigenic exposure following adenovirus vaccination [61] induces some effective anti-*Staphylococcus aureus* mucosal cellular immunity not induced by *i.m.* vaccination. Administration of MVA vectors expressing EapH1 and EapH2 by the same route did not appear to have this property (Fig. 4); the short antigen expression [62] following MVA infection may be insufficient to induce the mechanism(s) responsible.

At present, bundles of measures including prophylactic antibiotic therapy and topical antimicrobial use (decolonisation) to transiently reduce the human bioburden of colonising *S. aureus* have become a cornerstone of the prevention of *S. aureus* infections in clinical practice [46,53–57]. Such bundled measures have limited efficacy, are expensive to implement [63], and their longevity is likely to be limited due to the ongoing emergence of drug resistance [64–66]. Therefore, if confirmed in humans, the single dose intranasal vaccination strategy we describe here provides a potential route, independent of antimicrobial use, to the control the spread of multi-resistant *Staphylococcus aureus* [51].

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Author contributions

EA, EE, AF, YY and PVD performed viral vectored experiments, immune tests and statistical analysis. EE produced recombinant proteins. DHW and EA designed and made the constructs. DHW, CR, AM and YY provided input into study design. EA, EE, AF and DHW wrote the paper. All authors reviewed the manuscript. All authors attest they meet the ICMJE criteria for authorship.

Conflict of interest

DW, PvD, YY, EA, CR, EE, and AM are contributors to a patent related to the use of EapH proteins as vaccines.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2018.11.036.

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