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Heterogeneous early immune responses to the *S. aureus* EapH2 antigen induced by gastrointestinal tract colonisation impact the response to subsequent vaccination

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ABSTRACT

Introduction: *S. aureus* is a pathogen to which individuals are exposed shortly after birth, with immune responses to *S. aureus* increasing during childhood. There is marked heterogeneity between the anti- *S. aureus* immune responses of different humans, the basis of which is not fully understood.
Methods: To investigate development of anti-*S. aureus* immune responses, we studied *S. aureus* colonised mice under controlled conditions. Mice were either acquired colonised from breeding colonies, or experimentally colonised by exposure to a cage environment which had been sprayed with a *S. aureus* suspension. Colonisation was monitored by sequential stool sampling, and immunoglobulin levels against both whole fixed *S. aureus* and individual *S. aureus* antigens quantified. The immunological impact of colonisation on subsequent vaccination was investigated.
Results: Colonised BALB/c and BL/6 mice develop serum anti- *S. aureus* cell surface IgG1 antibodies. Responses were proportional to the cumulative *S. aureus* bioburden in the mice, and were higher in BALB/c mice, which have higher colonisation levels, than in C57BL/6 animals. We observed marked variation in the induction of anti-cell surface antibodies, even in genetically identical mice experimentally colonised with the same *S. aureus* clone. Heterogeneity was also evident when monitoring immune responses to the secreted *S. aureus* protein EapH2. Approximately 50% of colonised mice developed anti-EapH2 responses (responders); in other mice, responses were not significantly different to those in uncolonised mice (non-responders). Following vaccination with a replication deficient adenovirus expressing EapH2, less anti-EapH2 antibody was generated in non-responder than responder animals.
Conclusions: In genetically identical mice, *S. aureus* colonisation results in all-or-nothing antibody responses against some antigens, including EapH2. For antigens involved in colonisation success by microbes, apparently stochastic early immune responses may impact both vaccine responses and the establishment of an animal-specific microbiome.

1. Introduction

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium which causes a high burden of both hospital and community related disease [1]. The basis of human susceptibility to the pathogen is not completely understood [2], and the emergence of virulent and increasingly antibiotic resistant strains [3] poses an ongoing health and economic burden [4]. Consequently, the World Health Organization has categorised the organism as a high priority for antibiotic and vaccine development [5].

Despite its virulence, *S. aureus* is a common component of the human microbiome, with persistent carriage (defined as repeated asymptomatic isolation of the organism) described in approximately one third of the adult population [6]. These individuals are at increased risk of *Staphylococcus aureus* disease [7]. Longitudinal studies in adults show that all humans are exposed to the organism; the key feature separating persistent carrier from intermittent and non-carrier humans is the rate of loss after acquisition, which is very slow in individuals classified as persistent carriers, but much faster in members of the population who are intermittent or non-carriers [8]. In about 60% of persistent carriers, the

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organism can also be recovered from the gastrointestinal tract [9]. In neonates, high colonisation densities occur, with the organism recovered from over half of children under one [10].

Anti-*S. aureus* antibodies can be detected in serum in all humans from early childhood onward, with concentrations rising during the first two decades of life[11,12]. Wide variation in anti-*S. aureus* titres exists between adults [12–15]. Levels are slightly higher in persistent carriers than in other individuals [16], but the difference is small relative to the observed heterogeneity in human populations [4,11]. The initiation of anti-*S. aureus* immune responses is difficult to study in human populations due to very early exposure and the presence of maternal IgG, and the basis of the variations in the eventual response is unclear. However, this topic is of interest since the immune response generated may be important in natural protection against *S. aureus* in human populations since some epidemiological [17–19] and experimental data [20] suggests that immune responses against defined antigens (particularly toxins) may contribute to protection from severe *S. aureus* infection.

S. aureus colonises multiple mammalian species, including ruminants [21] and a range of wild rodent species [22]. We and others have recently described both naturally occurring and experimentally induced *S. aureus* carriage in laboratory mice [22,23]. In our observations, organism densities in the nares and the GI tract were strongly correlated, but isolation from faeces was a more sensitive and quantitative measure of carriage [24]. By longitudinal monitoring of faecal densities, we showed previously that naturally colonised mice, like human populations [8], lose colonisation at different rates, with some losing carriage rapidly while others carry organisms at a high level for a prolonged period [24]. We developed a non-invasive method of colonising mice, which involves spraying bedding with the organism [24], something which allows us to control the onset of *S. aureus* exposure and investigation of the induction by *S. aureus* colonisation of the early stages of the anti-*S. aureus* immune response.

In this work, we demonstrate markedly different colonisation-induced immune responses between genetically identical mice colonised with similar amounts of a *S. aureus* clone. Of note, we observe a high proportion of mice which fail to respond to colonisation by mounting responses to EapH proteins, inhibitors of mammalian serine proteases [25–27], mucosal vaccination against which accelerates the loss of *S. aureus* carriage [28]. We investigate how natural anti-EapH2 response status (none *vs* some) impacts subsequent vaccination response, and discuss the implications of the data for strategies aiming to control *S. aureus* carriage by vaccination.

2. Materials and methods

2.1. Animals and S. aureus colonisation

Six-week-old female BALB/c, specific opportunistic and pathogen free (SOPF) (certified *S. aureus* free) and specific pathogen free (SPF) (colonised with *S. aureus*) and C57BL/6 mice were obtained from Harlan (Blackthorn, UK). On arrival, animals were randomly distributed into individually ventilated cages and housed in groups of 3, 4, 5 or 6. For neonatal experiments six-week-old SOPF BALB/c male and female mice from Harlan were mated and bred in house, and timed-pregnant C57BL/6 females were obtained from Harlan. *S. aureus* colonisation involved cage environments, but not the mice themselves, being contaminated with a *S. aureus* spray [24]. Cages were cleaned and bedding replaced seven days later. Colonisation of neonatal mice was performed as follows: the bedding in the cage containing the pregnant female was sprayed on d19 of gestation, and when the pups were 1, 6, and 12 days old. The bedding was changed on day 19. Animals themselves were not sprayed. All mouse procedures were conducted in accordance with the Animal (Scientific Procedures) Act 1986 (UK Home Office Project licence 30/2825) and were approved by the University of Oxford Animal Care and Ethical Review Committee.

2.2. S. aureus carriage monitoring

'SaF_1' is representative *S. aureus* isolate obtained from colonised mice [24]. The strain USA300 JE2 (FPR3757) ('USA300') was obtained from ATCC. We studied three strains of mice: BALB/c mice naturally colonised with SaF, or SOPF BALB/c and C57BL/6 mice from Harlan, the latter two of which we confirmed to be *S. aureus* free by faecal culture [24]. SOPF BALB/c and C57BL/6 strains were colonised with SaF_1 or USA300 as above and stool samples collected from individual animals to quantify *S. aureus* [24]. detection limit for stools was estimated as 380 cfu/g (1 cfu/50 μl in a suspension of a mouse stool of average stool mass 0.0263 g in 500 μl PBS).

2.3. ELISA to determine serological response to S. aureus carriage

Serum was obtained from venous blood samples by centrifugation and stored at −20 °C. Anti-*S. aureus* Ig serum antibodies were detected by whole cell enzyme-linked immunosorbent assay (ELISA). Paraformaldehyde fixed *S. aureus* (Δ*spa* Newman strain, since a Δ*spa* SaF_1 strain was not available) were prepared and the assay was performed essentially as described previously [15,29] with the following exceptions: Alkaline phosphatase polyclonal goat anti mouse secondary antibodies to IgA α chain, IgM μ chain, IgG1 heavy chain, IgG2a heavy chain, IgG2b heavy chain, IgG3 heavy chain (all from AbCam) were used, followed by a washing step and incubation with SIGMAFAST p-Nitrophenyl phosphate Tablets (Sigma). Optical density was measured at 405 nm. All data shown is for 1:50 serum dilution, and is presented as EIA signal (A_{405}) minus background (mean + 2SD of no serum).

2.4. Vaccination study

Viral-vectored vaccines expressing *S. aureus* antigens of interest were produced using replication-deficient adenovirus human serotype 5 (AdHu5) and modified vaccinia Ankara (MVA) vectors as described previously [30,31]. The vaccine 'EapH1_H2' contained MAP (MHC analogue protein) domains of EapH1 (a.a. 24–141 of WP_001549607.1) and EapH2 (a.a. 24–144 of WP_000769689.1). A prime-boost strategy was adopted. Empty or irrelevant AdHu5 and MVA vectors were used as control vectors. Mice were injected intramuscularly (right hind leg) with either 25 μl PBS or 1×10^9 iu AdHu5 expressing either no transgene or the *S. aureus* antigen EapH1_H2 in 25 μl PBS. Eight weeks later, mice were injected intramuscularly (right hind leg) with either 25 μl PBS or 1×10^7 pfu of a MVA virus expressing either green fluorescent protein (GFP) (no *S. aureus* antigen) or EapH1_H2 in 25 μl PBS.

2.5. Luciferase immunoprecipitation system (LIPS) assay

LIPS assays [32] were used to determine specific IgG signal against *S. aureus* antigens EapH1 and EapH2. Briefly, plasmids expressing either EapH1 or EapH2 fused to Renilla luciferase were generated, transfected into HEK293 cells and cell lysates stored at −80 °C. Serially diluted mouse serum was incubated with relevant cell lysate for at least 1 h. Serum-lysate mixture was then added to Protein G UltraLink Resin (ThermoScientific) in filter plates (Merck Millipore, MSGVN2B50). After a further 1 h incubation and subsequent washing, assay substrate was added (Renilla luciferase assay system, Promega UK Ltd). Chemoluminescence was measured in a Luminometer (CLARIOstar®, BMG Labtech). Luminescence data

were log transformed prior to analysis. Background luminescence (no sera) was subtracted from luminescence values with sera added to give specific luminescence.

2.6. Statistical analysis

Area under curve (AUC) of longitudinal carriage data for each mouse was calculated using the trapezoidal method as an estimate of cumulative *S. aureus* exposure: if at a series of time points t_0, t_1, t_2, … t_n counts are x_0, x_1, x_2, … x_n, 'Long Term Carriage Burden' (LTCB) is estimated as

$$\mathrm{LTCB} = \sum_{k=1}^{n} \frac{x^k + x^{k-1}}{2\left(t^k - t^{k-1}\right)}$$

Grouped data are presented as means with standard error of mean (SEM). Scatterplot data are presented with linear regression and 95% confidence intervals (CIs), with p values for slope being significantly non-zero, and r^2 for goodness of fit, unless otherwise indicated. GraphPad Prism software version 6.03 (La Jolla, CA, USA) and IBM SPSS Statistics 22 were used for graphical presentation and statistical analyses.

3. Results

3.1. Serological response to natural S. aureus carriage

We have previously described a longitudinal study of three cohorts of 12, 24 and 12 BALB/c mice naturally colonised in a breeding colony with a CC15 *S. aureus* strain (SaF). On follow-up of these animals, some lost carriage and others retained it, leading to a range of cumulative *S. aureus* burdens in the animals [24]. To assess whether such exposure was immunogenic, we developed an anti-*S. aureus* cell surface EIA which could be performed on stored sera from these cohorts; attempts to evaluate specific mucosal immunoglobulins from nasal swabs and stool homogenates failed to detect any signals (data not shown). We computed long term carriage burden (LTCB), a metric of cumulative *S. aureus* burden, for each animal. In an alternative approach, we categorised mice as carriers or non-carriers based on the recovery of *S. aureus* from faeces on the day mice were bled. In all cohorts studied, when serological analysis was performed, some animals had *S. aureus* isolated from faeces, and some did not (Fig. 1), with culture positivity detected in 3/12, 11/24 and 5/12 in each cohort.

Anti-*S. aureus* IgG1 was detectable by EIA and was positively correlated with LTCB in all three experiments (Fig. 1A) ($p = 0.05$, $p < 0.0001$, in experiments 1 and 3 respectively; $p = 0.07$ in experiment 2, in which lower levels of IgG1 were detected). Similarly, significantly different anti-*S. aureus* antibody was detected in two of three experiments when comparing animals with *S. aureus* positive faeces on the day of serum sampling with those without ($p = 0.009$, 0.41, 0.017 respectively; Fig. 1B). Anti-*S. aureus* detection by other antibody isotypes were also investigated in experiments 1 and 2, but no significant relationship between LTCB and EIA signal was observed for other isotypes (Supp. Fig. 1).

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.11.063.

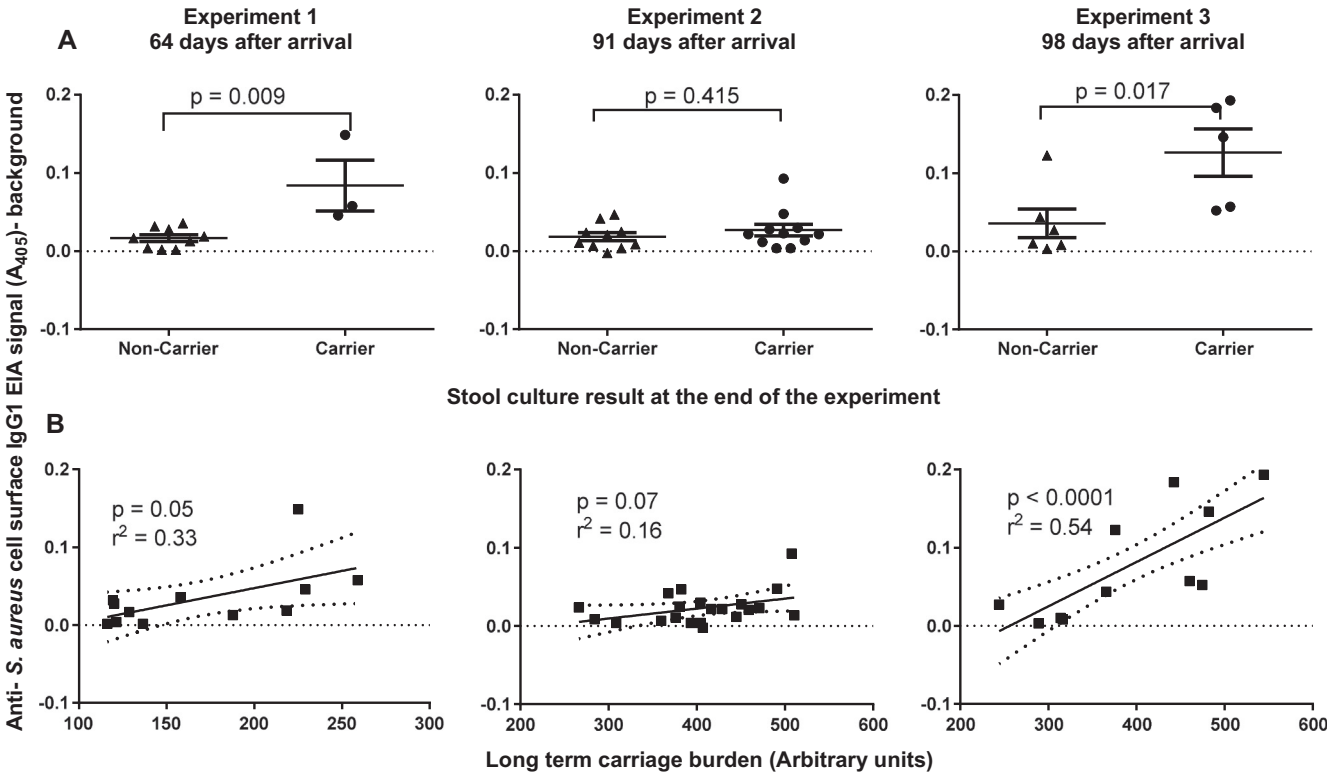

Fig. 1. Serological response to natural *S. aureus* carriage. Serum IgG1 antibody responses against *S. aureus* cell surface in 3 independent carriage monitoring experiments (i.e. in 3 groups of female BALB/c mice from Harlan) stratified by (A) carriage status and (B) Long Term Carriage Burden. Levels of IgG1 against the cell surface of fixed *S. aureus* (Δ*spa* Newman strain) are expressed as enzyme immunoassay (EIA) signal minus background. Experiment 1 was performed at 64 days, Experiment 2 at 91 days and Experiment 3 at 98 days after animals arrived at the unit. Mice arrived aged six weeks. In (A) animals were dichotomised according to carriage status (carrier or non-carrier) at the end of the experiment and EIA signal compared using Mann-Whitney test. In (B) Long Term Carriage Burden was calculated for day of experiment. Horizontal dotted lines show EIA – background of control mice (*S. aureus* naïve).

3.2. Serological response to experimentally induced S. aureus carriage

We have published data showing environmental contamination with *S. aureus* efficiently colonises both neonatal and adult SOPF BALB/c mice [24]. Here, we compare these conditions with the results of four additional regimes: experimentally colonising a second strain of *S. aureus* naïve mice, C57BL/6, both as neonates and adults, and with the results of colonising adult mice of both strains with a methicillin resistant *S. aureus* (MRSA) isolate, USA300 (Fig. 2; animal-level data shown in Supp. Fig. 2).

In both neonates and adults colonisation densities differed between mouse strains: bacterial density was significantly higher (approximate 2 log difference, $p < 0.01$ from after two weeks colonisation onwards, Fig. 2A and B) in BALB/c compared to C57BL/6 mice, with LTCB was significantly higher in BALB/c animals ($p < 0.01$, Fig. 2C). Adult mice were also colonised with a methicillin resistant *S. aureus* (MRSA) isolate, USA300, (Fig. 2B), and its colonisation profile appeared similar to SaF_1, with no significant difference in LTCB in animals colonised with SaF_1 *vs* USA300 in either BALB/c ($p = 0.65$, Fig. 2C) or C57BL/6 mice ($p = 0.48$, Fig. 2C). Thus, it is possible to experimentally colonise both BALB/c and C57BL/6 mice with an *S. aureus* isolate from a naturally colonised mouse and with an MRSA patient isolate, but densities were higher in BALB/c than C57BL/6.

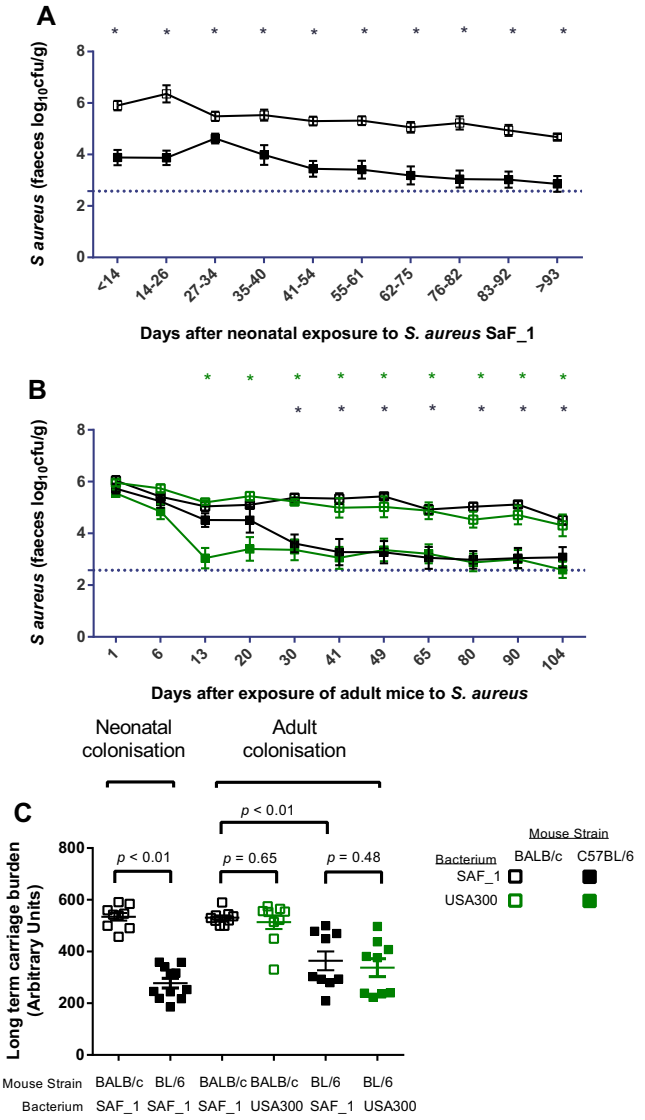

Fig. 2. Experimental *S. aureus* colonisation of naïve mice. Mice were confirmed *S. aureus* free by stool sampling. 6 mice colonised as neonates (A) received 4 SaF_1 doses, administered via aerosol by environmental contamination from −1 to 14 days old, whilst mice colonised as adults (B) received 1 dose at 6 weeks of age of either *S. aureus* SaF_1 strain (9 mice, black symbols) or USA300 (9 mice, green symbols). *S. aureus* colonisation levels were monitored by stool sampling from the day of final *S. aureus* exposure for at least 100 days. The detection limit is shown as a dashed line. * denotes $p < 0.01$ for comparison of BALB/c *vs.* C57BL/6 mice by *t*-test, with Bonferroni correction for multiple testing. (C) shows areas-under-the-curve (long term carriage burden) for the conditions illustrated in (A, B). *p*-values shown are calculated by Mann-Whitney tests. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Antibody induction by *S. aureus* carriage was analysed in these experimentally colonised mice. BALB/c mice exposed to *S. aureus* both as neonates and as adults elicited significantly higher serum anti-*S. aureus* IgG1, compared to non-exposed controls (Fig. 3A and C). In C57BL/6 mice, neonatal colonisation resulted in a significant induction of serum anti-*S. aureus* IgG1 ($p = 0.016$, Fig. 3B), however (unlike the situation in BALB/c), significant increases were not observed in C57BL/6 animals colonised as adults (Fig. 3D). Therefore, *S. aureus* GI tract colonisation induces a detectable serological response in BALB/c mice regardless of age of exposure, whereas in C57BL/6 mice where *S. aureus* bioburden was much lower, a serological response was only detected in animals exposed as neonates. We also noted heterogeneity in serological responses against the *S. aureus* cell surface, with some mice with having anti-cell surface immune responses similar to uninfected where others with similar LTCB had much higher EIA signals (Fig. 3).

3.3. Impact of S. aureus colonisation on immune response to individual antigens

Because of the heterogeneity observed, we asked whether *S. aureus* colonisation induces differential responses to individual *S. aureus* antigens. We were particularly interested in those which might be involved in the control of *S. aureus* colonisation, such as EapH1 and EapH2 [28]. We experimentally colonised BALB/c mice with two strains of *S. aureus*, or control (Fig. 4A) and subsequently measured responses against EapH1 and EapH2. 66 and 90 days after colonisation, no responses to EapH2 was detected in mock-colonised mice, whose cages were sprayed with PBS vehicle (Fig. 4B and E) while specific, stable responses to EapH2 were observed in 10/18 of *S. aureus* colonised mice (Fig. 4C, D and F). We termed these mice 'immuno-responders'. Immunoresponse occurred at similar frequencies with the two *S. aureus* strains tested: 5/9 SaF_1 colonised mice were immune-responders, as were 5/9 USA300 colonised mice, and in subsequent analyses we considered mice colonised with either strain together. Immunoresponse is not an EapH2 specific phenomenon, as immunoresponders are more likely to respond the *S. aureus* cell surface if they respond to EapH2, which is a secreted antigen (Fig. 4G, $p = 0.0014$), but two mice responded against the bacterial cell surface but not detectably against EapH2 (Fig. 4G). We also noted that the immune-response phenotype does not appear to be substantially explained by exposure: LTCB at day 66 did not differ significantly between immuno-responders and non-responders ($p = 0.8$, Mann-Whitney test), nor did the counts at any time point (Supp Fig. 3), nor was the frequency of immunoresponders significantly different across the six cages of colonised mice in this experiment (Fisher's Exact test, $p = 0.14$).

We investigated the consequences of these heterogeneous responses, and in particular whether responses generated by colonisation could influence vaccine responses. Therefore, we vaccinated a sub-group of colonised and un-colonised mice with viral vectored vaccines expressing EapH1 and EapH2 (Fig. 4A). After adenoviral prime vaccination, immuno-responders had signifi-

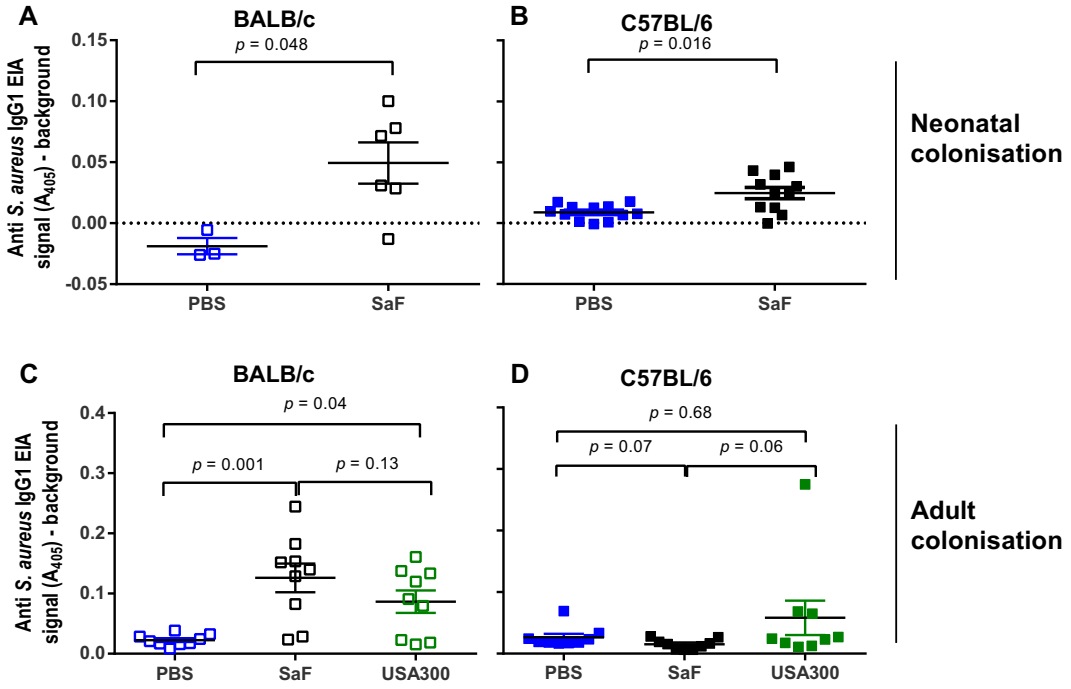

Fig. 3. Serological response to experimental *S. aureus* colonisation. Serum IgG1 antibody responses against *S. aureus* cell surface stratified by treatment and compared using Mann-Whitney test. Levels of IgG1 against the cell surface of fixed *S. aureus* (Δ*spa* Newman strain) are expressed as EIA signal minus background, (A) in BALB/c mice colonised as neonates, (B) in C57BL/6 mice colonised as neonates, (C) in BALB/c mice colonised as adults and (D) in C57BL/6 colonised as adults. Experiments performed at (A) 78 days, (B) 93 days and (C, D) 66 days after colonisation. 3–12 mice per control (PBS) group, 6–11 mice per colonised (SaF or USA300) group.

cantly increased anti-EapH2 response over non-responders ($p = 0.006$, *t*-test, Fig. 4D), showing that the natural response to EapH2 modifies subsequent vaccine responses. After MVA boost vaccination, anti-EapH2 responses were similar in both immuno-responders and non-responders ($p = 0.38$, *t*-test, Fig. 4D), since anti-EapH2 responses in carrier mice who were naturally immuno-responders did not increase further after boost vaccination (Fig. 4D and F).

Natural responses to the highly related EapH1 antigen was not observed in the same animals (Fig. 5), and consistent with this there was no differences in responses to EapH1 using the viral vector regime which expressed both EapH1 and EapH2 (Fig. 5).

In summary, murine colonisation with *S. aureus* induces highly variable immune responses in mice. EapH2 is a secreted antigen against which immune responses are generated during carriage, modifying the response to subsequent vaccination. Heterogeneity exists in responses which appear in this model as all-or-nothing responses despite the genetic identity of the mice and *S. aureus* strains administered. Non-responder mice respond following viral vector vaccination.

4. Discussion

We have shown that natural and experimental *S. aureus* colonisation elicits anti- *S. aureus* cell surface serum IgG1, which correlates positively with the cumulative *S. aureus* bioburden, but which also depends on the mouse strain and age at which mice are exposed. Our observations are compatible with those made using arrays [33] and multiplex EIA assays [34].

Cumulative *S. aureus* burden was lower in C57BL/6 mice than in Balb/c mice, but both were readily colonised both by a strain of S. aureus which likely jumped from human to mouse about twenty five years prior to isolation [24], and a human derived USA300 strain. Our observation of ready colonisation of C57BL/6 mice is compatible with the isolation of diverse *S. aureus* strains from C57BL/6 mice globally [22].

After colonisation, individual (genetically identical) BALB/c mice develop markedly different responses to the secreted neutrophil protease inhibitor [25–27], EapH2, with some mice generating detectable systemic serological responses ('immuno-responders') while other did not ('non-responders'). We observed strong correlation between anti-*S. aureus* cell surface IgG1 EIA signal and anti-EapH2 responses indicating that immuno-response may be a phenotype which applies to multiple antigens, both secreted (like EapH2) and to cell surface determinants. EapH1 responses were not seen in response to natural infection; it is possible that EapH1 is not sufficiently expressed *in vivo* to generate immune responses in this model. To test whether natural responses could be boosted, we used viral vectors, both of which have been used in humans [35], and which if expressing EapH1 and EapH2 are capable of eliciting partial protection to invasive *S. aureus* disease, and of accelerating the loss of carriage in experimental *S. aureus* carriage [28]. The viral vectors used generate potent antibody [30] and T cell responses [35]. Immuno-responders reach maximal antibody response with a single dose of adenoviral vector expressing the antigen. By contrast, non-responder animals require prime and boost (with MVA vector expressing the antigen) to reach maximal antibody responses, suggesting they have not been primed against the antigen.

The observed heterogeneity in immune responses to *S. aureus* in humans is poorly understood. One explanation might involve heterogeneous antigen expression by different *S. aureus* clones, leading to different individual exposure to *S. aureus* antigens. Another explanation might involve immune modulation by colonising *S. aureus*. *S. aureus* secretes a variety of potent immunomodulators, including protein A which narrowly focuses the B cell repertoire in humans [36] and which has recently been proven to influence murine carriage of *S. aureus* [33].

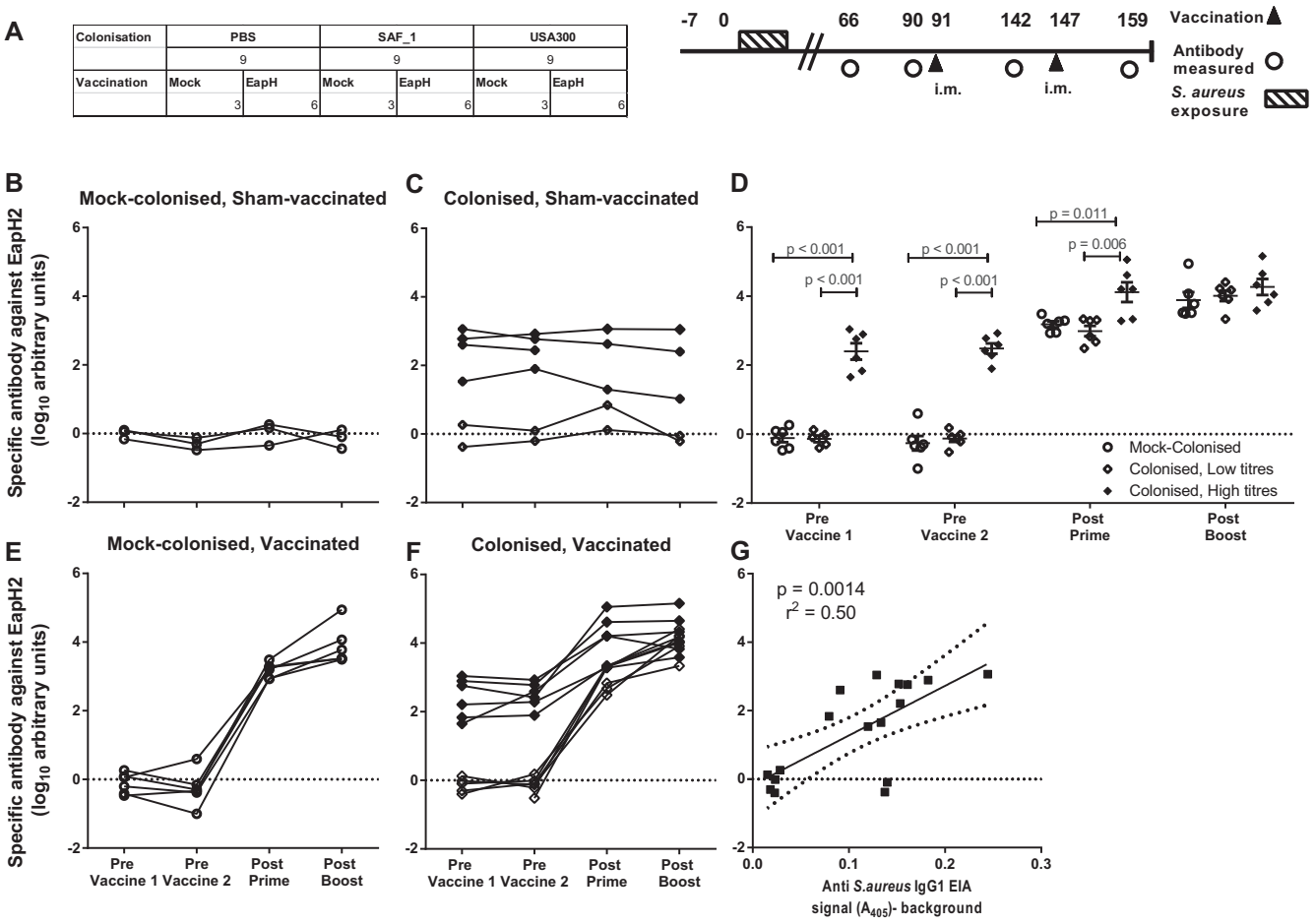

Fig. 4. Impact of experimental *S. aureus* carriage on vaccination with *S. aureus* antigen, EapH2. BALB/c mice colonised as adults were subsequently vaccinated intramuscularly in a prime-boost schedule with viral-vectored vaccines containing both *S. aureus* antigens EapH1 and EapH2. (A) Experimental design and schedule. (B, C, E, F) EapH2 antibody response stratified by experimental group over the course of experiment. (D) Comparison of EapH2 antibody levels in uncolonised and colonised animals which received vaccination. Sera were assayed at four time points: day 66 (Pre Vaccine 1), day 90 (Pre Vaccine 2, 1 day before vaccination), day 142 (Post-Prime), and day 159 (Post-Boost). Colonised animals which received vaccination have been dichotomised according to whether they eventually produced a high (filled symbols) or low EapH2 antibody (open symbols) response after colonisation. (G) EapH2 antibody responses in colonised animals at Pre Vaccine timepoint 1 displayed *vs.* IgG1 against the cell surface.

It is curious that large variations in immune responses are seen in the experimental design we have employed, in which factors such as murine genetics, *S. aureus* strain, and environmental conditions are controlled. Natural responses were not seen against a closely related protein, EapH1, and vaccine responses in EapH1 responder *vs.* non responder animals appeared identical. This argues that the decreased response in non-responder animals to vaccination reflects lack of priming, rather than some kind of a widespread immunosuppression in some animals by *S. aureus* carriage or other associated microflora.

It is possible that 'non-responders' do not develop an immune response to *S. aureus* if the number of productive antigen-antigen presenting cell, or lymphocyte-antigen presenting cells interactions are small; if so immunoresponder/non-responder status might be a stochastic phenomenon reflecting a low frequency pathogen – immune system interaction. Such low frequency interaction might reflect a low frequency of some microinvasion event necessary by bypass the restrictive immunological impact of interactions between the bacterium and pre-existing mucosal antibody [37], other gut flora or mucus [38], allowing access to mucosa-sampling dendritic cells [39]. Special environments for mucosal B cell – dendritic cell interaction exist in Peyer's Patches [40], and access to these environments may be also restricted in some animals, perhaps due to the impact of other microbiota [41], resulting in variations in immune responses [42].

The processes generating heterogeneous responses to carried microflora may have important sequelae. We would speculate that the early formation of neutralising antibody against bacterial virulence factors influencing the survival of carried microflora, such as the *S. aureus spa* [33] and EapH proteins [28] may impact the establishment of the microflora, including *S. aureus*, as well as (as we show here) modifying the response to vaccination against proteins expressed by the microflora.

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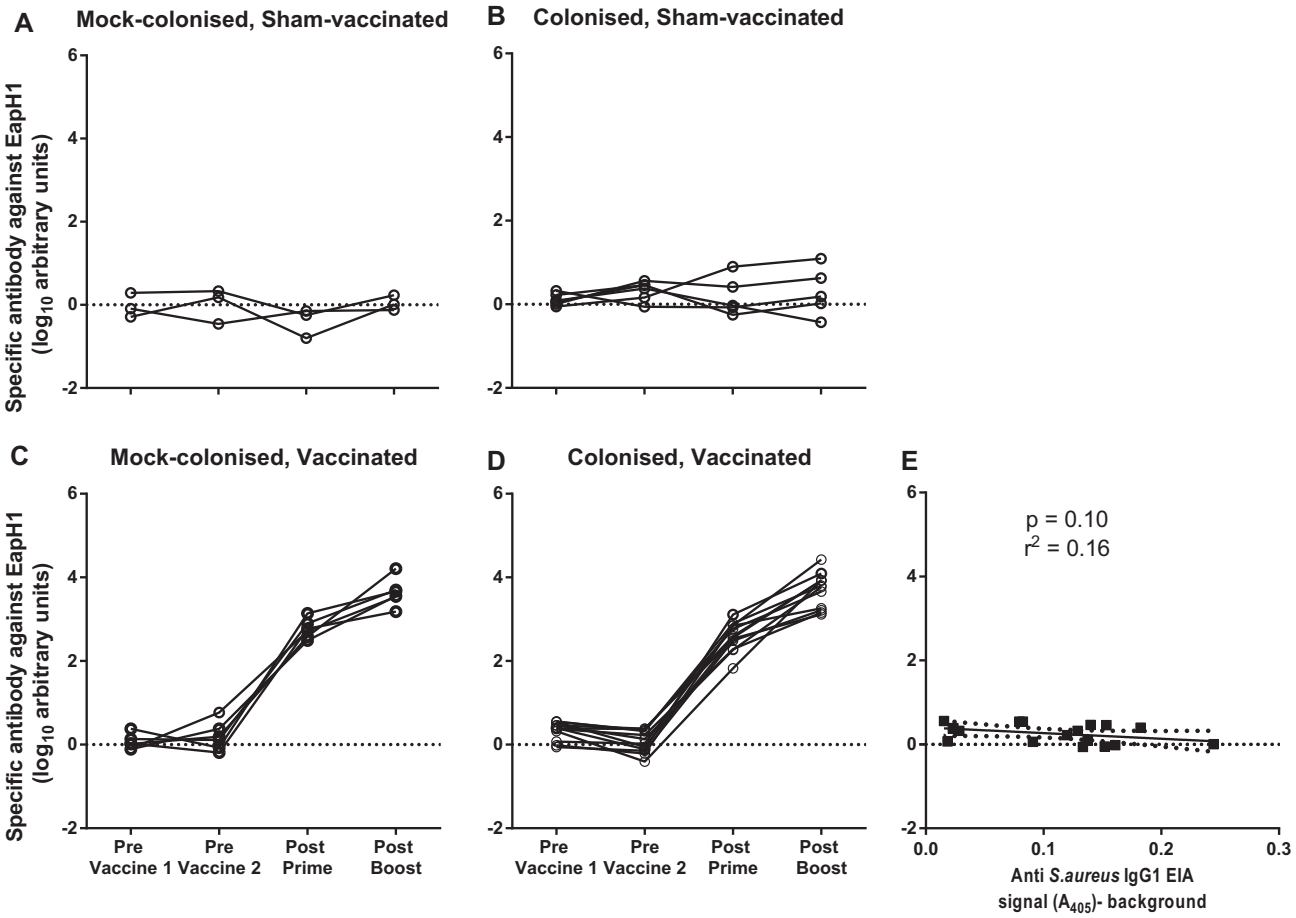

Fig. 5. Impact of experimental *S. aureus* carriage on vaccination with *S. aureus* antigen, EapH1. BALB/c mice colonised as adults and were subsequently vaccinated intramuscularly in a prime-boost schedule with viral-vectored vaccines containing both *S. aureus* antigens EapH1 and EapH2, as illustrated in Fig. 5. (A–D) EapH1 antibody response stratified by experimental group over course of experiment. (E) EapH1 antibody responses in colonised animals at Pre Vaccine timepoint 1 displayed against IgG1 against the cell surface.

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.

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