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BECC Adjuvanted Vaccine Provides Cross-Protection from Both Homologous and Heterologous Influenza A Infections

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Abstract

Influenza A virus (IAV) is a leading cause of respiratory disease worldwide often resulting in hospitalization or death. In this study, TLR4 immunostimulatory molecules, Bacterial Enzymatic Combinatorial Chemistry (BECC) 438 and BECC470 were found to be superior IAV vaccine adjuvants when compared to the classic adjuvant alhydrogel (alum) and Phosphorylated Hexa-Acyl Disaccharide (PHAD), a synthetic TLR4 agonist. BECC molecules allow for antigen sparing of a recombinant HA (rHA) protein, elicit a more balanced IgG1/IgG2a response, and were protective in a prime only dosing schedule. Importantly, BECC molecules afford protection from a heterologous IAV strain demonstrating that a cross-protective influenza vaccine is possible when the antigen is effectively adjuvanted.

Keywords

Vaccine; influenza; adjuvant; TLR4 agonist; cross-protection

Introduction

Seasonal influenza A causes significant annual morbidity and mortality worldwide, as well as severe economic losses [1]. In addition to normal seasonal infections, influenza viruses have a history of causing pandemics, as shown in 1918, 1957, 1968, and 2009 [2, 3];

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

outbreaks occur when influenza A viruses with novel antigenicity emerge and spread in a population with little preexisting immunity [4, 5]. Even normal seasonal outbreaks can cause significant morbidity and mortality. In the 2018–2019 influenza season there were over twice as many hospitalizations and deaths including greater than 35.5 million illnesses, 490,000 hospitalizations, and 34,000 deaths in the United States as compared to the previous five influenza seasons [6].

It is an annual challenge to determine the prevalent strain(s) of circulating influenza virus in an upcoming season. Yearly strain differences are largely caused by antigenic drift mutations in the immunodominant hemagglutinin (HA), a surface exposed spike protein and to a lesser extent the neuraminidase (NA), a surface enzyme required for viral replication [7]. The quadrivalent influenza virus vaccine most prevalent in use today consists of four different HA antigens (15 μg per HA antigen, depending on vaccine formulation) derived from individual influenza viruses; two influenza A viruses and two influenza B viruses and is administered to individuals greater than 6 months of age and is normally unadjuvanted. Vaccine adjuvants function by increasing the immunogenic capacities of poorly immunogenic antigens. In immunocompetent individuals, the use of an adjuvant also allows for antigen sparing, which creates a greater supply of vaccines [8], improves seroprotection against drifted strains [9], and helps promote heterologous cross protection against divergent strains of influenza virus [10]. In aged populations, immunosenescence (weakening of the immune system) leads to a progressive decline in the innate and adaptive immune responses. For individuals 65 and older, FluAd was specifically designed as a trivalent vaccine with a high dose of the HA antigen (15 μg of each HA antigen) formulated with the adjuvant MF59. MF59 is an oil-in-water emulsion of squalene oil, which helps create a more potent and durable immune response after vaccination in elderly individuals [11].

Immunoadjuvants are key components in vaccine formulations since they enhance and shape immune responses to vaccines. According to their mechanism of action, adjuvants are commonly classified as immunostimulating molecules, delivery systems, or a combination of both. Immunostimulating molecules function predominantly by activating innate immune receptors, such as Toll-like receptors (TLRs) generating signals that initiate a downstream adaptive immune response. Bacterial Enzymatic Combinatorial Chemistry (BECC) technology has been used to rapidly generate novel lipid A structures (Toll-like Receptor 4 ligands (TLR4Ls) for use as adjuvants [12]. BECC involves expression or deletion of enzymes from the lipid A synthesis pathways in Gram-negative bacteria, allowing for direct isolation/purification of TLR4Ls from a bacterial pellet without requiring further modification. In this study, we compare novel BECC-derived TLR4Ls, BECC438 and BECC470 to demonstrate their adjuvant potential in a prime only or prime-boost vaccination schedule, as compared to the adjuvant properties of alhydrogel (Alum) and Phosphorylated Hexa-Acyl Disaccharide (PHAD). Alum, an aluminum hydroxide wet gel suspension, induces a strong Th2 immune response. While the mechanisms of action of this widely used and FDA approved adjuvant have not been completely elucidated, it is thought that alum adjuvants by improving the attraction and uptake of antigen by antigen-presenting cells (APCs), allowing for extended release of antigen through a ‘depot’ effect [13–15]. More recently, TLR4Ls have been used as adjuvants that stimulate stronger

Th1 immune responses through pattern recognition receptors (PRR) [16]. MPLA (3-O-desacyl-4´-monophosphoryl lipid A), produced by GlaxoSmithKline is a biological extract of *Salmonella minnesota* (Re595) and contains a complex mixture of multiple acylated lipid A structures (3–7 fatty acids) arising from the chemical derivatization of MPLA from the parent extract [17–19]. PHAD, synthesized by Avanti Polar Lipids is a synthetic monophosphorylated hexa-acylated lipid A molecule that partially resembles GSK MPLA and is a commonly used control for MPLA. Both molecules can be used to induce Th1 immune response [20, 21]. BECC adjuvants, which were previously screened using reporter cell lines and flow cytometry for the ability to activate NFκB and cytokine production are capable of stimulating an innate immune response greater than PHAD but less than pyrogenic *E. coli* lipopolysaccharide (LPS) [12, 21]. In a *Yersinia pestis* rF1V protein vaccination model, C57BL6 mice showed balanced IgG1 and IgG2c levels and elicited a protective immune response against a lethal *Y. pestis* infection when adjuvanted with BECC438 [21]. Unlike other TLR4 ligand adjuvants which skew towards a Th1 response [22] and alum, which skews towards a Th2 response, BECC adjuvants are capable of driving a more balanced Th1/Th2 immune response [21].

BECC438 and 470 are created by expressing lipid A modifying enzymes that alter a backbone tetra-acylated, bis-phosphorylated *Yersinia pestis* lipid A [12]. BECC438 is a bis-phosphorylated structure with two secondary C16 acyl-chains added at the 2 and 2’ positions, the secondary C16 at the 2’ position has one unsaturation. This molecule is achieved by deletion of *msbB*, required for the addition of a C14 acyl-chain and repair of *pagP*, required for the addition of C16 acyl-chain [21, 23]. BECC470 is mono-phosphorylated with a C14 secondary acyl-chain added at the 3’ position and a secondary C16 acyl-chain added at the 2 position. It is engineered through repair of the *pagP* gene and addition of the *Francisella novicida lpxF* which removes the phosphate at the 4’ position. BECC molecules are capable of being manufactured reproducibly, inexpensively, and in large quantities making them advantageous for development as adjuvants for large scale vaccines, such as influenza virus vaccines.

For next generation influenza virus vaccines to be developed, it is critical that high-quality antigenic targets, such as influenza virus recombinant hemagglutinin (rHA) be used in combination with an adjuvant to increase their immunogenic capacities. Our experiments demonstrate that novel BECC adjuvants combined with an influenza virus rHA vaccine led to enhanced quantity and quality of antibodies as well as broad anti-viral protection in vivo.

Results

Determination of concentration of unadjuvanted rHA that confers protection to homologous challenge

To determine the concentration of rHA needed for vaccination studies, a concentration curve of antigen alone in both prime only (Figure 1A) or prime-boost (Figure 1B) vaccination schedule was performed in BALB/c mice (n=3). To assess the immune response in the antigen only vaccinated mice, total IgG titers (Figure 1C) were measured. This revealed elevated antibody production above sham for the 0.2 μg ($p = 0.0052$), 1.0 μg ($p = 0.0041$), and 5.0 μg ($p = 0.004$) rHA groups which demonstrated high levels of anti-rHA antibody

responses (Figure 1D). On day 28, mice were challenged by intranasal inoculation with influenza virus (A/Netherlands/02/09; NL/09 H1N1) and evaluated daily for protection from weight loss. In the prime only arm, no concentration of rHA provided protection from weight loss after challenge, whereas when administered in a prime-boost schedule, all concentrations of rHA were protective except for mice vaccinated with 0.04 μg rHA, which showed significant weight loss. Plaque assays were used to measure plaque forming units per gram (PFU/g) in day 7 post-challenge mouse lung homogenates (Figure 1E). Similar to antibody titer results, all prime only groups and the 0.04 μg prime plus boost group showed high virus levels in the lungs, as compared to the sham vaccination group. Vaccination groups that received 0.2, 1.0, or 5.0 μg prime-boost injections displayed one to two logs lower viral titer than the 0.04 μg prime plus boost group. Pathological scoring was performed on H&E stained lungs and inflammation scores calculated in a blinded study. All groups showed significant inflammation on histological examination, however the 0.2, 1.0, and 5.0 μg prime-boost groups showed minimal inflammation similar to the sham infection group (Figure 1F). While these data show that 0.04 μg rHA alone with a prime-boost vaccination schedule is not protective, they do suggest that this concentration of rHA elicits a minimal, but non-protective, immune response that can potentially be boosted through adjuvantation.

Adjuvant immune stimulation and protection from homologous challenge

To assess protection from influenza virus in a homologous challenge model where the antigen is derived from the same strain used for infection, BALB/c mice were immunized with 0.04 μg rHA formulated with 100 μg alum, 50 μg PHAD, 50 μg BECC438, or 50 μg BECC470 in a prime (day 0) - boost (day 14) schedule. Total serum IgG levels on day 28 were determined and showed that while there is a significant difference between the sham and PHAD groups ($p = 0.0016$), the BECC438 and BECC470 adjuvanted groups showed greatly increased IgG antibody titers when compared to sham ($p < 0.0001$ and $p = 0.0002$ respectively) (Figure 2A and 2D). rHA-specific IgG2a (Figure 2B) andIgG1 (Figure 2C) titers were also measured to assess the Th1- and Th2-type immune response, respectively. While antigen alone and alum adjuvanted groups did not show IgG2a production above sham vaccinated levels, IgG2a was significantly higher than sham for PHAD ($p = 0.0058$), BECC438 ($p = 0.0058$), and 470 ($p = 0.0054$) adjuvanted groups (Figure 2E). IgG1 production, however, was similar to sham for rHA only, alum, and PHAD groups, whereas BECC438 ($p < 0.0001$) and 470 ($p = 0.0001$) were both significantly elevated (Figure 2F). Following challenge with influenza virus A (NL/09), marked average weight loss was observed as approximately 30% in the sham group, 20% for the rHA only and alum groups, and 10% in the PHAD group. However, the BECC adjuvanted groups maintained a minimal weight superior to the sham infected group (Figure 2G). Virus titer values correspond with weight loss showing that both BECC adjuvanted groups showed no detectable viral titer in lung homogenates (Figure 2H). Histological inflammation scoring demonstrates that the sham vaccinated group, on average showed prominent to diffuse bronchiolar and periarterial inflammation with bronchial necrosis, along with the rHA only and alum groups. In contrast, the PHAD and BECC groups showed low inflammation scores with minimal bronchiolar or periarterial inflammation or small, focal alveolar consolidations (Figure 2I). Taken together, the BECC adjuvanted groups elicit a balanced Th1/Th2-type immune response and provide

superior protection from weight loss, virus replication, and lung pathology when challenged with a homologous IAV strain.

Adjuvant sparing capability observed for BECC438 and BECC470

To determine a minimal BECC adjuvant required for activity, BALB/c mice were immunized with 0.04 ug rHA in combination escalating amounts of BECC 438 or 470 (0.5, 5, or 50 μg) in a prime boost schedule. Total serum IgG levels from day 28 were determined and showed that total IgG production was below detection in sham mice and moderately elevated in the rHA only, 0.5 μg and 5 μg BECC438 or BECC470. Significantly higher antibody titers are observed in groups adjuvanted with 50 μg BECC438 ($p < 0.0001$) and 50 μg BECC470 ($p = 0.0002$) (Figure 3A and 3B). After homologous virus challenge, the average observed weight loss was similar for all groups (5–15%); whereas negligible weight loss was observed in the 50 μg BECC adjuvanted groups (Figure 3C). Plaque assays were used to measure plaque forming units per gram (PFU/g) of day 7 post-challenge mouse lung homogenate (Figure 3D). High viral titers ($>1\times10^6$ PFU/g) were observed in the sham and rHA only groups, whereas the 0.5 μg BECC adjuvanted groups showed a decrease of approximately two logs and lung homogenates from the 5 and 50 μg BECC groups displayed no plaques. The histological inflammation scores showed a similar pattern (Figure 3E). Histologically the sham and rHA only groups displayed moderate to prominent bronchiolar and periarterial inflammation with bronchiolar necrosis. Histological scoring showed dose-dependence with 5 μg BECC groups displaying decreased inflammation scores and correspondingly minimal to prominent bronchiolar or periarterial inflammation. The 50 μg BECC groups showed low to negligible inflammation and bronchiolar or periarterial inflammation (Figure 3E).

BECC adjuvant:rHA vaccine protect mice in a prime only dosing schedule

Current quadrivalent seasonal influenza vaccines are administered in a single dose and consist of four different HA antigens (15 μg per HA antigen). To determine if a prime-only BECC adjuvanted vaccine provides protection from homologous IAV challenge, mice were immunized with 5, 10, or 15 μg rHA, in combination with either 50 or 100 μg of BECC470 along with sham and 15 μg rHA only control groups with a single prime dose. For these studies, we chose to focus on BECC470 as it performed similarly or slightly better in our vaccine experiments compared to BECC438. Total serum IgG levels from day 28 were determined and showed that total IgG antibody production was minimal in sham mice and significantly higher titers in all other groups ($p < 0.0008$), as compared to rHA alone (Figure 4A and 4B). Interestingly, the difference in weight loss between the sham and rHA only groups, as compared to the BECC groups was striking. The rHA only group was similar to sham where protection from the challenge was not demonstrated. All other groups including the lowest rHA (5 μg) and BECC470 (50 μg) groups demonstrate minimal to no weight loss (Figure 4C). Virus titer was approximately 1×10^6PFU/g in both the sham and rHA only groups, whereas the BECC470 adjuvanted groups showed no detectable viral titer in lung homogenates (Figure 4D). Histological inflammation scoring showed that the sham group, on average, is similar to the HA only group. Microscopically they both presented with pronounced bronchiolar and periarterial inflammation, but with only mild bronchiolar necrosis. Inflammation scoring in all BECC470 adjuvanted groups, corresponding to viral

titer, showed low inflammation scores and little bronchiolar and periarterial inflammation (Figure 4E).

Extending protection from divergent strain

Due to poor genomic replication fidelity of the influenza virus and antigenic drift, especially in the highly immunogenic rHA surface protein, there is a requirement for a seasonal vaccine. A vaccine that provides protection across a broad array of antigenically variant rHA proteins and therefore, divergent influenza strains could improve vaccination efficacy rates and reduce disease prevalence. To test immune protection against a heterologous Sing/2015 (A/Singapore/GP1908/2015/IVR-180; Sing/2015 H1N1) challenge, we used similar formulations and a prime-boost dosing schedule as were used in the homologous NL/09 challenge above. Mice were immunized with 0.04 μg rHA formulated with 100 μg Alum, 50 μg PHAD, 50 μg BECC438, or 50 μg BECC470. Similar to the homologous challenge experiment, day 28 serum total IgG antibody production is negligible in sham mice, minimally elevated in the rHA only group and higher in the rHA in combination with alum or PHAD. The BECC438 ($p = 0.0002$) and BECC470 ($p < 0.0001$) adjuvanted groups; however, showed significantly higher IgG antibody titer, even when compared to PHAD (Figures 5A and 5B). Following challenge with IAV Sing/2015, both BECC adjuvanted groups only lost approximately 7% of starting weight (Figure 5C), which correlated with superior viral neutralization assay titers (Figure 5D). No detectable viral titer was observed for both BECC groups (Figure 5E), and histological inflammation scoring shows only moderate inflammation with scant to diffuse bronchiolar and mild to moderate periarterial inflammation (Figure 5F).

Discussion

Current seasonal influenza vaccines for individuals under 65 years of age are not formulated with an adjuvant. The trivalent vaccine (FLUAD), indicated for adults age 65 years and older was the first adjuvanted influenza vaccine marketed in the U.S and is formulated with MF-59 and high dose of the three included HA antigens. Vaccine adjuvants or immunostimulating molecules are required to increase immunogenic capacities and cross-protection capabilities of poorly immunogenic antigens in immunocompetent and immunosenescent individuals. Depending on the type of innate responses activated, adjuvants can alter the quality and quantity of adaptive immune responses and thereby enhance or modulate immune responses when compared with antigen alone. To develop effective next generation vaccines, such as for seasonal and pandemic influenza viruses, it is critical that high quality antigens are identified and used in combination with appropriate adjuvants to increase their immunogenic potential.

Here, we have characterized two novel BECC adjuvants which when formulated with a recombinant influenza HA protein were able to elicit a stronger and broader immune response, as compared to the known adjuvants, alum and PHAD. This enhanced response was seen in both homologous and heterologous challenge. Using a homologous challenge model, we showed a balanced Th1/Th2 immune response in mice vaccinated with BECC adjuvants. BECC adjuvanted vaccines also provided superior protection from weight loss,

virus titer reduction, and adverse lung pathology (Figure 2). A clear dose-dependent protection from an IAV challenge is also observed and it is encouraging to note that superior performance is attainable with >7-fold less antigen (Figure 2) and 10-fold less adjuvant (Figure 3). This is important to not only decrease the likelihood of an adverse immune reaction, but also spare adjuvant and allow the production of larger quantities of vaccine.

To improve patient compliance and widespread immunity, it is imperative that a seasonal vaccine is effective with a single dose. We have shown with a prime only dosing schedule, mice immunized with a BECC470 adjuvanted vaccine maintain high IgG antibody titers with minimal to negligible weight loss along with undetectable virus titer in the lung homogenate and low inflammation (Figure 4). It is also extremely advantageous for a vaccine to provide cross-protection from a wide range of influenza virus strains. As shown in Figure 5, immunization with Cal/09 rHA elicited a protective immune response to Sing/2015 which had been previously been shown to elicit no response in a Sing/2015 murine infection without the addition of the BECC molecules. H1N1/Michigan/2015's HA is antigenically identical to Sing/15's HA. Michigan/15 does not cross react with Cal/09 HA antisera [25]. This is a significant discovery that may allow manufacturers to move away from the annual, and all too often failing cycle of providing a highly efficacious vaccine, and instead produce a vaccine that protects against a wider spectrum of the influenza A virus.

A successful BECC adjuvanted rHA IAV vaccine would not only eliminate allergy concerns and protein production rate-limiting steps in eggs, but because cross-protection is elicited, it would relieve the need to formulate a new vaccine annually and significantly contribute to extensive community herd immunity. In addition, immunity wanes quickly after influenza virus vaccination which the BECC adjuvants may be able to extend based on increased immunogenicity during vaccination. Outside of influenza virus vaccines, a novel adjuvant showing a balanced Th1/Th2-type immune response and increased antigenicity may be an ideal formulation partner for a wide variety of seasonal and pandemic vaccine candidates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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- Seasonal influenza A causes significant morbidity and mortality worldwide
- Annually influenza vaccines are on average only 40–60% effective
- Adjuvanted vaccines enhance and shape immune responses to vaccines
- Novel BECC TLR4 ligands adjuvant flu vaccines to induce a balanced Th1/Th2 response
- BECC adjuvants protect from homologous and heterologous flu infection in mice

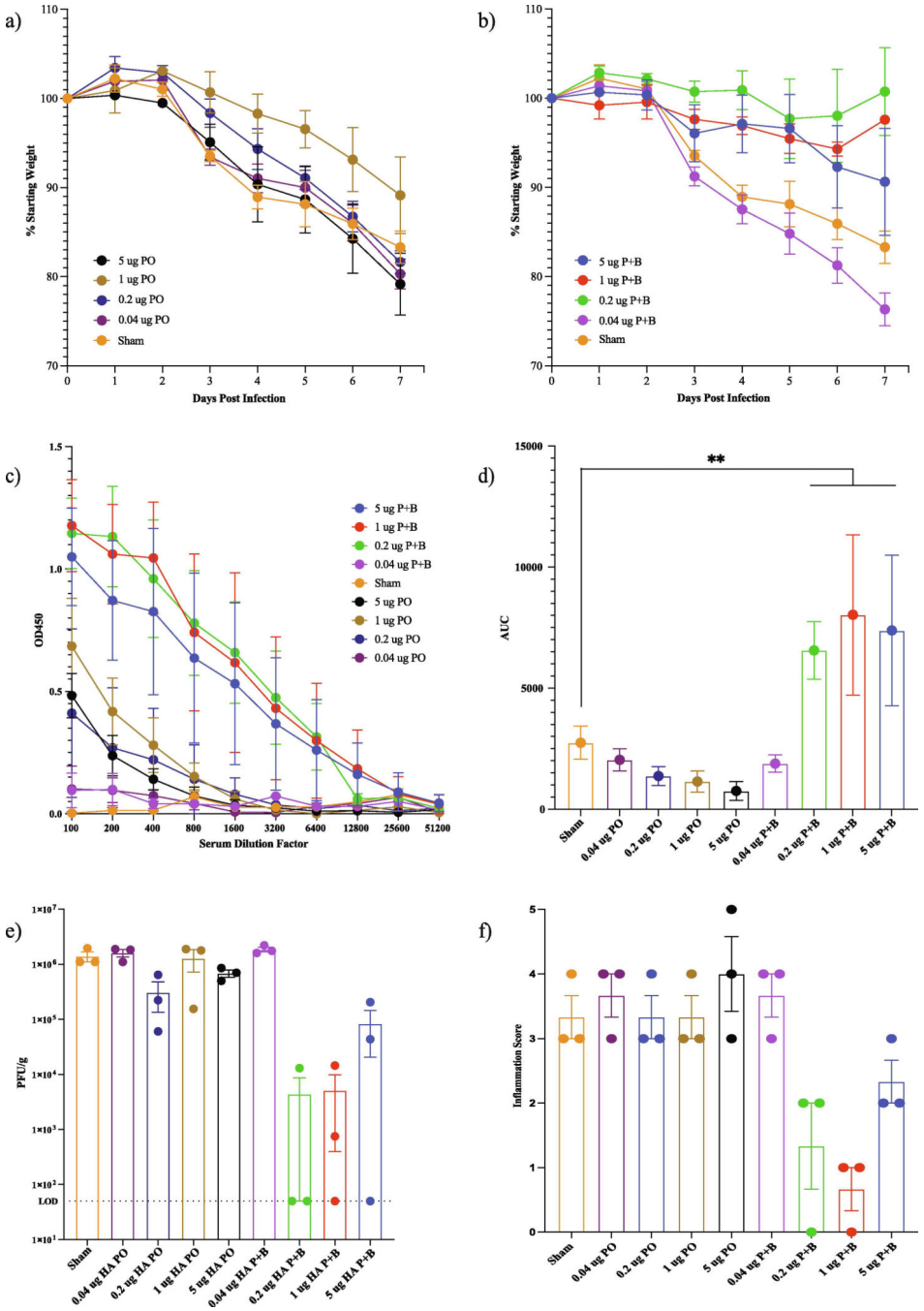

Fig. 1.

Determining Cal/09 HA antigen dose needed in vaccination for protection from NL/09 influenza A infection. 7-day weight loss in BALB/c mice (3 per group) after infection with 3200 PFU of NL/09 with either a) prime only (PO) vaccination schedule (Mean + SEM) or b) prime + boost (P+B) schedule (Mean + SEM). c) Pre-infection day-28 serum total IgG antibody titer in PO and P+B vaccination groups (Mean + SEM) with (d) corresponding area under the curve (AUC) (Mean ± standard error) (**$p < 0.006$.). e) Virus titer of lung homogenate 7-days post infection from PO and P+B groups (Mean + SEM) with (f)

pathology inflammation scoring of lung histology slides (Mean + SEM). Prism 9 used to calculate Mean + SEM and AUC ± standard error.

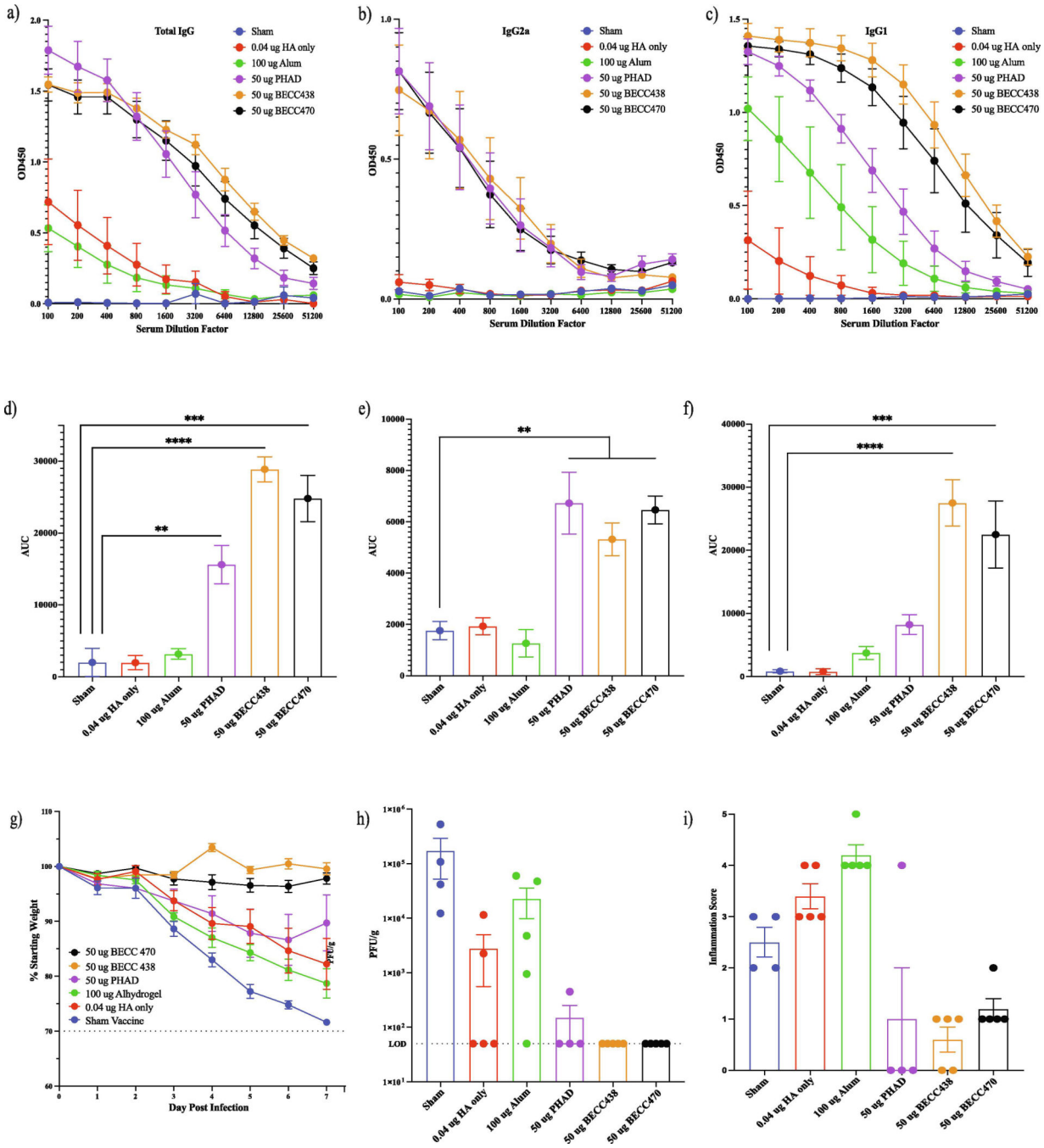

Fig. 2.

Homologous challenge protection, a) Pre-infection day 28 serum ELISA total IgG, (b) isotype specific IgG2a and (c) IgGl in prime + boost schedule (Mean + SEM). Area under the curve (AUC) (Mean ± standard error) of above serum ELISA curves for d) total IgG (**p = 0.0016, ****p < 0.0001, ***p = 0.0002), e) IgG2a (**p < 0.006) and f) IgGl (****p < 0.0001, ***p = 0.0001). g) 7-day weight loss in BALB/c mice (5 per group) after infection with 3200 PFU of NL/09 (Mean + SEM). h) Virus titer of lung homogenate 7- days post

infection (Mean + SEM) with i) pathology inflammation scoring of lung histology slides (Mean + SEM). Prism 9 used to calculate Mean + SEM and AUC ± standard error.

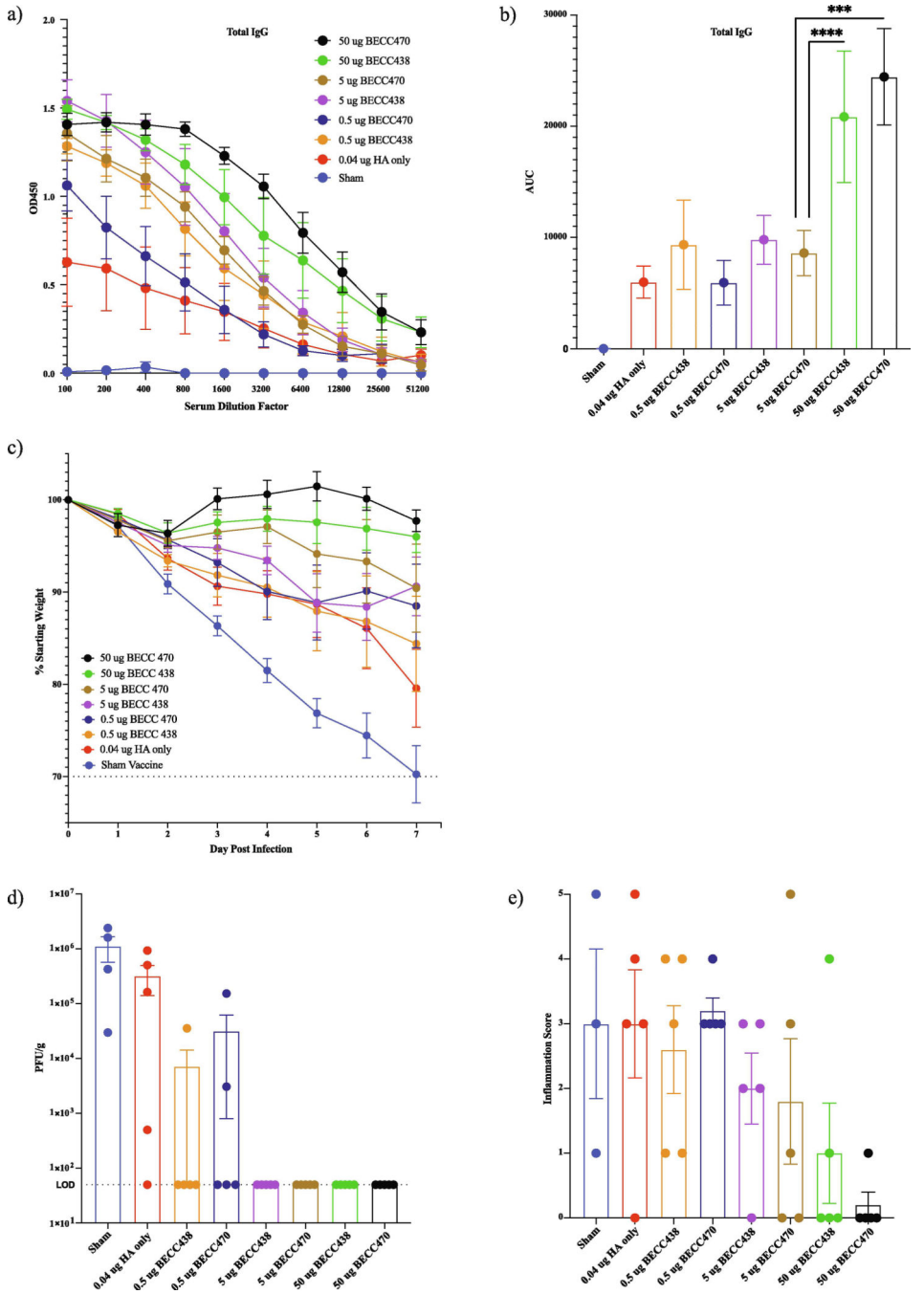

Fig. 3.

Adjuvant dose sparing, a) Pre-infection day-28 serum ELISA total IgG with 0.04 μg HA in combination with 0.5, 5 or 50 jig of BECC438 or BECC470 adjuvants in prime + boost schedule (Mean + SEM). b) Area under the curve (AUC) (Mean ± standard error) of ELISA curves in for total IgG antibody (****$p < 0.0001$, ***$p = 0.0002$). c) 7-day weight loss in BALB/c mice (5 per group) after infection with 3200 PFU of NL/09 (Mean + SEM). d) Virus titer of lung homogenate 7-days post infection (Mean + SEM) with e) pathology

inflammation scoring of lung histology slides (Mean + SEM). Prism 9 used to calculate Mean + SEM and AUC ± standard error.

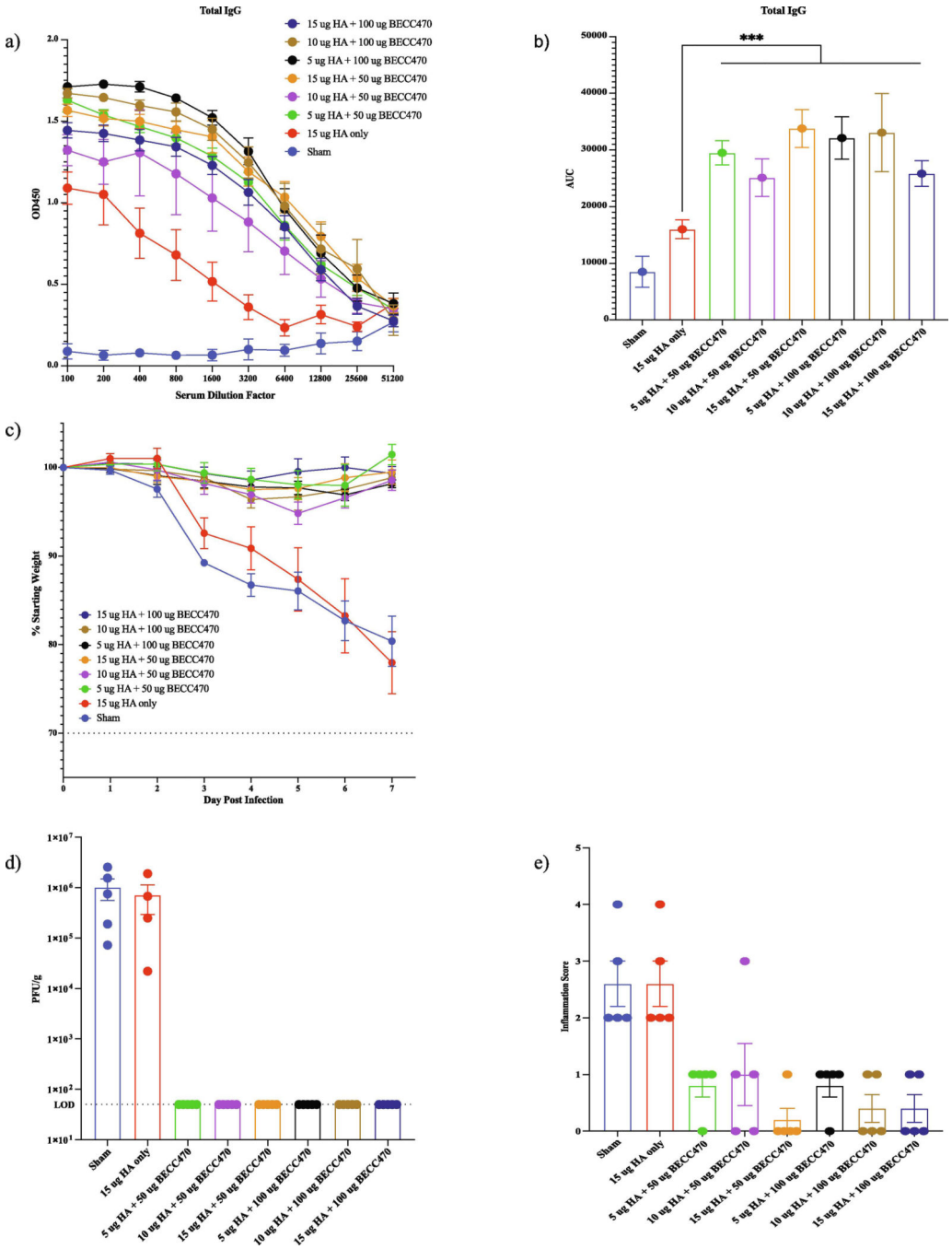

Fig. 4.

Prime only single vaccination, a) Pre-infection day 28 serum ELISA total IgG with 5, 10 or 15 μg HA protein in combination with 50 or 100 μg of BECC470 adjuvant in prime only vaccination schedule (Mean + SEM). b) Area under the curve (AUC) (Mean ± standard error) of ELISA curves for total IgG antibody (***$p < 0.0008$). c) 7-day weight loss in BALB/c mice (5 per group) after infection with 3200 PFU of NL/09 (Mean + SEM). d) Virus titer of lung homogenate 7-days post infection (Mean + SEM) with e) pathology

inflammation scoring of lung histology slides (Mean + SEM). Prism 9 used to calculate Mean + SEM and AUC ± standard error.

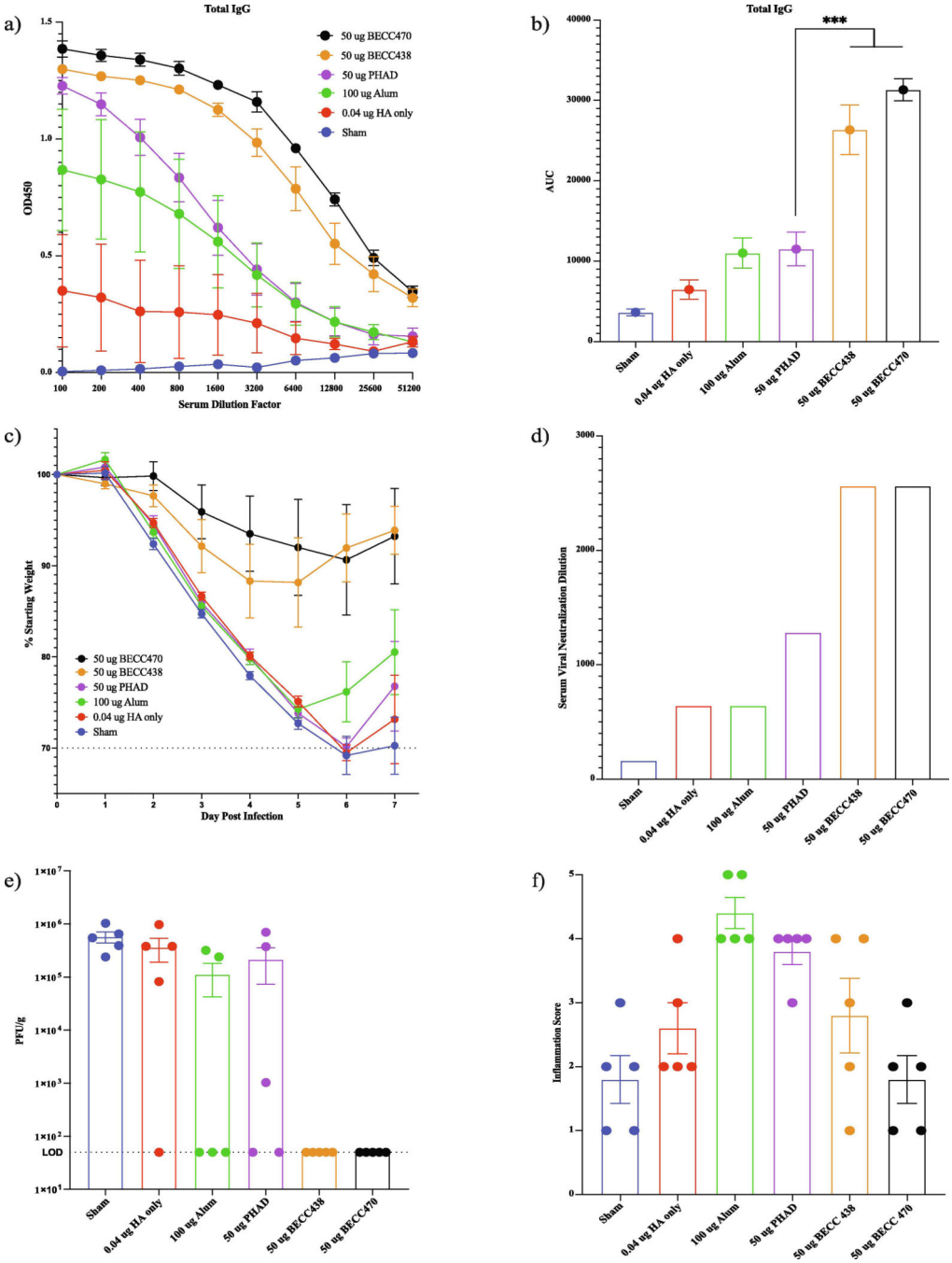

Fig. 5.

Protection from heterologous Sing/2015 Influenza A challenge, a) Pre-infection day 28 serum ELISA total IgG in prime + boost schedule (Mean + SEM). b) Area under the curve (AUC) of serum ELISA curves for total IgG antibody (***$p < 0.0003$). c) 7-day weight loss in BALB/c mice (5 per group) after infection with 51 PFU of Sing/2015 (Mean + SEM). d) Day 28 serum antibody viral neutralization assay, e) Virus titer of lung homogenate 7-days

post infection (Mean + SEM) with f) pathology inflammation scoring of lung histology slides (Mean + SEM). Prism 9 used to calculate Mean + SEM and AUC ± standard error.