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Archaeal glycolipid adjuvanted vaccines induce strong influenza-specific immune responses through direct immunization in young and aged mice or through passive maternal immunization

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ABSTRACT

Vaccine induced responses are often weaker in those individuals most susceptible to infection, namely the very young and the elderly, highlighting the need for safe and effective vaccine adjuvants. Herein we evaluated different archaeosome formulations as an adjuvant to the H1N1 influenza hemagglutinin protein and compared immune responses (anti-HA IgG and hemagglutination inhibition assay titers) as well as protection to an influenza A virus (strain A/Puerto Rico/8/1934 H1N1) homologous challenge to those generated using a squalene-based oil-in-water nano-emulsion, AddaVax™ in a murine model. The impact of age (young adult vs aged) on vaccine induced immune responses as well as the protection in pups due to the transfer of maternal antibodies was measured. Overall, we show that archaeal lipid based adjuvants can induce potent anti-HA responses in young and aged mice that can also be passed from vaccinated mothers to pups. Furthermore, young and aged mice immunized with archaeal lipid adjuvants as well as pups from immunized mothers were protected from challenge with influenza. In addition, we show that a simple admixed archaeosome formulation composed of a single sulfated glycolipid namely sulfated lactosylarchaeol (SLA; 6′-sulfate-β-D-Gal*p*-(1,4)-β-D-Glc*p*-(1,1)-archaeol) can give equal or better protection compared to AddaVax™ or the traditional antigen-encapsulated archaeosome formulations.

1. Introduction

Influenza infections cause high morbidity and mortality worldwide annually. While influenza poses a risk to all age groups, it most seriously affects the very young and elderly, two demographics in which influenza vaccines are often less effective [1–3]. While the ultimate goal of all vaccines is to induce life-long sterilizing immunity, this is a particularly difficult task for influenza vaccines because of the plasticity of influenza's immunogenic antigens and the resulting need to develop a new vaccine every year. As a result, there is a great need to improve upon influenza vaccines by identifying influenza antigens that are more functionally constrained and are therefore less likely to mutate [4]. Other ways to enhance influenza vaccines include re-designing the delivery platform with the use of viral vectors [5,6], DNA vaccines [7,8] and virus-like particles [9]. The current egg-based flu vaccine is available as a trivalent and quadrivalent formulation with a cocktail of two influenza A strains and one or two influenza B strains and these are also found to be combined with adjuvants that improve antibody and cellular immune responses [10,11]. In recent years, the oil-in-water emulsions MF59 and adjuvant systems 03 (AS03) have been used as adjuvants in both seasonal and pandemic influenza vaccines and have been shown to induce a stronger, longer lasting antibody response capable of enhancing the diversity and affinity of the antibody response compared with non-adjuvanted vaccines [12]; this highlights the potential of using other adjuvanted vaccines that enhance dose sparing and efficacy which hopefully will facilitate the approval of other novel adjuvants.

Archaeal lipid adjuvants are a class of adjuvants that have been previously shown to induce both antibody and cellular immune responses against multiple antigens, including listeriolysin (LLO),

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tyrosinase-related-protein-2 (Trp2), glycoprotein-100 (Gp100), Hepatitis-B surface antigen (HBsAg) and Hepatitis C virus e1e2 heterodimer (HCV E1E2) [13–16]; they were also capable of generating protective immunity against bacterial pathogens such as *Listeria monocytogenes, Trypanosoma cruzi* and *Mycobacterium tuberculosis* [13,17–19] and tumor growth in a B16 melanoma model [14,20]. Archaeal lipid adjuvants have been traditionally composed of total polar lipids derived from archaea such as the methanogen *Methanobrevibacter smithii* (MS), formulated into liposomes (archaeosomes) with encapsulated antigen. MS archaeosomes have been previously shown to effectively activate professional antigen presenting cells [21–23] and generate robust cellular and humoral immune response in both cancer and infection models [13,22,24], including breaking tolerance to cancer self-antigens in a melanoma model [14]. Additionally, MS archaeosomes exhibit high thermal and pH stability and low proton permeability when compared to conventional liposomes. MS archaeosomes also do not generate anti-lipid immune responses and can be used in a repeat boosting vaccination setting [24]. MS archaeosomes are however relatively complex and are composed of a wide array of naturally occurring lipid species that can potentially vary from batch to batch depending on archaeal growth characteristics. To simplify the formulation a semi-synthetic archaeosome has been developed composed of a sulfated saccharide group covalently linked to the free *sn*-1 hydroxyl backbone of an archaeal core lipid (sulfated S-lactosylarchaeol, SLA). Advantages to this simpler formulation include consistency of production, reduced costs and ease of synthesis while still retaining a similar level of adjuvanticity as observed with MS archaeosomes [16,23,25]. We have evaluated SLA archaeosomes using the model antigen OVA and we have shown them to be safe, effective and capable of inducing strong antigen-specific immune responses in mice and protection against a subsequent B16 melanoma tumor challenge. We have also demonstrated that a key step in their mechanism of action appears to be the recruitment of immune cells to the injection site and the subsequent trafficking of antigen to local draining lymph nodes [23]. However, the efficiency of antigen entrapment within archaeosome formulations is variable and relatively low (5–40%) which results not only in loss of antigen but also increased cost and varied amounts of archaeal lipid in the final vaccine formulations [26,27]. Recently we have developed a novel archaeosome formulation, whereby antigen is simply admixed with preformed SLA archaeosomes providing a convenient easy to mix format with no loss of antigen during the formulation process.

Herein, we compared immune responses induced by H1N1 influenza A virus (A/Puerto Rico/8/34, PR8) hemagglutinin (HA) alone, encapsulated inside MS or SLA archaeosomes, admixed with empty SLA archaeosomes or formulated with AddaVax™, a squalene-based oil-in-water nano-emulsion with a formulation similar to MF59® that is widely used in influenza vaccine formulations [28]. The impact of age (young adult vs aged) on HA-specific immune responses as well as the protection in pups due to the transfer of maternal antibodies was measured. Overall, we show that archaeal lipid based adjuvants, in particular a simple admixed formulation composed of a single semi-synthetic glycolipid, SLA, can induce potent anti-HA responses in young, aged and pregnant mice and that these mice (and their pups) are protected against subsequent challenge with H1N1 influenza A virus.

2. Materials and methods

2.1. Mice

6–8 week old female Balb/c mice were obtained from Charles River Laboratories (Saint-Constant, Canada). Vaccines were also assessed in an aged mouse model using female Balb/c mice that were obtained at 6–8 weeks of age and housed for 23 months before vaccination such that they were approximately 2 years of age at the time of first immunization. For mating, female mice were caged overnight for two nights with male Balb/c mice, 2 female mice to 1 male mouse [29,30]. Pups of immunized female mice were weaned at 3 weeks of age and separated by gender. Mice were maintained in individually ventilated cages with 5 female mice to a cage with easy access to food and water in a specific pathogen free small animal facility with automatically controlled light/dark cycles, humidity and temperature at the National Research Council Canada (NRC) in accordance with the guidelines of the Canadian Council on Animal Care. The animal use protocol (2016.08) was approved by the NRC Animal Care Committee. All mice were randomized upon entering our facility and all mice that entered these studies were monitored in a blinded method. For example, numerical group numbers were assigned to each cage and formulations were prepared by one individual and injected by another. Influenza challenge and the monitoring of body weights and clinical scores was carried out by another individual who had no knowledge of the vaccines being tested or the ordering of the groups.

2.2. Vaccine route and preparation

Female Balb/c mice were immunized by i.m. injection (50 μl) into the left tibialis anterior muscle with 2 μg of recombinant influenza H1N1 A/Puerto Rico/8/34 rHA protein (encoding the extracellular domain Met 1 – Gln 528, fused with a C-terminal polyhistidine tag), purity > 90% (Sino Biological Inc., USA) in combination with the commercial adjuvant AddaVax™ (1:1 v/v mixture squalene-oil-in-water emulsion, Invivogen) or with MS or sulfated lactosylarchaeol (SLA; 6′-sulfate-β-D-Gal*p*-(1,4)-β-D-Glc*p*-(1,1)-archaeol) archaeosomes. MS and SLA lipids were extracted and synthesized (respectively) at the National Research Council Canada as described previously [22,27,31]. MS and SLA archaeosomes with encapsulated HA protein were prepared by first dissolving 20 mg of total polar lipids for MS archaeosomes, or SLA lipid for SLA archaeosomes in chloroform/methanol; the archaeal lipid was next deposited as a thin film after removal of organic solvent under N_2 gas with mild heating. The vacuum was applied for at least 2 h to ensure total removal of trace solvents. Lipid film was hydrated with 1.0 mL of Milli-Q water containing 0.375 mg HA protein and was shaken for 2 hrs at 40 °C or until hydration was completed. Archaeosome vesicles were reduced in size using a tabletop ultrasonic water bath (Fisher Scientific FS60H, 130 W and operating frequency of 40 kHz) and high pressure, they were then left to anneal at 4 °C for 12 h in static conditions. Removal of free antigen was performed using ultracentrifugation at 50,000 rpm (222,592 g) for 2 hrs and the final formulation was filter sterilized through 0.22 μm filter units. For entrapped archaeosome formulation, quantification of the % antigen entrapped was conducted using Mini-PROTEAN TGX™ 12% pre-cast gel (Bio-Rad Laboratories, Mississauga, Ontario) for SDS polyacrylamide gel electrophoresis and densitometry. The concentration of encapsulated HA was determined by subjecting 10 μl of archaeosomes to SDS-PAGE electrophoresis in parallel with known amounts of antigen. Protein samples were electrophoresed at a constant 200 V for approximately 1 h. Protein was visualized by SYPRO red staining (Thermo Scientific). The density of the bands was determined by gel scanning and densitometry analysis using Alphaview Software (ProteinSimple, San Jose, CA, USA).

For the preparation of empty SLA archaeosomes admixed with HA protein, the same process was followed except the lipid film was hydrated in Milli-Q water without HA antigen. The HA solution was added to the empty archaeosomes at the desired amount

immediately before immunization so that a single dose contained 1 mg of SLA and 2 μg of antigen. All solutions were brought to a physiological pH of 7.4 in phosphate buffered saline (PBS).

2.3. Immunization schedule

Young adult (10–12 weeks) and aged Balb/c mice (23 months) were used to compare protective influenza virus-specific immune responses generated by MS and SLA archaeosomes; immunizations were given on day 0 and 21. To evaluate the impact of archaeosomes in pregnant mice, female mice were immunized once or twice with AddaVax – HA, SLA – HA encapsulated (enc) or SLA – HA admixed (adm). Briefly, all mice were mated on day 14 and 15 of the study; mice were immunized once on day 0 of the study or twice on day 0 and day 21. Serum anti-HA IgG in the mated females was assessed on day 28 and 58 (Supplemental Fig. 1). While all mice in the study were mated, only 18 out of 40 immunized mice became pregnant which was an expected pregnancy rate for 2 days of mating (unpublished observations). We chose a short mating period of 2 days to ensure that pups were born at the same time for consistency between groups. The pups of these immunized mice were selected randomly (6–13 mice per group, we strived to have equal male and female pups) and challenged with influenza at 7 weeks of age (4 weeks post weaning). Negative control groups include unimmunized naïve mice or mice injected with HA protein alone, SLA alone or AddaVax™ alone.

2.4. H1N1 influenza challenge

8–12 weeks post immunization (as specified in each figure caption) mice were challenged intranasally with 5×10^3 plaque-forming units (pfu) of the mouse-adapted H1N1 influenza A virus (A/Puerto Rico/8/34, PR8) in 50 μl PBS. Mouse body weights were measured prior to influenza challenge and daily afterwards for 12–14 days. Mice were euthanized once they reached the study humane endpoint of >20% loss in body weight combined with observed clinical signs of illness that include signs of respiratory distress, decreased mobility and piloerection (clinical data is not shown).

2.5. Assessment of IgG titers

Anti-HA total IgG titers in mouse serum were quantified by ELISA as described previously [16], with plates coated with 1 μg/mL of the HA protein used for immunization. Titers for IgG in serum were defined as the dilution that resulted in an absorbance value (OD 450) of 0.2 and calculated using XLfit software (ID Business Solutions, Guildford, UK). No detectable titers were measured in serum samples from naïve and adjuvant-treated (without antigen) control animals.

2.6. Hemagglutination inhibition assay (HAI)

The hemagglutination inhibition assay was performed as described previously [32]. Briefly, sera was pre-treated with a receptor destroying enzyme (RDE) (Denka SeikenCo, Japan) to prevent non-specific agglutination, then doubling dilutions (1/10, 1/20, 1/40, 1/80 etc.) were made for each serum sample across 8 wells in duplicate in a 96 well plate. Standardized influenza virus containing 4 HA units/well (as confirmed by back titration) were added and incubated for 30 min at room temperature. Finally 0.5% standardized chicken red blood cells (Innovative Research, Novi, Michigan) was added to a final concentration of 0.2% to all wells and incubated for 1 h at room temperature. Positive control sera (NIBSC, South Mimms, England, UK) as well as negative control sera from naïve mice were used for each plate. The HAI titer was defined as the reciprocal of the highest dilution of antiserum that completely inhibited hemagglutination.

2.7. Statistical analysis

Data were analyzed using GraphPad Prism® (GraphPad Software, San Diego, CA). Antibody titers were log transformed prior to testing and shown as geometric mean titer with the lower and upper 95% confidence interval shown in the graph and written in the text. One-way and two-way analysis of variance (ANOVA) followed by post-hoc analysis using Tukey's (comparison between all groups) multiple comparison tests were used as indicated in the figure legends. For all analyses, differences were considered to be not significant with $p > 0.05$.

3. Results

3.1. Archaeal lipid adjuvants protect against H1N1 influenza challenge in young adult mice

Mice were immunized with HA antigen encapsulated in MS or SLA archaeosome formulations by intramuscular (i.m.) injection on days 0 and 21; serum anti-HA IgG antibody responses were assessed on day 35 (Fig. 1A). Control mice received HA antigen alone or HA mixed with AddaVax™. All three adjuvanted vaccine groups developed serum anti-HA IgG titers that were significantly greater than those to HA antigen alone ($p < 0.0001$). There was no significant difference in anti-HA antibody titers in mice immunized with HA adjuvanted with AddaVax™ compared to encapsulated within SLA archaeosomes with a geometric mean titer (GMT) and lower and upper 95% confidence interval (95% CI) of 542,482 (434,782 & 676,860) and 204,390 (153,270 & 272,559) respectively ($p > 0.05$), although both of these vaccine formulations induced higher responses than those obtained with HA encapsulated within MS archaeosomes at 39,632 (24,116 & 60,144) ($p < 0.0001$ and $p < 0.01$, respectively). When an HAI assay was conducted on serum samples collected on day 49, no significant HAI titers were detected in serum from mice immunized with HA alone or HA encapsulated in MS archaeosomes. In contrast, significantly higher HAI titers were measured in serum from mice immunized with HA encapsulated in SLA archaeosomes, 210 (126 & 350), or admixed with AddaVax™, 337 (154 & 738), than in mice immunized with HA alone, 5.7 (4.2 & 7.9), or encapsulated in MS archaeosomes, 5.4 (4.6 & 6.3) ($p < 0.0001$) (Fig. 1B). Following challenge with the mouse-adapted H1N1 influenza strain PR8, unimmunized mice and those that received AddaVax alone had the most rapid weight loss (Fig. 2A) and met the study humane endpoint of > 20% weight loss with observed clinical signs of illness (including signs of respiratory distress, decreased mobility and piloerection) at days 6 and 7, HA alone immunized mice reached maximum weight loss at day 7 with 60% of the mice regaining weight and surviving indefinitely (Fig. 2B). Mice immunized with HA adjuvanted with AddaVax or encapsulated within SLA archaeosomes survived significantly longer compared to HA alone immunized mice ($p < 0.05$) with no weight loss (Fig. 2A) and with 100% survival (Fig. 2B). Mice immunized with HA encapsulated in MS archaeosomes had a mean maximum body weight loss of ~10% on day 6 and eventually fully recovered; this was significantly more body weight loss compared to mice immunized with either HA encapsulated in SLA archaeosomes ($p < 0.0001$) or admixed with AddaVax™ ($p < 0.01$). Thus, although both archaeosome formulations afforded complete protection against an influenza challenge, HA encapsulated in SLA archaeosomes was selected for further evaluation on account of the higher antibody responses, better HAI titers and lower weight loss compared to HA encapsulated in MS archaeosomes.

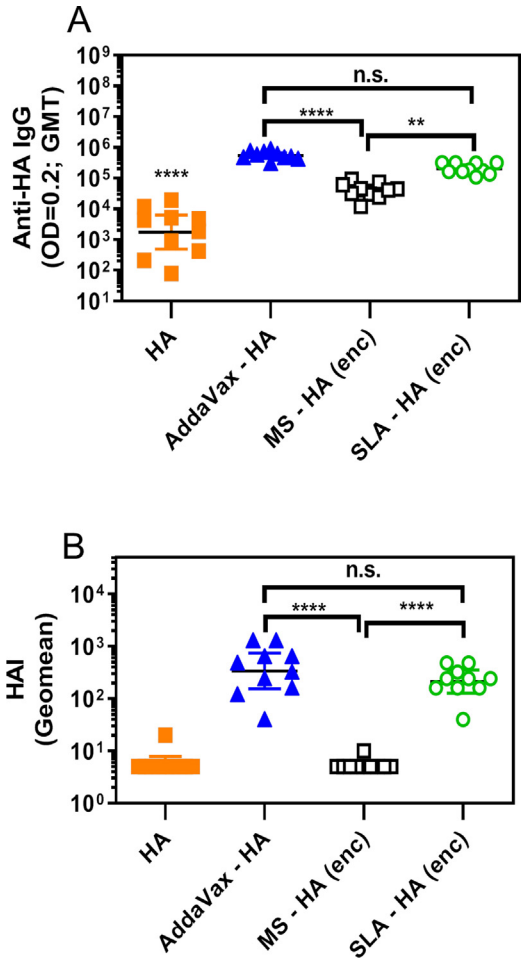

Fig. 1. Anti-HA IgG titers and Hemagglutination inhibition assay in immunized mice. Balb/c mice (n = 10/group) were immunized i.m. (left anterior tibialis) with HA protein ss μg) alone or HA protein formulated with AddaVax™ or encapsulated within archaeosomes composed of MS total polar lipids or SLA lipids on days 0 and 21. (A) Serum was obtained from all mice on day 35 and analyzed for anti-HA IgG antibody by ELISA. Individual data is presented with the GMT and 95% confidence interval. ****$p < 0.0001$, **$p < 0.01$, n.s. $p > 0.05$ (B) Serum was obtained from all mice on day 49 and analyzed for inhibition of HA induced agglutination. ****$p < 0.0001$, n.s. $p > 0.05$. All data was log transformed for one-way ANOVA analysis with multiple comparisons.

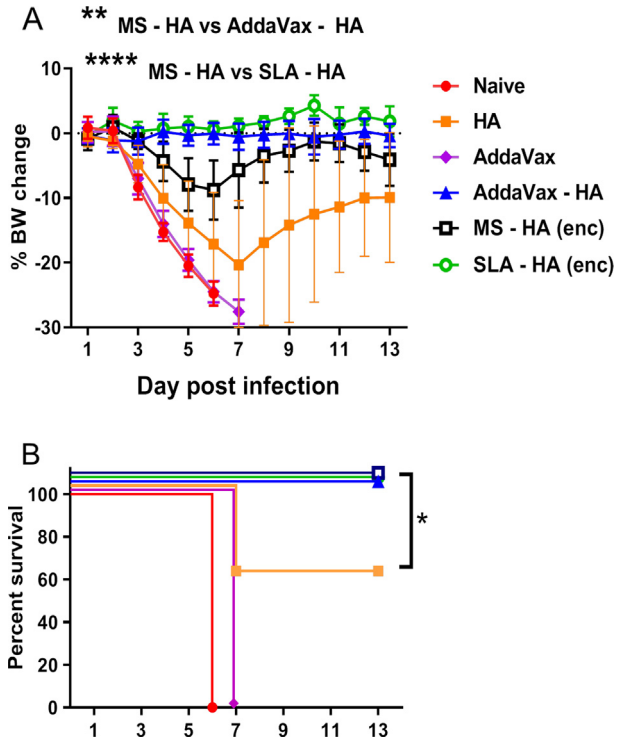

Fig. 2. Archaeosome adjuvants induce protection in mice against influenza challenge. Balb/c mice (n = 10/group) were immunized i.m. (left anterior tibialis) with HA protein (2 μg) alone or formulated with AddaVax™ or encapsulated within archaeosomes composed of MS total polar lipids or SLA lipids on days 0 and 21. At 59 days after initial vaccination, all mice as well as a naïve group were challenged with 5×10^3 PFU of Influenza strain A/Puerto Rico/8/1934 (H1N1). (A) Body weights were taken on the day before infection as well daily until day 13. Note that only body weights for surviving mice at each time-point are shown. A two-way repeated measure ANOVA with multiple comparisons (Tukey's correction) was used to assess differences in body weight after infection. MS – HA vs. SLA – HA ***$p < 0.0001$, MS – HA vs. AddaVax – HA **$p < 0.01$, SLA – HA vs. AddaVax – HA n.s., $p > 0.05$. B) Percent survival is shown for each group with Log-rank (Mantel-Cox) test. HA immunization alone vs SLA – HA (enc), AddaVax – HA or *M.smithii* – HA (enc) * $p < 0.05$.

3.2. Archaeal lipid adjuvants protect against H1N1 influenza strain PR8 challenge in aged mice

Elderly people are known to be especially susceptible to influenza infections with increased morbidity and mortality rates compared to young adults [33–35], therefore we also evaluated and compared the immunogenicity and protective efficacies between SLA archaeosomes and AddaVax™ adjuvanted vaccine in aged Balb/c mice. Aged mice (~2 years old) were immunized by i.m. injection on days 0 and 21 with HA antigen alone, HA encapsulated in SLA archaeosomes (SLA – HA (enc)), HA admixed with preformed empty SLA archaeosomes (SLA–HA (adm)) or HA formulated with AddaVax™. Young adult mice (10–12 weeks) immunized with AddaVax – HA, were used as an additional positive control. All of the adjuvanted formulations induced anti-HA antibody responses significantly greater than those observed with HA alone, ($p < 0.0001$) (Fig. 3). The use of SLA archaeosomes as an adjuvant induced equivalent anti-HA responses to AddaVax™, irrespective of whether HA was encapsulated or simply admixed with SLA archaeosomes ($p > 0.05$). Overall, antibody levels were lower in aged mice than in young adult mice ($p < 0.0001$). For example, anti-HA GMT with 95% CI in aged mice immunized with AddaVax – HA were approximately 20–30 fold lower than those in young adult mice 14 days post boost at 28,194 (13,077 & 60,788) compared to 694,928 (504,698 & 962,582) respectively ($p < 0.0001$). Moreover, no HAI titers were detected in aged mice immunized with any of the vaccine formulations (data not shown); at 28 days post boost the HAI assay did not detect any HAI titer for all aged mice (<10) whereas young mice immunized with AddaVax – HA generated an HAI titer of 399 (111 & 1433). Following the challenge of aged mice with H1N1 influenza strain PR8 (at 58 days after initial immunization), naïve mice as well as mice immunized with AddaVax, SLA or antigen alone rapidly lost body weight (Fig. 4A) and reached the study humane endpoint within 8 days (Fig. 4B). In contrast, aged mice immunized with AddaVax – HA, SLA – HA (enc) or SLA – HA (adm) had 78–88% survival. When we compared weight loss of AddaVax – HA immunized aged mice to that of the AddaVax – HA immunized young mice (positive control) the day 7 wt loss of aged mice was significantly greater ($p < 0.05$) (Fig. 4A), however there was no significant difference in survival between these two groups ($p > 0.05$) (Fig. 4B). Control young adult mice immunized with AddaVax – HA were all protected without any significant change in body weight as previously seen. Thus, while protection from influenza was reduced in aged mice compared to young mice especially in mice receiving the unadjuvanted vaccine formulation, both archaeosome adjuvant formulations

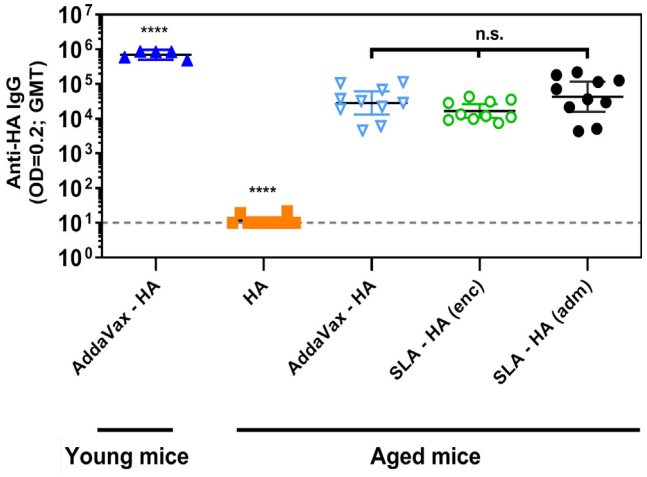

Fig. 3. Anti-HA IgG titers in aged immunized mice. Balb/c mice aged 23 months (n = 10/group) were immunized i.m. (left anterior tibialis) with HA protein (2 μg) alone or formulated with AddaVax™, encapsulated within SLA archaeosomes (SLA – HA (enc)), or admixed with SLA archaeosomes (SLA – HA (adm)) on days 0 and 21. Young Balb/c mice aged 10–12 weeks immunized twice with AddaVax – HA served as a positive control (n = 5). Negative controls include AddaVax™ alone, SLA alone or HA protein alone. Serum was obtained from all mice on day 35 and analyzed for anti-HA IgG Abs by ELISA. Individual data is presented with the GMT and 95% confidence interval. All data was log transformed for one-way ANOVA analysis. ****, $p < 0.0001$, compared to all other groups. n.s., $p > 0.05$, compared to the three indicated groups.

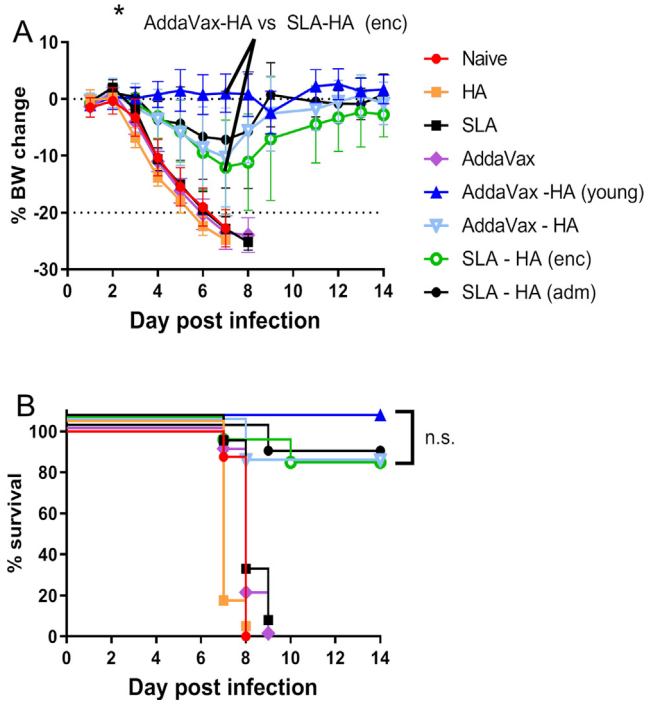

Fig. 4. Archaeosome adjuvants induce protection in aged mice against influenza challenge. Balb/c mice aged 23 months (n = 8–10/group) were immunized i.m. (left anterior tibialis) with HA protein (2 μg) alone or formulated with AddaVax™, encapsulated within SLA archaeosomes (SLA – HA (enc)) or admixed with SLA archaeosomes (SLA – HA (adm)) on days 0 and 21. Young Balb/c mice aged 10–12 weeks immunized twice with AddaVax – HA served as a positive control (n = 5). Negative controls include AddaVax™ alone, SLA alone or HA protein alone. All mice, as well as a naïve group were challenged with 5×10^3 PFU of Influenza strain A/Puerto Rico/8/1934 (H1N1) 58 days after initial vaccination. (A) Body weights were taken on the day before infection as well daily until day 14. Note that only body weights for surviving mice at each time-point are shown. (B) Percent survival is shown for each group with a Log rank (Mantel-Cox) test. One-way ANOVA was used to assess differences in body weight at the peak of weight loss at day 7. SLA – HA (enc) vs. AddaVax – HA (young), *$p < 0.05$.

were shown to be as effective as AddaVax™ to induce anti-HA IgG immune responses as well as protective immunity to an H1N1 influenza strain PR8 virus challenge.

3.3. Archaeal lipid adjuvants are effective during pregnancy and antibodies are transferred to mouse pups

Pregnant women and infants are particularly susceptible to influenza related complications [36], and are often over-represented in hospital admissions [36–40], therefore we also evaluated SLA archaeosome adjuvant compared to AddaVax™ in pregnant Balb/c mice. The transfer of maternal antibodies to the pups and the pups' protection from influenza challenge was assessed.

The female mice were mated on day 14 and 15 of the study; pups were born on day 33–35, weaned 3 weeks later and challenged with H1N1 influenza strain PR8 at 4 weeks post weaning (Fig. 5). To assess the transfer of maternal antibodies to the pups, serum anti-HA IgG was assessed at 6 weeks of age (75 days post their mother's initial vaccination) (Fig. 6). With any particular vaccine, there was no significant difference between male and female pups. Overall ranking of HA-specific responses (e.g., SLA – HA (adm) = AddaVax – HA > SLA – HA (enc) > HA alone) was the same as obtained in the mated female group although levels were about 10-fold lower (Supplemental Fig. 1). Likewise responses following a single immunization prior to mating were lower than those induced by two immunizations. Following infection with H1N1 influenza strain PR8, pups from naïve non-immunized mothers or from mothers that received HA alone, all succumbed to infection within one week. In contrast, pups from mothers immunized twice with any of the adjuvanted vaccine formulations had 100% protection. Pups from mice immunized once with SLA – HA (adm) or AddaVax –HA had 90% survival, while all pups of mothers immunized with SLA – HA (enc) succumbed to infection within one week (Fig. 7); this was associated with a notably decreased level of anti-HA antibodies which was greater than HA alone, a GMT and 95% CI of 293 (181 & 473) vs < 100 respectively ($p < 0.05$), but was significantly less than the titers observed from pups of mothers

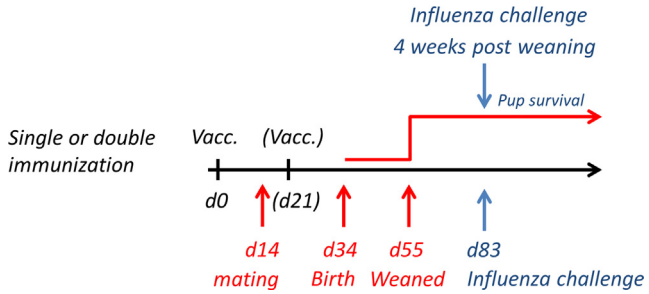

Fig. 5. Schematic of immunization and pregnancy in female mice and pup birth and weaning. Female Balb/c mice (n = 5/group) were immunized i.m. with HA protein alone (2 μg) or formulated with AddaVax™, encapsulated within SLA archaeosomes (SLA – HA (enc)) or admixed with SLA archaeosomes (SLA – HA (adm)) once on day 0 or twice on day 0 and 21. All female mice were housed with male mice (2 female to 1 male) for two days on day 14 and 15 and pups were tested for protection against an influenza challenge. Schematic depicting the timeline of vaccination, pregnancy, birth and testing of pup's for protection against influenza. Pups were born on day 33–35 after 1st immunization and were weaned on day 55 after 1st immunization. At 6 weeks of age, pups are tested for serum anti-HA antibodies and at 7 weeks for protection against an influenza challenge.

immunized with SLA – HA (adm), 935 (711 & 1229) ($p < 0.001$) or with AddaVax – HA, 2692 (2360 & 3071) ($p < 0.0001$) (Fig. 6A and Supplemental Table I). Notably, there was a correlation between mice having a higher anti-HA IgG titer and a reduced

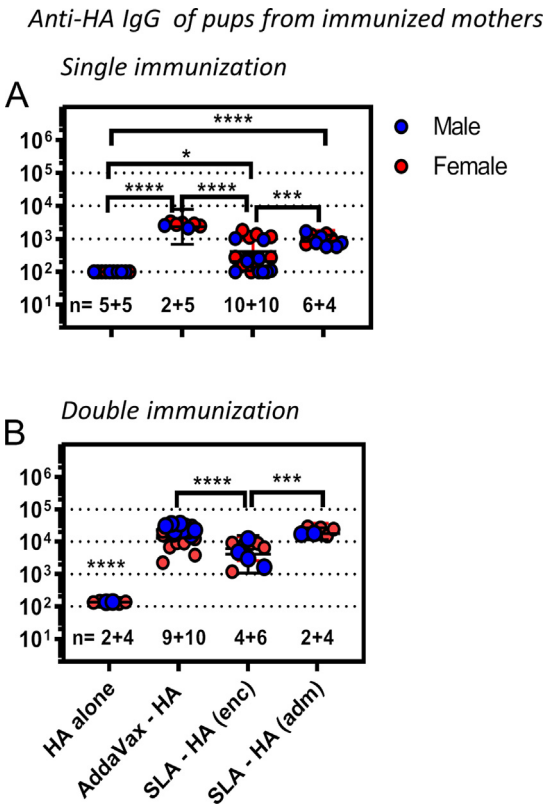

Fig. 6. Maternal antibody transfer of anti-HA IgG in pups of immunized female mice. Female Balb/c mice (n = 5/group) were immunized i.m. with HA protein alone (2 μg) or formulated with AddaVax™, encapsulated within SLA archaeosomes (SLA – HA (enc)) or admixed with SLA archaeosomes (SLA – HA (adm)) once on day 0 or twice on day 0 and 21. Pups were born on study day 33–35 and weaned 3 weeks later. Serum was collected at 6 weeks of age (study day 75) and analyzed for the presence of anti-HA IgG Abs by ELISA. Individual data for male and female pups is presented with the GMT and 95% confidence interval. The number of male mice + number of female mice is identified for each group in the figure (n = 6–20 mice/group). All data was log transformed and analyzed with a two-way ANOVA with multiple comparisons for effect of gender or treatment. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

weight loss at day 7 of infection in the pups ($r^2 = 0.83$). We also evaluated immune responses in the mated female group once pups had been weaned. As expected, anti-HA antibody levels were significantly lower in the mice immunized once compared to twice (Supplemental Fig. 1). Overall, the highest antibody titers were induced in mice immunized twice with SLA – HA (adm), 152,297 (64,795–357,964) or AddaVax – HA, 137,222 (63,458 & 294,730) and these were both significantly higher than responses obtained in mice immunized with SLA – HA (enc), 38,443 (11,189–132,090) ($p < 0.001$) or HA alone, 2,665 (652–10,887) ($p < 0.0001$) (Fig. 6A and Supplemental Table I). Eighty-three days after the start of the study, all female mice were challenged with H1N1 Influenza strain PR8. Naïve mice met their humane endpoint within 7 days, and a single immunization with HA protein alone afforded little protection for mice (20–40%). Single immunization with the archaeosome or SLA-adjuvanted vaccine formulations protected all mice from influenza challenge, with 100% survival and no significant loss in body weight observed. While mated females were protected from influenza with a single immunization of SLA – HA (enc), their pups were not; this could be due to a higher circulating titre of anti-HA antibody in the mated females before challenge compared to the pups, 1915 (503 & 7282) and 293 (181 & 473) respectively. Strong protection was induced in all mated females immunized twice with HA, regardless of whether the adjuvant was present or not (80–100%). Overall, very little loss of weight (Supplemental Fig. 2) or signs of illness by clinical score (data not shown) was observed for any of the adjuvanted – HA immunized groups, indicating the vaccines conferred not only enhanced survival but also decreased infection related morbidity.

Overall these results show that HA adjuvanted with archaeal lipid based adjuvants, in particular admixed with a simple semi-synthetic glycolipid (SLA), can induce potent anti-HA responses in young, aged and pregnant mice which can protect against subsequent viral infection and that these responses are equivalent to those obtained with a squalene oil-in-water based emulsion.

4. Discussion

Archaeosomes have previously been shown to be effective vaccine adjuvants capable of enhancing both humoral and cell mediated immune responses to multiple antigens, and generating protective immunity against bacterial pathogens and tumors in preclinical mouse models [13–15,17–20]. Additionally, the latest generation of archaeosomes, composed of SLA lipids, was shown to be equal to or superior to many other tested adjuvants including aluminum hydroxide, TLR3/4/9 agonists, oil-in-water and water-in-oil emulsions in enhancing responses to OVA or hepatitis B surface antigen (HBsAg) [16]. To date, SLA archaeosomes have not been evaluated in a viral challenge model and we therefore evaluated whether HA antigen formulated with SLA lipids could induce protective immunity against an H1N1 influenza strain PR8 challenge in mice. In addition, their activity was directly compared to AddaVax™ (a mimetic of MF59) in young, aged mice and pregnant mice.

SLA-based archaeosomes induce local cytokine production at the site of injection even in the absence of antigen [23]. When used to deliver entrapped antigen (ovalbumin) they also stimulate the recruitment of neutrophils and macrophages, enhance antigen uptake/retention at the vaccination site and subsequent trafficking of antigen-containing cells to local draining lymph nodes [23]. While this has previously only been demonstrated for entrapped antigen, similar effects will also likely occur with an admixed formulation since they are dependent on the archaeal lipids rather than the antigen itself. Therefore, the strong adjuvant effects of SLA archaeosomes on HA seen in the current study, whether using an admixed or an encapsulated formulation, are most likely also due to the combined effects of enhanced local cytokine secretion, immune cell infiltration and enhanced antigen uptake. In previous studies, we have compared SLA archaeosome formulations with multiple adjuvants including the AddaVax™ (a squalene-based oil-in-water emulsion similar to MF59) with both OVA and HBsAg [16]. In those studies, SLA and AddaVax™ both induced high levels of antigen-specific antibodies, although there were some differences in cellular responses. Likewise, in this study using HA as antigen, both SLA and AddaVax™ induced strong levels of anti-HA IgG antibodies compared to the unadjuvanted control. We did not assess antibody isotypes due to limitations in antigen supply. However, in previous studies we have shown that AddaVax™ induced a strong Th2-biased response, whereas SLA archaeosomes induced a more mixed Th1/Th2 response and it is likely that a similar bias occurred with HA. We also did not evaluate cellular responses to HA in this study as antibodies are considered the major mediator of protection to the influenza virus however it is possible that other immune components, such as CD8+ T cells could have played a role in providing resistance to infection. As expected, the anti-HA IgG responses were lower in aged mice in comparison to young adult mice. This has been shown previously in other vaccination models where aged mice were less able to induce antigen-specific IgG antibodies [41,42]. Importantly, SLA adjuvanted formulations (whether encapsulated or admixed) were able to function and enhance

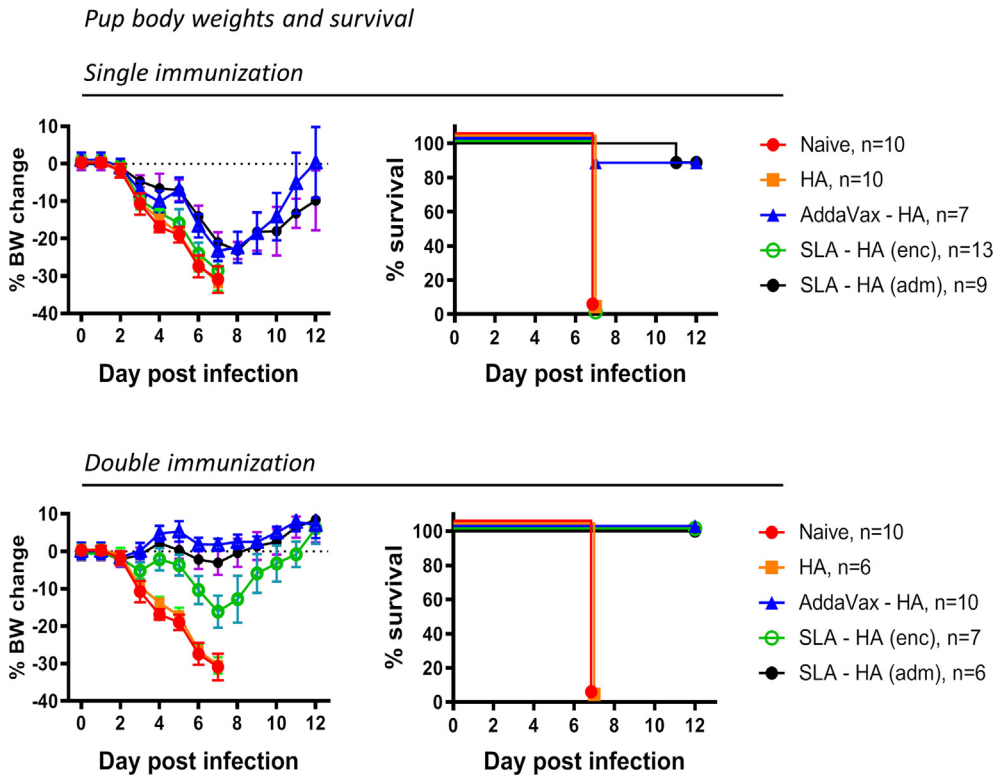

Fig. 7. Protection from influenza challenge in pups of immunized female Balb/c mice. Female Balb/c mice (n = 5/group) were immunized i.m. with HA protein alone (2 μg) or formulated with AddaVax™, encapsulated within SLA archaeosomes (SLA – HA (enc)) or admixed with SLA archaeosomes (SLA – HA (adm)) once on day 0 or twice on day 0 and 21. Pups were born on study day 33–35 and weaned 3 weeks later. The number of male mice + number of female mice is identified for each group in the figure (n = 6–13 mice/group). 7 week old pup were challenged with 5×10^3 PFU of Influenza strain A/Puerto Rico/8/1934 (H1N1) 83 days after the study start date, body weights were taken on the day before infection as well daily until day 12. Note that only body weights for surviving mice at each time-point are shown. Percent survival is shown for each group.

immune responses to the vaccine antigen in these older mice to a level sufficient to confer protection to viral challenge.

Archaeosomes, like most liposomes, have traditionally been formed by conventional lipid hydration methods, whereby an antigen solution is used to hydrate a dried layer of lipid to create a solution of liposomes with encapsulated antigen. The liposome solution is then further processed to achieve a uniform size of liposome and to remove free antigen. However, this can result in a high loss of antigen during formulation preparation and laborious preparation steps; typically entrapment efficiency is only 5–40% which may be prohibitive in the case of costly antigens. We have recently developed a simple admixed formulation that can induce strong humoral and cell-mediated immune responses in a convenient easy to mix format with no loss of antigen during the formulation process [43]. Herein, we found that in aged mice SLA archaeosomes prepared using the lipid hydration method as well as the admixed formulation (empty SLA archaeosomes and HA protein) were as effective as AddaVax™ to induce anti-HA IgG immune responses and protect against an influenza challenge. In pregnant mice, the encapsulated formulation generated weaker responses than the AddaVax™ or admixed SLA formulations in both the adult females and the resulting pups. Although not statistically significant, antibody titers were higher (>2.5 fold) with AddaVax – HA vs. SLA – HA (enc) in the young adult study. In contrast, the admixed formulation generated similar responses to AddaVax™ in both the aged and pregnancy models tested above. Therefore, when compared to the encapsulated formulation method, the SLA admixed formulation may offer enhanced adjuvant activity in addition to the manufacturing advantages mentioned above.

In Canada the MF-59 adjuvant vaccine, FLUAD, is also licensed for use in babies 6 months to <2 years of age [44,45], since it elicits higher immune responses compared to the unadjuvanted inactivated vaccine [46]. There isn't an approved vaccine for use in infants under 6 months of age, and studies have shown that infants in the first 8 weeks of life would benefit from maternally transferred influenza specific antibodies [47]. We found that HA adjuvanted with archaeal lipid based adjuvants, in particular admixed with a simple semi-synthetic glycolipid (SLA), can induce anti-HA responses in pregnant mice which can protect against subsequent viral infection in both pups and mated females and that these responses were equivalent to those obtained with a squalene oil-in-water based emulsion. While maternally transferred anti-HA antibodies are detected in the pups of immunized mice and are attributed with providing the pups protection from influenza infection it is also possible that cellular immune components (innate immune cells, CD8 T cells) were transferred from immunized mother to pup (passive cellular immunity) [48–52]. Subsequent studies could evaluate the effect of adjuvanted versus non-adjuvanted vaccines on the induction of passive cellular immunity and any associated effects this would have on the transfer of maternal antibodies, this could help to improve the design of influenza vaccination regimes for pregnant women and neonates.

In summary, we show that archaeal lipid based adjuvants, in particular a simple semi-synthetic glycolipid (SLA), can induce potent anti-HA responses in young, aged and pregnant mice which can protect against subsequent viral infection in both pups and mothers. In addition, these responses were equivalent to those obtained with a squalene oil-in-water based emulsion. Furthermore, a simple

admixed formulation induced strong immune responses with no loss of antigen during the formulation process.

Declaration of Competing Interest

Lakshmi Krishnan is an inventor on various archaeosome patents and patent applications. All other authors state no conflict of interest, nor competing financial interests.

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Author contributions

FS, BA and MM conceived the study. All authors contributed to the design and synthesis of the vaccines and/or the study. FS, BA, AP, RD, LD, GA, KW, BH performed the experiments. FS, BA, AP, RD, LD, BH, MM analyzed the data. FS, BA and MM took the lead in writing the manuscript. All authors provided critical feedback and helped to shape the research, analysis and manuscript.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.07.010.

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