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Single dose combination nanovaccine provides protection against influenza A virus in young and aged mice

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Immunosenescence poses a formidable challenge in designing effective influenza vaccines for aging populations. While approved vaccines against influenza viruses exist, their efficacy in older adults is significantly decreased due to the diminished capabilities of innate and adaptive immune responses. In this work, the ability of a combination nanovaccine containing both recombinant hemagglutinin and nucleoprotein to provide protection against seasonal influenza virus infection was examined in young and aged mice. Vaccine formulations combining two nanoadjuvants, polyanhydride nanoparticles and pentablock copolymer micelles, were shown to enhance protection against challenge compared to each adjuvant alone in young mice. Nanoparticles were shown to enhance *in vitro* activation of dendritic cells isolated from aged mice, while both nanoadjuvants did not induce proinflammatory cytokine secretion which may be detrimental in aged individuals. In addition, the combination nanovaccine platform was shown to induce demonstrable antibody titers in both young and aged mice that correlated with the maintenance of body weight post-challenge. Collectively, these data demonstrate that the combination nanovaccine platform is a promising technology for influenza vaccines for older adults.

Introduction

Influenza A virus (IAV) is a significant health threat to elderly populations, with up to 90% of influenza-related deaths occurring in patients 65 years and older.[1] In addition, hospitalization rates due to influenza are two-fold higher in older adults and account for approximately $10.4 billion in U.S. medical costs each year.[2, 3] Although the use of existing influenza vaccines may provide protection, these vaccines often have significantly lower efficacy and/or significant variability with respect to their performance in aged individuals.[1, 4] For example, older adults were particularly affected during the 2017-2018 influenza season (which had a high severity of 48.8 million cases in the U.S.), by accounting for 70% of influenza-associated hospitalizations and 90% of influenza-related deaths.[5]

In light of these major public health challenges, it is important to understand the changes in aging-related immune functions and use this information to design new and improved vaccines for older adults. Immunosenescence, the decline in immune function with age, constitutes defects in both innate and adaptive immunity.[6] These defects include the decline in haematopoietic stem cell function,[7] decreased B cell numbers,[8] reduced phagocytic ability of neutrophils,[9] reduced expression of transcription factors,[10] and thymic involution.[11] In addition, "inflamm-aging" is another consequence of immunosenescence, leading to the presence of an increased basal intrinsic inflammation in the immune system.[12, 13] All of these age-related immune deficiencies often lead to poor antibody production (which typically doesn't correlate with protection in older adults) and T cell responses post-vaccination.[14] Although recent studies have sought to improve responses in older adults through multiple or high dose immunizations,[15, 16] there is an urgent need to improve vaccines for older adults.[5] Thus, novel vaccine technologies may be required to induce protection against influenza in older adults.

Nanovaccines composed of polyanhydride nanoparticles and pentablock copolymers represent a novel platform for the design of subunit influenza vaccines. In particular, polyanhydride nanoparticles have been demonstrated to provide sustained release of encapsulated influenza antigens while enhancing antigen stability.[17] In addition, polyanhydride-based nanovaccines have been shown to induce anti-hemagglutinin (HA) antibody titers capable of neutralizing influenza virus, inducing antigen-specific CD4^{+} and CD8^{+} T cell responses, including lung-resident memory phenotypes[18] and providing protection against challenge.[4] Similarly, pentablock

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copolymers based on Pluronic F127 and polydiethylaminoethyl methacrylate (PDEAEM) represent another effective platform for affording sustained release of antigen, depositing the antigen in the cytosol, and promoting the rapid development of antibody titers.[19, 20] Our previous work has shown that combination nanovaccines consisting of both polyanhydride nanoparticles and pentablock copolymers provided the greatest protection against influenza in young mice compared to either nanoadjuvant alone.[21] That being said, older adults often require vaccines which stimulate the immune system without exacerbating the already inflamed state.[22] Cyclic dinucleotides (CDNs), which are STING activators, have been previously shown to rapidly induce antibody titers in aged mice, without displaying the inflammatory profile of traditional TLR agonist adjuvants.[23] In addition, CDN stimulation of dendritic cells has been shown to induce greater amounts of B cell activating factor (BAFF), which is important for germinal center maintenance and B cell survival.[24, 25]

Herein, we describe the formulation of a combination nanovaccine based on these two nanoadjuvants, incorporating the hemagglutinin (HA) and nucleoprotein (NP) antigens from H1N1 IAV, and demonstrate the ability of the combination nanovaccine to provide protection against seasonal influenza virus. These nanovaccines enhanced dendritic cell activation, while limiting detrimental inflammation. Additionally, the combination nanovaccine combined with CDNs induced measurable anti-HA antibody titers in aged animals and limited weight loss post-challenge. Furthermore, the data demonstrated that a single dose of the combination nanovaccine was shown to provide protection against influenza virus in both young and aged animals. Collectively, the data presented here demonstrates the ability of the combination nanovaccine to reduce clinical disease following IAV infection in aged animals.

Experimental

Polyanhydride nanoparticle synthesis

Monomers of 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) and 1,6-bis(*p*-carboxyphenoxy)hexane (CPH) were synthesized as previously described.[26, 27] Next, 20:80 CPTEG:CPH polymer was synthesized via melt polycondensation.[27] The resulting molecular weight (5,957 g/mol), composition (24:76), and purity of the polymer were characterized with ^{1}H nuclear magnetic resonance spectroscopy (^{1}H NMR; DXR 500, Bruker, Billerica, MA).

Nanoparticles containing 1 wt. % H1 hemagglutinin (HA) and 1 wt. % nucleoprotein (NP) were synthesized using a solid-oil-oil double emulsion technique as previously described.[28] Briefly, HA and NP proteins obtained from Sino Biological (Beijing, China) were dialyzed to nanopure water and lyophilized overnight. Next, 20 mg/mL of 20:80 CPTEG:CPH polymer containing 1 wt. % of each HA and NP was dissolved in methylene chloride. The solution was sonicated for 30 s to ensure that the polymer was dissolved and the protein evenly distributed. The solution was then poured into chilled pentane

(-10°C; 1:250 methylene chloride:pentane) and the resulting particles collected via vacuum filtration. Nanoparticle morphology and size (~200 nm) were verified with scanning electron microscopy (FEI Quanta 250, FEI, Hillsboro, OR).

Pentablock copolymer micelle synthesis

A novel pentablock copolymer (PDEAEM-POE-POP-POE-PDEAEM) was synthesized by atom transfer radical polymerization.[29] A difunctional macroinitiator prepared from commercially available Pluronic®-F127 was dissolved in tetrahydrofuran and reacted overnight with triethylamine and 2-bromoisobutyryl. The product was precipitated in n-hexane and characterized using ^{1}H NMR to confirm the end group functionalization. Next, the macroinitiator and the DEAEM monomer were used to synthesize the pentablock copolymer utilizing copper (I) oxide nanoparticles as the catalyst and N-propylpyrilidine methanamine as the complexing ligand.[30] The pentablock copolymer was characterized with ^{1}H NMR to determine purity and molecular weight (14,600 g/mol).

To formulate the micelles used in Fig. 1, a stock solution of 30% poly(vinyl alcohol) (PVA; 10 kDa) was prepared in phosphate buffer saline (PBS) and added to a chilled solution of the pentablock copolymer (4.1 wt. %) and Pluronic® F-127 (5.9 wt. %). Additional PBS containing HA, NP, and/or nanoparticles was added to the micelles resulting in a total of 25 wt. % polymer. The suspension was then sonicated for 30 s to ensure even distribution of the protein and/or nanoparticles throughout the micelle solution used. In order to improve injectability of the formulation, later experiments (Fig. 2-7) utilized a reduced concentration of polymer. The final formulation was prepared similarly with 7.5 wt. % PVA, 2.95 wt. % Pluronic® F-127 and 2.05 wt. % pentablock copolymer.

Inactivated virus

Influenza A/Puerto Rico/8/34 (H1N1) virus was purified from infected chicken embryo allantoic fluid. A sterile petri dish containing 2 mL of allantoic fluid (40,960 HAU/mL) was placed within 12 inches of a UV light inside a biosafety cabinet for 30 min. After inactivation, no evidence of infectious virus was found based on a MDCK plaque assay. UV-inactivated virus was frozen at -80°C until 1 h prior to immunizations, at which point the UV-inactivated virus was thawed at room temperature, diluted 1:2.5 in physiological saline, and kept on ice until administration.

Mice

Female (6-8 week old) BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). Aged (~18 mo) mice were obtained from the National Institute of Aging (Bethesda, MD) or Charles River. All mice were housed under specific pathogen-free conditions where all bedding, caging, water, and feed were sterilized before use. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Iowa State University and approved by the Iowa State University Institutional Animal Care and Use Committee.

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Immunizations

All mice were immunized subcutaneously with a total of 20 µg HA and 20 µg NP. In experiments using a two dose regimen (Fig. 1, 2, and 4), mice were immunized twice (day 0 and 21) with the following: Saline, Soluble (10 µg HA + 10 µg NP in PBS), Nanoparticle (5 µg HA + 5 µg NP encapsulated in 500 µg nanoparticles co-delivered with 5 µg HA + 5 µg NP in PBS), Micelle (10 µg HA + 10 µg NP in 100 µL of micelles), and Combination Nanoadjuvants (5 µg HA + 5 µg NP encapsulated in 500 µg nanoparticles co-delivered with 5 µg HA + 5 µg NP in 100 µL of micelles). In experiments using a single dose regimen (Fig. 2-4) mice were administered the following: Combination Nanovaccine (5 µg HA + 5 µg NP encapsulated in 500 µg nanoparticles co-delivered with 15 µg HA + 15 µg NP in 100 µL of micelles), inactivated virus (50 µL containing 819 HAU administered intramuscularly into the rear flank), or saline. In the study demonstrating vaccine efficacy in aged mice (Fig. 7), 5 µg of cyclic dinucleotides (CDNs; dithio-RP,RP-cyclic di-guanosine monophosphate) provided by Aduro Biotech (Berkeley, CA) were included in the combination nanovaccine.

Antibody titers

Antibody titers were determined using an enzyme-linked immunosorbent assay (ELISA). First, 100 µL of blood was isolated from the saphenous vein of each mouse and centrifuged for 10 min at 10,000 x g. The serum was separated and stored at 4°C until further analysis. High binding, flat-bottomed 96 well plates were coated with 100 µL/well of 0.5 µg/mL HA or NP protein in PBS. After incubating overnight at 4°C, the wells were emptied and blocked for 2 h at room temperature with 2% (w/v) gelatin in PBS with 0.05% Tween (PBS-T). Next, the plates were washed three times with PBS-T before adding 100 µL/well of PBS-T + 1% goat serum. Serum samples from immunized mice were added to each well beginning with a 1:200 dilution and performing 1:2 serial dilutions across the plate. After incubating overnight at 4°C, the plates were washed three times with PBS-T and incubated for 2 h at room temperature with 100 µL/well of 1:1000 Alkaline Phosphatase-conjugated anti-mouse IgG H&L (Jackson ImmunoResearch, West Grove, PA). The plates were again washed with PBS-T before adding 100 µL/well of substrate buffer (50 mM sodium carbonate, 2 mM magnesium chloride, pH 9.3) containing 1 mg/mL p-nitrophenylphosphate (Fisher Scientific, Pittsburgh, PA). The optical density (405 nm) was recorded after 30 min. Titer was defined as the reciprocal of the last serum dilution that produced an optical density greater than twice the value of background (i.e., average optical density of saline administered mice).

Virus microneutralization

Serum samples were diluted 1:3 with receptor destroying enzyme (RDE; Denka Seiken, Tokyo, Japan) diluted according to the manufacturer's instructions and incubated in a water bath at 37°C overnight. Next, the samples were heat inactivated by incubating in a water bath at 56°C for 1 h before adding media (DMEM with 1% Pen/Strep, Invitrogen, Carlsbad, CA) to bring the final serum dilution to 1:20. The serum samples (25 µL/well) were subsequently added to 96 well, round bottom plates in duplicate and 1:2 serial dilutions were performed down each column. Next, 25 µL of virus stock (A/PR/8/34; 2,000 $TCID_{50}$/mL) were added to each well. After incubating for 1 h at 37°C, the samples were transferred to 96 well plates seeded with 90% confluent Madin-Darby Canine Kidney (MDCK; BEI Resources, Manassas, VA) cells. The cells were incubated for 1 h at 37°C before washing with HBSS (Invitrogen) and adding 100 µL media supplemented with 10% FBS and 1 µg/mL TPK-trypsin (Fisher Scientific, Waltham, MA). After three days of incubation (37°C, 5% CO_2), 50 µL of each well was transferred to a 96 well, V-bottom plate. Next, 50 µL of 0.75% turkey red bloods cells (Lampire, Hershey, PA) in HBSS was added to each well. The plates were incubated at room temperature for 45 min before reading the plates. The microneutralization titer was taken to be the last dilution of serum with the appearance of a button.

Virus challenge

Original influenza virus stocks (A/PR/8/34) were obtained from the Influenza Division of the Center for Disease Control and Prevention. The virus was propagated in the allantoic cavity of embryonated hen's eggs, harvested after three days, and purified using a discontinuous sucrose gradient. The biological activity was determined using a hemagglutination assay and diluted to 10 HAU/mL before administering to mice. In the final experiment observing the vaccine efficacy of combination nanovaccines in aged mice, the virus stock was diluted to 100 HAU/mL before administering to mice.

Mice were challenged intranasally with live virus 35-42 days following the first immunization. Briefly, mice were anesthetized in a chamber with 3% isoflurane in 100% O_2 at a flow rate of 2.5 L/min. Next, 50 µL of the prepared virus was pipetted onto the nares of the nose before recovering from anesthesia. All mice were monitored twice daily after challenge with body weight collected once per day. Animals were removed from study after losing greater than 25% their original body weight. A subset of mice was euthanized three days post-challenge for bronchoalveolar lavage (BAL) and lung tissue. The total IgG anti-HA antibody titer was determined using ELISA as described above. The lung tissue was used to determine virus load.

Virus load

Lung tissue was extracted and homogenized before quantitative reverse-transcription polymer chain reaction (qRT-PCR). A commercially available one-step real-time multiplex RT-PCR kit (VetMAX™-Gold SIV Detection Kit; Life Technologies, Austin, TX), designed to target viral matrix and nucleoprotein genes, was used to amplify influenza viral RNA. The PCR reaction was set up in a 25 µL volume containing 12.5 µL of 2X multiplex RT-PCR buffer, 1.0 µL nuclease-free water,

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1.0 µL of influenza virus primer probe mix, 2.5 µL of multiplex RT-PCR enzyme mix and 8.0 µL of RNA template (i.e., extract) or controls. Xeno™ RNA Control supplied with the kit was included as an internal control for RNA purity to assess possible PCR inhibition from samples. Influenza Virus-Xeno™ RNA Control (1000 copies/µL) included in the kit was used as a positive amplification control (PAC). Nuclease-free water was used as a no amplification control. Thermocycling was performed in a 7500 Fast PCR System (Applied Biosystems, Foster City, CA) under the following conditions: reverse transcription at 48°C for 10 min, reverse transcriptase inactivation/initial denaturation at 95°C for 10 min, and 40 cycles of amplification and extension (95°C for 15 s and 60°C for 45 s).

Samples with cycle threshold (Ct) values < 38 were recorded as positive for influenza A viral RNA, whereas samples with Ct values > 38 were recorded as negative as per manufacturer's instructions. However, the data were collected until the end of run (40 cycles) and viral titers ($TCID_{50}$/mL) appearing after the cut-off were included in the final statistical data analyses.

Characterization of aged dendritic cell response *in vitro*

Spleens from aged mice were collected after euthanasia following IACUC protocols. Single cell suspensions of spleen cells were isolated after treatment with Collagenase D and mechanical grinding of the spleens with RPMI media. A pan dendritic cells (DCs) negative selection kit (Miltenyi Biotech, Bergisch Gladbach, Germany) was used to isolate $CD11c^+$ DCs from the total spleen cells using the manufacturer's protocol. Briefly, the spleen cells were incubated with the antibody cocktail and magnetic microbeads provided in the kit and run through AutoMACS ProSeparator (Miltenyi Biotech) to collect the negative selection pool of $CD11c^+$ DCs. Following isolation, $2.5x10^6$ - $5x10^6$ spleen DCs were stimulated for 48 h with the following treatment groups: nanoparticles (500 µg/mL), micelles (12.5 µg/mL), LPS (1 µg/mL) and unstimulated control with or without a soluble model antigen (ovalbumin; sOva, 20 µg/mL).

Following stimulation, the upregulation of costimulatory molecules were evaluated using flow cytometry. DCs were stained with antibodies for different co-stimulatory molecules, namely, CD80 (Biolegend, PerCP-Cy5.5, clone 16-10A1), CD11c (Biolegend, APC-Cy7, clone N418), MHCII (eBioscience, AF700, clone M5/114.15.2), CD86 (eBioscience, FITC, clone GL1) and CD40 (eBioscience, APC, clone 1C10). The cells were blocked prior to labelling by using 100 µg/mL of rat IgG (Sigma Aldrich) and 10 µg/mL of anti-CD16/32 (eBioscience). Supernatants from the splenic DCs stimulated with different treatment groups were collected and analyzed for the presence of cytokines and chemokines using MILLIPLEX® MAP mouse cytokine/chemokine magnetic bead panel (Millipore Sigma, Burlington, MA) on a Bio-Plex 200 system (Bio-Rad, Hercules, CA).

Statistics

Statistical significance ($p \leq 0.05$) among treatments was determined using GraphPad (Prism 7.00, GraphPad Software, La Jolla, CA). Briefly, the Kruskal-Wallis and Mann-Whitney tests were used to compare vaccine formulations in all antibody or virus load data. A repeated measures ANOVA was used to determine significance of weight loss post-challenge. The log rank Mantel-Cox test determined significance of survival data. Finally, statistical significance among treatment groups in dendritic cell experiments was determined using a Tukey T-test.

Results

Nanoadjuvants enhanced immune response post-vaccination

To assess the combination of nanoadjuvants (i.e., nanoparticles and micelles), young (6-8 wk old) BALB/c mice were subcutaneously immunized twice (day 0 and 21) with each dose containing 10 µg of recombinant HA and 10 µg of recombinant NP derived from A/PR/8/34 virus. Serum samples were collected 35 days after the first immunization and analyzed for anti-HA antibody titers. As shown in Fig. 1A, all nanoadjuvanted formulations induced significantly greater antibody titers in comparison to soluble antigen alone. In addition, mice receiving formulations containing the pentablock copolymer micelles demonstrated the greatest mean antibody titers.

At 42 days post-immunization, the mice were intranasally challenged with live A/PR/8/34 virus. Three days post-challenge, six mice within each group were euthanized and their lung viral load was quantified. The lungs of mice administered the nanoadjuvant formulations showed a significant reduction in viral load, with the lungs of animals administered the combination nanoadjuvant showing a seven-log reduction in mean viral load compared to mice administered soluble antigen (Fig. 1B). Furthermore, no virus was detected in most of the mice (5/6) receiving the combination nanoadjuvant formulation at three days post-challenge compared to mice administered nanoparticle (2/6) or micelle (4/6) formulations alone.

Finally, the remaining mice were weighed daily for eight days post-challenge. The saline-administered mice began to lose weight three days post-challenge, and continued to lose up to 20% their original body weight (Fig. 1C). In contrast, all vaccinated mice maintained their body weight post-infection regardless of nanoadjuvant formulation used and were significantly different compared to saline controls.

Assessing the efficacy of a single dose vaccine regimen using combination nanoadjuvants

Although the combination nanoadjuvants induced robust immune responses with a prime-boost regimen in young mice, vaccines that elicit protection in a single dose are ideal. To ascertain the efficacy of a single dose combination nanoadjuvant formulation against influenza virus infection, the responses of young mice receiving two doses (day 0 and 21) or a single dose of the combination nanoadjuvant formulation

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were compared. Regardless of vaccine regimen, all mice received a total of 20 μg HA and 20 μg NP. Serum was collected at day 32 post-vaccination and analyzed for the

100% survival compared to non-vaccinated, infected control mice with approximately 40% survival (Fig. 2C).
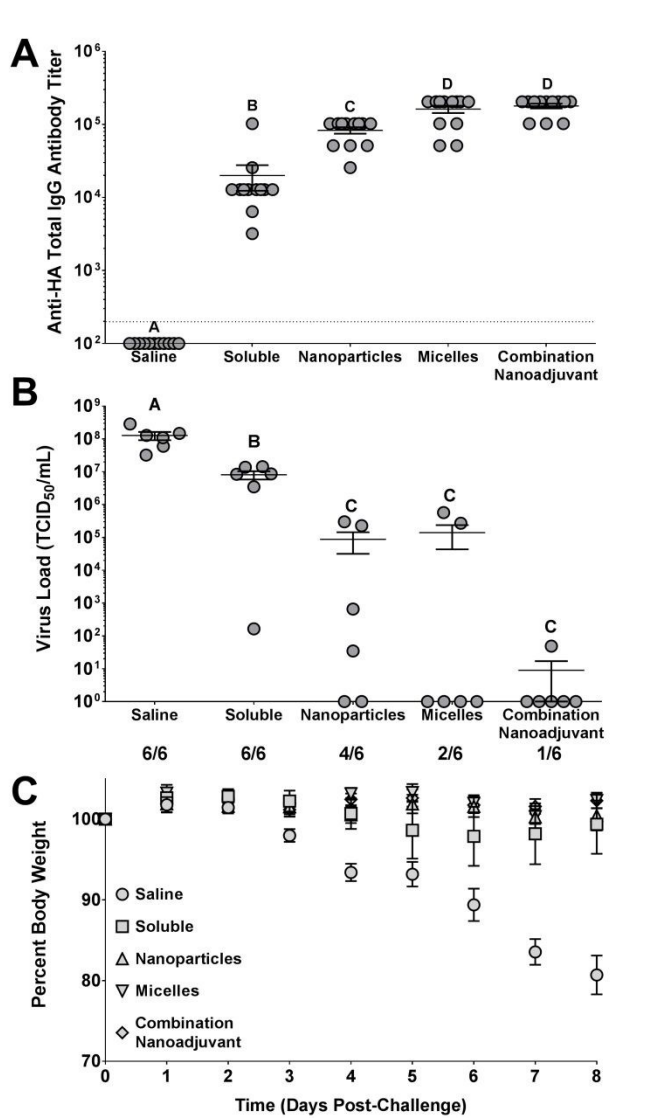
Fig. 1 Nanoadjuvants enhanced immune response after prime-boost vaccination in young mice. (A) Anti-HA total IgG antibody titers 35 days after primary immunization. Dotted line represents limit of detection. (B) Virus titers assessed three days post-challenge. Numbers below each group indicate number of mice with detectable viral load. (C) Body weight monitored for eight days post-challenge. Mice were challenge intranasally with 0.5 HAU/mouse. Statistical differences in titer were determined using Kruskal-Wallis and Mann-Whitney tests. Different letters indicate significant differences. n=12. Statistical differences in body weight were determined with a repeated measure ANOVA. n=6. $p \leq 0.05$. Error bars represent standard error of mean.

Fig. 2 Combination nanoadjuvants optimized for single dose regimen in young mice. (A) Antibody titer 32 days after immunization with a single dose or two dose (day 0 and 21) regimen. Dotted line indicates limit of detection. *indicates significant differences determined by Kruskal-Wallis and Mann-Whitney tests. (B) Body weight monitored for 14 days post-challenge. Mice were challenge intranasally with 0.5 HAU/mouse. Significant differences between single dose (*) or two dose (#) regimens and saline controls at individual time points are indicated. Different letters indicate significant differences overall as determined with a repeated measure ANOVA. (C) Survival of mice 14 days post-challenge. * indicates statistical differences via the log rank Mantel-Cox test. n=7. $p \leq 0.05$. Error bars represent standard error of mean.

presence of anti-HA and anti-NP antibodies (Fig. 2A). The young mice developed antibody responses against both HA and NP antigens, regardless of regimen.

Mice were challenged intranasally with live A/PR/8/34 at day 35, after which their body weight and survival were monitored for 14 days. Regardless of administration regimen, the young mice maintained body weight post-infection (Fig. 2B). In addition, the vaccinated young mice demonstrated
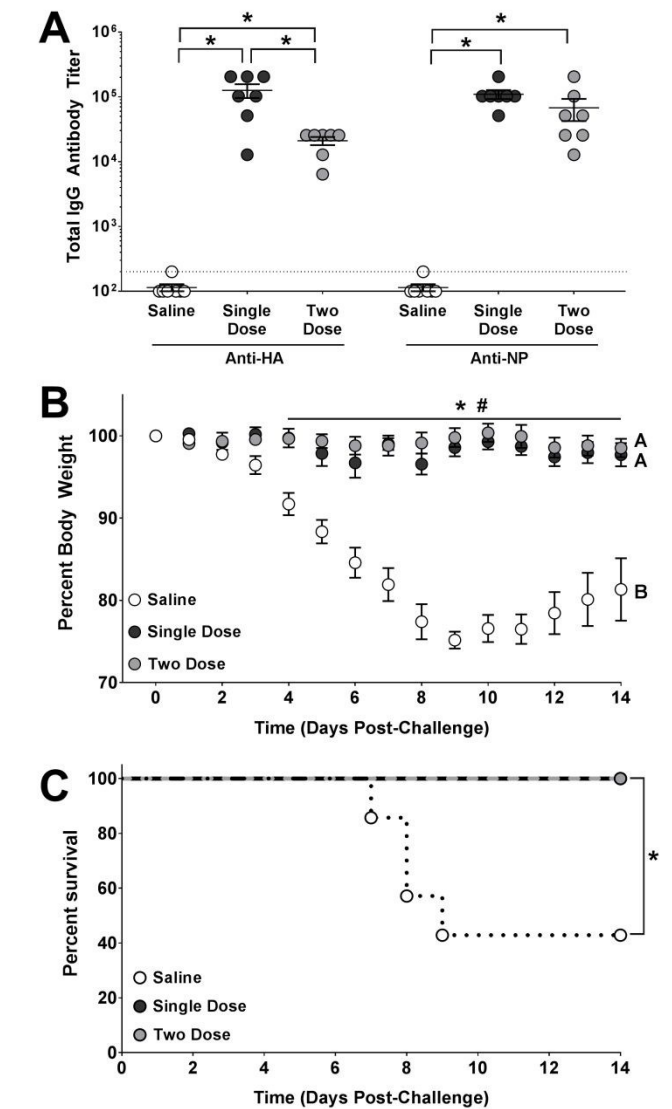
Combination nanovaccine protected young mice from IAV challenge

After demonstrating that a single dose nanoadjuvant formulation induced protection in young mice, the efficacy of the single dose combination nanovaccine was compared to that induced by inactivated influenza virus (i.e., similar to a

traditional influenza vaccines as opposed to recombinant antigens) and saline controls.

Serum microneutralization titers were measured at 35 days

Mice were challenged 42 days post-immunization and monitored for weight loss, virus load in the lungs three days post-challenge, and survival. While mice receiving saline lost
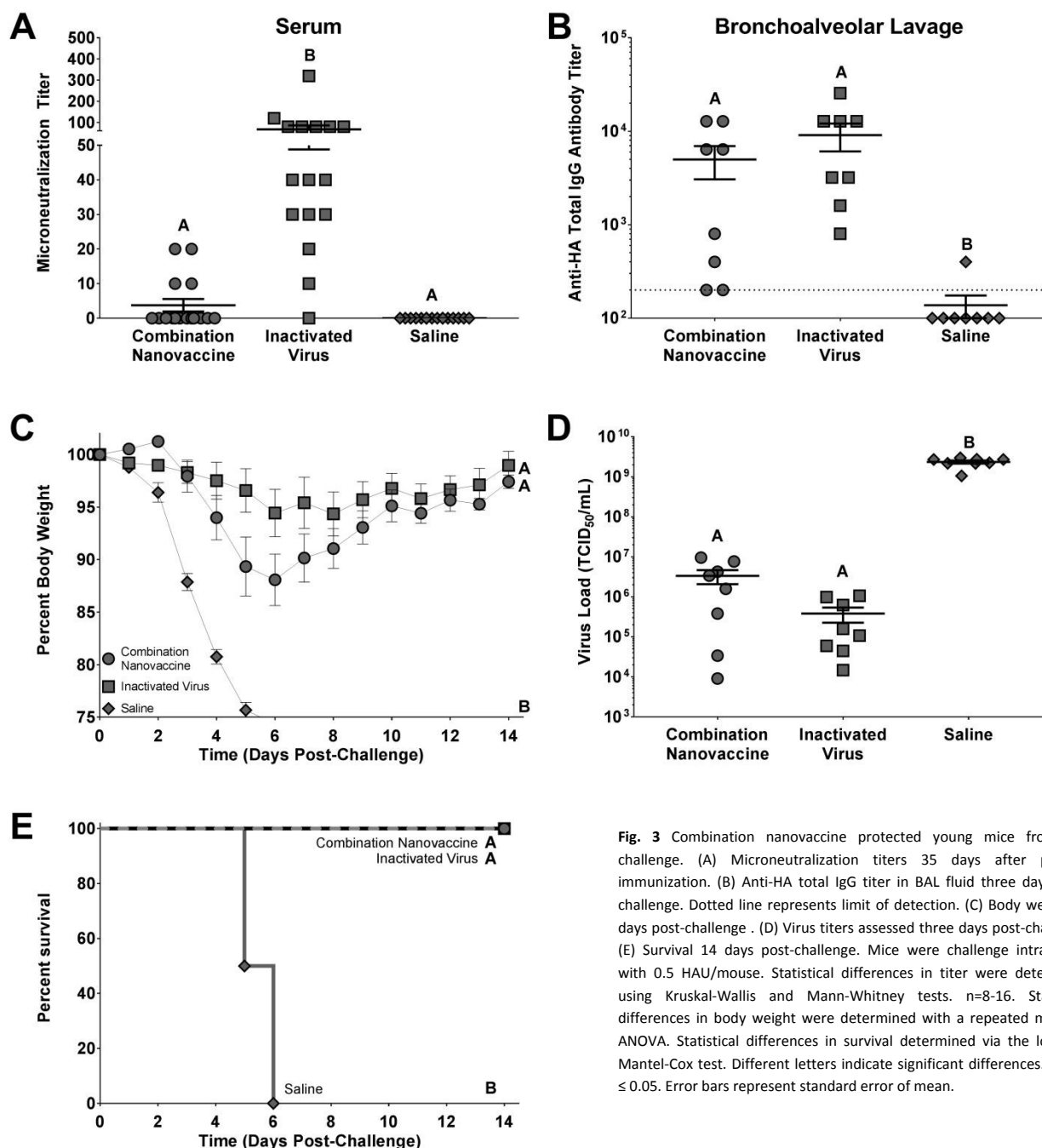
Fig. 3 Combination nanovaccine protected young mice from IAV challenge. (A) Microneutralization titers 35 days after primary immunization. (B) Anti-HA total IgG titer in BAL fluid three days post-challenge. Dotted line represents limit of detection. (C) Body weight 14 days post-challenge . (D) Virus titers assessed three days post-challenge. (E) Survival 14 days post-challenge. Mice were challenge intranasally with 0.5 HAU/mouse. Statistical differences in titer were determined using Kruskal-Wallis and Mann-Whitney tests. n=8-16. Statistical differences in body weight were determined with a repeated measure ANOVA. Statistical differences in survival determined via the log rank Mantel-Cox test. Different letters indicate significant differences. n=8. $p \leq 0.05$. Error bars represent standard error of mean.

post-immunization. While mice immunized with the inactivated IAV demonstrated a significant microneutralization response, combination nanovaccine-administered mice did not have a titer significantly greater than that of saline controls (Fig. 3A). However, when the anti-HA total IgG antibody titers were measured in BAL fluid three days post-challenge, animals immunized with either the combination nanovaccine or inactivated IAV had significantly higher anti-HA titers compared to saline controls (Fig. 3B).

25% of their body weight and were removed from study within 5-6 days, the combination nanovaccine protected mice from challenge (Fig. 3C). Weight loss in mice administered the combination nanovaccine and inactivated virus was limited to ~5-10%, and no significant differences were observed between the two groups. In addition, the combination nanovaccine and inactivated virus significantly reduced the virus load (i.e., ≥ 99%) in the lungs of mice post-challenge (Fig. 3D), and protected 100% of the mice in contrast to saline controls (Fig.

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Combination nanoadjuvant formulations for aged mice

Similar to nanoadjuvant optimization in young mice, we examined the immune responses of single and two dose regimens in aged mice. As expected, antibody production in aged mice was lower in comparison to that in young mice. While the two dose formulation induced greater anti-HA antibody production in aged mice compared to the single dose vaccine, both regimens generated similar levels of anti-NP antibody (Fig. 4A).

After virus challenge, the aged mice displayed significantly less weight loss post-challenge compared to non-immunized controls, regardless of regimen (Fig. 4B). Also, both the single dose and two dose regimens induced similar levels of protection (~85%) in aged mice (Fig. 4C).

Nanoparticles enhanced activation of dendritic cells isolated from aged mice

Following vaccination with the combination nanoadjuvants, we observed that aged mice (Fig. 4) had reduced serum antibody responses in comparison to young mice (Fig. 2). However, despite the absence of demonstrable antibody in aged mice, the combination nanovaccine formulation protected against virus challenge suggesting that other immune mechanisms (e.g., cell-mediated immunity) may be at play. Indeed, cell-mediated immunity (i.e., CD8^{+} T cells) has been suggested as a better correlate of protection in older adults compared to antibody.[31] However, it is known that age-related immune deficiencies, including poor dendritic cell (DC) activation, often result in poor cell-mediated immunity.[14] While the ability of nanoadjuvants to enhance DC activation in young mice has been demonstrated,[32, 33] we examined the ability of nanoadjuvants to activate DCs isolated from aged mice.

Splenic DCs isolated from aged mice were stimulated with the nanoadjuvants for 48 h and the upregulation of costimulatory markers was examined via flow cytometry. The upregulation of CD86, CD80, and CD40 in conventional DCs (CD11c^{+} MHCII^{+}) was found to be significantly enhanced when stimulated with nanoparticles (Fig. 5A). In contrast, we did not observe any significant upregulation of the cell surface markers when stimulated with micelles, which is consistent with our previous findings in young splenic DCs.[20] Additionally, two sub-populations of conventional DCs were analyzed: CD8α^{+} (Fig. 5B) and CD103^{+} (Fig. 5C). CD8α^{+} DCs have been shown to efficiently cross-present exogenous antigens to CD8^{+} T cells leading to an effective CTL response.[34, 35] The α4βE integrin CD103 expressed on DCs also plays an important role in the migration of DCs and the induction of T cell-mediated responses, especially in mucosal surfaces such as the lung epithelium.[36] The nanoparticles significantly enhanced the expression of co-stimulatory molecules on CD8α^{+} and CD103^{+} DCs (Fig. 5B & 5C, respectively) while micelles did not activate either DC subpopulation.

While secretion of pro-inflammatory cytokines is important for the activation of immune responses, they may prove

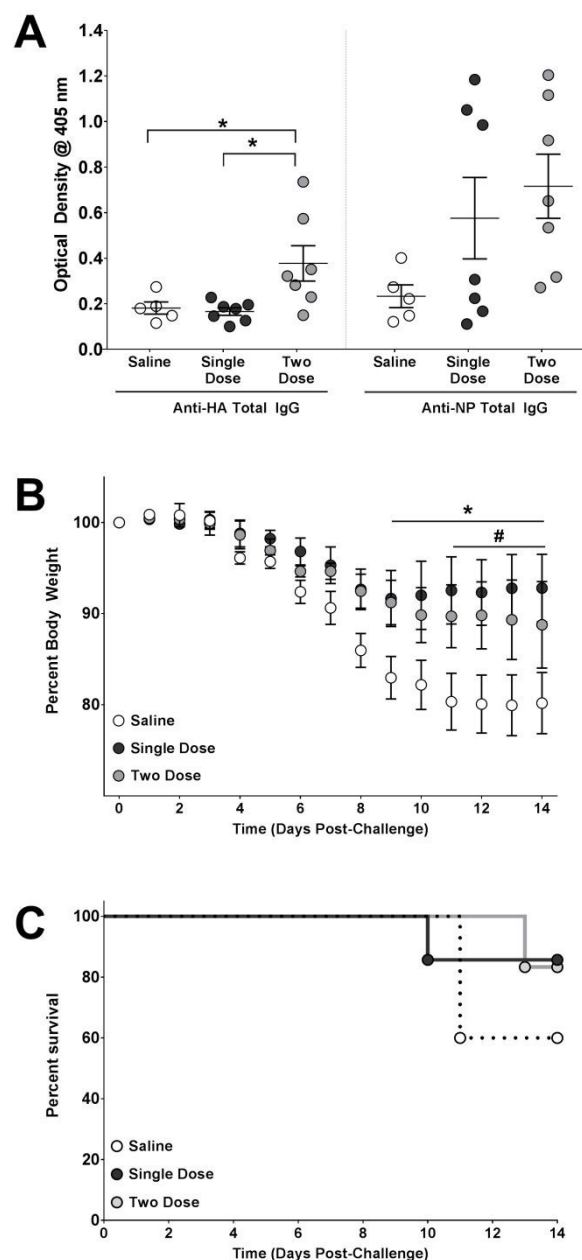detrimental in the already increased inflamed state of older

Fig. 4 Optimization of combination nanoadjuvants in aged mice. (A) Optical density of total IgG ELISA 32 days after immunization with a single dose or two dose (day 0 and 21) regimen. *indicates significant differences determined by Kruskal-Wallis and Mann-Whitney tests. (B) Body weight monitored for 14 days post-challenge. Significant differences between single dose (*) or two dose (#) regimens and saline controls at individual time points are indicated. No statistical significance observed overall. (C) Survival of aged mice 14 days post-challenge. No significant differences observed. Mice were challenge intranasally with 0.5 HAU/mouse. n=7. $p \leq 0.05$. Error bars represent standard error of mean.

adults (i.e., "inflamm-aging"). The supernatants harvested from DCs recovered from aged mice were examined for secretion of cytokines. The data demonstrated DCs from aged mice stimulated with nanoparticles or micelles did not significantly increase the secretion of pro-inflammatory

cytokines (Fig. 6A & B, respectively). In contrast, LPS (i.e., TLR4 agonist) significantly increased production of cytokines such as

minimal weight loss observed in control aged mice (Fig. 4), the virus dose was increased by ten-fold (5 HAU) to examine
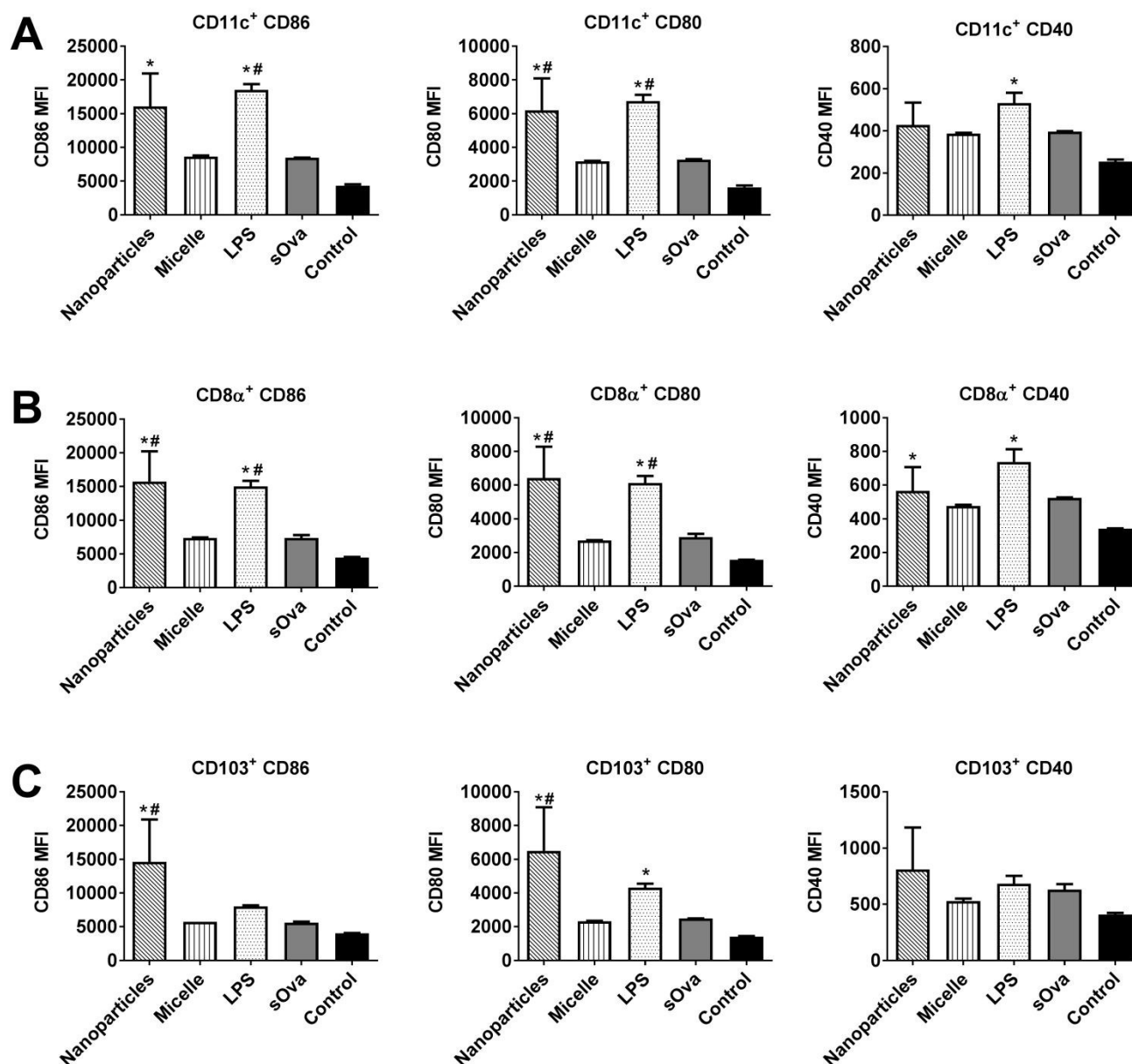
Fig. 5 Nanoparticles activated innate immune cells from aged mice. CD86, CD80, and CD40 mean fluorescence intensity (MFI) of (A) CD11c+, (B) CD8α+, and (C) CD103+ dendritic cells isolated from the spleens of aged mice. Statistical differences determined via Tukey t-test. * indicates significant difference compared to unstimulated control. # indicates significant difference compared to sOva. n=3. $p \leq 0.05$. Error bars represent standard error of mean.

IL-1β, IL-6, and TNFα.

Combination nanovaccine protected aged mice from IAV challenge

The nanoadjuvants were shown to stimulate to DCs from aged mice (Fig. 5&6), however, we noted that the antibody response of aged mice was not robust (Fig. 4). In this regard, CDNs have been shown to induce robust and long-lasting antibody titers following a single immunization.[23] Thus, CDNs (i.e., a STING agonist) was included in the combination nanoadjuvant formulation in order to improve the immune response following administration in aged mice. Due to the

vaccine efficacy.

Serum was collected from aged mice 35 days post-immunization and examined for the presence of anti-HA and anti-NP antibody titers. While the combination nanovaccine + CDN formulation and inactivated IAV induced significant anti-HA IgG antibody titers, the responses to NP were minimal to none (Fig. 7A&B). Although no significant differences were observed in microneutralization titers, the combination nanovaccine + CDN formulation induced an average titer that was an order of magnitude greater than that induced by the

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inactivated IAV control (Fig. 7C). Clinically, mice administered

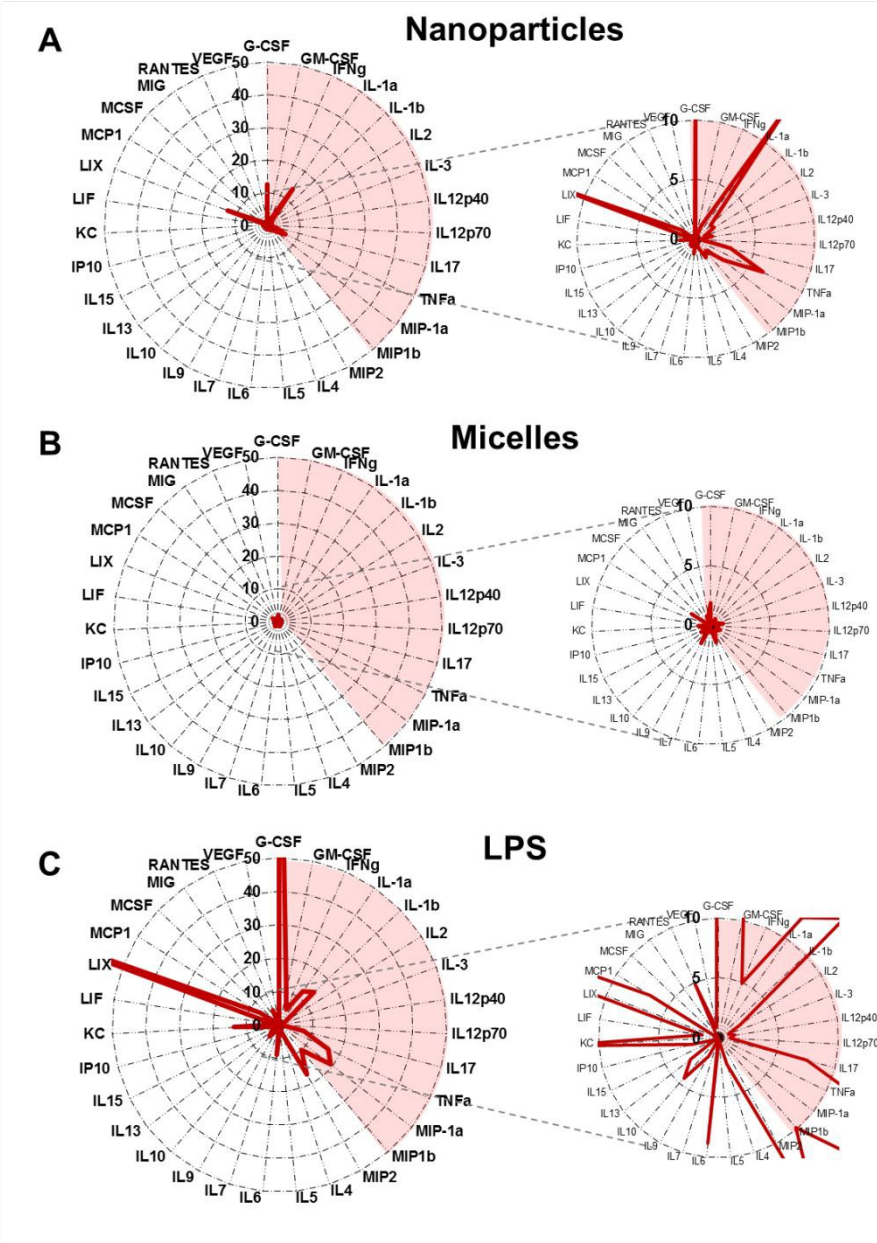previously shown success against infections with low

Fig. 6 Nanoadjuvants do not induce pro-inflammatory cytokine secretion from resting splenic DCs recovered from aged mice. Cytokine secretion (presented in red as fold change over an unstimulated control) in the supernatant of aged mice splenic DCs stimulated with (A) nanoparticles, (B) micelles, or (C) LPS. Cytokines that are considered inflammatory are shaded in pink. An expanded view of the data is presented on the right for clarification. Data was obtained from three independent pools of splenic DCs.

the combination nanovaccine + CDN formulation lost approximately 10-15% of their body weight and 60% of the mice survived the lethal challenge, as did the aged mice receiving the inactivated IAV control (Fig. 7D&E).

Discussion

Other than the use of higher dose vaccines (e.g., Fluzone High-Dose), the design and development of more effective vaccines for older adults is largely unmet. Immunosenescence in aging individuals often leads to poor immune responses after vaccination,[14, 37] and thus, novel vaccines that enhance immune responsiveness in elderly are urgently needed. While combination nanovaccines based on polyanhydride nanoparticles and pentablock copolymer micelles have

pathogenic H5N1 influenza virus in young animals,[21] the current work demonstrates the ability of nanovaccines to induce protection in both young and aged animals against seasonal H1N1 IAV.

Immunosenescence involves age-related immune deficiencies in both the innate and adaptive immune responses such as poor antigen presentation,[38] reduced T cell help,[39, 40] and low antibody production.[37] In this context, vaccine formulations composed of multiple adjuvants may engage immune signalling/activation pathways differently for optimal efficacy. For example, studies have shown elevated levels of inflammatory factors including IL-1β, TNF-α, IL-6, IL-17 in aged animals,[41, 42] which are linked to poorer vaccine
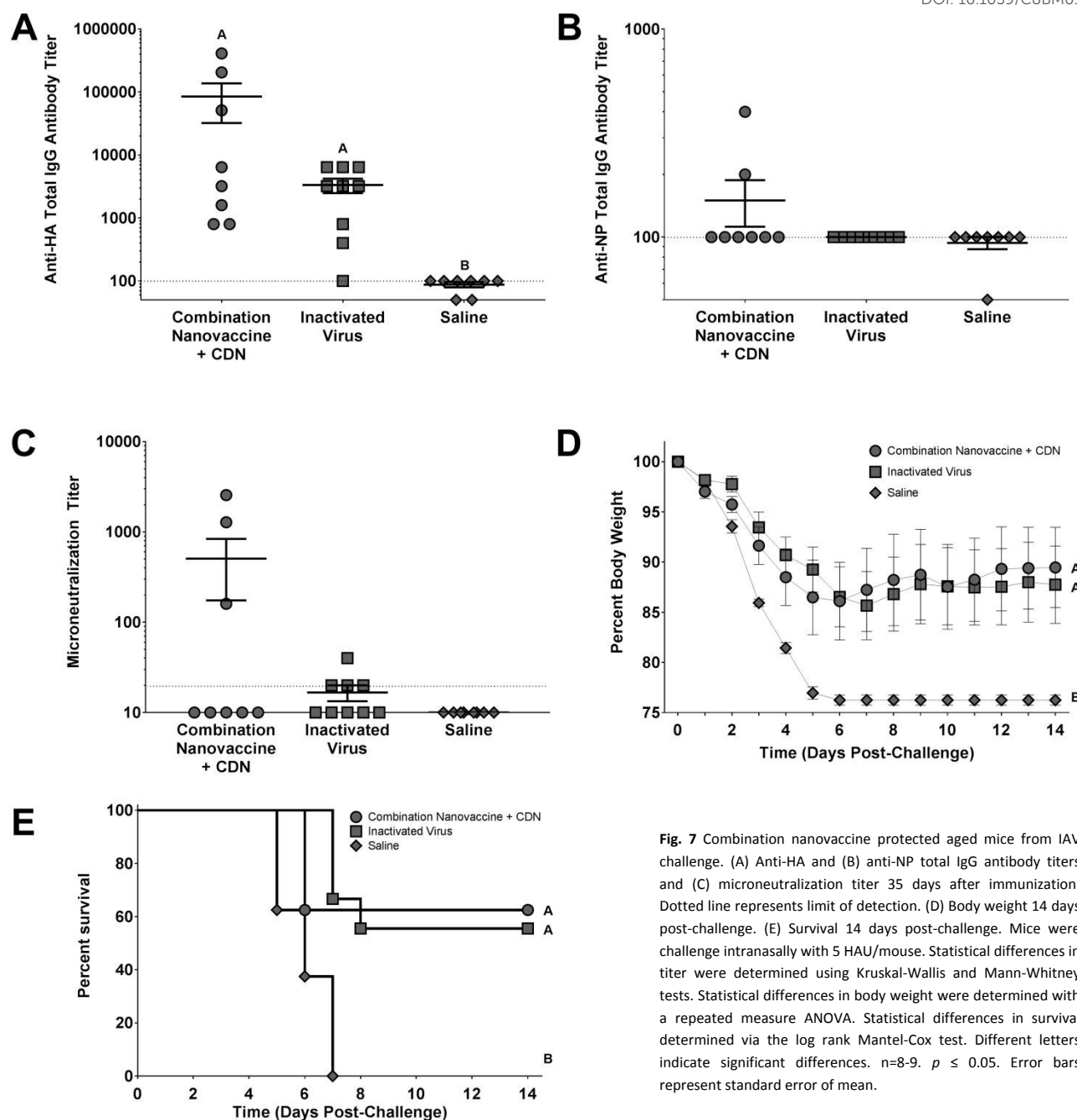
Fig. 7 Combination nanovaccine protected aged mice from IAV challenge. (A) Anti-HA and (B) anti-NP total IgG antibody titers and (C) microneutralization titer 35 days after immunization. Dotted line represents limit of detection. (D) Body weight 14 days post-challenge. (E) Survival 14 days post-challenge. Mice were challenge intranasally with 5 HAU/mouse. Statistical differences in titer were determined using Kruskal-Wallis and Mann-Whitney tests. Statistical differences in body weight were determined with a repeated measure ANOVA. Statistical differences in survival determined via the log rank Mantel-Cox test. Different letters indicate significant differences. n=8-9. $p \leq 0.05$. Error bars represent standard error of mean.

responses.[22] In addition, defects in cytokine production from DCs have been highly correlated with poor antibody responses.[43] While acute and transient inflammation is an important part of the defence and clearance of pathogens, chronic and persistent inflammation in elderly can be detrimental. An aged immune system requires a careful balancing of innate immune activation and optimal induction of adaptive immune responses. In this work, the polyanhydride nanoparticles were found to activate dendritic cells with little to no inflammation as represented by reduced pro-inflammatory cytokine secretion in comparison with a TLR4 agonist (Fig. 6). In addition, the expression of co-stimulatory

molecules such as CD80, CD86, CD40 are also known to be diminished in aged mice.[44] In this work, the nanoparticles significantly enhanced upregulation of co-stimulatory molecules in conventional, CD8α+, and CD103+ DCs (Fig. 5). Indeed, CD8α+ DCs have been shown to efficiently cross-present exogenous antigens to CD8+ T cells leading to an effective CTL response.[34, 35] Furthermore, CD103+ DCs play an important role in the uptake of antigen at the site of infection and delivery of antigen to the draining lymph node for the induction of T cell mediated responses, especially at mucosal surfaces such as the lung epithelium.[45]

In addition to enhanced DC responses, polyanhydride

nanoparticles have also been demonstrated to enhance the induction of antigen-specific cytotoxic T cells[46] and promote production of virus neutralizing antibodies.[4, 21] Likewise, pentablock copolymer micelles provide a matrix within which both antigen and nanoparticles are maintained as well as promote the rapid and sustained development of high antibody titers.[19] As a case in point, although animals administered the HA and NP in each adjuvant alone generated robust antibody titers (Fig. 1), combining the two nanoadjuvants (i.e., nanoparticles and micelles) produced the greatest mean titer and reduction in viral load in the lungs. Thus, we hypothesize that formulations utilizing combination adjuvants would effectively activate multiple immune pathways and would overcome immune deficits in older adults resulting in more efficacious vaccines.

The ability to formulate a single dose vaccine, especially for seasonal IAV vaccines, is ideal for improved patient compliance and efficiency of vaccine distribution to a naïve population. Previously, polyanhydride nanovaccines have been shown to induce elevated antibody titers with suboptimal doses of antigen.[47] However, the onset of these antibody responses are typically delayed due to the slow release kinetics of antigen from the particles. In addition, higher doses of antigen are often necessary in developing effective vaccines for older adults.[48, 49] In this work, we hypothesize that a large initial bolus of soluble antigen adjuvanted by the pentablock copolymer micelles may more effectively initiate the induction of an immune response while the sustained release of antigen from the nanoparticles promotes the maturation and development of immunological memory. In this manner, the combination nanovaccine formulated as a single dose resulted in similar efficacy as a two dose immunization regimen in both young and aged mice (Fig. 2 & 4).

Protection against IAV involves multiple immune pathways, especially in older adults, including mucosal immunity, non-neutralizing antibody, and T cell immunity. Generation of mucosal immunity is often important when considering respiratory pathogens such as IAV. Indeed, the pulmonary tract contains immunoreactive sites enriched with B and T cells that may respond quickly upon exposure.[50] Our data demonstrates that although serum antibody responses were diminished, robust titers were observed in the BAL fluid suggesting that perhaps some mucosal immunity played a role in subsequent protection from challenge (Fig. 3). In this regard, intranasal IAV nanovaccines have been shown to induce local and systemic immunity resulting in protection against both homologous and heterologous IAV challenge.[18] Thus, vaccination strategies that allow for intranasal administration of combination nanovaccines and enhance mucosal immunity in older adults may be explored in future studies.

Although anti-HA titers are important, it is known that vaccines incorporating multiple antigens, especially conserved viral proteins, may be necessary for protection against heterologous IAV strains.[51, 52] Recently, non-neutralizing antibodies in cooperation with antigen-specific CD8+ T cells were demonstrated to provide immunity against IAV challenge.[53, 54] With respect to the data presented in this work,

the aged mice generated a greater anti-NP antibody response compared to their anti-HA antibody response (Fig. 4) suggesting that while the combination nanovaccine provided protection in both young and aged mice, the mechanisms of protection in aged mice may rely on the breadth of the immune response (i.e., cell-mediated immunity).

While outside the scope of this work, it is vital to note that recent evidence suggests that T cell responses may correlate better to protection against IAV in older adults.[31] The T cells typically respond to epitopes present on conserved, internal viral proteins such as NP.[52, 55] As a result, CD8+ T cell responses play a central role in enhancing viral clearance[55] and greatly contribute to providing heterologous protection against subsequent infections.[52, 55] Therefore, vaccine formulations that enhance cell-mediated immune responses towards these conserved epitopes would be beneficial for older adults. Indeed, polyanhydride nanovaccines have previously been demonstrated to enhance cytotoxic T cell responses and promote development of immunological memory including tissue-resident memory T cells.[18, 46] In addition, while the combination nanovaccine induced low neutralizing antibody titers in young mice, 100% protection was achieved suggesting that cellular immune responses may be, in part, responsible for protection (Fig. 3). These cellular immune responses coupled with the demonstrable antibody response to NP antigen in aged mice (Fig. 4) suggest that the combination nanovaccine formulation induced cell-mediated immune mechanisms of protection to be explored in future studies.

Finally, we hypothesize that the appropriate selection of unique nanoadjuvants allows for the induction of robust immune responses in aged mice without exacerbating the state of inflammation in aged mice resulting in protective immunity against influenza virus. Indeed, the combination of multiple nanoadjuvants in this work was found to provide protection in aged mice against influenza virus as evidenced by minimizing weight loss and enhancing survival post-challenge relative to saline controls (Fig. 7). We speculate that in addition to the benefits previously discussed, the polyanhydride nanoparticles provide sustained delivery of antigen which may induce cross priming of CD8+ T cells and aid in the development of T cell memory. However, this sustained delivery of antigen often results in the delayed development of antibody responses.[56] Therefore, the inclusion of micelles and CDNs, which induce high antibody titers early post-immunization, are beneficial co-adjuvants.[19, 20, 23] Finally, the micelles may provide a depot of antigens and/or nanoadjuvants and encourage delivery of antigen to the cytosol.[20] These nanoadjuvants used concomitantly set the stage for the development of an efficacious single dose influenza virus nanovaccine for older adults.

Conclusions

Due to age-related immune deficiencies, the development of successful influenza vaccines for older adults is an unmet need. In this work, the efficacy of a combination nanovaccine based on polyanhydride nanoparticles and pentablock

copolymer micelles that contained the IAV HA and NP antigens was demonstrated in both young and aged mice. The combination of two nanoadjuvants was shown to induce elevated antibody titers resulting in reduced viral load and 100% protection in young mice. More significantly, the nanoparticles enhanced activation of DCs from aged mice, which may promote cell-mediated immune responses. Together, these data provide evidence that a combination nanovaccine platform based on pentablock copolymer micelles and polyanhydride nanoparticles is a promising technology to rationally design influenza vaccines for older adults.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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