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Vaccination against the M protein of *Streptococcus pyogenes* prevents death after influenza virus:*S. pyogenes* super-infection

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Abstract

Influenza virus infections are associated with a significant number of illnesses and deaths on an annual basis. Many of the deaths are due to complications from secondary bacterial invaders, including *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Streptococcus pyogenes*. The β-hemolytic bacteria *S. pyogenes* colonizes both skin and respiratory surfaces, and frequently presents clinically as strep throat or impetigo. However, when these bacteria gain access to normally sterile sites, they can cause deadly diseases including sepsis, necrotizing fasciitis, and pneumonia. We previously developed a model of influenza virus:*S. pyogenes* super-infection, which we used to demonstrate that vaccination against influenza virus can limit deaths associated with a secondary bacterial infection, but this protection was not complete. In the current study, we evaluated the efficacy of a vaccine that targets the M protein of *S. pyogenes* to determine whether immunity toward the bacteria alone would allow the host to survive an influenza virus:*S. pyogenes* super-infection. Our data demonstrate that vaccination against the M protein induces IgG antibodies, in particular those of the IgG1 and IgG2a isotypes, and that these antibodies can interact with macrophages. Ultimately, this vaccine-induced immunity eliminated death within our influenza virus:*S. pyogenes* super-infection model, despite the fact that all M protein-vaccinated mice showed signs of illness following influenza virus

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Conflict of Interest

J. Dale is the inventor of certain technologies related to the development of group A streptococcal vaccines. The University of Tennessee Research Foundation has licensed the technology to Vaxent, LLC, of which J. Dale is a member and Chief Scientific Officer. All other authors declare that they have no financial conflict of interest to disclose.

inoculation. These findings identify immunity against bacteria as an important component of protection against influenza virus:bacteria super-infection.

1. Introduction

Streptococcus pyogenes is a β-hemolytic, Gram-positive pathogen that causes significant morbidity and mortality on an annual basis [1]. These bacteria have the ability to colonize both the skin and the respiratory tract, and cause non-invasive diseases such as pharyngitis, impetigo, and scarlet fever [2;3]. However, when these bacteria gain access to sterile sites, invasive diseases like necrotizing fasciitis, sepsis, toxic shock syndrome, and pneumonia can develop. Post-infection sequelae, including acute post-streptococcal glomerulonephritis, and acute rheumatic fever can also occur [4]. Worldwide, *S. pyogenes* is associated with approximately 500,000 deaths per year [1], with up to 1,850 deaths in the US from invasive diseases alone [5]. While, it has recently been estimated that only 11–12% of invasive *S. pyogenes* infections exhibit lower respiratory tract complications [6], there is a surprising 38% case fatality rate associated with *S. pyogenes* pneumonia [7].

It is well-accepted that influenza viruses have the ability to predispose infected hosts toward secondary bacterial complications [8], and secondary bacterial infections contributed to the excess mortality observed during past influenza pandemics [9]. Of note, during the 1918 pandemic, as many as 90% of the 40–50 million estimated deaths were due to complications associated with secondary bacterial infections [10]. These secondary bacterial infections contribute to pneumonia and influenza-related deaths during seasonal influenza epidemics as well, with the excess mortality varying from season to season [11–14]. Influenza viruses and *S. pyogenes* share a common seasonality [15–17], and vaccines against *S. pyogenes* are not currently available [18]. As such, we rely on antibiotics [19], anti-viral agents [20], and vaccines against influenza virus [21] to limit the impact of influenza virus:*S. pyogenes* super-infections. Of these, antibiotics have demonstrated an ability to prevent infection with *S. pyogenes* when given prophylactically, but this practice is typically limited to defined populations like military recruits [22].

We recently developed a model of influenza virus:*S. pyogenes* super-infection in mice, using an H3N2 influenza virus and a serotype M3 isolate of *S. pyogenes* [23]. We have been evaluating the effects of vaccination against either influenza virus or *S. pyogenes* to determine the contribution of immunity against these pathogens toward preventing mortality after super-infection. Our previous work demonstrated that vaccination against influenza virus could limit progression toward a secondary bacterial infection with *S. pyogenes*, but that the protection was not complete. These results corroborate the findings of other lab-based [15] and clinical studies [24–26] that demonstrate that vaccination against influenza viruses can limit, but not eliminate, complications from secondary bacterial infections. To date, the benefits of vaccine-induced anti-bacterial immunity toward protection within models of influenza virus:bacteria super-infection have not been completely defined.

In the current study, we tested the hypothesis that vaccine-induced immunity against the bacteria *S. pyogenes* can prevent death after influenza virus:*S. pyogenes* super-infection. In order to test this, we used a vaccine that induces immunity toward the M protein of 6

different *S. pyogenes* serotypes, including the M3 serotype used in our studies [27]. This M protein vaccine has been used in both animal studies [28] and human clinical trials [29] to demonstrate safety and efficacy, but it has not been tested in an animal model of poly-microbial infection. We show that mice vaccinated against the M protein of *S. pyogenes* are protected after super-infection, and that this protection is evident despite significant illness associated with influenza virus inoculation. These findings are discussed in the context of host immune responses that can be targeted to prevent deadly influenza virus:*S. pyogenes* super-infections.

2. Methods and Material

2.1. Mice

Adult (6–8-week-old) female BALB/cJ mice were obtained from Harlan Laboratories (Indianapolis, IN). All animal experiments were performed following the guidelines established and approved by the Animal Care and Use committee at the University of South Dakota (Vermillion, SD).

2.2. Vaccination with S. pyogenes M-protein antigens

Groups of mice were vaccinated intramuscularly (i.m.) at 21 to 28 day intervals with 30 μg of a recombinant hexavalent *S. pyogenes* M protein vaccine in a 100 μL volume [28;29]. The vaccine was delivered in 2 mg/mL Alum (General Chemical, Berkeley Heights, NJ) as an adjuvant, and control mice received Alum alone.

2.3. Serum antibody titers

Sera collected from the orbital plexus of anesthetized mice were analyzed by ELISA, as described previously [28]. For these ELISAs, 96-well plates (Becton Dickinson and Company, Franklin Lakes, NJ) were coated with 5 μg/mL of the indicated M peptides (M1, M3, M5, M6, M19, and M24) diluted in 0.1 M sodium carbonate (pH 9.8), and IgG (H+L) was detected in serum samples. ELISA plates were washed using PBS containing 0.05% Tween 20 (PBS-T), and two-fold serial dilutions of individual mouse sera were incubated for 2 hours at 37°C. Plates were washed with PBS-T, and HRP-conjugated goat anti-mouse IgG (H+L) (Sigma, St. Louis, MO) was added to each well. After washing, HRP was detected using One-Step-TMB Turbo substrate (Thermo Scientific, Rockford, IL), the OD was measured at 450 nm using a Biotek EL808 plate reader (Biotek, Winooski, VT), and end-point titers were calculated. End-point titers are presented as the reciprocal serum dilution corresponding with the last well demonstrating an OD_{450} of 0.1 in the titration curve. Serum samples that did not result in an OD of 0.1 at the starting dilution of 1:100 were assigned a titer of 50 for the purpose of graphing and statistics. Vaccination was repeated to achieve an IgG (H+L) antibody titer of 1:3200 against the M3 serotype, and 5 separate inoculations with the vaccine were required.

When IgG1 and IgG2a antibodies were detected, the ELISA used was similar to the one described previously [30], with the exception that the antigen used for coating was the hexavalent M protein vaccine [27] diluted to 5 μg/mL in 0.1 M sodium carbonate (pH 9.8). For this ELISA, alkaline phosphatase-conjugated goat anti-mouse IgG1 and IgG2a

antibodies (Southern Biotech, Birmingham, AL) were used along with 1 mg/mL *para*-Nitrophenyl phosphate substrate (Sigma), to exploit the AP:*p*NPP enzyme:substrate reaction. One hour after addition of *p*NPP substrate, OD was measured at 405 nm, and mid-point titers are presented as the reciprocal serum dilution corresponding to 50% maximal binding calculated for each individual serum sample using its respective titration curve.

2.4. Nanoparticle Synthesis, Amine Quantitation, and Protein Conjugation

Fluorescein-encapsulated silica nanoparticles were synthesized according to previously described methods with minor alterations [31]. Briefly, Fluorescein isothiocyanate (FITC) isomer I and (3-aminopropyl)triethoxysilane (APTES) were stirred in absolute ethanol, producing FITC-APTES conjugate. Concurrently, cyclohexane, Triton X-100, *n*-hexanol, and water were combined to create a water-in-oil emulsion. The FITC-APTES conjugate solution, tetraethyl orthosilicate, and 14.5 M NH_4OH were added to this micro-emulsion. After stirring, 3-(trihydroxysilyl)propyl methylphosphonate, monosodium salt, (THPMP) was added, and mixed for 24 hours at RT. Ethanol was then added to disrupt the micro-emulsions, and nanoparticles were isolated by centrifugation. Nanoparticles were washed three times in ethanol and air-dried. Nanoparticle size was measured by TEM, and the presence of surface amines was confirmed by a qualitative ninhydrin test [32]. Further quantitation of surface amino-groups was performed by adapting methods that have been described previously to analyze proteins [33]. The concentration of amino-groups present was recalculated into their molar amount per 1 mg of nanoparticles.

Protein-nanoparticle conjugates were prepared using known coupling methods [34] incorporating a stock solution of 1 mg/mL succinic anhydride in *N*,*N*-dimethylformamide, and a qualitative ninhydrin test of the resulting nanoparticles confirmed the lack of amine functionality. The resulting carboxylic acid groups were activated by reaction with 2 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 1 mg *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS) (both solutions are 1 mg/mL in 0.1 M, pH 6.0 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer). The nanoparticle solution was diluted by 1 mL with MES and centrifuged. The precipitated nanoparticles were washed with 0.1 M, pH 7.4 phosphate buffered saline (PBS) and resuspended in PBS. Approximately 1 mg of hexavalent M protein (3 mg/mL in PBS) was shaken with the nanoparticle solution for 5 hours. The protein-nanoparticle conjugates were centrifuged, and washed with PBS.

2.5. Nanoparticle-coupled cellular uptake assay

FITC-labeled nanoparticles conjugated with the hexavalent M protein vaccine were resuspended in 0.5 mL PBS containing 0.2% BSA (Sigma, St. Louis, MO), and incubated with sera for 60 minutes at 37°C. One million J774A.1 BALB/c murine macrophage cells (American Type Culture Collection, Manassas, VA) were added, and the nanoparticle:serum mixtures were incubated with these cells for 60 minutes at 37°C. Cells were washed, and uptake was quantitated using an Accuri C6 flow cytometer (Accuri Cytometers Ltd., Ann Arbor, MI), with CFlow Plus software (Accuri).

2.6. Confocal slide preparation and imaging

Cells were fixed on slides and cover slips were added prior to imaging with an Olympus Fluoview 1000 Laser Scanning Confocal Microscope (Olympus America, Inc., Center Valley, PA). Samples were scanned using a 60× 1.4 numerical aperture oil-immersion objective and 488-nm argon laser, and images were collected using FluoView software (Olympus) for 3-D reconstruction.

2.7. Influenza virus:S. pyogenes super-infection model

The influenza virus:*S. pyogenes* super-infection model used for this study was similar to the one described previously by our group [23]. The influenza virus expressing hemagglutinin (HA) and neuraminidase (NA) from A/Hong Kong/1/68-H3N2, designated HK68, was kindly provided by Jonathan A. McCullers (St. Jude Children's Research Hospital, Memphis, TN) [30;35]. Mice in both vaccine groups were first challenged intranasally (i.n.) with a sub-lethal dose of HK68 virus (0.1 LD_{50}, $10^{4.75}$ $TCID_{50}$) on day 0. On day 7 after influenza virus inoculation, mice were randomly divided into groups that received the *S. pyogenes* isolate MGAS315 (serotype M3) at either the previously calculated 0.1 LD_{50} (10^6 CFU) or subsequent log dilutions (10^5, 10^4, 10^3 CFU) of bacteria. Dilutions of either virus or bacteria were delivered in PBS via the i.n. route (100 μL). Morbidity (weight loss) and mortality (survival) were observed during the days after influenza virus inoculation.

2.8. Statistical analyses

Figures were created using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA), and data were analyzed using Statmost programs (Dataxiom Software Inc. Los Angeles). Values were accepted as significant if $P<0.05$.

3. Results

3.1. Vaccination using hexavalent M vaccine induces antibodies toward S. pyogenes M serotype variants

Mice vaccinated with the hexavalent M protein vaccine (Figure 1) showed increased IgG (H+L) antibody titers against the M3 and M24 peptides, with optimal seropositivity (100% of titers being 1:100 or above), and the highest mean titers observed. Antibody titers against the M5 (39% seropositive), M6 (36% seropositive), M19 (64% seropositive), and M1 (75% seropositive) peptides were suboptimal in both the percent seropositivity and the mean titers. Despite this, antibody titers against the M5 peptide were the only ones that failed to achieve a significant increase ($P>0.05$) in vaccinated animals, when compared to unvaccinated mice. From this, we conclude that the hexavalent M protein vaccine was immunogenic in our mouse model, with 100% of vaccinated mice being seropositive toward the M3 protein expressed by MGAS315 [23]. To further evaluate the antibody-mediated immune response induced by vaccination, we measured the amount of serum IgG1 and IgG2a antibody isotypes against the hexavalent vaccine (Figure 2). Similar to results from total IgG ELISAs, antibodies of both the IgG1 and IgG2a isotypes were below the detectable limit in sera obtained from unvaccinated mice, while sera from vaccinated mice had significantly increased levels of hexavalent protein-reactive antibodies for both isotypes ($P<0.05$).

3.2. Antibodies against the S. pyogenes M protein enhance nanoparticle uptake by murine macrophages

The hexavalent vaccine used in this study was specifically designed to induce antibodies against opsonic epitopes from selected M proteins [18;36]. Detection of antibodies by ELISA demonstrated the specificity of these antibodies against individual M peptide antigens via their Fab region. However, to demonstrate that the Fc portions of these antibodies have the ability to enhance host effector responses, we developed an assay to measure uptake by macrophages. To achieve this, we coupled the hexavalent M protein construct to SiO_2 nanoparticles labeled with the fluorescent dye FITC via sulfo-NHS-activated carboxyl groups on the nanoparticles (Figure 3). These nanoparticles were 75–100 nm, as determined with TEM (Figure 3A).

Uptake of nanoparticles by a murine macrophage cell line (J774A.1) was quantitated using flow cytometry. Using both pooled (Figure 3B) and individual serum samples (Figure 3C), we found that fluorescent intensity was significantly increased in the presence of sera from vaccinated mice compared to sera from unvaccinated animals ($P<0.05$). As expected, fluorescence in the group that included nanoparticles pre-incubated with sera from unvaccinated mice demonstrated a mean intensity that was similar to that observed with cells incubated with nanoparticles alone. These data showed that vaccine-induced antibodies against the hexavalent M protein vaccine, which includes the M3 expressed by MGAS315, can bind to protein-conjugated nanoparticles and promote interactions with murine macrophages. Efforts to detect similar uptake using individual M3 peptides were not successful (data not shown), which we attributed to reduced availability of epitopes on peptide constructs bound to SiO_2 nanoparticles. In order to determine if the nanoparticles were internalized by macrophages, we used confocal microscopy (Figure 4, Supplemental Figure 1, and Supplemental Figure 2), which showed internalization in the presence of sera from vaccinated mice.

3.3. Vaccination against the S. pyogenes M protein prevents illness and eliminates mortality during an influenza virus:S. pyogenes super-infection

Previous work by our group [23;37] and others [15] demonstrated that vaccination against influenza virus could decrease, but not eliminate, death in models of influenza virus:bacteria super-infection. Specifically, despite the absence of weight loss following inoculation with influenza virus, overall survival was only 67–75% after inoculation of bacteria, depending on whether a formalin-inactivated or live, attenuated form of the vaccine was used. In the current study, we evaluated the impact of anti-M protein immunity toward preventing morbidity (Figure 5) and mortality (Figure 6) after super-infection. To do so, mice were inoculated with a sub-lethal dose of influenza virus (0.1 LD_{50}) seven days prior to secondary infection with one of four sub-lethal doses of *S. pyogenes* (10^6–10^3 CFU).

Mice in both the unvaccinated and vaccinated groups lost weight after influenza virus challenge, as expected [23]. Upon inoculation with MGAS315 at day 7, unvaccinated animals experienced a second period of weight loss that varied based on the dose of *S. pyogenes* delivered. These data demonstrate that weight loss was challenge dose-dependent in unvaccinated mice. Vaccinated mice lost weight, but only among mice receiving the two

highest doses of MGAS315 (10^5 and 10^6 CFU). The LD_{50} for unvaccinated mice was 7.4×10^4 CFU *S. pyogenes*, which was associated with 58% survival across all four challenge doses. In contrast, all the mice vaccinated against the M protein of *S. pyogenes* survived, resulting in an LD_{50} that was greater than the highest dose of bacteria delivered (10^6 CFU *S. pyogenes*).

4. Discussion

In this study, we tested the hypothesis that vaccine-induced immunity against *S. pyogenes* infection will prevent death in an influenza virus:*S. pyogenes* super-infection model. Our data show that a strong IgG antibody response against the streptococcal M protein, including antibodies of the IgG1 and IgG2a isotypes, fully protected against death. Importantly, the antibodies induced by vaccination also interacted with macrophages to promote uptake of hexavalent M protein-coated nanoparticles. Our findings are important for future consideration of anti-bacterial vaccination as a method toward limiting severe illnesses and deaths during influenza virus epidemics and pandemics, and provide evidence that a vaccine targeting the M protein of *S. pyogenes* would impact the incidence of diseases associated with this pathogen.

Vaccines to prevent erysipelas and scarlet fever can be traced back to the early 1900's [38;39]. Early studies by Rebecca Lancefield demonstrated immunogenic differences between *S. pyogenes* strains [40], in particular those associated with expression of the M protein (encoded by the *emm* gene) [41]. To date, over 120 M serotypes have been identified [42], although the majority of invasive diseases are associated with a more limited repertoire of M serotypes [43]. The M protein is a key antigen expressed by *S. pyogenes* and it has the ability to promote resistance to phagocytosis [44;45] while also being the antigen toward which antibody-mediated protective immunity is directed [46–49]. It is worth noting that during the influenza virus pandemic of 1918, whole-cell vaccines against bacteria were used to limit complications with secondary bacterial pneumonia, and there is evidence that deaths were prevented using this approach [50]. In fact, the manuscript by Chien et al. [50] cites a report from 1919 where a vaccine specifically designed to target hemolytic streptococci showed 100% protection against death in 144 vaccinated individuals that were infected with influenza virus [51]. Using more modern approaches, we show that a recombinant polypeptide vaccine against the M protein can provide complete protection against mortality in a model of influenza virus:*S. pyogenes* super-infection.

Our influenza virus:*S. pyogenes* super-infection model yields high mortality [23], which allows for a stringent test of vaccine-induced protection in the context of *S. pyogenes* infection. Here we report that vaccine-induced antibodies against the *S. pyogenes* M protein protect against an otherwise fatal influenza virus:*S. pyogenes* super-infection. Our data showed that IgG antibodies were induced by this vaccine, including those of the IgG1 and IgG2a isotypes, and that these antibodies are able to interact with murine macrophages, possibly using Fc receptors [52]. Variability in the antibody response against the individual M peptides has been reported previously in BALB/c mice [28] after vaccination with the hexavalent M protein construct used in this study. Since the vaccine administered was protein-based, and delivered to BALB/cJ mice with Alum as an adjuvant, a bias toward a

Th2-like, IgG1 antibody response could be expected [53]. However, our data also show a significant increase in IgG2a antibodies after vaccination, indicating that Th1-like immunity could be induced by this vaccine as well [54]. The importance of host:Fc interactions in the pathogenesis of *S. pyogenes* can be seen in the evolution of methods for masking the Fc portion of host antibodies by variants of this bacterial species. These include expression of proteins that either bind the Fc portion of antibodies, like Protein H [55] and Protein I [56], or degrade the Fc region directly, like EndoS [57] and IdeS [58;59]. Interestingly, a recent study demonstrated that antibody interaction with *S. pyogenes* could dictate the distribution of bacteria during an invasive *S. pyogenes* infection [60].

The protection that we observed using the hexavalent M protein vaccine was superior to the vaccine-induced protection that we previously reported in animals that were vaccinated against influenza virus [23]. This finding was surprising because all of the *S. pyogenes* M protein-vaccinated mice were susceptible to influenza virus infection prior to inoculation with bacteria. These findings indicate that this vaccine-induced immunity against *S. pyogenes* M protein can overcome influenza virus-mediated defects in host immune responses [8], ultimately protecting against secondary bacterial complications. To date, the literature describing clearance of *S. pyogenes* indicates that interactions with antibodies, complement proteins, and phagocytic cells (neutrophils and macrophages) are associated with protective immunity [49;61–63]. However, at the current time, immune correlates of protection against *S. pyogenes* are not well-defined [64]. This is in contrast to the influenza virus vaccine field where an antibody titer of 1:40, detected using the hemagglutination inhibiton assay, is an accepted correlate of protection [9;65]. These vaccine-induced correlates of protection are typically defined using multiple human vaccine studies [66;67], which have not been performed for these M protein vaccines [18]. Future evaluation of the protective response in our model will focus on understanding the specific antibody:host interactions that can result in protection, even within a virus-weakened host. The results from our *ex vivo* uptake assay provide evidence that Fc receptor interactions with murine macrophages may play a role [68;69], and future studies will evaluate the contribution of these cells toward the protection observed *in vivo*.

In addition, future work will consider the contributions of neutrophils [70;71] and complement components [72], toward protection, as well as the potential breadth of vaccine-induced immunity against distinct M peptides, which will require further optimization of the nanoparticle uptake assay. It is worth noting that when vaccines against influenza virus [23] and *S. pyogenes* M proteins (data presented here) were delivered individually, the influenza vaccine impacted body weight after influenza virus challenge, while the benefits of the hexavalent M protein vaccine were seen in survival after inoculation with *S. pyogenes*. Based on these findings, one would expect that vaccinating against both pathogens would yield optimal immunity as evidenced by lack of morbidity (weight loss) and mortality throughout the course of a super-infection. This situation is relevant to the clinical application of vaccines against *S. pyogenes* M protein, and can also be explored in future laboratory and clinical studies.

Historically, many labs studying super-infections evaluate the influenza virus-mediated effects on host immunity that predispose toward death, using either *S. pneumoniae* [73;74]

or *Staphylococcus aureus* [75–78] as secondary invaders [8]. The information obtained from these published super-infection models can be used to identify host responses toward influenza viruses that may allow for increased susceptibility to super-infection with *S. pyogenes*. These can include influenza virus-associated effects on epithelial cells [79;80], macrophages [81–84], and/or neutrophils [85;86], which combine to enhance bacterial pathogenesis. However, since *S. pyogenes* establishes distinct clinical diseases, and has unique pathogenic properties [87;88], future work is needed to consider this bacteria individually for its contribution to secondary infections [89;90].

Our results show that antibodies induced in mice after vaccination against *S. pyogenes* M protein can recognize the M protein vaccine linked to nanoparticles and induce uptake of these nanoparticles by a murine macrophage cell line (J774A.1). These experiments were designed to use M protein vaccine-coated nanoparticles as a replacement for viable streptococci in the classic bactericidal test of Lancefield [48]. Since the macrophage is only one cellular component associated with protection of *S. pyogenes* [91], there is more work required to define the immune mechanisms associated with vaccine-induced protection in our model. However, our previous evaluation of vaccine-induced correlates of protective immunity against influenza viruses has indicated contributions of Fc receptors, macrophages [92], and distinct IgG isotypes [30] toward influenza virus clearance. Applying this knowledge of immune function to *S. pyogenes* M protein:antibody interactions, we hypothesize that the clearance observed in our model is optimized by Fab recognition of the M protein epitopes [27] in conjunction with Fc binding to Fc receptors expressed by host effector cells [93]. This situation would allow the antibodies induced by vaccination with an M protein vaccine to link the infecting *S. pyogenes* isolate to host phagocytic cells, resulting in bacterial clearance despite defects in host immunity associated with antecedent influenza virus infection. It is worth noting that the study by Ely et al. [51] reported 100% protection against hemolytic streptococcus in influenza virus-infected humans that had been vaccinated against these bacteria. Similarly, more than 90 years later, we present a benefit associated with vaccine-induced anti-bacterial immunity toward preventing mortality in a model of influenza virus:bacteria super-infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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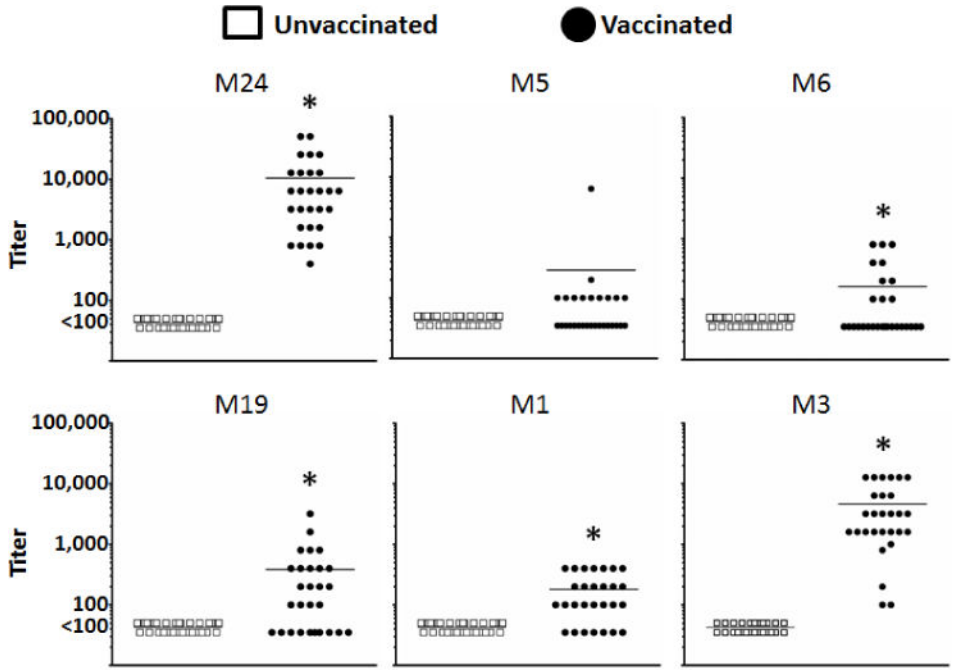

Figure 1.

Serum IgG antibody titers after vaccination with the hexavalent M protein vaccine. Mice (n=28 per group) were vaccinated with either the hexavalent M vaccine or alum, as a control (unvaccinated). Each data point represents the end-point titer of an individual serum sample against the designated M peptides (M24, M5, M6, M19, M1, or M3), individually. The mean titers of unvaccinated and vaccinated sera are represented as a single bar. *Indicates a significant difference between the vaccinated and unvaccinated sera ($P<0.05$) using a paired, two-tailed Student's t-test.

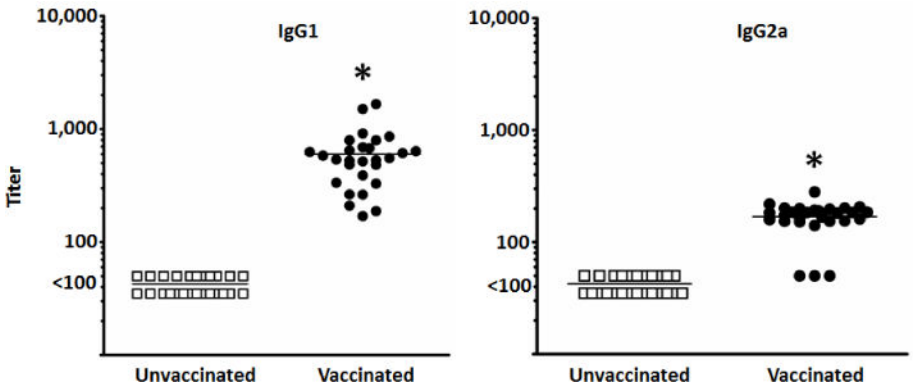

Figure 2.

Serum IgG1 and IgG2a antibody titers after vaccination with the hexavalent M protein vaccine. Mice (n=28 per group) were either vaccinated with the hexavalent M vaccine or alum, as a control (unvaccinated). Each data point represents the 50% maximal binding titer of an individual serum sample against the hexavalent M protein. The mean titers of unvaccinated and vaccinated sera are represented as a single bar. *Indicates a significant difference between the vaccinated and unvaccinated sera ($P<0.05$) using a paired, two-tailed Student's t-test.

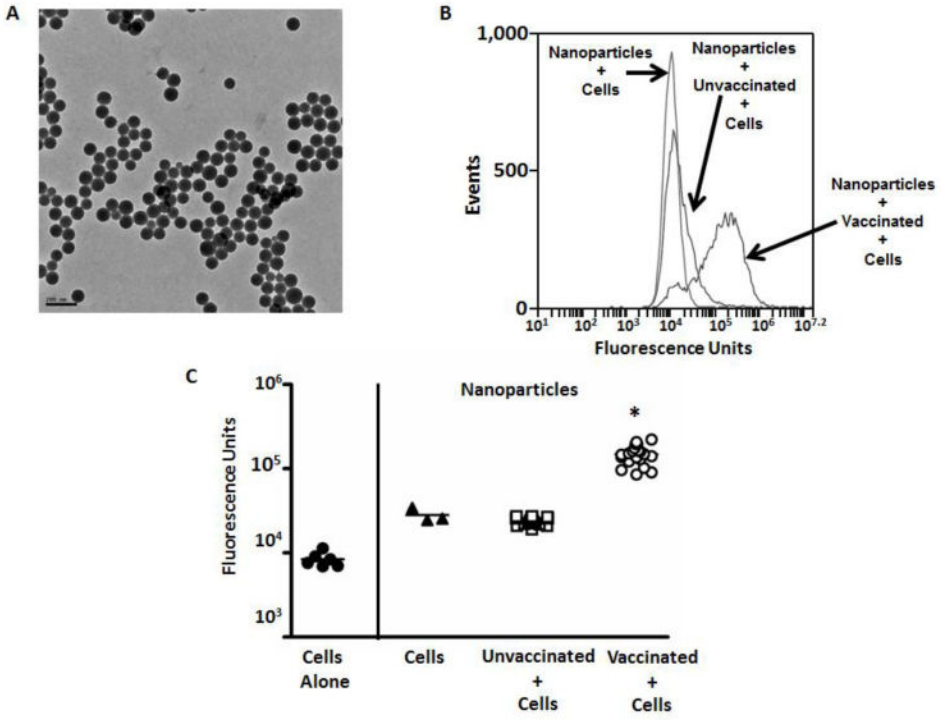

Figure 3.

Serum-mediated uptake of FITC-labeled SiO_2 nanoparticles that were linked to the hexavalent M protein. TEM images of the nanoparticles (A) are presented, with the bar indicating 0.2 μm. Uptake of fluorescent nanoparticles was quantitated using flow cytometry, and results for pooled sera (B) and individual sera (C) are presented. *Indicates a significant difference between the sera from vaccinated and unvaccinated animals ($P<0.05$) using one way ANOVA and *post hoc* Tukey's multiple comparison test.

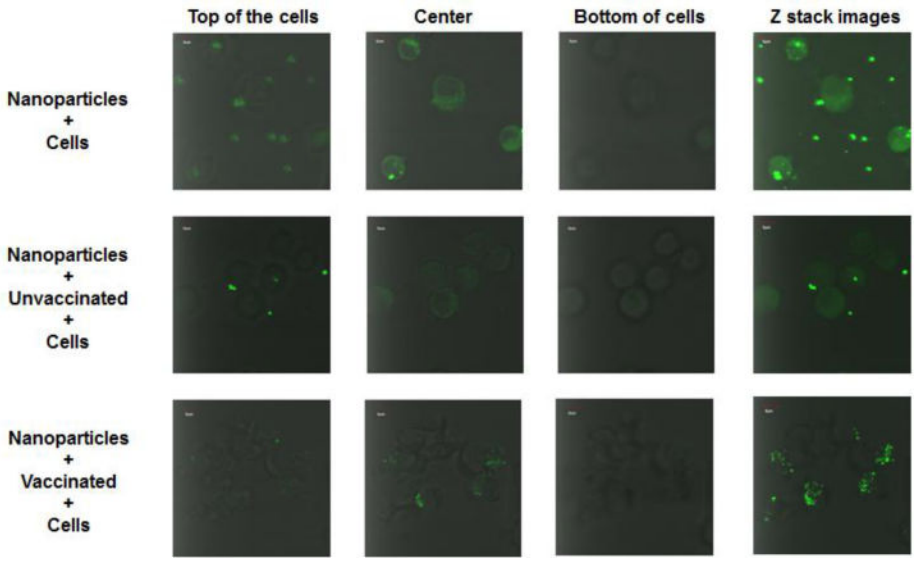

Figure 4.

Serum-mediated uptake of FITC-labeled SiO_2 nanoparticles that were linked to the hexavalent M protein, visualized using confocal microscopy. Confocal microscopy was used to visualize the uptake of fluorescent nanoparticles by J774A.1 macrophages. Images were taken from the top, center, and bottom of the cells. The entire stack of images in the Z plane are also presented.

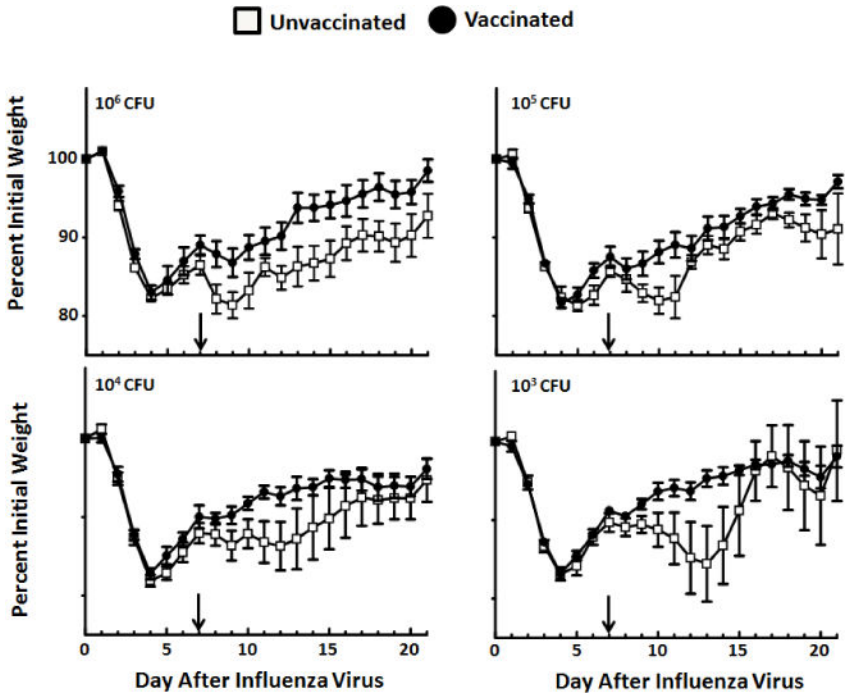

Figure 5.

Body weight after influenza virus:*S. pyogenes* super-infection. Mice from each vaccine group (n = 28 for vaccinated and n = 28 for unvaccinated) were randomly separated for challenge with *S. pyogenes* at four different sub-lethal doses (10^3–10^6 CFU). Each challenge dose included 7 mice per vaccine group. The arrow within the figure represents the day of MGAS315 infection (day 7 after influenza virus challenge). Weights are calculated as percent initial body weight for each individual mouse, with the mean ± SEM presented for each day after influenza virus infection.

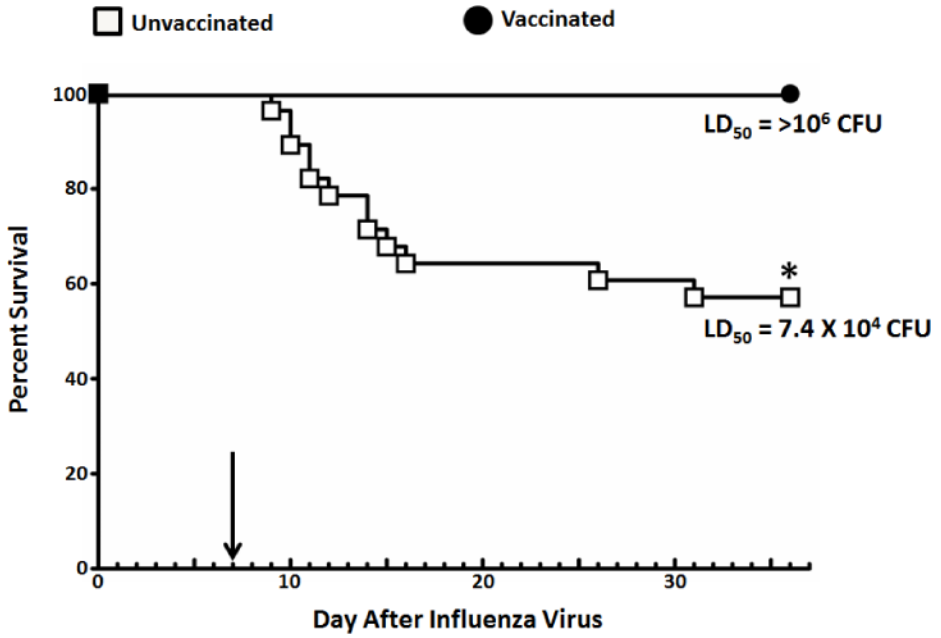

Figure 6.

Survival after influenza virus:*S. pyogenes* super-infection. Mice from each vaccine group (n = 28 for vaccinated and n = 28 for unvaccinated) were randomly separated for challenge with *S. pyogenes* at four different doses (10^3–10^6 CFU). For each challenge dose, n = 7 per vaccine group. This dose response was used to calculate an LD_{50} in both unvaccinated and vaccinated animals after super-infection. The arrow within the figure represents the day of MGAS315 inoculation (Day 7 after influenza virus inoculation). *Indicates a significant difference between vaccinated and unvaccinated mice (P <0.05) using a Log Rank chi-squared test on the Kaplan Meier survival data.