RESEARCH PAPER

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Single-dose monomeric HA subunit vaccine generates full protection from influenza challenge

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Recombinant subunit vaccines are an efficient strategy to meet the demands of a possible influenza pandemic, because of rapid and scalable production. However, vaccines made from recombinant hemagglutinin (HA) subunit protein are often of low potency, requiring high dose or boosting to generate a sustained immune response. We have improved the immunogenicity of a plant-made HA vaccine by chemical conjugation to the surface of the *Tobacco mosaic virus* (TMV) which is non infectious in mammals. We have previously shown that TMV is taken up by mammalian dendritic cells and is a highly effective antigen carrier. In this work, we tested several TMV-HA conjugation chemistries, and compared immunogenicity in mice as measured by anti-HA IgG titers and hemagglutination inhibition (HAI). Importantly, pre-existing immunity to TMV did not reduce initial or boosted titers. Further optimization included dosing with and without alum or oil-in water adjuvants. Surprisingly, we were able to stimulate potent immunogenicity and HAI titers with a single 15μg dose of HA as a TMV conjugate. We then evaluated the efficacy of the TMV-HA vaccine in a lethal virus challenge in mice. Our results show that a single dose of the TMV-HA conjugate vaccine is sufficient to generate 50% survival, or 100% survival with adjuvant, compared with 10% survival after vaccination with a commercially available H1N1 vaccine. TMV-HA is an effective dose-sparing influenza vaccine, using a single-step process to rapidly generate large quantities of highly effective flu vaccine from an otherwise low potency HA subunit protein.

Introduction

Inactivated virus vaccines have been extremely successful in controlling seasonal influenza,[1] and more recently, pandemic H1N1 influenza.[2] However, typical trivalent inactivated virus vaccines, which account for more than 90% of all immunizations, require egg-based production in specialized facilities that can take up to 7 years to develop.[3] Notably, not all influenza virus strains can be optimally adapted to egg production, including highly pathogenic pandemic H5N1 strains, even when reverse genetics is used to facilitate the reassortment process.[4] This serious problem has pushed significant changes in licensed production strategies, which now include both reverse genetics and cell-based production.[5-9] It is not yet clear, however, if these methods will accelerate vaccine production to meet emerging pandemic vaccine needs.[10] Current production capacity provides vaccines for approximately 800 million doses,[11,12] but protection from pandemic disease resulting from antigen shift will require greater production capacity.[13] Ideally, a pandemic response should also not compete for production of seasonal vaccines.[12] For example, the preventive vaccine requirements for a potentially emerging influenza such as H7N9[14] could still pose a significant production challenge.

Awareness of these emerging pandemic threats has stimulated development of new strategies for producing influenza vaccines that may be able to overcome existing production limitations. One highly promising area is the development of recombinant subunit vaccines, including recently licensed FluBlok® for seasonal influenza.[15] Production is based on overexpression of strain-specific hemagglutinin (HA) proteins by baculovirus in insect cells.[16] Since HA is the main antigenic determinant in inactivated virus vaccines, HA protein is an effective single antigen vaccine, and much easier to produce than the entire virus in eggs or cell culture. The major advantages are that speed of production is greatly improved, and that HA protein can be rapidly matched to current and future seasonal or pandemic strains. HA-based influenza vaccines have successfully been developed over the last decade from many sources, including bacterial[17] and plant expression systems.[18-20] Regardless of the source, a significant disadvantage is that HA protein is not robustly immunogenic on its own. Most formulations require high doses, repeat immunization, or adjuvant formulation for effective and protective immune activation in animal models[18,21] and in clinical trials.[22,23]

Our work has focused on developing an alternative plant-produced HA subunit vaccine with improved immunogenicity that can provide single-dose protection against virus challenge. Rapid

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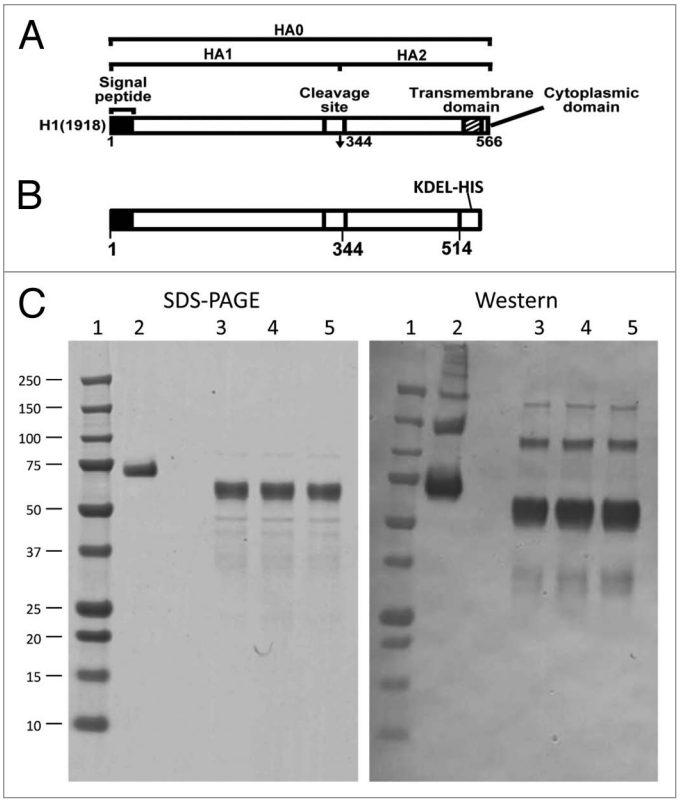

Figure 1. Expression of Hemagglutinin protein in plants and purification. (**A**) A schematic of the HA0 protein structure with described domains. (**B**) Schematic of HA0 protein expressed *in planta* lacking transmembrane and cytoplasmic domains and containing poly-His tag and HDEL retention sequence. (**C**) Three representative lots of HA protein analyzed by SDS-PAGE (left panel; lanes 3–5; 0.5 µg/lane). Recombinant HA protein from Sino Biological Inc. was used as a positive control (lane 2; 0.38 µg/lane). Precision plus protein molecular weight standards were used as size markers (lane 1). The same proteins were loaded at 7ng/lane and visualized by anti-HA Western, using an HA-specific monoclonal antibody (Sino Biological Inc.).

production of 500 g of H1N1 HA protein (A/California/04/09) was accomplished using an agroinfiltration expression system in plants. Initial testing suggested monomeric HA structure, with low vaccine potency. Our goal was to improve the potency of monomeric HA protein, using a chemical conjugation method to Tobacco Mosaic Virus (TMV). Although TMV is not pathogenic in humans, it is a highly potent rod-shaped antigen carrier that stimulates robust immunity to associated peptides and proteins.[24-28] In this study, we created TMV-HA conjugate vaccines from monomeric HA, and show one formulation that supports single dose protection against a lethal H1N1 virus challenge in mice.

Results

HA protein expression and purification

HA protein derived from H1N1 A/California/07/09 virus sequence was expressed using a magnICON® Agroinfiltration vector.[29-31] As shown in **Figure 1A**, HA0 was comprised of the native leader, H1 and H2 domains (truncated just before the transmembrane domain), followed by a six His/HDEL purification/Endoplasmic Reticulum (ER) retention signal (**Fig. 1B**), for a total expected mass of ~60Kd. *Nicotiana benthamiana* plants were infiltrated, and plant material was harvested 6–8 d post infiltration, extracted, and purified by column chromatography. **Figure 1C** shows typical results of three independent extraction/purification cycles, analyzed by SDS-PAGE and Western immunoblot analysis, showing purity and H1N1 antigen identity and immune reactivity. 500 g of this material was qualified by lot release for HA, under cGMP specifications, including potency, purity, and safety, consistent with WHO and USP guidelines. This HA protein was determined to be safe after administration to BALB/c mice, and immunogenic when given with Alum, but of relatively low potency by anti-HA enzyme-linked immunosorbent assay (ELISA) titer and by hemagglutination inhibition (HAI; data not shown).

HA-TMV conjugation

In order to improve the HA subunit protein immunogenicity, we used chemical conjugation to link HA protein to the surface of Tobacco Mosaic Virus (TMV), which has been shown in multiple studies to provide carrier and adjuvant effect to weakly immunogenic peptides and proteins[24,28,32]. TMV is an abundant, non-infectious plant virus, and has the same scale up potential in plants as HA protein, and has been modified to express a surface exposed lysine[28] for improved surface reactivity. Six chemistries were tested to determine the relative efficiency and ease of conjugation, and immunogenicity. Reactions performed with EGS ([ethylene glycolbis(succinimidylsuccinate)], azide/PEG (polyethylene glycol) or DCC (N, N'-Dicyclohexylcarbodiimide) according to manufacturers specifications, produced less than 10% conjugate products by SDS-PAGE, and were not tested further (data not shown). Three conjugation reactions generated acceptable conjugation characteristics, defined as the absence of free HA protein, at ratios of 1 mg HA protein to 1 mg of TMV virus at ~2 mg/ml total protein. As shown in **Figure 2A**, glutaraldehyde conjugation of HA to Keyhole Lympet Hemocyannin (KLH-HA) or to TMV (TMV-HA) generated high molecular weight aggregates that accumulated above the 190 kD marker, and in the gel stack after 8–16% SDS-PAGE separation, with an absence of free HA protein after 30 min reaction time. Strikingly similar results were observed using EDC/NHS (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide) using essentially the same protein ratios and reaction time (**Fig. 2B**). Linkage using Sulfo-SMCC (Sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate) also generated aggregates (**Fig. 2C**), but at much lower molecular mass than glutaraldehyde or EDC/NHS, and with residual free HA protein despite a longer conjugation time of 12 h. TMV coat dimers were also much more evident, suggesting end-to-end conjugation was also occurring. HA was conjugated to itself (HA-HA) under similar reaction conditions for each chemistry, to determine the characteristics of self-aggregation, and to serve as a TMV-free aggregation control in immunization studies. ELISA data was used to establish that >95% HA conjugated to self or carrier molecules was reactive to a commercially available anti-HA sera (Sino Biological Inc., data not shown).

Immunization with conjugated TMV-HA results in anti-HA titers

Initially, glutaraldehyde, EDC-NHS and SMCC conjugates, as well as controls, were used to vaccinate BALB/c mice with 15 µg HA concentration without adjuvant. In order to determine the effect of pre-existing immunity to TMV on the antigen specific response, three groups were pre-immunized with 2 × 25 µg doses of unreacted TMV two weeks apart, two weeks prior to the start of TMV-HA immunizations (T+). TMV preimmune titers were measured, and all mice had >8 µg/ml anti-TMV antibody reactivity at the start of immunization (**Fig. 3A**, right axis, patterned bars).

Ten days after a single injection, all conjugate vaccine groups, irrespective of type or pre-exposure to TMV, stimulated higher titers of anti-HA antibodies than HA given alone (all groups, $P < 0.05$), except for the SMCC HA-HA self conjugate, which had no IgG titer. All glutaraldehyde produced vaccines showed low but measurable titers after a single dose (**Fig. 3A**, left axis, solid bars), with statistically similar responses between the gold-standard KLH-HA compared with the experimental TMV-HA vaccine ($P = 0.11$). Despite the similarity of high molecular weight bands observed after glutaraldehyde or EDC/NHS conjugation by SDS-PAGE, the EDC/NHS TMV-HA vaccine formulations stimulated higher single dose anti-HA mean titers than that observed for glutaraldehyde TMV-HA, with a significantly improved response in the TMV primed group ($P = 0.02$). Statistically, IgG titers in all other groups were similar to each other. Interestingly, TMV pre-immunization seemed to boost single dose titers, especially after glutaraldehyde conjugation. SMCC TMV-HA conjugates stimulated low mean IgG titers, but were not detected after HA-HA conjugate vaccination, even after boosting.

Administration of a second 15 µg dose of conjugate HA resulted in 50–100 fold increases in anti-HA antibodies (**Fig. 3B**, left axis, solid bars), with no significant differences in titers in the TMV-HA group compared with the gold standard KLH-HA conjugate ($P = 0.13$). TMV pre-exposure had no effect on subsequent boosting of HA titers, even after four administrations of TMV (two TMV pre-immunizations, and two TMV-HA boosts). Glutaraldehyde and EDC/NHS HA-HA self-conjugates stimulated lower levels of boosted titers but, like all vaccine groups, were statistically better than HA alone ($P < 0.05$), demonstrating that aggregation of HA is sufficient to improve IgG titers compared with monomeric HA.

To determine the hemagglutination inhibition (HAI) potential of the antibody response, we performed HAI testing on the best performing glutaraldehyde and EDC/NHS vaccine sera, post vaccine 2. Although immune titers were similar across all groups, HAI was only detected in TMV-HA EDC/NHS vaccinated mice, with higher HAI titers observed in the TMV primed mice (**Fig. 3B**, right axis, patterned bars). Although HA-HA aggregation improved IgG titer compared with HA alone, the contribution of viral structure and viral RNA (albeit inactive) from TMV facilitated stimulation of potentially neutralizing HAI immunity.

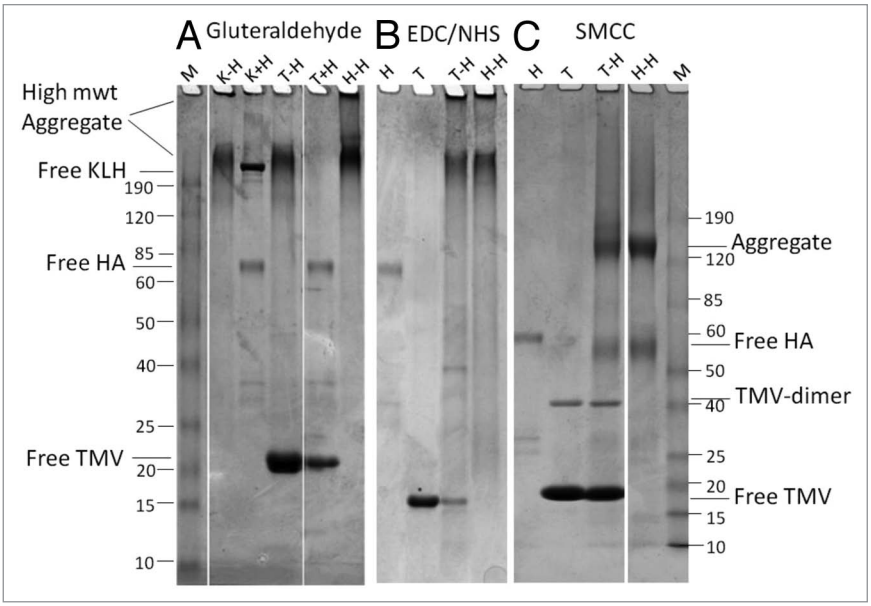

Figure 2. Conjugation of TMV to HA protein. Intact TMV 1295.10 was conjugated to HA protein in vitro as described, and analyzed on an SDS PAGE after staining with Coomassie Blue. (**A**) Glutaraldehyde, (**B**) EDC/Sulfo-NHS or (**C**) SMCC chemistries was successful in conjugating the HA protein to KLH (KLH-HA), TMV (TMV-HA) or as a self-conjugate (HA-HA). The mixture, as indicated by K+H or T+H, sampled before addition of the conjugation chemistry, shows an equimolar representation of both the proteins. The conjugate appears as a high molecular weight aggregate >190 kDa and in the gel stack. Successful conjugation was qualified by the complete absence of 'free' (unbound) HA protein as indicated. K-H (KLH-HA Conjugate), T-H (TMV-HA Conjugate), H-H (HA Self Conjugate).

Characterization of the conjugate vaccine

Due to potentially superior neutralizing antibody titer, as measured by HAI > 40 (as is considered the minimum HAI titer for protection), we further investigated the EDC/NHS TMV-HA conjugate by a number of measures. We had observed by time-course analysis of conjugate products that increased conjugation time above 30 min generated a higher mass band by SDS-PAGE analysis. This is shown in **Figure 4A** in a 60 and 90 min reactions (asterisk), along with an increased accumulation of very high molecular weight aggregate in the well (arrow). Increasing the reaction time to 90 min did not change the appearance of the conjugate product further, so only 30 and 60 min reaction times were compared in immunogenicity studies (**Fig. 5**).

We also characterized the EDC/NHS TMV-HA conjugate vaccines to determine the relative structure of the conjugation product. Rotational size was measured for TMV and TMV-HA using NanoSight optical analysis, with TMV demonstrating an average rotational mass of 83 nM (**Fig. 4B**). TMV-HA size had an increased average rotational mass of 139 nM, suggesting single TMV molecules coated with HA protein. Some heterogeneity

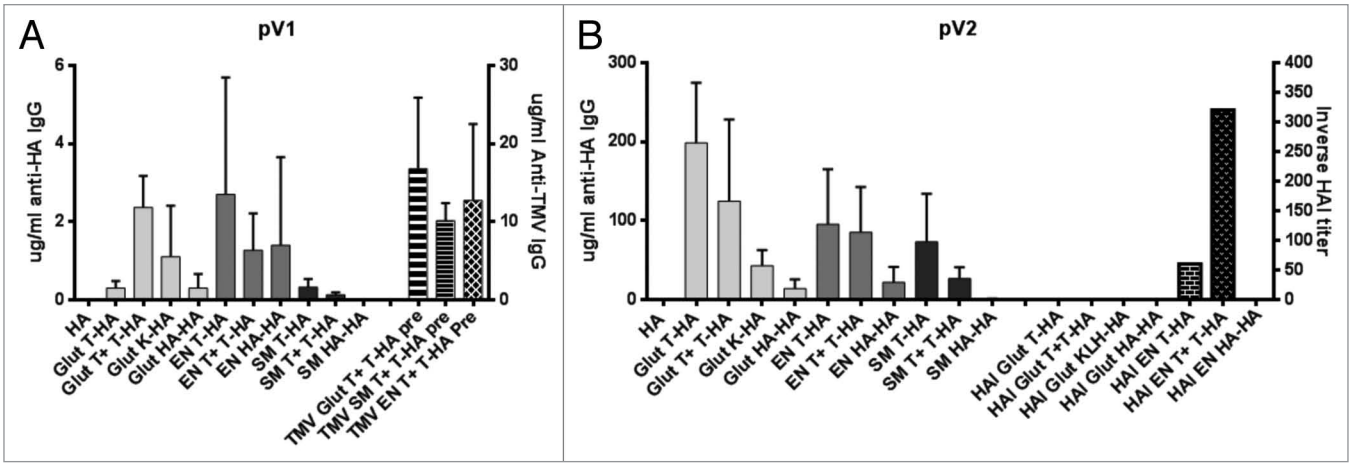

Figure 3. Conjugation to TMV confers immunizing activity to HA protein. Protein equivalent to 15 μg HA was injected subcutaneously in the right rear flank two weeks apart. (**A**) After a single dose (pV1), immunization, sera were analyzed for anti-HA antibodies at 10 d post injection by ELISA (solid bars, left Y axis). The data are presented as μg/ml anti HA IgG, compared with a known quantity of anti-HA-IgG (Sino Biological Inc.). Pre-existing anti-TMV IgG titers (patterned bars, right Y axis) are shown for the groups pre-immunized 2 times with 25 μg TMV (T+) before the start of TMV-HA immunizations. (**B**) After a second dose (pV2), IgG titers (solid bars, left Y axis) and HAI (patterned bars, right Y axis) were evaluated. Abbreviations: Glut (glutaraldehyde conjugated, light gray), EN (EDC/NHS conjugated, medium gray), SM (SMCC conjugated, dark gray), K-HA (KLH-HA conjugate), HA-HA (HA self-conjugate), T+ (pre immunized with TMV before vaccination), T-HA (TMV-HA conjugate).

was observed at higher masses, which we postulate are dimers of TMV created by end to end conjugation (also seen by SDS-PAGE and EM), in addition to association with HA protein. These results were verified by electron microscopy (**Fig. 4C**), where apparent diameter of the TMV-HA conjugate increased by 34% (~8 nM) after HA conjugation compared the average diameter of unconjugated TMV (18nM), whereas the expected rod length was maintained. Notably, most of the TMV-HA conjugates were visually evident as single virus particles. Taken together, these data characterize the EDC-NHS TMV-HA conjugate as HA protein coated onto the surface of TMV, rather than cross-linked mats of virus and HA protein. Additionally, we tested the ability of TMV-HA to confer the ability to crosslink red blood cells in a hemagglutination assay. Although HA monomer protein alone, or TMV alone, does not provide any hemagglutination activity, TMV-HA was able to agglutinate turkey RBCs (**Fig. 4D**).

Immunization with the EDC/NHS conjugate vaccine with or without adjuvants

We further characterized TMV-HA (EDC/NHS) conjugate vaccine efficacy by varying conjugation time, and HA dose, and by addition of either Alum (Alhydrogel; InVivogen) or a squalene oil-in-water based adjuvant (Addavax; InVivogen), similar to the MF59 adjuvant used to potentiate human influenza vaccine responses. We immunized BALB/c mice with vaccines prepared by 30 or 60 min conjugation, with 15 or 60 μg of HA protein in the TMV-conjugate vaccine, with or without additional adjuvant. We measured anti-HA IgG responses at 14 and 28 d post vaccine 1 (pV1; **Fig. 5A**), and saw a significant increase in the response at day 28 after a single dose. At day 14, only the 30 min conjugate given at 15 μg (30' TMV-HA 15) and the same vaccine given with squalene induced significantly more IgG than HA alone (*, $P = 0.004$). At day 28 after a single dose, all but two groups induced significantly more IgG than HA alone (*, $P < 0.05$). All of the groups were statistically similar to 60 μg of HA given with Alum, except one 60 min conjugate, 60 μg dose given with Alum, that was a very low responder ($P = 0.044$). At day 14 post vaccine 2 (**Fig. 5B**), all groups were significantly better than HA alone ($P < 0.005$) and all groups were similar to HA given with Alum except the 60 min conjugate given at 60 μg with Alum (lower responder, $P = 0.023$) and the 30 min conjugate given at 15 μg with squalene (high responder, $P = 0.016$). Similar results were seen 28 d post vaccine 2. Overall, we saw no statistically significant differences in immune response profiles with additional TMV-HA protein (15 μg vs. 60 μg HA dose), but did measure decreased immune responses with additional conjugation reaction time. Addition of a squalene-based adjuvant to the 15 μg and 30 min conjugate vaccine resulted in significantly higher titers of anti-HA IgG at both 14 and 28 d after a single vaccine dose, as well as robust HAI titers (shown numerically above bars of select groups tested). Surprisingly, the 30 min TMV-HA 15 μg vaccine without adjuvant also provided both robust IgG titers after a single dose (>10 μg/ml), and HAI at day 28. After a second dose, all IgG titers increased significantly, and HAI was still measured at greater than protective levels (>40) in selected groups.

Protection from H1N1 Viral Challenge

To determine the effectiveness of the immune response induced by TMV-HA vaccines, we initiated a study testing one or two doses of the TMV-HA vaccines in BALB/c mice, followed by H1N1 virus challenge. For single dose immunizations, mice were vaccinated by subcutaneous injection with 30 μg TMV-HA (15 μg total HA protein) with or without squalene adjuvant. For double dose immunizations, mice were given two vaccinations of 15 μg TMV-HA (7.5 μg total HA protein) two weeks apart, with or without squalene adjuvant. Mice were given PBS or HA protein as negative controls, and positive controls included HA protein given with a squalene adjuvant, or a commercially

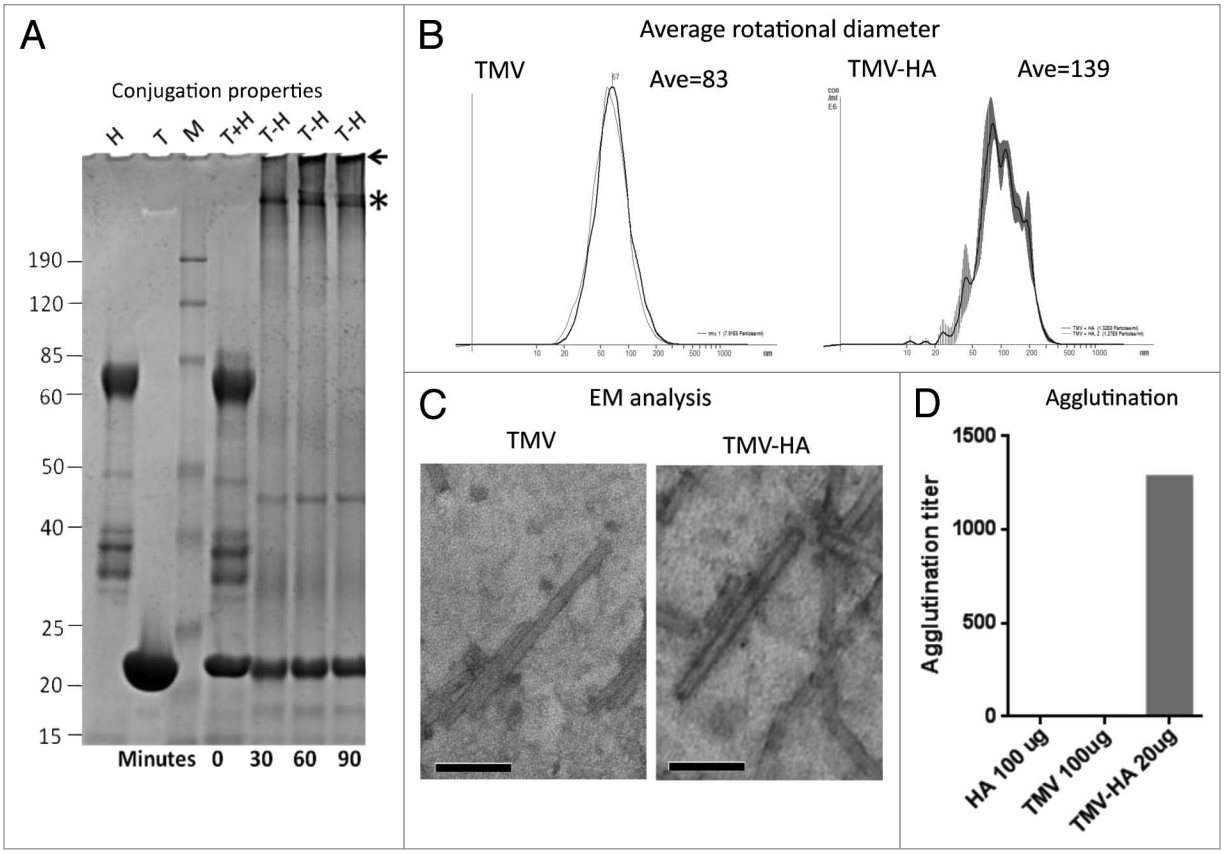

Figure 4. Physical characterization of the EDC/NHS TMV-HA conjugate. (**A**) Increased EDC/NHS conjugation time from 30 to 60 min caused additional high molecular weight bands to appear in the conjugated reactions (*), in addition to more accumulation in the well (arrow; T-H reactions). (**B**) Laser Optical scattering microscopy was used to determine the size of the vaccine particle in suspension. Average rotational size was measured for both TMV and TMV-HA, in triplicate. (**C**) 40 000× Electron microscopy imaging of TMV or the TMV-HA conjugate. Particle average diameter increased by 34% from 18.0 nM to 24.1 nM after HA conjugation. The diameter was derived as the average of 30 electron micrograph images, using the bar = 100 nM as a size standard. (**D**) TMV and HA were assessed independently at 100 µg, and the TMV-HA conjugate was assessed at 20 µg for the ability to agglutinate turkey RBCs. Data are shown as the inverse of the minimum titer required to stimulate agglutination.

available trimeric vaccine containing H1N1 inactivated virus. Serum from all animals was collected at day 26, and IgG and titers (**Fig. 6A and B**) were similar to that shown 28 d after a single dose (**Fig. 5A**). Mice were challenged on day 28 with 25MLD$_{50}$ of mouse adapted H1N1 influenza A/California/04/09 virus, and monitored for weight loss and neurological symptoms. Mice losing >25% body weight, or showing overt signs of neurological symptoms were euthanized. Surprisingly, 50% of mice receiving a 15 µg single dose TMV-HA vaccine without adjuvant survived virus challenge (large filled triangle). Mice receiving a 15 µg single dose TMV-HA vaccine plus squalene adjuvant showed minimal symptoms and 100% of mice survived (large filled diamond). Mice given PBS or HA alone did not survive challenge. HA plus squalene adjuvant (open triangle), or H1N1 (open circle) were partially protected at 20% and 10% respectively. Mice receiving two 7.5 µg doses of TMV-HA vaccine, with (large filled diamond) or without adjuvant (large filled triangle) were 100% protected from viral challenge, where HA and control groups fully succumbed. H1N1 vaccine protected only 50% of the mice after two doses (open circle). Survival was plotted by Kaplan-Meier analysis, and only TMV-HA groups (as shown as boxed p values) were significantly better than the H1N1 vaccine groups. HAI titer analysis (**Fig. 6A**) also show a positive correlation of HAI titers >40 in TMV-HA (no adjuvant) surviving mice compared with mice that did not survive viral challenge.

Discussion

Plant-based vaccine production of HA and other subunit vaccines has been in development for decades because of rapid prototyping, capacity, economy of scale, and the overall low cost.[33-40] Several previous studies have shown plant produced HA is effective in mouse and ferret models of influenza challenge, but always require at least two immunizations with adjuvant to stimulate HAI titers or protective immunity.[18,20,41] Disappointing results from a Phase I clinical trial reported only 5–10% seroconversion after 90 µg dosing with an HA vaccine, irrespective of adjuvant co-formulation,[42] or may require 2 doses of alum-adjuvanted vaccine for clinically significant immune protection.[22] Two immunizations require doubling production capacity for the number of immunizations needed, doubling physical access to vaccination centers, and increasing the time to achieve immune protection

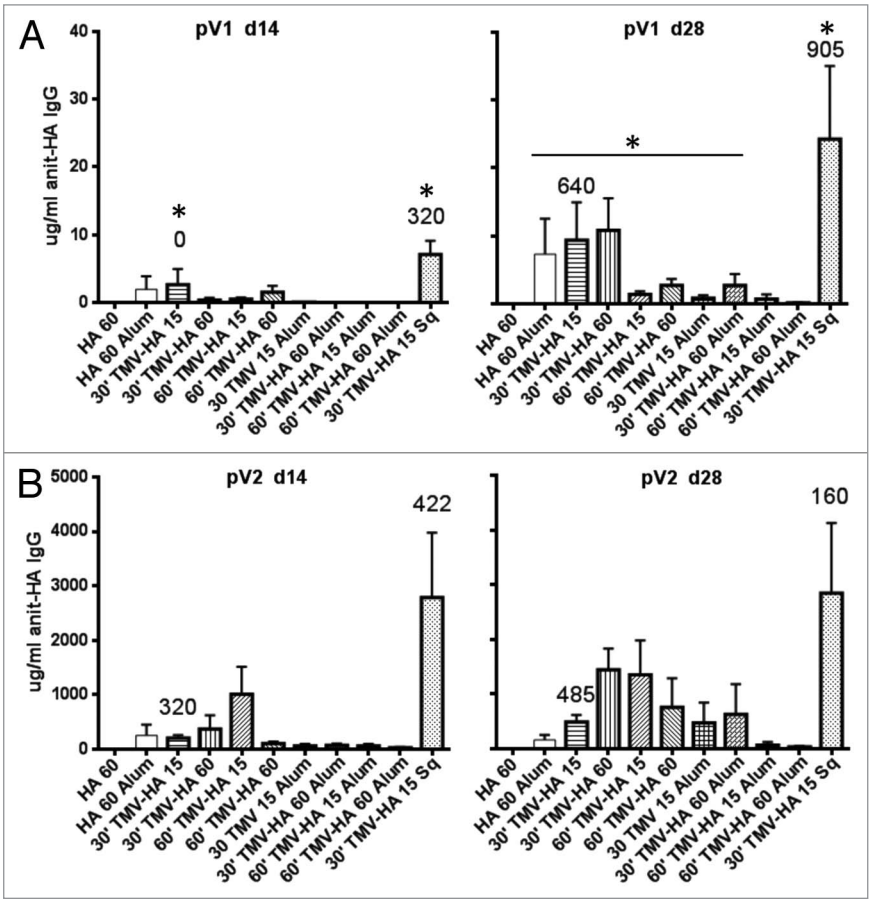

Figure 5. Vaccine efficacy by dose, conjugation time, or addition of adjuvants. Mice (n = 5) were vaccinated with indicated amounts (15 or 60 μg) of the TMV-HA conjugate protein conjugated for 30′ or 60′ (minutes) with or without adjuvant Alum (Alum) or with oil-in-water squalene adjuvant (Sq; AddaVax). ELISA analysis of anti-HA IgG titers in sera collected at 14 or 28 d after the first vaccination (**A** pV1) or after the second vaccine (**B** pV2) given at day 30. Asterisks in A represent IgG titers significantly greater ($P < 0.05$) than induced by HA 60 vaccination. Titers were measured against a known quantity of anti-HA standard (Sino Biological Inc.). Numbers above the bars are the HAI presented as a geometric mean titer.

after vaccination. In order to respond to pandemic influenza, an optimal vaccine should be made in weeks, based on a scalable production system, and provide single dose vaccine potency.

Based on our results, 500 g of monomeric HA protein was made at pilot scale in less than a month which, along with other HA vaccine sources, could provide significant vaccine protection from a potential pandemic influenza without competing for seasonal vaccine production. To our knowledge, this is the only demonstration of protective immunity after single dose monomeric HA subunit vaccination in mice. Monomeric HA is the easiest form of subunit protein to produce, but has typically been ineffective in vaccine settings.[17,43-45] Our ability to stimulate potent and effective single dose HA vaccines comes from the association of monomeric HA with TMV, a plant virus that in this context is acting both as a viral antigen display scaffold, and viral delivery mechanism. TMV can directly stimulate dendritic cell (DC) antigen uptake in vitro,[24] along with DC activation, migration of DCs to lymphoid organs and robust stimulation of antigen-specific B and T cells in vivo.[46] As clearly demonstrated in these studies, TMV also serves as an adjuvant to the conjugated subunit vaccine protein, which may have importance in geographic areas where cold-storage dependant adjuvants are impractical. Using TMV to deliver HA protein harnesses DC uptake and activation to stimulate effective antigen presentation, and influences the quality of the subsequent protective immune response. This is most clearly shown in experiments comparing the HAI titers stimulated by an HA-HA aggregate to TMV-HA using the same conjugation method: overall IgG titers were similar, but only TMV-HA stimulated HAI.

It is also important to note that the TMV virus as an antigen carrier supports repeated boosting without loss of immune activation, even in the presence of anti-TMV antibodies at similar or greater levels than anti-HA antibodies. In fact, early exposure to TMV potentiates the initial response to HA, in contrast to many virus carriers that are limited to one exposure before neutralization blocks boosting. This is an important characteristic, given most of the human population has been exposed to TMV through food and tobacco, and have pre-existing anti-TMV IgG titers.[47]

Conjugation method also has a significant impact on the quality of the immune response. Glutaraldehyde, which uses random vinyl addition to crosslink proteins, was effective in linking HA to TMV, and stimulated robust single dose titers. However, these conjugates failed to stimulate HAI, an important measure of potential for protection against pathogen challenge. SMCC, which directs association between free disulfides and amine groups, was the least effective at generating TMV-HA conjugates, as measured by the most free HA protein, with the longest reaction time. SMCC conjugates stimulated very low single dose IgG, and also failed to stimulate HAI titers, even after a boost. EDC/NHS, linking HA and TMV via amine and carboxyl groups, had less random association than glutaraldehyde, and less ordered association than SMCC, but generated rapid predictable association between the two proteins in a single step reaction, and induced both IgG and HAI titers.

Our data describes a novel subunit vaccine that protects mice from virus challenge after single dose vaccination. We show compelling data that TMV-HA fusions induce HAI neutralization properties of subunit HA protein, either by displaying a semi-crystalline array of antigen that stimulates a broader range of antibody targets, or by stimulating dendritic cell uptake and activation that enhances the effectiveness of antigen presentation. Although we have improved H1N1 HA subunit vaccine potency, we still need to demonstrate that these results will extend to successful protection using alternative clade HA proteins as fusions

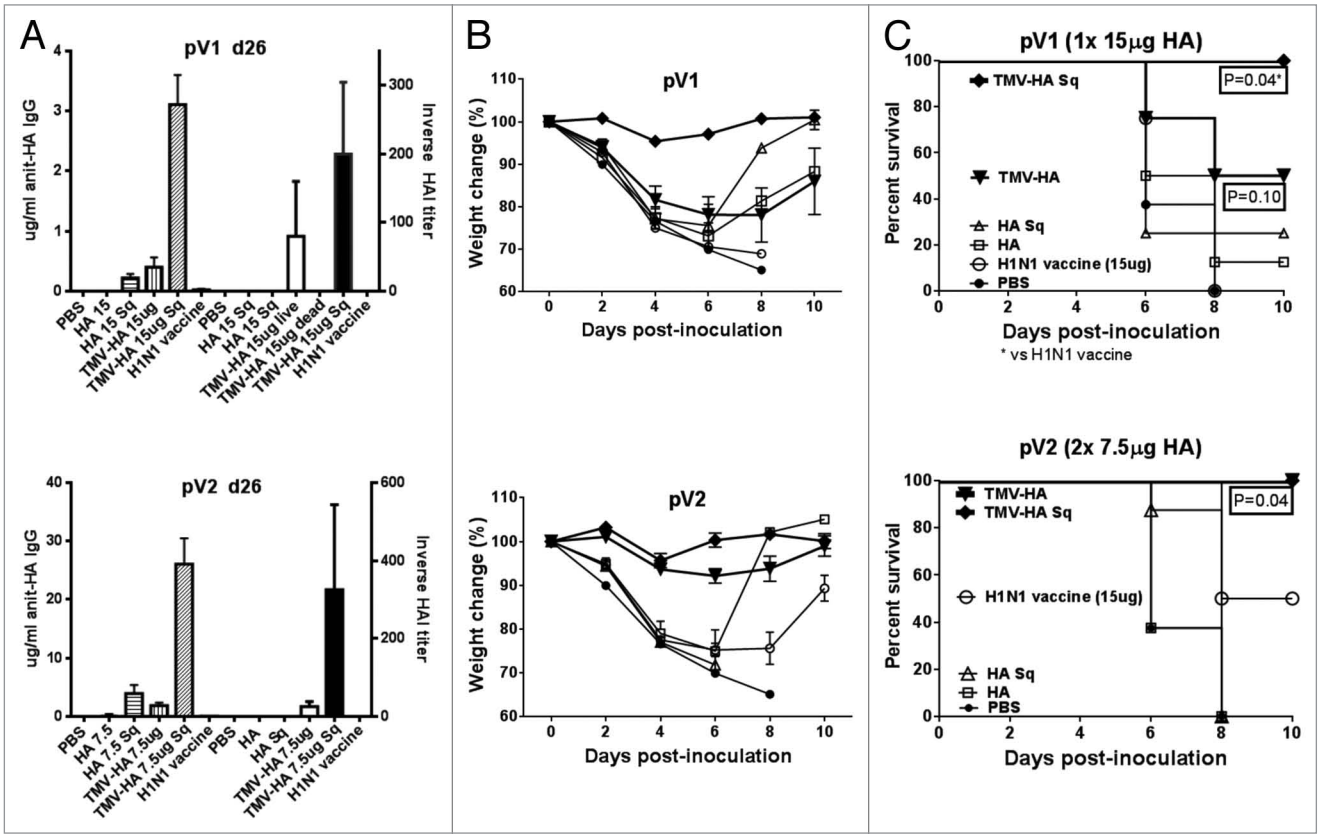

Figure 6. H1N1 virus challenge. Mice (n = 8) were vaccinated once, or twice 14 d apart, with 15 μg equivalent of HA protein, as free HA or TMV-HA, with and without squalene adjuvant, compared with a commercial H1N1 vaccine or PBS controls. On day 28, immunized mice were challenge with mouse adapted H1N1 influenza A/California/04/09 virus. (**A**) ELISA (shaded bars, left Y axis) and HAI analysis (open and closed bars, right Y axis) were performed on the sera drawn at day 26 post vaccination. (**B**) Weight change in each group of mice is represented as percent deviation from original weight. (**C**) Survival was recorded and plotted as a Kaplan-Meier curve, and groups with significant differences in survival by log-rank analysis against the H1N1 vaccine control are shown. Symbols, as indicated in C, are the same in B.

to TMV. Future studies should also test protection by virus challenge in ferrets, given this is the most closely aligned model to human efficacy. Future studies will include analysis of the role of TMV RNA in vaccine potency, and efficacy by mucosal immunization. Initial pilot studies indicate that intranasal delivery of un-adjuvanted TMV-HA further potentiates the immune response compared with subcutaneous vaccination, with the potential of substantial dose sparing. Ideally, a monomeric HA made in planta can be formulated into a rapid response, single dose influenza subunit vaccine that can be administered without needle delivery or additional adjuvant.

Materials and Methods

Expression and Purification of HA protein

HA sequence from H1N1 CA07/09 was cloned as HA0 into a magnICON® Agroinfiltration vector[31] and screened at small scale for HA expression by ELISA and Western. High yield HA recovery from Agroinfiltrated plants was accomplished by plant homogenization and clarification using physical separations of insoluble plant cellulosic compounds, proteins and membranes from soluble proteins followed by three chromatography steps. HA protein (final fill) was analyzed by SDS-PAGE and Western by standard methods. HA protein standards and an HA-specific monoclonal from Sino Biologicals Inc. were used to establish identity and purity. Potency was measured by single radial immunodiffusion (SRID), and safety was established by entotoxin and bioburden tests (not shown).

Conjugation and Vaccine preparation

Chemical conjugation of HA protein to TMV required presence of a surface reactive lysine at the N terminus. A well-characterized lysine-modified TMV used in this study was genetically engineered to express a single reactive N-terminal lysine (K) residue on the surface of the TMV virion.[28] The virus was expressed by transient infection of plants and purified as previously described.[24,32] Multiple conjugation chemistries were tested, in order to determine the most efficient method. Equal quantities of HA protein and TMV were mixed with 0.025% glutaraldehyde in PBS, and incubated end-over-end for 30–45 min at room temperature. HA protein was conjugated to TMV using EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) and Sulfo-NHS (*N*-hydroxysulfosuccinimide;

Thermo-Fisher Scientific) chemistry as per manufacturer's instructions.[48] Equal quantities of HA protein and TMV were mixed in 0.1M MES pH 5.0 in 0.5M NaCl. Freshly prepared EDC (Thermo-Scientific) was added to 2mM and the mixture was immediately vortexed, and Sulfo-NHS (Thermo-Scientific) was added to 5mM. This mixture was vortexed at room temperature for 30 min, or for indicated times. At the end of the incubation, the reaction was stopped by addition of Hydroxylamine-HCl to 10mM. For SMCC reactions, conjugation was initiated in a reaction mix of 5mM EDTA and 10 mg/ml Sulfo-SMCC (Thermo-Scientific) with 1 mg each TMV and HA in PBS in 1ml. Reactions were vortexed overnight at room temperature. Conjugation efficiency was evaluated by SDS-PAGE and Coomassie staining, and equal quantities of the unreacted proteins were run in parallel as controls. Reactions were considered complete when no free HA protein was visible. Vaccines were prepared after conjugation by removal of reactive agents by overnight dialysis against PBS in Slide-a-Lyzer (Thermo-Scientific) cartridges, and the protein quantity, determined by the bicinchoninic acid assay (BCA ;BioRad), was used to normalize the vaccine dose.

Vaccination and immune response evaluation in mice

BALB/c mice (Charles River) were housed at Touro University according to guidelines established in the Care and Use of Animals and according to IACUC approved protocols. Typically, mice were given a 100 – 200 µl subcutaneuous (s.c.) injection of 15 or 60 µg HA protein as conjugates, neat (unconjugated), or with either Alum (Alhydrogel; Invivogen, 1:1 ratio, final 1%) or a squalene based oil-in-water emulsion adjuvant Addavax (1:1 ratio;Invivogen), according to the manufacturer's directions. Vaccines were typically administered at two or four week intervals and tail vein bleeds were taken at indicated days for ELISA analysis.

The IgG immune response was determined by enzyme linked immunosorbent assay (ELISA) as previously described.[49] Briefly, 5µg/ml HA protein was used to coat Maxisorp ELISA plates (Nunc) in carbonate buffer. A dilution series of sera from HA vaccinated mice was then added, and detected with anti-Mouse HRP (Southern Biotech). Plates were developed using a tetramethyl benzidine substrate solution (TMB; BioFx, Owing Mills) and the reactions stopped by the addition of 1N sulfuric acid. Plate absorbance was read at 450 nm in a 96-well plate spectrophotometer (Molecular Devices). Anti HA titers reported in µg/ml were determined from a standard curve generated by a 3-fold serial dilution of a 100 ng/ml mouse anti-HA monoclonal antibody (Sino Biological Inc.).

Hemagglutination dose (HAD) was established for the agglutinating agent (the mouse adapted inactivated influenza virus H1N1 A/California/04/09) using serial 2-fold dilution and mixing with 0.5% turkey RBCs in PBS. Agglutination was recorded after 45 min incubation. HAI titers of the sera were examined using a microtitration method as described.[50] Briefly, serum samples were treated with receptor-destroying enzyme (RDE: Denka Seiken) overnight at 37 °C and heat-inactivated for 30 min at 56 °C, and then diluted 1:40 before the assay. Serial dilutions of samples were incubated for an hour with an equal volume of the agglutinating virus equivalent to 4xHAD in a 96-well plate. Control lanes containing virus or diluent alone were included on each plate. Equal volumes of 0.5% turkey RBCs in PBS were added to the wells and the plates were incubated for 30 or 45 min at RT. All wells were observed for the presence or absence of a button that indicates no agglutination. Serum samples prepared for the assay were also treated with packed red blood cells to remove non-specific hemagglutination-inhibiting materials. HAI titer was read as the reciprocal of the highest dilution of serum that inhibited hemagglutination. Serum HAI titers were evaluated using geometric mean titer (GMT).

Physical characterization of TMV-HA particles

Polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 8–16% Tris-glycine gels (BioRad), following the manufacturer's instructions. Benchmark protein standard (Invitrogen) was used as molecular weight reference. Protein quantitation was performed using the bicinchoninic acid (BCA) assay (Pierce) in a microtiter plate format following the manufacturers' instructions. For electron microscopy, grids (400 Mesh copper, carbon coated; Ted Pella) were floated on drops of TMV or TMV-HA diluted to 100–200 µg/ml in 0.1 M phosphate buffer, pH 7.0 and negatively stained with 1% phosphotungstic acid. All samples were examined on a Philips CM120 microscope, coupled to a digital camera. NanoSight analysis was performed as per the manufacturers protocol. Briefly, dilutions of TMV or TMV-HA samples were injected into the Nanosight LM10 using a 488 laser light source with a 1 ml sterile syringe. 60 s sample videos were recorded with a CCD camera, and particle motion was analyzed with Nanoparticle Tracking Analysis (NTA) 2.0 Analytical software to determine rotational diameter.[51] Samples were evaluated in triplicate, with average values reported.

Murine H1N1 virus challenge

Virus challenge studies were conducted under animal Biosafety level 2 (ABSL2) conditions and according to St. Jude Children's Research Hospital approved institutional procedures and protocols. Ten week old BALB/c mice were vaccinated as described, and at 26 d, serum was collected from each experimental group for ELISA and HAI analysis. At 28 d, mice were briefly anesthetized with 3% isoflurane for induction plus 1L-O_2 and challenged intranasally at 25MLD_{50}[52] with a mouse adapted 2009 pandemic influenza H1N1 virus, A/California/04/09, diluted in 50 µL of PBS. The mice were then monitored daily for body weight loss including neurologic signs and motor dysfunction for 10 d. The experimental end point was predetermined to be either 25% or more of body weight loss or any neurological signs or motor dysfunction. Mice were euthanized according to approved procedures by carbon dioxide asphyxiation followed by cervical dislocation. On the 10th day, all survivors were anesthetized as previously stated, and euthanized.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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