NS1-Truncated Live Attenuated Virus Vaccine Provides Robust Protection to Aged Mice from Viral Challenge

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Immunological changes associated with age contribute to the high rates of influenza virus morbidity and mortality in the elderly. Compounding this problem, aged individuals do not respond to vaccination as well as younger, healthy adults. Efforts to increase protection to this demographic group are of utmost importance, as the proportion of the population above the age of 65 is projected to increase in the coming decade. Using a live influenza virus with a truncated nonstructural protein 1 (NS1), we are able to stimulate cellular and humoral immune responses of aged mice comparable to levels seen in young mice. Impressively, a single vaccination provided protection following stringent lethal challenge in aged mice.

Influenza viruses are negative-sense, single-stranded RNA members of the Orthomyxoviridae family, with influenza A and B viruses causing significant clinical disease each year. At present, vaccination remains our best medical intervention at preventing influenza virus-related illness and death that occur during seasonal epidemics (47). While annual influenza epidemics can affect all age groups, there has historically been an increased risk of more serious infection in those who are over 65 years of age. While aged individuals seem to be less susceptible to infection with the novel swine influenza H1N1 virus (75), the virus still causes severe morbidity and mortality in this age group, with high rates of hospitalizations (16) and death for those over the age of 65 (44). Aged individuals are therefore still considered to be at risk for infection and are a top vaccination priority by the CDC’s Control Advisory Committee on Immunization Practices (15).

Trivalent influenza vaccines can be administered as either inactivated or live attenuated virus preparations and are formulated to protect against the influenza viruses that are expected to circulate during the coming influenza season. Only trivalent inactivated split vaccines are licensed for use in the elderly, though vaccine efficacy remains suboptimal for this group (2, 8, 9, 29, 32, 39, 57). The gradual decline of innate and adaptive immune responses in the aging, a process termed immunosenescence, is thought to largely contribute to decreased vaccine efficacy in elderly adults. Age-related immunological changes include aberrant interferon (IFN) and cytokine responses, as well as deficiencies in antigen presentation and T and B cell activation (9, 11, 13, 23, 43, 48, 49, 53, 56, 65, 77). Aged mice display many of these same immunological deficits (5, 24, 35, 46, 65, 68, 70, 77) and thus are a relevant model in which to study novel vaccine strategies for the aged. Because the elderly population is the fastest growing age demographic in the United States (33), it is critical that we develop improved methods of protection against influenza virus for these individuals.

Nonstructural protein 1 (NS1) of influenza A and B viruses antagonizes the host interferon response following infection and contributes to the virulence of viral strains (21, 28). It has been shown that viruses with partial deletions in NS1 protein are attenuated and do not cause disease, yet induce a protective adaptive immune response in mice (31, 69). Similar findings have been demonstrated in pigs (64, 72), horses (58), birds (66, 74), and macaques (3), and phase I trials in individuals aged 18 to 50 have shown that vaccine viruses attenuated through NS1 deletion are immunogenic in a small cohort of individuals (73). However, it remains unknown if this vaccine strategy can confer enhanced protection to immunosenescent aged mice.

Here we demonstrate that an NS1-truncated vaccine boosts cellular and humoral adaptive immune responses following vaccination, and to our knowledge, it is the first example by which a single vaccination can protect aged mice from stringent lethal challenge without the use of additional adjuvant. Furthermore, we demonstrate that immunization of nonreplicating vaccines at higher doses does not provide protection in aged mice, arguing that a novel vaccine strategy may be necessary to protect this rapidly growing age group from influenza virus infection.

MATERIALS AND METHODS

Viruses and cells. 293T and MDCK cells were obtained from ATCC and were maintained in Dulbecco’s modified Eagle’s medium (DMEM) and minimal essential medium (MEM) (both from Gibco), respectively, each supplemented with 10% fetal calf serum (HyClone) and 100 units/ml of penicillin-100 μg/ml of streptomycin (pen-strep; Gibco).

Recombinant influenza viruses were produced using an eight-plasmid influenza virus reverse genetics system as described previously (58). In order to generate NS1-truncated viruses, primers encoding stop codons and restriction sites were used to amplify fragments by PCR that were then subsequently ligated into pDZ vectors. Detailed construction of each plasmid has been described previously (52, 58). Plasmids were designed so that recombinant viruses expressed either the full-length NS1 protein (wild-type [WT] PR8) or the first 126 amino acids (NS1-126). NS1 truncations did not affect the sequence of nuclear export protein (NEP). Following rescue, virus stocks were grown in 10-day-old eggs and subsequently sequenced to confirm NS1 truncation.

A novel influenza virus was mouse-adapted via serial passage in mouse lungs and then passaged in 10-day-old eggs. In order to purify virus for enzyme-linked immunosorbent assays (ELISAs), virus stocks were grown in 10-day-old chicken eggs and the allantoic fluid was harvested 72 h later. Virus was pelleted by centrifuga-
tion at 10,000 rpm for 30 min at 4°C using an SW28 rotor, resuspended in 1 × NTE buffer (0.5 mM NaCl, 10 mM Tris–HCl [pH 7.5], 5 mM EDTA), and then partially purified over a 30% sucrose cushion.

**Plaque assay and immunostaining.** Virus stocks and lung titers were determined by plaque assay of 10-fold serial dilutions in phosphate-buffered saline (PBS), bovine serum albumin (BSA), and pen-strep on MDCK cells in the presence of 1 μg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin. Cells were incubated for 48 h at 37°C. Cells were then fixed with 4% formaldehyde, and plaques were visualized with a crystal violet counterstain or by immunostaining as described previously (50, 66).

**Animals.** All animal experiments were performed in accordance with the guidelines of the Mount Sinai School of Medicine Institutional Animal Care and Use Committee. Animals were allowed access to food and water *ad libitum* and kept on a 12-h light/dark cycle. Six- to 8-week-old (Jackson Laboratories) and 18-month-old (National Institute of Aging) female BALB/c mice were anesthetized for all procedures. Young mice were anesthetized with intraperitoneal (i.p.) injection of 0.1 ml of ketamine-xylazine (1.5 mg ketamine and 0.5 mg xylazine), while aged mice were anesthetized with 0.075 ml ketamine-xylazine (0.203 mg ketamine and 0.060 mg xylazine) (aged mice are more susceptible to ketamine-xylazine than young mice). For all experiments involving viral infections, animals were euthanized if they lost 25% of their initial body weight in accordance with institutional guidelines.

**Infection experiments of young and aged BALB/c mice.** Young and aged mice were inoculated with 10 PFU of WT PR8 virus and monitored for weight loss following infection. On day 4 postinfection, animals were sacrificed and lungs were harvested and mechanically homogenized (Fast-Prep-24; MP Biochemicals) in 1 ml of PBS. Lung homogenates were serially diluted in PBS containing 0.3% BSA. Plaque assays were performed on confluent MDCK cell monolayers in the presence of 1 μg/ml TPCK-treated trypsin. Another cohort was sacrificed on day 10, and lung CD8 T cell responses were assessed by FACS analysis.

**Vaccination and challenge of young and aged BALB/c mice.** Live attenuated influenza virus expressing the first 126 amino acids of NS1 (NS1-126) was diluted to a concentration of 10³, 10⁴, or 10⁵ PFU in a volume of 50 μl and administered intranasally. The monovalent 2009 pandemic influenza virus vaccine (Sanofi) was administered intramuscularly in a volume of 50 μl. Three weeks postvaccination, animals were challenged with 2,400 PFU of WT PR8 virus or 500 PFU of mouse-adapted pandemic A/Netherlands/602/09 (pH1N1) (approximately 100 50% lethal doses [LD₅₀]). Following each homologous challenge, mice were weighed daily and monitored for signs of disease for a 14-day period.

**Virus replication in the lungs and assessment of lung pathology.** Mice were vaccinated and challenged as described above. Four days postchallenge, mice were euthanized and lung tissues were harvested. In order to determine viral lung titers, lungs were mechanically homogenized by plaque assay as described above. To assess the degree of inflammation in the lungs following infection, mice were again vaccinated and challenged as above and sacrificed on day 4. Lungs were removed and fixed in 4% paraformaldehyde (PFA), paraffin embedded, sectioned, and stained with hematoxylin and eosin (H&E). Sections were evaluated and scored blindly for weight loss following infection. On day 4 postinfection, animals were euthanized if they lost 25% of their initial body weight in accordance with institutional guidelines.

**Flow cytometry and MHC-I tetramers.** Following sublethal infection or vaccination and lethal challenge (71), mice were euthanized and single cell suspensions from whole lungs were stained with rat anti-mouse CD8α (53-6.7), rat anti-mouse CD3e (145-2C11) (Becton, Dickinson), and/or major histocompatibility complex class I (MHC-I) tetramers. In order to assess influenza virus specific CD8 T cell activation, MHC-I tetramer N³¹⁴⁻¹⁵⁻²⁴⁻⁵⁻⁶⁻⁷⁻⁸⁻⁹⁻¹⁰⁻¹¹⁻¹²⁻¹³⁻¹⁴⁻¹⁵⁻¹⁶⁻¹⁷⁻¹⁸⁻¹⁹⁻²⁰⁻²¹⁻²²⁻²³⁻²⁴⁻²⁵⁻²⁶⁻²⁷⁻²⁸⁻²⁹⁻³⁰⁻³¹⁻³²⁻³³⁻³⁴⁻³⁵⁻³⁶⁻³⁷⁻³⁸⁻³⁹⁻⁴⁰⁻⁴¹⁻⁴²⁻⁴³⁻⁴⁴⁻⁴⁵⁻⁴⁶⁻⁴⁷⁻⁴⁸⁻⁴⁹⁻⁵⁰⁻⁵¹⁻⁵²⁻⁵³⁻⁵⁴⁻⁵⁵⁻⁵⁶⁻⁵⁷⁻⁵⁸⁻⁵⁹⁻⁶⁰⁻⁶¹⁻⁶²⁻⁶³⁻⁶⁴⁻⁶⁵⁻⁶⁶⁻⁶⁷⁻⁶⁸⁻⁶⁹⁻⁷⁰⁻⁷¹⁻⁷²⁻⁷³⁻⁷⁴⁻⁷⁵⁻⁷⁶⁻⁷⁷⁻⁷⁸⁻⁷⁹⁻⁸⁰⁻⁸¹⁻⁸²⁻⁸³⁻⁸⁴⁻⁸⁵⁻⁸⁶⁻⁸⁷⁻⁸⁸⁻⁸⁹⁻⁹⁰⁻⁹¹⁻⁹²⁻⁹³⁻⁹⁴⁻⁹⁵⁻⁹⁶⁻⁹⁷⁻⁹⁸⁻⁹⁹⁻¹⁰⁰⁻¹⁰¹⁻¹⁰²⁻¹⁰³⁻¹⁰⁴⁻¹⁰⁵⁻¹⁰⁶⁻¹⁰⁷⁻¹⁰⁸⁻¹⁰⁹⁻¹¹⁰⁻¹¹¹⁻¹¹²⁻¹¹³⁻¹¹⁴⁻¹¹⁵⁻¹¹⁶⁻¹¹⁷⁻¹¹十八个月] mice were intranasally inoculated with 10 PFU of WT PR8 virus or 500 PFU of mouse-adapted pandemic A/Netherlands/602/09 (pH1N1) (approximately 100 50% lethal doses [LD₅₀]). Following each homologous challenge, mice were weighed daily and monitored for signs of disease for a 14-day period.

**RESULTS**

**Aged mice display deficits in cellular and humoral immunity following influenza virus infection.** Young (6 to 8 weeks) and aged (18 months) mice were intranasally inoculated with 10 PFU of WT PR8 virus. As shown in Fig. 1, both sets of mice responded similarly to infection, with weight changes only statistically different on day 14 (Fig. 1A). Additionally, there was no statistical difference in survival (Fig. 1B). In order to confirm that viral replication kinetics were similar in the lungs of young and old mice, infected animals were euthanized on day four and lungs were harvested. Lung viral titers were similar in both young and aged mice at this time point (Fig. 1C).

To confirm the immune senescence seen in aged mice during influenza virus infections (4, 34, 70), we next wanted to assess cellular and humoral responses following infection with WT virus in young and aged mice. Mice were again infected with 10 PFU of WT PR8 virus, and levels of CD8+ T cell activation in the lung were assayed on day 10. Aged mice had statistically significant lower numbers of total CD8+ T cells in the lung (Fig. 2A). Additionally, aged mice had lower total cell numbers and percentages of CD8+ T cells specific for influenza virus nucleoprotein (NP) (Fig. 2B and
C). Development of humoral immunity was also compromised in aged mice. At day 21 postinfection, inoculated young and aged mice were bled and influenza virus-specific serum antibody titers were assessed by ELISA. Antibody titers in aged mice were statistically lower than those in inoculated young mice (Fig. 2D). Despite comparable replication kinetics in young and old animals, the immunological response to infection differs with age, with a waning degree of immunity in the aged mice compared to the young mice.

Live attenuated vaccine vectors are safe in young and aged mice and protect animals from stringent lethal challenge. Given that NS1-truncated viruses have successfully protected young mice from viral challenges (27, 69), we hypothesized that live attenuated viruses could potentially be successful vaccine vectors in aged mice. At day 21 postinfection, inoculated young and aged mice were bled and influenza virus-specific serum antibody titers were assessed by ELISA. Antibody titers in aged mice were statistically lower than those in inoculated young mice (Fig. 2D). Despite comparable replication kinetics in young and old animals, the immunological response to infection differs with age, with a waning degree of immunity in the aged mice compared to the young mice.

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aged mice. While it is known that NS1-126 viruses serve as safe and effective vaccines in young mice (78), the effect of infection in the context of immunosenescence is unknown, as these viruses are not as attenuated as those with larger NS1 truncations (27). NS1-126 viruses were rescued, sequenced to confirm NS1 deletion, and inoculated onto an MDCK monolayer culture to assess attenuation in an IFN-competent system. As expected, this virus displayed an attenuated plaque assay phenotype compared to WT virus. (B) Young and aged mice (n = 5 per dose) infected with 10, 10², or 10³ PFU of NS1-126. An additional group of aged mice was inoculated with 10⁴ PFU of the NS1-126 vaccine. Mice did not lose weight during the 14-day monitoring period. Aged mice are plotted in blue; young mice are plotted in green. (C) Young (n = 3) and aged (n = 3) mice were infected with 10⁴ PFU of NS1-126 virus. Fold induction of IFN-β was calculated based on mock-vaccinated, aged-matched controls (n = 1, young mouse; n = 1, aged mouse), normalized to tubulin expression. Aged mice are plotted in blue; young mice are plotted in green.

FIG 3 NS1-126 influenza virus vaccine displayed attenuated phenotypes in vitro and in vivo. (A) PR8 influenza viruses encoding the first 126 amino acids of the NS1 protein (NS1-126) display smaller plaques on MDCK cells compared to WT virus. (B) Young and aged mice (n = 5 per dose) infected with 10, 10², or 10³ PFU of NS1-126. An additional group of aged mice was inoculated with 10⁴ PFU of the NS1-126 vaccine. Mice did not lose weight during the 14-day monitoring period. Aged mice are plotted in blue; young mice are plotted in green. (C) Young (n = 3) and aged (n = 3) mice were infected with 10⁴ PFU of NS1-126 virus. Fold induction of IFN-β was calculated based on mock-vaccinated, aged-matched controls (n = 1, young mouse; n = 1, aged mouse), normalized to tubulin expression. Aged mice are plotted in blue; young mice are plotted in green.

only the first 126 amino acids of the interferon antagonist NS1 protein, we would expect interferon (IFN) expression to be elevated in the infected host (20, 28). Aberrant cytokine responses have been reported in aged mice following influenza infection (70), and we therefore wanted to assess whether these viruses were capable of stimulating IFN expression in aged mice. Despite their immunosenescent phenotype, IFN expression was upregulated in the lungs of young and aged mice by 48 h postvaccination as determined by RT-PCR (Fig. 3C).

Three weeks post vaccination, young and aged mice were challenged with 100 LD₅₀ of WT PR8 virus. Mock-infected mice succumbed to infection beginning on day 5. Young mice were protected from death at a dose of 10² PFU, though they lost an average of 8% of their body weight in the first week postinfection. In contrast, aged animals were unprotected from challenge, with 0% survival at this vaccine dose. Impressively, both young and old mice were completely protected from lethal challenge when vaccinated with 10³ PFU of NS1-126 virus, with little to no weight loss in any of the challenged animals. Complete 100% survival was seen in both groups (Fig. 4A and B).

In order to assess the degree to which the vaccine virus suppressed viral replication in the lungs, mice were vaccinated and challenged as described above, but were sacrificed on day 4 postinfection. At this time point, lung tissues were harvested and titers were quantified by plaque assay. A reduction in viral titers was seen in both young and old mice compared to mock-vaccinated, age-matched controls. Interestingly, there was no statistical difference between the lung titers seen in young and old mice vaccinated with 10² PFU, even though this dose was not protective for old mice. At the protective dose for aged mice (10³ PFU), virus was only detected at low levels in the lungs of one out of three mice postchallenge. No virus was detected in the lungs of young mice that were vaccinated with this dose (Fig. 4C). Lung titer correlated with the degree of inflammation and damage seen in the lung as quantified by a veterinary pathologist. Pathology scores were inversely correlated with the vaccine dose in both young and aged mice (Fig. 4D).

Aged mice mount protective immunological response following NS1-126 vaccination. We next wanted to investigate correlates of protection in aged mice following NS1-126 vaccination. Following vaccination, young and aged mice were bled and their sera were tested by ELISA for influenza virus-specific antibodies. As expected, both young and aged mice responded in a dose-dependent manner to NS1-126 vaccination, with 10³ PFU eliciting higher serum IgG titers compared to age-matched animals vaccinated with 10² PFU (P = 0.002 for young mice, P = 0.0089 for aged mice). Serum IgG was statistically higher in young mice compared to that in aged mice vaccinated with the same dose. Antibody titers were barely above background in aged animals that were vaccinated with 10² PFU. This is, of course, not surprising, as this group was not protected from lethal challenge. When looking at antibody titers at the doses that provided protection to young (10³ PFU) and aged (10² PFU) mice, no statistical difference is seen (Fig. 5A). We can therefore argue that aged mice, though requiring 10-fold more vaccine, were able to mount influenza virus-specific antibody responses that were comparable to those seen in young mice.

Interestingly, aged mice vaccinated with 10² or 10³ PFU did not generate hemagglutination inhibition (HI) responses, while...
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influenza vaccines in the United States (1, 37, 51, 59). Overall, there is a general weakness in many of the studies that test efficacy of vaccination in aged mice: protection from lethal challenge is not assessed or viral challenges are not very robust. Here, we demonstrated the immunogenicity of a NS1-truncated virus vaccine, and the ability of a single vaccination to protect against stringent lethal challenge of 100 LD50 of WT PR8 virus.

While we were able to demonstrate the stimulation of adaptive immune responses following vaccination of aged mice with the NS1-126 vaccine, it is interesting to note that HI titers were undetectable in pooled sera from vaccinated aged mice. Despite being HI negative, aged mice that were vaccinated with 10^3 PFU of the NS1-126 vaccine were completely protected from lethal challenge. These data might highlight a difference in the mechanism by which young and aged mice are protected from virus challenge, though it should be noted that discrepancies between HI titer and protection elicited from live attenuated vaccines have been appreciated in other studies (10, 45).

Although a single administration of a live virus vaccine has been shown by others to be immunogenic in aged mice, the efficacy of such vaccines has not yet been assessed in the context of a lethal virus challenge (7, 26). Only Ben-Yehuda et al. tested the ability of their vaccinia virus-based approach to protect aged mice from virus challenge, though instead of monitoring morbidity and mortality, viral lung titers were quantified on day three following infection with 10^6 50% egg infectious doses (7). We have shown here, however, that viral titers may not be an accurate predictor of survival (Fig. 4). In our study, aged mice vaccinated with 10^2 PFU had statistically similar virus titers in the lung after challenge compared to young mice vaccinated with the same dose. Aged mice succumbed to infection, however, and all young mice survived (Fig. 4B). While it has been shown that replicative efficiency of virus in the mouse lung is related to viral pathogenicity (55), it appears that titer may not be completely predictive in aged mice. Since inflammatory cytokine production has been implicated in morbidity following challenge (12), and aged mice are known to have aberrant cytokine responses compared to young mice (70), this may explain why similar lung titers on day 4 postchallenge in aged mice were not predictive of survival outcomes in our experiments.

It is also of note that the lung titer of one aged mouse vaccinated with 10^2 PFU NS1-126 on day 4 was at or below the limit of detection of the assay, though this was shown to not be a protective vaccine dose in aged mice (Fig. 4). Because naïve aged mice succumbed to infection as late as day 7 (Fig. 4), it is possible that lung titers would be higher if assessed at later time points.

While we have observed many of the immunological deficits that have been associated with influenza virus infection in aged mice (4, 34, 70), we were unable to detect dramatic differences in weight loss and symptom scores as reported by Toapanta et al. (70). As shown in Fig. 1, young and aged mice responded similarly to infection following a sublethal inoculation, though aged mice recovered more slowly from infection (Fig. 1). The more stringent WT PR8 dose used by Toapanta et al. could account for this dis-
crepancy, as well as differences that may exist between laboratory strains. We have, however, found that aged mice can be more sensitive to treatment, even though it seems to be virus dependent. While young and aged mice responded similarly to WT PR8 and pH1N1 virus infection, the LD50 value of aged mice infected with X31 (mouse-adapted H3N2) was determined to be 10-fold lower than that of young mice (data not shown).

In the context of a PR8 virus, the length of the NS1 protein is inversely related to its degree of replication in the mouse (27). We therefore hypothesized that, in order to stimulate immunity to the highest levels in an immunosenescent host, a virus with greater replicative capacity, and thus a smaller NS1 truncation, should be used. Although 10-fold more virus was required, vaccination with NS1-126 virus induced influenza-specific antibody titers and CD8 T cell activation in the lung to levels comparable to what was seen in young mice (Fig. 5), rescuing the decreased ability of these aged mice to produce humoral and cellular responses following infection (Fig. 2) (4, 34, 70). Despite its less attenuated nature compared to PR8 viruses with shorter NS1 segments, the NS1-126 virus vaccine was still safe in aged mice, causing no weight loss following vaccination in the doses we administered (Fig. 3).

Compared to viruses with full-length proteins, those with shorter NS1 segments are less able to antagonize the host IFN system. We hypothesized that the function of the NS1 protein would have to be disrupted enough to attenuate the virus, yet allow it to replicate to levels that would confer protection to a host with age-related immunological deficits. The NS1-126 virus vaccine encoding a truncated version of the NS1 protein stimulated IFN expression, even in the aged mouse. An induction greater than 10-fold was seen in the lung of aged mice by 48 h postvaccination (Fig. 3), and it is likely that other inflammatory cytokines are upregulated as well (60). It is of note that cytokine production in aged mice can be delayed compared to when it is detected in young mice (70), and therefore, it is possible that IFN would be expressed in aged mice to greater levels at later time points.

While more vaccine was required in the aged mouse, we argue that the protection provided by the NS1-126 vaccine is related more to quality of the vaccine than to the quantity. In addition to the immunostimulatory nature of replicating vaccines (18, 36), the IFN produced following vaccination with NS1-126 could have a self-adjuvanting effect. Indeed, type I interferons are known to enhance the development of adaptive immune responses (22, 40, 41, 67).

Inactivated vaccine preparations are currently used to immunize those over 50 years of age. In an effort to better protect the elderly, a high-dose version of the trivalent inactivated vaccine was FDA approved for use in the elderly starting in 2010. Clinical studies comparing standard-dose and high-dose formulations demonstrated statistically higher HI titers in response to high-dose vaccination (14, 17, 25, 38). It is interesting to note that higher doses of inactivated vaccines did not provide protection from homologous challenge in the aged mouse model. Animals vaccinated with 4 or 10 times the dose of young mice all succumbed to infection (Fig. 6A), even though aged antibody titers were comparable to those seen in young mice (Fig. 6B). This suggests that these antibodies might not be as potent as those produced following vaccination with NS1-126 virus, for example. Indeed, type I interferon has been shown to enhance humoral immunity, induce isotype switching, and promote affinity maturation (19, 41) and suggests that inactivated vaccines may not be as effective for this demographic group as originally hoped.

In conclusion, we show that vaccination with an NS1-truncated virus can rescue many of the age-related immunological deficits seen following infection in 18-month-old mice. Humoral and cellular responses were produced following infection, with a single administration resulting in complete protection from a robust challenge. These findings have implications for the development of improved vaccines for this vulnerable, rapidly growing segment of the population.

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