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VACCINES AND ANTIVIRAL AGENTS

Effect of an Adenovirus-Vectored Universal Influenza Virus Vaccine on Pulmonary Pathophysiology in a Mouse Model

Santosh Dhakal,[a] Jeffrey Loube,[b] Julia A. Misplon,[c] Chia-Yun Lo,[c] Patrick S. Creisher,[a] Kathleen R. Mulka,[d] Sharvari Deshpande,[a] Wayne Mitzner,[b] Sabra L. Klein,[a] Suzanne L. Epstein[c]

[a]W. Harry Feinstone Department of Molecular Microbiology and Immunology, The Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA
[b]Department of Environmental Health and Engineering, The Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA
[c]Division of Cellular and Gene Therapies, Office of Tissues and Advanced Therapies, Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, Maryland, USA
[d]Department of Molecular and Comparative Pathobiology, The Johns Hopkins School of Medicine, Baltimore, Maryland, USA

ABSTRACT Current influenza vaccines, live attenuated or inactivated, do not protect against antigenically novel influenza A viruses (IAVs) of pandemic potential, which has driven interest in the development of universal influenza vaccines. Universal influenza vaccine candidates targeting highly conserved antigens of IAV nucleoprotein (NP) are promising as vaccines that induce T cell immunity, but concerns have been raised about the safety of inducing robust CD8 T cell responses in the lungs. Using a mouse model, we systematically evaluated effects of recombinant adenovirus vectors (rAd) expressing IAV NP (A/NP-rAd) or influenza B virus (IBV) NP (B/NP-rAd) on pulmonary inflammation and function after vaccination and following live IAV challenge. After A/NP-rAd or B/NP-rAd vaccination, female mice exhibited robust systemic and pulmonary vaccine-specific B cell and T cell responses and experienced no morbidity (e.g., body mass loss). Both *in vivo* pulmonary function testing and lung histopathology scoring revealed minimal adverse effects of intranasal rAd vaccination compared with unvaccinated mice. After IAV challenge, A/NP-rAd-vaccinated mice experienced significantly less morbidity, had lower pulmonary virus titers, and developed less pulmonary inflammation than unvaccinated or B/NP-rAd-vaccinated mice. Based on analysis of pulmonary physiology using detailed testing not previously applied to the question of T cell damage, mice protected by vaccination also had better lung function than controls. Results provide evidence that, in this model, adenoviral universal influenza vaccine does not damage pulmonary tissue. In addition, adaptive immunity, in particular, T cell immunity in the lungs, does not cause damage when restimulated but instead mitigates pulmonary damage following IAV infection.

IMPORTANCE Respiratory viruses can emerge and spread rapidly before vaccines are available. It would be a tremendous advance to use vaccines that protect against whole categories of viruses, such as universal influenza vaccines, without the need to predict which virus will emerge. The nucleoprotein (NP) of influenza virus provides a target conserved among strains and is a dominant T cell target. In animals, vaccination to NP generates powerful T cell immunity and long-lasting protection against diverse influenza strains. Concerns have been raised, but not evaluated experimentally, that potent local T cell responses might damage the lungs. We analyzed lung function in detail in the setting of such a vaccination. Despite CD8 T cell responses in the lungs, lungs were not damaged and functioned normally after vaccination alone and were protected upon subsequent infection. This precedent provides important support for vaccines based on T cell-mediated protection, currently being considered for both influenza and SARS-CoV-2 vaccines.

KEYWORDS antibody, influenza A virus, influenza B virus, oxygen exchange, pulmonary function, recombinant adenovirus, T cell response, vaccine safety, universal influenza vaccine, adenovirus

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Address correspondence to Suzanne L. Epstein, suzanne.epstein@fda.hhs.gov.

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Seasonal influenza virus vaccines have variable effectiveness against circulating viruses each year, ranging from 20% to 60% in the United States in the past decade (https://www.cdc.gov/flu/vaccines-work/effectiveness-studies.htm). Current influenza vaccines, either live attenuated or inactivated, also do not provide protection against antigenically novel influenza A viruses (IAVs) of pandemic potential (1). Universal influenza vaccines could address these problems by providing broad protection against diverse strains and subtypes of influenza viruses. As per the U.S. National Institutes of Health (NIH), a universal influenza vaccine should provide greater than 75% protection against group 1 and 2 IAVs for more than 1 year in all age groups (1). A variety of universal influenza vaccine candidates are currently being studied in animal models, and clinical trials target various highly conserved antigens of IAVs (reviewed in reference 2). Broadly, cross-reactive activity against diverse virus strains has been shown for immune responses to the hemagglutinin (HA) stalk region (3–5), nucleoprotein (NP; see references below), and matrix 2 (M2) protein (6–8).

Efforts to develop universal influenza vaccines have been ongoing for decades. NP has long been known to be a prominent target of T cell immunity to influenza virus and a dominant antigen in CD8 T cell responses (9, 10). For vaccine purposes, NP has been evaluated as a promising target antigen, with T cell immunity playing an important role in broad protection (11–14). Nonneutralizing antibodies to NP have also been shown to play a role in protection (15). Forms of NP vaccines have included protein (16–18), DNA vaccines (11, 12), and DNA prime viral boost regimens with poxvirus or adenovirus vectors (13, 19).

The present study uses a recombinant adenovirus vector (rAd) expressing IAV NP (A/NP-rAd), a vector which has previously been studied either alone or in combination with rAd expressing M2. Intranasally delivered A/NP plus M2-rAd induces activated T cells and antibody (IgG and IgA) responses in the lungs that persist long term (20, 21) and can provide protection against diverse IAVs, including H1N1, H5N1, and H3N2 subtypes, in mice and ferrets (20, 22). The vaccine protects not only young adult but aged mice (21), reduces transmission to susceptible contacts (23), and is effective in mice with histories of previous respiratory infections, including rhinovirus, respiratory syncytial virus, and influenza A and B virus infections (24).

There has long been concern about the safety of inducing robust CD8 T cell responses in the lungs. Historically, nude mice lacking T cells survive better than wild-type mice shortly after infection, although they later succumb (25). In addition, transgenic mice with a repertoire dominated by CD8 T cells specific for influenza NP had increased pathology when challenged with high virus doses (26). In the case of a vaccine inducing a powerful CD8 T cell response, the concern has been raised that upon subsequent influenza virus infection, the enhanced T cell killing in the infected lungs might cause pulmonary tissue damage, exacerbating the effects of disease (26–28). This possibility has not been experimentally evaluated for such a vaccine.

Using a mouse model, we systematically evaluated the effects of adenovirus-vectored universal influenza vaccine on pulmonary functions after the initial vaccination and following IAV challenge. For this study, we chose intranasal A/NP-rAd alone rather than in combination with M2-rAd because of the dominance of the anti-NP CD8 T cell response in protection and because A/NP-rAd alone has been shown to be adequate to protect mice against lethal challenge (20). The study was designed to test for adverse effects on the lungs either due to immune and inflammatory responses to the adenoviral vector postvaccination or to T cell activity restimulated by challenge infection. Multiple measures of pulmonary function were used, including lung capacity, compliance (a measure of the ability of the lungs to inflate), resistance (a measure of frictional resistance to airflow in the lungs), and diffusion capacity (a measure of gas exchange), as well as examination of inflammation by histology.

RESULTS

Single-dose intranasal adenoviral influenza vaccine does not alter baseline pulmonary functions and lung integrity in the mice. The main safety issue posed above is the impact of potent T cell immunity when influenza virus infection is encountered after vaccination with intranasal adenoviral influenza vaccine. However, we also

examined the safety of this vaccination itself. When a vaccine is administered intranasally to anesthetized mice, it reaches not only the upper respiratory tract but the lungs, as do drugs delivered to human lungs by aerosol. After the intranasal adenovirus-vectored vaccine was given, mice appeared healthy and did not lose body mass, but we carried out a much more rigorous analysis of possible adverse effects on lung physiology. We performed *in vivo* pulmonary function testing (PFT) in mice vaccinated with A/NP-rAd or B/NP-rAd or given phosphate-buffered saline (PBS) as a control 6 days prior (Fig. 1A). There were no differences in total lung capacity, lung compliance (ability of the lungs to inflate), pulmonary resistance to airflow, or pulmonary diffusing capacity permitting gas exchange (Fig. 1B to E) between PBS-inoculated mice and mice that were vaccinated with either A/NP-rAd or B/NP-rAd. To determine whether vaccination with adenoviral influenza vaccine resulted in lung pathology, scoring of hematoxylin and eosin (H&E)-stained lung sections was carried out at 6 days after vaccination (Fig. 1F). There was significantly more inflammation in adenoviral vector-vaccinated mice compared with PBS-inoculated mice, but the inflammation was still minimal (mean cumulative inflammation score, 0 to 0.5, on a scale up to 9) (Fig. 1F and G). This slight inflammation was primarily perivascular. These data provide evidence that intranasal administration of the vaccine does not cause damage to the lungs.

Adenoviral universal influenza vaccine induces efficient B and T cell immunity and protects mice from influenza virus challenge. Adenovirus-based universal influenza vaccines induce both B and T cell immunity after intranasal immunization. To confirm responses in these cohorts of mice, antibody responses were evaluated at 4 or 5 weeks after vaccination (Fig. 2A). As expected, mice immunized with A/NP-rAd vaccine produced robust IgG antibody responses in serum (Fig. 2B) and IgA antibody responses in bronchoalveolar lavage (BAL) fluid (Fig. 2D) specific to NP from IAV, while mice immunized with B/NP-rAd vaccine produced antibodies specific to NP from influenza B virus (Fig. 2C and E).

We have previously studied the T cell immunity induced by this vaccination and have demonstrated in the lungs production of interferon gamma (IFN-γ), interleukin 12 (IL-12), and MKC; tetramer-positive cells; expression of T cell surface activation markers by flow cytometry (20); and cytolytic activity against infected targets (21). To confirm T cell immunity in the mouse groups in this study, IFN-γ responses were evaluated in the lungs after restimulation with influenza virus peptides (Fig. 2F). Note that NP366 is a target of the CD8 T cell response and the dominant epitope of A/NP for B6 mice, while NP260 is an NP T helper cell epitope. Mice immunized with A/NP-rAd had strong IFN-γ responses in the lungs after restimulation with NP366-PR8 and NP260-PR8 peptides, while mice vaccinated with B/NP-rAd had robust IFN-γ production in response to the stimulation with the B/NP peptide pool (Fig. 2F). Bold letters indicate residues in the protein sequence of the challenge virus that differ from the sequence in the vaccine by 1 amino acid in the NP-dominant CD8 epitope $NP_{366–374}$ (ASNEN**V**ETM versus ASNENMETM) and 1 in the major CD4 epitope $NP_{260–283}$ (ARSALILRGSVAHKSCLPACVYG**L** versus ARSALILRGSVAHKSCLPACVYGP). Alignment of NP amino acid sequences expressed by the challenge virus and the vaccine insert shows homology of 92% identity, 97% positive. Empirically, T cells induced by A/NP-rAd in B6 mice cross-react with the NP of the same sequence as the challenge virus (29).

To determine whether the induced immunity provided protection against IAV infection, vaccinated mice were challenged with ma2009 H1N1 virus (Fig. 3A). The infectious virus titers at 7 days postchallenge were significantly reduced in the lungs of mice immunized with A/NP-rAd compared with mice vaccinated with either PBS or B/NP-rAd (Fig. 3B). Mice vaccinated with either PBS or B/NP-rAd suffered morbidity after IAV challenge as evidenced by a 15 to 30% loss of body mass (Fig. 3C) and 2 to 4°C loss in body temperature (Fig. 3D), which was not observed among A/NP-rAd-vaccinated mice. The B/NP-rAd vaccine induces antibody and T cell responses against influenza B virus and does not provide protection after challenge with IAV. The independent set of mice that were vaccinated in parallel at the Food and Drug Administration (FDA) for antibody and T cell determinations was also challenged with the same virus and showed similar morbidity results (data not shown). Overall,

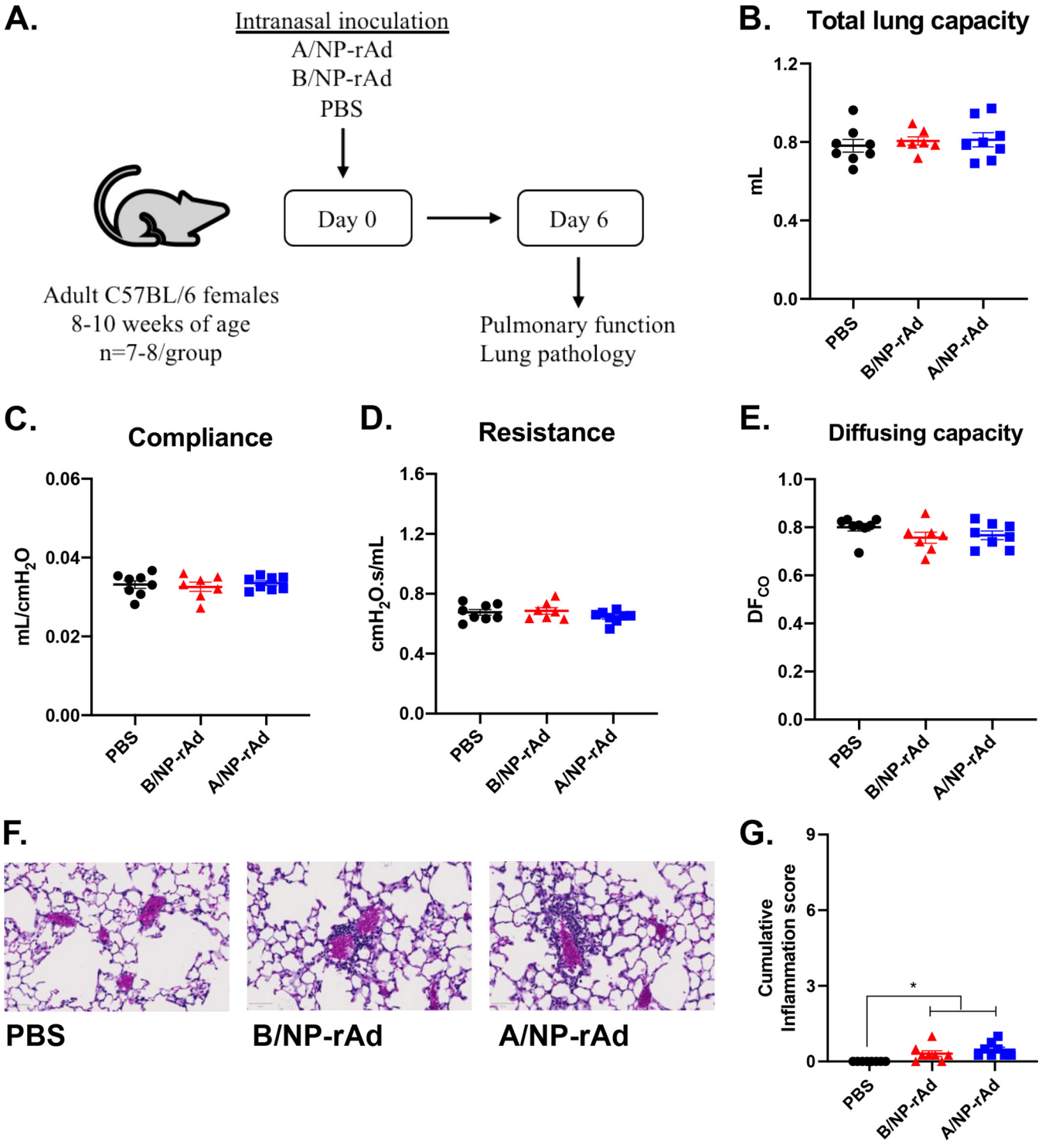

FIG 1 Adenoviral universal influenza vaccine does not alter pulmonary function and lung integrity after vaccination. (A) Adult (8- to 10-week-old) female mice were vaccinated once with A/NP-rAd, B/NP-rAd, or PBS by the intranasal route. Six days after vaccination, a subset of mice was utilized to measure pulmonary functions and euthanized for performing histopathology analysis of lung tissues. (B to E) Total lung capacity (B), pulmonary compliance (C), pulmonary resistance (D), and lung diffusion capacity (E) were measured for determining lung function. (F and G) Representative images of H&E-stained sections from each group taken at ×10 magnification (F) and cumulative inflammation scores in the lungs (G) are shown. Data represent mean ± standard error of the mean (SEM) from 7 to 8 mice/group, and significant differences (*, $P < 0.05$) between groups are denoted by asterisks based on one-way ANOVAs.

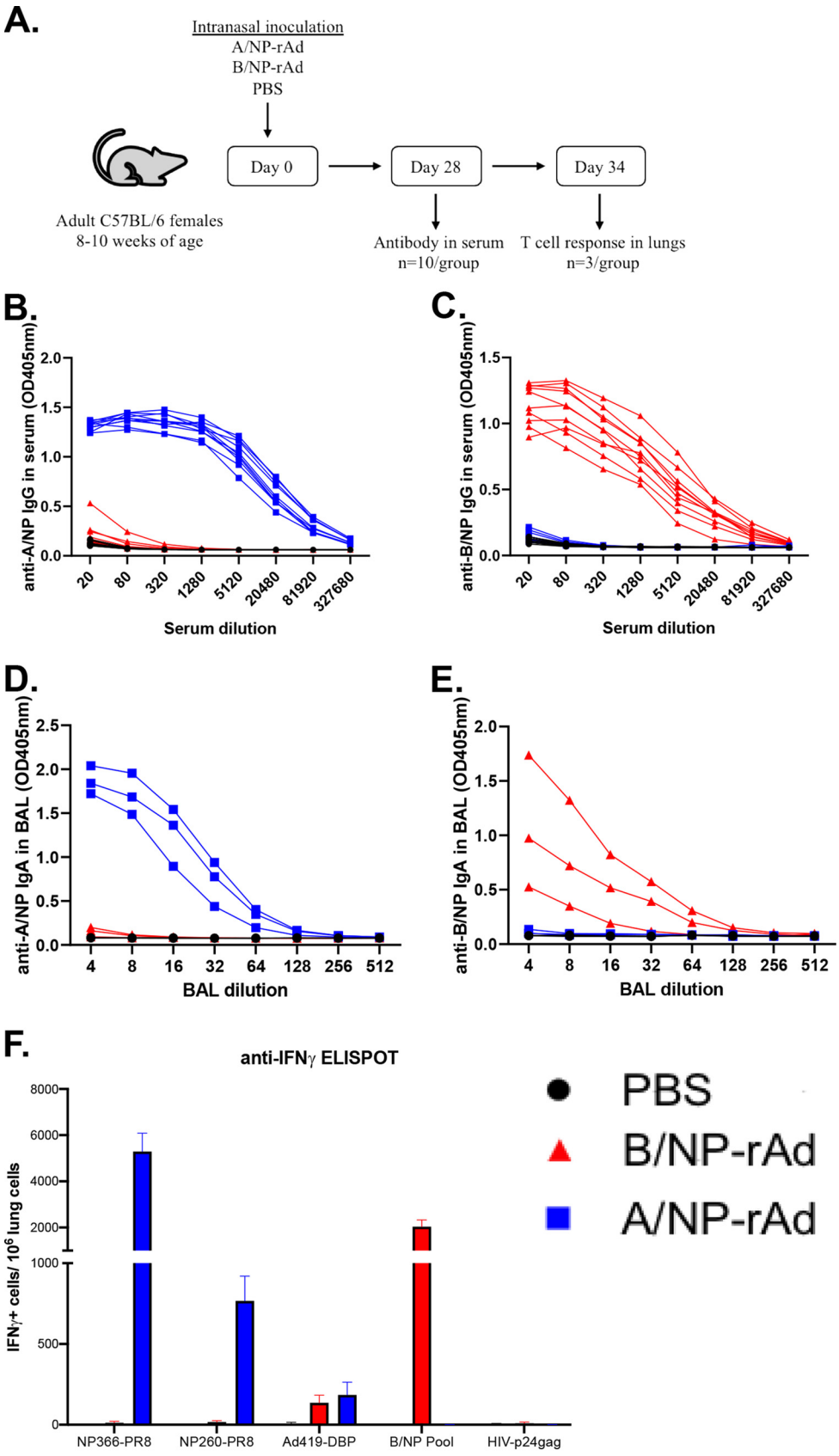

FIG 2 Single-dose intranasal adenoviral universal influenza vaccine induces antibody and T cell immunity. (A) Adult (8- to 10-week-old) female mice were vaccinated once with A/NP-rAd, B/NP-rAd, or PBS by the intranasal

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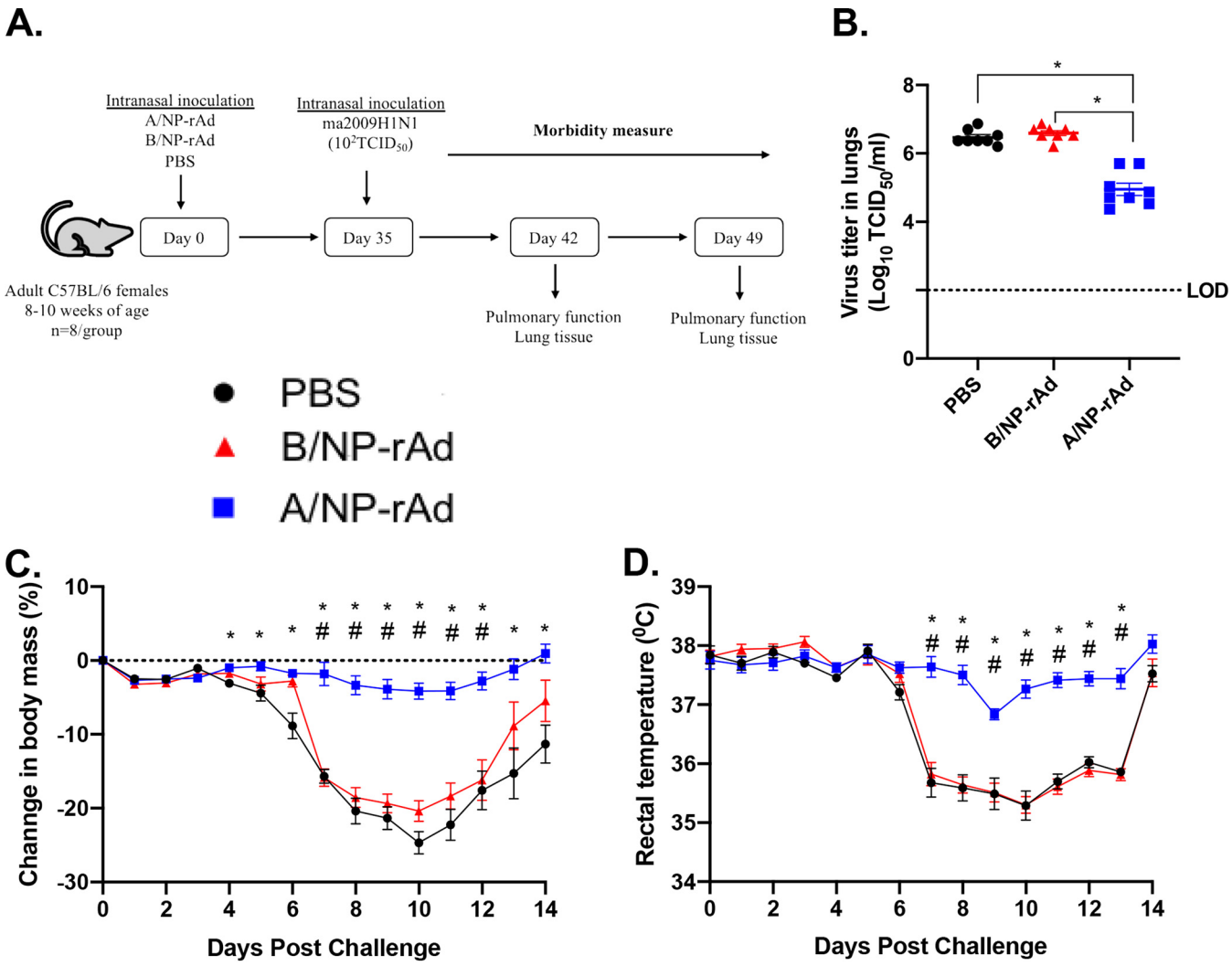

FIG 3 Single-dose intranasal adenoviral universal influenza vaccine protects mice from influenza virus infection. (A) Adult (8- to 10-week-old) female mice were vaccinated once with A/NP-rAd, B/NP-rAd, or PBS by the intranasal route. At 35 days after vaccination, mice were challenged with mouse-adapted 2009 H1N1 virus (10^2 TCID$_{50}$). A subset of mice ($n = 8$/group) was euthanized at 7 days postchallenge to determine virus titer in the lungs, and another subset ($n = 8$ to 9/group) was followed for 14 days for morbidity measures. (B to D) The infectious virus titer in the lungs at 7 days postchallenge (B), percentage change in the body mass (C), and rectal temperature (D) until 14 days postchallenge are shown. One of the 9 mice in PBS group reached the humane endpoint of 30% body mass loss on day 11 postchallenge, while all other mice recovered. Data represent mean ± standard error of the mean from 8 to 9 mice/group. In panel B, significant differences (*, $P < 0.05$) between groups are denoted by asterisks as determined by one-way ANOVA. Likewise, * and # in panels C and D represent significant differences between A/NP-rAd versus PBS and A/NP-rAd versus B/NP-rAd, respectively, based on repeated-measures ANOVA (mixed model).

these data confirm that single-dose intranasal administration of A/NP-rAd alone can induce robust antibody and T cell immunity and protect mice from IAV challenge.

Intranasal adenovirus-vectored universal influenza vaccine protects lung integrity and function after IAV challenge. When vaccinated mice are subsequently challenged with influenza virus, T cells recognizing influenza virus antigens are reacti-

FIG 2 Legend (Continued)
route. Twenty-eight days after vaccination, serum was collected to analyze antibody responses, while a subset of mice ($n = 3$/group) was euthanized at 34 days after vaccination to analyze T cell immunity in the lungs and IgA antibody responses in BAL fluid. (B to E) IgG antibody responses against A/NP (B) and B/NP (C) and IgA antibody responses against A/NP (D) and B/NP (E) were determined by ELISA. (F) T cell responses were measured by ELISPOT detecting IFN-γ-secreting cells in response to stimulation by indicated peptides. Data represent the average number of IFN-γ-positive lung cells per 10^6 cells from 3 mice per immunization group, with each sample run in triplicate. Bars show mean ± SEM for three animals. Converting to total responding lung cells per animal, results correspond to, for example, a CD8 response to the NP366 epitope in the lungs of A/NP-immunized mice of approximately 25,000 cells/mouse.

vated. To determine whether the T cell immunity in the lungs induced by adenoviral influenza vaccine resulted in pulmonary tissue damage upon IAV infection, we challenged vaccinated mice with IAV and performed pulmonary function tests and histopathological analysis at 7 and 14 days postchallenge (Fig. 4A).

The total lung capacity did not change as either a function of vaccination or IAV challenge in any of the mice (Fig. 4B). The dynamic lung compliance, which measures the ability of lungs to stretch and expand, was significantly reduced in PBS- and B/NP-rAd-vaccinated mice compared with A/NP-rAd vaccinated mice at 7 and 14 days post-IAV challenge (Fig. 4C). The pulmonary resistance, which measures the frictional resistance to airflow in the lungs, was significantly lower (i.e., better airflow) in the A/NP-rAd vaccine group than either PBS- or B/NP-rAd-vaccinated mice at 7 days postchallenge and than the B/NP-rAd group at 14 days postchallenge (Fig. 4D). The diffusing capacity (DFCO), which measures the ability of the lungs to exchange gas, was also significantly reduced in both PBS- and B/NP-rAd-vaccinated mice after IAV challenge during peak disease (7 days postchallenge) and the recovery (14 days postchallenge) phase compared with A/NP-rAd-vaccinated mice (Fig. 4E).

The scoring of H&E-stained lungs (Fig. 5A; Fig. S1) showed no difference in the cumulative inflammation score at 7 days postchallenge but significantly greater inflammation at 14 days postchallenge in the PBS-inoculated group compared with either A/NP-rAd or B/NP-rAd groups (Fig. 5B). At 14 days postchallenge, inflammation was greatest in PBS-treated mice, followed by B/NP-rAd, and lowest in the A/NP-rAd group (Fig. 5B). Perivascular inflammation (Fig. 5C) was greater in A/NP-rAd-inoculated mice at 7 days postchallenge, while PBS-inoculated mice had greater peribronchiolar (Fig. 5D) and alveolar (Fig. 5E) inflammation at 14 days postchallenge. Together, these data suggest that the T cell immunity in the lungs induced by adenoviral influenza vaccine did not result in pulmonary tissue damage upon IAV infection. Vaccination reduced overall inflammation but selectively and transiently enhanced perivascular inflammation without compromising lung functions, including gas exchange.

DISCUSSION

Local immune responses in the respiratory tract are a powerful source of protection because they are focused at the site of infection. Mucosal IgA antibody responses in the airways are an important correlate of protection against influenza viruses by neutralizing the virus at the site of entry (30). Cell-mediated immune responses induced by the highly conserved structural proteins, including NP, clear virus, reduce disease severity, and provide heterosubtypic immunity to influenza viruses (31–33). Adenovirus-vectored influenza vaccines, incorporating conserved influenza virus antigens like NP and M2, are effective vaccine platforms for inducing both mucosal and systemic antibody- and cell-mediated immunity and providing broad protection against a wide range of influenza virus subtypes (20, 34, 35). In this study, induction of robust pulmonary antibody (i.e., IgG and IgA) and T cell responses was observed in female mice vaccinated with either the A/NP-rAd or B/NP-rAd vaccines. The induction of strong T cell immunity in the lungs through vaccination is considered a double-edged sword because of concern that heightened pulmonary cytotoxic and inflammatory responses after subsequent infection with influenza virus could worsen disease (26–28). As far as we know, this is the first study empirically addressing the question with a panel of pulmonary function tests. We did not observe adverse effects on pulmonary function following either intranasal adenovirus-vectored universal influenza vaccination or IAV challenge.

Influenza virus infection can decrease alveolar fluid clearance, leading to pulmonary edema and acute respiratory distress syndrome (ARDS) (36, 37). To the extent that some alveoli are filled with fluid, there is less space for air inflation, and this volume loss will lead to decreased total lung compliance (38). In addition, the presence of interstitial fluid around airways leads to a decreased coupling between the airways and lung parenchyma causing narrowed airways with increased airflow resistance (39, 40).

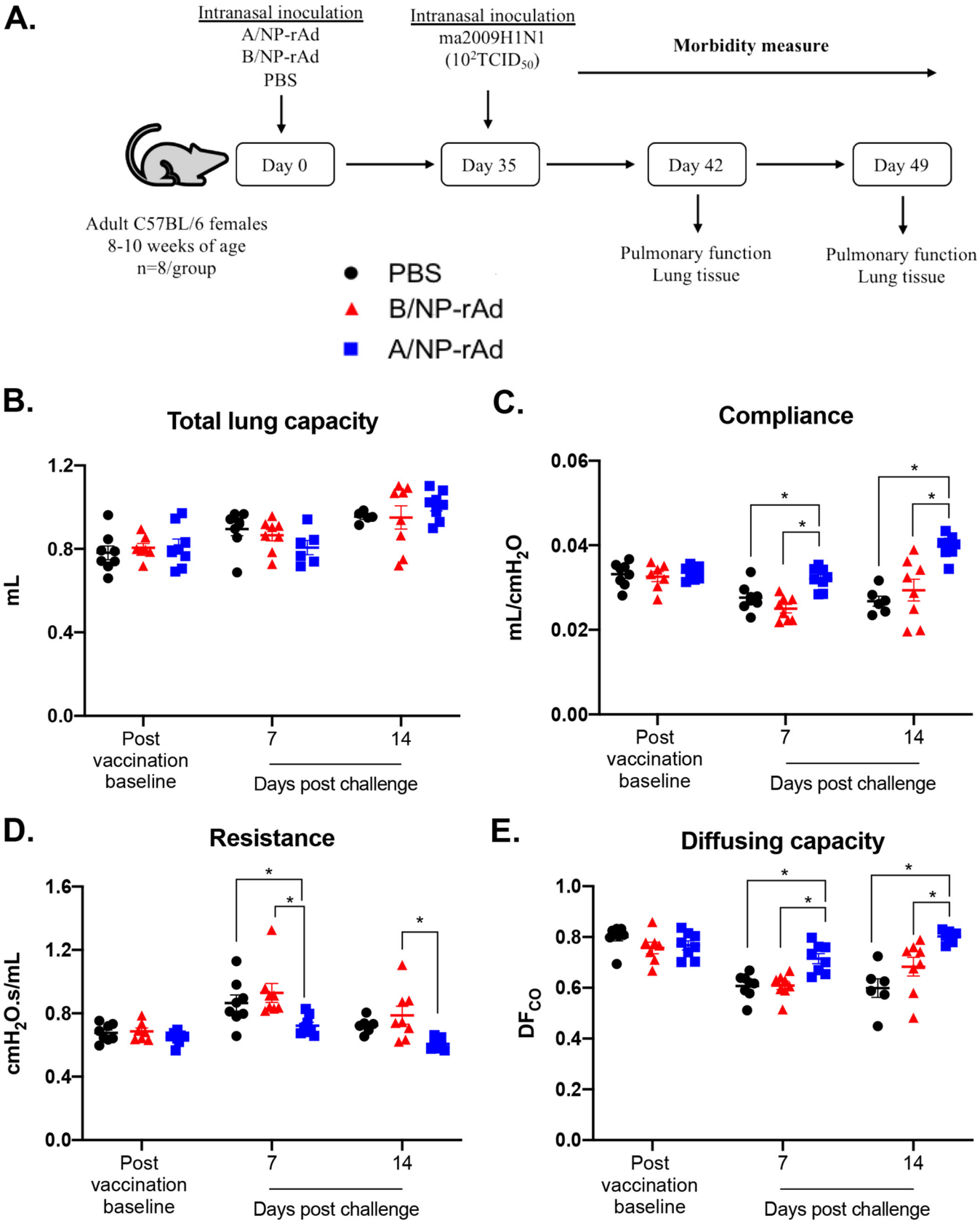

FIG 4 Adenoviral universal influenza vaccine maintains pulmonary functions after influenza virus challenge. (A) Adult (8- to 10-week-old) female mice were vaccinated once with A/NP-rAd, B/NP-rAd, or PBS by the intranasal route. At 35 days after vaccination, mice were challenged with mouse-adapted 2009

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In the current study, mice that received the A/NP-rAd vaccine had greater pulmonary compliance and lower airway resistance than mice that received either PBS or B/NP-rAd, suggesting that vaccination reduced pulmonary disease following IAV infection.

Reduced pulmonary function, in particular, the decrease in the ability to exchange gas, is of significant concern in influenza-associated ARDS (37). The excess water allowed to accumulate by influenza-induced damage lengthens the gas diffusion pathways, resulting in low oxygenation of blood. In humans, decreased gas exchange, as measured by the diffusing capacity for carbon monoxide (DLCO), can persist after recovery from IAV infection (41, 42). In mice, we measured a diffusing capacity which is equivalent to what is measured in humans (43, 44) and showed that gas exchange was significantly compromised in control mice but preserved in the A/NP-rAd group, even after IAV challenge. Moreover, the H&E staining of the lungs also indicated that the resolution of pulmonary inflammation was improved in A/NP-rAd-vaccinated mice compared with controls.

Regarding the particular vector used here, replication-deficient adenovirus vectors can incorporate large transgenes, are stable, can be grown to high titers, have adjuvant potential, and can stimulate cellular and humoral immune responses in a sustained manner (reviewed in reference 45). Prior immunity to the vector in humans could theoretically block antigen-specific immune responses, which could be circumvented by using rare serotypes of human adenovirus or adenovirus of nonhuman origin. Adenovirus-vectored vaccines against different infectious diseases, including hepatitis C virus (46), respiratory syncytial virus (RSV) (47), human immunodeficiency virus type 1 (HIV-1) (48, 49), Ebola virus (50), and IAV (51, 52) are being tested in different phases of clinical trials. Moreover, adenovirus vectors are one of the leading platforms for vaccines against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (53–56). However, the vaccine safety concern about an intensely focused local T cell response in the lungs could apply to any vector type if administered intranasally.

It is known that host-associated factors, including age and sex, impact the performance of seasonal and universal influenza vaccines (21, 57). Host genetics also have a major impact on immune responses to influenza antigens (58). These additional factors should be considered in future studies with respect to the intranasal adenovirus influenza vaccine and evaluation of pulmonary function after subsequent IAV infection. The balance of beneficial versus potentially harmful effects of vaccination could also differ with virus challenge dose. The challenge dose used here permitted survival of the animals for analysis 7 and 14 days later. The virus dose was sufficient to cause morbidity, thus establishing infection and sufficient to restimulate the T cells present in the lungs prior to challenge.

In the animal model studied here, we found that potent T cell immunity induced in the lungs by intranasal adenovirus influenza vaccine did not damage pulmonary tissue. Lung function and integrity were preserved, as measured by an extensive panel of lung physiology tests. Thus, the especially powerful protection offered by mucosal immunity at the site of infection, including potent T cell responses in the lungs, is promising as part of universal influenza vaccination. One cannot assume that outcomes would be the same in humans, but certain invasive measurements such as local lung T cell responses and lung viral titers cannot be made in humans. Studies in animal models provide context for interpreting those analyses that are feasible in humans.

Respiratory viruses cause a common category of diseases and are extremely contagious as shown by SARS-CoV-2. Vaccine candidates making use of all arms of the immune system may be needed, and local immunity in the respiratory tract has improved vaccine protection in animal models. Our results on the preservation of lung

FIG 4 Legend (Continued)
H1N1 virus (10^2 $TCID_{50}$). Pulmonary function tests were performed at 7 and 14 days postchallenge. (B to E) Total lung capacity (B), pulmonary compliance (C), pulmonary resistance (D), and lung diffusion capacity (E) were measured to determine lung functions. Data represent mean ± SEM from 6 to 8 mice/group, and significant differences (*, $P < 0.05$) between groups are denoted by asterisks based on two-way ANOVAs.

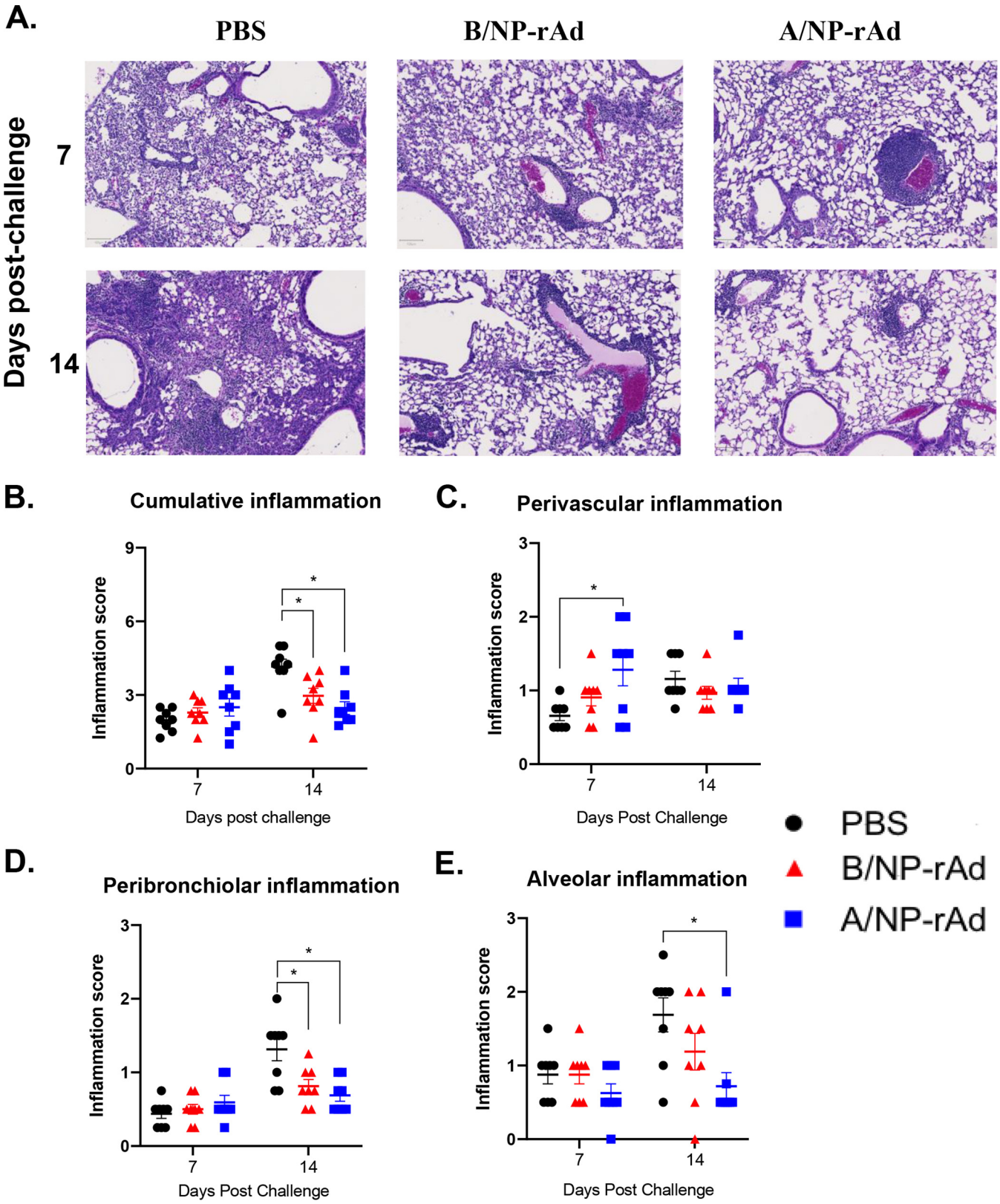

FIG 5 Adenoviral universal influenza vaccine does not exacerbate pulmonary inflammation after subsequent infection with influenza virus. Adult (8- to 10-week-old) female mice were vaccinated once with A/NP-rAd, B/NP-rAd, or PBS by the intranasal route. At 35 days after vaccination, mice were challenged with mouse-adapted 2009 H1N1 virus (10^2 $TCID_{50}$). A subset of mice was euthanized at 7 and 14 days postchallenge, and lungs were collected, fixed, and H&E stained. (A) Representative images of H&E-stained sections taken at ×4 magnification are shown and can be compared to the prechallenge

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function with little damage or inflammation are encouraging for the use of such strategies for vaccines against respiratory infections.

MATERIALS AND METHODS

Animals. All animal procedures were approved by the Johns Hopkins University Animal Care and Use Committee under animal protocol number MO18H250 or by the Food and Drug Administration (FDA) White Oak Campus Animal Care and Use Committee under protocol number 1991-06. Adult (7- to 8-week-old) female C57BL/6 (B6) mice were purchased from Charles River Laboratories. All mice were housed at 5 animals per cage under standard biosafety level 2 housing conditions at either Johns Hopkins Bloomberg School of Public Health or the FDA White Oak Campus Vivarium with *ad libitum* food and water.

Recombinant adenoviral vaccine preparation. Replication-deficient (E1 and E3 deleted) recombinant adenovirus-5 (rAd) vectors expressing conserved IAV nucleoprotein antigens from A/PR/8/34 (A/NP-rAd) or B/Ann Arbor/1/86 (B/AA) (B/NP-rAd) were constructed as described previously (13), and batches prepared at ViraQuest, North Liberty, IA. B/NP-rAd vaccines were used as a specificity control, not providing protection against IAV challenge.

Vaccination, challenge, and sample collection. After a week of acclimatization in the animal facilities, mice ($n = 25$/group at Johns Hopkins University, 10/group at FDA) were immunized either with phosphate-buffered saline (PBS) as negative control or with 10^{10} virus particles of A/NP-rAd or B/NP-rAd in PBS under ketamine/xylazine anesthesia. At Johns Hopkins, 6 days after vaccination, one subset of mice ($n = 8$/group) was used for the evaluation of pulmonary functions and euthanized to collect blood and lung samples for histopathology. Four weeks after vaccination, blood samples were collected from remaining mice. At 35 days postvaccination, mice were challenged with mouse-adapted A/California/04/09 (ma2009 H1N1) virus generated by reverse genetics by Andrew Pekosz, Johns Hopkins University, from a published sequence (59). Virus was given at a sublethal dose based on previous experience, 10^2 50% tissue culture infective dose ($TCID_{50}$) in 30 μl volume inoculated intranasally under ketamine/xylazine anesthesia. The second subset of mice ($n = 8$/group) was euthanized at 7 days postchallenge, and the third subset ($n = 8$ to 9/group) was followed for 14 days postchallenge to monitor changes in body mass, temperature, and mortality. Blood was collected, and lung function tests were carried out at both 7 and 14 days postchallenge. Left lung was collected for histopathology and right lung lobes for lung lysates to determine infectious virus titers. At the FDA, the cohort of vaccinated mice was evaluated for antibody responses and T cell immunity after vaccination ($n = 3$/group) and morbidity and mortality after virus challenge ($n = 7$/group).

Influenza virus titer determination in the lungs. Lung samples were homogenized in 500 μl of serum-free Dulbecco's modified Eagle's medium (DMEM), and the infectious virus titer was determined on the supernatants using the $TCID_{50}$ assay as previously described (60). Briefly, lung homogenates were 10-fold serially diluted and transferred into 96-well tissue culture plates, in replicates of six, confluent with Madin-Darby canine kidney (MDCK) cells. Plates were incubated at 32°C for 6 days, fixed with 4% formaldehyde solution, and stained with naphthol blue-black solution, and the $TCID_{50}$ was determined by using the Reed-Muench method (61).

Antibody and T cell responses. Serum samples were collected at 28 days after vaccination and tested for IgG antibodies against the nucleoproteins of influenza A and B viruses by enzyme-linked immunosorbent assay (ELISA). Bronchoalveolar lavage (BAL) fluid collected from a subset of mice 34 days after vaccination was tested by the same method for both IgG and IgA antibodies. Lung cells isolated at 34 days postvaccination from a subset of mice were used for IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assay after stimulation with the following peptides. $NP_{366–374}$ from A/PR/8 H1N1 with the sequence ASNENMETM (NP366-PR8) is the dominant CD8 epitope for B6 mice. $NP_{260–283}$ is an NP helper epitope with the sequence ARSALILRGSVAHKSCLPACVYGP from A/PR/8 (NP260-PR8). For testing the response to B/NP, a pool of overlapping peptides spanning the nucleoprotein from B/AA, composed of 17-mer amino acid (aa) peptides that overlap by 10 aa (B/NP pool) was used. The response to rAd was measured with adenovirus 5 DNA-binding protein peptide, $Ad_{419–427}$ FALSNAEDL (Ad419). A peptide from HIV-p24 $gag_{285–307}$ QGPKEPFRDYVDRFYKTLRAEQA was used as a negative control. All peptides were manufactured by GenScript Biotech Corporation, Piscataway, NJ. ELISPOT was performed as described previously (62). In brief, filter-bottom plates were coated with anti-interferon-γ (IFN-γ) antibody. The next day, plates were blocked. Cell populations were added and stimulated with indicated peptides (200,000 to 250,000 cells/well, except that NP_{147} was also plated at 1:4 dilution to ensure the strong response to NP_{147} was readable). Interferon that was produced and bound to the plates was detected with monoclonal anti-interferon antibody followed by alkaline phosphatase-labeled streptavidin and then substrate. Plates were read on an ELISPOT reader with ImmunoSpot software from Cellular Technology Limited, Cleveland, OH.

Pulmonary function tests. Pulmonary function analyses were performed in mice at 6 days postvaccination and at 7 and 14 days after IAV challenge. All measurements were done in mice anesthetized with a ketamine-xylazine (75 mg/kg-15 mg/kg) mixture. After tracheostomy with an 18-G stub needle,

FIG 5 Legend (Continued)
histopathology images in Fig. 1F. (B to E) Cumulative pulmonary inflammation (B) was determined on a scale of 0 to 3 each for perivasculitis (C), peribronchiolitis (D), and alveolitis (E). Data represent mean ± SEM from 8 mice/group, and significant differences between groups are denoted by asterisks (*, $P < 0.05$) based on two-way ANOVAs.

the lung diffusing capacity for carbon monoxide (DFCO) was measured using gas chromatography as previously described (43). After this measurement, mice were connected to a mechanical ventilator (Flexivent, Quebec, Canada), and measurements of dynamic lung compliance, airway resistance, and total lung pressure-volume (PV) curves were measured as previously described (63). The pressure-volume curve procedure involves degassing the lungs by absorbing oxygen, a process which stops the heart. After these curves were completed, lungs were excised for histologic assessment.

Histopathology. The left lung lobes were inflated with zinc-buffered formalin (Z-Fix; Anatech, Battle Creek, MI, USA) under a constant pressure of 25 cm H_2O and fixed for at least 24 h. Fixed lungs were then sliced into 3-mm blocks, embedded in paraffin, sectioned into 5 μm size, mounted on glass slides, and stained with hematoxylin and eosin (H&E) solution. Histopathological scoring to assess lung inflammation was done as described previously (64). Briefly, single-blinded observation was made by a veterinary pathologist on a scale of 0 to 3 (0, no inflammation; 1, mild inflammation; 2, moderate inflammation; and 3, severe inflammation) for perivascular, peribronchiolar, and alveolar inflammation. Thus, a cumulative inflammation score of 0 to 9 was possible for each mouse lung, depending on the severity of inflammation.

Statistical analyses. Statistical analyses were performed in GraphPad Prism 8. Antibody responses, T cell responses, virus titers, lung inflammation scores, and lung function test results were analyzed by one-way or two-way analyses of variance (ANOVAs) followed by Tukey's *post hoc* test for multiple comparisons. For morbidity measures, repeated-measures ANOVA or mixed-model analysis was performed. Mean differences were considered significantly different if P was <0.05.

Data availability. In compliance with the ASM journals data policy, all relevant data are readily available. While data supporting the conclusions is all contained within this paper, other related data will be provided in response to requests. The other data collected in the course of the study were additional parameters of pulmonary physiology measured by the instruments during the same run but not reported here.

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S.L.K., S.L.E., W.M., and S.D. conceived and designed the research; S.D., J.L., J.A.M., C.-Y.L., S.D., P.S.C., and K.R.M. performed the experiments; S.D., J.L., and J.A.M. analyzed data; S.D., S.L.E., W.M., S.L.K., J.A.M., C.-Y.L., and K.R.M. interpreted results of the experiments; S.D. and P.S.C. prepared figures; S.D. and S.L.K. drafted the manuscript; S.D., J.L., J.A.M., W.M., C.-Y.L., S.L.E., and S.L.K. edited and revised the manuscript; and all the authors approved the final version of the manuscript.

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