Successful respiratory immunization with dry powder live-attenuated measles virus vaccine in rhesus macaques

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Edited by Peter Palese, Mount Sinai School of Medicine, New York, NY, and approved January 6, 2011 (received for review December 2, 2010)

Measles remains an important cause of childhood mortality worldwide. Sustained high vaccination coverage is the key to preventing measles deaths. Because measles vaccine is delivered by injection, hurdles to high coverage include the need for trained medical personnel and a cold chain, waste of vaccine in multidose vials and risks associated with needle use and disposal. Respiratory vaccine delivery could lower these barriers and facilitate sustained high coverage. We developed a novel single unit dose, dry powder live-attenuated measles vaccine (MVDP) for respiratory delivery without reconstitution. We tested the immunogenicity and protective efficacy in rhesus macaques of one dose of MVDP delivered either with a mask or directly intranasal with two dry powder inhalers, PuffHaler and BD Solovent. MVDP induced robust measles virus (MeV)-specific humoral and T-cell responses, without adverse effects, which completely protected the macaques from infection with wild-type MeV more than one year later. Respiratory delivery of MVDP was safe and effective and could aid in measles control.

aerosol delivery | protective immunity | multifunctional T cells | antibody avidity

Measles is a highly contagious viral disease. Before the availability of measles virus (MeV) vaccines, more than 130 million cases and 7–8 million deaths occurred annually. Intensive immunization efforts with the live attenuated measles vaccine (LAMV) given by injection have resulted in substantial decreases in global measles disease. However, with an estimated 164,000 deaths in 2008 (1), measles continues to be an important cause of child mortality, especially in less-developed regions of the world. The key to prevention of measles is achieving and sustaining high levels of population immunity through vaccination, and substantial challenges for high coverage remain in many countries. Some of the challenges to providing a first dose of measles vaccine to at least 95% of each birth cohort, plus a second dose to older children, are related to the method of vaccine delivery.

Measles vaccine is given by injection, and this creates hurdles to sustained high coverage in many developing countries. First, there is often a shortage of the trained personnel needed for sterile reconstitution and safe injection of vaccine. Second, in most developing countries, lyophilized vaccine is in 5–10 dose vials that, after reconstitution, lose 30–50% potency in an hour at 37 °C (2), so unused doses must be discarded. Third, contaminated needles and syringes create risks for transmitting bloodborne disease and require safe disposal.

As a potential improvement, respiratory delivery of reconstituted liquid LAMV has been studied for more than three decades (3) but has never been licensed or widely used. Aerosol administration of aqueous vaccine is highly effective in boosting preexisting antibody and holds promise for use in older children (4–11), but primary humoral and cellular immune responses vary with the age of vaccinees (12–15).

We have developed a dry powder formulation of LAMV (MVDP) that is thermostable and can remain as powder throughout the shipping and immunization process, eliminating the need for reconstitution. In a rhesus macaque model (16), we show that MVDP delivered as a single dose with either of two dry-powder inhalers, PuffHaler and Solovent, is safe and provides durable protective immunity from measles. MVDP could facilitate high vaccine coverage in resource-limited areas.

Results

Characteristics and Delivery of the Dry Powder Live-Attenuated MeV Vaccine. CO_2-assisted nebulization with a bubble-dryer (CAN-BD) processing of liquid Edmonston–Zagreb strain of LAMV yielded a free-flowing MVDP (17, 18) with a low moisture content (0.7%), small particle size, and good stability (Table S1). The process had little effect on virus viability, and MVDP maintained potency for at least 2 y at 2–8 °C. A significant percentage of MVDP was in the range of 1–5 μm, appropriate for pulmonary delivery (19, 20).

Two unique dry-powder delivery devices, the PuffHaler (Aktiv-Dry and RPC Formatec) and the BD Solovent (BD Technologies; Fig. 1 *A* and *B*) (21–24) were used for delivery. To deliver MVDP to free-breathing macaques, the devices were connected with silicone-based masks (Puff-mask and Sol-mask) that covered the mouth and nose and provided a seal to reduce loss. For nasal administration, nasal prongs were attached to the downstream end of the PuffHaler disperser (Puff-nasal); the Solovent device was actuated directly into the nares (Sol-nasal).

Groups of three macaques were vaccinated once with 50 mg MVDP (~15,000 pfu) for PuffHaler and 23,000 pfu for Solovent) by Puff-mask, Sol-mask, Puff-nasal, or Sol-nasal. Groups of two macaques were vaccinated by s.c. injection with 1,000 pfu (standard, SC1000) or 100 pfu (low-dose, SC100) liquid LAMV (Table S2). Administration of MVDP by either inhalation or

Author contributions: W.-H.L., D.E.G., P.A.R., M.P., R.E.S., and S.W. designed research; W.-H.L., S.P.C., D.B., B.Q., C.S., K.P., R.J.A., and S.G. performed research; S.P.C., D.B., B.Q., C.S., and K.P. contributed new reagents/analytic tools; W.-H.L., P.A.R., and M.P. analyzed data; and W.-H.L., D.E.G., and S.W. wrote the paper.

Conflict of interest statement: D.B., B.Q., and R.E.S. are employees of Aktiv-Dry LLC that developed the vaccine processing and Puffhaler delivery. C.S. and K.P. are employees of BD Technologies that developed the Solovent delivery.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1017334108/-/DCSupplemental.

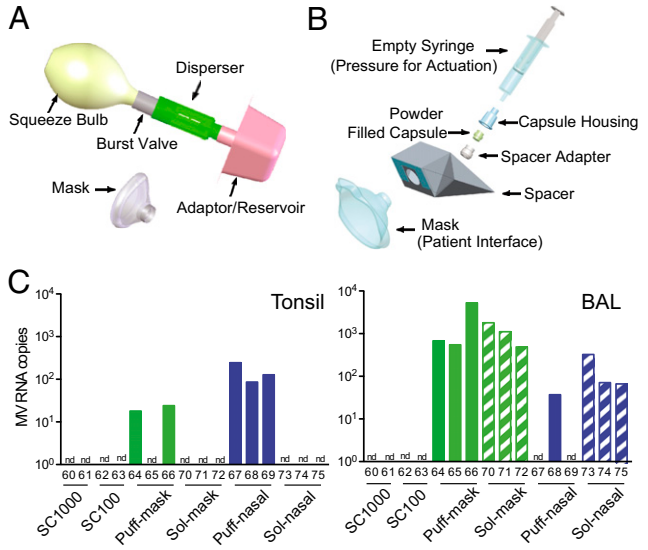

Fig. 1. Administration of MVDP. PuffHaler (*A*) or BD Solovent (*B*) inhalers were configured for aerosol delivery through a mask or a nasal interface and resulted in differential deposition and replication of MeV in the respiratory tract. (*A*) When the PuffHaler squeeze bulb is compressed to 2 psi, the silicone rubber burst valve pops open. The air rushes into the disperser through the powder in an aluminum foil blister and the aerosol cloud fills a collapsed plastic bag reservoir. The aerosol-filled bag is detached and affixed to a facemask from which the subject is allowed to breathe for 30 s to become vaccinated (Puff-mask). Alternately, the PuffHaler device is used without a reservoir and with a standard nasal prong (Puff-nasal). (*B*) The syringe of the BD Solovent device is used to pressurize the capsule containing the powder vaccine. As the pressure rises, the thin films sealing the capsule abruptly rupture, and the powder is expelled and captured in the disposable spacer for delivery through a silicone facemask (Sol-mask). Alternatively, the aerosol stream is delivered through a nasal adapter directly to the nares (Sol-nasal). (*C*) qRT-PCR analysis of MeV RNA in cells from tonsil swabs or BAL of individual monkeys at 5 d after immunization. Levels of MeV RNA were higher in monkeys immunized with a mask than with nasal interfaces (P = 0.0008). nd, not detected.

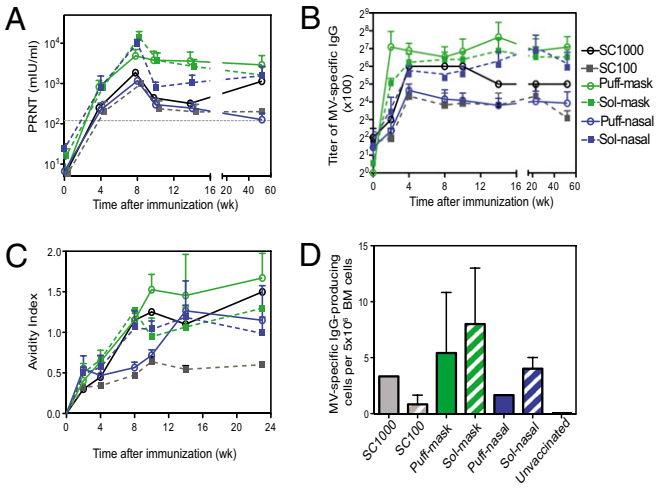

Fig. 2. MeV-specific antibody responses. (*A*) Neutralizing antibody, as measured by plaque-reduction neutralization. The predicted protective level of antibody (120 mIU/mL) is indicated with a dashed line. Values are plotted as geometric means + SEM. Significant differences were in Puff-mask vs. Puff-nasal, Sol-mask vs. SC100, Sol-mask vs. Puff-nasal ($P < 0.001$); in Puff-mask vs. SC100 (s.c. delivery of 100 pfu) and Sol-mask vs. Puff-nasal ($P < 0.01$) and in Sol-nasal vs. SC100 ($P < 0.05$). (*B*) MeV-specific IgG, as measured by EIA. Titers were determined by twofold serial dilution of samples and MeV-negative controls (mean OD + 2 SD). Values are plotted as geometric means + SEM. Significant differences were in Puff-mask, Sol-mask, and Sol-nasal vs. SC100 and Puff-nasal ($P < 0.001$). (*C*) Avidity of MeV-specific IgG assessed by disruption of antibody binding with 0–3 M NH_4SCN and calculation of an avidity index. Significant differences were between SC100 vs. Puff-mask ($P < 0.05$) and Sol-mask ($P < 0.05$). (*D*) Number of MeV-specific IgG-secreting cells in the bone marrow assessed by ELISpot assay using 5×10^5 bone marrow cells collected 12–14 mo after immunization. Assays had eight replicates. Bar graph shows the mean + SEM of the spot numbers for each vaccine group. One-way ANOVA followed by Bonferroni's multiple comparison test was used.

nasal delivery was well tolerated, with only a temporary decrease in breathing rate immediately following dosing.

Deposition and Replication of MVDP in the Respiratory Tract. MeV RNA was measured in the cells and supernatant fluids from tonsil swabs and bronchoalveolar lavage (BAL) specimens collected 5 d after immunization (Fig. 1 *C* and *D*). MeV RNA was detected in cells, but not supernatant fluids, from tonsil swabs or BAL in all animals that received respiratory MVDP, but not s.c. LAMV. Animals that received MVDP by mask showed high levels of MeV RNA in the BAL samples but not in tonsil swabs, indicating deposition and replication in the lower airways. Puff-nasal delivered vaccine to the upper airway with MeV RNA detected mostly in the tonsil swabs and Sol-nasal delivered vaccine to the lower airway with MeV RNA detected solely in BAL. Levels of total MeV RNA detected were higher in monkeys immunized with a mask than intranasally (P = 0.0008). Viral RNA was not detected in the blood of similarly vaccinated animals.

Antibody Responses After Immunization. To assess the MeV-specific humoral immune response, we measured the titers of neutralizing antibody, IgM, IgG and IgA; IgG avidity; and the numbers of IgG-producing cells in the bone marrow. Neutralizing antibodies were induced in all vaccinated monkeys (Fig. 2*A*) and developed with a similar time course. However, monkeys immunized with MVDP through Sol-mask, Puff-mask, or Sol-nasal had higher neutralizing titers than monkeys immunized through Puff-nasal or by injection (P = 0.036; Fig. 2*A* and Fig. S1). One of two monkeys that received low-dose s.c. LAMV and two of three monkeys in the Puff-nasal MVDP group had neutralizing antibodies below the predicted protective level of 120 mIU/mL at the time of wild-type MeV challenge.

To identify the classes of Ig-induced, MeV-specific IgM, IgG and IgA were assessed by enzyme immunoassay (EIA). IgM was detected at 4 wk in all monkeys immunized by Puff-mask, Sol-mask, and Sol-nasal but not in monkeys immunized by Puff-nasal (Table S3). IgG was induced in all vaccinated monkeys and those in the Puff-mask, Sol-mask, and Sol-nasal groups had higher titers than monkeys in the Puff-nasal or s.c. groups (Fig. 2*B*). Induction of IgG was more rapid in monkeys that received Puff-mask and Sol-mask MVDP than in monkeys that received Puff-nasal MVDP or s.c. LAMV (Fig. S2*A*). MeV-specific IgA was induced in monkeys immunized with Puff-mask or Sol-mask, but not in monkeys immunized s.c. IgA peaked at 2 wk after immunization (Fig. 2*B*), whereas IgG peaked at 8 wk after immunization. The avidity of the MeV-specific IgG matured over time in all groups except low-dose s.c. LAMV (Fig. 2*C*).

To evaluate humoral memory (25), bone marrow was assessed for the presence of antibody-secreting cells ~1 y after immunization. MeV-specific IgG-producing cells were present in all vaccinated animals (Fig. 2*D*), and the numbers correlated with the level of MeV-specific IgG in plasma.

Mask Administration of MVDP Elicited a Biphasic T-cell Response. To assess the timing and magnitude of the T-cell response, ELISpot assays were used to measure IFN-γ and IL-4 production after MeV peptide stimulation (Fig. 3). An increase in MeV-specific

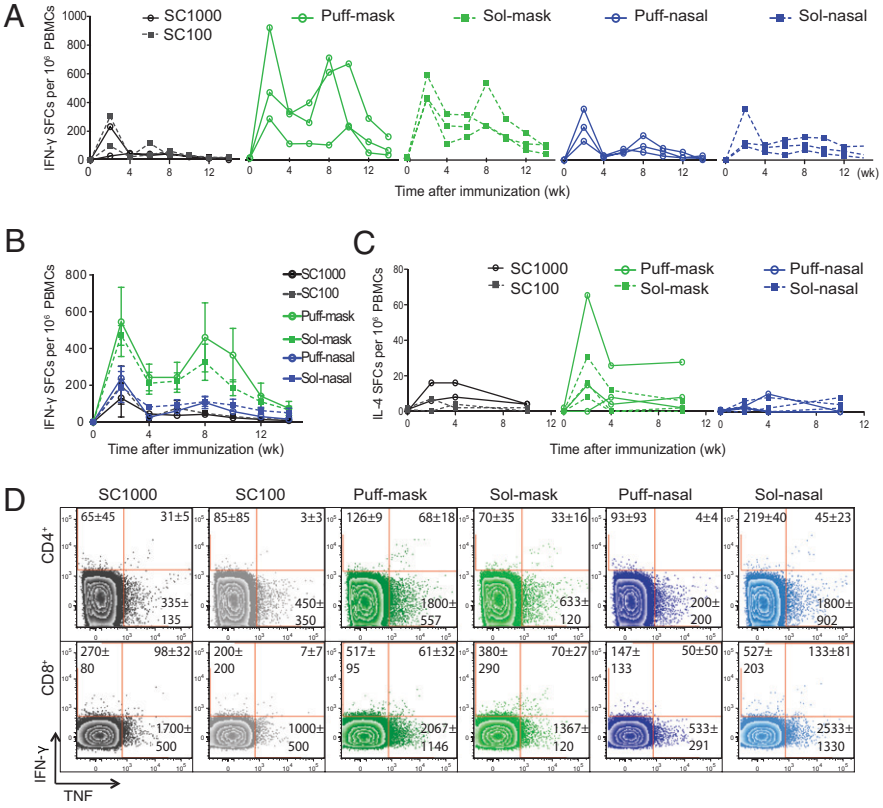

Fig. 3. Puff-mask and Sol-mask MVDP elicited robust biphasic T-cell responses. T-cell responses were assessed by IFN-γ and IL-4 ELISpot assays. PBMCs were stimulated with overlapping peptides from the hemagglutinin (H), fusion (F), or nucleoprotein (N). Numbers of specific spot-forming cells (SFCs) were calculated by subtracting nonspecific responses. Data are presented as the sum of the H, F, and N responses. (*A*) MeV-specific IFN-γ responses of individual monkeys. Data are plotted as the mean of the duplicates from each animal. (*B*) Result of MeV-specific IFN-γ ELISpot plotted as average numbers of SFCs per 10^6 PBMCs ± SEM for each group. Puff-mask vs. SC1000 (2 wk and 8 wk, $P < 0.001$; 10 wk, $P < 0.01$). Sol-mask vs. SC1000 (2 wk, $P < 0.01$; 8 wk, $P < 0.05$; two-way ANOVA with Bonferroni's multiple comparison test). (*C*) MeV-specific IL-4 response of individual monkeys. (*D*) Intracellular cytokine production of PBMCs responding to H, F, and N overlapping peptides analyzed 2 wk after vaccination. The quadrant values of the dot plots indicate the average net number (minus background) of cells/10^6 $CD4^+$ and $CD8^+$ T cells in each group with MeV-specific production of IFN-γ alone, IFN-γ and TNF, or TNF alone.

T cells was observed in all vaccinated animals. Higher numbers of IFN-γ (Fig. 3 *A* and *B*) and IL-4 (Fig. 3*C*)-producing T cells were present 2 wk after immunization in animals that received MVDP with a mask rather than nasal delivery or LAMV by injection (Fig. 3 *A*–*C*). In mask-immunized monkeys there was an unexpected reactivation of IFN-γ–producing cells 8 wk after immunization (Fig. 3 *A* and *B*). PBMCs collected 8 and 10 wk after immunization were negative for MeV-nucleoprotein (N) RNA by qRT-PCR.

Quality of the MeV-Specific T-Cell Responses to MVDP Delivered by Mask. To assess the quality of the T-cell responses defined by combinations of effector functions (26, 27), we used an eight-color antibody panel to evaluate MeV-specific expression of IFN-γ, TNF, IL-2, and CD107a in $CD4^+$ and $CD8^+$ T cells (Fig. S3). All monkeys developed $CD4^+$ and $CD8^+$ T-cell responses, as measured by TNF production 2 wk after immunization (Fig. 3*D*). Monkeys immunized with MVDP by Puff-mask had a higher frequency of $CD4^+$ but not $CD8^+$ T cells that produced both IFN-γ and TNF than monkeys that received low-dose s.c. LAMV or Puff-nasal MVDP (Fig. 4*A*, *Upper*). Puff-mask–immunized monkeys also developed more $CD4^+$ T cells that simultaneously produced IFN-γ, TNF, and IL-2 (Fig. 4*B*, *Lower*). Therefore, 2 wk after immunization, more multifunctional $CD4^+$ T cells were present in monkeys immunized with Puff-mask MVDP, but there were no significant differences in the $CD8^+$ T cells induced by different vaccines and devices. At 8 wk after immunization, MeV-specific memory $CD4^+$ and $CD8^+$ T cells were found in most monkeys immunized with MVDP (Fig. 4*B*).

To assess the frequency and quality of memory T cells, cells were expanded by repetitive stimulation with MeV antigens to detect low-frequency memory T cells directly ex vivo (Fig. 4 *C* and *D* and Fig. S4). More polyfunctional $CD4^+$ T cells capable of producing IFN-γ, TNF, and IL-2 were found in monkeys immunized with MVDP through a mask than LAMV through injection (Fig. 4 *C* and *D*). No significant differences in the frequency and quality of $CD8^+$ T cells were detected between immunization strategies.

MVDP Provided Protection Against Wild-Type MeV Challenge. To determine whether respiratory immunization with MVDP would protect monkeys against wild-type MeV challenge, all vaccinated monkeys and three MeV-seronegative monkeys were challenged intratracheally with the Bilthoven wild-type strain of MeV14-16 mo after vaccination (28). Protection was assessed by presence or absence of rash and by measuring viremia and respiratory shedding of MeV. Rashes developed in all unvaccinated animals and in none of the vaccinated animals. Viremia was detected in one Puff-nasal monkey, one low-dose s.c. monkey, and all unvaccinated monkeys by cocultivation of PBMCs with Vero/hSLAM cells (Fig. 5*A*). MeV-N RNA was detected by qRT-PCR in all monkeys positive for MeV by cocultivation, and in two additional monkeys in the Puff-nasal group (Fig. 5*B*). The viremias in vaccinated monkeys that were not protected from infection were lower than in unvaccinated monkeys. Replication of MeV in the respiratory tract was examined by RT-PCR on cells from nasal swabs. All monkeys negative for MeV in the blood were also negative in the respiratory tract (Table S4). Neither infectious MeV nor MeV-N RNA were detected in animals vaccinated with Puff-mask, Sol-mask, or Sol-nasal. Therefore, MVDP delivered to deep lung provided full protection against wild-type MeV infection.

Cellular and Humoral Responses After Challenge. To assess the impact of different vaccinations on the secondary immune response to MeV, T-cell responses and neutralizing antibody were assessed. Monkeys that were fully protected from wild-type MeV infection had minimal T-cell responses compared with monkeys that became infected, indicating that solid immunity was generated by MVDP through Puff-mask, Sol-mask, and Sol-nasal, as well as standard-dose LAMV by injection (Fig. 6*A*). The IFN-γ response

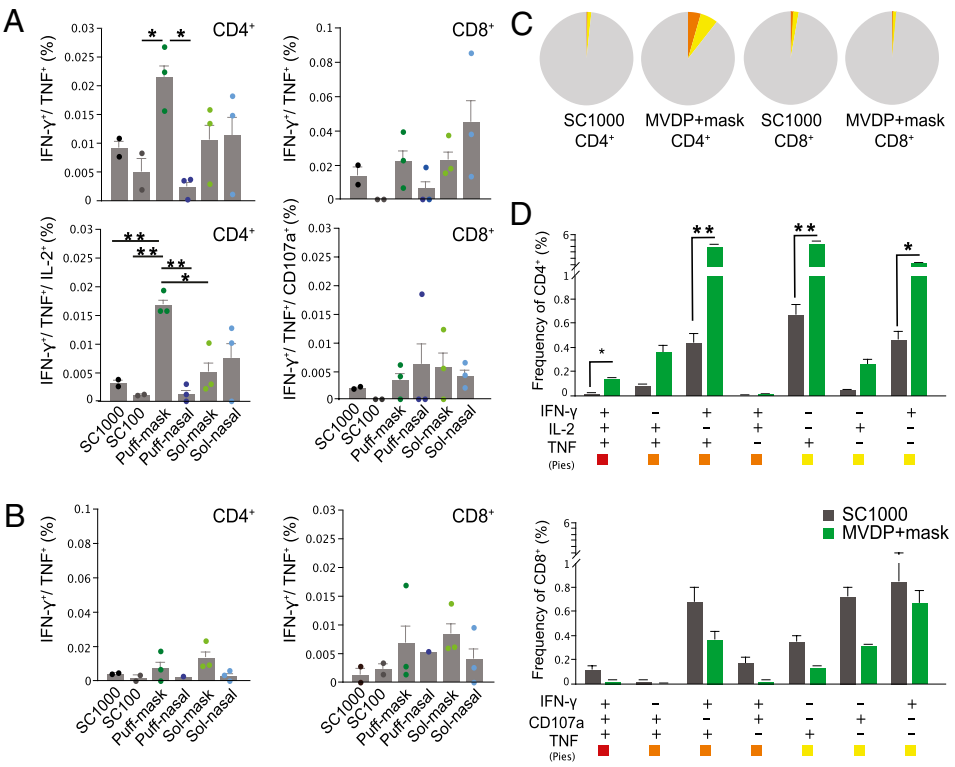

Fig. 4. Higher frequency and better quality of MeV-specific CD4+ T-cell response in monkeys receiving MVDP through a mask. Cytokine production by PBMCs stimulated with H, F, and N peptides was analyzed by FACS. (*A* and *B*) Mean frequencies + SEM of MeV-specific CD4+ and CD8+ T cells making both IFN-γ and TNF at 2 wk (*A Upper*) or 8 wk (*B*) after immunization. (*A Lower*) Mean frequencies + SEM of MeV-specific CD4+ T cells making IFN-γ, TNF, and IL-2, and CD8+ T cells making IFN-γ, TNF, and CD107a at 2 wk after immunization. (*C* and *D*) Comparison of the MeV-specific memory T-cell response (14 wk) after expansion in vitro for 2 wk of cells from monkeys immunized with MVDP through a mask or standard-dose liquid LAMV s.c. (*C*) Pie chart of the functional composition of the CD4+ and CD8+ T-cell response. Subsets of cells expressing each functional marker were analyzed by Boolean gating; seven subsets that express each possible combination of functions were identified. Subsets that simultaneously express zero (gray), one (yellow), two (orange), or three (red) different cytokines were grouped. (*D*) The frequency of each functional subset within CD4+ T cells (*Upper*) or CD8+ T cells (*Lower*) is shown in the bar chart. Statistics used SPICE software *t* test. $^{**}P < 0.01$; $^{*}P < 0.05$.

in vaccinated monkeys was higher than in unvaccinated monkeys 7 d after challenge, indicating a recall T-cell response (Fig. 6*B*). Independent of identifiable MeV infection, all vaccinated monkeys boosted levels of neutralizing antibodies (Fig. 6*C*).

Discussion

This study demonstrates that respiratory delivery of a single dose of dry powder live-attenuated MeV vaccine is capable of inducing durable, fully protective immunity comparable to injection of LAMV. This was accomplished through production of a stable fine-particle dry powder LAMV, development of two dry powder inhalers, and use of a highly relevant nonhuman primate model for measles. Our findings show that both the PuffHaler and Solovent devices efficiently deliver the vaccine to target respiratory tissues. When connected to masks, both devices delivered MVDP to the deep lung and induced more robust antibody and T-cell responses than nasal delivery or s.c. injection of LAMV.

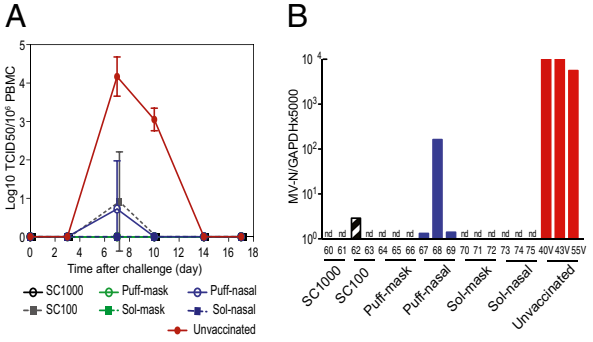

Fig. 5. Viremia after wild-type MeV challenge. (*A*) Infectious virus in the blood was detected by cocultivation of PBMCs with Vero/hSLAM cells. Animals vaccinated with Puff-mask, Sol-mask, Sol-nasal, and SC1000 were negative. Values are plotted as geometric means ± SEM. (*B*) Quantitation of viral load by N-specific qRT-PCR using RNA extracted from PBMCs. nd, not detected.

For immunogenicity, LAMV requires replication, but the types of cells infected in the respiratory tract remain unclear. Replication of vaccine strains of MeV in the upper and lower respiratory tract and in lung CD11c+ immune cells has been observed after respiratory delivery to macaques (29). Furthermore, protective levels of antibody can be induced with nebulized intratracheal LAMV, which suggests that viral replication in the lower respiratory tract is sufficient to induce protective immunity (30). In the present study, Sol-mask, Puff-mask, and Sol-nasal delivered MVDP to the lower respiratory tract, with viral RNA detected only in BAL, and induced robust humoral and cellular immune responses. Puff-nasal delivered MVDP to the upper respiratory tract, with viral RNA detected only in tonsil swabs, and induced limited immune responses. Therefore, devices used for nasal delivery can be effective in deeper lung delivery when paired with a properly sized particle. These observations emphasize the critical roles for successful respiratory measles immunization of small particle size and the device used for delivery. Previous studies using liquid LAMV delivered intranasally with a sprayer designed for deposition in the nose only (large droplets) did not demonstrate robust immune responses (31), whereas the powder used in this study was suitable for deep lung delivery. These results suggest that delivery of a small particle-size powder vaccine via the nasal route may be an option for measles vaccination.

In this proof-of-concept study, a dose–response for respiratory immunization by MVDP was not included. A previous attempt to immunize rhesus macaques with a dry powder vaccine gave suboptimal antibody responses (32) either because the vaccine was not immunogenic or the powder was not delivered to the respiratory tract. We used a dose equivalent to 5× the standard dose to minimize the likelihood of inadequate delivery. It has been estimated that successful measles immunization by liquid aerosol can be accomplished with 30–225 pfu (33). In this study, the packaged dose was ∼15,000 pfu for PuffHaler and 23,000 pfu for Solovent. It is difficult to quantify the number of live MeV particles that were actually inhaled, but if ∼1–2% of the packaged dose reached the pulmonary system (34), 150–450 pfu would have

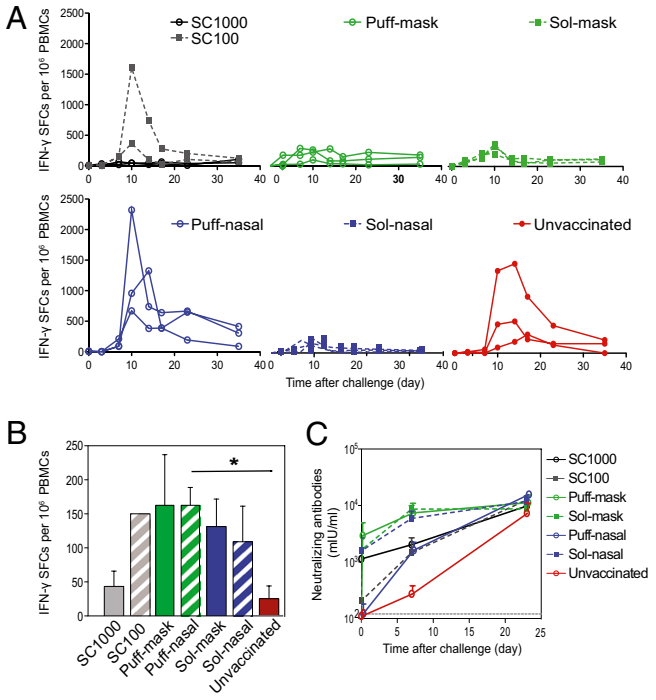

Fig. 6. Cellular and humoral immune responses following challenge. (*A*) MeV-specific T-cell responses to challenge assessed by IFN-γ ELISpot. PBMCs were stimulated with overlapping peptides from the hemagglutinin (H), fusion protein (F), or nucleoprotein (N). Numbers of specific spot-forming cells (SFCs) were calculated by subtracting nonspecific responses. MeV-specific T-cell response was the sum of H, F, and N responses. (*A*) MeV-specific IFN-γ response of individual monkeys. (*B*) Higher MeV-specific IFN-γ response in respiratory vaccinated monkeys than unvaccinated controls at 7 d after challenge due to reactivation of MeV-specific memory T cells. (*C*) Neutralizing antibody measured by plaque-reduction neutralization using the Edmonston strain of MeV. The predicted protective level (120 mIU/mL) is indicated with a dashed line. Values are plotted as geometric means + SEM.

been delivered to the lungs and nasopharynx. Future studies will be needed to evaluate the immune response to a range of doses and safety in immune-compromised or allergic individuals.

High-titer, high-avidity, durable neutralizing antibody is a strong correlate of vaccine-induced protection from measles (35). The numbers of MeV-specific IgG-producing cells in the bone marrow were highest in animals that received mask-delivered MVDP. Antibody titers in plasma before challenge were highest in the Sol-mask, Puff-mask, and Sol-nasal groups that had demonstrated deposition of MVDP in the lower airways.

T cells, especially $CD8^+$ T cells, are important for the control and clearance of MeV (36), but the role of T cells in protective immunity is less clear. T-cell responses induced by MVDP demonstrated qualitative and temporal differences from the T-cell responses induced by injection. Animals immunized with mask-delivered MVDP had more MeV-specific polyfunctional $CD4^+$ T cells and a reappearance of MeV-specific IFN-γ–producing T cells 8 wk after immunization. Biphasic MeV-specific T-cell responses after wild-type MeV infection have been observed in macaques primed with alphavirus replicon particle MeV vaccines (37, 38). This may be a feature of the dose or of lower respiratory tract delivery. A potential explanation for reappearance of MeV-specific T cells is recrudescence of MeV (37, 38). We did not detect MeV RNA in the PBMCs of MVDP-vaccinated monkeys, but cannot exclude the possibility of MeV replication in other tissues.

It is difficult to compare the relative efficacy of s.c. injection of LAMV and MVDP delivery into the lung. The number of animals that received injections was small ($N = 4$), two different doses levels were given (1,000 and 100 pfu), one of the animals had probably been exposed to MeV earlier, and the amount of live MeV delivered to target tissue in the MVDP groups is not known. However, in contrast to an earlier study that found lower levels of MeV-specific antibody in monkeys vaccinated with inhaled dry powder than liquid LAMV by injection (32), we found that MVDP delivered to the lower airways induced higher levels of long-lasting MeV-specific antibodies and T cells compared with s.c. vaccination. Differences in the formulation of the dry powder vaccine, dose, and delivery methods may account for the differences in the immune responses induced.

In summary, monkeys immunized with MVDP by Puff-mask, Sol-mask, Sol-nasal, and with standard-dose LAMV by s.c. injection developed MeV-specific immunity that was completely protective from challenge with wild-type MeV. This demonstration that respiratory delivery of a single dose of MVDP is capable of inducing durable, fully protective immunity comparable to injection of standard LAMV moves this approach to measles vaccination closer to being a practical tool for improving measles control.

Methods

Vaccine. MVDP was prepared from a measles-clarified virus pool (MCVP-3) consisting of Edmonston–Zagreb LAMV, *myo*-inositol, and other stabilizing excipients (Serum Institute of India). CAN-BD technology was used to process the liquid vaccine into a dry power (17, 18, 39, 40) (*SI Methods*).

Animals. Nineteen rhesus macaques (*Macaca mulatta*) negative for herpes B virus, tuberculosis, and antibody to MeV were obtained from Harlan Laboratories, Three Springs Scientific, or the Johns Hopkins University primate facility. For the immunization study, monkeys were at AVANZA Laboratories, and for the challenge study, monkeys were at Johns Hopkins University. All studies were performed in accordance with experimental protocols approved by the appropriate institutional animal care and use committees.

Immunization. Four groups of three monkeys were immunized with 50 mg MVDP through different delivery methods: Puff-mask (monkeys 64, 65, and 66), Puff-nasal (67, 68, and 69), Sol-mask (70, 71, and 72), and Sol-nasal (73, 74, and 75). Two monkeys (60 and 61) received 1,000 pfu, and two monkeys (62 and 63) received 100 pfu liquid LAMV by s.c. injection. After immunization, tonsillar swab, BAL samples, and heparinized blood were collected.

MeV Challenge and Virus Assays. The vaccinated monkeys and three MeV-naive monkeys (40V, 43V, 55V) were challenged intratracheally with 10^4 tissue culture 50% infectious doses of the Bilthoven strain of MeV (28) (gift from Albert Osterhaus, Erasmas University, Rotterdam) 14–16 mo after immunization. Monkeys were shaved and monitored for development of a rash. Heparinized blood was collected to assess viremia and immune responses. Viremia was quantitated by cocultivation of serially diluted PBMCs with Vero/hSLAM cells (41). Cultures were scored for cytopathic effect after 5 d, and data reported as the number of infected PBMCs per 10^6 PBMCs. MeV RNA in PBMC was detected by quantitative RT-PCR as previously described (38, 42, 43).

Antibody Assays. For plaque reduction neutralization (PRN), the Edmonston strain of MeV was mixed with serially diluted plasma and assayed for plaque formation on Vero cells. An internal standard calibrated to the international standard (66/202) was included in all assays, and data were normalized to the standard and expressed as milli-international units (mIU) (44). One monkey in the standard dose s.c. LAMV group (no. 60), identified to be MeV seronegative by enzyme immunoassay (EIA; OD = 0.15) before immunization, had a low PRN titer (32 mIU/mL) and an anamnestic antibody response to vaccination and was excluded from the antibody analyses. EIAs were used to measure MeV-specific IgM, IgG (45), and IgA (*SI Methods*).

To assess antibody avidity, increasing concentrations (0.5–3 M) of ammonium thiocyanate (NH_4SCN) were added to the EIA to disrupt the MeV–antibody interaction. The avidity index was defined as the concentration of NH_4SCN to elute 50% of the bound antibody.

To measure antibody-secreting cells in the bone marrow, cells isolated from bone marrow aspirates by density gradient centrifugation using Lympholyte Mammal (Cedarlane Laboratories) were incubated with Multiscreen ELISpot plates coated with MeV-infected Vero cell lysate or purified goat anti-monkey IgG, IgM, and IgA (Open Biosystems) for 6 h. Bound Ig was detected

with HRP-conjugated goat anti-monkey IgG (Nordic), developed with stable diaminobenzidine (DAB) solution and read on an ImmunoSpot plate reader (Cellular Technology).

T-Cell Assays. Enzyme-linked immunospot (ELISpot) assays were used to measure IFN-γ and IL-4–producing T cells. Plates were coated with antibody to human IFN-γ or human IL-4 (BD Biosciences). After plates were washed and blocked with RPMI-10, 1–5 × 10^5 fresh PBMCs were added along with 1 μg/mL pooled MeV peptides (20-mer overlapping by 11 amino acids) from the H, F, or N proteins or 5 μg/mL Con A. After 40 h of incubation, washed plates were incubated with biotinylated antibody to IFN-γ (Mabtech) or IL-4 (BD Biosciences) followed by HRP-conjugated avidin. Assays were developed with stable DAB solution and scanned. Data were analyzed using ImmunoSpot version 3.0 software. Results are presented as the number of spot-forming cells (SFCs) per 10^6 PBMCs. Function of MeV-specific T cells collected directly ex vivo or after in vitro expansion was assessed by intracellular cytokine staining with multiparameter flow cytometry (*SI Methods*).

Statistical Analyses. The significance of differences in IgG titer, PRNT, ELISpot, and viral load was assessed by ANOVA followed by Bonferroni's multiple comparison test (Prism 5; GraphPad Software). Differences in the functional properties of MeV-specific T cells were assessed by *t* tests (SPICE software, version 4.3; Mario Roederer and Joshua Nozzi, National Institute of Allergy and Infectious Diseases, Bethesda). In all analyses, we used a two-sided significance level of 0.05.

ACKNOWLEDGMENTS. We thank Ravindra Muley, Vivek Vaidya, and Rajeev Dhere (Serum Institute of India Ltd.); Brandyn Lau, Debra Hauer, and Annie Tsay (The Johns Hopkins University); Marcus Collins, Sun Bae Sowers, and Laura Walls (Centers for Disease Control); Lia Rebits, David Krank, Jim Searles, Pankaj Pathak, and Pradnya Bhagwat (Aktiv-Dry LLC); Jessica Burger, David McAdams, Lowry Lindsay, and Mark Hernandez (University of Colorado); and Vince Sullivan (BD Technologies) for expert technical assistance. This work was funded by Bill and Melinda Gates Foundation Grant RG3522 (to D.E.G.) and the Foundation for the National Institutes of Health Grant 1077 (to R.E.S.). W.-H.L. was partially supported by the Marjorie Gilbert Scholarship Fund.

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