Needle-free delivery of measles virus vaccine to the lower respiratory tract of non-human primates elicits optimal immunity and protection

Rik L. de Swart1,2, Rory D. de Vries1, Linda J. Rennick3, Geert van Amerongen1,4, Stephen McQuaid5, R. Joyce Verburgh1,5, Selma Yüksel1, Alwin de Jong1, Ken Lemon6,7, D. Tien Nguyen1, Martin Ludlow2,7, Albert D. M. E. Osterhaus1,7 and W. Paul Duprex1

Needle-free measles virus vaccination by aerosol inhalation has many potential benefits. The current standard route of vaccination is subcutaneous injection, whereas measels virus is an airborne pathogen. However, the target cells that support replication of live-attenuated measles virus vaccines in the respiratory tract are largely unknown. The aims of this study were to assess the in vivo tropism of live-attenuated measles virus and determine whether respiratory measles virus vaccination should target the upper or lower respiratory tract.

Four groups of twelve cynomolgus macaques were immunized with $10^6$ TCID$_{50}$ of recombinant measles virus vaccine strain Edmonston-Zagreb expressing enhanced green fluorescent protein. The vaccine virus was grown in MRC-5 cells and formulated with identical stabilizers and excipients as used in the commercial MV$_{E22}$ vaccine produced by the Serum Institute of India. Animals were immunized by hypodermic injection, intra-tracheal inoculation, intra-nasal instillation, or aerosol inhalation. In each group six animals were euthanized at early time points post-vaccination, whereas the other six were followed for 14 months to assess immunogenicity and protection from challenge infection with wild-type measles virus. At early time-points, enhanced green fluorescent protein-positive measles virus-infected cells were detected locally in the muscle, nasal tissues, lungs, and draining lymph nodes. Systemic vaccine virus replication and viremia were virtually absent. Infected macrophages, dendritic cells and tissue-resident lymphocytes predominated. Exclusive delivery of vaccine virus to the lower respiratory tract resulted in highest immunogenicity and protection. This study sheds light on the tropism of a live-attenuated measles virus vaccine and identifies the alveolar spaces as the optimal site for respiratory delivery of measles virus vaccine.

npj Vaccines (2017) 2:22; doi:10.1038/s41541-017-0022-8

INTRODUCTION

Measles remains a leading cause of childhood morbidity and mortality. Live-attenuated measles virus (MV) vaccines are safe and effective, and high coverage in two-dose regimens has successfully interrupted endemic transmission in large geographic areas. However, measles still caused an estimated 134,200 deaths in 2015, and vaccine refusal based on religious and/or philosophical objections has led to measles resurgence in Europe and the Americas.

Wild-type MV is one of the most contagious human pathogens, is spread by airborne transmission and causes an acute systemic disease. Measles is associated with transient but severe immune suppression, which results in increased susceptibility to opportunistic infections. Live-attenuated MV vaccines were introduced in the early 1960s at a time when worldwide millions of children were dying of measles every year. Introduction of MV vaccination has consistently reduced childhood mortality in every geographic region. Despite their success, live-attenuated MV vaccines also have limitations, including dependency on maintaining the cold-chain, requirement for trained health-care workers and the need for sterile hypodermic needles and concomitant safe waste disposal. Needle-free MV vaccination regimens were developed to address some of these issues and administration of the vaccine by aerosol inhalation (AI) has been considered as a promising technology. Initially, clinical trials using aerosolized measles vaccine were performed in Mexico and these demonstrated the feasibility of this vaccination route. Recent studies confirmed that aerosol measles vaccination was equivalent or superior to injection as booster vaccine. However, when used for primary immunization of infants, aerosol vaccination resulted in lower seroconversion levels than standard injection. A randomized, controlled trial of aerosol vaccination in infants 9–12 months of age confirmed that aerosol vaccination was immunogenic, but aerosol delivery induced lower seroconversion rates than subcutaneous (SC) injection (85% and 95%, respectively).

Regulatory authorities consider a vaccine and its administration route as a single entity. Consequently, both preclinical and clinical studies were required to achieve licensure for MV aerosol vaccination.
vaccination. To support licensing we previously compared administration of the MV Edmonston-Zagreb (MVEZ) vaccine strain via AI, dry powder inhalation and injection in non-human primates. Aerosol vaccination induced similar levels of neutralizing antibodies and T-cell responses as detected in the injection group, and protection from challenge infection with a wild-type MV was comparable. Dry powder aerosol vaccination was less effective than injection in this study. However, in a more recent study successful immunization of macaques by an aerosolized dry powder MV vaccine was reported, and this approach was shown to be safe in a phase I clinical trial in humans.

Despite the fact that live-attenuated MV vaccines have been used for almost 50 years, there is still a fundamental lack of understanding of the in vivo tropism of the virus and the molecular basis of attenuation remains elusive. This hampers rational development of next-generation delivery strategies that could be transformative in the developing world by mitigating challenges associated with virus thermostability. Live-attenuated MV must replicate in the host to induce protective immune responses, and alternative routes of administration may well deliver the vaccine to different target cells than those infected following injection. In recent years, we have used recombinant (r) MV strains expressing fluorescent proteins to study wild-type MV tropism in vitro and in vivo. These viruses express a fluorescent reporter protein from an additional transcription unit (ATU), which has the advantage that the entire cytoplasm of an infected cell is flooded with the reporter proteins facilitating sensitive detection of infected cells by flow cytometry, immunofluorescence and/or immunohistochemistry. We recently described an rMV based on the MVEZ vaccine strain expressing enhanced green fluorescent protein (EGFP), which was produced using a miniaturized (laboratory-scale) industrial process using cells and protocols provided by the Serum Institute of India. This reporter virus permitted detection of small numbers of MV-infected cells in the muscle of vaccinated non-human primates in the first week following injection. In addition, we optimized methodology for dose estimation and aerosol delivery in non-human primates. Based on these foundational studies we now report results of a large-scale vaccination study in cynomolgus macaques comparing four different routes of immunization. The aim of the study was to compare vaccine virus replication, tropism, immunogenicity and protection, and determine whether MV vaccination via the respiratory route should target the upper or lower respiratory tract (LRT).

RESULTS

Study design

Four groups of 12 cynomolgus macaques were immunized by intra-muscular (IM) injection, intra-tracheal (IT) inoculation, intra-nasal (IN) instillation, or AI of rMVEZEGFP(3). Six animals from each group were euthanized at early time points in the virus replication and tropism arm of the study, the other six were followed up for 14 months post-vaccination (MPV) to assess immunogenicity and protection from wild-type MV challenge infection (Fig. 1a). For logistical reasons the study was performed in two parallel sessions (1 and 2) comprising half of the animals from each of the groups (Fig. 1b and Supplementary Table 1). To comply with Dutch laws on the use of genetically modified...
organisms, vaccinations were performed in high efficiency particulate air (HEPA)-filtered negatively pressurized animal biosafety level (ABSL)-3 isolators.

Replication
Clinical specimens collected during the first 9 days post-vaccination (DPV) were screened for the presence of MV-infected (EGFP-positive) cells by combining flow cytometry (fluorescence-activated cell sorting (FACS)), ultraviolet (UV) microscopy and virus isolation to assess vaccine virus replication. FACS analysis of unstained broncho-alveolar lavage (BAL) cells was a sensitive method for detection of EGFP-positive cells in the LRT, as illustrated by a negative and positive sample at five DPV (Fig. 2a). The highest frequencies of EGFP-positive BAL cells were consistently detected in animals of groups 3 (IT) and 4 (AI), while infected cells were undetectable in the BAL of most animals in groups 1 (IM) or 2 (IN) (Fig. 2b). This was compatible with virus isolation data obtained from BAL cells: in 24 out of 48 animals EGFP-positive BAL cells were detected by FACS analysis, and we were able to isolate MV from BAL cells from 23 out of these 24 animals, predominantly at 3, 5, or 6 DPV (results not shown).

Morphologically, lung specimens collected from animals euthanized 3, 5, or 7 DPV to assess MV replication were processed for FFPE slices. Targeted pathological assessment of a wide range of formalin-fixed paraffin embedded (FFPE) tissues was performed using an infectious center assay to assess the level of protection to challenge infection (438 DPV = 0 days post-challenge; DPC), MV-infected cells were mainly cytokeratin-negative (Fig. 3b, c), and some MV-infected cells expressed CD68 demonstrating a macrophage lineage (Fig. 3d, e). Equivalent MV-infected cells were present in nasal epithelia in animals from group 2 (IN; data not shown). In conclusion, MV vaccine virus predominantly replicated in myeloid and lymphoid cells and rarely in epithelial cells.

Immunogenicity
Animals were followed for MV-specific immune responses for 14 MPV. Serum antibody responses were detected by enzyme linked immunosorbent assay (ELISA, Fig. 4a, b, d), virus neutralization (Fig. 4c) or indirect immunofluorescence for MV fusion (MV-F) or hemagglutinin (MV-H) glycoprotein-specific antibodies (Fig. 4e, f). Animals vaccinated by IT inoculation (blue symbols) consistently showed highest antibody levels, whereas samples from AI administered (green symbols) and IM (grey symbols) injected animals showed slightly lower responses. In all assays, lowest serum antibody levels were consistently observed in animals immunized by IN instillation (red symbols). Serological profiles were comparable to cellular immune responses measured at 35, 45, or 411 DPV in vitro PBMC stimulation assays consistently resulted in highest levels of interferon-gamma (IFN-γ) production in supernatants of cells infected with wild-type MV strain Bilthoven by IT inoculation with a dose of 10^6 TCID50 (based on titration in Vero-hCD150 cells), as described previously.27 At the moment of challenge infection (438 DPV = 0 days post-challenge; DPC), MV-specific serum antibody levels in the majority of vaccinated animals were above previously identified immunological correlates of protection of 120–200 mIU/ml.

Protection
Fourteen MPV, all twenty-four animals in the immunogenicity arm of the study, and four additional seronegative control animals, were challenged with wild-type MV strain Bilthoven by IT inoculation with a dose of 10^6 TCID50 (based on titration in Vero-hCD150 cells), as described previously.27 At the moment of challenge infection (438 DPV = 0 days post-challenge; DPC), MV-specific serum antibody levels in the majority of vaccinated animals were above previously identified immunological correlates of protection of 120–200 mIU/ml.

Needle-free measles vaccination of macaques
RL de Swart et al.

Published in partnership with the Sealy Center for Vaccine Development

npj Vaccines (2017) 22
were also detected in animals immunized by IN instillation (group 2), and two out of six animals immunized by IM injection (which also had the lowest serum antibody levels at time point C0) also showed substantial virus loads in BAL cells collected 3 or 6 DPC. Only two out of six IN immunized animals had detectable virus loads in PBMC. Measurement of MV-specific serum antibody responses at different time points post-challenge (Fig. 5e, f) demonstrated accelerated secondary immune responses in all
vaccinated animals as compared to slower primary responses in the challenge control animals (black symbols). Animals in group 4 (AI) showed the most rapid onset of a secondary immune response, with four out of six animals showing strong increases in specific antibody levels between 3 and 6 DPC (Fig. S2). In conclusion, all vaccinated animals were largely protected from systemic wild-type MV replication, but also showed secondary immune responses demonstrating the absence of sterilizing immunity after MV vaccination. One animal (I–31; group 3, session 1, IT) failed to recover from anesthesia after collection of clinical specimens 9 DPC. A full necropsy was performed to establish a possible cause of death. Replicating MV was not detected and no gross pulmonary lesions were observed. Histopathological examination showed a very mild neutrophilic tracheitis and bronchitis that may have been associated with the MV-specific immune response. No lesions in any tissues were found that could explain the cause of death. This led to the conclusion that the unexpected death resulted from anesthesia. In conclusion, immunogenicity and protection, as determined by inhibition of wild-type MV replication, were closely related. Lowest relative protection levels (as shown by challenge virus replication) were detected in animals immunized by IN instillation and in two out of six animals immunized by IM injection. Delivery of MV vaccine to the LRT (groups 3 and 4) resulted in high immunogenicity and this was associated with high levels of protection from wild-type MV replication.

**DISCUSSION**

Live-attenuated virus vaccines have had a profound impact on human and animal health. Development of measles vaccines started 50 years ago, based on the first MV isolate that was adapted to grow on embryonated chicken eggs and embry fibroblasts.29 Measles vaccines are safe and effective and have successfully interrupted endemic MV transmission in large parts of the world.30 However, neither the molecular basis of attenuation nor the in vivo tropism of live-attenuated measles virus vaccine are completely understood.

We have previously used rMVs generated using wild-type MV sequences obtained directly from clinical specimens, and therefore unmodified by tissue culture adaptation, for in vitro and in vivo pathogenesis studies. An ATU encoding a fluorescent reporter protein had a negligible effect on virulence and allowed sensitive detection of infected cells. Studies employing these rMVs have improved our understanding of wild-type MV entry,31 dissemination19, 20 and transmission.32 Furthermore, these viruses were essential in elucidating how wild-type MV causes immune suppression.7 The possibility of identifying single MV-infected cells as early as 2 days post-infection in tissues and organs21 suggested that it is possible to perform tropism studies following vaccination. This approach was piloted following immunization of macaques by IM injection and permitted the identification of the primary target cells in the muscle.25 Here we extended this approach, and identified the tropism of measles vaccine viruses after IN instillation, IT inoculation or AI. To facilitate these studies we optimized aerosol administration and dose estimation of MV vaccine to non-human primates.25

Repeated tissue culture passage of wild-type MV, which normally uses CD150 or nectin-4 as cellular entry receptors,33, 34 resulted in attenuated MV vaccine strains that may use the ubiquitously expressed protein CD46 as an entry receptor.35, 36 In addition, the ATU encoding a functional EGFP reporter gene successfully interrupted endemic MV transmission in large parts of the developing world, led us to perform a unique study in non-human primates comparing the immunogenicity of rMVES2 delivered via different routes of respiratory administration. The particle size of the aerosol (mass median aerodynamic diameter 3.5 μm) was chosen to allow deposition of a substantial fraction of the inhaled dose into the LRT.25 Robust humoral immune responses were induced by IM injection, IT inoculation or AI, whereas IN instillation induced poor antibody responses. Furthermore, direct delivery of live-attenuated vaccine to the LRT via IT inoculation or AI gave superior humoral and cellular responses compared to IM injection. These results demonstrate that the immunogenicity of MVES2 after primary immunization with equivalent dosage was comparable when the vaccine was delivered by AI or injection.14
Fig. 3  Tropism. Macaques were euthanized by exsanguination 3, 5, or 7 DPV. During necropsy, lungs were inflated with low-melting point agarose and allowed to solidify on ice. Lung slices were screened by UV microscopy for the presence of EGFP-positive cells. Seven micrometer sections were prepared from leveled, fixed, and paraffin embedded tissues to permit small numbers of EGFP-positive cells to be identified.

a Hematoxylin counterstained anti-EGFP immunohistochemical staining of a lung slice, showing evidence of EGFP-positive cells in brown (see inset, positive cells indicated by arrows); b, c EGFP (green)/4',6-diamidino-2-phenylindole (DAPI; blue) / cytokeratin (red) staining (single fluorescence scans shown in insets); d, e: EGFP (green)/DAPI (blue)/CD68 (red) staining (single fluorescence scans shown in insets). Arrow in panel e indicates CD68 and EGFP co-localization. Images shown were collected from animal T-41 (see Table S1, group 4A (AI), euthanized 5 DPV). Av alveoli, Br bronchiolo
All animals vaccinated via the IT and AI route still had MV-specific neutralizing antibodies at the moment of challenge infection at 14 MPV. Interestingly, these antibodies had waned in 6/6 IN vaccinated animals and 2/6 IM vaccinated animals. This was reflected post-challenge and infectious virus was more likely to be recovered from BAL cells obtained from animals without detectable antibodies (6/6 IN, 2/6 IM, 0/6 IT, 0/6 AI and 4/4 challenge controls). A significant boosting of antibodies, characteristic of a secondary immune response, was observed in all vaccinated animals and this was both faster and stronger than the primary immune responses observed in the challenge control animals. Rapid increases in MV-specific antibodies was most evident in animals vaccinated via AI. Although this study was not designed to assess vaccine safety, it is important to note that no indications for adverse events associated with these non-conventional vaccination routes were noted.

Even though aerosol delivery of live-attenuated MV vaccine offers promise, clinical studies in children have produced variable results. Although effective in inducing secondary immune responses as booster vaccination, primary vaccination of infants proved inferior to vaccination by standard injection contradictory to the results we obtained in non-human primates. Extending exposure time of infants to aerosol resulted in improved responses, suggesting delivery of insufficient vaccine as a potential explanation for the reduced effectiveness in infants with low tidal breathing volumes and irregular breathing patterns. These issues, and the fact that in our study immunogenicity of IT inoculation was superior to AI, highlight the need to determine the parameters for normalization of the dose delivered to the LRT during aerosol delivery, if the goal of aerosol MV vaccination is ever to be realized.

Dry powder inhalation has also been examined as an alternative to nebulized Al. This approach avoids the need for vaccine reconstitution and allows packaging of individual doses in a thermostable formulation, which may reduce dependence on cold chain maintenance. Microneedle patches containing measles vaccine are considered as another alternative, and have similar advantages. Microneedle patches were immunogenic in macaques, and studies with a combination of microneedle patches and an rMV vaccine strain encoding a fluorescent reporter protein could support further development and elucidate the tropism of live-attenuated MV vaccine in the dermis and/or epidermis of the skin.

Since live-attenuated virus vaccines typically contain a low antigenic dose, replication in the host is essential for induction of protective immune responses. Primary vaccine virus replication in professional antigen presenting cells (expressing MHC class II) in the LRT, as we show here, could facilitate the induction of robust

---

**Fig. 4** Immunogenicity. Virus-specific immune responses after immunization of non-human primates with rMVEZEGFP(3) via four different routes of administration. Panels a-f show MV-specific serum antibody responses measured by six different assays: a Serion IgG ELISA, with results expressed in milli-international units per ml (mIU/ml); b in house MV IgG ELISA, with results expressed in optical density at 450 nm (OD450); c focus reduction neutralization test (FRNT), with results expressed in international units per ml (IU/ml); d MV nucleoprotein (MV-N)-specific IgG ELISA, with results expressed in OD450; e MV fusion (MV-F) glycoprotein-specific FACS-measured IgG immunofluorescence (expressed in arbitrary fluorescence units, AFU); f MV hemagglutinin (MV-H) glycoprotein-specific FACS-measured IgG immunofluorescence (expressed in AFU). Results are shown as means ± standard error of six animals per group. Panels g-i show MV-specific cellular responses measured using PBMC collected 35 (g), 45 (h), or 411 (i) DPV. Briefly, PBMC were stimulated with UV-inactivated MV (UV-MV), mock antigen (UV-Vero) or medium. Culture supernatants were harvested after 2 days for measurement of the concentration of IFN-γ by ELISA. Bars represent the means, while symbols represent responses of individual animals. Results of statistical analysis (Two-way analysis of variance with Tukey’s multiple comparison test) are indicated by the following symbols if P-values were below 0.05: $ (IM vs. IT),! (IM vs. AI), and (IM vs. IN), # (IT vs. AI), % (IT vs. IN), * (AI vs. IN)
immune responses. This demonstrates that it is critical to understand replication and tropism of existing live-attenuated vaccines, as this will inform the optimal route of delivery of vaccines. In conclusion, we show that delivery of live-attenuated MV vaccines to the LRT is a biologically feasible method of measles vaccination. These studies support the notion that inferior immunogenicity following primary immunization of infants can be attributed to suboptimal vaccine virus delivery to the LRT in the short exposure time used in this trial.13 It remains to be seen whether the collective will exists to re-evaluate respiratory MV vaccination in clinical studies in infants.

METHODS

Ethics statement

Animal experiments were conducted in compliance with European guidelines (EU directive on animal testing 86/609/EEC) and Dutch legislation (Experiments on Animals Act, 1997). The study protocol was approved by Stichting Dier Experimenten Commissie Consult (DEC Consult, permit number EMC2646), a Dutch independent animal experimentation ethics review board. Animals were housed in groups, received standard primate feed and fresh fruit on a daily basis and had access to water ad libitum. In addition, their cages contained several sources of environmental enrichment, for example hiding places, hanging ropes, tires, and other toys. During the vaccination and challenge periods (0–24 DPV and 0–24 DPC) animals were housed in HEPA-filtered, negatively pressurized ABSL-3 isolator cages, both to ensure biological containment of the genetically modified viruses and to reduce stress during the repeated sample collection by using the squeeze mechanism for sedating the animals.

Animal welfare was observed on a daily basis, and all animal handling was performed under light anesthesia using ketamine and medetomidine (50/50 v/v, 0.2 ml/kg body weight, IM injection). After handling, atipamezole was administered to antagonize the effect of medetomidine (0.05 ml/kg body weight, IM injection). The manuscript was prepared in accordance with the "Animal Research: Reporting of In Vivo Experiments" (ARRIVE) guidelines.

Animal study design

Forty-eight male cynomolgus macaques (Macaca fascicularis) were immunized with $10^9$ TCID50 of rMV$^{EGFP(3)}$ administered via four different routes of administration: IM injection ($n=12$), IN instillation ($n=12$), IT inoculation ($n=12$) or AI ($n=12$) (Fig. 1a).

The standard route of MV vaccination is SC injection. However, the skin of macaques is more loosely connected to the subcutus than the skin of humans, therefore, we chose to use IM rather than SC injection to avoid redistribution of the vaccine over a large surface area. Several studies have demonstrated that both routes are safe and effective in humans.53, 54 IM injection was performed in 0.5 ml into the rectus femoris. IN instillation was included as a route of administration to assess tropism and immunogenicity if the complete vaccine dose was delivered to the URT only. IN instillation (0.5 ml) was performed by using a Gilson P200 pipette, dividing the volume over both nostrils. Immediately after instillation, the nostrils were gently squeezed from the outside to distribute the vaccine virus over the surfaces of the nasal cavity. IT instillation was included as a route of administration to assess tropism and immunogenicity if the complete vaccine dose was delivered to the LRT. Immediately before IT instillation the vaccine virus was diluted 1:10 in phosphate-buffered saline (PBS, Gibco), and 5 ml was inoculated IT through a flexible catheter inserted into the trachea just below the larynx. AI was performed as described.25 Briefly,
vaccine virus was nebulized using a vibrating mesh nebulizer (Aeroneb Pro, Aerogen Ireland Ltd., mass median aerodynamic diameter 3.5 µm), connected to a 22 mm T-piece and using a silicone pediatric resuscitation mask (Laerdal 0/0) as interface with the macaque. Bench studies performed using macaque breathing parameters demonstrated that approximately 25% of a nebulized dose was inhaled by the animal, whereas the remainder of the dose was lost as condensate in the nebulizer or T-piece or was exhaled. Therefore, 0.5 ml of a 4× concentrated vaccine virus stock was nebulized to deliver the same vaccine dose as in the other three groups. It should be noted that there is no control over the fate of the inhaled vaccine virus after inhalation: part may be lost when it gets swallowed, whereas the remainder will be deposited in the URT or the LRT. Six animals of each of the four vaccination groups were used to study viral tropism. There were euthanized 3 (n = 2), 5 (n = 2), or 7 (n = 2) DPV. The other six animals/group were followed up for 14 months to study immunogenicity, and were subsequently challenged with wild-type MV. An additional four unvaccinated animals were included as challenge controls. Due to the limitations of housing facilities and laboratory capacity, the additional four unvaccinated animals were included as challenge controls. The other six animals/group were followed up for 14 months to study immunogenicity, and were subsequently challenged with wild-type MV. An additional four unvaccinated animals were included as challenge controls. Due to the limitations of housing facilities and laboratory capacity, the other six animals/group were followed up for 14 months to study immunogenicity, and were subsequently challenged with wild-type MV. An additional four unvaccinated animals were included as challenge controls. The other six animals/group were followed up for 14 months to study immunogenicity, and were subsequently challenged with wild-type MV. An additional four unvaccinated animals were included as challenge controls. The other six animals/group were followed up for 14 months to study immunogenicity, and were subsequently challenged with wild-type MV. An additional four unvaccinated animals were included as challenge controls. The other six animals/group were followed up for 14 months to study immunogenicity, and were subsequently challenged with wild-type MV. An additional four unvaccinated animals were included as challenge controls. The other six animals/group were followed up for 14 months to study immunogenicity, and were subsequently challenged with wild-type MV. An additional four unvaccinated animals were included as challenge controls. The other six animals/group were followed up for 14 months to study immunogenicity, and were subsequently challenged with wild-type MV. An additional four unvaccinated animals were included as challenge controls. The other six animals/group were followed up for 14 months to study immunogenicity, and were subsequently challenged with wild-type MV. An additional four unvaccinated animals were included as challenge controls. The other six animals/group were followed up for 14 months to study immunogenicity, and were subsequently challenged with wild-type MV.

Sample size estimation, randomization, and blinding

In the replication and tropism arm of the study, the main outcome parameters were qualitative assessment and characterization of EGFP-positive cells at early time points after vaccination. We selected three time points of euthanasia (3, 5, and 7 DPV) based on the expected peak of virus replication as determined in previous measles vaccination studies in the macaque model, and chose to include two animals at each time point (leading to a total group size of 6). In the immunogenicity and protection arm of the study, the required group size was based on a power calculation using MV-specific serum antibody responses as main outcome parameter. Based on variation in antibody responses in previous non-human primate vaccination studies, we estimated that a group size of six animals would result in adequate statistical power.

Upon arrival from the supplier, animals were randomly assigned to groups of three animals. These groups were maintained as much as possible for the complete duration of the study, and only modified in the beginning if animals were not socially compatible. Stable groups of non-human primates should not be changed, and, therefore, the same group composition was used when the animals were moved into DM-III isolators for their vaccinations. Which isolator was used for each route of administration was selected randomly (without using a formal randomization procedure).

During sample collection, tubes were labeled with the last four digits of the animal identification chip number, and subsequently transported to the lab. Laboratory technicians were not aware which animal number corresponded to which of the different treatment groups.

Viruses

Animals were vaccinated with rMV2EGFP(3). Non-recombiant vaccine virus, MRC-5 cells and protocols for vaccine production and formulation were kindly provided by the Serum Institute of India. Generation, formulation, and characterization of the recombinant vaccine virus was described previously. The formulated vaccine virus had a history of one virus, MRC-5 cells and protocols for vaccine production and formulation passage in Vero-hCD150 cells (rescue) and was stored at 80 °C. The stock had a titer of 10^6.6 TCID50/ml, and was −20°C. PBMC were isolated from EDTA blood by density gradient centrifugation, and re-suspended in complete RPMI-1640 medium (Gibco Invitrogen, Carlsbad, CA) supplemented with 1-glutamine (2.0 mM), 10% (v/v) FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml). BAL was performed in animals of the immunogenicity arm at 3, 6, and 9 DPV and 3, 6, and 9 days DPC by IT infusion of 10 ml PBS through a flexible catheter followed by immediate recovery of the fluid. BAL samples were centrifuged, and BAL cells were resuspended in culture medium with supplements as described above. Throat brushes and nasal swabs were collected in virus transport medium as described elsewhere. For the virus tropism study, animals were euthanized by exsanguination under deep anesthesia using ketamine and medetomidine. During necropsy, tissue samples were collected by SBS and directly processed and screened for the presence of EGFP by UV microscopy, EGFP-positive samples were either transferred to 4% (v/v) paraformaldehyde in PBS (to preserve EGFP autofluorescence) for direct imaging, or to 10% (v/v) neutral-buffered formalin for paraffin embedding or to the laboratory for preparation of single-cell suspensions for flow cytometry. Direct imaging was performed by confocal laser scanning microscopy with a LSM700 system fitted on an Axio Observer Z1 inverted microscope (Zeiss), and images were generated using Zen software version 2010B SP1.

Virus detection

Isolation of MV vaccine or wild-type virus was performed on Vero or Vero-HC150 cells (kind gift of Dr Y. Yanagi, Fukuoka, Japan), respectively. All cell lines tested negative for mycoplasma spp. contamination. Virus isolation was performed by infectious-center test as previously described, monitoring for cytopathic effect and/or fluorescence 6 days post-isolation. Results of virus vaccine isolations are shown as positive or negative (regardless of load), results of wild-type MV isolation are shown as the number of infected cells per million. Flow cytometry was used to detect EGFP-positive MV-infected cells in PBMC or single-cell suspensions of lymphoid tissues. Immunohistochemistry (IHC) was performed as described previously. Briefly, GFP was visualized in IHC and dual-labeling indirect immunofluorescence with a polyclonal rabbit anti-EGFP (Invitrogen) or rabbit anti-MV (Novus Biologicals) antibody. Monoclonal mouse antibodies to the macrophage marker CD68 (Dako; clone KPI1) or the epithelial cell marker Cytokeratin (Abcam; clone AE1/AE3) were used to visualize the respective cell types. In dual-labeling immunofluorescence, antigen-binding sites were detected with a mixture of anti-mouse Alexa 568 and anti-rabbit Alexa 488 (Invitrogen). Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) hard-set mounting medium (Vector). Fluorescently stained slides were examined at x100, x200, x400, and x1000 magnifications on a fluorescence imaging microscope (Leica Microsystems).

Virus-specific immune responses

MV-specific antibody levels were measured in serum (virus neutralization) or EDTA plasma samples (all other serological assays). The ELISA classic Measles Virus IgG assay (Serion, Würzburg, Germany) was performed according to the instructions of the manufacturer. The in house MV-specific IgG ELISA, MV-N-specific IgG ELISA, and the MV-F and MV-H glycoprotein-specific indirect immunofluorescence assays were performed as described previously. In all cases, bound antibodies were detected by peroxidase-conjugated anti-human IgG conjugates that show excellent cross-reactivity with macaque IgG.

Virus neutralizing antibodies were detected using a fluorescent focus reduction neutralization assay, with some modifications. Briefly, Vero-HC150 cells were seeded in 96-well flat-bottom plates (Greiner) in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum (FBS) 4 days before the experiment, and refreshed with RPMI-1640 medium supplemented with 2% (v/v) FBS (R2F) the day before the experiment. Serial dilutions (2−2, each dilution tested in triplicate) of heat inactivated serum in R2F were mixed 1:1 (v/v) with 40 TCID50 of rMV2EGFP(3), and incubated for 90 min at 37 °C. Subsequently, the serum/virus mixtures were transferred to the Vero-HC150 monolayers and spinoculated for 15 min at 1200g. Cells were cultured at 37 °C for 2 h, after which fusion inhibitory peptide (FIP; Z-D-Phe-L-Phe-Gly-OH, Bachem, Heidelberg, Germany) was added to a final concentration of 0.2 mM to prevent cell-to-cell fusion. Two days later, plates were washed with PBS, fixed with 1% (w/v) paraformaldehyde in PBS, and single fluorescent cells were counted using an automated fluorescence imager (ImmunoSpot S6 analyzer, CTL, Bonn, Germany). Graphpad Prism 5.01 software was used to calculate sigmoidal dose-response curves, on basis of which a 50% reduction titer (EC50) was determined. Results were expressed in international units per ml using the WHO 3rd international reference serum for measles (NIBSC, South Mimms, United Kingdom).

Purified MV Edmonston with a concentration of approximately 1 mg/ml was inactivated by UV-irradiation (30 min, 15 W 312 nm), for cellular assays...
and is hereafter referred to as UV-MV. A Vero cell lysate was also UV-irradiated, and used as mock control antigen (hereafter referred to as UV-Vero). PBMC were thawed and allowed to recover overnight at 37°C in RPMI-1640 supplemented with 10% (v/v) human serum, 1% (v/v) macaque serum, and 3.5 ng/ml recombinant human IL-7 (ImmunoTools, Friesoythe, Germany). The following day cells were counted and plated in 96-well round-bottom plates (Greiner) at a concentration of 3 x 10^5 cells per well. Triplicate cultures were stimulated with UV-MV (final dilution 1:100), UV-Vero (final dilution 1:100) or medium. Two days later supernatants were harvested and IFN-γ concentrations were measured using a monkey IFN-γ ELISA (U-Cytech, Utrecht, Netherlands).

Statistical analyses

Longitudinal data were analyzed in GraphPad Prism version 7.0a, using grouped analysis: Two-way analysis of variance for repeated measures with Tukey's multiple comparisons test. P-values lower than 0.05 were considered significant. This generalized linear model was automatically parameterized, resulting in 20 degrees of freedom and n-values of 78.

Data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

ACKNOWLEDGEMENTS

We thank Connor Bamford, Thomas Carsillo, Jim Fink, Michael Gottlieb, Ana Maria Henao-Restrepo, Hettie Janssens, Bert Rima, Paul Rota, Steven Rubin, and all EMC animal caretakers for their contributions to these studies. We are grateful to Dr Dhere and Dr Vaidya of the Serum Institute of India (Pune, India), who donated vaccine virus, cells and protocols free of charge. We thank Dr. MacLoughlin of Aerogen Ltd. (Galway, Ireland), who donated nebulizers and performed bench tests free of charge. This study was funded by the Foundation for the National Institutes of Health, through the Bill and Melinda Gates Foundation Grand Challenges in Global Health initiative (grant number: DUPREX09GCGH0). Neither the funder, nor the companies had a role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

AUTHOR CONTRIBUTIONS

R.L.S. and W.P.D. obtained funding and designed research; R.L.S., R.D.V., L.J.R., G.A., R.J.V., J.V., A.J., J.L., D.T.N., M.L., and W.P.D. performed research; A.O. provided challenge model and infrastructure for non-human primate studies; S.M.Q. performed histopathological analyses; R.L.S., R.D.V., and W.P.D. wrote the manuscript; all authors reviewed and provided feedback to the manuscript.

ADDITIONAL INFORMATION

Supplementary Information accompanies the paper on the npj Vaccines website (doi:10.1038/s41541-017-0022-8).

Competing interests: The authors declare that they have no competing financial interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

REFERENCES

1. WHO. W. H. O. position on measles vaccines. Vaccine 27, 7219–7221 (2009).
2. Patel, M. K. et al. Progress Toward Regional Measles Elimination—Worldwide, 2000–2015. MMWR Morb. Mortal. Wkly. Rep. 64, 153–154 (2015).
3. Santibanez, S. et al. Long-term transmission of measles virus in Central and continental Western Europe. Virus Genes 50, 2–11 (2015).
4. Zipprich, J. et al. Measles outbreak—California, December 2014–February 2015. MMWR Morb. Mortal. Wkly. Rep. 64, 153–154 (2015).
5. Rota, P. A. et al. Measles. Nat. Rev. Dis. Primers 2, 16049 (2016).
6. Griffin, D. E. Measles virus-induced suppression of immune responses. Immunol. Rev. 236, 176–189 (2010).
7. de Vries, R. D. & de Swart, R. L. Measles immune suppression: functional impairment or numbers game? PLoS Pathog. 10, e1004482 (2014).
8. Simons, E. et al. Assessment of the 2010 global measles mortality reduction goal: results from a model of surveillance data. Lancet 379, 2173–2178 (2012).
9. Griffin, D. E. Current progress in pulmonary delivery of measles vaccine. Expert Rev. Vaccines 13, 751–759 (2014).
10. Sabin, A. B. et al. Successful immunization of infants with and without maternal antibody by aerosolized measles vaccine. II. Vaccine comparisons and evidence for multiple antibody response. JAMA 251, 2363–2371 (1984).
11. Dilraj, A., Sukhoo, R., Cutts, F. T. & Bennett, J. V. Aerosol and subcutaneous measles vaccines: measles antibody responses 6 years after re-vaccination. Vaccine 25, 4170–4174 (2007).
12. Low, N., Kraemer, S., Schneider, M. & Restrepo, A. M. Immunogenicity and safety of aerosolized measles vaccine: systematic review and meta-analysis. Vaccine 26, 383–398 (2008).
13. Low, N. et al. A randomized, controlled trial of an aerosolized vaccine against measles. N. Engl. J. Med. 372, 1519–1529 (2015).
14. de Swart, R. L. et al. Aerosol measles vaccination in macaques: preclinical studies of immune responses and safety. Vaccine 24, 6424–6436 (2006).
15. de Swart, R. L. et al. Measles vaccination of macaques by dry powder inhalation. Vaccine 25, 1183–1190 (2007).
16. Lin, W. H. et al. Successful respiratory immunization with dry powder live-attenuated measles virus vaccine in rhesus macaques. Proc. Natl Acad. Sci. U.S.A. 108, 2987–2992 (2011).
17. Agarkhedkar, S. et al. Safety and immunogenicity of dry powder measles vaccine administered by inhalation: a randomized controlled Phase I clinical trial. Vaccine 32, 6791–6797 (2014).
18. Duprex, W. P. et al. In vitro and in vivo infection of neural cells by a recombinant measles virus expressing enhanced green fluorescent protein. J. Virol. 74, 7972–7979 (2000).
19. de Swart, R. L. et al. Predominant infection of CD150+ lymphocytes and dendritic cells during measles virus infection of macaques. PLoS Pathog. 3, e1178 (2007).
20. de Vries, R. D. et al. In vivo tropism of attenuated and pathogenic measles virus expressing green fluorescent protein in macaques. J. Virol. 84, 4714–4724 (2010).
21. Lemon, K. et al. Early target cells of measles virus after aerosol infection of non-human primates. PLoS Pathog. 7, e1001263 (2011).
22. de Vries, R. D. et al. Measles immune suppression: lessons from the macaque model. PLoS Pathog. 8, e1002885 (2012).
23. Ludlow, M., McQuaid, S., Milner, D., de Swart, R. L. & Duprex, W. P. Pathological consequences of systemic measles virus infection. J. Pathol. 235, 265–265 (2015).
24. Rennick, L. J. et al. Live-attenuated measles virus vaccine targets dendritic cells and macrophages in muscle of nonhuman primates. J. Virol. 89, 2192–2200 (2015).
25. MacLoughlin, R. J. et al. Optimization and dose estimation of aerosol delivery to non-human primates. J. Aerosol. Med. Pulm. Drug Deliv. 29, 281–287 (2016).
26. Ono, N. et al. Measles viruses on throat swabs from measles patients use signaling lymphocytic activation molecule (CD150) but not CD46 as a cellular receptor. J. Virol. 75, 4399–4401 (2001).
27. van Binnendijk, R. S., Poelen, M. C., van Amerongen, G., de Vries, P. & Osterhaus, A. D. Protective immunity in macaques vaccinated with live attenuated, recombiant, and subunit measles viruses in the presence of passively acquired anti-bodies. J. Infect. Dis. 175, 524–532 (1997).
28. Cohen, B. J., Audet, S., Andrews, N. & Beeler, J., WHO working group on measles plaque reduction neutralization test. Plaque reduction neutralization test for measles antibodies: Description of a standardised laboratory method for use in immunogenicity studies of aerosol vaccination. Vaccine 26, 59–66 (2007).
29. Enders, J. F., Katz, S. L., Milovanovic, M. V. & Holloway, A. Studies on an attenuated virus for measles virus. J. Virol. 7, 7979 (2000).
30. Bankamp, B., Takeda, M., Zhang, Y., Xu, W. & Rota, P. A. Genetic characterization of wild-type measles virus with the hemagglutinin protein of the edmonston vaccine strain retains wild-type tropism in macaques. J. Virol. 86, 3027–3037 (2012).
38. Valdespino-Gomez, J. L. et al. Measles aerosol vaccination. *Curr. Top. Microbiol. Immunol.* **304**, 165–193 (2006).
39. Bellanti, J. A. et al. Immunologic studies of specific mucosal and systemic immune responses in Mexican school children after booster aerosol or subcutaneous immunization with measles vaccine. *Vaccine* **22**, 1214–1220 (2004).
40. Barria, M. I. et al. Localized mucosal response to intranasal live attenuated influenza vaccine in adults. *J. Infect. Dis.* **207**, 115–124 (2013).
41. Satti, I. et al. Safety and immunogenicity of a candidate tuberculosis vaccine MVA85A delivered by aerosol in BCG-vaccinated healthy adults: a phase 1, double-blind, randomised controlled trial. *Lancet Infect. Dis.* **14**, 939–946 (2014).
42. Manjaly Thomas, Z. R. & McShane, H. Aerosol immunisation for TB: matching route of vaccination to route of infection. *Trans. R. Soc. Trop. Med. Hyg.* **109**, 175–181 (2015).
43. Sabin, A. B. et al. Clinical trials of inhaled aerosol of human diploid and chick embryo measles vaccine. *Lancet* **2**, 604 (1982).
44. Dilraj, A. et al. Response to different measles vaccine strains given by aerosol and chick embryo measles vaccine. *Lancet* **2**, 604 (1982).
45. Bennett, J. V. et al. Aerosolized measles and measles-rubella vaccines induce better measles antibody booster responses than injected vaccines: randomized trials in Mexican schoolchildren. *Bull. World Health Organ.* **80**, 806–812 (2002).
46. Wong-Chew, R. M. et al. Induction of cellular and humoral immunity after aerosol or subcutaneous administration of Edmonston-Zagreb measles vaccine as a primary dose to 12-month-old children. *J. Infect. Dis.* **189**, 254–257 (2004).
47. Wong-Chew, R. M. et al. Immunogenicity of aerosol measles vaccine given as the primary measles immunization to nine-month-old Mexican children. *Vaccine* **24**, 683–690 (2006).
48. Wong-Chew, R. M. et al. Increasing the time of exposure to aerosol measles vaccine elicits an immune response equivalent to that seen in 9-month-old Mexican children given the same dose subcutaneously. *J. Infect. Dis.* **204**, 426–432 (2011).
49. LiCalys, C., Christensen, T., Bennett, J. V., Phillips, E. & Witham, C. Dry powder inhalation as a potential delivery method for vaccines. *Vaccine* **17**, 1796–1803 (1999).
50. de Swart, R. L. et al. Immunization of macaques with formalin-inactivated human metapneumovirus induces hypersensitivity to hMPV infection. *Vaccine* **25**, 8518–8528 (2007).
51. Edens, C., Collins, M. L., Ayers, J., Rota, P. A. & Prausnitz, M. R. Measles vaccination using a microneedle patch. *Vaccine* **31**, 3403–3409 (2013).