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A polyvalent inactivated rhinovirus vaccine is broadly immunogenic in rhesus macaques

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As the predominant aetiological agent of the common cold, human rhinovirus (HRV) is the leading cause of human infectious disease. Early studies showed that a monovalent formalin-inactivated HRV vaccine can be protective, and virus-neutralizing antibodies (nAb) correlated with protection. However, co-circulation of many HRV types discouraged further vaccine efforts. Here, we test the hypothesis that increasing virus input titres in polyvalent inactivated HRV vaccine may result in broad nAb responses. We show that serum nAb against many rhinovirus types can be induced by polyvalent, inactivated HRVs plus alhydrogel (alum) adjuvant. Using formulations up to 25-valent in mice and 50-valent in rhesus macaques, HRV vaccine immunogenicity was related to sufficient quantity of input antigens, and valency was not a major factor for potency or breadth of the response. Thus, we have generated a vaccine capable of inducing nAb responses to numerous and diverse HRV types.
HRV causes respiratory illness in billions of people annually, a socio-economic burden. HRV also causes pneumonia hospitalizations in children and adults, and exacerbations of asthma and chronic obstructive pulmonary disease (COPD). HRV was found to be the second leading cause of community-acquired pneumonia requiring hospitalization in US children, second only to respiratory syncytial virus, and the most common pathogen associated with pneumonia hospitalization in US adults. A vaccine for HRV could alleviate serious disease in asthma and COPD, reduce pneumonia hospitalizations and have widespread benefits for society on the whole. Decades ago, researchers identified inactivated HRV as a protective vaccine, defined virus-neutralizing antibodies (nAb) as a correlate of protection, and estimated duration of immunity.

Trials with monovalent HRV vaccine demonstrated that protection from homologous challenge and disease can be achieved with formalin-inactivated virus given intramuscularly (i.m.) or intranasally. Humoral immunity to heterologous virus types was not observed, though cross-reactive CD8 T cells can promote clearance. Limited cross-neutralizing antibodies can be induced by hyper-immunization in animals. The possibility of a vaccine composed of 50, 100 or more distinct HRV antigens has been viewed as formidable or impossible.

There are two main challenges, generating a broad immune response and the feasibility of composing such a vaccine. The Ab repertoire is theoretically immense, and many vaccines in clinical use are thought to work via a polyclonal Ab response. Deep-sequencing of human Ab genes following vaccination against influenza virus found thousands of Ab lineages. Whole pathogen and human Ab genes following vaccination against influenza virus are thought there are 150–170 serologically distinct HRV types.

Results
Immunogenicity of 10-valent HRV vaccine in mice. We first used BALB/c mice to test immunogenicity. We propagated HRVs in H1-HeLa cells and inactivated infectivity using formalin. Sera from naive mice had no detectable nAb against HRV-16 (Fig. 1). Alum adjuvant enhanced the nAb response induced by i.m. inactivated HRV-16 (Fig. 1). There was no effect of valency (comparing 1-, 3-, 5-, 7- and 10-valent) on the nAb response induced by inactivated HRV-16 or to the 3 types in the 3-valent vaccine (HRV-16, HRV-36 and HRV-78; Fig. 1). The 50% tissue culture infectious dose (TCID50) titres of the input viruses before inactivation (inactivated-TCID50) are provided in Supplementary Table 1. Original antigenic sin can occur when sequential exposure to related virus variants results in biased immunity to the type encountered first. In bivalent HRV-immune mice, we observed modest original antigenic sin following prime vaccination with 10-valent inactivated HRV, and boost vaccination partially alleviated the effect (Supplementary Fig. 1), similar to influenza virus. Collectively, these results prompted us to explore more fully the nAb response to polyvalent HRV vaccine.

Sufficient input HRV titre determines immunogenicity. In 1975, it was reported that two different 10-valent inactivated HRV preparations induced nAb titres to only 30–40% of the input virus types in recipient subjects. However, the input titres of viruses before inactivation ranged from 101.5 to 105.5 TCID50 per ml, and these were then diluted 10-fold to generate 10-valent 1.0 ml doses given i.m. as prime and boost with no adjuvant. We hypothesized that low input antigen doses are responsible...
for poor nAb responses to 10-valent inactivated HRV. We reconstituted the 1975 10-valent vaccine, as closely as possible with available HRV types, over a $10^1$ to $10^6$ inactivated-TCID$_{50}$ per vaccine dose, and we compared it with a 10-valent vaccine of the same types with input titres ranging from $>10^6$ to $>10^7$ inactivated-TCID$_{50}$ per dose. The reconstituted 1975 vaccine resulted in no detectable nAb after prime vaccination and, following boost vaccination, nAb to the five types that had the highest input titres (Fig. 2). The high titre vaccine resulted in nAb to 5 of 10 types after prime vaccination and all 10 types after the boost (Fig. 2). Following the boost vaccinations, there appeared to be a $10^4$ inactivated-TCID$_{50}$ per vaccine dose threshold for the induction of nAb in this model (Fig. 2b). Above this titre, there was no correlation between input load and nAb induction.

**Immunogenicity of 25-valent HRV vaccine in mice.** Injectable vaccines used in people are commonly given in a 0.5 ml dose. In our facility, the highest allowable i.m. vaccine volume in mice was 0.1 ml. We tested a 25-valent per 0.1 ml HRV vaccine in mice as a scalable prototype. The 25-valent inactivated HRV vaccine had a 7.4-fold lower average inactivated-TCID$_{50}$ per type per dose than the 10-valent composition (Supplementary Table 2) to accommodate the volume adjustment. The 10-valent inactivated HRV vaccine induced nAb to 100% of input types following the prime and the boost (Fig. 3a). The nAb induced by 10-valent inactivated HRV were persisting at 203 days post-boost (Supplementary Fig. 2). We used two different sets of virus stocks for the prime and boost 25-valent vaccinations in these experiments (Supplementary Table 2). The reason is that in the interim between the prime and boost vaccinations, we obtained higher titre virus stocks of eleven of the input types (HRV-32, -49, -58, -55, -41, -33, -39, -50, -1B, -21 and -60). The higher titre stocks of these eleven were used in the boost vaccination. The 25-valent inactivated HRV prime vaccination induced nAb to 18 of 25 (72%) virus types, and the 25-valent boost resulted in nAb against 24 of the 25 types (96%; Fig. 3b). The average nAb titre resulting from prime and boost was $2^7$ for 10-valent and $2^{6.8}$ for 25-valent. These data demonstrate broad neutralization of diverse HRV types with a straightforward vaccine approach.

**Figure 2 | Immunogenicity of inactivated polyvalent HRV is related to dose.** Mice (2 groups, 20 per group) were vaccinated with 10-valent HRV vaccine consisting of low inactivated-TCID$_{50}$ per dose input titres (x-axis), similar to the 1975 Hamory et al. study, plus alum (magenta open circles) or with 10-valent HRV vaccine with high inactivated-TCID$_{50}$ per dose input titres plus alum (blue open squares). Sera were collected 18 days after prime (a) and 18 days after boost (b), pooled for each group and nAb titres (y-axis) were measured against the indicated types in the vaccines. The dashed line represents LOD. Undetectable nAb were assigned LOD/2, and some symbols below LOD were nudged for visualization. Three independent experiments using low input titres showed similar results. There was a statistically significant association between input TCID$_{50}$ virus titre and a detectable nAb response following prime ($P = 0.01$) and boost ($P = 0.03$) vaccination (Fisher’s exact test).

**Figure 3 | Broad nAb responses against 10-valent and 25-valent inactivated HRV in mice.** The inactivated-TCID$_{50}$ input titres per dose are specified in Supplementary Table 2. (a) 20 mice were vaccinated then boosted at 50 days with 10-valent HRV. (b) 30 mice were vaccinated then boosted at 50 days with 25-valent HRV. Sera were collected at day 18 (prime) and day 68 (boost). nAb levels against the indicated types in the vaccines were measured in pooled sera. Error bars depict 95% confidence interval. Data shown represent one of three (10-valent) or two (25-valent) experiments with similar results. The dashed line represents LOD. Undetectable nAb were assigned LOD/2.
Immunogenicity of 25- and 50-valent HRV in RMs. To increase vaccine valency, we chose RMs and a 1.0 ml i.m. vaccine volume. Two RMs were vaccinated with 25-valent inactivated HRV, and two RMs were vaccinated with 50-valent inactivated HRV. Pre-immune sera in RM A and RM B had no detectable nAb against the 25 HRV types included in the 25-valent vaccine. The inactivated-TCID<sub>50</sub> titres per dose were higher in RMs than in mice (Supplementary Table 3). The 25-valent vaccine induced nAb to 96% (RM A) and 100% (RM B) of input viruses following the prime vaccination (Fig. 4a). The 50-valent vaccine induced nAb to 90% (RM C) and 82% (RM D) of input viruses following the prime vaccination (Fig. 4c). The breadth of nAb following prime vaccination in RM was superior to what we observed in mice, which may have been due to animal species differences and/or higher inactivated-TCID<sub>50</sub> input titres in the RM vaccines. Following boost vaccination, there were serum nAb titres against 100% of the types in 25-valent HRV-vaccinated RMs (Fig. 4b) and 98% (49 out of 50) of the virus types in 50-valent HRV-vaccinated RMs (Fig. 4d). The average nAb titre resulting from prime and boost in RMs was 2.3 for 25-valent and 2.8<sup>2</sup> for 50-valent. The nAb responses were type-specific, not cross-neutralizing, because there were minimal nAbs induced by the 25-valent vaccine against 10 non-vaccine types (Supplementary Fig. 3). The nAb response to 50-valent inactivated HRV vaccine was broad and potent in RMs.

HRV yield in WI-38 cells and semi-purification by HPLC. The HRV stocks used in our vaccinations were produced in H1-HeLa cells, a good substrate for HRV replication but not suitable for vaccine production. Adequate yields were obtained from WI-38 cells, a good substrate for HRV replication but not suitable for vaccine production. Adequate yields were obtained from WI-38 cells, which can be qualified for vaccine production. Adequate yields were obtained from WI-38 cells (Supplementary Fig. 4). Injectable vaccines require defined purity. As proof of principle, we semi-purified three HRV types by high-performance liquid chromatography and found uncompromised immunogenicity of trivalent inactivated semi-purified HRV in mice (Supplementary Fig. 5).

Discussion

In this study, we demonstrate that polyvalent inactivated HRV vaccine expanded to a 50-valent composition, with alum adjuvant, is immunogenic against approximately one-third of circulating HRV types. Although fifty HRV types is a minority, we hope to produce a comprehensive 83-valent HRV A vaccine.

Methods

Cell lines and viruses. H1-HeLa (CRL-1518) and WI-38 (CCL-75) cells were obtained from the American Type Culture Collection (ATCC) and cultured in minimal essential media with Richter’s modification and no phenol red (MEM) (Thermo Fisher) supplemented with 10% fetal bovine serum. The cell lines were not authenticated but are not commonly misidentified (International Cell Line Authentication Committee). We tested HeLa-H1 cells using the LookOut Mycoplasma detection kit (Sigma). HRV-7 (VR-1601), HRV-9 (VR-1745), HRV-11 (VR-1567), HRV-14 (VR-286), HRV-16 (VR-283), HRV-19 (V4-1129), HRV-24 (VR-1134), HRV-29 (VR-1809), HRV-30 (VR-1140), HRV-31 (VR-1795), HRV-32 (VR-342), HRV-36 (VR-509), HRV-38 (VR-511), HRV-40 (VR-541), HRV-41 (VR-1151), HRV-49 (VR-1156), HRV-52 (VR-1770), HRV-53 (VR-1166), HRV-55 (VR-1169), HRV-60 (VR-1473), HRV-64 (VR-1174), HRV-66 (VR-1176), HRV-76 (VR-1186), HRV-77 (VR-1187), HRV-78 (VR-1188), HRV-80 (VR-1190), HRV-81 (VR-1191), HRV-85 (VR-1193), HRV-88 (VR-1198), HRV-89 (VR-1199), HRV-96 (VR-1296), and HRV-105 (VR-1000) prototype strains were purchased from ATCC. HRV-1B, HRV-10, HRV-21, HRV-28, HRV-33, HRV-34, HRV-39, HRV-45, HRV-50, HRV-51, HRV-54, HRV-55 and HRV-94 strains were obtained from the Centers for Disease Control and Prevention. The HRVs in the study are species A, with the exception of HRV-14 (B), and represent A species broadly<sup>31,32</sup>.

HRV propagation and titration. HRV stocks were generated in H1-HeLa cells. Approximately 0.5 ml of HRV was inoculated onto subconfluent H1-HeLa cells using 0.5 ml T-25 flasks. Viral cell cultures and harvests were harvested at a T-180 flask. Following adsorption for 1 h at room temperature, the medium was replaced with fresh medium containing 10% FBS and 1% sodium bicarbonate. The cell cultures were kept at 33 °C and checked for viral titres every 2 days. HRV inactivation was performed by a method described above and clarified by brief centrifugation at 950 g for 10 min. The supernatant was transferred to cryovials, snap-frozen in liquid nitrogen and stored at −80 °C. For comparing HRV yield in WI-38 and H1-HeLa cells and WI-38 cells, T-75 flasks of subconfluent cell cultures infected with HRV were harvested after a multiplicity of infection of 0.1 TCID<sub>50</sub> per cell, and 20 ml of culture medium were discarded before scraping the cells in the remaining 5 ml followed by sonication. For all stocks, TCID<sub>50</sub> were determined by subconfluent H1-HeLa cells in 96-well plates with serially diluted samples, staining the cells 6 days post-infection with 0.1% crystal violet/20% methanol, scoring wells for CPE and calculating the end point titre using the Reed and Muench method<sup>38</sup>.

HRV purification. HRV stock was collected from H1-HeLa cell monolayers as described above and clarified by brief centrifugation at low speed to remove large cellular debris (931 g, 10 min, 4 °C). To remove excess albumin from the crude virus stock by affinity chromatography, the supernatant was loaded onto a HiTrap Blue HP column (GE Healthcare) using an AKTA Purifier system (GE Healthcare) according to the manufacturer specifications. Flowthrough was subsequently loaded through a HiTrap Capto Core 700 column (GE Healthcare) to refine the virus prep by size exclusion chromatography. The flowthrough from the HiTrap Blue HP and the HiTrap Capto Core 700 was captured using the AKTA Purifier system (GE Healthcare) with a 20 mM sodium phosphate buffer (pH 7.0). Flowthrough from size exclusion chromatography was dialyzed overnight with 0.1 M Tris-HCl buffer (pH 8.0), then loaded onto a HiTrap Q XL column (GE Healthcare) and separated into fractions by ion exchange chromatography. Virus-containing fractions were eluted using the AKTA Purifier system (GE Healthcare) with a 0.1 M Tris-HCl buffer (pH 8.0) and a sodium chloride gradient. Fractions showing high viral yield were collected at 280 mM NaCl. Virus was further purified by analytical ultracentrifugation and run in a closed rotor with 1/3 pulse per 1 ml of material. Sonication yielded higher titres than freeze–thaw. The suspension was clarified by centrifugation at 931 g for 10 min. The supernatant was transferred to cryovials, snap-frozen in liquid nitrogen and stored at −80 °C. For comparing HRV yield in WI-38 and H1-HeLa cells and WI-38 cells, T-75 flasks of subconfluent cell cultures infected with HRV were harvested after a multiplicity of infection of 0.1 TCID<sub>50</sub> per cell, and 20 ml of culture medium were discarded before scraping the cells in the remaining 5 ml followed by sonication. For all stocks, TCID<sub>50</sub> were determined by subconfluent H1-HeLa cells in 96-well plates with serially diluted samples, staining the cells 6 days post-infection with 0.1% crystal violet/20% methanol, scoring wells for CPE and calculating the end point titre using the Reed and Muench method<sup>38</sup>.

Mice and RMs. All experiments involving animals were performed at Emory University and the Yerkes National Primate Research Center in accordance with guidelines established by the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals. Animal facilities at Emory University and the Yerkes Center are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The Institutional Animal Care and Use Committee (IACUC) of Emory University approved these studies. Balb/c mice were obtained from Taconic (Germantown, NY). 

On the basis of our results and doses of early immunogenic HRV vaccines<sup>6,8</sup>, we estimate 10<sup>5</sup>–10<sup>6</sup> TCID<sub>50</sub> per mouse are required for a potential 83-valent HRV A formulation in a 0.5 ml dose containing alum adjuvant. In future studies, we hope to produce a comprehensive 83-valent HRV A vaccine. A limitation of our study is that we have not included HRV C antigens, which will be important for HRV vaccine development, especially for pediatric populations. Ongoing efforts are aimed at propagation of HRV C strains to high titre in cell lines and development of serology assays. Ultimately, our approach may lead to vaccines for rhinovirus-mediated diseases including asthma and COPD exacerbations and the common cold. Advancing valency may be applicable to vaccines for other antigenically variable pathogens.
Figure 4 | Broad nAb responses against 25-valent and 50-valent inactivated HRV in RMs. The inactivated-TCID$_{50}$ input titres per dose are specified in Supplementary Table 3. Two RMs (RM A and RM B) were vaccinated i.m. with 25-valent HRV and alum (a,b), and two RMs (RM C and RM D) were vaccinated i.m. with 50-valent HRV and alum (c,d). nAb titres against input virus types were measured in individual serum samples collected at day 18 (a,c). The RM received an identical boost vaccination at day 28, and sera were collected at day 46 for determining nAb titres post-boost vaccination (b,d). Error bars depict 95% confidence interval. The dashed line represents LOD. Undetectable nAb were assigned LOD/2.
blinded to outcome assessment. No statistical methods were used in predetermining sample sizes.

Young adult (3–5 kg, 2–4 years of age, 2 females and 2 males) Indian RMs (Macaca mulatta; RM) were maintained according to NIH guidelines at the Yerkes National Primate Research Center. Handling and movement of RMs was performed by qualified personnel who have received specific training in safe and humane methods of animal handling at the Yerkes National Primate Research Center. The initial exclusion criterion was pre-existing nAb against HRV. The studies were performed in strict accordance with US Department of Agriculture regulations and the recommendations in the Guide for Care and Use of Laboratory Animals of the NIH. The RMs were allocated in an un-blinded manner to two vaccine groups (25-valent and 50-valent), one male and one female per group. No statistical methods were used in predetermining sample sizes.

Vaccination. Before immunization, all HRV types were inactivated by addition of 0.025% formalin followed by incubation with stirring for 72 h at 37 °C, as previously described for HRV vaccine35. Complete inactivation of infectivity was confirmed by end point TCD50 titration in H1-HeLa cells. Formalin inactivation by this method resulted in greater immunogenicity in mice than alternative inactivation by β-propiolactone, suggesting formalin inactivation preserved antigenic determinants. Mice were vaccinated i.m. with inactivated HRV strains mixed with 100 μg of Alhydrogel adjuvant 2% (aluminium hydroxide wet gel suspension, alum; Sigma catalog AR8222 or Inovigen vaccine val-alu) according to instructions of the manufacturers. The total volume per mouse was 100 μl, administered in 50 μl in 50 μl each. Mice were given two identical doses (boost) at the time indicated in figure legends. RMs were vaccinated i.m. with inactivated HRV strains mixed with 500 μg of Alhydrogel adjuvant 2%. The total volume per RM was 1 ml, administered in one leg. RMs were boosted with an identical vaccination at four weeks.

Serum collection. In mice, peripheral blood was collected into microcentrifuge tubes from the submandibular vein. Samples were incubated at room temperature for 20 min to clot. The tubes were centrifuged 7,500 g for 7 min to separate serum. The serum samples were pooled from mice of each group and stored at –80 °C until used. Phlebotomy involving RMs was performed under either ketamine (10 mg kg−1) or Telazol (4 mg kg−1) anesthesia on fasting animals. Following anesthesia with ketamine or Telazol, the animals were bled from the femoral vein. Yerkes blood collection guidelines were followed and no more than 10 ml kg−1 of HRV infection medium were added to each well. The 96-well plates were incubated for 6 days at 32 °C. The mixture was transferred onto H1-HeLa cell monolayers in 96-well plates in 100 μl of HRV infection medium were added to each well. The 96-well plates were incubated for 6 days at 32 °C and 5% CO2 and then stained with crystal violet as described above. Wells were scored for the presence or absence of CPE. Neutralizing antibody end point titres and 95% confidence intervals were determined by the method of Reed and Muench38, as previously described for HRV13,39. The 95% confidence interval indicates variability of three technical replicates within a single nAb experiment. An example of the nAb assay and titre calculation is provided in Supplementary Fig. 7.

Data availability. Primary data that support the findings of this study are available from the corresponding author on request.

References
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Author contributions
S.L., M.T.N., M.G.C., J.B.J., E.A.S., A.E.K. and R.M.-L. performed experiments. K.R., Y.A.B., J.E.G. and P.S. provided reagents and advice. X.L. and D.D.E. provided rhinovirus types. S.L., M.T.N. and M.L.M. designed the experiments, analysed data and wrote the paper. The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: M.L.M. co-founded and serves as Chief Scientific Officer for Meissa Vaccines, Inc. S.L., M.T.N. and M.L.M are co-inventors in a patent application (PCT/US2016/037658) describing the rhinovirus vaccine reported in this paper. The vaccine technology has been optioned to Meissa Vaccines, Inc. by Emory University. The remaining authors declare no competing financial interests.

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