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A host-range restricted parainfluenza virus type 3 (PIV3) expressing the human metapneumovirus (hMPV) fusion protein elicits protective immunity in African green monkeys

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Abstract

Human metapneumovirus (hMPV) infection causes respiratory tract disease similar to that observed during human respiratory syncytial virus infection (hRSV). hMPV infections have been reported across the entire age spectrum although the most severe disease occurs in young children. No vaccines, chemotherapeutics or antibodies are presently available for preventing or treating hMPV infections. In this study, a bovine/human chimeric parainfluenza virus type 3 (b/h PIV3) expressing the human parainfluenza type 3 (hPIV3) fusion (F) and hemagglutinin-neuraminidase (HN) proteins was engineered to express hMPV fusion (F) protein from the second genome position (b/h PIV3/hMPV F2) with the goal of generating a novel hMPV vaccine. b/h PIV3/hMPV F2 was previously shown to protect hamsters from challenge with *wt* hMPV (Tang RS, Schickli JH, Macphail M, Fernandes F, Bicha L, Spaete J, et al. Effects of human metapneumovirus and respiratory syncytial virus antigen insertion in two 3' proximal genome positions of bovine/human parainfluenza virus type 3 on virus replication and immunogenicity. J Virol 2003;77:10819–28) and is here further evaluated for efficacy and immunogenicity in African green monkeys (AGMs). AGMs immunized intranasally and intratracheally with b/h PIV3/hMPV F2 generated hMPV- and hPIV3-specific humoral and cellular immune responses and were protected from *wt* hMPV infection. In a separate study, the host-range restriction of b/h PIV3/hMPV F2 replication relative to *wt* hPIV3 was performed in rhesus monkeys to demonstrate attenuation. These studies showed that b/h PIV3/hMPV F2 was immunogenic, protective and attenuated in non-human primates and warrants further evaluation in humans as a vaccine candidate for prevention of hMPV-associated respiratory tract diseases.

Keywords: PIV3 vectored-vaccines; Primates; Human metapneumovirus

1. Introduction

Human metapneumovirus (hMPV) is a respiratory pathogen that was first isolated in The Netherlands from nasopharyngeal aspirates taken from young children suffering from respiratory tract disease not attributable to human respiratory syncytial virus (hRSV), parainfluenza virus types 1–3, and influenza A and B viruses [1]. hMPV has been characterized as an enveloped, non-segmented, negative-strand RNA virus and classified as a member of the *Paramyxoviridae* family [1,2]. The hMPV gene constellation, genome organization and nucleotide sequences were most closely related to avian pneumovirus subtype C (APV/C) [3], the causative agent of severe and life-threatening upper respiratory tract infection in turkeys. hMPV is the newest addition to the *Pneumovirinae* subfamily that also includes other mammalian respiratory

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pathogens such as human and bovine RSV and pneumovirus of mice (PVM) [4].

hMPV is not a newly emerging virus but has been circulating in the human population for more than 43 years [1,5]. It has since also been identified in North [5–7] and South America [8] as well as Asia [9–11], Africa [12] and Australia [13] from patients as young as <1 year [5,6] and as old as 87 years of age [14]. hMPV infection occurs in the winter and peak incidence in the northern hemisphere are from December to February [6,15]. The clinical syndrome associated with hMPV infection is similar to that observed for RSV infection ranging from mild respiratory illness to bronchiolitis and pneumonia [1,5,7]. The impact of hMPV infections appears to be greatest for infants, the elderly and the immunocompromised [5–7,16,17] similar to populations at-risk for severe RSV infections. Seven to twelve percent of respiratory tract illnesses in young children can be attributed to hMPV infection [5,16] although the hMPV-associated disease rate was lower (4.5%) in young and elderly adults [17]. The full extent of disease burden and health care costs associated with hMPV infections are still undetermined. A live attenuated hMPV vaccine would be an appropriate addition to the vaccines currently under development against other pediatric respiratory tract diseases caused by hRSV and human parainfluenza virus type 3 (hPIV3) [18,19].

Two main genetic lineages of hMPV have been identified (A and B), that are further divided into two sublineages (A1, A2, B1 and B2). Group A is represented by hMPV/NL/1/00 (A1) and CAN 83 (A2), and group B by hMPV/NL/1/99 (B1) and CAN 75 (B2) [1,7,20]. Therefore, vaccination strategies against hMPV infections must be effective against both groups of hMPV. Comparison of hMPV sequences from Europe, Asia and South America showed no geographic clustering or antigenic drift between the A and B viruses [21]. hMPV-infected ferret and hamster sera neutralized homologous group viruses better than viruses belonging to the heterologous group [21,22]. This effect was also observed for African green monkey (AGM) sera [22,23]. However, hamsters and AGMs infected with hMPV from one group were effectively protected when challenged with heterologous group hMPV [22,23].

Although all the protective epitopes of hMPV have not been identified, the glycoproteins of hRSV and hPIV3 have been shown to play major roles in inducing immune protection [18,19]. Of the three hMPV surface glycoproteins, the predicted amino acid sequence of the F protein (95% identity) was the most highly conserved between the genetic lineages of hMPV while the SH (59% identity) and the G (37% identity) glycoproteins were poorly conserved [2,24]. The hMPV F protein is thus likely to be the major antigen that induces cross-protection in hamsters and AGMs [23]. We have previously shown that a chimeric PIV3 virus [25,26] expressing the hMPV/NL/1/00 F protein (b/h PIV3/hMPV F2), elicited hMPV serum neutralizing antibodies and protected hamsters from homologous hMPV challenge [27]. A similar approach using hPIV 1 as a vector to express group A hMPV F protein protected hamsters from both group A and group B hMPV infections [23].

In this study, the ability of b/h PIV3/hMPV F2 to induce protective immunity against hMPV infection was further evaluated in non-human primates, an animal more closely related to humans genetically. AGMs are highly permissive for both hMPV [22,23] and PIV3 [28] infections and provide a good model for assessing the immunogenicity and efficacy of the b/h PIV3/hMPV F2 vaccine candidate. For this reason, the host-range restricted replication of bPIV3, and its derivatives relative to hPIV3, cannot be evaluated in AGMs. However, the restricted replication of bPIV3 compared to hPIV3 was previously demonstrated in the respiratory tract of chimpanzees and rhesus monkeys and this attenuation phenotype was shown to correlate with the safety profile of bPIV3 in human infants [29–32]. We have previously shown that rbPIV3, the virus vector was restricted in replication in the upper and lower respiratory tract of rhesus monkeys compared to hPIV3, and that replacement of the bovine PIV3 F and HN gene with human PIV3 F and HN resulted in an intermediate level of attenuation [33]. Here, we compared the replication of the chimeric b/h PIV3/hMPV F2 virus with *wt* hPIV3, recombinant bPIV3 (rbPIV3) and a bovine parainfluenza virus expressing the human PIV3 F and HN genes (b/h PIV3) in the respiratory tract of rhesus monkeys. The results of our studies in African green and rhesus monkeys indicated that b/h PIV3/hMPV F2 elicited hMPV- and hPIV3-specific humoral and cellular immune responses, and was efficacious and attenuated. Thus, further evaluation of this vaccine candidate in humans is warranted to assess its potential as a pediatric vaccine for prevention of respiratory tract diseases caused by hMPV.

2. Materials and methods

2.1. Cells and viruses

Vero cells were maintained in Modified Eagle's Medium (MEM) (JRH Biosciences) supplemented with 2 Mm L-glutamine, non-essential amino acids (NEAA), antibiotics, and 10% FBS. b/h PIV3/hMPV F2, hMPV/NL/1/00, and hMPV/NL/1/99 were propagated in Vero cells. Virus stocks were prepared by infecting Vero cells at a multiplicity of infection (MOI) of 0.1 PFU/cell. b/h PIV3/hMPV F2 was harvested 3–4 days post-infection while the hMPV strains were harvested 7–9 days post-infection following incubation at 35 °C. The presence of trypsin does not enhance the growth of hMPV/NL/1/00 and hMPV/NL/1/99 in Vero cells and was omitted. Infected cell and media were collected together and stabilized with 10× SPG (2.18 M sucrose, 0.038 M KH_2PO_4, 0.072 M K_2HPO_4, 0.054 M L-glutamate) to a final concentration of 1×. The stabilized virus stocks were stored at −70 °C. hPIV3/JS, rbPIV3, b/h PIV3 and b/h PIV3/hMPV F2 titers were determined by plaque assays on Vero cells. Vero cells were infected with 10-fold serially diluted virus

samples and overlaid with EMEM/L-15 medium (JRH Biosciences) containing 1% methylcellulose and 2% fetal bovine serum. After 5–6 days of incubation at 35 or 37 °C, the overlay was removed and the cells were fixed with methanol. Following methanol fixation, b/h PIV3/hMPV F2 plaques were immunostained with a polyclonal ferret hMPV antiserum (MedImmune Vaccines Inc.) and a secondary HRP-conjugated goat anti-ferret IgG (Immunology Consultants Laboratory, USA). PIV3 plaques were immunostained with a primary bPIV3 polyclonal goat antibody (VRMD Inc., WA, USA) that cross-reacts with hPIV3 antigens and a secondary rabbit anti-goat IgG conjugated with horseradish peroxidase (DAKO Corporation, CA, USA). hMPV plaque assays were performed in the same way except that the overlay contained OptiMEM (Invitrogen) and the infected cells were incubated at 37 °C for 7 days prior to immunostaining. All primary and secondary antibodies were used at a dilution of 1:1000 except for the ferret hMPV antiserum that was used at 1:500 for immunostaining.

2.2. African green monkey studies

Experimentally-naïve African green monkeys (*Cercopithecus aethiops*) (2.6–6 years old weighing 3.3–5.8 kg) were screened for exposure to hMPV and PIV3 using a 50% plaque reduction neutralization assay (PRNA) or a hemagglutination inhibition (HAI) assay respectively (described below). The pre-screening was done with sera collected 21 days prior to initiation of the study. Only hMPV and PIV3 sero-negative animals were selected for the study. The Sierra Biomedical IACUC committee approved all protocols and procedures involving the use and care of monkeys in accordance with the regulations of the USDA Animal Welfare Act and in compliance with Sierra Biomedical Animal Welfare Assurance (A4112-01) filed with the National Institutes of Health. The monkeys were individually housed in biocontainment cages and generally maintained in a BSL-3 containment to minimize inappropriate spread of live viruses. The monkeys were infected intranasally and intratracheally with b/h PIV3/hMPV F2 (3.2×10^5 pfu/ml), hMPV/NL/1/00 (4.5×10^5 pfu/ml) or Opti-MEM (Invitrogen) containing 1× sucrose/phosphate/glutamate mixture (SPG) as placebo. On day 1, the animals received a nasal dose volume of 0.5 ml per nostril, and a tracheal dose volume of 1 ml. The placebo animal group received the same dose volume of Opti-MEM supplemented with 1× SPG. Prior to dosing monkeys were lightly sedated with a 1:1 (v/v) mixture of ketamine and diazepam at 10 mg/kg ketamine and 0.5 mg/kg diazepam.

On day 28, all animals were challenged intratracheally and intranasally with 5×10^5 PFU of *wt* hMPV/NL/1/00 in a dose volume of 1 ml at each site. Nasopharyngeal (NP) swabs were collected daily for 11 days post-immunization and post-challenge from each monkey using sterile cotton swabs, pre-moistened in Opti-MEM containing 1× SPG. Each cotton swab was placed into a tube containing 1.0 ml Opti-MEM with 1× SPG and immediately frozen on dry ice. Bronchoalveolar lavage (BAL) specimens were collected on alternate days post-immunization and post-challenge. During BAL collection, the monkeys were sedated using a ketamine/valium mixture to allow passage of a sterile laryngoscope and endotracheal tube. The feeding tube was passed through the endotracheal tube, and 5–10 ml (∼2 ml/kg) of warm sterile saline was instilled and gently suctioned back into the syringe promptly. The BAL samples were stabilized with 1× SPG, and two aliquots of the samples were prepared, frozen on dry ice and stored at −70 °C. Blood samples obtained from the femoral vein were collected on day 1 (prior to dosing), 7, 14, 21 (prior to challenge), 28, 35, 42, 49 and 56 (after challenge) for serological analysis. The sera were stored at −70 °C for measuring neutralizing antibody titers. Heparinized blood was separately collected on days 1, 28 and 56 for processing peripheral blood mononuclear cells. The animals were monitored for indications of fever by measuring changes in their body temperatures, signs of a cold, runny nose, sneezing, loss of appetite, and body weight. Virus present in the frozen NP and BAL specimens was quantitated by plaque assays on Vero cells. Plaques were visualized by immunostaining with hMPV-specific ferret polyclonal antibodies (MedImmune Vaccines Inc.) as described above.

2.3. Rhesus monkey studies

Experimentally-naïve rhesus monkeys (*Macaca mulatta*) ranging from 2.8 to 4.5 years of age and weighing 3.9–6.4 kg were screened for exposure to hMPV and PIV3 as described above. Screening was done on sera collected 3 and 2 weeks prior to initiation of study. Only hMPV and hPIV3 sero-negative animals were selected for this study. Care and use of monkeys were in accordance with guidelines described above for the AGM studies. Rhesus monkeys were individually housed in stainless-steel cages and groups of animals infected with the same virus were kept in separate animal rooms to minimize inappropriate spread of live viruses. Rhesus monkeys were infected intratracheally with b/h PIV3/hMPV F2 or *wt* hPIV3/JS in Study A and with rbPIV3 or b/h PIV3 in Study B. The route of inoculation was designed to replicate a previous bPIV3 attenuation study performed in rhesus monkeys [29]. The tracheal dose volume was 1 ml containing 5.3 $\log_{10}$ pfu of b/h PIV3/hMPV F2, 5.4 $\log_{10}$ pfu of hPIV3/JS, 5.0 $\log_{10}$ pfu of rbPIV3 or 5.0 $\log_{10}$ pfu b/h PIV3 in Opti-MEM (Invitrogen) stablilized with 1× SPG. Monkeys were lightly sedated prior to dosing. Nasopharyngeal (NP) swabs were collected daily for 13 days post-immunization while bronchoalveolar lavage (BAL) specimens were collected on alternate days post-immunization under light sedation as described for the AGM study. Samples were stabilized with 1× SPG, frozen on dry ice and stored at −70 °C. Blood was collected by venipuncture of the peripheral veins on days 1 (prior to dosing), 7, 14 and 21 and stored at −70 °C for serological analysis. The rhesus monkeys were also monitored for clinical signs of a cold. Virus titrations of the NP and BAL specimens were performed on Vero cells.

Plaques were visualized by immunostaining with PIV3 or hMPV-specific polyclonal antibodies and enumerated.

2.4. Plaque reduction neutralization assay (PRNA)

The primate sera were heat-inactivated at 56 °C for 1 h, two-fold serially diluted, and incubated with 100 PFU of hMPV in the presence of guinea pig complement for 4 h at 4 °C. The serum-virus mixtures were transferred to Vero cell monolayers and overlaid with 1% methyl-cellulose in Opti-MEM (Invitrogen) with 1% antibiotics. After 7 days of incubation at 37 °C, the monolayers were immunostained using hMPV ferret polyclonal antiserum to visualize plaques for counting. Neutralization titers were expressed as the reciprocal $\log_2$ of the highest serum dilution that inhibited 50% of viral plaques.

2.5. hPIV3 microneutralization assays

Microneutralization assays were performed on Vero cells. Serial two-fold dilutions of heat-inactivated primate serum, starting at 1:4, were incubated at 4 °C for 3 h with 500 $TCID_{50}$ of hPIV3. Serum-virus mixtures were transferred to cell monolayers in 96-well plates and incubated at 37 °C for 6 days, after which all wells were observed for CPE. Neutralization titers were expressed as the reciprocal of the highest serum dilution that inhibited CPE. Neutralization antibody titers of ≤1:4 (the lowest serum dilution tested) were assigned a reciprocal $\log_2$ titer of 2.

2.6. PIV3 hemagglutination inhibition assay

HAI assays were performed by incubating serial two-fold dilutions of primate serum at 25 °C for 30 min with eight HA units/0.05 ml of either bPIV3 or hPIV3. Subsequently, guinea pig red blood cells were added to each well and incubation continued for 90 min. Following incubation, each well was observed for hemagglutination. HAI titers were expressed as the reciprocal of the highest dilution of antiserum that inhibited virus-mediated agglutination of erythrocytes.

2.7. ELISPOT assays

Heparinized blood was collected from b/h PIV3/hMPV F2-, hMPV/NL/1/00- or placebo-treated African green monkeys on days 1, 28 and 56. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by Ficoll-Hypaque (Pharmacia) density gradient centrifugation and were frozen in liquid nitrogen. Thawed PBMCs were resuspended in complete medium [RPMI-1640 medium (Invitrogen) with 2 mM L-glutamine, antibiotics and supplemented with 10% fetal bovine serum (Hyclone)]. The cells were incubated for 4 h at 37 °C in a 5% CO_2 incubator and a viable cell count was performed after 4 h. Serial three-fold dilutions of PBMC suspensions (1×10^6 cells/ml) were prepared in complete medium. The diluted cells were seeded in duplicate wells starting at 1×10^5 cells/0.1 ml onto anti-human IFNγ antibody coated ELISPOT plates (BD Biosciences) pre-seeded with 0.1 ml of diluted virus in Vero cell lysate at 10^5 pfu/well or with uninfected Vero cell lysates. The viruses were grown in Vero cells as described above. The ELISPOT plates were incubated at 37 °C for 20 h. Following incubation, the cells were removed by multiple rounds of washes with PBS. The plates were developed for IFNγ ELISPOTs using biotin-conjugated anti-human IFNγ and streptavidin conjugated to horse radish peroxidase (BD Biosciences). Several washes were performed with PBS containing 0.05% Tween-20 followed by a final PBS wash before the addition of AEC chromogenic substrate. The developed spots were counted using a dissection microscope. Stimulation with 5 ng/ml of Phorbol Myristic Acid (PMA) (Sigma) + 500 ng/ml Ionomycin (Sigma) at each of the cell densities resulted in more than 200 spots/well.

3. Results

3.1. b/h PIV3/hMPV F2 replicated efficiently in the respiratory tract of AGMs

AGMs are highly permissive to PIV3 and hMPV infections resulting in high virus titers in the lower (LRT) and upper respiratory tract (URT) [22,23,28]. Three groups ($n = 4$) of hMPV- and hPIV3- sero-negative AGMs were immunized intranasally and intratracheally with b/h PIV3/hMPV F2, hMPV/NL/1/00 or placebo (Fig. 1) and shed viruses were measured by plaque assays. b/h PIV3/hMPV F2 was detected for 11 days in nasal swabs (Fig. 2A) with a mean peak titer of 6.0 $\log_{10}$ pfu/ml (Table 1), and for 8 days in the BAL with mean peak titers of 6.0 $\log_{10}$ pfu/ml. *wt* hMPV/NL/1/00 was shed for 11 days in the nasopharynx and 8 days in the trachea with mean peak titers of 4.4 and 5.4 $\log_{10}$ pfu/ml, respectively (Table 1, Fig. 2). The mean peak titer for hMPV/NL/1/00 in the nasopharynx was approximately 2 $\log_{10}$ higher than

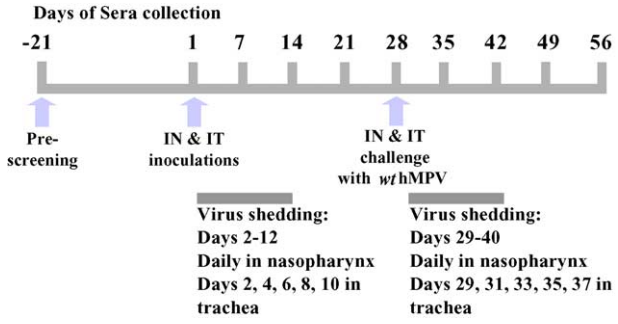

Fig. 1. Outline of the AGM primate study design from day 21 to day 56. Serum was collected at the days indicated. Three groups of four animals were inoculated intranasally (IN) and intratracheally (IT) on day 1 with *wt* hMPV/NL/1/00, b/h PIV3/hMPV F2 or placebo. On day 28, AGMs were challenged with *wt* hMPV/NL/1/00 by intranasal and intratracheal administrations.

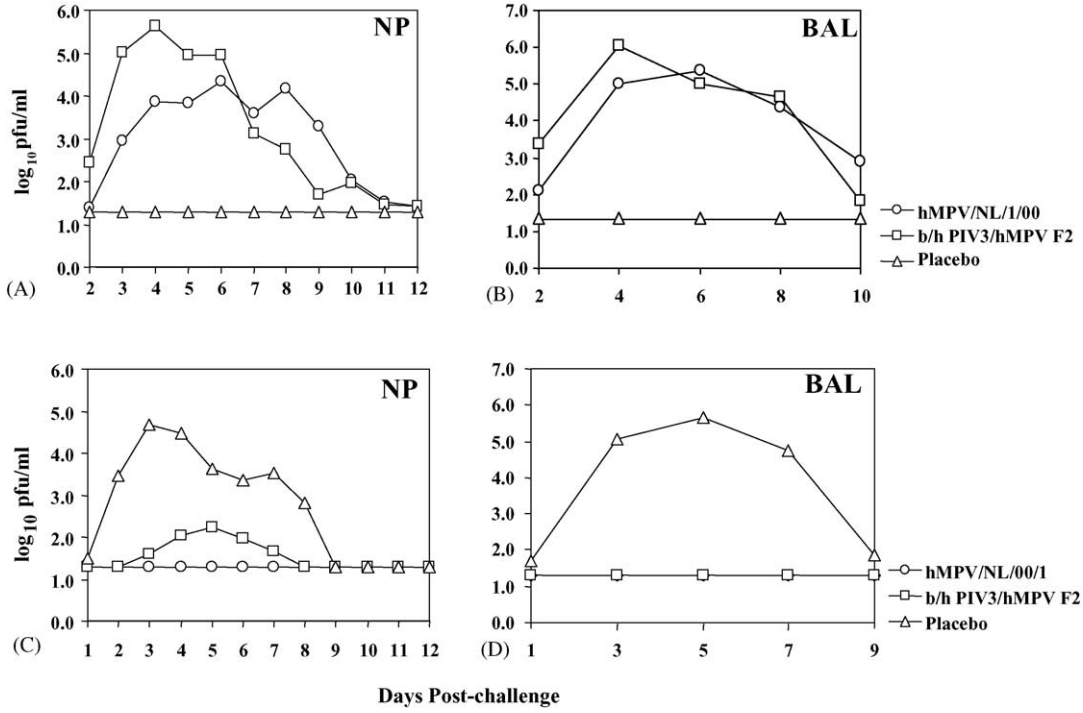

Fig. 2. Mean daily shedding of b/h PIV3/hMPV F2, *wt* hMPV/NL/1/00 and placebo in the URT and LRT of AGMs ($n = 4$ for each group) are shown for 12 days post-immunization and 10 days post-challenge. Virus titers were determined by plaque assays immunostained with hMPV-specific polyclonal antisera. The limit of detection is 1.3 $\log_{10}$ pfu/ml. (A) The mean daily virus titers shed in the URT of AGMs post-vaccination and (B) in the LRT are shown. Shedding of the challenge virus (*wt* hMPV/NL/1/00) in the URT (C) and LRT (D) of AGMs immunized with hMPV/NL/1/00, b/h PIV3/hMPV F2 and placebo are shown for 12 days post-challenge in the URT and 9 days in the LRT. hMPV/NL/1/00 (○), b/h PIV3/hMPV F2 (□), placebo (△).

hMPV CAN83 [23], another group A isolate of hMPV. No signs of rhinorrhea or fever were noted for any of the infected animals post-vaccination and after hMPV challenge (data not shown). Thus, b/h PIV3/hMPV F2 replicated efficiently in the URT and LRT of AGMs and achieved titers higher than *wt* hMPV/NL/1/00.

3.2. b/h PIV3/hMPV F2 immunization protected AGMs from wt hMPV challenge

The efficacy of b/h PIV3/hMPV F2 was evaluated following challenge with 5×10^5 $\log_{10}$ pfu of *wt* hMPV/NL/1/00 four weeks post-vaccination. Protection was defined as a reduction of at least 100-fold in hMPV titer in the URT and LRT compared to the placebo group. Immunization with b/h PIV3/hMPV F2 effectively protected AGMs from *wt* hMPV/NL/1/00 challenge (Table 1; Fig. 2C and D). None of the b/h PIV3/hMPV F2-immunized animals had detectable challenge virus in the LRT (Table 1). Two animals vaccinated with b/h PIV3/hMPV F2 shed low levels of challenge virus in the URT with peak titers of 3.4 and 3.5 $\log_{10}$ pfu/ml on days 6 and 5, respectively resulting in a mean peak titer of 2.1 $\log_{10}$ PFU/ml (Table 1). In contrast the mean peak titers for the placebo group were 4.7 and 5.6 $\log_{10}$ pfu/ml in the URT and LRT, respectively (Fig. 2C and D; Table 1). As expected, hMPV/NL/1/00-infected animals were fully protected (Table 1; Fig. 2C and D). Therefore, b/h PIV3/hMPV F2 protected AGMs effectively against hMPV infection in the LRT, the site of hMPV-associated bronchiolitis and pneumonia.

Table 1
Immunization of African green monkeys with b/h PIV3/hMPV F2 protects against *wt* hMPV/NL/1/00 challenge

Immunizing[a] virus	No. of animals	Mean peak titers[b] $\log_{10}$ pfu/ml			
		Pre-challenge		Post-challenge[c]	
		NP	BAL	NP	BAL
hMPV/NL/1/00	4	4.4 ± 0.4	5.4 ± 0.4	<1.3 ± 0.0	<1.3 ± 0.0
b/hPIV3/hMPV F2	4	6.0 ± 0.1	6.0 ± 0.5	2.3 ± 1.1	<1.3 ± 0.0
Placebo	4	<1.3 ± 0.0	<1.3 ± 0.0	4.7 ± 0.4	5.6 ± 0.6

[a] Animals were inoculated intranasally and intratracheally with $3–4 \times 10^5$ pfu of the indicated virus at each site.
[b] Mean of the highest titers obtained for each group of animals expressed as $\log_{10}$ pfu/ml ± standard deviation.
[c] Animals were challenged on day 28 with 5×10^5 pfu of hMPV/NL/1/00.

Table 2
Vaccination of African green monkeys with b/h PIV3/hMPV F2 generates hMPV neutralizing antibody titers

Immunizing virus	Day of serum collection[a]	Rise in hMPV neutralizing antibody titers (50% Reciprocal $\log_2 \pm$ S.D.)	
		hMPV A[b,c]	hMPV B[b,d]
hMPV/NL/1/00	28	11.1 ± 1.9[e]	4.0 ± 0.8[e]
	56	10.2 ± 1.5	3.7 ± 1.0
b/h PIV3/hMPV F2	28	7.1 ± 1.2[e]	2.7 ± 1.1[e]
	56	8.4 ± 1.2	3.9 ± 1.6
Placebo	28	0.0 ± 0.0	1.0 ± 1.3
	56	6.8 ± 2.0	2.9 ± 1.6

[a] Day 28 prior to hMPV/NL/1/00 (Al) challenge and day 56 (4 weeks post-challenge).
[b] hMPV/NL/l/00 and hMPV/NL/l/99 represent group A l and B l antigens in the neutralization assays.
[c] Average day 1 pre-sera = 2.2 ± 0.9.
[d] Average day 1 pre-sera = 5.1 ± 1.4.
[e] Neutralization antibody titers between hMPV A and hMPV B antigens is statistically significant ($P < 0.05$).

3.3. AGMs immunized with b/h PIV3/hMPV F2 produced protective hMPV serum antibodies

hMPV neutralizing antibody responses following immunization with b/h PIV3/hMPV F2 were quantified using a 50% PRNA (Table 2). AGMs infected with *wt* hMPV/NL/1/00 displayed a 11.1 $\log_2$ rise in neutralizing antibody titer against hMPV/NL/1/00 but only 4.0 $\log_2$ against hMPV/NL/1/99, a subgroup B hMPV, 4 weeks post-infection. The lower rise in hMPV neutralizing antibody titer detected in sera from b/h PIV3/hMPV F2-infected animals was not significant (Table 2). In both groups, antibodies against hMPV/NL/1/00 F protein, neutralizes hMPV/NL/1/00 virus more efficiently than hMPV/NL/1/99. Day 56 sera showed no further rise in hMPV neutralizing antibody titer against both subgroup A and B hMPV (Table 2). b/h PIV3/hMPV F2-treated group showed a small increase in hMPV neutralizing antibody titer against hMPV A and B after challenge that was not statistically significant. Therefore, b/h PIV3/hMPV F2 immunization resulted in a robust neutralizing antibody response against *wt* hMPV/NL/1/00 that also cross-neutralized *wt* hMPV/NL/1/99.

3.4. b/h PIV3/RSV F2 immunization of AGMs induced hPIV3 neutralizing and HAI serum antibodies

The ability of b/h PIV3/hMPV F2 to elicit hPIV3 neutralizing and hemagglutination inhibition serum antibodies was evaluated to determine whether vaccinated animals could also be protected from hPIV3 infection (Table 3). Both days 28 and 56 sera from animals immunized with b/h PIV3/hMPV F2 exhibited high hPIV3 neutralizing antibody titers of 7.2 $\log_2$. High titers of human PIV3-specific HAI antibody of 10.5 and 10.0 $\log_2$ were also detected for b/h PIV3/hMPV F2-infected sera from days 28 and 56, respectively. As expected lower bPIV3-specific HAI antibody titers of 8.5 and 7.3 $\log_2$ were observed at day 28 against the heterologous bovine PIV3 antigen. This suggested that immunization of AGMs with b/h PIV3/hMPV F2 would provide a protective antibody response against hPIV3 infection.

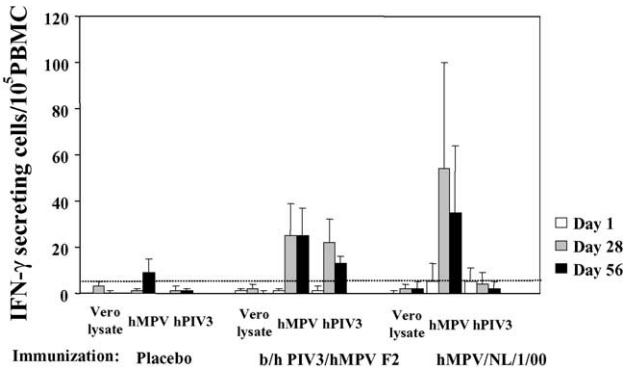

Fig. 3. Elispot assay using PBMCs from African green monkeys immunized with placebo, b/h PIV3/hMPV F2 or hMPV/NL/1/00 ($n = 4$ in each group). The frequencies of IFN-γ secreting T cells per 10^5 input cell stimulated with uninfected Vero cell lysate, *wt* hMPV/NL/1/00 or *wt* hPIV3/JS is shown with standard deviations. The number of vaccinated animals in each group is four. Day 1 = pre-vaccination, day 28 = pre-challenge, day 56 = post *wt* hMPV/NL/1/00 challenge.

3.5. Cellular immune response to hMPV and hPIV3 in b/h PIV3/hMPV F2 vaccinated African green monkeys

To determine the frequency of IFN-γ secreting T cells following immunization ELISPOT assays were performed on PBMC from AGMs inoculated with b/h PIV3/hMPV F2, *wt* hMPV/NL/1/00 or placebo. Fig. 3 shows the number of IFN-γ secreting T cells per 10^5 PBMC for each treatment group ($n = 4$). The frequency of IFN-γ secreting T cells from AGMs immunized with b/h PIV3/hMPV F2 was greater following stimulation with hMPV/NL/1/00 and hPIV3/JS compared with uninfected Vero cell lysate. PBMCs from hMPV/NL/1/00-infected AGMs also had an easily detectable level of IFN-γ secreting T cells when stimulated with *wt* hMPV although one animal had a weak T cell response resulting in the large error bars (Fig. 3). In the placebo animals, there was a clear T cell response following *wt* hMPV/NL/1/00 challenge that was reproducibly weaker than the hMPV/NL/1/00-treated animals. The placebo animals also showed a lower rise in hMPV neutralizing antibody titers after *wt* hMPV/NL/1/00 challenge. Perhaps these immune responses in the placebo treated animals are related (Table 2).

The ELISPOT assay was performed at 28 days post-immunization or 28 days post-challenge. Thus, the population of IFN-γ secreting T cells detected likely represented memory populations. However, it is interesting to note that the T cell response for both treatment groups on days 28 and 56 were comparable showing that *wt* hMPV challenge did

Table 3
Immunization of African green monkeys with b/h PIV3/hMPV F2 elicits hPIV3 neutralizing and HAI serum antibodies

Virus immunized	Day of sera collection	hPIV3 neutralizing antibody titer reciprocal $\log_2 \pm$ S.D.	Mean reciprocal $\log_2 \pm$ S.D. PIV3 HAI antibody titers	
			hPIV3[a]	bPIV3[a]
b/h PIV3/hMPV F2	1	<2	<2	<2
	28	7.2 ± 1.3	10.5 ± 0.6	8.5 ± 1.3
	56	7.2 ± 0.5	10.0 ± 1.2	7.3 ± 1.0
Placebo	1	<2[b]	ND	ND
	28	<2[b]	ND	ND
	56	<2[b]	<2[b]	<2[b]

[a] hPIV3/Wash/47885/57 and bPIV3/Kansas/15626/84 served as antigens in the HAI assay.
[b] One of the four placebo animals was positive for hPIV3 at pre-screening (day −21). The positive animal is not included in the mean.

not increase the frequency of IFN-γ secreting T cells. Thus, vaccination of AGMs with b/h PIV3/hMPV F2 elicits both hMPV- and hPIV3-specific T cell responses.

3.6. b/h PIV3/hMPV F2 displayed host-range restricted replication in rhesus monkey

bPIV3 is restricted for growth in the respiratory tract of rhesus monkeys and this phenotype correlated with attenuation and safety in humans [29,31,32]. To characterize the safety profile of b/h PIV3/hMPV F2, PIV3-seronegative rhesus monkeys were immunized intratracheally with b/h PIV3/hMPV F2 and *wt* hPIV3/JS (Study A) and with rbPIV3 and b/h PIV3 (Study B) and monitored for virus shedding. Daily observations were also conducted during the entire course of these studies (21 days) but no clinical signs, changes in food consumption, body weight and body temperature were noted.

In the nasopharynx, b/h PIV3/hMPV F2 was detected in three of the four animals for 12 days with a daily mean peak titer of $\sim 2.7 \log_{10}$ pfu/ml (Fig. 4A). The animal that did not shed detectable virus sero-converted to hPIV3 and showed high reciprocal hPIV3 HAI antibody titer of $10 \log_2$ on day 21 (data not shown). Low levels of rbPIV3 and b/h PIV3/hMPV F2 were detected for 14 days. In contrast, high titers ($\sim 4.2 \log_{10}$ pfu/ml) of *wt* hPIV3/JS were detected for 9 days (Fig. 4A).

In the trachea, b/h PIV3/hMPV F2 replication peaked on day 4 (Fig. 4B) and the mean peak titer was $3.6 \log_{10}$ pfu/ml (Fig. 5). The animal that did not shed detectable virus in the URT, also had no detectable virus in the LRT (Fig. 5). All the *wt* hPIV3-infected animals supported high levels of viral replication with a mean peak titer $\sim 2 \log_{10}$ higher than rbPIV3 and its derivatives (Fig. 5). Thus, rbPIV3 and its derivatives replicated to lower levels in the respiratory tract of rhesus monkeys compared to *wt* hPIV3/JS.

While differences in titer between hPIV3 and bPIV3 of $2 \log_{10}$ could be readily measured in the URT of rhesus monkeys, differences of $2.8–1.4 \log_{10}$ have been reported [29,30,34] in the LRT, demonstrating substantial variability in this attenuation model. However, the differences between hPIV3 and bPIV3 titer reported here are comparable to other studies [30,34]. Two sided Wilcoxon exact *P*-values showed a significant difference in the URT and LRT titers between b/h PIV3/hMPV F2 and *wt* hPIV3/JS groups ($P = 0.05$). However, the titers between b/h PIV3/hMPV F2 and b/h PIV3 or rbPIV3 in the URT and LRT were not significantly different ($P > 0.05$). Therefore, b/h PIV3/hMPV F2 replicated to the same level as rbPIV3, a virus that was attenuated and safe in human infants [31–33].

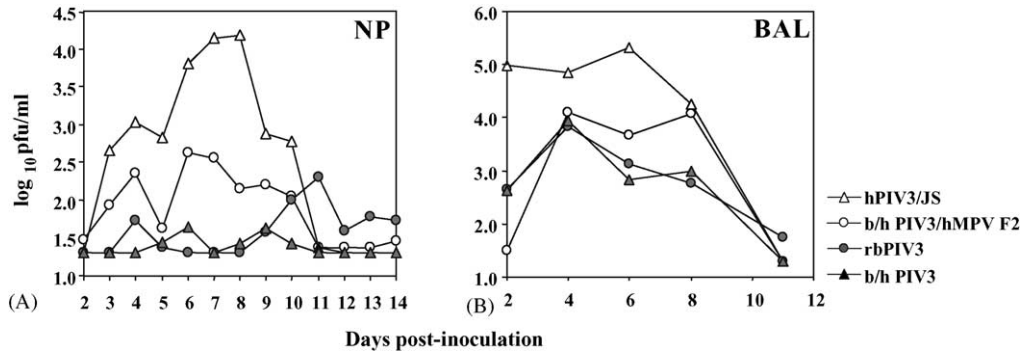

Fig. 4. Mean daily shedding of b/h PIV3/hMPV F2, *wt* hPIV3/JS (Study A) and rbPIV3, b/h PIV3 (Study B) in the URT and LRT of rhesus monkeys ($n = 4$ in each group). Virus titers were determined by plaque assays immunostained with hMPV or PIV3-specific polyclonal antisera. The limit of detection is $1.3 \log_{10}$ pfu/ml. (A) The mean daily virus titers shed in the URT of rhesus monkeys post-vaccination and (B) in the LRT are shown. hPIV3/JS (△), b/h PIV3/hMPV F2 (○), rbPIV3 (●) and b/h PIV3 (▲).

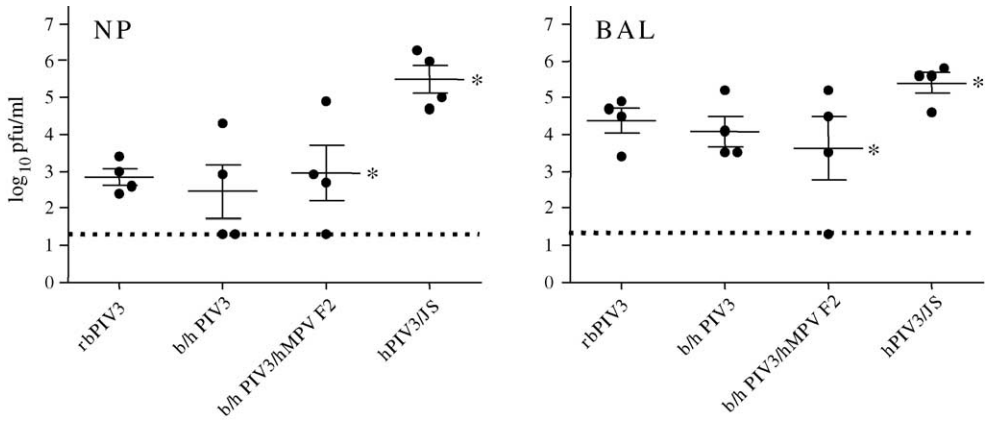

Fig. 5. Mean peak titers of rbPIV3, b/h PIV3, b/h PIV3/hMPV F2 and *wt* hPIV3/JS shed in the URT (NP) and LRT (BAL) of rhesus monkeys ($n = 4$ in each group). The limit of detection is 1.3 $\log_{10}$ pfu/ml. Two animals in the b/h PIV3 group did not shed the administered virus in the URT. One animal in b/h PIV3/hMPV F2 group did not shed vaccine virus in both the URT and LRT. The standard error of the mean for each treatment group is shown. The NP and BAL mean peak titers between b/h PIV3/hMPV F2 and hPIV3/JS are statistically different, $P = 0.0571$ (two-sided Wilcoxon exact P-values) and are indicated by asterisks. Differences between b/h PIV3/hMPV F2 and rbPIV3 or b/h PIV3 are not significant ($P > 0.05$).

4. Discussion

hMPV infection in young infants is a significant medical problem although the incidence of hMPV infection appears to be less common than that of hRSV and is comparable to that of hPIV3 [5,8]. No vaccines or anti-virals are currently available to prevent and treat respiratory disease caused by hMPV infections. In tissue culture-based assays, ribavirin and IVIG preparations containing high titers of hMPV-neutralizing antibody (10 $\log_2$/0.05 ml) were found to inhibit hMPV replication [35]. The utility of these reagents for treatment against hMPV infection in humans remains to be tested. The strategy presented here utilized an attenuated PIV3 virus vector to deliver the hMPV F protein with the aim of inducing both humoral and cell-mediated immunity against hMPV infection. This vectored approach has previously been shown to generate promising hRSV vaccine candidates [18,19] as well as to other respiratory pathogens such as measles virus [36] and the SARS coronavirus [37].

Two main genetic lineages of hMPV have been identified and immunization strategies must address questions about cross-protection between the two major groups of hMPV. Results from animal studies suggest that infection with one group of hMPV can provide cross-protection against infections by other hMPV subgroups [21–23]. The hMPV F protein that is highly conserved among the different lineages of hMPV, is sufficient to provide protection in animals against hMPV infection in the absence of the other hMPV surface glycoproteins, such as G and SH [23,27]. Furthermore, exposure to the hMPV F protein from group A hMPV protected animals from infection with group B hMPV [23]. In the present study, we did not challenge b/h PIV3/hMPV F2- immunized animals with subgroup B hMPV. However, sera from b/h PIV3/hMPV F2- and hMPV/NL/1/00-infected AGMs showed a rise in neutralization titer against hMPV/NL/1/99, about 40% of that seen with hMPV/NL/1/00. This level of cross-neutralization against hMPV/NL/1/99 was also seen in previous animal studies with *wt* hMPV/NL/1/00-infected hamsters and AGMs [22,23]. Despite lower titers of cross-neutralization antibodies against heterologous group hMPV, effective protection against heterologous group hMPV challenge were observed in hamsters and AGMs [22,23]. In this study, b/h PIV3/hMPV F2 was shown to elicit hMPV-specific as well as virus-specific antibodies and T cell responses in AGMs that received the vaccine virus. The hMPV-specific T cell response was lower in AGMs immunized with b/h PIV3/hMPV F2 compared to hMPV/NL/1/00-infected animals likely because only one hMPV antigen was expressed by the chimeric virus. Cellular immune responses have been demonstrated in human RSV infection [38] and are thought to contribute to clearing of RSV infection in mice [39,40]. It is likely that cell-mediated immunity also contributes to the effective protection and cross-protection against the different hMPV strains. Although sera from AGMs infected with b/h PIV3/hMPV F2 expressing a group A hMPV F glycoprotein showed lower neutralization titers against a group B hMPV/NL/1/99, the presence of both humoral and cellular immune responses suggest that the vaccinated AGMs may also be protected against group B hMPV infections.

A question of considerable interest in using virus vectors for delivery of antigens is whether the host will develop immunity to the vector itself. This is important not only for using the same vector to deliver different antigens for sequential vaccinations but also to fulfill the need for boosting in the event that the primary vaccination is insufficient to induce lasting protective immunity. The second point is particularly relevant when the target population is young infants because they have undeveloped immune systems and may need multiple doses to stimulate a robust protective response. It is not clear how long the immune response to hMPV and/or hPIV3 will persist in AGMs. It has been reported that PIV3 humoral immunity can last up to 4 months in hamsters [41]. We were

unable to detect any decay in PIV3 HAI antibody titer or hMPV neutralizing antibody titers in hamsters 6 months after immunization with 10^2 pfu or 10^3 pfu of b/h PIV3/hMPV F2 (data not shown). The apparent long-term immunity seen in hamsters may not reflect the immune status of PIV3 infections in young infants. A recent hPIV3 clinical trial in young infants showed that multiple doses of an attenuated hPIV3 did not result in inhibitory vector immunity provided the intervals between dose administrations were timed appropriately [42]. Only 24% of infants shed virus when a second dose of vaccine was administered 1 month later. In contrast, 62% of infants shed virus after the second dose when the interval between the primary and secondary dose was 3 months. Thus, a boosting regimen that maximizes both vaccine take and duration of immunity in young infants should be >1 month but <3 months. Maternally-derived HAI serum antibody titers as high as 5.7 $\log_2$ in 4–12 week-old infants did not appear to interfere with the replication of an attenuated hPIV3 (cp-45) vaccine strain [42]. Since we have now shown sufficient efficacy, immunogenicity and attenuation of b/h PIV3/hMPV F2 in primate models, it might be interesting to test the effect of boosting on the ability of b/h PIV3/hMPV F2 to more effectively protect the URT of AGMs against hMPV challenge.

The safety profile of b/h PIV3/hMPV F2 was evaluated in rhesus monkeys because the high permissiveness of AGMs for both hPIV3 [28] and b/h PIV3 precluded the use of this primate model for evaluating the host range restriction of bPIV3 replication relative to *wt* hPIV3. Rhesus monkeys do not show clinical signs of PIV3 infection [29]. However, clinical signs have been reported in AGMs infected with *wt* hPIV3 [28] but not for *wt* hMPV infections [23]. The lack of clinical signs observed in AGMs infected with b/h PIV3/hMPV F2 is an additional indication that the chimeric vaccine virus is attenuated.

The bPIV3 attenuation phenotype in humans resides in multiple genes [30]. While the bPIV3 F and HN genes contain some genetic determinants specifying attenuation [30,33], the major contributors to the attenuation phenotype were ascribed to the bPIV3 N and P proteins. The polygenic distribution of the bPIV3 attenuation markers predicts that the attenuation phenotype will be stable. Indeed it was shown that bPIV3 isolated from seronegative children was phenotypically stable and retained the host-range restricted replication in rhesus monkeys [31]. The stability of the inserted hMPV F has not been tested in vivo. However, b/h PIV3/RSV F2 an identical PIV3 vector expressing the RSV F gene at the second genome position, was found to retain RSV F expression after four serial passages in the respiratory tract of hamsters. Nonetheless, stable retention of the added hMPV F gene needs to be further evaluated in humans.

While the main goal of this study was to evaluate b/h PIV3/hMPV F2 as a potential hMPV vaccine, we also determined the level of hPIV3 serum HAI and neutralizing antibody titers following vaccination. Even though a direct hPIV3 challenge was not performed in this study, the magnitude of the rise in hPIV3 HAI and neutralizing antibodies in the primate sera from animals immunized with b/h PIV3/hMPV F2 correlated with antibody levels giving protection against LRT infection in rhesus monkeys and in infants [29,33,43]. We were also able to detect virus-specific T-cell responses in AGMs immunized with b/h PIV3/hMPV F2. The presence of both virus-specific humoral and T-cell responses suggested that the b/h PIV3 vectored hMPV vaccines might function as a bivalent vaccine for immunization against both hMPV and hPIV3 infections.

Finally, an hMPV vaccine such as b/h PIV3/hMPV F2 will most likely have to be co-administered with a vaccine against respiratory syncytial virus (RSV). A formulation consisting of b/h PIV3/hMPV F2 and b/h PIV3/RSV F2, that was also shown to be attenuated [34] and protective [44], or b/h PIV3/hMPV F2 formulated with an attenuated live RSV [18,45] should provide protection against hPIV3, hMPV as well as RSV in infants. In these formulations, interference between vaccine viruses needs to be evaluated. Alternatively, b/h PIV3 could be engineered to express both RSV and hMPV F. However, concerns about the genetic stability of a b/h PIV3 expressing three heterologous F proteins have not been thoroughly addressed. Further preclinical studies are needed to identify an efficacious, safe and stable trivalent vaccine for prevention of RSV, hMPV and hPIV3 infections in infants.

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