Research

Attenuation and efficacy of human parainfluenza virus type 1 (HPIV1) vaccine candidates containing stabilized mutations in the P/C and L genes

Emmalene J Bartlett*, Adam Castaño, Sonja R Surman, Peter L Collins, Mario H Skiadopoulos and Brian R Murphy

Address: Laboratory of Infectious Diseases, Respiratory Viruses Section, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Department of Health and Human Services, Bethesda, MD, USA

Email: Emmalene J Bartlett* - ebartlett@niaid.nih.gov; Adam Castaño - adam.castaano@gmail.com; Sonja R Surman - SBarbagallo@niaid.nih.gov; Peter L Collins - PCOLLINS@niaid.nih.gov; Mario H Skiadopoulos - mskiadopoulos@niaid.nih.gov; Brian R Murphy - bmurphy@niaid.nih.gov

* Corresponding author

Abstract

Background: Two recombinant, live attenuated human parainfluenza virus type 1 (rHPIV1) mutant viruses have been developed, using a reverse genetics system, for evaluation as potential intranasal vaccine candidates. These rHPIV1 vaccine candidates have two non-temperature sensitive (non-ts) attenuating (att) mutations primarily in the P/C gene, namely CR84G-HNT553A (two point mutations used together as a set) and CA170 (a short deletion mutation), and two ts att mutations in the L gene, namely LY942A (a point mutation), and LΔ1710-11 (a short deletion), the last of which has not been previously described. The latter three mutations were specifically designed for increased genetic and phenotypic stability. These mutations were evaluated on the HPIV1 backbone, both individually and in combination, for attenuation, immunogenicity, and protective efficacy in African green monkeys (AGMs).

Results: The rHPIV1 mutant bearing the novel LΔ1710-11 mutation was highly ts and attenuated in AGMs and was immunogenic and efficacious against HPIV1 wt challenge. The rHPIV1-CR84G/Δ170-HNT553ALY942A and rHPIV1-CR84G/Δ170-HNT553ALΔ1710-11 vaccine candidates were highly ts, with shut-off temperatures of 38°C and 35°C, respectively, and were highly attenuated in AGMs. Immunization with rHPIV1-CR84G/Δ170-HNT553ALY942A protected against HPIV1 wt challenge in both the upper and lower respiratory tracts. In contrast, rHPIV1-CR84G/Δ170-HNT553ALΔ1710-11 was not protective in AGMs due to over-attenuation, but it is expected to replicate more efficiently and be more immunogenic in the natural human host.

Conclusion: The rHPIV1-CR84G/Δ170-HNT553ALY942A and rHPIV1-CR84G/Δ170-HNT553ALΔ1710-11 vaccine candidates are clearly highly attenuated in AGMs and clinical trials are planned to address safety and immunogenicity in humans.

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Background

Human parainfluenza virus type 1 (HPIV1) is responsible for approximately 6% of pediatric hospitalizations due to respiratory tract disease with significant illness occurring predominantly in infants and young children [1]. Clinical manifestations range from mild disease, including rhinitis, pharyngitis, and otitis media, to more severe disease, including croup, bronchiolitis, and pneumonia [1-6]. Collectively, human parainfluenza virus serotypes 1, 2 and 3 (HPIV1, 2 and 3) are the second leading causative agents of pediatric hospitalizations due to respiratory disease following respiratory syncytial virus (RSV) [7,1]. However, a licensed vaccine is currently not available for the prevention of illness caused by any HPIV.

HPIV1 is an enveloped, non-segmented, single-stranded, negative-sense RNA virus belonging to the family Paramyxoviridae, genus Respirovirus, of which HPIV3 is also a member. The HPIV1 genome is 15,600 nucleotides in length and contains six genes in the order 3'-N-P/C-M-F-HN-L-5', which encode three nucleocapsid-associated proteins including the nucleocapsid protein (N), the phosphoprotein (P), and the large polymerase protein (L) and three envelope-associated proteins including the internal matrix protein (M) and the fusion (F) and hemagglutinin-neuraminidase (HN) transmembrane surface glycoproteins [8]. F and HN are the two viral neutralization antigens and are major viral protective antigens. The P/C gene of HPIV1 contains a second open reading frame (ORF) that encodes up to four accessory C proteins, C', C, Y1 and Y2, that initiate at four separate translational start codons in the C ORF and are carboxy co-terminal [1]. However, it is unclear whether the Y2 protein is actually expressed during HPIV1 infection [9]. The HPIV1 C proteins have recently been shown to act as antagonists of the innate immune response during virus infection by inhibiting type 1 interferon (IFN) production and signaling of IFN through its receptor [10].

Our laboratory is developing a live attenuated virus vaccine for HPIV1 for intranasal administration to infants and young children. The intranasal route of administration is needle-free and has the advantage of direct stimulation of local immunity as well as induction of a substantial systemic immune response [11]. Furthermore, compared to an inactivated vaccine, a live virus vaccine stimulates a broader spectrum of innate and adaptive immune responses [11]. The recent licensure of the trivalent live attenuated influenza virus vaccine (Flumist™) indicates that it is possible to achieve an acceptable balance between attenuation and immunogenicity with a live attenuated respiratory virus vaccine [12].

Reverse genetics provides a method for introducing attenuating mutations in desired combinations into wild type (wt) HPIV1 [13-16]. Temperature sensitive (ts) attenuating (att) and non-ts att mutations have been developed that, in combination, can enhance both the phenotypic and genetic stability of a HPIV1 vaccine candidate. The licensed cold-adapted influenza A viruses contain similar non-ts and ts att mutations [17,18]. In the case of HPIV1, non-ts att mutations have been introduced into the P/C gene that inactivate the anti-IFN activities of the C accessory proteins [10]. One of these mutations (CΔ170) is a deletion mutation that affects codon 170 of the HPIV1 C protein; deletion mutations are desirable because they are essentially free of same-site reversion and thus provide for enhanced genetic and phenotypic stability. The CΔ170 mutation inhibited both the production of Type 1 IFN and the signaling of IFN through its receptor and specified an att phenotype in hamsters and African green monkeys (AGMs) [10,16]. A second non-ts att mutation involves a pair of amino acid substitutions, R84G and HN1753A, that attenuates HPIV1 for AGMs when they are present together but not individually. This attenuating pair of mutations was not further genetically stabilized, i.e., it is possible to revert to a wt phenotype with a single nucleotide substitution at either mutation. A substitution at amino acid position 942 of L, L942A, generated a ts att mutation that was engineered for increased genetic and phenotypic stability by the strategy of identifying a codon whose amino acid assignment yielded a ts att phenotype and which would require three nucleotide substitutions for reversion [13]. A virus bearing this stabilized mutation was attenuated in both AGMs and hamsters [15].

The present study consists of two parts. First, we developed an additional ts att mutation involving a small deletion in the HPIV1 L protein. This mutation was originally identified as a ts att point mutation in the bovine PIV3 (BPIV3) L protein ([L1710]) [19]. The corresponding site in the HPIV1 L protein was identified as position 1710 by sequence alignment, and this codon and its downstream neighbor (codon 1711) were deleted to yield the LΔ1710–11 mutation. This gave us two genetically stabilized ts att mutations in L, the LΔ1710–11 and the L1742A mutations. In the second part of the study, the two non-ts att mutations in C, namely the C84G/HN1753A set and the CΔ170 mutation, were combined with each other and with either the LΔ1710–11 mutation or the L1742A mutation to develop two live intranasal HPIV1 vaccine candidates. Each of these vaccine candidates contained at least one genetically stabilized ts and non-ts att mutation. These viruses were evaluated for their in vitro attenuation phenotype and for replication, efficacy and immunogenicity in AGMs.

Results

Construction and recovery of mutant rHPIV1 viruses

Point and deletion mutations in the P/C, HN and L genes that attenuate HPIV1 for replication in the respiratory
tract of hamsters or AGMs are indicated in Table 1[13–16]. The C R84G mutation is a single nucleotide substitution mutation that affects both the P and C proteins and that results in amino acid substitutions of R84 to G in C, and E87 to G in P (Table 1) [15]. The C R84G mutation is attenuating in the upper respiratory tract (URT) of AGMs, but only in the presence of the HNT553A point mutation indicated in Table 1[15]. The C R84G and HNT553A mutations are each based on single nucleotide substitutions (Table 1), and thus the att phenotype would be lost by reversion at either position. The C A170 deletion mutation in HPIV1 involves a six-nucleotide deletion, a length that was chosen to comply with the "rule of six" [20]. This deletion results in a loss of two amino acids and substitution of a third at codon positions 168–170 in C (RDF to S), and a deletion of amino acids GF in P at codon positions 172–173 (Table 1) [16]. The changes in the C protein also would be present in the nested C', Y1, and Y2 proteins (not shown) [16]. The Y942A mutation in L has three nucleotide changes in codon 942 and specifies a genetically and phenotypically stabilized ts att phenotype [13].

In the present study, the L A1710–11 deletion mutation in HPIV1 was created at a site that corresponds by sequence alignment to a ts att point mutation originally identified in BPIV3 [19]. Importation of this BPIV3 point mutation has previously been shown to attenuate HPIV2 [21]. Here, the L A1710–11 mutation contains a six-nucleotide deletion that results in a deletion of amino acids AE at codon positions 1710–11 of the L gene of HPIV1 (Table 1).

The mutations in Table 1 were introduced into the HPIV1 antigenomic cDNA individually or in combinations to yield the panel of rHPIV1 viruses listed in Table 2. These viruses were recovered following transfection of cDNAs into HEp-2, BHK-T7 or Vero cells and biologically cloned in LLC-MK2 cells, and each was sequenced in its entirety to confirm the presence of the engineered mutation(s) and the absence of adventitious mutations. Unexpectedly, we were unable to isolate rHPIV1 containing the LA1710–11 mutation by itself and without adventitious mutations despite four attempts to do this using multiple replicates each time. However, we were able to recover virus bearing L A1710–11 in the presence of C R84G without adventitious mutations. Thus, our analysis of the phenotype of the L A1710–11 mutation was performed in the presence of the C R84G mutation, which is neither ts nor att [15].

**Characterization of rHPIV1s containing single att mutations**

We first sought to characterize the rHPIV1 mutants bearing the four single att mutations (the C R84G, HNT553A, and L Y942A pair of mutations [13]). The newly generated rHPIV1-L A1710–11 mutation by itself and without adventitious mutations was performed in the presence of C R84G, and rHPIV1-L Y942A, bearing the Y942A mutation in L was generated for the present study. We had previously generated and characterized a virus, rHPIV1-C R84G, HNT553A, and L Y942A, containing the Y942A mutation in combination with the C R84G, HNT553A pair of mutations [13]. The newly generated rHPIV1-L Y942A virus would permit evaluation of its specific contribution to the level of temperature sensitivity in vitro and attenuation in vivo. The rHPIV1 mutant bearing the individual att mutation L A1710–11 (rHPIV1-C R84G, L A1710–11) also

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**Table 1: Summary of the mutations introduced into the rHPIV1 genome**

| Gene | Mutation | ORF | nt changes wt → mutant | Type of mutation | Codon position | Amino acid change | # nt changes for reversion to wt |
|------|----------|-----|------------------------|-----------------|---------------|------------------|-------------------------------|
| P/C  | R84G     | C   | AGA → GGA              | point           | 84            | R → G            | 1                             |
|      |          | P   | GAG → GGG              | point           | 87            | E → G            | 1                             |
| ΔA170 |          | C   | AGG GAT TT C → AGC     | deletion        | 168–170       | RDF → S (D deletion; 3 nt deletions in the flanking R-F codons results in a S substitution) | 6 (insertions) |
| HN   | T553A    | HN  | ACC → GCC              | point           | 172–173       | GF deletion      | 6 (insertions) |
| L    | Y942A    | L   | GAG GAG → deletion     | deletion        | 553           | T → A            | 1                             |
| ΔA1710—11 |    | L   | GCT ATC → deletion     | point           | 942           | Y → A            | 3  |

*a* HPIV1 strain Washington/1964, GenBank accession no. NC_003461

*b* The nomenclature used to describe each mutation indicates the wt amino acid, the codon position and the new amino acid, or the position of the deletion (Δ), with respect to the C, HN or L protein.

*c* The nucleotides (nt) affected by substitution or deletion are shown underlined and in bold type.

*d* Designed for increased genetic stability by use of a deletion. Deletions involved six nt to conform to the rule of six [20].

*e* Designed for increased genetic stability by the use of a codon that differs by three nucleotides from codons yielding a wild type assignment.
Table 2: Level of temperature sensitivity of replication of rHPIV1 mutants in vitro.

| Virus              | Virus titer ± S.E. at 32°C | Mean reduction (log₁₀) in virus titer ± S.E. at the indicated temperature compared to 32°C |
|--------------------|-----------------------------|---------------------------------------------------------------------------------------------|
|                    |                             | 35°C             | 36°C             | 37°C             | 38°C             | 39°C             | 40°C             | Shut-off (°C) d |
| 1 HPIV1 wt         | 7.7 ± 0.1                   | 0.1 ± 0.1        | 0.1 ± 0.1        | 0.2 ± 0.1        | 0.7 ± 0.1        | 1.3 ± 0.1        | 3.0 ± 0.3        | -               |
| 2 rHPIV1-CR84G     | 9.2 ± 0.4                   | 0.4 ± 0.2        | 0.4 ± 0.6        | 0.8 ± 0.5        | 0.3 ± 0.4        | 1.8 ± 0.6        | 4.5 ± 0.9        | -               |
| 3 rHPIV1-CR84GHNT553A | 7.8 ± 0.1             | -0.3 ± 0.2       | -0.3 ± 0.2       | -0.2 ± 0.2       | 0.1 ± 0.2        | 0.7 ± 0.2        | 2.5 ± 0.6        | -               |
| 4 rHPIV1-LY942A    | 7.9 ± 0.3                   | 0.2 ± 0.2        | 0.7 ± 0.8        | 0.5 ± 0.2        | 1.0 ± 0.3        | 2.6 ± 0.7        | 4.5 ± 1.0        | -               |
| 5 rHPIV1-LY942A    | 8.0 ± 0.3                   | 0.2 ± 0.3        | 1.2 ± 0.3        | 2.6 ± 1.1        | 6.4 ± 0.4        | ≥6.8 f           | ≥6.8            | 37°C            |
| 6 rHPIV1-CR84GHNT553ALY942A | 7.4 ± 0.2       | 0.4 ± 0.4        | 0.5 ± 0.4        | 2.3 ± 0.4        | 4.0 ± 0.6        | 6.0 ± 0.4        | 6.4 ± 0.4        | 37°C            |
| 7 rHPIV1-CR84GLA1710–11 | 7.5 ± 0.7     | 0.8 ± 0.7        | 3.0 ± 0.6        | 4.8 ± 0.2        | ≥6.3            | ≥6.3            | ≥6.3            | 36°C            |
| 8 rHPIV1-CR84G/Δ1710HNT553ALY942A | 6.3 ± 0.1     | 0.3 ± 0.2        | 0.9 ± 0.6        | 2.0 ± 0.3        | 4.9 ± 0.2        | ≥5.1            | ≥5.1            | 38°C            |
| 9 rHPIV1-CR84G/Δ1710HNT553AL1710–11 | 6.4 ± 0.3     | 2.6 ± 0.6        | 4.0 ± 0.4        | ≥5.2            | ≥5.2            | ≥5.2            | ≥5.2            | 35°C            |

a Data are the mean of three to sixteen experiments.
b Viruses were titrated on LLC-MK2 cells at either permissive (32°C) or potentially restrictive (35°C – 40°C) temperatures for 7 days and virus titers are expressed as the mean ± standard error (S.E.). The limit of detection was 1.2 log₁₀ TCID₅₀/ml.
c Values in bold indicate restricted replication, where the mean log₁₀ reduction in virus titer at the indicated temperature vs 32°C was 2.0 log₁₀ or greater than the difference in titer of HPIV1 wt at the same temperature vs 32°C. A virus is designated as restricted replication at 35°C–40°C if replication at 35°C–40°C is observed.
d Underlined values indicate viral shut-off temperature, the lowest temperature at which restricted replication is observed.
e These data have been previously published [13] [15] [16] and are included here for the purposes of comparison.
f The symbol “≥” indicates that virus titers were at the limit of detection and therefore the reduction in virus titer versus 32°C is greater than or equal to the indicated value. There is no S.E. value for viruses at the limit of detection.

The level of temperature sensitivity of replication of the four viruses with single att mutations was first studied (Table 2, groups 3, 4, 5, and 7) and compared to that of rHPIV1 wt and rHPIV1-CR84G. Viruses containing only P/ C gene mutations with or without the HN mutation were non-ts, whereas each of the L gene mutations specified a ts phenotype in vitro. The single LY942A mutation specified a shut-off temperature of 37°C, a level of temperature sensitivity that is phenotypically silent on its own, as already noted. The level of replication of rHPIV1-L Y942A and rHPIV1-CR84GLA1710–11 in AGMs was next evaluated and compared to that of rHPIV1 wt and the other two single att mutants (Table 3, Groups 1, 3, 4, 5, 7). A rHPIV1 mutant was considered attenuated if it exhibited a significant (P < 0.05) reduction in replication in either the mean peak virus titer or the mean sum of the daily virus titers (a measure of the total amount of virus shed over the duration of the infection) in either the nasopharyngeal (NP) swab (representative of the upper respiratory tract, URT) or tracheal lavage (TL) samples (representative of the lower respiratory tract, LRT) compared to the HPIV1 wt group. We have previously demonstrated that rHPIV1-CR84G replicates to levels equivalent to HPIV1 wt in AGMs, whereas rHPIV1-CR84GHNT553A and rHPIV1-CR84GHNT553ALY942A were attenuated in AGMs [15,16]. Here, both rHPIV1-LY942A and rHPIV1-CR84GLA1710–11 were significantly attenuated in the URT and LRT of AGMs in comparison to HPIV1 wt. The levels of attenuation of rHPIV1-LY942A and rHPIV1-CR84GHNT553ALY942A were comparable, indicating that the LY942A mutation is an attenuating mutation by itself and that the attenuation specified by the LY942A mutation is not additive to that specified by the CR84GHNT553A att mutation. The rHPIV1-CR84GLA1710–11 mutant also was significantly attenuated in AGMs, reducing virus titer in
Table 3: Level of replication of HPIV1 vaccine candidates in the upper and lower respiratory tract of African green monkeys.

| Virus         | Shut-off temperature | No. of animals | Mean peak virus titer (log_{10} TCID_{50}/ml) | Mean sum of the daily virus titers (log_{10} TCID_{50}/ml) | att<sup>Δ</sup> |
|---------------|----------------------|----------------|---------------------------------------------|-----------------------------------------------------------|----------------|
| 1 HPIV1 wt    | -                    | 14             | 4.2 ± 0.2                                   | 26.4 ± 1.5                                                | -              |
| 2<sup>h</sup> HPIV1-CR84G | -                     | 4              | 3.6 ± 0.4                                   | 21.0 ± 1.7                                                | No             |
| 3<sup>h</sup> HPIV1-CR84GHN553A | -                     | 12             | 2.1 ± 0.2                                   | 10.5 ± 0.9                                                | Yes            |
| 4<sup>h</sup> HPIV1-C170 | -                     | 6              | 3.4 ± 0.5                                   | 14.8 ± 1.9                                                | Yes            |
| 5 HPIV1-LY942A | 37°C                  | 4              | 2.3 ± 0.1                                   | 16.9 ± 0.7                                                | Yes            |
| 6<sup>h</sup> HPIV1-CR84GHN553ALY942A | 37°C               | 8              | 2.4 ± 0.2                                   | 12.9 ± 1.0                                                | Yes            |
| 7 HPIV1-CR84GL1710–11 | 36°C           | 4              | 1.5 ± 0.4                                   | 8.6 ± 1.8                                                 | Yes            |
| 8<sup>h</sup> HPIV1-CR84G1710–11HN553ALY942A | 38°C           | 4              | 1.2 ± 0.3                                   | 5.9 ± 0.5                                                 | Yes            |
| 9<sup>h</sup> HPIV1-CR84G1710–11HN553AL1710–11 | 35°C           | 4              | 0.9 ± 0.3                                   | 6.3 ± 0.5                                                 | Yes            |

<sup>a</sup> Monkeys were inoculated i.n. and i.t. with 10<sup>6</sup> TCID<sub>50</sub> of the indicated virus in a 1 ml inoculum at each site. Data are representative of one to five experiments.

<sup>b</sup> Shut-off temperature is defined in footnote d, Table 2.

<sup>c</sup> Virus titrations were performed on LLC-MK2 cells at 32°C and expressed as the mean ± S.E. of the individual peak virus titers for the animals in each group irrespective of day. The limit of detection was 0.5 log<sub>10</sub> TCID<sub>50</sub>/ml.

<sup>d</sup> Mean sum of the daily virus titers: the sum of the titers for all of the days of sampling was determined for each animal individually, and the mean was calculated for each group. On days when virus was not detected, a value of 0.5 log<sub>10</sub> TCID<sub>50</sub>/ml was assigned for the purpose of calculation. The mean sum of the lower limit of detection was 5.0 log<sub>10</sub> TCID<sub>50</sub>/ml for NP swabs and 2.5 log<sub>10</sub> TCID<sub>50</sub>/ml for TL samples.

<sup>e</sup> Virus is designated att in the URT or LRT based on a significant reduction in either mean peak titer or mean sum of daily titers compared to the HPIV1 wt group (see footnote h).

<sup>f</sup> Nasopharyngeal (NP) swab samples were collected on days 1–10 post-infection.

<sup>g</sup> Tracheal lavage (TL) samples were collected on days 2, 4, 6, 8, and 10 post-infection.

<sup>h</sup> These data have been previously published [13] [15] [16] and are included here for the purposes of comparison.

<sup>i</sup> Underlined values indicate a statistically significant reduction compared to corresponding HPIV1 wt titer, P < 0.05 (Student-Newman-Keuls multiple comparison test).

The immunogenicity and protective efficacy resulting from immunization with rHPIV1s containing single att mutations were evaluated in AGMs by measuring post-immunization HPIV1 hemagglutination inhibiting (HAI) serum antibody titers and by challenging immunized and control animals with HPIV1 wt 28 days following immunization and determining challenge virus titers in the URT and LRT (Table 4). AGMs immunized with rHPIV1s containing single att mutations (Groups 3, 4, 5, and 7) developed post-immunization HAI serum antibodies and manifested resistance to replication of the challenge virus. The rHPIV1-CR84G1710–11 mutant, which showed a strong level of attenuation following immunization of AGMs, was protective only at a low level in the URT.

**Combination of three single att mutations into rHPIV1 to generate two live attenuated HPIV1 vaccine candidates**

Having identified the in vitro and in vivo properties of the four single att mutations, we used this information to generate two live attenuated HPIV1 vaccine candidates containing both non-ts and ts attenuating mutations. These vaccine candidates were designed to incorporate a backbone containing one stabilized non-ts attenuating mutation, C<sup>170</sup>, as well as the C<sup>R84G</sup>H<sup>N553A</sup> mutant. The addition of this second mutation (the C<sup>R84G</sup>H<sup>N553A</sup> att mutation) would be expected to increase the overall stability of the virus by increasing the total number of attenuating mutations present in the vaccine candidate. To generate the two live attenuated HPIV1 vaccine candidates, either the stabilized ts att L<sup>Y942A</sup> mutation or the L<sup>1710–11</sup> deletion mutation was added to the rHPIV1-C<sup>R84G</sup>A<sup>170H</sup>N<sup>553A</sup>L<sup>Y942A</sup> backbone. We then evaluated the resulting combination mutants, rHPIV1-C<sup>R84G</sup>A<sup>170H</sup>N<sup>553A</sup>L<sup>Y942A</sup> and rHPIV1-C<sup>R84G</sup>A<sup>170H</sup>N<sup>553A</sup>L<sup>1710–11</sup>, as potential vaccine candidates.

These two viruses were first evaluated for their level of temperature sensitivity of replication in vitro (Table 2). The level of temperature sensitivity of rHPIV1-C<sup>R84G</sup>A<sup>170H</sup>N<sup>553A</sup>L<sup>Y942A</sup> and rHPIV1-C<sup>R84G</sup>A<sup>170H</sup>N<sup>553A</sup>L<sup>1710–11</sup> (Groups 8 and 9 in Table 2) was equivalent to that of the corresponding L gene single-mutation viruses from which they were derived (namely rHPIV1-L<sup>Y942A</sup> and rHPIV1-C<sup>R84G</sup>A<sup>1710–11</sup>). Groups 5 and 7 in Table 2). This indicates that combining the non-ts and ts mutations in rHPIV1-C<sup>R84G</sup>A<sup>170H</sup>N<sup>553A</sup>L<sup>Y942A</sup> and rHPIV1-C<sup>R84G</sup>A<sup>170H</sup>N<sup>553A</sup>L<sup>1710–11</sup> did not significantly alter their over-
all level of temperature sensitivity of replication in vitro. A multiple cycle growth curve at 32°C demonstrated that each virus achieved titers in Vero cells that will allow efficient manufacture. Specifically, the rHPIV1-CR84G/Δ170HNT553ALY942A and rHPIV1-CR84G/Δ170HNT553AL1710-11 vaccine candidates reached peak titers of 7.9 and 7.2 log_{10} TCID_{50}/ml, respectively, in Vero cells (Figure 1).

The level of replication of rHPIV1-CR84G/Δ170HNT553ALY942A and rHPIV1-CR84G/Δ170HNT553AL1710-11
in AGMs were next evaluated and compared to that of rHPIV1 wt and the other two single att mutants (Table 3, Groups 1, 3, 4, 5, 7, 8, and 9). The rHPIV1-CR84G/Δ170HNT553ALT554ALY942A virus was strongly attenuated compared to rHPIV1 mutants bearing the corresponding single att mutations only in C/P, C/P/HN or L. The mean peak titer of rHPIV1-CR84G/Δ170HNT553ALT554ALY942A in the URT and LRT was reduced by 3.0 and 3.3 log₁₀ TCID₅₀/ml, respectively, in comparison to rHPIV1 wt (Table 3). Similarly, the additional of the HNT553A and CA170 mutations to rHPIV1-CR84G/Δ170HNT553ALT554ALT554ALY942A further attenuated the virus in AGMs, restricting virus replication in comparison to rHPIV1 wt by 3.1 and 3.4 log₁₀ TCID₅₀/ml in the URT and LRT, respectively (Table 3). Therefore these two HPIV1 vaccine candidates demonstrate strong attenuation phenotypes in vivo. Considering the 9 viruses in Table 3 together, a relationship was found to exist between level of temperature sensitivity of replication in vitro and the attenuation manifested in vivo, i.e., the lower the shut off temperature, the higher the level of in vivo attenuation (Figure 2). Evaluation of these data using the Spearman rank test gives correlation coefficients of 0.47 and 0.67 for the URT and LRT, respectively, based on the mean daily sum of virus titers for individual AGMs. This indicates a moderate positive correlation with a stronger association between the level of temperature sensitivity and virus replication in the LRT. However, as might be expected, viruses bearing only the non-ts attenuating P/C gene mutations, including the CA170 and the CR84G/Δ170HNT553A set of mutations, did not follow this pattern (Figure 2), and we would expect a higher correlation coefficient if these non-ts viruses were not included in the analysis.

The levels of immunogenicity and protective efficacy against HPIV1 wt challenge following immunization with rHPIV1-CR84G/Δ170HNT553ALT554ALY942A and rHPIV1-CR84G/Δ170HNT553ALT554ALT554ALY942A were also determined (Groups 8 and 9 in Table 4). The two vaccine candidates failed to induce detectable HAI antibodies. However, immunization with the rHPIV1-CR84G/Δ170HNT553ALT554ALY942A was protective against HPIV1 wt challenge in both the URT and LRT (Table 4). In contrast, immunization with rHPIV1-CR84G/Δ170HNT553ALT554ALT554ALY942A did not offer significant protection.

### Table 4: Immunogenicity and protective efficacy of rHPIV1 vaccine candidates in AGMs.

| Virus a | No. animals | Pre-challenge serum HAI titerb | Mean peak challenge virus titer (log₁₀ TCID₅₀/ml) c | Mean sum of the daily challenge virus titers (log₁₀ TCID₅₀/ml)d | Post-challenge serum HAI titerc |
|---------|-------------|--------------------------------|-----------------------------------------------------|---------------------------------------------------------------|-------------------------------|
| 1       | 12          | 6.7 ± 0.6 (12/12)              | 0.8 ± 0.2 0.7 ± 0.1                                  | 2.3 ± 0.2 2.4 ± 0.2                                           | 6.6 ± 0.5                     |
| 2       | 4           | 3.8 ± 0.2 (3/4)               | ≤0.5 ± 0.0 ≤0.5 ± 0.0                                | ≤2.0 ± 0.0 ≤2.0 ± 0.0                                         | 4.4 ± 1.2                     |
| 3       | 12          | 6.0 ± 0.6 (11/12)             | 0.6 ± 0.1 0.6 ± 0.1                                 | 2.1 ± 0.1 2.1 ± 0.1                                           | 7.9 ± 0.4                     |
| 4       | 6           | 5.5 ± 0.6 (6/6)               | ≤0.5 ± 0.0 ≤0.5 ± 0.0                                | ≤2.0 ± 0.0 ≤2.0 ± 0.0                                         | 6.5 ± 0.4                     |
| 5       | 4           | 6.3 ± 1.2 (4/4)               | 1.1 ± 0.2 1.2 ± 0.2                                 | 2.7 ± 0.3 2.8 ± 0.3                                           | 8.9 ± 1.1                     |
| 6       | 8           | 2.0 ± 0.0 (3/8)               | ≤0.5 ± 0.0 ≤0.5 ± 0.0                                | ≤2.0 ± 0.0 ≤2.0 ± 0.0                                         | 3.3 ± 0.7                     |
| 7       | 4           | 6.1 ± 1.8 (3/4)               | 3.4 ± 0.6 3.0 ± 0.6                                 | 8.4 ± 2.0 8.3 ± 1.3                                           | 6.9 ± 1.5                     |
| 8       | 4           | ≤1.0 ± 0.0 (0/4)              | 2.2 ± 0.2 1.8 ± 0.5                                 | 5.1 ± 0.3 4.3 ± 1.3                                           | 5.5 ± 1.6                     |
| 9       | 4           | ≤1.0 ± 0.0 (0/4)              | ≤0.5 ± 0.0 ≤0.5 ± 0.0                                | ≤2.0 ± 0.0 ≤2.0 ± 0.0                                         | 7.5 ± 1.4                     |
| 10      | 7           | 5.0 ± 0.6 (0/4)               | 4.5 ± 0.9 3.4 ± 0.4                                 | 11.8 ± 2.5 8.1 ± 1.3                                          | 7.5 ± 1.4                     |

a Monkeys were immunized i.n. and i.t. with 10⁶ TCID₅₀ of the indicated virus in a 1 ml inoculum at each site and were challenged on day 28 post-infection with rHPIV1 wt.

b HAI titers to HPIV1 were determined by HAI assay of sera collected at day 28 (pre-challenge) and day 56 (post-challenge) in separate assays. Titers are expressed as mean reciprocal log₁₀ S.E.; the limit of detection was 1.0 ± 0.0. The number of animals with a 4-fold or greater increase in pre-challenge antibody titers is shown in brackets for each group.

c Mean ± S.E of the individual peak virus titers for the animals in each group irrespective of day. Virus titrations were performed on LLC-MK2 cells at 32°C. The limit of detection was 0.5 log₁₀ TCID₅₀/ml. NP and TL samples were collected on days 2, 4, 6 and 8 post-challenge.

d Mean sum of the daily virus titers: the sum of the titers for all of the days of sampling was determined for each animal individually, and the mean was calculated for each group. On days when no virus was detected, a value of was 0.5 log₁₀ TCID₅₀/ml was assigned for the purpose of calculation.

Post-challenge serum HAI titers were also determined (Groups 8 and 9 were included here for the purposes of comparison.

*Underlined values indicate statistically significant reductions in mean peaks or sum of daily virus titers for HPIV1 wt titer compared to the corresponding non-immune group, P < 0.05 (Student-Newman-Keuls multiple comparison test).
against HPIV1 wt challenge in the AGMs (Table 4), i.e., it appeared overattenuated in this animal model. A relationship was found between the level of replication of the immunizing virus and its ability to induce resistance to replication of the challenge virus (Tables 3 and 4), and this is graphically displayed in Figure 3.

**Discussion**

The advent of a reverse genetics system for the generation of infectious parainfluenza viruses with full-length cDNA plasmids has greatly facilitated the development of live attenuated HPIV1 vaccine candidates [13-16]. The reverse genetics system for HPIV1 has allowed site-directed manipulation of the viral genome via cDNA intermediates, permitting the introduction of attenuating mutations in desired combinations into vaccine candidates. It has also been possible to genetically modify some of the attenuating mutations to optimize genetic and phenotypic stability of viruses bearing the mutations, both by the use of gene deletions and by using codons chosen for a low probability of reversion. This process enables us to optimize the safety profile of the live attenuated HPIV1 vaccine candidates before these viruses are tested in humans.

We are focusing our efforts on the development of live attenuated rHPIV1 vaccines since they have a number of advantages over inactivated or subunit vaccines, including the ability to: (i) induce the full spectrum of protective immune responses including serum and local antibodies as well as CD4+ and CD8+ T cells [11]; (ii) infect and replicate in the presence of maternal antibody permitting immunization of young infants [22,23]; (iii) cause an acute, self-limited infection that is readily eliminated from the respiratory tract; and (iv) replicate to high titers in cell substrates acceptable for products for human use, including qualified Vero cells, making manufacture of these vaccines commercially feasible. In the present study, two new rHPIV1 viruses containing single *att* mutations in L, L_{Δ1710–11} and LY_{942A}, were generated and characterized, and these *ts* *att* mutations were used in combination with previously described non-*ts* *att* mutations in the P/C gene and HN gene to generate two new live attenuated HPIV1 vaccine candidates.
A major result of the present study was the creation of the L\textsuperscript{Δ1710–11} mutation that was found to specify a strong ts att phenotype. The L\textsuperscript{Δ1710–11} mutation was originally identified as an attenuating point mutation, L\textsuperscript{T1711I}, in BPIV3 [19]. It was evaluated as a deletion mutation in the present study since a deletion mutation offers a higher level of genetic stability than a point mutation, a property that is desirable for mutations in a vaccine candidate. Indeed, since this deletion occurs in an ORF (in which the triplet nature of the codons must be maintained) and in a virus that conforms to the rule of six (in which the hexamer organization must be maintained), same-site reversion would require the precise restoration of six nucleotides. We unfortunately were not able to isolate a virus that conforms to the rule of six (in which the codons specifying the wild type phenotype codon in the three nucleotide changes to convert the GCG alanine to a cysteine and phenylalanine) all of which would require three amino acids were shown to specify a wild type phenotype for HPIV1, and, as such, it is a suitable mutation to include in a HPIV1 vaccine candidate. Further, since this deletion occurs in an ORF (in which the codons specifying the wild type phenotype codon in the three nucleotide changes to convert the GCG alanine to a cysteine and phenylalanine) all of which would require three amino acids were shown to specify a wild type phenotype for HPIV1, and, as such, it is a suitable mutation to include in a HPIV1 vaccine candidate.

The LY942A mutation was identified previously as an attenuating point mutation, L\textsuperscript{Y942A}, in BPIV3 [19]. It was evaluated as a deletion mutation in the present study since a deletion mutation offers a higher level of genetic stability than a point mutation, a property that is desirable for mutations in a vaccine candidate. Indeed, since this deletion occurs in an ORF (in which the codons specifying the wild type phenotype codon in the three nucleotide changes to convert the GCG alanine to a cysteine and phenylalanine) all of which would require three amino acids were shown to specify a wild type phenotype for HPIV1, and, as such, it is a suitable mutation to include in a HPIV1 vaccine candidate.

The LY942A and L\textsuperscript{Δ1710–11} mutations were used in conjunction with two of the non-ts mutations, the C\textsuperscript{R84G}HNT553A and C\textsuperscript{A170H}HNT553A mutations [16], to develop two live attenuated vaccine candidates for HPIV1, namely, rHPIV1-C\textsuperscript{R84G}/C\textsuperscript{A170H}HNT553A/\textsuperscript{LY942A} and rHPIV1-C\textsuperscript{R84G}/C\textsuperscript{A170H}HNT553A/\textsuperscript{Δ1710–11}. These vaccine candidates thus each contain three independent attenuating mutations (two non-ts att and one ts att mutation), two of which have been genetically stabilized. The combination of mutations present in these two vaccine candidates should enhance the genetic and phenotypic stability of the viruses, although this will require formal demonstration in a clinical trial using clinical grade virus preparations.

Evaluation of the two vaccine candidates revealed that they are reasonable candidates for further study in clinical trials. Both candidates replicated well in Vero cells, a characteristic that is important for manufacturing purposes. Both viruses also demonstrated a strong ts phenotype in vitro (shut-off temperature of ≤38°C) that was similar to that of their ts parent virus, but the two viruses differ in their level of temperature sensitivity in vitro. Since the level of temperature sensitivity of respiratory viruses [24], including HPIV1 as demonstrated here, correlates with level of attenuation, it was anticipated that this difference in the ts phenotype would be reflected in a difference in the level of attenuation and immunogenicity in vivo, and this indeed was seen. The HPIV1 vaccine candidates were both strongly attenuated in the URT and LRT of AGMs, with rHPIV1-C\textsuperscript{R84G}/C\textsuperscript{A170H}HNT553A/\textsuperscript{LY942A} replicating to slightly higher levels than the more ts rHPIV1-C\textsuperscript{R84G}/C\textsuperscript{A170H}HNT553A/\textsuperscript{Δ1710–11}. Both vaccines were weakly immunogenic and failed to induce a detectable level of serum HAI antibodies in AGMs. A low level of protective efficacy was observed in AGMs immunized with rHPIV1-C\textsuperscript{R84G}/C\textsuperscript{A170H}HNT553A/\textsuperscript{LY942A}, but the rHPIV1-C\textsuperscript{R84G}/C\textsuperscript{A170H}HNT553A/\textsuperscript{Δ1710–11} was not protective. This low level of
immunogenicity and efficacy was not unexpected since each vaccine was highly restricted in replication and since there is a strong correlation between the level of replication of vaccine virus and its immunogenicity and ability to restrict replication of HPIV1 challenge virus. These results can be interpreted to indicate that the two vaccine candidates are over-attenuated, but we think that this conclusion would be premature. It is likely that these viruses will be more immunogenic, and therefore more efficacious, in humans compared to AGMs since they should replicate more efficiently in humans. The reasons for this are two-fold. First, HPIV1 is a human virus, and it should replicate more efficiently in its natural host in which it causes disease than in AGMs in which it causes only an asymptomatic infection. The actual level of replication of HPIV1 in seronegative humans is unknown, but it replicates efficiently even in adults with pre-existing immunity [25,26]. Second, these vaccine candidates are highly attenuated in AGMs, and it is expected that the viruses should replicate somewhat more efficiently in humans and would be more immunogenic than in AGMs. It also is fortunate that the two vaccine candidates appear to differ somewhat in their level of attenuation, since this provides two chances to achieve an optimal balance between safety and efficacy.

Conclusion
The rHPIV1-Δ170HNT553ALY942A and rHPIV1-Δ170HNT553ALY942A Δ170HNT553AL1710–11 vaccine candidates are highly attenuated in AGMs. We plan to initiate studies in humans with the less attenuated vaccine candidate, rHPIV1-Δ170HNT553ALY942A Δ170HNT553AL1710–11. If this virus proves to be insufficiently attenuated in the target population of young seronegative infants (following an initial step-wise progression of safety testing in adults, seropositive children, and seronegative children), we would proceed to evaluate the more attenuated rHPIV1-Δ170HNT553AL1710–11 vaccine candidate. If rHPIV1-Δ170HNT553ALY942A Δ170HNT553AL1710–11 vaccine candidate is over-attenuated, then the ΔLY942A mutation would be deleted and the rHPIV1-Δ170HNT553ALY942A would be tested in humans. In this way, we will identify a HPIV1 vaccine candidate that exhibits a satisfactory balance between attenuation and immunogenicity for the target population of seronegative infants and young children.

Methods
Cells and viruses
LLC-MK2 cells (ATCCCL23) and HEp-2 cells (ATCCCL23) were maintained in Opti-MEM I (Gibco-Invitrogen, Inc. Grand Island, NY) supplemented with 5% FBS and gentamicin sulfate (50 μg/ml). Vero cells (ATCC CCL-81) were maintained in Opti-PRO SFM (Gibco-Invitrogen, Inc.) in the absence of FBS and supplemented with gentamicin sulfate (50 μg/ml) and L-glutamine (4 mM). BHK-17 cells, which constitutively express T7 RNA polymerase [27], were kindly provided by Dr. Ulla Buchholz, NIAID, and were maintained in GMEM (Gibco-Invitrogen, Inc.) supplemented with 10% FBS, gentamicin (1 mg/ml), MEM amino acids, and L-glutamine (2 mM). Biologically-derived wt HPIV1 Washington/20993/1964, the parent for the recombinant virus system, was isolated previously from a clinical sample in primary African green monkey kidney (AGMK) cells and passaged 2 additional times in primary AGMK cells [25] and once in LLC-MK2 cells [15]. This preparation has a wild type phenotype in AGMs, and will be referred to here as HPIV1 wt. It was previously described as HPIV1 LLC [15]. HPIV1 wt and rHPIV1 mutants were grown in LLC-MK2 cells in the presence of 1.2% Tryple select, a recombinant trypsin (Gibco-Invitrogen, Inc.), as described previously [8].

Construction of mutant HPIV1 cDNA
P/C, HN and L gene mutations (Table 1) were introduced into the appropriate rHPIV1 subgenomic clones [14] using the Advantage-HF PCR Kit (Clontech Laboratories, Palo Alto, CA) with a modified PCR mutagenesis protocol described elsewhere [28]. The entire PCR amplified subgenomic clone was sequenced using a Perkin-Elmer ABI 3100 sequencer with the Big Dye sequencing kit (Perkin-Elmer Applied Biosystems, Warrington, UK) to confirm that the subclone contained the introduced mutation and to confirm the absence of adventitious mutations introduced during PCR amplification. Full-length antigenomic cDNA clones (FLCs) of HPIV1 containing the desired mutations were assembled using standard molecular cloning techniques [8], and the region containing the introduced mutation in each FLC was sequenced as described above to confirm the presence of the introduced mutation and absence of adventitious changes. Each virus was designed to conform to the rule of six, which is a requirement by HPIV1 and numerous other paramyxoviruses that the nucleotide length of their genome be an even multiple of six for efficient replication [20].

Recovery of rHPIV1 mutant viruses
Three different recovery methods were used to generate rHPIV1 mutants that differed in the source of the T7 polymerase needed to synthesize RNA from the transfected virus-specific plasmids and, in one case, a different transfection method was used. First, using previously described procedures [8], rHPIV1 virus was recovered from HEp-2 cells that were transfected with plasmids encoding the antigenome and N, P, and L support proteins and infected with an MVA-T7 vaccinia virus recombinant as a source of T7 polymerase. Second, Vero cells were grown to 80% confluency and transfection experiments were performed using the AMAXA Cell Line Nucleo-
Characterization of the temperature sensitivity of the rHPIV1 vaccine candidates

The ts phenotype for each mutant rHPIV1 virus was determined by comparing its level of replication to that of HPIV1 wt at 32°C and at 1°C increments from 35°C to 40°C, as described previously [31]. Briefly, each virus was serially diluted 10-fold in 96-well LLC-MK2 monolayer cultures in L-15 media (Gibco-Invitrogen, Inc.) containing trypsin with four replicate wells per plate. Replicate plates were incubated at the temperatures indicated above for seven days, and virus infected wells were detected by hemadsorption with guinea pig erythrocytes. The virus titer at each temperature was determined in three to sixteen separate experiments and is expressed as the mean log10 TCID50/ml. The mean titer at an elevated temperature was compared to the mean titer at 32°C, and the reduction in mean titer was determined. The shut-off temperature of an rHPIV1 mutant is defined as the lowest temperature at which the reduction in virus titer compared to its titer at 32°C was 100-fold greater than the reduction in HPIV1 wt titer between the same two temperatures. A mutant is defined as having a ts phenotype if its shut-off temperature is ≤40°C.

Evaluation of replication of viruses in AGMs and efficacy against challenge

AGMs in groups of two to four animals at a time were inoculated intranasally (i.n.) and intratracheally (i.t.) with 10⁶ TCID₅₀ of either HPIV1 wt or mutant rHPIV1 in a 1 ml inoculum at each site. NP swab samples were collected daily from days 1 to 10 post-inoculation, and TL fluid samples were collected on days 2, 4, 6, 8 and 10 post-inoculation. The specimens were flash frozen and stored at -80°C and were subsequently assayed in parallel. Virus present in the samples was titered in dilutions on LLC-MK2 cell monolayers in 96-well plates and an undiluted 100 μl aliquot was also tested in 24-well plates. These were incubated at 32°C for 7 days. Virus was detected by hemadsorption, and the mean log₁₀ TCID₅₀/ml was calculated for each sample day. The limit of detection was 0.5 log₁₀TCID₅₀/ml. The mean peak titer for each group was calculated using the peak titer for each animal, irrespective of the day of sampling. The mean sum of the virus titers for each group was calculated from the sum, calculated for each animal individually, of the virus titers on each day of sampling, up to day 10. The sum of the lower limit of detectability was 5.0 log₁₀ TCID₅₀/ml for NP swabs and 2.5 log₁₀TCID₅₀/ml for TL samples.

On day 28 post-inoculation, the AGMs were challenged i.n. and i.t. with 10⁶ TCID₅₀ of HPIV1 wt in 1 ml at each site. NP swab and TL samples were collected for virus quantitation on days 2, 4, 6 and 8 post-challenge.
All animal studies were performed under protocol LID22, as approved by the National Institute of Allergy and Infectious Disease (NIAID) Animal Care and Use Committee (ACUC).

**Evaluation of immune responses in AGMs**

Serum was collected from each monkey on days 0 and 28 post-immunization and on day 28 post-challenge (day 56 post-immunization). HPIV1 HAI antibody titers were determined at 21°C, as described previously [32], using 0.5% v/v guinea pig erythrocytes and HPIV1 wt as the antigen. The HAI antibody titer was defined as the endpoint serum dilution that inhibited hemagglutination and is expressed as the mean reciprocal log₂ ± standard error (SE).

**Statistical Analysis**

The Prism 4 (GraphPad Software Inc., San Diego, CA) one-way ANOVA test, (Student-Newman-Keuls multiple comparison test) was used to assess statistically significant differences between data groups ($P < 0.05$). The R software programme [33] was used to perform a Spearman rank test to determine correlation between data sets.

**Competing interests**

Patent applications for the vaccine candidates described here have been filed by NIH. In addition, the vaccine candidates are being developed under a Cooperative Research and Development Agreement (CRADA) between NIAID and MedImmune. NIAID investigators work under CRADAs as part of the normal responsibilities of their NIAID, NIH employment. Through the execution of licensing agreements, the NIAID makes the vaccine candidates available to parties interested in their further development and commercialization.

**Authors’ contributions**

EB recovered viruses, performed in vitro and in vivo studies and drafted the manuscript. AC recovered virus and performed in vitro and in vivo studies. SRS recovered viruses and assisted with in vivo studies. PLC contributed to the study design and drafting of the manuscript. MHS and BRM supervised the study, participated in its design and planning and contributed to drafting of the manuscript. All authors read and approved the final manuscript.

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