Evaluation of the Safety and Immune Efficacy of Recombinant Human Respiratory Syncytial Virus Strain Long Live Attenuated Vaccine Candidates

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Abstract

Human respiratory syncytial virus (RSV) infection is the leading cause of lower respiratory tract illness (LRTI), and no vaccine against LRTI has proven to be safe and effective in infants. Our study assessed attenuated recombinant RSVs as vaccine candidates to prevent RSV infection in mice. The constructed recombinant plasmids harbored (5′ to 3′) a T7 promoter, hammerhead ribozyme, RSV Long strain antigenomic cDNA with cold-passaged (cp) mutations or cp combined with temperature-sensitive attenuated mutations from the A2 strain (A2cpts) or further combined with SH gene deletion (A2cptsDSH), HDV ribozyme (δ), and a T7 terminator. These vectors were subsequently co-transfected with four helper plasmids encoding N, P, L, and M2-1 viral proteins into BHK/T7-9 cells, and the recovered viruses were then passaged in Vero cells. The rescued recombinant RSVs (rRSVs) were named rRSV-Long/A2cpts and rRSV-Long/A2cptsDSH, respectively, and stably passaged in vitro, without reversion to wild type (wt) at sites containing introduced mutations or deletion. Although rRSV-Long/A2cpts and rRSV-Long/A2cptsDSH displayed temperature-sensitive (ts) phenotype in vitro and in vivo, all rRSVs were significantly attenuated in vivo. Furthermore, BALB/c mice immunized with rRSVs produced Th1-biased immune response, resisted wtRSV infection, and were free from enhanced respiratory disease. We showed that the combination of DSH with attenuation (att) mutations of cpts contributed to improving att phenotype, efficacy, and gene stability of rRSV. By successfully introducing att mutations and SH gene deletion into the RSV Long parent and producing three rRSV strains, we have laid an important foundation for the development of RSV live attenuated vaccines.

Keywords Human respiratory syncytial virus (RSV) · RSV long strain · Live attenuated vaccine · Safety · Protective immunity

Introduction

Human respiratory syncytial virus (RSV) is the most common cause of pneumonia and the key factor in pneumonia-related deaths among children under five years of age. It is also known to cause deaths in the elderly and immunocompromised people (Falsey et al. 2005; Jorquera et al. 2016; Troeger et al. 2018; PERCH 2019). The earlier formaldehyde-inactivated RSV vaccine (FI-RSV) did not prevent RSV infection but resulted in enhanced respiratory disease (ERD) in the vaccinated children (Kapikian et al. 1969). Therefore, safety is set as the priority for the development of RSV vaccine for infants, and avoiding ERD as well as stimulating effective immune protection are still the main challenges of RSV vaccine research. One
of the promising candidates is the RSV live attenuated vaccine (LAV), which has been successfully obtained by introducing mutations or/and deleting non-essential genes from the RSV genome (Connors et al. 1995; McFarland et al. 2018; McFarland et al. 2020a; McFarland et al. 2020b). Among the dispensable genomic regions are genes encoding small hydrophobic protein (SH), nonstructural proteins (NS), and M2 ORF protein 2 (M2-2) proteins. The deletions of NS1, NS2, or M2-2 are known to be promising strategies for obtaining intranasal RSV LAV against neonatal RSV infection (Luongo et al. 2013; McFarland et al. 2020b; Teng et al. 2020).

RSV LAV originating from the RSV Long parent strain has not yet been obtained; however, the RSV A2 parent has been widely used to construct RSV LAV candidates (Huang et al. 2009; Hu et al. 2014; Xu et al. 2018; Bouillier et al. 2019). RSV Long and A2 strains belong to the same GA1 clade and RSV group A. Moreover, their attachment glycoproteins (G) share 94% sequence identity (Johnson et al. 1987; Peret et al. 1998). Compared with the G protein, the fusion glycoprotein (F) is even more highly conserved among A and B RSV groups. A few RSV vaccine candidates have been developed by neutralizing antigenic F or G proteins from the RSV Long strain (Singh et al. 2007; Huang et al. 2009; Magro et al. 2012; Blanco et al. 2014). RSV LAV candidates from the RSV A2 parent have also been developed; however, different laboratory stocks of RSV parents differed in the level of attenuation (Lawlor et al. 2013; McFarland et al. 2018). Hence, we aimed to develop RSV LAV candidates from the RSV Long parent using a reverse genetics technology platform and determine whether their efficacy is comparable to that of RSV LAV from the RSV A2 strain.

The known examples of RSV LAV are mainly attenuated by point mutation, gene deletion, or by their combination. The original set of mutations included five cold-passaged (cp) mutations, and the temperature-sensitive (ts) point mutations identified by reverse genetics method (Crowe et al. 1994a, b, 1995). The replication ability of cp-248/404 in vitro was weakened at 37 °C and the virus remained attenuated in infants attending the clinical trials. However, viral replication in the nasal cavity caused serious nasal congestion. Another RSV LAV (rA2cp248/404/1030ΔSH) was generated by SH gene deletion (ΔSH) (Bukreyev et al. 1997).

Here, we cloned plasmids containing RSV antigenic cDNA from the wild-type RSV (wtRSV) Long strain harboring cp mutations, cp ts mutations, or cp ts ΔSH mutations, and named them pBRB-LSV-rLong/A2cp, pBRB-LSV-rLong/A2cpts, and pBRB-LSV-rLong/A2cptsΔSH, respectively. Then, we co-transfected each of the constructed recombinant plasmids together with four helper plasmids encoding nucleoprotein (N), phosphoprotein (P), large protein (L), and M2-1 into BHK/T7-9 cells expressing T7 RNA polymerase, and the recovered viral particles were blind passaged in Vero cells. The successfully rescued recombinant RSVs (rRSVs), rRSV-Long/A2cp, rRSV-Long/A2cpts, and rRSV-Long/A2cptsΔSH, were analyzed for their genetic stability by sequencing and for their attenuation (att) phenotypes by measuring replication activity on the basis of growth curves. The strains were also assessed for temperature sensitivity by measuring viral titers at different temperatures. Finally, BALB/c mice were intranasally inoculated with each rRSV and subsequently challenged with wrRSV. The immunogenicity, safety, and efficacy of these rRSVs were evaluated on the basis of serum antibody levels, neutralizing antibody titers, lung pathology, and viral replication activity in the upper and lower respiratory tracts.

Materials and Methods

Cells, Viruses, and Plasmids

HEp-2 cells and Vero cells (ATCC, Rockefeller, MD, USA) were grown in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco BRL, Gaithersburg, USA) containing 2 mmol/L l-glutamine and 10% fetal bovine serum (FBS, Gibco, Australia). BHK/T7-9 cells were kindly provided by Professor W. Y. Zhu (CDC, Beijing, China) and maintained in DMEM with 10% tryptose phosphate broth (FBS, Gibco, Australia). RSV Long and A2 strains belong to the same GA1 clade and RSV group A. Moreover, their attachment glycoproteins (G) share 94% sequence identity (Johnson et al. 1987; Peret et al. 1998). Compared with the G protein, the fusion glycoprotein (F) is even more highly conserved among A and B RSV groups. A few RSV vaccine candidates have been developed by neutralizing antigenic F or G proteins from the RSV Long strain (Singh et al. 2007; Huang et al. 2009; Magro et al. 2012; Blanco et al. 2014). RSV LAV candidates from the RSV A2 parent have also been developed; however, different laboratory stocks of RSV parents differed in the level of attenuation (Lawlor et al. 2013; McFarland et al. 2018). Hence, we aimed to develop RSV LAV candidates from the RSV Long parent using a reverse genetics technology platform and determine whether their efficacy is comparable to that of RSV LAV from the RSV A2 strain.

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Rescue and Identification of Recombinant RSVs

The construction and recovery of rRSV-Long/A2cp, rRSV-Long/A2cpts, and rRSV-Long/A2cptsΔSH were performed as reported previously (Xu et al. 2018). Briefly, to construct antigenic cDNA clones, each of the mentioned mutations and deletion was introduced into the full-length wrRSV cDNA. Specifically, the deletion included the entire SH gene or the majority of the downstream noncoding region of the SH gene, as well as silent nucleotide (nt) substitutions in the last three codons and the termination codon of the SH ORF, leaving the gene end signal intact.

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(Bukreyev et al. 2001). The resulting plasmids, containing antigenomic cDNAs flanked by T7 promoter-hammerhead ribozyme in the 5’ end and by HDV ribozyme-T7 terminator in the 3’ end (Fig. 1A), were named pBRB-RSV-rLong/A2cp, pBRB-RSV-rLong/A2cpts, and pBRB-RSV-rLong/A2cptsΔSH, respectively. For rescue of rRSVs, BHK/T7-9 cells were co-transfected with a plasmid harboring rRSV antigenomic cDNA and four helper plasmids (pCITE-N, pCITE-P, pCITE-L, and pCITE-M2-1) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The transfection mixtures were prepared as follows: rRSV cDNA plasmid 1.25 μg, pCITE-N 1 μg, pCITE-P 1 μg, pCITE-L 0.5 μg, pCITE-M2-1 0.25 μg. The mixtures of DNA-lipo-OptiMEM (Gibco BRL, Gaithersburg, MD, USA) were added into cells after a 20-min incubation. The cells were incubated at 33 °C in a 5% CO2 incubator. For the blind passage, Vero cells were incubated with 400 μL of the suspension from the harvested co-transfected cells at 33 °C for 2 h. Then, the infected Vero cells were incubated with DMEM containing 4% FBS at 33 °C and expanded or serially passaged. They were monitored by immunoplaque assay and real-time quantitative PCR (RT-qPCR) as described previously (van Elden et al. 2003; Fu et al. 2013; Jiao et al. 2017). For the immunoplaque assay, serially diluted RSV samples were grown to 80% confluency of HEp-2 cells in a 96-well plate in triplicate for 1 h at 37 °C, and then DMEM containing 0.9% methyl cellulose (Sigma) was added to the wells. Subsequently, the media were discarded and the cells were rinsed with DMEM without FBS. After 3 days of incubation at 37 °C under 5% CO2, the cell monolayer was fixed with 95% cold alcohol, and viral replication activity could be assessed using an goat anti-RSV antibody (Millipore, Billerica, MA, USA) incubated with horseradish peroxidase rabbit anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and visualized after adding TMB (Promega, Madison, WI, USA). RSV titers were expressed as plaque-forming units per mL (pfu/mL). For RT-qPCR, RNA samples from virus-infected cells were extracted using Trizol reagent (Invitrogen) according to the manufacturer’s instructions, and quantified on the basis of the obtained OD value. The reverse transcription was completed with the GoScript™

Fig. 1 Schematic diagram illustrating our strategy for the identification of recombinant human respiratory syncytial viruses (rRSVs). A Schematic diagram of rRSV genomes. The five missense mutations Val-267-Ile/N, Glu-218-Ala/F, Thr-523-Ile/F, Cys-319-Tyr/L, and His-1690-Tyr/L are independent attenuating genetic elements of cold-passaged (cp) mutation; Gln-831-Leu/248 and T-7605-C/404 are two independent temperature-sensitive (ts) mutations; ΔSH is a complete deletion of the SH gene. T7 Pro: T7 promoter, T7 Ter: T7 terminator, Le: Lead, Tr: Trail. B Identification of replication of the rescued rRSVs by immunoplaque assay. Vero cells were inoculated with suspensions from cells co-transfected with four helper plasmids (Mock), and with pBRB-RSV-rLong/A2cp, pBRB-RSV-rLong/A2cpts, or pBRB-RSV-rLong/A2cptsΔSH, respectively.
reverse RNA samples were incubated with oligo dT for 5 min at 70 °C and then placed for 5 min on ice. Following the addition of 15 µL of RT reaction mix, the samples were placed for 5 min at 25 °C, 1 h at 42 °C, and 15 min at 70 °C, and then stored at 4 °C. Subsequently, qPCR was performed by using a SYBR green probe (Tiangen Biotech, Beijing, China). The primers for the RSV N gene were as follows: forward primer, 5'-AGATCAACTCTGTATCGAC- CAGCAA-3' and reverse primer, 5'CGCATCA- TAATTAGGTATCAAT-3'. The thermal cycling conditions included 15 min at 95 °C, followed by 45 cycles of 15 s at 9 °C and 1 min at 60 °C. The specificity of the obtained qPCR products was verified by melting point analysis in the range from 45 to 95 °C.

**Preparation of rRSVs, wtRSV, and FI-RSV**

The preparation and purification of wtRSV and rRSVs were performed in line with a previously described method (Kohmann et al. 2009; Kwanten 2013; Jiao et al. 2017). Briefly, the viral samples were inoculated onto HEp-2 cells with 80% confluency and incubated for 3 days at 37 °C under 5% CO2. After the formation of syncytia, the cells were scraped off and centrifuged at 3000 rpm for 10 min at 4 °C, and the supernatants were collected. To further release intracellular viral particles, the cell pellet was exposed to three cycles of swift freezing/thawing (the cell pellet was immersed in liquid nitrogen for 10 s and then thawed using running tap water). The frozen/thawed pellet was centrifuged for 10 min at 3000 rpm and the supernatants were collected. The pooled supernatants were run through a 0.45 µm sterile filter (Merck Millipore, Co., Cork, Ireland), and purified by ultracentrifugation on a sucrose cushion gradient (10% sucrose, Sigma) at 17,000 rpm in a P28S rotor (Hitachi, Tokyo, Japan) for 2 h at 4 °C. The pellet was suspended in 300 µL 10% sucrose, divided into aliquots, and stored at −80 °C. The infectivity of the RSV was assessed using an immunoplaque assay as described above.

FI-RSV was prepared according to a previously described method (Kim et al. 1969). Briefly, RSV-infected cell lysates were clarified by centrifugation for 15 min at 550 ×g. Then, the clarified cell lysates were incubated with 37% formalin (Sigma) for 3 days at 36 °C in the proportion of 1:4000. The mixture was then pelleted by ultracentrifugation at 17,000 rpm in a P28S rotor for 1 h at 4 °C. The resulting pellet was resuspended in 1/25 of the original volume in serum-free DMEM and assayed for protein concentration using a BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA).

**In vitro Characterization of rRSVs**

The above mentioned immunoplaque assay and RT-qPCR were used to evaluate the replication activity and confirm the successful rescue of the rRSVs since passage 1 (P1) in Vero cells. Simultaneously, a GoScript™ reverse transcription (RT)-PCR kit was used to assess the genetic stability of the rescued rRSVs by amplifying fragments of viral DNA harboring the mutated nucleotides contributing to the att phenotype. The primers used for RT-PCR are shown in Supplementary Table S1. These characteristic DNA fragments were amplified for each rRSV sample from every odd-numbered passage from P1 to P9 and subsequently sequenced to monitor the possible reversion of individual point mutations.

The rRSV growth kinetics was also assayed using a previously described method (Collins and Bermingham 1999; Schickli et al. 2012). Briefly, HEp-2 cells were infected with wtRSV or rRSVs at a multiplicity of infection (MOI) of 0.1 in triplicate and incubated at 32 °C. Cells and supernatants were harvested at 24-h intervals post-infection, and viral titers were determined by immunoplaque assay as mentioned above.

The temperature-sensitive phenotype of rRSVs in vitro was evaluated by determining the efficiency of plaque formation at various temperatures, through a modified method from a previous report (Crowe et al. 1993). Briefly, a HEp-2 cell monolayer, grown in 24-well plates, was inoculated with tenfold serial dilutions of wtRSV and rRSV cultures, incubated for 5 days at 32 °C, 35 °C, 36 °C, 37 °C, 38 °C, 39 °C, and 40 °C, and then assayed for infectivity at each corresponding temperature using the immunoplaque assay.

The temperature stress test was performed as reported previously (Collins and Bermingham 1999; Schickli et al. 2012). Briefly, rRSVs were passaged at elevated non-permissive temperatures, twice at 37 °C, twice at 39 °C, and once at 40 °C. Next, HEp-2 cells were inoculated with rRSVs at 0.1 MOI in a 96-well plate. Following a 1-h incubation at 33 °C, the inoculum was collected by pipetting, and cells were supplied with 200 µL/well of DMEM with 2% FBS and incubated at 37 °C. Following a 5-day incubation at 37 °C, 100 µL of the media from each of the infected wells were transferred to an uninfected 96-well plate containing HEp-2 cells, in duplicate. After a 1-h incubation, the media were removed by pipetting and the cells were supplied with 200 µL/well of fresh medium. The plates were then incubated for 5 days at 37 °C for the second passage. The virus was similarly transferred and incubated at 39 °C to generate the third and fourth passages. The last passage was performed at 40 °C. For each
passage, one of the duplicate HEp-2 plates was immunostained to assess RSV infectivity.

**Replication of rRSVs in the Upper and Lower Respiratory Tracts of Mice**

Six-week-old female BALB/c mice (Charles River Laboratories, Beijing, China) were housed in five groups under pathogen-free conditions in microisolator cages at the animal quarters of Tsinghua University Medical Center (Beijing, China). The replication of rRSVs was evaluated in the upper and lower respiratory tracts of mice as previously described (Whitehead et al. 1998a). Briefly, mice were infected via intranasal (i.n.) route by rRSVs or wtRSV (at 1 × 10^6 pfu/mouse) and sacrificed by CO₂ inhalation for washes of nasal and lung tissues on days 2, 4, 6, or 8 post-infection. Viral titers in the harvested washes of nasal and lung tissues were determined using the immunoplaque assay and RT-qPCR as mentioned above. For RT-qPCR, viral RNA extracts from nasal and lung washes were obtained using Trizol reagent according to the manufacturer’s instructions, and all the remaining RT-qPCR procedures were repeated as described previously.

Nasal washes were collected as previously reported (Rostad et al. 2016). Briefly, the jaws and the head of each sacrificed mouse were disarticulated and sequentially removed. Next, 1 mL of Iscove’s Modified Dulbecco’s Medium (IMDM) (Gibco, Paisley, Scotland, UK) containing 15% glycerin mixed with 2% FBS-MEM (1:1, vol/vol) was pushed through each naris (total of 2 mL). All nasal washes were collected and stored at −80 °C.

The lungs were harvested from mice in each group as previously reported (Fu et al. 2014), weighed, placed in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (100 μL/0.1 g lung), and homogenized with a glass tissue grinder. The lung homogenates were centrifuged at 12,000 × g for 10 min and stored at −80 °C.

**Animal Vaccination and Challenge**

BALB/c mice were randomly grouped (five mice/group), lightly anesthetized with avertin, and then subjected to either i.n. immunization with rRSVs (1 × 10^6 pfu/mouse) or wtRSV (1 × 10^6 pfu/mouse) or intramuscular (i.m.) immunization with FI-RSV (1.875 μg/mouse) in 50 μL of PBS with 10% sucrose. Alternatively, the mice were subjected to i.m. injection containing only 50 μL PBS with 10% sucrose and were labeled as the negative control group. For the challenge experiments, the immunized or negative control BALB/c mice were further lightly anesthetized and infected with 1 × 10^6 pfu of wtRSV at day 28 after vaccination.

**Evaluation of RSV-Specific Serum IgG Responses by ELISA**

Blood samples were collected as previously reported (Fu et al. 2014). Briefly, the samples were collected from the retro-orbital plexus of mice with a capillary tube. After centrifugation at 6000 × g for 15 min, the obtained sera were stored at 4 °C. RSV-specific serum antibody responses (Jiao et al. 2017; Ma et al. 2018) were analyzed by adding tenfold dilutions of serum samples to the RSV-coated plates, which were then incubated for 1 h at 37 °C. Following three washes with PBS, the plates were incubated with HRP-conjugated anti-mouse IgG, IgG1, or IgG2 antibodies (1:5000 dilution, Santa Cruz Biotechnology) for 1 h, developed with 100 μL of TMB substrate solution (Sigma), stopped with 50 μL of 2 mol/L H₂SO₄, and analyzed at 450 nm using an ELISA plate reader (Tecan, Grodig, Austria). The titers of anti-RSV antibodies were expressed as the reciprocal of the maximal dilution of serum giving an absorbance reading greater than 0.2 absorbance units and being two-fold above the absorbance obtained for the negative control group.

**RSV-Specific Neutralizing Antibody Assay**

To analyze RSV-specific neutralizing antibody titer (Jiao et al. 2017), the samples of sera were heat-inactivated at 56 °C for 30 min and prepared by two-fold serial dilution in separate virus diluents. Fifty pfu of RSV virus suspensions (subgroup A wtRSV Long or subgroup B (WV VR1400)) were incubated with serially diluted samples for 1 h at 37 °C. Then, the neutralizing antibody titers were determined by immunoplaque assay as described above. Neutralization titers were expressed as the reciprocal of the dilution of serum giving a 50% reduction in the number of pfu in control wells.

**Pulmonary Histology of RSV-Infected Mice**

Histological analyses of lung tissues following RSV challenge were performed as described previously (Jiao et al. 2017). After mice were sacrificed by CO₂ inhalation, the lungs were fixed via 4% formalin infusion through the trachea. The solution was removed and the lungs were subsequently immersed in 4% formalin for 24 h, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Ten sections of 100 fields per mouse were examined. Sections from each mouse were blindly scored for the degree of pulmonary inflammatory changes including peribronchiolitis, perivasculitis, alveolitis, and interstitial pneumonitis. They were graded as follows: 0, clear; 1, slight; 2, moderate; 3, abundant; and 4, severe.
The eosinophilia in lung tissues of RSV-challenged mice was determined as previously described (Jiao et al. 2017). Briefly, the formalin-fixed, paraffin-embedded lung tissue sections were stained with specific stain kits (ZSGB-BIO, Beijing, China), and interpreted by assessment of both staining distribution and intensity with Image-Pro Plus software (Media Cybernetics, Washington, D.C., USA).

**Statistical Analyses**

Statistical analyses of data were performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Differences were evaluated using independent, two-sided Student’s t test. Results characterized by \( P < 0.05 \) were considered statistically significant.

**Results**

**Construction and Identification of rRSVs**

The plasmids encoding rRSV-Long/A2cp, rRSV-Long/A2cpts, and rRSV-Long/A2cpts\( \Delta \)SH were obtained by a stepwise assembly of the synthesized cDNA segments. The locations of \( cp \) and \( ts \) mutations are shown in Fig. 1A. The full-length antigenomic cDNAs of pBRB-\( \text{RSV-rLong/A2cp} \) and pBRB-\( \text{RSV-rLong/A2cpts} \) were both expected to be 18,815 bp in size, while that of \( \text{pBRB-RSV-rLong/immunoplaque assay as shown in Fig. 1B and by RT-PCR} \) the recovered rRSVs were subsequently blindly passaged in BHK/T7-9 cells, and antigenomic cDNA was co-transfected together with four plasmids encoding helper proteins to BHK/T7-9 cells, and corresponding lengths were confirmed by DNA sequencing (data not shown).

For the recovery of rRSVs, the plasmid containing RSV antigenomic cDNA was co-transfected together with four plasmids encoding helper proteins to BHK/T7-9 cells, and the recovered rRSVs were subsequently blindly passaged in Vero cells. The rescued rRSVs were identified by immunoplaque assay as shown in Fig. 1B and by RT-PCR (data not shown). These results demonstrated that we successfully rescued the rRSVs bearing the anticipated \( cp \), \( cpts \), and \( cpts\Delta \)SH mutations.

**In vitro Characterization of rRSVs**

The titers of rRSV-Long/A2cp increased rapidly during the initial passages from passage 1 (P1) to P3 (\( P < 0.01 \)) and remained constant after P3 as shown by immunoplaque assay and RT-qPCR. In contrast, the titers of rRSV-Long/A2cpts and rRSV-Long/A2cpts\( \Delta \)SH increased steadily until P4 (\( P < 0.01 \)), and fluctuated marginally after P4 (Fig. 2A, 2B). The sequencing of the DNA fragments enclosing the individual rRSV point mutations determined the genetic stability of the rescued viruses. All the introduced \( att \) mutations were stable, and no reversion was detected. The sequencing results for passages from P1 to P9 are shown in Supplementary Table S2. To further characterize rRSVs, the growth kinetics of rRSVs and \( \text{wrRSV in HEp-2 cells at 33 } ^{\circ} \text{C were assessed and compared between the two groups. The viral titers began to increase from 24 h post-inoculation and ultimately plateaued at 72 h post-inoculation for all the viruses. \( \text{wrRSV achieved the titer of } 4.5 \times 10^{7} \text{ pfu/mL, rRSV-Long/A2cp} \) 2.8 \( \times 10^{6} \text{ pfu/mL, rRSV-Long/A2cpts} \) 2.2 \( \times 10^{5} \text{ pfu/mL, and rRSV-Long/A2cpts\( \Delta \)SH} \) 1.1 \( \times 10^{5} \text{ pfu/mL, as shown in Fig. 2C. All the three rRSVs exhibited approximately 10- or 100-fold reduced growth kinetics compared with that of the parent strain. Altogether, all rRSVs bearing the \( att \) mutations were constructed successfully, showed a markedly attenuated phenotype in vitro, and their proliferation rates were significantly lower when compared with that of the \( \text{wrRSV parent.} \)

**Analysis of ts Mutation Phenotype of rRSVs In Vivo and In Vitro**

The \( ts \) phenotype of the rRSVs was examined by determining the efficiency of plaque formation by inoculation of tenfold viral dilutions at various temperatures in HEp-2 cells placed in TC24-well plates and comparing the results for \( \text{wrRSV} \) and rRSVs. The plates were incubated for 5 days at the specified temperatures in CO\( \text{2 incubators calibrated to } \pm 1 ^{\circ} \text{C and the average viral titers were measured by immunoplaque assay in HEp-2 cells at the corresponding temperatures (Table 1). Both rRSV-Long/A2cp and rRSV-Long/A2cpts\( \Delta \)SH exhibited reduced viral titers by more than 2\( \log_{10} \) at 37 \( ^{\circ} \text{C compared to the permissive temperature of 32 } ^{\circ} \text{C and were therefore considered } \text{ts at } \geq 37 ^{\circ} \text{C. As expected, the } \text{wrRSV as well as the precursor virus rRSV-Long/A2cp were not ts and showed no statistical titer reduction from 32 to 40 } ^{\circ} \text{C.} \)

We also intranasally infected mice with \( 1 \times 10^{6} \text{ pfu of rRSVs and measured viral titers in samples obtained from nasal washes and lung tissue homogenates on days 2, 4, 6, and 8 post-infection by using immunoplaque assay and RT-qPCR (Fig. 3A–3D). The results showed that the titers of all three rRSVs in the nasal wash specimens at day 4 were 0.52–1.06 \( \log_{10} \) total pfu, lower than that of \( \text{wrRSV} \) (\( P < 0.01–P < 0.001 \)), and the titers in the lung tissues at day 4 and day 6 were 0.51–2.51 and 1.55–3.99 \( \log_{10} \) pfu/g tissue, lower than the respective ones of \( \text{wrRSV} \) (\( P < 0.001 \)). The number of RNA copies of the three rRSVs in the nasal wash specimens at day 4 was 0.32–0.88 \( \log_{10} \) RSV copies/\( \mu \)g RNA, lower than that of \( \text{wrRSV} \) (\( P < 0.01–P < 0.001 \)); in addition, in the lung tissues at days 4 and 6 the three rRSVs exhibited 1.00–2.55 and 1.20–3.74 \( \log_{10} \) RSV copies/\( \mu \)g RNA, respectively, lower than the corresponding number of RNA copies of \( \text{wrRSV} \).
These results are consistent with the corresponding immunoplaque test results. Among the three rRSVs, the two rRSVs possessing ts mutations displayed a higher level of attenuation than rRSV-Long/A2cp in the lung tissues ($P < 0.001$).

We also found that the replication titers of each rRSV bearing ts mutations were significantly higher in the samples obtained from nasal washes than in those obtained from lung tissue homogenates ($P < 0.05$ or $P < 0.01$), as shown in Table 1. Consistent with their in vitro ts phenotypes, the two rRSVs harboring ts mutations displayed shut-off temperatures equal to 37 °C and are therefore more competent to multiply in the nasal cavity environment, characterized by lower temperatures (Table 1).

To determine both the genetic and phenotypic stability of rRSVs, rRSV-Long/A2cp was passaged in vitro at non-permissive temperatures to induce mutation, in line with the classical theory of the survival of the fittest. Briefly, rRSV-Long/A2cp was serially passaged twice at 37 °C, and then subjected to two further passages at 39 °C and one passage at 40 °C (Fig. 4). After each passage, one of the duplicate plates was immunostained with anti-RSV antibodies. Initially, after the expansion at 37 °C, all the wells exhibited positive RSV immunostaining. After P4 at 39 °C, more than 80% of the wells had positive RSV immunostaining, suggesting the presence of the temperature-sensitive intermediate (tsi) viruses at this temperature. At 40 °C, the control wells containing wrRSV all had positive immunostaining. In sharp contrast, only 20%–30% of the

**Fig. 2** In vitro characterization of recombinant human respiratory syncytial viruses (rRSVs). The replication titers during serial passages of rRSVs were monitored by immunoplaque assay (A) and by RT-qPCR (B) since passage 1 (P1). The growth curves for rRSVs and wild-type RSV (wrRSV) were established and compared (C). Each virus was harvested every other 24 h post-infection and titers were assayed by immunoplaque assay. All results are representative of three independent experiments. Data are shown as mean ± SD. *$P < 0.05$, **$P < 0.01$.

**Table 1** Characterization of the temperature sensitivity and attenuation (att) phenotypes of recombinant RSVs (rRSVs) in vitro and in vivo.

| Virus          | Mean virus titera ($\log_{10}$ pfu/ml ± SD) at the indicated temperature (°C) | Mean titer in miceb (Nasal wash ($\log_{10}$ total pfu ± SD) | Lung ($\log_{10}$ pfu/g tissue ± SD) |
|---------------|-------------------------------------------------|-------------------------------------------------|-----------------------------------|
|               | 32    | 35    | 36    | 37    | 38    | 39    | 40    | 32    | 35    | 36    | 37    | 38    | 39    | 40    | 32    | 35    | 36    | 37    | 38    | 39    | 40    |
| wrRSV         | 6.9 ± 0.2 | 6.9 ± 0.1 | 6.8 ± 0.1 | 7.1 ± 0.1 | 7.1 ± 0.1 | 6.8 ± 0.1 | 6.8 ± 0.1 | 3.1 ± 0.1 | 4.4 ± 0.2 |
| rRSV-Long/A2cp | 6.1 ± 0.1 | 6.1 ± 0.1 | 6.0 ± 0.1 | 6.0 ± 0.1 | 5.9 ± 0.1 | 5.8 ± 0.1 | 5.7 ± 0.1 | 2.6 ± 0.1 | 3.1 ± 0.1 |
| rRSV-Long/A2cps | 6.0 ± 0.1 | 5.2 ± 0.1 | 4.7 ± 0.1 | $< 1.0^b$ | $< 1.0$ | $< 1.0$ | $< 1.0$ | 2.3 ± 0.1 | 2.0 ± 0.1** |
| rRSV-Long/A2cpsASH | 5.4 ± 0.1 | 4.3 ± 0.2 | 4.2 ± 0.1 | $< 1.0^b$ | $< 1.0$ | $< 1.0$ | $< 1.0$ | 2.1 ± 0.1 | 1.9 ± 0.1* |

*a n = 3 replicates (at each temperature).

b Shut-off temperature is defined as the restrictive temperature at which a 100-fold or greater reduction compared to the titer observed at the permissive temperature of 32 °C and the lowest shut-off temperatures for each virus are italic.

c Groups of five mice were administered $1 \times 10^6$ pfu of the indicated virus intranasally under light anesthesia on day 0 and sacrificed on day 4. Virus titer was determined in the nasal washes ($\log_{10}$ total pfu) and lung tissues ($\log_{10}$ pfu/g tissue).

d The significant difference of RSV replication titers between nasal wash and lung tissue. All the results were shown as the representative of three independent experiments. Data were shown as mean ± SD. *$P < 0.05$, **$P < 0.01$. 

(P < 0.001). These results are consistent with the corresponding immunoplaque test results. Among the three rRSVs, the two rRSVs possessing ts mutations displayed a higher level of attenuation than rRSV-Long/A2cp in the lung tissues ($P < 0.001$).
wells containing rRSV-Long/A2cpts had positive RSV immunostaining. To investigate the nt changes at the ts markers, we analyzed the sequence of 1–2 kb cDNA fragments spanning from the start of the M2 gene through the L gene. RT-PCR was performed on five randomly chosen potential tsi revertants from RSV-positive wells to detect nt changes at the 248 and 404 ts sites of the L and M2 genes by sequence analysis. The characteristics of the biologically derived tsi viruses from the passaging of rRSV-Long/A2cpts at 39°C are listed in Supplementary Table S3. The tsi strain were characterized by nt changes causing the reversion to the wt nt or amino acid at the 248 or the 404 ts markers and the partial loss of temperature sensitivity.

Characteristics of RSV-Specific Immune Responses and Neutralizing Antibody Responses

To study the immunogenicity of the rRSVs, mice were immunized intranasally with 1 × 10^6 pfu of rRSVs or wtRSV. Mice inoculated with PBS were used as negative control. Serum samples were collected 21 days after immunization, and the levels of RSV-specific serum IgG were detected by ELISA (Fig. 5A). The mice in both immunized groups produced significant RSV-specific IgG responses compared to the negative control (P < 0.001), but there was no difference between the IgG levels induced by the three rRSVs (P > 0.05). Compared with wtRSV, similar immunogenicity was observed for rRSV-Long/A2cp and rRSV-Long/A2cptsD (P > 0.05). However, rRSV-Long/A2cpts was characterized by a slightly lower level of immunogenicity than that of wtRSV (P < 0.05).

The cross-protection against subgroup A wtRSV Long and subgroup B (WV VR1400) infection was also detected and the neutralizing antibody level was measured (Fig. 5B). Similar to the IgG responses, no significant difference in the levels of neutralizing antibodies was induced by immunization with the three rRSVs (P > 0.05). Analogically, when compared with wtRSV, similar neutralizing antibody responses and cross-protective effects were induced by rRSV-Long/A2cp and rRSV-Long/
RSV (Fig. 5C, 5D). In addition, the FI-RSV immunized response induced by intramuscular immunization with FI-RSV and wt RSV was significantly different from that of FI-RSV-immunized mice (P < 0.05 or P < 0.001). On the other hand, mice immunized by either rRSV-Long/A2cpts or rRSV-Long/A2cptsASH did not display significant differences in weight loss compared with that of wt RSV (P > 0.05), but a significant difference was observed between mice immunized with rRSV-Long/A2cpts and wtRSV on day 3 and day 4 after the challenge (P < 0.05). Moreover, the immunization with rRSV-Long/A2cpts resulted in weight changes different from those induced by rRSV-Long/A2cpts or rRSV-Long/A2cptsASH at day 3 after challenge (P < 0.05, data not shown). The slightly decreased neutralizing antibody response observed in mice after immunization with rRSV-Long/A2cpts when compared to other rRSVs, mentioned above, may be an explanation for this phenomenon. Altogether, rRSV-Long/A2cpts and rRSV-Long/A2cptsASH exhibited the best efficacy among the three rRSVs, which was reflected by weight changes after wtRSV challenge.

On day 5 after the challenge, the lung tissues were collected from the examined mice. Lung viral titers were analyzed by immunoplaque assay and RT-qPCR (Fig. 6B, C). Immunization with wtRSV and each rRSV resulted in decreased lung viral titers when compared to those observed in mice immunized with FI-RSV and mice from the negative control group (P < 0.001). On the other hand, there were no significant differences in lung viral titers among mice immunized with the three rRSVs (P > 0.05). However, the lung viral loads were significantly higher in mice from the rRSV-Long/A2cpts and rRSV-Long/A2cptsASH groups than in the wtRSV-immunized group (P < 0.05). Further, no observable differences existed between the lung viral titers of mice immunized with rRSV-Long/A2cptsASH and wtRSV after wtRSV challenge (P > 0.05). These results showed that all rRSV-immunized mice were protected against RSV infection; however, rRSV-Long/A2cptsASH provided the best protection.

**Absence of Pulmonary Pathology and Eosinophilia in rRSV-Immunized Mice Following RSV Infection**

Since the FI-RSV vaccine causes ERD in immunized children and animals, the pathological examination of lungs in primed mice after RSV challenge is an important safety index for the RSV vaccine candidates. To this purpose, we collected the mouse lung tissues for histological sections 5 days after the challenge, performed H&E staining, and evaluated the pulmonary pathology under a microscope (Fig. 7A). The alveolar walls in mice immunized with FI-RSV were thickened, and alveolar cavities

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The Efficacy of Intranasal Vaccination with rRSVs

To evaluate the efficacy of RSV vaccine candidates, changes in body weight and lung viral titers were examined in vaccinated mice after RSV challenge. The mice were challenged with wtRSV 28 days after rRSV immunization, and their body weight was monitored for 5 consecutive days (Fig. 6A). Weight loss in challenged mice immunized by wtRSV and the three rRSVs was significantly reduced from day 2 to day 5 compared to that of FI-RSV-immunized mice (P < 0.05 or P < 0.001). On the other hand, mice immunized by either rRSV-Long/A2cpts or rRSV-Long/A2cptsASH did not display significant differences in weight loss compared with that of wtRSV (P > 0.05), but a significant difference was observed between mice immunized with rRSV-Long/A2cpts and wtRSV on day 3 and day 4 after the challenge (P < 0.05). Moreover, the immunization with rRSV-Long/A2cpts resulted in weight changes different from those induced by rRSV-Long/A2cpts or rRSV-Long/A2cptsASH at day 3 after challenge (P < 0.05, data not shown). The slightly decreased neutralizing antibody response observed in mice after immunization with rRSV-Long/A2cpts when compared to other rRSVs, mentioned above, may be an explanation for this phenomenon. Altogether, rRSV-Long/A2cpts and rRSV-Long/A2cptsASH exhibited the best efficacy among the three rRSVs, which was reflected by weight changes after wtRSV challenge.

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**Absence of Pulmonary Pathology and Eosinophilia in rRSV-Immunized Mice Following RSV Infection**

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were compressed to form lung parenchyma. There was also a large number of inflammatory cells infiltrating around the blood vessels and bronchi. In negative control mice, the alveolar walls appeared thickened, the alveolar septa collapsed resulting in cavitation, and inflammatory cell infiltration was present around the blood vessels and bronchi. In the \textit{wt} RSV- and rRSV-Long/A2cp- and rRSV-Long/A2cptsDSH-immunized mice, thickening alveolar walls, cavitation, and increased levels of inflammatory cells were also observed in the lung tissues, albeit to a less extent. On the other hand, the immunization of mice with rRSV-Long/A2cp and rRSV-Long/A2cptsDSH resulted in clear as well as less destroyed alveoli, and only a few inflammatory cells accumulated around the bronchi.

Lung sections from all mice were scored for inflammation around airways, blood vessels, and interstitial and alveolar spaces as described in the Materials and Methods (Fig. 7B–7E). A considerably severe (score around 4) lung histopathology was observed in mice vaccinated with FI-RSV while a less severe lung histopathology (score around 2) was characteristic for mice in the negative control group. The least severe lung histopathology (score range, 1 to 2) was observed in at least two mice in each rRSV-vaccinated group (rRSV-Long/A2cp and rRSV-Long/A2cptsDSH) compared to the FI-RSV vaccination group \((P < 0.001)\) and the negative control group (either \(P < 0.05\), \(P < 0.01\), or \(P < 0.001\)). These results showed that vaccination with two of the three rRSVs resulted in protective immunity without causing any obvious signs of ERD, while the documented FI-RSV immunization-triggered ERD after viral challenge was confirmed. Altogether, the observed pulmonary pathology exhibited similar trends to those of the relative body weight loss among the experimental groups.

In the lungs of mice immunized with FI-RSV we also observed the infiltration of eosinophils, unlike in the lungs...
of mice from the rRSVs and wt RSV vaccination groups (P < 0.001) (Fig. 7F). In particular, the lungs of mice immunized with rRSV-Long/A2cptsD.SH displayed the least number of eosinophils in comparison with the wt RSV vaccination group and the negative control group (P < 0.05; P < 0.01). This result is consistent with the histopathological observations, and indicates decreased inflammation owing to a better protection against RSV infection through immunization with rRSV-Long/A2cptsD.SH.

**Discussion**

To our knowledge, this is the first report on the successful construction and rescue of rRSVs obtained from a parental strain other than A2 (parental strain Long) and bearing cp, cpts, or cptsD.SH mutations. Most of the cDNA-derived rRSVs are based on the cpRSV and cptsRSV parents, which are both biologically derived live attenuated viruses from the RSV A2 strain. Although differences exist in the genomic makeup as well as the encoded proteins (such as G protein) of wt RSV Long and RSV A2, both strains are classified as group A and genotype GA1 RSVs (Peret et al. 1998). Therefore, we aimed to examine the characteristics of RSV LAV candidates obtained from cDNA of the wt RSV Long parent rather than the biologically derived one, and used the rRSV-Long/A2cp parent, a counterpart of the biologically derived cpRSV.

The att phenotype and genetic stability are the critical features of live attenuated RSV vaccine candidates. We found that rRSV-Long/A2cp is non-ts, similar to the biologically derived cpRSV and rRSVA2cp (Crowe et al. 1994a, b, 1995; Whitehead et al. 1998b). Moreover, rRSV-Long/A2cpts and rRSV-Long/A2cptsD.SH share the same ts phenotype and shut-off temperature of around 37 °C, similar to their cpRSV derivative counterparts, cpts-248/404 and rA2cp248/404D.SH (Crowe et al. 1994a, 1995; Firestone et al. 1996; Karron et al. 2005; Lin et al. 2006; Schickli et al. 2012). We have monitored all the att mutation sites including the set of five cp mutations (Whitehead et al. 1998b) and ts mutations at the 248 and 404 ts markers in the serially passaged rRSVs from P1 to P9, and no reversion was observed at these sites. However, during the temperature stress test of rRSV-Long/A2cpts, all the randomly selected tsfi viruses had nt changes causing reversion to the wild-type amino acid or nt at the 248 or 404 ts markers. According to a previous in vitro analysis of MEDI-559 from MedImmune, an updated version of rA2cp248/404/1030D.SH and containing 39 silent nt substitutions (including TTA to CTG substitution at the 248 ts marker), MEDI-599 displayed a similar nt change at the 248 site as the resultant tsfi viruses (Schickli et al. 2012). In contrast to our observation, no changes were observed at the 404 ts marker located in MEDI-559 and no MEDI-559-infected plates showed positive RSV immunostaining at 38 °C. This phenomenon may be attributed to both the lower shut-off temperature of MEDI-559 (35 °C) and the lower passage temperature (37 °C) used in the cited study than the 39 °C passage conditions of our study.
Fig. 7 Absence of pulmonary histopathology and eosinophilia in mice vaccinated with recombinant human respiratory syncytial viruses (rRSVs) following RSV challenge. After the immunized BALB/c mice were challenged with wild-type RSV (wtRSV, $1 \times 10^6$ pfu/mouse) at day 28 post-immunization, lung tissues were collected at day 5 post-challenge and pulmonary histopathology was analyzed by hematoxylin and eosin staining (H&E) (A). The H&E-stained lung sections from each mouse were scored for inflammation, including peribronchiolar inflammation (B), perivascular inflammation (C), interstitial pneumonia (D), and alveolitis (E). Distribution and intensity of eosinophils were measured and analyzed using specific stains and Image-Pro Plus (F). IOD: integrated optical density. nc: negative control group. Data are shown as mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. 

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temperature applied here for rRSV-Long/A2cpts. Although no changes were reported at the MEDI-559 404 ts, reversion at the 404 locus is possible and did occur during the earlier clinical trials with cpts248/404 in young infants (Whitehead et al. 1998a; Wright et al. 2000). In our analysis of nt changes at the ts markers, cDNA from only five randomly chosen positive wells was sequenced and analyzed for the presence of gene fragments containing ts mutations. Despite its convenience, our method is not as comprehensive and accurate as high-throughput deep sequencing of the whole genomes. Therefore, further analysis might be needed to uncover some of the mutations.

The characteristics of the in vivo att phenotypes of rRSVs were uncovered by infecting mice intranasally. Compared with wt RSV, all three rRSVs had lower replication activity in vitro (Fig. 2 and Fig. 3), indicating that the cp mutation endowed all the three rRSVs with att phenotype from ‘host-range’ restrictions functioning only in vivo but not in vitro, similar to cp RSV in chimpanzees and humans (Whitehead et al. 1998a, b). Furthermore, the titers of the two rRSVs with ts mutations were reduced in the lower respiratory tract compared with those in the upper respiratory tract (Table 1), in sharp contrast with the titers of rRSV-Long/A2cp. These results demonstrate that restricted replication at physiological temperatures is attributed to the ts phenotypes of rRSV-Long/A2cpts and rRSV-Long/A2cptsASH. Moreover, similar levels of serum IgG and neutralizing antibodies were found among mice vaccinated with rRSV-Long/A2cp, rRSV-Long/A2cpts, and wt RSV (P > 0.05). However, despite the shared ts phenotype, rRSV-Long/A2cpts immunization, unlike rRSV-Long/A2cptsASH immunization, resulted in a slightly decreased antibody response compared to wt RSV immunization (P < 0.05). This result slightly differs from that of a previous report (Whitehead et al. 1999), possibly owing to an increased transcription of the downstream neighboring genes G and F in ASH RSV (Bukreyev et al. 1997). Notably, despite the inconsistency in comparison with wt RSV, there were no significant differences in IgG and neutralizing antibody responses among the three rRSVs, similar to the previous observations in chimpanzees (Whitehead et al. 1999).

The most dramatic reduction of lung viral loads occurred in mice vaccinated with rRSV-Long/A2cptsASH among the three rRSVs (P < 0.05). Similar results were observed for wt RSV-immunized mice. Therefore, compared with the other two rRSVs, rRSV-Long/A2cptsASH exhibited the best protective properties against RSV infection. Finally, analyses of lung pathology and eosinophilia in mice after viral challenge showed the absence of ERD following immunization by either of the rRSVs, especially by rRSV-Long/A2cptsASH. These results are likely attributed to the potent efficacy of rRSV-Long/A2cptsASH immunization. FI-RSV immunization is known to be followed by ERD; however, there are still uncertainties regarding the underlying mechanism of this phenomenon. Generally, the key factors are thought to be the poor functional activity of the induced RSV-specific antibodies and a Th2-biased CD4 T-cell response characterized by increasing cytokine levels. Previous studies have already confirmed that ERD does not occur after a natural RSV infection or inoculation with RSV LAV candidates (Wright et al. 2007), consistent with our results.

Altogether, rRSVs bearing either cp, cpts, or cptsASH mutations have been successfully constructed and rescued from a parent strain other than A2 (wtRSV Long). These data indicate that the proposed strategy allows to develop promising RSV LAV candidates, with att phenotype, immunogenicity, genetic stability, and safety similar to those characteristic for strains derived from the traditional RSV A2 parent. Although it is necessary to confirm these data in animal models more susceptible to human strains of RSV, such as cotton rats, which are 100-fold more permissive than BALB/c mice (Prince et al. 1978, 1999; Byrd and Prince 1997; Taylor 2017), our work has paved the way to improve the safety, immunogenicity, and efficacy of live attenuated RSV vaccine candidates derived from the wtRSV Long parent.

In summary, we have successfully constructed and rescued three rRSVs bearing cp, cpts, or cptsASH mutations based on a reverse genetics technology. We used the wtRSV Long parental strain, which differs from the standard RSV A2 strain. However, the three cDNA-derived RSVs exhibited biological and immunological similarities with their RSV A2 counterparts. Moreover, the results of in vitro and in vivo experiments showed that the rRSVs with the introduced mutations (cp, 248ts, and 404ts) from the RSV A2 strain exhibited optimal levels of attenuation, efficacy, and genetic stability, which could be further improved when combined with the SH gene deletion. Therefore, we can preliminarily conclude that the development of live attenuated RSV vaccines with RSV Long as parent strain is a promising strategy which needs to be further explored in the future.

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Author Contributions JSH conceived and designed the study. LNW, MX, YBZ, YYJ, and XLP conducted the experiments. LNW, MX, JMY, YHF, YPZ, WYZ, and ZJD analyzed the data. XLP and LNW drafted and revised the paper. All authors read and approved the final manuscript.
Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

Human and Animal Rights This study was conducted with the approval of the Institutional Animal Care and Use Committees of Tsinghua University (No. 17-DZJ1).

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