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SHORT COMMUNICATION

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Production of single-cycle infectious SARS-CoV-2 through a trans-complemented replicon

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Abstract

Single-cycle infectious virus can elicit close-to-natural immune response and memory. One approach to generate single-cycle severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is through deletion of structural genes such as spike (S) and nucleocapsid (N). Transcomplementation of the resulting ΔS or ΔN virus through enforced expression of S or N protein in the cells gives rise to a live but unproductive virus. In this study, ΔS and ΔN BAC clones were constructed and their live virions were rescued by transient expression of S and N proteins from the ancestral and the Omicron strains. ΔS and ΔN virions were visualized by transmission electron microscopy. Virion production of ΔS was more efficient than that of ΔN. The coated S protein from ΔS was delivered to infected cells in which the expression of N protein was also robust. In contrast, expression of neither S nor N was detected in ΔN-infected cells. ΔS underwent viral RNA replication, induced type I interferon (IFN) response, but did not form plaques. Despite RNA replication in cells, ΔS infection did not produce viral progeny in culture supernatant. Interestingly, viral RNA replication was not further enhanced upon overexpression of S protein. Taken together, our work provides a versatile platform for development of single-cycle vaccines for SARS-CoV-2.

KEYWORDS
COVID-19, live attenuated vaccine, replicon, SARS-CoV-2, single-cycle infectious virus

1 | INTRODUCTION

Vaccination remains the best solution to curtail the ongoing pandemic of COVID-19 by generating protective immunity against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection in the general population. This is however overshadowed by the emergence of vaccine-evasive SARS-CoV-2 variants and the high prevalence of breakthrough infections in vaccinees.[1,2] There is an urgent need to develop next-generation SARS-CoV-2 vaccines that can not only combat new variants but also elicit mucosal immunity that provides better protection against SARS-CoV-2 infection.[2]

Live attenuated vaccines (LAV) elicit a full-spectrum immune response resembling that triggered by natural infection, but safety concerns persist in areas such as the induction of cytokine storm and the emergence of virulent revertants.[3] To address these concerns, single-cycle infectious viruses are developed as LAV candidates. These viruses are highly attenuated but sufficiently immunogenic to elicit protective immune response against infection by their virulent counterparts. One or more essential structural components of the

virus have been disrupted so that the resulting mutant is defective to execute further viral life cycles after primary infection. Live virions are produced by supplying a functional copy of the disrupted gene in trans in the host cells. Single-cycle infectious viruses that serve as LAV candidates have been successfully demonstrated with influenza A virus,[4] vesicular stomatitis virus,[5] Rift Valley fever virus,[6] herpes simplex virus,[7] vaccinia virus,[8] flavivirus,[9] and simian immunodeficiency virus.[10]

Generation of single-cycle infectious SARS-CoV-2 has also been achieved by abrogating the expression of spike (S),[11,12] nucleoprotein (N),[13] or envelope (E) plus ORF3a.[14] Transcomplementation of the defective genome with an expression plasmid of the abrogated gene enables virion production. Single-cycle SARS-CoV-2 can express its own viral antigens within its limited life cycle. By manipulating the expression construct for transcomplementation, it may also be pseudotyped with other antigens of interest. For example, antigens, such as cognate S and N proteins from emerging variants of concern (VOCs), are coated and embedded directly in its virion to be delivered to the host cells for stimulation of immune response.

S and N proteins of SARS-CoV-2 are the major targets of neutralizing antibodies and T cell responses.[15,16] Importantly, both S and N proteins contain most of the nonsynonymous mutations in novel VOCs that confer immune evasion.[17] Thus, pseudo-typing single-cycle SARS-CoV-2 with S or N from an emerging VOC might give rise to an optimal vaccine candidate that elicits both VOC-targeting and potentially sterilizing immunity.

In the current study, we harnessed BAC recombineering to manipulate a BAC molecular clone carrying SARS-CoV-2 genome (BAC-S2-WT) and generated two mutants BAC-S2-ΔS and BAC-S2-ΔN, in which the expression of S and N, respectively, has been disrupted. Direct transfection of BAC-S2-WT into Vero-hTMPRSS2 cells produced up to 10^6 PFU/ml infectious titer of SARS-CoV-2. By using the same method, ΔS virion (ΔS-S-Flag) was successfully rescued through cotransfection of BAC-S2-ΔS with S-Flag expression construct. For simplicity, hereafter we used ΔS-S-Flag to refer to the ΔS virion rescued with Flag-tagged S protein of the Wuhan-Hu-1 strain. In contrast to ΔS virion, coexpression of N-Flag protein in BAC-S2-ΔN-transfected cells yielded significantly less ΔN virion (ΔN-N-Flag). Surprisingly, ΔS-S-Flag infected and replicated in Vero-hTMPRSS2 cells without the need of an additional dose of S protein. ΔS-S-Flag did not cause cytopathic effects (CPE) or plaques. ΔS-S-Flag was also capable of infecting human lung epithelial Calu-3 cells and eliciting type I interferon (IFN) response. Our results suggest the potential of ΔS-S-Flag as a candidate LAV and a surrogate model to study SARS-CoV-2 infection.

2 | MATERIALS AND METHODS

2.1 | Plasmids and antibodies

Plasmid pCAGEN-Flag-N with codon optimization on N-coding sequence was kindly provided by Dr. Kin-Hang Kok and Prof. Kwok-Yung Yuen (Department of Microbiology, The University of Hong Kong).[18] pCMV14-3×Flag-S (C-19) which expresses S protein of the ancestral Wuhan-Hu-1 strain of SARS-CoV-2 with codon optimization and C-19 deletion was a gift from Prof. Qi Jin, Prof. Jianwei Wang and Prof. Zhaohui Qian (Institute of Pathogen Biology, Chinese Academy of Medical Sciences and Peking Union Medical College).[19] pCAGEN-S-Omicron which expresses Flag-tagged S protein with C-19 deletion from SARS-CoV-2 Omicron BA.1 strain was kindly provided by Dr. Shuofeng Yuan and Mr. Jian-Piao Cai (Department of Microbiology, The University of Hong Kong). pCAGEN-Flag-N, pCMV14-3×Flag-S (C-19) and pCAGEN-S-Omicron were respectively the N-Flag, S-Flag and S-Omicron expression constructs described throughout the paper. pMOD4-GalK-G, kindly provided by Dr. Søren Warming (National Cancer Institute), was used as the template for galK expression cassette in BAC recombineering. Mito-GFP was purchased from BD Biosciences.

Anti-S (cat. No. PA5114451) and anti-N antibodies (cat. No. MA535943) were purchased from Thermo Fisher. Anti-β-tubulin antibody was purchased from Santa Cruz. Goat anti-mouse IgG H&L (10 nm Gold) preadsorbed (cat. No. ab27241) and goat anti-rabbit IgG H&L (15 nm Gold) preadsorbed (cat. No. ab27236) were from Abcam. Anti-Flag antibody (M2, cat. No. F3165) was from Sigma.

2.2 | Galk recombineering to generate BAC-S2-ΔS and BAC-S2-ΔN

BAC clone for SARS-CoV-2 Wuhan-Hu-1 has been described.[20] BAC recombineering was performed as described.[21] In brief, homologous recombination was performed to insert a galK expression cassette to replace S- or N-coding sequence. The galK expression cassette was amplified from pMOD4-GalK-G. Primers to amplify galK for replacing S were (capital letters represented sequence complementary to galK while others are the homology arms corresponding to regions just outside S- or N-coding sequence) 5′-caacagagtt gttatttcta gtgatgttct tgttaacaac taaacgaaca CCTGTTGACA ATTAATCATC GGCA-3′ (forward) and 5′-agttacagtt ccaattgtga agattctcat aaacaaatcc ataagttcgt TCAGCACTGT CCTGCTCCTT-3′ (reverse). Those for N were 5′-tatcatgacg ttcgtgttgt tttagatttc atctaaacga acaaactaaa CCTGTTGACA ATTAATCATC GGCA-3′ (forward) and 5′-aaagcgaaaa cgtttatata gcccatctgc cttgtgtggt ctgcatgagt TCAGCACTGT CCTGCTCCTT-3′ (reverse). The amplified products were treated with DpnI (New England BioLabs) and polymerase chain reaction (PCR) Clean-Up System (Promega). Electroporation was performed to introduce purified PCR product into BAC-S2-WT-carrying SW105 cells which were heat shocked at 42°C to express λ Red-encoded genes *exo*, *bet*, and *gam* for homologous recombination. Cells were grown on M63 minimal agar plate (M63 medium from MilliporeSigma) supplemented with galactose for 3 days at 32°C. Bacterial colonies were picked and purified by streaking on MacConkey agar plate. Correct clones with galK cassette were identified through PCR screening and Sanger sequencing. Subsequently, oligonucleotides designed to carry ΔS and ΔN sequence were synthesized by intergrated DNA technologies.

The sequence to generate double-stranded oligonucleotide for ΔS insertion to replace galK cassette was 5′-caacagagtt gttatttcta gtgatgttct tgttaacaac taaacgaaca acgaacttat ggatttgttt atgagaatct tcacaattgg aactgtaact-3′. That for ΔN was 5′- tatcatgacg ttcgtgttgt tttagatttc atctaaacga acaaactaaa actcatgcag accacacaag gcagatgggc tatataaacg ttttcgcttt-3′. Electroporation was performed as described above to introduce double-stranded oligonucleotides into the heat-shocked SW105 clone carrying correct BAC with galK cassette and then plated onto DOG-M63 minimal agar plate for 3 days at 32°C. Bacterial colonies were picked and purified by streaking on MacConkey agar plate. Correct clones were identified through PCR screening and Sanger sequencing. Purified BAC was then obtained from the correct SW105 clone through PureLink™ HiPure BAC Buffer Kit (Thermo Fisher).

2.3 | Cell culture and virion rescue

Vero-hTMPRSS2 cells were purchased from JCRB Cell Bank and cultured in dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin-streptomycin and 1 mg/ml G418 in CO_2-humidified incubator at 37°C. Cells were subcultured every 2–3 days to prevent over-confluency that might reduce transfection efficiency. Calu-3 cells were cultured in DMEM with 10% FBS and 50 U/ml penicillin-streptomycin. All experiments involving live SARS-CoV-2 (including WT, ΔS-S-Flag, and ΔN-N-Flag) were performed in the Biosafety Level 3 facility provided by Department of Microbiology, Li Ka Shing Faculty of Medicine.

For DNA transfection of Vero-hTMPRSS2 cells, GeneJuice (1.33 mg/ml suspension in 80%–90% ethanol; Novagen; Merck KGaA) was used with reference to the manufacturer's manual. To rescue recombinant SARS-CoV-2 WT from BAC clones, 10 μg of purified BAC-S2-WT was used. For BAC-S2-ΔS or BAC-S2-ΔN, 10 μg of bacmid was either transfected alone or together with 2 μg of S-Flag or N-Flag expression construct. In brief, 4.8×10^6 Vero-hTMPRSS2 cells were seeded in T75 flask 1 day before transfection. On the day of transfection, 1:3 volume of GeneJuice (DNA (μg): GeneJuice (μl)) was diluted in 960 μl OPTI-MEM (Thermo Fisher) and incubated for 5 min at room temperature. Next, purified DNA of BAC clone or a mixture of BAC and expression construct was added, mixed well with the GeneJuice/OPTIMEM and incubated for another 15 min at room temperature. In the meantime, the medium of Vero-hTMPRSS2 was changed to 12 ml OPTI-MEM. DNA/GeneJuice/OPTIMEM mix was then added dropwise to the Vero-hTMPRSS2. After 24 h, medium was changed to 12 ml serum-free DMEM. After another 48 h, culture supernatant was collected and precleared by low-speed centrifugation at 3000*g* for 10 min at 4°C. The 12 ml precleared supernatants were then spin-dialyzed with 100 kDa centrifugal filter purchased from Millipore and concentrated to 3 ml, which was finally aliquoted for storage in −80°C or used for further experiment.

2.4 | Infection, plaque assay, and fluorescent focus assay (FFA)

Vero-hTMPRSS2 or Calu-3 cells (2×10^5) were seeded into 24-well plates 1 day before infection. On the day of infection, media of Vero-hTMPRSS2 cells were changed to fresh serum-free DMEM. One hundred microliter of phosphate-buffered saline (PBS) or viruses were then added to Vero-hTMPRSS2 cells. Infected cells were collected at indicated time points and assayed as described below in 2.5.

For plaque assays, 4×10^5 Vero-hTMPRSS2 cells were seeded into 12-well plates 1 day before infection. On the day of infection, media of Vero-hTMPRSS2 cells were changed to fresh serum-free DMEM. Virus stocks were serially diluted 10-fold in serum-free DMEM. Diluted viruses were added to cells which were then incubated in CO_2 incubator at 37°C. After 1 h of virus adhesion, media were removed. One milliliter of 1% agarose in 0.5 × DMEM was added to overlay the infected Vero-hTMPRSS2 cells. The cells were incubated for 72 h. Next, 10% formalin was added to each well and incubated overnight. The fixative and the agarose overlay were then removed. The fixed cells were stained with 0.5% crystal violet. The plaques were photographed.

For FFA, 1.2×10^5 Vero-hTMPRSS2 cells were seeded into eight-well Millicell® EZ slides one day before infection. Like plaque assay, Vero-hTMPRSS2 cells were infected with serially diluted virus in serum-free DMEM for 1 h in CO_2 incubator at 37°C. Then, infection media were removed. 0.3 milliliter of 1% agarose in 0.5 × DMEM was added to overlay the infected Vero-hTMPRSS2 cells. The cells were incubated for 72 h. Next, 10% formalin was added to each well to inactivate and fix the infected cells overnight. The fixative and overlay were removed. The fixed cells were then washed once with PBS. 0.2% Triton X-100 in PBS was then added to the cells for permeabilization for 5 min followed by three washes with PBS. The cells were next blocked with 3% bovine serumalbumin (BSA) in PBS for 1 h. Anti-S (cat. No. PA5114451) at 1:100 dilution in PBS with 3% BSA was then added to each well and incubated overnight at 4°C with gentle agitation. After removing the primary antibody solution, the cells were washed three times with PBS. Secondary antibody anti-rabbit FITC (AP307F) at 1:200 dilution and 0.5 μg/ml 4',6-diamidino-2-phenylindole was then added and incubated for 1 h followed by three PBS washes. The stained cells were finally mounted with Vectashield mounting medium and observed with Olympus BX53 fluorescence microscope.

2.5 | Western blot analysis, reverse transcription-PCR (RT-PCR), and Quantitative reverse transcription-PCR (RT-qPCR)

For Western blot analysis, cells in 24-well plate were lysed with ×1 protein sample buffer and assayed through SDS-PAGE and immunoblotting as previously described.[22] For RNA extraction and purification from infected cells, RNeasy Mini Kit (Qiagen) was used following

manufacturer's protocol. For RNA extraction and purification from virion, QIAamp Viral RNA Kits (Qiagen) was used following manufacturer's protocol. For RT, PrimeScript™ RT Reagent Kit with gDNA Eraser was used following manufacture's protocol. For cellular RNA, 1 μg RNA was used for RT. For viral RNA, 7 or 14 μl was used for RT. Oligo-dT plus random hexamer were used as primer for RT. For qPCR, complementary DNA (cDNA) was subjected to be amplified by Taq polymerase with TB Green Premix Ex Taq II (Tli RNase H Plus) (Takara) following manufacture's protocol using CFX96 Touch Real-Time PCR Detection System (Bio-Rad). qPCR primers were: (1) viral RNA: 5′-ggcgaaatac cagtggctta-3′ (forward) and 5′-tgagttcacg ggtaacacca-3′ (reverse); (2) IFN- β: 5′-aggacaggat gaactttgac-3′ (forward) and 5′-tgatagacat tagccaggag-3′ (reverse); (3) CCL5: 5′-gcatctgcct ccccatatt-3′ (forward) and 5′-agcacttgcc actggtgtag-3' (reverse); (4) β-tubulin: 5′-aagatccgag aagaataccc tga-3′ (forward) and 5′-ctaccaactg atggacggag a-3′ (reverse); (5) β-actin (based on *Cercopithecus aethiops*): 5′-ggcatcctca ccctgaagta-3′ (forward) and 5′-gctggggtgt tgaaggtct-3′ (reverse). For RT-PCR on viral RNA genotyping, cDNA was subjected to PCR amplification by Q5 polymerase (NEB, Ipswich) followed by 1% agarose gel electrophoresis using 1 kb+ DNA ladder as marker. Primers for RT-PCR were 5′-ccaattcagt tgtcttccta ttct-3′ (forward) and 5′-gtagcgcgaa caaaatctg-3′ (reverse) for S gene as well as 5′-gcgttgttcg ttctatgaag ac-3′ (forward) and 5′-aaaatgtggt ggctctttca a-3′ (reverse) for N gene.

2.6 | Transmission electron microscopy (TEM) and immunogold staining

Viruses were fixed and inactivated with 10% formalin for 30 min. Fixed viruses were either directly negative-stained with uranyl acetate or pretreated for immunogold-staining followed by uranyl acetate staining as described.[23] In brief, for negative staining, 10 μl of fixed virus solution was mounted onto a hydrophilized formvar-carbon coated copper grid by grid floating. The grid was then transferred to 10 μl 2% uranyl acetate solution and floated for 1 min. After that, the grid was washed once with ddH_2O droplet and excess liquid on the grid was removed by filter paper. The grids were air-dried. The grids were examined with FEI Tecnai G2 20 scanning TEM at 100 kV.

For immunogold staining before negative staining, after mounting fixed virus solution onto hydrophilized TEM carbon-coated grid as mentioned above, grid was washed three times with 100 μl freshly prepared PBS followed by one time on 100 μl 50 mM glycine in PBS that fully quenches formalin. The grid was then transferred onto 1% BSA in PBS for 10 min blocking. Then, the blocked grid was transferred to a 10 μl droplet containing primary antibody for 1 ho to overnight. After that, the grid was washed three times with 100 μl freshly prepared 1% BSA in PBS. Next, the washed grid was transferred to 10 μl secondary gold-conjugated antibody in 1% BSA (1:1) and incubated for 20 min. After that, the grid was washed twice with 100 μl freshly prepared PBS and twice with 100 μl ddH_2O. Finally, the grid was floated on 10 μl of 2% uranyl acetate solution for 1 min, washed once with ddH_2O droplet and examined with FEI Tecnai G2 20 scanning TEM at 100 kV.

3 | RESULTS

3.1 | Generation of BAC-S2-ΔS and BAC-S2-ΔN molecular clones

To generate S- or N-defective SARS-CoV-2 mutant ΔS or ΔN, BAC recombineering using the galK selection method[20,21] was performed. The coding sequence of S or N was completely deleted (Figure 1A). The deleted regions in BAC-S2-ΔS and BAC-S2-ΔN were confirmed by Sanger sequencing (Figure 1B,C). The Sanger sequencing signals of the deletion were clean, indicating the homogeneity of BAC-S2-ΔS and BAC-S2-ΔN. Restriction mapping was also performed to verify the corresponding deletions in BAC-S2-ΔS and BAC-S2-ΔN (Figure 1 D–F). It was confirmed that BstBI/BamHI digestion failed to yield the 3417 and 1525 bp fragments from BAC-S2-ΔS, while both fragments were observed for BAC-S2-WT and BAC-S2-ΔN (Figure 1E). By using BamHI/SalI digestion, which can generate a 4852 bp fragment from BAC-S2-WT, we confirmed that restriction digestion of BAC-S2-ΔS and BAC-S2-ΔN failed to produce the 4852 bp fragment. Instead, a 3592 bp fragment was seen from BamHI/SalI digestion of BAC-S2-ΔN due to deletion of the N gene of 1260 bp (Figure 1F). This BamHI/SalI restriction pattern of BAC-S2-ΔS further confirmed the absence of both BamHI and BstBI sites of S gene in BAC-S2-ΔS. Thus, our results indicated successful deletion of S and N genes from the respective BAC-S2-ΔS and BAC-S2-ΔN clones.

3.2 | Production of ΔS-S-Flag and ΔN-N-Flag in Vero-hTMPRSS2 cells

To rescue SARS-CoV-2 from BAC molecular clones, we performed direct transfection with Vero-hTMPRSS2 cells, a proficient cell line for SARS-CoV-2 propagation.[24] Transfection efficiency was up to 50% as shown by mito-GFP reporter (Figure 1G). Moreover, transfection of BAC-S2-WT into Vero-hTMPRSS2 cells yielded SARS-CoV-2 of viral titer as high as 6.8×10^{12} copies/μl (Figures 1I) and 1.5×10^{6} PFU/ml (Figure 4D, lane 3), detected by RT-qPCR and plaque assay, respectively. Negative staining in TEM analysis revealed SARS-CoV-2 virion of around 100 nm diameter (Figure 2A), which was positively recognized by anti-S antibody in immunogold staining (Figure 2B). Thus, direct transfection of BAC molecular clones of SARS-CoV-2 into Vero-hTMPRSS2 is a feasible approach to derive SARS-CoV-2 virion.

Transcomplementation is required for virion production from BAC-S2-ΔS and BAC-S2-ΔN.[11–13] Both S and N were Flag-tagged and expressed well in Vero-hTMPRSS2 cells (Figure 1H). The C-terminal ER retention signal of S protein had been removed to facilitate virion production.[19] In addition, the coding sequence of S or N had also been codon-optimized to minimize the chance of RNA

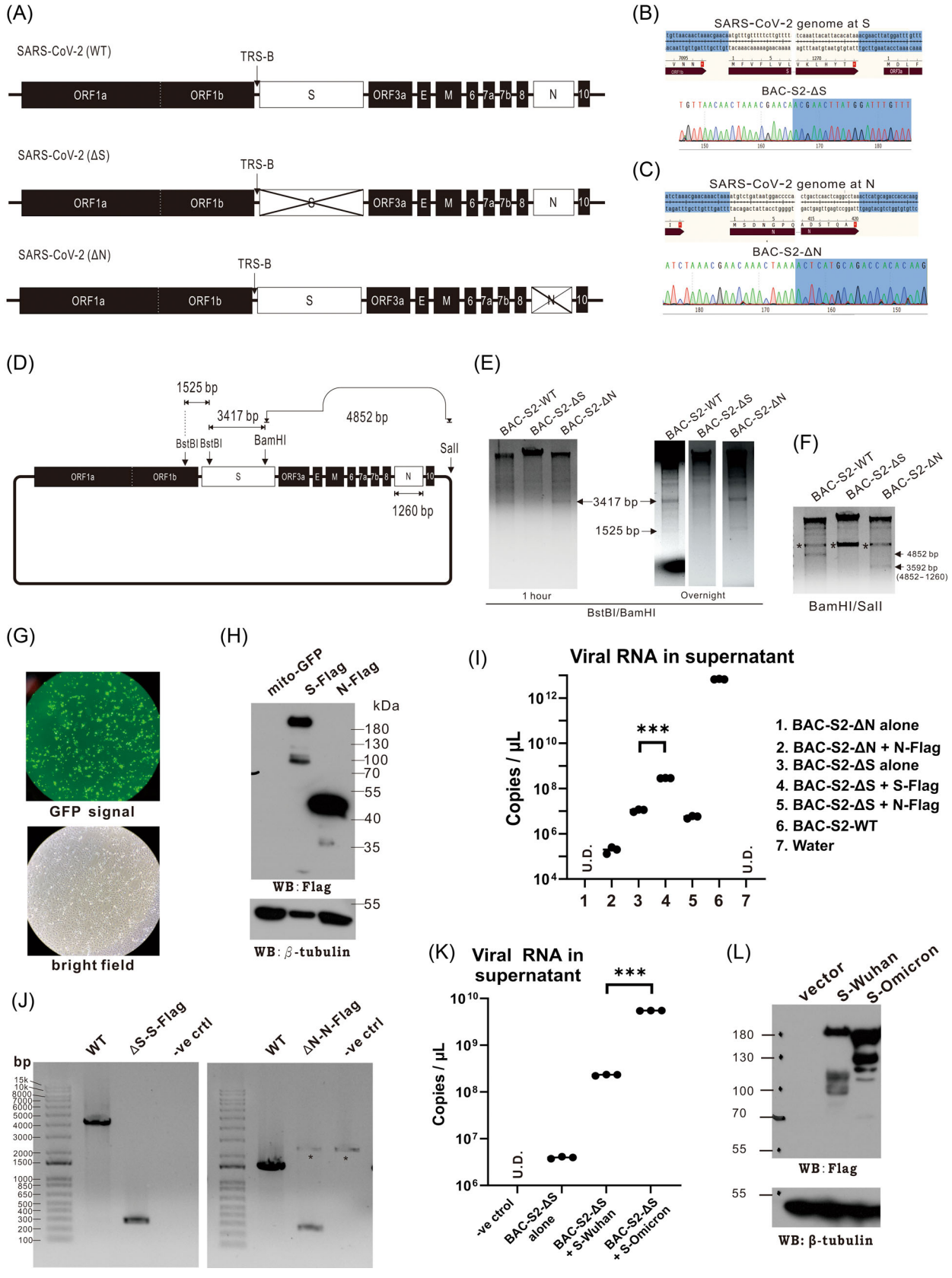

FIGURE 1 (See caption on next page)

recombination with the defective genome or RNA incorporation into progeny virion.

Vero-hTMPRSS2 cells were then transfected with BAC-S2-ΔS or BAC-S2-ΔN with or without transcomplementation with S-Flag or N-Flag expression construct. Virions released to the culture supernatant were collected and viral RNA in these virions was quantitatively analyzed by RT-qPCR. BAC-S2-ΔS alone was already capable of producing virions containing 1.1×10^7 copies/μl of viral RNA as detected by RT-qPCR. Upon co-expression of S-Flag, the amount of viral RNA in the virions was boosted further to 2.9×10^8 copies/μl (Figure 1I). This indicated successful rescue of ΔS-S-Flag virions. Cotransfection of BAC-S2-ΔS with N-Flag expression construct did not promote viral RNA accumulation in the culture supernatant, suggesting that BAC-S2-ΔS was trans-complemented specifically by S. In contrast, viral RNA was undetectable in the culture supernatant of BAC-S2-ΔN-transfected cells (Figure 1I). Cotransfection of N-Flag expression construct with BAC-S2-ΔN led to the detection of viral RNA at the level of 1.9×10^5 copies/μl (Figure 1I), suggesting a much lower efficiency of viral RNA accumulation in the ΔN-N-Flag virions. Moreover, ΔS-S-Omicron virion was also rescued with the expression of S protein from the Omicron strain (S-Omicron). S-Omicron and S protein from the ancestral Wuhan strain (S-Wuhan) were found to be expressed equally well in HEK293T-ACE-2 cells (Figure 1L). Importantly, we found that ΔS-S-Omicron was more efficiently rescued to a titer of 5.5×10^9 copies/μl upon expression of S-Omicron (Figure 1K). Next, the viral genome of ΔS-S-Flag or ΔN-N-Flag was further assayed by RT-PCR for the region of deletion. The deletion in ΔS-S-Flag or ΔN-N-Flag was confirmed with RT-PCR showing a band of reduced size at 302 or 277 bp (Figure 1J). The 4124 and 1537 bp bands for S and N genes of SARS-CoV-2 WT were absent from ΔS-S-Flag and ΔN-N-Flag, respectively, indicating good purity of ΔS-S-Flag and ΔN-N-Flag with no contamination with SARS-CoV-2 WT (Figure 1J). To conclude, ΔS-S-Flag and ΔN-N-Flag virions appeared to be successfully rescued from BAC-S2-ΔS and BAC-S2-ΔN cotransfected with S-Flag and N-Flag expression constructs, respectively. Whereas virion rescue of ΔS-S-Flag was more efficient than that of ΔN-N-Flag, the best efficiency was obtained with ΔS-S-Omicron.

3.3 | Characterization of ΔS-S-Flag and ΔN-N-Flag virions

To further characterize the integrity of ΔS-S-Flag and ΔN-N-Flag, TEM with negative staining was performed to visualize the virions. Both ΔS-S-Flag and ΔN-N-Flag virions, with a diameter of around 50–70 nm, were smaller than those of SARS-CoV-2 WT (Figure 2A). Immunogold staining against S protein was performed to confirm virion detection.[23] As shown by four different views from immunogold staining, SARS-CoV-2 WT, ΔS-S-Flag and ΔN-N-Flag virions can be stained with anti-S antibody and gold (15 nm)-conjugated secondary antibody (Figure 2B). When compared with virion samples, negative control (PBS/formalin) showed a scattered pattern of fewer gold particles, representing background signal derived from nonspecific interaction of antibodies or gold particles. For ΔS-S-Flag, views 3 and 4 (Figure 2B) were of similar size to a single viral particle as shown in negative staining views (Figure 2A). Views 1 and 2 of ΔS-S-Flag (Figure 2B) showed larger viral particle size probably arisen from virion aggregate. For ΔN-N-Flag, views 2–3 (Figure 2B) were consistent with

FIGURE 1 Production of SARS-CoV-2 wild type (WT), ΔS-S-Flag and ΔN-N-Flag from Vero-hTMPRSS2 cells. (A) Diagram of WT, S-deleted (ΔS) and N-deleted (ΔN) versions of SARS-CoV-2 genome. (B,C) Sanger sequencing was performed on the region of S and N on BAC-S2-ΔS and BAC-S2-ΔN, respectively. The upper panels represent the 5' and 3' ends of S or N gene of SARS-CoV-2 WT. The lower panels represent the Sanger sequencing signals of BAC-S2-ΔS and BAC-S2-ΔN at S and N genes, showing successful deletion of S- and N-coding sequence. (D) Expected fragment sizes for restriction digestion of BAC-S2-ΔS and BAC-S2-ΔN. (E) Restriction mapping of BACs with BstBI and BamHI. Two micrograms of BAC-S2-WT, BAC-S2-ΔS or BAC-S2- ΔN were incubated with 0.5 U of BstBI and BamHI in rCutsmart buffer (New England Biolabs) for 1h or overnight at 37°C. The resulting digests were resolved in 0.7% agarose gel. The gel was stained with ethidium bromide. The DNA bands were visualized with Molecular Imager GelDoc XR+ (Bio-Rad). (F) Restriction digestion was performed with BamHI and SalI and the time of incubation was 1 h at 37°C. A band at around 6000 bp derived from the digestion of the pBeloBAC11 backbone of BAC-S2-WT, BAC-S2-ΔS or BAC-S2-ΔN was asterisked (*). (G,H) Vero-hTMRPSS2 cells were transfected with mito-GFP, N-Flag and S-Flag expression constructs using GeneJuice reagent. After 48 h, mito-GFP-transfected cells were visualized by fluorescent microscopy for GFP signal (G). Moreover, protein samples of the transfected cells were extracted and assayed for expression of N-Flag or S-Flag through Western blot analysis (H). (I–K) Viral RNA was extracted from the culture supernatant of Vero-hTMPRSS2 cells without any transfection (negative control) or transfected with BAC-S2-WT, BAC-S2-ΔS alone, BAC-S2-ΔN alone, BAC-S2-ΔS plus S-Flag expression construct, BAC-S2-ΔS plus N-Flag expression construct, BAC-S2-ΔS plus S-Omicron construct or BAC-S2-ΔN plus N-Flag expression construct and assayed with RT-qPCR to quantify viral RNA (I, K) or with RT-PCR for genotyping (J). For RT-qPCR data, results are the means of copies/μl derived from three replicates and error bars indicate SD. Student's t test was performed to judge the statistical significance of the indicated groups. ***$p < 0.001$. U.D.: undetectable by RT-qPCR at 40 cycles. ddH_2O was used as negative control for qPCR reaction. For RT-PCR, *represents a nonspecific band amplified by N gene-specific primers. The nonspecific signal was also seen in water control (−ve ctrl). (L) Expression of S-Wuhan and S-Omicron in HEK293-ACE-2 cells 48 h after transfection respectively with pCMV14-3 × Flag-S (C-19) and pCAGEN-S-Omicron was confirmed by Western blot analysis. pCAGEN empty vector was used as negative control. RT-qPCR, reverse transcription quantitative polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; SD, standard deviation.

single viral particle, while views 1 and 4 might represent virion aggregate. For SARS-CoV-2 WT, views 2 and 4 (Figure 2B) were compatible with single viral particle, whereas views 1 and 3 could be ascribed to virion aggregate. All in all, ΔS-S-Flag and ΔN-N-Flag had intact virion structure reactive to anti-S antibody.

3.4 | Antigen delivery via ΔS-S-Flag infection

ΔS-S-Flag and ΔN-N-Flag virions should carry the respective S-Flag and N-Flag supplied in trans when they were produced from Vero-hTMPRSS2 cells. To determine if they can deliver S-Flag and N-Flag

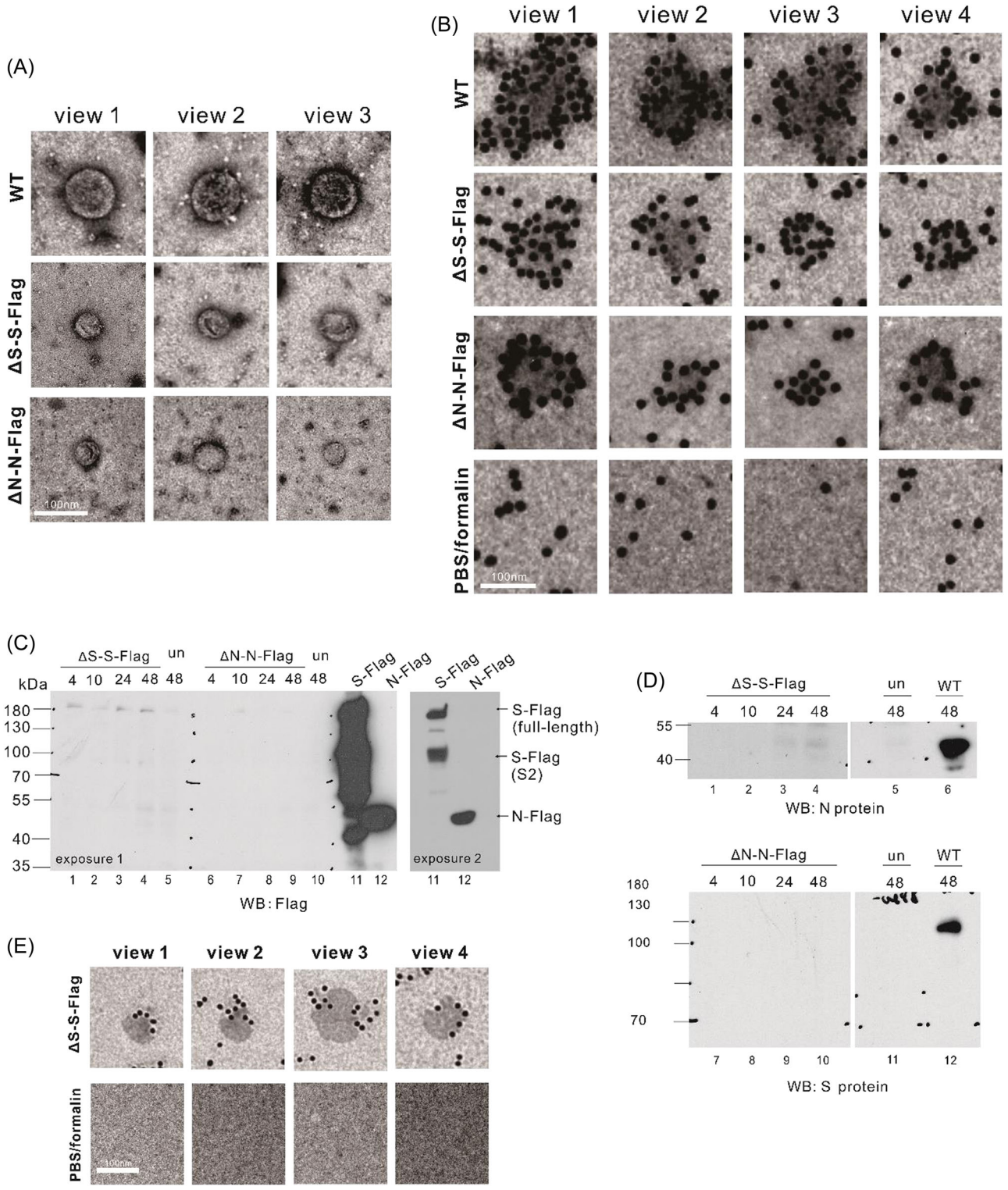

FIGURE 2 (See caption on next page)

to target cells, Vero-hTMPRSS2 cells were infected with ΔS-S-Flag and ΔN-N-Flag. S-Flag was detectable in ΔS-S-Flag-infected Vero-hTMRPSS2 cells and the signal persisted for 48 h (Figure 2C, lanes 1–4). It was noted that the major S-Flag signal in ΔS-S-Flag-infected cells was derived from full-length S (Figure 2C, lanes 1–4), while the S2 cleaved form at 100 kDa was weakly observed at 24 and 48 h (Figure 2C, lanes 3 and 4). In contrast, we did not detect any N-Flag signal in ΔN-N-Flag-infected cells (Figure 2C, lanes 6–9). The absence of N-Flag indicated that plasmid contamination during virus purification was unlikely. In other words, the S-Flag signal plausibly derived from the ΔS-S-Flag virion. In addition to Western blot analysis, FFA which detects viral antigen at a focal area of infected cells was performed using anti-S antibody and ΔS-S-Flag-infected Vero-hTMRPSS2 cells (Figure 3). We found that ΔS-S-Flag infection but not ΔS alone, which was the ΔS without S-Flag reconstitution, resulted in S-positive foci, the quantity of which correlated positively with the concentration of ΔS-S-Flag (Figure 3). Higher numbers of S-positive foci were shown in panels 5 and 8 compared to panels 11 and 14. The number of observed S-containing foci in panel 14 was slightly higher than that in panel 17, which was ΔS alone, or that in panel 20, which was the uninfected control. This further supported that ΔS-S-Flag virion infection can deliver S antigen to the target cells. To compare fluorescent intensity, fluorescent microscopy was performed with the same laser power (50%) and digital gain. The immunofluorescent signal of ΔS-S-Flag-infected cells at the dilution of 1:2 (Figure 3, panel 5) was significantly more intense than that of the uninfected control (panel 11) or ΔS alone at the same dilution (panel 17), but it was still weaker than SARS-CoV-2 WT at $1:10^6$ dilution (panel 2). The initial viral titer of SARS-CoV-2 WT at $1:10^6$ dilution was much lower than that of ΔS-S-Flag at 1:2 dilution (Figure 1I). Hence, although ΔS-S-Flag was able to deliver S protein to the infected cells, it was further attenuated.

We next sought to investigate viral protein expression during infection with ΔS-S-Flag and ΔN-N-Flag. Infected Vero-hTMPRSS2 cells were respectively tested for the expression of N and S antigens at 4-, 10-, 24-, and 48-h time points (Figure 2D). N protein was detected at 24- and 48-h postinfection with ΔS-S-Flag (Figure 2D, lanes 3 and 4), although the expression was weaker than in cells infected with SARS-CoV-2 WT (Figure 2D, lane 6). In contrast, ΔN-N-Flag infection did not give rise to detectable S protein over the 48-h course of infection (Figure 2D, lanes 7–10), while S protein was abundantly expressed from SARS-CoV-2 WT (Figure 2D, lane 12). Hence, ΔS-S-Flag can not only deliver S antigen to the target cells, but also express N antigen, whereas ΔN-N-Flag infection can neither deliver N-Flag nor express S antigen.

To verify the expression of S-Flag on ΔS-S-Flag virions, immunogold staining against Flag-tag was performed with ΔS-S-Flag and observed under TEM (Figure 2E). We found that ΔS-S-Flag virions can be stained with anti-Flag while the negative control (PBS/formalin) showed low background enrichment of gold particles. Thus, ΔS-S-Flag can deliver its coated S antigen to the target cells, but we were unable to detect either N-Flag or S in ΔN-N-Flag-infected cells.

3.5 | ΔS-S-Flag replication in the first cycle of infection unresponsive to further expression of S

ΔS-S-Flag can express N protein during infection (Figure 2D) and should be able to undergo single-cycle replication during primary infection. To confirm if ΔS-S-Flag replicates in a single cycle manner, viral replication kinetic was performed to detect virus progeny in the culture supernatant of ΔS-S-Flag-infected Vero-hTMPRSS2 cells (Figure 4A). Both ΔS-S-Wuhan and ΔS-S-Omicron were used in this experiment. We found that the viral titers of both versions of ΔS-S-Flag in the culture supernatant decreased with time. Viral titer of ΔS-S-Wuhan dropped below the detection limit of RT-qPCR at 24 and 48 h post infection while that for ΔS-S-Omicron dropped by more than 10-fold at 48 h when compared with 0 h. This indicated that neither ΔS-S-Wuhan nor ΔS-S-Omicron infection produced viral progeny, lending support to the notion that they underwent single-cycle RNA replication.

Viral RNA in the culture supernatant of ΔS-S-Wuhan- or ΔS-S-Omicron-infected Vero-hTMPRSS2 cells was undetectable or decreased (Figure 4A), but it was detected in the infected cells at 48 h

FIGURE 2 Characterization of ΔS-S-Flag and ΔN-N-Flag virions by transmission electron microscopy (TEM) and Western blot analysis. (A) Detection of ΔS-S-Flag and ΔN-N-Flag by negative staining. Formalin-fixed SARS-CoV-2 WT, ΔS-S-Flag and ΔN-N-Flag were negatively stained with uranyl acetate and mounted onto TEM grids. The samples were visualized with FEI Tecnai G2 20 scanning TEM at 100 kV. Three independent views were shown for each virus. The scale bar represents 100 nm. (B) Detection of ΔS-S-Flag and ΔN-N-Flag by immunogold staining against S protein. Formalin-fixed SARS-CoV-2 WT, ΔS-S-Flag and ΔN-N-Flag on TEM grid were stained with anti-S antibody followed by staining with secondary anti-rabbit antibody conjugated to gold (15 nm). The samples were then negatively stained with uranyl acetate and visualized with FEI Tecnai G2 20 scanning TEM at 100 kV. Four independent views were shown for each virus. The few gold particles present in the negative control are background signal coming from nonspecific interactions of the antibodies or the gold particles. The scale bar represents 100 nm. (C,D) Vero-hTMPRSS2 cells (2×10^5) were infected with 100 µl of PBS (un), ΔS-S-Flag or ΔN-N-Flag. At 4-, 10-, 24- and 48-h time-points, protein samples were extracted and assayed by Western blot analysis against anti-Flag (C) and either anti-N or anti-S (D). Positive controls were Vero-hTMPRSS2 cells overexpressing S-Flag or N-Flag (C; two exposures were shown) and Vero-hTMPRSS2 cells infected with SARS-CoV-2 WT (D). (E) Detection of ΔS-S-Flag by immunogold staining against S-Flag protein. Formalin-fixed ΔS-S-Flag virions on TEM grid were stained with anti-Flag antibody (M2) followed by staining with secondary anti-mouse antibody conjugated to gold (10 nm). The samples were then negatively stained with uranyl acetate and visualized with FEI Tecnai G2 20 scanning TEM at 100 kV. Four independent views were shown. The scale bar represents 100 nm. SARS-CoV-2, severe acute respiratory syndrome coronavirus-2.

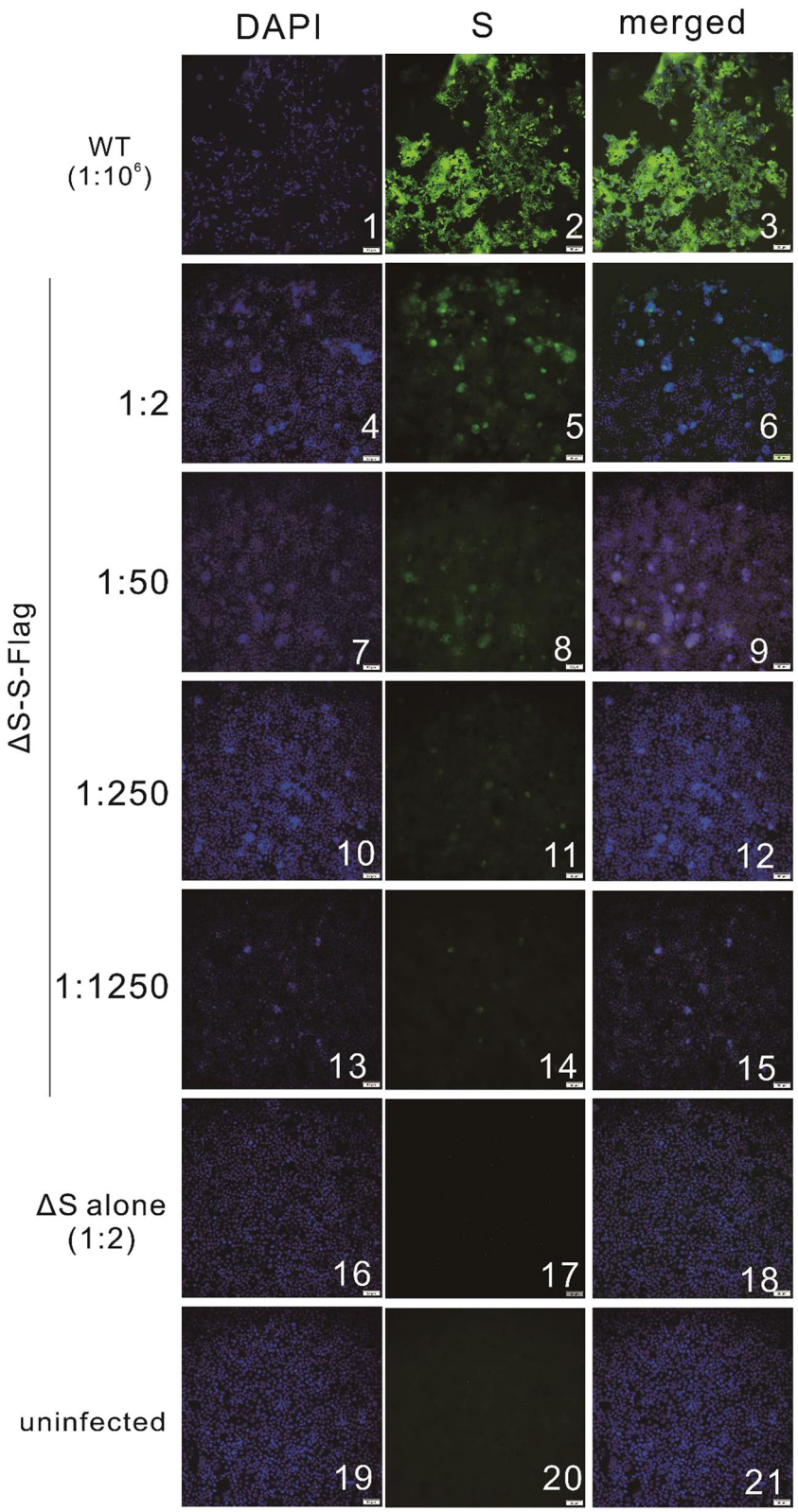

FIGURE 3 Characterization of S-positive foci in ΔS-S-Flag-infected Vero-hTMPRSS2 cells. Vero-hTMRPSS2 cells were infected with SARS-CoV-2 WT (1:10^6), serially diluted ΔS-S-Flag (1:2, 1:50, 1:250, and 1:1250), or ΔS alone (1:2). Uninfected cells served as another negative control. Cells were then overlayed with 1% agarose medium in 0.5 × DMEM. After 72 h, infected cells were fixed and immunostained with anti-S antibody and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Cells were visualized with Olympus BX53 fluorescence microscope at 50% of maximal light output power with excitation 405 nm for DAPI or 488 nm for S. DAPI signal was captured for 20 ms while that for S was 1 s. Panels 1, 4, 7, 10, 13, 16, and 19 represent individual counterstained DAPI views corresponding to the anti-S views in respective panels 2, 5, 8, 11, 14, 17, and 20. Panels 3, 6, 9, 12, 15, 18, and 21 are the merged view for DAPI and anti-S. The scale bars represent 50 µm. SARS-CoV-2, severe acute respiratory syndrome coronavirus-2.

(Figure 4B). To shed light on whether ΔS-S-Flag replicates in the infected cells, cellular RNA of infected Vero-hTMPRSS2 cells were collected at various time points for RT-qPCR analysis. We found that viral RNA was detected in Vero-hTMPRSS2 cells 4, 10, 24 and 48 h after infection with ΔS-S-Flag, while uninfected control showed undetectable signal for viral RNA with 40 cycles of qPCR reaction (Figure 4C). ΔS-S-Flag viral RNA was increased by 62-fold ($\log_{10}$ fold = 1.8) at 48 h postinfection, indicating vigorous RNA replication. Since RNA replication of SARS-CoV-2 WT was far more robust than that of ΔS-S-Flag (Figures 1 and 4), we next interrogated whether

ΔS-S-Flag replication might be further boosted by enforced pre-expression of S-Flag. Vero-hTMPRSS2 cells were transfected with S-Flag expression construct 24 h before ΔS-S-Flag infection. Interestingly, although S protein cannot be expressed from the defective ΔS-S-Flag genome (Figure 1J), extrinsic overexpression of S-Flag did not boost viral RNA replication of ΔS-S-Flag compared to that of ΔS-S-Flag alone (Figure 4C). Plaque assay indicated that ΔS-S-Flag did not form plaques in stark contrast to SARS-CoV-2 WT (Figure 4D, lane 1 vs. 3), consistent with limited viral life cycle of ΔS-S-Flag. In addition, plaque formation by ΔS-S-Flag was not enhanced by extrinsic overexpression of S-Flag (Figure 4D, lane 2). Thus, ΔS-S-Flag undergoes RNA replication with no need for further expression of S-Flag.

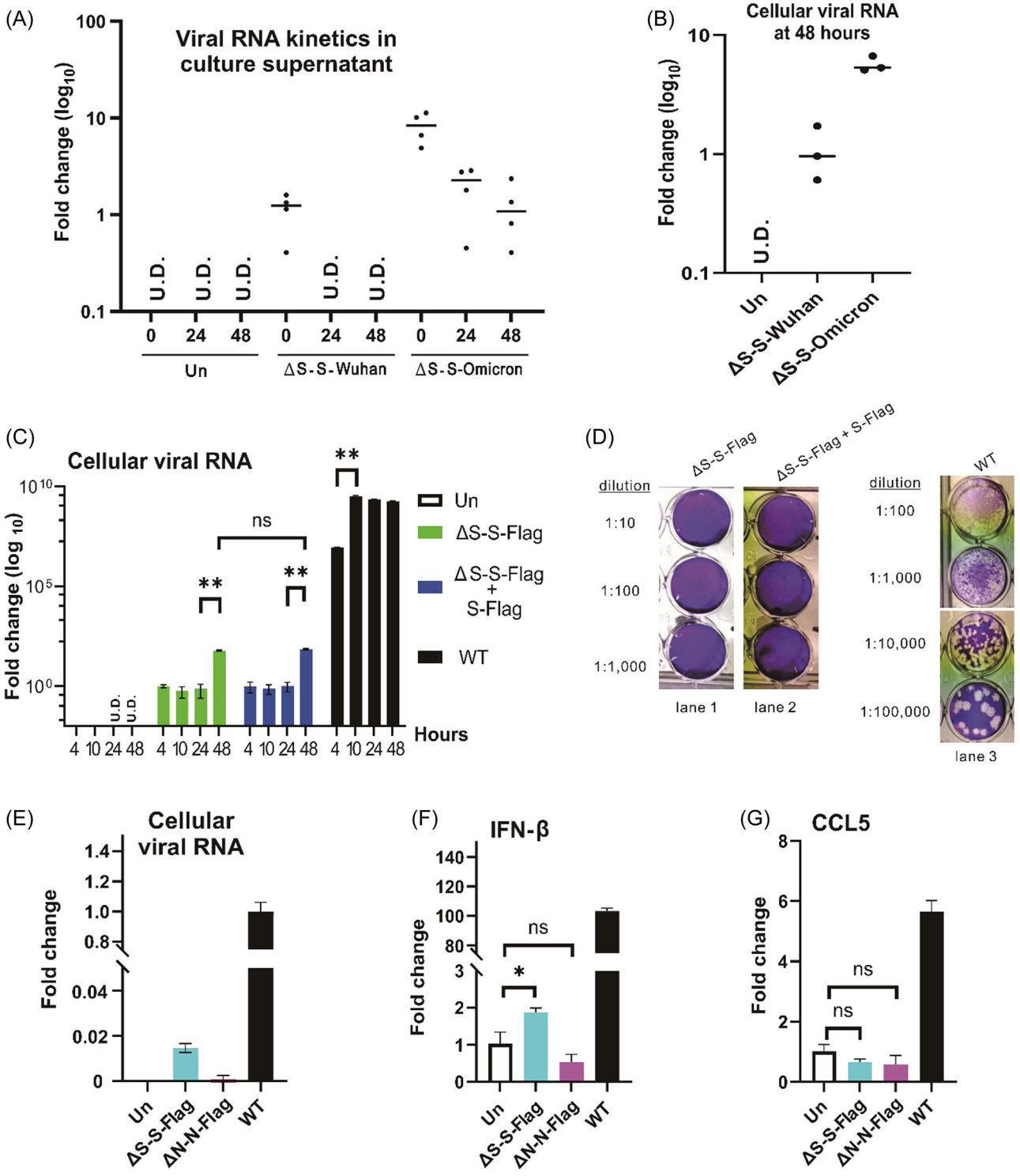

FIGURE 4 (See caption on next page)

3.6 | ΔS-S-Flag infection of human lung Calu-3 cells and induction of IFN-β expression

Finally, we interrogated if ΔS-S-Flag can infect human lung cells. Calu-3 cell line, which is permissive to SARS-CoV-2 infection,[20] was used. It was found that ΔS-S-Flag can infect Calu-3 cells with detectable viral RNA (Figure 4E). In addition, ΔS-S-Flag infection induced IFN-β expression by twofold compared to the uninfected control (Figure 4F). We also compared the expression of proinflammatory cytokine CCL5 and found that ΔS-S-Flag infection did not promote CCL5 expression (Figure 4G). To conclude, ΔS-S-Flag can infect human Calu-3 cells and induce IFN-β expression.

4 | DISCUSSION

In this study, we constructed and characterized a single-cycle infectious SARS-CoV-2 using a transcomplemented BAC clone in which the S region had been disrupted. This virus named ΔS-S-Flag can deliver S-Flag protein (Figure 2C, lanes 1–4; Figure 3) and express N protein (Figure 2D, lanes 3 and 4) in Vero-hTMPRSS2 cells. ΔS-S-Flag virions were immunostained with anti-Flag (Figure 2E). Interestingly, trans-complementation of the ΔS virus with S-Omicron might be more efficient (Figure 1K), consistent with the notion that S-Omicron might be better adapted to humans.[1] We provided the first evidence for efficient rescue of ΔS-S-Omicron virion, paving the avenue for its further development as a candidate LAV specifically targeting the Omicron variant and capable of eliciting mucosal and T cell response more robustly.

S-Flag protein expressed from Vero-TMPRSS2 cells was not completely cleaved (Figure 1H). Thus, ΔS-S-Flag virion may contain both uncleaved and cleaved forms of S-Flag, resembling those of SARS-CoV and SARS-CoV-2.[25] Although ΔS-S-Flag virions were smaller than those of SARS-CoV-2 (Figure 2A), the S-Flag protein on the virions appeared to be competent for receptor binding, proteolytic processing and membrane fusion required for SARS-CoV-2 entry.[19] We did not detect S2 form of S-Flag protein at initial time points of ΔS-S-Flag infection (Figure 2C, lanes 1 and 2), suggesting that it might be the minority on ΔS-S-Flag virion. S2 emerged 24 and 48 h after ΔS-S-Flag infection (Figure 2C, lanes 3 and 4), indicating that some of the delivered full-length S-Flag was processed by proteases such as TMPRSS2.

We found that ΔS-S-Flag can replicate in target cells (Figures 2 and 4) with limited viral life cycle (Figure 4A) and absence of CPE (Figure 4D, lanes 1 and 2). ΔS-S-Flag can also infect and replicate in human lung Calu-3 cells, resulting in detectable viral RNA and production of type I IFN (Figure 4E,F). Interestingly, ΔS-S-Flag infection was not further augmented by further expression of S-Flag in trans (Figure 4C). This is different from the observation that overexpression of S-Flag in trans can enhance viral rescue from BAC-S2-ΔS transfection (Figure 1I). S protein is only necessary for viral entry but not viral replication or packaging,[19] so BAC-S2-ΔS transfection alone can still undergo RNA replication (Figure 1I). However, the lack of S protein prevented assembly of a functional virion. Expression of S-Flag protein in trans can equip viral progenies with S protein for re-entering cells to give a second round of viral production. Expression of S-Flag is thus beneficial to BAC-S2-ΔS virion production since S protein is completely absent in BAC-S2-ΔS-transfected cells. However, in the case of ΔS-S-Flag infection, we

FIGURE 4 Further characterization of ΔS-S-Flag replication and induction of antiviral response. (A, B) 2×10^5 Vero-hTMPRSS2 cells were seeded into 24-well plates 1 day before infection. Culture media of Vero-hTMRPSS2 cells were changed to serum-free DMEM. One hundred microliters of ΔS-S-Wuhan or ΔS-S-Omicron was added to the cells. No virus was added to negative control (un). After 1h, the culture medium was removed. The infected cells were washed twice with sterile PBS. Then, 0.5 ml serum-free DMEM was replenished to the cells. 100 microliters of the culture media were then extracted 0, 24 or 48 h after infection for RT-qPCR assay to quantify viral RNA (A). The cellular RNA was extracted at 48 h to quantify viral RNA and β-actin mRNA. $\Delta\Delta C_T$ was calculated using β-actin as reference gene by taking the group of ΔS-S-Wuhan as 1 and converted to fold change which was finally plotted in terms of $\log_{10}$ fold (B). Experiment in (A) was performed in quadruplicate, which the analysis in (B) was carried out in triplicate. (C) Viral RNA replication of ΔS-S-Flag in Vero-hTMPRSS2 cells independently of expression of S-Flag in trans. Vero-hTMRPSS2 cells (2×10^5) were either left untransfected or transfected with an S-Flag expression construct 24 h before infection with ΔS-S-Flag virus (100 µl). SARS-CoV-2 WT (100 µl) was used as positive control. Cells were collected 4, 10, 24 or 48 h after infection. RNA was extracted. RT-qPCR was performed to quantify viral RNA and β-actin mRNA. $\Delta\Delta C_T$ was calculated using β-actin as reference gene by taking the group of ΔS-S-Flag alone at 4 h as 1 and converted to fold change which was finally plotted in terms of $\log_{10}$ fold. The results are the means of $\log_{10}$ fold change derived from three replicates and error bars indicate SD. Uninfected cells (Un) served as the control. U.D.: undetectable by RT-qPCR at 40 cycles. (D) Plaque assay. Vero-hTMPRSS2 cells (4×10^5) in 12-well plate were transfected with S-Flag expression construct. After 6 h, 10-fold serially diluted SARS-CoV-2 WT or ΔS-S-Flag was added to Vero-hTMPRSS2 cells. After 1 h, cells were overlayed with 1% agarose in DMEM. After another 72 h, cells were fixed with 10% formalin and stained with 0.5% crystal violet. (E–G) ΔS-S-Flag infects Calu-3 cells and provokes IFN-β expression. Calu-3 cells (2×10^5) were infected with 100 µl of ΔS-S-Flag, PBS (Un) or wildtype SARS-CoV-2 (WT). At 24 h postinfection, cellular RNA was extracted and subjected to RT-qPCR quantification of β-tubulin mRNA, viral RNA (E), IFN-β mRNA (F) and CCL5 mRNA (G). U.D.: undetectable for viral RNA by RT-qPCR at 40 cycles. $\Delta\Delta C_T$ was calculated for viral RNA, IFN-β mRNA and CCL5 mRNA using β-tubulin as reference gene. PBS group (Un) was normalized as 1 for IFN-β and CCL5 mRNA. WT was normalized as 1 for viral RNA. The results are the means of fold change derived from at least three replicates and error bars indicate SD. Statistical significance of the difference between the indicated groups was judged by Student's *t* test. mRNA, messenger RNA; ns, not significant; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; SD, standard deviation.

found that S-Flag can be delivered and remained intact in ΔS-S-Flag-infected cells (Figure 2C, lanes 1–4). The full-length S-Flag might reside in TMPRSS2-deprived endosome. As long as the amount of S-Flag was sufficient, expression of another dose of S-Flag is no longer beneficial to ΔS-S-Flag infection (Figure 4C). ΔS-S-Flag might plausibly sustain several limited viral life cycles until the initial S-Flag is diluted out. However, in principle it should not produce more virions than originally introduced.

The sizes of ΔS-S and ΔN-N virions were smaller than that of the WT virion (Figure 2A). Plausibly, expression of S or N in trans might affect the formation of virion. N and S proteins are the fundamental structural proteins of the SARS-CoV-2 virion. Expression of N or S protein through mammalian expression plasmid might generate different quantity of the respective protein when comparing to that expressed naturally from viral subgenomic RNA. Indeed, virus-like particle of SARS-CoV-2 generated by extrinsic expression of S, N, M, and E is smaller than 100 nm.[26,27] This suggests that SARS-CoV-2 virions generated by artificially expressed structural proteins might not be of the same size as that of the natural ones. Likewise, ΔS-S and ΔN-N virions generated respectively through extrinsic reconstitution of S and N are also of a smaller size.

Another group has also constructed SARS-CoV-2 ΔN with the N-coding sequence replaced by EGFP. Virion rescue was achieved in Caco-2 cells stably expressing N protein.[13] The resulting SARS-CoV-2-GFP/ΔN trVLP virus cannot express GFP signal in Caco-2 cells except when N protein was further supplied in trans. This is generally consistent with our observation that ΔN-N-Flag cannot express S protein in Vero-hTMPRSS2 cells (Figure 2D, lanes 7–10). We further tested if ΔN-N-Flag can deliver N-Flag protein to target cells and we did not detect N-Flag in infected Vero-hTMRPSS2 cells (Figure 2C, lanes 6–9). A possible reason is the low initial viral titer of ΔN-N-Flag (Figure 2C), leading to inefficient delivery of N-Flag protein which fell below the detection limit of Western blot analysis. Indeed, ΔN-N-Flag produced from cotransfection of BAC-S2-ΔN and N-Flag expression construct was not effective. Although SARS-CoV-2-GFP/ΔN trVLP can be propagated in Caco-2 cells expressing N protein,[13] its genome stability was low that deletion of ORF6, ORF7, ORF8 and the inserted EGFP emerged at passage 4. Thus, rescuing N-deficient single-cycle SARS-CoV-2 is more challenging, probably due to multifaceted roles of N protein in coronaviral life cycle, including viral gene transcription and viral packaging.[28] One solution can be generation of single-cycle SARS-CoV-2 with minimally deleted or mutated N protein instead of complete N deletion. It will also be of interest to see whether the use of Flag tag might affect the folding or function of the N protein. Removal of the Flag tag or substitution with another tag would provide the clue.

The rescue of ΔS has previously been reported by two other groups.[11,12] Their ΔS engineered to replace S gene with luciferase or EGFP reporter has been found to express the reporter gene in Huh7.5 cell stably expressing ACE-2 and TMPRSS2 (Hub7.5-AT). Consistently, ΔS-S-Flag infection resulted in N protein expression at 24 and 48 h postinfection in our study (Figure 2D, lanes 3 and 4). In contrast to no detection of the delivery of S protein to Huh7.5 AT cells by immunostaining in the other study,[12] we observed S-Flag delivery to Vero-hTMPRSS2 cells starting from 4 h postinfection by use of immunoblotting (Figure 2C, lanes 1–4) or FFA (Figure 3, panels 5, 8, 11 and 14). This difference is possibly due to the difference in experimental settings such as cells, version of virus and detection method. Our finding that ΔS-S-Flag infection is not promoted by prior overexpression of S further supports self-sufficiency of ΔS-S-Flag in the first infection cycle with the incorporated S-Flag. Further analysis of whether ΔS can deliver S protein and express other viral antigens in other physiologically relevant infection models such as primary lung epithelial cells and organoids is warranted. ΔS has also be shown to be rescued by vesicular stomatitis virus G protein in the other study and the rescued virus is susceptible to inhibition by remdesivir.[11] Although we already have obtained several lines of evidence in support of the unproductive single-cycle replication of the ΔS-S-Flag virus, the use of remdesivir or another antiviral in our future study might further confirm its replication in cells. Notably, a recent study on SARS-CoV-2 replicon showed that BAC-SARS-CoV-2 system can generate nonnatural spliced viral RNA species.[29] However, full-length viral genomic RNA is known to be packaged preferentially.[30] Whether the spliced RNA species might be packaged into ΔS-S-Flag or ΔN-N-Flag leading to further attenuation of the BAC-rescued single-cycle virus requires further analysis.

ΔS has been shown to be useful in neutralization assays to detect anti-SARS-CoV-2 antibodies[12] or to screen for anti-SARS-CoV-2 drugs,[11] suggesting that ΔS is a good and safe surrogate model of SARS-CoV-2 infection, to be handled in Biosafety Level 2 facility. Our finding that ΔS can deliver coated antigens, express other viral antigens and induce type I IFN production suggests that ΔS mimics natural SARS-CoV-2 infection. In addition, ΔS infection should be more capable of stimulating mucosal and T cell immunity as in the course of natural infection. In this regard, further investigations in cellular and animal models are required to optimize mass production of ΔS-S-Flag virion as well as to assess its immunogenicity and other properties as a candidate LAV. Particularly, its ability to stimulate mucosal immunity after intranasal inoculation should be characterized in full. In addition, further analysis of the ΔS-S-Omicron virion reported in this study is also required. It will be of particularly great interest to determine whether booster vaccination through intranasal inoculation of ΔS-S-Omicron might be safe and as effective as natural infection in offering strong protection against future infection.

AUTHOR CONTRIBUTIONS

Pak-Hin Hinson Cheung and Dong-Yan Jin conceptualized and designed the study. Pak-Hin Hinson Cheung performed experiments with the help of Zi-Wei Ye, Wai-Yin Lui, Chon Phin Ong, Pearl Chan, Tak-Wang Terence Lee, Tze-Tung Tang, Tin-Long Yuen, Sin-Yee Fung, Yun Cheng, and Ching-Ping Chan. All authors including Chi-Ping Chan contributed to data analysis. Pak-Hin Hinson Cheung and Dong-Yan Jin wrote the manuscript with input from all authors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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