SARS-CoV-2 Delta Spike Protein Enhances the Viral Fusogenicity and Inflammatory Cytokine Production

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SUMMARY

The Delta variant is now the most dominant and virulent SARS-CoV-2 variant of concern (VOC). In this study, we investigated several virological features of Delta spike protein (SP_{Delta}), including protein maturation and its impact on viral entry of cell-free pseudotyped virus, cell-cell fusion ability and its induction of inflammatory cytokine production in human macrophages and dendritic cells. The results showed that $SP\Delta C_{Delta}$ exhibited enhanced S1/S2 cleavage in cells and pseudotyped virus-like particles (PVLPs). We further showed that $SP\Delta C_{Delta}$ elevated pseudovirus infection in human lung cell lines and mediated significantly enhanced syncytia formation. Furthermore, we revealed that $SP\Delta C_{Delta}$-PVLPs had stronger effects on stimulating NF-κB and AP-1 signaling in human monocytic THP1 cells and induced significantly higher levels of pro-inflammatory cytokine, such as TNF-α, IL-1β and IL-6, released from human macrophages and dendritic cells. Overall, these studies provide evidence to support the important role of $SP\Delta C_{Delta}$ during virus infection, transmission and pathogenesis.

Keywords: COVID-19, SARS-CoV-2, Delta variant, Spike protein, cell-to-cell fusion, NF-κB pathway, proinflammatory cytokines

INTRODUCTION

The emergence of the highly pathogenic coronavirus disease 2019 (COVID-19) has been a major concern and threat to public health for two years. As of early November 2021, approximately 248 million COVID-19 cases and more than 5 million deaths were reported globally (WHO, 2021). COVID-19 is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is a member of betacoronaviridae with a single-stranded 30 kb positive-sense RNA genome encoding 29 proteins (Srivastava et al., 2021). Within 2 years, multiple variants of SARS-CoV-2 have emerged (Galloway et al., 2021; Greaney et al., 2021; Nonaka et al., 2021; Paiva et al., 2021; Resende et al., 2021; Santos and Passos, 2021; Tegally et al., 2020; Volz et al., 2021). Delta variant, which was first detected in India and derived from the Pango lineage B.1.617.2, is the most dominant variant of concern (VOC) and has accounted for approximately 99% of new cases of coronavirus worldwide (Banu et al., 2020; Ranjan et al., 2021; Sahoo et al., 2021; Worldometer, 2021). Previous studies suggested that Delta variant infection has a shorter incubation period but a greater viral load (> 1,000 times) than earlier variants (Li et al., 2021; Reardon, 2021). Further, the patients contracted with Delta variant have higher hospitalization rate and more severe outcomes (Twohig et al., 2021). Unfortunately, current vaccines only provide partial protection against the infection of Delta variant, because these vaccines are designed based on the original Wuhan-Hu-1 sequence (Mlcochova et al., 2021). Therefore, it is important to understand the molecular mechanisms of the increased transmissibility and immune evasion of these SARS-CoV-2 variants to facilitate the development of vaccines and therapeutic drugs against COVID-19.

VOCs are mainly classified based on the mutations on their spike protein (SP). The SP of coronavirus is responsible for viral attachment and entry to the host cells (Huang et al., 2020b).

The matured SP is cleaved to generate S1 and S2 subunits at specific cleavage sites. The S1 subunit (aa 14-685) is responsible for receptor binding through its receptor-binding domain (RBD). The S2 subunit (aa 686-1273) mediates membrane fusion to facilitate cell entry (Bertram et al., 2013; Hoffmann et al., 2020b; Peacock et al., 2021b). Mutations in SP have resulted in the high rates of transmission and replication of various variants (Zhang et al., 2021a; Zhou et al., 2021) and the immune evasion from antibody neutralization of various variants (McCallum et al.; Mlcochova et al., 2021; Zhang et al.). The SP of the Delta variant has eight mutations compared with the original virus, including T19R, Δ156– Δ157, and R158G in the N-terminus Domain (NTD), D614G, L452R and T478K at the RBD, P681R close to the furin cleavage site, and D950N at the S2 region (Cherian et al., 2021; Planas et al., 2021; Zhang et al., 2021a). It has been demonstrated that the mutations at the NTD of Delta SP alter the antigenic surface near the NTD-1 epitope, thus leading to the lack of binding affinity with the NTD neutralizing antibodies (Zhang et al., 2021a).

Furthermore, the P681R mutation closed to the furin cleavage has been demonstrated to aid the pathogenicity of the virus (Cherian et al., 2021; Liu et al., 2021; Saito et al., 2021). The newly identified furin cleavage site (681-PRRAR↓SV-687) at the S1/S2 site is reported to be critical for the pathogenesis of SARS-CoV-2 in mouse models, and also be responsible for the cell-cell fusion, which is absent in other group-2B coronaviruses (Coutard et al., 2020; Johnson et al., 2020; Xia et al., 2020). It was shown that P681R mutation at this cleavage site endow Delta variant with special features that facilitate the spike protein cleavage and viral fusogenicity (Peacock et al., 2021b; Saito et al., 2021). Study found that a chimeric Delta SARS-CoV-2 bearing the Alpha-SP replicated less efficiently than the wild-type Delta variant, and the reversion of Delta P681R mutation to wild-type P681 attenuated Delta variant replication as well

(Liu et al., 2021). These observations suggested that the P618R mutation that occurs in Delta variants contributes immensely to the high replication and transmissibility rate. We are therefore interested in further investigating how the P618R mutation impacts the high replication rate of Delta variants.

Like other high pathogenic viruses (influenza H5N1, SARS-CoV-1 and MERS-CoV), SARS-CoV-2 infection also induced excessive inflammatory response with the release of a large amount of pro-inflammatory cytokines (cytokine storm) that may result in Acute Respiratory Distress Syndrome (ARDS) and multiorgan damage (Zhu and al., 2020). Clinical studies showed that the high mortality of COVID-19 is related to cytokine release syndrome (CRS) in a subgroup of severe patients (Huang et al., 2020a), characterized by elevated levels of certain cytokines including IL-6, TNF-α, IL-8, IL-1β, IL-10, MCP-1 and IP-10 (Hadjadj et al., 2020; Huang et al., 2020a; Mehta et al., 2020; Xu et al., 2020; Yang et al., 2020). The observed cytokine production induced by SARS-CoV-2 infection or spike protein expression has been linked with nuclear factor kappa B (NF-κB) and and activator protein-1(AP-1) signaling pathways that can induce the expression of a variety of proinflammatory cytokine genes (Neufeldt et al., 2020b; Zhu et al., 2021). Like other RNA virus, SARS-CoV-2 was found activate NF-κB and AP-1 transcription factors following the sensing of viral RNAs or proteins by different pathogen pattern recognition receptors (PRRs) and associated signalling cascades, including RLRs and TLRs (Pantazi et al., 2021; Zhu et al., 2021). In addition, angiotensin II type 1 receptor (AT1)-MAPK signalling (Patra et al., 2020) and the cGAS-STING signalling (Neufeldt et al., 2020a) have been identified responsible for the activation of NF-κB and the elevated expression of IL-6 in the SARS-CoV-2 infected or SP expressing cells. However,

whether Delta variant or its SP may initiate stronger cytokine storm in patients that leading to more severe illness than other strains still required more investigation.

The current study aims to characterize the cleavage/maturation of various SPs of SARS-CoV-2, especially the Delta variant SP, and their effects on virus infection, cell-cell fusion, cytokine production and related signaling pathways. By using a SARS-CoV-2 SP pseudotyped lentiviral vector or viral-like particles (PVLPs), we demonstrated that SP_{Delta} enhanced S1/S2 cleavage, accelerated pseudovirus infection and promoted cell-cell fusion. We also showed that SP_{Delta} strongly activates NF-κB and AP-1 signaling in THP1 cells. Furthermore, we observed that $SP\Delta C_{Delta}$-PVLP stimulation promoted the production of several pro-inflammatory cytokines by human macrophages and dendritic cells.

RESULTS

SARS-CoV-2 Delta SP exhibited enhanced cleavage and maturation in cells and in the pseudotyped virus

To investigate the functional role of SARS-CoV-2 Delta SP, we first synthesized cDNA encoding SARS-CoV-2 Delta SP, as described in CDC's SARS-CoV-2 Variant Classifications and Definitions (CDC, 2021) (Fig. 1A) and inserted cDNA into a pCAGG-expressing plasmid, as described in the Materials and Methods. Previously described pCAGG-$SP\Delta C_{WT}$- and pCAGG-$SP\Delta C_{G614}$-expressing plasmids (Ao et al., 2021a) were also used in this study. Meanwhile, we constructed a pCAGG-$SP\Delta C_{Delta\text{-}PD}$ in which arginine (R) at the amino acid position of 681 and asparagine (N) at 950 in $SP\Delta C_{Delta}$ were reverted to the original proline (P) and aspartic acid (D) to test the effect of these amino acids on the function of $SP\Delta C_{Delta}$ (Fig. 1A). To enhance the

transportation of SP to the cell surface and increase the virus incorporation of SP, the cDNA encoding the C-terminal 17 aa in SARS-CoV-2 SP was deleted in all pCAGG-SPΔC plasmids (Ao et al., 2021a).

To examine the expression of various SPs in the cells and their incorporation in the SPΔC-pseudotyped viral particles (PVPs), each SPΔC-expressing plasmid was cotransfected with a multiple-gene deleted HIV-based vector encoding a Gaussia luciferase gene (ΔRI/ΔEnv/Gluc) and a packaging plasmid (pCMVΔR8.2) in HEK293T cells, as described previously (Ao et al., 2021a). After 48 hrs of transfection, the expression of each SPΔC in the transfected cells was analyzed by an indirect immunofluorescence (IF) assay using human SARS-CoV-2 S-NTD antibodies. The results revealed that all SPΔCs were well expressed in the transfected cells or cell surface (Fig. 1B). Meanwhile, the transfected cells and PVPs were lysed and processed with WB with anti-SP/RBD or anti-S2 antibodies, respectively (Fig. 1C, top and middle panels). Interestingly, the data clearly showed that in the cells, $SP\Delta C_{Delta}$ was more efficiently processed from the S precursor into S1 and S2 than $SP\Delta C_{WT}$ and $SP\Delta C_{G614}$ (Fig. 1C, compare Lane 3 to Lanes 1 and 2). In PVPs, the majority of $SP\Delta C_{Delta}$ presented as a mature form (S1 and S2) compared to $SP\Delta C_{WT}$ and $SP\Delta C_{G614}$ (Fig. 1C, compare Lane 7 to Lanes 5 and 6), indicating that $SP\Delta C_{Delta}$ undergoes a more efficient maturation process. Surprisingly, S1 of $SP\Delta C_{Delta}$ but not S2 appeared to migrate faster than S1 of $SP\Delta C_{WT}$ and $SP\Delta C_{G614}$ (Fig. 1C, top panel). The possible mechanism for this behavior is currently unknown.

The cleavage of the SARS-CoV-2 SP into S1 and S2 most likely occurs by furin, and the P681R mutation of the SP Delta was suggested to enhance S1/S2 cleavability (Liu et al., 2021; Peacock et al., 2021a). We therefore further tested whether a fusin protease inhibitor, a peptidyl

chloromethylketone (CMK), or the reverse change in R_{681} of $SP\Delta C_{Delta}$ to P would alter the maturation rate of the S protein. The $SP\Delta C_{Delta\text{-}PD}$-PVPs or $SP\Delta C_{Delta}$-PVPs packaged in the presence or absence of CMK were analyzed by WB with anti-RBD antibody and quantified by densitometry using ImageJ (https://imagej.nih.gov/ij/). The results showed that either CMK treatment or $SP\Delta C_{Delta\text{-}PD}$ clearly negatively impacted the maturation of $SP\Delta C_{Delta}$ (Fig. 1D).

Delta-SP mediates more efficient pseudovirus infection in a lung epithelial cell line and primary macrophages

To investigate the impact of $SP\Delta C_{Delta}$ on viral infection, we produced Gluc-expressing Delta-SP-PVPs (Fig. 2A) and tested the infectivity of different pseudoviruses in two human lung epithelial cell lines, Calu-3 and A549 cells. To increase susceptibility to SP-PVP infection, the $A549_{ACE2}$ cell line was generated by transducting a lentivirus expressing hACE2 and subsequent puromycin selection, as described in the Materials and Methods. hACE2 expression in $A549_{ACE2}$ cells was verified by WB (Fig. 2B). Then, both cell lines were infected with equivalent amounts (adjusted with p24 values) of the $SP\Delta C_{WT}$-, $SP\Delta C_{G614}$-, and $SP\Delta C_{Delta}$-PVPs for three hours and washed. At 24 and 48 hrs post infection (p.i.), the supernatants were collected, and the infection levels of pseudoviruses were monitored by measuring Gluc activity. The results showed that in both cell lines, the $SP\Delta C_{Delta}$-PVPs exhibited the highest infection efficiency, the $SP\Delta C_{G614}$-PVPs had a slightly lower infection efficiency than $SP\Delta C_{Delta}$, while the $SP\Delta C_{wt}$-PVPs showed a significantly lower infection efficiency (Fig. 2C). All of these results indicated that $SP\Delta C_{Delta}$-PVPs had a significantly more efficient virus entry step than $SP\Delta C_{wt.}$-PVPs in a single cycle replication system.

Next, we also checked the ability of SPΔC-PVPs to infect human differentiated macrophages and dendritic cells. Briefly, human monocyte-derived macrophages (MDMs) or dendritic cells (MDDCs) were infected with equal amounts (adjusted with HIV p24 levels) of $SP\Delta C_{wt}$-, $SP\Delta C_{G614}$-, and $SP\Delta C_{Delta}$-PVPs. At 48 and 72 hrs p.i., the Gluc activity in the supernatant from the infected cell cultures was monitored. The results showed that both human primary cells, especially MDMs, could be infected by SPΔC-PVPs, while $SP\Delta C_{Delta}$- and $SP\Delta C_{G614}$-PVPs displayed more efficient infection than $SP\Delta C_{wt}$-PVPs (Fig. 2D). All of these experimental observations indicate that $SP\Delta C_{Delta}$-PVPs have a stronger ability to target MDMs than $SP\Delta C_{wt}$-PVPs. The results also suggested that MDDCs can be targeted by SPΔC-PVPs but with less efficiency.

Delta-SP variant enhanced syncytia formation in lung epithelial A549 cells expressing ACE2

Previous studies have shown that SARS-CoV-2 SP is able to possess fusogenic activity and form large multinucleated cells (syncytia formation) (Bussania et al., 2020; Cattin-Ortola´ et al., 2020). We then asked whether Delta-SP could possess higher fusogenic activity than other variants. Briefly, 293T cells were transfected with $SP\Delta C_{WT}$, $SP\Delta C_{G614}$, $SP\Delta C_{Delta,}$ or $SP\Delta C_{DeltaPD}$ plasmids by Lipofectamine 2000. At 24 hrs of transfection, we mixed SPΔC-expressing 293T cells with $A549_{ACE2}$ cells at a ratio of 1:3. At 6 and 30 hrs post transfection, syncytial formation was observed under a microscope, and the results revealed that $SP\Delta C_{WT}$ and $SP\Delta C_{G614}$ induced similar levels and sizes of syncytia. Intriguingly, an increasing number of syncytia formations were observed in the coculture of $SP\Delta C_{Delta}$-expressing 293T and $A549_{ACE2}$ cells (Fig. 3A and B), indicating that SP from the Delta variant has a stronger fusogenic ability. However,

198 SPΔC$_{DeltaPD}$-expressing 293T/A549$_{ACE2}$ cell coculture displayed less syncytia formation than
199 SPΔC$_{Delta}$ (Fig. 3 B). A and B), suggesting the importance of P681R for the strong fusogenic
200 activity of SP from the Delta variant.

201 To further confirm the strong fusogenic ability of SPΔC$_{Delta}$, we also generated A549
202 cells stably expressing SPΔC$_{wt}$, SPΔC$_{G614}$ or SPΔC$_{Delta}$ (named A549-SPΔC$_{Delta}$, A549-SPΔC$_{G614}$
203 or A549-SPΔC$_{wt}$ cells) (Fig. 3C). Since A549-SPΔC$_{wt}$ and A549-SPΔC$_{Delta}$ cells displayed
204 similar levels of SPΔC expression based on a WB analysis, we then tested their fusogenic ability
205 by mixing A549-SPΔC$_{Delta}$ or A549-SPΔC$_{wt}$ cells with the A549$_{ACE2}$ cell line using a similar
206 experimental process as described above. Meanwhile, A549-SPΔC$_{Delta}$ or A549-SPΔC$_{wt}$ cells
207 were cocultured with A549 cells as a control. The results confirmed that the coculture of A549-
208 SPΔC$_{Delta}$ cells and A549$_{ACE2}$ cells formed large syncytia formation more efficiently than that of
209 A549-SPΔC$_{wt}$ cells (Fig. 3D), confirming the stronger fusogenic activity of the SP of the Delta
210 variant.

211

212 **Delta variant SP stimulates higher NFκB and AP1 signaling pathway activities**

213 The severity of COVID-19 is highly correlated with dysregulated and excessive release of
214 proinflammatory cytokines (Huang et al., 2020a). Given that the NFκB and AP1 signaling
215 pathways are among the critical pathways responsible for the expression of proinflammatory
216 cytokines and chemokines (Hojyo et al., 2020; Kawasaki and Kawai, 2014), we therefore
217 examined the activities of these two signaling pathways triggered by SPΔC in the monocyte cell
218 line THP1 and THP1-derived macrophages. First, we generated THP1-NF-κB-Luc and THP1-
219 AP-1-Luc sensor cell lines by transducing THP1 cells with a lentiviral vector encoding the
220 luciferase reporter gene driven by NFκB- or AP1-activated transcription response elements (Fig.

4A), as described in the Materials and Methods. To obtain THP1-derived macrophages, THP-1-NF-κB-Luc and THP1-AP-1-Luc sensor cell lines were treated with phorbol 12-myristate 13-acetate (PMA) (100 nM) for 3 days. Additionally, we produced genome-free SPΔC-PVLPs by cotransfecting each SPΔC-expressing plasmid with a packaging plasmid (pCMVΔR8.2) in 293T cells, and the expression of SPΔC in the purified PVLPs was verified by WB with an anti-RBD antibody (Fig. 4B). Then, different THP1 sensor cell lines and THP1-derived macrophages were treated with different SPΔC-PVLPs of same amount (adjusted by p24) for 6 hrs, and the luciferase activity in treated cells was measured by a luciferase assay system (Promega). Interestingly, we found that the NFκB activity induced by $SP\Delta C_{wt}$ and $SP\Delta C_{G614}$-PVLPs was slightly higher than that induced by the VLP control (Gag). However, the $SP\Delta C_{Delta}$-treated THP1 cells/macrophages produced significantly higher (3~7-fold) NFκB activity compared with $SP\Delta C_{wt}$ or $SP\Delta C_{G614}$ (Fig. 4C). Consistent with this finding, $SP\Delta C_{Delta}$ also triggered higher (~2-fold) AP1 signaling pathway activities in THP1/macrophages than $SP\Delta C_{wt}$ or $SP\Delta C_{G614}$ (Fig. 4D). These results indicated that $SP\Delta C_{Delta}$ triggered significantly stronger signals to activate the NFκB and AP1 pathways in the monocyte cell line THP1 and THP1-derived macrophages.

Delta variant SP stimulates higher proinflammatory cytokine production in human macrophages (MDMs) and dendritic cells (MDDCs)

Previous studies have shown that SARS-CoV-2 infection can stimulate the production of immunoregulatory cytokines (IL-6, IL-10) in human monocytes and macrophages (Boumaza et al., 2021). We further investigated whether SP of the Delta variant can induce higher levels of proinflammatory cytokine and chemokine in MDMs and MDDCs. Briefly, human MDMs and MDDCs were treated with the same amount (adjusted by p24) of SPΔC-PVLPs, including

SPΔC$_{wt}$-, SPΔC$_{G614}$-, SPΔC$_{Delta}$-PVLPs or control VLPs (Gag-VLPs). After 24 hrs of incubation, the cytokines released in the supernatants were determined by a MSD (Meso Scale Discovery) immunoassay. The results revealed that SPΔC$_{wt}$-PVLP stimulation did not result in a significant change in cytokine release from MDMs compared to the control VLPs (Fig. 5A). However, in MDMs, SPΔC$_{Delta}$-PVLPs induced significantly higher levels of several proinflammatory cytokines, such as IFN-γ, TNF-α, IL-1β, and IL-6, while SPΔC$_{G614}$-PVLPs also induced increase of these cytokines but overall to a less extent, when compared with SPΔC$_{wt}$-PVLPs (Fig. 5A). For example, the SPΔC$_{Delta}$-PVLPs elevated TNF-α level 61-fold in comparison with SPΔC$_{wt}$-PVLPs, contrastingly, SPΔC$_{G614}$-PVLPs only increased to approximately 33-fold. Nevertheless, all of SPΔC-PVLPs showed no stimulating effects on IL-2 and IL-8 production. Interestingly, SPΔC$_{Delta}$-PVLPs and SPΔC$_{G614}$-PVLPs also slightly increased anti-inflammatory cytokines IL-4, IL-10 and IL-13, indicating the negative feedback of inflammation may exist during these stimulations.

Surprisingly, in MDDCs, SPΔC$_{Delta}$-PVLP stimulation resulted in a significant increase in most pro-inflammation cytokines we tested, including IFN-γ, TNF-α, IL-1β, IL-2, IL-6, IL-8, and IL-12p70 (Fig. 5B). Among them, IL-6, TNF-α and IL-2 were the most increased cytokines (8~13-fold), followed by IFN-γ and IL-1β (5~6-fold). The levels of IFN-γ , IL-2, and IL-6 in the supernatans of SPΔC$_{G614}$-PVLPs treated MDDCs were higher than that of SPΔC$_{WT}$-PVLPs or control VLPs, also to a less extent. In contrast, IL-10 production appears to be negatively regulated by all SPΔC-PVLPs, including the control VLPs. Altogether, the above results suggested that SPΔC$_{Delta}$ could induce remarkably higher levels of most proinflammatory cytokines tested and small differentce in some anti-inflammatory cytokines than VLP-SPΔC$_{G614}$ or SPΔC$_{wt}$ in human MDMs and MDDCs.

DISCUSSION

The SARS-CoV-2 Delta variant has higher transmissibility and thus has become the predominant strain worldwide in 2021(Li et al., 2021). It is important to understand the mechanisms of the increased transmissibility and cytokine release triggered by this variant. In this study, we investigated the cleavage and maturation efficiency of the Delta variant Spike protein ($SP\Delta C_{Delta}$) during pseudovirus assembly and its impact on cell-free pseudovirus infection and cell-cell fusion activities. The results demonstrated enhanced cleavage and maturation of $SP\Delta C_{Delta}$ in the produced viral particles. Additionally, the studies clearly showed that $SP\Delta C_{Delta}$ mediated more efficient pseudovirus infection and mediated a significantly enhanced cell-cell fusion process. Furthermore, our analyses revealed that SP_{Delta}-PVLPs had stronger effects on stimulating NF-κB and AP-1 signaling in THP1 cells and elevated the production of several essential proinflammatory cytokines by human MDMs and MDDCs, compared with SP_{WT}-PVLPs.

SARS-CoV-2 transmission and pathogenesis require the polybasic cleavage site between S1 and S2 in its SP (Johnson et al., 2021; Peacock et al., 2021a). This furin protease cleavable site is critical for the maturation of SARS-CoV-2 and its biological functions (Boson et al., 2021; Hoffmann et al., 2018; Hoffmann et al., 2020a). In an attempt to understand the mechanisms by which the Delta variant is more infectious than other variants, our study revealed that $SP\Delta C_{Delta}$ was significantly more efficient in the processing of the precursor S into S1 and S2 compared with $SP\Delta C_{WT}$ and $SP\Delta C_{G614}$ in both the cells and PVPs. In the PVPs, the majority of $SP\Delta C_{Delta}$ was presented as mature forms (S1 and S2), while some portions of the precursor S of $SP\Delta C_{WT}$ and $SP\Delta C_{G614}$ were still present in the PVPs (Fig. 1C, right panel). To further investigate the importance of the furin cleavage site for the enhanced cleavage of the Delta variant, we produced $SP\Delta C_{Delta}$-PVPs packaged in the presence or absence of the furin protease inhibitor CMK. As

expected, CMK significantly inhibited the cleavage of the spike protein of the Delta variant.

Among the mutations in the Delta variant spike protein, an amino acid proline (P681) at the N-terminus of the polybasic cleavage site (RRAR) was changed to arginine (R), known as the P681R mutation (Saito et al., 2021). The P681R mutation is of great importance because it is part of a proteolytic cleavage site for furin and furin-like proteases. The P618R mutation clearly plays a critical role in the SP of the Delta variant to abrogate host *O*-glycosylation (Zhang et al., 2021b). To further investigate whether this altered polybasic cleavage site (RRRAR) is required for effective furin cleavage of SARS-CoV-2 SP, we reverted the arginine (R) of 681 and asparagine (N) of 950 back to the original proline (P) and aspartic acid (D) on SP of the Delta variant ($SP\Delta C_{Delta\text{-}PD}$). The WB results showed that the cleavage of the $SP\Delta C_{Delta\text{-}PD}$-PVPs was comparable to that of $SP\Delta C_{WT}$-, $SP\Delta C_{G614}$- or $SP\Delta C_{Delta}$-PVPs produced in the presence of CMK. Consistent with other reports (Peacock et al., 2021b; Saito et al., 2021), our results further demonstrated that the P618R mutation in $SP\Delta C_{Delta}$ is essential for the enhanced furin cleavage of Delta variant SP. Meanwhile, we also observed that S1 of $SP\Delta C_{Delta}$ appeared to migrate faster than S1 of $SP\Delta C_{WT}$ and $SP\Delta C_{G614}$. Although the mechanism is currently unknown, it could be related to the possible altered glycosylation content of $SP\Delta C_{Delta}$ because a previous study revealed that P681R could circumvent host *O*-glycosylation (Zhang et al., 2021b). In addition, multiple amino acid mutations and deletions present in S1 of Delta SP may also partially contribute to this alteration. More detailed studies are required to analyze the possible underlying mechanism(s).

In this study, we also observed that $SP\Delta C_{Delta}$ enhanced cell-free pseudovirus infection in $A549_{ACE2+}$ and Calu-3 cell lines and macrophages, indicating that $SP\Delta C_{Delta}$ is an important factor contributing to the increased infectiousness of the Delta variant. Additionally, it was

observed that the infection mediated by SPΔC$_{Delta}$-pseudovirus was only slightly higher than that mediated by SPΔC$_{G614}$-pseudovirus, suggesting that the G614 mutation present in SP$_{Delta}$ may be one of the main driving forces for the increased infectivity of the Delta virus (Daniloski et al., 2020; Zhang et al., 2020). This finding is in agreement with previous studies showing that the D614G mutation in SARS-CoV-2 SP contributes immensely to virus infectivity and replication (Daniloski et al., 2020; Zhang et al., 2020). It should be noted that our results were based on single-cycle SPΔC-pseudovirus replication; thus, the infection advantage of the native SARS-CoV-2 Delta variant needs further investigation.

It is well known that spike protein expressed at the surface of infected cells is sufficient to generate fusion with neighboring cells. Here, we further showed that significantly enhanced syncytia formation was observed when SPΔC$_{Delta}$-expressing 293T or A549 cells were cocultured with A549$_{ACE2}$ cells. This observation raised potential importance in terms of the SARS-CoV-2 Delta variant's virulence since cell-to-cell fusion may provide another efficient method of viral dispersal in the host, thus indicating its stronger transmission among the population, as described previously (Michael Rajah et al., 2021). This finding is in agreement with recent reports that B.1.617.2 SP mediates highly efficient syncytia formation compared with wild-type SP (Michael Rajah et al., 2021; Mlcochova et al., 2021; Planas et al., 2021; Zhang et al., 2021a). Moreover, this efficient cell-to-cell transmission ability of SPΔC$_{Delta}$ may enhance its resistance to host immune responses, such as antibody-mediated neutralization (Mlcochova et al., 2021; Planas et al., 2021). It is worth noting that we did not investigate the impact of TMPRSS2 on the cell-cell fusion process. For SARS-CoV-2, cleavage of S by furin at the S1/S2 site is required for subsequent cleavage by TMPRSS2 at the S2' site. Previous studies have demonstrated that TMPRSS2 could enhance the infectivity and fusogenic activity of different coronaviruses,

including SARS-CoV-2 (Buchrieser et al., 2020; Glowacka et al., 2011; Kleine-Weber et al., 2018; Matsuyama et al., 2010). Future investigations into the role of TMPRSS2 in SP Delta-induced syncytia formation and infection will provide a better understanding of the persistence, dissemination, and immune or inflammatory responses of Delta variants.

The severity of COVID-19 is highly correlated with dysregulated and excessive release of proinflammatory cytokines (Huang et al., 2020a). Hence, we also tested whether macrophages or dendritic cells act as major modulators of the immune response by producing a large amount of cytokines and chemokines to recruit immune cells and presenting antigens to them. The engagement of the spike protein of SARS-CoV-2 with the receptor ACE2 on THP1-derived macrophages is reported to initiate signaling pathways and activate the production of proinflammatory cytokines, including IL-6, TNF-α, and MIP1a (Pantazi et al., 2021). Here, we showed that NFκB and AP1 signaling pathway activities were also enhanced by $SP\Delta C_{Delta}$ compared with $SP\Delta C_{WT}$ in THP1 cells and THP1-derived macrophages, suggesting that $SP\Delta C_{Delta}$ might promote the inflammatory status of these cells. Similarly, the SP of SARS-CoV-1 has been discovered to activate NF-κB and stimulate the release of IL-6 and TNF-α (Wang et al., 2007).

A previous study reported that high plasma levels of TNFα, IL-1, IL-6, IL-8 and other inflammatory mediators were found in severe COVID-19 patients, and the serum IL-6, IL-8 and TNF-α levels were strong and independent predictors of disease progression, severity and death. (Del Valle et al., 2020; Huang et al., 2020a; Santa Cruz et al., 2021). Interestingly, we found that $SP\Delta C_{Delta}$ significantly enhanced the expression of several proinflammatory cytokines (TNF-α, IL-1β, and IL-6) in both MDMs and MDDCs (Fig. 5). Especially for MDDCs, increased levels of other proinflammatory cytokines, including IFN-γ , IL-2, IL-8, and IL-12p70 were also

359 detected (Fig. 5). However, SPΔC_{wt} only exhibited induction of IFN-γ in MDDCs, but did not
360 show any effect on other cytokine production of MDMs or MDDCs. This is agree with previous
361 study that revealed, upon SARS-CoV-2 infection, neither macrophage, nor dendritic cells
362 produce the pro-inflammatory cytokines (Niles et al., 2021). Mutations in Delta SP seem to be
363 the key points that cause the differential expression of cytokines. Given the fact that IFN-γ,
364 TNF-α, IL-1β and IL-12 are T-helper-1 (Th1) cytokines, it also suggests that the Th1/Th2
365 balance has further shifted to Th1 dominance after stimulation with SPΔC_{Delta}-PVLP. Along with
366 proinflammatory cytokines, three anti-inflammatory cytokines (IL-4, IL-10 and IL-13) were also
367 increased in SPΔC_{Delta}-PVLP treated macrophages compared with SPΔC_{WT}-PVLP-treated
368 macrophages. Consistently, higher secretion of T-helper-2 (Th2) cytokines such as IL-4 and IL-
369 10 has been reported in ICU patients than in non-ICU patients (Huang et al., 2020a). Their
370 functions are to suppress both inflammation and the TH1 cellular response, indicating that the
371 balances between pro- and anti-inflammation, as well as the balances between TH1 and TH2
372 cellular responses existing in patients, are important for the clinical outcomes of COVID-19
373 therapy. However, the lower IL-10 level in all PVLPs treated MDDCs is surprising and the
374 reason of this is unclear. In conclusion, SPΔC_{Delta}-treated macrophages and DCs are in a higher
375 inflammatory state and in a Th1-dominant Th1/Th2 balance.

376 Overall, we demonstrated that the SARS-CoV-2 Delta variant spike protein exhibited
377 enhanced cleavage and maturation, which may play an important role in viral infection and cell-
378 cell transmission. Furthermore, we revealed that SP_{Delta} had stronger effects on stimulating NF-
379 κB and AP-1 signaling in monocytes and the release of proinflammatory cytokines from human
380 macrophages and dendritic cells. All of these studies provide strong evidence to support the
381 important role of Delta SP during virus infection, transmission and pathogenesis.

MATERIALS AND METHODS

Plasmid constructs

The SARS-CoV-2 SP protein-expressing plasmids (pCAGGS-nCoVSPΔC and pCAGGS-nCoVSPΔC$_{G614}$) were described previously (Ao et al., 2021a). The gene encoding SPΔC$_{Delta}$ or SPΔC$_{Delta-PD}$ was synthesized (Genescript) and cloned into the pCAGGS plasmid, and each mutation was confirmed by sequencing. pEF1-SPΔCwt, pEF1-SPΔC$_{G614}$ or pEF1-SPΔC$_{Delta}$ was constructed by inserting the cDNA encoding SPΔCwt, SPΔC$_{G614}$ or SPΔC$_{Delta}$ through the *BamHI* and *NheI* sites into the pEF1-pcs-puro vector (Ao et al., 2008). The HIV RT/IN/Env trideleted proviral plasmid containing the Gaussia luciferase gene (ΔRI/E/Gluc) and the helper packaging plasmid pCMVΔ8.2 encoding the HIV Gag-Pol plasmids have been described previously (Ao et al., 2016; Zhang et al., 2016).

Cell culture, antibodies and chemicals

Human embryonic kidney cells (HEK293T), human lung (carcinoma) cells (A549), A549$_{ACE2}$, Calu-3 cells and THP1- sensor cells were cultured in Dulbecco's modified Eagle's medium or RPMI 1640 medium supplemented with 10% fetal bovine serum (F.B.S.) and 1% penicillin/streptomycin. To obtain human MDMs or MDDCs, human peripheral blood mononuclear cells (hPBMCs) from healthy donors were collected by sedimentation on a Ficoll (Lymphoprep; Axis-Shield) gradient, adherent to 24-well plates for 2 hrs, and then treated with macrophage colony stimulator (M-CSF) or granulocyte-macrophage-stimulating factor (GM-CSF) and IL-4 (R&D system) for 7 days.

The THP1-NF-κB-Luc and THP1-AP-1-Luc sensor cell lines were described previously (Ao et al., 2021b). To obtain THP1-derived macrophages, THP-1-NF-κB-Luc and THP1-AP-1-Luc

406 sensor cell lines were treated with phorbol 12-myristate 13-acetate (PMA) (200 ng/mL) for 3
407 days followed by 2 days of rest, as previously described (Starr et al., 2018). A549-expressing
408 human ACE2 ($A549_{ACE2}$) cells were generated by transducing A549 cells with the ACE2-
409 expressing lentiviral vector (pLenti-C-mGFP-ACE2) (Origene, Cat# PS100093) and then
410 selected with puromycin according to the manufacturer's procedure.
411 The rabbit polyclonal antibody against SARS-CoV-2 SP/RBD (Cat# 40592-T62) or human
412 SARS-CoV-2 S-NTD antibody (E-AB-V1030) was obtained from Sino Biological or
413 Elabscience. Mouse monoclonal antibody (1A9) against SARS-CoV-2 SP-S2 (Cat# ab273433)
414 was obtained from Abcam. Anti-HIVp24 monoclonal antibody was described previously (Ao et
415 al., 2007; Qiu et al., 2011). Anti-human ACE2 antibody (sc-390851) was obtained from Santa
416 Cruz Biotechnology Inc. Furin inhibitor I, a peptidyl chloromethylketone (CMK) (Cat# 344930),
417 was obtained from Millipore Sigma.
418
419 **Virus production and infection experiments**
420 SARS-CoV-2 SPΔC pseudotyped viruses (CoV-2-SPΔC-PVs, CoV-2-$SP\Delta C_{G614}$-PVs and CoV-
421 2-$SP\Delta C_{Delta}$-PVs) or pseudotyped virus-like particles (VLPs) were produced by transfecting
422 HEK293T cells with pCAGGS-$SP\Delta C_{WT}$, pCAGGS-$SP\Delta C_{G614}$ or pCAGGS-$SP\Delta C_{Delta}$ and
423 pCMVΔ8.2 with or without a Gluc-expressing HIV vector ΔRI/E/Gluc (Ao et al., 2021a). After
424 48 hrs of transfection, cell culture supernatants were collected, and VPs or VLPs were purified
425 from the supernatant by ultracentrifugation (32,000 rpm) for 2 hrs. The pelleted VPs or VLPs
426 were resuspended in RPMI medium, and virus titers were quantified by HIV-1 p24 amounts
427 using an HIV-1 p24 ELISA.

To measure the infection ability of SARS-CoV-2 SPΔC pseudotyped VPs, equal amounts of each SPΔC-PVs stock (as adjusted by p24 levels) were used to infect $A549_{ACE2}$, Calu-3 cells, human MDMs or MDDCs. After different time intervals (24, 48 and 72 hrs), the supernatants were collected, and the viral infection levels were monitored by measuring Gaussia luciferase (Gluc) activity. Briefly, 50 µl of coelenterazine substrate (Nanolight Technology) was added to 10 µl of supernatant, mixed well and read in a luminometer (Promega, U.S.A.).

To evaluate the effects of various SCoV-2 SPΔC-VLPs on the NF-κB and AP-1 signaling pathways, the same amount of each SPΔC-pseudotyped VLP stock (10 ng, as adjusted by the p24 levels) was directly added to THP1-NF-κB-Luc or THP1-AP1-Luc sensor cells. After 6 hrs, the cells were collected and subjected to luciferase assay as described previously (Ao et al., 2021b).

To test the effect of different SPΔC-VPs on cytokine production in MDMs and MDDCs, the same amount of each SPΔC-VP stock (20 ng, as adjusted by the p24 levels) was added to MDMs and MDDCs, and the supernatants were collected after 24 hrs. The cytokine (IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-α) levels in the supernatants were measured using the MSD V-PLEX proinflammatory Panel 1 (human) Kit (Mesoscale Discovery, USA, Cat# K15049D-1) following the manufacturer's procedure.

Generation of different SPΔC-expressing A549 stable cell lines

Production of lentiviral vectors expressing different SPΔC: 293T cells were cotransfected with pEF1-$SP\Delta C_{wt}$, pEF1-$SP\Delta C_{G614}$ or pEF1-$SP\Delta C_{Delta}$ with packaging plasmid Δ8.2 and VSV-G expressing plasmid. Forty-eight hours posttransfection, each lentiviral vector particle in the supernatant was collected. Then, each produced lentiviral particle was used to transduce A549 cells, and the transduced cells were selected with puromycin for one week. SPΔCwt/mutant

451 expression in the different transduced A549 cells was evaluated by WB using an anti-RBD
452 antibody.
453
454 **Immunofluorescence assay**
455 293T cells transfected with various SARS-CoV-2 SPΔC-expressing plasmids were grown on
456 glass coverslips (12 mm^2) in a 24-well plate. After 48 hrs, cells on the coverslip were fixed in 4%
457 paraformaldehyde for 5 minutes and permeabilized with 0.2% Triton X-100 in PBS. The cells
458 were then incubated with primary antibodies against the N-terminal domain of SARS-CoV-2 SP
459 followed by the corresponding FITC-conjugated secondary antibodies. The cells were viewed
460 under a computerized Axiovert 200 fluorescence microscope (ZEISS).).
461
462 **Syncytium formation assay**
463 293T cells were transfected with pCAGGS-SPΔC_{WT}, SPΔC_{G614}, SPΔC_{Delta} or SPΔ$C_{DeltaPD}$
464 plasmids using Lipofectamine 2000. After 24 hrs, the cells were washed, resuspended and mixed
465 with $A549_{ACE2}$ cells at a 1:3 ratio and plated into 48-well plates. For syncytium formation of the
466 stable cell line, A549-SPΔC_{WT} or A549-SPΔC_{Delta} cells were detached with 0.05% trypsin and
467 mixed with A549 or $A549_{ACE2}$ cells. At different time points, syncytium formation was observed,
468 counted and imaged by bright-field microscopy (Axiovert 200, ZEISS).
469
470 **Statistics**
471 Statistical analysis of cytokine levels, including the results of GLuc assay, Luciferase assay, and
472 various cytokine/chemokines assay, were performed using the unpaired t-test (considered
473 significant at $P \geq 0.05$) by GraphPad Prism 9 software.

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AUTHOR CONTRIBUTIONS

Experimental design, X. Y, Z.A. and M.J.O; Investigation, Z.A., M.J.O., and O.T.A; Writing-Original Draft Preparation, Z.A. and M. J. O. and O.T.A; Review, Z.A. M. J. O. and X.Y. Supervision, X.Y.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Ao, Z., Huang, G., Yao, H., Xu, Z., Labine, M., Cochrane, A.W., and Yao, X. (2007). Interaction of human immunodeficiency virus type 1 integrase with cellular nuclear import receptor importin 7 and its impact on viral replication. Journal of Biological Chemistry *282*, 13456-13467.

Ao, Z., Huang, J., Tan, X., Wang, X., Tian, T., Zhang, X., Ouyang, Q., and Yao, X. (2016). Characterization of the single cycle replication of HIV-1 expressing Gaussia luciferase in human PBMCs, macrophages, and in CD4(+) T cell-grafted nude mouse. Journal of virological methods *228*, 95-102.

Ao, Z., Yu, Z., Wang, L., Zheng, Y., and Yao, X. (2008). Vpr14-88-Apobec3G fusion protein is efficiently incorporated into Vif-positive HIV-1 particles and inhibits viral infection. PloS one *3*, e1995.

Ao, Z.-j., Chan, M., Ouyang, M., Abiola, T., Mahmoudi, M., Kobasa, D., and Yao, X.-J. (2021a). Identification and evaluation of the inhibitory effect of Prunella vulgaris extract on SARS-coronavirus 2 virus entry. PloS one, https://doi.org/10.1371/journal.pone.0251649

Ao, Z.-j., Wang, L.-j., Azizi, H., Olukitibi, T., Mahmoudi, M., Kobinger, G., and Yao , X.-j. (2021b). Development and Evaluation of an Ebola Virus Glycoprotein Mucin-Like Domain Replacement System as a New Dendritic Cell-Targeting Vaccine Approach against HIV-1. Journal of virology *95*, e0236820. PMID: 34011553.

Banu, S., Jolly, B., Mukherjee, P., Singh, P., Khan, S., Zaveri, L., Shambhavi, S., Gaur, N., Reddy, S., and Kaveri, K. (2020). A Distinct Phylogenetic Cluster of Indian Severe Acute Respiratory Syndrome Coronavirus 2 Isolates. Paper presented at: Open forum infectious diseases (Oxford University Press US).

Bertram, S., Dijkman, R., Habjan, M., Heurich, A., Gierer, S., Glowacka, I., Welsch, K., Winkler, M., Schneider, H., and Hofmann-Winkler, H. (2013). TMPRSS2 activates the human coronavirus 229E for cathepsin-independent host cell entry and is expressed in viral target cells in the respiratory epithelium. Journal of virology *87*, 6150-6160.

Boson, B., Legros, V., Zhou, B., Siret, E., Mathieu, C., Cosset, F.-L., Lavillette, D., and Denolly, S. (2021). The SARS-CoV-2 envelope and membrane proteins modulate maturation and retention of the spike protein, allowing assembly of virus-like particles. Journal of Biological Chemistry *296*.

Boumaza, A., Gay, L., Mezouar, S., Bestion, E., Diallo, A.B., and Michel, M.e.a. (2021). Monocytes and macrophages, targets of SARS-CoV-2: the clue for Covid-19 immunoparalysis. J Infect Dis *doi: 10.1093/infdis/jiab044*.

Buchrieser, J., Dufloo, J., Hubert, M., Monel, B., Planas, D., Rajah, M.M., and al, e. (2020). Syncytia formation by SARS-CoV-2-infected cells. The EMBO journal *39. e106267https://doi.org/10.15252/embj.2020106267*.

Bussania, R., Schneider, E., Zentilinb, L., Collesi, C., Ali, H., and al., e. (2020). Persistence of viral RNA, pneumocyte syncytia and thrombosis are hallmarks of advanced COVID-19 pathology. EBioMedicine *61*, 103104.

Cattin-Ortolá, J., Welch, L., Maslen, S.L., Skehel, J.M., Papa, G., James, L.C., and Munro, S. (2020). Sequences in the cytoplasmic tail of SARS-CoV-2 Spike facilitate expression at the cell surface and syncytia formation. bioRxiv *https://doi.org/10.1101/2020.10.12.335562.*

CDC (2021). SARS-CoV-2 Variant Classifications and Definitions. Centers for Disease Control and Prevention *https://*http://www.cdc.gov/coronavirus/2019-ncov/variants/variant-info.html?CDC_AA_refVal=https%3A%2F%2Fwww.cdc.gov%2Fcoronavirus%2F2019-ncov%2Fcases-updates%2Fvariant-surveillance%2Fvariant-info.html.

Cherian, S., Potdar, V., Jadhav, S., Yadav, P., Gupta, N., Das, M., Rakshit, P., Singh, S., Abraham, P., and Panda, S. (2021). Convergent evolution of SARS-CoV-2 spike mutations, L452R, E484Q and P681R, in the second wave of COVID-19 in Maharashtra, India. bioRxiv.

538 Coutard, B., Valle, C., de Lamballerie, X., Canard, B., Seidah, N.G., and Decroly, E. (2020). The spike
539 glycoprotein of the new coronavirus 2019-nCoV contains a furin-like cleavage site absent in CoV of the
540 same clade. Antiviral research *176*, 104742.
541 Daniloski, Z., Jordan, T.X., Ilmain, J.K., Guo, X., Bhabha, G., and Sanjana, N.E. (2020). The Spike D614G
542 mutation increases SARS-CoV-2 infection of multiple human cell types. Elife *10*, e65365.
543 Del Valle, D.M., Kim-Schulze, S., Huang, H.-H., and Beckmann, N.D.e.a. (2020). An inflammatory cytokine
544 signature predicts COVID-19 severity and survival. nature medicine *26*, 1636–1643.
545 Galloway, S.E., Paul, P., MacCannell, D.R., Johansson, M.A., Brooks, J.T., MacNeil, A., Slayton, R.B., Tong,
546 S., Silk, B.J., and Armstrong, G.L. (2021). Emergence of SARS-CoV-2 b. 1.1. 7 lineageâ€"united states,
547 december 29, 2020â€"january 12, 2021. Morbidity and Mortality Weekly Report *70*, 95.
548 Glowacka, I., Bertram, S., Müller, M.A., Allen, P., and Soilleux, E.e.a. (2011). Evidence that TMPRSS2
549 activates the severe acute respiratory syndrome coronavirus spike protein for membrane fusion and
550 reduces viral control by the humoral immune response. Journal of virology *85*, 4122-4134.
551 Greaney, A.J., Starr, T.N., Gilchuk, P., Zost, S.J., Binshtein, E., Loes, A.N., Hilton, S.K., Huddleston, J.,
552 Eguia, R., and Crawford, K.H.D. (2021). Complete mapping of mutations to the SARS-CoV-2 spike
553 receptor-binding domain that escape antibody recognition. Cell host & microbe *29*, 44-57. e49.
554 Hadjadj, J.r.m., Yatim, N., Barnabei, L., Corneau, A.l., Boussier, J., Smith, N.a., PÃra, H., Charbit, B.,
555 Bondet, V., and Chenevier-Gobeaux, C. (2020). Impaired type I interferon activity and inflammatory
556 responses in severe COVID-19 patients. Science *369*, 718-724.
557 Hoffmann, M., Hofmann-Winkler, H., and Pahlmann, S. (2018). Priming time: how cellular proteases arm
558 coronavirus spike proteins. In Activation of Viruses by Host Proteases (Springer), pp. 71-98.
559 Hoffmann, M., Kleine-Weber, H., and Pahlmann, S. (2020a). A multibasic cleavage site in the spike
560 protein of SARS-CoV-2 is essential for infection of human lung cells. Molecular cell *78*, 779-784. e775.
561 Hoffmann, M., Kleine-Weber, H., Schroeder, S., KrÃ¼ger, N., Herrler, T., Erichsen, S., Schiergens, T.S.,
562 Herrler, G., Wu, N.-H., and Nitsche, A. (2020b). SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2
563 and is blocked by a clinically proven protease inhibitor. cell *181*, 271-280. e278.
564 Hojyo, S., Uchida, M., Tanaka, K., Hasebe, R., Tanaka, Y., Murakami, M., Hirano, T., and (2020). How
565 COVID-19 induces cytokine storm with high mortality. Inflammation and Regeneration *40*, 37.
566 Huang, C.L., Wang, Y.M., Li, X.W., Ren, L., Zhao, J.P., Yi Hu, Y., and al., e. (2020a). Clinical features of
567 patients infected with 2019 novel coronavirus in Wuhan, China. THE LANCET *395*, 497-506.
568 Huang, Y., Yang, C., Xu, X.-f., Xu, W., and Liu, S.-w. (2020b). Structural and functional properties of SARS-
569 CoV-2 spike protein: potential antivirus drug development for COVID-19. Acta Pharmacologica Sinica *41*,
570 1141-1149.
571 Johnson, B.A., Xie, X., Kalveram, B., Lokugamage, K.G., Muruato, A., Zou, J., Zhang, X., Juelich, T., Smith,
572 J.K., and Zhang, L. (2020). Furin cleavage site is key to SARS-CoV-2 pathogenesis. bioRxiv.
573 Johnson, B.A., Xuping Xie, X.p., Kalveram, B., and Lokugamage, K.G., et al. (2021). Loss of furin cleavage
574 site attenuates SARS-CoV-2 pathogenesis. . Nature *591*, 293-299.
575 Kawasaki, T., and Kawai, T. (2014). Toll-like receptor signaling pathways. Front Immunol
576 *https://doi.org/10.3389/fimmu.2014.00461*.
577 Kleine-Weber, H., Elzayat, M.T., Hoffmann, M., and Pöhlmann, S. (2018). Functional analysis of potential
578 cleavage sites in the MERS-coronavirus spike protein. Scientific reports *8*, 16597
579 Li, B., Deng, A., Li, K., Hu, Y., Li, Z., Xiong, Q., Liu, Z., Guo, Q., Zou, L., and Zhang, H. (2021). Viral infection
580 and transmission in a large well-traced outbreak caused by the Delta SARS-CoV-2 variant. MedRxiv.
581 https://doi.org/10.1101/2021.07.07.21260122
582 Liu, Y., Liu, J.Y., Johnson, B.A., Xia, H.J., Ku, Z.Q., and al., e. (2021). Delta spike P681R mutation enhances
583 SARS-CoV-2 fitness over Alpha variant. BioRxiv *doi: 10.1101/2021.08.12.456173.* .

584 Matsuyama, S., Nagata, N., Shirato, K., Kawase, M., Takeda, M., and Taguchi, F. (2010). Efficient
585 activation of the severe acute respiratory syndrome coronavirus spike protein by the transmembrane
586 protease TMPRSS2. Journal of virology *84*, 12658-12664.
587 McCallum, M., Walls, A.C., Sprouse, K.R., Bowen, J.E., Rosen, L.E., Dang, H.V., Marco, A.D., Franko, N.,
588 Tilles, S.W., Logue, J.*, et al.* Molecular basis of immune evasion by the Delta and Kappa SARS-CoV-2
589 variants. Science *0*, eabl8506.
590 Mehta, P., McAuley, D.F., Brown, M., Sanchez, E., Tattersall, R.S., and Manson, J.J. (2020). COVID-19:
591 consider cytokine storm syndromes and immunosuppression. The Lancet *395*, 1033-1034.
592 Michael Rajah, M., Hubert, M., Bishop, E., Saunders, N., Robinot, R., Grzelak, L., Planas, D., Dufloo, J.,
593 Gellenoncourt, S., and Bongers, A. (2021). SARS-CoV2 Alpha, Beta and Delta variants display enhanced
594 Spike-mediated Syncytia Formation. The EMBO Journal, e108944.
595 Mlcochova, P., Kemp, S.A., Dhar, M.S., Papa, G., Meng, B., Ferreira, I.A.T.M., Datir, R., Collier, D.A.,
596 Albecka, A., and Singh, S. (2021). SARS-CoV-2 B. 1.617. 2 Delta variant replication and immune evasion.
597 Nature, 1-6.
598 Neufeldt, C.J., Cerikan, B., Cortese, M., Frankish, J., Lee, J.-Y., Plociennikowska, A., Heigwer, F., Joecks, S.,
599 Burkart, S.S., Zander, D.Y.*, et al.* (2020a). SARS-CoV-2 infection induces a pro-inflammatory cytokine
600 response through cGAS-STING and NF-κB. bioRxiv, 2020.2007.2021.212639.
601 Neufeldt, C.J., Cerikan, B., Cortese, M., Frankish, J., and Lee, J.-Y.e.a. (2020b). SARS-CoV-2 infection
602 inducesa pro-inflammatory cytokine response through cGAS-STING and NF-kB bioRxiv
603 *https://doi.org/10.1101/2020.07.21.212639*.
604 Niles, M.A., Gogesch, P., Kronhart, S., Ortega Iannazzo, S., Kochs, G., Waibler, Z., and Anzaghe, M.
605 (2021). Macrophages and Dendritic Cells Are Not the Major Source of Pro-Inflammatory Cytokines Upon
606 SARS-CoV-2 Infection. Frontiers in Immunology *12*.
607 Nonaka, C.K.V., Franco, M.M., GrÃ¤f, T., Mendes, A.V.A., de Aguiar, R.S., Giovanetti, M., and de Freitas
608 Souza, B.S. (2021). Genomic Evidence of a Sars-Cov-2 Reinfection Case With E484K Spike Mutation in
609 Brazil.
610 Paiva, M.H.S., Guedes, D.R.D., Docena, C.s., Bezerra, M.F., Dezordi, F.Z., Machado, L.C., Krokovsky, L.,
611 Helvecio, E., da Silva, A.F., and Vasconcelos, L.R.S. (2021). Multiple introductions followed by ongoing
612 community spread of SARS-CoV-2 at one of the largest metropolitan areas of Northeast Brazil. Viruses
613 *12*, 1414.
614 Pantazi, I., Al-Qahtani, A.A., Alhamlan, F.S., H., A., Matou-Nasri, S., George Sourvinos, G., Eleni Vergadi,
615 E., and Christos Tsatsanis, C. (2021). SARS-CoV-2/ACE2 Interaction Suppresses IRAK-M Expression and
616 Promotes Pro-Inflammatory Cytokine Production in Macrophages. Front Immunol *12*.
617 Patra, T., Meyer, K., Geerling, L., Isbell, S., F., D., Hoft, D.F., Brien, J., Pinto, A.K., B., R., Ray, R.B.*, et al.*
618 (2020). SARS-CoV-2 spike protein promotes IL-6 trans-signaling by activation of angiotensin II receptor
619 signaling in epithelial cells. PLoS Pathogens, *https://doi.org/10.1371/journal.ppat.1009128*
620 Peacock, T.P., Goldhill, D.H., Zhou, J., Baillon, L., and al., e. (2021a). The furin cleavage site in the SARS-
621 CoV-2 spike protein is required for transmission in ferrets. . Nat Microbiol *6*, 899-909.
622 Peacock, T.P., Sheppard, C.M., Brown, J.C., Goonawardane, N., Zhou, J., Whiteley, M., de Silva, T.I.,
623 Barclay, W.S., and Consortium, P.H.E.V. (2021b). The SARS-CoV-2 variants associated with infections in
624 India, B. 1.617, show enhanced spike cleavage by furin. bioRxiv.
625 Planas, D., Veyer, D., Baidaliuk, A., Staropoli, I., Guivel-Benhassine, F., Rajah, M.M., Planchais, C., Porrot,
626 F.o., Robillard, N., and Puech, J. (2021). Reduced sensitivity of SARS-CoV-2 variant Delta to antibody
627 neutralization. Nature *596*, 276-280.
628 Qiu, X., Alimonti, J.B., Melito, P.L., Fernando, L., Ströher, U., and Jones, S.M. (2011). Characterization of
629 Zaire ebolavirus glycoprotein-specific monoclonal antibodies. Clinical immunology *141*, 218-227.
630 Ranjan, R., Sharma, A., and Verma, M.K. (2021). Characterization of the Second Wave of COVID-19 in
631 India. medRxiv.

Reardon, S. (2021). How the Delta variant achieves its ultrafast spread. Nature *21*.

Resende, P.C., Bezerra, J.o.F., Vasconcelos, R., Arantes, I., Appolinario, L., MendonÃ§a, A.C., Paixao, A.C., Rodrigues, A.C.D., Silva, T., and Rocha, A.S. (2021). Spike E484K mutation in the first SARS-CoV-2 reinfection case confirmed in Brazil, 2020. Virological [Internet] *10*.

Sahoo, J.P., Mishra, A.P., and Samal, K.C. (2021). Triple Mutant Bengal Strain (B. 1.618) of Coronavirus and the Worst COVID Outbreak in India. Biotica Research Today *3*, 261-265.

Saito, A., Irie, T., Suzuki, R., Maemura, T., Nasser, H., Uriu, K., Kosugi, Y., Shirakawa, K., Sadamasu, K., and Kimura, I. (2021). SARS-CoV-2 spike P681R mutation, a hallmark of the Delta variant, enhances viral fusogenicity and pathogenicity. bioRxiv.

Santa Cruz, A., Mendes-Frias, A., I., O.A., Dias, L., A.R., M., and Carvalho, A. (2021). Interleukin-6 Is a Biomarker for the Development of Fatal Severe Acute Respiratory Syndrome Coronavirus 2 Pneumonia. Front Immunol *https://doi.org/10.3389/fimmu.2021.613422*.

Santos, J.C., and Passos, G.A. (2021). The high infectivity of SARS-CoV-2 B. 1.1. 7 is associated with increased interaction force between Spike-ACE2 caused by the viral N501Y mutation. bioRxiv, 2020.2012. 2029.424708.

Srivastava, S., Banu, S., Singh, P., Sowpati, D.T., and Mishra, R.K. (2021). SARS-CoV-2 genomics: An Indian perspective on sequencing viral variants. Journal of Biosciences *46*, 1-14.

Starr, T., Bauler, T.J., Malik-Kale, T., and Steele-Mortimer, O. (2018). The phorbol 12-myristate-13-acetate differentiation protocol is critical to the interaction of THP-1 macrophages with Salmonella Typhimurium. PloS one *https://doi.org/10.1371/journal.pone.0193601*

Tegally, H., Wilkinson, E., Giovanetti, M., Iranzadeh, A., Fonseca, V., Giandhari, J., Doolabh, D., Pillay, S., San, E.J., and Msomi, N. (2020). Emergence and rapid spread of a new severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) lineage with multiple spike mutations in South Africa. medRxiv.

Twohig, K.A., Nyberg, T., Zaidi, A., Thelwall, S., Sinnathamby, M.A., Aliabadi, S., and al., e. (2021). Hospital admission and emergency care attendance risk for SARS-CoV-2 delta (B.1.617.2) compared with alpha (B.1.1.7) variants of concern: a cohort study. THE LANCET Infectious Diseases *DOI:https://doi.org/10.1016/S1473-3099(21)00475-8*.

Volz, E., Mishra, S., Chand, M., Barrett, J.C., Johnson, R., Geidelberg, L., Hinsley, W.R., Laydon, D.J., Dabrera, G., and Oâ€™Toole, Ã.i. (2021). Transmission of SARS-CoV-2 Lineage B. 1.1. 7 in England: Insights from linking epidemiological and genetic data. medRxiv, 2020.2012. 2030.20249034.

Wang, W., Ye, L.-B., Ye, L., Li, B-Z., Gao, B., and al., e. (2007). Up-regulation of IL-6 and TNF-alpha induced by SARS-coronavirus spike protein in murine macrophages via NF-kappaB pathway. Virus Res *128*, 1-8.

WHO (2021). WHO Coronavirus (COVID-19) Dashboard. World Health Organization *https://covid19.who.int/*.

Worldometer (2021). COVID-19 Coronavirus Pandemic Worldometer *https://*http://www.worldometers.info/coronavirus/.

Xia, S., Lan, Q., Su, S., Wang, X., Xu, W., Liu, Z., Zhu, Y., Wang, Q., Lu, L., and Jiang, S. (2020). The role of furin cleavage site in SARS-CoV-2 spike protein-mediated membrane fusion in the presence or absence of trypsin. Signal Transduction and Targeted Therapy *5*, 92.

Xu, Z.-S., Shu, T., Kang, L., Wu, D., Zhou, X., Liao, B.-W., Sun, X.-L., Zhou, X., and Wang, Y.-Y. (2020). Temporal profiling of plasma cytokines, chemokines and growth factors from mild, severe and fatal COVID-19 patients. Signal transduction and targeted therapy *5*, 1-3.

Yang, L., Liu, S., Liu, J., Zhang, Z., Wan, X., Huang, B., Chen, Y., and Zhang, Y. (2020). COVID-19: immunopathogenesis and Immunotherapeutics. Signal transduction and targeted therapy *5*, 1-8.

678 Zhang, J., Xiao, T., Cai, Y., Lavine, C.L., Peng, H., Zhu, H., Anand, K., Tong, P., Gautam, A., and Mayer, M.L.
679 (2021a). Membrane fusion and immune evasion by the spike protein of SARS-CoV-2 Delta variant.
680 Science, eabl9463.
681 Zhang, J., Xiao, T., Cai, Y., Lavine, C.L., Peng, H., Zhu, H., Anand, K., Tong, P., Gautam, A., Mayer, M.L*., et*
682 *al.* Membrane fusion and immune evasion by the spike protein of SARS-CoV-2 Delta variant. Science *0*,
683 eabl9463.
684 Zhang, L., Jackson, C.B., Mou, H., Ojha, A., Peng, H., Quinlan, B.D., Rangarajan, E.S., Pan, A.,
685 Vanderheiden, A., and Suthar, M.S. (2020). SARS-CoV-2 spike-protein D614G mutation increases virion
686 spike density and infectivity. . Nature communications *11*, 1-9.
687 Zhang, L., Mann, M., Syed, Z., Reynolds, H.M., Tian, E., Samara, N.L., Zeldin, D.C., Tabak, L.A., and K.G.,
688 T.H. (2021b). Furin cleavage of the SARS-CoV-2 spike is modulated by O-glycosylation. PNAS *118*,
689 e2109905118.
690 Zhang, X., Ao, Z., Bello, A., Ran, X., Liu, S., Wigle, J., Kobinger, G., and Yao, X. (2016). Characterization of
691 the inhibitory effect of an extract of Prunella vulgaris on Ebola virus glycoprotein (GP)-mediated virus
692 entry and infection. Antiviral research *127*, 20-31.
693 Zhou, B., Thao, T.T.N., Hoffmann, D., Taddeo, A., Ebert, N., Labroussaa, F., Pohlmann, A., King, J., Steiner,
694 S., and Kelly, J.N. (2021). SARS-CoV-2 spike D614G change enhances replication and transmission. Nature
695 *592*, 122-127.
696 Zhu, H., Chen, C.Z., Sakamuru, S., Zhao, J., Ngan, D.K., Simeonov, A., Hall, M.D., Xia, M., Zheng, W., and
697 Huang, R. (2021). Mining of high throughput screening database reveals AP-1 and autophagy pathways
698 as potential targets for COVID-19 therapeutics. Scientific Reports *11*, 6725.
699 Zhu, N., D. Zhang, W. Wang, X. Li, B. Yang, J. Song, X. Zhao, B. Huang, W. Shi, and R. Lu. , and al., e.
700 (2020). A novel coronavirus from patients with pneumonia in China, 2019. New England Journal of
701 Medicine *382*, 727-733.

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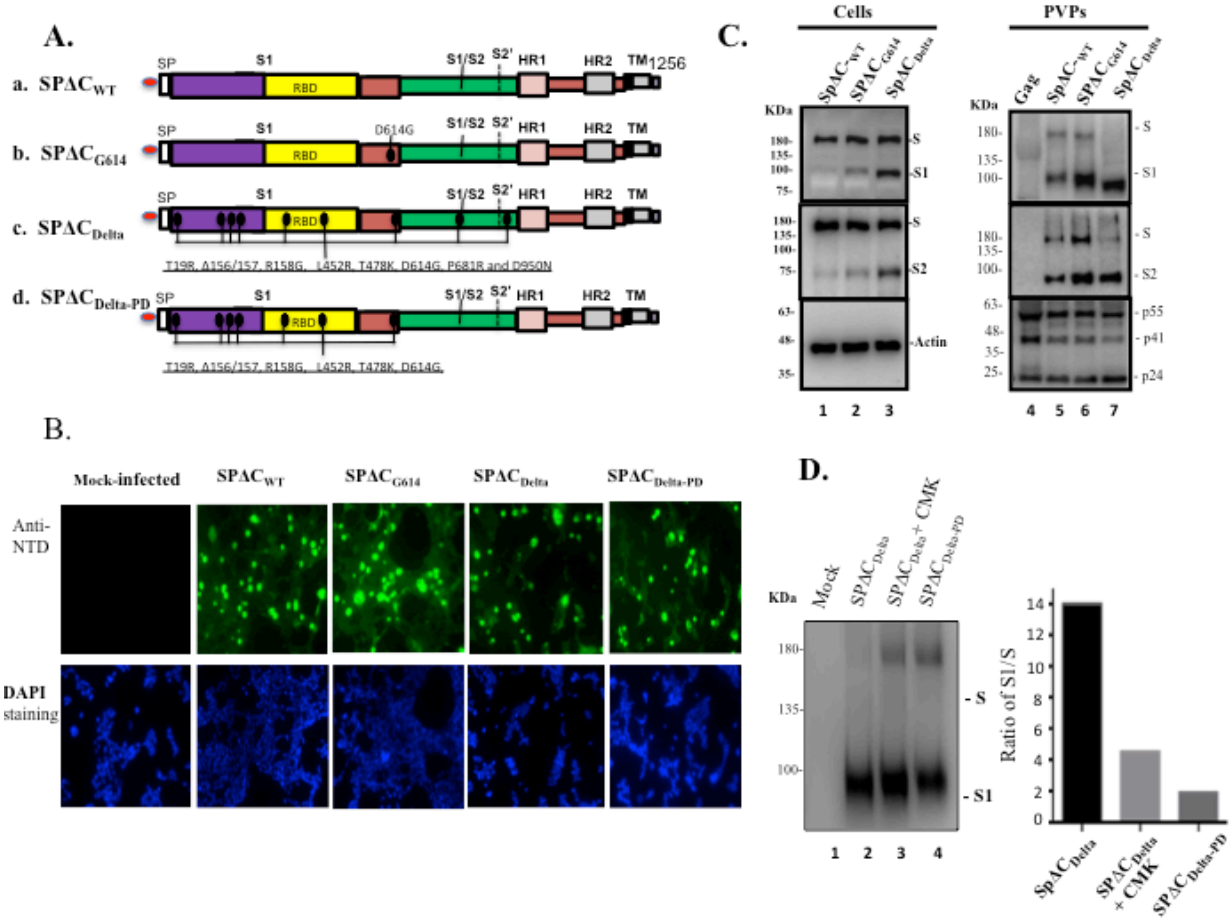

Figure 1. The expression and cleavage of SARS-CoV-2 Delta Spike protein (SP) in the cells and in the pseudotyped virus particles (PVPs). (A) Schematic representation of the structure domains and mutations of SARS-CoV-2 SP and its variants. A C-terminal 17aa of each of SARS-CoV-2 SPΔC was deleted in order to increase the incorporation of SP into the virrus particles. (B) 293T cells were transfected with pCAGGS-SPΔC$_{wt}$, -SPΔC$_{G614}$, -SPΔC$_{Delta}$ or -SPΔC$_{DeltaPD}$. The intracellular expression of each SPΔC in the transfected 293T cells were detected by immunofluorescence assay with SARS-CoV-2 S-NTD antibody. (C) 293T cells were transfected with each SPΔC-expressing plasmid, HIV ΔRI/Env^{-}/Gluc) and a packaging plasmid (pCMVΔ8.2) in HEK293T cells. As Gag-control, 293T cells were only transfected with

716 pCMVΔ8.2 plasmid. WB were used to detect the expression and cleavage of various SPΔC and
717 HIV Gag protein in cells and in PVPs. Full-length spike (S), cleaved S1 and S2 were annotated.
718 (D) $SP\Delta C_{Delta}$-PVPs were produced in the absence or in the presence of a furin inhibitor (CMK)
719 (25μM) and $SP\Delta C_{DeltaPD}$-PVPs were analyzed by WB using polyclonal anti-SP/RBD antibody
720 (Left panel). The ratio of S1 relative to the full length SP was also quantified by laser
721 densitometry as indication of SP processing efficiencies (Right panel). Panel C-D show the
722 representative WB image from three independent experiments.

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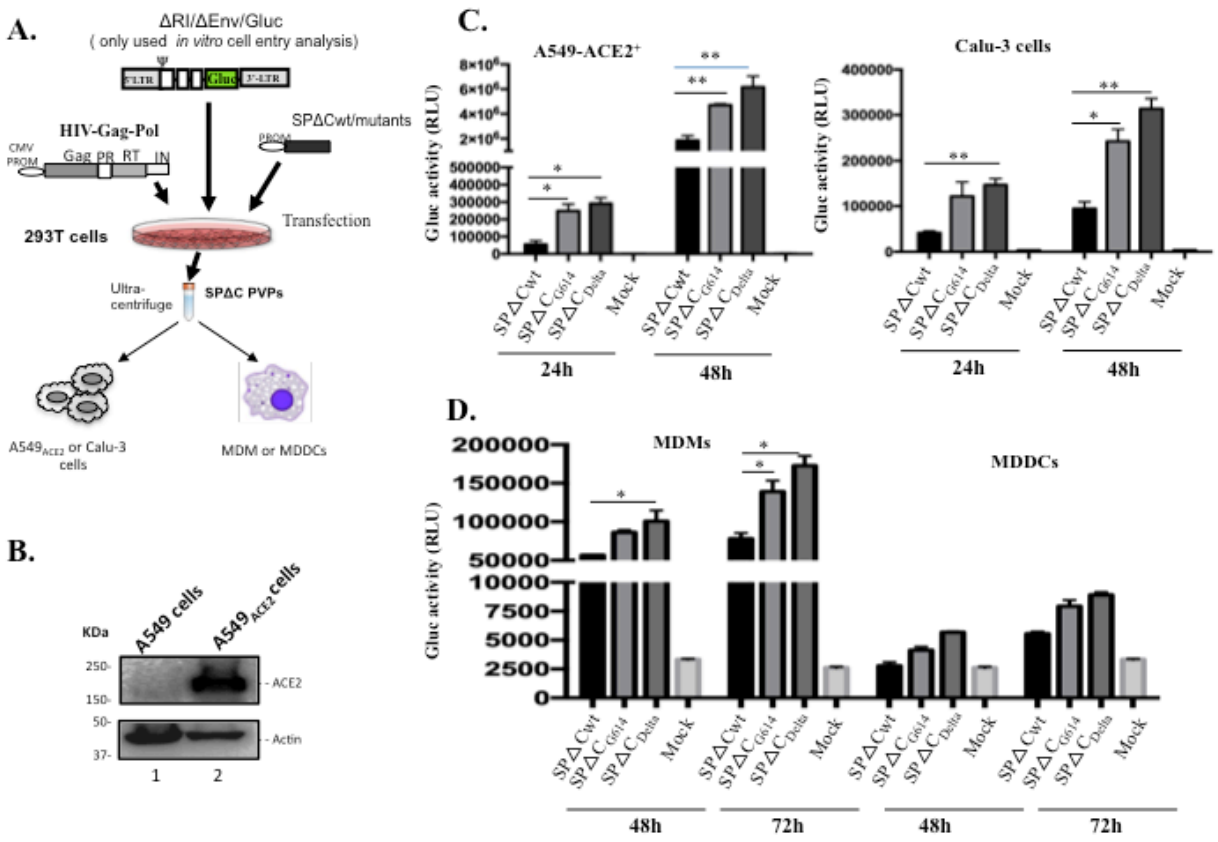

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725 **Figure 2. SP pseudotyped viruse infectivity assays on human lung cell lines, human**
726 **macrophages (MDMs) and dendritic cells (MDDCs).** (A) Schematic representation of the

727 procedures and the plasmids used for production of SARS-COV2-SPΔC-pseudotyped
728 lentivirus particles (SPΔC-PVPs). (B) an $A549_{ACE2}$ cell lins was generated by transducing
729 A549 cells with a lentiviral vector (pLenti-C-ACE2).The ACE2 expression in the transduced
730 cells was detected by WB using anti-ACE2 antibody. (C, D) $A549_{ACE2}$, Calu-3 cell lines,
731 human MDMs or MDDCs were infected with an equal amounts of $SP\Delta C_{wt}$, -$SP\Delta C_{G614}$, or -
732 $SP\Delta C_{Delta}$-PVPs carrying Gaussia luciferase (Gluc) gene (adjusted by P24). At different time
733 points, the Gluc activity in the supernatant of infected cultures was measured. The results are
734 the mean values ± standard deviations (SD) of two independent experiments. Statistically
735 significant differences (* $P \leq 0.05$; **, $P \leq 0.01$) versus the SPΔCwt were determined by
736 unpaired t test. No significant (ns) was not shown.

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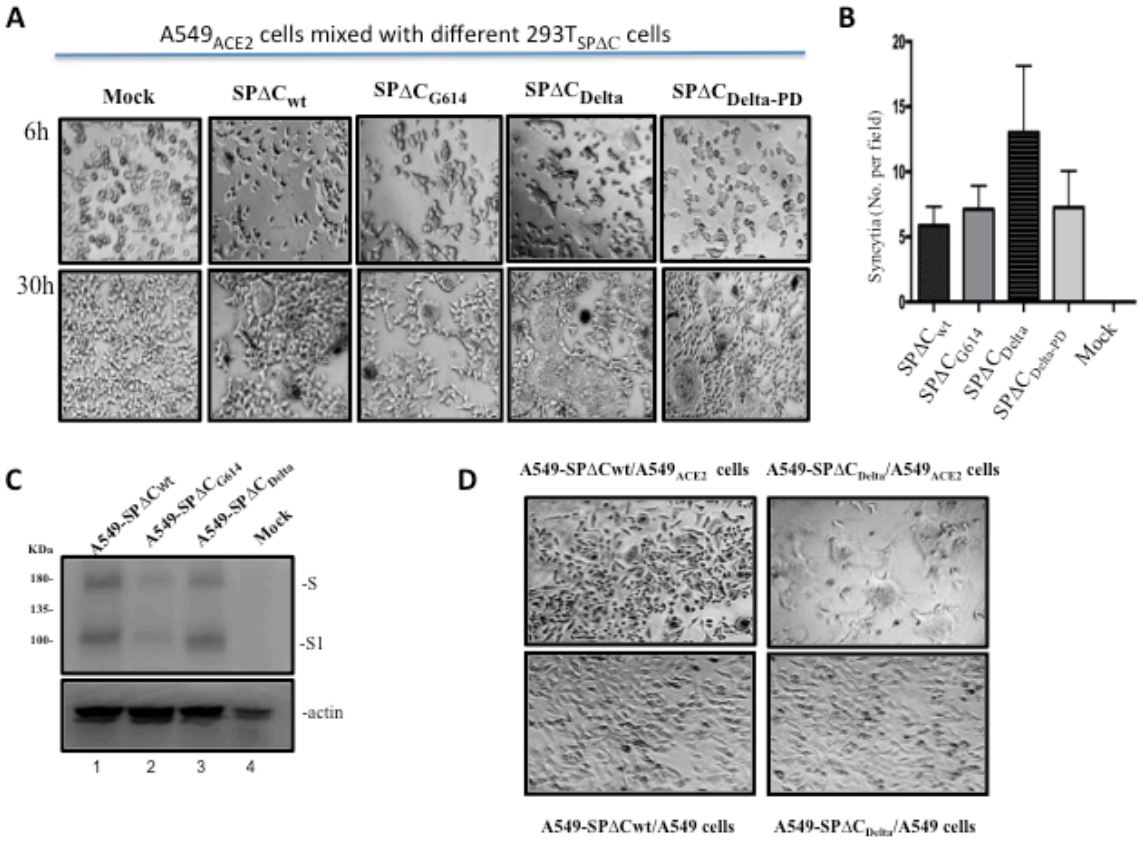

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739 **Figure 3. Delta-SP variant enhanced syncytia formation in lung epithelia cell line.** (A)
740 $A549_{ACE2}$ cells were cocultured with 293T cells transfected with SPΔC or variants. The syncytia
741 formation was monitored by microscopy at 6 hours and 30 hours after coculture. (B)
742 Quantification of the numbers of syncytia formation in the cocultures at 6 hrs under bright-field
743 microscopy. Results are the mean values ± standard deviations (SD) from two independent
744 experiments. C) Expression of $SP\Delta C_{wt}$, $SP\Delta C_{G614}$, or $SP\Delta C_{Delta}$ in corresponding A549 stably
745 cell lines was detected by WB using anti-SP/RBD antibody. D) $A549_{ACE2}$ cells or A549 cells
746 were cocultured with A549-$SP\Delta C_{wt}$ or A549-$SP\Delta C_{Delta}$ stable cells. The syncytia formation was
747 visualized by microscopy at 30 hours after co-culture.

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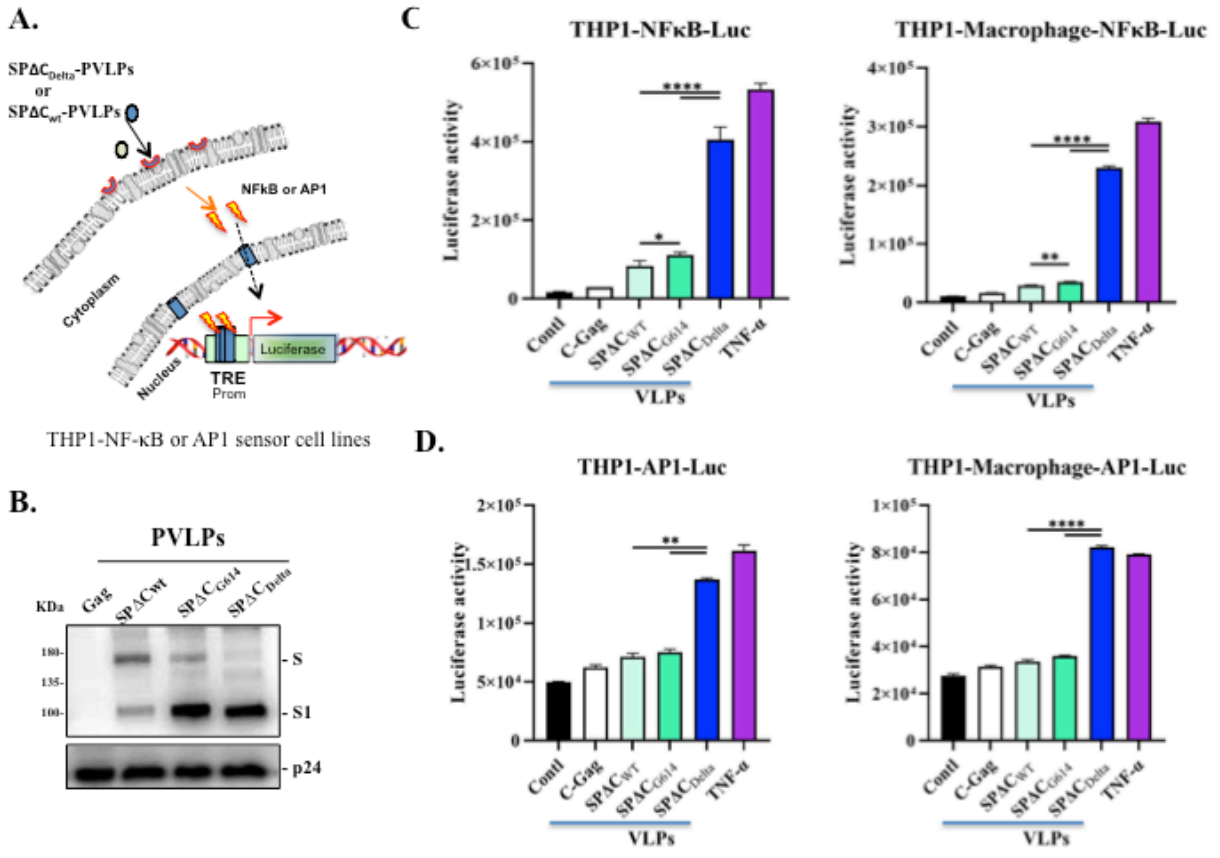

Figure 4. SPΔC$_{Delta}$-PVLPs stimulated NF-κB and Ap1-signal pathway in THP1 cells and THP1 derived macrophages. **(A)** The schematic diagram of NF-κB activity luciferase reporter assay. The THP1-NF-κB -Luc, or THP1-AP1-Luc sensor cell line were incubated with SPΔC$_{wt}$, -SPΔC$_{G614}$, or -SPΔC$_{Delta}$-PVLPs for 6 hrs, and the activation of NF-κB or AP1 signaling was detected by measurement of the luciferase activity. (B) WB detected the incorporation of SPΔC$_{wt}$, -SPΔC$_{G614}$, or -SPΔC$_{Delta}$ in PVLPs using SARS-CoV-2 S-NTD antibody. The GagP24 was detected using mouse anti-P24 antibody. (C, D) The THP1-NFκB-Luc or THP1-NFκB-Luc-derived macrophages, and THP1-AP-1-Luc cells or THP1-AP-1-Luc-derived macrophages were treated with equal amounts of SPΔC$_{wt}$, SPΔC$_{G614}$ or SPΔC$_{Delta}$-PVLPs (adjusted by P24) for 6 hrs, and the activation of NF-κB or AP-1 signaling was

detected by measurement of the Luc activity. Meanwhile, Gag-VLPs or TNF-α treatment were used as negative or positive control. The results are the mean values ± standard deviations (SD) of two independent experiments. Statistical significance was determined using unpaired t test, and significant p values are represented with asterisks (*P ≤0.05; **P ≤ 0.01; ***P≤0.001; **** P≤0.0001). No significant (ns) was not shown

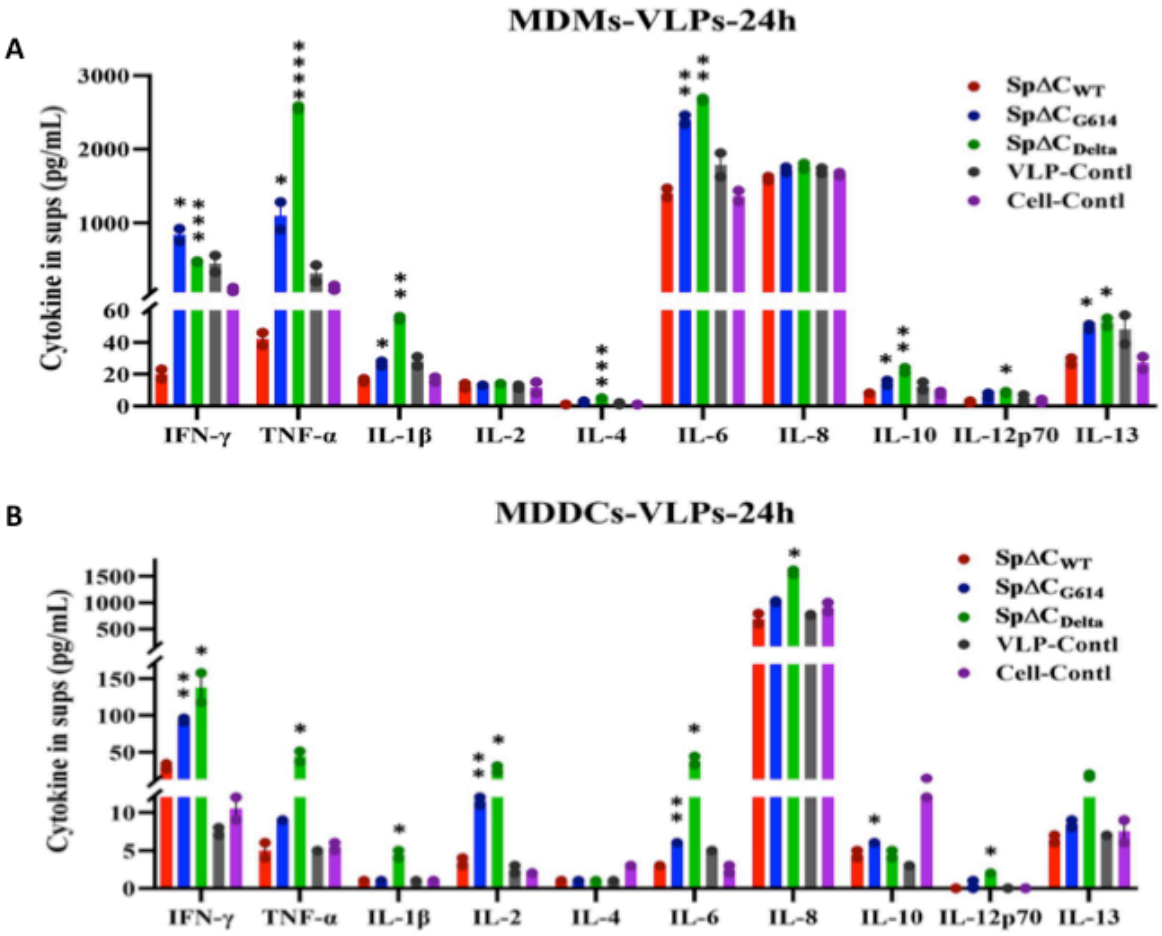

Figure 5. SpΔC$_{Delta}$- PVLPs stimulated proinflammatory cytokines release in human MDM and MDDCs. Human MDMs (A) or MDDCs (B) were treated with equal amount of SPΔC$_{wt}$, SPΔC$_{G614}$ or SPΔC$_{Delta}$ –PVLPs (adjusted by p24). Gag-VLPs treated and non-treated

MDMs or MDDCs were used as negative controls. After 24 hrs, the cell culture supernatants were collected and different cytokines, including IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-α were measured by MSD immunoassay (Meso Scale Discovery). The results are the mean values ± standard deviations (SD) of two biologic replicates. Statistical significance was determined using unpaired t test, and significant p values are represented with asterisks (*P ≤0.05; **P ≤ 0.01; ***P≤0.001; **** P≤0.0001). No significant (ns) was not shown.