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Pyroptosis of syncytia formed by fusion of SARS-CoV-2 spike and ACE2-expressing cells

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Dear Editor,

Coronavirus Disease 2019 (COVID-19) is an infectious disease associated with systematical multi-organ failure caused by SARS-CoV-2, which mainly infects the lung and upper respiratory system[1,2]. Massive multinucleated syncytia are commonly observed in autopsy of severe COVID-19 patients[3]. It has been reported that the interaction between Spike (S) protein and ACE2 not only mediated the fusion of virus with host cells, but also multinucleated giant cells formation[4–7]. However, the underlying molecular mechanisms of syncytia death are poorly understood.

To better observe the formation of syncytia, we established an in vitro cell-cell fusion system to mimic the fusion of SARS-CoV-2 infected cells with ACE2-expressing cells. The SARS-CoV-2-S-GFP expressing cells were imaged by high-content imaging with confocal laser scanning microscope. We found that SARS-CoV-2-S-GFP mainly appeared on the cell membrane surface[8], as it co-located with cell membrane marker PLCδ-PH (Supplementary Fig. S1a), but not with nuclear RFP (Supplementary Fig. S1e). In addition, S protein was detected as puncta in the cytoplasm with co-localization of Golgi marker GGA1 (Supplementary Fig. S1b), consistent with report showing that S protein was glycosylated in Golgi apparatus[9]. However, two endosome-related proteins Rab5a and Rab7a did not co-locate with the S protein (Supplementary Fig. S1c and d). Then, the SARS-CoV-2-S-GFP expressing cells (HeLa-S-GFP) were co-cultured with A549 expressing ACE2 cells (A549-PLC-RFP- ACE2) at a 1:1 ratio. Syncytia were observed 4 h later (Fig. 1a), and the cell-cell fusion occurred between the cell membranes because the nuclei were intact (Fig. 1b). In addition, syncytia formation in the co-culture system was dependent on the expression of Spike and ACE2 (Supplementary Fig. S2), and regardless of whether the cells were the same type (A549-S-GFP and A549-ACE2, Supplementary Fig. S3a) or different types (H1299-S-GFP and A549-ACE2, Supplementary Fig. S3b; HeLa-S-GFP and A549-ACE2, Fig. 1a), or even from different species (L929-S-GFP and A549-ACE2, Supplementary Fig. S3c).

Next, we aimed to investigate the fate of syncytia. We found that multiple bubbles were formed on the membrane of syncytia, which tended to rupture at 12 h after co-culture (Supplementary Fig. S3d and e). Then the real-time observation system was used to record the fusing process of SARS-CoV-2-S (HeLa-S) expressing HeLa cells with HeLa-ACE2 cells (Supplementary Movie S1). We found that syncytia were formed upon cell-cell fusion, grew in size steadily, and finally ruptured with LDH release, ATP decrease, and caspase-3/7 activity increase (Fig. 1c-g) as well as IL1β release in the case of THP-1-ACE2 cells co-culture with HeLa-Spike cells (Supplementary Fig. S4). Interestingly, the death was blocked by pan-caspase inhibitor ZVAD, although ZVAD did not affect the syncytia formation. As S protein priming by protease TMPRSS2 or Cathepsin L is essential for cell-cell fusion[7,10], we explored the expression of S protein under ZVAD treatment. We found that ZVAD did not affect S protein priming because the bands of S2 and S2' fragment were the same in control and in ZVAD-treated cells, and ZVAD treatment also failed to inhibit the entry of SARS-CoV-2-Spike pseudovirus into HeLa-ACE2 cells, while chloroquine (CQ) inhibited this event (Supplementary Fig. S5a and b). Next, we assessed the activation of molecules related to death. As shown in Fig. 1h, S2

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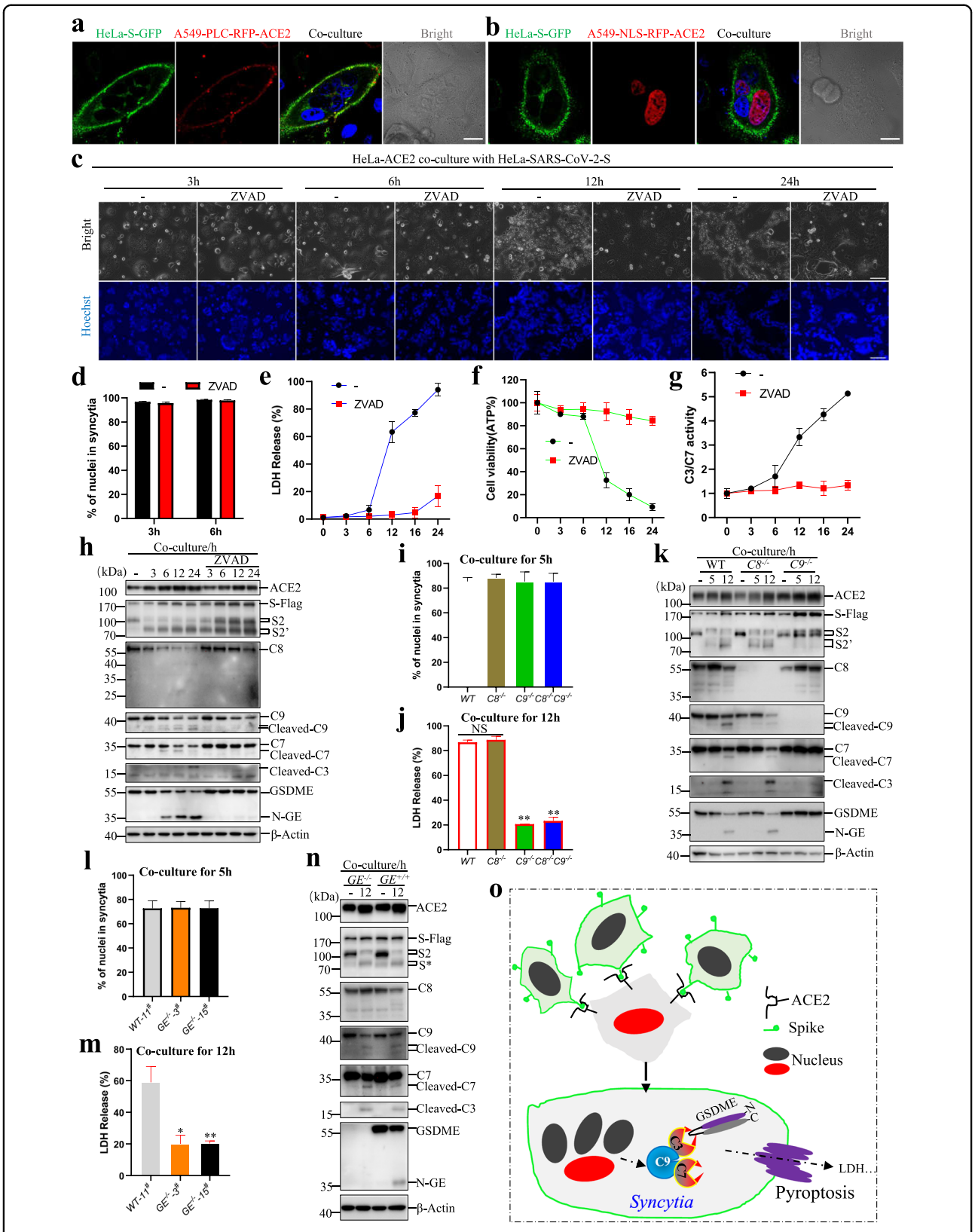

Fig. 1 (See legend on next page.)

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Fig. 1 Caspase-9/GSDME trigged pyroptosis of syncytia formed by fusion of SARS-CoV-2 Spike and ACE2-expressing cells. SARS-CoV-2-S-GFP HeLa cells were co-cultured with PLC-RFP-A549-ACE2 cells (**a**), or NLS-RFP-A549-ACE2 cells (**b**) at a 1:1 ratio. Four hours later, image was scanned by LSM780. The nucleus (blue) was stained by Hoechst; Bar, 20 μm. **c** The time-phase of cell-cell fusion progress, HeLa-ACE2 co-culture with HeLa-SARS-CoV-2-S cells, treatment with pan-caspase inhibitor, ZVAD (20 μM) or not; Images were obtained at 3 h, 6 h, 12 h 24 h; the nucleus(blue) was stained by Hoechst; Bar, 100 μm; then the cell-cell fusion was quantified (**d**); the LDH release (**e**), ATP level (**f**), the activity of caspase-3/7 (**g**) were detected at indicated time. Western blot analysis of the cells collected as indicated. ACE2, Flag, caspase-8 (C8), caspase-9 (C9), caspase-7 (C7), Cleaved-caspase-3 (Cleaved-C3), and GSMDE were probed. β-Actin as loading control (**h**, **k** and **n**). The cell-cell fusion was quantified after co-culture for 5 h as indicated (**i** and **l**). LDH release was measured after co-culture for 12 h as indicated (**j** and **m**). The model of SARS-CoV-2 Spike and ACE2 interaction induced syncytia pyroptosis (**o**). The data were shown as means ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, NS non-significance, and all the experiments were replicated more than three times.

fragment seemed to be modified because of a little "up-shift" in SDS-PAGE gel, and S2' was moderately induced upon cell-cell fusion. We also found that caspase-9 and caspase-3/7 were cleaved, which generally implies the activation of apoptosis pathway. Interestingly, however, we detected the cleavage of GSDME. It is known that activation of GSDME is mediated by caspase-3[11,12], and we confirmed the role of caspase in activating GSDME as ZVAD effectively blocked GSDME cleavage. Thus, it can be proposed that syncytia formation led to activation of caspase-9 to caspase-3/7 cascade. The activated caspase-3 cleaved GSDME and released N-GSDME to permeabilize cell membrane to execute syncytia pyroptosis.

To define the death pathway underlying syncytia pyroptosis, we knocked out caspase-8 ($C8^{-/-}$) or caspase-9 ($C9^{-/-}$) in HeLa cells by CRISPR-Cas9 and expressed ACE2 and SARS-CoV-2-S-Flag in these cells respectively, and then co-cultured these cells in pairs. We found that syncytia formed in $C8^{-/-}$, $C9^{-/-}$ and $C8^{-/-}C9^{-/-}$ cells, which showed no difference from WT cells (Fig. 1i and Supplementary Fig. S6a and b). In contrast, *C9* but not *C8* deletion blocked syncytia death (Fig. 1j). Further analysis showed that the cleavage of GSDME (N-GE) was abolished in $C9^{-/-}$ cells (Fig. 1k). Interestingly, the S2' fragment of SARS-CoV-2-S-Flag induced by cell-cell fusion was not observed in $C9^{-/-}$ cells, indicating a linkage between caspase-9 and SARS-CoV-2 S protein cleavage (Fig. 1k and Supplementary Fig. S6c).

To further confirm that GSDME was involved in the death of syncytia, we generated GSDME knock-out ($GE^{-/-}$-ACE2, $GE^{-/-}$-SARS-CoV-2-S-Flag) and WT HeLa cell lines ($GE^{+/+}$-ACE2, $GE^{+/+}$-SARS-CoV-2-S-Flag), and co-cultured them. Similarly, GSDME did not affect cell fusion to form syncytia (Fig. 1l and Supplementary S6d and e), but GSDME knock-out significantly inhibited the death of syncytia (Fig. 1m). In addition, GSDME knock-out did not affect the activation of caspases (Fig. 1n), confirming it is downstream of caspases.

Our data indicated that the death of syncytia induced by SARS-CoV-2 infection could be mediated by GSDME-dependent pyroptosis. The existing transcriptomic data[13] showed correlations of the expression between ACE2 and GSDME, especially in the small intestine and testis (Supplementary Fig. S7). The high-level expression of ACE2 and GSDME in testis could link to the destruction of male reproductive system by SARS-CoV-2 infection[14]. We analyzed single-cell-RNA-sequencing (scRNA-Seq) data from eight normal human lung transplant donors with a total of 42,225 cells[15]. As reported, the expression of ACE2 is concentrated in alveolar type 2 (AT2) cells, a special cell type with a small population in lung[16] and GSDME is also enriched in AT2 cells (Supplementary Fig. S8a and b). Thus, GSDME-dependent pyroptosis could occur in SARS-CoV-2 infected AT2 cells.

Although cell death induced by SARS-CoV-2 infection has been shown by several studies[17,18], in this study, we provide in vitro evidence showing that the syncytia formed by fusion of the cells expressing SARS-CoV-2 S protein and ACE2 respectively undergo pyroptosis (Fig. 1o). The pyroptosis is initiated by components of intrinsic apoptosis pathway and executed by caspase-3/7 mediated activation/cleavage of GSDME. Since scRNA-seq data showed that both ACE2 and GSDME were expressed in AT2 cells in human lung, we propose that GSDME-mediated syncytia death is a potential mechanism of the death of SARS-CoV-2 infection-caused syncytia. The lytic death of syncytia may contribute to the excessive inflammatory responses in severe COVID-19 patients.

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Author contributions
J.H., Y.Z., H.M. conceived and designed the experiments. H.M., Z.Z., H.L., P.Z., L.L. performed the experiments: generated the cell strains and performed immunofluorescent imaging, and the associated western blot analyses. S.W. and Y.L. analyzed the data from GTEx and scRNA-seq. J.W. helped with discussion and interpretation of results. H.M. and J.H. wrote the paper. All authors provided the final approval of the paper.

Conflict of interest
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