Exacerbation of RSV Infectivity by SARS-CoV-2 in an In-vitro Co-Infection Cellular Model

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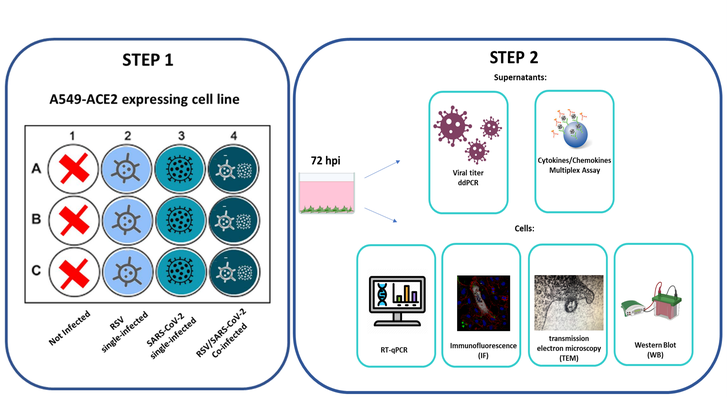
BACKGROUND

Co-infections are common and can seriously affect the course of each infection from its own natural history. Co-infecting pathogens with an analogous tropism, such as RSV and SARS-CoV-2, may antagonize or facilitate each other modulating host disease outcomes and microbe transmission relative to a single infection. Clinically, a severe phenotype has been occasionally reported in children with RSV/SARS-CoV-2 co-infections (Halabi KC, *Pediatr Pulmonol*. 2023). However, experimental models to study the molecular and immunological dynamics of co-infections are extremely limited. Herein, we propose an *in vitro* co-infection model to assess RSV/SARS-CoV-2 immune and viral evolution.

METHODS



Scheme 1. Experimental design of the *in-vitro* RSV/SARS-CoV-2 co-infection model.

RESULTS

1. Assessment of *in vitro* RSV and SARS-CoV-2 viral replication.

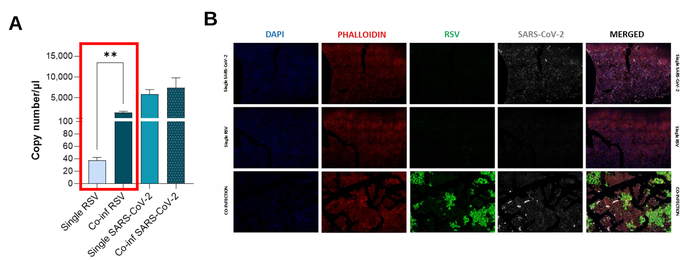
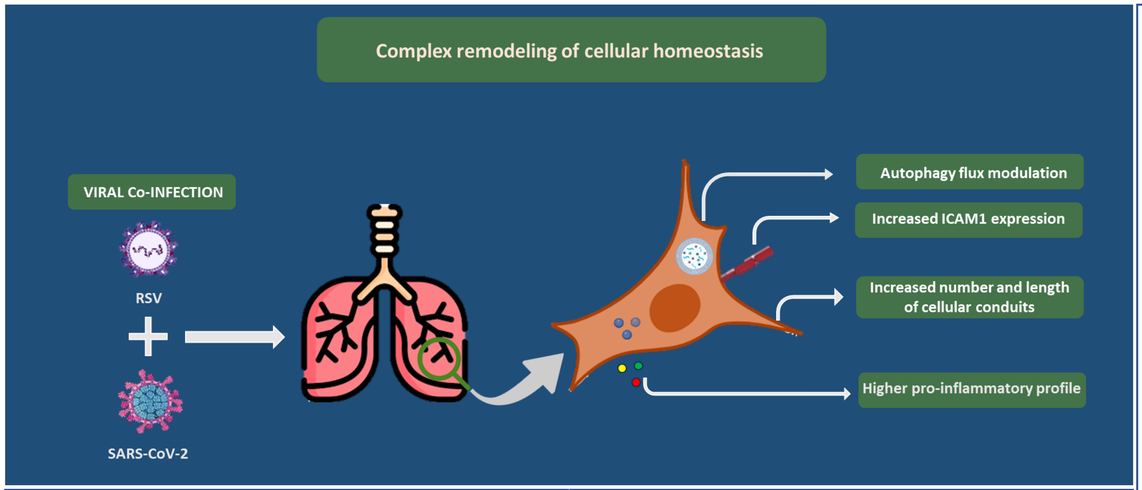


FIGURE 1. A) SARS-CoV-2 and RSV viral replication was assessed by digital droplet PCR in single infection and co-infection conditions at 72 hpi. Mean values±SEM are reported. ** p<0.01. **B)** Representative immunofluorescence images acquired in 40X resolution of combined F-RSV and N-SARS-CoV-2 proteins in uninfected, SARS-CoV-2 single-infected, RSV single-infected and co-infected A549-ACE2 cells (DAPI in blue, phalloidin in red, N-SARS-CoV-2 in grey, F-RSV in green).



2. Single cell *in vitro* co-infection assessment.

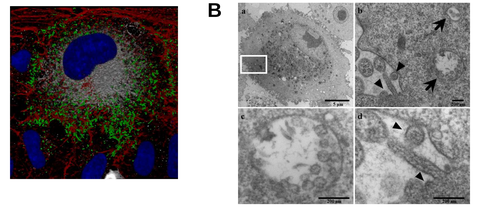


FIGURE 2. A) Representative IF image of combined RSV-F and SARS-CoV-2-N proteins in a co-infected A549-hACE2 cell (DAPI in blue, phalloidin in red, N-SARS-CoV-2 in grey, F-RSV in green). **B)** a. Representative TEM image of a A549 cell co-infected with SARS-CoV-2 and RSV; b. a greater enlargement of the outlined area of the figure (a) showing the morphological features of the co-infection with SARS-CoV-2 (arrows) and RSV (arrowheads); c. high magnification of a vesicle containing multiple SARS-CoV-2 assembled virions (average diameter ≈80 nm). Within virions, the nucleocapsid is visible as small electron-dense dots; d. higher magnification of RSV virion (arrowheads) budding from the surface of the infected cell, showing a spherical and the nucleocapsid which appears as electron-dense dots.

3. Viral receptor expression analyses.

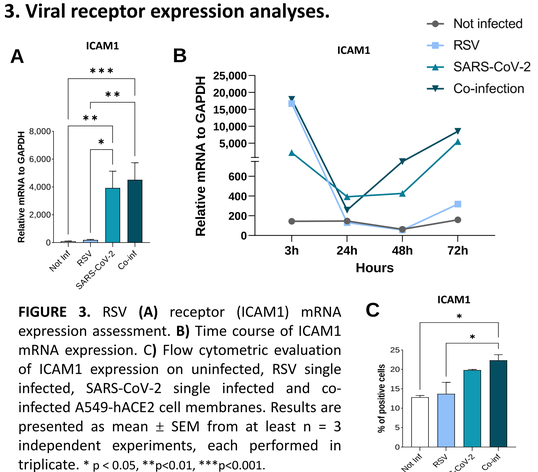


FIGURE 3. RSV **(A)** receptor (ICAM1) mRNA expression assessment. **B)** Time course of ICAM1 mRNA expression. C**)** Flow cytometric evaluation of ICAM1 expression on uninfected, RSV single infected, SARS-CoV-2 single infected and co-infected A549-hACE2 cell membranes. Results are presented as mean ± SEM from at least n = 3 independent experiments, each performed in triplicate. * p < 0.05, **p<0.01, ***p<0.001.

4. Analyses of viral-driven conduits by immunofluorescence.

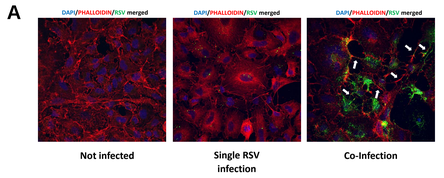


FIGURE 4. A) Representative IF images acquired in 40X resolution of F-RSV protein in uninfected, RSV single-infected and co-infected A549-hACE2 cells (DAPI in blue, phalloidin in red, F-RSV in green). White arrows show viral-driven conduits. B**)** Quantification of filopodia length per cell in not infected, RSV single infected (RSV) and co-infected (Co-Inf) conditions. Results are presented as mean ± SEM. * p < 0.05.

5. Analyses of A549-hACE2 transcriptome and secretome following single SARS-CoV-2, RSV infection or co-infection.

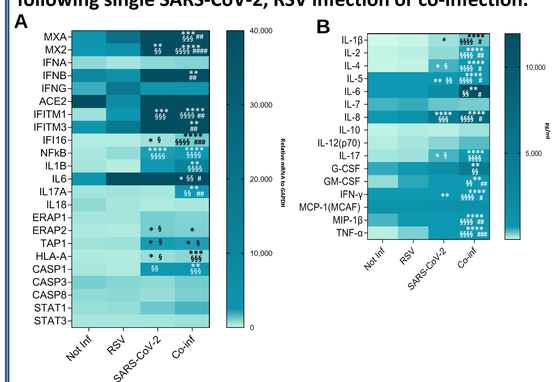


FIGURE 5. A) Antiviral, inflammatory, antigen presentation gene expression. **B)** Cytokine/chemokine in supernatants from A549-hACE2. Results are summarized in heatmaps. Vs not-infected * p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001; vs RSV § p < 0.05, §§ p<0.01, §§§ p<0.001, §§§§ p<0.0001; vs SARS-CoV-2 # p < 0.05, ## p<0.01, ### p<0.001, #### p<0.0001

6. Assessment of autophagy process in A549-hACE2 cell lines following RSV, SARS-CoV-2 single infection or co-infection

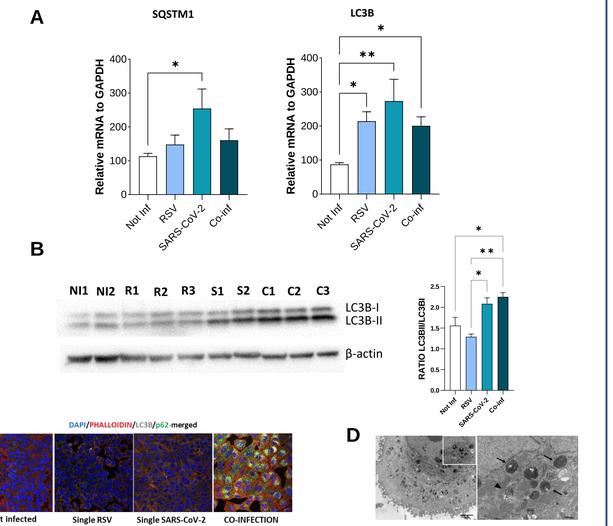


FIGURE 6. A) Significantly expressed autophagy mediator genes. Results are reported as mean ± SEM. * p < 0.05, **p<0.01. **B)** Western blot analysis of LC3B-I and LC3B-II isoforms in uninfected, RSV, SARS-CoV-2 single infected and co-infected conditions (left panels) and results reported as LC3BII and LC3BI ratio mean ± SEM histograms (right panel). * p < 0.05, **p<0.01. **C)** IF showing LC3B (grey) and p62 (green) in uninfected, RSV single infected, SARS-CoV-2 single infected and co-infected conditions. **D)** Left panel, representative TEM image of a A549-hACE2 cell co-infected with SARS CoV-2 and RSV with a large amount of widespread autophagosomes at different steps of the autophagic process (early/initial and late autophagic compartments); right panel, a greater enlargement of the outlined area of the left panel showing an early/initial autophagic vacuole (arrowhead) and late autophagic compartments (arrow).

CONCLUSIONS

The RSV/SARS-CoV-2 co-infection model displays a unique and specific viral and molecular fingerprint. In particular, in the co-infected condition we observed:

1. An increased replication rate of RSV;
2. The simultaneous infection of a single cell by both viruses;
3. Increased expression of ICAM1, exploited by RSV to enter the target cell;
4. Increased number and length of viral-driven conduits;
5. An enhanced pro-inflammatory profile;
6. A dramatic change in the autophagic flux.

Our findings give clues of augmented severity upon RSV infection in the context of a concomitant SARS-CoV-2 co-infection. The *in vitro* co-infection model may represent an attractive cost/effective approach to mimic both viral dynamics and host immune responses, providing readily-measurable targets predictive of co-infection progression.

ADDITIONAL KEY INFORMATION

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