Intractable COVID-19 and Prolonged SARS-CoV-2 Replication in a CAR-T-cell Therapy Recipient: A Case Study

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Abstract

A CAR-T-cell recipient developed severe COVID-19, intractable RNAemia, and viral replication lasting >2 months. Pre-mortem endotracheal aspirate contained $2x10^{10}$ SARS-CoV-2 RNA copies/mL and infectious virus. Deep sequencing revealed multiple sequence variants consistent with intra-host virus evolution. SARS-CoV-2 humoral and cell-mediated immunity were minimal. Prolonged transmission from immunosuppressed patients is possible.

Keywords: COVID-19, SARS-CoV-2 immune responses, SARS-CoV-2 RNAemia, SARS-CoV-2 intra-host variation, SARS-CoV-2 infectivity, Severe Acute Respiratory Syndrome Coronavirus-2

Introduction:

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in patients with hematologic malignancies results in poor coronavirus disease 2019 (COVID-19)-related outcomes[1]. Chimeric antigen-modified T-cell therapy (CAR-T-cell) recipients are at risk for severe COVID-19 because of chronic B-cell aplasia and hypogammaglobulinemia due to "on-target/off-tumor" effects of CAR-T-cells[2], which occur when CAR-T-cells kill normal B-cells that express the CAR-T-cell target antigen. Conditioning regimens for CAR-T-cell therapy can also cause lymphopenia and diminish B- and T-cell function. Although persistence of SARS-CoV-2 RNA in respiratory specimens is not thought to represent infectiousness[3, 4], it is possible that CAR-T-cell recipients can persistently shed infectious SARS-CoV-2. Similarly, SARS-CoV-2 RNAemia, which correlates with disease severity[5], is thought to be short-lived[6] but may be more protracted in immunosuppressed hosts.

We present a case of prolonged SARS-CoV-2 infection in a patient with multiple myeloma who received CAR-T-cells targeting the B-cell maturation antigen (BCMA), which is universally expressed on malignant plasma cells and on some normal plasma cells and mature B-cells and is involved in plasma cell survival and B-cell differentiation into plasma cells[7-10]. We characterize viral persistence, intra-host viral evolution, and immune profiles from longitudinal samples and demonstrate that the patient experienced high-level SARS-CoV-2 RNAemia for >2 months, viral diversification, and massive lung infection before succumbing to the infection.

Case report:

The patient was a 73-year-old male with treatment-refractory multiple myeloma. He had undergone an autologous hematopoietic cell transplant 2 years prior but developed recurrent disease and therefore underwent anti-BCMA CAR-T-cell therapy after fludarabine/cyclophosphamide lymphodepletion. Nasopharyngeal (NP) swab reverse transcriptase polymerase chain reaction (RT-PCR) testing for SARS-CoV-2 was negative 17 and 2 days before CAR-T-cell therapy. He received tocilizumab for cytokine release syndrome, a known toxicity of CAR-T-cell therapy [2], but not corticosteroids. He was

discharged home in stable condition. Twelve days after discharge (and 25 days after the CAR-T-cell infusion), he was admitted to the intensive care unit (ICU) with two days of a productive cough, dyspnea, anorexia, and lightheadedness. Temperature was 37.1°C; oxygen saturation was 86% on room air. Laboratory evaluation showed a white blood cell (WBC) count of $3.5x10^9$ cells/L (normal $3.8x10^9$-$10.6x10^9$), with an absolute lymphocyte count (ALC) and absolute neutrophil count (ANC) of $0.7x10^9$ cells/L (normal $0.8\ x10^9$-$3.65\ x10^9$) and $2.6x10^9$ cells/L (normal $2.24\ x10^9$-$7.68\ x10^9$), respectively. NP swab RT-PCR testing was positive for SARS-CoV-2 RNA (day 0); cycle thresholds were 20.1 and 21.5 for the nucleoprotein (N)1 and envelope (E) genes, respectively (**Figure 1-I**). Chest radiograph revealed bibasilar and mid-zone opacities. He received convalescent plasma (day 2) and remdesivir (days 5-10). During this hospitalization, he had escalating oxygen requirements, necessitating the use of non-invasive positive pressure ventilation (NIPPV) on day 5. He never required intubation, and his oxygen requirements gradually improved over the following week. On day 14, he no longer needed supplemental oxygen and was discharged in stable condition on day 17, with minimal residual dyspnea. Follow-up SARS-CoV-2 NP RT-PCR testing remained positive (days 15, 26, and 37). Cycle thresholds were unavailable.

Forty-one days after being diagnosed with COVID-19, he was readmitted to the ICU with a one-week history of weakness and four days of progressively worsening dyspnea, a minimally productive cough, and diarrhea. During the 24 days between hospital discharge and readmission, he had not left his residence except for obtaining outpatient SARS-CoV-2 PCR testing. Temperature was 36.9°C. Oxygen saturation was 81% on room air, and he was started on heated high-flow supplemental oxygen with improvement in his saturation to 97%. WBC count and ANC were normal, but ALC was $0.1x10^9$ cells/L. Lymphocyte subset testing showed 0 CD19+ B-cells and 32 CD3+ T cells/mm^3 (normal 856-2669/mm^3). Chest computed tomography demonstrated bilateral ground glass opacities (**Figure 1-I**).

Since the literature at the time of the patient's presentation suggested that prolonged PCR positivity indicated the presence of non-infectious RNA[3, 4], he initially received no specific treatments for COVID-19. Instead, an extensive evaluation was sent to identify other

infections, which included blood cultures (grew *Escherichia coli* that rapidly cleared with piperacillin-tazobactam), and negative tests for urine *Legionella* antigen, plasma PCR for adenovirus, cytomegalovirus, and Epstein Barr virus, serum for *Aspergillus* galactomannan and beta-D-glucan, non-SARS-CoV-2 respiratory virus PCR, and stool pathogen PCR. He required pressor support on day 50 for hypotension, and his respiratory status continued to worsen. He was intubated on day 55 and required mechanical ventilation with 80%-100% fraction of inspired oxygen for the remainder of his hospital stay. SARS-CoV-2 NP RT-PCR testing remained positive on day 55; cycle thresholds were 13.3 and 16 for the N1 and E genes, respectively. Bronchoalveolar lavage fluid from day 55 grew rare *Klebsiella pneumoniae*, which was not thought to be significantly contributing to his condition but was treated with meropenem, which did not improve his respiratory status.

Because no etiology aside from COVID-19 could be identified, he received another course of convalescent plasma (day 58) and dexamethasone (days 63-74) (**Figure 1-I**). He was unable to receive additional remdesivir due to limited availability. Serum from days 45, 56, and 71 was negative for SARS-CoV-2 IgG and IgA (Euroimmun assay[11]). Despite myeloma biomarkers showing an excellent response to CAR-T-cell therapy, his family ultimately decided to focus on comfort measures because of non-resolving respiratory failure. The patient passed away on day 74 after COVID-19 diagnosis.

Methods:

The patient was enrolled in the University of Pittsburgh's Acute Lung Injury Registry and Biospecimen Repository (IRB# PRO10110387). SARS-CoV-2 RNA quantification was performed using a sensitive (limit of detection 3 copies/reaction) quantitative RT-PCR (qRT-PCR) assay on plasma (days 4, 9, 13, 16, 67, and 71), and in endotracheal aspirate (ETA) fluid from day 72. SARS-CoV-2 Spike-specific deep next-generation sequencing (NGS) was performed on plasma (days 4, 13, 67, and 72) and ETA fluid (day 72). Virus isolation from plasma (days 4, 65, and 71) and ETA fluid (day 72) was attempted on Vero E6 cells, followed by indirect immunofluorescence and dideoxy sequencing of the Spike (S) gene. Total anti-SARS-CoV-2 IgG antibody titers directed against the S-protein receptor binding

domain (RBD) using an indirect enzyme-linked immunosorbent assay (ELISA), competitive ELISA using human ACE2, and pseudovirus neutralization assays were performed with samples from days 4, 9, 13, 16, and 72. Plaque reduction neutralization assays on cultured SARS-CoV-2 (laboratory strain) were also performed (days 4, 65, and 71). To characterize T-cell and B-cell responses, single-cell RNAseq (sc-RNASeq) was performed on peripheral blood mononuclear cells (PBMC) isolated from blood obtained on days 4 and 9. SARS-CoV-2 S-specific T-cell responses were determined by flow cytometric quantification of T-cell frequencies with intracellular staining of interferon-gamma (IFN)γ and CD107a, following co-culture with S-protein and nucleocapsid protein peptide pools. Assay details can be found in the **Supplementary Materials**.

Results:

SARS-CoV-2 RNA measurements:

High levels of SARS-CoV-2 RNA were detected in all plasma samples (**Figure 1-I**). RNAemia was greatest on day 4 (126,792 copies/mL) and showed a >10-fold decrease by day 9 (8,100 copies/mL), after administration of convalescent plasma and remdesivir. RNAemia remained readily detected but <100,000 copies/ml on days 13 (11,508 copies/mL), 16 (18,000 copies/mL), and 67 (14,720 copies/mL), then increased to 101,800 copies/mL at day 71 during administration of dexamethasone. Over 20 billion copies/ml of SARS-CoV-2 RNA ($2.78x10^{10}$ copies/mL) were detected by qRT-PCR in a serially diluted ETA sample from day 72.

Next generation sequencing of SARS-CoV-2 S gene in longitudinal samples:

Six different SARS-CoV-2 sequence variants were identified in longitudinal plasma and ETA fluid between days 4, 13, 67, and 72 (**Figure 2 and supplementary methods)**. On day 4, plasma viral S sequences matched the SARS-CoV-2 GH clade (containing D614G) that was circulating in Pittsburgh at the time. However, by day 13, while the patient was still in the hospital, additional mutations were detected, including a including a R190K and G1124D substitution, which were previously observed in 0.005% (N=9/189,163) and 0% (N=0/189,163) of GISAID SARS-CoV-2 sequences respectively. Comparison of sequences

between days 13 and 67 demonstrated the emergence of several additional mutations, such as a Y144 deletion and a D215G substitution, which were later identified in the United Kingdom (UK) and South African variants, respectively, several months after the patient's death [12, 13]. We also identified an N501T substitution on day 67, which was recently shown to enhance binding affinity to the ACE2 receptor[14]. Interestingly, a substitution at the same location (N501Y) is currently circulating in the UK and South Africa[12, 13]. Finally, mutations continued to emerge between days 67 and 72 (e.g., H146 deletion). The longitudinal emergence of multiple different and novel sequence variants is indicative of intra-host evolution of SARS-CoV-2.

Viral isolation and dideoxy sequencing:

Infectious SARS-CoV-2 was recovered from the day-72 ETA sample (**Figure 1-II**) with a titer of $1.125x10^6$ plaque forming units (PFU)/mL. Numerous SARS-CoV-2 virions were detected by electron microscopy in the ETA sample (**Figure 1-III**). Dideoxy sequencing of the S-gene of cultured virus showed mutations consistent with those identified by NGS (e.g., D614G substitution, Y144 deletion, and H146 deletion). Viral isolation from plasma was unsuccessful despite the presence of SARS-CoV-2 RNA.

Immune responses:

Analysis of PBMCs (days 4 and 9) by immunophenotyping and scRNA-Seq demonstrated absence of B-cells and near total depletion of T-cells, consistent with lymphodepleting chemotherapy with fludarabine and cyclophosphamide (**Supplementary figure 2B**). scRNA-Seq profiling showed interferon-stimulated genes in the monocyte lineage suggestive of a viral infection. We evaluated SARS-CoV-2-specific T-cell responses from days 4 and 67. The day 4 sample contained too few live T-cells (<1%) to assess. The day 67 PBMC sample showed somewhat higher levels of live T-cells (9.2%), with 1.72% and 0.79% of CD8+T cells expressing the cytotoxicity marker CD107a following stimulation with S protein and nucleocapsid peptide pools, respectively, suggesting that a small fraction of the T-cells were able to react to SARS-CoV-2 antigens ex-vivo (**Supplementary figure 3**).

However, the low number of events (<5000 per sample) acquired by flow cytometry prevents any conclusions from being drawn from these analyses.

Despite the administration of convalescent plasma on days 2 and 58, no IgG antibody targeting the S-protein RBD was detected at any time point, nor was there any antibody that competed with human ACE2 binding to RBD, possibly because the convalescent plasma used had a low titer of SARS-CoV-2 antibody, or there was rapid clearance of antibody bound to very higher numbers of virions (**Supplementary table 2)**. Additionally, SARS-CoV-2 plaque reduction neutralization assays showed no plaque reduction in the day 4 sample. However, pseudovirus assays showed ~50% inhibition at a 1:10 dilution in the day 13 sample (**Supplementary figure 1)**. After the second dose of convalescent plasma on day 58, samples from days 65 and 71 showed $\geq$ 50% plaque reduction at a 1:16 dilution. Since IgG antibody against the S-protein RBD was not detected at any time point (days 4, 9, 13, 16, 45, 56, 71 and 72), the observed plaque reduction suggests low titers of neutralizing antibodies or other proteins directed to non-RBD regions of the S-protein in the patient's plasma (**Supplementary table 2)**.

Discussion

We report here sustained SARS-CoV-2 RNAemia for >2 months and intra-host viral evolution in a patient with COVID-19 and both B- and T-cell depletion as a result of the therapies he had received for multiple myeloma. The initial clinical improvement and >10-fold reduction in plasma RNAemia (days 4-9) suggests a treatment response following convalescent plasma (day 2) and remdesivir (days 5-10). RNAemia was never completely suppressed, however, and we hypothesize that the absence of anti-SARS-CoV-2 humoral responses and the paucity of T-cell responses related to prior chemotherapy and anti-BCMA CAR-T-cells resulted in uncontrolled viral replication and overwhelming SARS-CoV-2 pneumonia (1.125×10^6 PFU/mL and 2.78×10^{10} RNA copies/mL in ETA) that likely contributed to death. The detection of a distinct SARS-CoV-2 sequence variant on day 13 of the first hospitalization with 3 coding mutations (**Figure 2**) compared to the initial viral variant detected on day 4 that matched the circulating GH clade argues for viral evolution within the

host and against superinfection or reinfection. Several additional sequence variants including those with coding mutations and deletions in the S gene that have yet to be detected in SARS-CoV-2 in Pittsburgh as of January 2021 were identified on day 67. Furthermore, additional sequence variants rapidly developed in the span of 5 days between days 67 and 72 while the patient was still in the hospital (**Figure 2**), further supporting continued intra-host viral evolution, since reinfection with 5 distinct variants is highly improbable. Taken together, these findings provide insights into the potential duration of continued SARS-CoV-2 replication and the plasticity of the SARS-CoV-2 S gene, as others have noted[15].

RNAemia has been described in immunocompromised patients with non-SARS-CoV-2 respiratory viruses. Respiratory syncytial virus (RSV) RNAemia developed in 30% of hematopoietic cell transplant recipients 2 days after onset of RSV pneumonia and was a predictor of mortality[16]. Emerging data suggest that SARS-CoV-2 RNAemia may be a marker of severity of COVID-19 pneumonia[5, 17], but sustained RNAemia for >2 months has not yet been described. We were unable to isolate replication-competent virus from plasma, which may be due to technical issues, or may suggest that RNAemia is not caused by the presence of virions in the plasma, but rather due to “spillover” of infected cells with SARS-CoV-2 RNA from the lung. The reduction in plasma RNAemia between days 4 and 9 (after administration of convalescent plasma on day 2 and of remdesivir between days 5 and 10) suggests that viral replication was inhibited by the therapies given. Unfortunately, measurement of plasma RNAemia is not readily available for clinical use in the Unites States, and it is not currently known whether suppression of SARS-CoV-2 RNAemia can improve clinical outcomes. The rebound of viremia back to 101,800 RNA copies/mL on day 71 occurred in the setting of the administration of dexamethasone. Whether corticosteroids should be avoided in heavily immunosuppressed individuals in whom prolonged SARS-CoV-2 replication is suspected warrants further study. Ultimately, randomized trials are needed to evaluate whether measurement of RNAemia should be used to determine clinical response to SARS-CoV-2 therapies.

We isolated virus from respiratory samples collected 72 days after COVID-19 onset, demonstrating the potential for infectivity late into the clinical course. In a prior study of immunocompetent patients with mild COVID-19, virus isolation from samples obtained after 8 days was unsuccessful[3]. Our findings suggest that certain severely immunosuppressed patients with COVID-19 may require isolation longer than the 20-day period currently proposed by the Centers for Disease Control[18]. Whether PCR cycle thresholds (which were consistently <25 in our patient) can be used to make decisions related to discontinuation of transmission-based precautions warrants further investigation. Importantly, replication-competent variants isolated in cell culture harbored deletions associated with increased transmissibility similar to what has now been identified in the UK months later[12]. These findings raise the possibility that the origin of the highly mutated UK and South African variants may have been persons with protracted infection.

Humoral and cell-mediated immunity after COVID-19 appear to be necessary to control SARS-CoV-2 infection [19, 20]. In our patient, PBMC analysis confirmed B-cell aplasia, which is compatible with negative assay results for IgGs against the S-protein RBD, or IgAs/IgGs using a clinically-approved assay. IgGs against the S-protein IBD were absent at all time points (days 4, 9, 13, 16, 45, 56, 71, and 72) despite the fact that the patient received convalescent plasma on days 2 and 58 of illness. This finding suggests either low titer of antibodies in the convalescent plasma or rapid clearance of antibodies in the context of high viral burden. Interestingly, there was evidence of low-level viral neutralization on days 65 and 71 (after the second dose of convalescent plasma) using live virus neutralization assays, which may suggest the presence of low titers of neutralizing antibodies directed to non-RBD regions of Spike or low titers of other antibodies in the convalescent plasma samples. scRNA-Seq profiling from days 4 and 9 showed upregulation of interferon-stimulated genes in monocytes, suggesting the presence of innate antiviral immunity, which was unable to control the infection. SARS-CoV-2-specific T-cell response assays did show some evidence of T-cell activation late into the disease course, although the T-cell lymphopenia related to antecedent chemotherapy limited our ability to draw conclusions

about the patient's T-cell responses. Overall, these findings support the hypothesis that the diminished T-cell responses and nearly absent B-cell responses led to uncontrolled SARS-CoV-2 infection.

In conclusion, our case highlights that immunocompromised patients with profound lymphocyte defects such as CAR-T-cell therapy recipients are at risk for prolonged SARS-CoV-2 replication. Other patients with severe lymphocyte deficiencies, such as anti-CD20 monoclonal antibody recipients, and hematopoietic cell or organ transplant recipients within the first few months of transplant, may also be at risk. Clinicians caring for such patients should be cautious not to attribute persistent detection of SARS-CoV-2 RNA from clinical samples of these patients to the presence of non-infectious virus and should consider revising their transmission-based precautions for these patients. Further work is needed to define the role of monitoring RNAemia in managing immunocompromised persons with COVID-19, the duration of infectivity in these patients, the importance of intra-host emergence of SARS-CoV-2 sequence variants in immune escape, and the immune phenotypes associated with recovery versus fatal infection. More effective antiviral agents to suppress SARS-CoV-2 replication are urgently needed, especially for immunodeficient hosts.

NOTES:

Author contributions: Drs. Hensley and Bain contributed equally to this article. Drs. Jacobs and Nambulli contributed equally to this article. All authors have seen and approved the manuscript and contributed significantly to the work. The manuscript has not been previously published nor is it being considered for publication elsewhere.

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Figure legends:

Figure 1. I) Clinical timeline showing CAR-T-cell infusion (days -25 to 0), first hospital admission (days 0-17), home stay (days 18-41), and second hospital admission (days 41-74). Day 0 denotes day of first positive nasopharyngeal (NP) swab PCR for SARS-CoV-2 RNA. The top panel shows the clinical course and timeline, with results of clinical SARS-CoV-2 NP swab RT-PCR testing and clinical IgG and IgA testing. The bottom graph shows serial plasma SARS-CoV-2 RNA quantification obtained using a research qRT-PCR assay, with viral quantification from endotracheal aspirate fluid using research assays (qRT-PCR and infectious viral titer). Inset shows a coronal view of a chest CT (computed tomography) from day 41. Green squares: convalescent plasma (CP); red squares: remdesivir; yellow squares: dexamethasone. **II) Indirect immunofluorescence of SARS-CoV-2 (Green) isolated in Vero E6 cells (Red/Blue) from day 72 endotracheal aspirate sample.** Green: antibody directed against SARS CoV-2 spike protein (Sinobiologicals). Blue: DNA counterstained with DAPI. Red: filamentous actin counterstained with phalloidin. A syncytium is shown in the inset (B) at higher magnification. Scale bars: 100 µm. **III) Electron microscopy of endotracheal aspirate sample obtained on day 72.** (A) Comparison of virus localization in a bronchial epithelial cell (top) and a transient secretory cell (bottom). Each image is shown as montaged 2D overviews of the whole cell in a 200 nm section (left), overlaid with colored dots to indicate positions of virions (center) and 3D tomographic reconstructions detailing virus populations within cytoplasmic compartments. (B) 2D overview of SARS-CoV-2–infected ciliated epithelial cell in a 200 nm semi-thick section. The apical side of the cell is characterized by numerous membrane-bound compartments that are filled with SARS-CoV-2 virions. (C) Montaged tomogram of the apical side of the cell shown in B; hundreds of virus particles are contained within smooth-walled cytoplasmic compartments. Inset in C: Detail of a single virion with spikes indicated by red dots. (D) Tomogram detail of a smooth-walled cytoplasmic compartment containing at least 30 SARS-CoV-2 virions within the shown 15 nm thick volume.

Figure 2. Mutations and deletions in the SARS-CoV-2 Spike gene identified in the patient's samples compared to the SARS-CoV-2 GISAID GH Clade circulating in Pittsburgh. The full genome at the top shows the GH clade sequence. The enlarged S gene shows all the mutations identified in the patient's samples compared with the GH clade. The sequence alignments in S compared to the GH clade are shown for each of the multiple (6) sequence variants (var) identified by deep next-generation sequencing (Illumina). All of the sequence variants were detected in plasma. Day 72 (*) shows the matching sequence variants identified in the endotracheal aspirate sample. The D614G substitution was found in all samples.
# indicates mutations detected as mixed populations <100%, but >20%.
E envelope; FP; fusion peptide; HR 1, heptad repeat 1; M, membrane; N, nucleocapsid; NTD, N-terminal domain; ORF, open reading frame; SP, signal peptide RBD receptor binding domain; RBM, receptor binding motif; TM, transmembrane domain; Var, sequence variant.

Figure 1

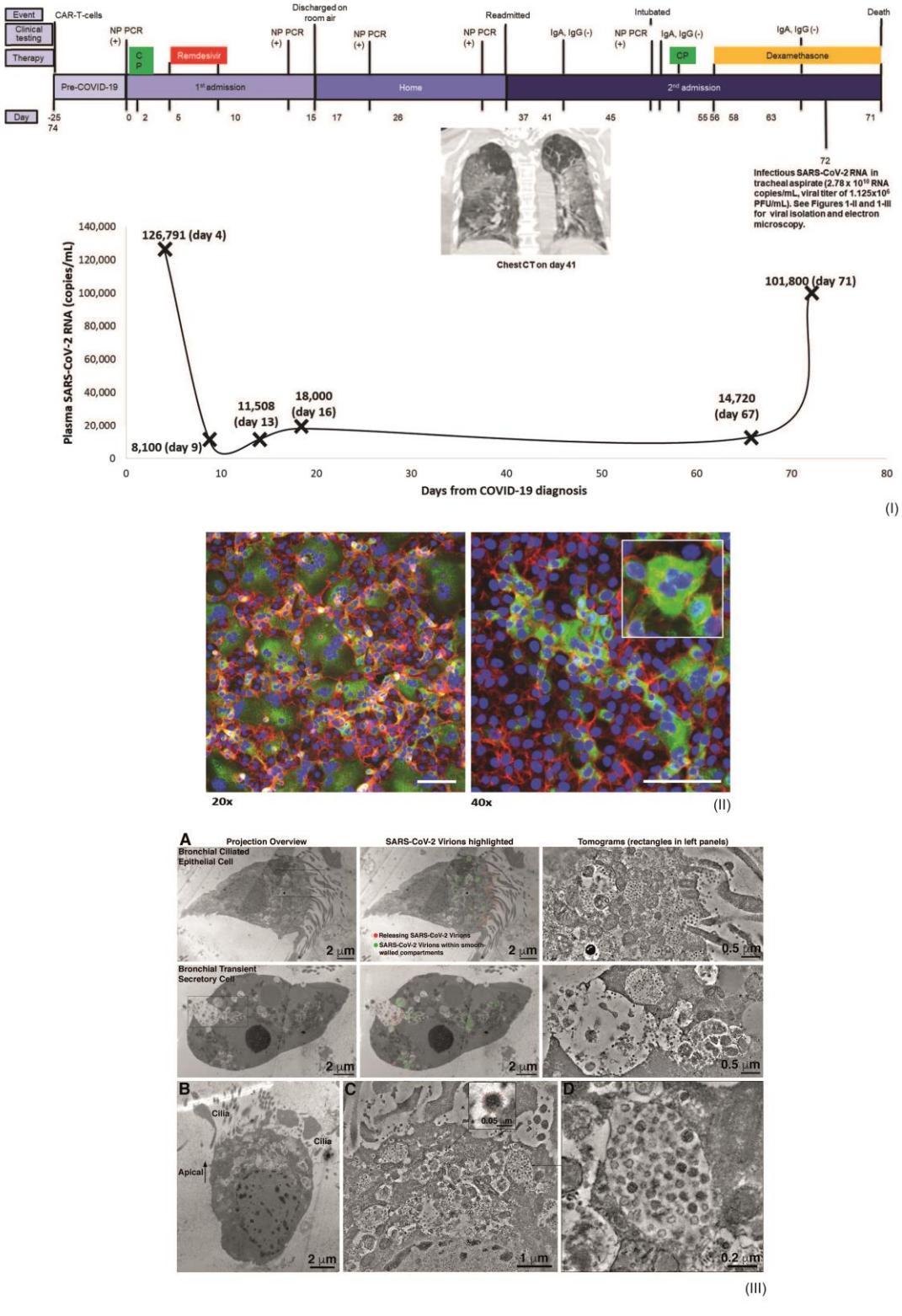

Figure 2