Residual SARS-CoV-2 viral antigens detected in GI and hepatic tissues from five recovered patients with COVID-19

We read with great interest the article published by Zuo et al, which highlighted the presence of SARS-CoV-2 RNA in stool samples during active and convalescence phases of COVID-19 infection. However, no study has reported the presence of viral antigens within GI and hepatic organs during the convalescent phase.

Using conventional immunohistochemistry, we detected SARS-CoV-2 nucleocapsid protein (NP) in the colon, appendix, ileum, haemorrhoid, liver, gallbladder and lymph nodes (figure 1A–K) from five patients who recovered from COVID-19, ranging from 9 to 180 days after testing negative for SARS-CoV-2 (online supplemental table 1). Notably, when multiple tissues were obtained from one patient (patients 1 and 4), all the tissues showed the presence of the viral antigen, suggesting widespread multiorgan involvement of the viral infection. Interestingly, for the colon, the viral antigen was only present in normal colonic crypts and polyps but not in the neoplastic tissues (figure 1Q). Similar negative staining in the hepatocellular carcinoma tumour region was also observed (figure 1R) albeit the positive staining in some of the scattered immune cells (figure 1D). Validating our findings, we detected SARS-CoV-2 spike protein (figure 1L–P) and RNA (figure 2B–F) in the above-mentioned tissues using conventional immunohistochemistry and RNAscope, respectively. However, we were unable to detect viral RNA in some patients’ tissues (online supplemental table 1), possibly because of higher RNA degradation rate as compared with protein and other patient-dependent factors such as disease severity, time since recovery and basal metabolic rate.

In addition, multiplex immunohistochemistry and RNAscope staining showed that some SARS-CoV-2-positive cells colocalised with ACE2 receptor and CD68 in the colon and liver (figure 2A,B). These cells were likely of monocyte lineage and liver-resident sinusoidal Kupffer cells, which therefore confirmed our earlier speculation that was based on cellular morphology. This suggests that SARS-CoV-2 might indeed infect these immune cells directly, as previously reported. Finally, on detection and validation of viral antigens in the tissues, we interrogated whether the tissues harboured an immune response to the virus. We performed ex vivo peptide stimulation assays whereby blood and tissues were incubated with a cocktail of the viral nucleocapsid, spike and membrane proteins, followed by flow cytometry analysis. Notably, SARS-CoV-2-specific CD38+Granzyme B+CD4+ T
cells were isolated from the tissues in a comparable fashion with matched blood samples (figure 2G), suggesting that SARS-CoV-2-specific memory T cells may be maintained in both blood and tissue over a period of time. Nevertheless, further study is warranted to compare the tissue immune microenvironment before and after the infection and to confirm whether the immune cells in the proximity of viral antigen are indeed specific to SARS-CoV-2.

Several groups have reported the phenomenon where patients who had recovered from mild or moderate COVID-19 later tested positive in nasopharyngeal swabs or sputum samples, raising concern for residual virus reservoirs and potential transmissibility in recovered individuals. Although respiratory transmission is responsible for most COVID-19 infections, there is increasing evidence of COVID-19 causing GI and hepatic manifestations, as studies reported the presence positive SARS-CoV-2 RNA in anal swabs and stool samples, despite nasopharyngeal or sputum specimens testing negative for the virus. These reports are in line with our findings of the intestinal tissues of patients with negative nasopharyngeal swab tests. Also, while SARS-CoV-2 viral antigen has been detected in the lung tissues of deceased patients despite negative nasopharyngeal swab tests, our findings constitute the first evidence of residual virus in extrapulmonary tissues during the convalescent phase, up to 6 months after recovery, in a non-postmortem setting. It seems that a negative swab result might not necessarily indicate complete viral clearance from the body. Yet unfortunately, we were unable to determine whether the viral antigens isolated from the tissues were infectious, as the virus was inevitably destroyed during tissue fixation. We also did not have access to anal swab, stool samples and viral isolation facility, which would have provided additional insight into the possibility of faecal–oral transmission. Regardless, based on these preliminary findings, we believe that further research in a larger cohort is warranted to explore the replication and infectivity of the virus in tissue specimens and to understand the GI and hepatic involvement in COVID-19.

Figure 2 Multiplex immunohistochemistry, RNAscope and flow cytometry analysis of tissues obtained from five patients with COVID-19. (A) Representative images of liver tissue stained using multiplex immunohistochemistry for DAPI (blue), SARS-CoV2 NP (red), ACE2 (green) and CD68 (magenta). #DP1: double positive cell 1; #TP1 and #TP2: triple positive cell 1 and 2, respectively. (B) Representative images of colon tissue stained using RNAscope for DAPI (blue), SARS-CoV-2 S gene (red), ACE2 (green) and CD68 (magenta) RNA. (C–F) Representative RNAscope images of haemorrhoid (C and D) and ileum (E and F) stained for DAPI (blue), SARS-CoV-2 S gene (red) and CD68 (green). Some SARS-CoV-2-positive cells were stained positive for CD68, as indicated by the white arrows (D and F). (G) SARS-CoV-2-specific CD4 T cells were identified from the CD45\(^+\)CD3\(^+\)CD4\(^+\)CD39\(^+\)CD103\(^+\)CD38\(^+\)Granzyme B\(^+\) population, where CD39, CD103 and CD38 select for immune cells with a memory phenotype, and granzyme B selects for immune cells with a functional phenotype. Representative pseudo-colour plots of CD38\(^+\)Granzyme B\(^+\) CD4\(^+\) T cells following stimulation with SARS-CoV-2 peptides in tissue and blood samples of patient 1 against healthy donor blood. Similar results were obtained from patient 2. The numbers indicate the percentages in the drawn gates. The plots shown are representative of at least two independent experiments. (A–F) Scale bar: 100 µm.

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CCLC, DG and XL contributed equally.

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REFERENCES


Supplemental material

Materials and Methods

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endogenous peroxidase blocking (Leica Biosystems, Newcastle). Next, the slides were incubated with primary antibodies against SARS-CoV-2 NP, ACE2 (Abcam, Cat# Ab108252, EPR4435(2)) and CD68 (Dako, Cat# DKO.M087601, PG-M1), followed by incubation with polymeric HRP-conjugated secondary antibodies (Leica Biosystems, Newcastle). Then, the samples were incubated with Opal fluorophore-conjugated tyramide signal amplification (TSA) (Akoya Biosciences, USA) at a 1:100 dilution. The slides were rinsed with wash buffer (BOND Wash Solution 10X Concentrate) after each step. Following TSA deposition, the slides were again subjected to heat-induced epitope retrieval to strip the tissue-bound primary/secondary antibody complexes prior to further labelling. These steps were repeated until the samples were labelled with all six markers and spectral DAPI (Akoya Biosciences, USA) at a 1:10 dilution. Finally, the slides were mounted in ProLong Diamond Anti-fade Mountant (Molecular Probes, Life Technologies, USA) and developed in the dark at room temperature for 24 h. Images were captured for each case under a Vectra 3 pathology imaging system microscope (Akoya Biosciences, USA), and then analysed and scored by a pathologist using inForm software (version 2.4.2; Akoya Biosciences) and HALO™ (Indica Labs).

**RNA extraction and RT-PCR.** Total RNA was extracted using an AllPrep FFPE DNA/RNA Kit (Qiagen, Germany) according to the manufacturer’s protocol. Extracted RNA was tested for SARS-CoV-2 envelope (E) and spike (S) genes using the RealStar® SARS-Cov-2 RT-PCR kit RUO (Altona Diagnostics, Germany) following the manufacturer’s instructions[8] on the CFX96 (BioRad) real time thermal cycler. Each RT-PCR reaction (total volume 30 µl) comprised 20 µl master mix from the kit and 10 µl template. Fluorescent-labelled probes were used to enable parallel identification and detection of target RNA. Samples in which both S and E genes showed positive amplification signal above the baseline within 45 cycles were considered positive. Amplification below the baseline were marked as ‘Undetectable’, and thus negative for the S and E genes. If positive amplification signal was only displayed for one of
the two targets, it was reported as a ‘Presumptive positive’. The limit of detection of the Altona RealStar® SARS-CoV-2 assay based on the E gene at 95% confidence was determined to be 14.85 copies/ml using Probit analysis. Each run contained a positive control for E and S genes, and a non-template control. The cycling conditions were according to the manufacturer’s instruction with a total of 45 amplification cycles.

**RNAscope.** RNAscope in situ hybridisation (Advanced Cell Diagnostics, USA) assay was performed according to standard manufacturer protocol,[9] on FFPE tissue sections. Deparaffinised tissues were subjected to peroxidase inhibition and pre-treatments, followed by incubation with specific probes (ACE2, Cat# 848151; CD68, Cat# 560591-C2; SARS-CoV2-S, Cat# 848561-C3) and Opal dyes prior to DAPI counterstain. RNAscope Multiplex Fluorescent Reagent Kit v2 (Cat# 323100) and Opal TSA Plus fluorophores (Akoya Biosciences, USA) were used for detection. Appropriate positive and negative controls were included in accordance to manufacturer’s recommendation. Images were acquired using a Vectra 3 pathology imaging system (Akoya Biosciences, USA).

**Ex vivo peptide stimulation assay.** To simulate SARS-CoV-2 infection and to examine SARS-CoV-2-specific immune cells, SARS-CoV-2 PepTivator peptide pools (Miltenyi Biotec, Germany) were used. Lyophilised peptide pools were reconstituted as per the manufacturer’s instructions. Blood and tissue samples were prepared as previously described.[5] A total of 1x10^6 cells were stimulated with 1 μg/mL peptides for 16 h at 37°C in 5% CO_2 in RPMI 1640 media (Gibco, USA) supplemented with 10% FBS (Hyclone) and 1% Penicillin-Streptomycin-Glutamine (Gibco, USA).[10, 11] Negative controls were left unstimulated. Brefeldin A (1 μg/ml, Sigma Aldrich, Germany) was added 2 h into the stimulation assay.

**Flow cytometry and Analysis.** Cells were stained with Zombie NIR Fixable Viability dye (BioLegend, USA) for 10 min at 4°C in the dark, washed with PBS at 300 g for 5 min at 4°C,
and blocked with Human TruStain FcX (BioLegend, USA) for 10 min at room temperature prior to staining with fluorescent-conjugated antibodies. Then the cells were surface stained with an antibody cocktail containing Pacific Orange-anti CD45 (Invitrogen, Cat# MHCD4530, HI30), BV605-anti CD103 (BD Biosciences, Cat# 743652, Ber-ACT8), BV750-anti CD4 (BD Biosciences, Cat# 566355, SK3), Alexa 532-anti CD3 (Invitrogen, Cat# 58-0038-42, UCHT1), PerCP-eFluor 710-anti CD38 (Invitrogen, Cat# 46-0388-42, HB7) and PE-CF594-anti CD39 (BioLegend, Cat# 563678, TU66) in Brilliant Stain Buffer (BD Biosciences, USA), and incubated for 30 min at 4°C in the dark.

Prior to intracellular staining, the cells were fixed and permeabilised with BD Cytofix/Cytoperm™ (BD Biosciences, USA). The cells were then stained with PE-Cy5.5-anti Granzyme B (Invitrogen, Cat# GRB18, GB11) in Brilliant Stain Buffer (BD Biosciences, USA), for 30 min at 4°C in the dark. Finally, the cells were washed with 1x BD Perm/Wash Buffer™ (BD Biosciences, USA) and resuspended in PBS containing 2% FBS for flow cytometry using Cytek Aurora spectral flow cytometer (Cytek Biosciences, USA). Data analysis was performed using FlowJo V.10 software (FlowJo LLC, USA).
Supplementary Table 1. Cohort characteristics.

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References


