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Review article

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SARS-CoV-2 reservoir in post-acute sequelae of COVID-19 (PASC)

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Supplementary Note

Approaches and methodologies to study SARS-CoV-2 reservoir

The concurrent use of different approaches on samples obtained from the same PASC cohort is likely to result in the most comprehensive understanding of SARS-CoV-2 reservoir and related biological factors in PASC (Supplementary Figure 1). If tissue and blood samples are collected from the same PASC study participants, identification of SARS-CoV-2 RNA or protein in tissue can be correlated with the identification of SARS-CoV-2 protein or immune responses in plasma. The comparison could inform how well potential blood-based biomarkers of PASC reflect tissue-based pathology. Careful selection of control study participants is also a priority because the presence of SARS-CoV-2 RNA or protein in asymptomatic individuals also requires further study.

Autopsy studies can identify SARS-CoV-2 RNA or protein in tissues that cannot be easily or safely obtained via biopsy from living patients. These include the brain, spinal cord, cardiopulmonary tissue, vascular tissue, spleen, and liver. Viral genome sequencing can be performed on tissue obtained via autopsy to clarify the role of SARS-CoV-2 mutations potentially associated with persistence in certain anatomical locations. For example, in their post-COVID autopsy study of SARS-CoV-2 persistence throughout the body and brain, members of our team (Stein *et al.*) measured the diversity and anatomic distribution of intra-individual SARS-CoV-2 spike gene variants via high-throughput single-genome amplification and sequencing (HT-SGS)[31]. Because the RNA required for optimal sequencing analysis and identification of SARS-CoV-2 RNA generally rapidly degrades, autopsy studies that prioritize short postmortem intervals, and either flash-freeze or preserve the fresh tissue in DNase/RNase blocking solution, are most likely to succeed in accurately identifying and characterizing SARS-CoV-2 activity in tissue. PASC tissue obtained at autopsy could also be analyzed for host immune activity or tissue damage, however postmortem changes may alter the tissue landscape [119]. It should be taken into consideration that presence of comorbid conditions and/or use of antiviral or immunosuppressive medications in individuals whose tissue is examined at autopsy might impact study interpretation related to SARS-CoV-2 persistence**.**

Positron emission tomography (PET) imaging can identify SARS-CoV-2 reservoirs in living PASC patients in tissue locations that cannot be accessed via biopsy. For example, ImmunoPET-CT imaging using ^{89}Zr-VRC01, a positron-labeled broadly neutralizing antibody against the HIV gp120 surface glycoprotein was successfully used to identify deep tissue HIV reservoirs [120] [121].The same approach can be applied for identification of SARS-CoV-2 protein via use of anti-SARS-CoV-2 monoclonal antibodies tagged with a positron-emitting radioisotope. Other agents such as the novel radiotracer [^{18}F]F-AraG can localize and characterize tissue-based activated T cell responses in PASC patients that can be correlated with SARS-CoV-2 reservoir in certain deep tissue body sites[122] [123]. If tissue samples (e.g., lymph node and gut) can be obtained from study participants undergoing imaging, identification of SARS-CoV-2 RNA, protein and cellular immune responses in such samples may be correlated with the imaging data, allowing for additional validation of study findings.

Tissue biopsy and surgical tissue studies can identify SARS-CoV-2 RNA and protein in samples collected from living PASC individuals in a manner that avoids postmortem autopsy changes because samples can be preserved immediately and optimally. Sample types that can be collected via biopsy or during surgery from PASC individuals include intestinal, lung, lymph node, skeletal, salivary gland, olfactory epithelium, hemorrhoid, tonsil, and appendix. SARS-CoV-2 RNA and protein can be identified in biopsy or surgically collected tissue samples via advanced microscopy, ddPCR, immunohistochemistry and other methods. Tissue samples collected at multiple longitudinal time points can undergo sequencing for SARS-CoV-2 genomes to inform the study of viral mutations that might facilitate persistence over time. Sequencing technologies such as spatial transcriptomics can be used to characterize host immune and gene expression changes near identified SARS-CoV-2 RNA or protein**.** Such analyses should provide clarity on whether host immune signaling is altered or dysregulated near identified viral RNA or protein to better understand mechanisms by which SARS-CoV-2 may evade the immune response to persist. Single-nucleus RNA sequencing can be used to determine if tissue damage or remodeling and vascular endothelial cell dysregulation is associated with SARS-CoV-2 RNA or protein persistence.

Ultrasensitive assays like Simoa [124] with high sensitivity to measure SARS-CoV-2 antigens can be used to detect viral antigens in blood or other body fluids that may reflect the presence of a SARS-CoV-2 reservoir in tissue. A growing number of platforms can also map seroreactivity to SARS-CoV-2 proteins in various PASC sample types. A benefit of these platforms is that in some cases mapping can be simultaneously performed to determine seroreactivity to a large number of other viral proteins, including those expressed by human herpesviruses and human endogenous retroviruses (HERVs) as well as non-viral pathogens. Examples of such high-throughput approaches include rapid extracellular antigen profiling (REAP), which detects antibody reactivity to the human exoproteome and viral glycoproteins [59]proteome-scanning libraries using phage immunoprecipitation sequencing (PhIP-seq)[125], and bacterial display such as Serimmune's SERA technology that can detect antibodies to 10^{10} linear epitopes.

The activity of adaptive immune cells can serve as biosensors of SARS-CoV-2 persistence. T cells have two key properties that allow them to exhibit potent biosensor capabilities of viral persistence: 1) ultrasensitive immune recognition; 2) the ability to 'record' environmental information. Key metrics for T cell interrogation should include markers of immune activation and proliferation, as described previously in the context of acute COVID-19[126] and the expression of coinhibitory receptors demarcating exhaustion, as described in other chronic infections [127] [128] **[129] [130]** which could also help explain a failure of the adaptive immune system to clear SARS-CoV-2. GC B cell responses can also be a useful biosensor for the presence of persistent SARS-CoV-2 antigens**.** It should be noted however that while the continued appearance of ASCs and circulating Tfh cells in samples collected after acute COVID-19 suggests SARS-CoV-2 viral and/or antigen persistence, one surprise from recent studies of SARS-CoV-2 mRNA vaccination has been the persistence of GC responses for at least 6 months in most individuals [131]. Thus, a key aspect of examining GC-dependent immune responses in PASC will be careful comparison not only of the presence of ASC or activated memory B cells, Tfh, and

Tex cells, but detailed examination of their qualitative features compared to post COVID-19 non-PASC and even mRNA vaccinees.

***In vitro* organoid systems and animal** models can be used to study SARS-CoV-2 persistence, immune evasion, and mechanisms of mutations contributing to infected cell survival. Organoid and/or cell culture lines exist for SARS-CoV-2 infection and PASC associated cells, including hepatocytes, cardiomyocytes, endothelial cells, neurons, and myeloid cells. Transcriptional profiling in these models can provide insight into the response to SARS-CoV-2 infection and SARS-CoV-2 antigens across potential reservoir sites. Infection of animals can also provide clarity on mechanisms of SARS-CoV-2 reservoir**,** provided appropriate homology with relevant human pathways and receptors. A variety of small animal models have been developed for coronavirus research including the golden Syrian hamster [132] and transgenic mice expressing the human ACE2 receptor [133]. Non-human primate models have also been used to document SARS-CoV-2 persistence. For example, Böszörményi *el al.* identified SARS-CoV-2 RNA (and in some cases sgRNA) in post-mortem tissues in 50% of macaques sacrificed 5-6 weeks after acute infection, including in heart, lungs, and tracheobronchial lymph nodes[25]. Further development of cell culture, organoid, and animal models is an important priority for the study of SARS-CoV-2 reservoir and related biological factors in PASC.

Because SARS-CoV-2 reservoir may directly contribute to inflammatory, immunomodulatory, coagulation, microbiome, autoimmune, and neuroimmune abnormalities in PASC individuals, studying these biological factors in concert with SARS-CoV-2 reservoir is imperative. This goal can be achieved by adding additional forms of analysis into SARS-CoV-2 reservoir studies. For example, lung tissue and bronchial lavage fluid can be collected from the same PASC cohort. Tissue could be analyzed for SARS-CoV-2 RNA and protein and fluid for upper respiratory microbiome composition and activity via metatranscriptomics. Findings can then be correlated to determine if persistence of SARS-CoV-2 correlates with potential microbiome dysbiosis in the general lung environment. Artificial intelligence approaches that leverage standardized research and clinical metadata should help with identifying key trends and associations**.**

Supplementary Figure 1

Legend: Diverse approaches and methodologies can be used in the study of SARS-CoV-2 reservoir. BioRender licensed software was not used to create the figure.

Autopsy Studies

Strengths

- Can identify SARS-CoV-2 RNA and protein in tissues that cannot be obtained safely via biopsy, including from the CNS
- Viral genome sequencing can identify SARS-CoV-2 mutations associated with persistence in certain anatomical locations
- Tissue cytopathology near identified RNA and protein can be assessed

Weaknesses

- Short postmortem interval is necessary for optimal tissue preservation
- Perimortem changes can alter the tissue's transcriptional landscape, meaning tissue is not optimized for transcriptome-based approaches that capture host immune & gene expression

Imaging Studies

Strengths

- Can identify SARS-CoV-2 spike protein and T cell activity in tissue locations in living patients that otherwise cannot be accessed via biopsy
- SARS-CoV-2 spike protein and T cell activity in a wide range of tissue sites can be measured simultaneously

Weaknesses

- High cost of analysis and imaging scanners only available in limited locations
- Radioligands are limited by penetration and specificity

Biopsy & Surgical Sample Studies

Strengths

- Samples can be preserved immediately and optimally for both genomic and protein analyses
- Allows for optimal use of sequencing technologies to characterize host immune and gene expression changes near identified SARS-CoV-2 RNA or protein (e.g., spatial transcriptomics)

Weaknesses

- Only certain tissue types can be safely obtained via biopsy, and tissue sample size must be small

Ultrasensitive Protein & Antibody Detection in Fuids

Strengths

- Analysis can be performed on body fluids (e.g., blood, saliva) which can be collected non-invasively and at routine study visits

Weaknesses

- Protein from SARS-CoV-2 reservoir in certain tissues or CNS sites may not enter body fluids or could be bound by antibodies. This could prevent recognition by relevant immunoassays
- In some cases it is known that circulating markers do not accurately reflect local responses (e.g., cytokines)

Adaptive Immune Cells as Biomarkers of Persistence

Strengths

- The adaptive immune response can act as a sensitive indicator of virus persistence, with single-molecule detection possible at the level of cognate viral epitopes displayed on the infected cell surface

Weaknesses

- Expensive and requires a large amounts of sample to isolate specific cell types

Organoid & Animal Studies

Strengths

- Can be directly infected and used to explore mechanisms of persistence, including how viral variants and mutations can contribute to the survival of infected cells

Weaknesses

- Lack of homology between animal model pathways and human pathways must be considered
- Culture and organoid models are incomplete biological systems

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