Differential transcriptomic landscapes of multiple organs from SARS-CoV-2 early infected rhesus macaques

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Resource

Keywords: SARS-CoV-2, multi-organ injuries, COVID-19, clinical complications

Posted Date: April 16th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-403788/v1>

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Abstract

SARS-CoV-2 infection causes complicated clinic manifestations with variable multi-organ injuries, however, the underlying mechanism, in particular immune responses in different organs, remains elusive. In this study, comprehensive transcriptomic alterations of 14 tissues from rhesus macaque infected with SARS-CoV-2 were analyzed. Compared to normal controls, SARS-CoV-2 infection resulted in dysregulation of genes involving diverse functions in various tissues/organs examined, with drastic transcriptomic changes in cerebral cortex and right ventricle. Intriguingly, cerebral cortex exhibited a hyperinflammatory state evidenced by significant upregulation of inflammation response-related genes. Meanwhile, expressions of coagulation, angiogenesis and fibrosis factors were also up-regulated in cerebral cortex. Neuronal receptor NRP1 expression showed a significant induction by SARS-CoV-2 in cerebral cortex, which might be responsible for a higher infectivity and consequent inflammatory response. Overall, our study depicts a multi-tissue/organ transcriptomic landscapes of rhesus macaque with early infection of SARS-CoV-2, and provides important insights into the mechanistic basis for COVID-19-associated clinical complications.

Introduction

The pandemic coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, is becoming a public health emergency and an unprecedented global threat worldwide. Together with SARS-CoV and MERS-CoV, SARS-CoV-2 belongs to the human coronavirus (CoVs) and displays a higher transmissibility than the other two CoVs. As one of the respiratory viruses, SARS-CoV-2 infection causes pneumonia and acute respiratory distress syndrome in the lungs^{1,2}. However, many infected patients had the symptoms manifested with injury in the liver³, heart⁴, vascular system⁵, kidney⁶, and nervous system⁷. The possible reason for such a multiple organ syndrome is the impeded exchange of O₂ and CO₂ in the virus infected lungs, leading to hypoxia and subsequent multiple organ damages. Moreover, SARS-CoV-2 could directly invade organs beyond the lungs^{8,9}. For a better clinical treatment or intervention, it is still needed to clarify whether those injuries in multiple organs are caused directly by SARS-CoV-2 infection or/and indirectly by the severe hypoxia in COVID-19 patients.

To date, a recent study based on proteomic analysis have characterized the genetic responses to SARS-CoV-2 infection in some organs of COVID-19 patient autopsies¹⁰. However, the underlying molecular features in the infected or consequently affected organs during early stage of infection remain obscure. Here, we established the transcriptome-scale comprehensive molecular profiles of 14 tissues (cerebral cortex, brainstem, cerebellum, medulla, left ventricle, right ventricle, muscle, lung, kidney, spleen, liver, intestine, stomach and reproductive organs) collected from each of three rhesus macaques infected by SARS-CoV-2 for 7 days and one uninfected control. The dysregulated RNA existed in at least one tissue of the infected rhesus macaques was counted, and a total of 18,730 RNAs were identified. This data resource uncovers the transcriptome-wide molecular changes in multiple organs during the early stage of

SARS-CoV-2 infection, which will improve our understanding of molecular basis for the multi-organ injuries in the infected individuals.

Results

Wide distribution and replication of SARS-CoV-2 in organs of rhesus macaques. SARS-CoV-2 is a positive-sense single-stranded RNA virus, and its replication is thought to occur in double-membrane vesicles, where negative-sense RNAs are produced. Based on this viral information, green and red fluorescence-labeled probes were designed to recognize sense and antisense viral RNAs, respectively. Combined with a RNAscope assay, the cellular replication of SARS-CoV-2 was well detected. Here, we used SARS-CoV-2 to infect rhesus macaque, and performed RNAscope to investigate both the *in situ* distribution and replication of SARS-CoV-2 in different tissues, including lung, heart, cerebral cortex, cerebellum, liver, kidney microvascular vessel and testis (Fig. 1 and Extended Data Fig. 1). The rhesus macaques were infected with SARS CoV-2 virus at 10^6 TCID₅₀, and the tissues were collected at 7 days post infection (dpi) in the ABSL3 laboratory¹¹. Upon SARS-CoV-2 infection, a severe lung interstitial inflammation with extrudate of proteins in alveoli was evidenced in all animals examined (Fig. 1a), implying a well-established rhesus macaque model for COVID-19. All RNAscope analyses for expression (Green fluorescence, FITC-labelled) and replication (Red fluorescence, Cy3-labelled) of SARS-CoV-2 were performed on formalin-fixed paraffin-embedded (FFPE) sections. The fluorescence signals were acquired and analyzed using TissueGnostics. We found that in addition to the severely infected lung epithelial cells showing a focal signal for both SARS-CoV-2 expressions and replications (Fig. 1a), myocardial cells in left and right atria and ventricles also displayed expression of SARS-CoV-2, mainly located at both side of enlarged nuclei (Fig. 1b). Likewise, abundant SARS-CoV-2 can be detected in testicular Sertoli cells (Extended Data Fig. 1a), probably due to their high expression of angiotensin-converting enzyme 2 (ACE2)¹². However, only a small portion of hepatocytes showed invasion of SARS-CoV-2 (Extended Data Fig. 1b). In the kidney cortical regions, sparse expression and replication of SARS-CoV-2 was observed in proximal, distal convoluted tubules, and collecting tubules (Extended Data Fig. 1c), as well as endothelial cells of the interstitial micro-vessels (Extended Data Fig. 1d). No SARS-CoV-2 signal was observed in Leydig and spermatocytes. Notably, we found a sparse signal of SARS-CoV-2 expression and replication in various cerebral cortex regions, including cerebral cortical neuronal cells (Fig. 1c) and cerebellar Purkinje cells (Fig. 1d). Collectively, these data showed a wide distribution and replication of SARS-CoV-2 in multiple organs of rhesus macaques, with rich levels in the lung, heart and testis (Supplementary Table 1), suggesting its higher infectivity and potential pathogenicity.

Distinct transcriptomic features of rhesus macaque organs. To investigate the organ-specific transcriptomes of the healthy control samples, we obtained the transcriptomic profiles by RNA-seq in each of 14 organs from non-infected control rhesus macaque (Fig. 2a). The tissue markers specifically for different organs were selected from Human Cell Landscape¹³ and used to validate the data quality. The markers with high expression levels in their corresponding organs were demonstrated (Fig. 2b, c, Extended Data Fig. 2a and Supplementary Table 2), justifying our collected samples suitable for further

analysis. We then performed principle components analysis (PCA) and hierarchical clustering for different organs and found that functionally closed organs were well clustered, such as tissues of cerebral cortex, brainstem, cerebellum and medulla of the brain organ, intestine and stomach of digestive system, and left ventricle, right ventricle and muscle related cardiovascular system (Fig. 2d and Extended Data Fig. 2b).

To further identify the organ-specific genes and their functions in rhesus macaque, we performed K-means clustering analysis for all tissues, and 8 clusters were categorized by their whole transcriptomic profiling (Fig. 2e,g, Extended Data Fig. 2c-h and Supplementary Table 3). The genes from different clusters displayed highly conserved enrichments in both their tissue-specific expressions and organ-related functions. For example, genes from Cluster 1 were highly expressed in the brain organ, especially medulla (Fig. 2e), and showed enrichment in nervous system related pathways, including locomotory behavior, positive regulation of axonogenesis, learning and so on (Fig. 2f), while the cardiovascular system related genes were mainly identified in Cluster 2 and shown high expression levels in heart and muscle organs (Fig. 2g,h). A similar enrichment mode was also observed in genes from Cluster 3 to 7, such as Cluster 3 in cerebellum with functional enrichment in synapse assembly, Cluster 4 in lung and kidney with smoothened signaling and hypoxia, Cluster 5 in liver with cholesterol metabolism, Cluster 6 in intestine with carbohydrate metabolic processing and Cluster 7 in spleen with innate immune and inflammatory responses (Extended Data Fig. 2c-g; 2i-m). Moreover, genes from Cluster 8 were ubiquitously expressed in all organs (Extended Data Fig. 2h) with enrichment of fundamental cellular functions, like translation and RNA processing (Extended Data Fig. 2n). These results illustrated tissue/organ-specific features of whole transcriptome in gene expression and functional enrichment.

Transcriptomic landscape of multiple tissues in SARS-CoV-2 infected rhesus macaques. To determine the SARS-CoV-2-induced transcriptional changes in 14 tissues, the tissues were collected from three rhesus macaques infected with the virus for 7 days and subjected to RNA sequencing. Based on the whole transcriptome profiles, lung samples from control and infected rhesus macaques were distinctly separated, while other control and infected tissues from the same organ were clustered together, suggesting that lung tissue underwent serious injury at the early stage of infection¹⁴ (Extended Data Fig. 3a and Supplementary Table 4). Intriguingly, post virus infection, the transcriptomic profiles from right ventricle was clustered with brain tissues except cerebral cortex which showed a similar expression pattern with uninfected right ventricle (Extended Data Fig. 3a). This distinct clustering result was further validated by t-distributed stochastic neighbor embedding (t-SNE) analysis (Extended Data Fig. 3b), suggesting a differential yet closely related responses to SARS-CoV-2 infection in right ventricle and cerebral cortex.

To further evaluate the differentially expressed genes (DEGs) in multiple organs post SARS-CoV-2 infection, we compared all the infected organ samples with their corresponding controls. Since the infected rhesus macaques include 2 male and 1 female, the DEG analysis for testis was only based on 2 male infected samples. We totally identified 18,730 dysregulated genes (fold change ≥ 2 and P value < 0.05) among all 14 tissues and most of them were located in cerebral cortex, cerebellum and right

ventricle (Fig. 3a and Supplementary Table 5). We further compared the expression level of *ACE2*, the first identified receptor for SARS-CoV-2¹⁵, in multi-tissues between control and infected groups (Fig. 3b). Intriguingly, among all the tissues tested, *ACE2* was highly expressed in intestine, which is consistent with previous reports^{16,17}, and remarkably increased in lung^{15,18} and testis¹² post infection (Supplementary Tables 4–5). Moreover, we performed both PCA and hierarchical clustering analysis for expression changes among all dysregulated genes in 14 organs, and found that right ventricle was more closer to cerebellum and brainstem but apart from cerebral cortex (Fig. 3c,d), suggesting that differential signaling pathways are involved in the individual tissue/organ response to SARS-CoV-2 infection.

Spatial response of cerebral cortex, cerebellum and right ventricle. We then performed Gene Ontology enrichment analysis to determine the up- or down-regulated genes and their related functions in each organ upon SARS-CoV-2 exposure (Fig. 3e and Extended Data Fig. 3c). The transcriptomic changes in each type of organ post SARS-CoV-2 infection were involved in diversely distinct functions. Interestingly, cerebellum and right ventricle shared similar transcriptomic changes in both gene dysregulation and annotated functions (Fig. 3c,d), such as up-regulated genes related to with skeletal system development, actin cytoskeleton organization and signal transduction pathways (Fig. 3e) and down-regulated ones involving cilium assembly and telomere maintenance pathways (Extended Data Fig. 3c). Beyond that, the other up-regulated genes of right ventricle were significantly annotated in chemical synaptic transmission, locomotory behavior, neurotransmitter secretion, nervous system development and learning pathways (Fig. 3e), suggesting that the homeostatic genes for nerve cells were significantly affected in right ventricle post SARS-CoV-2 infection, resulting in an enhanced secretion of neurotransmitter at early stage of viral infection. Moreover, specific transcriptomic changes in cerebral cortex post infection (Fig. 3c,d and Extended Data Fig. 3a,b) showed that the down-regulated genes were mainly related to synapse, locomotory behavior and learning terms (Extended Data Fig. 3c), while up-regulated ones participated in muscle contraction, transcription, angiogenesis and Nuclear factor-kappa B (NF-κB) signaling pathways (Fig. 3e). This data suggests that genes related to immune response were specifically induced in the cerebral cortex post SARS-CoV-2 infection, whereas genes functioning in the original learning and motor behavior functions were inhibited.

Relative to cerebellum and right ventricle, cerebral cortex showed an opposite tendency in dysregulated genes in terms of differential expression and functional annotation post SARS-CoV-2 infection (Fig. 3c-e and Extended Data Fig. 3b-d). Moreover, the same opposite changes were also observed on those significantly dysregulated genes in cerebral cortex relative to cerebellum and right ventricle (Extended Data Fig. 3e). A relatively higher proportion of intersection, especially between cerebral cortex and right ventricle, was also observed (Extended Data Fig. 3f). Specifically, some metabolic process related genes were up-regulated in cerebral cortex but down-regulated in cerebellum/right ventricle (Extended Data Fig. 3g), while nervous system development related genes exhibited a reverse pattern (Extended Data Fig. 3h). These results suggest that brain and heart undergo drastic transcriptomic reprogramming at the early stage of SARS-CoV-2 infection.

Elevated innate immune response of cerebral cortex. Since innate immune response is the first line of defense against SARS-CoV-2 infection, which triggers the production of interferons (IFN), pro-inflammatory cytokines and chemokines¹⁹, we further investigated the expression of interferon-related genes in each organ (Fig. 4a). Intriguingly, the dysregulated genes were enriched in cerebral cortex, cerebellum and right ventricle, and moreover, interferon gamma (IFN- γ) receptor 1 (*IFNGR1*) and interferon lamda (IFN- λ) receptor 1 (*IFNLR1*) expressions were up in cerebral cortex but down in cerebellum and right ventricle (Fig. 4b), a similar opposite pattern as the cluster analyses on whole transcriptome and dysregulated genes (Fig. 3d, Extended Data Fig. 3d,e). We then focused on interferon-stimulated genes (ISGs)²⁰ (Fig. 4c, Extended Data Fig. 4a,c and Supplementary Table 6), and observed that in cerebral cortex, a large proportion of ISGs showed an elevated expression with enrichment in the response pathways to IFN- γ and cytokine (Fig. 4d), while reversely the down-regulated ISGs were predominant in right ventricle and cerebellum and enriched in defense responses to viral and metabolic process, respectively (Extended Data Fig. 4b,d). Moreover, most of the up-regulated ISGs (112/259) in cerebral cortex showed down-regulation in both cerebellum and right ventricle (Fig. 4e) and mainly enriched in type I interferon production, response to cytokine and metabolic process pathways (Fig. 4f). These findings suggest that IFN- γ related genes were specifically up-regulated in the SARS-CoV-2 infected cerebral cortex.

IFN- γ , as the first macrophage-activating factor (MAF) identified so far, facilitates immune response by promoting proinflammatory cytokine synthesis, phagocytosis and antigen-presenting capacity in activated M1 macrophages^{21,22}. To elucidate the immune response of cerebral cortex caused by IFN- γ , we evaluated the expression of inflammatory factors from polarized macrophages, and found that *IL-23*, the key factor in Th1-antigen-specific responses, as well as *CD80* and *CD86*, the co-stimulatory factors for pro-immune responses, had a significantly higher expression in cerebral cortex post infection (Fig. 4g). In addition, anti-inflammation factors of M2 macrophages also showed high expression in the cortex probably for balancing the inflammatory response (Extended Data Fig. 4e). However, those genes were preferentially down-regulated in the infected cerebellum and right ventricle tissues (Fig. 4g and Extended Data Fig. 4e). To further determine the proportion of different stages of macrophages (M0: inactive macrophages, M1: pro-inflammatory macrophages, M2: anti-inflammatory macrophages), we employed CIBERSORT²³ to evaluate the amounts of immune cell types based on transcriptomic data of cerebral cortex, cerebellum and right ventricle tissues (Extended Data Fig. 4f). The cerebral cortex exhibited an increased proportion of macrophages within active M1 and M2 phases upon infection, while most macrophages in cerebellum and right ventricle were in inactive M0 (Fig. 4h), suggesting that cerebral cortex harbors a specifically elevated immune response in the early stage of SARS-CoV-2 infection.

Transcriptional regulatory network of hyperinflammation in cerebral cortex. We then analyzed the signaling network regulated by transcription factors (TFs), which might associate with the susceptibility or severity of SARS-CoV-2 infection²⁴. Among 745 TFs identified in rhesus macaque based on human transcriptional regulatory interactions of TRRUST database²⁵, 567 showed dysregulation in the infected tissues, especially in cerebral cortex, cerebellum and right ventricle (Extended Data Fig. 5a and

Supplementary Table 7). By matching the TF-target pairs and the dysregulated genes among these three infected tissues, we built the regulatory interaction network about the up-regulated TFs and their targets by Cytoscape²⁶ (Extended Data Fig. 5b-d). To further determine the infection-elicited TF network, four up-regulated TFs with their most interacting targets were retrieved to build a relatively simple regulatory interaction network for cerebral cortex (Fig. 5a), cerebellum (Fig. 5c) and right ventricle (Fig. 5e). The up-regulated targets in cerebral cortex post infection were significantly annotated in response pathways to inflammation and hypoxia (Fig. 5b), while in cerebellum and right ventricle the up-regulated ones were mainly annotated in transcription pathways (Fig. 5d,f). These findings suggest that SARS-CoV-2 might hijack the host transcriptional regulatory mechanism to induce hyper-inflammatory state *via* TFs in cerebral cortex, but not in cerebellum and right ventricle.

Hypercytokinemia, thrombosis, angiogenesis and fibrotic factors elevated in cerebral cortex. The hallmark of COVID-19 pathogenesis is the cytokine storm with higher levels of proinflammatory cytokines, such as interleukin-1 β (IL-1 β), interleukin-2 (IL-2), interleukin-6 (IL-6), tumor necrosis factor (TNF- α), granulocyte-macrophage colony-stimulating factor (GM-CSF), chemokines (C-C-motif chemokine ligand (CCL)-2, CCL-3 and CCL-5), also interleukin-2 (IL-2), interleukin-7 (IL-7) and, interleukin-10 (IL-10)²⁷. The severe cases with hyperinflammatory syndrome can include coagulation impairment²⁸, abnormal angiogenesis²⁹, cystic fibrosis³⁰ and even death^{27,31}. We then examined the expression of those factors using the transcriptomic data from various tissues of rhesus macaques, and observed their dysregulation in the early stage of infection, especially in cerebral cortex, cerebellum and right ventricle (Extended Data Fig. 6a and Supplementary Table 8).

The cytokines retrieved from M9809 gene set of Molecular Signatures Database in Gene Set Enrichment Analysis (GSEA)³², such as IL-7, CCL2, CXCL10 and IFNs, were significantly up-regulated in cerebral cortex, but markedly down-regulated in cerebellum and right ventricle post infection (Fig. 6a). Moreover, these cytokines were enriched in the pathways of cytokine-mediated signaling pathway and acute inflammatory response (Extended Data Fig. 6b), suggesting a state of hypercytokinemia or cytokine storm in the cerebral cortex. In addition, among the coagulation factors retrieved from gene set of GSEA, over half of them were dysregulated, and showed the most significant changes in three aforementioned tissues. Specifically, the coagulation factors with significant upregulation in cerebral cortex include coagulation factors prothrombin XIIIa (F13A1)^{33,34}, glycoprotein VWF binding to factor VIII (F8)^{33,34} and plasminogen activator inhibitor 1 (SERPINE1)³⁵ (Fig. 6b). These up-regulated cytokines were annotated in blood coagulation pathway (Extended Data Fig. 6c), indicating an impaired coagulation in cerebral cortex. Similarly, most angiogenesis factors, annotated in nCounter PanCancer Progression Panel (NanoString Technologies), were aberrantly regulated with significant changes in cerebral cortex relative to cerebellum and right ventricle (Fig. 6c), and enriched in angiogenesis and some signaling pathways (Extended Data Fig. 6d). suggesting an abnormal angiogenetic state in the virus infected cerebral cortex.

Fibrosis is usually divided into four stages: initiation is triggered by a cascade of immune response and stress, inflammation with activated inflammatory signaling pathways, proliferation with differentiation

and proliferation of fibroblasts, and modification with restructured extracellular matrix (ECM) composed of immune cells and fibroblasts. We have quantified the expression of 771 fibrosis-related genes from mCounter Fibrosis Panel (NanoString Technologies), and found that 567 genes were dysregulated in at least one tissue post viral infection. We built an interaction network from String database³⁶ using these altered genes by Cytoscape²⁶ (Fig. 6d and Extended Data Fig. 6e). Dysregulated fibrosis markers were dominant in cerebral cortex, cerebellum and right ventricle, while inflammatory fibrosis markers were significantly dysregulated in cerebral cortex (Fig. 6d), revealing an abnormal fibrotic response in cerebral cortex at the early stage of SARS-CoV-2 infection.

Thus, SARS-CoV-2 infection rapidly induces the abnormal state of hypercytokinemia, thrombosis, angiogenesis and fibrosis, in particular the cerebral cortex. As these imbalanced activities could facilitate the formation of microthrombi³⁷, SARS-CoV-2 patients have higher risk to develop microthrombi in brain^{38,39} and other systems as well⁴.

SARS-CoV-2 receptors might mediate the differential transcriptomic changes of cerebral cortex. We next examined the expression of reported receptors^{18,40-44} for SARS-CoV-2 in multi-tissues post infection and observed that Neuropilin-1 (*NRP1*) and Niemann-Pick disease type C intracellular cholesterol transporter 1 (*NPC1*) were significantly up-regulated in cerebral, but down-regulated in right ventricle (Fig. 7a,b). It has been reported that NRP1 expression is higher in nasal epithelium rendering susceptible to SARS-CoV-2 infection^{40,41}. It is likely that SARS-CoV-2 may enter the cerebral tissues by crossing the blood-brain barrier *via* an upper nasal transcribrial route⁴⁵. Thus, the higher expression of NRP1 in the cerebral tissue might serve as a potential receptor to promote SARS-CoV-2 infection in brain.

To elucidate the potential interactions among organs post infection, we used the ligand-receptor pairs from CellTalkDB⁴⁶ to examine their transcriptomic changes in the infected organs, and observed a close communication between cerebral cortex and right ventricle/cerebellum (Fig. 7c) with higher expression of ligands in right ventricle and cerebellum and its corresponding receptors in cerebral cortex. Those genes were mainly involved in the regulation of cell migration and axon guidance pathways (Fig. 7d), which is consistent with the findings of significantly up-regulated genes in these two organs (Fig. 3e). The results suggest a plausible mechanism through which the signaling molecules secreted by right ventricle and cerebellum could transmit to cerebral cortex through the circulatory system to target the receptors and induce the responses to SARS-CoV-2 infection (Fig. 7e).

Discussion

In this study, we established a transcriptomic atlas of 14 tissues collected from rhesus macaque infected with SARS-CoV-2 at 7 dpi. Through analyzing and comparing the dysregulated genes among various tissues, we observed the following typical features: (1) widespread and replication of SARS-CoV-2 in multiple tissues; (2) tissue/organ-specific responses and functional differences after virus infection; (3) innate immune response is enhanced in the cerebral cortex with elevated levels of cytokines, coagulation, angiogenesis and fibrotic factors in cerebral cortex; (4) hyperinflammatory state in the cerebral cortex; (5)

both neuronal receptor and secreted signaling might mediate the SARS-CoV-2 infection in central nerve system. Our study provides the first transcriptome profiling of various tissues from the living primate host post SARS-CoV-2 infection, and illustrates that wide-spread transcriptomic changes involving diverse functions exist in multi-organs, and an elevated immune response in cerebral cortex was the prominent feature at the early stage of SARS-CoV-2 infection.

Similar to the epidemic of SARS, COVID-19 patients often present with respiratory-like illnesses with highly chances to progress to severe pneumonia, suggesting that the lung is the primary tropism of SARS-CoV-2⁴⁷, even though accumulative studies reported that COVID-19 patients also suffer from injuries of liver, heart, kidney, and so on^{38,48–50}. However, systemic study about the effect of SARS-CoV-2 on multi-organs, especially nerve system and cardiovascular system in living host, is still lacking till recently. The tissue tropism of SARS-CoV-2 depends on susceptibility and permissiveness in a specific host cell. ACE2, as the primary cellular receptor for SARS-CoV-2, has been reported to be highly expressed in various organ types, including intestine and lung^{15–18}. Consistently, we also observed an enhanced *ACE2* expression in intestine and lung after SARS-CoV-2 infection. Through analyzing the enrichment of collected candidate receptors, we identified *NRP1* being highly expressed in SARS-CoV-2 infected cerebral cortex and right ventricles. This finding suggests that *NRP1* might serve as the receptor for SARS-CoV-2 and specifically mediate the infection in these tissues.

A rapid and coordinated immune response during viral infection leads to an enhanced secretion of various inflammatory factors, including interferons, pro-inflammatory cytokines and chemokines, which acts as a defense mechanism against the viral infection. Numerous reports suggest that individuals infected with SARS-CoV-2 have dysregulated cytokine production⁵¹. Consistently, we detected that interferon receptors and ISGs were up-regulated in cortex but down-regulated in cerebellum and right ventricle tissues. In addition, the expression of cytokines, coagulation, angiogenesis and fibrosis related factors was significantly elevated in cerebral cortex post infection, indicating the enhanced immune responses of nervous systems, in particular the cerebral cortex post SARS-CoV-2 infection.

Various studies have predicted and reported the adverse neurological and psychiatric outcomes occurring in COVID-19 patients. Moreover, a recent study using a large electronic health records network, provides further evidence for this kind of substantial neurological and psychiatric morbidity in COVID-19 patients⁵². In this study, we reveal that the significant transcriptomic changes in cerebral cortex at early stage of SARS-CoV-2 infection illustrate that brain injury might be the prominent feature in SARS-CoV-2 patients. Neuronal receptor pathway might mediate the exposure of brain to the virus in which beta coronaviruses could spread into and directly infect the brain when inhaled as droplets *via* the nasal epithelium^{45,53}. Moreover, *NRP1* serves as the receptor of SARS-CoV-2 with higher expression in nasal epithelium and cerebral cortex, which probably contribute to the brain injuries post infection. Additionally, signals secreted by cerebellum and right ventricle might also cooperatively contribute to the disease process in the cerebral cortex (Fig. 7e). Our findings extend our current understanding about the drastic

transcriptomic changes and further possible injuries in nerve and cardiovascular systems caused by SARS-CoV-2, which has potential significance in the guidance of clinical therapies of COVID-19 patients.

Methods

Animal experiments. Rhesus macaques of 3-4 years old were used in an animal biosafety level 3 (ABSL3) facility using HEPA-filtered isolators under pathogen-free conditions. All procedures in this study involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Institute of Laboratory Animal Science, Peking Union Medical College (BLL20001-8). All the experiments were complied with all relevant ethical regulations.

Upon intraperitoneally anesthetized by 2.5% avertin with 0.02 mL/g body weight, the rhesus macaques were inoculated intranasally with SARS-CoV-2 stock virus at a dosage of 10^6 TCID₅₀. The infected animals were continuously observed daily to record body weights, clinical symptoms, response to external stimuli and death. The rhesus macaques were dissected at 7 dpi. Major organs were grossly examined and then fixed in 10% buffered formalin solution, and paraffin sections (3-4 μ m in thickness) were prepared routinely.

RNAscope *in situ* hybridization (ISH). We used RNAscope Multiplex Fluorescent Assay v2 (Advanced Cell Diagnostics, ACD, Cat No.323100) to detect SARS-CoV-2 RNA in formalin fixed and paraffin embedded (FFPE) tissues. RNAscope probe with C1 channel (RNAscope® Probe-V-nCoV2019-S, ACD, Cat No.848561) is for targeting SARS-CoV-2 positive-sense (genomic) RNA and probe with C2 channel (RNAscope® Probe-V-nCoV2019-S-sense, ACD, Cat No. 845701) is for targeting SARS-CoV-2 negative-sense (a replicative intermediate indicating viral replication) RNA. Briefly, FFPE slides were pretreated by baking, de-paraffinizing, H₂O₂-blocking and digestion with Protease Plus. Then the sections were exposed to target probes and incubated at 40°C in a hybridization oven for 2 h. RNAscope detection was performed by successive incubation at 40°C with Opal-520 (A-520, FITC, 1:1500) and Opal-570 (A-570, Cy3, 1:1500) for 30 minutes each. Nuclei were counterstained with DAPI and mounted with ProLong Glass Antifade Mountant (P10144, Invitrogen) for TissueGnostics imaging analysis.

RNA extraction and rRNA depletion. Total RNAs were extracted from rhesus macaques with TRIzol (ThermoFisher, 15596018), and then subjected to DNase treatment (ThermoFisher, AM2238) and rRNA depletion with the Ribo-off rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme, N406-02) following the manufacturer's instructions.

RNA-seq. For each sample, the obtained ribominus RNA was used to construct libraries with the VAHTS® Universal V6 RNA-seq Library Prep Kit (Vazyme, NR604) following the manufacturer's instructions. Sequencing was performed on the Illumina HiSeq X-Ten sequencing system with paired end 150 bp read length.

Data processing and analysis. The quality of raw sequencing reads was checked using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), and then adapters were trimmed by

Cutadapt⁵⁴ (version 1.13). We used Trimmomatic⁵⁵ (version 0.36) to filter the reads shorter than 35 nt or containing ambiguous nucleotides. After quality control, the clean reads were aligned to the *Macaca mulatta* (rhesus macaque) genome from Ensembl⁵⁶ (release 100) using HISAT2⁵⁷ (version 2.0.5) with default parameters. Samtools⁵⁸ was used to filter and sort the mapped reads, then featureCounts program⁵⁹ was applied to summarize the gene counts. Reads per kilobase of exon model per million mapped reads (RPKM) was used to evaluate the genes expression in each sample. Clustering of organs in control group was performed using scaled RPKM by K-means algorithm⁶⁰ with 10,000 iterations. DEGs between infected and control were identified by edgeR⁶¹ in R programme using the threshold $|\log_2(\text{fold change})| \geq 1$ and $P \text{ value} < 0.05$.

For the functional annotation of gene sets, ClueGO⁶² of Cytoscape²⁶ and DAVID^{63,64} were applied to annotate Gene Ontology Consortium⁶⁵ biological process terms with threshold $P \text{ value} < 0.05$ and GO term fusion in ClueGO. The reads counts were normalized using bamCoverage of BEDtools⁶⁶ v2.26.0 and displayed in the genome by Integrative Genomics Viewer (IGV)⁶⁷. UpSetR⁶⁸ was used to display the overlapped genes in multiple organs.

To predict composition of immune cells in multiple organs, gene expression of RPKM in each sample as input processed using CIBERSORT algorithm²³. To Protein-protein interaction (PPI) network of fibrosis markers were built using STRING v11.0³⁶ using multiple proteins with default parameters. TFs and targets pairs of human were collected from TRRUST version2²⁵, and ligand-receptor pairs of human were used to build tissues communication from CellTalkDB database⁴⁶. The polar heatmap of dysregulated TFs was generated by TBtools⁶⁹.

Statistics and reproducibility. Statically significant differences between infected and control group were evaluated by Student's two-side unpaired t -test and wilcox test. Statistical significances are presented in the figures as single asterisk (*) when $P \text{ value} < 0.05$.

Declarations

Acknowledgments

This work was supported by grants from National Key R&D Program of China (2020YFC0848900), CAS Key Research Projects of the Frontier Science (QYZDY-SSW-SMC027), National Natural Science Foundation of China (31625016 and 81788101), K.C.Wong Education Foundation (GJTD-2019-08), Chinese Academy of Medical Sciences (CAMS) Initiative for Innovative Medicine (2016-I2M-2-001, 2016-I2M-2-006), and the Youth Innovation Promotion Association, CAS (2018133)□

Author contributions

Y.G.Y., W.M.T. and C.Q. conceived the project and supervised all the experiments; W.D. performed the virus infection and tissues collection with the help from F.L., F.Q., and J.L.; Y.Y. performed RNA extraction,

library construction assays with the help from Y.Q.S., W.J.L. and B.Z.; M.L. performed RNA scope analysis with the help of L.H.S., P.W., Z.Y.L. and Y.N; C.C.G. performed bioinformatics analysis with the help from Y.S.C.; Y.G.Y., W.M.T., C.Q., Y.L.Z., C.C.G., Y.S.C. and Y.Y. analyzed the data and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All NGS sequencing data in this study are available at National Genomics Data Center (NGDC), China National Center for Bioinformation under the accession number CRA004025 (shared URL: <https://bigd.big.ac.cn/gsa/s/j8I5Df17>). All data that support the findings of this study are available from the corresponding author upon reasonable request.

Code availability

There was no new code generation in this study.

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Figures

Figure 1

In situ hybridization of SARS-CoV-2 in organs of rhesus macaques. Representative RNAscope images of SARS-CoV-2 expression (Green, FITC-labelled) and replication (Red, Cy3-labelled) in a) Lung, b) Heart, c) Cerebral cortex, d) Cerebellum from a SARS-CoV-2 infected macaques of 7 dpi. Hematoxylin and Eosin (H&E) staining was used to refer the SARS-CoV-2 in situ distribution and replication in each organ. Scale bars, H&E 100 mm and ISH 20 mm.

Figure 2

Functional clusters of genes were identified by RNA-seq in multiple organs of rhesus macaque. a, Schematic diagram displaying 14 organs of rhesus macaque used in this study. b, c, Violin plots showing expression profiling for specific tissue marker genes of lung (b) and kidney (c) in all of 14 organs. d, Principal component analysis of gene expression patterns for 14 organs. e, Line plot showing the normalized expression pattern for genes from Cluster 1, which is determined by K-means clustering

analysis. The light lines represent normalized expression value for each gene from Cluster 1, while dark line represents mean of normalized expression value among all Cluster 1 genes. f, Barplot showing the enriched GO (Gene Ontology) terms for Cluster 1. g, Line plot showing the normalized expression pattern for genes from Cluster 2, which is determined by K-means clustering analysis. The light lines represent normalized expression value for each gene from Cluster 2, while the dark line represents mean of normalized expression value among all Cluster 2 genes. h, Barplot showing the enriched GO (Gene Ontology) terms for Cluster 2.

Figure 3

Multi-organ transcriptomic landscape of rhesus macaques post SARS-CoV-2 infection. a, Radar diagram showing the numbers of dysregulated genes across multiple organs. The red points represent number of

up-regulated genes in organ of SARS-CoV-2 infected rhesus macaques, while blue points stand for down-regulated genes. b, Expression level of ACE2 in 14 different organs for both control and infected rhesus macaques. Expression level for infected rhesus macaques is determined as mean $\pm$ standard deviation. Except testis, the standard deviations are obtained by 3 biological replicates, while testis only used 2 biological replicates for only 2 infected male rhesus macaques. c, Principal component analysis for significantly dysregulated genes, corresponding to a, across multi-organ transcriptomes. To each organ, fold changes of gene expressions after infection are used for principal component analysis. d, Heatmap showing the fold changes of the significantly dysregulated genes for all 14 organs. The fold change of each gene is then normalized by Z-score among organs. e. Bubble chart showing the enrichment of GO terms for significantly up-regulated genes identified in each organ. Bubble size represents number of identified up-regulated genes in each term for individual organ, and P values from non-significance to high significance are shown as blue to red.

Figure 4

Enhanced innate immune response in cerebral cortex after SARS-CoV-2 infection. a, Heatmap showing fold change of interferon genes in different organs from infected rhesus macaques compared to the control one. The white asterisk represents dysregulated genes with statistical significance (P value < 0.05). b, Genome browser showing the reads abundance along IFNGR1 and IFNL1 in cerebral cortex, cerebellum and right ventricle from control and infected rhesus macaques. c, Volcano plot showing the

difference in all expressed ISGs between control cerebral cortex and infected samples. Red points: up-regulated ISGs; blue points: down-regulated ISGs. d, Map of enriched GO functional terms for significantly up-regulated ISGs in cerebral cortex from infected rhesus macaques. Dot represents enriched GO term, while size of dot stands for its significance level (P value). Line means there are shared genes between two terms, while the width of lines stands for the counts of shared genes. e, Intersection analysis of dysregulated ISGs in cerebral cortex, right ventricle and cerebellum of infected rhesus macaques. Only up-regulated ISGs for cerebral cortex are selected, while down-regulated ISGs for cerebellum and right ventricle, respectively. f, Map of enriched GO functional terms for 112 common dysregulated ISGs in cerebral cortex, right ventricle and cerebellum of infected rhesus macaques (corresponding to e). Dot represents enriched GO term, while size of dot stands for its significance level (P value). Line means there are shared genes between two terms, while the width of lines stands for the counts of shared genes. g, Heatmap showing the fold changes of M1 macrophage related genes after infection for each of 14 organs, individually. The fold change of each gene is then normalized by Z-score among organs, while asterisk represents dysregulated genes with statistical significance (P value < 0.05) identified in each organ. h, Pie chart displaying estimated proportions for different types of polarized macrophage cells. M0, original macrophage cell; M1, proinflammatory polarization macrophage; M2, anti-inflammatory polarization macrophage.

Figure 5

Inflammatory transcriptional interaction network in cerebral cortex. a, Interaction network displaying the top four induced TFs with the most abundant targets in cerebral cortex post SARS-CoV-2 infection. Red diamonds were the induced TFs, and circular dots represented their targets. Light purple dots were up-regulated targets in cerebral cortex post infection, light green ones were down-regulated targets and light blue ones were the unchanged targets. The size of TFs represented of number of their interaction pairs,

and the size of targets represented their fold change post infection. b, Bubble chart showing the enrichment of GO terms for significantly dysregulated target genes in (a). Up column represented the up-regulated targets in (a), Down column was the down-regulated targets. c, Interaction network displaying the top four induced TFs with the most abundant targets in cerebellum post SARS-CoV-2 infection. The legend was the same as (a). d, Bubble chart showing the enrichment of GO terms for significantly dysregulated target genes in (c). e, Interaction network displaying the top four induced TFs with the most abundant targets in right ventricle post SARS-CoV-2 infection. The legend was the same as (a). f, Bubble chart showing the enrichment of GO terms for significantly dysregulated target genes in (e).

Figure 6

Factors of cytokine, coagulation, angiogenesis and fibrosis significantly induced in cerebral cortex post infection. a, Heatmap showing fold change of dysregulated cytokines among cerebral cortex, cerebellum and right ventricle organs from infected rhesus macaques compared to the control one. b, Heatmap showing fold change of dysregulated coagulation factors among cerebral cortex, cerebellum and right ventricle organs from infected rhesus macaques compared to the control one. The white asterisk represents dysregulated genes with statistical significance (P value < 0.05). c, Heatmap showing fold change of dysregulated angiogenesis factors among cerebral cortex, cerebellum and right ventricle

organs from infected rhesus macaques compared to the control one. d, Interaction network showing the stage-specific dysregulated fibrotic genes in cerebral cortex (left), right ventricle (middle) and cerebellum (right) post SARS-CoV-2 infection over four fibrosis stages, including initiation (green), inflammation (light blue), proliferation (orange) and modification (red). The interactions were built based on the STRING database using Cytoscape. The size of bubbles represented the value of fold change of dysregulated genes from infected rhesus macaques compared to the control one.

Figure 7

Neuronal receptor and signal transduction pathways leading to the cerebral cortex infection. a, Bubble chart showing the fold change of the identified receptors for SARS-CoV-2 infection from infected rhesus macaques compared to the control one. b, Genome browser showing the reads abundance along NRP1 and NPC1 in cerebral cortex, right ventricle and cerebellum from control and infected rhesus macaques. c, Interaction network of multiple organs displaying the tissues communication based on the ligand-receptor pairs. The size of nodes represented the number of significantly induced ligands or receptors post SARS-CoV-2 infection in each organ, the direction of arrows was the signaling transduction from ligand to receptor between two organs, and the width of arrows were the number of significantly induced ligand-receptor pairs post infection between two organs. d, enrichment of GO terms for significantly dysregulated transported ligands from cerebellum and right ventricle to cerebral cortex. e. Schematic diagram displaying the two potential pathways for cerebral cortex infection. (i) neuronal receptor pathway: NRP1 served as neuronal receptor of SARS-CoV-2 in nasal epithelium and cerebral cortex, contributed to cerebral cortex infection.; (ii) signal transduction pathway: secreted neurotransmitter from right ventricle post SARS-CoV-2 infection might induce hormones secretion, which transport via the circulatory system until to the cerebral cortex and induced response for virus infection.

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