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Human cardiac organoids to model COVID-19 cytokine storm induced cardiac injuries

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Abstract

Acute cardiac injuries occur in 20%–25% of hospitalized COVID-19 patients. Herein, we demonstrate that human cardiac organoids (hCOs) are a viable platform to model the cardiac injuries caused by COVID-19 hyperinflammation. As IL-1β is an upstream cytokine and a core COVID-19 signature cytokine, it was used to stimulate hCOs to induce the release of a milieu of proinflammatory cytokines that mirror the profile of COVID-19 cytokine storm. The IL-1β treated hCOs recapitulated transcriptomic, structural, and functional signatures of COVID-19 hearts. The comparison of IL-1β treated hCOs with cardiac tissue from COVID-19 autopsies illustrated the critical roles of hyper-inflammation in COVID-19 cardiac insults and indicated the cardioprotective effects of endothelium. The IL-1β treated hCOs thus provide a defined and robust model to assess the efficacy and potential side effects of immunomodulatory drugs, as well as the reversibility of COVID-19 cardiac injuries at baseline and simulated exercise conditions.

KEYWORDS
biomaterials, cardiac organoids, cardiac physiology, cardiology, engineered cardiac tissue, inflammation, regenerative medicine, RNAseq, seahorse, stem cell biology, tissue engineering

1 | INTRODUCTION

Besides pulmonary complications, one of the most prevalent complications of COVID-19 is acute cardiac injury (ACI) (Ruan et al., 2020; Zheng et al., 2020). 20%–25% of hospitalized COVID-19 patients suffer ACIs, which are associated with a poor prognosis and increased mortality rate (Szekely et al., 2020; Zhou et al., 2020). Despite the evidence of direct viral infection of the myocardium (Juan A. Perez-Bermejo1† et al., 2021; Lindner et al., 2020), a growing body of literature suggests COVID-19 induced cytokine storm is a major contributor to ACI (Giustino et al., 2020; Mehta et al., 2020; Zhu et al., 2020). Unlike the cytokine storm induced by Chimeric Antigen Receptor T (CART) Cell therapy (Fajgenbaum & June 2020; Murthy et al., 2019), the inflammatory profile of COVID-19 cytokine storm is marked by lymphopenia, granulocytosis, and a milieu of proinflammatory cytokines with a strong innate component. Several innate cytokines such as IL-1β, and IL-6 have been used clinically as markers of patient prognosis and offer viable options for pharmacologic intervention (Brodin, 2021; Jenner et al., 2021; Lucas et al., 2020).

Our laboratory has developed in vitro 3D human organoids (hCOs) that are composed of human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs), human cardiac fibroblasts (hcFBs), human umbilical vein endothelial cells (HUVECs), and human adipose derived stem cells (hADSCs) (Richards et al., 2017). These hCOs provide a powerful in vitro system to model cardiovascular pathologies. For example, they have been shown to recapitulate the transcriptomic, structural and functional hallmarks of myocardial infarction and cardiovascular disease exacerbated pharmacotoxicity (Richards et al., 2020). Recently, Mills et al. first used hCOs to identify novel drug targets for COVID-19 induced cytokine storm by using a high throughput cytokine screen (Mills et al., 2021). Here we implemented a biomimetic design and treated our hCOs with an upstream stimulus to induce a cytokine storm in our system. In doing so, we anticipated our model would provide a platform to investigate multiple aspects of cardiac pathology.

IL-1β is one of the first cytokines released in response to viral infection by macrophages and epithelial cells (Fajgenbaum & June, 2020). Importantly, its serum levels have strongly correlated with severe COVID-19 disease, despite a short half-life in serum (Brodin, 2021). Lung and heart tissues of COVID-19 patients have revealed increased expression of the IL-1β receptor (IL1R1), corroborating high levels of IL-1β signaling (Delorey et al., 2021). As an upstream cytokine, IL-1β is known to induce the release of downstream cytokines including IL-6 (Fajgenbaum & June 2020). Given all of the cell-types comprising the hCOs (e.g., HUVECs, hPSC-CMs, hcFBs) can produce cytokines in response to proinflammatory stimulation (Aoyagi & Matsui, 2011; Mako et al., 2010; Sandstedt et al., 2019), we postulated that IL-1β stimulation would induce a cytokine storm in our organoids, recapitulating the hyperinflammation affecting COVID-19 hearts and inducing functional and morphological changes consistent with clinical findings. As IL-1β may be considered a nonspecific upstream stimulus, we used clinical data and autopsy samples to validate the experimental design of using IL-1β treated hCOs to model COVID-19 specific cardiac pathologies. Herein, we report that treating hCOs with 1 ng/ml IL-1β induces the release of an array of proinflammatory cytokines that mirrors the profile of COVID-19 cytokine storm. IL-1β treated hCOs recapitulated transcriptomic, structural, and functional hallmarks of the COVID-19 induced acute cardiac injuries.

2 | RESULTS

2.1 | RNA seq analysis of IL-1β treated hCOs established their transcriptomic relevance with COVID-19 hearts

This study was designed on the growing premise that systemic inflammation appears to be the major driving force leading to COVID-19 ACI (Figure 1) (Bavishi et al., 2020; Zhu et al., 2020). As IL-1β is an upstream proinflammatory cytokine and a signature core COVID-19 cytokine, we treated hCOs with IL-1β (1 ng/ml) to simulate the COVID-19 ACIs using the treatment regimen depicted in Figure 2a. By using a single upstream stimulus, we aimed to generate a defined and robust model that can address the large variation found in patient serum samples (Wang et al., 2021). Four days was selected for IL-1β treatment because clinical data has indicated that it takes 12–96 h to transition from moderate to severe disease characterized by hypercytokinemia (Cappanera et al., 2021). We did not observe significant differences in hCO diameter and gross morphology after IL-1β treatment (Figure 2b). There was no significant difference between normalized TUNEL positivity between control and IL-1β treated hCOs (Figure 2c), consistent with minimal evidence of cardiomyocyte death in COVID-19 autopsies (Sang et al., 2021).

To establish system level relevance of IL-1β treated hCOs for modeling COVID-19 hearts, we performed RNA sequencing (RNA-seq) on hCOs with/without IL-1β treatment and compared their transcriptomic profiles to COVID-19 and healthy heart autopsy samples from publicly available datasets (Yang et al., 2021). We first plotted the top differentially expressed genes (DEGs) for our IL-1β treated hCOs (compared to control hCOs) and the COVID-19 hearts (compared to healthy controls). As seen in Figure 2d–e, IL1R1 was upregulated in both IL-1β treated hCOs and COVID-19 hearts, supporting the critical role of the IL-1β signaling in the COVID-19 induced hyperinflammation. Principal Component Analyses (PCA) showed control hCOs and healthy hearts grouped in the top right, while the IL-1β treated hCOs and COVID-19 hearts grouped together in the bottom left in the PC1/PC2 plot (Figure 2f), illustrating the high fidelity of IL-1β treated hCOs to model COVID-19 hearts. This is further supported by the heatmap of the shared top 20 unregulated and downregulated pathways between IL-1β treated hCOs (vs. control hCOs) and COVID-19 hearts (vs. healthy hearts). IL-1β treatment of hCOs led to the upregulation of several key pathways that were also upregulated in the COVID-19 hearts (Figure 2g). The top Gene Ontology (GO) terms included leukocyte

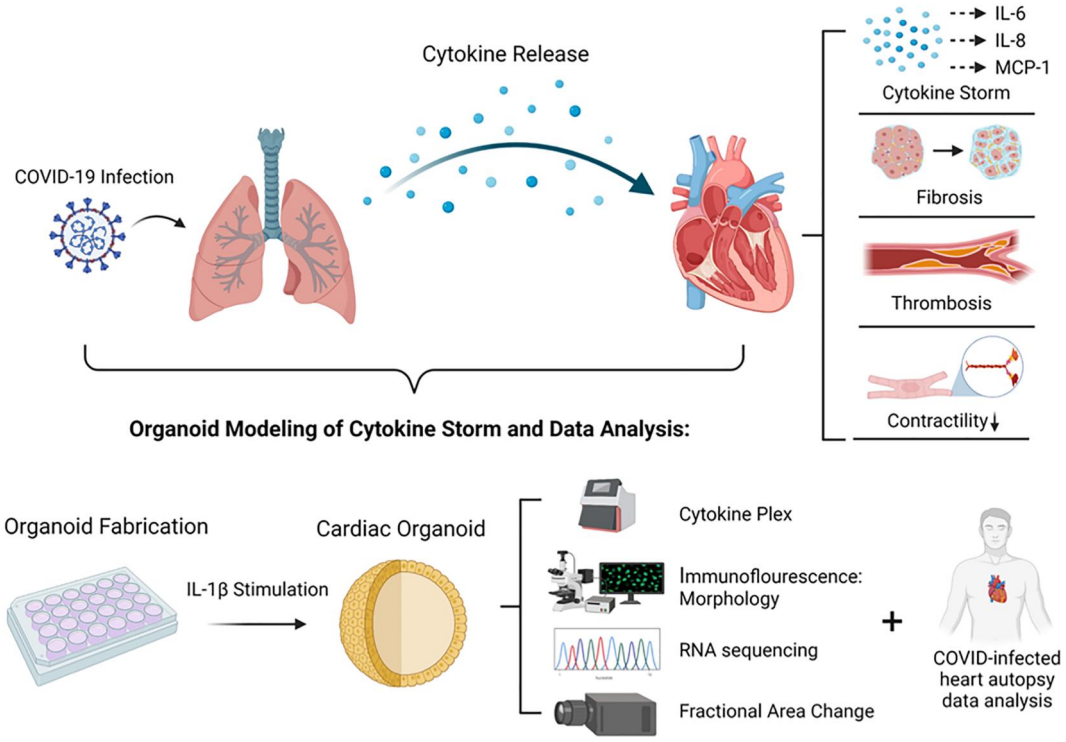

FIGURE 1 Experimental Design of the Study is Based on the Presumed Physiology of SARS-CoV-2 Infection. SARS-CoV-2 infects a patient's lungs, inducing cytokine production and release into the blood stream. Cytokines that have entered the blood stream affect the heart and induce fibrosis, thrombi, and reductions in contractile function. Human Cardiac Organoids (hCOs) treated with IL-1β were multiplexed for downstream cytokine release, assessed for alterations in morphology and transcriptomic signatures, and for reductions in contraction amplitude. Transcriptomic signatures of hCOs were also compared to COVID-19 autopsy samples. Created with BioRender.com [Colour figure can be viewed at wileyonlinelibrary.com]

differentiation (GO:0002521), chemotaxis (GO:0006935), and positive regulation of cytokine production (GO:0001819), indicating a similar proinflammatory transcriptomic profile between IL-1β treated hCOs and COVID-19 hearts. Moreover, the shared extracellular matrix organization and mesenchyme development indicated the IL-1β treated hCOs recapitulated cardiac fibrosis observed in COVID-19 patients (Roshdy et al., 2020). In Figure 2h, hCOs and COVID-19 hearts also shared common downregulated genes, which were aligned with key GO terms such as "muscle organ development" (GO:0007517), and "cardiac muscle tissue morphogenesis" (GO:0055008). The downregulated cardiomyocyte structure pathways are consistent with acute reductions in cardiac output (acute heart failure, cardiogenic shock in COVID-19 patients with acute cardiac injuries (Italia et al., 2021).

2.2 | IL-1β treated hCOs recapitulated cytokine profile from severe COVID-19 patient serum

We next examined whether the IL-1β treated hCOs were capable of secreting key proinflammatory cytokines found in the serum of severe COVID-19 patients. In particular, IL-6 is a pro-inflammatory cytokine thought to be a key marker of cytokine storm. Its levels have strongly correlated with both disease severity and mortality of COVID-19 patients (Aziz et al., 2020; Brodin, 2021; Jenner et al., 2021). To confirm IL-6 was released in response to IL-1β treatment, supernatant was collected on day 4 after IL-1β treatment and IL-6 levels were significantly upregulated, with a mean fold change of 6.5 (Supplementary Figure 1A–B), consistent with the measurements from the serum of severe COVID-19 patients (6-8-fold) and distinct from that of CART cytokine storm (75 fold) (Murthy et al., 2019). To assess the direct effects of IL-6 on our human cardiac organoids, IL-6 +/− its soluble receptor (sRα) were added to our cardiac organoids for 10 days at 10 ng/ml, 50 ng/ml and 100 ng/ml (Supplementary Figure 1C). No significant changes in contraction amplitude were found at any dose, with or without the addition of its soluble receptor, suggesting that in the context of the COVID-19 hearts, IL-6 may serve as a marker rather than the etiology for cardiac dysfunction. Given the increase of IL-6, supernatants from IL-1β treated hCOs collected were examined to determine the induced cytokine profile present using a bead-based multiplexed immunoassay system (Eve Tech, Canada). GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70 and MCP-1 were all upregulated (Figure 3a), consistent with previous reports detailing cytokine composition in the COVID-19 cytokine storm (Jenner et al., 2021; Liau et al., 2011; Lucas et al., 2020). Though TNFα, IFNγ, and IL-13 were measured, they were not as consistently upregulated in our samples (Supplementary Figure 2).

To support the multiplexing analyses, we evaluated the RNAseq data of the IL-1β treated hCOs and COVID-19 hearts on

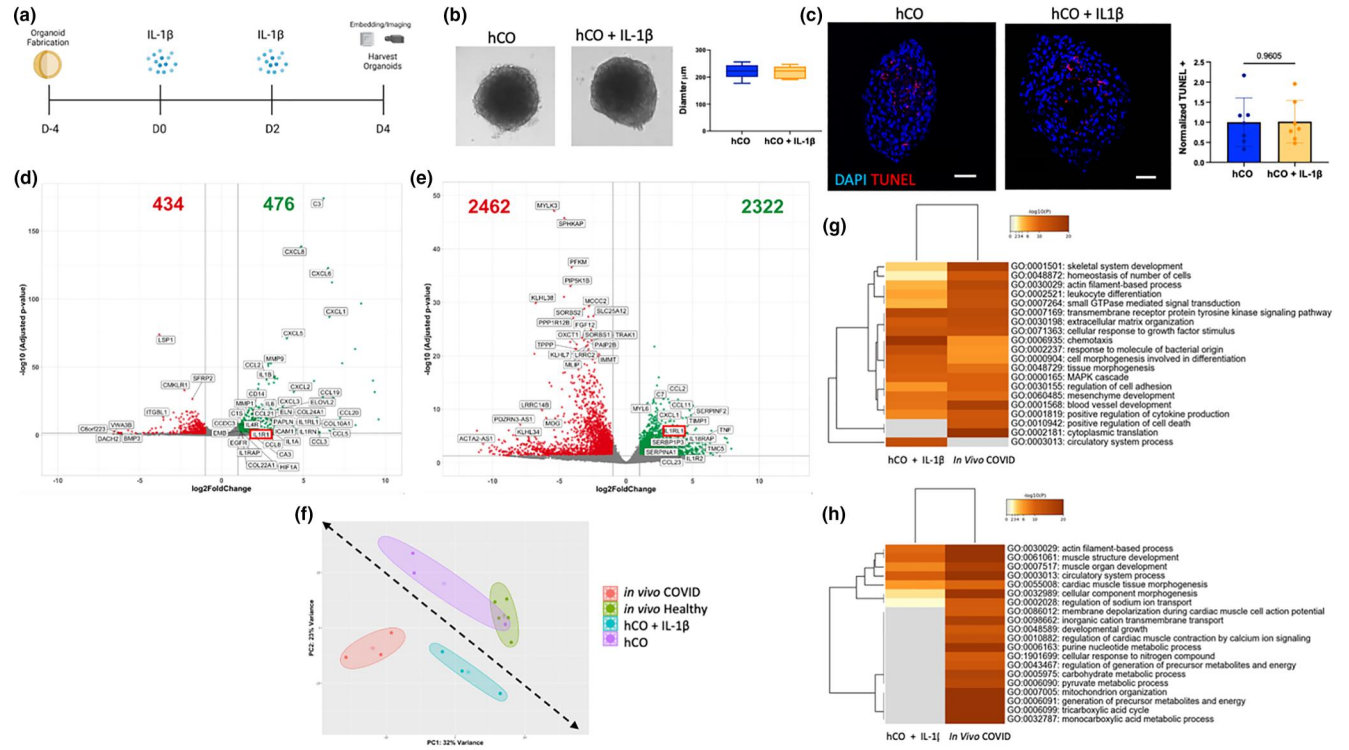

FIGURE 2 Transcriptomic Signature of Human Cardiac Organoids Stimulated with IL-1*β* Resemble that of COVID-19 Autopsy Samples. (a). Schematic of treatment regimen for human cardiac organoids. (b) (Left) Representative brightfield images of Organoids on D4 (Right) Diameters of organoids on Day 4 (mean +/− s.d., $n = 10$, $p = 0.9290$) (c) Representative images of immunofluorescent staining of human hCOs and hCOs + IL-1*β*. (Blue = DAPI, Red = TUNEL). Scale bar = 50 μm for each image. Quantification of Normalized TUNEL expression to the right (mean +/− s.d., $n = 6$, $p = 0.9605$). (d) Volcano plot illustrating DEGs between hCOs and IL-1*β* stimulated hCO. (e) Volcano plot illustrating differentially expressed genes (DEGs) of publicly available dataset from COVID-19 autopsy samples, relative to control patients. (f) Principal Component Analysis (PCA) showing clustering of hCOs and control patients, and COVID-19 patients with hCOs + IL-1*β*. (g) Pathway analysis comparing upregulated pathways in IL-1*β* stimulated human cardiac organoids with autopsy samples of COVID-19 patients. (h) Pathway analysis comparing downregulated pathways in pathways in IL-1*β* stimulated human cardiac organoids and autopsy samples of COVID-19 patients. Student's *t*-test used for all statistical analyses [Colour figure can be viewed at wileyonlinelibrary.com]

inflammatory GO terms and pathways using Gene Set Variation Analysis (GSVA) analyses. To account for the cellular composition differences between cardiac tissues and hCOs (e.g., without immune and neural cells), we utilized publicly available single cell RNAseq (scRNAseq) data from COVID-19 and healthy hearts to develop in silico hCOs (Delorey et al., 2021; Litvinukova et al., 2020). The in silico hCOs were constructed by selecting the specific cell types (i.e., ventricular cardiomyocytes, ventricular fibroblasts, cardiac endothelial cells, and cardiac pericytes) and compositions similar to those used in hCOs fabrication and aggregating them into an in silico organoid-like pseudo-bulk sample per donor (Supplementary Figure 3). hCOs showed significant increases in GSVA score upon IL-1*β* treatment under GO terms of "Innate Immune Response" (GO:0045087), "Cellular Response to IL-6" (GO:0071354), and "Cellular Response to TNF" (GO:0071356) (Figure 3b), mirroring the clinical findings of the upregulation of innate immunity, IL-6 and TNFα signaling observed in COVID-19 hearts (Aziz et al., 2020; Del Valle et al., 2020; Lucas et al., 2020). Though not significant, IL-1*β* treatment promoted the increase of a variety of proinflammatory pathways in hCOs such as "Adaptive Immune Response" (GO:0002250), "Cellular Response to IFNα" (GO:0035457) and "Cellular Response to IFNγ" (GO:0071346), with a similar trend observed in the in silico COVID hCOs and COVID-19 heart samples. Importantly, IL-1*β* treatment led to the elevation of key proinflammatory innate genes such as CXCL1, CCL2, and CXCL8 in the hCOs. IL-1*β* treatment also upregulated key clinical markers of inflammation such as IL-6 and FTH1 (Figure 3c). NF_KB1, a key transcriptional regulator of cytokine production and other key biological processes showed increasing trends with IL-1*β* treatment.

2.3 | IL-1*β* treated hCOs showed reduced cardiac function and pathological cardiac fibrosis

Given the similarities of the IL-1*β* treated hCOs to in vivo samples (i.e., in silico hCOs and heart samples) under immune-related Go terms, we next performed GSVA analyses for both cardiac structure and function related GO terms (Figure 4a). We observed a decrease with IL-1*β* treated hCOs of key cardiac structural and functional GO terms such as "Cardiac Myofibril Assembly" (GO:0055003) and "Cardiac Muscle Contraction" (GO:0060048), mirroring the in silico COVID-19 organoids and COVID-19 heart samples. To support the

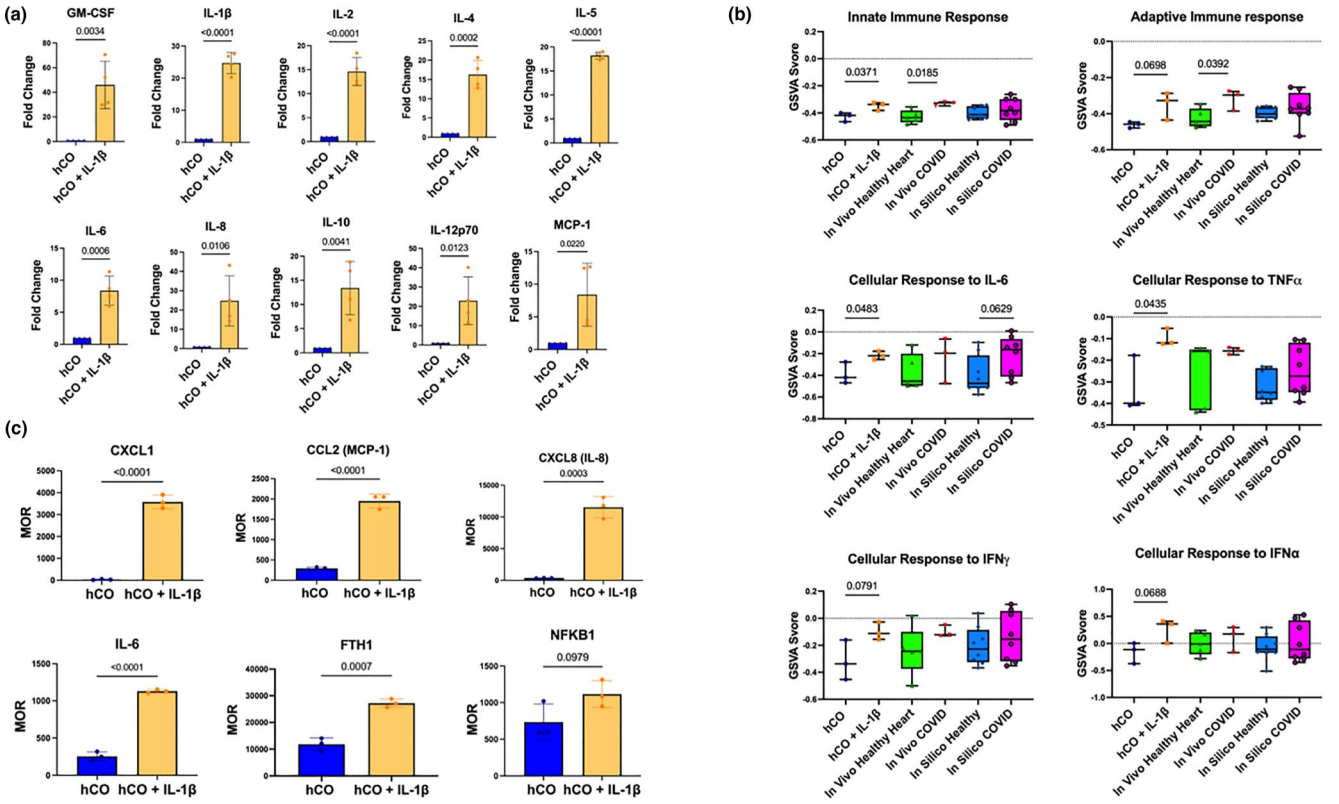

FIGURE 3 Inflammatory Profile of IL-1β Stimulated Human Cardiac Organoids Resembles that of COVID-19 Cytokine Storm. (a) Cytokine multiplex results showing fold change of cytokines in D4 supernatant as compared to hCOs; (mean +/− s.d., $n = 4$) (b) GSVA of hCOs and IL-1β stimulated hCOs ($n = 3$), Healthy *In Silico* and COVID *In Silico* ($n = 5$, and $n = 3$, respectively) and samples from Healthy and COVID autopsy samples ($n = 8$) assessing multiple GO terms pertaining to inflammation (mean +/− s.d.). Analysis performed using student's *t*-test. (c) Median of Ratios (MOR) of key genes upregulated upon IL-1β treatment; $n = 3$ (mean +/− s.d.). Student's *t*-test used for all statistical analyses [Colour figure can be viewed at wileyonlinelibrary.com]

results of RNAseq analyses, we examined the effects of IL-1β treatment on the cardiac structure and function of hCOs. As seen in Figure 4, IL-1β treated hCOs had distinct morphologies (Figure 4b), significantly reduced fractional area change (FAC) (i.e., contraction amplitude) (Figure 4c) and reduced sarcomere width (Figure 4d), consistent with the downregulated cardiac structure and function GO terms. This was also seen at higher doses of IL-1β (Supplementary Figure 4A–C). This was further supported by the significant downregulation of actinin α2 (ACTN2) and ß-myosin heavy chain (MYH7), while cardiac troponin I3 (TNNI3) and tropomyosin 1 (TPM1) trended downwards (Figure 4e).

To assess the validity of the cardiac findings within our hCO model, cardiac tissue was harvested from 95 autopsies of COVID-19 patients. 49 of these patients had confirmed SARS-CoV-2 infection in their cardiac tissue, with viral localization limited to interstitial spaces. 46 of these 95 autopsies had no detectable SARS-CoV-2 in the myocardium. Both SARS-CoV-2 negative (Figure 4f–g), and positive (Supplemental Figure 5A–B) hearts showed decreased α-sarcomeric actinin (α-SA) expression with increased cardiomyocyte destruction, indicating that COVID-19 induced cardiomyocyte damage is independent of viral infection of the myocardium.

Cardiac fibrosis has been reported in autopsies of COVID-19 patients (Roshdy et al., 2020). The in silico COVID-19 hCOs and COVID-19 heart samples showed significant increases in "Fibroblast Proliferation" (GO:0048144) scores, with the IL-1β treated hCOs showing increases with high variation (Figure 4h). Only the in silico COVID-19 hCOs showed significant increases in "Fibroblast Migration" (GO:0010761) scores, with the hCOs and COVID-19 samples showing increases but with high variation (Figure 4h). Notably, the immunofluorescent staining revealed that IL-1β treated hCOs showed significantly less α-SA expression, increased vimentin expression ($p = 0.0933$) and a significantly higher vimentin:α-SA ratio (Figure 4i), supporting the cardiac fibrosis. The significant increase in vimentin:α-SA ratio was also seen at higher doses of IL-1β (Supplemental Figure 4B). This is further supported by the upregulation of cardiac fibrosis hallmark genes such as angiotensinogen (AGT), matrix metalloproteinase 9 (MMP9), and α-smooth muscle actin (ACTA2), in the hCOs (Figure 4j).

2.4 | IL-1β treated hCOs showed prothrombotic vasculature

Severe COVID-19 patients can experience thrombotic events and vascular damage (Escher et al., 2020; Jin et al., 2020; Varga et al., 2020). To assess the vascular content of our hCOs, we stained

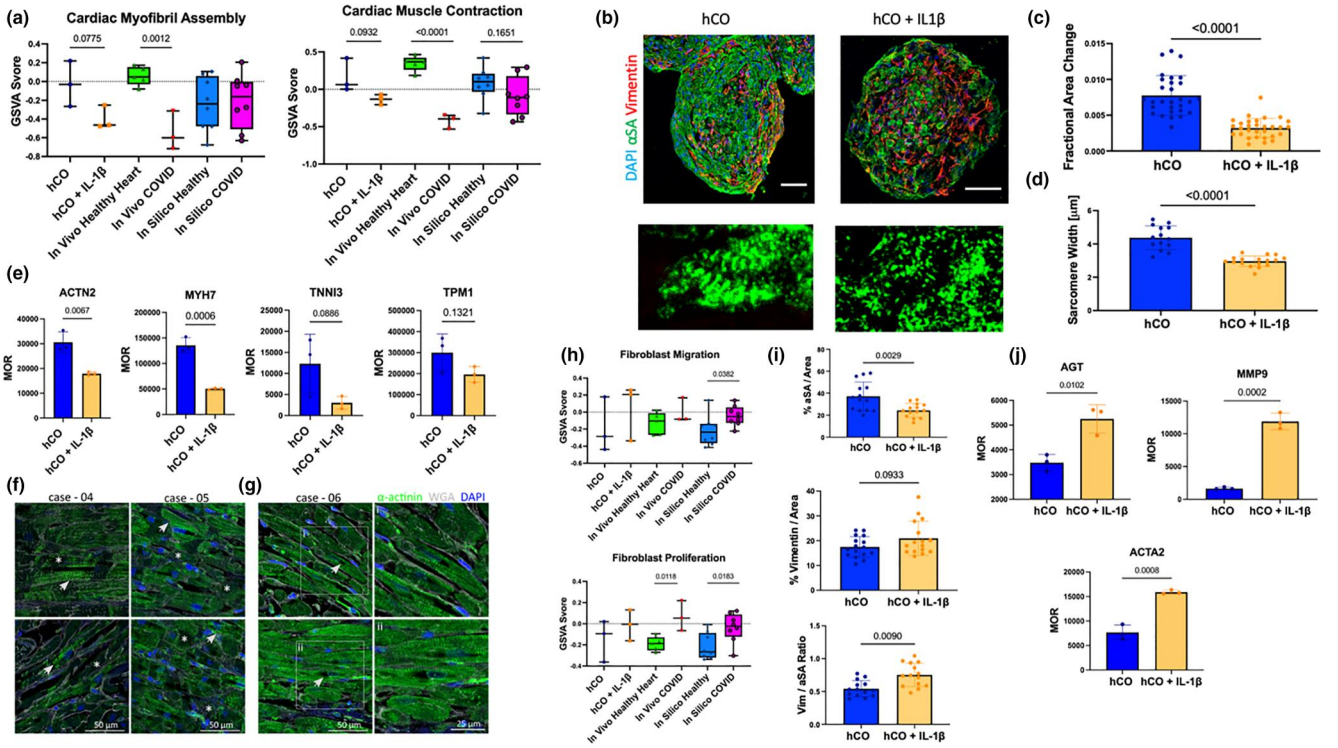

FIGURE 4 Human Cardiac Organoids Recapitulate Key Hallmarks of Cardiac Function and Fibrotic Response to COVID-19. (a) GSVA (GO Terms pertaining to cardiac function) of hCOs ($n = 3$), *In Silico* hcOs (Healthy: $n = 5$, COVID: $n = 3$), and in vivo samples ($n = 8$) (mean ± s.d.) (b) Immunofluorescent staining of hCOs (top left) or IL-1β stimulated hCOs (top right) on Day 4 (Green = α-SA, Red = Vimentin, Blue = DAPI). Higher magnification of sarcomeres for hCOs (bottom left), or IL-1β stimulated hCOs (bottom right) Scale bar = 50 μm. (c) FAC for hCOs, (mean ± s.d., $n = 29–30$, $p < 0.0001$). (d) Sarcomere width of hCOs (mean ± s.d., $n = 14–17$, $p < 0.0001$). (e) Key cardiac genes, Median of Ratios (mean ± s.d., $n = 3$). (f) Representative cardiac tissue (case-04 and case-05), both without cardiac SARS-CoV-2 infection. Normal sarcomeric structure (arrows), and sarcomeric disarray (asterisks) shown. (Blue = DAPI, Green = α-actinin, White = WGA). (g) Representative images of cardiac tissue from case-06 (without cardiac SARS-CoV-2 infection). Boxes indicate zoomed in regions on the right. Some cardiomyocytes lack nuclear staining (DAPI). Arrows denote putative region of nuclear localization. (h) GSVA (GO Terms pertaining to fibrosis and fibroblast behavior) of hCOs ($n = 3$), *In Silico* hCOs (Healthy: $n = 5$, COVID: $n = 3$), and in vivo samples ($n = 8$) (mean ± s.d). (i) Quantification of staining (Top) α-SA (mean ± s.d., $n = 16$, $p = 0.0029$ (Middle) Vimentin (mean ± s.d., $n = 16$, $p = 0.0933$). (Bottom). Vimentin:α-SA ratio ($n = 13–14$, $p = 0.0090$). (j) Key fibrosis genes, Median of Ratios (mean ± s.d., $n = 3$). Student's *t*-test used for all statistical analyses [Colour figure can be viewed at wileyonlinelibrary.com]

our organoids for Platelet Endothelial Cell Adhesion Molecule (PECAM/CD31) and von Willebrand Factor (vWF). The CD31 expression between vehicle and IL-1β treated was not significantly different, while the expression of vWF, a clotting factor, was significantly higher in the IL-1β treated organoids (Figure 5a). Notably, the endothelial cell content was largely depleted in our hCOs at higher IL-1β doses (Supplemental Figure 6A–B), which validated our IL-1β dose (1 ng/ml) used in the study. While transcriptomic analysis only showed weak indications of a coagulative transcriptome (GO:0050817) of the hCOs upon IL-1β treatment (Figure 5b), the upregulation of key prothrombotic genes such as Intercellular Adhesion Molecule 1 (ICAM1), E-Selectin, and Vascular Adhesion Protein (VAP1) was significant (Figure 5c), similar to COVID-19 patients (Jin et al., 2020). Additionally, downregulation of Claudin-5 (CLDN5) may lead to vascular permeability (Jin et al., 2020). Nitric Oxide Synthase 3 (NOS3) expression trended downwards in our IL-1β treated hCOs and indicates a potential decrease in the ability of endothelial cells to support CM function through nitric oxide (Juni et al., 2019). PLAU, a gene responsible for counteracting coagulation, was found to be significantly upregulated, and high levels of its signaling has been found to predict disease severity in COVID-19 patients (Kyriazopoulou et al., 2021).

Recent studies have indicated endothelial cells are the main target of SARS-CoV-2 viral infection in the myocardium, which then induce secretion of proinflammatory cytokines (e.g., IL-1β) (Brauninger et al., 2021). To assess how the endothelial cells affect IL-1β mediated damage to our hCOs, we removed HUVECs from our standard hCO formulation to prepare HUVEC Deficient Organoids (HDOs). Representative images of HDOs and HDOs treated with IL-1β (Figure 5d) showed increases in the vimentin:α-SA ratio and decreases in sarcomere width (Figure 5e), consistent with IL-1β treated HCOs. Unlike the hCOs (Figure 2c), TUNEL expression was significantly increased upon IL-1β treatment in the HDOs (Figure 5f), indicating a shift in mechanism in IL-1β mediated damage to the organoids. Further, Figure 5g indicates the HDOs had a functional reduction in contraction amplitude that was significantly more severe

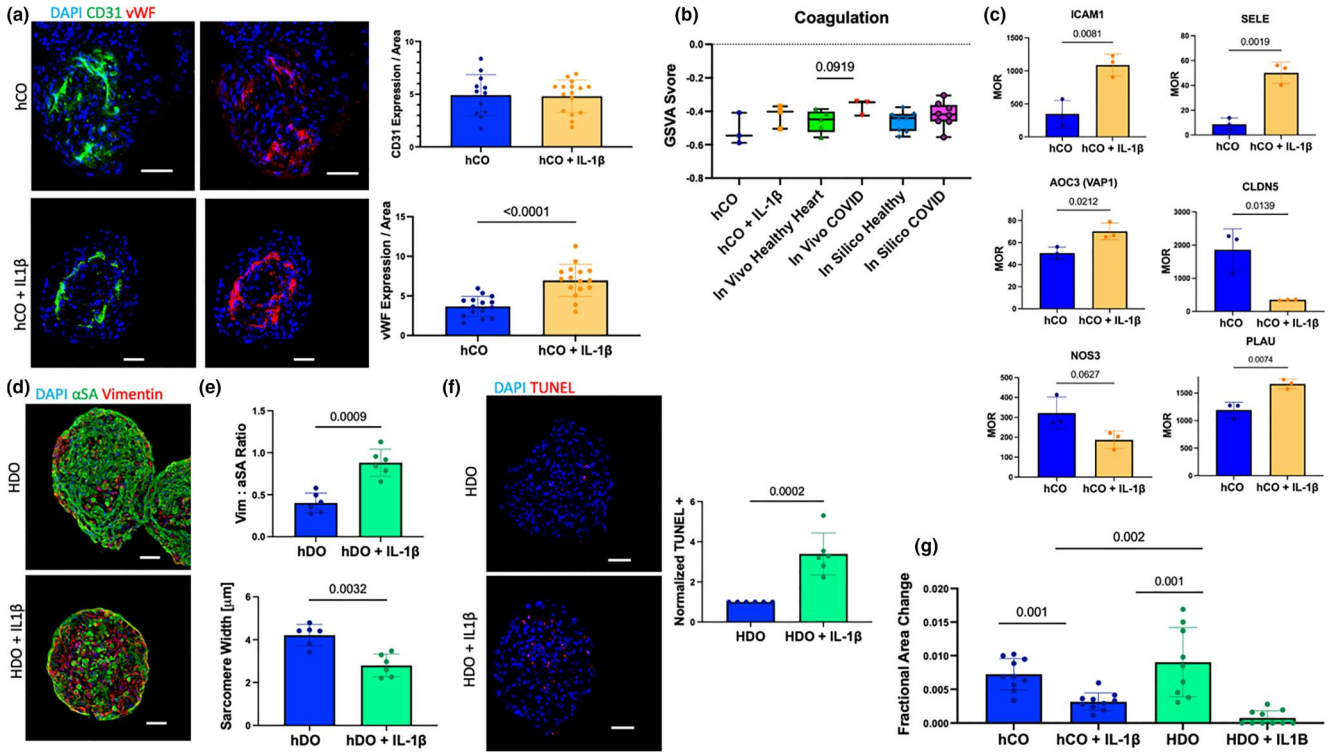

FIGURE 5 Human Cardiac Organoids Stimulated with IL-1β Recapitulate Hallmarks of Vascular Response to COVID-19. (a) Immunofluorescent staining of hCOs (top) or IL-1β stimulated hCOs (bottom) (Green = CD31, Red = vWF). Quantification (Top) reveals no difference in CD31 expression, (mean ± s.d., $n = 12$, and $n = 16$, $p = 0.9969$). (Bottom) reveals increase in vWF expression upon IL-1β stimulation, $p < 0.0001$ (mean ± s.d., $n = 14$, and $n = 16$, respectively). (b) GSVA (Coagulation) of hCOs, ($n = 3$), *In Silico* hCOs (healthy: $n = 5$, COVID: $n = 3$), and in vivo samples ($n = 8$) (mean ± s.d). (c) Key genes in the vascular response to inflammation/COVID-19. Median of Ratios (mean ± s.d, $n = 3$). (d) Representative Images of HDOs (Top) or IL-1β stimulated HDOs (Bottom) (Green = α-SA, Red = Vimentin, Blue = DAPI). (e) Quantification of Vimentin:α-SA ratio (left), and sarcomere width (right). (mean ± s.d., $n = 6$), $p = 0.0009$ and 0.0032, respectively. (f) TUNEL Staining of HDOs (top) or HDOs + IL-1β (bottom), (Red = TUNEL, Blue = DAPI). Quantification normalized to control, (mean ± s.d., $n = 6$, $p = 0.0002$). (g) FAC of hCOs or HDOs ± IL-1β on Day 4. FAC analysis reveals difference ($p = 0.002$) between IL-1β stimulated groups (mean/s.d., $n = 9$–10). Student's *t*-test was used for all statistical analyses. Scale bar = 50 μm for each image [Colour figure can be viewed at wileyonlinelibrary.com]

than that of the hCOs. Collectively, these experiments indicate the critical roles of endothelial cells in mitigating COVID-19 cytokine storm mediated damage.

2.5 | IL-1β treated hCOs are a viable platform to test immunomodulatory drugs

We leveraged our hCOs to examine the ability of clinically available immunomodulatory drugs to alleviate the IL-1β induced cardiac injuries to predict their clinical performance. We chose to assess 4 common immunomodulatory drugs (Figure 6a): an IL-1 receptor antagonist (IL-1RA) (similar mechanism of action as Anakinra); Tocilizumab, a monoclonal antibody against the IL-6 receptor; Baricitinib, a JAK/STAT inhibitor aimed at blocking cytokine receptors; and Dexamethasone, a potent glucocorticoid meant to inhibit the transcription of cytokines, now used commonly to treat hospitalized COVID-19 patients (Group et al., 2021). Organoids were treated with each drug concurrently with IL-1β on Day 0, and drug was replenished in the media with every media change/cytokine replenishment. As seen in Figure 6b, Dexamethasone was the only drug that was able to ameliorate the hCOs from the IL-1β induced reduction in organoid contractility (i.e., fractional area change). Tocilizumab was unable to improve contraction amplitude, consistent with its modest therapeutic benefits in clinic (Furlow, 2020). Baricitinib was also unable to improve contraction amplitude, which was also reported by Mills et al. when tested in their hCO system (Mills et al., 2021). While effective, Dexamethasone exacerbated the ratio of vWF/CD31, showing even higher levels of vWF than IL-1β treatment alone ($p = 0.0514$) (Figure 6c). This is consistent with the prothrombotic side effects of Dexamethasone observed in the clinical studies (Brotman et al., 2006). (Representative images of CD31/vWF staining for each condition can be found in Supplementary Figure 7A–B). Interestingly, although they were unable to spare the hCOs from IL-1β induced decreases in contraction amplitude, Tocilizumab and Baricitinib helped preserve sarcomere width (Figure 6d). Moreover, Dexamethasone showed a higher vimentin expression in the hCOs (Figure 6e–f), indicating an increased presence of fibroblasts. While initially unexpected, literature has suggested that Dexamethasone can induce fibroblast proliferation in certain instances (Warshamana, 1998).

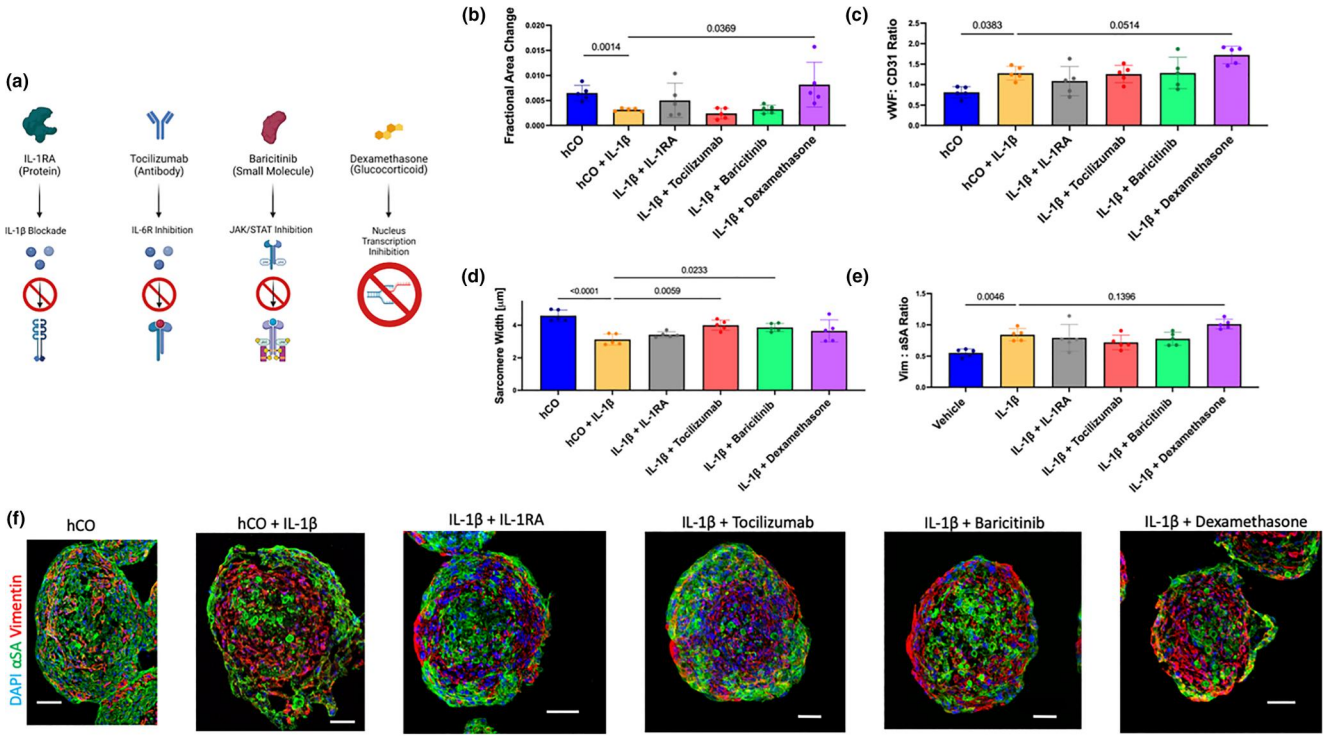

FIGURE 6 Human Cardiac Organoids Provide Platform for Immunomodulatory Drug Testing. (a) Schematic indicating the mechanism of action for each immunomodulatory drug tested with the human cardiac organoid system. (b) FAC on Day 4 for hCOs, IL-1β stimulated hCOs, or IL-1β stimulated hCOs with each immunomodulatory drug. (mean ± s.d., $n = 5$), analysis performed by Student's *t*-test. (c) vWF to CD31 ratio for hCOs, IL-1β stimulated hCOs, or IL-1β stimulated hCOs with each immunomodulatory drug, (mean ± s.d., $n = 5$), analysis performed by ANOVA. (d) Sarcomere width in μm for hCOs, IL-1β stimulated hCOs, or IL-1β stimulated hCOs with each immunomodulatory drug, (mean ± s.d., $n = 5$), analysis performed by ANOVA. (e) Vimentin:α-SA ratio for vehicle, IL-1β, or IL-1β with each immunomodulatory drug, (mean ± s.d., $n = 5$), analysis performed by ANOVA. (f). Representative Images of immunofluorescent staining for hCOs, IL-1β stimulated hCOs, or IL-1β stimulated hCOs with each immunomodulatory drug. (Green = α-SA, Red = Vimentin, Blue = DAPI). Scale bar for each image = 50 μm [Colour figure can be viewed at wileyonlinelibrary.com]

2.6 | IL-1β treated hCOs provide a valid platform for recovery studies

Significant interests surround the reversibility of COVID-19 ACIs and the extent to which patients may be susceptible to long-COVID as they recover from active infection and enter convalescence (Nalbandian et al., 2021). To investigate COVID-19 ACI reversibility, hCOs were conditioned with a recovery period (normal culture medium with no IL-1β) for 10 days after 4 days of IL-1β treatment (Figure 7a). It took 2 days of recovery before FAC was not statistically significant, despite their mean FAC values not leveling out until day 14. Representative images for the control hCOs and the hCOs recovered from IL-1β treatment are shown in Figure 7b. Despite similar mean FACs and vimentin:α-SA ratios for each treatment on day 14, the recovered hCOs after IL-1β treatment had a lower mean sarcomere width (Figure 7c) and reduced CD31 and vWF staining (Supplemental Figure 8).

To assess the functional recovery of hCOs on D14, we performed a "Stress Test" to simulate physiological exercise. While hCOs responded to norepinephrine (NE) stimulation by showing a significant increase in spontaneous beating rate, IL-1β treated hCOs did not respond to NE stimulation on Day 4 (Figure 7d–e). However, both groups responded to the NE stimulation on day 14 (Figure 7f–g), with each group showing a significant increase in the beating rate. Of note, levels of the adrenergic receptor β1 were not different via RNAseq analysis between vehicle and IL-1β, though levels of the β2 receptor were significantly increased (Figure 7h). These studies indicated the cardiac injuries caused by the COVID-19 cytokine storm could be reversed after a sufficient recovery period.

3 | DISCUSSION

The cytokine profile seen from the IL-1β treated hCOs suggests the organ level response to IL-1β closely mirrors that of the serum cytokine profile frequently reported in literature. Based on these findings, we shed light on the possibility that the inflammatory response of the myocardium either mirrors the systemic inflammatory profile measured via serum analysis, or that the myocardium itself contributes to the systemic inflammation found in severe COVID-19 patients as indicated in recent publications (Hartmann et al., 2021). By leveraging the innate inflammatory properties of our hCOs and stimulating them with IL-1β, we have formulated a model with a biomimetic approach to characterize multiple aspects of

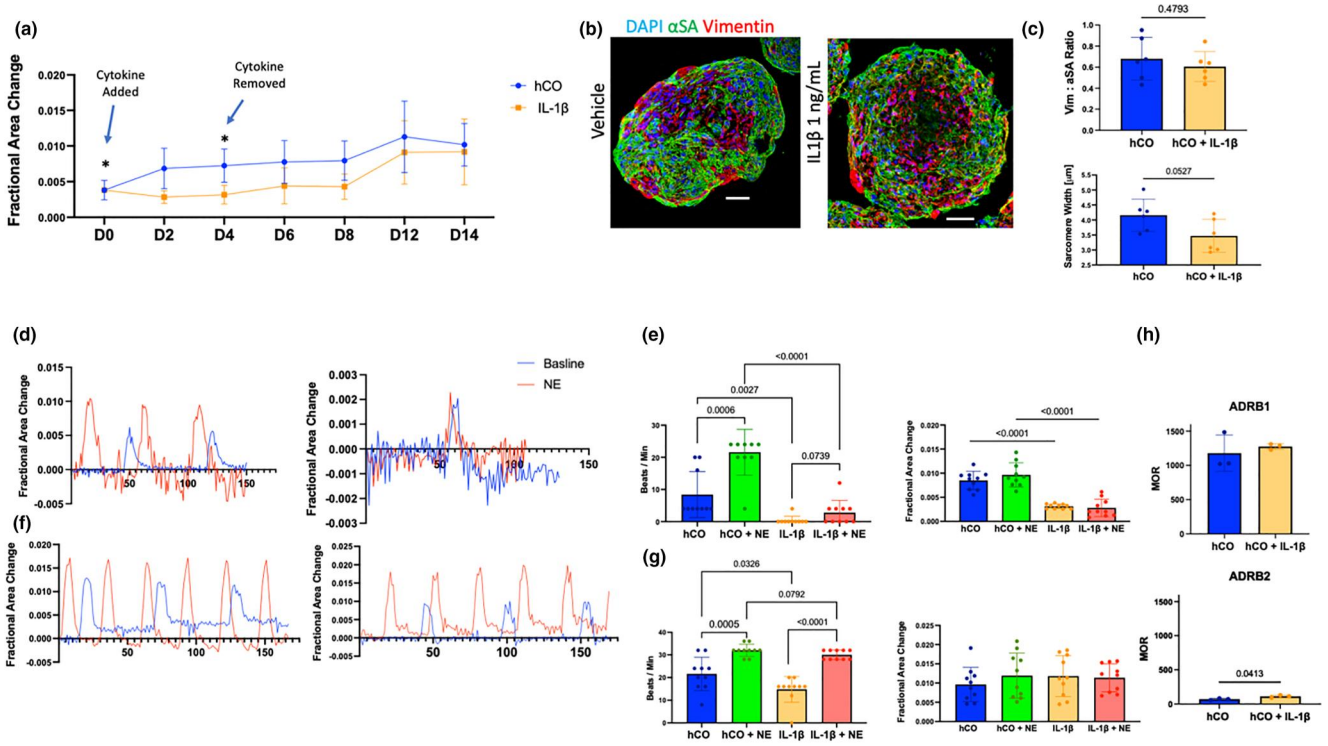

FIGURE 7 Human Cardiac Organoids Are a Viable Model to Test Recovery Studies. (a) FAC over time (mean ± s.d., $n = 9–10$, * = $p < 0.05$). (b) Immunofluorescent staining of organoids on D14 treated initially with vehicle or IL-1β (Green = α-SA, Red = Vimentin, Blue = DAPI, Scale bar = 50 μm). (c) (Top) Vimentin:α-SA ratio for D14 hCOs (Bottom) Quantification of sarcomere width for D14 organoids. ($n = 6$, $p = 0.4793$ and 0.0527, respectively. (d). FAC waveform of Day 4 hCOs (left) or IL-1β stimulated hCOS (right) in the presence (red) or absence (blue) of 1 μM NE. (e) (Left) Beats/minute of hCOs, hCOs with NE, IL-1β treated hCOs, or IL-1β treated hCOs with NE. (Right) FAC for each group before and after NE stimulation ($n = 10$, mean ± s.d.). (f) FAC waveform of Day 14 hCOs (left) or IL-1β treated hCOs (right) with (red) or without (blue) 1 μM Norepinephrine. (g) (Left) Beats/minute in hCOs, hCOs with NE, IL-1β treated hCOs, or IL-1β treated hCOs with NE. (Right) FAC ± NE stimulation ($n = 10$, mean ± s.d.). (h) Median of Ratios of beta 1 (top) and beta 2 (bottom) adrenergic receptors in hCOs, (mean ± s.d., $n = 3$). Student's *t*-test was used for all statistical analyses [Colour figure can be viewed at wileyonlinelibrary.com]

myocardial pathology besides contraction amplitude (cytokine release, fibrosis, endothelial dysfunction/thrombosis). Interestingly, recent studies have highlighted the dysfunctionality of peripheral monocytes in COVID-19 and suggested the organ level inflammatory response may be a major contributor to serum cytokine profiles (Knoll et al., 2021). Mills et al. recently published a study using a cytokine screen on hCOs to identify specific cytokine induced diastolic dysfunction (Mills et al., 2021). They ultimately used a cocktail of IL-1*β*, IFNγ, and poly(I:C) as their "cytokine storm" formulation and assessed effects of TNFα on hCOs. Our transcriptomic data may indicate that a multi-cellular response to IL-1*β* shares sufficient downstream mediators to capture hallmarks of both TNFα and IFNγ, despite the lack of a consistent upregulation of either gene. To the best of our knowledge, this is the first direct transcriptomic comparison of hCOs to human COVID-19 cardiac tissue samples, and illustrates that a defined, single input is sufficient to recapitulate several main transcriptomic signatures found in COVID-19 hearts.

Our study is also the first to illustrate the histologic changes in hCOs following cytokine exposure, and through comparisons to histology from 95 COVID-19 autopsies, show a key mechanism for cardiac dysfunction is the degradation/downregulation of contractile machinery, or cardiomyocyte destruction, pending the presence of a viable endothelium. Additionally, our histologic analyses provide insight into the "noncardiac" pathologies that may ensue from COVID-19 in the myocardium such as fibrosis and thrombosis. Cardiac fibrosis following COVID-19 has been reported, even in mild or asymptomatic patients, which may lead to cardiac complications such as arrhythmias later in life (Huang et al., 2020; Nalbandian et al., 2021). As long-term effects of COVID-19 continue to be assessed in patients, understanding the reversibility of cardiac dysfunction has become paramount, particularly in the context of acute convalescence. Currently, clinical guidelines recommend patients recovering from COVID-19 infection should not resume physical activity for at least 10 days after the onset of symptoms and 7 days after symptom resolution (Metzl et al., 2020). Our data indicates it takes up to 2.5 times of the exposure time for contractile machinery to be re-upregulated. Even at the point of functional recovery, sarcomere width was still reduced, supporting current clinical guidelines. Though the reversibility of these injuries may be increased due to the use of hPSC-CMs, reversibility of IL-1*β* mediated cardiac dysfunction has been shown both in vitro and in vivo (Anand Kumar, 1996; Jun-ichi Oyama, 1997; Juni et al., 2019).

Dexamethasone was the only drug able to significantly improve IL-1*β* treated hCO function. This implies, at least in the context our

system, inhibition of cytokine signaling is required at the transcriptional level to adequately spare hCOs from functional deficits. This protection may come at the cost of a pro-coagulative state and a propensity to induce fibroblast proliferation, which have also been observed (Brotman et al., 2006; Warshamana, 1998). IL-1RA (Anakinra analogue) treatment did not significantly spare function ($p = 0.2637$), but, unlike dexamethasone, did not significanty alter the stromal cells of our hCOs. Further studies are needed to determine if the comprehensive profile of either Dexamethasone or IL-1RA treated hCOs better correlates with clinical outcomes.

While recent studies have suggested that SARS-CoV-2 viral infection of endothelium leads to IL-1β production and myocardial dysfunction (Hartmann et al., 2021), the results from the HDO system indicates the endothelium also mitigates the COVID-19 cardiac insults, consistent with its well-established cardioprotective effects (Colliva et al., 2020). Interestingly, recent COVID-19 autopsy studies have shown direct SARS-CoV2 virial infection of myocardium is associated with significant reduction of endothelial cells and early death (Brauninger et al., 2021). As current COVID-19 clinical trials that target endothelium have been focusing on anti-coagulation therapies (Lopes et al., 2021), our data indicates the importance of endothelium protection to ameliorate the COVID-19 induced cardiac injuries.

Though IL-1β treated hCOs align with COVID-19 hearts, our findings can be more widely applied to other instances of cardiac inflammation. IL-1β has been shown to have key roles post myocardial infarction, and with inflammatory diseases such as myocarditis, and rheumatic disease (Bujak & Frangogiannis, 2009; Szekely & Arbel, 2018). In the post infarction heart, IL-1β is a key stimulator of the ensuing inflammatory response that ultimately leads to cardiac remodeling. In other inflammatory diseases, IL-1β leads to widespread inflammation, and directly can alter key cardiac proteins involved in contractions, calcium handling, and electrophysiology (Szekely & Arbel, 2018). Alterations of the dose of IL-1β, duration of treatment, culture conditions, and cellular composition of the hCOs may result in new, viable in vitro models for these conditions.

The hCOs in this study were formed from pre-differentiated cells that then self-assembled. A limitation is that the cellular composition and architecture of our hCOs does not perfectly recapitulate the heart (e.g., HUVECs, lack of chambers). Recent advances in cardiac organoid technology have employed a variety of strategies such as leveraging WNT signaling or modulating their spatial organization to result in differentiated cardiac organoids that self-assemble and spatially mirror natural cardiac architecture (Hoang et al., 2021; Hofbauer et al., 2021; Lewis-Israeli et al., 2021). Utilizing such strategies could provide more physiologically accurate representations of the heart and cardiac inflammation.

4 | CONCLUSIONS

In summary, our results showed IL-1β induced the release of a milieu of the proinflammatory cytokines from hCOs, with a similar profile to COVID-19 cytokine storm. Our data also validated the IL-1β treated hCOs' ability recapitulate the hallmarks of the transcriptome, structure, and function of COVID-19 hearts. We further demonstrated the IL-1β treated hCOs are an effective testing platform for immunomodulatory drugs and long-term reversibility of COVID-19 induced cardiac pathologies.

5 | METHODS

Fabrication of Organoids. We have previously described the fabrication of our organoids (Richards et al., 2017; Richards et al., 2020). Briefly, agarose molds fabricated from commercial master micromolds from Microtissues were used as molds for microtissue fabrication, with each mold containing a 7 x 5 matrix of recesses. Organoid cellular suspensions are composed of 55% hPSC–CMs, 24% hcFBs, 14% HUVECs, and 7% hADSCs in medium at a concentration of 2×10^6 cells per ml. To generate organoids with a diameter ~150 µm, 75 µl of the organoid suspension was added into the molds and allowed to settle for 15 min. Upon settling, 2 ml of medium was added to submerge the molds in a 12-well plate. Media was changed every 2 days for the entirety of the experiment. The organoids were allowed to form for 4 days, when it was denoted as D0 of the experiment. IL-1β treatment protocol was then initiated for 4 days. Organoid media is composed of a ratiometric combination of cell-specific medium reflecting the starting cell ratio of the organoid. The hPSC-CM-specific component was defined as glucose-containing F12/DEME medium with 10% FBS, 1% glutamine and 1% non-essential amino acids (Gibco). In the instance of the HUVEC Deficient Organoids (HDOs) the 14% of the HUVEC cell content was replaced with cardiomyocytes in its cell suspension.

Statistical Analysis. Differences between experimental groups were analyzed using Microsoft Excel (v13.7) and GraphPad Prism (v9.1.1) statistical tools. Sample distribution was assumed normal with equal variance. Statistical analysis was performed using a two-tailed Student's *t*-tests or one-way ANOVA with post-hoc Bonferroni-corrected *t*-tests and $p < 0.05$ was considered to be statistically significant. Sample sizes of biologically independent samples per group and the number of independent experiments are indicated in figure legends. Each measurement was taken from a distinct sample. Outliers were excluded with the ROUT method.

AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The main data supporting the results in this study are available within the paper and its Supplementary Information. The raw and analyzed datasets generated during the study are available from the corresponding authors on reasonable request. RNA-seq data will be made available from the NCBI GEO. Accession numbers will be provided as soon as they are provided to the corresponding authors.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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