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Modeling mechanisms underlying differential inflammatory responses to COVID-19 in type 2 diabetes using a patient-derived microphysiological organ-on-a-chip system†

Vinny Negi,[a] Dillon Gavlock,[b] Mark T. Miedel,[b] Jeong Kyung Lee,[a] Tongying Shun,[b] Albert Gough,[b] Lawrence Vernetti,[b] Andrew M. Stern,[b] D. Lansing Taylor[b] and Vijay K. Yechoor*[a]

Background: COVID-19 pandemic has caused more than 6 million deaths worldwide. Co-morbid conditions such as Type 2 Diabetes (T2D) have increased mortality in COVID-19. With limited translatability of *in vitro* and small animal models to human disease, human organ-on-a-chip models are an attractive platform to model *in vivo* disease conditions and test potential therapeutics. *Methods*: T2D or non-diabetic patient-derived macrophages and human liver sinusoidal endothelial cells were seeded, along with normal hepatocytes and stellate cells in the liver-on-a-chip (LAMPS – liver acinus micro physiological system), perfused with media mimicking non-diabetic fasting or T2D (high levels of glucose, fatty acids, insulin, glucagon) states. The macrophages and endothelial cells were transduced to overexpress the SARS-CoV2-S (spike) protein with appropriate controls before their incorporation into LAMPS. Cytokine concentrations in the efflux served as a read-out of the effects of S-protein expression in the different experimental conditions (non-diabetic-LAMPS, T2D-LAMPS), including incubation with tocilizumab, an FDA-approved drug for severe COVID-19. *Findings*: S-protein expression in the non-diabetic LAMPS led to increased cytokines, but in the T2D-LAMPS, this was significantly amplified both in the number and magnitude of key pro-inflammatory cytokines (IL6, CCL3, IL1β, IL2, TNFα, *etc.*) involved in cytokine storm syndrome (CSS), mimicking severe COVID-19 infection in T2D patients. Compared to vehicle control, tocilizumab (IL6-receptor antagonist) decreased the pro-inflammatory cytokine secretion in T2D-COVID-19-LAMPS but not in non-diabetic-COVID-19-LAMPS. *Interpretation*: macrophages and endothelial cells play a synergistic role in the pathophysiology of the hyper-inflammatory response seen with COVID-19 and T2D. The effect of Tocilizumab was consistent with large clinical trials that demonstrated Tocilizumab's efficacy only in critically ill patients with severe disease, providing confirmatory evidence that the T2D-COVID-19-LAMPS is a robust platform to model human *in vivo* pathophysiology of COVID-19 in T2D and for screening potential therapeutics.

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Introduction

The COVID-19 pandemic caused by the highly contagious SARS-CoV-2 virus resulted in millions of deaths worldwide, often with multiple organ failures.[1] It was observed that people with co-morbidities such as diabetes, hypertension, cardiovascular disease, and pulmonary disease are at higher risk of developing severe COVID-19 infection.[2,3] A meta-analysis of all the co-morbidities associated with COVID-19 revealed that diabetes is the second most common co-morbidity, with 2.5 times more prevalent among fatal cases than total cases.[4] The major cause of mortality was cytokine storm syndrome (CSS). At the molecular level, hyperglycemia was predicted to be the underlying cause, as diabetes patients with controlled blood glucose did not have severe symptoms.[5] Increased blood glucose could weaken the immune system and hyperactivation of inflammatory cytokines.[6] Furthermore, it was found that COVID-19 can, in turn, worsen diabetes and, in some cases, results in the development of diabetes.[7–9] Thus, there is an imperative need to determine drugs that are effective in COVID-19 infection in diabetic patients.

[a] *Diabetes and Beta Cell Biology Center, Division of Endocrinology and Metabolism, University of Pittsburgh, Pittsburgh, PA, USA. E-mail: yechoorv@pitt.edu*
[b] *Drug Discovery Institute and Department of Computational and Systems Biology, University of Pittsburgh, Pittsburgh, PA, USA*

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Many *in vitro* and *in vivo* systems exist to screen and select drugs for a disease; however, the translatability of such systems has been challenging because of the limited efficacy and efficiency of these conventional models. With the advent of organ-on-a-chip platforms and the use of primary human cells, these MPS systems act as a robust and reliable tool for drug screening and selection.[10–12] Thus, we sought to develop a T2D-COVID-19 organ-on-a-chip model to test some potential drugs for treating T2D patients with COVID-19. Given the central role of the liver in the T2D pathophysiology, a Liver-on-a-chip (LAMPS – liver acinus micro physiological system)[13] was modified to model T2D-COVID-19 disease. LAMPS typically consists of the sequential addition of four different cell types – hepatocytes, endothelial, macrophage, and stellate cells on a collagen-fibronectin matrix. A T2D-COVID-19 specific model was developed by introducing T2D patient-derived endothelial and macrophage cells over-expressing lentivirus-based SARS-CoV-2-S protein in parallel with non-diabetic derived cells and lentivirus control. Further, these LAMPS were perfused with T2D media consisting of high glucose, fatty acid, insulin, and glucagon, mimicking the T2D state compared to non-diabetic fasting media (Non).[14] The disease development was monitored by measuring secreted cytokines from LAMPS using Luminex multiplex assay to quantify cytokine storm syndrome (CSS). Overexpression of S-protein in T2D-LAMPS was sufficient to induce a hyperimmune state with a significant increase in pro-inflammatory cytokine secretion. Furthermore, tocilizumab – an IL6R antagonist, was able to mitigate these effects in T2D-LAMPS consistent with clinical observations, providing a proof-of-principle to the translatability of this platform. CSS is one of the causes of death in COVID-19 infection;[15,16] thus, modeling it and testing a broader scope of drugs creates the potential to identify new therapeutic strategies for reducing the morbidity and mortality of COVID-19.

Materials and methods

Cells source

Cryopreserved human hepatocytes (cat# HMCPQC, lot# HU8339) were obtained from ThermoFisher. Cryopreserved human normal (lot# HL160019) and T2D (lot# HL160035) liver sinusoidal endothelial cells (LSEC, cat# NPC-AD-LEC-P1) were obtained from LifeNet Health. Cryopreserved human normal (cat# 70025.1, lot# 210171802C) and T2D (cat# 70062, lot# 1010113250) peripheral blood mononuclear cells (PBMCs) were obtained from STEMCELL Technologies. LX-2 (RRID: CVCL_5792) was obtained from EMD Millipore. The clinical details of T2D patients are shown in Table 1.

Table 1 Clinical details of T2D patients

Cell type	Gender	Race	Age	COD
PBMCs	Female	Caucasian	52	N/A
LSECs	Female	Hispanic	47	ICH/stroke

Macrophage differentiation

Human monocytic cell line THP-1 was differentiated into macrophages using 200 ng ml^{-1} of PMA for 48 h cultured in RPMI with 10% FBS media. The human primary monocytes were isolated from PBMCs (peripheral blood mononuclear cells) using the EasySep human monocyte isolation kit (Cat# 19359) from STEMCELL Technologies according to the manufacturer's instructions. The percentage purity of the isolated cells was determined by staining them for surface marker CD14-FITC labeled (RRID: AB_2571928) and CD16-Alexa Flour 647 labeled (RRID: AB_492977) and acquired on BD LSR II flow cytometer. The monocytes were then cultured in the presence of GM-CSF (50 ng ml^{-1}) in RPMI 1640 with 10% FBS for 6 days. At the end of 6 days, the percentage polarization was determined by measuring M1 (CD80, RRID: AB_314501), M2 (CD163, RRID: AB_2291272), and total (CD11b, RRID: AB_314157) macrophage marker using flow cytometry. CD80 was FITC, CD163 was APC, and CD11b was PE-labeled. All fluorochrome-labeled antibodies were obtained from BioLegend.

Over-expression of SARS-CoV-2-spike protein

The lentivirus plasmid over-expressing SARS-CoV2-Spike protein (S-prot) was obtained from Addgene (141347, RRID: Addgene_141347). For the control plasmid (Ctrl), an empty vector with the same backbone was used. The lentivirus was packaged in HEK 293T cells. The cell culture supernatant was collected 48 h post-transfection and filtered through a 0.45-micron filter. Later, it was transduced into macrophages and endothelial cells with 8 μg ml^{-1} polybrene overnight. The next morning cell media was changed, and cells were trypsinized for seeding onto the respective LAMPS.

Non-diabetic fasting and T2D media

LAMPS were perfused with non-diabetic fasting (Non) or type 2 diabetic (T2D) media, as indicated in each experiment, to mimic non-diabetic (Non) and T2D conditions, respectively. As previously established, the media composition mimics normal and T2D conditions.[14,17] The composition is given as below in Table 2.

Table 2 Composition of non-diabetic fasting (non) and type 2 diabetic (T2D) media

Components	Non	T2D
Glucose	5.5 mM	20 mM
Insulin	10 pM	10 nM
Glucagon	100 pM	10 pM
Oleic acid	—	200 μM
Palmitic acid	—	100 μM
LPS	—	1 μg ml^{-1}
TGF-β	—	10 ng ml^{-1}
Glutamine (Gluta-Max)	2 nM	2 mM

LAMPS assembly and culture

LAMPS were assembled as previously described.[13,14,17,18] Briefly, a single chamber commercial microfluidic device (HAR-V single-channel device, SCC-001, Nortis, Inc. Seattle, WA) was used for LAMPS studies. For all steps involving injection of media and/or cell suspensions into LAMPS devices, 100–150 μl per device was used to ensure complete filling of fluidic pathways, chamber, and bubble traps. LAMPS were first coated with collagen/fibronectin matrix, followed by hepatocytes seeding. The next day, macrophage and endothelial cells over-expressing S-protein (S-prot) and control (Ctrl) were seeded along with LX-2 cells. The LAMPS were cultured overnight in a CO_2 cell incubator, and the desired media flow was established the next morning at a constant rate of 15 μl h^{-1} for 4–10 days, depending on the experiment. The tocilizumab (Toz) solution obtained from Selleckchem was added to a final concentration of 100 μg ml^{-1} for overnight culture and 24 h under flow, thus a total of about 36 h of incubation.

Multiplex immunoassay

Efflux collected from LAMPS every other day was stored at −20 °C until the completion of the experiment. The cytokines in the efflux were measured using the Human XL Cytokines Discovery (45-plex; R&D systems, cat# FCSTM18), according to the manufacturer's instructions at the University of Pittsburgh Cancer Proteomics Facility Luminex® Core Laboratory. All multiplex panels were run simultaneously to avoid run-to-run and operator error variability utilizing the xMAP platform licensed by Luminex®. The Luminex data obtained had many out-of-range values. First, those out-of-range values were estimated by an automated computational method developed in-house to optimize the parameters of the 5-parameter asymmetrical sigmoidal math model for calculating cytokine concentration from the asymmetrical sigmoidal math model. Next, the TIBCO Spotfire (TIBCO, Palo Alto, CA 94304, USA) software was used to display the log2 fold change of paired comparison.

Statistical analysis

Statistical comparisons between specific media treatment groups were made using PRISM (San Diego, CA) to perform either a two-tailed Student's *t*-test (for 2 groups) or a One-Way ANOVA analysis with Tukey's test (for more than 2 groups) with a significance level α of 0.05 unless stated otherwise. Each experiment was performed, having at least three replicates. Each cell culture experiment was repeated three independent times, and each LAMPS study was repeated twice.

Results

S-protein overexpression induced an inflammatory phenotype in macrophages

Macrophages are one of the major cells of the innate immune system that are infected by the SARS-CoV-2 virus through ACEII receptor and are significant contributors to cytokine storm and hyper-inflammation seen in severe COVID illness.[19,20] THP-1 cell line, a human monocyte–macrophage cell line, was first used to test the effects of the SARS-CoV2-Spike protein overexpression (S-prot) *in vitro*. THP-1 cells were differentiated into macrophages using standard protocols with PMA.[21] Lentiviral transduction of these differentiated THP-1 cells led to a robust expression of S-protein (Fig. 1A). Consequently, there was a significant increase in many pro-inflammatory cytokines, such as TNFα, IFNβ, and NFKB1, as measured by qRT-PCR (Fig. 1B). Interestingly, IL1β was unchanged, while IL6 was decreased (Fig. S1A†). These data suggested that the overexpression of S-protein in macrophages was sufficient to induce an inflammatory phenotype. However, to obtain a more robust modeling of the *in vivo* effects of SARS-CoV2 infection, we tested the effects of S-protein overexpression in primary human PBMC-derived macrophages.

We isolated classical monocytes ($CD14^+$ $CD16^-$)[22] from human peripheral blood mononuclear cells (PBMCs) (Fig. 1C). These monocytes were then differentiated into predominant M1 macrophages by culturing them in the presence of GM-CSF for 6 days.[23,24] At the end of day 6, the differentiation percentage was determined by measuring the markers for M1 macrophages (CD80), M2 macrophages (CD163), and all macrophages (CD1b) using flow cytometry (Fig. 1C). We found the majority of cells to be M1 phenotype. These M1 differentiated macrophages were transduced either with lentivirus to over-express S-protein or with control virus. Unlike the THP-1 cells, upon S-protein overexpression, human PBMC-derived macrophages did not display a significant increase in the pro-inflammatory cytokines (Fig. 1D). Instead, they showed a decrease in expression of M1 markers corresponding with an increase in M2 markers (Fig. S1B†). In addition to differences among macrophage subtypes, this contrasting result raised the possibility that the S-protein-induced proinflammatory phenotype could depend upon the complex physiological and pathophysiological conditions and cellular microenvironments not recapitulated during traditional cell culture conditions. Our previous studies with a liver microphysiological system supported this hypothesis.[17] Hence, we set up conditions to mimic normal non-diabetic fasting (Non – 5.5 mM glucose) state or T2D (20 mM glucose) state (Table 2) to test if overexpression of S-protein in primary macrophages in the context of the liver microphysiological system could induce pro-inflammatory states.

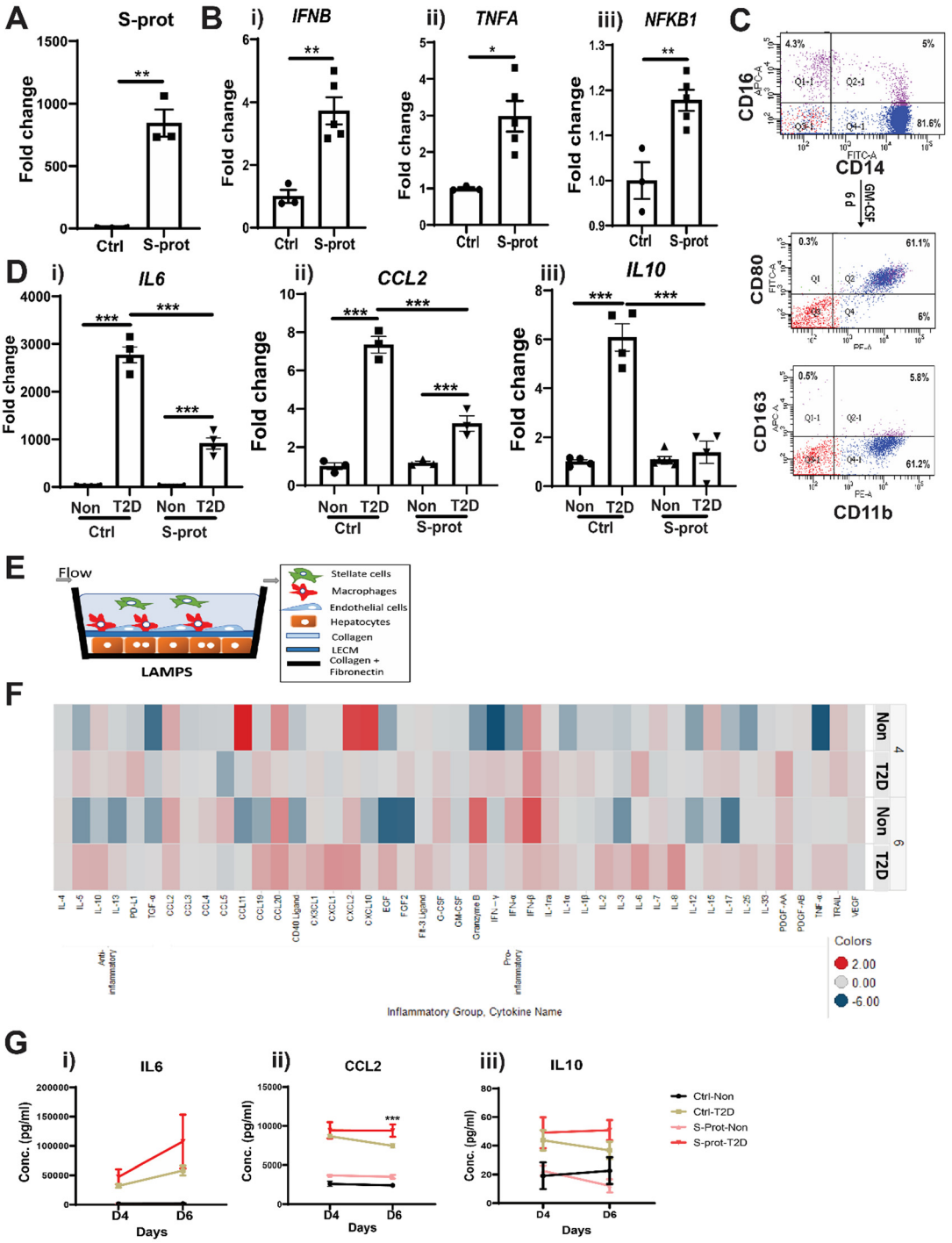

Fig. 1 S-protein expression induced an inflammatory phenotype in macrophages. THP-1 cells were differentiated into macrophages using PMA (200 ng ml^{-1}) for 48 h, followed by overexpression of lentivirus-based SARS-CoV-2-Spike protein (S-prot). An empty lentivirus vector was used as control. A. The overexpression was validated by qRT-PCR at 72 h post transduction and B. the proinflammatory cytokines and transcription factor i) IFNB, ii) TNFA, and iii) NFKB1 were measured at same time point. C. Classical monocytes (CD14^{+} CD16^{-}) were isolated from human peripheral blood mononuclear cells (PBMCs) and their percentage purity was determined using flow cytometry using CD14 and CD16 markers. These cells were then cultured in presence of GM-CSF (50 ng ml^{-1}) for 6 days to differentiate into M1 macrophages. The percentage purity of M1 macrophage was assessed by the presence of CD80-M1 marker but not CD163-M2 marker along with total macrophage maker CD11b. D. S-protein was overexpressed in these macrophages and were cultured in either non-diabetic (Non) or T2D media. After 72 h of transduction, the gene expression of pro-inflammatory-i) IL6, ii) CCL2 and iii) anti-inflammatory-IL10 were determined by qRT-PCR. E. Schematic demonstrates the sequential addition of these macrophages along with other cell types, namely hepatocytes, liver sinusoidal endothelial cells (LSECs), and stellate (LX-2) cells into LAMPS. F. Heat-map showing the log 2 fold change S-protein over control (S/C) of secreted cytokines in LAMPS in non-diabetic (Non) and T2D media for day 4 and 6. G. Line graph representing i) IL6, ii) CCL2, and iii) IL10 levels in LAMPS expressing S-protein (S-prot) and control (Ctrl) in non-diabetic (Non) or T2D media. Statistical significance was assessed using Student's two-tailed *t*-test and one-way ANOVA (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

Validating the setup of liver acinus micro-physiological system (LAMPS)

The LAMPS was setup as shown in Fig. S2A† and described in Materials and methods. The hepatocytes, endothelial cells, macrophages and stellate cells were added onto the device and flow of normal (NF – non-diabetic fasting) and T2D diabetic (LMS – late metabolic syndrome) media was setup at a rate of 15 μl h^{-1}. The efflux was collected every other day for 10 days; albumin and lactate dehydrogenase (LDH) were measured in the efflux to determine liver function (Fig. S2B†). The albumin levels were found to be lower in T2D media as compared to non-diabetic (non) media, this was consistent with previous NASH models (non-alcoholic steatohepatitis).[14,25] Conversely, LDH levels were higher in T2D media as compared to non, consistent with NASH induced cellular damage to liver. The secretome measurements in non-diabetic media were consistent with prior published reports[14,18] suggesting a functionally competent liver model that can be modified further to develop T2D-COVID-19-LAMPS.

Patient-derived LAMPS for modeling SARS-CoV2 infection

As previously reported,[14,17,18] LAMPS is constituted using four predominant liver cell types – hepatocytes, liver sinusoidal endothelial cells (LSECs), macrophages, and stellate cells, in the ratio normally present in the human liver. Here, we modified the LAMPS to incorporate macrophages differentiated from patient-derived PBMCs that expressed S-protein to model SARS-CoV2 infection and perfused it with either Non or T2D media (schematic in Fig. 1E) at a constant rate of 15 μl h^{-1} for 6 days. The efflux was collected every other day to measure cytokines (Materials and methods). 45 cytokines were measured in the efflux using Luminex the assay. There is an increase in cytokine efflux soon after flow is initiated that returns towards the baseline and achieves a steady level 4 days after the perfusion initiation in the LAMPS control (data not shown). There was an increase in pro-inflammatory cytokines with S-protein over control (S/C) from the LAMPS in T2D media compared to Non (Fig. 1F). An increase in a greater number of cytokines was observed on day 6, specifically in IL6 and CCL2, with no difference in the anti-inflammatory cytokine, IL10 (Fig. 1G). This was consistent with our hypothesis that T2D media conditions could promote the pro-inflammatory effects of S-protein overexpression in macrophages in the LAMPS, to recapitulate in part clinical observations from patients with diabetes and COVID-19.[26,27]

Endothelial cells and macrophages play a synergistic role in perpetuating a pro-inflammatory state upon S-protein overexpression

The pathophysiology of cytokine storm syndrome involves multiple cell types, in addition to immune cells, with endothelial cells playing a significant role.[28,29] Hence, we first tested the effect of overexpression of S-protein in endothelial *in vitro*. Upon lentivirus-mediated S-protein overexpression (Fig. S3A†), liver sinusoidal endothelial cells (LSECs) displayed a robust pro-inflammatory state with increased expression of ICAM1 and prothrombotic factors (F2, VAP1, VWF) in T2D media conditions (Fig. S3B†).

To test if S-protein overexpression in both primary endothelial cells and macrophages from a healthy individual could have a synergistic effect in perpetuating a pro-inflammatory state we incorporated these cells in LAMPS perfused with non-diabetic (Non) media. Interestingly, the combined infection of endothelial and macrophages with lentiviral vectors expressing S-protein led to a significant increase in cytokine efflux in non-diabetic media conditions (Fig. 2A) than observed with S-protein overexpression in macrophages alone (Fig. 1D). The key cytokines that are known to be involved in CSS[30] such as IL6, CCL2, 3, and 4 were increased, and anti-inflammatory cytokine IL10 was decreased (Fig. 2B). Other cytokines that were altered by S-protein alone are shown in Fig. S4A and B.†

T2D exacerbates the cytokine release from S-protein on macrophages and endothelial cells in LAMPS

To dissect the pathophysiology of the increased incidence of cytokine storm syndrome in COVID-19 patients with T2D,[31,32] we incorporated primary cells into LAMPS obtained from patients with and without T2D. T2D patients' derived LSECs and PBMCs-macrophages were used in LAMPS, and perfused with T2D media. In parallel, non-diabetic cells were perfused with non-diabetic media as control (Fig. 2C). Twelve of 45 cytokines were altered by T2D condition (T2D) as compared to non-diabetic condition (Non) (Fig. 2D and E). Specifically, key cytokines involved in CSS, such as IL6, CCL3, TNFα, and VEGF, were increased, and anti-inflammatory cytokines such as IL10 and PDL1 were decreased, others are represented in Fig. S4C and D.† Interestingly, the combination of S-protein overexpression in both macrophage and endothelial cells of T2D LAMPS (S-T2D) (Fig. 2C) significantly altered many more cytokines (23 out of 45) as compared to control virus overexpression in non-diabetic cells (C-Non). These included those that were altered by S-protein and T2D media alone, such as IL6, CCL3, and CCL19 (Fig. 3A and C). In addition, other cytokines were significantly increased only in combination, were IL1β, IL17, IL2, IL33, TNFα, VEGF, and GM-CSF (Fig. 3B and C), all of which have been implicated in the pathogenesis of CSS.[30] Furthermore, other cytokines that were altered by the combination are shown in Fig. S5A and B.† These results support the hypothesis that T2D-COVID-19-LAMPS is a reliable model to simulate COVID-19 infection in T2D patients and that the combination of T2D with COVID-19 infection significantly amplifies the cytokine response consistent with hyper inflammation and development of CSS.

Tocilizumab is more effective in reducing CSS in T2D-COVID-19-LAMPS as compared to non-diabetic-COVID-19-LAMPS

T2D-COVID-19-LAMPS developed in this study can be utilized to test many potential novel therapeutics. For proof of principle, we first tested an FDA-approved drug – tocilizumab (Toz), a humanized antibody against IL6R that inhibits IL6 signaling pathway, acting as an immunosuppressive drug, initially developed to treat inflammatory arthritis.[33,34]

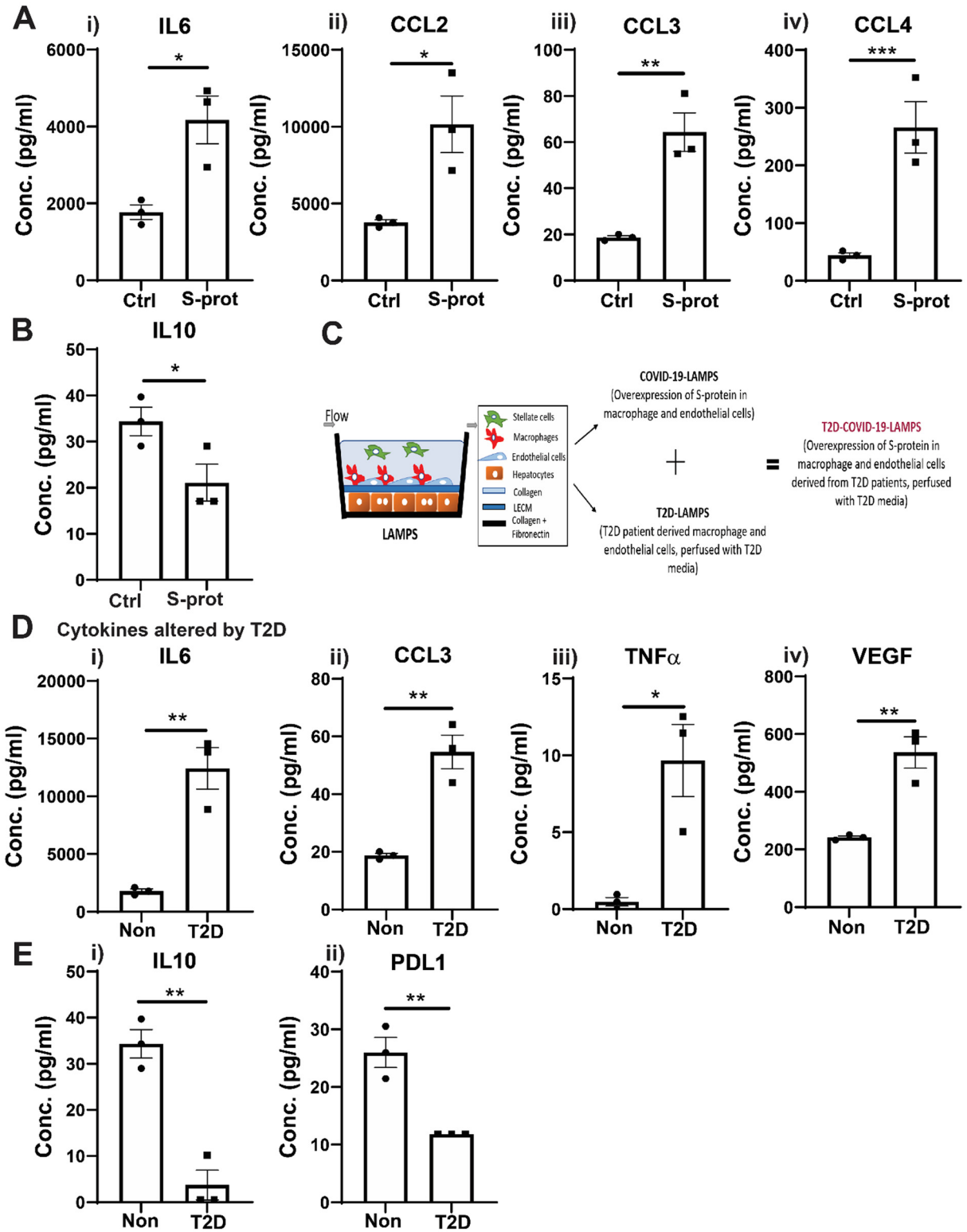

Fig. 2 Patient-derived liver acinus micro physiological system (LAMPS) for modeling SARS-CoV2 infection. S-protein was over-expressed in both macrophage and endothelial cells and were incorporated into LAMPS in non-diabetic media (non-diabetic-COVID-19-LAMPS). Cytokines were measured in the efflux using Luminex assay. Bar graph representing A. pro-inflammatory cytokines (i) IL6, ii) CCL2, iii) CCL3, iv) CCL4) and, B. anti-inflammatory cytokine-IL10 that were altered by S-protein (S-prot) with respect to control (Ctrl) lentivirus. C. Schematic representing the setup of COVID-19-LAMPS, T2D-LAMPS and T2D-COVID-19-LAMPS. Briefly, S-protein was overexpressed in both macrophages and endothelial cells in COVID-19-LAMPS; T2D patient derived macrophages and endothelial cells perfused in T2D media was used in T2D-LAMPS and both were combined to form T2D-COVID-19-LAMPS. D. Pro-inflammatory cytokines (i) IL6, ii) CCL3, iii) TNFα, and iv) VEGF) and E. anti-inflammatory cytokines (i) IL10, ii) PDL1), which were altered by T2D condition with respect to non-diabetic-LAMPS (Non) are shown as bar graph. Statistical significance was assessed using Student's two-tailed *t*-test (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

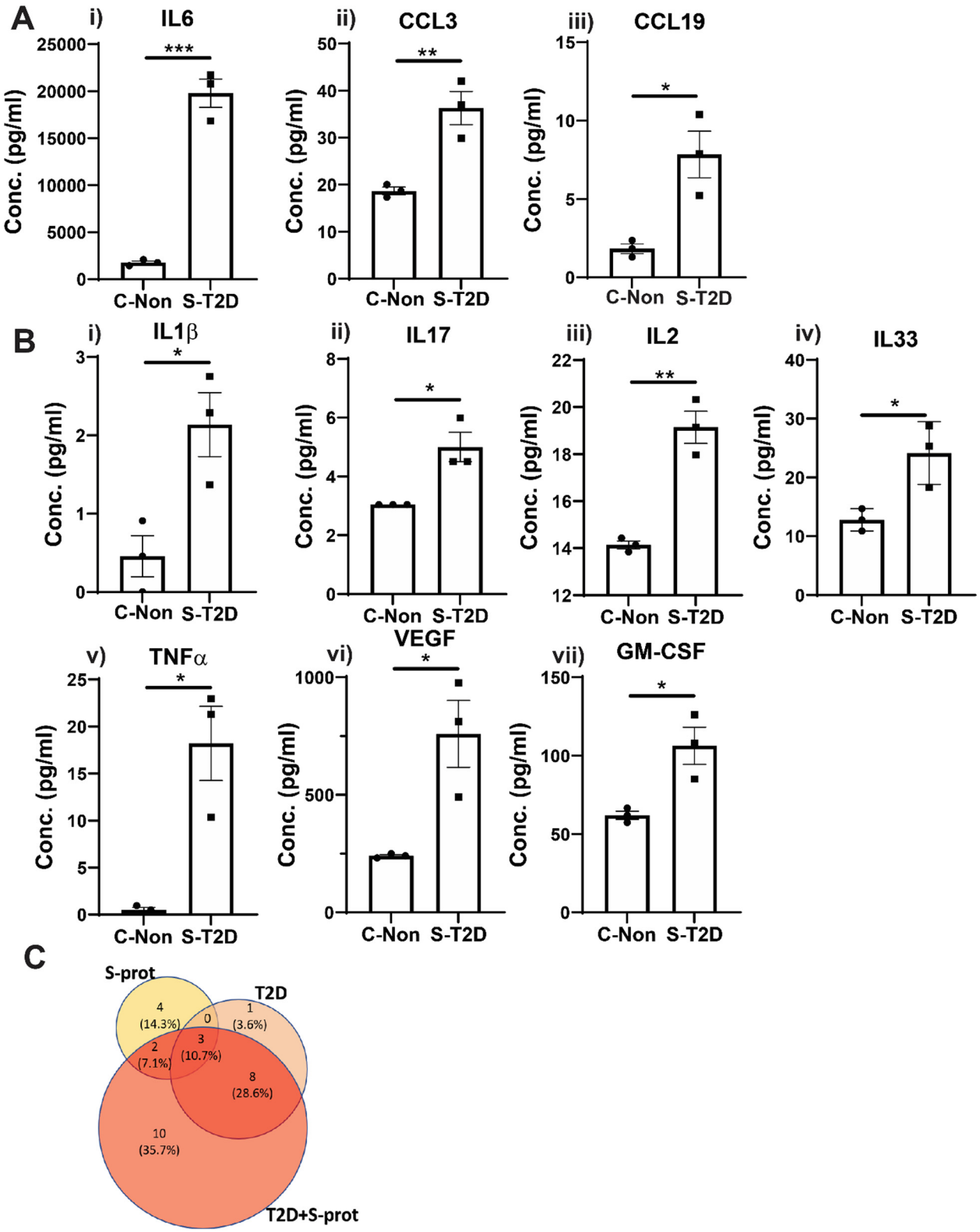

Fig. 3 T2D exacerbates the cytokine release from S-protein on macrophages and endothelial cells in LAMPS. A. Bar graph representing key cytokines (i) IL6, ii) CCL3, iii) CCL19) that were increased by T2D-COVID-19-LAMPS and were also increased by S-protein or T2D conditions alone. B. Bar graph representing cytokines (i) IL1β, ii) IL17, iii) IL2, iv) IL33, v)TNFα, vi) VEGF, and vii) GM-CSF) that were only increased in T2D-COVID-19-LAMPS but not by S-protein or T2D conditions alone. C. Venn diagram representing all of the cytokines that were altered by S-protein over-expression, T2D condition (media + T2D patient-derived cells) alone and in combination in LAMPS. Statistical significance was assessed using Student's two-tailed *t*-test (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

Recently tocilizumab has been used for COVID-19 infection[35–39] with variable outcomes. Interestingly, meta-analyses of many clinical trials demonstrated the benefits of tocilizumab mostly in severe cases of COVID-19 infection.[40–44] Using this finding as a guide, we hypothesized that Tocilizumab efficacy in severe COVID-19 could be secondary to a decrease in the cytokines induced by COVID-19 infection and this decrease would be seen only in severe complicated COVID-19 infection, such as that occurring in patients with T2D. To test this, we used the T2D-COVID-19-LAMPS model to assess the effect of tocilizumab on cytokine efflux as compared to non-diabetic-COVID-19-LAMPS. The LAMPS was incubated overnight in the presence of tocilizumab at 100 μg ml^{-1}, followed by an additional incubation of 24 h under flow, for a total of 36 h of incubation. The LAMPS were maintained under flow for 10 days and efflux was collected every other day. We found that at day 4, fold change of many cytokines altered by S-protein overexpression with respect to control (S/C) in non-diabetic LAMPS were not reduced by tocilizumab, rather they were further increased, as shown in the heat map in Fig. 4A. Interestingly, a greater number of cytokines were reduced in

A

	Normal-S/C-VC	Normal-S/C-Toz
CCL2	2.69	2.54
CCL3	3.44	4.43
CCL4	6.03	9.55
CCL5	1.14	1.94
CCL11	1.00	1.44
CCL19	3.94	4.19
CCL20	0.66	1.94
CD40	0.82	1.60
CX3CL1	3.24	2.77
CXCL1	0.85	2.06
CXCL2	0.69	2.75
CXCL10	0.68	18.04
EGF	0.67	2.68
FGF2	0.27	1.64
Flt-3 Ligand	0.96	1.48
G-CSF	1.05	0.86
GM-CSF	1.17	1.54
Granzyme B	1.05	0.67
IFN-alpha	1.14	1.52
IFN-beta	17.57	10.12
IFN-gamma	0.79	2.49
IL-1 beta	1.66	2.01
IL-1alpha	0.98	0.67
IL-1ra	2.41	2.79
IL-2	0.81	1.55
IL-3	1.33	1.00
IL-4	2.22	1.48
IL-5	0.92	1.65
IL-6	2.36	2.21
IL-7	1.44	3.20
IL-8	0.85	1.76
IL-10	0.61	2.46
IL-12p70	0.44	2.59
IL-13	1.56	0.93
IL-15	1.48	1.85
IL-17	1.00	1.47
IL-17E	0.03	3.75
IL-33	0.64	2.76
PDGF-AA	2.47	0.82
PDGF-AB	0.50	2.54
PDL1	1.10	1.62
TGF-alpha	2.43	1.29
TNF-alpha	8.41	3.28
TRAIL	3.86	5.07
VEGF	2.03	1.63

B

	T2D-S/C-VC	T2D-S/C-Toz
CCL2	1.41	0.93
CCL3	0.66	0.88
CCL4	1.28	0.86
CCL5	1.03	1.63
CCL11	1.53	1.23
CCL19	1.70	1.00
CCL20	0.81	1.09
CD40	1.69	0.95
CX3CL1	0.92	0.92
CXCL1	1.60	1.52
CXCL2	1.08	1.52
CXCL10	0.75	5.98
EGF	1.25	1.21
FGF2	1.95	1.83
Flt-3 Ligand	1.37	1.24
G-CSF	1.78	0.85
GM-CSF	1.26	0.91
Granzyme B	1.60	1.14
IFN-alpha	1.26	0.88
IFN-beta	0.30	0.30
IFN-gamma	1.68	1.57
IL-1 beta	2.35	1.51
IL-1alpha	1.72	1.33
IL-1ra	0.81	0.91
IL-2	1.29	1.20
IL-3	1.98	0.67
IL-4	1.19	1.23
IL-5	1.27	1.37
IL-6	1.59	0.89
IL-7	1.64	1.00
IL-8	2.25	2.49
IL-10	7.63	1.16
IL-12p70	0.26	1.67
IL-13	1.05	0.93
IL-15	1.08	0.87
IL-17	1.64	1.18
IL-17E	0.51	1.00
IL-33	1.80	1.05
PDGF-AA	1.82	1.78
PDGF-AB	1.62	0.97
PDL1	2.55	0.99
TGF-alpha	1.36	0.87
TNF-alpha	1.88	0.82
TRAIL	1.69	1.19
VEGF	1.42	0.91

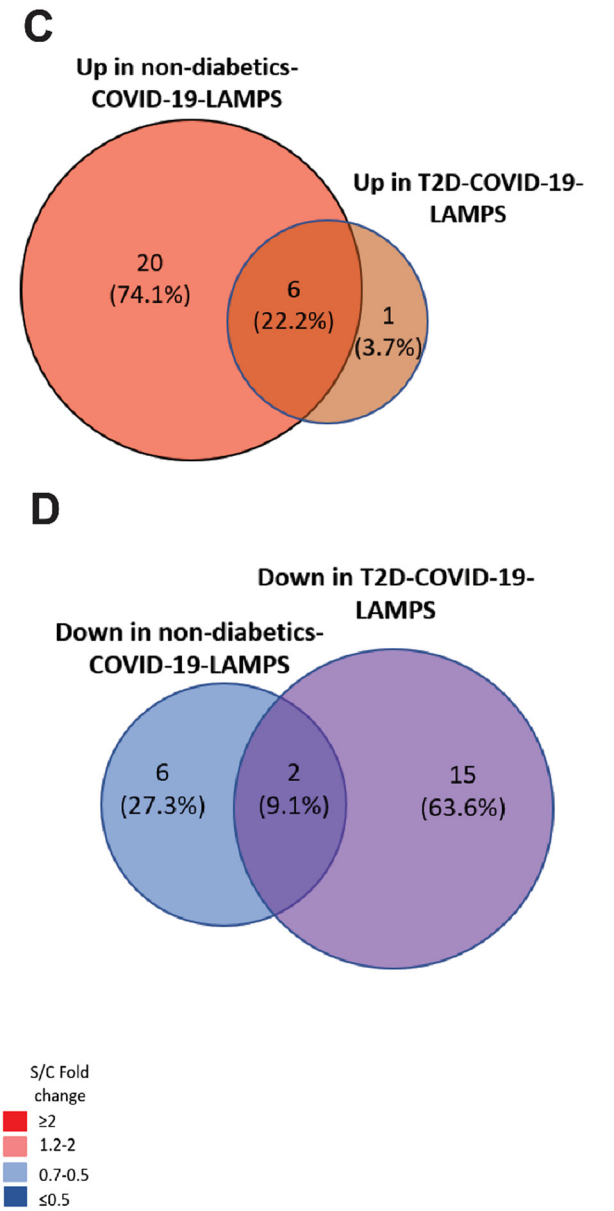

Fig. 4 Tocilizumab is more effective in reducing CSS in T2D-COVID-19-LAMPS as compared to non-diabetic-COVID-19-LAMPS. Heat map representing fold change of cytokines altered by S-protein over control (S/C) in A. non-diabetic-COVID-19-LAMPS and B. T2D-diabetic-COVID-19-LAMPS with VC and Toz. The fold change over 2 is represented as dark red, from 1.2–2 (light red), from 0.7–0.5 (light blue), and less than or equal to 0.5 (dark blue). Venn diagram representing the overlap of cytokines that were C. upregulated in non-diabetic-COVID-19-LAMPS and T2D-COVID-19-LAMPS by Toz and D. downregulated by Toz.

T2D-COVID-LAMPS by tocilizumab that were increased by S-protein, indicated by the fold change of S-protein over control (S/C) in Fig. 4B. Specifically, 26 out of 45 cytokines (57.75%) in non-diabetic-COVID-19-LAMPS and 7 cytokines (15.5%) in T2D-COVID-19-LAMPS, were increased by Toz (Fig. 4C), six cytokines that were increased by Toz in both T2D and non-diabetic LAMPS, were CCL5, CCL20, CXCL2, CXCL10, IL-12p70, and IL17E. Similarly, 8 cytokines (17.7%) in non-diabetes-COVID-19-LAMPS and 17 cytokines (37.7%) in T2D-COVID-19-LAMPS were reduced by Toz, where TGF-α and TNF-α were common in both (Fig. 4D). This indicates that Toz is effective in mitigating CSS in T2D-COVID-19-LAMPS but did not have a significant effect in non-diabetic-COVID-19-LAMPS. This recapitulates the observations in clinical settings wherein Toz was effective in improving outcomes only in severe, but not mild COVID-19 infections.

Discussion

The COVID-19 pandemic caused millions of deaths worldwide with a disproportionate number being in patients with underlying illnesses.[2,3] Despite the decrease in overall mortality with the advent of less virulent variants, such as the omicron variants, and increased vaccination rates, the risk of severe illness and mortality remains high in patients with significant co-morbidity.[45] In addition, many people who recover from COVID-19 infection develop the long-COVID syndrome and other complications.[46–48] Although vaccines have played a pivotal role in decreasing severe COVID infections and consequent mortality, their long-term efficacy appears to be limited requiring repeated boosters.[49] Thus, drugs treating COVID-19 infection are needed, especially for patients with existing co-morbidities such as diabetes, who have more severe infections and higher mortality rates. Many antiviral drugs, such as remdesivir, lopinavir, and interferon beta-1a were tested in COVID-19 patients but none of them were found to be effective in reducing mortality.[50] The combination of nirmatrelvir/ritonavir has been approved by FDA for the treatment of COVID-19 infection with other potential drugs under consideration.[51] Screening, selecting, and testing drugs and their combinations are very time consuming, tedious, and expensive. Conventional *in vitro* and *in vivo* animal models have proved less reliable in replicating human *in vivo* pathophysiology and disease conditions.[10,11] MPS-organ-on-a-chip technology has provided a robust and reliable way with translational potential owing to the use of human primary cells and flow as well as media conditions mimicking *in vivo* physiology.[10,11] An airway-on-a-chip that had been used to study influenza virus infection and screen drugs to combat the infection was used to determine the effect of retrovirus-based SARS-CoV-2-Spike on epithelial cell line and screen various drugs using virus entry as a readout.[52,53] Similarly, to study COVID-19 infection on intestine, a gut-on-a-chip was utilized to determine its underlying pathogenesis.[54] In this study, we modified LAMPS[13] to develop a T2D-COVID-19 liver organ-on-a-chip model to explore underlying mechanisms for the higher risk of CSS and severe disease in patients with T2D and COVID-19. To the best of our knowledge, this is the first report that has developed T2D-COVID-19 organ-on-a-chip model that mimics clinical features of cytokine storm syndrome of patients and we provide a proof-of-principle for drug screening using Tocilizumab, an FDA approved drug to dissect possible mechanisms underlying its efficacy only in severe COVID-19 infections.

LAMPS offers tremendous potential for a customizable liver-on-a-chip model. It has been used to mimic disease progression and test drugs in non-alcoholic fatty liver disease (NAFLD), test fluorescent biosensors that track the progression of NAFLD,[14,18] and to screen drugs for hepatotoxicity.[55,56] In this study, we modified LAMPS by introducing T2D patient-derived primary macrophages from PBMCs and LSECs, over-expressing Spike protein, and culturing them in T2D mimicking media and demonstrate their contribution in the development of a hyperimmune state in the context of T2D.

Macrophages are the primary line of defense as part of the innate immune system and are infected by SARS-CoV-2 virus *via* the ACE2 receptor.[57,58] In severe COVID-19 illness, they are often in a hyperactivated state and secrete many inflammatory cytokines, aggravating tissue damage.[20,59] The classically activated M1 macrophages are the primary pro-inflammatory subtype that is involved in the hyperimmune state in COVID-19 infection where a defect in the transition of M1 to M2 macrophage phenotype has been observed leading to a persistent pro-inflammatory state.[60] However, other studies have shown a variable M1/M2 activation propensities with COVID-19 infection,[61] though this may be related to the stage of disease, whether it is in the initial stages of COVID-19 infection (immunoparalysis with a predominant M2 macrophage state) *versus* a CSS associated hyperactivation state (predominant M1 macrophage state). While it is interesting to study the effects of T2D and COVID-19 infection on monocyte differentiation into M1/M2 phenotypes in the context of the LAMPS system, in this study we replicated the hyperactivated state by differentiating the monocytes into M1 macrophages using GM-CSF before incorporating them into LAMPS. While both resident and monocyte-derived macrophages could play a significant role in CSS, in the LAMPS model, we used PBMC derived M1 macrophages instead of liver resident macrophages-Kupffer cells, since previous studies demonstrated Kupffer cells did not express ACE2 receptor.[62] Many potential therapeutics to reduce macrophage mediated inflammation are under investigation,[63–65] indicating the significance of macrophages in COVID-19 infection. The LAMPS presented in this study could serve as a platform to screen and study the mechanisms of action and efficacy of these potential therapeutics targeting the macrophages as a treatment for severe COVID-19.

In addition to macrophages, endothelial cells also play a critical role in COVID-19 infection. They also express high levels of ACE2 on their surface, leading to endothelial activation, secretion of prothrombotic factors, and infiltration of leukocytes[28,29] though some studies have indicated that SARS-CoV2 induction of endothelial activation may be ACE2 independent and TLR4 dependent.[61] It was also observed that in severe infection, endothelial cells are the major culprit,[66] similar to our observation where S-protein over-expression in macrophage alone results in a slight increase in inflammatory cytokines, whereas over-expression in both leads to a synergistic and significant increase in cytokine levels. Similar to macrophages, endothelial dysfunction has also been considered to be a potential therapeutic target.[67,68] This study thus highlights the importance of studying the different cell types in a system, such as the LAMPS, that allows cell–cell interactions that mimic *in vivo* pathophysiology.

Cytokine storm syndrome (CSS) is one of the main causes of death in COVID-19 infection.[15] Many cytokines have been found to play a key role in CSS.[30] We found that in non-diabetic-COVID-19-LAMPS, six of these pro-inflammatory cytokines were increased, including pro-inflammatory cytokines that have been shown to contribute to the significant hyperimmune status in CSS, such as IL6, CXCL2, CXCL3, CXCL4, and three were down-regulated, including the anti-inflammatory cytokine IL10, as compared to non-diabetic-non-COVID-19 LAMPS. Furthermore, cytokines altered in T2D-COVID-19-LAMPS were more exaggerated than either S-protein expression or T2D state alone and consisted majority of the pro-inflammatory cytokines that were increased in CSS – namely, IL6, CCL3, TNF-α, VEGF, IL1b, IL17, IL2, GM-CSF, and IL33. This supports the clinical observation where T2D-COVID-19 patients have a severe infection as compared to non-diabetic patients,[4,5] thus validating the platform as a valuable tool to model CSS. Further, as a proof of principle, we tested an FDA-approved drug tocilizumab (Toz) which is known to improve severe COVID-19 infection. Congruent with clinical trials, we found that Toz was able to reduce more cytokines in T2D-COVID-19-LAMPS as compared to non-diabetic-COVID-19 LAMPS.

Despite the striking potential of T2D-COVID-19-LAMPS model, one of the limitation of the present study Is that only the S-protein expressing lentiviral vector was used without expression of the other components of the original SARS-CoV-2 virus. The spike protein present on the surface of the virus plays a key role in binding to the host cell surface receptor ACE2, mediating its entry into the host cell.[69,70] Mutation in this Spike protein has led to generation of more infectious and severe strains such as alpha, and delta causing more deaths. While using a patient derived-fully infectious SARS-CoV-2 virus may mimic more comprehensively the infections seen in patients, the expression of only S-protein was sufficient to demonstrate the importance of the synergy of macrophages and endothelial cells in the context of T2D for a hyperimmune state. Many variants of SARS-CoV-2 have evolved with numerous mutations predominantly in the S-protein due to their primary role in infecting the host and as a vaccination target. Various mutated Spike protein variants have been tested for their infectivity, severity, and protection against vaccination.[71–74] This signifies the critical role of Spike protein in COVID-19 infection and forms the primary basis of its use in our study. Another mutation in non-structural protein has also been shown to affect strain severity.[75,76] One such example is a mutation in non-structural protein-orf9b and orf6, which play immune-suppressive role in the alpha variant – leading to increasing expression of these proteins and hence increasing the severity of the strain.[77] We may, hence, be underestimating the effect, as the expression of the whole virus could lead to a more severe pathological response than S-protein alone. The other proteins can be tested in LAMPS either individually or in combination to include the whole infectious virus, in future studies, to determine the molecular mechanism of disease severity, and test potential therapeutics. Another limitation of the study was the measure of only cytokines as an indicator of the propensity of cytokine storm *in vivo*. However, measurement of other angiogenic, pro-thrombotic markers could also provide insight into the pathological states such as thrombotic microangiopathy that contributes to the severity of the disease. In addition, assessing the interactions between the different cell types, along with determining the changes in transcriptome and proteome at a single cell level in future studies could lead to a more complete understanding of the underlying molecular mechanisms. While this study focused on the liver-based LAMPS as a platform due to the focus on T2D, similar approaches could be used for an MPS system incorporating lung, kidney, or heart that are also critical to understanding the COVID-19 disease. The next steps include generating patient-specific iPS-derived hepatocytes in LAMPS, allowing patient-specific modeling and scalable human studies.

In conclusion, T2D is one of the most prevalent co-morbidities that result in severe COVID-19 infection. The presence of a reliable and rapid drug testing model would assist in a quick turnover rate for drug development. T2D-COVID-19-LAMPS is a liver-on-a-chip model that uses T2D patient-derived macrophages and endothelial cells over-expressing SARS-CoV-2-Spike protein and cultured in T2D-mimicking media. This model recapitulated the features of cytokine storm syndrome, which is severe in T2D-COVID-19 patients as compared to non-diabetic patients. Furthermore, tocilizumab, an IL6R antagonist was effective in reducing inflammatory cytokines in T2D-COVID-19-LAMPS, but not in non-diabetic-COVID-19-LAMPS, correlating with clinical trials (Fig. 5).[40–44] This highlights the translatability of the T2D-COVID-19-LAMPS and its further applicability to test potential new drugs. Moreover, its ease to manipulate into any other disease model in the future makes it an attractive platform for patient-specific disease modeling and drug discovery.

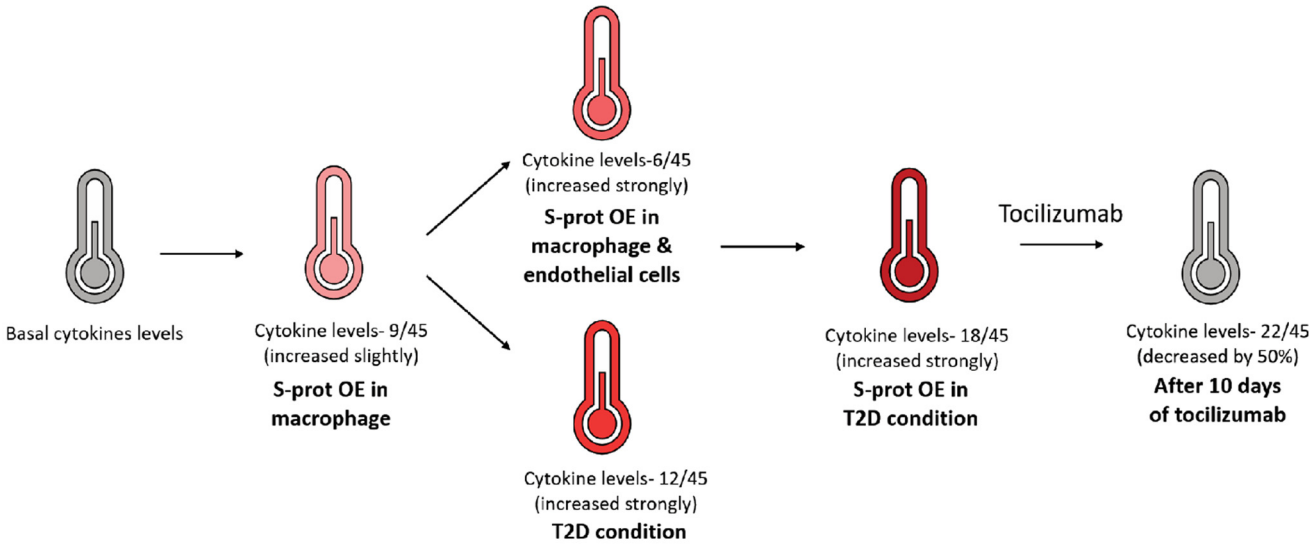

Fig. 5 Schematic summarizing the key findings of the study. Cytokines secreted by LAMPS were measured by 45-plex Luminex assay. S-protein overexpression in macrophages alone, increased 9 out of 45 cytokines slightly. With S-protein over-expression in both macrophages and endothelial cells, 6 out of 45 cytokines were increased strongly. Simultaneously, T2D patient-derived macrophage and endothelial cells in T2D media increased 12 out of 45 cytokines. Subsequently, combining S-protein overexpression in T2D condition increased 18 out of 45 cytokines. Lastly, administration of tocilizumab decreased about 50% of cytokines (22 out of 45), bringing it back to the basal level.

Data sharing statement

All the data supporting the findings of this study are available within the article and its ESI† files or from the corresponding author upon reasonable request.

Author contributions

Conceptualization, VKY, DLT, MTM, AMS; investigation and methodology, VN, JKL, DG, MTM, LV; formal analysis, VN, DG, TS, AG, AMS, VKY, writing, VN, VKY; funding acquisition, VKY, DLT, MTM, AMS; data verification and supervision: VN, VKY. All authors read and approved final version of the manuscript.

Conflicts of interest

All the authors have declared no conflicts of interests.

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