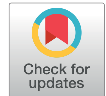

Indirect and Direct Effects of SARS-CoV-2 on Human Pancreatic Islets

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PATHOPHYSIOLOGY

Recent studies have shown that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection may induce metabolic distress, leading to hyperglycemia in patients affected by coronavirus disease 19 (COVID-19). We investigated the potential indirect and direct effects of SARS-CoV-2 on human pancreatic islets in 10 patients who became hyperglycemic after COVID-19. Although there was no evidence of peripheral anti-islet autoimmunity, the serum of these patients displayed toxicity on human pancreatic islets, which could be abrogated by the use of anti–interleukin-1β (IL-1β), anti–IL-6, and anti–tumor necrosis factor α, cytokines known to be highly upregulated during COVID-19. Interestingly, the receptors of those aforementioned cytokines were highly expressed on human pancreatic islets. An increase in peripheral unmethylated *INS* DNA, a marker of cell death, was evident in several patients with COVID-19. Pathology of the pancreas from deceased hyperglycemic patients who had COVID-19 revealed mild lymphocytic infiltration of pancreatic islets and pancreatic lymph nodes. Moreover, SARS-CoV-2–specific viral RNA, along with the presence of several immature insulin granules or proinsulin, was detected in postmortem pancreatic tissues, suggestive of β-cell–altered proinsulin processing, as well as β-cell degeneration and hyperstimulation. These data demonstrate that SARS-CoV-2 may negatively affect human pancreatic islet function and survival by creating inflammatory conditions, possibly with a direct tropism, which may in turn lead to metabolic abnormalities observed in patients with COVID-19.

Several reports have described the association between new hyperglycemia and severe acute respiratory syndrome

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coronavirus 2 (SARS-CoV-2) infection (1–4). The prevalence of hyperglycemia during coronavirus disease 2019 (COVID-19) infection is much higher than that observed in other viral infections such as SARS-CoV-1 (5) and hepatitis C (6,7). Mechanistic studies, although scant, have suggested an association with the inflammatory immune response and cytokine storm, which may impair glucose uptake and increase insulin resistance (8–10). Earlier reports suggested an extrapulmonary tropism of SARS-CoV-2 in several organs, including the heart, the brain, and the kidneys (11). If the virus also localizes to the pancreas, a potential direct toxic effect on β-cells could occur. We studied the indirect and direct effects of SARS-CoV-2 on human pancreatic islets in patients with COVID-19 with new-onset hyperglycemia. We hypothesized that a combination of inflammatory-mediated dysfunction of human pancreatic islets associated with direct injury/localization of the virus to β-cells initiates a cascade of inflammatory damage that may predispose to the onset of new hyperglycemia. Indirect and direct toxic effects may explain in part the increased evidence of new hyperglycemia in patients with COVID-19.

RESEARCH DESIGN AND METHODS

Study Design and Outcomes

Serum was obtained from 10 patients hospitalized for COVID-19 who were consecutively admitted and who developed new-onset hyperglycemia, and serum was obtained from 10 patients who had recovered from COVID-19 infection. All patients had a positive COVID-19 test and had no history of diabetes before their admission to hospital. Serum was also collected from a group of healthy control participants (ClinicalTrials.gov identifier NCT04463849). Clinical and demographic data of all participants are summarized in Table 1. Ethical permission was obtained from the local ethical research committee of Milan (Comitato Etico Milano Area 1, Milan, Italy), which granted the approval of the current study (approval no. 2020/ST/167), and informed consent was obtained from all study participants.

Islet-Specific Autoantibodies

Insulin, GAD, islet antigen 2 (IA-2), and ZnT8A autoantibodies were measured using fully validated ELISAs as previously described (12,13). Titers of insulin, GAD, IA-2, and ZnT8A antibodies, measured in duplicate in 25 μL serum, were expressed in units derived from in-house standard curves. According to the manufacturer's protocol, the values for anti-insulin and anti–IA-2 were considered negative when $<$10 IU/mL and were considered positive if $>$10 IU/mL. The threshold was set at 5 units/mL for anti-GAD and 15 units/mL for anti-ZnT8.

Human Pancreatic Islet In Vitro Studies

Human pancreatic islets were cultured with and without interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), IL-13, IL-6, and interferon-γ–induced protein 10 (IP-10) (please see *Recombinant Proteins and Interventional Studies*), and human pancreatic islet apoptosis and function were examined. Supernatants were collected to measure insulin release by ELISA. To mimic the effect of COVID-19 on the pancreas, human sera obtained from patients with acute COVID-19 and from patients who had recovered from COVID-19 were added to human islets, which were cultured as reported in the *Pancreatic Islets* section; serum from healthy control subjects or normal culture medium (with 10% FBS) was used as control. Culture supernatant was collected at 24 h, and insulin levels were assayed with a microparticle enzyme immunoassay (Iso-Insulin ELISA; cat. nos. 10-1113-01 and 10-1247-01; Mercodia, Uppsala, Sweden) with intra- and interassay coefficients of variation (CVs) of 3.0% and 5.0%. Islet lysates were collected, and cell death/apoptosis was assessed by ELISA after 24 h of culture (cat. no. 11544675001; Roche Diagnostics GmbH, Mannheim, Germany).

Recombinant Proteins and Interventional Studies

Human pancreatic islets were cultured for 24 h as per the manufacturer's instructions and were exposed to 15 pg/mL recombinant human IL-1β (R&D Systems, Minneapolis, MN), 100 pg/mL recombinant human TNF-α (R&D Systems), 20 pg/mL recombinant human IL-13 (R&D Systems), 30 pg/mL recombinant human IL-6 (R&D Systems), and 1 ng/mL recombinant human IP-10 (R&D Systems) or to serum obtained from patients with acute COVID-19 or from patients who had recovered from COVID-19 (long COVID-19). As a control, serum from healthy participants was added to the culture medium in place of 10% FBS for 24 h. Immunoneutralization studies were performed using the following inhibitors: anti–IL-1β (1 μg/mL) (Thermo Fisher Scientific, Waltham, MA), anti–IL-6 (10 μg/mL) (Roche), and anti–IL-13 (10 μg/mL) (Sigma Aldrich, St Louis, MO) in a 24 h in vitro culture.

Pancreatic Islets

Human purified pancreatic islets of Langerhans obtained from healthy participants were purchased from a commercial source (cat. no. 35002-04; Celprogen, Torrance, CA) and were cultured with standard medium as per the manufacturer's instructions and as previously described (1).

Receptome Analysis

RNA was extracted from 400 purified islets of healthy donors (n = 4) whose pancreases were not suitable for donation (see Human Checklist in Supplementary Material) using the Direct-zol RNA Mini Prep Plus Kit (cat. no. R2070; Zymo Research, Irvine, CA), and RNA sequencing was performed at the Center of Bioinformatics and Functional Genomics at Ospedale San Raffaele. Gene expression analysis was conducted using R software (version 3.6.1), with libraries edgeR_3.26.5, DESeq2_1.24.0, and pheatmap_1.0.12, and transcripts were normalized to reads per kilobase per million mapped reads expression units to

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Table 1—Baseline demographic and clinical characteristics of participants included in this study

	Controls	Acute COVID-19	Post COVID-19	*P*
N of participants	15	10	10	NS
Age, years ± SEM	45.9 ± 2.1	47.2 ± 3.1	43.0 ± 4.7	NS
Sex, M/F	10/5	4/6	7/3	NS
BMI, kg/m^2 ± SEM	23.4 ± 0.6	23.3 ± 0.6	24.8 ± 2.1	NS
Estimated HbA_{1c}				
mmol/mol ± SEM	34.1 ± 0.4	35.6 ± 1.4	38.0 ± 0.9	NS,* <0.05,† NS‡
%	5.5	5.4	5.6	
Autoantibodies, units/mL ± SEM				
Anti-insulin	2.1 ± 0.2	1.0 ± 0.4	2.5 ± 0.2	NS
Anti-GAD	1.0 ± 0.0	1.3 ± 0.3	1.0 ± 0.0	NS
Anti–IA-2	1.6 ± 0.6	1.0 ± 0.0	1.0 ± 0.0	NS
Anti-ZnT8	8.9 ± 0.0	9.0 ± 0.1	9.0 ± 0.0	NS
COVID-19 therapy, %				
Hydroxychloroquine	0	20	10	NS
Dexamethasone	0	10	0	NS
Antiviral	0	30	0	NS
Tocilizumab	0	0	0	NS
Antibiotics	20	20	20	NS
Heparin	0	60	0	NS

NS, not significant. *Controls vs. acute COVID-19. †Controls vs. post COVID-19. ‡Acute COVID-19 vs. post COVID-19.

estimate the relative abundance of transcripts. A rank analysis was next performed based on genes previously identified by transcriptome analysis (Affymetrix, Santa Clara, CA) for surface receptors expressed at a moderate/high level (cutoff >25) in human islets and β-cells.

Histopathology and Immunohistochemistry

Pancreatic tissues were retrieved from deceased patients who had COVID-19, from deceased control subjects, or from deceased patients with type 2 diabetes. Samples were obtained from a collaboration with the Department of Pathology at ASST Fatebenefratelli-Sacco Hospital, which routinely collects samples for clinical and research purposes. All patients provided informed consent upon hospital admission for sample collection at Sacco Hospital, Milan, Italy. Collected samples were fixed in 10% neutral buffered formalin (4% w/v formaldehyde and 0.05 M acetate buffer) and stored in 70% ethanol solution. Specimens were then processed for paraffin embedding as previously described. Hematoxylin-eosin (H-E) staining was performed on sections 3 μm in thickness after deparaffinization, rehydration, and antigen retrieval. Photomicrographs were taken using an Olympus BX41 microscope (Center Valley, PA). Pancreatic lymph nodes (PLNs) isolated from patients with COVID-19 were fixed, paraffin embedded, and processed for H-E staining and MECA-79 staining as previously described (2–4).

Detection of β-Cell Death by Analyzing β-Cell–Derived Unmethylated *INS* DNA

We first isolated DNA from 200 μL serum using the QIAGEN DNA Blood and Tissue Kit, and obtained DNA was bisulfite treated using the EZ DNA Methylation Kit (Zymo Research) as previously described (5). Next, we performed a droplet digital PCR as previously reported. We prepared

a 25-μL assay volume, which consisted of Droplet PCR Supermix (Bio-Rad Laboratories, Billerica, MA), 900 nmol/L primer, and 250 nmol/L probe. Two probes that target two methylation-sensitive sites of the human insulin gene (hg19_knownGene_uc021qcd.1; range chr11:2181009–2182439) at nucleotides 21814010 and 21814012, which are +396 and +399 from the transcription start site, were used together with 5 μL DNA. The mixture and droplet generation oil were loaded onto a droplet generator (Bio-Rad Laboratories), and the generated droplets were transferred to a 96-well PCR plate and sealed. PCR was run on a thermal cycler using the following conditions: 10-min activation at 95°C, 40 cycles of a two-step amplification protocol (30 s at 94°C denaturation and 60 s at 58°C), and 10-min inactivation step at 98°C. The PCR plate was transferred to a QX100 Droplet Reader (Bio-Rad Laboratories), and products were analyzed with QuantaSoft analysis software (Bio-Rad Laboratories). Discrimination between droplets that contained the target (positives) and those that did not (negatives) was achieved by applying a fluorescence amplitude threshold based on the amplitude read from the negative template control. For each sample, the ratio of unmethylated *INS* DNA/methylated *INS* DNA was calculated.

Electron Microscopy

Pancreatic paraffin-embedded sections of samples isolated from patients with COVID-19, from healthy control

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subjects, and from patients with type 2 diabetes were first fixed in a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.005 M sodium cacodylate buffer (pH 7.4), then postfixed in 1% OsO4 in 0.1 M sodium cacodylate buffer (pH 7.4). After dehydration in graded series of ethanol, sections were transferred to propylene oxide and embedded in Epon-Araldite as previously described (14). Next, sections 60 nm in thickness were cut and mounted on nickel grids with Formvar membranes, stained with uranyl acetate and lead citrate, and finally examined using a Morgagni 268D transmission electron microscope (Philips, Eindhoven, the Netherlands). Digital images were captured as previously described (15). For immunocytochemical analysis, pancreatic sections were treated successively with sodium metaperiodate for 30 min and with citrate buffer (pH 6) for 10 min at 98°C. After several rinses in 0.05 M Tris-buffered saline (pH 7.4), sections were incubated for 5 min in a solution of 1% ovalbumin, then transferred into a solution containing guinea pig anti-insulin antibody (Dako, Carpinteria, CA), diluted at 1:50, and incubated overnight at 4°C. The following day, sections were subjected to several rinses and then incubated for 1 h in a solution of colloidal gold anti-guinea pig antibodies (Jackson ImmunoResearch, Philadelphia, PA), diluted at 1:20. After incubation, sections were rinsed and finally stained with uranyl acetate and lead citrate.

SARS-CoV-2 Detection by RT-PCR

Total RNA isolated from postmortem pancreatic tissues obtained from hyperglycemic patients with COVID-19 was purified using the mirVana miRNA Isolation Kit (Thermo Fisher Scientific) as per the manufacturer's instructions. To quantify SARS-CoV-2 expression, Norgen's 2019-nCoV TaqMan RT-PCR Kit was used for the detection of SARS-CoV-2–specific RNA in a one-step real-time RT-PCR based on the use of TaqMan technology following the manufacturer's instructions (Norgen Biotek Corp., Thorold, Canada). RT-PCR reactions were performed in a 96-well format using the 7900HT Fast Real-Time PCR instrument (Applied Biosystems, Foster City, CA).

Immunofluorescence and Immunohistochemistry of Pancreatic Sections

To detect the SARS-CoV-2 virus, we tested an anti–SARS-CoV-2 spike protein S1 primary polyclonal antibody (cat. no. AHP3013; Bio-Rad Laboratories, Hercules, CA) (dilution 1:50) in the autopsied pancreatic samples. To evaluate in which cell types the abovementioned antibody could have potentially been positive, we tested in combination the following primary antibodies, respectively:

1. To highlight insular β-cells, an anti-insulin monoclonal antibody (cat. no. 14-9769-82; Thermo Fisher Scientific) (dilution 0.5 mg/mL)
2. To highlight exocrine acinar cells, an antitrypsin 1 monoclonal antibody (cat. no. ab200997; Abcam, Cambridge, U.K.) (dilution 1:2,000)
3. To highlight ductal cells, an antikeratin 19 monoclonal antibody (cat. no. LS-B108; Lifespan BioSciences, Seattle, WA) (dilution 5 μg/mL)

The anti–SARS-CoV-2 spike protein S1 primary antibody was revealed by means of a tetramethylrhodamine probe (tetramethylrhodamine goat anti-rat IgG; cat. no. AP136R; Merck Millipore) (dilution 1:70), whereas the *1*), *2*), and *3*) primary antibodies were revealed by a fluorescent probe (fluorescein isothiocyanate–conjugated goat anti-rabbit IgG; cat. no. AP187F; Merck Millipore) (dilution 1:70).

The three pairs of antibodies were observed using a confocal system (STELLARIS 5 Confocal Microscope; Leica Microsystems, Wetzlar, Germany) with a ×63 oil objective. The images were acquired in a multitrack mode, using consecutive and independent optical pathways.

Statistical Analysis

Continuous variables are presented as means with SEs, and categorical variables are presented as proportions. We used the independent samples *t* test to compare continuous variables and χ^2 test/Fisher exact test to compare categorical variables. For multiple comparisons, one-way or two-way ANOVA followed by Bonferroni post hoc test between the group of interest and all other groups was used.

Data and Resource Availability

All data generated during the current study are available from the corresponding author upon reasonable request.

RESULTS

In Vivo and In Vitro Evidence of Cell Death Associated With COVID-19

We first assessed whether the cohort of patients with COVID-19 or patients who had recovered from COVID-19 had developed related signs of anti-islet autoimmunity. Our data demonstrated that patients from both subgroups were negative for the commonly known anti-islet autoantibodies (Table 1), although significant dysregulation of glycometabolic control was evident. To evaluate the existence of β-cell death, we then assessed the relative amount of circulating unmethylated and methylated insulin DNA in the serum of patients with COVID-19, as well as in that of patients who had recovered from COVID-19 and that of control subjects. We used the droplet digital PCR method for analyzing unmethylated/methylated CpG sites in circulating insulin as previously reported by Usmani-Brown et al. (16). By comparing the averages of the ratios of unmethylated to methylated *INS* DNA, considering a ratio of 0.196 as a cutoff, with values >0.196 indicating β-cell death, a significant decrease in the ratio was found in the patients with acute COVID-19 but not

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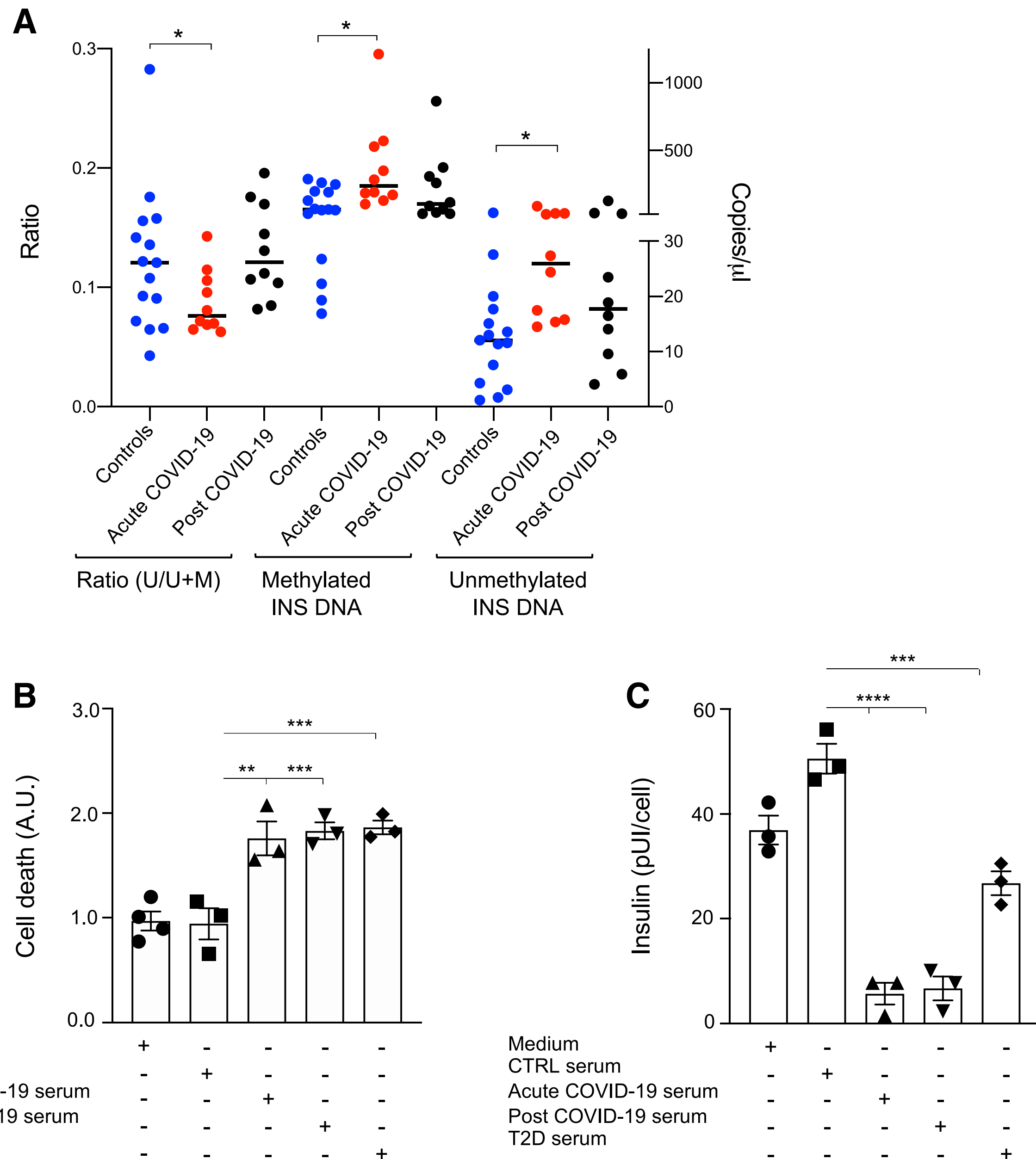

Figure 1—In vivo and in vitro evidence of cell death associated with COVID-19. *A*: Comparison of the ratio of unmethylated (U) *INS* DNA/U plus methylated (M) *INS* DNA was performed in patients with COVID-19 (acute COVID-19), in patients who had recovered from COVID-19 (post COVID-19), and in healthy control subjects. Ratio of 0.196 was considered as a cutoff (mean plus 2SD; 97.7th percentile) for the U/M+U calculation, with a ratio ≥0.196 indicating β-cell death. Comparison of the levels of U *INS* DNA as well as of M *INS* DNA (i.e., copies of U/µL and copies of M *INS* DNA/µL) was performed and measured by droplet digital PCR in serum samples from patients with COVID-19 (acute COVID-19 and post COVID-19) and in healthy control subjects. *B* and *C*: Rates of cell death and insulin secretion were analyzed for purified human islets upon in vitro challenge with serum from healthy control subjects, from patients with COVID-19 (acute COVID-19), from patients who had recovered from COVID-19 (post COVID-19), or from patients with type 2 diabetes (T2D). Data are representative of n = 15 samples from the subgroup of healthy control subjects and n = 10 from acute COVID-19 and from post COVID-19. Data are representative of a pool of n = 3–4 commercial islet preparations in three separate experiments (n = 3–5 pooled sera tested). Data are represented as mean ± SEM. Ordinary one-way ANOVA test with Bonferroni correction was used for calculating statistical significance between all groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. AU, arbitrary unit.

in those post COVID-19 as compared with control subjects (Fig. 1*A*). Conversely, a significant increase in the copies of unmethylated and methylated *INS* DNA was observed in patients with COVID-19, but not in those post COVID-19, as compared with control subjects (Fig. 1*A*). This may indeed reflect widespread tissue destruction in patients with COVID-19. To better understand whether the known abnormal COVID-19–related secretome (2) can

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alter human pancreatic islet function and survival, we next cocultured human pancreatic islets in vitro with serum obtained from patients with COVID-19 or post COVID-19. Human pancreatic islet apoptosis associated with a drastic reduction in insulin secretion was observed when using serum obtained from patients with COVID-19 or post COVID-19 (Fig. 1*B* and *C*). Interestingly, when challenging human pancreatic islets with the sera obtained from patients with type 2 diabetes, only a slight reduction in insulin secretion was evident, while a higher increase in human pancreatic islet death was observed (Fig. 1*B* and *C*).

Altered COVID-19–Associated Secretome Has a Toxic Effect on Human Pancreatic Islets

To mechanistically elucidate the effect that the COVID-19–associated secretome may have on human pancreatic islets, we first performed a receptome analysis on purified human pancreatic islets obtained from healthy donors. The receptome analysis revealed that human pancreatic islets expressed many cytokine receptors that bind cytokines found to be upregulated in the secretome of patients with COVID-19 (2). In particular, within the islet receptome, the receptors for TNF-α, IL-13, IL-1β, and IL-6 were found to be expressed by human pancreatic islets (Fig. 2*A*). Based on the previous results of secretome profiling in patients with COVID-19 (2) and on the results of this receptome analysis, we sought to challenge human islets either with each single cytokine or with a combination of the cytokines predominantly increased in serum of patients with COVID-19. Our data further confirmed the apoptosis-mediated effect of single and combined cytokines increased in serum of patients with COVID-19. Indeed, IL-1β added in combination with IL-6, IL-13, IP-10, and TNF-α had potently induced human pancreatic islet apoptosis (Fig. 2*B*). Paralleling the induction of apoptosis, a reduction in insulin secretion was also evident with each single cytokine (IL-1β, IL-6, IL-13, IP-10, and TNF-α) or with the combination of these five cytokines (Fig. 2*C*). To further probe whether the peripheral secretome is a primary cause of the human pancreatic islet damage and to determine the factor responsible for the observed human pancreatic islet injury, we conducted an immunoneutralization assay in which we targeted the cytokines found to be most highly increased in the serum of patients with COVID-19. Results showed a reduction in serum-mediated human pancreatic islet apoptosis after addition of the blocking/neutralizing antibodies anti–IL-1β and anti–IL-6 to the human pancreatic islet serum assay, while simultaneous immunoneutralization of three cytokines (IL-1β, IL-6, and IL-13) prevented islet apoptosis to a greater extent, reverting the percentage of apoptotic human pancreatic islets comparable to baseline conditions (Fig. 2*D*). Finally, the addition of anti–IL-1β, anti–IL-6, and anti–IL-13 also rescued human pancreatic islet insulin secretion (Fig. 2*E*). Taken together, these experiments indicate that high levels of IL-1β and IL-6 may play a determinant role in the β-cell dysfunction observed in patients with COVID-19, which may persist as a result of the persistent abnormal secretome.

Abnormalities in the Endocrine Pancreas Are Observed in Deceased Patients With COVID-19

We next sought to investigate whether we could detect features of abnormalities in pancreatic pathologic sections isolated from deceased patients with COVID-19 who had been newly hyperglycemic. Histologic examination of pancreatic sections stained with H-E showed mild lymphocytic infiltration of human pancreatic islets, with few lymphocytes in the exocrine pancreas (Fig. 3*A* and Supplementary Fig. 1*A*). Interestingly, RT-PCR analysis of samples obtained postmortem from patients with COVID-19 who had been newly hyperglycemic showed the presence of viral RNA within pancreatic tissues (Fig. 3*B*). Similarly, diffuse activation was observed within the PLNs retrieved from deceased patients with COVID-19 (Fig. 3*C*). Expansion of HEV-like vasculature was evident, as demonstrated by detection of peripheral node addressin via positive staining of MECA-79 in PLNs (Supplementary Fig. 1*B*), which has been previously described as a permissive feature that mediates lymphocyte trafficking within the PLNs during the onset of diabetes (17–19). Furthermore, viral RNA was detected by RT-PCR within the PLNs in several samples as well (Fig. 3*D*). Next, we performed ultrastructural analysis using transmission electron microscopy on pancreatic tissues retrieved from the same samples as well as from deceased participants without COVID-19 (controls) and from deceased patients with type 2 diabetes. Alterations in islet structures were observed in pancreatic tissues from patients with COVID-19 who had been newly hyperglycemic, which paralleled the alterations observed in patients who had type 2 diabetes (Fig. 3*E*–*G*). In particular, changes in β-cell structure and morphology were evident in all samples obtained from patients (i.e., with COVID-19 and with type 2 diabetes), as shown by a reduced number of mature insulin granules associated with features of β-cell damage compared with controls (Fig. 3*H*–*J*). Notably, a high proportion of immature granules presumably containing proinsulin was observed in the β-cells of patients with COVID-19 who had been newly hyperglycemic (Fig. 3*H*–*K*). β-cells from patients with COVID-19 who had been newly hyperglycemic were found to contain numerous circular granules and several granules with crystalline morphology, a typical feature of degeneration and hyperstimulation (Fig. 3*H*–*K*). Moreover, a conspicuous number of vacuoles, which may be suggestive of the presence of virus, were observed in β-cells (Fig. 3*L* and *M*) as well as in α-cells, δ-cells, endothelial cells, pericytes, and histiocytes (Supplementary Fig. 2*A*–*E*), although the viral origin of these vacuoles was not confirmed. We further conducted an extensive ultrastructural analysis on pancreatic sections from patients with COVID-19 (Fig. 3*N*). We observed a localization of the spike S1 protein of SARS-CoV-2 within endocrine cells, but

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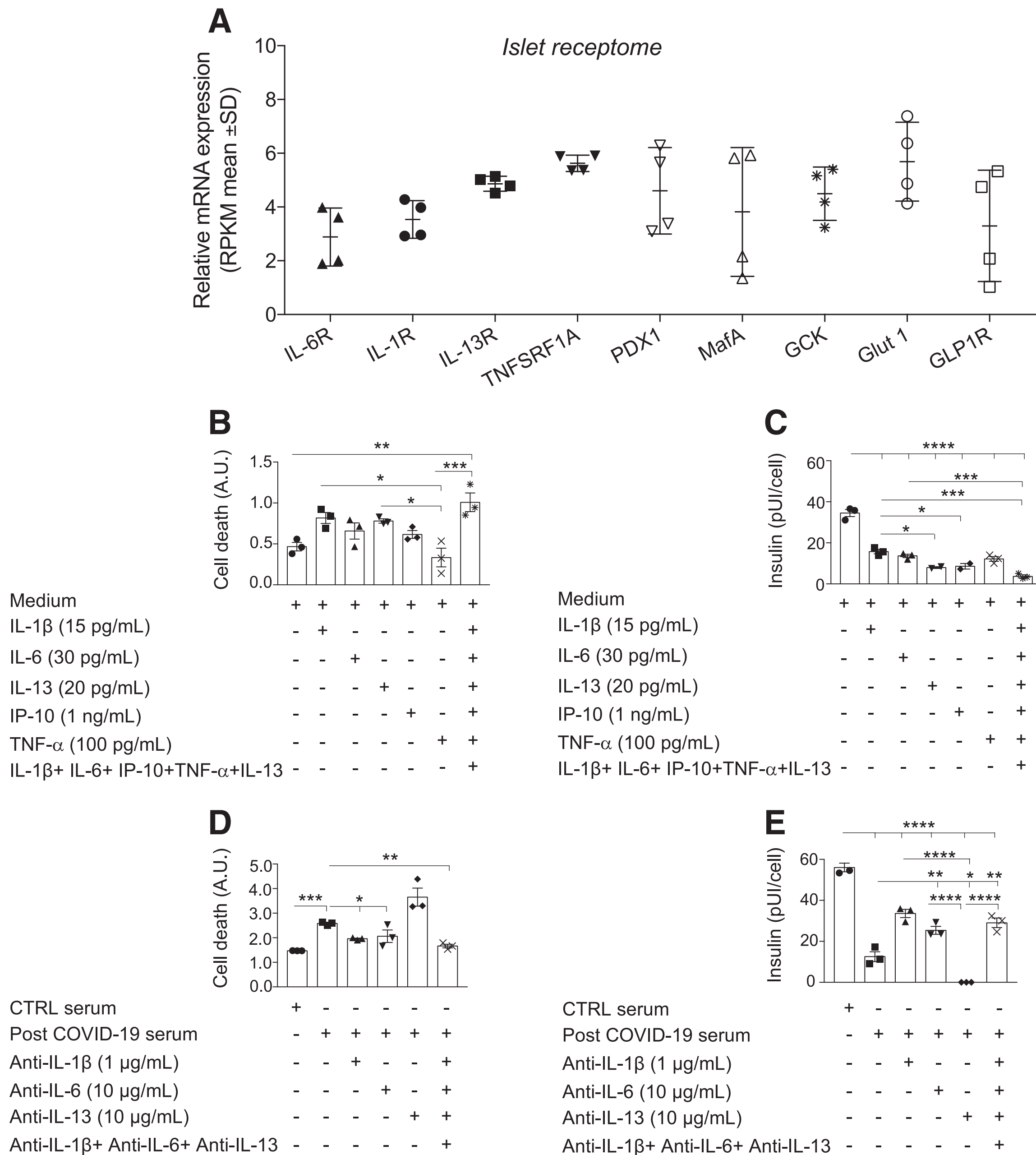

Figure 2—Altered COVID-19–associated secretome has a toxic effect on human pancreatic islets. *A*: Receptome analysis of human pancreatic islets depicting the expression of proinflammatory cytokine receptors (TNAFAR, IL-13R, IL-1R1, and IL-6R); mRNAs of GLP1R, Glut-2, MAFA, GCK, and PDX-1 are shown as well. Data are representative of four independent human islet isolations ($n = 4$ donors), represented as mean plus SD. *B* and *C*: Rates of cell death (*B*) and insulin secretion (*C*) were analyzed for purified human pancreatic islets upon in vitro challenge with a selected panel of cytokines (IL-1β, IL-6, IL-13, IP-10, and TNF-α) observed to be upregulated in the serum of patients with COVID-19. Effects were assessed when the cytokines were added alone or in combination. *D* and *E*: Rates of cell death (*D*) and insulin secretion (*E*) were analyzed for purified human pancreatic islets upon in vitro challenge with serum isolated from a patient who recovered from COVID-19 (post COVID-19) in the presence of neutralizing antibodies (anti–IL-1β, anti–IL-6, or anti–IL-13) alone or in combination, as compared with when challenged with serum from healthy control subjects. Data are representative of a pool of $n = 3$–4 commercial islet preparations in three separate experiments ($n = 3$–5 pooled sera tested). Data are represented as mean ± SEM. Ordinary one-way ANOVA test with Bonferroni correction was used for calculating statistical significance between all groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. AU, arbitrary unit; RPKM, reads per kilobase per million mapped reads.

also within the exocrine pancreas (Fig. 3*N*). Notably, we found a SARS-CoV-2 spike protein S1 staining discerning into β-cells, as clearly depicted by our confocal analysis showing a double immunostaining for SARS-CoV-2 spike protein S1 and insulin-positive cells of the autopsied pancreatic islets (Fig. 4*A*–*C*), where SARS-CoV-2 RNA was

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Figure 3—Abnormalities in the endocrine pancreas are observed in patients with COVID-19. *A*: Histologic examination of pancreatic section retrieved from a patient with COVID-19 stained with H-E showing mild islet lymphocytic infiltration. *B*: Bar graph showing the results of a SARS-CoV-2 RT-PCR assay using the 2019-nCoV_N1 and 2019-nCoV_N2 primer probe sets performed on RNA samples extracted from pancreatic sections from patients with COVID-19 (P1, P2, P3, and P4) and from healthy control subjects, showing detectable viral RNA in patient P1. RNA extracted from a Centers for Disease Control and Prevention (CDC)–positive control DNA plasmid (CDC^{+}) was used as a positive control, and RNA extracted from a CDC-negative sample (CDC^{-}) was used as a negative control. Cycle threshold (C_T)

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already confirmed by RT-PCR. Similarly, we detected SARS-CoV-2 spike protein S1 staining within pancreatic exocrine cells, as shown by the double immunostaining for SARS-CoV-2 spike protein S1 and trypsin-positive acinar cells (Fig. 4*D–F*) and also with CK19-positive ductal cells (Fig. 4*G–I*). We further performed immunohistochemistry, H-E staining, and islet size quantification as well as quantification of insulin expression on pancreatic samples. Our pathologic analysis revealed a substantial decrease in islet size and a slight reduction in insulin expression in pancreatic sections from patients with COVID-19 as compared with control participants (Supplementary Fig. 3*A–E*). Next, to assess β-cell death in pancreatic sections from patients with COVID-19, where the presence of viral infection had been ascertained (Fig. 3*M* and *N*), we performed caspase 3 immunostaining. We did not detect any positive staining for caspase 3 within the endocrine cells in the aforementioned pancreatic sections. It is well known that caspase 3 is just a part of canonic apoptotic cascade; β-cell death may be caspase 3 independent, or the process may be less evident (Supplementary Fig. 3*F* and *G*). Taken together, histologic and ultrastructural examinations of pancreatic islets from patients with COVID-19 who were newly hyperglycemic revealed mild pancreatic inflammation and altered β-cell structure, displaying characteristic features commonly reported and observed in patients with type 2 diabetes, and detection of viral RNA suggested pancreatic localization of SARS-CoV-2.

DISCUSSION

Several emerging clinical reports have described an increased incidence of patients with new-onset hyperglycemia associated with acute COVID-19 (20) or onset within a few weeks from recovery of the disease (21). In this study, we observed impaired function and survival of human pancreatic islets induced upon challenge with serum from patients with COVID-19 but also with that from patients with type 2 diabetes, and the triggered lethality may have been due to the presence of some circulating inflammatory factors as reported in literature (22). We would like to point out that the lethality of type 2 diabetes sera on human islets was largely described in cross-sectional and prospective studies, where elevated circulating inflammatory factors, including acute-phase proteins and proinflammatory cytokines, drove the inflammatory process during type 2 diabetes pathogenesis and led to islet inflammation and consequently β-cell death (23). This may confirm the high rate of cell death and the drastic decrease in insulin secretion that we observed upon challenge of human islets with sera from patients with type 2 diabetes. Importantly, IL-1β was retrieved within the several circulating proinflammatory cytokines found in the sera of patients with type 2 diabetes, possessing a pleiotropic effect on human islet survival and homeostasis. Extended exposure or high levels of IL-1β may trigger a proinflammatory cascade prominent in type 2 diabetes pathogenesis and disease progression (23,24). That being said, when applying a more rigorous one-way ANOVA with Bonferroni correction for multiple comparisons analysis, the effect of IL-1β was no longer statistically different. Another important observation is related to the absence of the autoantibodies in our samples obtained from patients with COVID-19 and from those who had recovered from COVID-19. This may also have been due to the short study follow-up since their diagnosis of SARS-CoV-2 infection. We acknowledge that this limited endured timeframe may not have been enough to allow us to see any defined aspects of the autoimmune process, including the generation of anti-islet autoantibodies. Notably, pathologic examination of pancreatic sections retrieved from newly hyperglycemic patients with COVID-19 revealed mild lymphocytic infiltration, which, combined with the detection of SARS-CoV-2–specific viral RNA, was suggestive of a direct tropism of SARS-CoV-2 for β-cells that in turn may have contributed to their dysfunction or death, as suggested by other recent studies (3,25). The expression of several cytokine receptors on human pancreatic islets is also indicative of islet susceptibility to COVID-19–associated cytokine-induced death. Interestingly, the detection of histopathologic alterations such as features of pancreatic inflammation and activation of pancreatic lymph nodes as well as altered pancreatic ultrastructure suggests broad and potentially long-term effects in patients with COVID-19. Surprisingly, we were also able to detect SARS-CoV-2 viral RNA in pancreatic samples from some hyperglycemic patients with COVID-19, which confirms the extrapulmonary tropism of SARS-CoV-2 (26–28). The ultrastructural finding of numerous cytoplasmic

values are shown. *C*: H-E staining of PLNs from a patient with COVID-19. *D*: Bar graph depicting the results of a SARS-CoV-2 RT-PCR assay using the 2019-nCoV_N1 and 2019-nCoV_N2 primer probe sets performed on RNA samples extracted from PLNs from a patient with COVID-19 (P1), showing detectable viral RNA in patient P1. RNA extracted from a CDC^+ control DNA plasmid was used as a positive control, and RNA extracted from a CDC^- sample was used as a negative control. C_T values are shown. Magnification in panels *A* and *D*, ×10 and panel *C*, ×20; scale bars in panels *A* and *D*, 100 μm and panel *C*, 100 μm. *E–J*: Transmission electron microscopic analysis of pancreatic tissue from patients with COVID-19 as compared with healthy control subjects and patients with type 2 diabetes, showing similar islet alterations in pancreatic tissues from patients with COVID-19 and patients with type 2 diabetes; arrows indicate the presence of insulin secretion granules; in healthy control subjects, arrows delineate insulin granules with different degrees of granulation; in diabetic samples as well as in COVID-19 samples, arrows delineate β-cells with several immature granules. Asterisks in panels *H*, *I*, and *J* show mature insulin granules. *K*: Quantification of the proportions of immature insulin granules per total mature insulin secretion granules; $n = 3$ cases per section were analyzed. *L* and *M*: Transmission electron microscopic analysis of pancreatic tissue from a patient with COVID-19 as compared with that from healthy control subjects depicting the presence of several vacuoles (shown by black arrows) in the vicinity of β-cells from a patient with COVID-19. *N*: SARS-CoV-2 spike S1 staining depicted within endocrine cells; arrows indicate the positive staining within endocrine cells of the pancreas; asterisk refers to an islet, and arrowheads indicate pancreatic acinar cells. AU, arbitrary unit.

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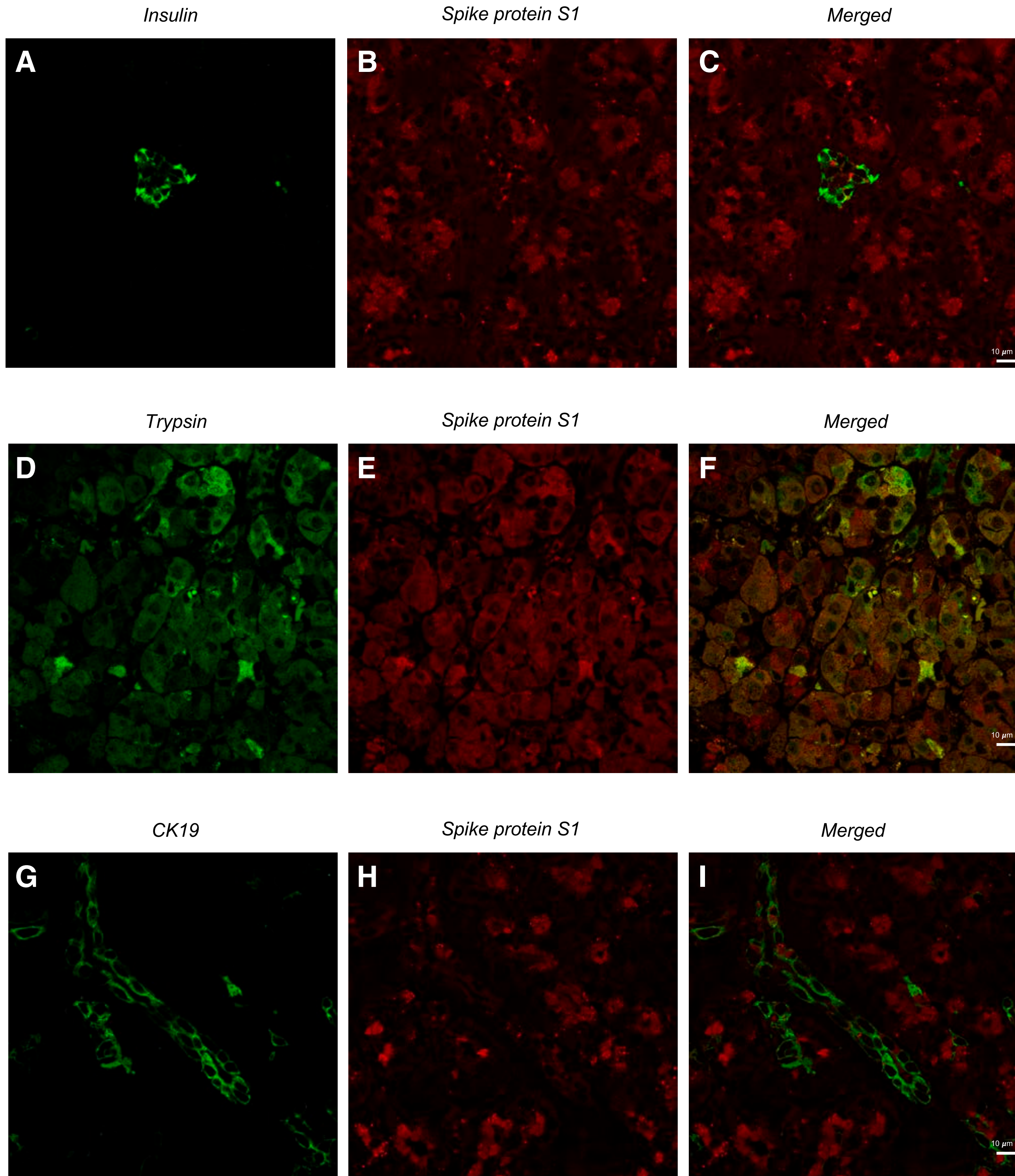

Figure 4—SARS-CoV-2 spike protein S1 localizes within endocrine pancreatic β-cells and exocrine pancreatic cells. *A–I*: Confocal microscopic analysis of pancreatic tissue from a patient with COVID-19 depicting the localization of SARS-CoV-2 spike protein S1 within β-cells (insulin-positive cells) (shown in panel *C*) and within exocrine cells, trypsin-positive cells (shown in panel *F*), and CK19-positive cells (as shown in *I*). Scale bars in panels *A–I*, 10 µm.

vacuoles in some β-cells, as well as in capillary endothelial cells, in pericytes, and in histiocytes, is suggestive of the presence of virus, even if viral particles have not been identified. In summary, this may suggest that the diabetogenic effect induced by SARS-CoV-2 infection can also be mediated by a possible direct viral cytotoxicity against human pancreatic islets. Evidence of SARS-CoV-2 viral particles within the pancreas has been reported

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recently (29–33), as has the expression of the SARS-CoV-2 cell entry receptor ACE2 in pancreatic ductal epithelium and microvasculature, while minor expression of ACE2 mRNA transcript has been observed in the endocrine pancreas (34). Other recent reports have demonstrated the presence of the canonic SARS-CoV-2 cell entry machinery, such as ACE2, TMPRSS2, and DPP4, in addition to direct evidence of SARS-CoV-2 infection, within pancreatic ducts, acinar cells, and endothelial cells and in close proximity to the islets of Langerhans, within the islets, and within insulin-producing β-cells (25,30). We did not find increased levels of unmethylated β-cell–derived *INS* DNA, but the timing of killing may have been well before the samples were collected, and there was a dramatic increase in *INS* DNA derived from non–β-cells, which may have obscured this sign of β-cell killing. Although data from the literature supported/considered that the increased frequency of unmethylated *INS* CpG sites in β-cells and the ratio of unmethylated/ methylated *INS* DNA released into the circulation upon cell death are a reflection of β-cell death (35), there have been several limitations regarding the use unmethylated *INS* DNA as a reliable marker of in vivo β-cell death, particularly the limited interlaboratory validation of the indicated methodology and also the fact that stressed β-cells may methylate insulin DNA and die without releasing demethylated insulin (36). Several other caveats are related to the findings from the literature indicating that other cell types may contain limited/lower levels of circulating unmethylated *INS* and raised the concern that unmethylated *INS* DNA may not be exclusively restricted to β-cells and therefore may not solely reflect on β-cell death (37,38). To our knowledge, this is the first report showing the coexistence of an exaggerated peripheral inflammation and a direct tropism of SARS-CoV-2 virus, which together may drive β-cell dysfunction/damage. Our data suggest that a dysregulation of cytokines and a proinflammatory environment may synergistically act in concert with pancreatic localization of SARS-CoV-2 to promote abnormal glycometabolic control (Supplementary Fig. 4). In conclusion, in the current study, we suggest that new-onset hyperglycemia in patients with COVID-19 may be due to the proinflammatory milieu initiated by a cytokine storm in combination with a direct localization of SARS-CoV-2 within pancreatic β-cells.

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