Brief Communication

Highlight article

ISGylation is increased in the peripheral blood mononuclear cells derived from symptomatic COVID-19 patients

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Impact Statement

Persistent elevation of interferons (IFNs) (interferonopathy) leads to the induction of cytokine storm (CS) and inflammation in patients with chronic viral infection; conditions that are common in patients severely infected with SARS-CoV-2 virus. However, if the IFN pathway is dysregulated and, in turn, leads to severe symptoms in COVID-19 patients is not known. Here, we report significant increases in protein ISGylation (conjugates of ISG15 (Interferon-Stimulated Gene 15)) and MX-1 (myxovirus resistance protein-1) protein levels, both induced by type-I IFN, in the peripheral blood mononuclear cells (PBMCs) from symptomatic (SARS-CoV-2-positive with symptoms) but not in asymptomatic patients (SARS-CoV-2-positive with no symptoms) and uninfected individuals (SARS-CoV-2-negative). Knowing that ISGylation augments CS and intestinal inflammation in colon cancers, our results provide increased ISGylation in PBMCs as a warning sign for dysregulated inflammation in symptomatic patients that can help identify patients who may benefit from immunosuppressive therapy to mitigate inflammation in COVID-19 patients.

Abstract

Cytokine-driven hyper inflammation has been identified as a critical factor behind poor outcomes in patients severely infected with SARS-CoV-2 virus. Notably, protein ISGylation, a protein conjugated form of Type 1 IFN-inducible ubiquitin-like protein ISG15 (**I**nterferon-**S**timulated **G**ene **15**), induces cytokine storm (CS) and augments colonic inflammation in colitis-associated colon cancers in mouse models. However, whether ISGylation is increased and causally responsible for CS and hyper inflammation in symptomatic COVID-19 patients is unknown. Here, we measured ISGylation levels in peripheral blood mononuclear cells (PBMCs) from 10 symptomatic (SARS-CoV-2-positive with symptoms) and asymptomatic (SARS-CoV-2-positive with no symptoms) COVID-19 patients, and 4 uninfected individuals (SARS-CoV-2-negative), using Wes^{Tm} assay. Strikingly, we note significant increases in protein ISGylation and MX-1 (myxovirus-resistance protein-1) protein levels, both induced by type-I IFN, in symptomatic but not in asymptomatic patients and uninfected individuals. Knowing that ISGylation augments CS and intestinal inflammation in colon cancers, we propose that increased ISGylation may be an underlying cause of CS and inflammation in symptomatic patients.

Keywords: COVID-19, PBMCs, cytokine storm, ISGylation, interferon, Interferon-Stimulated Gene 15

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Introduction

Coronavirus disease 2019 (COVID-19), caused by the SARS-CoV-2 virus, is continuing to spread and kill humans globally. Accumulating evidence suggest that induction of the cytokine-driven hyper inflammation results in poor outcomes in symptomatic COVID-19 patients. However, the underlying reason(s) behind the cytokine storm (CS) and hyper inflammation is unclear. One likely explanation to this viropathology may be differences in the induction of the Type I interferon (IFN) pathway in COVID-19 patients.

Type-I IFNs induce the expression of > 500 genes, which are collectively called ISGs (IFN-stimulated genes). One of the earliest ISGs induced by IFN is ISG15 (Interferon-Stimulated Gene 15), a known anti-viral protein.[1] ISG15 is post-translationally conjugated to cellular proteins in a process called ISGylation.[2] Elevated levels of both free ISG15 and ISGylation are correlated with the resistance to viral

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infection in cells grown in culture.[3] Given the importance of the anti-viral response governed by ISG15/ISGylation, it is not surprising that humans harbor strategies to sustain IFN-mediated induction of the ISG15 pathway to achieve an anti-viral state, and viruses have evolved strategies to counteract them (reviewed in Perng and Lenschow[4]). Although induction of IFN establishes an anti-viral state in humans, some viruses continue to replicate in the presence of IFN in patients with a chronic viral infection, suggesting failure of IFNs to establish an anti-viral state in these patients.[5] Persistent elevation of IFNs (interferonopathy) leads to the induction of cytokine storm and inflammation causing multi-organ dysfunction in patients with chronic viral infections; conditions that are common in severely ill COVID-19 patients.[5] Why the IFN pathway is dysregulated (causing persistent elevation of IFNs) in patients with chronic viral infections, and if it is dysregulated in COVID-19 patients, is not known. Since ISGylation augments CS and intestinal inflammation in colon cancer patients,[6] we presumed that increased ISGylation due to the dysregulation of the IFN pathway may be an underlying cause of CS and hyper inflammation in symptomatic patients. We therefore measured levels of ISGylation in symptomatic and asymptomatic patients finding that ISGylation is significantly elevated in COVID-19 symptomatic patients.

Materials and methods

Collection of samples, analyses of viral loads and genetic variants

Patients admitted to the emergency room at the University Medical Center, New Orleans, with suspected COVID-19 viral infection (symptomatic or asymptomatic patients) were enrolled in this study. Information on the demographics, genetic variants, and symptoms associated with COVID-19 in patients enrolled in this study is provided in Table 1. Blood samples were collected after informed consent and tested for COVID19 status by RT-PCR. To characterize the SARS-COV-2 variant classification, frozen nasal swab samples (Puritan sterile polyester tipped swabs #25-806 1PD SOLID) were received, thawed, and placed in 2 ml of viral transport media for 2 h. Specimens were accessioned and RNA extraction was performed using the Zymo Quick DNA/RNA Viral MagBead kit (Zymo #R2141) automated on a Tecan Fluent liquid handling workstation. Resulting viral RNA was used for library generation and next generation sequencing using the Illumina COVID-seq workflow per manufacturer's instructions (ARCTIC v3). Libraries were pooled and loaded on an Illumina NextSeq550Dx in RUO mode, with 74 cycles of paired end sequencing using a 150 cycle mid output reagent cartridge and flow cell. Data processing and QC was performed using the DRAGEN COVID-Seq Test (EUA) v.1.3.0 on the cloud-based BaseSpace sequence analysis hub hosted by Illumina. Lineage assignment was similarly performed using the DRAGEN COVID Lineage app v.3.5.3. Sample collection and processing was performed using the protocol approved by the Institutional Review Board Committee of the Louisiana State University Health Sciences Center-School of Medicine, New Orleans (Kuali IRB # 667).

Preparation of PBMCs

Samples of venous blood (30 ml) were diluted with Hanks' balanced salt solution (HBSS) (1:4) (Lonza, Walkersville, MD, USA) and were separated with Ficoll-Paque-Plus (GE Healthcare, Piscataway, NJ, USA). Samples were centrifuged at 2000 r/min for 15 min at room temperature. The interface containing PBMCs were harvested and washed 2X with HBSS. The red blood cell lysis was performed by resuspending the pellet in 2 ml sterile Ammonium-Chloride-Potassium lysis buffer (ACK Lysing Buffer, Lonza) and incubated for 5 min at room temperature. PBMCs were counted and frozen at concentrations of 3.0×10^6 cells/ml in RPMI-1640 media (Lonza) containing 20% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA, USA) and 10% Dimethyl Sulfoxide (Fisher Scientific, Waltham, MA, USA). PBMCs were kept in liquid nitrogen until use. Normal PBMCs were purchased from Zen-Bio Inc (Durham, NC, USA).

Immunodetection by the Wes™ assay

PBMCs were lysed in 4% SDS lysis buffer (50 mM Tris-HCl, pH 7.5, 4% SDS), sonicated, and boiled for 10 min at 100ºC. Lysates were clarified *via* centrifugation at 13,000 r/min for 2 min. Protein concentrations were determined by measuring absorbance at 280 nm using a Beckman Coulter Spectrophotometer, and proteins were adjusted to equal protein amounts. Immunodetection of ISGylation, β-actin (Abcam, ab6276) and MX-1 (Cell Signaling, 37849s) was performed using the Wes™ system from ProteinSimple (San Jose, CA, USA) as described in Schwartzenburg *et al.*[7]

Results and discussion

ISGylation is increased in the peripheral blood mononuclear cells derived from symptomatic patients

We have noted that when the IFN/ISG15 pathway is deregulated, high molecular weight ISG15 conjugates (66 kDa and above) are predominantly accumulated in the human tissues and cells of ataxia telangiectasia (A-T), amyotrophic lateral sclerosis (ALS), and traumatic brain injury (TBI)-exposed ALS patients.[7] To test if the IFN/ISG15 pathway is deregulated in COVID-19 patients, we measured the levels of ISGylation (ISG15 conjugates from 66 kDa and above) in PMBCs isolated from three groups: uninfected individuals (SARS-CoV-2-negative) ($n = 4$), asymptomatic (SARS-CoV-2-positive with no symptoms) ($n = 10$), and symptomatic (SARS-CoV-2-positive with symptoms) ($n = 10$) patients using Wes™ assay and anti-ISG15-specific antibodies as described in Schwartzenburg *et al.*[7] (see Table 1 for the information on demographics, genetic variants, and symptoms associated with COVID-19 in these patients). A representative Wes™ image of ISGylation in two PBMC samples from each group is shown in Figure 1(a). The bar graph in Figure 1(b) shows values of the ratio between ISGylation/β-actin in individual PBMCs isolated from uninfected subjects and COVID-19 patients. The bar graph in Figure 1(c) shows mean values of the ratio between ISGylation/β-actin in PBMCs

from each group. When normalized to β-actin, ISGylation levels were significantly increased in symptomatic compared to asymptomatic patients ($p = 0.017$). These results suggest that ISGylation is predominantly increased in the symptomatic patients.

MX-1 protein has previously been identified as a sensitive and selective substitute to measure an IFN-α/β-induced state in preclinical and paraclinical models.[8] To test if the IFN pathway is indeed elevated, we measured MX-1 protein in the same samples shown in Figure 1. A representative Wes image of MX-1 is shown in Figure 2(a) and the bar graphs in Figure 2(b) (samples are loaded in the same order as in Figure 1(b)) and (c) shows values of the ratio between MX-1/β-actin in PBMCs from each subject, and mean values of the ratio between ISGylation/β-actin in PBMCs isolated from each group, respectively. When normalized to β-actin, MX-1 protein levels were significantly increased in symptomatic compared to asymptomatic COVID patients ($p = 0.019$).

Table 1. Demographics, genetic variants, and symptoms associated with COVID-19 in patients enrolled in the study.

Patient #s	Enrollment date	Age	Sex	Was the patient admitted (Y/N)	How many days ago patient showed symptoms	Pre-existing conditions	Variant
Asymptomatic							
5	1/19/2021	63	M	Y	Unknown	Y	*
6	7/2/2020	51	M	Y	Unknown	Y	*
7	9/3/2020	49	M	Y	Unknown	Y	*
8	8/31/2020	52	M	Y	Unknown	Y	*
9	7/20/2020	57	M	Y	Unknown	Y	*
10	9/1/2020	34	M	Y	Unknown	N	*
11	12/4/2020	42	M	Y	Unknown	Y	*
12	12/8/2020	56	M	Y	Unknown	N	B.1.234
13	7/13/2020	62	M	Y	Unknown	Y	*
14	1/12/2021	45	F	N	Unknown	Y	*
Symptomatic							
15	2/2/2021	56	M	Y	3	N	*
16	1/21/2021	52	M	Y	9	Y	B.1.311
17	1/8/2021	39	M	Y	8	N	*
18	7/12/2020	48	M	N	6	Y	*
19	6/2/2020	39	M	Y	15	Y	*
20	2/11/2021	40	M	Y	9	N	*
21	1/4/2021	63	F	Y	7	Y	B.1.2
22	7/22/2020	69	F	Y	4	Y	B.1.595
23	2/19/2021	40	M	Y	7	Y	*
24	2/18/2021	30	M	Y	8	N	*

Patient #s	Symptoms								
Asymptomatic	Fever	Cough	Sore throat	Shortness of breadth	Wheezing	Chest pain	Palpitation	Tiredness	Achy muscles
5	N	N	N	N	N	N	N	N	N
6	N	N	N	N	N	N	N	N	N
7	N	N	N	N	N	N	N	N	N
8	N	N	N	N	N	N	N	N	N
9	N	N	N	N	N	N	N	N	N
10	N	N	N	N	N	N	N	N	N
11	N	N	N	N	N	N	N	N	N
12	N	N	N	N	N	N	N	N	N
13	N	N	N	N	N	N	N	N	N
14	N	N	N	N	N	N	N	N	N
Symptomatic									
15	Y	Y	N	N	N	Y	N	N	N
16	N	Y	N	N	N	N	N	N	N
17	Y	Y	N	Y	N	N	N	N	N
18	Y	Y	Y	Y	N	N	N	N	N
19	Y	Y	Y	Y	N	N	N	N	N
20	Y	Y	N	Y	N	N	N	N	Y
21	Y	Y	N	N	N	Y	N	N	N
22	Y	Y	Y	Y	N	N	N	Y	N
23	Y	Y	Y	Y	N	Y	N	N	Y
24	Y	Y	N	Y	N	Y	Y	Y	Y

(Continued)

Table 1. (Continued)

Patient #s	Symptoms							
Asymptomatic	Vomiting	Diarrhea	Stomach pain	Lack of appetite	Loss of smell	Headache	Confusion	COVID-19 pneumonia
5	N	N	N	N	N	N	N	N
6	N	N	N	N	N	N	N	N
7	N	N	N	N	N	N	N	N
8	N	N	N	N	N	N	N	N
9	N	N	N	N	N	N	N	N
10	N	N	N	N	N	N	N	N
11	N	N	N	N	N	N	N	N
12	N	N	N	N	N	N	N	N
13	N	N	N	N	N	N	N	N
14	N	N	N	N	N	N	N	N
Symptomatic								
15	Y	N	N	N	N	Y	N	Y
16	N	N	N	N	N	N	N	N
17	N	N	N	N	Y	Y	N	N
18	N	Y	Y	N	N	N	N	N
19	N	Y	Y	N	N	N	N	Y
20	N	N	N	N	N	Y	N	Y
21	Y	N	N	N	N	Y	N	N
22	N	N	N	N	N	Y	N	Y
23	Y	Y	Y	Y	Y	N	N	Y
24	N	N	Y	N	N	Y	N	N

N: No symptoms were developed; Y: Symptoms developed.
COVID-related death: None; Health Care worker: None; Diagnosed with ARDS: None; Intubated and MV: None; All samples were collected pre-COVID-19 vaccination.
*Unable to obtain complete sequence.

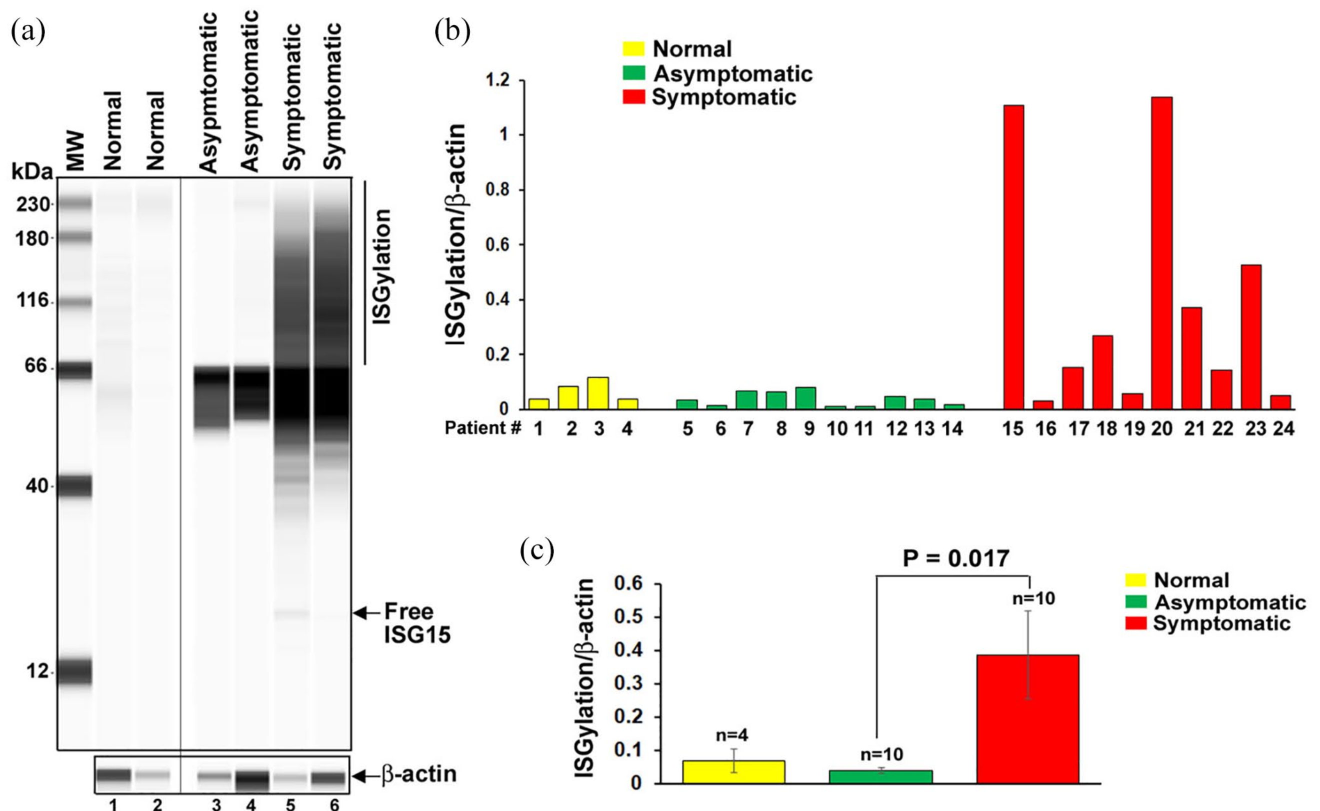

Figure 1. ISGylation is increased in the peripheral blood mononuclear cells derived from symptomatic patients. (a) ISGylation levels in normal subjects (Lanes 1, 2), asymptomatic (Lanes 3, 4) and symptomatic (Lanes 5, 6) patients were assessed using Wes™ (ProteinSimple) with ISG15-specific antisera as described in Materials and Methods. For loading controls, the same samples were probed using an antibody against β-actin. Data are shown in a gel view. (b) Intensities of ISGylation (area under peaks spanning from 66 to 230 kDa comprising high molecular weight ISG15 conjugates), and β-actin for all samples were quantitated using Compass for Simple Western software. The bar graph shows values of the ratio between ISGylation/β-actin, and each bar represents normal subjects/patients tested for ISGylation. (c) The bar graph shows mean values of the ratio between ISGylation/β-actin in PBMCs from each group.

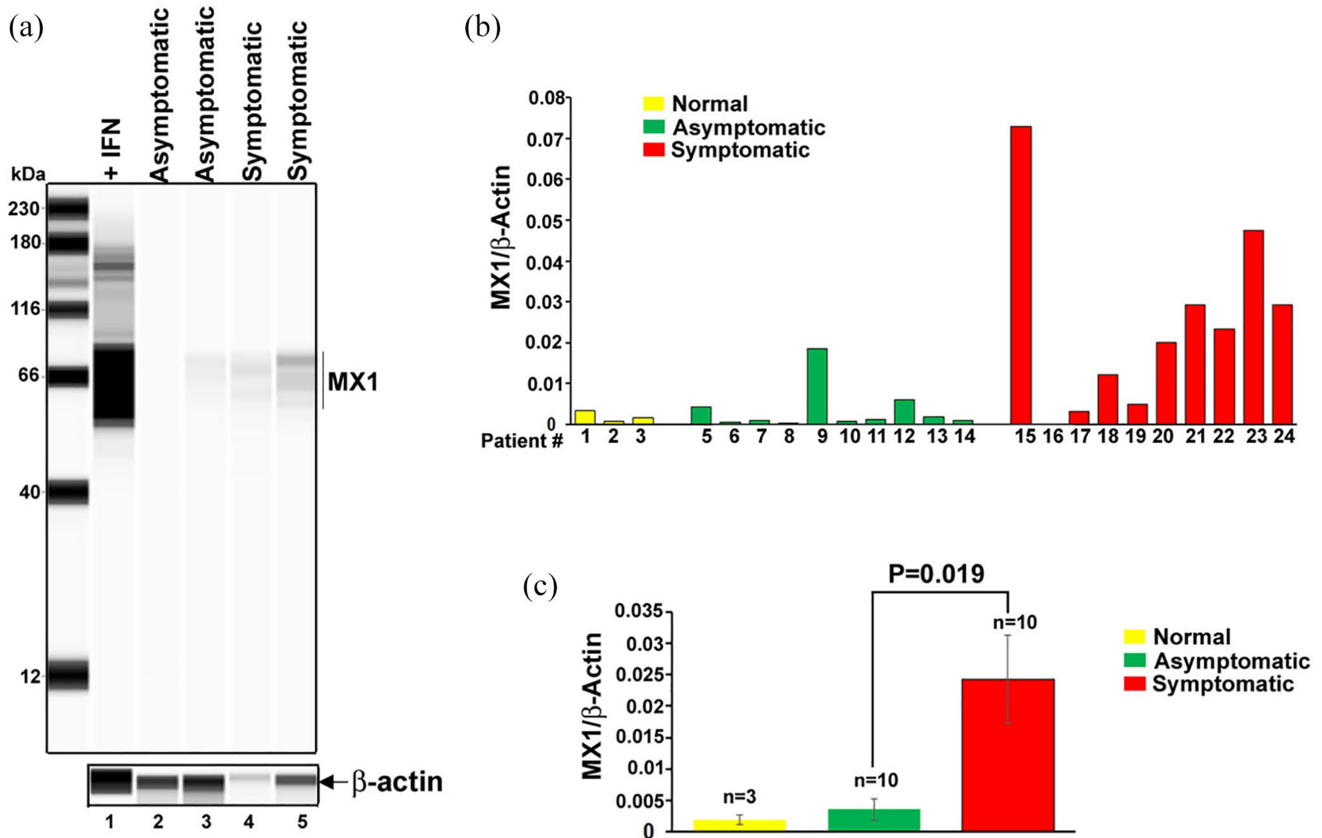

Figure 2. MX-1 protein levels are increased in the peripheral blood mononuclear cells derived from symptomatic patients. (a) MX-1 levels in asymptomatic (Lanes 2, 3) and symptomatic (lanes 4. 5) patients were assessed using Wes™ (ProteinSimple) with ISG15-specific antisera as described in Figure 1 legends. Neuroblastoma SH-SY5Y cells treated with IFN-β are shown as positive control for IFN-mediated MX-1 induction. For loading controls, the same samples were probed using an antibody against β-actin. Data is shown in a gel view. (b) Intensities of MX-1 (area under peaks spanning from 59–79 kDa comprising MX-1 immunoreactive smear), and β-actin for all samples were quantitated using Compass for Simple Western software. The bar graph shows values of the ratio between MX-1/β-actin, and each bar represents normal subjects/patients tested for MX-1. (c) The bar graph shows mean values of the ratio between MX-1/β-actin in PBMCs from each group.

Together, results shown in Figures (1) and (2) reveal that the IFN pathway is induced, and consequently, levels of IFN-inducible ISGylation and MX-1 are increased in symptomatic but not in asymptomatic group of patients.

The IFN response is tightly regulated in most cell types. In humans, free ISG15 binds to and stabilizes ISG15 deconjugating enzyme USP18 (a.k.a. UBP43), which in turn attenuates IFN-α/β signaling.[9] Based on these results, Speer *et al.* suggested that one of the roles of ISGylation at early stages of viral infection in humans may be to sequester free ISG15, thereby allowing IFN signaling to occur. At later stages, USP18-mediated de-ISGylation releases free ISG15, which in turn stabilizes USP18 and attenuates IFN-α/β signaling and inflammation.[10] This model suggest that protein ISGylation may be increased at early stages and decreased at later stages of viral infection. In the current study, we noted variable levels of ISGylation (increased/decreased levels of ISGylation) in symptomatic patients tested ~3–15 days post-infection (Figure (1) and Table 1). Whether variable levels of ISGylation are causally correlated with the COVID-19 disease onset, progression, and/or recovery in these symptomatic patients is not known. Knowing that persistent induction of IFN production (interferonopathy) leads to CS and inflammation in autoimmune diseases (e.g. Sjögren's syndrome),[11] chronic viral infections,[11] and persistent induction of ISGylation leads to inflammation in colon cancer patients,[6] we propose that sustained elevation of ISGylation may lead to CS and inflammation in severely ill patients (e.g. in patients with Acute Respiratory Distress Syndrome (ARDS)). Notably, none of the patients tested in our study showed life-threatening COVID-19 symptoms (e.g. ARDS) or succumbed to the disease suggesting they may not have experienced severe CS. Further investigations on the status of ISGylation and inflammatory cytokines in COVID patients with ARDS is required to confirm if sustained ISGylation causes CS and inflammation in COVID-19 patients.

In our study, both asymptomatic and symptomatic patients were positive for SARS-Cov-2 using a clinical RT-TCR test. Interestingly, we have noted decreased ISGylation and MX-1 levels in asymptomatic compared to symptomatic patients. We do not know if decreased levels of ISGylation and MX-1 are due to the faster viral clearance, low viral infection levels, and/or lack of IFN induction in asymptomatic patients. Nevertheless, our current results that ISGylation is elevated in symptomatic patients provide increased ISGylation in PBMCs as a biomarker to help identify symptomatic patients who may benefit from immunosuppressive therapy to mitigate ISGylation-mediated inflammation.

AUTHORS' CONTRIBUTIONS

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; J.S. and R.R. conducted the experiments, A.Z. conducted biobanking of specimens and isolated PBMCs, H.K., J.S., and L.M. obtained IRB approval and patient consent for collecting biospecimens (blood and nasal swabs) from COVID-19 patients, J.C. performed variant analysis, and S.D. conceived the idea, analyzed data, and wrote the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICAL APPROVAL

Sample collection and processing was performed using the protocol approved by the Institutional Review Board Committee of the Louisiana State University Health Sciences Center-School of Medicine, New Orleans (Kuali IRB # 667).

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