INTERFERON LAMBDA GENE EXPRESSION IN IRAQI COVID-19 PATIENTS

*M.S. Jabbar, N.M. Utba**

Department of Biology, College of Science, University of Baghdad, Jadriyah intersection, Al- Jadriyah, Baghdad, 10071, Iraq.

* Corresponding author: nawalutba9@gmail.com, nawalutba@scbaghdad.edu.iq

Abstract. Purpose: type III interferons (IFNλ) are an early line of defense in upper respiratory tract infections, such as severe acute respiratory syndrome coronavirus 2 (SARS-COV-2). They have a crucial role in the control of the innate immune system and the modulation of immunological responses during the course of acute viral infection and tissue inflammation. The present study was aimed at evaluating the expression of IFNλ genes in Iraqi coronavirus disease (COVID-19)-infected patients. Materials and methods: ninety patients presented with COVID-19 and 50 healthy controls were recruited. Blood samples were obtained from the participants. Haematological and biochemical analyses were performed on the blood samples. IFNλ gene expression was assessed in peripheral blood mononuclear cells (PBMCs) of all the participants by real-time polymerase chain reaction (RT-PCR). Results: all COVID-19 patients had elevated relative expression of IFNλ-I, IFNλ-II, and IFNλ-III genes compared to controls, by 1.85 ± 0.25-, 39.9 ± 15.07-, and 4.001 ± 1.23-fold, respectively. According to the severity of the disease (moderate, severe, and critical), the relative expression of each IFN type was likewise elevated. However, the rise did not reach a significant level. On the other hand, there was a significant difference ($p < 0.05$) in the mean of relative expression between IFN-I, IFN-II, and IFN-III in total and each category of severity. Conclusion: the findings show that IFNλ gene expression was upregulated in COVID-19 disease and neither age, sex, nor underlying diseases impacted the variations in expressions.

Keywords: IFNλ, COVID-19, gene expression, Iraqi patients, real-time PCR.

Introduction

The COVID-19 pandemic has posed an unparalleled challenge to public health, food systems, and the global workforce, resulting in a significant loss of human life worldwide. As of May 2, 2023, coronavirus disease (COVID-19) cases have been identified in nearly every country worldwide. The virus infected approximately 690 million people globally, with a mortality toll of approximately 7 million. According to the World Health Organization (WHO), there were 25,375 deaths and 2,465,545 confirmed cases of COVID-19 in Iraq between January 3, 2020, and April 19, 2023 (WHO, 2023).

The innate immune response, which is triggered by COVID-19, is the body's first line of defense against severe acute respiratory syndrome coronavirus 2 (SARS-COV-2). Interferons (IFNs) are among the innate immune response components. Host cells produce and release IFNs in response to the presence of various viruses (Schultze & Aschenbrenner, 2021). Based on receptor complex recognition and protein structure, IFNs are usually classified into three classes: type I IFN, type II IFN, and type III IFN. All three classes of IFNs are crucial for combating viral infections and regulating the immune system (Choi & Shin, 2021). IFNλ, also known as type III interferons, is a family of cytokines that includes four molecules: IFN-λ1, IFN-λ2, IFN-λ3 (also called IL29, IL28A, and IL28B, respectively), and IFN-λ4. These cytokines were first identified in 2003 and have anti-viral properties similar to type I interferons. However, their effect is milder, and they act primarily as the initial defense against viruses in the epithelium (Kotenko & Durbin, 2017). When a virus like SARS-CoV-2 infects the body, it is detected by different receptors in the innate immune system, such as cytoplasmic RNA sensors (RIG-I and MDA5) and TLRs (TLR3, TLR4, TLR7, and TLR8). These receptors activate transcription factors, such as NF-κB and IRF3, which upregulate proinflammatory genes and IFNs (Farooq *et al*., 2022). In response to viral infection, type IFNs (IFN-α and -β) and type III IFNλ are released. These IFNs bind to the IFNα/β receptor and IFNλ, respectively, activate the JAK-STAT signaling pathway and in-

crease the expression of interferon-stimulated genes (Choi & Shin, 2021). In addition to producing an anti-viral immunological response, inhibiting the replication of the virus, IFN normally promotes apoptosis to help protect host cells against the spread of the virus. However, SARS-CoV-2 can inhibit the synthesis of the anti-viral type I IFN response by expressing factors, such as ORF6, delaying the onset of the initial response. This delay, alongside viral spread in host cells, causes disease progression and aggravates inflammation (Tain *et al*., 2020). On the other hand, type III IFNλ has wider consequences for respiratory infections like COVID-19. They play a critical role in preserving a well-adjusted antiviral response in the respiratory tract (Galani *et al*., 2017).

Type III IFNλ is stimulated at a lesser viral load than type I IFNs, helping to reduce the primary infection by stimulating cellular resistance against the virus, dealing with the virus load, and preventing disease progression (Galani *et al*., 2017). IFN-λ plays an essential role in regulating viral replication. IFN-λ are widely known for their antiviral effects, but they also aid in the immune response to bacterial infections (Bierne *et al*., 2012; Cohen & Prince, 2013). Due to their ability to activate a more limited range of genes in a specific group of target cells expressing IFNλR, IFN-λ is a promising therapeutic agent (Hermant &Michiels, 2014). The effects of IFN-λ are mostly focused on viruses that target the respiratory system, gastrointestinal tract, urogenital tract, and liver (Lazear *et al*., 2015). Concerning SARS-CoV-2 infections, the protective effects of IFN signaling are demonstrated by studies showing that severe COVID-19 is associated with decreased IFN signaling, the presence of autoantibodies blocking the action of specific IFNs, and genetic variants that impair IFN signaling (Galbraith *et al*., 2022). However, the studies that focused on IFNλ role in COVID-19 disease are very few.

Therefore, this study was conducted to investigate the *IFNλ* gene expression in Iraqi COVID-19 patients.

Materials and Methods

Subjects

A total of 90 unvaccinated COVID-19 patients were enrolled in this case-control study, including 30 critical cases, 30 moderate cases, and 30 severe cases, in addition to 50 healthy volunteers. The patients were admitted for diagnosis and treatment to the Dar Al-Salam Field Hospital in Baghdad during the period from November 2021 to May 2022. The diagnosis of COVID-19 patients was confirmed by positive nasopharyngeal swabs using the polymerase chain reaction (PCR) technique on the first day of admission and by a chest computerized tomography scan. The WHO Interim Guidance for determining disease severity was followed to enroll the patients. Information regarding age, sex, and underlying diseases (diabetes and cardiovascular) was documented for the patients and controls. The control group consisted of individuals who had not experienced any respiratory infections within the previous four months and had no underlying medical conditions. For the control group, only those who tested negative for CRP had an erythrocyte sedimentation rate (ESR) of less than 20 mm/h and had negative results on a nasopharyngeal swab were included.

Blood sample collection and preparation

An aliquot of 10 mL of blood was collected from both moderate patients and controls. However, for severe and critical patients, the blood was collected after 4-7 days of hospitalization. A plain tube was used to draw 2 mL of blood. After allowing the blood to clot, the tube was centrifuged at 3000 rpm for 15 minutes at a temperature of 20 °C. The resulting serum was collected and stored at a temperature of -20 °C until it was assessed. An aliquot of 8 mL of the remaining material was transferred to 50 mL centrifuge tubes, which contained 15 mL of lymphocyte separation medium (1.077 g D =/ml, at +20 °C), after being placed in an EDTA tube for an hour. Then, without mixing, the diluted blood was layered onto the 15 mL lymphocyte separation medium along with an equivalent volume of phosphate-buffered saline (PBS). The separation process was performed

by centrifugation at 800 g for 20 min at 20 °C. The lymphocytes and other mononuclear cells were concentrated in the interphase (white, cloudy layer) between the plasma and the separation solution. The white interphase was transferred completely with a sterile Pasteur pipette in a new sterile 50 mL-size tube. Then, PBS was used to fill up the tube and washed twice at 300 g for 5-10 min. The cell pellet was re-suspended in the PBS, transferred in a triazole tube, and kept frozen at -20 ℃ until assessment.

Haematological and biochemical analyses

Some haematological (hemoglobulin level, platelet count, erythrocyte sedimentation rate [ESR], white blood cell [WBC] count, and lymphocyte count) analysis was conducted. Also, biochemical (Fasting blood sugar [FBS], alanine aminotransferase [ALT], aspartate aminotransferase [AST], alkaline phosphatase [ALP], blood urea nitrogen [BUN], creatinine, serum electrolyte sodium [Na], potassium [K], and chloride [Cl], ferritin, lactate dehydrogenase [LDH], and D dimer) parameters were assayed for patients and controls.

IFNλ gene expression by real-time polymerase chain reaction (RT-PCR)

RNA was extracted from peripheral blood mononuclear cells (PBMCs) and real-time PCR was conducted to evaluate the type III IFNλ gene expression in all participants. The isolated RNA was converted to cDNA using the ProtoScript® First Strand cDNA Synthesis Kit (NEB/UK). The relative expression was evaluated by using SYBR green and normalized to the expression of 18S rRNA (housekeeping gene). All PCR reactions were performed in triplicate. The nucleotide sequences of primers used are presented in Table 1 (Saha *et al.*, 2017).

Etherical approval

The Ethics Committee of the College of Science at the University of Baghdad approved this research (Ref.: CSEC/0122/0157) which conformed to the standards set by the latest revision of the Declaration of Helsinki. Before participation of all individuals (Patients and control) were enrolled through a written informed consent.

Statistical analysis

Categorical variables were expressed as numbers and percentages. The Pearson Chi-square test was used to determine significant differences. Mean values with their standard errors (SEM) were used to express other variables, and significant differences were determined by analysis of variance (ANOVA) followed by LSD. The Spearman rank-order correlation analysis was used to evaluate correlations between variables. A probability value (p) of less than or equal to 0.05 was considered statistically significant. The statistical analysis was performed using IBM SPSS Statistics 25.0 (Armonk, IBM Corp., NY).

Results

Demographic characteristics of the subjects

The COVID-19 patients were classified into three groups based on age (≤ 29, 30-60, and > 60 years old), and the disease was significantly ($p < 0.001$) more frequent (55, 66.7%) in the >60 years old group, followed by the 30-40 years old group (21, 21.3%) and 14 cases (12%), in ≤ 29 years old group (Table 2). However, the distribution of healthy controls (HC) according to age group was 20 (40%), 16 (32%), and 14 (28%) in the three age groups, respectively. It should be noted that there were significantly more patients than controls ($p<0.05$). The COVID-19 disease was more common in male patients than female patients (63.3 and 36.7%, respectively, $p<0.05$), whereas the difference between them in HC patients (64 and 36%, respectively) was significant. Also, 53.9% of patients had chronic diseases, while others did not. The results showed significant differences ($p < 0.01$).

Laboratory characteristics of the subjects

The laboratory parameters listed in Table 3 were analyzed for COVID-19 patients.

Some parameters were within the expected range, while others were not. Means of WBC count and ESR in patients were increased, while lymphocyte count was lower than HC (12.9 ±

± 6.4 vs 8.32 ± 3.6 10^9/L), (37.5 ± 15.0 *vs* 14.6 ± 5.2), and (0.86 ± 0.67 *vs*.1.98 ± 0.82), respectively. Means ±SD of FBS (182.8 ± 83.6 *vs*. 85.9 ± 14.8 mg/dL), BUN (31.8 ± 16.9 *vs*. 14.7 ± 11.1mg/dL), ferritin (56.0 ± 10.7 *vs*. 4.1 ± 6.8 mg/L), CRP (92.9 ± 90.2 *vs*. 2.2 ± ± 1.7 mg/L) and HDL (92.9 ± 90.2 *vs*. 2.2 ± ± 1.7 U/L in patients were above than HC and the reference range, while other parameters are within the normal reference range.

Table 1

The nucleotide sequences of the primers used in this study

Primer name	Description	Sequence 5`- 3`
18S rRNA (Housekeeping gene)	Forward	GTAACCCGTTGAACCCCATT
	Reverse	CCATCCAATCGGTAGTAGCG
IFNλ-II (IL-28 A)	Forward	AGGGCCAAAGATGCCTTAGA
	Reverse	TCCAGAACCTTCCAGCGTCAG
IFNλ-III (IL-28B)	Forward	TAAGAGGGCCAAAGATGCCTT
	Reverse	CTGGTCCAAGACATCCCCC
IFNλ-I (IL-29)	Forward	GCCCCCAAAAAGGAGTCCG
	Reverse	AGGTTCCCATCGGCCACATA

Note: Source: Saha *et al*., 2017

Table 2

Characteristics of COVID-19 patients and controls

Characteristics		COVID-19 patients N=90(%)	Control N=50(%)	*P*- value
Age group/years	≤ 29,	14 (12)[a]	14 (28)	<0.05
	30-60	21(21.3)[b]	16 (32)	
	> 60	55 (66.7)[c]	20 (40)	
	P-value	<0.001		
Gender	males	57 (63.3)[a]	32 (64)	
	females	33 (36.7)[b]	18 (36)	
	P-value	<0.05		
Underlying diseases	have	53 (58.9)	0 (0)	<0.001
	haven't	37 (41.1)	100(100)	
	P-value	NS		

Note: p: Probability of least significant difference or Pearson Chi-square test; NS: Non-significant.

Table 3

Laboratory parameters of COVID-19 patients and controls

Laboratory parameter	COVID-19 patients (Mean ± SD)	HC (Mean ± SD)	Reference range **	Status / p-value
Hb g/ml	12.2 ± 1.8	12.4 ± 1.7	11.6-16.6	Normal
Platelets x10^9/L	281.7 ± 125.09	273.6 ± 81.52	135-317	Normal
WBC x10^9/L	12.9 ± 6.4	8.32 ± 3.6	3.4-9.6	Increased
Lymphocytes x10^9/L	0.86 ±0.67	1.98 ± 0.82	1.0-4.8	Decreased
ESR (mm/hour)	47.5 ± 15.0	14.6 ± 5.2	0-29	Increased
FBS mg/dl	182.8 ± 83.6	85.9 ± 14.8	70-140	Increased (p < 0.001)
BUN mg/dl	31.8 ± 16.9	14.7 ± 11.1	7-20	Increased (p < 0.001)
Creatinine mg/dl	1.2 ± 1.8	0.7 ± 0.28	0.74-1.35	Normal
D-dimer ng/mL FEU	479.1 ± 157.9	278 ± 71.4	220-500	Normal
Ferritin mg/L	56.0 ± 10.7	4.1 ± 6.8	1.8-4.6	Increased

End of table 3

Laboratory parameter	COVID-19 patients (Mean ± SD)	HC (Mean ± SD)	Reference range **	Status / p-value
CRP mg/L	92.9 ± 90.2	2.2 ± 1.7	< or =8.0	Increased (p < 0.001)
AST U/L	31.4 ± 27.4	21.5 ± 9.8	8-48	Normal
ALT U/L	31.1 ± 24.7	20.4 ± 11.8	7-55	Normal
ALP U/L	102.3 ± 54.5	80.3 ± 29.9	40-129	Normal
LDH U/L	551.8 ± 228.6	188.9 ± 50.4	122-222	Increased p < 0.001
Na mmol/L	134.7 ± 11.5	138.5 ± 3.8	136-144	Normal
CL mmol/L	102.1 ± 7.1	102.6 ± 5.7	97-105	Normal
K mmol/L	4.4 ± 0.72	4.3 ± 0.64	3.7 to 5.1	Normal

Note: HC: Healthy control; COVID-19: Coronavirus disease

Expression levels of IFNλ genes in all the subjects

IFN-I, IFN-II, and IFN-III mean relative expression levels were higher in total COVID-19 patients than in controls by 1.85 ± 0.25-, 39.9 ± 15.07-, and 4.001 ± 1.23-fold, respectively (Table 4). The relative expression of IFNλ-1 mRNA was higher in severe COVID-19 patients (2.30 ± 0.62) compared to moderate (1.65 ± 0.33) and critical (1.78 ± 0.499) COVID-19 patients. However, compared to severe (30.62 ± 17.02) and critical (21.80 ± 7.74) COVID-19 patients, the relative expression of IFNλ-II mRNA was higher in moderate COVID-19 patients (62.07 ± 35.66). Specifically, compared to moderate (4.011 ± 1.59) and critical (0.442 ± 0.212) COVID-19 patients, the relative expression of IFNλ-III mRNA was higher in severe COVID-19 patients (6.705 ± 5.4). Although there was a slight increase in the three IFNλ expressions across the three types of infection. There was a significant difference ($p < 0.05$) between their folds in each infection type.

Stratified gene expression of IFNλ according to the characteristics of patients

To assess the influence of some of the characteristics of COVID-19 cases on the expression of IFNλ genes in PBMCs, the patients were stratified according to age, sex, and underlying diseases. The results showed that the gene expression of IFNλ was not influenced by these parameters, and no significant differences were observed between the means of IFNλ-I, IFNλ-II, and IFNλ-III in each stratum (Table 5).

Spearman's rank-order correlation analysis

The correlation coefficient was estimated using a Spearman rank-order correlation analysis between IFNλ-I, IFNλ-II, and IFNλ-III with WBC, ESR, lymphocyte count, FBS, BUN, fer- ritin, CRP, and LDH. The analysis revealed non-significant positive or negative correlations between them, as shown in Table 6. So that, the present study demonstrated that there was no significant correlation between IFNλ gene expression and inflammatory markers (WBC,

Table 4

Expression of IFNλ gene in COVID-19 patients and controls

Studied group	IFNλ Folding $2^{-\Delta\Delta Ct}$ (Mean ± SEM)			P-value
	INFλ-I	***INFλ-II***	***INFλ-III***	
Total COVID-19 Patients	1.85 ± 0.25	39.9 ± 15.07	4.001 ± 1.23	<0.05
Moderate COVID-19 patients	1.65 ± 0.33	62.07± 35.66	4.011 ± 1.59	<0.05
Severe COVID-19 patients	2.30 ± 0.62	30.62 ± 17.02	6.705 ± 5.4	<0.05
Critical COVID-19 patients	1.78 ± 0.499	21.80 ± 7.74	0.4429 ± 0.212	<0.05
Controls	1	1	1	
***P*-value**	> 0.05	> 0.05	> 0.05	

Table 5

Fold change of IFNλ-I, IFNλ-II, and IFNλ-III in PBMCs in COVID-19 patients

Characteristics		Fold change (Mean ± SEM) of IFNλ		
		IFNλ-I	IFNλ-II	IFNλ-III
Age	< 29	0.89 ± 0.37	16.22 ± 7.6	0.19 ± 0.078
	30-60	1.81 ± 0.26	27.45 ± 7.6	2.32 ± 0.94
	> 60	1.68 ± 0.27	20.03 ± 4.7	2.35 ± 1.12
	P value	0.48	0.44	0.43
Sex	male	1.73 ± 0.39	19.7 ± 5.3	2.92 ± 2.15
	female	1.79 ± 0.7	29.8 ± 4.9	2.51 ± 1.41
	P value	0.11	0.11	0.13
Chronic diseases	yes	1.99 ± 0.5	23.7 ± 4.8	2.41 ± 0.99
	no	2.2 ± 0.44	21.3 ± 3.33	2.2 ± 1.0
	P value	0.25	0.22	0.28

Note: PBMC: Peripheral blood mononuclear cells; COVID-19: Coronavirus disease

Table 6

Spearman rank-order correlation analysis between IFNλ-I, IFNλ-II, and IFNλ-III with haematological and biochemical parameters

Parameter	Correlation coefficient (rs)	Two-tailed p-value
IFNλ-I with IFNλ-II	-0.00558	0.96
IFNλ-I with IFNλ-III	0.09705	0.48
IFNλ-III with IFNλ-II	-0.22944	0.095
IFNλ-I with WBC count	-0.06228	0.63
IFNλ-II with WBC count	-0.15415	0.26
IFNλ-III with WBC count	-0.020221	0.14
IFNλ-I with ESR	0.19365	0.33
IFNλ-II with ESR	0.12004	0.38
IFNλ-III with ESR	0.150	0.11
IFNλ-I with Lymphocytes count	0.0065	0.95
IFNλ-II with Lymphocytes count	-0.09695	0.48
IFNλ-III with Lymphocytes count	-0.07815	0.57
IFNλ-I with FBS	0.07239	0.58
IFNλ-II with FBS	-0.04002	0.77
IFNλ-III with FBS	-0.11443	0.40
IFNλ-I with BUN	0.24731	0.05
IFNλ-II with BUN	-0.17258	0.21
IFNλ-III with BUN	-0.10959	0.43
IFNλ-I with Ferritin	0.2077	0.10
IFNλ-II with Ferritin	-0.02146	0.82
IFNλ-III with Ferritin	0.25871	0.058
IFNλ-I with CRP	0.15491	0.22
IFNλ-II with CRP	0.24845	0.07
IFNλ-III with CRP	-0.06511	0.63
IFNλ-I with LDL	-0.00423	0.98
IFNλ-II with LDL	0.28721	0.12
IFNλ-III with LDL	-0.15308	0.41

ESR, lymphocyte count, and CRP), which may indicate that IFNλ was not associated with the inflammatory responses in COVID-19.

Discussion

The immune system plays an essential role in defense against viral infections. One of these infections is COVID-19. Therefore, the present study focused on the role of one immune system component, IFN, in COVID-19. Virally infected host cells produce and release small proteins called interferons, which play a role in immune protection against viruses. The current findings demonstrated that three categories of COVID-19 (moderate, severe, and critical) and total PBMC had higher relative expression levels of IFN-I, IFN-II, and IFN-III mRNA than healthy controls. However, IFNλ-II demonstrated a highly significant increase ($p<0.05$) over others in each infection type, especially in moderate patients. This increase in IFNλ-II may protect the patients from developing a severe or critical type of infection. Several studies have suggested that type III IFNs may play a more crucial role in initial defense mechanisms at barrier surfaces, such as the urogenital, gastrointestinal, and respiratory tracts compared to type I IFNs (Wack *et al*., 2015; Galani *et al*., 2015; Lazear *et al*., 2015; Utba, 2019). It was observed that the mRNA levels of all the types III IFNλ studied were induced by SARS-COV-2 in PBMCs. However, the IFNλ-II showed highly significant expression in PBMCs than others (IFNλ-I and III) in moderate, severe, or critical COVID-19 patients. It was therefore postulated that IL-28A may play an important defensive role during the SARS-COV-2 infection.

IFNλs significantly reduce the pathogenicity of COVID-19 by maintaining an effective immune response against the virus in the respiratory tract at the lowest levels of the virus before type I IFNs, preventing the primary infection by promoting intracellular resistance to the virus, and managing the viral load (Galani *et al*., 2017; Shalash& Utba, 2017). When a patient has a high viral load or is particularly vulnerable to virus entry, the immune system responds by producing increasing amounts of type I IFNs, which can cause tissue damage. Some viruses that cause inflammation can decrease type IFNλs. Patients using medications that alter the cytokine equilibrium or viral clearance pathway, or those with genetic predispositions to produce more or fewer type IFNλs, may also exhibit a greater immune response. However, administering recombinant or pegylated formulae of IFNλ can suppress the replication of the virus whereas preventing the development of a "cytokine storm" (Davidson *et al*., 2016; Galani *et al*., 207). IFNs activate T helper 1, cytotoxic T cell, and antibody responses, which are necessary for establishing a long-term immune response, rather than jeopardizing adaptive immunity (Koltsida *et al*., 2011; Ye *et al*., 2019). Thus, IFNλs cooperate with type I IFNs to optimize antiviral immunity for the best infection protection while minimizing the associated adverse effects.

COVID-19 was more frequent in those over 60 years old, especially in severe and critical types. Due to this, older people do not have the same robust immunological responses as younger people. Age-related changes have an impact on the host's immunity, which negatively impacts the host's capacity to fight diseases, including respiratory infections. Also, they are more frequent in males than females. Although the sample size of patients was relatively small (90 cases), the data suggested that the advanced age of the elderly can make them more susceptible to COVID-19. Previous studies have demonstrated that the severity and prognosis of COVID-19 are strongly influenced by the patient's age along with the length of hospitalization, with a higher risk of death being more common among people over 60 compared to younger adults (Speretta & Leite,2020; Starke *et al*., 2020; Al-Bayatee &Ad'hiah, 2021). This may be related to immunosenescence and cellular senescence, which can lead to diminished resistance to SARS-CoV-2 and increased systemic inflammation due to irreversible cell cycle arrest (Zhou *et al*., 2021). IFN expression was unaffected by age, according to research on the gene expression of IFNλ-I, IFNλ-II, and IFNλ-III in peripheral blood mononuclear cells, which did not find any age-

related variations. Furthermore, gender and chronic disease did not seem to have an impact on IFNλ folding.

Conclusion

The findings of this study demonstrated that an increased level of IFNλ mRNA in PBMCs of COVID-19 patients with severe symptoms, especially IFNλ-II, which exhibited significantly higher expression in PBMCs of COVID-19 patients with severe symptoms and in moderate cases than in other infection types. This Important and novel finding suggests that it may be possible for IFNλ especially IFNλ -II to prevent the disease from progressing to a severe or critical stage. Age, sex, or underlying diseases of patients had little effect on the differences in IFNλ expression.

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