Brief report

Dried blood spot proteome identifies subclinical interferon signature in neonates with type I interferonopathy

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Background: Type I interferonopathy is characterized by aberrant upregulation of type I interferon signaling. The mRNA interferon signature is a useful marker for activation of the interferon pathway and for diagnosis of type I interferonopathy; however, early diagnosis is challenging.

Objective: This study sought to identify the proteomic interferon signature in dried blood spot (DBS) samples. The aim was to evaluate the usefulness of the interferon signature for neonatal screening and to gain insight into presymptomatic state of neonates with inborn errors of immunity (IEIs).

Methods: DBS samples from healthy newborns/adults, patients with type I interferonopathy or other IEIs as well as from neonates with viral infections, including some samples obtained during the presymptomatic neonatal period, were examined by nontargeted proteome analyses. Expression of interferon-stimulated genes (ISGs) was evaluated and a DBS-interferon signature was defined. Differential expression/pathway analysis was also performed.

Results: The ISG products IFIT5, ISG15, and OAS2 were detected. Expression of IFIT5 and ISG15 was upregulated significantly in individuals with type I interferonopathy. We defined the sum of the z scores for these as the DBS-interferon signature, and found that patients with IEIs other than type I interferonopathy, such as chronic granulomatous disease (CGD), also showed significant elevation. Additionally, neonatal samples of type I interferonopathy and CGD patients showed high interferon signatures. Pathway analysis of neonatal CGD samples revealed upregulation of systemic lupus erythematosus–like pathways.

Conclusion: Upregulation of the interferon pathway exists already at birth—not only in neonates with type I interferonopathy but also in other IEIs, including CGD. (J Allergy Clin Immunol 2025;156:473-9.)

Key words: *Inborn errors of immunity, interferonopathy, signature, proteome, dried blood spot, CGD, WAS, newborn, neonate*

Abbreviations used
AGS: Aicardi-Goutières syndrome
BTK: Bruton tyrosine kinase
CGD: Chronic granulomatous disease
CHS: Chediak-Higashi syndrome
CMV: Cytomegalovirus
DADA2: Deficiency of adenosine deaminase 2
DBS: Dried blood spot
HA20: A20 haploinsufficiency
IEIs: Inborn errors of immunity
IFN-I: Type I interferon
ISG: Interferon-stimulated gene
NADPH: Nicotinamide adenine dinucleotide phosphate
PRAAS: Proteasome-associated autoinflammatory syndromes
ROS: Reactive oxygen species
SAAD: SAMD9L-associated autoinflammatory disease
SAVI: STING-associated vasculopathy with onset in infancy
SCID: Severe combined immunodeficiency
WAS: Wiskott-Aldrich syndrome

INTRODUCTION

Type I interferonopathy is an autoinflammatory syndrome characterized by excessive production of type I interferons (IFN-I).[1,2] Typically, it manifests during early infancy and follows a progressive course to cause permanent damage, although Janus kinase inhibitors appear to be an effective treatment.[3] However, early diagnosis, ideally before symptoms emerge, is necessary to minimize damage.

Analysis of expression of interferon-stimulated genes (ISGs)—that is, the interferon signature—at the mRNA level is useful for diagnosis and evaluation of disease activity in those with type I interferonopathy.[4,5] Excessive production of interferon has been reported in cases of inborn errors of immunity (IEIs) other than classical type I interferonopathy,[6] and we have shown that the interferon signature is useful for assessing pathophysiology in patients with unknown inflammatory conditions;[7] however, early diagnosis of type I interferonopathy remains challenging as a result of mRNA instability, which can cause difficulties with respect to sample collection and shipping; such restrictions preclude implementation of interferon signature analysis on a large scale.

Recently, we reported that proteomic analysis of dried blood spot (DBS) samples allows semiquantitative analysis of thousands of blood proteins.[8,9] DBS is used as part of national newborn screening programs around the world because it is inexpensive, dried blood is easy to handle, and sampling is minimally invasive.[10,11] DBS samples also provide a valuable resource that allows evaluation of a patient's central pathology soon after birth, with minimum interference by infection, secondary inflammation, and treatment regimens. This is important because the immune status, especially inflammatory status, of IEI patients can change rapidly after birth.

Here, we investigated whether levels of interferon-stimulated proteins in DBS samples could be evaluated and used to identify individuals with type I interferonopathies. We also analyzed DBS samples from patients with other IEI, which were also obtained soon after birth, to evaluate the activity of the interferon pathway and to gain insight into their immune conditions at the presymptomatic stage.

RESULTS AND DISCUSSION

In this report, we used a previously described method based on data-independent acquisition liquid chromatography–coupled mass spectrometry[8,9] to perform nontargeted proteome analysis of DBS samples from healthy controls (20 neonates and 6 adults), disease controls (congenital cytomegalovirus [CMV] infection, n = 5; neonatal herpes simplex virus infection, n = 1; and neonates with maternal anti-Ro/SSA antibodies, n = 3), patients with rheumatic diseases (systemic lupus erythematosus, n = 3; juvenile dermatomyositis, n = 1; and Sjögren syndrome, n = 1), and IEI patients with Aicardi-Goutières syndrome (AGS; n = 2), STING-associated vasculopathy with onset in infancy (SAVI; n = 3), proteasome-associated autoinflammatory syndromes (PRAAS; n = 2), deficiency of adenosine deaminase 2 (DADA2; n = 4), SAMD9L-associated autoinflammatory disease (SAAD; n = 1), A20 haploinsufficiency (HA20; n = 2), chronic granulomatous disease (CGD; n = 9), Wiskott-Aldrich syndrome (WAS; n = 9), Bruton tyrosine kinase (BTK) deficiency (n = 7), severe combined immunodeficiency (SCID: adenosine deaminase deficiency, n = 2; and X-linked SCID, n = 2), and Chediak-Higashi syndrome (CHS; n = 5). These samples included neonatal samples from 3 patients with CGD, 2 each with SAVI, PRAAS, HA20, and WAS, and 1 each with DADA2 and SAAD. The classification of IEIs by the International Union of Immunological Societies includes only a limited number of diseases (ie, AGS, SAVI, and DADA2) that are classified as type I interferonopathies;[12] however, because significant enhancement of interferon signaling is also observed in patients with diseases such as PRAAS, HA20, and SAAD, the applicable range is expanding.[1-3,13,14] In the present study, these IEIs were also included as type I interferonopathies.

First, to obtain the proteomic interferon signature from DBS samples, we compared postdiagnosis samples (obtained after disease onset) from 2 AGS patients with those obtained from 5 healthy newborns (Fig 1, *A*). Canonical pathway analysis of upregulated genes in AGS patients using Ingenuity Pathway Analysis (Qiagen, Hilden, Germany) revealed upregulation of IFN-α/β signaling pathways (Fig 1, *B*). These results were compatible with the pathophysiology of AGS.[1,2] Next, we focused on ISGs: 28 representative genes were reported as ISGs, including 6 genes belonging to the interferon signature.[4,5] Among the 28 ISGs, we detected expression of 3 genes in DBS samples, namely *IFIT5, ISG15,* and *OAS2*. The distribution of each of the 3 gene products is shown in a volcano plot comparing AGS patients with normal controls in Fig 1, *C*. To develop a quantitative index for the proteomic interferon signature, expression of the 3 proteins was normalized to that of β-actin (internal control);[9] the levels of IFIT5 and ISG15 were significantly higher in samples from individuals with type I interferonopathy (ie, AGS, SAVI, PRAAS, DADA2, and SAAD), while levels of *OAS2* did not differ significantly (Fig 1, *D*). Therefore, we defined the sum of the z scores for IFIT5 and ISG15 levels as the proteomic interferon signature—that is, the ISG score. The ISG scores for type I interferonopathy samples were significantly higher than those for the controls (neonatal control, mean score = 0.00; type I interferonopathy, mean score = 85.36; $P < .0001$; Fig 1, *E*). The same tendency was apparent when we compared the same samples with adult control samples, although the difference was not significant. In addition, we analyzed the mRNA interferon signature of

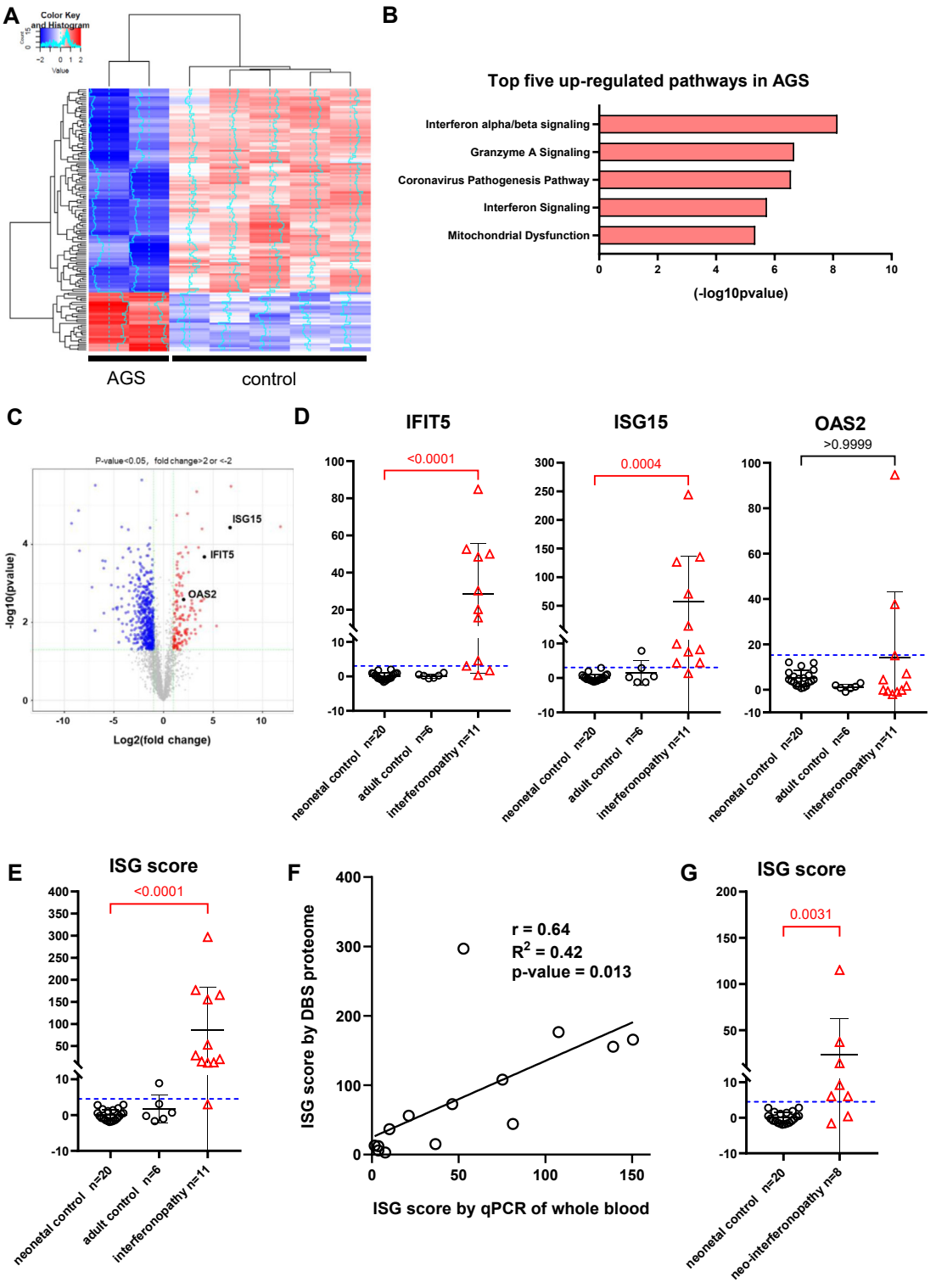

FIG 1. Comparison of AGS patients with controls and expression of ISGs in patients with type I interferonopathy. Comparison of 2 AGS patients with 5 neonatal controls **(A-C)**. Heat map *(A)*, top 5 upregulated canonical pathways in AGS *(B)*, and volcano plot showing distribution of ISGs *(C)*. Expression of ISGs in patients with type I interferonopathy (AGS, SAVI, PRAAS, DADA2, and SAAD) **(D-G)**. *Z* scores for IFIT5, ISG15, and OAS2 *(D)*. Sum of *z* scores for IFIT5 and ISG15 is defined as ISG score *(E)*. Correlation between ISG score for DBS proteome and results of real-time quantitative PCR *(F)*. ISG score for samples from neonates with type I interferonopathy (SAVI, PRAAS, DADA2, SAAD, and HA20) *(G)*.

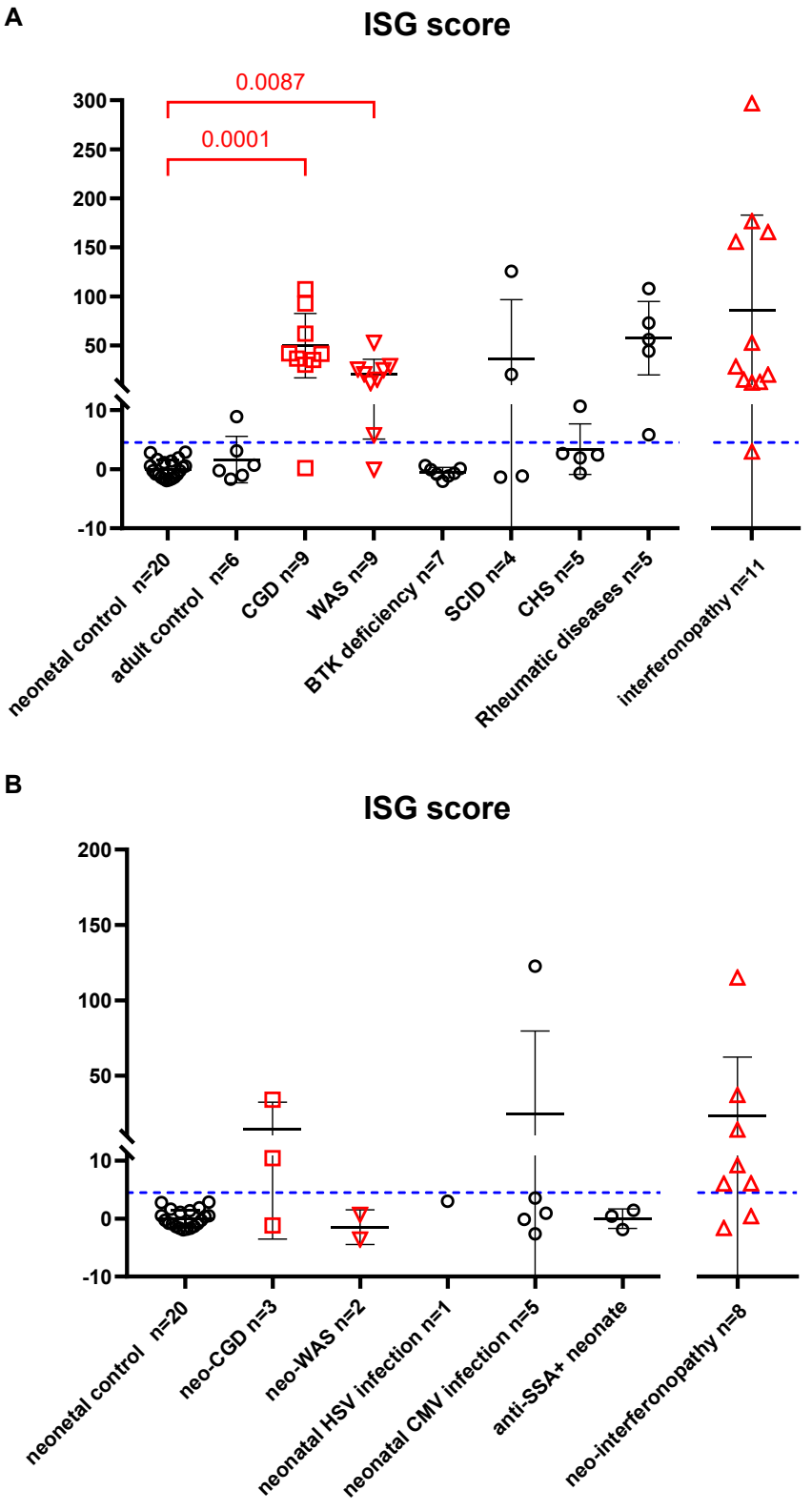

FIG 2. ISG score for various IEIs. ISG scores for other IEI, including CGD, WAS, BTK deficiency, SCID, and CHS. Patients with CGD and WAS have significantly higher values than normal controls **(A)**, and samples from neonates with CGD tend to have higher ISG scores **(B)**.

TABLE I. Samples used in this study

Characteristic	Total no. of samples	Sample type	No. with positive score	No. with negative score
Neonatal control	20		0	20
Adult control	6		1	5
Interferonopathy	11	AGS = 2; SAVI = 2; PRAAS = 2; DADA2 = 4; SAAD = 1	10	1 (DADA2 = 1)
CGD	9	(*CYBB* = 7; *CYBA* = 1; *NCF1* = 1)	8	1
WAS	9		8	1
BTK deficiency	7		0	7
SCID	4	ADA deficiency = 2; X-linked SCID = 2	1	3
CHS	5		0	5
Rheumatic diseases	5	SLE = 3, JDM = 1; SjS = 1	5	0
Neointerferonopathy	8	SAVI = 2; PRAAS = 2; DADA2 = 1; SAAD = 1; HA20 = 2	6	2 (SAVI = 1; HA20 = 1)
Neo-CGD	3	(*CYBB* = 2; *CYBA* = 1)	2	1
Neo-WAS	2		0	2
Neo-HSV infection	1		0	1
Neo-CMV infection	5		1	4
Anti-Ro/SSA^{+} neonate	3		0	3

Neonatal average cutoff, +3SD = 4.49.
ADA, Adenosine deaminase; *HSV,* herpes simplex virus; *JDM,* juvenile dermatomyositis; *neo-,* neonatal sample; *SD,* standard deviation; *SjS,* Sjögren syndrome; *SLE,* systemic lupus erythematosus.

several patients, including those with type I interferonopathy,[7] and found a significant positive correlation between the proteomic and mRNA ISG scores (Fig 1, *F*).

Type I interferonopathy often manifests as autoinflammatory symptoms that develop during early infancy; such symptoms include chilblains and recurrent fever. To evaluate whether the ISG score is upregulated at birth in patients with type I interferonopathy and whether it will be useful for early detection, we analyzed neonatal DBS samples that had been used for public newborn screening of metabolic disorders. Although the number of samples available was small (Fig 1, *G*), we found that the ISG scores for neonatal samples from patients with type I interferonopathy (ie, SAVI, PRAAS, DADA2, SAAD, and HA20) were significantly higher than those for healthy neonates (samples from neonates with type I interferonopathy, mean score = 23.35, $P = .0031$). This suggests that interferon signaling is upregulated at birth in patients with type I interferonopathy, demonstrating the usefulness of the DBS ISG score for neonatal screening of these disorders.

Some IEIs possess both immunodeficiency and inflammatory pathologies. CGD patients often exhibit autoinflammatory symptoms such as arthritis, inflammatory bowel disease, and nephritis;[15,16] WAS is complicated by various autoimmune phenotypes, including enhancement of IFN-I signaling and production of autoantibodies.[4,17] Some studies of CGD mouse models suggest a relationship between autoinflammatory features or upregulation of the IFN-I pathway along with decreased production of reactive oxygen species (ROS) production, which is associated with nicotinamide adenine dinucleotide phosphate (NADPH) dysfunction.[18-20] Little is known about the role of IFN-I in human CGD, although a few reports have shown enhanced interferon signaling at the mRNA level.[7,21] Therefore, we next analyzed the ISG score of IEI samples other than type I interferonopathies; these included CGD, WAS, BTK deficiency, SCID, and CHS (Fig 2, *A*). We found that patients with CGD and WAS also had significantly higher ISG scores than controls (CGD, mean score = 49.74, $P = .0001$; WAS, mean score = 20.51, $P = .0087$). Unexpectedly, 2 of 3 neonatal CGD samples showed a very high ISG score compared to neonatal controls (Fig 2, *B*). There was no apparent increase in the ISG score for samples obtained from controls, except for one individual with congenital CMV infection who experienced moderate hearing loss in the right ear from birth, who was diagnosed via a CMV-DNA–positive urine sample; however, the patient has not manifested any other apparent symptoms or features associated with congenital CMV infection at 5 months of age. Table I summarizes the sample set and the results of DBS-interferon signature analysis. These results indicate that increased interferon signaling occurs in CGD patients before they show infectious symptoms, suggesting that the DBS ISG score may identify patients with interferonopathy in a broad sense, including not only classical type I interferonopathy but also other IEIs with activation of the interferon pathway.

To gain further insight into the autoinflammatory mechanism of CGD, we compared expression of genes in neonatal CGD samples with that in healthy neonates. We found 119 upregulated and 61 downregulated differentially expressed genes (Fig 3, *A*). The results of canonical pathway analysis are shown in Fig 3, *B*. The downregulated differentially expressed genes in CGD patients included those related to ROS and IL-12 production pathways. These results seem compatible with CGD pathogenesis, which is associated with a defect in ROS production caused by loss of NADPH function.[22] Also, ROS production via NADPH is essential for production of IL-12.[23,24] In contrast, pathway analysis revealed upregulation of complement cascade, binding and uptake of ligands by scavenger receptors, acute phase response signaling, Fc-gamma receptor–dependent phagocytosis, and systemic lupus erythematosus in B-cell signaling pathways. Although direct upregulation of interferon pathways could not be detected, these results suggest that activation of interferon signaling, as in systemic lupus erythematosus, might potentially exist in CGD patients soon after birth.

In conclusion, we identified a proteomic interferon signature in DBS samples and showed its usefulness for early screening of type I interferonopathy. Combined with genetic analysis, the

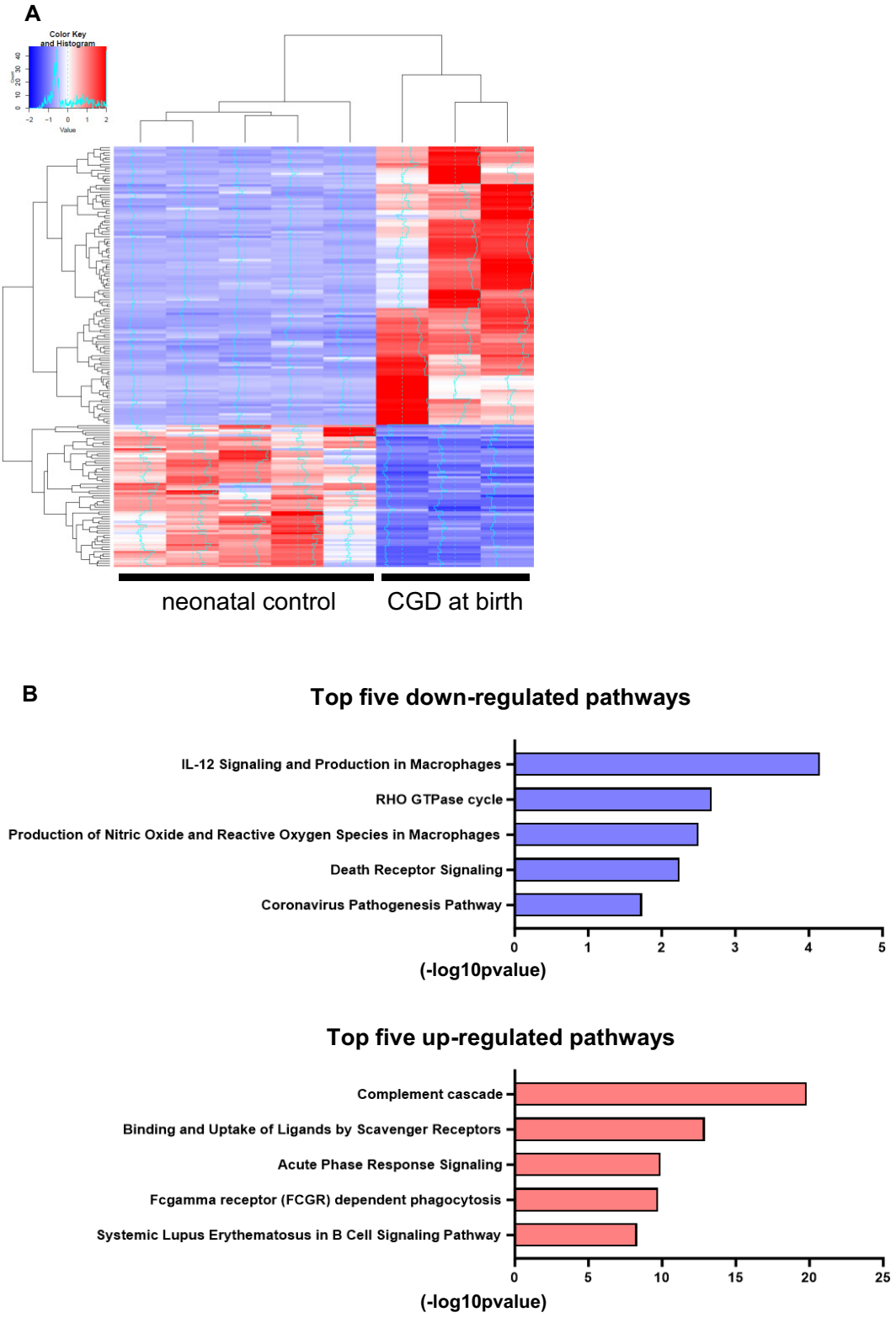

FIG 3. Gene expression analysis comparing neonatal CGD and neonatal control samples. Heat map showing expression in neonatal CGD samples and in neonatal controls **(A)**, and top 5 downregulated and upregulated canonical pathways in neonatal CGD samples **(B)**.

proteomic interferon signature may be helpful for diagnosis of not only type I interferonopathies but also for other IEIs associated with enhancement of the interferon pathway, including CGD, at an early stage. Like the mRNA interferon signature, the proteomic interferon signature might reflect disease activity in patients with type I interferonopathy; however, further analysis is needed. These data also suggest that differential expression analysis of the DBS proteome is useful for analysis of the

pathophysiology of IEIs at the neonatal stage, with little susceptibility to secondary modification.

DISCLOSURE STATEMENT

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Clinical implication: The DBS-interferon signature reflects upregulation of the interferon pathway, showing that patients with IEIs such as CGD other than type I interferonopathy also exhibit high ISG scores at birth.

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METHODS

Preparation and digestion of insoluble protein fraction

Soluble fractions were reduced and the proteins in DBS digested as previously described,[E1] with a slight modification. Briefly, a disc (3.2 mm diameter) punched from a DBS was suspended in 1 mL of Na_2CO_3 solution and shaken vigorously for 5 minutes at room temperature in the presence of a 5 mm zirconia bead (Tomy Seiko, Tokyo, Japan) using a Tissue Lyser (Qiagen) oscillating at 25 Hz. The remaining disc was removed by centrifugation (3,000 × *g,* 3 minutes, 4°C), the supernatant (approximately 850 μL) centrifuged (17,400 × *g,* 15 minutes, 4°C), and the precipitate resuspended in 200 μL of 100 mmol sodium carbonate aqueous solution by vigorous vortexing at room temperature. After centrifugation (17,400 × *g,* 5 minutes, 4°C), the supernatant was removed completely and the precipitate dissolved in 100 μL of 12 mmol sodium deoxycholate, 12 mmol *N*-lauroylsarcosine, and 100 mmol Tris-Cl pH 8.0 by vigorous vortexing for 1 minute, followed by sonication for 10 minutes using Bioruptor II (Cosmo-Bio, Tokyo, Japan) in high-power mode (cycles of 30 seconds on/30 seconds off). A 500 ng aliquot of trypsin/lys-C mix (Promega, Madison, Wis) was added to each protein extract, and the samples were digested overnight at 37°C. After precipitation, the detergent was removed by acidification with 30 μL 5% trifluoroacetic acid, followed by sonication with a Bioruptor II in high-power mode (5 minutes; 30 seconds on/30 seconds off). The mixture was then shaken for 5 minutes and centrifuged (15,000 × *g,* 5 minutes). The supernatant was desalted using GL-Tip SDB (GL Sciences, Tokyo, Japan) according to the manufacturer's instructions, then dried using a centrifugal evaporator (miVac Duo concentrator; Genevac, Ipswich, United Kingdom). Dried peptides were redissolved in 3% ACN/0.1% formic acid.

Liquid chromatography–tandem mass spectrometry

Digested peptides were injected directly onto a 75 μm × 20 cm PicoFrit emitter (New Objective, Woburn, Mass) packed in house with C_{18} core-shell particles (Capcell Core MP; 2.7 μm, 160 Å material; Osaka Soda, Osaka, Japan) at 50°C, and the peptides were separated using an UltiMate 3000 RSLCnano LC system (Thermo Fisher Scientific, Waltham, Mass), with an 80-minute gradient and a flow rate of 100 nL/min. Peptides eluted from the column were analyzed on an Orbitrap Exploris 480 Mass Spectrometer (Thermo Fisher Scientific) with overlapping window DIA-MS.[E2,E3] MS1 spectra were collected at 30,000 resolution in the range of 495 to 785 *m/z,* with an automatic gain control target of 3×10^6 and the maximum injection time set to auto. MS2 spectra were collected at 30,000 resolution in the range 200-1800 *m/z,* with an automatic gain control target of 3×10^6, the maximum injection time set to auto, and stepped normalized collision energies of 22%, 26%, and 30%. The MS2 isolation width was set to 4 *m/z,* and overlapping window patterns of 500-780 *m/z* were used for window placements optimized by Skyline v20.2.0.34 (University of Washington, Seattle).[E4]

MS data analysis

MS files were compared with human spectral libraries using Scaffold DIA v2.2.1 (Proteome Software, Portland, Ore). Human spectral libraries were generated from the human protein sequence database (UniProt ID UP000005640, reviewed, canonical) using Prosit.[E5,E6] The scaffold DIA search parameters were as follows: experimental data search enzyme, trypsin; maximum missed cleavage sites, 1; precursor mass tolerance, 9 ppm; and fragment mass tolerance, 9 ppm. The protein identification threshold was set at <1% for both peptides and protein false discovery rates. Proteins and peptides were quantified using the EncyclopeDIA algorithm in Scaffold DIA.[E7]

ISG scoring of DBS proteome

There are 28 representative interferon-related genes that are useful for evaluating the activity of the interferon pathway: *CXCL10, DDX60, EPST11, GBP1, HERC5, HERC6, IFI27, IFI44, IFI44L, IFI6, IFIT1, IFIT2, IFIT3, IFIT5, ISG15, LAMP3, LY6E, MX1, OAS1, OAS2, OAS3, OASL, RSAD2, RTP4, SIGLEC1, SOCS1, SPATS2L,* and *USP18*.[E8] Among these, the expression levels of 3 genes—*IFIT5, ISG15,* and *OAS2,* which can be measured by DBS proteome analysis—were normalized to the expression level of β-actin.[E9] The *z* score was calculated on the basis of 20 samples from healthy newborns, and the sum of the *z* scores for *IFIT5* and *ISG15* was defined as the ISG score.

ISG scoring by real-time quantitative PCR of whole blood

Total RNA was extracted from human blood samples collected in PAX gene Blood RNA tubes (PreAnalytix, Hombrechtikon, Switzerland), and real-time quantitative PCR was performed with TaqMan Gene Expression Master Mix and probes, and a Step One Plus Real Time PCR system as previously described. The expression levels of each gene were measured in triplicate and normalized to the level of β-actin. The results are expressed relative to a single control calibrator. The median relative quantity of 6 ISGs (*SIGLEC1, IFI27, RSAD2, ISG15, IFIT1,* and *IFI44L*) was defined as the ISG score for each patient.[E10]

Statistical analysis

The levels of expression of genes identified in the DBS proteome were normalized by Perseus software platform (maxquant.net/perseus/). Differentially expressed genes between 2 groups were selected as those with a $P < .01$ and fold change ≥4 cutoff, and pathway analysis was performed by Ingenuity Pathway Analysis with these differentially expressed genes. Statistical differences among 3 or more groups were analyzed by 1-way ANOVA on ranks (ie, Kruskal-Wallis test), followed by Dunn multiple comparisons test. Significant differences between 2 groups were assessed by the Mann-Whitney test. Correlation analysis was performed by simple linear regression. Statistical significance was defined as a $P < .05$ by GraphPad Prism v10.2.3 software (GraphPad Software, La Jolla, Calif).