NFKB2 haploinsufficiency identified via screening for IFNα2 autoantibodies in children and adolescents hospitalized with SARS-CoV-2-related complications

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ABSTRACT

Background. Autoantibodies against type I interferons (IFNs) occur in approximately 10% of adults with life-threatening COVID-19. The frequency of anti-IFN autoantibodies in children with severe sequelae of SARS-CoV-2 infection is unknown.

Objective. To quantify anti-Type I IFN autoantibodies in a multi-center cohort of children with severe COVID-19, Multisystem Inflammatory Syndrome in Children (MIS-C), and mild SARS-CoV-2 infections.

Methods. Circulating anti-IFNa2 antibodies were measured by a radioligand binding assay. Whole exome sequencing (WES), RNA-sequencing, and functional studies of peripheral blood mononuclear cells were used to study any patients with levels of anti-IFNα2 autoantibodies exceeding the assay's positive control.

Results. Among 168 patients with severe COVID-19, 199 with MIS-C, and 45 with mild SARS-CoV-2 infections, only one had high levels of anti-IFNα2 antibodies. Anti-IFNα2 autoantibodies were not detected in patients treated with intravenous immunoglobulin prior to sample collection. WES identified a missense variant in the ankyrin domain of *NFKB2*, encoding the p100 subunit of NF-kB essential for non-canonical NF-kB signaling. Her peripheral blood mononuclear cells exhibited impaired cleavage of p100 characteristic of NFKB2 haploinsufficiency, an inborn error of immunity with a high prevalence of autoimmunity.

Conclusions. High levels of anti-IFNα2 autoantibodies in children and adolescents with MIS-C, severe COVID-19, and mild SARS-CoV-2 infections are rare, but can occur in patients with inborn errors of immunity.

Clinical implications. Anti-IFNα2 autoantibodies should prompt diagnostic evaluation for inborn errors of immunity if identified in children or adolescents.

Capsule Summary. In contrast to studies of adults with COVID-19, this multicenter study of 412 pediatric patients with severe COVID-19, MIS-C, or mild SARS-CoV-2 infections shows that anti-IFNα2 autoantibodies are unlikely to cause severe COVID-19 in the general pediatric population, but can be associated with an inborn error of immunity.

Key words: anti-interferon autoantibody, COVID-19, MIS-C, NFKB2, inborn errors of immunity

Abbreviations: IFN, interferons; COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; Multisystem Inflammatory Syndrome in Children, MIS-C; WES, whole exome sequencing; NF-κB, nuclear factor kappa B; APS-1, autoimmune polyendocrine syndrome type 1.

Introduction

Neutralizing autoantibodies against Type I IFNs occur in approximately 10% of adults with life-threatening coronavirus disease-2019 (COVID-19).[1,2] Less is known about levels of anti-IFN antibodies in the pediatric populations. Small studies of seven to 59 children identified autoantibodies to several tissue antigens in patients with Multisystem Inflammatory Syndrome in Children (MIS-C), a post-infectious inflammatory disorder typically occurring within two to six weeks of SARS-CoV-2 infection.[3–6] Although inborn errors of immunity can be associated with autoantibodies,[7] it is unknown if anti-IFN autoantibodies are associated with severe COVID-19 or MIS-C in the general pediatric population. Treatment of patients with MIS-C with intravenous immunoglobulin (IVIG), which can contain autoantibodies to tissue antigens, has been thought to confound the measurement of endogenous autoantibodies.[8] Here, we present results from a multicenter study investigating anti-IFNα2 autoantibodies in a large cohort of children and adolescents with MIS-C, severe COVID-19, or mild SARS-CoV-2 infections.

Results and Discussion

This study included 412 patients: 199 patients with MIS-C, 168 patients hospitalized for COVID-19 in an intensive care or step-down unit (henceforth referred to as severe COVID-19), and 45 with SARS-CoV-2 infections managed as outpatients. All but two patients were under 21 years of age (**Table 1**). IVIG was administered to 137 (68.8%) patients with MIS-C and to nine (5%) with severe COVID-19 prior to specimen collection. Critical care was required for 85.4% of patients with MIS-C and 69.6% of patients with severe COVID-19 (**Table 1**). Five (3%) patients hospitalized for COVID-19 died; all with MIS-C survived.

Anti-IFNα2 autoantibodies were measured using an established radioligand binding assay (methods in the online repository at www.jacionline.org). The antibody index indicates the ratio of IFNα2 protein precipitated by patient plasma normalized to the assay's positive control.[2]

Plasma from eight healthy adults served as negative controls (mean antibody index of 0.011). Positive disease controls included six individuals with anti-IFNα2 autoantibodies due to autoimmune polyglandular syndrome type 1 (APS-1), a disease known to cause neutralizing anti-Type I IFN autoantibodies.[9,10] In our cohort, only one patient had an antibody index (3.73) exceeding that of the assay's positive control and the levels previously found in adults with severe COVID-19 due to neutralizing anti-IFNα2 antibodies[2] (**Fig. 1**). This patient's sample was obtained prior to treatment with IVIG. Additionally, none of the other 137 patients who received IVIG prior to specimen collection had high levels of anti-IFNα2 autoantibody, thus excluding IVIG as a confounding source of autoantibodies. Our study was not designed to quantify the incidence of anti-IFNα2 autoantibodies in children with COVID-19 or MIS-C, as not all eligible children consented to enrollment. However, the largest study of anti-IFN autoantibodies in adults identified anti-IFNα2 autoantibodies in 88 out of 987 patients (8.9%) with severe COVID-19,[1] which is higher than the 0.5% (n=1) with autoantibodies and severe COVID-19 in our cohort.

The patient with high levels of anti-IFNa2 autoantibodies was an adolescent female with acute hypoxemic respiratory failure due to severe COVID-19. Neutralizing antibodies to SARS-CoV-2 were not detected until the fifth day of hospitalization, rising to levels comparable to that of other patients with severe COVID-19 by day 12.[11] Her respiratory failure resolved after two weeks of hospitalization, but she subsequently developed left ventricular dysfunction treated with milrinone in the setting of persistently elevated inflammatory markers, prompting diagnostic consideration of MIS-C. Compared to other patients similarly treated for MIS-C, the patient's peripheral blood mononuclear cells exhibited reduced expression of differentially expressed genes in pathways of neutrophil degranulation, innate immune signaling, SARS-CoV-2 IFN-stimulated genes, and oncostatin M, an enhancer of Type I IFN signaling (**Fig. 2A**). Reduced IFN signaling is found in adults with acute COVID-19, particularly those with anti-Type I

interferon autoantibodies.[1,2] However, the reduced neutrophil degranulation and innate immune signaling in our patient contrasts with that of neutrophil and monocyte activation found in adults with severe COVID-19[6,12,13] and children with MIS-C,[4,14] suggesting additional factors beyond anti-IFNα2 autoantibodies contributing to her immune dysregulation.

In addition to prolonged hospitalization for COVID-19 followed by MIS-C, this patient had a history of influenza A pandemic H1N1/09 viral pneumonia requiring non-invasive ventilation. Immunologic evaluation after recovery from that hospitalization was notable only for reduced levels of IgG and IgA, although titers to tetanus and pneumococcus were normal (**Table E1** in the online repository at www.jacionline.org). Genetic testing and immunoglobulin replacement were not initiated then as she had no prior significant infections. Immunologic evaluation after recovery from COVID-19 and MIS-C in 2020 revealed panhypogammaglobulinemia, with reduced titers to pneumococcal subtypes. Whole exome sequencing identified a heterozygous variant in the ankyrin domain of *NFKB2* (p.Thr684Pro), encoding the p100 subunit of NF-κB essential for non-canonical NF-kB signaling. This variant is absent from the gnomAD database and is predicted to be pathogenic with a CADD score of 27.6. Structural modeling indicates that the Thr684Pro variant causes steric clash (**Fig. 2B**). After stimulation with anti-CD3 and CD28 antibodies, the patients peripheral blood mononuclear cells exhibited increased p100 levels and reduced p52 levels, indicative of impaired cleavage of p100 into its active form (**Fig. 2C**). This finding is consistent with the importance of the ankyrin domain for p100 ubiquitinylation and cleavage.[15] All previously published missense mutations affect the protein's C-terminal degron domain essential for p100 processing.[16,17] The patient's pan-hypogammaglobulinemia, anti-IFNα2 autoantibodies, and susceptibility to severe viral infections indicate the deleterious effect of her NFKB2^{Thr683Pro} variant. Her residual levels of p52 likely contributed to the sporadic nature of her infections, which emerged only when exposed to newly emerged pathogens during two

pandemics. Similarly, all three previously reported patients with NFKB2 haploinsufficiency and COVID-19 required critical care.[7,18,19]

Autoimmunity, including anti-cytokine antibodies, occurs in up to 80% of patients with NFKB2 haploinsufficiency.[17] Although we did not measure the neutralizing capacity of our patient's anti-IFNα2 autoantibodies, her antibody index exceeds that of autoantibodies with neutralizing capacity in published studies using the same assay in independent cohorts.[2,20] Additionally, differentially expressed genes from her whole blood transcriptome were reduced in pathways downstream of Type I IFN signaling compared to other patients with MIS-C, further supporting a neutralizing effect of her anti-IFNα2 autoantibodies. At the time of this study, she had no clinical evidence of autoimmunity beyond the anti-IFNα2 autoantibodies. As our study is limited by the identification of only one patient with NFKB2 haploinsufficiency, future studies with larger cohorts are needed to determine the prevalence and levels of anti-IFN autoantibodies in patients with this disease.

Our study underscores the rarity of high levels of anti-IFNα2 antibodies in most children. While the majority of adults with severe COVID-19 and autoantibodies to Type 1 IFNs have anti-IFNα2 autoantibodies, Bastard et al. have shown that a subset of individuals have only anti-IFN-ω antibodies,[1] for which we did not screen. In samples collected prior to the COVID-19 pandemic, neutralizing autoantibodies to IFN-α were identified in less than 0.3% of individuals younger than 69 years of age,[1,21] compared to 1.1% of adults aged 70 through 79 years and 3.4% in those over 80 years of age.[21] Neutralizing autoantibodies to IFN occur in 10% of individuals with severe COVID-19, the majority of whom were over 75 years of age. Other than APS-1, no inborn errors of immunity, including defects in the NFKB2 pathway, have been reported in adults with severe COVID-19 and autoantibodies to Type I IFNs. Autoantibodies occur more frequently in the elderly due to progressive B cell dysfunction, differentiation of age-associated B cells into autoantibody-producing plasma cells, and the release of self-antigens

from tissue damage. In children, anti-IFN autoantibodies may instead reflect early onset B cell dysfunction with underlying immune dysfunction. In support of this, a recently published study of 31 individuals with known inborn errors of immunity identified neutralizing autoantibodies against IFNα2 and IFN-ω in one child with a combined immunodeficiency and another with immune dysregulation.[7] This previously published cohort had three additional pediatric patients with anti-nuclear antibodies, reflecting the spectrum of autoantibodies in patients with inborn errors of immunity. Thus, diagnostic studies for genetic causes of immune dysregulation are merited in children with anti-cytokine antibodies.

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Figure Legends

Figure 1. High levels of anti-IFNα2 autoantibodies in children and adolescents with MIS-C, severe COVID-19, and mild SARS-CoV-2 infections are rare. Levels of anti-IFNα2 measured by radioligand binding assay. The dotted line represents the antibody index of the anti-Myc assay positive control. P denotes the patient with high levels of anti-IFNa2 autoantibodies. APS-1, autoimmune polyendocrine syndrome Type 1, used as positive disease controls. Median and interquartile ranges are shown for the controls (n= 8) and APS-1 (n=6) cohorts.

Figure 2. **A**. Differentially expressed genes ($\geq$2-fold change, false discovery rate [FDR] <0.05) determined via bulk RNA-sequencing of whole blood from the patient with increased levels of anti-IFNa2 autoantibodies, compared to five disease controls (patients with MIS-C who had comparable ages, disease severity, and treatment). The patient and one of the disease controls (labeled 2) had two samples available at the midpoint of their hospitalizations. **B.** Top, schematic of NFKB2 with the patient's variant indicated with a red triangle in ankyrin repeat domain 6 (ANK6). RHD, Rel homology domain; DD, death domain with the degron domain needed for p100 processing noted in orange. Bottom, steric clash (indicated by red disks) is predicted to arise from the patient's substitution of a bulky proline residue for threonine 684. **C**. Representative immunoblot of full-length p100 and processed p52 in peripheral blood mononuclear cells from the patient (P) and two healthy controls (C1 and C2) with and without anti-CD3 stimulation for two days. Bar graphs show densitometric quantitation of indicated

proteins pooled from two experiments in which PBMCs from the patient and four controls, with and without two days of anti-CD3 stimulation. *p<0.05, **p<0.01 by Student's t test.

Table 1. Patient characteristics of children with MIS-C, severe COVID-19 requiring ICU or step-down unit hospital care, and outpatients with mild SARS-CoV-2 infections evaluated for anti-IFNa2 autoantibodies.

	MIS-C n=199	**Severe COVID-19** n=168	**Mild SARS-CoV-2 infections** n=45
Male sex (%)	118 (59.3)	83 (49.4)	24 (53.3)
Median age in years (IQR)	10.9 (7.5 – 14.7)	13.6 (6.3 – 17.2)	5.5 (2.2 – 11.1)
Race and ethnicity (%)			
White, non-Hispanic	63 (31.7)	62 (36.9)	14 (31.1)
Black, non-Hispanic	74 (37.2)	41 (24.4)	5 (11.1)
Hispanic or Latino	47 (23.6)	46 (27.4)	15 (33.3)
Other race, non-Hispanic	8 (4.0)	14 (8.3)	2 (4.4)
Unknown	7 (3.5)	5 (3.0)	9 (20.0)
Previously healthy (%)	134 (67.3)	48 (28.6)	42 (93)[1]
Pre-existing conditions (%)			
Obesity[2]	29 (14.6)	50 (29.8)	8 (17.8)
Asthma	22 (11.1)	34 (20.2)	1 (2.2)
Cardiovascular	4 (2.0)[3]	15 (8.9)[4]	0
Interventions (%)			
ICU admission	170 (85.4)	117 (69.6)	0
Shock requiring vasopressors	90 (45.2)	16 (9.5)	0
Mechanical ventilation	38 (19.1)	60 (35.7)	0

[1]Among the outpatients, one had asthma requiring inhaled steroids, one had sickle cell trait, and one had a seizure disorder.
[2]Because height was not available for many outpatients, weight-for-age of >95% percentile for age was used as a proxy to assign potential obesity status in lieu of body mass index.
[3]Congenital heart disease (n=4)
[4]Congenital heart disease (n=7), systemic hypertension (n=6), acquired heart disease (n=2)

Figure 1

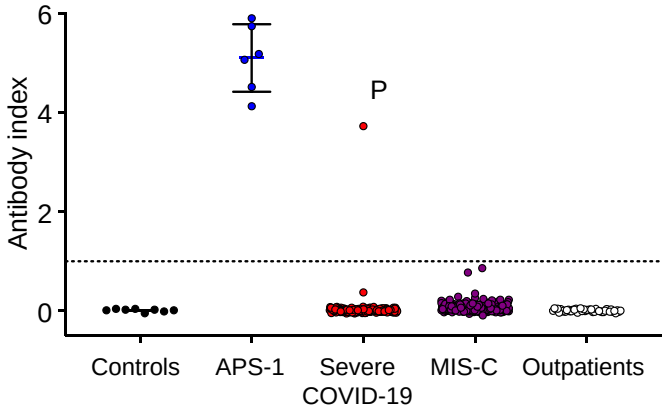
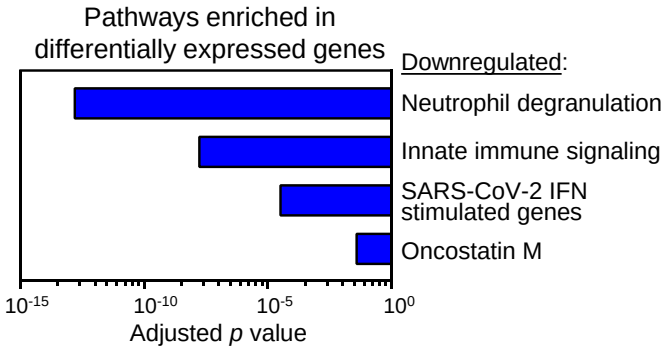
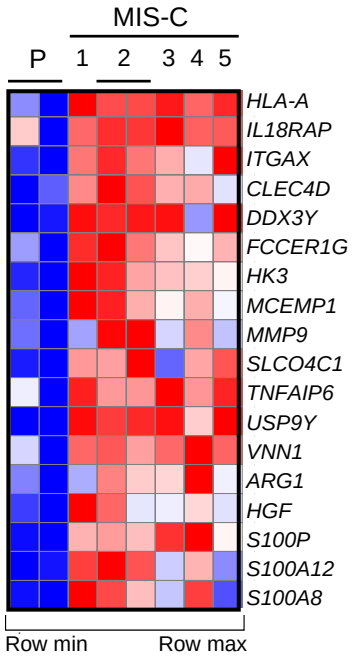

6
4
2
0
Antibody index
P
Controls
APS-1
Severe COVID-19
MIS-C
Outpatients

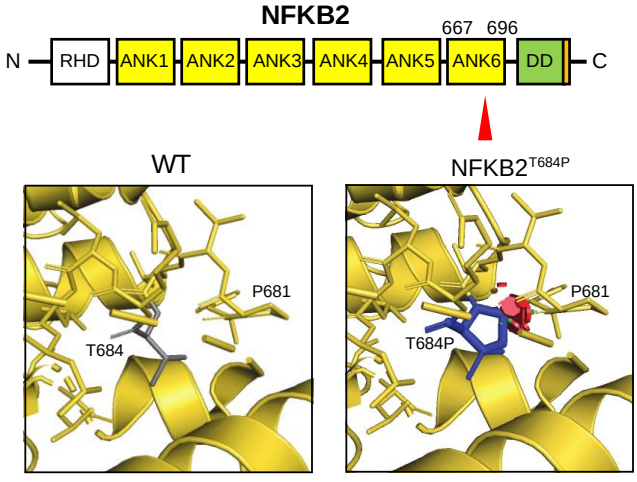
Figure 2

A.

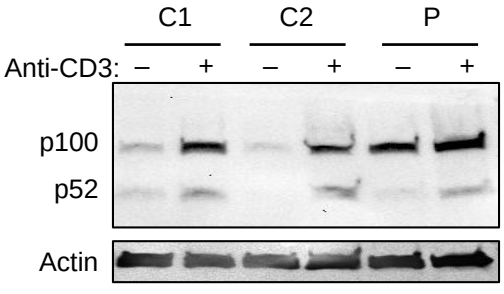

Pathways enriched in differentially expressed genes
Downregulated:
Neutrophil degranulation
Innate immune signaling
SARS-CoV-2 IFN stimulated genes
Oncostatin M
10-15
10-10
10-5
100
Adjusted p value


MIS-C
P 1 2 3 4 5
HLA-A
IL18RAP
ITGAX
CLEC4D
DDX3Y
FCCER1G
HK3
MCEMP1
MMP9
SLCO4C1
TNFAIP6
USP9Y
VNN1
ARG1
HGF
S100P
S100A12
S100A8
Row min
Row max

B.


NFKB2
667 696
N
RHD
ANK1
ANK2
ANK3
ANK4
ANK5
ANK6
DD
C
WT
NFKB2T684P
P681
T684
P681
T684P

C.


C1
C2
P
Anti-CD3: – + – + – +
p100
p52
Actin

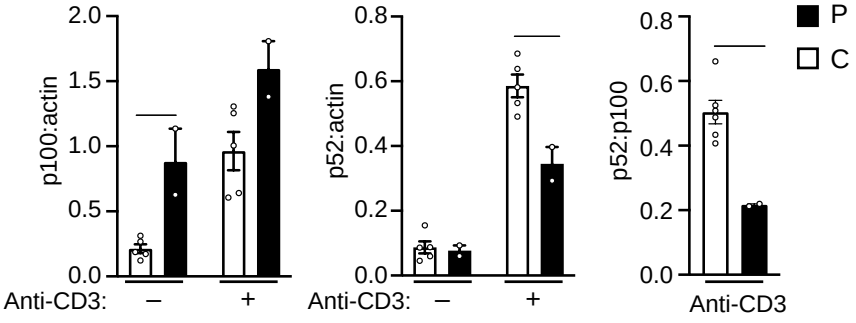

p100:actin
2.0
1.5
1.0
0.5
0.0
Anti-CD3: – +
p52:actin
0.8
0.6
0.4
0.2
0.0
Anti-CD3: – +
p52:p100
0.8
0.6
0.4
0.2
0.0
Anti-CD3
P
C

Table E1. Immunologic evaluations in the patient with *NFKB2*^{Thr684Pro}. This patient had two immunologic evaluations: after recovery from influenza A(H1N1)pdm09 virus pneumonia in early childhood and after recovery from SARS-CoV-2 during adolescence. The bolded values represent clinically relevant values outside the reference ranges shown in parentheses.

	Patient's approximate age group at the time of laboratory testing	
	Young childhood after recovery from influenza A(H1N1)pdm09 virus pneumonia (reference range)†	Older adolescence after recovery from severe COVID-19 (reference range)†
Hemogram		
White blood cells	11.28 (5.41 – 9.7)	7.78 (5.52 – 9.29)
Neutrophils	5.31 (2.58 – 5.95)	3.51 (3.04 – 6.06)
Lymphocytes	4.92 (1.23 – 2.76)	3.09 **(**1.17 – 3.10)
Platelets	406 (187 – 376)	384 (189 – 342)
Lymphocyte subsets		
$CD3^+$, 10^3 cells/µL	3879 (1000 – 2600)	3166 (1000 – 2600)
$CD3^+CD4^+$, 10^3 cells/µL	2070 (225 – 1100)	1649 (530 – 1500)
$CD3^+CD8^+$, 10^3 cells/µL	1616 (3330 – 1100)	1367 (330 – 1100)
$CD19^+$, 10^3 cells/µL	705 (270 – 860)	251 (110 – 570)
Naïve, % $CD19^+$	74.0 (48.4 – 79.7)	72.3 (48.4 – 79.7)
Unswitched memory, % $CD19^+$	10.4 (7.0 – 23.8)	**6.6** (7.0 – 23.8)
Switched memory, % $CD19^+$	12.4 (8.30 – 27.8)	16.7 (8.30 – 27.8)
Plasmablasts	Not done	0.3 (0.1 – 2.4)
Marginal zone-like B cells, %$CD19^+$	Not done	19.6 (11.8 – 59.7)
$CD3^-CD56^+$, 10^3 cells/µL	591 (70 – 480)	**62 (**70 – 480)
Immunoglobulins		
IgG	**501** (639 – 1434)	**369 (**639 – 1344)
IgM	182 (40 – 240)	**28 (**40 – 240)
IgA	**61** (70 – 312)	**24 (**70 – 312)
Vaccine titers		
Positive titers to pneumococcal subtypes	13 out of 14 (>7)	5 out of 23 (>14)
Tetanus	>7.0 (>0.15)	0.44 (>0.15)

#Naïve, $CD19^+CD27^-IgD^+$, unswitched memory $CD19^+CD27^+IgD^+$, switched memory $CD19^+CD27^+IgD^-$, plasmablast $CD19^+CD24^{lo}CD38^{hi}$.

†Reference ranges are matched the patient's age group and are derived from healthy controls, as determined by the Clinical Immunology Laboratory at Boston Children's Hospital.

Supplementary Methods

Study design and subjects. Patients were recruited through the prospectively enrolling multicenter Overcoming COVID-19 Study in the United States.[1,2] A total of 412 patients were enrolled in one of the following independent cohorts: 199 patients hospitalized with MIS-C, 168 patients hospitalized for COVID-19 in either an intensive care or step-down unit (referred to as severe COVID-19 in this study), and 45 outpatients with SARS-CoV-2 infections associated with mild or no symptoms. The demographic and clinical data are summarized in Table 1. U.S. Centers for Disease Control and Prevention (CDC) case definitions were used to define MIS-C;[3] those with acute COVID-19 had a positive antigen test or nucleic acid amplification test.[4] All patients with MIS-C had positive SARS-CoV-2 serologies and/or positive SARS-CoV-2 testing by reverse transcriptase quantitative PCR (RT-qPCR). All patients with severe COVID-19 or outpatient SARS-CoV2 infections had positive RT-qPCR testing for SARS-CoV-2. For outpatients, samples were collected from 36 to 190 days after the positive RT-qPCR test (median: 70 days after positive test; IQR 56 – 81).

To maintain deidentification of clinical data from the patient with the NFKB2^{Thr684Pro} variant, the approximate age, rather than exact age, is provided. Informed consent was provided by participants or legal guardians. All protocols were approved by the Institutional Review Board at Boston Children's Hospital, which served as the single IRB for the study (IRB-P00033157). Autoimmune Polyglandular Syndrome Type 1 (APS1) positive control samples were previously published and collected as described in Ferre et al.[5] All patients with APS1 were enrolled in research study protocols approved by the NIAID, NIH Clinical Center, and NCI Institutional Review Board Committee and provided with written informed consent for study participation. All patients recruited at the NIH gave passive consent for use of their medical record for research purposes, thus allowing eligible participants to opt-out of study inclusion (protocol #11-I-0187). Healthy, pre-COVID-19 control plasma were obtained from the New York Blood Center, where they were collected under informed consent, including usage for research.

Anti-IFNa2 antibody radioligand binding assay. A sequence-verified plasmid encoding the IFNA2 cDNA sequence with a Flag-Myc tag (Origene#RC221091) was used as template in T7-promoter-based *in vitro* transcription/translation reactions (Promega, Madison, WI: #L1170) with ^{35}S-methionine (Perkin Elmer, Waltham, MA; #NEG709A). IFNA2 protein was purified via Nap-5 columns (GE Healthcare, Chicago, IL; #17–0853-01), incubated with 2.5 µL of study participant plasma or 1 µL of anti-myc positive control antibody (CellSignaling, Danvers, MA; #2272), followed by immunoprecipitation with Sephadex protein A/G beads (Sigma Aldrich, St. Louis, MO; #GE17-5280–02 and #GE17-0618–05, 4:1 ratio) in 96-well polyvinylidene difluoride filtration plates (Corning, Corning, NY; #EK-680860). A Microbeta Trilux liquid scintillation plate reader (Perkin Elmer) was used to measure the radioactive counts (cpm) of immunoprecipitated protein samples. The antibody index was calculated as follows: (sample cpm value – mean blank cpm value)/(positive control antibody cpm value – mean blank cpm value).

Whole exome sequencing. Whole exome sequencing and candidate variant analysis was done as previously described.[6]

Bulk RNA-sequencing. Whole blood was collected in PAXgene tubes (Qiagen, Germantown, MD). mRNA was extracted using the PAXgene blood RNA Kit (Qiagen, Germantown, MD), followed by globin mRNA depletion and polyA capture. Barcoded non-directional libraries were sequenced using the Illumina NovoSeq platform, generating paired-end 150 bp reads. Differential gene expression analysis was performed using Partek Flow software (Partek, Chesterfield, MO).

Assessment of NFKB2 activation. 1 x 10^6 peripheral blood mononuclear cells, corresponding to 120 µg of protein, were stimulated with anti-CD3 (clone OKT3, ThermoFisher Scientific) and anti-CD28 (clone 28.2, ThermoFisher Scientific) for two days, then lysed, transferred to nitrocellulose membranes and immunoblotted with using antibodies against p100/p52 (CellSignaling, Danvers, MA, catalogue #4882) and b-actin (CellSignaling, Danvers,

MA, clone 13E5), both used at 1:1000 dilution in 5% milk. Images were acquired on an iBright Imager (Thermo Fisher Scientific) and quantified using ImageJ2.[7]

Statistical analysis. Pathway analysis was done using Ingenuity Pathway Analysis (Qiagen, Germantown, MD) on differentially expressed genes with a fold change of at least two-fold and false discovery rate of less than 0.05.

References for online methods

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6. Platt CD, Zaman F, Bainter W, Stafstrom K, Almutairi A, Reigle M, et al. Efficacy and economics of targeted panel versus whole exome sequencing in 878 patients with suspected primary immunodeficiency. J Allergy Clin Immunol. 2021; 147(2):723-726.

7. Rueden CT, Schindelin J, Hiner MC, DeZonia BE, Walter AE, Arena ET, et al. ImageJ2: ImageJ for the next generation of scientific image data. BMC Bioinformatics. 2017;18:529.