Supplemental data to
Inherited human IFN-γ deficiency underlies mycobacterial disease

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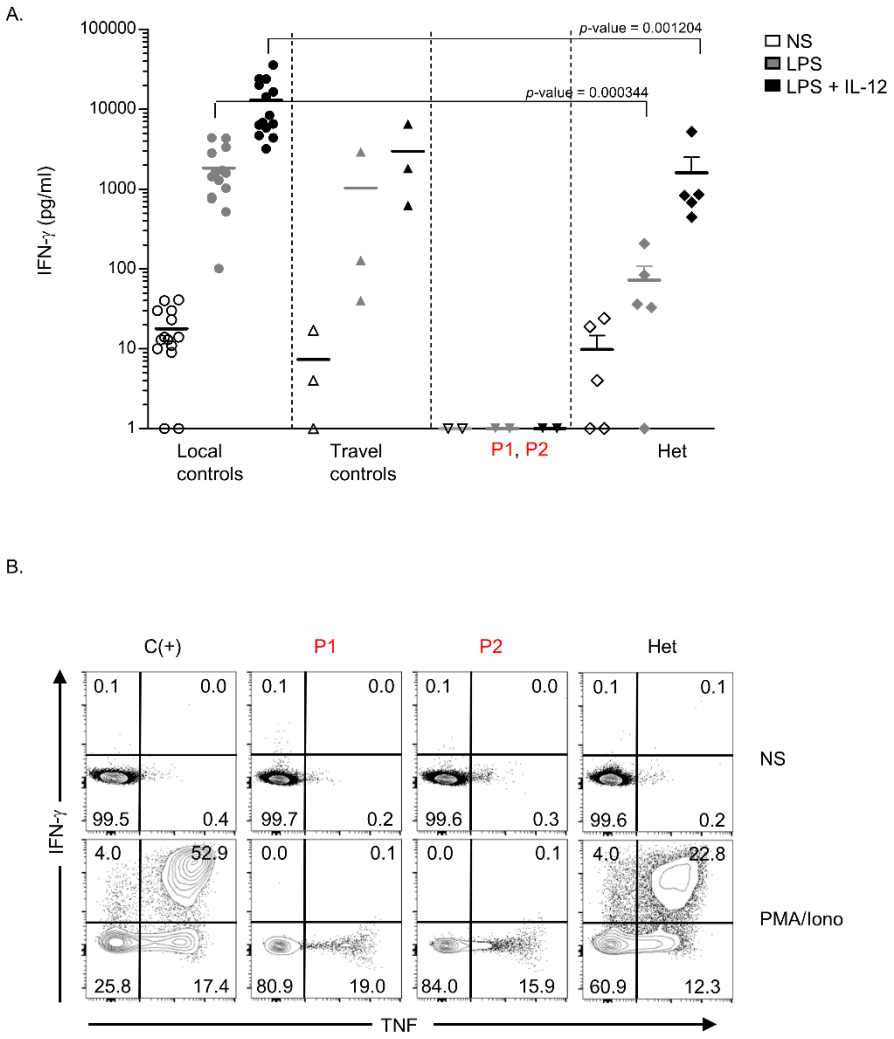
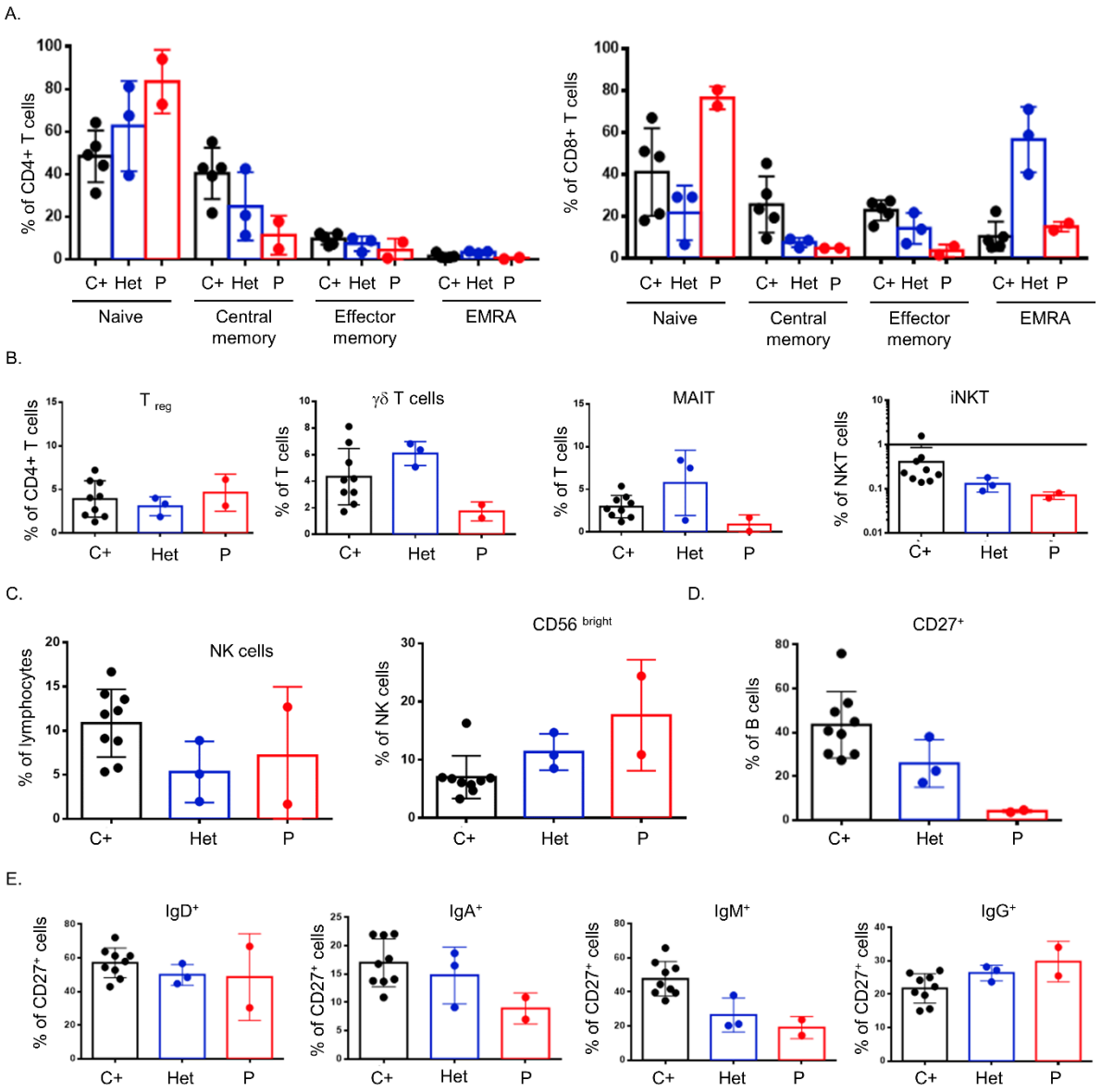
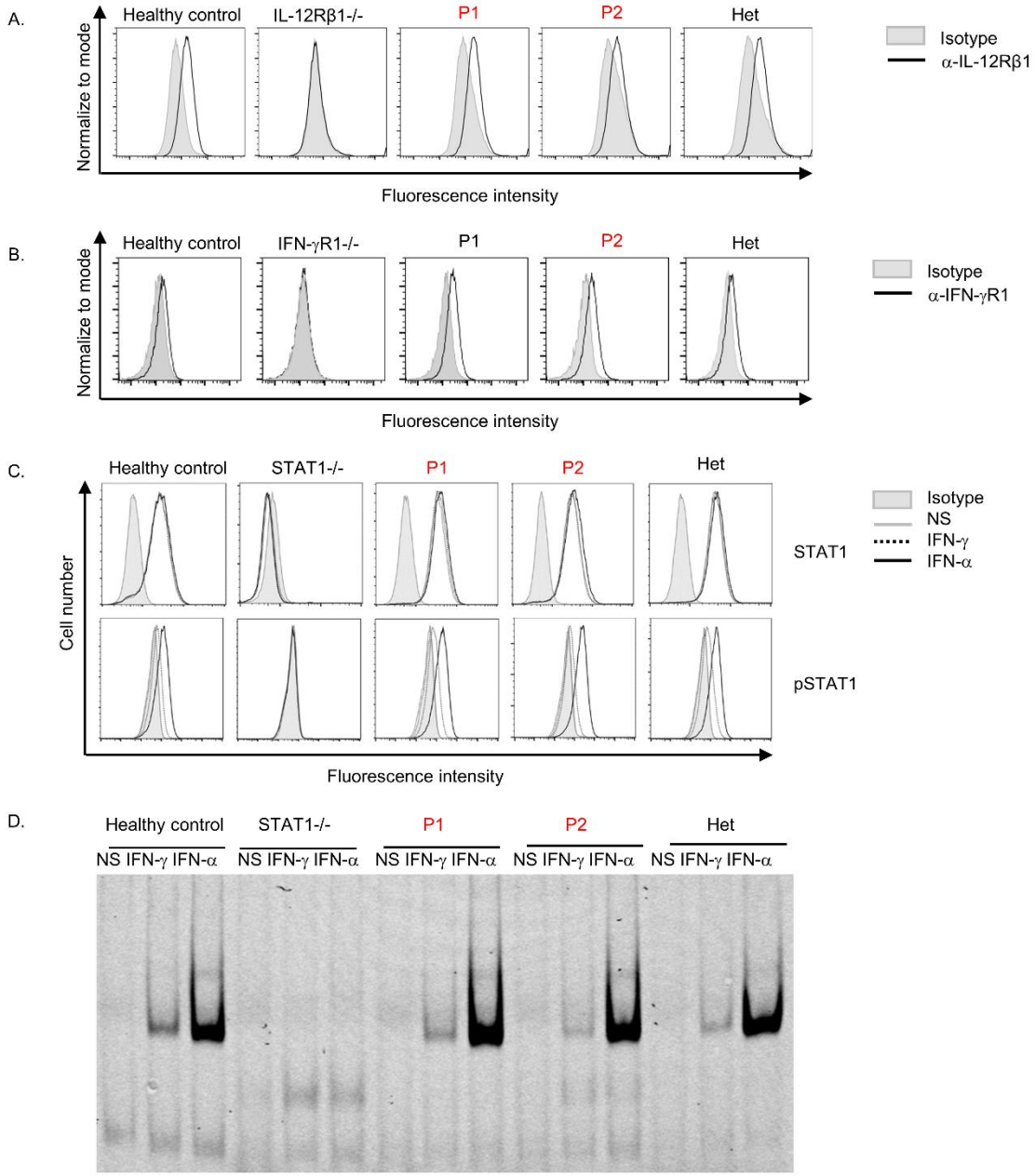


Supplemental Figure 1. Abolition of the production and secretion of IFN-γ by the cells of IFN-γ-deficient patients. A. Induction of IFN-γ secretion in a whole-blood assay for local controls, travel controls, patients (P1 and P2) and heterozygous relatives, following stimulation with live LPS alone or in combination with IL-12 (20 ng/ml). The nonparametric Wilcoxon test was used to calculate p-value between heterozygous relatives and controls (local and travel). **B.** IFN-γ production by PHA-activated T cells from P1 and P2, one heterozygous relative and one healthy travel donor in the absence of stimulation (NS), or following stimulation with phorbol myristate acetate-ionomycin (PMA/Iono).



Supplemental Figure 2. Immunophenotyping of subpopulations of T, B and NK cells in IFN-γ deficient patients. A. Frequency of naïve ($CD45RA^+CCR7^+$), central memory ($CD45^-CCR7^+$), effector memory ($CD45^-CCR7^-$) and TEMRA ($CD45^+CCR7^+$) cells among $CD4^+$ T cells and **B.** T $CD8^+$ cells of patients (P1, P2), healthy controls (C+) and a heterozygous relative (Het). **C.** Subpopulations of T cells showing the frequency of T_{reg} in the $CD4^+$ T-cell compartment, and the frequency of γδ T, MAIT and iNKT cells among the T cells of patients (P1, P2), healthy controls (C+) and a heterozygous relative (Het). **D.** Frequency of $CD27^+$ cells within the B-cell compartment. **E.** Frequency of IgD^+, IgA^+, IgM^+, and IgG^+ cells within the $CD27^+$ B-cell compartment.



Supplemental Figure 3. Functional test for the IFN-γ immunity in IFN-γ-deficient patients. **A.** IL-12Rβ1 expression on the surface of EBV-B cells from a healthy control, a patient with AR complete IL-12Rβ1 deficiency (IL-12Rβ1$^{-/-}$), the patients (P1 and P2) and a heterozygous relative (het). **B.** IFN-γR1 expression on the surface of EBV-B cells from a healthy control, a patient with AR complete IFN-γR1 deficiency (IFN-γR1$^{-/-}$), the patients (P1 and P2) and a heterozygous relative (het). **C.** EBV-B cells from a healthy control, a patient with complete STAT1 deficiency (STAT1$^{-/-}$), the patients (P1 and P2), and a heterozygous relative (het) were stimulated by incubation for 30 minutes with 10^5 IU/ml IFN-γ or IFN-α or left unstimulated and subjected to FACS analysis with specific antibodies against p-Y701-STAT1 (upper panel) or the N-terminal part of STAT1 (STAT1, lower panel).

Supplemental Table 1: Immunological phenotype of the patients, based on an analysis of whole-blood samples

	P1 Age 6 months	**P2** Age 5 months	Normal range*
$CD3^+$	4,442	3,394	1,900-5,900/mm^3
$CD3^+CD4^+$	3,508	2,554	1,400-4,300/mm^3
$CD3^+CD8^+$	828	814	500-1,700/mm^3
$CD16^+CD56^+$	852	522	160-950/mm^3
$CD19^+$	3,126	1,636	610-2,600/mm^3

T-cell proliferation in response to mitogen and antigen

	P1	**P2**	Normal range
PHA (3-day culture)	18330	13320	(16540-15720)
Tuberculin (6-day culture)	ND	16	>10 cpm/10*^{3}

Immunoglobulin levels

	P1	**P2**	Normal range*
IgG (mg/dl)	1780	1290	(240-880)
IgA (mg/dl)	188	150	(10-50)
IgM (mg/dl)	223	195	(20-100)

*according to the age of the patients

Supplemental Table 2: Distribution of non-synonymous variants according to their amino-acid position and frequencies, for the *IFNG*, *IFNGR1* and *IFNGR2* genes

			IFNG	*IFNGR1*	*IFNGR2*
Gene region	Coding[a]	N variants	47	286	172
		Length (bp)	498	1467	1011
		Ratio (var/bp)	0.09	0.20	0.17
	Non-coding[b]	N variants	101	237	237
		Length (bp)	866	4146	2809
		Ratio (var/bp)	0.12	0.06	0.08

Note: Data were obtained from the gnomAD browser.

[a] Only non-synonymous coding variants were considered.

[b] Non-coding regions covered in the exomes of the gnomAD database.

Bp: base pairs; N: number; var: variant

Supplemental data

Biological data for known PID in two new MSMD patients

Both patients (P1 and P2) were studied for probable PID predisposing to mycobacterial infections (2,7,77). They had normal counts of polymorphonuclear cells (PMNs) and monocytes (data not shown). The capacity of PMNs to produce reactive oxygen species was normal, as shown by dihydrorhodamine (DHR) tests and stimulation with phorbol myristate acetate (PMA). No IFN-γ was detected in the plasma of the two patients (data not shown). The levels of IL-12Rβ1 and IFN-γR1 protein expressed on the surface of EBV-B cells were similar to those for a healthy control (**Supplemental Figure 2A and 2B**). The phosphorylation of STAT1 in response to IFN-γ and IFN-α was strictly normal (**Supplemental Figure 2C**). The ability of IFN-γ to stimulate the formation of gamma-activating sequence (GAS)-binding nuclear complexes, as assessed by EMSA, was similar in the EBV-B cells of patients and a healthy control (**Supplemental Figure 2D**).

WES data for the two MSMD patients

WES data for regions covering a total of 12.16 Mb identified 19 homozygous coding variants that were non-synonymous or splice variants, with a MAF <0.001, common to both patients (**Figure 1C**). Only two of these variants were present in the linked chromosome intervals. The first variant was located on chromosome 7 and was irrelevant to MSMD. It was a stop-gained, c.494C>A or p.S165* mutation in exon 6 of the *CD36* gene (rs3694405900, gnomAD frequency of 4.06e-06). This gene encodes platelet glycoprotein IV (92), a major receptor for *Plasmodium*-infected erythrocytes (93). *CD36* mutations are commonly associated with platelet glycoprotein deficiency (94, 95) and refractoriness to platelet transfusion. The frequency of CD36 is high, particularly in Asia and Africa, whereas MSMD has a much lower

frequency. Evidence of an association of CD36 with susceptibility to malaria has been obtained, but this association has yet to be confirmed (92).

PCR and qPCR primers and probes

We extracted mRNA with the RNeasy Mini Kit (Qiagen) and treated it with DNase (Roche) before reverse transcription. A probe spanning exons 2 and 3 of *IFNG* was then used for qPCR (Hs00174143_m1, 1-2 exon boundary, Thermo Fisher Scientific), and *GUSB* (4310888E, Thermo Fisher Scientific) probes was used for normalization. qPCR was performed with Applied Biosystems Taqman assays. The relative expression of genes was calculated with the formula $2^{-\Delta Ct}$, with normalization of the results relative to the values obtained for the transcript of the housekeeping gene, *GUSB*. Representative results from two independent experiments were compared (mean ± SEM).

The following primers were used to amplify the region containing exon 1 or exon 4 of the cDNA: cIFNG-F: GGCTTAATTCTCTCGGAAACG and cIFNG-R: ATATTGCAGGCAGGACAACC. For amplification and Sanger sequencing of the genomic DNA region containing the mutation, we used the following primers: gIFNG-3F: GCTAATTTTCCTGTTGCTTGC; and gIFNG-3R: TAGAGTTTCAGCCATGAGTTG.

Whole-blood/PBMC activation and ELISA for cytokines

Venous blood samples from healthy controls and patients were collected into heparin-containing collection tubes (76, 77). These samples were diluted 1:2 in RPMI 1640 (GibcoBRL) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (GibcoBRL). We then dispensed 1 ml of each diluted blood sample into each of five wells (1 ml/well) of a 48-well plate (Nunc). These samples were incubated for 48 hours at 37°C, under an atmosphere containing 5% CO_2/95% air, and under three different sets of activation conditions: with

medium alone, with live BCG (*M. bovis*-BCG, Pasteur substrain) at a MOI of 20 BCG cells/leukocyte, or with BCG plus recombinant (rh) IL-12 (20 ng/ml; R&D Systems), BCG plus IFN-γ (Imukin), LPS alone, LPS plus IL-12, or LPS plus IFN-γ (76, 77). The supernatants were then collected and subjected to ELISA with the human IFN-γ (Sanquin), IL12p40 (R&D), IL-12p70 (R&D) and TNF (Sanquin) ELISA kits, according with the manufacturer's instructions. The statistic values were determined by computing *p*-values from a nonparametric Mann-Whitney-Wilcoxon Test on the samples of heterozygous relatives and of controls (local or travels). PBMCs were isolated on Ficoll and stimulated with PHA or anti-CD3 Abs ± IL-12p70. The supernatant was collected 48 hours later, and the levels of IFN-γ in the supernatant were determined by ELISA. We assessed IL-12p70 production by stimulating PBMCs for 24 h with LPS+IFN-γ, and then performing ELISA for IL-12p70 on the supernatant.

HLA-DR induction

SV40-fibroblasts from a healthy control and a previously reported patient with AR complete IFN-γR1 deficiency (Patient I.1 from Jouanguy *et al.*, (44)) were plated at a density of 300,000 cells/well in six-well plates, with 2 ml DMEM supplemented with FCS per well. The day after plating, the SV40-fibroblasts were incubated for 48 hours with recombinant IFN-γ1b (Imukin, Horizon Pharma) at a final concentration of 1000 IU/ml or with 200 µL of supernatant from transfected HEK293T cells. Cells were then harvested by incubation with 0.05% trypsin and stained with anti-FITC-HLA-DR antibody (L243, Biolegend), or the corresponding isotype antibody, together with the Aqua Dead Cell Stain Kit (Thermo Fisher Scientific). Cells were then acquired on a FACS Gallios machine (Beckman-Coulter) and analyzed with FlowJo software.

Immunophenotyping

Immunophenotyping was performed by flow cytometry, with mAbs against CCR7 (G043H7, Sony), CD3 (UCHT1, BD), CD4 (RPA-T4, BD), CD8 (RPA-T8, BD), CD16 (3G8, BD), CD19 (4G7, BD), CD25 (MA-251, BD), CD27 (O323, Sony), CD45RA (Hl100, BD), CD56 (B159, BD), CD161 (DX12, BD), FOXP3 (259D/C7, BD), IgA (IS11-8E10, Miltenyi Biotec), IgD (IgD26, Miltenyi Biotec), IgG (G18-145, BD), IgM (PJ2-22H3, Miltenyi Biotec), TCR-iNKT (6B11, BD), TCR-γδ (11F2, Miltenyi Biotec), and TCR-Vα7.2 (REA179, Miltenyi Biotec). Cells were also stained with the Aqua Live/Dead Cell Stain Kit (Thermo Fisher Scientific). When required, for intracellular staining, cells were fixed and permeabilized with a fixation/permeabilization kit (eBioscience or BD) after extracellular staining. Samples were analyzed with a FACS Fortessa X20 (BD) flow cytometer. Data were then analyzed with FlowJo 10.1r5 software.

Cell-line culture and functional test

EBV-B cells were cultured in RPMI supplemented with 10% heat-inactivated pooled fetal bovine serum (FBV, GIBCO BRL), referred to as complete medium. Staining of IL-12Rβ1 and IFN-γR1 on surface of EBV-B cells was performed as previously described (59, 96). EBV-B cells were left unstimulated or were stimulated with 10^5 IU/ml IFN-γ (Imukin, Boehringer Ingelheim) and 10^5 IU/ml IFN-α2b (Intron A, Schering Plough) for 20 minutes. Intracellular staining was performed by incubation with the Cytofix/Cytoperm Plus Fixation/Permeabilization kit (BD) with anti-STAT1-PE antibodies (BD), anti-Y701-pSTAT1-Alexa468 (BD) antibodies or an equivalent concentration of isotype-matched control mAb (Becton Dickinson) in 2% FBS in PBS for 1 hour at 4°C. Compensation was performed on single-stained non-stimulated samples. Staining was assessed on a Galios cytometer (Beckman

Coulter), and the results were analyzed with FlowJo v10 (Tree Star). The IRDye 700 GAS probe was used for EMSA, which was performed as previously described (16).

Phage immunoprecipitation-sequencing (PhIP-Seq)

For antibody profiling by PhIP-Seq, plasma samples were obtained from the patients when they were two years old. For comparison, and as additional controls, we also tested 10% liquid IVIg from pooled human plasma (Privigen® CSL Behring AG), human IgG-depleted serum (Supplier No HPLASERGFA5ML, Molecular Innovations, Inc.), and plasma samples from two unrelated healthy adult subjects and one unrelated healthy four-year-old child. These samples were collected at Sidra Medicine, with written informed consent and in accordance with a study protocol approved by the Clinical Research Ethics Board of Sidra Medicine. Each plasma sample and Privigen® were assayed in duplicate. PhIP-Seq was carried out as previously described (92), but with the following modifications. The total IgG levels in the plasma samples were determined with a Human IgG total ELISA Ready-SET-Go kit (Thermo Fisher Scientific). Diluted plasma samples containing approximately 4 μg of total IgG were incubated at 4°C overnight with 2×10^{10} plaque-forming units (PFUs) of a modified version of the original VirScan phage library, which was kindly provided by Stephen Elledge (Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA). This modified T7 phage library was used to display a total of 115,753 peptides, each 56 amino acids (aa) long, including the same viral peptides as the original VirScan phage library and additional peptides derived from the protein sequences of various microbial B-cell antigens and allergens made available from the IEDB (www.iedb.org). Subsequent steps, including the pulldown of the phage-antibody complexes with Protein A- and G- Dynabeads, washes to remove unbound phages, phage lysis, PCR amplification, indexing and pooling of the samples, were performed as previously described (97). We also sequenced the phage library before (input library sample)

and after immunoprecipitation with beads alone (mock IP). Mock IP was performed on 46 technical repeats and used for downstream statistical analysis and background correction. Single-end sequencing was performed with the NextSeq Kit v2 (Illumina) and the NextSeq500 system (Illumina) with 75 cycles to generate approximately two million reads per IP sample (technical repeat) and ~20 million reads for the input library samples. We quantified the enrichment of each peptide, as previously described (97). We mapped the reads to the original library sequences with Bowtie 2, counted the mapped reads for each sequence ID, performed adjustment based on library size (i.e., the total mapped read counts per sample), and used a zero-inflated generalized Poisson model to estimate the *p*-values reflecting enrichment for each of the peptides. We considered peptides to be significantly enriched only if the –log10 *p*-value (peptide enrichment score) was at least 2.3 in both replicates. Species-specific score values were calculated for each plasma sample by counting the significantly enriched peptides for a given species sharing less than a continuous seven-residue subsequence, the estimated size of a linear epitope. We corrected for the nonspecific binding of peptides to the capture matrix, by also calculating species-specific background score values in the absence of antibody, by counting the peptides enriched in the 90th percentile of the mock IP samples and using these values for background subtraction from all scores for serum or plasma samples containing antibodies. We then performed a differential enrichment analysis to identify peptides displaying enrichment in patients. We analyzed peptides that were significantly enriched in at least one of the patients (i.e. 1308 peptides) and performed principal component analysis, using peptide enrichment scores, to identify differentially enriched peptides.

Supplemental references

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