Supplemental material

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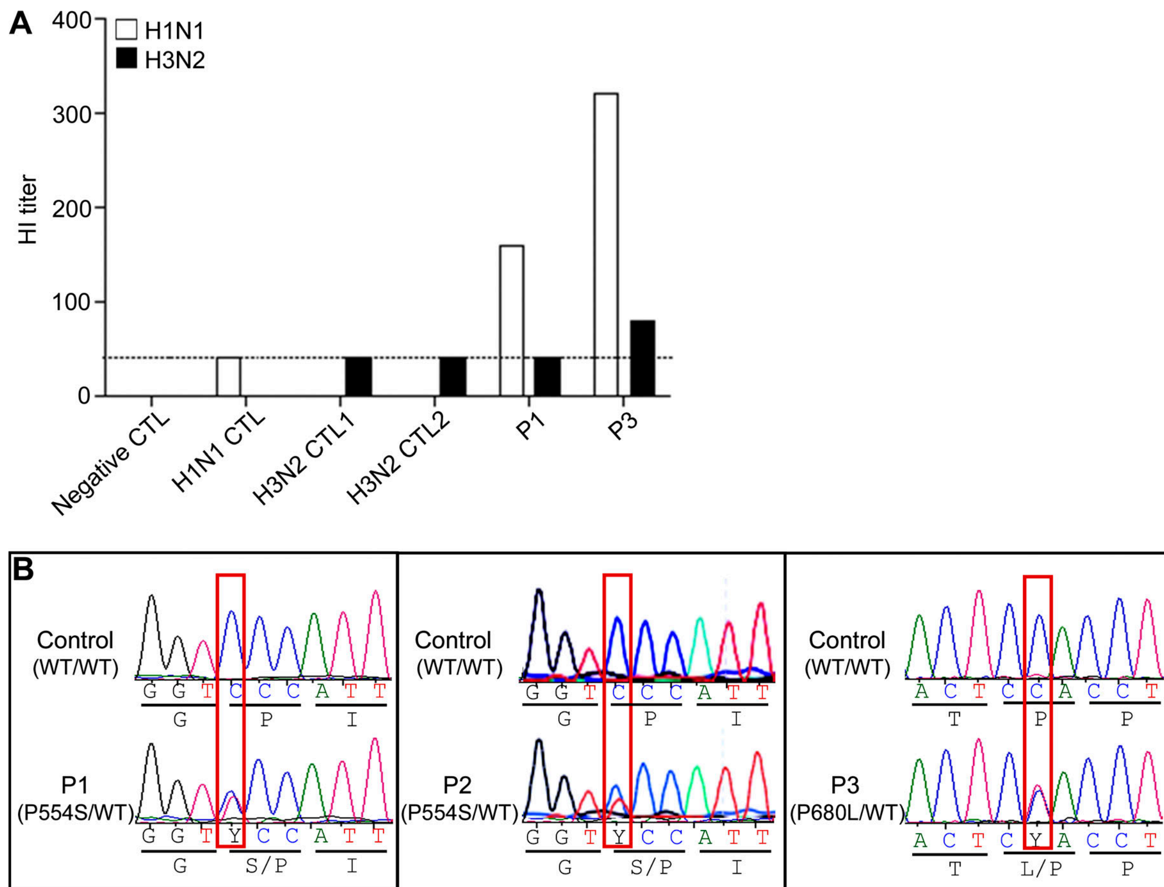

Figure S1. **Heterozygous *TLR3* mutations in three unrelated IAV-ARDS patients. (A)** HI assay using serum from P1 and P3. Serum from patients and controls was used to assess the inhibition of turkey red blood cell hemagglutination by H1N1 and H3N2 IAV. This inhibition indicates the presence of neutralizing antibodies directed against H1N1 or H3N2 IAV. **(B)** Confirmation of the TLR3 mutations by Sanger sequencing. The single nucleotide substitutions for each patient relative to the healthy control sequence are indicated by red squares.

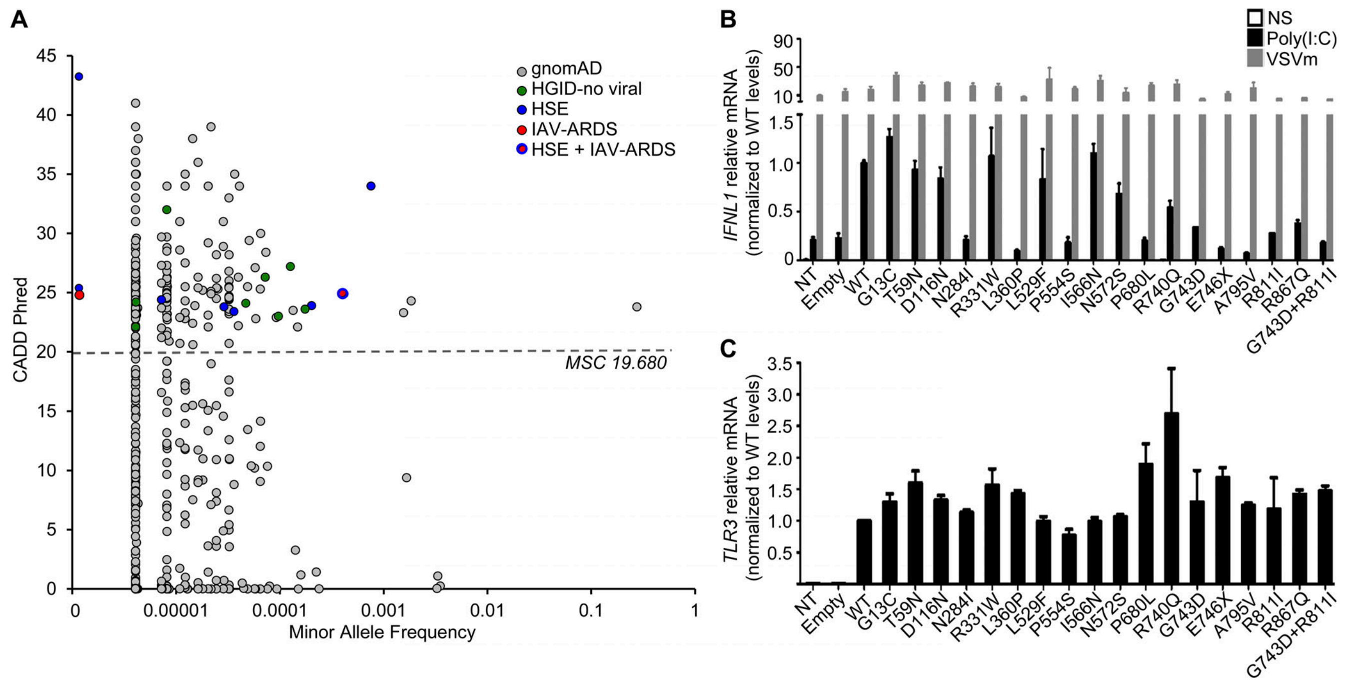

Figure S2. **Expression and function of various mutant *TLR3* alleles from the HGID database. (A)** Representation of all nonsynonymous *TLR3* variants reported in the gnomAD database or found to be private variants in the HGID database of IAV-ARDS, HSE, and other nonviral infection-related exomes. The 17 *TLR3* variants with a MAF < 0.001 and a CADD > MSC of the 99% interval confidence seen in the HGID database are highlighted in green (nonviral infection–related exomes), blue (HSE), or red (IAV-ARDS). The MAF and CADD PHRED score of each variant are shown. **(B)** Assessment of *IFNL1* mRNA induction by RT-qPCR in P2.1 cells left untransfected (NT) or stably transfected with empty vector, HA-tagged *TLR3* WT, or *TLR3* variants found in the laboratory in-house WES database. The levels of mRNA expression were normalized relative to that in TLR3 WT-transfected P2.1 cells. We used cutoffs of 0.5 for hypomorphic variants, and 0.25 for LOF variants, based on previously confirmed deleterious variants. **(C)** TLR3 mRNA levels were determined by RT-qPCR in P2.1 TLR3-deficient fibrosarcoma cells with or without transfection with empty vector, HA-tagged *TLR3* WT, or *TLR3* variants. Mean values ± SD from three independent experiments are shown in B and C, with technical duplicates in each experiment. VSVm, M51R VSV mutant.

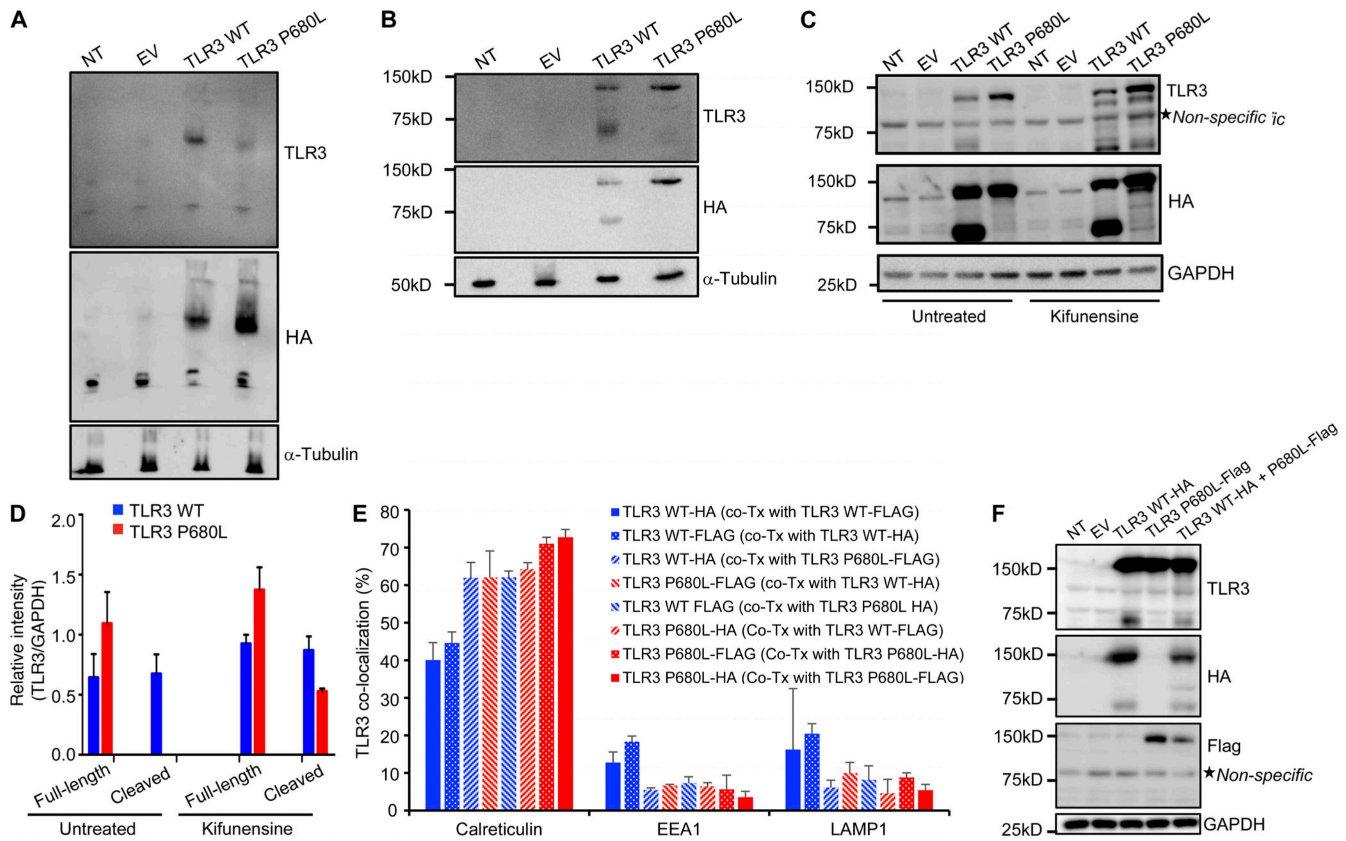

Figure S3. **The P680L TLR3 is misfolded and retained in the ER. (A and B)** Native-PAGE (A) and SDS-PAGE (B) analysis of TLR3. P2.1 cells not transfected (NT) or stably transfected with empty vector (EV), HA-tagged *TLR3* WT, or P680L were subjected to Native-PAGE or SDS-PAGE followed by immunoblotting with TLR3 antibody, C-ter HA antibody. α-Tublin was used as a loading control for immunoblotting. **(C)** TLR3 was detected by SDS-PAGE analysis after chemical treatment. P2.1 TLR3-deficient fibrosarcoma cells with or without transfection with empty vector, HA-tagged WT, or P680L TLR3 were treated or untreated with 166 μM kifunensine for 48 h, then subjected to SDS-PAGE and immunoblotted with TLR3 antibody and C-ter HA antibody. GAPDH was used as a loading control for immunoblotting. **(D)** Semi-quantification results of the TLR3 immunoblots using ImageJ, for extracts from TLR3-deficient P2.1 cells with stable transfection of HA-tagged WT or P680L TLR3 that were treated or untreated with 166 μM kifunensine for 48 h. The relative intensity of full-length or cleaved forms of TLR3 relative to that of GAPDH is presented. Mean values ± SD were calculated from three independent experiments. **(E)** Immunofluorescence imaging of HeLa cells following transient cotransfection with HA- or FLAG-tagged TLR3 WT- and P680L-containing plasmids. Intracellular distribution was assessed by colocalization with a subcellular marker: anti-calreticulin antibody for the ER, anti-EEA1 antibody for early endosomes, and anti-LAMP1 antibody for lysosomes. The data shown are representative of results from two independent experiments. For each independent experiment, 5–12 cells were analyzed per condition. **(F)** TLR3 was detected by SDS-PAGE analysis, for extracts from HEK293 cells with or without transfection with empty vector, HA-tagged WT TLR3, and FLAG-tagged P680L TLR3 separately or together. Cell extracts were subjected to SDS-PAGE and immunoblotted with TLR3 antibody, C-ter HA antibody, or C-ter FLAG antibody. GAPDH was used as a loading control for immunoblotting. The data shown in A–C and F are each representative of results from three independent experiments.

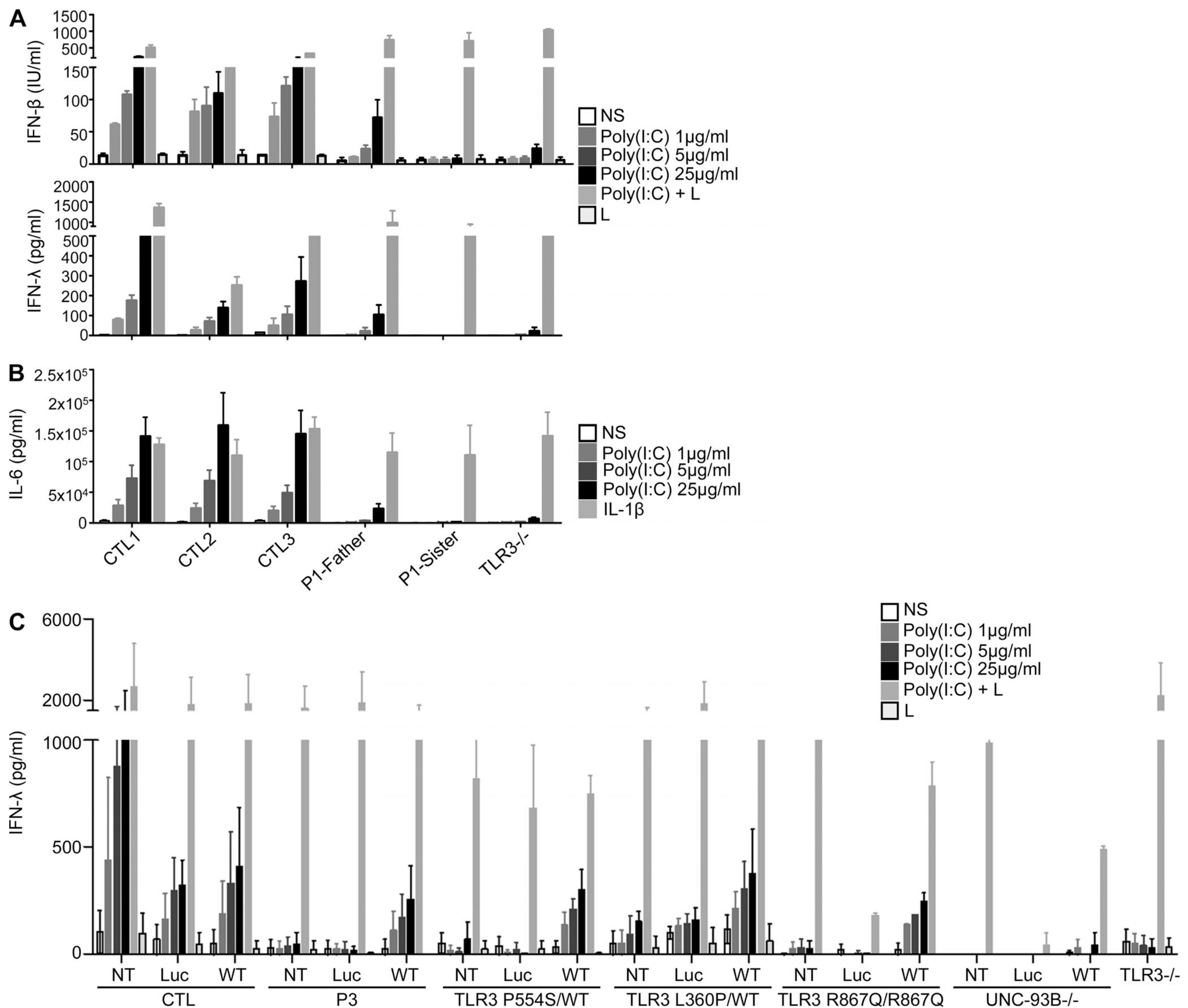

Figure S4. **Impaired poly(I:C) responses in the TLR3 P554S or P680L heterozygous SV40-fibroblasts from P1 family members or P3. (A and B)** Production of IFN-β, IFN-λ (A), and IL-6 (B) in SV40-fibroblasts from three healthy controls (CTL1/2/3), the father and sister of P1 who are heterozygous for the P554S *TLR3* mutation, and a $TLR3^{-/-}$ HSE patient, 24 h after stimulation with 1, 5, or 25 µg/ml poly(I:C), or with 25 µg/ml poly(I:C) in the presence of lipofectamine (poly(I:C)+L; A), lipofectamine alone (L; A), or IL-1β (B), as assessed by ELISA. **(C)** Production of IFN-λ as assessed by ELISA, in SV40-fibroblasts from a CTL, P3 (TLR3 P680L/WT), TLR3 P554S/WT, TLR3 L360P/WT, TLR3 R867Q/R867Q, and $UNC\text{-}93B^{-/-}$ HSE patients left untransfected, or transfected with the pTRIP vector encoding Luc or WT *TLR3*, 24 h after stimulation with 1, 5, or 25 µg/ml poly(I:C), or with 25 µg/ml poly(I:C) in the presence of lipofectamine or lipofectamine alone. NS, not stimulated. Mean values ± SD were calculated from two independent experiments, with biological duplicates in each experiment.

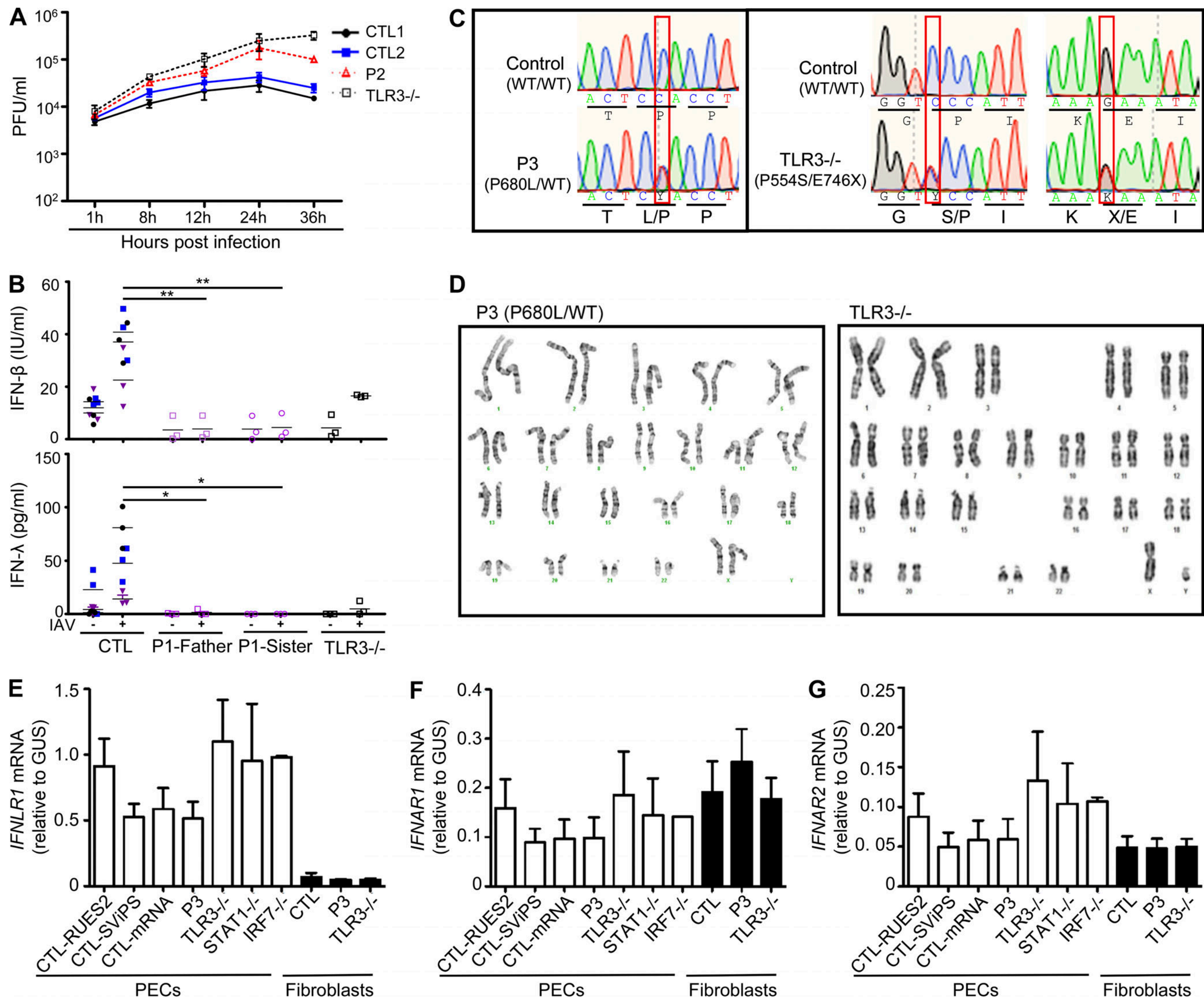

Figure S5. **Enhanced susceptibility to IAV in TLR3 P554S heterozygous SV40-fibroblasts and characterization of TLR3-mutated patient iPSCs. (A)** IAV replication, quantified by plaque assays, in SV40-fibroblasts from two CTLs, P2, and TLR3$^{-/-}$, 1, 8, 12, 24, and 36 h after infection at a MOI of 5. **(B)** Production of IFN-β and IFN-λ in the absence of infection or after 24 h of infection with IAV at a MOI of 5, in SV40-fibroblasts from three CTLs (CTL1–3), the father and sister of P1 who are heterozygous for the P554S *TLR3* mutation, and a TLR3$^{-/-}$ HSE patient, as assessed by ELISA. *, $P < 0.05$; **, $P < 0.01$. **(C)** The genotype of patient-specific iPSCs was confirmed by Sanger sequencing. The two TLR3-deficient iPSC lines carried the same mutations as the original fibroblasts from P3 and from a TLR3$^{-/-}$ patient with HSE. G, gylcine; S/P, serine/proline; I, isoleucine; K, lysine; X/E, stop codon/glutamic acid. **(D)** Karyotype analysis was performed to check the genomic stability of iPSCs. No aberrations were detected in TLR3 P680L/WT or TLR3$^{-/-}$ iPSCs. **(E–G)** Levels of mRNA for IFN receptor-encoding genes in fibroblasts and iPSC-derived PECs. Relative mRNA levels for IFNLR1 (E), IFNAR1 (F), and IFNAR2 (G) in PECs or fibroblasts, as measured by RT-qPCR. Mean values ± SD were calculated from three (A and B) or two (E–G) independent experiments, with biological duplicates in each experiment.

Table S1. **Ranking of the top 10 enriched mutated IRF7-connected genes in the IAV-ARDS cohort versus controls**

Gene	No. of carriers[a] in control cohorts (1,485 exomes)	No. of carriers[a] in IAV-ARDS cohort (25 exomes)	P value[b]	OR[c]	CI_2.5%	CI_97.5%
TLR3	11	3	0.00052	24.82	5.05	96.83
HLA-DRB5	16	2	0.0312	8.52	1.27	33.99
PML	56	3	0.0687	3.87	0.88	11.93
MX1	31	2	0.0738	5.35	0.82	20.22
TRIM21	6	1	0.1129	9.75	0.48	70.50
BCL2A1	5	1	0.1138	9.58	0.48	67.91
RNASEL	24	2	0.1186	4.35	0.63	17.62
STAT1	53	0	0.1284	NA	NA	NA
INTS6	13	1	0.1426	7.40	0.39	42.94
OAS3	35	2	0.1685	3.43	0.52	12.85

We considered a total of 168 genes closely connected to IRF7 (IRF7 connectivity P value < 0.01) in the analysis. NA, not applicable; OR, odds ratio; CI_2.5%, 2.5% confidence interval; CI_97.5%, 97.5% confidence interval.

[a]For each gene, carriers are defined as individuals carrying at least one mutant allele with the following criteria: MAF < 0.001 in 1000 Genomes, gnomAD, and our in-house WES database; CADD > MSC (99% confidence MSC).

[b]P values were obtained using a likelihood ratio test adjusted for the first three principal components.

[c]Odds ratios were obtained from the coefficients of the adjusted likelihood ratio test. The boundaries of the 95% confidence interval for the odds ratio are also shown in the last two columns.

Table S2. **Number of rare TLR3 nonsynonymous variant carriers in the flu cohort and in other infectious disease cohorts of the laboratory in-house WES database**

	Flu	HSE	In-house (excluding flu, HSE)
No. of exomes	25	245	1,485
No. of variant carriers	3	8	11
P values (versus in-house) for the no. of variant carriers	0.00052	0.0022	–

Variants were considered if they had a MAF < 0.001 in 1000Genomes, gnomAD, and our in-house WES database. Variants were considered if they had a CADD score higher than the 99% confidence MSC for TLR3 (19.68). Ethnic-adjusted TLR3 mutation enrichment assessment was performed using logistic regression adjusting on the three first components of the PCA. The number of variant carriers is indicated for flu, HSE, and in-house cohorts, in the three columns corresponding to these cohorts.

Table S3. **Viral serological tests in patients and family members**

Virus seropositivity	Patients		P1 family member		P2 family member[a]	P3 family member			
	P1	P3	Father	Sister	Father	Father	Mother	Brother	Sister
Influenza A	+	+/−	+	+	+	+	+	+	+
Influenza B	−	+	+	−	ND	+	+	+	+
RSV	+	+	+	+	+	+	+	+	+
HSV	−	+/−	−	−	−	+	+	−	−
VZV	−	+	+	+	+	+	+	+	+
CMV	−	+	−	−	+	−	+	−	−
EBV	−	+	−	−	+	−	+	+	−
HAV	−	+	−	−	ND	+	−	+	+
Measles	−	+	+	+	+	+	+	+	+
Mumps	−	+	+	+	+	+	+	+	+
Rubella	−	+	−	+	+	+	+	+	+
B19	−	+	−	+	ND	+	+	+	+

CMV, cytomegalovirus; HAV, hepatitis A virus; VZV, varicella zoster virus.
[a]P2 was not included in the table due to her IVIG treatment.

Table S4. **Number of genes harboring rare nonsynonymous or essential splicing variants in the three patients, as revealed by WES**

Patient	No. of genes	No. of variants	No. of variants			Inheritance model		
			Sub	Del	Ins	He	"Comp"	Ho
P1	213	238	176	41	21	180	38	20
P2	316	407	290	76	41	255	33	28
P3	197	209	144	40	25	171	22	16

Variants were considered if they had a MAF < 0.001 in 1000 Genomes, ExAC, and our in-house WES database, and a CADD score higher than the gene-specific MSC of the 99% confidence interval. Nonsynonymous variants include missense, frameshift, in-frame, stop gained, stop lost, start gained, and start lost. "Comp," possible compound heterozygosity, with two or more variants of the same gene, ExAC, the Exome Aggregation Consortium; Sub, substitution; Del, deletion; Ins, insertion; He, heterozygous; Ho, homozygous.

Table S5. **List of rare TLR3 variants found in our in-house WES database for various infectious diseases**

	Flu	HSE	In-house (excluding flu, HSE)	1000G	dbSNP	gnomAD	SIFT	Poly phen2	CADD score	Experimental result
G13C			1	0	–	0.0000969	0.02	0.572	23	N
T59N		1		0.0002	rs143307508	0.0002020	0.01	0.282	23.9	N
D116N			1	0.0006	rs140176180	0.0001747	0.01	0.237	23.6	N
N284I		1		0	rs5743316	0.0000361	0	1	23.4	D
R331W			1	0	–	0.0000469	0	0.023	24.1	N
L360P		1		0	–	0.0000289	0	1	23.8	D
L529F			1	0	rs201222071	0.0001263	0	1	27.2	N
P554S	2	4	2	0	rs121434431	0.0004151	0	0.962	24.9	D
I566N			1	0	–	0.0000041	0.1	0.638	22.1	N
N572S			1	0	–	0.0000041	0	0.999	24.3	N
P680L	1			0	–	0	0	1	24.9	D
R740Q			1	0	–	0.0000081	0.02	1	32	N
G743D		1		0	–	0.0000072	0.18	0.908	24.4	H
E746X		1		0	–	0	NA	NA	44	D
A795V			2	0	rs373118024	0.0000722	0.14	0.97	26.3	D
R811I		1		0	–	0	0	0.992	26.6	H
R867Q		1	1	0.0004	rs199768900	0.0007540	0	0.78	34	H

Variants were considered if they had a MAF < 0.001 in 1000 Genomes, gnomAD, and our in-house WES database of non-flu and non-HSE patients. Variants were considered if they had a CADD score higher than the 99% confidence MSC of TLR3 (19.68). The variants were experimentally tested in TLR3-deficient cell lines. The numbers of carriers of each variant are indicated for flu, HSE, or in-house cohorts, in the three columns corresponding to these cohorts. D, functionally deleterious mutations; H, severely hypomorphic mutations; N, neutral mutations; 1000G, 1000 Genomes; dbSNP, Single Nucleotide Polymorphism Database; SIFT, sorting intolerant from tolerant.