Inherited STING gain of function mutation in a familial inflammatory syndrome with lupus-like manifestations.

Supplemental information

Case reports

The index patient was referred to the pediatric immunology unit of the Necker-Enfants-Malades Hospital (Paris, France) at the age of two years because of febrile attacks and failure to thrive. She was born to unrelated French Caucasian parents. She had experienced febrile episodes (38-39°C lasting 2-4 days) and demonstrated an intermittent squamous malar rash from the age of 1 year. Clinical examination and biological findings (Table 1 and supplemental Table 1) revealed a systemic inflammatory condition associated with lung disease and autoantibodies to nucleic acids, thus defining a juvenile lupus. Type-I IFN concentration was elevated in the serum (but normal in cerebrospinal fluid) (supplemental Table 1). Chest CT scan revealed interstitial lung disease (Figure 1c, left panel) and fibrosis (Figure 1c, right panel) in parenchymal and mediastinal views respectively. Lung biopsy showed macrophagic alveolitis, follicular hyperplasia, presence of B cell germinal centers and moderate interstitial fibrosis (Fig. 1d). Brain CT scan and MRI were normal. She was treated with steroids, anti-CD20 monoclonal antibodies and mycophenolate mofetil without obvious therapeutic response.

The proband's father (II-5) and paternal uncle (II-6) are monozygotic twins, and experienced failure to thrive from childhood, together with recurrent febrile attacks beginning in the teenage years, malar rash, interstitial lung disease, polyarthralgia and polyarthritis, in association with elevated acute-phase reactants, positive Antinuclear Antibodies (ANA) and rheumatoid factor (Table 1). Patients II-5 and II-6 were treated with steroids, anti-TNF, methotrexate and anti-CD20 with only partial improvement of their condition. Patient II-5 died at the age of 29 years of fulminant necrotizing fasciitis. The paternal grandfather of the proband (I-4) had long-lasting, elevated ESR and CRP, intermittent arthralgia and is chronically underweight (weight -3SD, height -1.5 SD).

Supplemental Methods

Exome sequencing

Exome sequencing was performed by the Centre National de Génotypage, Institut de Génomique, CEA. After quality control by the DNA Bank Laboratory, genomic DNA (3μg) was captured using in-solution enrichment methodology (Human All Exon v5 – 50 Mb, Agilent Technologies, CA, USA). Library preparation and exome enrichment protocol (~20.000 targeted genes) was performed on an automated platform, using NGSx (Perkin Elmer Inc, MA, USA) and Bravo (Agilent Technologies, CA, USA) robots respectively, according to manufacturer's instructions (SureSelect, Agilent Technologies CA, USA). After normalisation and quality control, exome enriched libraries were sequenced on a HiSEQ 2000 (Illumina Inc., CA, USA) as paired-end 100b reads. Sequencing was performed in order to provide a mean cover of at least 60 to 70X for each sample. Image analysis and base calling was performed using Illumina Real Time Analysis (RTA) Pipeline. Sequence quality parameters were assessed daily throughout the 12 days sequencing run. Sequences were aligned to the reference human genome hg19 using the Burrows-Wheeler Aligner. Downstream processing was carried out with the Genome Analysis Toolkit (GATK), SAMtools, and Picard, following documented best practices (http://www.broadinstitute.org/gatk/guide/topic?name=best-practices). Variant calls were made with the GATK Unified Genotyper. All calls with read coverage of ≤2X or a Phred-scaled SNP quality score of ≤20 were removed from consideration. All variants were annotated using a software system developed by the Paris Descartes University Bioinformatics platform. All the annotation processes were based on the latest release of Ensembl database.

Pedigree information suggested an autosomal dominant pattern of inheritance; hence, variants common between the proband (III-2) and the paternal uncle (II-6), and absent in the healthy mother, were of interest. Polymorphisms with a frequency of <1% in the population previously reported in public databases such as 1000genomes project, EVS, dbSNP, as well as variants reported in-house (4047 exomes), were excluded. The exomes were mined for variations including substitutions, deletions, and insertions in the coding regions, splice sites and essential splice site in the flanking introns. Silent mutations in the coding regions were excluded in this analysis.

Structural analysis

Experimental 3D structures of STING in complex with or without c-di-GMP (22) were extracted from the RCSB database, analyzed and compared using Chimera (https://www.cgl.ucsf.edu/chimera/).

RNA and DNA preparation

DNA was isolated from the red blood pellet after Ficoll preparation using the QiAamp DNA blood Mini kit. PCR was performed using Go Taq Flexi DNA polymerase (Promega) and amplified in a 25ul reaction for 40 cycles. PCR products were purified on a sephadex G-50 columns (GE healthcare) and the sequencing reaction was performed using BigDye Terminator v3.3 cycle sequencing kit (Applied Biosystems) and read using the 3500XL

genetic analyser (Applied Biosystems). Forward primers were used for sequencing (supplemental table 2). All sequence data was analysed using ApE and 4peaks software.

Total RNA was isolated from PBMCS, and T-lymphoblasts using the RNeasy mini Kit and cDNA was prepared using the Quantitect Reverse Transcription Kit after depleting genomic DNA. Day 14 cultured T lymphoblasts were derived from PBMCS, stimulated with Dynabeads Human T-activator CD3/CD28 (Invitrogen) and IL2 (100U/ml).

Gene expression analysis
The expression of a set of 6 interferon-stimulated genes (ISGs) in PBMC and T-lymphoblasts was assessed by q-RT-PCR using Taqman Gene Expression Assays and normalized against GAPDH. Primer-Probes used were as follows IFI27 - Hs01086370_m1, IFI44L-Hs00199115_m1, IFIT1 - Hs01675197_m1, RSAD2 - Hs00369813_m1, SIGLEC1-Hs00988063_m1, ISG15 – Hs01921425_s1, GAPDH- Hs03929097_g1 labeled with FAM dye and amplified in a universal reaction mix. For non- type I regulated genes Primer probes used were as follows *CXCL9* Hs00171065_m1, *IL-12A* Hs01073447_m1, and *IL-6* Hs00985639_m1. Real Time quantitative PCR was performed in duplicate using the LightCycler VIIA7 System (Roche). The RQ value is equal to $2^{\Delta\Delta ct}$ where $\Delta\Delta ct$ is calculated by (CT $_{target}$- CT$_{GAPDH}$) test sample - (CT $_{target}$- CT$_{GAPDH}$) calibrator sample. Each value is derived from three technical replicates.

Western Blotting
1 well of transfected 293FT was lysed in 100µl of sample buffer (2% SDS, 10% Glycerol, 0.05M Tris-HCl pH 6.8, 0.025% bromophenol blue, 0.05M DTT). Cellular protein lysates were resolved on 4%-20% SDS-PAGE gels (Biorad), transferred on nitrocellulose membrane (Biorad) and the membrane was blocked with 5% non-fat milk. Proteins were blotted with monoclonal anti-STING (R&D MAB7169) and monoclonal anti-alpha Tubulin (eBioscience clone DM1A).

Plasmids
The p.V155M was introduced in pUNO1-hSTING (Invivogen) by site-directed mutagenesis using QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies). The STING coding sequences were cloned by PCR in pMSCV-hygro(+) (Addgene) by PCR. IFNß-pGL3 plasmid was obtained from the lab of Olivier Schwartz, Pasteur Institute.

Interferon reporter assay
293FT cells were plated in a 24-well plate. The next day, cells were transfected with 500ng of total DNA comprising IFNß-pGL3 and the empty vector pMSCV-hygro(+) or pMSCV-hygro(+) encoding the STING variants with TransIT-293 (Mirus). The next day, medium was removed and 2'3'-cGAMP (InvivoGen) was delivered with Lipofectamine 2000 (Invitrogen) transfection (1µg 2'3'-cGAMP:1µl Lipofectamine 2000) in a final volume of 500µl (final concentrations: 4µg/ml, 1.3µg/ml, 0.4µg/ml). Fresh medium was added in the case of non-stimulated cells. After 24 hours cells were washed with PBS and lysed with Passive Lysis

Buffer (Promega) and 10µl of the lysate were used to perform the Luciferase assay. Luciferase activity was measured using Luciferase Assay Reagent (Promega). Luminescence was acquired on a FLUOstar OPTIMA microplate reader (BMG labtech). Statistical analyses were performed in Prism (GraphPad).

Confocal Microscopy

Primary fibroblasts obtained from the patient's skin biopsy were stimulated in a 24 well plate with 4µg/ml 2'3'-cGAMP (InvivoGen) and Lipofectamine 2000 (Invitrogen) for 8hours. Cells were fixed with 4% paraformaldehyde and blocked with PBS-2%BSA. Permeablization was carried out in PBS-2%BSA-0.5% saponin. Cells were then incubated in the primary antibodies, STING (R&D MAB7169), and Rab6 (Santa Cruz sc-310) followed by fluorophore conjugated secondary antibodies. Prolong Gold antifade reagent with DAPI (Molecular Probes) was used as the mounting solution. Images were acquired on LeicaSP8 40X oil immersion objective. Images were analysed using ImageJ and Imaris software. 3D reconstruction of STING localisation around the nucleus is depicted in the supplemental Figure 4.
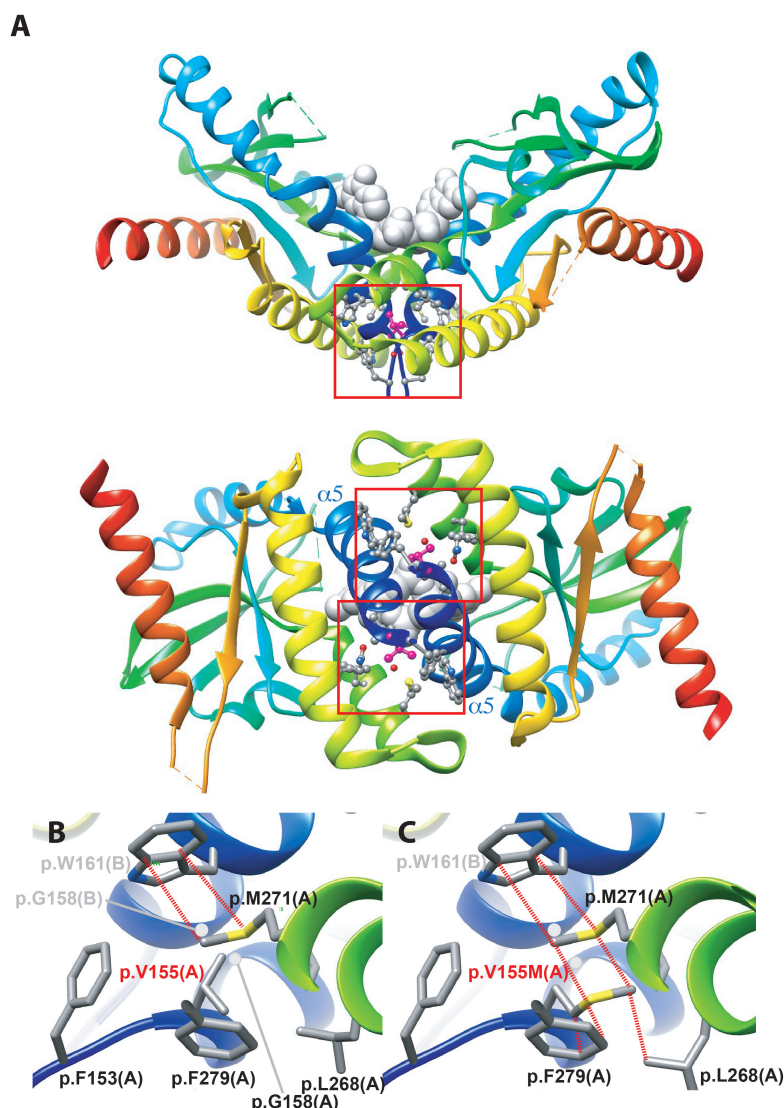
Supplementary Figure 1: 3D structure of the p.V155M mutant predicts enhanced stability of the dimer.

Ribbon representation of the STING C-Terminal Domain (CTD) 3D structure (protein database (pdb) identifier 4EF4) in complex with c-di-GMP (gray spheres) in two orthogonal views (14). (A) The first highly hydrophobic helix (a5) of the STING CTD (dark blue), the helix a7 (green) and the pV155 (pink), with its immediate neighborhood boxed . (B) Focus on the wild-type p.V155 and (C) mutant p.V155M STING. The sulfur atom (yellow) and methyl group (gray) of p.V155M are predicted to interact on the one hand with the p.F279 aryl and the p.L268 alkyl groups, and on the other hand with the methyl group and sulfur atom of p.M271, of the same subunit (designated as A in supplemental Figure 1B). p.M271 sulfur atom in turn interacts with the p-electron cloud of the pW161 indole group of the other subunit (B in supplemental Figure 1B). The stabilization of the p.M271 side chain should favor the sulfur-aromatic interaction.
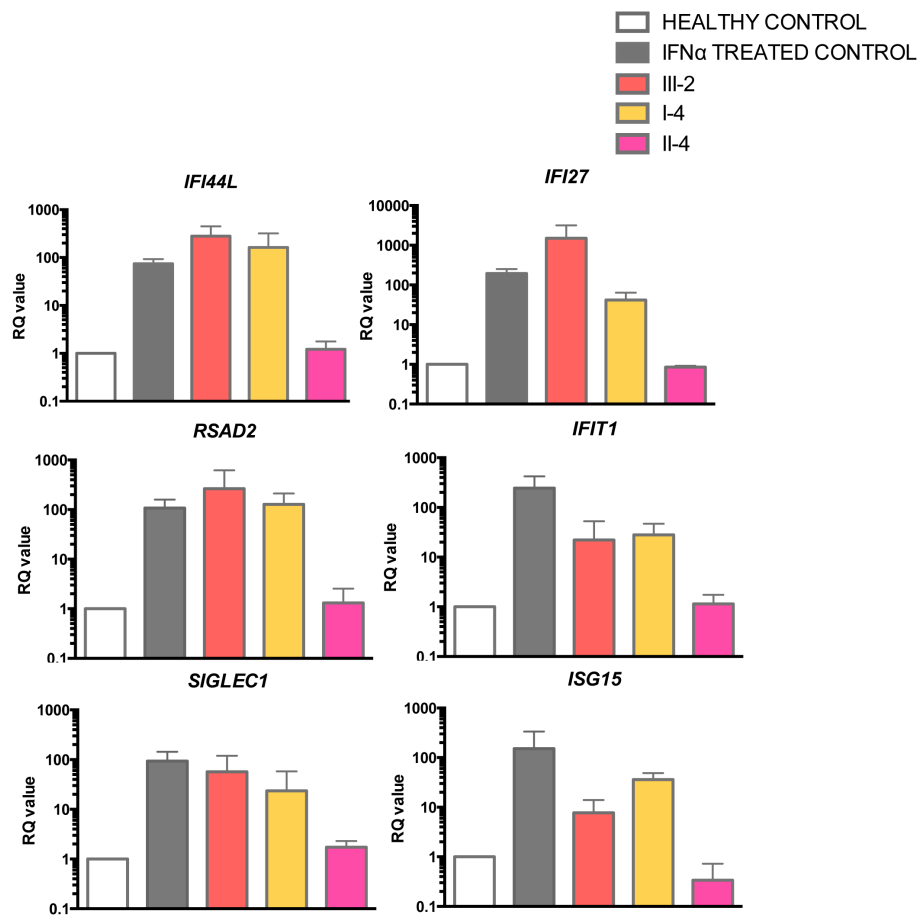
Supplementary Figure 2: **Functional consequences of the p.V155M mutation *ex vivo*.**
Interferon stimulated gene expression levels in PBMCs measured by q-RT-PCR in patients carrying the V155M mutation, healthy family members and unrelated healthy controls. RQ values for a set of six genes (*IFI27, IFIT1, RSAD2, SIGLEC1, IFI44L, ISG15*) measured in PBMCs are shown.
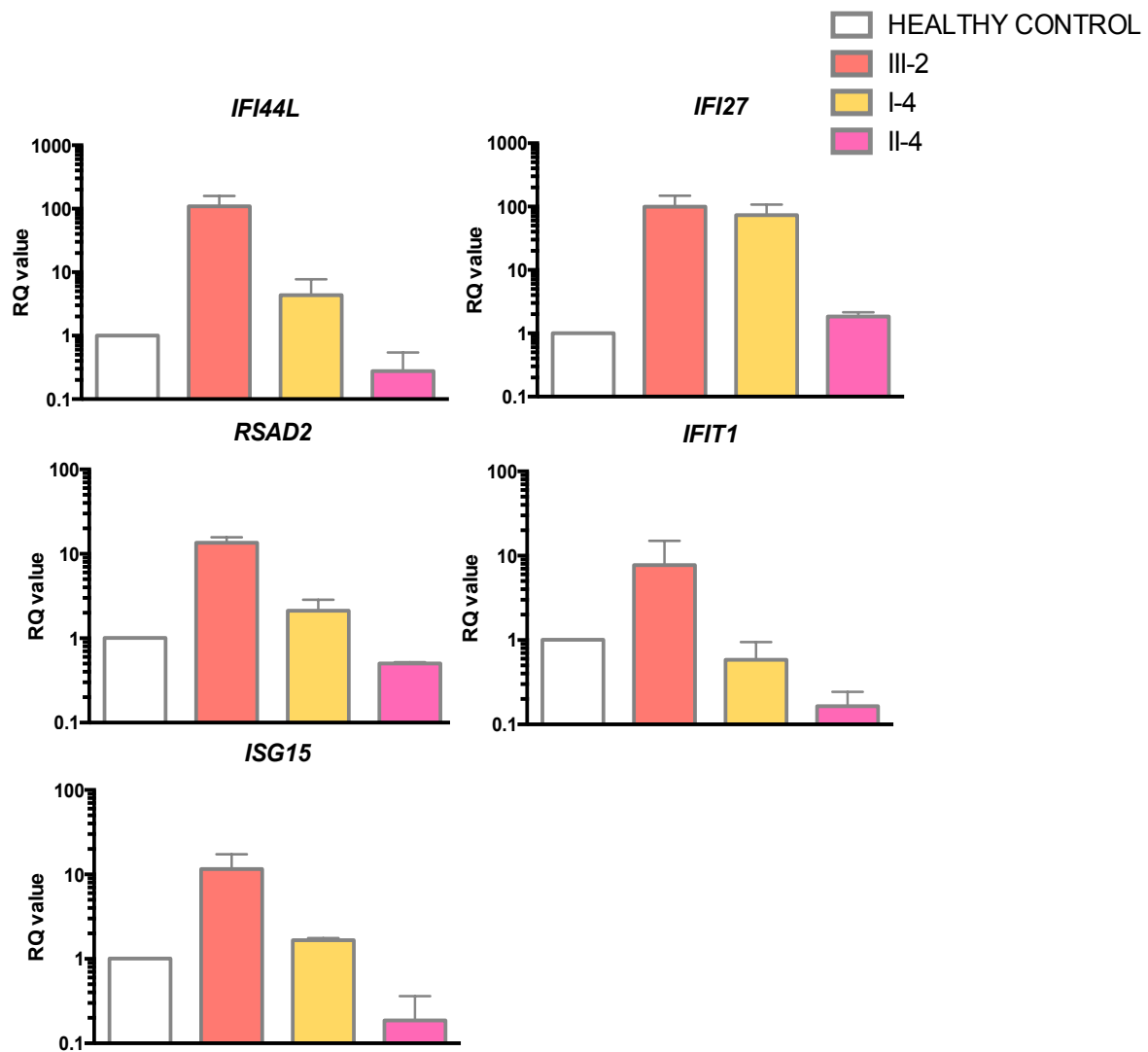
Supplementary Figure 3: **Functional consequences of the p.V155M mutation ex vivo**.
IFN stimulated gene expression levels in T cells expanded exvivo measured by q-RT-PCR
in patients carrying the V155M mutation, healthy family members and unrelated healthy
control. RQ values for a set of five genes (*IFI27, IFIT1, RSAD2, IFI44L, ISG15*) measured
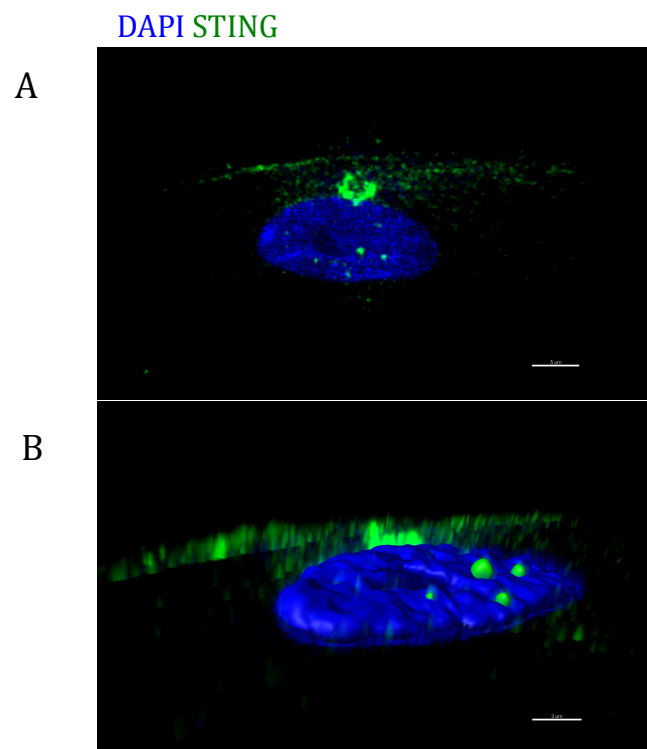in T lymphoblasts are shown.
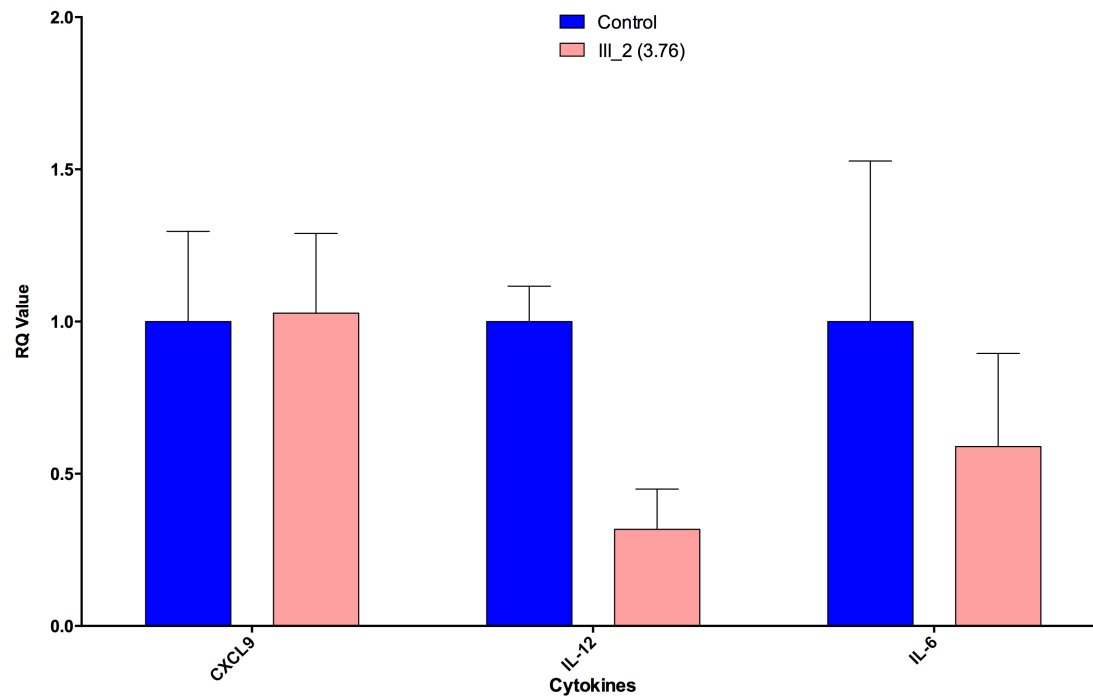
Supplementary Figure 4: Normal expression of non- type I regulated genes
Non Type1 regulated genes were measured in patient III-2 with age in years at time of testing stated in brackets. RQ values for CXCL9, IL12 and IL6 normalised to *HPRT1 and I8s* . Data assessed with Applied Biosystems StepOne Software V2.1 and Applied Biosystems Data Assist Software V3.01.

Supplemental Figure 5

Supplementary Figure 5 : 3D reconstruction of STING localisation around the nucleus.
A. 2D image of patient fibroblast (III-2) at steady state B. 3D reconstruction showing perinuclear localisation of STING in patient fibroblast (III-2) at steady state. Scale bar 15µm.

Supplementary Table 1:

Hematological and Immunological findings in index Patient (III-2)

	III-2	normal range
Hematological findings		
Hemoglobin	8-10.2	11.5-13.5 g/dl
Leucocytes	8-16.2	6-17 x10exp9/l
PMN	3.1-12.3	1.5-8.5 x10exp9/l
Lymphocytes	1.7-9.2	2-8 x10exp9/l
Platelets	550-890	175-500x10exp9/l
ESR	35-90	< 25 mm
CRP	20-200	< 6 mg/L
Immunoglobulin levels		
IgG	13.8-14.2	5.5-10.2 g/l
IgA	2.1-4.8	0.4-1.4 g/l
IgM	0.8-1.5	0.5-1.5 g/l
Antibody response		
post- immunisation	positive	positive
Auto-antibodies		
ANA	1/200-1/800	< 1/100
Anti DNA	4.1-10.2	< 5.5 UI/ml
Anti SmB	45-375	< 225cpm
Serum IFNα	5-18	<2 UI/l
Immunophenotype		
T CD3+	3060	1400-3700
T CD4+	1935	700-2200
T CD8+	945	490-1300
T Naive CD4+ (CD31+CD45RA+/CD4+)(%)	74	57-65
Naive CD8+ (CCR7+CD45RA+/CD8+)(%)	88	52-68
T Central Memory CD8+ (CCR7+ CD45RA-/CD8+)(%)	1	3-4
T Effector Memory CD8+ (CCR7- CD45RA-/CD8+)(%)	3	11-20
T EMRA CD8+ (CCR7- CD45RA+/CD8+)(%)	8	16-28
B CD19+	1560	390-1400
B memory (CD27+/CD19+)(%)	1.8	>10
B switched memory (IgM-IgD-/CD27+CD19+)(%)	1.8	>10
NK CD16+CD56+	225	130-720

Supplementary Table 2
Primer used to amplify STING

EXONS	PRIMER SEQUENCE	PRODUCT SIZE
EXON3	CCCTGCTCTGTTTTCAGCA	683
	CGCATTTGGGAGGGAGTAGT	
EXON4	CTGCTGTTAAACGGGGTCTG	592
	CCCAGAGAACTCCTCCTCCT	
EXON5	AGGGGGAGGTAGAAGCTCTG	574
	TTGGGCAAGTCACTTAATGG	
EXON6	GGCGCTGACATAGACATCTG	804
	CGTCCACTTTCCTACGGTGT	
EXON7	TAAATTTAGCCCAGCCGTGT	686
	CATACTGGCCTGGGACTCTT	
EXON8	GGCCTGAACCACTTCTACCTC	848
	CCTCCTCCTCCTCTCCATTC	