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Disease-associated mutations identify a novel region in human STING necessary for the control of type I interferon signaling

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Capsule summary

Three new mutations in *TMEM173* are reported in patients presenting STING-associated autoinflammation. These mutations confer constitutive activation independent of ligand stimulation, revealing a novel functionally important region of the protein.

Key words

Stimulator of interferon genes (STING), *TMEM173*, interferon, type I interferonopathy, STING associated vasculopathy with onset in infancy (SAVI)

Abstract

Gain-of-function mutations in *TMEM173* encoding STING (stimulator of interferon genes) underlie a recently described type I interferonopathy. We report three individuals variably exhibiting the core features of STING-associated vasculopathy with onset in infancy (SAVI) including systemic inflammation, destructive skin lesions and interstitial lung disease. Molecular and *in vitro* data demonstrate that the pathology in these patients is due to substitutions at positions 206, 281 and 284 of the human protein. These mutations confer cGAMP-independent constitutive activation of type I interferon signaling through TBK1 (TANK binding kinase). Structural analysis indicates that these three amino acids lie in a discrete region of the protein, thereby implicating a novel cluster of amino acids in STING as functionally important in the regulation of type I interferon signaling.

Key Messages

- p.Cys206, p.Arg281 and p.Arg284 are new mutations in *TMEM173* identified in patients with STING-associated vasculopathy with onset in infancy (SAVI).
- These mutations confer constitutive activation independent of ligand stimulation, thereby revealing a novel, functionally important region of the protein.

Abbreviations

3D: Three-dimensional; AMP: Adenosine monophosphate; ANA: Antinuclear antibodies; BSA: Bovine serum albumin; c: Complementary DNA; CBD: cGAMP binding domain; cGAMP: 2'3'-cyclic GMP-AMP; cGAS: Cyclic GMP-AMP synthase; cm: Centimeter; CT: Computed tomography; CTD: C-terminal cyclic dinucleotide-binding domain; DNA: deoxyribonucleic acid; ER: Endoplasmic reticulum; ERGIC: ER-Golgi intermediate compartment; ExAC: Exome Aggregation Consortium; GMP: Cyclic guanosine monophosphate; HEK: Human embryonic kidney; IFN: Interferon; IFNα: Interferon alpha; IFNAR: Interferon alpha receptor; IFNß: Interferon beta; IRF3: Interferon regulatory factor 3; ISGs: Interferon-

stimulated genes; JAK1: Janus kinase 1; Kg: Kilogram; mAb: Monoclonal antibody; MAFFT: Multiple alignment using fast fourier transform; P: Patient; pcDNA: plasmid cDNA; PCR: Polymerase chain reaction; PDB: Protein Data Bank; RCSB: Research Collaboratory for Structural Bioinformatics; RNA: Ribonucleic acid; p: Protein; SAVI: STING associated vasculopathy with onset in infancy; SD: Standard deviation; STAT1: Signal transducer and activator of transcription 1; STING: Stimulator of interferon genes; TBK1: TANK binding kinase; TBS: Tris-buffered saline; Tm: Melting temperature; *TMEM173*: Transmembrane protein 173; TNF: Tumor necrosis factor; TYK2: Tyrosine kinase 2; WB: Western blot; WT: Wild type.

Introduction

Gain-of-function mutations in *TMEM173* encoding STING (stimulator of interferon genes) have been described to cause an autoinflammatory syndrome[1–8] belonging to a recently defined class of disorders referred to as the type I interferonopathies[9,10]. This phenotype, known as STING associated vasculopathy with onset in infancy (SAVI), is characterized by early-onset systemic inflammation with fever, a severe skin vasculopathy leading in some cases to extensive tissue loss, and interstitial lung disease resulting in pulmonary fibrosis and end-stage respiratory failure[1–5,7,8]

To date, twenty-three patients from fifteen families have been reported with gain-of-function mutations in STING[1–8], a key adaptor molecule in the cytosolic DNA-sensing pathway, expressed in various endothelial and epithelial cell types, as well as in haematopoetic cells, such as T cells, macrophages and dendritic cells. Once stimulated, STING induces the transcription of type I interferons (IFN) and the expression of a set of IFN-stimulated genes (ISGs), thus establishing an antiviral state[11–13]. In all of these published cases the heterozygous substitution involves one of three amino acids at positions 147, 154, 155, and 166, being located either in the linker connecting the transmembrane domain of STING to the cyclic dinucleotide domain (CTD) (p.Val147), or within the N-terminal dimerization region of the CTD (p.Asn154, p.Val155 and p.Gly166)[14,15]. It has been suggested that these disease-associated residues play non-redundant roles in retaining the protein on the endoplasmic reticulum (ER), causing STING to constitutively localize to the ER-Golgi intermediate compartment (ERGIC) and activate downstream signaling through the TANK binding kinase (TBK1) - Interferon regulatory factor 3 (IRF3) axis[15,16].

We describe three individuals variably exhibiting the characteristic features of STING-associated autoinflammation. However, in contrast to earlier reports, our data show that the pathology in these patients is due to substitutions at positions 206, 281 and 284 of the human protein, thus implicating a novel region of STING as functionally important in the regulation of type I IFN signaling.

Methods

Patient and study approval

The study was approved by the Comité de Protection des Personnes (ID-RCB/EUDRACT: 2014-A01017-40) and undertaken with written informed parental consent.

Genetic analysis

DNA was extracted from whole blood samples using standard methods. Whole-exome sequencing was performed on genomic DNA from patient 2 (P2) and her mother using SureSelect Human All Exon kit (Agilent Technologies) for targeted enrichment and Illumina HiSeq2000 for sequencing. Sanger sequencing was performed on DNA from patient 1 (P1), patient 3 (P3) and their parents, and from P2 and her mother to confirm the variant found by exome sequencing (Primers in Table EII). Variant frequency data in controls was derived from the Exome Aggregation Consortium (ExAC)[17].

Structural analysis

The experimental 3D structures of STING were extracted from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) and analyzed using Chimera (https://www.cgl.ucsf.edu/chimera/).

Construct generation

Site-directed mutagenesis was performed to generate the desired *TMEM173* variants by using the Q5® Site-Directed Mutagenesis Kit (E0554S from NEB) following the manufacturer's instructions. pMSCV-hygro(+) (Addgene) carrying wild type (WT) *TMEM173* gene was used as a template for the PCR reactions[18]. This plasmid carries the haplotype most frequently recorded in the general population (p.Arg71, p.Gly230, p.Arg232, p.Arg293)[19]. Mutagenic primer sequences were designed using the NEBase Changer software (http://nebasechanger.neb.com/). Cycling parameters are indicated in Table EI. NEB 5-alpha Competent *E. coli* were transformed with the newly synthesized pcDNA, and colonies were screened for the presence of the desired variants (primers in Table EII).

Cell culture procedures

Human embryonic kidney (HEK) 293T cells were grown in 96-well plates at 37°C in 5% CO_2 in DMEM (GIBCO #31966-021) supplemented with 10% (v/v) fetal bovine serum (GIBCO #10270), 100 U/ml penicillin and 100 mg/ml streptomycin (GIBCO #15070-063). At 70% confluency, HEK293T cells were co-transfected with 60 ng of vector pMSCV- hygro(+) either empty or encoding the *TMEM173* variants, 40 ng of IFNß promoter-driven firefly luciferase reporter plasmid (IFNß-pGL3) and 1.4 ng of constitutively expressed renilla luciferase reporter plasmid (pRL-TK) by using TransIT-293 (Mirus #MIR2700 from Euromedex). 24 hours later, cells were stimulated by transfecting 1.3-12 µg/ml 2'3'-cGAMP_STING ligand (tlrl-nacga23, InvivoGen) using lipofectamine 2000 (#11668027 from Thermo Fischer). 24 hours after cGAMP stimulation, cells were lysed with passive lysis buffer 5X (#E194A from Promega) containing protease and phosphatase inhibitors. One third of the lysate for each condition was used for the luciferase assay, and the remaining material for protein analysis by western blotting (WB).

Luciferase assay

The IFNß-pGL3 plasmid was used to measure IFNß promoter activity, and the renilla reporter control plasmid to normalize for transfection efficiency. Luciferase assays were performed using the Dual-Glo® Luciferase Assay System (E2940 from Promega) following the manufacturer's protocol. Luminescence was acquired on a FLUOstar OPTIMA microplate reader (BMG LABTECH). Firefly luciferase activity was normalized against renilla luciferase activity.

Western blot

Bolt LDS Sample Buffer (4X) (#B0008 Novex Life Technologies) and Bolt Sample Reducing agent (10X) (#B0009 Novex Life Technologies) were added to protein lysates, and samples were resolved on 4-12% Bis-Tris Plus gels (# NW04122BOX Invitrogen) transferred to nitrocellulose membrane (#IB23002 Invitrogen). When the phosphorylation status of the protein was investigated, membranes were blocked in 5 % BSA in TBS, and primary phospho-antibodies were incubated overnight in the blocking solution. Otherwise, membranes were blocked with 5 % milk in TBS and primary antibodies were incubated overnight in the blocking solution. Proteins were

blotted with monoclonal anti-STING (R&D MAB7169), anti-Phospho IRF3 Rabbit mAb (Ser396, #4947 Cell Signaling), anti-IRF3 Rabbit mAb (#11904 Cell Signaling) and anti-cofilin Rabbit mAb (#5175 Cell Signaling). Primary antibodies were detected with horseradish peroxidase–conjugated secondary antibody (#7074S and 7076S, Cell Signaling). All western blot images were captured and quantified with a ChemiDoc MP imager and Image Lab software (Bio-Rad Laboratories, Hercules, CA) after adding Clarity Western ECL substrate (Biorad).

STAT1 phosphorylation assay staining

Ficolled PBMCs were treated or not with ruxolitinib 1 μM for 45 minutes at 37°C. Cells were fixed and permeabilized according to PerFix EXPOSE Kit from Beckman Coulter (#B26976) recommendations. Cells were stained for pSTAT1 PE-anti-STAT1 pY701, and cell surface marker (PE-Cy^{TM} 7-CD19, APC-CD3, BV421-CD8) for 1 hour at room temperature. Flow cytometry analysis was performed on a Gallios Beckman Coulter flow cytometer. Results were analyzed using Kaluza software v1.3.

Results

Clinical presentation (Table I, Fig 1)

P1 (F1058) is a 7-year-old female born to non-consanguineous parents of white European ethnicity. She was delivered at term weighing 3.2kg. She presented at the age of 3 months with feeding difficulties and respiratory distress necessitating oxygen therapy (Table I). Computed tomography (CT) scan of the lungs at age 1.5 years revealed extensive ground glass abnormalities, and there was interstitial fibrosis on lung biopsy without signs of vasculitis (Fig 1, *A*). Telangiectatic skin lesions on the cheeks and nose were noted from the age of 4 months (Fig 1, *B*). She has not experienced any involvement of the digits. Limited skin biopsy did not reveal any sign of vasculitis. Elevated transaminases and hepatomegaly prompted a liver biopsy at age 2 years, when the presence of necrotizing granulomatous hepatitis was suggested (intralobular granulomas characterized by necrotizing zones with mixed inflammatory infiltrates). She has demonstrated no further evidence of liver disease. She had a gastrostomy fitted at age 2 years because of poor weight gain, and subsequently suffered recurrent skin infections with poor healing around the entry

site. In light of a continued oxygen requirement and raised inflammatory markers she was treated with immunosuppressants (steroids, methotrexate and anti-TNFα therapy) starting at age 23 months, but with limited efficacy. She remains oxygen dependent with a severe deficit of lung function and markedly limited exercise tolerance.

P2 (F1125) is a 25-year-old female born to non-consanguineous parents of white European ethnicity. There is no family history of note. She demonstrated intra-uterine growth retardation (birth weight at term -3 SD), with subsequent failure to thrive (adult weight / height of 143 cm and 34 kg) requiring a gastrostomy to be placed by age 1 year until puberty. She suffered recurrent bacterial infections of the upper respiratory tract, leading to a severe septicemia at the age of five months (due to *Pseudomonas aeruginosa*) and palatal and nasal septum necrosis. Screening for primary immune deficiencies was negative. A widespread livedo was noted from birth, which worsened gradually with an impressive purpuric aspect in patches on both cheeks and buttocks, and a marked dark purple marbling on the limbs (Fig 1, *C* and *D*). When last seen at the age of 25 years she had lost a large part of the external nares, and demonstrated extreme acrocyanosis of the hands and feet. She has never experienced fevers, and systemic markers of inflammation and autoantibody titers have been consistently normal until recent antinuclear antibodies (ANA) were detected at a low level (1/160). Lung function is normal. She is not currently treated.

P3 (F1802) is a 15-year-old male born to non-consanguineous parents of white European ethnicity. A fixed erythema of the cheeks was noted shortly after birth. Telangiectasia of the cheeks, buttocks and limbs were observed for the first time at age 10 years. From the age of 14 years he has suffered recurrent acrocyanosis moderately influenced by cold temperature, with painful acral erosions that heal as atrophic scars on the ear helices and digits. Lesional gluteal skin biopsies revealed dilated capillaries without obvious vasculitis or thrombosis. He has been treated with nifedipin, pentoxyfillin and acetyl salicylic acid, with limited efficacy. He has never experienced fevers or other features of systemic disease. Blood analysis, including markers of inflammation and autoantibody profile, has been consistently normal. Recent chest CT revealed no evidence of interstitial lung disease. Lung function testing and a six minute exercise tolerance test were normal.

Identification of three new variants in STING

A summary of the molecular data relating to these three patients is given in Figure 2 and Table I. A multiple sequence alignment of STING proteins from various species is provided in Supplementary Figure E1.

P1 (F1058) was observed to carry a c.842G>A (p.Arg281Gln) variant in exon 7 of *TMEM173* (Fig 2, *A*) which has not been recorded on more than 120,000 control alleles on the ExAC database. Both parents were WT at this nucleotide, and microsatellite testing confirmed that the substitution had arisen *de novo*. The arginine at position 281 is conserved in mouse, but is an alanine in *Xenopus tropicalis* and a glutamate in *Danio rerio*. We note a similarly limited degree of conservation of the valine at position 147, substitution of which for a leucine has been previously shown to act as a gain-of-function mutation[1].

P2 (F1125) was observed to carry a c.850A>G (p.Arg284Gly) variant in exon 7 of *TMEM173* (Fig 2, *A*) which has not been recorded on more than 120,000 control alleles on the ExAC database. The mother was WT for this variant. DNA was not available from the father. The arginine is conserved to *Nematostella vectensis*.

P3 (F1802) was observed to carry a c.617G>A (p.Cys206Tyr) variant in exon 6 of *TMEM173* (Fig 2, *A*) which has not been recorded on more than 120,000 control alleles on the ExAC database. Both parents were WT at this nucleotide, and microsatellite testing confirmed that the substitution had arisen *de novo*. The cysteine at this position is generally well conserved, except in *Danio rerio* where it is replaced by an alanine.

3D structural analysis reveals a new mutation hotspot in STING

In order to gain further insight into the effect of the three STING substitutions that we observed in the patients described above, we examined the experimental 3D structures of the CTD of STING (Fig 2, *B*). p.Cys206 is buried within the 3D structure, whilst p.Arg281 and p.Arg284 lie on the surface of the protein. However, these three amino acids are in close proximity and are aligned, with p.Arg284 located in between

p.Cys206 and p.Arg281 (Fig 2, *C* and *D*). The substitution of the cysteine for a tyrosine is predicted to induce a steric clash, which might locally destabilize the 3D structure of the helix bundle and affect the positioning of the 281 / 284 arginine residues.

p.Cys206, p.Arg281 and p.Arg284 mutations in STING induce over-activation of IFNß transcription

In order to determine if these mutations were gain-of-function, we used a luciferase-based IFNß transcription reporter assay to measure their impact on type I IFN induction[1,15,16]. As previously shown, the p.Val155Met and p.Asn154Ser mutants induce reporter activity in the absence of ligand (Fig 2, *E*)[1,2,15]. We observed a similarly robust activation with patient-associated substitutions at p.Cys206Tyr, p.Arg281Gln and p.Arg284Gly (Fig 2, *E*), supporting the hypothesis that these variants are constitutively active.

To investigate whether these three variants lie within a novel functional cluster in STING, we went on to mutate other residues spatially close to the amino acids at 206, 281 and 284. Specifically, we changed p.Asp205 to p.Asp205Tyr and p.Asp205His, p.Leu265 to p.Leu265Tyr, p.Phe269 to p.Phe269Tyr, p.Gln273 to p.Gln273Ala and p.Gln273Leu, and p.Glu282 to p.Glu282Ala and p.Glu282Asp (Fig 3, *A* and *B*). All these amino acids are exposed at the surface of the protein, except p.Leu265 which is buried and in contact with p.Cys206. In contrast to our patient-associated variants, substitution of any of these residues, except for the aspartate at position 205, behaved in a manner similar to WT (Fig 3, *C* and Table EIII). In contrast, substitution of the p.Asp205 resulted in an upregulation of IFNß reporter activity as for the gain-of-function variants (Fig 3, *C* and Table EIII).

Altogether, these results suggest the identification of a novel region implicated in STING regulation, involving at least four amino acids. The fact that all of the different substitutions tested at these residues (two, four, three and three at p.Asp205, p.Cys206, p.Arg281 and p.Arg284 respectively) result in increased IFNß activity indicates that subtle conformational changes are sufficient to disrupt the normal control of the pathway. The specificity of this effect was emphasized by the fact that

four other amino acids, p.Leu265, p.Phe269, p.Gln273 and p.Glu282, located in close proximity in the same tertiary structure did not induce a gain-of-function when substituted.

STING gain-of-function induced by p.Cys206Tyr, p.Arg281Gln and p.Arg284Gly is independent of cGAMP binding

While all previously described STING-associated mutations are located close to the cGAMP binding site, the three novel mutations described here lie on the surface of the protein, without direct contact with this binding site (Fig 2, *B*). In order to determine if the gain of function observed in association with our new mutations resulted from ligand independent constitutive activation, or reflected an increased sensitivity to low levels of endogenous cGAMP, we generated double mutants involving the arginine at 232 which is essential for cGAMP binding[14,15]. For every previously and newly described mutation we observed a similarly robust upregulation of signaling, suggesting that despite clearly distinct localization in the tertiary structure they all confer ligand-independent constitutive activation (Fig 4, *A*).

Furthermore, using an antibody against phosphorylated residue p.Ser396 of IRF3, we recorded increased phosphorylation of IRF3 in cells expressing the three patient-associated mutations, comparable with the previously described p.Val155Met. Consistent with our luciferase results, this increased IRF3 phosphorylation was not impaired by introducing a second mutation at p.Arg232Ala, which abrogates cGAMP binding and IRF3 phosphorylation in the WT state (Fig 4, *B* and E2, *A* and *D*).

STING gain-of-function induced by p.Cys206Tyr, p.Arg281Gln and p.Arg284Gly is independent of the membrane fusion pathway

Recently, it has been reported that a STING-dependent but cGAS-independent pathway can induce type I IFN expression in response to membrane fusion of enveloped RNA viruses[20–22]. In this model, the arginine at 169 in human STING was shown to be important for stimulation of IFN expression by liposomes but not cGAMP, demonstrating that activation by these two stimuli can be mechanistically separated. p.Arg169 is surface exposed, located just outside the STING dimerization

domain and facing away from the cGAMP-binding pocket. To determine if the arginine at position 169 was crucial in the constitutive activation that we observed with our patient-associated mutations, we substituted an alanine for the wild-type arginine at 169 (p.Arg169Ala) and expressed the double-mutants in our *in vitro* system. We observed no effect of the 169 substitution with mutations at 206, 281 and 284, or with the previously described mutations at 154 and 155, indicating that human disease-associated constitutive activation of STING is not dependent on this alternative pathway (Fig 5, *A*). A similar result was obtained with the double mutant p.Asp205His and p.Arg169Ala (Fig E2, *B* and *E*). Consistent with these data, constitutively activated constructs bearing the second mutation p.Arg169Ala demonstrated phosphorylated IRF3 at p.Ser396 in the basal state (Fig 5, *B* and E2, *B* and *E*).

Constitutive activation of newly identified disease-causing STING mutants is dependent on IRF3 phosphorylation

Phosphorylation of STING at p.Ser366 by TBK1 is critical for the recruitment and activation of IRF3 by STING and the subsequent activation of IFN signaling[23,24]. To investigate whether SAVI-associated variants were dependent on TBK1-mediated STING phosphorylation and IRF3 recruitment, we introduced a mutation of serine to alanine at position 366 (p.Ser366Ala) into our mutant constructs. In all cases, this substitution led to an abolition of IFNß activation despite similar expression of single and double STING mutants (Fig 6, *A* and *B*). This was associated with loss of IRF3 phosphorylation, indicating that patient-associated mutations are dependent on phosphorylation at residue 366 for subsequent downstream activation of IRF3 (Fig 6, *B* and Fig E2, *C* and *D*) and transcription of ISGs.

STING constitutive activation leads to increased phosphorylation of STAT1 in T and B cells of P3 and normalize after ruxolitinib treatment *in vitro*

Type I interferons bind to a specific IFNα receptor (IFNAR), thereby activating Janus kinase 1 (JAK1) and Tyrosine kinase 2 (TYK2), which then induce phosphorylation of Signal transducer and activator of transcription 1 (STAT1). We investigated STING constitutive activation in CD4, CD8 and CD19 cells from P3, and observed

increased levels of STAT1 phosphorylation compared to a control (Fig 7). Consistent with published data, this upregulation normalized after 45 minutes of treatment with ruxolitinib *in vitro* (Fig 7)[1,5].

Discussion

Since its identification in 2008 as an adaptor molecule in the cytosolic DNA-sensing pathway, STING has emerged as a central player in antiviral immunity, autoinflammation and cancer[11,13]. Thus, an understanding of the mechanism of action and control of STING in health and disease is of considerable scientific and medical importance.

Here we describe three novel variants seen in association with a phenotype consistent with previously described cases of STING-associated autoinflammation. In two of these patients we were able to show that the amino acid substitutions arose *de novo*, whilst in the third the mother was WT but DNA was unavailable from the father. These substitutions, involving evolutionarily well-conserved residues, have not been described in publically available databases comprising more than 120,000 alleles. As for other molecularly confirmed cases of STING-associated autoinflammation, all three substitutions were associated with the *in vitro* induction of IFNß reporter activity. Considering these observations, it is very likely that the variants we describe represent pathogenic gain-of-function mutations.

In contrast to all previously published cases of type I interferonopathy related to mutations in STING, the three variants reported here do not lie in the linker region connecting the N-terminal transmembrane domain of STING to the CTD or in the first a-helix (a5) of the CTD involved in dimerization. The function of the arginine residues at 281 and 284 is currently undefined. However, these residues are exposed at the surface of the molecule, so that they are unlikely to involve the intramolecular interaction of the STING dimer. A recent survey of STING mutations in the COSMIC database led to the demonstration that a substitution of the arginine at 284 to methionine (p.Arg284Met) was able to induce an IFNß reporter to a significantly greater extent than WT STING[25]. Alternative substitutions of arginine 284 for lysine or threonine (p.Arg284Lys, p.Arg284Thr) also represented hyperactive mutants. The

results of these experiments were further supported by demonstrating increased IFNß in tissue culture supernatants, and an enhanced ability to inhibit viral replication. Of note, the mutation identified in P2 affects this same residue, substituting the arginine for a glycine. Thus, multiple types of amino acid substitution at 284 confer a gain-of-function, indicating a high degree of specificity for the function of an arginine at this position.

Similar to the arginine at 284, we found that different substitutions of the arginine at 281 and the cysteine at 206 also resulted in constitutive activation. It is of note that these three amino acids are aligned at the 3D level, with the arginine at 284 located in between the cysteine at 206 and the arginine at 281. The substitution of the cysteine for a tyrosine at 206 is predicted to lead to steric hindrance and locally disturb the fold, which might affect the positioning of the arginine at 284. However, substituting other less bulky amino acids at 206 had the same effect, highlighting the specificity of the WT cysteine at this position.

In order to define the extent of a putative functionally important region involving the amino acids that we identified through human disease genetics, we went on to mutate a selection of other residues predicted to lie in close proximity with these disease-associated mutations - specifically, p.Leu265, p.Phe269, p.Gln273 and p.Glu282. We also derived a number of conservative and non-conservative substitutions at Asp205, which lies in contact with the p.Arg284. Substitutions at these residues behaved in a similar manner to WT protein, except for those at position 205, where we observed enhanced signaling in our *in vitro* assay similar to the patient-associated mutations. Of note, constitutive activation has been described previously at the equivalent residue in murine STING[12,26].

In regards of cGAMP-dependency, the involvement of a cGAMP-independent signaling pathway and TBK1 downstream signaling, our data did not suggest any difference in the behavior of variants at 206, 281 and 284 compared to previously described disease-associated mutations at 147, 154 and 155. Recently, Dobbs et al. suggested that the latter mutations may disrupt the retention of STING in the ER, resulting in translocation to the ERGIC and activation of TBK1 / IRF3 in the absence of cGAMP binding[15]. Furthermore, these mutations appeared to result in continued

activation, presumed due to a failure of STING degradation. Our data clearly implicate a novel region of STING as important in type I IFN signaling, leading us to speculate that the surface-exposed arginine residues at 281 and 284 may also be involved in ER retention, or in binding to a negative regulator of STING[25].

Our results indicate that mutations at residues 206, 281 and 284 of human STING are responsible for the disabling autoinflammation observed in the three patients that we describe. Given the favorable effects seen with JAK1/2 inhibition in other patients with STING gain-of-function mutations, these patients may similarly benefit from such a therapeutic approach[5]. Alternative potential treatment strategies might include ER exit blocking agents, palmitoylation inhibitors or TBK1 antagonists[15,27–29]. Considering that a p.Ser366Ala substitution abrogated the constitutive activation seen with every patient-associated mutation tested in our *in vitro* model, TBK1 blockade might be relevant to all SAVI patients.

A major aspect of the pathology due to mutations in *TMEM173* appears to relate to a dysfunction of endothelium consequent upon an upregulation of type I IFN signaling. The explanation for variable expression observed between, and indeed within families, will require further study, with such observations likely reflecting differential environmental exposures and / or modifying genetic factors. Indeed, whilst the cGAS-STING cytosolic DNA signaling pathway is essential for the induction of an effective anti-viral response, human genetics is teaching us about the risk of inflammatory disease associated with variation in this system. Polymorphisms in STING, and other molecules involved in cytosolic nucleic acid recognition, can confer differences in signaling to a type I IFN response - a non-binary situation where a balance is struck between anti-viral priming and the risk of autoinflammation[19,30]. Elucidation of the mechanisms of STING regulation is thus of great importance in understanding both microbial pathogenesis and inflammation. The findings presented here suggest a previously unappreciated aspect of the control of STING in this context.

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Conflict of interest

The authors declare no conflict of interest.

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Figure Legends

TABLE I. Clinical and molecular data.

ANA: Antinuclear Antibodies; ANCA: Anti-neutrophilic cytoplasmic antibodies; F: Female; M: Male

FIG 1. Clinical and radiological phenotypes. **A**. High resolution chest CT scan of P1 demonstrating evolution of bilateral alveolar and interstitial disease with ground-glass lesions and interlobular septa thickening (at ages 3, 5 and 8). Improvement of interstitial lung disease between ages 3 and 5 years was observed after oral steroid treatment. **B**. Telangiectatic skin lesions on the cheeks and nose of P1. **C**. Purpuric aspect to patches on both cheeks of P2 at age 1.5 years. **D**. Widespread livedo and marked dark purple marbling on the limbs of P2 at age 1.5 years. y: years.

FIG 2. Constitutively activated mutations of *TMEM173*. **A**. Schematic representation of the *TMEM173* protein highlighting the four transmembrane domains (TM1, 2, 3 and 4), the cGAMP binding domain (CBD) and the C terminal tail domain (CTD), as well as four phosphorylation sites (P) at p.S345, p.S358, p.S366 and p.S379. In black, previously described mutations in exon 5 (p.V147L; p.V147M; p.N154S; p.V155M). In red, 3 novel mutations in exons 6 and 7 (p.C206Y: P3; p.R281Q: P1; p.R284G: P2). **B**. Ribbon representation of the 3D structure of the STING CTD dimer in complex with cyclic-di-GMP (PDB reference 4ef4), indicating the position of the three novel amino acids found mutated in this study, as well as amino acids previously reported with mutations and involved in the CTD dimer interface, at the bottom of the ligand binding site. **C**. Focus on the 3D aligned positions of p.C206, p.R281 and p.R284. **D**. Surface representation of the 3D structure of the STING CTD dimer (orthogonal view relative to panel B), showing the accessibility of the three amino acids with patient-associated substitutions (p.C206, p.R281 and p.R284). **E**. IFNß-Luc reporter assay. HEK293T cells were transfected with IFNß-Firefly Luc, Renilla Luciferase and indicated human STING plasmids (EV: Empty vector; WT: Wild type STING; p.N154S p.V155M; p.C206Y; p.R281Q; p.R284G) and stimulated with increasing doses of cGAMP (none; 4 µg/µl; 12 µg/µl). Luciferase activity was determined by Dual-luc assay 24 hours post-stimulation. Mean and SDs shown, representative of 5 different experiments in triplicate.

FIG 3. Analysis of substitutions conferring constitutive activation of STING. **A**. and **B**. Localization of amino acids on surface and ribbon representations of the STING dimer 3D structure (PDB reference 4ef4)[31]. Colored amino acids in which mutations confer constitutive activation: p.D205 in green, p.C206 in yellow, p.R281 in red and p.R284 in blue. Amino acids where substitutions did not confer constitutive activation are shown in grey. **C**. IFNß-Luc reporter assay. HEK293T cells were transfected with IFNß-Firefly Luc, Renilla Luciferase and indicated human STING plasmid (EV: Empty vector; WT: Wild type STING; p.N154A; p.V155M; p.D205Y or H; p.C206Y, F, L or S; p.R281Q, K or V; p.R284G, H or K) and stimulated with increasing doses of cGAMP (none; 4 µg/µl; 12 µg/µl). Luciferase activity was determined by Dual-luc assay 24 hour post-stimulation. Mean and SDs shown, representative of 3 different experiments in triplicate.

FIG 4. Constitutive activation conferred by p.C206Y, p.R281Q and p.R284G is independent of cGAMP binding. **A**. IFNß-Luc reporter assay. HEK293T cells were transfected with IFNß-Firefly Luc, Renilla Luciferase and indicated human STING plasmid (EV: Empty vector; WT: Wild type STING; p.V155M; p.C206Y; p.R281Q; p.R284G with or without a second mutation p.R232A) and stimulated with increasing doses of cGAMP (none; 4 µg/µl; 12 µg/µl). Luciferase activity was determined by Dual-luc assay 24 hours post-stimulation. Mean and SDs shown, representative of 4 different experiments in triplicate. **B**. Immunoblot (IB) analysis of STING, phosphorylated IRF3 (p.S396), total IRF3 and cofilin in whole cell lysates of HEK293T cells transfected with empty vector (EV), wild type (WT) human STING with or without cGAMP stimulation, p.V155M, p.C206Y, p.R281Q and p.R284G (unstimulated), either alone or in combination with a second mutation at p.R232A. Of note, in HEK293T cells transfected with WT plasmids, the amount of STING was greater at basal state than upon stimulation with cGAMP, which is consistent with a model of STING degradation, possibly through an autophagic-mediated mechanism[24].

FIG 5. Constitutive activation conferred by p.C206Y, p.R281Q and p.R284G mutants is independent of non-canonical pathway STING engagement. **A**. IFNß-Luc reporter assay. HEK293T cells were transfected with IFNß-Firefly Luc, Renilla Luciferase, and indicated human STING plasmid (EV: Empty vector; WT: Wild type

STING; p.N154S; p.V155M; p.C206Y; p.R281Q; p.R284G with or without a second mutation p.R169A) and stimulated with increasing doses of cGAMP (none; 4 µg/µl; 12 µg/µl). Luciferase activity was determined by Dual-luc assay 24 hours post-stimulation. Mean and SDs shown, representative of 4 different experiments in triplicate. **B**. Immunoblot (IB) analysis of STING, IRF3 phosphorylated at p.S396, total IRF3 and cofilin in whole cell lysate of HEK293T cells transfected with empty vector (EV), wild type (WT) human STING with or without cGAMP stimulation, p.N154S, p.V155M, p.C206Y, p.R281Q and p.R284G unstimulated, with or without double mutant p.R169A.

FIG 6. Activation of newly identified disease-causing STING mutants relies on IRF3 phopshorylation. **A**. IFNß-Luc reporter assay. HEK293T cells were transfected with IFNß-Firefly Luc, Renilla Luciferase, and indicated human STING plasmid (EV: Empty vector; WT: Wild type STING; p.V155M; p.C206Y; p.R281Q; p.R284G with or without a second mutation p.S366A) and stimulated with cGAMP (12 µg/µl) or not. Luciferase activity was determined by Dual-luc assay 24 hours post-stimulation. Mean and SDs shown, representative of 3 different experiments in triplicate. **B**. Immunoblot (IB) analysis of STING, IRF3 phosphorylated at p.S396, total IRF3 and cofilin in whole cell lysate of HEK293T cells transfected with empty vector (EV), wild type (WT) human STING with or without cGAMP stimulation, p.V155M, p.C206Y, p.R281Q and p.R284G unstimulated, with or without a second mutation p.S366A.

FIG 7. STAT1 phosphorylation status in CD4, CD8 and CD19 cells before and after treatment with ruxolitinib *in vitro*. Increased basal STAT1 phosphorylation was observed in CD4, CD8 and CD19 cells from P3, which normalized after *in vitro* treatment with the JAK1/2 blocker ruxolitinib.

TABLE EI. Primer sequences used for site directed mutagenesis.

TABLE EII. *TMEM173* primer sequences.

TABLE EIII. Summary data of substitutions of STING tested *in vitro*. WT: Wild

type.

FIG E1. Multiple sequence alignment of STING proteins. Sequences are designated using UniProt identifiers. The regular secondary structures, as observed from experimental 3D structure (pdb 4ef4) are reported at the top. MAFFT and ESPript were used for building and rendering of the multiple alignment[32,33]. BOVIN: Bovine; XENTR: *Xenopus tropicalis*; DANRE: *Danio rerio,* NEMVE: *Nematostella vectensis*.

FIG E2. Double mutant p.D205H with p.R232A, p.R169A or p.S366A. **A**, **B** and **C**. IFNß-Luc reporter assay. HEK293T cells were transfected with IFNß-Firefly Luc, Renilla Luciferase, and indicated human STING plasmid (EV: Empty vector; WT: Wild type STING; p.D205H with or without a second mutation p.R232A, p.R169A or p.S366A respectively) and stimulated with increasing doses of cGAMP (none; 4 µg/µl; 12 µg/µl). Luciferase activity was determined by Dual-luc assay 24 hours post-stimulation. Mean and SDs shown, representative of 2 different experiments in triplicate. **D** and **E**. Immunoblot (IB) analysis of STING, IRF3 phosphorylated at p.S396, total IRF3 and cofilin in whole cell lysate of transfected HEK293T transfected with wild type (WT) human STING with or without cGAMP stimulation and p.D205H unstimulated, with or without double mutant p.R232A, p.S366A or p.R169A.

***** Uncharacterized band. Of note, in HEK293T cells transfected with WT plasmids, the amount of STING was greater at basal state than upon stimulation with cGAMP, which is consistent with a model of STING degradation, possibly through an autophagic-mediated mechanism[24].