Impaired STING activation due to a variant in the E3 ubiqitin ligase AMFR in a patient with severe VZV infection and hemophagocytic lymphohistiocytosis

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Research Article

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Abstract

Varicella zoster virus (VZV) is a neurotropic alphaherpesvirus exclusively infecting humans, where it causes two distinct pathologies: varicella (chickenpox) upon primary infection and herpes zoster (shingles) following reactivation. In susceptible individuals, VZV can give rise to more severe clinical manifestations, including disseminated infection, pneumonitis, encephalitis, and vasculopathy. Here we describe a 3-year-old boy with severe VZV infection involving the central nervous system, subsequently triggering longstanding hemophagocytic lymphohistiocytosis (HLH). We found that the patient carries a rare monoallelic variant in *autocrine motility factor receptor AMFR* encoding an ubiquitin ligase involved in innate cytosolic DNA sensing and interferon (IFN) production through the cyclic GMP-AMP synthase – stimulator of IFN genes (cGAS-STING) pathway. Peripheral blood mononuclear cells (PBMCs) from the patient exhibited impaired signaling downstream of STING in response to the cGAS agonists 2'3'-cGAMP and dsDNA, as well as decreased IFN induction in response to herpes virus. VZV replication in patient PBMCs was found to be slightly increased compared to healthy controls. Overexpression of the variant *AMFR* p.R594C resulted in decreased K27-linked STING ubiquitination compared to expression of WT *AMFR*. This work links defective AMFR-STING signaling to severe VZV disease and hyperinflammation and suggests a direct role for cGAS-STING in control of viral infections in humans.

Introduction

Varicella zoster virus (VZV) is a neurotropic alphaherpesvirus exclusively infecting humans, where it causes two distinct pathologies: varicella (chickenpox) upon primary infection and herpes zoster (shingles) following reactivation [1]. VZV spreads via inhalation of infectious droplets, and infects mononuclear cells in the tonsils, leading to viremia through infected T cells and dissemination to the skin to replicate and cause the characteristic vesicular eruptions known as varicella [1]. Importantly, VZV establishes latency in sensory neuronal ganglia with the potential to cause reactivation to the skin as zoster or to the central nervous system (CNS) manifesting as meningitis, encephalitis, cerebellitis or vasculopathy with stroke [2, 3]. About 20% of patients hospitalized with chickenpox experience neurological complications [4], suggesting a correlation between impaired systemic control of the infection and development of severe varicella with dissemination to the CNS. Knowledge on the pathogenesis of VZV infection and immunity remains incomplete, and although implementation of a VZV vaccine with the Oka strain in certain countries have resulted in reduced frequency of VZV cases, this infectious disease remains a worldwide health problem [5].

VZV is an enveloped DNA virus, which can infect epithelial cells, peripheral blood mononuclear cells (PBMCs), and neurons [1, 6]. The genome of VZV comprises 71 known *open reading frames (ORF)s*, of which the *VZV latency-associated transcript (VLT)* transcript is the only viral gene transcribed during latency, together with a *VLT-ORF63* fusion transcript [7, 8]. Unrelated clinical VZV isolates exhibit only little variability in virulence [9], suggesting that defective host immunity is the major determinant of disease severity rather than differences between virus strains. The precise determinants of protective immunity towards VZV is incompletely understood, in part because VZV is a strictly human pathogen and limited

data are available from humanized mice studies [10]. Studies regarding inborn errors of immunity (IEI) that cause increased susceptibility to VZV are therefore of particular importance. From these studies, it is clear that cellular immunity mediated by natural killer (NK) cells and T cells play a particular important role during VZV infection, as individuals with IEI affecting NK and T cell function may present with severe and disseminated VZV infections [11–13]. Moreover, genetic defects in interferon-γ-receptor 1 (IFNGR1) and tyrosine kinase (TYK) 2 interfere with macrophage function and were found to predispose to disseminated infection with mycobacteria and VZV [14, 15]. Finally, genetic defects in the innate cytosolic DNA sensor RNA polymerase III (POL III) were identified and demonstrated to cause selectively increased susceptibility to VZV CNS infection and pneumonitis in otherwise healthy children and adults [16–18].

The innate immune system utilizes pattern recognition receptors (PRRs) to detect pathogen–associated molecular patterns (PAMP)s in order to mount protective immune responses. This includes production of cytokines and interferons (IFN)s, the latter exhibiting antiviral activity through their ability to induce IFN-stimulated genes (ISGs) [19]. Different classes of PRRs are involved in recognition of virus infections, including membrane-associated Toll-like receptors (TLR)s, cytosolic RNA-sensing retinoic acid-inducible gene 1 (RIG-I)-like receptors, and finally cytosolic DNA sensors [19]. Within the group of DNA sensors, TLR9 detects unmethylated DNA, RNA polymerase III (POL III) recognizes AT-rich DNA, while absent in melanoma (AIM)2, gamma-interferon-inducible protein 16 (IFI16) and cyclic GMP-AMP synthase (cGAS) senses double-stranded (ds) DNA in a sequence-independent manner [20, 21]. Among these, cGAS has emerged as the main cytosolic DNA sensor, signaling through the adaptor molecule Stimulator of Interferon Genes (STING) to induce mainly type 1 IFN responses but also to some extent nuclear factor kappa B (NF-kB) activation [22].

Recent reports have described the occurrence of haemophagocytic lymphohistiocytosis (HLH) during VZV infection in the setting of IEI [23]. HLH is a rare but life-threatening clinical syndrome characterized by uncontrolled activation of immune cells and severe systemic inflammation [24]. Familial (or primary) HLH (fHLH) is a genetic disorder most often caused by autosomal recessive loss of function mutations in genes that disrupt the cytotoxic activity of NK and CD8 + T cells, including *PRF1, UNC13D, STX11, STXBP2, RAB27A, LYST, AP3B1, SH2D1A* and *BIRC4* [25]. While autosomal recessive primary HLH presents early in childhood, hypomorphic mutations in HLH genes are generally associated with fHLH onset in adulthood [26]. Sporadic (or secondary) HLH develops without a known underlying genetic cause, but rather presents in association with malignancies, autoimmune or auto inflammatory diseases, or triggered by infections [27, 28]. Interestingly, both familial and sporadic HLH might present with an infectious trigger, and herpesviruses are the most common causes of virus-induced HLH, with Epstein-Barr-virus (EBV) and cytomegalovirus (CMV) accounting for more than 50% of virus-associated HLH [27]. Similarly, VZV can trigger HLH upon primary infection or reactivation, although this is mostly reported in patients with underlying immunosuppression or fHLH [23, 29–31].

Here we describe a patient with severe clinical manifestations of VZV infection and HLH, whom we studied in great detail in order to understand the underlying genetic and immunological pathogenesis. We hypothesize that severe VZV disease in some otherwise healthy individuals may be caused by monogenic

inborn errors of IFN immunity, not necessarily displaying complete clinical penetrance. Based on the current knowledge on VZV infection pathogenesis, innate and adaptive immune circuits, and HLH-associated molecules, we searched whole exome sequencing (WES) data for rare gene variants that may underlie susceptibility to severe VZV infection and/or HLH. In this patient we identified a monoallelic variant in the gene encoding the ubiquitin ligase autocrine motility factor receptor (AMFR) which regulates cGAS-STING signaling through STING poly-ubiquitination. We propose that defects in AMFR, which impair cGAS-STING-mediated sensing of foreign DNA, confer enhanced susceptibility to severe disseminated VZV infection and risk of secondary hyperinflammation.

Methods

WES and bioinformatics; confirmation by Sanger sequencing

(See supplementary material)

In vitro stimulations of PBMCs

Patient and control PBMCs were thawed and seeded in RPMI supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin (cRPMI) at a density of $5*10^5$ cells/well for infection experiments, and $1*10^6$ cells/well for stimulation with 2'3'-cGAMP and dsDNA, and incubated overnight at 37°C and 5% CO_2. Cells were infected with 50 HAU of Sendai virus (SeV) (Cantell strain, Charles River), 3 MOI of HSV-1 (KOS Strain) or 0.2 MOI of cell-free (CF) VZV debris (ROKa strain) or CF debris mock, containing the same chemicals as used to propagate CF VZV debris. For CF VZV debris infection, cells were washed twice 4 hrs post infection and incubated in Hank's Balanced Salt Solution (HBSS) for the remaining time. Following 24 hrs of infection, supernatants were harvested for Mesoscale and cells lysed for RNA isolation. In addition, PBMCs were stimulated with 100 µg/mL of 2'3-cGAMP (Invivogen, #tlrl-nacga23-1) or 2 µg/mL of transfected ht (herring testes) dsDNA for 3 hrs following cell lysis for Western blotting.

RNA isolation qRT-PCR

Total RNA was purified using Nucleospin 96 RNA core kit (Macherey-Nagel, #740466.4) and reverse transcribed into cDNA using Iscript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad, #1725035) with relevant primers as described in supplementary methods.

Mesoscale measurements of cytokines and interferons in supernatants

Expression of IFNs (IFNα2a, IFNβ, IFNλ1 and IFNγ) and proinflammatory cytokines (IL-1β and TNFα) were measured in cell culture supernatants using U-PLEX assays (Meso Scale Diagnostics) according to manufacturer's protocols on a Meso Quickplex SQ 120 instrument.

STING immunoprecipitation

HEK293T cells were seeded at a density of 2.5 *10^6 cells in 6 cm petri dish and following overnight incubation, transfected with 1 ug of the following plasmids: pcDNA3/FLAG-STING, pRK5/HA-K27-Ubiquitin and pcDNA3/Myc-AMFR WT or pcDNA3/Myc-AMFR R594C. Controls transfected only with FLAG-STING, HA-K27-Ubiquitin and pcDNA3-Empty vector (No AMFR control), or HA-K27-Ubiquitin, AMFR WT and FLAG-Empty vector (No STING control) were included. See supplementary methods for experimental details.

Western blotting

For PMBC stimulations, cells were washed twice with PBS and lysed in RIPA buffer (Thermo Fischer Scientific, #89901) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fischer Scientific, #78440) and Benzonase Nuclease (Sigma Aldrich, #E1014-25KU). Protein concentrations were measured using Pierce BCA protein Assay Kit (Thermo Scientific, #23227) and cell lysates were denatured at 95°C for 5 min with 50 mM DTT (Sigma Aldrich, #43816-10ML) and 4xLaemmli buffer (Bio-rad, #1610747). Samples were subjected to SDS gel electrophoresis and transferred to a PVDF-membrane using the Transfer-Blot Turbo systems. (See supplementary methods for specifications on antibodies).

Statistics

The Mann-Whitney rank sum test (Graphpad Prism 9) was used to determine if there was a statistical significant difference between inductions in patient and pooled controls.

Results

Case description: 3 year old boy with disseminated VZV infection and prolonged hemophagocytic lymphohistiocytosis.

The patient was a 3-year-old boy admitted to hospital and diagnosed with severe disseminated varicella infection with high fevers and impaired consciousness. Initially the boy was treated with intravenous acyclovir, but due to progressive disease he was transferred to a tertiary center, where extensive and progressive systemic inflammation, splenomegaly and hemophagocytosis in the bone marrow was identified. CNS infection was suspected but lumbar puncture performed several days into the disease course did not reveal pleocytosis or presence of VZV in the CSF. HLH with fever, hemophagocytosis in the bone marrow, and paraclinical findings fulfilling the HLH2004 criteria was diagnosed and treated according to protocol. WES was performed and initially analyzed for presence of variants in a gene panel consisting of 291 PID and HLH related genes, but no known disease causing mutations were identified and immunological analysis also did not demonstarte any significant abnormalities in cytotoxic NK cell or T cell function consistent with fHLH. Despite reasonable response to treatment, the boy experienced

long-standing inflammation with elevated inflammatory markers and persistent finding of activated macrophages with hemophagocytosis in the bone marrow, until after 40 weeks at which point therapy was finally discontinued. When reexamined four months later hemophagocytosis had disappeared. Aciclovir prophylaxis was discontinued 16 months after the initial infection and the boy finally recovered and is presently without any infections or inflammation at the age of 8 years. However, based on the severe and prolonged infectious and inflammatory disease course, we hypothesize that the patient may have an unrecognized IEI predisposing to severe VZV infection, which in turn led to the development of hyperinflammation and secondary HLH.

Identification of a variant in the E3 ubiquitin ligase AMFR predicted to be deleterious

As the initial diagnostic sequencing analysis did not identify any mutations within a panel of known PID and HLH related genes, we went on and performed a full and unbiased WES to look for the presence of rare genetic variant that might have contributed to disease in the patient. We based the analysis on a hypothesis of autosomal dominant (AD) inheritance with possible incomplete penetrance. During the analysis, variants were kept if they were rare (frequency < 0.1%), predicted to be pathogenic as defined by a CADD score above 15 and above the mutation significance cutoff (MSC) score, and had relevance to antiviral immunity and/or inflammation. Remaining variants (Supplementary Table 1) were manually inspected to establish the biological function of each variant-encoding gene. This approach led to the identification of a rare, monoallelic missense variant in the *AMFR* gene, predicted to be deleterious by various bioinformatic prediction tools, including Polymorphism phenotyping v2 (PolyPhen-2), sorting intolerant from tolerant (SIFT) and CADD. In addition, the gene damage index (GDI) Phred score indicated low tolerance for damaging loss of functional variants within the *AMFR* gene (Fig. 1A). The variant was confirmed by Sanger sequencing of patient genomic DNA (Fig. 1B) and relatives were also Sanger sequenced revealing the presence of the variant in the infant sister and in the brother and mother, suggesting AD inheritance with incomplete penetrance (Fig. 1B-C). The variant, *AMFR* c.1780C > T results in an amino acid substitution of the highly conserved arginine to cysteine residue at position 594 within the G2BR domain of the molecule (Fig. 1A and D). AMFR/gp78 is an E3 protein ubiquitin ligase embedded in the endoplasmic reticulum (ER) membrane, important in degradation of misfolded proteins in the ER-associated degradation (ERAD) process [32]. The protein consists of five N-terminal transmembrane domains, a RING domain, a Cue domain and C terminal G2BR and VIM domains (Fig. 1E). Upon microbial DNA challenge, AMFR catalyzes K27-linked polyubiquitination of STING, and this ubiquitin chain creates an anchoring platform for recruiting and activating Serine/Threonine-protein kinase (TBK)1, resulting in IFN regulatory factor (IRF)3 phosphorylation and IFN-β induction [33]. Examination of PBMC lysates revealed normal AMFR protein levels in patient PBMCs compared to healthy controls (Fig. 1F). These data together suggested a potentially disease causing role of the identified AMFR variant and prompted further functional analyses for functional validation and establishment of the molecular mechanism involved in increased susceptibility to VZV infection and development of HLH in the patient.

Antiviral immune responses and VZV replication in patient PMBCs

To evaluate the functional impact of the AMFR variant in patient cells, we next measured antiviral and inflammatory responses in patient PBMCs (harvested when the patient was asymptomatic) infected with cell free (CF) VZV in vitro. However, surprisingly we observed VZV-induced production of IFN-α2a, IFN-β, IFN-λ1, IFNγ, and IL-1β to be similar in patient and healthy controls, although IFN-β induction in patient cells was in the lower part of the spectrum (Fig. 2A-E), whereas TNF-α production in response to VZV infection was increased in patient PBMCs compared to healthy controls (Fig. 2F). To examine the degree of viral control in patient cells, we measured VZV open *ORF* mRNA induction in CF VZV-infected PBMCs using expression of these ORFs as a measure of viral replication. Here, we observed a tendency towards increased *ORF* expression in patient cells, as the means of *ORF9* and *ORF40* mRNA expression were higher in the patient compared to the means of VZV infected pooled healthy controls, although without reaching statistical significance (Fig. 2G-I).

Interestingly, when we infected patient PBMCs with the closely related alphaherpesvirus HSV-1, we found statistical significantly decreased expression of IFN-α2a, IFN-β, IFN-γ and TNF-α in cell culture supernatants of patient PBMCs compared to healthy control (Fig. 3A-F). Moreover, this trend was not observed when patient PBMCs were subjected to infection with SeV, a single stranded RNA virus, in which case patient cells responded with normal production of type I, II and III IFNs and proinflammatory cytokines as compared to healthy controls (Fig. 3A-F). Collectively these data indicate a specifically impaired antiviral immune response to HSV-1 and possibly VZV, the latter being more difficult to ascertain due to the highly cell-associated nature of VZV, as well as a tendency towards increased VZV replication in patient PBMCs compared to healthy controls.

Patient cells exhibit impaired signaling downstream of STING in response to DNA ligands

Based on our observation of impaired antiviral immune response in patient cells, combined with the identification of an *AMFR* variant with the potential to interfere with cGAS-STING signaling, we next investigated STING-related signaling pathways in patient PBMCs. To this end we stimulated cells with 2’3’-cGAMP or transfected dsDNA for 3 hrs and performed immunoblotting on whole cell lysates. This experimental set-up revealed markedly reduced phosphorylation of STING and the downstream pathway components TBK1 and (to a minor extent) IRF3 in patient PBMCs compared to controls (Fig. 4A-B). Importantly, induction of ISG15 protein was clearly reduced in patient PBMCs (Fig. 4A-B).

Reduced K27-linked STING polyubiquitination in HEK293T cells expressing mutant AMFR

To further investigate the mechanisms underlying decreased STING phosphorylation and activation in patient cells, and to clarify the functional role of the identified AMFR p.R594C variant on STING signaling,

we went on to express Myc-tagged *AMFR WT* and the *AMFR p.R594C* variant in HEK293T cells together with tagged STING and K27-ubiquitin. We then examined the impact of the AMFR variant upon STING K27-linked polyubiquitination in a two-step immunoprecipitation (IP) assay of STING. When we examined the immunoprecipitates by western blotting, we found markedly reduced STING K27-polyubiquitination in cells expressing AMFR p.R594C compared to AMFR WT transfected cells, and this observation was consistent after the first as well as the second IP (Fig. 4C). Collectively these data suggested that the AMFR p.R594 variant in the patient resulted in impaired STING ubiquitination and signaling, causing decreased downstream phosphorylation and activation of TBK1 and IRF3, ultimately resulting in reduced antiviral responses and possibly impaired control of VZV infection in the patient.

Discussion

The major finding of the present study is the description of a novel predisposition to severe viral infection involving abnormal AMFR-mediated polyubiquitination and decreased activation of STING in a child with severe disseminated VZV infection and HLH. To our knowledge, this is the first report of a defect in STING signaling in humans causing increased susceptibility to viral infection.

The cGAS-STING pathway is now appreciated as the major driver of the antiviral IFN response, after the initial discovery of STING [34], and subsequent discovery of cGAS [35]. Other DNA sensors, including RNA POL III, have been identified by many independent groups, but their respective roles in innate immunity are not fully clarified, and may be dependent on cell type and cellular context [16, 36, 37]. cGAS recognizes foreign and host-derived dsDNA in a sequence independent manner and catalyzes the formation of 2’3’-cGAMP that serves as an activator of the ER-bound adaptor protein STING, which traffics to the Golgi, where it recruits TBK1 thus leading to phosphorylation of STING on serine 366, and this in turn leads to recruitment of IRF3 [38]. This positions IRF3 for phosphorylation by TBK1 allowing dimerization, nuclear translocation and induction of IFN-β gene expression [39]. The role of ubiquitination in STING biology is well documented and includes multiple types of ubiquitin linkage [40]. For instance, K63 ubiquitination by TRIM56, TRIM32, and Mul1 is important for activation of signaling [41–43] and is targeted by HSV-1 to facilitate CNS infection [44]. Moreover, K48 ubiquitination by RNF5 and RNF90 has been reported to promote STING degradation and to down-regulate signaling [45, 46]. Finally, K27-linked ubiquitination of STING by AMFR promotes STING ER-to-Golgi trafficking [33], which is a rate-limiting step in STING signaling [47], and promotes activation of the pathway and antiviral defense [33]. AMFR/g78 is an E3 protein ubiquitin ligase embedded in the ER membrane, important for degradation of misfolded proteins in the ER-associated degradation response (ERAD).

Upon microbial DNA challenge, AMFR catalyzes K27-linked polyubiquitination of STING, and this ubiquitin chain creates an anchoring platform for recruiting and activating TBK1, resulting in IRF3 phosphorylation and IFN-β induction [33]. The missense variant causing an amino acid substitution from arginine to cysteine is localized at the well-preserved position 594 within the G2BR domain, which is responsible for binding to the E2 ubiquitin conjugating enzyme UBE2G, thus facilitating interaction between the RING domain and E2 enzyme and subsequently ubiquitin transfer. Deletion of the G2BR

domain was reported to abolish ERAD function of AMFR [48], and a pathogenic variant in this domain would therefore be expected to cause disturbed AMFR-mediated functions, most notably defective ubiquitination and activation of STING [33] as also demonstrated in the present study. Whether the AMFR-STING defect is relatively specific to VZV (and HSV-1), or alternatively involves other DNA viruses remains to be resolved. Collectively, the precise role of cGAS STING-mediated DNA sensing and IFN induction in humans remains to be determined.

The existence of defective innate DNA sensing predisposing to VZV infection, possibly through impaired signaling in response to the presence of viral cytosolic DNA, is similar to the previously reported IEI affecting another cytosolic DNA sensor RNA Polymerase III, which has been described in children and adults with severe VZV CNS encephalitis, vasculopathy and recurrent VZV meningoencephalitis [16–18]. Therefore, defective DNA sensing may be a common theme in VZV predisposition and might act in a partially cell type dependent manner. In the case of POL III deficiency, with CNS involvement constituting a prominent feature, we hypothesized that a particularly important role of POL III in recognizing the AT-rich VZV genome may be at play in certain cell types, such as neuronal cells, in which cGAS expression is low/absent [16, 17]. On the other hand, one study identified an important role of STING in mounting type I and Type III IFN responses to VZV in human dermal fibroblasts and HaCaT keratinocytes with potential implications for varicella pathogenesis and suggesting an important role of cGAS-STING in the skin [49]. Recently, the cGAS-STING DNA sensing pathway was demonstrated to be required for IFN induction and VZV restriction during VZV infection in THP1 cells and the VZV protein ORF9 was reported to antagonize cGAS-STING signaling and IFN production [50]. The observation that POL III dominates as VZV sensor in mononuclear cells might in fact explain why we do not observe significantly decreased IFN induction in PBMCs in response to VZV, and only could measure a modest increased in VZV replication in this cell type from the patient.

Previously, Goldbach-Mansky and associates described a vascular and pulmonary syndrome in patients with gain-of-function variants in the STING-encoding gene TMEM173 and suggested the name STING-associated vasculopathy with onset in infancy (SAVI) for this autoinflammatory interferonopathy [51]. The authors identified three different mutations in exon 5 of TMEM173 in these six patients and observed constitutive STAT-1 phosphorylation and increased constitutive and inducible type I IFN expression in patient cells [51]. Since then, several other publications have described additional patients and extended the clinical phenotype [52, 53]. Moreover, other IEIs also affecting IFN production, such as STAT2 deficiency, translate into partially overlapping clinical manifestations together with the interferonopathy signature [54, 55]. However, despite the description of numerous IEIs affecting pathogen and PAMP receptors and their downstream IFN-inducing signaling pathways causing susceptibility to severe viral infections, defects in the cGAS-STING pathway in humans, has not been previously reported. The clinical and cellular phenotype described here is significantly different from the one ascribed to SAVI, and notably we did not observe increased IFN levels in vitro under the given conditions examining patient cells harvested after acute illness. However, we cannot entirely exclude that elevated ISGs may have been present in the circulation of the patient during the acute episode, and thus we cannot rule out a degree of

autoinflammation/interferonopathy caused by the AMRF variant and possibly associated to the development of VZV-triggered HLH in the patient.

In conclusion, the present work contributes with information on the question on the role of DNA sensors in host defense in human immunology. Together with our previous work on POL III, the available data suggest that POL III and cGAS-STING may each play non-redundant roles in VZV immunity in humans depending on the cell type and tissue involved. Collectively, the precise role of cGAS STING-mediated DNA sensing and IFN induction in humans remains to be further studied. Identification of human IEIs involving these molecules remain a powerful tool for establishing the contributions of relevant immune signaling pathways in humans and for gaining valuable insights into how dysregulated cGAS-STING signaling may lead to human disease, ranging from autoinflammatory interferonopathy to severe viral infection.

Declarations

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Authorship contributions and statement

THM and MMT conceived the idea; NF, cared for patients and/or collected and isolated patient material; MMT, MCT, AKH, JL and JH performed experiments and analyzed data together with THM BZ, SRP and KA. THM wrote the first draft of the manuscript with input from MMT, NF, and KA; all authors contributed, read, commented and approved the final version of the manuscript.

Conflicts of interests/Competing interests

The authors declare no conflicts of interests or competing interests.

Data and material availability statement

All data related to this study will be shared at the request of other investigators for purposes of replicating procedures and results, according to national and international GDPR rules and following individual DTA and MTA rules with relevant investigators.

Ethics

The patient, family, and healthy controls were included following oral and written consent in accordance with The Helsinki Declaration and national ethics guidelines and after approval from the Danish National Committee on Health Ethics (# 1-10-72-275-15), the Data Protection Agency, and Institutional Review Board.

Consent to participate and consent for publication

The patient and family have provided consent to participate and consent for publication.

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Figures

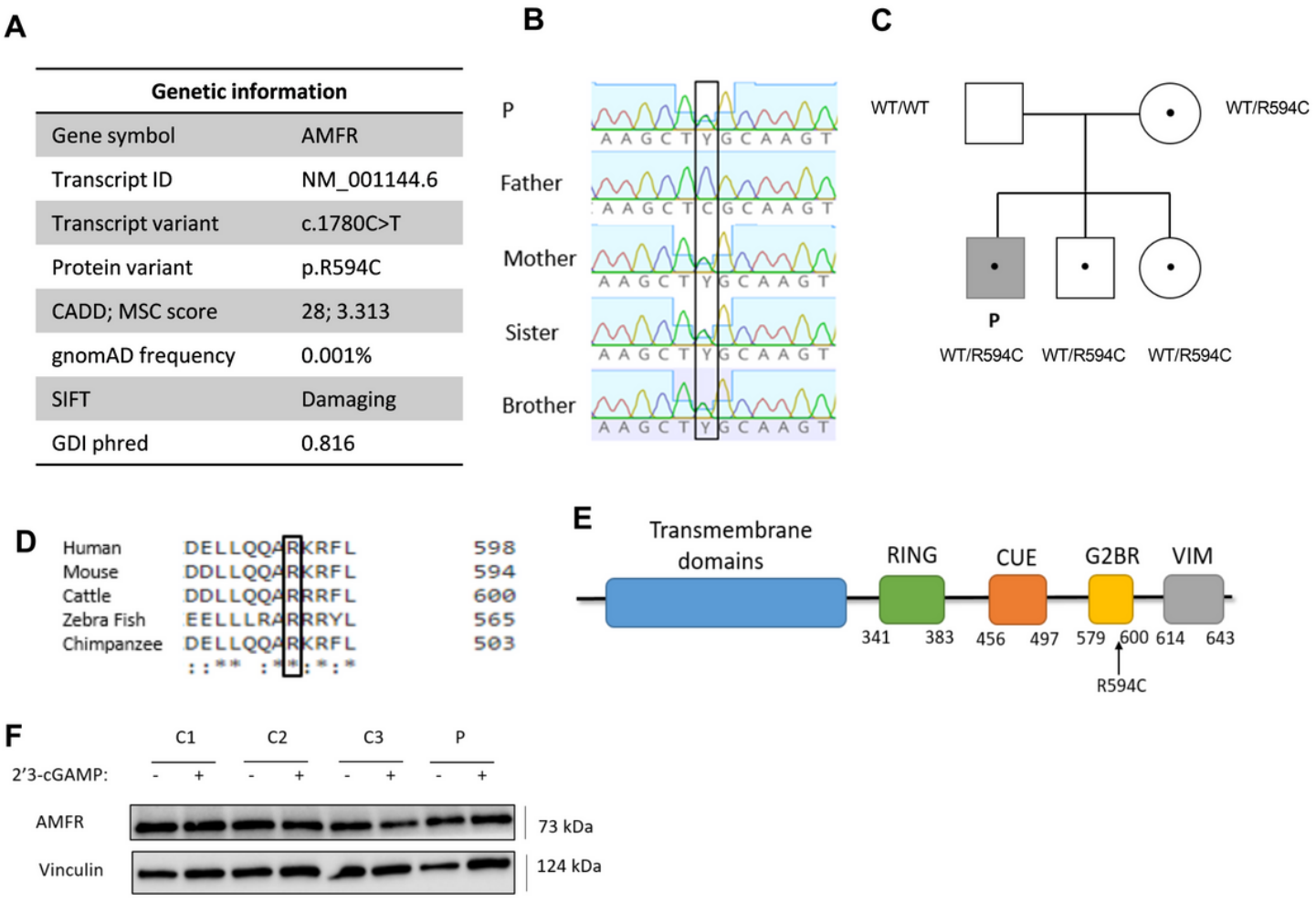

Figure 1

Identification of a rare genetic variant in *AMFR* in a patient with severe VZV infection and HLH. A) Characteristics of the monoallelic *AMFR* variant. **B)** Sanger sequencing confirming the presence of the *AMFR R594C* variant in the patient (P) and the patient's mother and siblings. **C)** Pedigree showing heterozygous inheritance of the *AMFR R594C* variant inherited from the mother. Dot indicates carriers of the variant. **D)** Protein alignment showing conservation of Arginine at position 594 in AMFR across different species. **E)** Protein structure of AMFR with localization of the R594C variant in the G2BR domain. **F)** Western blotting showing similar AMFR protein levels in PBMC lysates from P1 and three healthy controls, unstimulated or stimulated with 100 ug/mL 2'3-cGAMP. Vinculin was used as loading control. CADD, Combined Annotation Dependent Depletion; MSC, Mutation Significance Cutoff; SIFT, Sorting Intolerant From Tolerant; gnomAD, The genome aggregation database; GDI, Gene Damage Index; RING, Really Interesting New Gene; CUE, Coupling of ubiquitin conjugation to ER degradation; G2BR, Ube2g2-binding region; VIM, p97/VCP-interacting motif.

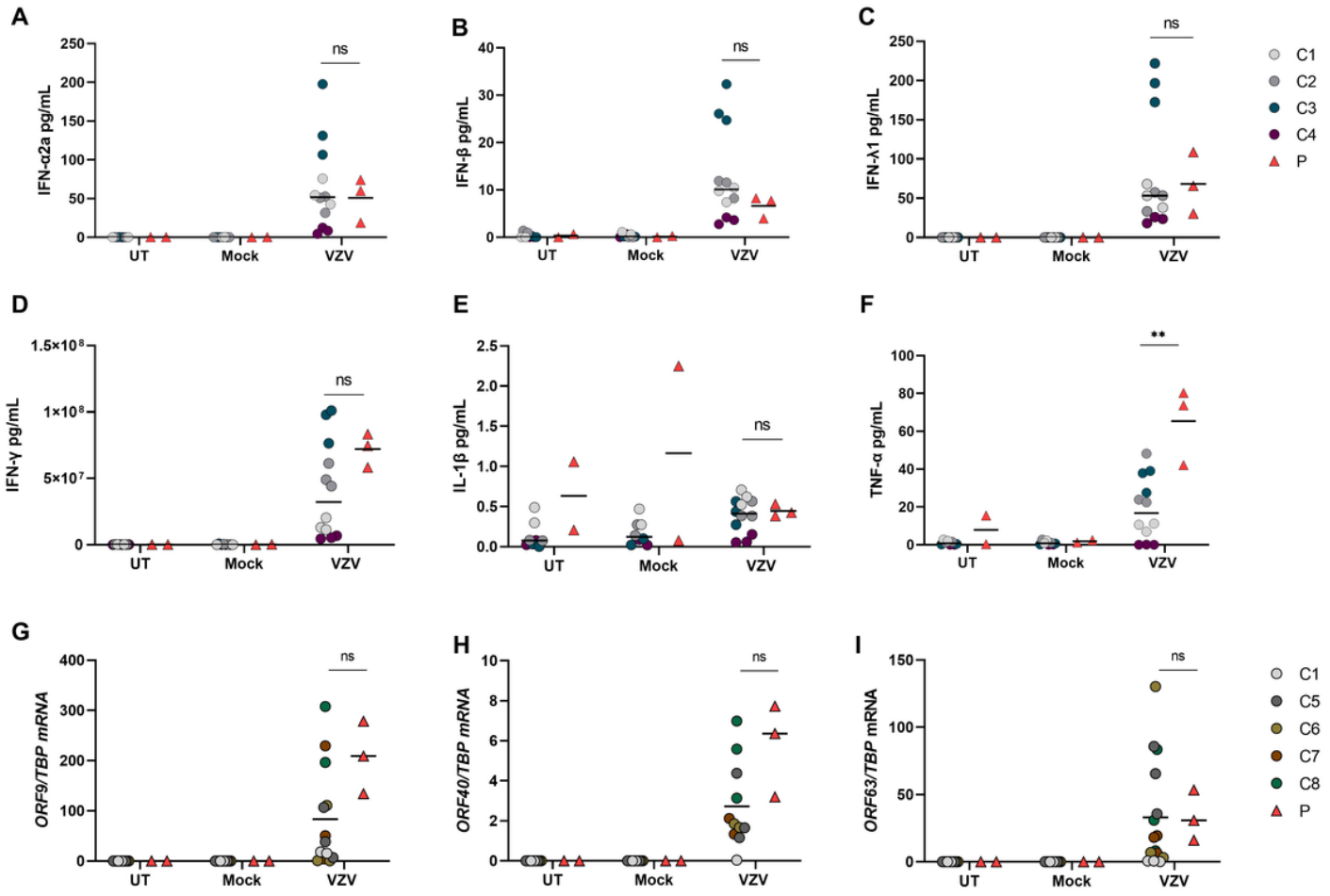

Figure 2

Antiviral and proinflammatory immune responses and viral replication in patient cells infected with cell-free VZV. PBMCs from P and healthy controls were infected with cell-free (CF) VZV debris (moi o.2) for 24 hrs and supernatants were examined for the expression of IFN-α2a **(A),** IFN-β1 **(B)** IFN-λ1 **(C),** IFN-γ **(D),** IL-1β (**E**) or TNF-α (**F**) using Mesoscale U-plex assays. **G-I)** VZV infected PBMCs were lysed and RNA purified and subjected to RT-qPCR for the expression of VZV *open reading frame gene* (*ORF*)9 **(G)**, *ORF40* **(H)** and *ORF63* **(I)**. *ORF* mRNA were normalized to *TBP* levels, and statistics calculated using the nonparametric Mann-Whitney Rank-sum test. Ns, non-significant; ** ≤0.01. The experiment is representative of three independent experiments.

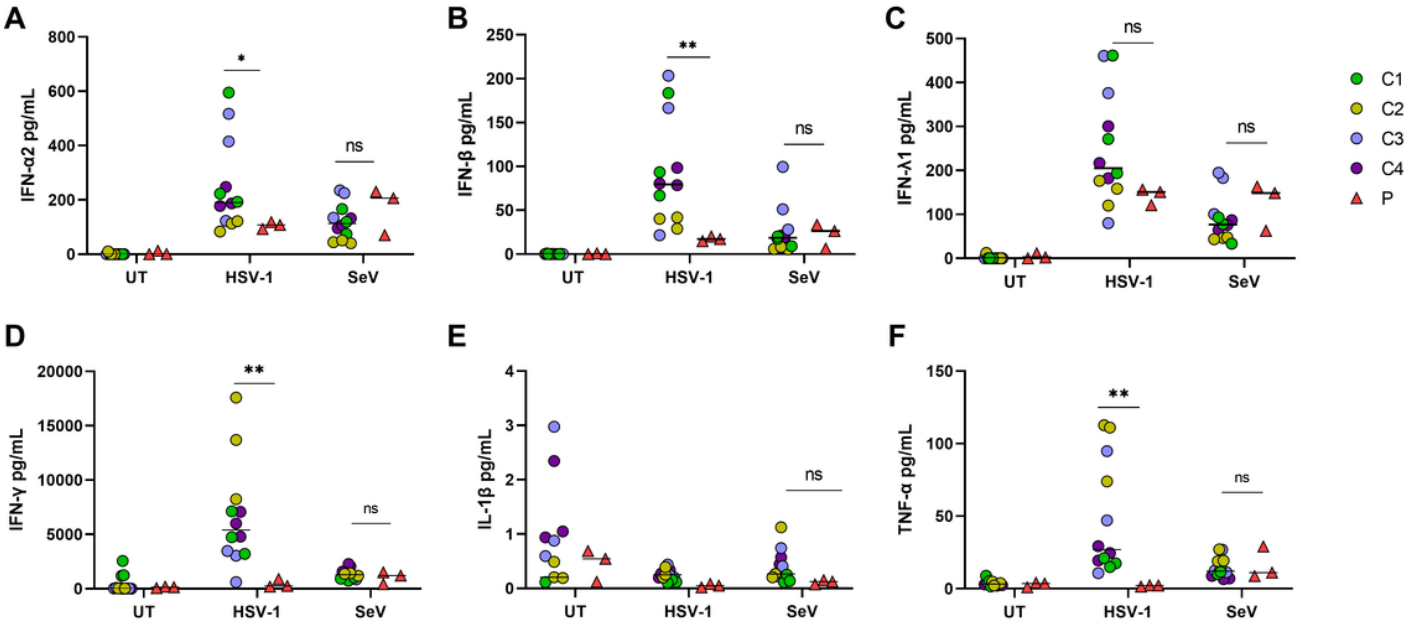

Figure 3

Induction of IFNs and proinflammatory cytokines in patient PBMCs in response to HSV-1 and SeV infection. PBMCs were infected with HSV-1 (MOI 3) or SeV (50 HAU), and supernatants harvested 24 hrs post infection, and expression of IFN-α2a **(A),** IFN-β1 **(B)** IFN-λ1 **(C),** IFN-γ **(D),** IL-1β (**E**) or TNF-α (**F**) were measured using Mesoscale U-plex assays. Statistical significance was calculated using the Mann-Whitney Rank-sum test. Ns, non-significant; * ≤ 0.05, ** ≤ 0.01. The experiments were performed twice.

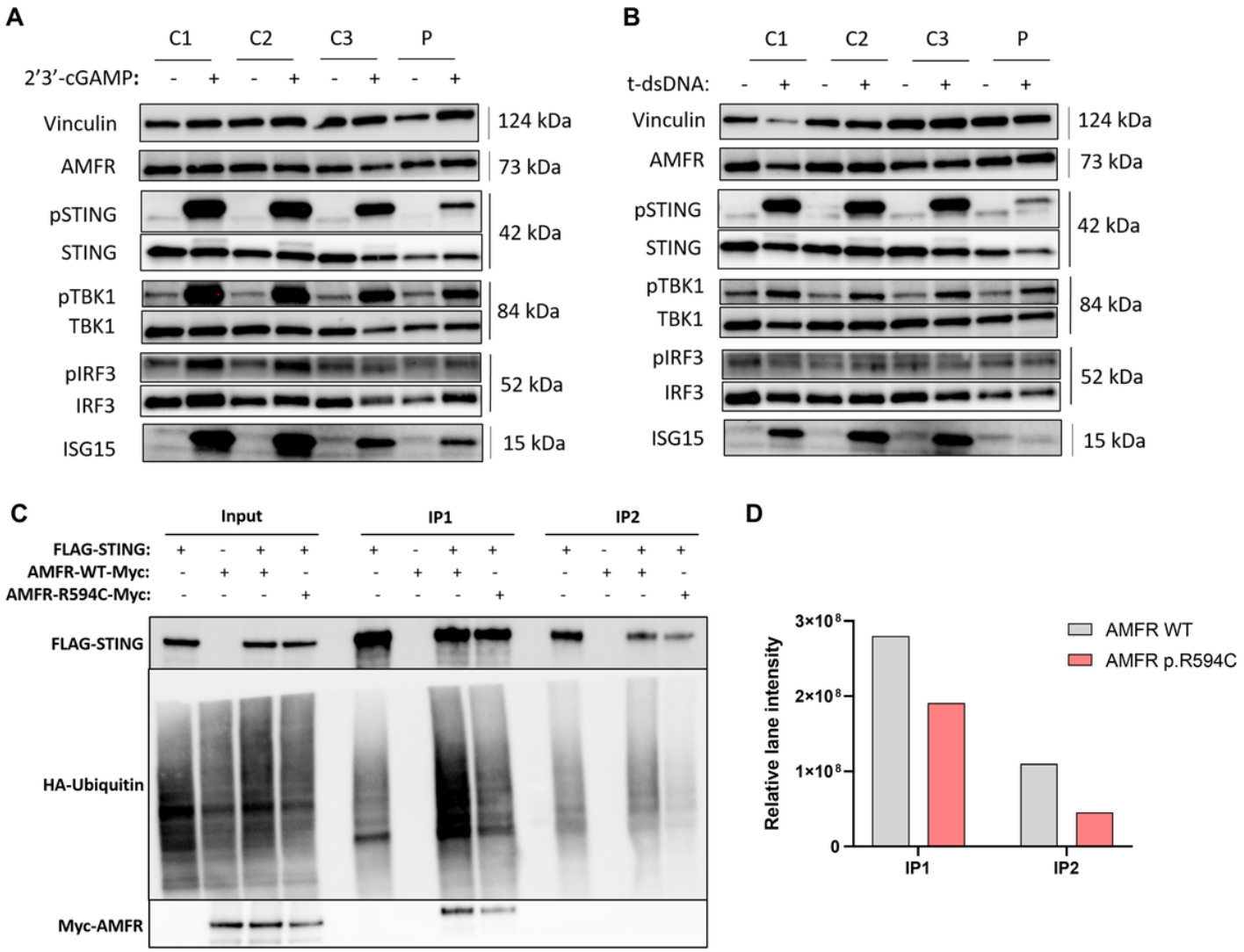

Figure 4

Reduced STING signaling in patient cells and decreased K27-linked ubiquitination of STING in HEK cells overexpressing AMFR R594C compared to WT. PBMCs from the patient and three healthy controls (C1-C3) were stimulated with 100 ug/mL of 2'3'-cGAMP **(A)** or 2 ug/mL transfected dsDNA **(B)** for 3 hrs and lysates were subjected to Western blotting for the expression levels of AMFR, pSTING, STING, pTBK1, TBK1, pIRF3, IRF3, ISG15, and Vinculin (loading control). **C)** HEK293T cells were transfected with 1 ug of FLAG-STING, HA-K27 ubiquitin, and AMFR-Myc-WT or AMFR-Myc-R594C for 24 hrs, after which STING was immunoprecipitated twice with anti-FLAG-M2 beads. Lysates were immunoblotted for expression of FLAG-STING, HA-(K27) Ubiquitin and Myc-AMFR. **D)** Quantification of HA-ubiquitin expression in IP1 and IP2 were performed in ImageLab. The experiments are representative of three independent experiments.

Supplementary Files

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- SupplementaryMaterial280622final.docx