Multisystem Inflammation and Susceptibility to Viral infections in Human ZNFX1 Deficiency

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Key words

ZNFX1, type I interferon, susceptibility to viral infections, HLH-like disease, virally induced hepatitis, thrombotic microangiopathy, leukoencephalopathy, brain calcification, interstitial lung disease

Abstract

Background: The recognition of viral nucleic acids is one of the primary triggers for a type I interferon-mediated antiviral immune response. Inborn errors of type I interferon immunity can be associated with increased inflammation and/or increased susceptibility to viral infections, as a result of dysbalanced interferon production. NFX1-type zinc-finger-containing 1 (ZNFX1) is an interferon-stimulated double-strand RNA sensor that restricts the replication of RNA viruses in mice. ZNFX1's role in the human immune response is not known.

Objective: We studied 15 patients from 8 families with an autosomal recessive immunodeficiency characterized by severe infections by both RNA and DNA viruses and virally triggered inflammatory episodes with hemophagocytic-lymphohistiocytosis-like disease, early-onset seizures, as well as renal and lung disease.

Methods: Whole exome sequencing was performed on 13 patients from 8 families. We investigated the transcriptome, post-transcriptional regulation of interferon-stimulated genes (ISGs) and predisposition to viral infections in primary cells from patients and controls stimulated with synthetic double-stranded nucleic acids.

Results: Deleterious homozygous and compound heterozygous *ZNFX1* variants were identified in all 13 patients. Stimulation of patient-derived primary cells with synthetic double-stranded nucleic acids was associated with a deregulated pattern of expression of ISGs and alterations in the half-life of ISGs mRNA and was associated with poorer clearance of virus infections by monocytes.

Conclusion: ZNFX1 is an important regulator of the response to double-stranded nucleic acids stimuli following viral infections. ZNFX1 deficiency predisposes to severe viral infections and a multisystem inflammatory disease.

Clinical Implications

ZNFX1 deficiency should be considered in patients with severe viral infections and signs of virally triggered hemophagocytic-lymphohistiocytosis-like disease with hepatitis, encephalopathy, interstitial lung disease, and/or microangiopathy.

Capsule summary

ZNFX1 deficiency in humans affects the type I interferon response and predisposes to severe viral infections and multisystem inflammatory damage.

Abbreviations

ADV: Adenovirus

ARDS: Acute respiratory distress syndrome

BM: Bone marrow

CASP8: Caspase 8

CCL4: C-C Motif chemokine ligand 4

CCL5: C-C Motif chemokine ligand 5

CMV: Cytomegalovirus

CSF: Celebrospinal fluid

CXCL10: C-X-C Motif chemokine ligand 10

DRB: 6-dichlorobenzimidazole 1-β-D-ribofuranoside

ds: double-stranded

EBV: Epstein-Barr virus

FADD: Fas Associated via death domain

FLAIR: Fluid-attenuated inversion recovery

GFP: Green fluorescent protein

HHV6: Human herpes virus type 6

HLH: Hemophagocytic lymphohistiocytosis

HSCT: Hematopoietic stem cell transplantation

IFN: Interferon

IL-6: Interleukin 6

ISG: Interferon-stimulated gene

LDH: Lactate dehydrogenase

MAVS: Mitochondrial antiviral signaling protein

MDA5: Melanoma differentiation-associated protein 5

MOF: multiorgan failure

MOI: Multiplicity of infection

MPGN: Membranoproliferative glomerulonephritis

MRI: Magnetic resonance imaging

NFκB: Nuclear factor kappa B

NK: Natural killer cells

PAMP: Pathogen associated molecular pattern

PBMC: peripheral blood mononuclear cell

PCR: Polymerase chain reaction

PML: Promyelocytic leukemia protein

RIG-I: Retinoic acid-inducible gene I

SAP: SLAM-associated protein

SHISA5: Shisa family member 5

STING: Stimulator of interferon response cGAMP interactor

TMA: Thrombotic microangiopathy

TLR3: Toll-like receptor 3

VSV: Vesicular stomatitis virus

VZV: Varicella zoster virus

WES: Whole Exome Sequencing

XIAP: X-linked inhibitor of apoptosis

ZNFX1: NFX1-type zinc-finger-containing 1

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Introduction

Studies of patients showing susceptibility to specific viral infections have helped to elucidate critical pathways in innate and adaptive immunity. Pathogenic variants in genes that disrupt type I and III interferon (IFN) immune responses (e.g. *TLR3*, *UNC93B*, *IRF7*, and *IRF9*) have been found in patients with severe herpes simplex virus type 1 encephalitis, influenza A, and SARS-CoV2 infections (1-5).

NFX1-type zinc-finger-containing 1 (ZNFX1) is a highly conserved IFN-stimulated double-strand (ds)RNA sensor that restricts the replication of RNA viruses in mice (6) and contributes to trans-generation inheritance in *C. elegans* by binding to mRNA complexed with short, non-coding RNAs (7). ZNFX1 expression is low in uninfected cells but is rapidly upregulated in response to viral infections and exposure to type I IFNs (8). ZNFX1 binds to viral RNA and interacts with the mitochondrial antiviral signaling protein (MAVS), promoting the expression of IFN-stimulated genes (ISGs). Signaling downstream of ZNFX1 does not depend on two other MAVS-associated cytosolic viral sensors (retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5)) (6). Although studies of ZNFX1-deficient mice and cell lines identified a role for the protein in sensing dsRNA, Furthermore,the protein's putative role in the human immune response was undefined.

Here, we describe the clinical and molecular features of biallelic ZNFX1 deficiency in 13 patients and two clinically affected (but not genotyped) siblings from eight unrelated kindreds. This early-onset disease is characterized by susceptibility to viral infections, multi-organ dysfunction, and a high mortality rate indicating the critical role of ZNFX1 in human immunity. Our experimental data demonstrate that ZNFX1 is required for the balanced induction of ISGs downstream of double stranded nucleic acid sensing in human primary cells.

Methods

Study participants

Written, informed consent was provided on behalf of all study participants by their parents. The 15 patients described hereafter came from Iraq (n=3 patients), Syria (n=2), Turkey (n=4), Germany (n=2), Australia (n=2), Egypt (n=1), and Canada (n=1). For details of individual patients, please refer to the “Patient Clinical History” section and the accompanying Tables in the Supplementary Appendix.

Whole exome sequencing (WES)

WES was performed on 13 of the affected individuals and in their parents and siblings as specified. DNA was extracted from blood samples collected in EDTA tubes. Standard methods were used to generate the WES library and to filter and prioritize nuclear single-nucleotide variants and indel variants (see the Methods section of the Supplementary Appendix).

Functional assays

Quantitative polymerase chain reaction (qPCR) assays, Western blots, immunofluorescence imaging, viral infection, flow cytometry, and transcriptomic analysis were performed according to the standard protocols detailed in the Methods section of the Supplementary Appendix.

Results

Severe inflammatory disease and increased susceptibility to viral infections

We investigated 15 patients from 8 families. The patients were abnormally susceptible to viral infections and presented with early-onset, systemic, severe, acute inflammatory disease associated with major dysfunctions of the liver, brain, kidneys, and lungs (Table I, Table S1 and Fig. 1A and B). The severe infections were caused by RNA viruses (influenza A virus (-ssRNA, n=5), influenza B virus (-ssRNA, n=1), parainfluenza virus (-ssRNA, n=1), respiratory syncytial virus (-ssRNA, n=2), norovirus (+ssRNA, n=2), rotavirus (dsRNA, n=1)) or DNA viruses (human herpes virus 6 (n=3), adenovirus (n=2), and cytomegalovirus (n=1)) (Table I). Hence, most of these pathogens were negative single-strand (ss)RNA or DNA viruses. It is noteworthy that live virus vaccines also caused severe vaccine strain infections in 2 patients (measles and VZV, respectively) (Table S2). A rotavirus infection relapsed within a few weeks in patient (P) 2.2, and HHV6 was detectable (with a variable copy number) for 5 months in P5.2. Although ongoing disease manifestations in these two patients can be attributed to persistent or relapsing viral infections, other patients showed progressive disease even after the virus had been cleared (P8.1) in the apparent absence of infectious agents (P1.2 and P6.1).

The mortality rate was high: 11 of the 15 patients died in childhood, with 7 deaths before the age of 3 months (Fig. 1A, Table S1). The mean age at death was 3.6 years (median: 1.1 year; range: 3 months to 15 years). Inflammatory episodes with hepatitis and cytopenia were fatal in seven cases (age at death: 0.3 to 8 years). Sepsis was reportedly the cause of death in P6.1 (at the age of 9 years). P1.2 died of necrotizing pulmonary aspergillosis at the age of 15, five years after lung transplantation. The cause of death was unknown for P1.1 and P3.1 (Clinical histories are shown in Supplementary Appendix).

Infections leading to severe inflammatory diseases were the initial presentation in 9 of the 15 patients and were present in 12 of the 15 patients at some point in the course of disease. The systemic inflammatory disease was characterized by episodes of cytopenia and hepatitis. The cytopenia was characterized by anemia in 1 individual and anemia with thrombocytopenia in 12 individuals. In 8 individuals, anemia and anemia with thrombocytopenia were combined with a high leukocyte count (23.8–50.0x10^9/L), neutrophilia, and lymphocytosis. Other initial presentations were seizures (n=3 patients), renal disease (n=2), and lung disease (n=1).

Fourteen patients had hepatic disease, as evidenced by elevated serum liver enzyme levels (n=12 patients), hepatomegaly (n=13), elevated serum LDH levels (n=10), coagulopathy (n=7), and hepatic encephalopathy (n=1) (Table S3). In 11 patients, hepatic disease was associated with systemic inflammatory disease. Three patients met the criteria for acute liver failure (Table S3). Histologic assessment of the liver showed heterogeneous, non-specific changes, such as necrosis and extramedullary hematopoiesis (in P1.2), necrosis and lymphocytic infiltration (in P2.1), necrosis and nodular regenerative hyperplasia (in P4.2), and centrilobular necrosis (in P5.2) (Fig. S1).

Six of the 12 patients with systemic inflammatory disease met the diagnostic criteria for hemophagocytic lymphohistiocytosis (HLH), including hemophagocytosis in bone marrow aspirates (Table II, Fig. 1C). Some patients experienced more than one HLH- or HLH-like episodes, which were associated with hepatitis and leukocytosis. The latter is much less common in classical HLH. Natural killer (NK) cell degranulation and/or cytotoxicity was normal in all patients with HLH. The level of perforin expression was in the lower normal range in P5.1 and P5.2; this was probably due to a heterozygous p.Ala91Val variant in *PRF1* also carried by their healthy father (data not shown). Spontaneous remission of systemic inflammation was observed in some

patients, immunosuppressants were administered to others - with varying degrees of success (Table S1). The JAK inhibitor ruxolitinib was administered in one patient (P5.2) and had a beneficial but transient effect.

Neurological involvement was observed in 10 patients, of whom 7 experienced recurrent seizures. In three cases, the seizures occurred during an episode of HLH (P4.1, P7.1, and P8.1). Three patients showed developmental regression (P4.2, P7.1, and P8.1). Neuroimaging evidenced multiple focal calcifications in three patients (P1.2, P1.3, and P8.1), ischemic lesions (diffusion restriction on MRI) in four (P3.2, P4.1, P5.1, and P7.1), and T2 hyperintense lesions in five (P1.2, P3.2, P4.2, P 5.2, and P7.1) (Fig. 1D and Fig. S2). Leptomeningeal enhancement was observed in P3.2 and P4.1 during an episode of HLH. Autism spectrum disorder was diagnosed in two patients (P4.2 and P8.1).

Lung disease was present in 13 of the 15 patients. Acute respiratory distress syndrome occurred in 7 patients and was mostly associated with viral infections. Recurrent lower respiratory tract infections were observed in six cases (P1.1, P1.2, P1.3, P2.1, P2.2, and P8.2). One patient experienced two episodes of respiratory syncytial virus bronchiolitis with respiratory failure within the space of a few weeks (P2.2). Six patients had pulmonary hemorrhage. Chest CT and a histopathologic assessment of lung biopsies from P1.2 and P1.3 showed interstitial pneumonitis and cholesterol pneumonitis, respectively (Fig. 1E and Fig. S3).

There was evidence of renal involvement in 12 patients, including histologically proven thrombotic microangiopathy (TMA) in P2.1, P5.2, and P8.1 (Table I, Fig. 1F and Fig. S4). We variously observed hemolytic uremic syndrome (P2.1), membranoproliferative glomerulonephritis (P6.1), nephrotic syndrome (P4.2, P6.1, and P8.1), mild proteinuria (P2.2), and transiently elevated creatinine with glomerulosclerosis, tubular atrophy and interstitial fibrosis at autopsy (P1.2). Renal

failure (in the context of multiorgan failure) occurred in another 4 patients. Taken together, although peripheral destruction might have contributed to the bi-cytopenia e.g. in cases with TMA, we think that HLH overweighs as the driving force for the bi-cytopenia.

One patient (P4.2) underwent allogeneic hematopoietic stem cell transplantation (HSCT) at the age of 3 years. Five years later, he is in good health but still has a significant developmental delay. Brain MRI of this patient showed that the white matter changes present at the age of 32 months had stabilized at the age of 42 months (i.e. 6 months after HSCT) and had even regressed 5 years after HSCT (Fig. 2). Although the patient has made developmental progress since the HSCT, he continues to show developmental delay and has been diagnosed with autism. Another survivor (P2.1, now aged 14) has renal disease but never experienced HLH-like disease. The third and fourth survivors P7.1 (now aged 3) and P8.2 (now aged 7 years) are stable, although both show severe neurologic impairments. P7.1 is receiving immunoglobulin replacement therapy but P8.2 is not receiving any immunomodulatory treatments at all.

Biallelic ZNFX1 variants in the patients

We identified 11 biallelic *ZNFX1* variants in 13 patients by WES (i.e. in all eight families studied; Fig. 3A). There were five truncating variants and six missense variants. In all patients, *ZNFX1* was the only candidate gene that segregated with the disease. Only one variant (p.C1264S) is listed in the Genome Annotation Database (https://gnomad.broadinstitute.org/) as being heterozygous, with a frequency of 1.22×10^{-5}. All missense variants were predicted to be deleterious by several tools, including CADD, PROVEAN Polyphen-2 and SIFT (Table S4).

ZNFX1 is a 1918 amino-acid multidomain protein comprising a large helicase domain with an ATP-binding site (9) and a DEAD helicase box (10), six zinc fingers, and a coiled-coil region (Fig. 3B). The large helicase domain is homologous to the human RNA helicase Aquarius involved in RNA splicing (11). The spatial distribution of the patients' four missense variants within the RNA helicase motif are shown in the three-dimensional model of ZNFX1 in Fig. 3C.

ZNFX1 mRNA is ubiquitously expressed in human tissues, albeit predominantly in the hematopoietic system (Fig. S5). Low ZNFX1 protein expression was noted in fibroblasts under resting conditions, while a rapid upregulation was observed after 24 h of stimulation with transfected poly(I:C) or poly(dA:dT) (Fig. 3D). ZNFX1 could not be detected in whole cell extracts of fibroblasts from two of the patients carrying biallelic stop codons (p.R900Mfs*5/p.H542Cfs*41 in P2.1 and p.K133*/p.K133* in P3.2), while low levels of ZNFX1 could be detected in extracts from stimulated dermal fibroblasts isolated from P5.1 bearing one missense variant (p.C1264S) and one C-terminally truncating variant (E1727Kfs*11). Conceivable lower molecular weight forms of ZNFX1 were not detected with this approach.

Impaired viral clearance and skewed ISG-expression in ZNFX1-deficiency.

Since ZNFX1-deficiency was associated with severe viral infections in the patients, we evaluated the capability of patient's cells to initiate an antiviral interferon response leading to elimination of infection with vesicular stomatitis virus (VSV) or influenza virus in vitro. Indeed, after pre-stimulation through transfection of poly(I:C),) (lyovec poly(I:C)), P2.1 monocytes were less efficient in clearing VSV when compared with control monocytes (Fig. 4A and B). In notable contrast, the baseline expression of ISGs seen in peripheral blood isolated from patients (Fig onceivable limited sample A), was higher than in controls. This difference was biologically relevant since it was associated

with a moderate resistance of unstimulated patient monocytes to the VSV and influenza virus infections (Fig. S6B).

The defective ability of the patient's monocytes to establish a fully competent antiviral defense program in monocytes following stimulation with intracellular poly(I:C) could not be attributed to a generally weak response to intracellular double-stranded nucleic acids. In patients derived dermal fibroblasts stimulated with intracellular poly(I:C) or poly(dA:dT), we found an enhanced expression of the interferon sensitive genes (ISGs) *IFIT1* and *OAS2* (Fig S7A and C). Transfection with poly(dA:dT) also caused increased rate of expression of *IFIT2*, while transfection with poly(I:C) did not affect the expression pattern of this ISG. On the other hand, patients' fibroblasts exposed to poly(I:C) in solution failed to increase the expression of *IFIT1* and *IFIT2* to the levels observed in control fibroblasts under the same conditions (Fig S7B).

Transcriptomic analysis of dermal fibroblasts derived from 4 patients and 4 controls (treated with intracellular or soluble dsRNA or dsDNA) confirmed qPCR data showing increased rate of expression of ISGs in response to intracellular double stranded nucleic acids (Fig. S7A and C) as evidenced by overexpression of ISGs involved in antiviral responses (Fig. 4C). Although treatment with soluble poly(I:C) (non lyovec poly(I:C) confirmed qPCR data (Fig. S7B) showing a marked reduction in the expression of most ISGs involved in antiviral defense (Fig. 4C), it was associated with elevated levels of expression of ISGs known to modulate the p53-dependent apoptosis pathways (PML and SHISA5). Analysis of pathways belonging to the canonical sensing of intracellular and extracellular double stranded nucleic acid sensing revealed that intracellular poly(I:C) caused a heightened fold expression in ISGs belonging to the RIG-I-MAVS pathway in patients' fibroblasts compared to controls' fibroblasts (Fig. 4D). Consistent with an upregulation of this pathway, we observed elevated transcript levels of cytokines such as IL-6, CXCL10, CCL4, CCL5 and IFNβ. Stimulation with

soluble poly(I:C) instead resulted in lower fold induction of type I interferons and other NF-κB responsive ISGs (Fig 4E) in patients' fibroblasts. Interestingly, the rates of expression of transcripts encoding known apoptosis-inducing proteins (FADD and CASP8) were upregulated in poly(I:C) stimulated patients' fibroblasts, consistent with the known role of some components of the TLR3 signaling pathway in inducing dsRNA-induced cell death through caspase-8. Stimulation of patients' fibroblasts with intracellular poly(dA:dT) resulted in higher expression of ISGs belonging to the STING pathway compare to controls' fibroblasts, including downstream type I interferons and interferon responsive cytokines and chemokines (Fig. 4F).

Therefore, absence of ZNFX1 in primary fibroblasts results in hyper-responses to double-stranded nucleic acid stimulation. In the case of intracellular RNA and DNA, this results in enhanced interferon responses, while extracellular soluble RNA induces a transcriptome pattern corresponding to apoptosis via Caspase 8, lowering other interferon responses. Overall, dysregulation of interferon responses prevents acquisition of protection from infections following pre-stimulation. These results place ZNFX1 as an essential protein in balancing viral sensing.

ZNFX1 is required for a balanced post-transcriptional regulation of ISGs.

Since previous work has demonstrated that ZNFX1 in lower eukaryotes binds to endogenous transcripts and regulates their processing by miRNA, we evaluated whether post-transcriptional mechanisms might influence the differential rate of expression of some ISGs detected in patients' fibroblasts. Therefore, to understand the mechanism underlying higher ISG expression rates after extended (18 hrs) stimulation with double stranded nucleic acids (Fig. 5A), we examined whether the absence of ZNFX1 promotes the stability of ISG mRNAs in response to intracellular poly(dA:dT). To this end, we added 6-dichlorobenzimidazole 1-β-D-ribofuranoside

(DRB, an inhibitor of transcription elongation by RNA-Polymerase-II) to the cultures 18 hrs after poly(dA:dT) transfection. The levels of ISG mRNAs at 0, 30, 60 and 90 min after DRB treatment initiation were higher in patients than in controls; this indicated that ISG mRNAs were more stable in the absence of ZNFX1 (Fig. 5B). Secretion of IFNβ and CXCL10 from fibroblasts in response to stimulation with poly(I:C)Lyovec and poly(dA:dT)Lyovec was elevated in patients, when compared with healthy controls (Fig. 5C and D). Finally, supplementation of fibroblasts with a ZNFX1 WT construct lowered secretion of IFNβ and CXCL10.

Collectively, these findings demonstrate that ZNFX1 is important for viral defense and acts as a buffer in keeping a balanced interferon response to double-stranded nucleic acids, via a program of post-transcriptional regulation, towards a less inflammatory, but more protective response, placing it as an essential protein in balancing the innate immune response.

Discussion

To the best of our knowledge, this is the first report on human ZNFX1 deficiency. This deleterious deficiency is associated with susceptibility to viral infections, and subsequent multi-organ dysfunction and inflammation. The consistent clinical phenotype observed among 15 patients from 8 unrelated families with distinct ethnic backgrounds suggests that ZNFX1 is the causative gene for this disease.

Compared to prior studies in ZNFX1 deficient mice (6), our patients exhibit a broader range of virally induced disease that includes both RNA and DNA viruses, suggesting that ZNFX1 has additional roles beyond sensing cytosolic viral dsRNA in humans.

We show that transfection with synthetic double stranded RNA and DNA oligos, mimicking infections with DNA and RNA viruses, causes an upregulation of

inflammatory pathways, but pre-treatment with intracellular delivered dsRNA does not protect patients derived monocytes from infection. This lack of protection may be due to the complex gene signature seen in the patients' fibroblasts following treatment with nucleic acids, which on the other hand promotes interferon associated inflammation, but on the other hand interferes with mechanisms of antiviral response. Previous work has demonstrated that ZNFX1 deficiency does not predispose mice and human cell lines to DNA virus infections (6). Therefore, damage caused by DNA viruses in six of the patients might be directly linked to an insufficient resolution of the interferon-response to the infection and not excessive viral load. Extracellular dsRNA-mimicking oligos also cause a hyper-response, although in this case, the signature corresponds to apoptosis with increased expression of FADD and Caspase 8 and lower expression of inflammatory cytokines.

Consistent with increased susceptibility to viral infections, a respiratory syncytial virus infection recurred in one patient within a few weeks and two patients suffered from vaccine strain infections (measles and varicella zoster virus (VZV), respectively). These are extremely rare events in immunocompetent hosts (12) but are well documented in patients with defective type I and III IFN immune responses (13-17). Amelioration of CNS manifestations after HSCT points to an immune driven disease. While these observations do not fully exclude a tissue-specific role of ZNFX1 in neurons, liver-, lung- and renal cells, our clinical observations and in vitro data clearly show that ZNFX1-deficiency has an impact on the immune system. In this regard, CNS manifestations could be both caused by HLH activity or viral infection.

For many viral infections, the severity of clinical disease is thought to be associated with a high viral load (18-20). In addition to cell-autonomous impairment of inflammation control, poor viral control might also contribute to the immune disease observed in patients with ZNFX1 deficiency. Thus, viral infections with RNA viruses

(positive (+)ssRNA: norovirus; negative (-)ssRNA: influenza A, RSV, parainfluenza virus, and influenza B virus) were directly linked to HLH or to HLH-like manifestations in seven patients. Multiple organ involvement might be suggestive of hyperinflammation caused by viral escape and viraemia. However, a persistent viral load was observed in only some of the patients with ZNFX1 deficiency; the others continued to display an immune disease either after viral clearance or in the absence of an identified pathogen.

Occurrence of complement-mediated TMA has recently been reported in a cohort of patients with therapy-refractory HLH (21). TMA has been described also as a dose-dependent adverse reaction to recombinant type I IFNs in the treatment of viral hepatitis and multiple sclerosis (22-26). The overexpression of inflammatory genes seen in ZNFX1-deficient patients' cells after exposure to intracellular dsDNA and dsRNA might therefore be implicated in the pathogenesis of TMA observed in these patients.

Our observation that in the absence of ZNFX1 the half-life of ISGs is increased following extensive stimulation (24hrs) with intracellular DNA offers an attractive mechanism, which is in line with previous work showing the essential role of ZNFX1 in post-transcriptional regulation of mRNA in lower eukaryotes (7, 27). Nevertheless, whether ZNFX1 is directly involved in regulating the half-life of ISGs, or whether regulation of ISG mRNAs stability is a result of alternative mechanisms, that are secondary to its possible role in sensing nucleic acids, remains unclear. Furthermore, because of limited sample availability, fibroblasts from patients carrying biallelic missense mutations in ZNFX1 were not included in functional studies, therefore no conclusions on phenotype to genotype association could be drawn.

Viral infections in patients with ZNFX1 deficiency were associated with HLH-like episodes. HLH is characterized by fever, hepatosplenomegaly, pancytopenia,

hyperferritinemia, severe coagulopathy, and hypercytokinemia. Viral infections are known to be major HLH triggers (28). To date, variants in 6 different genes (*PRF1, UNC13D, STXBP2, STX11, RAB27A*, and *LYST*) are known to directly impact perforin-mediated cytotoxicity and thereby cause HLH (29). Variants in other genes (*SH2D1A, CD48, BIRC4, NLRC4, HAVCR2* (TIM-3)*, CDC42, RC3H1*, HEM1 and *AP3B3A*) have been linked to HLH and HLH-like disease (30-35). *ZNFX1* must now be added to this list.

Clinical observations in patients with ZNFX1 deficiency have revealed an interplay between inflammation and immunodeficiency. At present, there are few treatment options for individuals with ZNFX1 deficiency. In our study, treatment with immunosuppressants (including a JAK inhibitor) led to only transient benefit. In one patient, HSCT arrested the HLH-like episodes and was followed by improvements in neurological development. We recommend that (i) variants in ZNFX1 should be included in genomic screens for patients suffering from severe viral infections and HLH, and (ii) HSCT should be evaluated as a treatment for patients with ZNFX1 deficiency.

We show that ZNFX1 is important for sensing of viral derived double-stranded nucleic acids in humans. Our data furthermore implicates a role for ZNFX1 in post-transcriptional regulation of ISGs', as previously found for other proteins, such as ZAP (36). Higher expression of ISGs was seen in peripheral blood of ZNFX1 patients, together with a lower predisposition to infection, suggesting that a persistent status of hyperinflammation might on one hand provide some levels of protection from viral infections but might on the other hand contribute to multi-organ damage. The mechanism by which ZNFX1 regulates the stability of mRNA remains elusive, but its ability to bind dsRNA is suggestive of a RNA interference mechanism mediated by small RNAs, as shown in lower eukaryotes.

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Table I: Clinical characteristics

ID	Gender	Viruses eliciting severe disease	HLH or HLH-like disease	Organs affected: Liver	CNS	Kidney	Lung	Age last FU	Outcome
P1.1	F	-	-	+	-	-	+	2y 8mo	Dead
P1.2	F	Influenza A	-	+	+	+	+	15y	Dead
P1.3	M	Influenza A	+	+	+	-	+	1y 2mo	Dead
P2.1	F	-	-	+	-	+	+	14y	Alive
P2.2	M	RSV Influenza A	+	+	(+)	+	+	3 mo	Dead
P3.1	F	NA	NA	NA	+	NA	NA	5 mo	Dead
P3.2	M	Norovirus HHV6	+	+	+	+	+	1y	Dead
P4.1	F	ADV	+	+	+	+	+	8 mo	Dead
P4.2	M	ADV Parainfluenza	+	+	+	+	-	8y	Alive
P5.1	M	HHV6 (+CMV)	+	+	+	+	+	3 mo	Dead
P5.2	F	HHV6 (+Sapovirus, Rhinovirus)	+	+	-	+	+	1y 4mo	Dead
P6.1	M	Sepsis, germ not identified	-	+	-	+	+	9y	Dead
P7.1	F	Vaccine strain measles (+EBV) Influenza B	+	+	+	+	+	3y	Alive
P8.1	F	CMV	+	+	+	+	+	8y	Dead
P8.2	M	Vaccine strain VZV Influenza A	+	+	+	-	+	7y	Alive
Summary	Female:male: 8:7	(-)ssRNA viruses: 10 (+)ssRNA viruses: 2 dsRNA viruses: 1 dsDNA viruses: 7	n=10	n=14	n= 11	n=11	n= 13		Alive:dead: 4:11

CNS: central nervous system; CMV: cytomegalovirus; EBV: Epstein Barr virus; FU: follow-up; HLH: hemophagocytic lymphohistiocytosis; mo: months; NA: not available; y: year(s).

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Patient ID	Fever	Splenomegaly	Hemoglobin (g/dL) minimal	Platelet count, minimal	Leukocytes, maximal	Hemophagocytosis	Hyperferritinemia (≥500 mg/l)	Hypertriglyceridemia (fasting level: ≥3.0 mmol/l) or hypofibrinogenemia (≤1.5 g/l)	Elevated soluble CD25 (≥2400 U/ml)	Low NK and/or T cell cytotoxicity	NK cell degranulation	Perforin/SAP/XIAP expression	HLH-criteria fulfilled	HLH according to an assessment by the attending physicians assessment (age at onset in years)	HLH trigger
P1.1	NA	NA	low	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	No HLH	Not applicable
P1.2	Yes	No	6.8	245-425 (age 8-14 y)	9.2	NA	Yes (553, age 14 y)	No Fib: 1.99-6.47, age 8-14 y TG 1.2 age 9 y	NA	NA	NA	NA	NA (2/5; NA: 3))	No HLH	Not applicable
P1.3	Yes	Yes	7.1	29	17.4	Not in bone marrow (age 11 mo)	Yes (4511)	Yes TG: 3,32, coagulation defect	Yes, 3957	Normal	Normal CD107 expression	NA	**Yes** (6/8)	**HLH** (6 mo)	Rotavirus and norovirus
P2.1	NA	NA	14.7	213	9	NA	No (68)	Yes TG: 4.9 Fib level normal	NA	Not done	Not done	NA	NA (1/3; NA: 5)	No HLH	Not applicable
P2.2	Yes	Yes	6.0	6	25.58	NA	Yes (34616)	No Fib: 1.9 TG 0.8	NA	Not done	Not done	NA	NA (4/5; NA: 3)	**HLH-like** (3 mo)	Influenza A
P3.1	NA	NA	NA	low	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	Not applicable
P3.2	Yes	No	8.8	34	9.12	NA	No (220)	No Fib 1.55	No, 2122	Not done	Not done	NA	No (2/6; NA: 2)	**HLH-like** (1 y)	HHV6
P4.1	No	Yes	6.7	15	33	in liver (autopsy)	Yes (4890)	Yes TG: 9.8 Fib not decreased: 5.4	No	Normal	Normal CD107 expression	Normal	**Yes** (5/8)	**HLH** (7 mo)	ADV
P4.2	Yes	Yes	7.5	62	23.8	No	No (141)	Yes TG: 5.8 Fib not decreased: 4.9	NA	Normal	Normal CD107 expression	Normal	NA (4/7; NA: 1)	**HLH-like** (22 mo)	ADV, Parainfluenza
P5.1	Yes	No	7.5	54	36.6	No (CSF analyzed)	Yes (82148)	Yes Fib: 0.78 TG not increased: 1.2	Yes, 3'185	Not done	Normal CD107 expression	13% in NK cells (Ref>5%)l	**Yes** (5/8)	**HLH** (2 mo)	HHV6, low-level CMV viremia
P5.2	Yes	Yes	6.8	14	36.7	Yes (in BM)	Yes (37474)	Yes Fib: 1.3 TG 5.5	Yes, 12'000	Not done	Normal CD107 expression	20% in NK cells (Ref>5%)	**Yes** (7/8)	**HLH** (9 mo)	HHV6
P6.1	Yes	Yes	9.1	63	17.3	NA	NA	NA	NA	NA	NA	NA	NA (3/3; NA:5)	No HLH	Not applicable
P7.1	Yes	Yes	low	42	50	NA	Yes (12000)	No	No	Normal NK cytotoxicity	Normal CD107a expression		NA (4/7; NA:1)	**HLH-like** (5 mo)	
	Yes	Yes	7.5	20	>50	Yes (BM)	Yes (23000)	No	NA				**Yes** (5/7; NA:1)	**HLH** (1 y)	Vaccine strain measles and low-level EBV viremia

	Yes	Yes	low	low	high	No (CSF)	Yes (2546)	No	NA		.	**.	NA (4/7; NA:1)	**HLH-like** (2 y 9 mo)	Influenza B and S. aureus
P8.1	Yes	Yes	5.5	25	Low (0.2)	No	Yes (6,000)	Yes TG 11.82 mmol/l	Yes 14,682	Normal	NA	ND	**Yes** 6/8	**HLH** (8 y)	No infectious agent found
P8.2		No	Yes	5.2	18	No (bone marrow)	Yes (2805)	No	Yes (2534)	Reduced NK cytotoxicity but tested with low NK cell number	NA	NA	NA (4/6, NA:2)	**HLH-like** (2 mo)	No infectious agent found
		Yes	Yes	6.5	95	NA	Yes (582)	No	No (1164)		NA	NA	No (4/8)	**HLH-like** (6 y)	Influenza A

Diagnostic criteria for hemophagocytic lymphohistiocytosis; according to the HLH-2004 study group's criteria (Henter et al.) (37)

**Perforin expression was in the lower normal range in P5.1 and P5.2; this was probably due to the concomitant presence of a heterozygous p.Ala91Val variant in PRF1, which was also carried by the healthy father (data not shown). In the (male) patient P5.1, SAP and XIAP expression was measured by flow cytometry and was normal.*

***An abnormally low percentage of perforin-expressing NK cells, although this might reflect the relative expansion of a CD56 bright NK cell population. The sample was collected during a period of acute illness and when the NK cell count was low (in a context of viral infection/HLH). It is noteworthy that the NK cell count subsequently normalized but perforin release was not retested.*

ANC: absolute neutrophil count; BM: bone marrow; Fib: Fibrinogen level; NA: not available; TG: triglycerides (fasting level).

Figure legends:

Fig. 1: Severe viral infections and inflammatory disease in patients with ZNFX1 deficiency. Panel A: Kaplan-Meier survival curve for patients; dashes indicate the age of patients who are alive. **Panel B:** Overall inflammatory organ involvement with or without a proven link to infections; number of patients affected. HLH, hemophagocytic lymphohistiocytosis; MOF, multiorgan failure; MPGN, membranoproliferative glomerulonephritis; ARDS, acute respiratory distress syndrome. **Panel C:** May-Gruenwald-Giemsa staining (light microscope, magnification × 1000) of a bone marrow aspirate from P5.2. A macrophage with engulfed leukocytes is shown: its nucleus is indicated by an arrowhead, and the engulfed leukocytes are indicated by an arrow. **Panel D:** A CT image of P1.2's brain at the age of 15 years, showing calcification of the basal ganglia and white matter abnormalities (white arrowheads). **Panel E:** A high-resolution CT image of P1.2's lungs at the age of 9 years and 11 months, showing bilateral diffuse ground glass attenuation, subpleural thickening, and septal thickening. **Panel F:** Jones staining of a kidney biopsy, highlighting TMA lesions in P5.2. The arrow indicates a small arteriole with endothelial cell swelling and a fibrin/red blood microthrombus obliterating the lumen. Two glomeruli with capillary lumen dilatation and red blood cell stasis are indicated by asterisks. Acute tubular lesions with epithelial cell necrosis, lumen debris and interstitial hemorrhage are observed (scale bar: 50 µm).

Fig. 2: Regression of white matter changes in P4.2's brain following HSCT. Axial fluid-attenuated inversion recovery (FLAIR) MR images at the ages of 32 months (A, D), 42 months (B, E), and 8 years (C, F), demonstrating an initial increase in

periventricular and deep white matter changes 6 months after HSCT (B, E) and then marked regression seen at last follow-up (5 years after HSCT) (C, F).

Fig. 3: Biallelic *ZNFX1* variants lead to the loss of protein expression in response to stimulation by intracellular nucleic acids. Panel A: The pedigrees of the eight families. Patients carrying homozygous or compound heterozygous deleterious variants in *ZNFX1* are indicated by solid symbols. Healthy individuals carrying heterozygous variants are indicated by dotted symbols. Affected persons with an unknown genotype are indicated by open red symbols, while unaffected individuals are indicated by open diamonds. Circles indicate females, and squares indicate males. Slashes over symbols indicate deceased patients. N/A indicates that sequencing was not performed. **Panel B:** Predicted domains and identified variants in the ZNFX1 amino acid sequence. The eleven deleterious variants identified are indicated by arrows. The domain homologous to the RNA helicase Aquarius (PDB 4PJ3) is highlighted in orange, with an insert shown in yellow. **Panel C:** A ribbon diagram of a homology model of ZNFX1 (183–1255), based on the structural template RNA helicase Aquarius (PDB ID: 4PJ3) is shown. Locations of the four missense variants within this domain are shown as teal spheres in the present study. **Panel D:** A protein immunoblot for ZNFX1 in dermal fibroblasts from a healthy donor (CTRL) and from P5.1, P3.2, and P2.1, under resting conditions and 24 hours after transfection with the nucleic acids poly(dA:dT) or poly(I:C). Beta actin was used as a loading control.

Fig. 4: Biallelic defects in ZNFX1 deregulate ISGs expression and protection to viral infections in response to treatment with nucleic acids. Panel A and B: Flow cytometry analysis of monocytes from P2.1 and healthy control (CTRL) pre-treated for 12 hours with different concentrations of lyovec-poly(I:C) and subsequently infected

with VSV-GFP for 5 h. Representative plots of a single experiment (**Panel A**) and mean percentage of VSV-GFP positive monocytes relative to the unstimulated condition (no lyovec-poly(I:C)) for four repeats (**Panel B**). Error bars refer to ± SD, n=4. pValues were calculated using two-way ANOVA, Sidak's multiple comparisons test. **Panel C:** Transcriptomic analysis results for selected ISGs involved in antiviral responses summarized in heat map showing mean difference in fold induction of ISGs expression from resting conditions, in dermal fibroblasts from four patients (P1.2, P2.1, P3.2 and P5.2), over 4 different age matched, healthy controls. 3 different stimulations were used: 18hrs of intracellular poly(I:C) (Lyovec Poly (I:C)), 6hrs of soluble poly(I:C) (Poly (I:C)) or 18hrs of transfected poly(dA:dT) (Lyovec Poly (dA:dT)). **Panels D, E and F:** The same data was used to study activity of canonical double-stranded nucleic acids sensing pathways according to the Kyoto encyclopedia of genes and genomes (KEGG). Colored highlights indicate the rate of gene expression fold-induction in patients over controls: red highlight indicated increase, blue highlight indicate decrease and white boxes indicate no difference. Results from stimulation with Lyovec-poly(I:C) is shown in **panel D**, with soluble poly(I:C) in **panel E** and with Lyovec-poly(dA:dT) in **panel F**.

Fig. 5: Increased ISG expression in response to transfected poly(dA:dT) in biallelic defects in ZNFX1 is associated with increased mRNA stability. Panel A: The mRNA expression levels of *OAS1, OAS2* and *MX1* (representative ISGs) by skin fibroblasts from P1.2, P2.1, P3.2, P5.2 (red squares) and four healthy controls (CTRL, black circles) at baseline (0 hours) and at different time points (6, 12, 18, 24 and 30 hours) after stimulation with transfection reagent-complexed poly(dA:dT). **Panel B:** Mean values of mRNA stability of representative ISG mRNAs in fibroblasts from four

healthy control and four patients (P1.2, P2.1, P3.2 and P5.2). Gene transcription was inhibited by the addition of 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB) 18hrs after transfection with Lyovec-poly(dA:dT). qPCR was performed at the indicated time points after DRB addition. The amount of mRNA at each time point was normalized against ribosomal 18S RNA and represented relative to the amount at the time of DRB addition (time 0). The half-life ($t_{1/2}$) of each mRNA (red for P1.2 and black for CTRL) was calculated using nonlinear regression analysis. A representative result of 3 independent experiments is shown. Concentrations of IFNβ (**Panel C**) and CXCL10 (**Panel D**) in the supernatant of dermal fibroblasts from 3 healthy controls (CTRL, black bars) and 3 patients (Patients, red bars (P1.2, P3.2, P5.2)) following 18hrs of stimulation with poly(I:C)Lyovec or poly(dA:dT)Lyovec. Fibroblasts were transfected with plasmids expressing ZNFX1 or GFP. Shown is the mean of 3 repeats for each of the 3 samples (n=9) with error bars showing standard deviation. p-values were calculated using ordinary one-way ANOVA (0.12 (ns), 0.033 (*), 0.002 (**), < 0.001 (***)).