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Interstitial lung disease in immuno-compromised children and a novel monogenic defect

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Inhaltsverzeichnis

Abkürzungsverzeichnis

AD	Autosomal dominant transmission
ADA	Adenosine deaminase
AGS7	Aicardi-Goutieres syndrome 7
ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia
APLAID	Auto-inflammation and phospholipase Cγ2 (PLCγ2)-associated antibody deficiency and immune dysregulation
AR	Autosomal recessive transmission
ATM	Ataxia Telangiectasia, Mutated
BO	Bronchiolitis obliterans
CD	Cluster of differentiation
CD40LG	CD40 Ligand
CGD	Chronic granulomatous disease
CID	Combined immunodeficiencies
CLL	Chronic lymphocytic leukemia
CML	Chronic myelogenous leukemia
CVID	Common variable immunodeficiency
CYBA	Cytochrome B-245 Alpha Chain
CYBB	Cytochrome b-245 beta chain
def	deficiency
DKC	Dyskeratosis congenita
DNMT3B	DNA methyltransferase 3b
EDA	Anhidrotic ectodermodysplasia
FOXP3	Forkhead box protein P3
GOF	Gain-of-function
HIV	human Immunodeficiency virus
ICF	Immunodeficiency, Centromeric region instability, Facial anomalies syndrome
ID	Immunodeficiency
Ig	Immunoglobulin
IKBA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IL	Interleukin
IL2RG	Interleukin 2 Receptor Subunit Gamma

ILD	Interstitial lung disease
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy X-linked
JMML	Juvenile myelomonocytic leukemia
LOF	Loss of function
MCM4	Minichromosome maintenance
MDA	Melanoma differentiation-associated protein
MHC	Major histocompatibility complex
MIRAGE	Myelodysplasia, infection, restriction of growth, adrenal hypoplasia, genital phenotypes, enteropathy
NBS	Nijmegen breakage syndrome
NCF	Neutrophil cytosolic factor
NFKB1	Nuclear factor-kappaB1
PAP	Pulmonary alveolar proteinosis
PAS	Periodic acid–Schiff
PHT	Pulmonary hypertension
PIK3CD	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Delta
PLAID	PLCγ2 associated antibody deficiency and immune dysregulation
PLCG2	phospholipase C gamma 2
RF	Respiratory failure
RFXAP	Regulatory Factor X Associated Protein
SAMD9	Sterile Alpha Motif Domain Containing 9
SCID	Severe combined immunodeficiency
SID	Secondary Immunodeficiency
STAT	Signal transducer and activator of transcription
TACI	Transmembrane activator calcium modulator and cyclophilin ligand interactor
TERC	Telomerase RNA Component
TERT	Telomerase Reverse Transcriptase
TNFRSF13B	Tumor Necrosis Factor Receptor Superfamily Member 13B
TNFRSF1A	TNF Receptor Superfamily Member 1A
TRAPS	TNF receptor-associated periodic syndrome
TTC7A	Tetratricopeptide repeat domain 7A gene
UNC13D	Protein unc-13 homolog D
ZNFX1	NFX1-type zinc finger-containing 1

Publikationsliste

My cumulative dissertation:

1. **Multisystem inflammation and susceptibility to viral infections in human ZNFX1 deficiency**

   Stefano Vavassori, Janet Chou, Laura Eva Faletti, Veronika Haunerdinger Lennart Opitz, Pascal Joset, Christopher J Fraser, Seraina Prader, Xianfei Gao, Luise A Schuch, Matias Wagner, Julia Hoefele, Maria Elena Maccari, Ying Zhu, George Elakis, Michael T Gabbett, Maria Forstner, Heymut Omran, Thomas Kaiser, Christina Kessler, Heike Olbrich, Patrick Frosk , Abduarahman Almutairi, Craig D Platt, Megan Elkins, Sabrina Weeks, Tamar Rubin, Raquel Planas, Tommaso Marchetti, Danil Koovely, Verena Klämbt, Neveen A Soliman, Sandra von Hardenberg, Christian Klemann, Ulrich Baumann, Dominic Lenz, Andreas Klein-Franke, Martin Schwemmle, Michael Huber, Ekkehard Sturm, Steffen Hartleif, Karsten Häffner, Charlotte Gimpel, Barbara Brotschi, Guido Laube, Tayfun Güngör, Michael F Buckley, Raimund Kottke, Christian Staufner, Friedhelm Hildebrandt, Simone Reu-Hofer, Solange Moll, Achim Weber, Hundeep Kaur, Stephan Ehl, Sebastian Hiller, Raif Geha, Tony Roscioli, Matthias Griese, Jana Pachlopnik Schmid.

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2. **Interstitial lung disease in immuno-compromised children**

   Xianfei Gao, Katarzyna Michel, and Matthias Griese

   Diagnostics 2023, Volume 13, Issue 1, 64

1. Ihr Beitrag zu den Veröffentlichungen

1.1 Beitrag zu Paper I: Multisystem inflammation and susceptibility to viral infections in human ZNFX1 deficiency

In the modern clinical period, gene therapy or molecular therapy has established itself as an essential treatment for complex syndromes by providing effective and long-lasting remission with minimal side effects. Genetic diagnosis has become clinicians' ultimate goal in pursuing better therapies and outcomes. The International Union of Immunological Societies (IUIS) updates the phenotypical classification of human inborn errors of immunity every two years, discovering approximately 56 to 65 new inborn errors in the past five years. However, many patients still remain undiagnosed even after genetic analysis, indicating the presence of unknown inborn errors that need further exploration.

To identify the underlying genetic mutations responsible for these unknown syndromes, an analysis was conducted on immunocompromised children with significant lung disease between 1997 and 2020 at the Department of Pediatric Pneumology at the Dr. von Hauner Children’s Hospital of the University of Munich. These children had undergone Whole exome sequencing (WES) but did not exhibit any known disease-causing gene mutations. Among them, five patients from two different families displayed similar multiple-system inflammations and were found to have pathogenic variants in the ZNFX1 gene (NFX1-type zinc finger–containing 1). The main clinical issue observed in these patients was severe infections and significant inflammatory responses, even in the absence of identifiable pathogens. Extensive clinical information regarding their medical history, organ manifestations, laboratory examinations, imaging, biopsies, and genetic analysis was collected and presented in a structured format.

After analyzing previous research, it has been determined that NFX1-type zinc finger-containing 1 (ZNFX1) is a highly conserved interferon-stimulated double-stranded RNA (dsRNA) sensor. This sensor restricts the replication of RNA viruses in mice and contributes to trans-generational inheritance in Caenorhabditis elegans by binding to mRNA complexed with short, noncoding RNAs. The expression of ZNFX1 is low in uninfected cells but is rapidly increased in response to viral infections and exposure to type I interferons. ZNFX1 binds to viral RNA and interacts with the mitochondrial antiviral signaling (MAVS) protein, leading to the expression of interferon-stimulated genes (ISGs). The signaling downstream of ZNFX1 is independent of two other MAVS-associated cytosolic viral sensors, retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5). Additional studies involving ZNFX1-deficient mice and cell lines have identified a role for the protein in sensing dsRNA. Thus mutations in the ZNFX1 gene have the potential to explain the abnormal immune condition experienced by these five patients.

For a deeper analysis of these cases, bio-samples (such as blood, fibroblast, and biopsy tissue) were collected in cooperation with the University Hospital of Munster, the Faculty of Medicine at the University of Freiburg, and the University Hospital Tubingen for detailed analysis of the underlying mechanisms.

However, five patients are too little to build a supportive cohort. Fortunately, ten additional patients from 6 families with other variations in ZNFX1 were contacted via gene-matcher. These patients´ data were recruited from the University Children’s Hospital Zurich, Boston Children’s Hospital, the Queensland Children’s Hospital, Prince of Wales Hospital, Security Forces Hospital, University Hospital Heidelberg, Cantonal Hospital Aarau, University of Geneva, Sydney Children’s Hospital, and University of New South Wales.

A comprehensive summary of the clinical characteristics was generated in addition to the detailed clinical data in the supplement of the publication. Then the functional experiment was performed. ZNFX1 deficiency predisposes to severe viral infections and a multisystem inflammatory disease which was exhibited in paper I below.

1.2 Beitrag zu Paper II: Interstitial lung disease in immuno-compromised children

Interstitial lung diseases (ILD) are a diverse group of conditions characterized by inflammation or fibrosis in the pulmonary tissue, resulting in diffuse parenchymal lung disorders. The prevalence of interstitial lung disease increases rapidly, with poor survival rates of 3-5 years. ILD affects people of all ages but it has been found to be particularly common among immunocompromised children due to their weakened immune systems making them more susceptible to infection and other illnesses.

Interestingly, interstitial lung disease often presents as a primary symptom in many immunodeficiency diseases, reflecting the immune system dysfunction in the lungs, which leads to altered white cell composition and the accumulation of extracellular matrix (ECM), ultimately exacerbating fibrosis.

Specifically in childhood, interstitial lung disease and immunodeficiency are rare, making it difficult to make a timely diagnosis. Currently, there are no accurate early diagnosis predictors. However, respiratory disorders such as infections, pulmonary hypertension, acute respiratory distress syndrome, and interstitial lung disease often serve as the initial symptoms in most immunodeficiency diseases. Therefore, a comprehensive analysis of pulmonary disorders, particularly interstitial lung disease, and immune defects is urgently needed.

Furthermore, interstitial lung disease has been shown to contribute to a worse prognosis in immunodeficiency patients, causing a significant reduction in their quality of life. Research suggests that early and effective management of respiratory symptoms can reduce mortality rates and improve the quality of life for immunodeficiency patients. However, there is still a lack of large-scale studies to thoroughly analyze the relationship and impact of interstitial lung disease on immunodeficiency.

In the study of Paper II below, we examined the clinical relationship between childhood interstitial lung disease and immunodeficiency in children. We collected data on gender, age at diagnosis, consanguinity, family history, gestational age, use of oxygen supplementation or mechanical ventilation during the neonatal period, and lung disease outcomes, in order to elaborate on the connection between interstitial lung disease and immunodeficiency.

Genetic diagnosis was found to be the most accurate method for identifying these complex syndromes. In some cases, interstitial lung disease was the primary or secondary phenotype associated with specific genetic defects in immunodeficiency conditions. In our cohort, the incidence of interstitial lung disease in children with primary immune compromise was higher compared to existing literature.

2. Einleitung

2.1 Immune system of the lungs

The internal surface of the lungs are exposed to the environment and needs a well-balanced immune system to protect the body from pathogens and toxicants. However both the innate and acquired immune system of the lung acts as a double-edged sword in defense [1]. It is known that overshooting immune dysregulation or immune deficiencies may act as inducers or enhancers for acute and chronic pulmonary diseases [2, 3] .

Immunodeficiency diseases including primary immunodeficiency diseases and secondary immunodeficiency diseases are due to defects of the innate or adaptive pathways of the immune system. They may be responsible for an increasing frequency or severity of infections and/or immune dysregulation disorders [4, 5]. Specific or more frequent infections are used as warning signs of immune deficiency [6]. Especially respiratory infections are common manifestations [7, 8]. However, the spectrum of disorders extends beyond infections. Among others, interstitial lung diseases represent a chronic pulmonary complex of different types of disorders [9]. Thus, early and accurate diagnosis of lung conditions is key for targeted treatment and the possibility of an improved prognosis of the respiratory disorders of children with immunodeficiency diseases [10-15].

2.2 Classification of lung disease

The pulmonary diagnoses varied widely and were extracted in a standardized way based on the clinical letters and then classified according to the updated etiologic classification system of the chILD-EU register [16]. We further differentiated twelve different groups which included opportunistic/recurrent infections, bronchiolitis obliterans, asthma, bronchiectasis, Interstitial lung diseases, acute respiratory distress syndrome (ARDS), respiratory failure, pneumothorax, pulmonary hypertension, diffuse alveolar damage, pleural dis-ease, and post-transplant disorders.

2.3 Classification of immunodeficiencies

The immunodeficiencies, in childhood also a broad group of rare diseases, were either based on inborn errors of immunity and classified as primary, or on hemato-oncologic diseases or immunosuppressive treatments and classified as secondary.

2.3.1 Primary immunodeficiency

The recent classification of the primary immunodeficiencies differentiates more than 450 different molecularly defined entities [17, 18]. Many of these genetic mutations are known and are used for precise diagnosis. Here we use this classification for categorizing our patients´ diagnoses.

We categorized all patients with immune deficiencies and treated in the Department of Pediatric Pneumology of the Dr. von Hauner Children’s Hospital from 1997 and 2020, according to the system published by the International Union of Immunological Societies (IUIS) for the inborn errors of immunity.

The diagnosis of primary immunodeficiency may become more specific during the progression of the disease and in particular based on immunological tests, however, it becomes more definite when disease-causing variants in responsible genes are detected. However, there are still many cases with a clinical-immunological diagnosis only, in the absence of an identified genetic cause.

A well-defined genetic cause indicates not only potential inheritance but also pathological processes which might be much better addressed by novel treatments [19].

However, there are still blanks left for many primary immunodeficiency diseases with unknown genetic muta-tions or clinical features [16, 17]. As early diagnosis may be essential for a better prognosis, any sign that may point into the direction of an inborn error of immunity needs to be recorded and considered critically.

The type of interstitial lung disease may sometimes be used as an indicator of a specific genetic mutation. It can thus support an early and precise diagnosis of a primary immunodeficiency possibly with a complex syndrome phenotype [16.17]. Molecularly unresolved interstitial lung diseases may point out a precise genetic defect or clear diagnosis, and a novel mutation might be considered and searched.

In our paper “Multisystem inflammation and susceptibility to viral infections in human ZNFX1 deficiency”, we make a contribution to a novel primary disease caused by the genetic mutation ZNFX1 which is classified in the group of defects of innate immunity [18] .

The ZNFX1 deficient patients suffered from multi-system inflammation. Enlightened by the previous research in mice which showed that ZNFX1 participated in the interferon immune response to RNA viruses, the cellular mechanisms of the patients were investigated. It turned out that ZNFX1 plays an essential role in regulating the interferon response as part of the innate immune system. Abnormal ZNFX1 drives a more pronounced inflammatory but less protective response which leads to the phenotype of our patients.

2.3.2 Secondary immunodeficiency.

Secondary immunodeficiencies are due to a superimposed impairment of the immune response by factors extrinsic to the immune system. These factors are heterogeneous, including prematurity and aging, infections including human immunodeficiency virus, and treatments with systemic corticosteroids, chemotherapy, other immunosuppression, radiotherapy, or from the disease processes. Lastly, diseases like lymphoma, leukemia, myeloma, malnutrition, protein-losing, enteropathy, or nephropathy are associated with secondary immunodeficiency [20].

2.4 Lung disease in immunodeficiency

In the past the main focus has been on infectious pulmonary complications. However, lung disease may not only manifest as an airway disease including chronic bronchitis, bronchiectasis, obliterating bronchiolitis or asthma, but also as diffuse parenchymal or interstitial lung disease, including pulmonary hypertension or lymphoproliferative disease [8].

As both, immunodeficiency and pulmonary complications other than infections were believed to be rare, there is some difficulty to gather a larger cohort of these diseases.

The outcome of lung disease at the end of follow-up was significantly better in patients without interstitial lung disease than in those with interstitial lung disease. This may be because interstitial lung diseases often are progressive lung diseases that influence the outcome of immunodeficiencies and induce a worse outcome than other chronic pulmonary conditions [21, 22]

As expected, in children with primary immunodeficiencies, both, consanguinity and a family history of interstitial lung disease were more frequent. In the case of consanguineous marriages, this may be due to recessive inheritance and thus an increased likelihood of homozygote states by the carrier. As primary immunodeficiencies are likely caused by genetic mutations and these familial risks may further increase.

In many of the immunodeficiencies, the accompanying lung disease came from several categories. Although the entire spectrum of lung diseases may be linked to immunodeficiencies, possibly due to the small of cases

described, some of the IUIS-categorized conditions may be associated with specific pulmonary conditions (Table 1)[18].

Table 1 The spectrum of pulmonary diseases acts as clinical features in genetically defined primary immunodeficiency

Pulmonary diseases as associated clinical features	Immunodeficiency disease	Genetic defect/presumed pathogenesis	Inheritance
Lung granulomas	RHOH deficiency	RHOH	AR
Pulmonary alveolar proteinosis	ADA deficiency	ADA	AR
	SLC7A7 deficiency	SLC7A7 AR	AR
	GATA2 deficiency	GATA2	AD
	Pulmonary alveolar proteinosis	CSF2RA	XL
		CSFR2B	AR
	OAS1 deficiency	OAS1	AD GOF
	Pulmonary alveolar proteinosis	AutoAb to GM-CSF	-
Sino-pulmonary infections	MHC class I deficiency	B2M	AR
	PU1 deficiency	SPI1	AD
	NOS2 deficiency	NOS2	AR
	TWEAK deficiency	TNFSF12	AD
	BACH2 deficiency	BACH2	AD
	Ataxia-telangiectasia	ATM	AR
	COPG1 deficiency	COPG1	AR
Respiratory infections	MHC class II deficiency group A, B, C, D	CITA	AR
		RFXANK	AR
		RFX5	AR
		RFXAP	AR
	X-link reticulate pigmentary disorder	POLA1	XL
	Tricho-Hepato-Enteric Syndrome	TTC37	AR
		SKIV2L	AR
	KMT2A deficiency	KMT2A	AD

	X-linked magnesium EBV and neoplasia	MAGT1	XL
	Cystic fibrosis	CFTR	AR
	TLR3 deficiency	TLR3	AD/AR
	Ficolin 3 deficiency	FCN3	AR
Recurrent sino-pulmonary infections	IKAROS deficiency	IKZF1	AD DN
	IL-21 deficiency	IL-21	AR
	SASH3 deficiency	SASH3	XL
	AIOLOS deficiency	IKZF3	AD
	RASGRP1 deficiency	RASGRP1	AR
	NFKB1 deficiency	NFKB1	AD
	NFKB2 deficiency	NFKB2	AD
	IKAROS deficiency	IKZF1	AD
	RAC2 deficiency	RAC2	AR
	NFAT5 haploinsufficiency	NFAT5	AD
	Kabuki syndrome	KMT2D	AD
		KDM6A	AD
	HELIOS deficiency	IKZF2	AD AR
Pneumocystis	IKAROS deficiency	IKZF1	AD DN
	CARD11 deficiency	CARD11	AR LOF
	IL-21R deficiency	IL-21R	AR
	AD-HIES STAT3 deficiency	STAT3	AD LOF
	MTHFD1 deficiency	MTHFD1	AR
	Hepatic veno-occlusive disease with immunodeficiency	SP110	AR
	AIOLOS deficiency	IKZF3	AD
	NOS2 deficiency	NOS2	AR
Recurrent respiratory infections		POLD1	AR

	Polymerase δ deficiency	POLD2	AR
	HELIOS deficiency	IKZF2	AD AR
	POLE1 deficiency	POLE1	AR
	IL-10 deficiency	IL-10	AR
	AIOLOS deficiency	IKZF3	AD
	FOXN1 haploinsufficiency	FOXN1	AD
	Chromosome 11q deletion syndrome	11q23del	AD
	ZNF341 deficiency AR-HIES	ZNF341	AR
	ERBIN deficiency	ERBB2IP	AD
	Loeys-Dietz syndrome	TGFBR1	AD
		TGFBR2	AD
	CARD11 deficiency	CARD11	AD LOF
	CRACR2A deficiency	CRACR2A	AR
	Activating de novo mutations in nuclear factor, erythroid 2-like	NFE2L2	AD
	SEC61A1 deficiency	SEC61A1	AD
	BOB1 deficiency	POU2AF1	AR
	IL10R deficiency	IL10RA	AR
		IL10RB	AR
Severe lung disease	NSMCE3 deficiency	NSMCE3	AR
Pulmonary abscesses	AD-HIES STAT3 deficiency	STAT3	AD LOF
	IL6ST deficiency (partial)	IL6ST	AR
	ZNF341 deficiency AR-HIES	ZNF341	AR
	Ficolin 3 deficiency	FCN3	AR

Pneumatoceles	AD-HIES STAT3 deficiency	STAT3	AD LOF
	IL6ST deficiency (partial)	IL6ST	AR/AD
	ZNF341 deficiency AR-HIES	ZNF341	AR
Pulmonary aspergillus	AD-HIES STAT3 deficiency	STAT3	AD LOF
	IL6ST deficiency (partial)	IL6ST	AD
Bronchiectasis	IL6ST deficiency (partial)	IL6ST	AD
	FNIP1 deficiency	FNIP1	AR
	ARHGEF1 deficiency	ARHGEF1	AR
Interstitial pneumonitis	STAT5b deficiency	STAT5B	AR
	FNIP1 deficiency	FNIP1	AR
	CTLA4 haploinsufficiency	CTLA4	AD
	COPA defect	COPA	AD
	ITCH deficiency	ITCH	AR
	AR STING-associated vasculopathy, infantile-onset	TMEM173	AR GOF
Lung fibrosis	FNIP1 deficiency	FNIP1	AR
	Hermansky-Pudlak syndrome, type 2	AP3B1	AR
	Cystic fibrosis	CFTR	AR
	HCK GOF	HCK	AD GOF
	DKCX1	DKC1	XL
	DKCA1	TERC	AD
	DKCA2	TERT	AD
	DKCA3	TINF2	AD
	DKCA4	RTEL1	AD

	DKCA5	TINF2	AD
	DKCA6	ACD	AD
	DKCB1	NOLA3	AR
	DKCB2	NOLA2	AR
	DKCB3	WRAP53	AR
	DKCB4	TERT	AR
	DKCB7	ACD	AR
COPD	NFKB1 deficiency	NFKB1	AD
Fatal pulmonary autoimmunity	PD-1 deficiency	PDCD1	AR
Inflammatory lung disease	AD STING-associated vasculopathy, infantile-onset	TMEM173	AD
	MASP2 deficiency	MASP2	AR
Recurrent respiratory papillomatosis	NLRP1 GOF	NLRP1	AD GOF
Lung dysfunction	IL6ST deficiency	IL6ST	AR
Pulmonary hypertension	PSMB9 GOF	PSMB9	AD GOF

AR autosomal recessive inheritance, *AD* autosomal dominant inheritance, *LOF* loss-of-function, *GOF* gain-of-function,

2.4.1 Interstitial lung diseases in immunodeficiency

Interstitial lung diseases (ILDs) are a group of disorders characterized by inflammation and scarring in the interstitium, which is the tissue that surrounds and supports the air sacs (alveoli) in the lungs. These conditions can have various causes, including exposure to environmental toxins or occupational hazards, certain medications, autoimmune diseases, and infections [23].

Immunodeficiency refers to a weakened immune system that fails to adequately protect against infectious agents such as bacteria, viruses, fungi, or parasites. It can be congenital (present from birth) or acquired later in life due to factors like HIV infection or medical treatments such as chemotherapy [30].

When an individual has both immunodeficiency and ILD simultaneously it creates unique challenges for diagnosis and management. The compromised immune response makes these patients more susceptible to opportunistic infections that may directly cause lung damage leading to ILD development [13].

One example of an immunodeficient state predisposing individuals towards developing ILDs is common variable immunodeficiency disorder (CVID). CVID affects approximately 1 in every 25-50 thousand people worldwide; however, its prevalence among those diagnosed with interstitial lung disease remains uncertain due to limited data availability [31].

The histological spectrum of interstitial lung diseases included most frequently non-specific interstitial pneumonitis (NSIP), lymphoid interstitial pneumonitis (LIP), follicular bronchiolitis, granulomatous and lymphocytic interstitial lung disease (GLILD), desquamative interstitial pneumonitis (DIP) and pulmonary alveolar proteinsis (PAP) [16].

The clinical presentation of a patient with interstitial lung disease should induce consideration of immunodeficiencies especially primary immunodeficiencies in the differential diagnosis if the etiology of the pulmonary condition is unknown [23]. Interstitial lung disease in particular GLILD is a prominent pulmonary complication mediated by immune disorders [24]. The early diagnosis and better management of interstitial lung disease may help to pursue a better outcome. We did not find a difference between immunodeficiencies with interstitial lung disease and immunodeficiencies without interstitial lung disease patients with respect to the outcome of lung disease at the end of follow-up. This may be due to the limitation of our study cohort, as we did not include a final study visit at the end of follow-up; also a selection bias, focusing on more severely affected patients, may play a role.

The clinical evidence for childhood interstitial lung disease is not outstanding or specific. In clinical practice, children with interstitial lung disease may only present with dry cough, shortness of breath, hypoxemia, pulmonary rales, and some other non-specific respiratory phenotypes[25]

2.4.2 The bronchoalveolar lavage fluid in patients with lung diseases and immunodeficiency

The bronchoalveolar lavage is a relatively non-invasive and well-tolerated procedure [26]. However, most frequently bronchoalveolar lavage cannot diagnose interstitial lung disease alone. The bronchoalveolar lav-age cellular differentiations are combined with thoracic imaging and clinical manifestation for accurate interstitial lung disease diagnosis [27].

In our cohort, bronchoalveolar lavage was performed in 59% of the patients (129/217). The numerically higher percentage of eosinophils in bronchoalveolar lavage fluid of patients with immunodeficiency and interstitial lung disease might point towards immune dysregulation in those patients.

In 129 patient bronchoalveolar lavage samples, the micro-biological test results of bronchoalveolar lavages were available. Among the frequently recovered organism were Pneumocystis (12% (16/129)), Cytomegalovirus (CMV)(5% (7/129)), followed by Viridans streptococci, Haemophilus influenza, and Streptococcus with a frequency of 4% (5/129) each. It must be considered that all patients were pre-treated empirically with antibiotics. Unfortunately, immuno-phenotyping was not consistently conducted on bronchoalveolar lavage fluid, hence no lymphocyte differentiation data were available.

2.4.3 Computed tomography in patients with lung diseases and immunodeficiency

Computed tomography is very useful to evaluate and diagnose rare and/or severe lung diseases [28]. Computed tomography can reduce the frequency of invasive investigations such as lung biopsy [29]. Many studies recommended that chest computed tomography should be performed routinely even under a negative result of X-ray, in order to make an accurate diagnosis of lung disease in immunodeficiency patients and enhance the outcome [30, 31] .

In our cohort, chest computed tomographies were done in about 89% of the subjects and 80% of these were consistent with interstitial lung disease. Computed tomography is a sensitive technique to detect interstitial lung disease. This was further supported by a high rate of concordance of radiological findings and the results of the lung biopsy. Histopathological examination confirmed a suspected interstitial lung disease in 95% of cases. However, computed tomography cannot differentiate the type of interstitial lung disease, thus lung biopsies do not always appear to be redundant. On computed tomography imaging interstitial thickening, pulmonary fibrosis, pleuropulmonary elastosis, or pleuroparenchymal fibroelastosis were the most common findings in interstitial lung disease patients with immunodeficiency.

2.4.4 Lung biopsy in patients with lung diseases and immunodeficiency

The lung biopsy is an important standard for the diagnosis of acute and chronic pulmonary complex if the diagnosis cannot be made by other means [32]. However, the invasiveness of lung biopsy sometimes restrains open biopsy techniques in critically ill patients. On the other hand, an over-restriction of lung biopsy may lead to an inaccurate diagnosis, relay an effective treatment, and induce an unexpected outcome [33]. Cryobiopsies are becoming a valid alternative.

In our cohort lung biopsies were done at a relatively high frequency of 66% of the interstitial lung disease patients. This was most likely due to a highly selected cohort of subjects with significant pulmonary problems, presenting after various diagnostic efforts and empirical therapeutic trials had been made. The biopsies led to an interstitial lung disease diagnosis in 95% of the cases. A precise diagnosis may also be important for novel treatments; e.g. presence of fibrosis in a biopsy may support treatment with anti-fibrotic drugs like nintedanib or pirfenidone.

As is known, GLILD is considered a partial lung condition in the common variable immunodeficiency[34] . Histopathology of interstitial lung disease may be used as a sign for an early and precise diagnosis of the complex syndrome that caused it. However, as the proportion of lung biopsies in patients with interstitial lung disease is not high, it is a limitation in the analysis of the relationship between pulmonary histopathology and genetic defect in primary immunodeficiency.

2.5 Presence of interstitial lung disease in genetically defined primary immunodeficiency

Clinical case series in immunocompromised children report variable rates of interstitial lung disease prevalence (64% (39/61 pediatric and adult cases [34]), 15% [35], 34%[36], 26% (18/69 cases[37]), 11% (3/28 cases [38]), 7% (46/637 cases [39], 15% (8/54 cases [31]), 13% (78/623 cases [40]), 30% (24/80 cases [41]), 11% (40/370 cases [42]), 8% (6/73 cases [43]), 7% (114/1647 cases [44]), 10% (22/219 cases [45]), 40% (29/73 [46]), 18% (9/50 cases [47]), 9% (138/1518 cases [48]), 22% (16/73 cases [49]), 20% (29/148 cases [50]), and 12% (4/33 cases [51]). A recent pediatric study reported a very low rate of interstitial lung disease, e.g. 1% (11/796 cases [52]).

The interstitial lung disease may act as a major clinical feature for the diagnosis primary immunodeficiency. Patients with disease causing mutations in CTLA4 (AD) , ITCH (AR), TMEM173(AR GOF), COPA(AD), HCK(AD GOF), OAS1 (AD GOF), HCK (AD GOF), DKC1 (XL), TERC (AD), TERT (AD), TINF2 (AD), ACD (AD), RTEL1 (AD), NOLA 3 (AR), NOLA 2 (AR), WRAP53 (AR), TERT (AR), ACD (AR) have been shown to express interstitial lung disease pattern [53].

In 18 out of 25 conditions we did not observe interstitial lung disease involvement of the lungs in agreement with the literature, whereas in 7 conditions we newly observed an interstitial lung disease. These diseases were caused by genetic variants in CD40, 10p13-p14DS, HELLS, TNFRSF13B, CYBA, and NCF2. The microorganisms found in patients and corresponding and related mutated genes are shown in the table 2.

Table 2 Gene defect and Microorganism present in the affected patient.

Immunodeficiency disease	Gene defect	Inheritance	Microorganism present in the affected patient
TACI deficiency	TNFRSF13B	AR	Unknown
Autosomal recessive CGD	NCF2	AR	Cytomegalovirus (CMV), haemophilus influenzae
Immunodeficiency with centromeric	HELLS	AR	Pneumocystis

instability and facial anomalies			
Autosomal recessive CGD	CYBA	AR	Aspergillus
CD 40 deficiency	CD 40	AR	CMV
CD 40 deficiency	CD 40	AR	Pneumocystis
Chromosome 10p13-p14 deletion syndrome	10p13-p14DS	AD	unknown

3. Zusammenfassung

Hintergrund: Interstitielle Lungenerkrankungen und Immundefizienzen sind beides seltene Erkrankungsgruppen. Es gibt im Kindesalter kaum Analysen von klinischen Daten und korrespondierenden genetischen Varianten.

Methode: In dieser Arbeit wurden Patienten mit primären und sekundären Immundefizienzen untersucht, um die besonderen pulmonalen klinischen Merkmale, insbesondere die interstitiellen Lungenveränderungen zu ermitteln.

Ergebnisse: Patienten mit primärer Immundefizienz hatten signifikant häufiger eine Blutsverwandtschaft und eine Familienanamnese mit Hinweisen auf interstitiellen Lungenerkrankungen. Dies kann darauf hinweisen, dass die Blutsverwandtschaft auf gemeinsame genetische Faktoren die für beide Erkrankungen von Relevanz sein können, hinweist. Die Computertomographie der Lungen und die Lungenbiopsie sind grundlegende und wesentliche Untersuchungen für die Diagnose interstitieller Lungenerkrankungen. Bei 49 % der Patienten mit primärer Immundefizienz wurde eine genetische Variante identifiziert, die die Ätiologie der Erkrankung erklärt. Unter diesen monogenen Defekten wurde die ZNFX1 Mutation von uns als krankheitsverursachender Gendefekt beschrieben. Darüber hinaus wurden für PLCG2, NCF2, CYBA, HELLS, TNFRSF13B, 10p13-p14DS, CD40 genetische Defekte beschrieben, die interstitielle Lungenerkrankungen als pulmonale Manifestationen von uns erstmals belegen.

Schlussfolgerung: Interstitielle Lungenerkrankungen sind ein wichtiges klinisches Merkmal einiger diagnostizierter primärer und sekundärer Immundefekte. Insbesondere bei Patienten mit primären Immundefekten sollten interstitielle Lungenerkrankungen gezielt beachtet werden.

4. Abstract (English)

Background: Interstitial lung diseases and immunodeficiency are both rare diseases. Reports on the analysis of clinical data and genetic mutations in these two conditions are scarce.

Method: This research assessed primary immunodeficiency patients to investigate the pulmonary clinical disease features, especially the presence of interstitial lung disease in immunodeficiency.

Result: Patients with primary immunodeficiency had significantly more frequently a history of consanguinity and a family history of interstitial lung diseases. This indicated the promise of consanguinity genetic factors for both conditions. The computed tomography and lung biopsy are basic and essential examinations for interstitial lung diseases diagnosis. The genetic mutation supporting was identified in 49% of the patients with primary immunodeficiency. Among these monogenic defects the ZNFX1 gene was linked by us as a disease causing genetic defect. In addition, for PLCG2, NCF2, CYBA, HELLS, TNFRSF13B, 10p13-p14DS, CD 40 genetic defects may present with interstitial lung disease as pulmonary disease manifestations, not previously reported.

Conclusion: Interstitial lung diseases represent major clinical features so far in children not very much acknowledged. Particularly in patients with primary immunodeficiencies, interstitial lung disease should be targeted for attention.

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5. Paper I

Multisystem inflammation and susceptibility to viral infections in human ZNFX1 deficiency

A full list of authors and affiliations appears at the end of the article.

Abstract

Background: 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-stranded RNA sensor that restricts the replication of RNA viruses in mice. The role of ZNFX1 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, and renal and lung disease.

Methods: Whole exome sequencing was performed on 13 patients from 8 families. We investigated the transcriptome, posttranscriptional 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 the mRNA of ISGs and also associated with poorer clearance of viral 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.

Keywords

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

Studies of patients showing susceptibility to specific viral infections have helped to elucidate critical pathways in innate and adaptive immunity. Pathogenic variants of genes that disrupt type I and III interferon immune responses (eg, *TLR3, UNC93B, IRF7, 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 interferon-stimulated double-stranded RNA (dsRNA) sensor that restricts the replication of RNA viruses
in mice[6] and contributes to transgeneration inheritance in *Caenorhabditis elegans* by binding to mRNA complexed with short, noncoding RNAs.[7] ZNFX1 expression is low in

uninfected cells but is rapidly upregulated in response to viral infections and exposure to type I interferons.[8] ZNFX1 binds to viral RNA and interacts with the mitochondrial antiviral signaling (MAVS) protein, promoting the expression of interferon-stimulated genes (ISGs). Signaling downstream of ZNFX1 does not depend on 2 other MAVS-associated cytosolic viral sensors (retinoic acid-inducible gene I [*RIG-I*] and melanoma differentiation– associated protein 5 [*MDA5*]).[6] Furthermore, although studies of ZNFX1-deficient mice and cell lines identified a role for the protein in sensing dsRNA, 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 2 clinically affected (but not genotyped) siblings from 8 unrelated kindreds. This early-onset disease is characterized by susceptibility to viral infections, multiorgan 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 in this article came from Iraq (n = 3), Syria (n = 2), Turkey (n = 4), Germany (n = 2), Australia (n = 2), Egypt (n = 1), and Canada (n = 1). For details on individual patients, please refer to the Patient Clinical History section and the accompanying tables in this article's Online Repository (at www.jacionline.org).

WES

Whole exome sequencing (WES) was performed on 13 of the affected individuals and on 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 as well as to filter and prioritize nuclear single-nucleotide variants and indel variants (see the Methods section of the Online Repository at www.jacionline.org).

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 Online Repository.

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 (Fig 1, A and B, Table I, and see Table E1 in the Online Repository at www.jacionline.org). The severe infections were caused by RNA viruses (influenza A virus [negative single-stranded RNA (ssRNA) (n = 5)], influenza B virus [negative ssRNA (n = 1)], parainfluenza virus [negative ssRNA (n = 1)], respiratory syncytial virus [negative ssRNA (n = 2)], norovirus [positive ssRNA] (n = 2)], and rotavirus [dsRNA (n = 1)]) or DNA viruses (human herpes virus type 6 [n = 3], adenovirus [n = 2], and cytomegalovirus [n = 1]) (Table I). Hence, most of these pathogens were negative ssRNA or DNA viruses. It is noteworthy that live virus vaccines also caused severe vaccine strain infections in 2 patients (measles and varicella zoster virus,

respectively) (see Table E2 in the Online Repository at www.jacionline.org). A rotavirus infection recurred in patient 2.2 (P2.2) within a few weeks , and human herpes virus type 6 (with a variable copy number) was detectable in P5.2 for 5 months. Although ongoing disease manifestations in these 2 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 during childhood, with 7 deaths occurring before patients reached the age of 3 months (Fig 1, A, and see Table E1). The mean age at death was 3.6 years (median = 1.1 years; range = 3 months to 15 years). Inflammatory episodes with hepatitis and cytopenia were fatal in 7 cases (age at death = 0.3-8 years). Sepsis was reportedly the cause of death in P6.1 (at the age of 9 years). P1.2 died of necrotizing pulmonary aspergillosis 5 years after lung transplantation (ie, at the age of 15 years) . The cause of death was unknown for P1.1 and P3.1 (for their clinical histories, see the Patient Clinical History section in the Online Repository at www.jacionline.org).

Infections leading to severe inflammatory diseases were the initial presentation in 9 of the 15 patients and were present in 12 of the 15 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 (23.8-50.0 × 10^9/L), neutrophilia, and lymphocytosis. Other initial presentations were seizures (in 3 patients), renal disease (in 2 patients), and lung disease (in 1 patient).

A total of 14 patients had hepatic disease, as evidenced by elevated serum liver enzyme levels (n = 12 patients), hepatomegaly (n = 13), elevated serum lactate dehydrogenase levels (n = 10), coagulopathy (n = 7), and hepatic encephalopathy (n = 1) (see Table E3 in the Online Repository at www.jacionline.org). In 11 patients, hepatic disease was associated with systemic inflammatory disease. A total of 3 patients met the criteria for acute liver failure (see Table E3). Histologic assessment of the liver showed heterogeneous, nonspecific 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) (see Fig E1 in the Online Repository at www.jacionline.org).

Of the 12 patients with systemic inflammatory disease, 6 met the diagnostic criteria for hemophagocytic lymphohistiocytosis (HLH), including hemophagocytosis in bone marrow aspirates (Table II[9] and Fig 1, C). Some patients experienced more than 1 HLH or HLH-like episode associated with hepatitis and leukocytosis, with the latter being much less common in classical HLH. Levels of natural killer cell degranulation and/or cytotoxicity were normal in all patients with HLH. The level of perforin expression was in the lower normal range in P5.1 and P5.2, which was probably due to a heterozygous p.Ala91Val variant in the *PRF1* gene that was also carried by their healthy father (data not shown). Spontaneous remission of systemic inflammation was observed in some patients, whereas immunosuppressants were administered to others—with varying degrees of success (see Table E1). The Janus kinase inhibitor ruxolitinib was administered to 1 patient (P5.2); it had a beneficial but transient effect.

Neurologic involvement was observed in 10 patients, 7 of whom experienced recurrent seizures. In 3 cases, the seizures occurred during an episode of HLH (P4.1, P7.1, and P8.1). A total of 3 patients showed developmental regression (P4.2, P7.1, and P8.1). Neuroimaging showed evidence of multiple focal calcifications in 3 patients (P1.2, P1.3,

and P8.1), ischemic lesions (diffusion restriction on magnetic resonance imaging) in 4 patients (P3.2, P4.1, P5.1, and P7.1), and T2 hyperintense lesions in 5 patients (P1.2, P3.2, P4.2, P 5.2, and P7.1) (Fig 1, D and see Fig E2 in the Online Repository at www.jacionline.org).
Leptomeningeal enhancement was observed in patients P3.2 and P4.1 during an episode of HLH. Autism spectrum disorder was diagnosed in 2 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 6 patients (P1.1, P1.2, P1.3, P2.1, P2.2, and P8.2). One patient (P2.2) experienced 2 episodes of respiratory syncytial virus bronchiolitis with respiratory failure within the space of a few weeks. A total of 6 patients had pulmonary hemorrhage. Chest computed tomography and a histopathologic assessment of lung biopsy specimens from P1.2 and P1.3 showed interstitial pneumonitis and cholesterol pneumonitis, respectively (Fig 1, E and see Fig E3 in the Online Repository at www.jacionline.org).

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 1, F, and see Fig E4 in the Online Repository at www.jacionline.org). 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 levels 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, the data indicate that although peripheral destruction might have contributed to the bicytopenia (eg, in cases with TMA, we think that HLH outweighs as the driving force for the bicytopenia).

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 magnetic resonance imaging of this patient showed that the white matter changes present at the age of 32 months had stabilized at the age of 42 months (ie, 6 months after HSCT) and had even regressed 5 years after HSCT (Fig 2, A–F). 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 years) 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 treatment at all.

Biallelic *ZNFX1* variants in the patients

By using WES, we identified 11 biallelic *ZNFX1* variants in 13 patients (ie, in all 8 families studied; Fig 3, A). There were 5 truncating variants and 6 missense variants.
In all of the patients, *ZNFX1* was the only candidate gene that segregated with the disease. Only 1 variant (p.C1264S) is listed in the Genome Annotation Database (https://gnomad.broadinstitute.org/) as being heterozygous and occurring at a frequency of 1.22×10^{-5}. All missense variants were predicted to be deleterious by several tools, including combined annotation-dependent depletion (CADD), PROVEAN PolyPhen-2, and the algorithm Sorting Intolerant from Tolerant (SIFT) (see Table E4 in the Online Repository at www.jacionline.org).

ZNFX1 is a 1918–amino acid multidomain protein comprising a large helicase domain

with an ATP-binding site[10] and a DEAD helicase box,[11] 6 zinc fingers, and a coiled-coil region (Fig 3, B). The large helicase domain is homologous to the human RNA helicase Aquarius that is involved in RNA splicing.[12] The spatial distribution of the patients' 4 missense variants within the RNA helicase motif are shown in the 3-dimensional model of ZNFX1 in Fig 3, C.

ZNFX1 mRNA is ubiquitously expressed in human tissues, albeit predominantly in the hematopoietic system (see Fig E5 in the Online Repository at www.jacionline.org). Low ZNFX1 protein expression was noted in fibroblasts under resting conditions, whereas a rapid upregulation was observed after 24 hours of stimulation with transfected poly(I:C) or poly(dA:dT) (Fig 3, D). ZNFX1 could not be detected in whole cell extracts of fibroblasts from 2 of the patients carrying biallelic stop codons (p.R900Mfs*5/p.H542Cfs*41 in P2.1 and p.K133*/p.K133* in P3.2), whereas low levels of ZNFX1 could be detected in extracts from stimulated dermal fibroblasts isolated from P5.1, who bears 1 missense variant (p.C1264S) and 1 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

Because ZNFX1 deficiency was associated with severe viral infections in the patients, we evaluated the capability of patient cells to initiate an antiviral interferon response leading to elimination of infection with vesicular stomatitis virus (VSV) or influenza virus *in vitro*. Indeed, after prestimulation through transfection of poly(I:C), (LyoVec poly(I:C)), the monocytes of P2.1 were less efficient in clearing VSV than the control monocytes were (Fig 4, A and B). In notable contrast, the baseline expression of ISGs seen in peripheral blood isolated from the patients (see Fig E6, A in the Online Repository at www.jacionline.org) was higher than in the controls. This difference was biologically relevant because it was associated with a moderate resistance of unstimulated patient monocytes to the VSV and influenza virus infections (see Fig E6, B).

The defective ability of the patient 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 patient-derived dermal fibroblasts stimulated with intracellular poly(I:C) or poly(dA:dT), we found an enhanced expression of the ISGs *IFIT1* and *OAS2* (see Fig E7, A and C in the Online Repository at www.jacionline.org). Transfection with poly(dA:dT) also caused an increased rate of expression of *IFIT2,* whereas transfection with poly(I:C) did not affect the expression pattern of this ISG. On the other hand, patient fibroblasts exposed to poly(I:C) in solution failed to increase the expression of *IFIT1* and *IFIT2* to the levels observed in the control fibroblasts under the same conditions (Fig E7, B).

Transcriptomic analysis of dermal fibroblasts derived from 4 patients and 4 controls (treated with intracellular or soluble dsRNA or double-stranded DNA) confirmed the qPCR data showing an increased rate of expression of ISGs in response to intracellular double-stranded nucleic acids (Fig E7, A and C), as evidenced by overexpression of ISGs involved in antiviral responses (Fig 4, C). Although treatment with soluble poly(I:C) (no LyoVec poly(I:C) confirmed qPCR data; see Fig E7, B) showing a marked reduction in the expression of most ISGs involved in antiviral defense (Fig 4, C), it was associated with elevated levels of expression of ISGs known to modulate the p53-dependent apoptosis pathways (promyelocytic leukemia protein [PML] and Shisa family member 5 [SHISA5]). Analysis of pathways belonging to the canonic 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 patient fibroblasts compared with that in the control fibroblasts (Fig 4, D). Consistent with an upregulation of this pathway, we observed elevated transcript levels of cytokines such as IL-6, C-X-C motif chemokine ligand 10 (CXCL10), C-C motif chemokine ligand 4, C-C motif chemokine ligand 5, and IFN-β. Stimulation with soluble poly(I:C) instead
resulted in lower fold induction of type I interferons and other nuclear factor κB–responsive ISGs (Fig 4, E) in patient fibroblasts. Interestingly, the rates of expression of transcripts encoding known apoptosis-inducing proteins (FADD and caspase 8) were upregulated in poly(I:C)-stimulated patient fibroblasts, which is consistent with the known role of some components of the TLR3 signaling pathway in inducing dsRNA-induced cell death through caspase 8. Stimulation of the patient fibroblasts with intracellular poly(dA:dT)

resulted in higher expression of ISGs belonging to the STING pathway than in the control fibroblasts, including downstream type I interferons and interferon-responsive cytokines and chemokines (Fig 4, F).

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

ZNFX1 is required for a balanced posttranscriptional regulation of ISGs

Because previous work has demonstrated that ZNFX1 in lower eukaryotes binds to endogenous transcripts and regulates their processing by microRNA, we evaluated whether posttranscriptional mechanisms might influence the differential rate of expression of some ISGs detected in the patient fibroblasts. Therefore, to understand the mechanism underlying higher ISG expression rates after extended (18-hour) stimulation with double-stranded nucleic acids (Fig 5, A), we examine 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, an inhibitor of transcription elongation by RNA polymerase II, to the cultures 18 hours after poly(dA:dT) transfection. The levels of ISG mRNAs at 0, 30, 60, and 90 minutes after initiation of the 6-dichlorobenzimidazole
1-β-D-ribofuranoside treatment were higher in the patients than in the controls, which indicated that ISG mRNAs were more stable in the absence of ZNFX1 (Fig 5, B). Secretion of IFN-β 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 that in healthy controls (Fig 5, C and D). Finally, supplementation of fibroblasts with a ZNFX1 wild-type construct lowered secretion of IFN-β 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 posttranscriptional regulation, toward 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 as well as

subsequent multiorgan dysfunction and inflammation. The consistent clinical phenotype observed among the 15 patients from 8 unrelated families with distinct ethnic backgrounds suggests that ZNFX1 is the causative gene for this disease.

Compared with ZNFX1-deficient mice in prior studies,[6] our patients exhibited 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 have shown that although transfection with synthetic dsRNA and double-stranded DNA (dsDNA) oligos (mimicking infections with DNA and RNA viruses) causes an upregulation of inflammatory pathways, pretreatment with intracellularly delivered dsRNA does not protect patient-derived monocytes from infection. This lack of protection may be due to

the complex gene signature seen in the patient fibroblasts following treatment with nucleic acids, which on the one 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 or human cell lines to DNA virus infections.[6] Therefore, the damage caused by DNA viruses in 6 of the patients might be directly linked to an insufficient resolution of the interferon response to the infection and not to excessive viral load. Extracellular dsRNA-mimicking oligos also cause a hyperresponse, 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 1 patient within a few weeks and 2 patients experienced vaccine strain infections (measles and varicella zoster virus, respectively). These are extremely rare events in immunocompetent hosts,[13] but they are well documented in patients with defective type I and III interferon immune responses.[14–18] Amelioration of central nervous system manifestations after HSCT points to an immune-driven disease. Although these observations do not fully exclude a tissue-specific role of ZNFX1 in neurons, liver cells, lung cells, 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, central nervous system manifestations could be caused by either HLH activity and/or viral infections.

For many viral infections, the severity of clinical disease is thought to be associated with a high viral load.[19–21] 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 (norovirus [positive ssRNA] and influenza A virus, RSV, parainfluenza virus, and influenza B virus [negative ssRNA]) were directly linked to HLH or to HLH-like manifestations in 7 patients. Multiorgan involvement might be suggestive of hyperinflammation caused by viral escape and viremia. However, a persistent viral load was observed in only some of the patients with ZNFX1 deficiency, whereas 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.[22] TMA has also been described as a dose-dependent adverse reaction to recombinant type I interferons in the treatment of viral hepatitis and multiple sclerosis.[23–27] The overexpression of inflammatory genes seen in cells of ZNFX1-deficient patients after exposure to intracellular double-stranded sDNA and dsRNA might therefore be implicated in the pathogenesis of TMA that is observed in these patients.

Our observation that in the absence of ZNFX1 the half-life of ISGs is increased following extensive stimulation (for 24 hours) with intracellular DNA offers an attractive mechanism that is in line with the findings of previous work showing the essential role of ZNFX1 in posttranscriptional regulation of mRNA in lower eukaryotes.[7,28] Nevertheless, whether ZNFX1 is directly involved in regulating the half-life of ISGs, or whether regulation of ISG mRNA 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, and 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.[29] To date, variants in 6 different genes *(PRF1, UNC13D, STXBP2, STX11, RAB27A,* and *LYST)* are known to directly affect perforin-mediated cytotoxicity and thereby cause HLH.[30] 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.[31–36] *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 Janus kinase inhibitor) led to only transient benefit. In 1 patient, HSCT arrested the HLH-like episodes and was followed by improvements in neurologic development.

We recommend that (1) variants in ZNFX1 be included in genomic screens for patients experiencing severe viral infections and HLH and (2) HSCT be evaluated as a treatment for patients with ZNFX1 deficiency.

We have shown that ZNFX1 is important for sensing of viral-derived double-stranded nucleic acids in humans. Our data furthermore implicate a role for ZNFX1 in posttranscriptional regulation of ISGs, as was previously found for other proteins such as ZAP.[37] Higher expression of ISGs was seen in the peripheral blood of patients with

ZNFX1, together with a lower predisposition to infection, suggesting that a persistent status of hyperinflammation might on the one hand provide some levels of protection from viral infections but might on the other hand contribute to multiorgan 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.

Supplement Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

CXCL10 C-X-C Motif chemokine ligand 10

dsRNA Double-stranded RNA

HLH Hemophagocytic lymphohistiocytosis

HSCT Hematopoietic stem cell transplantation

ISG Interferon-stimulated gene

MAVS Mitochondrial antiviral signaling protein

MDA5 Melanoma differentiation-associated protein 5

PML Promyelocytic leukemia protein

qPCR Quantitative PCR

RIG-I Retinoic acid–inducible gene I

SAP SLAM-associated protein

SHISA5 Shisa family member 5

ssRNA Single-stranded RNA

STING Stimulator of interferon response cGAMP interactor

TMA Thrombotic microangiopathy

TLR3 Toll-like receptor 3

VSV Vesicular stomatitis virus

WES Whole exome sequencing

XIAP X-linked inhibitor of apoptosis

ZNFX1 NFX1-type zinc finger–containing 1

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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.

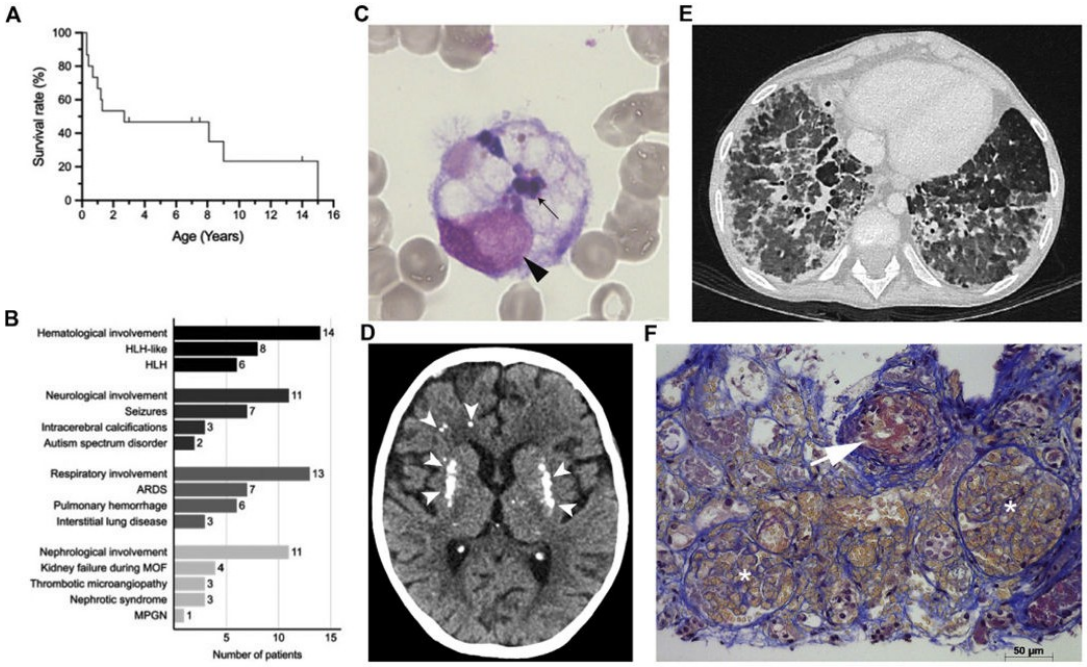

FIG 1.

Severe viral infections and inflammatory disease in patients with ZNFX1 deficiency. **A,** Kaplan-Meier survival curve for patients; dashes indicate ages of patients who are alive. **B,** Overall inflammatory organ involvement with or without a proven link to infections; number of patients affected. **C,** May-Gruenwald-Giemsa staining (light microscopy; magnification,

×1000) of a bone marrow aspirate from P5.2. A macrophage with engulfed leukocytes is shown: its nucleus is indicated by an ar, and the engulfed leukocytes are indicated by an arrow. **D,** Computed tomography image of the brain of P1.2 at the age of 15 years showing calcification of the basal ganglia and white matter abnormalities (*white ars*). **E,**

A high-resolution computed tomography image of the lungs P1.2 at the age of 9 years and 11 months, showing bilateral diffuse ground glass attenuation, subpleural thickening, and septal thickening. **F,** Jones staining of a kidney biopsy specimen, 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). *ARDS,* Acute respiratory distress syndrome; *MOF,* multiorgan failure; *MPGN,* membranoproliferative glomerulonephritis.

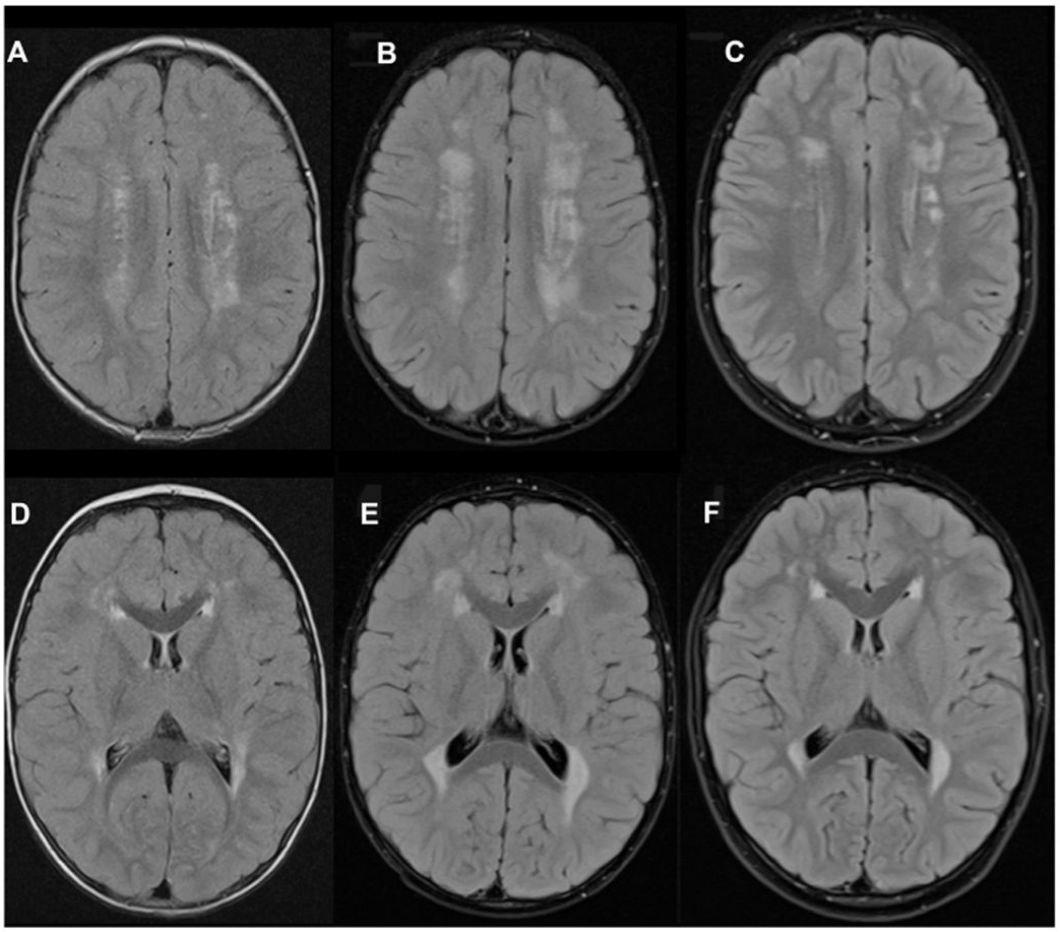

FIG 2.
Regression of white matter changes in the brain of patient P4.2 following HSCT. Axial fluid–attenuated inversion recovery. Magnetic resonance images at the ages of 32 months (**A** and **D**), 42 months (**B** and **E**), and 8 years (**C** and **F**) demonstrating an initial increase in periventricular and deep white matter changes 6 months after HSCT (**B** and **E**) and then marked regression seen at last follow-up (5 years after HSCT) (**C** and **F**).

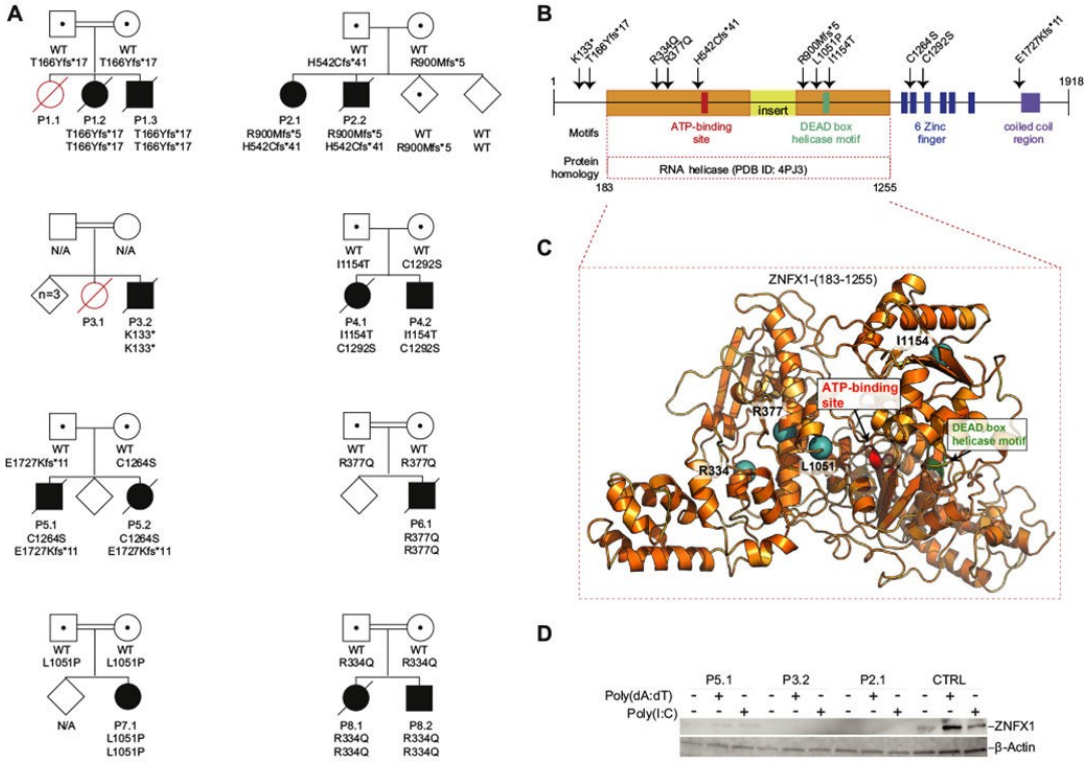

FIG 3.

Biallelic *ZNFX1* variants lead to the loss of protein expression in response to stimulation by intracellular nucleic acids. **A,** The pedigrees of the 8 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, whereas unaffected individuals are indicated by open diamonds. Circles indicate females, and squares indicate males. Slashes over symbols indicate deceased patients. N/A (meaning not available) indicates that sequencing was not performed. **B,** Predicted domains and

identified variants in the ZNFX1 amino acid sequence. The 11 deleterious variants identified are indicated by arrows. The domain homologous to the RNA helicase Aquarius (Protein Data Bank identifier 4PJ3) is highlighted in orange, with an insert shown in yellow. **C,**

A ribbon diagram of a homology model of ZNFX1 (183-1255), based on the structural template RNA helicase Aquarius (Protein Data Bank identifier 4PJ3) is shown. Locations of the 4 missense variants within this domain are shown as teal spheres in the present study. **D,** A protein immunoblot for ZNFX1 in dermal fibroblasts from a healthy donor

(control [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). β-Actin was used as a loading control.

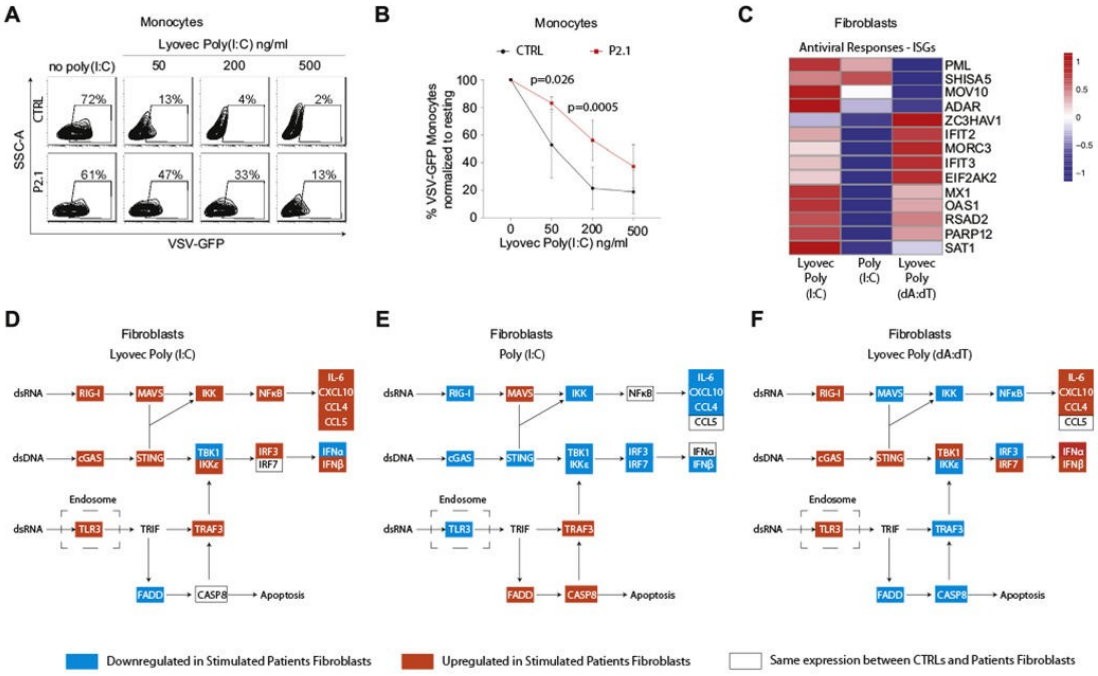

FIG 4.

Biallelic defects in ZNFX1 deregulate ISGs' expression and protection against viral infections in response to treatment with nucleic acids. **A** and **B,** Flow cytometry analysis of monocytes from P2.1 and a healthy control (CTRL) pretreated for 12 hours with different concentrations of LyoVec-poly(I:C) and subsequently infected with VSV–green fluorescent protein (GFP) for 5 hours. Representative plots of a single experiment showing VSV-GFP signal versus area of side scattered signal (SSC-A) (**A**) and mean percentage of

VSV-GFP-positive monocytes relative to the unstimulated condition (no LyoVec-poly(I:C)) for 4 repeats (**B**). Error bars refer to the SD (n = 4). *P* values were calculated by using

2-way ANOVA and the Sidak multiple comparisons test. **C,** Transcriptomic analysis results for selected ISGs involved in antiviral responses are summarized in a heat map showing the mean difference in fold induction of ISGs expression from resting conditions in dermal fibroblasts from 4 patients (P1.2, P2.1, P3.2, and P5.2) over that in dermal fibroblasts from 4 different age-matched, healthy controls. Three different stimulations were used: 18 hours of intracellular poly(I:C) (LyoVec Poly (I:C)), 6 hours of soluble poly(I:C) (Poly (I:C)), or
18 hours of transfected poly(dA:dT) (LyoVec Poly (dA:dT)). **D-F,** The same data were used to study the activity of canonic double-stranded nucleic acids sensing pathways according to the Kyoto Encyclopedia of Genes and Genomes. Colored highlights indicate the rate of fold induction of gene expression in patients over that in the controls: red highlights the indicated increase, blue highlights the indicated decrease, and white boxes indicate no difference.

Results from stimulation with LyoVec-poly(I:C) is shown in (**D**), with soluble poly(I:C) in (**E**) and LyoVec-poly(dA:dT) in (**F**). *CASP8,* Caspase 8.

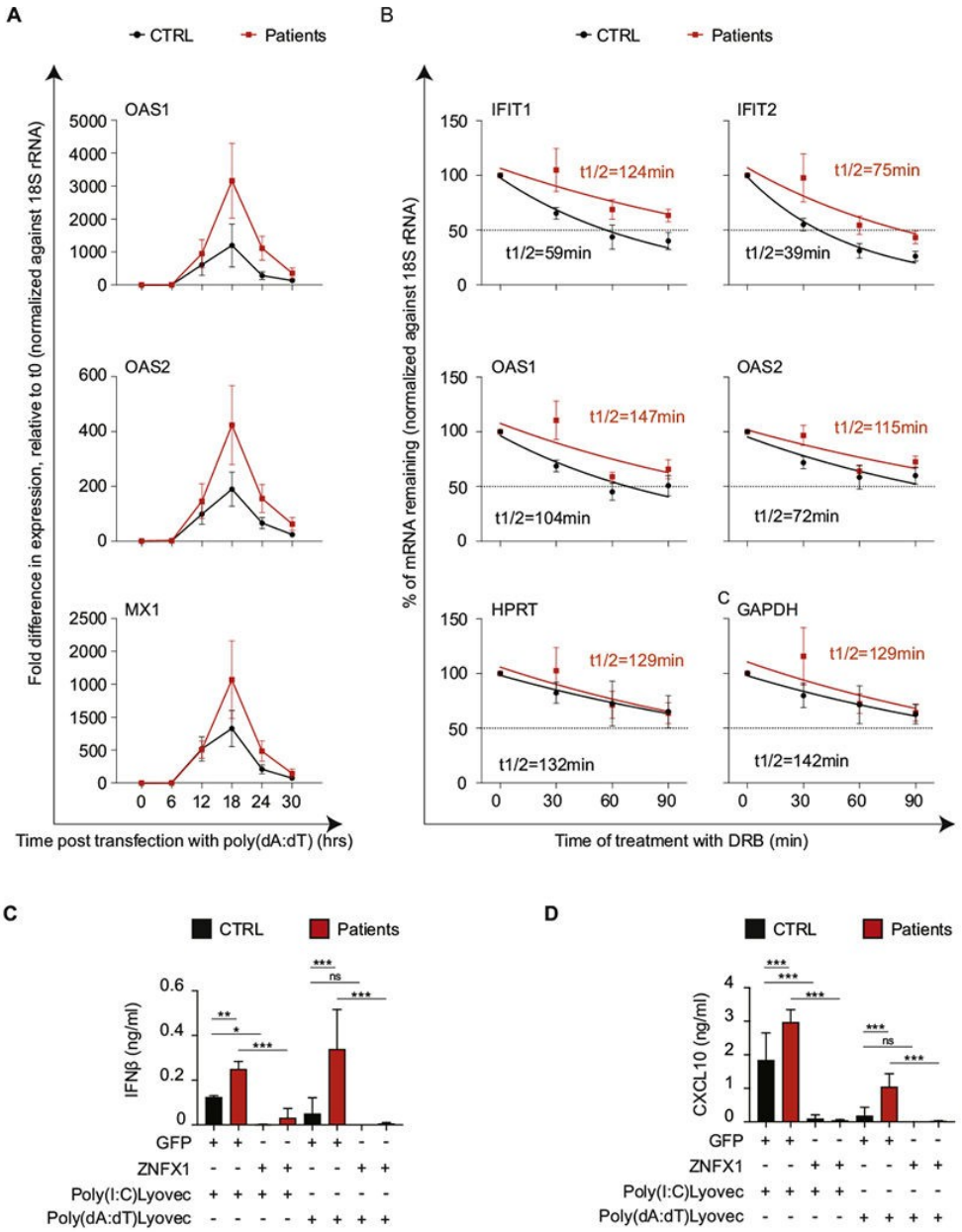

FIG 5.

Increased ISG expression in response to transfected poly(dA:dT) in biallelic defects in ZNFX1 is associated with increased mRNA stability. **A,** The mRNA expression levels of *OAS1, OAS2,* and *MX1* (representative ISGs) by skin fibroblasts from P1.2, P2.1, P3.2, and P5.2 (*red squares*) and 4 healthy controls (CTRLs) (*black circles*) at baseline (zero hours) and at different time points (6, 12, 18, 24. and 30 hours) after stimulation with transfection reagent–complexed poly(dA:dT). **B,** Mean values of mRNA stability of representative ISG mRNAs in fibroblasts from 4 healthy controls and 4 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) 18 hours after transfection with LyoVec-

poly(dA:dT).qPCR was performed at the indicated time points after the addition of DRB. 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 zero). The half-life ($t^{1/2}$) of each mRNA (*red for P1.2 and black for CTRL*) was calculated by using nonlinear regression analysis. A representative result of 3 independent experiments is shown. Concentrations of IFN-β (**C**) and CXCL10 (**D**) in the supernatant of dermal fibroblasts from 3 healthy controls (CTRL [*black bars*]) and 3 patients (P1.2, P3.2, P5.2 [*red bars*]) following 18 hours of stimulation with poly(I:C)LyoVec or poly(dA:dT)LyoVec. Fibroblasts were transfected with plasmids expressing ZNFX1 or green fluorescent protein. Shown is the mean of 3 repeats for each of the 3 samples(n = 9), with error bars showing the SD. *P* values were calculated by using ordinary 1-way ANOVA as follows: ns = 0.12; **P* = .033l; ***P* = .002; and ****P* < .001.

TABLE I.

Clinical characteristics

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

ADV, Adenovirus; *CMV,* cytomegalovirus; CNS, central nervous system; *dsDNA,* double-stranded DNA; F, female; *FU,* follow-up; *HHV6,* human herpes virus type 6; *HLH,* hemophagocytic lymphohistiocytosis; *ID,* identifier; M, male; *NA,* not available; *RSV,* respiratory syncytial virus; SAP, SLAM-associated protein; *VZV,* varicella zoster virus.

TABLE II.

teria for hemophagocytic lymphohistiocytosis

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 level (≥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 [y])	HLH trigger
NA	low	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	No HLH	Not applicable
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
Yes	7.1	29	17.4	Not in bone marrow (age 11 mo)	Yes (4,511)	Yes TG: 3,32, coagulation defect	Yes; 3,957	Normal	Normal CD107 expression	NA	Yes (6/8)	HLH (6 mo)	Rotavirus and norovirus
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
Yes	6.0	6	25.58	NA	Yes (34,616)	No Fib: 1.9 TG 0.8	NA	Not done	Not done	NA	NA (4/5; NA: 3)	HLH-like (3 mo)	Influenza A
NA	NA	low	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	Not applicable
No	8.8	34	9.12	NA	No (220)	No Fib 1.55	No; 2,122	Not done	Not done	NA	No (2/6; NA: 2)	HLH-like (1 y)	HHV6
Yes	6.7	15	33	In liver (autopsy)	Yes (4,890)	Yes TG: 9.8 Fib not decreased: 5.4	No	Normal	Normal CD107 expression	Normal	Yes (5/8)	HLH (7 mo)	ADV
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
No	7.5	54	36.6	No (CSF analyzed)	Yes (82,148)	Yes Fib: 0.78 TG not increased: 1.2	Yes; 3,185	Not done	Normal CD107 expression	13% in NK cells (ref >5%)*	Yes (5/8)	HLH (2 mo)	HHV6, low-level CMV viremia
Yes	6.8	14	36.7	Yes (in BM)	Yes (37,474)	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

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 level (≥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 [y])	HLH trigger
Yes	9.1	63	17.3	NA	NA	NA	NA	NA	NA	NA	NA (3/3; NA:5)	No HLH	Not applicable
Yes	low	42	50	NA	Yes (12,000)	No	No	Normal NK cytotoxicity	Normal CD107a expression		NA (4/7; NA:1)	HLH-like (5 mo)	
Yes	7.5	20	>50	Yes (BM)	Yes (23,000)	No	NA				Yes (5/7; NA:1)	HLH (1 y)	Vaccine strain measles and low-level EBV viremia
Yes	low	low	high	No (CSF)	Yes (2,546)	No	NA			†	NA (4/7; NA:1)	HLH-like (2 y 9 mo)	Influenza B and *Staphylococcus aureus*
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
No	Yes	5.2	18	No (BM)	Yes (2,805)	No	Yes (2,534)	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 (1,164)		NA	NA	No (4/8)	HLH-like (6 y)	Influenza A

BM, bone marrow; *CMV*, cytomegalovirus; *CSF*, cerebrospinal fluid; *Fib*, Fibrinogen level; *HHV6*, human herpes virus type 6; *HLH*, hemophagocytic lymphohistiocytosis; *ID*, available; *NK*, natural killer; *ref*, reference; *SAP*, SLAM-associated protein; *TG*, triglycerides (fasting level).

for hemophagocytic lymphohistiocytosis, according to the HLH-2004 study group's criteria (Henter et al[9]).

n was in the lower normal range in P5.1 and P5.2; this was probably due to the concomitant presence of a heterozygous p.A1a91Val variant in PRF1, which was also carried by the not shown). In the (male) patient P5.1, SAP and XIAP expression was measured by flow cytometry and was normal.

w 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 K 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.

6. Paper II:

Article

Interstitial Lung Disease in Immunocompromised Children

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Abstract: Background: The range of pulmonary complications beyond infections in pediatric im- munocompromised patients is broad but not well characterized. Our goal was to assess the spectrum of disorders with a focus on interstitial lung diseases (ILD) in immunodeficient patients. Methods: We reviewed 217 immunocompromised children attending a specialized pneumology service during a period of 23 years. We assigned molecular diagnoses where possible and categorized the under- lying immunological conditions into inborn errors of immunity or secondary immunodeficiencies according to the IUIS and the pulmonary conditions according to the chILD-EU classification system. Results: Among a wide array of conditions, opportunistic and chronic infections were the most frequent. ILD had a 40% prevalence. Of these children, 89% had a CT available, and 66% had a lung biopsy, which supported the diagnosis of ILD in 95% of cases. Histology was often lymphocyte predominant with the histo-pattern of granulomatous and lymphocytic interstitial lung disease (GLILD), follicular bronchiolitis or lymphocytic interstitial pneumonitis. Of interest, DIP, PAP and NSIP were also diagnosed. ILD was detected in several immunological disorders not yet associated with ILD. Conclusions: Specialized pneumological expertise is necessary to manage the full spectrum of respiratory complications in pediatric immunocompromised patients.

Keywords: interstitial lung disease; ILD; diffuse parenchymal lung disease; primary immunodefi- ciency; PID; secondary immunodeficiency; SID; genetic defect

1. Introduction

The lung is a complex parenchymal tissue ensuring proper gas exchange. While continuously perfused with blood through the capillary network, the large internal sur- face of the organ is exposed to air-born micro-organisms and many other environmental factors. A robust immunological balance is necessary to keep this delicate system fully functioning [1,2]. Host defense and multiple immunological, inflammatory and structural reactions involve, on the one hand, the airways contacting the outside world and, on the other hand, the interstitial organ compartment. These defense processes can resolve or lead to chronic immune cell-shaped specific tissue reactions, including fibrotic tissue repair processes or organ destruction with respiratory failure [3]. Due to this fragile balance of immune tolerance and response, it is obvious that the lungs are an important target organ in immunocompromised patients; pulmonary complications have been shown to represent the main clinical manifestations of immunodeficiencies and are an important cause of death [4–6]. Childhood immunodeficiencies are a broad group of rare diseases either caused by inborn errors of immunity, classified as primary immunodeficiency, or by hemato-oncologic diseases or immunosuppressive treatments leading to secondary immun- odeficiency. The recent classification of the primary

immunodeficiencies differentiates more than 400 different molecularly defined entities [7]. Such fine granular classification has not yet been used to address the frequency and type of different pulmonary complications in children.

In the past, the focus was predominantly on infectious pulmonary complications. However, lung disease may clinically not only manifest as airway disease, including

bronchitis, bronchiectasis, obliterating bronchiolitis (BO) or asthma, but also as diffuse parenchymal or interstitial lung disease (ILD), including pulmonary hypertension (PHT) or lymphoproliferative disease (PTLD) [8]. Infrequently, pleural disease or pneumothorax is observed. Depending on the extent, all conditions may lead to respiratory failure with diffuse alveolar damage or acute respiratory distress syndrome (ARDS).

As both immunodeficiency and its pulmonary complications are rare, an overview from a specialized pediatric pneumology unit may be helpful to highlight some useful perspectives for the immunologist [9–11]. Our goal was to provide details on the clinical characteristics, including the age of onset, results of broncho-alveolar lavage, lung biopsy, chest computer tomography (CT), as well as the outcome of pediatric immunocompromised patients and pulmonary disease. Specifically, we focused on ILD manifestations within the different entities of immunodeficiency. Our findings indicate a high rate of various non-infectious complications and provide insight into the management of these patients in clinical practice.

2.Materials and Methods

We included all immunocompromised children assessed for significant lung disease between 1997 and 2020 in the Department of Pediatric Pneumology at the Dr. von Hauner Children's Hospital of the University of Munich. Clinical information was collected retrospectively from the pneumological clinics' charts, and patient files were updated for follow-up information.

Data on gender, age at investigation, consanguinity, family history, gestational age, O_2 supplement or mechanical ventilation during the neonatal period, as well as information on genetic and immunologic diagnostics and lung disease outcome were collected. Imaging studies were evaluated by pediatric radiologists with long-standing expertise in chest imaging, especially in pediatric interstitial lung diseases. Flexible bronchoscopy, including bronchoalveolar lavage (BAL), was performed if clinically indicated using 1 mL warmed normal saline per kilogram body weight 3 to 4 times [12]. BAL was performed in the most affected lobe or middle lobe if diffuse and examined for cell differentiation and microbiologically. In cases where a lung biopsy was obtained, the tissue was investigated by light microscopy, routine stain (hematoxylin and eosin stain (HE), Elastica van Gieson, PAS, iron) and bombesin, where indicated [13].

The immunodeficiencies were categorized using the system published by the International Union of Immunological Societies (IUIS) for inborn errors of immunity [7]. The primary immunodeficiencies included combined deficiencies, combined immunodeficien- cies with syndromic features, antibody deficiencies, immune dysregulation, congenital defects of phagocyte number or function, defects of intrinsic and innate immunity, autoin- flammatory syndromes and bone marrow failure. The secondary immunodeficiencies were due to malignancies or immunosuppressive treatment, including leukemias, lymphoma, other cancers, and transplantations.

The pulmonary conditions were categorized by the updated etiologic classification system of the chILD-EU register [14]. Currently, the histopathological description of lung

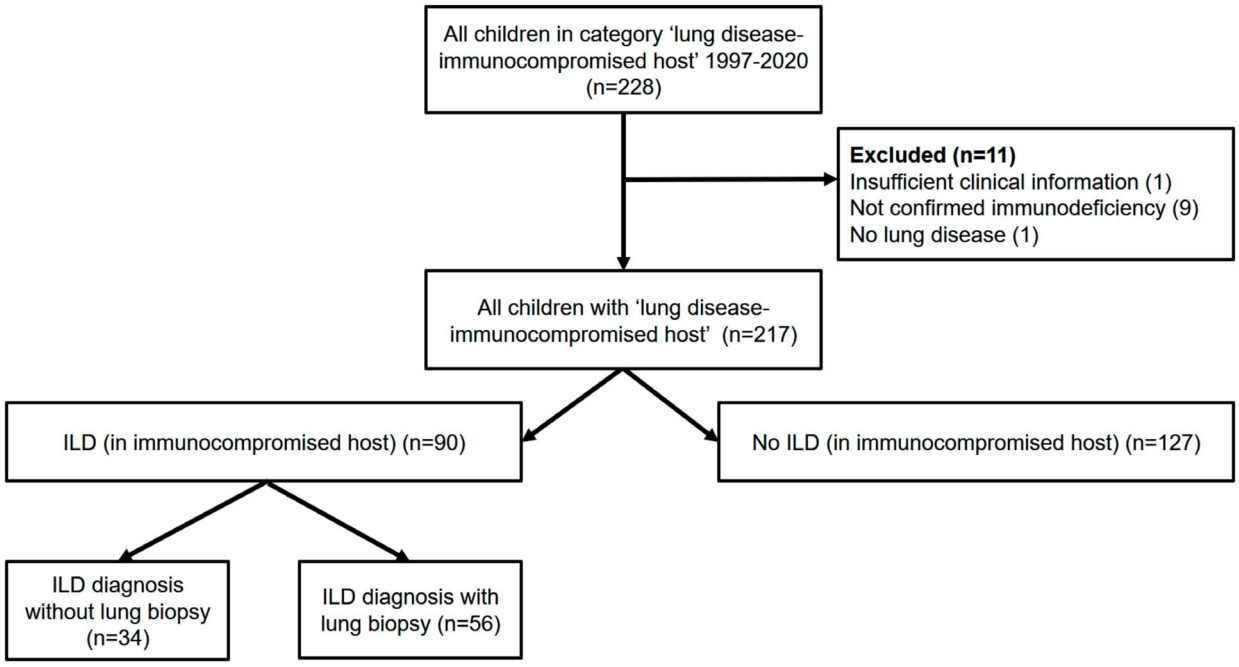
biopsies helps to categorize and distinguish specific parenchymal reaction patterns dominated by certain cell types or tissue components [15]. These most frequently include non-specific interstitial pneumonitis (NSIP), lymphoid interstitial pneumonitis (LIP), follicular bronchiolitis, granulomatous and lymphocytic interstitial lung disease (GLILD), desqua- mative interstitial pneumonitis (DIP) and alveolar proteinosis (PAP). NSIP histopathology consists of varying degrees of chronic inflammation and interstitial fibrosis, expanding the alveolar walls temporally and spatially uniformly and preserving lung architecture. The inflammation consists of lymphoid cells, mainly CD3-positive T lymphocytes, and small aggregates of CD20-positive B lymphocytes. The degree of infiltration is less than that in LIP, although potential overlap is recognized. Follicular bronchiolitis is characterized by lymphoid follicles around the bronchioles. DIP is characterized pathologically by a uniform involvement of lung parenchyma with an intra-alveolar accumulation of alveolar

macrophages. Mild chronic lymphocytic inflammation and mild-moderate interstitial fibro- sis may be present. PAP is a sometimes patchy intra-alveolar accumulation of amorphous, PAS-positive granular eosinophilic material that is lipid-rich (surfactant) and can contain cholesterol clefts and foamy macrophages [15].

3. Result

3.1 Characteristics of the Immunodeficiency Population and Spectrum of Associated Lung Diseases

The local pulmonary database retrieved 228 children, adolescents and young adults allocated to the disease category immunocompromised (Supplemental Table S1); 217 cases had sufficient information for review (Figure 1). Overall, more boys than girls were affected (60% vs. 40%), the majority (90%) of children were born as mature newborns, and less than 10% had respiratory problems at birth. Disease onset was at a median age



of 2 years (Table 1).

Figure 1. Overview of patients included and excluded.

Table 1. Clinical characteristics of patients with immunodeficiency and with ILD or without ILD.

	All Immunodeficiency Patients	Immunodeficiency with ILD	Immunodeficiency without ILD	Comparison between with/without ILD p
Total number	217	90 (41%)	127 (59%)	
Sex (male/female)	129 (59%)/88 (41%)	53 (59%)/37 (41%)	76 (60%)/51 (40%)	0.888 *
Age at onset of lung disease in years (range)	2.0 (0.0–20.1)	2.9 (0.0–15.2)	1.5 (0.0–20.1)	0.116 **
Follow-up duration in years (range)	4.9 (0.0–30.2)	4.9 (0.1–19.4)	4.9 (0.0–30.2)	0.893 **
ILD family history (yes/no)	10 (9%)/97 (91%)	8 (12%)/59 (88%)	2 (5%)/38 (95%)	0.233 *

A broad spectrum of lung diseases was identified (Table 2). Opportunistic and chronic infections were most frequent, occurring across all groups of immunodeficiencies at a rate of 65%. In the 129 BAL samples available from this group, viral, fungal and bacterial infections occurred. The most common opportunistic infections were caused by *Pneumocystis jirovecii* (12%). Cytomegalovirus was the second most common pathogen (5%). Bacteria, including *Viridans streptococci*, *Streptococcus pneumoniae* and *Haemophilus influenzae*, were also common causes of infection in this group (4%). Interstitial lung diseases were the second most common pulmonary complication, occurring at a rate of 40%. Respiratory failure was identified in more than 25% of the patients. Other less frequent conditions included ARDS, diffuse alveolar damage, pulmonary hypertension, bronchiolitis obliterans, bronchiectasis, PTLD, pneumothorax, asthma and pleural disease (Supplemental Table S2).

Comparing primary and secondary immunodeficiencies, the frequency of bronchiolitis obliterans was higher in the latter, whereas opportunistic and recurrent infections were more frequently observed in the group of primary immunodeficiencies. Interestingly, ILD frequency was the same in both groups. Next, we focused on the group of immunocompromised children with ILD.

3.2 *Comparison of Immunodeficient Children with and without ILD*

The patients were divided into two groups: (1) those with immunodeficiency and ILD and (2) those with immunodeficiency without ILD. More than 40% of the immunodeficient children were diagnosed with ILD. No significant differences in the clinical characteristics were evident (Table 1), including the cellular composition of broncho-alveolar lavage (Table 3). The numerically higher percentage of eosinophils in the BAL fluid of patients with immunodeficiency and ILD might point towards immune dysregulation in those patients; however, the difference was not statistically significant. Overall, patients had elevated percentages of neutrophils (normal < 3%) and eosinophils (normal < 0.5%) in their lavages, independent of the presence of ILD. This differentiation was based on cytology results, as the immunophenotyping of BAL cells was not regularly conducted.

Table 2. Immunodeficiency types and associated lung diseases.

	Lung Disease; n (% of Immunodeficiency Group)											
Immunodeficiency Type (n)	**Interstitial Lung Disease**	**Pulmonary Hypertension**	**Infections (Opportunistic/Recurrent)**	**Bronchiolitis Obliterans**	**Bronchiectasis**	**PTLD**	**Respiratory Failure**	**ARDS**	**Diffuse Alveolar Damage**	**Pneumothorax**	**Asthma**	**Pleural Disease**
All immunodeficiencies (217)	90 (41)	11 (5)	142 (65)	32 (15)	23 (11)	3 (1)	58 (27)	15 (7)	3 (1)	9 (4)	21 (10)	12 (6)
Primary immunodeficiencies (120)	52 (44)	6 (5)	88 (73)	6 (5)	19 (16)	-	31 (26)	9 (8)	-	1 (1)	13 (11)	4 (3)
Combined immunodeficiencies (22)	9 (41)	-	18 (82)	-	2 (9)	-	8 (36)	3(14)	-	1 (5)	1 (5)	-
Well-defined syndromes (15)	4 (27)	2 (13)	11 (73)	2 (13)	4 (27)	-	1 (7)	2 (13)	-	-	1 (7)	-
Antibody deficiencies (21)	4 (19)	1 (5)	17 (81)	1 (5)	8 (38)	-	2 (10)	1 (5)	-	-	6 (29)	-
Immune dysregulation (5)	2 (40)	-	3 (60)	1 (20)	-	-	1 (20)	-	-	-	-	-
Defects of phagocytes (30)	18 (60)	1 (3)	20 (67)	2 (7)	3 (10)	-	10 (33)	-	-	-	3 (10)	2 (7)
Defects of innate immunity (7)	3 (43)	-	6 (86)	-	1 (14)	-	4 (57)	2 (29)	-	-	-	1 (14)
Autoinflammatory syndromes (17)	10 (59)	2 (12)	12 (71)	-	1 (6)	-	4 (24)	1 (6)	-	-	2 (12)	1 (6)
Bone marrow failure (3)	2 (67)	-	1 (33)	-	-	-	1 (33)	-	-	-	-	-
Secondary immunodeficiencies (97)	38 (39)	5 (5)	54 (56)	26 (27)	4 (4)	3 (3)	27 (28)	6 (6)	3 (3)	8 (8)	8 (8)	8 (8)
ALL (15)	4 (27)	-	11 (73)	1 (7)	1 (7)	10(7)	5 (33)	1 (7)	-	-	1 (7)	1 (7)
AML (10)	3 (30)	-	6 (60)	1 (10)	-	-	3 (30)	-	1 (10)	-	-	1 (10)
Cancer, other (10)	4 (40)	-	4 (40)	1 (10)	-	-	-	1 (10)	-	1 (10)	1 (10)	-
CLL (2)	1 (50)	-	1 (50)	-	-	-	-	-	-	-	1 (50)	1 (50)
CML (1)	-	-	-	-	-	-	1 (100)	-	-	-	-	1 (100)
HIV (2)	1 (50)	-	1 (50)	-	-	-	-	-	-	-	-	-
Hodgkin lymphoma (3)	-	-	2 (67)	-	-	-	-	-	-	-	1 (33)	-
JMML (3)	2 (66)	1 (33)	2 (67)	1 (33)	-	-	1 (33)	1 (33)	-	-	-	-
MDS (5)	4 (80)	1 (20)	2 (40)	1 (20)	-	-	2 (40)	-	-	1 (20)	2 (40)	1 (20)
Non-Hodgkin lymphoma (1)	-	-	1 (100)	-	-	-	-	-	-	-	-	-
Other therap. intervention (1)	-	-	1 (100)	-	-	-	-	-	-	-	-	-
Transplant-heart (3)	2 (67)	-	2 (67)	-	-	1 (33)	-	-	-	-	-	1 (33)
Transplant-heart and lung (6)	3 (50)	3 (50)	2 (33)	2 (33)	-	-	2 (33)	-	1 (17)	-	-	-
Transplant-kidney (1)	-	-	1 (100)	-	-	-	1 (100)	-	-	-	-	-

Table 2. *Cont.*

	Lung Disease; n (% of Immunodeficiency Group)											
Immunodeficiency Type (n)	**Interstitial Lung Disease**	**Pulmonary Hypertension**	**Infections (Opportunistic/Recurrent)**	**Bronchiolitis Obliterans**	**Bronchiectasis**	**PTLD**	**Respiratory Failure**	**ARDS**	**Diffuse Alveolar Damage**	**Pneumothorax**	**Asthma**	**Pleural Disease**
Transplant-lung (4)	1 (25)	-	3 (75)	1 (25)	1 (25)	1 (25)	2 (50)	-	-	1 (25)	-	-
Transplant-stem cell (30)	14 (47)	-	15 (50)	18 (60)	2 (7)	1(3)	10 (33)	3 (10)	1 (3)	5 (17)	2 (7)	2 (7)

Table 3. Features of ILDs in immunodeficient children.

	All Immunodeficiency Patients	Immunodeficiency with ILD	Immunodeficiency without ILD	Comparison between with/without ILD P *
Cell concentration (/µL)	405.3 ± 1209.4 (114)	343.5 ± 308.1 (28)	425.4 ± 1383.0 (86)	0.216
Macrophages (%)	60.1 ± 27.9 (138)	64.7 ± 23.6 (46)	57.8 ± 29.6 (92)	0.299
PMN (%)	19.4 ± 25.9 (138)	16.3 ± 19.2 (46)	21.0 ± 28.7 (92)	0.626
Lymphocytes (%)	16.2 ± 17.0 (138)	13.5 ± 14.0 (46)	17.5 ± 18.2 (92)	0.437
Eosinophils (%)	2.1 ± 6.6 (138)	4.0 ± 10.5 (46)	1.2 ± 2.9 (92)	0.101
Mast cells (%)	0.2 ± 1.0 (138)	0.2 ± 0.8 (46)	0.3 ± 1.2 (92)	0.995
Plasma cells (%)	0.04 ± 0.3 (138)	0.03 ± 0.1 (46)	0.05 ± 0.4 (92)	0.388
Cell viability (%)	79.0 ± 23.0 (105)	80.4 ± 25.9 (23)	78.5 ± 22.2 (82)	0.283
Cell recovery (/µL)	53.0 ± 24.2 (107)	50.7 ± 28.5 (30)	53.9 ± 22.5 (77)	0.539 **

Data are means ± SD (n); * Mann–Whitney test, except "Cell recovery", which was assessed with ** *t*-test.

3.2 Features of ILDs in Immunodeficient Children

Within the group of patients who had developed an ILD, those with primary immunodeficiency more frequently had a family history of ILD and consanguinity (Table 4), pointing towards a potential genetic predisposition and risk factors for ILD. Gender dis- tribution, age at disease onset, neonatal history and outcome of lung disease were not different when comparing primary and secondary immunodeficiency.

In 90% of the children with ILD, a CT scan was performed, and in 80% of the studies, the features were consistent with an ILD (Table 5). Two-thirds of all children with ILD had a lung biopsy, which supported the diagnosis of ILD in 95% of cases. There were three ILD cases not supported by lung biopsy. Histopathological diagnosis in these patients included a normal transplanted lung, chronic bronchitis and a DAD with bronchiolitis obliterans. If genetic testing was performed, a monogenic condition known to be associated with ILD was identified in 76% of the patients. In more than two-thirds of the cases, the diagnosis of ILD was supported by two or three different diagnostic tests (Table 5).

Table 4. History, neonatal period and long-term course of patients with ILD and underlying immunodeficiency.

	Sex (m/f)	ILD Family History (y/n)	Consanguinity (y/n)	Age at Onset of Lung Disease (Years)	Gestational Age (Week)	O_2 Supplement in Neonatal Period (y/n)	Mechanical Ventilation in Neonatal Period (y/n)	Follow-Up Duration (Years)	Outcome of Lung Disease			
									Sick-Better	Sick-Same	Sick-Worse	Dead
All types of immunodeficiency	53/37	8/59	20/46	4.4 ± 4.4	31–41 (40)	6/58	4/60	6.0 ± 5.2	39	23	7	19
Primary immunodeficiency	28/24	8/38	18/28	3.5 ± 4.2	31–41 (40)	4/38	2/40	6.8 ± 5.8	22	15	4	10
Combined immunodeficiencies	8/1	0/6	3/4	1.4 ± 3.2	34–41 (40)	0/8	0/8	2.4 ± 1.7	6	1	0	1
Well-defined syndromes	3/1	0/3	1/2	6.1 ± 5.5	37–40 (40)	1/1	1/1	3.6 ± 4.0	1	1	1	1
Antibody deficiencies	2/2	0/4	0/4	5.2 ± 6.9	37–40 (38)	0/4	0/4	6.3 ± 4.2	1	1	0	0
Immune dysregulation	2/0	0/2	0/2	2.7 ± 2.8	39–40 (40)	1/1	0/2	4.4 ± 3.8	1	0	1	0
Defects of phagocytes	4/14	3/13	9/6	4.5 ± 3.9	31–41 (40)	0/12	0/12	8.9 ± 5.2	8	5	2	2
Defects of innate immunity	2/1	2/1	3/0	0.6 ± 0.2	40 (40)	1/2	1/2	5.2 ± 8.1	0	0	0	3
Autoinflammatory syndromes	5/5	3/7	2/8	2.5 ± 2.8	34–40 (40)	1/8	0/9	9.4 ± 7.3	3	5	0	2
Bone marrow failure	2/0	0/2	0/2	7.7 ± 9.5	40 (40)	0/2	0/2	3.6 ± 4.0	0	1	0	1
Secondary immunodeficiency	25/13	0/21	2/18	5.5 ± 4.4	32–40 (40)	2/20	2/20	5.0 ± 4.3	17	8	3	9
Comparisons between primary and secondary immunodeficiency	0.255 *	0.042 *	0.018 *	0.612 **	0.901 **	1.0 *	0.603 *	0.445 **	0.217 *	0.412 *	1.000 *	0.596

Data are numbers or means ± SD. Comparisons were made between primary and secondary immunodeficiency by * chi-square tests, ** ANOVA.

Table 5. ILD diagnosis supported by the results of the diagnostic tests used in the cohort of 90 patients with immunodeficiency.

	Numbers; %
Chest CT completed (yes/nk; % yes of all patients)	80/10; 89
ILD consistent with CT diagnosis (yes/no; % yes of patients with this test)	64/16; 80
Lung biopsy completed (yes/nk; % yes of all patients)	59/31; 66
Lung biopsy diagnosis proving ILD (yes/no; % yes of patients with this test)	56/3; 95
Genetic testing completed (yes/nk; % yes of all patients)	44/46; 49
Gene identified known to be associated with ILD (yes/no; % yes of patients with this test)	34/10; 76
ILD supported by genetics and lung biopsy (yes/no; % yes of patients with these tests)	21/28; 75
ILD supported by genetics and CT (yes/no; % yes of patients with these tests)	26/38; 68
ILD supported by lung biopsy and CT (yes/no; % yes of patients with these tests)	43/56; 77
ILD supported by genetics, biopsy and CT (yes/no; % yes of patients with these tests)	18/26; 69
ILD diagnosis only according to clinical records	3/87; 3

nk = not known or not available.

The spectrum of histopathological ILD patterns in the lung biopsies of the immunode- ficient patients was broad. Typical lymphocyte-dominated conditions were most prevalent and included GLILD, follicular bronchiolitis, LIP and NSIP, and constituted a total of 41% of all biopsies (Table 6). Other histological patterns included cholesterol pneumonia, DIP, PAP, lung fibrosis and pulmonary hemosiderosis, among others. Lung fibrosis was indicated in 13 patients, 3 of whom suffered from primary and 10 from secondary immunodeficiency (data not shown).

Table 6. Histopathological ILD diagnosis observed in 56 patients with immunodeficiencies and a lung biopsy.

Immunodeficiency (n, Percentage of Histopathological ILD Diagnosis in Immunodeficiency Subcategories)	Gender (Male/Female)	Histopathological Diagnosis and Pattern (n)
Primary immunodeficiency (32, 27%)		
Combined deficiencies (5)	4/1	NSIP (1), GLILD (1), Interstitial pneumonia (1), Intra-alveolar haemorrhage (1), Follicular bronchiolitis (1)
Well-defined syndromes (2)	2/0	Interstitial pneumonia (1), CPI (1)
Antibody deficiencies (2)	2/0	GLILD (1), Interstitial pneumonia (1)
Immune dysregulation (2)	2/0	LIP (2)
Defects of phagocytes (11)	1/10	Cholesterol pneumonia (1), DIP (2), PAP (7), Interstitial pneumonia (1)
Autoinflammatory syndromes (8)	4/4	LIP (1), Follicular bronchiolitis (1), NSIP (2), Interstitial pneumonia (1), DIP (1), PAP (1), Lung hypoplasia (1)
Bone marrow failure (2)	2/0	NSIP (1), Lung fibrosis (1)
Secondary immunodeficiency (24, 25%)		
ALL (3)	2/1	GLILD (1), NSIP (1), Follicular bronchiolitis (1)

Table 6. *Cont.*

Immunodeficiency (n, Percentage of Histopathological ILD Diagnosis in Immunodeficiency Subcategories)	Gender (Male/Female)	Histopathological Diagnosis and Pattern (n)
AML (1)	1/0	PAP (1)
Cancer (2)	2/0	BPD (1), DIP (1)
JMML (2)	2/0	Follicular bronchiolitis (1), Pulmonary hemosiderosis (1)
MDS (2)	2/0	Lung fibrosis (1), NSIP (1)
Transplanted (14)	9/5	LIP (1), DIP + NSIP (1), DAD (1), Lung fibrosis (4), Cholesterol pneumonia (2), NSIP (5)

3.2 ILD in Genetically Defined Primary Immunodeficiency: Experience from a Single Pediatric Pneumology Center and Review of Literature

The frequency of ILD observed in patients with immunodeficiency and genetically identified causes observed in our cohort is depicted in Table 7. For comparison, we per- formed a literature review of genetically determined immunodeficiency conditions present in our cohort and extracted the associated pulmonary conditions (Table 7). Whereas oppor- tunistic infections were the most frequently reported, ILD was prevalent in multiple but not all disorders. In 18 out of 25 conditions, we did not observe ILD involvement of the lungs in agreement with the literature, whereas, in 7 conditions, we observed an ILD. These diseases were caused by genetic variants in CD40, 10p13-p14DS, HELLS, TNFRSF13B, CYBA and NCF2. The patients in this group presented with an ILD-typical phenotype; however, susceptibility to opportunistic pathogens, including Cytomegalovirus, *Pneumocystis jirovecii* and *Aspergillus*, was coincidental, suggesting a possible role of microorganisms in the resulting lung disease. Of note, all these conditions were mainly described in single case reports or small series, increasing the likelihood that ILD manifestations might have been missed previously.

Table 7. Comparison between our cohort and literature regarding the percentage of the presence of ILD in genetically defined primary immunodeficiency.

Immunodeficiency Subcategories (Number ot Patients with ILD/Number of Patients with Genetically Defined Immun-odeficiency)	Disease Genetically Defined in Our Cohort (No.)	No. ot Cases with ILD in Our Cohort (ILD Percentage)	Pulmonary Diseases Other than ILD (n)	Prevalence ot ILD (%) in Primary Immunodeficiency Genetic Defect, as Reported in the Literature (May 1999 to May 2022)	Gene Identified, Known to Be Associated with a Condition Presenting with an ILD
Combined deficiencies (4/8)	ADA (2)	1 (50%)	ARDS, Respiratory failure, Opportunis-tic/recurrent infections (1)	44% [16] **	Y
	CD40 (2)	2 (100%)	Opportunistic/recurrent infections (1)	0% [17] **, [18] *	N
	CD40LG (1)	0 (0%)		20% [19] ***, 0% [20] ***	N
	IL2RG (1)	1 (100%)		7% ILD [21] ***	Y
	DOCK8 (1)	0 (0%)	Bronchiolitis obliterans, Bronchiectasis (1)	0% [22] ***, [23] ***	N

Immunodeficiency Subcategories (Number of Patients with ILD/Number of Patients with Genetically Defined Immunodeficiency)	Disease Genetically Defined in Our Cohort (No.)	No. of Cases with ILD in Our Cohort (ILD Percentage)	Pulmonary Diseases Other than ILD (n)	Prevalence of ILD (%) in Primary Immunodeficiency Genetic Defect, as Reported in the Literature (May 1999 to May 2022)	Gene Identified, Known to Be Associated with a Condition Presenting with an ILD
Well-defined syndromes (4/14)	RFXAP (1)	0 (0%)	Opportunistic/recurrent infections (1)	50% [24] *	Y
	ATM (3)	1 (33%)	Bronchiectasis, Pulmonary hypertension (1), Bronchiectasis, Opportunistic/recurrent infections (1)	50% [25] *, 26% [26] ***, 14% [27] ***	Y
	10p13-p14DS (1)	1 (100%)		0% [28] ***, [29] *	N
	MCM4 (1)	1 (100%)		50% [30] **, 0% [31] **	Y
	DNMT3B (1)	0 (0%)	Opportunistic/recurrent infections (1)	0% [32] ***	N
	IKBA (1)	0 (0%)	Opportunistic/recurrent infections (1)	0% [33] *, [34] **	N
	NBS1 (1)	0 (0%)	Bronchiectasis, Opportunistic/recurrent infections (1)	0% [35] *. [36] *, [37] ***	N
	TTC7A (1)	0 (0%)	Opportunistic/recurrent infections (1)	0% [38] *, [39] *, [40] ***	N
	DiGeorge (4)	0 (0%)	Opportunistic/recurrent infections (2), Asthma, Pulmonary hypertension (1), Opportunistic/recurrent infections, ADRS (1)	0% [41] ***, 100% [42] *	Y
Antibody deficiencies (1/4)	HELLS (1)	1 (100%)		0% [43] **, [44] *	N
	TNFRSF13B (1)	1 (100%)		0% [45] **, [46] *	N
	BTK (1)	0 (0%)	Opportunistic/recurrent infections, Respiratory failure (1)	0% [47] *, [48] ***	N
	NFKB1 (1)	0 (0%)	Opportunistic/recurrent infections, ARDS (1)	12% [49] ***, 0% [50] **	Y
	PIK3CD (1)	0 (0%)	Bronchiectasis, Opportunistic/recurrent infections (1)	0% [51] **, [52] **	N
Immune dysregulation (2/5)	FOXP3 (2)	1 (50%)	Bronchiolitis obliterans, Respiratory failure (1)	23% [53] ***,	Y
	STAT3 GOF (1)	1 (100%)		36% [54] ***, 100% [55] *	Y
	IL10 (1)	0 (0%)	Opportunistic/recurrent infections (1)	0% [56] **, [57] ***	N
	UNC13D (1)	0 (0%)	Opportunistic/recurrent infections (1)	0% [58] **, [59] *	N

Immunodeficiency Subcategories (Number of Patients with ILD/Number of Patients with Genetically Defined Immunodeficiency)	Disease Genetically Defined in Our Cohort (No.)	No. of Cases with ILD in Our Cohort (ILD Percentage)	Pulmonary Diseases Other than ILD (n)	Prevalence of ILD (%) in Primary Immunodeficiency Genetic Defect, as Reported in the Literature (May 1999 to May 2022)	Gene Identified, Known to Be Associated with a Condition Presenting with an ILD
Defects of phagocytes (18/21)	CSF2RA (15)	15 (100%)		100% [60] **, [61] ***	Y
	CYBA (3)	2 (67%)	Bronchiolitis obliterans, Opportunistic/recurrent infections, Asthma (1)	0% [62] **, [63] *, [64] ***, [65] ***	N
	CYBB (1)	0 (0%)	Opportunistic/recurrent infections (1)	0% [62] ***, [63] ***, [64] ***, [65] ***	N
	NCF4 (1)	0 (0%)	Bronchiectasis (1)	0% [65]*	N
	NCF2 (1)	1 (100%)		0% [62] **, [63] *, [64] ***, [65] ***	N
Defects of innate immunity (3/7)	MDA5 def (LOF). IFIH1 (1)	0 (0%)	Respiratory failure (1)	0% [66] *, 100% [67] *	Y
	STAT1 (AD LOF) (1)	0 (0%)	Bronchiectasis, Opportunistic/recurrent infections (1)	5% [68] ***	Y
	TCIRG1 (1)	0 (0%)	Opportunistic/recurrent infections (1)	0% [69]***	N
	ZNFX-1 (4)	3 (75%)	Opportunistic/recurrent infections (1)	13% [70] **, 50% [71] *	Y
Autoinflammatory syndromes (10/16	COPA (7)	7 (100%)		100% [72] ***, [73] ***	Y
	OAS1 (1)	1 (100%)		100% [74] **, [75] *	Y
	PLCG2 (1)	1 (100%)		0% [76] **, [77] *, [78] ***	N
	STING (1)	1 (100%)		100% [79] ***, 85% [80] **	Y
	AG57.IFIH1 (1)	0 (0%)	Opportunistic/recurrent infections, Respiratory failure, ARDS (1)	0% [81] *, [82] *, [83] **	N
	MEFV (2)	0 (0%)	Opportunistic/recurrent infections (2)	0% [84] ***, [85] ***, [86] ***	N
	TMEM173 (1)	0 (0%)	Opportunistic/recurrent infections (1)	100% [79] ***, 85% [80] **	Y
	TNFRSF1A (2)	0 (0%)	Asthma (1), Opportunistic/recurrent infections (1)	0% [87] ***	N
Bone marrow failure (2/3)	SAMD9 (1)	0 (0%)	Opportunistic/recurrent infections, Respiratory failure (1)	0% [88] **, [89] **	N
	TERC (1)	1 (100%)		50% [90] *	Y
	TERT (1)	1 (100%)		16% [90] ***, 56% [91] **	Y

* Case reports on 1 to 5 patients, ** Cohorts with 6 to 20 patients, *** Cohorts with more than 20 patients.

3. Discussion

Our data on lung diseases in immunodeficiencies confirmed that opportunistic and recurrent infectious diseases are still among the most prevalent pulmonary complications in an immunocompromised host; however, the data clearly demonstrate that formerly less frequently diagnosed conditions need to be considered carefully in clinical practice. This is particularly true for ILDs during childhood, which were identified in more than 40% of all patients. Of great interest and importance is an accurate etiological differentiation of the ILDs, as they represent an extremely broad spectrum of various disorders. Of note, many other but less frequent pneumological disorders, including bronchiolitis obliterans and pulmonary hypertension, must also be differentiated.

There are several lessons to be learned from our study. (1) Respiratory complications in primary and secondary immunodeficiencies are important problems and need to be carefully addressed by clinicians; (2) the spectrum of pulmonary

differential diagnosis beyond infectious complications is broad, including various forms of ILDs; (3) GLILD is a useful umbrella term alerting for ILD, but in immunodeficiencies, there are also other ILDs than GLILD; (4) traditional histopathological analysis can give important clues not only for differential treatments but also supporting advanced diagnostic multi-omics in the near future; (5) the limitation of cross-sectional analysis needs to be overcome by longitudinal studies, e.g., in registries to assess the course and stages of molecular entities with the help of CT imaging, lung function testing and deep clinical follow-up; and (6) importantly, close collaboration between immunologists and pulmonologists and other involved subspecialties will likely make an important difference.

Overall, 18% of the patients included died, and 15% became worse during the observa- tion time. Even treatment patients with immunodeficiency still suffered from high rates of pulmonary infections (primary immunodeficiency 73%, secondary immunodeficiency 56%) or non-infectious chronic lung disease. While respiratory diseases started at a median age of about 2 years (range 0 to 20), neonatal respiratory disease was not a risk factor for later lung affection. Beyond suppurative infectious lung disease, various kinds of obstructive lung diseases, including bronchiolitis obliterans, spontaneous and recurrent pneumothorax, acute respiratory distress syndrome (ARDS), acute and chronic respiratory insufficiency, partial and global respiratory failure, and diffuse alveolar damage (DAD), were noted (see Table 2, Supplemental Table S2). Less frequent conditions to differentiate diagnostically in the wide spectrum of pulmonary affections were PTLD, subpleural and pleural fibrosis, pleurisy, pleural effusion, pleural empyema, pulmonary hypertension, portopulmonary hypertension, stenosis of the pulmonary artery, and pronounced obliterative vasculopathy. For the pneumologist, ILD may be the presenting condition, and the underlying immunodeficiency is not yet diagnosed [92]. Several of the ILDs we identified in our sample are not typically expected in immunodeficiency, i.e., conditions not linked to GLILD. Such conditions included restrictive lung diseases such as cholesterol pneumonia, DIP, pulmonary hemosiderosis, pulmonary hemorrhage, bronchopulmonary dysplasia, PAP or NSIP. None of these histological patterns corresponded to a single disease entity. As an example, the NSIP pattern was found in connective tissue diseases, drug-induced ILD, hypersensitivity pneumonitis, HIV infection, chronic infection, chronic aspiration, previous acute lung injury and idiopathic NSIP [15].

All of these conditions are rare, and making such a diagnosis or not may contribute to the wide variation of ILD frequencies reported in several case series of immunocompromised children. While a recent pediatric study reported a very low rate of ILD, e.g., 1% (11/796 cases [93]), all other reports indicate higher rates (64% (39/61 pediatric and adult cases [94]), 15% [95], 34% [96], 26% (18/69 cases [97]), 11% (3/28 cases), 7% (46/637 cases [98]), 15% (8/54 cases [99]), 13% (78/623 cases [100]), 30% (24/80 [101]), 11% (40/370 cases [102]), 8% (6/73 cases [103]), 7% (114/1647 cases [104]), 10% (22/219 cases [105]), 40% (29/73 [106], 18% (9/50 cases [107]), 9% (138/1518 cases [108]), 22% (16/73 cases [109]), 20% (29/148 cases [110]), and 12% (4/33 cases [111]). It is clear that such differences result from selection bias due to differences in criteria for diagnosis, different age groups investi- gated, variable underlying diseases or selection bias from the researcher's perspective and interest, i.e., observing primarily from an immunological or pneumological viewpoint, and also knowledge about the conditions and the existence of such complications. More exact estimates could be collected in population-based prospective studies using appropriate inclusion and exclusion criteria and case definitions.

In our cohort, lung biopsies were conducted at a relatively high frequency in 66% of the ILD patients. This was most likely due to a highly selected cohort of subjects with significant pulmonary problems, presenting after various diagnostic efforts and empirical therapeutic trials had been made. The biopsies led to an ILD diagnosis in 95% of the cases. A precise diagnosis may also be important for novel treatments, e.g., the presence of fibrosis in a biopsy may support treatment with anti-fibrotic drugs such as nintedanib or pirfenidone.

Hurst et al., 2017 generated a consensus statement for CVID, introducing GLILD defined as a "distinct clinico-radio-pathological ILD occurring in patients with CVID, as- sociated with a lymphocytic infiltrate and/or granuloma in the lung, and in whom other conditions have been considered and where possible excluded" [112]. As the authors pointed out later, there is still complex terminology for ILD in CVID and no consensus [113]. We believe that GLILD may be a useful umbrella term alerting for ILD in immunodeficien- cies. Using the category of GLILD as a practical approach for currently available treatments also appears appropriate, as the ILD associated with immunodeficiencies often represents some form of benign lymphoproliferative pathology, and the ILD may simply be a manifes- tation of some immune dysregulation [112,114]. However, the traditional histopathological analysis as conducted here can give important additional diagnostic clues and, in the near future, may also support advanced diagnostic tissue-based multi-omics [115].

Chest CTs were performed in about 89% of the subjects, and 80% of these were consistent with an ILD. CT is a sensitive technique to detect ILD. This was further supported by a high rate of concordance with radiological findings and the results of lung biopsies. Histopathological examination confirmed a suspected ILD in 95% of cases. However, CTs cannot differentiate the type of ILD; thus, lung biopsies do not always appear to be redundant. On CT imaging, interstitial thickening, pulmonary fibrosis, pleuropulmonary elastosis or pleuroparenchymal fibroelastosis were the most common findings.

An important strength of this study was the use of the advanced contemporary classification system for inborn errors of immunity, which focuses on distinct genetic disease categories. In our study, 49% of the patients with primary immunodeficiency had an underlying monogenic defect supporting their diagnosis. For seven conditions, we provided new evidence for ILD pulmonary manifestations. Another strength includes the collection of rare and clinically significant conditions, i.e., about 10 new cases annually over a period of more than 2 decades. However, this study was a cross-sectional analysis, and precise follow-up was lacking. Other limitations include its retrospective, single- center design and a selection of more severely affected patients submitted to our pediatric pneumology department. Longitudinal studies, e.g., in registries following the course of well-defined molecular entities, may use pre-structured CT imaging, lung function testing and deep clinical follow-up to overcome such shortcomings. Lastly, close collaboration between all involved subspecialties will likely make an important difference in unraveling the details of lung targeting in immunodeficiencies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/diagnostics13010064/s1, Table S1: Immunodeficiency groups and diagnosis; Table S2: Definition of final lung diseases diagnosis.

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Abbreviations

AD	Autosomal dominant transmission
ADA	Adenosine deaminase
AGS7	Aicardi-Goutieres syndrome 7
ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia
APLAID	Auto-inflammation and phospholipase Cγ2 (PLCγ2)-associated antibody deficiency and immune dysregulation
AR	Autosomal recessive transmission
ATM	Ataxia Telangiectasia, Mutated
BO	Bronchiolitis obliterans
CD	Cluster of differentiation
CD40LG	CD40 Ligand
CGD	Chronic granulomatous disease
CID	Combined immunodeficiencies
CLL	Chronic lymphocytic leukemia
CML	Chronic myelogenous leukemia
CVID	Common variable immunodeficiency
CYBA	Cytochrome B-245 Alpha Chain
CYBB	Cytochrome b-245 beta chain
def	deficiency
DKC	Dyskeratosis congenita
DNMT3B	DNA methyltransferase 3b
EDA	Anhidrotic ectodermodysplasia
FOXP3	Forkhead box protein P3
GOF	Gain-of-function
HIV	human Immunodeficiency virus
ICF	Immunodeficiency, Centromeric region instability, Facial anomalies syndrome
ID	Immunodeficiency
Ig	Immunoglobulin
IKBA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IL	Interleukin
IL2RG	Interleukin 2 Receptor Subunit Gamma
ILD	Interstitial lung disease
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy X-linked
JMML	Juvenile myelomonocytic leukemia
LOF	Loss of function
MCM4	Minichromosome maintenance
MDA	Melanoma differentiation-associated protein
MHC	Major histocompatibility complex
MIRAGE	Myelodysplasia, infection, restriction of growth, adrenal hypoplasia, genital phenotypes, enteropathy

NBS	Nijmegen breakage syndrome
NCF	Neutrophil cytosolic factor
NFKB1	Nuclear factor-kappaB1
PAP	Pulmonary alveolar proteinosis
PAS	Periodic acid–Schiff
PHT	Pulmonary hypertension
PIK3CD	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Delta
PLAID	PLCγ2 associated antibody deficiency and immune dysregulation
PLCG2	phospholipase C gamma 2
RF	Respiratory failure
RFXAP	Regulatory Factor X Associated Protein
SAMD9	Sterile Alpha Motif Domain Containing 9
SCID	Severe combined immunodeficiency
SID	Secondary Immunodeficiency
STAT	Signal transducer and activator of transcription
TACI	Transmembrane activator calcium modulator and cyclophilin ligand interactor
TERC	Telomerase RNA Component
TERT	Telomerase Reverse Transcriptase
TNFRSF13B	Tumor Necrosis Factor Receptor Superfamily Member 13B
TNFRSF1A	TNF Receptor Superfamily Member 1A
TRAPS	TNF receptor-associated periodic syndrome
TTC7A	Tetratricopeptide repeat domain 7A gene
UNC13D	Protein unc-13 homolog D
ZNFX-1	NFX1-type zinc finger-containing 1

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