Přílohy disertační práce:

1.) **Smetanova J,** Milota T, Rataj M, Bloomfield M, Sediva A, Klocperk A. „*Accelerated Maturation, Exhaustion, and Senescence of T cells in 22q11.2 Deletion Syndrome"*. J Clin Immunol. 2022 Feb; 42(2): 274-285. doi: 10.1007/s10875-021-01154-9.

2.) Parackova Z, Milota T, Vrabcova P, **Smetanova J,** Svaton M, Freiberger T, Kanderova V, Sediva A. „*Novel XIAP mutation causing enhanced spontaneous apoptosis and disturbed NOD2 signalling in a patient with atypical adult-onset Crohn's disease"*. Cell Death Dis. 2020 Jun 8;11(6):430. doi: 10.1038/s41419-020-2652-4.

3.) Milota T, Kotaska K, Lastuvka P, Klojdova I& **PID Clinical Group.** „*High Prevalence of Likely Passively Acquired Anti-TPO and Anti-GAD Autoantibodies in Common Variable Immunodeficiency"*. J Clin Immunol. 2022 Feb; 42(2): 427–429. doi: 10.1007/s10875-021-01171-8.

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ORIGINAL ARTICLE

Accelerated Maturation, Exhaustion, and Senescence of T cells in 22q11.2 Deletion Syndrome

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Abstract

Purpose 22q11.2 deletion syndrome (22q11.2DS) is a primary immunodeficiency characterized chiefly by the hypoplasia of the thymus resulting in T cell lymphopenia, increased susceptibility to infections, and higher risk of autoimmune diseases. The irregular thymic niche of T cell development may contribute to autoimmune and atopic complications, whereas the compensatory mechanism of homeostatic T cell proliferation and continuous immune stimulation may result in T cell senescence and exhaustion, further aggravating the immune system dysregulation.

Methods We used flow cytometry to investigate T cell maturation, delineation, proliferation, activation, and expression of senescence and exhaustion-associated markers (PD1, KLRG1, CD57) in 17 pediatric and adolescent patients with 22q11.2DS and age-matched healthy donors.

Results 22q11.2DS patients aged 0–5 years had fewer naïve but more effector memory T cells with a tendency to approach normal values with increasing age. Young patients in particular had a higher percentage of proliferating T cells and increased expression of PD1, KLRG1, and CD57, as well as cells co-expressing several exhaustion-associated molecules (PD1, KLRG1, Tbet, Eomes, Helios). Additionally, high-risk 22q11.2DS patients with very low numbers of CD4 T cells had significantly higher percentage of Th1 and Th17 T cells, driven in part by higher proportion of mature T cell forms.

Conclusion The low thymic output and accelerated T cell differentiation remain the principal features of 22q11.2DS patient immunity, especially in young patients of < 5 years. Later in life, homeostatic proliferation drives expression of T cell exhaustion and senescence-associated markers, suggesting functional aberrations in addition to numeric T cell deficiency.

Keywords DiGeorge syndrome · 22q11.2 deletion syndrome · thymus · immunodeficiency · T cells · PD1 · CD57 · differentiation · maturation

Introduction

Chromosome 22q11.2 deletion syndrome (22q11.2DS) with an estimated incidence of 1 in 3000–6000 infants represents one of the most common genetic disorders with a wide range of clinical manifestations. The 22q11.2 deletion causes a broad spectrum of clinically overlapping syndromes, sometimes named after those authors who first described them, such as Sedlackova, Shprintzen, Takao or DiGeorge, sometimes after their phenotype, such as velocardiofacial or conotruncal anomaly face [1–3]. DiGeorge syndrome (DGS) in particular was the first to be described in association with its effect on the immune system [4, 5]. While other genetic aberrations, including monogenic mutations of *TBX1* [6, 7], 10p deletions [8] or *CHD7* mutations [9], and environmental triggers such as prenatal exposure to retinoic acid or maternal diabetes can also cause a clinical phenotype very similar to 22q11.2DS [10] the 3 Mb 22q11.2 deletion is the most common cause of this phenotype [2, 11].

Clinical symptoms of 22q11.2DS are diverse and affect several organ systems. Its main hallmarks are congenital heart defects, craniofacial dysmorphia, and hypoparathyroidism-induced hypocalcaemia [12, 13], which are usually

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the first to prompt immunologic and genetic evaluation. Less apparent but no less characteristic are the variable degrees of thymic dys- or aplasia, which result in a T cell lymphopenic primary immunodeficiency with increased susceptibility to infections, as well as other non-infectious complications [13–15].

According to the severity of T cell lymphopenia, 22q11.2DS patients are sometimes classified as partial (less than 1500/μl T cells during the first three years of life) or complete (less than 50/μl T cells), with features of severe combined immunodeficiency [16]. However, recent studies highlighted that the disease exists on a spectrum—even within the partial 22q11.2DS category, patients with particularly low CD4 T cells counts have up to 3,3 times higher risk of severe infections [17] and low naïve CD4 and CD8 T cells were associated with persistent hypoparathyroidism and serious bacterial or fungal infections in a small study of 18 patients [18] although another larger study saw no such correlation but instead noted association between low T cell counts and autoimmune complications [13].

We and others have previously shown that the decreased thymic output results in early maturation of those T cells which do develop, leading to reduced number of recent thymic emigrants (RTE), non-memory T cells [13, 15, 19, 20] and low detectable T cell receptor excision circles (TRECs) [15, 21]. These cells divide through homeostatic proliferation, which together with the natural involution of thymic function over time leads to gradual normalization of T cell counts in older 22q11.2DS patients [22, 23], albeit with a limited repertoire of T cell receptors (TCR) [24] and altered delineation of helper T cells into unique functional lineages. Significantly higher production of IFNγ by Th1 cells was previously identified in pediatric patients with a 22q11.2 deletion syndrome and, conversely, significantly increased production of IL-4 by Th2 cells in adult patients [25], which may be partly responsible for the predisposition of DGS patients to autoimmune and infectious diseases.

Additionally, the frequent infections in 22q11.2DS patients may further drive the T cell differentiation and the recurrent exposure to antigens may finally result in their functional exhaustion. We have previously shown altered temporal changes in the expression pattern of PD1, one of the major T cell exhaustion markers [26], on 22q11.2DS T cells [20]; however, the principal focus of that study was follicular helper T cells, where PD1 may play a different role. To our knowledge, no publications describe exhaustion of T cells in patients with primary immunodeficiency caused by a thymic pathology, as most studies on this topic focus on chronic viral infections such as hepatitis C, HIV [27, 28] or intrinsic T cell defects [29]. Furthermore, the repeated cellular division cycles of homeostatic proliferation reduce telomere length [15], leading to state of cellular senescence. Senescent T cells have a unique functional phenotype and may be identified through expression of the CD57 and KLRG1 receptors [30, 31]. These features have been previously described in patients with activated phosphatidylinositol-3-kinase delta syndrome [32], but not in patients with thymic dysplasia.

This study aims to assess the senescence, exhaustion, and T cell differentiation in healthy children and pediatric and adolescent patients with 22q11.2DS, which may contribute to the immune deficiency seen in 22q11.2DS.

Materials and Methods

Patients

Patients followed at the Department of Immunology, 2nd Faculty of Medicine, Charles University and University Hospital in Motol who had a verified 22q11.2 deletion [16] and corresponding healthy age and sex-matched donors (HD) were enrolled into the study, which was approved by the Ethical Committee of the Motol University Hospital in Prague, Czech Republic. All participants or their legal guardians signed a written informed consent in accordance with the Declaration of Helsinki. Patients and HDs were divided into two groups according to their age: <5 years of age (group I) and >5 years of age (group II). Additionally, in some analyses, patients with 22q11.2DS were subdivided into groups according to their lowest recorded number of CD4 T cells; the high risk (HR) group included patients with CD4 T cells less than 400×10^6/L, whereas the standard risk (SR) group included patients with $400–1200\times10^6$/L CD4 T cells.

Flow Cytometry

Peripheral blood from patients and HDs was collected into EDTA-coated tubes. Peripheral blood mononuclear cells (PBMC) were then isolated using a Ficoll-Paque gradient (GE Healthcare Biosciences, Uppsala, Sweden). PBMCs were stained by fluorescent conjugated monoclonal antibodies against surface membrane markers. After fixation and permeabilization with eBioscience™ Foxp3/Transcription Factor Fixation/Permeabilization kit (Thermo Fisher Scientific, Waltham, CA, USA), cells were stained for intracellular markers according to previously published protocol [20]. The list of used antibody-fluorochrome conjugates is shown in Supplementary Table S1. The samples were measured on a BD LSRFortessa flow cytometer (BD Biosciences, San Diego, CA, USA). Gating strategy is summarized in Supplementary Figure S1.

Data Analysis

After measurement, the data was analyzed using the FlowJo software (version 10.6.1, BD Biosciences). Statistical analysis was perfmormed in GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California, USA) using an unpaired t test with Welch's correction. The differences were considered statistically significant when p values were in the following range: $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****).

Results

Clinical Features of the DiGeorge Syndrome Cohort

A total of 17 22q11.2DS patients (10 male, 7 female) and 17 HDs (7 male, 10 female) were enrolled into the study and divided into two age groups, 0–5 and 5+ years of age, to reflect the gradual normalization of immune status in 22q11.2DS patients with age. Age group I included 8 patients with 22q11.2DS (age 1,9 ± 1,4 years, mean ± standard deviation) and 7 HDs (age 2,0 ± 1,4 years), group II consisted of 9 patients with 22q11.2DS (age 15,5 ± 6,8 years) and 10 HDs (age 13,8 ± 7,1 years). 6 22q11.2DS patients (patient 8, 10, 14, 15, 16 and 17, age 16,6 ± 8,9 years) were considered high-risk (HR), whereas 11 patients (patient 1–7, 9, 11–13, age 5,0 ± 5,4 years) were standard-risk (SR) (Table 1).

As expected, congenital heart disease ($n=15/17$), hypoparathyroidism ($n=4/17$), and growth retardation ($n=6/17$) were the main clinical features of the cohort, which are summarized in Table 1. Other symptoms included neurologic complications ($n=9/17$) such as speech delay ($n=3/17$), psychomotoric retardation ($n=2/17$) or seizures ($n=4/17$); and allergic diseases—atopic dermatitis ($n=3/17$), bronchial asthma ($n=3/17$), allergic rhinoconjunctivitis ($n=1/17$), and drug allergy ($n=1/17$). Autoimmune complications represented by autoimmune thyroiditis were seen in 3 patients, with elevated thyroid stimulating hormone (TSH) but no autoantibodies or decrease of thyroid function in further 3/17. Scoliosis was seen in 3/17 patients.

Significantly reduced relative and absolute counts of T cells (CD3+) as well as helper (CD3+CD4+) and cytotoxic T cells (CD3+CD8+) in 22q11.2DS patients were apparent particularly in age group I when compared to HDs (Table 2). In older patients over 5 years of age (group II), the absolute T cell counts were almost normalized nearing the HD values, although relative CD3 and CD4 counts were still lower in 22q11.2DS patients. The B cells (CD19+) and NK cells (CD3-CD16+CD56+) remained unaffected in both age groups. The normal B cell counts were also reflected in normal distributions of IgG, IgA, and IgM serum levels which were comparable to HDs, with a tendency towards a gradual increase of IgG with age in 22q11.2DS patients, as already noted in our previous works [13, 20] (Table 2).

Accelerated Maturation of T Cells

As expected, the low thymic output of patients with 22q11.2DS resulted in lower proportion of recent thymic emigrants (RTEs, CD4+CD45RA+CD31+) within CD4 T cells; however, the difference was significant only in age group I ($p=0.015$) and no significant difference was found in age group II (Fig. 1A, representative FACS plots can be seen in Supplementary Figure S2, XY plots showing correlation with age can be seen in Supplementary Figure S3). An identical trend was seen in naïve (CD45RA+CCR7+) CD4 cells, which were also significantly decreased in young 22q11.2DS patients ($p=0.006$), but only insignificantly lower in age group II ($p=0.206$).

In contrast, significantly increased mature forms of CD4 T cells were apparent, including central memory (CM, CD45RA-CCR7+, $p=0.013$), effector memory (EM, CD45RA-CCR7-, $p=0.003$) and terminally differentiated effector memory re-expressing CD45RA (TEMRA, CD45RA+CCR7-, $p=0.004$). Again, the differences between 22q11.2DS patients and HDs became less pronounced with age and were not significant in age group II, with the exception of TEMRA cells (22q11.2DS mean 2.10%, HD mean 0.81, $p=0.027$).

While a similar trend of reduction of naïve and expansion of mature forms was also present in the CD8 T cell compartment, the differences were only significant for effector memory cells in age group I ($p=0.041$) (Fig. 1B).

Thus, pediatric patients with 22q11.2DS have decreased RTEs and naïve CD4+T cells, with a compensatorily expanded memory compartment. These differences largely normalize by age of five to ten years.

Altered Helper T Cell Lineages

We further divided CD4 helper T cells into subpopulations according to expression of chemokine receptors which functionally correspond to the different helper T cell lineages: Th1 (CXCR3+CCR6-), Th2 (CXCR3-CCR6-CCR4+CRTH2+), Th1/17 (CXCR3+CCR6+) and Th17 (CXCR3-CCR6+) [33].

In the younger patients in group I, significantly higher proportion of all helper T cell lineages was apparent (Fig. 2A)—Th1 ($p=0.028$), Th2 ($p=0.0006$), Th1/17 ($p=0.019$), and Th17 ($p=0.0007$). No significant changes were seen in the older patients in group II. The increased expression of CXCR3, CCR6, CCR4, and CRTH2 chemokine receptors on CD4 T cells of the youngest patients with 22q11.2DS likely reflects their accelerated

Table 1 Clinical description of the 22q11.2DS patient cohort. *F* female, *M* male, *PMR* psychomotor retardation, *AIT* autoimmune thyroiditis, *TSH* thyroid-stimulating hormone, *ANCA* antineutrophil cytoplasmic antibodies, *ANA* antinuclear autoantibodies, *AD* atopic dermatitis, *ARC* allergic rhinoconjunctivitis, *GER* gastroesophageal reflux, *UTI* urinary tract infection, *URTI* upper respiratory tract infection, *OMA* otitis media acuta, *SMX-TMP* sulfamethoxazole-trimethoprim prophylaxis, *SCIG* subcutaneous immunoglobulin prophylaxis

Patient	Age (years)	Age group	Sex	Congenital heart disease	Hypo-parathyroidism	Growth retardation	Neurologic complications	Autoimmune complications	Allergy	Other	Infections
1	0.1	I	M	Yes	Yes	Yes	No	No	No	No	None by the time of testing
2	0.4	I	F	Yes	Yes	No	No	No	No	GER	1 × UTI
3	0.8	I	F	Yes	No	No	Seizures	AIT, ANCA +	No	Coloboma	URTI
4	1.3	I	F	No	No	Yes	No	No	No	No	None by the time of testing
5	2.5	I	M	Yes	Yes	No	No	No	No	No	URTI, 2 × croup
6	3.0	I	M	Yes	No	No	Seizures	No	No	No	URTI, bronchitis with ATB
7	3.3	I	F	Yes	No	Yes	Unsteady gait	No	No	No	URTI, 2 × bronchitis with ATB
8	4.0	I	F	Yes	No	Yes	Seizures, speech delay	No	No	No	URTI, dental caries, SMX-TMP, SCIG
9	5.3	II	M	No	No	Yes	Weakness	No	No	No	URTI, bronchitis
10	7.4	II	F	Yes	No	Yes	No	Elevated TSH	AD	No	URTI, bronchitis, SMX-TMP
11	10.6	II	M	Yes	Borderline	No	Strabism	No	No	Hepatopathy	Dental caries
12	12.0	II	M	Yes	No	No	Speech delay	AIT	AD	Anorectal atresia, scoliosis	URTI, 1 × bronchitis with ATB
13	16.0	II	M	Yes	No	No	Speech delay	Elevated TSH	Asthma	No	URTI, 1 × pneumonia, 1 × OMA
14	18.0	II	F	Yes	No	No	No	ANA +	Drug allergy	No	URTI
15	21.1	II	M	Yes	Yes	No	PMR, seizures	AIT	No	Scoliosis	URTI, chronic rhinitis, conjunctivitis, OMA with ATB
16	23.6	II	M	Yes	No	No	No	No	Asthma	Scoliosis	URTI, bronchitis with ATB, Chlamydia pneumonia, skin infections, SCIG
17	25.7	II	M	Yes	No	No	PMR	Elevated TSH	ARC, asthma, AD	No	URTI, bronchitis, skin infections

Table 2 Basic immunologic parameters of the 22q11.2DS cohort. Mean values ± standard deviation (SD) are displayed, significant differences ($p \leq 0{,}05$) marked as bold. *DGS* DiGeorge syndrome, *HD* healthy donor, *lympho* lymphocytes

Age group	I			II		
	22q11.2DS	**HD**	*p*-value	**22q11.2DS**	**HD**	*p*-value
CD3 (% of lympho)	**50.75 ± 5.26**	**71.29 ± 5.56**	**< 0.0001**	**63.78 ± 10.57**	**72.90 ± 7.19**	**0.047**
CD3 (× 10⁹/L)	**1.41 ± 0.61**	**3.87 ± 1.01**	**0.0003**	1.28 ± 0.79	1.66 ± 0.39	0.2209
CD4 (% of lympho)	**33.0 ± 5.45**	**44.14 ± 8.26**	**0.0123**	**32.56 ± 7.21**	**44.00 ± 6.60**	**0.0024**
CD4 (× 10⁹/L)	**0.9 ± 0.37**	**2.44 ± 0.91**	**0.0035**	0.67 ± 0.42	1.01 ± 0.30	0.0663
CD8 (% of lympho)	**12.5 ± 2.78**	**26.57 ± 13.82**	**0.0358**	25.44 ± 7.5	22.70 ± 4.67	0.3617
CD8 (× 10⁹/L)	**0.35 ± 0.17**	**1.10 ± 0.17**	**< 0.0001**	0.52 ± 0.34	0.53 ± 0.13	0.9139
CD19 (% of lympho)	**30.63 ± 5.68**	**15.57 ± 5.06**	**0.0001**	13.56 ± 3.54	12.20 ± 3.82	0.4334
CD19 (× 10⁹/L)	0.81 ± 0.29	0.80 ± 0.19	0.94	0.25 ± 0.09	0.29 ± 0.13	0.4824
CD16/56 (% of lympho)	16.5 ± 8.19	11.86 ± 7.24	0.265	20.67 ± 11.27	12.50 ± 7.06	0.084
CD16/56 (× 10⁹/L)	0.39 ± 0.24	0.69 ± 0.42	0.133	0.43 ± 0.31	0.27 ± 0.11	0.1701
IgG (g/L)	7.49 ± 1.98	7.24 ± 2.47	0.835	12.75 ± 3.89	9.79 ± 1.96	0.0631
IgA (g/L)	0.54 ± 0.35	0.89 ± 0.58	0.193	1.84 ± 1.09	1.50 ± 0.63	0.4315
IgM (g/L)	0.74 ± 0.47	0.38 ± 0.18	0.077	**0.55 ± 0.26**	**0.92 ± 0.44**	**0.0384**

maturation compared to HD, as EM and TEMRA T cells—elevated in young 22q11.2DS patients—generally show higher proportion of delineated cells with expression of either CXCR3 and/or CCR6 (Fig. 2B). With increasing age of 22q11.2DS patients and normalization of their T cell developmental stages (Fig. 1A), these differences equalize (Fig. 2A, XY plots showing correlation with age can be seen in Supplementary Figure S4).

Interestingly, the balance within the most differentiated TEMRA CD4 T cells (which are the only subpopulation to remain significantly elevated in 22q11.2DS patients compared to HDs regardless of age) was shifted slightly (but not statistically significantly) towards a higher proportion of CCR6 + CD4 T cells (both Th1/17 and Th17) early in life, but skewed towards CXCR3 + CCR6- Th1 T cells in older years ($p = 0.0365$) (Fig. 2B).

A highly significant expansion of all delineated T cells was apparent in high-risk (HR) 22q11.2DS patients with very low CD4 T cell numbers (less than 400×10^6/L) compared to the standard-risk (SR) group—spanning Th1 ($p = 0.047$) (Fig. 2C), Th1/17 ($p = 0.011$), and Th17 cells ($p = 0.0001$).

Changes in T cell Th1, Th1/17, Th2, and Th17 lineages are thus largely secondary to precocious T cell maturation present in patients with 22q11.2DS and any results should be interpreted accordingly. Nevertheless, a bias towards CXCR3 + Th1 and CXCR3 + CCR6 + Th1/17 T cells is preserved even within the mature forms and may thus shape the immune milieu of DGS.

Amplified T Cell Proliferation in Younger 22q11.2DS Patients

The status of steady-state ex vivo T cell proliferation and activation was measured using the Ki-67 and HLA-DR markers, respectively. Significantly higher steady-state proliferation of CD4 T cells was detected in younger 22q11.2DS patients ($p = 0.034$) (Fig. 3, XY plots showing correlation with age can be seen in Supplementary Figure S5). The older patients of age group II did not show a significant difference in the number of Ki-67 + CD4 T cells. No significant difference in the number of Ki-67 + CD8 cells was found between patients and HDs in any age group, although a similar trend was apparent.

In contrast, the expression of the HLA-DR activation marker on CD4 + T cells was not significantly different between 22q11.2DS patients and HDs in any age group. Slightly increased numbers of activated CD8 + HLA-DR + cells were found in DGS patients in age group I, but the difference did not reach significance.

Our data reinforces the notion that CD4 and CD8 T cell compartments of the youngest pediatric patients with 22q11.2DS undergo increased proliferation as part of homeostatic expansion, which may be accompanied by basal activation compared to controls.

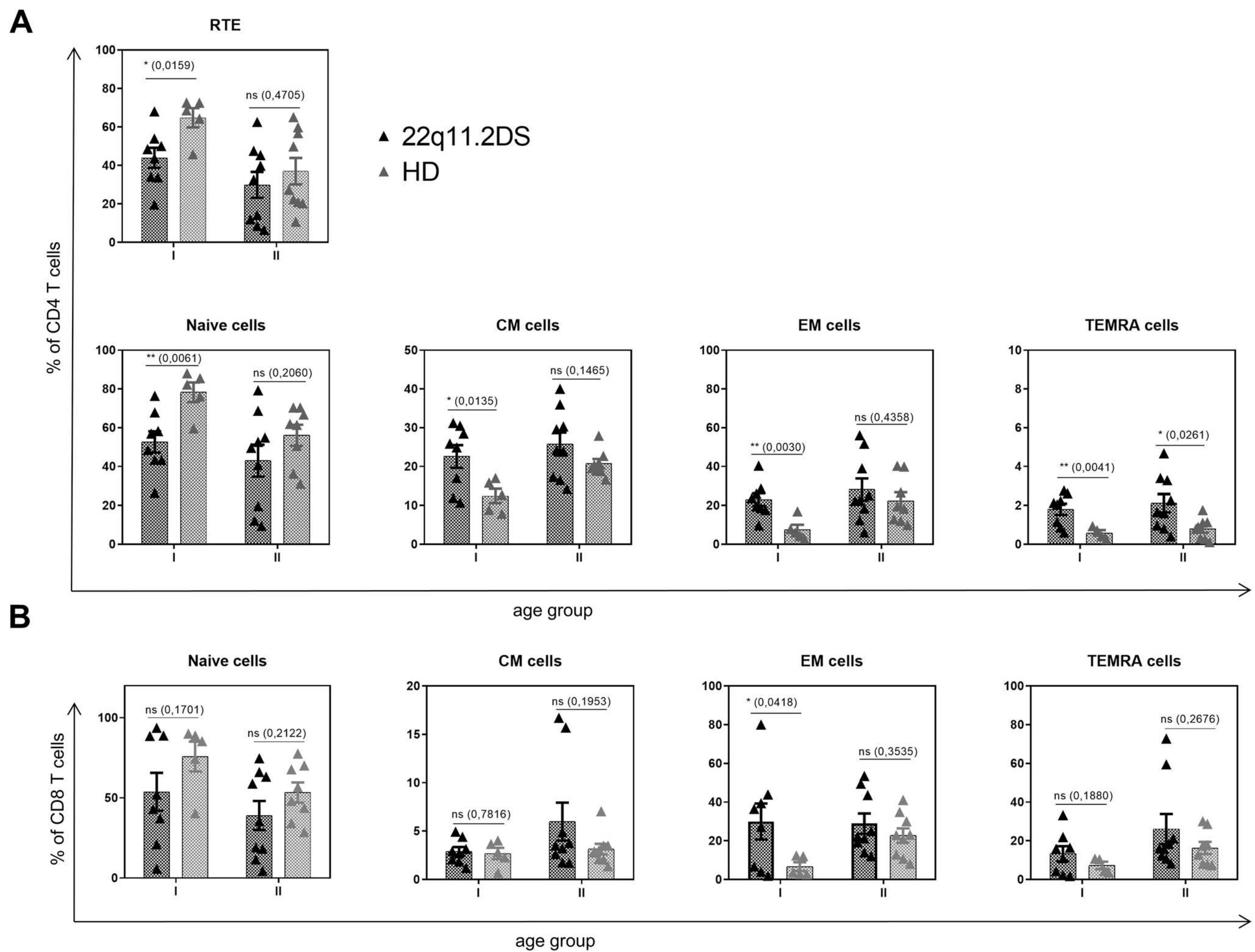

Fig. 1 Differentiation of T cells. Recent thymic emigrants (RTE, CD45RA + CD31 +), naïve (CD45RA + CCR7 +), central memory (CM, CD45RA-CCR7 +), effector memory (ER, CD45RA-CCR7-), and terminally differentiated (TEMRA, CD45RA + CCR7-) CD4 **A** and CD8 **B** T cells in patients with 22q11.2DS and healthy donors (HD). Boxes depict Mean and Standard Error of the Mean (SEM), *p* values in brackets

Increased Expression of PD1, KLRG1, and CD57 on 22q11.2DS T Cells

Exhausted and senescent states of T cells caused by excessive antigenic stimulation and homeostatic proliferation are associated with more frequent infections. Significatly higher expression of the exhaustion marker PD1 was found on CD4 T cells of 22q11.2DS patients in age group I ($p = 0.007$) (Fig. 4A), but the difference became less pronounced in older patients of age group II. The same trend of expression was also apparent in CD8 T cells; however, the difference did not reach significance.

As expected, the expression of senescence markers CD57 and KLRG1 was also markedly altered especially in young patients with 22q11.2DS. Significantly higher proportion of KLRG1 + CD4 T cells was found in both age group I ($p = 0.001$) and age group II ($p = 0.0376$), with an identical trend in CD8 T cells in age group I ($p = 0.0083$). This was also mirrored in the expression pattern of CD57, where a significantly higher proportion of CD57 + CD4 T cells ($p = 0.049$) and CD57 + CD8 T cells ($p = 0.039$) was found in 22q11.2DS patients in age group I, but not in older patients of age group II.

In general, high-risk 22q11.2DS patients had higher proportion of PD1 +, KLRG1 + and CD57 + CD4 and CD8 T cells when compared to standard risk 22q11.2DS patients, however, the difference only reached significance in the percentage of PD1 + CD8 T cells ($p = 0.037$) (Fig. 4B).

We also compared the expression of PD1, KLRG1, and CD57 at individual stages of CD4 and CD8 T cell differentiation (Fig. 3C). The most consistent finding was elevated expression of senescence-associated marker KLRG1 in 22q11.2DS patient cells across all developmental stages (naïve, EM, TEMRA), which was more pronounced in

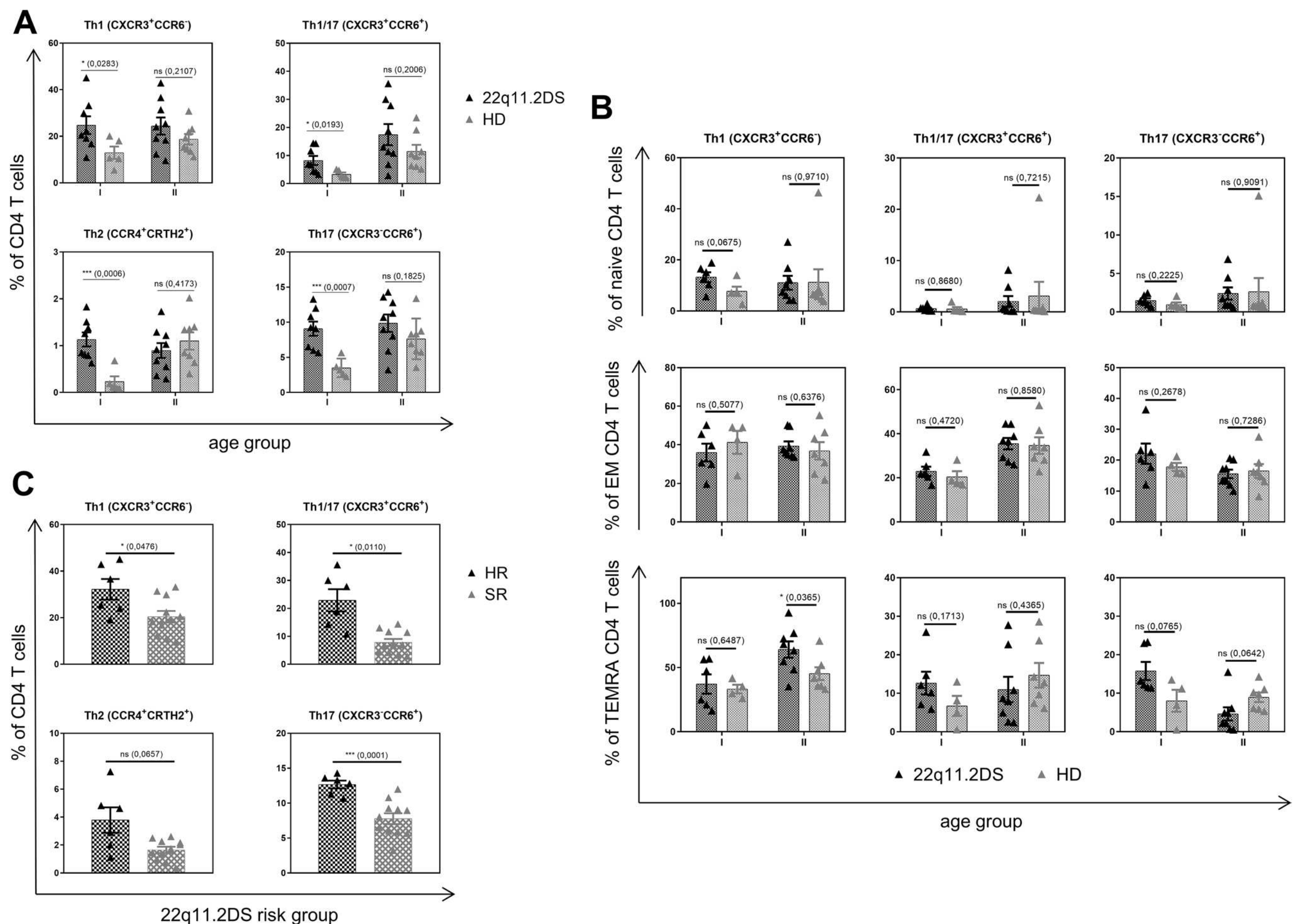

Fig. 2 Helper T cell subpopulations. **A** Th1 (CXCR3+CCR6-), Th1* (CXCR3+CCR6+), Th2 (CXCR3-CCR6-CCR4+CRTH2+), and Th17 (CXCR3-CCR6+) expressed as a percentage of total CD4 T cells, in 22q11.2DS patients and healthy donors (HD). **B** Th1, Th1*, Th2, and Th17 expressed as a percentage of naive (CD45RA+CCR7+), effector memory (EM, CD45RA-CCR7-) and terminally differentiated (TEMRA, CD45RA+CCR7-) CD4 T cells, in 22q11.2DS patients and HDs. **C**Th1, Th1*, Th2, and Th17 expressed as a percentage of CD4 T cells in high risk (HR) and standard risk (SR) 22q11.2DS patients. Boxes depict Mean and Standard Error of the Mean (SEM), *p* values in brackets

younger patients' CD8 T cells and older patients' CD4 T cells (individual *p*-values shown in the figure).

Co-expression of Exhaustion-Associated Molecules in 22q11.2DS Patient Cells

Finally, because the expression of individual markers of exhaustion may be misleading and not reflect truly exhausted cells, we also compared the proportions of cells co-expressing different sets of these molecules, including the surface expression of PD1 and KLRG1 and the expression of transcription factors Eomes and Helios in CD4 T cells and Eomes and Tbet in CD8 T cells.

As expected from data shown earlier, manually gating for phenotypically exhausted populations (PD1$^+$, KLRG1$^+$, PD1$^+$KLRG1$^+$, PD1$^+$Eomes$^+$ and PD1$^+$Helios$^+$ in CD4 T cells, PD1$^+$, KLRG1$^+$, PD1$^+$KLRG1$^+$, Tbet^{low}Eomes^{hi} and PD1$^+$Tbet^{low}Eomes^{hi} in CD8 T cells) revealed that these populations were most prevalent in patients with 22q11.2DS, and generally more so in age group II (Fig. 5A, B). Interestingly, limiting the evalutaion to cells positive exclusively for one marker and negative for others (or a combination of several positive/negative markers), we saw that 22q11.2DS patients had fewer CD4 and CD8 cells quadruple negative for all evaluated markers (Fig. 5C, D). Situation with single/double/triple positive cells was more complicated, however, as healthy donor CD4 T cells had generally highest proportion of Helios+cells, regardless of other markers. In the CD8 compartment, fewer surprising results were apparent—cells positive for KLRG1, PD1 and Eomes but negative for Tbet were highest in both 22q11.2DS cohorts, as expected.

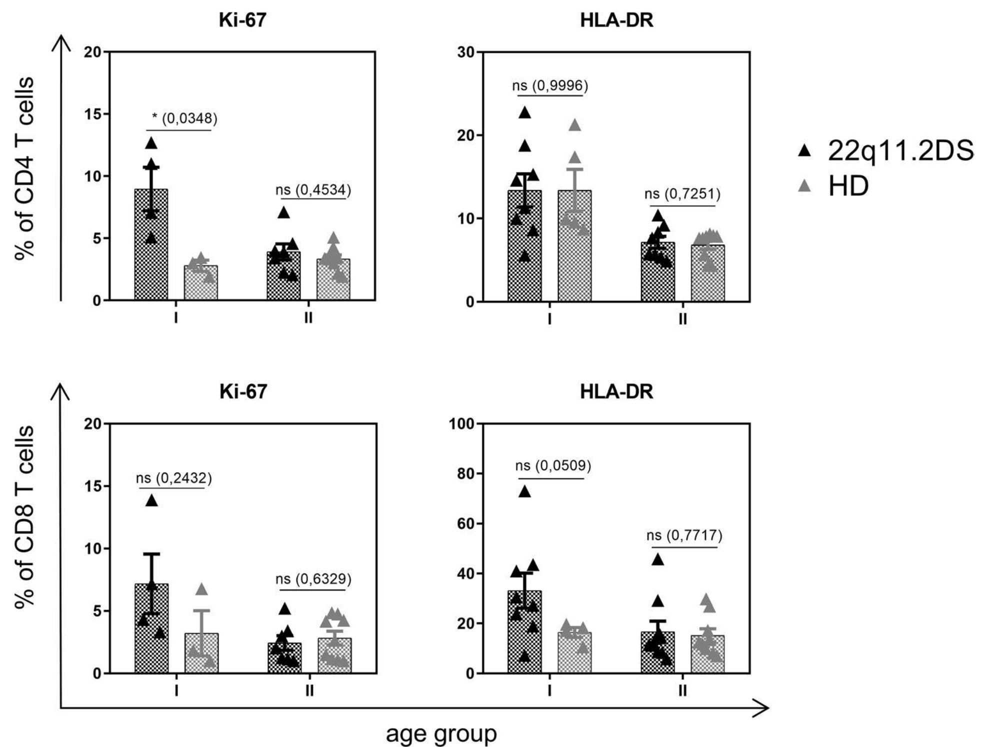

Fig. 3 T cell activation and proliferation. Percentage of proliferating (Ki-67 +) and activated (HLA-DR +) CD4 and CD8 T cells in patients with 22q11.2DS and healthy donors (HD). Boxes depict Mean and Standard Error of the Mean (SEM), *p* values in brackets

Discussion

In this manuscript, we focused on the detection of T cells, their subpopulations, proliferative status and expression of senescence and exhaustion-associated markers in pediatric and adolescent patients with 22q11.2DS. Our results spanning the basic analysis of humoral (B cell counts and IgG, IgA and IgM serum levels) and cellular immunity (NK, T helper and cytotoxic T cell counts) are in line with several publications showing that 22q11.2DS patient T cell numbers are reduced in early childhood, but in most cases develop towards almost physiological values between 5 and 20 years of age [13, 15].

The pathway through which the T cell compartment reconstitutes in patients with 22q11.2DS remains surprisingly obscured. While the physiologically decreasing number of T cells with age in the general population [34] certainly contributes to the relative normalization of the originally low 22q11.2DS T cell counts, extrathymic maturation of T cells in 22q11.2DS patients, for example in the tonsils, gut or elsewhere [35–37], may also play a role. Additionally, homeostatic clonal proliferation of T cells is ubiquitous in the healthy population and is vital in compensating the physiologic involution of thymic epithelium over time [38], but may also serve to replenish the T cell compartment in those with impaired T cell genesis, such as has been shown previously in immunodeficient *Rag* knockout mice [39]. This process drives T cells towards expression of a memory phenotype and functional characteristics of effector memory cells, including the expression of IFN-γ and others [40]. The predominant memory phenotype has been described previously in patients with 22q11.2DS [15, 20] and here we verify and expand the knowledge to individual memory subpopulations, with especially striking changes in young patients under 5 years of age, where we documented a higher proportion of proliferating Ki67 + cells. The proliferation may, in part, be driven by frequent exposure to bacterial and viral antigens during childhood, as we have also previously shown that between 2–5 years of age healthy but not 22q11.2DS children develop the bulk of their T-dependent class-switched memory B cells [41], but nevertheless it indubitably contributes to the compensatory T cell proliferation.

Homeostatic proliferation is not without its drawbacks, however. It may be associated with higher prevalence of autoimmune diseases, as was described in non-obese diabetic (NOD) mice [42, 43], and may thus also contribute to the autoimmune complications seen in patients with 22q11.2DS [13]. Indeed, a T helper 1 (Th1) response characterized by potent production of IFN-γ is associated with numerous autoimmune disorders, including inflammatory bowel disease [44], multiple sclerosis [45] and others. In common variable immunodeficiency (CVID), Th1 response is amplified in patients with complicated disease [46] and the higher proportion of IFN-γ producing cells has been also shown in patients with 22q11.2DS previously [25]. Our study also reveals an elevated proportion of Th1 CD4 T cells in pediatric 22q11.2DS patients, as well as between

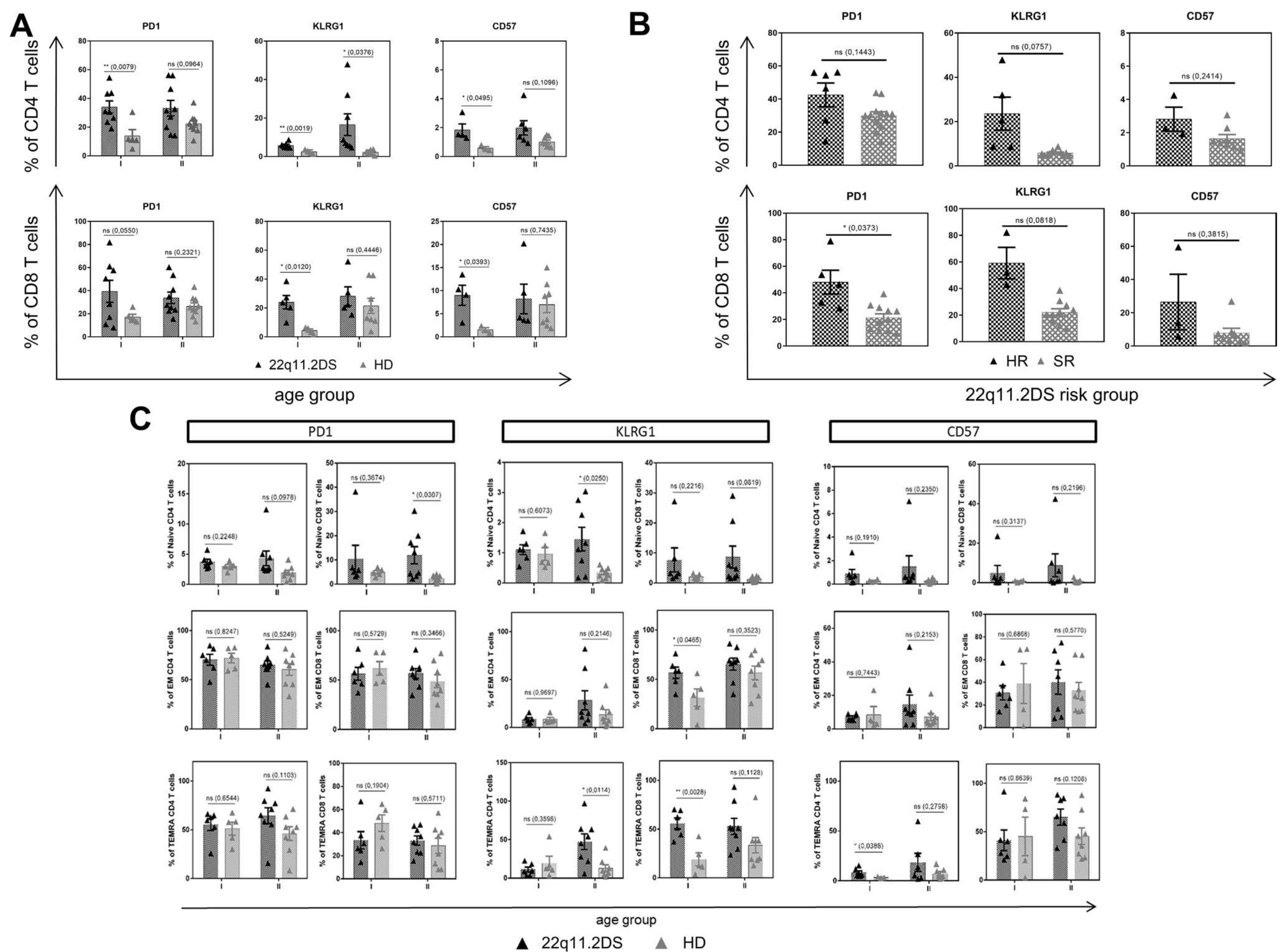

Fig. 4 Senescence and exhaustion of T cells. **A** Expression of PD1, KLRG1 and CD57 on the surface of DGS patient and healthy donor (HD) CD4 and CD8 T cells. **B** Expression of PD1, KLRG1 and CD57 on the surface of CD4 and CD8 T cells in high risk (HR) and standard risk (SR) 22q11.2DS patients. **C** Expression of PD1, KLRG1 and CD57 in naïve (CD45RA + CCR7 +), effector memory (EM, CD45RA-CCR7-) and terminally differentiated (TEMRA, CD45RA + CCR7-) CD4 and CD8 T cells, in DGS patients and HDs

standard and high risk 22q11.2DS patients, however, similarly elevated Th2 and Th17 responses suggest that these differences are driven by precocious maturation of 22q11.2DS T cells, as we show in detailed comparison between naïve and effector memory T cells.

The increased rate of T cell proliferation along with high antigenic load through more frequent infections may result in functional exhaustion with limited cytotoxicity and altered production of cytokines, or maturational senescence with impaired proliferative capacity. Surprisingly, only a limited number of publications dealing with T cell senescence and exhaustion in patients with primary immunodeficiencies exist, as this topic is mostly discussed in the context of chronic viral infections such as hepatitis C or HIV [47, 48]. We have previously shown an exhausted phenotype of follicular CD8 T cells in patients with CVID and lymphadenopathy [49], featuring higher expression of PD1 and TIGIT, as well as the senescence marker CD57, and a recent study of 32 CVID patients expanded these findings to chronically activated CD8 T cell in peripheral circulation [50]. Several groups have now shown features of T cell senescence in patients with activated phosphatidylinositol-3-kinase delta syndrome (APDS) [29, 32]. This study shows for the first time that both CD4 and CD8 T cell compartments show phenotypic features of exhaustion and senescence in young patients with 22q11.2DS, and that these changes are, to certain extent, preserved even within individual T cell maturational stages and congruent with previous reports of shortened telomeres in 22q11.2DS patients [15]. This may result in reduced sensitivity and immune response to antigens and, at a later age, susceptibility to cancer due to impaired immune surveillance [51], a topic not yet fully explored in 22q11.2DS.

Of note, however, there is substantial variance in our data and more robust cohorts would further strengthen our hypothesis, may reveal significant differences where we only

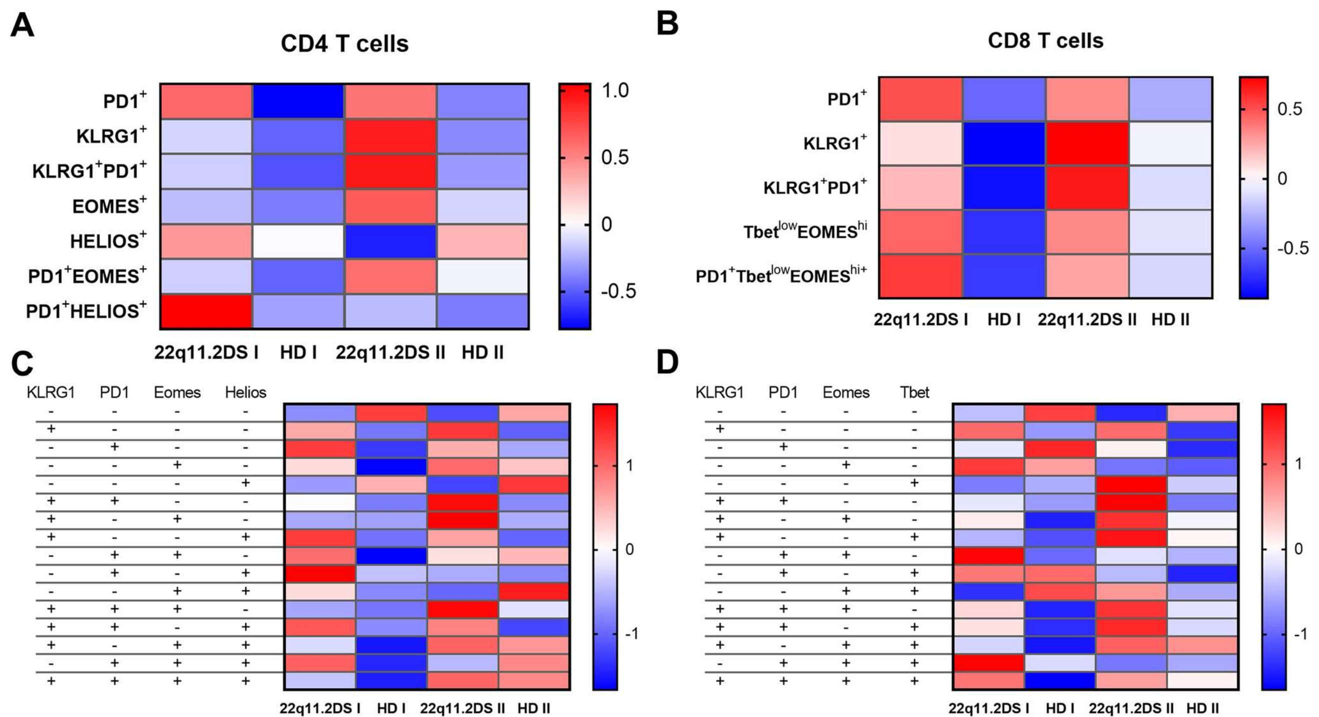

Fig. 5 Exhaustion marker coexpression patterns. **A** Heatmap showing the proportions of CD4 T cells (co-)expressing PD1, KLRG1, Eomes and Helios and **B** CD8 T cells (co-)expressing PD1, KLRG1, Eomes and Tbet in 22q11.2DS patients and healthy donors (HD) in age groups I and II. **C** Heatmap showing the proportions of cells with all possible combinations of markers positivities/negativities in CD4 T cells and **D** CD8 T cells in 22q11.2DS patients and healthy donors (HD) in age groups I and II. Proportions are normalized on per-population basis, colors of cells represent *Z*-scores, with scale shown next to each heatmap

saw trends or discover distinct subgroups of patients. While functional assays and unbiased analysis of the coexpression patterns of a wider spectrum of inhibitory markers would be helpful in verifying true exhaustion of 22q11.2DS T cells, such analyses present unique challenges in pediatric patients due to their higher required volume of biomaterial. Rapid changes of T cell phenotype during childhood also require more stringent age-matching, further eroding the cohorts already difficult to recruit given the rarity of primary immunodeficiency diseases. Regardless, despite the limitations of this pilot study, the results provide important insights into mechanisms of T cell homeostasis in patients with 22q11.2DS and rationale for apt design of further studies. More research into the functional impact of these changes along with modern unbiased methods of analysis is warranted and may in the future enable T cell invigoration therapy especially for high risk patients with 22q11.2DS.

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We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal.

Author Contribution JS performed experiments, analyzed data, interpreted results, and wrote the manuscript. MR performed experiments. MB provided biological material from healthy controls and reviewed the manuscript. TM analyzed data, interpreted results, and co-wrote the manuscript. AS participated in the collection of biological material from patients and reviewed the manuscript. AK designed the study, participated in the collection of biological material from patients, interpreted results, and co-wrote the manuscript.

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Data Availability Data is available from authors upon reasonable request. Material is not available.

Code Availability Not applicable.

Declarations

Ethics Approval The study was approved by the Ethical Committee of the Motol University Hospital in Prague, Czech Republic (# EK-657/21). All participants or their legal guardians signed a written informed consent in accordance with the 1964 Declaration of Helsinki.

Consent to Participate Written informed consent was obtained from all individual participants included in this study, or from their legal guardians.

Consent for Publication No identifiable information is published as part of this study.

Conflict of Interest The authors declare no competing interests.

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Novel *XIAP* mutation causing enhanced spontaneous apoptosis and disturbed NOD2 signalling in a patient with atypical adult-onset Crohn's disease

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Abstract

X-linked inhibitor of apoptosis (XIAP) is the most potent human inhibitor of apoptosis, and is also involved in NOD2-dependent NFκB and MAPK signalling cascade activation. The absence or defective function of XIAP leads to the development of a rare and severe primary immunodeficiency known as X-linked lymphoproliferative syndrome type 2 (XLP-2), which is characterized by a triad of clinical manifestations, including a high incidence of haemophagocytic lymphohistiocytosis (HLH), lymphoproliferation and inflammatory bowel disease (IBD), usually with very early onset. Here, we present a novel *XIAP* mutation identified in a patient with atypical adult-onset IBD complicated by relapsing HLH, splenomegaly and sarcoid-like disease. The c.266delA mutation in the *XIAP* gene creates a premature stop codon, and causes a severe reduction in XIAP protein expression. The mutation is also associated with impaired spontaneous and staurosporine- and PMA-induced apoptosis accompanied by significantly increased expression of pro-apoptotic genes. We also confirmed the negative impact of this particular *XIAP* mutation on NOD2-dependent NFκB and MAPK activation, while NOD2-independent activation was found to be unaffected. Moreover, we assume that the mutation has an impact on the overproduction of IL-12 and IFNγ, the shift towards the Th1 immune response and increased numbers of central memory and effector memory CD4+ and CD8+ T cells. All these changes contribute to immune dysregulation and the clinical manifestation of XLP-2.

Introduction

X-linked inhibitor of apoptosis (XIAP) or baculoviral IAP repeat-containing protein 4 (BIRC4), localized on the X chromosome, is a part of human IAP family. The protein consists of three different domains: (1) three baculoviral IAP repeat (BIR) domains, which are characteristic of all IAPs, (2) UBA domains that allow binding to ubiquitin and (3) a zinc-binding domain C-terminal RING finger domain, which is associated with E3 ubiquitin ligase activity[1].

One of the major roles of XIAP is the prevention of apoptotic cell death, which is achieved by binding and inhibiting the activity of caspases 3, 7 and 9[2]. In addition to its anti-apoptotic functions, XIAP is also involved in other signalling pathways and cellular responses, mostly because of the ubiquitylation activity through its RING domain[3,4]. XIAP is involved in intracellular pattern-recognition receptor signalling that senses peptidoglycan products, NOD1 and 2[5], leading to NFκB and mitogen-activated protein kinase (MAPK) cascade activation[6–8]. In mouse and human models, the absence of XIAP leads to defective

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secretion of proinflammatory cytokines after stimulation with NOD ligands[9,10]. Interestingly, NOD2 was the first identified susceptibility gene for Crohn's disease (CD), a typical condition associated with XIAP deficiency[11].

XIAP deficiency is a rare primary immunodeficiency, also known as X-linked lymphoproliferative syndrome type 2 (XLP-2), caused by mutations in the *XIAP (BIRC4)* gene. The estimated incidence is 1–2 cases per million of live-born children. Nevertheless, the real prevalence seems to be higher as the diagnosis of XIAP deficiency may be overlooked or misclassified. Current assessments suggest that up to 4% of early-onset IBD may represent XIAP-deficient patients[12].

Disease onset usually manifests in the first few years of life, and is characterized by a key triad of clinical symptoms consistent with a high incidence of haemophagocytic lymphohistiocytosis (HLH), often triggered by Epstein–Barr (EBV) infections, and characterized by splenomegaly and inflammatory bowel disease (IBD), particularly with features of CD[13]. HLH is a life-threatening condition characterized by hyperinflammation, in which activated T lymphocytes and macrophages accumulate in organs, and produce and induce massive production of proinflammatory cytokines, particularly IFNγ[14], resulting in tissue damage and multiorgan failure that typically affects the liver and bone marrow[15]. IBD in XIAP-deficient patients usually presents with very early onset[16]; however, adult onset has also been described[17], and is characterized by a complicated course, necessity of extensive surgical procedures and unresponsiveness to standard treatment, including biological treatment. These patients have also significantly increased mortality rate, dying within a few years upon manifestation or diagnosis of IBD[18]. In comparison with XLP-1, hypogammaglobulinaemia may accompany XIAP deficiency; however, it is less frequent. Moreover, no lymphoma has been reported, which approximately 30% of XLP-1 patients develop. On the other hand, XLP-1 does not present with higher risk of IBD[19]. Currently, haematopoietic stem cell transplantation is the only causal therapy of XLP-2, although attempts to develop targeted gene therapy seem to be promising[20].

Here, we report a novel XLP-2-causing mutation in the XIAP BIR1 domain, leading to a premature stop codon and a loss of protein expression, which results in impaired lymphocyte apoptosis and NOD2-dependent signalling with clinical manifestations that include a complicated course of IBD, unresponsiveness to standard treatment, including biologics (infliximab and vedolizumab) and relapsing HLH.

Results

Case report

A 32-year-old patient was born to non-consanguineous Caucasian parents. The patient presented without any health complications or abnormalities during the prenatal, perinatal and postnatal periods, and was diagnosed at 17 years of age with CD based on the clinical presentation and histological verification, which revealed nonspecific granulation tissue composed of multinucleated giant cells and lymphocytic infiltration in the submucosa of the colon. Complex examination, including ultrasonography of the abdomen, also revealed splenomegaly. Standard therapy with chimeric monoclonal anti-TNFα antibody (infliximab) at a standard dose of 5 mg/kg was initiated. However, the course of the CD was complicated by the development of an intra-abdominal abscess compressing the bladder, which required surgical intervention. Then, the biological therapy was switched to fully human monoclonal anti-TNFα (adalimumab), which successfully led to CD remission. Three years later (at the age of 20), the patient was admitted to the hospital for fever, elevation of inflammatory markers (including C-reactive protein), progressive splenomegaly, anaemia, leukocytopenia and decreased platelet count. Further testing revealed hypertriglyceridaemia, elevated transaminases and increased serum concentrations of ferritin. The results from extensive infectious diagnostic work identified the EBV as a possible trigger. The evaluation of bone marrow biopsy samples confirmed the suspicion of HLH. Thus, according to the Histocyte Society standards, the HLH diagnostic criteria were fulfilled, and adequate therapy started with a high-dose corticosteroid regimen (1000 mg of Solu-Medrol per day) for 3 consecutive days and intravenously administered cyclosporine at a dosage of 2 mg/kg/day, which led to normalization of the blood count values and inflammatory marker, liver transaminase, triglyceride and ferritin levels (Supplementary Table 1 and Table 1). Later, the therapy was switched to peroral corticosteroids and cyclosporin as long-term maintenance therapy. Despite this effort, HLH relapse occurred 4 years later (at the age of 24), and no infection or any other trigger was identified. Moreover, the patient developed mediastinal lymphadenopathy, histologically verified as epithelioid granuloma with images indicative of a sarcoid-like disease. Clinical manifestations and therapy are illustrated in Supplementary Fig. 1A. Suspicions about the primary aetiology arose despite the patient's age, and genetic testing was indicated. WES was performed because of the broad differential diagnosis of HLH and monogenic causes of CD, and the results revealed a novel c.266delA mutation in the *XIAP* (*BIRC4*) gene. This finding was subsequently confirmed by Sanger sequencing (Fig. 1a). Further genetic counselling with the patient's family members revealed that the patient's mother as a healthy carrier and two healthy siblings were without the mutation (Fig. 1b). When we searched the patient's pedigree, we also identified the mother's brother as a potentially affected family member who died of severe

Table 1 Laboratory values of the patient samples.

Immunology	Patient's values	Referential value
IgG (g/l)	13.00	7.65–13.60
IgG1 (g/l)	7.13	4.9–11.4
IgG2 (g/l)	4.13	1.50–6.40
IgG3 (g/l)	0.316	0.2–1.1
IgG4 (g/l)	0.342	0.08–1.4
IgA (g/l)	2.03	0.91–2.9
IgM (g/l)	↓ 0.38	0.47–1.95
IgE (IU/ml)	↑2.161	0–150
C3 (g/l)	0.98	0.83–2.25
C4 (g/l)	0.22	0.14–0.35
Tetanus (IU/ml)	1.01	0.1
Haemphilus (IU/ml)	9.00	6.00
ANA	neg	–
ANCA	pos (p-ANCA)	–
RF IgG (IU/ml)	4.4	0–22
RF IgA (IU/ml)	2.3	0–22
RF IgM (IU/ml)	2.7	0–22
aTRG (IU/ml)	2.48	0–10
ASCA IgG (IU/ml)	↑ 43.713	0–10
ASCA IgA (IU/ml)	↑ 12.36	0–10

infection-induced sepsis accompanied by splenomegaly and lymphadenopathy (major symptoms of HLH); however, biological material was not available for genetic testing to confirm the diagnosis or the cause of death.

Novel c.266delA mutation leads to a premature stop codon and loss of function of the XIAP molecule

A novel c.266delA frameshift mutation in the *XIAP* gene of the patient, leading to a premature stop codon after the translation of 41 amino acids (p. Asn89fs*41), was detected by whole-exome sequencing (WES) and confirmed by Sanger sequencing (Fig. 1a). The mother of the patient was confirmed to be a healthy heterozygous carrier (Fig. 1b). The mutation is in the first BIR domain of the protein, as shown in the scheme of the XIAP protein in Fig. 1c. The results from a Western blot analysis showed no XIAP expression in the patient PBMCs and reduced expression of XIAP in the mother's samples compared with healthy donors (Fig. 1d). We also observed reduced expression of the housekeeping protein β-actin, a finding in agreement with a previously reported role of XIAP in cytoskeleton regulation with reduced β-actin expression[21]. Expression of HSP90 and tubulin, additional housekeeping proteins, was comparable to controls (Supplementary Fig. 1E).

XIAP LOF mutation results in augmented apoptosis

As XIAP is an important molecule in apoptosis regulation, we decided to verify the XIAP LOF by analyzing spontaneous as well as induced apoptosis by staurosporine and PMA. We measured the activation of caspase-3 and -7 with a FAM-FLICA caspase-3,7 assay kit, and noticed elevated numbers of CD3 lymphocytes that were positive for activated caspase-3 and -7 in patient samples. Not only was staurosporine and PMA-induced apoptosis, but also spontaneous apoptosis was markedly enhanced in the patient's T lymphocytes (Fig. 2a, b). The augmented spontaneous apoptosis was confirmed by Annexin V and DAPI staining, verifying the results of the FLICA experiments (Fig. 2c, d).

In addition, we analyzed the expression of pro-apoptotic (*BAX* and *BAK*) and anti-apoptotic (*Bcl2*) genes. The ratio of *BAK/Bcl2* and *BAX/Bcl2* was highly increased in both induced and spontaneous apoptotic patient cells (Fig. 3a). Interestingly, the genes involved in caspase-independent apoptosis, *ENDOG* and *AIMF1*, were reduced in the samples (Fig. 3b). When a caspase inhibitor Z-VAD-FMK was applied, both patient and control samples displayed reduced apoptosis (Supplementary Fig. 2A, B). These observations suggest an enhanced caspase-dependent apoptosis. To test whether there was a compensatory mechanism critical for defective *XIAP* expression, we analyzed the presence of the *BIRC2* (*cIAP*) gene in patient cells. However, we did not observe enhanced compensatory *cIAP* expression in patient cells compared with healthy controls (Fig. 3c).

XIAP LOF abrogates NOD2 signalling

Moreover, XIAP is involved in NOD2 signalling; hence, we investigated whether the pathway was affected. Stimulation of NOD2 with muramyl dipeptide (MDP) leads to activation of NFκB and MAPK. We focused on the phosphorylation of the MAP kinases p38 and Erk (Fig. 4a), and observed diminished levels of kinase phosphorylation in response to MDP in patient monocytes detected by flow cytometry. Western blot analysis of MAPK activation confirmed this assessment (Fig. 4b). Furthermore, we examined the NFκB pathway activation after MDP stimulation, expressed as IκB (inhibitor of κB) degradation, and NFκB phosphorylation by flow cytometry and Western blot. Degradation of IκB leads to NFκB activation and its translocation to the nucleus. As anticipated, we detected neither inhibited IκB degradation in the patient's samples (Fig. 4c, d) nor NFκB phosphorylation in response to MDP stimulation. However, the patient's cells were able to phosphorylate MAPKs, as well as activate the NFκB pathway in response to PMA or TNFα stimulation

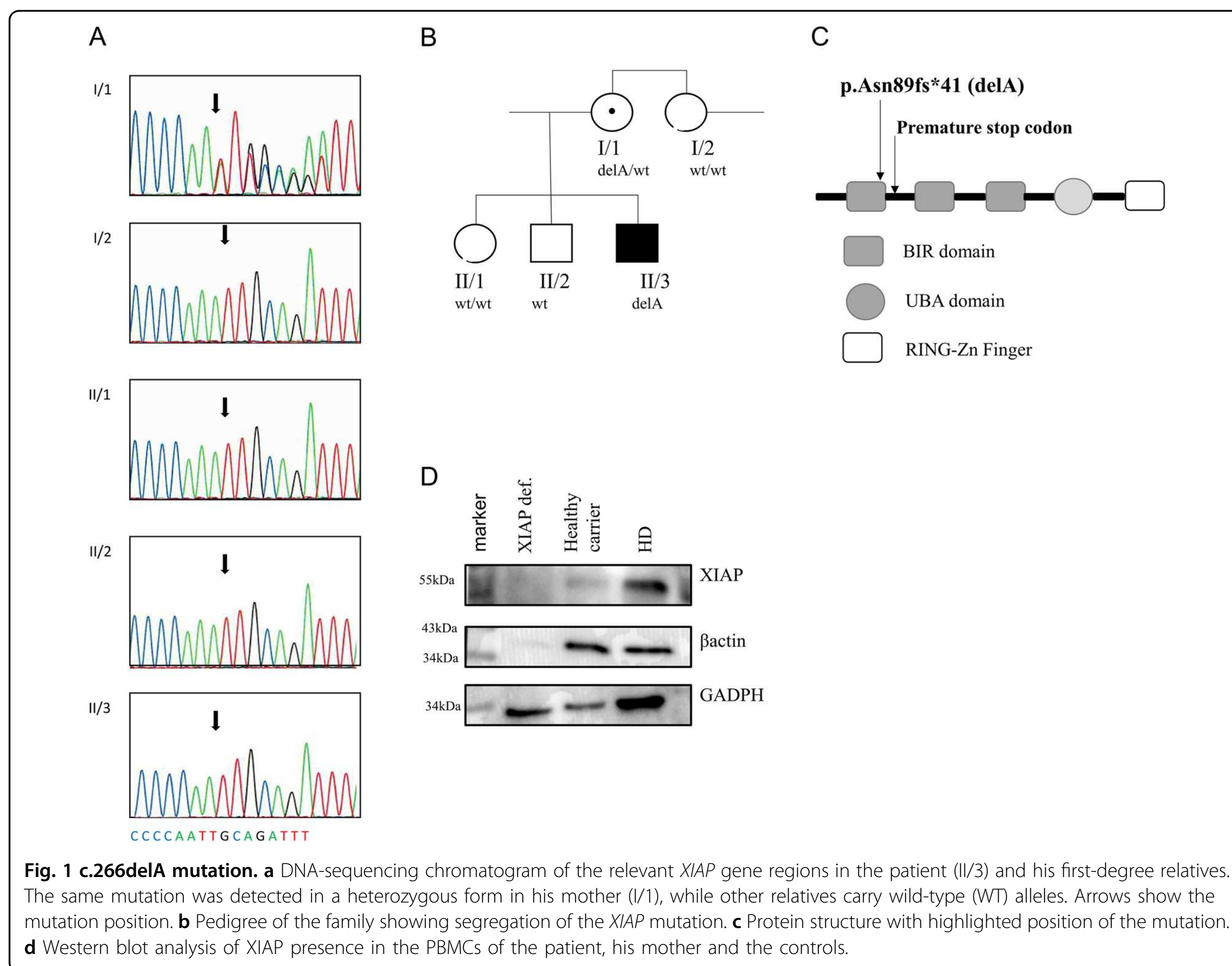

Fig. 1 c.266delA mutation. a DNA-sequencing chromatogram of the relevant *XIAP* gene regions in the patient (II/3) and his first-degree relatives. The same mutation was detected in a heterozygous form in his mother (I/1), while other relatives carry wild-type (WT) alleles. Arrows show the mutation position. **b** Pedigree of the family showing segregation of the *XIAP* mutation. **c** Protein structure with highlighted position of the mutation. **d** Western blot analysis of XIAP presence in the PBMCs of the patient, his mother and the controls.

(Supplementary Fig. 3A), suggesting that only the NOD2 pathway was affected. Next, we assessed cytokine production (IL-1β, IL-6 and TNFα) after stimulation of patient PBMCs with MDP and lipopolysaccharide (LPS) (Fig. 4e) using the Luminex method. The patient's cells produced decreased levels of cytokines after MDP stimulation compared with the healthy controls; however, in response to LPS stimulation, the patient's PBMCs produced comparable levels of cytokines, confirming defective NOD2 signalling in the patient's cells.

XIAP deficiency affects T-cell homoeostasis

To test whether XIAP deficiency and impaired apoptosis influenced the distribution of the patient's B- and T-cell subpopulations, we analyzed these subsets. The gating strategies used to distinguish between naive, central memory (CM), effector memory (EM), terminal effector T cells re-expressing CD45RA (TEMRA), recent thymic emigrants (RTEs) and B-cell subsets, are illustrated in Supplementary Fig. 4. The analysis showed a shift towards mature stages of CD4+ and CD8+ T cells in the patient samples (Fig. 5a, b). We found a noteworthy increase in the count of CM and EM, and a reduction in naive forms of the T cells; however, the percentage of RTEs was unaffected. Consequently, we analyzed the patient's T-lymphocyte ability to produce IFNγ by flow cytometry. The patient's T cells produced higher levels of IFNγ even in the unstimulated state, which was significantly elevated upon PMA stimulation. The percentage of IFNγ-producing CD4+ T cells (20.1%) was considerably higher than that of the healthy donors (5.3%) (Fig. 5d, e). In addition, analysis of activation marker expression on T cells, HLA-DR as a marker of chronic activation, and CD69 as the earliest activation marker, revealed a shift towards late stages of activation. HLA-DR expression was threefold higher on the CD8+ T cells and twofold higher on CD4+ T cells than it was in the healthy controls (Fig. 5a, b). CD69 expression was unaffected (Supplementary Fig. 3C). T-cell proliferation was negligibly decreased (55.2% patients; controls 74.7% after PMA and ionomycin stimulation) (Supplementary Fig. 3D). Moreover, we also observed higher production of IL-12 in response to LPS in

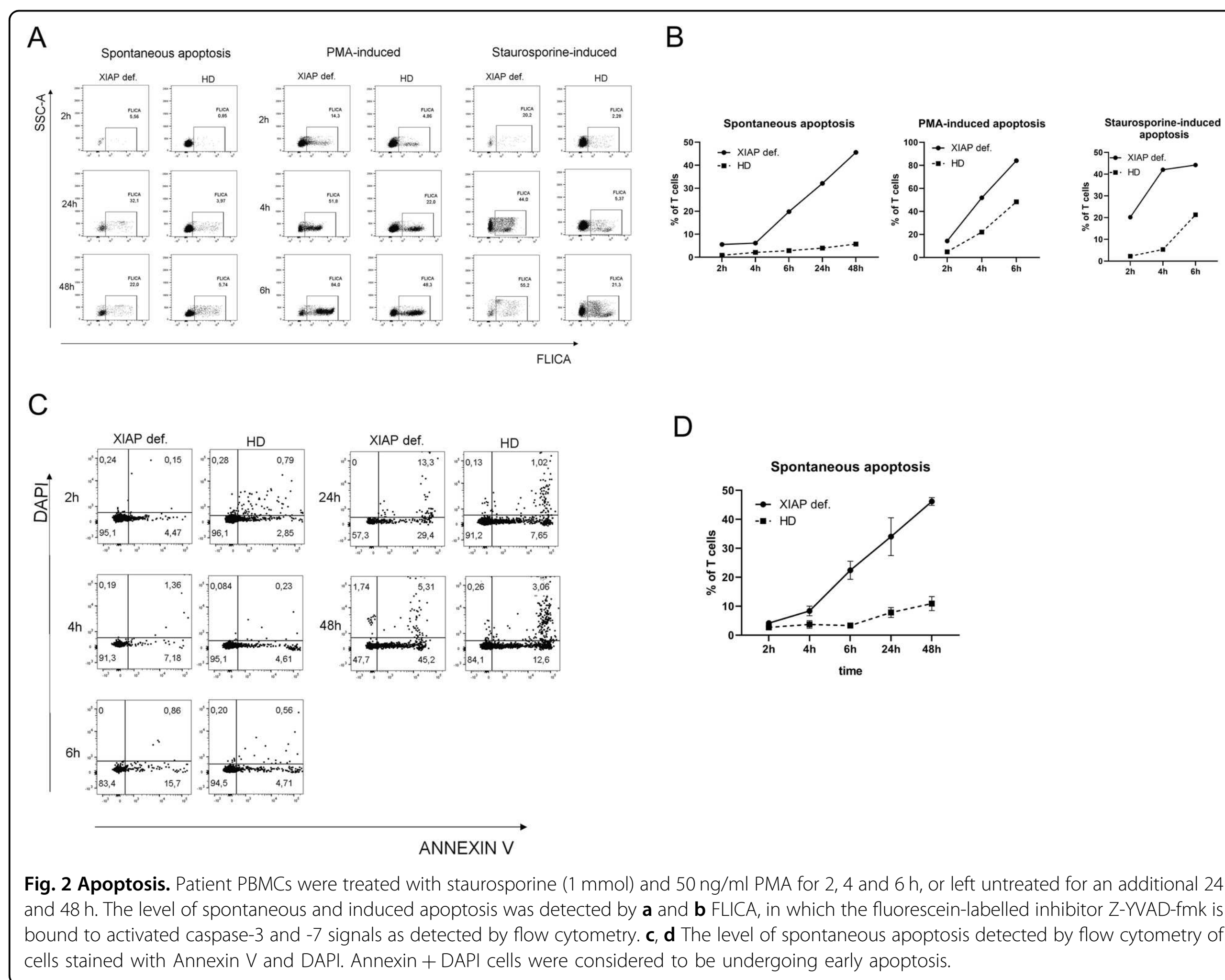

Fig. 2 Apoptosis. Patient PBMCs were treated with staurosporine (1 mmol) and 50 ng/ml PMA for 2, 4 and 6 h, or left untreated for an additional 24 and 48 h. The level of spontaneous and induced apoptosis was detected by **a** and **b** FLICA, in which the fluorescein-labelled inhibitor Z-YVAD-fmk is bound to activated caspase-3 and -7 signals as detected by flow cytometry. **c**, **d** The level of spontaneous apoptosis detected by flow cytometry of cells stained with Annexin V and DAPI. Annexin + DAPI cells were considered to be undergoing early apoptosis.

patient PBMCs, supporting a Th1-polarizing environment (Fig. 5f). No significant differences were found in the B-cell department (Fig. 5c).

Discussion

Here, we report the case of a patient who developed adult-onset IBD refractory to treatment and complicated by several episodes of HLH, and for whom WES revealed a novel previously unpublished c.266delA mutation in the *XIAP* (*BIRC4*) gene that led to its loss of function. HLH and IBD are the most common first manifestations of XIAP deficiency, which usually occurs in the first few years of life, and for which the potentially lethal outcome requires HSCT[13]. Adult-onset HLH and IBD associated with XIAP deficiency, although rare, have also been described[17,22]. In a large cohort of 54 XIAP-deficient patients, IBD manifestation was the main clinical feature in 17 of them. The remaining patients usually manifested with HLH as a major disease complication. The average age at the time of diagnosis of IBD was 11 years (range 3 months–41 years) compared with patients manifested with HLH (average age 6.5 years and range 0.1–23 years). In our patient, IBD manifested at the age of 17 and HLH at the age of 20. The majority of the first HLH attacks was associated with EBV infection; however, HHV6 and HSV1 were identified as potential triggers as well. IBD-related complications were the main cause of death in three of them at the average age 24 years (range 4–42 years) and after 4 years of disease duration (range 0–7 years). Interestingly, only four patients presented in a form of the adult-onset IBD[22,23].

Most of the *XIAP* mutations identified in XLP-2 patients are nonsense mutations, frameshift mutations or deletions that cause severe aberrations in the encoded protein or loss of its expression. They are distributed along all coding exons[10,13,24–26]. Neither type nor position of the mutation, as well as residual protein expression, do not correlate with the clinical manifestation and severity of the disease[23].

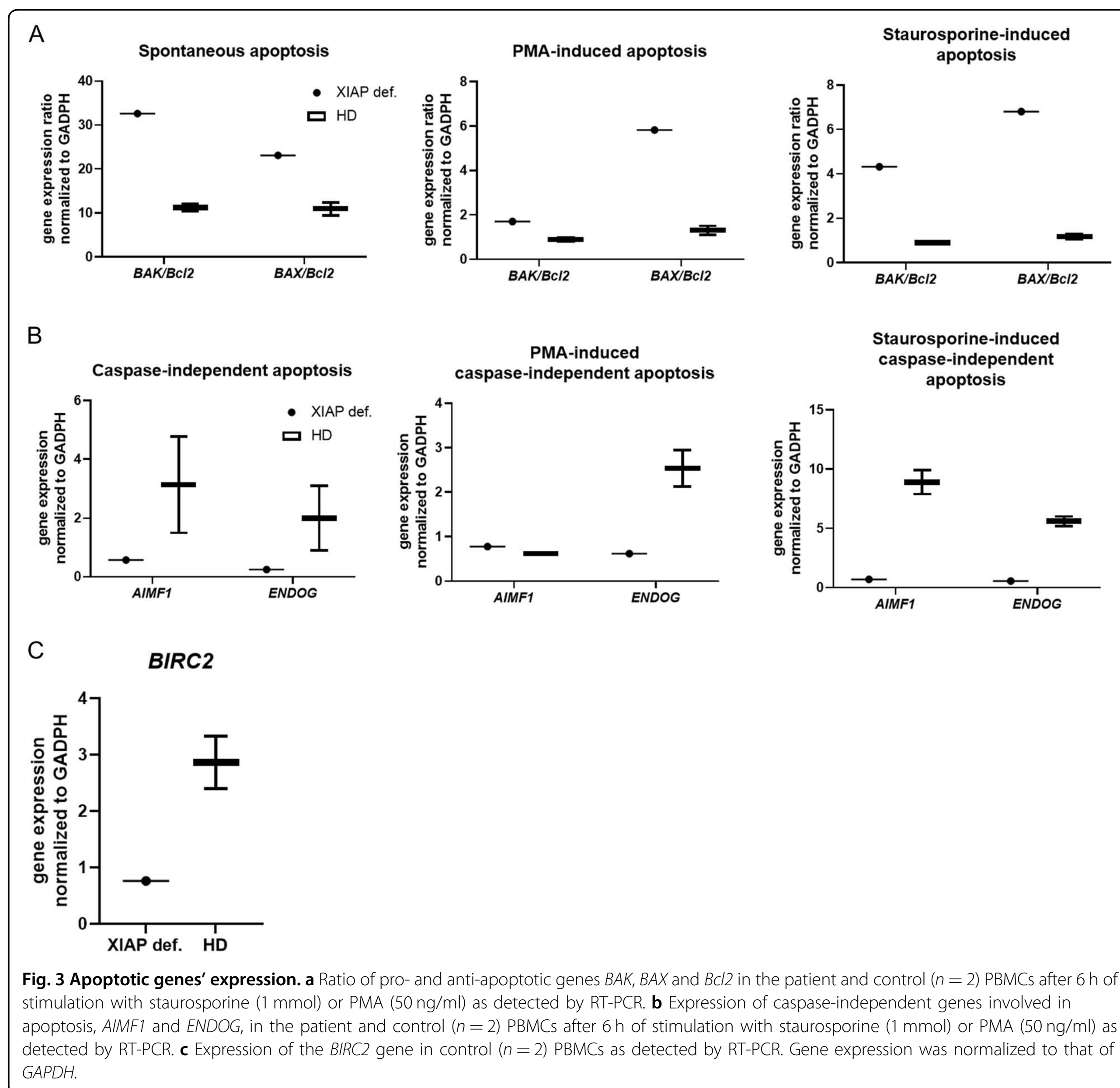

Fig. 3 Apoptotic genes' expression. a Ratio of pro- and anti-apoptotic genes *BAK*, *BAX* and *Bcl2* in the patient and control ($n = 2$) PBMCs after 6 h of stimulation with staurosporine (1 mmol) or PMA (50 ng/ml) as detected by RT-PCR. **b** Expression of caspase-independent genes involved in apoptosis, *AIMF1* and *ENDOG*, in the patient and control ($n = 2$) PBMCs after 6 h of stimulation with staurosporine (1 mmol) or PMA (50 ng/ml) as detected by RT-PCR. **c** Expression of the *BIRC2* gene in control ($n = 2$) PBMCs as detected by RT-PCR. Gene expression was normalized to that of *GAPDH*.

We report a novel deletion mutation c.266delA, resulting in a premature stop codon (p. Asn89fs*41), loss of protein expression and, as a consequence, a patient suffering from XLP-2 and lower expression in his mother, who is a healthy carrier of the mutation.

XIAP-deficient T cells are characterized by a high susceptibility to apoptosis ex vivo in response to apoptotic stimulus or upon activation[17,23,27]. Indeed, we observed an enhanced level of apoptosis in response to staurosporine, an inducer of apoptosis, as well as upon activation by PMA. Interestingly, we also observed increased spontaneous apoptosis in patient lymphocytes, which was reduced when a caspase inhibitor was applied. However, the sensitivity to apoptosis of T cells was found to have no influence on circulating blood lymphocyte numbers in patients[27]. Accordingly, circulating T-cell numbers were in the normal range in the patient, although we observed a shift to their more mature stages. Considering T-lymphocyte function, the expansion and proliferation of virus-specific T lymphocytes might be compromised in XIAP deficiency. XIAP-deficient patients suffer from an increased risk of EBV infections, and in a mouse model[28], XIAP and cIAP1 were required for the survival and expansion of virus-specific T cells. In addition, defective NOD2 signalling might also contribute to a higher risk of EBV infection[29,30]. Apoptosis may be further ameliorated

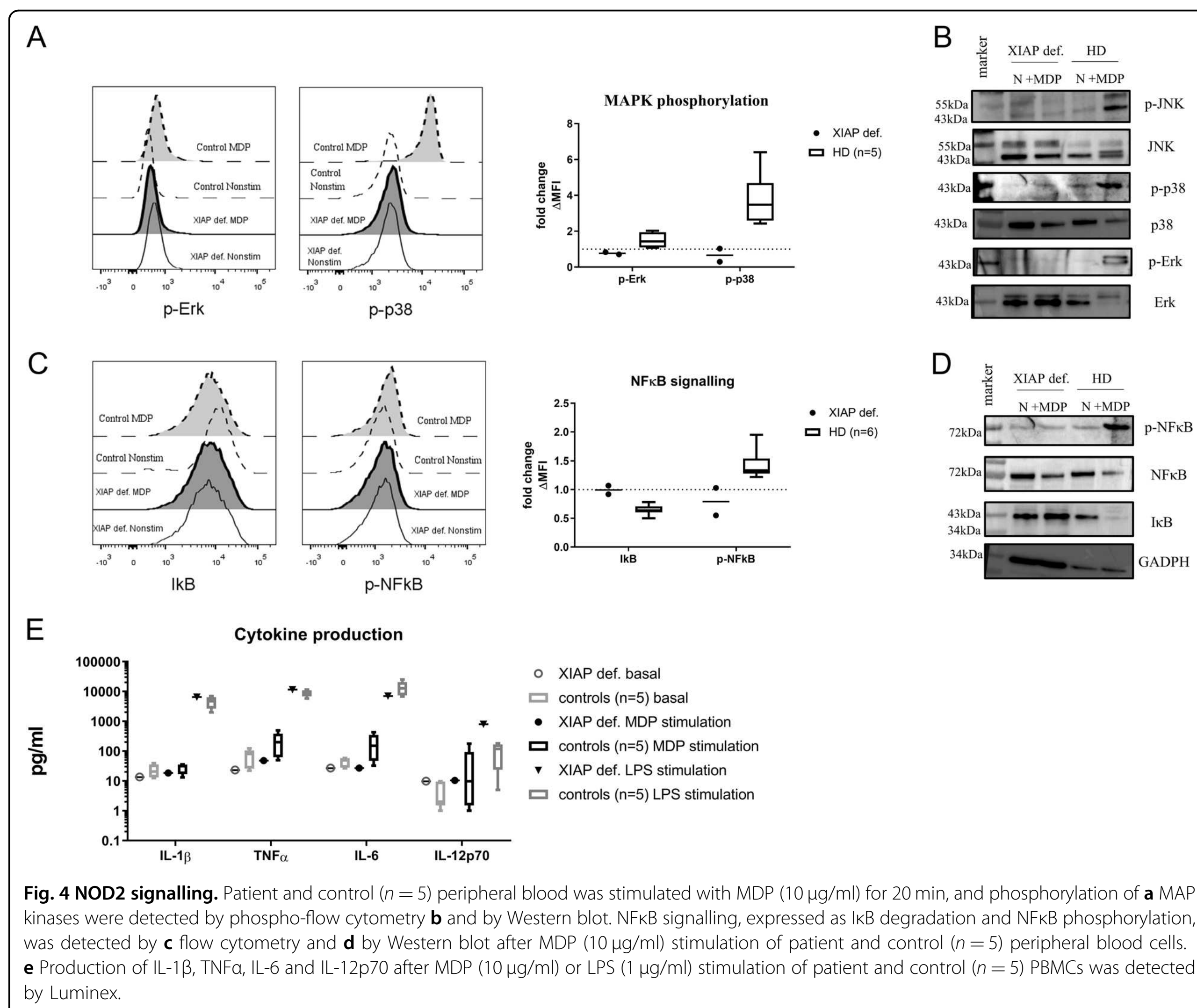

Fig. 4 NOD2 signalling. Patient and control ($n = 5$) peripheral blood was stimulated with MDP (10 μg/ml) for 20 min, and phosphorylation of **a** MAP kinases were detected by phospho-flow cytometry **b** and by Western blot. NFκB signalling, expressed as IκB degradation and NFκB phosphorylation, was detected by **c** flow cytometry and **d** by Western blot after MDP (10 μg/ml) stimulation of patient and control ($n = 5$) peripheral blood cells. **e** Production of IL-1β, TNFα, IL-6 and IL-12p70 after MDP (10 μg/ml) or LPS (1 μg/ml) stimulation of patient and control ($n = 5$) PBMCs was detected by Luminex.

by increased production of IFNγ, which further enhances the expression of pro-apoptotic genes (such as *BAX*, *BAK1* and/or *XAF1*)[31].

The aforementioned shift in the spectrum of T lymphocytes to their more mature stages seems to be related to the alteration of the apoptosis process. It has been previously reported that T lymphocytes at different stages of development have different sensitivities to apoptosis, possibly resulting from different expression of pro- and anti-apoptotic proteins[32,33]. These differences may lead to a significant reduction in naive and the subsequent survival of the mature memory forms of T cells, including CM and EM T cells, as observed in the patient.

HLH is the most severe and life-threatening manifestation in patients with XIAP deficiency, but the exact mechanism by which mutated *XIAP* results in HLH manifestations is not entirely clear. The mechanism differs from other genetic disorders associated with HLH, such as XLP-1, in which the impaired cytotoxic responses by CD8+ lymphocytes and NK cells result in exaggerated amounts of IFNγ and the activation of macrophages, thus explaining the positive effect of the IFNγ blockade on the outcome of HLH[15]. The patient's T lymphocytes produced markedly higher levels of IFNγ in comparison with the healthy donors, even though XIAP deficiency was not connected with defects in the cytotoxic responses by CD8+ lymphocytes or NK cells, as is typical in XLP-1[27]. The shift towards the Th1 immune response and increased production of IFNγ was further supported by the overproduction of IL-12, a crucial cytokine for Th1 polarization[34]. Observations in a mouse model propose, as a possible explanation, that HLH is due to NLRP3 inflammasome dysregulation and increased proinflammatory cytokine production[35,36]. Although it is still unclear whether XIAP in humans also acts as an NLRP3 inhibitor, impairment to this control might represent a key pathological mechanism. XIAP-deficient mice also develop splenomegaly when treated with an activator of

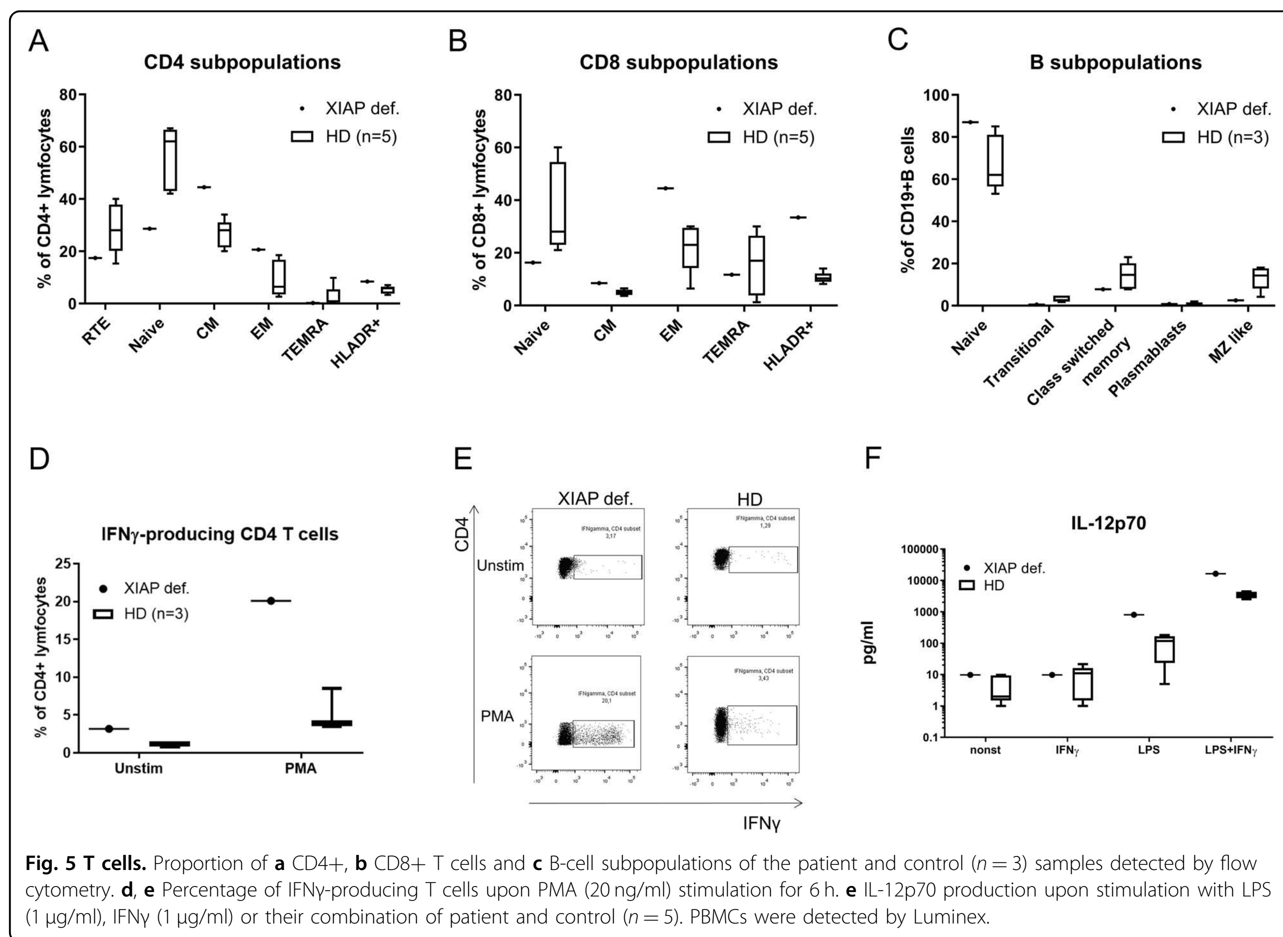

Fig. 5 T cells. Proportion of **a** CD4+, **b** CD8+ T cells and **c** B-cell subpopulations of the patient and control ($n = 3$) samples detected by flow cytometry. **d**, **e** Percentage of IFNγ-producing T cells upon PMA (20 ng/ml) stimulation for 6 h. **e** IL-12p70 production upon stimulation with LPS (1 μg/ml), IFNγ (1 μg/ml) or their combination of patient and control ($n = 5$). PBMCs were detected by Luminex.

the NLRP3 inflammasome[36]; therefore, this mechanism may explain two of three typical pathologies associated with XIAP deficiency. Interestingly, mutations in the human NLRC4 inflammasome were identified in patients suffering from recurrent HLH and autoinflammation, supporting a role of the inflammasome in HLH[37,38]. However, we observed only slightly higher IL-1β and TNFα production in the patient in response to LPS stimulation.

In line with previous reports, the patient displayed diminished proinflammatory cytokine production after NOD2 ligand stimulation[12,39,40], thus connecting the potential role for altered NOD2 signalling with IBD in XIAP patients. NOD2 mutations represent a strong genetic risk factor for CD[11], as NOD2-impaired secretion of cytokines and an altered gut microbiome may disturb intestinal homoeostasis. Like HLH, IFNγ is one of the most important cytokines in CD pathophysiology. Indeed, as shown here for the XIAP-deficient patient, altered NOD2-mediated signalling and high IFNγ production by T cells might explain, in an analogy to CD, the gastrointestinal IBD-like presentation as a feature of XIAP deficiency.

Taken together, our data reveal a novel mutation in a patient suffering from recurrent HLH, IBD and splenomegaly, typical conditions associated with XIAP deficiency. The deletion mutation leads to loss of XIAP expression, and it functions as a negative regulator of apoptosis. The absence of XIAP clearly leads to enhanced cell death, which may amplify inflammation. XIAP deficiency negatively influences MDP-induced NOD2 signalling, with implications for IBD. Changes in innate immunity, highlighted together with the role of IFNγ, contribute to XLP-2 pathogenesis and complex clinical presentation. Whereas HSCT in patients with the early onset of the disease represents a method of choice, in adult patients, such as in the index patient in our study, the therapeutic options are more limited. Emapalumab, a monoclonal antibody that targets IFNγ, was approved for the treatment of relapsed/refractory HLH[14] with a possible influence on the symptoms of CD, and anti-IL-12/23 (ustekinumab) therapy[41] is also available. The overlap in pathogenetic mechanisms gives hope for the use of this strategy to treat XIAP deficiency.

Patient and methods

Informed written consent was obtained from all subjects involved in the study and all controls in accordance with the Declaration of Helsinki, and according to the procedures established by the Ethical Committee of our institution.

Whole-exome sequencing

WES was performed on a NextSeq 500 instrument (Illumina, San Diego, CA), and sequencing libraries were prepared using the SureSelectXT Human All Exon V6 + UTR kit (Agilent Technologies, Santa Clara, CA). Sequencing reads were aligned against the human reference genome hg19 by BWA[42], and variant calling was performed using SAMtools[43] and VarScan 2[44] and their annotation using SnpEff[45].

Apoptosis

Peripheral blood was collected from the patient and healthy volunteers into EDTA-coated tubes. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque (GE Healthcare Biosciences, Uppsala, Sweden). The obtained cells were resuspended in RPMI 1640 medium with a sodium bicarbonate buffer system supplemented with 2% autologous serum, 1% penicillin and streptomycin and 1% GlutaMAX (Thermo Fisher Scientific, Waltham, CA, USA). PBMCs (10^6/ml) stimulated with staurosporine (1 mmol) (Abcam, Cambridge, UK) for 4 and 6 h, PMA (50 ng/ml) (Sigma-Aldrich, Darmstadt, Germany) for 4 h or left untreated for 4, 6, 24 and 48 h. When indicated, 20 µM Z-VAD-FMK was added in the culture 30 min before apoptosis induction. Then, the cells were washed in Annexin V binding buffer and stained with Annexin V–Dyomics 647 (EXBIO) and DAPI (Thermo Fisher Scientific).

FLICA staining

Active caspase-3 and -7 were detected using a FLICA caspase-3 and 7 assay kit (Thermo Fisher Scientific). PBMCs were stimulated as described above prior to treatment with the fluorescein-labelled inhibitor Z-YVAD-fmk (10 µM) for 1 h at 37 °C and CD3-A700 (clone MEM-57) (EXBIO, Prague, Czech Republic). The cells were washed three times and analyzed by flow cytometry with a FACS Fortessa flow cytometer (BD Biosciences, San Diego, CA, USA).

Phospho-flow cytometry

Detection of MAPK and NFκB activation was performed according to a previously published protocol[46]. Briefly, peripheral blood was stimulated with 10 µg/ml MDP (InvivoGen, San Diego, CA, USA) for 20 min at 37 °C or left unstimulated. Subsequently, the cells were fixed using 4% formaldehyde for 10 min at 25 °C, erythrocytes were lysed using 0.1% Triton X-100 (Sigma-Aldrich) for 15 min at 37 °C and the leukocytes were permeabilized using 80% ice-cold methanol for 30 min.

The following antibodies were used: CD3—A700 (clone MEM-57), CD14—PEDy594 (EXBIO) and CD19—PC7 (clone J3-119) (Beckman Coulter, USA, Brea, USA), phospho38 (Thr180)—A647 (#4552 S), phosphoErk1/2 (Thr202/Tyr204)—A488 (#4374 S), phosphoSAPJ/JNK (Thr183/185)—PE (#5755 S) (Cell Signaling, Denvers, MA, USA), phosphoNFκB—A647 (#4887) and anti-IκB—A488 (#5743) (both from Cell Signaling).

Cytokine production

Cytokines were detected using a multiplex Luminex cytokine-fluorescent bead-based immunoassay (Merck Millipore, Beerlengton, MA, USA) with cell-free supernatants. A total of 2×10^5 PBMCs were stimulated with MDP (10 µg/ml) (InvivoGen), *E. coli* LPS (1 µg/ml) (Sigma-Aldrich) or left untreated for 24 h.

T- and B-cell analysis

Immunophenotyping of T and B cells was performed according to a previously published protocol[47], and the gating strategy is shown in Supplementary Fig. 2B.

For IFNγ-producing cell detection, we applied an already-published protocol[46].

T-cell proliferation

The proliferation of CD3+ T lymphocytes was determined according to a previously published protocol[48].

RT-PCR

PBMCs were stimulated as stated in the 'Apoptosis' section. RNA isolation, reverse transcription and RT-PCR were performed according to a previously published protocol[49]. TaqMan primer/probe sets (Thermo Fisher Scientific) were used. The sample data were matched to a standard curve generated by amplifying serially diluted products using the same PCR, and normalized to *GAPDH* (TIB Molbiol, Berlin, Germany) to obtain the relative expression value. Real-time assays were run on an FX96 cycler (Bio-Rad). The primer/probe sets are available from the authors upon request.

Western blotting

Detection of proteins was performed according to a previously published protocol[46]. The membranes were incubated with the following primary antibodies: anti-XIAP (clone D2Z8W), anti-β-actin (clone D6A8), anti-GAPDH (clone D16H11), IκB anti (clone L35A5), anti-NFκB (clone D14E12) (all from Cell Signaling), anti-tubulin (clone TU-07), anti-HSP-90 (clone MBH90AB) (both from Exbio), anti-Erk1/2 (ab17942), anti-p-Erk1/2 (ab76299), anti-p-p38 (ab4822), anti-p38 (ab170099),

anti-p-JNK1/2/3 (ab124956), anti-JNK1/2/3 (ab208035) and anti-p-NFκB (ab76302) (all from Abcam) overnight, followed by incubation with peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies for 2 h. The membranes were developed using SuperSignal West Femto (Thermo Fisher Scientific).

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Author contributions
Z.P. designed the study and experiments, performed the experiments, analyzed the data, interpreted the results and wrote the paper. T.M. designed the experiments, interpreted the results, and provided patient information. P.V. performed the RT-PCR. J.S. acquired the data regarding apoptosis and T-cell proliferation. M.S. provided the NGS results. T.F. provided the Sanger sequencing data. V.K. performed the T- and B-cell analysis. A.S. reviewed and edited the paper.

Conflict of interest
The authors declare that they have no conflict of interest.

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LETTER TO EDITOR

High Prevalence of Likely Passively Acquired Anti-TPO and Anti-GAD Autoantibodies in Common Variable Immunodeficiency

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Common variable immunodeficiency disorder (CVID) is one of the most frequent inborn errors of immunity characterized by decreased immunoglobulin production, impaired specific antibody response, and higher susceptibility to infections along with immune system dysregulation and higher prevalence of non-infectious complications [1]. Despite the markedly impaired antibody production, diseases hallmarked by the presence of autoantibodies, such as autoimmune hemolytic anemia (AIHA) or immune thrombocytopenic purpura (ITP), are among the most commonly diagnosed autoimmune complications in CVID patients [2]. The mainstay of CVID management is a regular, long-term immunoglobulin replacement therapy (IRT). Importantly, the immunoglobulin solutions used for IRT were shown to contain various specific antibodies and may even be responsible for IRT-associated adverse events, such as self-limiting acute hemolysis triggered by passivelly transmitted antierythrocyte alloantibodies [3]. Therefore, we initiated a prospective observational trial to determine the prevalence, clinical significance, and origin of the spectrum of autoantibodies in CVID patients on IRT. Twenty-three different autoantibodies were screened in 38 CVID patients receiving IRT (baseline characteristics in Supplementary table 1) and in the immunoglobulin solutions used for IRT (Supplementary table 2). No patient serum samples prior to IRT were available for the analysis. Apart from the spectrum of autoantibodies, the following parameters were measured: the glucose and insulin metabolism, i.e., fasting serum concentration of C-peptide and glycosylated hemoglobin A1c, and thyroid gland function, i.e., free thyroxine and thyroid-stimulating hormone. Additionally, neck ultrasonography was performed to evaluate structural changes of the thyroid gland. The enrolled CVID patients were prospectively followed for the meantime of 2 years (±0.64, range: 0.25–2). The selected laboratory parameters were also compared to a cohort of 40 newly diagnosed type 1 diabetes (T1D) and 50 autoimmune thyroiditis (AIT) patients.

Anti-thyroid peroxidase (anti-TPO) and anti-glutamic acid decarboxylase (anti-GAD) autoantibodies markedly prevailed. We identified 68.4% ($n=26/38$) anti-TPO-positive patients and 55.3% ($n=21/38$) anti-GAD-positive subjects among the CVID patients. 36.8% of patients ($n=14/38$) were both anti-TPO and anti-GAD positive. Only 7% ($n=1/14$) and 60% ($n=12/20$) of patients with anti-GAD and anti-TPO positivity, respectively, who were followed for ≥1 year (the spectrum of autoantibodies assessed at least twice) remained positive for the duration of the study. The mean titer of anti-GAD was 3.22 kU/L (±3.94 SD) in CVID patients, which was significantly lower ($p<0.0001$) compared to the levels of anti-GAD 22.0 kU/L (±26.1 SD) in T1D at the time of T1D diagnosis (Table 1). The presence of anti-GAD antibodies in CVID patients was not associated with a disturbed insulin production or an impaired glucose metabolism. The serum levels of C-peptide (891 pmol/L, ±601 SD) were comparable to anti-GAD-negative CVID patients (924 pmol/L, ±781 SD, $p=0.177$). On the other hand, significant differences were found when C-peptide serum levels were compared to T1D patients (mean 266.8 pmol/L, ±235 SD, $p<0.0001$). Similar results were also observed with serum levels of glycosylated hemoglobin A1c (g-Hgb),

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Table 1 Concentrations of anti-thyroid peroxidase (TPO) and anti-glutamic acid decarboxylase (GAD) autoantibodies in TPO-positive Common variable immunodeficiency patients (CVID+, threshold limit for positivity of anti-TPO: > 60 kU/mL) and anti-GAD CVID+(threshold limit for positivity of anti-GAD: > 0.9 kU/mL) compared to autoimmune thyreoditis (AIT) and type 1 diabetes (T1D) patients, */#significant differences between groups ($p<0.05$, tested by the Mann–Whitney test); concentrations of anti-TPO and anti-GAD autoantibodies in immunoglobulin replacement therapy (IRT) solutions—10% intravenous IgG (IVIG)-I (Kiovig, Takeda, Vienna, Austria), 10% IVIG-II (Privigen, CSL Behring, Marburg, Germany), 16.5% subcutaneous IgG (SCIG)-16.5 (Gammanorm, Octapharma, Anderlecht, Belgium), and 20% SCIG-20 (Hizentra, CSL Behring, Marburg, Germany)

Group	Anti-TPO level	Group	Anti-GAD level
Patient cohorts			
CVID+	109.7 kU/L (±97.4)*	CVID+	3.22 (±3.94)#
AIT	713 kU/L (±520)*	T1D	22.0 kU/L (±26.1)#
IRT solutions			
IVIG-I	156.55 (19.62	IVIG-I	24.48 (13.94)
IVIG-II	142 (43.3)	IVIG-II	3.24 (1.51)
SCIG-16.5	123.6 (7.43)	SCIG-16.5	7.28 (3.80)
SCIG-20	145.4 (16.96)	SCIG-20	3.39 (3.73)

which were comparable between anti-GAD-positive CVID patients (32 mmol/mol, ± 4.04 SD) and negative patients (32 mmol/mol, ± 5.86 SD, $p=0.65$). Conversely, the levels of g-Hgb were significantly higher in newly diagnosed T1D patients (68 mmol/mol, ± 33.06 SD) than in anti-GAD-positive CVID patients ($p<0.0001$). The serum concentrations of C-peptide as well as g-Hgb remained unchanged from the baseline for the duration of the study.

The mean titer of anti-TPO was 109.7 kU/L (± 97.4 SD), which was significantly lower ($p<0.0001$) in CVID patients compared to the mean levels of anti-TPO 713 kU/L (± 520 SD) in AIT patients (Table 1). The serum concentrations of both TSH and fT4 in anti-TPO-positive CVID patients (TSH: 2.08 mIU/L, ± 1.17 SD, fT4: 14.49 pmol/L, ± 1.53 SD) did not differ from the anti-TPO-negative CVID patients (TSH: 1.67 mIU/L, ± 0.75 SD, $p=0.218$, fT4: 15.17, ± 3.47 SD, $p=0.518$) and the concentration of TSH levels in anti-TPO-positive CVID patients did not differ from the AIT patients (1.40 mIU/L, ± 3.11 SD, $p=0.826$). The level of fT4 in anti-TPO-positive CVID patients (14.49 pmol/L, ± 1.53 SD) was even significantly lower than in the AIT group (16.71, ± 2.57 SD, $p<0.0001$). Nevertheless, the values were within normal ranges (11.50–22.70 pmol/L). Neither TSH nor fT4 altered during the follow-up period. Moreover, insignificant differences ($p=0.593$) were also observed in the proportion of ultrasonographic (USG) evidence of AIT. The USG diagnosis of AIT was considered in only three out of 26 anti-TPO-positive CVID patients. All patients were females, median age 53 years (± 2.08 SD), median CVID duration 7 years (± 8.43 SD). Two of them had other non-infectious complications, including autoimmune and lymphoproliferative manifestations. On the other hand, one CVID patient probably developed AIT according to USG but no anti-TPO autoantibodies were detected.

Furthermore, the anti-GAD and anti-TPO were detected in very high levels in all the tested IRT. The titers of anti-GAD were ranging from 3.24 kU/L (± 1.51 SD, range: 0.9–4.74) in 10% IVIG-I to 24.48 kU/L (± 13.94 SD, range: 12.45–40.54) in 10% IVIG-II and anti-TPO from 123.6 kU/L (± 7.43 SD, range: 114.30–138.20) in 16.5% SCIG to 156.55 kU/L (± 19.62 SD, range: 108.70–165.40) in 10% IVIG-I (Table 1).

To our best knowledge, this is the first report investigating the content of a broad spectrum of autoantibodies in therapeutics used for IRT and their clinical relevance. We identified a high prevalence of anti-TPO autoantibodies and anti-GAD autoantibodies in the majority of the screened CVID patients receiving IRT. Higher amounts of anti-GAD antibodies have also been detected in immunoglobulin therapeutics in previously published studies [4]. Moreover, we found an increased concentration of anti-TPO autoantibodies in all investigated immunoglobulin therapeutics that may be passively transferred to the patients' blood circulation. Despite the fact that IRT may cause autoantibody transfer, we found no evidence that this mechanism would contribute to the clinical manifestation of the respective autoimmune diseases, compromising the safety of IRT. Our findings are consistent with previous observations that pancreatic islet-specific autoantibodies are not major effectors in β-cell damage [5]. On the other hand, the presence of autoantibodies may interfere with the diagnosis of autoimmune disease in IRT receivers and must, therefore, be interpreted with caution and correlated to other diagnostic signs.

While acknowledging this study's limitations, including particularly the limited number of enrolled patients, cohort heterogeneity, short duration of the study, and suboptimal IRT dosage regimens, we conclude that anti-GAD and anti-TPO antibodies are not accurate tools for the screening or diagnosis of T1D and AIT in CVID patients on regular immunoglobulin substitution therapy. Instead, the measurement of glycemia, C-peptide, and g-Hgb, respectively, thyroid ultrasound may be the screening method of choice.

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Declarations

Ethics Approval The study was approved by the Ethical Committee of Motol University Hospital.

Consent to Participate Only patients with signed informed consent are approved by the Ethical Committee of Motol University Hospital.

Consent for Publication All co-authors have reviewed the manuscript and have agreed with the submission in the current form.

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Risk Factors for Severe COVID-19 and Hospital Admission in Patients With Inborn Errors of Immunity - Results From a Multicenter Nationwide Study

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Despite the progress in the understanding how COVID-19 infection may impact immunocompromised patients, the data on inborn errors of immunity (IEI) remain limited and ambiguous. Therefore, we examined the risk of severe infection course and hospital admission in a large cohort of patients with IEI. In this multicenter nationwide retrospective survey-based trial, the demographic, clinical, and laboratory data were collected by investigating physicians from 8 national referral centers for the diagnosis and treatment of IEI using a COVID-19-IEI clinical questionnaire. In total, 81 patients with IEI (including 16 with hereditary angioedema, HAE) and confirmed SARS-CoV-2 infection were enrolled, and were found to have a 2.3-times increased (95%CI: 1.44–3.53) risk ratio for hospital admission and a higher mortality ratio (2.4% *vs.* 1.7% in the general population). COVID-19 severity was associated with the presence of clinically relevant comorbidities, lymphopenia, and hypogammaglobulinemia, but not with age or BMI. No individuals with HAE developed severe disease, despite a hypothesized increased risk due to perturbed bradykinin metabolism. We also demonstrated a high seroconversion rate in antibody-deficient patients and the safety of anti-spike SARS CoV-2 monoclonal antibodies and convalescent plasma. Thus, IEI except for HAE, represent significant risk factors for a severe COVID-19. Therefore, apart from general risk factors, immune system dysregulation may also be involved in the poor outcomes of COVID-19. Despite the study limitations, our results support the findings from previously published trials.

Keywords: inborn errors of immunity, COVID-19, SARS-CoV-2, risk factors, mortality, hospital admission

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INTRODUCTION

Inborn errors of immunity (IEI) are a heterogeneous group of rare disorders characterized by impaired immune system function, manifesting as increased susceptibility to infections and a broad spectrum of non-infectious complications including autoimmunity, autoinflammatory diseases, allergy, and/or malignancy (1, 2). In contrast to the general population, IEI predispose patients to severe, chronic, and recurrent infections, usually with complicated courses (3). Therefore, the novel coronavirus SARS-CoV-2 raised new concerns in patients with IEI patients. This single stranded mRNA virus was originally described in a patient with acute respiratory failure in the Chinese city of Wuhan; the disease was later named COVID-19 (4). To date, more than 250 million cases and 5 million deaths have been reported to be caused by COVID-19 (5). The main clinical features comprise fever, cough, malaise, fatigue, sore throat, and dyspnea that may rapidly progress to acute lung injury with acute respiratory failure (6, 7). Higher age (> 75 years), severe obesity, male sex, arterial hypertension, and cardiovascular or respiratory tract disease were determined as the main risk factors for a severe disease course and poor outcome (8, 9). Higher age (> 65 years) was also associated with an increased risk of reinfection (10), which is further emphasized by the emergence of novel virus variants (11). Complicated COVID-19 infection with increased hospital admission and mortality rates compared to the general population was also described in immunocompromised patients such as those with cancer or solid organ transplants. However, many of these patients had other risk factors (higher age, significant comorbidities) associated with worse outcomes. Interestingly, children who underwent hematopoietic stem cell transplantation showed a similar risk ratio for severe COVID-19 compared to the general population (12). Despite the progress in understanding how COVID-19 may impact patients with IEI, studies focused on the clinical presentation and outcome of COVID-19 in these patients remain limited, and present ambiguous results (13–17) that may reflect inter-population differences. Therefore, we initiated a retrospective multicenter study focused on the clinical presentation and outcome of COVID-19 in patients with IEI. The study included patients with hereditary angioedema (HAE) due to C1 inhibitor deficiency, as the bradykinin overproduction in the kallikrein-kinin cascade was proposed as a possible mediator involved in the respiratory complications of COVID-19 infection and, as such, a risk factor for severe COVID-19.

METHODS

Study Design

The study was designed as a multicenter retrospective survey-based study and was conducted for the period from March 2020 to October 2021. Data were obtained from 8 national referral centers for the diagnosis and treatment of IEI including patients with hereditary angioedema (HAE), who were evaluated separately. The study only included patients fulfilling the following inclusion criteria: 1) fulfilled diagnostic criteria for IEI, 2) PCR SARS-CoV2 positivity, the testing was indicated according to the recommendations of the healthcare authorities for the general population, 3) patient´s consent provided before inclusion. The study design is summarized in **Figure 1**. This study was approved by Ethics Committee of the University Hospital in Hradec Kralove, the Czech Republic.

Data Collection

Demographic and patient-specific data (gender, age, body mass index - BMI), the clinical presentation of IEI (diagnosis, manifestation, and therapy), comorbidities, the clinical presentation of COVID-19 (date of infection, symptoms, disease-related complications, therapy, and outcome), and laboratory parameters (lymphocyte subpopulations, serum IgG/IgA/IgM levels) as close as possible to the time of COVID-19 diagnosis were collected. Patients were classified based on the European Society for Immunodeficiency diagnostic criteria (18) and/or genetically confirmed diagnosis. Patients with humoral immunodeficiency and excluded secondary causes of hypogammaglobulinemia who did not meet diagnostic criteria were classified as unclassified primary antibody deficiency (unPAD). Subsequently, seroconversion was assessed as the presence of anti-SARS-CoV-2-specific IgG/IgA/IgM antibodies after recovery, the patients who received anti-spike SARS-CoV-2 monoclonal antibodies were excluded. The specific antibodies were detected using chemiluminescent immunoassays (LIAISON SARS-CoV-2 S1/S2 IgG, DiaSorin, Saluggia, Italy) or enzyme-linked immunosorbent assays (Elisa SARS-CoV-2 IgG, EUROIMMUN, Lübeck, Germany; COVID-19 RBD IgG, TestLine Clinical Diagnostics Ltd., Brno, Czech Republic). All data were collected by investigating physicians using a uniform COVID-19-IEI clinical questionnaire. The patients were divided into 3 groups - 1) asymptomatic, 2) symptomatic with mild/moderate disease, 3) symptomatic with a severe disease course (requiring hospital admission). The results were compared with data from the general population, obtained from the Ministry of Health, the Czech Republic (19) and the Czech Statistical Office (20).

Statistical Analysis

Mean and standard deviation (SD) were calculated for the continuous data (age, BMI, laboratory parameters). The statistically significant differences of the means were assessed by Mann-Whitney test for unpaired data with non-normal distribution. The normality was tested using Shapiro-Wilk normality test. Proportion, risk ratio (relative risk, RR) and 95% confidence interval were calculated for attributive data (gender, prevalence, hospital admission, risk factors). The statistically significant differences of the proportions were evaluated by Chi-squared test. Statistical significance was reached when p value < 0.05. Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA)

RESULTS

Cohort Characteristics

COVID-19 infection was diagnosed in 81 patients with IEI (47 females, 34 males) including 16 cases of HAE in a multicenter cohort of 805 patients with IEI (including 182 HAE patients) followed up at 8 national referral centers in the Czech Republic,

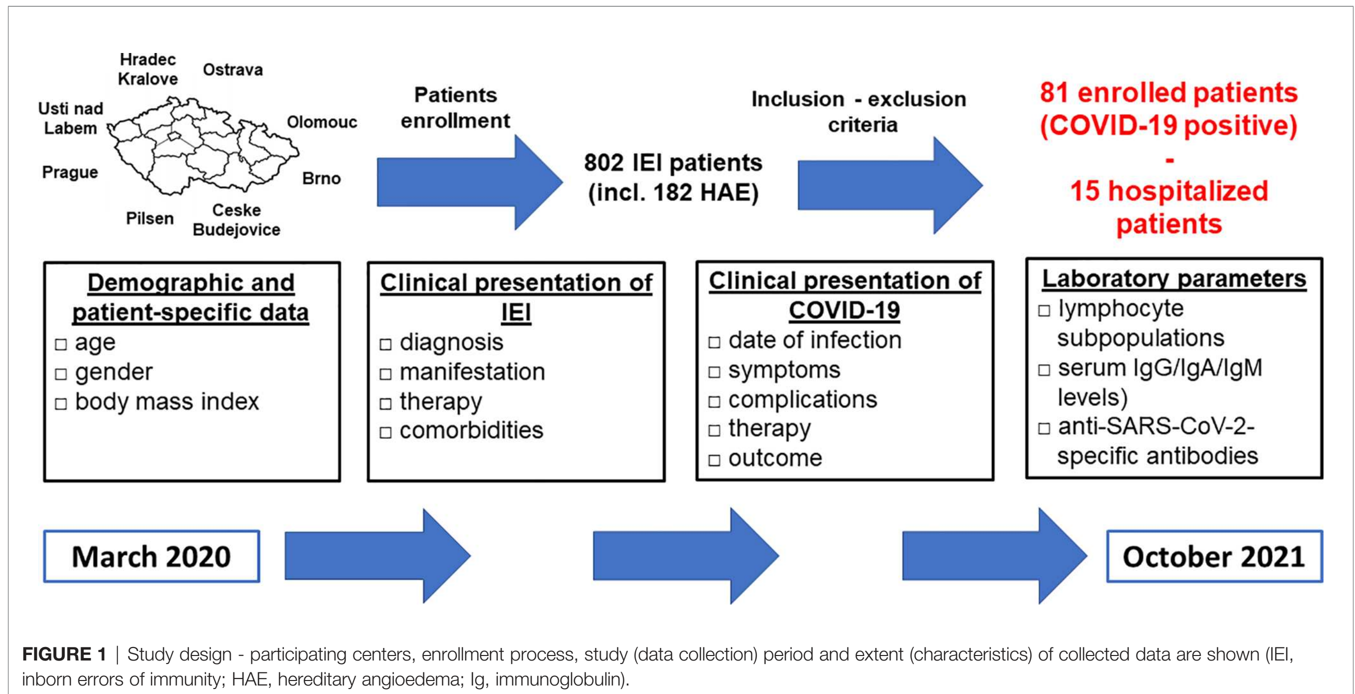

FIGURE 1 | Study design - participating centers, enrollment process, study (data collection) period and extent (characteristics) of collected data are shown (IEI, inborn errors of immunity; HAE, hereditary angioedema; Ig, immunoglobulin).

indicating an incidence of 10.1%, compared to that of 16.6% in the general population (1.77 million cases of COVID-19 infection in the 10.69 million population). Interestingly, these data suggest a significantly (p ˙ 0.0001) higher risk (RR= 1.66, 95%CI: 1.33–1.99) of COVID-19 infection in the general population. The risk of infection remained almost unchanged when HAE was excluded from the analysis (RR= 1.59, 95%CI: 1.27 – 2.00). The mean patient age was 42.41 years (± 16.07 SD) and mean BMI was 25.88 kg/m^2 (± 6.43 SD). The largest proportion of infection among patients with IEI (COVID-IEI) was observed in common variable immunodeficiency (CVID: 58%, n = 47/81), followed by that in HAE (19.8%, n= 16/81), unPAD (5%, n = 4/81), activated PI3K delta syndrome (APDS: 3.7%, n = 3/81), and Good´s syndrome (GS: 2.5%, n = 2/81). Other IEI were represented by only one patient, including late-onset combined immunodeficiency (LOCID), hyper IgE syndrome (HIES), Kabuki syndrome (KS), STAT-1 gain-of function chronic mucocutaneous candidosis (CMC), Wiskott-Aldrich syndrome (WAS), X-linked agammaglobulinemia (XLA), X-linked lymphoproliferative syndrome type 1 and type 2 (XLP-1, 2), and X-linked hyper IgM syndrome (**Figure 2**). The COVID-IEI cohort included 47 (58.5%) females and 34 males (41.5%). The most common comorbidities and IEI-related complications reported in COVID-IEI patients were chronic lung (29.6%, n = 24/81) and cardiovascular diseases (23.5%, n = 19/81), followed by enteropathy (13.6%, n =11/81), obesity (13.6%, n = 11/81), hepatopathy (8.6%, n = 7/81), hematologic malignancy (8.6%, n = 7/81), non-malignant lymphoproliferation (7.4%, n = 6/81), and serious autoimmunity (7.4%, n = 6/81) (complete list in **Figure 3**). IRT was indicated in 75.3% of patients (n = 61/81) including all CVID and APDS patients among others. Intravenous (10% IgG solutions), subcutaneous (10%, 16.5%, 20% resp. IgG solutions) and facilitated subcutaneous remedies (10% IgG solutions) were used.

Nineteen patients (23.4%) were using immunosuppressive therapy prior to COVID-19 infection. Glucocorticosteroids (GC: 73.7%, n = 14/19), direct PI3K inhibitors (15.8%, n = 3/19), and rituximab (10.5%, n = 2/19, in one case as a component of combined chemotherapy along with cyclophosphamide, doxorubicin, and prednisone) were the most frequent immunomodulatory drugs.

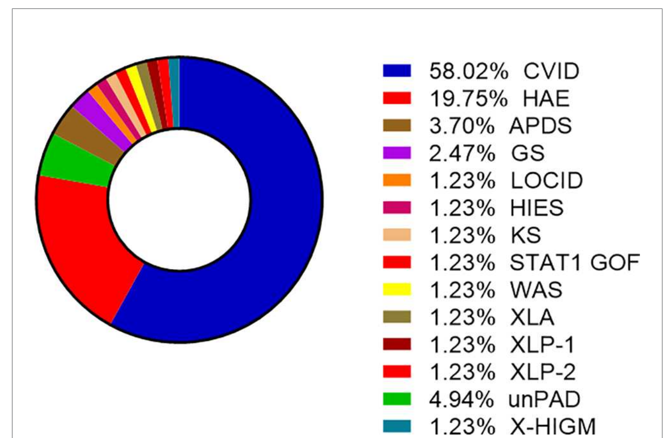

FIGURE 2 | Proportion of enrolled patients (n= 81) according to the type of Inborn errors of immunity (IEI), other IEI include GS, LOCID, HIES, KS, STAT-1 GOF, WAS, XLA, XLP1/2 (CVID, common variable immunodeficiency; HAE, hereditary angioedema; APDS, activated phosphoinositide 3-kinase δ syndrome; GS, Good´s syndrome; LOCID, late-onset combined immunodeficiency; HIES, hyper IgE syndrome; KS, Kabuki syndrome; CMC, STAT-1 gain-of-function chronic mucocutaneous candidiasis; WAS, Wiskott-Aldrich syndrome; XLA, X-linked agammaglobulinemia; XLP-1/2, X-linked lymphoproliferative syndrome type 1/2; unPAD, unclassified hypogammaglobulinemia; X-linked hyper IgM syndrome).

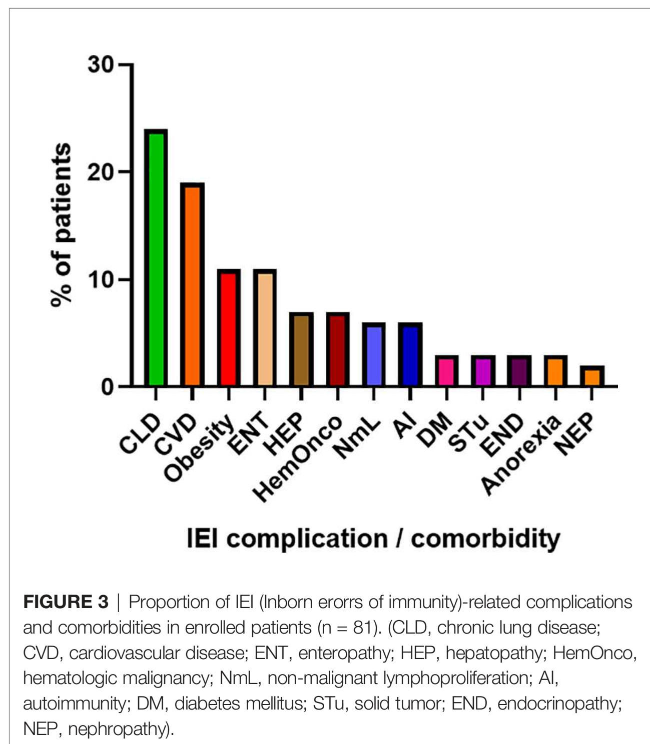

FIGURE 3 | Proportion of IEI (Inborn erorrs of immunity)-related complications and comorbidities in enrolled patients (n = 81). (CLD, chronic lung disease; CVD, cardiovascular disease; ENT, enteropathy; HEP, hepatopathy; HemOnco, hematologic malignancy; NmL, non-malignant lymphoproliferation; AI, autoimmunity; DM, diabetes mellitus; STu, solid tumor; END, endocrinopathy; NEP, nephropathy).

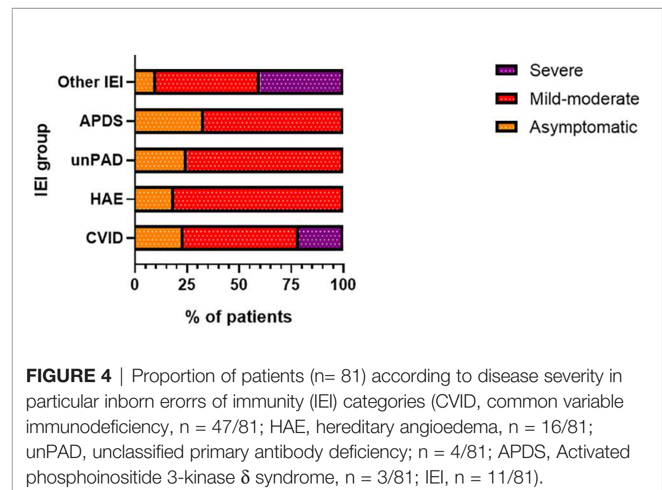

FIGURE 4 | Proportion of patients (n= 81) according to disease severity in particular inborn erorrs of immunity (IEI) categories (CVID, common variable immunodeficiency, n = 47/81; HAE, hereditary angioedema, n = 16/81; unPAD, unclassified primary antibody deficiency; n = 4/81; APDS, Activated phosphoinositide 3-kinase δ syndrome, n = 3/81; IEI, n = 11/81).

Asymptomatic infection was observed in 21% (n = 17/81) of the patients. The main manifestations in symptomatic patients included fever (45.3%, n = 29/64), dyspnea (31.3%, n = 20/64), cough (26.6%, n = 17/64), flu-like symptoms (26.6%, n = 17/64), loss of smell/taste (26.6%, n = 17/64), upper respiratory tract symptoms (25%, n = 16/64), fatigue (23.4%, n = 15/64), and headache (9.4%, n = 6/64). All the reported symptoms are summarized in **Figure 4**. Pneumonia developed in 19 patients, in whom diagnosis was confirmed by imaging methods. Ischemic (heart attack) or thromboembolic (pulmonary vein embolism) events occurred in two patients. Two patients (APDS and XLP-2) were infected by SARS-CoV-2 despite appropriate vaccination. Both of them manifested with the flu-like and upper respiratory tract infection symptoms. None of them required hospital admission. The anti-spike SARS-CoV-2-specific monoclonal antibody, bamlanivimab, was used in 1 hospitalized patient whereas convalescent plasma was indicated in 4 patients. Two other applications of bamlanivimab and casirivimab/imdevimab were administered in an outpatient regimen. None of these patients required hospital admission. Generally, administration was not associated with any adverse event. None of the IEI patients was fully vaccinated (completed vaccination schedule) prior to infection due to limited availability of the vaccines at the beginning of the COVID-19 pandemic. Only a single patients received 1 dose from 2-dose vaccine scheme. The remaining patients were vaccinated after infection.

Risk of Severe Course for COVID-19 and Hospital Admission

Overall, 15 patients with IEI required hospital admission, corresponding to 18.5%. In contrast to the risk for infection, patients with IEI showed a significantly (p = 0.0011) increased risk (RR) for hospital admission, which was 2.3-times higher (95%CI: 1.44 - 3.53). The risk for hospital admission further increased when HAE patients were excluded (RR= 2.91, 95%CI: 1.83 – 4.37, p < 0.0001). In the general Czech population, the total of 139 600 COVID-19 positive individuals needed hospital admission by the time of the study period, equaling 7.9%. In our study, the highest number of hospital admissions was reported in amongst the CVID patients. In total, 10 out of 47 patients with CVID (21.3%) were hospitalized. This corresponds to 2.68 RR (95%CI: 1.51 – 4.4). Other patients requiring hospital admission were diagnosed with LOCID, GS, KS, HIES, and WAS (one patient in each group). No hospitalization was documented in the remaining groups (**Figure 5**). The major symptom leading to hospital admission was dyspnea (66.7%, n = 10/15). Two patients experienced newly diagnosed atrial fibrillation and heart attack. Eight patients (53.3%) required intensive care, the remaining patients were hospitalized at standard care departments. High-flow nasal oxygen was needed in 5 patients (33.3%) and artificial ventilation was required in 2 patients (13.3%). Other medications (mono- or combined therapy) included the use of antibiotics (46.7%, n = 7/15), GC (40%, n = 6/15), and antivirotics such as remdesivir and/or favipiravir (33.3%, n = 5/15). Two patients with other significant comorbidities died due to severe complications. This corresponds to a mortality rate of 2.4% in the COVID-IEI cohort and 13.3% among hospitalized patients. In contrast, 1.7% mortality was reported in the general population (30.90 thousand COVID-19 associated deaths out of 1.766 million infected individuals).

Risk Factors Associated With Hospital Admission

We did not find statistically significant differences in age (45.33 ± 15.32 SD *vs.* 41.36 years ± 16.57 SD), gender ratio, or BMI (body mass Index: 27.17 ± 8.52 *vs.* 25.59 kg/m^2 ± 5.90 SD) value between hospitalized and non-hospitalized patients. However, we found significant differences in the absolute number of total lymphocyte counts (1.18 E9/L ± 0.84 SD *vs.* 1.75 E9/L ± 0.84 SD, p = 0.016), T cells (CD3+) specifically (0.92 E9/L ± 0.58 SD *vs.*

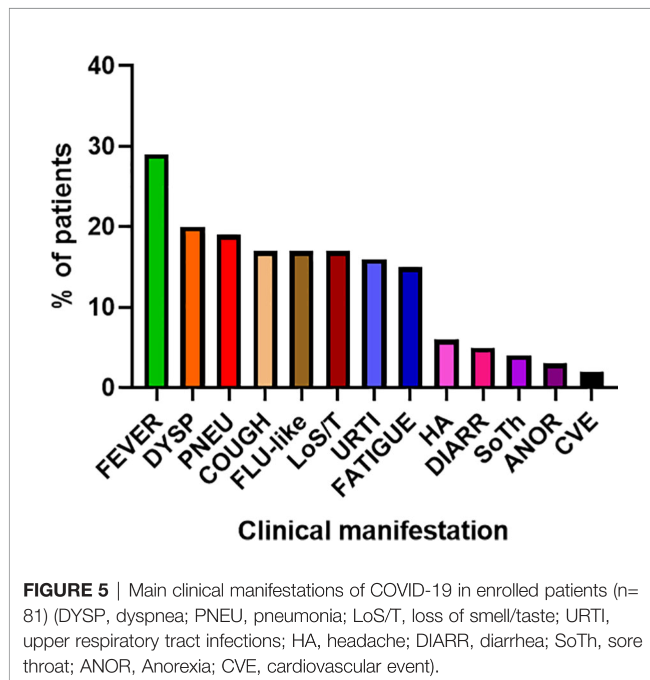

FIGURE 5 | Main clinical manifestations of COVID-19 in enrolled patients (n= 81) (DYSP, dyspnea; PNEU, pneumonia; LoS/T, loss of smell/taste; URTI, upper respiratory tract infections; HA, headache; DIARR, diarrhea; SoTh, sore throat; ANOR, Anorexia; CVE, cardiovascular event).

1.40 E9/L ± 1.0 SD, p = 0.03). The differences in the CD4+ and CD8+ T cells were not statistically significant. Hospitalized individuals also had a significantly lower number of B cells (CD19+: 0.068 E9/L ± 0.06 SD *vs.* 0.17 E9/L ± 0.15 SD, p = 0.004), NK cells (CD3- CD56+: 0.12 E9/L ± 0.16 SD *vs.* 0.2 E9/L ± 0.17 SD, p = 0.01) along with serum levels of IgA (0.3 g/L ± 0.64 SD *vs.* 0.76 g/L ± 1.14 SD, p = 0.04) and IgM (0.15 g/L ± 0.19 SD *vs.* 0.50 g/L ± 0.66 SD, p = 0.0095) in contrast to IgG values, which were comparable between patient groups (**Table 1**). When looking separetely at CVID sub-cohort, representing the largest IEI group, we found significant differences in B cells counts only (0.05 E9/L ± 0.06 SD vs. 0.17 E9/L ± 0.13 SD). Analyzing the CVID patients receiving IRT, we did not revealed significant differences between serum IgG trough levels of the hospitalized (6.65 IgG ± 2.1 SD) and non-hospitalized CVID individuals (6.87 IgG ± 2.5 SD). Regarding comorbidities and IEI-related complications, chronic lung disease (53.3%, n = 8/15) and cardiovascular diseases (33.3%, n = 5/15) were the most prevalent. Further, more than half of the patients (60%, n = 9/15) had at least two comorbidities/ complications (**Table 2**).

The Seroconversion Rate Upon Recovery

Seroconversion was assessed in 59.3% (n = 48/81) of the enrolled patients after 2.67 months (mean time, range: 1 – 7 months) from recovery. The patients who were exposed to anti-spike SARS-CoV-2 monoclonal antibodies and/or convalescent plasma were excluded from the seroconversion analysis. Anti-SARS-CoV-2 specific antibodies were detected in 68.8% (n= 33/48) of the recovered patients, including 7/9 (77.8%) asymptomatic patients, 21/31 (67.7%) outpatients, and 5/8 (62.5%) hospitalized patients. Surprisingly, a high seroconversion rate of 76% (n = 19/25) was also observed in patients with CVID patients who were expected to show heavily impaired production of antigen-specific antibodies.

DISCUSSION

IEI are rare diseases that predispose patients to increased risk of infections and their severe course. Therefore, patients with IEI were also regarded as a risk population for severe COVID-19. However, data from different studies indicated ambiguous conclusions. A multicenter study from Israel showed a lower incidence of COVID-19 infection among patients with IEI (1.2%) compared with that in the general population (2.5%). These data are consistent with our results, showing a 1.66-fold higher risk of COVID-19 infection in the general population of the Czech Republic. Here, the reported incidence was 10.1% and 16.6% in the IEI cohort and the general population, respectively. The risk of infection remained almost unchanged when HAE was excluded from the analysis. Nevertheless, these results may be biased due to the cohort size, thus they should be interpreted carefully. Several factors may affect the prevalence, such as tighter adherence of IEI patients to preventive measures, including stricter infection-avoidance behavior or early vaccination, compared to the general population. However, the authors reported a high number of asymptomatic patients (n = 6/20) and a only single case of pneumonia (13), which was in contrast to the United Kingdom Primary Immunodeficiency Network registry-based study. In this study, we observed 20% (n = 12/60) infection-fatality ratio and a 37.5% inpatient mortality rate. A higher age and comorbidities such as chronic lung disease, cardiovascular disease, and diabetes were clinically important risk factors. Poor outcome was also associated with low lymphocyte count (14). Similarly, we observed a 2.3-fold increased risk ratio for hospital admission, which was the highest rate for patients with CVID patients (21.3%). Hospital admission was also associated with higher mortality (13.3% *vs.* 2.4% in the

TABLE 1 | Laboratory parameters associated with risk of hospital admission.

Parameter	Hosp+	Hosp-	p-value
Lymphocyte count (10E9/L, ± SD)	1.18 ± 0.84	1.75 ± 0.84	0.016
T cell count (10E9/L, ± SD)	0.92 ± 0.58	1.40 ± 1.0	0.03
B cell count (10E9/L, ± SD)	0.06 ± 0.06	0.17 ± 0.15	0.004
NK cell count (10E9/L, ± SD)	0.12 ± 0.16	0.2 ± 0.17	0.01
Serum IgA (g/L, ± SD)	0.3 ± 0.64	0.76 ± 1.1	0.04
Serum IgM level (g/L, ± SD)	0.15 ± 0.19	0.51 ± 0.66	0.01

(SD, standard deviation; Hosp+, hospitalized patients; Hosp-, non-hospitalized patients).

TABLE 2 | Characteristics of hospitalized patients.

Diagnosis	Gender	Age (yrs.)	BMI (kg/ m^2)	Comorbidity	Immuno-suppressants	IRT	Treatment level	Oxygen therapy	Other treatment	Outcome
CVID	M	51	35.1	AH, DM, obesity	0	Yes	ICU	HFNO	REM	resolved
CVID	F	25	22.9	CLD	GC, RTX	Yes	SC	N/R	GC, CP, SMA	resolved
CVID	F	59	19.0	Absent	0	Yes	SC	N/R	Symptomatic	resolved
CVID	F	32	49	Obesity	0	Yes	SC	NC	GC, ATB, REM, CP	resolved
CVID	F	50	34.2	AH, CLD, obesity	GC	Yes	ICU	AV	ATB	died
CVID	M	27	12.9	AH, CLD, anorexia	0	Yes	SC	NC	ATB	died
CVID	F	65	22.6	CLD, HEP, THRO	0	Yes	SC	N/R	GC	resolved
CVID	F	40	24.4	HEP, CED, LYMPH	GC	Yes	ICU	HFNO	GC, ATB	resolved
CVID	F	46	30.1	CLD, LYMPH, obesity	GC	Yes	SC	NC	GC, ATB, REM	resolved
CVID	F	69	26	CLD	0	Yes	ICU	HFNO	GC, ATB	resolved
LOCID	F	34	24.3	CLD, SPLE	0	Yes	ICU	HFNO	ATB, REM, FAV, CP	resolved
GS	M	68	25.8	AH, DM, PCa	0	Yes	ICU	HFNO	CP	resolved
KS	M	23	32.9	NEPH, HPIT, CHD, AIHA	0	Yes	ICU	AV	Symptomatic	resolved
HIES	F	46	28.7	CLD	0	Yes	ICU	NC	REM	resolved
WAS	M	45	19.8	NEPH	GC	Yes	SC	N/R	Symptomatic	resolved

(IRT, immunoglobulin replacement therapy; yrs, years; BMI, body mass index; m, male; f, female; CVID, common variable immunodeficiency; LOCID, late-onset combined immunodeficiency; GS, Good´s syndrome; KS, Kabuki syndrome; HIES, hyper IgE syndrome; WAS, Wiskott-Aldrich syndrome; AH, arterial hypertension; DM, diabetes mellitus; CLD, chronic lung disease; HEP, hepatopathy; THRO, thrombocytopenia; CED, celiac disease; LYMPH, lymphadenopathy; SPLE, splenomegaly; PCa, prostate cancer; NEPH, nephropathy; HPIT, hypopituitarism; CHD, congenital heart defect; AIHA, autoimmune hemolytic anemia; SC, standard care; ICU, intensive care unit; N/R, not required; NC, nasal cannula; HFNO, high-flow nasal oxygen; AV, artificial ventilation; ATB, antibiotics; REM, remdesivir; FAV, favipiravir; GC, glucocorticoids; CP, convalescent plasma; SMA, anti-spike SARS-CoV-2 specific monoclonal antibodies; RTX, rituximab).

entire IEI cohort). However, we could not prove the impact of either age or BMI. Apart from low lymphocyte counts, we observed significant differences between hospitalized and non-hospitalized patients with IEI in the number of T, B, and NK cells along with reduced serum levels of IgA and IgM. Nevertheless, we found significant differences in B cells counts only when looking at CVID sub-cohort separately. Surprisingly, we did not observe any differences in serum IgG levels (measured as trough levels in patients on regular immunoglobulin substitution). A high mortality rate was also observed in a robust international multicenter retrospective web-based survey, reaching 10% (n = 9/94) but all adult patients had other pre-existing comorbidities such as a higher age, chronic lung, cardiovascular, or chronic renal disease and others. Hospital admission was required in 63% of the patients (n = 59–94). An asymptomatic course of SARS-CoV-2 infection was reported in only 11% of the patients with IEI (16). Based on these results, we assumed an important role of IEI itself, which is also supported by other studies (15, 17, 21) and meta-analyses (22). Additionally, anti-spike SARS-Cov2 monoclonal antibodies and convalescent plasma seem to be safe and possibly effective treatment options for patients with IEI. However, these observations need to be verified in clinical trials. More than half of the patients in each patient group (asymptomatic, outpatient, hospitalized) also exhibited humoral seroconversion after recovery. A high seroconversion rate was also observed in patients with CVID who show impaired production of antigen-specific antibodies. High seroconversion rate was later reported in IEI patients, including severe antibody deficiencies, following SARS-CoV-2 vaccination and may may provide safe and effective solution for IEI patients. However, the immunogenicity varies between the specific type of IEI (23).

With regard to patients with HAE, they showed neither SARS-CoV-2-associated hospital admission nor death despite previously proposed role of the bradykinin overproduction in the kallikrein-kinin cascade as a possible co-mediator of COVID-19-related pulmonary complications (24–26). However, three cohorts of patients with HAE who contracted COVID-19 did not confirm this hypothesis (17, 27). Thus, our observations support these findings. This could be also explained by a small number of risk factors for severe COVID-19 disease.

There are several limitations of this study. These primarily include the retrospective study design, survey-based data collection, lower number of enrolled patients, and the heterogeneity of the cohort. Therefore, the results should be interpreted carefully in the context of other studies. However, the strengths of this study include its nation-wide multicenter design including national referral centers for the management of patients with IEI. Our data also represent a substantial sample of Central European population with IEI.

CONCLUSION

Our study presents the results from a multicenter nationwide retrospective survey-based study including 81 patients with IEI (including 16 with HAE) in whom SARS-CoV-2 infection was confirmed. We revealed a lower risk of COVID-19 infection but a higher risk ratio for hospital admission and mortality compared to that in the general population. A severe course of COVID-19 was reported mainly in patients with significant comorbidities. However, we also assume that immune system dysregulation contributed to these outcomes based on the

findings of lymphopenia and hypogammaglobulinemia as the risk factors for severe course of the disease. The use of anti- spike SARS-Cov2 monoclonal antibodies and convalescent plasma provide safe and effective therapeutic options for patients with IEI. In line with the previous studies, we did not observe an increased risk of COVID-19 in patients with HAE despite the expected role of bradykinin overproduction in COVID-19 associated respiratory complications. COVID-19 infection also resulted in a high seroconversion rate after recovery even in antibody-deficient patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the University Hospital in Hradec Kralove, the Czech Republic. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

TM and MS (main authors) made substantial contributions to the conception and design of the study, analysis and interpretation of data, drafted the manuscript. JS, MB, JV, ZC, JL, RH, JN, IM, JH, DJ, BH, VN, and IK (co-authors) made substantial contributions to the acquisition of data, analysis and interpretation. AS and PK (senior authors) made substantial contributions to the conception and design of the study, reviewed the manuscript critically for important intellectual content, gave final approval of the version to be published.

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Original Article

Clinical Outcomes, Immunogenicity, and Safety of BNT162b2 Vaccine in Primary Antibody Deficiency

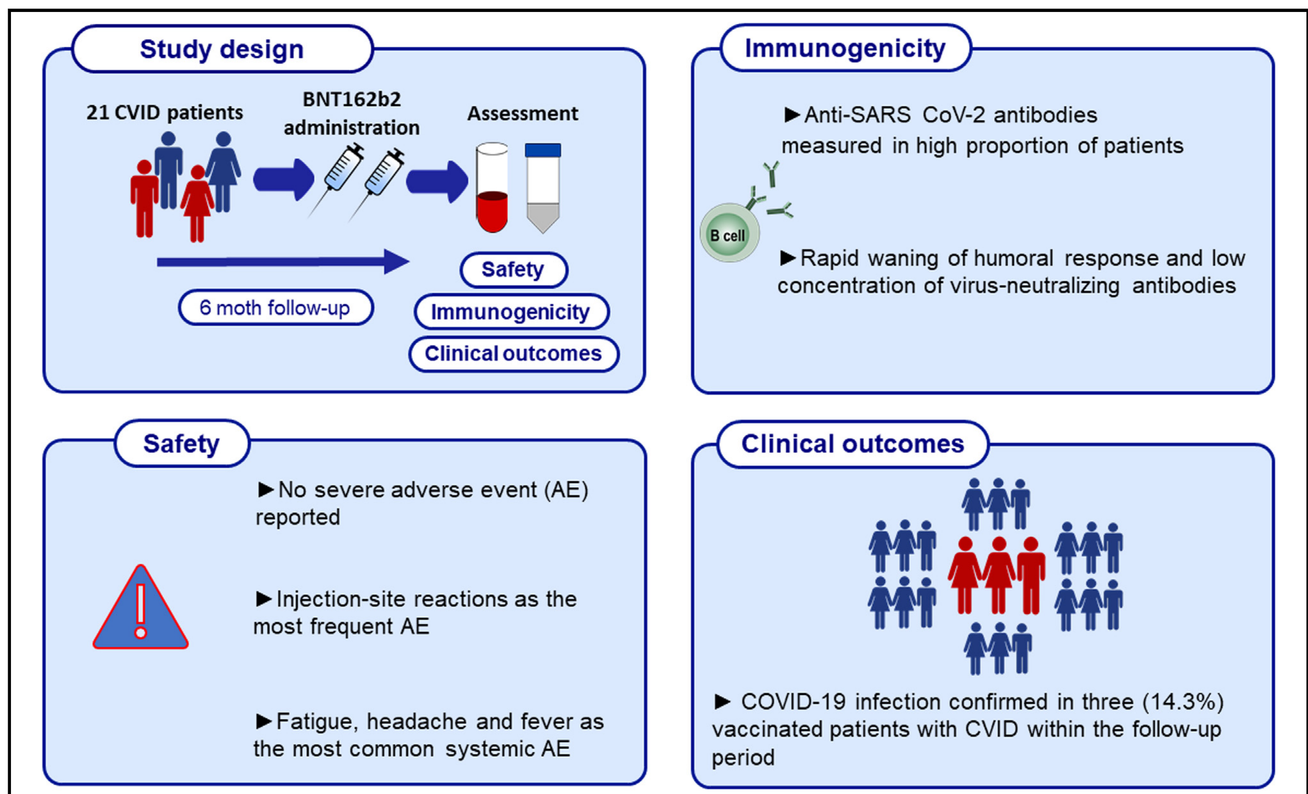
Tomas Milota, PhD[a,*], Jitka Smetanova, MSc[a,*], Aneta Skotnicova, MSc[b,*], Michal Rataj, MSc[a], Jan Lastovicka, MSc[a], Hana Zelena, PhD[c], Zuzana Parackova, PhD[a], Martina Fejtkova, PhD[b], Veronika Kanderova, PhD[b], Eva Fronkova, PhD[b], Katerina Rejlova, PhD[b], Anna Sediva, PhD[a], and Tomas Kalina, PhD[b] *Prague and Ostrava, Czech Republic*

What is already known about this topic? Primary antibody deficiency, such as common variable immunodeficiency, may lead to an impaired postvaccination response to protein and/or polysaccharide antigens, but the specific T-cell immune response may be preserved.

What does this article add to our knowledge? Anti-SARS-CoV-2 mRNA vaccine induces a measurable humoral response in a high proportion of patients with antibody deficiency, but it is limited by the low titer of virus-neutralizing antibodies, rapid waning of anti−receptor-binding domain SARS-CoV-2−specific antibodies, and impaired specific T-cell immune response.

How does this study impact current management guidelines? Our study suggests reasonable booster vaccination in shorter intervals than recommended for the general population.

VISUAL SUMMARY



Abbreviations used
AE- Adverse event
APRIL- A proliferation-inducing ligand
BAFF- B-cell-activating factor
CVID- Common variable immunodeficiency
HC- Healthy control
IB- Immunoblot
IEI- Inborn error of immunity
IRT- Immunoglobulin replacement therapy
RBD- Receptor-binding domain

BACKGROUND: Common variable immunodeficiency (CVID) is characterized by an impaired postvaccination response, high susceptibility to respiratory tract infections, and a broad spectrum of noninfectious complications. Thus, patients with CVID may be at high risk for COVID-19, and vaccination's role in prevention is questionable.

OBJECTIVE: We evaluated the clinical outcomes, safety, and dynamics of humoral and T-cell immune responses induced by the mRNA vaccine BNT162b2 in CVID.

METHODS: This prospective observational cohort study focused on the clinical outcomes (proportion of infected patients and disease severity), safety (incidences of adverse events and changes in laboratory parameters), and dynamics of humoral (specific postvaccination and virus-neutralizing antibody assessment) and T-cell immune responses (anti-SARS-CoV-2−specific T-cell detection) in 21 patients with CVID after a two-dose administration of BNT162b2. The patients were observed for 6 months.

RESULTS: Humoral response was observed in 52% of patients (11 of 21) at month 1 after vaccination but continuously decreased to 33.3% at month 6 (five of 15). Nevertheless, they had a remarkably lower anti-SARS-CoV-2 neutralizing antibody titer compared with healthy controls. The T-cell response was measurable in 46% of patients with CVID (six of 13) at month 1 and persisted over the study period. Mild infection occurred in three patients within the follow-up period (14.3%). The vaccine also exhibited a favorable safety profile.

CONCLUSIONS: The BNT162b2 vaccine elicited a measurable antibody response in a high proportion of patients, but it was limited by low titer of virus-neutralizing antibodies and rapid waning of anti−receptor-binding domain SARS-CoV-2−specific antibodies. T-cell response was detected in one-third of patients and remained stable within the follow-up period. Vaccination has favorable safety and clinical-related outcomes in preventing severe COVID-19. **© 2022 American Academy of Allergy, Asthma & Immunology** **(J Allergy Clin Immunol Pract 2023;11:306-14)**

Key words: *Common variable immunodeficiency; COVID-19; mRNA vaccine; Post-vaccination response; Immunogenicity; Safety*

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INTRODUCTION

The COVID-19 outbreak has affected over 500 million individuals and caused over 6 million deaths worldwide since its emergence in 2019.[1] Severe COVID-19 infections with poorer outcomes have been reported in immunocompromised patients, such as those with inborn errors of immunity (IEIs).[2,3] Conversely, Marcus et al[4] reported that neither more severe disease nor excess hospital admissions existed in a cohort of patients with IEIs and implied a high awareness level, extra precautions, and even self-isolation as possible explanations. However, preventive measures provide only short-term protection; therefore, vaccination may offer a long-term effective and safe solution.

Currently, two mRNA vaccines (BNT162b2, also known as Comirnaty [Pfizer {Manin, Germany}/BioNTech {Puurs, Belgium}]; and mRNA-173, also known as Spikevax [Moderna {Madrid, Spain}]), one recombinant-subunit vaccine (Nuvaxovid [Novavax {Jevany, the Czech Republic}]), and two viral-vector vaccines (AZD1222, also known as Vaxzevria [AstraZeneca]; and Ad26.COV2.S, also known as COVID-19 Vaccine [Janssen/Johnson & Johnson {Beerse, Belgium}]) have been approved by the European Medicines Agency for COVID-19 prevention. The vaccines induce high levels of immunogenicity and efficacy, ranging from 66.9% to 90.4%, and demonstrate a favorable safety profile, with a dominant prevalence of local reactions in the general population.[5-9] However, severe adverse events (AEs), such as thromboembolic events or pericarditis and myocarditis, have been reported.[10,11] In addition, evidence regarding their use in specific patient populations is limited, particularly those with heavily impaired antibody production, disturbed cellular immunity, and/or immune system dysregulation.

Common variable immunodeficiency (CVID) is the most prevalent IEI, characterized by impaired vaccination-induced specific antibody production,[12,13] immune dysregulation, and partially impaired T-cell phenotype and function.[14] Therefore, this study investigated the immunogenicity, safety, and clinical outcomes of the mRNA vaccine BNT162b2 in a cohort of patients with CVID.

MATERIALS AND METHODS

Ethics statement

This study was approved by the Motol University Hospital Ethics Committee (No. EK-753.1.3/21, issued June 10, 2020) and conducted according to the guidelines of the Declaration of Helsinki. We obtained informed consent from all patients.

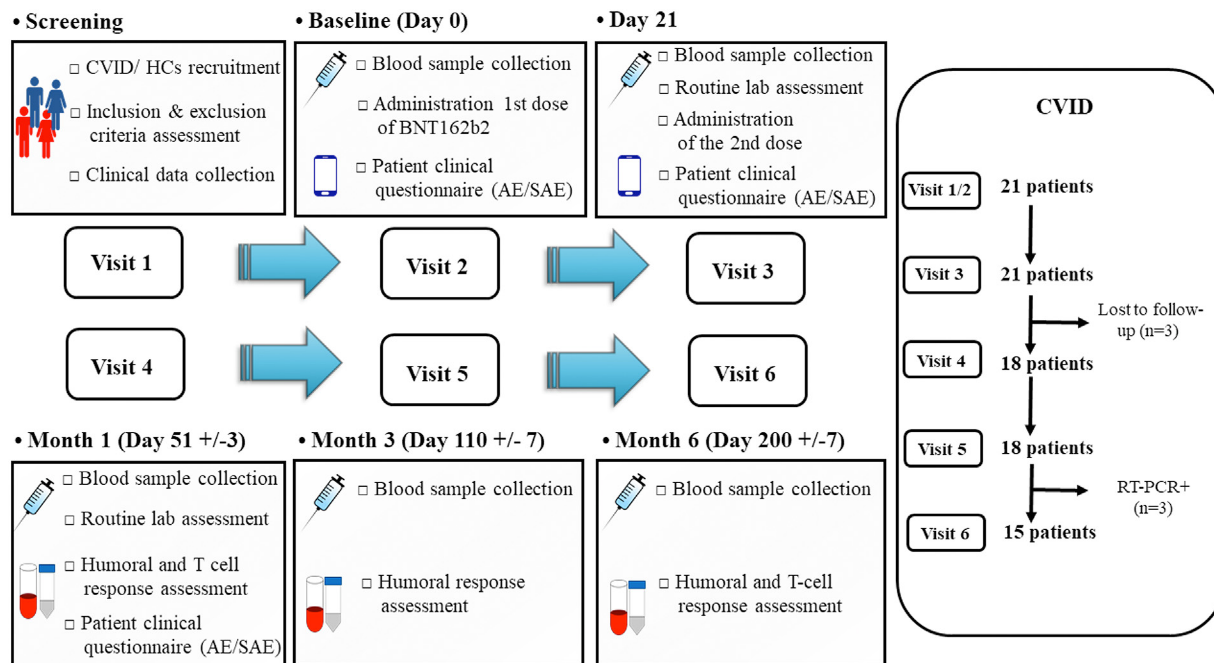

FIGURE 1. Study time line and profile of study participants. *AE*, adverse events; *CVID*, common variable immunodeficiency; *HCs*, healthy controls; *SAE*, severe adverse events.

Study design

This prospective observational study focused on the immunogenicity, safety, and efficacy of the mRNA vaccine BNT162b2. The study followed Strengthening the Reporting of Observational Studies in Epidemiology recommendations.[15] Patients received two standard doses (0.3 mL/30 µg mRNA) intramuscularly within the recommended 3-week interval and were observed for 6 months after vaccination. The follow-up period was divided into six consecutive visits. Figure 1 illustrates the study design. This study was conducted from March to November 2021.

Study population

This study included 21 adult patients with CVID meeting European Society for Immunodeficiency/International Consensus Document diagnostic criteria.[12,13] Vaccination was indicated by the attending immunologist and performed by specialized vaccination centers. Patients with pre-vaccination RT-PCR—confirmed SARS-CoV-2 infection or severe noninfectious complications were excluded. Their results were compared with those of 23 corresponding sex- and age-matched healthy controls (HCs).

B- and T-cell immunophenotyping

B-cell subpopulations (including CD21^{low}, naive, transitional, marginal zone—like, class-switched cells, and plasmablasts) and T-cell subpopulations (recent thymic emigrants, naive, central memory, effector memory, effector memory expressing CD45RA, and activated T cells) were analyzed using antibody-fluorochrome conjugates for fluorescence-activated cell sorting.

Humoral response assessment

We used ELISA COVID-19 receptor-binding domain (RBD) IgG kits (TestLine Clinical Diagnostics, Brno, Czech Republic) to measure anti-SARS-CoV-2 IgG titers (positive cutoff value > 18 U/mL). We measured anti-nucleocapsid, anti-Spike 2, anti-protein E, anti-angiotensin-converting enzyme 2, and RBD antibodies (positive cutoff value > 180 U/mL) using the immunoblot (IB) assay (Microblot-Array COVID-19 IgG, TestLine Clinical Diagnostics).

The virus neutralization test was performed according to a previously published protocol[16] using the SARS-CoV-2 strain extracted from a clinical sample (hCoV-19/Czech Republic/NRL_9640/2020|EPI_ISL_626593) and CV-1 cells (African green monkey kidney fibroblasts). Serum samples were diluted to a final serum concentration of 1:10-1:2560. Thereafter, uninfected cells were stained with neutral red dye. Virus neutralization test results were expressed in the form of a virus-neutralization titer, representing an inverted value of the highest sample dilution neutralizing the virus's cytopathic effect by greater than 50%. We determined positivity using a titer of 20 or greater.

Responders were defined as individuals in whom anti-RBD SARS-CoV-2—specific antibodies exceeding the positive cutoff level were detected by IB and ELISA at month 1. The humoral response was also determined by measuring serum B-cell—activating factor (BAFF, R&D Systems, Minneapolis, Minn), a proliferation-inducing ligand (APRIL, Abcam, Cambridge, United Kingdom), and IFN-alfa (Thermo Fisher Scientific [Waltham, MA]) levels using ELISA.

We also tested the presence of anti-RBD SARS-CoV-2—specific antibody in immunoglobulin replacement therapy (IRT) solutions used for immunoglobulin substitution in enrolled patients with CVID (Kiovig and HyQvia, Takeda Manufacturing, Vienna, Austria, with expiration dates of June 2022 to December 2022; and Hizentra, CSL Behring, Marburg, Germany, with expiration dates of September 2022 to January 2023). All solutions were diluted with a 5% BSA (lyophilized IgG-free powder, Merck KGaA, Darmstadt,

Germany) solution to a 1% concentration, corresponding to the IgG concentration in human plasma.

T-cell response

The T-cell response was assessed as previously described.[17,18] Briefly, PBMCs of patients with CVID and HCs were cryopreserved. Upon thawing and overnight rest, they were stimulated with 4 μL of BD Fast Immune CD28/CD49d (BD Biosciences, San Jose, Calif) and PepMix SARS-CoV-2 S-RBD/ NCAP (JPT Peptide Technologies, Berlin, Germany) or anti-human CD3 low endotoxin as a positive control. After incubation, PBMCs were stained with antibody-fluorochrome conjugates for fluorescence-activated cell sorting. Subsequently, cells were stained for viability with LIVE/ DEAD Violet Viability Dye (Invitrogen, Waltham, Mass). Finally, samples were measured on a BD LSR II flow cytometer to detect intracellular production of the cytokines IL-2, IFN-gamma, and TNF-α in CD4$^+$ T cells. CD4$^+$ T cells were gated as lymphocytes (forward scatter low/side scatter low), CD3$^+$ and CD4$^+$ whereas they were negative for ViViD, CD20, and CD14. We analyzed data using FlowJo software (version 10.6.1, BD Biosciences).

Only patients who responded to nonspecific anti-CD3 stimulation were considered for further analysis of antigen-specific responses. A positive response required greater than a 1.5-fold increase above the nonstimulated controls and detection of greater than 20 responding cells, as previously described for sensitive cytomegalovirus-specific T-cell detection.[19] All combinations of IL-2, IFN-gamma, and TNF-α production upon stimulation were analyzed for the total CD4$^+$ T-cell response.

Safety assessment

Adverse events were reported using the Patient Clinical Questionnaire, focusing on local (injection-site) and systemic (fever, headache, myalgia, and arthralgia) reactions and emergency medication (eg, analgesic or antipyretic drugs). Pain intensity was self-assessed by patients on a 100-point patient global assessment visual analog scale. Severe AEs were defined as acute conditions requiring hospital admission or urgent medical intervention after vaccination. Furthermore, hematologic, immunologic, and biochemical parameters were measured using routine and standardized laboratory tests for safety assessment. All assessed parameters are listed in Table E1 (in this article's Online Repository at www.jaci-inpractice.org).

Clinical outcomes

The primary outcome of vaccination was defined as the proportion of patients with CVID in whom SARS-CoV-2 infection was not confirmed by RT-PCR. Testing using RT-PCR was indicated in cases in which respiratory tract infection symptoms were present or after risk contact with a SARS-CoV-2–positive person. Secondary efficacy was defined as COVID-19 severity in RT-PCR–positive patients with CVID, who were divided into four groups: asymptomatic, mild (symptomatic treatment only), moderate (antiviral drugs and/or anti-SARS-CoV-2 monoclonal antibodies), and severe (hospital admission).

Statistical analysis

We calculated means and SDs for continuous data (age, body mass index, and laboratory parameters). Statistically significant differences in means were assessed using Mann-Whitney and Wilcoxon tests for nonnormally distributed unpaired and paired data, respectively. Normality was tested using Shapiro-Wilk normality test. Proportions were calculated for attributive data, and statistically significant differences in proportions were evaluated using Fisher exact test. We determined correlations using Spearman's rank correlation coefficient (r). Statistical significance was set at P less than 0.5. Statistical analyses were performed using GraphPad Prism (version 8, GraphPad Software, San Diego, Calif).

RESULTS

Patient characteristics

A total of 21 patients with CVID (14 women and 7 men) were enrolled in the study, mean age 46.3 years (±9.7 years). Mean disease duration was 9.1 years (±7.49 years). All patients underwent regular IRT with intravenous immunoglobulin (23.8%; n = 5 of 21) or subcutaneous immunoglobulin (76.2%; n = 16 of 21) administration, mean dose 287.6 mg/kg per month (±69.6 mg/kg per month). Mean serum IgG trough level was 6.13 g/L (±1.59 g/L). Antibiotic prophylaxis was indicated in three patients with CVID (14.3%), including cotrimoxazole and macrolides. Noninfectious complications were observed in 16 patients (76.2%); the most prevalent complications were bronchial asthma (28.6%; n = 6 of 21), autoimmune thyroiditis (23.8%; n = 5 of 21), enteropathy (23.8%; n = 5 of 21), chronic lung disease (19%; n = 4 of 21), splenomegaly (19%; n = 4 of 21), vitiligo (19%; n = 4 of 21), immune thrombocytopenic purpura (9.5%; n = 2 of 21), lymphadenopathy (9.5%; n = 2 of 21), and sarcoid-like disease (9.5%; n = 2 of 21). Other complications were present in fewer than two patients, and six patients exhibited more than two complications. Three patients (14.3%) were receiving active immunosuppression with glucocorticosteroids at the time of vaccination and during the follow-up period for generalized lymphadenopathy with sarcoid-like disease features, mixed connective tissue disease, and granulomatous lymphocytic interstitial lung disease. Two patients (9.5%) had previously received immunosuppressive therapy that had been terminated 5 and 8 years, respectively, before vaccination. In those patients, immunosuppression was indicated for inflammatory bowel disease-like enteropathy and Burkitt lymphoma, and included sulfasalazine and GMALL (German Multicenter Study Group for Adult Acute Lymphoblastic Leukemia) combined chemotherapy. Table I lists patient characteristics.

Humoral immune response

One month after the second BNT162b2 dose, anti-RBD SARS-CoV-2–specific antibodies were detected in 52.4% of patients (n = 11 of 21) and in all individuals in the HC group (n = 23 of 23) using the IB assay (574.4 ± 454 U/mL) (Figure 2, *A*). The humoral immune response was confirmed using ELISA (Figure 2, *B*). Other anti-SARS-CoV-2–specific antibodies, such as nucleocapsid protein and anti-Spike S2, were detected in two patients; however, they neither developed symptomatic infection nor had positive RT-PCR results for SARS-CoV-2, suggesting asymptomatic infection.

The humoral response was comparable to that of the HCs (870 ± 225.0 U/mL). However, patients with CVID had significantly lower virus-neutralizing antibody titers (49.4 ± 81.5 vs 960 ± 1093 U/mL; $P < .0001$) (Figure 2, *C*), which persisted at month 3 in 44.4% of patients (n = 8 of 18). However, they were significantly lower than those of the HCs (436.3 ± 415.4 vs 900.3 ± 232.7% U/mL; $P = .0002$). In contrast, the level of neutralizing antibodies correlated with the concentration of anti-RBD SARS-CoV-2–specific antibodies (r = 0.82; $P = .0001$).

TABLE I. Patient characteristics

Characteristics	All	Responders	Nonresponders	P
Demographics				
Sex, female (%)	14/21 (66.7%)	6/10 (60%)	4/11 (36.4%)	NS
Age, y (SD)	46.3 (9.7)	39.1 (8.10)	51.36 (8.13)	**.003**
Disease duration, y (SD)	9.1 (7.49)	7.4 (8.13)	10.8 (6.78)	NS
Disease characteristics				
Noninfectious complications (patients, n [%])	16/21 (76.2%)	7/10 (70%)	9/11 (81.8%)	NS
Baseline immunosuppression (patients, n [%])	3/21 (14.3%)	1/10 (10%)	2/11 (18.2%)	NS
Previous immunosuppression (patients, n [%])	2/21 (9.5%)	0/10 (0%)	2/11 (18.2%)	NS
Treatment characteristics				
Immunoglobulin replacement therapy dose (mg/kg per mo (SD)	288 (69.6)	262 (60.52)	310.9 (71.62)	NS
Serum IgG (trough g/L [SD])	6.13 (1.59)	6.28 (1.94)	6.0 (1.27)	NS
Antibiotic prophylaxis (patients, n [%])	3/21 (14.3%)	1/10 (10%)	2/11 (18.2%)	NS
Laboratory parameters				
Serum IgA, g/L (SD)	0.1 (0.09)	0.13 (0.13)	0.08 (0.03)	NS
Serum IgM, g/L (SD)	0.19 (0.18)	0.29 (0.21)	0.1 (0.06)	.002
Lymphocytes (E9/L [SD])	1.49 (0.7)	1.52 (0.52)	1.42 (0.85)	NS
$CD4^+$ (% of $CD3^+$ cells [SD])	57.69 (7.38)	59.20 (5.36)	56.75 (8.62)	NS
$CD4^+$ (absent, E9/L cells [SD])	0.55 (0.2)	0.56 (0.15)	0.54 (0.25)	NS
$CD4^+$ naive (% of $CD4^+$ cells [SD])	15.51 (13.3)	22.44 (16.24)	11.18 (9.89)	NS
$CD4^+$ Treg (% of $CD4^+$ cells [SD])	12.27 (5.54)	15.04 (6.28)	10.54 (4.6)	NS
$CD8^+$ (% of $CD3^+$ cells [SD])	38.46 (8.17)	36.40 (6.07)	39.75 (9.41)	NS
$CD8^+$ (absent, E9/L cells [SD])	0.43 (0.26)	0.36 (0.18)	0.5 (0.33)	NS
$CD8^+$ naive (% of $CD8^+$ cells [SD])	23.47 (9.58)	26.0 (7.42)	21.89 (10.88)	NS
$CD8^+$ senescent (% of $CD8^+$ cells [SD])	49.23 (16.44)	41.0 (18.75)	54.38 (13.56)	NS
$CD19^+$ (% of lymphocytes [SD])	11.19 (6.79)	8.52 (3.06)	12.86 (8.09)	NS
$CD19^+$ (absent, E9/L cells [SD])	0.16 (0.13)	0.18 (0.11)	0.15 (0.16)	NS
$CD19^+$ transitional (% of CD19 cells [SD])	7.48 (15.94)	2.34 (1.42)	10.7 (20.10)	NS
$CD19^+$ naive (% of $CD19^+$ cells [SD])	68.46 (22.86)	56.0 (18.41)	76.25 (22.84)	NS
$CD19^+$ class-switched (% of $CD19^+$ cells [SD])	5.0 (3.2)	7.58 (3.09)	3.39 (2.09)	.009
$CD19^+$ $CD21^{low}$ (% of CD19 cells [SD])	15.0 (21.16)	11.64 (6.99)	17.1 (26.96)	NS
T-cell response				
Cellular response at month 1 (patients, n [%])	5/13 (38.5%)	3/8 (37.5%)	2/5 (40%)	NS
Cellular response at month 6 (patients, n [%])	8/12 (66.7%)	5/7 (80%)	3/5 (60%)	NS

NS, nonsignificant.
Statistically significant differences ($P \leq .05$) between responders and nonresponders are marked in bold.

Only one patient with a history of immunosuppressive therapy developed humoral and T-cell immune response. The antibody titer decreased further at 6 months and was detected in only 33.3% of patients (5 of 15), whereas it persisted in all HC subjects (n = 23 of 23). Mean anti-RBD–specific IgG level was 218.2 U/mL (±268.5 U/mL) in patients with CVID and 1056 U/mL (±360.4 U/mL) in HCs, with statistically significant differences ($P < .0001$) (Figure 2, *A*).

Responders and nonresponders did not differ according to sex, disease duration, proportion of antibiotic prophylaxis, IRT dose, or IgG trough levels; however, we observed significant differences between age and serum IgM concentration. The responder group was composed of significantly younger patients with CVID (39.1 ± 8.1 vs 51.36 ± 8.13 years; $P = .003$) who had significantly higher serum IgM levels (0.29 ± 0.21 vs 0.1 ± 0.06 g/L; $P = .002$). The humoral-response level (serum anti-RBD SARS-CoV-2–specific antibody concentration) was also negatively correlated with higher age (r = −0.61; $P = .003$), as well as serum IgM (r = 0.46; $P = .036$). The humoral response was not influenced by ongoing or previous immunosuppression or the presence of noninfectious complications. We observed no significant differences in T-cell phenotypes. However, responders had a significantly higher proportion of $CD19^+$ class-switched B cells (7.58% ± 3.09% vs 3.39% ± 2.09%; $P = .009$) (Table I). The number of $CD19^+$ class-switched B cells correlated with the concentration of anti-RBD SARS-CoV-2–specific antibodies (r = 0.58; $P = .04$). Three patients were excluded based on RT-PCR–confirmed SARS-CoV-2 infection and three patients were lost to follow-up during the study.

In addition, we assessed APRIL, BAFF, and IFN-alfa as potential markers of humoral response that contribute to B-cell maturation, survival, and class switch. However, we observed no significant differences in serum BAFF concentration (1,841 ± 527 vs 2,496 ± 1,183 pg/mL; $P = .61$), APRIL (8.4 ± 5.39 vs 7.54 ± 10.7 pg/mL; $P = .21$), and IFN-alfa (25.06 ± 8.41 vs 21.72 ± 6.22 pg/mL; $P = .3$) between responders and nonresponders at month 1.

We tested all used IRT products to exclude the presence of anti-RBD SARS-CoV-2–specific antibodies leading to a false interpretation of vaccination response. Specific antibody levels

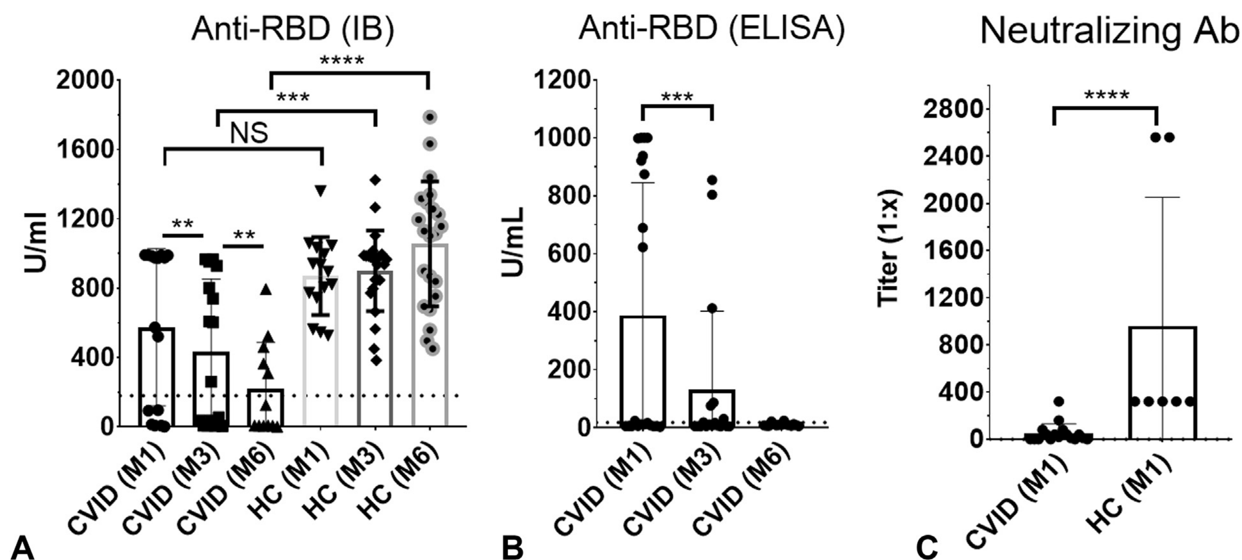

FIGURE 2. Humoral immune response in patients with common variable immunodeficiency (CVID) and in healthy controls (HCs). (**A**) Serum concentrations of anti–receptor-binding domain (RBD) SARS-CoV-2–specific antibodies at months 1 (M1), 3 (M3), and 6 (M6) measured by immunoblot (IB) (positive cutoff value >180 U/mL marked as dotted line in A) or (**B**) ELISA (positive cutoff value >18 U/mL marked as dotted line) assays. (**C**) Virus-neutralizing antibody titer (Ab) at month 1 (positive cutoff value >1:20). $*P < .05$, $**P < 0.01$, $***P < .001$, $****P < .000$. *NS*, not significant.

did not exceed the positive cutoff value (>18 U/mL) in any products (Kiovig: 7.38 ± 0.4; HyQvia: 6.22 ± 1.1; and Hizentra: 7.95 ± 0.66 U/mL).

T-cell immune response

One month after the second dose, $CD4^+$ T cells in 46% of patients (n = 6 of 13) responded to the S-RBD antigen in a short *ex vivo* stimulation and cytokine-production assays (Figure 3). Among the HCs, 73% responded (n = 8 of 11); however, the percentage of responding $CD4^+$ T cells did not differ significantly between cohorts. Fifty percent of patients with CVID with T-cell immune responses (three of six) also responded with specific antibody production. Then, we investigated the $CD4^+$ T-cell response's persistence 6 months after vaccination, which was detectable in 50% of CVID patients (n = 6 of 12) vs 60% of HCs (n = 9 of 15). Both antibody and T-cell responses were present in five of them (Figure 3). The T-cell assessment may be limited by impaired T cell functionality after a freeze–thaw cycle and impaired cell viability of the T cells in CVID patients, resulting in lower overall assay sensitivity. Five patients with CVID were excluded owing to low cell viability and/or unresponsiveness to CD3 stimulation.

Clinical outcomes

Of the 21 vaccinated patients, 18 (85.7%) neither developed COVID-19 symptoms nor tested positive after a risk contact with an SARS-CoV-2–infected person. At the end of the follow-up at month 6 (November 2021), COVID-19 was confirmed in three vaccinated patients with CVID (14.3%; two women and one man, aged 38.33 ± 7.57 years; range, 33-47 years). Two had CVID-associated noninfectious complications (splenomegaly, chronic enteropathy, and autoimmune thyroiditis). The infection was mild in all three patients. Major symptoms were fever, arthralgia, and myalgia, which were present in all patients. Two

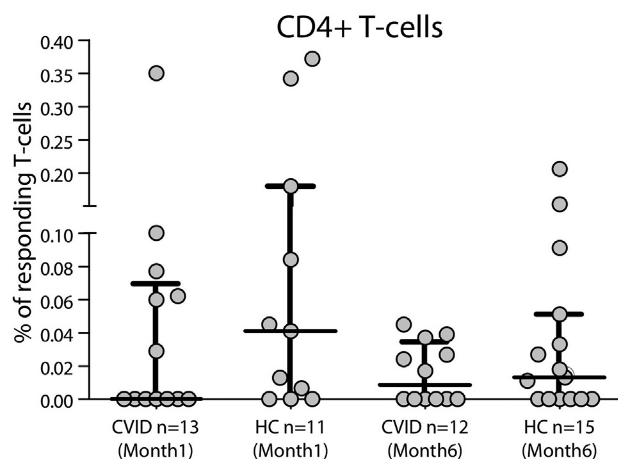

FIGURE 3. T-cell immune response. The proportion of IFN-gamma/TNF-α/IL-2–producing $CD4^+$ T cells (total count) in patients with common variable immunodeficiency (CVID) and in healthy controls (HC) measured at months 1 and 6.

patients reported cough, rhinitis, gastrointestinal symptoms, and fatigue. One patient developed a loss of smell. No patients required antiviral treatment or hospital admission. Two were classified as responders to vaccination; however, the humoral response persisted in a single patient at month 3. The three patients did not have a cellular response. None had a history of immunosuppressive therapy.

Safety

Adverse events were reported in 90% of patients with CVID after the first and second doses (n = 19 of 21). The most common event was local pain at the injection site (20 of 21),

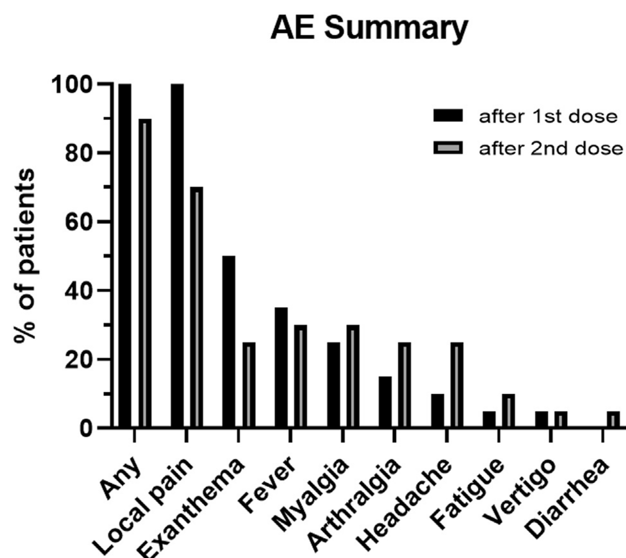

FIGURE 4. Adverse events (AEs) observed in study participants: incidence of AEs (as a percentage of 21 enrolled patients) after the first and the second dose. No severe AEs were reported.

followed by fatigue (10 of 21), headache (7 of 21), fever (5 of 21), myalgia (3 of 21), and arthralgia (2 of 21) after the first dose. A similar AE spectrum was observed after the second dose. Figure 4 shows the AE incidence. Mean durations of AEs after the first and second doses were 3.55 (±2.19 SD) and 2.95 (±2.04) days, respectively. The 100-point patient global assessment visual analog scale was 18.25 (±21.96) and 16.75 (±22.38) points. We also evaluated a broad spectrum of laboratory parameters, including biochemical, hematologic, immunologic, and inflammatory parameters. We detected significantly increased soluble CD25 levels after both vaccine doses. No changes in total blood count, liver or renal function, and coagulation were observed, and no autoantibodies were detected during the follow-up period. Vaccination did not increase inflammatory markers, except for soluble IL-2R (sCD25) levels, which were significantly increased after both vaccine doses. Table E1 provides a complete overview of these parameters.

DISCUSSION

Common variable immunodeficiency is characterized by recurrent and chronic respiratory tract infections and a broad spectrum of noninfectious complications, including chronic lung disease. Therefore, patients with CVID are potentially at high risk for severe COVID-19, which is associated with poor outcomes.[2,3] Despite an impaired specific antibody response to proteins, as well as polysaccharide antigens, in patients with CVID,[13,20] T-cell−mediated immunity is intact in most patients with CVID.[21] Therefore, patients with CVID may benefit from vaccination against COVID-19, which may induce a specific T-cell response.[22]

Owing to the COVID-19 outbreak, issues have been raised regarding the immunogenicity, safety, and efficacy of vaccination in patients with IEI. However, studies on the immunogenicity and safety of vaccination in a broad spectrum of IEIs have shown encouraging results. Amodio et al[23] revealed humoral and cellular responses in 86% and 76% of 21 patients with IEI, respectively, with no correlation with patient age, in contrast to the study by Hagin et al.[24] Both authors also reported a lower humoral response than that in the general population, and only four of 12 patients did not develop a cellular response. Similar findings were reported by Delmonte et al,[25] in which specific anti-SARS antibodies were detected in 63 of 74 patients with IEI (85.1%). Furthermore, van Leeuwen et al[26] demonstrated a negative correlation between the presence of noninfectious complications and immunosuppression in a large study of 505 patients, including 196 patients with CVID; however, those patients were vaccinated with the mRNA-1273 vaccine. Another significant limitation of previously published studies is the lack of prospective follow-up and limited vaccine safety data in patients with IEIs.

In the current study, a specific antibody response was observed in 52.4% of patients 1 month after vaccination, and anti-RBD SARS-CoV-2 antibody levels were comparable to those in HCs. Nevertheless, seemingly favorable humoral responses differed significantly in qualitative properties and persistence over time. The neutralizing antibody titer, which is predictive of the protection level,[27,28] suggested a qualitative insufficiency, which is consistent with a previous study reporting a reduced capacity to produce virus-neutralizing antibodies in CVID.[29] In addition, the humoral response was not influenced by baseline or previous immunosuppression or the presence of noninfectious complications. However, this might have been limited by the small number of patients included in the study. Responders were further characterized by a lower age (<40 years) and higher proportion of class-switched B cells, which is consistent with a previous study reporting an increased number of $CD21^{low}$ B cells, suggesting a possible dysregulation in the immune response to vaccination.[30] Moreover, we demonstrated that a higher serum IgM concentration is a novel potential positive response predictor. However, we observed no differences in CD4 and CD8 subsets between responders and nonresponders.

In addition, although a specific T-cell response was detected at month 1 in less than half of the patients with CVID in the current study, the proportion of responders was not significantly different from that observed in HCs. There were also no differences in the number of anti-SARS-CoV-2−specific T cells in CVID and HCs responders. The proportion of both patients with CVID and HCs with persistently measurable Spike-specific T-cell responses remained the same at month 6. We excluded five patients with CVID from the T-cell response analysis owing to an absent anti-CD3 response and/or low viability, which might have been related to T-cell abnormalities in CVID.[14] Moreover, the T-cell−specific response assessment is currently limited by the need for an *ex vivo* functional stimulation, which is negatively influenced by the freeze−thaw cycles. Long-term persistent antigen-specific memory T cells tend to redistribute to lymphoid tissues including bone marrow.[31,32] The lower sensitivity of the different anti-SARS-CoV-2−specific T-cell assays was also observed in other studies.[33]

Based on the immunophenotyping findings and the central role of APRIL and BAFF in the survival and maturation of B cells and their dysregulation in CVID,[34] we examined the serum concentration of both cytokines as potential response markers; however, we observed no significant differences in levels between responders and nonresponders at month 1. Moreover, no differences were found in serum IFN-alfa concentrations, which can promote isotype switching.[35]

Despite the promising antibody response rate at month 1, the proportion of responders rapidly decreased to 44.4% and 33.3% at 3 and 6 months, respectively. Anti-SARS-CoV-2 antibody titers were also significantly lower than those in HCs at the end of the study. The results were not influenced by IRT, and no specific anti-RBD antibodies were detected in IRT solutions. Previous studies used different methods to assess humoral and cellular responses. Therefore, our findings must be compared and interpreted with caution. Moreover, the specific anti-SARS antibody level that can predict immune protection remains unknown, and the efficacy of vaccination needs to be confirmed by long-term observation.

Although a high vaccine efficacy level has been observed in the general population, in our study, three of 21 patients (14.3%) tested positive for SARS-CoV-2 infection by RT-PCR. All patients were infected 6 months after the second-dose administration, when the SARS-CoV-2 Delta variant prevailed (November 21). The infected patients had a mild course, none developed a T-cell immune response, and the humoral response persisted in a single patient upon infection. Therefore, our data support booster vaccination in intervals shorter than 6 months for patients with CVID, as recommended for the general population.[36,37]

Moreover, studies on vaccination safety in patients with IEIs showed a favorable vaccination profile. In our study, AEs occurred in all patients with CVIDs, including injection site reactions, fatigue, headaches, and fever. No severe AEs were reported. The spectra of reactions after the first and second doses were comparable. Notably, we observed no changes in coagulation, including D-dimers, because a higher risk for thromboembolic events was described 15 to 21 days after BNT162b2 vaccine administration.[10] Among the tested parameters, only soluble CD25 was significantly increased in patients with CVID after vaccination.

To the best of our knowledge, this is the first study to investigate the long-term persistence of postvaccination responses and clinical outcomes in patients with CVID, providing data on a 6-month follow-up. We revealed that the anti-SARS-CoV-2 mRNA vaccine BNT162b2 induces a humoral response in a high proportion of patients with CVID. However, the vaccine induces lower anti-SARS-CoV-2 neutralizing antibody levels in patients with CVID compared with the general population. Importantly, the antibody response was not persistent and continuously decreased 3 months after vaccination, whereas the $CD4^+$ T-cell response persisted. We also demonstrated satisfactory clinical outcomes after vaccination in patients with CVID. No SARS-CoV-2 infections were reported within 5 months of the follow-up period, and only three patients (14.3%) tested positive for COVID-19; however, those patients had mild symptoms. Therefore, the BNT162b2 vaccine has a favorable safety profile in a proportion of patients with CVID. Moreover, our study suggests reasonable booster vaccination in shorter intervals than those recommended for the general population[38,39] that would elicit seasonal immune response even in CVID patients. However, several issues need to be addressed. The booster vaccine efficacy may be influenced by a predominant SARS-CoV-2 variant. Although original mRNA anti-SARS-CoV-2 vaccines are less effective in preventing infection by the Omicron variant,[40] a booster vaccination may reduce severe outcomes including hospital admission in the general population[41] as well as in immunocompromised[42] and other vulnerable populations.[43] New generations of multivalent mRNA vaccines could provide better efficiency against novel variants.[44] High vaccination and infection frequencies also pose the question of hybrid immunity, which may represent another important factor in reducing the risk for infection.[45,46]

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TABLE E1. Hematologic, biochemical, immunologic, and inflammatory parameters at different time points

Parameter	Day 0 (n = 21)	Day 21 (n =21)	*P**	Mo 1 (n = 18)	*P*†	Reference values
Complete and differential blood count						
Leukocytes, E9/L	6.55 ± 2.59 (2.7-11.7)	6.83 ± 2.99 (2.2-14.3)	NS	6.67 ± 2.42 (2.3-11.9)	NS	4.0-10.0
Neutrophils, E9/L	4.34 ± 2.16 (1.8-9.96)	4.62 ± 2.53 (2.38-12.28)	NS	4.45 ± 2.05 (1.47-9.13)	NS	2.2-7.0
Lymphocytes, E9/L	1.49 ± 0.7 (0.66-3.29)	1.42 ± 0.69 (0.58-3.5)	NS	1.44 ± 0.59 (0.54-2.82)	NS	0.8-4.0
Monocytes, E9/L	0.51 ± 0.23 (0.23-1.09)	0.51 ± 0.21 (0.23-1.06)	NS	0.51 ± 0.15 (0.21-0.78)	NS	0.08-1.2
Red blood cell count	5.06 ± 0.58 (3.66-6.05)	5.0 ± 0.56 (3.8-6.06)	NS	4.97 ± 0.55 (3.73-5.98)	NS	3.8-5.2
Hematocrit (ratio)	0.42 ± 0.04 (0.33-0.47)	0.42 ± 0.04 (0.34-0.48)	NS	0.41 ± 0.03 (0.33-0.47)	NS	0.35-0.47
Hemoglobin, g/L	148.8 ± 13.83 (112-166)	144.6 ± 14.26 (113-165)	NS	143.8 ± 13.59 (109-162)	NS	120-160
Platelets, E9/L	209.9 ± 72.12 (92-358)	212.5 ± 60.76 (102-301)	NS	212.6 ± 67.95 (108-375)	NS	150-400
Coagulation						
Activated partial thromboplastin time	26.44 ± 3.34 (19.9-31.7)	28.75 ± 3.43 (21.0-34.7)	NS	27.84 ± 3.35 (20.8-32.7)	NS	25.20
Prothrombin time	11.49 ± 0.61 (10.2-12.5)	12.49 ± 1.15 (10.7-14.9)	NS	11.84 ± 0.74 (10.6-13.4)	NS	12.20
Prothrombin time-international normalized ratio	0.94 ± 0.05 (0.83-1.03)	0.98 ± 0.06 (0.87-1.13)	NS	0.97 ± 0.06 (0.87-1.08)	NS	0.80-1.20
D-dimers	176.8 ± 166.6 (34-790)	159.5 ± 167 (28-791)	NS	186.3 ± 121.1 (19-465)	NS	0-249
Biochemistry						
Alanine aminotransferase	0.59 ± 0.36 (0.22-1.28)	0.58 ± 0.41 (0.25-1.77)	NS	0.63 ± 0.45 (0.23-1.74)	NS	0.17-0.84
Aspartate aminotransferase	0.42 ± 0.19 (0.18-0.79)	0.39 ± 0.17 (0.18-0.82)	NS	0.45 ± 0.2 (0.19-0.92)	NS	0.16-0.72
γ-Glutamyl transferase	0.67 ± 0.84 (0.13-3.99)	0.61 ± 0.69 (0.12-3.28)	NS	0.65 ± 0.69 (0.17-3.31)	NS	0.14-0.68
Alkaline phosphatase	1.74 ± 0.85 (0.79-4.33)	1.64 ± 0.61 (0.79-3.1)	NS	1.67 ± 0.79 (0.81-3.89)	NS	0.66-2.20
Total bilirubin	15.37 ± 9.78 (6.60-48.2)	14.34 ± 8.83 (6.30-44.7)	NS	14.03 ± 9.18 (4.1-46.5)	NS	5.0-21.0
Direct bilirubin	4.41 ± 3.0 (2.0-14.3)	4.25 ± 2.85 (2.0-13.9)	NS	4.14 ± 2.91 (2.0-13.1)	NS	0.0-21.0
Lactate dehydrogenase	3.47 ± 0.82 (2.39-5.86)	3.71 ± 1.0 (2.41-6.45)	NS	3.80 ± 0.86 (2.33-5.9)	NS	1.67-4.1
Amylase	1.25 ± 0.63 (0.45-2.88)	1.12 ± 0.55 (0.47-3.29)	NS	1.09 ± 0.5 (0.49-2.83)	NS	0.3-2.28
lipase	0.76 ± 0.30 (0.47-1.54)	0.68 ± 0.20 (0.42-1.2)	NS	0.75 ± 0.37 (0.47-2.1)	NS	0.12-1.0
Uric acid	325.2 +-94.62 (56-497)	318.2 ± 98.10 (64-572)	NS	318.2 ± 97.62 (50-522)	NS	140-340
Urea	4.72 ± 1.57 (2.60-8.60)	4.56 ± 1.78 (1.80-9.10)	NS	4.96 ± 1.53 (2.20-8.20)	NS	2.0-6.7
Creatinine	63.95 ± 15.09 (37-97)	63.0 ± 12.79 (41-84)	NS	62.57 ± 14.04 (41-95)	NS	42-80
Creatine kinase	1.16 ± 0.59 (0.25-2.81)	1.23 ± 0.63 (0.40-2.70)	NS	1.66 ± 1.28 (0.44-6.06)	NS	0.41-2.85
Myoglobin	28.24 ± 10.86 (16.3-64.3)	36.01 ± 16.94 (20.0-72.6)	NS	38.20 ± 28.16 (19.7-151.6)	NS	12.0-76.0
Immunology						
IgA	0.10 ± 0.09 (0.07-0.47)	0.11 ± 0.09 (0.07-0.44)	NS	0.10 ± 0.07 (0.07-0.35)	NS	0.91-2.9
IgM	0.19 ± 0.18 (0.06-0.72)	0.17 ± 0.16 (0.04-0.64)	NS	0.18 ± 0.17 (0.04-0.69)	NS	0.47-1.95
Complement component 3	1.08 ± 0.25 (0.62-1.71)	1.05 ± 0.28 (0.61-1.69)	NS	0.98 ± 0.31 (0.42-1.63)	NS	0.83-2.25
Complement component 4	0.24 ± 0.06 (0.14-0.37)	0.24 ± 0.06 (0.15-0.38)	NS	0.24 ± 0.06 (0.15-0.38)	NS	0.14-0.35
Anti-cardiolipin autoantibodies	2.66 ± 1.38 (1.6-6.1)	4.27 ± 2.42 (2.6-11.4)	NS	3.09 ± 1.89 (1.7-7.9)	NS	0.0-11.0
RF-IgG	4.62 ± 5.46 (1.2-26.0)	2.1 ± 1.23 (0.9-6.0)	0.0003	3.3 ± 1.58 (1.4-7.0)	0.0012	0.0-20.0
RF-IgA	0.92 ± 0.94 (0.14-3.4)	0.53 ± 0.26 (0.4-1.5)	0.047	0.8 ± 0.58 (0.4-3.1)	0.01	0.0-20.0
RF-IgM	0.57 ± 0.76 (0.1-3.5)	0.47 ± 0.88 (0.1-4.1)	0.017	0.48 ± 0.66 (0.1-3.1)	NS	0.0-20.0
Anti-nuclear autoantibodies (patients, n [%])	1	1	NS	1	NS	Positive
Anti-neutrophil cytoplasmic antibodies (patients, n [%])	0	0	NS	0	NS	Positive
Antimitochondrial autoantibodies (patients, n [%])	0	0	NS	0	NS	Positive

(continued)

TABLE E1. *(Continued)*

Parameter	Day 0 (n = 21)	Day 21 (n =21)	*P**	Mo 1 (n = 18)	*P*†	Reference values
Anti-smooth muscle antibodies (patients, n [%])	0	0	NS	0	NS	Positive
Liver kidney microsome autoantibodies (patients, n [%])	0	0	NS	0	NS	Positive
Gastric parietal cell autoantibodies (patients, n [%])	0	0	NS	0	NS	Positive
Inflammatory markers						
High sensitive C-reactive protein	5.44 ± 5.87 (0.5-25.1)	6.88 ± 6.21 (0.5-20.7)	NS	5.96 ± 5.64 (0.5-21.1)	NS	0.0-5.0
Ferritin	93.9 ± 93.4 (10.9-389.5)	93.1 ± 73.7 (8.1-305.3)	NS	172.3 ± 428.3 (13.9-1988)	NS	10.0-291
Soluble CD25 molecule	630.9 ± 352.3 (7.0-1248)	815.5 ± 415.9 (2.4-1725)	0.0024	767.3 ± 390.2 (260-1544)	0.044	0.0-710
IL-6	5.41 ± 2.6 (2.0-10.0)	5.19 ± 3.17 (2.0-13.2)	NS	4.01 ± 1.94 (2.0-9.0)	NS	0.0-5.9

RF, rheumatoid factor.
Statistically significant differences ($P \leq .05$) between baseline and day 21 (*) or month 1 (†) are marked in bold.

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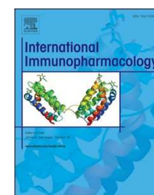

Content and specificity of the Anti-SARS-CoV-2 antibodies in solutions for immunoglobulin replacement therapy

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ABSTRACT

Background: Specific antibodies are important for post-vaccination and post-infection immune responses against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). The role of antibodies in preventing and treating Coronavirus disease 2019 (COVID-19) in high-risk populations has been highlighted through the use of virus-specific monoclonal antibodies, which has raised the question of immunoglobulin replacement therapy (IRT) used in immunocompromised patients.
Methods: Virus-specific anti-receptor-binding domain (RBD) and anti-nucleocapsid protein (NCAP) antibodies (assessed using a chemiluminescence assay and virus-neutralizing antibodies (virus neutralization test against Delta and Omicron variants)) were analyzed in 20 batches of 10 % (100 mg/mL) immunoglobulin solutions for intravenous IRT from two commercially available producers between January 2022 and March 2023 for clinical use.
Results: Anti-RBD and anti-NCAP antibodies were detected in all 20 batches of assessed IRT solutions (mean concentrations of 2817 IU/mL and 2380 IU/mL, respectively). Notably, the concentration of the virus-specific antibodies increased continuously during the follow-up period (from 822.5 IU/mL to 4066.4 IU/mL and 102 IU/mL to 3455.9 IU/mL). These antibodies demonstrated high virus-neutralizing activity against the Delta variant (mean titers of 436 and 325) but were limited to the Omicron variant (mean titers 78 and 70). The differences observed between the two brands were not statistically significant.
Conclusion: IRT solutions contain high concentrations of anti-SARS-CoV-2 specific antibodies, which may prevent COVID-19; however, the efficacy can be influenced by variable virus-neutralizing activities against different viral strains. Therefore, appropriate IRT should be combined with other approaches, such as vaccination or pre- and post-exposure prophylaxis. Passively transmitted specific antibodies may also lead to false-positive serological test results.

1. Introduction

Vaccines against severe acute respiratory syndrome Coronavirus-2 (SARS-CoV-2) inducing a robust, specific antibody production play an important role in preventing severe outcomes of coronavirus disease 2019 (COVID-19) [1]; their immunogenicity may be reduced in high-risk patients, particularly those with inborn errors of immunity (IEI), such as X-linked agammaglobulinemia and common variable immunodeficiency [2,3] or secondary immunodeficiency, including individuals with cancers and transplant recipients [4] in whom anti-SARS-CoV-2 antiviral therapies and monoclonal antibodies (mAbs) are among the main therapeutic strategies [5]. Anti-SARS-CoV-2 specific mAbs, including bamlanivimab/etesevimab, casirivimab/imdevimab, and sotrovimab have been shown to be effective in treating COVID-19, while tixagevimab/cilgavimab has been demonstrated to be effective in the pre-exposure prevention of severe COVID-19 in immunocompromised patients [6].

The increasing number of infected and vaccinated individuals, including blood plasma donors, raises questions regarding the effectiveness of immunoglobulin replacement therapy (IRT) in preventing

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COVID-19, which is the main therapeutic approach in treating primary and secondary antibody deficiencies. Currently, there is limited evidence to support its efficacy. Therefore, we analyzed the content and specificity of anti-SARS-CoV-2 antibodies in various commercially available brands and batches of immunoglobulin solutions used in clinical practice within a 15-month follow-up period.

2. Methods

Virus-specific and virus-neutralizing antibodies were assessed in different batches of 10 % (100 mg/mL) immunoglobulin solution brands (Kiovig, Takeda Manufacturing Vienna, Austria and Privigen, CSL Behring, Marburg, Germany) intended for intravenous immunoglobulin (IVIG) replacement therapy. These solutions were commercially available from January 2022 to March 2023. All solutions were diluted with a 5 % bovine serum albumin (lyophilized IgG-free powder, Merck KGaA, Darmstadt, Germany) solution to a concentration of 1 %, which corresponds to the immunoglobulin G concentration in human plasma. Anti-receptor-binding domain (RBD) and anti-nucleocapsid protein (NCAP) SARS-CoV-2 specific antibodies were determined using a chemiluminescence assay (KleeYa, Diatron, Budapest, Hungary). The virus neutralization test (VNT) was performed according to a previously published protocol [7] using SARS-CoV-2 strains derived from the following clinical samples: Delta 128940/21 and Omicron 55174/22. Virus stock suspensions were prepared by amplification on the CV-1 cell line (African green monkey kidney fibroblasts). Serum samples were diluted to final concentrations of 1/10 to 1/2560. Uninfected cells were stained with a neutral red dye. The VNT results were expressed as a virus-neutralization titer, representing an inverted value of the highest sample dilution that neutralized the cytopathic effect of the virus by more than 50 %.

The concentrations of the diluted immunoglobulin solutions were compared to the serum levels of anti-RBD specific and virus-neutralizing antibodies detected in 12 vaccinated healthy individuals 1 month after booster vaccination (third dose) with mRNA vaccine BNT162b2 (Comirnaty; BioNTech Manufacturing GmbH, Mainz, Germany).

The mean and 95 % confidence intervals (95 % CI) were calculated for the virus-specific and virus-neutralizing antibody concentrations. Statistically significant differences in the means were assessed using the Mann–Whitney test for non-normally distributed unpaired data. Normality was tested using the Shapiro–Wilk normality test. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA).

This research involved no human or animal subjects; therefore, ethical approval was not required.

3. Results

Virus-specific and virus-neutralizing antibodies were assessed in 20 batches of 10 % (100 mg/mL) immunoglobulin solution for intravenous IRT from two different producers. The solutions had expiration dates ranging from September 2023 to September 2024 (IVIG-I) and March 2024 to September 2025 (IVIG-II). Anti-RBD and anti-NCAP SARS-CoV-2 antibodies were detected in all batches. The mean concentration of anti-RBD antibody was 2610 IU/mL (95 % CI: 2089–3130). The differences in mean concentrations between the two brands were not statistically significant (IVIG-I:2817 IU/mL, 95 % CI: 2131–3502 vs. IVIG-II:2380, 95 % CI: 1446–3314). The concentration of anti-RBD antibodies increased from 822.5 IU/mL (expiration date of batch 09–23) to 4066.4 (09–24) and 102 IU/mL (03–24) to 3455.9 (09–25) in IVIG-I and IVIG-II, respectively (Fig. 1A and D). However, the concentrations of anti-NCAP antibodies were significantly lower ($p < 0.0001$) than those of anti-RBD antibodies, measuring −49.1 IU/mL (23.4–74.9) without differences between brands as follows: 46.7 IU/mL (95 % CI: 5.8–87.6) and 51.8 IU/mL (11.7–92) in IVIG-I and IVIG-II, respectively. Similar to anti-RBD, anti-NCAP antibodies increased from 19.5 IU/mL (09–23) to 186.7 IU/mL (09–24) and 13.0 IU/mL (03–24) to 117.7 IU/mL (09–25)

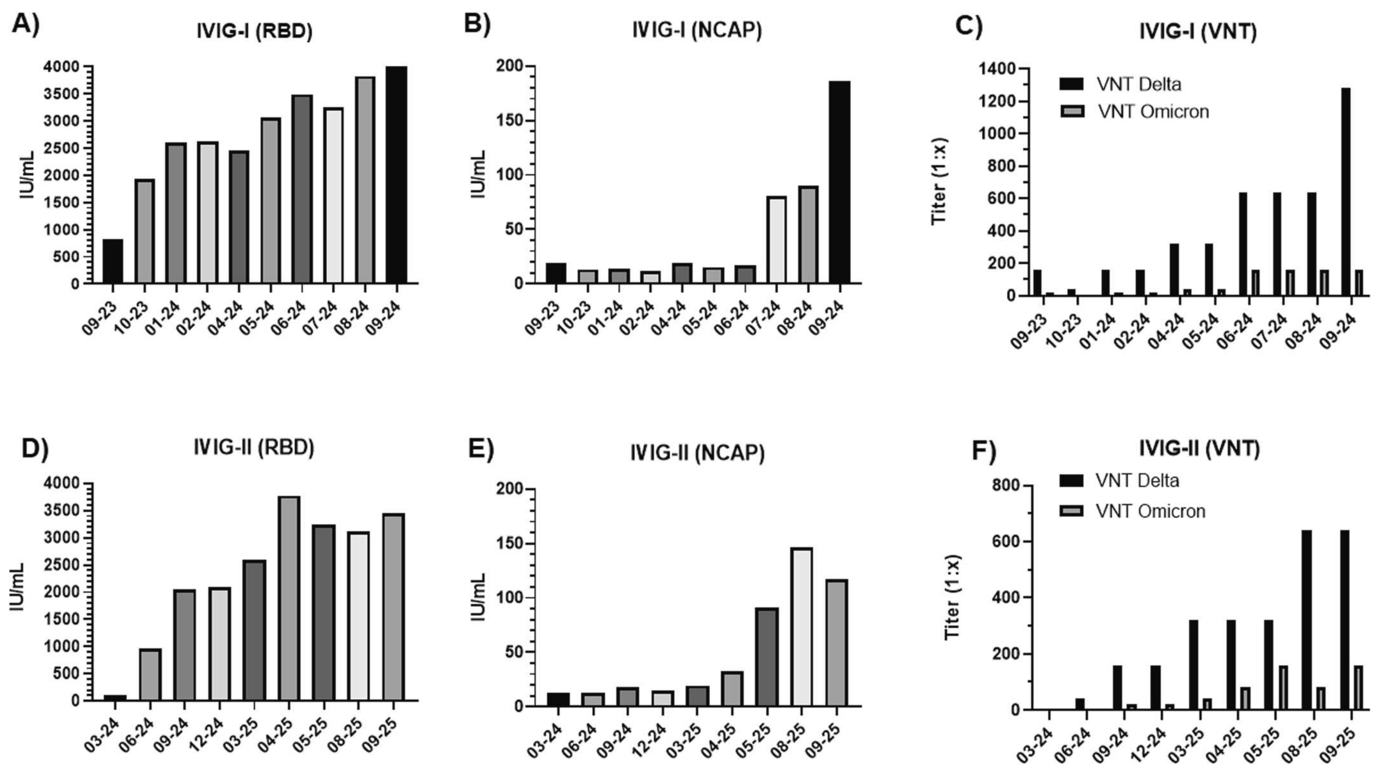

Fig. 1. Concentrations (IU/mL) of the anti-receptor-binding domain and anti-nucleocapsid protein SARS-CoV-2 specific antibodies and titers (1:x) of virus-neutralizing antibodies against Delta and Omicron variants in 2 different immunoglobulin solution for intravenous immunoglobulin replacement therapy (IVIG-I: A–C, IVIG-II: D–F). SARS-CoV-2, severe acute respiratory syndrome coronavirus-2.

in IVIG-I and IVIG-II, respectively (Fig. 1B and 1E). However, the highest increase occurred in batches with expiration dates from June 2024 to September 2024 (from 17 IU/mL to 186.7 IU/mL) and March 2025 to September 2025 (19.5 IU/mL 117.7 IU/mL) in IVIG-I and IVIG-II, respectively.

Virus-neutralizing antibodies against Delta and Omicron were detected in most of the assessed batches of IRT solutions. However, anti-delta-neutralizing antibodies were significantly more abundant ($p < 0.0001$). The mean titers of anti-Delta and anti-Omicron antibodies were 386.7 (95 % CI: 232.3–541) and 74.4 (41.8–107.1), respectively. The differences in titers of both antibodies between IVIG and I (anti-Delta:436.0, 95 % CI: 169.5–702.5 and anti-Omicron:78.0, 95 % CI: 26.9–129.1) and IVIG-II (anti-Delta:325.0, 95 % CI: 142.3 – 507.7 and anti-Omicron:70.0, 95 % CI:17.9–122.1) were insignificant. Similar to virus-specific antibodies, the titers of anti-delta and anti-Omicron antibodies increased throughout the batches during the follow-up period. However, the increase in anti-delta antibodies was more rapid; the antibodies reached 1:160 (09–23) to 1:1280 (09–24) and 1:40 (03–24) to 1:640 (08–25) in IVIG-I and IVIG-II, respectively. Anti-Omicron antibodies were detected at a titer of 1:20 at the beginning of the follow-up period (batch with expiration period 09–23) and reached the maximum titer in batch 06–24 of IVIG-I. Anti-Omicron antibodies were detected later in IVIG-II than in IVIG-I. They were detected in batch 09–24 at a titer of 1:20, which subsequently increased to a maximum of 1:160 in batch 05–25 (Fig. 1C and 1F). Except for concentrations of anti-RBD antibodies, the titers of virus-neuralizing antibodies detected in immunoglobulin solutions were comparable to the serum concentrations of antibodies found in vaccinated healthy individuals 1 month after booster vaccination. The concentrations and titers of virus-specific and virus-neutralizing antibodies are summarized in Table 1.

4. Discussion

Humoral responses and virus-specific antibodies play an important role in combating SARS-CoV-2 infection[8]. Because IRT is the main therapeutic approach for patients with IEI affecting humoral immunity and secondary antibody deficiencies [9,10], there is an open question regarding the efficacy of IRT in preventing and treating COVID-19. Our study extends existing evidence on the concentrations and specificity of anti-SARS-CoV-2 antibodies in IRT solutions for intravenous administration.

We detected high titers of anti-SARS-CoV-2 antibodies in all commercially available immunoglobulin solutions used in routine clinical practice between January 2022 and March 2023 (expiration period reaching from September 2023 to September 2024 and March 2024 to September 2025 for IVIG-I and IVIG-II, respectively). The concentrations of anti-RBD specific antibodies were increasing within the follow-up period (maximum concentrations 4066.4 IU/mL and 3455.9 IU/mL and mean concentrations 2817 IU/mL and 2380 IU/mL in IVIG-I and IVIG-II, respectively) along with anti-NCAP antibodies (maximum concentrations 186.7 IU/mL and 117.7 IU/mL and mean concentrations 46.7 IU/mL and 51.8 IU/mL in IVIG-I and IVIG-II, respectively). When examining the variant specificity using VNT, we found significantly higher titers of neutralizing antibodies against the Delta variant than those against Omicron. Comparable concentrations of virus-neutralizing antibodies against Delta and Omicron variants were found in IRT solutions and vaccinated healthy individuals.

We assume that varying concentrations of the strain-specific antibodies may limit the pre-exposure and therapeutic use of immunoglobulin therapeutics in clinical practice. At the beginning of the pandemic, SARS-CoV-2 antibodies were not detected in IVIG solutions produced before January 2020 [11]. The study by Diez et al., investigating the neutralization capability of IVIG solutions manufactured before the pandemic, showed neutralization activity against SARS-CoV and SARS-CoV-2, while the activity against MERS-CoV was limited [12]. Additionally, significant regional differences were also found in the concentrations of anti-SARS-CoV-2 antibody concentrations. Specific antibodies were detected early in healthy plasma donors from Spain and the USA. Subsequently, the antibody concentrations in plasma pools dramatically increased 10–50 times regardless of geographic origin, matching SARS-CoV-2 exposure [13].

However, we also recognize the important contribution of vaccinated plasma donors based on our results showing significantly higher concentrations of anti-RBD antibodies than those of anti-NCAP that occur after infection.

Similarly, limited efficacy of passively transmitted specific antibodies through IRT might be expected against various strains based on the evidence of the use of virus-specific mAbs and other seasonal viral infections. Despite the retained capability to reduce the risk of hospital admission or death, the efficacy of the original mAbs against novel viral strains may be limited compared with other variants [14]. It has been demonstrated that novel viral variants, such as Omicron, can evade neutralization [15]. In real clinical practice, the evidence of the efficacy of IVIG in critically ill patients is inconclusive [16,17]. During the Omicron wave in 2022, patients with X-linked agammaglobulinemia on regular IRT had low plasma concentrations of virus-specific and virus-neutralizing antibodies against the Omicrion variant [18]. The limited efficacy of IVIG or immune plasma against seasonal viral infections, such as influenza, has been shown in many clinical trials [19,20]. Moreover, the significance of passively transmitted antibodies from IRT should be interpreted regarding the capability of vaccines to induce a T-cell immune response compared to vaccination. Specific cellular immunity represents another crucial component of post-vaccination immunity; however, T-cell immunity assessment is less widespread in routine clinical practice [21]. Therefore, active vaccination against influenza should be provided to patients with antibody deficiencies [22]. Based on our findings and previous experience with other seasonal viral infections, we believe that vaccination against SARS-CoV-2 using a vaccine reflecting an actual epidemiological situation should still be indicated in most of the high-risk patients despite IRT.

Another aspect of passively transmitted antibodies is related to false-positive laboratory results from serological tests. Interference between the results of several serological tests and passively transmitted specific antibodies, such as autoantibodies or pathogen-specific antibodies, has been previously reported. This may limit the use of serological tests to diagnose post-infection or post-vaccination responses [23,24]. The risk

Table 1
Summary characteristics of the concentrations (IU/mL) of anti-receptor-biding domain (RBD) and anti-nucleocapsid protein (NCAP) SARS-CoV-2 specific antibodies, and titers (1:x) of virus-neutralizing antibodies (VNT) Delta and Omicron variants in 2 different brands of immunoglobulin solutions for intravenous immunoglobulin replacement therapy (IVIG-I, IVIG-II), serum levels of specific antibodies in vaccinated healthy individuals one month after booster vaccination (VACCINATED), mean values and 95 % confidence intervals dysplayed (95 %CI), the differences between IVIG and I and IVIG-II, ALL and VACCINATED not statistically significant ($p > 0.05$); CI, confidence interval; N/A, not applicable.

Parameter	ALL	Vaccinated	p-value	IVIG-I	IVIG-II	p-value
Anti-RBD IU/mL (95 %CI)	2610 (2089–3130)	1949 (482.4–139.3)	0.02	2817 (2131–3502)	2380 (1446–3314)	0.45
Anti-NCAP IU/mL (95 %CI)	49.1 (23.4–74.9)	N/A	N/A	46.7 (5.8–87.6)	51.8 (11.7–92)	0.72
Delta VNT titer (95 %CI)	386.7 (232.3–541)	986.7 (219.8–1753)	0.19	436 (169.5–702.5)	325 (142.3–507.7)	0.72
Omicron VNT titer (95 %CI)	74.4 (41.8–107.1)	204 (35.28–372.7)	0.10	78 (26.9–129.1)	70 (17.9–122.1)	0.92

of passive transmission bias was low in the first studies that focused on the immunogenicity of the anti-SARS-CoV-2 vaccine in patients receiving IRT [3,25], suggesting lower concentrations of virus-specific antibodies in the IRT solutions.

In conclusion, although commercially available IRT solutions contain high concentrations of anti-SARS-CoV-2 antibodies and provide protection for patients with immunoglobulin substitution, their efficacy is limited due to variable virus-neutralizing activity against different viral strains. Therefore, appropriate IRT should be combined with other approaches, such as vaccination or pre- and post-exposure prophylaxis, to address the current epidemiological situation. Further studies are required to demonstrate the efficacy of IRT in preventing and treating COVID-19.

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CRediT authorship contribution statement

Tomas Milota: Methodology, Visualization, Writing – original draft. **Jitka Smetanova:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing – original draft. **Hana Zelena:** Investigation, Methodology, Validation. **Michal Rataj:** Investigation, Methodology, Validation. **Jan Lastovicka:** Investigation, Methodology, Validation. **Jirina Bartunkova:** Conceptualization, Data curation, Investigation, Methodology, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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