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ABSTRACT

Incomplete genotype-phenotype correlations challenge the management of non-SCID *FOXN1* immunodeficiency. We describe the detailed clinical course of three distinct newborns with four novel *FOXN1* mutations identified by TREC—NBS. For comprehensive immune characterization advanced flow cytometry-based immunophenotyping was employed alongside high-resolution single-cell RNA sequencing. In our cohort, we detected heterozygous *FOXN1* mutations in P1 (c.1178delG; p.Gly393Ala*fs**157) and P2 (c.830+1G>*T*; p.?), and compound heterozygous *FOXN1*-mutations in P3 (c.1318C>T; p.Gln440* and c.668T>G; p.?). Despite slow and partial recovery from T-cell lymphocytopenia in P3, clinical signs for classical 'nude SCID` were incomplete. Compared to a healthy cord blood control, a distinct B-cell population was identified in the *FOXN1*-deficient patients expressing immature B-cell markers and lower HLA-II mRNA levels. In summary, our cohort of three newborns with four novel *FOXN1* variants highlights heterogeneous immunological courses and broader thymic dysfunction implications in this rare disease. Structured management strategies are essential for those identified by NBS-programs.

1. Introduction

Forkhead box N1 (FOXN1) encodes a transcription factor essential for thymic epithelial cell, hair follicle, and keratinocyte development [1] Biallelic loss-of-function mutations result in thymic aplasia and alopecia, while thymus transplantation has proven effective in restoring immune function [2,3] Conversely, individuals carrying heterozygous *FOXN1* mutations usually exhibit milder T-cell lymphocytopenia and susceptibility to infections during childhood, while preserving $CD8^+$ T-cell lymphocytopenia into adulthood [4]

Increasingly, these patients are being identified worldwide through newborn screening programs based on the detection of T-cell receptor excision circles (TREC—NBS) [5,6] Managing and interpreting these cases remains challenging, as affected newborns often appear otherwise healthy, and the long-term clinical prognosis remains uncertain. Recently described dominant negative effects of certain variants, as well as different consequences of compound heterozygous ones broadens the spectrum of *FOXN1* associated diseases and complicates their clinical management further [2,3,7]

Since the implementation of TREC—NBS in Germany in 2019, we have identified three unrelated individuals with four previously unreported *FOXN1* mutations. To elucidate the immunological consequences associated with these variants, we provide a detailed description of their longitudinal clinical courses alongside comprehensive immune

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characterization.

2. Methods

Following detection of three distinct newborns in national TREC—NBS program, T-cell lymphocytopenia was confirmed by multi-colour flow-cytometry. Subsequent panel-based exome sequencing led to the identification of four previously unreported *FOXN1* mutations in these newborns. To comprehensively characterize the clinical and immunological consequences of these variants, detailed clinical data, longitudinal flow cytometric immunophenotyping, stimulated T-cell proliferation assays, and TCRVβ repertoire analyses were systematically evaluated over an observation period extending through May 2025.

In addition, single-cell RNA-sequencing (scRNA-seq) was performed on sorted CD45+ lymphocytes from peripheral blood mononuclear cells (PBMCs) obtained from two of the three patients and from a healthy cord blood (CB) donor.

2.1. Ethics statement

The study protocol was accepted by the institutional review board of the University of Düsseldorf (study numbers: 2020_1201, 2020_1201_1, 2020_1201_2, 2021_1716, 2019–383) and is registered as Clinical Trial: DRKS00032712. The study is in accordance to the Declaration of Helsinki. Informed consent was obtained in all cases.

2.2. Single-cell RNA sequencing

2.2.1. PBMC isolation

CB (1:1) and peripheral blood (PB) of paediatric patients (1:5) were diluted with sterile 1xPBS (Gibco) and mononuclear cells (MNCs) were isolated by density gradient centrifugation (Lymphocyte separation Medium 1077, PromoCell). Remaining erythrocytes were lysed with ice-cold ammonium chloride solution (pH = 7.4, University Clinic Düsseldorf) and washed two times afterward. MNCs were counted and cryo-preserved for scRNA-seq analyses.

2.2.2. Single-cell RNA sequencing (scRNA-seq) using BD Rhapsody

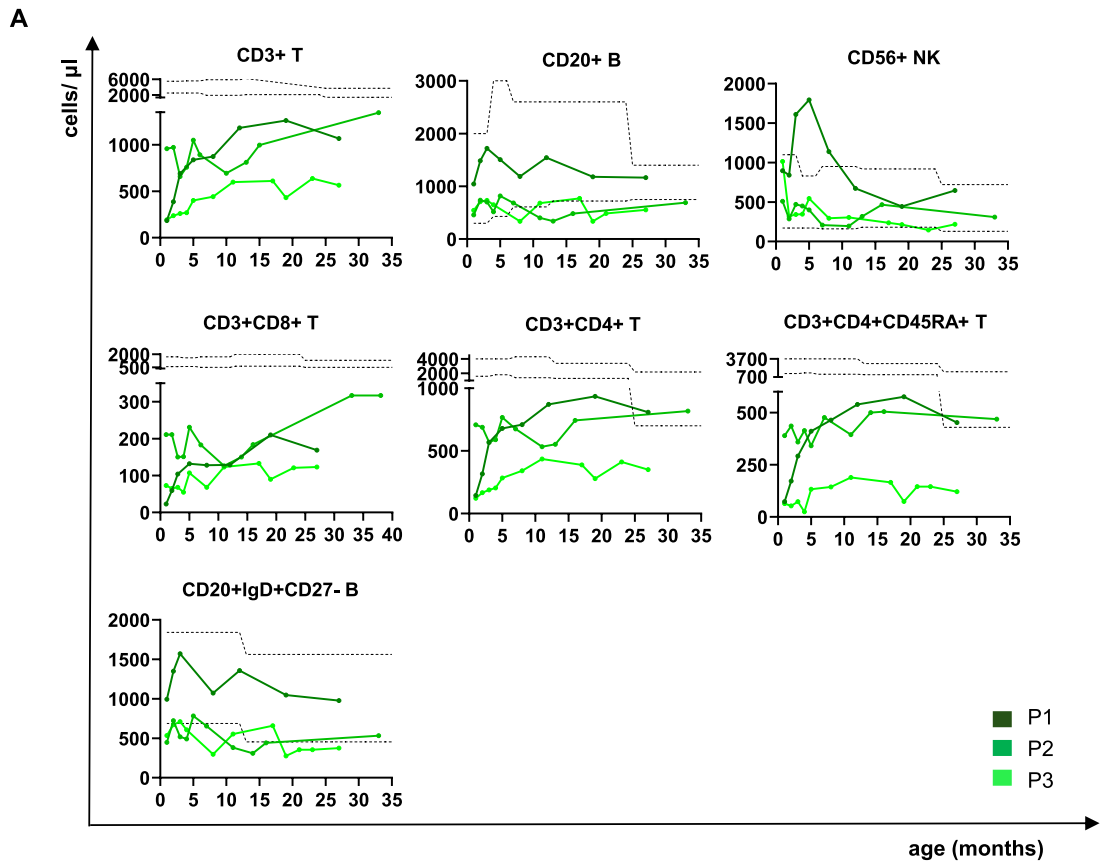
The two patient samples (P1 and P3) and one CB were thawed and stained with CD45-APC—Cy7 (HI30). CD45+ lymphocytes were sorted and for each of the three sample four multiplexing tags were used. All cells were pooled with identical cell numbers. The pooled samples were stained with the BD AbSeq Immune Discovery Panel plus additional AbSeq antibodies (anti-CD34 (clone: 581), anti-CD117 (clone: YB5.B8), anti-CD1a (clone: HI149), anti-CD94 (clone: HP-3D9), CD336 (NKp44, clone: p44–8), CD294 (CRTH2, clone: BM16) (all from BD Bioscience). The Rhapsody device was loaded with a total of 40.000 multiplexed and AbSeq-stained MNCs. The following single-cell capture and cDNA synthesis as well as exonuclease treatment was performed according to the manufacturer`s instructions and published protocol [8]. After these steps, the cDNA sample was stored at 4 °C for the library preparation and NGS. Library preparation and sequencing was performed at PRECISE, DZNE, Bonn (NovaSeq_S4, 200 bp. v1.5). The BD Rhapsody Sequence Analyses Pipeline (https://bd-rhapsody-bioinfo-docs.genomics.bd.com/top_introduction.html) was used with whole transcriptome analyses (WTA) and AbSeq Oligo labelling.

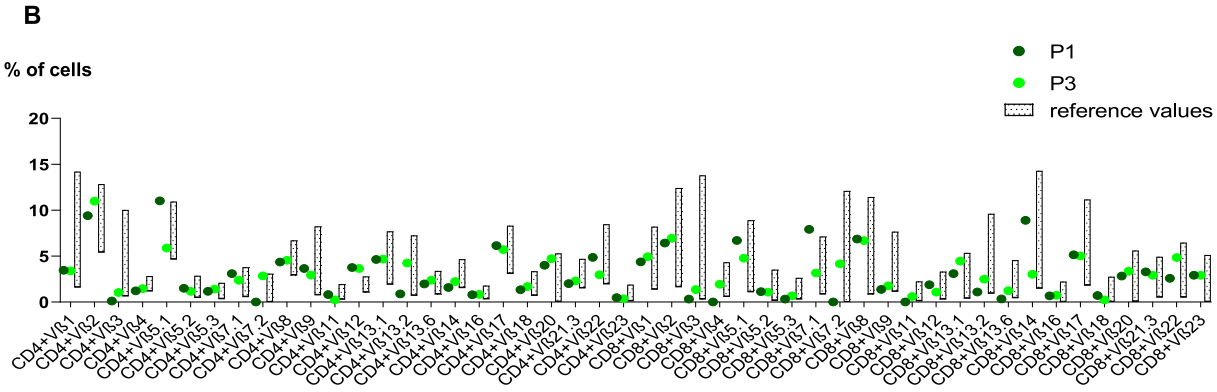
2.2.3. scRNA-seq analyses

Seurat (Version_4.3.0) was used for scRNA-seq analyses [9,10] with R (Version 4.2.1) [11] and R studio (Versions 2022.07.2) [12] After quality analyses with Seurat, a total of 3717 cells and 3685 cells remained post-filtering with a mitochondrial percentage (percent.mito) cut-off at 25, nFeature_RNA between 200–10,000, and n_count_RNA > 500. Despite equal sample loading, most cells from the patient samples were lost during processing and quality controls. One aspect was the high mitochondrial content in the patient samples, another factor might

Table 1
Characteristics of FOXN1 patients identified through TREC-based newborn screening (P1–P3). Shown are gender, TREC levels (copy number of T-cell receptor excision circles in newborn screening), genetic and clinical findings, implemented treatments, T-cell proliferation capacity (measured by CFSE = carboxyfluorescein succinimidyl ester assay), TCR Vβ repertoire, response to vaccinations, and outcome at the last follow-up (FU).

Patient	Gender	TREC	Familiy history	Ethnicity	Genetics	Clinical findings	Treatments	CFSE	TCR Vß	Vaccinations	Outcome at last FU
P1	male	absent	Father: asthma bronchiale and frequent infections in infancy, naildystrophy frequent in his familiy, A mother's cousin diagnosed with cystic fibrosis	Turkish	c.1778delG; p.Gly393Alafs*157 (het)	nail dystrophy, transient hypogammaglobulinemia	TMP-SMX and fluconazole until age 5M	normal	single CD8 families missing (age 1 M)	protective titers against inactivated vaccines, live vaccines without adverse events	alive and well at 27M
P2	male	Low (not absent)	Mother's grandmother: Hashimoto thyroiditis	mixed Croatian and German	c.830+1G>T; p.? (het)	beningn familial macrocephaly, transient hypogammaglobulinemia, transient neutropenia in first 2 years of life (nidar 414 neutrophiles/µl)	none	normal	not performed	basic immunization not yet completed due to incompliance	alive and well at 33M
P3	female	absent	Mother: atopic dermatitis and asthma bronchiale, two episodes of pneumonia after pregnancy. Maternal grandmother: multiple sclerosis, Materinal aunt and her children: long QT syndrome	German	c.1318C>T; p.Gln440* (het.) and c.668T>G; p.? (het.)	nail dystrophy, sparse hair growth, patent foramen ovale	TMP-SMX still continued, fluconazole until age 17M	normal	normal (age 3 M)	protective titers against inactivated vaccines, live vaccines not yet administered due to persistent low T-cell counts	alive and well at 27M





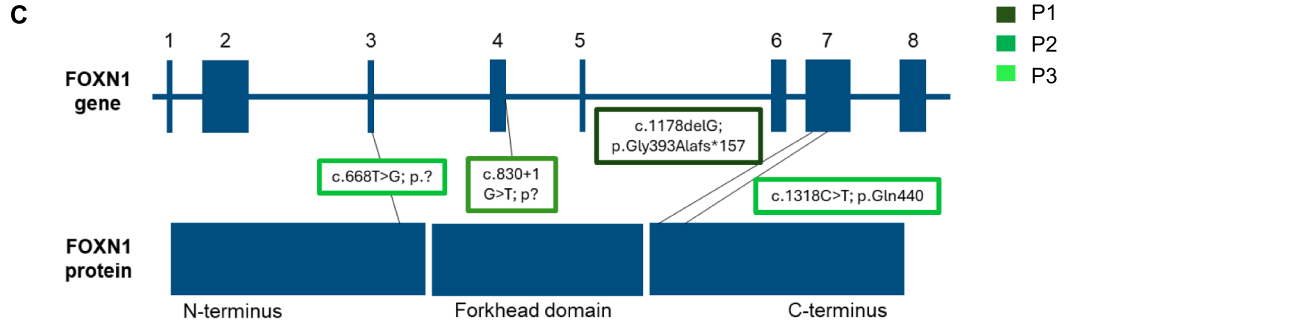


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Fig. 1. Immune characterization of *FOXN1* patients. A Lymphocyte subsets in *FOXN1* patients. Longitudinal analysis of the number of peripheral $CD3^+$ T cells, $CD20^+$B cells, CD56+ NK cells, $CD3^+CD8^+$/ $CD3^+CD4^+$ and $CD3^+CD4^+CD45RA^+$ T-cell subsets, and the $CD20^+IgD^+CD27$- naïve B-cell subset. Patients P1, P2, and P3 are represented in dark, medium, and light green, respectively. Age-matched reference values are shown as a black dashed line (*Huck et al., Clinical Immunology, 2009* for $CD20^+IgD^+CD27^-$ B cells; and *Shearer et al., Journal of Allergy and Clinical Immunology, 2003* for the remaining lymphocyte subsets). B TCR Vβ repertoire analysis in P1 and P3, analysed by flow cytometry. TCR Vβ repertoire was analysed in $CD4^+$ and $CD8^+$ subsets for these two patients (P1 = dark green dots, P3 = light green dots). The bars indicate the reference values for each Vβ family applied (*McLean-Tooke et al., Clinical & Experimental Immunology, 2008*) C *FOXN1* gene mutations identified in the patients. Their positions are illustrated on both the *FOXN1* gene (subdivided into the exons 1–8) and the encoded *FOXN1* protein (with its N-terminal, forehead and C-terminal domain).

be that the FOXN1 mutations might lead to increased cell death after freezing. RunPCA was used with VariableFeatures, ElbowPlot, and JackStrawPlot to determine 9 dimensions (dims) for RunUMAP. Clustering was performed with FindNeighbors (9 dims) with a resolution of 0.45 for the scRNA-seq data. Cluster markers were calculated with FindAllMarkers and the top 10 differentially expressed genes are displayed. The R code used for the scRNA-seq analyses was generously provided by Dr. Jonas Schulte-Schrepping [13] and modified for the purpose of this study. The code for Ab-seq data was taken from https://satijalab.org/seurat/archive/v3.2/multimodal_vignette.html.

2.2.4. Data and material availability

Sequencing data of scRNA-seq are being uploaded and will be deposited at the European Genome-phenome Archive (EGA). The Accession Number is EGAS50000001199.

3. Results

3.1. Clinical findings

All three patients (P1–P3) were term newborns, born to non-consanguineous parents, appearing clinically well at birth. In all cases, abnormal TREC—NBS results prompted referral to our centre: TREC copies were undetectable in P1 and P3 and markedly reduced in P2.

Details on ethnicity, family history and clinical findings of all three patients are provided in Table 1. Notably, family history revealed atopic conditions (P1 and P3) and autoimmune diseases (P2 and P3) in two out of three families each, and nail dystrophy was reported as a frequent finding in the family of P1 additionally. Clinically, nail dystrophy was observed in P1 and P3, whereas sparse hair growth was noted only in P3.

3.2. Immune characterization

Immunophenotyping confirmed T-cell lymphocytopenia affecting $CD8^+$, $CD4^+$, and naïve $CD4^+$ subsets in all three patients (Fig. 1A). Over time, T-cell counts increased significantly in P1 and P2, while remaining persistently low in P3, without occurrence of immune deregulatory symptoms in any of the patients. $CD20^+$ B-cell counts, including memory (IgD^-CD27^+) B-cells, were borderline low in P2 and P3 but within age-appropriate ranges in P1. $CD56^+/CD16^+$ NK-cells demonstrated normal counts in all cases, except for a significant increase of unclear aetiology during the first 5 months in P1. Furthermore, P1 and P3 both developed transient hypogammaglobulinemia, which was self-limiting in both cases and did not necessitate any immunoglobulin substitution. (Table 1)

Functional T-cell proliferation in response to PHA, anti-CD3, and anti-CD3/CD28 stimulation was preserved in all patients (Table 1). Only minor skewing of TCR Vβ families was noted in the $CD8^+$ compartment of P1, while it was generally normal in P3 (Fig. 1B). In P2, TCRVβ repertoire was not analysed because this patient exhibited only mild T-cell lymphocytopenia.

3.3. Genetics

Targeted exome sequencing revealed heterozygous *FOXN1* mutations in P1 (c.1178delG; p.Gly393Alafs157*)* and P2 (c.830+1G>T; p.?), both predicted to cause loss of function through nonsense-mediated decay or splice disruption respectively (Fig. 1C). P3 carried two compound heterozygous variants: a maternally inherited nonsense mutation (c.1318C>T; p.Gln440) likely triggering mRNA decay, and a paternally inherited missense or splice-disrupting variant (c.668T>G; p.?), most probably resulting in cryptic splicing and impaired protein function. Initially, the combination of biallelic mutations and profound T-cell lymphocytopenia in P3 raised concern for a null genotype and indication for definitive therapy. However, gradual immunological improvement made corrective treatment unnecessary. Although the father was clinically asymptomatic, his variant may contribute to the pronounced phenotype in the child.

3.4. Clinical management

Prophylactic treatment with fluconazole and trimethoprim-sulfamethoxazole (TMP-SMX) was initiated in P1 and P3 (Table 1). In P1, both agents were discontinued following immunological improvement by 5 months of age. In P3, fluconazole was stopped at 17 months, while TMP-SMX prophylaxis remains ongoing due to persistently low T-cell counts. Both patients demonstrated protective antibody responses to inactivated vaccines, enabling the safe administration of live vaccines in P1. In contrast, P3's profound T-cell lymphocytopenia precluded the use of live vaccines. In P2, prophylaxis was not initiated given the mild and improving T-cell lymphocytopenia. However, routine immunizations including live vaccines remain incomplete due to non-compliance. To date, none of the patients have experienced infectious or immunological complications under these precautionary measures.

3.5. Single cell RNA-sequencing

Notably, the number of viable cells retained for scRNA-seq analysis was markedly reduced in *FOXN1*-deficient patients, despite identical sample processing compared to healthy CB. (supplementary Figure S1) While almost no T cells could be detected (Fig. 2A and B), the analysis revealed striking transcriptional differences between B cells from both FOXN1 patients and CB.

FOXN1 B cells showed significantly higher expression of genes controlling B-cell identity and signalling, including the lineage-defining transcription factors EBF1 and PAX5, [14,15] the survival-associated regulator MEF2c, [16] and modulators of BCR signalling such as BANK1 and LYN (Fig. 2C) [17] Additional upregulated genes (AFF3, FCHSD2, RALGPS2, SIPA1L3) were linked to transcriptional control, cytoskeletal organisation and intracellular signalling [18–21] In contrast, CB B cells preferentially expressed genes involved in antigen presentation and humoral immune function, including CD74, HLA class II genes (DRB1, DPB1, DPA1, DRA), as well as core BCR components (CD79A, IGHM), the proliferation-associated gene POLA1, and MS4A family members (Fig. 2C) [22,23]

FOXN1 B cells further displayed increased expression of activation-associated pathways, including BTLA, IL4R, complement receptors (CR1, CR2), innate sensing via TLR1, NFκB regulation by TANK, and prominent upregulation of mTOR, indicating enhanced metabolic and activation readiness (Fig. 2D and E) [24–27]

Together, these data suggest that FOXN1 B cells are transcriptionally biased towards signalling modulation and activation, whereas CB B cells favour antigen presentation and proliferative capacity.

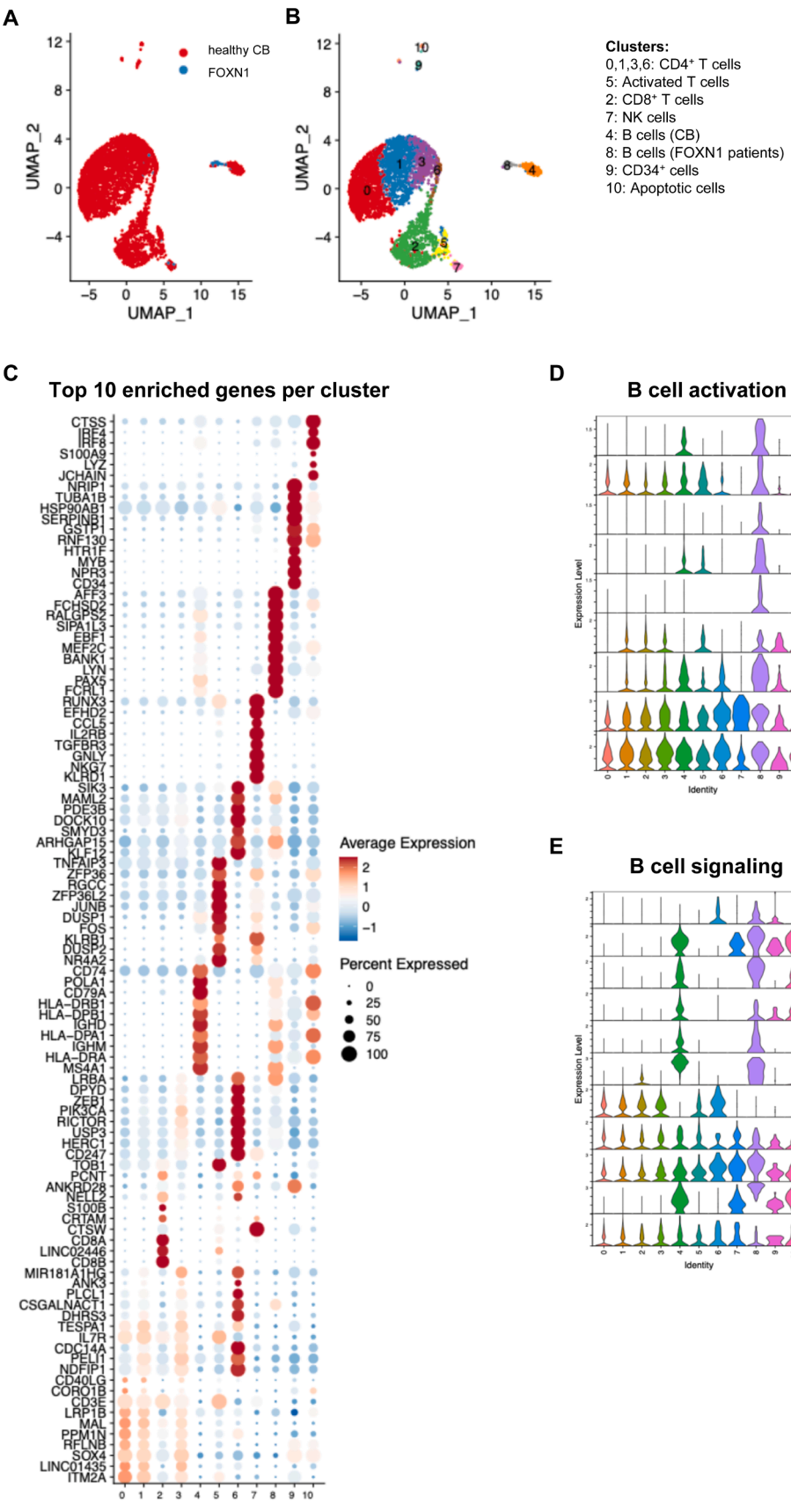

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Fig. 2. Single-cell RNA sequencing of PBMCs from *FOXN1* patients (P1 and P3) and a healthy cord blood donor (CB). A UMAP visualization shows cells from the *FOXN1* patients (blue) vs. healthy donor (red), along with B lymphocyte subset populations (clusters 0–10) indicated by colour: $CD4^+$ T cells: cluster 0, 1, 3, 6; activated T cells: cluster 5; $CD8^+$ T cells: cluster 2; NK cells: cluster 7; B cells of CB donor: cluster 4; B cells of FOXN1 patients: cluster 8; $CD34^+$ cells: cluster 9; apoptotic cells: cluster 10. C Dot plot displays the top 10 enriched genes per cluster. D Violin plots show expression levels of genes involved in B-cell activation (BTLA, IL4R, CR2, CR1, TLR10, IRAK4, TANK, CBLB, CD56) E and signalling (MTOR, SYK, BLNK, BTK, CD79B, CD79A, PLCG1, AKT2, INPP5D, LYN, RFTN1).

4. Discussion

Our cohort of three individuals carrying four novel *FOXN1* mutations detected through TREC—NBS exhibited a broad phenotypic spectrum of non-SCID *FOXN1* immunodeficiency. Clinical manifestations ranged from mild, transient T-cell lymphocytopenia to more severe and persistent forms, underscoring the need for highly individualized management strategies.

Specifically, the two patients with heterozygous *FOXN1* variants (P1 and P2) fit well within the established spectrum of transient T-cell lymphocytopenia with or without nail dystrophy. In contrast, one patient with biallelic *FOXN1* mutations (P3) displayed more pronounced T-cell lymphocytopenia yet did not require thymus transplantation. This observation suggests a larger phenotypic range than widely recognized and highlights the complexity of clinical decision-making in *FOXN1* deficiency [28] Because FOXN1 is an intracellular protein expressed in thymic epithelial cells and cannot be readily assessed in affected patients, the identification of reliable biomarkers of disease severity will be essential. Moreover, the development of evidence-based treatment guidelines – similar to those established for thymic disorders such as 22q11.2 deletion syndrome – remains critical to ensure optimal care [29,30]

At the cellular level, our scRNA-seq data reveal a previously undescribed B-cell subset in *FOXN1*-deficient patients with an immature transcriptional signature and a shift toward an activation-ready state, with increased expression of genes involved in co-stimulatory signalling and innate-like activation. These changes suggest that B cells in *FOXN1*-deficient patients might be primed for rapid responses, potentially reducing their reliance on classical T-cell-dependent activation pathways. In contrast, CB B cells displayed a transcriptional program dominated by genes associated with antigen presentation and proliferation, reflecting their dependence on T-cell cooperation.

This divergence highlights the plasticity of B-cell functional states and illustrates how severe thymic dysfunction could reshape immune programming. Emerging evidence further suggests that thymic disorders may exert direct effects beyond T-cell compartments, influencing populations such as innate lymphoid cells and $CD56^{dim}$ NK cells, which opens the possibility of alternative explanations for the distinct B-cell phenotype observed [31] One recent study in patients with 22q11.2 deletion syndrome using scRNA sequencing demonstrated widespread transcriptional alterations across multiple hematopoietic lineages, including B cells. Notably, B cells exhibited marked gene-expression remodelling, supporting the concept that B-cell abnormalities in thymic disorders are not solely attributable to reduced T-cell help but reflect broader immune reprogramming. In line with this observation, the immature and activation-biased B-cell state identified in FOXN1-deficient patients in our cohort is likely a secondary consequence of thymic dysfunction rather than a primary B-cell–intrinsic defect [32] Future investigations should address whether this distinct B-cell population in *FOXN1*-deficiency influences antibody repertoire diversity or predisposes to dysregulated responses, thereby informing therapeutic strategies aimed at restoring balanced immunity.

In conclusion, the management of newborns with *FOXN1* immunodeficiency identified via TREC-screening remains challenging, as uncertainty regarding optimal care for non-SCID conditions persist. Ongoing investigation into the underlying immunological alterations, improved risk stratification, and the development of evidence-based management protocols remain critical future challenges for clinical immunology.

CRediT authorship contribution statement

Lea Graafen: Writing – original draft, Visualization, Resources, Investigation, Data curation. **Arndt Borkhardt:** Writing – review & editing, Resources. **Julian Reiß:** Methodology, Investigation. **Stavrieta Soura:** Resources. **Hans-Jürgen Laws:** Resources, Investigation. **Markus Uhrberg:** Writing – review & editing, Resources. **Stefan Paulusch:** Methodology. **Elena De Domenico:** Methodology, Investigation. **Marc D Beyer:** Writing – review & editing, Methodology, Investigation, Data curation. **Sabrina B. Bennstein:** Writing – original draft, Visualization, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Sujal Ghosh:** Writing – original draft, Supervision, Resources, Conceptualization.

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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.imlet.2026.107142.

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