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A multiomics approach to primary immunodeficiencies in human

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I. Abbreviations

AD	autosomal dominant
ADA	adenosine deaminase
ALPS	autoimmune lymphoproliferative syndrome
AMI	acute myocardial infarction
APC	antigen presenting cells
AR	autosomal recessive
ATF6	activating transcription factor 6
BiP	binding immunoglobulin protein
BUN	blood urea nitrogen
CBC	complete blood count
CGD	chronic granulomatous disease
CHOP	C/EBP homologous protein
CID	combine immunodeficiency
CSR	class switch recombination
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte–associated antigen 4
DC	dendritic cell
DM	diabetes mellitus
ECM	extracellular matrix
eIF2α	eukaryotic initiation factor 2α
ER	endoplasmic reticulum
ERAD	endoplasmic-reticulum-associated protein degradation
ERSE	ER stress response element
FHL	familial hemophagocytic lymphohistiocytosis
FTT	failure to thrive
G-CSF	granulocyte-colony stimulating factor

GO	Gene Ontology
GOF	gain-of-function
GRP	glucose regulated protein
GSEA	Gene Set Enrichment Analysis
HPV	Human Papillomavirus
HSCT	hematopoietic stem cell transplantation
HYOU1	Hypoxia up-regulated protein 1
ICAM	intercellular adhesion molecule
IEI	inborn error of immunity
Ig	immunoglobulin
IL-1R	interleukin-1 receptor
INF-γ	interferon gamma
IRE1α	type 1 transmembrane protein inositol requiring 1
ITGAL	integrin Subunit Alpha L
ITGB2	integrin Subunit Beta 2
IUIS	International Union of Immunological Societies
IVIG	intravenous immunoglobulin
JNK	c-Jun amino-terminal kinase
LAD	leukocyte adhesion deficiency
LFA-1	lymphocyte function-associated antigen 1
LOF	loss-of-function
NK cell	natural killer cell
Ox-LDL	oxidizedl ow-density lipoprotein
PAMP	pathogen-associated molecular pattern
PD-1	programmed cell death 1 receptor
PERK	protein Kinase R-Like ER Kinase
PID	primary immunodeficiency disorder

PIDD	primary immunodeficiency disorder
PIRD	primary immune regulatory disorder
RNA-seq	RNA sequencing
ROS	reactive oxygen species
SCID	severe combined immunodeficiency
SCIG	subcutaneous immunoglobulin
scRNA-seq	single-cell RNA sequencing
SLE	systemic lupus erythematosus
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TCR	T cell receptor
TLN1	talin-1
TLR	Toll-like Receptor
UPR	unfolded protein response
VCAM-1	vascular cell adhesion molecule 1
VLA-4	very late antigen-4
WAS	Wiskott–Aldrich syndrome
WES	whole exome sequencing
WGS	whole genome sequencing
XBP1	X-box protein-1

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III. Summary (In French)

Mon travail de thèse, composé de deux projets, examine les déficits immunitaires primitifs chez l'homme, en se concentrant sur les rôles de deux gènes : *HYOU1* et *ITGAL*. Dans les deux projets, nous avons étudié des patients nés de parents consanguins et les membres de leur famille. Ces patients ont été étudiés en raison de la gravité de leurs symptômes ou de l'absence de réponse aux traitements. En effectuant un séquençage de l'exome et une analyse de ségrégation, notre équipe a découvert deux variants chez les patients : l'un dans la protéine 1 régulée par l'hypoxie (*HYOU1*) et l'autre dans la sous-unité alpha-L de l'intégrine (*ITGAL*). Mon objectif était de démontrer une corrélation génotype-phénotype et d'étudier l'impact de ces variants dans les voies biologiques associées, proposant ainsi des stratégies thérapeutiques potentielles pour des traitements futurs. J'ai d'abord démontré l'absence de la protéine, puis j'ai cherché à décrire plus en détail le phénotype et à explorer les voies dérégulées en fonction des connaissances actuelles sur la fonction de ces gènes par une approche multi-omique.

Projet 1 : Des analyses multi-omiques révèlent un arrêt de la différenciation des cellules B et une hypogranulation des neutrophiles chez un patient atteint d'une immunodéficience causée par un nouveau variant faux-sens homozygote dans *HYOU1*.

Introduction :

La protéine 1 régulée par l'hypoxie (HYOU1), également connue sous le nom d'ORP150 (Oxygen Regulated Protein 150) et GRP170 (Glucose Regulated Protein 170), est une protéine anti-apoptotique impliquée dans la protection des cellules lors de situations de stress physiologique, en particulier dans le stress du réticulum endoplasmique (Rao et al., 2021). Cette protéine de 150 kDa est régulée à la hausse en raison du stress du réticulum endoplasmique (RE), de l'hypoxie, de l'ischémie et de la carence en glucose, ce qui lui confère un rôle crucial dans la gestion du stress du RE et la prévention de l'apoptose (Kusaczuk & Cechowska-Pasko, 2013). De plus, HYOU1 est impliquée dans la réponse aux protéines non repliées (UPR), qui est activée par l'accumulation de protéines non ou mal repliées dans le RE (Kusaczuk & Cechowska-Pasko, 2013).

Dans cet article, je présente un nouveau patient présentant un nouveau variant homozygote du gène *HYOU1*, située dans le domaine de liaison aux protéines, et présentant des symptômes d'immunodéficience et de troubles métaboliques. J'ai cherché à étudier ce patient par une approche multi-omique afin de mieux comprendre le rôle du gène *HYOU1* dans la physiopathologie de la maladie.

Résultats :

Le patient dont il est question dans ce projet est un garçon de 5 ans d'origine iranienne, présentant une diarrhée chronique avec des exacerbations intermittentes associées à des épisodes d'hypoglycémie depuis la petite enfance. Il a été évalué pour un déficit immunitaire suspectée à l'âge de 6 mois en raison d'un retard de croissance, ainsi que d'épisodes de diarrhée et de fièvre. Il est actuellement traité par immunoglobuline intraveineuse et facteur de stimulation des colonies de granulocytes (G-CSF).

En raison de la consanguinité des parents, notre équipe a effectué un séquençage de l'exome chez le cas index, ses parents et son jeune frère sain, révélant un variant faux-sens homozygote, c.1331C>A (NM_001130991.3) ; p.Pro444His (NP_001124463.1) dans le gène *HYOU1*. L'analyse in silico a confirmé la pathogénicité prédite de ce variant sur la structure protéique, entraînant un conflit stérique avec les résidus voisins.

Lors de l'immunophénotypage, notre équipe a observé une hypogranularité dans les neutrophiles et une réduction marquée du nombre de cellules B. J'ai confirmé la diminution significative de l'expression de HYOU1 dans les fibroblastes dermiques du patient par rapport aux témoins par western blot. Cependant, *HYOU1* était exprimé de manière significative au niveau transcriptionnel.

En adoptant une approche multi-omique, j'ai cherché à étudier l'impact de ce variant dans les voies biologiques associées au sein des cellules mononucléés du sang périphérique (PBMC), de la moelle osseuse et des fibroblastes dermiques, en utilisant le séquençage d'ARN en bulk, la protéomique et le séquençage d'ARN en cellules uniques (scRNA-seq).

J'ai découvert une dérégulation de certaines voies de l'immunité innée, telles que les voies de signalisation du TNF, la signalisation par les interleukines et les voies d'activation des granulocytes, lors de l'analyse transcriptomique des PBMC du patient par rapport

aux témoins. L'analyse protéomique des PBMC a également révélé une dérégulation de l'inflammation médiée par la voie de signalisation des chimiokines et des cytokines.

De plus, j'ai induit un stress du RE dans les fibroblastes dermiques du patient et des témoins, puis comparé leur profil transcriptomique après six heures d'exposition à la tunicamycine, révélant une régulation négative significative du transcrit de HYOU1 dans des conditions de stress et suggérant son implication dans une boucle rétroactive autorégulatrice. Dans le scRNA-seq du sang, j'ai observé un groupe distinct composé de neutrophiles du patient avec un profil transcriptionnel différent de celui attendu chez les individus recevant du G-CSF. Enfin, dans le scRNA-seq de la moelle osseuse, j'ai montré que le développement de la lignée des cellules B est arrêté avant le stade pro-B chez le patient.

Conclusion :

Dans cette étude, un nouveau variant du gène *HYOU1* est rapporté. Nos recherches, utilisant une approche multi-omique, apportent des informations clés sur l'impact biologique de ce variant. La démonstration de l’arrêt du développement des cellules B établit un lien clair entre la mutation génétique et le phénotype immunologique du patient. De plus, ces résultats soulignent l’importance de la réponse aux protéines non repliées (UPR) dans le développement des cellules B. Notre expérience de stress du RE a démontré une régulation négative de *HYOU1* dans des conditions de stress, suggérant son implication dans une boucle rétroactive autorégulatrice. Dans l’ensemble, ces résultats impliquent HYOU1 dans la régulation immunitaire et les réponses cellulaires au stress, fournissant de nouvelles perspectives sur les mécanismes moléculaires sous-jacents à la présentation clinique du patient. Ce travail contribue à une meilleure compréhension des déficits immnitaires associés aux variants de HYOU1, ce qui pourrait guider de futures stratégies thérapeutiques.

Projet 2 : L'étude d'une famille atteinte d'épidermodysplasie verruciforme révèle le rôle de la sous-unité α-L de l'intégrine dans l'infection par le Virus du Papillome Humain (HPV)

Introduction :

L'antigène 1 associé à la fonction lymphocytaire (LFA) est une intégrine principalement présente sur les lymphocytes T, les neutrophiles et les monocytes. Comme les autres intégrines, elle est composée d'une sous-unité α (αL, CD11a, codée par le gène ITGAL) et d'une sous-unité β (β2, CD18, codée par le gène ITGB2) (Walling and Kim, 2018). LFA-1 se lie à ICAM-2 et joue un rôle crucial dans l'adhésion des leucocytes à l'endothélium, participant ainsi à la migration des leucocytes vers le site d'infection (Walling and Kim, 2018). Dans ce projet, nous discutons d'une famille présentant un variant nonsens de *ITGAL*, qui présente une épidermodysplasie verruciforme résistante ainsi qu'une infection récurrente par le HPV.

Résultats :

La patiente de cette étude est une femme de 38 ans d'origine iranienne, née de parents consanguins, présentant des lésions eczémateuses disséminées, caractérisées par des papillomatoses à sommet plat ou bosselé, rappelant l'épidermodysplasie verruciforme, principalement sur ses extrémités. Le père de la patiente et son oncle paternel présentent également des symptômes similaires. En raison de la persistance de ses symptômes, notre équipe a réalisé un séquençage de l'exome pour cette famille, qui a révélé un variant homozygote nonsens (c.1492C>T, p.Gln498*) dans la sous-unité alpha L de l'intégrine (*ITGAL*) chez la patiente et son père. Les biopsies obtenues à partir des lésions d'épidermodysplasie verruciforme et des lésions verruqueuses ont montré une infection par les β-HPV et le sous-type 159 du HPV (γ-HPV), respectivement. En immunophénotypage, notre équipe a noté que la fréquence des cellules T CD4 est significativement dérégulée chez les patients par rapport aux témoins. De plus, l'absence de CD11a et l'expression normale de CD18 chez la patiente et son père ont été confirmées par cytométrie en flux. Les analyses par western-blot ont montré une absence de protéine ITGAL dans les cellules T CD8+ de la patiente et de son père. Dans l'analyse protéomique, j'ai observé une régulation négative significative de la voie de liaison des

molécules d'adhésion. Dans le séquençage d'ARN à cellule unique (scRNA-seq) du sang, ITGAL était principalement exprimé par les cellules T. En somme, ces résultats suggèrent que ce variant d'ITGAL a un impact sur la réponse immunitaire, entraînant des altérations de la fonction d'adhésion, qui est essentielle à la migration des cellules T vers le site de l'infection.

Conclusion :

Cette étude identifie un nouveau variant non-sens (c.1492C>T, p.Gln498*) dans le gène *ITGAL*, contribuant au dysfonctionnement immunitaire chez deux patients présentant une épidermodysplasie verruciforme récurrente et sévère. L'absence de la protéine ITGAL, confirmée par western-blot et cytométrie en flux, perturbe probablement l'adhésion et la migration des lymphocytes T, soulignant ainsi son rôle crucial dans des réponses immunitaires efficaces. Nos résultats suggèrent que ce variant de *ITGAL* altère la fonction immunitaire, entraînant une infection chronique et la formation de lésions, et offrent des perspectives qui pourraient guider de futures études fonctionnelles et le développement de thérapies ciblées.

1. Introduction

Considering the importance of genomics in the discovery, diagnosis and management of the inborn errors of immunity (IEIs), our laboratory initiated the Genomax platform established in 2011 aiming at conducting an approach based on next generation sequencing for immune related genetic disorders. Since 2019, we have commenced a cohort consisted of roughly 1000 patients from Iran, where the rate of consanguinity is rather high - ranging from 23% in Gilan to 78% in Sistan and Balouchestan (Jalal Abbasi-Shavazi et al., 2008). The patients enrolled into this cohort are submitted by collaborator clinicians and are suspected for an underlying IEI. The first step in our approach in this cohort includes panel sequencing according to the latest update given by the International Union of Immunological Societies (IUIS) of the IEIs. Should no pathogenic germline variant be detected in the results of panel sequencing, we will perform whole exome sequencing (WES) for the proband and his family (including parents, sibling, and other affected family members in case of consent). In turn, we will search for candidate genes by applying a filtering strategy based on allele frequency in reference population, zygosity, and predictive tools of pathogenicity to identify novel candidate genes for IEIs. After identification of the candidate genes, we aim to characterize the impact of the recognized variant and design experiments to better investigate whether the identified variant could lead to a dysfunctional protein responsible of the immune deficiency. Our approach to this investigation includes multiomics analyses, as well as functional assays indicating the pathogenicity of the discovered variants.

This thesis is focused on the multiomics analyses in two Iranian probands, who had a potentially pathogenic variant in either of these two genes: *HYOU1* and *ITGAL.* Although there have been previous reports of individuals with pathogenic variants in *HYOU1* demonstrating immune deficiency, the investigated patient demonstrates eccentric phenotype which adds to the phenotypic spectrum of this type IEI. On the other hand, defects in *ITGAL* have not yet been reported as an IEI; therefore, making *ITGAL* a novel IEI candidate gene.

To better understand the role of *HYOU1* and its potential in causing immune deficiency, I will introduce HYOU1 role and function followed by a description of defects in apoptosis

leading to IEIs, prior to presenting the results of this project. In turn, I will describe the IEIs in integrins and introduce immune related pathways in which *ITGAL* plays a key role, followed by the results aiming to prove the pathogenicity of the identified *ITGAL* variant.

2. Introduction to Primary Immunodeficiencies and Literature Review

Primary immunodeficiencies (PIDs) or inborn errors of immunity (IEIs) are a group of inherited immune disorders which manifest as increased susceptibility to any immune dysregulations (Tangye et al., 2020). The term ‘primary’ addresses the genetic background of these disorders and distinguishes them from the secondary immunodeficiencies resulting from external factors (e.g. infections, chemotherapy, malnutrition, and drug-induced immunocompromisation (Al-Herz et al., 2014; Notarangelo, 2010a). The pivotal factor in identification of an IEI lies in the discovery of an immunological abnormality rather than a clinical phenotype (Casanova & Abel, 2021; Notarangelo et al., 2020).In this regard, the phenotypic spectrum of IEI is expanded to any dysregulation in immune system, not only covering susceptibility to infections, but to include autoimmunity, autoinflammation, allergy, and predisposition to cancer (Tangye et al., 2020). Although traditionally classified as rare diseases, IEI tend to be underreported, particularly in regions with limited access to diagnostic tools and high rate of consanguinity (Picard et al., 2017).

Studying IEIs is one of the best approaches to elucidate the normal pathways of immune regulation and defense against pathogens. It contributes to shedding light on gene function and paves the way for identification of mechanisms and pathways underlying the development and initiation of immune response (Notarangelo et al., 2020). Furthermore, investigating IEIs contributes to development of therapeutic approaches in diseases other than primary immunodeficiencies therein the discovered pathway is involved.

2.1 History of primary immunodeficiencies

Prior to Pasteur’s presentation of microbial theory of diseases (germ theory) in 1865, the individuals who were deceased after an episode of fever were simply considered weak. Back in that time, owing to lack of knowledge regarding microorganisms and their impact on physiology and disease processes, fever was the only general sign of infection. In turn, the germ theory revolutionized the understanding of infectious diseases and their etiology. This theory was reinforced when Robert Koch discovered mycobacterium tuberculosis as the causative agent of tuberculosis in 1882 (Casanova & Abel, 2018). Since the establishment of the germ theory, it was widely accepted that life-threatening infections

were caused only due to invasion of the host by pathogens. Despite this, the question of interindividual variability in response to infections, or fever at the time, remained unresolved. Although it did not explain the different fate of individuals after infectious diseases in the same family, despite living in the same environmental condition and exposure to the same microbe, such as death of three daughters of Pasteur's out of his five children who were living in the hygienic conditions of the upper-class of the society, the germ theory remained the major explanation for death due to 'fever', which was later recognized to be a symptom of infection (Alcaïs et al., 2009).

After almost 30 years of Koch's discovery, in the work presented by Charles Nicolle from 1911 to 1917, the simultaneous existence of symptomatic and asymptomatic infections in the human described as 'unapparent infection'. Nicolle showed that replicating microbes are present in the tissues and organs of asymptomatic individuals who would never demonstrate the symptom of infection and are therefore not in incubation period (Alcaïs et al., 2009). Although Nicolle was awarded the Nobel prize in 1928 for detection of lice as the transmitter of epidemic typhus, he considered his major contribution to science to be the identification of unapparent infection (Casanova, 2015). The observation that only a small group of individuals demonstrate the symptoms of an infectious diseases following contamination with the germ, and even fewer individuals who are infected experience life-threatening infections expire due to infection paved the way for consideration of factors required for disease development other than contamination with a germ administration.

Prior to advent of antibiotics, most of individuals with IEIs died in infancy or childhood, as did many others without inherent disorders of immune system. However, the broad administration of antibiotics contributed to cure in those with healthy immune system. In turn, the question of interindividual variations in the course of infections with pathogens persisted, for a minority of children presented recurrent and multiple (or more severe) infection despite antibiotic treatment (Casanova & Abel, 2021). These recurrent infections were mainly caused by the same or similar pathogens, which later were discovered to be linked to the defective component in the immune system.

The first description of a phenotype which led to the definition of PIDs was reported by Bruton in 1952 (Casanova & Abel, 2021). The reported patient manifested 19 episodes

of pneumococcal infections coupled with agammaglobulinemia, and his condition improved upon intramuscular injection of γ-globulins (Notarangelo et al., 2020). In 1958, Hitzig et al. reported Swiss-type agammaglobulinemia in infants with life-threatening infections with no lymphocytes or γ-globulins. This condition was later introduced as severe combined immune deficiency (SCID) (Casanova & Abel, 2021). These two conditions laid the groundwork for the future discovery of T and B cells and the importance of humoral and cellular immunity in defense against pathogens (Casanova & Abel, 2021).

The first reports describing disorders of innate immunity were published. Kostmann et al reported the first cases of congenital neutropenia in 14 patients, with clinical and morphological description of agranulocytosis for six of them (Carlsson & Fasth, 2001a), he described this condition as ‘hereditary reticulosis’ in 1950. Later in 1956, he used the term ‘infantile genetic agranulocytosis’ (Carlsson & Fasth, 2001a). This condition is now described as Kostmann syndrome and is characterized by recurrent bacterial infections in the first months of life, accompanied by severe neutropenia and a maturation arrest after the promyelocyte stage (Carlsson & Fasth, 2001a). However, at that time neutropenia was not considered as an immune disorder, and it therefore was classified as a hematological disorder until 1999 when it was included in the international classification of PIDs (Notarangelo et al., 2020). In 1954, Janeway et al. described the first case of recurrent infection alongside an increased level of immunoglobulins (Notarangelo et al., 2020). In 1957, it was discovered that a defect in phagocytes causes this granulomatosis disorder (Notarangelo et al., 2020). In 1954, Aldridge et al. described a X-linked syndrome characterized by eczema, thrombocytopenia, and immunodeficiency in three brothers (Notarangelo et al., 2020).

Thymic hypoplasia, later defined as DiGeorge syndrome, was described in 1968 and characterized failure of formation of thymus due to defects in third and fourth pharyngeal arcs (Casanova & Abel, 2021; Notarangelo et al., 2020). As a result, the development of T cells could not be completed and impairment of cell mediated immunity and antibody response has been compromised (Notarangelo, 2010b). Mendelian disorders of compliment were described from 1960s onward as PID shortly after the formulation of other types in primary immune disorders (Casanova & Abel, 2021). Together with

anatomical defects leading to infections, the increasingly growing descriptions of the mentioned phenotypes contributed to bringing attention to intrinsic conditions of impairment in immunity, hence the term ‘primary immune deficiency’ was introduced (Fudenberg et al., 1970a). In the first classification of PIDs in 1970, the molecular basis of PIDs had not yet been elucidated (Casanova & Abel, 2021). Consequently, the PIDs were categorized into 14 classes according to their mode of inheritance, the class of deficient cells, and non-immunological clinical features accompanying the conditions. Despite their efforts, the authors expressed their dissatisfaction for the classification in their report and left variable immunodeficiencies as ‘largely unclassified’, which in light of the advancements in molecular immunology now seems justified (Fudenberg et al., 1970a).

Although the identification of new primary immunodeficiency syndromes continued, the next major breakthrough in the study of primary immunodeficiencies was building the bridge between clinical phenotype and underlying genetic cause. The initial variants linked to a PID were found in 1986 and 1987, in the adenosine deaminase (*ADA*) and purine nucleoside phosphorylase (*PNP*) genes, both of which result in SCID (Notarangelo et al., 2020).

With advancements in molecular biology and discovering genes encoding the key proteins in the development and function of immune cells, our knowledge on underling etiology of PIDs expanded, which further elucidated the role and interrelation of immunological components with one another. For instance, *IL2RG* which encodes the interleukin-2 receptor (IL2R) common gamma chain (γ_c) is commonly shared among all receptors of IL-2 family (Notarangelo et al., 2020). Consequent to defects in IL-2 family signaling, development of T cells and natural killer (NK) cells is impaired and B cell function is additionally impacted (Notarangelo et al., 2020).

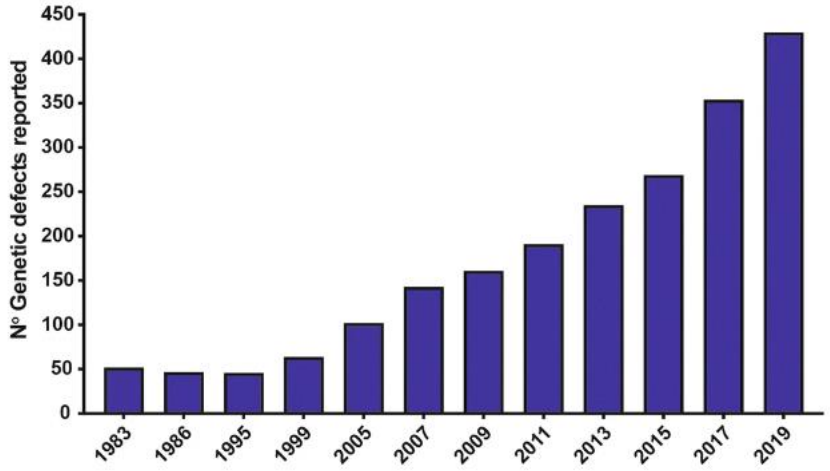

Figure 1. *The number of genes classified as IEI (PID) in each update of IUIS from 1983 to 2019. Figure adapted from Tangye et al., Journal of Clinical Immunology, 2020.*

Recent advancements in sequencing techniques facilitated genetic investigations in immune related diseases and contributed to an increase in the rate of discovery of novel PIDs. Therefore, the spectrum of PIDs was drastically broadened over the fifteen years (Figure 1) and included dysregulation in immune system happening as a result of monogenic mutations. Consequently, the PIDs were recognized as primary immunodeficiency disorders (PIDDs) and primary immune regulatory disorders (PIRDs) (Chan & Torgerson, 2020). In the 2019 update given by the IUIS, the term IEI replaced PID to advocate a broader range of phenotypes and encompass the imbalances of immune system such as autoimmunity, autoinflammations, allergies and malignancies (Tangye et al., 2020).

2.2 Definition and Overview

Inborn errors of immunity are defined as a group of monogenic genetic disorders which manifest as conferred predisposition to infectious diseases, autoimmunity, autoinflammatory diseases, allergy, bone marrow failure, and/or malignancy (Tangye et al., 2020).

From the early description of PIDs heterogeneity of their genetic inheritance was apparent. PIDs are generally monogenic conditions and have different patterns on inheritance – autosomal recessive, autosomal dominant, and X-linked. Most of the disease-causing variants could impact through complete (amorphic) or partial

(hypomorphic) loss-of-function (LOF), while some cause gain-of-function (GOF, hypermorphic). The very first described SCIDs were inherited through X-linked or autosomal recessive (AR) patterns. Moreover, IEIs can have complete (e.g. *RAG1*/*2*) or incomplete penetrance (e.g. *STAT3*). The majority of these variants cause diseases through LOF, whether amorphic or hypomorphic variants, and some variants exert their effect through GOF (Notarangelo et al., 2020).

At the time of discovery and initial classification, IEIs were traditionally classified as rare diseases when considered individually, as the prevalence was reported to be less than 1~50,000 (Notarangelo, 2010b). Nevertheless, with the growing discovery of IEIs and the expansion of the phenotypical spectrum of IEIs, as a group, they collectively have a prevalence of 1/2000 in the United States (Prince et al., 2020). The most common IEI is now considered to be IgA deficiency affecting 1~500 Caucasians (Murphy et al., 2022). It must also be considered that the prevalence of IEIs is higher in populations with high consanguinity rates and tends to be underdiagnosed in populations with limited access to diagnostic tools(Notarangelo, 2010b; Notarangelo et al., 2020).

2.2.1 Immune Components Affected in PIDs

Genetic mutations underlying IEIs cause defect in all classes of immune cells and pathways involved in the immunity (Tangye et al., 2022).

The fact of similarity of pathogens has been one of the earliest observations which has led to the classification of primary immunodeficiencies. With further studies and discoveries in the underlying molecular and cellular mechanism, it was highlighted that the type of pathogen is associated with class and function of the impaired component in the immune system (Murphy et al., 2022):

- **Defects in B cells and/or complement system:** recurrent respiration tract infections, chronic gastrointestinal infections, and meningitis with polysaccharide-encapsulated organisms are suggestive of deficiencies in humoral immunity or complements. The most common pathogens are *S. pneumoniae*, *H. influenzae* type b, and *N. meningitidis*, as well as *Giardia*, *Cryptosporidia*, and *Campylobacter*.

- **Defects in granulocytes:** recurrent infection in the soft tissue and/or invasive skin infections leading to abscesses are often indicative of defects in neutrophils. The most frequent organisms in causing infection in neutrophil disorders are *S.aureus*, gram-negative bacilli, *Aspergillus*, and *Nocardia*.
- **Defects in cell-mediated immunity:** infection with opportunistic pathogens and severe infections with typically “benign” viruses often indicate a deficiency in the T cell compartment. Moreover, defects in NK cells could possibly result in severe herpes virus infection.
- **Other disorders:** certain other types of infections are caused by impairment in components shared within classes. For instance:
    - Leukocyte adhesion deficiency (LAD), comprising of three types according to the mutated gene, leading to impairment in lymphocytes. Individuals with this condition are prone to severe viral and bacterial infections.
    - Hyperimmunoglobulin E syndrome (Job syndrome) resulting from defects in intracellular signalling pathways leading to predisposition to bacterial infections of respiratory tract and soft tissue.

The recurrent pathogens and type of infection is often taken into consideration during clinical evaluation as it facilitates the investigation in the genes whose function is related to the type of immunity implicated in fighting against a certain pathogen.

While consideration of type of infection may be beneficial in the diagnosis of the PID, it must be borne in mind that infections are the common symptoms of many PIDs and patients with other rarer types of immune dysregulation must be assessed for a presence of underlying PID in case of clinical suspicion. It must also be considered that the phenotypic impact of PIDs is quite diverse: Patients with some PIDs are prone to a variety of microbial infections, whereas some PIDs are causing predisposition to only one single infection (one gene one infection) (Alcaïs et al., 2009). While others are complex predisposition in other, where damaging mutations in multiple genes could lead to one infection (multiple genes, one infection) (Alcaïs et al., 2009).

2.2.2 Clinical Manifestations of PIDs

The clinical manifestations of IEIs are reflection of diversity of the genes underlying the condition. The typical clinical manifestation of IEIs is recurrent severe infections which according to the genes could manifest in infancy or later in life during adulthood. The most common infections are recurrent respiratory tract infection, recurrent gastrointestinal infection, failure to thrive (FTT) - in infants - and/or weight loss (Casanova & Abel, 2021; Tangye et al., 2022). Other more specific manifestations of IEIs include delayed wound healing and unexplained bronchiectasis (Casanova & Abel, 2021).

The non-infectious clinical manifestations of PIDs mainly include autoimmune disorders and malignancy (Tangye et al., 2022). These range from autoimmune thyroiditis to autoimmune haematological disorder such as haemolytic anemia, autoimmune thrombocytopenia, or autoimmune neutropenia (Tangye et al., 2022). Lymphoma (Hodgkin and non-hodgkin) is the most frequent malignancy in IEIs (Casanova & Abel, 2021; Murphy et al., 2022).

2.2.3 Epidemiology and Diagnosis

An underlying IEI is often suspected when the individual demonstrates “warning signs” of the primary immunodeficiencies (Table 1). The age at the time of diagnosis may vary according to the type of IEI and its involved immune component, for instance mild to moderate antibody defects, are often diagnosed in adulthood (García et al., 2010; Nelson & Lewis, 2010). The diagnosis of IEI could be made following a comprehensive clinical history, family history of recurrent infections, infant mortality, cancer, and autoimmune diseases (Cunningham-Rundles, 2010). Paraclinical investigations typically begins with laboratory assessments including complete blood count (CBC) with differential, a chemistry panel (often consisted of electrolytes glucose, blood urea nitrogen (BUN), creatinine, and albumin), urinalysis, inflammation markers, and measuring immunoglobulin levels. The investigation may be followed by flow cytometry of blood to assess the number and percentage of subtypes of immune cells (Kanegane et al., 2018). In case of suspected immunodeficiency, panel sequencing and whole exome sequencing

are progressively more used to confirm the diagnosis and identify the defect in the gene involved (Simon et al., 2020).

Table 1. Signs of PID created by the Jeffrey Modell Foundation (Jeffrey Modell Foundation).

Warning signs in children
1. ≥ 4 new ear infections within 1 year
2. ≥ 2 serious sinus infections within 1 year
3. ≥ 2 months on antibiotics with little effect
4. ≥ 2 pneumonias within 1year
5. Failure of an infant to gain weight or grow normally
6. Recurrent, deep skin or organ abscesses
7. Persistent thrush in mouth or fungal infection on skin
8. Need for IV antibiotics to clear infections
9. ≥ 2 deep-seated infections including septicemia
10. A family history of PID
Warning signs in adults
1. ≥ 2 new ear infections within 1 year
2. ≥ 2 new sinus infections within 1year, in the absence of allergy
3. 1 pneumonia per year for > 1 year
4. Chronic diarrhea with weight loss
5. Recurrent viral infections (colds, herpes, warts, condyloma)
6. Recurrent need for IV antibiotics to clear infections
7. Recurrent, deep abscesses of the skin or internal organs
8. Persistent thrush or fungal infection on skin or elsewhere
9. Infection with normally harmless tuberculosis-like bacteria
10. A family history of PID

2.2.4 Management and Treatment

Management and treatment of IEIs largely depends upon the type and affected class of immune cells. Nonetheless, it mostly concerns the prevention of infections and management of complications (e.g. bronchiectasis, autoimmunity, malignancy), which includes lifelong regiments of prophylactic antibiotics or antifungals. In many cases with impaired development and/or function of B cells, regular administration of intravenous immunoglobulin (IVIG) or subcutaneous immunoglobulin (SCIG) are a pivotal part of the clinical plan (Orange et al., 2006). Granulocyte-colony stimulating factor (G-CSF) is used in severe neutropenia to stimulate neutrophil production. Administration of interferon-gamma (INF-γ) has been shown to reduce the number of infectious patients with chronic granulomatous disease (CGD) (Holland, 2013).

Owning to the large class of cells involved in SCIDs, hematopoietic stem cell transplantation (HSCT) could be more beneficial in treatment of these conditions, and provide cure (Dvorak & Cowan, 2008). Recent advances in gene therapy have shown to offer promising results in certain SCIDs such as ADA-SCID, SCID-X1, CGD, and Wiskott–Aldrich syndrome (WAS) (Pinto & Neves, 2022; Segundo & Condino-Neto, 2021).

Different variants in the same gene can cause multiple clinical manifestation. An example is that gain of function variants in *STAT1* lead to a PIRD whereas the loss of function variants results in PIDD (Notarangelo et al., 2020). Variability of clinical features is not confined to LOF and GOF variants. Another example of this variability is multiple variants in *RAG1/2*. Amorphic variants in *RAG1/2* result in the complete arrest of B and T cell development. While severely hypomorphic variants in *RAG1/2* lead to Omenn syndrome (Notarangelo et al., 2020), less severely hypomorphic variants are linked to autoimmune disorders and CGD.

2.3 Classification of PIDs

The classification of PIDs has evolved substantially over time as genetic research has uncovered new mutations and pathways involved in immune dysfunction (Tangye et al., 2020). PIDs are conventionally categorized according to the primarily involved component, rather than the clinical phenotype. Nevertheless, the phenotypical report of IUIS is also provided to aid clinicians in the diagnosis of PIDs (Bousfiha et al., 2022).

Since 1970, a group of immunologists has been categorizing the recognized primary immunodeficiencies (Fudenberg et al., 1970b). Currently, the IUIS Expert Committee updates the list of IEIs and their classification nearly every two years. This classification is based on both genotypic (Tangye et al., 2022) and phenotypic characteristics (Bousfiha et al., 2022) provided for the clinicians and focusing on clinical and laboratory aspects of IEIs.

Over the years, the IUIS Expert Committee has classified the PIDs according to the primarily involved component. The rigorous criteria for classification for novel genes causing IEI comprise (Casanova et al., 2014a; Tangye et al., 2022):

1. The patient's candidate genotype is monogenic and does not occur in individuals without the clinical phenotype (acknowledging that some conditions have incomplete penetrance).
2. Experimental studies establish that the genetic variant impairs, destroys, or alters expression or function of the gene product.
3. The causal relationship between the candidate genotype and the clinical phenotype must be confirmed via a relevant cellular phenotype, including — where possible — rescue of a functional defect.

The latest IUIS update on IEIs, published in 2022, constitutes a total of 485 IEIs with 55 of them being added to the latest update of the IUIS, making the study of PIDs an increasingly growing field of research (Tangye et al., 2022). The current update classifies these disorders according to their genotypic features into ten main categories, each further divided into subcategories:

I. Immunodeficiencies affecting cellular and humoral immunity:
   1. T-B+ Severe Combined Immune Deficiency (SCID)
   2. T-B- SCID
   3. Combined Immunodeficiency (CID), Generally Less Profound than SCID
II. Combined immunodeficiencies with associated or syndromic features:
   1. Immunodeficiency with Congenital Thrombocytopenia
   2. DNA Repair Defects Other Than Those Listed in category I
   3. Thymic Defects with Additional Congenital Anomalies
   4. Immuno-osseous Dysplasias
   5. Hyper IgE Syndromes (HIES)
   6. Defects of Vitamin B12 and Folate Metabolism
   7. Anhidrotic Ectodermodysplasia with Immunodeficency (EDA-ID)
   8. Calcium Channel Defects
   9. Other Defects
III. Predominantly antibody deficiencies:
   1. Severe Reduction in All Serum Immunoglobulin Isotypes with Profoundly Decreased or Absent B Cells, Agammaglobulinemia

2. Severe Reduction in at Least 2 Serum Immunoglobulin Isotypes with Normal or Low Number of B Cells, CVID Phenotype
3. Severe Reduction in Serum IgG and IgA with Normal/Elevated IgM and Normal Numbers of B cells, Hyper IgM
4. Isotype, Light Chain, or Functional Deficiencies with Generally Normal Numbers of B Cells

IV. Diseases of immune dysregulation:

1. Familial Hemophagocytic Lymphohistiocytosis (FHL syndromes)
2. FHL Syndromes with Hypopigmentation
3. Regulatory T Cell Defects
4. Autoimmunity with or without Lymphoproliferation
5. Immune Dysregulation with Colitis
6. Autoimmune Lymphoproliferative Syndrome (ALPS, Canale-Smith syndrome)
7. Susceptibility to EBV and Lymphoproliferative Conditions

V. Congenital defects of phagocyte number or function:

1. Congenital Neutropenias
2. Defects of Motility
3. Defects of Respiratory Burst
4. Other Non-Lymphoid Defects

VI. Defects in intrinsic and innate immunity:

1. Mendelian Susceptibility to mycobacterial disease (MSMD)
2. Epidermodysplasia verruciformis (HPV)
3. Predisposition to Severe Viral Infection
4. Herpes Simplex Encephalitis (HSE)
5. Predisposition to invasive Fungal Diseases
6. Predisposition to Mucocutaneous Candidiasis
7. TLR Signaling Pathway Deficiency with Bacterial Susceptibility
8. Other Inborn Errors of Immunity Related to Non-Hematopoietic Tissues
9. Other Inborn Errors of Immunity Related to Leukocytes

VII. Autoinflammatory disorders:

1. Type 1 interferonopathies

2. Defects affecting the inflammasome
3. Non-inflammasome Related Conditions

VIII. Complement deficiencies

IX. Bone marrow failure

X. Phenocopies of inborn errors of immunity

2.4 Impact of Genetic Mutations on Immune Function

The IEIs result from genetic mutations which disrupt essential processes and pathways playing a role in production, development, and function of components of immune system; hence, the broad range of immunological defects (Bousfiha et al., 2022; Notarangelo et al., 2020). This section reviews how pathological variants in genes encoding essential proteins lead to IEIs, and the affected key pathways and processes.

2.4.1. From ‘Gene’ to ‘Immune System’

The immune system is formed by a complex network of cells, tissues, and signaling molecules functioning in harmonious interrelation, and protects the body against invading pathogens while recognizing the ‘self’ from ‘not-self’ (Casanova & Abel, 2018). Following a genetic mutation that causes an uncompensable imbalance in this delicately regulated system, a cascade of events leads to a major imbalance which manifests as an IEI. Such drastic impacts could be seen in exemplary LOF of RAG1/2 genes, resulting in Omenn Syndrome (Notarangelo, 2010a).

2.4.1.1 Types of Genetic Mutations in IEIs

The IEIs could be caused by a variety of mutations including large-scale mutation (large deletions and large insertion) and small-scale mutation (insertions, deletions, and substitutions mutations) (Tangye et al., 2022). Some of the most frequent types include(Casanova et al., 2014b; Casanova & Abel, 2021; Tangye et al., 2020):

- **Missense mutations:** the defect is followed by a single nucleotide change that leads to substitution one amino acid in the encoded protein, and the impact depends on the site of the amino acid (impacting a certain protein domain or 3D structure), as well as the characteristics of substituted amino acids.

- **Nonsense mutations:** these mutations are characterized by a single nucleotide change that introduces a premature stop codon.
- **Frameshift mutations:** this class of mutation is caused by indels (insertions or deletions) leading to an alteration in the reading frame. Thus, a completely different translation of the protein will be generated.
- **Splice site mutations:** DNA alterations in the splicing site that result in producing errant transcripts leading to dysfunctional protein.

These mutations can lead to production of non-functional or dysfunctional proteins and result in abruption in development of immune cells, signaling, and function of immune components, causing a defect in the immune cells and key pathways involved in immunity.

2.4.1.2 Inheritance Patterns and Genetic Penetrance

The phenotypes of IEIs are inherited through different patterns. These modes of inheritance include (Tangye et al., 2022):

- **Autosomal recessive (AR) inheritance:** in this pattern, two copies of defective variant are required to lead to immune deficiency. This is the most frequent type of inheritance in the IEIs.
- **Autosomal dominant (AD) inheritance:** in this pattern, only one pathogenic allele is adequate for disease manifestation.
- **X-linked inheritance:** the defective gene is located on the X chromosome, consequently, the phenotype mostly occurs in males. The females tend to show a milder phenotype owning to the inactivation of one of X chromosomes.

Intriguingly, genetic penetrance is a key factor in determining the clinical phenotype and its severity. The IEIs can have complete or incomplete penetrance. The penetrance of many IEIs and the underlying mechanisms of incomplete penetrance are unknown (Casanova & Abel, 2021), however, environmental factors are considered to play a possible role in the incomplete penetrance (Casanova & Abel, 2013).

2.4.2 Introduction to Key Pathways and Processes Affected by Genetic Mutations

The variants that lead to IEIs are those whose encoding genes are implicated in essential pathways required for cell development and signalling. (Notarangelo, 2010b). Such pathways are highly conserved and crucial for effective response in both innate and adaptive immunity (Notarangelo, 2010a). In this section, I review some of the most important pathways whose disruption leads to an IEI.

2.4.2.1 Developmental Pathways

T and B cells development: The development and differentiation of B and T cells from hematopoietic stem cells take place in the bone marrow and thymus, respectively (Murphy et al., 2022). Pathogenic variants in any of the genes involved in earlier phases of this process could potentially lead to a severe IEI, possibly a SCID (Figure 2). For instance, pathogenic variants in *RAG1*/*2* result in SCID with lack of functional T and B cells (Villa et al., 1998).

Phagocyte development: phagocytes are a group of immune cells whose role in innate immunity is to protect the organism against pathogen and remove the dead cells of the body. This group is consisted of neutrophils, monocytes, mast cells, and dendritic cells (Murphy et al., 2022). Pathogenic variants in genes encoding essential proteins for their development could impair innate immunity and result in neutropenia and CGD (Tangye et al., 2022). For example, *ELANE* which encodes neutrophil elastase is essential for neutrophil function (Dale et al., 2000). Pathogenic variants in this gene results in maturation arrest in the neutrophils, causing congenital neutropenia (Dale et al., 2000).

NK cell development: NK cells have a major role in the early defense against viruses and tumors, therefore, pathogenic variants affecting their development lead to increased susceptibility to viral infections. For instance, NK cell deficiency is reported in individuals with a pathogenic variant in *MCM4*. Patients with this condition are clinically characterized with an increased risk of herpesvirus and cancer (Casey et al., 2012).

2.4.2.2 Signalling Pathways

Cytokine Signaling: Cytokines have a pivotal role in the regulation of immune responses, and their signaling pathways are among the most defective signalling pathways in the IEIs. Also, pathogenic variants in *STAT1* or *STAT3* impact downstream cytokine signaling pathways, resulting in immune dysregulation and increased susceptibility to infections (Dupuis et al., 2001; Holland et al., 2007).

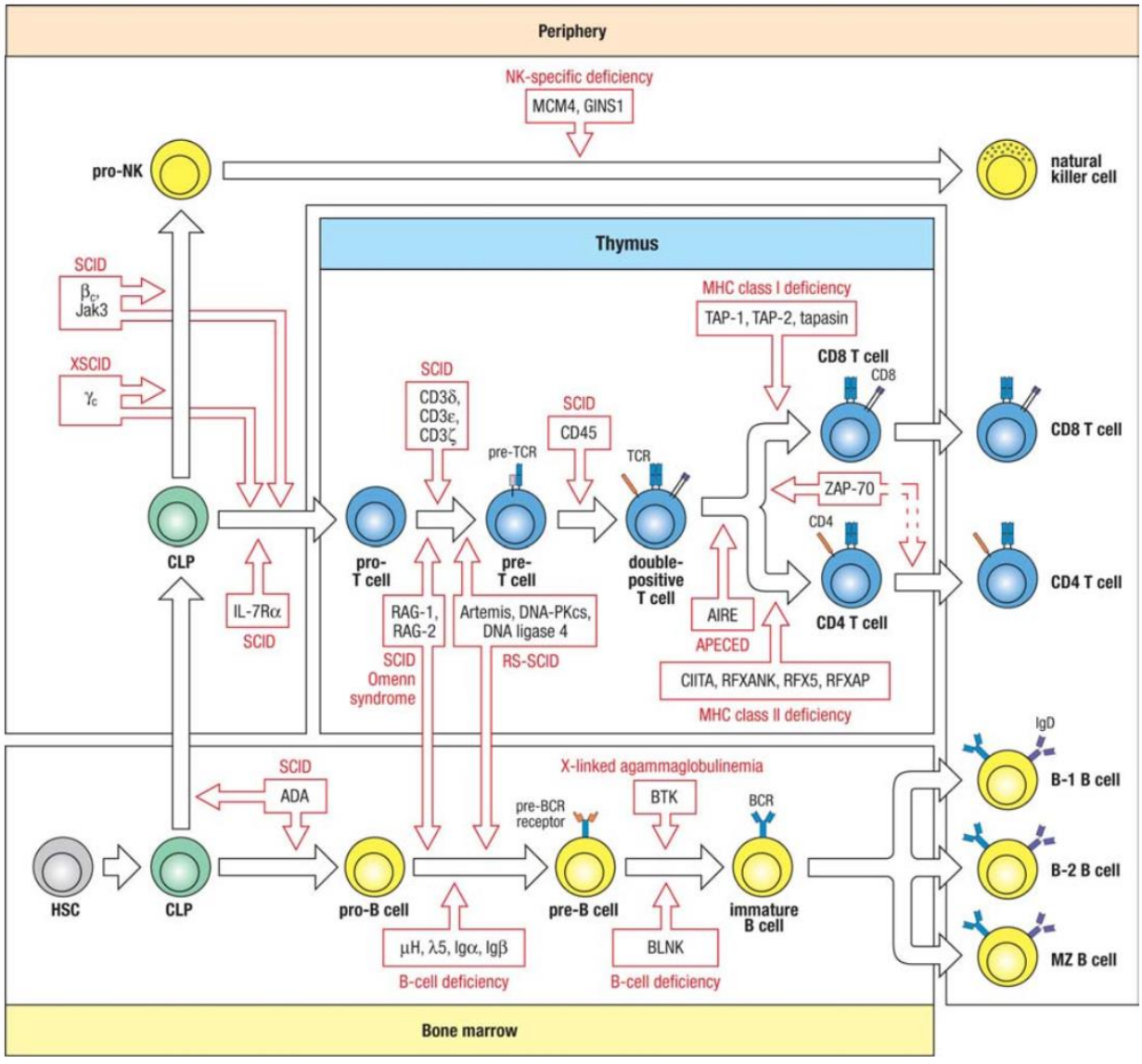

Figure 2. *Development of T and B cells and critical genes in their differentiation. Figure adapted from Murphy et al., Janeway's immunobiology, 10th ed., W.W. Norton & Company, 2022.*

Toll-like Receptor (TLR) Signaling: TLRs are a class of pattern recognition receptors that play an essential role in the innate immune response. Pathogenic variants in

components of the TLR signaling pathway could increase the susceptibility to bacterial, viral, and fungal infections. As an example, pathogenic variants in the *MYD88* gene, which encodes a critical adaptor protein in TLR and interleukin-1 receptor (IL-1R) signaling, result in a rare form of primary immunodeficiency characterized by increased susceptibility to pyogenic bacterial infections, such as invasive pneumococcal disease (von Bernuth et al., 2008). Another example of TLR related IEIs is *TIRAP*, .which encodes another adapter protein in the TLR pathway. Pathogenic variants in this gene lead to an early-onset IEI as to its bridging role between TLR2/4 and MyD88 (Tangye et al., 2022).

the ability of TIRAP to interact and collaborate with several signaling molecules in a context-dependent manner means this protein is a major regulator of cell signaling.

NF-κB Signaling: The NF-κB pathway is a proinflammatory signaling pathway which regulates immune cell activation and inflammation through the production of certain cytokines such as TNF-α, IL-1β, and IL-6. Activation of NF-κB alerts the immune system to the presence of pathogens**.** Pathogenic variants in genes encoding components of NF-κB pathway, such as *NFKB1* and *IKBA*, lead to IEIs characterized by recurrent infections, as well as autoimmune and autoinflammatory manifestations (e.g. small vessel vasculitis), and abnormalities in B cell maturation resulting in pronounced antibody deficiency (Kaustio et al., 2017).

2.4.2.3 Immune Effector Functions

Phagocytosis and Respiratory Burst: The role of phagocytes in innate immune responses includes pathogen engulfment and digestion. During the process of respiratory or oxidative burst, activated phagocytes produce reactive oxygen species (ROS) which degrade the engulfed pathogens. Variants affecting the respiratory burst commonly result in IEIs such as CGD. For instance, pathogenic variants in the *CYBB* gene, which encodes the gp91phox subunit of NADPH oxidase, impair the ability of phagocytes to produce ROS. The resulting defect in phagocyte respiratory burst and innate immune response lead to recurrent infections with catalase-positive organisms (Rae et al., 1998).

Complement Activation: Having a major role the innate immune response, the complement system including a large number of plasma proteins, are involved in

defending against foreign pathogens. Activated complement system participates in pathogen clearance, opsonization, chemotaxis and activation of leukocytes, and inflammation (Murphy et al., 2022). The complement system also plays a major role in adaptive immunity by modifying the T cell responses. Complement deficiencies as a result of mutations in genes encoding components of the complement pathway, e.g. the *C1Q* gene, are associated with increased susceptibility to encapsulated bacteria, such as Neisseria meningitidis. Patients with C1q deficiency, are at increased risks of developing recurrent bacterial infections and early-onset autoimmune diseases particularly systemic lupus erythematosus (SLE) (Botto et al., 1998).

Cytotoxicity: Cytotoxic T lymphocytes (CTLs) and NK cells, mediators of cell-mediated cytotoxicity, are responsible for killing infected or transformed cells through the release of cytotoxic granules containing perforin and granzymes. As an example, pathogenic variants in *PRF1* have been known to cause familial hemophagocytic lymphohistiocytosis (FHL), a fatal disease of early childhood which is characterized by significant reduction of absence in the cytotoxic T cell and NK cell activity (Stepp et al., 1999).

2.4.2.4 Regulation of Immune Responses

Immune Checkpoints: The immune checkpoint molecules or negative regulators of immune system prevent overactivation of immune responses and maintain immune homeostasis. Pathogenic variants in cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) are associated with immune dysregulation and deficiency and a complex syndrome of autoimmune disorders and lymphoproliferation, and immunodeficiencies. (Schubert et al., 2014). Additionally, pathogenic variants in *LRBA*, which plays a role in regulating CTLA-4 trafficking, result in immune dysregulation syndrome with features of autoimmunity and recurrent infections (Lo et al., 2015).

Apoptosis: Apoptosis or programmed cell death is a physiological process designed to protect the organism against infection or aberrant cells. Apoptosis protects the organism against infection, cancer, and autoimmune disorders by removing autoreactive cells. Defect of apoptosis is known to be a hallmark of cancer (Hanahan, 2022). Defects in apoptotic processes are previously characterized as IEIs. Of these, pathogenic variants

in the *FAS* and *FASLG* genes are causal for defects in apoptotic processes. This consequently leads to disrupted lymphocyte homeostasis and disorders such as autoimmune lymphoproliferative syndrome, which is characterized by uncontrolled lymphocyte proliferation, autoimmunity, and an increased risk of lymphoma (Magerus et al., 2021). It is also noteworthy to mention that pathogenic variants in *CASP10*, an initiator of extrinsic apoptotic pathway in collaboration with caspase-8, results in autoimmune lymphoproliferative syndrome, which manifests with lymphadenopathy, splenomegaly, and autoimmunity (Matas Pérez et al., 2021).

2.4.2.5 DNA Repair and Stability

V(D)J Recombination and Class Switch Recombination: V(D)J recombination is an essential mechanism of the adaptive immune system, which generates antigen receptor diversity in developing B and T lymphocytes. By affecting V(D)J recombination, mutations in genes involved in this process, such as *RAG1*, *RAG2*, and *DCLRE1C* (which encodes Artemis), both T and B cell functions are severely impaired which result in SCID or (CID) (Tangye et al., 2022; Villa et al., 1998). Class switch recombination (CSR) is a DNA recombination process that replaces the constant region of immunoglobulin (Ig) for the isotype that can best protect against the pathogen thereby allowing B cells to produce different antibody isotypes (Murphy et al., 2022).

Another example of immune dysregulation as a result of defects in CSR is hyper-IgM syndrome. Patients with hyper-IgM syndrome are characterized by increased IgM levels and a decrease in the other classes of antibodies, as well as B cell lymphoma (Jhamnani et al., 2018).

DNA Damage Response: Detection and repair of DNA damage is essential for maintaining genome stability and cellular homeostasis in all cells. Pathologies in this process is known to be hallmark of cancer. Considering the processes such as V(D)J recombination and CSR in immune cell development, the ability of DNA repair is of paramount importance. This importance is exemplified in the pathogenic variants in *DCLRE1C,* encoding the DNA-repair enzyme ARTEMIS, whose defects lead to an SCID in human. Pathogenic variants in other genes encoding proteins in the DNA repair such

as ATM and NBN, lead to immunodeficiency syndromes ataxia-telangiectasia and Nijmegen breakage syndromes, respectively (Lavin, 2008; Yilmaz Demirdag & Gupta, 2023).

2.5 Importance of Gene Studies in IEIs

Thanks to Gene technology and genomics, diagnosis and management of diseases such as cancer and IEIs have changed dramatically. Advanced genomic technologies have made the genetic underpinnings of IEIs possible to help better understand the responsible mechanisms that helps early detection of the disease and developing new targeted therapies (Casanova & Abel, 2018).

For many years, the diagnosis of IEIs was based on clinical evaluations and immunological assessments including the patient's immunoglobulin levels, lymphocyte counts, and responses to vaccines. Although these methods have been useful in identifying some immunodeficiencies, in case of atypical features or overlapping symptoms they were inconclusive. The advent of genomic techniques such as whole exome sequencing (WES) and whole genome sequencing (WGS) have yielded powerful tools to identify the underlying genetic variants responsible for these complex disorders enabling us to differentiate between different types of IEIs (Vorsteveld et al., 2021). They have served categorizations of IEIs, as the clinical manifestations caused by multiple variants in one gene might differ (Tangye et al., 2022). Moreover, by finding rare variants in genes previously not associated with immunodeficiencies, the genotypic spectrum of these diseases has expanded significantly (Bousfiha et al., 2022).

3. HYOU1: A Chaperone Implicated in Handling Cell Stress

Hypoxia up-regulated protein 1 (HYOU1), also known as oxygen-regulated protein 150 (ORP150) and glucose-regulated protein 170 (GRP170), is a molecular chaperone which belongs to heat shock protein family (HSP) and plays a major role in handling cell stress in response to physiological stress (Kusaczuk & Cechowska-Pasko, 2013). HYOU1 is expressed in response to physiological stressors, such as hypoxia, low glucose, oxidative stress and imbalanced calcium levels (Rao et al., 2021). HYOU1 has been reportedly overexpressed in response to tunicamycin and non-steroid anti-inflammatory drugs such as celecoxib (H. Wang et al., 2015). HYOU1 can be intracellular or extracellular and serves different roles according to its site (H. Wang et al., 2015)

HYOU1 is highly conserved among species highlighting its significance for the survival of the living organism (Takeuchi, 2006). Additionally, attempts for producing a *HYOU1* knocked-out mouse model have failed, indicating its indispensable role in cell and organism's homeostasis (Kitao et al., 2001).

3.1 Overview of Endoplasmic Reticulum Stress and Unfolded Protein Response

Endoplasmic reticulum (ER) is the main site for synthesis, modification, and folding of proteins in the cell. The oxidating condition and high-calcium setting of ER makes it susceptible to accumulation of misfolded or unfolded proteins, in conditions where there is an increased load of protein synthesis (Kusaczuk & Cechowska-Pasko, 2013). This condition, known as ER stress, happens in physiological and pathological conditions and results in decreased folding capacity (Rao et al., 2021). A self-regulatory mechanism is established in the ER to ensure the balance of protein folding capacity and demand and to restore ER homeostasis subsequent to ER stress. This mechanism is composed of a series of reactions called unfolded protein response (UPR) (Read & Schröder, 2021). Once UPR is triggered, a sequence of events will take place: 1) inhibition of translation (through phosphorylation of eIF2α) 2) elevated folding capacity 3) degradation of misfolded proteins (through ER-associated degradation [ERAD]) 4) apoptosis (in case of severe and uncontrollable stress). The first three signaling pathways are intended to re-

establish balance and physiological condition to the ER, while the last pathway is designed to sacrifice the cell to preserve unaffected cells (H. Wang et al., 2015).

The initiators for each of the three restorative signaling pathways UPR are: 1) type 1 transmembrane protein inositol requiring 1 (IRE1α), 2) eukaryotic initiation factor 2α (eIF2α) kinase (also known as protein kinase R-like endoplasmic reticulum kinase [PERK]), and 3) activating transcription factor 6 (ATF6) (Hetz, 2012). These three proteins are bound to the binding immunoglobulin protein (BiP, also known as 78 kDa glucose-regulated protein [GRP78]) in physiological condition. GRP78 has a higher affinity for unfolded proteins; thus, consequent to accumulation of unfolded proteins, GRP78 separates from UPR initiators leading to a series of events resulting in multiple cellular processes (Figure 3) (Read & Schröder, 2021)

- **Activation of ATF6** results in its cleavage in Golgi apparatus, and production of a 50 kDa ATF6 protein, which in turn binds to ER stress response element (ERSE) in glucose regulated proteins (GRPs) genes, including *HYOU1*, and leads to an increase in their transcription (Kusaczuk & Cechowska-Pasko, 2013).
- **Activation of PERK** phosphorylates eIF2α, which serves a twofold purpose – general translational block, to stop the increasing the load of proteins, and stimulating the translation of *ATF4*. ATF4 also binds to ERSE in GRP genes and enhances their transcription (H. Wang et al., 2015).
- **Activation of IRE1α** leads to splicing of *X-box protein-1* (*XBP1)* mRNA and generation of spliced *XBP1*, resulting in the transcription of genes involved in Endoplasmic-reticulum-associated protein degradation (ERAD) pathway. Activation of IRE1α also results in the recruitment of TRAF and consequent formation of IRE1–TRAF2–ASK1 complex (Nishitoh et al., 2002), which activates c-Jun amino-terminal kinase (JNK) and p38, thus, initiating apoptosis (Read & Schröder, 2021).

Through activation and production of glucose regulated proteins which aim to block ER stress signals and enhance destruction of misfolded proteins accumulated in the ER (ERAD pathway activation), the cell attempts to manage ER stress and re-establish ER homeostasis (Kusaczuk & Cechowska-Pasko, 2013). Nevertheless, if case of severe

stress or impairment in the mentioned pathways aiming for cell survival, the process of apoptosis is initiated (Hartl & Hayer-Hartl, 2009).

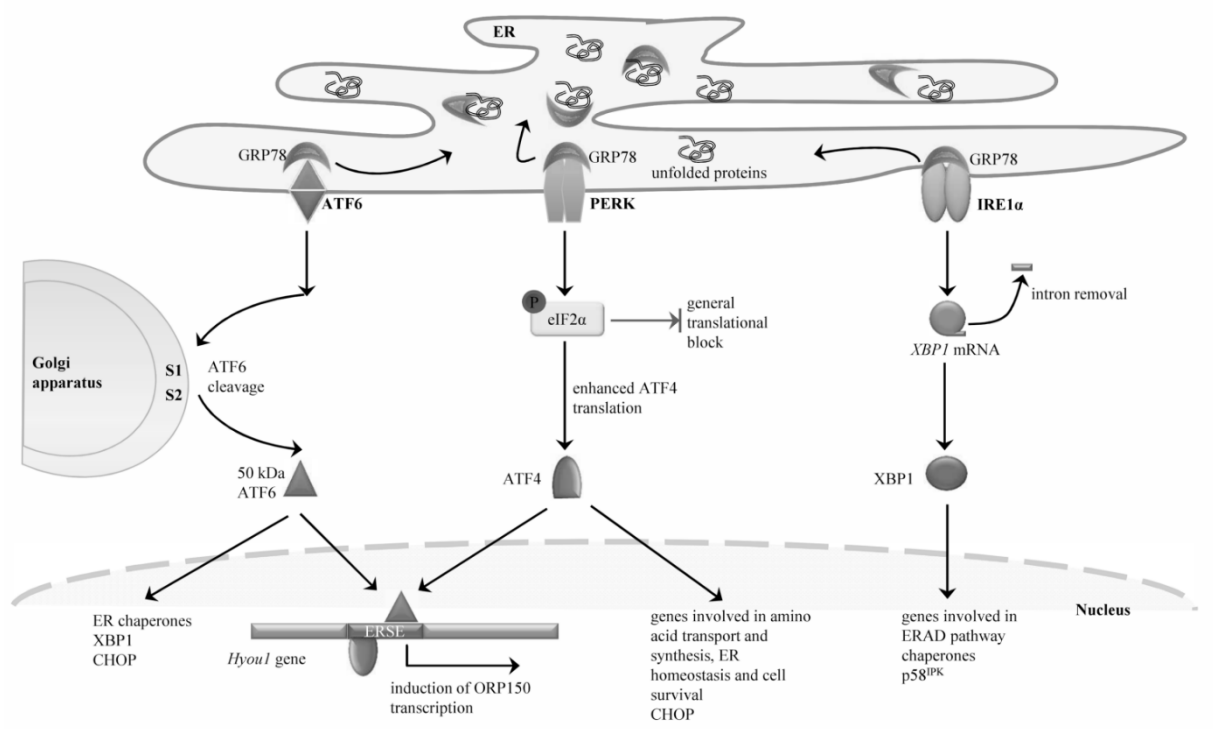

***Figure 3**. Signaling pathway of UPR and transcription activation of HYOU1. Adapted from Kusaczuk and Cechowska-Pasko, Current Pharmaceutical Design, 2013.*

3.2 HYOU1 Structure and Function

3.2.1 HYOU1 Structure

HYOU1 is located on chromosome 11.q23.3 and encodes a 999 amino acid-long protein (H. Wang et al., 2015). In the promoter region of *HYOU1* (between intron 1 and exon 2) a CCAAT-rich cis-acting element, known as ER stress element (ERSE), is sited and it is suggested that expression of HYOU1 is regulated by UPR through this ERSE region (Kaneda et al., 2000).

HYOU1 protein is consisted of six domains including a signal peptide domain (Figure 4), an ATPase domain (essential for its chaperone function), a peptide binding domain (enabling the protein to interact with a wide range of client proteins), acidic loop domain, α-helical domain, and ending with a KNDEL motif, hence, its recognition as an ER located

protein (Hartl & Hayer-Hartl, 2009; Takeuchi, 2006). Furthermore, presence of NH_2-terminus facilitates targeting of HYOU1 to mitochondria, making HYOU1 an ER and mitochondrial localized protein (Arrington & Schnellmann, 2008). Owning to its structural similarities, HYOU1 is also considered to be a member of HSP70 family (Kusaczuk & Cechowska-Pasko, 2013).

Initially, HYOU1 was considered to be solely ER-localized, however in 2008, Arrington and Schnellmann showed its presence in mitochondria and highlighted upregulation of both ER and mitochondrial *HYOU1* in response to stressful conditions (Arrington & Schnellmann, 2008).

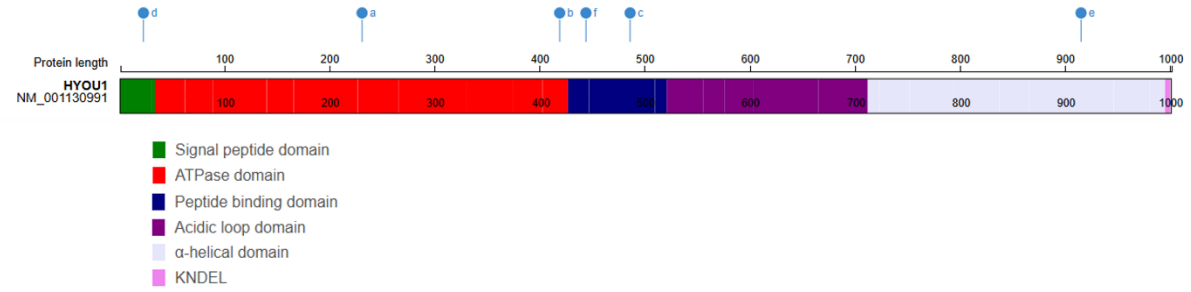

Figure 4. *HYOU1 domains and reported variants. Each bubble corresponds to one variant. a, p.Tyr231His; b, p.Ala419Pro; c, p.Arg486Cys; d, p.Leu23Phe; e. p.Arg915Gln; f, p.Pro444His.*

3.2.2 Role and Function of HYOU1

HYOU1 was first extracted from rat astrocytes and described by Kuwabara et al. in 1996 (Kuwabara et al., 1996). It was believed that it contributes to adaptive stress resulting from hypoxia and playing a protective role against cell death (Kuwabara et al., 1996).

Since its description, HYOU1 has been the focus of extensive research, aimed at identifying its cellular functions and studying its protective and anti-apoptotic roles. The major role of HYOU1 is believed to aid the ER overcome the excessive protein load by facilitating protein folding and restore ER function (Ron & Walter, 2007; Rutkowski & Hegde, 2010). It is noteworthy that chaperones are typically involved in a variety of cellular processes, such as protein folding, refolding of de-natured proteins, protein transport throughout the cell as well as proteolytic degradation (Hartl & Hayer-Hartl, 2009). In addition to the conventional roles of chaperones, association of HYOU1 with cell death prevention and UPR further revealed a broader range of cellular function of this protein

(H. Wang et al., 2015). Studies in renal tubular epithelial cells of rat showed that reduced levels of HYOU1 have been associated with increased cell death, while elevated levels appear to confer cytoprotective effects (Bando et al., 2004). Moreover, in vivo studies showed that the cell death was reduced by overexpression of *HYOU1* in rat cardiomyocytes exposed to hypoxia-reoxygenation (Aleshin et al., 2005). In vivo studies demonstrated the importance of HYOU1 is cell rescue upon stress conditions. Studying heterozygous *HYOU1* deficient mice indicated glutamate-induced cell death in hippocampal neurons (Kitao et al., 2001) and increased renal injury in response to ischemia/reperfusion (Bando et al., 2004).

Furthermore, it has been shown that HYOU1 plays a role in insulin secretion and wound healing (Arrington & Schnellmann, 2008).

The exact mode of HYOU1 involvement in UPR and its role in preventing apoptosis is not clear, however, several possible modes of action are considered for HYOU1 (Kusaczuk & Cechowska-Pasko, 2013):

1. **Direct interaction with proapoptotic C/EBP homologous protein (CHOP)**. A CHOP-binding element has been identified in the promoter region of *HYOU1* (28, kus), which suggests preventing apoptosis through binding of HYOU1 to proapoptotic CHOP.
2. **Binding to IP_3 channels** leading to their inactivation and consequent decrease in calcium levels of cytosol. HYOU1 also directly binds to Ca^{2+} ions in the cytosol. Both of these mechanisms lead to suppression of calpain activation which induces a cascade of events leading to mitochondrial-mediated apoptosis.
3. **promoting IRS-1 and Akt phosphorylation,** as prosurvival proteins, which in turn activate PEPCK and G6Pase regulatory pathways.
4. **Direct binding to UPR initiators:** PERK, IRE1α, and ATF6
5. **Contributing to ATP/ADP nucleotide exchange of GRP78**, impacting the substrate binding abilities of GRP78.
6. **Involvement in maturation of VEGF**, contributing to angiogenesis

7. Due to its structural similarities and evidence of binding to proteins process in the ER, the classical role of involvement in protein folding, transport and maturation which prevents ER in the first place.

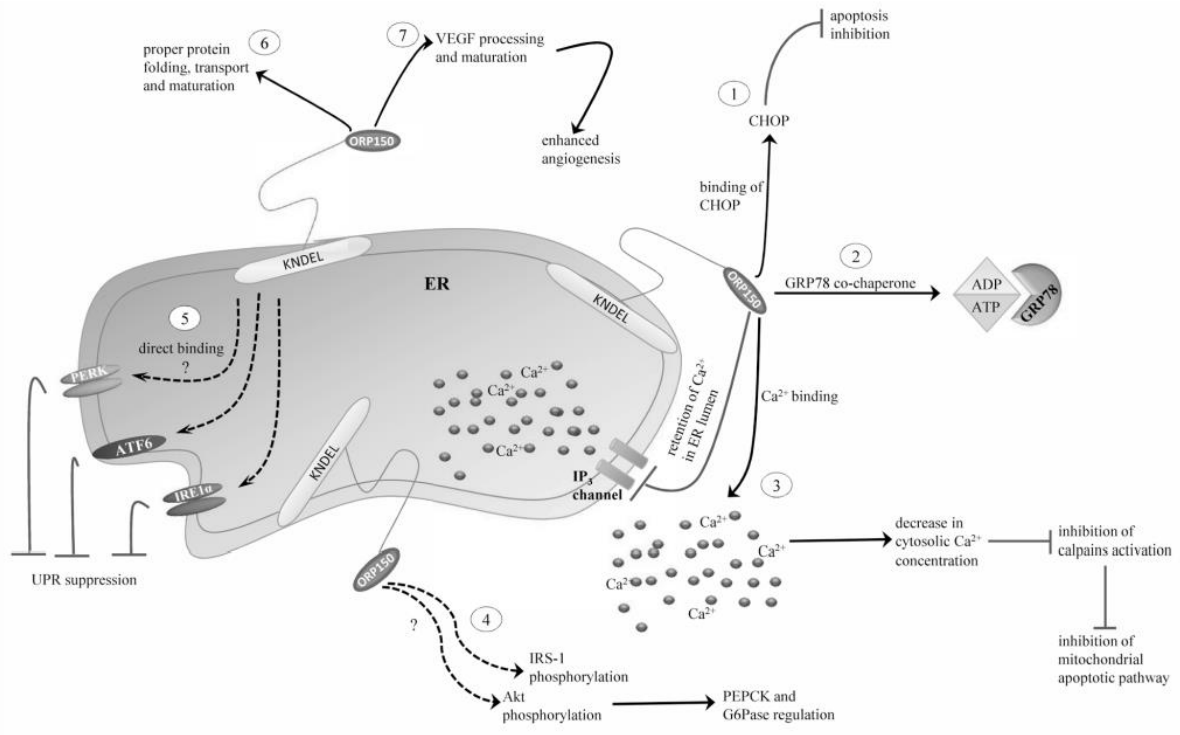

Figure 5. *HYOU1 function and possible modes of action. Adapted from Kusaczuk and Cechowska-Pasko, Current Pharmaceutical Design, 2013.*

In addition to being induced by activation of UPR signaling pathway, HYOU1 was shown to bind to the UPR initiator proteins in human endothelial cells (Sanson et al., 2009) and PERK and eIF2α in hepatocytes (Y. Wang et al., 2011). These findings suggest that GRP78 may not be the exclusive sensor for ER stress and HYOU1 may be another sensor whose binding to misfolded proteins results in the activation of the UPR.

The chaperoning abilities of HYOU1 extend beyond intracellular environment, as it allows HYOU1 to bind to extracellular proteins and stabilize protein antigens (Arnouk et al., 2010).

3.3 HYOU1 in Human Diseases

Thus far, HYOU1 implication in certain diseases has been shown (Rao et al., 2021). The most frequently associated diseases with HYOU1 are diabetes mellites,

neurodegenerative diseases, cardiovascular diseases, and cancer (Kusaczuk & Cechowska-Pasko, 2013; Rao et al., 2021; H. Wang et al., 2015).

3.3.1 HYOU1 in Diabetes Mellitus

Insulin is considered the key modulator of glucose homeostasis. Pathologies in secretion of insulin or defects of insulin signaling leads to Diabetes mellitus (DM). Insulin is exclusively secreted by β-cells of pancreas. In type 1 DM (T1DM), there is insufficient secretion of insulin due to destruction of pancreatic β-cells (Kusaczuk & Cechowska-Pasko, 2013). Whereas type 2 DM (T2DM) is characterized by insufficient response to insulin in tissues, known as insulin resistance (Kusaczuk & Cechowska-Pasko, 2013). This leads to an increase in insulin production and secretion in order to mitigate insulin resistance. In turn, elevated insulin production and secretion could overload the ER, and stimulate chronic UPR activation, leading to β-cells apoptosis (Kusaczuk & Cechowska-Pasko, 2013).

Several studies indicated the importance of HYOU1 in DM (Kusaczuk & Cechowska-Pasko, 2013; Rao et al., 2021). Studies investigating expression of HYOU1 in mouse MIN6 beta cells in stress conditions suggest that HYOU1 may be directly involved in the synthesis and folding of insulin considering its chaperoning abilities (Kobayashi et al., 2000; Kobayashi & Ohta, 2005). It has also been shown that overexpression of sense HYOU1 lowers insulin resistance in diabetic C57BL6 mice, whereas overexpression of antisense HYOU1 causes insulin resistance in non-diabetic control mice (Nakatani et al., 2005). In a study of 91 patients with T1DM compared with 37 healthy controls, HYOU1 autoantibodies were significantly elevated in the sera of diabetic patients, however there was a correlation between the anti-HYOU1 autoantibody levels and HbA_{1c} (Nakatani et al., 2006).

Although the direct role and interaction of HYOU1 with CHOP has not been studied in context of DM, some studies demonstrated the presence of transcription factor CHOP in the process of ER-stress mediated death of β-cells, suggesting that HYOU1 may play an antiapoptotic role through direct association with CHOP (Arrington & Schnellmann, 2008; Oyadomari et al., 2002).

3.3.2. HYOU1 in Neurodegenerative Diseases

Many neurodegenerative disorders are characterized by aggregation and deposition of unfolded or misfolded proteins in neurons, in particular Parkinson's disease and Alzheimer's disease (Lindholm et al., 2006). Accumulation of misfolded proteins can cause both increased toxicity and impaired physiological function resulting in disturbances in the signaling system of the cell and adverse effects on neuronal connectivity which may lead to cell death (Soto, 2003). Neuronal cell death can lead to the loss of both pre- and post-synaptic neurons finally resulting in a reduction of synaptic connections. Studies have shown strong association between increase of endoplasmic reticulum (ER) stress-regulated proteins detected in cell line models and postmortem brain tissues of patients with Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and Huntington disease (Lindholm et al., 2006).

In Alzheimer's disease, production and deposition of the β-amyloid peptide (Aβ) is considered a major pathogenetic factor. On the other hand, the enzymes involved in clearance of Aβ are dysregulated (Kusaczuk & Cechowska-Pasko, 2013). HYOU1 and other ER chaperones have been found to reduce levels of amyloid-beta peptides, though the exact mechanism remains unclear (Rao et al., 2021). HYOU1 is also implicated in maintaining cellular homeostasis and protecting neurons from excitotoxicity, a neuropathological process, describing the toxic actions of excitatory neurotransmitters which can occur due to overstimulation by excitatory neurotransmitters (Kusaczuk & Cechowska-Pasko, 2013).

Further studies have demonstrated that HYOU1 plays a neuroprotective role in models of ischemia and excitotoxicity (Kusaczuk & Cechowska-Pasko, 2013). In neurons subjected to excitatory stimuli, overexpression of HYOU1, may prevent ER stress and rescue neurodegeneration, possibly by reducing cytosolic calcium elevation, suppressing proteolytic activity, and finally preventing cell death (Kusaczuk & Cechowska-Pasko, 2013). This effect has been observed in transgenic mice, where HYOU1 overexpression reduces neuronal apoptosis and infarct size following ischemia (Kusaczuk & Cechowska-Pasko, 2013).

3.3.3 HYOU1 in Cardiovascular Diseases

Atherosclerosis is a chronic condition characterized by the hardening and narrowing of arteries due to the formation of plaques composed of lipoproteins, primarily in the subendothelium (Roy et al., 2022). The oxidized LDL (oxLDL) has antigenic potential and contributes to atherosclerosis associated inflammation, activating both innate and adaptive immunity (Roy et al., 2022). The oxLDL initiates inflammatory responses with recruitment of macrophages, which phagocyte the lipoproteins and become foam cells. Under these circumstances, apoptosis can occur in different cell types such as endothelial cells, T lymphocytes and macrophages (Roy et al., 2022). This in turn results in progression of atherosclerosis and increasing the risk of cardiovascular events such as myocardial infarction and stroke (Roy et al., 2022). Oxygen deprivation in atherosclerotic lesions, along with ER stress, plays a significant role in the disease's progression (Yang et al., 2020). ER stress-inducing agents can promote lipid accumulation in macrophages, exacerbate lipid metabolism dysregulation, and promote vascular cell apoptosis, thus increasing the risk of plaque rupture and thrombosis (Yang et al., 2020).

Studies have shown the role of HYOU1, a chaperone protein, in protecting cells against the damaging effects of ER stress and apoptosis in atherosclerosis (Kusaczuk & Cechowska-Pasko, 2013; Rao et al., 2021). HYOU1 is considerably expressed in atherosclerotic plaques, especially in macrophages, and its synthesis increases under hypoxic conditions in the presence of modified lipoproteins (Kusaczuk & Cechowska-Pasko, 2013). HYOU1 also protects vascular cells from oxLDL-induced apoptosis by regulating calcium release from the endoplasmic reticulum and inhibiting apoptotic pathways (Kusaczuk & Cechowska-Pasko, 2013).

On the other hand, HYOU1, due to its anti-ER stress and anti-apoptotic effects, by suppressing oxidative stress and calcium signaling contributes to cell survival (Kusaczuk & Cechowska-Pasko, 2013). Therefore, HYOU1 is considered as a potential therapeutic target for halting or decreasing progression of atherosclerosis. It has been shown that plasma levels of HYOU1 is elevated in patients who died after acute myocardial infarction (AMI) compared to survivors (Kusaczuk & Cechowska-Pasko, 2013). This suggests that HYOU1 might be considered as a prognostic marker for mortality.

3.4 HYOU1 in Immune System

Owing to its broad role in managing ER stress and involvement in multiple inflammatory response, HYOU1 plays a modulating role in both innate and adaptive immunity response towards pathogen by a variety of mechanism including:

- **Alarmin function:** HYOU1 is secreted from stressed cell and functions as an alarmin by informing the immune system of stress stress and possible cell death, thus, initiating immune response (Henderson et al., 2010).
- **Interactions with Dendritic Cells (DCs):** Through interaction with DCs, HYOU1 enhances the secretion of pro-inflammatory cytokines, and upregulates the expression of MHC class II, CD86 and CD40 molecules, which results in activariotn of both innate and immune responses (Facciponte et al., 2007; Manjili et al., 2006)
- **Cytokine production:** The interaction of HYOU1 and DCs results in production of pro-inflammatory cytokines such as IL-6, IL-12, and TNF-α (Manjili et al., 2006).
- **Chaperoning Pathogen-Associated Molecular Patterns (PAMPs):** Extracellular HYOU1 has been shown to interact with CpG oligodeoxynucleotides, which are microbial DNA mimetic recognized by TLR9. Moreover, it has been revealed that extracellular HYOU1 directly interacts with TLR9. This leads to TLR9 activation and consequent activation of the MyD88-dependent signaling pathways (Zuo et al., 2012).
- **Processing and developing immunoglobulins:** HYOU1 has been shown to bind to immunoglobulins in the ER (Lin et al., 1993), suggesting its HYOU1 in processing and development of immunoglobulins.

Additionally, following cell stress leading to apoptosis, or cell death as a result of pathogen invasion, HYOU1 is expected to be upregulated, particularly in immune cell in response to inflammation due to increase in cell activity requiring for fighting against pathogens (H. Wang et al., 2015)

3.5 HYOU1 as an Inborn Error of Immunity

The diverse clinical manifestations associated with *HYOU1* variants, and its involvement in multiple classes of immune cells - including neutrophils, B cells, and DCs - highlights

its crucial role in maintaining immune homeostasis through managing cell stress. Therefore, *HYOU1* can be considered a compelling candidate gene since the pathogenic variants in the gene have given phenotypes similar to previously known IEIs. Also, evidence has linked the implication of HYOU1 in known pathways (e.g. UPR) involved in other IEIs with neutropenia.

In 2017, Haapaniemi et al. reported a 45-year-old woman of Finnish ethnicity with compound heterozygous variants in *HYOU1* (NM_001130991.3): c.1255G>C; (p.Ala419Pro) and c.691T>C (p.Tyr231His). The reported patient manifested with failure to thrive, neutropenia, recurrent herpes infection, episodes of hypoglycemia, minor facial dysmorphia, and reduced B cells. Studying patient's neutrophils revealed an increase in the numbers of mitochondria with high reactive oxygen species production in patient neutrophils (Haapaniemi et al., 2017).

In WES performed for a 5-year-old Iranian patient included in our previously described cohort of IEIs, we discovered a novel missense variant in *HYOU1* (NM_001130991.3): c.1331C>A (p.Pro444His). The symptoms of the patient described in this thesis started at birth and include being small for gestation age (SGA), FTT, chronic diarrhea, episodes of hypoglycemia, neutropenia, and reduced B cells. The description of this phenotype alongside the introducing the novel variant contributes to the spectrum of HYOU1 function and aids to further establish a genotype-phenotype correlation for the spectrum of HYOU1 protein and function.

While working on this project, two other reports of patients with suspected IEIs with novel variants in *HYOU1* were published. One patient with rather more severe clinical phenotype was reported with a homozygous missense variant *HYOU1* (NM_001130991.3): c.1456C>T (p.Arg486Cys) reported by Jafari Khamirani et al. in 2022 (Jafari Khamirani et al., 2022). Her symptoms began at the age of 2 months with urinary tract and respiratory infections, leading to hospital admission, leading to her expiration at the age of three months and two weeks (Jafari Khamirani et al., 2022). Arab et al., also reported a 4-year-old Iranian patient with compound heterozygous variants in *HYOU1* (NM_001130991.3): c.69G>C (p.Leu23Phe) and c.2744G>A (p.Arg915Gln) manifesting

with chronic lung infection and refractory thrush (Arab et al., 2022). However, both report a clinical phenotype and no descriptive functional investigations.

3.6 Other Inborn Errors of Immunity Associated with Unfolded Protein Response and Apoptosis

Apart from previous evidence that defects in *HYOU1* cause an IEI (Arab et al., 2022; Haapaniemi et al., 2017; Jafari Khamirani et al., 2022), the role and function of *HYOU1* closely resemble those of other genes whose defects lead to an IEI with a similar phenotype. This makes *HYOU1* a suitable candidate gene for IEIs. Herein, we review a few of the classified IEI genes with similar implication of their encoding protein, in which a pathologic variant results in congenital neutropenia.

***ELANE*:** Pathogenic variants in *ELANE* are responsible for the majority of cases of severe congenital neutropenia in Caucasians, mainly because of its AD inheritance (Hauck & Klein, 2013). *ELANE* encodes neutrophil elastase, and its defects lead to congenital neutropenia (Hauck & Klein, 2013). It is suggested that pathogenic variants in *ELANE* cause an accumulation of misfolded neutrophil elastase in the ER, leading to ER stress and initiation of UPR, which eventually leads to apoptosis (Köllner et al., 2006; Nanua et al., 2011).

***HAX1*:** Pathological variants in *HAX1* cause severe neutropenia due to maturation arrest at the promyelocyte stage (Carlsson & Fasth, 2001b). Previous studies demonstrated the role of HAX1 in balancing pro-apoptotic and anti-apoptotic signaling pathways (Hauck & Klein, 2013). Following interaction of HAX1 with presenilin-associated rhomboid-like protein (PARL) and HtrA serine peptidase 2 (HTRA2), the pro-apoptotic BAX (BCL2-associated X protein) pathway is activated (Chao et al., 2008; Cilenti et al., 2004). Anti-apoptotic function of HAX1 is performed through binding to ATPase Ca^{2+} transporting cardiac muscle slow twitch 2 (ATP2A2), thereby reducing its levels and balancing ER calcium (Vafiadaki et al., 2009).

***JAGN1*:** Truncating variants in *JAGN1* result in the maturational arrest of granulocytes at the promyelocyte/myelocyte stage. Non-hematological features may include failure to thrive and skin abscesses (Hojabri et al., 2023). These variants lead to defects in the

protein trafficking between the ER and the Golgi apparatus (Khandagale et al., 2021; Klein, 2011), thus impairing the neutrophils' homeostasis.

***G6PC3*:** Patients with pathogenic variants in *G6PC3* demonstrate neutropenia, which is associated with thrombocytopenia in some cases, as well as congenital heart defects, urogenital defects, failure to thrive, and endocrine disorders (Hauck & Klein, 2013). Studies suggest that defects in G6PC3 lead to a decrease in glucose levels, and consequent energy availability in neutrophils. This results in the disruption of protein folding and leads to accumulation of misfolded proteins in the ER, which makes the cells susceptible to apoptosis (Boztug et al., 2009).

3.7 Hypothesis and Aim of Studying HYOU1 as an Inborn Error of Immunity

Considering the role of HYOU1 in cell protection and handling cell stress, in addition to its implication in essential cellular responses such as UPR, we hypothesized that the *HYOU1* variant c.1331C>A (p.Pro444His) is responsible for the clinical features observed in our patient.

Considering the difference of clinical manifestation and importance of studying different variants and introducing the various clinical manifestations resulting from IEIs, we aimed to further characterize this phenotype and the pathways implicated as a consequence of this variant using a multiomics approach.

4. Manuscript I: Multiomics analyses reveal B cell differentiation arrest and neutrophil hypogranulation in a patient with an immunodeficiency caused by a novel homozygous missense variant in *HYOU1*

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Key Words: *HYOU1*, Immunodeficiency, multiomics, B cell deficiency, neutrophil hypogranulation

4.1 Abstract

The Hypoxia Upregulated Protein 1 (*HYOU1*) encodes an endoplasmic reticulum chaperone which is expressed in response to endoplasmic reticulum stress and contributes to cellular protection by restoring cell homeostasis. In this study, we report a patient with a novel homozygous variant in *HYOU1* (NM_001130991.3:c.1331C>A, p.Pro444His), who was born to related parents and presented with combined immunodeficiency, failure to thrive, and hypoglycemia. We undertook a multiomics analysis combining transcriptomics, proteomics, and single cell RNA sequencing analyses, demonstrating a drastic reduction in B cell count and hypogranulation of neutrophils in conformity with the findings of immunophenotyping. Additionally, we showed that despite the *HYOU1* transcript being expressed and stable, the patient has HYOU1 deficiency at the protein level. Moreover, single cell RNA sequencing of bone marrow revealed that the B cell differentiation process is prematurely arrested at pre-pro B cell stage. In conclusion, this work brings new insights into genotype-phenotype correlations by providing a detailed characterization of the B-cell deficiency and neutrophil hypogranulation as phenotypic features of an immunodeficiency due to *HYOU1* variants.

4.2 Introduction

Hypoxia Upregulated Protein 1 (HYOU1), also known as ORP150 (Oxygen Regulated Protein 150) and GRP170 (Glucose Regulated Protein 170), belongs to the Heat Shock Protein 70 (HSP70) family which is involved in protecting cells in physiological stress situations (Kusaczuk and Cechowska-Pasko, 2013). Located on chromosome 11.q23.3, *HYOU1* encodes a 150 kDa protein which is found to be upregulated as a result of endoplasmic reticulum (ER) stress, hypoxia, ischemia, and glucose deficiency, hence its crucial role in managing ER stress and preventing apoptosis (Kusaczuk and Cechowska-Pasko, 2013; Rao et al., 2021). Moreover, HYOU1 is implicated in the Unfolded Protein Response (UPR) which is activated by the accumulation of unfolded and misfolded proteins in the ER. Once initiated, UPR serves as a mechanism to manage ER stress and rescues cells from apoptosis (Kusaczuk and Cechowska-Pasko, 2013). HYOU1 is already known to be involved in various ER stress diseases such as Diabetes Mellitus, neurodegenerative disorders, and cardiovascular disorders (Rao et al., 2021).

In 2017, Haapaniemi et al. reported a novel case of an immunometabolic syndrome presenting with recurrent infections and stress-induced hypoglycaemia accompanied by granulocytopenia as well as B-cell and dendritic cell deficiency (Haapaniemi et al., 2017). By exome sequencing, they identified compound heterozygous variants in *HYOU1*. Their evaluations showed an altered cell metabolism possibly resulting from impairments of the UPR and mitochondrial function (Haapaniemi et al., 2017). These findings were in line with the hitherto known functions of HYOU1 in protein folding and secretion in the ER (Rao et al., 2021). Both variants reported in the mentioned case are located within the HSP70 domain of HYOU1 (Haapaniemi et al., 2017). Later, Jafari Khamirani et al. reported another case born to healthy consanguineous parents presenting with pancytopenia and immunodeficiency. This patient had a homozygous missense variant in the peptide binding domain of HYOU1 and came to attention at 2 months of age due to recurrent infections coupled with high-grade fever, eventually leading to the patient's death at three months or age (Jafari Khamirani et al., 2022). In 2022, Arab et al. reported a new patient with compound heterozygous variants in *HYOU1* presenting with chronic lung infection and severe neutropenia (Arab et al., 2022).

Herein, we discuss a new patient with a novel homozygous variant in *HYOU1*, also located in the HSP70 domain, presenting symptoms of immunodeficiency and metabolic disorder. We sought to investigate this patient by a multiomics approach to further understand the role of HYOU1 in the pathophysiology of the disease. Our findings showed severe B cell deficiency and neutrophil hypogranulation as key features of the phenotype of this patient, thereby extending the phenotypic spectrum of *HYOU1*-related immunodeficiencies.

4.3 Results

Clinical phenotype

The Patient (Figure 1A) is a 5-year-old male of Iranian ethnicity presenting with chronic diarrhoea with intermittent exacerbations associated with episodes of hypoglycaemia since early infancy. He was the first child of consanguineous (first cousins) parents and was born early-term and small for gestational age (SGA) with a birth weight of 1,900 g (<-3SD), length of 41 cm (<-3SD), and head circumference of 29 cm (<-3SD). His mother had poor weight gain during pregnancy. His parents reported no family history of note, and he has a healthy younger brother (II.2). The patient received a complete set of vaccinations at birth – bacilli Calmette–Guérin (BCG) vaccine, oral polio vaccine (OPV), and Hepatitis B vaccine (HBV) without any adverse events followed by pentavalent vaccine (Diphteria, Tetanus, Pertusis, Hepatitis B, and Haemophilus Influenza type B) at the age of two months. Although there is no documented evidence of hypoglycaemia before 6 months of age, his mother reported several episodes of floppiness and poor feeding. His foetal growth retardation was preserved throughout his infancy and childhood, which alongside episodes of diarrhoea and fever, led to being evaluated at 6 months of age for a suspected immunodeficiency. On the first investigation, a complete blood count and serum immunoglobulin tests revealed neutropenia (720 cell/ml) and low levels of Immunoglobulins (IgG 123 mg/dl, IgM 81 mg/dl, IgA 15 mg/dl, IgE 4 IU/ml) which confirmed the presence of an immunodeficiency. On chest X-Ray obtained at 6 months of age, no thymus shadow was observed. At that time, Intravenous immunoglobulin (IVIG) replacement therapy was initiated with a provisional diagnosis of agammaglobulinemia. Despite regular IVIG replacement, he had several episodes of severe diarrhoea and hypoglycaemia, as well as recurrent oral aphthae, suggesting a combined immunodeficiency. When the patient was 12 months of age, Computed Tomography (CT) of the abdomen revealed mild hepatomegaly with diffuse fatty infiltration in the liver. Duodenal biopsy at 16 months of age showed mild duodenitis and a significant decrease in superficial lymphoplasma cells.

To correct neutropenia, pegylated filgrastim (100 μgr/kg every 14 days) was administered at 3 years 8 months of age, which resulted in a decrease in the frequency of diarrhoea episodes and consequent hospitalisations.

A peripheral blood smear at 4 years of age showed hypogranularity of neutrophils alongside segmentation anomalies in the nuclei, especially hyposegmentation (Figure 1B).

A histopathological study of the patient's bone marrow at 4 years of age showed a non-blastic rich marrow, alongside a predominance of the granular line without maturation blocking with a reserved compartment of 45% of neutrophils, and the presence of megakaryocyte line, as well as a collapse in erythroid line. Rare pyknosis forms were also observed. Nonetheless, the histopathological study of the bone marrow prior to G-CSF administration indicated a blockade of the granular line at the promyelocyte stage with the presence of a few myeloblasts, in the absence of other precursors (myelocyte and metamyelocyte). Polymorphonuclear neutrophils were rarely observed, with an increase in monocytes. The erythroid lineage was reportedly relatively diminished but with normal maturation.

At 4 years and 2 months of age, another episode of fever, diarrhoea, and poor feeding occurred. Given the history of COVID-19 exposure in the family, followed by a positive PCR of nasopharyngeal swab for SARS-COV-2, he received rehydration and IVIG, which resulted in an amelioration of his condition. No antiviral therapy was administered. Prothrombin time, partial thromboplastin time and international normalized ratio drawn during the episode of SARS-COV-2 infection were normal.

During the last evaluation at 4 years and 6 months of age, his weight was 8,500 g (-8.2SD), height 80 cm (-5.7SD), and head circumference 43 cm (<-3SD). The patient had minor facial dysmorphism compared to his healthy brother and dental caries. The patient had reportedly met developmental milestones (including gross motor, fine motor, language, cognitive, social-emotional and behavioural development) for his age. The remainder of the examination was normal. Throughout these years, he had mild microcytic anaemia, thrombocytosis, and hypoglycaemia on laboratory testing. Between 6 months and 4 years and 6 months of age, blood levels of electrolytes, phosphorus, magnesium, urea nitrogen, albumin, and total protein were normal, as were the results of tests of lipid profile and thyroid function; other test results are shown in Table 1. At the moment, he is being treated with monthly IVIG, peg-filgrastim, and prophylactic cotrimoxazole.

Genetic analyses

Given the severity of the symptoms in the patient, exome sequencing was performed on the patient, healthy brother and both parents to investigate any potential causal variants. Applying successive filtering steps based on variants' functional consequences, frequency and autosomal recessive inheritance we identified a missense variant, c.1331C>A (NM_001130991.3); p.Pro444His (NP_001124463.1) in the hypoxia up-regulated 1 (*HYOU1*) gene that is predicted to be deleterious by various bioinformatic prediction tools (Table 2). The remaining candidate variants and their annotation are shown in Table 1S. Sanger sequencing confirmed the homozygous status of this variant in the patient, the heterozygous status in his parents, and the wild-type homozygous status of his brother (Figure 1C).

Effect of Pro444His on the structure of HYOU1

Structural modelling confirmed the predicted pathogenicity of this variant on protein structure. Proline (444) can effectively stop the formation of the β-sheet, as a result of this variant the β-sheet tends to extend, thus possibly impairing the function (Figure 1D). Given that this variant is located on the protein binding domain of HYOU1 (Takeuchi, 2006), the variant leads to potential dysfunctions in interactions of HYOU1 with other proteins.

HYOU1 expression in dermal fibroblasts of the patient

Western blot analyses indicated a significant decrease in the expression of HYOU1 as compared to controls (Figure 1E). Quantification of these results showed that the expression of HYOU1 represented approximately 10% of the expression level in controls ($p=0.0102$, Figure 1F). This finding was in line with the results of proteomic analysis, in which the expression of HYOU1 compared to controls was approximately 20% ($p < 0.0001$, Figure 1G). Quantitative RT-PCR revealed no significant difference in the expression of *HYOU1* in the patient as compared to controls (Figure 1H).

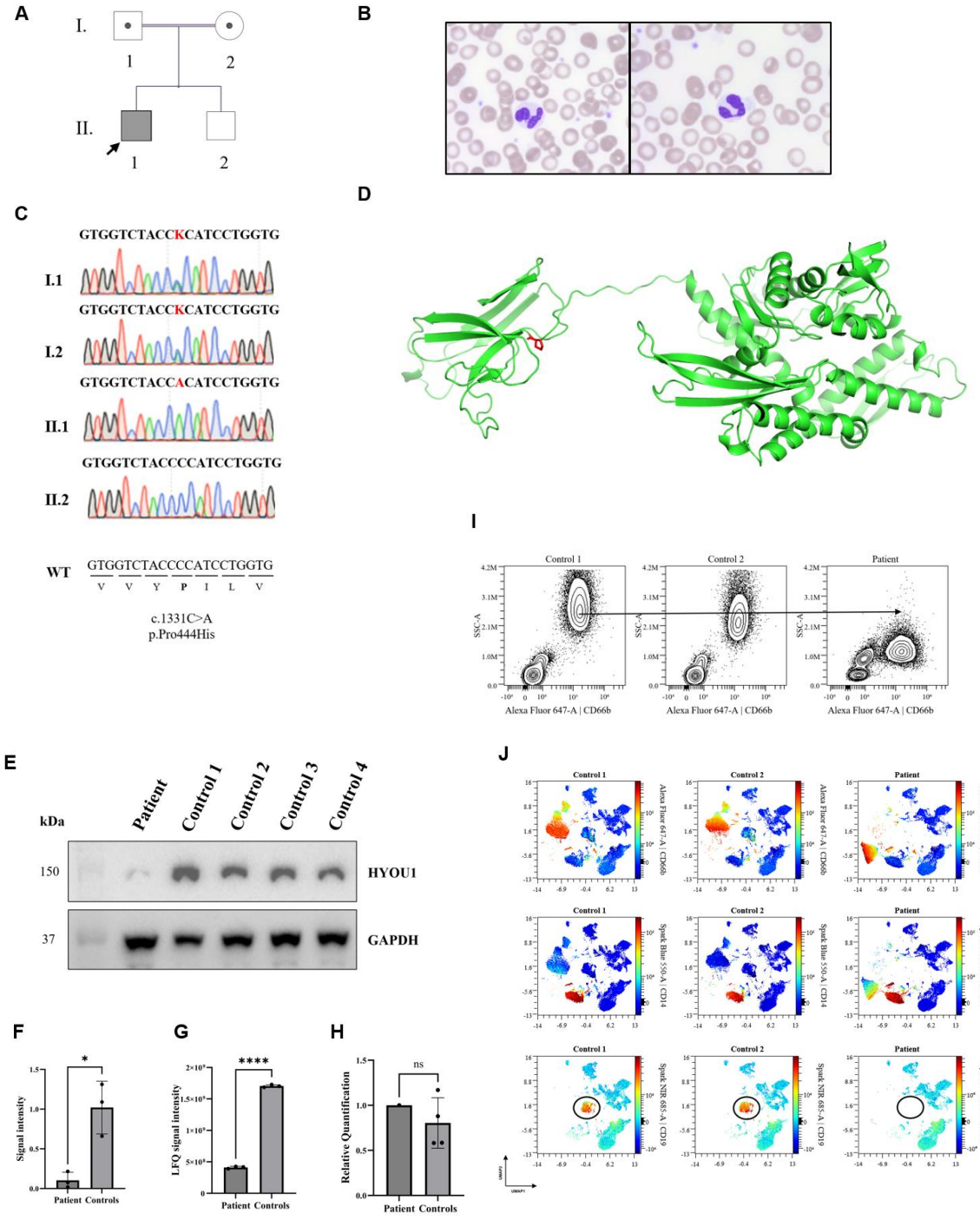

Figure 1.(A) *Pedigree of the patient. Roman numbers and Arabic numerals indicate generations and subjects, respectively. Double lines connecting the parents connote consanguinity. Squares, male subjects; circles, female subjects; filled (grey) symbol represents patient; white symbols represent unaffected family members; Dotted symbols represent carriers of the pathogenic variant; the arrow denotes to the proband. All members of the family were subjected to whole-exome sequencing.* ***(B)*** *The peripheral blood smear of the patient shows hypogranuled neutrophils.* ***(C)*** *Sanger traces of the HYOU1 NM_001130991.3:c.1331C>A, p.Pro444His variant in the family confirms the homozygous status of the proband and heterozygous status of the parents for the mutation. The electropherograms denote single base pair substitutions of Pro444His in HYOU1.* ***(D)*** *Structural modelling of HYOU1. HYOU1 is shown in green and Pro444His mutation is displayed in red.* ***(E)*** *Western blot analyses of HYOU1 protein expression in dermal fibroblasts of the patient and four controls. HYOU1 protein is absent in the patient. GAPDH was used as the internal control.* ***(F)*** *Signal intensity of HYOU1 protein relative to the expression of GAPDH (p-value 0.0102)* ***(G)*** *LFQ signal intensity of HYOU1 in the proteomics study of the dermal fibroblasts of patient and controls (p-value<0.0001).* ***(H)*** *Quantitative real-time PCR of dermal fibroblasts of the patient and controls revealed no difference of HYOU1 expression (p-value= 0.57).* ***(I)*** *Flow cytometry scatter plot depicting granulocytes in the patient and two controls, labelled with CD66a (Alexa Fluor 647) on the x-axis and side scatter (SSC) on the y-axis. The patient's granulocytes have considerably lower SSC-A indicating lower granularity.* ***(J)*** *The UMAP (Uniform Manifold Approximation and Projection) visualization depicts the analysis of white blood cells obtained from flow cytometry data of the patient and two controls coloured based on expression levels of CD66b (Alexa Fluor 647-A), CD14 (Spark Blue 550-A), and CD19 (Spark NIR 685-A). The Granulocytes of the patient are different in the expression of CD14 and CD66a. Almost no B cell is observed in the patient.*

Immunological phenotype

Compared to age-matched controls, immunophenotyping using flow cytometry revealed a significant decrease in the B cell compartment ($CD19^{+}CD20^{+}$) of the patient (0.14% and 0.2% detected in two different sampling of leukocytes in the patient, normal range 10.21%-20.12%) (Table 3). All B-cell subsets were drastically reduced (Table 3). We observed a less profound phenotype in the T cells of the patient. While a subtle increase in the percentage of $CD8^{+}$ T cells was observed the patient versus controls, the percentage of naive $CD8^{+}$ ($CD45RA^{++}CCR7^{+}CD27^{+}CD28^{+}$) and central memory CD8+ (CD45RA-CCR7+) was reduced and that of terminally differentiated effector memory cells (TEMRA; $CD45RA^{++}CCR7^{-}CD27^{-}CD28^{-}$) was increased. We observed a more notable increase in the $CD4^{+}$ T cells of the patient, with an increase in naive $CD4^{+}$ T cells ($CD45RA^{++}CCR7^{+}CD27^{+}CD28^{+}$). Moreover, the percentage of γδT cells was slightly lower in the patient versus controls, with an increase in the percentage of naive cells ($CCR7^{-}CD45RA^{++}$). Among NK cells, mature NK cells ($CD56^{+}CD16^{+}$) were decreased in favour of terminal NK cells ($CD56^{-}CD16^{+}$).

Although the number and percentage of patient's neutrophils were normal, we observed two additional atypical populations in the granulocytes of the patient (Figures 1I, 1J), characterized by significantly lower-than-expected granules (SSC^{lo}).

Finally, we also found an increase in the percentage of plasmacytoid dendritic cells ($CD123^{+}CD11c^{-}$) in the patient compared to controls.

Transcriptomics and proteomic analysis of peripheral blood mononuclear cells (PBMC)

The most downregulated genes observed in the transcriptomics data were B cell marker genes and genes involved in B cell activation (*MS4A1*, *CD19*, *IGHD*, *CD79A*, *IGHM*). Similarly, proteomics analysis revealed a drastic downregulation of B cell-related proteins (MS4A1, IGHM, IGHA1) in addition to a significant downregulation of the humoral immune response pathway in the patient compared to the controls (Figures 2A, 2B, and 2C). In transcriptomics, we noticed the alteration of certain innate immunity pathways. Among these TNF signalling pathways, signalling by interleukins, and granulocyte activations were upregulated in the patient compared to the controls (Figure 2D), while in proteomics

data, the inflammation mediated by chemokine and cytokine signalling pathway was downregulated in the patient (Figures 2B). We also observed downregulation in the STAT3 - a signal transducer for GCSF in the proteomics data (Figure 2A). In transcriptomics, *IL18R1* was significantly upregulated in the patient in comparison with the control which indicates the activation of INFγ regulated cytokines (Figure 2C). We also observed an upregulation of granulocyte activation as well as secretory granules membrane pathways in the transcriptomics analysis (Figure 2D) and the key neutrophil attractant *CXCL2* was significantly upregulated in the patient (Figure 2C) and is an important marker of neutrophil migration (p= 0.039, log-fold-change= 2.03).

Transcriptomic analysis of bone marrow

In bulk transcriptomics analysis of bone marrow cells of the patient compared to control, certain genes associated with interferon response (*IRF1*, *GBP5*, *CD69*) were upregulated as well as genes encoding subunits of IFNβ and IFNγ receptors (*IFNAR1*, *IFNGR1*). Genes encoding proteins in azurophilic granules, gelatinase granules, and specific granules (*ELANE*, *MMP9*, and *LCN2*, respectively) were upregulated while the cell cycle proteins (*MKI67*, *TOP2A*) coding genes were downregulated (Figure 2E). The apoptosis and neutrophil degranulation signalling pathways were upregulated in the patient in comparison with the controls (Figure 2F). Moreover, the TNF signalling pathway was upregulated in the patient in conformity with the findings of the RNA sequencing in the PBMC (Figure 2D). In line with the failure to thrive (FTT) observed in the patient, osteoclast differentiation was upregulated (Figure 2F) (Nandi et al., 2022).

Transcriptomic analysis of dermal fibroblasts during cellular stress

To compare ER stress and its regulation mechanisms such as UPR, we performed RNA sequencing on the dermal fibroblasts of the patient and healthy controls treated with and without tunicamycin (0.5 µg/ml). Prior to stress induction, we observed that the 'IREα activates chaperons', 'UPR', and 'XBP1s activates chaperone' pathways were upregulated in the patient, indicating higher stress levels without the presence of a stress-inducing factor (Figure S2A). However, the expression of HYOU1 was not significantly altered compared to the controls (p-value= 0.054 LogFC2= 1.02). After six hours of

exposure to tunicamycin, we compared the treatment effect in the patient to the treatment effect in the controls. We observed that UPR genes (*HSPA5* and *ERN1*) were downregulated, in addition to significant downregulation of *HYOU1* in the patient in the tunicamycin-treated cells of the patient in comparison to the mock-treated cells. The majority of significantly downregulated genes in the patient were ER stress regulator genes such as *TRIB3*, *DDIT3*, *TXNIP*, and *HERPUD1*. *SESN2* was among the genes significantly downregulated and is related to reactive oxygen species (ROS) (Figure S2B). The UPR inducer *ATF3* was also significantly downregulated in the patient. Gene Set Enrichment Analysis (GSEA) revealed that response to endoplasmic reticulum stress process was significantly downregulated in patient versus controls (Figure S2A).

Single-cell RNA-sequencing

To further understand the impact of the HYOU1 Pro444His variant, we performed single-cell RNA sequencing (scRNA-seq) on unsorted cells obtained from peripheral blood and bone marrow samples of the patient and healthy controls. Following quality control and normalization, 28,757 cells were obtained in total in blood samples (Figure 3A; n=15,737 composed of three biological replicates for the patient and n=13,021 cells in the four controls). After performing Principal Component Analysis (PCA) for the screening of significantly correlated genes, we selected 20 Principal Components (PCs) for further analysis. We distributed the cells in the Uniform and Manifold Approximation and Projection (UMAP). Graph-based clustering revealed 11 clusters of cells in the blood (Figure S3). We used the following markers to identify the clusters: *CD19*, *CD79A*, *CD79B*, and *MS4A1*(*CD20)* for B cells, *CD3D*, *CD3E*, *CD3G*, *CD4*, *CD8A*, *CD8B* and *TRAC* for T cells, *GNLY, GZMB*, *GZMA*, and *PRF1* for NK cells and cytotoxic cells *CD14*, *CD68*, *FCN1*, and *VCAN* for monocytes, and *FCGR3B* (*CD16b), ALPL, and CXCR2* for neutrophils (Figure S3A, S4). *HYOU1* was expressed in all clusters of the patient and controls, except in controls' neutrophils (Figure S3B).

There was a drastic reduction of B cells in the patient (n=0 for a total of three biological replicates) compared to the controls (n=2,541 for a total of four controls) (Figure 3A). In addition, we observed that the majority of the neutrophil and monocyte populations was from the patient (patient=5,326, controls=1,381), which could be due to treatment with

GCSF (Figure 3A). A more in-depth analysis of neutrophils showed two completely distinct clusters of neutrophils in the patient and controls (Figure 3B). One cluster was composed solely of patient's neutrophils (neutrophil cluster 2, NC2), whereas the other was composed of both the patient's and controls' neutrophils (neutrophil cluster 1, NC1). While *HYOU1* expression was not different between the two neutrophil clusters, the patient's neutrophils expressed significantly more *HYOU1* (p-value= 1.42E-6, fold change 4.83). Comparing the NC1 to the NC2, we noticed that gelatinase (*LYZ* and *HPSE*) and secretory (*CR1*, *CD14*, *ITGAM*) granules genes were overexpressed. There was no significant difference in the expression of genes encoding azurophilic granules (*ELANE* and *MPO*). Consistent with our previous findings in immunophenotyping, *CD14* was significantly over-expressed in patient's neutrophil compared to that of the controls (p-value 5.68E-137, fold change 13.90) possibly due to GCSF administration (Montaldo et al., 2022; Pedersen et al., 2016). Additionally, Toll-like receptor genes including toll-like receptors (*TLR2* and *TLR6*) were over-expressed in the patient (Figure 3C). We performed differential expression analysis to further characterize the differences between these two clusters. GSEA revealed the IFN-γ signalling and IFN α/β signalling pathways being significantly up-regulated in NC1 compared to the NC2 (Figure S5).

The majority of cells in both the patient and controls were T cells. Our analysis did not show any significant difference between the number of T cells between the patient and the controls (Figure 3A). *HYOU1* was up-regulated in patient's T cells in comparison with the controls (p-value 9.8E-29, fold change 2.03). GSEA of the patient's T cells revealed that T cell activation and regulation of apoptotic signalling pathways were significantly up-regulated compared to the controls (Figure S6).

In the scRNA-seq analysis of the bone marrow, after quality control and normalization, 5,157 cells were obtained in total (n=2,673 in the patient and n=2,484 in the control). We added three more controls from the publicly available data published by Oetjen et al., 2018 (number of cells after quality control and normalization: Control C2 = 2,496, Control W = 3,396, Control B = 2,945). We performed PCA and used 20 PCs for further analyses. We used Harmony to correct the batch effect for run and sex. Graph-based clustering revealed 13 clusters of cells in bone marrow. We used azimuth (Hao et al., 2021) to

annotate the clusters using known markers of cell populations shown in Figure 4A. *HYOU1* was not majorly expressed by a distinct cluster.

There were more granulocyte and monocyte populations in the patient than in the controls (patient=1354, 4 controls=1203, Figure S7).

Trajectory analysis in the B cell lineage according to automatic annotation using Azimuth (Figure 4B) revealed that the continuum of B cell differentiation in the patient was almost blocked before the pro B cell stage (Figure 4C, 5A) although *HYOU1* was expressed throughout the whole B cell continuum (Figure 5B).

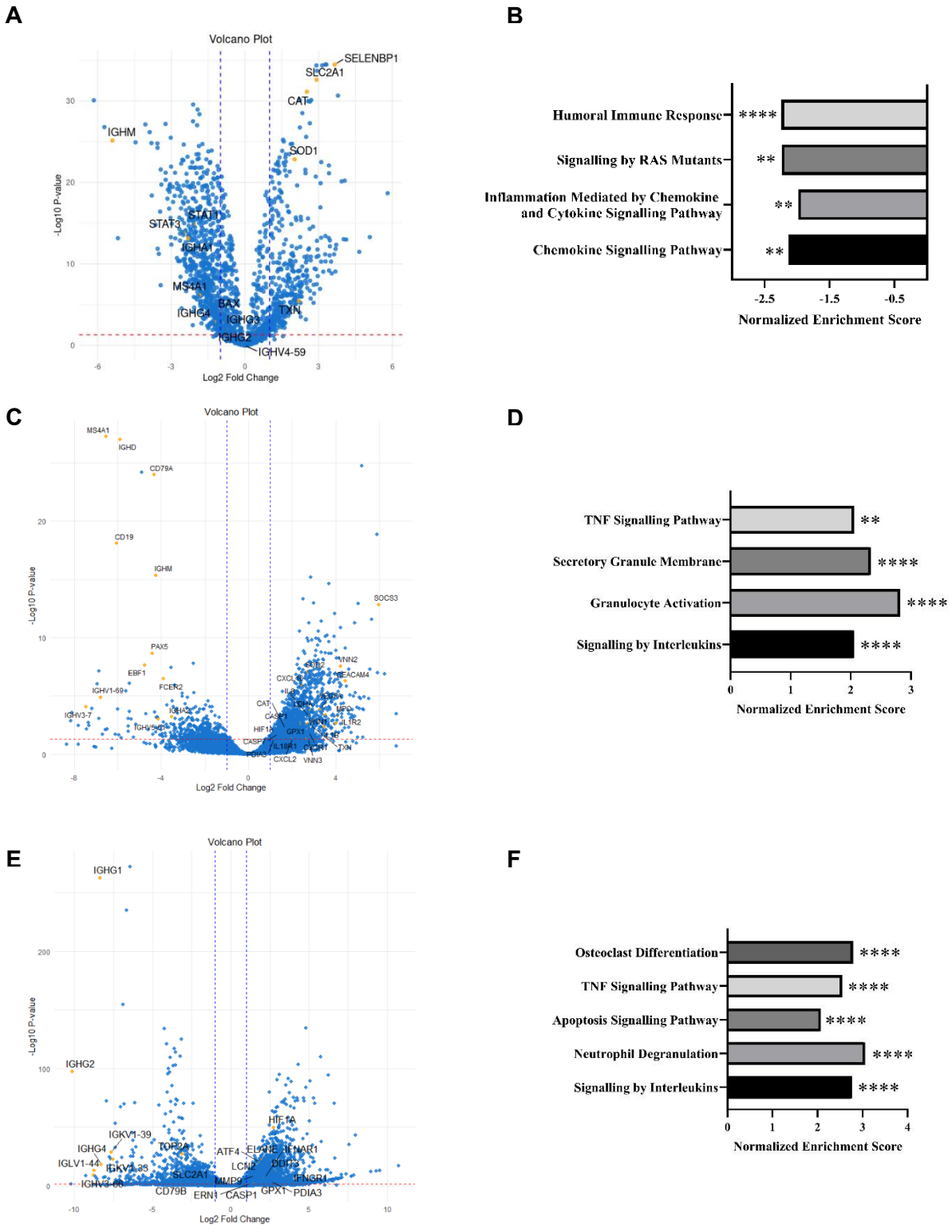

Figure 2. *Multiomics analyses* ***(A,C,E)*** *Volcano plots demonstrating up and downregulated genes and proteins in the patient compared to the controls, in proteomics of PBMC, bulk transcriptomics of PBMC, and bulk transcriptomics of bone marrow, respectively. using log2 fold change and P-value cutoffs of 1 and 0.05, respectively.* ***(B,D,F)*** *Dysregulated canonical pathways respectively in the PBMC proteomics, PBMC bulk transcriptomics, and bone marrow bulk transcriptomics of patient compared to controls, **, P < 0.01; ****, P < 0.0001.*

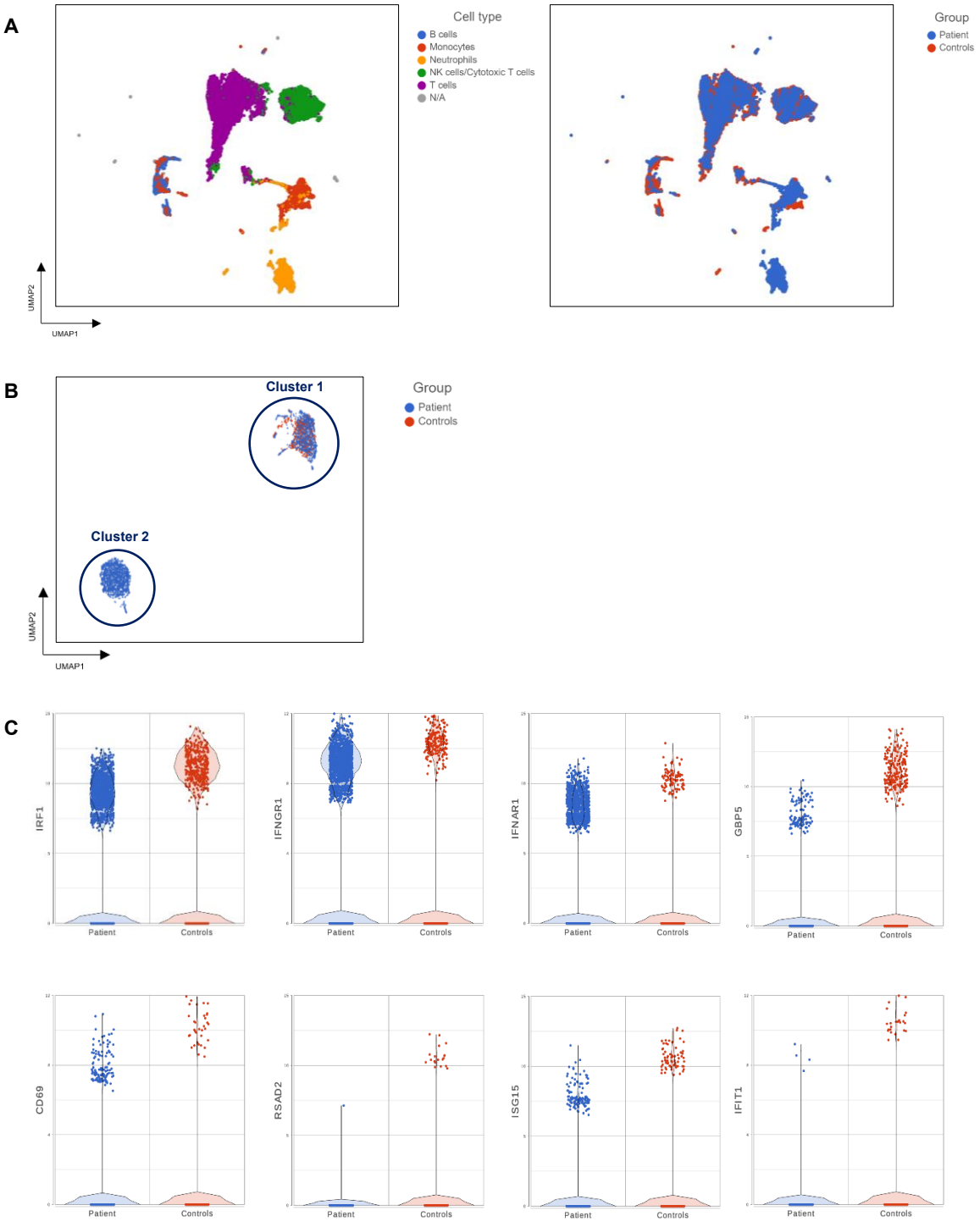

Figure 3. (A) *UMAP plot showing blood cells by cell type (left, colours correspond to cell type, right colours correspond to samples)* ***(B)*** *Reclustering of blood neutrophils shown in UMAP plot (blue, patient cells; red, control cells)* ***(C)*** *Dot plots showing the differentially expressed genes belonging to the response to the interferon-gamma and response to type I interferon pathways in neutrophil cluster 1 compared to the neutrophil cluster 2.*

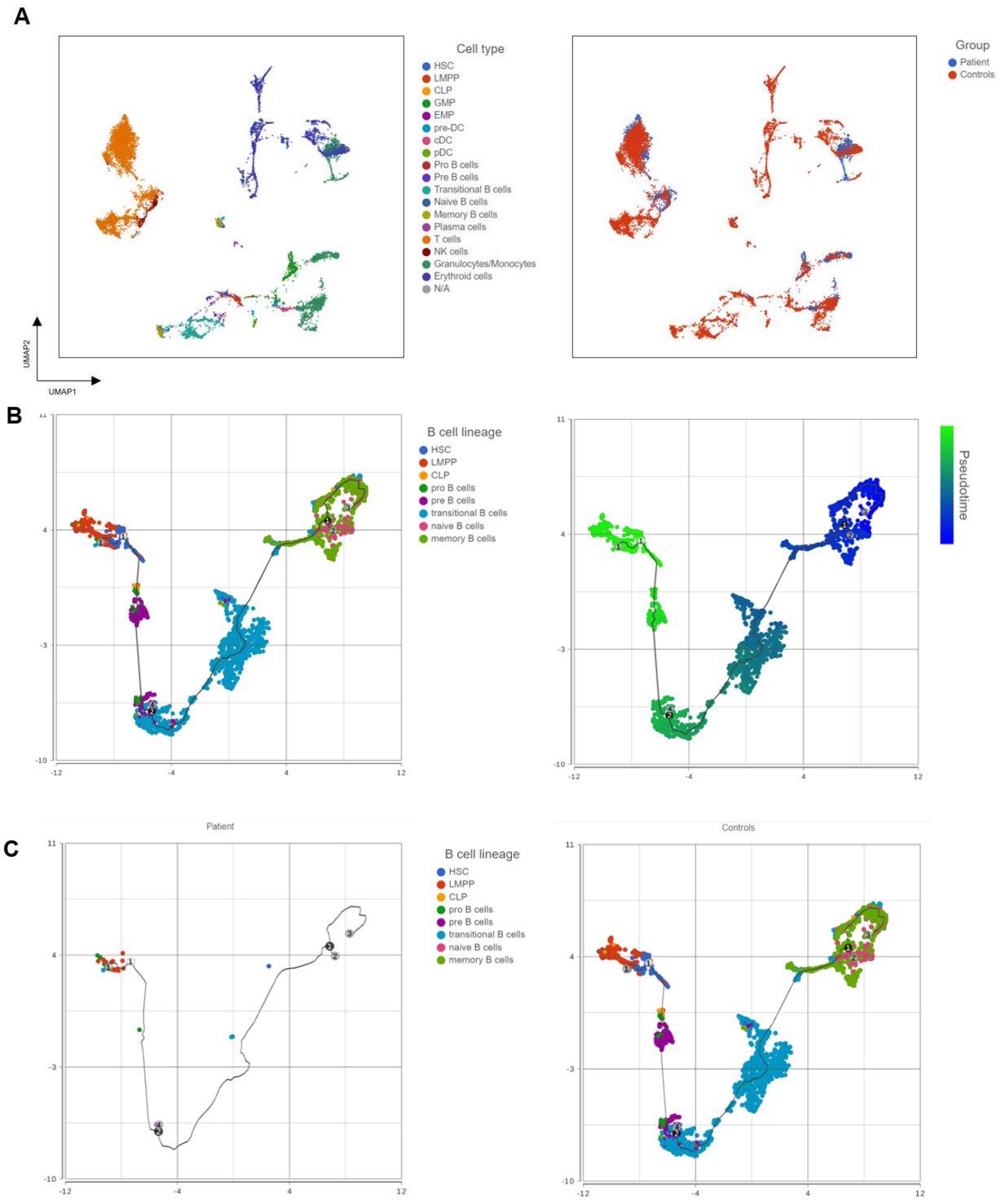

Figure 4. (A) *UMAP plot showing single-cell transcriptome of bone marrow cells in patient and control. (left, colours correspond to cell type, right colours correspond to samples)* ***(B)*** *Trajectory analysis of the B cell lineage of the patient and controls (left) Pseudotime trajectory denoting the differentiation of B cell lineage (right).* ***(C)*** *The B cell lineage of the patient (left) and the controls (right) according to annotation.*

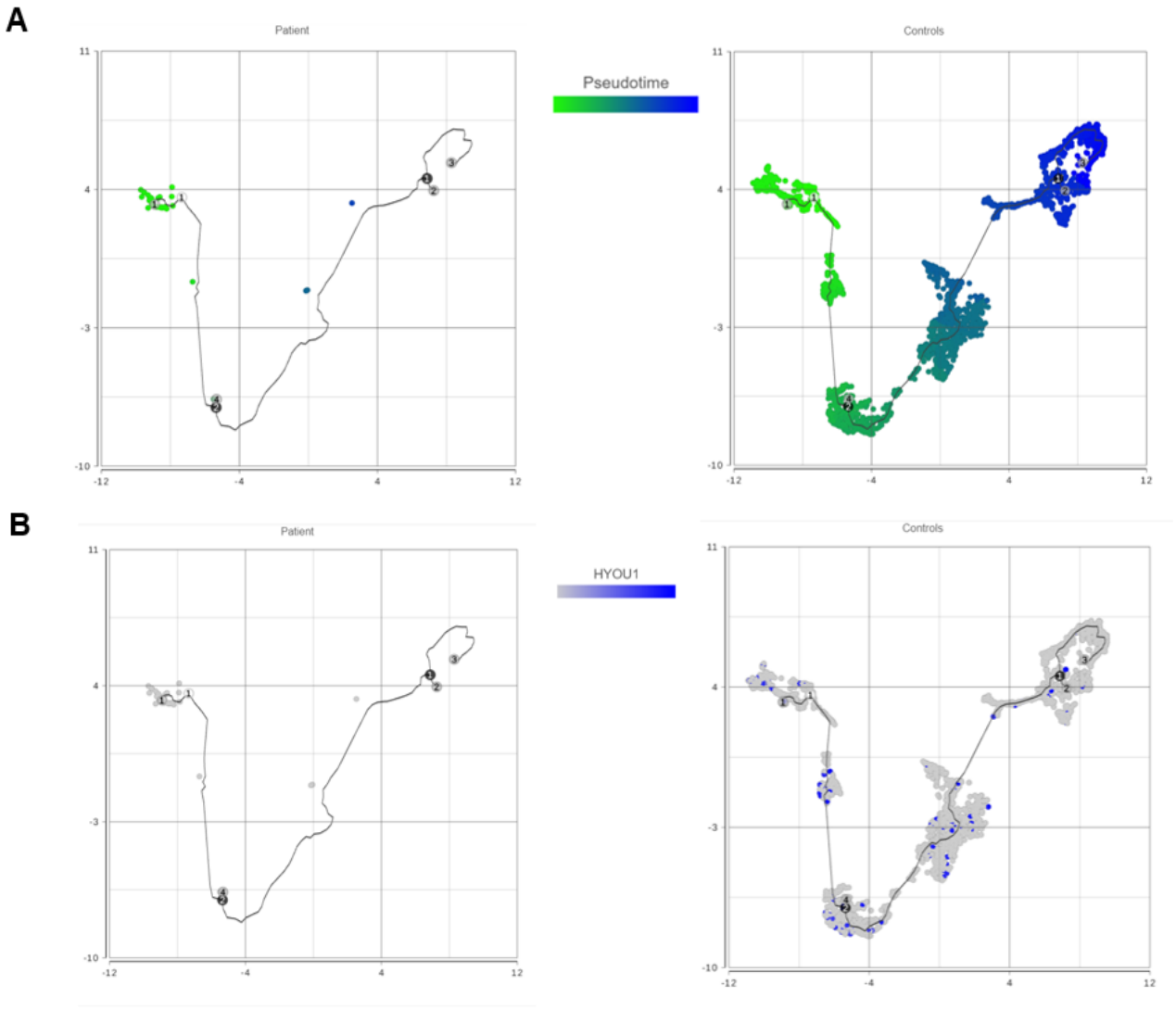

Figure 5. (A) *The B cell lineage of the patient (left) and the controls (right) according to pseudotime analysis* ***(B)*** *Expression of HYOU1 in the B cell lineage of the patient and controls.*

4.4 Discussion

In this study, we report a patient with a novel pathogenic variant in *HYOU1* who presented with chronic diarrhoea, FTT, and episodes of hypoglycaemia. All previous case reports of patients with a variant in *HYOU1* (Haapaniemi et al., 2017; Arab et al., 2022; Jafari Khamirani et al., 2022) showed recurrent infections accompanied by neutropenia (Table 4). The patient reported by Jafari Khamirani et al. died one month after showing the first symptoms (respiratory tract infection) at 3 months of age, unlike the other three patients. Similarly to our patient, Haapaniemi et al. also reported SGA, FTT, and episodes of stress-induced hypoglycaemia. All patients except the one reported in the present study had experienced respiratory infections. When considering the three published cases and the present one, anaemia and skeletal anomalies were reported in three out of four patients, including the present case. Moreover, three of the patients had reduced numbers of B cells, including the present case. Although the majority of clinical manifestations were shared among the patients, the differences could be explained by the protein domain localization of the variants. Moreover, two of the patients reported before (Haapaniemi et al., 2017; Arab et al., 2022) carried compound heterozygous mutations in multiple domains of *HYOU1*. In total, neutropenia and B-cell deficiency leading to recurrent infection are the prominent attributes of this phenotype and the differences in the phenotypic spectrum could be due to the patient specific variants or caused by somatic mutations in modifying genes.

When HDF cells were stressed using tunicamycin, we observed downregulation of *HYOU1* transcript coupled with downregulation of response to the endoplasmic reticulum pathway, signifying the inability of *HYOU1* mutated cells to cope with stress – physiological or induced. This also suggests that HYOU1 is involved in a self-feedback loop. While overexpression of *HYOU1* is already linked to ER stress-related diseases (Grootjans et al., 2016), we demonstrated that this variant which leads to the absence of the HYOU1 protein is associated with UPR activation and apoptotic pathways.

UPR activation is known to play an important role in plasma cell differentiation through *XBP1* splicing. Owing to extensive protein synthesis and high levels of antibody production in plasma cells, ER expansion takes place prior to immunoglobulin synthesis secretion during plasma cell differentiation. Plasma cell differentiation is known to be

XBP1-dependant and defects in XBP1 or UPR lead to a decrease in antibody production, while XBP1 deficiency results in the absence of plasma cells (Grootjans et al., 2016). Other studies have shown that UPR is activated in pro-B cells and pre-B cells (Grootjans et al., 2016; Zhang et al., 2005), which is in line with the stage wherein the arrest of B cell differentiation occurs in the patient.

UPR and XBP1 activation are pivotal to plasma cell differentiation and immunoglobulin secretion (Gass et al., 2008), as well as early lymphopoiesis in the stage of pro-B cells (Zhang et al., 2005). Although the exact place of HYOU1 implication in the UPR is not known, HYOU1 is known to play a cytoprotective and antiapoptotic role in response to ER stress (Kusaczuk and Cechowska-Pasko, 2013). Here we showed that the absence of HYOU1 is associated with differentiation arrest at the pro-B cell stage, leading to the very low number of B cells in the bone marrow and blood.

Moreover, we showed that the neutrophils were hypogranuled in the BM prior to G-CSF administration, and hypogranulation of neutrophils continued after receiving G-CSF. Although the expression of certain genes in neutrophils was altered as previously described by Montaldo et al. (Montaldo et al., 2022) and Pedersen et al. (Pedersen et al., 2016), interferon-related genes were down-regulated in response to G-CSF. These data show that not all changes in neutrophil transcripts are caused by G-CSF. Altogether, these findings indicate that the hypogranulation and lack of response to IFNs are inherent characteristics of patient's neutrophils and that the *HYOU1* variant rather impacts neutrophils' morphology, granule content, and function than numbers. Previous studies support the synergistic influence of UPR activation upon IFN-β synthesis (Smith et al., 2008), suggesting that impairment in UPR activation and XBP1 splicing plays a role in the downregulation of response to type I interferon in the neutrophils of the patient.

Moreover, previous studies demonstrated that the UPR is a pivotal modulator of skeletal metabolism and homeostasis and is crucial for managing physiological stress caused by production of extracellular matrix proteins (Rao et al., 2021). The UPR is also involved in the differentiation of osteoblasts, chondrocytes and osteoclasts. Defects in the UPR were shown to be associated with multiple skeletal disorders such as osteoporosis and congenital skeletal disorders (Horiuchi et al., 2016). Upregulation of the osteoclast differentiation pathway in the RNA-seq of bone marrow of the patient in this study

suggests that FTT and SGA in the patient could be associated with the role of the UPR in the differentiation of osteoclast.

4.5 Conclusion

Taken together, we demonstrated that the HYOU1 Pro444His affects neutrophil function and morphology and that the development of B cells is arrested at the stage of pre-pro B cells. These features may contribute to impairment in innate and adaptive immunity in patients with *HYOU1* variants.

4.6 Materials and Methods

Subjects, study approval, and sampling

The subjects of this study were members of a consanguineous family of Iranian ethnicity. The parents and brother of the proband were healthy. All subjects (or their legal guardians) gave written informed consent to participate in this study, which was conducted according to the principles of the Helsinki Declaration and approved by the institutional review board of Tehran University of Medical Sciences (Tehran, Iran).

Bone marrow biopsy and skin biopsy were obtained from the proband. Blood sampling was carried out for proband and his family. In total, sampling for proband's blood was done three times over the course of two years to be used as biological replicates. Peripheral Blood Mononuclear Cells (PBMC) were isolated within 24 hours of sampling using Ficoll-Paque density gradient centrifugation.

Whole exome sequencing

Genomic DNA was isolated from peripheral blood of the proband (II.1), his parents (I.1, I.2) and his healthy brother (II.2) using standard protocols. Exome sequencing was performed on a NextSeq500 sequencer (Illumina, San Diego, CA, USA) as described previously (Castro et al., 2020). The following filtering steps were applied for variant identification in the proband, i.e. selecting only (i) variants with a depth of coverage of more than or equal to 10 reads; (ii) protein-altering variants (excluding noncoding or synonymous variants), (iii) variants with allele frequencies < 0.001 in the 1000 Genomes Project, the Genome Aggregation Database (gnomAD), and an internal database including ~1000 exomes, and (iv) variants homozygous in the proband, heterozygous in parents, heterozygous or wildtype in the healthy brother. Raw exome files are available at National Center for Biotechnology Information (accession no. pending).

Sanger sequencing

The candidate variant was confirmed by Sanger sequencing as described earlier (Castro et al., 2020) and using the following primers: 5'-GCGGGACCCTTTTCTGTTGTCATC-3' (forward) and 5'-GGATCTCCTGAGGGCTATGTCACAG-3' (reverse) for both amplification and sequencing.

Whole transcriptome and proteome analyses

Bulk RNA sequencing was performed on bone marrow, human dermal fibroblasts (HDF) and PBMCs of the patient and were compared to the same sample types of healthy controls. Total RNA was extracted using the RNeasy Mini Kit (74106, Qiagen, Hilden, Germany). Total RNA-seq library preparation, sequencing, and analyses were performed as previously described (Castro et al., 2020). Up- and down-regulated genes were selected based on the adjusted p-value (<0.05) and the log-fold-change (≥1). Raw RNA-seq data have been deposited in the EMBL-EBI ArrayExpress archive (accession no. pending).

Proteomics analyses were performed on HDFs and PBMCs. Protein extraction, enzymatic digestion, HPLC separation, analysis of the resulting peptides with nano-liquid chromatography/tandem mass spectrometry (nano-LC-MS/MS), and software-based protein quantification using MaxQuant were performed using the previous protocols described (Castro et al., 2020). Up- and down-regulated proteins were selected based on the adjusted p-value (<0.05) and the log-fold-change (≥1). The complete proteomics dataset has been deposited into the ProteomeXchange Consortium via the PRIDE partner repository (accession no. pending).

Gene Ontology and pathway analyses of differentially expressed genes and proteins were performed using the WEbGestalt toolkit (Liao et al., 2019) (www.webgestalt.org). The functional databases of biological process non-redundant, cellular process non-redundant, and molecular process non-redundant were used for Gene Ontology analyses. The functional databases of KEGG, Panther, and Reactome were used for pathway analyses. Galaxy was used for creating heatmaps (Galaxy community, https://galaxyproject.org/community/).

Single-cell RNA sequencing

Five hundred µl of whole blood and bone marrow were incubated twice for 10 min at room temperature with RBC lysis solution 1X (Miltenyi, Bergisch Gladbach, North Rhine-Westphalia, Germany) to remove red blood cells. Cells were then washed and centrifuged at 500 g and subsequently resuspended in DPBS (14190144, Gibco™, Thermo Fisher

Scientific, Waltham, MA, USA) containing 2% FBS for cell counting using Trypan Blue and Countess™ II FL (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA).

Single-cell RNA sequencing (scRNA-seq) libraries were prepared using the Chromium Single Cell 3' Library and Gel Bead kit v3.1, Chip G kit, Single Index kit T set A and the Chromium Controller according to the manufacturer's instructions (10X Genomics, Pleasanton, CA, USA). Cells were loaded at a volume to target around 5,000 cells per sample. Size distribution and concentration of cDNA and final libraries were verified on an Agilent Bioanalyzer High Sensitivity chip (Agilent Technologies, Santa Clara, CA, USA). Libraries were sequenced using an Illumina NextSeq 2000 instrument according to the manufacturer's guidelines (Illumina, San Diego, CA, USA) with a sequencing depth of at least 30,000 reads per cell. Raw scRNA-seq data have been deposited in the EMBL-EBI ArrayExpress archive (accession no. pending).

Raw sequencing data were processed using Cell Ranger analysis pipeline, v6.1.2 (Zheng et al., 2017). The "mkfastq" command was used to generate FASTQ files and the "count" command to generate raw gene-barcode matrices aligned to the 10X Genomics GRCh38 Ensembl build 84 genome (version 1.2.0). The "--force-cells" command was used to run cellranger count and include low- Unique Molecular Identifiers (UMI) barcodes in order to capture neutrophils from 3' or 5' Single Cell Gene Expression data.

Partek® Flow® software v10.0 was used for single-cell RNA sequencing analyses. The following filtering criteria were used to remove the low-quality cells with (i) <200 genes and/or (ii) <300 UMI counts and/or (iii) >8% mitochondrial gene counts. In blood, this resulted in a total of 28,757 cells, 13,021 cells for the patient (merged from three independant samplings) and 15,736 cells for the controls (four controls including patient's healthy brother). In the bone marrow, the total residual cells were 5,139 cells, 2,668 for the patient and 2,471 for the control. Normalization was conducted using the following parameters: counts per million, add 1.0 Log 2.0. Principal component analysis (PCA) was performed and Harmony was used on the PCA space for the removal of the batch effect. Graph-based clustering was conducted using 20 principal components, followed by Uniform Manifold Approximation and Projection (UMAP) analysis. Azimuth was used for automatic annotation of cells. Differential gene expression was performed after annotation, comparing the normalized data of patient versus the controls using an

analysis of variance (ANOVA) model. The filtering criteria for differentially expressed genes used for pathway analysis were adjusted p-value (<0.05) and fold change (≥1.5). Monocle 3 was use with Partek Flow for trajectory analysis of B cell lineage.

Structural modelling

The crystal structure of lid-truncated apo BiP in the Protein Data Bank (Berman 2000) (PDB) was used as template (PDB id: 7a4u (Preissler et al., 2020)) to model the HYOU1 protein structure. We used the SWISS-MODEL (Waterhouse et al., 2018) web server, which includes the predicted model from AlphaFold2 (Jumper et al., 2021), to build the wild-type HYOU1 protein structure. Mutagenesis analysis was performed using 'mutagenesis' add-on in the PYMOL (Version 2. 0 Schrödinger, LLC) program. The backbone-independent rotamer library was used when modelling the mutated side-chains.

Human dermal fibroblasts isolation, culture, and stress induction

Human dermal fibroblast (HDF) isolation of the proband was performed within three days of the skin biopsies. The biopsy was transferred to a sterile petri dish. Dermis and hypodermis were removed and discarded. Epidermis was cut into small pieces and placed in 25 cm^2 flasks. Culture media (Dulbecco's Modified Eagle Medium (10566016, Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) (DMEM) supplemented with 10% inactivated foetal bovine serum (FBS), 40 U/ml penicillin, and 50 mg/ml streptomycin was added to cover the surface after 15 minutes. The media was changed after 24 hours. The cells were passed at 80% confluency using trypsinization. Control HDFs were purchased from Promocell (Heidelberg, Germany).

Half a million HDFs of the patient and three controls at the fifth passage were each transferred to 100 mm dishes and treated with DMEM (10566016, Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), 40 U/ml penicillin, and 50 mg/ml streptomycin, 24 hours prior to stress induction. ER stress was induced using 0.5 µg/ml Tunicamycin (3516, TOCRIS, Bristol, UK) dissolved in dimethyl sulfoxide (D4540, Sigma-Aldrich, St. Louis, MO, USA) (DMSO) and the same volume of DMSO was used for Mock-treated samples. The cells were then harvested at

6 hours after being treated using TrypLE™ Express Enzyme (12605010, Gibco™, Thermo Fisher Scientific, Waltham, MA, USA). This experiment was done in three biological replicates.

Western blotting

Frozen cell pellets of 10^6 HDFs were thawed on ice and treated with lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.1% Na deoxycholate, 1 mM dithiothreitol, 1 mM EDTA, and protease inhibitor cocktail; Roche, Basel, Switzerland). Cells were incubated for 20 min on ice; 20 µg of each sample was added to 5 µl of 5x loading buffer. Samples were heated for 15 min at 95°C, loaded on a 4-20% pre-cast gradient gel (NB10420, NuSep, Germantown, MD, United States), and subjected to electrophoresis at 150 V for 70 minutes. Protein transfer was performed using TransBlot® Turbo™ Transfer System (Bio-Rad Laboratories, Hercules, California, USA) following the manufacturer's instructions. The membrane was blocked for 1 h with Tris-buffered saline, Tween 20 (0.05%), and non-fat milk (5%), and was incubated at 4°C overnight with anti-HYOU1 antibody (1:5000 dilution, ab134944, abcam, Cambridge, UK). Anti-GAPDH antibody (1:10000, MAB374, Sigma-Aldrich, St. Louis, MO, USA) was used for the detection of housekeeping protein, incubating for 2 h at room temperature. The membrane was incubated with secondary antibodies (Goat Anti-Rabbit [H+L]-HRP conjugates (1721019, Bio-Rad Laboratories, Hercules, California, USA) and Goat Anti-Mouse IgG [H+L]-HRP conjugates (1706516, Bio-Rad Laboratories, Hercules, California, USA) both at dilution of 1:10000 for 1 h. The ChemiDoc XRS+ system (Bio-Rad Laboratories, Hercules, California, USA) and Clarity Western ECL (1705061, Bio-Rad Laboratories, Hercules, California, USA) were used for signal detection. Western blot quantification was performed using ImageJ (Schneider et al., 2012).

Quantitative real-time PCR

Total RNA was extracted from frozen dry pellets of 10^6 HDFs with the RNeasy Mini Kit (74106, Qiagen Qiagen, Hilden, Germany) and later reverse transcribed using Maxima™ H Minus cDNA Synthesis Master Mix (M1662, Thermo Scientific™, Thermo Fisher Scientific, Waltham, MA, USA). Quantitative real-time PCR was run on QuantStudio™ 3

(Thermo Fisher Scientific, Waltham, MA, USA) using PowerTrack™ SYBR Green Master mix (A46109, Thermo Fisher Scientific, Waltham, MA, USA). The following PCR conditions were used: 10 min at 96°C for denaturing followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. The *ACTB* (βactin) was used as a house keeping gene. The following primers were used: *HYOU1*, 5'-GCACCATTGTGACCTACCAGA-3' (forward) and 5'-CCAGGGTACGGTCAAATCC-3' (reverse); *ACTB,* 5'-CACCATTGGCAATGAGCGGTTC-3' (forward) and 5'-AGGTCTTTGCGGATGTCCACGT-3' (reverse).

Flow cytometry of blood

Cells from 250 µl of whole blood were washed twice with 2 ml of 1X PBS, spun at 350 x g for 5 min at room temperature. The samples were then stained with 1 ml of 1:1000 diluted Live/Dead Blue for 20 min at room temperature. Samples were then washed with 2 ml of FACS buffer (PBS 1X, FBS, 0.5%, EDTA, 2 mM Hepes 10 mM) and collected by centrifugation at 300 x g for 5 min at room temperature. Five microliters of Human TruStain FcX™ and 5 µl of True-Stain Monocyte Blocker™ were added to each sample, vortexed, and incubated for 5 min. The mix of 40 antibodies was added in 3 batches (see Table 2S for details). After staining for 30 min on ice, 3 ml of red blood cell lysis buffer (Versalyse, Beckman Coulter Life Sciences, Indianapolis, IN) were added to each sample. After 10 min of incubation at room temperature, the cells were immediately centrifuged at 300 x g for 5 min at 4°C. Samples were washed twice with 2 ml of FACS buffer, centrifuged and resuspended in 300 µl of FACS buffer. The resuspended material was then analyzed using a Cytek® Aurora 5L spectral flow cytometer (Cytek Biosciences, Fremont, CA). A total of 300,000 CD45-positive live cells were acquired. Whole blood samples were analyzed by using the OMIQ software from Dotmatics (www.omiq.ai, www.dotmatics.com). Downsampling was performed on 300,000 CD45+ cells. In this composite file, cells were pre-gated as follows: singlets, live, time and CD45+.Computational analysis was carried out on FlowSOM (Van Gassen et al., 2015) to delineate the total number of clusters generated. Cell cluster annotations were performed using OMIQ. All frequencies of clusters in the bar plots delineated were calculated by dividing each cluster by all clusters.

Statistics

Graphs were generated using GraphPad Prism v10.2.3 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com) and R packages ggplot (Wickham, 2016)) and ggrepel (Slowikowski et al, 2024). T-test was used for statistical comparison. The corresponding p-value is given for each figure in its legend. A p-value<0.05 was considered as significant.

Tables

Table 1. Clinical laboratory results of the patient compared with age-adjusted reference values.

	Patient at 6 months of age	Reference range at 6 months	Patient at 54 months of age	Reference range at 54 months
WBC (cells/ml)	9360	6200-14500	6540	5300-11500
Lymphocytes	7660	1400–22000	4820	1500-8500
Neutrophils	720	500-9500	480	1500-7500
Monocytes	1400	400–1200	1210	200-1000
Eosinophils	0	<810	0	<810
Basophils	0	<210	NA	<210
Haemoglobin (g/dl)	**8.3**	10.3-12.4	**9.4**	10.5-12.7
Red Blood Cells (x 10^6 cells/ml)	4	4.1-5	3.97	4-4.9
MCV (fL)[a]	**67**	70-90	77.8	74-94
Platelets (cells/ml) x 10^3	**216**	275-566	269	204-405
C-Reactive Protein (mg/L)	negative	<10	negative	<10
Erythrocyte sedimentation rate (mm/h)	15	<10	9	<10
IgG (mg/dl)	**123**	195-794	**205**	504-1464
IgM (mg/dl)	81	9-212	NA[e]	
IgA (mg/dl)	15	1-59	NA	
IgE (IU/ml)	4	<1	NA	
Anti-Tetanus antibodies (IU/mL)	2.1	>0.1	NA	
Anti-Diphtheria antibodies (IU/mL)	**<0.1**	>0.1	NA	
ASCA	NA		negative	negative
P-ANCA	NA		negative	negative
Stool calprotectin (mcg/g)	NA		**198**	<50
Triglyceride (mg/dl)	NA		52	32-116
Cholesterol (mg/dl)	NA		106	44-181
AST (U/L)[b]	NA		**80**	10-30
ALT (U/L)[c]	NA		**65**	10-40
Ferritin (ng/ml)	NA		154	24-336
BUN (mg/dl)[d]	NA		16	7-17
Cr (mg/dl)	NA		0.5	0.2-0.8
Na (mEq/l)	NA		138	136-146
K (mEq/l)	NA		4.8	3.5-5
Ca (mEq/l)	NA		9.2	8.5-10.5
P (mEq/l)	NA		6.4	4.0-7.0
Chloride (mEq/l)	NA		**100**	102-112
T4 (µg/dl)	NA		12.2	7-13.1
TSH (mIU/ml)	NA		5.7	0.55-7.1
25OH VitD3 (ng/ml)	NA		**57**	30-50
PT (sec)	NA		14.5	11-15
PTT (sec)	NA		50	45-54
INR	NA		1.06	0.9-1.2

Reference values were those of the local diagnostic laboratory. [a] MCV, mean corpuscular volume; [b] AST, aspartate transferase; [c] ALT, alanine transaminase; [d] BUN, blood urea nitrogen; [e] NA, not available.

Table 2. Candidate variant annotations and predicted impact

Genomic position (hg19)	chr11:118922538
Gene symbol	*HYOU1*
HGVSc: nucleotide change	NM_001130991.3: c.1331C>A
HGVSp: protein change	NP_001124463.1: p.Pro444His
SIFT (score)[a]	deleterious (0)
PolyPhen (score)[b]	Probably damaging (1)
CADD (phred score)[c]	29.8
GERP++_RS[d]	4.96
MutationTaster	disease causing
PhastCons[e]	0.95

[a] Function prediction tool based on protein sequence conservation among homologs. Variants with scores between 0 and 0.05 are considered deleterious.
[b] Polyphen2_HDIV_score: variants with scores between 0.85 and 1.0 are predicted to be damaging with high confidence.
[c] CADDv1.4 scores range from 1 to 99, with a higher score indicating greater deleteriousness.
[d] GERP++_RS score: DNA conservation score. Scores range from 1 to 6.18. The larger the score, the more conserved the site.
[e] PhastCons score: probabilities of negative selection and range between 0 and 1.

Table 3. Immunological parameters of the patient at 5 years of age

	Sample 1[a]	**Sample 2**[a]	**Normal range**[b]
Red blood cells (10^6 cells/µL)[1]	5.15		3.9-5.3
Hemoglobin (g/dL)[1]	11.4	8.4	11.5-14.5
Hematocrit (%)[1]	38	26.8	34-40
Mean corpuscular volume (fL)[1]	73.8	75.7	76-90
Mean corpuscular hemoglobin (pg)[1]	22.1	23.7	25-30
Mean corpuscular HGB (g/dL)[1]	30	31.3	32-36
Red cell distribution width-CV (%)[1]	26.5	22.4	11.5-15
Platelet count (10^3/µL)[1]	235	144	150-450
White blood cells (10^3 cells/µL)[1]	9.3	7.23	5-14.5
Granulocytes			
Granulocytes (%)[1]	41	44.3	
Neutrophils (10^3 cells/µL)[1]	3.72	3.19	1.5-8
Neutrophils (%)[4]	40	44.2	28-54
Basophils (10^3 cells/µL)[4]		0.01	0-0.2
Basophils (%)[1]		0.1	0-1
Eosinophils (10^3 cells/µL)[4]	0.93	0	0-0.7
Eosinophils (%)[1]	1	0	0-3
Monocytes			
Monocytes (10^3 cells/µL)[4]	0.372	1.28	100-1500
Monocytes (%)[1]	4	17.7	0-5
Lymphocytes			
Lymphocyte (cells/µL)[2]	5115	**2750**	2280-3820
Lymphocyte (%)[1]	55	**38**	17-67
$CD3^+$ (cells/µL)[2]	3313	3703	1424-2664
$CD3^+$ (%)[2]	84.84	92	60.05-74.8
$CD56^+$ (cells/µL)[2]	466	326	
$CD56^+$ (%)[2]	12.73	8.2	
$CD4^+$ (cells/µL)[2]	1694	2158	686-1358
$CD4^+$ (%)[2]	43.49	54.2	26.17-40.76
$CD8^+$ (cells/µL)[2]	1440	1353	518-1125
$CD8^+$ (%)[2]	36.88	34	19.68-34.06
CD4/CD8 ratio[2]	1.17	1.6	0.87-1.94
$CD4^+CD8^+$[4]	0.26	0.14	0-1
$CD19^+$ (cells/µL)[2]	5	6	280-623
$CD19^+$ (%)[2]	0.14	0.2	10.21-20.12
% in $CD4^+$ T cells			
Naive ($CD45RA^+CCR7^+$)[2]	78.3	77.7	45.56-75.28
Central Memory ($CD45RA^-CCR7^+$)[2]	6.2	6.9	22.06-46.46

Effector Memory ($CD45RA^-CCR7^-$)[2]	13.2	12.7	2.08-8.78
$CD45RA^+$ Effector Memory ($CD45RA^+CCR7^-$)[2]	2.2	3.7	0-1.06
Treg ($CD25^+CD127^-$)	5.9	6.2	5.81-10.68
$PD1^+$ ($CD279^+$)[4]	4.6	1	14-32
Follicular Helper (CD45RA-CCR7+CD27+CD28+)	0.5	0.8	
% in $CD8^+$ T cells			
Naïve ($CD45RA^+CCR7^+$)[2]	39.7	32.9	41.58-77.90
Central Memory ($CD45RA^-CCR7^+$)[2]	0.2	0.8	12.08-30.54
Effector Memory ($CD45RA^-CCR7^-$)[2]	11.1	13.2	1.58-13.18
$CD45RA^+$ Effector Memory ($CD45RA^+CCR7^-$)[2]	48.8	51.6	1.70-24.62
$PD1^+$ ($CD279^+$)[4]	4.5	0.6	15-47
$CD57^{+4}$	23.3	32.9	6-32
% in $CD19^+$ B cells			
Switched Memory (IgD^-CD27^+)[2]	Not detectable	Not detectable	7.76-19.9
Unswitched Memory (IgD^+CD27^+)[4]	Not detectable	Not detectable	5-20
Naive (IgD^+CD27^-)[2]	Not detectable	Not detectable	48.36-75.84
Naive ($IgD^+CD27^-IgM^+$)[4]	Not detectable	Not detectable	
% in Monocytes			
Classical ($CD14^+CD16^-$)[4]	71.4	57	69-87
Transient ($CD14^+CD16^+$)[4]	2	1.6	2-19
Resident ($CD14^{lo}CD16^+$)[4]	0.5	6.8	2-8
% in $CD16^+CD56^+$ NK cells			
$CD56^{bright}CD16^-$ ($CD56^+$)[4]	12.8	8.6	1-12
$CD56^{bright}CD16^+$ ($CD56^{++}CD16^+$)[4]	77.5	58.6	0-6
$CD56^{dim}/CD16^+$ ($CD56^+CD16^+$)[4]	9.6	32.8	72-96
$NKG2C^+$ ($CD94^+$)[4]	8.2	7.1	3-23

[a] Samples 1 and 2 were taken 2 months apart

[b] Normal range values are either from (1) Gregory's Pediatric Anesthesia, Editor(s):George A. Gregory, Dean B. Andropoulos, 2011, DOI:10.1002/9781444345186 Blackwell Publishing Ltd. (2) Ding et al. J Allergy Clin Immunol. 2018 Sep;142(3):970-973.e8. doi: 10.1016/j.jaci.2018.04.022. Reference values for peripheral blood lymphocyte subsets of healthy children in China. (3) Tosato, F et al. Cytometry A. 2015 Jan;87(1):81-5. doi: 10.1002/cyto.a.22520. Lymphocytes subsets reference values in childhood. or (4) the local diagnostic laboratory.

Table 4. Review and comparison of genetic and clinical data of previously reported cases with *HYOU1* variants and the patient reported in this study.

	Haapaniemi et al.	Jafari Khamirani et al.	Arab et al.	Patient reported in this study
Ethnicity	Finnish	Iranian	Iranain	Iranian
Age	45 years	3 months	4 years	6 years
Sex	Female	Female	Female	Male
Zygosity	Compound Heterozygous	Homozygous	Compound Heterozygous	Homozygous
Variants[a]	p.Ala419Pro p.Tyr231His	p.Arg486Cys	p.Leu23Phe p.Arg915Gln	p.Pro444His
Small for gestation age	+	-	-	+
Failure to thrive	+	-	-	+
Recurrent respiratory tract infection	+	+	+	-
Recurrent diarrhoea	+	+	-	+
Neurodevelopmental delay/regression	-	+	-	-
Herpes infection	+	-	-	-
Pectus carinatum	+	+	-	-
Facial dysmorphism	+	-	-	+
Hypoglycemia	+	-	-	+
Neutropenia	+	+	+	+
Reduced B-cells	+	+ (panleukopenia)	-	+
Anaemia	+	+	-	+
Thrombocytopenia	+	+	-	+

[a] Based on NCBI Reference Sequence NP_001124463.1

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4.8 Supplementary Material

Tables

Table S1. Annotation, predicted impact, and population frequencies of candidate variants after segregation analysis.

Variant annotation, predicted impact and population frequencies							
Position	chr1:158326324	chr2:242003078	chr10:73447482	chr11:6567244	chr11:117787994	chr11:124857105	chr13:113888287
Gene symbol	CD1E	SNED1	CDH23	DNHD1	TMPRSS13	CCDC15	CUL4A
ref	C	C	G	G	G	T	T
alt	G	T	A	A	A	G	C
dbSNP	rs756511340	rs374311632	rs727502923	-	rs1480573177	-	rs142598917
Consequence	missense	missense	splice region and intron variant	missense	splice region and intron variant	missense	missense
HGVSc: nucleotide change	NM_001042583.3:c.941C>G	NM_001080437.3:c.2446C>T	NM_001171930.2:c.2059+6G>A	NM_144666.2:c.5075G>A	NM_001077263.3:c.452-5C>T	NM_025004.3:c.983T>G	NM_001008895.4:c.752T>C
HGVSp: protein change	NP_001036048.1:p.Thr314Ser	NP_001073906.1:p.Arg816Trp	-	NP_653267.2:p.Gly1692Asp	-	NP_079280.2:p.Ile328Ser	NP_001008895.1:p.Met251Thr
Genotype							
Patient	1/1	1/1	1/1	1/1	1/1	1/1	1/1
Patient's Brother	0/1	0/0	0/1	0/1	0/0	0/0	0/1
Patient's Father	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Patient's Mother	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Predictive scores of pathogenicity							
SIFT (score)	Deleterious (0.04)	Tolerated (0.17)		Deleterious (0)		Deleterious (0)	Deleterious (0.03)
PolyPhen (score)	Benign (0.228)	Possibly damaging (0.903)		Probably damaging (1)		Probably damaging (0.996)	Benign (0.018)
CADD (phred score)	10.46	23.6	7.58	24.3	9.029	22.5	22.2
GERP++_RS	2.44	3.52		5.56		2.62	4.76
MutationTaster	0.999999	0.999704		0.999615		0.995605	1
phastCons20way_mammalian	0.10243	0.58386		0.66667		0.49956	0.55488
ClinVar ClinSig			Uncertain significance				
Allele Frequencies							
gnomAD combined populations	0.00003209	0.00006952	0.00003235		0.000004142		
gnomAD_AFR	0	0.00006674	0.00006582		0		
gnomAD_AMR	0	0	0.00005815		0		
gnomAD_ASJ	0	0.000403	0.0001001		0		
gnomAD_FIN	0	0	0		0		
gnomAD_NFE	0	0.0001086	0.00002683		0		

gnomAD_number of homozygous	0	0	0	0			
In house allele count	0	0	0	0	0	0	0
In house allele frequency	0	0	0	0	0	0	0
In house number of homozygous	0	0	0	0	0	0	0
In house number of heterozygous	1	0	0	0	0	0	0

Table S2. Antibodies used for immunophenotyping

#	Antigen	Fluorochrome	supplier	reference	clone
1	CD123	Super Bright 436	Thermo Fisher	62-1239-42	6H6
2	CD45	PerCP	Biolegend	304026	HI30
3	HLA-DR	PE Fire 640	Biolegend	307676	L243
4	CD14	SPARK Blue 550	Biolegend	367148	63D3
5	CD16	BUV496	BD	612944	3G8
6	CD3	BV510	Biolegend	344828	SK7
7	TCR-□□	PerCP-eFluor 710	Thermo Fisher	46-9959-42	B1.1
8	CD45RA	BUV395	BD	740315	5H9
9	CD197 CCR7	BV421	Biolegend	353208	G043H7
10	CD56	BUV737	BD	612766	NCAM 16.2
11	CD8	BUV805	BD	612889	SK1
12	CD19	SPARK NIR 685	Biolegend	302270	HIB19
13	CD2	PerCP-Cy5.5	Biolegend	309226	TS1/8
14	CD4	SPARK YG 581	Biolegend	344670	SK3
15	IgD	BV480	BD	566138	IA6-2
16	CD11c	Pacific Blue	Biolegend	301626	3,9
17	CD127	APC-R700	BD	565185	HIL-7R-M21
18	CD38	APC Fire 810	Biolegend	303550	HIT2
19	CD141	BB515	BD	565084	14A
20	CD39	BUV661	BD	749967	TU6
21	CD27	APC-Cy7	Biolegend	356424	M-T271
22	CD28	BV650	Biolegend	302946	CD28.2
23	IgM	BV570	Biolegend	314517	MHM-88
24	IgG	BV605	BD	563246	G18-145
25	CD185 (CXCR5)	BV750	BD	747111	RF8B2
26	CD183 (CXCR3)	PE-Cy7	Biolegend	353720	G025H7
27	CD95 (FAS)	PE-Cy5	Biolegend	305610	DX2
28	CD57	FITC	Biolegend	359604	HNK-1
29	CD159c (NKG2c)	PE	Biolegend	375004	S19005E

30	CD337 (NKp30)	PE DAZZLE 594	Biolegend	325232	P30-15
31	CD279 (PD-1)	BV785	Biolegend	329930	EH12.2H7
32	CD196 (CCR6)	BV711	Biolegend	353436	G034E3
33	CD24	PE-Alexa Fluor 610	Thermo Fisher	MHCD2422	SN3
34	CD25	PE-Alexa Fluor 700	Thermo Fisher	MHCD2524	CD25-3G10
35	CD20	Pacific Orange	Thermo Fisher	MHCD2030	HI47
36	CD66b	Alexa647	Biolegend	392911	6/40C
37	CD195 (CCR5)	BUV563	BD	741401	2D7/CCR5
38	CD185 (CXCR5)	BV750	BD	747111	RF8B2
39	CD314 (NKG2D)	BUV615	BD	751232	1D11
40	DAPI	DAPI	Thermo Fisher	D1306	

Figures

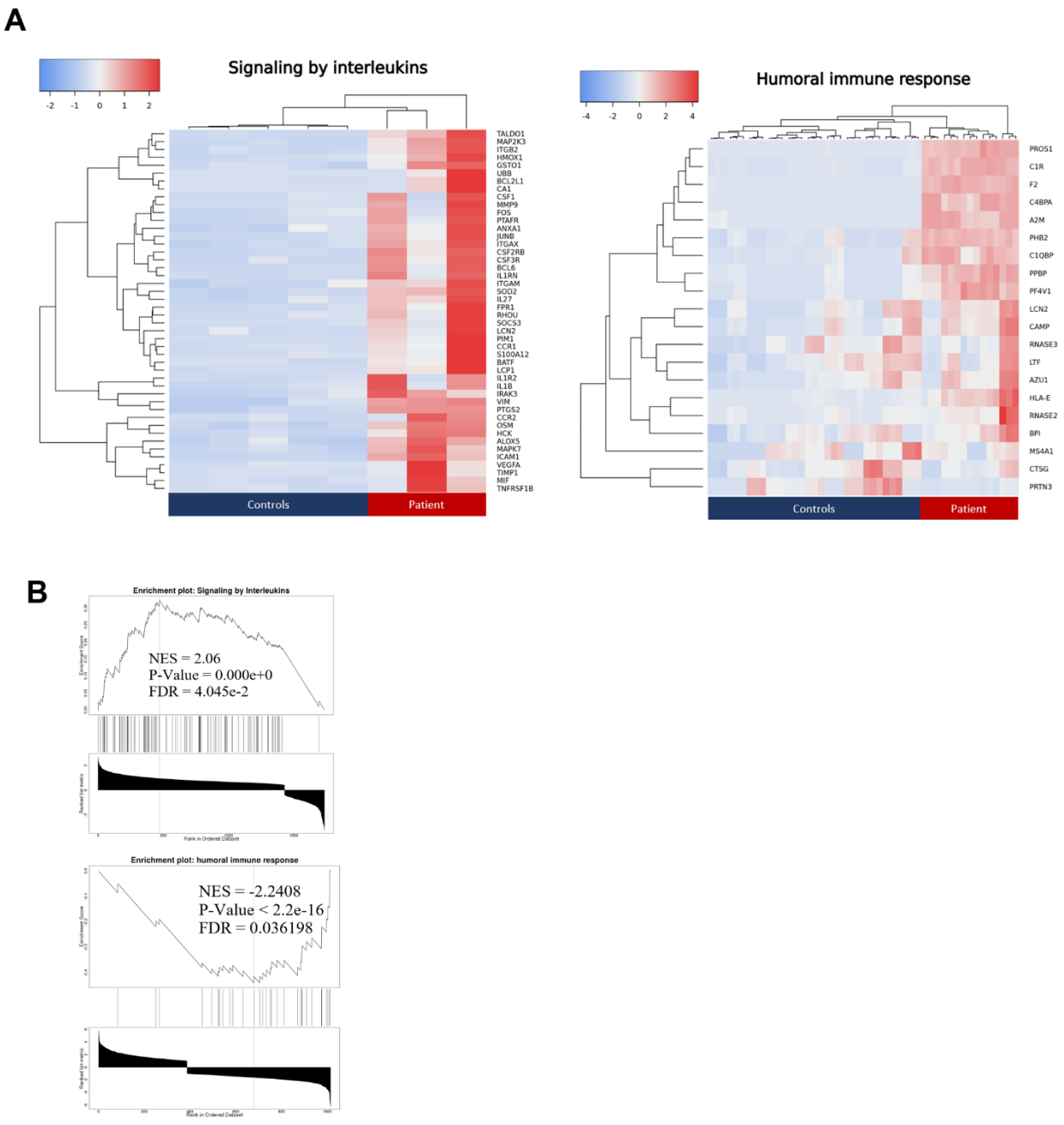

Figure S1. ***(A)*** *Heatmaps showing differentially regulated pathways in the patient compared to the controls.* ***(B)*** *Gene Set Enrichment Analysis plots displaying the enrichment of signalling by interleukin pathway in bulk transcriptomics of PBMC and negative enrichment of humoral immune response pathway in proteomics of PBMC. NES, Normalized enrichment score.*

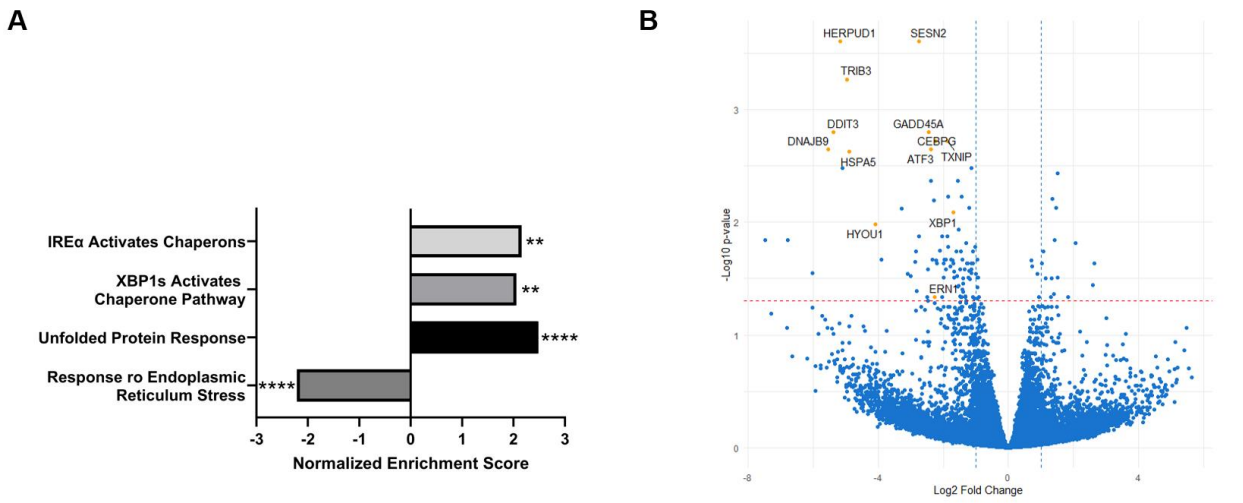

Figure S2. *(A) Dysregulated canonical pathways in the patient's HDF compared to controls' HDF, **; P < 0.01; ****; P < 0.0001. (B) Volcano plot showing the downregulated genes in the patient's HDF in response to tunicamycin treatment compared to the controls' in the same condition.*

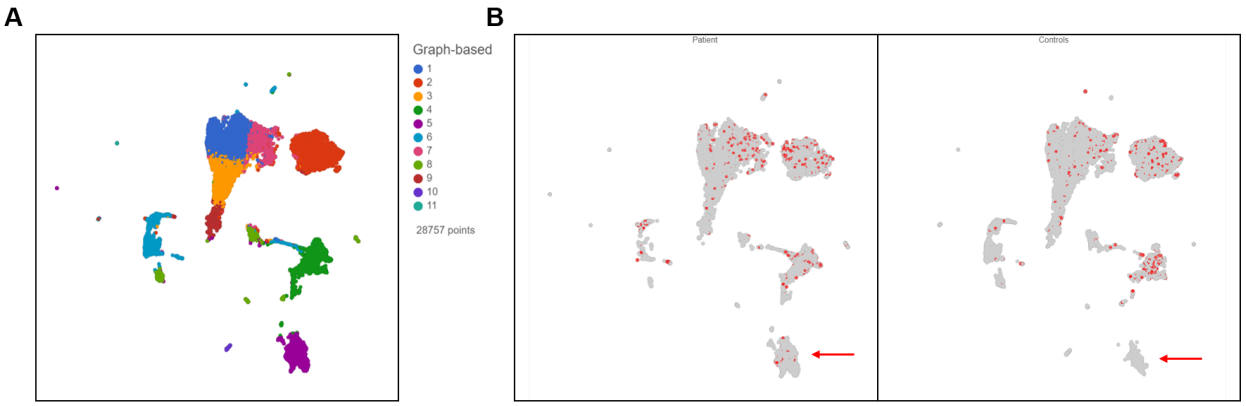

Figure S3. (A) *UMAP plot displaying the clusters according to graph-based clustering.* ***(B)*** *Graph showing the normalized counts for the patient (left) and the controls (right), cells expressing HYOU1 are shown in red.*

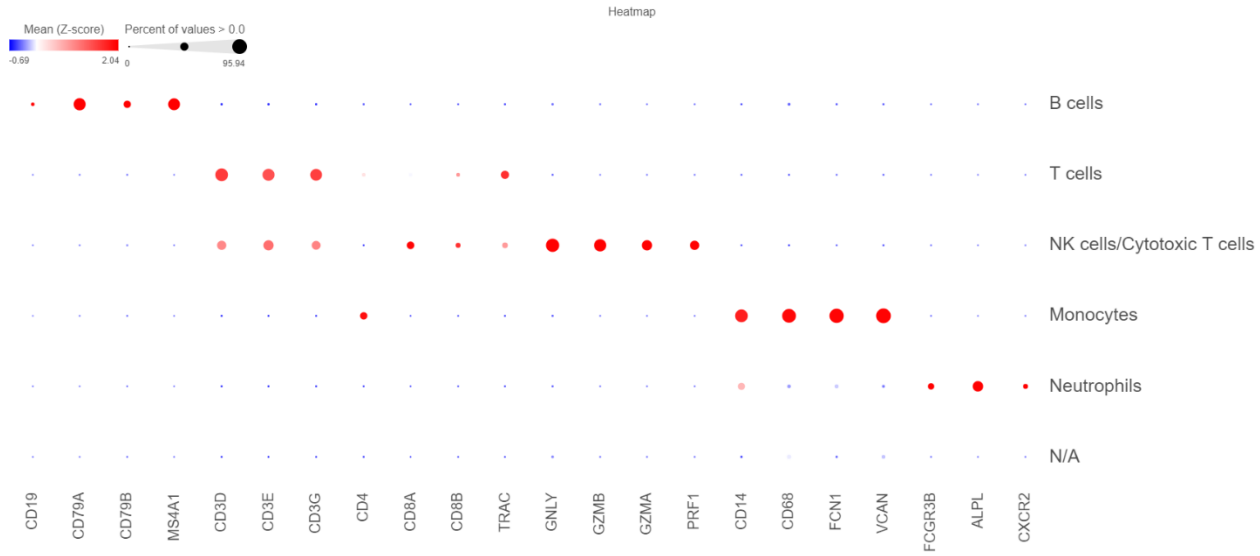

Figure S4. *Normalized expression of canonical gene markers demonstrated in bubblemap plots according to the annotated cell type.*

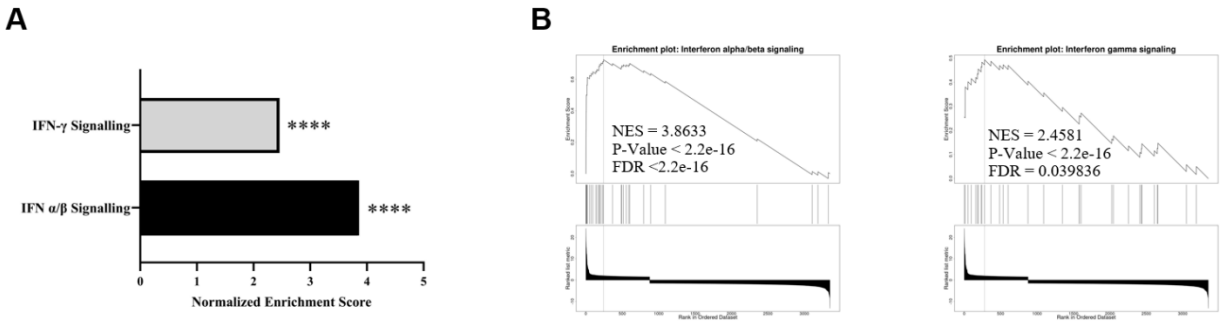

Figure S5*. **(A)** Upregulated canonical pathways in NC1 cluster compared to NC2 cluster, ****, P < 0.0001. **(B)** Gene Set Enrichment Analysis plots displaying the enrichment of IFN α/β signalling and IFN-γ signalling pathways in the NC1 compared to the NC2. FDR, false discovery rate; NES, normalized enrichment score.*

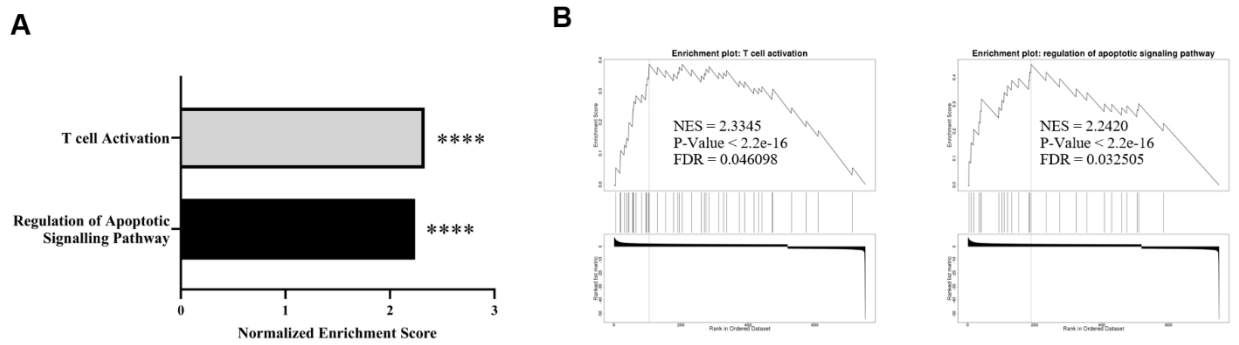

Figure S6. (A) *Upregulated canonical pathways in the patient's T cells compared to controls' T cells, ****, P < 0.0001.* ***(B)*** *Gene Set Enrichment Analysis plots displaying the enrichment of T cell activation and regulation of apoptotic signalling pathways in patient's T cells comparing to the controls. FDR, false discovery rate; NES, normalized enrichment score.*

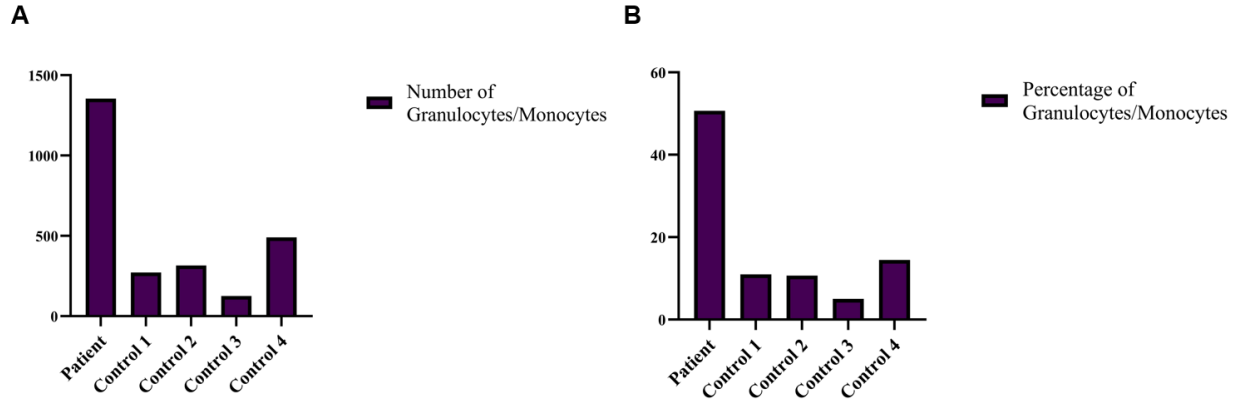

Figure S7. (A) *Bar graph representing the number of cells from single cell RNA-seq classified as granulocytes and monocytes in the bone marrow of different samples.* ***(B)*** *Bar graph representing the percentage of granulocytes and monocytes in each sample in single cell RNA-seq data.*

5 Defects of Integrins in Inborn Errors of Immunity

5.1 Introduction to Integrins and Their Role in Immune Function

Integrins are a family of transmembrane adhesion receptors, which mediate cell adhesion, migration, proliferation and intracellular signaling, thus, play a fundamental role in immune responses. Integrins are composed of a transmembrane α subunit and β subunit. These two combine to form heterodimers, enabling the cell immune cells to interact with extracellular matrix (ECM) components and different classes of cells. The combination of α and β subunits which form the heterodimer determines the ligand binding characteristics and the function of each integrin (Hynes, 2002).

Integrins are crucial in the immune system, as they are involved in recruitment of leukocytes, formation of immune synapses, and other processes required for effective immune surveillance and response to pathogens (Humphries et al., 2006).

In this section, I review the primary integrins involved in immune function, the previously described integrin-related IEIs, their underlying genetic mutations, as well as the possible impact of integrin defects on immune system function and introduce a novel candidate gene for IEIs.

5.1.1 Overview of Integrins in Immune Cells

Integrins are classified based on the composition of their α and β subunits. Integrins play a key role in regulating both innate and adaptive immune cell functions, particularly in leukocyte adhesion and migration to the site of infection. While α subunits typically pair with a distinct β subunit, β subunits can form an integrin by coupling with multiple α subunits. For instance, β2 subunit participates in the formation of the following integrins:

1. **LFA-1 (Lymphocyte function-associated antigen 1, $\alpha_L\beta_2$; CD11a/CD18)**: This integrin is found on multiple classes of leukocytes, including B cells, T cells, neutrophils and monocytes. LFA-1 mediates adhesion to intercellular adhesion molecules (ICAMs) on endothelial cells, and other immune cells, therefore, playing an important role in immune cell activation as well as migration (Humphries et al., 2006) (Figure 6).

2. **Mac-1 (Macrophage-1 antigen, $\alpha_M\beta_2$; CD11b/CD18)**: Mac-1 plays a role in neutrophil and monocyte migration and phagocytosis. Through binding to ICAM-1, fibrinogen, and complement C3b, Mac-1 facilitates immune clearance of pathogens (Solovjov et al., 2005) (Figure 6).
3. **CR4 (Complement receptor 4, $\alpha_X\beta_2$; CD11c/CD18)**: CR4 is expressed on macrophages and dendritic cells and is important in binding and phagocytosis of complement-opsonized pathogens including parasites (Maxson et al., 2018).

Another important integrin family in immune function is β1 integrins, such as VLA-4 (Very late antigen-1, $\alpha_4\beta_1$). VLA-4 is a leukocyte ligand for endothelial vascular cell adhesion molecule-1 (VCAM-1) and fibronectin which interacts with VCAM-1 at the surface of activated lymphocytes and monocytes and mediates cell migration to the site of infection and formation of immune synapses with APCs (Berlin et al., 1995).

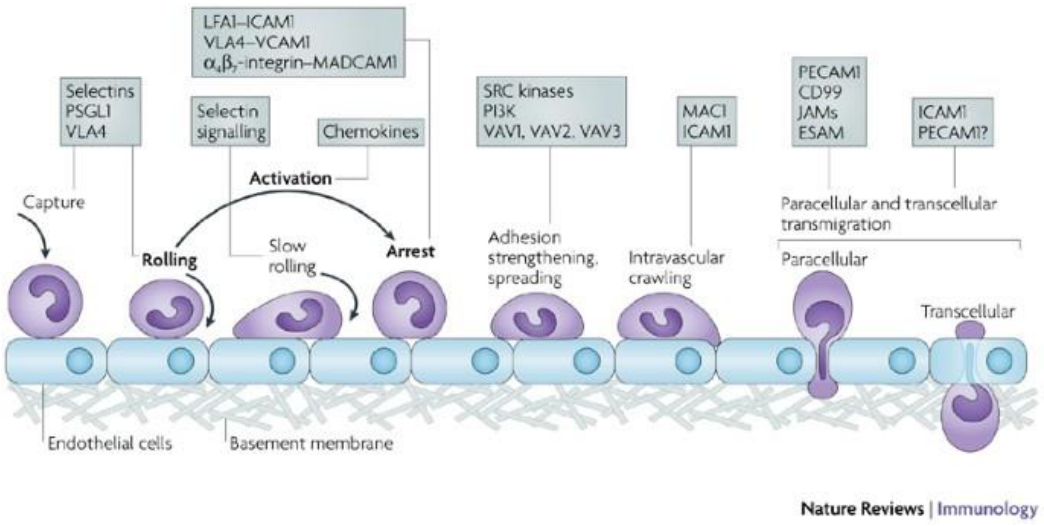

Figure 6. *Leukocyte adhesion process and implicated molecules in the process. Adapted from Ley et al, 2007 Nat Rev Immunology.*

Leukocyte's ability to adhere to the endothelium, migrating to the site of inflammation, and commencing proper immune response is dependent on the proper function of integrins. Consequently, these functions could potentially be compromised by any changes which results in an impairment in the function of the integrin. This is exemplified by the well-known leukocytes adhesion deficiency (LAD) syndromes, which are the subcategorized

into three types according to the integrin whose function is impaired (Walling & Kim, 2018).

5.1.2 Leukocyte Adhesion Deficiency Syndromes

A class of autosomal recessive IEIs known as leukocyte adhesion deficiencies is characterized by abnormalities in leukocyte adherence and migration brought on by integrin malfunction. Pathogenic variants in genes encoding integrins cause these deficits. The three main categories of LAD include:

1. **LAD Type I (LAD-I)**: This type is brought on by pathogenic variants in the *ITGB2* gene, which encodes the integrin beta chain-2 protein (CD18) and combines with several distinct α chains to form different integrin heterodimers (Anderson & Springer, 1987). Leukocytes' capacity to adhere to the endothelium and move to infection sites is compromised consequent to pathogenic variants in *ITGB2*.
2. **LAD Type II (LAD-II)**: In this type of LAD, the defective gene is *SLC35C1*, which affects fucosylation and the synthesis of selectin ligands required for leukocyte rolling on endothelial cells (Etzioni et al., 1992).
3. **LAD Type III (LAD-III)**: Pathogenic variants in *FERMT3* lead to this type of LAD. *FERMT3* gene encodes protein kindlin-3 and is essential for integrin activation (Kuijpers et al., 2009). Integrin expression in LAD-III is not affected consequent to mutations. However, it leads to an impairment in the activation of beta-integrins, resulting in impaired adhesion and migration of immune cells (Kuijpers et al., 2009).

5.1.2.1 LAD Type I: Molecular Defects and Clinical Manifestations

Pathogenic variants in the *ITGB2* , LAD-I is the most frequent integrin-related PID and results from mutations in the *ITGB2* gene, the gene encoding the β subunit (CD18) of the β2 integrins such as LFA-1, Mac-1, and CR4 (Anderson & Springer, 1987). Leukocyte adhesion is hampered by the absence of functioning CD18, especially when the cells are firmly adhering to the vascular endothelium. This stops leukocytes from migrating to the infection sites, particularly neutrophils (Walling & Kim, 2018).

- **Clinical Features**: Patients with LAD-I typically present in infancy with recurrent bacterial infections, consequent to defects in neutrophils. Such infections are frequently localized to the skin and mucous membranes, with the most common clinical features being delayed in umbilical cord sloughing, severe gingivitis, and the absence of pus at the sites of infection (Gorjipour et al., 2019). Impaired wound healing also occurs as a result of the inability of neutrophils to migrate tissues (Gorjipour et al., 2019).
- **Diagnosis and Treatment**: Flow cytometry, which finds the lack or markedly decreased expression of CD18 on leukocytes, is used to diagnose LAD-I. Mutations in the *ITGB2* gene are confirmed by genetic testing. Allogenic HSCT is the only corrective therapy available for these individuals, and treatment usually consists of intensive antibiotic therapy to manage infections (Mesa-Núñez et al., 2022).

5.1.2.2 LAD Type II: Defective Leukocyte Rolling

Pathogenic variants in the *SLC35C1* gene, which encodes for a GDP-fucose transporter in the Golgi apparatus and is required for the fucosylation of selectin ligands, result in LAD-II (Etzioni et al., 1992). LAD-II mainly hinders leukocyte rolling on endothelial cells, an early stage of migration, in contrast to LAD-I, which affects solid adhesion.

- **Clinical presentation**: Individuals with LAD-II often present with recurrent infections, mental retardation, short stature, and distinct facial features. The Bombay blood phenotype (inexpression of ABO antigens), which is present in all LAD-II patients but incredibly uncommon in the general population, is an important diagnostic marker, resulting from defective fucosylation of blood group antigens (Sturla et al., 2005).
- **Diagnosis and Treatment**: Diagnosis is based on the absence of functional selectin ligands on leukocytes and the identification of *SLC35C1* pathogenic variants. Oral fucose supplementation has been shown to induce the expression of fucosylated selectin ligands on neutrophils and core fucosylation of serum glycoproteins. This has been associated with signs of improvement such as

infection, fever and neutrophil counts in some cases by restoring the function of selectin ligands (Marquardt et al., 1999).

5.1.2.3 LAD Type III: Integrin Activation Defect

LAD-III is caused by mutations in the *FERMT3* gene. This gene encodes protein kindlin-3, which is required for the activation of integrins (Kuijpers et al., 2009). In contrast to LAD-I, where β2 integrins are defected, LAD-III patients express β1, β2, and β3 integrins yet, integrin activation is impaired in these individuals, resulting in defects in leukocyte adherence and platelet aggregation. The lack of β2 integrin function results in elevated neutrophil count as these cells fail to adhere to or migrate across the endothelium. Additionally, Integrin αIIbβ3, which is expressed at a high level in platelets, plays a central role in platelet functions (Kuijpers et al., 2009).

- **Clinical Features**: Similar to other LADs, LAD-III is characterized by recurrent bacterial infections. However, LAD-III is distinguished from other types of LADs by a bleeding tendency due to impaired platelet aggregation (Kuijpers et al., 2009). This thrombasthenia-like bleeding disorder results from the inability of platelet integrins in binding to fibrinogen and formation of stable clots (Kuijpers et al., 2009).
- **Diagnosis and Treatment**: The diagnostic tools for LAD-III include flow cytometry to evaluate integrin expression and function on leukocytes and platelets, and genetic testing to verify pathogenic variants in *FERMT3*. For severe cases of LAD-III, HSCT is considered to be the most effective treatment (Kuijpers et al., 2009).

5.2 ITGAL Gene and Its Potential Role in Primary Immunodeficiencies

The *ITGAL* gene encodes integrin α_L chain (CD11a), which coupled with CD18 forms LFA-1, playing a key role in adhesion and of leukocytes including T-cells and neutrophils (Kuijpers et al., 2009). Although pathogenic variants in *ITGAL* have not been as an IEIs in humans thus far, evidence from related pathways and functions strongly suggests that defects in ITGAL and consequent disruption of its downstream processes may contribute to immune deficiency. Herein, we will review the structure, function, and known

immunological roles of the *ITGAL* gene, and discuss its potential role in PIDs and the impact of immune cell adhesion defects in other genetic diseases.

5.2.1 Structure and Function of ITGAL

The *ITGAL* gene is located on chromosome 16p11.2 and encodes the α chain of lymphocyte function-associated antigen-1 (LFA-1), a heterodimeric integrin composed of *CD11a* (α chain) and *CD18* (β chain) (Walling & Kim, 2018). LFA-1 plays a key role in immune cell adhesion as well as interactions between T cells and APCs, which are essential for the formation of immune synapse and subsequent T cell activation (Walling & Kim, 2018).

The integrin family, to which LFA-1 belongs, consists of transmembrane receptors that link the extracellular matrix or cell-surface ligands to the intracellular cytoskeleton (Hyun et al., 2019). The α chain of LFA-1 (CD11a) interacts with the β chain (CD18) to form a functional integrin that binds to intercellular adhesion molecules (ICAMs) on other cells, including immune and endothelial cells (Walling & Kim, 2018). ICAM-1, the main ligand of LFA-1, is expressed on endothelial cells, APCs, and T cells, and contributes to rolling, adhesion, and transmigration of leukocytes during immune responses (Gérard et al., 2021). The domains of ITGAL protein are shown are Figure 7.

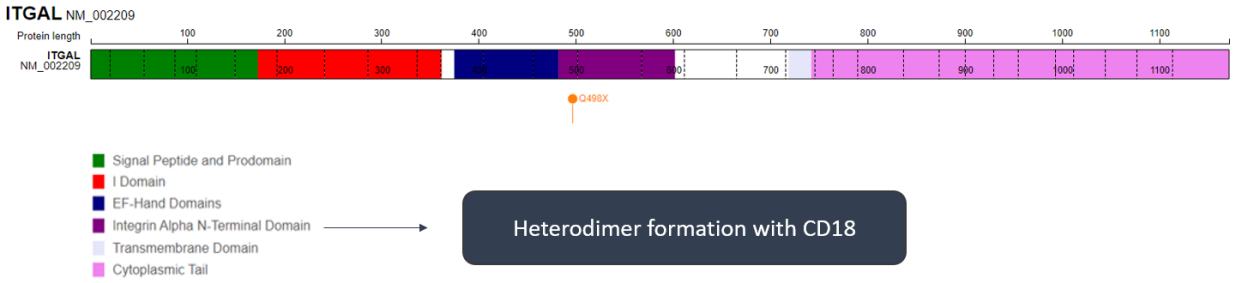

Figure 7. *Domains of ITGAL (CD11a) protein. The bubble represents the discovered variant in the binding domain with CD18.*

5.2.2 LFA-1 and its Role in Immune Cell Function

Being expressed on several immune cell types, including T cells, B cells, neutrophils, monocytes, and NK cells, LFA-1 plays multiple roles in immune responses, through participating in intracellular interactions related to both innate and adaptive immunity (Gérard et al., 2021). The ability of T cells to adhesion to APCs is of paramount importance for the activation of naive T cells and the subsequent T cell priming (Gérard et al., 2021).

In addition to its role in T cell activation, LFA-1 is essential for cytotoxic immune responses and it has been shown that it initiates the immunological synapse that forms between the cytotoxic T or NK cell and the cancer cell (Reina & Espel, 2017). A defect in LFA-1 may potentially reduce the function of immune system and increase susceptibility to infections or malignancies.

In light of the high level of expression of LFA-1 in neutrophils and monocytes and its role in adhesion and migration (Hyun et al., 2019), it is also possible that neutrophil recruitment might be affected by impaired LFA-1 function, resulting in recurrent infections due to compromised innate immune responses.

5.2.3 Immune Synapse Formation and ITGAL

Immune synapse formation is a key event in T cell activation, leading to subsequent immune responses following TCR signalling. LFA-1 also plays a important role in the stabilization of the immune synapse through interacting with ICAM-1 on APCs (Gérard et al., 2021). Thus, as a result of defects in LFA-1, immune synapses formation may fail, leading to reduced TCR signaling and impaired T cell activation, and consequent dysregulation in T cell priming.

In addition to its role in T cell activation, formation of immune synapse is critical for other immune cell functions, such as the cytotoxic activity of CTLs and NK cells. Studies in mice models have showed that LFA-1 enhances the early regulation of NK cells following infection with *T. gondii* and promotes NK cell cytotoxicity (Gao et al., 2022). Pathologies of immune synapses formation as a result of LFA-1 absence could potentially result in

defective cytotoxic responses, as previously seen in other IEIs with an impairment in immune synapse formation, such as defects in *DOCK8* (Dupré et al., 2002).

5.3 ITGAL as a Candidate Gene of Inborn Error of Immunity

Given the essential role of LFA-1 in immune cell adhesion, migration, and interaction as discussed earlier, pathogenic variants in *ITGAL* could possibly cause a novel form of IEI, previously not reported. Defects in CD11a could potentially impair multiple functions of several classes of immune cell, such as trafficking, antigen presentation, and cytotoxic responses. Impairment in such immune responses can possibly manifest as recurrent infections, autoimmunity, or susceptibility to cancer (Gérard et al., 2021; Hyun et al., 2019; Walling & Kim, 2018).

5.4 Hypothesis and Aim of Studying ITGAL as a Novel Inborn Error of Immunity

In our previously described cohort of IEIs, a 35-year-old woman with a history of recurrent wart and epidermodysplasia verruciform lesions was found to have homozygous missense variant *ITGAL* (NM_002209.3): c.1492C>T (p.Gln498*). We confirmed the homozygous status for this variant in ITGAL in the proband and her father and hypothesized that this variant is responsible for susceptibility to EV and wart. Considering the crucial role of ITGAL as well as implication of other integrins in IEIs, we aimed to investigate the downstream impact of this variant using a multiomics approach composed of RNA sequencing, proteomics and single-cell RNA sequencing to study the pathways altered as a result of this variant and provide insight into mechanisms by which this variant contributes to the described clinical phenotype.

6 Manuscript II: *A Study of a Family with Epidermodysplasia Verruciformis Reveals Defects of the Integrin α-L Subunit Associated with susceptibility to Human Papillomavirus (HPV) Infection*

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6.1 Abstract

Lymphocyte function-associated antigen 1 (LFA-1) is an integrin expressed by lymphocytes and other leukocyte and plays a pivotal role in leukocyte adhesion and migration to the site of infection. LFA-1 is composed of subunit αL (encoded by ITGAL) and subunit β2 (encoded by ITGB2), which form this heterodimeric integrin. In a cohort of inborn errors of immunity, our team discovered nonsense homozygous variant ITGAL (NM_002209.3): c.1492C>T (p.Gln498*) in a patient with susceptibility to epidermodysplasia verruciformis (EV) and wart, and her father presenting the same symptoms. We confirmed the heterozygous status of proband's healthy mother, showing that this variant is inherited in an autosomal recessive pattern. We aimed to investigate the downstream impact of this variant using a multiomics approach composed of RNA sequencing, proteomics and single-cell RNA sequencing and study the pathways altered as a consequence of this variant. In our study, we demonstrated the absence of ITGAL protein in patients, using western blot and flow cytometry. Moreover, proteomics analysis showed impairments in multiple immune-related pathways such as those related to adhesion and cell motility. Additionally, we showed an elevation in the expression of other integrins, suggesting a compensatory response aiming to counterbalance the defective integrins.

6.2 Results

Clinical description

The proband is a 35-year-old female of Iranian ethnicity, born to consanguineous parents. She has reported eczema and dermal allergies since childhood, which exacerbated at the age of 24. She presents with disseminated eczematous lesions, characterized by flat-topped or bumpy papillomatoses, reminiscent of epidermodysplasia verruciformis (EV), mainly on her extremities. Additionally, she experiences severe pruritus and itching. Furthermore, she has suffered from fungal superinfections of her eczematous lesions on her foot and between her fingers. Despite receiving various treatments over the past decade, including avoidance measures, antihistamines, antidepressants, anti-inflammatories, immunosuppressive medications, and intravenous immunoglobulins, her symptoms have only improved marginally, by 20-30%.

Laboratory analysis revealed mild leukocytosis in addition to elevated IgE levels (>2000 µg/dL), with a normal eosinophil count and hypergammaglobulinemia. Her lymphocyte subset is within normal range, with only Natural Killer (NK) cells at the lower limit of normal, and lymphocytosis evident in peripheral blood smear. Skin biopsy confirms the diagnosis of epidermodysplasia verruciformis.

The proband's father also experiences similar symptoms, including severe eczema, which improved after the age of 60. He exhibits disseminated flat-topped or raised bumpy papules, primarily on his toes and feet, accompanied by psoriatic-like, scaly lesions on his legs. However, he has never undergone biopsies for these lesions. Similar to the proband, he denies a history of recurrent respiratory or gastrointestinal infections. During childhood, he suffered from tinea capitis and other fungal infections of the scalp, which were treated with radiation. Later in life, he developed basal cell carcinoma lesions on his scalp, which were surgically removed. The proband’s paternal uncle also reports a history of disseminated papillomatoses, EV-like lesions, eczema, and allergies.

Human Papillomavirus typing

Biopsies were obtained from the normal skin and EV lesions of the proband and her father (Figure 1A), as well as the wart-like lesions of the proband and eczematoid lesions of the father. The results of Human papillomavirus (HPV) typing are shown in Table 1.

Table 1. Results of HPV typing in the proband and father, according to the site of biopsy.

Proband	**Detected HPV type(s)**	**Father**	**Detected HPV type(s)**
Normal skin	HPV5; 23; 38	Normal skin	HPV5; 8; 23; 24; 38 HPV57
EV lesion	HPV5; 8; 23; 38	EV lesion	HPV5; 8; 23; 24; 38; 99
Wart-like lesions	HPV5; 8; 22; 23; 38, γ-HPV169	Eczema	HPV8; 23; 38; 99

The results of HPV typing confirm the diagnosis of EV, as it shows replication of HPV5 and HPV8 which are typically linked to EV (Patel et al., 2010) and γ-HPV (type 169) which is associated with wart (Hošnjak et al., 2016).

Genetic Analyses

Due to persistency of her symptoms, whole exome sequencing was performed for the proband, and her parents in order to identify any likely causal variant (Figure 1B). We applied successive filtering steps based on variants' functional consequences, frequency and autosomal recessive inheritance which revealed a nonsense homozygous variant c.1492C>T, (p.Gln498*) in the Integrin Subunit Alpha L (*ITGAL, NM_002209.3*). Sanger sequencing confirmed the homozygous status of the proband and her father for this variant. Proband's symptomless mother was heterozygous for this variant (Figure 1C), confirming that this variant is inherited in an autosomal recessive pattern.

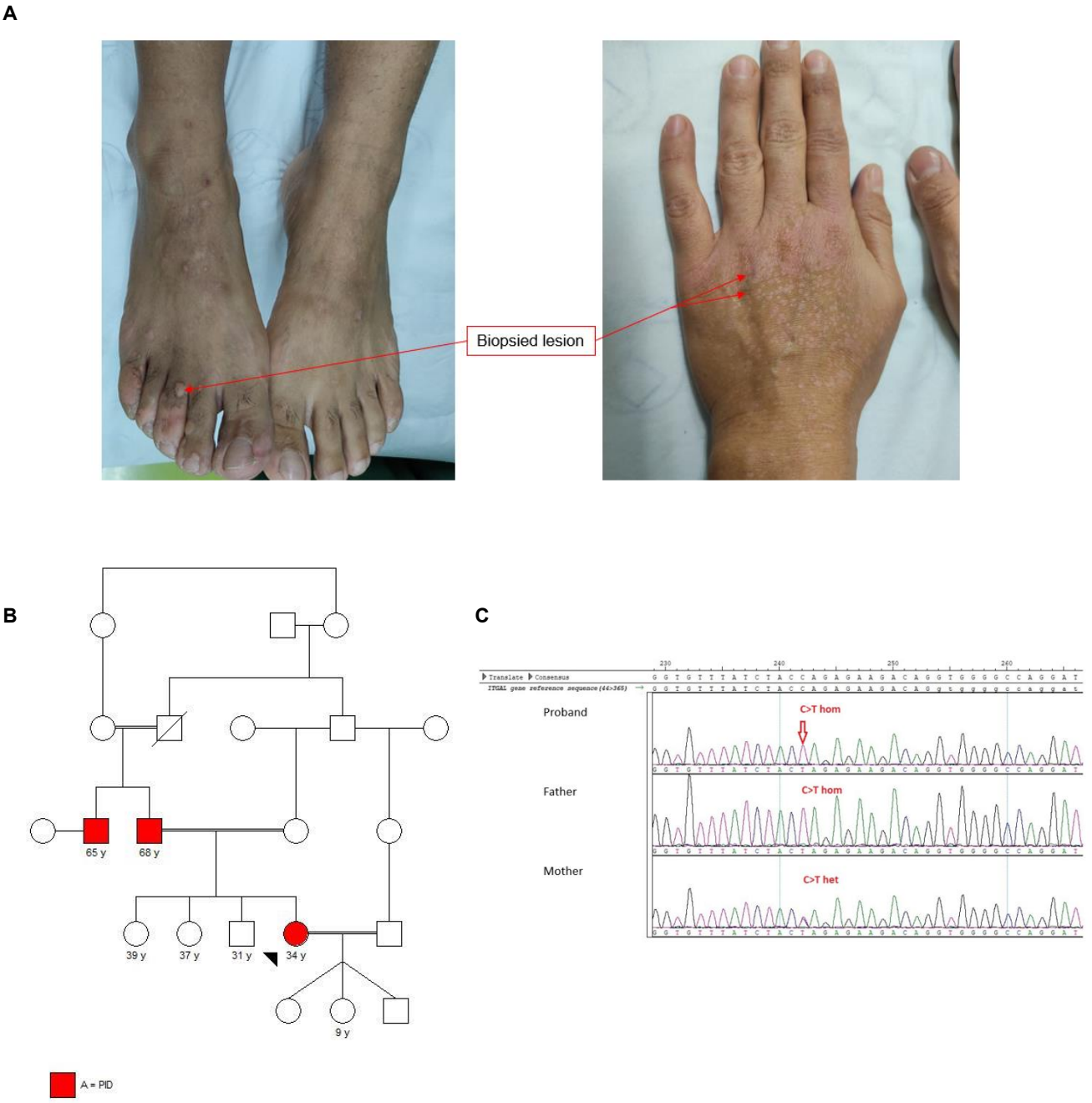

Figure 1. ***(A)*** *Biopsied lesions from the proband, wart (left) and EV (right).* ***(B)*** *Pedigree of the family. Double lines indicate consanguinity. Squares, male subjects; circles, female subject; filled (red) symbols represent affected individuals; white symbols represent unaffected family members; the arrow denotes to the proband. The proband, father, and mother were subjected to whole-exome sequencing.* ***(C)*** *Sanger traces of the ITGAL NM_002209.3: c.1492C>T, p.Gln498* variant in the family confirms the homozygous status of the proband and her father, and heterozygous status in the mother.*

Immunophenotyping

Compared with age-matched controls, immunophenotyping using flow cytometry revealed a significant decrease in the percentage of naïve CD4 T cell compartment ($CD4^+CD45RA^+CD197^+$) of the proband, while the frequency of CD8 T cells was not significantly altered in the patients compared to the controls. Moreover, there was a remarkable increase in the percentage of CD4 Treg cells ($CD4^+CD25^+CD127^{low}$) and CD4 activated T cells ($CD4^+CD38^+$). Additionally, the percentage of T helper 17 cells ($CD4^+CCR6^+$) were significantly elevated in the patients compared to the controls (Figure 2). The $CD337^+$ NK cells ($CD16^+CD56^+CD337^+$) of the patients were significantly decreased in comparison with the controls (Figure 2).

In the B cell compartment, there was an abnormal distribution of the memory cell and naïve cells in the patients versus the controls, with the percentage B memory cells ($CD19^+IgD^-CD27^-$) being considerably higher, and percentage of naïve B cells ($CD19^+IgD^+CD27^-$) being remarkably decreased (Figure 2).

LFA-1 expression in CD8+ T cells of the patients

We isolated $CD8^+$ T cells from fresh blood of the proband, father, and two healthy controls, and cultured with $CD3^+CD28^+$ beads for expansion. After one week the cells were harvested and Western blot was performed to study the expression of ITGAL in the patients. Western blot did not show any ITGAL expression in the patients, while the band is clearly visible in the controls (Figure 3A), and housekeeping protein (GAPDH) was normally expressed. Qualification of these results did not register any signal for ITGAL in the patients either (Figure 3B). No label-free quantification (LFQ) signal was registered for ITGAL in the proband and father, however, these results are not statistically significant, since in one of the controls, there was an absence of LFQ signal.

To verify the absence of ITGAL using another method and study the impact of this variant upon its other interacting integrins, the expression of CD11a (ITGAL), CD18 (integrin subunit β_2, the β subunit of LFA-1), CD11b, and CD11c was assessed using flow cytometry. The results demonstrated that although there is significant reduction in the expression of CD11a ($P < 0.0001$ in HI111 antibody, < 0.0001 in G-25.2 antibody).

Interestingly, the percentage of $CD11c^+$ $CD8^+$ T cells was significantly lower in the patients compared to the controls (Figure 3C, P = 0.0013). Moreover, CD11b expression was reduced in the $CD8^+$ T cells of the patients in comparison with the controls (Figure 3C, P = 0.0044).

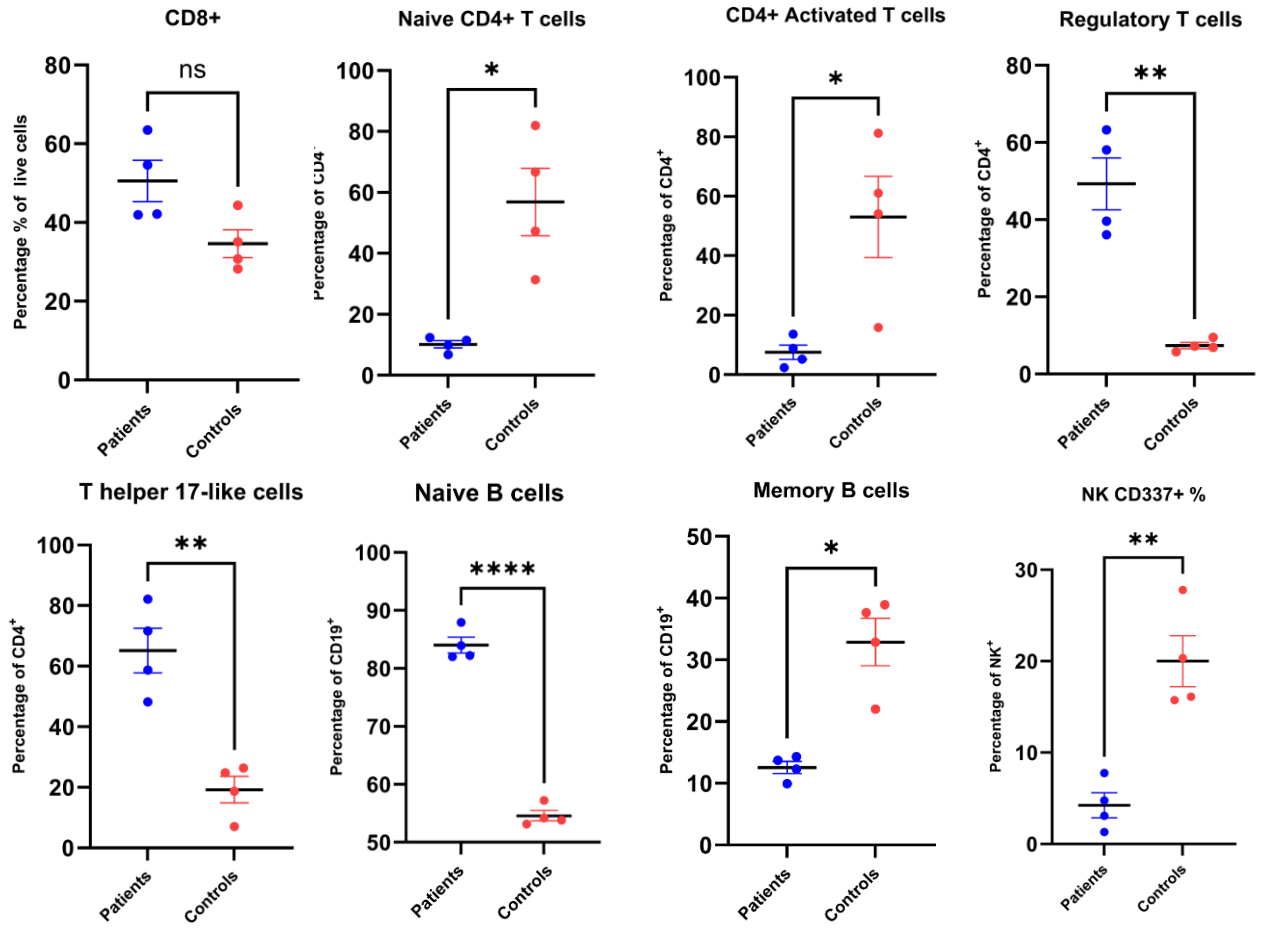

Figure 2. *The differences in the frequency of immune cells in the peripheral blood of the patients and controls. ns, not significant; *, P < 0.05; **, P < 0.01; ****, P < 0.0001.*

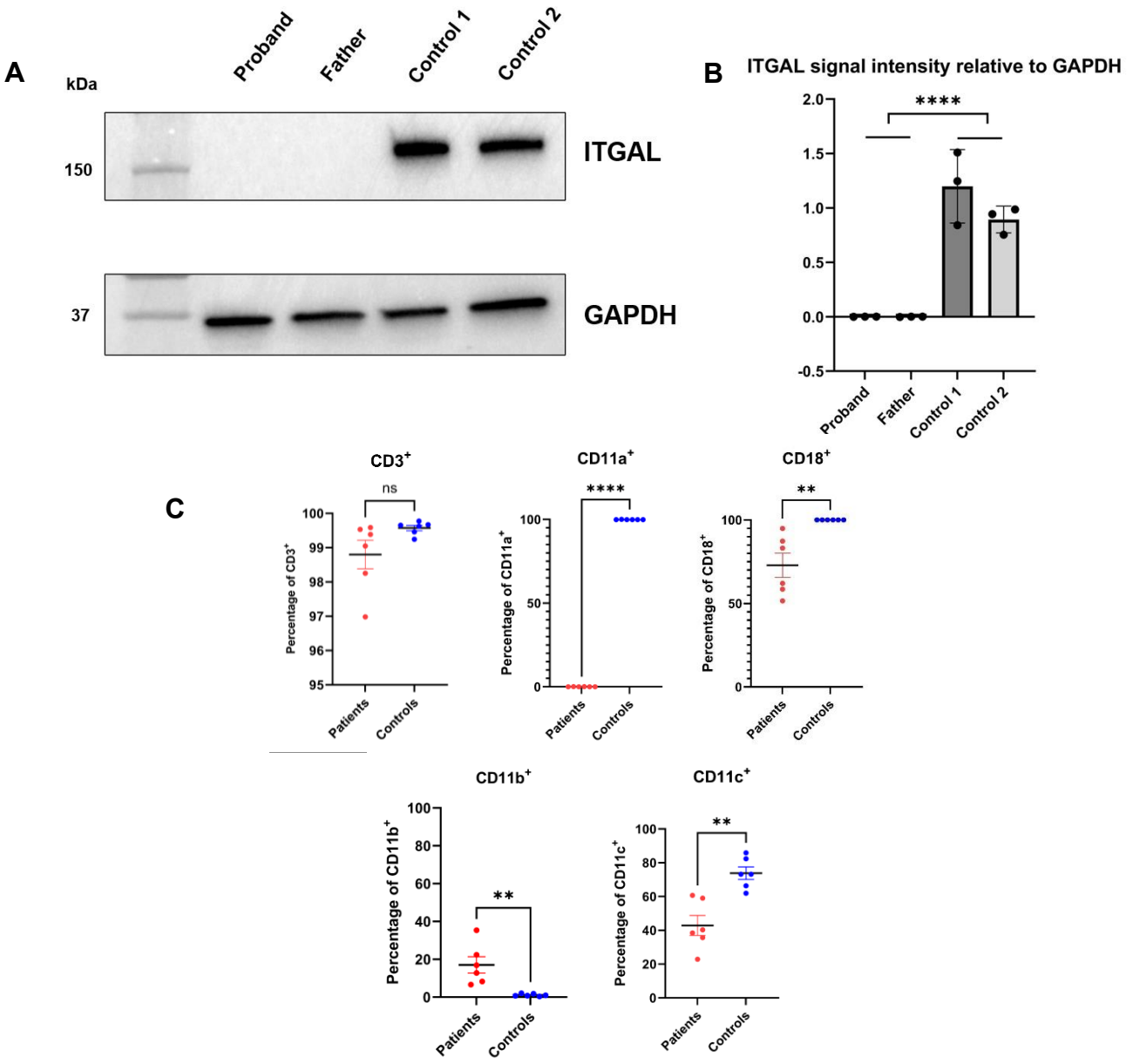

Figure 3. ***(A)*** *Western blot analyses of ITGAL protein expression in CD8+ T cells of the patients and two controls. ITGAL protein is absent in the patients. GAPDH was used as the internal control.* ***(B)*** *Signal intensity of ITGAL protein relative to the expression of GAPDH (p-value < 0.0001).* ***(C)*** *Frequency of expression of CD3, CD11b, CD11c, and LFA-1 (CD11a and CD18) cells in the patients and controls. ns, not significant; **, P < 0.01; ****, P < 0.0001.*

Combined RNA sequencing, proteomics, and single-cell RNA sequencing analyses

RNA sequencing (RNA-seq) and nano-liquid chromatography/tandem mass spectrometry was performed in peripheral blood mononuclear cells (PBMC). Single-cell RNA sequencing (scRNA-seq) was performed on peripheral blood cells of the patients (two biological replicates from the father, and one biological replicate from the proband) and two sex- and age-matched controls. Following quality control and normalization of single-cell RNA seq data, 9179 cells were obtained in total in blood samples, 5605 cells belonging to the patients (proband 3036 cells, father 2569 cells) and 3574 cells belonging to the controls. We performed Principal Component Analysis (PCA) and used harmony to remove batch and sex effect. We distributed the cells in the Uniform and Manifold Approximation and Projection (UMAP, Figure 4A). Graph-based clustering revealed 4 clusters of cells in the blood (Figure 4B), two of which were annotated as T cells, and the other two as B cells and NK cells according to the highly expressed genes in each cluster (Figure 4C). Additionally, we used following markers to annotate the cells: *CD79A*, *CD79B*, and *MS4A1* for B cells, *CD3D*, *CD3E*, *CD3G*, *CD4*, *CD8A*, *CD8B*, and *TRAC* for T cells, *GNLY, GZMB*, *GZMA*, and *PRF1* for natural killer cells and cytotoxic cells (Figure S1). *ITGAL* and *ITGB2* were majorly expressed by T cells and NK cells (Figure 4D). However, their expressions were not significantly altered in patients compared to the controls (Figure 4E).

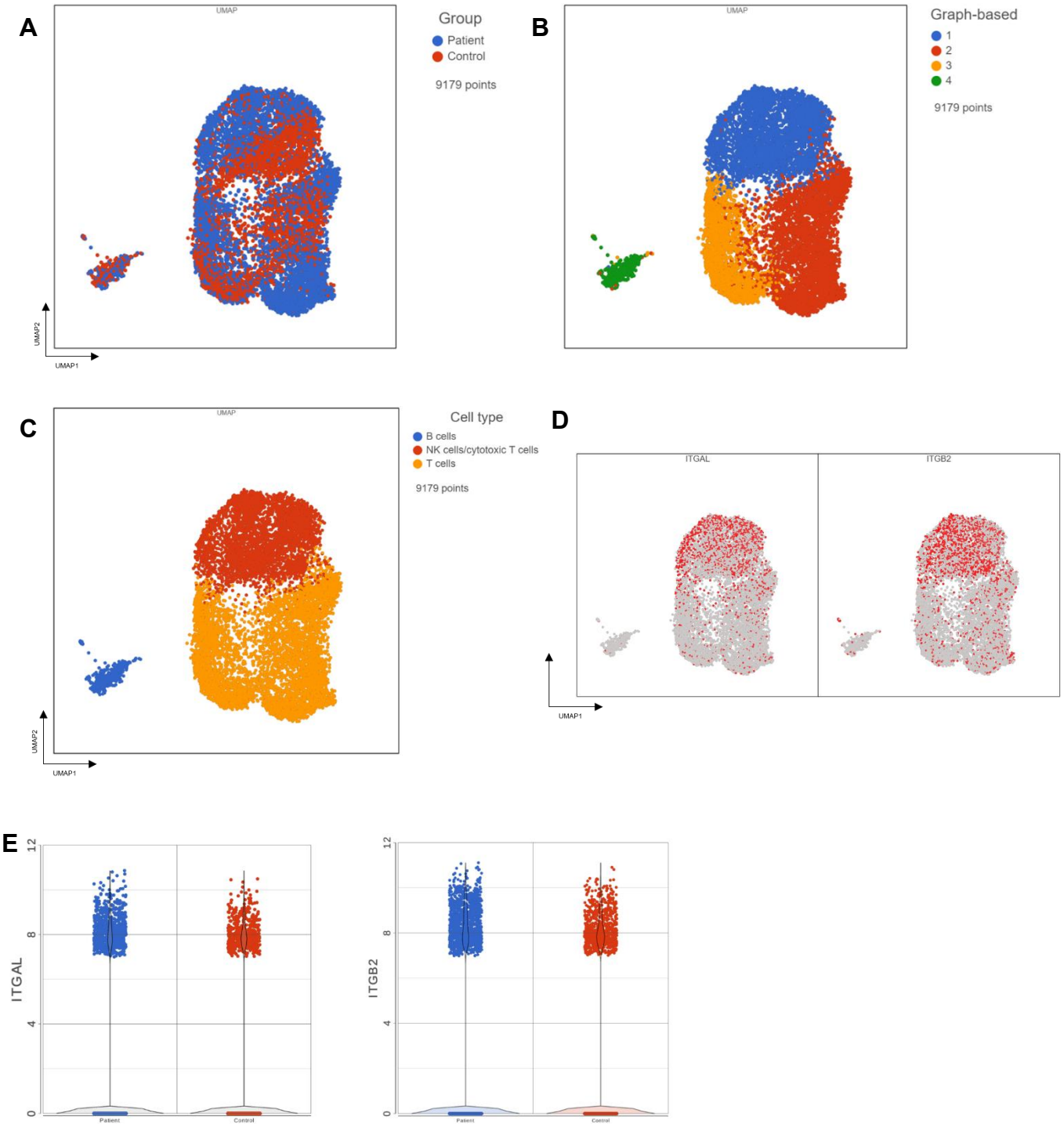

Figure 4. ***(A)*** *UMAP plot showing blood cells by group cell type (blue, patient cells; red, control cells).* ***(B)*** *UMAP plot displaying the clusters according to graph-based clustering (colors correspond to clusters).* ***(C)*** *UMAP plot showing blood cells by cell type (colors correspond to cell types).* ***(D)*** *Graphs showing the normalized counts of ITGAL (left) and ITGB2 (right) for the patients and the controls.* ***(E)*** *Dot plots showing the expression of ITGAL and ITGB2 genes in the patients and controls (blue, patient cells; red, control cells).*

ITGAL Gln498* Leads to Alterations in T Cell Activation and Cytokine Signaling

scRNA-seq revealed an upregulation of *IL2RA* (CD25) and *ZAP70* in the T cells and NK cells of the patients versus the controls, both of which encode proteins pivotal to T cell activation (Figure 5).

There was also an upregulation of *IFNG* (encoding interferon-γ), while *TNF* (encoding Tumor Necrosis Factor) was downregulated in the T cells and NK cells of the patients. In addition, *IL6R* and *IL2RG*, both encoding cytokine receptors involved in immune cell signalling, were upregulated in the T cells and NK cells of the patients (Figure 5). Furthermore, *DOCK10* and *DOCK11*, encoding proteins which regulate Rho GTPase signalling pathway were overexpressed in the T cells and NK cells of the patients in scRNA-seq (Figure 5). The transcription factors *STAT1* and *STAT2*, which are involved in cytokine signaling and immune regulation, were also upregulated in T cells and NK cells of the patients (Figure 5). Additionally, FOXP3 was upregulated in bulk RNA-seq in the patients' cells versus those of the controls (Figure 6A).

ITGAL Gln498* Impacts Expression Patterns of Immune Synapse and Adhesion Molecules

In proteomics analysis, ICAM1 - the main ligand binding to LFA-1 - was found to be downregulated in patients' PBMC compared to those of the controls (Figure 6B). *ICAM1* was also downregulated in the T cells and NK cells of the patients in scRNA-seq. Several integrins were also differentially expressed across the proteomics analysis or scRNA-seq analysis (Figures 6-7). *ITGAX*, *ITGA4*, *ITGB1*, and *ITGB7* were upregulated in the T cells and NK cells of the patients compared to the controls, while *ITGAV* was downregulated in the same dataset (Figure 7). In proteomics analysis, ITGA2B, ITGA6, and ITGA2 were downregulated in the patients compared to the controls (Figure 6B). *ITGB3* and *ITGB5* were found to be downregulated in bulk RNA-seq (Figure 6A). Moreover, the expression of *ITGAL* was not significantly altered in bulk RNA-seq (Figure 6A).

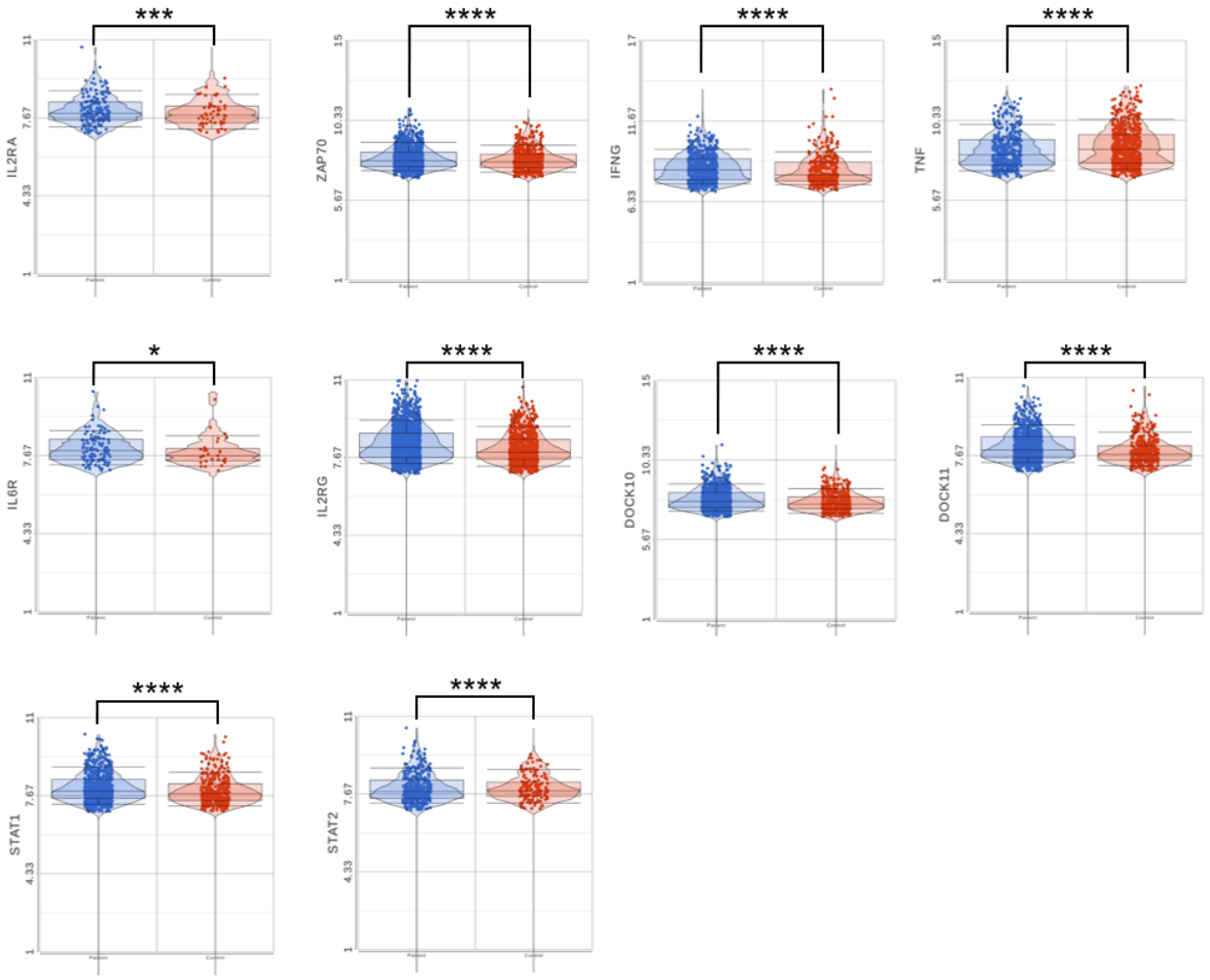

Figure 5. *Dot plots showing the differentially expressed genes involved in T cell activation and cytokine signaling. Each dot represents one cell expressing the gene shown on Y axis (blue, patient cells; red, control cells). Statistical significance was determined by ANOVA. *, P < 0.05; ***, P < 0.001; ****, P < 0.0001.*

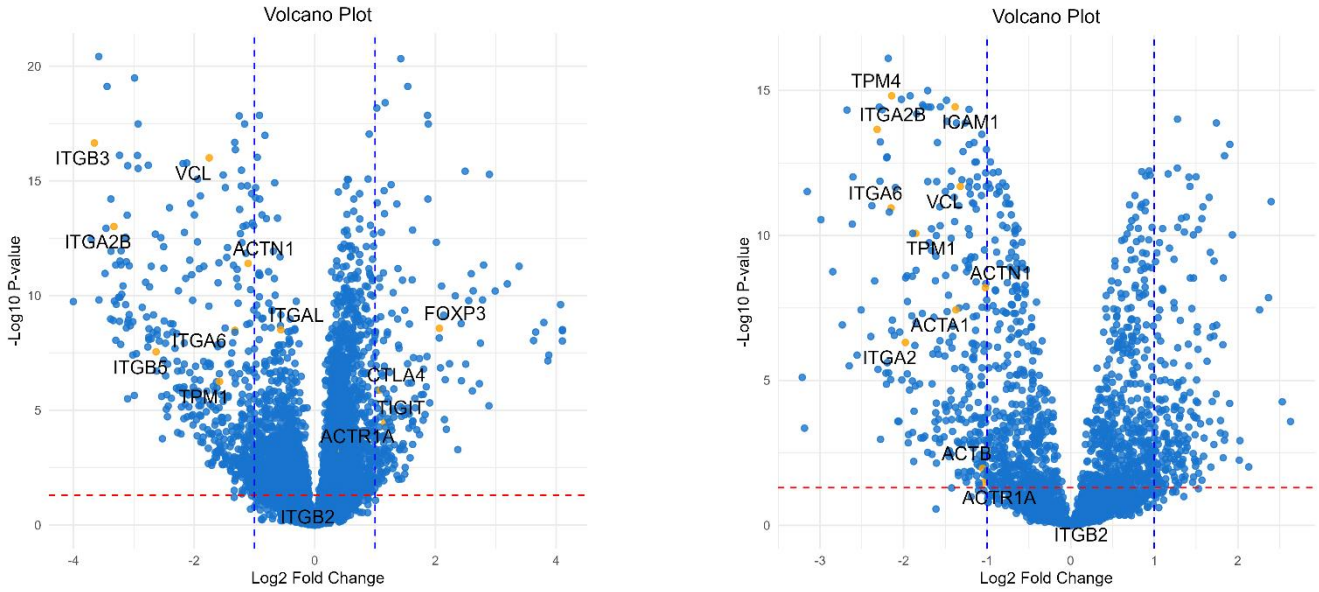

Figure 6. *Volcano plots demonstrating differentially expressed genes and proteins in the patient compared to the controls, in* ***(A)*** *RNA-seq of PBMC and* ***(B)*** *proteomics of PBMC. The blue and red dashed lines represent the threshold for Log-fold-change (<1 or >-1) and p-value (0.05), respectively.*

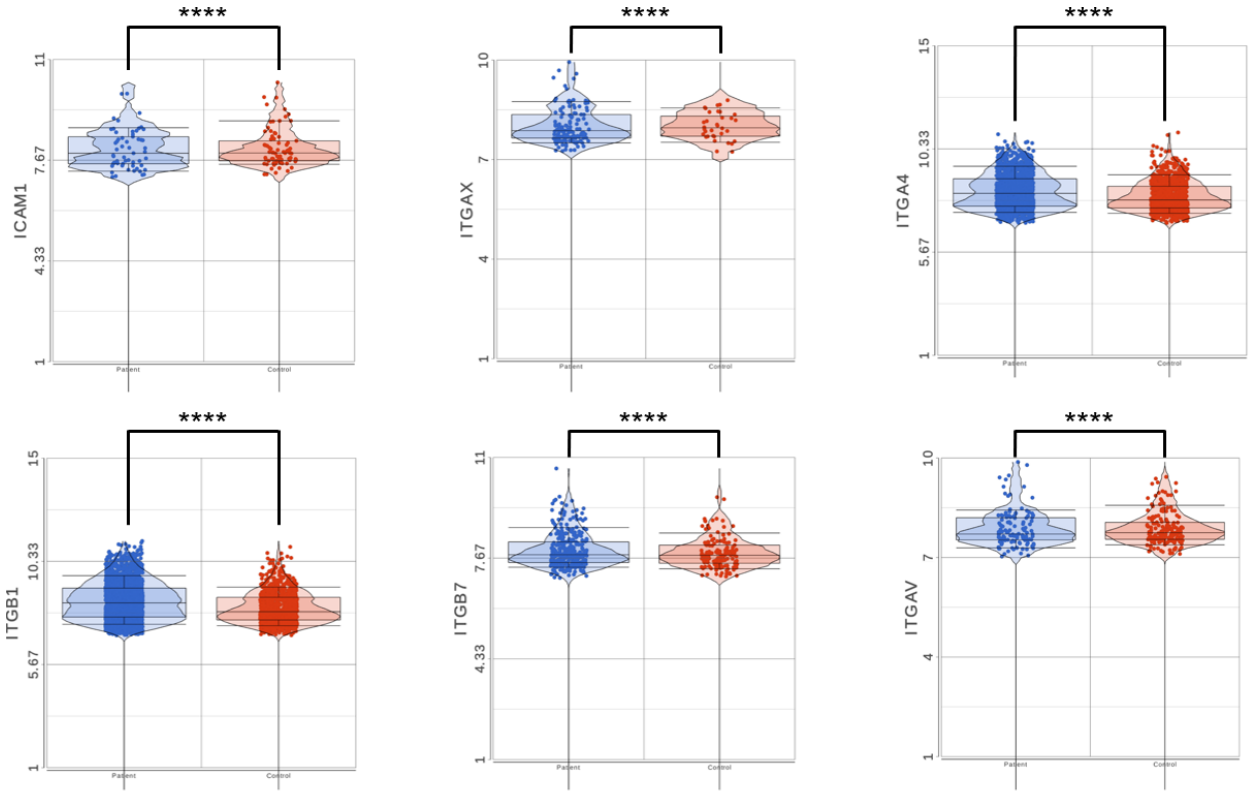

Figure 7. *Dot plots showing the differentially expressed genes encoding immune synapse and adhesion molecules. Each dot represents one cell expressing the gene shown on Y axis (blue, patient cells; red, control cells). Statistical significance was determined by ANOVA. ****; P < 0.0001.*

ITGAL Gln498* Affects the Expression of T Cell Receptor proteins

Within the T cell receptor (TCR) signaling pathway, scRNA-seq data showed upregulation of *CD247*, a crucial component of the TCR complex, in T cells and NK cells of the patients in the scRNA-seq data (Figure 8A). Additionally, *TRBV14* which encodes the V region of the variable domain of T cell receptor beta chain that participates in the antigen recognition was significantly downregulated in the T cells and NK cells of the patients (Figure 8A).

ITGAL Gln498* Disturbs Cytoskeletal Protein Expression and Leads to Cellular Structural Modifications

Certain proteins related to cytoskeletal and cell motility were differentially expressed across the datasets. Cytoskeletal proteins TPM1 (Tropomyosin 1) and ACTN1 (Alpha-Actinin 1) were downregulated consistently across proteomics (Figure 6B). Moreover, *PXN* (encoding paxillin), *VLC* (vinculin) and *ACTN4* were upregulated in the T cells and NK cells of the patients in scRNA-seq (Figure 8B). VCL, TPM4, ACTB, and ACTA1 were found to be downregulated in the proteomics data of the patients in comparison to the controls (Figure 6B). Additionally, certain genes belonging to the actin dynamics signalling pathway such as *PAK1* and *PTK2* were significantly downregulated in the scRNA-seq in T cells and NK cells of the patients versus the controls (Figure 8B).

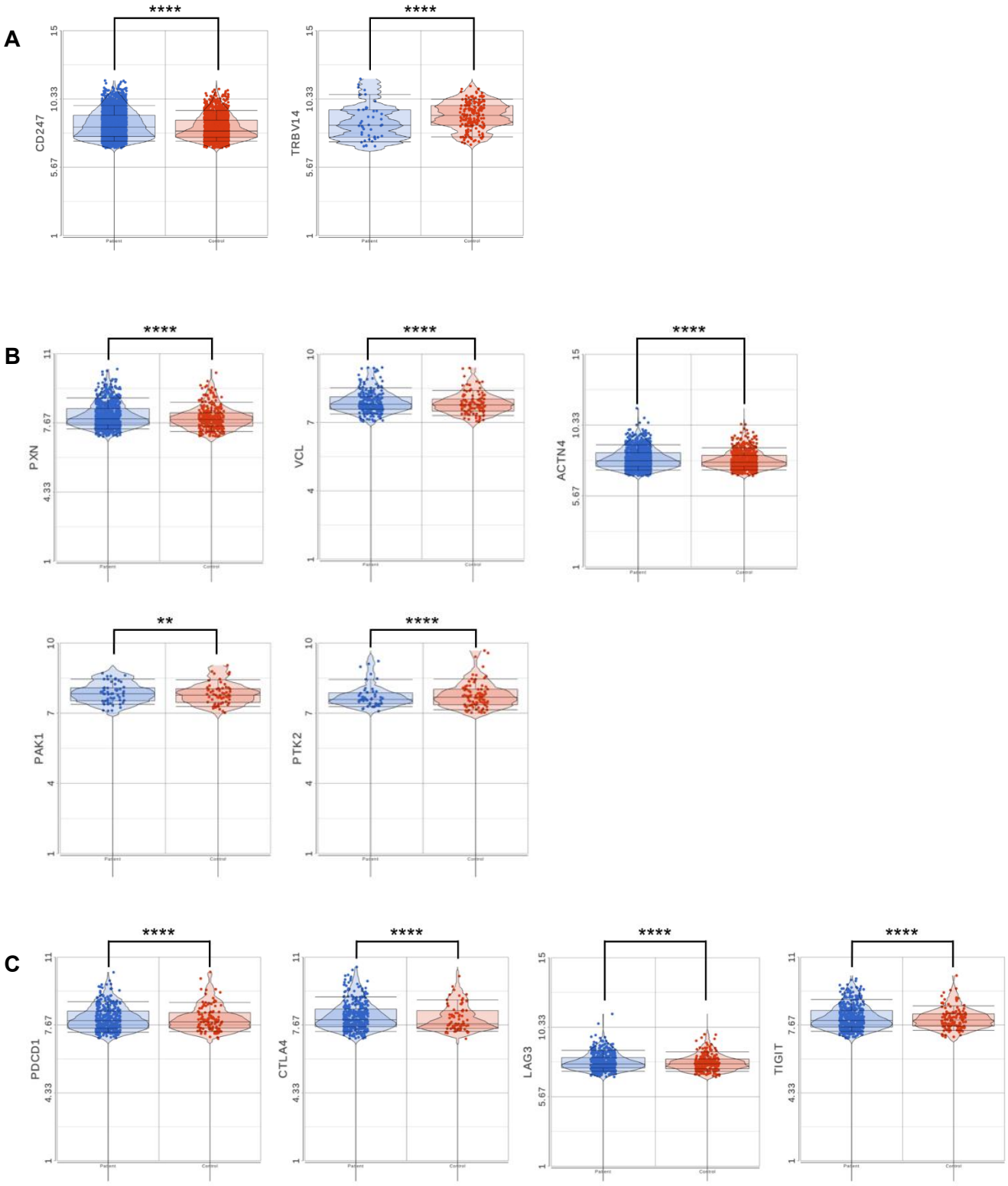

Figure 8. *Dot plots showing the differentially expressed genes related to* ***(A)*** *T cell receptors* ***(B)*** *Cytoskeleton and cellular structure (C) T cell exhaustion. Each dot represents one cell expressing the gene shown on Y axis (blue, patient cells; red, control cells). Statistical significance was determined by ANOVA. **, P < 0.01; ****, P < 0.0001.*

ITGAL Gln498* Alters the Expression of Immune Cell Markers and Exhaustion-Related Gene

PDCD1 (PD-1), a marker of immune cell exhaustion, was upregulated in the T cells and NK cells of the patients in comparison with the controls in scRNA-seq (Figure 8C). Moreover, genes encoding immune checkpoint proteins *CTLA4*, *LAG3*, and *TIGIT* were found to be upregulated in the T cells and NK cells of the patients compared to the controls, with *CTLA4* and *TIGIT* also being upregulated in bulk RNA sequencing of the patients compared to the controls (Figure 6A).

Pathway Enrichment Analyses

In Gene Ontology (GO) analysis of proteomics data, several molecular functions were identified as significantly downregulated, including integrin binding and extracellular matrix structural constituent (Figure 9A). In the cellular component category, there was a notable downregulation in proteins associated with actin filaments, endoplasmic reticulum lumen, cell-substrate junction, protein complexes involved in cell adhesion, and the external side of the plasma membrane (Figure 9B). For biological processes, there was significant downregulation in pathways involved in ERK1 and ERK2 cascades, positive regulation of cell adhesion, cell-substrate junction organization, homotypic cell-cell adhesion, wound healing, and cell-substrate adhesion (Figure 9C).

KEGG pathway analysis in proteomics data revealed downregulation in the PI3K-Akt signaling pathway, focal adhesion, neutrophil extracellular trap formation, and ECM-receptor interactions (Figure 10A).

Pathway analysis using Reactome demonstrated significant downregulation of pathways involved in p130Cas linkage to MAPK signaling for integrins, integrin signaling, extracellular matrix organization, integrin cell surface interactions, and response to elevated platelet cytosolic Ca^{2+} levels (Figure 10B).

Despite dysregulation in many immune-related genes in scRNA-seq and bulk RNA-seq which was discussed above, no pathway was significantly altered in the results of GSEA in bulk RNA-seq and between the cell clusters identified in scRNA-seq.

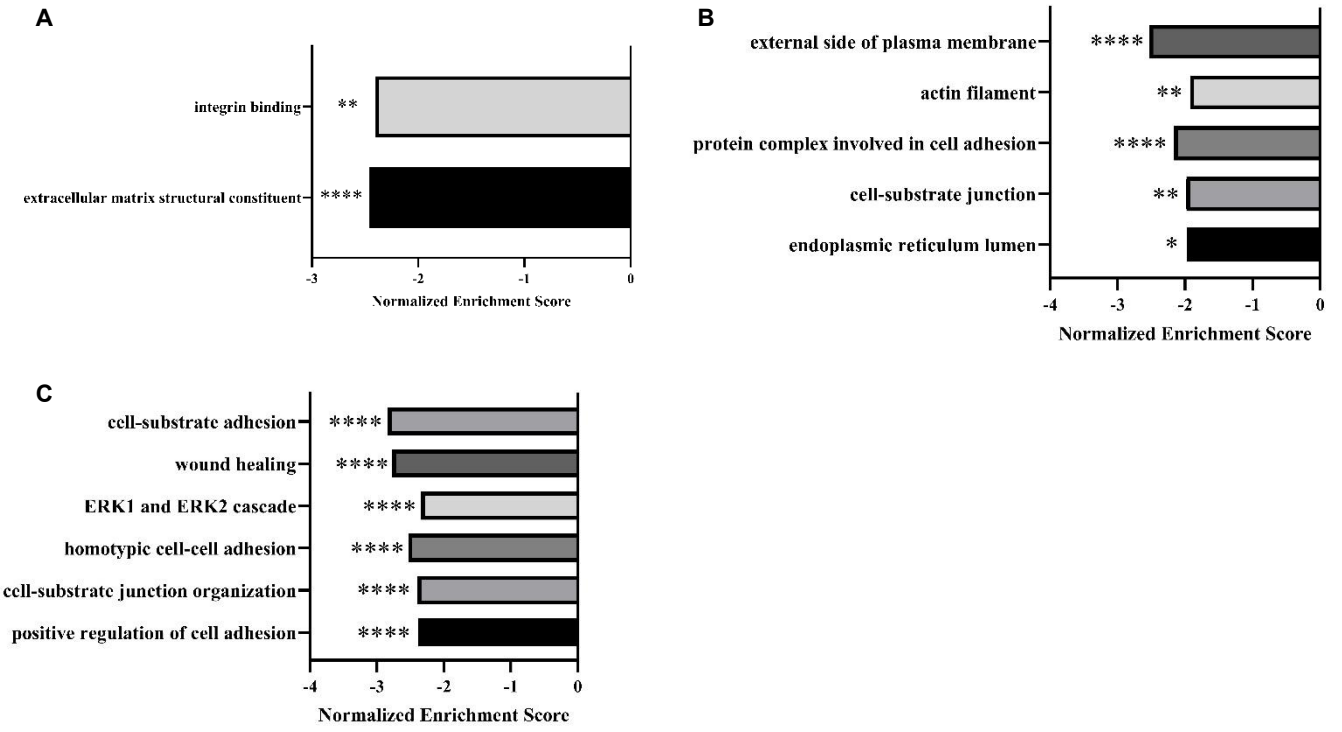

Figure 9. *Dysregulated Gene Ontology processes in the PBMC proteomics of patients compared to controls.* ***(A)*** *Molecular functions.* ***(B)*** *Cellular components.* ***(C)*** *Biological processes. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.*

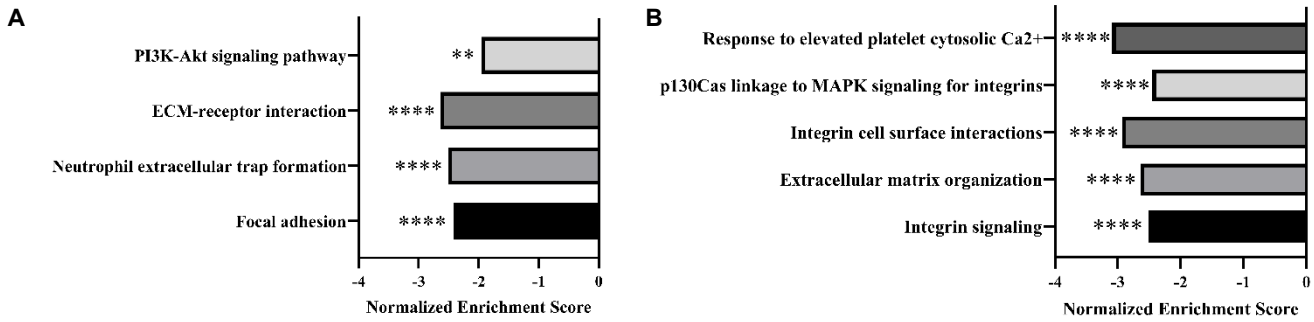

Figure 10. *Dysregulated pathways in the PBMC proteomics of patients compared to controls.* ***(A)*** *KEGG.* ***(B)*** *Reactome. **, P < 0.01; ****, P < 0.0001.*

6.3 Discussion and Conclusion

In this study, we reported a novel candidate gene *ITGAL* in a family with affected individuals with resistant wart and EV lesions, discovered in our cohort of IEIs. We further investigated the expression of ITGAL in the patients and demonstrated lack of ITGAL expression in their T cells. We found upregulation of T cell activation regulated genes in scRNA-seq, suggesting a counterbalance for the probable reduced stability in immune synapse formation due to the defective LFA-1. We also observed modulation of inflammatory markers, such as *TNF* and *IFNG*, as well as the elevation of certain markers such as *IL6R*, *IL2RG*, and *IL2RA* which might be aimed for maintaining cell functionality. Furthermore, upregulation of *CTLA4*, *LAG3*, and *TIGIT* indicate chronic T cell activation and probable exhaustion. This hypothesis is further supported by an overexpression of *PDCD1* (PD-1) which is the main inhibitory receptor regulating T-cell exhaustion (Jiang et al., 2015). Additionally, we showed downregulation of *ICAM1* in the T cells, suggesting the impairment of cell-cell interactions in the T cells and NK cells of the patients. Several genes modulating cytoskeletal remodeling following the activation of LFA-1 were downregulated in the proteomics data, while expression of other integrins such as *ITGAX* was increased in scRNA-seq, suggesting adjustment in attempt to compensate for lack of LFA-1 downstream signaling. Altogether, these findings show the impairment of LFA-1 activation, and indicate the compensatory mechanism utilized to maintain cell function and motility, thus emphasizing the concept of redundancy of immune and motility-related pathways and molecules.

As expected, genes belonging to MAPK/Erk signaling pathway, AKT signaling pathway, and actin dynamics signaling pathways were differentially expressed, indicating the absence of LFA-1 function and downstream activation of cellular processes essential to T cell function. While previous studies discussed crucial role of LFA-1 in human and mice (Walling and Kim, 2015; Hou and Yuki, 2023), the phenotype of the patients reported in this study seems to be rather mild, even milder than LAD type I, wherein the beta subunit of LFA-1 is defected. This could be owing to two reasons. The alpha subunit is unique to LFA-1, while the beta subunit combines with multiple alpha subunits and participates in forming multiple integrins. Hence, it is expected that pathogenic variants in *ITGB2* result in defects in more integrin - Mac-1 (αMβ2) and CR4 (αXβ2) - leading to a more severe

phenotype compared to a defect in the αL subunit (encoded by *ITGAL*) which only leads to a defect in LFA-1. Additionally, it is already reported that certain types of integrins are overexpressed to compensate for the lack of expression of other integrins in animal studies. For instance, it has been shown that in the absence of VLA-4 (α4β1), the migratory ability of Tregs towards the nervous system is maintained using LFA-1 (Glatigny et al., 2015). Intriguingly, *ITGA4* and *ITGB1* (encoding α4 and β1 subunits of VLA-4, respectively) were overexpressed in the T cells and NK cells of the patients. This suggests a bidirectional relationship between the expression of LFA-1 and VLA-4.

Overall, the clinical phenotype and our findings demonstrate major redundancy in the role of LFA-1 in immune system. While further functional studies are needed to verify the redundancy of LFA-1 and investigate its role in protection against HPV.

6.4 Material and Method

Subjects, study approval, and sampling

The subjects of this study were members of a consanguineous family of Iranian ethnicity. The mother and husband of the proband were healthy. All subjects gave written informed consent to participate in this study, which was conducted according to the principles of the Helsinki Declaration and approved by the institutional review board of Isfahan University of Medical Sciences (Isfahan, Iran).

Blood sampling was carried out for the proband, her father, her mother, and her husband. In total, sampling for proband's blood was done two times over the course of one years to be used as biological replicates. Peripheral Blood Mononuclear Cells (PBMC) were isolated within 24 hours of sampling using Ficoll-Paque density gradient centrifugation.

Whole exome sequencing

Genomic DNA was isolated from peripheral blood of the proband (II.1), his parents (I.1, I.2) and his healthy brother (II.2) using standard protocols. Exome sequencing was performed on a NextSeq500 sequencer (Illumina, San Diego, CA, USA) as described previously (Castro et al., 2020). The following filtering steps were applied for variant identification in the proband, i.e. selecting only (i) variants with a depth of coverage of more than or equal to 10 reads; (ii) protein-altering variants (excluding noncoding or synonymous variants), (iii) variants with allele frequencies < 0.001 in the 1000 Genomes Project, the Genome Aggregation Database (gnomAD), and an internal database including ~1000 exomes, and (iv) variants homozygous in the proband and father, and heterozygous or wildtype in the healthy mother.

Sanger sequencing

The candidate variant was confirmed by Sanger sequencing as described earlier (Castro et al., 2020) and using the following primers: 5'- CAAACACCTGCCTCTCCCCAC-3' (forward) and 5'-GAGGATTTGCTATTGCCTCTCTGTCG-3' (reverse) for both amplification and sequencing.

Whole transcriptome and proteome analyses

Bulk RNA sequencing was performed on PBMCs of the proband and father and were compared to the same sample types of healthy controls. Total RNA was extracted using the RNeasy Mini Kit (74106, Qiagen, Hilden, Germany). Total RNA-seq library preparation, sequencing, and analyses were performed as previously described (Castro et al., 2020). Up- and down-regulated genes were selected based on the adjusted p-value (<0.05) and the log-fold-change (≥1).

Proteomics analyses were performed on HDFs and PBMCs. Protein extraction, enzymatic digestion, HPLC separation, analysis of the resulting peptides with nano-liquid chromatography/tandem mass spectrometry (nano-LC-MS/MS), and software-based protein quantification using MaxQuant were performed using the previous protocols described (Castro et al., 2020). Up- and down-regulated proteins were selected based on the adjusted p-value (<0.05) and the log-fold-change (≥1).

Gene Ontology and pathway analyses of differentially expressed genes and proteins were performed using the WEbGestalt toolkit (Liao et al., 2019) (www.webgestalt.org). The functional databases of biological process non-redundant, cellular process non-redundant, and molecular process non-redundant were used for Gene Ontology analyses. The functional databases of KEGG, Panther, and Reactome were used for pathway analyses.

Single-cell RNA sequencing

Five hundred µl of whole blood and bone marrow were incubated twice for 10 min at room temperature with RBC lysis solution 1X (Miltenyi, Bergisch Gladbach, North Rhine-Westphalia, Germany) to remove red blood cells. Cells were then washed and centrifuged at 500 g and subsequently resuspended in DPBS (14190144, Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) containing 2% FBS for cell counting using Trypan Blue and Countess™ II FL (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA).

Single-cell RNA sequencing (scRNA-seq) libraries were prepared using the Chromium Single Cell 3’ Library and Gel Bead kit v3.1, Chip G kit, Single Index kit T set A and the

Chromium Controller according to the manufacturer's instructions (10X Genomics, Pleasanton, CA, USA). Cells were loaded at a volume to target around 5,000 cells per sample. Size distribution and concentration of cDNA and final libraries were verified on an Agilent Bioanalyzer High Sensitivity chip (Agilent Technologies, Santa Clara, CA, USA). Libraries were sequenced using an Illumina NextSeq 2000 instrument according to the manufacturer's guidelines (Illumina, San Diego, CA, USA) with a sequencing depth of at least 30,000 reads per cell.

Raw sequencing data were processed using Cell Ranger analysis pipeline, v6.1.2 (Zheng et al., 2017). The "mkfastq" command was used to generate FASTQ files and the "count" command to generate raw gene-barcode matrices aligned to the 10X Genomics GRCh38 Ensembl build 84 genome (version 1.2.0). The "--force-cells" command was used to run cellranger count and include low- Unique Molecular Identifiers (UMI) barcodes in order to capture neutrophils from 3' or 5' Single Cell Gene Expression data.

Partek® Flow® software v10.0 was used for single-cell RNA sequencing analyses. The following filtering criteria were used to remove the low-quality cells with (i) <200 genes and/or (ii) <300 UMI counts and/or (iii) >8% mitochondrial gene counts and/or (iv) >50% or <10% ribosomal protein genes. Normalization was conducted using the following parameters: counts per million, add 1.0 Log 2.0. Principal component analysis (PCA) was performed and Harmony was used on the PCA space for the removal of the batch effect. Graph-based clustering was conducted using 20 principal components, followed by Uniform Manifold Approximation and Projection (UMAP) analysis. Annotation of cells was done according to gene markers. Differential gene expression was performed after annotation, comparing the normalized data of patient versus the controls using an analysis of variance (ANOVA) model. The filtering criteria for differentially expressed genes used for pathway analysis were adjusted p-value (<0.05) and fold change (≥1.5).

CD8+ T cell isolation and culture

EasySep™ Human CD8+ T cell Isolation Kit (Catalog # 17953, STEMCELL™ Technologies, Vancouver, BC, Canada) was used according to the manufacturere's protocol to isolate CD8+ T cells from fresh blood. The cells were expanded in culture

media (Roswell Park Memorial Institute 1640 Medium (61870036, Gibco™,Thermo Fisher Scientific, Waltham, MA, USA) (RPMI 1640) supplemented with 10% inactivated foetal bovine serum (FBS), 40 U/ml penicillin, and 50 mg/ml streptomycin and Dynabeads® Human T-Activator CD3/CD28 (Catalog # 11131D, Gibco, ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions with incubation at 37°C and 5% CO_2.

Western blotting

Frozen cell pellets of 10^6 CD8+ T cells were thawed on ice and treated with lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.1% Na deoxycholate, 1 mM dithiothreitol, 1 mM EDTA, and protease inhibitor cocktail; Roche, Basel, Switzerland). Cells were incubated for 20 min on ice; 20 µg of each sample was added to 5 µl of 5x loading buffer. Samples were heated for 15 min at 95°C, loaded on a 4-20% pre-cast gradient gel (NB10420, NuSep, Germantown, MD, United States), and subjected to electrophoresis at 150 V for 70 minutes. Protein transfer was performed using TransBlot® Turbo™ Transfer System (Bio-Rad Laboratories, Hercules, California, USA) following the manufacturer's instructions. The membrane was blocked for 1 h with Tris-buffered saline, Tween 20 (0.05%), and non-fat milk (5%), and was incubated at 4°C overnight with anti-ITGAL antibody (1:5000 dilution, ab52895, abcam, Cambridge, UK). Anti-GAPDH antibody (1:10000, MAB374, Sigma-Aldrich, St. Louis, MO, USA) was used for the detection of housekeeping protein, incubating for 2 h at room temperature. The membrane was incubated with secondary antibodies (Goat Anti-Rabbit [H+L]-HRP conjugates (1721019, Bio-Rad Laboratories, Hercules, California, USA) and Goat Anti-Mouse IgG [H+L]-HRP conjugates (1706516, Bio-Rad Laboratories, Hercules, California, USA) both at dilution of 1:10000 for 1 h. The ChemiDoc XRS+ system (Bio-Rad Laboratories, Hercules, California, USA) and Clarity Western ECL (1705061, Bio-Rad Laboratories, Hercules, California, USA) were used for signal detection. Western blot quantification was performed using ImageJ (Schneider et al., 2012).

Flow cytometry of blood

Cells from 250 µl of whole blood were washed twice with 2 ml of 1X PBS, spun at 350 x g for 5 min at room temperature. The samples were then stained with 1 ml of 1:1000 diluted Live/Dead Blue for 20 min at room temperature. Samples were then washed with 2 ml of FACS buffer (PBS 1X, FBS, 0.5%, EDTA, 2 mM Hepes 10 mM) and collected by centrifugation at 300 x g for 5 min at room temperature. Five microliters of Human TruStain FcX™ and 5 µl of True-Stain Monocyte Blocker™ were added to each sample, vortexed, and incubated for 5 min. The mix of 40 antibodies was added in 3 batches (see Table S1 for details). After staining for 30 min on ice, 3 ml of red blood cell lysis buffer (Versalyse, Beckman Coulter Life Sciences, Indianapolis, IN) were added to each sample. After 10 min of incubation at room temperature, the cells were immediately centrifuged at 300 x g for 5 min at 4°C. Samples were washed twice with 2 ml of FACS buffer, centrifuged and resuspended in 300 µl of FACS buffer. The resuspended material was then analyzed using a Cytek® Aurora 5L spectral flow cytometer (Cytek Biosciences, Fremont, CA). A total of 300,000 CD45-positive live cells were acquired. Whole blood samples were analyzed by using the OMIQ software from Dotmatics (www.omiq.ai, www.dotmatics.com). Downsampling was performed on 300,000 CD45+ cells. In this composite file, cells were pre-gated as follows: singlets, live, time and CD45+.Computational analysis was carried out on FlowSOM (Van Gassen et al., 2015) to delineate the total number of clusters generated. Cell cluster annotations were performed using OMIQ. All frequencies of clusters in the bar plots delineated were calculated by dividing each cluster by all clusters.

Staining Protocol for CD11a, CD11b, CD11c, and CD18

Cell surface staining was performed using fluorophore-conjugated monoclonal antibodies in table S2. One hundred microliters of a mix containing 10% of Brilliant Stain Buffer Plus and 5% of Human True Stain FcX was added on 0.5 x10^6 cells in a 5 ml tube. The samples were gently vortexed and incubated for 30 minutes at 4°C in the dark.

After incubation, the cells were washed twice with FACS (PBS 1X, FBS, 0.5%, EDTA, 2 mM Hepes 10 mM) collected by centrifugation at 300 x g for 5 min. The cell pellets were then resuspended in 400 µL of PBS with 2% FBS with DAPI for flow cytometry acquisition.

Flow Cytometry Acquisition

The stained samples were analyzed using a Cytek Aurora 5L spectral flow cytometer (Cytek Biosciences, Fremont, CA). For each sample, a minimum of 10,000 events were acquired. Fluorescence-minus-one (FMO) controls and single-stained controls were included to set the gates and compensate for any spectral overlap.

Data Analysis

Flow cytometry data were analyzed using FlowJo™ software (TreeStar Inc., USA). Cells were gated based on forward and side scatter to exclude debris and DAPI positive cells to remove dead cells. The expression levels of CD11a, CD11b, CD11c, and CD18 were determined as the percentage of positive cells within the gated live-cell population. The expression of each integrin subunit was quantified.

Statistical Analysis

The percentage of positive cells for each marker were compared across samples. Statistical analysis was performed using GraphPad Prism v.10 (GraphPad Software, USA).

Statistics

Graphs were generated using GraphPad Prism v10.2.3 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com) and R packages ggplot (Wickham, 2016)) and ggrepel (Slowikowski et al, 2024). T-test was used for statistical comparison. The corresponding p-value is given for each figure in its legend. A p-value<0.05 was considered as significant.

6.5 References

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6.6 Supplementary Materials

Tables

Table S1. Antibodies used for immunophenotyping of blood.

#	Antigen	Fluorochrome	supplier	reference	clone
1	CD123	Super Bright 436	Thermo Fisher	62-1239-42	6H6
2	CD45	PerCP	Biolegend	304026	HI30
3	HLA-DR	PE Fire 640	Biolegend	307676	L243
4	CD14	SPARK Blue 550	Biolegend	367148	63D3
5	CD16	BUV496	BD	612944	3G8
6	CD3	BV510	Biolegend	344828	SK7
7	TCR-□□	PerCP-eFluor 710	Thermo Fisher	46-9959-42	B1.1
8	CD45RA	BUV395	BD	740315	5H9
9	CD197 CCR7	BV421	Biolegend	353208	G043H7
10	CD56	BUV737	BD	612766	NCAM 16.2
11	CD8	BUV805	BD	612889	SK1
12	CD19	SPARK NIR 685	Biolegend	302270	HIB19
13	CD2	PerCP-Cy5.5	Biolegend	309226	TS1/8
14	CD4	SPARK YG 581	Biolegend	344670	SK3
15	IgD	BV480	BD	566138	IA6-2
16	CD11c	Pacific Blue	Biolegend	301626	3,9
17	CD127	APC-R700	BD	565185	HIL-7R-M21
18	CD38	APC Fire 810	Biolegend	303550	HIT2
19	CD141	BB515	BD	565084	14A
20	CD39	BUV661	BD	749967	TU6
21	CD27	APC-Cy7	Biolegend	356424	M-T271
22	CD28	BV650	Biolegend	302946	CD28.2
23	IgM	BV570	Biolegend	314517	MHM-88
24	IgG	BV605	BD	563246	G18-145
25	CD185 (CXCR5)	BV750	BD	747111	RF8B2
26	CD183 (CXCR3)	PE-Cy7	Biolegend	353720	G025H7
27	CD95 (FAS)	PE-Cy5	Biolegend	305610	DX2
28	CD57	FITC	Biolegend	359604	HNK-1
29	CD159c (NKG2c)	PE	Biolegend	375004	S19005E
30	CD337 (NKp30)	PE DAZZLE 594	Biolegend	325232	P30-15
31	CD279 (PD-1)	BV785	Biolegend	329930	EH12.2H7
32	CD196 (CCR6)	BV711	Biolegend	353436	G034E3
33	CD24	PE-Alexa Fluor 610	Thermo Fisher	MHCD2422	SN3
34	CD25	PE-Alexa Fluor 700	Thermo Fisher	MHCD2524	CD25-3G10
35	CD20	Pacific Orange	Thermo Fisher	MHCD2030	HI47
36	CD66b	Alexa647	Biolegend	392911	6/40C
37	CD195 (CCR5)	BUV563	BD	741401	2D7/CCR5

38	CD185 (CXCR5)	BV750	BD	747111	RF8B2
39	CD314 (NKG2D)	BUV615	BD	751232	1D11
40	DAPI	DAPI	Thermo Fisher	D1306	

Table S2. Antibodies used for CD11a, CD11b, CD11c, and CD18 staining of CD8+ T cell.

Monoclonal antibodies	Dilution	clone	Company
CD3 APC-H7	100	SK7	Becton Dickinson
CD11a BV605	400	G-25-2	Becton Dickinson
CD11a PE	200	HI111	Biolegend
CD11b A488	200	ICRF 44	Becton Dickinson
CD11c PE-Cy7	100	B-ly6	Becton Dickinson
CD16 BV480	200	3g8	Becton Dickinson
CD18 BV750	200	L130	Becton Dickinson
CD19 Percp-Cy5.5	50	HIB19	Becton Dickinson
CD49a BV421	100	TS2/7	Becton Dickinson
CD56 RB780	50	B159	Becton Dickinson
DAPI	1 000 000		Becton Dickinson
Human TruStain FcX	5%		Biolegend

Figure

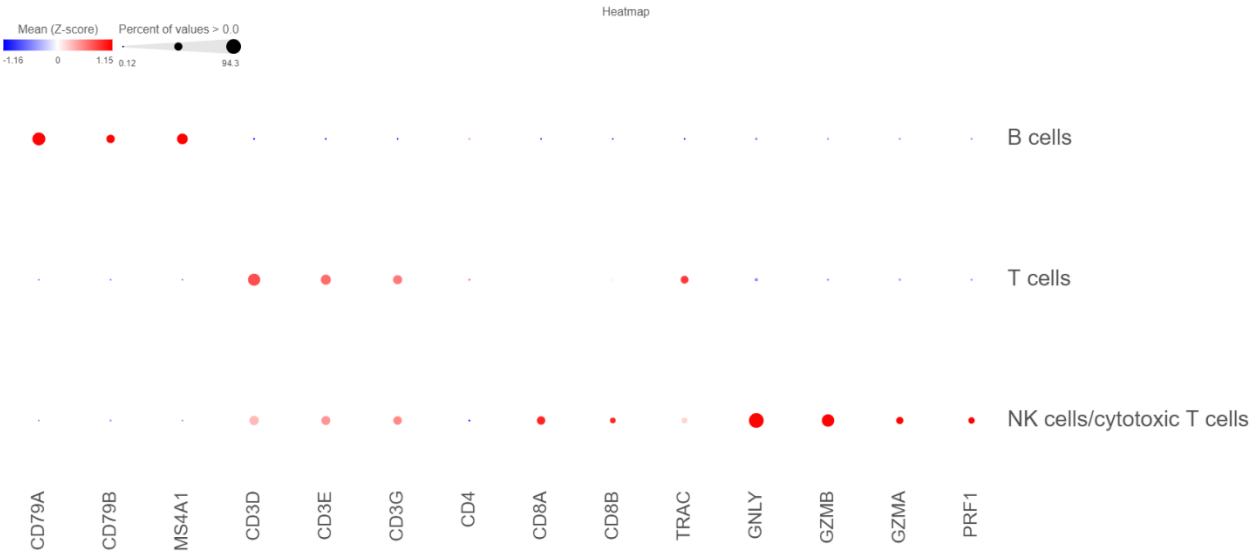

Figure S1. *Normalized expression of canonical gene markers demonstrated in bubblemap plots according to the annotated cell type.*

7 Discussion and Conclusion

In the first project, a novel missense variant in *HYOU1* was reported in a patient with neutropenia, manifesting with chronic diarrhea and FTT. In our investigations, we demonstrated hypogranularity in the neutrophils of the patients, suggesting that defects in HYOU1 impacts neutrophil function as well as numbers. We also showed that the maturation arrest of B cell lineage takes place before the stage of pro-B cells. Additionally, our findings suggest that HYOU1 is expressed in a self-regulatory loop. Our findings contribute to the expansion of HYOU1 scope beyond its known cellular stress repose role, and highlight its crucial role in immune regulation, particularly in B cell development, and aid in a better understanding of its mechanisms of expression.

In the second project, we reported a novel candidate gene ITGAL in a family with affected individuals with resistant wart and EV lesions. We further investigated the expression of ITGAL in the patients and demonstrated lack of ITGAL expression in their T cells. We found upregulation of multiple T cell regulating genes in scRNA-seq, with downregulation of pivotal T-cell related pathways in proteomics data, suggesting a counterbalance for the defects in T cell function such as probable reduced stability in immune synapse formation due to the defective LFA-1 and impairment in adhesion and migration. These findings coupled with relatively mild phenotype in the patients compared to other types of LADs suggest that expression of LFA-1 is gravely compensated by other integrins, enabling the immune system to re-establish the balance to some extent.

Nevertheless, functional studies are required to confirm these findings and suggest therapeutic approaches.

7.1 Significance of Genetic Discoveries in Novel PID Subtypes

Different variants in genes whose defects lead to IEIs, could potentially result in different clinical phenotypes (Tangye et al., 2022). Consequently, it is important to report novel variants and their associated phenotype. In addition to broadening the phenotypic spectrum of an IEI, reporting novel variants could potentially shed light on the previously known function of the protein. Additionally, in the latest update of the IUIS for the IEIs, defects in *HYOU1* are classified as a phagocyte disorder. This study emphasizes on the

non-redundant role and importance of HYOU1 in development of B cell lineage and serves to broaden its clinical spectrum in the categorization of IEIs.

As discussed earlier, LADs are a group of genetic disorders resulting from components involved in leukocyte adhesion and migration to the site of infections. LADs types I-III are caused due to defects in *ITGB2*, *SLC35C1*, and *FERMT3*, respectively (Harris et al., 2013). Considering the crucial role of LFA-1 in cell adhesion and migration of leukocytes, as well as the results of our study which showed downregulation of motility-related pathways, defects in ITGAL could possibility be considered as a new type of LAD, expanding this category of IEIs.

7.2 Future Therapeutic Strategies for Defects in *HYOU1* and *ITGAL*

Therapeutic approaches for patients diagnosed with IEI are mainly general treatments for amplifying the function of immune system, such as antibiotic treatment, administration of G-CSF, or treatments aimed for compensating the role of defective immune component, for instance administration of IVIG in B cell deficiencies (Segundo & Condino-Neto, 2021). However, with the emergence and progress of precision medicine, more tailored therapeutic approaches are suggested. Targeted therapies for IEIs consist of three main categories: HSCT, gene therapy, and the application of biologics or compounds that target a particular cell function or immunological pathway (Pinto & Neves, 2022). Since there is currently little information available on gene therapy, HSCT is still regarded as the first line curative treatment for IEIs with severe clinical manifestation such as SCIDs and WAS (Pinto & Neves, 2022; Segundo & Condino-Neto, 2021). Stem cell gene addition therapy (GT) is a promising field of treatment for IEIs, as lentiviral are being used for GT in ADA deficiency, X-linked SCID, and WAS (Lankester et al., 2021; Segundo & Condino-Neto, 2021). Another field of rapidly emerging therapeutics is biologics and small molecules that target the immune pathway or the molecular defect (Pinto & Neves, 2022). Advancement in genomics and rapid discovery of genes render this possible as recognition of the specific gene to target, or inhibition of a certain molecule is pivotal to this process (Casanova & Abel, 2018; Pinto & Neves, 2022). This method is exemplified in utilizing Jak1/2-kinase inhibitors in patients with GOF variants in *STAT1* (Pinto & Neves, 2022).

Nevertheless, in severe forms of IEIs, HSCT is still considered the most promising and remains the only curative treatment (Arnold et al., 2021).

For the patient with defect in *HYOU1*, GT could be a potential treatment approach, especially in case the patient's condition exacerbates over time. However, we are far from standardizing GT for rarer forms of CIDs, since this therapeutic approach is only being tested for SCIDs (Pinto & Neves, 2022; Segundo & Condino-Neto, 2021).

With regards to therapeutic options for the patients with a nonsense variant in *ITGAL,* and consideration of their mild phenotype, such therapeutic approaches must be considered with caution, as HSCT and GT may not be cost beneficial considering their high risks of adverse events and cost. Functional studies are needed to validate our findings and may propose a possible pathway-related treatment approach.

7.3 Future Directions in Research Studying the Role of *HYOU1* and *ITGAL* in Inborn Errors of Immunity

The next step in expanding our understanding of HYOU1's role in immunity and IEI is verifying our finding using functional studying. We performed CRISPR-Cas9 in Ramos cell and generated six knock-in for the Pro444His variant in the HYOU1, yet none of the colonies survived even to a point that we could perform proliferation assays. The next possible approach could be performing CRISPR-Cas9 in pre-pro B cells, and differentiating them into pro B cells, or performing signaling assays to study the affected pathways in these cells. Moreover, it is noteworthy to repeat that attempts for generation of a null HYOU1 mice model have failed. The majority of studies of mice are done on heterozygous $HYOU1^{+}/HYOU1^{-}$ mice (Kusaczuk & Cechowska-Pasko, 2013). By using a $HYOU1^{LoxP/LoxP}$ model of mice we can better study the impact of HYOU1 on B cell development.

To better describe the impact of *ITGAL* defects on predisposition to wart, performing spatial single-cell RNA sequencing of EV lesions could also be informative of the type of immune cells recruited to the site of infection as well as the differences in gene expression consequent to this variant in *ITGAL*. Also, in our datasets, while proteomics showed downregulation of pivotal immune-related pathways and genes involved in these

pathways were upregulated in bulking RNA-seq and scRNA-seq, performing GSEA demonstrated that these pathways are not enriched on RNA level. Nonetheless, we must bear in mind that sampling was done in the absence of any major infectious episode. The next step in studying the role of ITGAL as an IEI, could be isolating T cells and neutrophils of the patients, and performing binding and signaling assays to study downstream process, followed by bulk RNA-seq and/or scRNA-seq and flow cytometry assays to study the impact of this variant on T cell priming and T cell activation. Adhesion assay and migration assay could also be performed to help this condition to be categorized as a LAD.

7.4 Conclusion

In this thesis, I describe two families with immune impairments, using a multiomics approach to identify the immune-related pathways and clarify molecular changes and mechanisms underlying their phenotypes. I sought to link genotype and phenotype through demonstrating impairments in physiological processes that ultimately result in clinical manifestations, which may overlap with other disorders despite having different underlying causes. The vastness of these underlying causes was reflected in the complex nature of the immune impairments of the two families I studied. This highlights the importance of a comprehensive approach composed of descriptive and functional assessments for uncovering the intricate molecular relationships between genotype and phenotype. Although the findings of multiomics studies need to be functionally verified to contribute to prognostic or therapeutic approaches, they offer a valuable starting point worthy of deeper investigation indicating a helpful direction for future functional studies. Eventually, this brings us closer to impactful clinical applications and targeted interventions, paving the way ‘from bench to bedside’.

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A multiomics approach to primary immunodeficiencies in human

Abstract:

Primary immunodeficiencies, or inborn errors of immunity, are a group of disorders caused by monogenic mutations in genes playing a key role in the development and function of the immune system. In this thesis, a multiomics approach was taken to study two genes associated with these conditions, further elucidating the mechanisms by which pathogenic variants impair the immune system. The first subject was *HYOU1*, defined as a gene whose defects cause primary immunodeficiency. We observed hypogranularity in the patient's neutrophils and revealed a maturation arrest in the B cell lineage before the pro-B cell stage. The second subject was *ITGAL*, a potential candidate gene not previously described in relation to primary immunodeficiencies. We demonstrated that the studied variant is inherited in an autosomal recessive pattern, and pathway analyses revealed impairment of multiple adhesion and motility-related pathways. Moreover, we showed an elevation in the expression of other integrins, suggesting a compensatory response to counterbalance the defective integrins.

Résumé :

Les déficits immunitaires primitifs sont un groupe de troubles causés par des mutations monogéniques dans des gènes jouant un rôle clé dans le développement et la fonction du système immunitaire. Dans cette thèse, une approche multiomique a été adoptée pour étudier deux gènes associés à ces conditions, afin d'élucider davantage les mécanismes par lesquels les variants pathogènes nuisent au système immunitaire. Le premier sujet était *HYOU1*, défini comme un gène dont les défauts causent un déficit immunitaire primitif. Nous avons observé une hypogranularité dans les neutrophiles du patient et révélé un arrêt de maturation dans la lignée des cellules B avant le stade des cellules B pro. Le deuxième sujet était *ITGAL*, un gène candidat potentiel non précédemment décrit en relation avec les déficits immunitaires primitifs. Nous avons démontré que le variant étudié est hérité selon un mode autosomique récessif, et les analyses de voies ont révélé un dysfonctionnement de plusieurs voies liées à l'adhésion et à la motilité. De plus, nous avons montré une élévation de l'expression d'autres intégrines, suggérant une réponse compensatoire visant à contrebalancer les intégrines défectueuses.