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Dissertation zum Erwerb des Doctor of Philosophy (Ph.D.) an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

Inborn errors of immunity predisposing to primary immunodeficiency and immune dysregulation caused by CD137 and RASGRP1 deficiencies

vorgelegt von

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Jahr

2022

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Date of defense:	May 9, 2022

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List of Abbreviations

ADA:	Adenosine deaminase
AICD:	Activation induced cell death
AIHA:	Auto immune haemolytic anemia
AKT:	Protein kinase B
ALPS:	Auto immune lymphoproliferative syndrome
ANA:	Anti-nuclear antibodies
aPAP:	Autoimmune pulmonary alveolar proteinosis
APC:	Antigen presenting cells
BFM:	Berlin-Frankfurt-Muenster
CADD:	Combined annotation dependent depletion
CAR:	Chimeric antigen
CD:	Cluster of differentiation
CD137L:	CD137 ligand
CTLA4:	Cytotoxic T-lymphocyte associated protein 4
CTPS1:	Cytidine 5-prime triphosphate synthetase 1
CFSE:	Carboxyfluorescein succinimidyl ester
CMV:	Cytomegalovirus
CSR:	Class switch recombination
CTL:	Cytotoxic lymphocyte
CVID:	Common variable immune deficiency
DAG:	Diacylglycerol
DCs:	Dendritic cells
DLBCL:	Diffuse large B-cell lymphoma
DN:	Double negative
DNA:	Deoxyribonucleic acid
EBV:	Epstein Barr virus
ERK:	Extra cellular signal-related kinase
ERT:	Enzyme replacement therapy
FTT:	Failure to thrive
cDNA:	Complementary DNA
gDNA:	Genomic DNA
GOF:	Gain-of-function
GDP:	Guanine diphosphate
GTP:	Guanine triphosphate
HD:	Healthy donor
HLH:	Haemophagocytic lymphohistiocytosis
HSCT:	Haematopoietic stem cell transplantation
HSV:	Herpes simplex virus
IEI:	Inborn errors of immunity
IFN:	Interferon
IL:	Interleukin
IL7R:	Interleukin-7 receptor
ITK:	Interleukin-2-inducible T-cell kinase
IVIg:	Intra venous immune globulin
IUIS:	International Union of Immunological Societies
ITP:	Immune thrombocytopenic purpura
LOF:	Loss-of-function
LPD:	Lymphoproliferative disease
LYST:	Lysosomal trafficking regulator
MEK:	Mitogen-activated protein kinase kinase
MAPK:	Mitogen-activated protein kinase
MFI:	Mean fluorescence intensity
MHC:	Major histocompatibility complex
MSD:	Matched sibling donor
MRI:	Magnetic resonance imaging
NEF:	Nucleotide exchange factor
NFkB:	Nucleotide factor kappa light chain enhancer of B-cells
NHL:	Non Hodgkin lymphoma

NK: Natural killer
PBMCs: Peripheral blood mononuclear cells
PCP: Pneumocystis carinii pneumonia
PD-1: Programmed cell death protein 1
PHA: Phytohemmagglutinin-L
PID: Primary immune deficiency
PMA: Phorbol myristate acetate
RAB27A: Ras-related protein 27A
RAF: Rapidly accelerated fibrosarcoma
RAS: Rat sarcoma virus
RASGRP1: Ras guanyl nucleotide-releasing protein 1
SCID: Severe combined immune deficiency
SCN: Severe congenital neutropenia
SH2D1A: SH2 domain containing 1A
SLE: Systemic lupus erythematosus
STXBP2: Syntaxin binding protein 2
TCR: T-cell receptor
TNFRSF9: Tumor necrosis factor receptor superfamily
TRAF: TNF receptor associated factor
TREC: T-cell receptor excision circle
TRG: T-cell receptor gamma
TTP: Thrombotic thrombocytopenia purpura
UNC13D: Unc-13 homolog D
VPD450: Violet cell proliferation dye 450
WAS: Wiskott Aldrich syndrome
WES: Whole exome sequencing
WGS: Whole-Genome sequencing
WT: Wild type

Cumulative thesis publication list

First Author Publications:

1. CD137 deficiency causes immune dysregulation with predisposition to lymphomagenesis.

Somekh I[*], Thian M[*], Medgyesi D, Gülez N, Magg T, Gallón Duque A, Stauber T, Lev A, Genel F, Unal E, Simon AJ, Lee YN, Kalinichenko A, Dmytrus J, Kraakman MJ, Schiby G, Rohlfs M, Jacobson JM, Özer E, Akcal Ö, Conca R, Patiroglu T, Karakukcu M, Ozcan A, Shahin T, Appella E, Tatematsu M, Martinez-Jaramillo C, Chinn IK, Orange JS, Trujillo-Vargas CM, Franco JL, Hauck F, Somech R[#], Klein C[#], Boztug K[#].

Blood. 2019;134(18):1510-1516.

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2. Novel Mutations in RASGRP1 are Associated with Immunodeficiency, Immune Dysregulation, and EBV-Induced Lymphoma.

Somekh I[*], Marquardt B[*], Liu Y, Rohlfs M, Hollizeck S, Karakukcu M, Unal E, Yilmaz E, Patiroglu T, Cansever M, Frizinsky S, Vishnvenska-Dai V, Rechavi E, Stauber T, Simon AJ, Lev A, Klein C, Kotlarz D[#], Somech R[#].

J Clin Immunol. 2018;38(6):699-710

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3. Exploring Genetic Defects in Adults who were Clinically Diagnosed as Severe Combined Immune Deficiency during Infancy.

Somekh I, Lev A, Lee Y, Hendel E, Barel O, AJ Simon, Somech R.

Immunol Res. 2021;69:145-152.

1. Introduction:

1.1 Genetic Diagnosis in Inborn errors of immunity

Inborn errors of immunity (IEI) predispose affected individuals to life-threatening infections, autoimmunity and malignant diseases (1). The increase in the diagnosis of monogenetic PIDs has been enormous, in scale with the advancement in genetic diagnostic tools, including Whole Exome Sequencing (WES) and Whole Genome Sequencing (WGS), which have nowadays become a common practice in the diagnosis of primary immune deficiencies (PIDs) (2). According to the most recent update by the International Union of Immunological Societies (IUIS) expert committee, 26 novel monogenetic gene defects causing immune deficiencies had been reported in the past year (3). In fact, during the past decade, monogenetic defects have more than doubled, from 191 IEIs identified in 2011 to 430 listed in 2019 (4). The advent of genetic diagnostic tools has enabled the exact genetic diagnosis, improving the understanding of molecular mechanisms, which gradually lead to tailored genomic personalized therapies, based on the impaired mechanisms (5). To achieve this progress, many steps were taken that shaped our understanding of PIDs as well as advanced diagnosis and treatment. An ample example of the progress made, is the treatment of Severe Combined Immune Deficiency (SCID), one of the prototypic PIDs. It had once been managed by providing a sterile environment and can today be cured by modes of hematopoietic stem cell transplantation (HSCT) (6-8), gene therapy (6,9,10) and enzyme replacement therapy (ERT) (6,11). Severe congenital neutropenia (SCN), discovered by Kostmann in 1956 was characterized as an autosomal recessive disorder involving a bone marrow failure and early onset life threatening bacterial infections (12). Fifty years later, the causative genetic defect in *HAX1* was identified by Klein et al, in descendants from the original family (13). Wiskott, in 1937, reported 3 brothers who were presented with thrombocytopenia, eczema, bloody diarrhea and recurrent ear infections (14). In 1954, Aldrich reported a similar phenotype with an X-linked recessive inheritance (15), later termed Wiskott-Aldrich syndrome (16-17). *WAS*, the causative gene was subsequently identified following sequencing members of the original family (18). These are a few examples of milestones that led the path to associate clinical phenotypes with monogenic PIDs.

1.2 Epstein-Barr virus associated PIDs and immune dysregulation

Epstein-Barr Virus (EBV), a common virus infecting humans, is mostly associated with asymptomatic infections or with infectious mononucleosis (IMN) syndrome, a self-limited disease with signs of fever, fatigue and lymphadenopathy (19). However, in PIDs, EBV is one of the major pathogens which can predispose to lethal infections and is associated with a wide scope of life-threatening conditions, including hemophagocytic lymphohistiocytosis (HLH), B-cell lymphoproliferative disease (LPD) and a chronic acute EBV infection of T cells. (19-20). EBV targets B-cell lymphocytes through the interaction of its major glycoprotein with CD21 (referred to as the "EBV receptor"). Under normal circumstances, T-cell mediated immune response is paramount in controlling the virus. Therefore, T-cell associated PIDs, with impaired T-cell development, activation or function may result in a massive proliferation of the virus in the host B-lymphocytes, eventually leading to B-cell lymphoproliferative disease, and in some cases to malignant disorders (21-23). Examples of such defects include monogenetic PIDs in *RASGRP1, ITK, CD27, CD70, CD137, CTPS1*. Impaired T-cell cytotoxic functions which are essential in eliminating the virus, can result in constant activation and proliferation of T-cells, with the secretion of pro-inflammatory cytokines including IFNγ, leading to macrophage activation and ultimately to hemophagocytic lymphohistiocytosis (HLH). Genetic defects predisposing to EBV-mediated HLH include *LYST, SH2D1A, STXBP2, RAB27A, STX11, UNC13D* (19). Identification of the genetic basis for PIDs and understanding the underlying mechanisms for immune deficiency and lymphoproliferation are important for early diagnosis, intervention and the detection of potential targets of antineoplastic medications (24-25).

2. CD137 deficiency – immune dysregulation and EBV-associated lymphomagenesis

2.1 Biology of CD137

CD137, originally cloned in 1989 (26), is a member of the TNF receptor superfamily (TNFRSF) of co-stimulatory receptors. It plays an intricate role in key processes of the immune system. It is induced on activated CD4+ and CD8+ T cells upon engagement of the T-cell receptor (TCR) by peptide-major histocompatibility complex (MHC) complexes (27-29). It is also expressed in regulatory T cells (Tregs) (30) and in follicular helper T-cells (Tfh) (31). However, it was later discovered to have roles in non-T-cells, as it is also expressed in activated natural killer (NK) cells (32), activated dendritic cells (DCs) (33-34), activated B-cells (35), and also in neutrophils (36), activated monocytes (37), eosinophils (38) and mast cells (39). Its ligand – CD137L – is expressed mainly by antigen presenting cells (APCs), including B-cells, macrophages, monocytes and DCs (40-41). Upon binding to its ligand, CD137 triggers TNF receptor-associated factor (TRAF) 2, leading to polyubiquitination of k-63 and activating NF-κB (42-45). The result is the promotion of T-cell proliferation and effector functions (46). Further features following CD137 signaling such as enhancement of cytotoxicity (47), mitochondrial capacity (48-49), prevention of activation-induced cell death (AICD) have also been shown (50). Its expression in activated B-cells is important, promoting B-cell proliferation and survival upon engagement with CD137L (35). These functions have made CD137 molecule an attractive therapeutic candidate and it has been utilized in cancer immunotherapy (51-54). It is also used in chimeric antigen receptor (CAR) T-cells as a co-stimulatory molecule to eliminate tumor B-cells in refractory acute lymphoblastic leukemia (ALL) patients (55-58).

Studies of murine models have shown CD137/4-1BB deficient mice to have features of auto-immunity along with dysregulated CD28-dependent T-cell proliferation (59-60). Core T-cell immune responses, including cytotoxic lymphocyte (CTL) mediated cytotoxicity are impaired in response to influenza virus, while reduced IFNγ levels in response to choriomeningitis virus were observed (61-63). These mice have been also shown to develop an SLE-like disease, characterized by auto-antibodies, skin lesions, renal disease and early mortality (64). All together, these have shown the role

of CD137 in immune homeostasis, and also as a potential therapeutic agent.

2.2 Identification of patient-associated mutations in CD137 locus – clinical phenotypes and genetic diagnoses

Patient 1: A 2-year-old male patient born to consanguineous 1st-degree cousins of Turkish origin, presented with a primary intestinal mass with metastases in the liver and kidneys, as was observed in MRI scans. He was diagnosed with Burkitt lymphoma. Medical history was also relevant for recurrent ear infections, hepatosplenomegaly and hypogammaglobulinemia, necessitating intravenous immune globulin (IVIg) treatment. The patient was treated according to Non-Hodgkin's lymphoma Berlin-Frankfurt-Muenster (NHL BFM) 2000 protocol with cyclophosphamide, dexamethasone, methotrexate and rituximab, due to CD20 positivity. The patient is currently in remission. WES employed, identified a large deletion in the *TNFRSF9* gene encoding the immune checkpoint molecule CD137/4-1BB (c.1_545+1716del). Genetic evaluation of the family members revealed an identical deletion in *TNFRSF9* in an asymptomatic 7-year-old sibling. Parents were found to be heterozygous carriers.

Patient 2: A 6-year-old male patient, from consanguineous 1st degree cousins of Palestinian origin, suffered from recurrent infections, mainly repeated episodes of pneumonia since the age of 3 years. At the age of 6 years, auto immune lymphoproliferative syndrome (ALPS)-like manifestations, including autoimmune hemolytic anemia (AIHA), immune thrombocytopenic purpura (ITP) and positive anti-nuclear antibodies (ANA) titers were present, along with enlarged lymph nodes and hepatosplenomegaly. The patient was treated with immune-suppressive agents, including sirolimus, cellcept (mycophenolic acid) and glucocorticoids. However, the patient developed an EBV-lymphoproliferative disorder (LPD) and subsequently was diagnosed with Hodgkin's lymphoma. He underwent genetic analysis with WES which revealed a homozygous missense mutation in *TNFRSF9/CD137/4-1BB* (c.452C>T). Genetic evaluation of family members found an eight-year old asymptomatic sibling who harbored an identical homozygous missense variant. Parents were heterozygous carriers for the *TNFRSF9* missense mutation.

Patient 3: An 11-year-old male patient of consanguineous (3rd degree cousins) Turkish parents

suffered from recurrent ear and respiratory infections from early childhood. At the age of 10 years, diffuse lymphadenopathy was noted along with hypergammaglobulinemia, and a positive direct coombs test. A sub-mandibular lymph node was biopsied and the patient was diagnosed with EBV-positive Hodgkin's lymphoma. The patient was treated according to the German Society of Pediatric Oncology and Hematology Hodgkin Lymphoma (GPOH HD) Trial 95 protocol (adriamycin, vincristine, etoposide, prednisone), and is currently in remission. WES revealed a splice-site mutation in *TNFRSF9* (c.101-1G>A) disrupting the splice-acceptor site of exon 3, causing exons 3 and 6 skipping, as revealed by cDNA sequencing results. Genetic evaluation of family members identified an identical homozygous mutation in an older sibling (a 14-year-old sister who suffered from recurrent tonsillitis until the age of 10 years).

Patient 4: A 33-year-old male patient of Colombian origin of non-consanguineous parents, who suffered from multiple episodes of pneumonia, recurrent sino-track infections, hypogammaglobulinemia necessitating IVIg treatment, and impaired humoral responses after vaccinations. He was clinically diagnosed with common variable immune deficiency (CVID). The patients underwent WES which identified a homozygous splice site mutation in *TNFRSF9* (c. 100+1G>A), disrupting the donor splice site, resulting in the skipping of exon 2, as revealed by cDNA sequencing. Patient mutations were confirmed by Sanger sequencing. Family members (parents and a sister) were heterozygous carriers. Patients' *TNFRSF9* mutations were predicted to be damaging, as evaluated by Combined Annotation Dependent Depletion (CADD), SIFT, and PolyPhen scores.

2.3 Impact of patient associated mutations on CD137 expression and implications for T-cell function

Due to its role in immune homeostasis, together with previous data in murine models, and also the shared clinical findings in the studied patients which included signs of immunodeficiency, autoimmunity and immune dysregulation, we hypothesized that the patients' mutations identified in *TNFRSF9* could account for the patients' phenotypes. In order to determine whether patient-associated mutations are deleterious for protein function, we first assessed the expression of CD137 on activated T-, B-, and NK- cells by flow cytometry:

a) To determine CD137 expression in activated T-cells, Patient and healthy donor (HD) control peripheral blood mononuclear cells (PBMCs) were stimulated (beads-coupled anti-CD3 Ab and soluble anti-CD28 Ab for 48 hours) and stained for activation markers (CD69, CD25) and CD137. We found CD137 expression to be abrogated in activated patient T-cells, confirming the lack of expression in patient T-cells.

b) CD137 expression in activated CD25+CD86+ B-cells was assessed by 24-hour stimulation of B-cells with anti-IgM + CD40L. CD137 expression was found to be impaired in activated patients' B-cells.

c) CD137 expression was also assessed in activated CD56+ NK cells upon 48 hours stimulation of PBMCs with IL-2, showing a lack of expression in patient activated NK cells.

   Lack of CD137 expression in these cell subsets were also demonstrated in asymptomatic siblings who harbored identical homozygous mutations.

   These findings, showing markedly reduced or abrogated expression in activated cells confirmed a loss of function of CD137 in the patients studied, and are in line with CD137 expressions in activated T- B- and NK- cell subsets.

2.4 The T-cell phenotype in *CD137* mutated patients

Next, we aimed to study core T-cell functions in the CD137-deficient patients. Since CD137 is a widely studied T-cell co-stimulatory molecule and has been shown to have a role in T-cell functions, including proliferation, activation, cytotoxicity, we assessed these functions in CD137 deficient patients and hypothesized that T-cell functions would be impaired. We examined core T-cell functions in patients' cells, including T-cell activation, T-cell proliferation, T-cell cytotoxicity, TCR repertoire, and TCR (T-cell receptor) signaling pathways. T-cell immune phenotyping was performed.

T-cell immune-phenotyping: Immune phenotyping showed normal CD4+ and CD8+ T-cell subsets in the patients studied. However, reduced subsets of Tregs (CD3+CD4+CD25+CD127-) were found. These findings are in line with its expression in Tregs, which was previously shown in-vitro (27), and could in part account for the autoimmune features which the CD137 deficient patients exhibited. We also assessed Tfh (CD3+CXCR5+CD45RO+) subsets in the patients. CD137 has been shown to be

expressed in Tfh cells (28), and B-cell functions were impaired in patients, as discussed further. We hypothesized that these subsets would be reduced in the CD137 deficient patients, since Tfh cells are known to provide co-stimulatory signals to B-cells through the interaction of the CD40 co-stimulatory molecule with its ligand on B-cells. Tfh subsets were found to be low in the CD137 deficient patients.

T-cell activation: We studied T-cell activation markers (CD69 and CD25) in patients PBMCs upon 48-hour stimulations with: a) anti-CD3 coupled beads, b) anti-CD3 + CD137L, c) anti-CD3 + soluble anti-CD28. We hypothesized that T-cell activation would be impaired in patients' T-cells in response to all stimulations. Furthermore, we presumed that upon addition of CD137L, an upregulation would be observed in HDs only, and not in CD137 deficient patients. We found an impaired T-cell activation in patients in response to anti-CD3 coupled beads, and also no effect upon the addition of CD137L. However, upon the addition of anti-CD28 co-stimulatory molecule, patient cells had a completely normal upregulation of activation markers, similar to HD cells. These findings are in line with the reported T-cell functions of CD137, and also could provide an explanation for patients' immunodeficiency signs. Remarkably, it was found that the addition of CD28, a T-cell co-stimulatory molecule, could completely compensate the lack of CD137 in T-cell activation.

T-cell proliferation: We aimed to assess T-cell proliferation in CD137 deficient patients, as previously studied in mice models. We assessed T-cell proliferation by labeling PBMCs with carboxyfluorescein succinimidyl ester (CFSE), or with CellTrace Violet Cell Proliferation dye (VPD450) and 96-hour stimulations with: a) anti-CD3 coupled beads, b) anti-CD3 + soluble anti CD137, c) anti-CD3 + neutralizing anti-CD137monoclonal antibody, d) anti-CD3 + OX40, and e) anti-CD3 + soluble anti-CD28. We hypothesized that proliferation would be compromised in patients' cells, that addition of CD137 neutralizing antibodies would affect proliferation in HD cells, and that functions could potentially be compensated upon addition of co-stimulatory molecules (OX40 and CD28).

Similar to T-cell activation assay results, patients' cells exhibited impaired proliferation capacity in response to a) anti-CD3 coupled beads, and b) anti-CD3 + soluble anti-CD137 stimulations, compared to HD T-cells. Upon blocking of CD137 by adding a neutralizing anti-CD137 monoclonal antibody (c), a dose-dependent reduction in T-cell proliferation was noted in HD T-cells, but not in patient

cells. Interestingly, upon addition of other co-stimulatory molecules, including OX-40 and anti-CD28, a partial and complete compensation of T-cell proliferation was observed in patient cells, respectively. These findings show impaired T-cell functions in patients, which are amenable to correction with the presence of T-cell co-stimulatory agents.

T-cell cytotoxicity (and NK-cell degranulation): NK-cell CTL functions were shown to be impaired in murine models. We therefore hypothesized these functions to be defected in the patients' studied. We assessed T-cell cytotoxicity in phytohemagglutinin-L (PHA)+IL-2 stimulated T-lymphoblasts, and NK-cell degranulation in IL-2 activated CD56+ NK cells, upon stimulation with K562 cells. We found no difference between patients' and HD cells as both showed similar T- and NK-cell degranulation capacities, measured by CD107a surface staining. Since the patients studied were found to have EBV persistence, including extremely elevated EBV viral loads and EBV-LPD, we aimed to check EBV-specific CTL cytotoxicity, assessed by the percentage of specific lysis of autologous EBV-LCLs by their respective effector CD8 T-cells. A significant reduction in EBV-specific CTL cytotoxicity was found in patient cells, which may suggest of EBV-susceptibility in these patients. Specific cytotoxicity impairment with normal general cytotoxicity, as found here has previously been shown in FERMT3-deficient patients (65).

TCR repertoire: Diversity of the TCR repertoire is central for T-cell function. Here, we addressed the question whether patient-associated mutations impact on TCR diversity. Analyzing the TCR repertoire with next generation sequencing (NGS) enables a high throughput and an extremely high resolution in its evaluation (66). We measured TCR repertoire utilizing NGS, employing primers in conserved regions (V and J genes) in the T-cell receptor gamma (*TRG*) locus. TCR repertoire analysis showed a restricted TCR diversity in patients' cells, with significant clonal expansions, showing a reduced diversity in the patients studied. These findings may further explain the propensity of CD137 patients to both viral and bacterial infections which were noted from early childhood, including with cytomegalovirus (CMV), herpes simplex virus (HSV), adenovirus, EBV, and streptococcus pneumonia; a reduced TCR diversity results in improper recognition of foreign antigens and a therefore weakened immune response. Moreover, clonal selections, as were demonstrated in CD137 patients having clonal expansions, could explain auto-reactive phenomena.

TCR signaling: The CD137 plays a role in several TCR signaling pathways, including NF-κB, pERK, PI3K/AKT signaling. The CD137 signalosome involves the engagement of TRAF proteins upon binding of CD137 to its ligand, leading to polyubiquitination of target proteins (k-63, k-48) and activation of NF-κB. We therefore assessed phospho-ERK, phospho-AKT and phospho-P65 levels in anti-CD3+anti-CD28 stimulated T-lymphoblasts, both by flow cytometry and by western blotting. We found no differences in these phospho-proteins in patients' cells, with a normal expression upon stimulation. The unharmed TCR signaling is not surprising, as TCR-mediated signaling is not directly affected by CD137, and could therefore be intact in CD137 deficient patient cells.

Restoration of T-cell functions: We showed impaired T-cell functions in the CD137 deficient patients studied, which in line with the shared clinical phenotypes of the patients, previous mice data resembling patient-clinical phenotypes of immunodeficiency, autoimmunity and immune dysregulation suggested the causative role of CD137 deficiency in the patients. We therefore aimed to restore T-cell functions by performing a gene-rescue experiment, with exogenous expression of wild-type (WT) CD137 in patient T-cells. Upon reconstitution of CD137 in patient cells, defected T-cell functions were restored, including anti-CD3 mediated T-cell activation (as measured by CD25 surface expression), as well as T-cell proliferation, upon anti-CD3 + anti-CD 137 stimulation (as measured by VPD450 dilution). These results lend additional evidence that the abnormal T-cell functions observed in the patients studied are caused by CD137 deficiency, and could be restored in-vitro.

2.5 The B-cell phenotype in *CD137* mutated patients

CD137 was previously thought of as a T-cell restricted molecule. However, its expression in additional cell subsets, including activated B-cells, suggested a wider role, including its function for B-cell survival and proliferation functions. We therefore assessed B-cell functions, including activation, proliferation, class switch recombination (CSR), and plasma blast differentiation. B-cell immune phenotyping was performed, measured by flow cytometry. We hypothesized B-cell function to be perturbed in a similar manner to T-cell functions mentioned above.

B-cell immune-phenotyping: Memory B-cells (CD19+CD20+CD27+), class switched B-cells (CD19+CD20+CD27+IgD-), and transitional B-cells (CD19+ CD38+IgM+) were measured by flow

cytometry, showing reduced memory and class switched B-cell subsets in patients, whereas increased transitional B-cells were noted.

B-cell activation: We aimed to assess B-cell activation in patients' PBMCs (expression of CD86 and CD25 in CD19+ B-cells) upon 24 hour stimulations with T-cell dependent stimuli (CD40L+IL-4), and T-cell independent stimuli (CpG). We found markedly reduced B-cell activation in patients' B-cells in response to both 17stimuli. Furthermore, sorted naïve B-cells were stimulated with T-cell dependent stimuli (CD40L+IL21) and T-cell independent stimuli (CpG), showing reduced activation in patients' B-cells in response to all stimuli. These findings show that the B-cell functional phenotype observed in the patients is both secondary to the T-cell dysfunction (evident by the reduced Tfh subsets and the impaired B-cell functions in response to T-cell dependent stimuli), but also a primary B-cell function (evident by the impaired CD137 expression in patients' B-cells, and abnormal functional B-cell responses in response to T-cell independent stimuli).

B-cell proliferation: B-cell proliferation (measured by CFSE or VPD450 dilution) was also determined upon T-cell dependent (CD40L+IL4 or CD40L+IL21) stimuli and T-cell independent (CpG) 72-hour stimuli. Proliferation was found to be decreased in patients' B-cells in response to all stimuli. Furthermore, proliferation of sorted naïve B-cells (upon stimulations with CD40L+IL4 and with CpG), were impaired in patients' cells. These findings, together with the lack of expression in patients' B-cells once again show that the B-cell phenotype is both T-cell dependent but also a primary B-cell defect in the *CD137* loss of function (LOF) mutations.

B-cell CSR: CSR, a B-cell specific rearrangement that results in the switching to antibodies of secondary isotypes (IgG, IgA, and IgE) was assessed as another B-cell functional output. Patient 4 was clinically diagnosed with CVID, while patients had recurrent infections which we therefore assessed CSR. We measured CSR in T-cell dependent and T-cell independent stimulated PBMCs (CD40L+IL4, CD40L+IL21 and CpG, respectively), as well as in stimulated sorted naïve B-cells by flow cytometric expression of IgA+IgG+ or CD19+CD27+IgD- B-cells, and found to be significantly reduced in patients' B-cells.

Plasmablast differentiation: In-vitro plasmablast differentiation (determined by measuring CD27+CD38+ expression in CD3-CD19+ B-cells, stimulated with CpG, CD40L+IL4, CD40L+IL21),

showed a significant reduction in CD27+CD38+ expression in patients' B-cells.

Together, these findings show a B-cell phenotype in CD137-deficient patients, with impaired activation, proliferation, CSR and plasmablast differentiation in response to both T-cell dependent and T-cell independent stimuli.

2.6 Summary and conclusions

To summarize, in this study we identified 4 patients with distinct mutations in *TNFRSF9* encoding the checkpoint T-cell co-stimulatory molecule CD137. Patients presented with recurrent infections, autoimmunity and EBV-associated lymphoproliferative disease. Interestingly, 3 siblings of 3 of the patients studied harbored identical homozygous mutations, however were very mildly symptomatic or asymptomatic, suggesting an incomplete penetrance in CD137 deficiency. Studying of the asymptomatic siblings found markedly reduced CD137 expressions in activated T- B- and NK- cell subsets, similar to the patients studied, while an intermediate functional T- and B-cell phenotype with a partial impairment in the functions assessed. There is currently no single factor that has been identified to drive the phenotype in the patients while having a much weaker impact in the affected siblings. An incomplete penetrance has been previously described in PIDs with immune dysregulation such as CTLA4 haplo-insufficiency (67-68). Core T- and B- cell functions were perturbed in the patients' studied. Remarkably, T-cell functions were partially or completely restored following the addition of other co-stimulatory molecules, including OX-40 and CD28, respectively. Finally, impaired T-cell functions (activation and proliferation) were rescued following exogenous expression of CD137 in patient T-cells. Together, our findings (69) and also the findings of Alosaimi et al (70) which reported 2 patients which harbored *TNFRSF9* mutations with similar clinical findings and those of Rodriguez et al (71), which characterized a patient with concomitant *PIK3CD* and *TNFRSF9* mutations which caused chronic active EBV infection of T-cells lend evidence of this recently identified PID.

3. RASGRP1 deficiency – immune dysregulation and EBV-induced lymphoma

3.1 RASGRP1 biology

RAS are tightly regulated proteins which cycle between "active" mode when they are bound to guanine triphosphate (GTP), and "inactive" mode when bound to guanine diphosphate (GDP) (72). Loss of regulation predisposes to RASopathies. RAS guanyl nucleotide exchange factors (RasGEFs) play a critical role in the regulation of these processes, by catalyzing the release of GDP, thereby stabilizing RAS in a nucleotide-free state in which it can associate with GTP to an "active" mode (73-74). Ras guanyl nucleotide-releasing protein 1 (RASGRP1), a member of the RAS superfamily, is a nucleotide exchange factor containing a diacylglycerol (DAG)-binding C1 domain. It activates Ras through the exchange of GDP to GTP (75). This enables the activation of the RAS-RAF-MEK-ERK signaling pathway, a core pathway in the immune system, with a role in the development of B- and T-cells (76). *Rasgrp1*-/- mice were first reported in 2000 by Dower et al. to have impaired thymocyte development, defective thymocyte differentiation, abnormal proliferation, and impaired signaling, disturbing RAS-RAF-MEK-ERK signaling pathway (77). Further murine studies showed that RASGRP1-deficient mice had impaired T-cell development, defective positive selection of T-cells, reduced αβ T cells, CD4 T-cell lymphopenia, auto immune features, including an SLE-like disease or autoimmune pulmonary alveolar proteinosis (aPAP). Increased auto reactive CD4+ T-cells were found with an excessive production of auto-antibodies (78-81). Interestingly, RASGRP1 expression was found to be reduced in patients with SLE (82-83) and with rheumatoid arthritis (84). Germline mutations in *RASGRP1* were found to cause CD4 T-cell lymphopenia, impaired T- B- and NK-cell functions, defected cytoskeletal dynamics and an EBV LPD (85-88).

3.2 Identification of patient-associated mutations in RASGRP1 locus – clinical phenotypes and genetic diagnoses

We studied 3 patients from 2 consanguineous families. Clinical characteristics were as follows:

Patient 1: A 14-year-old female of Turkish origin, presented with recurrent respiratory infections

since infancy, and a herpes zoster infection in early childhood. Failure to thrive (FTT) was also noted. Family history was relevant for two siblings who died during infancy from pneumonia. Initial laboratory workup revealed a persistent CD4 T-cell lymphopenia from the age of 3 years. At the age of 6 years, the patient appeared with signs of fatigue, weight loss, lymphadenopathy, hepatosplenomegaly, and a subscapular mass. Biopsy of the subscapular mass revealed an EBV-positive diffuse large B-cell lymphoma (DLBCL) on histopathology. The patient was treated with chemotherapy (NHL BFM protocol). The patient responded to treatment achieving a remission, however, she experienced two relapses and subsequently underwent an allogeneic HSCT from a matched sibling donor (MSD) at the age of 8 years. In light of consanguinity, family history, clinical signs and CD4 T-cell lymphopenia, the patient underwent WES revealing a homozygous inversion mutation in *RASGRP1* (c.649_650inv, p.E217R). Parents were found to be heterozygous carriers.

Patients 2-3: Patients 2 and 3, male 1st degree cousins from a Palestinian origin, presented during infancy with severe coombs positive hemolytic anemia and immune thrombocytopenia, and were diagnosed with Evans syndrome. They were treated with glucocorticoids, blood transfusions and IVIg. The patients suffered from recurrent pyogenic and viral infections, including persistently elevated EBV viral load titers (up to 1 million copies/ml). Clinical signs of autoimmunity were noted in both patients, including lymphadenopathy and hepatosplenomegaly, as well as auto-immune hepatitis, severe posterior uveitis and an elevated ANA titer in patient 3. Patient 2 developed thrombotic thrombocytopenia purpura (TTP) including seizures and coagulopathy, which he succumbed to at the age of four years. Patient 3 was diagnosed with DLBCL at the age of 3 years, treated according to R-CHOP and currently in remission. The patients underwent WES identifying a homozygous 4-nucleotide deletion resulting in a frameshift in *RASGRP1* (c.1111_1114del, p.D371Ifs*7). Patients' mutations were verified by Sanger sequencing. Parents were found to be heterozygous carriers.

3.3 Impact of patient-associated mutations on RASGRP1 expression and function

In light of the clinical phenotypes in the patients studied, including CD4 T- cell lymphopenia, recurrent infections, autoimmunity and LPD, in concordance with previously reported data in RASGRP1-deficient patients and *rasgrp1-/-* mice, we hypothesized that the *RASGRP1* mutations

identified in the patients studied account for the clinical phenotype. We therefore initially tested RASGRP1 expression in cell lines expressing patients' mutations, RAS-RAF-MEK-ERK signaling pathway, and RASGTPase activity.

RASGRP1 expression: RASGRP1 knockout Jurkat T-cell line overexpressing WT RASGRP1 and patients' variants were generated, showing an abrogated expression of RASGRP1 in the D371Ifs*7 mutation (patients 2 and 3 variant) by immune-blotting.

Signaling: The RAS-signaling pathway was checked (upon PMA stimulation with assessment of phospho-ERK). We hypothesized that pERK levels would be reduced in patients' mutations, in line with previous reports in RASGRP1 deficient humans and murine models. Phospho ERK was perturbed in cells expressing patients' mutations (E217R and D371Ifs*7), confirming a LOF.

GTPase activity: An active RAS-GTPase pull-down assay was performed (Jurkat cells overexpressing patients' mutations and WT-RASGRP1), showing reduced levels of RAS-GTPase activity in cells overexpressing the patients' mutations, as compared with WT.

3.4 T-cell phenotype in *RASGRP1* mutated patients

RASGRP1-deficient patients and *rasgrp1-/-* mice were reported to have T-cell developmental defects affecting T-cell fractions (CD4 T-cell lymphopenia and inverted CD4:CD8 ratio) and functions (77-79). We screened T-cell functions in the RASGRP1-deficient patients, including T-cell receptor excision circle (TREC) levels, T-cell proliferation, and TCR repertoire which we hypothesized would be impaired in the RASGRP1-deficient patients.

Immune-phenotyping: Immune phenotyping performed in patients' PBMCs revealed a marked CD4 T-cell lymphopenia, with high levels of CD8 T-cell subsets, and an inverted CD4:CD8 ratio. B-cell and NK-cell numbers were normal. These findings resemble mouse studies.

T-cell proliferation: T-cell proliferation (assessed by thymidine incorporation upon stimulations with anti-CD3 and PHA) showed a markedly reduced T-cell proliferation in patients.

TCR assessment: Murine models demonstrated the central role of RASGRP1 in T-cell development including pre-TCR signaling and positive αβ T-cell selection, with reduced αβ T-cells while elevated γδ CD8 T-cell subsets were reported (79). We therefore assessed TCR re-arrangement in the

RASGRP1-deficient patients by evaluating TREC levels, and TCR repertoire. Single joint TREC copy numbers were checked by real-time PCR (qRT-PCR) from gDNA of patients. TREC levels in patients 2 and 3 were markedly reduced compared to HDs. Further, we evaluated TCR repertoire (Patients 2 and 3), first by the standard vβ surface expression (flow cytometric analysis). Patients were found to have abnormal clonal expansions. Patient 2 had a clonal expansion of vβ13.1 and under expression of vβ 1, 2, 4, 5.1, 5.3, 7.2, 12, 13.2, 14, 16, 18, 21.3, while Patient 3 had an over expression of vβ 1, 7.1, 8, 12, 21,3 and under expression of vβ 4, 5.2, 7.2, 13.2, 13.6, 14. 16, 17, 22). We also assessed TCR repertoire utilizing NGS, employing primers in conserved regions (V and J genes) in the T-cell receptor gamma *(TRG)* locus. TCR repertoire analysis showed a reduced TCR diversity in patients' cells, with significant clonal expansions. Simpson's D-diversity index score (evaluating repertoire diversity), was significantly reduced in patients 2 and 3, while Shannon's H' index score, which assesses repertoire-evenness (89) was significantly higher in patients 2 and 3. These findings show an impaired TCR repertoire in the RASGRP1-deficient patients with restricted diversity and clonal expansions, providing additional insights to RASGRP1-deficient patients' susceptibility to viral and bacterial infections, and autoimmune features with presence of auto-antibodies. These manifestations are not only a result of reduced CD4 T-cell numbers, but also their restricted repertoire.

3.5 Summary and conclusions

In summary, we identified 3 patients from two pedigrees harboring 2 distinct mutations in the Ras activating nucleotide exchange factor, *RASGRP1*. Patients' mutations were validated by Sanger sequencing, and found to affect RASGRP1 expression in heterologous Jurkat T-cell models, also reducing Ras-GTPase activity and abrogating phospho-ERK upregulation upon PMA stimulation. A dominant T-cell phenotype was observed, including CD4 T-cell lymphopenia, inversion of CD4:CD8 T-cell ratio, impaired T-cell proliferation, abnormal TREC levels, high TCRγδ subsets, and an abnormal TCR repertoire with a restricted diversity. Clinically, patients presented with recurrent infections, features of autoimmunity including auto-antibodies, and an EBV-LPD including high EBV viral loads. 2 patients developed DLBCL and one patient deceased at the age of 4 years.

These resemble findings in RASGRP-deficient mice, which show a prominent T-cell phenotype,

including CD4+ T-cell lymphopenia, with expansion of CD8+ T-cells, low TCRαβ T-cells with an increased subset of TCRγδ cells, impaired ERK signaling and features of autoimmunity. Moreover, RASGRP1-deficient patients showed similar clinical findings including recurrent infections, autoimmune features, enlarged lymph nodes, hepatosplenomegaly and an EBV-LPD, along with CD4-T cell lymphopenia and perturbed ERK signaling. Our findings extended the clinical, genetic and immunological scope of this recently described PID (90).

4. Exploring genetic defects in adults who were clinically diagnosed as SCID during infancy

In this study, we studied two adult patients, who were diagnosed with SCID during infancy and treated with a MSD HSCT, by performing WES and identifying the genetic defects underlying their PID. We attempted to highlight the importance of achieving a genetic diagnosis in aspects of treatment, surveillance, understanding disease mechanisms, and also future planning.

4.1 Clinical phenotypes and genetic diagnoses

Both patients studied are from consanguineous parents of Israeli origin.

Patient 1: A male patient, currently 19 years old, presented during infancy with recurrent infections (respiratory and gastro-intestinal), including opportunistic infections with a pneumocystis jiroveci pneumonia, and a candida albicans fungemia. FTT, lymphadenopathy and a diffuse seborrheic dermatitis were also noted. Laboratory workup revealed T-cell lymphopenia and hypogammaglobulinemia. He was diagnosed with SCID and underwent a full matched non-conditioned sibling donor HSCT at the age of 6 months. At the age of 17 years, WES was performed revealing a homozygous missense mutation in *IL7RA* (c.417T>C; p.L14S). The mutation was verified by Sanger sequencing.

Patient 2: A female patient, currently 24 years old, presented during infancy with recurrent infections, FTT and a generalized erythematous rash. Family history was relevant for a sibling who died at the age of 4 years. On laboratory workup T- and B-cell lymphopenia as well as hypogammaglobulinemia were noted. The patient was diagnosed with SCID, and underwent an allogeneic non-conditioned full MSD HSCT at the age of 3 years, with complete engraftment. The patient has since had an infection with varicella, recurrent episodes of pneumonia and developed bronchiectasis. T- and B-cell counts are low and the patient is treated with IVIg and prophylactic antibiotic therapy. At the age of 22 years, the patient underwent WES identifying a homozygous missense mutation in *RAG2* (c.283G>A; p.G95R). The mutation was verified by Sanger sequencing.

4.2 Immune workup – post transplantation

The patients underwent immune workup during the time period after HSCT. Patient 1 has had normal TREC levels, TCRvβ repertoire, intact proliferative responses and also normal B-cell counts. These confirm a good cellular and humoral immune reconstitution in the patient. In contrast, Patient 2 has consistently found to have low T- and B-cell counts, abnormal TREC levels, and subnormal proliferative responses in response to mitogen stimulations, which are in line with a partial immune reconstitution, requiring routine administration of IVIg replacement therapy and prophylactic antibiotics.

4.3 Significance of retrospective genetic diagnosis

As described above, the underlying genetic diagnosis was made many years after patients were clinically diagnosed and treated. The importance of achieving a genetic diagnosis has several aspects**:**

a. Disease understanding and prediction of outcome: SCID is one of the hallmarks of PID affecting the cellular and the humoral immune system (91). Patients are prone to severe infections, including opportunistic infections which put them at risk. Therefore, definitive early treatments with HSCT (92-93), or ERT (94-95), or gene therapy (96-98) are required. Survival rates have increased to above 90% when HSCT is done in the 1st year of life (99). Several monogenetic defects are known to cause SCID; *IL7RA* (encoding IL7R protein), is known to cause a T-B+NK+ SCID (100-101). B-cell functions are typically preserved in these patients. *RAG1* and *RAG2* LOF mutations were found to cause a T- B-NK+ SCID (102-104). T-lymphocyte development is arrested in DN2/DN3 stages. These cells compete with donor lymphocytes, therefore preventing a smooth cellular reconstitution, as observed in patient 2. Therefore, conditioned HSCT to clear the occupying cells is important for achieving an intact immune reconstitution (105-106). Conditioning regimens do have major multi-systemic side effects which should be taken into account. Determining a genetic diagnosis, as in these cases enables us to understand the present clinical and immune phenotype of the patients, and also to predict future outcomes.

b. Genetic counseling and future family planning: Monogenic PIDs may also impact future descendants, particularly in cases of consanguineous marriages. Therefore, establishing a genetic

diagnosis is crucial in estimating future recurrences in the family. This information may provide parents proper tools in future family planning and obtaining prenatal genetic diagnostic tests as needed.

c. Surveillance in affected patients and family members: Establishing an exact diagnosis is helpful in surveillance, both pre- and post-HSCT. For example, in ADA-deficient SCID patients, extra-hematological manifestations are not corrected by HSCT and therefore require routine checkups (107). Another critical factor is to determine the genetic status of a donor prior to HSCT, particularly a MSD which might harbor the identical genetic defect and is asymptomatic at the time of HSCT.

d. Personalized therapeutic options: Nowadays, the general aim is to provide personalized, tailored therapy based on the patient's genetic status. Such examples include: a) gene therapy, for example in SCID cases, as recently reported for infants with X-linked SCID (98); b) targeted therapy aimed at the molecular defect. One such example, are CD137 monoclonal antibodies (urelumab and utomilumab) which are currently tested in cancer immune therapy trials (108-109); c) enzyme replacement therapy, in cases of a missing enzymatic defect, as in ADA-deficient SCID patients (94-95).

e. Psychological and ethical aspects: Identification of a genetic diagnosis may as well be accompanied by psychological and ethical considerations. On the one hand, there is a clear advantage in achieving a genetic diagnosis on the medical management**.** On the other hand, psychologically an asymptomatic carrier status may cause anxiety for the individual and even a stigma, especially in cases of matchmaking. These are also important considerations.

4.4 Summary and conclusions

Herein, we studied two SCID patients that were genetically diagnosed years after being clinically diagnosed and treated with HSCT, showing the impact of genetic diagnosis, also in the case of the studied patients, but also on a wider perspective. This study shows how establishing a genetic diagnosis and genomic personalized medicine impact the whole landscape in the understanding and treatment of disease, in all layers.

Summary and perspective

The main theme of this work has been the identification of novel genetic defects causing primary immunodeficiencies and immune dysregulation.

We described 3 patients from two consanguineous families who were found to have homozygous mutations in the Ras nucleotide exchange factor, RASGRP1, perturbing a core and tightly controlled pathway, the Ras-pathway (72, 76). All patients had a prominent T-cell phenotype, including CD4 T-cell lymphopenia, impaired T-cell functions and a restricted TCR repertoire. Patients presented with recurrent infections, auto immune features and developed lymphoproliferative disorders. These findings resembled *rasgrp1-/-* mice which were found to have SLE-like features with auto-antibodies and lymphoproliferation (77-81). The RAS-RAF-MEK-ERK pathway is a well-studied pathway. Activating mutations in this process (e.g., HRAS, KRAS, NRAS and others) have been described in malignant cells (76). Interestingly, upon the inactivation of this pathway, as shown above in *RASGRP1* LOF mutations, demonstrated by markedly reduced phospho-ERK levels, patients developed a LPD, however in a different mechanism. While activating mutations lead to enhanced signaling and survival and ultimately lead to cancer, here, the defective T-cell functions which cause an inability to regulate EBV, eventually leads to LPD. This is an example of how two opposing processes may lead to similar outcomes.

In CD137 deficient patients, a similar picture was observed. We described 4 patients from 4 families harboring distinct mutations in *TNFRSF9/4-1BB/CD137*, encoding the T-cell co-stimulatory molecule, CD137. Patients had impaired T-cell functions including activation, proliferation, cytotoxicity and a restricted repertoire. However, the immune phenotype was not only restricted to T-cells, but also involved B-cell functions which were impaired, both in a T-cell dependent and T-cell independent manner. Patients' presented with recurrent infections, autoimmunity and developed an EBV-associated LPD. Interestingly, 3 siblings (in 3 of the families) harbored identical homozygous mutations in *TNFRSF9* with a lack of expression in CD137, and an intermediate T- and B- cell phenotype, however without clinical symptoms, showing an incomplete penetrance in this genetic defect. We currently do not know the reason for the incomplete penetrance. We did check expression of various immune checkpoints, past exposure to EBV (serology and viral loads), however we could not pinpoint a single

factor responsible for driving the patients phenotype while not affecting asymptomatic siblings with identical *TNFRSF9* mutations. We also demonstrated that anti-CD28 co-stimulation in-vitro resulted in correction of perturbed T-cell functions (while the addition of other co-stimulatory molecules, such as OX-40, resulted in partial compensation). This may highlight the strong co-stimulatory effects of CD28, which was developed as a therapeutic drug (theralizumab / TGN1412) and was withdrawn after inducing severe inflammatory effects and multi-organ failure (110-111). CD137 is currently in use in the field of onco-immunology in CAR-T cell constructs (55-58) and also monoclonal antibodies in cancer clinical trials (51-54, 108-109).

In the last project described herein, we studied two SCID patients who underwent an unconditioned MSD HSCT during infancy. Two decades after they were diagnosed and treated, the patients underwent WES identifying the genetic defect causing their PID (*IL7RA* in Patient1 and *RAG2* in Patient 2). The study demonstrated the significant advancement in the field of PIDs and genetic-diagnoses, which enable today to strive to provide tailored therapeutic regimens based on the individuals genetic status. Of the two patients studied, Patient 1 has a good immune reconstitution with normal T- and B- cell counts and function, while Patient 2 has low T- and B- cell counts, requiring IVIg and prophylactic antibiotics. We can currently understand the compromise in the long-term immune reconstitution in Patient 2, as RAG2 deficient patients require conditioned HSCT's to eliminate cells which compete with donor cells and result in an abnormal immune reconstitution (105-106). This study also emphasized the significance of obtaining a genetic diagnosis for future family planning, for disease surveillance and expected outcomes. Last, the study depicts ethical, psychological and social issues which are important to be considered: For example, identifying asymptomatic carriers in an autosomal recessive disorder on the one hand may result in anxiety and also a social "stigma", which could impact matchmaking, while on the other hand the identification of asymptomatic carriers may exclude a homozygous disease-prone genetic status, diminish anxiety, as the individual would not need to be as concerned of developing a future phenotype and would also reduce the need for surveillance.

All together, the above studies demonstrate the identification of novel genetic defects causing PIDs and presdisposing to immune dysregulation, and EBV-associated LPD. These studies have a role in

the understanding of mechanisms involved in disease, enabling development of tailored therapies, and also the perspective of genetic diagnoses and family counseling.

Own contributions

CD137 deficiency and predisposition to lymphomagenesis

In the above-mentioned study, I contributed to the analysis of WES data finding the *CD137* genetic defects in patients 1 and 2. I supervised medical care of patient 2 and obtained cellular and genetic material needed for the studies. Similarly, for patients 2, 4, and patients 1 and 3. I analyzed and interpreted clinical data for all patients, including imaging and pathology. I established the CD137 protein expression assays in stimulated T- B- and NK-cells, demonstrating the impaired expression in patients 1, 2 and 4. I carried out T- and B- cell activation and proliferation assays in patients 1, 2 and 4 in response to various stimuli, finding the effects of CD28 co-stimulatory rescue of the T-cell defects in patients. I generated T cell lymphoblasts from patients' 1, 2 and 4 PBMCs and performed phospho-signaling assays (pERK, pAKT, NFkB). I generated EBV-LCLs from patients 1, 2 and 4 and performed signaling assays. I performed NK-cell degranulation and T-cell cytotoxic tests for patients 1, 2 and 4. I wrote the manuscript together with Marini Thian, Raz Somech, Christoph Klein and Kaan Boztug.

RASGRP1 novel mutations and EBV-associated lymphomagenesis

In the above-mentioned study, I contributed to the analysis of WES data finding the *RASGRP1* genetic defects in patients 2 and 3. I obtained cellular and genetic material from patients 2 and 3, analyzed clinical data for all patients, including imaging and pathology. I analyzed immunological data for patients 2 and 3, including T-cell proliferation, TCR repertoire by flow cytometry and NGS. I prepared the Tables and Figures of Patients 2, 3. I wrote the initial draft manuscript with Raz Somech and revised the manuscript together with Benjamin Marquardt and Daniel Kotlarz.

Exploring genetic defects in adults who were clinically diagnosed as severe combined immune deficiency during infancy

I initiated the study together with Raz Somech. I analyzed patients' historical medical records, clinical and laboratory data, genetic results and immune data. I wrote the manuscript together with Raz Somech.

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Brief Report

IMMUNOBIOLOGY AND IMMUNOTHERAPY

CD137 deficiency causes immune dysregulation with predisposition to lymphomagenesis

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KEY POINTS

- **CD137 deficiency is a novel inborn error of immunity with immune dysregulation and EBV-associated lymphomagenesis.**
- **Our study highlights the key role of CD137 for immune homeostasis with relevance to immunodeficiency and cancer immunotherapy.**

Dysregulated immune responses are essential underlying causes of a plethora of pathologies including cancer, autoimmunity, and immunodeficiency. We here investigated 4 patients from unrelated families presenting with immunodeficiency, autoimmunity, and malignancy. We identified 4 distinct homozygous mutations in *TNFRSF9* encoding the tumor necrosis factor receptor superfamily member CD137/4-1BB, leading to reduced, or loss of, protein expression. Lymphocytic responses crucial for immune surveillance, including activation, proliferation, and differentiation, were impaired. Genetic reconstitution of CD137 reversed these defects. CD137 deficiency is a novel inborn error of human immunity characterized by lymphocytic defects with early-onset Epstein-Barr virus (EBV)-associated lymphoma. Our findings elucidate a functional role and relevance of CD137 in human immune homeostasis and antitumor responses. (*Blood*. 2019;134(18):1510-1516)

Introduction

Antigen receptors and associated signaling machineries function to sense and rapidly react to threats that endanger the organisms such as foreign invaders or altered self-structures. Coreceptors play fundamental roles in regulating and fine-tuning the signal strength of antigen receptors. Defective function of these immune receptors may lead to elevated susceptibility to infections, autoimmune manifestations, and cancer.[1] Epstein-Barr virus (EBV) is one of the most prevalent viruses that infects humans and maintains lifelong latency.[2,3] In individuals with impaired T-cell immunity, EBV infection may result in lymphoproliferative disease, or malignant lymphomas of T-, B-, or natural killer (NK)-cell origin.[3] To date, germline genetic mutations affecting *CD27*, *PRKCD*, *RASGRP1*, *MAGT1*, *SH2D1A*, *ITK*, and others have been identified to cause immune dysregulation with EBV-associated diseases.[2] Studies of these patients have provided mechanistic insight into pathways required for robust host immune surveillance against EBV infection and associated lymphomas. Here, we report an inborn deficiency of tumor necrosis factor (TNF) receptor superfamily member 9 (TNFRSF9)/CD137/4-1BB with marked immune dysregulation and predisposition to EBV-associated lymphoma.

Study design

Patients

The study was approved by the institutional review boards of the Medical University of Vienna (Vienna, Austria; EK499/2011),

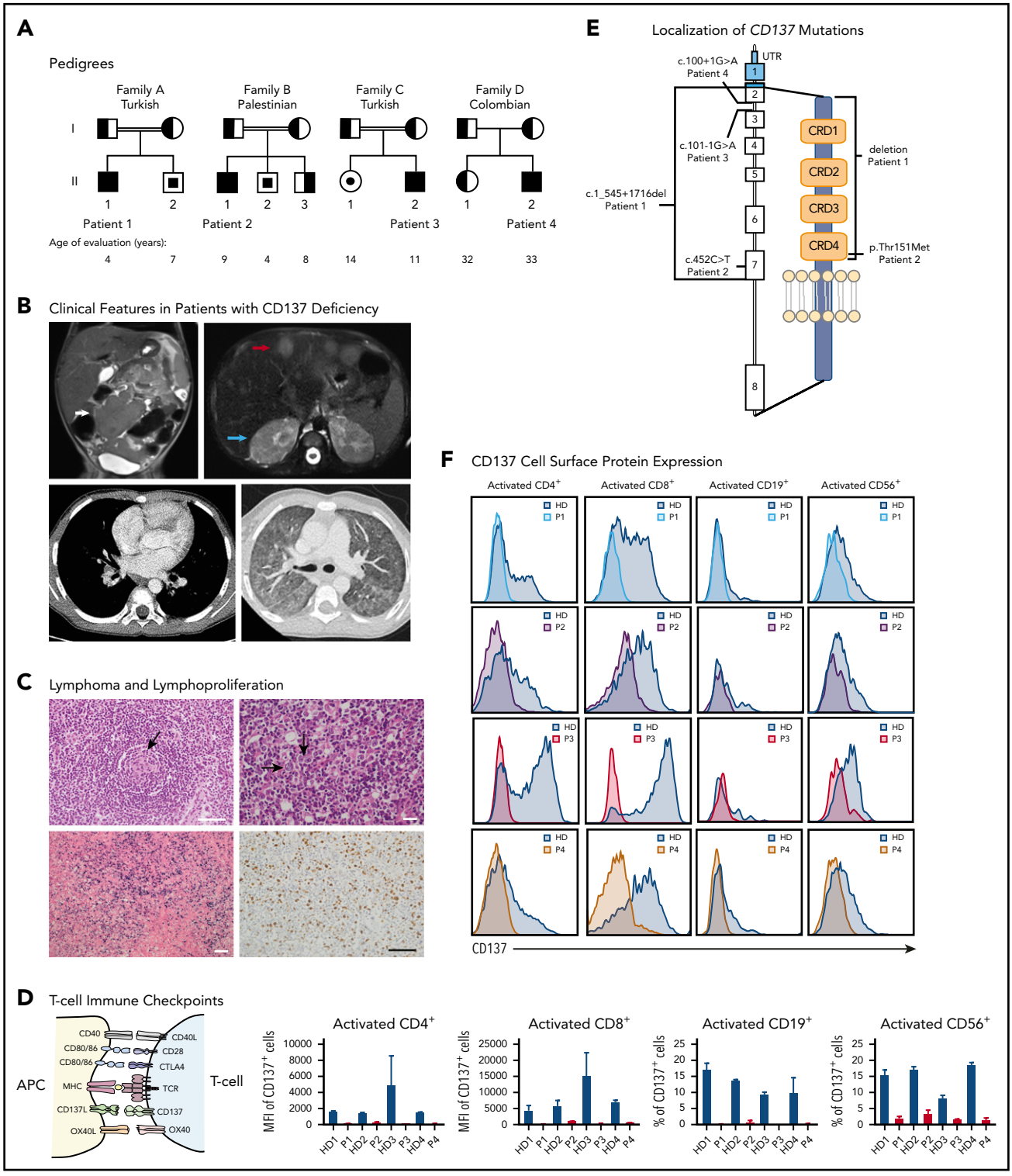

Figure 1. Genetic and clinical presentation of 4 patients with immune dysregulation. (A) Pedigrees showing 4 families with affected individuals (patients) harboring *TNFRSF9* mutations. Solid symbols indicate affected persons who were homozygous for the mutant allele; half-solid symbols, heterozygous persons; center solid symbols, unaffected persons homozygous for the mutant allele; circles, female family members; squares, male family members; double lines, consanguinity. (B) Radiographic features in patients with CD137 deficiency. Top left, Coronal T2-HASTE MR demonstrating a lobulated mass of the small intestine (white arrow) in patient 1 (P1). Top right, axial T2-HASTE STIR MR image of upper abdomen depicting hyperintense multiple metastatic liver nodules (red arrow) and bilateral renal cortical hypointense metastatic nodules (blue arrow) in patient 1. Bottom left, Chest computer tomography (CT) scan indicating right-sided hilar lymph nodes in patient 3 (P3). Bottom right, chest CT scan revealing bilateral ground-glass opacities in patient 2 (P2). (C) Top left, sections of right inguinal lymph node tissue (hematoxylin-and-eosin staining) showing numerous small regressive germinal centers (black arrow) in patient 2 (scale bar, 10 μm). Bottom right, Ki-67 immunostain in patient 2, positive in 20% to 30% of the cells exhibiting a moderate lymphocyte proliferative activity (scale bar, 10 μm). Bottom left, in situ hybridization for EBV-encoded small RNAs (EBER) displaying numerous positive cells in blue in patient 3 (scale bar, 200 μm). Top right, Multinucleated Reed-Sternberg cells (black arrows) typical of Hodgkin lymphoma (scale bar, 20 μm) in a resection of a submandibular lymph node in patient 3. (D) Schematic illustration displaying the interaction of ligands expressed on antigen-presenting cells (APCs) with their respective receptors on activated T cells, including the interaction

the Ludwig Maximilian University of Munich (Munich, Germany), Sheba Medical Center (Tel HaShomer, Israel), and the Baylor University College of Medicine (Houston, TX). All study participants provided written informed consent. Informed assent was obtained for children.

Experimental methodologies

All methods are detailed in the supplemental Materials and methods (available on the *Blood* Web site).

Results and discussion

Clinical phenotypes

We studied 4 patients from 4 unrelated families, 3 of whom were from consanguineous background (Figure 1A). Patients had early childhood recurrent infections of bacterial and viral origin, and signs of autoimmunity (Figure 1B-C; supplemental Figure 1; supplemental Table 1). Sinopulmonary and herpes virus infections were common. All patients exhibited hepatomegaly, splenomegaly, and/or lymphadenopathy. Signs of autoimmunity, including hemolytic anemia, were present in some patients. We found abnormal immunoglobulin levels in all patients (supplemental Table 2). Patients 1 and 3 developed EBV-related B-cell lymphoma, patient 2 had an autoimmune lymphoproliferative syndrome–like phenotype, and patient 4 was diagnosed as having common variable immune deficiency. Therapeutic regimens included chemotherapy, immunosuppression, antibiotic prophylaxis, and regular immunoglobulin substitution (supplemental Table 1).

Genetic evaluation and loss-of-function mutations in *TNFRSF9*

To elucidate the disease etiology, we performed whole-exome sequencing and identified distinct homozygous variants in *TNFRSF9* encoding the costimulatory immune checkpoint CD137/4-1BB (Figure 1D-E; supplemental Figure 2; supplemental Table 3). Parents were heterozygous carriers in all cases. All *TNFRSF9* variants were absent in gnomAD, and predicted to be deleterious using common prediction algorithms (supplemental Table 4). Patient 1 was homozygous for a large deletion in *TNFRSF9*, and patient 2 harbored a homozygous missense mutation affecting evolutionarily conserved residues (supplemental Figure 3). Patient 3 was homozygous for a *TNFRSF9* mutation disrupting the splice-acceptor site of exon 3, resulting in the skipping of exons 3 and 6. It remains unclear why alternative splice variants are present in this patient, including a smaller fraction of transcripts that has both exon 3 and exon 6 of *TNFRSF9* skipped (supplemental Figure 4). However, it is possible that this specific splice-acceptor site regulates the splicing of nearby exons, as demonstrated by variants in *ERBB4*.[4] Additional investigations in the splicing effects of this genetic variant were, however, beyond the scope of this study. Patient 4 was homozygous for a mutation in the splice-donor site of exon 2 causing skipping of exon 2 (supplemental Figure 4). All mutations resulted in markedly reduced or abrogated expression of CD137 on activated T, B, and NK cells, indicating a loss-of-function phenotype (Figure 1F; supplemental Figure 5). However, the loss of CD137 did not affect CD137L protein expression in patients' T cells (supplemental Figure 7D). Recent studies showed that CD137 can be transferred from Hodgkin and Reed-Sternberg cells to neighboring cells by trogocytosis.[5] We thus investigated the expression of *CD137L* in T cells by reverse transcription polymerase chain reaction and found that *TNFSF9*/*CD137L* messenger RNA is expressed intrinsically in T cells (supplemental Figure 7D).

Interestingly, in 3 of the pedigrees, 1 healthy sibling each was also homozygous for the same *TNFRSF9* mutation. Accordingly, they had abrogated or reduced CD137 expression, without overt clinical disease. It is currently unknown whether CD137 deficiency may be aggravated by infections or other extrinsic challenges. Incomplete penetrance is well known for pathogenic immune system mutations,[6] especially for defects with predominant immune dysregulation, as exemplified by CTLA-4 haploinsufficiency.[7]

Immune-cell phenotypes

Patients had variable lymphocyte abnormalities (supplemental Table 2). All patients had elevated proportions of transitional and immature B cells but markedly reduced memory B cells and plasmablasts. Decreased NK-cell counts were observed in patients 2 and 3. Patients 1, 2, and 3 had reduced follicular helper T cells (T_{FH}) (Figure 2A; supplemental Figure 6).

Functional T-cell defects in CD137-deficient patients

Cd137-deficient mice have impaired T-cell survival, proliferation, and cytotoxicity.[8-10] We thus hypothesized that human CD137 deficiency may also hamper T-cell differentiation and function. Indeed, T-cell proliferation responses to various stimuli were reduced in all patients (Figure 2B). Surprisingly, patients' T cells showed impaired proliferation to anti-CD3 stimulation alone. We hypothesized that CD137L is functionally important and required to elicit a normal cellular response to CD3. By blocking CD137 in healthy donor (HD) peripheral blood mononuclear cells (PBMCs) stimulated with anti-CD3, we showed a dose-dependent reduction in T-cell proliferation (supplemental Figure 7A). Indeed, CD137 receptor/ligand interaction plays a functional role in T cells. Remarkably, the addition of anti-CD28–mediating T-cell receptor (TCR) costimulation restored proliferative functions, and partial compensation was seen upon OX40 costimulation (Figure 2B). We also observed reduced T-cell activation in patients 1 to 3, amenable to correction upon additional CD28 costimulation. Patient 4 had normal T-cell activation (Figure 2C; supplemental Figure 7B), consistent with a milder T-cell proliferation defect (Figure 2B). Collectively, our findings highlight the importance of CD137 in immune homeostasis costimulation and, intriguingly, the lack of CD137 costimulation may be compensated in the presence of other costimulators.

Figure 1 (continued) between CD137L and CD137. (E) Localization of *CD137* mutations in our patients. *CD137* gene and protein domains with the 4 newly identified mutations are indicated. (F) CD137 protein expressions in activated ($CD25^+$) $CD4^+$ and $CD8^+$ T cells, activated ($CD86^+$) $CD19^+$ B cells, and activated (interleukin 2 [IL-2] stimulated) NK cells ($CD56^+$) in patients and healthy donors (HDs) demonstrating complete loss or markedly reduced expression in patients' cells, measured by flow cytometry. All error bars indicate plus or minus standard error of mean (SEM). CRD, cysteine-rich domain; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; P4, patient 4; UTR, untranslated region.

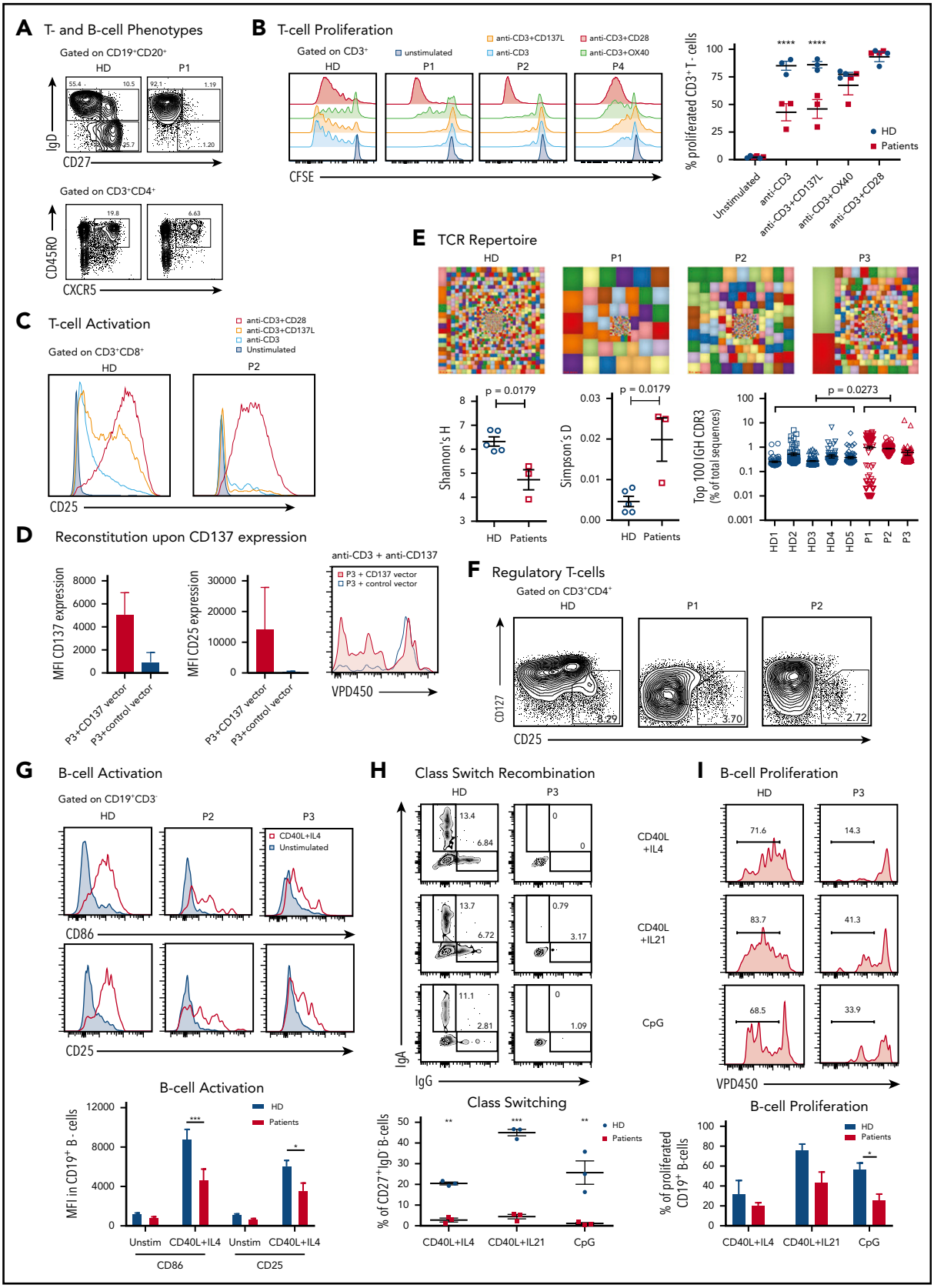

Figure 2. Immunological and functional phenotypes in CD137-deficient patients. (A) T- and B-cell features in our patients: immunophenotyping revealed decreased frequencies of class-switched ($CD27^+$ immunoglobulin D–negative [IgD^-]) B cells and T_{FH} ($CD45RO^+CXCR5^+$) cells in patient 1 (P1) compared with an HD, as measured by flow cytometry. (B) T-cell proliferation with carboxyfluorescein succinimidyl ester (CFSE) fluorescence cell incorporation assay 4 days poststimulation exhibiting reduced $CD3^+$ T-cell proliferation in response to anti-CD3 and anti-CD3 in combination with CD137L in patients 1, 2 (P2), and 4 (P4) with partial and complete restoration upon OX40 and CD28

To prove the causative role of lack of CD137 for the observed phenotypes, we performed a gene-rescue experiment in T cells from patient 3. Upon exogeneous expression of wild-type CD137, T-cell proliferation and activation defects were restored (Figure 2D; supplemental Figure 7C).

TCR repertoire analysis in patients 1 to 3 showed significant clonal expansion associated with reduced diversity (Figure 2E). CD137 is expressed in regulatory T cells (Tregs) and has been shown to play a role in Treg function, survival, and expansion.[10,11] We therefore assessed Treg frequencies in PBMCs and observed lower Treg frequencies in our patients (Figure 2F).

In Cd137-deficient mice, NK-cell and cytotoxic T-lymphocyte (CTL) function were diminished. Indeed, EBV-specific CTL cytotoxicity was reduced in patient CTLs compared with HDs (supplemental Figure 8A), suggesting that CD137 deficiency results in susceptibility to EBV and its related lymphomagenesis. However, CTL and NK-cell degranulation, as well as downstream TCR-signaling pathways, were intact in CD137-deficient patients (supplemental Figure 8B-E). This is reminiscent of a study investigating human FERMT3-deficient patients where there was specific cytotoxicity impairment but not general cytotoxicity.[12]

B-cell defects in CD137-deficient patients

CD137 is expressed in activated human B cells,[13] T_{FH},[10] and follicular dendritic cells.[14] CD137 has been shown to be essential for B-cell function, including activation, affinity maturation, proliferation, and class switch recombination (CSR) through its interaction with CD137L in germinal centers.[14-16] We thus hypothesized that these functions may be impaired in patient B cells. Indeed, CD137 expression was abrogated in B cells from all patients (Figure 1F), and B-cell activation was impaired (Figure 2G). Correspondingly, we mimicked T-cell–dependent and –independent stimulation on patient B cells and found defective CSR, proliferation, and lower frequencies of plasmablasts (Figure 2H-I; supplemental Figure 9). Patients' B cells consistently showed maturation and differentiation defects with a marked reduction in memory B cells, plasmablasts, and CSR (Figure 2A,H; supplemental Figure 9A). In accordance with our data, it has been shown that CD137L signaling is required for proper activation and maturation of B cells and humoral responses.[14] Alternatively, the maturation defects in B cells could also be an indirect effect of the lack of CD137 signaling in T_{FH} cells, resulting in the reduction of these cells and, therefore, the lack of help by these specific T-cell subsets to B cells. However, because CD137 is also expressed on activated B cells (Figure 1F), although to a smaller extent than in activated T cells, we believe that the lack of CD137 on patient B cells may contribute to their proliferation and survival directly.[13] Additionally, despite normal T_{FH} cell frequency in patient 4, B-cell development was still impaired (supplemental Figure 6), supporting the notion of a primary B-cell maturation defect. Furthermore, sorted naïve B cells also showed reduced activation, proliferation, and CSR (supplemental Figure 10). Together, these findings highlight the role of CD137 in proper differentiation and function of B cells as previously reported.[13]

CD137 is an appealing target for immunotherapy, both for autoimmunity and malignancies. It functions as an immune suppressor, enhancing Treg expansion and ameliorating T_H17 autoimmune effects.[17] Conversely, CD137 is a potent immune stimulator that has been found to modulate the tumor microenvironment, enhancing T- and NK-cell cytotoxicity and their infiltration into tumors.[18,19] CD137 agonistic monoclonal antibodies are currently in cancer immunotherapy trials, including combinations with checkpoint inhibitors to selectively activate tumor-targeting CTLs and NK cells aiming to provide a robust antitumor response.[20,21] CD137 signaling is also used in chimeric antigen receptor T-cell immunotherapy.[22] Whenever CD137 signals are enhanced, an enhanced cytotoxic response has been shown in various in vitro and in vivo models.[23-25] As the binding of CD137 to CD137L triggers a bidirectional signaling,[26] loss of CD137 expression in patients may also lead to lack of CD137L function. Cd137l-deficient mice develop B-cell lymphomas.[15] Consistently, 2 patients developed EBV-associated B-cell lymphomas and 1 patient displayed EBV-associated lymphoproliferation, implying that CD137 deficiency is a predisposing factor for malignant transformation. The phenotypes observed in our patients show some resemblance to that of 2 other recently reported CD137-deficient patients (supplemental Table 1).[27] Our findings further strengthen the role of CD137 as an appealing target for cancer immunotherapy.

Collectively, we identified novel inherited germline mutations in *TNFRSF9* that allow dissection of the essential role of this costimulatory molecule in regulating human immune homeostasis. In vitro cellular phenotypes were rescued by CD28 costimulation, possibly allowing for the development of targeted therapeutics in vivo in affected patients. CD137 deficiency should be considered in patients with dysregulated immune systems presenting with autoimmunity and autoimmune lymphoproliferative syndrome–like, common variable immune deficiency, and/or EBV-related lymphoma.

Figure 2 (continued) costimulation, respectively (****$P < .0001$; 2-way analysis of variance [ANOVA]). (C) Representative surface expression of CD25 on T cells as measured by flow cytometry 4 days poststimulation in patient 2 compared with an HD, demonstrating a T-cell activation defect with a compensatory effect upon CD28 costimulation. (D) Rescue of T-cell proliferation and activation via CD25 expression in patient 3 (P3) by exogenous expression of wild-type CD137. (E) Analysis of T-cell receptor γ (TRG) repertoire diversity with a tree-map representation for patients 1, 2, and 3, and age-matched healthy controls. Each colored square represents a unique clone and its size reflects its productive frequency within the repertoire. Simpson's D diversity index and Shannon's H index quantify repertoire clonality. (F) Flow cytometric expression of Tregs, displaying reduced Treg rates in patients 1 and 2 compared with an HD. (G) Top, Flow cytometric expression of $CD86^+$ and $CD25^+$ of $CD19^+CD3^-$ cells 1 day poststimulation with CD40L in combination with IL4 showed impaired activation in patients 2 and 3. Bottom, quantification of B-cell activation, showing significantly lower B-cell activation in patient cells compared with HDs (*$P < .05$; ***$P < .001$; 2-way ANOVA). (H) Top, class-switched IgG^+ and IgA^+ of $CD19^+$ cells upon various stimulations, displayed impaired class switch recombination in patient 3 in response to T-cell–dependent and –independent stimuli. Bottom, Quantification of class-switched ($CD27^+IgD^-$) $CD19^+$ cell rates showing significantly lower frequencies in the patients (**$P < .01$; ***$P < .001$; 2-way ANOVA). (I) Top, B-cell proliferation measured by violet proliferation dye (VPD450) 4 days poststimulation showing reduced proliferating B cells in patient 3 in response to T-cell–dependent and –independent stimuli. Bottom, quantification of $CD19^+$ proliferating cells, showing decreased rates in patients (*$P < .05$; 2-way ANOVA). All error bars indicate plus or minus SEM. CDR, complementarity-determining region; CpG, cytosine guanine dinucleotide; IGH, immunoglobulin heavy; Unstim, unstimulated.

Acknowledgments

The authors thank the patients and their families for participating in the study; Raúl Jimenez-Heredia (Ludwig Boltzmann Institute for Rare and Undiagnosed Diseases [LBI-RUD]), Laurène Pfajfer (LBI-RUD), Wendy Aloo (Ludwig Maximilian University of Munich [LMU]), Carlos E. Muskus (Universidad de Antioquia [UdeA]), and Laura Naranjo (UdeA) for excellent technical assistance; Kemal Deniz (Erciyes University), Burcu S. Gorkem (Erciyes University), and Julio C. Orrego (UdeA); and James R. Lupski, Richard A. Gibbs, and Zeynep H. Coban-Akdemir for sequencing through the Baylor-Hopkins Center for Mendelian Genomics.

This work was supported by the European Research Council (ERC; Consolidator grant 820074 "iDysChart" [K.B.] and an ERC Advanced grant [C.K.]), the Jeffrey Modell Foundation (JMF; R.S.), the Care for Rare Foundation, the German Research Foundation (Gottfried-Wilhelm-Leibniz Program, CRC1054 [C.K.]), and the Else Kröner-Fresenius-Stiftung (Forschungskolleg Rare Diseases of the Immune System [C.K.]). I.S. was supported by the Care for Rare Foundation and has been a scholar of the Else Kröner-Fresenius-Stiftung. M. Thian was supported by a Cell Communication in Health and Disease (CCHD; Medical University of Vienna) doctoral fellowship and a DOC fellowship (25225) of the Austrian Academy of Sciences. A.G.D. was supported by a Deutscher Akademischer Austauschdienst/German Academic Exchange Service (DAAD) Fellowship (Thematic Program on Rare Diseases and Personalized Therapies). The Baylor-Hopkins Center for Mendelian Genomics was supported by the National Institutes of Health, National Human Genome Research Institute/National Heart, Lung, and Blood Institute grant UM1HG006542.

Authorship

Contribution: I.S. and M. Thian designed, performed, and analyzed experiments for all patients; D.M. and A.K. performed transfection of CD137 in patient cells; A.L. and A.J.S. performed and analyzed immune and genetic experiments on P2; Y.N.L. performed TCR repertoire sequencing and analysis; N.G., T. Stauber, F.G., E.U., G.S., J.M.J., E.Ö., Ö.A., T.P., M.K., A.O., C.M.T.-V., and J.L.F. provided patient samples and interpreted clinical, pathology, and/or imaging data; M.R. conducted and analyzed next-generation sequencing of P1 and P2; D.M., T.M., M.J.K., and F.H. helped supervise the study and gave intellectual input; J.D. performed variant filtering, Sanger validation, and identified the CD137 mutation in P3; R.C. conducted immunophenotyping in patients' PBMCs; A.G.D., T. Shahin, E.A., and M. Tatematsu provided technical and experimental help; C.M.-J., I.K.C., and J.S.O. conducted and analyzed next-generation sequencing of P4; K.B., C.K., and R.S. conceptualized, initiated, and supervised the study; and I.S., M. Thian, R.S., C.K., and K.B. wrote the manuscript, which was reviewed and approved by all authors.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Footnotes

Submitted 17 March 2019; accepted 2 September 2019. Prepublished online as *Blood* First Edition paper, 9 September 2019; DOI 10.1182/blood.2019000644.

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The online version of this article contains a data supplement.

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CD137 deficiency causes immune dysregulation with predisposition to lymphomagenesis

Supplementary Appendix

Table of Contents

Supplementary Patient Clinical Histories

Patient 1 (AII-1, Fig. 1A) was born to consanguineous Turkish parents, presented with Burkitt's lymphoma (Fig. 1B) at 2 years of age. A lobulated mass in the small intestine was observed (Fig. 1B). Subsequent imaging demonstrated renal and hepatic metastases (Fig. 1B, Fig. S1A), and a lesion in the right para-spinal area infiltrating the adjacent para-vertebral muscle (Fig. S1A). The patient was treated according to the NHL BFM 2000 treatment regimen, including cytoreductive prophase treatment with cyclophosphamide, dexamethasone, and intravenous methotrexate (block AA, BB and CC). Rituximab treatment was initiated due to CD20 positivity. A reduction in tumor size was observed and the patient is currently in remission at the age of 3 years. He also suffered from recurrent ear infections, hepatosplenomegaly and hypogammaglobulinemia (Tables 1 and S1). The patient has a 7-year old sibling (AII-2, Fig. 1A) without obvious clinical phenotype and no family history of immunodeficiency.

Patient 2 (BII-1, Fig. 1A) was born to consanguineous Palestinian parents. He first presented with recurrent episodes of pneumonia (Fig. S1B) during the first 3 years of life. Bi-lateral ground glass opacities indicative of a chronic lung disease were subsequently demonstrated (Fig. S1C). He displayed autoimmune lymphoproliferative syndrome (ALPS)-like disease manifestations at the age of 6 years. Hepatosplenomegaly (Fig. S1D) and lymphadenopathy (Fig. S1E, S1F, S1G) were noted in physical examination and signs of autoimmunity including autoimmune hemolytic anemia (AIHA) and immune thrombocytopenic purpura (ITP) along with positive anti-nuclear antibodies (ANA) were detected (Tables 1 and S1). An Epstein-Barr virus (EBV)-related lymphoproliferative disorder with a monoclonal T-cell population was demonstrated in lymph node pathology (Fig. S1H) which was EBER positive (Fig. S1I). The patient was treated with sirolimus and glucocorticoids which he responded well to. Cellcept therapy has recently been initiated due to persistence of

autoimmune features (i.e. AIHA, ITP, ANA positivity) upon tapering of glucocorticoid treatment. Co-trimoxazole antibiotic prophylaxis is routinely administered as well. The patient has two siblings (BII-2, Fig. 1A) without overt clinical symptoms and no family history of immunodeficiency.

Patient 3 (CII-2, Fig. 1A), a boy born to consanguineous Turkish parents, manifested with herpes labialis and a lower respiratory tract infection at the age of six years. Hepatosplenomegaly (Fig. S1J) and lymphadenopathy were found during physical examination. Episodes of pneumonia, recurrent tonsillitis, otitis media and chronic suppurative otitis media were noted since the age of eight years (Table 1). Additional features included atopic dermatitis and xerosis. Laboratory evaluation revealed a positive direct Coombs test and hypergammaglobulinemia (Table S1), necessitating IVIG substitution therapy. At ten years of age, the patient was evaluated due to diffuse lymphadenopathy (bilateral cervical, submandibular and axillary nodes). An infectious etiology was ruled out. Submandibular lymph node biopsy exhibited large, prominent, multinucleated Reed-Sternberg cells (Fig. 1B) with EBER-positive lymph node histopathology (Fig. 1C). Immunohistochemistry showed weak CD20 staining and positive CD30 staining. Non-malignant nodular splenic lesions were observed, associated with lymphoproliferation. A diagnosis of Hodgkin lymphoma, stage 1, was established and the patient was treated according to GPOH-HD 95 therapy protocol, including two cycles of chemotherapy with adriamycin, vincristine, etoposide and prednisone. He is currently in remission. He is treated with immunoglobulin substitution and amoxicillin prophylaxis (Table 1). The patient has one sibling who is currently 14 years old (CII-1, Fig. 1A). She experienced recurrent tonsillitis until the age of ten years. Immunoglobulin levels were slightly elevated. Other than that, the patient has no remarkable family history of immunodeficiency.

Patient 4 (DII-2, Fig. 1A), a 33 year old male was born to non-consanguineous Colombian parents, presented with recurrent otitis media and sinusitis since the age of

eight. Between the age of 20-22 years, five episodes of pneumonia were documented. Laboratory workup revealed decreased serum IgG, IgM and IgA (Table S1) and no detectable IgG serum antibodies against rubella. The presumptive diagnosis of CVID was established and IVIG replacement therapy was initiated (Table 1). During the following 3 years, the patient was hospitalized with granulomatous pleuropneumonia, while *H. pylori* erythematous gastritis and chronic sinusitis (Fig. S1K) were noted. The patient has one healthy 32 year old sibling (DII-1, Fig. 1A). She suffered from recurrent infections during childhood. No family history of immunodeficiency is otherwise known.

Supplementary Materials and Methods

Study Subjects

All procedures were performed upon informed consent and assent from patients, first-degree relatives, and healthy donor controls in accordance with the ethical standards of the institutional and/or national research committees and with the current update of the Declaration of Helsinki.

Genetic Analysis

Whole-exome sequencing was performed to determine the underlying genetic defect in *TNFRSF9* in all four patients. Genomic DNA was isolated from whole blood of patients and their first-degree relatives for generation of whole-exome libraries using the SureSelect XT Human All Exon V5+UTR or V6+UTR kit (Agilent Technologies, USA). Barcoded libraries were sequenced on a NextSeq 500 platform (Illumina, USA) with an average coverage depth of 100x. Bioinformatics analysis and subsequent filtering identified rare sequence variants. *TNFRSF9*/*CD137* mutations were confirmed by Sanger sequencing.

In silico analysis of the exon 2: c.100+1G>A substitution was performed with the bioinformatics tools NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/) and

Fruitfly (http://www.fruitfly.org/seq_tools/splice.html). These tools predicted that the mutation affected the donor splice site at the end of exon 2. Moreover, multiple alignments of DNA sequences of *CD137* orthologous from several species show that exon2: c.100 +1G is a highly conserved region. We used the SIFT, PolyPhen-2 and CADD algorithms to assess these *in silico*[1].

Expansion of T cells

Patient or control blood was subjected to a Ficoll density gradient centrifugation, after which peripheral blood mononuclear cells (PBMCs) were collected from the interface. T cells were expanded by stimulation of the PBMCs with irradiated feeder cells (PBMCs from healthy donors), PHA (1µg/ml) and IL-2 (100U/mL) in RPMI medium containing 5% human serum. Cell lines were tested negative for mycoplasma by PCR using VenorGeM Mycoplasma Detection Kit (MP0025, Sigma-Aldrich).

Flow Cytometry

For analysis of cell surface markers, PBMCs were used as starting material or 100 µl of whole blood in EDTA was lysed using RBC Lysis Buffer (eBioscience). Cells were washed twice in FACS buffer (PBS with 5% FBS), and resuspended in 100 µl of FACS buffer with antibodies for 30 min on ice. Cells were washed twice in FACS buffer, resuspended in 300 µl of FACS buffer.

Western Blot

Whole cell lysates were prepared from control- and patient-derived expanded T cells or B-LCLs, loaded on 10% polyacrylamide TRIS-HEPES-SDS gel and separated by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane using iBlot system (Invitrogen). Blots were probed overnight with 1:1000 dilution of specific antibodies, and 1:8000 dilution of anti-HSP90α/β (Santa

Cruz) as a loading control. Bands were revealed using Amersham ECL Select Western Blotting Detection Reagent (GE Healthcare).

T- and B-cell stimulation

PBMCs were labeled with either CellTrace Violet Cell Proliferation dye VPD450 (ThermoFisher) or CFSE 5µM (ThermoFischer), washed with PBS with 10% FCS in a ratio of 1:1. Labeled PBMCs were stimulated with anti-CD3-coupled beads (Bio-anti-CD3, OKT3 from eBioscience coupled with anti-Biotin MACSiBeads from Miltenyi Biotec) at a ratio of 10:1 with and without 1 µg ml^{-1} soluble anti-CD28 (CD28.2, eBioscience) or anti-CD3 with anti-CD137, 10 µg/ml (R&D Systems) or CD137L, 20 ng/ml (R&D Systems) and stained for cell surface markers. For blocking experiments of CD137 in T cells, a neutralizing anti-CD137 monoclonal antibody (BBK-2 clone, Invitrogen) was added at increasing concentrations (1-2µg/mL) or mouse IgG1, kappa isotype control P3.6.2.8.1 (Invitrogen) to anti-CD3-coupled beads and stained for cell surface markers. For B-cell proliferation assays PBMCs were stimulated with CpG ODN 2006, 50nM (ODN7909, Invivogen), or CD40 ligand 200ng/ml (R&D Systems) in combination with rhIL4 100 ng/ml, rhIL21 20ng/ml (R&D Systems) or anti-IgM 20µg/ml (Southern Biotech). For sorting of CD3 T cells and naïve B cells, PBMCs were stained for CD3, CD19, CD27 and IgD: PE-CY7 anti-CD3 (Beckman Coulter), PerCP/Cy5.5 anti-CD19 (HIB19, Biolegend), BV480 anti-IgD (IA6-2, BD), PE anti-CD27 (L128, BD), and afterwards sorted by FACS for $CD3^+$, $CD19^+CD27^-IgD^+$ or $CD19^+CD27^+$ populations, respectively. Sorted PBMCs were then labeled with either CellTrace Violet Cell Proliferation dye VPD450 (ThermoFisher) or CFSE, stimulated as mentioned above, and re-stained for cell surface markers.

Flow cytometry-based CTL cytotoxicity test

Assays to quantify the cytotoxic activity of CTLs were done by stimulating PBMCs with 30Gy irradiated autologous B-LCLs at 5:1 ratio in complete RPMI (Gibco)

with 10ng/mL IL-7 (Peprotech) for 14 days. At day 7, 100U/mL IL-2 (Peprotech) was added. Two rounds of stimulation were performed every 14th day. CD8^{+} T cells were sorted with MagniSort Human CD8^{+} T-cell Enrichment Kit according to manufacturer's instructions. Autologous EBV B-LCLs were stained with VPD450 (ThermoFisher) and used as targets. CD8+ T cells were incubated for 4 hours at different ratios of effector and target cells. Cytotoxicity was evaluated by flow cytometry gating on CD19^{+} and VPD450^{+} cells.

Flow cytometry-based CTL and NK-cell degranulation test

NK- and CTL degranulation was assessed by CD107a surface staining without (medium-cultured cells) and 3 hours after stimulation with K562 cells at a ratio of 1:1 as previously described[2]. The erythroleukemic cell line K562 (ATCC, CCL-243) was used as a target cell line. NK cells were cultured in medium containing 600U/ml IL-2 (Novartis) for 48 h to assess degranulation of activated NK cells. CTL (cytotoxic T lymphoblasts) degranulation was evaluated in T-lymphoblasts 48 h after stimulation with 1.25 mg/ml 1- phytohemagglutinin-L (PHA-L, Sigma) and 200U/ml IL-2 (Novartis). CTL degranulation was calculated by the difference in median fluorescence intensity of CD107a of CTLs stimulated with CD3/CD28-coated microbeads (ThermoFisher Scientific) at a ratio of 1:10 for 3 h and medium cultured
cells.

Flow cytometry and antibodies

For immunophenotyping the following antibodies were used: BV480 anti-CD45 (clone HI30, BD), APC-Fire 780 anti-CD3 (SK7, Biolegend), BUV395 anti-CD4 (RPAT4, BD), BUV496 anti-CD8 (RPAT8, BD), APC anti-CD45RA (BUV737, BD), BB515 anti-CD45RO (UCHL1, BD), APC anti-CD127 (A019D5, Biolegend), PE anti-CD25 (M-A251, BD), APC R-700 anti-CD27 (M-T271, BD), BB700 anti-CD28 (L293, BD), BV711 anti-HLA-DR (L243, Biolegend), BV421 anti-CCR7 (G043H7, Biolegend), BV786 anti-

CCR6 (11A9, BD), PE-CF594, anti-CXCR3 (1C6, BD), BV650 anti-CD38 (HB-7, Biolegend), BUV395 anti-CD19 (SJ25C1, BD), PE-Cy7 anti-CD20 (2H7, BD), BB515 anti-IgD (IA6-2, BD), BV421 anti-IgM (G20-127, BD), APC anti-CD38 (HB7, BD), BUV737 anti-CD21 (B-Ly4, BD), BV786 anti-CD27 (L128, BD), PE anti-CD10 (HI10a, BD), BUV496 anti-CD3 (UCHT-1, BD), BUV737 anti-CD8 (SK1, BD), PE-CF594 anti-CD56 (NCAM16.2, BD), BB515 anti-CD57 (NK-1, BD), BB700 anti-CD15 (M5E2, BD), APC anti-CD16 (3G8, Biolegend), HLA-DR anti-BV711 (L243, Biolegend), BV785 anti-CD123 (6H6, BD), BV421 anti-CD11c (B-ly6, Biolegend), PE anti TCR g-d (5A6.E9, Life Tech), APC-R700 anti-TCR a-b, (IP26, Biolegend), PE-Cy7 anti-CD33 (P67.6, BD), and BV711 anti-CXCR5 (J2252D4, Biolegend).

CD137 and CD137L expression in T cells was measured upon 48 hour stimulation of PBMCs with anti-CD3 and anti-CD28. Cells were stained with PacB anti-CD3 (clone SK7, biolegend), BV711 anti-CD4 (SK3, BD), APC-Fire 750 anti-CD8 (RPA-T8, Biolegend), PE anti-CD25 (M-A251, BD), APC anti-CD137 (4B4-1, BD) or APC anti-CD137L (5F4, Biolegend). Expression in B cells was measured upon 24 hour stimulation of PBMCs with anti-IgM in combination with CD40L (RnD). PBMCs were stained with PacB anti-CD3 (clone SK7, Biolegend), PerCP/Cy5.5 anti-CD19 (HIB19, Biolegend), BV421 anti-CD86 (FUN-1, BD), CD25 (M-A251, BD), APC anti-CD137 (4B4-1, BD). Expression in activated NK cells was measured upon 48 hours stimulation of PBMCs with IL-2 600U/ml (Novartis). PBMCs were stained with FITC anti-CD56 (NCAM16.2, BD), PacB anti-CD3 (SK7, Biolegend), APC anti-CD137 (4B4-1, BD).

Proliferative responses were measured by labeling PBMCs with 2.5 mM carboxyfluorescein diacetate succinimidyl ester (CFSE, ThermoFisher), 7-aminoactinomycin D (7-AAD, 2.5 mg/ml, BD), PacB-anti-CD3 (clone SK7, biolegend), PE-anti-CD25 (M-A251, BD), APC-Fire 750 anti-CD8 (RPA-T8, Biolegend)) and BV711 anti-CD4 (SK3, BD) 4 days after stimulation. Class-switch recombination and plamablast numbers were measured 4 days after stimulation. PBMCs were stained with BUV395 anti-CD3 (clone SK7, BD), PerCP/Cy5.5 anti-CD19 (HIB19, Biolegend),

BV421 anti-IgD (IA6-2, BD), PE anti-CD27 (L128, BD), BUV661 anti-CD38 (HIT2, BD). Degranulation of NK cells and CTLs (cytotoxic T lymphocytes) was determined by surface staining with PacB-anti-CD3 (clone SK7, 1:100), APC-H7-anti-CD8 (SK1, 1:100), PE-Dazz-anti-CD56 (NCAM16.2, BD), FITC anti-CD56 (NCAM16.2, BD), and PE-anti-CD107a (H4A3, 1:50). T-cell signaling was performed in T cell blasts. PBMCs were stimulated with 5 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 µM ionomycin (Sigma-Aldrich) and expanded with IL-2 (100U/ml) for 9 days. T-lymphoblasts were stimulated by cross-linking of anti-CD3 (UCHT1) 2.5 µg/ml and/or 10 µg/ml anti-CD28 (CD28.2) with 10 µg/ml goat anti-mouse IgG all from BD for the indicated time. Cells were fixed and permeabilized with fixation and permeabilization kit from Molecular Probes in 90% methanol. The permeabilized cells were stained with the following intracellular antibodies detecting: ERK1/2 phosphorylated at T202 and Y204 (A647 anti-pERK1/2, BD), NFκB P65 phosphorylated at S529 (A647 anti-NFκB P65, BD), AKT phosphorylated at S473 (A488 anti-pAKT, (M85-61, BD)), isotype control (A488 mouse IgG κ1 isotype control, BD).

TCR Repertoire and Analysis

Cell surface marker expression of peripheral blood mononuclear cells (PBMCs) was analyzed by immunofluorescent staining with monoclonal antibodies and flow cytometry (Epics V; Coulter Electronics, Hialeah, FL). Signal joint T-cell receptor excision circles (sjTREC) copy numbers were determined by employing quantitative real-time PCR (qRT-PCR) of genomic DNA (gDNA, 0.5 µg) extracted from whole blood of our patients. Surface expression of individual T cell receptor Vβ (TCR-Vβ) gene families was assessed using a set of 24 Vβ-specific fluorochrome-labeled monoclonal antibodies (Beckman Coulter, USA) and flow cytometry. Next-generation sequencing (NGS) T-cell receptor (TCR) libraries were generated from gDNA of patients and controls using primers for conserved regions of V and J genes in the *TRG* (T-cell receptor gamma) locus according to the manufacturer's protocol (Lymphotrack,

Invivoscribe Technologies, Carlsbad, CA). Quantified libraries were pooled and sequenced using Mi-Seq Illumina technology (Illumina, USA). FASTA files from the filtered sequences were submitted to the IMGT HighV-QUEST webserver (http://www.imgt.org), filtered for productive sequences (no stop codons or frameshifts), and analyzed Repertoire diversity was calculated using Shannon's H diversity index and Simpson's D index of uneveness.

Shannon's H index: $H' = -\sum_{i=1}^{R} p_i \ln p_i$

Simpson's D index of unevenness: $\lambda = \sum_{i=1} p_i^2$

Retroviral Construction and Transfection Protocol

N-terminally tagged CD137 was generated using retroviral pfMIG 3981 (IRES-GFP) vector. For the reconstitution experiments constructs with either wild-type CD137 or empty vector (GFP only) were used. Before electroporation of the Patient 3 and healthy donor PBMCs, retroviral vector was digested with Xba I enzyme to generate 3 kb DNA fragment containing CD137-IRES-GFP gene sequences. Digested DNA was purified using QIAquick PCR purification kit (Qiagen) according to manufacturer instructions. Patient 3 and healthy donor PBMCs were electroporated with 2µg of the digested DNA mixture using Amaxa Human T cell Nucleofector Kit (Lonza), program V-024, according to manufacturer instructions. Transfection efficiency was assessed by GFP expression 24 hours after electroporation.

Statistical Analysis

Statistical evaluation of experimental data was performed using Prism version 6 (GraphPad Software, USA). Probability (*P*) values < 0.05 were considered statistically

significant. *P* values and statistical tests are indicated in figure legends, where applicable.

Supplementary References

1. Adzhubei I, Jordan DM, Sunyaev SR. Predicting Functional Effect of Human Missense Mutations Using PolyPhen-2. Current protocols in human genetics / editorial board, Jonathan L Haines [et al]. 2013 Jan;0 7:Unit7 20.

2. Bryceson YT, Pende D, Maul-Pavicic A, Gilmour KC, Ufheil H, Vraetz T, et al. A prospective evaluation of degranulation assays in the rapid diagnosis of familial hemophagocytic syndromes. Blood. 2012 Mar 22;119(12):2754-63.

Table S1. Clinical and genetic manifestations of CD137-deficient patients

Patients	P1	P2	P3	P4	*Pa	*Pb
Mutation	c.1_545+1716del	NM_001561.5: c.452C>T; p.Thr151Met	NM_001561.5: c.101 -1G>A	NM_001561.5: c.100 +1G>A	NM_001561.5: c.325G>A p.Gly109Ser	NM_001561.5: c.325G>A p.Gly109Ser
Initial clinical manifestation	abdominal distention	recurrent respiratory infections	lower respiratory tract infection	recurrent infections from childhood	sinopulmonary infections, bronchiectasis, pneumococcal septicemia	recurrent sinopulmonary infections, generalized lymphadenopathy, EBV viremia
Age of initial Clinical manifestation	2 years	4 years	6 years	8 years	3 years	6 years
Consanguinity	parents are 1st degree cousins	parents are 1st degree cousins	parents are 3rd degree cousins	no known consanguinity	parents are 1st degree cousins	parents are 1st degree cousins
Infectious complications during disease course	recurrent ear infections EBV viremia	recurrent pneumonia EBV viremia	recurrent labial herpes, recurrent tonsillitis, recurrent otitis media, recurrent pneumonia EBV viremia	recurrent ear infections, recurrent pneumonia, pleuropneumonia, chronic sinusitis EBV viremia	pneumococcal septicemia sinopulmonary infections EBV viremia	sinopulmonary infections EBV viremia
Identified Pathogens	CMV, EBV	*adenovirus,* EBV, HSV	EBV, HSV	EBV	*streptococcus pneumoniae,* EBV	EBV
Hepatosplenomegaly	+	+	+	+	splenomegaly	splenomegaly
Lymphadenopathy	-	+	+	-	+	+
Malignancy	CD20 positive Burkitt lymphoma	-	EBV-positive Hodgkin's lymphoma	-	-	EBV-positive Hodgkin´s lymphoma, relapse and progression to DLBCL

Treatment of B-cell malignancies	cytoreductive prophase treatment: cyclophosphamide, dexamethasone, methotrexate (AA, BB, CC blocks), rituximab	NA	etoposide, doxorubicin, vincristine	NA	NA	5 cycles: doxorubicin, bleomycin, vincristine, etoposide, prednisone, cyclophosphamide along with rituximab; upon relapse daratumumab, bortezomib, dexamethasone and rituximab
AIHA	-	+	+	-	-	-
ITP	-	+	-	-	-	-
Other features	-	ALPS like symptoms, EBV associated lymphoproliferation	short stature	distal peptic esophagitis, erythematous gastritis	hemophagocytic lymphohystiocytosis	-
Treatment of immunodeficiency/Infections	IVIG, co-trimoxazole, fluconazole	sirolimus, cellcept, co-trimoxazole	IVIG, antibiotic prophylaxis	SCIg	IVIG, rituximab	IVIG
Outcome	responded well to chemotherapy treatment protocol and rituximab, currently stable under IVIG and antibiotic prophylaxis	well on cellcept treatment and co-trimoxazole antibiotic prophylaxis	good response to chemotherapy with clinical remission. Currently stable under IVIG and antibiotic prophylaxis	stable under SCIg replacement treatment with resolution of infections	sinopulmonary infections improved upon IVIg treatment, resolution of viremia with rituximab. Currently undergoing HSCT from a healthy HLA-matched sibling	clinical remission and resolution of viremia with chemotherapy and rituximab treatment. Respiratory tract infections improved with IVIg treatment

CMV: Cytomegalovirus. EBV: Epstein-Barr virus. HSV: Herpes simplex virus. NA: Not applicable. AIHA: Autoimmune hemolytic anemia. ITP: Immune thrombocytopenic purpura.

ALPS: Autoimmune lymphoproliferative syndrome. IVIG: Intravenous immunoglobulin. SCIg: Subcutaneous immunoglobulin. HSCT: hematopoietic stem cell transplantation.

DLBCL: diffuse large B cell lymphoma.

*Pa, Pb reported by Alosaimi et al. *J Allergy Clin Immunol*, 2019.

Table S2. Immunologic characteristics of patients with CD137 deficiency

Variable	Patient 1		Patient 2		Patient 3		Patient 4	
	Value	Reference Range	Value	Reference Range	Value	Reference Range	Value	Reference Range
Age of evaluation	4 years		9 years		11 years		33 years	
Absolute lymphocyte count (cells/mm^3)	3390	2340-5028	3597	1300-5000	1590	1300-3000	2680	1200-4100
Lymphocyte subset								
CD3+ (cells/mm^3)	1551	1239-2611	2950	700-4200	1272	1000-2000	1860	780-3000
CD4+ (cells/mm^3)	1074	870-2144	1295	600-2100	858	500-1300	965	100-2300
CD45RO+ CCR7+ (%)	**7.5**	13.88-48.12	**14.6**	22.06-46.46	45.5	24.24-52.73	**11.5**	18-95
CD45RO+ CCR7- (%)	**78.7**	0.94-6.46	**59.3**	2.08-8.78	**26.5**	3.4-11.17	**55.8**	1-23
CD45RO- CCR7+ (%)	**11.6**	46.14-84.4	**22**	45.56-75.28	**24.5**	39.72-69.59	**27**	16-100
CD45RO- CCR7- (%)	1.1	0-1.36	0.1	0-1.06	**2.43**	0.1-1.29	5.7	0-6.8
CD8+ (cells/mm^3)	**407**	472-1107	**1439**	200-1100	333	300-800	772	200-1200
CD45RO+ CCR7+ (%)	**0.9**	5.18-31.66	**3.4**	12.08-30.54	**4.9**	13.21-37.89	7.3	1-20
CD45RO+ CCR7- (%)	**62.4**	0.7-11.22	**43.3**	1.58-13.18	15.2	1.53-15.39	29.3	14-98
CD45RO- CCR7+ (%)	**3.8**	36.8-83.16	**34.8**	41.58-77.9	**77.1**	41.41-73.04	23.7	41.41-73.04
CD45RO- CCR7- (%)	29.7	0.84-33.02	15.3	1.7-24.62	2.79	2.01-21.65	36.2	2.01-21.65
CD3-CD16+CD56+ (cells/mm^3)	176	155-565	**54**	120-483	**76**	100-700	417	90-600
CD19+ (cells/mm^3)	846	434-1274	**360**	50-300	238	200-500	278	100-500
IgD+ CD27- (%)	**93**	73-89	**94.6**	67.8-89	**93.7**	67.8-89	**95.5**	58-72.1
IgD+ CD27+ (%)	**2.2**	5.7-14.3	**2.1**	5-16.2	**1.83**	5-16.2	**1.9**	13.4-21.4
IgD- CD27+ (%)	**1.2**	3-10.3	**0.9**	4-14	**0.79**	4-14	**1.8**	9.2-18.9
CD27+ CD38+ (%)	0.76	0.5-7.06	**0.70**	0.9-7.36	**0.37**	0.7-5.67	**0.61**	0.9-7.36

Immunoglobulins								
IgG (mg/dL)	**413**	701-1157	**1599**	540-1550	**2290**	824-1300	**550**	968-2514
IgM (mg/dL)	105	42-80	**714**	40-240	**267**	44-142	**33.6**	103-397
IgA (mg/dL)	68.7	34-108	**49**	52-274	130	71-161	**31**	103-397
IgE (IU/mL)	26.4	2-199	0	0-200	7.77	<100	173	116-551
Complement								
C3(mg/dL)	N/A		**68.9**	90-180	105	90-180	N/A	
C4(mg/dL)	N/A		**72.4**	10-40	**9.3**	10-40	N/A	
T-cell repertoire								
αβ+ (%)	94		79		83		95.3	
γδ+ (%)	**2**	4.94-17.98	**2**	6.92-19.84	**1.1**	8.1-20.76	3.5	
TRECs	N/A		800	>400	N/A		N/A	
Auto-immune workup								
Positive auto-antibodies			ANA positive (1:180)		d. Coombs positive			
EBV/CMV status								
EBV DNA Viral Load (PCR)	**652 copies/ml**		**7.1 X10^5 copies/ml**		**9.1 X10^4 IU/ml**		**610 Geq/ml**	
CMV DNA Viral Load (PCR)	**82 copies/ml**		negative		NA		negative	
EBV Serology	NA		EBNA-1 (IgG): positive		*EBNA-1 (IgG): positive *VCA (IgM): negative *VCA (IgG): positive		NA	

CMV Serology	NA	anti-CMV (IgG): negative	*anti-CMV (IgM): **2.44** (>1.1 U/ml) *anti-CMV (IgG): positive avidity = 0.837 positive	NA
Common vaccine serologies				
Rubella Serology	&anti-rubella (IgG): 101.3 IU/ml (positive)	MMRV serology (IgG): positive	anti-rubella (IgG): 59.3 IU/ml (positive)	*anti-rubella (IgG): **0.94 uL/mL (negative)** Sister: anti-rubella (IgG): 15.2 uL/mL (positive)
Varicella Serology	&anti-VZV (IgG): 1141 IU/ml (positive)		NA	NA
Hepatitis Serology	&anti-HBs (IgG): 201.4 uL/ml (positive)	anti-HAV (IgG): positive anti-HBs (IgG): **negative**	NA	NA
Tetanus Serology	&anti-tetanus (IgG): 2.8 U/ml (positive)	NA	anti-tetanus (IgG): 2.8 U/ml (positive)	NA

Listed are reference ranges or laboratory values for the patient's age group. Abnormal values in **bold**.

CMV Cytomegalovirus; EBNA Epstein Barr Nuclear Antigen; EBV Epstein Barr Virus; HAV Hepatitis A virus; HBV Hepatitis B virus; MMRV Measles, Mumps, Rubella, Varicella; NA Not Applicable; VCA Viral-Capsid Antigen; VZV Varicella Zoster Virus.

*Prior to IVIg / SCIg replacement therapy; &On IVIg replacement therapy

Table S3. Summary of homozygous autosomal recessive mutations in patients

Patient	Gene	Chromosome	Position	Reference	Variant in patient	Mutation type	Protein variant in patient
1	*ZXDB*	X	57620724	T	A	MISSENSE	p.Val748Asp
1	*TNFRSF9*	1	7995072-8000054	NA	NA	DELETION	NA
1	*PARK7*	1	8022845-8031023	NA	NA	DELETION	NA
2	*XIST*	X	73048904	A	G	SPLICE REGION	NA
2	*AFF2*	X	148048444	C	T	MISSENSE	p.Thr1013Met
2	*TNFRSF9*	1	7995165	C	T	MISSENSE	p.Thr151Met
2	*METTL18*	1	169763046	C	A	SPLICE REGION	NA
2	*GPC1*	2	241375361	GGCT	G	DELETION	p.Leu11del
2	*DNAH5*	5	13794053	G	A	MISSENSE	p.Gly2668Arg
2	*CD36*	7	80293747	A	G	MISSENSE	p.Tyr212Cys
3	*ASB12*	X	63445095	C	G	MISSENSE	p.Ala146Pro
3	*TNFRSF9*	1	7998889	C	T	SPLICE SITE ACCEPTOR	NA
3	*HS6ST1*	2	129075797	A	C	MISSENSE	p.Val114Gly
3	*IRX4*	5	1880839	C	T	MISSENSE	p.Arg136Lys
3	*ZNF808*	19	53058034	C	T	MISSENSE	p.Pro622Leu
4	*TNFRSF9*	1	799954	G	A	SPLICE SITE DONOR	NA

Inclusion criteria:
CADD more than 15
SIFT deleterious
Polyphen probably_damaging

Table S4. Predicted impact scores of *CD137* variants

Patient	Mutation	Chromosome	Position	PolyPhenCat	SIFTcat	CADD
1	c.1_545+1716del	1	7995072-8000054	NA	NA	NA
2	c.452C>T	1	7995165	Probably_damaging	Deleterious	24.9
3	c.101 -1G>A	1	7998889	NA	NA	27
4	c.100 +1G>A	1	799954	NA	NA	25

Supplementary Figures legends

Figure S1. Clinical features, radiology, pathology, T cell clonality. (A) Coronal T2-HASTE image (Patient 1) demonstrating multiple hypointense metastatic renal cortical masses (black arrows). A mass lesion is observed on the right paraspinal area infiltrating the adjacent paravertebral muscle (red arrow). (B) Chest CT scan demonstrating consolidation in the anterior aspect of the right middle lobe (RML), nodular opacity in the lingula and posterior mediastinal adenopathy (Patient 2). (C) Coronal reconstructions of a follow-up CT four months later demonstrating ground glass opacities in both lungs (Patient 2). (D) Splenomegaly (Patient 2) shown in coronal reconstruction of CT scan. (E) CT scan of the upper neck showing cervical lymphadenopthy (Patient 2). (F) Contrast enhanced computerized tomography (CT) demonstrating mediastinal and axillary lymphadenopathy (Patient 2). (G) Coronal reconstruction of Positron Emission Tomography showing increased uptake in the cervical, axillary and mediastinal lymph nodes. An infiltrate in the right middle lobe (RML) is demonstrated. (H) TCR-gamma spectratyping of lymph node biopsy (Patient 2) revealing two dominant peaks, supporting the presence of a monoclonal T-cell population. (I) In situ hybridization for EBV-encoded small RNAs (EBER) displaying numerous positive cells (Patient 2). Sections used are from right inguinal lymph node tissue. (J) Abdominal CT scan (Patient 3) demonstrating hepatomegaly. (K) MR demonstrating chronic sinusitis in Patient 4 with obstruction in Lt. sinus.

Figure S2. Genetic analysis. Sanger sequencing chromatograms confirming the WES findings in Family B, Family C and Family D. For Family A, WES reads in Patient 1 are compared to a healthy control showing no NGS coverage for several exons of *TNFRSF9* and *PARK7*.

Figure S3. *TNFRSF9* sequence homology in species. *TNFRSF9* sequence alignment showing the conservation of amino acid threonine at position 151 across eight species (red frame). The variant in Patient 2 (p.Thr151Met) is stated.

Figure S4. Effect of variants in the patients. (A) (upper left) Amplified gDNA utilizing forward and reverse primers to amplify exon 6 in *TNFRSF9* for Family A and controls, depicting the genomic deletion in patient 1 (AII-1) and brother (AII-2). Full length cDNA of Patient 3 (CII-2) (upper middle panel) and Patient 4 and sister (DII-1) (upper right panel) were amplified and loaded on agarose gels, depicting smaller sized bands. * and ** in middle panel depicts two aberrantly spliced transcripts. (B) Schematic illustration showing the consequences for the splice site variants in Patient 3 and Patient 4. cDNA sequencing results revealed a skipping of exon 2 in Patient 4, and a skipping of exons 3 and 6 for Patient 3. (C) Chromatograms of Sanger sequencing of the PCR product marked * and ** in figure A showing skipping of exon 3 and exons 3 and 6 in P3.

Figure S5. CD137 expressions. Flow-cytometric expression of CD137 in unstimulated and anti-CD3/CD28 stimulated T-cells for patients, siblings and heathy donors (HDs) are shown.

Figure S6. (A) Peripheral blood B-cell immunophenotyping: Memory ($CD19^+CD20^+CD27^+$), class-switched ($CD19^+CD20^+CD27^+IgD^-$) and transitional B-cell ($CD19^+CD38^+IgM^+$) frequencies are compared in patients and siblings, measured by flow-cytometry. (B) Peripheral blood T-cell immunophenotyping: Follicular helper T-cell (T_{FH}) frequencies are shown in patients and siblings, measured by flow-cytometry.

Figure S7. CD137 and CD137L inhibition in T-cell function. (A) Effect of CD137 blockade on proliferation of T-cells in healthy donors (HDs). Anti-CD137 monoclonal neutralizing antibody (BBK-2 clone) or isotype control was added to anti-CD3 stimulation at increasing concentrations showing a dose dependent inhibition of T-cell proliferation (as

measured by VPD450 dilution). Error bars indicate ± SEM. (B) T-cell activation (CD25 expression): Flow-cytometric expression of $CD25^+$ is depicted in patients and HDs upon 96 hour stimulation with anti-CD3, anti-CD3+CD137L and anti-CD3+CD28. (C) Restoration of T-cell activation upon CD137 expression. CD137 expression, CD25 T-cell activation and rate of proliferated T cells (stimulated with anti-CD3) upon exogenous expression of wild-type CD137 are shown in Patient 3. (D) *TNFSF9* mRNA expression (top panel) and CD137L surface expression (bottom panel) was measured in unstimulated and anti-CD3+anti-CD28 stimulated T-cells. *HPRT* was used as a control.

Figure S8. Cytotoxicity, degranulation and T cell receptor mediated signaling. (A) EBV-specific CTL cytotoxicity measured by the percentage of specific lysis of autologous EBV B-LCL target cells by their respective $CD8^+$ T-cells at different effector to target ratios. Patient CTL cytotoxicity showed significant reduction compared to HDs (* *P* value<0.05, Two-way ANOVA). (B) CTL degranulation (CD107a) was measured in stimulated $CD8^+$ T-cells by flow cytometry. (C) NK-cell degranulation in patients and HDs is compared in unstimulated and stimulated PBMCs, measured by flow-cytometry. (D) Immunoblotting showing expression of IκBα, phospho-p65, total p65, phospho-AKT and total AKT upon anti-CD3+CD28 stimulation in expanded T cells. HSP90 was used as a loading control. Intracellular phospho-AKT and NF-κB1 phospho-p65 levels in unstimulated and stimulated T-cell blasts, measured by flow cytometry. (E) MAPK signaling is shown measured by immunoblotting. Immunoblotting expression of total ERK1/2 and phospho-ERK1/2 upon anti-CD3+CD28 stimulation in expanded T-cells. HSP90 was used as a loading control. Intracellular phospho-ERK levels in unstimulated and stimulated peripheral $CD4^+$ and $CD8^+$ T-cells is depicted, measured by flow cytometry.

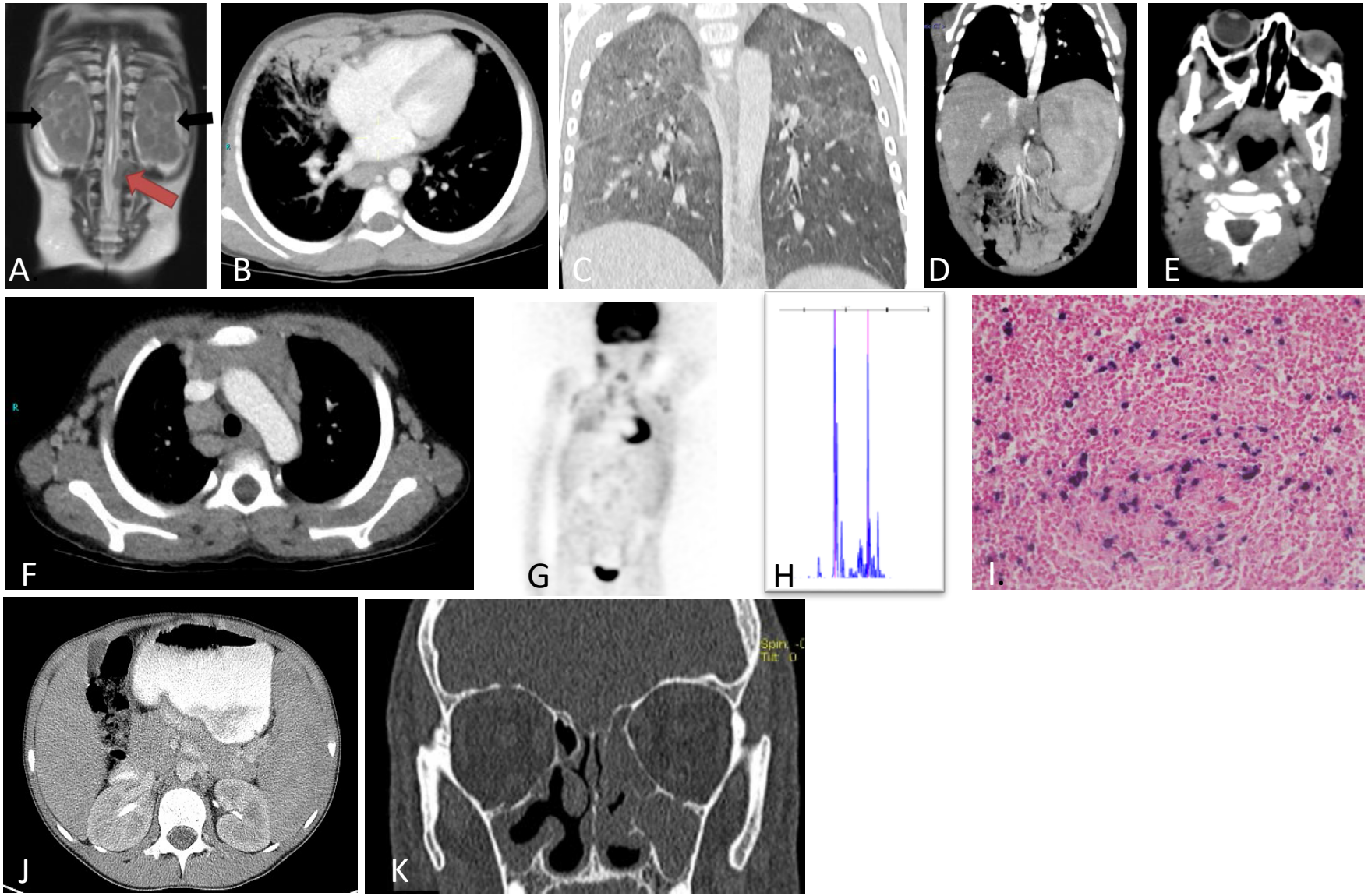
Figure S9. *In vitro* plasmablast differentiation. (A) Plasmablast ($CD3^-CD19^+CD27^+CD38^+$) frequencies are shown in unstimulated PBMCs and upon 96 hour stimulation with CpG,

CD40L+IL4 and CD40L+IL21, showing a significant difference between HDs and Patients. (* *P* value<0.05, ** *P* value<0.01, **** *P* value<0.0001, Two-way ANOVA). (B) CD137L expression in $CD19^+$ B-cells upon CpG stimulation measured by flow cytometry, showing expression of CD137L in both HD and Patient 3 stimulated cells.

Figure S10. Naïve B-cell function. Naïve B-cells ($CD19^+CD3^-IgD^+CD27^-$) were sorted and B-cell functions were assessed in our patients. (A) $CD86^+$ B-cell activation was measured 1 day post-stimulation with CpG, showing reduced B-cell activation in P3 and P4. (B) naïve B-cell proliferation measured by violet proliferation dye (VPD450) 5 days post-stimulation showing reduced rate of proliferating B-cells in P3 and P4 in response to T-cell dependent (CD40L + IL21) and independent (CpG) stimuli. (C) Class switch recombination in sorted naïve B-cells. Gating of class switched IgG^+ and IgA^+ of sorted naïve B-cells upon CD40L + IL21 and CpG stimuli, depicted reduced CSR in P3 and P4. (D) Reduced memory B cells rates in P2 and P4 upon CpG stimulation of sorted naïve B-cells.

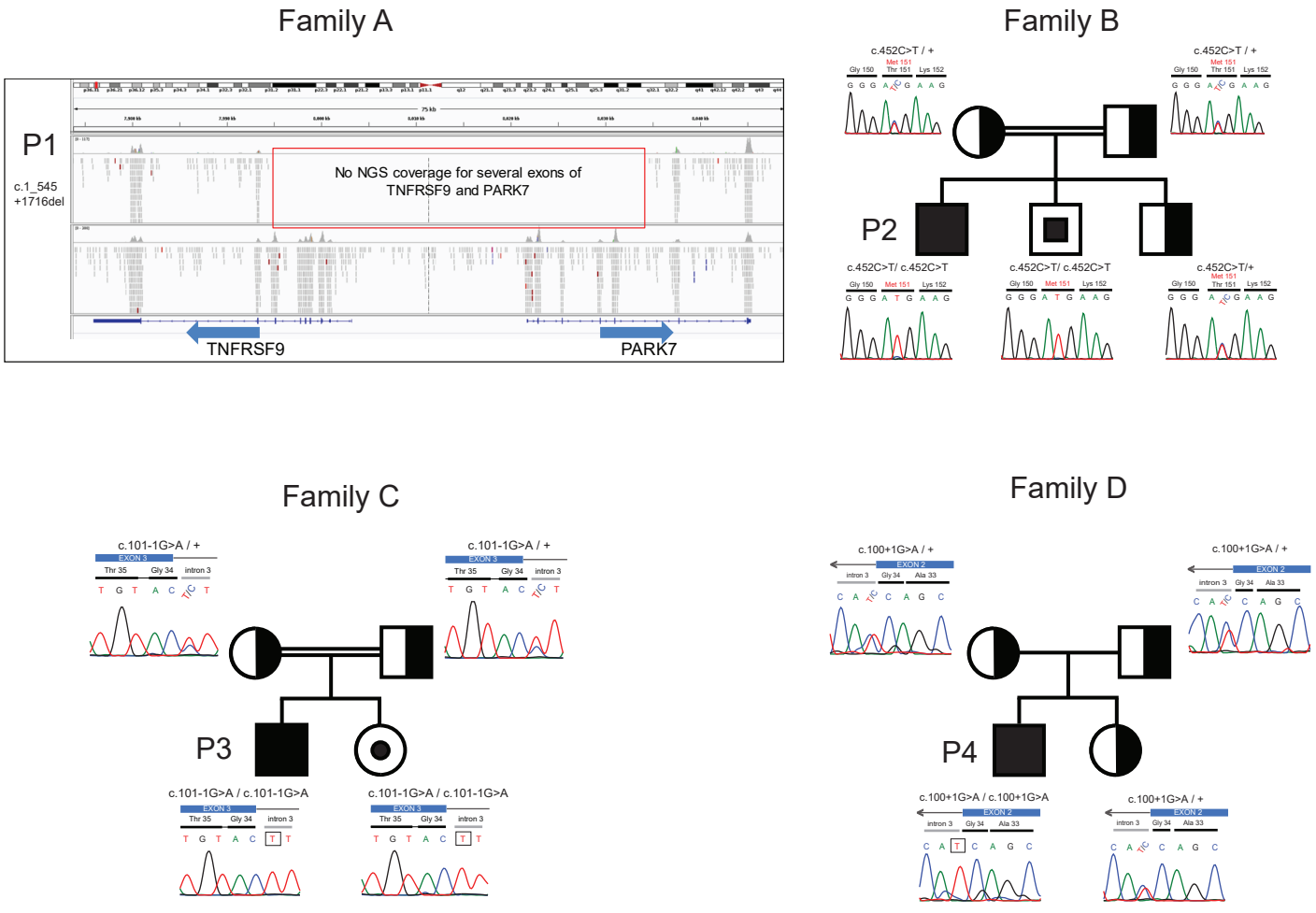
Supplementary Figure 1

Clinical Features, radiology, pathology，T-cell clonality



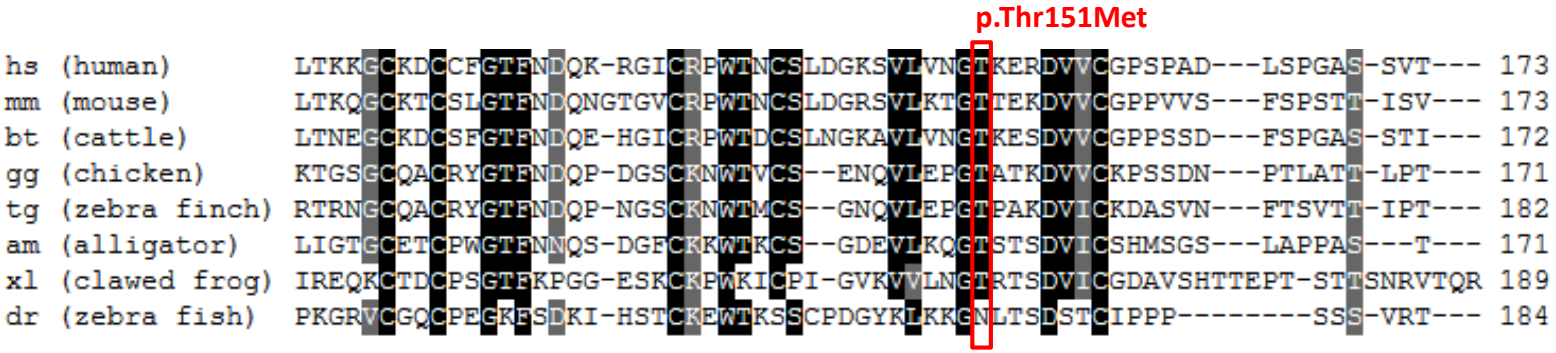
Supplementary Figure 2

Genetic analysis



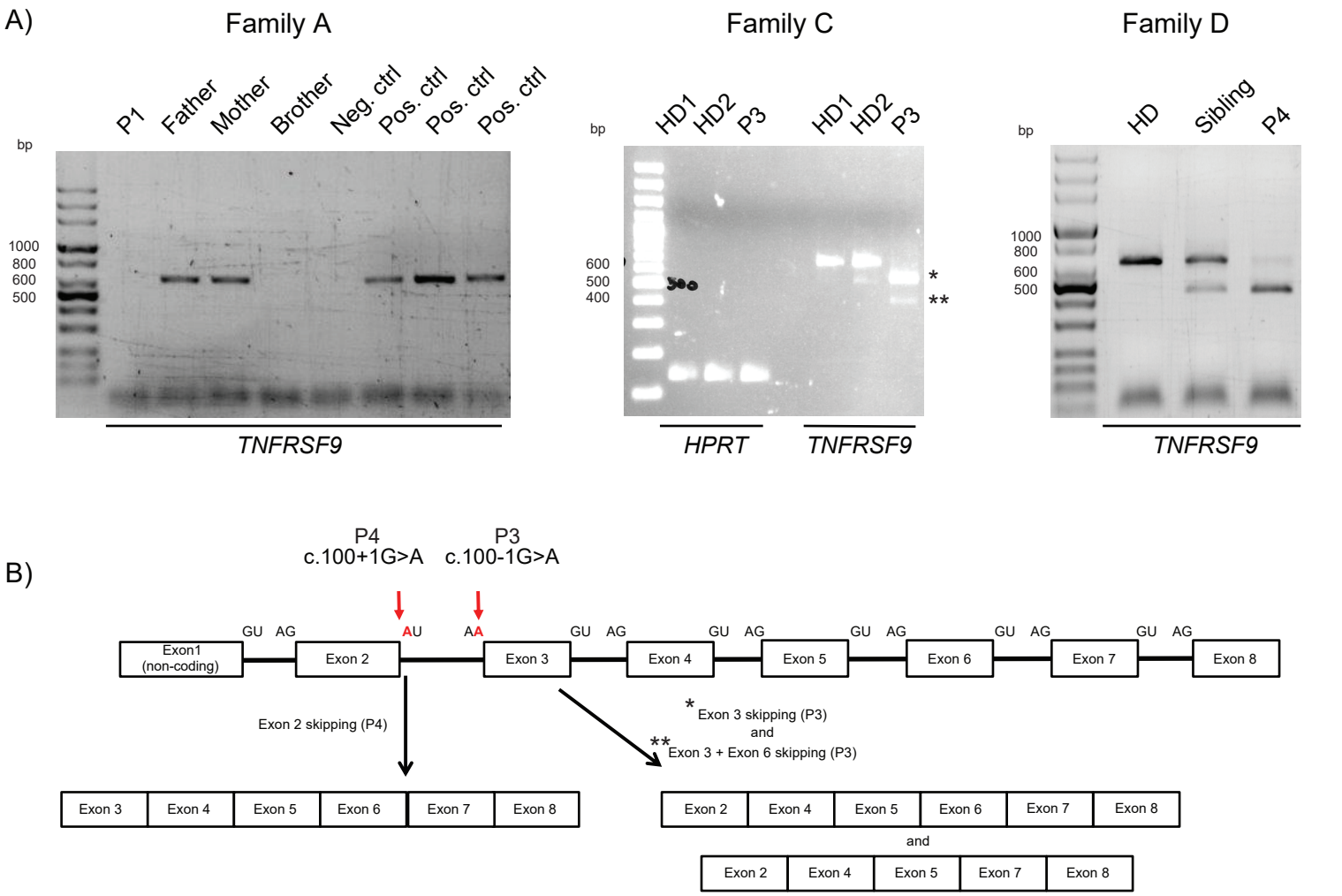
Supplementary Figure 3

TNFRSF9 sequence homology in species



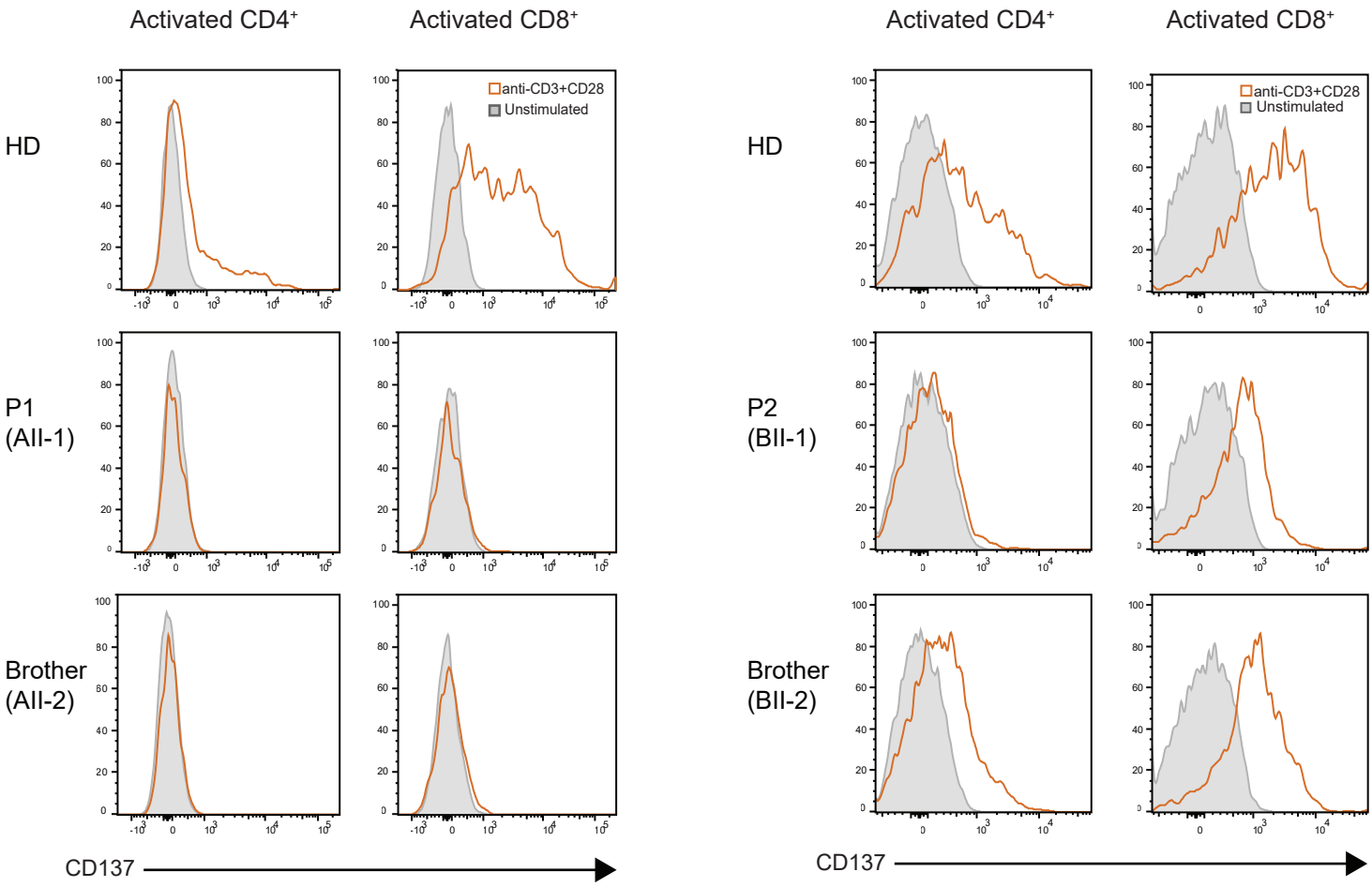
Supplementary Figure 4

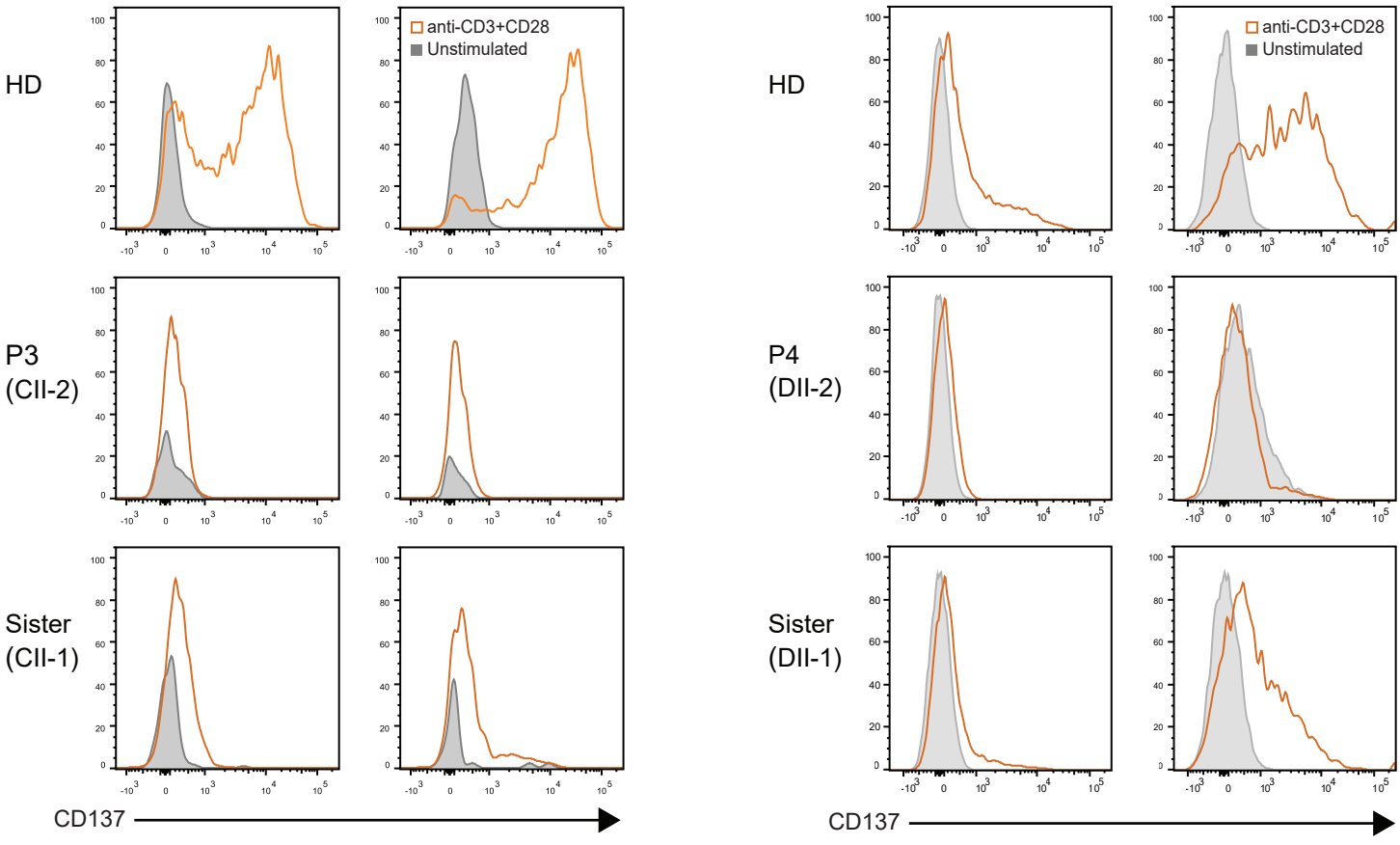
Effects of variants in the patients



Supplementary Figure 5

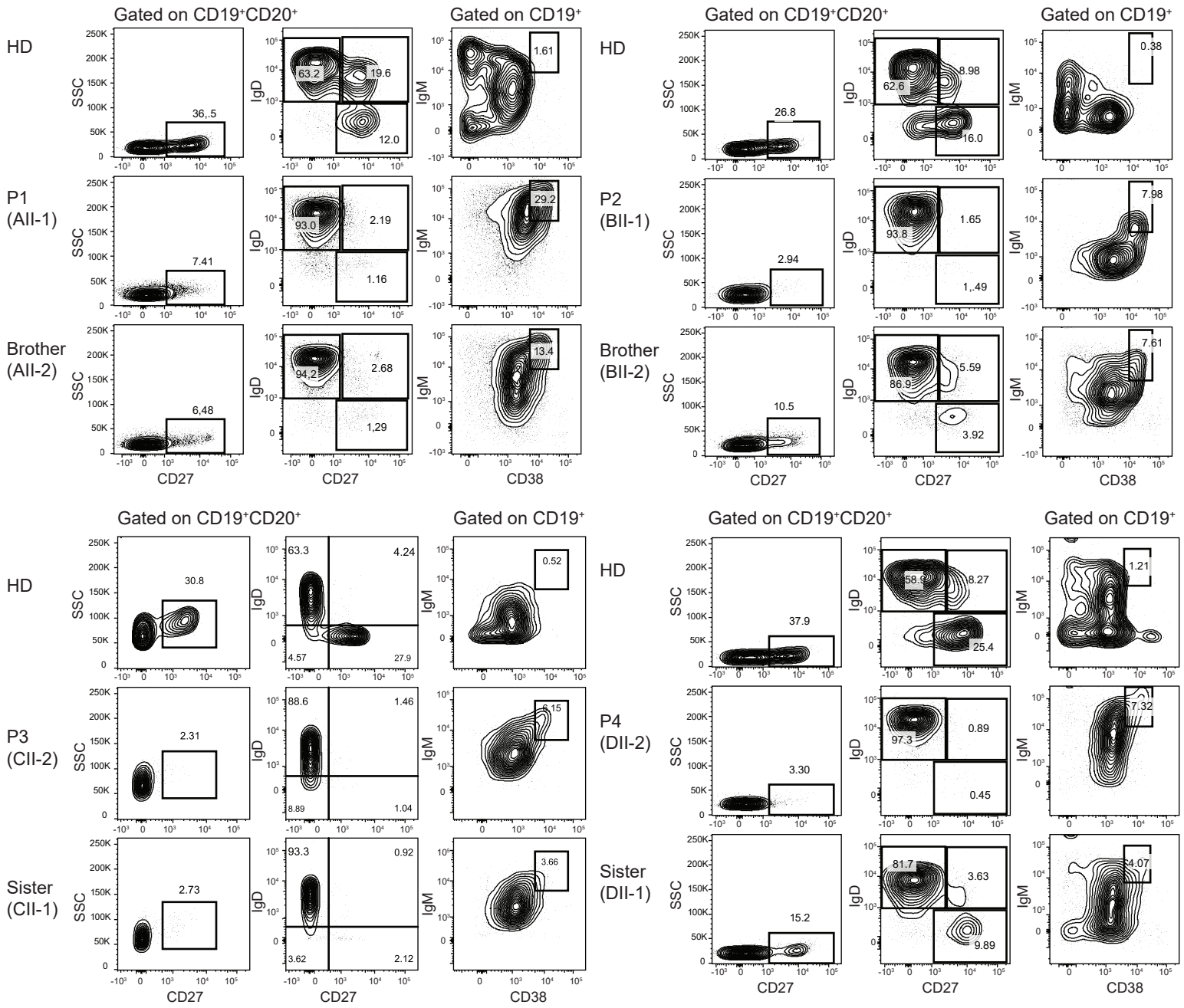
CD137 expression





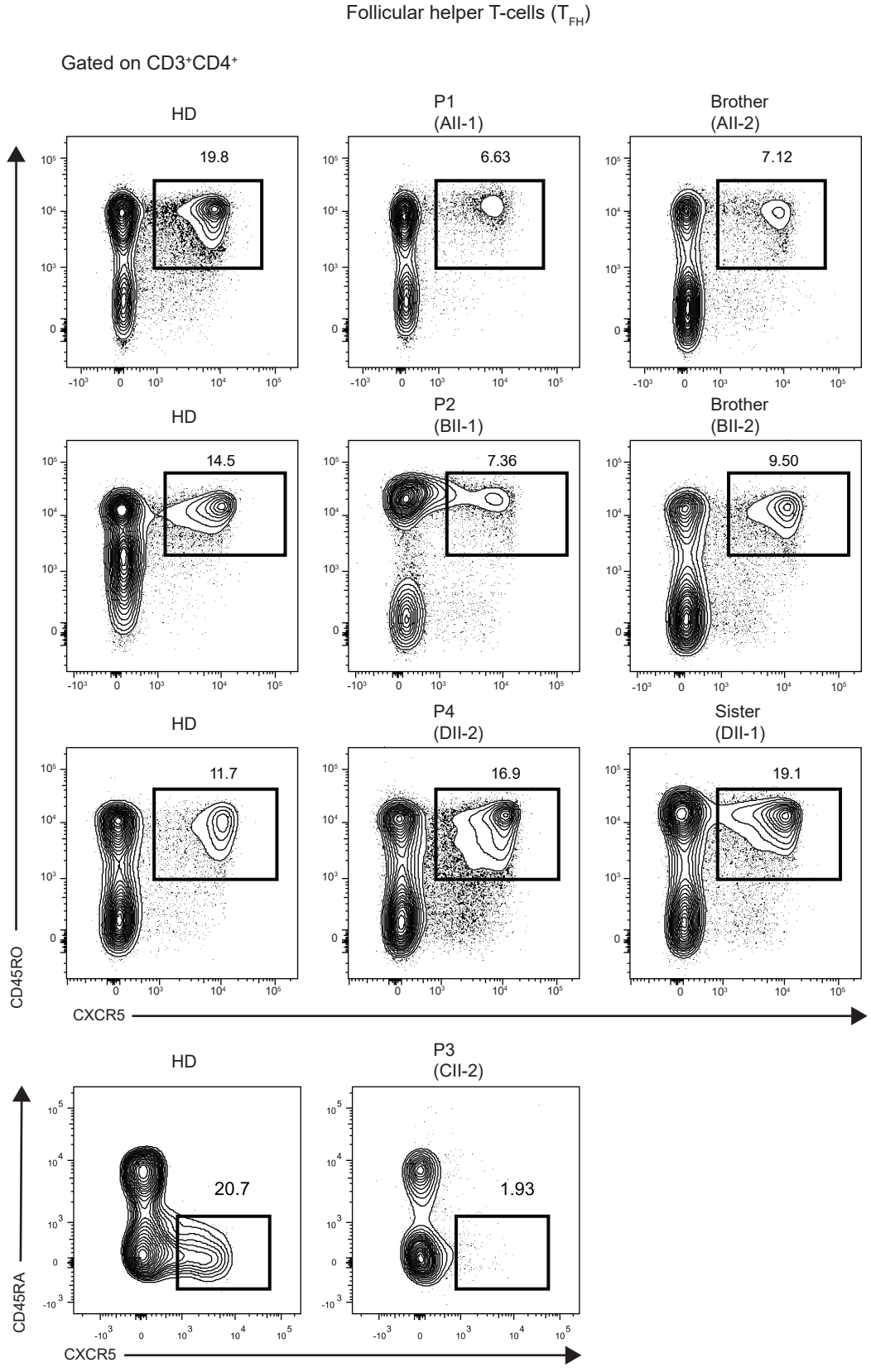
Supplementary Figure 6a

Peripheral blood B-cell immunophenotyping



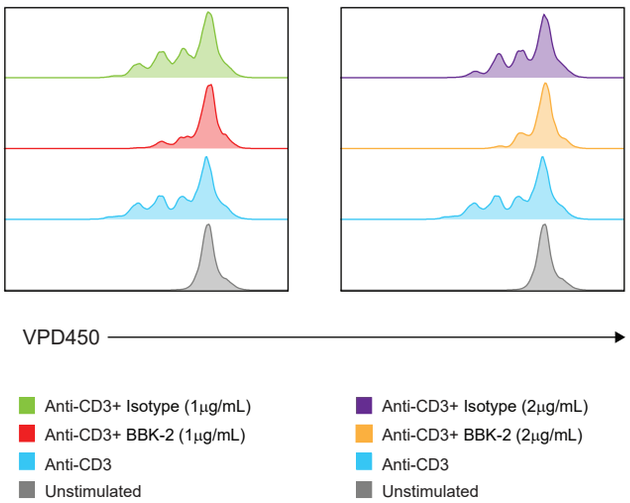
Supplementary Figure 6b

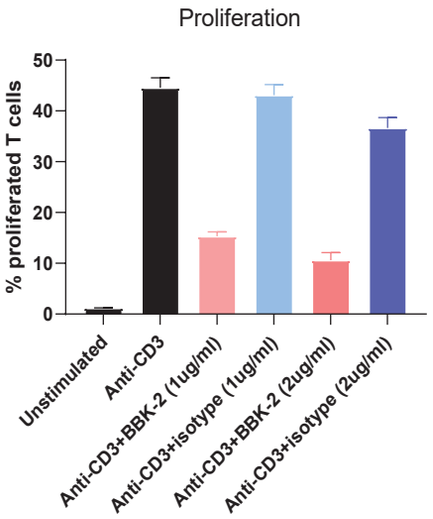
Peripheral blood T-cell immunophenotyping



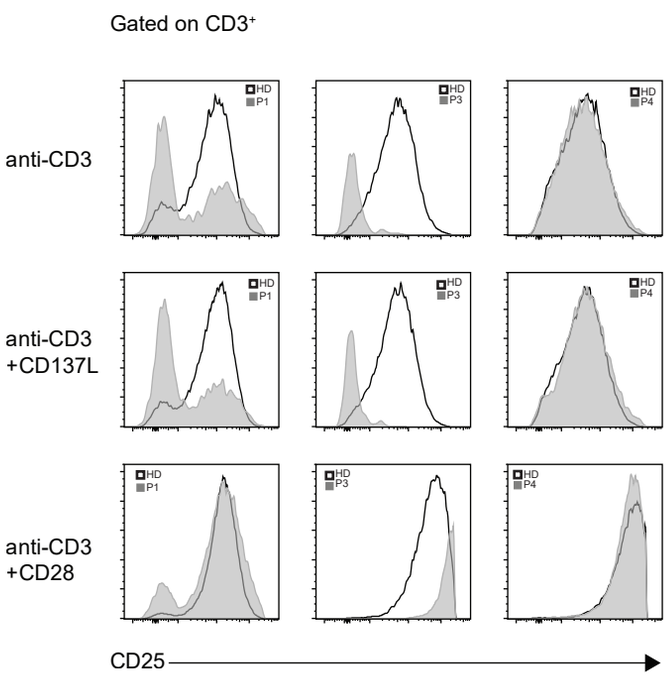
Supplementary Figure 7

A Blocking CD137 in HD T-cells

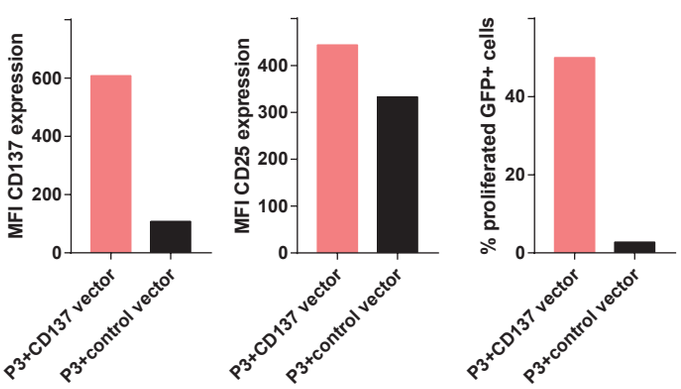




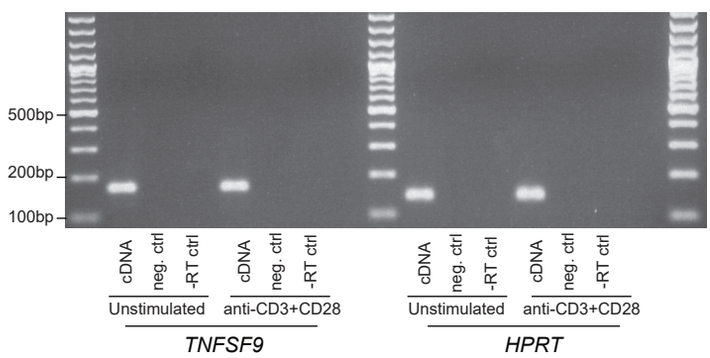
B T-cell activation (CD25 expression)

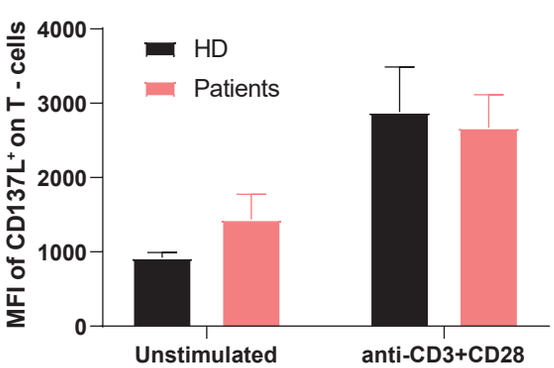


C Reconstitution of CD137 upon stimulation with anti-CD3 alone



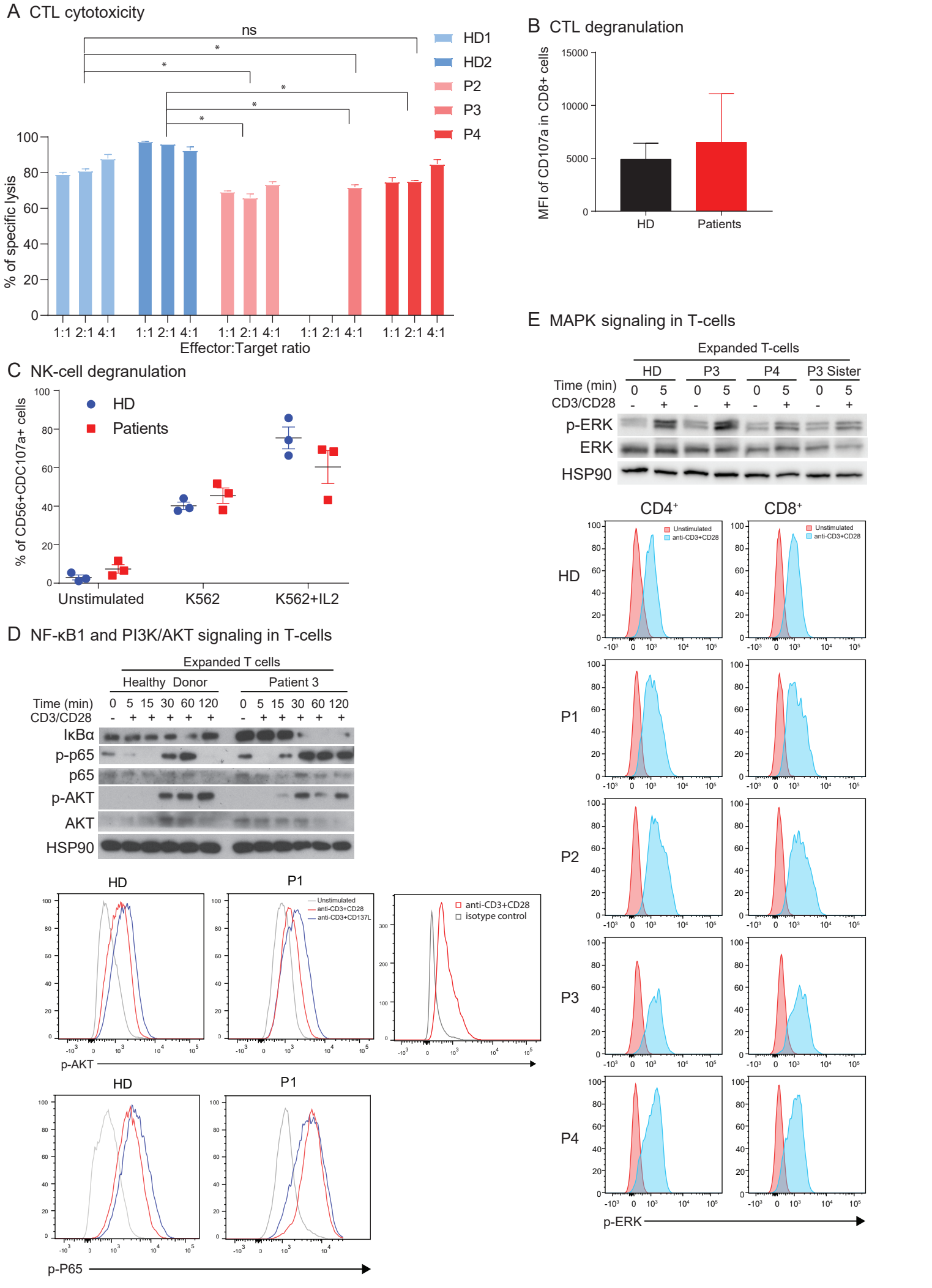
D *TNFSF9* and CD137L expression in T-cells





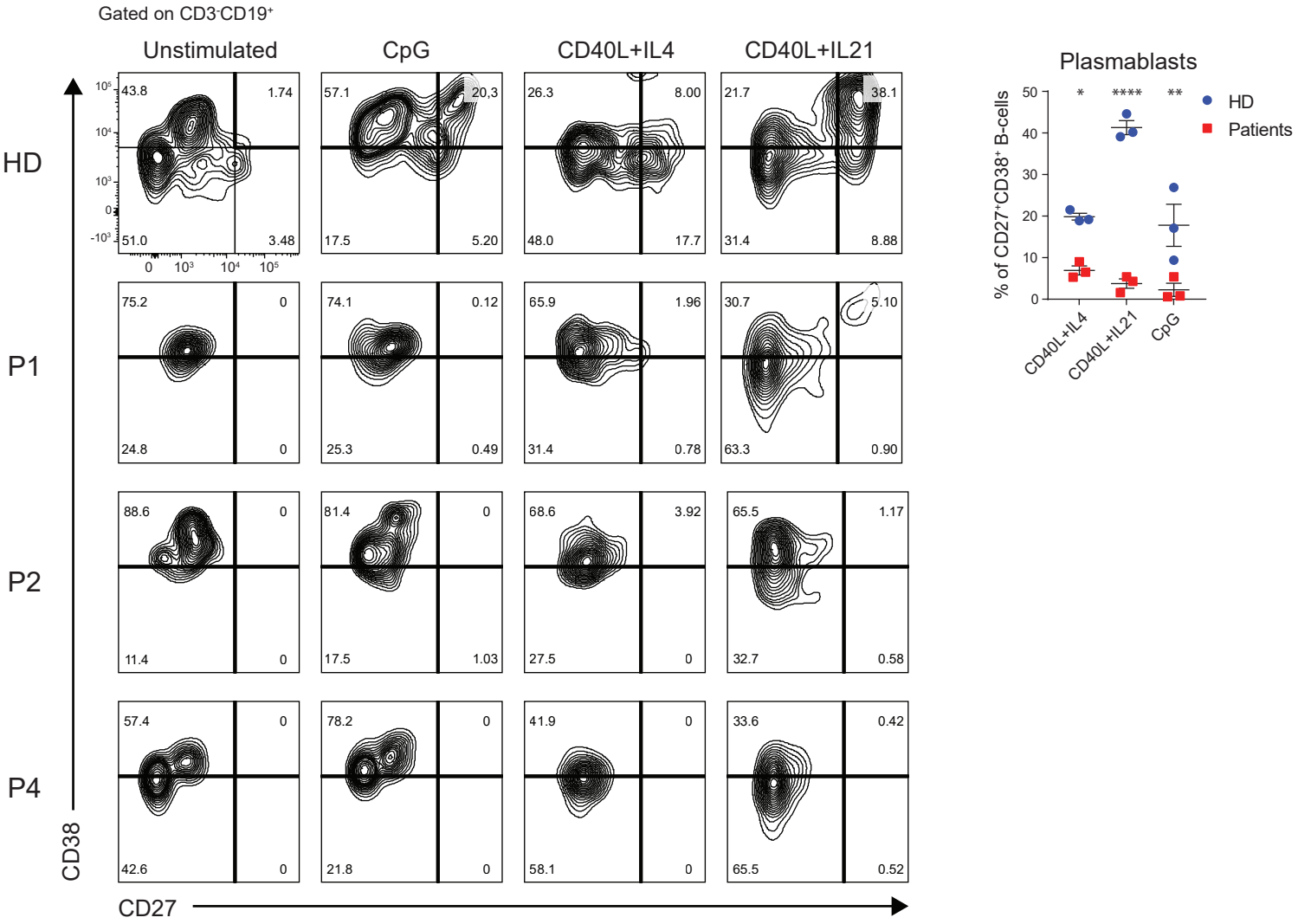
Supplementary Figure 8

CTL cytotoxicity, NK-cell degranulation and T-cell receptor mediated signaling

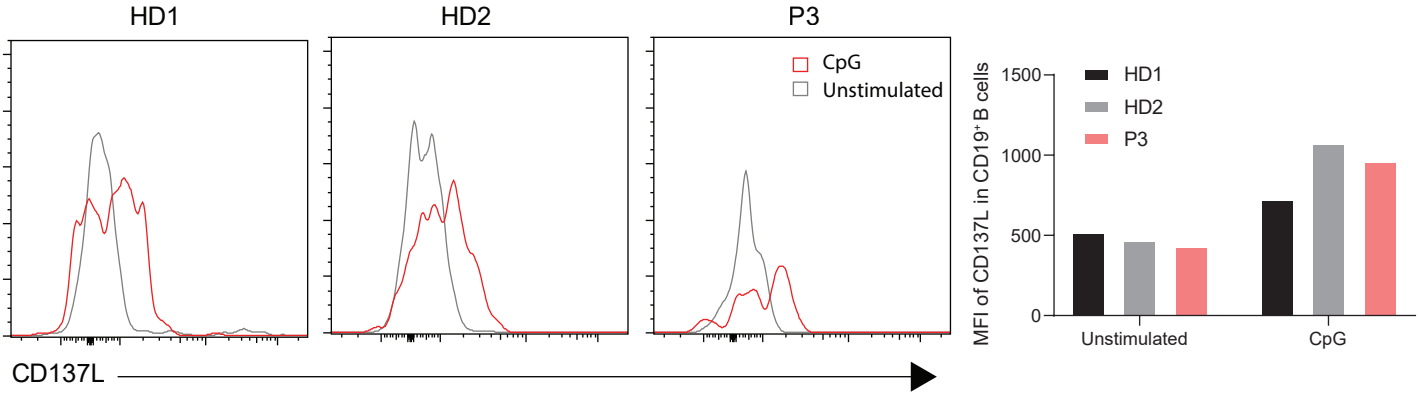


Supplementary Figure 9

A *In vitro* plasmablast differentiation

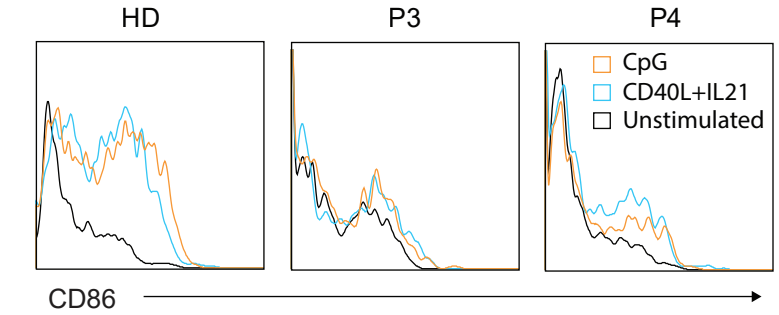


B CD137L expression on CD19⁺ B-cells

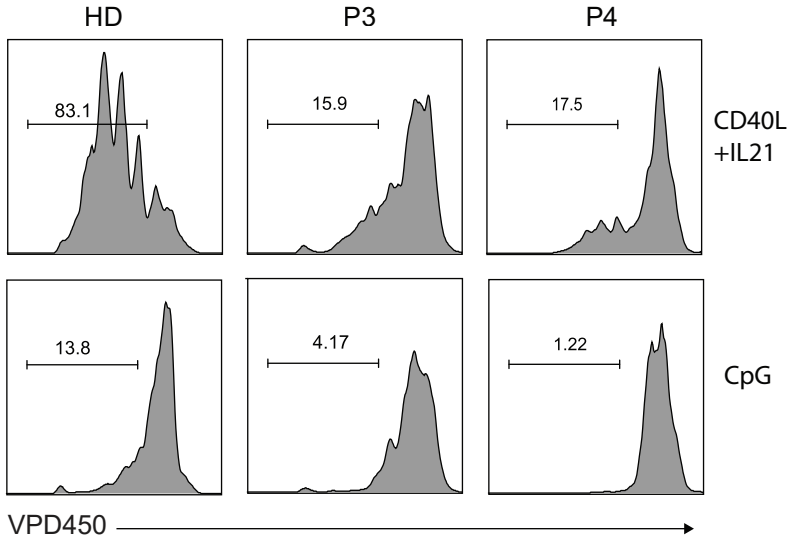


Supplementary Figure 10

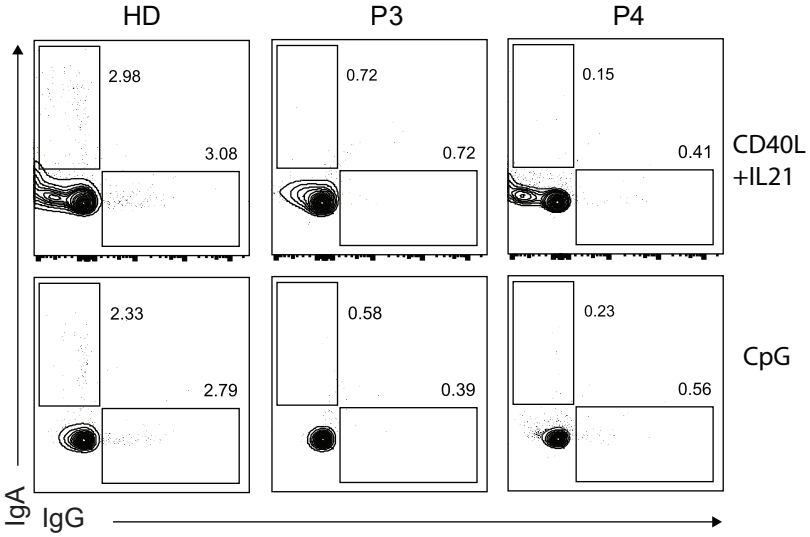
A CD86 cell surface expression in sorted naive B-cell



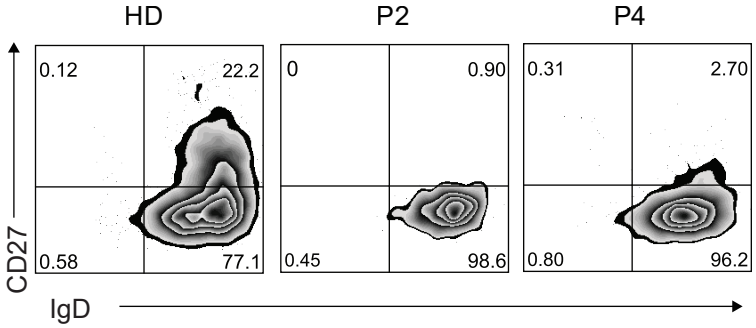
B Sorted naive B-cell proliferation



C Class switch recombination in sorted naive B-cells



D CpG stimulation in sorted naive B-cells



Journal of Clinical Immunology (2018) 38:699–710
https://doi.org/10.1007/s10875-018-0533-8

ORIGINAL ARTICLE

Novel Mutations in *RASGRP1* are Associated with Immunodeficiency, Immune Dysregulation, and EBV-Induced Lymphoma

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Received: 22 February 2018 / Accepted: 9 July 2018 / Published online: 20 July 2018

Abstract

Purpose RAS guanyl-releasing protein 1 (RASGRP1) deficiency has recently been shown to cause a primary immunodeficiency (PID) characterized by $CD4^+$ T cell lymphopenia and Epstein-Barr virus (EBV)-associated B cell lymphoma. Our report of three novel patients widens the scope of RASGRP1 deficiency by providing new clinical and immunological insights on autoimmunity, immune cell development, and predisposition to lymphoproliferative disease.

Methods One patient of Turkish origin (P1) and two Palestinian patients (P2, P3) were evaluated for immunodeficiency. To decipher the molecular cause of disease, whole exome sequencing was conducted. Identified mutations were validated by immunological and biochemical assays.

Results We report three patients presenting with similar clinical characteristics of immunodeficiency and EBV-associated lymphoproliferative disease. In addition, P2 and P3 exhibited overt autoimmune manifestations. Genetic screening identified two novel loss-of-function mutations in *RASGRP1*. Immunoblotting and active Ras pull-down assays confirmed perturbed ERK1/2 signaling and reduced Ras-GTPase activity in heterologous Jurkat cells with ectopic expression of RASGRP1 mutants. All three patients had $CD4^+$ T cell lymphopenia. P2 and P3 showed decreased mitogen-induced lymphocyte proliferation, reduced T cell receptor excision circles, abnormal T cell receptor (TCR) Vβ repertoires, and increased frequencies of TCRγδ cells. TCR gamma repertoire diversity was significantly reduced with a remarkable clonal expansion.

Conclusions RASGRP1 deficiency is associated with life-threatening immune dysregulation, severe autoimmune manifestations, and susceptibility to EBV-induced B cell malignancies. Early diagnosis is critical and hematopoietic stem cell transplantation might be considered as curative treatment.

Keywords Autoimmunity · EBV · lymphoproliferation · PID · RASGRP1 · T cell development

Ido Somekh, Benjamin Marquardt, Daniel Kotlarz, and Raz Somech contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10875-018-0533-8) contains supplementary material, which is available to authorized users.

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Introduction

Primary immunodeficiencies (PID) comprise a wide spectrum of rare genetic disorders that affect host immunity and predispose susceptibility for life-threatening infections, autoimmunity, and/or malignancies [1]. PID show phenotypic variabilities and many patients lack molecular diagnosis. Advanced next-generation sequencing technologies have expedited the diagnosis of PID, thus facilitating our knowledge of complex immune phenotypes and highlighting novel pathways controlling human immunity [2–4]. A prime example is the inborn defect in *RASGRP1* that has recently been described to cause severe immunodeficiency [5–8].

Mutations in the RAS superfamily pathway are known to have a significant role in human disease and specifically in lymphoproliferative disorders [9]. The intricate regulation of Ras activity in response to growth factors or extracellular stimuli is critical for the context of biological function and Ras proteins dynamically switch between inactive GDP-bound and active GTP-bound conformation. RASGRP1 is a guanine-nucleotide exchange factor (GEF) that converts Ras-GDP to Ras-GTP enabling activation of the RAS-REF-MAPK-ERK pathway, known to control key immune cell functions [10, 11]. RASGRP1-deficient mice have been previously shown to exhibit a marked T cell lymphopenia and immunodeficiency [12]. Importantly, the recent discovery of patients with biallelic loss-of-function mutations in *RASGRP1* presenting with immunodeficiency, impaired cytoskeletal dynamics, Epstein-Barr virus (EBV)-positive B cell lymphoma, and epidermodysplasia verruciformis has proven the fundamental role of RASGRP1 in human immune defense and lymphoproliferation [5–8] (Table 1). Here, we reinforce the knowledge about immunodeficiency, autoimmunity, and abnormal cell proliferation following EBV infections in patients with mutations in *RASGRP1*. Our results extend the clinical spectrum of RASGRP1 deficiency and provide new insights into this recently identified primary immunodeficiency.

Methods

Patients and Clinical Data

All procedures were performed upon informed consent and assent from patients, first-degree relatives, and healthy donor controls in accordance with the ethical standards of the institutional and/or national research committees and with the current update of the Declaration of Helsinki.

Immunological Evaluation

Cell surface marker expression of peripheral blood mononuclear cells (PBMC) was analyzed by immunofluorescent staining with monoclonal antibodies and flow cytometry (Epics V; Coulter Electronics, Hialeah, FL). Signal joint T cell receptor excision circles (sjTREC) copy numbers were determined by employing quantitative real-time PCR (qRT-PCR) of genomic DNA (gDNA, 0.5 μg) extracted from patients' PBMC [13]. Surface expression of individual T cell receptor Vβ (TCR Vβ) gene families was assessed using a set of 24 Vβ-specific fluorochrome-labeled monoclonal antibodies (Beckman Coulter, USA) and flow cytometry [14]. Next-generation sequencing (NGS) T cell receptor (TCR) libraries were generated from gDNA of patients and controls using primers for conserved regions of V and J genes in the *TRG* (T cell receptor gamma) locus according to the manufacturer's protocol (Lymphotrack, Invivoscribe Technologies, Carlsbad, CA). Quantified libraries were pooled and sequenced using Mi-Seq Illumina technology (Illumina, USA). FASTA files from the filtered sequences were submitted to the IMGT HighV-QUEST webserver (http://www.imgt.org), filtered for productive sequences (no stop codons or frameshifts), and analyzed [15]. Repertoire diversity was calculated using Shannon's and Gini-Simpson's diversity indices [16].

$$\text{Shannon}'\text{s entropy index}: \quad H' = -\sum_{i=1}^{R} p_i \ln p_i$$

And Gini-Simpson′s index of unevenness :

$$1\text{-} = 1-\sum_{i=1}^{R_2} p_i = 1-1/{}^2\mathrm{D}$$

Exome Sequencing Analysis and Sanger Sequencing

NGS was performed at the Dr. von Hauner Children's Hospital NGS facility. Genomic DNA was isolated from whole blood of patients and their first-degree relatives for generation of whole exome libraries using the SureSelect XT Human All Exon V5+UTR or V6+UTR kit (Agilent Technologies, USA). Barcoded libraries were sequenced on a NextSeq 500 platform (Illumina, USA) with an average coverage depth of 100x. Bioinformatics analysis and subsequent filtering identified rare sequence variants. *RASGRP1* mutations were confirmed by Sanger sequencing.

CRISPR-Cas9-Mediated Engineering of RASGRP1 Knockout Cell Lines

Two genomic loci in *RASGRP1* (transcript ENST00000310803.9) were designated for gene disruption by inducing double-strand breaks in exon 3 (T1: 5'-GTGTCGAAGTAACCAACTGT-3′) and exon 16 (T2: 5'-GTCTTGGTCAGAAAGCGGGC-3′). Genome editing of Jurkat cells (ATCC, USA) was performed employing the Alt-R® CRISPR-Cas9 system (IDT technology, Belgium)

Table 1 Clinical manifestations of newly identified and previously published RASGRP1-deficient patients

Patients	Pa (Ref. 5)	Pb (Ref. 6)	Pc (Ref. 7)	Pd (Ref. 7)	Pe (Ref. 8)	Pf (Ref. 8)	P1	P2	P3
Demographics and genetics									
Mutation	c.726C>T	c.771G>A	c.641C>T; c.946A>T	c.641C>T; c.946A>T	c.1910_1911 insAG	c.1910_1911 insAG	c.649_650inv	c.1111_1114del	c.1111_1114del
Origin	Turkish	Iraqi	n.k.	n.k.	n.k.	n.k.	Turkish	Palestinian	Palestinian
Age of presentation	n.k.	6 months	2 years	1 month	5 years	7 years	<1 year	2 months	4 months
Sex	Male	Female	Female	Male	Male	Female	Female	Male	Male
Consanguinity	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes
Infectious manifestations									
Recurrent infections	+	+	+	+	+	+	+	+	+
Recurrent pneumonia	+	–	+	+	+	+	+	+	+
Abscess	–	+	–	–	–	–	–	+	–
Pathogens	EBV, *herpes*	Human *Papilloma virus*	*Candida albicans, Haemophilus influenzae, Moraxella catarrhalis, Mycoplasma pneumonia, Staph. aureus*	*Aspergillus,* CMV, EBV, *Mycoplasma pneumonia, Mycobacterium tuberculosis, Staph. aureus*	EBV, *Mycobacterium tuberculosis*	EBV, *Pneumocystis jirovecii*	*Herpes zoster, Molluscum contagiosum*	CMV, EBV	CMV, EBV, HSV1, *Staph. coagulase-negative*
Lymphoproliferation and malignancy									
Hepatosplenomegaly	+	+	+	+	n.k.	n.k.	+	+	+
Lymphadenopathy	+	+	+	+	n.k.	n.k.	+	+	+
EBV-associated lymphoproliferation	+	+	–	–	+	+	+	+	+
Malignancy	+	+	–	–	+	+	+	–	+
Autoimmunity									
AIHA	–	+	+	+	–	–	–	+	+
ITP	–	+	+	+	–	–	–	+	+
Other								TTP	Posterior uveitis, AIH
Additional clinical manifestations									
Failure to thrive	+	+	n.k	n.k.	n.k.	n.k.	+	+	+
Other	Finger clubbing	Disseminated warts, chronic diarrhea	Leiomyoma-adrenal and liver	–	–	Adrenal EBV-smooth muscle tumor	–	Steatorrhea, bloody diarrhea	–

Table 1 (continued)

Patients	Pa (Ref. 5)	Pb (Ref. 6)	Pc (Ref. 7)	Pd (Ref. 7)	Pe (Ref. 8)	Pf (Ref. 8)	P1	P2	P3
Outcome	Allogeneic HSCT, remission	Exitus following lymphoma complications	Remission	Remission	Autologous HSCT, remission	Exitus at age 11 years following relapse of Hodgkin lymphoma	Autologous HSCT, remission	Exitus at age 4 years due to TTP	Remission, awaiting HSCT

n.k. not known; *TTP* thrombotic thrombocytopenic purpura; *ITP* immune thrombocytopenic purpura; *AIHA* autoimmune hemolytic anemia; *AIH* autoimmune hepatitis; *EBV* Epstein-Barr virus; *CMV* cytomegalovirus; *HSV* Herpes simplex virus; *Staph.* Staphylococcus

according to the manufacturer's instructions. Briefly, equimolar amounts of crRNA and ATTO™ 550 (ATTO-TEC, Germany) fluorescent dye-labeled tracrRNA were incubated for 5 min at 95 °C in TE buffer and slowly cooled down to room temperature. The RNA duplexes were electroporated together with Cas9 nuclease into the target cells using SG Cell Line 4D-Nucleofector® X Kit and the 4D-Nucleofector™ System (Lonza, Switzerland). After 48 h in a tissue incubator, red fluorescent protein (RFP)-positive cells were single-sorted into 96-well plates on a BD FACSAria (BD Bioscience, USA). Upon clonal cell expansion *RASGRP1*$^{-/-}$ knockout was confirmed by immunoblotting.

Construction of Expression Vectors, Cell Culture, Transfection, and Lentiviral Transduction

All biochemical assays were performed on Jurkat cells that were routinely tested negative for mycoplasma contamination. Jurkat cells and their derivatives were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 200 mM L-glutamine (all from Gibco, Life Technologies, USA) in a humidified incubator at 37 °C with 5% CO_2. Human *RASGRP1* was amplified from a Mammalian Gene Collection sequence-verified cDNA clone (cat. no. MHS6278-211690246, accession: BC109297; Dharmacon GE Healthcare, USA). Mutations in *RASGRP1* encoding p.E217R (P1), p.D371Ifs* (P2/P3), and p.R246* [5] were introduced by site-directed PCR mutagenesis using specific primer pairs. Sequence confirmed cDNAs were cloned into the lentiviral pRRL vector harboring IRES-murine CD24 (mCD24) as selection marker.

RASGRP1$^{-/-}$ Jurkat cells were reconstituted with RASGRP1 wild-type or mutant variants by transduction with lentiviral particles according to previously published protocols [17]. Briefly, vesicular stomatitis virus G glycoprotein (VSV-g)-pseudotyped lentiviral particles were generated by transfection of HEK293T cells (DSMZ, Germany). Using polyethyleneimine (Polysciences, USA) as a transfection agent, cells were incubated with 5 μg lentiviral vector, 12 μg pcDNA3.GP.4xCTE (which expresses HIV-1 gag-pol), 5 μg pRSV-Rev, and 1.5 μg pMD.G (which encodes VSV-g) in the presence of 25 μM chloroquine (Sigma, USA) for 12 h. Supernatants containing viral particles were collected every 24 for 72 h and concentrated by ultracentrifugation. Viral titers were determined in transduced HEK293T cells by FACS-based detection of expression of mCD24. Next, *RASGRP1*$^{-/-}$ Jurkat cells were transduced with lentiviral particles by spinoculation at 900 g for 4 h at 32 °C. To establish stable cell lines, transduced cells were sorted based on mCD24 expression using a BD FACSAria cell sorter (BD Bioscience, USA).

Immunoblotting and Active Ras Pull-Down Assay

To study protein expression and downstream MAPK signaling 1×10^6 Jurkat cells were serum-starved for 12 h in RPMI 1640 medium, followed by stimulation with 50 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma, USA) for indicated time points. Cells were washed once with ice-cold PBS containing 10 mM sodium fluoride and 100 mM sodium orthovanadate (both Sigma, USA) and subsequently lysed in 1X cell lysis buffer (Thermo Scientific, USA) containing 1 mM PMSF (Alpha Diagnostic, USA) and 1X PIC (Sigma, USA). To study Ras small GTPase activation, the Active Ras Pull-Down and Detection Kit (Thermo Scientific, USA) was used according to the manufacturer's instructions. Briefly, cell lysates were incubated for 1 h at 4 °C with a GST-Raf1-RBD fusion-protein, containing a human Ras binding domain, and pulled down using glutathione resin. After washing the resin, bound Ras was eluted by heat incubation at 95 °C in 2X SDS sample buffer for 10 min. Relative protein concentration was determined by Bradford assay. Pull-down samples and cell lysates were fractionated under reducing conditions by SDS–PAGE and proteins were blotted onto polyvinylidene difluoride membranes (GE Healthcare, UK) using the Trans-Blot Turbo Transfer System (Bio-Rad, Germany). Membranes were blocked in 5% BSA (Applichem, USA) in PBS prior antibody staining. Antibodies used for detection are indicated in Supplementary Table S3. Membranes were developed using a chemiluminescent substrate (Thermo Scientific, USA) and images were captured using a ChemiDoc XRS+ System (Bio-Rad, Germany).

Statistics

Statistical evaluation of experimental data was performed using Prism version 6 (GraphPad Software, USA). Figure 3b shows mean ± SD. Figure 3d shows individual data points including mean ± SEM. Probability (p) values were calculated using a two-tailed unpaired t test and p values < 0.05 were considered statistically significant.

Results

Clinical Presentation of RASGRP1-Deficient Patients

In our study, one patient of Turkish origin (P1) and two Palestinian patients (P2, P3) from consanguineous families shared many clinical characteristics and were referred to our centers due to suspected immunodeficiencies (Fig. 1a).

Patient 1 (P1, A.II-6; Fig. 1a) presented with fatigue, weight loss, and a subscapular mass at the age of 6 years. Medical history showed failure to thrive, recurrent pulmonary infections since infancy and a herpes zoster infection (Table 1). Laboratory testing indicated a persistent $CD4^+$ T cell lymphopenia since the age of 3 years. Family history revealed two siblings who died of pneumonia during infancy. Thoracic and abdominal computed tomography (CT) scans indicated hepatosplenomegaly as well as para-aortic and inguinal lymphadenopathy. Histopathologic examination from the left subscapular mass biopsy revealed an EBV-positive diffuse large B cell lymphoma (DLBCL) stage III. NHL BFM 90 protocol [18] was started, but the patient had a relapse with involvement of mediastinal, para-aortic, abdominal, and tonsillary lymph nodes as well as bone marrow. She received three courses of rituximab, ifosfamide, carboplatin, and etoposide but had a second relapse for which she was treated with two courses of R-CHOP [19] and achieved remission. She underwent autologous hematopoietic stem cell transplantation (HSCT) at the age of 8 years prior to genetic diagnosis, and she is currently showing a stable clinical course at the age of 14 years.

Patient 2 (P2, B.III-1; Fig. 1a) and patient 3 (P3, C.III-7; Fig. 1a) manifested during infancy with Evans syndrome consisting of immune thrombocytopenia and severe Coombs-positive hemolytic anemia, necessitating frequent blood transfusions, glucocorticoids treatment, and intravenous immunoglobulin infusions. Both P2 and P3 also presented with recurrent episodes of pneumonia, failure to thrive, lymphadenopathy, and hepatosplenomegaly (Table 1). In addition, P2 had a prolonged steatorrhea, an episode of dental abscess, and elevated EBV and CMV (cytomegalovirus) viral loads. High levels of β2-glycoprotein were observed (Table 2). Subsequently, he was admitted to the intensive care unit at the age of 4 years due to seizures and coagulopathy, suggestive of thrombotic thrombocytopenic purpura (TTP), which he had succumbed to. Postmortem examination showed severe coagulopathy, bronchial hyperplasia, and a pulmonary EBV infection. P3 had additional autoimmune manifestations, including right-sided severe posterior uveitis requiring treatment with intravitreous methotrexate, autoimmune hepatitis with a prominent population of small T and B cells, and plasma cell infiltrates in liver biopsy. An elevated titer of antinuclear antibody (ANA) was detected (Table 2). At the age of 3 years, the patient developed DLBCL. He was treated according to the R-CHOP protocol [19] inducing a reduction in EBV blood viral load (1×10^6 down to 2.5×10^4 copies/ml) and remission. The patient is currently awaiting allogeneic HSCT.

Identification of Novel Biallelic *RASGRP1* Mutations

In view of the consanguinity in both unrelated pedigrees, monogenic diseases following a homozygous recessive trait were suspected in our patients. Whole exome sequencing identified novel biallelic *RASGRP1* mutations in the affected children (Supplementary Tables S1 and S2). P1 had an

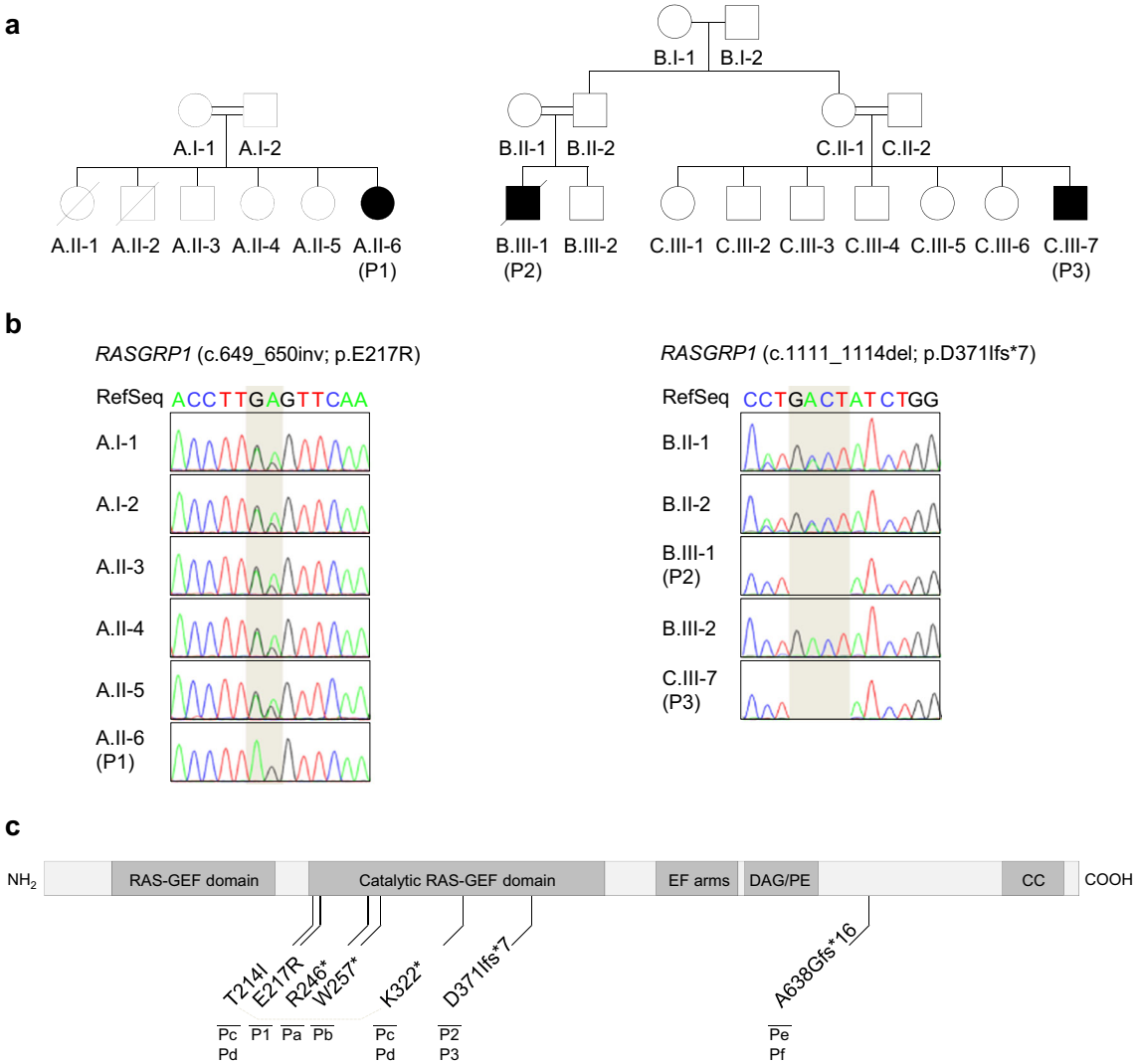

Fig. 1 Identification of patients with novel biallelic loss-of-function mutations in *RASGRP1*. **a** Pedigrees of two unrelated kindred with three patients: P1 (A.II-6), P2 (B.III-1), and P3 (C.III-7). Double lines indicate consanguinity (first-degree cousins); filled black circles or squares depict the patients and diagonal lines indicate deceased individuals. **b** Sanger sequencing chromatograms confirmed segregation of the identified *RASGRP1* sequence variants in first-degree relatives: P1, c.649_650inv; P2 and P3, c.1111_1114del. **c** Schematic illustration of the RASGRP1 protein and its domains, indicating the newly identified and previously published mutations (DAG/PE, diacylglycerol/phorbol-ester binding; CC, coiled coil domain)

inversion (NM_005739.3; c.649_650inv, p.E217R), P2 and P3 had a frameshift mutation (NM_005739.3; c.1111_1114del, p.D371Ifs*7) leading to a premature termination in exon 9. Segregation of the identified mutations with the disease phenotype was confirmed by Sanger sequencing (Fig. 1b). The *RASGRP1* variant p.E217R identified in P1 is rare and has not been described in the genome aggregation database (gnomad) [20]. The amino-acid substitution was predicted by the Polymorphism Phenotyping v2 (PolyPhen-2) tool [21] to affect protein function (score of 0.999). The mutation p.D371Ifs*7 from P2 and P3 has been reported as a heterozygous single nucleotide polymorphism in gnomad (rs761476720) but not homozygous (245,490 total alleles). Our patients shared many features that have recently been reported in mice and particularly in other patients with RASGRP1 deficiency, thus confirming our hypothesis that the homozygous *RASGRP1* variants are causative for the patients' disease. PID syndromes with similar characteristics of immune dysregulation, such as lipopolysaccharide-responsive and beige-like anchor protein (LRBA) deficiency and autoimmune lymphoproliferative syndrome (ALPS) were ruled out by genetic analysis.

Analysis of RASGRP1 Protein Expression and Function

The effect of the newly identified mutations on protein expression of RASGRP1 was determined by immunoblotting in a heterologous Jurkat cell model due to lack of primary patient material. We generated *RASGRP1*$^{-/-}$ Jurkat T cell lines by

employing CRISPR/Cas9-mediated genetic engineering and lentiviral overexpression of wild-type or mutant *RASGRP1* alleles. The previously reported variant p.R246* [5] has been used as control. Whereas, we could observe a substantial expression of wild-type RASGRP1 and the variant p.E217R, the mutants p.D371Ifs*7 and p.R246* could not be detected by Western blot analysis using commercially available antibodies (Fig. 2a). Importantly, Jurkat cells overexpressing the newly identified RASGRP1 mutants exhibited a decreased phosphorylation of ERK1/2 upon treatment with PMA (Fig. 2a), confirming loss-of-function of RASGRP1 in P1, P2, and P3. Correspondingly, RasGTPase activity upon PMA stimulation was reduced in Jurkat cells with knockout of *RASGRP1* or overexpression of RASGRP1 mutants in comparison to unmodified Jurkat cells or wild-type RASGRP1 (Fig. 2b).

Loss-of-Function of RASGRP1 is Associated with T Cell Dysfunction, TCR Clonality, and Autoimmunity

Immunophenotyping of PBMC from patients P1, P2, and P3 revealed $CD4^+$ T cell lymphopenia and elevated $CD8^+$ T cells, whereas frequencies of $CD19^+CD20^+$ B cells and $CD16^+CD56^+$ natural killer (NK) cells were normal (Table 2). Moreover, P2 and P3 showed a markedly increased proportion of TCRγδ cells. T cell proliferation was reduced in P2 and P3 in response to phytohemagglutinin (PHA) and anti-CD3 (Fig. 3a). T cell receptor excision circles (TRECs) of P2 and P3 were significantly lower than in age-matched controls, suggesting an impaired production of naïve T cells. Serum IgG levels were elevated for both patients, whereas antibody responses to vaccinations were normal. A laboratory workup for autoimmunity revealed increased serum levels of ANA for P3 and high levels of β2-glycoprotein in P2. Tests for human immunodeficiency virus were negative.

The TCR profile for P2, P3 was assessed using flow cytometry-based TCR Vβ assays (Fig. 3b). Abnormal TCR Vβ repertoire patterns could be observed in both patients. For P2, the TCR Vβ repertoire depicted a clonal expansion of Vβ 13.1 and underexpression of most of the other Vβ's (1, 2, 4, 5.1, 5.3, 7.2, 12, 13.2, 14, 16, 18, 21.3). This trend was consistent during a year of follow-up. For P3, the TCR Vβ repertoire demonstrated an oligoclonal pattern with overexpression of Vβ 1, 7.1, 8, 12, and 21.3 and underexpression of the other Vβ's (4, 5.2, 7.2, 13.2, 13.6, 14, 16, 17, 22). To further characterize the TCR repertoire, high-throughput immunosequencing of the TRG repertoire was performed on PBMC from P2, P3 and four age-matched healthy donor (HD) controls. The two patients had similar numbers of unique productive TRG sequences as controls (P2: 7813; P3: 1870; HD: average 4243, 2068–6558), but significantly higher total productive sequences due to significant clonal expansion (P2: 298246; P3: 384054; HD: average 177648, 142900–207469; $p < 0.01$) (Fig. 3c). The distribution of clones within the patients' repertoires was significantly less even than that of control samples ($p = 0.006$), as measured by the Simpson's D diversity index (Fig. 3d). Since Simpson's D index measures the likelihood that two clones drawn randomly from the repertoire would be identical, increased Simpson's D indices suggested a more clonal (less diverse) T cell repertoire in our patients. Reciprocally, Shannon's H' index measures the evenness of a repertoire, evaluating its diversity and the abundance of each individual clone. The reduced Shannon's H' scores indicate a less evenly distributed repertoire in our patients as compared to controls (Fig. 3d). Together, our data suggest RASGRP1-deficient patients have a clonal T cell expansion, reciprocally diminishing their overall T cell receptor repertoire diversity.

Discussion

RASGRP1 is a key regulator of immune responses, including pre-TCR development [22], positive selection of TCRαβ cells [23, 24], invariant NK cell development [22] and functions [25], as well as B cell signaling, proliferation, and development [5, 26]. Inborn defects in *RASGRP1* have recently been described to cause severe illness in six patients [5–8]. The main clinical findings of published RASGRP1-deficient patients included recurrent infections, hepatosplenomegaly, lymphadenopathy, EBV-associated lymphoproliferation and B cell lymphoma [5–8], as well as autoimmune features [6, 7]. Here, we report two novel *RASGRP1* mutations in three patients from two unrelated consanguineous kindred. All three patients exhibited clinical signs of immunodeficiency, including failure to thrive, recurrent pulmonary infections, and EBV-associated lymphoproliferation. Two patients (P1, P3) developed EBV-positive B cell lymphoma. In addition, the patients suffered from increased susceptibility to herpes virus infections (Herpes simplex virus (HSV), Varicella zoster virus (VZV), CMV, and EBV), and pyogenic infections, including recurrent pneumonia, empyema, recurrent ear infections, as well as dental and skin abscesses. Both P2 and P3 exhibited overt autoimmune manifestations, including severe recurrent autoimmune hemolytic anemia (AIHA) and thrombocytopenia, while uveitis and hepatitis were observed in P3. The clinical manifestations of our patients with RASGRP1 deficiency help to define phenotypes expected to be observed with these inborn defects, e.g., autoimmunity, impaired T cell development, susceptibility to infections, and lymphoproliferation.

Immunologically, RASGRP1 deficiency has been characterized by CD4 T cell lymphopenia and impaired CD8 signaling and proliferation, impaired B cell development, proliferation, and signaling, as well as NK cytotoxic function abnormalities [5, 6]. Patients P2 and P3 also exhibited CD4 T cell lymphopenia, inverted

Table 2 Immunological and autoimmune workup of newly identified and previously published RASGRP1-deficient patients

Patients	Pa (Ref. 5)	Pb (Ref. 6)	Pc (Ref. 7)	Pd (Ref. 7)	Pe (Ref. 8)	Pf (Ref. 8)	P1	P2	P3
Complete blood count (normal range)[3]									
Hemoglobin [g/dL] (11.5–13.5)	n.k.	*8.2*	*low*	*low*	n.k.	n.k.	*11.3*	*3.5*	*3.0*
Platelets [10^3 cells/μl] (150–450)	n.k.	*35*	*low*	*low*	n.k.	n.k.	*253*	*11*	*5*
WBC [10^3 cells/μl] (4.1–8.9)	n.k.	*1.8*	n.k.	n.k.	4.5	6.6	6.3	7	7.2
Lymphocytes [10^3 cells/μl])1.3–5(	2.7	1.3	3.67	1.98	1.2–1.4	*0.7*	3.4	3.9	3.3
Lymphocyte subsets (normal range)[3]									
CD3 [10^3 cells/μl] (0.7–4.2)	2.6	1.8	2.6	1.5	0.86–1.03	0.6	2.0	3.3	3.9
CD4 [10^3 cells/μl] (0.6–2.1)	*0.2*	*0.2*	0.75	*0.4*	*0.3–0.46*	*0.23*	*0.25*	*0.3*	*0.58*
CD8 [10^3 cells/μl] (0.2–1.1)	*1.9*	*1.5*	*1.6*	0.6	*0.33–0.55*	*0.32*	*1.6*	*1.1*	*2.9*
CD4:CD8 ratio (0.7–2.8)	*0.11*	*0.14*	*0.47*	*0.62*	*0.9–0.8*	*0.7*	*0.15*	*0.25*	*0.2*
CD19 [10^3 cells/μl] (0.2–1.3)	*0.08*	*0**	0.63	0.3	*0.18–0.19*	*0.056*	0.89	n.k.	n.k.
CD20 [10^3 cells/μl] (0.06–0.5)	n.k.	n.k.	n.k.	n.k.	n.k.	n.k.	n.k.	0.47	0.43
CD16/CD56 [10^3 cells/μl] (0.07–1.2)	0.35	0.16	0.049	0.12	0.11	*0.049*	0.58	0.38	0.11
Immunoglobulins (normal range)[3]									
IgG [g/dL] (540–1550)	1600	466	*2920*	1194	1450	1130	1120	1160	*2260*
IgM [g/dL] (40–240)	306	118	*263*	158	140	70	76.6	52	191
IgA [g/dL] (47–249)	766	**<7**	*496*	137	60	172	145	90	*288*
IgE [g/dL] (0–200)	NK	<1	0.7	1.5	n.k.	n.k.	17.3	ND	< 5
T cell proliferation (normal range)[3]									
PHA6	*abnormal*	*34,746* (96,090-358,179)	n.k.	n.k.	*29 (> 50)*	*2.8* (> 50)	n.k.	*7409* (62,936)	*7424* (111,749)
Anti-CD3	*abnormal*	n.k.	n.k.	n.k.	n.k.	*3.2* (30)	n.k.	*499* (4088)	*8273* (36,328)
Complement									
C3 (90–180)	n.k.	n.k.	n.k.	n.k.	n.k.	n.k.	n.k.	133	*195*
C4 (10-14)	n.k.	n.k.	n.k.	n.k.	n.k.	n.k.	n.k.	12	*21*
T cell repertoire									
αβ+ [%]	*62*	n.k.	*low*	*low*	n.k.	n.k.	n.k.	*37*	*62*
γδ+ [%]	*17*	n.k.	*elevated*	*elevated*	n.k.	1.8	n.k.	*21*	*38*
TRECs •(> 400)	n.k.	n.k.	n.k.	n.k.	n.k.	n.k.	n.k.	*116*	*62*

Table 2 (continued)

Patients	Pa (Ref. 5)	Pb (Ref. 6)	Pc (Ref. 7)	Pd (Ref. 7)	Pe (Ref. 8)	Pf (Ref. 8)	P1	P2	P3
Autoimmune workup									
Positive autoantibodies	negative	n.k.	ANA positive, anti-RO/SSA, Coombs positive, anti-TPO, TGAb	ANA positive, Anti-C3d, Anti-IgG	n.k.	n.k.	n.k.	β2-glycoprotein (IgG), Coombs positive	ANA positive (1:180), Coombs positive

Abnormal values in italics

n.k. not known; *TPO* thyroid peroxidase; *TGAb* anti-thyroglobulin antibodies

*After rituximab treatment •Copies per 0.5 μg DNA

CD4:CD8 ratios, increased frequencies of TCRγδ cells, reduced TRECs, and abnormal TCR Vβ repertoires. In addition, we could detect a significant clonal expansion and reduced repertoire diversity in our patients' T cells. PHA-induced lymphocyte proliferation and anti-CD3 mitogen proliferation responses were decreased in P2, P3.

RASGRP1 has been widely associated with autoimmune manifestations. RASGRP1-deficient mice are prone to autoimmunity and develop a lymphoproliferative syndrome with features of systemic lupus erythematosus (SLE) [27, 28] or autoimmune pulmonary alveolar proteinosis (aPAP) [29]. These phenotypes are associated with perturbed positive selection of T cells resulting in increased frequencies of autoreactive $CD4^+$ T cells, activation of B cells, and production of autoantibodies [27]. The link between RASGRP1 function and autoimmunity has also been explored in human diseases. Expression of RASGRP1 has been shown to be reduced in patients with SLE [30, 31] and rheumatoid arthritis [32]. Furthermore, genome-wide association studies have linked variants in *RASGRP1* to type 1 diabetes mellitus [33] and IgA nephropathy [34]. Importantly, two of the recently reported RASGRP1-deficient patients presented with AIHA, immune thrombocytopenia, and ANA and other autoantibodies [7]. Both, P2 and P3 also produced autoantibodies and presented with overt autoimmune manifestations, such as Coombs positive AIHA and thrombocytopenia, lymphadenopathy, and splenomegaly. As seen in RASGRP1-deficient mice, P3 had elevated ANA titers as well as hypergammaglobulinemia and elevated complement levels. Clinically, P3 had severe uveitis successfully treated with intravitreous methotrexate and exhibited signs of autoimmunity in liver biopsy. Liver function tests improved under treatment of high-dose glucocorticoids indicating an underlying autoimmunity. These prominent manifestations in our and previously reported RASGRP1-deficient patients demonstrate that autoimmunity should be considered in RASGRP1 deficiency.

Mouse studies have shown that RASGRP1 has a critical role in T cell development, including pre-TCR signaling and positive selection of αβ T cells, in particular in those expressing TCR with low affinity to self-peptide major histocompatibility complex (MHC) molecules [22]. *Rasgrp1*$^{-/-}$ mice exhibit decreased αβ T cells, accompanied by impaired ERK1/2 signaling [12, 23, 24] but have increased frequencies of γδ $CD8^+$ T cells [21]. Similarly, P2, P3 and previously reported patients [5, 7] had an abnormal TCR Vβ repertoire and markedly increased TCRγδ-positive T cells. Further characterization of the TRG repertoire diversity by employing immunosequencing revealed a substantial clonal expansion and reduced repertoire diversity of T

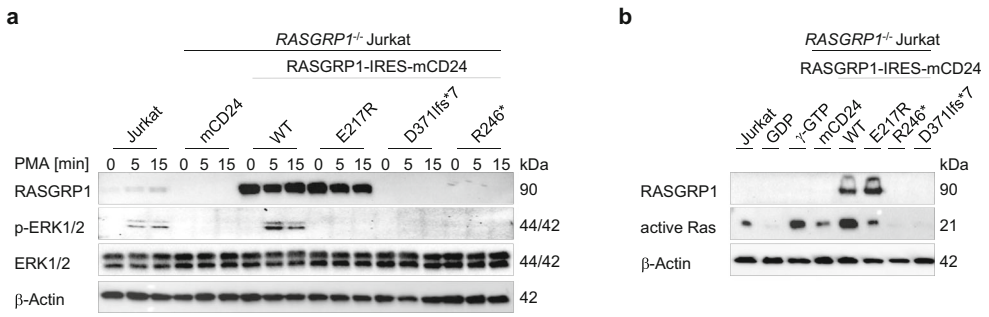

Fig. 2 Loss-of-function of mutated RASGRP1 in heterologous Jurkat T cell models. **a** Representative immunoblotting ($n = 3$) showed impaired phosphorylation of ERK1/2 (Thr202/Tyr204) upon treatment with PMA in Jurkat cells with *RASGRP1* knockout or ectopic expression of mutated RASGRP1 variants (E217R, D371Ifs*7, R246*) in comparison to wild-type RASGRP1 cells. **b** Active Ras-GTPase pull-down assay of PMA-stimulated Jurkat cells revealed reduced Ras-GTPase activity in cells overexpressing RASGRP1 mutants (E217R, D371Ifs*7, R246*). GDP indicates the negative control, γ-GTP was used as positive control. Staining with β-actin was used as loading control

cells. TREC were significantly decreased in P2 and P3, suggesting impaired TCR re-arrangement/maturation and thymic output in RASGRP1 deficiency [35]. Defective T cell development, $CD4^+$ T cell lymphopenia, and increased levels of $CD8^+$ T cells in RASGRP1-deficient patients might be attributed to impaired ERK1/2 signaling, which is known to be implicated in lineage commitment to CD4 [36–38].

In vitro studies on heterologous models showed that expression of a dominant-negative form of RASGRP1 suppressed apoptosis following B cell receptor ligation [39]. Accordingly, lymphoproliferation has been demonstrated as a predominant feature of RASGRP1 deficiency in mice [26, 40, 41] and human [30]. Four of the six previously reported patients presented with EBV-associated B cell malignancies [5, 6, 8]. In line, P1 and P3 developed EBV-associated DLBCL and P2 had high EBV viral loads but no malignancy has been reported prior to his death at the age of 4 years. Defects of cytotoxic T lymphocytes and NK cells can result in dysregulated and ineffective immune responses and are associated with EBV complications, such as hemophagocytic lymphohystiocytosis (HLH) and B cell malignancies [42–44]. P3 had a reduction in EBV blood viral load upon treatment with rituximab. Even though clinical remission of EBV-lymphoma may be achieved using high-dose chemotherapy regimens, HSCT should be

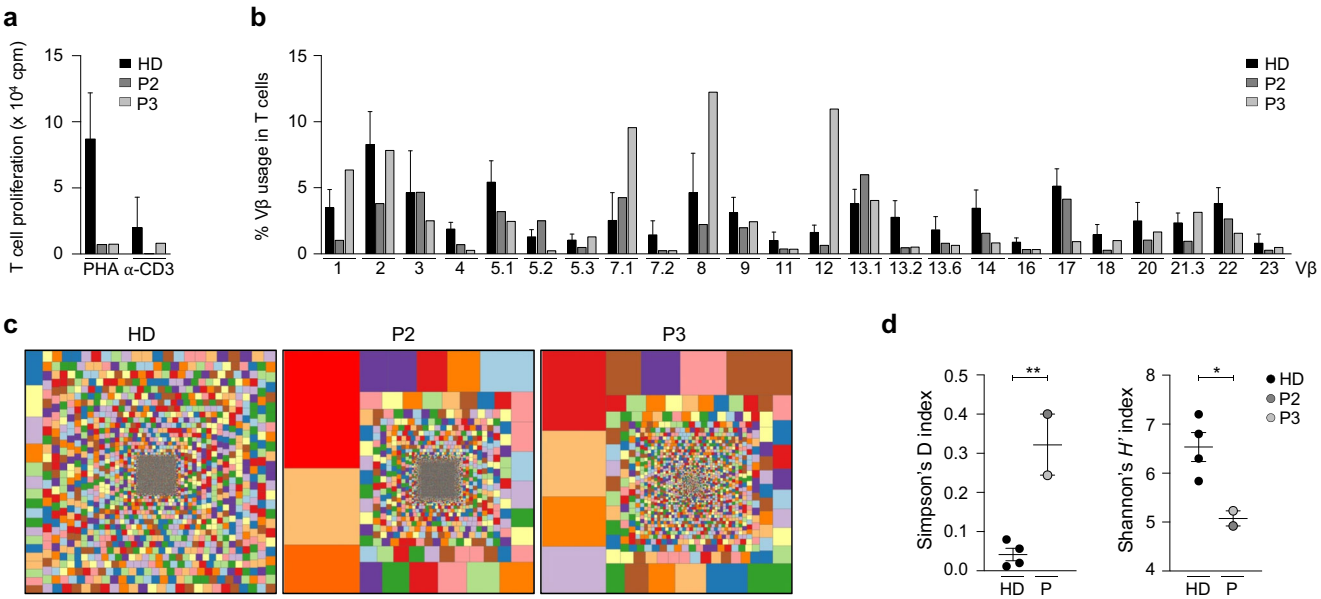

Fig. 3 RASGRP1 deficiency is associated with altered T cell clonal expansion and repertoire diversity. **a** Thymidine incorporation assays documented reduced T cell proliferation in P2 and P3 upon stimulation with (PHA) or anti-CD3, as compared to healthy donors (HD). **b** Flow cytometry-based TCR Vβ spectratyping of peripheral T cells from P2 and P3. P2 showed a clonal expansion of Vβ 13.1 and underexpression of most of the other Vβ's, while P3 exhibited overexpression of Vβ 1, 7.1, 8, 12, and 21.3 and underexpression of other Vβ's (4, 5.2, 7.2, 13.2, 13.6, 14, 16, 17, 22). Error bars indicate ± SD. **c** Analysis of TRG repertoire diversity with a treemap representation for P2, P3, and age-matched HD controls. Each colored square represents a unique clone and its size reflects its productive frequency within the repertoire. **d** Simpson's D diversity index and Shannon's *H'* index. Error bars indicate ± SEM. $*p < 0.05$. $**p < 0.01$

considered for patients with RASGRP1 deficiency as curative treatment.

Conclusions

In summary, our study further defines the clinical scope of RASGRP1 deficiency and reinforces the existing knowledge about the central role of RASGRP1 function in immunity. Moreover, our report may raise the awareness of RASGRP1 deficiency for an on-time diagnosis by providing a review of new and previously reported patients and phenotypes. In view of the underlying PID and the risk of developing malignancies, early diagnosis is critical and HSCT might be advocated to restore the immune system of affected patients with RASGRP1 deficiency.

Acknowledgements The authors thank the patients and their families for their collaboration and for their expert care by the interdisciplinary pediatric teams. The study would not have been possible without additional support by the German Academic Exchange Program (DAAD) and the Care-for-Rare Foundation.

Author Contributions I.S. and B.M. analyzed and interpreted results and I.S., B.M., and D.K. drafted the manuscript. B.M. designed, performed, and analyzed experiments for the patients. A.L., A.J.S., and E.R. performed the experiments for P2, P3. Y.L. and M.R. conducted NGS. S.H. analyzed NGS results. M.K., E.U., E.Y., T.P., and M.C. followed, diagnosed, and treated P1. R.S., T.S., V.V.D., and S.F. followed, diagnosed, and treated P2, P3. C.K., D.K., and R.S. designed and supervised the experiments.

Funding Information This work was supported by grants from the Jeffrey Modell Foundation (JMF) (Raz Somech), the German Research Foundation (DFG CRC1054, Leibniz Program) (Christoph Klein, Daniel Kotlarz), and the Else Kröner–Fresenius-Stiftung (Christoph Klein). Daniel Kotlarz has been a scholar funded by the Daimler und Benz Stiftung, Reinhard-Frank Stiftung, and Else Kröner–Fresenius-Stiftung.

Compliance with Ethical Standards

All procedures were performed upon informed consent and assent from patients, first-degree relatives, and healthy donor controls in accordance with the ethical standards of the institutional and/or national research committees and with the current update of the Declaration of Helsinki.

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

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Immunologic Research (2021) 69:145–152
https://doi.org/10.1007/s12026-021-09179-3

ORIGINAL ARTICLE

Exploring genetic defects in adults who were clinically diagnosed as severe combined immune deficiency during infancy

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Received: 28 September 2020 / Accepted: 3 February 2021 / Published online: 18 February 2021

Abstract

Genetic diagnostic tools including whole-exome sequencing (WES) have advanced our understanding in human diseases and become common practice in diagnosing patients with suspected primary immune deficiencies. Establishing a genetic diagnosis is of paramount importance for tailoring adequate therapeutic regimens, including identifying the need for hematopoietic stem cell transplantation (HSCT) and genetic-based therapies. Here, we genetically studied two adult patients who were clinically diagnosed during infancy with severe combined immune deficiency (SCID). Two unrelated patients, both of consanguineous kindred, underwent WES in adulthood, 2 decades after their initial clinical manifestations. Upon clinical presentation, immunological workup was performed, which led to a diagnosis of SCID. The patients presented during infancy with failure to thrive, generalized erythematous rash, and recurrent gastrointestinal and respiratory tract infections, including episodes of *Pneumocystis pneumonia* infection and *Candida albicans* fungemia. Hypogammaglobulinemia and T-cell lymphopenia were detected. Both patients were treated with a 10/10 HLA matched sibling donor unconditioned HSCT. Retrospective genetic workup revealed homozygous bi-allelic mutations in *IL7RA* in one patient and in *RAG2* in the other. Our study exemplifies the impact of retrospectively establishing a genetic diagnosis. Pinpointing the genetic cause raises several issues including optimized surveillance and treatment, understanding disease mechanisms and outcomes, future family planning, and social and psychological considerations.

Keywords IL7RA · IL7Rα · RAG2 · Primary immunodeficiency · PIDs · Retrospect · Severe combined immunodeficiency · SCID · WES

Introduction

Studying patients with rare genetic diseases of the immune system can save lives, prevent suffering, and also teach us about basic pathways of immune system function in health and disease [1]. Nowadays, the advent of genetic diagnostic tools, such as whole-exome sequencing (WES) and whole-genome sequencing (WGS), has progressed our understanding and the diagnosis of suspected genetic diseases, specifically in the field of primary immune deficiencies (PIDs) [2]. Establishing an exact diagnosis is of paramount importance to tailor adequate therapeutic regimens, including, in some cases, the need for hematopoietic stem cell transplantation (HSCT) and genetic-based therapies. Furthermore, genetic diagnosis may prevent further incidences in the same family [3]. However, for the current approach to become standard, many steps had to be completed. In 1956, Kostmann first described a new recessive severe congenital neutropenia [4], whose genetic etiology was unknown at the

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time. Fifty years later, genomic samples from the original Kostmann family were sequenced, confirming *HAX1* as the genetic cause for the disease [5]. In 1937, Wiskott described 3 brothers who presented shortly after birth with thrombocytopenia, bloody diarrhea, eczema, and recurrent ear infections [6]. Aldrich reported a sex-linked thrombocytopenia with an identical phenotype [7], later termed as Wiskott-Aldrich syndrome (WAS) [8, 9]. Members of the original family were recruited and underwent genomic sequencing, identifying *WAS* as the cause for their disease [10]. These are some of the milestones that paved the way to linking clinical phenotypes to a monogenetic PID syndrome. Early clinical diagnosis of PIDs remains a key goal. Nevertheless, a lack of relevant literature, evidence, and capabilities often delay the genetic diagnosis. Thus, individuals who have defied clinical diagnosis or who have an unclear diagnosis may benefit from novel genetic diagnostic modalities that enable a more definitive diagnosis, and therefore more appropriate management [11].

Herein, we describe two individuals with overt signs of PID, who were clinically diagnosed during infancy with severe combined immune deficiency (SCID). The overall prevalence of SCID is approximately 1 in 58,000 births [12]. Nevertheless, in areas with high rate of consanguine marriages, the prevalence is much higher [13]. SCID is caused by mutations in genes critical in the development of T, B, and natural killer (NK) cells and is classified according to the presence or absence of these cells, as a result of the underlying genetic defect. Regardless of the underlying genetic defect, all those affected are susceptible to severe infections and failure to thrive during infancy, unless their immune system is restored by means of HSCT or, in some specific cases, gene therapy or enzyme replacement therapy. Here, both patients presented during infancy with clinical characteristics of SCID and were treated with HLA sibling-matched unconditioned HSCT in the first year of life. They are both alive and well. Many years after the definitive treatment, they underwent WES and were subsequently diagnosed with bi-allelic mutations in *IL7RA* and *RAG2*, respectively, providing a genetic and molecular explanation for their infantile clinical phenotype.

Methods

Patients and clinical data

All the procedures were performed following informed consent from the patients and first-degree relatives, in accordance with the ethical standards of the institutional and/or national research committees and with the current update of the Declaration of Helsinki.

Immunological evaluation

Cell surface marker expression of peripheral blood mononuclear cells (PBMC) was analyzed by immunofluorescent staining with monoclonal antibodies and flow cytometry (Epics V; Coulter Electronics, Hialeah, FL). Signal joint T-cell receptor excision circles (TREC) copy numbers were determined by employing quantitative real-time PCR (qRT-PCR) of genomic DNA (gDNA, 0.5μg) extracted from patients' PBMCs [14]. Surface expression of individual T-cell receptor Vβ (TCR-Vβ) gene families was assessed using a set of 24 Vβ-specific fluorochrome-labeled monoclonal antibodies (Beckman Coulter, USA) and flow cytometry.

Genetic evaluation

Genomic DNA was isolated from fibroblast cell lines of patients and from whole blood of their parents. Primary fibroblasts were cultured from skin biopsy specimens in the patients. Fibroblasts were grown to 80% confluence in 48 h in 6-well or 24-well plates coated with fibronectin. DNA was prepared for a generation of whole-exome libraries using the SureSelect XT Human All Exon V5+UTR or V6+UTR kit (Agilent Technologies, USA). Barcoded libraries were sequenced on a NextSeq 500 platform (Illumina, USA) with an average coverage depth of 100x. Bioinformatics analysis and subsequent filtering identified rare sequence variants. *IL7RA* and *RAG2* mutations were confirmed by Sanger sequencing.

Results

Clinical description and outcome

Patient 1 (P1) is currently a 19-year-old male from consanguineous parents. He presented during infancy with failure to thrive, a generalized erythematous rash, a diffuse seborrheic dermatitis, lymphadenopathy, and recurrent gastrointestinal and respiratory tract infections, including an episode of *Pneumocystis pneumonia* infection and *Candida albicans* fungemia (Table 1). The stigmata of a multi-systemic disease with an opportunistic infection suggested a severe PID, Omenn phenotype. Hypogammaglobulinemia and T-cell lymphopenia were found (Table 2), suggesting a diagnosis of SCID. At age 6 months, the patient underwent a non-conditioned HSCT from a 10/10 HLA matched sibling, with a rapid engraftment at day 21 post-transplant, and with no signs of graft vs host disease. The patient is well and under routine follow-up. Patient 2 (P2) is currently a 24-year-old female, from a consanguineous family with a history of a sibling who deceased at age 4 years. She presented during infancy with developmental delay, fever of unknown origin, erythrodermic rash and lymphadenopathy. Recurrent

Table 1 Clinical manifestations of patients studied

	P1	P2
Family history	Parents 2nd-degree cousins	Parents 3rd-degree cousins 4-year-old sibling deceased
Age of presentation	6 weeks	3 months
Initial manifestation	Fever, rash	Generalized erythematous rash
Additional findings during infancy	FTT, recurrent respiratory and GI tract infections, *Pneumocystis pneumonia*, *Candida albicans* fungemia	FTT, recurrent respiratory and GI tract infections
Clinical diagnosis	SCID	SCID
Genetic diagnosis	NM_002185, c.417T>C; p.L14S	NM_000536, c.283G>A; p.G95R
Treatment	Full matched non-conditioned sibling HSCT	Full matched non-conditioned sibling HSCT
Subsequent infections	None	Varicella, recurrent pneumonia, bronchiectasia
Pathogens	None	*Streptococcus pneumonia*, *Mycobacterium shimoidei*, *Haemophilus influenzae*
Current treatment	None	IVIg, co-trimetophrim, azithromycin
Current status	Well	Well

FTT failure to thrive, *GI* gastrointestinal, *SCID* severe combined immune deficiency, *HSCT* hematopoietic stem cell transplantation, *IVIg* intra-venous immune globulin

gastrointestinal and respiratory tract infections were noted (Table 1). Her initial laboratory workup revealed hypogammaglobulinemia and both T- and B-cell lymphopenia (Table 2). She underwent a non-conditioned HSCT from a 10/10 HLA matched sibling donor at age 3 months, with initially complete engraftment and a 100% donor chimerism (Table 1). The patient suffered from varicella at age 4 years, recurrent lung infections, and bronchiectasis. She is alive and well, however has low T- and B-cell counts and is treated routinely with Ig replacement therapy (IVIg) due to hypogammaglobulinemia (Table 2), as well as with prophylactic antibiotics (Table 1).

Immunological workup

Both patients underwent routine immune workup following treatment with HSCT. A recent workup for patient 1 revealed normal T-cell receptor excision circle (TREC) levels, adequate lymphocyte counts, good responses to mitogen stimulation tests, intact immunoglobulin (Ig) levels, and excellent antibody responses to vaccinations (Table 2 and Table S1). These results confirm full restoration of cellular and humoral immune function. Surprisingly, donor chimerism was routinely screened and found to be mixed (within 18–25%) (Table S2). For P2, routine evaluation consistently showed full donor chimerism until the age of 23 years. During the past several months, donor chimerism has dropped to 11% despite a full donor chimerism in sorted T-cells (Table S2). Nonetheless, her immune workup revealed low TREC levels (Table 2), a partial lymphocytic response to mitogen stimulation tests and sub-normal Ig levels, depicting a partial immune reconstitution. Therefore, she was placed on gamma globulin replacement therapy and antibiotic prophylaxis. Both patients are clinically well.

Genetic results

In light of consanguinity in both patients and a SCID phenotype, monogenetic disease was suspected. Since extensive genetic tests were not performed at presentation, both patients underwent genetic evaluation years after their initial clinical presentation and treatment. At age 17 years P1 underwent WES, which revealed a homozygous missense mutation in *IL7RA* (NM_002185, c.417T>C; p.L14S). At age 22 years, P2 underwent WES, which revealed a homozygous missense mutation in *RAG2* (NM_000536, c.283G>A; p.G95R) (Fig. 1, upper panel). Both mutations occurred in evolutionary highly conserved amino acid residues in different vertebrate species (Fig. 1, lower panel). The variants were validated by Sanger sequencing and segregated with the disease phenotype. In both cases, parents were found to be heterozygote carriers for the specific mutation (Fig. 1). Both variants have been previously described [15–20].

Discussion

In SCID patients, both humoral and cellular immune functions are compromised. As a result, patients are susceptible to both severe bacterial infections and also to opportunistic infections, caused by *Pneumocystis pneumonia*, *Cytomegalovirus*, and *Candida albicans*. Immune workup typically shows profound lymphopenia, low or absent immune globulin levels, and low or

Table 2 Immune workup—at presentation and currently

	P1		P2	
Age of evaluation	6 months	18 years	3 months	24 years
WBC (cells/μl) (4100–10,800)	8550	5640	*1570*	3220
Lymph. Abs. (cells/μl) (1000–4800)	1490	1470	*714*	873
Neut. Abs. (cells/μl) (1800–7700)	3980	3470	*518*	1920
Lymph subsets				
CD3 (cells/μl) (700–4200)	*298*	951	*93*	690
CD4 (cells/μl) (440–1400)	*231*	521	N.A	419
CD8 (cells/μl) (160–880)	*134*	430	N.A	245
CD20 (cells/μl) (50–300)	*348*	182	*47*	70
CD56 (cells/μl) (108–300)	N.A	249	*502*	148
Mitogen stimulation responses (patient/healthy same day control)				
No mitogen (CPM)	N.A	192/151	N.A	2044/523
PHA 6 μg/ml (CPM)	N.A	36672/38466	N.A	50393/81093
PHA 25μg/ml (CPM)	N.A	35088/39775	N.A	91043/120767
CD3 Mitogen (CPM)	N.A	21848/17406	N.A	61070/72325
T-cell receptor evaluation				
TREC (copies/0.5μg DNA) (>400)	N.A	428	N.A	7
TCRvβ	N.A	Polyclonal	N.A	Polyclonal
Immune globulins				
IgG (mg/dl) (700–1600)	*603*	*1670*	N.A	*521
IgM (mg/dl) (40–280)	*66*	117	N.A	*<18.8
IgA (mg/dl) (70–500)	*9.5*	*<26.0*	N.A	*<27.9
Vaccination antigen-specific responses				
Inactive vaccines	N.A	Anti-HBV (IgG) positive	N.A	*Irrelevant
Attenuated vaccines	N.A	Anti-measles (IgG) positive	N.A	*Irrelevant
	N.A	Anti-VZV (IgG) negative	N.A	*Irrelevant
	N.A	Anti-rubella (IgG) negative	N.A	*Irrelevant

Abnormal values in italics

*Under IVIg replacement treatment

N.A not applicable, *PHA* phytohemagglutinin, *TREC* T-cell receptor excision circles, *TCR* T-cell receptor, *HBV* hepatitis B virus, *VZV* varicella zoster virus, *CPM* counts per minute

absent TREC levels, which reflect impaired TCR rearrangement and thymic output [21]. In some cases, a characteristic rash is presented resulting in a clinical diagnosis of Omenn syndrome [19]. Without adequate treatment, patients with SCID usually succumb in the first year of life. The overall survival rate following HSCT from a full HLA matched sibling donor exceeds 90% [22, 23]. With the introduction of newborn screening programs for SCID, early diagnosis and definitive treatment with either HSCT or other modes of therapy such as enzyme replacement therapy and gene therapy have improved prognosis and survival rate [24, 25]. We studied 2 individuals who presented during infancy with overt signs of PID and subsequently were diagnosed as SCID with Omenn like features (erythroderma and lymphadenopathy). They both were successfully treated with a non-conditioned 10/10 HLA matched sibling donor HSCT. Almost two decades later, they both underwent genetic evaluations and were diagnosed with homozygous biallelic mutations in SCID genes, one in *IL7RA* and the other in *RAG2*. This finding provides a genetic etiology for the observed earlier clinical phenotype. *IL7RA* gene, encoding the IL7R*a* protein, plays a role in lymphocyte development, and its deficiency is a typical example of T- B+ NK+ SCID [24]. An Omenn-like phenotype has been described [25]. *RAG2*, one of the two identified recombinating activating genes, binds to the RSS (recombination signal sequence). This enables VDJ recombination. RAG2-deficient SCID is a prototype example of T- B- NK+ SCID [26]. In many RAG2-deficient patients, a clinical presentation of Omenn syndrome is reported [27]. Both our patients underwent a full HLA matched sibling donor unconditioned HSCT during infancy. In both cases, the genetic defect was identified retrospectively, many years after the HSCT. Though the retrospective diagnosis did not alter the definitive therapy (HSCT), it yielded several important advantages, as discussed below.

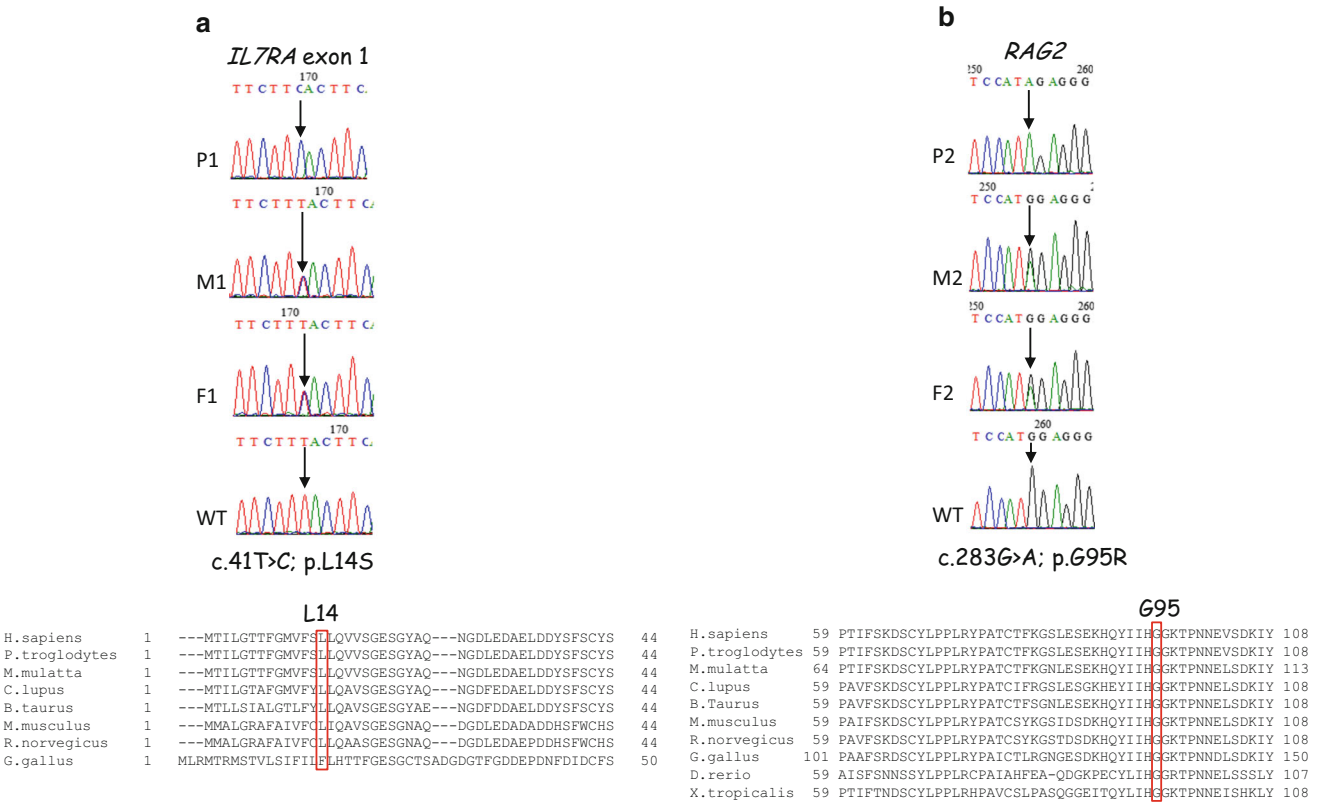

Fig. 1 Identification of patients with bi-allelic loss-of-function mutations in *IL7RA and RAG2*. Sanger sequencing chromatogram confirmed segregation of the identified homozygous missense mutations in *IL7RA* (NM_002185, c.417T>C; p.L14S) (**a**) and in *RAG2* (NM_000536, c.283G>A; p.G95R), (**b**), for P1 and for P2, respectively. Both mutations occurred in evolutionary highly conserved amino acid residues in different vertebrate species (lower panel). For both patients, parents were found to be heterozygote carriers for the specific mutation

Genetic diagnosis and disease outcome

While HSCT is considered the standard treatment for SCID, there are SCID patients that lack adequate immune reconstitution in unconditioned transplants. *RAG1* and *RAG2*-deficient SCID patients show an arrest in T-lymphocyte development with occupation of DN2/DN3 cells. These cells compete with donor T-lymphocyte progenitors for thymic sites and therefore compromise long-term immune function [28, 29]. In IL7Rα-deficient SCID patients, preserved intrinsic B-cell function is reported; consequently, patients usually develop B-cell lymphocyte function, in the presence of functioning donor T-lymphocytes [30]. These differences may explain the discrepancy in immune reconstitution between our two patients. Recovery of B-cell function is not expected without a pre-transplant conditioning. Thus, not surprisingly, P1 does not require monthly infusions of IVIg, in contrast to P2. Pre-transplant myeloablative conditioning is also important in other forms of SCID. However, long-term effects of alkylating agents including growth retardation, late-onset endocrinologic deficiencies, dental abnormalities, pulmonary fibrosis, pancreatic insufficiency, and increased mortality with alkylating agents are to be considered [31, 32]. Notably, HSCT does not correct some extra-hematologic abnormalities associated with PIDs, for instance neurologic abnormalities, such as mental retardation, motor dysfunction, and sensorineural hearing deficit [33, 34]. These examples illustrate how establishing the genetic diagnosis in retrospect contributes to the understanding of the disease mechanism, the prediction of long-term outcomes, determining prognosis, and the establishment of a definitive treatment regimen according to the identified genetic defect.

Genetic diagnosis and genetic counseling

Identification of genetic defects of the immune system has increased almost 9-fold during the past 4 decades, with a rise of ~50 to ~450 in the number of genetic PIDs reported by the IUIS/WHO committee in 2019 [35]. Genetic diagnosis is of utmost importance and relevance in regions where high rates of consanguinity are observed. In Israel, rates of consanguineous marriages and genetic founder effects are high compared to other developed countries. Consequently, higher rates of PIDs are observed [20]. The incidence of SCID in Israel has been estimated as 4.25:100,000 births [13], with a higher incidence of autosomal recessive SCID. This contrasts with the

higher incidence of X-linked SCID worldwide. The Israeli SCID newborn screening program detected genetic founder effects for mutations in *DCLRE1C* (ARTEMIS-deficient SCID) and *IL7RA* [36]. Genetic counseling has become an integral component of the management of PID. Establishing a genetic diagnosis is of great aid in understanding the pattern of inheritance and of thereby understanding the risk of future recurrences in the family. Obtaining this information can facilitate future family planning and provide essential information for parents including prenatal genetic testing.

Future surveillance and therapy for affected persons

In many PIDs, multi-systemic involvement affects various organs in addition to the immune system. These are not always corrected by HSCT, as in adenosine-deaminase (ADA)-deficient SCID. Therefore, for genetically affected individuals, intricate surveillance is often necessary. Determining the genetic status of a family member can eliminate the need for routine surveillance, sparing much time and distress, and also financial resources. Furthermore, screening the genetic status of a sibling can help determine suitability to HLA matched HSCT. This is critical prior to transplant, to rule out the possibility of a HLA matched HSCT from a sibling with an identical genetic defect who is clinically asymptomatic at the time of transplantation. Hence, characterizing donor genetic status can achieve better outcomes upon HSCT.

Genetic-based therapies

Genetic diagnosis enables establishing a direct therapeutic effect through several means of treatment: (A) Gene therapy (human gene transfer). The use of gene therapy in PIDs has progressed significantly since the first clinical trial in 1990 [37]. Recent examples include lentiviral vector gene therapy with Busulfan conditioning, as was reported in 8 infants with X-linked SCID [38]. Additional PIDs for which ongoing gene therapy trials are being conducted include Wiskott-Aldrich syndrome and chronic granulomatous disease (CGD) [39]. (B) Targeted therapy directed at the molecular defect is an attractive option. It may act by inhibiting overactive (gain of function) or by enhancing underactive (loss of function) cellular pathways [40]. An example is activated PI(3)K delta syndrome (APDS), which is caused by gain of function mutations, and characterized by immune deficiency, autoimmunity, lymphadenopathy, and accumulation of senescent T-cells. APDS was shown to be caused by augmented mTOR signaling, which resulted in increased S6 and AKT phosphorylation. The use of mTOR inhibitors, rapamycin, and leniolisib/CDZ173 was shown to reduce S6 and AKT phosphorylation [41–43]. Another example is CD137/4-1BB, an immune co-stimulatory checkpoint molecule whose loss-of-function mutations result in immune deficiency and EBV-associated lymphoproliferation [44–46]. CD137 agonist monoclonal antibodies have been investigated in cancer immunotherapy trials [47, 48]. These are only a few examples of how genetic diagnosis has changed and advanced the notion of targeted therapy, thus enhancing the effectiveness and precision of therapeutic agents. Furthermore, these agents are also in use for other indications. For instance, Abatecept, which is used to treat CTLA4 deficiency [49], is FDA approved for the treatment of refractory rheumatoid arthritis [50]; rapamycin is used for the prevention of organ transplant rejection [51]. None of these therapeutic approaches would be possible without a genetic diagnosis.

Psychological aspects of genetic diagnosis

The psychological impact of acquiring personal and familial genetic information may have both positive and negative aspects. On one hand, some individuals would prefer not to be aware of their genetic status. This may be especially true in asymptomatic carriers who are not directly affected. Some reasons for this preference are decreased anxiety and the avoidance of a "stigma" of a genetic defect, which could have implications later in life. However, on the other hand, the knowledge may have positive consequences: obtaining the genetic status can reduce anxiety that arises from uncertainty regarding the expected inheritance of the genetic defect. Moreover, in some communities, being a close relative of a patient with a suspected genetic disease may hamper the chance of appropriate matchmaking. Therefore, discovering the exact genetic basis for PID and determining the risk of each family member to inherit the defect affect not only the patient but also the social well-being of all the family members.

Conclusions

In summary, the patients presented exemplify the impact of recent genetic diagnostic measures as a tool for retrospectively establishing genetic diagnoses. Pinpointing the genetic cause entails multiple benefits and aspects regarding optimized surveillance and treatment, disease mechanisms, prognosis, family planning, and social and psychological repercussions.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12026-021-09179-3.

Acknowledgements We thank the families for participating in this study. We thank the Jeffrey Modell Foundation, Israel Ministry of Health, and the Israeli Science Foundation for their financial support. Prof. Raz Somech, Dr. Ayal Hendel, and Dr. Yu nee Lee gratefully acknowledge the funding support from the Israeli Science Foundation (ISF) under the Israel Precision Medicine Program (IPMP), grant agreement No. 3115/19.

Authors' contribution I.S. and R.S. conceptualized the designed study and drafted the manuscript which was reviewed and approved by all authors. R.S. treated the patients studied. A.L., A.H., Y.N.L., and A.J.S.

performed and analyzed immune and genetic experiments. O.B. analyzed WES data.

Declarations

Conflict of interest The authors declare no competing interests.

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Acknowledgements:

I wish to thank all colleagues who participated in the projects summarized in this thesis.

I would like to thank the members of the Klein Lab for their warm welcome, encouragement, and for sharing their knowledge and experience with me.

I wish to thank Florian Gothe for great help and fruitful discussions.

I wish to thank Thomas Magg for his supervision during my work at the Klein Lab.

I would like to thank Marini Thian for the fruitful work and friendship in the challenging journey.

I would like to thank Marcin Łyszkiewicz for thoroughly reading the PhD thesis and for his important intellectual input.

Last, I wish to thank my supervisors and mentors:

Christoph Klein, for the opportunity to join his group, for his inspiration and for the unlimited support which enabled me to pursue this journey.

Raz Somech, for his mentorship, dedication and endless support.

Natalia Ziętara, for her guidance, fruitful discussions and great support.

This work is dedicated to my loving family which believed and supported me throughout the path.

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I hereby declare, that the submitted thesis entitled

Inborn errors of immunity predisposing to primary immunodeficiency and immune dysregulation caused by CD137 and RASGRP1 deficiencies

is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

Petah Tikva, 04.12.2021
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is congruent with the printed version both in content and format.

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Education

2002-2009: The Joyce and Irving Goldman Medical School, Ben Gurion University, Israel
2012-2013: School of Continued Medical Education, Pediatrics, Sackler School of Medicine, Tel Aviv University

Clinical experience and training

2010: Internship, Carmel Hospital, Haifa
2011-2016: Residency, Schneider Children's Medical Center of Israel, Pediatrics
2015-2016: Chief resident, Schneider Children's Medical Center of Israel
2019-present: Fellow, Schneider Children's Hospital, Hematology Oncology

Degrees and certificates

2010: M.D. The Joyce and Irving Goldman Medical School, Ben Gurion University, Israel
2016: USMLE board certified physician
2019: Expert in Pediatrics

MD Thesis

Distribution, dynamics and antibiotic resistance patterns of Streptococcus pneumoniae serotypes causing acute otitis media in children in southern Israel during the 10 year-period before the introduction of the 7-valent pneumococcal conjugate vaccine.

Pediatric doctoral thesis

Laboratory and clinical characterization of LPS Responsive Beige-Like Anchor Protein (LRBA) deficient patients.

Scientific presentations

2011 Distribution, dynamics and antibiotic resistance patterns of Streptococcus pneumoniae serotypes causing acute otitis media in children in southern Israel during the 10 year-period before the introduction of the 7-valent pneumococcal conjugate vaccine. HIPAK (Israeli Clinical Paediatric Society), Tel Aviv, Israel. **Best presentation award** (oral presentation).

2016 Laboratory and clinical characterization of LPS Responsive Beige Like Anchor Protein (LRBA) deficient patients. Annual Meeting - Israel society of Immunology, Tel Aviv, Israel (oral presentation)

2017 Novel Mutations in *RASGRP1* are Associated with Immunodeficiency, Immune Dysregulation, and EBV-Induced Lymphoma. Annual Meeting - Israel society of Immunology and Hemato-Oncology, Tel Aviv, Israel (oral presentation).

2017 A homozygous *PTPN6* variant causes a novel Hyper-IgE syndrome-like primary immunodeficiency – iTarget Kubus Annual Conference, Munich, Germany (poster presentation).

2017 Novel Mutations in *RASGRP1* are Associated with Immunodeficiency, Immune Dysregulation, and EBV-Induced Lymphoma. Annual Meeting – American Society of Hematology –ASH, Atlanta, Georgia, USA (poster presentation).

2017 Pneumonia in Pediatric Population. Invited guest lecture, Fourth Hospital of Yulin, Yulin, China (oral lecture)

2017 The clinician scientist, a distinct and disappearing entity. Invited guest lecture, Shaanxi provincial people's hospital of Xi'an, Xi'an, China (oral lecture)

2018 Hoyeraal-Hreidarrson syndrome caused by an intronic branch-point mutation in *DKC1*. Annual Meeting – Padiatrische Immunologie (API), Innsbruck, Austria
(oral lecture)

2018 CD137 deficiency causes immune dysregulation with predisposition to lymphomagenesis. Invited guest lecture, CEMM research center for molecular medicine, Vienna, Austria (oral presentation)

2018 From bench to bedside , pediatric clinical conference, Edith and Wolfson Medical Center, Holon, Israel (oral presentation)

2018 CD137 deficiency and Lymphoma predisposition, EKFS annual retreat PhD Students, Munich, Germany (oral presentation).

2019 CD137 deficiency causes immune dysregulation with predisposition to lymphomagenesis. Annual Meeting – American Society of Hematology –ASH, Orlando, Florida, USA. **Abstract award winner** (poster presentation).

2019 EBV associated PIDs and CD137 deficiency. Annual Meeting - Israel society of Immunology, Kfar Blum, Israel (oral presentation)

2020 Age-Dependent Sensory Impairment in COVID-19 Infection and its Correlation with ACE2 Expression. European society for Pediatric Infectious Diseases (ESPID) **Late breaking abstract** (oral presentation)

2020 Exploring Genetic Defects in Adults who were Clinically Diagnosed as Severe Combined Immune Deficiency during Infancy. European society for Immunodeficiencies -ESID (poster presentation).

2020 Age-Dependent Sensory Impairment in COVID-19 Infection and its Correlation with ACE2 Expression. Pediatrics conference. Mayanei HaYeshua Medical Center, Bnei Brak, Israel (oral presentation).

2020 CD137 deficiency causes immune dysregulation with predisposition to lymphomagenesis. Annual Global Summit on Hematologic Malignancies, Lisbon, Portugal (poster presentation).

2020 GATA2 deficiency in a patient with Myelodysplastic Syndrome. Pediatric Leukemia Meeting (virtual), (oral presentation).

2021 Distinct cases of Fanconi anemia, Israeli society for pediatric hemato-oncology (ISPHO) Kedma, Israel (oral presentation).

E. ACADEMIC AND PROFESSIONAL AWARDS

(Prizes, fellowships, grants, scholarships, etc)

2011 Distribution, dynamics and antibiotic resistance patterns of Streptococcus pneumoniae serotypes causing acute otitis media in children in southern Israel during the 10 year-period before the introduction of the 7-valent pneumococcal conjugate vaccine. HIPAK (Israeli Clinical Paediatric Society), Tel Aviv, Israel. **Best presentation award** (oral presentation).

2016-2017 Else Kröner-Fresenius-Stiftung scholar (research fellow)

2018 Care for Rare foundation Travel Grant (Klein Laboratory, Munich, Germany)

2019 CD137 deficiency causes immune dysregulation with predisposition to lymphomagenesis. Annual Meeting – American Society of Hematology –ASH, Orlando, Florida, USA. **Abstract award winner** (poster presentation).

2020 COVID-19 research grant awarded by the Ministry of Science & Technology, Israel [grant number 3-16983]. (Ido Somekh and Raz Somech).

2021 The Israeli Society of Pediatrics, Sackler School of Medicine, **Dani Moran Scholarship for best published article.** (CD137 deficiency causes immune dysregulation with predisposition to lymphomagenesis).

Scientific Publications

Total Number: 28
First Author: 15

Main Publications

Somekh I*, Thian M*, Medgyesi D, Gülez N, Magg T, Gallón Duque A, Stauber T, Lev A, Genel F, Unal E, Simon AJ, Lee YN, Kalinichenko A, Dmytrus J, Kraakman MJ, Schiby G, Rohlfs M, Jacobson JM, Özer E, Akcal Ö, Conca R, Patiroglu T, Karakukcu M, Ozcan A, Shahin T, Appella E, Tatematsu M, Martinez-Jaramillo C, Chinn IK, Orange JS, Trujillo-Vargas CM, Franco JL, Hauck F, Somech R, Klein C, Boztug K. CD137 deficiency causes immune dysregulation with predisposition to lymphomagenesis. **Blood.** 2019;134:1510-1516. **IF 17.5**

Somekh I, Shohat T, Boker LK, Simões EAF, Somekh E. Reopening Schools and the Dynamics of SARS-CoV-2 Infections in Israel: A Nationwide Study. **Clin Infect Dis.** 2021 Jan 18:ciab035. doi: 10.1093/cid/ciab035. Epub ahead of print. **IF 9.1**

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Łyszkiewicz M, Ziętara N, Frey L, Pannicke U, Stern M, Liu Y, Fan Y, Puchałka J, Hollizeck S, **Somekh I**, Rohlfs M, Yilmaz T, Ünal E, Karakukcu M, Patiroğlu T, Kellerer C, Karasu E, Sykora KW, Lev A, Simon A, Somech R, Roesler J, Hoenig M, Keppler OT, Schwarz K, Klein C. Human FCHO1 Deficiency Reveals Role for Clathrin-Mediated Endocytosis in Development and Function of T Cells.
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Lev A, Lee YN, Sun G, Hallumi E, Simon AJ, Zrihen KS, Levy S, Beit Halevi T, Papazian M, Shwartz N, **Somekh I**, Levy-Mendelovich S, Wolach B, Gavrieli R, Vernitsky H, Barel O, Javasky E, Stauber T, Ma CA, Zhang Y, Amariglio N, Rechavi G, Hendel A, Yablonski D, Milner JD, Somech R. Inherited SLP76 deficiency in humans causes severe combined immunodeficiency, neutrophil and platelet defects.
J Exp Med. 2021 ;218:e20201062. doi: 10.1084/jem.20201062. **IF 10.9**

Full Publication List

2021

Lev A, Lee YN, Sun G, Hallumi E, Simon AJ, Zrihen KS, Levy S, Beit Halevi T, Papazian M, Shwartz N, **Somekh I**, Levy-Mendelovich S, Wolach B, Gavrieli R, Vernitsky H, Barel O, Javasky E, Stauber T, Ma CA, Zhang Y, Amariglio N, Rechavi G, Hendel A, Yablonski D, Milner JD, Somech R. Inherited SLP76 deficiency in humans causes severe combined immunodeficiency, neutrophil and platelet defects. **J Exp Med**. 2021 ;218:e20201062. doi: 10.1084/jem.20201062. **IF 10.9**

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2020

Łyszkiewicz M, Ziętara N, Frey L, Pannicke U, Stern M, Liu Y, Fan Y, Puchałka J, Hollizeck S, **Somekh I**, Rohlfs M, Yilmaz T, Ünal E, Karakukcu M, Patiroğlu T, Kellerer C, Karasu E, Sykora KW, Lev A, Simon A, Somech R, Roesler J, Hoenig M, Keppler OT, Schwarz K, Klein C. Human FCHO1 Deficiency Reveals Role for Clathrin-Mediated Endocytosis in Development and Function of T Cells. **Nat Commun.** 2020;11:1031. doi: 10.1038/s41467-020-14809-9. **IF 14.9**

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2018

Somekh I*, Marquardt B*, Liu Y, Rohlfs M, Hollizeck S, Karakukcu M, Unal E, Yilmaz E, Patiroglu T, Cansever M, Frizinsky S, Vishnvenska-Dai V, Rechavi E, Stauber T, Simon AJ, Lev A, Klein C, Kotlarz D, Somech R. Novel Mutations in RASGRP1 are Associated with Immunodeficiency, Immune Dysregulation, and EBV-Induced Lymphoma. **J Clin Immunol.** 2018;38:699-710.doi: 10.1007/s10875-018-0533-8. **IF 6.7**

Marcus N, Stauber T, Lev A, Simon AJ, Stein J, Broides A, **Somekh I**, Almashanu S, Somech R. MHC II Deficient Infant Identified by Newborn Screening Program for SCID. **Immunol Res.** 2018;66:537-542. doi: 10.1007/s12026-018-9019-2. **IF 2.6**

2011

Somech I, Dagan R, Givon-Lavi N, Porat N, Raiz S, Leiberman A, Puterman M, Peled N, Greenberg D, Leibovitz E. Distribution, dynamics and antibiotic resistance patterns of Streptococcus pneumoniae

serotypes causing acute otitis media in children in southern Israel during the 10 year-period before the introduction of the 7-valent pneumococcal conjugate vaccine. **Vaccine.** 2011;29:4202-9. doi: 10.1016/j.vaccine.2011.03.103. **IF 3.1**

Case Reports

Cansever M, Zietara N, Chiang SCC, Ozcan A, Yilmaz E, Karakukcu M, Rohlfs M, **Somekh I**, Canoz O, Abdulrezzak U, Bryceson Y, Klein C, Unal E, Patiroglu T. A Rare Case of Activated Phosphoinositide 3-Kinase Delta Syndrome (APDS) Presenting With Hemophagocytosis Complicated With Hodgkin Lymphoma. **J Pediatr Hematol Oncol.** 2019. **IF 1.0**

Letters to The Editor

Somekh I, Somekh E, Pettoello-Mantovani M, Somech R. Reply. **J Pediatr.** 2020 Feb;217:220. doi: 10.1016/j.jpeds.2019.10.002. Epub 2019. **IF 4.4**

Reviews

Somekh I, Somekh E, Pettoello-Mantovani M, Somech R. The Clinician Scientist, a Distinct and Disappearing Entity. **J Pediatr.** 2019;212:252-253. **IF 4.4**

Somekh I, Somech R. CURRENT KNOWLEDGE ON COVID-19 IN CHILDREN - CAUTIOUS OPTIMISM. **Harefuah**. 2020;159:315-319. **IF 0.2**

Somekh I, Somech R, Pettoello-Mantovani M, Somekh E. Changes in Routine Pediatric Practice in Light of COVID-19. **J Pediatr** 2020;S0022-3476. doi: 10.1016/j.jpeds.2020.05.053. **IF 4.4**

Guen CG, Hentgen V, Dubos F, Kochert F, Balençon M, Levy C, **Somekh I**, Somekh E, Ferrara P, Pettoello-Mantovani M, Cohen R. French Pediatric Societies Call for School to Stay Open amid the Coronavirus Disease 2019 Pandemic. **J Pediatr.** 2021;234:293-295.e2. doi: 10.1016/j.jpeds.2021.04.001. Epub 2021. **IF 4.4**

Text Book Chapters

Somekh I. EBV-associated Primary Immune Deficiencies and Immune Dysregulation. Rare Diseases in Children – Focusing on pediatric Immunology & Neurology (2020).

Credits for Manuscript Reviews

2020: Effect of Vitamin K2 and Vitamin D3 on Bone Mineral Density in Children with Acute Lymphoblastic Leukemia. A Prospective Cohort Study (Journal of Pediatric Endocrinology and Metabolism).

2021: Chronic lymphocytic leukemia-induced paraneoplastic hypercalcemia treated with ibrutinib (Erciyes Medical Journal)

2021: Management of Patients with Factor VII Deficiency in Surgery: Single Center Experience (Erciyes Medical Journal).

2021: The diagnostic value of prepepsin in acute appendicitis and reference ranges for healthy children (Journal of Tropical Pediatrics)

2021: Prospective study of COVID-19 screening for primary school children attending school in Liège, Belgium (JAMA Network Open).

2021: Feasibility of specimen self-collection in young children undergoing SARS-CoV-2 surveillance for in-person learning. (JAMA Network Open).