MEDICAL UNIVERSITY OF VIENNA

Identification and Characterization of Primary Immunodeficiencies with Defective Class Switch Recombination and Autoimmunity

Doctoral thesis at the Medical University of Vienna
for obtaining the academic degree

Doctor of Philosophy

Submitted by

Dr.med.univ. Elisabeth Salzer

Supervisor:

Ass.-Prof. Priv.-Doz. Dr.med. Kaan Boztug

CeMM Principal Investigator /
Associate Professor, Dept. of Pediatrics and
Adolescent Medicine, Medical University of Vienna
Director, Vienna Center for Rare und Undiagnosed Diseases (CeRUD)

Vienna, March 2015

Declaration

The work of this thesis was accomplished at several academic institutions with the assistance of different collaborators. In the following paragraphs, all contributions are listed in detail.

Chapter 3.1 was published by Salzer, Daschkey et al., 2012. The author of this thesis performed most of the experiments for family A, analyzed and interpreted data, prepared figures and contributed to the manuscript. Sharon Coo, Elisabeth Förster-Waldl, Kirsten Bienemann, Markus G.Seidel, and Arndt Borkhardt cared for the patients, had clinical, therapeutic, and/or diagnostic responsibilities. Elisangela Santos-Valente and Martina Schwendinger helped with exome sequencing and bioinformatic data analyses of family A. Svenja Daschkey, Kirsten Bienemann and Michael Gombert performed exome sequencing and bioinformatic analyses in families B & C. Sharon Choo and Markus G. Seidel arranged and the author of the thesis together with Elisangela Santos-Valente, Sebastian Ginzel, Oskar A. Haas, Gerhard Fritsch, Winfried F. Pickl, Svenja Daschkey and Kirsten Bienemann performed immunophenotypical, functional, and immunogenetic analyses of the patients. Kaan Boztug took overall responsibility for the research performed in this study and wrote substantial parts of the manuscript.

Chapter 3.2 was published by Salzer, Santos-Valente et al., 2013. The author of the thesis and Elisangela Santos-Valente performed most of the experiments, analyzed data and wrote the manuscript together with Kaan Boztug. Stefanie Klaver and Sol A Ban contributed to Western Blot and qPCR analyses. Winfried F. Pickl performed routine immune phenotypic characterization of the patient. Wolfgang Emminger, Andreas Heitger, Klaus Arbeiter, Franz Eitelberger, Markus G. Seidel, Wolfgang Holter, Arnold Pollak and Elisabeth Förster-Waldl provided clinical care and critically reviewed clinical patient data. Leonhard Müllauer and Renate Kain performed histopathological analyses. Kaan Boztug took overall responsibility for the research performed in this study and wrote substantial parts of the manuscript.

Chapter 3.3 was published by Salzer et al., 2014. The author of the thesis performed all experimental work except for B cell class switch and activation assays which were performed by Heiko Sic, Hermann Eibel and Marta Rizzi in Freiburg and T cell proliferation assays which were performed by Winfried F. Pickl. Aydan Kansu, Aydan Ikincioğullari, Esin Figen Dogu, Zarife Kuloğlu, Arzu Meltem Demir and Arzu Ensari provided clinical care of the patient and performed routine clinical interventions. Peter Májek performed computational modeling and *in silico* prediction algorithms. Sol A Ban performed TCR Vb spectratyping of the patient and Nina Prengemann and Elisangela Santos-Valente assisted in experimental procedures and performed SNP chip based homozygosity mapping. Kaan Boztug conceived this study, provided laboratory resources and together with the author of the thesis planned, designed and interpreted experiments. The author of the thesis and Kaan Boztug wrote the first draft and the revised version of the manuscript.

All chapters of the thesis were written by the author. Kaan Boztug and Ivan Bilic provided critical input to writing of the thesis.

Reprint permission for all figures in the introduction was obtained from the Nature publishing group through Copyright Clearance Center's Rights Link service.

Table of content

DECLARATION ..... **II**

TABLE OF CONTENT ..... **IV**

LIST OF FIGURES ..... **VI**

ABSTRACT ..... **VII**

DEUTSCHSPRACHIGE ZUSAMMENFASSUNG ..... **IX**

PUBLICATIONS ARISING FROM THIS THESIS ..... **XI**

ABBREVIATIONS ..... **XII**

ACKNOWLEDGMENTS ..... **XIV**

1. INTRODUCTION **1**

1.1. HUMAN GENETICS 1

1.2. EXOME SEQUENCING 2

1.2.1. Strategies to identify rare disease causing variants *2*

1.3. LYMPHOCYTE DEVELOPMENT 3

1.3.1. B cell development *4*

1.3.2. T cell development *5*

1.4. SECONDARY LYMPHOID ORGANS AND GERMINAL CENTERS 6

1.4.1. Structure of a germinal center *6*

1.4.2. B cell fate within the germinal center *7*

1.4.3. Follicular dendritic cells *9*

1.4.4. T_{FH} cells within the germinal center *10*

1.5. INHERITED DISORDERS OF THE IMMUNE SYSTEM 10

1.5.1. (Severe) Combined Immunodeficiencies *12*

1.5.2. Primary antibody deficiencies *14*

1.6. CENTRAL AND PERIPHERAL TOLERANCE 17

1.6.1. Central tolerance *17*

1.6.2. Peripheral tolerance *18*

1.7. AUTOIMMUNITY AND AUTO INFLAMMATION 18

1.7.1. Innate Immune Activation-Auto inflammation *18*

1.7.2. Activation of the adaptive immune system - Autoimmunity *19*

1.8. LYMPHOPROLIFERATION.....20
1.8.1. EBV-driven lymphoproliferation.....21
1.9. HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS (HLH).....22
1.10. CLINICAL DIAGNOSIS OF PATIENTS WITH PRIMARY IMMUNODEFICIENCIES ..23
1.11. THERAPY OF PRIMARY IMMUNODEFICIENCIES- CURATIVE APPROACHES 24
1.11.1. Hematopoietic stem cell transplantation (HSCT).....24
1.11.2. Gene therapy.....25
1.12. THERAPY OF PRIMARY IMMUNODEFICIENCIES- SUPPORTIVE/ NON-CURATIVE APPROACHES.....26
1.12.1. Enzyme replacement therapy.....26
1.12.2. Immunoglobulin substitution.....26
1.12.3. Antimicrobials.....26
1.12.4. Antifungals.....27
2. AIM OF THE THESIS.....28
3. RESULTS AND DISCUSSION.....29
3.1. COMBINED IMMUNODEFICIENCY WITH LIFE-THREATENING EBV-ASSOCIATED LYMPHOPROLIFERATIVE DISORDER IN PATIENTS LACKING FUNCTIONAL CD27.....29
3.2. B-CELL DEFICIENCY AND SEVERE AUTOIMMUNITY CAUSED BY DEFICIENCY OF PROTEIN KINASE C δ 36
3.3. EARLY-ONSET INFLAMMATORY BOWEL DISEASE AND COMMON VARIABLE IMMUNODEFICIENCY–LIKE DISEASE CAUSED BY IL-21 DEFICIENCY.....43
4. DISCUSSION 65
4.1. COMBINED IMMUNODEFICIENCY WITH LIFE THREATENING EBV-ASSOCIATED LYMPHOPROLIFERATIVE DISORDER IN PATIENTS LACKING FUNCTIONAL CD27.....65
4.1.1. CD27 biology.....65
4.1.2. CD27 deficiency in the context of other PIDs with lymphoproliferation.....66
4.2. B CELL DEFICIENCY AND SEVERE AUTOIMMUNITY CAUSED BY DEFICIENCY OF PROTEIN KINASE C δ. 67
4.2.1. PKCδ biology.....67
4.2.2. PKCδ activation.....68
4.2.3. Human PKCδ deficiency 70
4.2.4. PKCδ signaling and SLE.....70

4.3. EARLY-ONSET INFLAMMATORY BOWEL DISEASE AND COMMON VARIABLE IMMUNODEFICIENCY-LIKE DISEASE CAUSED BY IL-21 DEFICIENCY.....72
4.3.1. Discovery and biology of the IL-21/IL-21 receptor system 72
4.3.2. Human IL-21R and IL-21 deficiency.....73
4.3.3. Concluding remarks 74
4.4. CONTRIBUTION TO THE FIELD OF PRIMARY IMMUNODEFICIENCIES 76
5. REFERENCES.....71
6. CURRICULUM VITAE.....90

List of Figures

Figure 1: B cell maturation in the germinal center (taken from Klein & Dalla-Favera, 2008). 9
Figure 2 Mechanisms of T cell deficiencies (taken from Liston et al, 2008). ..14
Figure 3 Gene defects in B cell development (taken from Cunningham-Rundles & Ponda, 2005) 16
Figure 4 PKCδ related signalling pathways. 69

Abstract

Primary immunodeficiencies (PIDs) are a complex and heterogeneous group of diseases. In most cases these patients are attributed to a certain PID according to their clinical presentation as a genetic diagnosis can only be established in approximately 40% of the cases. The aim of this thesis was to identify novel monogenic disorders leading to primary immunodeficiencies using state of the art technologies such as exome sequencing and homozygosity mapping. We focused on a cohort of patients with putative autosomal recessive, monogenic forms of PID, presenting with autoimmunity, lymphoproliferation and defective class-switch recombination. In this group of patients we were able to identify 3 novel primary immunodeficiencies caused by biallelic loss of function mutations in *CD27*, *PRKCD* and *IL21*, respectively, leading to 3 publications in peer-reviewed journals.

For *CD27* deficiency we described the largest to-date published cohort of patients with this disease. As all patients shared the same causative missense mutation affecting CD27 (p. Cys53Tyr), but displayed diverse clinical presentations, we were able to provide a clinical overview of the disease spectrum. Moreover, we could show that the amount of invariant natural killer cells inversely correlated with Epstein Barr driven lymphoproliferative disease in these patients.

In another patient we were able to identify a splice site mutation in *PRKCD* encoding protein kinase C delta, which led to complete absence of the protein and resulted in a primary immunodeficiency with severe autoimmunity. The patient displayed glomerulonephritis, was positive for various autoantibodies and was previously diagnosed with systemic lupus erythematosus. We could show that in accordance with a previously published role of *PRKCD* in literature, the patient exhibited increased mRNA levels of *IL6* after stimulation with Phorbol myristate acetate and showed decreased phosphorylation of the PKCδ target myristoylated alanine-rich C-kinase substrate (MARCKS). The findings from this study enabled to propose treatment with Tocilizumab, a humanized anti-IL-6 receptor monoclonal antibody currently in use for the treatment of rheumatoid arthritis.

In a consanguineous family with a history of deaths due to inflammatory bowel disease we identified a missense mutation in *IL21* (p.Leu49Pro) in a boy. With the help of *in silico* simulations we demonstrated that the mutated residue is highly conserved and that any change at amino acid position Leu49 would reduce the stability of the native state. Building on this finding we utilized recombinant wild type and mutated IL-21 protein to demonstrate a loss of function phenotype of the mutant, displaying strongly reduced STAT3 phosphorylation upon *in vitro* cell stimulation. Moreover, as recombinant IL-21 is in clinical trials, we were able to propose an alternative potential curative treatment option for this patient.

Taken together the discovery of these three novel PIDs contributed to the understanding of the multifaceted regulatory mechanisms of the immune system and highlighted essential players in these complex signaling networks.

Deutschsprachige Zusammenfassung

Primäre Immundefekte (PID) sind eine komplexe und heterogene Gruppe von Erkrankungen. In den meisten Fällen wird die Diagnose klinisch gestellt. Eine genetische Ursache kann nur in etwa 40% der Fälle gefunden werden. Ziel dieser Arbeit war es, neue monogenetische primäre Immundefekte zu entdecken und zu charakterisieren. Wir konzentrierten uns auf eine Kohorte von Patienten mit vermeintlich autosomal-rezessiven, monogenen Formen von PID mit Autoimmunität, Lymphoproliferation und defektem B Zell Klassenwechsel. In dieser Patientengruppe gelang es uns drei neue primäre Immundefekte identifizieren.

Für CD27-Defizienz beschrieben wir die bisher größte veröffentlichte Kohorte. Da wir bei allen Patienten die gleiche Mutation in *CD27* (p. Cys53Tyr) fanden, konnten wir die vielfältigen klinischen Präsentationen, sowie eine klinische Übersicht des Krankheitsspektrums publizieren. Darüber hinaus konnten wir zeigen, dass die Anzahl invarianter natürlicher Killerzellen invers mit Epstein-Barr-assoziierter lymphoproliferativer Erkrankung korreliert.

Bei einem anderen Patienten entdeckten wir eine „splice-site“ Mutation in *PRKCD*, welche zur kompletten Abwesenheit des Proteins Proteinkinase C Delta führte und einen primären Immundefekt mit signifikanter Autoimmunität zur Folge hatte. Klinisch zeigte der Patient Glomerulonephritis, war positiv für verschiedene Autoantikörper und wurde mit systemischem Lupus erythematodes diagnostiziert. Wir konnten zeigen, dass der Patient erhöhte *IL6* mRNA-Level nach Stimulation mit Phorbolmyristatacetat hatte. Aufgrund dieser Ergebnisse ist es uns möglich, die Behandlung mit Tocilizumab, einem humanisierten anti-IL-6-Rezeptor Antikörper, vorzuschlagen.

Bei einem weiteren Patienten mit sehr früh einsetzender chronisch entzündlicher Darmerkrankung identifizierten wir eine missense-Mutation in Interleukin-21 (*IL21*) (p.Leu49Pro). Durch *in-silico*-Simulationen konnten wir zeigen, dass die Mutation an einer evolutionär stark konservierten Position des Proteins ist. Mithilfe rekombinanter Produktion von wildtyp und mutiertem IL-21-Protein konnten wir den Verlust der Funktion des mutierten Proteins am Beispiel defekter STAT3-Phosphorylierung in *in vitro* Zellstimulations-experimenten zeigen. Da rekombinantes IL-21 derzeit in klinischen Studien

getestet wird, ist es uns gelungen, eine alternative Behandlung für dieser Patienten vorschlagen.

Die Entdeckung dieser Krankheitsbilder leistete einen Beitrag zum Verständnis komplexer Regulationsmechanismen des Immunsystems.

Publications arising from this thesis

Salzer E, Kansu A, Sic H, Majek P, Ikinciogullari A, Dogu FE, Prengemann NK, Santos-Valente E, Pickl WF, Bilic I, Ban SA, Kuloglu, Demir AM, Ensari A, Colinge J, Rizzi M, Eibel H, Boztug K, **Early-onset inflammatory bowel disease and common variable immunodeficiency-like disease caused by IL21 deficiency.** J Allergy Clin Immunol. 2014 Apr 17

Salzer E*, Santos-Valente E***,** Klaver S, Ban SA, Emminger W, Prengemann NK, Garncarz W, Müllauer L, Kain R, Boztug H, Heitger A, Arbeiter K, Eitelberger F, Seidel MG, Holter W, Pollak A, Pickl WF, Förster-Waldl E#, Boztug K#.**B-cell deficiency and severe autoimmunity caused by deficiency of protein kinase C δ.** Blood. 2013 Apr 18 (* and # equal contribution)

Salzer E*, Daschkey S*, Choo S, Gombert M, Santos-Valente E, Ginzel S, Schwendinger M, Haas OA, Fritsch G, Pickl WF, Förster-Waldl E, Borkhardt A#, Boztug K#, Bienemann K, Seidel MG#. **Combined immunodeficiency with life-threatening EBV-associated lymphoproliferative disorder in patients lacking functional CD27.** Haematologica. 2013 Mar,13 (* and # equal contribution)

Abbreviations

ADA	adenosine deaminase
AID	activation-induced deaminase
AIRE	Auto-immune regulator
ALPS	Autoimmune lymphoproliferative syndrome
AP3B1	Adaptor-related protein complex 3, beta 1 subunit
APECED	Autoimmune polyendocrine syndrome type 1
APRIL	a proliferation inducing ligand
ATM	Ataxia-telangiectasia mutant
BAFF	B cell activating factor of the TNF family
BCL	B cell lymphoma
BCMA	B cell maturation antigen
BCR	B cell receptor
BLIMP	B lymphocyte induced maturation protein
BLNK	scaffolding protein B-cell linker
BM	Bone marrow
BTK	Bruton's tyrosine kinase
CCR	CC-chemokine receptor
CD	cluster of differentiation
CD40L	CD40 ligand
CGD	Chronic granulomatous disease
CID	Combined immunodeficiency
CVID	Common variable immunodeficiency
CXCL	CXC-motif chemokine receptor ligand
CXCR	CXC-motif chemokine receptor
DCLRE1C	DNA cross-link repair 1C
DN	Double negative
DNA	Deoxyribonucleic acid
DOCK	Dedicator of cytokinesis
DP	Double positive
DZ	Dark zone
EBI2	Epstein-Barr virus induced gene 2
EBV	Epstein-Barr virus
FasL	Fas Ligand
FOXP3	Forkhead box P3
GC	Germinal center
HLA	Human leukocyte antigen
HLH	Hemophagocytic lymphohistiocytosis
aHSCT	allogeneic hematopoietic stem cell transplantation
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNKT	invariant Natural killer cells
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy and X-linked syndrome

IRF	Interferon regulatory factor
ITK	Interleukin-2 inducible T cell kinase
ITP	Immune thrombocytopenia
JNK	c-Jun N-terminal kinase
LIG	Ligase
LPD	Lymphoproliferative disease
LYST	Lysosomal trafficking regulator
LZ	Light zone
MHC	major histocompatibility complex
MRE11	Meiotic recombination 11-homolog
NBS1	Nijmegen breakage syndrome
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	non-homologous end joining enzymes
NKT	natural killer T cells
PAD	primary antibody deficiency
PI3	Phosphoinoside 3 kinase
PID	Primary immunodeficiency / primäre Immundefekte
PKC	Protein kinase C
PLC	Phospholipase C
PNP	purine nucleoside phosphorylase
PRF1	Perforine 1
RAB27A	Ras-related protein Rab27-A
RAG	Recombination activating genes
RIC	Reduced intensity conditioning
SCID	Severe combined immunodeficiency
SH2D1A	SH2 domain containing 1A
SIN	self-inactivating
SLE	Systemic lupus erythematosus
SNV	Single nucleotide variant
SPENCDI	Spondyloenchondrodysplasia with immune dysregulation
STAT	Signal transducer and activator of transcription
STX11	Syntaxin 11
STXBP2	Syntaxin binding protein 2
TACI	transmembrane activator and CAML interactor
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
TEC	Thymic epithelial cells
TFH	T follicular helper cells
Th	T helper cells
TNF	Tumor necrosis factor
TNFRSF6	TNF receptor superfamily 6, Fas
Tregs	regulatory T cells
UNC13D	Unc-13 homolog D
UNG	Uracil-N-glycosylase
WAS	Wiskott Aldrich syndrome
XIAP	X-linked inhibitor of apoptosis protein

Acknowledgments

In many perspectives, the time that I could spend in the laboratory at the Center for Molecular Medicine was unique. To be able to interact and work in such a stimulating, creative and motivated environment was and is a privileged situation that I am very grateful for. In professional terms, this was the biggest adventure that I have undertaken so far and there are too many colleagues and friends to be mentioned here explicitly.

I would not want to miss the last four years both from a professional and a personal perspective. However, there are people that were simply outstanding in the way they influenced my personal as well as professional development in these last years.

I would like to express my special appreciation and thanks to my supervisor, Kaan Boztug, who has supported me throughout my thesis and has guided me to sharpen and develop my scientific tool set. Over the years I got to know him as one of the hardest-working people I know and I was inspired by his ambitious passion about research and science. He has taught me that persistence and hard work eventually pay off and that knowledge and passion are universal trades that will enable you to do things you never thought you would be able to do.

I would also like to thank my PhD committee consisting of Giulio Superti-Furga and Ulrich Jäger for following my progress and the valuable input and advice I have received from them.

Moreover, I would like to express my thanks to Wojtek Garncarz, without whom I could not imagine the last years. His commitment and his high standards have shaped the lab from the beginning and are also a significant factor for its success. I am also happy and grateful that I got to know Ivan Bilic, with whom I had heated scientific discussions and who always made me see the bigger picture.

Together with Elis, Nina, Katharina, Cecilia and Tatjana we mastered crises, burn-outs, weddings, babies, ballroom-dancing, waterfalls, conferences and of course many scientific obstacles we encountered and I am very grateful to know all of you. I would also thank Sola and Stavi who taught me a lot about teaching and supervising and who will both become excellent medical doctors in near future.

Of course I would also like to thank Johannes, my family and friends for their patience in waiting for me to reply to phone calls, showing up at dinners and keeping me up-to-date about the really important things in life ☺.

1. Introduction

1.1. Human Genetics

The understanding of the basic laws of inheritance is essential to study genetic disorders in man. Single gene disorders are rare and often called Mendelian diseases as Gregor Mendel first observed gene segregation patterns by studying selected traits in garden pea *Pisum sativum* (Mendel, 1865). From his work he deduced that basic units of heredity - genes come in pairs, where always one is inherited from each parent. Moreover, he was able to distinguish dominant or recessive traits, by recognizing mathematical patterns in the mode of inheritance.

Mendel's laws of inheritance are stated as follows (adapted from (Mendel, 1865):
1) The Law of Segregation: Each inherited trait is defined by a gene pair. Each gene within a parental pair is called allele. Alleles randomly segregate to the sex cells – gametes so that each gamete contains only one allele of the parental pair. Therefore, during fertilization the resulting offspring inherits one allele from each parent.
2) The Law of Independent Assortment: Genes for different traits are sorted independently from one another so that the inheritance of one trait is not dependent on the inheritance of others.
3) The Law of Dominance: An organism with alternate forms of a gene will express the form that is dominant.

In humans, all genes, except for the X and Y chromosomal ones in males, are present in 2 copies in the cell. Depending on the location of a gene and whether one or two intact copies are needed, a disease phenotype will manifest if a person carries one or two „disease"- alleles. Monogenic disorders can be inherited in four basic modes: autosomal-dominant, autosomal-recessive, X-linked and mitochondrial. Traditionally, monogenic disorders were identified using linkage analyses and candidate gene sequencing. Using this technique loci underlying approximately one-third of

Mendelian disorders have been identified (McKusick, 2007). However, in very small, uninformative families, it used to be extremely difficult to identify rare Mendelian diseases due to several reasons such as: incomplete penetrance, locus heterogeneity as well as substantially reduced reproductive fitness (Antonarakis & Beckmann, 2006).

1.2. Exome Sequencing

Before the development of next-generation sequencing technologies, linkage analyses, candidate gene sequencing and positional cloning were the strategies applied to identify disease-causing variants. These strategies mostly focused only on coding areas of the genome while neglecting regulatory regions. However, overall, they have proven to be highly successful in gene discovery. With the development of next-generation DNA sequencing technologies, cost of DNA sequencing decreased dramatically and enabled fast detection of almost all coding variants within a person's genome (Bamshad et al, 2011; Mamanova et al, 2010). Although exome sequencing does not systematically assess non-coding alleles and regulatory regions, it can be applied for the identification of Mendelian disorders, as most disease-causing variants identified until now disrupt the amino acid sequence of proteins (Bamshad et al, 2011). Today, the major challenge of "gene hunting" using exome sequencing is to distinguish disease-related alleles from the background of non-pathogenic polymorphisms and sequencing errors.

1.2.1. Strategies to identify rare disease causing variants

Exome sequencing of an individual typically yields approximately 20,000 single nucleotide variants (SNVs) out of which more than 95% are known polymorphisms. There are different strategies to identify the disease-causing variant depending on the mode of inheritance, pedigree structure, locus heterogeneity for an investigated trait and inheritance vs. *de novo* emergence of a phenotype (Bamshad et al, 2011). In the past years, since the development of next generation sequencing technologies, causative variants for many Mendelian disorders have been identified. In most of the cases the novel disease-causing variant was identified by filtering against publically available databases, such as dbSNP and the 1000 Genomes project for

variants present with a minor allele frequency below 0.01% (Bamshad et al, 2011). This strategy is very powerful especially for rare Mendelian disorders affecting few individuals within a family. In addition, detected SNVs can be further categorized using prediction algorithms, which calculate their potential effect on protein function. Dependent on the mode of inheritance different numbers of cases need to be sequenced in order to identify the disease causing mutation. For recessive disorders in consanguineous families, sequencing of one affected person with the smallest regions of homozygosity should initially be adequate. For non-consanguineous families sequencing of both parents and the affected child is a potent approach to identify potentially disease-causing variants. In any case, optimization of the filtering process tailored to the approach is crucial for the identification of these variants.

1.3. Lymphocyte development

During embryogenesis hematopoietic precursor cells populate the bone marrow (BM). These cells originate from the fetal liver and consist of cells stemming form the aorta-gonad-mesonephros, a part of the mesoderm (Muller et al, 1994) (Pieper et al, 2013). It is important to consider that hematopoietic stem cells (HCS) do not commit all at once into a certain lineage but as development progresses undergo a certain narrowing towards a specific lineage (Rothenberg, 2000). Long-term reconstituting hematopoietic stem cells (HSC) differentiate into multipotent progenitor cells branching into the lymphoid or the erythro-myeloid lineage (Akashi et al, 2000; Kondo et al, 1997). Due to the expression of *c-KIT* (Waskow et al, 2002) and *FLT3* (Sitnicka et al, 2002) HCSs further mature to common lymphoid progenitors (CLP), thereby loosing long-term self-renewal capacity (Busslinger, 2004). CLPs are a heterogeneous cell population and can give rise to T, B, natural killer (NK) and dendritic cells, depending on the environmental cues. *IL7*, *IL7R* and common γ-chain expression is essential for further differentiation of CLPs (Carvalho et al, 2001; Miller et al, 2002).

The bone marrow provides the appropriate support for the development of B, NK and dendritic cells, while T cells can only develop if a progenitor cell enters a specialized T cell development organ – the thymus.

1.3.1. B cell development

Multiple transcription factors regulate the generation of B lymphocytes from hematopoietic stem cells. The pro-B cell stage represents the earliest stage of committed B cell development (Hardy et al, 1991; Li et al, 1996). Upon induction of *CD19* expression, rearrangement of D and J segments of the heavy chain locus is completed by recombination activating genes (*RAG*) 1 and 2 (Hardy et al, 1991; Li et al, 1996). This stage is followed by a second rearrangement where an upstream V segment is connected to the DJ region (late pro-B cells) (Busslinger, 2004). During this step terminal deoxynucleotidyl transferase (TdT) adds additional nucleotides between the junctions of the rearranged gene segments, which enhances the diversity of the B cell antigen receptor repertoire (Janeway, 2008).

The productively rearranged heavy chain pairs with invariant surrogate light chains (l5 and VpreB) are induced by the transcription factors E2A and EBF. This leads to the formation of a pre-B cell receptor (pre-BCR) on the cell surface (Busslinger, 2004). The productive rearrangement and surface expression of the pre-BCR prevents further heavy chain rearrangement by allelic exclusion, thereby preventing expression of two different BCRs on one cell and leads to proliferation of pre-B cells. Signaling through the pre-BCR requires BLNK and Bruton's tyrosine kinase (BTK) (Janeway, 2008).

After several division rounds large pre-B cells become resting small pre-B cells which re-express *RAG1* and *RAG2* to initiate rearrangement of the immunoglobulin light chain (Meffre et al, 2000). This process is initiated on one allele with joining of V and J segments. Light chains also display isotypic exclusion, so that either the k or l light chain is expressed by one single cell. Productive light chain rearrangement results in the expression of immunoglobulin M (IgM) on the surface of the immature B cell (Meffre et al, 2000). The newly formed B cell receptor complex consisting of IgM and Igα and Igβ is first tested for tolerance to self-antigens in the bone marrow. Cells that react with self-antigens can either undergo apoptosis, clonal deletion, receptor editing, immunological ignorance or anergy. This process is termed central tolerance. Immature B cells with weak reactions to self-antigen are allowed to leave the bone marrow via sinusoids to secondary lymphoid organs to complete development (Janeway, 2008).

1.3.2. T cell development

During embryonic development, the thymus develops from endoderm-derived structures, the third pharyngeal pouch and the third branchial cleft, respectively. It is located above the heart behind the sternum (Hollander et al, 2006). The thymus is populated with hematopoietic cells, thymocytes, intrathymic dendritic cells and macrophages, respectively. It consists of an outer cortex and an inner medulla.

Progenitors enter the thymus at the cortico-medullary junction and move towards the outer cortex (Anderson et al, 1996). Notch ligands expressed on intrathymic epithelial cells guide progenitors to commit them through differentiation (Janeway, 2008; Radtke et al, 1999).

T cell differentiation is defined by surface expression of CD4 and CD8. Initially, progenitors lack most of the surface molecules characteristic for T cells, also CD4 and CD8 and have non-rearranged T cell receptor genes, but express CD3. In the DN1 stage, lymphoid progenitors migrate to the thymus and are predestined to give rise to T cells. However, in this stage they are still not fully committed and are termed “intermediates” (Rothenberg, 2000). It has been shown that these cells can also develop into NK cells as well as dendritic cells (Res et al, 1996). The next commitment towards T lineage occurs with the abrupt induction of members of the Ets and bHLH class A family of transcription factors. In this stage progenitors express the surface markers CD25, CD24 and c-Kit, respectively. The development into NK and DC is strongly attenuated at this stage although still possible. (Rothenberg et al, 1999). There is evidence that TCRαβ and TCRγδ precursors become distinct in this stage before TCR rearrangement (Rothenberg, 2000).

The rearrangement of the T cell receptor β chain starts during the DN2 phase with Db to Jβ rearrangement. At this stage T cells start expressing CD25 (Rothenberg, 2002). Subsequently expression of c-Kit and CD44 is reduced, which marks the entry into the DN3 phase. B-selection occurs in this stage as a rearranged β chain is expressed on the surface together with a surrogate α-chain, which enables the assembly of a pre-T cell receptor (pre-TCR), similar to the pre-BCR. With the expression of the pre-TCR, further rearrangement of the β-locus is stopped and, through an intermediate DN4 and immature single positive stage, expression of both CD4 and CD8 on the cell surface is

induced, marking the entry in the double positive (DP) phase (Rothenberg, 2002). Large DP cells proliferate and then become small-DP cells, where they only express the TCR at low levels. During this phase positive selection takes place, where the TCR is tested for the ability to bind self-antigens at low level. Positively selected cells, mature and start expressing high levels of the TCR and loose the expression of either the CD4 or the CD8 molecule (Rothenberg, 2002).

1.4. Secondary lymphoid organs and germinal centers

Secondary lymphoid organs contain follicles which, under pathogen free conditions are mainly populated by naive B cells (Victora & Nussenzweig, 2012). Approximately one week after antigen exposure, germinal centers (GCs) develop in these areas and form secondary follicles. During this stage, naive B cells form now the outer border of a GC, the so-called B cell mantle (MacLennan, 1994).

1.4.1. Structure of a germinal center

Germinal centers were first described by Flemming in 1884 as micro anatomical regions of secondary lymphoid organs, that contained dividing cells (Nieuwenhuis & Opstelten, 1984). GCs form a specialized microenvironment within secondary lymphoid organs where B cells undergo proliferation, somatic hypermutation and antigen-affinity driven selection processes (Shlomchik & Weisel, 2012). During this process, BCR affinity plays an essential role in the differentiation step of activated B cells into memory B cells as well as long-lived plasma cells. Anatomically GCs can be separated into dark (DZ) and light zone (LZ). Whereas the dark zone consists mainly of B cells with a high nucleus-to-cytoplasm ratio, LZ B cells are embedded in a network of follicular dendritic cells and T cells (Nieuwenhuis & Opstelten, 1984). These regions are surrounded by a mantle and marginal zone. Phenotypically the cells of the light and dark zone can be distinguished by flow cytometry using markers against the CXC motif chemokine receptor 4 (CXCR4), CD83 and CD86. LZ B cells express $CXCR4^{lo}CD38^{hi}CD86^{hi}$, whereas DZ B cells are $CXCR4^{hi}CD38^{lo}CD86^{lo}$ (Victora et al, 2010). Tingible

body macrophages, which phagocyte dying B cells, are found in almost all GC compartments (Victora & Nussenzweig, 2012).

1.4.2. B cell fate within the germinal center

The dark zone consists of antigen-activated B cells, differentiating into centroblasts while undergoing consecutive rounds of proliferation also termed clonal expansion. During that time somatic hypermutation takes place, enabling higher specificity of the BCR for a certain antigen. Subsequently those centroblasts with best binding to the antigen become centrocytes and migrate further to the light zone of the GC (Klein & Dalla-Favera, 2008). Once B cells engage an antigen via the BCR with sufficient affinity, they up regulate CC-chemokine receptor (CCR)-7 and move to the outer zone of the germinal center, the T cell zone. There activated B cells engage and are stimulated by CXC-chemokine receptor (CXCR)-5 expressing T helper (Th) cells or pre-follicular T helper cells to proliferate further (Fazilleau et al, 2009). Following T cell help, B cells initiate formation of a follicle leading to a GC or initiate extrafollicular plasma cell responses. Until now it is not completely understood which factors determine this differentiation decision (Vinuesa et al, 2009).

During extrafollicular plasma cell responses, B cells up regulate B lymphocyte induced maturation protein (BLIMP)1, maintain expression of the Epstein-Barr virus induced gene (*EBI2*, *GPR183*) and migrate to junction zones or move to lymph node medullary cords. Here they form clusters of proliferating plasmablasts and by this an extrafollicular response. Some cells undergo isotype switching, but generally these cells are of low affinity and rather short lived. Dependent on the stimulus, extrafollicular responses can last for several weeks and sometimes give rise to somatically mutated autoantibodies (see below) (William et al, 2002). Under normal circumstances, these cells die of an apoptotic death within the secondary lymphoid tissue (Tarlinton et al, 2008).

On the other hand, B cells that up regulate B cell lymphoma (BCL) 6 and reduce EBI2 expression upon interferon-regulatory factor (IRF)-8 expression, differentiate into GC B cells and are targeted to follicles in a CXCR5-dependent manner (Vinuesa et al, 2009). Therefore, most of the B cells in the germinal center display an activated phenotype characterized by the increase

in size, polarized morphology and rapid division (Victora & Nussenzweig, 2012). They express high levels of Fas and n-glycolylneuraminic acid as well as high levels of CD38, but loose surface IgD expression. As described above, GC B cells up regulate BCL-6 which is critical for the formation of GCs as mice lacking Bcl-6 cannot form germinal centers and lack high-affinity antibodies (Dent et al, 1997; Ye et al, 1997). BCL-6 also silences the anti-apoptotic molecule BCL-2 maintaining a pro-apoptotic state, which is fundamental in preventing autoimmunity due to defective somatic hypermutation. It also represses p53 and ATR in order to increase the GC B cell tolerance to DNA damage due to AID activity and rapid proliferation (Victora & Nussenzweig, 2012). In addition, BCL-2 reduces expression of Blimp-1, a master regulator of plasma cell differentiation from the GC B cells. It also down-regulates BCR and CD40 signaling, thus guiding B cells towards response to selective signals (Victora & Nussenzweig, 2012).

Since germinal centers are anatomically divided into a dark and a light zone, people sought to decipher whether this also corresponds to functional polarization (Allen et al, 2007). Although light and dark zone cells were similar in complexity and size, gene expression differed in important points. These differences enable to distinguish $CXCR4^{lo}CD83^{hi}CD86^{hi}$ light and $CXCR4^{hi}CD83^{lo}CD86^{lo}$ dark zone cells by flow cytometry.

Marginal zone B cells represent the first line of defense and are capable of mounting a T cell independent response. It has been shown that dedicator of cytokinesis (DOCK) 8 is critical for their development as patients with defective DOCK8 lack marginal zone B cells and do not produce protective antibodies after vaccination (Engelhardt et al, 2009; Randall et al, 2009; Zhang et al, 2009).

Classically cell division is thought to happen in the dark zone whereas cell selection is mainly restricted to the light zone. In line with this hypothesis, it has been shown, that light zone B cells seem to be in an activated state mirrored by the expression of CD69 and CD40, BCR stimulation, as well as nuclear factor (NF)-κB and c-Myc engagement (Victora et al, 2010), whereas mitotic cells can be detected to a higher extent in the dark zone (Hanna, 1964). However, the precise mechanisms guiding cells into one or the other

zone remain to be elucidated. This process is illustrated and summarized in Figure 1 taken from Klein & Dalla-Favera, 2008.

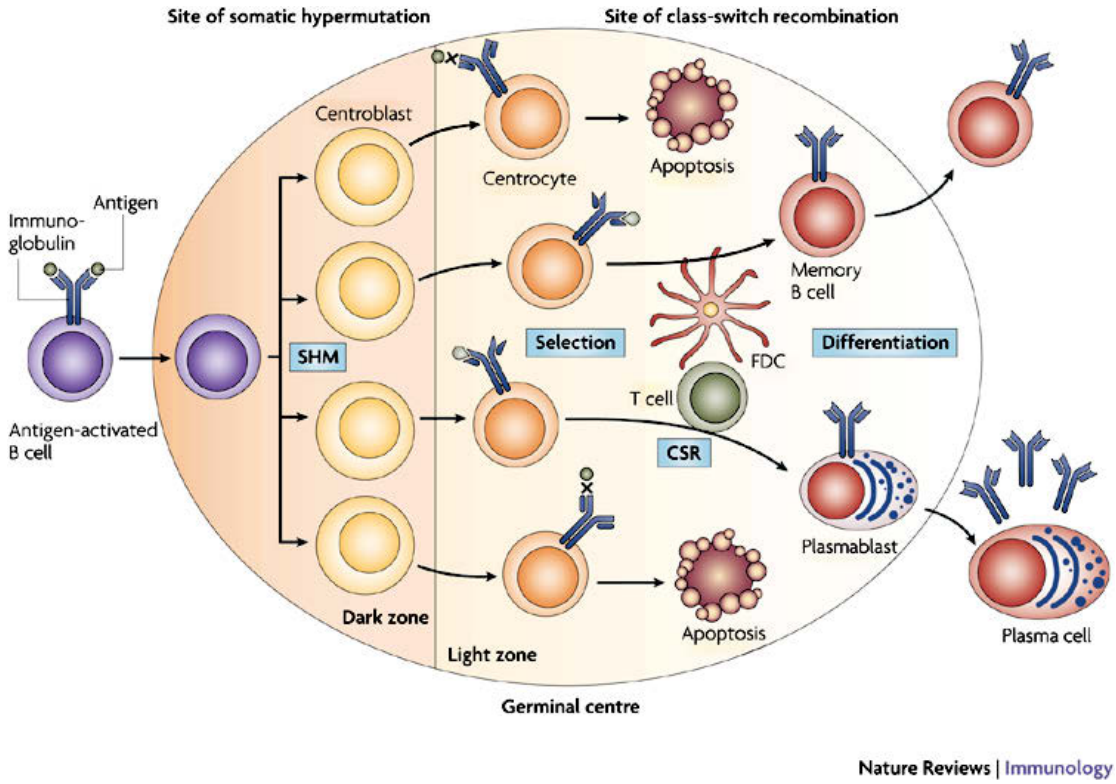

Figure 1: B cell maturation in the germinal center (taken from Klein & Dalla-Favera, 2008).

1.4.3. Follicular dendritic cells

Follicular dendritic cells (FDCs) mainly reside in the light zone of the germinal center. Their function is to attract germinal center B cells and serve as antigen pool during the germinal center reaction, as they can retain intact antigen on their surface for longer periods of time (Mandel et al, 1981). This trapping of immune complexes on the surface in the form of icosomes mainly relies on complement receptors 1 and 2 (Barrington et al, 2002; Szakal et al, 1985). It has also been shown that follicular dendritic cells secrete CXCR5 and CXCL13/BLC, important chemo attractants for B cells to guide them to the germinal centers (Cyster et al, 2000). In addition, follicular dendritic cells express ICAM-1 and VCAM-1, which are essential for germinal center maintenance and dark and light zone polarization (Haynes et al, 2007). Moreover, the secretion of interleukin (IL)-6 and B cell activating factor of the tumor necrosis factor (TNF) family (BAFF), may be important for a physiologic

germinal center reaction (Kopf et al, 1998; Nishikawa et al, 2006; Wu et al, 2009).

1.4.4. T_{FH} cells within the germinal center

Although there are only a small number of T cells within the germinal center, they are critical for affinity maturation of B cells and maintenance of the germinal center. It has been shown that patients with loss-of-function mutations affecting *CD40* or *CD40L* fail to develop germinal centers and exhibit increased levels of non-class switched cells (Allen et al, 1993; Ferrari et al, 2001). Especially the population of T-follicular helper (T_{FH}) cells plays a crucial role within the germinal center. T_{FH} are characterized by the expression of CD4 as well as the B cell zone homing factor CXCR5. On the other hand they down regulate the T cell zone homing factors CCR7 and IL7R. Accordingly, they can be found in B cell follicles and germinal centers, where they interact with antigen-specific B cells in order to facilitate B cell differentiation (Tangye et al, 2013). Upon interaction of naïve CD4 T cells with antigen presenting dendritic cells in the T cell zone, these primed cells up regulate BCL-6 as well as CXCR5 to become early T_{FH} cells. Subsequently, they migrate to the B cell zone where they encounter signal transducer and activator of transcription (STAT) 3 activating cytokines such as IL6, IL12, IL21 and IL27, secreted among others by follicular dendritic cells. This results in up regulation of SAP, MAF, BATF and IRF4, major regulators of the T_{FH} lineage (Tangye et al, 2013). In addition, T and B cell interactions including CD40-CD40L, ICOS-ICOSL and CD28-CD86 are crucial in this developmental phase. It has been shown that not only naive CD4 T cells but also NKT as well as γδT cells can develop into T_{FH} cells in a similar manner (Tangye et al, 2013). Whereas a lot of effort was taken to understand the role of T_{FH} cells in the germinal center, less attention has been paid to CD8 or TH17 cells until now (Victora et al, 2010).

1.5. Inherited disorders of the immune system

Primary immunodeficiencies (PID) are considered a heterogeneous group of inherited diseases that can affect either innate or adaptive immune system separately, as well as their intricate interplay (Al-Herz et al, 2014).

Importantly, PIDs have to be distinguished from secondary or acquired immunodeficiencies triggered by pathogens, malignant diseases, immune modulatory treatments or environmental factors (Duraisingham et al, 2014).

Until 3 years ago approximately 200 PID-causing genes have been identified (Al-Herz et al, 2011). In recent years the number increased rapidly. To date, mutations in more than 245 genes have been identified to cause a PID in men (Al-Herz et al, 2014). Nevertheless, even with the identification of a gene defect, the precise pathomechanism of the disease often remains elusive at first but represents a unique opportunity to understand specific aspects of the immune system (Fischer, 2007).

In general, PIDs are under-diagnosed as the primary manifestation can be highly variable but most of the time involves increased susceptibility to infections which can only be evaluated in retrospect (McCusker & Warrington, 2011). However, if unrecognized, the disease can be fatal due to high risk of infections or due to the occurrence of autoimmune phenomena or malignancy. PIDs are classified according to the primarily affected component of the immune system (Geha, 2007). Defects of the innate immune system include phagocyte disorders, defects in Toll-like receptor mediated signaling and complement disorders. Antibody deficiency syndromes and combined immunodeficiencies (CID) are considered as defects of the adaptive immune system. A unifying clinical presentation is increased susceptibility to recurrent infections and severe infections, or sometimes both, with characteristic susceptibility to certain pathogens, depending on the nature of the immune defect. Moreover, certain forms of PIDs might present with immune dysregulation or even a more complex phenotype where immunodeficiency represents only one of multiple components of the patient's disease (Notarangelo, 2010). The adaptive immune system, comprising the lymphoid compartment, has developed most recently in evolutionary history. Although acting slower than the innate immune response, this system has the ability not only to develop and tailor the highly specific immune answer to foreign, but also to memorize, leading to fast eradication of antigens upon a second encounter. Therefore, in order to understand the development of primary immunodeficiencies, understanding lymphocyte development is crucial. On

the other hand, also the discovery of PID contributed in an essential manner to the understanding of the immune system as we see it today.

1.5.1. (Severe) Combined Immunodeficiencies

Severe combined immunodeficiencies (SCID) represent the most severe forms of T cell immunodeficiencies with an intrinsic impairment of T cell development, sometimes associated with a severe impairment of other hematopoietic lineages.

The disease was first described by Glanzmann and colleagues in 1950 (Glanzmann & Riniker, 1950). SCID results in dramatic susceptibility to pathogens, including especially infections with opportunistic bacteria, viruses and fungi. SCID patients are classified according to the absence or presence of B and NK cells in addition to the T cell defects. As most of the patients succumb to infections within the first year of life, the necessity for a curative treatment is evident. In 1968, the first successful allogeneic bone marrow transplantation was performed in a child with SCID due to a defect in the common γ-chain receptor (Gatti et al, 1968). To date, globally four different molecular mechanisms have been shown to lead to SCID in humans (Fischer et al, 2005). One group includes mutations affecting the common γ-chain of several cytokine receptors. This disease is X-linked and results in absence of mature T and NK cells but presence of CD19+ B cells.

Another mechanism involves increased apoptosis of lymphoid precursors due to defective purine metabolism caused by deficiency of adenosine deaminase (*ADA*), first described in 1972 (Giblett et al, 1972). Later is has been discovered that lymphoid precursors are especially sensitive to the accumulation of deoxyadenosine triphosphate. In addition, it could be shown that the severity of the SCID phenotype corresponds to the residual activity of *ADA* (Hershfield, 2003).

The third group of mutations responsible for a SCID phenotype in humans are defects in pre-TCR/TCR signaling (Liston et al, 2008). For example, somatic rearrangement of both TCR and BCR is essential for lymphocyte differentiation and function. Accordingly, complete loss of function mutations in the recombination-activating genes (*RAG*) *1* and *RAG2* result in SCID with

presence of the NK lineage whereas hypomorphic mutations may result in Omenn syndrome (Schwarz et al, 1996; Villa et al, 1998). Taken together, these groups correspond to the majority of SCID patients. Hypomorphic mutations in SCID genes often result in oligoclonal and poorly functioning T cells. Often, the disease is associated with inflammatory and autoimmune manifestations, which can mask the immune defect at first (Felgentreff et al, 2011; van der Burg & Gennery, 2011).

Over the last years an increasing number of this type of diseases have been identified which seem to affect late stages of T cell development. In addition to significant immune dysregulation and severe impairment in pathogen defense, they can also be associated with an increased risk of malignancy. Often, CIDs are furthermore associated with additional B cell defects, either intrinsic or caused by defective T-helper cell activity. Mechanisms of T cell deficiencies are summarized in figure 2 taken from Liston et al, 2008.

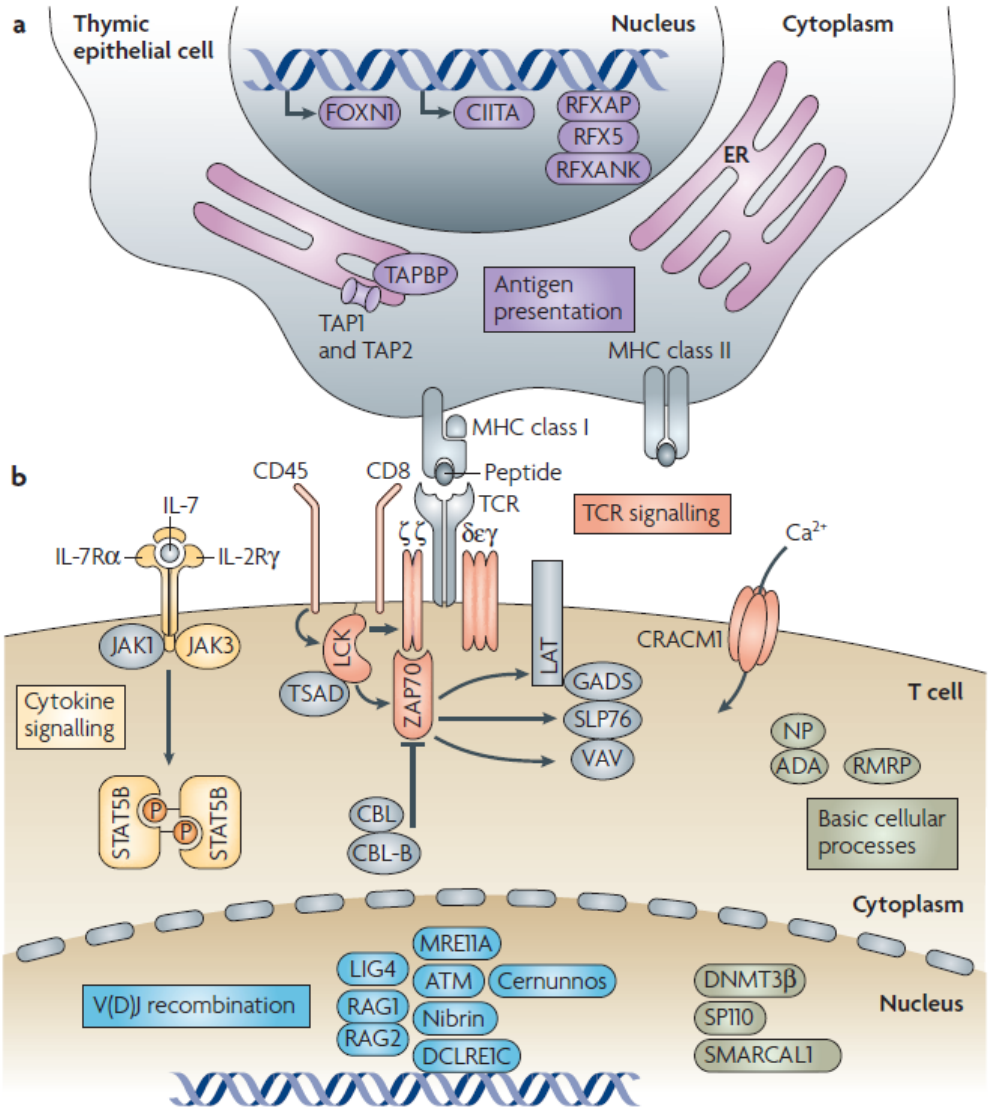

Figure 2 Mechanisms of T cell deficiencies (taken from Liston et al, 2008).

1.5.2. Primary antibody deficiencies

Primary antibody deficiencies (PAD) can result from B cell intrinsic defects but also as a secondary effect due to functional impairments in other immune cells such as T or innate immune cells. In general, most of the defective genes highlighted processes involved in B cell development and antibody production (Durandy et al, 2013). However, especially PADs often lack genotype phenotype correlation. This is mirrored by the fact that different mutations in the same gene can lead to distinct phenotypes even with different modes of inheritance (Durandy et al, 2013). Moreover, although most PIDs follow a monogenic pattern of inheritance, some PADs seem to have a

more complex genetic basis, also with variable penetrance as exemplified for mutations in the *TNFRS13B* gene (Salzer et al, 2009). Possibly due to this complexity in inheritance and variability in phenotypes the etiology of several PADs is still not known.

The most common PAD is caused by mutations in the Bruton's tyrosine kinase (*BTK*) gene (Vetrie et al, 1993). It was first described in 1952 and is the most common reason for X-linked agammaglobulinemia. Intrinsic B cell defects marked for example by defective expression of the pre-BCR result in absence of mature B cells, concomitant with absence of all immunoglobulin isotypes. This phenotype has been described for mutations in the *l5* chain (Minegishi et al, 1998), the *µ-chain* (Yel et al, 1996), the scaffolding protein B cell linker (*BLNK*) (Minegishi et al, 1999b), the regulatory subunit 1 (p85a) of Phosphoinositide-3-Kinase (*PIK3R1*) (Conley et al, 2012) as well as the *pre-BCR* and *BCR co-receptors Ig*a (Minegishi et al, 1999a) and *Igb* (Ferrari et al, 2007).

Another group of antibody deficiencies is represented by defects in B cell survival and homeostasis which is critically regulated by the two cytokines B cell activating factor (BAFF) and a proliferation inducing ligand (APRIL) (Durandy et al, 2013). Whereas BAFF binds to the BAFF-receptor (BAFFR), the trans-membrane activator and CAML interactor (TACI) and the B cell maturation antigen (BCMA), APRIL only bind to TACI and BCMA. Although BAFFR and TACI mutations have been reported to cause PADs in humans many of the described variants can also be found in healthy individuals.

Defects in B cell activation may result in pan-hypogammaglobulinemia with normal numbers of circulating B cells (Durandy et al, 2013). One of the major pathways in B cell activation is BCR-induced Ca^{++} signaling which is regulated by B cell surface molecules that modulate intensity and threshold of signal transduction (Durandy et al, 2013). An important regulator of signal transduction is the CD19 complex consisting of CD19, CD21, CD81 and CD225. In line with this observation, mutations in CD19 (van Zelm et al, 2006), CD21 (Thiel et al, 2012) and CD81 (van Zelm et al, 2010) have been described to cause hypogammaglobulinemia due to defective B cell activation. Interestingly, although defects in CD20 do not affect BCR mediated

calcium signaling, the defect still results in partial hypogammaglobulinemia (Kuijpers et al, 2010).

The immunoglobulin class switch recombination pathway is initiated by interaction of CD40 on B cells and CD40-ligand on activated T_{FH} cells. This interaction induces the activation-induced deaminase (AID) to generate deoxyribonucleid acid (DNA) lesions by deaminating cytosines to uracils. Subsequently, uracils in a DNA strand are recognized and processed by uracil-N-glycosylase (UNG) leading to double strand breaks. Double strand breaks are detected, among others by Ataxia-telangiectasia mutant (ATM) and the MRN complex (MRE11-RAD50-NBS1) and repaired by the non-homologous end joining pathway (NHEJ) leading to class switch recombination. Defects in this pathway usually lead to normal or increased serum IgM levels but reduction or absence of IgG, IgA and IgE. So far mutations affecting CD40 (Ferrari et al, 2001), CD40L (Allen et al, 1993), AID (Revy et al, 2000), and UNG (Imai et al, 2003) have been described. Mutations affecting ATM (Savitsky et al, 1995), MRE11 (Stewart et al, 1999) and NBS1 (Carney et al, 1998; Varon et al, 1998) have also been described but result in syndromes with a broader clinical picture, apart from CSR defects.

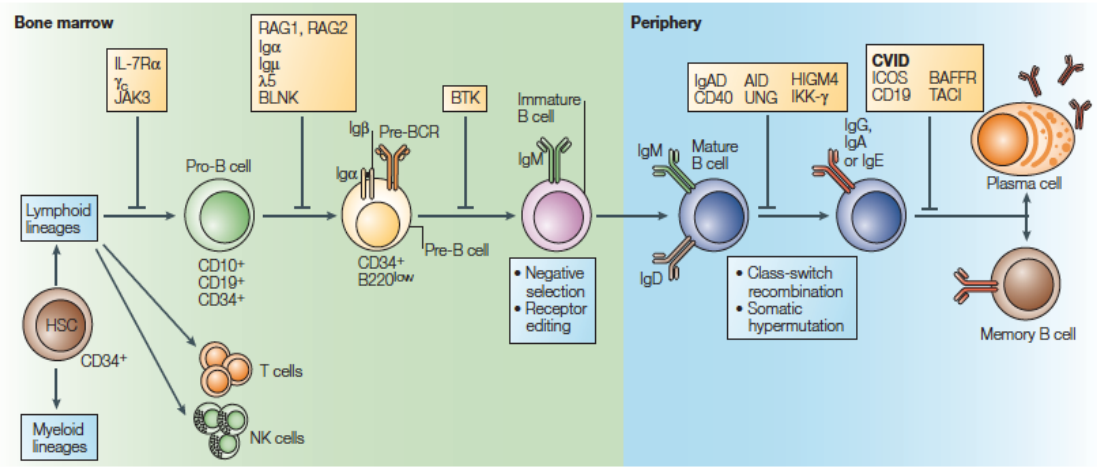

Figure 2 | **Protein and gene defects in B-cell development and function.** Haematopoietic stem cells (HSCs) give rise to

Figure 3 Gene defects in B cell development (taken from Cunningham-Rundles & Ponda, 2005)

1.6. Central and peripheral tolerance

Immune tolerance refers to the absence of an immune response to substances or tissues, which are capable of eliciting immune reactions. Dependent on the location of the induction, classifications distinguish central (bone marrow/thymus) from peripheral (lymph node) tolerance. Physiological development of the immune system and the body requires immune tolerance. Whereas central tolerance describes how the immune system learns to distinguish between self and non-self, peripheral tolerance is necessary to prevent over-reactivity of the immune system towards environmental factors such as commensal bacteria or allergens. Even though central tolerance mechanisms work well, not all self-reactive cells can be eliminated as not all self-antigens are expressed in the thymus. Consequently, peripheral tolerance mechanisms are necessary to induce tolerance in these T-lymphocytes (Hogquist et al, 2005).

1.6.1. Central tolerance

Central tolerance is essential for lymphocytes to focus the immune response on pathogens instead of healthy tissue. It takes place either in the bone marrow (B cells) or in the thymus (T cells). During a process called positive selection progenitor T cells able to bind to MHC molecules, are positively selected to differentiate into mature T cells. However, during positive selection self-reactive T cells are also enriched, which increases the risk for autoimmunity. Progenitors with the strongest avidity to self-peptide/MHC complexes are therefore either eliminated during negative selection or made self-tolerant. Thus only weakly reactive progenitors are allowed to mature to take part in immune responses to foreign pathogens (Hogquist et al, 2005).

The process of clonal deletion mainly characterizes central tolerance. This process results in apoptosis of T cells with high affinity for self-antigens (Palmer, 2003). Another process which has mainly been described for B cells is receptor editing. This is a tolerance mechanism where self-antigen binding induces antigen receptor rearrangement either in the T-cell receptor alpha chain or in B cells within the immunoglobulin light chain locus. Another possible tolerance mechanism is anergy, which describes an absent response to antigen even under optimal conditions (Hogquist et al, 2005).

The following three cell subsets are thought to be induced due to high-affinity TCR binding to self-peptides in the thymus: $CD4^+CD25^+FOXP3^+$ cells (Tregs), CD8αα intestinal epithelial lymphocytes and natural killer (NKT) cells (Hogquist et al, 2005). The importance of these processes has been highlighted by mutations in AIRE (Ahonen et al, 1990; Leonard, 1946) and FOXP3 (Powell et al, 1982) in humans (and will be discussed later).

1.6.2. Peripheral tolerance

Although central tolerance mechanisms are efficient, self-reactivity cannot be controlled in all circumstances, highlighting the need for additional tolerance mechanisms. In peripheral tolerance, self-reactive T cells become either unresponsive in a process called anergy or are deleted upon encounter of a self-antigen outside of the thymus (Xing & Hogquist, 2012). These mechanisms are especially important at the time when lymphocytes encounter self-antigens outside of the thymus, such as in the case of developmental antigens, food antigens or antigens only present in times of chronic infection (Xing & Hogquist, 2012).

When T cells encounter a costimulatory signal mediated by CD28 ligation in addition to a TCR signal, they become activated and start to produce cytokines such as IL-2 (Xing & Hogquist, 2012). Activation of the IL-2 receptor complex leads to PI3K/AKT and mTOR mediated T cell proliferation. In case of anergy, mTOR activation is actively suppressed thereby blocking proliferation (Chi, 2012).

1.7. Autoimmunity and auto inflammation

Autoimmunity and auto inflammatory diseases are diseases, which may lead to similar clinical manifestations such as tissue damage and inflammation. However, molecularly they differ in their mechanism. In auto inflammatory disorders the innate immune system directly causes tissue damage for example via altered cytokine production. In autoimmune disorders, the adaptive immune system gets activated and causes tissue damage via autoantibody production or T cell auto reactivity (Doria et al, 2012).

1.7.1. Innate Immune Activation-Auto inflammation

As the innate immune system represents a first line of defense and lacks

memory, it relies on the ability of germline encoded receptors to distinguish between foreign and self-antigens (Cheng & Anderson, 2012). This response is mediated by both complement system and cellular components such as dendritic cells as well as monocytes/macrophages. This response is tightly interconnected with the adaptive immune system and can amplify adaptive immune responses.

To date, syndromes known to result from defects of the adaptive immune system, are for example C1q deficiency, spondyloenchondrodysplasia with immune dysregulation (SPENCDI) and Aicardi-Goutières Syndrome (AGS) (Doria et al, 2012)

1.7.2. Activation of the adaptive immune system - Autoimmunity

B and T cells are unique in their specificity for antigens. Syndromes have been described which highlight the necessity of central and peripheral tolerance mechanisms of the adaptive immune system.

The immune dysregulation, polyendocrinopathy, enteropathy and X-linked (IPEX) syndrome was described in 1982 by Powell (Powell et al, 1982). Clinically, the majority of patients present with watery diarrhea, eczema, diabetes, thyroid autoimmunity, exaggerated response to viral infections and/or an overall increased susceptibility to infections (Moraes-Vasconcelos et al, 2008). Molecularly this syndrome is caused by mutations in the *FOXP3* gene, a key factor for regulatory T cell function. However, also activated T cells transiently express *FOXP3* (Zheng et al, 2010; Zhou et al, 2009). Suppression of immune responses is a complex process and different mechanisms have been proposed (Tang & Bluestone, 2008).

Autoimmune polyendocrine syndrome type 1, also known as APECED is a highly variable disease affecting multiple organs. The diagnosis of APS1 has to include at least two of the three hallmark conditions: autoimmune adrenal insufficiency, autoimmune hypoparathyroidism, and mucocutaneous candidiasis. Descriptions of this syndrome date back to 1910 when the first cases in Finland were described (Ahonen et al, 1990; Leonard, 1946). Most common mutations found in these patients are in the *autoimmune regulator* gene (*AIRE*). This gene is expressed in thymic epithelial cells (TEC) where it regulates expression of self-antigens to be presented to developing

thymocytes during positive selection (Derbinski et al, 2005; Derbinski et al, 2001). Recently, some studies have also described *AIRE* expression outside of the thymus in lymph nodes and the spleen (Gardner et al, 2009). It has also been shown that autoantibodies to IL17A and IL17F in these patients are associated with the development of candidiasis (Puel et al, 2010). This is in line with the observation that TH17 responses are essential for fungal immunity (Puel et al, 2011).

Immune dysregulation is also a common feature of some patients with combined immunodeficiencies or patients with leaky-SCID phenotypes (Notarangelo, 2009). For example in patients with *PNP* deficiency, autoimmunity is present in 30% (Notarangelo, 2009). Most commonly patients present with autoimmune hemolytic anemia, however, immune thrombocytopenia (ITP) or neutropenia have been reported as well. It is thought that these complications arise from hyperactive B cells due to loss of T cell regulation (Markert, 1991; Rich et al, 1979). Moreover, patients with *ADA* deficiency or partial DiGeorge anomaly after thymus transplantation can present with autoimmune cytopenias (Notarangelo, 2009). Interestingly, also V(D)J recombination and non-homologous end joining defects can lead to autoimmune cytopenias (Notarangelo, 2009).

Moreover, patients with CVID commonly present with autoimmune manifestations. It has been shown that, depending on country and study, autoimmune manifestations range between 22-48% (Notarangelo, 2009). Also in these patients cytopenias are common and might be the first manifestation of the disease. It has been shown that patients with decreased numbers of regulatory T cells or decreased switched memory B cells together with high $CD19^{hi}$ $CD21^{lo}$ B cells have a higher risk of developing autoimmune cytopenia. However, there is no correlation with organ specific autoimmunity but with splenomegaly (Notarangelo, 2009).

1.8. Lymphoproliferation

In Autoimmune lymphoproliferative syndrome (ALPS) proliferation of double negative T cells (DNT) result from loss of Fas-mediated apoptosis. Canale and Smith first described ALPS in 1967 (Canale & Smith, 1967) as lymphoproliferation simulating malignant lymphoma. However the term ALPS

was not used until 1992 when Sneller and colleagues published a „novel lymphoproliferative syndrome resembling murine *lpr/gld* disease“ (Sneller et al, 1992). ALPS criteria include persistent lymphadenopathy and/or splenomegaly in the absence of infectious or malignant etiologies, autoantibodies (anti-cardiolipin, anti-nuclear antibodies and a positive direct Coombs test), overt autoimmune disease and the presence of DNT cells. Most commonly these patients present with autoimmune cytopenias (Oliveira et al, 2010). The disease is frequently caused by mutations in the *FAS* gene (*TNFRSF6*) and is inherited in an autosomal dominant way. However, although less frequently, also mutations affecting *FASLG*, *CASP8*, *CASP10*, *NRAS*, *CTLA4* (ALPS1-5) are known to cause ALPS or a similar phenotype (Al-Herz et al, 2014). The severity depends on the genotype and can vary even among different family members (Jackson et al, 1999). In addition, these patients have an increased risk for developing malignancies of about 10-20% (Straus et al, 2001). Molecularly the disease can be explained by an inappropriate cessation of the immune response (Bidere et al, 2006). Physiologically, a high antigen load can stimulate Fas mediated cell death via up regulation of Fas ligand (FasL) on T cells. In addition, after successful clearance of the antigens, T cells undergo passive apoptosis due to lymphokine withdrawal. In case of defective Fas signaling, activated T cell accumulate and cause autoimmune phenomena.

1.8.1. EBV-driven lymphoproliferation

Epstein-Barr virus (EBV), which was discovered 40 years ago when studying Burkitt’s lymphoma, is common in humans and causes infectious mononucleosis in adults. It is a member of the Herpes virus family and persists in the vast majority of people as an asymptomatic infection (Young & Rickinson, 2004). However in patients with underlying immunodeficiencies it may lead to severe immune dysregulation presenting as fatal mononucleosis, lymphoproliferative disease (LPD), Hodgkin and Non-Hodgkin lymphoma, lymphomatoid granulomatosis, hemophagocytic lymphohistiocytosis (HLH) as well as dysgammaglobulinemia (Chen, 2011). These manifestations are especially common in patients with defects in important T cell receptor pathway genes, T and B cell interactions or defects in proteins important for

the cytotoxic pathway in lymphocytes. Therefore these conditions have been termed hemophagocytic syndromes or familial (primary) HLH (see below). Moreover, a rather heterogeneous group of patients with mutations in *DCLRE1C* (Moshous et al, 2001), *RAG1* and *RAG2* (Schwarz et al, 1996), *SH2D1A* (Coffey et al, 1998), *XIAP* (Rigaud et al, 2006) as well as the *LIG4* (O'Driscoll et al, 2001) gene, can present with lymphoproliferation and immune dysregulation after EBV infection. Within recent years additional immunodeficiencies presenting with lymphoproliferation have been discovered such as *STK4* (Nehme et al, 2012), *MAGT1* (Li et al, 2011) and *ITK* (Huck et al, 2009) deficiency.

1.9. Hemophagocytic lymphohistiocytosis (HLH)

HLH can be a life-threatening clinical condition characterized by extensive and uncontrolled activation of immune cells, especially macrophages. This over activation leads to significant overproduction of IFNγ , IL-6 and IL-10 mirrored clinically by symptoms including splenomegaly, cytopenias, prolonged fever, liver failure, seizures and abnormal NK cell function (Faitelson & Grunebaum, 2014). Laboratory alterations include elevated soluble CD25, CD168 as well as CD107 surface expression on NK and cytotoxic T cells (Jordan et al, 2011). An important, albeit rare, differential diagnosis includes visceral leishmaniasis, which can present with similar clinical symptoms.

HLH can occur in different clinical contexts and primary (genetic) HLH has to be distinguished from secondary HLH.

Primary HLH is associated with mutations in *PRF1, UNC13D, STX11, STXBP2, RAB27A, AP3B1* (Hermansky-Pudlak type 2) *ATXBP2, SH2D1A (SAP), XIAP, ITK, IL2Rγ, PNP* and *LYST.* Furthermore patients with more complex syndromes such as DiGeorge Syndrome, Wiskott-Aldrich syndrome, chronic mucocutaneous candidiasis, X-linked agammaglobulinemia can present with HLH (Faitelson & Grunebaum, 2014).

In secondary HLH, no family history or genetic cause is evident. It is hypothesized, that HLH is triggered by concurrent infections (EBV) or other medical conditions (malignancy, rheumatologic disorders) (Faitelson & Grunebaum, 2014).

Among triggering infections, EBV infections are most commonly associated with HLH. In this setting the disease course varies between spontaneously resolving and severely life-threatening necessitating HSCT. Among others, especially patients with XLP are at high risk to develop EBV-HLH (Faitelson & Grunebaum, 2014).

1.10. Clinical diagnosis of patients with primary immunodeficiencies

It is well known that PID patients have increased risk for infections, however often non-infectious manifestations such as autoimmunity or auto inflammation may initially be dominant clinical feature. Therefore, PIDs are under-diagnosed as the primary manifestation is highly variable (McCusker & Warrington, 2011). Due to their multifaceted clinical presentations, patients often present to various medical and surgical specialists before the correct diagnosis is made.

Nevertheless, increased susceptibility to infections is the hallmark of the disease. Mostly, patients present with chronic or reoccurring, rarely with unusually severe life threatening infections. Guidelines for diagnosis include infections with opportunistic pathogens, localization/severity of the infection and type of pathogen. In patients with defects in cellular immunity virus and fungal infections are common, whereas patients with antibody deficiencies, are more likely to present with infections with encapsulated bacteria or enteroviruses (Bousfiha et al, 2013). Infections with encapsulated bacteria are also common in patients with complement deficiencies, where they lead to meningitis, bacteremia or septic arthritis. Phagocytic syndromes are often mirrored by infections of skin, lymph nodes, spleen and liver (Lederman, 2000). As specified above, PID patients can also present with signs of autoimmunity/auto inflammation. These clinical manifestations can either be limited to one organ such as thrombocytopenia or autoimmune thyroiditis, or affect several organs. However, although certain manifestations seem to be autoimmune they rather reflect persistent infections such as enterovirus infection or dermatomyositis (Lederman, 2000). PID may present in a syndromic complex due to additional extra-immune manifestations. Examples are patients with Wiskott-Aldrich syndrome, which exhibit thrombocytopenia,

eczema, T and B cell dysfunctions (Aldrich et al, 1954; Wiskott, 1937). This enables clinicians to diagnose the patient prior to onset of immunodeficiency symptoms.

1.11. Therapy of primary immunodeficiencies- curative approaches

1.11.1. Hematopoietic stem cell transplantation (HSCT)

The first successful bone marrow transplantations were performed in 1968 in two SCID patients and one patient with WAS (Bortin, 1970; Gatti et al, 1968). Since then, constant progress has been made to improve transplant-related morbidity and mortality (Filipovich, 2008). High resolution tissue typing, cooperating donor registries and pre- transplant conditioning has led to increased survival of PID patients. Moreover, genetic diagnoses for several PIDs provide additional rationale for transplantation, especially in diseases with natural disease course survival rates below 20 years of age (Filipovich, 2008). For example, severe forms of PID, such as SCID patients, profit from HSCT and this treatment has increased survival rates in these patients to up to 90%.

Dependent on the underlying immunodeficiency, different conditioning regimens have been applied. Albeit HSCT is applied for PID patients since 40 years, there is no clear consensus about donor source or conditioning regimens (Filipovich, 2008). According to the Center for International Blood and Marrow Transplantation (CIBMTR) as well as the European Blood and Marrow Transplant network the type of SCID, predicts early (1–3 years) survival, with better results in T-B+NK- forms and worst outcomes in ADA, specifically with the use of unrelated donors (Myers et al, 2002). A good prognostic factor is young age at transplant (under 1 year, and preferably in the first weeks of life) (Myers et al, 2002).

Myeloablative conditioning regimens often lead to significant transplant-related morbidity and mortality. On the other hand, use of reduced-intensity conditioning (RIC) did not lead to severe acute toxicity in patients with pre-HSCT comorbidities, with the additional advantage of reducing or even avoiding long-term sequelae, especially infertility and growth retardation. On

the other hand, RIC are associated with increased probability of mixed donor chimerism and graft rejection. However, mixed donor engraftment is likely to correct for many primary immunodeficiency disorders, but still donor lymphocyte infusion second to HSCT procedures are sometimes required to increase donor chimerism.

Major adverse risk factors are pre-existing co-morbidities and infections prior to HSCT and infections post HSCT. Therefore, early genetic diagnosis maybe even in an asymptomatic state can improve success and the appropriate conditioning regimen can increase the positive outcome of HSCT in PID patients.

1.11.2. Gene therapy

As primary immunodeficiencies constitute a large and heterogeneous group of diseases, gene therapy is only indicated in distinct forms of PID. In addition, in approximately one third of patients no HLA-matched donor can be found. Particularly in those patients gene therapy may represent the best potential curative treatment option. In gene therapy the goal is to *ex vivo* correct for/restore gene expression in autologous cells and transplant them back into the patient. Such cells with restored function would revert disease symptoms in the host. To date gene therapy trials have been performed for ADA-SCID (Aiuti et al, 2009; Gaspar et al, 2011), patients with mutations in the common γ-chain (Gaspar et al, 2011), patients with WAS (Boztug et al, 2010) and CGD patients (Kang et al, 2011) among others. Whereas gene therapy for ADA-SCID was well tolerated, adverse effects including leukemia were observed with other diseases for reasons still not completely understood (Kildebeck et al, 2012).

In early trials mainly retroviral vectors with strong promoters were used to insert the functional copy into the cells. However as these vectors have a tendency to integrate in the vicinity of gene promoters they can also increase expression of potential oncogenes. Therefore in the last decade, systems were changed to self-inactivating (SIN) lentiviral vectors with endogenous promoters driving transgene expression. In addition these vectors do not depend on active replication of cells for infection and integration (Kildebeck et al, 2012).

Albeit partially successful clinical trials with viral vector systems, transgene silencing due to DNA methylation, insertional oncogenesis and absence of endogenous gene regulation let to efforts to find alternative approaches such as gene targeting using zinc finger nucleases (Porteus & Baltimore, 2003).
Taken together, improvements in the design of viral vectors and the development of new tools for precise gene targeting represent promising strategies for gene therapy trials and might be able to reduce genotoxic side effects.

1.12. Therapy of primary immunodeficiencies- supportive/ non-curative approaches

1.12.1. Enzyme replacement therapy

Unlike other PIDs, in ADA deficiency, another treatment option exists, being enzymatic replacement therapy (ERT) with pegylated bovine ADA (Gaspar et al, 2009). ERT is used as an initial therapy for patients where no related HLA-identical donor is available or where due to risk assessment of the physician ERT was chosen to be the most beneficial treatment option.

1.12.2. Immunoglobulin substitution

Since the 1950s Immunoglobulin (Ig) substitution has been performed for patients with hypogammaglobulinemia. Over the past years the use of Ig has been extended to patients with partial antibody deficiency and patients with combined immunodeficiencies (Group of Pediatric, 2013). This treatment leads to longer life expectancy by reducing infection frequency and lung damage. However, as a human blood product it also bears a risk of infection. It can be administered either subcutaneously or intravenously, with similar efficacy and safety (Group of Pediatric et al, 2013).

1.12.3. Antimicrobials

Although there is limited evidence for prophylactic antibiotics in many PIDs they are widely used. Mostly, common practices are based on knowledge about infecting organisms or studies with patients suffering from acquired immunodeficiencies (Group of Pediatric et al, 2013).
Clear indications for prophylactic antimicrobial therapy are patients with chronic granulomatous disease, since they have a high risk of developing

infections with *Staphylococcus aureus*, *Nocardia* and *Burkholderia species*. Patients with humoral immunodeficiencies are at risk of developing bronchiectasis due to chronic lung damage. In this group of patients as well as in patients with hyper-IgE syndrome azithromycin is applied. In T cell immunodeficiencies including SCID patients prophylactic co-trimoxazole is used due to the high risk of *Pneumocystis Jeroveci* infections (Group of Pediatric et al, 2013).

1.12.4. Antifungals

Fungal infections can be challenging to diagnose. However, this type of prophylaxis is mainly limited to patients with CGD, as they show a high risk for developing invasive fungal infections (Group of Pediatric et al, 2013).

2. Aim of the thesis

The aim oft this thesis was to identify novel monogenic disorders leading to primary immunodeficiencies using state of the art technologies such as exome sequencing and homozygosity mapping.

The identification and functional characterization of the underlying genetic defects will help to understand the pathogenesis of these diseases and may open novel therapeutic opportunities for targeted therapy of affected individuals. Moreover, the identification of key-molecules in the development of autoimmunity and/or lymphoproliferation will help to understand the pathophysiology and pathogenesis not only of these monogenic diseases, but can be extrapolated to a large group of diseases involving autoimmunity.

3. Results and Discussion

3.1. Combined immunodeficiency with life-threatening EBV-associated lymphoproliferative disorder in patients lacking functional CD27

Elisabeth Salzer,[1*] Svenja Daschkey,[2*] Sharon Choo,[3] Michael Gombert,[2] Elisangela Santos-Valente,[1] Sebastian Ginzel,[2] Martina Schwendinger,[1] Oskar A. Haas,[4] Gerhard Fritsch,[5] Winfried F. Pickl,[6] Elisabeth Förster-Waldl,[7] Arndt Borkhardt,[2#] Kaan Boztug,[1,7#] Kirsten Bienemann,[2#] and Markus G. Seidel[8,9#]

[1]CeMM Research Center for Molecular Medicine, Austrian Academy of Sciences, Vienna, Austria; [2]Pediatric Oncology, Hematology and Clinical Immunology, Medical Faculty, Heinrich Heine University, Düsseldorf, Germany; [3]Department of Allergy and Immunology, Royal Children's Hospital, Melbourne, Australia; [4]Medgen.at GmbH, Vienna, Austria; [5]Children's Cancer Research Institute, Vienna, Austria; [6]Institute of Immunology, Medical University Vienna, Austria; [7]Department of Pediatrics and Adolescent Medicine, Division of Neonatology, Pediatric Intensive Care and Neuropediatrics, Medical University Vienna, Austria; [8]St. Anna Children's Hospital, Medical University Vienna, Austria; and [9]Pediatric Hematology Oncology, Medical University Graz, Austria

(*&# equal contribution)

Combined immunodeficiency with life-threatening EBV-associated lymphoproliferative disorder in patients lacking functional CD27

Elisabeth Salzer,[1*] Svenja Daschkey,[2*] Sharon Choo,[3] Michael Gombert,[2] Elisangela Santos-Valente,[1] Sebastian Ginzel,[2] Martina Schwendinger,[1] Oskar A. Haas,[4] Gerhard Fritsch,[5] Winfried F. Pickl,[6] Elisabeth Förster-Waldl,[7] Arndt Borkhardt,[2#$] Kaan Boztug,[1,7#$] Kirsten Bienemann,[2#] and Markus G. Seidel[8,9#$]

[1]CeMM Research Center for Molecular Medicine, Austrian Academy of Sciences, Vienna, Austria; [2]Pediatric Oncology, Hematology and Clinical Immunology, Medical Faculty, Heinrich Heine University, Düsseldorf, Germany; [3]Department of Allergy and Immunology, Royal Children's Hospital, Melbourne, Australia; [4]Medgen.at GmbH, Vienna, Austria; [5]Children's Cancer Research Institute, Vienna, Austria; [6]Institute of Immunology, Medical University Vienna, Austria; [7]Department of Pediatrics and Adolescent Medicine, Division of Neonatology, Pediatric Intensive Care and Neuropediatrics, Medical University Vienna, Austria; [8]St. Anna Children's Hospital, Medical University Vienna, Austria; and [9]Pediatric Hematology Oncology, Medical University Graz, Austria

ABSTRACT

CD27, a tumor necrosis factor receptor family member, interacts with CD70 and influences T-, B- and NK-cell functions. Disturbance of this axis impairs immunity and memory generation against viruses including Epstein Barr virus (EBV), influenza, and others. CD27 is commonly used as marker of memory B cells for the classification of B-cell deficiencies including common variable immune deficiency. Flow cytometric immunophenotyping including expression analysis of CD27 on lymphoid cells was followed by capillary sequencing of *CD27* in index patients, their parents, and non-affected siblings. More comprehensive genetic analysis employed single nucleotide polymorphism-based homozygosity mapping and whole exome sequencing. Analysis of exome sequencing data was performed at two centers using slightly different data analysis pipelines, each based on the Genome Analysis ToolKit Best Practice version 3 recommendations. A comprehensive clinical characterization was correlated to genotype. We report the simultaneous confirmation of human CD27 deficiency in 3 independent families (8 patients) due to a homozygous mutation (p. Cys53Tyr) revealed by whole exome sequencing, leading to disruption of an evolutionarily conserved cystein knot motif of the transmembrane receptor. Phenotypes varied from asymptomatic memory B-cell deficiency (n=3) to EBV-associated hemophagocytosis and lymphoproliferative disorder (LPD; n=3) and malignant lymphoma (n=2; +1 after LPD). Following EBV infection, hypogammaglobulinemia developed in at least 3 of the affected individuals, while specific anti-viral and anti-polysaccharide antibodies and EBV-specific T-cell responses were detectable. In severely affected patients, numbers of iNKT cells and NK-cell function were reduced. Two of 8 patients died, 2 others underwent allogeneic hematopoietic stem cell transplantation successfully, and one received anti-CD20 (rituximab) therapy repeatedly. Since homozygosity mapping and exome sequencing did not reveal additional modifying factors, our findings suggest that lack of functional CD27 predisposes towards a combined immunodeficiency associated with potentially fatal EBV-driven hemophagocytosis, lymphoproliferation, and lymphoma development.

Introduction

CD27 is part of the tumor necrosis factor receptor family and critical for B-, T- and NK-cell function, survival, and differentiation, respectively.[1-4] After binding to its specific ligand CD70, CD27 plays a co-stimulatory role highly relevant for anti-viral responses, anti-tumor immunity, and alloreactivity.[5] CD27 is routinely used as marker for class-switched and non-class-switched memory B cells ($CD27^+IgD^-$ and $CD27^+IgD^+$) relevant for the classification of B-cell deficiencies including common variable immune deficiencies (CVIDs).[6] Recently, Peperzak *et al.* showed that CD27 signaling is crucial for sustained survival of $CD8^+$ effector T cells in mice,[7] and *Cd27*-/- mice show impaired primary and memory $CD4^+$ and $CD8^+$ T-cell responses.[4] Thus, it may be hypothesized that constitutional lack of CD27 in humans may cause a combined primary immunodeficiency.

In immunocompetent hosts, primary Epstein Barr virus (EBV) infection is often asymptomatic, whereas in primary immunodeficiencies such as IL-2-inducible T-cell kinase (ITK) deficiency, X-linked lymphoproliferative syndromes (XLP1, XLP2), primary hemophagocytic lymphohistiocytosis syndromes (HLH) and others, EBV infection may lead to persistent symptomatic viremia.[8-11] Although EBV-specific immunity involves virus-specific humoral components, $CD8^+$ effector T cells are considered essential for long-term virus control.[12]

 doi:10.3324/haematol.2012.068791
The online version of this article has a Supplementary Appendix.
* ES and SD contributed equally to this work; # AB, KBoztug, KBienemann and MGS contributed equally to this work.
Manuscript received on May 3, 2012. Manuscript accepted on July 5, 2012.
Correspondence: arndt.borkhardt@med.uni-duesseldorf.de

Here, we report 8 pediatric patients from 3 independent pedigrees lacking functional CD27. Clinical manifestations comprised EBV-associated lymphoproliferative disorder (EBV-LPD, n=3) with or without HLH, malignant lymphoma (n=2; +1 after LPD) or absence of overt clinical phenotype (n=3).

Design and Methods

Patients

Material from patients and healthy donors was obtained upon obtaining informed consent in accordance with the Declaration of Helsinki. Family A was analyzed in Vienna, Austria, while Families B and C were assessed in Düsseldorf, Germany. The respective institutional review boards approved the study.

Flow cytometric analysis

Analysis of CD27 surface expression and B-cell class switch was performed as described previously.[13]

Information on DNA isolation, primer design, and functional analyses is available in the *Online Supplementary Appendix*.

Homozygosity mapping

Genome-wide genotyping based on Affymetrix® Genome-Wide Human SNP Array 6.0 was performed for all 5 family members of Family A. For homozygosity mapping, DNA of each core family member was diluted to 50 ng/μL in 12 μL. The protocol was carried out according to the manufacturer's instructions. Raw data were analyzed using genotyping console version 4.0.1.8.6. Loss of heterozygosity (LOH) analysis was performed as well as genotyping, followed by more detailed analysis using PLINK. Homozygous regions in all family members were detected using PLINK (*http://pngu.mgh.harvard.edu/~purcell/plink/*) using a window size of 5000bp with a minimum of 50 SNPs within this region.[14] Adjacent intervals were considered to represent a single interval when the distance between the intervals was less than 1MB in size. Based on these data, homozygous regions present exclusively in the most severely affected patient, as well as homozygous regions present in all 3 affected siblings, were detected (*Online Supplementary Tables S1 and S2*).

Exome sequencing and data analysis

Exome sequencing for Family A was performed in Vienna, while Families B and C were assessed in Düsseldorf, Germany.

For Family A, a 50 base-pair paired read multiplexed whole exome sequencing (WES) run was performed for the most severely affected patient on an Illumina HiSeq2000 Sequencer running on HiSeq Control Software (HCS) 1.4.8, Real Time Analysis Software (RTA) 1.12.4.2. For WES, DNA was diluted to 20 ng/μL in 57 μL and sample preparation was carried out using Illumina TruSeq DNA Sample Preparation Guide and the Illumina TruSeq Exome Enrichment Guide version 2. The multiplexed pool of 6 samples including the patient was run on all lanes of the flowcell. Demultiplexing and raw image data conversion was performed using Consensus Assessment of Sequence and Variation version 1.7 (CASAVA). Reads were aligned with BWA using the algorithm for short reads (up to ~200bp),[15] a gapped global alignment with maximum of a 1bp gap open to a human genome 19 (hg19) reference was performed. Insertion/deletion realignment and GATK base quality score recalibration was performed.[16] Single nucleotide variants (SNVs) and insertions/deletions were called using Unified Genotyper and GATK Variant quality score recalibration (1000Genomes, HapMap, dbSNP131) was performed. All thresholds for GATK tools were based on the GATK Best Practice Variant Detection version 3 recommendations. SNV and insertion/deletion lists were uploaded to SeattleSeq Annotation database. Variants present in 1000 Genomes and dbSNP were excluded and lists were filtered for nonsense, missense and splice-site variants within the homozygous regions detected in the most severely affected patient.

For Families B and C, a similar approach was taken with minor differences referring to the usage of single reads with 100 cycles, and alignment with BWA for long reads and using dbSNP132 dataset. Resulting variation calls were annotated by NGS-SNP17 using a local copy of the ENSEMBL databases, PolyPhen2,[18] SIFT[19] and ConDel[20] before being imported into an SQL database.

Results

Patient reports and immunological findings

Patient 1 (Family A, Figure 1A), a 5-year old girl from a Turkish consanguineous family, presented at the age of 17 months after severe primary infectious mononucleosis with suspected hemophagocytic lymphohistiocytosis (HLH). A brief summary of her clinical phenotype was recently described.[21] She developed fulminant EBV-LPD and systemic inflammatory response syndrome, and was treated repeatedly with high-dose steroids and rituximab over four years. Flow cytometry revealed undetectable $CD27^+$ lymphocytes (Figure 1B), an increase in transitional and $CD21^{low}$ B cells, and near-absent invariant natural killer T (iNKT) cells (Table 1 and *Online Supplementary Figure S1*). EBV-specific T cells were detectable (Table 1), and T-cell receptor Vβ spectratyping showed no major abnormalitites in $CD4^+$, but moderate oligoclonality in $CD8^+$ T cells (*data not shown*). Two of her siblings (Patients 2 and 3; Figure 1A), were clinically asymptomatic but showed borderline hypogammaglobulinemia (Table 1 and *Online Supplementary Appendix*). Specific IgG antibody responses against EBV-viral capsid antigen were positive in Patient 1 before initiation of immunosuppressive and immunoglobulin substitution therapy; Patients 2 and 3 showed low-normal antibody responses against viral protein and bacterial polysaccharide antigens including CMV, EBNA1, and vaccine antigens (Table 1).

Patient 4 (Family B; Figure 1A) presented at 18 months of age with EBV-LPD and HLH, treated according to the HLH-2004 protocol (including dexamethasone, etoposide, cyclosporine-A) plus an anti-CD20 antibody (rtuximab). Although immunoglobulin levels were normal in Patient 4 at his initial presentation, he became hypogammaglobulinemic four months later. Nine months after initial presentation, EBV-LPD relapsed without signs of hemophagocytosis. He again received HLH treatment and rituximab, followed by matched unrelated cord blood transplantation. His younger sister, Patient 5 (Figure 1A), is 16 months old. She was diagnosed with absent CD27 expression and EBV-infection only after CD27 deficiency had been identified in her brother.

Patient 6 (Family C; Figure 1A) presented at the age of 15 years with EBV-LPD. He responded to rituximab but EBV-viremia recurred three months later. Although he was hypergammaglobulinemic at diagnosis, and his peripheral B cells were again detectable four months after rituximab treatment, immunoglobulin levels slowly decreased. Approximately 20 months after initial presentation, a relapse of EBV-LPD occurred, progressing into T-cell lymphoma within four months, and requiring treatment with

rituximab and chemotherapy (R-CHOP, *Online Supplementary Appendix*) followed by matched unrelated cord blood transplantation. Flow cytometric analysis at relapse revealed absent CD27$^+$ lymphocytes and very low iNKT cells (Table 1 and *Online Supplementary Figure S1*). Only limited clinical history and no immunological data are available for the patient's 2 older sisters (Patients 7 and 8). Both died of suspected EBV-driven lymphoma at two and 22 years of age, respectively (Figure 1A), and the diagnosis of CD27 deficiency was established retrospectively from Guthrie card DNA (*see below*).

Genetics

Because of the variability of phenotypes in Family A, we performed homozygosity mapping and whole-exome sequencing (WES) after earlier detection of the *CD27*

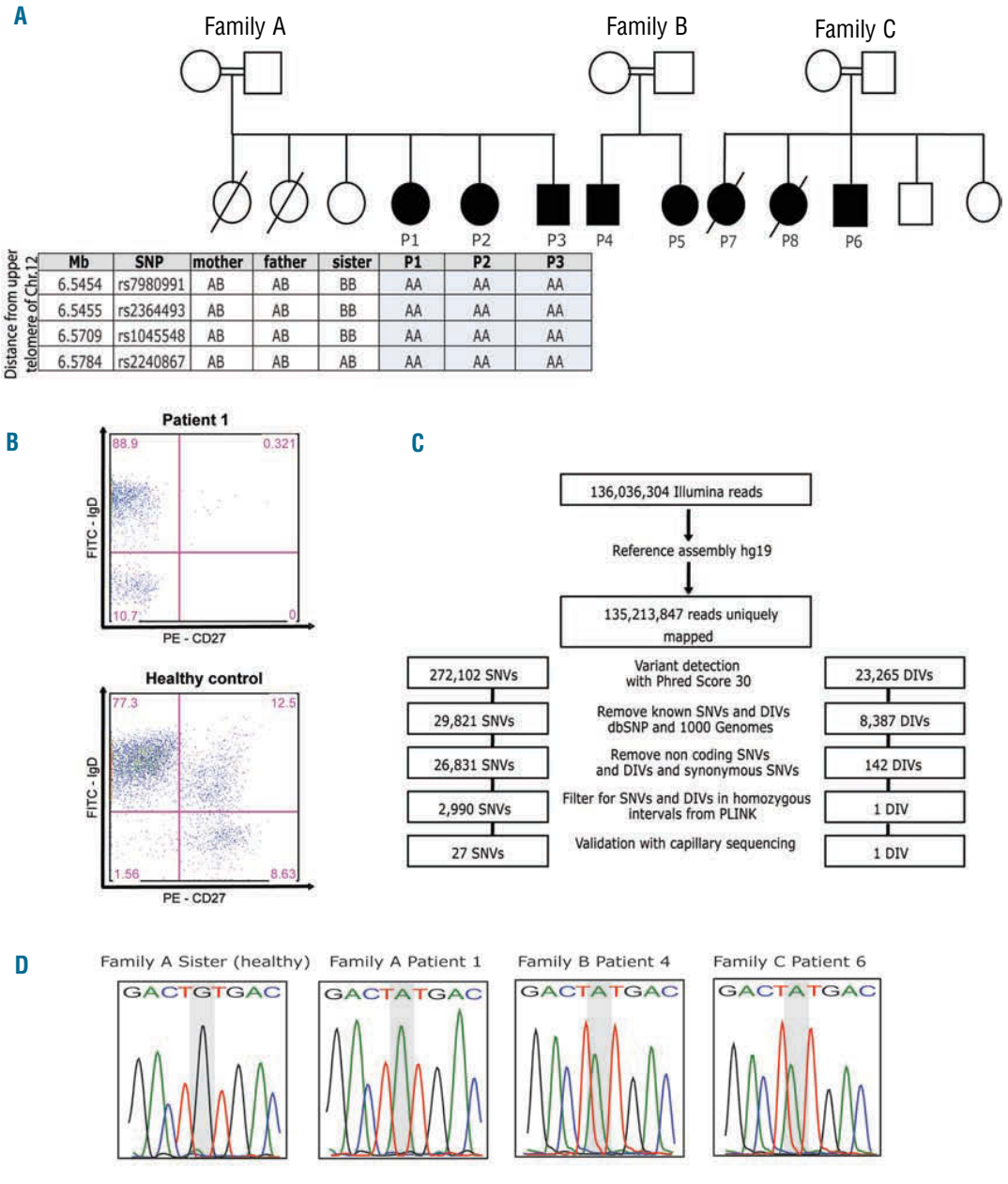

Figure 1. Identification of CD27 deficiency in three kindreds. Three pedigrees with CD27 deficiency were identified. (A) Family A was analyzed by homozygosity mapping, revealing a homozygous interval on Chromosome 12. Two siblings of Patient 1 (Family A) had died at nine months and three days of age, respectively, of unknown causes without molecular diagnosis in Turkey; no material for immunological or genetic analyses was available. (B) Flow cytometry showed absent surface expression for CD27 in lymphoid cells including B-cell subsets, shown here in Patient 1 for IgD *vs.* CD27 staining on CD19-gated B lymphocytes. (C) Exome sequencing was performed in Patient 1 and filtered for homozygous intervals detected exclusively in Patient 1. None of the total of 28 additional single nucleotide or deletions/insertions variants (SNVs or DIVs, respectively) was validated using capillary sequencing, suggesting that the mutation in *CD27* alone is causative of the phenotype in Patient 1. (D) Sanger sequencing confirmed the same mutation in *CD27* (c. G158A, p. Cys53Tyr) in Patients 1-3 from Family A, Patients 4 and 5 from Family B, and Patients 6-8 from Family C, respectively.

mutation by conventional Sanger sequencing (*Wolf et al., manuscript in preparation*), in order to exclude other contributing genetic conditions or, conversely, to define whether the mutation in *CD27* alone was sufficient for the development of a phenotype. Single nucleotide polymorphism (SNP)-array based homozygosity mapping in Family A revealed four intervals which were present only in the affected sibling (Patient 1; *Online Supplementary Table S1*). However, WES did not reveal any additional relevant genetic aberrations (Figure 1C). Analyses of all 3

Table 1. Patients' characteristics and immunophenotypic details of CD27-deficient individuals.

Patient	1	2	3	4	5	6
Center	Vienna	Vienna	Vienna	Melbourne	Melbourne	Melbourne
Gender	Female	Female	Male	Male	Female	Male
Ethnic origin	Turkish	Turkish	Turkish	Lebanese	Lebanese	Lebanese
Current age	5.5 years	14 years	3.5 years	4 years	16 months	19 years
Age at onset	15 months	NA	NA	1 year	1 year	15 years
Symptoms	Recurrent EBV-LPD, SIRS	None, asymptomatic	None, asymptomatic	EBV-LPD, EBV-HLH	Fever, EBV+ ulcers	EBV-LPD, recurrent sinusitis lymphoma
Treatment	IG; Steroids; Rituximab*	None	IG† from age 4-20 months	HLH-2004, cord-HSCT	None	Rituximab, R-CHOP, cord-HSCT
IgG before treatment, g/L (reference range)	4.51 (4.45-15)	8.43 (6.98-11.9)	2.9 (0.55-7.99)	4.67 (2.86-16.8)	11.6 (2.86-16.8)	32.4 (5.18-17.8)
IgA before treatment, g/L (reference range)	0.55 (0.21-2.03)	0.67 (0.22-2.74)	0.09 (0-0.64)	1.1 (0.19-1.75)	1.2 (0.19-1.75)	3.93 (0.33-2.67)
IgM before treatment, g/L (reference range)	0.581 (0.36-2.28)	1.34 (0.19-0.99)	0.56 (0.09-0.77)	0.75 (0.43-1.63)	3.05 (0.43-1.63)	0.85 (0.32-1.35)
EBV plasma load, copies/ mL min-max	1e2-2e6¶	ND	0-3.6e2‡	0-5e6¶	8e6	0-5e6¶
EBNA Ab	NA (IVIG)	Positive	Positive	ND	ND	ND
Response to vaccination antigens§	Tetanus low Pneumococcus not vaccinated HiB low TBE low-normal Rubella not vaccinated	Tetanus normal Pneumococcus normal HiB normal TBE normal Rubella normal	Tetanus low-normal Pneumococcus low-normal HiB low, TBE normal Rubella normal	Tetanus normal Pneumococcus ND HiB normal TBE not vacc. Rubella ND	Tetanus low-normal Pneumococcus ND HiB normal, TBE not vacc. Rubella ND	Tetanus normal Pneumococcus no response** HiB normal TBE not vacc. Rubella ND
$CD19^+$ B cells, number/μL§§	490-1100	520	750-1040	1120	1700	84
IgD^-CD27^+ B cells§§	Absent	Absent	Absent	Absent	Absent	Absent
IgD^+CD27^+ B cells§§	Absent	Absent	Absent	Absent	Absent	Absent
Transitional B cells % $CD19^+$§§	↑47	ND	↑24	ND	ND	2.6
$CD21^{low}$ B cells, % $CD19^+$§§	↑38	ND	↑18	ND	ND	0.4
$CD4^+$ T cells, number/μL§§	1340	1540	1460	1660	2400	796
$CD8^+$ T cells, number/μL§§	2150	1210	1280	1250	1580	2724
NK cells, number/μL§§	320	260	310	270	250	293
iNKT cells, % of $CD3^+$	0.01%-0.02%‡‡	0.08%	0.09%	ND	0.02	<0.01%
in vitro T-cell proliferation*	Normal	ND	ND	Normal	ND	Normal
NK cell function†	Mildly reduced	ND	ND	Moderately reduced	Mildly reduced	Mildly reduced
EBV-specific T cells§	present: 0.2% of $CD4^+$ and 9.6% of $CD8^+$ T cells, with highly increased IFNγ secretion of $CD8^+$ T cells	ND	ND	ND	ND	ND

NA: not applicable; EBV-LPD: EBV-associated lymphoproliferative disorder; SIRS: systemic inflammatory response syndrome; EBV-HLH: EBV-associated hemophagocytic lymphohistiocytosis; IG: immunoglobulin replacement; CyA: cyclosporin A; cord-HSCT: allogeneic cord blood hematopoietic stem cell transplantation; R-CHOP: rituximab-cyclophosphamide-doxorubicin-vincristine-prednisolone; ND: not determined; reference range: age-specific ranges of immunoglobulin levels in parenthesis; Pneumococcus: pneumococcal polysaccharide vaccine; HiB: Haemophilus influenzae B vaccine; TBE: tick-borne encephalitis vaccine; IFNγ: interferon-γ. §See Online Supplementary Appendix for reference ranges and sources concerning vaccine antibody responses. Briefly, anti-Tetanus IgG was considered 'normal' when >1 IU/mL, 'low-normal' when 0.1-1 IU/mL. Anti-Pneumococcus polysaccharide IgG antibodies were compared before and after vaccination in Patient 6 and measured repeatedly >6-12 months after IgG substitution therapy in Patient 3, being considered normal when >10-30mg/L. Anti-Haemophilus influenzae B IgG were considered normal when >0.16-1mg/L. Anti-TBE IgG were measured as 'Vienna units' and considered normal when >155 VIEunits. Anti-Rubella IgG antibodies were only qualitatively analyzed. §§B and T cell normal ranges and memory B-cell subset reference values were applied according to previous publications (see Online Supplementary Appendix). *In vitro T-cell proliferation was measured as standard lymphocyte function test after 3-day incubation with phytohemagglutinin (in Patients 1, 4 and 6), and concanavalin A, pokeweed mitogen, CD3, Tetanus antigen, and staphylococcal superantigens (Patient 1).† For NK-cell function, cytotoxicity assay with peripheral blood mononuclear cells against NK-sensitive target cells (K562) in six different effector and NK:target-cell ratios (Online Supplementary Design and Methods), and in Patient 1 also CD107 degranulation. ‡For cytokine secretion assay, EBV LMP2A peptivator® (Miltenyi, Bergisch Gladbach, according to the manufacturer's instructions). §Two courses of rituximab in one year. ||Stopped two years ago. ¶Always positive when B cells present. #Variable, asymptomatic. **Performed when patient became hypogammaglobulinemic. ‡‡Performed first during severe EBV-LPD and SIRS (0.01%), and later during clinical remission (0.02%; Online Supplementary Figure S1, right panel). Normal range of iNKT cells was considered >0.02% of $CD3^+$ T cells, and borderline 0.01-0.02%.*

individuals lacking CD27$^+$ cells (Patients 1-3) revealed a total of two overlapping, homozygous candidate intervals (*Online Supplementary Table S2*), including an interval on Chromosome 12 containing the *CD27* gene. The missense mutation in *CD27* (c. G158A, p. Cys53Tyr) was found homozygous in 3 of 4 siblings in this family and heterozygous in both parents (Figure 1D and *Online Supplementary Figure S2*).

The parental consanguinity and shared ethnic background (Lebanese) in Families B and C suggested a common autosomal recessive genetic alteration. Therefore, WES of Patients 4 and 6 was performed which identified the same missense mutation (c. G158A) in *CD27* as the only novel shared homozygous single nucleotide variant predicted to be probably damaging or deleterious by different prediction tools (*Online Supplementary Appendix* and *Online Supplementary Figures S2 and S3*). The mutation was confirmed by Sanger Sequencing in all cases (Figure 1D). It is located within a motif of the ligand-binding domain evolutionarily conserved among different species and various TNFR family members (*Online Supplementary Figure S4*). Retrospective analysis of Patients 7 and 8, who had died years earlier, using DNA obtained from Guthrie newborn screening cards, confirmed the same *CD27* mutation (*Online Supplementary Figure S2*). Unfortunately, no specimens for immunological analyses are available from those patients.

Discussion

In this study, clinical and laboratory observations revealed a novel CD27-linked immunodeficiency predisposing towards an EBV-associated, potentially fatal disease. In parallel, van Montfrans *et al*. recently identified a different homozygous mutation in *CD27* (c.G24A, p.Trp8X) in 2 brothers of a consanguineous Moroccan family, of whom one died from severe infectious mononucleosis at a young age and the other recovered with persistent EBV-viremia and secondary hypogammaglobulinemia.[22] The clinical courses of Patients 1-8 and the patients reported by van Montfrans *et al*. suggest that the immunological/environmental context of the primary EBV infection may play a role in the first occurrence of hypogammaglobulinemia and the severity of the clinical symptoms in CD27-deficient patients,[22] although longitudinal observation of a larger number of patients and, ideally, preemptive monitoring of asymptomatic family members will be needed to confirm this hypothesis. Immunoglobulin levels were normal or borderline-low at onset of symptoms in symptomatic patients and at the time of diagnosis in asymptomatic patients, respectively. To date, hypogammaglobulinemia developed in Patients 1, 4 and 6 who have had relapsing symptomatic EBV infection. In addition, van Montfrans *et al*. observed normal peripheral B-cell differentiation, undisturbed germinal center reactions, and normal T-cell independent B-cell responses, absence of CD27.[22] No EBV-seronegative CD27-deficient individual has been identified until present. It, therefore, remains unclear whether hypogammaglobulinemia is due to the loss of CD27 function alone or, more likely, the result of chronic EBV infection. While 'Timing and Tuning' was described necessary for co-stimulatory signals by Nolte *et al*.,[5] and other TNF- or immunoglobulin receptors (e.g. herpes virus entry mediator [HVEM], CD30, OX40 [CD134], 4-1BB [CD137] or CD28) have partially overlapping functions,[23] the human data presented here suggest that CD27 might not be essential but unique among co-stimulatory molecules in its relevance for the primary immune response against EBV. One could hypothesize that absent function of CD27 affected the normal silencing of *EBV* gene expression in infected cells, thus perturbing the establishment of EBV persistence. Whether there is a cellular reservoir of class-switched/germinal center-derived B cells or potentially other, normally also CD27-expressing, cell types with EBV persistence despite lack of functional CD27 is unclear. Together, the identification of CD27 deficiency in 4 independent families, and the observation that no additional mutations in genes other than CD27 could be identified by WES, suggest that CD27 deficiency alone, either due to a complete lack (p.Trp8X) or perhaps only a deficient surface expression (p.Cys53Tyr), causes disease with a broad clinical variability.

Immunological consequences

The severe reduction in iNKT cells in CD27-deficient patients during massive EBV-LPD (i.e. Patients 1 and 6; Table 1 and *Online Supplementary Figure S1*) may further support a primary role of iNKT cells for EBV-LPD pathogenesis as described previously in SAP, XIAP and ITK deficiency,[9,24,25] implicating that the CD70-CD27 axis acts as a co-stimulatory requirement for development and/or maintenance of iNKT cells, or it may be a secondary phenomenon.

Similar to other subtle T-cell disorders, such as SAP-, XIAP- and ITK deficiency, our data suggest that immunity against other viral infections does not seem as severely compromised in human CD27 deficiency.

Other clinically relevant consequences of CD27 dysfunction might include: i) decreased memory formation to viral (including vaccine protein) antigens;[4,26] and ii) perturbed anti-tumor immunity of T cells,[27] γδT cells,[28] and NK cells,[29,30] potentially leading to an increased risk of other malignomas in addition to EBV-lymphomas. It is likely that more individuals with dysfunctional CD27 will be identified among patient cohorts with hypogammaglobulinemia (with or without EBV-LPD) and absent CD27-expressing memory B cells, potentially leading to the recognition of CD27 deficiency as a novel, albeit probably a rare, combined immunodeficiency.[31-33] Of note, Patients 1 and 3 also showed expansion of transitional and CD21^{low} B cells, which is reportedly associated with increased risks of lymphadenopathy, splenomegaly, and granuloma formation in CVID, similar to XLP patients.[34]

Conclusions

CD27 deficiency should be considered in all patients with hypogammaglobulinemia or unusually severe causes of EBV infection in order to allow for an individualized treatment based upon the experience with this condition so far. Our results illustrate that modern genomic technologies such as WES may identify and confirm disease-causing mutations in monogenetic recessive diseases even with limited numbers of affected individuals. Future studies to clarify the cellular pathomechanistic consequences of CD27 deficiency are warranted.

Acknowledgments

The authors thank the patients' families and the staff of the

immunology outpatient clinics and hematology-oncology wards for their co-operation, Drs. H. Gadner, A. Heitger, and G. Mann (Vienna) for their support in experimental analyses, clinical advice, and helpful comments, Dr. S. Ehl and his team (Freiburg) for critical review of the manuscript and HLH diagnostics including NK cytotoxicity assays, and Dr. W. Schwinger (Graz) for TCR Vβ spectratyping. The authors from Vienna thank Drs. M. Eibl and H. Wolf for referral of Family A and constructive discussions.

Funding

Funding of parts of this work was provided by the Deutsche Forschungs Gemeinschaft (to KBi) and the START Programme of the Austrian Science Fund (FWF): [Y595B13] (to KBo).

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Bigler RD, Bushkin Y, Chiorazzi N. S152 (CD27). A modulating disulfide-linked T cell activation antigen. J Immunol. 1988; 141(1):21-8.
2. Camerini D, Walz G, Loenen WA, Borst J, Seed B. The T cell activation antigen CD27 is a member of the nerve growth factor/tumor necrosis factor receptor gene family. J Immunol. 1991;147(9):3165-9.
3. Gravestein LA, Amsen D, Boes M, Calvo CR, Kruisbeek AM, Borst J. The TNF receptor family member CD27 signals to Jun N-terminal kinase via Traf-2. Eur J Immunol. 1998;28(7):2208-16.
4. Hendriks J, Gravestein LA, Tesselaar K, van Lier RA, Schumacher TN, Borst J. CD27 is required for generation and long-term maintenance of T cell immunity. Nat Immunol. 2000;1(5):433-40.
5. Nolte MA, van Olffen RW, van Gisbergen KP, van Lier RA. Timing and tuning of CD27-CD70 interactions: the impact of signal strength in setting the balance between adaptive responses and immunopathology. Immunol Rev. 2009;229(1):216-31.
6. Klein U, Rajewsky K, Kuppers R. Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. J Exp Med. 1998;188(9):1679-89.
7. Peperzak V, Xiao Y, Veraar EA, Borst J. CD27 sustains survival of CTLs in virus-infected nonlymphoid tissue in mice by inducing autocrine IL-2 production. J Clin Invest. 2010;120(1):168-78.
8. Dupre L, Andolfi G, Tangye SG, Clementi R, Locatelli F, Arico M, et al. SAP controls the cytolytic activity of CD8+ T cells against EBV-infected cells. Blood. 2005; 105(11):4383-9.
9. Huck K, Feyen O, Niehues T, Ruschendorf F, Hubner N, Laws HJ, et al. Girls homozygous for an IL-2-inducible T cell kinase mutation that leads to protein deficiency develop fatal EBV-associated lymphoproliferation. J Clin Invest. 2009;119(5):1350-8.
10. Williams H, Crawford DH. Epstein-Barr virus: the impact of scientific advances on clinical practice. Blood. 2006;107(3):862-9.
11. Filipovich AH. Hemophagocytic lymphohistiocytosis and other hemophagocytic disorders. Immunol Allergy Clin North Am. 2008;28(2):293-313.
12. Moosmann A, Bigalke I, Tischer J, Schirrmann L, Kasten J, Tippmer S, et al. Effective and long-term control of EBV PTLD after transfer of peptide-selected T cells. Blood. 2010;115(14):2960-70.
13. Kuzmina Z, Greinix HT, Weigl R, Kormoczi U, Rottal A, Frantal S, et al. Significant differences in B-cell subpopulations characterize patients with chronic graft-versus-host disease-associated dysgammaglobulinemia. Blood. 2011;117(7): 2265-74.
14. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet. 2007;81(3):559-75.
15. Li H, Durbin R. Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics. 2010;26(5):589-95.
16. DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, Hartl C, et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet. 2011;43(5):491-8.
17. Grant JR, Arantes AS, Liao X, Stothard P. In-depth annotation of SNPs arising from resequencing projects using NGS-SNP. Bioinformatics. 2011;27(16):2300-1.
18. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, et al. A method and server for predicting damaging missense mutations. Nat Methods. 2010;7(4):248-9.
19. Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc. 2009;4(7):1073-81.
20. Gonzalez-Perez A, Lopez-Bigas N. Improving the assessment of the outcome of nonsynonymous SNVs with a consensus deleteriousness score, Condel. Am J Hum Genet. 2011;88(4):440-9.
21. Seidel MG. CD27: A new player in the field of common variable immunodeficiency and EBV-associated lymphoproliferative disorder? J Allergy Clin Immunol. 2012;129(4):1175.
22. van Montfrans JM, Hoepelman AI, Otto S, van Gijn M, van de Corput L, de Weger RA, et al. CD27 deficiency is associated with combined immunodeficiency and persistent symptomatic EBV viremia. J Allergy Clin Immunol. 2012;129(3):787-93.
23. Croft M. Costimulation of T cells by OX40, 4-1BB, and CD27. Cytokine Growth Factor Rev. 2003;14(3-4):265-73.
24. Pasquier B, Yin L, Fondaneche MC, Relouzat F, Bloch-Queyrat C, Lambert N, et al. Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked lymphoproliferative syndrome gene product. J Exp Med. 2005;201(5):695-701.
25. Rigaud S, Fondaneche MC, Lambert N, Pasquier B, Mateo V, Soulas P, et al. XIAP deficiency in humans causes an X-linked lymphoproliferative syndrome. Nature. 2006;444(7115):110-4.
26. Xiao Y, Peperzak V, Keller AM, Borst J. CD27 instructs CD4+ T cells to provide help for the memory CD8+ T cell response after protein immunization. J Immunol. 2008;181(2):1071-82.
27. Song DG, Ye Q, Poussin M, Harms GM, Figini M, Powell DJ Jr. CD27 costimulation augments the survival and antitumor activity of redirected human T cells in vivo. Blood. 2012;119(3):696-706.
28. DeBarros A, Chaves-Ferreira M, d'Orey F, Ribot JC, Silva-Santos B. CD70-CD27 interactions provide survival and proliferative signals that regulate T cell receptor-driven activation of human gammadelta peripheral blood lymphocytes. Eur J Immunol. 2011;41(1):195-201.
29. De Colvenaer V, Taveirne S, Delforche M, De Smedt M, Vandekerckhove B, Taghon T, et al. CD27-deficient mice show normal NK-cell differentiation but impaired function upon stimulation. Immunol Cell Biol. 2011;89(7):803-11.
30. Sugita K, Robertson MJ, Torimoto Y, Ritz J, Schlossman SF, Morimoto C. Participation of the CD27 antigen in the regulation of IL-2-activated human natural killer cells. J Immunol. 1992;149(4):1199-203.
31. Chapel H, Lucas M, Lee M, Bjorkander J, Webster D, Grimbacher B, et al. Common variable immunodeficiency disorders: division into distinct clinical phenotypes. Blood. 2008;112(2):277-86.
32. Alachkar H, Taubenheim N, Haeney MR, Durandy A, Arkwright PD. Memory switched B cell percentage and not serum immunoglobulin concentration is associated with clinical complications in children and adults with specific antibody deficiency and common variable immunodeficiency. Clin Immunol. 2006;120(3):310-8.
33. Cunningham-Rundles C. Common variable immunodeficiency. Curr Allergy Asthma Rep. 2001;1(5):421-9.
34. Wehr C, Kivioja T, Schmitt C, Ferry B, Witte T, Eren E, et al. The EUROclass trial: defining subgroups in common variable immunodeficiency. Blood. 2008;111(1):77-85.

3.2. B-cell deficiency and severe autoimmunity caused by deficiency of protein kinase C δ

Elisabeth Salzer,[1*] Elisangela Santos-Valente,[1*] Stefanie Klaver,[1,2] Sol A. Ban,[1] Wolfgang Emminger,[3] Nina Kathrin Prengemann,[1] Wojciech Garncarz,[1] Leonhard Mu¨llauer,[4] Renate Kain,[4] Heidrun Boztug,[5] Andreas Heitger,[5] Klaus Arbeiter,[3] Franz Eitelberger,[6] Markus G. Seidel,[5] Wolfgang Holter,[5] Arnold Pollak,[3] Winfried F. Pickl,[7] Elisabeth Förster-Waldl,[3] and Kaan Boztug[1,3]

[1]CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria; [2]Instituto de Ciencias Biomedicas, Universidade de Sao Paulo, Sao Paulo, Brazil; [3]Department of Pediatrics and Adolescent Medicine, [4]Clinical Institute of Pathology, and [5]Department of Pediatrics, St. Anna Kinderspital and Children's Cancer Research Institute, Medical University of Vienna, Vienna, Austria; [6]Department of Pediatrics and Adolescent Medicine, Klinikum Wels-Grieskirchen Wels, Austria; and [7]Christian Doppler Laboratory for Immunomodulation and Institute of Immunology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria

(*equal contribution)

blood

2013 121: 3112-3116
Prepublished online January 14, 2013;
doi:10.1182/blood-2012-10-460741

B-cell deficiency and severe autoimmunity caused by deficiency of protein kinase C δ

Elisabeth Salzer, Elisangela Santos-Valente, Stefanie Klaver, Sol A. Ban, Wolfgang Emminger, Nina Kathrin Prengemann, Wojciech Garncarz, Leonhard Müllauer, Renate Kain, Heidrun Boztug, Andreas Heitger, Klaus Arbeiter, Franz Eitelberger, Markus G. Seidel, Wolfgang Holter, Arnold Pollak, Winfried F. Pickl, Elisabeth Förster-Waldl and Kaan Boztug

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

Regular Article

IMMUNOBIOLOGY

B-cell deficiency and severe autoimmunity caused by deficiency of protein kinase C δ

Elisabeth Salzer,[1] Elisangela Santos-Valente,[1] Stefanie Klaver,[1,2] Sol A. Ban,[1] Wolfgang Emminger,[3] Nina Kathrin Prengemann,[1] Wojciech Garncarz,[1] Leonhard Müllauer,[4] Renate Kain,[4] Heidrun Boztug,[5] Andreas Heitger,[5] Klaus Arbeiter,[3] Franz Eitelberger,[6] Markus G. Seidel,[5] Wolfgang Holter,[5] Arnold Pollak,[3] Winfried F. Pickl,[7] Elisabeth Förster-Waldl,[3] and Kaan Boztug[1,3]

[1]CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria; [2]Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brazil; [3]Department of Pediatrics and Adolescent Medicine, [4]Clinical Institute of Pathology, and [5]Department of Pediatrics, St. Anna Kinderspital and Children's Cancer Research Institute, Medical University of Vienna, Vienna, Austria; [6]Department of Pediatrics and Adolescent Medicine, Klinikum Wels-Grieskirchen Wels, Austria; and [7]Christian Doppler Laboratory for Immunomodulation and Institute of Immunology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria

Key Points

- PRKCD deficiency causes a novel primary immunodeficiency with B-cell deficiency and severe autoimmunity.
- Protein kinase C δ may represent a key factor controlling immune homeostasis and autoimmunity.

Primary B-cell disorders comprise a heterogeneous group of inherited immunodeficiencies, often associated with autoimmunity causing significant morbidity. The underlying genetic etiology remains elusive in the majority of patients. In this study, we investigated a patient from a consanguineous family suffering from recurrent infections and severe lupuslike autoimmunity. Immunophenotyping revealed progressive decrease of $CD19^+$ B cells, a defective class switch indicated by low numbers of IgM- and IgG-memory B cells, as well as increased numbers of $CD21^{low}$ B cells. Combined homozygosity mapping and exome sequencing identified a biallelic splice-site mutation in *protein C kinase δ* (*PRKCD*), causing the absence of the corresponding protein product. Consequently, phosphorylation of myristoylated alanine-rich C kinase substrate was decreased, and mRNA levels of nuclear factor interleukin (IL)-6 and IL-6 were increased. Our study uncovers human PRKCD deficiency as a novel cause of common variable immunodeficiency-like B-cell deficiency with severe autoimmunity. (*Blood*. 2013;121(16):3112-3116)

Introduction

Primary B-cell immunodeficiencies (B-PID) constitute a heterogeneous group of immunodeficiencies characterized by defective production of antigen-specific antibodies and predisposition to recurrent and severe infections.[1] A high proportion of patients display autoimmune features.[2]

Fine-tuned B-cell receptor (BCR) signaling is crucial for controlling immune homeostasis, as aberrant BCR signaling predisposes patients to autoimmunity.[3]

In the last decade, several Mendelian defects causing B-PID have been identified.[3,4] Nonetheless, the molecular etiology of these disorders remains elusive in the majority of patients. The advent of high-throughput genomic technologies will be instrumental in defining the spectrum of molecular aberrations underlying primary B-cell deficiencies.

Here we investigated the molecular cause of a common variable immunodeficiency (CVID)-like B-PID with progressive B-cell lymphopenia, an immunoglobulin class switch defect, aberrant immunoglobulin levels, and severe autoimmunity. Combined homozygosity mapping and exome sequencing identified a biallelic mutation in *protein C kinase δ* (*PRKCD*) encoding protein kinase C δ as the molecular cause of this novel PID.

Methods

A detailed description of all experimental methods can be found in the supplemental Methods on the *Blood* website.

Subjects

This study has been approved by the ethics committee of the Medical University of Vienna, Austria. Biological material was obtained on informed consent in accordance with the Declaration of Helsinki. The patient was followed up and treated at the Klinikum Wels-Grieskirchen,

Submitted October 9, 2012; accepted December 28, 2012. Prepublished online as *Blood* First Edition paper, January 14, 2013; DOI 10.1182/blood-2012-10-460741.

E.S. and E.S.-V. contributed equally to this study.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

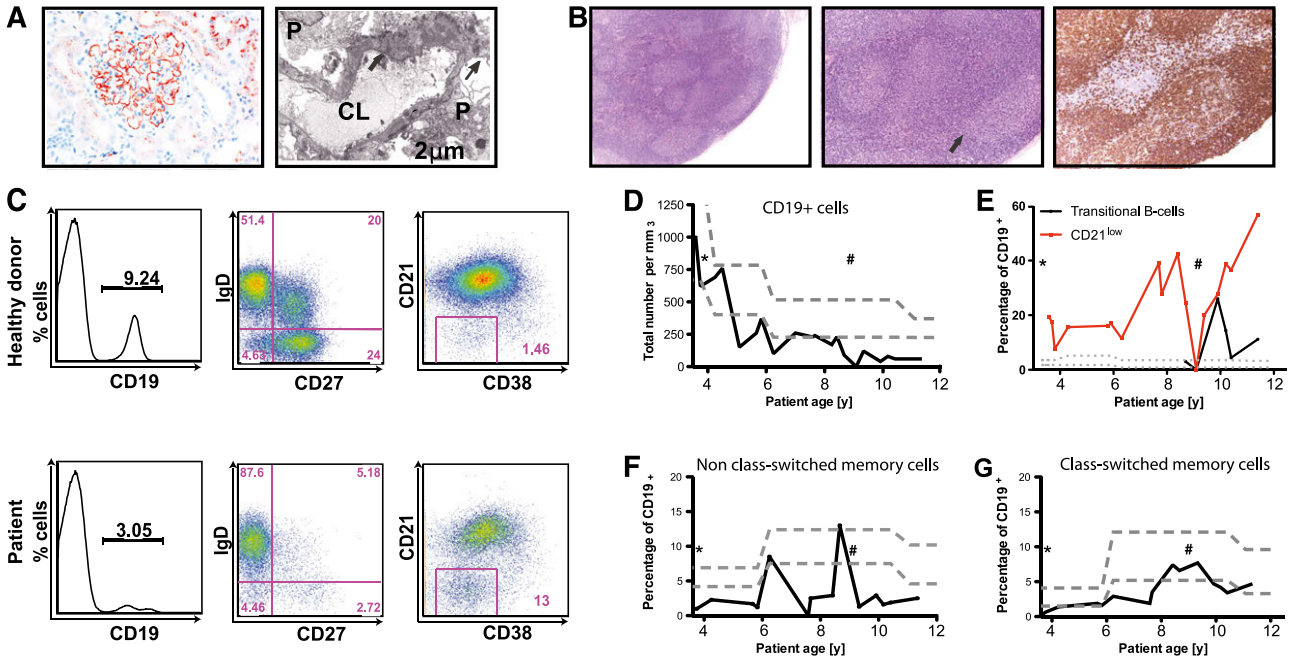

Figure 1. Clinical and immunological characterization of the index patient. (A) First renal biopsy was performed at the age of 15 months. Granular deposition of IgG along the periphery of the capillary loops (left) as seen in membranous nephropathy (MGN) was confirmed by transmission electron microscopy (TEM) (right), which showed electron dense deposits between basement membrane and podocytes (P), as well as deposits partially in resolution and incorporated by basement membrane material (arrows), consistent with MGN stage I to III (CL, capillary loop). (B) Histopathological analysis of a lymph node biopsy revealed unspecific, reactive follicular hyperplasia (arrow) but not the characteristic lymph node changes of autoimmune lymphoproliferative syndrome (ALPS) associated with *CD95/FAS* mutations (ALPS type 0/1a). The left and middle panels show hematoxylin and eosin stains, and the right panel shows anti-CD20 staining. (C) Representative FACS plots illustrating the aberrant B-cell phenotype including B-cell lymphopenia, decreased IgM- and IgG memory B cells, and increased numbers of $CD21^{low}$ B cells. (D) Longitudinal analysis illustrates progressive decrease of $CD19^{+}$ B cells and (E-G) persistence of the aberrant distribution of B-cell subsets. *First episode of nephrotic syndrome. #Treatment with anti-CD20. The dotted lines indicate the age-related 25th and 75th percentiles of the corresponding cells.[23]

St. Anna Kinderspital Vienna, and the Department of Pediatrics and Adolescent Medicine of the Medical University, Vienna, Austria.

Flow cytometry–based immunophenotyping

Flow cytometry analysis of peripheral blood mononuclear cells was performed on a Beckton-Dickinson LSR Fortessa or FACS Calibur.

Genetic analysis

Sanger sequencing was performed according to standard methods; single nucleotide polymorphism–based homozygosity mapping and exome sequencing were performed as described previously with minor modifications.[5]

Immunoblot analysis

Immunoblot analyses were performed with the following antibodies: anti-human PRKCD (Cell Signaling, Frankfurt am Main, Germany), anti-phospho (clone D13E4) and total myristoylated alanine-rich C kinase substrate (MARCKS) (clone D88D11; both from Cell Signaling), and anti-GAPDH (clone 6C5; Santa Cruz Biotechnology, Heidelberg, Germany).

Quantitative polymerase chain reaction analysis

mRNA levels of interleukin (*IL*)-*6* and nuclear factor (*NF*)-*IL6* in Epstein-Barr virus–transformed B cells from the patient and his father, upon stimulation with phorbol myristate acetate, were measured by quantitative polymerase chain reaction analysis.

T-cell Vβ spectratyping

T-cell receptor Vβ spectratyping was performed according to Pannetier et al[6] with minor modifications.

Results and discussion

Clinical and laboratory characterization

The index patient (now 12 years of age) was born to consanguineous parents (first-degree cousins) of Turkish origin (supplemental Figure 1). His father was diagnosed with Behçet's disease and mild autoimmune thyreoiditis at 40 years of age. The mother is asymptomatic. The patient's medical history is characterized by multifaceted manifestations of recurrent severe infections and autoimmunity as specified below.

Infections. From the first year of life onward, the patient experienced repeated episodes of infections, including urinary tract infections, gastroenteritis, upper and lower respiratory tract infections, and otitis media, prompting tonsillectomy and adenoidectomy within the first 4 years of life. Frequency and severity of infections decreased after commencement of immunoglobulin substitution at the age of 4 years.

Autoimmunity and immune dysregulation. The first manifestation of autoimmunity occurred at 15 months of age, when the patient presented with nephrotic syndrome. Renal biopsy revealed membranous glomerulonephritis (Figure 1A; supplemental Figure 2). Partial remission was achieved with steroid treatment with remaining mildly impaired renal function (low-grade proteinuria, hematuria; supplemental Table 1). By 3 years of age, hepatosplenomegaly (supplemental Figure 3) and generalized lymphadenopathy became apparent, prompting an in-depth diagnostic workup, which revealed low-grade viremia of human herpes virus subtypes 6 and 7. Herpes viremia was transient, whereas lymphadenopathy persisted. Several lymph node biopsies revealed nonspecific reactive follicular hyperplasia (Figure 1B). Bone marrow aspiration did

not reveal any signs of malignancy (not shown). In the following years, additional manifestations of autoimmunity including relapsing polychondritis developed. Latent hypothyroidism was detected; organ-specific autoantibodies were absent. At the age of 8 years, aseptic endocarditis and pulmonary embolism were diagnosed, and laboratory investigations suggested the diagnosis of antiphospholipid syndrome (positivity of anti-nuclear antibodies, anti-dsDNA, and anti-cardiolipin IgG antibodies; supplemental Table 2), prompting anticoagulation therapy and low-dose steroid therapy.

Immunological workup. Detailed laboratory evaluations were first performed after manifestation of glomerulonephritis at 15 months of age and revealed low IgG levels, whereas levels of IgA and IgM were above the normal range (supplemental Figure 4). B-cell studies showed a reduction of $CD19^+$ B cells, decreased relative proportions of non–class-switched ($CD19^+CD27^+IgD^+$) and class-switched ($CD19^+CD27^+IgD^-$) memory B cells, and increased numbers of $CD21^{low}$ B cells (Figure 1C; supplemental Table 3). Longitudinal analyses showed progressive decline of total $CD19^+$ B cells (Figure 1D), increased relative proportion of $CD21^{low}$ B cells (Figure 1E), and decreased frequencies of memory B cells (Figure 1F-G). T-cell studies showed mildly decreased T-cell proliferative responses (supplemental Table 2) in the absence of obvious immunophenotypic aberrations (supplemental Table 3) or skewing of the T-cell receptor Vβ repertoire (supplemental Figure 5). Impaired B-cell function was suggested by the absence of isohemagglutinins. Overall, findings were compatible with a CVID-like phenotype, although the formal criteria including decreased levels of at least 2 classes immunoglobulins[7] were not fulfilled.

Treatment. Because of recurrent respiratory tract infections including pneumonia, immunoglobulin G replacement was initiated at 4 years of age, leading to a decrease of infection frequency. At the age of 9 years, anti-CD20 therapy (2 courses of 375 mg/m^2 each) was performed to alleviate autoantibody production. Despite transient normalization of the previously increased IgM levels (supplemental Figure 4), autoantibodies persisted. Since the age of 8 years, the patient has been under continuous treatment with mycophenolate-mofetil and low-dose steroids. Other treatment includes enalapril (angiotensin-converting enzyme inhibitor), anticoagulants, thyroid hormone replacement, and immunoglobulin replacement. With this treatment, the boy has a reasonably good quality of life, without the need for hospitalization or intravenous antibiotics during the last 3 years.

Routine genetic investigation. A genetic workup revealed no mutations in the *ICOS*, *BAFFR*, *TACI,* or *FOXP3* genes, respectively. Surface expression of CD40/CD40 ligand was normal (data not shown). A heterozygous variant in *CTLA4* was discovered in both the index patient and his father (rs231775). Homozygosity for this variant has been associated with Graves's disease, rheumatoid arthritis, and systemic lupus erythematous,[8] whereas heterozygosity is associated with autoimmune thyreoiditis[9] but not systemic lupus erythematous.[10] The clinical presentation of this patient with multiple features of immune dysregulation including glomerulonephritis, lymphadenopathy, relapsing polychondritis, and antiphospholipid syndrome in the context of a CVID-like immune phenotype could thus not be reconciled with the heterozygous *CTLA4* variant alone. Hence, we initiated further genetic investigations to detect the molecular background of the patient's disease.

Mutation identification in the *PRKCD* gene

Given the consanguinity in the family, a monogenetic defect with autosomal recessive inheritance was assumed. To uncover the underlying genetic cause, we performed single nucleotide polymorphism array–based homozygosity mapping (Figure 2A; supplemental Table 4) and exome sequencing. Hits from exome sequencing were filtered for homozygous intervals present exclusively in the patient and validated by Sanger sequencing (supplemental Figure 6; supplemental Table 4). Only 2 of these hits showed perfect segregation with the disease: *UBXN1* (c. G686A, p. Thr229Met) and *PRKCD* (c.1352+1G>A) (Figure 2B and supplemental Figure 1, respectively).

While no obvious role for UBXN1 in the patient's disease pathogenesis could be recognized (Supplemental Materials), PRKCD was considered a plausible candidate, because it has a well-established role in B-cell signaling[11,12] and the corresponding $Prkcd^{-/-}$ knockout mouse exhibits various autoimmune manifestations together with generalized lymphadenopathy.[13] The murine model also shows splenic lymphocyte hyperproliferation,[13] reminiscent of human autoimmune lymphoproliferative syndrome.[14] Western blot analysis revealed the absence of PRKCD in the patient, whereas expression was decreased in a heterozygous parent compared with a healthy control (Figure 2C). Lower expression in the heterozygous carrier does not seem to be sufficient to cause disease, because the parents do not present with the characteristic clinical features seen in the patient.

Functional consequences of PRKCD deficiency

PRKCD is a member of the protein kinase C family critical for regulation of cell survival, proliferation, and apoptosis.[15] In B lymphocytes, PRKCD is involved in BCR-mediated signaling downstream of Bruton's tyrosine kinase and phospholipase Cγ2.[11] PRKCD is expected to have an essential function in B-cell tolerance, because the corresponding knockout mouse shows immune-complex glomerulonephritis, splenomegaly, and lymphadenopathy associated with B-cell expansion and defective B-cell tolerance to self-antigen.[13] Autoimmunity in $Prkcd^{-/-}$ mice has been linked to defective proapoptotic extracellular signal-regulated kinase signaling during B-cell development.[16] Recently, *PLCγ2* mutations have been identified in CVID(-like) B-cell deficiency with autoimmunity, highlighting the importance of this pathway for B-cell homeostasis.[17,18]

To assess functional consequences of PRKCD deficiency, expression of MARCKS, a major PKC target,[19] was evaluated. Immunoblot analysis in Epstein-Barr virus–immortalized patient B-cell lines showed reduced total levels of MARCKS, despite contrary literature findings.[20] Importantly, MARCKS phosphorylation at Ser167/170, which is critical for translocation of MARCKS from the plasma membrane to the cytoplasm mediating reduction of cell proliferation,[21] was abrogated in the patient (Figure 2D). Thus, deficiency of pMARCKS may be related to the lymphoproliferation in the patient.[21]

On phosphorylation of NF-IL6 at Ser240 by PRKCD, the DNA binding capability of NF-IL6, and consequently IL6 production, is markedly reduced.[22] Accordingly, we observed increased mRNA levels of *NF-IL6* and *IL6* in the PRKCD-deficient patient after phorbol myristate acetate stimulation (Figure 2E), similar to hyperactive NF-IL6 signaling observed in $Prkcd^{-/-}$ mice.[13]

In summary, we describe PRKCD deficiency as a novel primary CVID-like B-cell deficiency. The index patient of this study exhibited features of immune dysregulation including lymphoproliferation (splenomegaly and lymphadenopathy) and autoimmunity (glomerulonephritis, antiphospholipid syndrome, and relapsing polychondritis) similar to the murine knockout model.

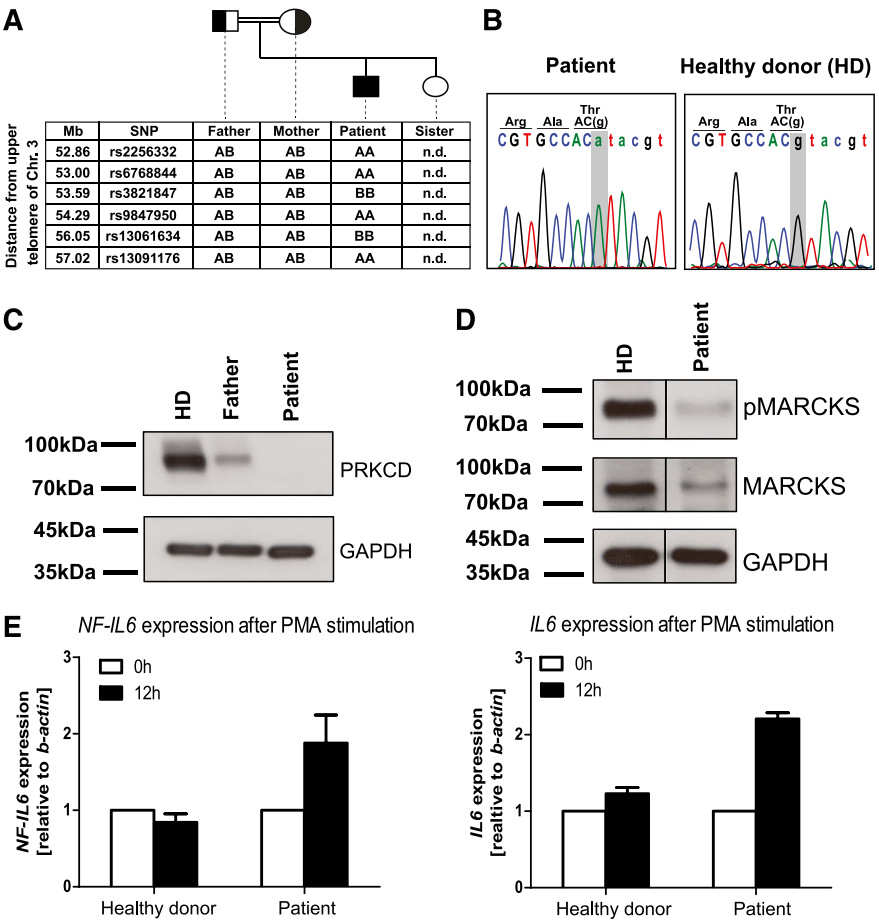

Figure 2. Identification of human PRKCD deficiency as a monogenetic B-cell deficiency associated with autoimmunity. (A) Single nucleotide polymorphism array–based homozygosity mapping was performed and revealed several homozygous candidate intervals, including an interval on chromosome 3p21.31. (B) Sanger sequencing validated a splice site mutation in *PRKCD*, encoding for protein kinase C δ which was homozygous in the patient. (C) Western blot analysis showed absent expression of the corresponding protein product in the patient compared with decreased expression in the heterozygous father and normal expression in a healthy control. (D) Western blot analysis showed defective phosphorylation of MARCKS, a downstream target of PRKCD. (E) Quantitative polymerase chain reaction analysis showed hyperactive NF-IL6 signaling on stimulation using phorbol myristate acetate, as indicated by increased mRNA levels of *NF-IL6* and *IL6*.

However, neither peripheral B-cell lymphopenia nor defective class switch observed in our patient was assessed in the mouse. It cannot be excluded that the known heterozygous variant in *CTLA4* in the patient may act as a disease-modifying factor. Future studies will need to comprehensively characterize the clinical and immunological phenotype in a cohort of PRKCD-deficient patients and further dissect the molecular pathophysiology of aberrant PRKCD-signaling in B-cell homeostasis and autoimmunity.

Acknowledgments

The authors thank the family for their participation in this study; Georg Ebetsberger-Dachs, Ulrike Habeler, and Christoph Male for clinical care; Karoly Lakatos for providing CT scan pictures; Linda Stöger for help with quantitative polymerase chain reaction analyses; Raphael Ott for technical assistance with library preparation for exome sequencing; Ulrike Koermoeczi and Arno Rottal for expert technical assistance with flow cytometric analyses; and Ciara Cleary for proofreading the manuscript.

This work was supported by an intramural grant from the CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences (K.B.) and START Programme of the Austrian Science Fund (FWF) grant Y595B13 (K.B.).

Authorship

Contribution: E.S., E.S.-V., S.K., S.A.B., N.K.P., and W.G. performed all experimental work except serial routine immunological characterization performed by W.F.P. W.E., H.B., A.H., K.A., F.E., M.G.S., W.H., A.P., and E.F.-W. provided clinical care and critically reviewed clinical and immunological patient data. L.M. and R.K. performed histopathological analyses. K.B. conceived this study with help from E.F.-W.; planned, designed, and interpreted experiments; provided laboratory resources; guided E.S., E.S.-V., S.K., S.A.B., N.K.P., and W.G.; and wrote the initial draft of the manuscript with assistance from E.S., E.S.-V., S.K., S.A.B., N.K.P., and E.F.-W. All authors critically reviewed the manuscript and agreed to its publication.

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

The current affiliation for M.G.S. is Division of Pediatric Hematology-Oncology, Department of Pediatrics and Adolescent Medicine, Medical University of Graz, Graz, Austria.

Correspondence: Kaan Boztug, CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, and Department of Pediatrics and Adolescent Medicine, Medical University of Vienna, Lazarettgasse 14, AKH BT 25.3, A-1090 Vienna, Austria; e-mail: kboztug@cemm.oeaw.ac.at; and Elisabeth Förster-Waldl, Department of Pediatrics and Adolescent Medicine, Medical University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna, Austria; e-mail: elisabeth.foerster-waldl@meduniwien.ac.at.

References

1. Conley ME, Dobbs AK, Farmer DM, et al. Primary B cell immunodeficiencies: comparisons and contrasts. *Annu Rev Immunol.* 2009;27:199-227.
2. Yong PF, Thaventhiran JE, Grimbacher B. "A rose is a rose is a rose," but CVID is Not CVID common variable immune deficiency (CVID), what do we know in 2011? *Adv Immunol.* 2011;111:47-107.
3. van der Burg M, van Zelm MC, Driessen GJ, et al. New frontiers of primary antibody deficiencies. *Cell Mol Life Sci.* 2012;69(1):59-73.
4. Al-Herz W, Bousfiha A, Casanova JL, et al. Primary immunodeficiency diseases: an update on the classification from the international union of immunological societies expert committee for primary immunodeficiency. *Front Immunol.* 2011; 2:54.
5. Salzer E, Daschkey S, Choo S, et al. Combined immunodeficiency with life-threatening EBV-associatedlymphoproliferative disorder in patients lacking functional CD27 [published online ahead of print July 16, 2012]. *Haematologica*.
6. Pannetier C, Even J, Kourilsky P. T-cell repertoire diversity and clonal expansions in normal and clinical samples. *Immunol Today.* 1995;16(4): 176-181.
7. Conley ME, Notarangelo LD, Etzioni A; Representing PAGID (Pan-American Group for Immunodeficiency) and ESID (European Society for Immunodeficiencies). Diagnostic criteria for primary immunodeficiencies. *Clin Immunol.* 1999; 93(3):190-197.
8. Gough SC, Walker LS, Sansom DM. CTLA4 gene polymorphism and autoimmunity. *Immunol Rev.* 2005;204:102-115.
9. Kimura H, Kato Y, Shimizu S, et al. Association of polymorphism at position 49 in exon 1 of the cytotoxic T-lymphocyte-associated factor 4 gene with Graves' disease refractory to medical treatment, but not with amiodarone-associated thyroid dysfunction. *Thyroid.* 2009;19(9):975-981.
10. Barreto M, Santos E, Ferreira R, et al. Evidence for CTLA4 as a susceptibility gene for systemic lupus erythematosus. *Eur J Hum Genet.* 2004; 12(8):620-626.
11. Guo B, Su TT, Rawlings DJ. Protein kinase C family functions in B-cell activation. *Curr Opin Immunol.* 2004;16(3):367-373.
12. Zouali M, Sarmay G. B lymphocyte signaling pathways in systemic autoimmunity: implications for pathogenesis and treatment. *Arthritis Rheum.* 2004;50(9):2730-2741.
13. Miyamoto A, Nakayama K, Imaki H, et al. Increased proliferation of B cells and auto-immunity in mice lacking protein kinase Cδ. *Nature.* 2002;416(6883):865-869.
14. Teachey DT. New advances in the diagnosis and treatment of autoimmune lymphoproliferative syndrome. *Curr Opin Pediatr.* 2012;24(1):1-8.
15. Griner EM, Kazanietz MG. Protein kinase C and other diacylglycerol effectors in cancer. *Nat Rev Cancer.* 2007;7(4):281-294.
16. Limnander A, Depeille P, Freedman TS, et al. STIM1, PKC-δ and RasGRP set a threshold for proapoptotic Erk signaling during B cell development. *Nat Immunol.* 2011;12(5):425-433.
17. Ombrello MJ, Remmers EF, Sun G, et al. Cold urticaria, immunodeficiency, and autoimmunity related to PLCG2 deletions. *N Engl J Med.* 2012; 366(4):330-338.
18. Zhou Q, Lee GS, Brady J, et al. A hypermorphic missense mutation in PLCG2, encoding phospholipase Cγ2, causes a dominantly inherited autoinflammatory disease with immunodeficiency. *Am J Hum Genet.* 2012;91(4): 713-720.
19. Hartwig JH, Thelen M, Rosen A, et al. MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium-calmodulin. *Nature.* 1992;356(6370):618-622.
20. Gallant C, You JY, Sasaki Y, et al. MARCKS is a major PKC-dependent regulator of calmodulin targeting in smooth muscle. *J Cell Sci.* 2005; 118(Pt 16):3595-3605.
21. Ramsden JJ. MARCKS: a case of molecular exaptation? *Int J Biochem Cell Biol.* 2000;32(5): 475-479.
22. Trautwein C, van der Geer P, Karin M, et al. Protein kinase A and C site-specific phosphorylations of LAP (NF-IL6) modulate its binding affinity to DNA recognition elements. *J Clin Invest.* 1994;93(6):2554-2561.
23. Morbach H, Eichhorn EM, Liese JG, et al. Reference values for B cell subpopulations from infancy to adulthood. *Clin Exp Immunol.* 2010; 162(2):271-279.

3.3. Early-onset inflammatory bowel disease and common variable immunodeficiency–like disease caused by IL-21 deficiency

Elisabeth Salzer,[1] Aydan Kansu,[2] Heiko Sic,[3]Peter Majek,[1] Aydan Ikinciogullari,[4] Figen E. Dogu,[4] Nina Kathrin Prengemann,[1] Elisangela Santos-Valente,[1] Winfried F. Pickl,[5] Ivan Bilic,[1] Sol A Ban,[1] Zarife Kuloglu,[2] Arzu Meltem Demir,[2] Arzu Ensari,[6] Jacques Colinge,[1] Marta Rizzi,[3] Hermann Eibel, [3] and Kaan Boztug[1,7]

[1]CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna; the Departments of [2]Pediatric Gastroenterology, [4]Pediatric Immunology, and [6]Pathology, Ankara University; [3]the Center for Chronic Immuno- deficiency, University Medical Center, Freiburg; [5]the Christian Doppler Laboratory for Immunomodulation and Institute of Immunology, Center for Pathophysiology, Infectiology and Immunology, and [7]the Department of Pediatrics and Adolescent Medicine, Medical University of Vienna.

Early-onset inflammatory bowel disease and common variable immunodeficiency–like disease caused by IL-21 deficiency

Elisabeth Salzer, MD,[a] Aydan Kansu, MD,[b] Heiko Sic, PhD,[c] Peter Májek, PhD,[a] Aydan Ikincioğullari, MD,[d] Figen E. Dogu, MD,[d] Nina Kathrin Prengemann, MSc,[a] Elisangela Santos-Valente, MD, MSc,[a] Winfried F. Pickl, PhD,[e] Ivan Bilic, PhD,[a] Sol A Ban,[a] Zarife Kuloğlu, MD,[b] Arzu Meltem Demir, MD,[b] Arzu Ensari, MD,[f] Jacques Colinge, PhD,[a] Marta Rizzi, PhD,[c] Hermann Eibel, PhD,[c] and Kaan Boztug, MD[a,g] *Vienna, Austria, Ankara, Turkey, and Freiburg, Germany*

Background: Alterations of immune homeostasis in the gut can result in development of inflammatory bowel disease (IBD). Recently, Mendelian forms of IBD have been discovered, as exemplified by deficiency of IL-10 or its receptor subunits. In addition, other types of primary immunodeficiency disorders might be associated with intestinal inflammation as one of their leading clinical presentations.

Objective: We investigated a large consanguineous family with 3 children who presented with early-onset IBD within the first year of life, leading to death in infancy in 2 of them.

Methods: Homozygosity mapping combined with exome sequencing was performed to identify the molecular cause of the disorder. Functional experiments were performed to assess the effect of IL-21 on the immune system.

Results: A homozygous mutation in *IL21* was discovered that showed perfect segregation with the disease. Deficiency of IL-21 resulted in reduced numbers of circulating $CD19^+$ B cells, including IgM^+ naive and class-switched IgG memory B cells, with a concomitant increase in transitional B-cell numbers. *In vitro* assays demonstrated that mutant $IL\text{-}21^{Leu49Pro}$ did not induce signal transducer and activator of transcription 3 phosphorylation and immunoglobulin class-switch recombination.

Conclusion: Our study uncovers IL-21 deficiency as a novel cause of early-onset IBD in human subjects accompanied by defects in B-cell development similar to those found in patients with common variable immunodeficiency. IBD might mask an underlying primary immunodeficiency, as illustrated here with IL-21 deficiency. (J Allergy Clin Immunol 2014;133:1651-9.)

Key words: *IL-21, early-onset inflammatory bowel disease, common variable immunodeficiency, exome sequencing*

A tightly regulated balance of the immune system is critical for immune homeostasis and, if altered, may be responsible for the development of inflammatory bowel disease (IBD).[1] Genome-wide association studies to identify genetic factors of complex IBD traits have suggested that both genetic and environmental factors contribute to the disease.[2-4] The majority of susceptibility loci identified have revealed genes important in immunologic processes.[5] More recently, early-onset Mendelian forms of IBD have been recognized, as illustrated by the discovery of IL-10 (receptor) deficiency.[6,7]

Intriguingly, patients with various primary immunodeficiency disorders can also exhibit intestinal inflammation as one of their leading symptoms.[8] For instance, qualitative or quantitative neutrophil defects, such as chronic granulomatous disease[9] or G6PC3 deficiency,[10] have been associated with chronic intestinal inflammation. In addition, patients with partial T-cell deficiency, Wiskott-Aldrich syndrome,[11,12] LPS-responsive beige-like anchor (LRBA) deficiency,[13] or defects in the development of regulatory T cells[14] might present with colitis. Similarly, primary B-cell deficiencies, including the subgroup of common variable immunodeficiencies (CVIDs), frequently display an IBD-like phenotype.[15,16] These observations indicate that multiple immunopathologic processes can result in IBD and point to the gastrointestinal tract as a particularly vulnerable site for aberrations of immune homeostasis.

We here describe IL-21 deficiency as a novel primary immunodeficiency, which associates a CVID-like B-cell deficiency with early-onset IBD.

METHODS

Patients and ethics

This study has been approved by the responsible local ethics committee. Biological material was obtained on informed consent in accordance with the Declaration of Helsinki. The patient was followed up and treated at the Departments of Immunology and Gastroenterology, respectively, at Ankara University in Turkey.

From [a]CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna; the Departments of [b]Pediatric Gastroenterology, [d]Pediatric Immunology, and [f]Pathology, Ankara University; [c]the Center for Chronic Immunodeficiency, University Medical Center, Freiburg; [e]the Christian Doppler Laboratory for Immunomodulation and Institute of Immunology, Center for Pathophysiology, Infectiology and Immunology, and [g]the Department of Pediatrics and Adolescent Medicine, Medical University of Vienna.

Supported by funding from the Austrian Science Fund (FWF; grant no. P24999 to K.B.) and the German ministry for Research and Technology, the German Cancer Research fund (grant 1098935, both to H.E.), as well as the German Federal Ministry of Education and Research (BMBF 01 EO 0803; to M.R.).

Disclosure of potential conflict of interest: E. Salzer, N. K. Prengemann and K. Boztug's institution has received support from the Austrian Science Fund. M. Rizzi has received funding from the Novartis Foundation for Therapeutic Research. H. Eibel's institution has received funding from the DFG, German Cancer Research Fund. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication September 24, 2013; revised January 16, 2014; accepted for publication February 5, 2014.

Available online April 17, 2014.

Corresponding author: Kaan Boztug, MD, CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna/Department of Pediatrics and Adolescent Medicine, Medical University of Vienna, Lazarettgasse 14 AKH BT 25.3, A-1090 Vienna, Austria. E-mail: kboztug@cemm.oeaw.ac.at.

0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2014.02.034

Abbreviations used
APC: Allophycocyanin
CD40L: CD40 ligand
CVID: Common variable immunodeficiency
DMEM: Dulbecco modified Eagle medium
IBD: Inflammatory bowel disease
IL-21R: IL-21 receptor
MD: Molecular dynamics
NK: Natural killer
PE: Phycoerythrin
SNP: Single nucleotide polymorphism
SNV: Single nucleotide variant
STAT: Signal transducer and activator of transcription
TCR: T-cell receptor
WT: Wild-type

Flow cytometry–based immunophenotyping

Flow cytometric analyses were performed on a BD LSR Fortessa, BD FACSCanto, or BD FACSCalibur. In brief, PBMCs from the patient and a healthy control subject were isolated by using Ficoll density gradient centrifugation and stained for 20 minutes at 4°C with mouse anti-human antibodies: CD3–allophycocyanin (APC)-H7 (clone SK7, BD Biosciences, Vienna, Austria), CD4-APC (clone RPA-T4, BD Biosciences), CD8-V500 (clone RPA-T8, BD Biosciences), CD19-PerCPCy5.5 (clone HIB19; eBioscience, Vienna, Austria), CD21–phycoerythrin (PE; clone B-ly4, BD-Biosciences), CD27-Brilliant violet (clone M-T271, BD Biosciences), CD38-PECy7 (clone HIT2, BD Biosciences), IgD–fluorescein isothiocyanate (clone IA6-2, BD-Biosciences), IgM-APC (clone G20-127, BD Biosciences), T-cell receptor (TCR) αβ (fluorescein isothiocyanate, clone WT31, BD Biosciences), and TCRγδ (PE, clone 11F2, BD Biosciences).

Activation of B cells, in-depth B-cell phenotyping, and stimulation were carried out, as published previously.[17-19]

Genetic analyses

Genomic DNA sequencing of *IL10*, *IL10RA*, and *IL10RB* was performed according to standard methods (see Table E1 the Methods section in this article's Online Repository at www.jacionline.org for details). Single nucleotide polymorphism (SNP)–based homozygosity mapping and exome sequencing were performed as described previously[20] with minor modifications (see the Methods section in this article's Online Repository).

Recombinant protein production

Recombinant protein production with the HEK-EBNA system was carried out, as described previously.[21,22] In brief, HEK-EBNA cells were seeded in a 10-cm dish in complete Dulbecco modified Eagle medium (DMEM; PAA, Invitrogen, Vienna, Austria) and transfected with 5 μg of vectors encoding N-terminally His-tagged IL-21^{WT} or IL-21^{Leu49Pro}.

Transfected cells were selected with complete DMEM containing 1 μg/mL puromycin (Sigma-Aldrich, Vienna, Austria). Once confluence was reached, cells were set on serum-free DMEM to initiate IL-21 production. Supernatants were collected after 1 week, filter sterilized, and frozen at −80°C. Trichloroacetic acid precipitation was performed at −20°C overnight to test successful IL-21 production. Subsequently, proteins were washed with 80% acetone and 100% acetone once each, air-dried, and resuspended in Laemmli buffer. Equal volumes of sterile-filtered supernatants were loaded on a 12% acrylamide gel, and the membrane was probed against the His-tag of the produced proteins to assess equal production and stability of wild-type (WT) and mutant IL-21 protein, respectively. Subsequently, stimulation experiments were carried out with equal volumes of supernatants and thus equal amounts of mutant and WT IL-21, respectively.

Stimulation of Jurkat cells

Jurkat cells (5×10^6 per condition) were serum starved to reduce global phosphorylation levels and stimulated with equal volumes of IL-21^{WT}, IL-21^{Leu49Pro}, or 10 ng/mL commercially available recombinant IL-21 (eBioscience), respectively, for 30 minutes. Cells were washed once with cold PBS and spun down, and the pellet was snap-frozen in liquid nitrogen. Protein was isolated with cell lysis buffer containing 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 2 mmol/L EDTA, 1% Triton X-100 (pH 7.1), and complete protease inhibitor cocktail (Sigma-Aldrich). Samples were run on an 8% acrylamide gel at a constant voltage of 110 V for 2 hours and blotted at 350 mA for 1.5 hours. Membranes were blocked with 5% BSA–Tris-buffered saline containing 0.05% Tween (Sigma-Aldrich) and incubated with the respective antibodies overnight at 4°C.

Immunoblot analysis

The primary antibodies used for Western blot analysis were rabbit anti-human signal transducer and activator of transcription (STAT) 3 and phospho-STAT3 (Tyr701, 58D6; both from Cell Signaling, Frankfurt am Main, Germany). Primary antibodies directed against His (AD1.1.10) and a mouse anti-human glyceraldehyde-3-phosphate dehydrogenase antibody (clone 6C5; both from Santa Cruz Biotechnology, Heidelberg, Germany) were used. Horseradish peroxidase–conjugated goat anti-rabbit (Bio-Rad Laboratories, Vienna, Austria) and goat anti-mouse (BD Biosciences) secondary antibodies were detected by using a chemiluminescent substrate (Amersham ECL Prime Western Blotting Detection Reagent, GE Life Sciences, Vienna, Austria). Detection was performed by using autoradiography with Hyperfilm ECL (Fisher Scientific, Vienna, Austria).

T-cell proliferation analysis

T-cell proliferation assays were carried out, as described previously.[20]

T-cell Vβ spectratyping

TCR Vβ spectratyping was performed, according to the method of Pannetier et al,[23] with minor modifications (see Table E2 in this article's Online Repository at www.jacionline.org), as described previously[20] (see the Methods section in this article's Online Repository).

In silico analyses of IL-21

In brief, molecular dynamics (MD) simulations of WT and mutant IL-21 were performed by using the coarse-gained model FREADDY[24] implemented in the MOIL[24,25] molecular modeling package with different degrees of flexibility. All simulations predicted the same qualitative behavior. For details, please see the Methods section in this article's Online Repository.

RESULTS

Clinical characterization of the patient

The index patient (currently 11 years of age) was born to healthy Turkish consanguineous parents (first-degree cousins). Two of 8 of the patient's siblings died of severe diarrhea before 1 year of age (2 months and 8 months, respectively). The index patient's disease is characterized by both early-onset IBD and a CVID-like immunodeficiency, as specified below.

IBD. The patient initially presented to the hospital at 2 months of age with persistent, mucoid, nonbloody diarrhea and recurrent oral aphthous ulcers. Additionally, he exhibited signs of chronic disease, such as fatigue and failure to thrive, with body weight being constantly less than the third percentile (not shown). He did not show any general skin lesions, perianal lesions, fistulas, uveitis, or signs of arthritis, respectively. During the following years, diarrhea persisted, and both malnutrition and finger clubbing became apparent. Results of microbiologic analyses in

feces for *Cryptosporidium* species, *Giardia lamblia*, rotavirus, and adenovirus were negative. Colonoscopy revealed macroscopically a loss of vascular pattern and mucosal erythema, as well as edema and aphthous ulcers (Fig 1, *A*). Biopsy specimens showed eosinophil and neutrophil infiltration, focal cryptitis, focal active colitis, and noncaseating granuloma, as seen in patients with typical Crohn disease (Fig 1, *B*). As a part of a local study protocol for patients with early-onset IBD, magnetic resonance cholangiopancreatography was performed, which did not reveal any abnormalities. The patient was evaluated for familial Mediterranean fever, therefore exons 2 and 10 of Mediterranean fever gene (*MEFV*) were sequenced, and a heterozygous carrier status of a common mutation (p. Met694Val) was detected. On the basis of colonoscopic and histologic findings, the patient was given a diagnosis of Crohn disease. Treatment with mesalazine, omega-3 fatty acids, and supplementary enteral feeding was initiated, but chronic diarrhea persisted. The patient, now 11 years of age, is still malnourished, with body weight persistently less than the third percentile (data not shown).

Immunodeficiency. At the age of 4 years, the patient was referred for assessment of a potential underlying immunodeficiency. Laboratory examinations revealed increased IgE levels (119 kU/mL), while IgG levels (338 mg/dL) and isohemagglutin levels were reduced (titer 1:2) (Table I). In retrospect, the patient experienced recurrent and severe upper and lower respiratory tract infections at an unusually high frequency from the age of 1 year onward. Not all criteria for CVID diagnosis were met as IgG was the sole type of reduced immunoglobulin (Table I), the patient was therefore given a diagnosis of a CVID-like immunodeficiency.[26,27] Intravenous immunoglobulin replacement together with prophylactic trimethoprim-sulfamethoxazole therapy was started. At present, the patient exhibits signs of severe chronic pulmonary infections, such as finger clubbing.

Detailed immunologic work-up. The patient was vaccinated according to general recommendations, receiving vaccinations against BCG, hepatitis B virus, diphtheria-pertussis-tetanus-polio, and measles. Anti–hepatitis B virus surface antigen titers were found to be borderline low, and antibody titers against BCG were negative. He was also evaluated for autoantibodies, such as anti-neutrophil antibodies, anti-neutrophil cytoplasmic antibodies, thyroglobulin antibodies, and anti-gliadin IgA and IgG, and results were negative at 9 years of life. PCR-based evaluation for cytomegalovirus and human immunodeficiency virus was negative. CD40, CD40 ligand (CD40L), and forkhead box protein 3 (FOXP3) expression was evaluated by means of flow cytometry to exclude known hereditary immunodeficiency syndromes; results were found to be normal (data not shown). *Cryptosporidium* species in feces was absent at age 6 years and 9 years, respectively. Neutrophil oxidative burst assays at age 5 years to assess potential chronic granulomatous disease did not show any abnormalities (data not shown). Because of the severity and early onset of IBD in this patient, the *IL10*, *IL10RA*, and *IL10RB* genes were sequenced, but no mutation could be detected (data not shown).

Mutation identification in the *IL21* gene

Given the consanguinity in the family and the occurrence of early-onset IBD in 3 of 8 siblings, a monogenetic defect with autosomal recessive inheritance was suspected (Fig 1, *C*). SNP array–based homozygosity mapping (see Tables E3 and E4 in this article's Online Repository at www.jacionline.org) and exome sequencing were then performed to identify the underlying genetic defect. Hits from exome sequencing were filtered for homozygous intervals present in the patient and validated by capillary sequencing (see the Methods and Results sections in this article's Online Repository at www.jacionline.org). A total of 2 hits which showed perfect segregation among the core family members were validated. No obvious role for the variant in *ENPEP* in the patient's disease could be detected (see the Results section in this article's Online Repository). The second hit, a homozygous mutation in *IL21* (c.T147C, p.Leu49Pro), showed perfect segregation under the assumption of autosomal recessive inheritance with full penetrance and could not be detected in existing SNP databases, such as ENSEMBL, dbSNP, or UCSC (date of accession: December 30, 2013; Fig 1, *C* and *D*). In light of the marked IBD phenotype in all 3 affected subjects in this family, we intersected exome data from the patient with known SNPs predisposing for IBD (taken from http://www.genome.gov). However, no known SNPs in *IL23R* or *NOD2* associated with IBD could be detected in the patient (data not shown).

The mutated residue IL-21^{Leu49} is highly conserved throughout evolution (Fig 1, *E*). Prediction of the protein-folding stability of the IL-21^{Leu49Pro} variant using the CUPSAT protein stability analysis tool[25] suggested that any change in IL-21^{Leu49} would reduce the stability of the native state. Notably, the strongest destabilization of the native state of protein folding was predicted by changing IL-21^{Leu49} to proline (see Table E5 in this article's Online Repository at www.jacionline.org). CUPSAT predictions were supported by using coarse-grained MD simulations of IL-21^{WT} and IL-21^{Leu49Pro}. In the simulation of IL-21^{WT}, IL-21^{Leu49} remains fully buried within the protein core, whereas the protein core of the IL-21^{Leu49Pro} mutant is predicted to be unstable in the original orientation, undergoing a transition to the outer side of helix A into the gap between helices A and C (Fig 1, *F*) within 10 ns (Fig 1, *G*). This transition is accompanied by an extension of the α-helical part of the helix A (the C-terminal end of the A is a 3_{10} helix in the WT fold) and slight modification of the relative position of helices A and C (Fig 1, *F*). Because the crystal structure of IL-21 bound to the IL-21 receptor (IL-21R; PDB id 3TGX) demonstrates that helices A and C of IL-21 are critical for receptor binding and initiation of signaling of the Janus kinase–Stat signaling cascade,[28] the predicted conformational change caused by the *IL21*^{Leu49Pro} mutation most likely decreased the affinity of IL-21 binding to IL-21R.

Functional consequences of IL-21 deficiency

Assuming that the patient's disease is most likely caused by defective IL-21 function that impairs adaptive immunity,[29,30] a more detailed analysis of the patient's immunologic phenotype was performed. Compared with healthy control subjects, the patient had fewer circulating CD19$^+$ B cells and almost no CD19$^+$CD27$^+$IgD$^+$ marginal zone–like and CD19$^+$CD27$^+$IgD$^-$ class-switched memory B cells (Fig 2, *A*, and see Fig E1 in this article's Online Repository at www.jacionline.org), resulting in a relative increase in CD19$^+$CD38^{hi}CD23$^-$ transitional B-cell numbers (Fig 2, *B*). Accordingly, very few IgG$^+$ or IgA$^+$ cells were detected in

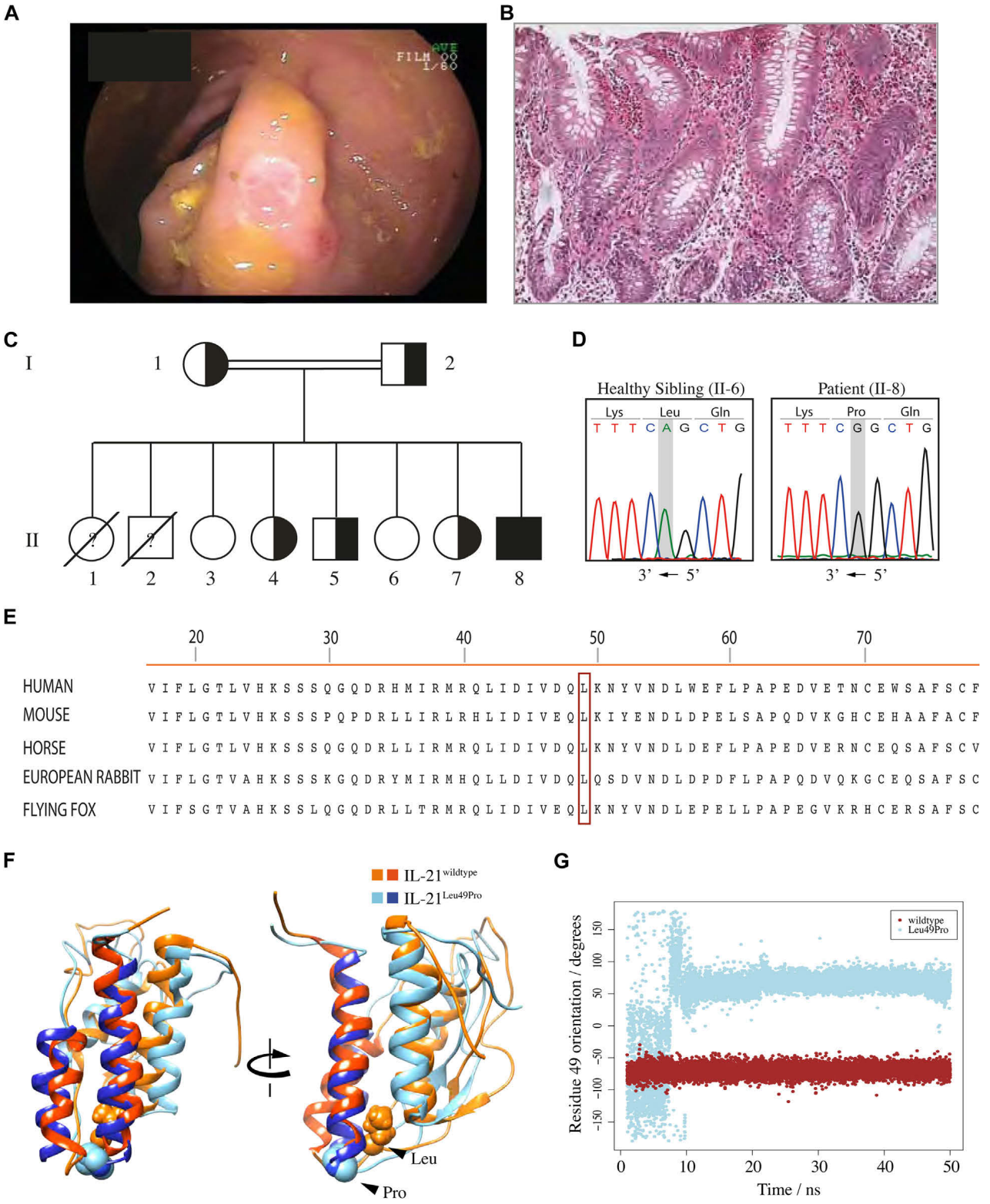

FIG 1. Identification of IL-21 deficiency. **A,** Colonoscopy. **B,** Histology consistent with Crohn disease. **C,** Pedigree of index family. *Solid portions of symbols* mark IL-21^{Leu49Pro} segregation pattern. **D,** Sanger chromatograms displaying the missense mutation in *IL21* in the patient (II-8) and a healthy sibling (II-6). **E,** Amino acid sequence conservation of IL-21^{Leu49Pro} among different species. **F,** Structure of mutant IL-21^{Leu49Pro} *(blue)* overlapped with IL-21^{WT} *(brown)*. **G,** Orientation of residue 49 in simulations of WT *(brown)* and IL-21^{Leu49Pro} *(blue)*.

TABLE I. Laboratory data of the index patient

	5 y	6 y (IVIG)
Immunoglobulins		
IgG (mg/dL)	338	567
IgM (mg/dL)	96	84
IgA (mg/dL)	67	61
IgE (kU/mL)	119	
IgG_1 (mg/dL)	230	
IgG_2 (mg/dL)	60	
IgG_3 (mg/dL)	7	
IgG_4 (mg/dL)	6	
Complement		
CH50 (U/mL)	+	+
C3 (mg/dL)	0.992	
C4 (mg/dL)	0.674	
Antibodies		
Cryptosporidium species	Negative	
Giardia lamblia	Negative	
Rotavirus	Negative	
Adenovirus	Negative	
Cytomegalovirus	Negative	
HIV	Negative	
Isohemagglutinin anti-B	1/2	
BCG + PPD		Negative
Anti-HBs	Positive	Positive
ANCA		Negative
ANA		Negative

ANA, Antinuclear antibody; *ANCA*, antineutrophil cytoplasmic antibody; *IVIG*, intravenous immunoglobulin; *HBs*, hepatitis B virus surface antigen.

peripheral blood of the patient compared with control values (Fig 2, *C*). Analysis of T-cell subsets revealed normal relative proportions of $CD3^+CD4^+$ T_H cell and $CD3^+CD8^+$ cytotoxic T-cell subsets (data not shown) but an increase in $CD3^+$ TCRγδ cell numbers (see Fig E2 in this article's Online Repository at www.jacionline.org). The cell receptor Vβ repertoire was found to be polyclonal (see Fig E3 in this article's Online Repository at www.jacionline.org), and cell proliferation induced by various stimuli was found to be normal (Fig 2, *D*, left panel), except for tetanus toxoid stimulation, despite the fact that the patient has a history of tetanus toxoid vaccinations (Fig 2, *D*, right panel).

IL-21^{Leu49Pro} does not induce IL-21R signaling, proliferation, and activation of B cells

The effects of Leu49Pro on the activity of the cytokine were analyzed by comparing *in vitro* B- and T-cell responses induced by equal amounts of recombinant mutant (IL-21^{Leu49Pro}) or WT (IL-21^{WT}) protein produced in HEK-EBNA cells (see Fig E4 in this article's Online Repository at www.jacionline.org). A Jurkat T-cell line was used to test STAT3 phosphorylation to exclude donor-dependent differences in T-cell responses. Stimulation with either commercially available recombinant IL-21 (IL-21^{rec}) or IL-21^{WT} phosphorylated the STAT3 tyrosine residue 705, whereas IL-21^{Leu49Pro} did not induce STAT3 phosphorylation at this position (Fig 3, *A*). Differences between IL-21^{WT} and IL-21^{Leu49Pro} in activating B cells were tested on normal donor cord blood–derived B cells.

Proliferation of total $CD19^+$ (Fig 3, *B*) and transitional cord blood–derived $CD19^+$ (Fig 3, *C*) B cells, as well as proliferation of memory and plasmablast subsets (see Fig E5 in this article's Online Repository at www.jacionline.org), were strongly decreased on activation with mutant IL-21^{Leu49Pro}.

IL-21–deficient B cells can be activated and undergo class-switch recombination

PBMCs were stimulated for 3 days with CD40L and IL-21 or alternatively with CD40L and IL-4 and tested for the expression of the activation markers CD69, CD86, and CD95 and class-switch recombination indicated by surface expression of IgG and IgA, respectively, to exclude intrinsic defects affecting the patient's B cells.

As expected, the expression pattern of the activation markers was normal in the B cells of the patient (Fig 4, *A*). Similar to control B cells, they proliferated and differentiated into IgG^+/IgA^+ cells in response to IL-21, strongly suggesting that the patient's B cells have an intact capacity to develop into plasma cells and memory B cells in response to IL-21R signaling (Fig 4, *B*).

DISCUSSION

Combining homozygosity mapping with exome sequencing, we uncovered IL-21 deficiency as a novel monogenetic cause of severe, early-onset IBD associated with a CVID-like primary immunodeficiency.

IL-21 belongs to the type I cytokine family and acts on many cells of the hematopoietic system.[31] IL-21 is predominantly produced by antigen-activated $CD4^+$ T cells and activated B-helper neutrophils.[32] B-helper neutrophils secrete IL-21 to promote T cell–independent B-cell responses against microbes.[32] Therefore our finding that IL-21 deficiency causes early-onset IBD suggests that IL-21 plays a critical role in the cooperation between the innate and adaptive immunity in the gut.

IL-21R is expressed by T, B, and natural killer (NK) cells, subsets of myeloid cells, and keratinocytes.[33-36] IL-21 binding to IL-21R induces primarily STAT1 and STAT3 phosphorylation.[30] Therefore it differs from other type I cytokines, such as IL-2, IL-7, IL-9, and IL-15, because these cytokines induce STAT3 and STAT5 activation, whereas IL-4 activates STAT6.[37,38] IL-21 does not seem to influence T_H1 or T_H2 polarization in $CD4^+$ T cells,[39,40] but it appears to be critical for the proliferation and activation of both naive and memory $CD8^+$ T cells.[40] In NK cells, IL-21 contributes to phenotypic and functional maturation by inducing the expression of killer immunoglobulin-like receptors, perforin expression, and IFN-γ secretion.[41,42] In CD40L-activated B cells, IL-21 strongly promotes proliferation, immunoglobulin class-switch recombination, and immunoglobulin secretion[39,43] while inhibiting the IL-4–driven induction of germline transcripts from the IgE constant region.[44,45] Accordingly, *Il21r*$^{-/-}$ knockout mice show dysgammaglobulinemia characterized by low IgG_1 but higher IgE levels.[39] In human subjects IL-21R deficiency has recently been reported to result in primary immunodeficiency characterized by defective B-cell class-switching, aberrant T-cell cytokine production, and NK cell cytotoxicity.[46]

Surprisingly, in contrast to the patients reported with IL-21R deficiency, the IL-21–deficient patient described here had early-onset IBD, which masked primary immunodeficiency found at a later age. In addition, 2 of the patient's siblings died of IBD within the first year of life, underlining the consistency of early-onset IBD presentation in this pedigree. Furthermore, to date, the patient has

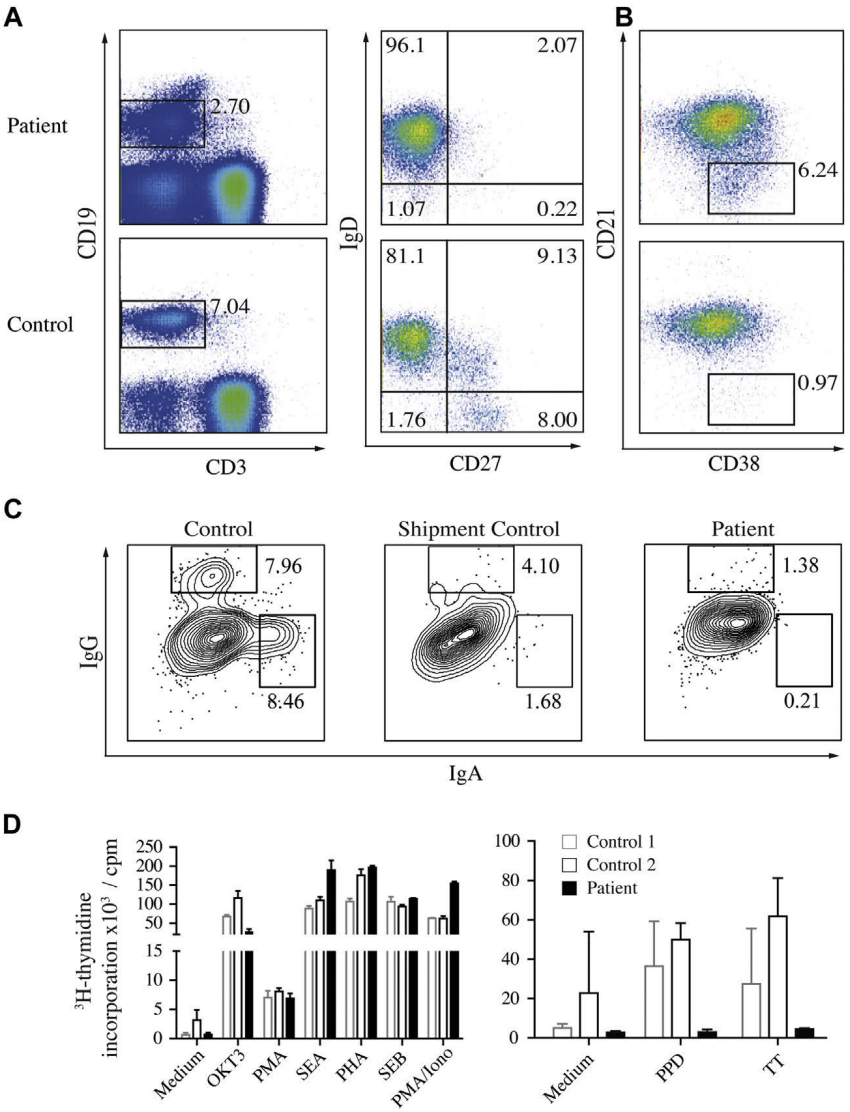

FIG 2. Immunologic phenotype of the patient. **A** and **B,** Proportion of total B cells and $IgD^{+}CD27^{+}$ and $IgD^{-}CD27^{+}$ memory B cells in peripheral blood from the patient (Fig 2, *A, upper panel*) and transitional B cells (Fig 2, *B*) compared with a healthy donor. **C,** Percentage of IgG^{+} and IgA^{+} class-switched cells in peripheral blood from patients and control subjects. **D,** T-cell proliferation: stimulation for 3 *(left panel)* and 7 *(right panel)* days. *Iono,* Ionomycin; *PMA,* phorbol 12-myristate 13-acetate; *PPD,* purified protein derivative; *SEA,* staphylococcal enterotoxin A; *SEB,* staphylococcal enterotoxin; *TT,* tetanus toxoid.

not shown any signs of cholangitis and was negative for *Cryptosporidium* species infection, one of the most striking observations in 3 of 4 of the described IL-21R–deficient patients.[46] However, 2 of the 4 patients with IL-21R deficiency also presented with diarrhea, which was possibly attributed to *Cryptosporidium* species infection and therefore not thought to be a primary cause of defective IL-21R–dependent signaling. *Cryptosporidium* species infections are common in patients with combined immunodeficiencies[47] and may have altered the clinical features of the immunodeficiency in the reported IL-21R–deficient patients. In addition, we cannot exclude underlying differences in the gut microbiota, which are essential nongenetic players in the development of IBD.[48,49] Future studies will need to address the complex interplay of the microbiome on the course of monogenetically determined immunodeficiency disorders.

The primary immunodeficiency caused by mutation in the *IL21* gene was characterized by reduced relative numbers of B cells and dramatically reduced class-switched memory B-cell populations, increased IgE levels, and hypogammaglobulinemia in peripheral blood. These findings are in line with the observations from murine models[50] and human IL-21R deficiency.[46] Although proliferation of $IL\text{-}21^{Leu49Pro}$–expressing T cells appeared normal on stimulation with several common stimuli, stimulation with specific agents, such as tetanus toxoid, illustrated specific defects in T-cell proliferation, thus recapitulating the findings described for IL-21R deficiency.[46]

Interestingly, approximately 20% of patients with CVID have autoimmunity, which often results in colitis and is associated with severely increased morbidity.[14] It has been hypothesized that IL-21 has immunosuppressive activities by inducing IL-10 secretion.[51] Similar to IL-10 or IL-10 receptor deficiency, IBD in the IL-21–deficient patient manifested in the first year of life and was characterized by a severe phenotype.[6,7,12,52] It has been shown previously that synergistic stimulation of bone marrow– or spleen-derived NK cells with IL-21 and IL-2 or IL-15 leads to increased IL-10 secretion, suggesting that the IBD might be

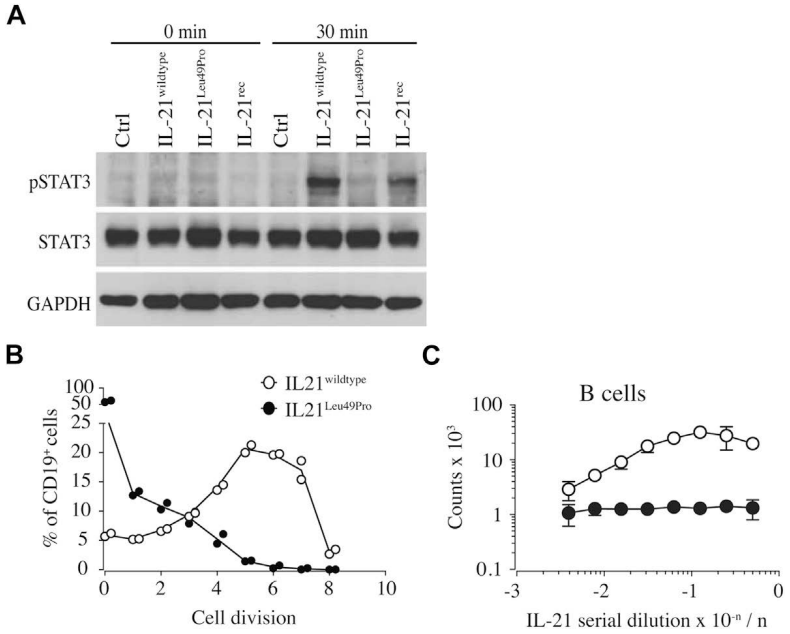

FIG 3. Functional consequences of IL-21 deficiency. **A,** Western blot of phospho-STAT3 in Jurkat cells after stimulation with IL-21^{WT}, IL-21^{Leu49Pro}, and commercially available IL-21 (IL-21^{rec}). *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase. **B,** Flow cytometry–based proliferation analysis of carboxyfluorescein succinimidyl ester–labeled cord blood–derived CD19$^+$ B cells after stimulation with IL-21^{WT} *(open circles)* and IL-21^{Leu49Pro} *(solid circles)*. **C,** IL-21–dependent proliferation of cord blood–derived B cells 6 days after stimulation with IL-21^{WT} *(open circles)* or IL-21^{Leu49Pro} *(solid circles)*.

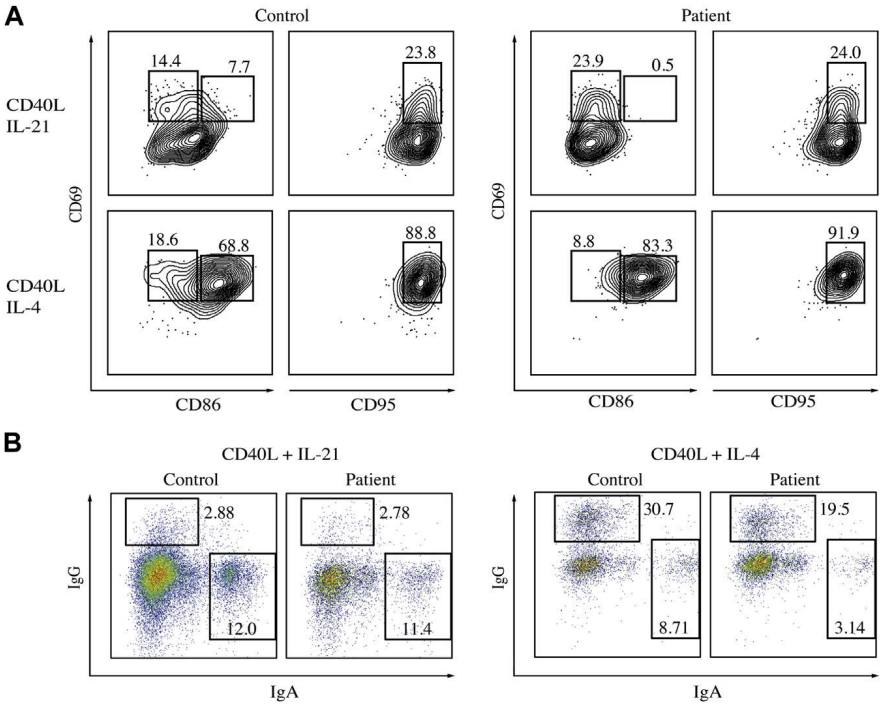

FIG 4. Normal activation and class-switch recombination potential of IL-21–deficient B cells. **A,** Flow cytometric analysis of PBMCs from control subjects and patients stimulated with either CD40L and IL-21 *(upper panel)* or CD40L and IL-4 *(lower panel)*. CD19$^+$ B cells were stained for the activation markers CD69, CD86, and CD95 after 3 days of stimulation. **B,** Capacity of CD19$^+$ B cells from control subjects and patients to undergo class-switch recombination into IgG$^+$ and IgA$^+$ cells after 6 days of stimulation.

due to decreased IL-10 secretion caused by IL-21 deficiency.[37] Nevertheless, the molecular mechanisms of how IL-21 deficiency leads to gut inflammation remain elusive.

IL-21 has emerged as a critical cytokine regulating multiple arms of the immune system (reviewed by Leonard and Spolski[30]). More recently, interfering with IL-21 signaling has been proposed

as a treatment option for patients with various autoimmune diseases, including systemic lupus erythematosus[53] and rheumatoid diseases.[54,55] However, our observation that lack of functional IL-21 is associated with an immunodeficiency with considerable morbidity raises an important concern for such therapeutic strategies.

In principle, IL-21 deficiency might be amenable to allogeneic hematopoietic stem cell transplantation to correct for the disease[6,52]; however, given the currently stable clinical condition, this has not been performed in the patient to date but remains an option for the future. In contrast to IL-21R deficiency, treatment with recombinant IL-21 might represent an alternative experimental strategy for treatment of IL-21 deficiency in a similar manner, as has been proposed for metastatic cancer, including metastatic melanoma[56] and renal cell carcinoma.[57-59] This might be particularly attractive for scenarios in which the patient's clinical status is incompatible with allogeneic hematopoietic cell transplantation.

In conclusion, here we identify deficiency of IL-21 as a novel cause of primary immunodeficiency and early-onset IBD, thereby further underlining the critical importance of tight control of immune homeostasis for inflammatory processes in the gut. Future studies will show whether targeting the affected pathways is a therapeutic option for IL-21 deficiency and related disorders.

We thank the family for their participation in this study and all clinicians who helped to manage the patient through the course of the disease. We also thank Linda Stöger for exclusion of coding mutations in *IL10*, *IL10RA*, and *IL10RB*, as well as Raphael Ott for assistance with library preparation for exome sequencing and Cecilia Domínguez Conde for critical proofreading of the manuscript. In addition, we would like to thank Frank Zaucke and his team for providing the HEK-EBNA system for recombinant protein production.

Clinical implications: IL-21 deficiency is a novel cause of early-onset IBD and a CVID-like primary immunodeficiency. The observation that lack of IL-21 is associated with an immunodeficiency raises an important concern for therapeutic strategies interfering with IL-21 in patients with immune-mediated diseases.

REFERENCES

1. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 2010;464:59-65.
2. Brant SR. Update on the heritability of inflammatory bowel disease: the importance of twin studies. Inflamm Bowel Dis 2011;17:1-5.
3. Cho JH. The genetics and immunopathogenesis of inflammatory bowel disease. Nat Rev Immunol 2008;8:458-66.
4. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. Nature 2007;448:427-34.
5. Khor B, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. Nature 2011;474:307-17.
6. Glocker EO, Kotlarz D, Boztug K, Gertz EM, Schaffer AA, Noyan F, et al. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. N Engl J Med 2009;361:2033-45.
7. Glocker EO, Frede N, Perro M, Sebire N, Elawad M, Shah N, et al. Infant colitis—it's in the genes. Lancet 2010;376:1272.
8. Arkwright PD, Abinun M, Cant AJ. Autoimmunity in human primary immunodeficiency diseases. Blood 2002;99:2694-702.
9. Marks DJ, Miyagi K, Rahman FZ, Novelli M, Bloom SL, Segal AW. Inflammatory bowel disease in CGD reproduces the clinicopathological features of Crohn's disease. Am J Gastroenterol 2009;104:117-24.
10. Begin P, Patey N, Mueller P, Rasquin A, Sirard A, Klein C, et al. Inflammatory bowel disease and T cell lymphopenia in G6PC3 deficiency. J Clin Immunol 2013;33:520-5.
11. Dupuis-Girod S, Medioni J, Haddad E, Quartier P, Cavazzana-Calvo M, Le Deist F, et al. Autoimmunity in Wiskott-Aldrich syndrome: risk factors, clinical features, and outcome in a single-center cohort of 55 patients. Pediatrics 2003;111:e622-7.
12. Glocker E, Grimbacher B. Inflammatory bowel disease: is it a primary immunodeficiency? Cell Mol Life Sci 2012;69:41-8.
13. Alangari A, Alsultan A, Adly N, Massaad MJ, Kiani IS, Aljebreen A, et al. LPS-responsive beige-like anchor (LRBA) gene mutation in a family with inflammatory bowel disease and combined immunodeficiency. J Allergy Clin Immunol 2012;130:481-8.e2.
14. Barzaghi F, Passerini L, Bacchetta R. Immune dysregulation, polyendocrinopathy, enteropathy, x-linked syndrome: a paradigm of immunodeficiency with autoimmunity. Front Immunol 2012;3:211.
15. Bussone G, Mouthon L. Autoimmune manifestations in primary immune deficiencies. Autoimmun Rev 2009;8:332-6.
16. Agarwal S, Smereka P, Harpaz N, Cunningham-Rundles C, Mayer L. Characterization of immunologic defects in patients with common variable immunodeficiency (CVID) with intestinal disease. Inflamm Bowel Dis 2011;17:251-9.
17. Warnatz K, Schlesier M. Flowcytometric phenotyping of common variable immunodeficiency. Cytometry B Clin Cytom 2008;74:261-71.
18. Warnatz K, Salzer U, Rizzi M, Fischer B, Gutenberger S, Bohm J, et al. B-cell activating factor receptor deficiency is associated with an adult-onset antibody deficiency syndrome in humans. Proc Natl Acad Sci U S A 2009;106:13945-50.
19. Kienzler AK, Rizzi M, Reith M, Nutt SL, Eibel H. Inhibition of human B-cell development into plasmablasts by histone deacetylase inhibitor valproic acid. J Allergy Clin Immunol 2013;131:1695-9.
20. Salzer E, Daschkey S, Choo S, Gombert M, Santos-Valente E, Ginzel S, et al. Combined immunodeficiency with life-threatening EBV-associated lymphoproliferative disorder in patients lacking functional CD27. Haematologica 2013;98:473-8.
21. Agarwal P, Zwolanek D, Keene DR, Schulz JN, Blumbach K, Heinegard D, et al. Collagen XII and XIV, new partners of cartilage oligomeric matrix protein in the skin extracellular matrix suprastructure. J Biol Chem 2012;287:22549-59.
22. Bechtel M, Keller MV, Bloch W, Sasaki T, Boukamp P, Zaucke F, et al. Different domains in nidogen-1 and nidogen-2 drive basement membrane formation in skin organotypic cocultures. FASEB J 2012;26:3637-48.
23. Pannetier C, Even J, Kourilsky P. T-cell repertoire diversity and clonal expansions in normal and clinical samples. Immunol Today 1995;16:176-81.
24. Elber R, Roitberg A, Simmerling C, Goldstein R, Li H, Verkhiver G, et al. MOIL: a program for simulations of macromolecules. Computer Physics Communications 1995;91:159-89.
25. Parthiban V, Gromiha MM, Schomburg D. CUPSAT: prediction of protein stability upon point mutations. Nucleic Acids Res 2006;34:W239-42.
26. Conley ME, Dobbs AK, Farmer DM, Kilic S, Paris K, Grigoriadou S, et al. Primary B cell immunodeficiencies: comparisons and contrasts. Annu Rev Immunol 2009;27:199-227.
27. Yong PF, Thaventhiran JE, Grimbacher B. "A rose is a rose is a rose," but CVID is Not CVID common variable immune deficiency (CVID), what do we know in 2011? Adv Immunol 2011;111:47-107.
28. Hamming OJ, Kang L, Svensson A, Karlsen JL, Rahbek-Nielsen H, Paludan SR, et al. Crystal structure of interleukin-21 receptor (IL-21R) bound to IL-21 reveals that sugar chain interacting with WSXWS motif is integral part of IL-21R. J Biol Chem 2012;287:9454-60.
29. Skak K, Kragh M, Hausman D, Smyth MJ, Sivakumar PV. Interleukin 21: combination strategies for cancer therapy. Nat Rev Drug Discov 2008;7:231-40.
30. Leonard WJ, Spolski R. Interleukin-21: a modulator of lymphoid proliferation, apoptosis and differentiation. Nat Rev Immunol 2005;5:688-98.
31. Lichtmann AHAAK. Cellular and molecular immunology. 5th ed. Philadelphia: Saunders; 2003.
32. Puga I, Cols M, Barra CM, He B, Cassis L, Gentile M, et al. B cell-helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen. Nat Immunol 2012;13:170-80.
33. Parrish-Novak J, Dillon SR, Nelson A, Hammond A, Sprecher C, Gross JA, et al. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. Nature 2000;408:57-63.
34. Brandt K, Bulfone-Paus S, Foster DC, Ruckert R. Interleukin-21 inhibits dendritic cell activation and maturation. Blood 2003;102:4090-8.
35. Brandt K, Bulfone-Paus S, Jenckel A, Foster DC, Paus R, Ruckert R. Interleukin-21 inhibits dendritic cell-mediated T cell activation and induction of contact hypersensitivity in vivo. J Invest Dermatol 2003;121:1379-82.

36. Distler JH, Jungel A, Kowal-Bielecka O, Michel BA, Gay RE, Sprott H, et al. Expression of interleukin-21 receptor in epidermis from patients with systemic sclerosis. Arthritis Rheum 2005;52:856-64.
37. Hou J, Schindler U, Henzel WJ, Ho TC, Brasseur M, McKnight SL. An interleukin-4-induced transcription factor: IL-4 Stat. Science 1994;265:1701-6.
38. Lin JX, Migone TS, Tsang M, Friedmann M, Weatherbee JA, Zhou L, et al. The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. Immunity 1995;2:331-9.
39. Ozaki K, Spolski R, Feng CG, Qi CF, Cheng J, Sher A, et al. A critical role for IL-21 in regulating immunoglobulin production. Science 2002;298:1630-4.
40. Zeng R, Spolski R, Finkelstein SE, Oh S, Kovanen PE, Hinrichs CS, et al. Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function. J Exp Med 2005;201:139-48.
41. Sivori S, Cantoni C, Parolini S, Marcenaro E, Conte R, Moretta L, et al. IL-21 induces both rapid maturation of human CD34+ cell precursors towards NK cells and acquisition of surface killer Ig-like receptors. Eur J Immunol 2003; 33:3439-47.
42. Brady J, Hayakawa Y, Smyth MJ, Nutt SL. IL-21 induces the functional maturation of murine NK cells. J Immunol 2004;172:2048-58.
43. Pene J, Gauchat JF, Lecart S, Drouet E, Guglielmi P, Boulay V, et al. Cutting edge: IL-21 is a switch factor for the production of IgG1 and IgG3 by human B cells. J Immunol 2004;172:5154-7.
44. Kuhn R, Rajewsky K, Muller W. Generation and analysis of interleukin-4 deficient mice. Science 1991;254:707-10.
45. Suto A, Nakajima H, Hirose K, Suzuki K, Kagami S, Seto Y, et al. Interleukin 21 prevents antigen-induced IgE production by inhibiting germ line C(epsilon) transcription of IL-4-stimulated B cells. Blood 2002;100:4565-73.
46. Kotlarz D, Zietara N, Uzel G, Weidemann T, Braun CJ, Diestelhorst J, et al. Loss-of-function mutations in the IL-21 receptor gene cause a primary immunodeficiency syndrome. J Exp Med 2013;210:433-43.
47. Rodrigues F, Davies EG, Harrison P, McLauchlin J, Karani J, Portmann B, et al. Liver disease in children with primary immunodeficiencies. J Pediatr 2004;145:333-9.
48. Elinav E, Strowig T, Kau AL, Henao-Mejia J, Thaiss CA, Booth CJ, et al. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. Cell 2011; 145:745-57.
49. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature 2012;491:119-24.
50. Rankin AL, MacLeod H, Keegan S, Andreyeva T, Lowe L, Bloom L, et al. IL-21 receptor is critical for the development of memory B cell responses. J Immunol 2011;186:667-74.
51. Spolski R, Kim HP, Zhu W, Levy DE, Leonard WJ. IL-21 mediates suppressive effects via its induction of IL-10. J Immunol 2009;182:2859-67.
52. Kotlarz D, Beier R, Murugan D, Diestelhorst J, Jensen O, Boztug K, et al. Loss of interleukin-10 signaling and infantile inflammatory bowel disease: implications for diagnosis and therapy. Gastroenterology 2012;143:347-55.
53. Li J, Pan HF, Cen H, Tian J, Ma Y, Tao JH, et al. Interleukin-21 as a potential therapeutic target for systemic lupus erythematosus. Mol Biol Rep 2011;38: 4077-81.
54. Sarra M, Franze E, Pallone F, Monteleone G. Targeting interleukin-21 in inflammatory diseases. Expert Opin Ther Targets 2011;15:695-702.
55. Yuan FL, Hu W, Lu WG, Li X, Li JP, Xu RS, et al. Targeting interleukin-21 in rheumatoid arthritis. Mol Biol Rep 2011;38:1717-21.
56. Sivendran S, Glodny B, Pan M, Merad M, Saenger Y. Melanoma immunotherapy. Mt Sinai J Med 2010;77:620-42.
57. Hashmi MH, Van Veldhuizen PJ. Interleukin-21: updated review of Phase I and II clinical trials in metastatic renal cell carcinoma, metastatic melanoma and relapsed/refractory indolent non-Hodgkin's lymphoma. Expert Opin Biol Ther 2010;10:807-17.
58. Monteleone G, Pallone F, Macdonald TT. Interleukin-21 as a new therapeutic target for immune-mediated diseases. Trends Pharmacol Sci 2009;30:441-7.
59. Schmidt H, Brown J, Mouritzen U, Selby P, Fode K, Svane IM, et al. Safety and clinical effect of subcutaneous human interleukin-21 in patients with metastatic melanoma or renal cell carcinoma: a phase I trial. Clin Cancer Res 2010;16: 5312-9.

METHODS

DNA isolation

Genomic DNA was isolated from whole blood by using a commercially available kit (Wizard Genomic DNA Purification Kit; Promega, Vienna, Austria), according to the manufacturer's instructions.

Sequencing of *IL10, IL10RA,* and *IL10RB*

Sanger sequencing of *IL10*, *IL10RA*, and *IL10RB* was performed according to standard methods. Primers are listed in Table E1. Primers marked with a footnote were described previously in Glocker et al.[E1]

Homozygosity mapping

SNP-based homozygosity mapping with Affymetrix 6.0 arrays (Affymetrix, Santa Clara, Calif) was performed on genomic DNA from the index patient to map homozygous intervals, as described previously, according to the Affymetrix Genome-Wide Human SNP Nsp/Sty 6.0 protocol.[E2] In brief, 10 μL of 50 ng/μL DNA was digested with restriction enzymes and ligated to adaptors. The adaptor-ligated DNA fragments were amplified with a generic primer recognizing the adaptor sequences. The amplified DNA was fragmented, labeled, and hybridized to the Affymetrix Genome-Wide Human SNP 6.0 Array. Results were analyzed with Affymetrix Genotyping Console software.

Homozygosity Mapper (www.homozygositymapper.org/) and PLINK[E3] whole-genome data analysis toolset with a window size of 5000 bp (a minimum of 50 SNPs within this region) were used to detect homozygous regions.

Exome sequencing

Sample preparation was done with the Illumina TruSeq DNA Sample Preparation Guide and the Illumina TruSeq Exome Enrichment Guide version 3 (Illumina, San Diego, Calif). The genomic DNA (1 μg) was sheared to fragments of 200 to 300 bp. Blunt ending, adenylation, and adapter ligation to allow the fragments to hybridize onto the flow cell were carried out. Subsequently, exons of the DNA fragments were enriched, and clusters were generated with the Illumina cBot Cluster Generation System according to the TruSeq PE Cluster Kit version 3 Reagent Preparation Guide. The DNA fragment clusters ran in a multiplexed pool with 5 other samples distributed on 3 lanes of the flow cell.

Data analysis was performed as previously described.[E2] In brief, reads were aligned by using Burrows-Wheeler Aligner to the human genome 19. Insertion/deletion realignment was performed, as was Genome Analysis Toolkit (GATK version 1.4)–based quality score recalibration. For single nucleotide variant (SNV) and deletion/insertion variant calling, Unified Genotyper and GATK Variant quality score recalibration were performed. SNV and deletion/insertion variant lists were uploaded to the SeattleSeq Annotation database (accessed January 2012). Variants present in 1000 Genomes and dbSNP build 135 were excluded, and the lists were filtered for nonsense, missense, and splice-site variants present within the homozygous regions only found in the patient.

Variant validation

Validation of the variants found on the final list of hits was performed by using capillary sequencing on genomic DNA from the patient. The primers for each position were designed with PrimerZ (http://genepipe.ngc.sinica.edu.tw/primerz/), excluding 60 bp adjacent to the target position and with the product size ranging between 100 and 500 bp. All primers were purchased from Sigma-Aldrich.

PCR amplification of the detected variants was performed with the Expand High Fidelity PCR System (Roche, Basel, Switzerland). Capillary sequencing was carried out with the Big Dye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany), and the analysis took place on a 3130xl Genetic Analyzer (Applied Biosystems). Sequencher DNA Software version 4.10.1 (Gene Codes, Ann Arbor, Mich) was used for sequence analysis. Segregation analysis of the validated hits was performed in the parents and healthy siblings.

T-cell CDR3 Vβ spectratyping

TCR Vβ spectratyping was performed according to the method of Pannetier et al.[E4] The primers used were described previously,[E5] with the exceptions indicated in Table E2.

Sequences were acquired with an ABI 3130xl Sequencer (Applied Biosystems) and analyzed with ABI GeneMapper software, version 4.0.

RESULTS

Homozygosity mapping

Homozygous intervals detected in the patient with Homozygosity Mapper (Table E3) and PLINK (Table E4). The interval on chromosome 4 in which *IL21* was detected (gray shading) was found with both methods.

Exome sequencing

Filtering of exome sequencing data for unknown missense, nonsense, or splice-site variants present within the homozygous regions found in the patient revealed 23 SNVs, of which 10 could be validated by using capillary sequencing. For hits in *LOC100132767* (chromosome 16: 29606607 G→R), *CNTNAP4* (chromosome 16: 76311602 G→T), and *C17orf100* (chromosome 17: 6555546 C→G), no unique sequences could be obtained (all positions refer to hg19). Under the assumption of an autosomal recessive trait with full penetrance, perfect segregation with the disease was found for the variant in *IL21*, which is discussed in detail in the main article, as well as in the gene *ENPEP* encoding aminopeptidase A (BP-1/6C3; chromosome 4: 111431495 C→A). The detected variant in *ENPEP* results in an amino acid change from alanine to glutamine at position 430 of the protein. Although it had previously been hypothesized that *ENPEP* might play a role in early B-cell development,[E6] the corresponding knockout mouse showed normal T- and B-cell numbers, displayed normal T cell–dependent and T cell–independent antibodies, and had normal serum immunoglobulin levels.[E7] Therefore this variant was not further considered to be of pathogenic relevance to the patient's disease.

Modeling of *IL21*

In silico modeling of the variant detected in *IL21* was done by using http://cupsat.tu-bs.de/jsp/nmrpredict.jsp. This tool enables the prediction of an amino acid change at a certain position of the protein. Of all possible amino acid changes, the exchange to proline, which was detected in the patient, was predicted to be the most unfavorable (gray shading in Table E3).

To obtain a qualitative hypothesis about the difference in protein structure and dynamics of the IL-21 variants, we performed MD simulations of IL-21^{WT} and IL-21^{Leu49Pro}. Simulations were performed with the coarse-grained model FREADY[E8] implemented in the MOIL[E9,E10] molecular modeling package. We initiated the MD simulations from the NMR structure of IL-21 (PDB id 2OQP)[E11] and let it run for 50 ns at 300 K. Simulations were performed with different degree of flexibility for the protein, ranging from fully free MD simulations to simulations in which backbone dihedral angles of residues further than a flexibility cutoff from residue 49 were constrained to remain similar to the NMR structure. The flexibility cutoff was varied from 5 to 9 Å. All simulations over a range of flexibility cutoffs, including the fully free simulation, resulted in the same qualitative behavior. Results displayed in Fig 1 consider simulations with a flexibility cutoff of 9 Å. At this cutoff

value, the overall folding of IL-21 is highly preserved throughout both simulations (backbone Root-mean-square deviation [RMSD] of WT simulation, approximately 3 Å; backbone RMSD of mutant stimulation, approximately 4 Å; template-modeling [TM] scores,[E12] ≤0.93 and 0.9, respectively), and the local conformational transition at the mutation point is clearly observable (Fig 1, *F*). Protein structures were aligned and visualized with the UCSF Chimera tool.[E13]

REFERENCES

E1. Glocker EO, Kotlarz D, Boztug K, Gertz EM, Schaffer AA, Noyan F, et al. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. N Engl J Med 2009;361:2033-45.

E2. Salzer E, Daschkey S, Choo S, Gombert M, Santos-Valente E, Ginzel S, et al. Combined immunodeficiency with life-threatening EBV-associated lymphoproliferative disorder in patients lacking functional CD27. Haematologica 2013;98:473-8.

E3. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 2007;81:559-75.

E4. Pannetier C, Even J, Kourilsky P. T-cell repertoire diversity and clonal expansions in normal and clinical samples. Immunol Today 1995;16:176-81.

E5. Pannetier C, Even J, Kourilsky P. T-cell repertoire diversity and clonal expansions in normal and clinical samples. Immunol Today 1995;16:176-81.

E6. Welch PA. Regulation of B cell precursor proliferation by aminopeptidase A. Int Immunol 1995;7:737-46.

E7. Lin Q, Taniuchi I, Kitamura D, Wang J, Kearney JF, Watanabe T, et al. T and B cell development in BP-1/6C3/aminopeptidase A-deficient mice. J Immunol 1998;160:4681-7.

E8. Majek P, Elber R. A coarse-grained potential for fold recognition and molecular dynamics simulations of proteins. Proteins 2009;76:822-36.

E9. Elber R, Roitberg A, Simmerling C, Goldstein R, Li H, Verkhiver G, et al. MOIL: a program for simulations of macromolecules. Computer Physics Communications 1995;91:159-89.

E10. Parthiban V, Gromiha MM, Schomburg D. CUPSAT: prediction of protein stability upon point mutations. Nucleic Acids Res 2006;34: W239-42.

E11. Bondensgaard K, Breinholt J, Madsen D, Omkvist DH, Kang L, Worsaae A, et al. The existence of multiple conformers of interleukin-21 directs engineering of a superpotent analogue. J Biol Chem 2007;282:23326-36.

E12. Zhang Y, Skolnick J. Scoring function for automated assessment of protein structure template quality. Proteins 2004;57:702-10.

E13. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al. UCSF Chimera—a visualization system for exploratory research and analysis. J Comput Chem 2004;25:1605-12.

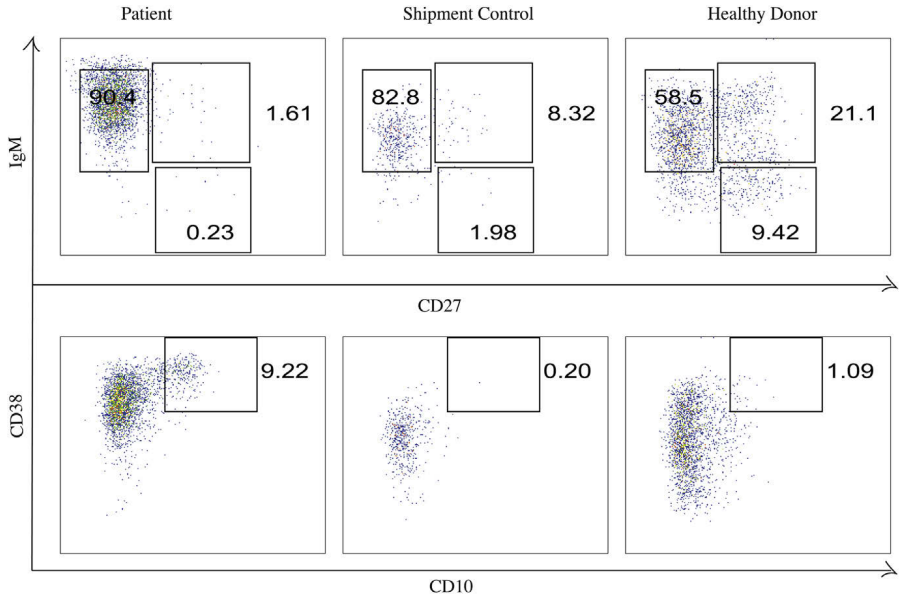

FIG E1. Flow cytometric analysis gated on $CD19^+$ cells displaying decreased IgM^+CD27^+ non–class-switched memory B cells in the patient compared with a healthy shipment control, as well as a healthy donor *(top)* and increased immature early transitional $CD38^+CD10^+$ B cells *(bottom)*.

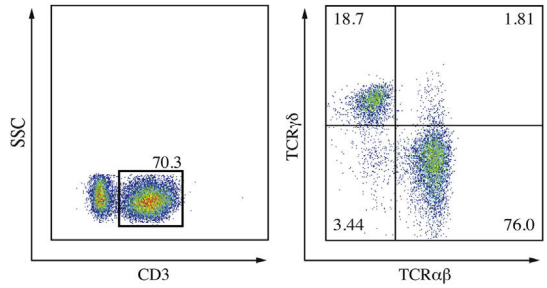

FIG E2. Flow cytometric analysis displaying increased proportion of $TCR\gamma\delta^{+}CD3^{+}$ cells. *SSC,* Side scatter.

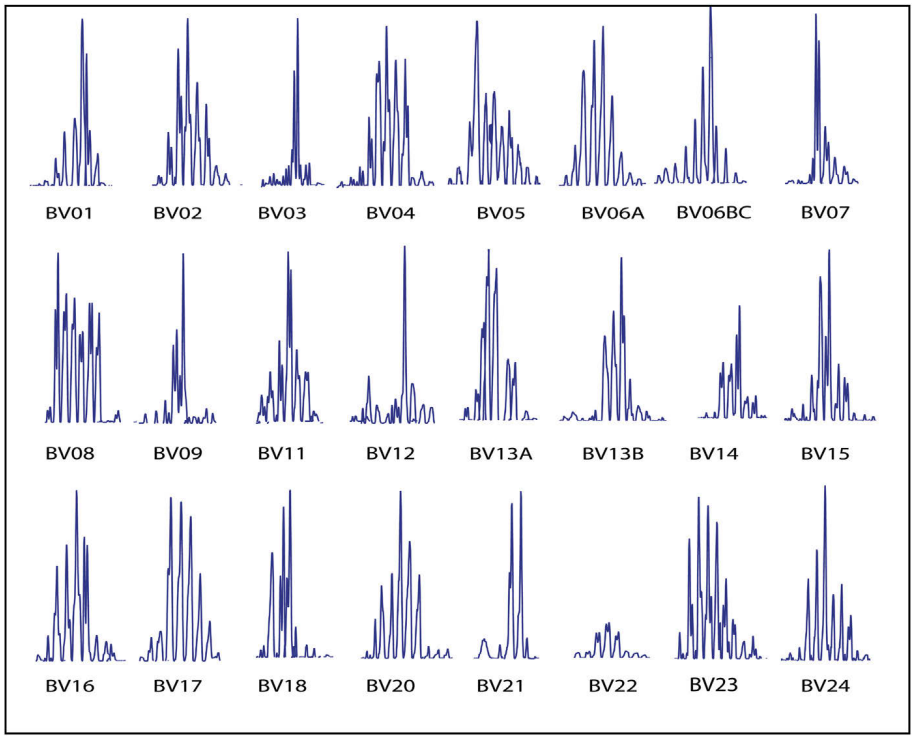

FIG E3. TCR Vβ spectratyping analysis. Analysis of the patient shows a normal polyclonal use of the TCR Vβ repertoire.

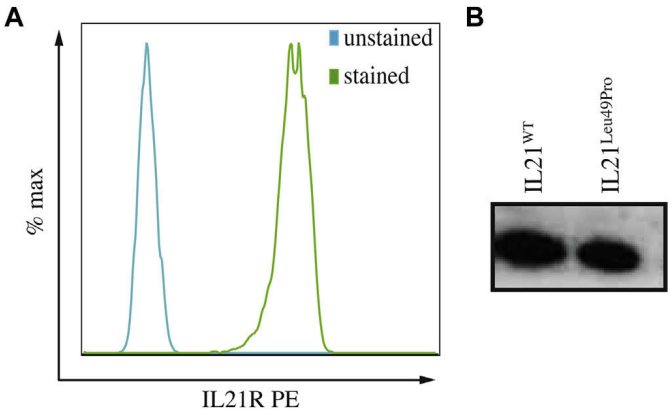

FIG E4. IL-21 production by HEK293 cells. **A,** Flow cytometric analysis of IL-21R expression on Jurkat cells. **B,** Western blot analysis of supernatant of IL-21–producing HEK293 cells blotted for the His-tag attached to both with IL-21^{Leu49Pro} and IL-21^{WT}.

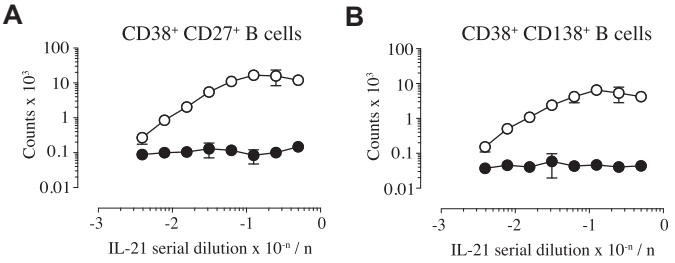

FIG E5. Proliferation capacity of the cord blood–derived plasmablast and memory B-cell populations in response to WT *(open circles)* or mutant *(solid circles)* IL-21. Data were gated on $CD19^{+}$ cells and analyzed for expression of either CD38 and CD27 **(A)** or CD38 and CD138 **(B)**.

TABLE E1. Primer sequences

Gene exon	Orientation	Sequence (5′-3′)
IL10 exon 1	F	GCCGGGAAACCTTGATTG
	R	GGAATAGGTGTTGGGGATGG
IL10 exon 2	F	TCCCATCACTGTTGAATCCTC
	R	GACTGAGCCCTTTGTAAACCC
IL10 exon 3	F	ATCGCTAGAACCAAGCTGTCC
	R	GTGTCTTTGCTGTGTCTGTGG
IL10 exon 4	F	ACCAGCTTGTCCCCTAAGTG
	R	GAATGGGGCCTATTGAGTCC
IL10 exon 5.1	F	CATGAGCATGAGGGAGGG
	R	TCCGAGACACTGGAAGGTG
IL10 exon 5.2	F	ATCTTGTCTCTGGGCTTGGG
	R	TGCAGAATTCATTCACCCAC
IL10 exon 5.3	F	CCTAAATTTGGTTCTAGGCCG
	R	GGCTTCCTTTCTCTGAAATGC
IL10 exon 5.4	F	TGGATCACTTGAGGTCAGGA
	R	CCTGGGGGTAGGGGGTAG
IL10RA exon 1*	F*	GACAGTGGTTCCCCGTCC*
	R*	CACTGGATGGAGAACTTTAATGG*
IL10RA exon 2*	F*	GAACCTCCCTTTCTTCTTTGG*
	R*	AGGCAGGTATCTTCCCATGC*
IL10RA exon 3*	F*	GGCCTCTTGCGTCTCCC*
	R*	GCAGACATGGTGAGCTATGG*
IL10RA exon 4*	F*	ATTCTGGAGGCAAAGTCTCG*
	R*	AGTTCCCAATGGCACACAAG*
IL10RA exon 5*	F*	CTAAAGGCCCACCAGCTCTC*
	R*	ACGCGTTTTGGATTGCAC*
IL10RA exon 6*	F*	AATGGATTTCATGGGACCAG*
	R*	ACTGGCTGGGAGGAAAAGAG*
IL10RA exon 7.1*	F*	CGAGCTCTCCTCCTGGG*
	R*	CCTCAGGTAACCCTGGAATG*
IL10RA exon 7.2*	F*	TGACAGTGGCATTGACTTAGTTC*
	R*	GTCCAGGCAGAGGAGCAG*
IL10RA exon 7.3*	F*	CCTGGGCAGCTTTAACTCAG*
	R*	AGGTTCCCCATGTGACCATC*
IL10RA exon 7.4*	F*	GCTGAAGTCAGCTCAGACCC*
	R*	CAGTGCCCAGTGGCTTATC*
IL10RA exon 7.5	F	TGGTCATAACTCAGCCCTTTG
	R	GTCATGGCTGGATTCCCTG
IL10RA exon 7.6	F	AACAAAGGCAGTTCAGTCCAC
	R	AACCGAGTCCTCCATGAGC
IL10RB exon 1*	F*	AGGGTAAAGAAGACCCTCAAA*
	R*	CCTAGTTGCGTCTCAGCAG*
IL10RB exon 2*	F*	AGCCATAGAGGAGAACCAAGT*
	R*	ACCTAGAGATGACAGCAGTGG*
IL10RB exon 3	F	TTAACACAGTTTCCACTCCCG
	R	AAGGCCATCCATTTGTGG
IL10RB exon 4	F	TCCGTGGACTAATTGTTCTGC
	R	AGTCCATAAGGTGCTGCCAC
IL10RB exon 5	F	CCCTGAACTGAGAGGAGCAC
	R	TGAATGAGCTGCCTCAGAAG
IL10RB exon 6*	F*	GGATTGTGATGGTTAAAATGC*
	R*	CCCTTTTACAAATAGCCTTCC*
IL10RB exon 7.1*	F*	ATAGATTTTCCAGCCAGGAGT*
	R*	GCCCTGTTTCTCACAATTAAA*
IL10RB exon 7.2	F	CACATCTAGAACTCCCAGACCC
	R	TCACTTTGTCACCCAGGC
IL10RB exon 7.3	F	GATGGCGCATGCCTATAATC
	R	TGGACATCAAGATGGCAAAC

F, Forward; *R*, reverse.
*Primers according to Glocker et al.[E1]

TABLE E2. Newly designed primers for TCR Vβ spectratyping

Primer name	Sequences
Primers for variable regions	
BV02	ACATACGAGCAAGGCGTCGA
BV04	CATCAGCCGCCCAAACCTAA
BV07	CAAGTCGCTTCTCACCTGAATGC
BV17	TGTGACATCGGCCCAAAAGAA
BV21	GGAGTAGACTCCACTCTAAG
BV24	CCCAGTTTGGAAAGCCAGTGACCC
Primers for constant regions	
CβB1 (used for BV05, BV06BC, BV20)	CGGGCTGCTCCTTGAGGGGCTGCG
FAM-marked constant primer	ACACAGCGACCTCGGGTGGG

TABLE E3. Homozygous intervals detected in the index patient (II-8) using Homozygosity Mapper software

Chromosome	Start	End	SNP start	SNP end
4	83395044	88826972	rs7664077	rs2009152
4	90025980	96699508	rs7691983	rs10014317
4	96719160	109475974	rs11930626	rs6835794
4	109481858	118949490	rs7666063	rs2389493
4*	118949878*	127531669*	rs2254215*	rs313142*
4	127534757	146750966	rs313139	rs13149290
5	2757959	4885009	rs1391124	rs3913374
5	163400151	167294665	rs11135295	rs13175544
5	167303317	169376419	rs1862198	rs259917
5	169516018	173192744	rs10073750	rs791346
16	27166296	54177001	rs16976570	rs2689265
16	70928314	75081551	rs1774433	rs4243109
16	76351875	78526427	rs11864692	rs8055841
16	78527004	80747410	rs2738716	rs3100179
16	80754387	84729485	rs4580175	rs7193019
17	410451	10054798	rs9748016	rs1024370
17	11245099	13841519	rs16944600	rs4792419

*Interval which contains IL21.

TABLE E4. Homozygous intervals detected in the index patient (II-8) by using PLINK

Chromosome	Start	End	SNP start	SNP end
1	49222528	50674403	SNP_A-2011849	SNP_A-8467861
2	72347041	74123827	SNP_A-2313026	SNP_A-8687775
2	184851796	185944619	SNP_A-2237199	SNP_A-8537410
2	189306009	190951583	SNP_A-2053595	SNP_A-8472550
3	41234516	42417982	AFFX-SNP_5800238	SNP_A-2263719
3	96053560	97183817	SNP_A-1884420	SNP_A-8495492
4	1052009	2647131	SNP_A-8420001	SNP_A-8512601
4	2674271	4333327	SNP_A-2168327	SNP_A-8684751
4	33409962	34724345	SNP_A-8629522	SNP_A-1976751
4	83403068	88824216	SNP_A-8389116	SNP_A-1977976
4	88859131	90003726	SNP_A-8588059	SNP_A-8458421
4	90034030	98611720	SNP_A-8621918	SNP_A-8561152
4	98665087	109472301	SNP_A-8680070	SNP_A-8687642
4	109483856	113784482	SNP_A-1847528	SNP_A-8371169
4	113811255	116726594	SNP_A-8386473	SNP_A-2252669
4*	116729292*	127525092*	SNP_A-8648720*	SNP_A-8445435*
4	127539506	129888651	SNP_A-8662340	SNP_A-8630365
4	129965026	142021835	SNP_A-4272521	SNP_A-8662873
4	142066636	146764719	SNP_A-1979175	SNP_A-1932018
5	36344	2738455	SNP_A-8392711	SNP_A-4273394
5	3642021	4819918	SNP_A-8664849	SNP_A-1897861
5	44719866	45888504	SNP_A-8604529	SNP_A-4282307
5	56755502	58101645	SNP_A-1854819	SNP_A-8396567
5	97874728	99039123	SNP_A-8607625	SNP_A-2274520
5	163392787	167296428	SNP_A-8708191	SNP_A-2272719
5	167309980	169374909	SNP_A-1904490	SNP_A-2197407
5	170274844	172957916	SNP_A-2260629	SNP_A-1895904
6	26091336	27189517	SNP_A-4273092	SNP_A-2258495
7	62550165	63692343	SNP_A-8346354	SNP_A-8330375
7	123016059	124061924	SNP_A-4266575	SNP_A-8520938
9	133988447	137109245	SNP_A-8379710	SNP_A-1908201
9	138059244	139666473	SNP_A-8496411	SNP_A-8690526
9	139839904	141071475	SNP_A-8477786	SNP_A-8302801
10	22320193	23336057	SNP_A-8553730	SNP_A-8349587
10	68522925	69871319	SNP_A-2304575	SNP_A-4275777
12	37857751	38957140	SNP_A-2298756	SNP_A-1914010
12	88317831	89373308	SNP_A-4268475	SNP_A-8471938
14	24239341	26640934	SNP_A-2037635	SNP_A-1807016
14	27450705	28791689	SNP_A-8623427	SNP_A-8319810
14	90646020	92838444	SNP_A-8646350	SNP_A-2246935
15	42335477	43395847	SNP_A-2004120	SNP_A-2130382
15	72094856	73124491	SNP_A-8685433	SNP_A-2276311
16	27842014	35205717	SNP_A-8582579	SNP_A-8595671
16	46534977	54175313	SNP_A-8350504	SNP_A-8413639
16	54355601	55425383	SNP_A-8319132	SNP_A-8390684
16	55442987	57154547	SNP_A-8424883	SNP_A-2279806
16	67040335	68326200	SNP_A-1812292	SNP_A-1806333
16	69534688	70836370	SNP_A-4244436	SNP_A-2004827
16	71103393	75046184	SNP_A-8610073	SNP_A-8364418
16	76355255	78517027	SNP_A-8560561	SNP_A-1806599
16	78527065	82919690	SNP_A-4192918	SNP_A-2027051
16	83379737	84730830	SNP_A-8401789	SNP_A-8280151
17	6689	3205886	SNP_A-8398136	SNP_A-2284710
17	3210368	5000130	SNP_A-4196849	SNP_A-8299342
17	5133734	11236566	SNP_A-8287174	SNP_A-2224114
17	11259037	13837051	SNP_A-4202679	SNP_A-1945664
17	19442146	20554628	SNP_A-8344319	SNP_A-8546621
17	28687196	29796197	SNP_A-1816266	SNP_A-8616647
20	32773809	33831187	SNP_A-1941912	SNP_A-8383932

*Interval which contains IL21.

TABLE E5. Prediction and effect of an amino acid change in IL-21 at position Leu49

Amino acid	Overall stability	Torsion	Predicted ΔΔG (kcal/mol)
GLY	Destabilizing	Unfavorable	−2.51
ALA	Destabilizing	Unfavorable	−1.92
VAL	Destabilizing	Favorable	−1.01
ILE	Destabilizing	Favorable	−1
MET	Destabilizing	Unfavorable	−1.56
PRO	Destabilizing	Unfavorable	−3.92
TRP	Destabilizing	Unfavorable	−1.53
SER	Destabilizing	Unfavorable	−1.78
THR	Destabilizing	Favorable	−1.41
PHE	Destabilizing	Favorable	−1.43
GLN	Destabilizing	Unfavorable	−1.64
LYS	Destabilizing	Unfavorable	−1.65
TYR	Destabilizing	Favorable	−1.44
ASN	Destabilizing	Unfavorable	−1.69
CYS	Destabilizing	Favorable	−1.44
GLU	Destabilizing	Unfavorable	−1.72
ASP	Destabilizing	Unfavorable	−1.8
ARG	Destabilizing	Unfavorable	−1.6
HIS	Destabilizing	Unfavorable	−1.51

4. Discussion

In the following chapters afore mentioned discoveries are described and discussed in more detail, referring to the basic biology background of the discovered diseases followed by discussing novel findings and placing them into context of prior knowledge from the literature.

4.1. Combined immunodeficiency with life threatening EBV-associated lymphoproliferative disorder in patients lacking functional CD27.

4.1.1. CD27 biology

CD27 is expressed on early thymocytes, naïve CD4 and CD8 cells (Nolte et al, 2009) and is commonly used as a marker for memory B cells (Klein et al, 1998). CD27 is a member of the TNF receptor (TNFR) superfamily and is known to interact with its unique ligand, CD70, a membrane-bound homotrimeric type II membrane protein (Lens et al, 1998). Binding of CD70 induces CD27 trimerization and thus initiation of intracellular signaling (Nolte et al, 2009). Moreover, it has been shown that upon binding of CD70, a truncated form of CD27 is released possibly cleaved by a membrane-linked protease (Loenen et al, 1992). Interestingly, increased levels of soluble CD27 have been found in patients with autoimmune diseases or viral infections (Lens et al, 1998).

On the other hand, CD27 activation induces intracellular signaling via TNFR-associated factor (TRAF) 2 and 5, which both get ubiquitinated after CD70 binding (Nolte et al, 2009). Consequently, both canonical and non-canonical NFkB pathways get activated (Ramakrishnan et al, 2004). However, it has been shown that the c-Jun terminal kinase (JNK)-signaling cascade (Akiba et al, 1998; Gravestein et al, 1998) as well as intracellular mediators of apoptosis (Prasad et al, 1997; Spinicelli et al, 2002) get activated upon CD70 binding (Nolte et al, 2009). In a mouse model it has been demonstrated that stimulation with an agonistic CD27 antibody induces proliferation and differentiation of T and B cells. Moreover, CD27 signaling seems to result in induction of TH1 differentiation in mice (Nolte et al, 2009).

4.1.2. CD27 deficiency in the context of other PIDs with lymphoproliferation

The first description of CD27 deficiency consisted of two brothers of a consanguineous Morrocan family who presented with EBV-associated immunodeficiency and lack of expression of CD27 on memory B cells. Capillary sequencing of *CD27* revealed a homozygous stopgain mutation (p.W8X) (van Montfrans et al, 2012). One patient presented with aplastic anemia and died of gram-positive sepsis. The other patient exhibited hypogammaglobulinemia with impaired specific antibody production. Both patients however showed lymphadenopathy, hepatosplenomegaly and lacked seroconversion for EBV-nuclear antigen (van Montfrans et al, 2012).

In our 8 patients described, the phenotype varied from asymptomatic borderline hypogammaglobulinemia to EBV-LPD, with progression to T and B cell lymphomas (Salzer et al, 2013). These clinical features resemble other syndromes with increased susceptibility to EBV-LPD such as, among others, ITK deficiency (Huck et al, 2009), XIAP- (Rigaud et al, 2006) and SAP deficiency (Coffey et al, 1998). Interestingly, in all of these diseases including CD27 deficiency patients exhibit severely reduced to absent numbers of iNKT cells during active EBV-LPD (Ghosh et al, 2014). Therefore a critical role for iNKT cells has been suggested in controlling EBV infections (Chung et al, 2013). Recently our lab published a patient with ITK deficiency diagnosed prior to EBV infection and without LPD, who presented with CD4 lymphopenia and showed absence of iNKT cells, indicating the necessity of ITK for iNKT cell development (Serwas et al, 2014). Nevertheless, how and at which point during development CD27 influences iNKT cells remains to be clarified.

Interestingly, all 8 patients showed the exact same genetic mutation but presented high variability in the clinical presentation as well as spectrum of diseases. Clinical presentation of CD27 deficiency ranged from asymptomatic hypogammaglobulinemia to the development of both T and B cell malignancies (Salzer et al, 2013). As already discussed in the manuscript, it is hypothesized that “timing and tuning” of co-stimulatory signals in the course of the infection may be crucial to shape the immune response towards control or lymphoproliferation (Nolte et al, 2009). However, the precise mechanism by which CD27 facilitates EBV-LPD remains to be discovered.

4.2. B cell deficiency and severe autoimmunity caused by deficiency of protein kinase C δ.

4.2.1. PKCδ biology

The protein kinase C (PKC) family of serine/threonine kinases executes key roles in a plethora of cellular processes, including cell proliferation, apoptosis, and differentiation (Wu-Zhang et al, 2012). The PKC family can be divided in 3 subfamilies: conventional PKCs (cPKC), novel PKCs (nPKC) and atypical PKCs (aPKC) (Wu-Zhang et al, 2012).

PKCδ is a 78kDa protein with 676 amino acids, belongs to the novel PKC group and is a calcium-independent, phospholipid-dependent, serine/threonine kinase. The protein consists of a regulatory and a catalytic domain. PKCδ also contains five variable regions (V), where the variable region 3 (V3) acts as a hinge region between catalytic and regulatory domains. The C1 motif contains DAG (diacylglycerol)/PMA (Phorbol 12-myristate 13-acetate) binding sequences, which enable PKCδ binding to membranes (Cho, 2001). Although PKCδ has a C2-like region, this domain lacks the essential Ca^{2+} coordinating acidic residues that allow classical PKCs to bind Ca^{2+} (Pappa et al, 1998). C3 and C4 domains are needed for ATP/substrate binding and thus for the catalytic activity of the enzyme.

A pseudo substrate between C1 and C2 motifs retains PKCδ in an inactive conformation, thus blocking access to the substrate-binding pocket. When PKCδ is activated by proteolytic cleavage, a 40kDa fragment is generated which can translocate to mitochondria and/or nucleus (Cho, 2001; Hurley & Misra, 2000; Steinberg, 2004). Three specific sites need to be phosphorylated so that the kinase can be fully active: auto-phosphorylation at Ser643 (turn motif), Thr505 (activation loop of the kinase domain) and Ser662 (hydrophobic region)(Steinberg, 2004). Moreover, PKCδ can be phosphorylated by tyrosine kinases at eight Tyr residues. Tyr155 phosphorylation seems to be important for the inhibitory effect of PKCδ on cell proliferation (Sun et al, 2000; Szallasi et al, 1995). Tyrosine phosphorylation at the hinge and activation regions results in PKCδ activation and differential subcellular distribution onto membranes (Blake et al, 1999; Konishi et al, 1997). Conversely, phosphorylation of Tyr155 and Tyr187 promote the anti-apoptotic effect of

PKCδ resulting in an increase in cell proliferation in response to PMA (Kronfeld et al, 2000).

4.2.2. PKCδ activation

PKCδ is activated downstream of a variety of stimuli including stress response to oxidative stress, DNA damage or ultra violet radiation (Zhao et al, 2012) (Figure 4). It is involved in signaling downstream of the BCR, interferon (IFN) receptors (Deb et al, 2003; Uddin et al, 2002) the insulin receptor (Braiman et al, 1999) and many others. PKCδ can be phosphorylated by various kinases leading to different phosphorylation patterns and possibly resulting in differential activation of downstream targets. After DAG binding, Ser/Thr phosphorylation by PDK1 and other upstream kinases including PKCζ and mTOR (mammalian target of rapamycin), but also auto-phosphorylation (Durgan et al, 2007) leads to full kinase activation. Tyr residues of PKCδ are phosphorylated by Src kinase family members (SRC, LYN, FYN, LCK), PYK2 or growth factor receptors, fine-tuning kinase activity and downstream effects (Kikkawa et al, 2002) (Basu & Pal, 2010).

Although PKCδ is a known regulator of pleiotropic functions, the true effect of absence of PKCδ in humans was only uncovered when we identified biallelic mutations in *PRKCD* as the molecular cause of a novel primary immunodeficiency disorder with severe, SLE-like systemic autoimmunity (Salzer et al, 2013).

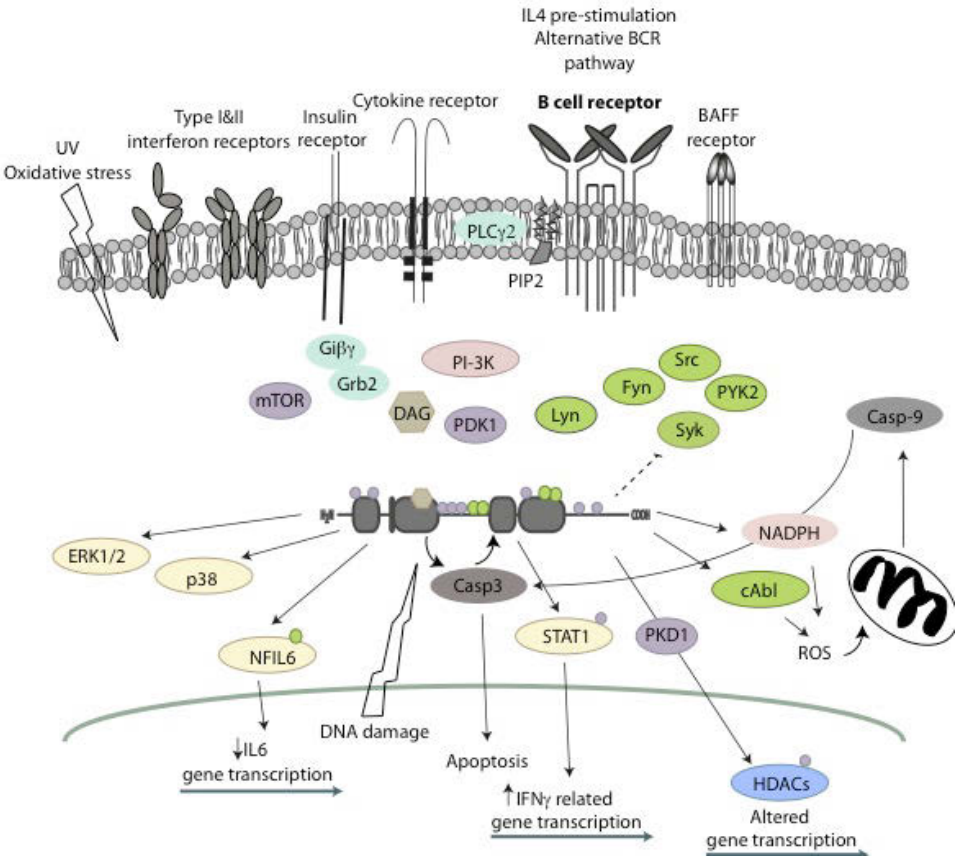

Figure 4 PKCδ related signaling pathways as described in the text. Tyrosine kinases and tyrosine phosphorylation sites are depicted in green; serine/threonine kinases and phosphorylation sites in purple.

4.2.3. Human PKCδ deficiency

Since our group initially described PKCδ deficiency in 2013 (Salzer et al, 2013), four additional patients from 2 unrelated kindreds have been published (Belot et al, 2013; Kuehn et al, 2013). All detected mutations were biallelic and led to loss of function or loss of expression of the corresponding protein product: c.1352+1G>A (splice-site),p.G510S and R614W.

Common features in all five patients include hepatosplenomegaly, lymphoproliferation and positive autoantibodies. Four patients also presented with lymphoproliferative features and/or kidney involvement. All patients developed symptoms before the age of 10 years. Recurrent infections were seen in 2 patients (Kuehn et al, 2013; Salzer et al, 2013). Immune phenotyping of peripheral blood leukocytes revealed mostly normal T cell numbers and function (Miyamoto et al, 2002). Circulating B cell counts were variable and reduced numbers of memory B cells were detected. Immunoglobulin levels varied as only 2 patients presented with elevated IgM levels. In peripheral lymphoid organs of two patients predominant B cell infiltration was observed. Treatment involved the use of corticosteroids and other immunosuppressants such as mycophenolate mofetil or rapamycin.

As specified above, all patients presented with systemic lupus erythematosus (SLE) or SLE-like autoimmunity and 4 out of 5 exhibited lymphoproliferative features and/or kidney involvement.

4.2.4. PKCδ signaling and SLE

Among others, PKCδ acts downstream of BTK and PLCγ2 (Guo et al, 2004). Recently, this pathway has been implicated in a novel PID with significant autoimmunity, displaying activating mutations in the *PLCγ2* gene, described by two independent groups in 2012 (Ombrello et al, 2012; Zhou et al, 2012). Moreover human PKCδ deficiency resembles the corresponding knock out mouse model, described in 2002 (Miyamoto et al, 2002). These mice showed lymphadenopathy, immune complex glomerulonephritis, splenomegaly and B cell infiltrations in several organs. Experimental studies revealed, as also demonstrated in PKCδ-deficient patients, increased production of IL-6, possibly leading to B cell hyperproliferation. In this case an interesting therapeutic opportunity would be the use of Tocilizumab an anti-IL-6 antibody

already in clinical use for selected rheumathoid arthritis patients (Balsa et al, 2015).

SLE is a complex and to date still poorly understood disease and can be considered as the showcase for a systemic autoimmune mediated disease.

In SLE, tolerance breakdown of both T and B cells can be observed (Wahren-Herlenius & Dorner, 2013). Already for some time it has been hypothesized that apoptosis defects may play an important role in the pathogenesis of SLE (Belot & Cimaz, 2012). In this model T and B cells are resistant to death signals during central or peripheral tolerance induction. Regarding T cells this hypothesis is evidenced by the fact that mutations in genes involved in the Fas pathway lead to ALPS or ALPS-like syndromes in humans (Oliveira et al, 2010). Interestingly, 4 out of 5 PKCδ deficient patients were clinically diagnosed with ALPS before the causative gene defect was discovered (Belot et al, 2013; Kuehn et al, 2013). For B cells, given the phenotype of Pkcδ knock out mice, a potential role for PKCδ in the pathogenesis of SLE was hypothesized. Given the multiple activities of PKCδ, it is not surprising that an altered PKCδ function contributes probably through several mechanisms to the immune dysregulation in SLE, although currently only few of them are sufficiently defined. Taken together the discovery of PKCδ deficient patients shed light on crucial functions of this kinase and define PKCδ as a critical regulator of immune homeostasis in man.

4.3. Early-onset inflammatory bowel disease and common variable immunodeficiency-like disease caused by IL-21 deficiency

4.3.1. Discovery and biology of the IL-21/IL-21 receptor system

The IL-21 receptor (IL-21R) was discovered by 2 groups in the year 2000 (Ozaki et al, 2000; Parrish-Novak et al, 2000), whereby they additionally identified the highly conserved WSXWS motive in the extracellular domain typically seen in type I cytokine receptors. The IL-21 cytokine, on the other hand, is composed of four a-helices (A-D), arranged in up-up-down-down constellation (Hamming et al, 2012). Due to the recent report of the crystal structure of the IL-21/IL-21R complex by Hamming et al. it is now known that the WSXWS motif as well as glycosylation of the IL-21R are critical for receptor binding and the interaction between IL-21 and the IL-21 receptor (IL-21R) takes place between amino-acids of helices A, C and a small part of the CD loop of IL-21 (Hamming et al, 2012). The IL-21R forms a heterodimeric complex with the common-γ chain, which is shared by IL-2, IL-4, IL-7, IL-9 and IL-15 (Rochman et al, 2009). Although the majority of binding energy is provided by the IL-21R, the interaction with the common-γ chain has been shown to be indispensable for signal transduction (Asao et al, 2001), evidenced by the fact that IL-21 mutants which cannot interact with the common-γ chain are agonists of IL-21 signaling (Kang et al, 2010).

IL-21 signals vial the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway (Rochman et al, 2009). IL-21 predominately activates STAT3 and 1, which distinguishes it from other cytokines of the common-γ chain-family but can phosphorylate STAT5A and B as well. Upon phosphorylation STATs dimerize and enter the nucleus to promote transcription of various target genes. Target genes of the phosphorylated STATs include*, Gzma, Gzmb, Il10, Socs1 and 3, Ifng, Cyclins a/b/e, Cxcr3 and 6, Bcl3, Jak3, Bim, Bcl6, Maf, Prdm1, Rorgt, Eomes, Il21*, and *Il21R* (Kotlarz et al, 2014). Thus IL-21 is critically involved in cell survival, cell-cycle regulation, cell migration, cellular activation, cytotoxicity and regulating its own expression (Kotlarz et al, 2014). Apart from the JAK-STAT pathway, IL-21 can also mediate activation of mitogen activated protein (MAP) kinase pathways

or the Phosphoinositide (PI)-3 kinase pathway (Spolski & Leonard, 2014) via activation of JAK2 and 3.

The IL-21R is expressed on all hematopoietic cells, including epithelial cells, fibroblasts and keratinocytes. IL-21 is predominately produced by activated CD4 T cells (Kotlarz et al, 2014; Leonard & Spolski, 2005). Consistently, IL-21 exerts pleiotropic functions on almost all cells of the immune system (Spolski & Leonard, 2014). IL-21 is essential in the development of T_{FH} cells (Bauquet et al, 2009), T_{H17} cell differentiation (Linterman et al, 2010) and germinal center formation (Ozaki et al, 2004). Moreover, it is crucial for immunoglobulin class-switch and plasma cell differentiation of B cells (Ettinger et al, 2005). IL-21 is important for function and survival of CD8 T cells (Silver & Hunter, 2008) and has been shown to modulate NKT cell function (Coquet et al, 2007). Interestingly, IL-21 has been shown to be involved in the suppression of regulatory T cells by FOXP3 suppression (Li & Yee, 2008). More recently it became clear that IL-21 also plays important roles in the myeloid compartment by acting on macrophages (Vallieres & Girard, 2013), B-helper neutrophils (Puga et al, 2012) and dendritic cells (Wan et al, 2013).

4.3.2. Human IL-21R and IL-21 deficiency

In 2013, Kotlarz et al. (Kotlarz et al, 2014; Kotlarz et al, 2013) identified human IL-21R deficiency in two unrelated families with 4 affected individuals in total. The identified mutations in the *IL21R* gene were a missense mutation (c.G602T, p.Arg2201Leu) in patients 1 and 2 and a 6 base pair deletion (c.240_245delCTGCCA, p.Cys81_His82del) in patients 3 and 4.

Interestingly, all patients presented with severe liver disease due to chronic cholangitis caused by cryptosporidium infection (Kotlarz et al, 2014; Kotlarz et al, 2013). Two out of four patients were initially diagnosed with idiopathic liver fibrosis and listed for liver transplantation. Only in retrospect it became clear that these patients also suffered from a combined immunodeficiency mirrored by recurrent respiratory and gastrointestinal infections. Immunophenotyping of peripheral blood lymphocytes of these four patients showed normal T, B, and NK cell numbers (Kotlarz et al, 2014; Kotlarz et al, 2013). Detailed evaluation of B cells, however, revealed an increase of naïve B cells and a reduction of

memory-switched B cells in three patients. All four patients also showed increased levels of IgE (Kotlarz et al, 2014; Kotlarz et al, 2013).
Experimental studies showed defective IL-21-induced STAT activation leading to defective IL-21 responses in lymphoid cells. Moreover, these patients also exhibited defective T cell proliferation towards specific antigens (Kotlarz et al, 2014; Kotlarz et al, 2013).
In a recent review by Kotlarz et al. (Kotlarz et al, 2014), three additional, to date still unpublished IL-21R deficient patients are mentioned. One patient presented with pulmonary tuberculosis, hepatosplenomegaly, chronic inflammatory skin disease and invasive fungal and viral infections as well as increased IgM levels and reduced class-switched CD19 cells. This patient died due to post HSCT complications. The detected mutation was a splice site conferring c.153-1G>T (Kotlarz et al, 2014). Another patient showed recurrent upper and lower respiratory tract infections and additionally presented with a B cell class switch defect. The patient was positive for *Pneumocystis jirovecii,* indicating a T cell deficiency. However, he did not show any cryptosporidium infection. This patient was transplanted at the age of 8 years. The detected mutation was a missense mutation (c.G602A, p.Arg201Gln). For the third patient no clinical description is available (Kotlarz et al, 2014).
In summary, the to date described IL-21R-deficient patients exhibit a T cell defect with reduced proliferation to specific antigens such as tetanus toxoid, but also a B cell defect, which is characterized by defective class switch and proliferation.
The up to now only case of IL-21 deficiency, described in the results section of this thesis on the other presented with early-onset inflammatory bowel disease and a CVID-like primary immunodeficiency. This patient also exhibited high IgE levels and a defective class switch in B cells, which was reversible *in vitro* after stimulation with wild type IL-21 (Salzer et al, 2014).

4.3.3. Concluding remarks

Interestingly, in contrast to other common γ-chain immunodeficiencies, IL21 or IL21R receptor deficiencies do not seem to be associated with a SCID phenotype. Still, all patients display a clear immunodeficiency with an increased susceptibility to infections and a higher mortality during childhood.

Whereas IL-21R deficiency can be associated with severe liver disease due to cryptosporidium infection, the IL-21-deficient patient presented with inflammatory bowel disease but no cryptosporidium could be detected to date. Regarding the critical role of IL-21 in the modulation of immune homeostasis, early diagnosis and treatment is important and can be lifesaving.

In the case of IL-21R deficiency HSCT before cryptosporidium infection is crucial, as all patients transplanted with positive cryptosporidium suffered from severe transplant-related morbidities or died shortly after transplantation. For patients with IL-21 deficiency recombinant IL-21 represents an elegant alternative option, especially when the patient is not eligible for aHSCT.

4.1. Contribution to the field of primary immunodeficiencies

Primary immunodeficiencies are complex and diverse diseases. Although the incidence and prevalence of severe forms is not high, the identification of underlying genetic causes teaches us about non-redundant molecular mechanism of immune defense and host-pathogen interactions (Ochs & Hitzig, 2012).

Before the advancement of sequencing technologies, PIDs were only recognized in severe forms or as parts of characteristic syndromes (Milner & Holland, 2013). In the past years, the development of high throughput sequencing technologies enabled the discovery of PIDs with specific or subtle phenotypes, which often manifest during the first decade of life or during adolescence. Interestingly, many recently discovered PIDs do not have a corresponding animal model, reflecting the fact that the immune system is shaped in a constant intricate interplay with pathogens and environmental factors.

Here, we employed high-throughput sequencing technologies in combination with homozygosity mapping in a group of patients presenting with immunodeficiency, autoimmunity and / or lymphoproliferation and defective class-switch. We were able to identify two novel PIDs and increase the spectrum of patients with CD27 deficiency, for which before only a single family was published. Moreover, in the case of PKCδ and IL-21 deficiency we went on to discover the underlying mechanisms to explain the clinical picture of the disease.

We believe, that our work of the past four years has not only provided a molecular diagnosis for in total 10 patients, suffering from a previously unknown disease but has also helped to understand basic mechanisms of the immune system. Moreover, with the discovery of the underlying defect, therapy options have come into place, which had not been considered before, especially in the cases of PRKCD and IL-21 deficiency. We hope, that follow-up studies will help to better characterize these diseases and to identify the best possible treatment option for these patients.

5. References

Ahonen P, Myllarniemi S, Sipila I, Perheentupa J (1990) Clinical variation of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) in a series of 68 patients. *N Engl J Med* **322:** 1829-1836

Aiuti A, Cattaneo F, Galimberti S, Benninghoff U, Cassani B, Callegaro L, Scaramuzza S, Andolfi G, Mirolo M, Brigida I, Tabucchi A, Carlucci F, Eibl M, Aker M, Slavin S, Al-Mousa H, Al Ghonaium A, Ferster A, Duppenthaler A, Notarangelo L, Wintergerst U, Buckley RH, Bregni M, Marktel S, Valsecchi MG, Rossi P, Ciceri F, Miniero R, Bordignon C, Roncarolo MG (2009) Gene therapy for immunodeficiency due to adenosine deaminase deficiency. *N Engl J Med* **360:** 447-458

Akashi K, Traver D, Miyamoto T, Weissman IL (2000) A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* **404:** 193-197

Akiba H, Nakano H, Nishinaka S, Shindo M, Kobata T, Atsuta M, Morimoto C, Ware CF, Malinin NL, Wallach D, Yagita H, Okumura K (1998) CD27, a member of the tumor necrosis factor receptor superfamily, activates NF-kappaB and stress-activated protein kinase/c-Jun N-terminal kinase via TRAF2, TRAF5, and NF-kappaB-inducing kinase. *The Journal of biological chemistry* **273:** 13353-13358

Al-Herz W, Bousfiha A, Casanova JL, Chapel H, Conley ME, Cunningham-Rundles C, Etzioni A, Fischer A, Franco JL, Geha RS, Hammarstrom L, Nonoyama S, Notarangelo LD, Ochs HD, Puck JM, Roifman CM, Seger R, Tang ML (2011) Primary immunodeficiency diseases: an update on the classification from the international union of immunological societies expert committee for primary immunodeficiency. *Front Immunol* **2:** 54

Al-Herz W, Bousfiha A, Casanova JL, Chatila T, Conley ME, Cunningham-Rundles C, Etzioni A, Franco JL, Gaspar HB, Holland SM, Klein C, Nonoyama S, Ochs HD, Oksenhendler E, Picard C, Puck JM, Sullivan K, Tang ML (2014) Primary immunodeficiency diseases: an update on the classification from the international union of immunological societies expert committee for primary immunodeficiency. *Front Immunol* **5:** 162

Aldrich RA, Steinberg AG, Campbell DC (1954) Pedigree demonstrating a sex-linked recessive condition characterized by draining ears, eczematoid dermatitis and bloody diarrhea. *Pediatrics* **13:** 133-139

Allen CD, Okada T, Cyster JG (2007) Germinal-center organization and cellular dynamics. *Immunity* **27:** 190-202

Allen RC, Armitage RJ, Conley ME, Rosenblatt H, Jenkins NA, Copeland NG, Bedell MA, Edelhoff S, Disteche CM, Simoneaux DK, et al. (1993) CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. *Science* **259:** 990-993

Anderson G, Moore NC, Owen JJ, Jenkinson EJ (1996) Cellular interactions in thymocyte development. *Annu Rev Immunol* **14:** 73-99

Antonarakis SE, Beckmann JS (2006) Mendelian disorders deserve more attention. *Nat Rev Genet* **7:** 277-282

Asao H, Okuyama C, Kumaki S, Ishii N, Tsuchiya S, Foster D, Sugamura K (2001) Cutting edge: the common gamma-chain is an indispensable subunit of the IL-21 receptor complex. *J Immunol* **167:** 1-5

Balsa A, Tovar Beltrán JV, Cáliz Cáliz R, Mateo Bernardo I, García-Vicuña R, Rodríguez-Gómez M, Belmonte Serrano MA, Marras C, Loza Cortina E, Pérez-Pampin E, Vila V. Patterns of use and dosing of tocilizumab in the treatment of patients with rheumatoid arthritis in routine clinical practice: the ACT-LIFE study. Rheumatol Int. 2015 Mar 15 (EPUB ahead of print)

Bamshad MJ, Ng SB, Bigham AW, Tabor HK, Emond MJ, Nickerson DA, Shendure J (2011) Exome sequencing as a tool for Mendelian disease gene discovery. *Nat Rev Genet* **12:** 745-755

Barrington RA, Pozdnyakova O, Zafari MR, Benjamin CD, Carroll MC (2002) B lymphocyte memory: role of stromal cell complement and FcgammaRIIB receptors. *J Exp Med* **196:** 1189-1199

Basu A, Pal D (2010) Two faces of protein kinase Cdelta: the contrasting roles of PKCdelta in cell survival and cell death. *TheScientificWorldJournal* **10:** 2272-2284

Bauquet AT, Jin H, Paterson AM, Mitsdoerffer M, Ho IC, Sharpe AH, Kuchroo VK (2009) The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nat Immunol* **10:** 167-175

Belot A, Cimaz R (2012) Monogenic forms of systemic lupus erythematosus: new insights into SLE pathogenesis. *Pediatric rheumatology online journal* **10:** 21

Belot A, Kasher PR, Trotter EW, Foray AP, Debaud AL, Rice GI, Szynkiewicz M, Zabot MT, Rouvet I, Bhaskar SS, Daly SB, Dickerson JE, Mayer J, O'Sullivan J, Juillard L, Urquhart JE, Fawdar S, Marusiak AA, Stephenson N, Waszkowycz B, M WB, Biesecker LG, G CMB, Rene C, Eliaou JF, Fabien N, Ranchin B, Cochat P, Gaffney PM, Rozenberg F, Lebon P, Malcus C, Crow YJ, Brognard J, Bonnefoy N (2013) Protein kinase cdelta deficiency causes mendelian systemic lupus erythematosus with B cell-defective apoptosis and hyperproliferation. *Arthritis and rheumatism* **65:** 2161-2171

Bidere N, Su HC, Lenardo MJ (2006) Genetic disorders of programmed cell death in the immune system. *Annu Rev Immunol* **24:** 321-352

Blake RA, Garcia-Paramio P, Parker PJ, Courtneidge SA (1999) Src promotes PKCdelta degradation. *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research* **10:** 231-241

Bortin MM (1970) A compendium of reported human bone marrow transplants. *Transplantation* **9:** 571-587

Bousfiha AA, Jeddane L, Ailal F, Al Herz W, Conley ME, Cunningham-Rundles C, Etzioni A, Fischer A, Franco JL, Geha RS, Hammarstrom L, Nonoyama S, Ochs HD, Roifman CM, Seger R, Tang ML, Puck JM, Chapel H, Notarangelo LD, Casanova JL (2013) A phenotypic approach for IUIS PID classification and diagnosis: guidelines for clinicians at the bedside. *J Clin Immunol* **33:** 1078-1087

Boztug K, Schmidt M, Schwarzer A, Banerjee PP, Diez IA, Dewey RA, Bohm M, Nowrouzi A, Ball CR, Glimm H, Naundorf S, Kuhlcke K, Blasczyk R, Kondratenko I, Marodi L, Orange JS, von Kalle C, Klein C (2010) Stem-cell gene therapy for the Wiskott-Aldrich syndrome. *N Engl J Med* **363:** 1918-1927

Braiman L, Alt A, Kuroki T, Ohba M, Bak A, Tennenbaum T, Sampson SR (1999) Protein kinase Cdelta mediates insulin-induced glucose transport in primary cultures of rat skeletal muscle. *Molecular endocrinology* **13:** 2002-2012

Busslinger M (2004) Transcriptional control of early B cell development. *Annu Rev Immunol* **22:** 55-79

Canale VC, Smith CH (1967) Chronic lymphadenopathy simulating malignant lymphoma. *J Pediatr* **70:** 891-899

Carney JP, Maser RS, Olivares H, Davis EM, Le Beau M, Yates JR, 3rd, Hays L, Morgan WF, Petrini JH (1998) The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: linkage of double-strand break repair to the cellular DNA damage response. *Cell* **93:** 477-486

Carvalho TL, Mota-Santos T, Cumano A, Demengeot J, Vieira P (2001) Arrested B lymphopoiesis and persistence of activated B cells in adult interleukin 7(-/)- mice. *J Exp Med* **194:** 1141-1150

Chen MR (2011) Epstein-barr virus, the immune system, and associated diseases. *Frontiers in microbiology* **2:** 5

Cheng MH, Anderson MS (2012) Monogenic autoimmunity. *Annu Rev Immunol* **30:** 393-427

Chi H (2012) Regulation and function of mTOR signalling in T cell fate decisions. *Nat Rev Immunol* **12:** 325-338

Cho W (2001) Membrane targeting by C1 and C2 domains. *The Journal of biological chemistry* **276:** 32407-32410

Chung BK, Tsai K, Allan LL, Zheng DJ, Nie JC, Biggs CM, Hasan MR, Kozak FK, van den Elzen P, Priatel JJ, Tan R (2013) Innate immune control of EBV-infected B cells by invariant natural killer T cells. *Blood* **122:** 2600-2608

Coffey AJ, Brooksbank RA, Brandau O, Oohashi T, Howell GR, Bye JM, Cahn AP, Durham J, Heath P, Wray P, Pavitt R, Wilkinson J, Leversha M, Huckle E, Shaw-Smith CJ, Dunham A, Rhodes S, Schuster V, Porta G, Yin L, Serafini P, Sylla B, Zollo M, Franco B, Bolino A, Seri M, Lanyi A, Davis JR, Webster D, Harris A, Lenoir G, de St Basile G, Jones A, Behloradsky BH, Achatz H, Murken J, Fassler R, Sumegi J, Romeo G, Vaudin M, Ross MT, Meindl A, Bentley DR (1998) Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. *Nat Genet* **20:** 129-135

Conley ME, Dobbs AK, Quintana AM, Bosompem A, Wang YD, Coustan-Smith E, Smith AM, Perez EE, Murray PJ (2012) Agammaglobulinemia and absent B lineage cells in a patient lacking the p85alpha subunit of PI3K. *J Exp Med* **209:** 463-470

Coquet JM, Kyparissoudis K, Pellicci DG, Besra G, Berzins SP, Smyth MJ, Godfrey DI (2007) IL-21 is produced by NKT cells and modulates NKT cell activation and cytokine production. *J Immunol* **178:** 2827-2834

Cunningham-Rundles C, Ponda PP (2005) Molecular defects in T- and B-cell primary immunodeficiency diseases. *Nat Rev Immunol* **5:** 880-892

Cyster JG, Ansel KM, Reif K, Ekland EH, Hyman PL, Tang HL, Luther SA, Ngo VN (2000) Follicular stromal cells and lymphocyte homing to follicles. *Immunol Rev* **176:** 181-193

Deb DK, Sassano A, Lekmine F, Majchrzak B, Verma A, Kambhampati S, Uddin S, Rahman A, Fish EN, Platanias LC (2003) Activation of protein kinase C delta by IFN-gamma. *J Immunol* **171:** 267-273

Dent AL, Shaffer AL, Yu X, Allman D, Staudt LM (1997) Control of inflammation, cytokine expression, and germinal center formation by BCL-6. *Science* **276:** 589-592

Derbinski J, Gabler J, Brors B, Tierling S, Jonnakuty S, Hergenhahn M, Peltonen L, Walter J, Kyewski B (2005) Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. *J Exp Med* **202:** 33-45

Derbinski J, Schulte A, Kyewski B, Klein L (2001) Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat Immunol* **2:** 1032-1039

Doria A, Zen M, Bettio S, Gatto M, Bassi N, Nalotto L, Ghirardello A, Iaccarino L, Punzi L (2012) Autoinflammation and autoimmunity: bridging the divide. *Autoimmun Rev* **12:** 22-30

Duraisingham SS, Buckland M, Dempster J, Lorenzo L, Grigoriadou S, Longhurst HJ (2014) Primary vs. secondary antibody deficiency: clinical features and infection outcomes of immunoglobulin replacement. *PloS one* **9:** e100324

Durandy A, Kracker S, Fischer A (2013) Primary antibody deficiencies. *Nat Rev Immunol* **13:** 519-533

Durgan J, Michael N, Totty N, Parker PJ (2007) Novel phosphorylation site markers of protein kinase C delta activation. *FEBS letters* **581:** 3377-3381

Engelhardt KR, McGhee S, Winkler S, Sassi A, Woellner C, Lopez-Herrera G, Chen A, Kim HS, Lloret MG, Schulze I, Ehl S, Thiel J, Pfeifer D, Veelken H, Niehues T, Siepermann K, Weinspach S, Reisli I, Keles S, Genel F, Kutukculer N, Camcioglu Y, Somer A, Karakoc-Aydiner E, Barlan I, Gennery A, Metin A, Degerliyurt A, Pietrogrande MC, Yeganeh M, Baz Z, Al-Tamemi S, Klein C, Puck JM, Holland SM, McCabe ER, Grimbacher B, Chatila TA (2009) Large deletions and point mutations involving the dedicator of cytokinesis 8 (DOCK8) in the autosomal-recessive form of hyper-IgE syndrome. *J Allergy Clin Immunol* **124:** 1289-1302 e1284

Ettinger R, Sims GP, Fairhurst AM, Robbins R, da Silva YS, Spolski R, Leonard WJ, Lipsky PE (2005) IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells. *J Immunol* **175:** 7867-7879

Faitelson Y, Grunebaum E (2014) Hemophagocytic lymphohistiocytosis and primary immune deficiency disorders. *Clin Immunol* **155:** 118-125

Fazilleau N, Mark L, McHeyzer-Williams LJ, McHeyzer-Williams MG (2009) Follicular helper T cells: lineage and location. *Immunity* **30:** 324-335

Felgentreff K, Perez-Becker R, Speckmann C, Schwarz K, Kalwak K, Markelj G, Avcin T, Qasim W, Davies EG, Niehues T, Ehl S (2011) Clinical and immunological manifestations of patients with atypical severe combined immunodeficiency. *Clin Immunol* **141:** 73-82

Ferrari S, Giliani S, Insalaco A, Al-Ghonaium A, Soresina AR, Loubser M, Avanzini MA, Marconi M, Badolato R, Ugazio AG, Levy Y, Catalan N, Durandy A, Tbakhi A, Notarangelo LD, Plebani A (2001) Mutations of CD40 gene cause an autosomal recessive form of immunodeficiency with hyper IgM. *Proc Natl Acad Sci U S A* **98:** 12614-12619

Ferrari S, Zuntini R, Lougaris V, Soresina A, Sourkova V, Fiorini M, Martino S, Rossi P, Pietrogrande MC, Martire B, Spadaro G, Cardinale F, Cossu F, Pierani P, Quinti I, Rossi C, Plebani A (2007) Molecular analysis of the pre-BCR complex in a large cohort of patients affected by autosomal-recessive agammaglobulinemia. *Genes and immunity* **8:** 325-333

Filipovich A (2008) Hematopoietic cell transplantation for correction of primary immunodeficiencies. *Bone marrow transplantation* **42 Suppl 1:** S49-S52

Fischer A (2007) Human primary immunodeficiency diseases. *Immunity* **27:** 835-845

Fischer A, Le Deist F, Hacein-Bey-Abina S, Andre-Schmutz I, Basile Gde S, de Villartay JP, Cavazzana-Calvo M (2005) Severe combined immunodeficiency. A model disease for molecular immunology and therapy. *Immunol Rev* **203:** 98-109

Gardner JM, Fletcher AL, Anderson MS, Turley SJ (2009) AIRE in the thymus and beyond. *Curr Opin Immunol* **21:** 582-589

Gaspar HB, Aiuti A, Porta F, Candotti F, Hershfield MS, Notarangelo LD (2009) How I treat ADA deficiency. *Blood* **114:** 3524-3532

Gaspar HB, Cooray S, Gilmour KC, Parsley KL, Adams S, Howe SJ, Al Ghonaium A, Bayford J, Brown L, Davies EG, Kinnon C, Thrasher AJ (2011a) Long-term persistence of a polyclonal T cell repertoire after gene therapy for X-linked severe combined immunodeficiency. *Science translational medicine* **3:** 97ra79

Gaspar HB, Cooray S, Gilmour KC, Parsley KL, Zhang F, Adams S, Bjorkegren E, Bayford J, Brown L, Davies EG, Veys P, Fairbanks L, Bordon V, Petropoulou T, Kinnon C, Thrasher AJ (2011b) Hematopoietic stem cell gene therapy for adenosine deaminase-deficient severe combined immunodeficiency leads to long-term immunological recovery and metabolic correction. *Science translational medicine* **3:** 97ra80

Gatti RA, Meuwissen HJ, Allen HD, Hong R, Good RA (1968) Immunological reconstitution of sex-linked lymphopenic immunological deficiency. *Lancet* **2:** 1366-1369

Geha RS, Notarangelo LD, Casanova JL, Chapel H, Conley ME, Fischer A, Hammarstrom L, Nonoyama S, Ochs HD, Puck JM, Roifman C, Seger R, Wedgwood J (2007) Primary immunodeficiency diseases: an update from the International Union of Immunological Societies Primary Immunodeficiency Diseases Classification Committee. *J Allergy Clin Immunol* **120:** 776-794

Ghosh S, Bienemann K, Boztug K, Borkhardt A (2014) Interleukin-2-Inducible T-Cell Kinase (ITK) Deficiency - Clinical and Molecular Aspects. *J Clin Immunol* **34:** 892-899

Giblett ER, Anderson JE, Cohen F, Pollara B, Meuwissen HJ (1972) Adenosine-deaminase deficiency in two patients with severely impaired cellular immunity. *Lancet* **2:** 1067-1069

Glanzmann E, Riniker P (1950) [Essential lymphocytophthisis; new clinical aspect of infant pathology]. *Ann Paediatr* **175:** 1-32

Gravestein LA, Amsen D, Boes M, Calvo CR, Kruisbeek AM, Borst J (1998) The TNF receptor family member CD27 signals to Jun N-terminal kinase via Traf-2. *European journal of immunology* **28:** 2208-2216

Group of Pediatric I, Zelazko M, Liberatore D, Galicchio M, Perez N, Regairaz L, Oleastro M, Danielian S, Beroznik L, Di Giovani D, Riganti C, Diaz H, Cantisano C, Zorrilla LC, Nievas E (2013) Comment on: advances in primary immunodeficiency diseases in Latin America: epidemiology, research, and perspectives. Ann. N.Y. Acad. Sci. 1250: 62-72 (2012). *Annals of the New York Academy of Sciences* **1306:** 71-72

Guo B, Su TT, Rawlings DJ (2004) Protein kinase C family functions in B-cell activation. *Curr Opin Immunol* **16:** 367-373

Hamming OJ, Kang L, Svensson A, Karlsen JL, Rahbek-Nielsen H, Paludan SR, Hjorth SA, Bondensgaard K, Hartmann R (2012) Crystal structure of interleukin-21 receptor (IL-21R) bound to IL-21 reveals that sugar chain interacting with WSXWS motif is integral part of IL-21R. *The Journal of biological chemistry* **287:** 9454-9460

Hanna MG, Jr. (1964) An Autoradiographic Study of the Germinal Center in Spleen White Pulp during Early Intervals of the Immune Response. *Lab Invest* **13:** 95-104

Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K (1991) Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *J Exp Med* **173:** 1213-1225

Haynes NM, Allen CD, Lesley R, Ansel KM, Killeen N, Cyster JG (2007) Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1high germinal center-associated subpopulation. *J Immunol* **179:** 5099-5108

Hershfield MS (2003) Genotype is an important determinant of phenotype in adenosine deaminase deficiency. *Curr Opin Immunol* **15:** 571-577

Hogquist KA, Baldwin TA, Jameson SC (2005) Central tolerance: learning self-control in the thymus. *Nat Rev Immunol* **5:** 772-782

Hollander G, Gill J, Zuklys S, Iwanami N, Liu C, Takahama Y (2006) Cellular and molecular events during early thymus development. *Immunol Rev* **209:** 28-46

Huck K, Feyen O, Niehues T, Ruschendorf F, Hubner N, Laws HJ, Telieps T, Knapp S, Wacker HH, Meindl A, Jumaa H, Borkhardt A (2009) Girls homozygous for an IL-2-inducible T cell kinase mutation that leads to protein deficiency develop fatal EBV-associated lymphoproliferation. *J Clin Invest* **119:** 1350-1358

Hurley JH, Misra S (2000) Signaling and subcellular targeting by membrane-binding domains. *Annual review of biophysics and biomolecular structure* **29:** 49-79

Imai K, Slupphaug G, Lee WI, Revy P, Nonoyama S, Catalan N, Yel L, Forveille M, Kavli B, Krokan HE, Ochs HD, Fischer A, Durandy A (2003) Human uracil-DNA glycosylase deficiency associated with profoundly impaired immunoglobulin class-switch recombination. *Nat Immunol* **4:** 1023-1028

Jackson CE, Fischer RE, Hsu AP, Anderson SM, Choi Y, Wang J, Dale JK, Fleisher TA, Middelton LA, Sneller MC, Lenardo MJ, Straus SE, Puck JM (1999) Autoimmune lymphoproliferative syndrome with defective Fas: genotype influences penetrance. *Am J Hum Genet* **64:** 1002-1014

Janeway C (2008) Immunobiology.

Jordan MB, Allen CE, Weitzman S, Filipovich AH, McClain KL (2011) How I treat hemophagocytic lymphohistiocytosis. *Blood* **118:** 4041-4052

Kang HJ, Bartholomae CC, Paruzynski A, Arens A, Kim S, Yu SS, Hong Y, Joo CW, Yoon NK, Rhim JW, Kim JG, Von Kalle C, Schmidt M, Kim S, Ahn HS (2011) Retroviral gene therapy for X-linked chronic granulomatous disease: results from phase I/II trial. *Molecular therapy : the journal of the American Society of Gene Therapy* **19:** 2092-2101

Kang L, Bondensgaard K, Li T, Hartmann R, Hjorth SA (2010) Rational design of interleukin-21 antagonist through selective elimination of the gammaC binding epitope. *The Journal of biological chemistry* **285:** 12223-12231

Kikkawa U, Matsuzaki H, Yamamoto T (2002) Protein kinase C delta (PKC delta): activation mechanisms and functions. *Journal of biochemistry* **132:** 831-839

Kildebeck E, Checketts J, Porteus M (2012) Gene therapy for primary immunodeficiencies. *Current opinion in pediatrics* **24:** 731-738

Klein U, Dalla-Favera R (2008) Germinal centres: role in B-cell physiology and malignancy. *Nat Rev Immunol* **8:** 22-33

Klein U, Rajewsky K, Kuppers R (1998) Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. *J Exp Med* **188:** 1679-1689

Kondo M, Weissman IL, Akashi K (1997) Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* **91:** 661-672

Konishi H, Tanaka M, Takemura Y, Matsuzaki H, Ono Y, Kikkawa U, Nishizuka Y (1997) Activation of protein kinase C by tyrosine phosphorylation

in response to H2O2. *Proceedings of the National Academy of Sciences of the United States of America* **94:** 11233-11237

Kopf M, Herren S, Wiles MV, Pepys MB, Kosco-Vilbois MH (1998) Interleukin 6 influences germinal center development and antibody production via a contribution of C3 complement component. *J Exp Med* **188:** 1895-1906

Kotlarz D, Zietara N, Milner JD, Klein C (2014) Human IL-21 and IL-21R deficiencies: two novel entities of primary immunodeficiency. *Current opinion in pediatrics* **26:** 704-712

Kotlarz D, Zietara N, Uzel G, Weidemann T, Braun CJ, Diestelhorst J, Krawitz PM, Robinson PN, Hecht J, Puchalka J, Gertz EM, Schaffer AA, Lawrence MG, Kardava L, Pfeifer D, Baumann U, Pfister ED, Hanson EP, Schambach A, Jacobs R, Kreipe H, Moir S, Milner JD, Schwille P, Mundlos S, Klein C (2013) Loss-of-function mutations in the IL-21 receptor gene cause a primary immunodeficiency syndrome. *J Exp Med* **210:** 433-443

Kronfeld I, Kazimirsky G, Lorenzo PS, Garfield SH, Blumberg PM, Brodie C (2000) Phosphorylation of protein kinase Cdelta on distinct tyrosine residues regulates specific cellular functions. *The Journal of biological chemistry* **275:** 35491-35498

Kuehn HS, Niemela JE, Rangel-Santos A, Zhang M, Pittaluga S, Stoddard JL, Hussey AA, Evbuomwan MO, Priel DA, Kuhns DB, Park CL, Fleisher TA, Uzel G, Oliveira JB (2013) Loss-of-function of the protein kinase C delta (PKCdelta) causes a B-cell lymphoproliferative syndrome in humans. *Blood* **121:** 3117-3125

Kuijpers TW, Bende RJ, Baars PA, Grummels A, Derks IA, Dolman KM, Beaumont T, Tedder TF, van Noesel CJ, Eldering E, van Lier RA (2010) CD20 deficiency in humans results in impaired T cell-independent antibody responses. *J Clin Invest* **120:** 214-222

Lederman HM (2000) The Clinical Presentation of Primary Immunodeficiency Diseases. *Clinical Focus on* **2**

Lens SM, Tesselaar K, van Oers MH, van Lier RA (1998) Control of lymphocyte function through CD27-CD70 interactions. *Seminars in immunology* **10:** 491-499

Leonard MF (1946) Chronic idiopathic hypoparathyroidism with superimposed Addison's disease in a child. *J Clin Endocrinol Metab* **6:** 493-506

Leonard WJ, Spolski R (2005) Interleukin-21: a modulator of lymphoid proliferation, apoptosis and differentiation. *Nat Rev Immunol* **5:** 688-698

Li FY, Chaigne-Delalande B, Kanellopoulou C, Davis JC, Matthews HF, Douek DC, Cohen JI, Uzel G, Su HC, Lenardo MJ (2011) Second messenger

role for Mg2+ revealed by human T-cell immunodeficiency. *Nature* **475:** 471-476

Li Y, Yee C (2008) IL-21 mediated Foxp3 suppression leads to enhanced generation of antigen-specific CD8+ cytotoxic T lymphocytes. *Blood* **111:** 229-235

Li YS, Wasserman R, Hayakawa K, Hardy RR (1996) Identification of the earliest B lineage stage in mouse bone marrow. *Immunity* **5:** 527-535

Linterman MA, Beaton L, Yu D, Ramiscal RR, Srivastava M, Hogan JJ, Verma NK, Smyth MJ, Rigby RJ, Vinuesa CG (2010) IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. *J Exp Med* **207:** 353-363

Liston A, Enders A, Siggs OM (2008) Unravelling the association of partial T-cell immunodeficiency and immune dysregulation. *Nat Rev Immunol* **8:** 545-558

Loenen WA, De Vries E, Gravestein LA, Hintzen RQ, Van Lier RA, Borst J (1992) The CD27 membrane receptor, a lymphocyte-specific member of the nerve growth factor receptor family, gives rise to a soluble form by protein processing that does not involve receptor endocytosis. *European journal of immunology* **22:** 447-455

MacLennan IC (1994) Germinal centers. *Annu Rev Immunol* **12:** 117-139

Mamanova L, Coffey AJ, Scott CE, Kozarewa I, Turner EH, Kumar A, Howard E, Shendure J, Turner DJ (2010) Target-enrichment strategies for next-generation sequencing. *Nat Methods* **7:** 111-118

Mandel TE, Phipps RP, Abbot AP, Tew JG (1981) Long-term antigen retention by dendritic cells in the popliteal lymph node of immunized mice. *Immunology* **43:** 353-362

Markert ML (1991) Purine nucleoside phosphorylase deficiency. *Immunodefic Rev* **3:** 45-81

McCusker C, Warrington R (2011) Primary immunodeficiency. *Allergy Asthma Clin Immunol* **7 Suppl 1:** S11

McKusick VA (2007) Mendelian Inheritance in Man and its online version, OMIM. *Am J Hum Genet* **80:** 588-604

Meffre E, Casellas R, Nussenzweig MC (2000) Antibody regulation of B cell development. *Nat Immunol* **1:** 379-385

Mendel G (1865) Versuche über Plflanzenhybriden. *Verhandlungen des naturforschenden Vereines in Brünn* **Bd. IV für das Jahr 1865:** 3–47

Miller JP, Izon D, DeMuth W, Gerstein R, Bhandoola A, Allman D (2002) The earliest step in B lineage differentiation from common lymphoid progenitors is critically dependent upon interleukin 7. *J Exp Med* **196:** 705-711

Milner JD, Holland SM (2013) The cup runneth over: lessons from the ever-expanding pool of primary immunodeficiency diseases. *Nat Rev Immunol* **13:** 635-648

Minegishi Y, Coustan-Smith E, Rapalus L, Ersoy F, Campana D, Conley ME (1999a) Mutations in Igalpha (CD79a) result in a complete block in B-cell development. *J Clin Invest* **104:** 1115-1121

Minegishi Y, Coustan-Smith E, Wang YH, Cooper MD, Campana D, Conley ME (1998) Mutations in the human lambda5/14.1 gene result in B cell deficiency and agammaglobulinemia. *J Exp Med* **187:** 71-77

Minegishi Y, Rohrer J, Coustan-Smith E, Lederman HM, Pappu R, Campana D, Chan AC, Conley ME (1999b) An essential role for BLNK in human B cell development. *Science* **286:** 1954-1957

Miyamoto A, Nakayama K, Imaki H, Hirose S, Jiang Y, Abe M, Tsukiyama T, Nagahama H, Ohno S, Hatakeyama S, Nakayama KI (2002) Increased proliferation of B cells and auto-immunity in mice lacking protein kinase Cdelta. *Nature* **416:** 865-869

Moraes-Vasconcelos D, Costa-Carvalho BT, Torgerson TR, Ochs HD (2008) Primary immune deficiency disorders presenting as autoimmune diseases: IPEX and APECED. *J Clin Immunol* **28 Suppl 1:** S11-19

Moshous D, Callebaut I, de Chasseval R, Corneo B, Cavazzana-Calvo M, Le Deist F, Tezcan I, Sanal O, Bertrand Y, Philippe N, Fischer A, de Villartay JP (2001) Artemis, a novel DNA double-strand break repair/V(D)J recombination protein, is mutated in human severe combined immune deficiency. *Cell* **105:** 177-186

Muller AM, Medvinsky A, Strouboulis J, Grosveld F, Dzierzak E (1994) Development of hematopoietic stem cell activity in the mouse embryo. *Immunity* **1:** 291-301

Myers LA, Patel DD, Puck JM, Buckley RH (2002) Hematopoietic stem cell transplantation for severe combined immunodeficiency in the neonatal period leads to superior thymic output and improved survival. *Blood* **99:** 872-878

Nehme NT, Pachlopnik Schmid J, Debeurme F, Andre-Schmutz I, Lim A, Nitschke P, Rieux-Laucat F, Lutz P, Picard C, Mahlaoui N, Fischer A, de Saint Basile G (2012) MST1 mutations in autosomal recessive primary immunodeficiency characterized by defective naive T-cell survival. *Blood* **119:** 3458-3468

Nieuwenhuis P, Opstelten D (1984) Functional anatomy of germinal centers. *Am J Anat* **170:** 421-435

Nishikawa Y, Hikida M, Magari M, Kanayama N, Mori M, Kitamura H, Kurosaki T, Ohmori H (2006) Establishment of lymphotoxin beta receptor signaling-dependent cell lines with follicular dendritic cell phenotypes from mouse lymph nodes. *J Immunol* **177:** 5204-5214

Nolte MA, van Olffen RW, van Gisbergen KP, van Lier RA (2009) Timing and tuning of CD27-CD70 interactions: the impact of signal strength in setting the balance between adaptive responses and immunopathology. *Immunol Rev* **229:** 216-231

Notarangelo LD (2009) Primary immunodeficiencies (PIDs) presenting with cytopenias. *Hematology Am Soc Hematol Educ Program***:** 139-143

Notarangelo LD (2010) Primary immunodeficiencies. *J Allergy Clin Immunol* **125:** S182-194

O'Driscoll M, Cerosaletti KM, Girard PM, Dai Y, Stumm M, Kysela B, Hirsch B, Gennery A, Palmer SE, Seidel J, Gatti RA, Varon R, Oettinger MA, Neitzel H, Jeggo PA, Concannon P (2001) DNA ligase IV mutations identified in patients exhibiting developmental delay and immunodeficiency. *Mol Cell* **8:** 1175-1185

Ochs HD, Hitzig WH (2012) History of primary immunodeficiency diseases. *Current opinion in allergy and clinical immunology* **12:** 577-587

Oliveira JB, Bleesing JJ, Dianzani U, Fleisher TA, Jaffe ES, Lenardo MJ, Rieux-Laucat F, Siegel RM, Su HC, Teachey DT, Rao VK (2010) Revised diagnostic criteria and classification for the autoimmune lymphoproliferative syndrome (ALPS): report from the 2009 NIH International Workshop. *Blood* **116:** e35-40

Ombrello MJ, Remmers EF, Sun G, Freeman AF, Datta S, Torabi-Parizi P, Subramanian N, Bunney TD, Baxendale RW, Martins MS, Romberg N, Komarow H, Aksentijevich I, Kim HS, Ho J, Cruse G, Jung MY, Gilfillan AM, Metcalfe DD, Nelson C, O'Brien M, Wisch L, Stone K, Douek DC, Gandhi C, Wanderer AA, Lee H, Nelson SF, Shianna KV, Cirulli ET, Goldstein DB, Long EO, Moir S, Meffre E, Holland SM, Kastner DL, Katan M, Hoffman HM, Milner JD (2012) Cold urticaria, immunodeficiency, and autoimmunity related to PLCG2 deletions. *N Engl J Med* **366:** 330-338

Ozaki K, Kikly K, Michalovich D, Young PR, Leonard WJ (2000) Cloning of a type I cytokine receptor most related to the IL-2 receptor beta chain. *Proc Natl Acad Sci U S A* **97:** 11439-11444

Ozaki K, Spolski R, Ettinger R, Kim HP, Wang G, Qi CF, Hwu P, Shaffer DJ, Akilesh S, Roopenian DC, Morse HC, 3rd, Lipsky PE, Leonard WJ (2004) Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *J Immunol* **173:** 5361-5371

Palmer E (2003) Negative selection--clearing out the bad apples from the T-cell repertoire. *Nat Rev Immunol* **3:** 383-391

Pappa H, Murray-Rust J, Dekker LV, Parker PJ, McDonald NQ (1998) Crystal structure of the C2 domain from protein kinase C-delta. *Structure* **6:** 885-894

Parrish-Novak J, Dillon SR, Nelson A, Hammond A, Sprecher C, Gross JA, Johnston J, Madden K, Xu W, West J, Schrader S, Burkhead S, Heipel M, Brandt C, Kuijper JL, Kramer J, Conklin D, Presnell SR, Berry J, Shiota F, Bort S, Hambly K, Mudri S, Clegg C, Moore M, Grant FJ, Lofton-Day C, Gilbert T, Rayond F, Ching A, Yao L, Smith D, Webster P, Whitmore T, Maurer M, Kaushansky K, Holly RD, Foster D (2000) Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature* **408:** 57-63

Pieper K, Grimbacher B, Eibel H (2013) B-cell biology and development. *J Allergy Clin Immunol* **131:** 959-971

Porteus MH, Baltimore D (2003) Chimeric nucleases stimulate gene targeting in human cells. *Science* **300:** 763

Powell BR, Buist NR, Stenzel P (1982) An X-linked syndrome of diarrhea, polyendocrinopathy, and fatal infection in infancy. *J Pediatr* **100:** 731-737

Prasad KV, Ao Z, Yoon Y, Wu MX, Rizk M, Jacquot S, Schlossman SF (1997) CD27, a member of the tumor necrosis factor receptor family, induces apoptosis and binds to Siva, a proapoptotic protein. *Proc Natl Acad Sci U S A* **94:** 6346-6351

Puel A, Cypowyj S, Bustamante J, Wright JF, Liu L, Lim HK, Migaud M, Israel L, Chrabieh M, Audry M, Gumbleton M, Toulon A, Bodemer C, El-Baghdadi J, Whitters M, Paradis T, Brooks J, Collins M, Wolfman NM, Al-Muhsen S, Galicchio M, Abel L, Picard C, Casanova JL (2011) Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. *Science* **332:** 65-68

Puel A, Doffinger R, Natividad A, Chrabieh M, Barcenas-Morales G, Picard C, Cobat A, Ouachee-Chardin M, Toulon A, Bustamante J, Al-Muhsen S, Al-Owain M, Arkwright PD, Costigan C, McConnell V, Cant AJ, Abinun M, Polak M, Bougneres PF, Kumararatne D, Marodi L, Nahum A, Roifman C, Blanche S, Fischer A, Bodemer C, Abel L, Lilic D, Casanova JL (2010) Autoantibodies against IL-17A, IL-17F, and IL-22 in patients with chronic mucocutaneous candidiasis and autoimmune polyendocrine syndrome type I. *J Exp Med* **207:** 291-297

Puga I, Cols M, Barra CM, He B, Cassis L, Gentile M, Comerma L, Chorny A, Shan M, Xu W, Magri G, Knowles DM, Tam W, Chiu A, Bussel JB, Serrano S, Lorente JA, Bellosillo B, Lloreta J, Juanpere N, Alameda F, Baro T, de Heredia CD, Toran N, Catala A, Torrebadell M, Fortuny C, Cusi V, Carreras

C, Diaz GA, Blander JM, Farber CM, Silvestri G, Cunningham-Rundles C, Calvillo M, Dufour C, Notarangelo LD, Lougaris V, Plebani A, Casanova JL, Ganal SC, Diefenbach A, Arostegui JI, Juan M, Yague J, Mahlaoui N, Donadieu J, Chen K, Cerutti A (2012) B cell-helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen. *Nat Immunol* **13:** 170-180

Radtke F, Wilson A, Stark G, Bauer M, van Meerwijk J, MacDonald HR, Aguet M (1999) Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* **10:** 547-558

Ramakrishnan P, Wang W, Wallach D (2004) Receptor-specific signaling for both the alternative and the canonical NF-kappaB activation pathways by NF-kappaB-inducing kinase. *Immunity* **21:** 477-489

Randall KL, Lambe T, Johnson AL, Treanor B, Kucharska E, Domaschenz H, Whittle B, Tze LE, Enders A, Crockford TL, Bouriez-Jones T, Alston D, Cyster JG, Lenardo MJ, Mackay F, Deenick EK, Tangye SG, Chan TD, Camidge T, Brink R, Vinuesa CG, Batista FD, Cornall RJ, Goodnow CC (2009) Dock8 mutations cripple B cell immunological synapses, germinal centers and long-lived antibody production. *Nat Immunol* **10:** 1283-1291

Res P, Martinez-Caceres E, Cristina Jaleco A, Staal F, Noteboom E, Weijer K, Spits H (1996) CD34+CD38dim cells in the human thymus can differentiate into T, natural killer, and dendritic cells but are distinct from pluripotent stem cells. *Blood* **87:** 5196-5206

Revy P, Muto T, Levy Y, Geissmann F, Plebani A, Sanal O, Catalan N, Forveille M, Dufourcq-Labelouse R, Gennery A, Tezcan I, Ersoy F, Kayserili H, Ugazio AG, Brousse N, Muramatsu M, Notarangelo LD, Kinoshita K, Honjo T, Fischer A, Durandy A (2000) Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell* **102:** 565-575

Rich KC, Arnold WJ, Palella T, Fox IH (1979) Cellular immune deficiency with autoimmune hemolytic anemia in purine nucleoside phosphorylase deficiency. *Am J Med* **67:** 172-176

Rigaud S, Fondaneche MC, Lambert N, Pasquier B, Mateo V, Soulas P, Galicier L, Le Deist F, Rieux-Laucat F, Revy P, Fischer A, de Saint Basile G, Latour S (2006) XIAP deficiency in humans causes an X-linked lymphoproliferative syndrome. *Nature* **444:** 110-114

Rochman Y, Spolski R, Leonard WJ (2009) New insights into the regulation of T cells by gamma(c) family cytokines. *Nat Rev Immunol* **9:** 480-490

Rothenberg EV (2000) Stepwise specification of lymphocyte developmental lineages. *Current opinion in genetics & development* **10:** 370-379

Rothenberg EV (2002) T-lineage specification and commitment: a gene regulation perspective. *Seminars in immunology* **14:** 431-440

Rothenberg EV, Telfer JC, Anderson MK (1999) Transcriptional regulation of lymphocyte lineage commitment. *BioEssays : news and reviews in molecular, cellular and developmental biology* **21:** 726-742

Salzer E, Daschkey S, Choo S, Gombert M, Santos-Valente E, Ginzel S, Schwendinger M, Haas OA, Fritsch G, Pickl WF, Forster-Waldl E, Borkhardt A, Boztug K, Bienemann K, Seidel MG (2013a) Combined immunodeficiency with life-threatening EBV-associated lymphoproliferative disorder in patients lacking functional CD27. *Haematologica* **98:** 473-478

Salzer E, Kansu A, Sic H, Majek P, Ikinciogullari A, Dogu FE, Prengemann NK, Santos-Valente E, Pickl WF, Bilic I, Ban SA, Kuloglu Z, Demir AM, Ensari A, Colinge J, Rizzi M, Eibel H, Boztug K (2014) Early-onset inflammatory bowel disease and common variable immunodeficiency-like disease caused by IL-21 deficiency. *J Allergy Clin Immunol* **133:** 1651-1659 e1612

Salzer E, Santos-Valente E, Klaver S, Ban SA, Emminger W, Prengemann NK, Garncarz W, Mullauer L, Kain R, Boztug H, Heitger A, Arbeiter K, Eitelberger F, Seidel MG, Holter W, Pollak A, Pickl WF, Forster-Waldl E, Boztug K (2013b) B-cell deficiency and severe autoimmunity caused by deficiency of protein kinase C delta. *Blood* **121:** 3112-3116

Salzer U, Bacchelli C, Buckridge S, Pan-Hammarstrom Q, Jennings S, Lougaris V, Bergbreiter A, Hagena T, Birmelin J, Plebani A, Webster AD, Peter HH, Suez D, Chapel H, McLean-Tooke A, Spickett GP, Anover-Sombke S, Ochs HD, Urschel S, Belohradsky BH, Ugrinovic S, Kumararatne DS, Lawrence TC, Holm AM, Franco JL, Schulze I, Schneider P, Gertz EM, Schaffer AA, Hammarstrom L, Thrasher AJ, Gaspar HB, Grimbacher B (2009) Relevance of biallelic versus monoallelic TNFRSF13B mutations in distinguishing disease-causing from risk-increasing TNFRSF13B variants in antibody deficiency syndromes. *Blood* **113:** 1967-1976

Savitsky K, Bar-Shira A, Gilad S, Rotman G, Ziv Y, Vanagaite L, Tagle DA, Smith S, Uziel T, Sfez S, Ashkenazi M, Pecker I, Frydman M, Harnik R, Patanjali SR, Simmons A, Clines GA, Sartiel A, Gatti RA, Chessa L, Sanal O, Lavin MF, Jaspers NG, Taylor AM, Arlett CF, Miki T, Weissman SM, Lovett M, Collins FS, Shiloh Y (1995) A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science* **268:** 1749-1753

Schwarz K, Gauss GH, Ludwig L, Pannicke U, Li Z, Lindner D, Friedrich W, Seger RA, Hansen-Hagge TE, Desiderio S, Lieber MR, Bartram CR (1996) RAG mutations in human B cell-negative SCID. *Science* **274:** 97-99

Serwas NK, Cagdas D, Ban SA, Bienemann K, Salzer E, Tezcan I, Borkhardt A, Sanal O, Boztug K (2014) Identification of ITK deficiency as a novel genetic cause of idiopathic CD4+ T-cell lymphopenia. *Blood* **124:** 655-657

Shlomchik MJ, Weisel F (2012) Germinal center selection and the development of memory B and plasma cells. *Immunol Rev* **247:** 52-63

Silver JS, Hunter CA (2008) With a little help from their friends: interleukin-21, T cells, and B cells. *Immunity* **29:** 7-9

Sitnicka E, Bryder D, Theilgaard-Monch K, Buza-Vidas N, Adolfsson J, Jacobsen SE (2002) Key role of flt3 ligand in regulation of the common lymphoid progenitor but not in maintenance of the hematopoietic stem cell pool. *Immunity* **17:** 463-472

Sneller MC, Straus SE, Jaffe ES, Jaffe JS, Fleisher TA, Stetler-Stevenson M, Strober W (1992) A novel lymphoproliferative/autoimmune syndrome resembling murine lpr/gld disease. *J Clin Invest* **90:** 334-341

Spinicelli S, Nocentini G, Ronchetti S, Krausz LT, Bianchini R, Riccardi C (2002) GITR interacts with the pro-apoptotic protein Siva and induces apoptosis. *Cell death and differentiation* **9:** 1382-1384

Spolski R, Leonard WJ (2014) Interleukin-21: a double-edged sword with therapeutic potential. *Nature reviews Drug discovery* **13:** 379-395

Steinberg SF (2004) Distinctive activation mechanisms and functions for protein kinase Cdelta. *The Biochemical journal* **384:** 449-459

Stewart GS, Maser RS, Stankovic T, Bressan DA, Kaplan MI, Jaspers NG, Raams A, Byrd PJ, Petrini JH, Taylor AM (1999) The DNA double-strand break repair gene hMRE11 is mutated in individuals with an ataxia-telangiectasia-like disorder. *Cell* **99:** 577-587

Straus SE, Jaffe ES, Puck JM, Dale JK, Elkon KB, Rosen-Wolff A, Peters AM, Sneller MC, Hallahan CW, Wang J, Fischer RE, Jackson CM, Lin AY, Baumler C, Siegert E, Marx A, Vaishnaw AK, Grodzicky T, Fleisher TA, Lenardo MJ (2001) The development of lymphomas in families with autoimmune lymphoproliferative syndrome with germline Fas mutations and defective lymphocyte apoptosis. *Blood* **98:** 194-200

Sun X, Wu F, Datta R, Kharbanda S, Kufe D (2000) Interaction between protein kinase C delta and the c-Abl tyrosine kinase in the cellular response to oxidative stress. *The Journal of biological chemistry* **275:** 7470-7473

Szakal AK, Gieringer RL, Kosco MH, Tew JG (1985) Isolated follicular dendritic cells: cytochemical antigen localization, Nomarski, SEM, and TEM morphology. *J Immunol* **134:** 1349-1359

Szallasi Z, Denning MF, Chang EY, Rivera J, Yuspa SH, Lehel C, Olah Z, Anderson WB, Blumberg PM (1995) Development of a rapid approach to identification of tyrosine phosphorylation sites: application to PKC delta phosphorylated upon activation of the high affinity receptor for IgE in rat

basophilic leukemia cells. *Biochemical and biophysical research communications* **214:** 888-894

Tang Q, Bluestone JA (2008) The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol* **9:** 239-244

Tangye SG, Ma CS, Brink R, Deenick EK (2013) The good, the bad and the ugly - TFH cells in human health and disease. *Nat Rev Immunol* **13:** 412-426

Tarlinton D, Radbruch A, Hiepe F, Dorner T (2008) Plasma cell differentiation and survival. *Curr Opin Immunol* **20:** 162-169

Thiel J, Kimmig L, Salzer U, Grudzien M, Lebrecht D, Hagena T, Draeger R, Volxen N, Bergbreiter A, Jennings S, Gutenberger S, Aichem A, Illges H, Hannan JP, Kienzler AK, Rizzi M, Eibel H, Peter HH, Warnatz K, Grimbacher B, Rump JA, Schlesier M (2012) Genetic CD21 deficiency is associated with hypogammaglobulinemia. *J Allergy Clin Immunol* **129:** 801-810 e806

Uddin S, Sassano A, Deb DK, Verma A, Majchrzak B, Rahman A, Malik AB, Fish EN, Platanias LC (2002) Protein kinase C-delta (PKC-delta) is activated by type I interferons and mediates phosphorylation of Stat1 on serine 727. *The Journal of biological chemistry* **277:** 14408-14416

Vallieres F, Girard D (2013) IL-21 enhances phagocytosis in mononuclear phagocyte cells: identification of spleen tyrosine kinase as a novel molecular target of IL-21. *J Immunol* **190:** 2904-2912

van der Burg M, Gennery AR (2011) Educational paper. The expanding clinical and immunological spectrum of severe combined immunodeficiency. *Eur J Pediatr* **170:** 561-571

van Montfrans JM, Hoepelman AI, Otto S, van Gijn M, van de Corput L, de Weger RA, Monaco-Shawver L, Banerjee PP, Sanders EA, Jol-van der Zijde CM, Betts MR, Orange JS, Bloem AC, Tesselaar K (2012) CD27 deficiency is associated with combined immunodeficiency and persistent symptomatic EBV viremia. *J Allergy Clin Immunol* **129:** 787-793 e786

van Zelm MC, Reisli I, van der Burg M, Castano D, van Noesel CJ, van Tol MJ, Woellner C, Grimbacher B, Patino PJ, van Dongen JJ, Franco JL (2006) An antibody-deficiency syndrome due to mutations in the CD19 gene. *N Engl J Med* **354:** 1901-1912

van Zelm MC, Smet J, Adams B, Mascart F, Schandene L, Janssen F, Ferster A, Kuo CC, Levy S, van Dongen JJ, van der Burg M (2010) CD81 gene defect in humans disrupts CD19 complex formation and leads to antibody deficiency. *J Clin Invest* **120:** 1265-1274

Varon R, Vissinga C, Platzer M, Cerosaletti KM, Chrzanowska KH, Saar K, Beckmann G, Seemanova E, Cooper PR, Nowak NJ, Stumm M, Weemaes CM, Gatti RA, Wilson RK, Digweed M, Rosenthal A, Sperling K, Concannon

P, Reis A (1998) Nibrin, a novel DNA double-strand break repair protein, is mutated in Nijmegen breakage syndrome. *Cell* **93:** 467-476

Vetrie D, Vorechovsky I, Sideras P, Holland J, Davies A, Flinter F, Hammarstrom L, Kinnon C, Levinsky R, Bobrow M, et al. (1993) The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. *Nature* **361:** 226-233

Victora GD, Nussenzweig MC (2012) Germinal centers. *Annu Rev Immunol* **30:** 429-457

Victora GD, Schwickert TA, Fooksman DR, Kamphorst AO, Meyer-Hermann M, Dustin ML, Nussenzweig MC (2010) Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. *Cell* **143:** 592-605

Villa A, Santagata S, Bozzi F, Giliani S, Frattini A, Imberti L, Gatta LB, Ochs HD, Schwarz K, Notarangelo LD, Vezzoni P, Spanopoulou E (1998) Partial V(D)J recombination activity leads to Omenn syndrome. *Cell* **93:** 885-896

Vinuesa CG, Sanz I, Cook MC (2009) Dysregulation of germinal centres in autoimmune disease. *Nat Rev Immunol* **9:** 845-857

Wahren-Herlenius M, Dorner T (2013) Immunopathogenic mechanisms of systemic autoimmune disease. *Lancet* **382:** 819-831

Wan CK, Oh J, Li P, West EE, Wong EA, Andraski AB, Spolski R, Yu ZX, He J, Kelsall BL, Leonard WJ (2013) The cytokines IL-21 and GM-CSF have opposing regulatory roles in the apoptosis of conventional dendritic cells. *Immunity* **38:** 514-527

Waskow C, Paul S, Haller C, Gassmann M, Rodewald HR (2002) Viable c-Kit(W/W) mutants reveal pivotal role for c-kit in the maintenance of lymphopoiesis. *Immunity* **17:** 277-288

William J, Euler C, Christensen S, Shlomchik MJ (2002) Evolution of autoantibody responses via somatic hypermutation outside of germinal centers. *Science* **297:** 2066-2070

Wiskott A (1937) Familiärer, angeborener Morbus Werlhofii? *Monatsschrift für Kinderheilkunde***:** 212-216

Wu Y, El Shikh ME, El Sayed RM, Best AM, Szakal AK, Tew JG (2009) IL-6 produced by immune complex-activated follicular dendritic cells promotes germinal center reactions, IgG responses and somatic hypermutation. *Int Immunol* **21:** 745-756

Wu-Zhang AX, Murphy AN, Bachman M, Newton AC (2012) Isozyme-specific interaction of protein kinase Cdelta with mitochondria dissected using live cell fluorescence imaging. *J Biol Chem* **287:** 37891-37906

Xing Y, Hogquist KA (2012) T-cell tolerance: central and peripheral. *Cold Spring Harbor perspectives in biology* **4**

Ye BH, Cattoretti G, Shen Q, Zhang J, Hawe N, de Waard R, Leung C, Nouri-Shirazi M, Orazi A, Chaganti RS, Rothman P, Stall AM, Pandolfi PP, Dalla-Favera R (1997) The BCL-6 proto-oncogene controls germinal-centre formation and Th2-type inflammation. *Nat Genet* **16:** 161-170

Yel L, Minegishi Y, Coustan-Smith E, Buckley RH, Trubel H, Pachman LM, Kitchingman GR, Campana D, Rohrer J, Conley ME (1996) Mutations in the mu heavy-chain gene in patients with agammaglobulinemia. *N Engl J Med* **335:** 1486-1493

Young LS, Rickinson AB (2004) Epstein-Barr virus: 40 years on. *Nat Rev Cancer* **4:** 757-768

Zhang Q, Davis JC, Lamborn IT, Freeman AF, Jing H, Favreau AJ, Matthews HF, Davis J, Turner ML, Uzel G, Holland SM, Su HC (2009) Combined immunodeficiency associated with DOCK8 mutations. *N Engl J Med* **361:** 2046-2055

Zhao M, Xia L, Chen GQ (2012) Protein kinase cdelta in apoptosis: a brief overview. *Archivum immunologiae et therapiae experimentalis* **60:** 361-372

Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY (2010) Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature* **463:** 808-812

Zhou Q, Lee GS, Brady J, Datta S, Katan M, Sheikh A, Martins MS, Bunney TD, Santich BH, Moir S, Kuhns DB, Long Priel DA, Ombrello A, Stone D, Ombrello MJ, Khan J, Milner JD, Kastner DL, Aksentijevich I (2012) A hypermorphic missense mutation in PLCG2, encoding phospholipase Cgamma2, causes a dominantly inherited autoinflammatory disease with immunodeficiency. *Am J Hum Genet* **91:** 713-720

Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martinez-Llordella M, Ashby M, Nakayama M, Rosenthal W, Bluestone JA (2009) Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol* **10:** 1000-1007

6. Curriculum vitae

ELISABETH SALZER

E-mail: esalzer@cemm.oeaw.ac.at

SCHOOL AND UNIVERSITY EDUCATION

Postgraduate education

PhD Program N094 at the Medical University of Vienna 10/2010- present
Ce-M-M- PhD Program

Medical school

Medical University of Vienna 10/2004- 07/2010
Programme of Medicine (with distinction)
Completed 02.07. 2010 (MD)

High School

Austrian (Vienna) high school qualifying for university admission 06/2004 (with distinction)

INTERNATIONAL EXPERIENCE

Melbourne, Australia –Clinical elective
Pediatric emergency medicine at Monash Medical Centre 07/2009

Lausanne, Switzerland- Erasmus scholarship
Completion of all clinical rotations of the 5th year at CHUV,
University of Lausanne 2008/2009

Boston, USA- Observership
Pediatric cardiology, Children's Hospital Boston 07/2008

Berlin, Germany- Clinical elective
Pediatric hematology/oncology, Charité, Berlin 07/2007

RESEARCH

Research Center for Molecular Medicine (Ce-M-M-) 10/2010- present

Research group: Kaan Boztug

Anna Spiegel Center for Translational Research 10/2007- 07/2010

Pediatric Nephrology Research group

Research group: Christoph Aufricht

Diploma Thesis

Inflammation and Cytoprotection during in vitro Peritoneal Dialysis completed 05/10

Completed with distinction

PUBLICATIONS

Erman B, Bilic I, Hirschmugl T, **Salzer E**, Çağdaş DA, Esenboga S, Akcoren Z, Sanal O, Tezcan I, Boztug K. **Combined immunodeficiency with CD4 lymphopenia and sclerosing cholangitis caused by a novel loss-of-function mutation affecting IL21R.**
Haematologica. 2015 Mar 13

Woutsas S, Aytekin C, **Salzer E**, Conde CD, Apaydin S, Pichler H, Memaran-Dadgar N, Hosnut FO, Förster-Waldl E, Matthes S, Huber WD, Lion T, Holter W, Bilic I, Boztug K. **Hypomorphic mutation in TTC7A causes combined immunodeficiency with mild structural intestinal defects.**
Blood. 2015 Mar 5

Willmann KL, Klaver S, Doğu F, Santos-Valente E, Garncarz W, Bilic I, Mace E, **Salzer E**, Conde CD, Sic H, Májek P, Banerjee PP, Vladimer GI, Haskoloğlu S, Bolkent MG, Küpesiz A, Condino-Neto A, Colinge J, Superti-Furga G, Pickl WF, van Zelm MC, Eibel H, Orange JS, Ikincioğulları A, Boztuğ K. **Biallelic loss-of-function mutation in NIK causes a primary immunodeficiency with multifaceted aberrant lymphoid immunity.**
Nat Commun. 2014 Nov

Ban SA, **Salzer E**, Eibl MM, Linder A, Geier CB, Santos-Valente E, Garncarz W, Lion T, Ott R, Seelbach C, Boztug K, Wolf HM. **Combined immunodeficiency evolving into predominant CD4+ lymphopenia caused by somatic chimerism in JAK3.**
J Clin Immunol. 2014 Nov

Boztug K, Järvinen P, **Salzer E**, Racek T, Mönch S, Garncarz W, Gertz ME, Schäffer AE, Antonopoulos A, Haslam SM, Ziesenitz L, Puchałka J, Diestelhorst D, Appaswamy G, Lescoeur B, Giambruno R, Bigenzahn JW, Elling U, Pfeifer D, Domínguez Conde C, Albert M, Welte K, Brandes G, Sherkat R, van der Werff ten Bosch J, Rezaei N, Etzioni A, Bellanné-Chantelot C, Superti-Furga G, Penninger JM, Bennett KL, von Blume J, Dell A, Donadieu J, Klein C **JAGN1 deficiency causes aberrant myeloid cell homeostasis and congenital neutropenia**
Nat Genet. 2014 Sep

Salzer E, Kansu A, Sic H, Májek P, Ikincioğullari A, Dogu FE, Prengemann NK, Santos-Valente E, Pickl WF, Bilic I, Ban SA, Kuloğlu Z, Demir AM, Ensari A, Colinge J, Rizzi M, Eibel H, Boztug K. **Early-onset inflammatory bowel disease and common variable immunodeficiency-like disease caused by loss-of-function mutation in *IL21***
J Allergy Clin Immunol. 2014 Apr 17

Salzer E*, Santos-Valente E***,** Klaver S, Ban SA, Emminger W, Prengemann NK, Garncarz W, Müllauer L, Kain R, Boztug H, Heitger A, Arbeiter K, Eitelberger F, Seidel MG, Holter W, Pollak A, Pickl WF, Förster-Waldl E#, Boztug K#.**B-cell deficiency and severe autoimmunity caused by deficiency of protein kinase Cδ.**
Blood. 2013 Apr 18 (* and # equal contribution)

Salzer E*, Daschkey S*, Choo S, Gombert M, Santos-Valente E, Ginzel S, Schwendinger M, Haas OA, Fritsch G, Pickl WF, Förster-Waldl E, Borkhardt A#, Boztug K#, Bienemann K, Seidel MG#. **Combined immunodeficiency with life-threatening EBV-associated lymphoproliferative disorder in patients lacking functional CD27.**
Haematologica. 2013 Mar 13 (* and # equal contribution)

Kratochwill K, Boehm M, Herzog R, Lichtenauer AM, **Salzer E**, Lechner M, Kuster L, Bergmeister K, Rizzi A, Mayer B, Aufricht C.
Alanyl-glutamine dipeptide restores the cytoprotective stress proteome of mesothelial cells exposed to peritoneal dialysis fluid;
Nephrol Dial Transplant. 2012 Mar 27

Riesenhuber A., Vargha R., Kratochwill K., Kasper D.C., **Salzer E**.; Aufricht C.
Peritoneal Dialysis Fluid induces p38 dependent Inflammation in Human mesothelial cells;
Peritoneal Dialysis International. 2011 May 31

ADDITIONAL CLASSES AND SEMINARS

Seminars

Summer School of the Clinical Immunology Society (CIS) 09/2014
Miami Beach, Florida

Bedside Teaching Seminar in Pediatrics, Cologne, Germany 04/2010
Symptoms, Signs and Interpretation in Pediatrics
John B. Watkins/Clifford Lo (CHB, Harvard Medical School, Boston)

Bedside Teaching Seminar in Internal Medicine, Innsbruck, Austria 01/2009
Patient based Review of Internal Medicine-
Exercise in Art and Science of Clinical diagnosis
Steven Mackey (Columbia University, New York)

AWARDS AND SCHOLARSHIPS

Awards

Austrian Society of Pediatric and Adolescent Medicine 09/2013
Best Poster Presentation

Young Scientists Association of the Medical University of Vienna 06/2013
Best Poster Presentation

Austrian Society of Allergy and Immunology 11/2012
Best Poster Presentation

Young investigator award for diploma thesis of the Austrian 10/2010
Society of pediatric and adolescent medicine

National award for school leaving exam thesis in humanities 06/2004

Scholarships

Excellence Scholarship of the Medical University of Vienna 2009/2010
2005-2008
Erasmus Scholarship 2008/2009
Research scholarship of the Medical University of Vienna 2007-2010

MUSIC EDUCATION

Violin studies

Diploma in Violin 05/2010

Qualifying for education in violin studies

LANGUAGES

German (mother tongue), English, French

HOBBIES

Music, playing the violin, rock climbing, skiing, running