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Molecular Investigation of Primary Immunodeficiency Disorders with Susceptibility to Immune Dysregulation

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Submitted by

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Declaration

This doctoral thesis was written in a cumulative form and includes three manuscripts in which the author of the current thesis is co-author. Most of the work presented here was performed in the laboratory of Kaan Boztug at CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria. The exact contributions of Elisangela Santos-Valente and of the other co-authors for the respective manuscripts are described below.

The manuscript #1 (chapter 3.1) entitled **"A Novel Mutation in the Complement Component 3 Gene in a Patient with Selective IgA Deficiency"** was published in the Journal of Clinical Immunology by Santos-Valente E., Reisli I. *et al*, J Clin Immunol. 2013 Jan; 33(1): 127-33.

The author of this thesis co-designed the study, performed the molecular analysis, created the figures, and wrote the manuscript with assistance of the other co-authors. Ismail Reisli and Hasibe Artaç, from the Necmettin Erbakan University, Konya, Turkey, performed the clinical characterization of the patient and the routine laboratory exams. Özden Sanal was responsible for the quantification of specific antibody titers against *Streptococcus pneumoniae*, carried out at the Hacettepe University, Ankara, Turkey. Raphael Ott contributed to the capillary sequencing of the family members at CeMM, Vienna, Austria. Kaan Boztug designed and supervised the study, and guided the writing of the manuscript.

The manuscript in chapter 3.2 entitled **"B-cell deficiency and severe autoimmunity caused by deficiency of protein kinase C δ"** was published in Blood by Salzer E., Santos-Valente E., *et al*. Blood. 2013 Apr 18; 121(16): 3112-6.

In this project, the author of the present thesis performed the experimental work concerning the detailed B-cell immunophenotyping, array-based homozygosity mapping, whole exome sequencing (WES) and validation/segregation of WES hits in family members. The author also wrote the manuscript together with Elisabeth Salzer, Ellisabeth Förster-Waldl and Kaan Boztug. Elisabeth Salzer, Stefanie Klaver, Sol A Ban, Nina K. Prengemann and Wojciech Garncarz performed experimental work at CeMM, Vienna, Austria. Routine laboratory analysis were carried out by Winfried F. Pickl from the Christian Doppler Laboratory for Immunomodulation and Institute of Immunology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria; Wolfgang Emminger, Klaus Arbeiter and Arnold Pollak from the Department of Pediatrics and Adolescent Medicine,

Medical University of Vienna; Heidrun Boztug, Andreas Heitger, Markus G. Seidel and Wolfgang Holter from the Department of Pediatrics, St. Anna Kinderspital and Children's Cancer Research Institute, Medical University of Vienna, Vienna, Austria, and Franz Eitelberger from the Department of Pediatrics and Adolescent Medicine, Klinikum Wels-Grieskirchen Wels, Austria. Leonhard Müllauer and Renate Kain performed histopathological analyses at the Clinical Institute of Pathology, St. Anna Kinderspital and Children's Cancer Research Institute, Medical University of Vienna, Vienna, Austria. Elisabeth Förster-Waldl, from the Department of Pediatrics and Adolescent Medicine, Medical University of Vienna, provided clinical care, critically reviewed clinical and immunological patient data and helped conceiving the study. Kaan Boztug conceived the study, planned, designed, and interpreted experiments, wrote the initial draft of the manuscript with assistance of the author of this thesis, Elisabeth Salzer, Stefanie Klaver, Sol A Ban, Nina K. Prengemann and Elisabeth Förster-Waldl.

Chapter 3.3 comprises the third manuscript: **"Biallelic loss-of-function mutation in NIK causes a primary immunodeficiency with multifaceted aberrant lymphoid immunity"** by Katharina L. Willmann, Stefanie Klaver, Figen Dogu, Elisangela Santos-Valente, *et al*. Nat Commun. 2014 Nov 19;5:5360.

The author of this thesis performed array-based homozygosity mapping, WES, validation/segregation of WES hits in family members and B-cell immunophenotyping. She also drafted the initial experimental design together with Kaan Boztug, gave input into the manuscript writing and critically reviewed the manuscript together with the other authors. Figen Dogu, Sule Haskologlu, Musa Gökalp Bolkent and Aydan Ikinciogullari from the Department of Pediatric Immunology and Allergy, Ankara University Medical School, Ankara, Turkey were responsible for the clinical data and clinical care of the patients, including allogeneic hematopoietic stem cell transplantation (aHSCT). Alphan Küpesiz, from the Department of Pediatric Hematology, Akdeniz University Medical School, Antalya, Turkey, performed aHSCT of patient 1. Katharina L. Willmann, Stefanie Klaver, Wojciech Garncarz, Ivan Bilic, Elisabeth Salzer and Cecilia Dominguez Conde, performed experimental work at CeMM, Vienna, Austria. Emily Mace, Pinaki P. Banerjee and Jordan S. Orange performed analyses of NK cell at the Center for Human Immunobiology, Baylor College of Medicine and Texas Children's Hospital, Houston, Texas, USA. Menno C. van Zelm, from the Department of Immunology, Erasmus MC, University Medical Center, Rotterdam The Netherlands, performed T-cell immunophenotyping and immunoglobulin sequence analysis. Winfried F. Pickl

performed T-cell proliferation assays at the Christian Doppler Laboratory for Immunomodulation and Institute of Immunology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria. Gregory I. Vladimer assisted with imaging and Peter Májek performed computational modeling and *in silico* prediction algorithms at CeMM. Giulio Superti-Furga and Jacques Colinge, from CeMM, and Antonio Condino-Neto, from the Department of Immunology, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil, contributed with critical scientific discussions. Kaan Boztug conceived the study and, together with Katharina L. Willmann, Stefanie Klaver and the author of this thesis, planned, designed and interpreted experiments. Katharina L. Willmann, Stefanie Klaver, Ivan Bilic and Kaan Boztug wrote the manuscript with input from the author of the present thesis, Hermann Eibel, Menno C. van Zelm, Jordan S. Orange, Giulio Superti-Furga, Emily Mace, Cecilia Dominguez Conde and Aydan Ikinciogullari.

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The chapters in the Results section were reprinted with permission from Elsevier (chapter 3.1), The American Society of Hematology (chapter 3.2) and Nature Publishing Group (chapter 3.3), respectively.

Table of Content

List of Figures

Abstract

The immune system is crucial for defense against external pathogens and a fine tuned balance of its functions is necessary for immune homeostasis. Inborn errors of the immune system result in primary immunodeficiency disorders (PIDs), characterized by a predisposition to infections, malignancies and autoimmune manifestations. Increasing identification and therefore prevalence of PIDs have encouraged intense investigation in this field in the past decades. With the help of modern genetic techniques, the study and molecular characterization of numerous PIDs have substantially contributed to recognition and understanding of important players in the immune system. The work presented here was carried out with the purpose of recognizing critical genes in the immune system by investigating the underlying genetic mutations in PID patients with recurrent infections and immune dysregulation. Additionally the significance of the specific findings in immune response and regulation was functionally assessed.

Three manuscripts describing one previously unknown mutation and two novel genes causing PID in humans resulted from the work accomplished during the course of this PhD. In the first manuscript we demonstrated the importance of the C345C domain of complement factor 3 (C3) in human immune response to encapsulated bacteria by discovering a previously unknown mutation within this domain (c. C4554G, p. Cys1518Trp) in a patient suffering from severe pneumococcal infections. The second publication discloses a novel PID marked by a CVID-like B-cell deficiency and severe autoimmunity as a result of mutation in the *protein kinase C delta* (*PRKCD*) gene encoding PKCδ (c.13521+1G>A). As a result, our study establishes direct implication of PKCδ in B-cell homeostasis and immune regulation in humans. The third paper of this thesis covers the detection of a hitherto unidentified disorder caused by homozygous mutation of the *MAP3K14* gene encoding NF-κB-inducing kinase (NIK) (c. C1694G, p. Pro565Arg) in patients affected by a pervasive combined immunodeficiency. This finding determines non-redundant functions for NIK in human lymphoid immunity.

Collectively, the investigations within this thesis reveal previously unknown pivotal roles for the implicated domains/molecules in immunity against foreign antigens, immune homeostasis and tolerance in humans, and therefore contribute to a broader comprehension of the genetic mechanisms involved in pathogenesis of PIDs with susceptibility to immune dysregulation.

Zusammenfassung

Das Immunsystem ist essentiell für die Abwehr von Pathogenen. Die diversen Funktionen des Immunesystems unterliegen Kontrollmechanismen, die eine Immunhomöostase generieren. Angeborene Defekte des Immunsystems, sogenannte primäre Immundefekte (PIDs), sind durch Prädisposition für Infektionen, Tumoren und Autoimmunität charakterisiert. Eine vermehrte Identifikation und Prävalenz von PIDs während der letzten Jahrzehnte resultierte in einer erweiterten Forschung in diesem Bereich, welche durch die Entwicklung von neuen Technologien zur Genomsequenzierung vereinfacht wurde. Die Identifikation und molekulare Charakterisierung diverser PIDs haben substantiell dazu beigetragen wichtige Faktoren des Immunsystems zu ermitteln. Die hier eingereichte Arbeit basiert auf der Identifikation von genetischen Mutationen mittels Single Nukleotid Polymorphismus-Array basiertem Mapping von homozygoten Regionen und Exome Sequenzierung in PID Patienten mit wiederkehrenden Infekten und Immundisregulation. Weitergehend wurde die Signifikanz der genetischen Ergebnisse für die relevante Erkrankung funktionell getestet.

Die hier eingerichtet PhD Arbeit resultierte in der Publikation von drei Manuskripten welche neue genetische Defekte beschreiben. Im ersten Manuskript wird die Relevanz der C345C Domäne vom Komplementfaktor C3 für die humane Immunantwort gegen Bakterien demonstriert. Dies wurde durch die Identifikation einer bisher unbekannten Mutation in dieser Domäne (c. C4554G, p. Cys1518Trp) bei einem Patienten mit wiederkehrenden Pneumokokkeninfektionen erreicht. Das zweite Manuskript beschreibt einen neuen PID Subtyp welcher durch CVID-ähnlicher B-Zelldefizienz und schwerer Autoimmunität gekennzeichnet ist. Der Patient weist eine Mutation in dem Gen *protein kinase C delta* (*PRKCD*; c.13521+1G>A) auf. In unserer Studie konnten wir die direkten Auswirkungen der durch das Gen kodierten PKCδ auf B-Zellhomöostase und Immunregulation zeigen. Die dritte Publikation beschreibt die Entdeckung einer neuen Krankheitsursache für einen kombinierten Immundefekt, welcher durch homozygote Mutationen in dem Gen *MAP3K14* (c. C1694G, p. Pro565Arg) ausgelöst wird. Die Untersuchungen zeigen diverse nicht redundante Funktionen der durch das Gen kodierten NF-κB-inducing kinase (NIK) in humaner Immunbiologie.

Zusammengefasst beschreibt die vorgelegte Arbeit diverse neue krankheitsauslösende Mutationen in zwei neuen und einem bekannten Gen mit

wichtigen Rollen in humaner Immunität, Immunhomöostasis und Immuntoleranz. Die Arbeit trägt zu einem erweiterten Verständnis von genetischen Ursachen in der Pathogenese von PIDs mit Anfälligkeit für Immundisregulation bei.

Publications arising from this thesis

Santos-Valente E*, Reisli I*, Artaç H, Ott R, Sanal Ö, Boztug K. **A Novel Mutation in the Complement Component 3 Gene in a Patient with Selective IgA Deficiency.** J Clin Immunol (2013) 33:127–133. (* equal contribution)

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;121(16):3112-6. (* and [#] equal contribution)

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Abbreviations

AD	Autosomal dominant
ADA	Adenosine deaminase
aHSCT	Allogeneic hematopoietic stem cell transplantation
aHUS	Atypical hemolytic-uremic syndrome
AICDA	Activation-induced cytidine deaminase
AIRE	Auto-immune regulator
ALPS	Autoimmune lymphoproliferative syndrome
ANA	Anaphylatoxin
AP-1	Activator protein 1
APC	Antigen-presenting cell
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
APRIL	A proliferation inducing ligand
AR	Autosomal recessive
BAFF	B-cell activating factor of the TNF family
BCMA	B-cell maturation antigen
BCR	B-cell receptor
BLNK	B-cell linker protein
BTK	Bruton's tyrosine kinase
CCR	CC-chemokine receptor
CD	Cluster of differentiation
CF	Complement factor
CID	Combined immunodeficiency
CLP	Common lymphoid progenitor
CMV	Cytomegalovirus
CNV	Copy number variation
CR	Complement receptor
CSR	Class switch recombination
CVID	Common variable immunodeficiency
CXCR	CXC-motif chemokine receptor
DAF	Decay-accelerating factor
DAG	Diacylglycerol
DAMP	Damage- or danger-associated molecular pattern
DC	Dendritic cell
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
dsDNA	Double stranded DNA
EBV	Epstein-Barr virus
EDA-ID	Anhidrotic ectodermal dysplasia with immunodeficiency
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ESID	European Society for Immunodeficiencies
ETP	Early T-cell progenitor

FOXP3	Forkhead box P3
GATA3	GATA binding protein 3
GC	Germinal center
GEF	Guanine exchange factor
GOF	Gain-of-function
HAX-1	HCLS1 associated protein X-1
HIGM	Hyper IgM syndrome
HSV	Herpes simplex virus
ICOS	Inducible T-cell co-stimulator
IFN	Interferon
Ig	Immunoglobulin
IKK	IκB serine kinase
IL	Interleukin
IP3	Inositol 1,4,5-trisphosphate
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
IRAK-4	Interleukin-1 receptor associated kinase 4
IRF	Interferon regulatory factor
IκB	Inhibitor of κB
JAGN1	Jagunal homolog 1
JAK3	Janus kinase 3
JNK	c-Jun N-terminal kinase
KD	Kinase domain
LTβR	Lymphotoxin-β receptor
MAC	Membrane attack complex
MAP	Mitogen-activated protein
MASP	Mannan-binding lectin serine peptidase
MCP	Membrane cofactor protein
MG	Macroglobulin
MHC	Major histocompatibility complex
MMF	Mycophenolate mofetil
mTEC	Medullary thymic epithelial cells
mTOR	Mechanistic target of rapamycin
MYD88	Myeloid differentiation primary response 88
MZ	Marginal zone
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	Nuclear factor of activated T cells
NGS	Next generation DNA sequencing
NIK	NF-κB-inducing kinase
NK	Natural killer
NRD	Negative regulatory domain
PAMP	Pathogen-associated molecular pattern
PAX5	Paired-box protein 5
PD-1	Programmed cell death 1
PDK1	Pyruvate dehydrogenase kinase, isozyme 1
PI3K	Phosphoinoside 3 kinase

PID	Primary Immunodeficiency disorder
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein Kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern-recognition receptors
RAG	Recombination activating genes
RANK:	Receptor activator of nuclear factor kappa-B
RASGRP	RAS guanyl-releasing protein
RNA	Ribonucleic acid
RUNX1	Runt-related transcription factor 1
SCID	Severe combined immunodeficiency
Ser	Serine
SHM	Somatic hypermutation
sIgAD	Selective IgA deficiency
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
TACI	Transmembrane activator and CAML interactor
TBK1	TANK-binding kinase 1
TCR	T-cell receptor
TED	Thio-ester containing domain
TFH	Follicular helper T cell
Th	Helper T cell
Thr	Threonine
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFRSF	TNF receptor superfamily
TRA	T-cell receptor α
TRAF3	Tumor necrosis factor receptor associated factor 3
Treg	Regulatory T cell
TRIF	TIR domain-containing adaptor protein inducing IFN-β
TWEAK	TNF-like weaker inducer of apoptosis
Tyr	Tyrosine
UNG	Uracil-N-glycosylase
WAS	Wiskott-Aldrich syndrome
WES	Whole exome sequencing
WGS	Whole genome sequencing
XL	X-linked
XLA	X-linked agammaglobulinemia
ZAP-70	Zeta chain of T cell receptor associated protein kinase 70kDa

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1 THESIS BACKGROUND

Since the most ancient single-cellular organisms, the complexity of the immune system has evolved as a defense tool against external threats. Throughout evolution, two connecting branches with different roles developed: the innate and the adaptive immune system. The early-evolved innate immune system accounts for a first-line set of protection mechanisms that constitutes the first defenses of the body (reviewed in (Sirisinha, 2014; Travis, 2009)). The characteristics of the innate immunity are fast and short-lasting immune responses directed to a wide range of microorganisms. Eventually, once the infection is cleared, activated innate mechanisms return to baseline levels (Murphy et al, 2008).

Upon species evolution, the organisms became more complex and the lifespan longer, which demanded the development of more specialized tools against external threats in vertebrates, the adaptive or acquired immunity (Sirisinha, 2014). The adaptive immunity is distinguished from the innate branch by a diverse yet highly specific repertoire for microbe recognition and by the formation of immunological memory (Murphy et al, 2008). The classical distinction between innate and acquired immunity is however currently being challenged, since the discovery that some innate immune cells have characteristics previously attributed to cells of the adaptive immune system (reviewed in (Netea et al, 2015)).

Inborn defects of one or more components of the immune system result in conditions characterized by increased susceptibility to infections, malignancies and autoimmunity, known as primary immunodeficiency disorders (PIDs) (reviewed in (Gupta & Louis, 2013; Salavoura et al, 2008)). The first recognized PIDs were congenital neutropenia (Kostmann, 1950; Kostmann, 1956) and X-linked agammaglobulinemia (XLA) (Bruton, 1952). Only four decades after the first XLA description, mutations in the *Bruton tyrosine kinase* (*BTK*) gene were discovered to cause the phenotype observed of lack of antibodies, soluble effectors of the adaptive immunity, also known as immunoglobulins (Vetrie et al, 1993).

Following these first descriptions, an increasing number of individuals affected by PIDs have been reported. Due to vast progress in diagnostic tests during the past 30 years, and particularly with the advent of high throughput techniques, a continuously rising number of distinct PIDs have been recognized (Bousfiha et al, 2015; Picard et al, 2015). Precise molecular characterization from many of those disorders has immensely contributed to the recognition of critical components in host defense and

immune regulation and therefore to extensive scientific progress in this area (reviewed in (Milner & Holland, 2013; Quintana-Murci et al, 2007)).

According to the European Society for immunodeficiencies (ESID) database (esid.org/Working-Parties/Registry/ESID-Database-Statistics, accessed on 06.10.2015), 19,355 PID patients have been registered by June 2014. In the same year, the Jeffrey Modell Foundation Network identified 77,193 individuals diagnosed with PID (Modell et al, 2014). It is estimated that the incidence of PIDs can reach 1:2000 live births and up to 6 million people worldwide may have a congenital defect of the immune system (Bousfiha et al, 2013).

Also contributing to the rapid expansion of PIDs are several disorders with autoimmunity and autoinflammation recognized as immunodeficiencies in recent classifications (Bousfiha et al, 2015; Picard et al, 2015). In addition to the increasing prevalence, the severity of many PIDs urges precise diagnosis and proper treatment in order to reduce morbidity and/or mortality. Prototypical examples are the severe combined immunodeficiencies (SCID), defined by profound defects in T and B lymphocytes, whose patients suffer early in life from severe opportunistic infections and failure to thrive, and mostly the disease is lethal if treatment is not promptly established (reviewed in (Sponzilli & Notarangelo, 2011)).

1.1 Innate immunity

The innate immune system encompasses several defense elements, including among others, epithelial barriers, effector cells, pattern-recognition receptors (PRRs) and soluble molecules, as for instance proteins of the complement system. Most of the cells participating in the innate immune responses are derived from a common myeloid progenitor in the bone marrow. Exceptions of this rule are the natural killer (NK) cells and other recently discovered innate lymphoid cells (reviewed in (Spits & Di Santo, 2011)), which originate from a lymphoid progenitor. One of the key effector mechanisms of innate immunity is phagocytosis, in which cells such as monocytes, neutrophils and dendritic cells (DCs), internalize and enzymatically destroy foreign particles (Murphy et al, 2008).

The quick response of the innate immune system is made possible due to germline-encoded PRRs present in many innate immune cells, which identify motifs commonly found in several pathogenic microorganisms and triggers immediate responses from effector cells (reviewed in (Medzhitov & Janeway, 2000)). PRRs comprehend secreted receptors such as the mannose-binding lectin (MBL), as well as receptors with endocytic (scavenger and macrophage mannose receptors) or signaling functions (Toll-like receptors, RIG-I receptors) (reviewed in (Kumar et al, 2011)). The structures recognized by PRRs are known as pathogen-associated molecular patterns (PAMPs). PAMPs are not present in host cells but occur in pathogen proteins important for their life cycle. Examples of such molecules include components of bacterial wall, bacterial DNA and double-stranded RNA, often found in viruses (Murphy et al, 2008). In addition to the PAMPs, the PRRs also recognize danger signals released by injured, stressed or dying cells, known as damage- or danger-associated molecular patterns (DAMPs) (reviewed in (Krysko et al, 2012)).

Toll-like receptors (TLRs) are representative examples for PRRs, which recognize a diverse set of PAMPs and DAMPs, and connect the innate and the acquired immunity. In humans, cell surface TLRs, including TLR1, 2, 4, 5 and 6, are able to perceive PAMPs from bacteria, fungi and protozoa. TLR3, 7, 8 and 9, on the other hand, are found in intracellular compartments and are specialized in the recognition of intracellular PAMPs such as nucleic acids from bacteria and viruses (Kumar et al, 2011). In resting cells, such TLRs are mainly found inside the endoplasmic reticulum (ER), and upon stimulation they are recruited to endolysosomes where they are able to initiate downstream signaling. The transportation of such TLRs from the ER to

endocytic compartments depends for instance on the ER-membrane protein UNC93B (Kim et al, 2008).

The structure of TLR includes leucine-rich repeats, important for the recognition of PAMPs, and a cytoplasmic Toll/interleukin-1 receptor (TIR) domain responsible for intracellular signaling. Downstream signaling requires the recruitment of TIR domain-containing adaptor molecules including MYD88 (myeloid differentiation primary response 88) and TRIF (TIR domain-containing adapter protein inducing IFN-β) among others, and results in activation of transcription factors and induction of inflammatory responses (Kumar et al, 2011).

All TLRs, except TLR3 signal through the adaptor molecule MYD88, that recruits interleukin-1 receptor–associated kinase 4 (IRAK4), and triggers production of pro-inflammatory cytokines via activation of nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) and mitogen-activated protein (MAP) kinases pathways. TLRs 3 and 4 signal through TRIF to activate NF-κB and IRF3 (interferon regulatory factor 3), with subsequent production of type I interferon (IFN) in addition to pro-inflammatory cytokines. IRF3 phosphorylation also involves TRAF3 (tumor necrosis factor receptor-associated factor 3) and inhibitors of NF-κB kinases (IKKs) such as TBK1 (TANK-binding kinase 1) and IKKε (inhibitor of NF-κB kinase, subunit epsilon) (Kumar et al, 2011).

Mutations in several elements involved in TLR signaling have been demonstrated as a cause of PIDs and thus reveal the importance of such molecules and pathways in specific immune responses in humans. For instance, critical roles for UNC93B, TRIF, IRF3, TRAF3, TLR3 and TBK1 in controlling central nervous system infection by herpes simplex virus 1 (HSV-1) have been demonstrated with the discovery of patients bearing mutations in one of the above-mentioned molecules. These patients have a very high predisposition to childhood HSV-1 encephalitis but not to any other severe infectious disease (Andersen et al, 2015; Casrouge et al, 2006; Herman et al, 2012; Perez de Diego et al, 2010; Sancho-Shimizu et al, 2011; Zhang et al, 2007).

Similarly, autosomal recessive (AR) mutations in *MYD88* and *IRAK4* reveal that the TIR-MYD88-IRAK4 signaling pathway has a non-redundant role in immunity against pyogenic bacteria in humans. In contrast to corresponding mouse models, which are susceptible to a variety of microorganisms (reviewed in (von Bernuth et al, 2012)), MYD88- and IRAK4-deficient patients present with severe and recurrent infections mainly caused by *S. pneumoniae* and *S. aureus*, while being not susceptible to other bacteria, viruses, fungi and parasites (Picard et al, 2003a; von Bernuth et al, 2008).

1.1.1 The complement system

Besides cells and receptors, soluble proteins of the complement system are also vital for innate immune responses. These heat-labile plasma proteins were first recognized in the 19th century as having the ability to kill bacteria, complementary to the role of antibodies. The complement system is of great importance for host defense against infections by inducing inflammation, microbe opsonization (targeting of the pathogen for destruction), osmotic lysis of pathogen membranes, and clearance of apoptotic cells and immune complexes. In addition, the complement system is also able to initiate a crosstalk between the innate and the adaptive immunity (reviewed in (Dunkelberger & Song, 2010)).

Three pathways have been traditionally described to activate the complement system: the classical, the alternative and the lectin pathway (Figure 1). They all converge to the cleavage of the third component of the complement (C3) and thus the generation of C3 convertases, which cleave the inactive C3 into two active fragments: C3a and C3b (Dunkelberger & Song, 2010). Apart from the already established pathways, increasing evidences propose additional routes for complement activation through C3- and C5-independent cleavage of other complement components (reviewed in (Merle et al, 2015a)).

The classical pathway is typically triggered when the C1q serine protease recognizes complement-fixing antibodies present on pathogenic surfaces. C1q is able to recognize diverse targets including C-reactive protein, but also PAMPs such as lipopolysaccharide, and dsDNA expressed on the surface of dying cells, as well as components of the adaptive immune system such as IgM and IgG (Merle et al, 2015a). Target recognition by C1q allows activation of the other two components of C1 complex, C1r and C1s, and subsequent cleavage of C4 and C2 (Figure 1). Together, the resulting fragments C4b and C2a form the C3 convertase of this pathway (C4bC2a) (Dunkelberger & Song, 2010).

The alternative pathway is continually active in normal hosts since it is initiated by spontaneous cleavage of the thioester bond in C3, resulting in the hydrolyzed C3 form ($C3(H_2O)$). This process triggers structural modifications that expose the binding site for complement factor B (CFB) on $C3(H_2O)$ (Merle et al, 2015a). Binding of $C3(H_2O)$ to CFB allows its cleavage in Ba and Bb by factor D (CFD). Bb remains bound to the $C3(H_2O)$, resulting in the initial C3 convertase of this pathway: $C3(H_2O)$Bb. The resulting opsonin C3b is able to bind any surface nearby, facilitating

the recognition and phagocytosis of apoptotic or pathogenic cells. It also binds to CFB and CFD allowing the formation of an additional C3 convertase in this pathway: C3bBb. Factor P (properdin) is important for stabilization of this complex, amplifying the activation of the alternative pathway (Dunkelberger & Song, 2010; Merle et al, 2015a).

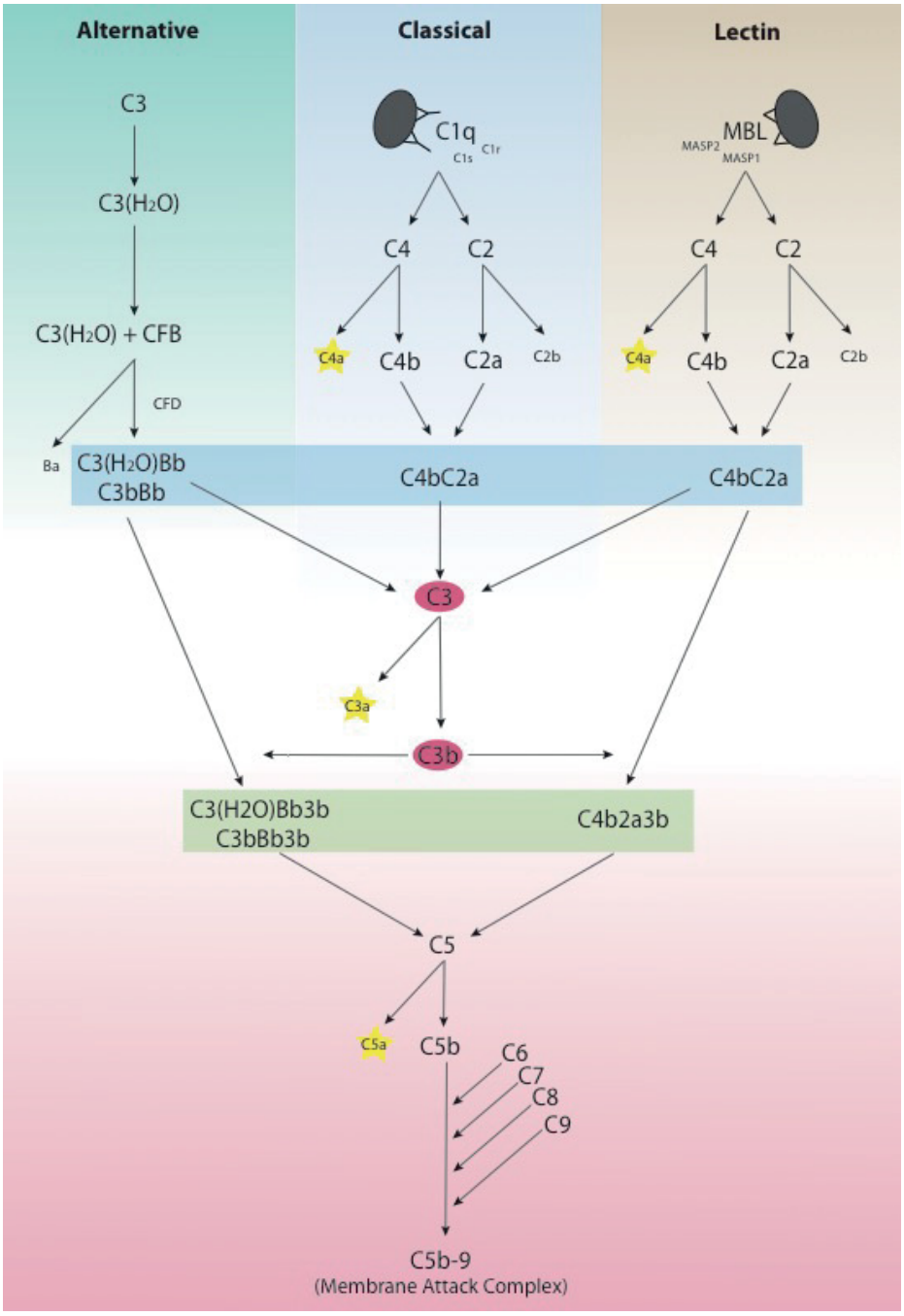

Figure 1. Complement activation pathways CFB: complement factor B; CFD: complement factor D; MASP: mannan-binding lectin serine peptidase. Grey oval symbols: pathogens displaying surface complement-fixing antibodies (classical pathway) or PAMPs (alternative pathway).

The initiation of the lectin pathway does not require antibodies but PRRs such as MBL, which binds PAMPs commonly found in bacteria, yeasts and in some viruses and parasites. The binding activates MBL-associated serine proteases (MASPs, mannan-binding lectin serine peptidase) and, similarly to the C1 complex proteases, the MASPs cleave C2 and C4, generating C4bC2a (Dunkelberger & Song, 2010).

The C3b generated in the complement pathways further associates with the C3 convertases to generate the C5 convertases: C3(H2O)Bb3b, C3bBb3b and C4b2a3b, respectively. The C5b fraction generated associates with C6 and starts the assembly of the membrane attack complex (MAC), which contains C5b, C6, C7, C8 and C9 and forms pores on the microorganism membrane to induce osmotic lysis (Dunkelberger & Song, 2010). C3b is therefore crucial for inducing cell lysis through the generation of C5 convertases and subsequent formation of the membrane attack complex (Figure 1).

Besides the cell lysis caused by the MAC, the complement cascade can also induce inflammation. The potent inflammatory process triggered by the complement activation is mediated by anaphylatoxins (C3a, C4a and C5a). Those cleavage products of C3, C4 and C5, respectively, possess intense proinflammatory and leukocyte-chemoattractant activity. Only cells bearing receptors for anaphylatoxins, for instance monocytes/macrophages, granulocytes, DCs, mast cells and some nonmyeloid cells, are able to respond to their presence (Dunkelberger & Song, 2010).

The third main effect of the complement system is opsonization of cell surfaces by proteolytic fragments called opsonins. Opsonins such as the C3b and its cleavage products (iC3b, C3c, C3dg and C3d) are recognized by complement receptors of phagocytes and other peripheral blood cells and enable the respective opsonin-coated pathogen, apoptotic cell or immune complex to be eliminated (Dunkelberger & Song, 2010).

Due to constant activation and positive feedback amplification, the complement system is able to generate intense inflammatory responses, which can be immensely destructive to the host tissues if not controlled. Therefore, regulatory elements to guarantee homeostatic balance are necessary and assure that full complement activation takes place only in the presence of pathogens containing PAMPs or complement-fixing antibodies, and that complement activation is restricted to the site of inflammation (Dunkelberger & Song, 2010).

Permanent activity of the alternative pathway is controlled by rapid inactivation of C3b by factor I (CFI), or binding and subsequent inactivation of C3b by host cells (Merle et al, 2015a). In addition, degradation of C3b and C4b into inactive fragments by CFI prevents assembly of the C3 convertase in a process that requires membrane cofactor protein (MCP), complement receptor 1 (CR1), factor H (CFH) and C4 binding protein (C4BP). MCP and CR1 are present on host cell surfaces, ensuring that inflammation is restricted to pathogen surfaces. Some protective inhibitors including the decay-accelerating factor (DAF or CD55), CR1, CFH and C4BP also have decay-accelerating activity and precipitate dissociation of the convertases (Dunkelberger & Song, 2010).

Furthermore, activation of the classical pathway is hampered by C1 inhibitor (C1INH), which binds to C1s and C1r, and promote dissociation of the C1 complex. Likewise, proteins homologous to MASP-1 and MASP-2, which also bind to MBL but are not able to induce complement responses, restrict the lectin pathway. C1INH can also control activation of the lectin pathway by inhibiting MASP-1 and MASP-2 (Merle et al, 2015a).

Complement functions are additionally regulated through prevention of the MAC assembly in host cells. The mechanisms involved in this process include binding of plasma proteins such as vitronectin (S-protein) to the C5b67 complex and inhibition of C9 binding to the C5b678 complex by CD59 (protectin). Moreover, the displacement of the N-terminal arginine residue of anaphylatoxins by carboxypeptidades, restricts their proinflammatory activity (Dunkelberger & Song, 2010).

Examples of a connection between complement system and adaptive immunity are complement-fixing antibodies (IgM, IgG1, IgG3 and, to a lesser extend, IgG2) and anaphylatoxins. As mentioned above, antibodies with complement-fixing functions are able to activate the classical pathway when bound to antigens in antigen-antibody immune complexes (reviewed in (Daha et al, 2011)). Besides their main inflammation-inducing function, anaphylatoxins are also able to regulate polyclonal immune responses, restrict the secretion of proinflammatory cytokines and contribute to trafficking and migration of B cells (Dunkelberger & Song, 2010).

Complement receptors are also important for effective humoral responses, with roles in antigen presentation to B cells, amplification of antigen receptor-mediated B-cell activation, B-cell survival and B-cell differentiation into plasma and memory cells (Dunkelberger & Song, 2010). Both transmembrane complement receptors 1 (CR1

or CD35), expressed in several peripheral cell types, and 2 (CR2 or CD21), expressed mostly in B-lymphocytes and follicular DCs, bind complement opsonins and enable the clearance of immune complexes. CR1 also binds C3b and C4b with high affinity and, in B cells, it seems to have a role in antigen presentation and inhibition of B-cell activation (reviewed in (Erdei et al, 2009)). CR2 is mainly found in complex with CD19 and CD81 or with CR1, and its main ligand is the final C3 cleavage fragment C3d. The CD19/CD21/CD81 trimer forms the B-cell co-receptor complex, in which CD21 serves as a link between antigen recognition via C3d and B-cell intracellular signaling via CD19. When bound to C3d, this complex enhances the signaling through the B-cell receptor (BCR) and amplifies B cell activation (Dunkelberger & Song, 2010; Erdei et al, 2009).

Additionally, link between the complement system and T-cell responses, survival, activation and proliferation have also been demonstrated (Capasso et al, 2006; Dunkelberger & Song, 2010; Ghannam et al, 2014).

Due to the central roles of complement in innate immunity against infections and in the adaptive immunity, inadequacy of any of its component's functions can induce a variety of clinical manifestations depending on the defective protein (reviewed in (Skattum et al, 2011)). To date, deficiency of several proteins of the complement system has been described to cause PIDs (Bousfiha et al, 2015; Picard et al, 2015).

Even though the clinical presentation of complement deficiencies is variable, characteristic clinical manifestations include enhanced susceptibility to infections by encapsulated bacteria, meningococcal meningitis, autoimmunity, angioedema and disorders of the kidneys and eyes. Autoimmune manifestations occur predominantly in patients with deficiency in the early components of the complement cascade such as C1q, C1r, C1s, C2, C3 and C4 and include, among others, systemic lupus erythematosus (SLE), glomerulonephritis, vasculitis, rheumatoid arthritis and anti-phospholipid syndrome (Grumach & Kirschfink, 2014; Skattum et al, 2011).

The association of increased complement function and autoimmunity may result from uncontrolled inflammatory process and subsequent tissue damage. In cases of diminished complement function, autoimmune manifestations may arise from defective clearance of immune complexes and inadequate exclusion of maturing self-reactive lymphocytes (reviewed in (Ballanti et al, 2013; Grumach & Kirschfink, 2014; Merle et al, 2015b)).

1.2 Adaptive immunity

Complementary to innate immunity, adaptive immunity is activated and supports the recovery from the infection. After phagocytosis of pathogens, innate immune cells migrate to lymphoid tissues, where they are able to induce adaptive immune responses. The classic components of acquired immunity are immunoglobulins, lymphocyte receptors and proteins of the major histocompatibility complex (MHC) classes I and II. The adaptive immune responses are mainly driven by T and B lymphocytes, derived from a common lymphoid progenitor, which drive the cellular and humoral immune responses, respectively (Murphy et al, 2008).

The hallmark of acquired immunity is a virtually unlimited diversification of pathogen recognition by B- and T-cell receptors, which is achieved by arbitrary recombination of the receptor gene segments in the bone marrow (B cells) or thymus (T cells) during lymphocyte development. Further selection and editing processes ensure that the generated receptors are capable of recognizing co-receptors and foreign protein fragments while reacting weakly to self-antigens. In addition, adaptive immune responses generate long-living memory cells that allow endurance of specific protective immunity after the threat is eliminated and enable faster and more intense response in subsequent encounters with the same pathogen (Murphy et al, 2008).

Activation of B and T cells requires antigen presentation and co-stimulation provided by cells with antigen-presenting functions, such as DCs, macrophages and B cells. Antigen-presenting cells (APCs) are capable of processing antigens and displaying peptide fragments on the cell surface. Adequately activated T and B cells proliferate and give rise to effector and memory cells (Murphy et al, 2008).

1.2.1 Cellular immunity

The T-cell development comprises several consecutive events in the thymus and periphery that will produce various types of mature T cells, which can be differentiated according to their effector functions, antigen specificity and T-cell receptor (TCR) constitution (αβ or γδ). Common lymphoid progenitors (CLPs) that migrate to the thymus and commit to the T-cell lineage are known as early T-cell progenitors (ETP). The commitment requires signaling through NOTCH1, while chemokine receptors such as CCR9 and probably also CCR7 and PSGL1 (P-selectin

glycoprotein ligand-1) are crucial for thymic colonization (reviewed in (Carpenter & Bosselut, 2010; Ken Murphy, 2008)).

ETPs are immature cells included in the group of double negative thymocytes (DN), which lack surface expression of CD4 and CD8. DN cells undergo a gradual process of loss of multipotency and T-cell lineage commitment throughout which they experience four successive stages (DN1 to DN4) according to the expression of CD44 and CD25. The commitment process is concluded in the DN2 stage and is characterized by the repression of genes related to other lineages and activation of genes related to T-cell differentiation including *NOTCH1*, *RUNX1* and *GATA3*. Before the commitment process is concluded, such cells can still engage in other lineages like B cells, DCs, natural killer cells and macrophages (Luc et al, 2012) (reviewed in (Carpenter & Bosselut, 2010; Naito et al, 2011)).

In DN3 thymocytes, recombination-activating genes (RAG) 1 and 2 mediate the rearrangement of TCR variable, diversity and joining (VDJ) gene segments to generate pre-TCRs with diverse antigen specificities. The pre-TCR in those cells is not yet exposed on the cell surface and comprises a rearranged TCRβ chain, two CD3 chains and the pre-Tα (instead of the TCRα). In this stage, the function of the pre-TCR in precursors bearing TCRαβ (majority of T cells) is evaluated in a process known as β-selection. This process requires not only successful signaling through pre-TCR but also signaling through interleukin-7 receptor (IL7R) and the presence of other factors including CXCR4 and NOTCH1. In thymocytes giving rise to γδ T cells, whose function seem to be related to stress surveillance (Hayday, 2009), the control of the TCR rearrangement also takes place in the DN3 stage, though already in the TCRγδ (Carpenter & Bosselut, 2010).

Decisive role of IL7R signaling pathway in T-cell development is endorsed by T^-B^+ severe combined immunodeficiencies (SCID) resulting from mutations in *IL7Rα* (*IL7 receptor subunit alpha*), in *IL2RG*, which encodes the common cytokine receptor gamma (γc) chain of the IL2, IL4, IL7, IL9, IL15 and IL21 receptors, or in *JAK3* (*Janus kinase 3*), an intracellular tyrosine kinase associated with the γc chain (Noguchi et al, 1993; Puel et al, 1998; Russell et al, 1995).

The discovery of T^-B^- SCID resulting from *RAG1/2* mutations in humans (Schwarz et al, 1996) confirmed previous observations in mice (Shinkai et al, 1992) about the pivotal functions of those genes in lymphocyte development and maturation of both T and B-cell lineages. Defective RAG1/2 disrupts V(D)J rearrangement of TCR and BCR gene segments (Oettinger et al, 1990; Schatz et al, 1989), lead to reduced T

and B cell clones, severe inflammatory responses and increased susceptibility to life-threatening infections (Schwarz et al, 1996).

After β-selection, αβ thymocytes initiate expression of CD4 and CD8 co-receptors and become double positive T cells (DP) (CD4+CD8+). In DP T cells the rearrangement of *TRA* (*T-cell receptor α*) occurs and the receptor is evaluated for its function and self-reactivity. Cells surpassing this checkpoint will further differentiate and acquire specific functions, of e.g. helper (CD4+) or cytotoxic cells (CD8+), according to expression of distinct groups of genes and to the surface MHC subclass co-receptor displayed (Carpenter & Bosselut, 2010). For instance, cells carrying TCRs which respond to MHCII differentiate into CD4+ (helper) T cells and those whose TCRs are MHCI-restricted give rise to CD8+ (cytotoxic) T cells (reviewed in (Singer et al, 2008)).

In peripheral lymphoid organs, naïve T cells encounter antigens and eventually become effector cells. The nature of the antigen as well as the microenvironment where the encounter takes place result in distinct types of effector cells with adjusted responses to individual classes of pathogens. Examples of such differentiation are the CD4+ T helper 1 (Th1) cells, which produce mainly IFN-γ and act in the response against intracellular pathogens, and Th2 cells, which secrete several other cytokines and possess important roles in immunity to extracellular agents (Naito et al, 2011).

Since TCRs are able to recognize only contiguous peptide sequences that can be hidden in the intact protein, T-cell activation requires antigen processing by APCs and antigen presentation in the context of MHC molecules (Murphy et al, 2008). The MHC is a cluster of genes encoding cell-surface glycoproteins known as MHC molecules, of which MHCI and MHCII are the most prominent representatives. They contain an extracellular structure that carries peptides acquired during the intracellular synthesis and assembly of the protein, or taken up by endocytosis/phagocytosis and processed through the cell's protein degradation machinery. The nature of the trapped peptide varies according to the class of the molecule. MHCI molecules display antigens derived from viral proteins originated in the cytosol. On the other hand, peptides from intracellular vesicles, as the ones derived from phagocytized pathogens, are associated with MHCII molecules. Proteins of the MHCI are expressed in all cells, while MHCII molecules are found only in cells that can be activated by T cells such as DCs, macrophages and B cells (Murphy et al, 2008).

Furthermore, TCR activation requires associated proteins that in conjunction with the receptor form the T-cell receptor complex. Such molecules (CD3γ, CD3δ, CD3ε and CD3ζ chain) are necessary for transporting the receptor to the cell surface and are responsible for initiation of intracellular signaling upon antigen binding, since the TCR chains do not possess such ability (Murphy et al, 2008). The combined immunodeficiency present in the majority of individuals carrying mutations in *CD3G*, *CD3D*, *CD3E* or *CD3Z* genes confirm their essential role in T-cell development (Arnaiz-Villena et al, 1992; Dadi et al, 2003; de Saint Basile et al, 2004; Rieux-Laucat et al, 2006; Soudais et al, 1993).

Co-receptors CD4 and CD8 are also required for effective T-cell responses to antigen by enhancing signal transduction downstream the TCR. During antigen recognition, the association of the CD4 and CD8 with the respective MHC molecules enables the co-receptor-associated intracellular tyrosine kinase LCK to initiate TCR signaling in a complex process involving ZAP-70 (zeta chain of T cell receptor associated protein kinase 70kDa) and subsequent phospholipase C-γ (PLC-γ) activation, which engage important signaling cascades involved in cell activation and proliferation (Murphy et al, 2008).

1.2.2 Humoral immunity

Development of B lymphocytes from hematopoietic precursors occurs in several consecutive stages characterized by specific lineage markers (Murphy et al, 2008) as depicted in figure 2. In mammals, the early stages of B-lymphocyte development (pro-, pre- and immature B cells) occur inside the bone marrow and are marked by the rearrangement of immunoglobulin gene segments (reviewed in (Pieper et al, 2013)).

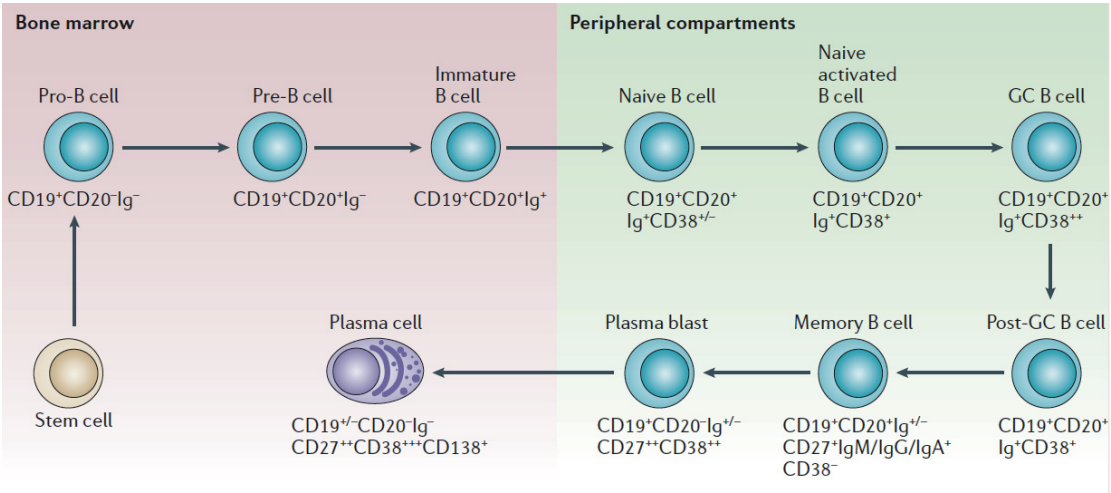

Figure 2. Overview of B-cell development (taken from (Edwards & Cambridge, 2006))
GC: germinal center.

The earliest cell committed to B-lymphocyte lineage is the pro-B cell and the commitment relies on the transcription factor PAX5 (paired-box protein 5). The stage of pro-B cell is characterized by the rearrangement of the heavy chain gene segments, encoding the μ chain, in a process dependent on RAG1/2 (Pieper et al, 2013) and that occurs in two steps: 1) in the early pro-B cells: arrangement of diversity (D) and joint (J_H) gene segments; 2) in the late pro-B cells: arrangement of variable (V_H) gene segments and combination to already rearranged DJ_H segments. The complete H-chain is part of the pre-BCR, which contains surrogate light chains (λ5 and VpreB) and whose function is tested before the cells enter the next developmental stage. Regular function of pre-BCR and BCR requires, among other molecules, BLNK (B-cell linker or B-cell adapter containing SH2 domain protein) and BTK (Bruton tyrosine kinase), which are essential to connect the BCR signaling to downstream events, mainly through PLC-γ activation (Pieper et al, 2013). BTK is a key molecule downstream the BCR and pre-BCR, that phosphorylates PLCγ2 and ultimately promotes the activation of NFAT, NF-κB and the extracellular signal-regulated kinases ERK1 and ERK2. BLNK acts as a scaffold protein for several signaling molecules including BTK and PLCγ2 (reviewed in (Hendriks et al, 2014)).

The roles of BTK and B-cell linker protein (BLNK) in B-cell development in humans were confirmed through the study of PIDs with predominant antibody deficiency. Inadequate function of BTK (X-linked agammaglobulinemia, XLA) or BLNK cause profound defect in B-cell differentiation after the stage of pre-B cells and result in mature B-cell lymphopenia, severe reduction of all immunoglobulin subtypes and predisposition to recurrent bacterial infections early in life (Bruton, 1952; Minegishi et

al, 1999; Vetrie et al, 1993). The phenotypes displayed by such patients validate the important functions of the respective protein products in the signaling through the pre-BCR and BCR, and consequently in B-cell maturation in the bone marrow (Murphy et al, 2008; Pieper et al, 2013).

Only cells bearing productively rearranged pre-BCR are able to enter to the next stage of pre-B cell, in which the light chain gene segments, encoding the κ and λ chains, are rearranged. Analogous to the rearrangement of heavy chain genes, this process also requires RAG1/2. The resulting molecule is an immunoglobulin M (IgM) formed by two heavy (Igμ) and two light chains (Igκ or Igλ) and corresponds to the BCR expressed on the surface of immature B cells (Figure 2) (Pieper et al, 2013).

Functional BCR with no strong self-reactivity can trigger changes in the gene expression profile and entitle cell migration to peripheral lymphoid organs, such as spleen, lymph nodes and others. Several cell types present in the spleen secrete BAFF, which deliver survival signals to the now termed transitional B cells through BAFFR. This group will further differentiate into follicular and marginal zone (MZ) B-cells (Pieper et al, 2013).

Follicular B cells constitute the majority of the cells in secondary lymphoid organs. During infections, encounter with antigens stimulate follicular B cells to proliferate and form germinal centers (GC) of secondary follicles. In the GC additional mechanisms of BCR diversification dependent on T follicular helper (T_{FH}) cells, such as class switch recombination (CSR) and somatic hypermutation (SHM), take place (explained below). Stimulated follicular B cells subsequently develop into high-affinity and class-switched memory B cells or plasma cells (Murphy et al, 2008; Pieper et al, 2013).

The marginal zone (MZ) region of the spleen surrounds the follicles and contains the human MZ B cells. This region, in which blood-borne pathogens are captured, is rich in macrophages and also contains some T cells. MZ B cells mount immune responses independent of T cells and exhibit surface IgM bearing antigen-independent SHM. Those cells constitute a first line defense against microorganisms present in the blood since they rapidly mature to IgM-secreting short-lived plasma cells upon antigen experience (Pieper et al, 2013) (Murphy et al, 2008).

The survival and homeostasis of B cells in the periphery relies not only on functional BCR but also on the B-cell activator of the TNF family (BAFF). BAFF interacts with the TNF-receptor superfamily members BAFFR (BAFF receptor), TACI (transmembrane activator and CAML interactor) and BCMA (B-cell maturation

factor). This complex receptor-ligand system additionally contains a proliferation-inducing ligand (APRIL), which binds TACI and BCMA but not BAFFR (Pieper et al, 2013). The essential requirement for BAFF in regulating peripheral B-cell survival and apoptosis is supported by the reduced peripheral B cells and defective B-cell responses found in Baff-deficient mice (Schiemann et al, 2001), by the disturbed B-cell survival, mainly of the later stages of B cells, observed in BAFFR-deficient patients (Warnatz et al, 2009) and by the variable antibody deficiency phenotypes in mice and humans carrying *TNFRSF13B* mutations, encoding TACI (Castigli et al, 2005; Salzer et al, 2005; von Bulow et al, 2001).

Differently from the TCRs, which require processing and MHC antigen presentation, B-cell receptors are able to identify and respond to extracellular antigens in their native form, upon recognition of so-called antigenic determinants or epitopes. While some antigens can directly trigger B-cell responses, others require assistance of T_{FH} lymphocytes to induce B-cell differentiation into plasma cells. T-cell-independent (also known as thymus-independent) antigens are characterized by repeating epitopes able to cause massive cross-linking of surface IgM and sufficient for B-cell activation. T-cell-independent B-cell responses are important to protect the host mainly against extracellular bacteria (Murphy et al, 2008).

Response to thymus-dependent antigens in GC requires antigen internalization, degradation and further peptide expression on cell surface associated to MHCII molecules. The associated presentation of antigens and MHCII on the surface of B cells enables that antigen-specific T_{FH} cells recognize the degraded antigen fragments. The T-B-cell interaction triggers in T_{FH} cells synthesis and expression of CD40L. Binding of CD40L to CD40 expressed on B cells provides a second activating signal to those cells and induces proliferation, survival, development of germinal centers and class switching (Murphy et al, 2008; Sun, 2011).

While somatic mutations that rearrange genes of BCR heavy and light chains are independent of antigen stimulation and generate the primary antibody repertoire of B cells, secondary DNA modifications in activated B cells in the GC are antigen-dependent. Class switch recombination (CSR) and affinity maturation, from which somatic hypermutation (SHM) is the major component, are responsible for generating diversification of the immunoglobulin repertoire. Both of these are dependent on the DNA-editing enzyme activation-induced cytidine deaminase (AID or AICDA) expressed upon CD40 signaling (Kinoshita & Honjo, 2001; Murphy et al, 2008; Pieper et al, 2013).

T_{FH}-cell-dependent B-cell-activation, through CD40L-CD40 interaction, generates long-lived class-switched memory B cells and antibody-producing (plasma) cells. During CSR, the primary constant region of the heavy chain (Cμ) is replaced with C regions of different isotypes (Cγ, Cε or Cα) by a DNA recombination mechanism that modifies the functional activity of the antibody without changing antigen specificity. SHM on the other hand alters the rearranged variable region of the immunoglobulin, involved in antigen recognition, by introducing point mutations in the light and heavy V chains. This process therefore gives rise to immunoglobulins with improved affinity to specific antigens. Consequently, antibodies directed to thymus-dependent antigens tend to have higher affinity and demonstrate more flexible functions compared to antibodies against thymus-independent antigens (Kinoshita & Honjo, 2001; Murphy et al, 2008; Pieper et al, 2013).

X-linked-CD40L and AR-CD40 deficiency have been described in individuals with hyper IgM syndromes (HIGM), combined immunodeficiencies characterized by early onset of recurrent bacterial and opportunistic infections, often associated with liver and/or biliary tract disease. Immunological findings include severely reduced IgG and IgA levels with normal or increased IgM. Peripheral class-switched (IgD^-) and memory ($CD27^+$) B cells as well as germinal centers are characteristically absent, confirming the influence of CD40-CD40L signaling pathway to the thymus-dependent GC reactions (Allen et al, 1993; Ferrari et al, 2001; Gulino & Notarangelo, 2003).

Recognition of HIGM associated with germinal center hyperplasia and absence of CSR and SHM in subjects bearing mutations in *activation-induced cytidine deaminase* (*AICDA*) denotes the crucial requirement of this DNA-editing enzyme for both mentioned processes and for terminal B-cell differentiation (Revy et al, 2000). Beside AICDA, CSR also requires uracil DNA glycosylase (UNG) to remove the cytosine deaminated into uracil by AICDA. Defective UNG function also leads to HIGM with severe CSR impairment (Imai et al, 2003).

Besides the first signal for activating naïve cells, provided by the interaction of the receptor with the respective antigen, additional associated stimuli are required for optimal lymphocyte activation, among them signals provided by the B-cell co-receptor complex and the so-called second signals (Murphy et al, 2008).

Active complement components bound to antigens are also able to amplify B-cell activation through engagement of the B-cell co-receptor complex. When antigens in complex with C3d or C3dg bind to the BCR, CD21 brings together the complex and the antigen receptor. Subsequent signaling through CD19 activates intracellular

events that enhance B-cell responses (Dunkelberger & Song, 2010; Murphy et al, 2008). The non-essential function of CD19, CD21 and CD81 in B-cell development and their relevant roles in B-cell activity are endorsed by the B-cell-PID observed in individuals with mutations in the respective genes. Despite normal counts of peripheral B lymphocytes, such patients exhibit impaired affinity maturation and antibody responses (Thiel et al, 2012; van Zelm et al, 2006; van Zelm et al, 2010).

Co-stimulatory pathways, which deliver inhibitory or activating downstream signals according to the molecules involved, provide the second signals for lymphocyte activation. Positive co-stimulatory molecules include B7 (CD80/CD86), CD40, ICOSL and OX40L expressed on the surface of APCs, including B cells, respectively interacting with CD28, CD40L, ICOS and OX40 in T cells. Negative pathways include B7 interaction with CTLA4 on the surface of activated T cells, and interaction of PD-1 (programmed cell death 1) expressed in activated T and B cells with PD-L1/2 in APCs. Balance between such positive and negative are important for controlling lymphocyte activation (reviewed in (Huang & Yang, 2011)).

Inducible T cell co-stimulator (ICOS), expressed on activated T cells including T_{FH} cells, interacts with ICOSL that is constitutively expressed in a broad range of cells, including B cells. Signaling downstream ICOSL is necessary for T-cell-dependent terminal-B-cell differentiation in GC, which is corroborated by the predominant antibody deficiency caused by *ICOS* mutations in humans, who present with poorly formed GC as well as markedly decreased $CD27^{+}IgD^{-}$-class-switched memory B cells, reminiscent of CD40 and CD40L deficiency (Grimbacher et al, 2003; Warnatz et al, 2006).

Another evidence for the significance of co-stimulatory pathways in immunity is the human autosomal recessive deficiency of OX40, which prompt impaired immune response to human herpes virus 8 and reduced memory CD4+T cells (Byun et al, 2013).

1.2.3 Overview on mechanisms of immune tolerance

TCR and BCR gene rearrangements allow the generation of receptors able to recognize a great variety of antigens, including self-antigens. In order to avoid aberrant immune responses to cause damage to the host, induction of self-tolerance is necessary (Murphy et al, 2008). In both thymus and bone marrow, mechanisms of

central tolerance take place, resulting in the selection of immature T and B lymphocytes to continue the maturation process. The aim of such process is to direct the adaptive immune responses towards pathogen elimination instead of host tissue damage. Since not all self-reactive lymphocytes are eliminated in the thymus and bone marrow, peripheral mechanisms are also required to induce tolerance when self-reactive lymphocytes recognize the corresponding host antigen in the periphery (reviewed in (Hogquist et al, 2005)).

In the thymus, TCRs that are able to recognize MHC molecules associated with self-antigens are positively selected to undergo further differentiation. Negative selection mechanisms, mostly deletion, eliminate progenitors with strong self-reactivity. In addition to cell death, receptor editing, anergy or immunological ignorance are other possible fates for self-reactive lymphocytes (Hogquist et al, 2005; Murphy et al, 2008).

Disruption of tolerance mechanisms in several PIDs may result in manifestations of immune dysregulation such as autoimmunity, lymphoproliferation and susceptibility to malignancies**.** Association of PIDs with autoimmune features can result not only from a breakdown of central or peripheral tolerance but also from abnormal inflammatory responses due to inefficient pathogen elimination (reviewed in (Notarangelo et al, 2006)).

Positive and negative selections in the thymus for instance are orchestrated by the autoimmune regulator (AIRE), which controls the transcription of self-antigens in medullary thymic epithelial cells (mTECs) and allows clonal deletion of autoreactive T cells (Gupta & Louis, 2013). Disturbed selection of thymocytes resulting from mutations in *AIRE* prompts a syndrome with severe immune dysregulation called autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) (Nagamine et al, 1997). The phenotype is variable, but the patients characteristically present with chronic mucocutaneous candidiasis, chronic hypoparathyroidism and adrenal insufficiency (reviewed in (De Martino et al, 2013)).

Defective CD40-CD40L signaling is also implicated in susceptibility to autoimmune manifestations in mice and humans, for instance as a result of altered clonal deletion of autoreactive thymocytes, inadequate development of regulatory T (T_{reg}) cells, as well as impaired peripheral B-cell tolerance (reviewed in (Jesus et al, 2008)). Additionally, increased inflammatory responses due to impaired pathogen clearance may be involved in the sclerosing cholangitis observed in patients with CD40L or CD40 deficiency (Notarangelo et al, 2006).

T_{reg} cells are self-reactive T cells positively selected to mature and migrate to the periphery where they control immune responses to self-antigens. The commitment to the regulatory T-cell lineage depends on the expression of the transcription factor forkhead box P3 (FOXP3) (reviewed in (Fontenot & Rudensky, 2005; Gupta & Louis, 2013)). Mutations in *FOXP3* result in a severe disease known as immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome (Bennett et al, 2001), marked by very early onset of severe diarrhea, eczema and polyendocrinopathy, caused by the absence of T_{reg} cells (reviewed in (Gambineri et al, 2008)).

Experimental data demonstrate that not only thymic DCs and mTECs but also B lymphocytes found in the thymus are implicated in central T-cell tolerance (reviewed in (Yamano et al, 2015)). The thymic B cells hold particular features that distinguish them from their peers in spleen and lymph nodes, namely high expression of MHCII and co-stimulatory molecules CD80 and CD86, as well as expression of Aire. Those characteristics are induced upon B-cell interaction with $CD4^+$T cells, require CD40 signaling and entitle thymic B cells to have non-redundant roles in negative selection of $CD4^+$T cells and in induction of T_{reg} cells (Yamano et al, 2015).

Similarly to APECED and IPEX, the autoimmune lymphoproliferative syndromes (ALPS) are also characterized by autoimmunity caused by disruption in tolerance mechanisms (Gupta & Louis, 2013). ALPS comprise different disorders in which programmed cell death of activated lymphocytes is defective. The characteristic manifestations of affected subjects include lymphadenopathy, splenomegaly and autoimmunity, predominantly autoimmune cytopenias (reviewed in (Turbyville & Rao, 2010)).

Central B-cell tolerance takes place in the bone marrow where, similar to the T-cell process, B lymphocytes are selected to continue or not the maturation process. The first screen for self-reactivity occurs on occasion of BCR testing in immature B cells, during which cells bearing productive antigen receptors with weak self-reactivity receive signals that enable their migration to the periphery, while strongly self-reactive cells are eliminated (reviewed in (Meffre, 2011)). The increased frequency of autoreactive B-cell clones observed in XLA patients endorses the requirement of functional BCR signaling through BTK in reduction of polyreactive and antinuclear clones at this stage (Ng et al, 2004).

In addition to BCR signaling, regulation of central B-cell tolerance also relies on self-antigen recognition by TLRs expressed on B-lymphocyte surface. The increased

autoreactive mature B cells observed in subjects bearing mutations in *UNC93B*, *IRAK4* and *MYD88* support this finding. These individuals however do not suffer from autoimmune diseases and their autoreactive B cells do not secrete antinuclear or autoreactive antibodies (Isnardi et al, 2008). On the other hand, increased frequency of autoreactive antibodies associated with autoimmunity in AICDA-deficient HIGM patients indicates the influence of this enzyme in B-cell tolerance (Meyers et al, 2011).

Although B-cell tolerance in the bone marrow eliminates most of the antinuclear and polyreactive B cells, a second removal of autoreactive B cells in the periphery (between the stages of new emigrant and mature naïve cells) is necessary, since healthy donors can exhibit up to 5.6% and 11% of new emigrant B cells with polyreactive or anti-nuclear antibodies, respectively (Meffre, 2011). In the periphery, BCRs binding strongly to self-antigens can be excluded from B-cell follicles, while those with intermediate affinity undergo functional anergy through enhanced receptor internalization. Additionally, differentiation of self-reactive B cells into plasma cells is inhibited in the presence of high concentration of self-antigens inside the germinal centers (Gupta & Louis, 2013).

Peripheral B-cell tolerance relies, among other factors, on CD4+T cell signals and on regulated BAFF expression. The important roles of helper T cells in eliminating self-reactive mature naïve B cells are validated by the increased expression of autoreactive antibodies in CD40L- and MHCII-deficient patients (Herve et al, 2007). Analogously, increased Baff expression in mice disturbs the peripheral B-cell homeostasis and prompts autoimmune disorders, by inhibiting new migrant/transitional B cells with autoreactive properties to be eliminated and possibly also by promoting proliferation of seal-reactive clones (Mackay et al, 1999). Individuals carrying mutations in CD40L, MHCII and AICDA also exhibit increased serum levels of BAFF, which may explain their high proportion of autoreactive mature naïve B cells (Herve et al, 2007; Meyers et al, 2011).

1.3 Immune system intracellular signaling

Apart from extracellular events, the recognition of threats by surface receptors expressed by immune cells prompts responses that involve transmission of intracellular signals initiated by the receptor itself or by associated transmembrane proteins. Downstream signaling in response to receptor activation is carried out by diverse pathways, which differ according to the cell type, stimuli, etc. Distinct surface receptors exhibited by cells from innate or adaptive immune system, such as Toll-like receptors (TLR), antigen receptors (TCR and BCR) and cytokines receptors, are able to engage variable protein complexes and lead to diverse cellular responses. Transmission of intracellular signals downstream those surface receptors enroll networks that include, among others, phospholipid-cleaving enzymes called phospholipase C (PLC) and mitogen-activated kinases (MAPKs) (Murphy et al, 2008).

Cleavage of plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP2) and generation of the so-called second messengers, IP3 (inositol 1,4,5-trisphosphate) and DAG (diacylglycerol), are the predominant results of PLC activation. IP_3 and DAG engage important intracellular pathways, which will induce transcription of specific genes involved in cell activation and proliferation. Among others, the activator protein 1 (AP-1), the transcription factor nuclear factor kappa B (NF-κB), and the nuclear factor of activated T cells (NFAT), respectively, are engaged downstream IP_3 and DAG (reviewed in (Koss et al, 2014; Murphy et al, 2008)).

Phospholipase C, gamma (PLC-γ), one of the six PLC families (β, γ, δ, ε, η and ζ), is recruited and phosphorylated in important signaling pathways initiated by a variety of extracellular stimuli. Following antigen signaling and PLC-γ activation, the cascades leading to nuclear translocation of AP-1 and NF-κB involve activation of a variety of DAG-binding proteins including RAS guanyl-releasing protein (RASGRP) and protein kinase C (PKC), respectively (Murphy et al, 2008).

Two isoforms of PLC-γ are known, PLC-γ1 and PLC-γ2. PLC-γ1 is ubiquitously expressed, activated mainly by growth factors and has important functions in T- and NK-cell activity. PLC-γ2, on the other hand, is expressed in cells of the hematopoietic lineage, is activated by immune cell receptors (except TCR activation, which involves PLC-γ1) and has significant roles in B cells, NK cells, mast cells and platelets (Koss et al, 2014; Murphy et al, 2008). Recently reported human *PLCG2* mutations, encoding PLC-γ2, establish a connection between PLC-γ2-dependent pathways and

B cell homeostasis, since the described patients suffer from antibody deficiency with or without autoimmunity (Ombrello et al, 2012; Zhou et al, 2012).

The MAPKs are serine-threonine kinases involved in a number of cell activities such as proliferation, differentiation, survival and death (reviewed in (Kim & Choi, 2010)). The signaling cascade of all MAPKs shares similar features starting with the activation of a small G protein by a guanine-nucleotide exchange factor (GEF), systematic activation of three other kinases, activation of nuclear transcription factors and induction of gene expression (Murphy et al, 2008).

The three-kinase relay system consists of sequential activation of kinases that can phosphorylate and activate several other proteins and transcription factors, starting with MAP3K (MAP kinase kinase kinase or MAPKKK), then MAP2K (MAP kinase kinase or MAPKK) and MAPK (Murphy et al, 2008). In mammals, the MAPKs comprise ERK (extracellular signal-regulated kinase), p38, and JNK (c-Jun NH2-terminal kinase), each of them found in several isoforms (Kim & Choi, 2010). The RAS-RAF-MEK-ERK pathway is a well-known example of MAP kinase cascade activated in T and B cells in response to antigen signaling. After DAG activation of RASGRP, this GEF activates small G protein RAS, which in turn phosphorylates RAF (MAPKKK). The following kinases in this cascade are MEK (MAPKK) and ERK (MAPK). Phosphorylated ERK translocates to the nucleus and is responsible for the phosphorylation of transcription factors that activates genes involved in cell proliferation and differentiation (Murphy et al, 2008).

1.3.1 Protein kinase C family

The protein kinase C (PKC) family consist of 10 isoforms of conserved serine-threonine kinases that are critical signal transduction mediators in several pathways involved in cell proliferation, differentiation, migration, survival and apoptosis. PKC proteins contain five variable regions (V1 to V5) alternating with 4 constant regions (C1 to C4). In the protein structure a variable amino-terminal regulatory domain is linked to a carboxi-terminal kinase domain (KD) by a hinge region (V3). The regulatory domain contains two conserved membrane-targeting regions, C1 and C2, and a pseudosubstrate sequence. The catalytic domain comprises conserved regions C3 and C4, which are essential for ATP/substrate binding and catalytic activity (reviewed in (Duquesnes et al, 2011; Rosse et al, 2010)).

The PKCs can be divided in 3 groups according to the domain composition and activation pattern: conventional or classical (cPKC), novel (nPKC) and atypical (aPKC). PKCα, PKCβ1 and PKCβ2 are classical PKCs, activated in response DAG and phospholipids binding to C1 domain and phospholipids binding to C2 domain, in a Ca^{2+}-dependent process. DAG also activates the group of nPKCs, which consists of PKCδ, PKCε, PKCθ and PKCη. Those forms however do not respond to Ca^{2+} (Rosse et al, 2010) since the C2 domain of nPKCs, called novel C2 or C2-like domain lacks important residues for Ca^{2+} sensitivity (Pappa et al, 1998). The group of aPKCs contains the isoforms PKCι/λ and PKCζ and their activation occurs independently of both DAG and Ca^{2+} (Rosse et al, 2010).

Since nPKCs are regulated by only one cofactor they exhibit higher affinity to DAG compared to conventional isoforms granted by the change of a conserved tyrosine in position 22 by tryptophan. This modification causes increased sensitivity to DAG in nPKCs independently of the membrane recruitment by the classical C2 domain (Ca^{2+}-regulated) (reviewed in (Duquesnes et al, 2011; Newton, 2010)).

In non-stimulated cells, PKCs are typically found in the soluble fraction as inactive folded proteins, with the pseudosubstrate domain localized in the substrate-binding pocket (reviewed in (Steinberg, 2004)). Upon cell stimulation, different combination of tyrosine residues are phosphorylated according to the isoform and the stimuli (reviewed in (Kikkawa et al, 2002)). Full PKC maturation and activation however requires consecutive phosphorylation of 3 conserved serine/threonine residues: activation loop, turn and hydrophobic motif (reviewed in (Griner & Kazanietz, 2007)).

Upon cell activation and cleavage of membrane inositol phospholipids into DAG and IP_3, the latter diffuses to the cytosol, engages endoplasmic reticulum receptors and open calcium channels, increasing concentration of intracellular calcium (reviewed in (Murphy et al, 2008)). The regulatory domain of mature cPKC is recruited to the plasma membrane where C2 domain binds to Ca^{+2}, facilitating the protein interaction to the membrane and the C1 domain interaction with DAG (Steinberg, 2004). PKC-DAG interaction results in conformational changes that displace the pseudosubstrate from its localization and allow protein activation (reviewed in (Duquesnes et al, 2011; Rosse et al, 2010)). Simplified mechanisms of activation and inactivation of PKCs are illustrated in figure 3.

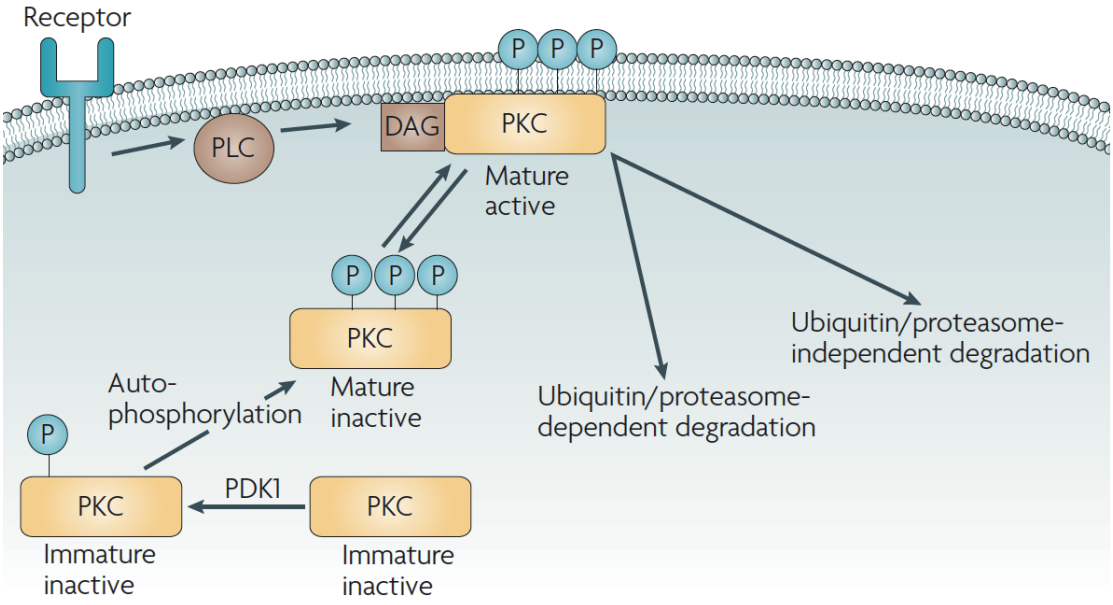

Figure 3. PCK activation and inactivation mechanisms (taken from (Griner & Kazanietz, 2007)) PLC: phospholipase C; DAG: diacylglycerol; PKC: protein kinase C.

Most cell types can co-express several PKC isoforms and the function specificity of the isoforms *in vivo* depends on their interaction to anchoring proteins in the membrane, which place the kinases close to their activators and/or substrates (Steinberg, 2004). Unlike other PKCs with expression restricted to some cell types such as the nPKC PKCθ, mainly found in muscle and hematopoietic cells, the delta isoform (PKCδ) is expressed in diverse cells and tissues, indicating a broad role of PKCδ in the organism (Kikkawa et al, 2002). In response to diverse stimuli, PKCδ can localize in different organelles in the cell, especially in the nucleus and mitochondria (reviewed in (Yoshida, 2007)). Among multiple physiological functions, PKCδ has been demonstrated to reduce proliferation, and induce cell cycle arrest and apoptosis (Steinberg, 2004).

1.3.2 NF-κB pathways

The NF-κB family comprises a group of essential inducible transcription factors downstream cell signaling pathways that controls survival, differentiation and proliferation of a variety of cells, including cells of the immune system. In mammals, the NF-κB family is composed by c-rel, RelA (p65), RelB, NF-κB1 (p50) and NF-κB2 (p52). Each family member can be combined in homo or heterodimers with individual

functional characteristics. The NF-κB pathways can be divided in canonical and non-canonical (Figure 4), in both of which the transcription regulation is mediated by binding of a different set of activated dimers to kappaB (κB) consensus elements present in target genes (reviewed in (Gerondakis et al, 2014; Hayden & Ghosh, 2012)).

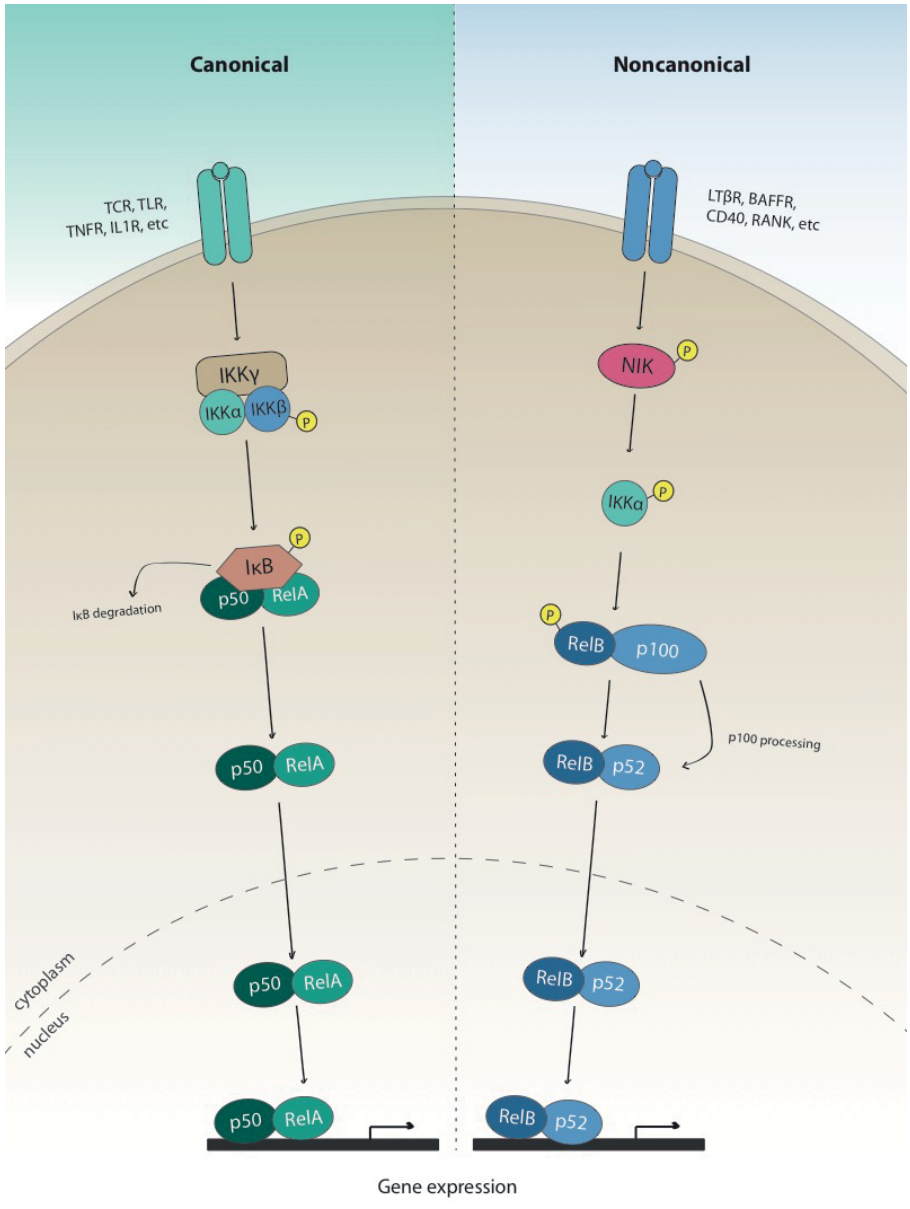

Figure 4. Classical and alternative NF-κB pathways (adapted from (Sun, 2012)) TCR: T-cell receptor; TLR: Toll-like receptor; TNFR: Tumor necrosis factor receptor; IL1R: Interleukin-1 receptor; LTβR: Lymphotoxin-β receptor; BAFF: B-cell activator of the TNF-α family receptor; RANK: Receptor activator of nuclear factor kappa-B; IKK: IκB serine kinase; IκB: inhibitor of κB.

The canonical or classical NF-κB pathway is activated through a number of receptors such as antigen receptors, pattern recognition receptors (e.g. TLR) and cytokine receptors (e.g. TNFR, IL1R). As a result of the variety of receptors involved, engagement of classical NF-κB pathway occurs in many cell types and its activation typically induces inflammation, cell survival and proliferation (Hayden & Ghosh, 2012).

Most NF-κB subunits are found inactive in the cytosol bound to IκB (NF-κB inhibitor). Activation of IκB serine kinase β (IKKβ) promotes phosphorylation, ubiquitination and degradation of the IκB, enables dimer translocation to the nucleus and subsequently gene transcription regulation. The IKKβ is found in a complex with other IKKs, IKKα and IKKγ (NF-κB essential modulator, NEMO), the latter being a regulatory protein critical for activation of the canonical NF-κB pathway (Hayden & Ghosh, 2012).

Defect of key components from NF-κB cascades inducing PID in humans illustrates the multifaceted roles of such pathways in the immune system. Although IKKα also have NF-κB-independent functions in epidermal differentiation (Descargues et al, 2008), the anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) caused by gain-of-function (GOF) mutations in *IKBA* (*NF-κB inhibitor, alpha*) or X-linked (XL) mutations in *NEMO* (*IKBKG*) are examples of such functional diversity. EDA-ID is a combined immunodeficiency (CID) characterized by early onset of skin, teeth and sweat glands abnormalities associated with recurrent infections due to diverse pathogens (Courtois et al, 2003; Doffinger et al, 2001; Zonana et al, 2000). Mutations in *IKBKB,* encoding IKKβ, have also been described to compromise NF-κB signaling and to result in a disease with a SCID phenotype with defects in innate and adaptive immunity (Pannicke et al, 2013).

Compared to the classical NF-κB pathway, the activation of the alternative or non-canonical pathway is restricted to a limited number of receptors, including members of the TNF receptor superfamily LTβR, BAFFR, CD40, RANK (receptor activator of nuclear factor kappa-B), TWEAKR, TNFR2, CD30 and CD27. This group of receptors contains a motif for TRAF binding (mainly TRAF2 and 3), that recruits TRAF to the receptor complex and allows its degradation and subsequent NF-κB activation (Sun, 2011). The functions of the alternative pathway are also more limited than the canonical since they have been so far mainly implicated in the development of secondary lymphoid organs, differentiation of T lymphocytes and thymic epithelial cells, and maturation and survival of B cells (reviewed in (Sun, 2012)).

The non-canonical NF-κB pathway is also slower than the classical pathway since its activation depends on the synthesis and accumulation of a specific mitogen-activated protein kinase kinase kinase (MAP3 kinase), the NF-κB-inducing kinase (NIK), which in unstimulated cells is suppressed by TRAF3 containing ubiquitination complex. Upon receptor activation, TRAF3 degradation and NIK accumulation, in conjunction with NIK-dependent IKKα (inhibitor of NF-κB kinase α) phosphorylation lead to processing of p100 into p52 independently of IKKβ or NEMO (Sun, 2012; Xiao et al, 2001).

NIK phosphorylates Ser176 of IKKα (Ling et al, 1998) and both activated proteins induce phosphorylation of p100 (Senftleben et al, 2001) at serine 866 and 870 (Liang et al, 2006). The active proteolytic processing of p100 is inducible and involves disruption of regulatory domains present in the carboxi-terminal portion of the protein, which seem to prevent the translocation of p100 to the nucleus by holding the molecule in a structural conformation protecting the nuclear localization sequence. p100 processing allows generation of p52-RelB dimers, the hallmark dimers of the non-canonical NF-κB signaling, which are able to enter the nucleus and to induce gene transcription (reviewed in (Sun, 2012)).

1.4 PIDs as resources for immune system investigation

Experimental animal models have immensely contributed to uncovering diverse aspects of immunity, however potential different roles for specific molecules or pathways among species and the distinct conditions of infections (e.g. natural versus experimental) prevent many of the animal findings to be directly extrapolated to humans (reviewed in (Casanova & Abel, 2004; Conley & Casanova, 2014)). This idea is sustained by several PIDs whose phenotype in mice does not completely overlap with the manifestations observed in humans, for example deficiency in IL-7 receptor (Puel et al, 1998), IRAK4 (Picard et al, 2003a), MYD88 (von Bernuth et al, 2008), PIK3R1 (Conley et al, 2012), IKBKB (Pannicke et al, 2013), JAGN1 (Boztug et al, 2014), INO80 (Kracker et al, 2015), and others.

Therefore, investigation of humans presenting with PIDs, enables direct evaluation of protein functions in humans (reviewed in (Quintana-Murci et al, 2007)). These studies complement the knowledge acquired by experimental models. Assessment of the link between human genes and their products to specific cellular processes has been boosted since the sequencing of the first human genomes in the early 2000s and subsequently, it has become a major interest in biomedical research (reviewed in (Lander, 2011)).

The study of human cells by means of *in vitro* experiments allows the functional examination of specific immune mechanisms in our species. Several genetic loss-of-function-based screenings such as gene RNA interference (Berns et al, 2004), insertional mutagenesis in near haploid cell lines (Carette et al, 2009) and genome-editing (Shalem et al, 2014; Wang et al, 2014) allow the functional study of particular genes in human cells. All those methods however carry technical issues that limit their widespread use (Carette et al, 2009; Jackson et al, 2006; Wright et al, 2014; Zhang et al, 2014).

Spontaneous genetic defects in humans on the other hand allow the study of infections as they occur in nature and enable a detailed observation of the phenotype during the course of the disease in genetically variable hosts. Naturally occurring inborn defects of the immune system constitutes therefore unique opportunities for understanding essential features of immunity in humans, and the use of molecular genetic techniques in the past decades allowed the uncovering of several important players not only in immune responses against external agents but also in immune regulation (reviewed in (Casanova & Abel, 2004; Fudenberg et al, 1971)).

2 AIMS OF THIS THESIS

The general aim of this thesis is to contribute to the generation of knowledge regarding mechanisms involved in human immune responses to infections and immune regulation, possibly with future clinical applications. The specific aims of this work are the following:

- to investigate underlying genetic mutations involved in human primary immunodeficiencies, using biological material from the affected individuals (and family members) and derived cell lines;
- to characterize inheritance pattern and molecular cause of hitherto unknown primary immunodeficiencies in patients from consanguineous families employing modern genetic techniques such as single nucleotide polymorphism-array based whole genome homozygosity mapping and exome sequencing;
- to evaluate the roles of the newly described mutations in immune response and regulation and the consequences of the defect in the pathways involved, placing the generated knowledge into the context of previously characterized process from animal or *in vitro* studies.

3 RESULTS

3.1 A novel mutation in the complement component 3 gene in a patient with selective IgA deficiency

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* equal contribution

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ORIGINAL RESEARCH

A Novel Mutation in the *Complement Component 3* Gene in a Patient with Selective IgA Deficiency

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Abstract
Purpose Immunological and molecular evaluation of a patient presenting with recurrent infections caused by *Streptococcus pneumoniae* and low complement component 3 (C3) levels.
Methods Immunological evaluation included complement components and immunoglobulin level quantification as well as number and function of T cells, B cells and neutrophils. Serotype-specific immunoglobulin G antibodies against *S. pneumoniae* capsular polysaccharides were quantified by ELISA in serum samples before and after vaccination with unconjugated polysaccharide vaccine. For the molecular analysis, genomic DNA from the patient and parents were isolated and all exons as well as exon-intron boundaries of the *C3* gene were sequenced by Sanger sequencing.
Results A 16-year-old male, born to consanguineous parents, presented with recurrent episodes of pneumonia caused by *S. pneumoniae* and bronchiectasis. The patient showed severely reduced C3 and immunoglobulin A levels, while the parents showed moderately reduced levels of C3. Mutational analysis revealed a novel, homozygous missense mutation in the *C3* gene (c. C4554G, p. Cys1518Trp), substituting a highly conserved amino acid in the C345C domain of C3 and interrupting one of its disulfide bonds. Both parents were found to be carriers of the affected allele. Vaccination against *S. pneumoniae* resulted in considerable clinical improvement.

Elisangela Santos-Valente and Ismail Reisli contributed equally and should be considered *aequo loco.*

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Conclusions We report a novel homozygous mutation in the *C3* gene in a patient with concomitant selective IgA deficiency who presented with a marked clinical improvement after vaccination against *S. pneumoniae*. This observation underlines the notion that vaccination against this microorganism is an important strategy for treatment of PID patients, particularly those presenting with increased susceptibility to infections caused by this agent.

Keywords Complement deficiency · complement component 3 · *Streptococcus pneumoniae* · primary immunodeficiency

Abbreviations

C3	Complement component 3
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
IgA	Immunoglobulin A
IgG	Immunoglobulin G
PID	Primary immunodeficiency

Introduction

Primary immunodeficiencies (PIDs) are a heterogeneous group of inherited disorders of the immune system leading to enhanced susceptibility to infections [1]. The complement system is a crucial component of innate immunity and one of the main effector mechanisms of antibody-mediated immunity (reviewed in [2]).

Inherited complement deficiencies represent immunodeficiencies characterized by susceptibility to invasive infections by encapsulated bacteria such as *Streptococcus pneumoniae* (reviewed in [3–5]). The third component of the complement system (C3) is indispensable to all the known pathways of complement activation. C3 deficiency (OMIM: 120700) is a rare PID, leading to predisposition to recurrent pyogenic infections [1, 4].

A few biallelic defects in the *C3* gene have been described in patients suffering not only from *S. pneumoniae* infections [6–12] but also from autoimmune and immune-complex-related disorders, in particular affecting the kidney [13–15]. A similar phenotype can also be observed in patients with deficiency of complement factor H or I, respectively [5].

Here, we describe a patient with selective immunoglobulin A (IgA) deficiency presenting with recurrent airway infections caused by *S. pneumoniae* and bronchiectasis with no autoimmune or immune complex manifestations. Our molecular analyses revealed that the patient suffers from C3 deficiency caused by a novel, homozygous mutation in the *C3* gene.

Methods

Ethics Committee

This study has been approved by the Ethics Committee at the Medical University of Vienna, Austria. The patient and the other family members gave informed consent to the genetic analysis described here. Clinical data from the patients were provided in anonymized form by the responsible physician(s).

Determination of Antibody Titers Against *Streptococcus pneumoniae*

Measurement of capsular polysaccharide serotype-specific immunoglobulin G (IgG) antibodies against *S. pneumoniae* was performed by enzyme-linked immunosorbent assay (ELISA) in serum samples before and 6 weeks after vaccination with *PNEUMO 23*® (unconjugated polysaccharide vaccine against *S. pneumoniae*) as described previously with minor modifications [16]. All sera were analyzed in duplicates in the same ELISA run. In order to eliminate antibodies to cell wall polysaccharides, microtiter plates were coated with capsular polysaccharide antigens (from the American Type Culture Collection (ATCC), Rockville, MD) and the samples were pre-incubated overnight with species-specific common cell wall polysaccharide from *S. pneumoniae* (CWPS; C-polysaccharide purified; Statens Serum Institute, Denmark). Antibody concentrations are indicated as the percentage of reference serum, the hyperimmune plasma pool (U.S. Pneumococcal Reference serum FDA7 CBER, Bethesda, MD) in units per milliliter (U/mL), where the reference plasma pool represents 100 U/mL for each serotype. Since patients with high pre-immunization titers may not generate a drastic increase after immunization, the final concentration of antibodies after immunization (regardless of increase from pre-immunization concentration) was taken into account. A minimal concentration of 20 U/mL in at least 50 % of the serotypes tested was considered as a positive response to the vaccination. This criterion was selected according to results obtained in 40 healthy Turkish children (age range: 5 to 15 years; median: 10 years and mean, 9.7 years) (O. Sanal, unpublished data).

Molecular Analysis

Genomic DNA was isolated from whole blood obtained from the patient and parents using a commercially available kit (Wizard® Genomic DNA Purification Kit, Promega Corporation) according to the manufacturer's instructions.

The primers used for sequencing of the *C3* gene were previously described by Goldberg et al. [10] with one additional pair covering part of exon 41 and the 3'UTR. This additional pair has the following sequences: forward 5′-

ctcagctacatcatcgggaag-3′ and reverse 5′-ccttggctaaagaagtcagca-3′. All primers were purchased from Sigma Aldrich, Austria. Capillary sequencing was performed with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Germany) and analyzed on a 3130×1 Genetic Analyzer (Applied Biosystems). For sequence analysis, Sequencher DNA Software 4.10.1 (Gene Codes Corporation, USA) was used. The nucleotide variations found were further sequenced on both parents in order to evaluate the segregation. PolyPhen2 (Polymorphism Phenotyping v2, http://genetics.bwh.harvard.edu/pph2/) and SIFT (J. Craig Venter Institute, http://sift.jcvi.org/) were used to predict the effect of the mutation on protein function.

Phylogenetic conservation was assessed using protein sequences from Ensembl (http://www.ensembl.org) and UniProt (http://www.uniprot.org/) and aligned using UniProt multiple sequence alignment tool. For the protein modeling, we used Molsoft ICM Browser Pro software and a crystal structure model of the C3 convertase (2WIN) from the Protein Database website (http://www.rcsb.org/pdb/).

Results

Clinical Characterization of Patient and Family

At the age of 16 years, a male Turkish patient born to consanguineous parents (first-degree cousins) as the third of eight children, was admitted to hospital with a history of fever, cough and respiratory distress for 48 h. Physical examination revealed weight and height within normal range, a wound scar resulting from a thoracotomy performed at the age of 6 months, and crackles and bronchial respiratory sounds. A chest X-ray revealed pneumonia (Fig. 1) but no specific infectious agent could be isolated.

With regards to the past medical history, the patient had needed a tube thoracostomy due to pleural effusion at the age of 6 months and had suffered from recurrent lower respiratory infections with a frequency of 4 to 5 times per year. At the age of 8 years, bronchiectasis was detected and posterolateral segmentectomy in the left lower lobe was performed. Subsequently, the frequency of infections was reduced however, in a period of 6 years (from 14 to 20 years) he still presented with two episodes of otitis media and 8 episodes of lower respiratory tract infections for which hospitalization was necessary. At the age of 20 years, the patient was vaccinated for *S. pneumoniae* and has not suffered from severe infections or needed hospitalization since (7 years of follow up so far).

The patient has never presented with hematuria, hypertension or other clinical feature indicative of renal involvement or autoimmune disorder.

Notably, two of his brothers had died during the neonatal period for unknown reasons. His parents, three brothers and two sisters are healthy (see Fig. 2a and Supplementary Figure 1 for pedigrees).

Laboratory Evaluation

The patient had normal red cell counts but elevated erythrocyte sedimentation rate (52 mm/h; reference values: 0–15 mm/h) and C-reactive protein levels (66 mg/dL; reference values: 8.5–10.6 mg/dL), respectively. Total leukocyte (7500/mm^3) and lymphocyte counts (3500/mm^3) were within the normal range. Routine urine analysis and renal function were unremarkable (see Table 1 for blood urea nitrogen and creatinine levels; urine analysis data are not shown). Immunoglobulin M, G and E levels were within the normal range as well as the IgG subclasses, while serum IgA levels were reduced (Table 1). As there were no signs or symptoms of autoimmunity, autoantibody levels were not evaluated.

Six weeks after vaccination for *S. pneumoniae* (20 years of age), the patient demonstrated a positive antibody response (Table 2).

C3 levels were severely decreased in the patient, varying from 8 to 19 mg/dL at 6 different measurements (Table 1; reference values: 90–180 mg/dL), with absent complement hemolytic activity measured using the CH50 test. The other complement levels (C1, C2, C4, C5, C6, C7, C8, C9, Factor H and Factor I) were found to be normal (see Table 1 for C4; other data not shown). The proliferative response of peripheral blood lymphocytes to phytohemagglutinin and the spontaneous and stimulated nitroblue tetrazolium test were normal (Table 1). The parents showed normal levels of IgA and normal CH50, but reduced C3 levels (63 mg/dL and

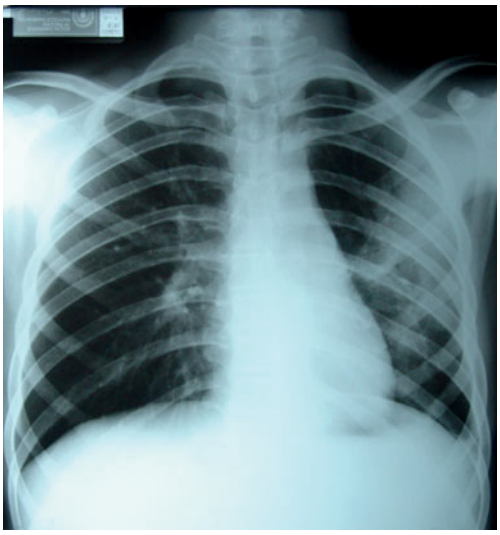

Fig. 1 Lung radiography of the patient at admission showing pneumonia in the left lung and bronchial wall thickening on the right lung

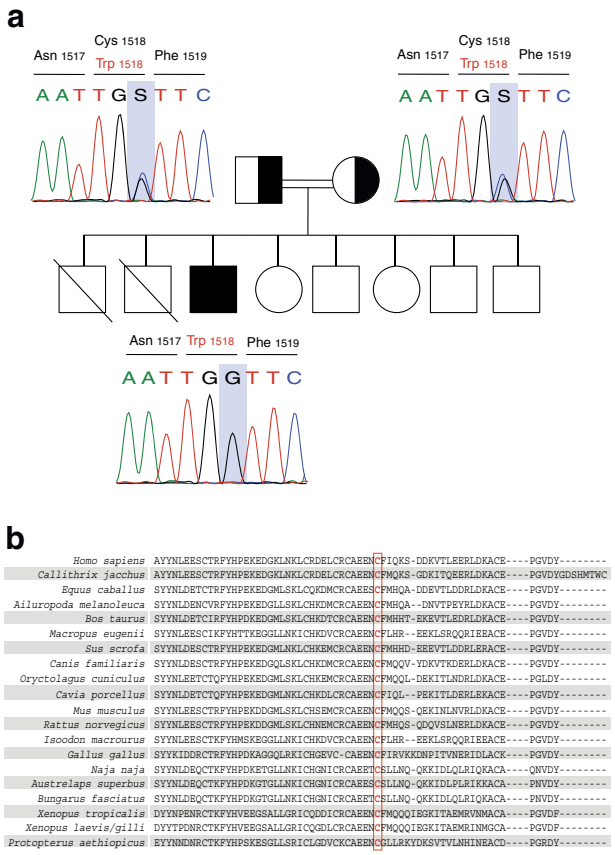

Fig. 2 Pedigree and genetic analysis of the core family (*2a*) and phylogenetic conservation of the cysteine 1518 in C3 (*2b*). **a** Perfect segregation of the *C3* mutation (c. C4554G, p. Cys1518Trp) is shown in the patient and parents. Females are represented as *circles* and males as *squares*. *Filled and half-filled symbols* represent homozygous and heterozygous individuals, respectively. The point mutation is marked with a *grey bar*. The segregation in the siblings is not shown. **b** Phylogenetic conservation of the mutated amino acid in C3 in vertebrates. Cysteine 1518 is indicated with a *red box*

71 mg/dL), respectively. Details of the laboratory findings of the patient and his parents are shown in Table 1.

Mutation Identification in the *C3* Gene

The patient was evaluated for an underlying mutation in the *C3* gene. Sanger sequencing identified a homozygous missense mutation in exon 38 (c. C4554G, p. Cys1518Trp). Molecular segregation analysis showed perfect segregation, with both parents and three siblings being carriers of one affected allele (Fig. 2a and Supplementary figure 1). To date, this variant has neither been annotated as a polymorphism in NCBI (http://www.ncbi.nlm.nih.gov/), nor in UCSC Genomic Bioinformatics site (http://genome.ucsc.edu/) or Ensembl (http://www.ensembl.org), respectively. Polyphen2 and Sorting Intolerant from Tolerant (SIFT) analysis predict that the substitution of cysteine to tryptophan at position 1518 of C3 is very likely to affect protein function, with the maximum score in both analyses (1.000 in Polyphen2 and 0.000 in SIFT, respectively). As indicated in Fig. 2b, the cysteine residue at position 1518 is conserved among vertebrates [17] and it has been shown to form a disulfide bridge with cysteine 1590 [17, 18]. In Fig. 3, a detail of the C345C domain in complex with Factor B (model based on the PDB 2WIN) is depicted and the localization of the disulphide bridge is shown.

Discussion

The complement system is a protein network crucial for both innate and adaptive immune responses. C3 is the convergence point for all the known complement activation cascades, resulting in cleavage of C3 into C3a (anaphylatoxin) and C3b (reviewed in [2]). The latter is an important product for opsonization of bacteria including encapsulated bacteria such as *S. pneumoniae* [18, 19], for amplification of complement activation through the alternative pathway, where the association of C3b with complement factor B is essential, and for cell lysis through the formation of C5 convertase (reviewed in [2]). Patients with C3 deficiency frequently develop severe episodes of recurrent pneumonia, meningitis or sepsis. Clinically, these patients present at an early age with overwhelming infections caused by *S. pneumoniae* [4, 5]. Autoimmunity and other immune manifestations, frequently affecting the kidney, are also observed in C3-deficient patients [13–15].

Since the parents of the patient described here were first-degree cousins and the index patient's severe clinical manifestations were unlikely to be explained by the diagnosis of isolated IgA deficiency, an autosomal recessive disorder was suspected. We discovered and here describe a novel, homozygous missense mutation in *C3,* altering a highly conserved amino acid found in the first position of the C345C domain of the C3 protein which is hypothesized to function as a binding site for factor B, as required for C3 convertase formation [20–22]. This domain is known to undergo large rearrangements upon activation and is present in the C3b molecule [23, 24]. Furthermore, this cysteine residue forms one of the disulfide bonds in the C3 protein [20, 25], thus its loss will likely affect protein folding and/or stability.

After vaccination for *S. pneumoniae*, our patient showed a marked clinical improvement. In line with this observation, it has been shown that mice depleted for C3 by intraperitoneal injection of cobra venom factor which are immunized against *S. pneumoniae* have reduced sepsis when colonized with this bacterium compared to control or neutrophil-depleted mice [26]. Previous studies have illustrated that C3-deficient patients are able to mount adaptive immune responses to conjugated vaccines against *S. pneumoniae* [8, 9].

As mentioned, in addition to C3 deficiency the patient showed selective IgA deficiency (sIgAD). sIgAD is the

Table 1 Description of the laboratory findings of the patient and parents

	Patient[1]	Father	Mother	Reference values
Blood urea nitrogen (mg/dL)	26			20–40
Creatinine (mg/dL)	0,8			0,6–1,2
CH50[a] (U/mL)	0	71	71	>15
C3 levels (mg/dL)	8–19	63	71	90–180[2]
C4 levels (mg/dL)	20			10–40
IgA (mg/dL)	<5.8	230	341	44–244
IgG (mg/dL)	1811			640–2010
IgG1	1220			315–855
IgG2	668			64–495
IgG3	125			23–196
IgG4	25			11–157
IgM (mg/dL)	86			52–297
IgE (IU/mL)	46			0–100
Anti-A titer	1/64			1/10
Anti-B titer	1/128			1/10
Tuberculin test (mm)	10			5–10
In vitro PHA[b] (%)	61			65.8±9.2
Unstimulated NBT[c] (%)	50			0–38
Stimulated NBT[c] (%)	70			60–90
Total lymphocyte counts (cell/mm^3)	3500			1700–5700
T lymphocytes (CD3+) (%)	68			55–79
(cells/mm^3)	2040			1100–4100
T helper cells (CD4+) (%)	38			28–51
(cells/mm^3)	1140			600–2400
Cytotoxic T cells (CD8+) (%)	26			16–42
(cells/mm^3)	780			400–1500
Natural killer cells (CD16+56+) (%)	7			5–28
(cells/mm^3)	210			200–1000
B lymphocytes (CD19+) (%)	20			10–28
(cells/mm^3)	600			200–1400

[a]Total hemolytic complement activity test. [b] In vitro lymphocyte stimulation with 20 μg/mL of phytohemagglutinin for 72 h. The values refer to the percentages of the blastic transformation of lymphocytes (enlarged nucleus, condensed chromatin and/or with pores in the cytoplasm) evaluated using light microscopy. [c] Nitroblue tetrazolium semiquantitative test for evaluating neutrophil oxidative burst, values refer to the percentages of activated neutrophils presenting with respiratory burst activity

[1]Patient examination at the age of 16 years

[2]Normal values in age-matched Turkish subjects

most common form of primary immunodeficiency defined by decreased levels of IgA in the presence of normal levels of other immunoglobulin isotypes (reviewed in [27]). Patients are predisposed to recurrent sinopulmonary infections, gastrointestinal disorders, autoimmune diseases, atopy and malignancies [27, 28]. Amongst the gastrointestinal disorders, giardiasis, malabsorption, lactose intolerance, celiac disease, ulcerative colitis and nodular lymphoid hyperplasia can be found [27]. Respiratory tract infections are the most frequent morbidities in sIgAD patients [28], however, bronchiectasis is a rare complication [29]. Although sIgAD is often asymptomatic, patients with concomitant IgG2 deficiency may present with impaired antibody responses against polysaccharide antigens and show predisposition to more severe bacterial infections [28].

Table 2 Specific IgG antibody levels in the patient before and after *S. pneumoniae* vaccine (U/mL). Reference values: ≥20 U/mL

Serotypes	3	6B	14	19F	23F	7F
Before vaccination	22	16	32	19	16	18
6 weeks after vaccination	29	>100	46	65	68	20

Besides C3 deficiency, other deficiencies of adaptive or innate immunity can also lead to increased susceptibility to infections caused by *S. pneumoniae*, albeit with differences in the clinical and laboratory findings (reviewed in [4, 12]). Two interesting examples of such innate immune deficiencies are IRAK4 and MYD88 deficiency, respectively. IRAK4- or MYD88-deficient patients are predisposed to recurrent invasive infections with *S. pneumoniae*, especially meningitis (reviewed in [12]). These patients also frequently present with impaired ability to increase plasma C-reactive protein and to mount fever in response to infection, with spontaneous improvement in adolescence (reviewed in

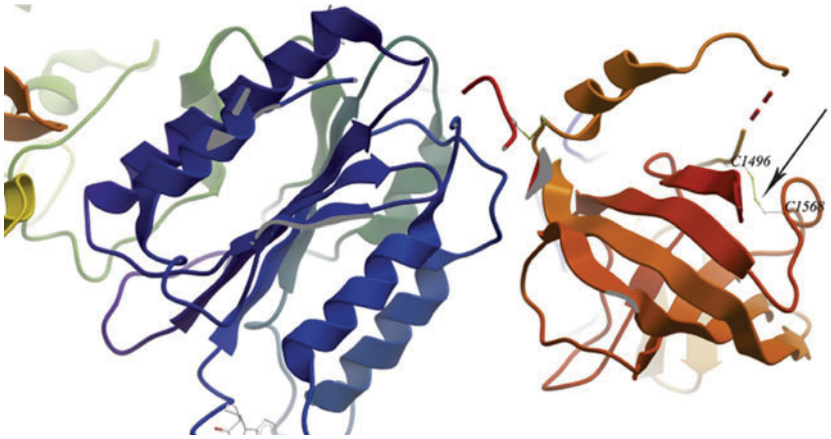

Fig. 3 Details of the structure of C345C domain of C3 and factor B based on the PDB model 2WIN. The figure shows the C345C domain on the *right hand side* (in red/orange) and factor B on the *left hand side* (in blue/green). Note that, in the PDB model, the residues Cys1518 and Cys1590 correspond to Cys1496 and Cys 1568, respectively. The arrow points the disulphide bridge formed by both cysteine residues

[12]). By contrast, our patient presented mainly with pneumonias, and similar to other C3-deficient patients [4, 5], he showed high levels of CRP and had episodes of fever, with infectious episodes persisting throughout adolescence.

Taken together, the following observations support our hypothesis that the clinical phenotype of our patient was - at least predominantly - caused by the underlying deficiency in C3 rather than associated with sIgAD: 1) sinusitis or gastrointestinal disorders are absent in the patient; 2) bronchiectasis is observed although our patient presented with normal IgG2 levels and normal antibody responses to polysaccharide antigens; 3) the patient displays a marked and relatively specific susceptibility to infections with encapsulated bacteria such as *S. pneumoniae*; and 4) there was a marked clinical amelioration upon vaccination against *S. pneumoniae*.

Conclusions

We here report a novel homozygous mutation in *C3* in a patient with recurrent and severe infections caused by *Streptococcus pneumoniae* and associated IgA deficiency. The case presented here highlights the importance of a more thorough evaluation of sIgAD patients when the clinical presentation is unusual or more severe than expected. In case of severe infections caused by encapsulated agents such as *S. pneumoniae*, a careful evaluation of complement components is mandatory. Our data lend further support to the concept that vaccination against this microorganism is critical for immunodeficient patients, in particular for inherited complement deficiencies.

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

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

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* equal contribution

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

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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,

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

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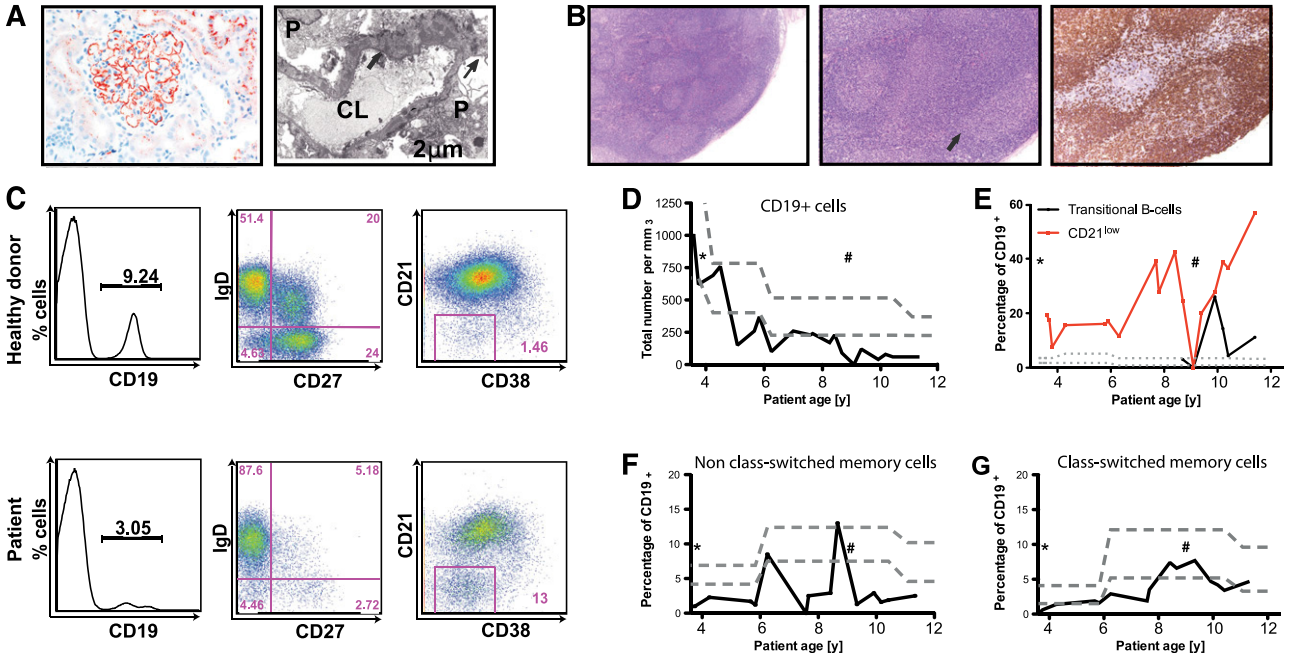

Figure 1. Clinical and immunological characterization of the index patient. (A) First renal biopsy was performed at the age of 15 months. Granular deposition of IgG along the periphery of the capillary loops (left) as seen in membranous nephropathy (MGN) was confirmed by transmission electron microscopy (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.

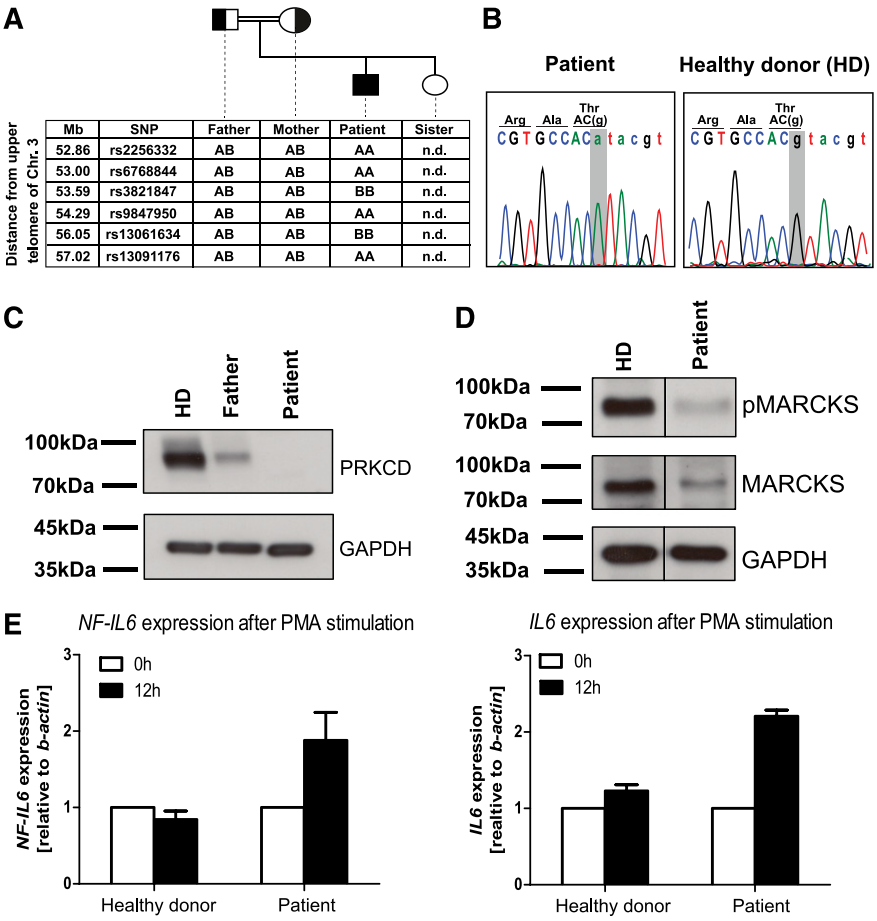

Figure 2. Identification of human PRKCD deficiency as a monogenetic B-cell deficiency associated with autoimmunity. (A) Single nucleotide polymorphism array–based homozygosity mapping was performed and revealed several homozygous candidate intervals, including an interval on chromosome 3p21.31. (B) Sanger 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.

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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.

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3.3 Biallelic loss-of-function mutation in NIK causes a primary immunodeficiency with multifaceted aberrant lymphoid immunity

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Biallelic loss-of-function mutation in *NIK* causes a primary immunodeficiency with multifaceted aberrant lymphoid immunity

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Primary immunodeficiency disorders enable identification of genes with crucial roles in the human immune system. Here we study patients suffering from recurrent bacterial, viral and *Cryptosporidium* infections, and identify a biallelic mutation in the *MAP3K14* gene encoding NIK (NF-κB-inducing kinase). Loss of kinase activity of mutant NIK, predicted by *in silico* analysis and confirmed by functional assays, leads to defective activation of both canonical and non-canonical NF-κB signalling. Patients with mutated *NIK* exhibit B-cell lymphopenia, decreased frequencies of class-switched memory B cells and hypogammaglobulinemia due to impaired B-cell survival, and impaired ICOSL expression. Although overall T-cell numbers are normal, both follicular helper and memory T cells are perturbed. Natural killer (NK) cells are decreased and exhibit defective activation, leading to impaired formation of NK-cell immunological synapses. Collectively, our data illustrate the non-redundant role for NIK in human immune responses, demonstrating that loss-of-function mutations in *NIK* can cause multiple aberrations of lymphoid immunity.

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Primary immunodeficiency disorders represent unique models to identify factors essential for host defense and immune homeostasis. In humans, development of mature B cells from immature precursor cells is critically dependent on signalling pathways downstream of B-cell receptor (BCR) and on tumour necrosis factor-α (TNFα) receptor superfamily members including BAFF receptor (BAFFR), TACI and CD40 (reviewed in ref. 1). BAFFR signals are needed to mature beyond the transitional B-cell stage[2], while lymphotoxin-α1/β2 (LTβ) and CD40 ligand (CD40L) are required for thymic and secondary lymphoid organ structure, respectively[3]. CD40-mediated signalling additionally orchestrates processes dependent on $CD4^+$ T-helper cells such as class-switch recombination (CSR) and somatic hypermutation (SHM) in the germinal centre (GC) reaction and $CD8^+$ cytotoxic T-cell memory[4].

BAFFR, CD40 and LTβ receptors transmit signals through the non-canonical nuclear factor-κB (NF-κB) pathway (reviewed in ref. 5), which induces proteolytic processing of p100 to p52 (ref. 6). Together with RelB, p52 forms a heterodimer that upon nuclear translocation functions as transcriptional activator of a subset of NF-κB target genes[5]. Processing of p100 depends on the phosphorylation of the serine residues 866 and 870, which is controlled by the MAP3 kinase–kinase–kinase NIK (NF-κB inducing kinase, MAP3K14)[6] through NIK's substrate IκB kinase α (IKKα)[7]. Non-canonical NF-κB signalling is controlled by TNF receptor associated factor (TRAF) proteins TRAF2 and NIK's negative regulator TRAF3, whereby a TRAF3-containing complex continuously targets NIK for degradation under steady-state conditions[5]. On receptor activation, TRAF3 is degraded and NIK protein levels can accumulate, allowing NIK to phosphorylate and activate downstream effectors.

To date, human patients carrying mutations in *MAP3K14* have not yet been described. In *Nik* mutant mice (*aly*; alymphoplasia)[8,9] and knockout animals[3], lymph nodes, Peyer's patches as well as splenic and thymic structures are severely disorganized. In addition, B-cell numbers are reduced and immunoglobulin (Ig) serum levels are decreased leading to humoral immunodeficiency. Although the function of NIK in B lymphocytes has been well established, the role of NIK-dependent signalling for T and natural killer (NK) lymphocytes is less well understood.

Here we report a combined immunodeficiency syndrome caused by biallelic mutations in the gene encoding NIK, encompassing B-cell lymphopenia and impaired memory B-cell differentiation. We also identify abnormal NK-cell development and function, as well as aberrant T-cell responses, indicating that biallelic loss-of-function mutations in *NIK* cause a hitherto unrecognized, pervasive combined immunodeficiency syndrome.

Results

Identification of a homozygous mutation in *MAP3K14*. We studied a large consanguineous pedigree with two patients (termed P1 and P2) who showed signs of combined immunodeficiency including recurrent, severe bacterial and viral infections and *Cryptosporidium* infection (Supplementary Fig. 1a,b and Supplementary Tables 1 and 2; see Supplementary Note for further clinical course details). Investigation for known genetic aetiologies of defective CSR including CD40 and CD40L deficiencies and gain-of-function *PIK3CD* mutations[10,11] was performed; however, no mutation was identified.

Immunological assessment in both affected patients revealed decreased immunoglobulin levels (Supplementary Table 1) and decreased numbers of both B and NK cells, while T-cell numbers were within normal age-adjusted ranges (Supplementary Table 3). As decreased immunoglobulin levels and B-cell numbers suggested impaired B-cell development and function, we performed flow cytometry-based immunophenotyping to assess the relative frequencies of $CD27^+$ memory B-cell populations. Both patients showed a relative reduction of total $CD19^+$ B cells in peripheral blood (Fig. 1a). Absolute blood cell counts revealed B lymphopenia in P2, while B-cell numbers in P1 were in the age-matched lower normal range (Supplementary Table 3). Patients had decreased $CD19^+CD27^+IgD^+$ marginal zone-like/innate B cells and $CD19^+CD27^+IgD^-$ class-switched memory B cells compared with controls[12], suggesting defects in late stages of B-cell development and activation (Fig. 1a).

Given the consanguineous background, an autosomal-recessive inheritance mode was assumed. To unveil the presumed monogenetic cause of disease, single-nucleotide polymorphism (SNP) array-based homozygosity mapping of P1 and P2 (Fig. 1b and Supplementary Table 4) was combined with exome sequencing (ES) (Fig. 1c) of P1. Single-nucleotide variants (SNVs) and insertion/deletion variants resulting from ES were filtered for those present inside homozygous candidate intervals shared between both affected patients. Synonymous and non-coding variants were excluded. We identified a single homozygous variant on chromosome 17q21 in *MAP3K14* (c. C1694G, p. Pro565Arg) present in both patients (Fig. 1d and Supplementary Fig. 1), which was not detected in dbSNPbuild137, 1000Genomes, ENSEMBL, UCSC, NCBI or EVS (Exome Variant Server) public SNP databases. The Pro565 residue of NIK is located within the kinase domain of the protein (Fig. 1e) and is highly conserved throughout evolution (Fig. 1f). The exchange from proline to arginine at this position was predicted as highly deleterious using the functional prediction algorithms Polyphen-2 and SIFT with maximum scores (1.0 and 0.0, respectively).

Effects of $NIK^{Pro565Arg}$ on kinase function. Pro565 forms part of the APE motif within a helix in the activation segment of the kinase[13]. This motif is conserved in NIK from various vertebrate phyla as well as in orthologous serine/threonine kinases (Fig. 1f). An exchange of a non-polar, conformationally rigid amino acid as in $NIK^{Pro565Arg}$ may have an impact on protein folding and function. The protein stability analysis tool CUPSAT predicted that the overall stability of $NIK^{Pro565Arg}$ may be compromised (Supplementary Table 5). Coarse-grained molecular dynamics simulation of $NIK^{wild\text{-}type}$ and $NIK^{Pro565Arg}$ showed conformational changes within the kinase domain. Notably, the nearby Thr559 residue has been reported to form a hydrogen bond with Lys517 in the catalytic loop (Fig. 2a) and mutation of Thr559 has been found to reduce kinase activity[14,15]. In the $NIK^{wild\text{-}type}$ simulation, Pro565 remains buried within the protein in the vicinity of the ATP-coordinating centre, allowing hydrogen bond formation between Lys517 and Thr559 (Fig. 2a). In the $NIK^{Pro565Arg}$ simulation, the mutated arginine residue transitions towards the protein surface, thereby increasingly contacting the surrounding solvent and repositioning adjacent helices. In the simulation, Arg565 prevents Thr559 from forming a hydrogen bond with Lys517 in the ATP-coordinating centre, thereby impairing the kinase activity of NIK (Fig. 2b,c).

To experimentally assess the effect of the mutation, we analysed the kinase activity of $NIK^{Pro565Arg}$ compared with $NIK^{wild\text{-}type}$ and the catalytically inactive mutant $NIK^{Lys429Ala/Lys430Ala}$ (ref. 16) by testing NIK-dependent phosphorylation of IKKα. Recombinantly expressed $NIK^{wild\text{-}type}$, but not $NIK^{Pro565Arg}$ or $NIK^{Lys429Ala/Lys430Ala}$, could phosphorylate both endogenous (Fig. 2d) and co-expressed IKKα (Supplementary Fig. 2) in HEK293 cells. These data demonstrate that $NIK^{Pro565Arg}$

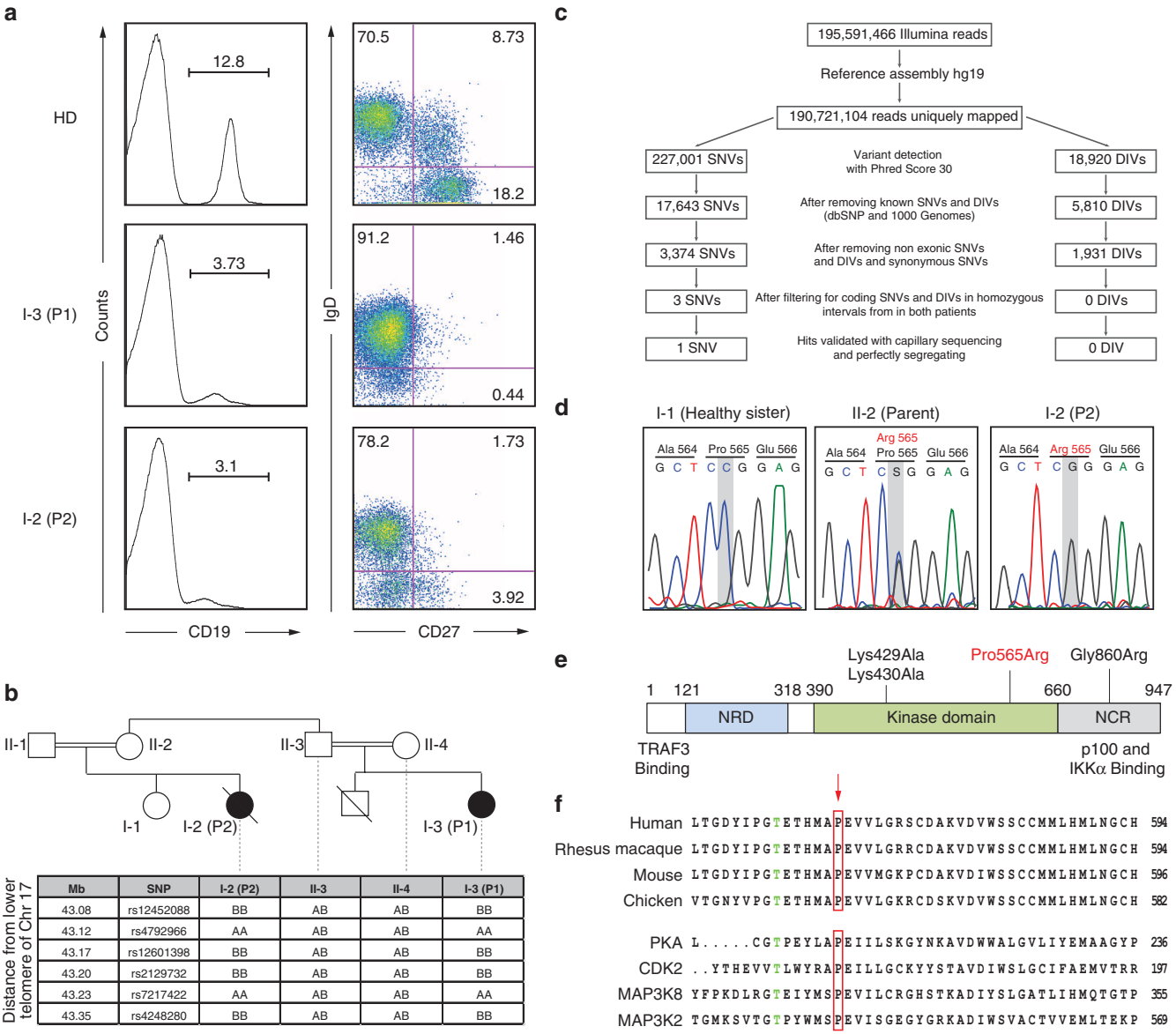

Figure 1 | Identification of *MAP3K14/NIK* mutation in patients with defective B cells. (**a**) Flow cytometry plots illustrating decreased CD19$^+$ B cells and decreased CD27$^+$IgD$^-$ class-switched memory B cells in P1 and P2. Plots representative of three independent experiments. (**b**) SNP array based homozygosity mapping revealed several homozygous candidate intervals shared between both patients, including an interval on chromosome 17q21, described in the box. (**c**) Scheme of exome sequencing workflow and filtering strategy. SNVs, single nucleotide variants; DIVs, deletions and insertions variants. (**d**) Capillary DNA sequencing of the regions adjacent to the nonsense mutation in *MAP3K14* in P2 and core family members. Chromatograms shown for a healthy sister of P2, the mother of P2 and P2. The mutated residue is indicated by a grey box. (**e**) Schematic representation of the NIK protein domain structure. NRD, negative regulatory domain (blue); kinase domain (green); NCR, non-catalytic region (grey)[79]. Red label indicates the amino acid change in P1 and P2. Black labels indicate the catalytic inactive mutant NIK$^{Lys429Ala/Lys430Ala}$ and the murine *aly/aly* mutant (Gly860Arg). (**f**) Amino acid sequence conservation of the region adjacent to Pro565 across species as well as a panel of human serine/threonine kinases. Red arrow indicates Pro565 mutated in P1 and P2; Thr559 printed in green.

represents a loss-of-function mutation with respect to abolished kinase activity towards its direct target IKKα.

Defective non-canonical and canonical NF-κB signalling. Processing of p100 into p52 and nuclear translocation of the p52/RelB complex is an essential step following NIK activation[6]. Therefore, we studied activity of the non-canonical NF-κB pathway in response to activation with BAFF and LTβ, respectively. In patient-derived Epstein–Barr virus-immortalized lymphoblastoid cell lines (B-LCL), total NIK protein levels (which are tightly controlled via proteolysis[5]) were unaffected (Fig. 3a). However, levels of its immediate downstream target IKKα were elevated and p100 accumulated already before BAFF-mediated BAFFR ligation (Fig. 3a), possibly reflecting pre-activation of the NF-κB pathway in B-LCL by viral proteins[17]. Despite p100 accumulation, p52 protein levels were decreased (Fig. 3a), resulting in a severely reduced nuclear content of p52 and a lower nuclear content of RelB in patient-derived cells (Fig. 3b), demonstrating functional insufficiency of the non-canonical NF-κB pathway in patient-derived cells.

Next, we tested the effect of NIK^{Pro565Arg} in NF-κB activation upon LTβ stimulation, which is strictly dependent on NIK[18]. LTβ can activate non-canonical NF-κB signalling as well as the IKKα-IKKβ-NEMO complex mediating nuclear translocation of

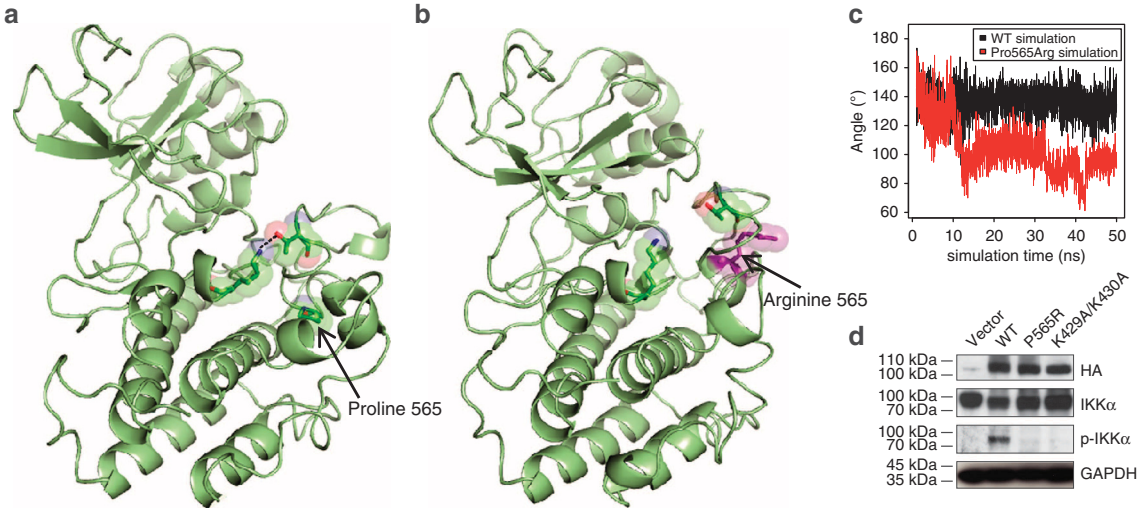

Figure 2 | NIK^{Pro565Arg} is structurally altered and catalytically impaired. (**a**) Structure prediction of NIK$^{wild\text{-}type}$ catalytic domain from coarse-grained molecular dynamics simulation. Pro565, Thr559 and Lys517 residues are displayed as sticks and transparent molecular spheres. Dashed line represents hydrogen bond between Lys517 and Thr559. (**b**) Structure prediction of NIK^{Pro565Arg} from coarse-grained molecular dynamics simulation. Arg565 residue is displayed as sticks and transparent molecular spheres (violet). (**c**) Molecular dynamics simulation of wild-type NIK and NIK^{Pro565Arg}. Profile of the angle of Thr559-CA, Thr559-CM and Lys517-CM. The angle of Thr559-CA, Thr559-CM and Lys517-CM constantly approaches 150° (close to the value observed in the NIK$^{wild\text{-}type}$ experimental X-ray structure), while it rapidly transitions to 90° (hydrogen bonding unfavourable) in the simulation of NIK^{Pro565Arg}. CA, alpha carbon; CM, centre of side chain mass. (**d**) Analysis of kinase activity of NIK variants expressed in HEK293 cells. HA-tagged NIK variants NIK$^{wild\text{-}type}$, NIK^{Pro565Arg} and the catalytically inactive mutant NIK$^{Lys429Ala/Lys430Ala}$ were transfected into HEK293 cells and levels of total and phosphorylated IKKα were detected by immunoblot. Anti-HA and anti-GAPDH blots were used as loading controls for NIK and total protein, respectively. Blots are representative of three independent experiments. All uncropped blots can be seen in Supplementary Fig. 9.

canonical NF-κB complexes[19]. Stimulation of patient fibroblasts with the NIK-independent canonical NF-κB activator TNFα led to a rapid decay of IκBα, a hallmark event of canonical NF-κB signalling (Fig. 3c). In contrast, patient cells failed to induce IκBα decay after LTβ stimulation (Fig. 3d). Consistent with these findings, immunofluorescent staining showed defective nuclear translocation of both p52 and p50 in patient primary fibroblasts upon LTβ stimulation (Fig. 4a,b).

To demonstrate the causative role of NIK^{Pro565Arg} for deficient non-canonical NF-κB signalling, we performed retroviral-mediated gene transfer of *MAP3K14* into patient fibroblasts. Expression of NIK$^{wild\text{-}type}$ reactivated non-canonical NF-κB signalling manifesting in nuclear translocation of p52 (Fig. 4c,d), demonstrating that the presence of functional NIK protein is the limiting factor for p100 activation.

Although NIK may have IKKα-independent functions[20], the main function of the protein comprises the catalytic activity as a kinase and activation of the signalling cascade leading to NF-κB translocation. As the NIK^{Pro565Arg} mutant is catalytically inactive and therefore deleterious to these functions, the phenotype caused by NIK^{Pro565Arg} is hereafter also referred to as functional NIK deficiency.

Reduced survival of mature B cells. The spontaneous *Map3k14* mouse *aly* mutant[8,9] and *Map3k14* knockout mice[3] show reduced mature B-cell numbers and decreased Ig serum levels, resulting in defects in both antibody and cellular immune responses. In addition, non-canonical NF-κB signalling mediated by Nik controls CSR, in particular to IgA isotype[21]. Accordingly, patients bearing NIK^{Pro565Arg} had severely reduced total B-cell counts and impaired generation of CD27$^+$IgD$^-$ class-switched memory B cells in the peripheral blood (Fig. 1a), leading to chronically reduced IgA titres in both patients and to reduced IgG levels in P1 at the age of 10 months (Supplementary Table 1) prompting intravenous immunoglobulin substitution. This led us to test whether NIK is involved in CSR and SHM, processes essential for the generation of high-affinity antibodies.

To study the occurrence of SHM in B cells, we analysed the mutation frequency in rearranged variable regions of the Ig heavy chain (*IGHV*) genes by cloning and sequencing the *IGHV3* and *IGHV4* rearranged gene families of both γ- and α-chain immunoglobulin transcripts (Cγ and Cα). The percentage of mutations within the analysed *IGHV* regions was significantly reduced in NIK^{Pro565Arg}-bearing patients compared with age-matched healthy donors, although not as severely as in CD40L-deficient patients who showed near-complete absence of mutations in *IGHV* Cα and completely lacked Cγ transcripts (Fig. 5a)[22].

Next, we investigated activation and CSR capacity of NIK^{Pro565Arg} B cells by stimulating peripheral blood mononuclear cells (PBMCs) with a range of stimuli. Patient B cells were able to respond to stimulation with CD40L and IL4 by upregulating the activation markers CD95 and CD69, as well as the costimulatory molecule CD86, although to a lesser extent than B cells from a healthy donor (Fig. 5b). Notably, patient B cells were only partially able to upregulate the activation marker CD25, suggesting impaired IL2-mediated survival and proliferation[23]. As NIK^{Pro565Arg} B cells were largely able to upregulate the aforementioned activation markers, we further tested their proliferation capacity and ability to undergo CSR in response to CD40L and IL4 stimulation. Indeed, we observed a progressive increase in the percentage of DAPI (4′,6-diamidino-2-phenylindole)-negative blasts in NIK^{Pro565Arg} mutant cells (Supplementary Fig. 3a) over a course of 9 days, consistent with activation and proliferation of cells upon stimulation with CD40L and IL4 (ref. 24). Furthermore, B cells with mutated NIK underwent CSR to IgG *in vitro*, albeit with reduced frequency at day 6 when compared with controls (Fig. 5c), probably due to delayed lymphocyte proliferation (Supplementary Fig. 3a). Concomitant to proliferative outgrowth of lymphocytes at day 9,

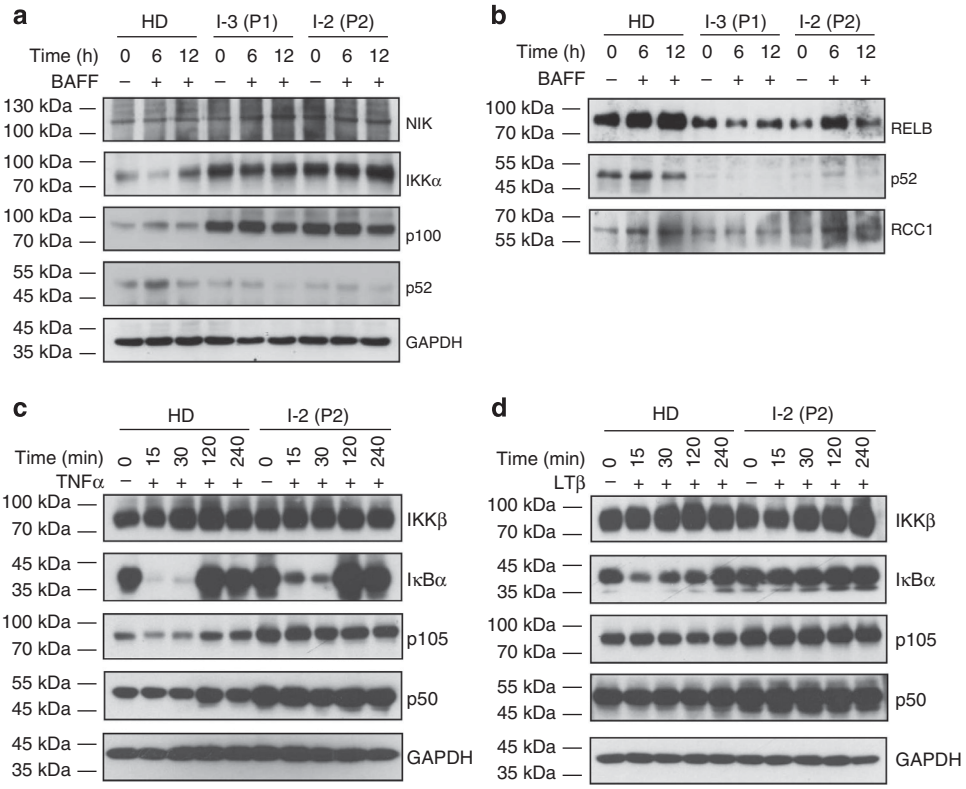

Figure 3 | Defective canonical and non-canonical NF-κB pathway function. (**a**) Immunoblot analysis of whole cell lysates of B-LCL stimulated with hBAFF. Healthy donor, P1 and P2 derived B-LCL were used. Blots were probed for non-canonical NF-κB pathway components NIK, IKKα, p100/p52 and sample processing control GAPDH. (**b**) Immunoblot analysis of nuclear extracts of B-LCL stimulated with hBAFF in healthy donor, P1- and P2-derived B-LCL. RCC1 was used as nuclear sample processing control. (**a**,**b**) Blots are representative of two independent experiments. All uncropped blots can be seen in Supplementary Fig. 9. (**c**) Immunoblot analysis of whole cell lysates of healthy donor- and P2-derived fibroblasts after TNFα stimulation. Blots were probed for canonical NF-κB pathway components IKKβ, IκBα, p105/p50 and GAPDH as loading control. (**d**) Immunoblot analysis of whole cell lysates of healthy donor- and P2-derived fibroblasts after LTβ stimulation. Blots were probed for canonical NF-κB pathway components IKKβ, IκBα, p105/p50 and loading control GAPDH. (**c**,**d**) Blots represent one experiment. All uncropped blots can be seen in Supplementary Fig. 9.

CSR to IgG was restored to levels comparable to control cells (Fig. 5c). NIK^{Pro565Arg} mutant B cells did not proliferate after *in vitro* stimulation with CD40L/IL21 (Fig. 5d and Supplementary Fig. 3b), despite intact expression of IL21 receptor on patient B cells (Supplementary Fig. 4a) nor after *in vitro* stimulation with anti-IgM/CpG or anti-IgM/CpG/BAFF-Fc (Supplementary Fig. 3c,d). These data demonstrated the inability of NIK^{Pro565Arg} B cells to respond to BCR, TLR9, IL21R and/or BAFFR stimulation. Only CD40L/IL4 stimulation, known to mediate survival and proliferation of primary B cells[25,26], could induce cell proliferation (Fig. 5d and Supplementary Fig. 3a), suggesting that intact NIK is required to relay signals essential for survival and proliferation of activated mature B-cell populations.

Given the partial phenotypic overlap of functional NIK deficiency and IL21 (receptor) deficiency[27,28] (for example, colitis, susceptibility to *Cryptosporidium* infection, hypogammaglobulinemia and decreased frequencies of class-switched B cells, defective antigen-specific T-cell proliferation and impaired NK-cell cytotoxicity), we sought to exclude an involvement of NIK in IL21-mediated signalling in B cells. Indeed, although classical signalling via STAT3 (signal transducer and activator of transcription 3) phosphorylation was readily observed after stimulating sorted B cells with IL21, no activation of p100 processing could be detected (Supplementary Fig. 5).

As NIK is an integral component of the non-canonical NF-κB pathway downstream of the BAFFR, which plays a key role in mature B-cell survival[29], we investigated whether functional NIK deficiency resembles phenotypes found in BAFFR deficiency[2,30]. Similar to findings in *Baffr*$^{-/-}$ mice[30], B cells of both patients showed lower cell surface expression of CD21, involved in pro-survival signalling on B cells[31] (Fig. 5e). This observation prompted us to investigate whether the NIK^{Pro565Arg} mutant affects the expression of anti-apoptotic genes *BCL2*, *BCL2L1* and *MCL1* by quantitative reverse transcriptase–PCR in sorted naïve mature CD19$^+$CD27$^-$IgD$^+$ B cells. From the transcripts tested, *BCL2* expression was markedly downregulated in patient naive B cells compared with heterozygous parent and healthy donor controls (Fig. 5f and Supplementary Fig. 4b). To exclude that functional NIK deficiency ablated BAFFR expression, we analysed surface BAFFR levels by flow cytometry on PBMCs stimulated *in vitro* with CD40L and IL4 for 9 days. BAFFR expression on B cells from P1 was comparable to B cells from control parent or healthy donor (Supplementary Fig. 4c).

BAFFR-deficient patients display a partial block in development beyond the transitional CD19$^+$CD21$^{low/intermediate}$ B-cell stage[2]. As NIK^{Pro565Arg} patient peripheral B cells had overall a CD19$^+$CD21$^{low/intermediate}$ phenotype, we tested the expression of the alternative transitional B-cell markers IgM, CD10, CD38 and CD5. Transitional B cells (defined as CD19$^+$IgM^{hi}IgD$^+$ and CD19$^+$CD23$^-$CD27$^-$CD5$^+$IgM^{hi}, respectively) were increased, in particular the CD19$^+$CD38$^+$CD10$^-$ transitional T2 population, indicating a partial block in B-cell maturation (Supplementary Fig. 4d,e). Taken together, the NIK^{Pro565Arg} loss-of-function mutant causes a partial block of B-cell development between transitional and naive mature B-cell stages accompanied by impaired survival of mature peripheral B cells.

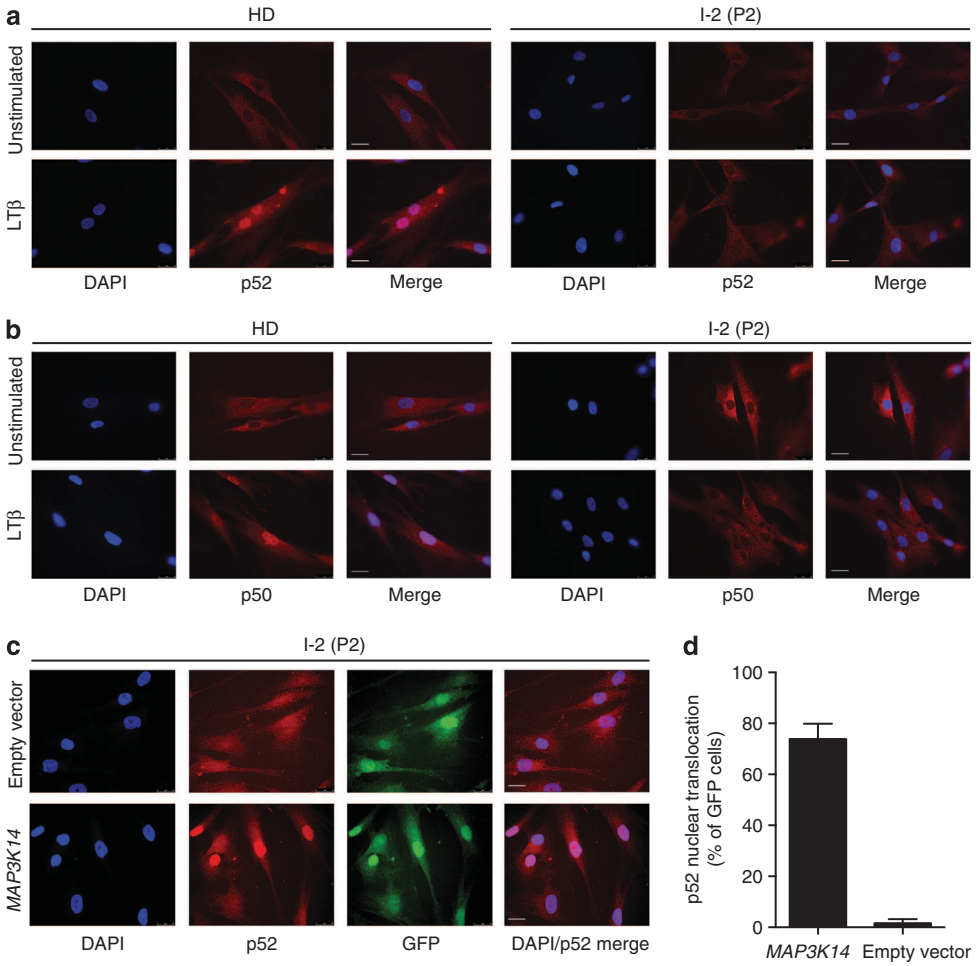

Figure 4 | Defective nuclear translocation of canonical and non-canonical NF-κB and rescue by ectopic expression of NIK$^{wild\text{-}type}$. (**a**,**b**) Immunofluorescence analysis of healthy donor and P2 fibroblasts stimulated with LTβ and stained with DAPI, (**a**) anti-p100/p52 and (**b**) anti-p105/p50 antibodies, respectively. White bar indicates 25 μm. (**c**) Immunofluorescence analysis of P2 fibroblasts after retroviral transduction with *MAP3K14* or empty vector, both coexpressing GFP. Cells were stained with DAPI, anti-GFP and anti-p100/p52 antibody. White bar indicates 25 μm. (**d**) Quantification of reconstitution experiment in P2 fibroblasts shown in **c**. GFP-positive cells (representing cells transduced with *MAP3K14* or empty vector, respectively) were scored for p52 nuclear translocation events, indicated as mean percentage of total (± s.e.m.). 86 (*MAP3K14*) and 97 (empty vector) cells were scored, respectively. (**c**,**d**) Images and quantification are representative of two independent experiments.

Aberrant T-cell phenotype and antigen-specific proliferation. Next, we assessed the effect of NIK^{Pro565Arg} on T cells. Overall $CD3^+CD4^+$ helper T-cell and $CD3^+CD8^+$ cytotoxic T-cell subset distribution was unaffected (Supplementary Fig. 6a), T-cell receptor Vβ repertoires were polyclonal (Supplementary Fig. 7) and regulatory T cells were unaltered (Supplementary Fig. 6b). Upon stimulation of PBMCs with T-cell proliferation stimuli such as anti-CD3 antibody (clone OKT3), phorbol 12-myristate 13-acetate, Staphylococcal enterotoxin A, Staphylococcal enterotoxin B or phytohaemagglutinin, normal proliferative responses were observed (Fig. 6a). In contrast, when the antigen-specific stimuli tetanus toxoid or purified protein derivatives of *Mycobacterium tuberculosis* were used, proliferative responses were severely reduced (Fig. 6b) despite prior tetanus and Bacillus Calmette–Guérin vaccination. These observations prompted us to assess the presence of naive and memory T-cell subsets in both patients. The relative proportions of $CD4^+$ effector memory T cells (T_{EM}) (markers $CD4^+CD45RA^-CCR7^-$ or $CD4^+CD45RA^-CD27^-$, respectively) were comparable between patients and healthy controls and were within the normal age-matched range[32] (Supplementary Fig. 6c,d and Supplementary Table 3). Relative numbers of $CD8^+$ memory T cells from both patients were also within the highly variable normal range[32]. However, P1 exhibited a remarkable expansion of $CD8^+$ T_{EM} cells and terminally differentiated effector memory T cells (T_{EMRA}, identified as $CD8^+CD45RA^+CD27^-$ or $CD8^+CD45RA^+CCR7^-$; Supplementary Fig. 6c,d), possibly attributable to the persistent cytomegalovirus (CMV) viremia in P1 (Supplementary Table 2). Increased IL7R/CD127 expression on $CD8^+$ T cells identifies long-lived memory cells[33,34]. Similar to Nik-deficient mice[35], we found dramatically reduced CD127 expression on $CD8^+$ memory T cells, on $CD8^+$ T_{CM} and on $CD8^+$ T_{EM} in both P1 and P2 (Fig. 6c), pointing towards impaired memory responses to viral infections observed in P1 (Fig. 6b and Supplementary Table 2).

Interaction of inducible co-receptor ICOS with ICOS ligand (ICOSL) is important for the differentiation of follicular helper T_{FH} cells and for memory responses of both T and B cells[36]. T_{FH} cells localize to GC reactions within secondary lymphoid organs where they interact with B cells to aid antibody production and maturation[37]. T_{FH} cell numbers are reduced in ICOS-deficient common variable immunodeficiency and in CD40L or CD40 deficiency[37]. Indeed, we found decreased proportions of T_{FH} cells (identified as $CXCR5^+CD45RA^-$) in both patients compared

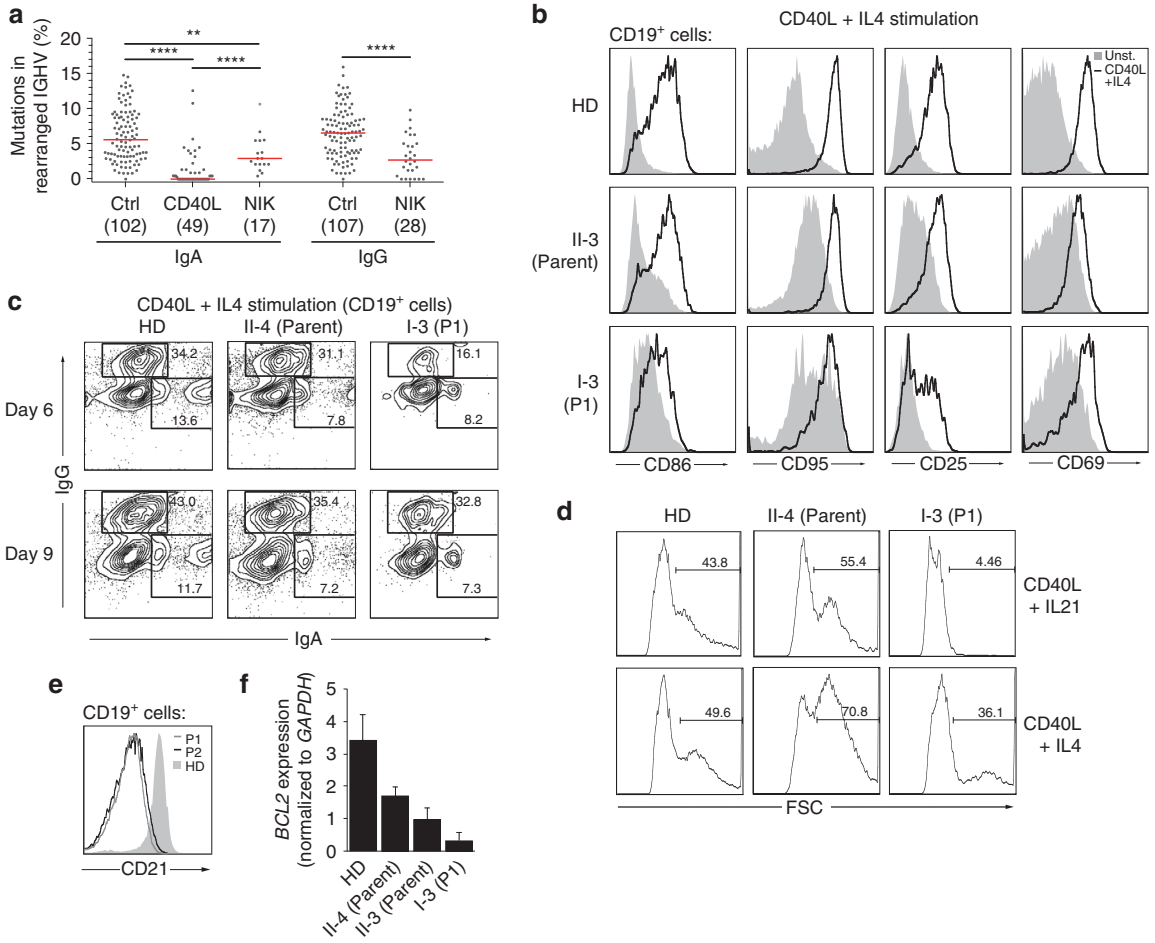

Figure 5 | Aberrant B-cell phenotype. (**a**) Analysis of mutation frequency in percent mutated bases in rearranged variable regions of switched Ig heavy chains (*IGHV*) of IgG and IgA isotypes in healthy donors and patients. Analysed sequences from both patients are represented combined. The number of sequences analysed is indicated in brackets. (**b**) Flow cytometric analysis of the expression of activation markers CD86, CD95, CD25 and CD69, 1 day after *in vitro* stimulation with CD40L and IL4. Plots are representative of three independent experiments. (**c**) Flow cytometry analysis of the expression of class-switch markers IgG and IgA at day 6 and 9 after *in vitro* stimulation with CD40L and IL4. (**d**) *In vitro* proliferation of primary lymphocytes after stimulation with CD40L and IL21 or CD40L and IL4. The forward scatter gate indicates percentages of proliferating blasts in parent or patient P1 cultures after 9 days. (**e**) Flow cytometry analysis of the CD21 expression on CD19$^+$ B cells of a healthy donor (filled grey), P1 (grey line) and P2 (black line). (**c**–**e**) Plots represent one experiment. (**f**) Quantitative reverse transcriptase-PCR analysis of *BCL2* expression in sorted peripheral blood naive B cells. *BCL2* transcript expression was normalized to *GAPDH* expression. Mean fold enrichment from one experiment is shown. Error bars denote ± s.d. from three technical replicates.

with an age-matched healthy donor (Fig. 6d) and with previously reported healthy donors[38]. As T_{FH} cell development is dependent on ICOSL expression on B cells which is controlled by non-canonical NF-κB signalling[39], we hypothesized that NIK^{Pro565Arg} causes reduced ICOSL expression leading to impaired T_{FH} generation. Thus, we stimulated PBMCs with CD40L for 36 h and monitored ICOSL expression by flow cytometry. Patient, control parent and healthy donor B cells responded to CD40L stimulation by inducing CD69 expression; however, although control cells were able to upregulate ICOSL, the patient cells failed to do so (Fig. 6e). In summary, NIK^{Pro565Arg} patients exhibit defective differentiation into T_{FH} and impaired function of memory T-cell subsets.

Decreased numbers and functional impairment of NK cells. Consistently low NK-cell numbers in both patients (Supplementary Table 3) along with the susceptibility to CMV, for which NK-cell-mediated defense is relevant, prompted a detailed phenotypic analysis of NK cells. Both patients bearing the NIK^{Pro565Arg} mutant had low numbers of NK cells in the peripheral blood, particularly P1 (Fig. 7a). Despite their low frequency, NK cells showed normal expression of the cell-surface markers perforin, CD16, CD69, CD57 and NKG2C, hallmarks of acquisition of cytotoxic function (Supplementary Fig. 8). Both CD56^{bright} and CD56^{dim} NK cells, representing subsequent stages of NK cell development, were present (Fig. 7a and Supplementary Fig. 8). In addition, patients expressed markers associated with pre-terminal NK-cell developmental stages, including CD117, CD27, CD11a, KIR2DL4 and CD94, at levels comparable to healthy donors (Supplementary Fig. 8). The notable exception was CD62L, which was expressed by a markedly lower proportion of NK cells with NIK^{Pro565Arg} (Fig. 7b).

To determine whether the NK cells present were able to exert cytolytic function and cytokine secretion, we performed activation with phorbol 12-myristate 13-acetate and ionomycin. Notably, production of interferon-γ and TNFα were markedly reduced in patient NK cells, in comparison with stimulated healthy donor cells (Fig. 7c). Although patient NK cells expressed comparable levels of perforin compared with

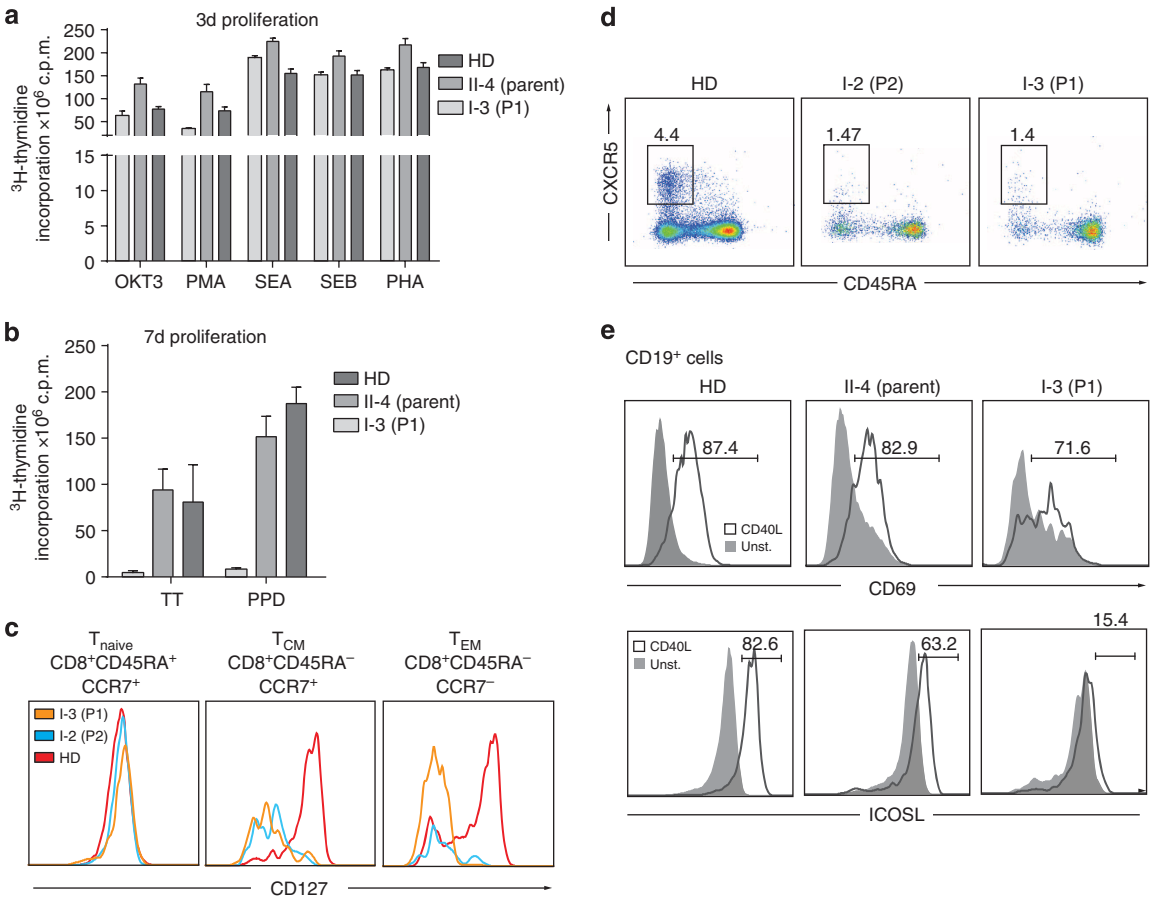

Figure 6 | Effects of NIK^{Pro565Arg} on T cells. (**a**) Proliferative response of T cells determined by [^{3}H]-thymidine incorporation assay after stimulation with various stimuli after 3 days (OKT3, anti-CD3 antibody (clone OKT3); PMA, phorbol 12-myristate 13-acetate; SEA, Staphylococcal enterotoxin A; SEB, Staphylococcal enterotoxin B; PHA, phytohaemagglutinin) and (**b**) after 7 days stimulation with specific antigens tetanus toxoid (TT) and purified protein derivatives of *M. tuberculosis* (PPD). Mean fold enrichment from one experiment is shown. Error bars denote ± s.d. from three technical replicates. (**c**) Flow cytometric analysis of the CD127 expression in healthy donor (red), P1 (orange) and P2 (blue) in T_{naive} ($CD8^+CD45RA^+CCR7^+$), T_{CM} ($CD8^+CD45RA^-CCR7^+$) and T_{EM} ($CD8^+CD45RA^-CCR7^-$) cell populations. (**d**) Flow cytometric analysis of the T_{FH} cell subset in patient and age matched (9 years) healthy donor. T_{FH} cells defined as $CD4^+CXCR5^+CD45RA^-$. (**d**,**e**) Plots represent one experiment. (**e**) Flow cytometry analysis of CD69 and ICOSL upregulation on CD40 stimulation on peripheral B cells. PBMCs from healthy donor, parent or P1 were kept unstimulated (grey) or *in vitro* stimulated for 36 h with CD40L (black line). Plots are representative of two independent experiments.

healthy donor cells (Supplementary Fig. 8), they degranulated at a significantly lower frequency as measured by the expression of CD107a (LAMP1) on the cell surface after stimulation (Fig. 7c).

To further define the cytolytic potential of these cells, we evaluated key components of cytotoxicity by quantitative confocal microscopy. Patient-derived NK cells failed to accumulate F-actin at the immunological synapse following incubation with susceptible targets (Fig. 7d,e). In addition, lytic granules failed to polarize to the lytic synapse (Fig. 7e). In concert with the flow cytometric analyses, these data suggest a marked inability of NIK^{Pro565Arg} NK cells to become activated and exert cytolytic function.

Discussion

TNFα receptor family signalling is essential for B-cell immunity in humans as illustrated by deficiencies in CD40L, CD40 and BAFFR[40]. NIK is an integral component of the non-canonical NF-κB pathway downstream of these receptors[5].

Studies in the mouse *aly* mutant[8,9] and *Nik* knockout mice[3] described B-cell deficiency due to disorganized lymph nodes, Peyer's patches and splenic architecture, accompanied by B-cell lymphopenia and low serum Ig levels due to compromised CSR and SHM[41]. We here identify patients with biallelic mutation in NIK, leading to loss-of-function of the kinase function of NIK. We show that human functional NIK deficiency recapitulates phenotypes described in the mouse studies including B-cell lymphopenia, impaired CSR and SHM, decreased marginal zone and memory B cells, and hypogammaglobulinemia. Although ethic considerations prevented us from obtaining patient biopsies to further investigate secondary lymphoid organ structures, the absence of lymph nodes on repeated clinical examinations suggests that secondary lymphoid organs may be disturbed on loss-of-function of NIK, similar to the observations in mouse models[3,8,41].

To extend the murine studies on NIK function in B cells, we investigated the survival properties of patient peripheral blood B cells. *In vitro* stimulation of BAFFR together with BCR and TLR9 resulted in absence of B cells, suggesting a profound survival defect. This is supported by earlier studies showing that Nik overexpression or expression of the NikΔT3 mutant in mice (resistant to Traf3-mediated degradation) leads to increased survival of B cells[42]. Here we find significantly decreased expression of the anti-apoptotic gene *BCL2* in peripheral NIK^{Pro565Arg} B cells, leading to reduced survival. This is most probably the result of impaired BAFF signalling, as NIK is an integral molecule downstream of BAFFR required for B-cell

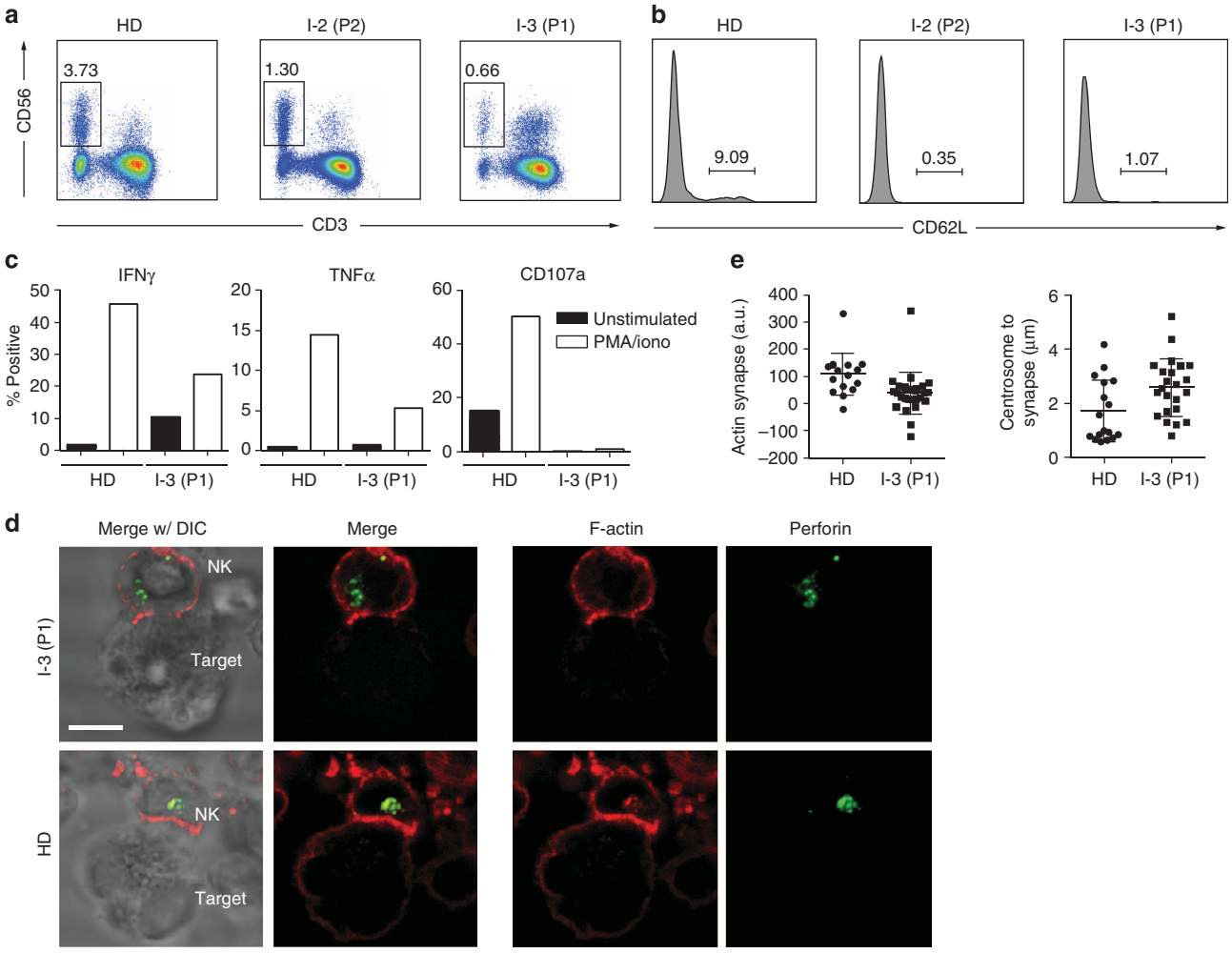

Figure 7 | Functional impairment of NIK-deficient NK cells. (**a**) Flow cytometric analysis of NK cells in patients and healthy donor. NK cells defined as $CD56^+CD3^-$. (**b**) Histogram of expression of CD62L on NK cells of patients and healthy donor (gated on $CD56^+CD3^-$ cells). (**c**) Functional response to PMA and ionomycin of NK cells from NIK-deficient patients and healthy donor. PBMCs from healthy donor (HD, left) or patient 1 (P1, right) were incubated with vehicle control (black) or PMA and ionomycin (white) then fixed, permeabilized and analysed by flow cytometry for intracellular expression of IFNγ, TNFα and CD107a. (**a**–**c**) Data represent one experiment. (**d**) Immunofluorescence analysis of mature immunological synapse formation by NK cells. Immunofluorescence detection of perforin (green) and F-Actin by phalloidin (red) in an NK-cell conjugate from Patient 1 (P1, top panel) or healthy donor (HD, bottom panel). K562 cells were used as target cells. DIC, differential interference contrast. White bar indicates 5 μm. Image is representative of one experiment. (**e**) Quantification of immunological synapse formation. $n=16$ (healthy donor) and $n=27$ (P1) for actin synapse quantification, $n=17$ (healthy donor) and $n=23$ (P1) for centrosome to synapse distance quantification; mean ± s.d. shown. Differences are significant as determined by student's two-sided *t*-test ($P=0.0073$ and $P=0.0190$, respectively).

survival[2,29,30]. Recently, a common variant in *BAFFR* has been shown to modulate NF-κB signalling albeit without effects on survival and subset composition of B cells[43]. Although we identified this variant in our patients, its presence could not explain the B-cell defects described in this study. BAFFR signalling also activates the expression of the B-cell maturation marker CD21 on transitional B cells[2,30,44]. CD21-deficient mice display severely impaired GC B-cell development and T-cell-dependent B-cell responses[45] due to reduced GC B-cell survival[46]. CD21 deficiency in humans leads to reduced class-switched memory B cells and hypogammaglobulinemia[47]. Therefore, the reduced CD21 expression on peripheral patient B cells we observed may provide an additional explanation for B-cell survival defects in $NIK^{Pro565Arg}$ patients.

Stimulation with CD40L and IL4, a cytokine with potent anti-apoptotic activity mediated by Stat6-dependent upregulation of Bcl-xL[26], led to CSR with nearly normal frequency, but delayed kinetics, indicating that at least a proportion of patient B cells was responsive to these stimuli, and that the CSR process itself is largely functional. As CD40 stimulation can signal both via canonical and non-canonical NF-κB pathways leading to AID expression and Igγ germline transcription[48], our findings indicate that CD40L-mediated, NIK-independent NF-κB signalling contributes to CSR. Delayed CSR kinetics might also be explained by an increased proportion of transitional B cells in patient PBMCs, which reacted more slowly to the B-cell activation stimuli, similar to BAFF-deficient B cells[2].

As the clinical phentoype suggested a combined immunodeficiency and because recent studies have focused on the role of NIK in T cells[35,49,50], we aimed at investigating T-cell functions and T-cell interplay with B cells in patients carrying $NIK^{Pro565Arg}$. Previous studies indicate that NIK-dependent NF-κB signalling is required for ICOSL expression on activated B cells, directing T_{FH} differentiation via interaction with ICOS[39]. T_{FH} cells in turn stimulate B-cell differentiation by expressing CD40 and IL21 (ref. 37). This intimate cell–cell communication

leads to the formation of GCs, structures essential for generation of high-affinity antibody responses. Abrogated ICOSL upregulation on B cells and reduced T_{FH} cells in the here described patients probably contribute to impaired GC formation; however, *in situ* analysis of GCs were precluded by ethical constraints.

Another lymphocyte communication process dependent on CD40 signalling via $CD4^+$ T-cell help is $CD8^+$ T-cell memory maintenance. Subsequent stimulation of IL7R expression is characteristic and essential for $CD8^+$ memory T-cell survival[4]. Therefore, lack of IL7R expression on both Nik-deficient mouse[35] and human (this study) $CD8^+$ memory T cells may contribute to the inability of T cells with $NIK^{Pro565Arg}$ to respond to tetanus toxoid and tuberculin despite prior vaccination. Recently, data from ICOSL-deficient patients has implicated ICOSL costimulation in maintenance of memory populations[51], possibly providing an additional explanation for memory defects in patients carrying $NIK^{Pro565Arg}$.

Patients exhibiting defects in canonical NF-κB signalling such as NEMO[52,53] or IKKβ deficiency[54] and IκBα hypermorphism[55] also show antibody deficiencies. Consistent with multiple roles of canonical NF-κB signalling, they show pleiotropic defects throughout the adaptive and innate immune system and developmental defects. Patients with heterozygous mutations in *NFKB2/p100* have recently been described with B-cell deficiency and autoimmunity[56]. Although showing similar manifestations, functional NIK deficiency is more severe than the heterozygous *NFKB2* mutation in humans (this study) and mice[5]. This may be due to the increased p100 levels in $NIK^{Pro565Arg}$ B-LCL that we detected, potentially caused by compensatory increase of NIK-independent canonical NF-κB signalling, which can upregulate p100 expression[19]. In line with this, unprocessed p100 is known to specifically sequester and inhibit RelB[5], leading to a severe signalling defect.

CD40-dependent IL12 secretion by monocytes is crucial in the defense against mycobacteria. The disseminated Bacillus Calmette–Guérin infection observed in P1 illustrates mycobacterial susceptibility similar to NEMO[57] and IKKβ-deficient patients[54]. As NIK can signal via both non-canonical and canonical pathway, NF-κB response to mycobacteria may depend on NIK, suggesting that functional NIK deficiency causes defective CD40 signalling in monocytes as well.

Recently, impaired NK-cell function has been recognized for several primary immunodeficiencies (reviewed in ref. 58) most often manifesting in susceptibility to infection by herpesviruses (for example, CMV). NK-cells were persistently decreased in patients carrying $NIK^{Pro565Arg}$. Interestingly, CD62L expression, a marker linked to mature NK-cell subsets with stronger cytolytic functions[59], was downregulated on patient NK cells. Indeed, although they acquired appropriate developmental and maturity markers, including perforin, patient cells failed to become activated as shown by impaired cytokine secretion, degranulation and polarization of lytic granules towards targets. NIK has not been linked to NK-cell function so far. As canonical NF-κB signalling has been implicated in NK-cell activation[60], these signals may depend on NIK and therefore mirror NK-cell phenotypes such as those found in CD40L or NEMO deficiencies.

Interestingly, despite the potential defects in secondary lymphoid organ organization discussed above, allogeneic haematopoietic stem cell transplantation (aHSCT) improved the overall condition of patient P1, suggesting that functional NIK deficiency is amenable to aHSCT to at least partially correct the disease. The fatal outcome for P2 (who had received aHSCT without prior conditioning and succumbed shortly after a second aHSCT including conditioning) suggests that at least reduced-intensity conditioning is indicated for successful treatment using aHSCT.

In sum, we identify functional NIK deficiency as a novel, pervasive combined primary immunodeficiency syndrome. Our data revealed an unexpectedly broad range of phenotypic aberrations affecting B-, T- and NK-lineages, and thus highlight essential roles for NIK and adequate control of non-canonical NF-κB signalling for generation and maintenance of the human immune system.

Methods

Patient and ethics. This study has been approved by the ethics committee at the Medical University of Vienna, Austria. Biological material was obtained on informed consent in accordance with the Declaration of Helsinki. Clinical data from the patients was provided in anonymized form by the responsible physician(s). The patients were evaluated, followed up and treated at the Department of Immunology at Ankara University in Turkey, covered by the local ethics agreement.

Homozygosity mapping. Affymetrix 6.0 SNP-based homozygosity mapping was performed in both patients and in both parents of P1, to map homozygous intervals common to both patients but not present in the parents.

Ten microlitres of 50 ng μl^{-1} DNA from the patients were used. The protocol was carried out according to the Affymetrix Genome-Wide Human SNP Nsp/Sty 6.0 protocol. The results were analysed using the Affymetrix Genotyping Console software and PLINK whole genome data analysis toolset (http://pngu.mgh.harvard.edu/~purcell/plink/) as previously described[61].

Exome sequencing. A multiplexed 50-bp paired-end read ES was carried out for P1 on Illumina HiSeq2000 Sequencer running on HiSeq Control Software 1.4.8, Real Time Analysis Software 1.12.4.2. The sample preparation used 1 μg of genomic DNA fragmented using Illumina TruSeq DNA Sample Preparation Guide and the Illumina TruSeq Exome Enrichment Guide version 3. The DNA fragment clusters generated ran in a multiplexed pool with five other samples distributed on three lanes of the flow cell.

The data analysis was carried out as previously described[62] using Burrows–Wheeler Aligner to align the reads to the human genome 19. Insertion/deletion realignment was performed as well as GATK (Genome Analysis Toolkit) base quality score recalibration. For SNV and insertion/deletion calling, Unified Genotyper and GATK Variant quality score recalibration was performed. SNV and insertion/deletion lists were uploaded to SeattleSeq Annotation database. Known variants (present in 1000Genomes or dbSNP build 137, date of accession: 2 January 2012) were excluded and the lists were filtered for nonsense, missense and splice-site variants present within the homozygous regions detected in both patients and absent in the parents. In addition, ENSEMBL, UCSC, NCBI and EVS public SNP databases (date of accession: 20 February 2012) were interrogated for presence of the variant.

The validation and segregation of the variants found in the final hit list from ES were performed using capillary sequencing on genomic DNA from both patients and family members as described below.

Variant validation by capillary sequencing. Primers for the variants detected with whole ES were designed using ExonPrimer software from the Helmholtz Center Munich (http://ihg.gsf.de/ihg/ExonPrimer.html) and PrimerZ[63], respectively, and ordered from Sigma Aldrich, Austria. PCR amplification of the detected variants was performed using Expand High Fidelity PCR System (Roche, Basel, Switzerland).

Capillary sequencing of amplicons was performed on the Applied Biosystems 3130xl Genetic Analyzer capillary sequencer running 3130xl Genetic Analyzer Data Collection Software v3.0, using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Germany). Sequence Analysis Software Version 5.2 was used for analysis of the sequences and heterozygous signals with ambiguity code were indicated when 25% of the signal intensity was exceeded. Reads were aligned to reference sequences using the Sequencher software, version 4.10.1.

***In silico* analyses and modelling of NIK protein structure.** The algorithms SIFT[64], PolyPhen2 (ref. 65) (website accessed: 27 March 2012) and CUPSAT[66] (website accessed: 9 December 2013) tools were used to predict the effect of the identified mutation on protein function.

To obtain a hypothesis about the change in protein structure and dynamics of the NIK variant compared with the wild-type, molecular dynamics (MD) simulations of $NIK^{wild\text{-}type}$ and $NIK^{Pro565Arg}$ were performed using the coarse-grained model FREADY[67] implemented in MOIL[68] molecular modelling package. We initiated the MD simulations from the crystal structure of NIK (PDB ID 4G3D, chain A[14]) and let it run for 50 ns at 300 K. In the MD simulations, residues farther than 9 Å from the mutated residue were fixed to the experimental structure. Protein structures were aligned and visualized using MacPyMol (The PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC).

Flow cytometry-based immunophenotyping and cell sorting. Immunophenotyping characterization was performed on a BD LSR Fortessa, BC FACS Canto or BD FACS Calibur. In brief, PBMCs from the patients, parents and healthy donors were isolated using Ficoll density gradient centrifugation and either stored frozen in liquid nitrogen and thawn at a later time point or immediately stained for 20 min at 4 °C with mouse anti-human antibodies using the following volumes for one million cells in 100 µl: 2 µl CD3-APC-H7 (SK7), 3 µl CD4-APC (RPA-T4), 5 µl CD8-V500 (RPA-T8), 5 µl CD8-APC-H7 (SK1), 3 µl CD21-PE (B-ly4), 10 µl CD25-PE (M-A251), 1 µl CD27-BV421 (M-T271), 10 µl CD27-FITC (L128), 2.5 µl CCR7-PE-CF594 (150503), 3 µl IgD-FITC (IA6-2), 10 µl CD86-FITC (2331, FUN-1), 5 µl CD95-PECy7 (DX2), 20 µl IL21R-PE (17A12) (all from BD Biosciences); 3 µl CD56-PE (N901) (from Beckman-Coulter); 2 µl CD3-BV711 (OKT3), 1.5 µl CD4-BV510 (OKT4), 0.2 µl CD45RA-BV605 (HI100), 5 µl CD127-BV421 (A019D5) (all from BioLegend); 2 µl CD3-APC (SK7), 3 µl CD4-PerCP-Cy5.5 (RPA-T4), 3 µl CD19-PerCPCy5.5 (HIB19), 5 µl CD69-APC (FN50), 3 µl ICOSL-PE (B7-H2; clone MIH12), 2.5 µl Foxp3-FITC (PCH101) (all from e-Bioscience); and 10 µl CXCR5-APC (51505; R&D Systems).

T and NK cells were evaluated using around 1×10^6 PBMCs. The analysis of the B-lymphocyte compartment was performed using around 4×10^6 cells as previously described[2,69,70]. NK cells were stained as described previously[71]. For intracellular staining, PBMCs were activated for 3 h with 50 mg ml^{-1} ionomycin and 5 mg ml^{-1} phorbol myristate acetate in the presence of Brefeldin A and antibodies to CD107a and cell surface markers[72]. Cells were then fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences).

All analyses were performed using FlowJo X (TreeStar Inc.) and data were graphed with Prism 6.0 (GraphPad Software).

Flow cytometry-based sorting of peripheral B-cell populations stained with anti-CD19-PerCP-Cy5.5, anti-CD27-BV421, anti-CD3-APC-H7 and anti-IgD-FITC as described above was performed on ultra-high-speed six-way digital cell sorter from Beckmann Coulter at the Medical University Vienna Flow Cytometry Core Facility.

Magnetic microbeads based sorting of peripheral B cells was performed using anti-CD20 paramagnetic labelling according to the manufacturer's instructions (130-091-104, Miltenyi Biotec, Bergisch-Gladbach, Germany).

Quantitative real-time PCR analysis. Extraction of RNA from sorted B cells was performed using RNeasy kit from Qiagen, first-strand complementary DNA synthesis was done using Expand Reverse Transcriptase from Roche using both oligo-dT and random hexamer primers, and gene expression was analysed by quantitative PCR using Kappa Sybr Fast qPCR MasterMix ABI Bioprism from Kappa Biosystems on 7900HT Fast Real-Time PCR System from Applied Biosciences according to manufacturers' instructions.

Intron-spanning primers were used for the gene expression analysis. The primer sequences are as follows: *BCL2*-forward 5′-CCGGGAGATGTCGCCCCTGGT GGA-3′, *BCL2*-reverse 5′-AGGCCGCATGCTGGGGCCGTA-3′; *MCL1*-forward 5′-TCGTAAGGACAAAACGGGAC-3′, *MCL1*-reverse 5′-ACCAGCTCCTACT CCAGCAA-3′; *BCL2L1*-forward 5′-GAATGACCACCTAGAGCCTTGG-3′, *BCL2L1*-reverse 5′-TGTTCCCATAGAGTTCCACAAAAG-3′; *GAPDH*-forward 5′-TGATGGCATGGACTGTGGTC-3′, *GAPDH*-reverse 5′-TTCACCACCATGGA GAAGGC-3′.

Cell culture and stimulation conditions. Healthy donor, patient and family members PBMCs (isolation as above) and Epstein–Barr virus transformed B-cell line were maintained in RPMI-1640 medium supplemented with 10% of inactivated FCS (Life Technologies, Gibco), 50 U ml^{-1} penicillin, 50 µg ml^{-1} streptomycin and 292 µg ml^{-1} L-glutamin (all from Gibco) at 37 °C in a humidified atmosphere with 5% CO_2. PBMC stimulation conditions and reagents were trimeric human CD40L and human BAFF-Fc, both produced as described[2], and human recombinant IL21 (ebiosciences) used at 20 ng ml^{-1}; B-LCL stimulation conditions were hBAFF (R&D Systems; 2149-BF-010) used at 50 ng ml^{-1} for 6–12 h; and primary fibroblast stimulation conditions were Lymphotoxin α1/β2 (R&D Systems, 678-LY-010) used at 50 and 100 ng ml^{-1} from 15 min to 4 h and TNFα (14-8329-62; eBioscience) used at 20 ng ml^{-1} from 15 min to 4 h.

Primary fibroblasts and HEK293 cells were cultured in glucose-rich DMEM (PAA), supplemented and cultured as above.

B-cell activation assays were performed by stimulating PBMCs with CD40L and IL21 as described[2] or using CD40L and IL4 (100 ng ml^{-1}, ImmunoTools) in Iscoves's modified DMEM medium (Invitrogen), supplemented with 10% heat-inactivated FCS (Biowest), 100 U ml^{-1} penicillin (Invitrogen), 100 µg ml^{-1} streptomycin (Invitrogen), 1 µg ml^{-1} insulin (Sigma-Aldrich), 1 µg ml^{-1} reduced glutathione (Sigma-Aldrich), 2.5 µg ml^{-1} apo-transferrin (Sigma-Aldrich), 2 mM glutamine (Life Technologies, Gibco) and 1% non-essential amino acids (Gibco). Culture was started with equal number of cells and samples were analysed at days 3, 6 and 9 by flow cytometry.

Immunoblot analysis. Protein was isolated using cell lysis buffer containing 20 mM Tris (pH7.5), 150 mM NaCl, 2 mM EDTA, 1% TritonX-100 (pH7.1) and complete protease inhibitor cocktail (Sigma Aldrich). Polyvinylidene difluoride or nitrocellulose membranes were prepared according to standard methods. Primary antibodies used for immunoblot analysis of NF-κB pathways were: rabbit anti-human IKKα (2682), phospho-IKKα/β (2697), p100/p52 (4882), NIK (4994), RelB (clone C1E4), TRAF3 (4729), p65 (clone D14E12), p105/p50 (3035) and mouse anti-human IκBα (clone L35A5), all purchased from Cell Signaling and used at 1:1,000 dilution. For detection of tagged recombinant proteins, anti-human c-Myc (551101, BD Biosciences) was used at 1:1,000 dilution and horseradish peroxidase-coupled anti-HA (H6533, Sigma-Aldrich) was used at 1:3,000 dilution. For loading controls, mouse anti-human GAPDH (clone 6C5; Santa Cruz Biotechnology) and anti-human RCC1 (clone E-6; Santa Cruz Biotechnology) were used at 1:1,000 dilution. Horseradish peroxidase-conjugated goat anti-rabbit (Bio-rad) and goat anti-mouse (BD Biosciences) secondary antibodies were used at 1:10,000 or 1:50,000 dilution and detected using a chemiluminescent substrate (Amersham ECL Prime Western Blotting Detection Reagent, GE Life Sciences) together with Hyperfilm ECL (Fischer Scientific).

Kinase assay. HEK293 cells were transfected in six-well plates with NIK- and/or IKKα-tagged expression vectors generated by gateway recombination using the pTO (carboxy-terminal streptavidin–haemagglutinin tag) or pCS2 (amino-terminal 6 × myc tag) destination vectors[73], lysed 24 h post transfection in lysis buffer containing 20 mM HEPES (ph7.9), 20% glycerol, 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5 mM β-glycerophosphate, 1 mM phenylmethyl sulphonyl fluoride, 5 mg ml^{-1} apronitin, 10 mM NaF, 5 mg ml^{-1} leupeptin and 5 mM Na_3VO_4, and subsequently subjected to SDS–PAGE and immunoblot analysis.

Amplification and sequence analysis of *IGH* transcripts. IgA and IgG transcripts were amplified from cDNA of thawed PBMCs using subgroup-specific forward primers in the leader sequence of *IGHV3* and *IGHV4* in combination with a Cα or Cγ consensus reverse primer[22,74]. All PCR products were cloned into the pGEM-T easy vector (Promega) and prepared for sequencing on an ABIPRISM 3130xl (Applied Biosystems). Obtained sequences were analysed with the IMGT database (http://www.imgt.org/) for *IGHV*, *IGHD* and *IGHJ* use, and mutation analysis[75].

T-cell proliferation analysis. T-cell proliferation assays were carried out as described previously[62,76].

T-cell CDR3 Vβ spectratyping. TCR Vβ spectratyping was performed as previously described[77] with minor modifications. The primers used were as before with the following exceptions: Primers for variable regions, BV02-5′-ACATACG AGCAAGGCGTCGA-3′, BV04-5′-CATCAGCCGCCCAAACCTAA-3′, BV07-5′-CAAGTCGCTTCTCACCTGAATGC-3′, BV17-5′-TGTGACATCGGCCCAAA AGAA-3′, BV21-5′-GGAGTAGACTCCACTCTAAG-3′, BV24-5′-CCCAGTTT GGAAAGCCAGTGACCC-3′; primers for constant regions (used for BV05, BV06BC, BV20), CβB1- 5′-CGGGCTGCTCCTTGAGGGGCTGCG-3′; FAM-marked constant primer-5′-ACACAGCGACCTCGGGTGGG-3′.

Sequences were acquired using an ABI 3130xl Sequencer (ABI Applied Biosystems) and analysed using GeneMapper software version 4.0.

Immunostaining of lymphotoxin-stimulated fibroblasts. Fibroblasts of patient (P2) and healthy donor were stimulated with 100 ng ml^{-1} of lymphotoxin α1/β2 (R&D Systems, 678-LY-010) for 4 h. After stimulation, cells were fixed with 4% formaldehyde in PBS for 30 min and then blocked and permeabilized with solution containing 10% FCS plus 0.1% Triton X-100. Cells were immunostained with DAPI and rabbit antibodies against NFκB2 (p100/p52) (Cell Signaling, 3017) and NF-κB (p105/p50) (Cell Signaling, 3035) at a dilution of 1:100, respectively, and afterwards with anti-rabbit Alexa Fluor 546-conjugated antibody at a dilution of 1:500 (Life Technologies, A10040). Images were acquired on a Leica AF6000 fluorescent microscope using Leica LASAF software for acquisition. Images were taken at × 64 magnification.

Reconstitution assay. cDNA encoding for wild-type human *MAP3K14* was cloned into a bicistronic retroviral pMMP vector coexpressing *MAP3K14* and enhanced green fluorescent protein (eGFP) marker gene via IRES sequence. RD114-pseudotyped retroviral particles were generated by transfection into HEK293 cells using the calcium chloride transfection method (8 µg retroviral vector DNA, 12 µg gag/pol DNA, 5 µg RD114 DNA) in the presence of 25 µM chloroquin (Sigma-Aldrich, C6628) for 12 h. Supernatants containing viral particles were collected after 24, 36 and 48 h. Viral titration was performed on HT-1080 cells. Patient and normal donors fibroblast cells were transduced with retroviral particles in the presence of 8 µg ml^{-1} polybrene (Santa Cruz, sc-134220) for 12 h. Transduction efficiency was determined by eGFP expression by FACS analysis and was between 45% and 70%. After transduction, immunofluorescence studies were performed as above with additional staining against GFP used at a dilution of 1:100 (antibody sc-69779, Santa Cruz). Slides were visualized as above. Data was graphed using Prism 6.0 (GraphPad Software).

Confocal microscopy of immunological synapses. Cell conjugates were formed for the evaluation of the immunological synapse by confocal microscopy as previously described[78]. Following fixation and permeabilization, conjugates were incubated with anti-perforin Alexa Fluor 488 (Biolegend) at a dilution of 1:50 and Phalloidin Alexa Fluor 568 at a dilution of 1:100. Images were acquired on a Leica SP8 laser scanning confocal microscope. Excitation was by tunable white light laser and detection of emission by hybrid gallidium (HyD) detectors. Acquisition was controlled by Leica LASAF software and images were subsequently exported to Volocity software (PerkinElmer) for analysis. Data were graphed using Prism 6.0 (GraphPad Software).

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Author contributions

K.L.W., S.K., E.S.-V., W.G., E.M., I.B., E.S., C.D.-C., H.S and P.P.B. performed experiments. F.D., S.H., M.G.B. and A.I. provided clinical care of the patients, provided clinical data and were involved in clinical care including allogeneic HSCT of both patients. A.K. performed HSCT of P1. E.M., P.P.B. and J.S.O. performed NK-cell analyses. M.C.vZ. performed T-cell phenotyping and Ig sequence analysis. P.M. performed computational modelling and *in silico* prediction algorithms. W.F.P. performed T-cell proliferation assays. G.V. assisted with imaging. G.S.-F., A.C.-N. and J.C. were involved in critical scientific discussions. K.B. conceived this study, provided laboratory resources, and together with K.L.W., S.K. and E.S-V. planned, designed and interpreted experiments. K.L.W, S.K., I.B. and K.B. wrote the manuscript with input from H.E., M.C.vZ., J.S.O, G.S.-F., E.M., C.D.-C., E.S.-V. and A.I. All authors critically reviewed the manuscript and agreed to its publication.

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4 DISCUSSION

4.1 Application of modern genetic technologies in PID investigation

The use of molecular approaches in the investigation of PIDs for the past 30 years has disclosed relevant information about the function of critical molecules and pathways in immune response to infections and immune regulation, which eventually contributed to the development of improved targeted therapies for PID patients (reviewed in (Conley & Casanova, 2014; Picard & Fischer, 2014)).

In human populations or families, the influence of genetic elements in healthy and disease can be investigated with approaches such as linkage and association studies, which can identify the chromosomal region or genetic factors linked to a given phenotype (Abel & Dessein, 1998; Casanova & Abel, 2004). More specifically, evaluation of definite alleles carried by single individuals can reveal particular vulnerability to infections (Quintana-Murci et al, 2007). This individual approach had a great impulse with the development of a simple and efficient method of nucleotide sequencing in the 1970s, based on transcription of specific regions of the DNA using DNA polymerase and deoxynucleoside triphosphates as chain-terminating inhibitors (Sanger et al, 1977). Despite this advance, until mid 1980s only few genes had been mapped to cause primary immunodeficiencies (1986).

Between 1984 and 2009, the identification of hundreds of PID-associated genes was possible on account of techniques including cytogenetic tools, positional cloning, candidate gene approach and particularly Sanger sequencing. Cytogenetic methods such as fluorescence *in situ* hybridization and restriction fragment length polymorphism allowed the detection of chromosomal abnormalities in patients with Di George syndrome and of the gene causing X-linked GCD, respectively (Baehner et al, 1986; Greenberg et al, 1988; Royer-Pokora et al, 1986). Additionally, the use of positional cloning enabled mapping of *BTK* in XLA patients (Vetrie et al, 1993) and of *WAS* as a cause for Wiskott-Aldrich syndrome (Kwan et al, 1991). Candidate gene approaches also revealed for example deficiency of RAG1 and RAG2 in AR T-B-NK+ SCID patients (Schatz et al, 1989; Schwarz et al, 1996). Employment of Sanger sequencing, especially after the development of automated DNA Sanger sequencing analysis, enabled identification of several other PID genes (Smith et al, 1986) (reviewed in (Al-Herz et al, 2014; Picard & Fischer, 2014)).

Since the emergence of the high throughput or next generation DNA sequencing (NGS) methods less than a decade ago (Ng et al, 2009; Wheeler et al, 2008), the recognition of new genetic defects has been transformed. NGS technologies entitle fast and relatively cheap genetic investigation, especially under circumstances in which evaluation of several candidate genes are necessary (Picard & Fischer, 2014). High-throughput methods including whole exome sequencing (WES), whole genome sequencing (WGS), genetic linkage (GL) analysis, homozygosity mapping, analysis of copy number variations (CNV) and others, are proven efficient in molecular characterization of several conditions with unknown genetic background (reviewed in Conley & Casanova, 2014; Picard & Fischer, 2014)).

The uncovering of molecular defects with the use of NGS methods is accelerated when consanguineous families or isolated populations are investigated. In such cases, filtering of the data is facilitated by the similarities or differences expected (reviewed in (Conley & Casanova, 2014)). In consanguineous families and/or in families with multiple affected individuals for example, the use of WES in combination or not with homozygosity mapping has been proven very useful for uncovering several new disease-related genes (Picard & Fischer, 2014).

Nowadays, NGS technologies are more efficient than Sanger sequencing in cases where there are no or several candidate genes. However, since high-throughput methods also possess limitations such as, as for instance, the coverage, not always ideal, of only exonic regions in WES, Sanger sequencing is still considered the gold standard method to evaluate single or few candidate genes and, due to its low error rates, also to validate variants found using NGS methods. Despite existing limitations, the effectiveness of such techniques in identifying genetic variants not only in well-known genes, but also in genes with poorly characterized functions is endorsed by a long list of recently described PIDs upon use of NGS approaches (Picard & Fischer, 2014).

One important question raised however with the uncovering of such enormous number of new gene variants using high-throughput methods is the functional significance of those findings. To answer this query, several approaches are employed to ensure the causative link to the disease. Bioinformatics tools for instance can predict *in silico* the pathogenic consequences of a given amino acid substitution in the protein function and stability (Adzhubei et al, 2013; Ng & Henikoff, 2003; Parthiban et al, 2006). Additionally, searching public single nucleotide polymorphism (SNP) databases for the variants found will further assist the evaluation of the possible outcome. Besides such predictive tools, the evaluation of

healthy individuals or others with the same molecular defect, and particularly the implementation of functional *in vitro* assays are important for validating the functional consequences of the variants found, proving causative link with the observed phenotype.

On that account, regardless of obvious ethical issues inherent to human research that prevent invasive experiments, the direct study of the immune system in humans, particularly with employment of a variety of modern techniques available, enables more extensive and straightforward data regarding crucial factors in immune responses to be generated, which is undoubtedly valuable for direct clinical applications (reviewed in (Casanova & Abel, 2004; Milner & Holland, 2013)).

Considering all the above-mentioned facts, modern molecular techniques were employed in this doctoral thesis to investigate the underlying genetic mutations in PID patients from consanguineous families and to functionally evaluate the newly identified mutations. The work resulting from this doctoral thesis is further discussed in more details in the following chapters.

4.2 A novel mutation in the complement component 3 gene in a patient with selective IgA deficiency

4.2.1 Complement component 3 and the C345C domain

The complement component 3 (C3) has a central role in complement activation and function and therefore is of great value to host defense against infections, clearance of immune complexes and apoptotic cells (Dunkelberger & Song, 2010; Merle et al, 2015a). The gene encoding the human C3 contains 41 exons (Fong et al, 1990) and the resulting protein is 187kDa (Tack & Prahl, 1976). C3 is member of the α2-macroglobulins (α2M) family (reviewed in (Borth, 1992)) and its structure comprises two chains (β: residues 1-645, and α: residues 650- 1641) with 13 macroglobulin (MG) domains (Janssen et al, 2005). Figure 5 shows the protein domains in the C3 and its cleavage products.

Following proteolytic activation, C3 is cleaved into C3a (ANA) and C3b (Dunkelberger & Song, 2010; Merle et al, 2015a), in a process that triggers major structural reorganization in the protein, mainly involving MG7, MG8, anchor, C345C, TED (Thioester containing domain) and CUB domains. Those conformational changes expose the thioester group (Cys988-Gln991) in the TED domain from C3b and allows its binding to pathogenic or apoptotic surfaces (Janssen et al, 2006). Changes in the C3 structure after activation also expose putative CFB binding sites in C3b (Janssen & Gros, 2007).

The C-terminal domain C345C or Netrin-like of C3 corresponds to exons 38 to 41 of *C3* gene and is connected to MG8 domain by an anchor region (Janssen et al, 2005). It is a common domain to C3, C4 and C5, but not to other α2M proteins (reviewed in (Janssen & Gros, 2007)) and contains three of the 13 disulfide bonds (Figure 5) that keep the C3 protein folding and stability (Cys1496-Cys1568, Cys1515-Cys1639 and Cys1615-Cys1624) (Dolmer & Sottrup-Jensen, 1993; Huber et al, 1980). C345C has been proposed to interact with some other components of the complement system, such as CFB (Torreira et al, 2009), properdin (Alcorlo et al, 2013), CFI (Wu et al, 2009) and possibly C5 (Laursen et al, 2011), which evinces its significant role in C3 activation and activity regulation.

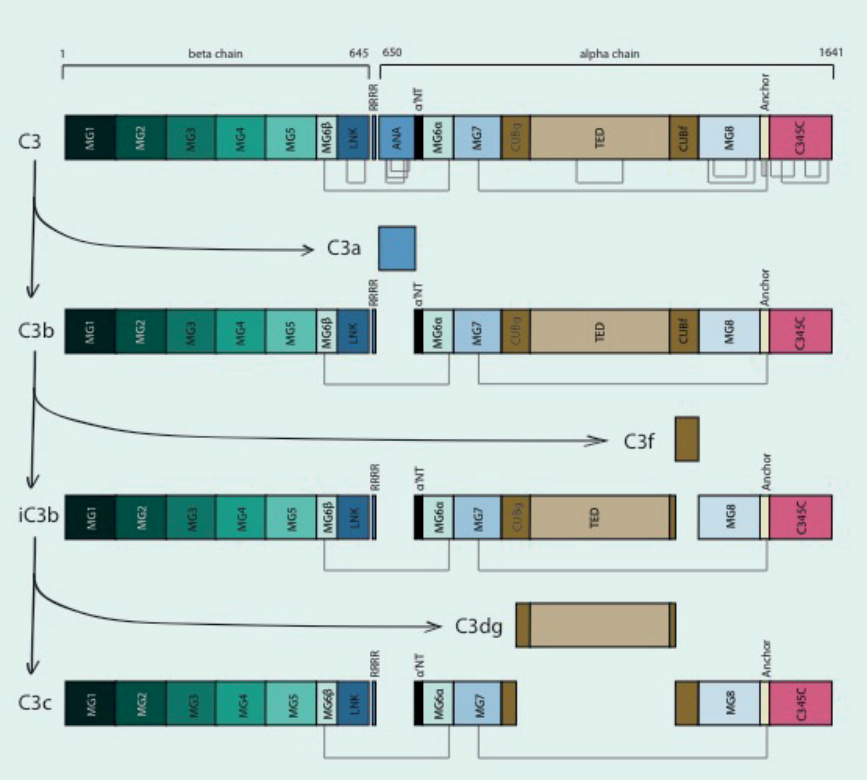

Figure 5. Structural domains of C3 protein and its cleavage products (adapted from (Janssen et al, 2005)) MG: macroglobulin domain; LNK: linker domain; RRRR: processing site present in the inactive C3, composed of four arginine residues (646-RRRR-649); ANA: anaphylatoxin; α'NT: N-terminal region of the α-chain in C3b. Grey lines: disulfide bonds.

4.2.2 Homozygous mutation in the C345C domain of *C3* impairs immune response to encapsulated bacteria

In the first manuscript of this thesis we investigated a subject suffering from severe and recurrent respiratory infections caused to *S. pneumonia*, in whom we uncovered a new homozygous mutation in exon 38 of *C3,* inside the C345C domain (c. C4554G, p. Cys1518Trp).

Deficiency of C3 (OMIN 120700) was first described in the early 1970's (Alper et al, 1972), and the main clinical manifestations of AR or compound heterozygous mutation in *C3* are recurrent and severe pyogenic infections early in life. The most common infections are pneumonia and meningitis due to encapsulated bacteria, including *Neisseria meningitidis*, *Streptococcus pneumonia* and *Haemophilus influenzae*. The predominance of such pathogens demonstrates the important function of C3 in opsonization of those microorganisms. In addition, immune-complex

disorders such as glomerulonephritis, vasculitis and SLE-like disease have also been described (reviewed in (Grumach & Kirschfink, 2014; Okura et al, 2015)).

Currently, only 29 families have been described worldwide and the majority of these patients have homozygous mutations (Okura et al, 2011); few cases of compound heterozygous C3 deficiency however were also published (Katz et al, 1995; Kida et al, 2008; Okura et al, 2011). Additionally, AD gain-of-function defects have been described, mainly associated with atypical hemolytic-uremic syndrome (aHUS) (Grumach & Kirschfink, 2014; Okura et al, 2015).

Since the phenotype exhibited by our patient was in accordance to the literature findings for C3 deficiency (Grumach & Kirschfink, 2014), our molecular investigation comprised Sanger sequencing of the *C3* gene. In addition to the complement C3 deficiency, the patient also presented with selective IgA deficiency (sIgAD), with reduced serum IgA levels, while other immunoglobulin isotypes were within the normal range. Manifestations of immune dysregulation, described in some cases of C3 and of sIgAD deficiency, were however absent in our patient.

As shown in the first manuscript and already discussed above, the C345C domain is important for regulation and activation of the complement cascade. Additionally, the cysteine mutated in our patient (Cys1518) is highly conserved among other species and forms one of the C3 disulfide bridges. Therefore, mutations in this amino acid likely prompt protein instability and consequent inefficient responses to pathogens primarily eliminated via complement activation such as encapsulated bacteria.

Until now, C345C region was implicated in rare cases of human aHUS, caused by GOF mutations in *C3*, yet without association to bacterial infections (Azukaitis et al, 2014; Sartz et al, 2012). From the hitherto known AR C3 deficiencies, only one case displayed a splice-site mutation in C345C, which resulted in recurrent infections and SLE (Tsukamoto et al, 2005). Together with this study, our observations substantiate the valuable functions of the C345C domain of C3 in regulation of human complement activity.

4.2.3 C3 deficiency in the context of other PIDs prompting pneumococcal infections

The phenotype marked by pneumococcal infections exhibited by our patient also overlaps with other PIDs. Despite defects in early components of the classical complement pathway, patients bearing B-cell (XLA, HIGM, CVID, selective IgG

subclass, IgA and anti-polysaccharide antibody deficiencies, etc.) or T-cell defects (Di George syndrome, SCID, etc.) are also identified as high-risk groups for infections caused by this pathogen (reviewed in (Picard et al, 2003b)).

Likewise, increased susceptibility pneumococcal diseases are observed in individuals with mutations in genes involved in other cell functions and pathways such as *IRAK4* (Picard et al, 2003a)*, MYD88* (von Bernuth et al, 2008), *HOIL1* (Boisson et al, 2012), *PIK3CD* (Angulo et al, 2013; Lucas et al, 2014) and *TWEAK* (Wang et al, 2013). Nevertheless, such disorders are also characterized by other associated characteristics that are absent in our patient.

Toll/IL1R signaling impairment resulting from IRAK4 or MYD88 deficiency for instance results in early-onset, recurrent and life-threatening infections predominantly caused by pyogenic bacteria, including *S. aureus* and *S. pneumoniae*. The manifestations included respiratory tract infections, meningitis, septicemia, arthritis, osteomyelitis, etc., whose frequency and severity reduced with age. Specific characteristics of IRAK4 and MYD88 deficiency are weak inflammatory responses displayed by the affected individuals, who presented with body temperature and C-reactive protein levels not compatible with the severity of the clinical picture (Picard et al, 2003a; von Bernuth et al, 2008).

Similarly, AR mutations in *HOIL1*, part of the E3 ligase complex LUBAC (linear ubiquitination chain assembly complex), predispose the affected subjects to severe pyogenic infections. Moreover, HOIL1-deficient patients present with autoinflammation syndrome and muscular amylopectinosis (Boisson et al, 2012).

Similarly to the phenotype of C3 deficiency, gain-of-function mutations in the *PI3K catalytic subunit delta* (*PIK3CD*), prompt recurrent respiratory infections due to *S. pneumonia* and *H. influenza*. This disorder however is also characterized by a combined immunodeficiency, with chronic viremia mainly due to cytomegalovirus (CMV) and Epstein-Barr virus (EBV), lymphoproliferative disease, and reduced CSR in some patients (Angulo et al, 2013; Lucas et al, 2014), which do not correspond to the C3-deficiency phenotype.

AD mutations in the TNF-like weak inducer of apoptosis (TWEAK) prompts antibody deficiency associated with frequent infections, including pneumonia and pneumococcal meningitis, since early age. Different from C3-deficient patients however, they also presented with multiple warts and chronic thrombocytopenia or intermittent neutropenia, and the laboratory findings revealed impaired antibody

production following protein and polysaccharide vaccines, reduced immunoglobulin levels and increased TCRαβ double negative T cells (Wang et al, 2013).

In summary, the observation of susceptibility to pneumococcal infections in such diverse PIDs demonstrates the complex immune responses to those agents. Although overlapping clinical manifestations are observed, C3 deficiency represents a unique disorder of the complement function which prompts not only predisposition to those agents but also increased frequency of autoimmune and autoinflammatory disorders (Grumach & Kirschfink, 2014). Our findings in this manuscript draw attention to the critical role of the C345C domain in the function of C3 regarding immune response to encapsulated bacteria. Further studies are however necessary to assess the roles of this domain in immune regulation.

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

4.3.1 Protein kinase C delta (PKCδ)

PRKCD gene is localized in the short arm of chromosome 3 (Huppi et al, 1994) and the corresponding 78kDa serine/threonine kinase, PKCδ, is 676 residues long (Kikkawa et al, 2002) (Gschwendt et al, 1986; Ono et al, 1987). As a novel PKC, PKCδ contains the C2-like domain, without the calcium-binding region, preceding the pseudosubstrate (reviewed in (Duquesnes et al, 2011)). Figure 6 shows the structural domains of PKCδ and its phosphorylation sites.

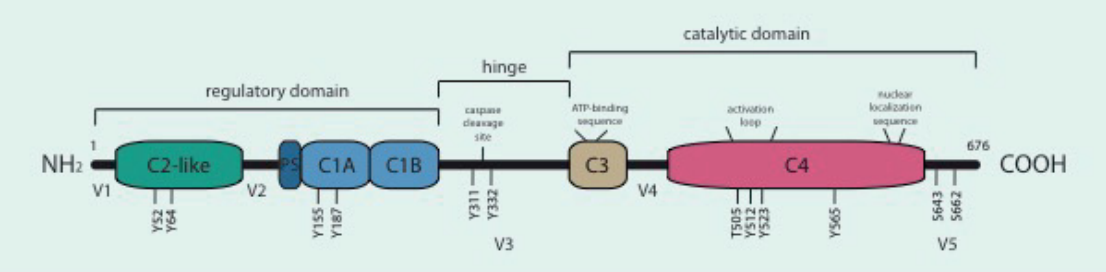

Figure 6. Structural domains and phosphorylation sites of PKCδ (adapted from (Steinberg, 2004)) V: variable domains; C: constant domains; Y: Tyrosine; T: Threonine; S: Serine.

Besides the novel C2 domain, the regulatory region of PKCδ contains a C1 domain common to other PKCs, composed by cysteine-rich tandem repeats (C1A and C1B). The C1 domain also contains hydrophobic residues that penetrate the membrane and bind DAG and PMA (phorbol 12-myristate 13-acetate) (reviewed in (Cho, 2001)) and in nPKC isoforms C1B has higher affinity to DAG/phorbol ester than C1A (Kikkawa et al, 2002).

Translocation of PKCδ to cell membranes follows similar steps as described for the cPKC, except for the Ca^{2+} dependency (reviewed in (Steinberg, 2004)). Activation of PKCδ on the other hand is complex and is regulated not only by the binding to DAG but also by phosphorylation of serine/threonine and tyrosine residues and by proteolytic formation of a catalytic fragment (Kikkawa et al, 2002).

As for other PKCs, priming phosphorylation of serine/threonine residues, activation loop, turn motif and hydrophobic motif precede the binding of PKCδ to DAG/phorbol ester. Phosphorylation of those residues is necessary for full catalytic activity of the enzyme (Kikkawa et al, 2002), while keeping the enzyme resistant to degradation (Steinberg, 2004). The first of the three residues is Thr505, localized inside the catalytic domain in C4, which is common to other PKC isoforms and is phosphorylated by PDK1 (Kikkawa et al, 2002). The second and third motifs are common to conventional and novel PKCs: the turn motif (Ser643) is autophosphorylated while the hydrophobic motif (Ser662) is phosphorylated by PKCζ and also via a pathway involving mTOR (mechanistic target of rapamycin) (Kikkawa et al, 2002; Rybin et al, 2003; Steinberg, 2004).

Further phosphorylation of different tyrosine residues (Tyr52, 64, 155, 187, 311, 332, 512, 523 and 565) regulate kinase activity of PKCδ depending on the initial stimulus (reviewed in (Yoshida, 2007)). Many of those residues present in the human PKCδ are conserved among species, but are not found in other PKC members. Therefore, the regulatory mechanism by tyrosine phosphorylation seems specific for each isoform (Steinberg, 2004). Phosphorylation of Tyr311 and Tyr332 in the hinge region of PKCδ supposedly exposes a cleavage site for caspase 3 to form a 40KDa catalytic active fragment of PKCδ in the nucleus. The proteolytic cleavage of PKCδ in response to several pro-apoptotic agents activates this isoform and results in increased apoptosis (Emoto et al, 1995; Ghayur et al, 1996).

Both full-length and catalytically active PKCδ can be found in the nucleus in response to certain stimuli, although the active fragment is found in this compartment in much higher concentration (Steinberg, 2004). Under normal conditions, quick export of full-length PKCδ back to the cytoplasm occurs to regulate PKCδ activation and guarantee cell survival (DeVries-Seimon et al, 2007). Prevention of PKCδ nuclear translocation mediated by BAFF for instance, regulates apoptosis and consequently promotes B-cell survival (Mecklenbrauker et al, 2004). Differences observed in the cellular localization and target substrates between full-length and active fragment suggest they have different functions and/or act at distinct stages during apoptosis process (Steinberg, 2004).

Aside from proapoptotic functions, under certain circumstances PKCδ can also exert antiapoptotic activity (reviewed in (Basu & Pal, 2010)), which indicates the complex and not yet fully understood roles for PKCδ.

4.3.2 Human PKCδ deficiency prompts B-cell immunodeficiency and autoimmunity

In the second manuscript deriving from this thesis, we studied a patient who presented with recurrent infections affecting mainly the respiratory tract from the first year of life and multiple features of autoimmunity and lymphoproliferation, including membranous glomerulonephritis, relapsing polychondritis, antiphospholipid syndrome, autoreactive antibodies and expansion of lymphoid organs. Immunological investigation revealed impaired B-lymphocyte function with absent isohemagglutinins, low IgG levels, progressive reduction of $CD19^+$ B cells, decreased proportions of class-switched ($CD27^+IgD^-$) and non-class-switched ($CD27^+IgD^+$) memory B cells, and expansion of $CD21^{low}$ B-cell population. Slight reduction in T-cell lymphoproliferative response was also observed.

In both patient and his father, who presented with Behçet's disease and mild autoimmune thyroiditis, a heterozygous CTLA4 gene polymorphism (49A/G, rs231775) was discovered, which has been implicated in autoimmune thyroiditis (Kimura et al, 2009), type 1 diabetes (Chen 2013) and latent autoimmune diabetes of adults (Dong et al, 2014). Graves's disease, SLE and rheumatoid arthritis (Gough et al, 2005) are observed in carriers of the homozygous variant. Furthermore, recently described AD *CTLA4* mutations in humans resulted in multiple autoimmune manifestations and immunodeficiency. Common findings to most affected individuals encompass hyper activation of T cells, suppression of regulatory T-cell function, reduction of circulating B-cell and lymphocyte infiltration in non-lymphoid organs (Kuehn et al, 2014; Schubert et al, 2014). Collectively, those findings impart the significance of CD28-CTLA4 signaling balance in immune system, particularly lymphocyte, homeostasis.

The clinical and laboratory pictures exhibited by the patient implied a CVID-like antibody deficiency (Bonilla et al, 2015). The group of CVID/CVID-like diseases include antibody deficiencies characterized by reduced memory B cells, variable reduction of immunoglobulins and susceptibility to infections and autoimmunity (reviewed in (Abolhassani et al, 2014)). Several genes encoding surface or intracellular molecules, directly or indirectly related to peripheral B-cell development have been implicated in disorders with those characteristics (Alangari et al, 2012; Angulo et al, 2013; Borte et al, 2014; Castigli et al, 2005; Chen et al, 2013; Grimbacher et al, 2003; Kuijpers et al, 2010; Li et al, 2015; Lopez-Herrera et al, 2012; Lucas et al, 2014; Ombrello et al, 2012; Salzer et al, 2014; Salzer et al, 2005;

Thiel et al, 2012; van Zelm et al, 2006; van Zelm et al, 2010; Warnatz et al, 2009; Zhou et al, 2012), yet the genetic origin of the majority of CVIDs remains unknown. In addition, similarly our patient, a group of CVID patients with impaired BCR-mediated calcium response also demonstrate severe reduction of class switched memory B cells and increased number of $CD21^{low}$ B cells, as well as high frequency of lymphadenopathy, splenomegaly and autoimmune complications (Foerster et al, 2010; Wehr et al, 2008).

On account of such similarities, defects in molecules implicated in CVID or other diseases with phenotype resembling our patient were excluded and further workup exploiting modern genetic technologies including SNP-array based whole genome homozygosity mapping and WES was carried out in the attempt to reveal the molecular origin of the disease. Our molecular investigation disclosed perfectly segregating mutations in *UBX domain protein 1 (UBXN1)* and in *PRKCD* gene (c.13521+1G>A). Since UBXN1, in contrast to PKCδ, had neither been implicated in B-cell homeostasis nor in immune regulation by the time of our publication, its involvement on the disease origin was not investigated. Further molecular analysis regarding PKCδ function in the affected subject revealed absent protein expression and reduced PKCδ function compared to healthy control.

The phenotype observed in our patient is reminiscent of Pkcδ knockout mice, which present with disturbed B-cell homeostasis and immune dysregulation, mainly characterized by B-cell hyper activation phenotype, abnormal B-cell expansion of lymphoid organs, poorly formed germinal centers and B-cell zones in spleen and lymph nodes, respectively, reduced apoptosis, B-cell infiltration in multiple organs and glomerulonephritis (Mecklenbrauker et al, 2002; Miyamoto et al, 2002). The restriction of B-cell activity promoted by PKCδ in mouse models seems to be related to constraint of IL6 transcription (Miyamoto, 2002), which is supported by the phenotypical similarities with Il6-transgenic mice (Suematsu et al, 1989) and by the increased *IL6* and *NF-IL6* mRNA levels in our patient's B cells after stimulation.

The involvement of PKCδ in B-cell apoptosis was endorsed by the identification of a critical proapoptotic Ca^{2+}-driven Erk pathway, mediated by PKCδ and RASGRP, which is involved in regulation of B-cell development (Limnander et al, 2011). Moreover, a recent publication demonstrated PKCδ phosphorylation downstream BCR and BAFFR, and negative regulation of BCR signaling and activation of the Ca^{2+}-Erk proapoptotic pathway by PKCδ. Deficiency of this kinase also resulted in uncontrolled expansion of autoreactive and hyperresponsive B cells (Limnander et al, 2014). In humans, the link between Erk signaling and B-cell function/homeostasis

was demonstrated with the recognition of CVID-like B cell-deficiency (associated or not with autoimmunity) in humans carrying AD mutations in *PLCG2* (Ombrello et al, 2012; Zhou et al, 2012).

Another contemporary study demonstrated the main mechanism of pro-apoptotic activity of PKCδ via induced proteasomal degradation of HAX-1 mediated by SCF^{FBXO25} ubiquitin ligase. In response to different apoptotic stimuli, PKCδ phosphorylates FBXO25 at serine 178 in the nucleus, and both FBXO25 and PKCδ later co-localize with HAX-1 to the mitochondrial compartment. Phosphorylation of HAX-1 at serine 210 by PKCδ enables HAX-1 ubiquitination by SCF^{FBXO25} and its consequent degradation. Disruption of this apoptotic mechanism was implicated in the pathogenesis and growth of B-cell lymphoma (Baumann et al, 2014).

4.2.3 Further evidences implicating PKCδ in immune regulation in humans

Since our description of human PKCδ deficiency, 5 other patients from 3 unrelated kindreds have been published, with loss-of-function mutations in *PRKCD* (Belot et al, 2013; Kiykim et al, 2015; Kuehn et al, 2013). Analogous to our patient, the patient described by Kuehn *et al* presented with frequent infections in early childhood, intermittent fevers, lymphoproliferation and autoimmune features, reminiscent of ALPS-like disease. Laboratory workup revealed B-cell hyperproliferation, defective B-cell apoptosis, reduced class-switched memory B cells, increased immunoglobulin levels, autoantibody production and impaired NK cell activity (Kuehn et al, 2013). Belot *et al* described three siblings diagnosed with juvenile-onset SLE, who carried *PRKCD* mutation, whose clinical manifestations included nephritis, arthritis, vasculitis and lymphoproliferation. Immunological investigation uncovered diminished B-cell apoptosis, increased B-cell proliferation, as well as high frequency of naïve and reduced proportion of memory B cells (Belot et al, 2013). Recent publication by Kiykim *et al* depicts a PKCδ-deficient patient with periodic fever and cutaneous lupus from infancy, who exhibited increased B-cell number, reduced memory B cells and increased naïve and $CD21^{low}$ B cells. In this patient, cytolytic activity of NK cells was moderately diminished, resembling the individual described by Kuehn *et al* and, likewise our patient, he also demonstrated mildly decreased T-cell proliferation (Kiykim et al, 2015).

In short, the immunosuppressive treatment applied to our patient consisted of mycophenolate mofetil (MMF) and low-dose steroids, which maintained satisfactory

disease control for over 3 years of follow up. Immunosuppressants were also employed to treat the other reported PKCδ-deficient patients and included steroids, rapamycin, MMF and hydroxychloroquine. Three out of the 5 patients displayed exceptional clinical and laboratory improvement with rapamycin (Kuehn et al, 2013), hydroxychloroquin (Kiykim et al, 2015) and MMF (Belot et al, 2013), respectively, which demonstrate the efficacy of such medicaments in controlling at least partially the immune dysregulation triggered by loss of function of PKCδ. Since increased production of IL6 may have important roles in increased B-cell proliferation and activity in the context of PKCδ deficiency, the use of anti-IL6 antibody, shown to be effective in treatment of rheumatoid arthritis (Balsa et al, 2015), could be an alternative to limit the immune dysregulation observed in PKCδ-deficient patients.

Despite variability in the clinical and laboratory features, all the individuals hitherto described with loss-of-function mutations in PKCδ presented with early-onset generalized lymphoproliferation, lupus or lupus-like manifestations, autoimmunity, and anomalies in the B-cell compartment, marked by increased B-cell activity and cell arrest in the stage of naïve B cells. Additionally, restriction of B-cell inflammation and proliferation with the use of immunomodulatory agents was effective in controlling features of immune dysregulation in the majority of the patients.

In sum, our investigation of a patient with B-cell PID and autoimmunity caused by loss-of-function mutation in *PRKCD*, together with the subsequent mentioned publications, validates the findings in animal models, supports the key functions of PKCδ in regulation of B-lymphocyte proliferation, activation, maturation, survival and tolerance in humans and demonstrates definite connection between breakdown of B-cell homeostasis and lupus pathogenesis.

4.4 Biallelic loss-of-function mutation in NIK causes a primary immunodeficiency with multifaceted aberrant lymphoid immunity

4.3.1 NF-κB-inducing kinase

NF-κB-inducing kinase (NIK) is a serine/threonine kinase (Malinin et al, 1997) encoded by the *MAP3K14* gene localized at chromosome 17q21 (Aronsson et al, 1998). The resulting 947-amino-acid-long protein, with approximately 100kDa, shows significant sequence similarity to other MAPKs (Malinin et al, 1997). The protein structure is composed by TRAF-binding domain, negative regulatory domain (NRD), kinase domain (KD) and a C-terminal region without catalytic function (Liao et al, 2004; Liu et al, 2012; Xiao & Sun, 2000), arranged in a two-lobe structure with the N- and C-terminal lobes connected by a hinge region (Liu et al, 2012). Figure **7** shows the domains of NIK protein.

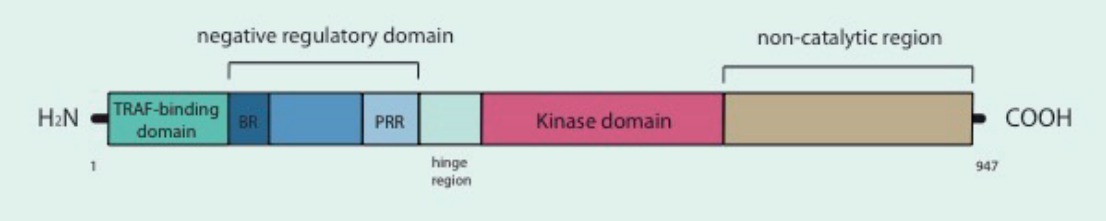

Figure 7. Structural domains of NIK (adapted from (Liu et al, 2012)) BR: basic region; PRR: proline- rich repeat.

The N-terminal region of NIK between residues 30 and 120 mediates its strong binding to TRAF3 C-terminal domain and is important for negative regulation of the alternative NF-κB pathway (Liao, 2004). The amino terminal NRD (residues 121 to 318) inhibits NIK signaling function by interacting with the C-terminal portion of NIK and preventing its interaction with IKKα (Xiao & Sun, 2000).

The KD (residues 390 to 660) contains essential residues to NIK activation and function. Lysine 429 and 430 for instance are essential for NIK autophosphorylation (Malinin et al, 1997), and phosphorylation of Thr559, inside the activation loop, is essential for NIK-dependent IKKα phosphorylation (Lin et al, 1998). Auto- or transphosphorylation in the activation loop of NIK is necessary for triggering the alternative NF-κB pathway (Lin et al, 1998). Although the carboxi terminal region of

NIK does not hold catalytic activity, it is essential for NIK function (Xiao & Sun, 2000) and interacts with other crucial elements in NIK signaling cascade such as IKKα (Lin et al, 1998) and TRAF2 (Malinin et al, 1997).

Regulation of NIK endogenous levels is important to control activation of the alternative NF-κB pathway. Although expressed in several tissues, its endogenous concentration in resting cells is typically low (Malinin et al, 1997) due to interaction of newly produced NIK with TRAF3 and subsequent targeting to proteasomal degradation. (Liao et al, 2004). NIK is able to control the activation of non-canonical NF-κB pathway by induction of *TRAF2* and *TRAF3* expression (Sasaki et al, 2008). The corresponding adaptor proteins, together with cIAP1 or cIAP2, form a NIK ubiquitin ligase. In unstimulated cells, TRAF3 brings TRAF2 and cIAP1/2 close to NIK, which enables NIK polyubiquitination mediated by cIAP1/2 and consequent proteasomal degradation (Vallabhapurapu et al, 2008). Recent data demonstrate that carboxyl terminus of HSC70-interacting protein (CHIP) works together with TRAF3 to promote NIK degradation (Jiang et al, 2015).

In response to non-canonical stimuli, the ubiquitin ligase is mobilized to the activated receptor where TRAF2 is subject to selfubiquitination. This process prompts ubiquitination of cIAP1/2 by TRAF2, which enables cIAP1/2-mediated TRAF3 ubiquitination and proteasomal degradation. Reduction of TRAF3 levels prevents NIK association to the cIAP1/2-TRAF2 complex and therefore the newly synthesized NIK accumulates in the cells, which allows its autophosphorylation (Vallabhapurapu et al, 2008).

Moreover, in non-stimulated cells NIK is likely kept in an inactive structural conformation where the active KD is protected by the NRD (Liu 2012). Upon cytokine stimulation, conformational changes may relocate or remove the NRD rendering the kinase active (Liu et al, 2012; Rosebeck et al, 2011). Another possible regulatory mechanism of the non-canonical NF-κB pathway activation is negative regulation of NIK stability through IKKα phosphorylation of serine residues 809, 812 and 815 in the C-terminal region of activated NIK, and consequent targeting of the kinase to degradation in a TRAF-cIAP ubiquitin ligase-independent mechanism (Razani et al, 2010).

Apart from its crucial role in the alternative NF-κB pathway, in some circumstances, NIK may also influence the classical pathway (reviewed in (Sun, 2012)). Interconnection between classical and alternative pathways can also be demonstrated by participation of IKKα in both networks (reviewed in (Hayden &

Ghosh, 2012)), as well as by the implication of non-canonical receptors, such as CD40 (Pone et al, 2012) and LTβR (Yilmaz et al, 2003), in activation of the canonical pathway.

4.3.2 Human loss-of-function mutation in *NIK* results in extensive combined immunodeficiency affecting B, T and NK cells

In the third manuscript of this thesis, we studied two cousins born to a consanguineous family with clinical phenotype of combined immunodeficiency, including recurrent and severe infections caused by bacteria, viruses and *Cryptosporidium*. Laboratory assessment revealed mainly restricted B-cell development and activation. After exclusion of mutations in genes known to result in CSR defects, homozygosity mapping and WES were carried out, assuming a monogenetic basis for the disease. As a result, a biallelic mutation showing perfect segregation with the disease was found in *MAP3K14* in both patients, affecting a highly conserved protein residue (c. C1694G, p. Pro565Arg). Functional analysis confirmed the loss of NIK kinase activity consequent to this amino acid substitution. Further investigation detected impaired NF-κB signaling, B-cell development, mature B-cell survival, follicular helper T-cell differentiation and memory T-cell function, as well as defective NK-cell activation and function.

Similar to Nik-deficient mice (Miyawaki et al, 1994; Shinkura et al, 1996), NIK loss-of-function mutation in humans impaired B- and T-lymphocyte maturation, differentiation and survival. Other important findings in mice, which could be suggested but not confirmed in our patients, are structural defects in secondary lymphoid organs (Miyawaki et al, 1994), including lack of germinal centers (Shinkura et al, 1996) and marginal zone in the spleen (Koike et al, 1996), and absent lymph nodes (Miyawaki et al, 1994). Similar observations are also made in mice with mutations in other components of the non-canonical NF-κB pathway, such as Ltβr (Futterer et al, 1998) and NF-κB2 (p100) (Caamano et al, 1998; Franzoso et al, 1998), confirming the crucial role of this pathway in the organogenesis of secondary lymphoid organs in this species. Regardless of the suggested abnormalities, one of the NIK-deficient patients responded to allogeneic hematopoietic stem cell transplantation.

Despite the presence of mature lymphocytes in the mouse models and normal numbers of T cells in NIK-deficient patients, combined immunodeficiency, hindered CSR/SHM and increased susceptibility to infections are observed in all affected

subjects (Miyawaki et al, 1994; Shinkura et al, 1996), including our patients. Impaired affinity maturation was also demonstrated in mice bearing Ltβr deficiency, in addition to extensive T and B lymphocyte infiltration in several organs (Futterer et al, 1998). A less severe phenotype is present in mice bearing mutations in *NF-κB2*, downstream of Nik, in which B cells, despite moderate functional deficiency, present with fairly normal class switching and normal maturation (Caamano et al, 1998; Franzoso et al, 1998).

4.3.3 NIK deficiency in the context of other PIDs affecting NF-κB pathways

To date, several elements of the NF-κB pathways have been implicated in human PIDs with diverse clinical and laboratory manifestations (Boisson et al, 2015; Boisson et al, 2012; Courtois et al, 2003; Doffinger et al, 2001; Fliegauf et al, 2015; Pannicke et al, 2013; Zonana et al, 2000). This variability reveals the myriad of pathways and functions involving NF-κB.

Specifically in alternative NF-κB pathway, description of autosomal-dominant *NF-κB2* mutation in patients presenting with childhood-onset CVID, autoimmunity and adrenal insufficiency, revealed the pivotal role of this pathway in human peripheral B-cell maturation and immune tolerance (Chen et al, 2013). Resembling NIK-deficient patients, yet with a milder phenotype, subjects with truncated NF-κB2 displayed recurrent bacterial and viral respiratory infections, as well as reduced immunoglobulin isotypes, switched-memory B cells and marginal-zone-like B cells (Chen et al, 2013). Autoimmune complications were also observed in some of the affected individuals, reminiscent of mice bearing a non-processible form of p100 (Tucker et al, 2007). Although the non-canonical NF-κB pathway had been linked to regulation of self-tolerance (Akiyama et al, 2008; Chin et al, 2003), autoimmunity were neither observed in half of the NF-κB2-deficient subjects (Chen et al, 2013) nor in our NIK-deficient patients.

Molecular defects of molecules engaging the non-canonical NF-κB pathway upstream NIK, such as BAFFR, TWEAK, CD27, CD40 and CD40L, respectively, also implicate this pathway in human B-cell PIDs. Mutations in the corresponding genes, analogously to *NIK* mutation, prompt repetitive infections, reduction of immunoglobulin isotypes and impaired antibody responses (Allen et al, 1993; Ferrari et al, 2001; van Montfrans et al, 2012; Wang et al, 2013; Warnatz et al, 2009).

In addition to those similarities, human BAFFR deficiency, likewise NIK-deficiency, also results in impaired development of mature B cells, increased percentage of transitional B cells, and reduced marginal zone-like and switched memory B cells (Warnatz et al, 2009), therefore supporting the crucial role of BAFFR signaling in the human B-cell survival and homeostasis in the periphery. On the other hand, BAFFR-deficient patients display a B-cell restricted phenotype with a late-onset mild disease and normal vaccine responses (Warnatz et al, 2009), differing from the patients carrying $NIK^{Pro565Arg}$ mutation who also presented with disturbed T-cell memory responses and follicular helper T-cell differentiation.

Although TWEAK physiologic functions are not yet fully understood, this protein seems to be involved in several process of cell proliferation (Lynch et al, 1999), apoptosis (reviewed in (Nakayama et al, 2002)) and modulation of immune responses (Maecker et al, 2005). Apart from the characteristics already mentioned in this thesis, AD TWEAK deficiency is also marked by other findings common to NIK and BAFFR deficiency such as restricted B-cell proliferation and survival beyond the naïve stage which, at least in part, may be caused by association of the mutant protein with BAFF and consequent hindering of alternative NF-κB activation (Wang et al, 2013).

Signaling downstream the transmembrane receptor CD27 upon ligation to CD70 has multifaceted roles in activation, survival and differentiation of a variety of cells including T, B and NK cells (reviewed in (Nolte et al, 2009)). Human CD27 deficiency prompts not only B-cell phenotype but also combined immunodeficiency with EBV-associated lymphoproliferation, which indicates its non-redundant roles in human B- and T-cell function, particularly in immune response to EBV (van Montfrans et al, 2012).

The NIK deficiency phenotype of repeated infections, including susceptibility to *Cryptosporidium* and defective CSR/SHM, with reduced immunoglobulins and compromised antibody responses, is reminiscent of combined immunodeficiency resulting from defects in CD40/CD40L (reviewed in (Davies & Thrasher, 2010)). Patients with mutations in the respective genes additionally exhibit infections caused by other opportunistic agents, such as *Pneumocystis jiroveci*, *Toxoplasma gondii* and *Mycobacteria*, as well as autoimmune manifestations (Davies & Thrasher, 2010), which were not observed following *MAP3K14* mutation. Also unlike the mentioned disorders, defective NIK function did not abolish CSR process, since CD40-mediated CSR could be demonstrated in $NIK^{Pro565Arg}$ B cells in response to stimulation with CD40L and IL4.

Although not directly involved in NF-κB pathways, deficiency of IL21R also prompts a phenotype comparable to NIK and to CD40/CD40L deficiency, including recurrent infections, reduced B-cell proliferation and class switching, and predisposition to *Cryptosporidium* infection (Kotlarz et al, 2013). Furthermore, IL21R deficiency results in diminished NK-cell cytotoxic activity (Kotlarz et al, 2013), as observed in our patients with functional NIK deficiency. Despite similar phenotype, human IL21 deficiency does not prompt defects in T or NK cells but a CVID-like B-cell deficiency, with early-onset inflammatory bowel disease and without *Cryptosporidium* infection (Salzer et al, 2014).

The findings described in the third manuscript resulting from this thesis demonstrate therefore non-redundant roles for NIK not only in function of NF-κB pathways and in mature B-cell survival, but also in T-cell proliferative responses and in NK-cell activity.

4.5 Conclusion & future prospects

In this thesis we uncovered hitherto unexplored critical roles in humans for the C345C domain of C3 in the protein function and immune response to encapsulated bacteria, for PKCδ in B-cell homeostasis and immune regulation, and for NIK in lymphoid immunity. Our work therefore incorporates meaningful knowledge in the area human immunology and human primary immunodeficiencies, regarding genetic mechanisms involved in the pathogenesis of PIDs with susceptibility to immune dysregulation. Those findings additionally encourage the execution of further studies to more comprehensively characterize the clinical and immunological phenotypes of such patients, and ultimately contribute to the development of improved targeted therapies for such disorders.

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7 CURRICULUM VITAE

Elisangela Calheiro dos Santos Valente, MD, MSc

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Education and training

10/2010 – ongoing	**PhD candidate** in Assoc.-Prof. Priv.-Doz. Dr.med. Kaan Boztug's Laboratory at CeMM Center for Molecular Medicine of the Austrian Academy of Sciences, 1090 Vienna, Austria (Fully funded PhD studentship). Topic: Molecular Genetics of Primary Immunodeficiencies.
09/2010	**Master of Science**, Department of Pediatrics, Division of Allergy and Immunology of the Federal University of Sao Paulo, Brazil. Title of the thesis: "Assessment of Nutritional Status Concerning Retinol, Beta Carotene And Zinc In Common Variable Immunodeficiency Patients".
02/2006 - 01/2008	**Specialist in Allergy and Immunology**, Division of Pediatric Allergy and Immunology from the Department of Pediatrics of the Federal University of Sao Paulo, Brazil. Board certified by the Brazilian Society for Allergy and Immunology in 2009.
02/2004 - 01/2006	**Specialist in Pediatrics**, University Hospital of Taubate, Brazil. Board certified by the Brazilian Society for Pediatrics in 2007.
01/1998 - 11/2003	**Medical doctor**, Medical University of Taubate, Brazil.

Voluntary internship

03/2006 08/2010	At the Pediatric Infectious Disease Laboratory of the Federal university of Sao Paulo, working on research and diagnostic of primary immunodeficiencies.

dos Santos-Valente EC, da Silva R, de Moraes-Pinto MI, Sarni RO, Costa-Carvalho BT. Assessment of nutritional status: vitamin A and zinc in patients with common variable immunodeficiency. **J Investig Allergol Clin Immunol.** 2012;22(6):427-31.

Santos-Valente E*, Reisli I*, Artaç H, Ott R, Sanal Ö, Boztug K. A novel mutation in the complement component 3 gene in a patient with selective IgA deficiency. **J Clin Immunol.** 2013 Jan;33(1):127-33. (* 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;98(3):473-8. (* and # equal contribution)

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;121(16):3112-6. (* and # equal contribution)

da Silva R, dos **Santos-Valente** EC, Burim Scomparini F, Saccardo Sarni RO, Costa-Carvalho BT. The relationship between nutritional status, vitamin A and zinc levels and oxidative stress in patients with ataxia-telangiectasia. **Allergol Immunopathol (Madr).** 2014 Jul-Aug;42(4):329-35.

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Awards

University Extension Program Award for "*Projeto Trote Solidário da Faculdade de Medicina da Universidade de Taubate*" ["Project Solidary College Initiation of the Medical School of University of Taubate"] at the 30th Scientific Meeting of Medical Students – Federal University of Maranhao, MA, Brazil – 2000.

Prêmio Saúde Brasil [Health Brazil Award] – National Contest for University Medical Students on Socially Responsible Work Interacting with the Community – 2003.

Scientific meetings

15th Biennial Meeting of the European Society for Immunodeficiencies (ESID) – Florence - Italy – 2012.

29th Annual Meeting of the Arbeitsgruppe Paediatrische Immunologie and 3rd Meeting of the DGFI Study Group Pediatric Immunology – Ittingen - Switzerland – 2012.

29th Annual meeting of the Arbeitsgruppe Paediatrische Immunologie (Pediatric Immunology Working group) and 3rd Meeting of the Deutsche Gesellschaft für Immunologie (German Society for Immunology) Study Group Pädiatrische Immunologie (Pediatric Immunology) - Ittingen - Switzerland – 2012.

11th European Society for Immunodeficiencies (ESID) Spring Meeting – Prague - Czech Republic – 2012.

First North American Primary Immune Deficiency National Conference – Philadelphia – USA - 2010.

2nd Latin American Society for Immunodeficiencies (LASID)/ Brazilian Group for Immunodeficiencies (BRAGID) Summer School in PID and Registry Meeting – Costa do Sauípe – Brazil - 2010.

8th Summer School in Primary Immunodeficiency Disorders from Clinical Immunology Society – Miami - USA – 2009.

13th Biennial Meeting of the European Society for Immunodeficiencies (ESID) – 's-Hertogenbosch - Netherlands – 2008.

13th International Congress of Immunology - ImmunoRio 2007 – Rio de Janeiro - Brazil - 2007.

6th Latin American Congress of Pediatrics Allergy and Immunology – Porto Alegre - Brazil - 2006.

Work experience

04/2015 - ongoing	English-Portuguese translator/reviser for mySugr Apps for diabetic patients, mySugr GmbH, 1060 Vienna.
2009 – 2010	Attending physician at the neonatal ICU of the public hospital “Amparo Maternal” in Sao Paulo, Brazil.
2007 – 2010	Founder and head of the Pediatric Allergy and Immunology Outpatient Clinic of University Hospital of Taubate, Brazil.
2007 – 2010	Attending physician at the neonatal ICU of University Hospital of Taubate, Brazil.
2006 – 2010	Attending physician at the Emergency Care Unit of the private hospital “Sao Lucas” in Taubate, Brazil.

Languages

Portuguese (native speaker), English (C1), German (B2), Spanish (A2).