MEDICAL UNIVERSITY OF VIENNA

Identification and Molecular Characterization of Novel Immune Homeostasis Regulators in Patients with Primary Immunodeficiency and Predominant Autoimmunity

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Submitted by

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Declaration

This thesis is written as a cumulative thesis. The work described here was carried out in different laboratories. The contributions of individual scientists are described below.

Chapter 2.2. includes the publication of Serwas, NK et. al. 2015. The majority of the published data was acquired by the author of this thesis. This includes homozygosity mapping and library preparation for exome sequencing, variant validation, flow cytometry analysis and protein 3D structure modelling. Aydan Kansu, Zarife Kuloglu, Arzu Demir, Aytac Yaman, Reha Artan, Ersin Sayar and Arzu Ensari provided care for the index patient and performed clinical routine interventions. Laura Yaneth Gamez Diaz conducted the Western Blot for lipopolysaccharide-responsive and beige-like anchor protein (LRBA). Elisangela Santos-Valente provided critical input on the clinical analysis of the index patient. Bodo Grimbacher and Kaan Boztug supervised the work performed in their respective laboratories. The first draft of the manuscript was written by Nina Serwas and finalized with the help of Kaan Boztug.

In chapter 2.4. the submitted manuscript of Serwas, NK et al is included. The work of this manuscript was performed by several scientist. The author of this thesis performed most of the experiments including immune phenotyping of the patient, assessment of serum cytokines, T cell receptor spectratyping, Western Blot for differentially expressed in FDCP 6 homolog (DEF6) expression, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) expression analysis with quantitative polymerase chain reaction (PCR) and flow cytometry, cloning and transfection of DEF6 and interferon regulatory factor 4 (IRF4), mutagenesis, T cell proliferation analysis, CTLA-4 trafficking assessment, *ex vivo* regulatory T cell (T_{reg}) suppression assay, reconstitution assay, imaging of primary T cells and data analysis. Zhenhua Sui subcloned DEF6 and RAB11, performed the guanine nucleotide exchange factor (GEF) activity assay, confocal microscopy on transfected HEK293 cells and primary cells and the co-immunoprecipitation of DEF6 and RAB11. Elisangela Santos-Valente performed the genetic analysis of the patient, Nima Memaran, Elisabeth Förster-Waldl and Wolf-Dietrich Huber provided clinical care for the two patients and performed clinical routine investigations. Özlem Yüce Petronczki performed the staining of primary cells. Malini Mukherjee and Pinaki Banerjee analyzed the NK cell defect of the patient. Renate Kain interpreted and analyzed histological pictures of

the two patients. Jean-Nicholas Schickel and Salomé Glauzy support the author of this thesis in performing the T_{reg} suppression assay and did immune phenotype analysis of different T_{reg} subsets. Elisabeth Salzer performed different flow cytometry analyses, Ana Krolo performed the co immunoprecipitation of DEF6 and IRF4, Ivan Bilic provided critical input, Jens Thiel assessed the functional impact of the variant in CD21, Jordan Orange, Eric Meffre, Amnon Altman and Kaan Boztug supervised the work conducted in their respective laboratories. Nina Serwas and Kaan Boztug wrote the manusript.

All other chapter of this thesis were written by the author of this thesis.

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Abstract

The prevalence of autoimmune disorders is constantly rising in the developed world. The underlying molecular mechanisms for such altered immune homeostasis resulting in immune response towards self is only barely understood. Genome-wide association studies have uncovered several susceptibility loci for human autoimmune disorders, however, such studies often remain associative without mechanistic proof. In the past, primary immunodeficiencies (PIDs) with severe and early-onset autoimmune phenotypes have been of tremendous importance to uncover critical regulatory mechanisms in immune homeostasis. One recent example is the identification of mutations affecting the protein lipopolysaccharide-responsive and beige-like anchor protein (LRBA), which normally protects the inhibitory protein cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) from lysosomal degradation. The aim of this thesis was to genetically and mechanistically analyze patients with PID and a predominant autoimmune phenotype to ultimately gain novel mechanistic insights into immune homeostasis. To this end, patients were clinically characterized and analyzed with homozygosity mapping and whole exome sequencing. Variants in candidate genes were validated and functionally characterized.

Genetic characterization of a patient presenting with early-onset inflammatory bowel disease revealed a novel mutation affecting the gene *LRBA*. The patient presented mainly with an autoimmune phenotype without alterations in the B-cell compartment. A second family with two affected siblings revealed a hitherto unknown monogenetic defect in a gene encoding a guanine nucleotide exchange factor (GEF) highly expressed in T cells. The work showed that the identified mutation leads to a reduction in exchange activity towards the small GTPase cell division control protein 42 (CDC42). Furthermore, a link between the mutated GEF and the regulation of CTLA-4 expression and trafficking was established, both of which were severely reduced in activated T cells of the affected individuals. Ultimately, treatment of one patient with CTLA-4-Ig lead to complete remission of the autoimmune phenotype.

Collectively, the work of this thesis could identify a novel mutation in the gene *LRBA* leading to early-onset IBD. Furthermore, a novel regulator of CTLA-4 biology affecting both, trafficking and transcription was uncovered which was followed by a comprehensive analysis to define its precise role in immune homeostasis.

Deutschsprachige Zusammenfassung

In den Industriestaaten nimmt die Prävalenz von Autoimmunerkrankungen konstant zu. Trotzdem sind zugrundeliegende Mechanismen der Immunhomöostase, welche in solchen Krankheiten meist defekt sind, nicht vollständig aufgeklärt. Genom-weite Assoziationsstudien konnten potentielle Risiko-Loci identifizieren, welche in einer erhöhten Anfälligkeit für Autoimmunerkrankungen resultieren können. Solche Studien bleiben jedoch oft nur assoziativ und haben meist keine mechanistische Grundlage. Im Gegensatz dazu kann die Identifikation von monogenetischen Formen der primären Immundefekte (PIDs) mit schwerer Autoimmunität neue Regulatoren der Immunhomöostase identifizieren. Ein Beispiel dafür ist die Identifikation einer Form der PID, welche durch Mutationen des Proteins *lipopolysaccharide-responsive and beige-like anchor protein* (LRBA) ausgelöst wird. LRBA schützt das inhibitorische Protein *cytotoxic T-lymphocyte-associated protein 4* (CTLA-4) vor lysosomalem Abbau schützt. Das Ziel dieser Arbeit war es Patienten mit PID und assoziierter Autoimmunität genetische mittels *homozygosity mapping* und Exomsequenzierung zu charakterisieren. Identifizierte Varianten sollten dann funktionell validiert werden.

Die genetische Charakterisierung einer Patientin mit frühkindlicher chronisch entzündlichen Darmerkrankung konnte als Krankheitsursache eine Mutation in *LRBA* identifizieren. Die Patientin zeigte, im Gegensatz zu publizierten LRBA-defizienten Patienten, eine prädominante Autoimmunität ohne offensichtlichen Defekt des B Zell-Subtypenverteilung. In einer zweiten Familie mit zwei erkrankten Schwestern konnte ein bisher unbeschriebener monogenetischer Defekt in einem Gen, welches einen Guanin Nukleotid Austauschfaktor (GEF) kodiert, identifiziert werden. Dieser GEF ist hauptsächlich in T Zellen exprimiert. Die Mutation resultiert in einer reduzierten Austauschaktivität gegenüber der kleinen GTPase *cell division control protein 42 homolog* (CDC42). Durch funktionelle Charakterisierung von Primärmaterial einer der Patientinnen konnte eine Verbindung zwischen dem GEF und Expression und Transport des Proteins CTLA-4 identifiziert werden. CTLA-4 Transport und Expression sind in aktivierten T Zellen der Patientin stark reduziert. Die Behandlung einer der beiden Patientinnen mit rekombinantem CTLA-4-Immunoglobulin führte zur Remission der schweren Autoimmunität.

Zusammengefasst konnte in dieser Arbeit eine neue Mutation in *LRBA* als Krankheitsursache für früh-einsetzende chronisch entzündliche Darmerkrankung identifiziert werden. Des Weiteren konnte ein neuer Regulator der Expression und des Transportes von CTLA-4 identifiziert und charakterisiert werden.

Publications arising from this thesis

Nina Kathrin Serwas, Aydan Kansu, Elisangela Santos-Valente, Zarife Kuloğlu, Arzu Demir, Aytaç Yaman, Laura Yaneth Gamez Diaz, Reha Artan, Ersin Sayar, Arzu Ensari, Bodo Grimbacher, Kaan Boztug. **Atypical manifestation of LRBA deficiency with predominant IBD-like phenotype.** Inflamm Bowel Dis **21**: 40-47

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Abbreviations

ACPA	Anticitrullinated protein/peptide antibodies
ADAM17	Disintegrin and metalloproteinase domain-containing protein 17
AHR	Aryl hydrocarbon receptor
AIRE	Autoimmune regulator
ALPS	Autoimmune lymphoproliferative syndrome
ALT	Alanine transaminase
AMA	Anti-mitochondrial antibody
ANA	Antinuclear antibody
ANCA	Anti-neutrophil cytoplasmic antibodies
APC	Antigen presenting cell
ASD	Atrial septal defect
ASMA	Anti-smooth muscle antibody
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AVSD	Atrioventricular septum defect
BCR	B cell receptor complex
BEACH	Beige and Chediak−Higashi syndrome
BLNK	B-cell linker protein
BSA	Bovine serum albumin
BTK	Bruton tyrosine kinase
CADD	Combined Annotation Dependent Depletion
CARD11	Caspase recruitment domain-containing protein 11
CASP	Caspase
CCL3	Chemokine (C-C motif) ligand 3
cDC	Conventional DC
CDC42	Cell division control protein 42
CLIP	Class II-associated Ii peptide
CLP	Common lymphoid precursor
CNS	Conserved non-coding sequence
CR2	Complement receptor 2
CsA	Cyclosporine A

cSMAC	Central supra-molecular activation cluster
cTECs	Cortical thymic epithelial cells
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CVID	Common variable immunodeficiency
D	Diversity
DAG	Diacylglycerol
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
DEF6	Differentially expressed in FDCP 6 homolog
DIV	Deletion/insertion variant
DMARD	Disease-modifying antirheumatic drug
DMSO	Dimethyl sulfoxide
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
dsDNA	Double stranded DNA
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
EBV	Eppstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmatic reticulum
ERK	Extracellular signal-regulated kinase
ExAC	Exome Aggregation Consortium
FADD	FAS-associated death domain protein
FBS	Fetal bovine serum
FCS	Fetal calf serum
FOXP3	Forkhead box P3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic DNA
GEF	Guanine nucleotide exchange factor
GFP	green fluorescent protein
GRB2	Growth factor receptor-bound protein 2

GWAS	Genome-wide association study
H&E	Hematoxylin and eosin
HD	Healthy donor
HEK	Human embryonic kidney
HLA	Human leukocyte antigen
HLH	Hemophagocytic lymphohistiocytosis
HOIL-1	Heme-oxidized IRP2 ubiquitin ligase 1
HOIP	HOIL-1-interacting protein
HRP	Horse radish peroxidase
HSCT	Hematopoietic stem cell transplantation
IBD	Inflammatory bowel disease
IBP	IRF4 binding protein
IFN	Interferon
Ig	Immunoglobulin
Igα	B-cell antigen receptor complex-associated protein alpha chain
Igβ	B-cell antigen receptor complex-associated protein beta chain
Ii	Invariant chain
IKK	IκB kinase
IL	Interleukin
Iono	Ionomycin
IP3	Inositol-1,4,5-trisphosphate
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
ITK	Interleukin-2-inducible T-cell kinase
IVIG	Intravenous immunoglobulins
IκBα	NF-κB inhibitor α
J	Joining
JaCoP	Just Another Colocalization Plugin
LAT	Linker for activation of T cells

LCK	Lymphocyte-specific protein tyrosine kinase
LKM	Liver Kidney Microsomal
LPS	Lipopolysaccharide
LRBA	Lipopolysaccharide-responsive and beige-like anchor protein
LYST	Lysosomal-trafficking regulator
MAF	Minor allele frequency
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MIIC	MHC class II compartment
mRNA	Messenger ribonucleic acid
mTEC	Medullary thymic epithelial cell
MTOC	Microtubule organization center
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response gene 88
n.s.	Not significant
NBEA	Neurobeachin
ND	Normal donor
NEMO	NF-κB essential modulator
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor κB
NHEJ	Nonhomologous end-joining
NK cells	Natural killer cells
NKT cells	Natural killer T cells
NP-40	Nonyl phenoxypolyethoxylethanol
ORAI-1	Calcium release-activated calcium channel protein 1
P	Patient
PAGE	Polyacrylamide gel electrophoresis
PAK	P21 activated kinase 1
PAMP	Pathogen-associated molecular pattern
PBD	Protein binding domain
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
pDC	Plasmacytoid DC

PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinase
PID	Primary immunodeficiency
PKC	Protein kinase C
PLCγ	Phospholipase Cγ
pLI	Probability of loss-of-function intolerance
PMA	Phorbol-12-myristate-13-acetate
PMSF	Phenylmethane sulfonyl fluoride
PRR	Pattern recognition receptor
pSMAC	Peripheral supra-molecular activation cluster
PSMB11	Proteasome subunit beta type-11
PtdIns(3,4,5)P3	Phosphatidylinositol-3,4,5-triphosphate
PtdIns(4,5)P2	Phosphatidylinositol-4,5,-bisphosphate
pT_{regs}	Peripherally derived T_{regs}
qPCR	Quantitative PCR
RA	Retinoic acid
RAC1	Ras-related C3 botulinum toxin substrate 1
RAG	Recombination activating gene
RAR	Retinoic acid receptor
RASGRP1	RAS guanyl nucleotide releasing protein
RF	Rheumatoid factor
RIP1	Receptor interacting kinase 1
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
ROI	Region of interest
RORγt	RAR-related orphan receptor γt
SAVI	STING-associated vasculopathy with onset in infancy
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulfate
SLAT	SWAP-70-like adaptor of T cells
SLP76	SH2 domain containing leukocyte protein of 76kDa
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant

SOS	Son of sevenless homolog
SP	Single positive
STIM1	Stromal interaction molecule 1
STING	Stimulator of interferon genes
SWAP70	Switch-associated protein 70
SYK	Spleen tyrosine kinase
TAP	Transporter associated with antigen presentation
TCR	T cell receptor
TGF	Tumor growth factor
Th	T helper
TICAM-1	TIR-containing adaptor molecule
TIR	Toll/interleukin-1 receptor
TIRAP	TIR-associated protein
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TRA	Tissue-restricted antigen
TRAF	TNF receptor associated factor
Treg	Regulatory T cell
Tresp	Responder T cell
TTC7A	Tetratricopeptide repeat domain 7A
TTG	Tissue Transglutaminase
tT_{regs}	Thymus-derived T_{regs}
V	Variable
VpreB	Immunoglobulin I chain
XIAP	X-linked inhibitor of apoptosis protein
ZAP70	Zeta-chain-associated protein kinase 70

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1. Introduction

The immune system comprises the diverse defense mechanisms of an organism against the various damaging substances of the environment such as pathogens or toxins (Murphy et al, 2012). Upon encounter of such an agent, the immune system is activated through a diversity of receptors which elicit a certain response that should protect the organism from potential devastating effects (Takeuchi & Akira, 2010). Evolutionarily, two distinct arms of the immune system developed: (1) the fast, but unspecific response of innate immunity, and (2) the specific, long-lasting response of the adaptive immunity. Whereas the innate immunity always initiates a defined sequence of pathogen recognition and pathogen-specific effector function, the adaptive immune system has the ability to memorize the experienced antigen/pathogen repertoire and thus, is able to respond to pathogens faster and more efficient upon second encounter. Importantly, both arms of the immune system have to be kept under tight control to prevent collateral damage of healthy tissue which may result in autoimmune or autoinflammatory diseases. If the adaptive immune system elicits an immune response towards self-antigens, the memory property comprises the additional risk to deteriorate the disease phenotype due to constant immune activation directed against self-antigens, which may result in chronic autoimmune diseases. Thus, several check points evolved to keep this powerful system under control.

Primary immunodeficiencies (PIDs) are inherited conditions of failure in the immune system and can affect almost any parts of the immune system (Picard et al, 2015). Some forms of PID present with a severe dysregulation of immune homeostasis, which may result in severe autoimmune or autoinflammatory disease (Grimbacher et al, 2016). The identification and molecular characterization of such PID patients has led to the discovery and elucidation of many immune homeostasis processes and broadened our general understanding of the function and importance of several components of the immune system (Grimbacher et al, 2016).

In the following chapters of this thesis, I will introduce several important concepts of the immune system. Furthermore, I will outline some key mechanisms which are critical for the prevention of immune reaction against self and introduce some examples of autoimmune diseases. Finally, I will give some important examples of

PIDs with severe, early-onset autoinflammatory or autoimmune diseases which have been critical to elucidate fundamental immune homeostasis mechanisms.

1.1. Activation of the immune system

An invading pathogen can be detected by cells of the innate immune system such as neutrophils, macrophages and dendritic cells (DCs). These cells use certain receptors that can recognize and bind to so-called pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (Takeuchi & Akira, 2010; Venereau et al, 2015). These receptors are called pattern recognition receptors (PRRs) and can exist as soluble receptors, receptors associated with the cell membrane, and/or intracellularly located receptors. Some of the membrane-bound receptors have the property to be endocytosed when bound to their ligands and thus are termed scavenger receptors (Gough & Gordon, 2000). An important group of PRRs is the group of Toll-like receptors (TLRs) (Kopp & Medzhitov, 2003). Humans express several distinct TLRs which recognize different PAMPs. TLR-1:TLR-2 and TLR-2:TLR-6 heterodimer recognize peptidoglycans and lipoproteins (Kopp & Medzhitov, 2003), TLR-3 recognizes double-stranded ribonucleic acid (RNA) (Xagorari & Chlichlia, 2008), and TLR-4, which is the most studied TLR, binds lipopolysaccharide (LPS) (Kopp & Medzhitov, 2003). TLR-5 plays a role in immune defense against fungi as it binds flagellin (Kopp & Medzhitov, 2003), whereas TLR-7 is involved in the detection of viral single stranded RNA (Xagorari & Chlichlia, 2008). TLR-8 binds to G-rich oligonucleotides which can also appear during a viral infection (Xagorari & Chlichlia, 2008). TLR-9 recognizes the unmethylated CpG deoxyribonucleic acid (DNA) of bacteria (Kopp & Medzhitov, 2003). Once bound to their ligands, all TLRs use a similar signaling cascade. The Toll/interleukin-1 receptor (TIR) homology domain of the respective TLR binds to the TIR domain of the protein myeloid differentiation primary response gene 88 (MyD88). MyD88 bears a death domain which recruits the protein interleukin-1 receptor-associated kinase (IRAK). The kinase function of IRAK activates TNF receptor associated factor 6 (TRAF6) which leads to the activation of the transcription factor nuclear factor κB (NF-κB) (Kopp & Medzhitov, 2003). In addition to this well-established signaling pathway, other adapters have been shown to interact with the TIR domain of the TLRs. Among them are the TIR-associated protein (TIRAP) (Fitzgerald et al, 2001; Horng et al, 2001) and TIR-containing adaptor molecule (TICAM-1) (Oshiumi et al, 2003;

Yamamoto et al, 2002). The activation of NF-κB results in a transcriptional program that leads to expression of proinflammatory cytokines and chemokines which further activate and recruit immune cells (Kawai & Akira, 2010). Furthermore, signaling through TLRs results in the expression of costimulatory molecules, such as CD80 or CD86, on the surface of macrophages and dendritic cells (Akira et al, 2001). Those cells can present ingested antigens in cooperation with these costimulatory molecules to cells of the adaptive immune system and activate them.

1.1.1. Antigen presentation

Antigen presentation is one of the important processes that links innate and the adaptive immune system. The general principle of this process is the binding of short peptides to major histocompatibility complex (MHC) molecules, presentation of the bound peptide-MHC complex on the cell surface and subsequent recognition of it by cells of the adaptive immune system. Two main classes of MHC molecules exist.

MHC class II molecules are mainly expressed by professional antigen-presenting cells such as the above mentioned dendritic cells and macrophages, but also on B cells (Neefjes et al, 2011). Other cells can induce expression of MHC class II upon encounter of interferon γ (IFNγ) (Romieu-Mourez et al, 2007). MHC class II molecules consist of an α and a β-chain, which are assembled in the endoplasmatic reticulum (ER). To stabilize the complex, the MHC molecules associate with the invariant chain (Ii). After transport to the MHC class II compartment (MIIC), Ii is cleaved. A small peptide, the class II-associated Ii peptide (CLIP), remains in the binding pocket of the MHC class II molecule to further stabilize the assembled protein. Early endosomes which contain ingested antigens and proteases fuse with the MIIC. Consequently, CLIP is replaced by a processed antigen (Neefjes et al, 2011).

In contrast to the restricted expression of MHC class II molecules, MHC class I molecules are expressed by all nucleated cells of the human body, and constantly present the proteins that are expressed inside the cells. This includes all proteins that are produced in a cell in steady state, but also those that are produced once the cell has been infected with a pathogen and proteins that are expressed by this invading pathogen. Proteins are degraded by specific proteases, called the immunoproteasome, and transported into the ER by the transporter associated with antigen presentation (TAP). In the ER, MHC class I molecules assemble, are loaded

with the peptide, and subsequently transported to the cell membrane (Neefjes et al, 2011). Malignant cells are also constantly forced to present their expressed proteins on their MHC class I molecules. This renders them recognizable by the immune system. Thus, downregulation of MHC class I molecules is an escape mechanism often seen in cancer and viral infections to prevent the presentation of mutated or viral peptides to the adaptive immune cells. Downregulation of MHC molecules, however, can be recognized by natural killer (NK) cells (Vivier et al, 2008). NK cells recognize several ligands on their target cells with activating and inhibitory receptors which results in a distinct information about the health status of the target cell (Vivier et al, 2008). The absence of MHC class I molecules results in the activation of the NK cells which consequently kills its target cell through a release of perforin and granzyme B.

1.2. Adaptive Immunity

The response of the adaptive part of the immune system is highly specific and efficient. Furthermore, the adaptive immune system can memorize the encounter of a specific antigen and react much faster upon second encounter. The general principle of memory makes use of somatic modifications of the genomic DNA of lymphocytes.

1.2.1. Principles of memory and diversity

The idea that the adaptive immune system can elicit highly specific responses to an enormous amount of different antigens and at the same time memorize the encounter of them was proposed in the clonal selection theory (Burnet, 1959). The main idea of this theory is that every T and B cell expresses a unique receptor that is necessary for their activation upon pathogen encounter. Activated lymphocytes will expand and generate effector lymphocytes that express the same receptor. To prevent immune reaction against self, autoreactive lymphocytes are eliminated from the circulation (Burnet, 1959).

The receptors of B and T cell consist of heavy and light chains with constant and variable regions. However, the genes encoding the variable regions are not present in a functional state in germ-line DNA. During B and T cell development, up to three independent gene clusters are combined together to form a functional receptor. The

gene clusters consist of segments of variable (V), diversity (D, not present in the immunoglobulin light and T cell receptor α and β chains), and joining (J) regions (Schatz & Ji, 2011). The so-called V(D)J recombination is initiated by the recombination activating gene (RAG) which can bind to DNA sequences close to the respective V, D and J segments. At these specific recombination signal sequences, RAG recombinase complex cleaves the DNA to generate double strand breaks (Schatz & Ji, 2011). For proper rejoining of DNA ends, RAG interaction with the cleaved DNA ends persists. (Hiom & Gellert, 1997) The assembly of the cut DNA sites happens through the DNA repair machinery of the non-homologous end joining (NHEJ) pathway (Roth, 2014). The massive diversity of the B and T cell receptors is generated through the large number of V, D, and J segments. Additional diversity is gained through the removal of nucleotides by endonucleases during the joining process. Furthermore, the enzyme terminal deoxynucleotidyl transferase can add several nucleotides called N regions to the cleaved ends which results in even increased diversity (Roth, 2014).

Patients with inherited defects in any parts of this recombination machinery have highlighted its importance in the defense against pathogens. The first identified genetic etiologies for severe combined immunodeficiency (SCID) caused by genes involved in V(D)J recombination were homozygous germline mutations in the lymphocyte-specific genes *RAG1* or *RAG2* (Schwarz et al, 1996). Patients present with the absence of B and T cells as they are not able to produce functional B or T cell receptors (Schwarz et al, 1996). Homozygous germline mutations in genes encoding proteins involved in the NHEJ machinery, such as mutation in the gene encoding DNA ligase IV, (which encodes the ligase necessary for relegation of the separated DNA parts) not only lead to SCID and absence of T and B cells, but also to developmental delay and increased risk for malignancy as NHEJ is a process which is not only important in immune cells but in almost all cells of the body (O'Driscoll et al, 2001; van der Burg et al, 2006).

1.2.2. Cells of the adaptive immune system – B cells

B cells originate from the common lymphoid precursor (CLP) in the bone marrow. During B cell maturation, the CLP develops to a pro B cell in which the recombination of the different segments of the heavy chain locus occurs. During the early pro B cell stage D and J segments rearrange to so-called DJ segments. In the

late pro B cell stage those newly formed DJ segments rearrange with a V segment (Alt et al, 1984). The completion of the VDJ rearrangements at the heavy chain locus marks the beginning of the large pre B cell stage. During this stage, the arranged heavy chain is tested for successful expression on the surface of the B cell. To this end, it is assembled with a surrogate light chain, consisting of the proteins immunoglobulin I chain (VpreB) and λ5, to a pre-B cell receptor (Kudo & Melchers, 1987; Sakaguchi & Melchers, 1986; Schiff et al, 1992). The maturation of B cell into small pre B cell stage would only occur if proper signaling through this receptor is achieved. In this stage, the light chain V and J segments rearrange while further rearrangements of the heavy-chain locus are inhibited (Melchers et al, 1999). Expression of the newly formed B cell receptor consisting of rearranged heavy and light chains marks the entry to the immature B cell stage. Before the B cell leaves the bone marrow as a mature B cell, the antigen receptor is tested for self-reactivity against soluble and stromal-bound self-antigens. If a B cell recognizes self, this cell will either be deleted, becomes anergic, or will be forced towards a second round of V(D)J recombination (Nemazee, 2006). The B cells that complete the selection step mature, leave the bone marrow, and enter the circulation.

Survival and differentiation of the mature naïve B cell depends on signaling through the B cell receptor (BCR) (Lam et al, 1997). Activation of the BCR complex leads to the recruitment and activation of the tyrosine-protein kinase LYN (Monroe, 2006). LYN phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) of the BCR associated proteins B-cell antigen receptor complex-associated protein alpha chain (Igα) and beta chain (Igβ). This leads to activation and recruitment of the proteins Bruton tyrosine kinase (BTK) and spleen tyrosine kinase (SYK), which are both central kinases in B cell signaling (Turner et al, 1995; Vetrie et al, 1993). SYK can phosphorylate and activate the guanine nucleotide exchange factor VAV3 which in turn activates the small GTPase Ras-related C3 botulinum toxin substrate 1 (RAC1) (Inabe et al, 2002). RAC1 is important for the recruitment of phosphatidylinositol 3-kinase (PI3K) (Inabe et al, 2002). In addition to PI3K, both SYK and BTK recruit and activate phospholipase Cγ2 (PLCγ2). PLCγ2 and PI3K both have the same substrate which is phosphatidylinositol-4,5,-bisphosphate (PtdIns(4,5)P_2). PLCγ2 converts PtdIns(4,5)P_2 to inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 binds and opens endoplasmatic Ca^{2+}-channels and, thus, increases the concentration of this second messenger in the cytoplasm of the

B cell (Berridge, 1981). Ca^{2+} is needed for the activation of the phosphatase calcineurin which is important for the dephosphorylation of the transcription factor nuclear factor of activated T-cells (NFAT). Phosphorylated NFAT is only present in the cytoplasm of the cell. Once the protein is dephosphorylated, it can be shuttled to the nucleus and bind to the promoters its target genes. In parallel, the generated DAG activates several members of the protein kinase C (PKC) family which in turn, and in cooperation with the increase of cytoplasmic Ca^{2+} concentration, activate the mitogen-activated protein kinase (MAPK) pathways and the transcription factor NF-κB (Niiro & Clark, 2002). PI3K, in contrast to PLCγ2, phosphorylates PtdIns(4,5)P_2 to phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P_3). PtdIns(3,4,5)P_3 recruits and subsequently activates the serine/threonine-protein kinase AKT and induces the activation of the mammalian target of rapamycin (mTOR) pathway (Seda & Mraz, 2015). The strength of the different signals affects metabolism and gene transcription, and thus result in a defined outcome for the B cell of either maintenance, proliferation or differentiation.

Several PIDs have been described to be caused by mutations in genes encoding B cell relevant proteins (Figure 1). For example, biallelic germline mutations in the gene *immunoglobulin lambda-like-1* encoding λ5, a component of the surrogate light chain, result in B cell deficiency and agammaglobulinemia due to failure of B cell maturation (Minegishi et al, 1998). Mutations in the gene *BTK*, which is located on the X chromosome, lead to severe X-linked agammaglobulinemia due to defective BCR signaling (Vetrie et al, 1993). On the other hand, over activation of B cells may also lead to severe forms of autoimmunity as seen in patients with autosomal dominant gain-of-function mutations in the gene *PLCG2* (Ombrello et al, 2012). Mutations in genes involved in the regulation of NF-κB activation, such as components of the linear ubiquitination chain assembly complex, can also lead to PID associated with autoinflammation (Tokunaga, 2013). As an example, homozygous germline mutations in the genes *RBCK1* or *RNF31* encoding the proteins HOIL-1 (Heme-oxidized IRP2 ubiquitin ligase 1) and HOIP (HOIL-1-interacting protein) respectively, result in an inappropriate activation of NF-κB (Boisson et al, 2015; Boisson et al, 2012). HOIP and HOIL-1 are both needed to form the complex of the so far only known E3 complex that exerts M1-linked linear ubiquitination (Kirisako et al, 2006; Tokunaga, 2013). HOIP which elicits the ubiquitin ligase function and HOIL-1, one of its accessory proteins, target the NF-κB essential

modulator (NEMO) and the receptor interactin kinase 1 (RIP1). Linear ubiquitination is needed for the activation of the IκB kinase (IKK) which leads to subsequent degradation of inhibitory proteins. Consequently, deficiency of HOIL-1 or HOIP leads to a reduction of NF-κB signaling and proinflammatory cytokine transcription in lymphocytes (Boisson et al, 2015; Boisson et al, 2012). Surprisingly, those deficiencies lead to an overactivation of monocytes which results in an elevated production of interleukin 6 (IL-6). The reason for this proinflammatory phenotype of monocytes is not yet understood.

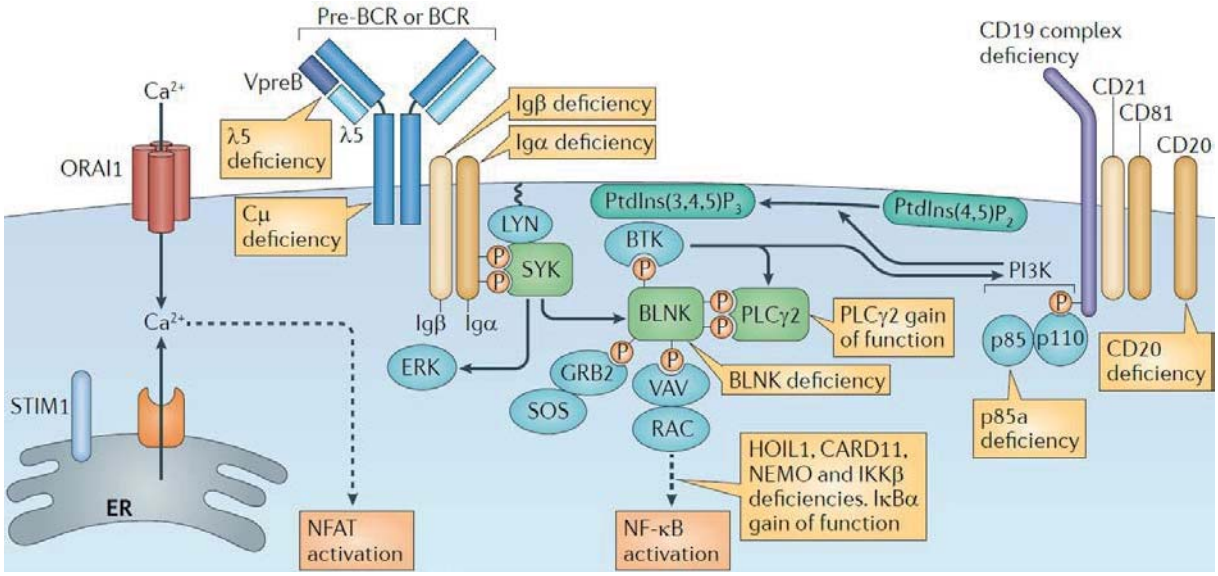

Figure 1 – B cell receptor signaling and primary immunodeficiencies. Several PIDs have been identified in the BCR signaling pathway including developmental defects such as λ5 deficiency, or PIDs caused by mutations in genes important in BCR signaling such as PLCγ2 and NEMO deficiency. (BLNK: B-cell linker protein; SOS: Son of sevenless homolog; ERK: Extracellular signal-regulated kinase; ORAI1: Calcium release-activated calcium channel protein 1; STIM1: Stromal interaction molecule 1; CARD11: Caspase recruitment domain-containing protein 11, IκBα: NF-κB inhibitor α). Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology (Durandy et al, 2013), copyright (2013).

Apart from mutations in proteins involved in development and signaling downstream of the BCR, mutations in BCR co-receptors have been described. As an example, patients with biallelic germline mutations in the gene encoding CD19, a common marker of B cells and co-receptor of the BCR, suffer from recurrent infections caused

by antibody deficiency (van Zelm et al, 2006). Deficiency of CD21, also known as complement receptor 2 (CR2), causes hypogammaglobulinemia (Thiel et al, 2012; Wentink et al, 2015). CD21, which exists in a complex with CD19, binds to C3d and thus lowers the threshold of B cell activation (Holers, 2014). Despite the fact that CD21 and CD19 form a complex, deficiencies in either of the two proteins result in a substantial difference of disease phenotype (Thiel et al, 2012; van Zelm et al, 2006; Wentink et al, 2015). The phenotype of CD21 deficiency is arguably milder than the one of CD19 deficiency. On the cellular level, this is explained by the substantial defect of BCR signaling in CD19-deficient B cells. In contrast, CD21-deficient B cells are able to signal through their BCR if stimulated with a comparable signal strength (Wentink et al, 2015).

1.2.3. Cells of the adaptive immune system – T cells

Similar to B cells, T cells also develop from the CLP. However, the development does not happen in the bone marrow as it does for B cells. The primed CLP migrates to the thymus where it enters at the cortical region and immediately initiates the developmental program of T cells. Upon the entry to the thymus, CLP differentiates to the double-negative (DN) 1 stage of T cell maturation (Figure 2). The DN cells neither express T cell receptor (TCR) genes nor the co-receptors CD4 or CD8 (Rothenberg et al, 2008). While migrating through the cortical thymus, DN1 cells develop to DN2 cells (Figure 2) which start to express T cell specific genes such as *CD3G*, *ZAP70* and *LCK* (Rothenberg et al, 2008). During DN1 and DN2 stages cells proliferate, which is halted when they start the transition to the DN3 stage (Rothenberg et al, 2008). DN3 cells initiate the expression of the RAG proteins, which is accompanied by the rearrangements of the D and J segments of the TCR β locus (Rothenberg et al, 2008). In the next step of TCR gene rearrangement, one of the V segments is joint to the DJ segment of the β chain. Subsequently, the TCRβ chain is tested for successful rearrangement by expression and assembly with the invariant pre-Tα chain and the co-receptor CD3, a process called β-selection (Michie & Zuniga-Pflucker, 2002). The outcome of this early signaling defines whether the cell undergoes apoptosis or initiates further proliferation and the rearrangement of the V and J gene segment in the TCRα locus, whereby the latter induces the progression to the double positive (DP) stage (Michie & Zuniga-Pflucker, 2002).

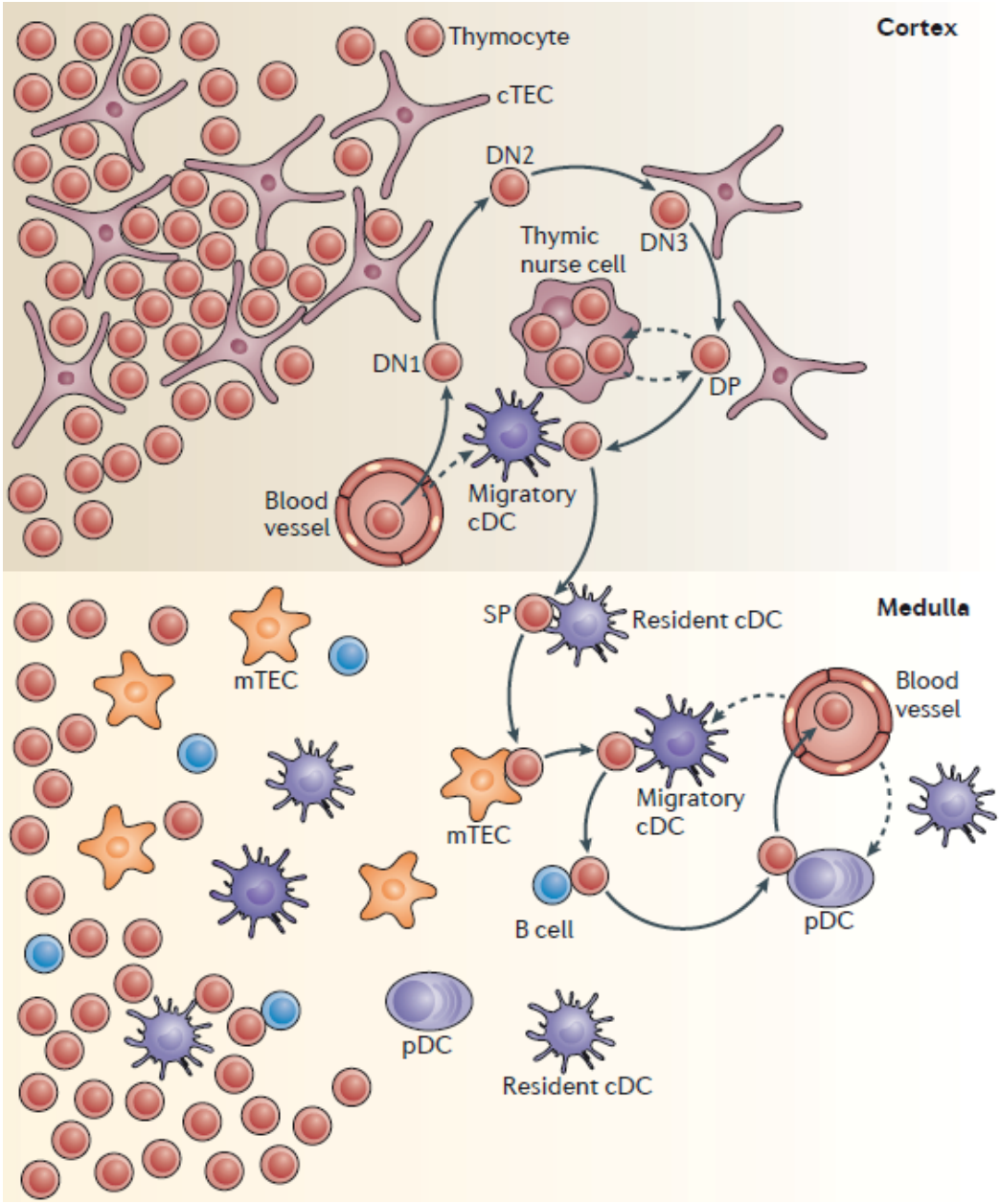

Figure 2 – T cell development and selection in the thymus. Upon leaving the blood vessel and entering the cortical zone of the thymus, thymocytes migrate through the cortex and develop from DN1 cells to DN2 and DN3 cells. Thymic nurse cells support the β-selection of DN3 cells and the positive selection at the double positive (DP) stage. During positive selection, DP thymocytes recognize the TCRs of cortical thymic epithelial cells (cTECs) which express a unique peptide-MHC ligandome. Already at this stage interaction with migratory conventional DCs (cDCs) might lead to a negative selection of thymocytes. After positive selection, DP thymocytes develop into single positive (SP) thymocytes, which undergo negative selection to prevent recognition of self-ligands in the periphery. This is mainly regulated by autoimmune regulator (AIRE) expressing medullary thymic epithelial cells (mTECs), but also by migratory and resident cDCs and plasmacytoid DCs (pDCs). After successful negative selection, thymocytes leave the thymus as mature naïve T cells. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology (Klein et al, 2014), copyright (2014).

On the transcriptional level, cells in the DP stage initiate the expression of CD4, CD8, CD3 and ζ-chain. After the TCRα locus is successfully rearranged, the DP cell further starts to express the TCRαβ complex which is subsequently tested in a process called positive selection. In this process, DP thymocytes interact with cortical thymic epithelial cells (cTECs, Figure 2). cTECs display a unique peptide-MHC-ligandome on their surfaces which is substantially different from the ligandome of any other cell, as they express the proteasome subunit beta type-11 (PSMB11). PSMB11 differs from the proteasomal subunits of the immunoproteasome in all other nucleated cells leading to a different antigen cleavage and thus a substantial different peptide-MHCI-ligandome (Klein et al, 2014). MHCII molecules of cTECs are loaded by antigen processing through the cTEC-specific proteases thymus-specific serine protease and cathepsin L which also results in a peptide-MHC-ligandome that is unique and different from other cells (Klein et al, 2014). A weak interaction of the newly formed TCRαβ complex of the DP thymocytes is needed to proceed to the next developmental stage.

The identity of the interacting MHC molecule defines the commitment of the DP thymocyte to the differentiation and lineage commitment to either the CD4 single positive (SP), or the CD8 SP thymocyte (Klein et al, 2014). SP thymocytes undergo negative selection, an important process to prevent self-reactivity. During this process, the cells interact mainly with medullary thymic epithelial cells (mTECs, Figure 2). Those cells express the transcription factor AIRE which initiates the expression of tissue-restricted antigens (TRAs). TRAs are loaded onto the MHCI molecules through the classical immunoproteasome and onto MHCII through autophagy (Klein et al, 2014). Interaction of a SP thymocyte with the TRA-MHC complex initiates tolerance mechanisms by deletion of reactive clones through apoptosis or induction of the regulatory T cell program (Malhotra et al, 2016), a subset of T cell which will be discussed later. Negative selection is supported by DCs and B cells, however, their role in the process of negative selection is only at the beginning of being understood (Klein et al, 2014). Once thymocytes successfully underwent positive and negative selection, they leave the thymus as mature naïve helper ($CD4^+$) or cytotoxic ($CD8^+$) T cells.

Several primary immunodeficiencies have been described that manifest with a T cell developmental defect, and thus reduced or absent numbers of T cells (or T cell subsets). One example is the above mentioned SCID caused by mutation in the

genes encoding the RAG proteins (Schwarz et al, 1996). Another form of SCID is caused by biallelic mutation in the *CD3D* or *CD3E* genes, respectively (Dadi et al, 2003; Soudais et al, 1993). Absence of any of the CD3 chains leads to a non-functional TCR complex. Signaling through this complex is already required at the state of β-selection. Thus, patients with mutations in any of this genes lack peripheral mature T cells. However, in contrast to RAG-deficient SCID, the B-cell compartment of patients with mutations in the CD3-encoding genes is unaffected.

Another form of primary immunodeficiency is caused by biallelic mutations in the gene *AIRE* (Nagamine et al, 1997). Patient present with a severe autoimmune reaction against endocrine and sometimes ectodermal tissues (Gallo et al, 2013). The underlying pathological mechanism is caused by defective negative selection of SP thymocytes which results in an unwanted T cell response towards self-antigens in the periphery (Gallo et al, 2013). However, an early characteristic of AIRE-deficient patients is the appearance of autoantibodies against proinflammatory cytokines such as IFNα or IL-17 (Meager et al, 2006). The molecular reason why those autoantibodies evolve so early and frequently in the disease pathology is not yet fully understood.

Already during development, TCR signaling is important in the selection processes in the thymus. Furthermore, the activation of T cells critically depends on TCR signaling. This signaling is initiated by the binding of the TCR to its specific MHC-peptide agonist. It has been shown that a single specific MHC-peptide can lead to activation of TCR signaling (Huang et al, 2013). Early work proposed that the binding of TCR and MHC-peptide needs to be accompanied by a second signal to successfully activate the TCR signaling cascade (Lafferty & Cunningham, 1975). This second signal is provided through the binding of the co-stimulatory molecule CD28 to its ligands CD80 or CD86 (Azuma et al, 1993; Hathcock et al, 1993; Linsley et al, 1990). Of note, the co-inhibitory protein CTLA-4 competes with CD28 for the binding of CD80 and CD86 and thus prevents the activation of the T cell receptor cascade (Azuma et al, 1993; Hathcock et al, 1993; Linsley et al, 1991). The role of CTLA-4 is discussed later in more detail. The TCR signaling cascade is initiated through the recruitment of the lymphocyte-specific protein tyrosine kinase (LCK), which is associated with the co-receptors CD4 and CD8 (Veillette et al, 1988) and the formation of so-called TCR-microclusters (Saito et al, 2010). These microclusters evolve to a unique signaling structure called the immunological synapse (IS), which

is formed between the MHC-bearing antigen presenting cell and the T cell (Grakoui et al, 1999). This structure forms the spatial basis for the transmission of the signal to the T cells (Grakoui et al, 1999). The IS consists of a central and peripheral supra-molecular activation cluster (cSMAC, pSMAC). While signaling molecules concentrate at the cSMAC, adhesion molecules that promote the tight contact between the T cell and the APC accumulate at the pSMAC (Chen & Flies, 2013). Accumulation and activation of LCK at the cSMAC leads to the phosphorylation of the ITAMs of the TCR-ζ- chains, CD3ε, and the subsequent activation of these molecules (van Oers et al, 1996).

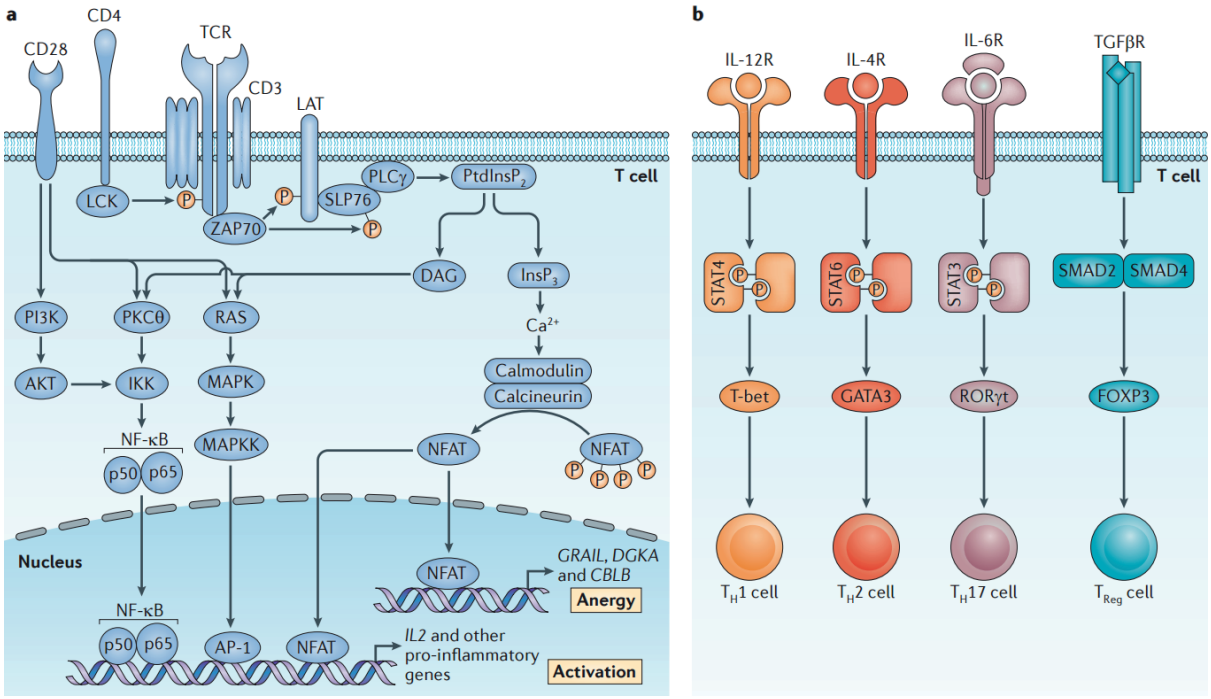

Figure 3 – TCR signaling. (**a**) The binding of the TCR to the peptide-MHC complex and CD28 to its ligands CD80 or CD86 results in the recruitment and activation of LCK which in turn phosphorylates the TCR-ζ-chains and the kinase ZAP70. Activation of ZAP70 results in the phosphorylation of LAT and SLP76 which in turn recruits and activates PLCγ1 which hydrolyses PtdIns(4,5)P_2 (PtdInsP$_2$) to DAG and IP3 (InsP$_3$) similar as PLCγ2 in B cells. DAG together with the co-stimulatory signals mediated through CD28 results in the activation of the MAPK and NF-κB pathways. Together, these signaling events lead to the activation and nuclear translocation of the transcription factors NF-κB, AP-1, and NFAT. (**b**) The generation of different helper cell subsets from naïve T cells is mediated through the specific signaling of cytokine receptors, which turn on the signature transcription factors of these helper T cell subtype. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology (Pollizzi & Powell, 2014), copyright (2014).

The phosphorylation of those ITAMs results in the recruitment of zeta-chain-associated protein kinase 70 (ZAP70) which subsequently is phosphorylated and activated by LCK (Brownlie & Zamoyska, 2013). ZAP70 is a protein tyrosine kinase that phosphorylates the linker for activation of T cells (LAT, Figure 3a). LAT serves as a signaling platform for a multitude of proteins including PLCγ1, VAV, growth factor receptor-bound protein 2 (GRB2), SOS1, and the SH2 domain containing leukocyte protein of 76kDa (SLP76) (Balagopalan et al, 2010). GRB2 and SOS1 are important for the activation of the MAPK pathway. SLP76 further interacts with PI3K and interleukin-2-inducible T-cell kinase (ITK) (Balagopalan et al, 2010). ITK can phosphorylate DEF6 (Hey et al, 2012) which in turn acts, similar to VAV, as a GEF for the small G-proteins CDC42, RAC1 and RHOA which are important for actin cytoskeleton remodeling after T cell activation (Balagopalan et al, 2010; Mavrakis et al, 2004). The above mentioned co-stimulatory signal, which is provided by the binding of CD28 to CD80 or CD86 results in the enhanced activation of PI3K and translocation of PKCθ which in turn activates the NF-κB pathway (Boomer & Green, 2010). The orchestrated activation of NF-κB and MAPK pathways and PLCγ1/IP3/Ca^{2+} mediated nuclear translocation of NFAT results in the initiation of a transcriptional program that activates and differentiates the naïve T cell in one of the T cell subsets described below.

Several primary immunodeficiencies caused by defective T cell signaling have been described (Notarangelo, 2014). As an example, biallelic germline mutations in the gene encoding one of the central kinases LCK lead to CD4 T cell lymphopenia and profound TCR signaling defects which ultimately result in recurrent respiratory tract infection in the patients (Hauck et al, 2012). The genetic characterization of this disease points towards the importance of LCK in the development and maintenance of $CD4^+$ T cells. Another form of PID involving T cell signaling is caused by biallelic germline mutations in the gene *ITK* (Huck et al, 2009). Patients with ITK deficiency present with reduced numbers of $CD4^+$ T cells which also suggests a central role for ITK in CD4+ T cell development and/or maintenance (Serwas et al, 2014). In contrast to LCK-deficient patients, ITK deficiency also results in the absence of natural killer T (NKT) cells. Those cells express an invariant TCR consisting of the Vα24Jα18 and Vβ11 chains, which are important for the recognition of phospholipid antigens presented on CD1d (Godfrey et al, 2010). Absence of NKT cells is associated with an increased risk of developing Eppstein-Barr virus (EBV)-driven

lymphoma (Worth et al, 2016), which is a common feature of ITK-deficient patients (Ghosh et al, 2014). The identification of several mutations affecting the *ITK* gene revealed that mutations are not concentrated to a specific domain of the protein product, but rather spread all over the gene (Linka et al, 2012; Serwas et al, 2014). Separate analysis of specific patient mutations revealed novel insights into the role of specific parts of the protein in TCR signaling. Ectopic expression of mutated ITK in *Itk*$^{-/-}$ T cells derived from mice revealed that only wildtype and ITK^{D500T,F501L,M503X}, a mutation found in an Indian ITK-deficient patient, could rescue defective Ca^{2+}-flux in the murine cells (Linka et al, 2012) suggesting that the last 120 amino acids of ITK are not essential for the role of it in this process.

Another PID was discovered in patients with homozygous frameshift mutations in the gene encoding LAT (Keller et al, 2016). As pointed out above, LAT is a central linker of several proteins during TCR signaling. *Lat*$^{-/-}$ mice reveal a severe block of T cell development at the early DN T cell stage and have essentially no peripheral T cells (Zhang et al, 1999). Despite this, LAT-deficient patients were able to produce a certain amount of T cells although numbers were substantially decreased. Those T cells further were unable to signal through the MAPK pathway (Keller et al, 2016). A further report identified a patient with defective MAPK signaling which resulted from homozygous germline mutations affecting the RAS guanyl nucleotide releasing protein (RASGRP1) (Salzer et al, 2016). Patients suffer from $CD4^+$ T cell lymphopenia and proliferation defects. In contrast to LAT, RASGRP1 is also expressed in B cells. Thus, RASGRP1 deficiency further results in a MAPK pathway defect in these cells (Salzer et al, 2016). Strikingly, this report was also able to uncover a connection between RASGRP1 and the transport of vesicles towards the immunologic synapse, as it interacts with the motor protein dynein light chain. The disruption of this interaction in the patient resulted in reduced NK- and T-cell mediated cytotoxicity (Salzer et al, 2016).

As mentioned above, signaling through the TCR results in an activation of several transcription factors which activate certain transcriptional programs in T cell. CD8 T cells develop upon TCR signaling towards cytotoxic T lymphocytes (CTLs). These cells express high levels of FAS ligand, perforin and granzyme due to the activation of the transcription factors eomesodermin and T-bet. The cytotoxic proteins are stored in specialized granules which can be exocytosed towards a virus-infected target cell (Kaech & Cui, 2012). In CD4 T cells, the defined interplay of these factors

with each other and the additional activation of other transcriptional regulators through signaling of cytokine receptors (Figure 3b) result in the development of diverse T cell subsets such as T helper 1 (Th1), Th2, Th17, Th9, Th22, and Treg cells (Raphael et al, 2015). T cell subsets differ in their effector mechanisms and cytokine production.

Th1 cells have been shown to preferentially develop from naïve CD4 T cells if those cells are stimulated with IL-12 coupled with strong TCR signaling. This results in the activation of the transcription factor T-bet (Figure 3b). Th1 cells itself secrete IFNγ and tumor necrosis factor alpha (TNFα) which are important cytokines for the defense against intracellular pathogens (Raphael et al, 2015). Th2 cells are generated if the naïve CD4 T cell is stimulated through the TCR in cooperation with IL-4 signaling. This results in the activation of the transcription factor GATA3 (Figure 3b). Th2 cells secrete the cytokines IL-4, IL-5, and IL-13 which play a major role in the immune response against multicellular parasites such as helminths. Th2 cells are also involved in the unwanted immune response generated in allergies, which is mediated through IL-5 and IL-13 (Raphael et al, 2015).

In contrast to Th1 and Th2 cells, other T cell subsets such as Th17, Th22, and Th9, have been named after their signature cytokine. Th17 cells, as the name suggests, produce substantial amounts of the cytokine IL-17. The interplay of the cytokines tumor growth factor (TGFβ), IL-6, IL-1 and IL-23 in cooperation with TCR signaling results in the activation of the Th17 transcriptional program. The key transcription factor in this subset of cells is retinoic acid receptor (RAR)-related orphan receptor γt (RORγt). Th17 cells are critically important for the defense against fungal pathogens. They also contribute to immunity against extracellular bacteria (Raphael et al, 2015). However, Th17 cells have also been recognized as an important player in some forms of autoimmune diseases (Raphael et al, 2015). Several PIDs have been previously described to affect Th17 homeostasis. This includes the identification of biallelic mutations affecting the genes encoding IL-17 receptor A (Puel et al, 2011), IL-17 receptor C (Ling et al, 2015), and autosomal dominant mutations affecting IL-17F (Puel et al, 2011). All of these diseases affect the function of the signature cytokine of Th17 cells. Furthermore, biallelic mutations in the gene *RORC* which encodes the key transcription factor of Th17 cells RORγt have been described (Okada et al, 2015). Patients with a deficiency of the above mentioned proteins

present with recurrent infection of the fungal pathogen *Candida albicans* (Ling et al, 2015; Okada et al, 2015; Puel et al, 2011). Interestingly, patients with RORγt deficiency present an additional susceptibility towards mycobacteria which unraveled an additional, yet unexplored important role for RORγt in the production of the Th1 cytokine IFNγ (Okada et al, 2015).

Th22 cells were initially identified in human skin (Duhen et al, 2009). Upon TCR, IL-6 and TNFα signaling, naïve CD4 T cells develop towards the Th22 lineage, which results in the expression of the signature cytokine IL-22, and their key transcription factor aryl hydrocarbon receptor (AHR). Th9 cells develop from naïve CD4 T cells when stimulated with the cytokines IL-4 and TGFβ, which leads to the upregulation of the transcription factors IRF4 and PU.1 (Raphael et al, 2015). Th9 cells seem to play a role in autoimmune diseases, though this is not yet clearly defined as they can also produce the anti-inflammatory cytokine IL-10 (Raphael et al, 2015).

Another subset of T cells which develops upon the combination of signaling through TCR signaling and exposure to the predominant anti-inflammatory cytokine TGFβ are regulatory T cells (T_{regs}), which are described in more detail in the following chapter.

1.2.4. Keeping adaptive immunity under control - regulatory T cells

One of the key concepts of adaptive immunity is memory formation, which exacerbates autoimmunity in the case of self-recognition. Thus, some mechanisms have evolved to prevent damage of self. A critical role in this is attributed to a specific subset of T cells, namely regulatory T cells (T_{regs}). These cells were discovered in 1995 as a subset of CD4 T cell that express high levels of the IL-2 receptor CD25 (Sakaguchi et al, 1995). T_{regs} can either develop in the thymus during negative selection (thymus-derived T_{regs}; tT_{regs}), or in the periphery in a non-inflamed environment (peripherally derived T_{reg} cells, pT_{regs} (Abbas et al, 2013; Lin et al, 2013)).

The development of tT_{regs} in the thymus requires a very strong interaction of the TCR of a thymocyte with a self-antigen loaded MHC molecule presented by an mTEC. Instead of ultimate induction of apoptosis in the thymocyte, the interaction leads to an upregulation of forkhead box P3 (FOXP3) transcription, which is the key transcription factor of regulatory T cells ((Fontenot et al, 2003; Hori et al, 2003; Khattri et al, 2003) (Figure 3b)). FOXP3 initiates the development of thymocytes

towards the T_{reg} lineage. The generation of pT_{regs} from naïve CD4 T cells also requires the initiation of FOXP3 expression. This is achieved if naïve CD4 T cells receive a very strong signal through their TCR but no or only very little co-stimulatory signal. A certain cytokine milieu such as an increase in TGFβ and retinoic acid (RA) concentration can also contribute to the induction of FOXP3 (Chen et al, 2003). The signaling that is induced by such cytokines leads to an activation of a particular inter-exonic conserved non-coding sequences (CNS) in the *FOXP3* gene. As an example, CNS1 contains binding sites for the RA receptor (Zheng et al, 2010). Binding of RA to CNS1 is required for the development of pT_{regs}, but dispensable for the generation of tT_{regs} (Zheng et al, 2010). The essential role of FOXP3 in the development of tT_{regs} and pT_{regs} is confirmed by mice and patients with mutations in the gene encoding this protein which results in a complete absence of T_{regs} (Brunkow et al, 2001; Fontenot et al, 2003; Hori et al, 2003; Wildin et al, 2001).

T_{regs} suppress the immune system through several intertwined mechanisms. They use up the extracellular IL-2, which is essential for cell proliferation of activated cells, by expression of high levels of the high-affinity IL-2 receptor CD25. Furthermore, T_{regs} express two extracellular enzymes CD39 and CD73 which together convert extracellular adenosine triphosphate (ATP) to adenosine, another component needed for efficient cell proliferation, (Plitas & Rudensky, 2016). Additionally, T_{regs} secrete cytokines which rather dampen the immune response such as IL-10 and TGFβ (Plitas & Rudensky, 2016). The above mentioned inhibitory protein CTLA-4 which can compete with CD28 for binding of CD80 and CD86 is also expressed by T_{regs}. It has been shown that T_{regs} utilize CTLA-4 to reduce the amount of available CD80 and CD86 on antigen presenting cells by trans-endocytosis (Qureshi et al, 2011). CTLA-4 is stored in vesicles of these cells and travels to the membrane where it only stays transiently before it is endocytosed again. If at the short time of membrane location CTLA-4 has bound to one of the costimulatory molecules, both molecules are endocytosed together which removes the CD80 or CD86 from the antigen-presenting cell (Qureshi et al, 2011). Endocytosed vesicles containing CTLA-4-CD80/86 complex are further processed to either late endosomes and lysosomes which degrade their internal content, or are fused to RAB11-positive recycling endosomes which are able to fuse again to the membrane resulting in CTLA-4 recycling (Qureshi et al, 2012; Sansom, 2015). To prevent the sorting of CTLA-4 to lysosomal degradation, the cytoplasmic tail of CTLA-4 is bound to the

protein LRBA (Lo et al, 2015). Both LRBA and CTLA-4 are essential for the prevention of autoimmune disease in humans (and mice) as discussed below (Kuehn et al, 2014; Lo et al, 2015; Schubert et al, 2014; Tivol et al, 1995; Waterhouse et al, 1995). However, a recent study has shown that deletion of CTLA-4 from T_{regs} in adult mice protects those animals from experimental autoimmune encephalomyelitis (EAE), a commonly used mouse model of autoimmune disease (Paterson et al, 2015). The molecular mechanism of this protection is not yet fully understood.

1.3. Autoimmune and autoinflammatory diseases

As outlined in the previous chapters, the immune system evolved several mechanisms to defend against invading pathogens or malignancies. One important function of the immune system is to keep itself under tight control. To this end, it is crucial to discriminate self and non-self. Several strategies have evolved that prevent immune reactions against self. This includes the tightly regulated development of B and T cells and the existence and function of T_{regs} as discussed above. However, some patients develop diseases which are caused by defects in one of these regulatory mechanisms resulting in autoimmune or autoinflammatory diseases. To understand the molecular etiology of these diseases, it is important to discriminate between autoimmunity and autoinflammation. An autoimmune reaction arises mostly through a defect in the adaptive immune system which results in autoreactive T and/or B cells (Conrad et al, 2007). Autoinflammatory diseases suggest a defect in the innate arm of the immune system which results in an inappropriate activation of immune cells and consequently in a release of pro-inflammatory cytokines (van Kempen et al, 2015). Autoimmunity and autoinflammation can also occur together as autoimmune diseases can lead to an enhanced release of pro-inflammatory cytokines. On the other hand the pro-inflammatory cytokine milieu in autoinflammatory diseases increases the likelihood for the production of autoreactive cells. The importance of the discrimination between autoimmunity and autoinflammation is reflected in the varying degree of success of therapeutic agents. For example, treatment of an autoinflammatory form of rheumatoid arthritis with an antibody directed against tumor necrosis factor α (TNFα) results in a good clinical response whereas the same treatment for patients with phenotypically similar

systemic-onset juvenile idiopathic arthritis yields no or only partial improvement (Correll & Binstadt, 2014; Singh et al, 2009). In contrast, treatment of autoimmune diseases like anti-neutrophil cytoplasmic antibodies (ANCA) positive vasculitis with drugs targeting adaptive immunity, such as rituximab (a monoclonal anti-CD20 antibody), result in significant treatment success (Dumoitier et al, 2015; Kao et al, 2014). Despite intense study, the molecular triggers for the onset of autoimmune or autoinflammatory diseases are not well understood. It has been known that several single nucleotide polymorphisms (SNPs) increase the risk of autoimmune disease onset (Stanford & Bottini, 2014). Genome-wide association studies revealed susceptibility loci in several immune genes which can be grouped by their involvement in different pathways, such as the T cell receptor, the janus kinase, or the NF-κB pathway (Messemaker et al, 2015). These studies only reveal a certain gene signature of the disease. The effect sizes of the associated polymorphisms are very low and increase the risk of disease onset minimally. Furthermore, the onset of the disease is not only dependent on the genetic background but can also be triggered through environmental factors such as smoking or diet (Pollard, 2015). However, they provide a valuable insight into potential disease mechanisms.

1.3.1. Rheumatoid arthritis

The most common autoimmune/autoinflammatory disorder in the elderly population is rheumatoid arthritis. The prevalence in Europe and North America has been estimated to be 0.5 – 1 % (Minichiello et al, 2016), with most of them being female (Scott et al, 2010). The disease course is characterized by severe synovitis that leads to pain, remodeling of the bone, and subsequently restrained ability to move. Classification criteria, from which a disease score is calculated, includes: 1) a combination of number and location of affected joints (maximal 5 points), 2) disease duration (maximal 1 points), 3) serological abnormalities (see below, maximal 3 point), and 4) elevated acute-phase reactants (maximal 1 point) (Aletaha et al, 2010). Definitive diagnosis of rheumatoid arthritis is given if a patient has a disease score greater than 6 points (Aletaha et al, 2010). Location of affected joints includes the inflammation status of small versus large joints such as shoulder, hip, knee or elbow. Synovitis in smaller joints results in a higher score. The score for disease duration differentiates whether symptoms are present for shorter or longer than 6 weeks (Aletaha et al, 2010). Serological abnormalities include the presence of

antibodies against constant portions of patients' antibodies. Those self-reactive antibodies are called rheumatic factors (RF). Additionally, it has been shown that patients suffering from rheumatoid arthritis frequently develop autoantibodies against citrulinated proteins (anticitrullinated protein/peptide antibodies, ACPAs) (Schellekens et al, 1998). The presence and quantity of RF and ACPA define the score for this subcategory (Aletaha et al, 2010). The score concerning an elevated acute phase reactants is defined by an elevated C-reactive protein concentration or erythrocyte sedimentation rate (Aletaha et al, 2010).

One of the current model of rheumatoid arthritis -onset includes a damage of the airway through smoking which induces the production of ACPAs (Klareskog et al, 2013). Proteins that are commonly citrullinated include collagen, vimentin and fibrinogen (Gudmann et al, 2015). Specific MHC molecules that are associated with the onset of rheumatoid arthritis (see below) might be optimal for the presentation of such peptides, which in turn results in the activation of DCs and subsequently B and T cells. ACPAs are also able to activate osteoclasts, a specific type of macrophages which is important for bone remodeling (Klareskog et al, 2013). Activated T cells amplify the immune reaction in the joints and secrete TNFα and IL-6. Accordingly, in addition to Th22 and Th17 cells, macrophages also accumulate in the joints of patients (Li et al, 2013; Zhang et al, 2012).

Twin studies revealed a heritability of rheumatoid arthritis of about 53-65% depending on the analyzed cohort (MacGregor et al, 2000). Several genome-wide association studies (GWAS) have been performed to identify susceptibility loci that increase the personal risk of disease initiation. So far, more than 200 of those loci have been identified (Kirino & Remmers, 2015). Among them are SNPs located in the genes *PTPN22*, *CTLA4*, *IRF5*, *BLK*, and in multiple human leukocyte antigen (HLA) class II alleles (Kirino & Remmers, 2015). The identity of those genes and their known role in adaptive immunity (*PTPN22*, *CTLA4*, *BLK*, HLA) underlines the importance of this arm of the immune system for the onset of the disease. Interestingly, genes involved in innate immunity, such as *IRF5*, are associated with an increased risk of disease onset. IRF5, which can be activated by TLR-5, might thus play a role in the amplification of the initial disease (Kirino & Remmers, 2015).

Several treatment strategies have evolved. However, no drug has been identified that is able to cure patients with rheumatoid arthritis so far (Smolen & Aletaha, 2015). Several synthetic drugs, so-called disease-modifying antirheumatic drugs

(DMARDs), are used to keep patients symptom-free. An example of DMARDs is methotrexate. The mechanism of action of methotrexate is not yet fully understood. However, the drug increases T cell apoptosis and reduces T cell proliferation (Wessels et al, 2008).

In the past 20 years, there has been an increase in the use of biologicals. This includes antibodies directed against TNFα (infliximab), IL-6 receptor (tocilizumab) and IL-1 receptor ((anakinra, an IL-1receptor antagonist) (Smolen & Aletaha, 2015)). Furthermore, targeted therapy using rituximab, a monoclonal antibody against the B cell-specific membrane protein CD20, is also being used (Smolen & Aletaha, 2015). Another therapy includes the use abatacept, a drug that blocks co-stimulation. Abatacept is a CTLA-4-immunoglobulin (Ig) fusion protein, and is able to bind to the co-stimulatory molecules CD80 or CD86 and thus prevents the signaling through the co-receptor CD28 (see chapter 1.2.3.).

1.3.2. Type 1 diabetes mellitus

Another autoimmune disorder is type 1 diabetes mellitus. Patients present with high blood glucose levels due to an absence of the protein insulin. Insulin is produced in the pancreas and is the main regulator of glucose concentration in the blood (Saltiel & Kahn, 2001). The prevalence of type 1 diabetes mellitus was calculated to be 0.17 % (van Belle et al, 2011). Diagnosis is made if a patient suffers from ketosis or ketoacidosis and needs continuous insulin replacement therapy (Kawasaki et al, 2014). Type 1 diabetes can be triggered through development of autoimmunity directed against insulin or insulin-producing cells which mediates the destruction of pancreatic β-cells (van Belle et al, 2011). Thus, a subgroup of patients who have confirmed presence of anti-islet autoantibodies are further classified as autoimmune-mediated type 1 diabetes mellitus (Kawasaki et al, 2014).

The genetic contribution on the onset of disease was estimated with twin studies. Monozygotic twins show a 50 % concordance rate (Kyvik et al, 1995) In general, type 1 diabetes mellitus is seen as a disease which is triggered through a combination of genetic and environmental factors (Bluestone et al, 2010). Presently, there is no monogenetic form of type 1 diabetes mellitus. However, some PIDs with predominant autoimmune phenotype such as AIRE deficiency, FOXP3 deficiency (van Belle et al, 2011), CTLA-4 haploinsufficiency (Schubert et al, 2014) and LRBA deficiency (Charbonnier et al, 2015; Lo et al, 2015; Serwas et al, 2015) may present

frequently with this disease. GWAS identified several loci which are associated with a risk for diabetes mellitus type 1 disease onset, such as the *HLA B39* allele, the *insulin* gene, *CTLA4*, *PTPN22*, *IL2RA*, and *CLEC16A*. Some of these loci overlap with rheumatoid arthritis-associated risk genes suggesting a common mechanistic pathway that contributes to the disease onset (Stankov et al, 2013).

Type 1 diabetes mellitus is a non-curable disease so far. The current therapies concentrate on the replacement of insulin to control the blood glucose levels, and immune suppression to downscale the autoimmune reaction in the pancreas. Current research focuses on the prevention of disease onset and β-cell destruction (Creusot et al, 2016). This approach relies on the definition of biomarkers and the specific suppression of the autoimmune reaction in the pancreas. One option is the use of immunosuppressive drugs such as abatacept, a drug which is also used in rheumatoid arthritis, or low-dose IL-2 which specifically expand the regulatory T cell population in the patients (Creusot et al, 2016). Another alternative includes cell-specific therapies such as the extraction, expansion and transfusion of patients' regulatory T cells (Bluestone et al, 2015; Creusot et al, 2016). However, the efficiency of these therapies have yet to be formally tested.

1.3.3. Monogenic forms of PIDs with autoinflammation

A subgroup of patients develops severe autoinflammatory and/or autoimmune disease characteristics in early childhood. In these patients, the genetic contribution to the disease onset is generally very high as the young age reduces the exposure to environmental influences. Some of the diseases seen in such patients are caused by mutations in only a single gene. Those diseases are classified as primary immunodeficiencies (PIDs) with immune dysregulation (Picard et al, 2015). The rapid advances of next generation sequencing technologies enables the quick diagnosis of known PIDs with immune dysregulation and the discovery of novel yet undefined disease etiologies (Carneiro-Sampaio & Coutinho, 2015).

As an example, the genetic analysis of a group of PID patients with an undefined inflammatory syndrome has uncovered the regulatory mechanisms involved in the prevention of autoinflammation (Giannelou et al, 2014). Autosomal dominant mutations in *TMEM173* which encodes the stimulator of interferon genes (STING) have been described (Liu et al, 2014). The mutations lead to a constant activation of

STING resulting in a global increase of STING-responsive cytokines and an autoinflammatory syndrome called STING-associated vasculopathy with onset in infancy (SAVI) (Liu et al, 2014). STING is involved in the sensing mechanism for cytosolic double-stranded DNA and subsequent transcription of type I interferon genes (Dempsey & Bowie, 2015). The mutations identified in SAVI patients lead to a constitutively active form of STING and thus permanent transcription of the IFN-β gene (Liu et al, 2014).

Another group of primary immunodeficiencies present with associated autoimmunity due to defective apoptosis. This group of diseases is summarized with the term autoimmune lymphoproliferative syndromes (ALPS) (Oliveira, 2013). Patients suffering from this disease show a defective clearance of activated cells leading to uncontrolled expansion of those cells and an accumulation of double negative T cells in the blood. The underlying defects affect the pathway mediating the initiation of apoptosis (Oliveira, 2013). Most of the mutations can be found in the gene *TNFRSF6* encoding the FAS receptor. The mutations mostly affect only one allele and are of dominant interfering origin (Fisher et al, 1995). Both, germline as well as somatic mutations have been described (Oliveira, 2013). Furthermore, mutations affecting the gene encoding the ligand of FAS, the genes encoding the downstream caspases 10 (CASP10) and 8 (CASP8) and the FAS-associated death domain protein (FADD) also cause ALPS (Oliveira, 2013; Picard et al, 2015). Furthermore, some patients deficient for the protein kinase δ and LRBA have been described to present with an ALPS-like phenotype of autoimmunity and lymphoproliferation (Kuehn et al, 2013; Revel-Vilk et al, 2015; Salzer et al, 2013). However, the underlying mechanisms for this cases remains elusive and needs further investigation.

Immune dysregulation can also been seen in another group of PIDs called familial hemophagocytic lymphohistiocytosis (HLH) syndromes (Picard et al, 2015). Patients present with elevated pro-inflammatory blood cytokines, high and long fever periods, cytopenias, and sometimes hemophagocytosis. On the cellular level, immune cells are highly activated even in an absence of a pathogen (Janka & Lehmberg, 2013). Causative mutations affect genes encoding proteins involved in the vesicular transport and fusion machinery of cytotoxic T and NK cells, such as *UNC13D, STX11, RAB27A* or *STXBP2*, (Sieni et al, 2014). The most common form of familial HLH is caused by mutation affecting one of the content proteins of those vesicles, namely perforin (Sieni et al, 2014). These defects affect either cytotoxic vesicle

content or transport which results in defective killing of target cells. Two pathological mechanisms have been proposed to be causal for the immune dysregulation seen in HLH patients (Janka & Lehmberg, 2013). On the one hand, virus-infected antigen-presenting cells cannot be killed by cytotoxic T or NK cells. This results in a constant production of pro-inflammatory cytokines such as IL-6, which keeps other immune cells such as macrophages activated. On the other hand, cytotoxic T and NK cells fulfill an important role in contraction of the immune system after pathogen clearance. The inability to kill activated cells results in an overactivation and a phenotypic immune dysregulation (Janka & Lehmberg, 2013). However, the relative contribution of those two mechanisms has not yet been determined.

Another group of patients with an autoinflammatory phenotype is caused by biallelic mutation in either of the three genes *IL10*, *IL10RA* or *IL10RB* (Glocker et al, 2010; Glocker et al, 2009). Absent IL-10 signaling results in neonatal inflammatory bowel disease (Glocker et al, 2010; Glocker et al, 2009). The resulting phenotype resembles previously published mouse models and can be reasonably explained by an imbalance of the pro- and anti-inflammatory cytokines (Glocker et al, 2010; Glocker et al, 2009; Kuhn et al, 1993). Strikingly, as this disease is caused by a component of the hematopoietic system, it can be cured through hematopoietic stem cell transplantation (Glocker et al, 2009). However, recent investigations uncovered an increased susceptibility to B cell lymphoma for patients with mutation in the genes encoding IL10 receptors (Neven et al, 2013). The underlying disease pathology is not yet understood.

1.3.4. Monogenic forms of PIDs with autoimmunity

Several distinct PIDs with associated autoimmunity have been instrumental in uncovering important regulatory pathways in the prevention of autoimmune diseases. One such example is PID caused through mutation in the gene *AIRE,* which encodes the transcriptional master regulator for thymic antigen expression during negative selection (see above).

Furthermore, several mutations affecting components of the regulatory T cell compartment result in PIDs with associated autoimmunity. As an example, mutations in the X chromosomal encoded gene *FOXP3* results in immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (Bennett et al, 2001; Brunkow et al, 2001; Wildin et al, 2001). FOXP3 is the key transcription factor in T_{regs}

which was confirmed by the absence of those cells in patients with IPEX syndrome (Brunkow et al, 2001; Wildin et al, 2001) and male *scurfy* mice, which carry a frameshift mutation in the murine ortholog *Foxp3* (Fontenot et al, 2003; Hori et al, 2003). Thus, the discovery of IPEX syndrome underlined the importance of T_{regs} in the prevention of autoimmune diseases in humans.

As outlined in previous chapters, a key protein for T_{regs} function is CTLA-4, a transmembrane protein that resides in preformed vesicles. Mice deficient for Ctla4 develop fatal autoimmune disease (Tivol et al, 1995; Waterhouse et al, 1995). As stated in previous chapters, several human autoimmune diseases such as rheumatoid arthritis and diabetes mellitus type 1 have been associated with polymorphisms in the *CTLA4* gene (Gough et al, 2005). Recently, CTLA-4 haploinsufficiency has been described as a novel primary immunodeficiency with severe autoimmunity (Kuehn et al, 2014; Schubert et al, 2014). Similar to *Ctla4*$^{-/-}$ mice, patients suffer from lymphoproliferation and lymphocyte organ infiltration (Kuehn et al, 2014; Schubert et al, 2014; Tivol et al, 1995; Waterhouse et al, 1995). However, a main difference between mice and humans is that heterozygous mice do not develop a disease phenotype (Waterhouse et al, 1995). The mechanism how CTLA-4 prevents unwanted lymphoproliferation has been long under debate as there is evidence not only for a cell-intrinsic, but also cell-extrinsic effect of CTLA-4 (Walker & Sansom, 2015). The cytoplasmic domain of CTLA-4 contains a immunoreceptor tyrosine-based inhibition motif (ITIM), which are the counterparts of ITAMs (Ravetch & Lanier, 2000). The evidences for an intrinsic role are vague and unprecise as many contradicting findings were made. As an example, CTLA-4 was shown to disrupt ZAP70 microclusters (Schneider et al, 2008).

However, other studies have shown that this is not the case (Calvo et al, 1997; Schneider et al, 2001). In contrast to the unknown intrinsic functions of CTLA-4, the extrinsic role of it was clearly defined. It was shown to compete with the co-stimulatory protein CD28 for the binding of CD80 and CD86 (Linsley et al, 1991). The affinity of CTLA-4 to CD80 and CD86 is up to 20-fold higher as the affinity of CD28 to these proteins (Collins et al, 2002). Once CTLA-4 binds CD80 or CD86 it subsequently trans-endocytoses the receptors and thus sequesters the costimulatory ligand from the antigen presenting cell (Qureshi et al, 2011). How the trafficking and recycling of CTLA-4 is regulated has remained elusive until recently.

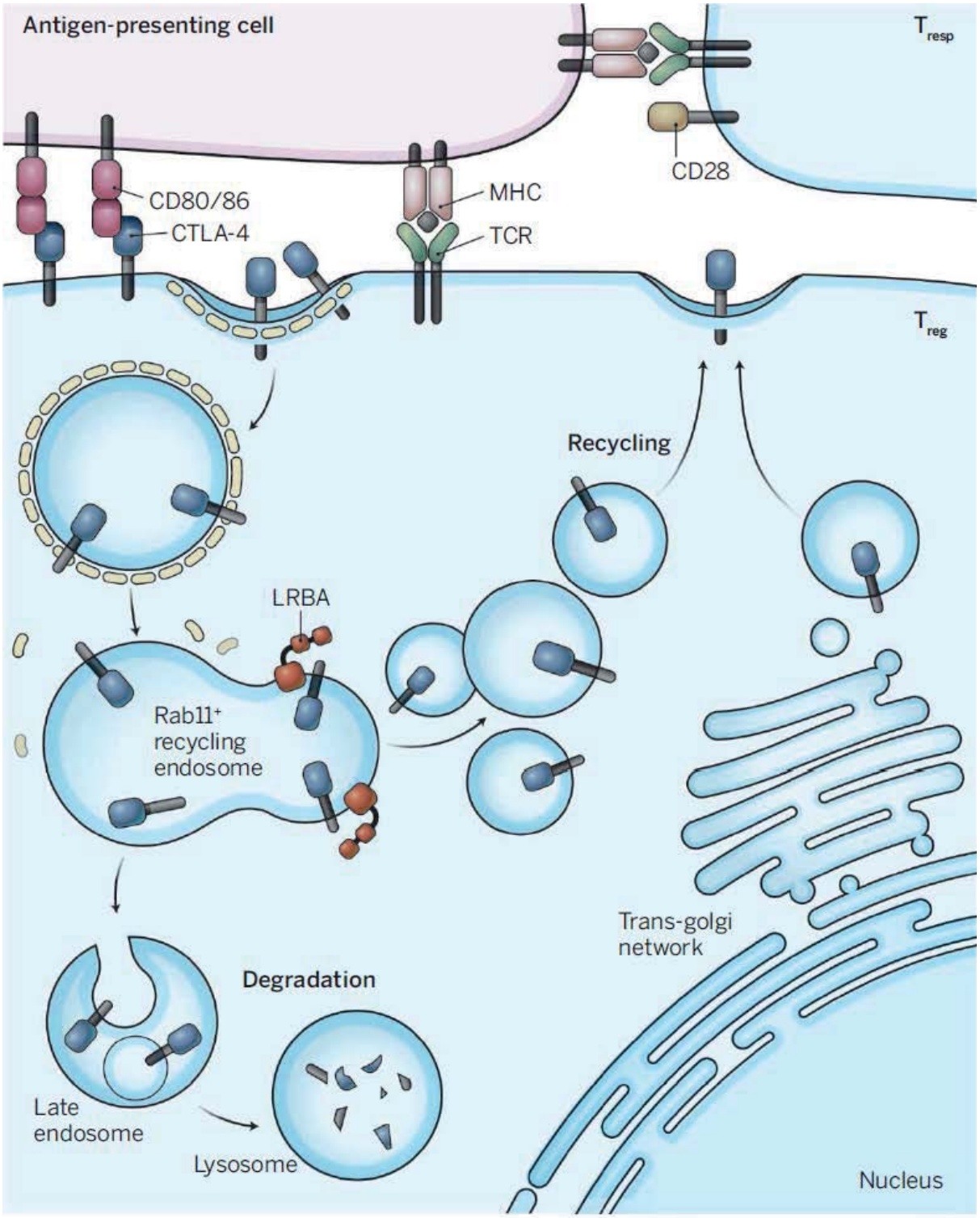

Figure 4 – CTLA-4 recycling. CTLA-4 is brought to the membrane of regulatory T cells where it interacts with CD80 or CD86 and thus, prevents that CD80 or CD86 bind the co-stimulatory molecule CD28 on the responder T cell (T_{resp}). CTLA-4 is constantly endocytosed. Endocytosed vesicles fuse with RAB11+ recycling endosomes and a brought back to the membrane. Alternatively CTLA-4 can be degraded in lysosomes. The degradation is circumvented by binding of LRBA to the cytoplasmic tail of CTLA-4. From (Sansom, 2015). Reprinted with permission from AAAS.

Patients deficient in LRBA also present with a severe autoimmune phenotype similar to CTLA-4-haploinsufficent patients (Alangari et al, 2012; Burns et al, 2012; Charbonnier et al, 2015; Lopez-Herrera et al, 2012; Revel-Vilk et al, 2015; Seidel et al, 2015; Serwas et al, 2015). This, and the observation that CTLA-4-Ig therapy reverts the phenotype of the patients led to the hypothesis that LRBA plays a significant role in the regulation of CTLA-4 (Lo et al, 2015). Indeed, it was shown that LRBA interacts with the intracellular domain of CTLA-4 and prevents the degradation of it once it is endocytosed (Figure 4). Thus, deficiency of LRBA resembles CTLA-4 haploinsufficiency as CTLA-4 in LRBA-deficient cells is constantly shuttled to lysosomal degradation, which results in a CTLA-4 deficit on the cells.

1.4. Aims of the thesis

The aims of this thesis were to identify and molecular characterize novel causes of primary immunodeficiency with predominant autoimmunity. To this end, homozygosity mapping via 6.0 SNP chips (Affymetrix®) and exome sequencing on an Illumina® platform was applied on selected patients presenting with the early-onset autoimmunity. Identification of mutations in known or novel genes was accompanied by critical literature review and subsequent functional analyses revealing the causality and molecular mechanism of the mutation for the observed phenotype.

2. Results

2.1. LRBA deficiency as a novel disease etiology

The protein LPS-responsive beige like anchor protein, which is encoded by the gene *LRBA,* was first described in 2001 (Wang et al, 2001). LRBA is a 319 kDa protein which consists of a large N-terminal domain of unknown function, a beige and Chediak−Higashi syndrome (BEACH) domain, and a C-terminal WD40 domain which is important for protein interaction (Wang et al, 2001). LRBA can be upregulated in B cells and macrophages upon LPS stimulation (Wang et al, 2001). Furthermore, it was shown that LRBA- green fluorescent protein (GFP) fusion protein co-localizes with the trans-golgi, lysosomes, and endocytosed vesicles (Wang et al, 2001). The expression of LRBA is upregulated in several cancers (Wang et al, 2004). Approximately a decade after the initial identification of *LRBA*; three groups independently describe a total of 11 patients suffering from common variable immunodeficiency that present with biallelic loss-of function mutations in the *LRBA* gene (Alangari et al, 2012; Burns et al, 2012; Lopez-Herrera et al, 2012). The described patients present with qualitative and/or quantitative B-cell defects. Furthermore, the patients suffer from diverse forms of autoimmunity (Alangari et al, 2012; Burns et al, 2012; Lopez-Herrera et al, 2012). The included manuscript in chapter 2.2. describes the identification of a patient bearing a novel mutation in *LRBA*. The predominant phenotype of this patient was severe diarrhea that appeared at the age of 6 months, and was later diagnosed as inflammatory bowel disease. In contrast to previously described LRBA-deficient patients, the mutation in *LRBA* does not lead to an absence of the protein product suggesting that the protein might have residual function. Furthermore, the here-identified patient presented no abnormalities in the B cell compartment (Serwas et al, 2015).

2.2. Atypical Manifestation of LRBA Deficiency with Predominant IBD-like Phenotype

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Atypical Manifestation of LRBA Deficiency with Predominant IBD-like Phenotype

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Background: Inflammatory bowel diseases (IBDs) denote a heterogeneous group of disorders associated with an imbalance of gut microbiome and the immune system. Importance of the immune system in the gut is endorsed by the presence of IBD-like symptoms in several primary immunodeficiencies. A fraction of early-onset IBDs presenting with more severe disease course and incomplete response to conventional treatment is assumed to be inherited in a Mendelian fashion, as exemplified by the recent discovery of interleukin (IL)-10 (receptor) deficiency.

Methods: We analyzed a patient born to consanguineous parents suffering from severe intestinal manifestations since 6 months of age and later diagnosed as IBD. Eventually, she developed autoimmune manifestations including thyroiditis and type I diabetes at the age of 6 and 9 years, respectively. Combined single-nucleotide polymorphism array-based homozygosity mapping and exome sequencing was performed to identify the underlying genetic defect. Protein structural predictions were calculated using I-TASSER. Immunoblot was performed to assess protein expression. Flow cytometric analysis was applied to investigate B-cell subpopulations.

Results: We identified a homozygous missense mutation (p.Ile2824Pro) in lipopolysaccharide-responsive and beige-like anchor (LRBA) affecting the C-terminal WD40 domain of the protein. In contrast to previously published LRBA-deficient patients, the mutant protein was expressed at similar levels to healthy controls. Immunophenotyping of the index patient revealed normal B-cell subpopulations except increased $CD21^{low}$ B cells.

Conclusions: We describe a patient with a novel missense mutation in *LRBA* who presented with IBD-like symptoms at early age, illustrating that LRBA deficiency should be considered in the differential diagnosis for IBD(-like) disease even in the absence of overt immunodeficiency.

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Key Words: LRBA, inflammatory bowel disease, autoimmunity, exome sequencing

The gastrointestinal tract represents the largest interface of the organism with the environment and is constantly confronted with foreign antigens and bacteria, which may elicit either beneficial or pathogenic effects. Whether the outcome is beneficial is determined by a variety of systems, including the immune system (reviewed in Ref. 1). Effector functions of the immune system in the gut are tightly regulated as inadequate activation may have destructive effects on the bowel (reviewed in Ref. 2). Inflammatory bowel diseases (IBDs) represent a group of diseases resulting from pathologically increased activation of host defense systems leading to severe inflammation and diarrhea (reviewed in Ref. 3). Conversely, primary immunodeficiency disorders including common variable immunodeficiencies (CVIDs) are also associated with IBD-like manifestations.[4] It has been hypothesized that for IBDs, disease onset is both partially environmentally and partially genetically driven (reviewed in Ref. 5). Recent studies have identified monogenetic causes of IBD, which may explain early disease onset in particular cases where the relative contribution of host genetics will arguably be the highest (reviewed in Ref. 6). For instance, mutations affecting the interleukin (*IL*) *10* (*receptor*),[7,8] *ADAM17*,[9] *XIAP*,[10] and *TTC7A*[11] genes have recently been identified as monogenic causes of very early-onset IBD (onset before 6 yr of age[12]). The diagnosis in these cases is difficult due to the unusual phenotype and lack of specific laboratory signs of intestinal inflammation (reviewed Ref. 6). A large proportion of patients with very early- or early-onset IBD (symptoms before 10 yr of age[13]) remain

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molecularly unclassified.[14,15] Early detection of such diseases and identification of the underlying causative genetic aberration(s) may improve the treatment strategies and enable further understanding of the pathogenic mechanisms underlying IBD.

We here describe, for the first time, a patient with very early-onset intestinal manifestations and diagnosed as IBD later in her life in whom we identified a biallelic mutation affecting the CVID-related gene *lipopolysaccharide-responsive and beige-like anchor* (*LRBA*).

MATERIAL AND METHODS

Patient

The described study was performed according to the Helsinki Declaration and approved by the local ethics committee. All investigated individuals signed informed consent documents. The patient was treated at the Department of Pediatric Gastroenterology at Ankara University and at the Department of Pediatric Gastroenterology at Akdeniz University in Turkey.

Immunohistochemistry

Staining against CD3 was performed using an anti-CD3 antibody (DAKO, United Kingdom) combined with streptavidin–peroxidase method. Hemosiderin staining was used to detect iron deposition in hepatocytes (Prussian blue staining).

Homozygosity Mapping

Homozygous regions were mapped using Affymetrix 6.0 SNP arrays (Affymetrix, High Wycombe, United Kingdom) as previously described[16] with minor modifications. In brief, genomic DNA was digested using the enzymes NspI and StyI (New England Biolabs, Frankfurt, Germany). Fragmented DNA was purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, Vienna, Austria) and ligated to adapters, which were labeled and hybridized to the chips. Analysis was done using Genotyping console (Affymetrix) and the online tool homozygositymapper.org[17] (accession date February 10, 2014).

Exome Sequencing

The index patient's exome was sequenced applying the Nextera exome enrichment kit (Illumina, Eindhoven, the Netherlands) according to manufacturer's recommendation. In brief, 50 ng of genomic DNA (gDNA) extracted from whole blood were subjected to transposase-based in vitro shotgun library preparation (tagmentation), which introduced adapters into the genomic DNA, while fragmenting it into 300 to 500 bp segments.[18] The adapter sequence was used to amplify fragmented gDNA in a limited cycle polymerase chain reaction. The DNA was enriched for exonic fragments, which were also amplified. Clusters were generated on a cBot Cluster Generation System (Illumina) applying the SE cluster kit v3 (Illumina) and sequenced on an Illumina HiSeq 2000 (Illumina) applying 3-plexed 50 bp single-end sequencing. Sequences were demultiplexed and aligned to the human genome 19 with Burrows-Wheeler Aligner version 0.5.9. Insertion/deletion realignment, quality score recalibration, and variant calling were done applying the genome analysis toolkit version 1.6[19]. Annotation of single nucleotide variants, insertion and deletions were performed with ANNOVAR.[20] Common variants listed in dbSNP 137 were excluded from further analysis. Validation of the identified variants in *LRBA* was performed using conventional Sanger sequencing.

FACS Analysis

Peripheral blood monocytes were isolated from shipped blood samples after 2 days using Ficoll Paque PLUS (VWR International GmbH, Vienna, Austria) and stored in 90% FBS (PAA Laboratories GmbH, Pasching, Austria) and 10% dimethyl sulfoxide (Sigma-Aldrich Handels GmbH, Vienna, Austria) in liquid nitrogen. After thawing of peripheral blood monocytes, surface molecules were blocked in RPMI (PAA Laboratories GmbH) supplemented with 10% FBS. Staining was performed 30 minutes on ice using the following antibodies: CD19-PerCP-Cy5.5 (eBiosciences; Vienna, Austria); CD3-APC-H7; CD4-APC; CD8-V450; IgD-FITC; IgM-APC; CD27-Brilliant violet; CD38-PECy7; CD21-PE (all: Becton Dickinson Austria GmbH; Vienna, Austria); CD14-PECy7; CD56-PE (both: Beckman Coulter; Vienna, Austria). Cells were analyzed on a LSR Fortessa (Becton Dickinson Austria GmbH; Vienna, Austria).

Protein 3D Structure Modeling

The protein model covering the beige and Chediak-Higashi (BEACH)–WD40 domain of LRBA (amino acids 2073–2863; NP_006717.2) was calculated using I-TASSER.[21] Secondary structures were assigned with the program ICM-Browser (Molsoft LLC, San Diego, CA). Phylogenetic conservation analysis was performed using Polyphen-2 (version 2.2.2r398).[22]

Western Blot

Patient granulocytes were isolated applying density centrifugation with Ficoll Paque PLUS (VWR International GmbH; Vienna, Austria) and stored at −80°C. Healthy donor granulocytes were isolated from fresh blood stimulated or not with 100 ng/mL LPS (Sigma-Aldrich, Handels GmbH) and directly lysed. Lysis was performed using RIPA buffer (1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, and 10 mM Tris-HCl pH 7.5). 50 μg of protein were loaded on a gradient of 12% and 8% polyacrylamide gel and subjected to gel electrophoresis. Cell lysates were blotted onto an immobilon polyvinylidene difluoride membrane (Roche, Grenzach-Wyhlen, Germany) for 4 hours at 45 V and then stained with the primary antibodies anti-LRBA (Sigma-Aldrich Handels GmbH) and anti-tubulin (Abcam, Cambridge, United Kingdom) as well as the secondary anti-rabbit antibody coupled to horseradish peroxidase.

RESULTS

The index patient is a female born at term in 1998 to healthy consanguineous parents of Turkish origin. Her sister is healthy except for hypothyroidism without detectable autoantibodies (Fig. 1A).

Symptoms of nonmucoid and nonbloody diarrhea commenced at the age of 6 months (Fig. 1B). Persisting diarrhea was

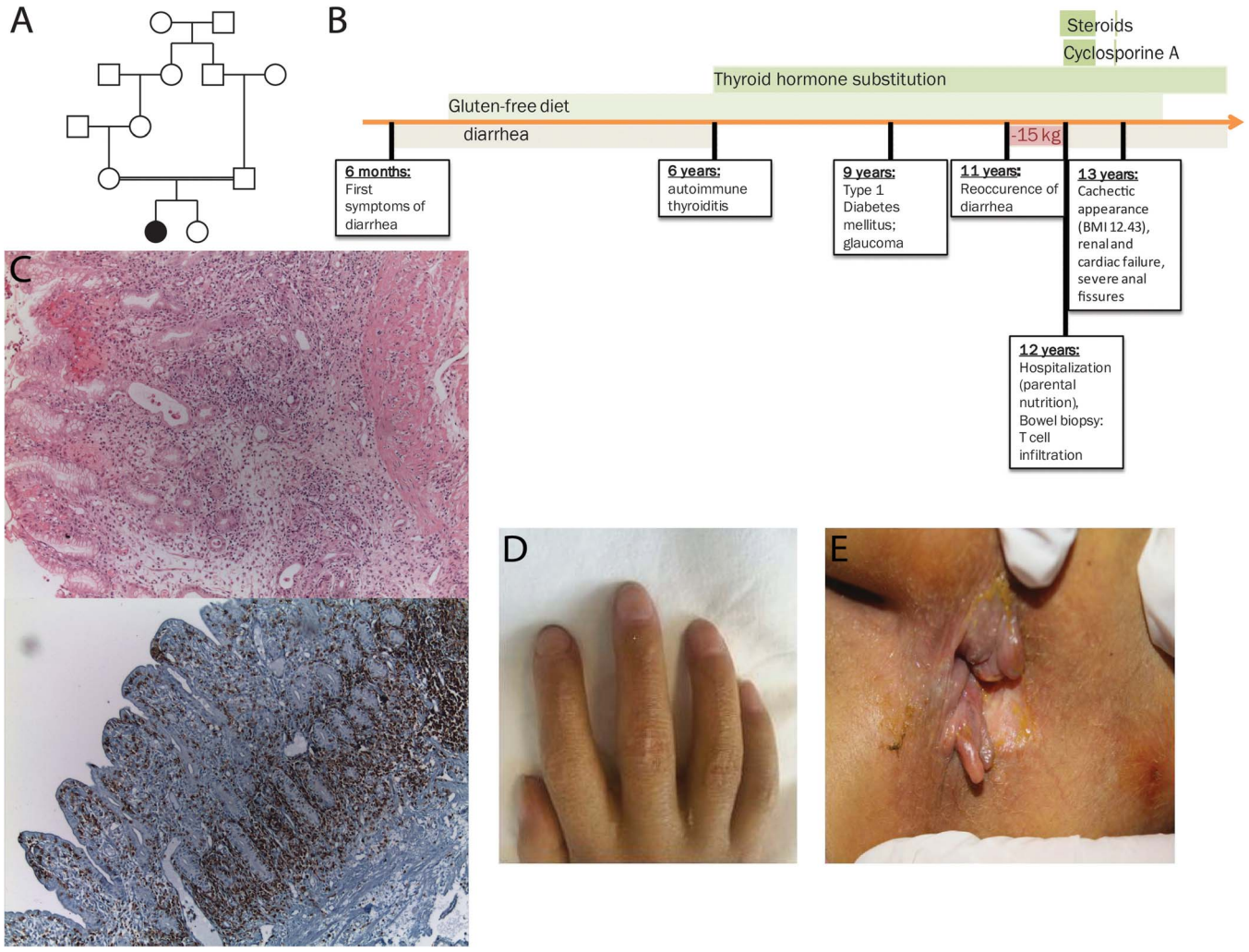

FIGURE 1. Clinical history and presentation of the index patient. A, Pedigree showing the consanguinity in the family. (B) Diarrhea started shortly after birth and was later accompanied by autoimmunity. C, (upper panel) Duodenal mucosa shows erosion and regeneration together with diffuse mixed cellular inflammatory infiltration in the lamina propria (×200 H&E stain). C, (lower panel) $CD3^+$ T cells are present in the lamina propria and surface epithelium in duodenal mucosa (×100). Patient presents with (D) finger clubbing and (E) persisting severe anal fissures and skin tags.

accompanied by severe edema due to hypoalbuminemia prompting recurrent albumine infusions. Other routine laboratory tests were normal. Known causes of persistent diarrhea such as congenital lactase deficiency, congenital chloride diarrhea, microvillous inclusion disease, primary intestinal lymphangiectasia, and food-induced enteropathy had been excluded by clinical history and appropriate laboratory tests. At the age of 1.5 years, a diagnosis of celiac disease was considered after endoscopic biopsy revealed diffuse villous atrophy, crypt hyperplasia, and intraepithelial lymphocytosis in duodenum (see Fig., Supplemental Digital Content 1, http://links.lww.com/IBD/A645). However, introduction of gluten-free diet did not induce remission of the disease, thus making this diagnosis highly unlikely. In addition, tissue transglutaminase antibodies were normal (0.4 U/mL; reference value <10 U/mL), and screening for HLA DQ2 and DQ8 was negative when assessed later in her life.

At the age of 6 years, she was diagnosed with autoimmune thyroiditis based on the presence of autoantibodies, and thyroid hormone therapy was initiated. Three years later, the index patient presented with type 1 diabetes mellitus. From 9 to 11 years of age, the patient remained clinically stable despite some episodes of diarrhea, which did not require hospitalization.

At the age of 11 years, the clinical course deteriorated with increasing diarrhea and a weight loss of 15 kg within a year. When she was 12 years old, another endoscopy was performed also showing diffuse villous atrophy, crypt hyperplasia, and intraepithelial lymphocytosis in duodenum. In addition, a colonoscopy revealed crypt epithelium injury and regenerative inflammation in the histology examination. Reanalysis of this biopsy at the age of 13 years showed T-cell mediated epithelium destruction based on autoinflammatory processes (Fig. 1C).

Those findings suggested autoimmune enteropathy or IBD. Autoimmune polyglandular syndrome, IPEX-like syndrome, and mitochondrial disease were excluded through Sanger sequencing of *AIRE*, *IL2RA*, *CD25*, *TYMP*, and *POLG* genes, respectively. Steroid therapy to treat the deterioration of the intestinal manifestations was started and resulted in clinical improvement. After 3 months, recurrence of intense diarrhea motivated the addition of cyclosporine A (CsA) to her therapy. This combined therapy induced partial improvement and was continued for 5 months.

At the age of 13 years, the patient presented with cachexia (21 kg, 130 cm), severe diarrhea, finger clubbing (Fig. 1D), and long-standing severe anal fissure and skin tags (Fig. 1E). The finger clubbing had not been previously noticed, whereas the anal fissures and skin tags had already been present for 2 to 3 years. It remains unclear whether the finger clubbing may have been related to therapy with CsA and/or the autoimmune thyroiditis (despite euthyroid state under therapy). Those clinical manifestations, together with the previously described colonoscopy results, suggested the diagnosis of IBD and prompted reinitiation of treatment with steroids and CsA.

After 30 days of combined therapy, the patient developed renal, respiratory and cardiac failure, bicytopenia, pleural effusion, pericardial effusion, ascites, splenomegaly, deranged coagulation tests, and direct hyperbilirubinemia. Additional laboratory investigations evidenced thrombotic microangiopathy and thrombocytopenia associated with multiple organ failure possibly associated with CsA treatment. In light of these severe reactions, the combined immunosuppressive therapy was immediately discontinued, with subsequent recovery of the patient. After this crisis, she showed lactate and ammonium elevations, pseudo-obstruction, and metabolic acidosis attacks intermittently. Magnetic resonance imaging revealed cerebral and cerebellar atrophy, whereas MR spectroscopy was normal. Electromyogram showed demyelinating polyneuropathy. Autoantibodies other than thyroid autoantibodies (ANA, AMA, ASMA, TTG IgA, ANCA, anti dsDNA, LKM) were negative.

The presence of the thrombocytopenia and anemia prompted bone marrow aspiration which showed no abnormalities, suggesting that the bicytopenia was caused by peripheral destruction. Liver biopsy revealed fibrosis in portal area, periportal fibrosis, perisinusoidal fibrosis, patchy cholestatic findings, and hemosiderosis (Fig. 2A).

The clinical picture continued to deteriorate with persistent diarrhea and lack of weight gain despite enteral and parenteral nutrition support. Additional invasive examinations could not be performed because of her poor clinical condition.

To exclude that her symptoms were caused by an underlying CVID, we performed extensive immunophenotyping. The patient, however, did not at any time presented with either recurrent/severe infections or serum reduction of specific immunoglobulin subtypes. B- and T-lymphocyte counts were also within normal range (Fig. 2B). The evaluation of specific subgroups of B lymphocytes revealed normal numbers of class-switched IgD^- and $CD27^+$ B cells (Fig. 2B). Interestingly, she presented with increased numbers of $CD21^{low}$ B cells.

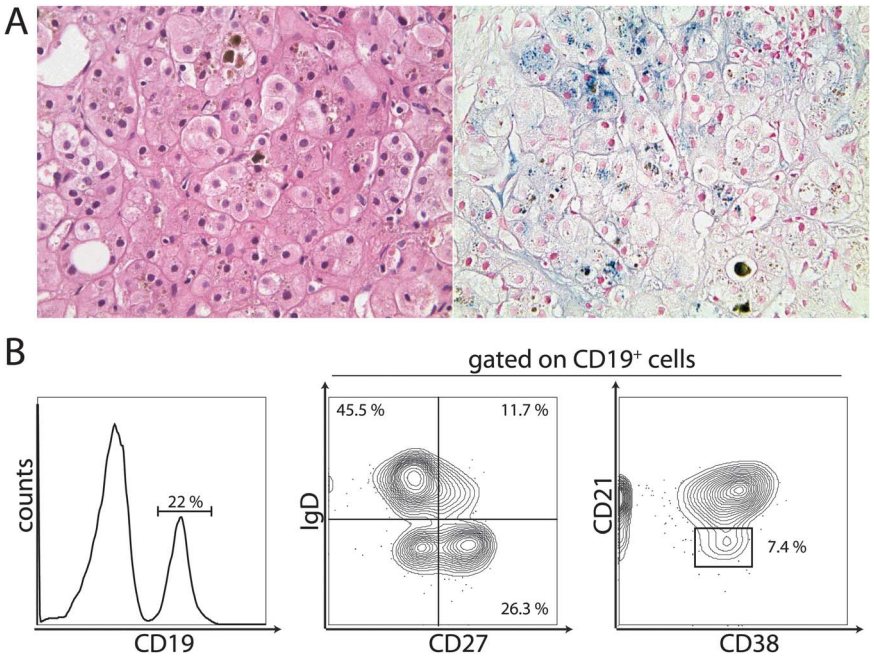

FIGURE 2. Histological and immunological analysis of the patient. A, (left panel) Cholectasis manifested as bile plugs in canaliculi and pseudo-acinar cholestatic changes in hepatocytes without inflammation or ductular reaction (×400 H&E stain). A, (right panel) Additional hemosiderin depositions are present in hepatocytes (×400 Prussian blue stain). B, The patient shows normal numbers of absolute and class-switched ($CD27^+$, IgD^-) B cells but elevated $CD21^{low}$ B cells.

Giving the early disease onset, a monogenetic cause for the disease was suspected. As the patient was born to consanguineous parents, we assumed an autosomal recessive mode of inheritance. Thus, we performed homozygosity mapping using Affymetrix 6.0 Genotyping SNP arrays. Calculations using homozygosity mapper[17] revealed 2 homozygous stretches with the maximal homozygosity score 1000 each on chromosomes 4 and 7 (Fig. 3A). The patient's DNA was subjected to exome sequencing which revealed a total of 43,772,399 reads that could be mapped uniquely to the genome (98.38% of total reads), resulting in a mean coverage of 19 reads per base. Exome sequencing revealed 5 variants in 4 genes (Table 1) fulfilling the criteria of novel nonsense, missense, or splice-site variants located inside the homozygous candidate intervals, among them 2 single nucleotide exchanges of neighboring nucleotides in the gene encoding LPS-responsive and beige-like anchor protein (LRBA; NP_006717.2). The variants affecting adjacent nucleotide positions (c.A8470C; c.T8471C) in LRBA lead to an amino acid exchange within the C-terminus of the protein (p.Ile2824Pro). Both variants were validated using conventional Sanger sequencing and showed perfect segregation under the assumption of autosomal-recessive inheritance with full penetrance (Fig. 3B). The mutated residue Ile-2824 is highly conserved throughout vertebrate evolution (Fig. 3C). Protein 3D structure modeling of the C-terminal region (amino acids 2073-2863) predicted the exchanged amino acid to be located in one of the 5 WD40 domains at

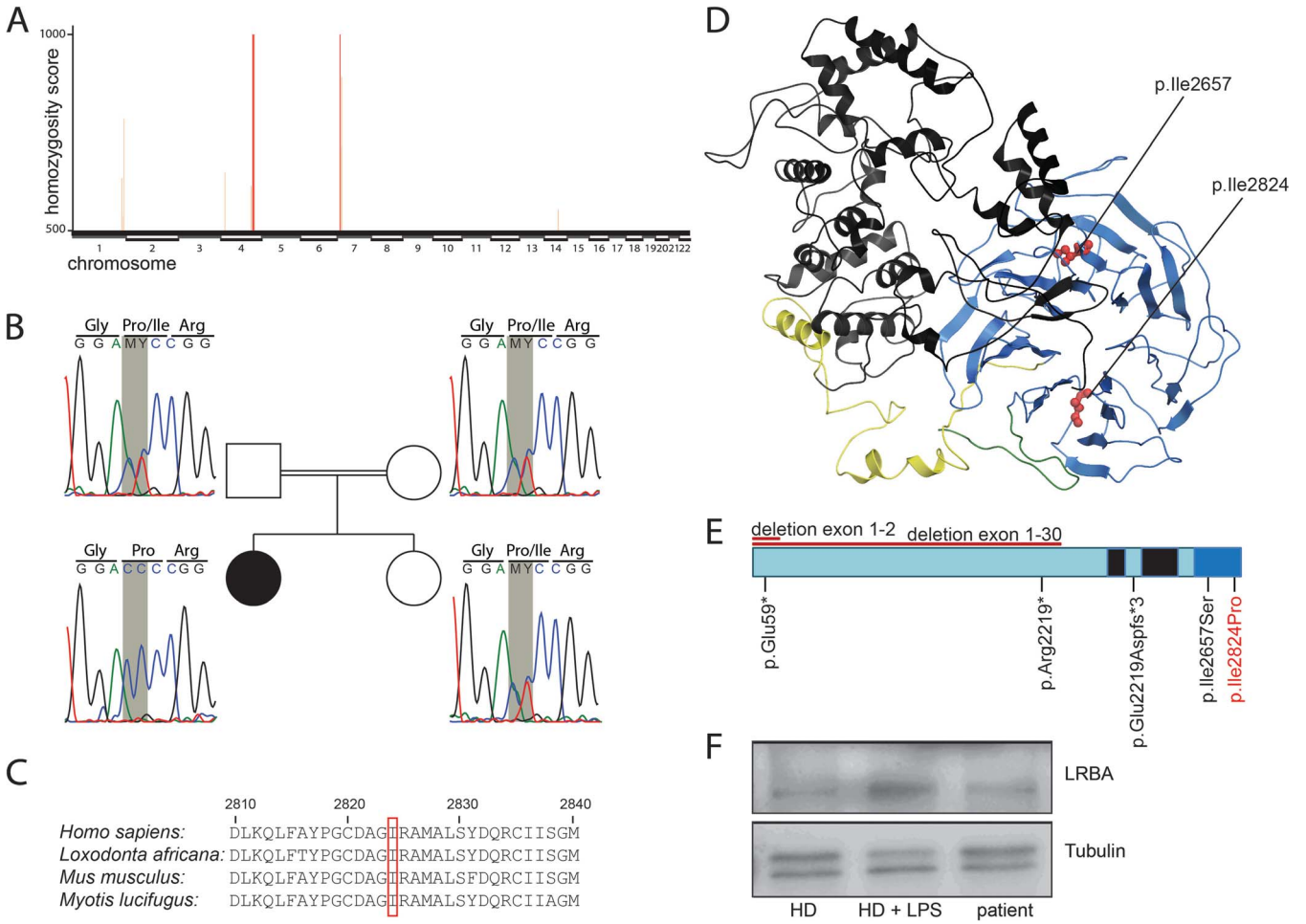

FIGURE 3. Identification of a homozygous *LRBA* mutation as underlying genetic defect of the index patient's phenotype. A, Two homozygous intervals with maximal homozygosity scores of 1000 (red) were identified in the patient. B, Inside the candidate intervals, a homozygous missense mutation in *LRBA* (c.A8470C; c.T8471C; p.Ile2824Pro) was identified, which could be validated by Sanger sequencing and segregated with the disease. C, The mutated residue is highly conserved throughout vertebrates. D, Protein 3D structure modeling of the BEACH (black) and WD40 (blue) domains revealed that the mutated amino acid of the index patient Ile-2824 is located inside the second last β-sheet of the WD40 domain β-propeller whereas the previously published mutated amino acid Ile-2857 is located in close proximity to the BEACH domain structure (yellow: Linker between BEACH and WD40). E, All published mutations (black annotation) lead to an absence of the protein product. The index patient mutation is annotated in red (black: BEACH domain; dark blue: WD40 domain). F, The mutation p.Ile2824Pro allows protein expression at similar levels as the healthy donor.

TABLE 1. Identification of 5 Variants Within the Homozygous Intervals

Gene	Function	Nucleotide	Amino Acid	Chromosome	Position	Reference	Observed
ABCE1	SNV	c.C727G	p.P243A	4	146033407	C	G
LRBA	SNV	c.T8471C	p.I2824T	4	151199035	A	G
LRBA	SNV	c.A8470C	p.I2824L	4	151199036	T	G
SCIN	SNV	c.A274T	p.I92F	7	12666242	A	T
DNAH11	SNV	c.A3200G	p.H1067R	7	21640493	A	G

the C-terminus (Fig. 3D, E). Polyphen-2 calculations predicted the mutation as probably damaging with a score of 0.985 (maximum 1). Immunoblot analysis showed that the mutation in LRBA allowed for protein expression at a similar level as in a healthy control (Fig. 3F).

DISCUSSION

Recently identified monogenic forms of IBD such as IL-10 (receptor) deficiency,[7,8] XIAP deficiency,[10] TTC7A deficiency,[11] and ADAM17 deficiency[9] are associated with very early and severe onset of the disease. These patients often do not respond well to conventional therapy (reviewed in Ref. 6).

Here, we describe a female patient whose main symptom was very early-onset and treatment-resistant nonmucoid, nonbloody diarrhea. She developed signs of autoimmunity, such as thyroiditis, at the age of 6 and diabetes mellitus type 1 at the age of 9 years. Histological analysis of duodenal biopsies revealed T-cell mediated epithelial destruction, thus enabling a diagnosis of IBD at the age of 13 years. Retrospectively, the diagnosis of early-onset IBD could be considered if early supporting evidence of intestinal inflammation had been available. However, as she was under treatment at a rural hospital until the age of 13, such evidence was not available.

Exome sequencing covering more than 98% of coding genomic region revealed no variants or mutations with minor allele frequency of less than 1% in the *IL10*, *IL10RA*, *IL10RB*, *XIAP*, *ADAM17*, or *TTC7A* genes, respectively. Further genes related to polyglandular autoimmune syndrome or other diseases that might explain the phenotype of the patient were also not uncovered by combined homozygosity mapping and exome sequencing. Heterozygous mutations were excluded from the analysis as they would lead to symptoms in other variant-bearing family members. Homozygous missense variants in the 4 genes, *ABCE1*, *LRBA*, *SCIN*, and *DNAH11*, were identified (Table 1). *ABCE1* encodes the protein adenosine triphosphate-binding cassette subfamily E member 1, which is a negative regulator of RNAse L.[23] *SCIN* encodes the protein adseverin, which is an actin capping and serving protein.[24] Deleterious mutations in *DNAH11* are causative for situs inversus totalis.[25] Regarding the index patient, none of these 3 genes can be easily linked to her phenotype. Mutations in the fourth gene *LRBA* have been recently identified as a cause of a CVID associated with autoimmunity and IBD-like disease,[26–28] which prompted us to further investigations.

LRBA was first identified as a lipopolysaccharide responsive gene in B cells and macrophages whose protein structure is similar to the lysosomal-trafficking regulator LYST.[29] Both LYST and LRBA belong to the group of BEACH domain-containing proteins, which consists of 9 human proteins (reviewed in Ref. 30). Apart from mutations in *LRBA*, 3 other BEACH domain-containing genes have been implicated in autosomal recessive Mendelian disorders. Homozygous *LYST* mutations lead to Chediak–Higashi syndrome[31]; homozygous *Neurobeachin-like 2* mutations result in gray platelet syndrome[32–34]; and biallelic *WD repeat domain 81* mutations result in a cerebellar ataxia, mental retardation, and dysequilibrium syndrome.[35] Two of these Mendelian disorders, namely Chediak–Higashi syndrome and LRBA deficiency, result in reduced immune functions.

The functional role of BEACH domain-containing proteins remains elusive. It has been speculated that these proteins are involved in membrane dynamics and vesicular transport (reviewed in Ref. 30). LRBA has been shown to colocalize with lysosomes, ER, and the Golgi complex, respectively.[29] Furthermore, it has been implicated as a negative regulator of apoptosis as it is overexpressed in several cancers.[36] This is in concordance with the increased apoptosis in B cells, which has been described in LRBA-deficient patients.[27] Elevated cell death might be due to defective autophagy.[27] Interestingly, our patient did not show reduced numbers of B cells (Fig. 2B). Whether autophagy is affected in the index patient could not be determined.

So far, only a limited number of patients with LRBA deficiency have been published.[26–28] Interestingly, all previously described patients bear mutations that lead to absence of protein expression.[26–28] One of the identified patients presented with a missense mutation in *LRBA* located inside the WD40 domains (p.Ile2657Ser) similar to the index patient (p.Ile2824Pro).[27] The reason why the mutation p.Ile2657Ser results in an absent protein in contrast to the mutation p.Ile2824Pro might be explained by the different location of the amino acids in the protein. Protein 3D structure modeling of the BEACH-WD40 domain of LRBA revealed that the amino acid Ile-2657 is located in close proximity to the BEACH domain (Fig. 3D), which might potentially be crucial for protein stability. However, the amino acid Ile-2824 is located between the last 2 β-sheets of the WD40 β-propeller (Fig. 3D) and does not

significantly influence the stability of the protein, as it is still detectable (Fig. 3F). Because there is currently no simple test for intact function of LRBA protein, we cannot formally assess whether the mutation described here allows for residual protein function.

LRBA-deficient patients present with heterogeneity of clinical symptoms with no clear genotype to phenotype correlation. Common features of LRBA-deficient patients are quantitative and/or qualitative B-cell defects as well as autoimmunity.[26–28] Nine of 11 published patients to date present with autoimmune IBD-like manifestations.[26,27] Another common feature is recurrence of pulmonary infections. The patient described here differs from those previously described because her leading symptom was a potential IBD-like disease, starting at the age of 6 months and diagnosed as IBD when she was 13 years old. Further autoimmune features only manifested later in her life.

B-cell phenotyping at the age of 14 years revealed increased numbers of $CD21^{low}$ B cells. This subgroup of B cells has been associated with autoimmunity in patients suffering from CVID[37] but has not been reported for LRBA deficiency to date. Overall B-cell numbers and numbers of class-switched B cells were not affected in the index patient, and she did not show any pulmonary complications. Also in contrast to other LRBA-deficient patients,[26,27] the index patient does not fulfill the formal criteria for a CVID diagnosis.[38,39]

To our knowledge, this is the first LRBA-mutant patient presenting exclusively with gastrointestinal symptoms in the first few months of life. Only one previously reported patient bears some similarities to the patient described here as he also initially showed nonbloody diarrhea.[26] The patient however presented in addition with autoimmunity and Epstein–Barr virus-associated lymphoproliferative disease very early in his life.[26] We speculate that the differences in phenotype might be due to a hypomorphic nature of the variant in LRBA, as the missense mutation identified may allow for residual function of the corresponding gene product, although we cannot formally rule out the effects of the other variants found and of hidden intronic variants, which are not covered by exome sequencing.

Taken together, we describe for the first time a patient with a missense mutation in *LRBA* allowing for detectable protein expression (and potentially residual function) presenting exclusively with gastrointestinal manifestations at very young age, later diagnosed as IBD. LRBA deficiency thus represents an important molecular differential diagnosis for severe persisting IBD-like disease and related conditions.

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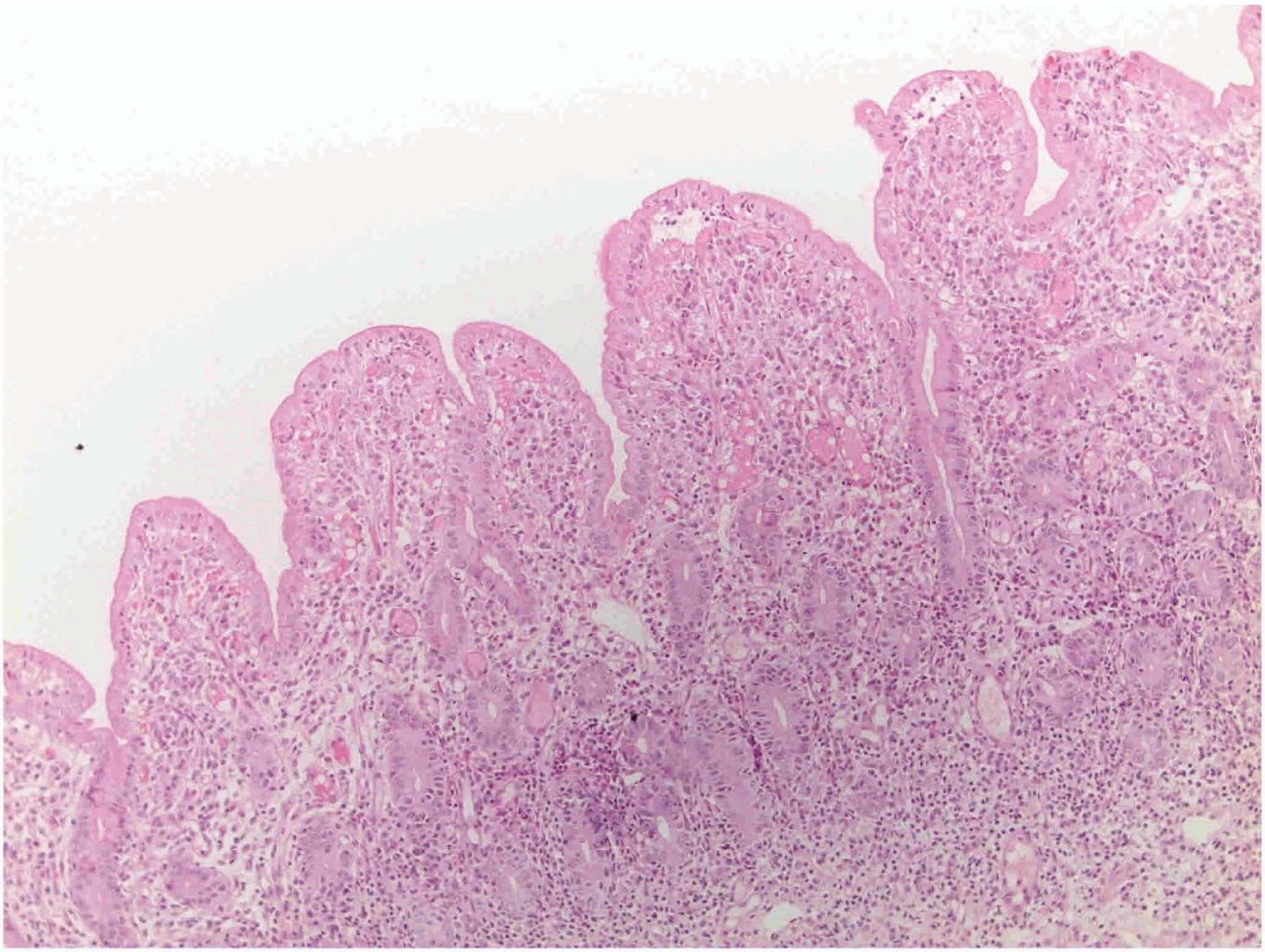

Supplementary Figure 1 - Endoscopy at the age of 18 months revealed diffuse villous atrophy and crypt hyperplasia together with intraepithelial lymphocytosis

2.3. The critical role of CTLA-4 in the prevention of autoimmunity

As previously outlined in the introduction, the protein CTLA-4 plays a central role in the prevention of autoimmune diseases. CTLA-4 haploinsufficiency has been described to cause early-onset autoimmunity (Kuehn et al, 2014; Schubert et al, 2014). Moreover, trafficking defects affecting the recycling of CTLA-4 have been shown to result in severe and early-onset autoimmunity (Lo et al, 2015). The included submitted manuscript in chapter 2.4. describes a family with two patients who suffer from severe early-onset autoimmunity. Genetic investigations of the two patients and the extended pedigree revealed a missense mutation in the gene *DEF6* (CCDS4802, c.G991A, p.E331K) which segregated perfectly with the disease. DEF6 is a guanine nucleotide exchange factor for the small GTPases RHOA, RAC1 and CDC42 (Becart & Altman, 2009). Alternative names of DEF6 are IRF4 binding protein (IBP) (Gupta et al, 2003b) or SWAP-70-like adaptor of T cells (SLAT) (Tanaka et al, 2003). The role of Def6 in mice remain controversial as *Def6*$^{-/-}$ mice, depending on their genetic background, are either protected from autoimmune disease (Canonigo-Balancio et al, 2009; Vistica et al, 2012), or develop fatal systemic autoimmunity (Chen et al, 2008; Fanzo et al, 2006). The submitted work included in chapter 2.4. set out to understand the role of DEF6 in human autoimmune disease. The assessment of the cellular and clinical phenotype revealed an absence of soluble CTLA-4 in the serum of one patient. Thus, the expression and trafficking of CTLA-4 were investigated which showed a substantial defect. The trafficking defect was amenable to rescue upon ectopic expression of wildtype DEF6. The mechanistic link between DEF6 and CTLA-4 was established through the identification of the interaction and co-localization of DEF6 with the small GTPase RAB11 which is important for CTLA-4 trafficking and recycling. Mutated DEF6 was unable to interact with RAB11. Ultimately, the severe autoimmune phenotype of one patient could be rescued by administration of CTLA-4-Ig (Serwas et al, submitted).

2.4. Severe systemic autoimmunity caused by germline mutations affecting DEF6, a novel CTLA-4 regulator

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Severe systemic autoimmunity caused by germline mutations affecting DEF6, a novel CTLA-4 regulator

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ABSTRACT

Studying monogenetic disorders with predominant autoimmunity may unravel key regulators of immune homeostasis. We identified a novel primary immunodeficiency with life-threatening multi-organ autoimmunity caused by biallelic germline mutations in *differentially expressed in FDCP6 homolog (DEF6)*. Mutant DEF6 exhibited reduced interaction with interferon regulatory factor 4 (IRF4) and subsequent reduction of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) expression in conventional T cells. Furthermore, CTLA-4 trafficking was reduced and amenable to rescue upon ectopic DEF6 expression. We identified the small GTPase RAB11, previously implicated in recycling of CTLA-4, as a novel interactor of DEF6, and observed that this interaction was abrogated by the DEF6 mutation. Consistent with these observations, treatment with CTLA-4-Ig enabled sustained disease remission. Our findings elucidate an essential role of DEF6 in prevention of autoimmunity by regulating the function of the checkpoint protein CTLA-4, identifying a potential target for autoimmune and/or cancer therapy.

Though immune dysregulation and autoimmunity are hallmarks of multiple human diseases, underlying molecular pathological mechanisms are still not fully understood[1]. Studying monogenetic disorders with predominant autoimmunity offers an attractive strategy to identify core regulators of immune homeostasis[2].

Some key regulatory units have been identified which help to adjust the immune response, such as regulatory T cells (T_{regs})[3], and the checkpoint protein CTLA-4[4,5]. CTLA-4 competes with CD28, a costimulatory molecule expressed by T cells, for interaction with their shared ligands CD80/CD86 on antigen presenting cells (APCs), and is therefore one of the key proteins needed to inhibit unwanted T-cell responses[6]. Binding of CTLA-4 to CD80/CD86 results in transendocytosis of both molecules into the T_{reg} and sequesters the costimulatory ligand from the APC[7]. The interaction of CTLA-4 with Lipopolysaccharide-responsive and beige-like anchor protein (LRBA) is critical for the prevention of CTLA-4 degradation and the initiation of CTLA-4 recycling[8]. CTLA-4 trafficking and recycling is regulated by the small GTPase RAB11, as endocytosed CTLA-4 resides in $RAB11^+$ recycling endosomes which are subsequently exocytosed[9]. While the importance of CTLA-4 in regulation of immune homeostasis has been suggested by several SNPs conferring increased risk of autoimmunity[10], the recent identification of humans with heterozygous deleterious mutations in *CTLA4* leading to CTLA-4 haploinsufficiency and the identification of LRBA-deficient patients with severe autoimmunity has highlighted the importance of this regulatory system[4,5,11-13].

DEF6, also known as IRF4 binding protein (IBP)[14] or SWAP-70-like adaptor of T cells (SLAT)[15], is a guanine nucleotide exchange factor (GEF) which, together with its homolog switch-associated protein 70 (SWAP70) represent a unique GEF subfamily in which the PH domain is N-terminal to the DH domain, contrary to “conventional” GEFs that display the opposite arrangement of these two domains[14-16]. DEF6 acts downstream of the T-cell receptor (TCR) and can be phosphorylated by the tyrosine-protein kinases LCK[16] and ITK[17]. It targets the small GTPases CDC42, RAC1 and RHOA[16], mediating Ca^{2+}-influx and NFAT1 activation[18]. Apart from its GEF function, DEF6 interacts with the transcription factor IRF4 and is important for its function[19,20]. Recently, DEF6 was also described as an effector for the small GTPase RAP1 and its role in adhesion[21]. Studies in Def6-deficient (*Def6*$^{-/-}$) mice have shown its implication in various immune processes including immunological synapse formation[22], Th1/Th2 lineage differentiation[18], IL17 and IL21 production[20], phagocytosis[23], T-cell proliferation[24], and Ca^{2+} flux[25]. Yet, it is still controversial which of these effects are primary or secondary. The role of DEF6 in autoimmunity has remained elusive. *Def6*$^{-/-}$ mice on C57BL/6 background are resistant to experimental autoimmune encephalitis[26] and uveitis[27]. However, *Def6*$^{-/-}$ mice on a BALB/c

background crossed to DO11.10 TCR transgenic mice develop early-onset large vessel vasculitis[20], and *Def6*$^{-/-}$ mice on mixed 129xC57BL/6 background develop fatal systemic autoimmunity[22].

We identified a novel primary immunodeficiency comprising early-onset systemic autoimmunity and CD8-cell lymphopenia caused by biallelic mutations in the gene encoding DEF6, offering a unique opportunity to dissect the role of this molecule for human autoimmunity. Mechanistically, we identify the small GTPase RAB11, which is involved in CTLA-4 recycling, as a novel interactor of DEF6. Our results illustrate that germline mutations in *DEF6* result in reduction of CTLA-4 trafficking in conventional T cells. Thus, we identify a previously unknown role of DEF6 in the biology of CTLA-4, a master regulator of immune homeostasis[4,5,28].

RESULTS

Severe autoimmunity and absence of serum CTLA-4

We studied an index patient (P1) born to consanguineous Pakistani parents (**Fig. 1a**) who presented with severe watery diarrhea in the first month of life. Endoscopy revealed atrophy of gastric mucosa with numerous apoptotic cells (**Fig. 1b**). Complete villous atrophy and pronounced T- and eosinophilic cell infiltration were observed in the small intestine (**Fig. 1c** and **Supplementary Fig. 1a**). Further disease features included hepatosplenomegaly (**Fig. 1d**), dilated cardiomyopathy (**Fig. 1e**), and increased susceptibility to viral and bacterial infections suggesting PID (**Supplementary Information**). Immune phenotyping revealed reduced $CD8^+$ T cells (**Fig. 1f**) and slightly reduced $CD25^{high}CD127I^{ow}FoxP3^+$ regulatory T-cell (T_{reg}) numbers (**Fig. 1g** and **Supplementary Table 1**), T-cell receptor Vβ spectratyping revealed no oligoclonal restriction (**Supplementary Fig. 1b**). T-cell proliferation upon stimulation with various mitogens (PHA, PMA, anti-CD3) was intact (data not shown). Immunoglobulin levels were in the lower normal range (**Supplementary Table 2**), only few $CD19^+CD27^+IgD^-$ class-switched B cells were detected (**Supplementary Fig. 1c**), and specific antibody responses were reduced (**Supplementary Table 3**). Clinical signs of autoimmunity were paralleled by detectable anti-neutrophil cytoplasmic antibodies (ANCA) and autoantibodies against cardiolipin, smooth muscle protein and β2-glycoprotein (**Supplementary Table 4**). Patient-derived NK cells were unable to form organized immunological synapses (**Fig. 1h**), accompanied by significantly increased microtubule organization center (MTOC) to granule distances (**Supplementary Fig. 1d**), reduced actin density at contact sites (**Supplementary Fig. 1e**), and defective granule

exocytosis (**Fig. 1i**). Notably, MTOC to synapse distance was unaltered (**Supplementary Fig. 1f**). No systemic increase of pro-inflammatory cytokines (**Supplementary Fig. 1g**) but absence of soluble CTLA-4 and CCL3 was observed (**Fig. 1j**). Family history was remarkable for a sibling (P2) with an undefined autoimmune/autoinflammatory disorder associating bowel inflammation, hepatomegaly, cholestasis, and cardiac ventricular septal defect. Immunological investigations had not been performed in similar depth, but P2 had recurrent infections (**Supplementary Information**), exhibited reduced numbers of $CD8^+$ T cells (**Fig. 1f** and **Supplementary Table 1**), and died at 10.5 months of age due to multi-organ failure.

Homozygous germline mutations in *DEF6*

To identify the underlying molecular disease etiology, we combined exome sequencing and homozygosity mapping (**Supplementary Fig. 2, Supplementary Table 5** and **Supplementary Information**) and identified a homozygous missense variant in *DEF6* (CCDS4802, c.G991A, p.E331K) affecting the highly conserved PH-DH domain (**Fig. 2 a,b**). The variant was predicted damaging by Polyphen, SIFT and CADD (**Supplementary Table 6**). According to the ExAC database[29] no loss-of-function mutations in *DEF6* have been described in homozygous state yet. Furthermore, expected-to-observed mutation ratio for *DEF6* was reasonably high (Z-score: 3.48). Probability of loss-of-function intolerance (pLI) was calculated as 0.98[29]. Thus, we considered the variant $DEF6^{E331K}$ as the potential cause for the disease phenotype. We found that the mutated amino acid in $DEF6^{E331K}$ had no effect on protein expression (**Fig. 2c**), however, GEF activity of $DEF6^{E331K}$ towards its known target CDC42[19,30,31] was 50 % reduced (**Fig. 2 d,e**). This is in line with defective NK-cell synapse formation (**Fig. 1 h,i** and **Supplementary Fig. 1 d,e**), a process critically dependent on CDC42 function[32,33].

$DEF6^{E331K}$ reduces CTLA-4 transcription

DEF6 has been described to regulate the activity of the transcription factor IRF4[20]. We aimed to investigate whether $DEF6^{E331K}$ is still able to interact with IRF4. Thus, we overexpressed both proteins and pulled down mutated and wildtype DEF6 through a GFP-tag. We could show that mutated DEF6 interacted with IRF4 to a much lesser extent than its wildtype counterpart (**Fig. 3a**). Given the lack of CTLA-4 in P1's serum (**Fig. 1j**), which is one of the target genes of IRF4[34], we assumed that IRF4 is not able to function properly as a transcription factor. To investigate the impact of DEF6 on *CTLA4* transcription, peripheral blood mononuclear cells (PBMCs) were stimulated with anti-CD3/CD28 antibody-coated beads for 16 hours. Upregulation of *CTLA4* mRNA was markedly higher in healthy control than patient cells (19-vs.

6-fold, **Fig. 3b**). Comparable results were seen in sorted naïve $CD4^+$ T cells (**Fig. 3c**). The defect in *CTLA4* mRNA expression was not observed when cells were stimulated with phorbol-12-myristate-13-acetate (PMA) and ionomycin (**Fig. 3d**), which is in concordance with the literature on the circumvention of *Def6*$^{-/-}$ in mice[20,22]. Flow cytometry after 22 hours of stimulation confirmed the qPCR results at the protein level (**Fig. 3e**). The comparable shedding of the early activation marker CD62L and in-depth analysis of short-term (3 days) proliferative capacities of patient T cells excluded a general delay in T-cell activation (**Fig. 3e** and **Supplementary Fig. 3a**). However, 48 hours after stimulation, the difference between patient and healthy control CTLA-4 protein levels was reduced and no longer statisctically significant, suggesting a compensatory mechanism, potentially reflecting the strong stimulus used (**Fig. 3 f,g**). Additionally, T-cell proliferation upon 6-day strong stimulation with anti-CD3/CD28 antibodies revealed reduced proliferative capacity (**Supplementary Fig. 3b**).

DEF6^{E331K} affects CTLA-4 trafficking

Although our data revealed complete absence of soluble CTLA-4 in the serum of P2 (**Fig. 1j**), both mRNA and protein expression levels of CTLA-4 were markedly reduced but not absent after stimulation (**Fig. 3**). Analysis of the spatial distribution of CTLA-4 and DEF6 in stimulated T cells revealed a concentration of DEF6 on the sites of CTLA-4 expression (**Supplementary Fig. 4a**), suggesting that DEF6 may affect CTLA-4 availability through mechanisms such as trafficking and recycling. To test this, we performed a CTLA-4 trafficking assay as previously described[8] involving stimulation of PBMCs with PMA/ionomycin for 1 hour to induce mobilization of CTLA-4 containing vesicles to the cell surface. We observed a significant reduction of CTLA-4 trafficking in DEF6-mutant $CD4^+$ T cells (**Fig. 4 a,b**). In parallel, we did not observe an overt difference in total CTLA-4 levels in $CD4^+$ T cells (**Fig. 4c**), possibly reflecting the short stimulation period (in contrast to the expression defect observed in cells stimulated for 22 hours; **Fig. 3e**). Somewhat surprisingly, CTLA-4 trafficking in $FoxP3^+$ T_{regs} was comparable in healthy control and patient cells (**Fig. 4 d,e**). A small, but significant reduction of total CTLA-4 was detected in patient T_{regs} (**Fig. 4f**). However, *ex vivo* suppression assays revealed no difference in suppressive capabilities of T_{regs} (**Supplementary Fig. 4 b,c**), reminiscent of findings in *Def6*$^{-/-}$ mice[20,26]. To confirm the importance of DEF6 in regulating CTLA-4 trafficking, we used transfection to reconstitute PBMCs from P1 with either wild-type DEF6 or mutated DEF6^{E331K}-GFP fusion protein. The observed trafficking defect of CTLA-4 in $CD4^+$ T cells was reversed by DEF6 but not by DEF6^{E331K} (**Fig. 4 g,h** and **Supplementary Fig. 4 d,e**). Together, these results demonstrate that DEF6 plays a role in promoting cell surface trafficking of CTLA-4 in

conventional $CD4^+$ T cells but not in T_{regs} and, moreover, that CTLA-4 trafficking to the cell surface is impaired in *DEF6*-mutated cells.

RAB11 is a novel interactor of DEF6

Given our finding that DEF6 regulates trafficking of CTLA-4 to the cell surface, we hypothesized that DEF6 interacts with the small GTPase RAB11, which has been linked to CTLA-4 trafficking[9]. Indeed, we found that DEF6 and RAB11 partially co-localize when expressed in transfected HEK293T cells (**Supplementary Fig. 5a**). Next, we imaged simultaneously the localization of endogenous DEF6, RAB11 and CTLA-4 in normal control and patient cells stimulated for 48 hours with anti-CD3 plus anti-CD28 antibodies and performed line scans to visualize the degree of co-localization (**Fig. 5 a, b**). In line with a previous study[9], we observed prominent co-localization of CTLA-4 with RAB11 (a marker of recycling endosomes) in stimulated patient cells; in contrast, such co-localization was largely absent in the patient cells (**Fig. 5 a,b**). To quantify RAB11 and CTLA-4 co-localization in stimulated PBMCs, defined regions of interest with the highest CTLA-4 expression (**Fig. 5c**) were analyzed using the JaCoP tool[35] (**Fig. 5d**). Quantification revealed a significant reduction of the Pearson's coefficient in the patient cells from 0.5 to 0.3 (**Fig. 5d**), suggesting an impact of the *DEF6* mutation on the fusion of $CTLA\text{-}4^+$ and $RAB11^+$ vesicles. We further imaged the relative localization of endogenous DEF6 and RAB11 in unstimulated or stimulated healthy control and patient PBMCs (**Supplementary Fig. 5 b,c),** and found that co-localization was comparable in patient and healthy control cells and independent of whether PBMCs were rested or activated for 48 hours through the TCR (**Supplementary Fig. 5 b-d**), suggesting no or only mild impact of $DEF6^{E331K}$ on RAB11 localization.

To validate the importance of DEF6-RAB11 co-localization, we performed a co-immunoprecipitation assay. Indeed, GFP-tagged RAB11 and MYC-tagged wildtype DEF6 could be co-immunoprecipitated (**Fig. 5d**). By contrast, $DEF6^{E331K}$ was unable to interact with RAB11 (**Fig. 5d**) despite similar localization. This lack of interaction is consistent with the idea that the mutation may result in defective DEF6-GEF activity leading to reduced recycling of CTLA-4, and that DEF6 might possess GEF activity towards RAB11. Indeed, consistent with the fact that GEFs for small G proteins interact preferentially with the dominant negative (GDP-bound) form of their target G proteins[36], we found that DEF6 interacted strongly with dominant negative (S25N) RAB11, but very weakly (if at all) with constitutively active (Q70L) RAB11 (**Fig. 5e**).

Collectively, our data reveal a previously unknown co-localization and interaction of DEF6 with the small GTPase RAB11, which is required for trafficking of CTLA-4.

Treatment of DEF6-mutated patient with CTLA-4 replacement therapy

The severe autoimmunity associated with CTLA-4 haploinsufficiency results from a 50% reduction of CTLA-4 expression[4,5]. LRBA-deficient patients with reduced CTLA-4 surface expression exhibit a dramatic improvement in response to treatment with CTLA4-Ig[8]. Since the *DEF6*-E331K mutation was associated with reduced CTLA-4 expression (~2-3-fold) and trafficking (~2-fold) in conventional T cells, we assumed that CTLA-4-Ig treatment may ameliorate autoimmunity in P1. Thus, upon clinical deterioration, P1 received CTLA4-Ig at 4 weekly intervals starting at 15 months of age (**Supplementary Fig. 6a**). Subsequently, bowel inflammation decreased markedly as reflected by fecal calprotectin values (**Fig. 6a**), furthermore, persisting perianal lesions recovered and did not recur (**Supplementary Fig. 6b**). Lymphocytic infiltration and incomplete villous atrophy of the duodenum improved within 1 month of treatment (**Fig. 6 b,c**). P1 was consequently discharged and treated as an out-patient (**Supplementary Fig. 6a**). To date, ~2.5 years after treatment initiation, no overt signs of autoimmunity have reoccurred. However, recurrent infections requiring antibiotic treatment have persisted (**Supplementary Fig. 6a**), further underlining the role of DEF6 in immune defense. Nevertheless, treatment of functional DEF6 deficiency with CTLA-4-Ig improved the health status of P1 and possibly prevented fatal outcome of the disease which had been seen in P2, thus confirming the key role of DEF6 in regulating CTLA-4.

DISCUSSION

The role of Def6 in autoimmunity in mouse models has been controversial. *Def6*$^{-/-}$ mice on C57BL/6 background are resistant to experimental autoimmune encephalitis[26] and uveitis[27]. However, *Def6*$^{-/-}$ mice on a BALB/c background crossed to DO11.10 TCR transgenic mice develop early-onset large vessel vasculitis[20], and *Def6*$^{-/-}$ mice on mixed 129xC57BL/6 background develop fatal systemic autoimmunity[22]. In humans, the intronic SNP rs10807150 in *DEF6* altering gene expression has been associated with the onset of systemic lupus erythematosus[37]. Strikingly, the patients described here presented with severe autoimmune symptoms fatal in one patient, illustrating a critical role for DEF6 in preventing autoimmunity in

humans. This role involved DEF6-mediated regulation of CTLA-4 at two independent levels, *i.e*. its transcription and trafficking in conventional but not regulatory T cells, suggesting that regulatory mechanisms of CTLA-4 expression and trafficking may be distinct in these cell types. The apparent lack of effect of the *DEF6* mutation on T_{regs}[5,8] may be explained by the finding that the only DEF6 homolog, SWAP70, is expressed in regulatory but not conventional T cells[38]; thus, SWAP70 might compensate for the loss of DEF6 function in T_{regs}. A substantial role for CTLA-4 in conventional T cells has already been proposed previously. For example, mice with a T_{reg}-specific *Ctla4* deletion have a significantly prolonged lifespan in comparison to germline *Ctla4*$^{-/-}$ mice[39,40] suggesting an additional level of importance for CTLA-4 in conventional T cells. Mice with a specific deletion of *Ctla4* in conventional T cells have not been investigated so far. However, reduction of CTLA-4 protein levels in mice results in organ T-cell infiltration despite normal T_{reg} function[40,41]. Future studies are needed to further substantiate the importance of CTLA-4 in conventional T cells and discriminate the different pathways involved in T_{regs} versus conventional T cells.

Our data reveal that DEF6 co-localizes and interacts with the small GTPase RAB11, which has important roles in CTLA-4 trafficking and recycling[9]. Additionally, deficiency in LRBA has been identified as disease-causing for sever autoimmunity in PID patients[8,11-13]. LRBA has been shown to interact with CTLA-4 and prevent its degradation[8]. LRBA-deficient patients show partial phenotypic resemblance to our two index patients, although LRBA deficiency is arguably milder (**Supplementary Information**). Our results reveal a novel and yet unexplored layer of regulation in CTLA-4 biology, which may involve activation of the small GTPase RAB11 by DEF6, a GEF that functions downstream of the TCR[16]. This further supports the notion that CTLA-4 transcription and regulation has to be tightly regulated to prevent autoimmune or malignant disease[4,5,8,28].

The work presented herein thus underlines the power of identifying novel genetic causes for PIDs as a way to uncover novel immunoregulatory pathways[2]. Given the novel role of DEF6 as a regulator of the availability of the immune checkpoint protein CTLA-4, future studies should address whether DEF6 and related proteins are amenable to manipulation for targeted therapeutic intervention in immune-mediated disorders or potentially even in cancer immunotherapy.

METHODS

Patients and ethics. This study has been approved by the ethics committee at the Medical University of Vienna (MedUni Vienna), Austria (study number EK499/2011). Both patients P1 and P2 were evaluated, followed up and treated at the Children's hospital of the MedUni Vienna. The CD21-deficient patient was previously published[42]. Clinical routine investigations on P1 and P2, such as endoscopy, staining and evaluation of biopsies, ultrasound and computed tomography scan investigations, vaccination response analysis, autoantibody detection and autopsy were performed at different departments of the MedUni Vienna. Biological material of patients and healthy donors (HD) was obtained on informed consent in accordance with the Declaration of Helsinki.

Flow cytometry-based immunophenotyping and cell sorting. Immunophenotyping was performed on a BD LSRFortessa^{TM} or BD FACSCanto^{TM}II. Peripheral blood mononuclear cells (PBMCs) were isolated from patients or HD blood with a Ficoll gradient and were either used fresh or cryo-preserved in liquid nitrogen. Staining of surface antigens was performed after blocking in FBS-containing medium for 30 minutes at 4°C, intracellular antigens were stained applying the fixation/permeabilization kit for intracellular antigens or transcription factors (Affymetrix, eBioscience). All analyses were performed using FlowJo X (TreeStar Inc.) and Prism 6.0 (GraphPad Software). Analysis of NK cells degranulation was performed as described previously[43]. In brief, $1x10^6$ PBMCs were resuspended in 1 ml of RPMI+10% FCS and stimulated in a humidified CO_2 incubator with 10 ng/ml phorbol myristate acetate (PMA) and 1 µg/ml ionomycin in the presence of 10 µg/ml Brefeldin A for 4 hrs at 37 degree. An antibody against CD107a was present during the whole time of the incubation[44]. Cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences). Flow cytometry-based sorting of regulatory ($CD3^+CD4^+CD25^+CD127^{low}$) and responder ($CD3^+CD4^+CD25^-CD127^{high}$) T cells for the analysis of regulatory T cell function was performed on a BD FACSAria^{TM}. Magnetic microbeads based sorting of naïve $CD4^+$ T cells was done by negative depletion applying the Magnisort kit (eBioscience) according to manufacturer's instructions.The following antibodies were used for FACS: From Beckman Coulter: CD14-PECy7 (clone RMO52), CD16-FITC (clone 3G8), CD19-PECy7 (clone J3-119),CD3-FITC (clone UCHT1), CD3-PC5.5 (clone UCHT1), CD4-PE (clone 13B8.2), CD4-PECy7 (clone SFCI12T4D11), CD45RA (clone ALB11), CD45RO (clone UCHL1), CD56-PE (clone N901), CD56-PECy5 (clone N901), CD8-PECy7 (clone SFCI21), TCRvα24-PC-7 (clone C15), TCRvβ11-FITC (clone C21); from eBisocience: CD152-PE (clone 14D3), CD19-PerCPCy5.5 (clone HIB19), CD28-APC (clone CD28.2), CD3-APC (clone SK7), CD3-APC (clone UCHT1), CD4-eFluor450 (clone RPA-T4), CD4-PerCPCy5.5 (clone RPA-T4), CD62L-FITC (clone DREG56), CD69-APC (clone FN50), FOXP3-FITC (clone

PCH101), IgE-APC (clone Ige21), IgG-PE (polyclonal); from BD Biosciences: CD16-PECy7 (clone 3G8), CD19-PECy7 (clone SJ25C1), clone CCR7-PE-CF594 (clone 150503), CD20-FITC (clone 2H7), CD21-PE (clone B-ly4), CD25-PE (clone M-A251), CD27-BV (clone M-T271), CD27-PE (clone M-T271), CD27-PECy7 (clone M-T271), CD27-V450 (clone M-T271), CD3-APC-H7 (clone SK7), CD38-PECy7 (clone HIT2), CD4-APC (clone RPA-T4), CD4-BV421 (clone RPA-T4), CD4-BV605 (clone RPA-T4), CD44-PerCPCy5.5 (clone G44-26(C26)), CD45RA-AF700 (clone HI100), CD45RO-FITC (clone UCHL1), CD56-AF700 (clone B159), CD56-V450 (clone B159), CD62L-APC (clone DREG-56), CD62L-PE-CF594 (clone DREG-56), CD69-APC (clone L78), CD69-PECy7 (clone L78), CD69-PECy7 (clone FN50), CD8-V450 (clone RPA-T8), CD8-V500 (clone RPA-T8), CD8-FITC (clone HIT8a), CD86-FITC (clone 2331), Igκ-PE (clone G20-193), IgD-APC-H7 (clone IA6-2), IgD-FITC (clone IA6-2), IgG-APC (clone G18-145), IgM-APC (clone G20-127), IgM-V450 (clone G20-127), TCRαβ-FITC (clone WT31), TCRαβ-PE (clone T10B9.1A-31), TCRγδ-APC (clone B1), TCRγδ-PE (clone 11F2); from Miltenyi Biotec: IgA-PE (clone IS11-8E10).

Cytokine blot. Presence or absence of cytokines was detected with the Human Antibody Array 3.0 Kit (Panomics) according to manufacture instructions. In brief, patient or HD serum was separated from blood in specific serum tubes by centrifugation (1000x g, 10 mins) and stored at -80 °C until use. Blood donation of patient was performed 5 months after last IVIg treatment and before initiation fo CTLA-4-Ig replacement therapy. Healthy donor serum sample was a pool from up to 7 donors. Serum was diluted 1:10 for incubation with the antibody-coated membrane. Cytokines were detected by staining with biotin-conjugated antibodies against the indicated cytokines and HRP-conjugated streptavidin. Two replicates were performed.

T-cell CDR3 Vβ spectratyping. T cell receptor Vβ spectratyping was performed as described elsewhere[45].

Genetic analysis. DNA of P1 was extracted from whole blood with the Wizard® Genomic DNA Purification kit (Promega). DNA of P2 was extracted after death from stored histology slides. DNA of relatives was extracted either as described for P1 or from saliva samples with the QIAamp® DNA mini kit. Homozygous intervals were determined applying Affymetrix® SNP-based homozygosity mapping as published elsewhere[45] and used as a filter for detected variants. Genomic DNA of P1 was exome sequenced and analyzed for novel non-sense, missense and frameshift variants as previously described[46]. After library prep with the Illumina True Seq and Exon Enrichment kit, the sample was multiplexed with 3 other samples and loaded on two lanes of one flow cell. DNA was sequenced on an Illumina HiSeq2000 Sequencer and analyzed as previously described[45]. Variant calling was done as previously described[46].

Validation of variants was done applying standard capillary sequencing. Analyses of conversation and mutational impact were done with the predition tools Polyphen-2[47] and SIFT[48]. CADD scores were calculated as described elsewhere[49]. Genes that bear mutations were analyzed and investigated in the ExAC browser for loss-of-function intolerance and predicted-to-observed mutation rates[50].

Cell culture and stimulation conditions. Epstein–Barr virus (EBV) transformed B-cell line were generated from patient or normal donor derived PBMCs by incubating them with EBV containing supernatant (minimum 125 transfecting units per 1 Mio PBMCs) for 2 hours. T cells were depleted by adding 1 µg/ml cyclosporine A. B-cell lines were maintained in RPMI-1640 medium supplemented with 10% of inactivated FCS (Life Technologies, Gibco), 50 U/ml penicillin, 50 mg/ml streptomycin and 292 mg/ml L-glutamine (all from Gibco) at 37 °C in a humidified atmosphere with 5% CO_2. Transformed B cells were expanded and frozen for later analyses in 10% DMSO/90% FBS. For CTLA-4 transcription and protein expression analysis PBMCs or naïve $CD4^+$ T cells were either stimulated with CD3 and CD28 antibody bearing dynabeads (Gibco) at a 1:1 bead to cell ratio, or PMA (25 ng/ml) and ionomycin (1 µg/ml) at 37 °C in a humidified atmosphere with 5% CO_2 for the indicated time.

Cloning of *DEF6*. *DEF6* and *IRF4* cloning plasmid were bought from DNASU[51] and further subcloned in the expression vectors pcDNA-GFP and pTO-STREP-HA. Mutagenesis of *DEF6* was achieved with the Q5® Site-Directed Mutagenesis Kit (New England Biolabs) according to manufactors instructions. The sequences of the plasmids were verified after every cloning step through capillary sequencing.

DEF6 expression analysis. EBV-transformed B-cell lines were lysed in IP-buffer (20mM Tris (pH7.5), 150mM NaCl, 2mM EDTA, 1% TritonX-100 (pH7.1) and complete protease inhibitor cocktail (Sigma Aldrich)) and analyzed by Western blot with primary antibodies against DEF6 (polyclonal mouse; H00050619-B01; Abnova) and GAPDH (monoclonal mouse; sc-365062; Santa Cruz Biotechnology).

Guanine nucleotide exchange assay. The GEF assay measuring the activation of CDC42 was performed as previously described[31]. Briefly, COS-7 cells were transfected with constructs encoding the PH-DHL domain of DEF6 and $DEF6^{E331K}$ (amino acids 217-631) using Lipofectamine® 3000 (Invitrogen) according to manufacturer's instructions. After culture for 24 hours at 37°C, cells were washed twice in ice-cold PBS and lysed in Rac/Cdc42 (p21) binding domain (PBD) assay buffer (25 mM Tris, pH8.0, 25 mM $MgCl_2$, 30 mM NaCl, 0.5% NP-40, 1 mM Dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml leupeptin, 5 µg/ml aprotinin). After centrifugation at 13,000 x g (10 min at 4°C), the supernatants were mixed with

p21 activated kinase 1 (PAK)-PBD beads (Cytoskeleton Inc.) and incubated for 1 hour at 4°C. Beads were washed three times and bound proteins were analyzed by SDS-PAGE. Bound (activated) Myc-tagged CDC42 was detected by immunoblotting using a mouse anti-Myc monoclonal antibody (clone 9E10; Santa Cruz). 3 independent replicates were performed.

Immunofluorescence. PBMCs of P1 and healthy controls were either stimulated with antibodies against CD3 and CD28 as described above ore left untreated. After a stimulation period of 48 hours cells were harvested and adhered to poly-L-lysine (Sigma) coated cover slips by incubation of cells for 5 to 10 mins at 37 °C, 5% CO_2. Cells were immediately fixed in 4 % Paraformaldehyde (PFA) solution. Permeabilization was done by incubation with 0.5% Triton-X100 (Sigma). Cells were blocked with 4 % BSA (Roth). For staining the following antibodies were used: antibodies against DEF6: (polyclonal mouse; H00050619-B01; Abnova; and DEF6-antisera (rabbit) as previously described[15]), antibody against CTLA-4 (monoclonal mouse IgG2a; 550405, BD Biosciences), antibody against RAB11 (rabbit IgG; 700184; ThermoFisher Scientific), anti-mouse antibody (goat IgG, Alexa Fluor 488-coupled, A-11029, Life Technologies), anti-rabbit antibody (goat IgG, Alexa Flour 555-coupled, A-21429, Life Technologies). Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Roth) for 10 mins. Coverslips were mounted in Prolong® Gold antifade reagent (Life Technologies). For epifluorescence analysis, randomly selected fields were examined with a Leica DMI 6000B fluorescence microscope equipped with a X63 objective. Confocal microscopy was performed on a Leica SP8, Olympus FluoView FV10i or Zeiss LSM700 laser scanning confocal microscope as described below.

Confocal microscopy. Cell conjugates of primary NK cells and K562 cells were formed, stained and assessed for the evaluation of the immunological synapse formation by confocal microscopy as previously described[45]. RAB11 and DEF6 co-localization after ectopic expression was assessed as follows. Plasmid DNAs encoding MYC-tagged DEF6 and FLAG-tagged RAB11 were transfected into HEK293 cells grown on coverslips using Lipofectamine® 3000. After incubation for 24 hours at 37°C, cells were fixed in 4% PFA for 15 min, permeabilized in PBS containing 0.3% Triton X-100 for 30 min, and blocked with 2% BSA (PBS/0.1% Triton X-100) for 30 min. The cells were then incubated with primary antibodies overnight at 4°C followed by secondary antibodies for 2 hours at room temperature. The primary antibodies included mouse anti-FLAG (clone M2; Sigma-Aldrich) and rabbit anti-MYC (71D10; Cell signaling). The secondary antibodies were Alexa 488-coupled goat anti-mouse and Alexa 546-coupled goat anti-rabbit (Life technologies). The coverslips were mounted in ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). Triple staining and analysis of endogenous RAB11, CTLA-

4 and DEF6 was performed after stimulation with antibodies against CD3 and CD28 for 48 hours. Cells were fixed, permabilized and blocked as described for HEK293T cells. Cells were stained with primary antibodies rabbit anti DEF6 (rabbit anti-sera prepared as previously described[15]), mouse anti-CTLA4 (clone BN13, BD Biosciences) and goat anti-RAB11 (clone C-19, Santa Cruz) at 4°C overnight. After wash with PBS/0.1% Triton, cells were stained with secondary antibodies, Alexa 488 conjugated donkey anti-rabbit IgG, Alexa 555 conjugated donkey anti-goat IgG and Alexa 647 conjugated donkey anti-mouse IgG (Life technologies). Cells were cytospined onto slides and mounted with Prolong ProLong Diamond Antifade Mountant with DAPI (Life technologies). HEK293T cells and triple stainings were observed using an Olympus FluoView FV10i confocal microscope. All pictures were taken using a 60x/1.35 Oil objective. Line scan analysis of triple stainings were done with ImageJ. For co-localization analysis, randomly chosen cells (for RAB11 DEF6), or randomly chosen CTLA-4 expressing cells (for CTLA-4 RAB11) were acquired with a Zeiss LSM700. For representative RAB11 DEF6 co-localization images, primary cells were analyzed with the co-localization tool of ImageJ. Threshold for both channels was set to 100 (of 255) intensity units. Quantification of RAB11 and DEF6 co-localization was done on the contact area of cells with the coverslips on a region of interest (ROI) of the size of 12.4 µm x 12.4 µm. Quantification of RAB11 and CTLA-4 co-localization was done at the plane of maximal CTLA-4 expression in a ROI of 4.96 µm x 4.96 µm. The thickness of slices was set to 0.4 µm for both analysis. Pearson's coefficient for both analysis was calculated with the JaCoP tool of ImageJ[35].

C3d binding. Assessment of WT and mutated C3d binding ability to CD21 on patient and healthy control EBV-transformed B cell lines was done as previously described[42].

Quantitative real time PCR analysis. Extraction of RNA from PBMCs or sorted naïve $CD4^+$ T 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 analyzed by quantitative PCR using Kappa Sybr Fast qPCR MasterMix ABI Bioprism from Kappa Biosystems on a 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: *CTLA4*-forward: 5'-GTAATTGATCCAGAACCGTGCC-3', *CTLA4*-reverse: 5'-GGCTGTGCCATTCCCTAACT-3', *GAPDH*-forward: 5'-CATCACCATCTTCCAGGAGC-3', *GAPDH*-reverse: 5'-GGATGATGTTCTGGAGAGCC-3'. *CTLA4* expression increase upon stimulation was normalized to *GAPDH* expression applying the ΔΔct method. All qPCR

experiments were done in minimum 3 technical and 2 biological (different blood donation time points) replicates.

Cell line generation. Flp-InTM-293 TRex cells (Invitrogen) with inducible expression of STREP-HA-IRF4 were generated as described previously[52]. In brief, Flp-InTM-293 TRex cells were co-transfected with pTO-STREP-HA-IRF4 and a Flp recombinase vector pOG44 (Invitrogen). 24 hours post transfection cells were selected with 15 µg/ml blasticidin and 100 µg/ml hygromycin B. Cells were frozen in liquid nitrogen until usage.

T-cell proliferation analysis. T-cell proliferation was assessed using cell proliferation dye eFluor® 450 (eBioscience, Affymetrix) according to manufactures recommendations. PBMCs or isolated naïve $CD4^+$ T cells were stimulated with CD3/CD28 antibody bearing human T cell activation dynabeads (Gibco) at a 1:1 ratio, or with antibodies against CD3 (clone OKT3, 1µg/ml) and CD28 (clone CD28.2, 1 µg/ml, both eBioscience) for the indicated time. Cells were washed and stained with different surface antibodies as described above and analyzed on a LSR Fortessa. Experiments were done in 3 biological (different blood donation time points) replicates.

CTLA-4 trafficking assay. The analysis of CTLA-4 trafficking was done as previously described[8] with minor modifications. In brief, PBMCs were stimulated with PMA (20 ng/ml) and ionomycin (1µM) for 60 mins with or without the presence of CTLA-4 antibody (1:20). Unstimulated PBMCs incubated with the CTLA-4 antibody served as a negative control for trafficking. For total CTLA-4 analysis, CTLA-4 was stained intracellularly. Analysis of CTLA-4 expression was performed on $CD3^+CD4^+$ (conventional $CD4^+$ T cells) or $CD3^+CD4^+CD25^+FOXP3^+$ (regulatory T cells) PBMCs. For analysis, geometric means of CTLA-4 staining in negative controls were subtracted from trafficking CTLA-4 geometric means. Several healthy controls were used. Normalization was done to compare trafficking CTLA-4 in healthy individuals and patients as previously described[8]. Experiments on material derived from P1 were done in at least 3 technical replicates at minimum two independent blood donations. Experiments on the CD21-deficient patient was done once in 3 technical replicates.

***Ex vivo* regulatory T cell suppression assay.** Function of regulatory T cells was assessed as previously described[5]. Experiment was done at two independent blood donation time points.

Reconstitution assay. PBMCs of patient and up to 4 healthy controls were isolated in parallel as described above. The cells were directly used for transfection with the vectors pcDNA-GFP-DEF6 or pcDNA-GFP-DEF6^{E331K} applying the Amaxa® Nuclefector® kit for human primary T cell transfection according to manufactures protocol. Cells were left resting for 48h and stimulated to assess exocytosis of CTLA-4 as described above. During analysis, only GFP^+

cells expressing the transgene were considered. Experiments were performed in two biological replicates.

Co-immunoprecipitation. The co-immunoprecipitation of DEF6 and RAB11 was done as follows. HEK293 cells were transfected with DNA plasmids encoding Myc-DEF6, Myc-DEF6^{E331K}, FLAG-RAB11, FLAG-RAB11^{S25N} and/or FLAG-RAB11^{Q70L} using Lipofectamine® 3000. After incubation for 48 hours at 37°C, the cells were treated with 20 mM pervanadate for 5 min and lysed using radioimmunoprecipitation assay buffer (RIPA) buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% nonyl phenoxypolyethoxylethanol (NP-40), 0.02% sodium dodecyl sulfate (SDS), 1 mM phenylmethane sulfonyl fluoride (PMSF), 10 µg/ml leupeptin, 5 µg/ml aprotinin). After centrifugation at 13,000 x g (10 min at 4°C), the supernatants were incubated with EZview™ Red anti-MYC Affinity Gel or EZview™ Red anti-FLAG® M2 Affinity Gel (Sigma-Aldrich) overnight at 4°C. The beads were washed three times, and bound proteins were analyzed by SDS- polyacrylamide gel electrophoresis (PAGE). Proteins were detected by immunoblotting using mouse anti-FLAG and anti-MYC antibodies. Experiments were performed in 3 independent replicates. Co-immunoprecipitation of DEF6 and IRF4 was done in IRF4-Flp-In^{TM}-293 TRex cells. In brief, in cells seeded at 50 % confluence IRF4 expression was induced with doxycycline (1 µg/ml) for 36 hours. 12 hours after initial induction the cells were transfected with pcDNA-GFP-DEF6 or pcDNA-GFP-DEF6^{E331K} vectors using the calcium phosphate co-precipitation method. Controls were either left uninduced and/or left untransfected. The pulldown was performed with GFP-Trap®_MA beads (chromotek) according to manufacturer's recommendations. Whole cell lysates and immune-precipitated proteins were analyzed by Western Blot with antibodies against IRF4 (rabbit, 4964S, Cell Signaling Technologies), DEF6 (polyclonal mouse; H00050619-B01; Abnova), and CAD protein (rabbit, 11933S, Cell Signaling Technologies).

Statistical testing. A normal distribution of data was assumed and the appropriate test was applied. If not stated otherwise, error bars represent the s.d.

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AUTHOR CONTRIBUTIONS

K.B. conceived and designed the study. N.K.S., Z.S., E.S.-V., Ö.Y.P., M.M., P.B., J.-N.S., S.G., E.S., A.K., J.T. performed and analyzed all experiments. N.M., E.F.-W., W.-D.H., R.K. provided clinical care for the two patients and performed clinical routine investigations. J.O., E.M., A.A. and K.B. performed extensive supervision of the performed experiments. N.K.S. and K.B. wrote the initial draft of the manuscript with input from I.B. and A.A. The draft was finalized by N.K.S. and K.B.. All authors read the manuscript and agreed on publishing.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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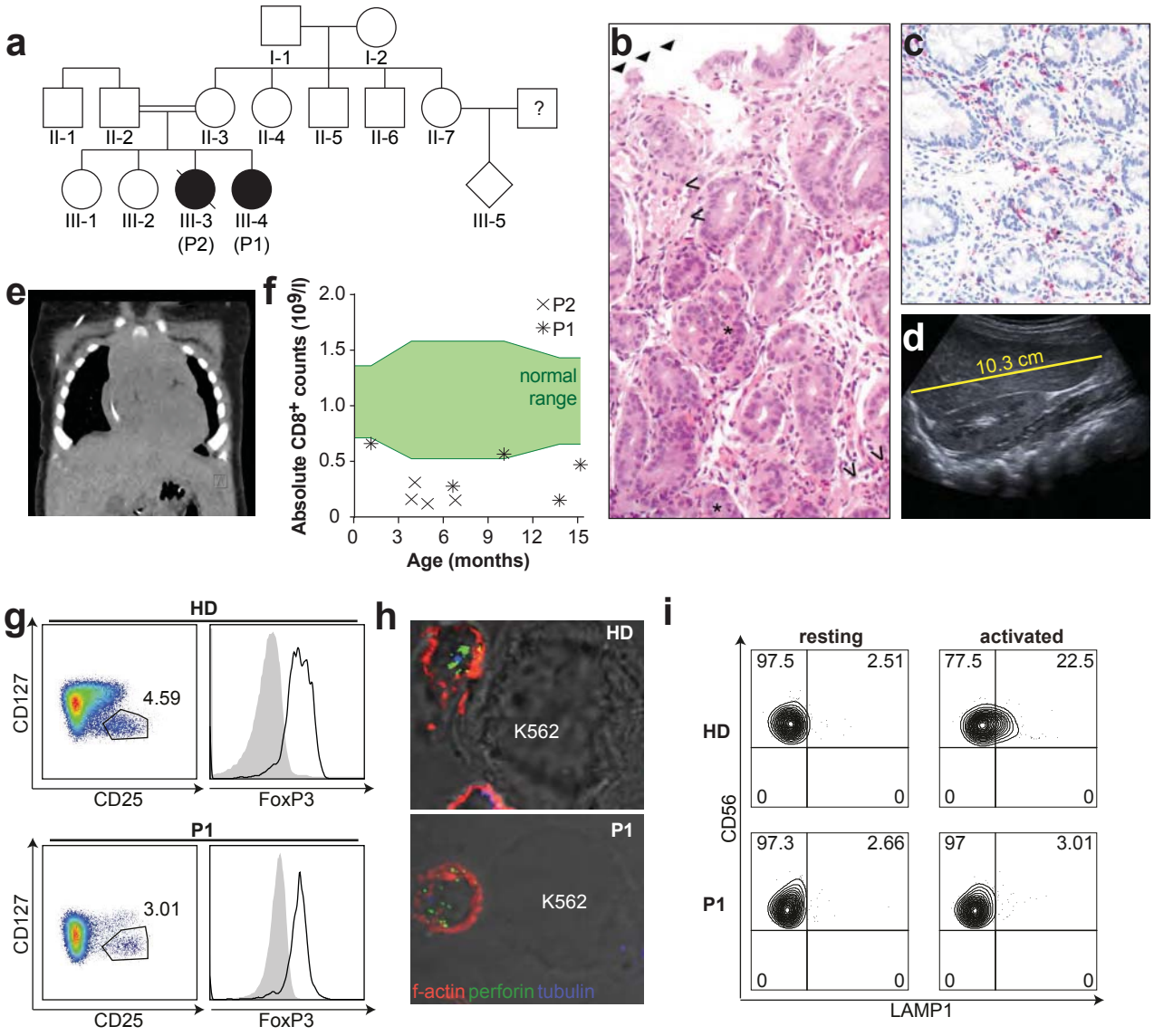

Figure 1 Severe early-onset autoimmunity affecting gut, liver and heart in two patients. (**a**) Pedigree of affected patients is shown. (**b**) Gastric biopsy of P1 at the age of 40 days shows numerous apoptotic cells in the glandular epithelium (*), with mucosal atrophy and regeneration. Lamina propria contained eosinophilic and polymorphonuclear leukocytic infiltrates (>H&E stain). (**c**) Colon biopsy of P1 reveals T-cell infiltration (red:anti-CD3). (**d**) Ultrasound of P1 reveals hepatosplenomegaly. (**e**) Dilated cardiomyopathy of P1 is visualized through CT scan. (**f**) Both patients present reduced cytotoxic T cells. (**g**) Assessment of regulatory T cells ($CD25^{+}CD127^{low}$) revealed a slight reduction in P1. The gated cells are FoxP3 positive (right panels, n>3). (**h**) NK-cell immunological synapse formation towards K562 cells is disturbed in the P1 (> 12 analyzed conjugates per individual). (**i**) P1 presents with reduced NK cell exocytosis as marked by reduced LAMP1 staining. (**j**) Serum cytokine blot shows reduced/absent levels of CTLA-4 and CCL3 (n=2). (HD: healthy donor).

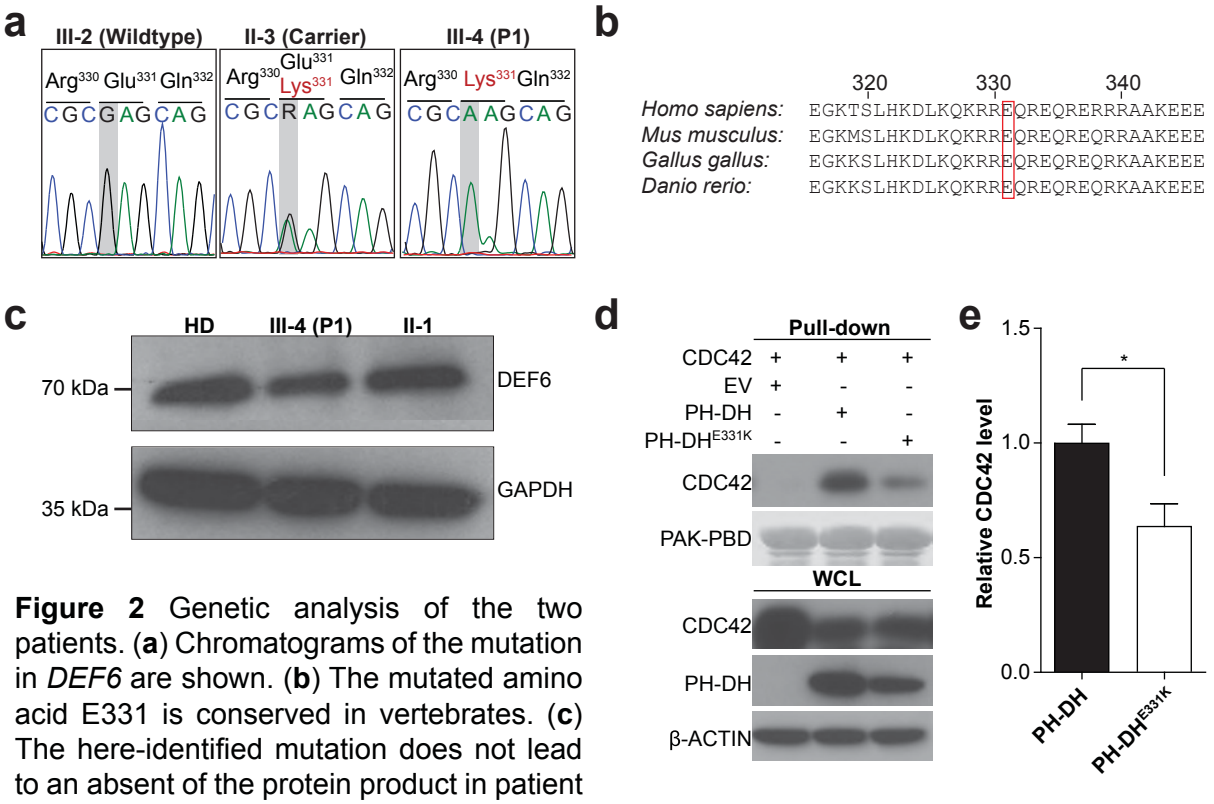

Figure 2 Genetic analysis of the two patients. (**a**) Chromatograms of the mutation in *DEF6* are shown. (**b**) The mutated amino acid E331 is conserved in vertebrates. (**c**) The here-identified mutation does not lead to an absent of the protein product in patient cells as exemplified here in a EBV-transduced B cell line (n>3). (**d**, **e**) Representative blots (**d**) and quantification (**e**) of GEF activity assay of wildtype and mutated PH-DH domain of DEF6 towards CDC42 reveals 50% reduction (p-value: 0.0472, error bars show s.e.m.; EV: empty vector, n=3).

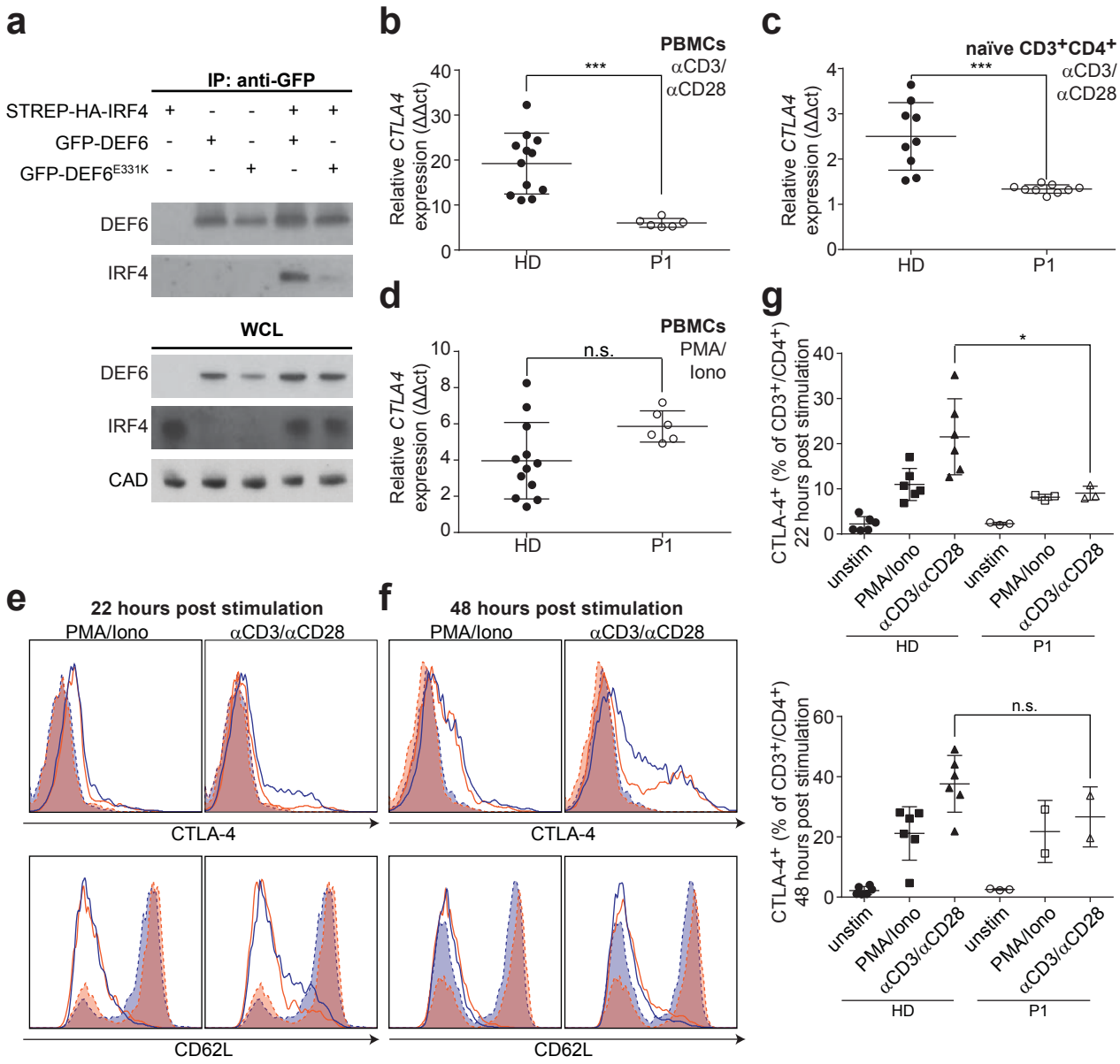

Figure 3 *DEF6*-mutated primary T cells show reduced CTLA-4 expression. (**a**) Co-immunoprecipitation of GFP-tagged DEF6 or DEF6^{E331K} in HEK Flp-In^{TM}-293 TRex-IRF4-STREP-HA cells reveals an interaction of both proteins which is dependent on the identified mutation (3 replicates). CAD protein serves as a loading control. (**b**) Relative upregulation (ΔΔct) of *CTLA4* mRNA expression in PBMCs compared to *GAPDH* expression after 18 hours stimulation with anti-CD3/CD28-coated beads (αCD3/αCD28) is reduced (p-value: 0.0002, one representative of two biological replicates with six technical replicates each, HD shows data of two unrelated healthy donors). (**c**) Similar analysis as in (**b**) with negative selected naïve CD4$^+$ T cells (p-value: 0.0003, three biological replicates with three technical replicates each). (**d**) Relative increase in *CTLA4* mRNA expression as in (**b**) in PBMCs after 18 hours stimulation with PMA and ionomycin (PMA/Iono) is not significantly changed (p-value: 0.0535, two biological replicates with three technical replicates each). (**e**,**f**) Representative FACS blots of total CTLA-4 (top) and CD62L (bottom) protein expression in purified naïve CD4 T cells after 22 (**e**) or 48 hours (**f**) stimulation with anti-CD3/CD28 antibody-coated beads or PMA and ionomycin reveal reduced CTLA-4 protein expression after 22 hours despite equivalent shedding of CD62L (blue: healthy control; orange: P1; tinted: unstimulated; non-filled: stimulated). (**g**) Quantification of (**e**) and (**f**) after 22 (upper panel, p-value: 0.043) or 48 hours (lower panel, p-value: 0.207, two to three biological replicates). HD: healthy donor, P1: patient 1

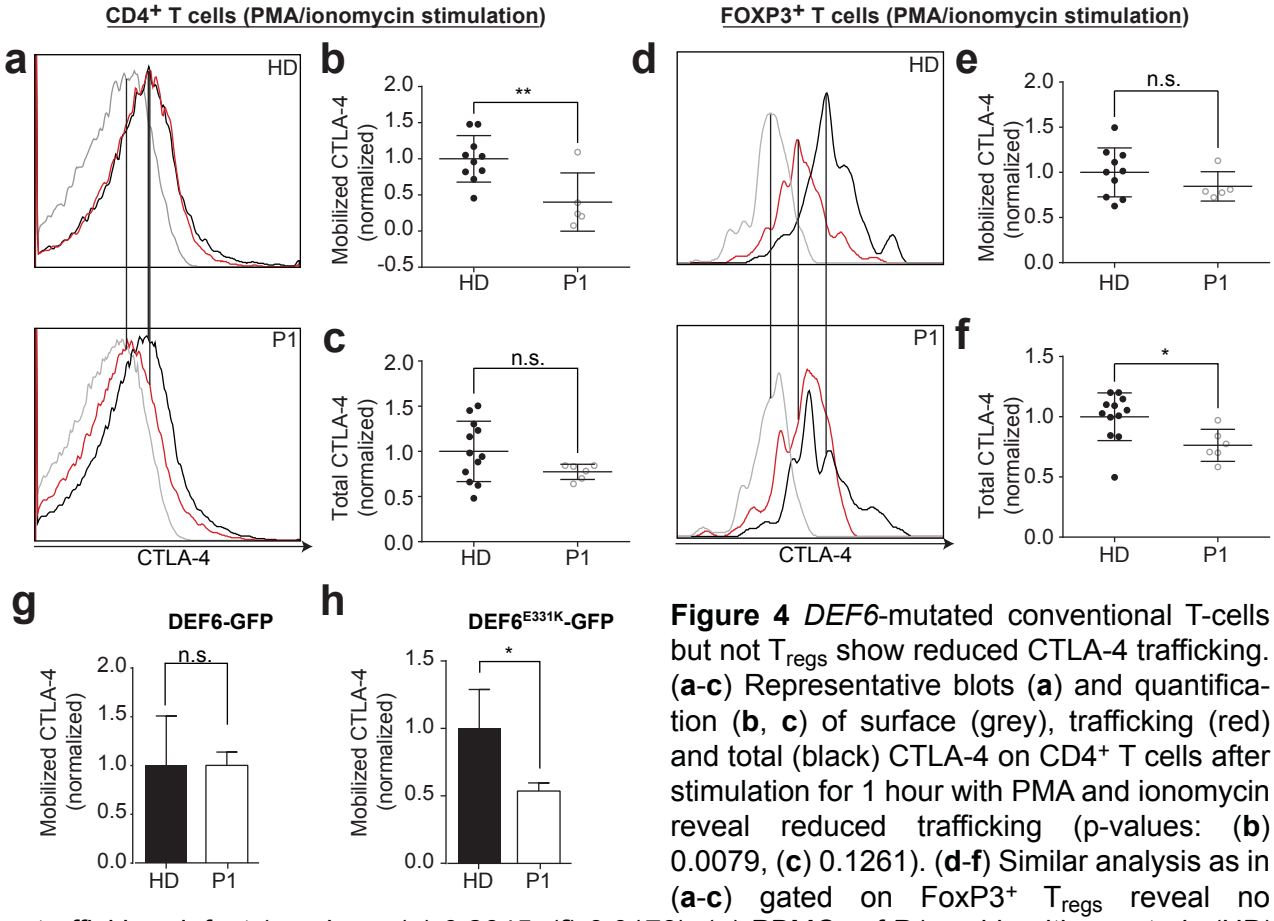

Figure 4 *DEF6*-mutated conventional T-cells but not T_{regs} show reduced CTLA-4 trafficking. (**a**-**c**) Representative blots (**a**) and quantification (**b**, **c**) of surface (grey), trafficking (red) and total (black) CTLA-4 on $CD4^+$ T cells after stimulation for 1 hour with PMA and ionomycin reveal reduced trafficking (p-values: (**b**) 0.0079, (**c**) 0.1261). (**d**-**f**) Similar analysis as in (**a**-**c**) gated on $FoxP3^+$ T_{regs} reveal no trafficking defect (p-values: (**e**) 0.2645, (**f**) 0.0178). (**g**) PBMCs of P1 and healthy controls (HD) transfected with a pcDNA-GFP-DEF6 construct exhibit similar trafficking (p-value: 0.9955, gated on $CD3^+CD4^+GFP^+$ PBMC). (**h**) Transfection of patient and healthy control PBMCs with pcDNA-GFP-$DEF6^{E331K}$ does not rescue the reduced CTLA-4 trafficking (p-value: 0.0126). All experiments shown in Figure 4 were performed in two biological replicates.

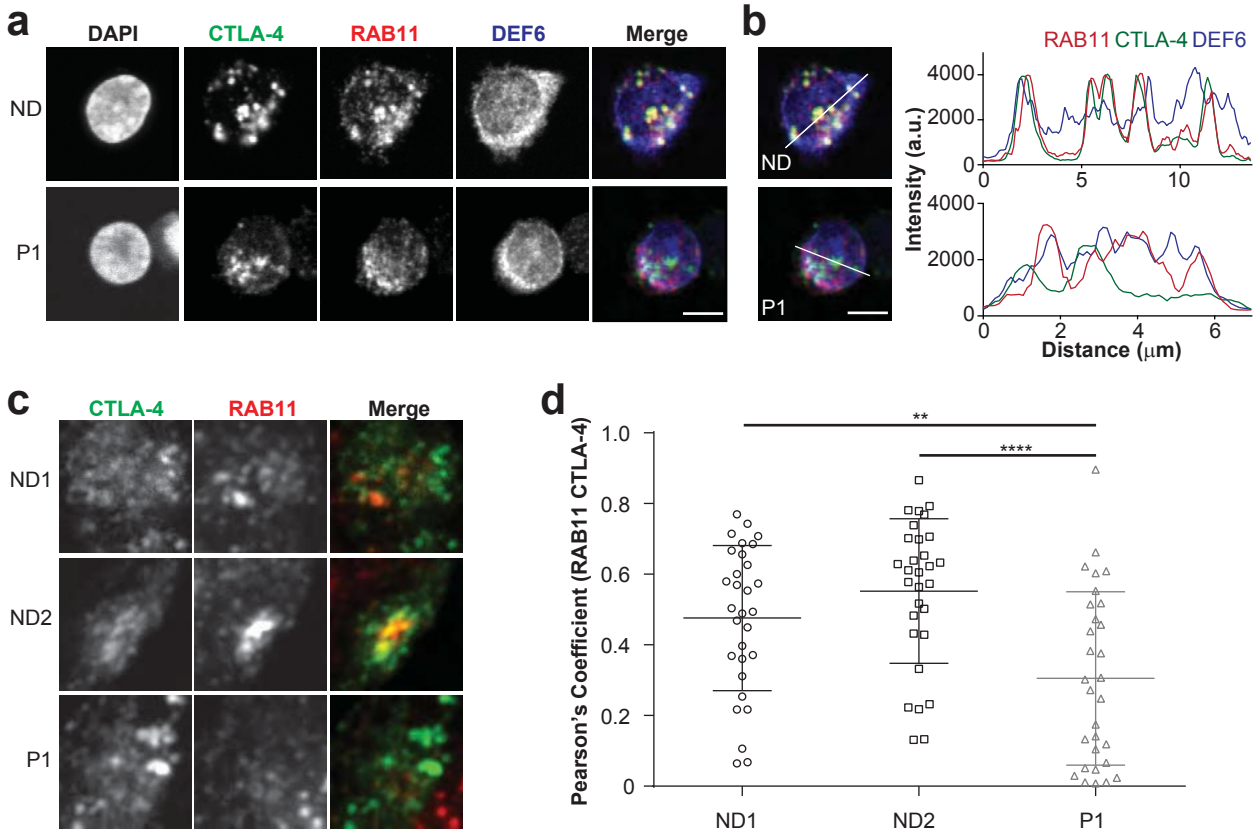

Figure 5 RAB11 is a novel interactor of DEF6. (**a**) Representative image of endogenous DEF6, CTLA-4 and RAB11 staining in activated PBMCs of healthy controls (ND) and P1 reveal a loss of RAB11-CTLA-4 co-localization in patient cells (scale bar: 5 µm, representative images of 30-40 analyzed cells). (**b**) Line scans of the RAB11+ vesicles shown in (**a**) reveal a high overlap of RAB11 with CTLA-4+ vesicles in activated PBMCs of normal donor (upper panels), which is not present in patient cells (lower panels). (**c**) Close-up analysis of 4.96 µm x 4.96 µm large region of interest (ROI) in stimulated PBMCs, which were selected based on highest CTLA-4 expression reveal a reduced RAB11-CTLA-4 co-localization in the patient cells. (**d**) Quantification of RAB11 and CTLA-4 co-localization in selected ROIs with the JaCoP tool of ImageJ reveals a significant reduction of the Pearson's coefficient in the patient (p-values: **: 0.005; ****: <0.0001). (**e**) Co-immunoprecipitation of MYC-tagged DEF6 and GFP-tagged RAB11 reveal a novel interaction which is abrogated by the mutation E331K (EV: empty vector). (**f**) DEF6 preferentially interacts with FLAG-tagged dominant negative RAB11^{S25N} but not with the FLAG-tagged constitutive active RAB11^{Q70L}.

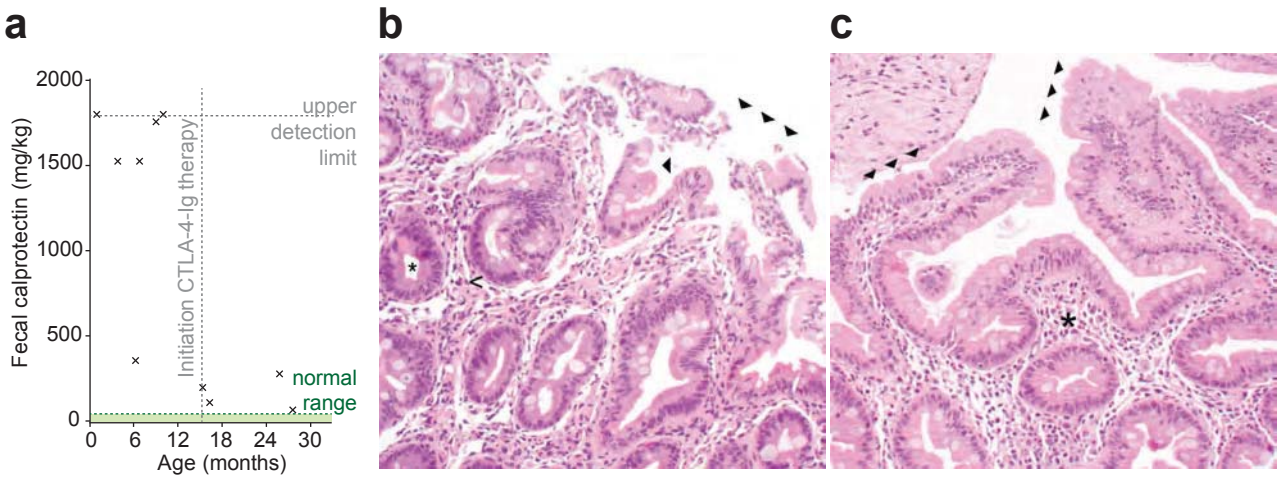

Figure 6 CTLA-4-Ig administration reverts the autoimmune phenotype. (**a**) Fecal calprotectin values reveal therapy-dependent reduction of bowel inflammation. (**b**) Duodenal biopsies at the age of 5 months showed incomplete villous atrophy with villi focally reduced and plump (closed arrows). The inflammatory infiltrate contains clusters of eosinophilic granulocytes (>) and only few crypts with isolated apoptotic figures (*). (**c**) At the age of 16 months (1 months of therapy with abatacept) duodenal biopsies showed presence of villi (arrows) and no signs of acute inflammation in the lamina propria (*).

SUPPLEMENTARY INFORMATION

Patients description.

Patient 1 (P1, index patient) was born in January 2012 as the fourth child of healthy consanguineous parents of Pakistani origin. Two older sisters are reported healthy. A third sister is described below (P2). P1 presented oligohydramnion in prenatal ultrasounds and intrauterine growth retardation. A Caesarean section was performed at 38 weeks of gestation due to rupture of the membrane and pathological dopplersonographic measurements. Weight and length at birth were below third percentile (1435 g; 39 cm; head circumference was 25.5 cm). No abnormalities were observed during the perinatal period, apart from mild respiratory distress syndrome. P1 presented hypertelorism, inward mamillae, growth retardation (below third percentile) and abnormal fatty tissue distribution.

At 23 days of life severe watery diarrhea was observed, associated with vomiting and electrolytes imbalances (hypernatremia and hyperchloremic acidosis; Na: 159 mmol/l, pH 7.1; base excess: -18) and massive increase of inflammation markers (C reactive protein concentration: > 20 mg/dl). Total parenteral nutrition was initiated with no obvious improvements of diarrhea. Hydrolyzed formula also did not improve P1's health status. Viral, bacterial, parasitic or allergic causes of the diarrhea were excluded upon repeated testing. Massive bowel inflammation was suggested by increased stool calprotectin (**Fig. 6a**). Endoscopy revealed atrophy of gastric mucosa with numerous apoptotic cells. Complete villous atrophy was also observed in the small intestine. Microvillus inclusion disease and an underlying metabolic disease were excluded. The duodenum showed massive infiltration of eosinophils and T cells (**Fig. 1c**, and **Supplementary Fig. 1a**). Plasma and goblet cell numbers were reduced. Colonic mucosa showed normal appearance. Subsequently, P1 developed perianal dermatitis (**Supplementary Fig. 6b**) requiring antimycotic or steroid-containing ointments, astringent agents, or airflow. However, the treatment had no effect on the dermatitis and deep, indurated, painful ulcers evolved. Enteral feeding was offered, but led to massive vomiting.

Thus, therapy with corticosteroids (prednisone 1mg /kg/day) was initiated which showed slight improvement of the disease status. Intravenous cyclosporine was added to the therapy. Enteral feeding was gradually increased through a jejunostomy. Vomiting ceased and stool consistency improved substantially. Parenteral nutrition could be withdrawn. With subsequent reduction of cyclosporine, watery diarrhea recurred leading to a loss of about 1.5kg within two weeks.

At the age of 2 months P1 presented hepatomegaly (**Fig. 1d**) and laboratory liver abnormalities (elevated γ-glutamyl transferase (450 U/l) and liver enzymes (AST/ALT: 100 U/l)), which were treatable with ursodeoxycholic acid.

Chest computer tomography imaging of P1 revealed a dilated cardiomyopathy (**Fig. 1e**), treatment with phosphodiesterase inhibitor and acetylsalicylic acid was initiated. Due to suspected vasculitis the patient further received intravenous immunoglobulins. P1 developed a biventricular hypertrophy, with an atrial septal defect (ASD). Treatment with enalapril, atenolol, spironolactone, and furosemide resulted in improved ventricular function, however, biventricular hypertrophy persisted and arterial hypertension developed.

P1 presented with recurrent bacterial, viral and fungal infections (*Streptococcus pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermis*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, rhinoviruses, influenza B, and respiratory syncytial virus, rotavirus) in concordance with an underlying primary immunodeficiency. Immune phenotyping revealed slightly decreased numbers of $CD27^+$ memory B cells (**Supplementary Fig. 1c**) and $CD8^+$ T cells (**Fig. 1f** and **Supplementary Table 1**). Due to reduced immunoglobulins after birth (**Supplementary Table 2**), the patient was supplemented with intravenous immunoglobulins (IVIG) for two months in which IgG/IgM/IgA levels were closely monitored. IVIG administration was paused until the age of 10 months when IVIG supplementation was reinitiated due to reduced specific immunoglobulin titers after vaccination with various agents (polio, diphtheria, tetanus) (**Supplementary Table 3**). Autoantibodies against ANCA (1:160), cardiolipin (IgG:12.1-12.9 U/ml), beta2-glycoprotein (IgG: 28.8-49.8 U/ml; IgM: 6.4-8 U/ml), and smooth muscle could be detected (**Supplementary Table 4**).

Patient 2 (P2, deceased sister) is the third child of the above mentioned parents. P2 was born at 35 weeks of gestation in November 2008 due to premature rupture of the membrane. She showed intrauterine growth retardation (weight at birth: 1260 g; length at birth: 38 cm (both: below third percentile for age); and head circumference at birth: 29.5 cm (at third percentile)) without catch-up postnatally (weight at 2 months of age: 1696 g; length at 2 months of age: 40 cm (both: below third percentile for age). She presented with a cleft palate. Enteral feeding was difficult due to clinical signs that were interpreted as necrotizing enterocolitis. Neither diet with extensively hydrolyzed formula nor with elemental formula improved her clinical condition, necessitating

parenteral nutrition. Colonoscopy at the age of 138 days revealed rectal ulcers and remarkably few plasma cells and increased numbers of apoptotic cells in the descending and transverse colon. Rectal fissures and ulcers were detected. Mucosal membrane showed normal conditions.

Besides the gastrointestinal symptoms, the patient was found to have an atrioventricular septum defect (AVSD) which was treated with pulmonary artery banding and VSD patch closure. She developed increasing heart insufficiency, pulmonary hypertension and a third-grade atrioventricular (AV) block requiring pacemaker treatment.

Her liver presented severe hepatomegaly and signs of progressing cholestasis , siderosis, steatosis and hypertriglyceridemia (430 mg/dl). Furthermore, she developed a metabolic acidosis and hypokalemia. Screening for an underlying metabolic disorder was negative. Liver failure was reported as P2 presented massive jaundice, anasarca and ascites. Histology of the liver revealed autolysis.

Clinical signs and symptoms suggestive of an undefined immunodeficiency appeared as P2 presented with recurrent infections and sepsis due to gram-negative bacteria (*Enterobacter aerogenes*, *Klebsiella oxytoca*), gram-positive bacteria (*Staphylococcus epidermidis*, *Enterococcus faecalis*), and fungi (*Malassezia furfur*). Cytotoxic T-cell numbers were reduced in comparison to age-matched controls (**Fig. 1f** and **Supplementary Table 1**).

Eventually, the patient died at the age of 10.5 months due to a multiple organ dysfunction syndrome. Autopsy revealed pulmonary artery and biventricular dilation as well as right ventricular hypertrophy. She showed pleural effusion and congestion. Thymus was comparably small and T cell depleted (**Supplementary Fig 1h**).

Genetic analysis

Genomic DNA of P1 was exome sequenced and analyzed as previously described[1]. After demultiplexing and alignment of the 227,048,916 reads, 97.8% of reads could be uniquely mapped (**Supplementary Fig. 2a**). Filtering for missense, nonsense, splice-site and frameshift variants inside the homozygous intervals (**Supplementary Table 5)** with a minor allele frequency (MAF) of below 0.01 and validation by Sanger sequencing resulted in 20 single nucleotide variants and 1 deletion/insertion variant. Only two of these variants, affecting the

genes *DEF6* (CCDS4802, c.G991A, p.E331K) and *CD21* (CCDS31007, c.G1295A, p.G432E), segregated with the disease (**Fig. 2a, Supplementary Fig. 2b**). Both variants were predicted to be damaging with several prediction programs (**Supplementary Table 6**). According to the ExAC database probability of loss-of-function intolerance (pLI) and expected-to-observed mutation ratios (Z-score) for *CD21* were calculated as 0[2]. We analyzed the expression of $CD21^{G432E}$ and $DEF6^{E331K}$ which showed no alterations (**Fig. 2c** and **Supplementary Fig. 2c**). CD21 binding capability to its ligand C3d was not lost although the variant is located in the extracellular proportion of the protein (**Supplementary Fig. 2d**). In contrast, the variant in DEF6 has an impact on protein function as the variant results in a reduction of GEF activity (**Fig. 2d,e**). Loss-of-function mutations in *CD21* can result in a relatively mild form of common variable immunodeficiency[3, 4]. Phenotypically the index patients differ from published CD21-deficient patients as those present with an arguably milder phenotype of hypogammaglobinemia but no alteration in the lymphocyte compartment, auto-antibodies and normal vaccination responses[3, 4] (**Fig. 1f** and **Supplementary Table 1-4**). Given the distinct clinical and immunological disease phenotype and our observed lack of functional consequences of the $CD21^{G432E}$ variant on expression and binding of C3d, we considered the variant in *CD21* as not causative for the phenotype of the two index patients. Furthermore, analysis of CTLA-4 trafficking and expression in a previously published CD21 deficient patient[3] did not show any alteration (**Supplementary Fig. 2e-g**), thus excluding an impact of non-functional CD21 on those processes. Taken together, the genetic data and the analysis of the CD21 protein suggest that the phenotype of the two patients is caused by the *DEF6* mutation E331K.

Supplementary Tables

Supplementary Table 1. Immune phenotyping of the two patients

Patient	**P1**		**P2**		
Age	**1 month**	**10 months**	**4 months**	**5 months**	**7 months**
ALC (cells/mm³) (normal range)	6040 (4054-7048)	6250 (3320-7006)	**2230** (3320-7006)	**1450** (3320-7006)	**1930** (3320-7006)
Lymphocyte subsets					
CD3 (%)	77 (62.7-81.6)	68 (51.8-74.2)	62 (51.8-74.2)	**78** (51.8-74.2)	67 (51.8-74.2)
(cells/mm³)	4650 (3180-5401)	4250 (2284-4776)	**1380** (2284-4776)	**1130** (2284-4776)	**1290** (2284-4776)
CD4 (%)	65 (42.8-65.7)	53 (34.9-53.1)	**56** (34.9-53.1)	**67** (34.9-53.1)	**61** (34.9-53.1)
(cells/mm³)	**3930** (2330-3617)	3310 (2284-4776)	**1250** (2284-4776)	**970** (2284-4776)	**1170** (2284-4776)

CD8 (%)	**11** (15-23)	**11** (12.8-27.1)	**7** (12.8-27.1)	**8** (12.8-27.1)	**8** (12.8-27.1)
(cells/mm³)	**660** (712-1361)	690 (524-1583)	**160** (524-1583)	**120** (524-1583)	**150** (524-1583)
CD19 (%)	8 (7.4-21.3)	19 (17-37.2)	18 (17-37.2)	**12** (17-37.2)	18 (17-37.2)
(cells/mm³)	480 (315-1383)	1190 (776-2238)	**400** (776-2238)	**170** (776-2238)	**350** (776-2238)
CD16+56 (%)	12 (4.2-14.8)	12 (4-15.1)	15 (4-15.1)	11 (4-15.1)	18 (4-15.1)
(cells/mm³)	730 (201-870)	750 (230-801)	330 (230-801)	**160** (230-801)	350 (230-801)
CD45RA	78 3630	82 3490			63 810
CD45RO	6 280	9 380			14 180
TCR αβ	75 4530	62 3880			62 1190
TCR γδ	2 (0.7-4.1) 120	**2** (2.8-5.8) 130			**2** (2.8-5.8) 40

Reference values in brackets were taken from[48]. Values outside reference range are marked in bold (ALC, absolute lymphocyte count; TCR, T cell receptor).

Supplementary Table 2. Immunoglobulin levels of patients

Patient	**P1**			**P2**	
Age	**1 month**	**4.5 months**	**10 months**	**4 months**	**6 months**
IgG	160 (↓)	432	959	599.9	551.2
IgA	0.87	69 (↑)	234 (↑)	41.9	109 (↑)
IgM	16.9	90.9 (↑)	474 (↑)	66.7	292.1 (↑)

Immunoglobulin (Ig) concentration were tested at least 4 weeks after the last intravenous Ig treatment (all: mg/dl)

Supplementary Table 3. Vaccination response of patient 1

P1 age	**4 months**	**8 months**	**9 months**
Clostridium tetani (>=0.4 IU/ml)	0.73 IU/ml	0.06 IU/ml	0.05 IU/ml
Corynebacterium diphtheriae (>=0.4 IU/ml)	0.05 IU/ml	0.02 IU/ml	0.02 IU/ml
Streptococcus pneumonia (>=1:200)	1:76	1:20	1:20
Haemophilus influenzae (>=1 µg/ml)	0.76 µg/ml	0.06 µg/ml	0.07 µg/ml
Bordetella pertussis (>=11 VE)		0.6 VE	

The patient was vaccinated three times with Prevenar 13® (Pfizer: pneumococcal polysaccharide conjugated vaccine) and INFANRIX hexa® (GlaxoSmithKline: *Corynebacterium diphtheriae*, *Clostridium tetani*, *Bordetella pertussis*, *Haemophilus influenzae type 1B*, hepatitis B virus, poliovirus) at the age of 3, 4 and 10 months. The higher values at the age of 4 months might be caused by the presence of maternal antibodies.

Supplementary Table 4. Analysis of P1's serum for autoantibodies

P1 age	**10 months**	**12 months**	**13.5 months**
ANCA	Positive (1:160)	Positive (1:40)	n.d.
Cardiolipin (IgG)	n.d.	Positive (12.1 U/ml)	Positive (12.9 U/ml)
Beta2-glycoprotein			
IgG	Elevated (10.5 U/ml)	Positive (28.8 U/ml)	Positive (49.8 U/ml)
IgM	Normal (4.9 U/ml)	Positive (8 U/ml)	Positive (6.4 U/ml)
Smooth muscle antibodies	positive	n.d.	n.d.

Patient's serum was tested for the presence of different autoantibodies (n.d.: not done)

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138 **Supplementary Table 5**. Homozygous intervals in genomic DNA of P1

Chromosome	Start	End	SNP Start	SNP End
1	*73055268*	*74483797*	*rs17545392*	*rs17094731*
1	**163053580**	**163084690**	**rs1395967**	**rs2841980**
1	***163084690***	***165677673***	***rs2841980***	***rs12027066***
1	**165677673**	**165732482**	**rs12027066**	**rs16859005**
1	***165732482***	***167373146***	***rs16859005***	***rs1917533***
1	*167373146*	*167391783*	*rs1917533*	*rs17418864*
1	**206716779**	**206724659**	**rs6685780**	**rs12058254**
1	***206724659***	***208285746***	***rs12058254***	***rs4844407***
1	**208285746**	**209183453**	**rs4844407**	**rs2807404**
1	**214138105**	**219253706**	**rs12089247**	**rs1256614**
2	*69237583*	*70558085*	*rs3890796*	*rs17615728*
2	*97339781*	*98764640*	*rs11891926*	*rs6744080*
3	*49443081*	*50863104*	*rs13096474*	*rs2675836*
3	*126469403*	*127709698*	*rs9848317*	*rs2878602*
3	*162418405*	*163527155*	*rs13083759*	*rs13096751*
4	**9825814**	**10840650**	**rs1996335**	**rs12645385**
4	***10840650***	***12578250***	***rs12645385***	***rs10029767***
4	**12578250**	**13182756**	**rs10029767**	**rs7685975**
4	***13182756***	***15598478***	***rs7685975***	***rs11726647***
4	**15598478**	**15673337**	**rs11726647**	**rs11723554**
4	***15673337***	***17206547***	***rs11723554***	***rs12650417***
4	**17206547**	**17389207**	**rs12650417**	**rs6821103**
5	*63139240*	*64214745*	*rs16892337*	*rs6885916*
6	**16516192**	**18930193**	**rs2299088**	**rs520750**
6	***18930193***	***21144119***	***rs520750***	***rs17835633***
6	**21144119**	**21157038**	**rs17835633**	**rs4712586**
6	***21157038***	***22022732***	***rs4712586***	***rs17201607***
6	*22022732*	*22030361*	*rs17201607*	*rs7751013*
6	***22030361***	***22256381***	***rs7751013***	***rs9366441***
6	**22256381**	**25953684**	**rs9366441**	**rs199725**
6	***25953684***	***28321544***	***rs199725***	***rs1474589***
6	**28321544**	**28444165**	**rs1474589**	**rs29233**
6	***28444165***	***29611229***	***rs16894128***	***rs29233***
6	**29611229**	**29811709**	**rs29233**	**rs16896081**
6	***29811709***	***31071645***	***rs16896081***	***rs9263600***
6	**31071645**	**31337872**	**rs9263600**	**rs5006725**
6	**31340433**	**32359821**	**rs2844558**	**rs17577980**
6	***32359821***	***33389381***	***rs17577980***	***rs211456***
6	**33389381**	**33426699**	**rs211456**	**rs9380355**
6	***33426699***	***33653448***	***rs9380355***	***rs749338***
6	*33653448*	*33661035*	*rs749338*	*rs3818527*
6	***33661035***	***38365384***	***rs3818527***	***rs9380744***
6	**38365384**	**38669184**	**rs9380744**	**rs17622621**
6	***38669184***	***40055134***	***rs17622621***	***rs847778***
6	**40055134**	**40066516**	**rs847778**	**rs10947854**
6	**40102591**	**40111427**	**rs4573070**	**rs10947856**
6	***40111427***	***42462055***	***rs10947856***	***rs9369362***
6	*42462055*	*42467237*	*rs9369362*	*rs9381184*
7	*85879503*	*86880460*	*rs12539847*	*rs2214930*
7	*113639779*	*115089985*	*rs4730617*	*rs7810594*
7	*157830676*	*159119220*	*rs11760246*	*rs1985369*
8	**37472540**	**37473070**	**rs9297257**	**rs713190**
8	***37473070***	***40284521***	***rs713190***	***rs12679092***
8	**40284521**	**41519462**	**rs12679092**	**rs515071**
8	***41519462***	***43778914***	***rs515071***	***rs8185971***
8	**43778914**	**47704399**	**rs8185971**	**rs17061715**
8	***47704399***	***50659324***	***rs17061715***	***rs7465257***
8	**50659324**	**56081879**	**rs7465257**	**rs2199921**
8	***56081879***	***59268021***	***rs2199921***	***rs16923384***
8	**59268021**	**59767223**	**rs16923384**	**rs4738722**
8	***59767223***	***60838202***	***rs4738722***	***rs10106496***
8	**60838202**	**61098558**	**rs10106496**	**rs997574**
8	***61098558***	***62476530***	***rs997574***	***rs6471958***
8	**62476530**	**62503456**	**rs6471958**	**rs7834148**
8	***62503456***	***66302199***	***rs7834148***	***rs1473603***
8	**66302199**	**66356528**	**rs1473603**	**rs16932103**
8	***66356528***	***69009268***	***rs16932103***	***rs3812458***
8	**69009268**	**70003448**	**rs3812458**	**rs2042731**
8	**124861459**	**125686049**	**rs12677352**	**rs1549784**
8	***125686049***	***127744421***	***rs1549784***	***rs1906879***
8	**127744421**	**129300304**	**rs1906879**	**rs11785559**
8	***129300304***	***131630914***	***rs11785559***	***rs12549418***
8	**131630914**	**131654000**	**rs12549418**	**rs10093085**
10	**118947165**	**119141161**	**rs7908165**	**rs7902237**

10	***119141161***	***123096468***	***rs7902237***	***rs10466213***
10	**123096468**	**123096719**	**rs10466213**	**rs10466214**
11	*20088839*	*21156093*	*rs7126674*	*rs7937535*
11	***21192532***	***22217447***	***rs10833472***	***rs4589299***
11	**22217447**	**22621802**	**rs4589299**	**rs16909745**
11	***22621802***	***24247859***	***rs16909745***	***rs7115582***
11	**24247859**	**24758856**	**rs7115582**	**rs1906099**
12	*37857751*	*39110284*	*rs3956186*	*rs1095575*
13	*56329717*	*57434594*	*rs9597255*	*rs7333137*
15	**33288154**	**33908018**	**rs343917**	**rs10519838**
15	***33908018***	***36553702***	***rs10519838***	***rs12907765***
15	**36553702**	**36559831**	**rs12907765**	**36559831**
15	***36559831***	***38488839***	***36559831***	***rs2134333***
15	**38488839**	**39492267**	**rs2134333**	**rs10520122**
15	***39492267***	***43301476***	***rs10520122***	***rs2126602***
15	**43301476**	**39492267**	**rs2126602**	**rs4923956**
15	***43338652***	***46268141***	***rs4923956***	***rs939395***
15	**46268141**	**46629194**	**rs939395**	**rs11858584**
15	**47686590**	**47774480**	**rs11070582**	**rs7165573**
15	***47774480***	***49443741***	***rs7165573***	***rs7181656***
15	**49443741**	**49561455**	**rs7181656**	**rs16962236**
15	***49561455***	***52406658***	***rs16962236***	***rs2414132***
15	**52406658**	**54787358**	**rs2414132**	**rs579664**
15	***54787358***	***56057024***	***rs579664***	***rs7179377***
15	**56057024**	**57253829**	**rs7179377**	**rs34262415**
15	***57253829***	***59131331***	***rs34262415***	***rs2054097***
15	**59131331**	**60284511**	**rs2054097**	**rs12595347**
15	***60284511***	***63093555***	***rs12595347***	***rs8040709***
15	**63093555**	**63112145**	**rs8040709**	**rs2270689**
15	***63112145***	***65706569***	***rs2270689***	***rs7171289***
15	*65706569*	*65723582*	*rs7171289*	*rs8041604*
15	*72003161*	*73348186*	*rs2173875*	*rs11856466*
15	**80624283**	**81362307**	**rs11629706**	**rs7176655**
15	***81362307***	***86159692***	***rs7176655***	***rs41423246***
15	**86159692**	**88036774**	**rs41423246**	**rs2584162**
16	*31906572*	*35205717*	*rs12446802*	*rs2200012*
17	*28444183*	*29722809*	*rs6505162*	*rs731759*
17	*43954686*	*44988703*	*rs916793*	*rs4968247*
20	**52685208**	**53545724**	**rs290447**	**rs407147**
20	***53545724***	***57562102***	***rs407147***	***rs2273359***
20	**57562102**	**57810138**	**rs2273359**	**rs7270917**
20	***57810138***	***60762198***	***rs7270917***	***rs6062129***
20	***61382667***	***62032035***	***rs2243399***	***rs3746372***
20	*62032035*	*62912463*	*rs3746372*	*rs2243399*
22	**17796971**	**27200942**	**rs5994255**	**rs9613266**
22	***17810053***	***25700895***	***rs17808489***	***rs4083400***
22	**25700895**	**25931580**	**rs4083400**	**rs576895**
22	***25931580***	***27191942***	***rs576895***	***rs4822799***
22	**27191942**	**27200942**	**rs4822799**	**rs9613266**

DNA was analyzed with 6.0 SNP chips. Intervals were calculated using homozygositymapper.org [5] (bold) and PLINK [6] (italics).

Supplementary Table 6. Characteristics of the two segregating variants

Gene	Position	Ref.	Obs.	Protein	Polyphen-2	SIFT	CADD	ExAC allele counts	ExAC Z-score	ExAC pLI
CD21	chr1 207644154 (rs781255614)	G	A	CCDS31007; c.G1295A; p.G432E	1.000	0.02	25.4	Het:74, hom:0 (16502) MAF: 0.004		0
DEF6	chr6 35286024 (rs541285645)	G	A	CCDS4802; c.G991A; p.E331K	0.978	0.14	23.6	Het: 21, hom: 0 (8886) MAF: 0.002		0.98

Prediction scores were calculated with Polyphen-2 [7], SIFT [8] and CADD [9]. Variant counts (in brackets: total analysed alleles) and frequencies were downloaded from ExAC database (accession date 1st June 2016)[44] for the South Asian population (Ref.: reference; Obs.: observed; het: heterozygous; hom: homozygous; pLI: probability of loss of function intolerance).

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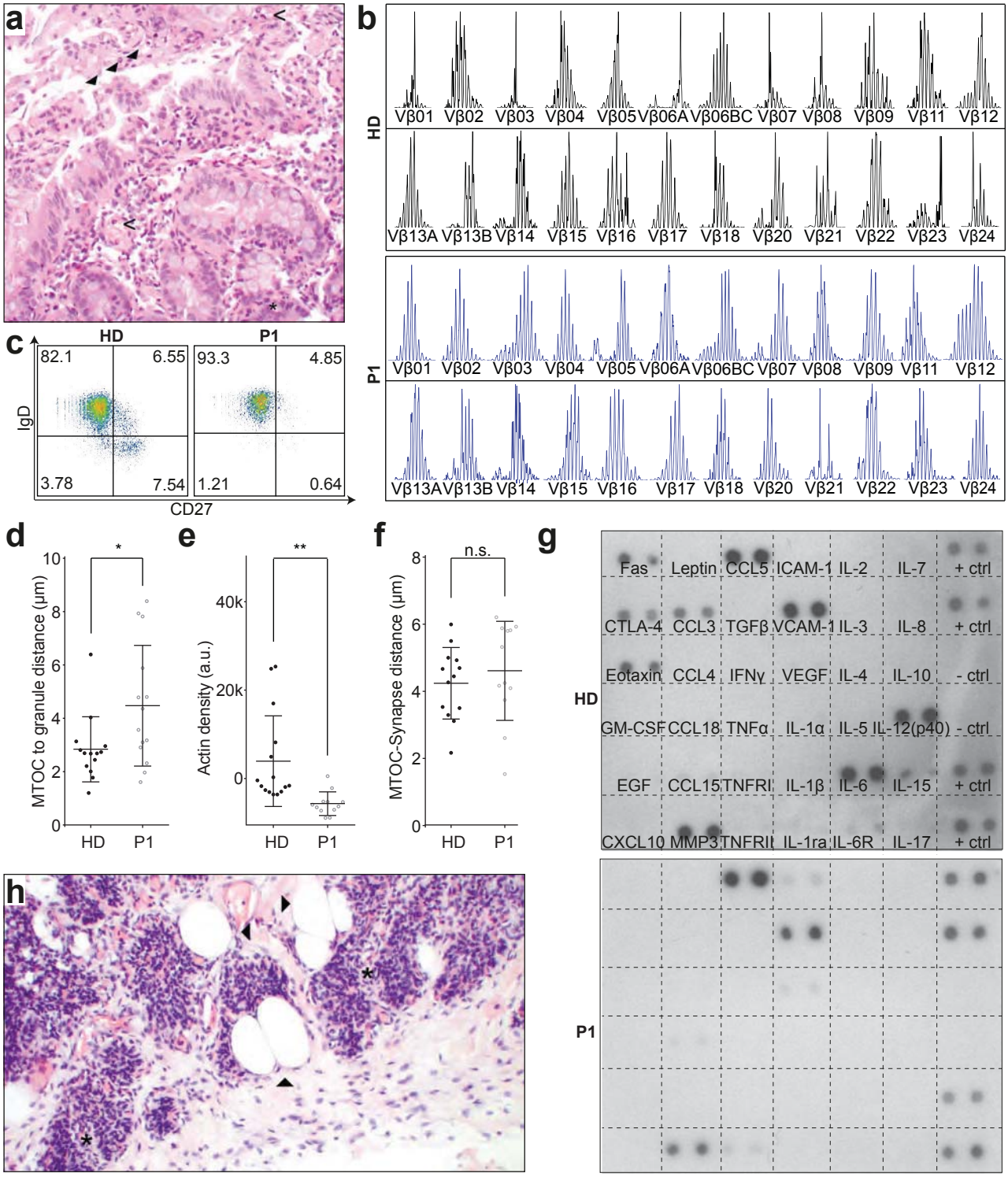
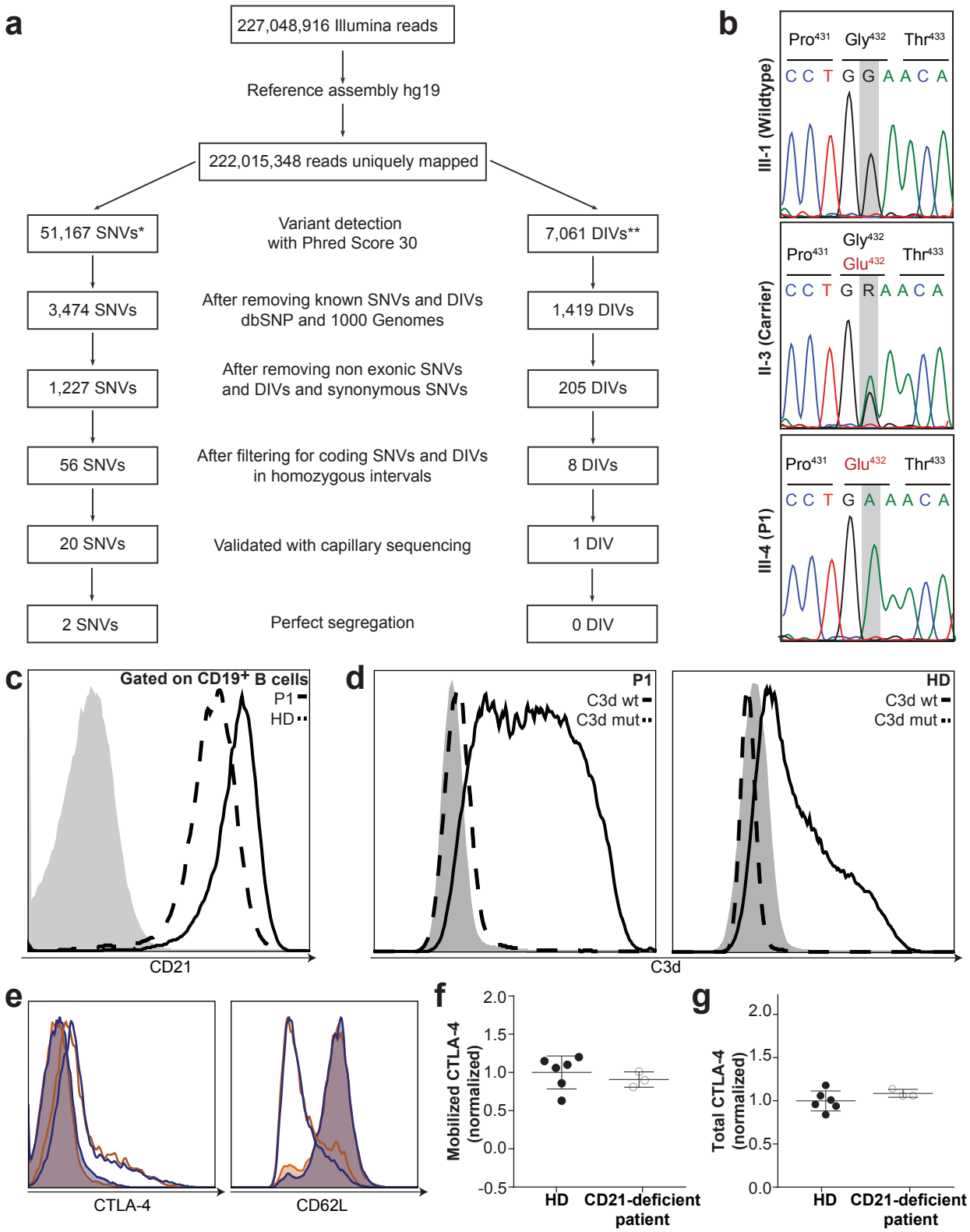
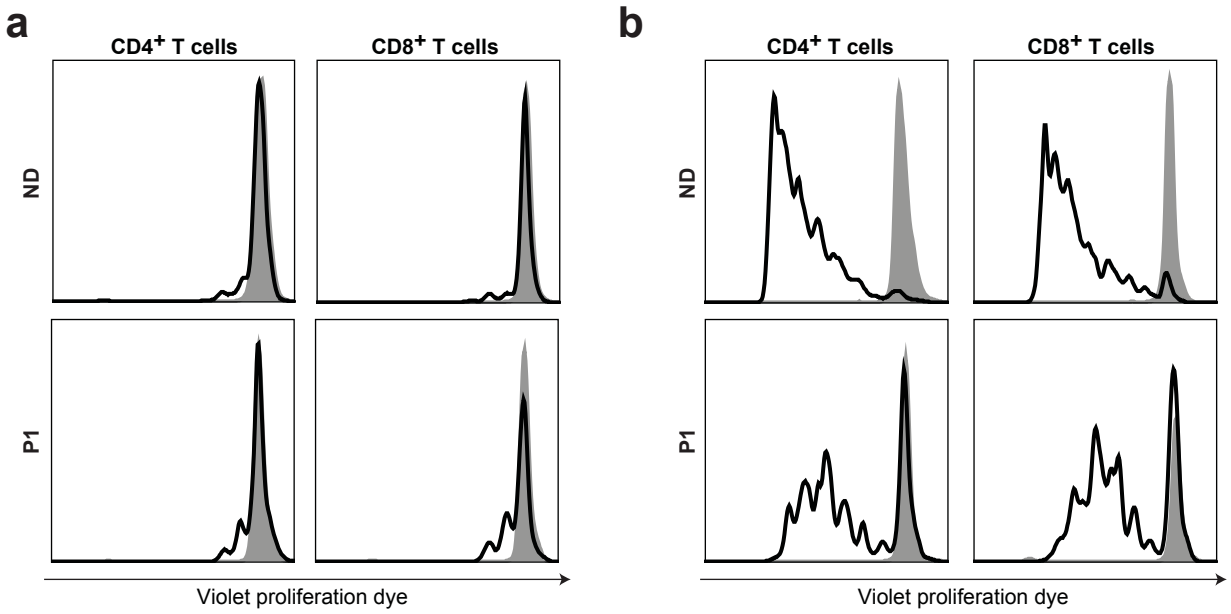


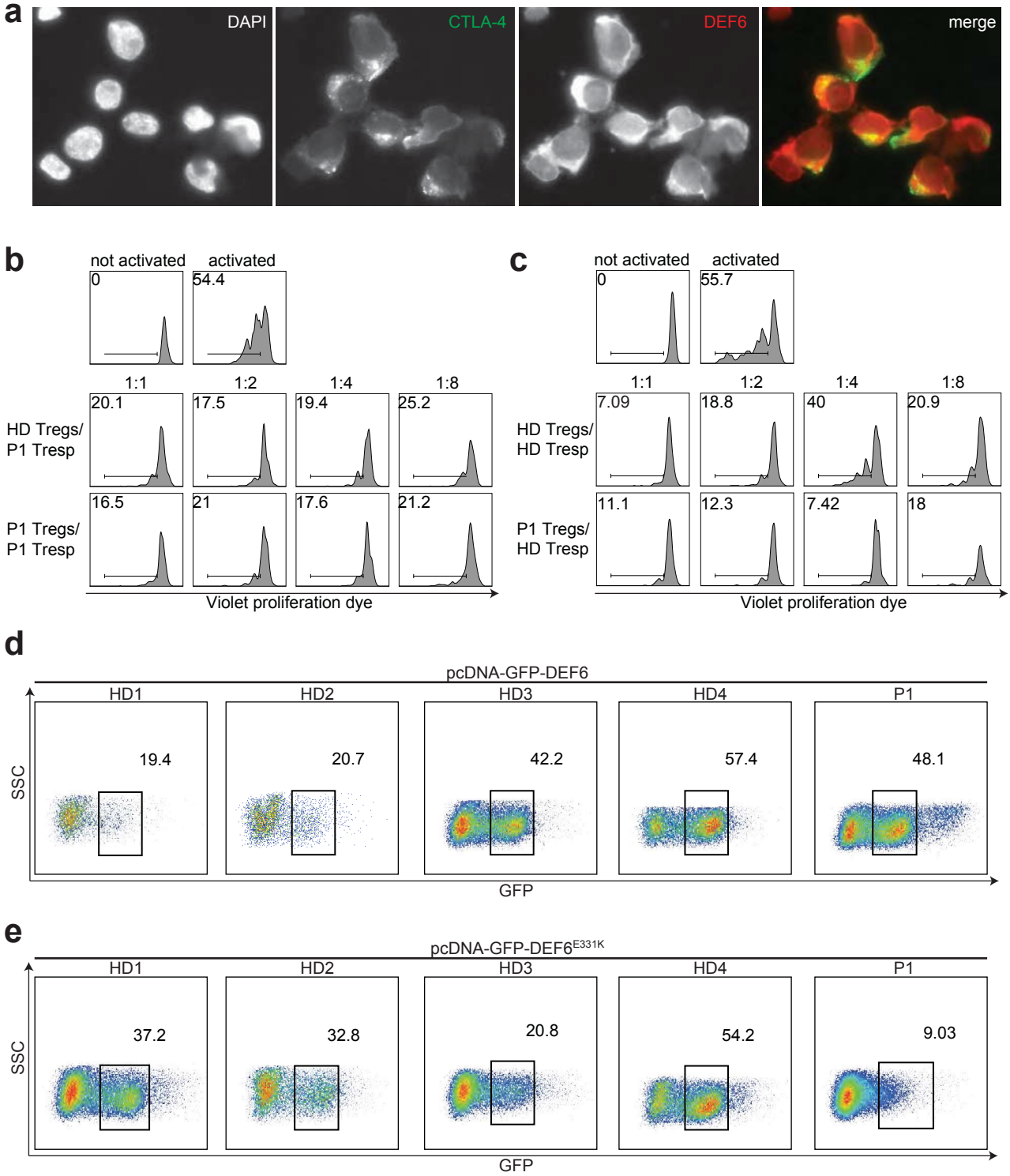
Supplementary Figure 1 Extended phenotype of the two DEF6-mutated patients. (**a**) Duodenal biopsies of P1 at the age of 40 days showed complete villous atrophy (closed arrows) and an imbalance of the leukocytic infiltrate with few plasma cells but increased eosinophilic granulocytes (<). Apoptotic bodies were clearly visible in the epithelium of the crypts (*) (H&E stain). (**b**) T cell spectratyping revealed no oligoclonal restriction. (**c**) P1 presented reduced CD19+CD27+ memory B cells (n>3). (**d**) Immunological synapse formation of NK cells is disturbed in P1 as visualized through increased MTOC to granule distance (p-value: 0.0251) and (**e**) reduced actin density at contact area (a.u.: arbitrary units; p-value: 0.0041). (**f**) Despite defective NK cell synapse, MTOC to synapse distance is not altered. (**g**) Serum cytokine blot of HD (top) and P1 (bottom) showed no systemic increase of proinflammatory cytokines (n=2). (**h**) Autopsy of the thymus of P2 showed signs of involution, reduction in size and the corticomedullary demarcation appeared obscured (*). There was decreased cellularity and increase of adipocytes in fibrous septae between shrunken thymic lobules (closed arrows). (HD: healthy donor).



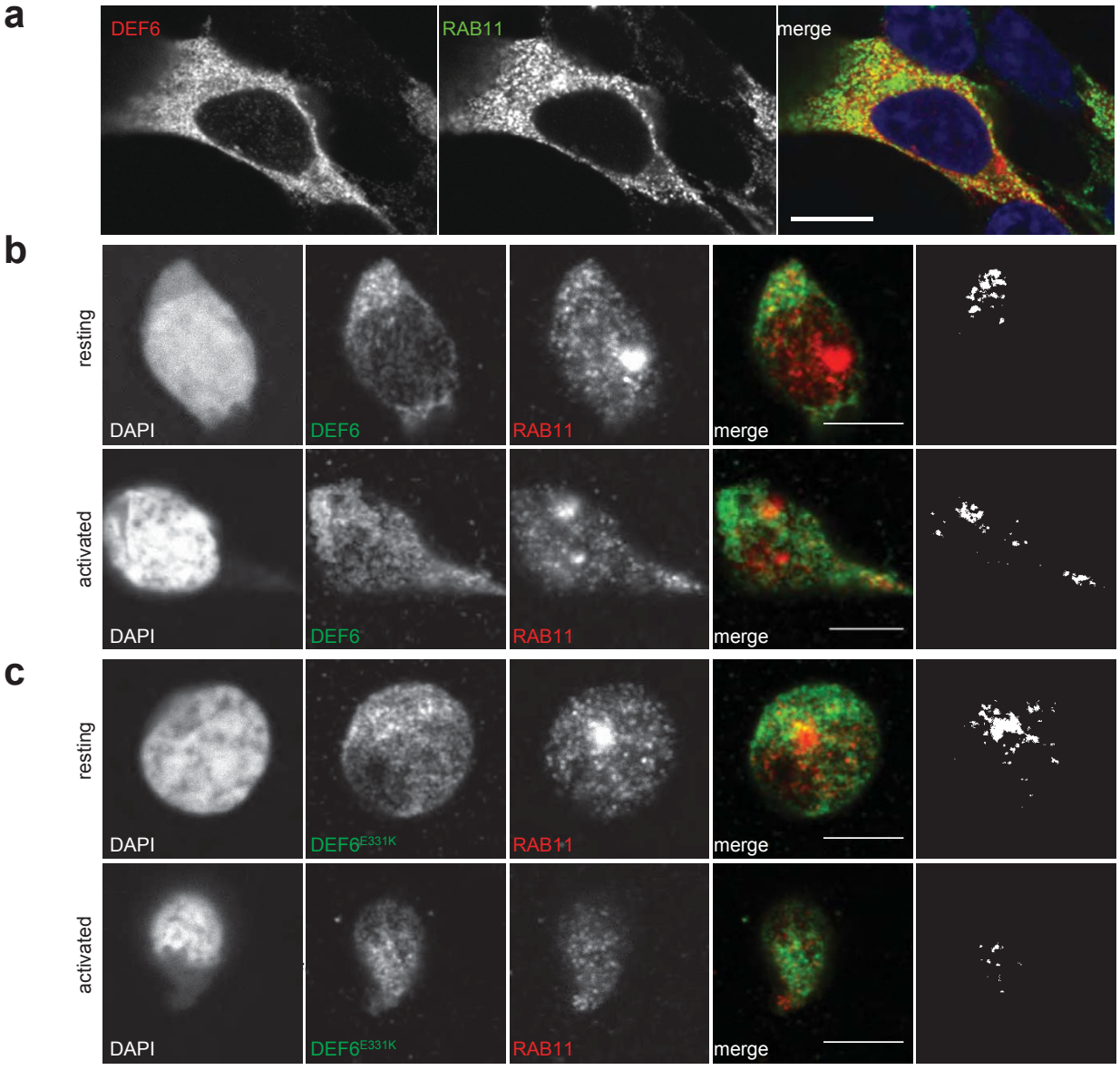
Supplementary Figure 2 Genetic analysis of P1 and analysis of the variant in *CD21*. (**a**) Exome sequencing of P1 revealed 58,228 variants (*SNV: single nucleotide variants, **DIV: deletion/insertion variant), of which only two fulfilled the filtering criteria and segregated with the disease. (**b**) Chromatograms of the variant in *CD21* are shown. (**c**) The *CD21* variant does not lead to an absent of the protein product (n>3). (**d**) Although the variant is located in the extracellular domain of *CD21*, the variant does not alter C3d binding. (**e**) Representative FACS blots of total CTLA-4 (top) and surface CD62L (bottom) protein expression in purified naïve $CD4^+$ T cells after 22 hours stimulation with anti-CD3/anti-CD28 antibody-coated beads is normal in a CD21-deficient patient (blue: healthy control; orange: CD21-deficient patient; tinted: unstimulated; non-filled: stimulated, 3 technical replicates). (**f**,**g**) Quantification of trafficking (**f**) and total (**g**) CTLA-4 on $CD4^+$ T cells show no abnormalities in a CD21-deficient patient (3 technical replicates).



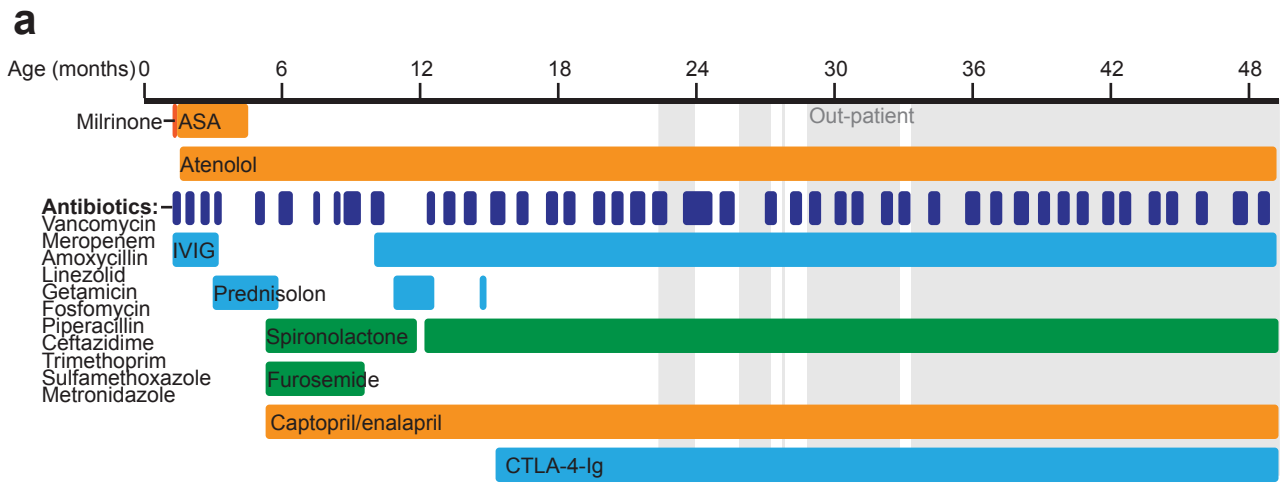
Supplementary Figure 3 Short-term T cell proliferation is unaltered whereas long-term proliferation is decreased in patient's T cells. (**a**) Analysis of $CD4^+$ and $CD8^+$ T cell proliferation after 3 days of activation did not reveal any obvious differences between patient and healthy control (n>3). (**b**) Assessment of T cell proliferation after 6 days of stimulation reveals a slight reduction proliferated T cells in the patient (n>3).

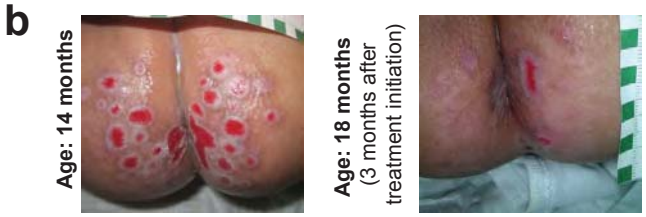


Supplementary Figure 4 CTLA-4 and DEF6 localization and *ex vivo* T_{reg} suppression analysis. (**a**), Staining of CTLA-4 and DEF6 in activated PBMCs of a healthy donor reveal a concentration of DEF6 to sites of CTLA-4 expression. (**b**) Proliferation of T responder cells (Tresp) of the patient could be suppressed by patient and healthy donor derived Tregs. (**c**) Patient Tregs were able to suppress proliferation of T responder cells derived from the patient herself or healthy donors.(**d**, **e**) PBMCs were transfected with pcDNA-GFP-DEF6 (d) or pcDNA-GFP-DEF6^{E331K} (**e**) using the Amaxa® Nucleofector® kit for primary human T cells. Cells were left resting for 48 hours before the assay and gated with forward/sideward scatter and CD3 expression. Due to different expression efficiency, slightly different gating was used for each of the transfections.



Supplementary Figure 5 DEF6 and RAB11 localization. (**a**) Overexpression of DEF6 and RAB11 in HEK293T cells reveals a partial co-localization of this two proteins (scale bar: 5 µm). (**b**, **c**) Analysis of resting and activated (stimulation with antibodies against CD3 and CD28) PBMCs of a healthy control (**b**) and patient (**c**) confirms the partial co-localization of DEF6 and RAB11 (scale bar: 5 µm, right panels: co-localizing pixels, representative images of >30 analyzed cells). (**d**) Pearson's Coefficient calculated with the JaCoP tool of ImageJ identifies a partial co-localization of RAB11 and DEF6 in resting and activated PBMCs of patient and healthy controls (error bars show s.e.m.).





Supplementary Figure 6 *DEF6*-mutated immunodeficiency is responsive to CTLA-4-Ig therapy. (**a**) Timeline of therapeutic interventions in P1 is shown. Further medication include darbepoetin alfa, esomeprazole, cholestyramine (grey background: time spent as out-patient; white background: time spent as in-patient (longer than 2 weeks)). (**b**) Perianal fissures of P2 before (top) and after (bottom) therapy initiation present a marked improvement of patient quality of life.

3. Discussion

In the result section of this thesis, I present two papers that characterize one recently described and one novel primary immunodeficiencies. First, chapter 2.2. describes a novel phenotype of LRBA deficiency. Chapter 2.4. contains a submitted manuscript discovering and characterizing DEF6-mutant patients. All three patients that are described in this work present with severe (fatal) autoimmunity. Our genetic and functional analysis pinpoints the disease phenotype of all three patients towards a mechanism connected to CTLA-4 biology. Whereas in LRBA deficiency, work after our publication could show the functional link towards CTLA-4 (Lo et al, 2015), the submitted manuscript in chapter 2.4. contains the identification of this novel disease and unravels a mechanistic link towards CTLA-4 biology.

3.1. Clinical phenotype of LRBA-deficient patients

LRBA deficiency was initially described in 2012 by 3 independent groups as a common variable immunodeficiency affecting the B cell compartment (Alangari et al, 2012; Burns et al, 2012; Lopez-Herrera et al, 2012). The initial 11 patients presented with different forms of autoimmunity, such as cytopenias and inflammatory bowel disease, and functional defects of B cells including hypogammaglobulinemia (Alangari et al, 2012; Burns et al, 2012; Lopez-Herrera et al, 2012). In early 2015, several additional patients with LRBA deficiency were reported, among them the paper included in chapter 2.2 which describes a young girl whose main manifestation was IBD starting at the age of 6 months (Serwas et al, 2015). Another report describes patients with LRBA deficiency that present with an ALPS-like phenotype (Revel-Vilk et al, 2015). A further group identified a patient with regulatory T cell deficiency in and IPEX-like syndrome (Charbonnier et al, 2015). All of these reports added specific characteristics to the phenotypic spectrum of the disease pinpointing the clinical phenotype to an autoimmune syndrome. Ultimately, in mid-2015, the group of Michael B. Jordan was able to provide the mechanistic reason for the resemblance of LRBA-deficiency towards CTLA-4 haplo-insufficiency as LRBA is important for CTLA-4 recycling (Lo et al, 2015). Thus, the identification of several patients with predominant autoimmune disease such as IBD-, ALPS- or IPEX-like manifestations could be explained through the involvement of LRBA in this immune

regulatory pathway. The variable phenotype of LRBA-deficient patients has been confirmed in two cohort studies analyzing a total of 61 LRBA-deficient patients (Alkhairy et al, 2016; Gamez-Diaz et al, 2016). Most of the patients present with an autoimmune phenotype.

However, other disease manifestations such as recurrent respiratory infections and EBV-driven lymphoma have been described (Alkhairy et al, 2016; Gamez-Diaz et al, 2016). These phenotypes might be linked to the recurrent B cell phenotype seen in LRBA-deficient patients. Several patients presented with hypogammaglobulinemia and reduced numbers of class-switch B cells. However, this phenotype does not occur in all patients (Alkhairy et al, 2016; Gamez-Diaz et al, 2016). On the cellular level, LRBA-deficient B cells reveal reduced survival and increased apoptosis (Lopez-Herrera et al, 2012). The mechanistic link of this phenotype to the function of LRBA phenotype has not yet been provided. It also remains elusive why this phenotype is not seen in all patients with LRBA mutations. Of note, the patient described in chapter 2.2. presents with a severe autoimmune phenotype but an apparent lack of B cell involvement in the disease. The presence of LRBA protein product and the mutation located in the C-terminal domain of the protein suggest an involvement of the N-terminal domain in the role of LRBA in B cells. However, this needs further in-depth characterization.

A subgroup of LRBA-deficient patients present with neurologic symptoms mainly affecting the eye and the optic nerve (Alkhairy et al, 2016; Gamez-Diaz et al, 2016). The reason for this phenotype has not been defined. A potential explanation for this might be found at the genetic locus of *LRBA*. This locus contains not only *LRBA* but also a second gene called *MAB21L2* (Tsang et al, 2009). *MAB21L2* has been linked to eye development (Yamada et al, 2004). Monoallelic or biallelic mutations affecting different domains of the protein have been described to result in major eye malformations. All identified mutations in *MAB21L2* reduce the ability of the corresponding protein product to bind RNA in comparison to wildtype protein. The identified monoallelic mutations affect the N-terminal region of the protein and result in increased stability of the mutated protein product. The biallelic mutations do not have an effect on the protein stability (Rainger et al, 2014). It might be possible that the LRBA-deficient patients with neurological symptoms affecting the eye have a mutation in LRBA that also affects the function or expression of *MAB21L2*. Further studies need to be conducted to clarify this topic.

The assumption that the main role of LRBA lies within the hematopoietic systems is supported by the fact that 4 patients could be successfully treated with hematopoietic stem cell transplantation (HSCT) (Gamez-Diaz et al, 2016; Seidel et al, 2015; Tesi et al, 2016). This is surprising as LRBA is widely expressed according to the protein atlas database (Uhlen et al, 2015). It might be hypothesized that other BEACH-domain containing proteins such as lysosomal-trafficking regulator (LYST) or neurobeachin (NBEA) take over the function of LRBA in other tissues (Cullinane et al, 2013). However, this needs further investigations.

3.2. Functional role of LRBA

As outlined above, LRBA has a central role in preventing autoimmunity through its interaction with the inhibitory protein CTLA-4 (Figure 4). The BEACH domain of LRBA is able to interact with the cytoplasmic ITIM (YVKM) motif of CTLA-4 and thus preventing the binding of the protein AP-1 which sorts CTLA-4 for lysosomal degradation (Lo et al, 2015). Genetic analysis of the patient which is described in chapter 2.2 revealed a missense mutation affecting the C-terminal end of the WD40 domain of LRBA. Despite retained stability of the protein product, the patient presented with severe autoimmunity suggesting an impact of this amino acid on binding of LRBA and CTLA-4. However, it has been published that the PH-BEACH domain is sufficient to bind CTLA-4, whereas the C-terminal WD40 domain is not able to bind it (Lo et al, 2015). Whether the mutation that was identified in the paper attached in chapter 2.2. leads to an abrogation of binding of CTLA-4 and LRBA still needs to be determined.

The initial description of the protein LRBA reports three different isoforms (α, β and γ) that are differentially transcribed throughout the different tissues of the body (Wang et al, 2001). The mutation that has been identified in the patient reported in chapter 2.2. is only present in one of this isoforms, which is termed LRBA-α, as the other isoforms are shorter and do not cover the mutated nucleotides. The expression of LRBA-α has been shown to be weak in different B-cell lines and very low in comparison to the other isoforms in spleen tissue. LRBA-α was not detectable in lung tissue and bone marrow expression analysis, whereas both of this tissues expressed the β-isoforms and lung even the γ-isoform of this protein (Wang et al, 2001). Thus, it is not surprising that the identified mutation does not lead to a B-cell

intrinsic defect. It might be speculated that β- and γ-LRBA have an important role in the epithelial barrier function of lung tissue as the *C. elegans* homolog of LRBA, SEL-2, has been shown to function in endosomal trafficking of polarized epithelial cells (de Souza et al, 2007). Whether or not mutations in *LRBA* affecting the β- or γ isoform contribute to the recurrent pulmonary infections seen in several patients remains elusive.

Apart from its expression in B cells, LRBA has been described to be upregulated in macrophages upon LPS treatment. Overexpression experiments further showed that the LRBA specifically localizes to vesicular, endosomal and lysosomal compartments upon LPS treatment (Wang et al, 2001). As LRBA interacts with the ITIM motif of CTLA-4 (Lo et al, 2015), it might be hypothesized that it can also interact with other ITIM-bearing proteins and thus prevent their lysosomal degradation. Whether this hypothesis holds true, and whether lysosomal degradation of any other protein contributes to the phenotype of LRBA-deficient patients needs further investigations.

The interest in the functional role of LRBA has increased tremendously after identification of LRBA deficiency 4 years ago. However, as outlined above, the only function of LRBA which has been clearly defined is the interaction with CTLA-4.

3.3. CTLA-4 and its role in immune homeostasis

In addition to CTLA-4 haploinsufficiency, the identification of LRBA-deficient patients further strengthens the central role of CTLA-4 in immune homeostasis. It seems that a tight regulation of the amount and availability of CTLA-4 is needed to prevent not only autoimmunity, but also malignancies as increased CTLA-4 expression leads to reduced activation of T cells. This results in the escape of tumors from the immune surveillance. Taking this into consideration, it is not surprising that targeting of CTLA-4 with an antibody results in the reactivation of tumor-specific T cells (Gubin et al, 2014). However, the efficacy and precise clearance of tumors with high mutational burden in patients was unexpected and lead to a revolution in the field of cancer immunobiology (Sharma & Allison, 2015). The use of antibodies against CTLA-4 bears the risk of overdosing which results in a lack of the inhibitory function of CTLA-4 in unaffected organs. Thus, one of the most common adverse effects is the infiltration of activated T cells into unaffected organs (Pardoll, 2012). Strikingly, the phenotype of patients treated with CTLA-4 antibodies resembles the autoimmune

phenotype of CTLA-4-haplosinsufficient and LRBA-deficient patients, suggesting that the presence or absence of CTLA-4 is responsible for the reaction against self. However, both LRBA-deficient (Alkhairy et al, 2016; Gamez-Diaz et al, 2016) and CTLA-4-haplosinsufficient patients (Kuehn et al, 2014; Schubert et al, 2014) present with additional phenotypes affecting other cellular compartments. Some patients also present with agammaglobulinemia and a common variable immunodeficiency (CVID)-like phenotype (Kuehn et al, 2014; Schubert et al, 2014). The underlying mechanisms is completely uncharacterized and needs future investigations.

Similar to CTLA-4-haplosinsufficient and LRBA-deficient patients, the patients described in chapter 2.4. present with relative lack of CTLA-4. The work shows that CTLA-4 transcription and trafficking is reduced in patient-derived cells. Comparable to patient suffering from the two other above-mentioned deficiencies, the reduced availability of CTLA-4 resulted in severe T cell infiltration into the colon of the patients. Treatment of the surviving patient with the immunomodulatory drug CTLA-4-Ig resulted in sustained remission of the disease, which suggested a mechanistic link between LRBA-CTLA-4 and DEF6.

3.4. DEF6 deficiency as a novel disease etiology

To define the molecular cause of disease in the two severely affected patients described in chapter 2.4. combined homozygosity mapping and exome sequencing was performed, a method which is well-established for the identification on autosomal-recessive diseases with full penetrance in consanguineous pedigrees (Dobbs et al, 2015; Salzer et al, 2013; Willmann et al, 2014). The analysis enabled us to identify a mutation in the gene *DEF6* which segregated with the disease in the pedigree. A second variant was identified in the gene encoding CD21, a B cell co-receptor which reduces the threshold of B cell activation (Holers, 2014). Our analysis revealed that the altered protein was still expressed and able to bind its ligand C3d suggesting that CD21 is still functional. Additionally, the phenotype of CD21-deficiency is substantially different from the phenotype of the here-described DEF6 deficient patients (Thiel et al, 2012; Wentink et al, 2015). However, it is possible that the variant in CD21 contributes to the disease phenotype of the patient. I excluded the effect of CD21 on CTLA-4 trafficking and expression as I performed the analysis

also on peripheral blood mononuclear cells (PBMCs) of a previously published CD21-deficient patient (Thiel et al, 2012).

3.5. The role of DEF6 in humans and mice

DEF6 is a guanine nucleotide exchange factor downstream of the TCR. *Def6*$^{-/-}$ mice have been previously described by two research groups (Canonigo-Balancio et al, 2009; Chen et al, 2008; Fanzo et al, 2006; Vistica et al, 2012). Surprisingly, *Def6*$^{-/-}$ mice show remarkable differences in their phenotype depending on the genetic background of the mouse strains. As an example, *Def6*$^{-/-}$ on a mixed 129xC57BL/6 background are prone to spontaneously develop an autoimmune syndrome with splenomegaly, hypergammaglobulinemia and autoantibody production (Fanzo et al, 2006). Although 129xC57BL/6 wildtype mice are generally prone to develop autoimmune disease (Bygrave et al, 2004), the gene-trap mediated deletion of Def6 resulted in an earlier and more severe onset of symptoms than wildtype or heterozygous littermates (Fanzo et al, 2006). On the other hand, *Def6*$^{-/-}$ mice on a C57BL/6 background do not develop autoimmune responses and are even resistant to experimental autoimmune encephalomyelitis (Canonigo-Balancio et al, 2009). The reasons for this contrary findings are not yet understood. Thus, human DEF6 deficiency enables us to shed light on the role of DEF6 in autoimmune disease.

The discovery of the mechanistic link between DEF6 and CTLA-4 raises new explanatory possibilities for this discrepancy. As stated above, CTLA-4 is a critical molecule in humans. Patients with biallelic mutations in *CTLA4* have not yet been described which further underlines the importance of this molecule. Reduction of CTLA-4 as seen in CTLA-4 haploinsufficiency, in which only one allele is mutated and thus leaving one remaining healthy gene, results in severe autoimmunity (Kuehn et al, 2014; Schubert et al, 2014). In contrast to humans, *Ctla4*$^{-/-}$ mice have been described. These mice develop fatal autoimmunity early in life. However, their heterozygous littermates are healthy (Tivol et al, 1995; Waterhouse et al, 1995). This suggest that mice can cope with a 50 % reduction of Ctla-4, whereas humans have a very narrow window of tolerance regarding CTLA-4 levels and are very sensitive to a reduction. This model is further supported by several GWAS that identified SNPs in CTLA-4 which affect the relative cell surface expression of CTLA-4 (Li et al, 2012). The severe autoimmune phenotype in LRBA deficiency is also caused by a relative

reduction of CTLA-4 cell surface expression (Lo et al, 2015). Concordantly, the relative CTLA-4 reduction seen in *Lrba*$^{-/-}$ mice on a C57BL/6 also does not result in an autoimmune phenotype (Laura Yaneth Gamez-Diaz, personal communication). In summary, *Def6*$^{-/-}$, *Ctla4*$^{-/+}$ and *Lrba*$^{-/-}$ mice present a comparable phenotype in regards of autoimmune manifestations which does not necessary reflect the phenotype seen in the corresponding human diseases.

3.6. DEF6 in regulatory and conventional T cells

Our analysis of DEF6-mutated T cells reveals differences between CTLA-4 trafficking in conventional vs. regulatory T cells. Whereas the trafficking was severely reduced in conventional T cells, regulatory T cells seemed to be unaffected by this defect. This raises the question whether CTLA-4, although acting in a similar fashion in both cell types (Wang et al, 2012), might be regulated through different pathways in these two cell subsets. DEF6, which acts downstream of the T cell receptor complex, is expressed in both cell subsets and might get activated in both T cell subsets. One hypothesis for the different outcomes in the absence of functional DEF6 takes into consideration that the only homolog of DEF6, switch-associated protein 70 (SWAP70), is not expressed in conventional T cells but in regulatory T cells (Chandrasekaran et al, 2016). Therefore, there is a possibility that SWAP70 is able to perform similar tasks as DEF6 and can compensate the loss of DEF6 in regulatory T cell.

The functional role of CTLA-4 on conventional T cells is still under debate. It has been shown that the general function of CTLA-4 on non-T_{reg} cells is similar to its role in regulatory T cells as it has been shown that CTLA-4 on conventional T cells is also able to induce transendocytosis of CD80 and CD86 (Wang et al, 2012). However, the outcome of a conventional T cell specific *Ctla4* knockout has not been investigated so far. The importance of CTLA-4 on those cells has been shown in mice with a regulatory T cell specific deletion of *Ctla4*. Those mice survive longer than their germline knockout littermates (Jain et al, 2010; Wing et al, 2008). One can conclude that the difference in lifespan reflects the role of CTLA-4 in conventional T cells. The novel immunodeficiency described in chapter 2.4. suggests that the trafficking and expression of CTLA-4 in conventional T cells is also of importance in

humans to prevent severe systemic autoimmunity. However, further studies will be needed to confirm this hypothesis.

3.7. DEF6 in other cellular compartments

The manuscript in chapter 2.4. describes a novel function of DEF6 which links it to the inhibitory protein CTLA-4. Other attributes of DEF6 have been described previously. This includes its role in cell morphology as it is a guanine nucleotide exchange factor for RAC1, RHOA and CDC42 (Mavrakis et al, 2004; Oka et al, 2007). The identified mutation DEF6^{E331K} results in a reduction of GEF activity towards CDC42. Thus, it might also be possible that the GEF activity towards the other small GTPases is affected. So far, no investigations on cell motility have been made on patient material. As the patients with mutations in DEF6 do not only present with a severe autoimmune phenotype, but also with recurrent infections, it might be possible that a reduced migratory activity contributes to the increased susceptibility towards pathogens. Further studies will be necessary to test this idea.

DEF6 has been described to be phosphorylated by ITK (Hey et al, 2012). The functional role of this phosphorylation is not yet clear. As outlined in the introduction, ITK deficiency has been described previously (Huck et al, 2009; Serwas et al, 2014). Patients suffer from recurrent infections and $CD4^+$ T cell lymphopenia (Serwas et al, 2014). Autoimmunity has not yet been described as a disease feature of ITK-deficient patients. The DEF6-mutated patients rather show a $CD8^+$ T cell lymphopenia. In mice, it has been further shown that DEF6 is necessary for the initial expansion of those cells (Feau et al, 2013). The characterization of the functional role of the ITK-mediated phosphorylation might shed light on the differences in phenotype of DEF6- and ITK-deficient patients.

DEF6 has also been described to play a role in innate immunity such as in Fcγ receptor-mediated phagocytosis (Mehta et al, 2009) and TLR-4 signaling (Chen et al, 2009). Analysis of innate immune cells derived from the DEF6-mutated patient did not reveal any obvious defects.

An important function of DEF6 is the interaction with the transcription factor IRF4 (Gupta et al, 2003a). In chapter 2.4. it is shown that DEF6^{E331K} presents a reduced ability to bind IRF4. It is further shown that mRNA and protein levels of CTLA-4, one

of the targets of IRF4 are reduced. It might be possible that other target genes of IRF4 are affected in expression which needs further in-depths investigation.

3.8. Targeted treatment approach for DEF6 deficiency

One striking result of the paper presented in chapter 2.4. is the application of precision medicine for the surviving patient. The identification and molecular characterization of functional DEF6 deficiency resulted in a treatment recommendation. Upon a severe deterioration of the patient's phenotype, the decision was made to try an off-label medication. As the link between DEF6 and CTLA-4 was established, CTLA-4-Ig was the medication of choice which was administered to the patient in a 4-weekly circle. The initiation of the therapy resulted in a complete remission of the autoimmune phenotype providing further *in vivo* relevance for a functional link of DEF6 and CTLA-4. Another possibility would have been to perform HSCT, as DEF6 is mostly expressed in the immune system (Uhlen et al, 2015), and the observed phenotype seems to affect the hematopoietic system. However, HSCT bears a certain risk for patients and it is not clear whether HSCT would cure the phenotype of the patient.

3.9. Regulation of CTLA-4 trafficking

The identification and molecular characterization of functional DEF6 deficiency sheds light on the complex mechanisms that are necessary to keep our immune system under control. As CTLA-4 is one of the most potent inhibitory molecules of the immune system, its expression and surface localization has to be under strict control as too much, or too little of this protein can result in severe, sometimes fatal outcomes (Gubin et al, 2014; Kuehn et al, 2014; Schubert et al, 2014). Recent work has identified the important role of LRBA in the recycling of CTLA-4 (Lo et al, 2015). The phenotype of the patient described in chapter 2.2. thus is most likely caused by a reduced availability of CTLA-4 on the surface of T cells. The phenotype of the patient described in chapter 2.4. is also caused through a relative absence of CTLA-4 which unraveled a novel regulatory pathway involved in CTLA-4 trafficking and recycling. DEF6, which is downstream of the TCR (Gupta et al, 2003a), might be a central guanine nucleotide exchange factor to activate the recycling of CTLA-4

(Figure 5). Activation of DEF6 might lead to the initiation of the fusion of preformed RAB11-positive recycling endosomes and endocytosed CTLA-4. DEF6 thus acts in cooperation with LRBA which prevents lysosomal degradation as it binds the ITIM motif of CTLA-4 (Lo et al, 2015). The LRBA-CTLA-4 interaction persists until CTLA-4 is brought back to the membrane.

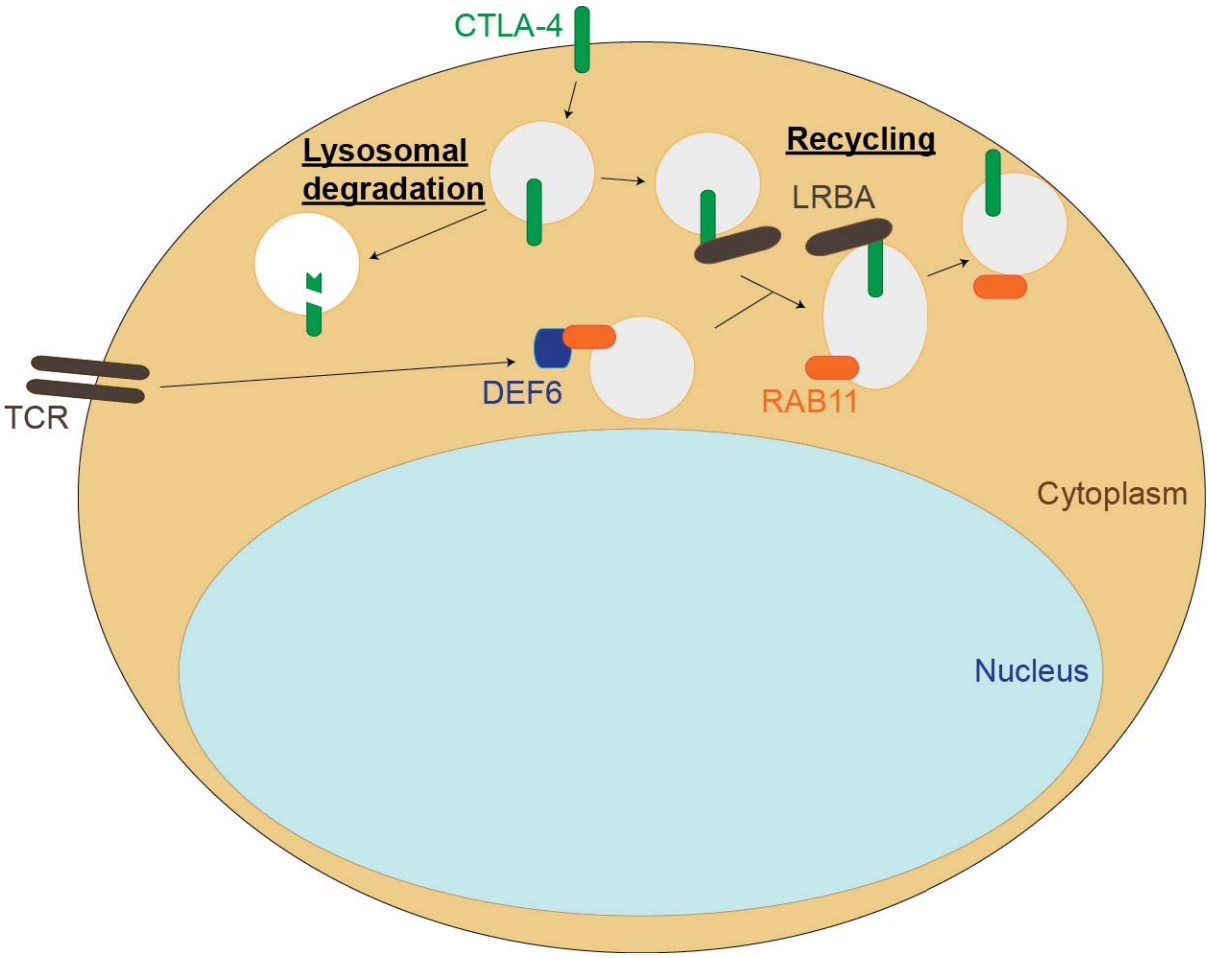


Figure 5 – Simplified potential mechanism of CTLA-4 regulation including DEF6 and LRBA.

In summary, the work performed in this thesis suggest that CTLA-4 haploinsufficiency, LRBA deficiency as well as functional DEF6 deficiency are related disease etiologies affecting a similar pathway. Strikingly, all of these diseases can be (mechanistically) linked to CTLA-4, and thus treated with CTLA-4 replacement therapy, providing a beautiful example for genome-informed precision medicine.

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Curriculum Vitae

Nina Kathrin Serwas

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Education and Practical Experience

Since Sept. 2011	Predoctoral Fellow in Boztug Laboratory, CeMM - Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria Thesis: “Identification and Molecular Characterization of Novel Immune Homeostasis Regulators in Patients with Primary Immunodeficiency and Predominant Autoimmunity”
July 2015	Research internship in the laboratory of Eric Meffre, Yale University, New Haven, CT, USA
Oct. 2006 – July 2011	Diploma study *Molecular Biomedicine*, University of Bonn, Germany (GPA: 1.2 [1 = best; 5 = failed]) Thesis: “Soluble and membrane bound Fas ligand in natural killer cells” (graded with 1.0)
Aug. 1999 – June 2006	Secondary education at Gymnasium Soltau, Germany (GPA: 1.8 [1 = best; 5 = failed])

Active Participation on Conferences

14th – 15th May 2012	European Society for Immunodeficiencies (ESID) Prague Spring Meeting 2012, Prague, Czech Republic Presentation: Heterozygous *CEBPE* mutation in a patient suffering from specific granule deficiency
13th – 14th May 2013	ESID Prague Spring Meeting 2013, Prague, Czech Republic Presentation: Massively parallel sequencing for rapid and accurate newborn screening of patients with severe combined immunodeficiencies (SCIDs)
19th – 20th June 2013	9th Young Scientist Association (YSA) PhD Symposium, Vienna, Austria Presentation: Mutations in *CEBPE* result in dramatic changes of protein content in neutrophils
13th – 14th Sept. 2013	Arbeitsgemeinschaft Pädiatrische Immunologie (Working party pediatric immunology; API) Annual Meeting 2013, Dusseldorf, Germany Presentation: Mutations in *CEBPE* result in dramatic changes of protein content in neutrophils
9th – 11th May 2014	API Annual Meeting 2014, Ittingen, Switzerland Presentation: Identification of ITK deficiency as a novel genetic cause of idiopathic CD4+ T-cell lymphopenia
11th – 12th June 2014	10th Young Scientist Association (YSA) PhD Symposium, Vienna, Austria Organization Committee

17th – 21th Jan. 2015 Midwinter Conference – Advances in Immunobiology, Seefeld in Tyrol, Austria
Poster: Identification of a hypomorphic *LRBA* mutation as a cause of an IBD-like phenotype without affecting B-cell homeostasis

6th – 9th May 2015 European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) – 48th Annual Meeting 2015, Amsterdam, The Netherlands
Presentation: Identification of a hypomorphic *LRBA* mutation as a cause of an IBD-like phenotype without affecting B-cell homeostasis

10th – 11th June 2015 11th YSA PhD Symposium, Vienna, Austria
Organization Committee

2nd – 4th Sep 2015 9th European Workshop on Immune-Mediated Inflammatory Diseases, Amsterdam, The Netherlands
Invited lecture: Identification of monogenetic defects in patients with early-onset inflammatory bowel disease pinpoints core regulators of mucosal immune homeostasis

6th – 9th Sep 2015 4th European Congress of Immunology, Vienna, Austria
Poster: Detection of rare Mendelian variants involved in basic immunological mechanisms in a cohort of 139 pediatric inflammatory bowel disease patients

29th Sep – 4th Oct 2015 7th International Conference on Autoimmunity: Mechanisms and Novel Treatments, Crete, Greece
Poster: Unraveling the genetic causes of severe autoinflammation and autoimmunity in the gut

19th – 20th Feb 2016 1st Symposium of the CeRUD – Vienna Center for Rare and Undiagnosed Diseases / 3rd International Rare and Undiagnosed Diseases Meeting, Vienna, Austria
Poster: *CEBPE*-mutant specific granule deficiency causes aberrant granule organization and substantial proteome alterations in neutrophils

12th– 13th May 2016 EU-LIFE Scientific Workshop – Inflammation & Immunity in Health and Diseases, Vienna, Austria
Poster: Primary immunodeficiency with severe multi-organ autoimmunity uncovers novel regulator of CTLA-4 trafficking in conventional activated T cells

10th – 11th June 2016 12th YSA PhD Symposium, Vienna, Austria
Organization Committee

Publications

Cipe FE, Aydogmus C, **Serwas NK**, Tuğcu D, Demirkaya M, Biçici FA, Hocaoglu AB, Doğu F, Boztuğ K. (2015) **ITK Deficiency: How can EBV be Treated Before Lymphoma?**
Pediatr Blood Cancer **62:** 2247-8

Kuloglu Z, Kansu A, **Serwas NK**, Demir A, Yaman A, Ensari A, Boztug K. (2015) **INFLAMMATORY BOWEL DISEASE-LIKE PHENOTYPE IN A YOUNG GIRL WITH PROLIDASE DEFICIENCY: A NEW SPECTRUM OF CLINICAL MANIFESTATION**.
Genet Couns **26**: 205-211

Dobbs K, Domínguez Conde C, Zhang SY, Parolini S, Audry M, Chou J, Haapaniemi E, Keles S, Bilic I, Okada S, Massaad MJ, Rounioja S, Alwahadneh AM, **Serwas NK**, Capuder K, Çiftçi E, Felgentreff K, Ohsumi TK, Pedergnana V, Boisson B, Haskoloğlu Ş, Ensari A, Schuster M, Moretta A, Itan Y, Patrizi O, Rozenberg F, Lebon P, Saarela J, Knip M, Petrovski S, Goldstein DB, Parrott RE, Savas B, Schambach A, Tabellini G, Bock C, Chatila TA, Comeau AM, Geha RS, Abel L, Buckley RH, İkincioğulları A, Al-Herz W, Helminen M, Doğu F, Casanova JL, Boztuğ K, Notarangelo LD. (2015) **Inherited DOCK2 Deficiency in Patients with Early-Onset Invasive Infections**.
N Engl J Med **372**: 2409-2422

Seidel MG, Hirschmugl T, Gamez-Diaz L, Schwinger W, **Serwas NK**, Deutschmann A, Gorkiewicz G, Zenz W, Windpassinger C, Grimbacher B, Urban C, Boztug K. (2015) **Long-term remission after allogeneic hematopoietic stem cell transplantation in LPS-responsive beige-like anchor (LRBA) deficiency.**
J Allergy Clin Immunol **135**: 1384-1390 e1381-1388

Beser OF, Conde CD, **Serwas NK,** Cokugras FC, Kutlu T, Boztug K, Erkan T. (2015) **Clinical features of interleukin 10 receptor gene mutations in children with very early-onset inflammatory bowel disease**.
J Pediatr Gastroenterol Nutr **60**: 332-338

Serwas NK, Kansu A, Santos-Valente E, Kuloğlu Z, Demir A, Yaman A, Gamez Diaz LY, Artan R, Sayar E, Ensari A, Grimbacher B, Boztug K. (2015) **Atypical manifestation of LRBA deficiency with predominant IBD-like phenotype.**
Inflamm Bowel Dis **21**: 40-47

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

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

Salzer E, Santos-Valente E, Klaver S, Ban SA, Emminger W, **Prengemann NK**, Garncarz W, 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. (2013) **B-cell deficiency and severe autoimmunity caused by deficiency of protein kinase C δ.**
Blood **121**: 3112-3116

Awards and Prizes

May 2015	Young Investigator Award of the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN)
October 2015	Aegean Conference Travel Award in recognition of an excellent research contribution

Special Commitments and Interests

Since Mar. 2009	Member of the German Society for Biochemistry and Molecular Biology (Gesellschaft für Biochemie und Molekularbiologie, GBM)
Since Oct. 2013	Member of the Young Scientist Association (YSA) Board Organization of the 10th, 11th and 12th YSA PhD Symposium (2014-2016)
Interests:	Immunology, riding motorcycles, saxophone, running, cooking