Defining the pathomechanisms of immune dysfunctions by dissecting monogenic *TGFB1* and *RASGRP1* deficiencies

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Benjamin Marquardt

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Erstgutachter: Prof. Dr. Heinrich Leonhardt
Zweitgutachterin: Prof. Dr. Bettina Kempkes

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"ALL AT PRESENT KNOWN IN MEDICINE IS ALMOST NOTHING IN COMPARISON WITH WHAT REMAINS TO BE DISCOVERED."

— RENÉ DESCARTES (1596-1650) —

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1 SUMMARY

Children with primary immunodeficiency disorders (PID) suffer from severe diseases which are characterized by an increased susceptibility to infections but may present with variable phenotypes (e.g., allergies, autoinflammation). PID are caused by rare monogenic germline mutations affecting the development and/or function of the immune system. To date, mutations in more than 430 genes have been identified to cause a PID [1]. The affected individuals often require intensive, lifelong medical care because treatment options are limited. Therefore, the elucidation of molecular mechanisms in PID is critical for the development of new treatment strategies for children with life-threatening immune diseases.

In this PhD thesis, I have characterized two monogenic entities of pediatric PID. First, we have reported biallelic loss-of-function mutations in transforming growth factor beta 1 (*TGFB1*) as a novel cause for PID. Three patients with TGF-β1 deficiency presented with severe immunodeficiency, inflammatory bowel disease (IBD) and encephalopathy. Immunological analysis revealed defective T cell differentiation and activation. Biochemical assays confirmed a disturbed secretion, stability, and bioavailability of the mutant *TGFB1* alleles. Our studies highlighted an indispensable role of TGF-β1 for intestinal immune homeostasis and neurological development in humans. Second, we have identified novel homozygous mutations in Ras guanyl releasing protein 1 (*RASGRP1*) in three children from two unrelated families. The patients showed severe immune dysregulation, including signs of autoimmunity and Epstein-Barr-Virus (EBV)-associated B cell lymphoproliferation. Immunological studies pointed to perturbed T cell development and plasticity in our RASGRP1-deficient patients, as indicated by $CD4^+$ T cell lymphopenia, abnormal T cell receptor (TCR) Vβ repertoires, reduced TCR excision circles (TREC) and increased frequencies of TCRγδ cells. As molecular pathomechanism, altered MAPK/ERK signaling could be detected in RASGRP1-deficient T cells.

In summary, we identified novel germline mutations in *TGFB1* and *RASGRP1* causing severe PID. Our findings contribute to a better understanding of pathological pathways in PID, highlighting TGF-β1 and RASGRP1 as critical regulators of immune development and homeostasis. Our studies lay the foundation for the optimization of personalized treatment for children with PID but also provide critical insights for common immune- and autoinflammatory-related disorders.

1 ZUSAMMENFASSUNG

Kinder mit primären Immundefekten (PID) zeigen häufig einen schweren Krankheitsverlauf, der im Wesentlichen durch ein erhöhtes Infektionsrisiko charakterisiert ist, aber auch heterogene Krankheitsausprägungen aufweisen kann (z.B. Allergien oder Autoinflammation). PID werden durch seltene angeborene Gendefekte, die vorwiegend die Entwicklung und/oder Funktion des Immunsystems stören, verursacht. Bis heute wurden mehr als 430 genetische Entitäten als Ursache für PID identifiziert [1]. In vielen Fällen benötigen die Erkrankten eine intensive und teilweise lebenslange medizinische Betreuung, weil nicht immer kurative Therapieoptionen zur Verfügung stehen. Daher ist die Erforschung der zugrundeliegenden molekularen Mechanismen von PID entscheidend für die Entwicklung neuer Behandlungskonzepte von Kindern mit diesen lebensbedrohlichen immunologischen Erkrankungen.

Diese Dissertation beschreibt zwei monogenetische Erkrankungen die frühkindliche PID verursachen. Im ersten Fall beschreiben wir biallelische *loss-of-function*-Mutationen im Gen *transforming growth factor beta 1* (*TGFB1*). Drei Patienten mit TGF-β1-Defizienz zeigten eine schwergradige Immunschwäche, chronische-entzündliche Darmerkrankung und Enzephalopathie. Immunologische Untersuchungen konnten einen Defekt in der Differenzierung und Aktivierung von T Zellen nachweisen. Funktionelle biochemische Experimente zeigten eine gestörte Sekretion, Stabilität und Bioverfügbarkeit der mutierten TGF-β1-Proteine. Damit verdeutlichten unsere Studien die unverzichtbare Rolle von TGF-β1 für die intestinale Immunhomöostase und neurologische Entwicklung im Menschen. Im zweiten Fall haben wir neue homozygote Mutationen im *Ras guanyl releasing protein 1* (*RASGRP1*)-Gen in drei Kindern von zwei nicht verwandten Familien identifiziert. Die Patienten zeigten einen schweren Immundefekt und entwickelten eine Autoimmunerkrankung bzw. Epstein-Barr-Virus (EBV)-assoziierte B-Zell Lymphoproliferation. Unsere Studien wiesen auf eine gestörte T-Zell Entwicklung hin, was sich durch eine reduzierte $CD4^+$ T-Zellzahl, ein abnormales *T cell receptor* (TCR) Vβ Repertoire, eine verringerte Anzahl an *TCR excision circles* (TREC) sowie eine erhöhte Frequenz von TCRγδ-positiven T-Zellen widerspiegelte. Als molekularen Pathomechanismus konnten wir einen Defekt im MAPK/ERK-Signalweg in RASGRP1-defizienten T-Zellen beobachten.

Zusammenfassend haben wir neue Keimbahnmutationen in den Genen *TGFB1* und *RASGRP1* als Ursachen für PID identifiziert. Unsere Ergebnisse tragen zu einem besseren Verständnis der Krankheitsentstehung auf molekularer Ebene in PID bei und zeigen TGF-β1 bzw. RASGRP1 als kritische Regulatoren der Immunentwicklung und -homöostase auf. Diese Studien legen das Fundament für die Optimierung von personalisierten Behandlungen für Kinder mit PID, liefern gleichzeitig aber auch wichtige grundlegende Erkenntnisse für andere häufige immunologische und autoinflammatorische Erkrankungen.

2 INTRODUCTION

2.1 Primary immunodeficiency disorders

Primary immunodeficiency disorders (PID) comprise a heterogeneous collection of inherited diseases which are commonly caused by mutations in genes involved in innate or adaptive immune mechanisms [2]. Although many rare immune disorders typically harbor a monogenic origin which follows a classical Mendelian inheritance pattern, the pathomechanisms are sometimes complex and polygenic [3]. From a clinical point of view, the majority of PID patients presents with an increased susceptibility to infections, as well as higher morbidity and mortality [4]. Nonetheless, PID phenotypes are extremely variable and may include allergy, lymphoproliferation, chronic inflammation, autoimmunity, and cancer [5]. The disease onset strongly depends on the underlying immunodeficiency and manifests mainly in early childhood, but in some cases PID can occur during adulthood [6]. Since the second half of the 20th century the number of clinically classified PID has increased steadily and up to date more than 430 disorders have been characterized [1]. Even though PID are still considered rare diseases their true incidence and prevalence remain difficult to predict [7]. Recent epidemiological studies suggest a noticeable trend pointing to a global surge in the incidence of PID, particularly in countries with increased frequency of consanguineous marriages [8-12]. The International Union of Immunological Society's (IUIS) Expert Committee on Primary Immunodeficiencies regularly provides an elaborate and up-to-date catalogue of 'inborn errors of immunity', listing genes, disease names, as well as their clinical and laboratory features [13]. Based on this catalogue, nine main categories for PID have been defined documenting the most frequently observed phenotypes: 1) combined immunodeficiencies, 2) combined immunodeficiencies with syndromic features, 3) antibody deficiencies, 4) diseases of immune dysregulation, 5) defects of phagocyte number or function, 6) defects in intrinsic and innate immunity, 7) autoinflammatory diseases, 8) complement deficiencies, and 9) phenocopies of inborn errors of immunity.

Clinically, the identification of PID is still challenging, since in many cases no clear genotype-phenotype correlations can be defined [3]. However, recent advances in next-generation sequencing (NGS) technologies have fostered the elucidation of genetic backgrounds in PID patients, converging to 10-15 newly identified PID genes per year [14]. Moreover, complementary approaches utilizing single cell transcriptome analysis [15], proteome analysis [16], multi-parameter tissue imaging [17], and mass cytometry (CyTOF) [18]

have facilitated the identification of abnormal gene expression and dysregulated pathways in immunocompromised patients allowing a more detailed categorization of PID characteristics. Since many PID constitute life-threatening conditions, early diagnosis is key to offer suitable treatment options for the affected patients. Standard therapies commonly include antimicrobial and anti-inflammatory drugs, immunoglobulin supplementation, biologicals, or other immunosuppressive agents [19-21]. Hematopoietic stem cell transplantation (HSCT) might be considered as curative treatment for the underlying immunodeficiency, with high success rates and lifelong remission [22]. Recent advances in gene editing of patient-derived hematopoietic stem cells or the use of induced pluripotent stem cells (iPSC) may provide other suitable therapy options in the future [23, 24].

2.1.1 Lymphoproliferative disorders

Individuals living with a PID routinely present with a pronounced immune dysregulation and thus many patients are subjected to a higher risk for secondary diseases, in particular malignancies [25]. The most common malignancies observed in PID comprise digestive tract cancers, virus-induced cancers, and lymphoproliferative disorders (LD), including leukemia and lymphoma [26, 27]. Interestingly, the occurrence of solid tumors in PID did not differ from the frequency observed in the general population, therefore an improper recognition of cancer cells by the host's immune system might be the main cause for malignancy in PID rather than abnormal cell growth [28]. The probability for the development of cancer in PID patients was postulated to be 10,000-fold higher than in healthy individuals [29]. Latest studies suggest an overall 8 to 11-fold increased risk for hematologic malignancies in PID with a 4-25% risk in immunodeficient children where non-Hodgkin B cell lymphoma seems to be the most prominent type [30]. Intriguingly, clinical investigations have revealed that many PID are characterized by fundamental abnormalities in the lymphoid system, what may explain the high occurrence of LD [31]. Above all, mutations in immunity-related genes contribute significantly to LD in PID patients [5], especially, when monogenic defects interfere with DNA repair mechanisms or abrogate cellular immune surveillance [25]. As another pathomechanism, oncogenic viruses such as human papilloma virus, Kaposi's sarcoma-associated herpesvirus, or Epstein-Barr virus (EBV) have been found to promote malignancy in PID, as they are capable of converting normal cells into hyperproliferating cancer cells, e.g. B cell lymphoma [32-35]. Current estimates predict that up to 98% of the world's population are positive for EBV, highlighting its extreme virulence [36, 37]. Most remarkably, PID that are characterized by dysfunctional T or NK cells, as for instance CD27 deficiency or

CD70 deficiency, often involve a high risk for EBV-induced lymphoproliferation [38]. Consequently, delicate genetic profiling of PID patients, including the functional analysis of target genes, provides valuable understanding on the (i) molecular pathomechanisms, (ii) biomarkers and predictors, and (iii) druggable targets of PID-related LD.

2.1.2 Inflammatory bowel diseases

Inflammatory bowel diseases (IBD) comprise chronic and relapsing inflammatory disorders of the gastrointestinal tract [39] with Crohn's disease (CD) and ulcerative colitis (UC) being the most common forms [40]. CD can affect any part of the gastrointestinal tract and shows a discontinuous, transmural pattern which is often accompanied by stenoses and fistulae. In contrast, UC mostly affects superficial epithelial layers of the (sub-)mucosa and is foremost confined to the colon, while emanating from the rectum [41, 42]. In certain scenarios, the clinical and endoscopic features do not specifically match to CD or UC but rather show an overlapping phenotype of both entities [43]. This indifferent form, usually referred to as 'IBD unclassified', is more frequently observed in very young patients with an onset of disease below 6 years of age. Importantly, these children often present with a more severe and refractory disease activity than adults [44].

Common symptoms of IBD comprise abdominal pain, chronic diarrhea, and gastrointestinal bleeding; in addition, the disease may present with extraintestinal manifestations affecting joints, skin, and eye [42]. The peak age of onset of classic IBD lies between the second and fourth decade of life without a gender-specific prevalence [45]. Epidemiologically, IBD have become a worldwide concern, particularly in westernized and highly industrialized regions, with a global prevalence of more than 0.3% [46, 47]. The pathogenesis of IBD is complex and not completely understood but multiple factors have been associated with disease appearance, such as environmental, microbial, immunological and genetic determinants [48] (Fig. 1). Moreover, given the diverging incidence and prevalence of IBD between countries and populations, geographical[49], ethnical[50], and socioeconomic[51] parameters are also most likely contributing to the development of IBD. Interestingly, IBD has always been speculated to retain a familial background [52] and genome-wide association studies have suggested more than 200 genetic risk loci for IBD, but causality of these loci remains elusive [53]. However, based on the discrete and severe phenotypes in children with very early onset IBD (VEO-IBD), a higher contribution of the familial background has been proposed [54, 55]. At present, more than 50 distinct monogenic defects have been identified to

cause IBD [56], in particular encompassing genes which encode for critical components of the human immune system, such as interleukin 10 (IL-10) or the IL-10 receptor [57]. *Vice versa*, many PID show an onset with a clinical presentation reminiscent of IBD, especially in children less than 2 years of age [58, 59]. Likewise, recent reports indicated that approximately 5-50% of PID patients present with a pronounced gastrointestinal disease that usually differs from classic or late onset IBD in terms of its pathogenesis and unresponsiveness to conventional treatment [60].

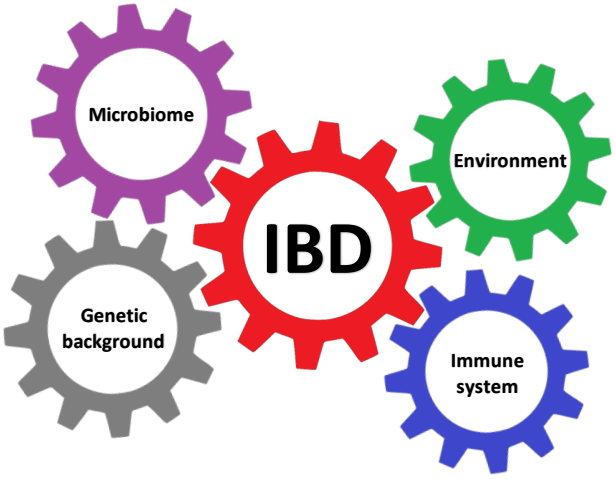

Figure 1: The pathogenesis of inflammatory bowel diseases.
IBD are a group of complex disorders and the pathogenesis is associated with immunological, environmental, and microbial determinants which are strongly influenced by the genetic background of an individual. In young children with very early onset IBD, the disease might originate from monogenic defects that mainly affect genes implicated in immune function and pathogen defense.

2.2 Transforming growth factor beta 1

In the late 1970's, Joseph De Larco and George Todaro isolated a mixture of low molecular weight polypeptides from supernatants of murine cell cultures which induced cell proliferation *in vitro* [61]. Todaro found out that human tumor cell lines released a similar type of polypeptides, which caused profound morphologic changes on rat and human fibroblasts, while this effect was completely reversible upon factor deprivation [62]. Few years later, two molecules with similar size could be purified from previously mentioned cell extracts which have been termed 'transforming growth factor alpha' (TGF-α) and 'transforming growth factor beta' (TGF-β) [63]. The classification into 'α' or 'β' was based on the interaction with the epidermal growth factor (EGF) receptor and the requirement of EGF or an EGF-like polypeptide for functional activity [64]. In 1985, an American research unit at Genentech, San Francisco was successful in sequencing and cloning the cDNA of TGF-β1 which has been isolated from human blood platelets [65]. Interestingly, Roberts *et al.* showed that TGF-β1

exhibits dichotomous functions on cell proliferation which depends on the local microenvironment, particularly on growth factors and the expression of surface receptors of a cell at a given time [66]. Based on these studies, two additional isoforms, TGF-β2 and TGF-β3, sharing up to 82% sequence homology with TGF-β1, could be discovered [67]. Remarkably, although all three isoforms are essential during pre-natal development, only TGF-β1 is critically involved in regulation of the immune system at later stages [68]. Over the last decades more than 30 structurally-related molecules of the modern TGF-β superfamily have been characterized, including bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), or activins [69]. Up to date, TGF-β1 has been considered as the prototype of this unique cytokine family, controlling a myriad of fundamental functions such as embryonic development, angiogenesis, cell differentiation, tissue repair, and immune homeostasis [70]. Moreover, TGF-β1 has been also linked to certain pathologies, in particular abnormal skeletal and connective tissue disorders, fibrosis, and cancer [71].

2.2.1 Expression, structure, and secretion of TGF-β1

TGF-β1 is expressed by a large set of cells, especially epithelium, fibroblasts, and immune cells [72]. It is originally translated as a 390 amino acid pre-pro precursor, comprising a N-terminal signal peptide, the latency-associated peptide (LAP) domain, and the C-terminal mature growth factor [73] (Fig. 2). Within the endoplasmic reticulum (ER), the signal peptide is removed and two pro-TGF-β1's are tethered together via three interchain disulfide bonds [74]. Following N-glycosylation in the trans-Golgi network, the pro-TGF-β1 homodimer is cleaved by the protease furin, resulting in the formation of the small latent complex (SLC) by non-covalent association of homodimers of LAP and mature TGF-β1 [75, 76]. In 2011, Shi *et al.* published the crystal structure of the SLC and demonstrated that mature TGF-β1 is constrained and immobilized within the LAP homodimer by the 'straitjacket domain', which not only prevents interaction of the cytokine with its cognate receptor but also protects it from rapid degradation [77] (Fig. 4a). Of note, in some cells the SLC can be conjugated with latent TGF-β binding proteins (LTBPs) to form large latent complexes (LLC) which enable extracellular storage of the cytokine by binding to components of the extracellular matrix (ECM) [78]. To date, four human isoforms of LTBPs have been identified, although only LTPB-1, -3, and -4 bind to TGF-β1 [79]. In T cells, the SLC can be similarly linked to the lymphocyte-specific surface protein GARP (glycoprotein A repetitions predominant), which plays an important role in controlling TGF-β1 bioavailability in an immunity-related context [80, 81].

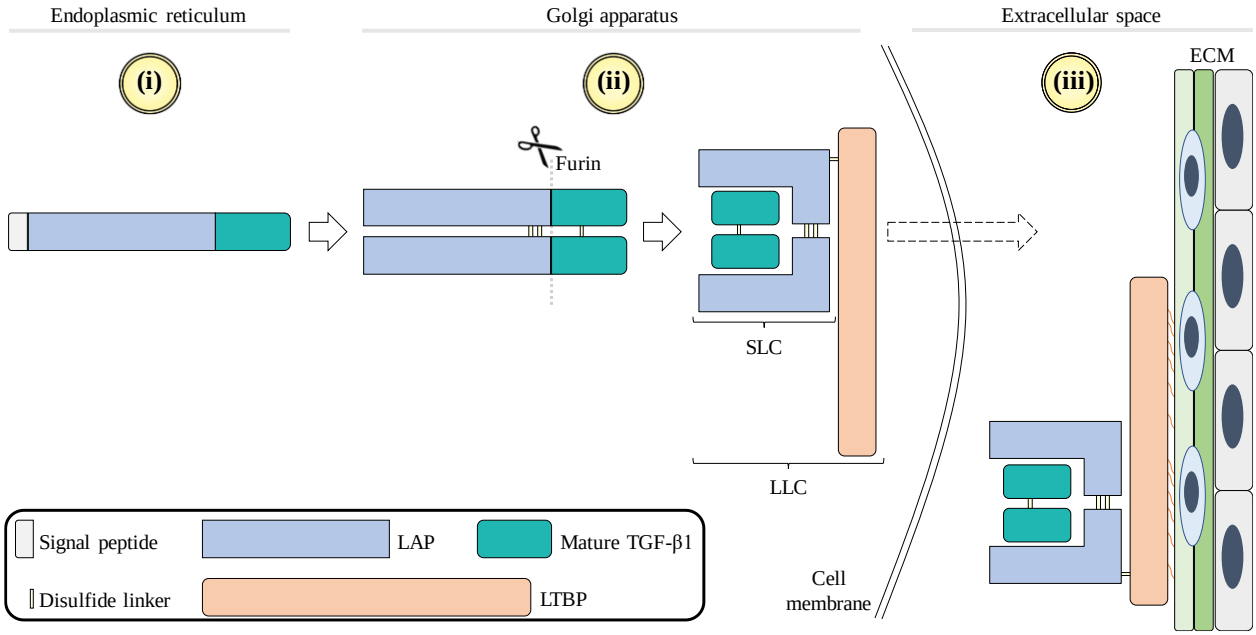

Figure 2: Assembly and secretion of TGF-β1.
(**i**) The pre-pro-TGF-β1 precursor is translated at the endoplasmic reticulum, comprising the N-terminal signal peptide, latency-associated peptide (LAP) domain and C-terminal mature growth factor. Two precursors form a pre-TGF-β1 homodimer via disulfide bonds. (**ii**) During Golgi translocation, the signal peptide is removed and dimeric pre-TGF-β1 is cleaved by the proprotein convertase furin, releasing two homodimers of LAP and mature TGF-β1 which non-covalently associate to the small latent complex (SLC). (**iii**) Prior to TGF-β1 secretion, the SLC can be covalently linked to a latent TGF-β1 binding protein (LTBP), resulting in the formation of the large latent complex (LLC) which is usually stored in the extracellular matrix (ECM).

2.2.2 TGF-β1-mediated signaling

All members of the TGF-β family signal through a dedicated transmembrane protein serine/threonine kinase receptor system comprising seven type I and five type II receptors in humans [82]. In few cases, TGF-β signaling is co-regulated by type III receptors, which lack intrinsic kinase activity but mediate binding of ligands to type II receptors [83]. With respect to TGF-β1, signals will be exclusively mediated through a unique combination of type II (TβR-II) and type I (TβR-I) receptors [84] (Fig. 3). Upon ligand binding, a conformational change in TβR-II enables its constitutively active kinase to phosphorylate threonine and serine residues in the cytoplasmic motif of a proximal type I receptor, engaging its TβR-I kinase activity [85, 86]. Consequently, activated TβR-I kinase promotes the signal by phosphorylation of cytoplasmic SMAD (small mothers against decapentaplegic [87]) proteins, which are signal-driven transcription factors [88].

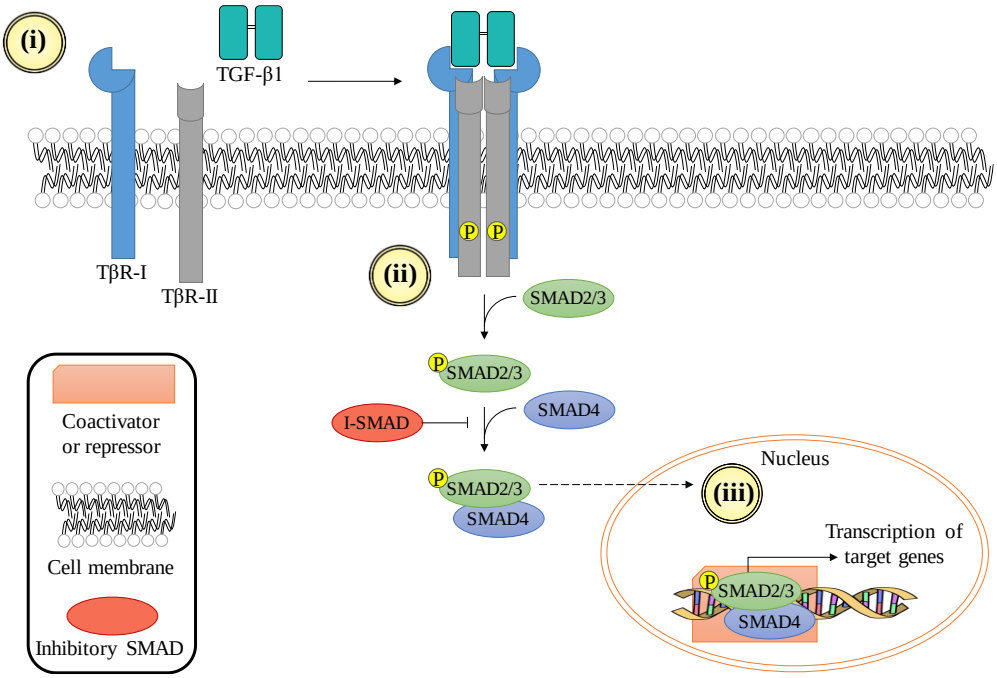

Figure 3: Canonical TGF-β1 signaling.
(**i**) Free TGF-β1 binds to a heterotetrameric TβR-II and TβR-I complex, inducing its intracellular kinase activity. (**ii**) Phosphorylation of receptor-regulated SMAD2 and SMAD3 allow the association with the common mediator SMAD4, promoting nuclear transport of the phospo-SMAD complex. (**iii**) Within the nucleus, SMAD2/3/4 complexes associate with specific DNA binding sites, activating the transcription of TGF-β1-associated target genes.

To date, eight human SMADs have been identified which are categorized based on their function into (i) receptor-regulated signal-transducing SMADs (R-SMADs 1, 2, 3, 5, and 8), (ii) the common scaffolding SMAD4, and (iii) inhibitory SMADs (I-SMADs 6 and 7) that antagonize TGF-β1 signaling [89]. In case of TGF-β1, specifically SMAD2 and SMAD3 are phosphorylated and rapidly associate with SMAD4, facilitating nuclear translocation of the SMAD2/3/4 complex that binds to dedicated DNA sequences and activates the expression of TGF-β1 target genes [83]. The specificity of TGF-β1 signaling is modulated by interaction of nuclear SMADs with lineage-determining transcription factors, including co-activators, co-repressors, or chromatin remodeling factors [88]. Accordingly, TGF-β1 is able to orchestrate the differential expression of few hundred genes in many distinct tissues [90]. In addition to the classic canonical signaling, other non-SMAD pathways can be stimulated by TGF-β1, encompassing (i) the mitogen-activating protein kinases ERK, p38, and JNK, (ii) the PI3K/AKT/mTOR pathway, or (iii) other GTPases; thus, providing additional complexity and fine-tuning of TGF-β-mediated cellular responses [91].

2.2.3 Activation of latent TGF-β1

Under physiological conditions, TGF-β1 is produced and secreted as a latent complex which prevents its interaction with the TGF-β1 receptor [92] (Fig. 2). Disassembly of this complex results in release of free TGF-β1 molecules, a process that is commonly referred to as 'TGF-β1 activation' and strongly depends on cellular and environmental settings. Activation of latent (LAP-bound) TGF-β1 can be induced by a variety of events which promote protein denaturation, e.g., heat, acidic or basic pH, mechanical force, or ionizing radiation [93]. However, the physiological significance of these mechanisms is controversially discussed [94].

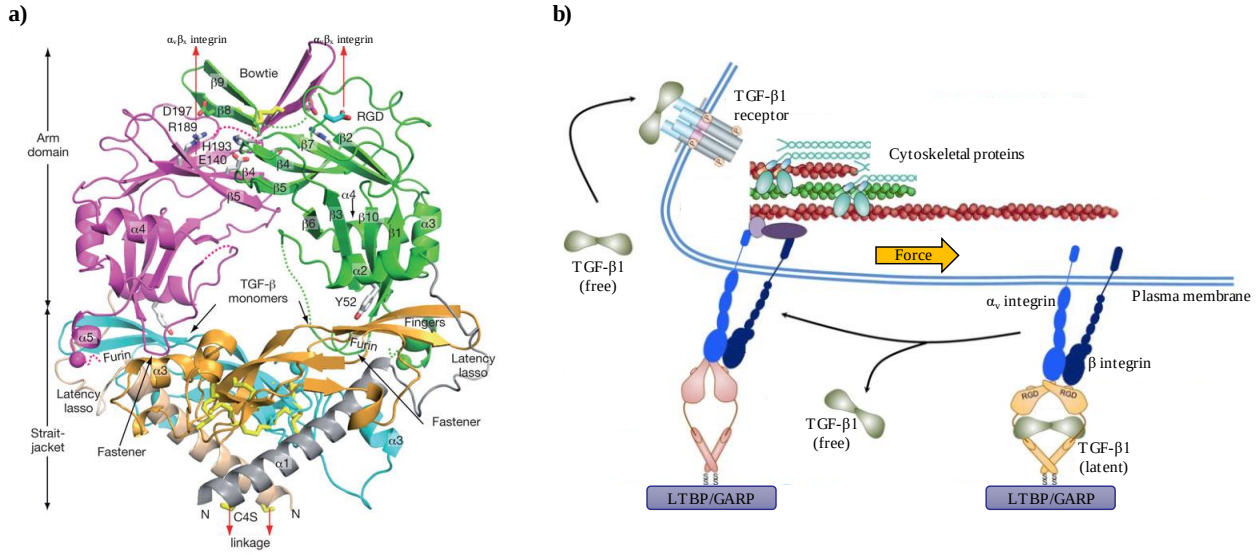

Figure 4: Structure and activation of latent TGF-β1.
a) Basic architecture of latent TGF-β1 indicating bowtie, arm, and straitjacket domains. Disordered segments are dashed, red arrows show the directions of forces during activation by integrins. Disulfide bonds are displayed in yellow and key side chains are shown in stick representation, including Asp of the RGD motif in cyan. Image adapted from Shi *et al.*, Nature 474, 2011 [77]. **b**) Simple model of TGF-β1 activation by shear force (from right to left): The LAP prodomain of latent TGF-β1 interacts via its RGD motif with $\alpha_v\beta_x$ integrins on a neighboring cell. Traction is transferred from cytoskeletal proteins towards the latent complex, resulting in release of mature (free) TGF-β1. Image adapted from Hinz B., Matrix Biology 47, 2015 [95].

On the other hand, the *in vivo* activation of latent TGF-β1 is complex and remains to some extent unresolved. Based on cleavage motif analysis, a series of proteases (e.g., matrix metalloproteinases, plasmin, thrombospondin 1, calpain, and cathepsin D) have been postulated to process the SLC under physiological conditions [74, 96, 97]. Besides proteolysis, deglycosylation, reactive oxygen species (ROS), and integrins can activate latent TGF-β1 *in vivo* [76, 93]. In particular integrins have been shown to play an elementary role for the physiological activation of complex-bound TGF-β1, since they undergo a strong interaction with a consecutive arginine-glycine-aspartic acid (RGD) sequence found in TGF-β propeptides, LTBPs, and other ECM proteins [98, 99]. Specifically integrin $\alpha_v\beta_6$ and $\alpha_v\beta_8$, which are mainly

expressed on epithelia, dendritic cells, as well as B and T lymphocytes, are predominantly involved in the activation process of latent TGF-β1 [93], whereas integrins $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$ have been shown to mediate TGF-β1 release from myofibroblasts [100]. Mechanistically, the most globally used model suggests that traction force and shear stress are transferred from the cellular actin cytoskeleton via integrins to a vicinal latent complex involving LTBPs [95] or GARP [101], resulting in relaxation of the straight jacket conformation and release of mature TGF-β1 [77] (Fig. 4b).

2.2.4 Physiological functions of TGF-β1 – A master regulator of immunity

TGF-β1 is a pleiotropic cytokine that regulates growth and tissue development but also plays a major role for immunity [102]. During embryonal development, TGF-β1 controls transformation of stem cells into functional cells of neuronal, hematopoietic, mesenchymal, and epithelial lineages, thereby giving rise to specialized body tissues and organs [69]. At later stages, TGF-β1 is pivotal for both tissue homeostasis and regeneration upon damage by controlling re-epithelialization, angiogenesis, and fibroplasia [103]. Importantly, Shull *et al.* highlighted the indispensable role of TGF-β1 for the maintenance of immune homeostasis, because *Tgfb1*$^{-/-}$ mice presented with a lethal multiorgan inflammation characterized by excessive lymphocyte activation and proliferation [104]. In consecutive studies, knockout models of crucial components involved in TGF-β1 signal transduction or cytokine activation showed overlapping autoimmune-driven inflammatory phenotypes with fatal outcomes [105, 106]. As a result, TGF-β1 was found to mediate strong anti-inflammatory effects by downregulating various pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-6, or inflammation-related chemokines [107]. In particular, TGF-β1 has been recognized as central component of immunotolerance by controlling proliferation, differentiation, activation, and survival of almost any lymphocyte lineage (Fig. 5). Depending on cell type and context, TGF-β1 can either constrain or drive lymphocyte effector functions, as it stimulates resting (immature) cells while having a proliferation-dampening influence on activated immune cells [108]. Especially T lymphocytes are highly responsive to the growth factor: TGF-β1 regulates early thymopoiesis, including positive and negative selection [109], and promotes survival and expansion of naïve T cells, natural killer T (NKT) cells [106], central memory T cells [110], as well as natural thymic and peripherally induced regulatory T cells (Tregs) [111]. Moreover, TGF-β1 supports the differentiation of naïve $CD4^+$ T cells into specialized T helper subsets such as Th9 or Th17 cells, which have elementary immune functions [93, 112, 113]. In addition to its effects on T cell development and homeostasis, TGF-β1 modulates a vast number of other

immune-related events including phagocytosis, production of immunoglobulins, and chemotaxis [105].

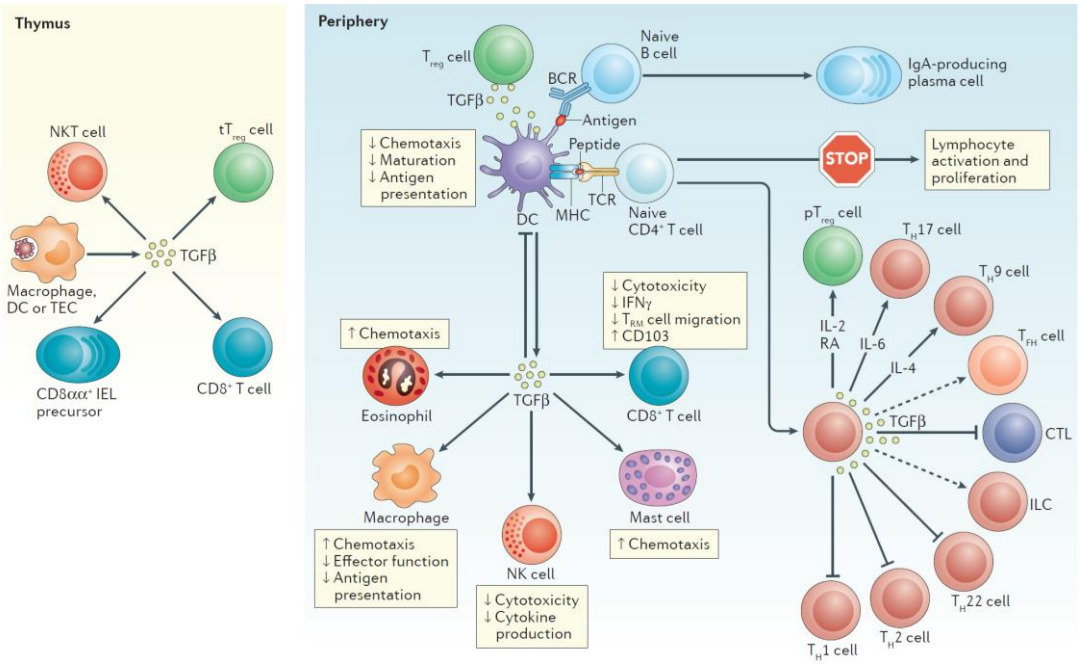

Figure 5: TGF-β1 regulates immune cells in the thymus and periphery.
a) In the thymus, TGF-β1-mediated signaling is crucial for the development of conventional T cells, Tregs, and NKT cells, but also for precursors of intraepithelial lymphocytes (IELs). **b**) In the periphery, TGF-β1 regulates the activation, proliferation and differentiation of immune cells in both lymphoid and non-lymphoid tissues where it acts in combination with other factors, such as IL-2, IL-4, IL-6 or retinoic acid (RA) to support the development of peripherally induced regulatory T cells, Th9 or Th17 cells. By contrast, TGF-β1 can also inhibit the development of effector subsets, such as Th1 or Th2 cells. In addition, TGF-β1 controls effector function and chemotaxis of other immune cells such as granulocytes or antigen-presenting cells. BCR, B cell receptor; ID, inhibitor of DNA binding; ILC, innate lymphoid cell; TCR, T cell receptor; TEC, thymic epithelial cell; T_{FH}, T follicular helper. Image adapted from Chen W. & Dijke P., Nat Rev Immunol. 16(12), 2016[105].

2.2.5 The role of TGF-β1 in human disease

Dysfunction of TGF-β1 and its signaling has been attributed to a large set of human diseases. For example, increased protein expression has been reported in IBD, atherosclerosis, cancer, and fibrosis [114-116]. Particularly in IBD, many patients showed significantly higher levels of the cytokine than healthy individuals, pointing to a malfunction in TGF-β1 homeostasis in the gut [117]. Similarly, reduced levels of phosphorylated SMAD3 and increased expression of the inhibitory SMAD7 in the inflamed intestinal mucosa of IBD patients suggested that perturbed TGF-β1 signaling contributes to the development of gastrointestinal inflammation [118]. On a genetic basis, *TGFB1* polymorphisms have been associated with an increased disease severity in cystic fibrosis [119], susceptibility for schizophrenia [120], and a greater risk for the development of cancer [121, 122]. Rare alleles in *TGFB1*, transmitted in an autosomal dominant way, are known to cause Camurati-Engelmann disease (CED), which is characterized by abundant release of active, mature

TGF-β1 due to a destabilized SLC [123]. CED patients present with atypical bone morphology (hyperostosis and sclerosis of the long bones and skull), muscular weakness, pain, and other mobility restraints [124]. Loeys-Dietz syndrome (LDS), in contrast, is associated with increased availability of free TGF-β1 but is caused by heterozygous mutations in *TGFBR1/2*, *SMAD2/3*, or *TGFB2/3* [125, 126]. LDS patients show perturbed cardiovascular, craniofacial, neurocognitive and skeletal development in association with abnormal connective tissue formation. Furthermore, autosomal dominant mutations in endoglin (*ENG*) and activin receptor-like kinase 1 (*ALK1*) result in increased TGF-β1 and vascular endothelial growth factor (VEGF) levels and have been documented in hereditary hemorrhagic telangiectasia (HHT) which is characterized by multisystemic vascular dysplasia as well as recurrent hemorrhage [127, 128]. Since both *ENG* and *ALK1* encode for constituents of the TGF-β superfamily receptor complex, HHT is thought to be the result of impaired growth factor signaling caused by compromised interaction of TGF-β ligands and the receptor complexes [129]. In addition, heterozygous mutations in *SMAD4* and the ECM protein fibrillin-1 (*FBN1*) were identified as cause for Myhre syndrome [130] and Marfan syndrome [131], respectively. Myhre patients are characterized by short stature, short hands and feet, facial dysmorphism, muscular hypertrophy, deafness and cognitive delay, as a consequence of defective TGF-β1-mediated transcriptional control. Individuals with Marfan syndrome present with a multisystem connective tissue disorder, including cardiac and skeletal abnormalities as well as vision problems, which is speculated to be a result of increased bioavailability of TGF-β1 as the mutated fibrillin-1 fails to stabilize the SLC [71]. Taken together, perturbations affecting TGF-β1 bioavailability and TGF-β signaling may cause severe developmental abnormalities in bones and connective tissue or impair psychomotor abilities. Moreover, mouse studies have highlighted the critical role of TGF-β1 in controlling immunity and inflammation.

2.3 Ras guanyl releasing protein 1

Ras guanyl releasing protein 1 (RASGRP1) belongs to a group of proteins functioning as guanine nucleotide exchange factors (GEFs) [132] which catalyze the exchange of a G-protein bound guanosine diphosphate (GDP) to guanosine triphosphate (GTP) in manifold biochemical processes [133]. Besides RASGRP1, three additional isoforms (RASGRP2-4) with a high degree of sequence conservation have been identified, and each of it interacts with distinct members of the Ras superfamily [134]. In general, Ras proteins are commonly considered as molecular on/off switches, as they cycle between active GTP-bound and inactive GDP-bound states. Accordingly, Ras proteins regulate important cellular processes such as proliferation, differentiation, and survival [135, 136]. However, the intrinsic GDP/GTP exchange rate of Ras proteins is relatively slow and consequently, the transmission of cellular signals would be limited by low nucleotide cycling turnover [137]. For that reason, GEFs, such as RASGRP1, act as catalysts to increase the GDP/GTP exchange rate by several orders of magnitude. As a result, activated GTP-bound Ras can initiate signaling via the MAPK/ERK protein kinase cascade with high efficiency [138], regulating the expression of more than 160 transcription factors such as Ets-1, c-Jun, c-Myc, and NF-κB [139].

2.3.1 Expression, structure, and activation of RASGPR1

In humans, RASGRP1 is predominantly expressed in lymphocytes as well as in some cells of the brain, kidney and skin [140]. The structure of RASGRP1 (Fig. 6) includes a catalytic region, comprising a CDC25 homology domain and Ras exchange motif (REM), which specifically interact with Ras [133]. In addition, the protein harbors a pair of calcium-binding EF hands and a C1 domain which both bind to the second messengers calcium (Ca^{2+}) and diacylglycerol (DAG) [132]. The function of the ca. 200 residual carboxy-terminal amino acids of RASGRP1, containing a coiled coil (CC) motif, has been recently shown to be critical for membrane localization, protein stability, and Ras activation [141]. Surprisingly, the crystal structure analysis of RASGRP1 revealed that the inactive molecule is preserved in an autoinhibited dimeric state, which does not allow membrane localization and Ras interaction, however, Ca^{2+} and DAG can conjointly induce conformational changes in both the CDC25 and EF hands motifs of RASGRP1, enabling Ras binding [142].

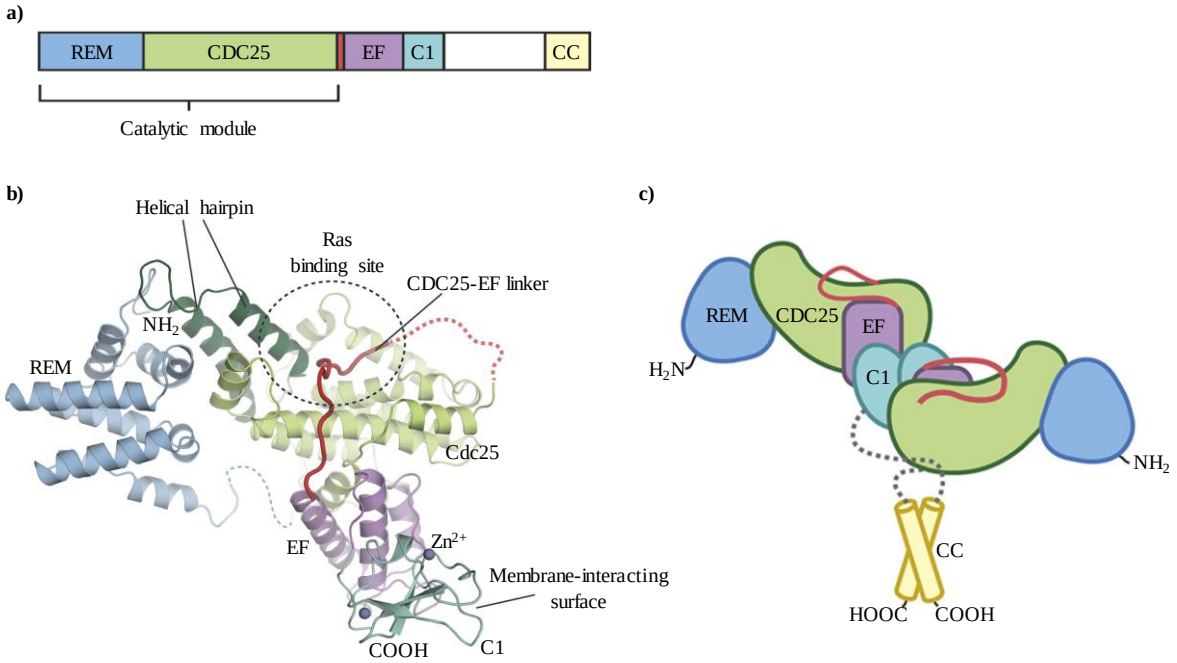

Figure 6: Crystal structure and basic protein architecture of RASGRP1.
a) The catalytic core of RASGRP1 includes the Ras exchange motif (REM) and CDC25 domain, which are followed by a regulatory module containing the EF domain, membrane binding C1 domain, and a predicted coiled coil (CC) motif. **b**) Crystal structure of the first four domains of RASGRP1. Two zinc ions in the C1 domain are shown as gray spheres. The CDC25-EF linker (red) traverses the Ras-binding site on the CDC25 domain. Linkers that could not be modeled due to poor electron density are shown with dotted lines. **c**) Dimeric, autoinhibited RASGRP1 may be stabilized by the coiled coil motifs. The residues between the C1 domain and the coiled coil (gray) are predicted to be primarily unstructured. Image adapted from Iwig *et al.*, eLife 2:e00813, 2013 [142].

2.3.2 RASGRP1-mediated signaling

30 year ago, IL-2 has been defined as potent driver of Ras signaling in T lymphocytes which is paramount for subsequent cell proliferation and differentiation [143]. Yet, the complete repertoire of molecules which are involved into T cell-specific signaling cascades, including the Ras-MAPK/ERK axis remained largely unexplored. At that time, there was consensus that Ras activation in T cells is primarily initiated via son of sevenless 1 (SOS1), a GEF which is abundantly expressed in various cell lineages [144]. Consequently, it has been proposed that SOS1 is recruited to the plasma membrane upon T cell receptor (TCR) stimulation, leading to activation of Ras by mediating exchange of GDP with GTP [145]. However, the SOS1-indepentend activation of Ras has been recently proven in T cells. First, lymphocyte cytosolic protein 2 (SLP-76) and phospholipase C gamma 1 (PLC-γ1) were shown to be essential for sustained Ras activation, because T cells deficient of these molecules were not able to signal through Ras [146]. And second, T cells subjected to DAG analogs such as phorbol 12-myristate 13-acetate (PMA) exhibited increased Ras signaling, suggesting that Ras activation requires a second messenger-mediated stimulus [147]. Based on sequence homology with other known Ras activating proteins, Ebinu *et al.* successfully identified RASGRP1 as a

potent Ras activator in T cells [132]. Here, RASGRP1 acts downstream of TCR signaling in response to increased intracellular Ca^{2+} and DAG levels [142]. Interestingly, in absence of TCR ligands, RASGRP1 is maintained in an inactive, dimeric conformation preventing DAG binding and consequently any interaction with Ras [134]. Upon TCR engagement the phosphorylation of cytoplasmic proteins and other adaptor molecules promotes an increase in intracellular pH as well as the formation of a multi-nucleated signaling complex with subsequent activation of PLC-γ1 [148, 149]. This enzyme rapidly hydrolyzes phospholipids located on the plasma membrane, generating DAG and inositol-3-phosphate, while the latter triggers the release of Ca^{2+} from the ER. Although the process is not completely understood, latest models proposed that the coordinated increase in pH, DAG, and Ca^{2+} are key for the full activation of RASGRP1 [134]. Ultimately, a conformational change in RASGRP1 activates and directs the GEF to the plasma membrane where it catalyzes the formation of active, GTP-loaded Ras [150] (Fig. 7).

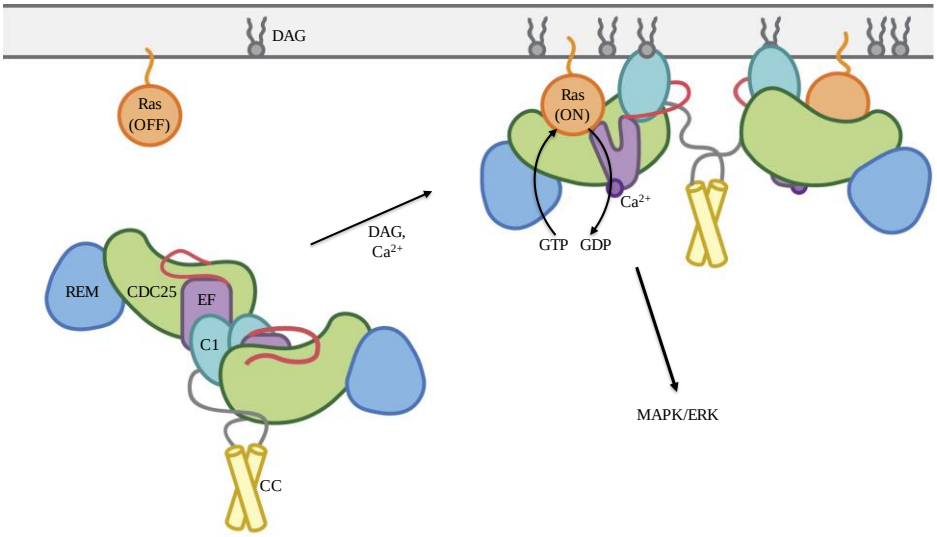

Figure 7: Model of RASGRP1 activation.
Inactive RASGRP1 (left) is stabilized by the C1-dimer interface, which prevents membrane localization and blocks the Ras binding site between the CDC25-EF linker (red). This autoinhibited form of RASGRP1 is activated by diacylglycerol (DAG) binding which disrupts C1 dimerization but allows membrane targeting, while Ca^{2+} binding to EF causes a conformational change which enables Ras interaction. Catalytically active RASGRP1 facilitates Ras-GTP formation, which drives MAPK/ERK signaling. Image adapted from Iwig *et al.*, eLife 2:e00813, 2013 [142].

2.3.3 The role of RASGRP1 in immunity

Mouse studies have demonstrated that RASGRP1 is a crucial factor for the development of T and B lymphocytes. Accordingly, genetic knockout of *Rasgrp1* in mice resulted in severe immunodeficiency characterized by pronounced T cell lymphopenia [151]. Strikingly, RASGRP1-deficient mice developed a late-onset T cell-dependent autoimmune lymphoproliferative disease associated with impaired thymocyte migration and enhanced B cell

proliferation [152-156]. In this context, RASGRP1 has been shown to exhibit pro-survival functions in T cells by facilitating sustained ERK1/2 activation [157]. MAPK signaling has been shown to be exceptionally important during early T cell maturation, because it controls the transition of thymic progenitors from double negative to double positive into single positive T cells [158-160]. Moreover, RASGRP1 has been implicated in transcriptional regulation of genes that are central for T cell proliferation and survival, such as TCRα, CD69, and Bcl-2 [161, 162]. In addition to the critical role in controlling T cell plasticity, RASGRP1 has been demonstrated to regulate the development and function of B cells [163], traditional NK [164] and invariant NKT (iNKT) cells [165], as well as the generation of TCRαβ^{+} CD8αα intraepithelial lymphocyte progenitors [166]. Thus, RASGRP1 exhibits pleiotropic functions that are fundamental for immune surveillance and pathogen clearance. Besides its immunologic relevance, Shahani *et al.* recently documented that RASGRP1 plays a role in brain formation and function, since RASGRP1-deficient mice presented with abnormal striatal motor activity associated with defective nerve cell-specific Ras signaling [167].

2.4 Aim of this study

PID are life-threatening diseases. The elucidation of the underlying molecular causes of PID are critical for the clinical diagnosis and may have implications for the management of patients. In particular, in-depth studies of perturbed immunological mechanisms in monogenic PID contribute to a better understanding of important pathways controlling development and function of the human immune system. This knowledge is of high relevance for the development of patient-specific therapies. The overall goal of this PhD thesis was to characterize patients who were suffering from severe immunodeficiency caused by biallelic germline mutations in *TGFB1* and *RASGRP1*.

Objective 1

The first objective was to analyze the underlying immunological pathomechanisms in patients with TGF-β1 deficiency who presented with acute immunodeficiency, VEO-IBD and encephalopathy. Patients were characterized by performing immunological assays on primary cells, including immunophenotyping of peripheral blood mononuclear cells (PBMC) and analysis of T cell function and activation. Secretion, stability, and bioavailability of the TGF-β1 mutant variants were assessed in heterologous cellular models by biochemical assays.

Objective 2

The second objective was to characterize three patients with novel homozygous mutations in *RASGRP1* who showed severe immune dysregulation, including autoimmunity and EBV-associated B cell lymphoma. Here, we conducted immunological studies to (i) analyze the development and distribution of immune cell subsets and (ii) investigate defects in lymphocyte-specific signaling pathways. In particular, we characterized clonal TCR diversity, performed mitogen-induced proliferation assays, and analyzed MAPK/ERK signaling in heterologous Jurkat cells overexpressing patient-specific RASGRP1 mutants.

3 RESULTS

3.1 Manuscript I: Human TGF-β1 deficiency causes severe inflammatory bowel disease and encephalopathy

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LETTERS

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Human TGF-β1 deficiency causes severe inflammatory bowel disease and encephalopathy

Daniel Kotlarz[1,17], Benjamin Marquardt[1,17], Tuva Barøy[2,3], Way S. Lee[4], Liza Konnikova[5,6,7], Sebastian Hollizeck[1], Thomas Magg[1], Anna S. Lehle[1], Christoph Walz[8], Ingo Borggraefe[1], Fabian Hauck[1], Philip Bufler[1], Raffaele Conca[1], Sarah M. Wall[5], Eva M. Schumacher[9], Doriana Misceo[2,3], Eirik Frengen[2,3], Beint S. Bentsen[9,10], Holm H. Uhlig[11], Karl-Peter Hopfner[12], Aleixo M. Muise[13,14,15], Scott B. Snapper[5,6,16], Petter Strømme[3,9] and Christoph Klein[1*]

Transforming growth factor (TGF)-β1 (encoded by *TGFB1*) is the prototypic member of the TGF-β family of 33 proteins that orchestrate embryogenesis, development and tissue homeostasis[1,2]. Following its discovery[3], enormous interest and numerous controversies have emerged about the role of TGF-β in coordinating the balance of pro- and anti-oncogenic properties[4,5], pro- and anti-inflammatory effects[6], or pro- and anti-fibrinogenic characteristics[7]. Here we describe three individuals from two pedigrees with biallelic loss-of-function mutations in the *TGFB1* gene who presented with severe infantile inflammatory bowel disease (IBD) and central nervous system (CNS) disease associated with epilepsy, brain atrophy and posterior leukoencephalopathy. The proteins encoded by the mutated *TGFB1* alleles were characterized by impaired secretion, function or stability of the TGF-β1–LAP complex, which is suggestive of perturbed bioavailability of TGF-β1. Our study shows that TGF-β1 has a critical and nonredundant role in the development and homeostasis of intestinal immunity and the CNS in humans.

TGF-β1 is translated as a precursor protein, which consists of an N-terminal signal peptide, the latency-associated peptide (LAP) and the C-terminal mature growth factor (TGF-β1). After proteolytic cleavage, LAP and TGF-β1 form the noncovalent small latent complex (SLC)[8]. The stabilization, secretion, deposition in the extracellular matrix and activation of SLCs are regulated by covalent association with latent TGF-β-binding proteins (LTBPs), resulting in formation of large latent complexes (LLCs)[9]. Multiple factors are known to control the release of active TGF-β1, for example, proteases, reactive oxygen species and integrins. Active TGF-β1 binds to a heterotetrameric transmembrane complex composed of TGF-β receptor type 1 (TGFBR1) and TGFBR2, which results in the phosphorylation of signal-transducing SMAD molecules and transcription of target genes[10].

Dysfunction of TGF-β1 signaling has been implicated in several human diseases, including cancer, cardiovascular diseases, fibrosis, atherosclerosis and developmental defects[1]. Heterozygous gain-of-function mutations in *TGFB1* are associated with Camurati–Engelmann disease (CED), which is characterized by osteosclerotic lesions in the long bones and skull[11]. Increased TGF-β1-mediated signaling due to mutations in *TGFBR1* and *TGFBR2* has been documented in patients with Loeys–Dietz syndrome, which is characterized by connective tissue disorders and arterial aneurysms[12]. Here we report that biallelic loss-of-function mutations in *TGFB1* result in very early-onset IBD and CNS dysfunction.

Patient 1 (also referred to as P1 or A.II-1), who was born to nonconsanguineous parents from Malaysia (Fig. 1a), presented in the first months of life with bloody diarrhea and subsequently developed severe perianal abscesses and fistulae. Colonoscopy confirmed the diagnosis of chronic active pancolitis associated with diffuse erythema, superficial ulcerations and multiple pseudopolyps (Fig. 1b). Histology showed crypt abscesses and inflammatory infiltrations of the epithelium with mucosal ulcerations (Fig. 1b). In addition, P1 showed eosinophilic esophagitis and esophageal candidiasis. He was refractory to nutrition regimens and intensive conventional anti-inflammatory therapy, including mesalazine, steroids, azathioprine, methotrexate, infliximab, adalimumab and tacrolimus. At the age of 4 years, a total colectomy with ileostomy was performed. P1 also showed global developmental delay associated with impaired speech and cognitive dysfunction. Generalized skeletal muscle atrophy and muscular hypotonia were present, but neither pyramidal tract signs nor evidence for movement disorders were detected. Cranial magnetic resonance imaging (MRI) indicated global brain atrophy and posterior leukoencephalopathy (Fig. 1c). Electroencephalography (EEG) analysis showed a moderate global encephalopathic pattern lacking normal background activity and continuous mixed alpha and beta activity. No interictal

[1]Dr. von Hauner Children's Hospital, Department of Pediatrics, University Hospital LMU Munich, Munich, Germany. [2]Department of Medical Genetics, Oslo University Hospital, Oslo, Norway. [3]Faculty of Medicine, University of Oslo, Oslo, Norway. [4]Department of Pediatrics, University Malaya Medical Center, Kuala Lumpur, Malaysia. [5]Division of Gastroenterology, Hepatology and Nutrition, Boston Children's Hospital, Boston, MA, USA. [6]Harvard Medical School, Boston, MA, USA. [7]Department of Pediatric and Newborn Medicine, Brigham and Women's Hospital, Boston, MA, USA. [8]Institute of Pathology, Ludwig-Maximilians-Universität München, Munich, Germany. [9]Division of Pediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway. [10]Department of Pediatric Research, Pediatric Liver Kidney Alimentary Nutrition and Transplantation Research Group, Oslo University Hospital, Oslo, Norway. [11]Translational Gastroenterology Unit and Department of Pediatrics, University of Oxford, Oxford, UK. [12]Department of Biochemistry and Gene Center, Ludwig-Maximilians-Universität München, Munich, Germany. [13]SickKids Inflammatory Bowel Disease Center and Cell Biology Program, Research Institute, Hospital for Sick Children, Toronto, Ontario, Canada. [14]Division of Gastroenterology, Hepatology and Nutrition, Department of Pediatrics, University of Toronto, Hospital for Sick Children, Toronto, Ontario, Canada. [15]Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada. [16]Division of Gastroenterology, Brigham and Women's Hospital, Boston, MA, USA. [17]These authors contributed equally: Daniel Kotlarz and Benjamin Marquardt. *e-mail: christoph.klein@med.uni-muenchen.de

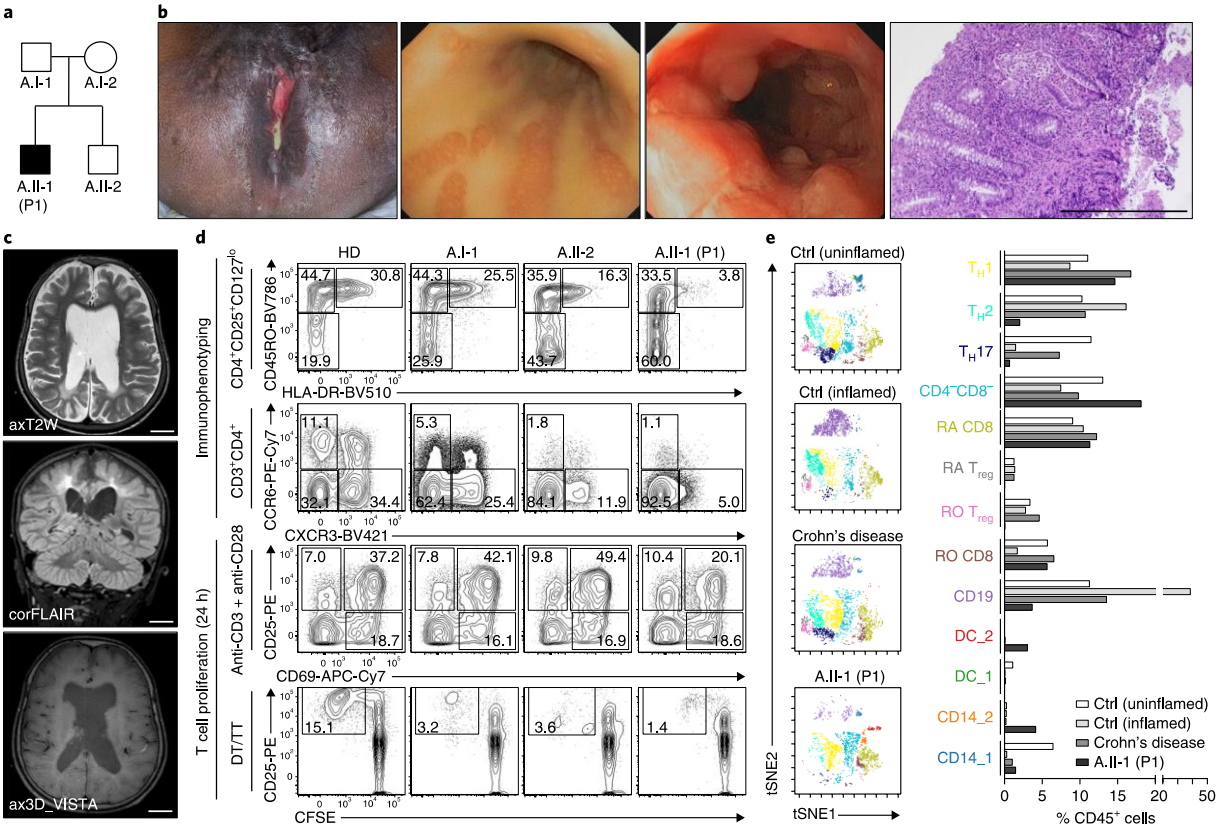

Fig. 1 | Identification of a biallelic *TGFB1* mutation in patient 1 with very early-onset inflammatory bowel disease and global neurological defects. **a**, Pedigree of patient 1 (P1, A.II-1) born to a nonconsanguineous Malaysian family (family A). **b**, Images showing severe perianal disease with purulent discharge (leftmost image) in P1, massive suppuration (second image from left) and superficial ulcerations and multiple pseudopolyps (third image from left) in the colon, as revealed by endoscopy, and crypt abscesses and inflammatory infiltrations of the epithelium with mucosal ulceration, as documented by histology (rightmost image; scale bar, 500 µm). **c**, MRI images of the brain indicating global brain atrophy and posterior periventricular leukencephalopathy in P1. Scale bars, 2 cm. **d**, Representative immunophenotypic analysis of $CD4^+CD25^+CD127^{lo}$ T_{reg} cells, $CCR6^-CXCR3^+$ T_H1 and $CCR6^+CXCR3^-$ T_H17 T cells and assessment of $CD3^+CD4^+$ T cell activation and proliferation in response to anti-CD3/anti-CD28 or specific antigens (DT/TT, diphtheria and tetanus toxoid). Immunophenotyping was performed in two independent experiments. **e**, CyTOF analysis of the composition of lamina propria mononuclear cells derived from two control (Ctrl) patients without IBD (uninflamed and inflamed), a patient with Crohn's disease and P1. Left, clusters of $CD45^+$ viSNE[27] plots were manually gated and color-coded for various populations on the basis of similar marker expression. Right, graphical representations depicting percentages of the indicated immune cell populations. CyTOF analysis was performed once owing to limited availability of patient material.

epileptic discharges were recorded. Oligoclonal IgG bands and increased levels of IL-1β and IL-8 in the cerebrospinal fluid were suggestive of inflammatory processes.

P1 had a history of recurrent upper and lower respiratory tract infections and chronic cytomegalovirus (CMV) retinitis. Laboratory studies showed leukocytosis, thrombocytosis and hypochromic anemia. Serum levels of IgG (4,044 mg/dl; normal: 576–1,507) and IgE (2,665 IU/ml; normal: <90) were high, whereas IgA and IgM levels were within normal ranges. Immunophenotypic analysis of peripheral blood mononuclear cells (PBMCs) showed decreased proportions of activated memory regulatory T (T_{reg}) cells, as well as $CCR6^-CXCR3^+$ T helper 1 (T_H1) and $CCR6^+CXCR3^-$ T_H17 T cells (Fig. 1d). T cell activation following stimulation with anti-CD3 and anti-CD28, and T cell proliferation in response to specific antigens (diphtheria and tetanus toxoid), were reduced as compared to healthy donors and first-degree relatives (Fig. 1d). Mass cytometry (CyTOF) analysis of colonic lamina propria mononuclear cells showed a decreased frequency of $CD45RO^+$ and $CD45RA^+FOXP3^+$, $CCR6^-CXCR3^-$, $CCR6^+CXCR3^-$ and $CD103^+$ T cells as compared to patients without IBD (control patients without (uninflamed) or with (inflamed) inflammation) and a patient with Crohn's disease (Fig. 1e and Supplementary Fig. 1). TGF-β1 exerts both stimulatory and inhibitory immunomodulatory effects[6]; however, we cannot exclude the possibility that some of the clinical and immunological features we observed could have been influenced by infections or drug-associated immunosuppression. P1 is currently in stable clinical condition at the age of 11 years.

To elucidate the genetic etiology, we performed whole-exome sequencing and identified a compound heterozygous mutation in *TGFB1* (ENST00000221930.5) (c.[328C>T];[1159T>C], p.[Arg110Cys];[Cys387Arg]). Segregation of the sequence variant with the disease phenotype was confirmed by Sanger sequencing, which indicated that the heterozygous mutation located in the sequence encoding the LAP domain was inherited from the mother, whereas the mutation in the sequence encoding the mature TGF-β1 domain was inherited from the father (Fig. 2a and Supplementary Fig. 2).

In contrast to CED[11], radiographs of P1 were consistent with osteopenia (data not shown).

We also identified a homozygous missense mutation in *TGFB1* (c.133C>T, p.Arg45Cys) in a second pedigree with two affected individuals (patients 2 and 3) born to consanguineous parents from Pakistan (Supplementary Fig. 3a,b). The p.Arg45Cys substitution is located in the LAP domain of the pre-pro-TGF-β1 precursor (Fig. 2a). Patient 2 (also referred to as P2 or B.II-1) had a small head circumference (2.5th centile) and bloody diarrhea at 3 months of age. Neurological development reached a plateau at 9–10 months of age and subsequently regressed. At 19 months of age, P2 developed refractory complex partial and myoclonic seizures. EEG analysis showed a pathological pattern similar to that for hypsarrhythmia. Cerebral MRI showed volume loss, cortical atrophy and thinning of the corpus callosum. Between 19 and 20 months of age, P2 started to lose her abilities to communicate and became increasingly spastic. Despite optimization of her diet via a nasogastric tube, P2 failed to thrive and died at the age of 25 months while hospitalized for suspected septicemia.

Patient 3 (also referred to as P3 or B.II-4) had microcephaly at birth (head circumference <2.5th centile). At 3 months of age, he presented with failure to thrive and bloody diarrhea. Colonoscopy and histology showed chronic active inflammation with abscesses and crypt branching (Supplementary Fig. 3c). Psychomotor regression, muscular hypotonia and hyperreflexia were diagnosed at 12 months of age. At 25 months of age, P3 had complex partial seizures and a hypsarrhythmia-like EEG pattern. Cerebral MRI examination showed gross cortical atrophy, delayed myelination and marked thinning of the corpus callosum (Supplementary Fig. 3d). He developed spasticity and contractures and lost visual and social contact, as well as the ability to perform voluntary movements. Moreover, P3 had fungal dermatitis, scabies skin infection and an episode of severe varicella infection. An influenza A viral infection triggered renal and subsequent multi-organ failure, causing his death at the age of 39 months. Immunophenotyping of PBMCs showed normal numbers and distributions for T cells, B cells and NK cells but a reduced ability of $CD4^+$ and $CD8^+$ T cells to proliferate after stimulation with anti-CD3 (data not shown).

We studied the structural consequences of the amino acid substitutions in the mutant TGF-β1 proteins by analyzing the crystal structure of latent TGF-β1 (Protein Data Bank (PDB) accession 3RJR)[13]. The substitutions we identified may perturb the interaction of TGF-β1 with the pro-domain or with the TGF-β1 cysteine knot (Fig. 2b). Arg110 maps to a region denoted as the 'fastener', which locks the interaction between the pro-domain and the growth factor domain (Fig. 2c, left). The β-sheet harboring Arg110 forms a 'super β-sheet' with the growth factor domain. Thus, both a proper stable interaction, as well as an integrin-binding-mediated release of the growth factor domain, could be affected. Arg45 maps to the interface between the TGF-β1 dimer and the pro-domain, suggesting that the substitution p.Arg45Cys alters the interaction between these two functional elements (Fig. 2c, middle). Correct folding requires the presence of the pro-domain and may therefore be affected by the p.Arg45Cys substitution. We predict that the p.Cys387Arg alteration perturbs the correct formation of the cysteine knot in the growth factor domain, as the substitution will result in an unpaired cysteine and will prevent formation of the disulfide bond to Cys322 (Fig. 2c, right). Furthermore, the unpaired Cys322 might also affect correct formation of the other disulfide bonds by disulfide scrambling. Thus, p.Cys387Arg presumably affects folding or stability of the TGF-β1 growth factor domain dimer.

To validate the predicted consequences of the mutations on the biosynthesis and function of the TGF-β1–LAP complex, we used heterologous HEK293T cells that were transduced with lentiviral particles encoding wild-type (WT) TGF-β1 or mutant TGF-β1 variants. The CED-causing TGF-β1 variant Arg218Cys was used as a control[11]. Immunoblotting of cell lysates confirmed stable expression of TGF-β1–LAP homodimers in cells that had been transduced with lentivirus expressing either WT or mutant TGF-β1 (Fig. 2d). Latent and mature TGF-β1 could be detected in conditioned medium from HEK293T cells overexpressing the TGF-β1 Arg45Cys, Arg110Cys or Arg218Cys variant. The TGF-β1 variants Arg45Cys and Arg110Cys showed reduced levels of secreted TGF-β1. In contrast, the Cys387Arg mutant could not be detected in supernatants, suggesting that abrogation of the disulfide bond prevented proper assembly and secretion. Correspondingly, ELISAs showed that (i) only the Arg218Cys variant was detected in the mature form under cell culture conditions (without acidification with HCl), indicating a gain of function for this mutant[11], (ii) mature TGF-β1 was released from the SLC after HCl treatment in the cases of the Arg45Cys and Arg110Cys variants, although at lower levels than for WT TGF-β1, and (iii) secretion of the Cys387Arg variant was completely abrogated (Fig. 2e). To analyze downstream effects on TGF-β1-mediated signaling, we examined conditioned medium in HEK293T cells expressing a SMAD-sensitive luciferase reporter. Cells expressing the Arg45Cys and Arg110Cys variants exhibited reduced luciferase activity as compared to the activity with WT TGF-β1, whereas no activity could be detected for the Cys387Arg mutant (Fig. 2f). To assess the stability of WT and mutant SLCs, we monitored re-association of LAP and TGF-β1 over time. The SLCs in supernatants from HEK293T cells were destabilized by HCl treatment and subsequently allowed to reassemble after neutralization with NaOH. In contrast to WT TGF-β1, all of the mutants showed compromised re-association capacity, suggesting reduced stability of the SLC (Fig. 2g). To assess TGF-β1 signaling in mucosal tissue, we performed CyTOF analysis on colonic biopsies from P1. As compared to patients without IBD (uninflamed and inflamed controls), the mean expression values of phosphorylated SMAD2 and SMAD3 (p-SMAD2/3) were reduced in lamina propria mononuclear $CD45^+$, $CD19^+$ and $CD3^+$ cells from P1 (Fig. 2h), whereas TGF-β1-independent STAT6 phosphorylation was normal (Supplementary Fig. 4). Reduced levels of phosphorylated SMAD2/3 were also seen in $CD45^+$ and $CD19^+$ cells from an unrelated patient with Crohn's disease, confirming impaired SMAD3 activity in the mucosal tissue of patients with IBD[14]. Taken together, all of the newly identified mutated *TGFB1* alleles seem to have deleterious consequences with respect to TGF-β1 complex formation, secretion and/or bioavailability for signal transduction, as well as direct effects on downstream SMAD2/3 signaling in vivo.

The role of TGF-β signaling in human disease has been controversial. Although increased TGF-β activity has been linked to cancer, fibrosis and progressive diaphyseal dysplasia, decreased TGF-β activity has been associated with early tumorigenesis, vascular dysplasia, developmental defects and atherosclerosis[1]. Our studies highlight a nonredundant role of TGF-β1 in controlling intestinal immune homeostasis and CNS function, whereas other organ systems apparently were not affected. These findings are reminiscent of those in patients with IL-10 or IL-10 receptor deficiency who present predominantly with infantile IBD[15,16], even though IL-10 is known to mediate pleiotropic stimulatory and suppressive functions in the immune system.

The role of TGF-β1 in immunity has previously been documented in experimental models. Mice that have a constitutive disruption of *Tgfb1*[17] or a T cell–specific deletion of *Tgfb1*[18] or that express dominant-negative TGFBR2[19] develop a lethal wasting syndrome, including severe colitis. In patients with Crohn's disease, intestinal tissue or mucosal T cells are characterized by increased activation of SMAD7, an inhibitor of TGF-β1 signaling[14]. Treatment with SMAD7-specific antisense oligonucleotides holds promise to alleviate colitis in mice[20] and in patients[21] by restoring TGF-β1 signaling.

The role of TGF-β1 in the brain is less well understood. Brionne et al. have reported that lack of TGF-β1 expression in mice

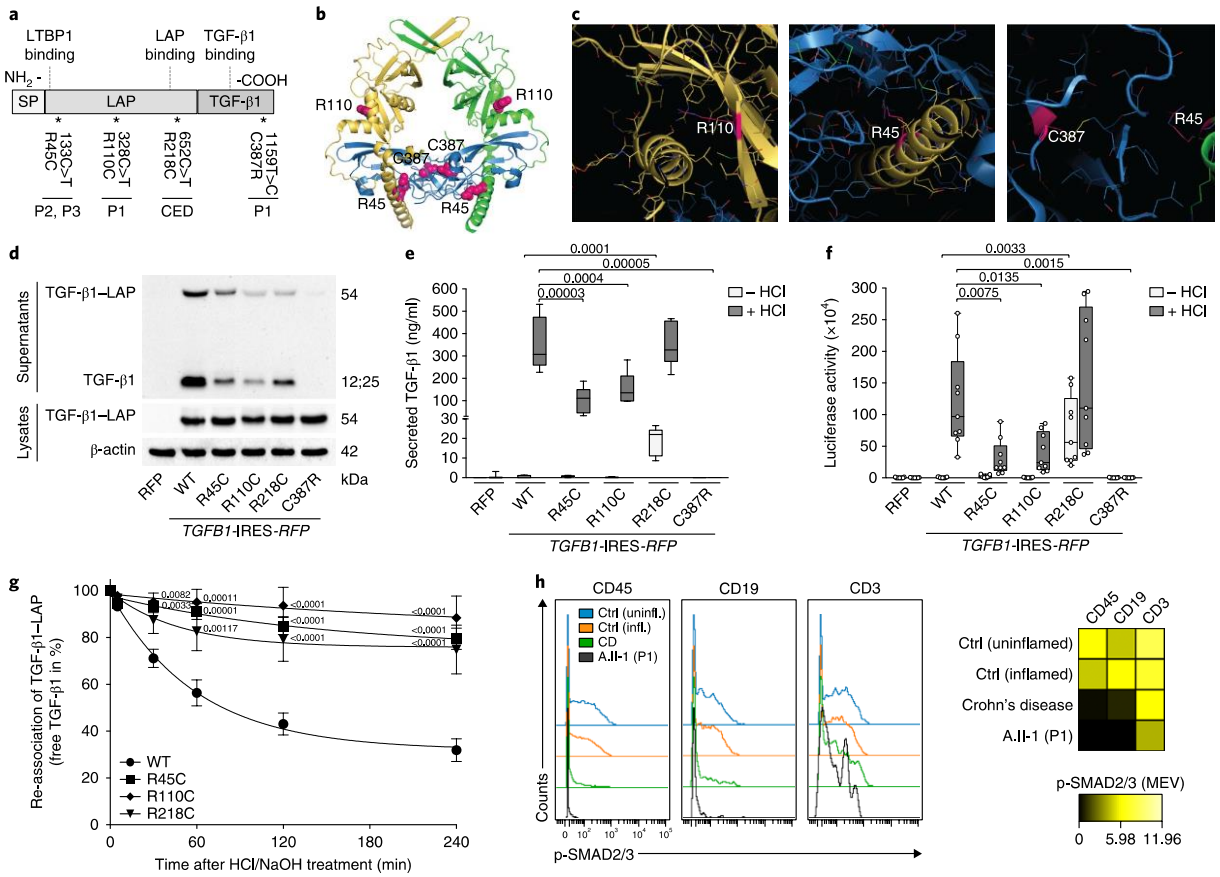

Fig. 2 | Effects of *TGFB1* mutations on the biosynthesis and bioavailability of TGF-β1. a, Schematic illustration showing the distribution of identified alterations relative to the TGF-β1 structure depicting the N-terminal signal peptide (SP), the latency-associated peptide (LAP) and the C-terminal mature growth factor (TGF-β1). The mutations identified in patients, including a previously described gain-of-function mutation in *TGFB1* causing CED[11], are depicted for the DNA and protein sequences. **b**, Structural visualization of the identified TGF-β1 alterations using the crystal structure of latent TGF-β1 (PDB accession 3RJR)[13]. The structure is depicted as a ribbon model with highlighted secondary structure. Color code: yellow and green, pro-domain dimer; blue, TGF-β1 dimer; magenta spheres, mutation sites. **c**, Detailed views of the altered sites (stick model, with altered side chains highlighted in magenta). **d**, Representative immunoblot ($n = 3$) for TGF-β1 levels in lysates and conditioned medium from HEK293T cells that stably overexpressed WT and mutant TGF-β1 variants. RFP, red fluorescent protein; R45C, TGF-β1 Arg45Cys; R110C, TGF-β1 Arg110Cys; R218C, TGF-β1 Arg218Cys; C387R, TGF-β1 Cys387Arg. **e**, ELISA determining the TGF-β1 levels in conditioned medium from HEK293T cells that stably overexpressed WT and mutant TGF-β1 variants ± HCl treatment for the release of mature growth factor from latent complexes. Samples of ten biologically independent cell culture experiments were analyzed. Box-and-whisker plots: center line, median; box limits, upper and lower quartiles; whiskers, quartile range. *P* values (indicated in the graphs) were calculated using a two-tailed unpaired *t* test with Welch's correction. **f**, SMAD-dependent luciferase reporter assays in HEK293T cells that were stimulated with conditioned medium from cells stably overexpressing WT or mutant TGF-β1. Samples of nine biologically independent cell culture experiments were analyzed. Box-and-whisker plots: center line, median; box limits, upper and lower quartiles; whiskers, quartile range; individual data points as overlays. *P* values were calculated using a two-tailed unpaired *t* test with Welch's correction. **g**, Analysis of the re-association capacity of mutant TGF-β1 variants and LAP over time. Data shown represent the means ± s.e.m. of 11 independent cell culture experiments. *P* values were calculated using two-way repeated-measures ANOVA with Dunnett's correction for multiple comparisons. **h**, CyTOF analysis of SMAD2/3 phosphorylation (p-SMAD2/3) in lamina propria mononuclear cells derived from patients without IBD (uninflamed, inflamed), a patient with Crohn's disease (CD) and P1 (A.II-1). Histogram plots of baseline p-SMAD2/3 levels (left) and median expression values (MEV) of p-SMAD2/3 (right) are shown for the indicated hematopoietic cell (CD45⁺, CD19⁺ or CD3⁺) populations. CyTOF analysis was performed once owing to limited availability of patient material.

results in neuronal cell death and microgliosis[22]. Tissue-specific deletion of *Tgfb1* in the mouse CNS prevents lethal hyperinflammation but leads to progressive defects in synaptic plasticity and loss of microglia[23]. Decreased plasma levels of TGF-β1[24] and reduced neuronal expression of TGFBR2[25] have been documented in patients with Alzheimer's disease. Genetic polymorphisms altering TGF-β1 expression have been associated with increased risk for conversion of mild cognitive impairment in patients with Alzheimer's disease[26]. Our studies suggest that TGF-β1 may have a neuroprotective role, but the mechanisms remain unknown.

Human TGF-β1 deficiency is a life-threatening disease, yet clinical management remains challenging. In view of the documented role of TGF-β1 in T cells, allogeneic hematopoietic stem cell transplantation might be considered to alleviate intestinal inflammation. We opted not to pursue this approach given the severe neurological comorbidities. Substitution with recombinant TGF-β1 may represent an

alternative experimental approach, but currently no such product is available for therapeutic use, and controlling tissue- and context-specific bioavailability of TGF-β1 is challenging, in particular in the CNS.

In conclusion, our study demonstrates a nonredundant role of TGF-β1-mediated signaling for intestinal immune homeostasis and neurological development in humans.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41588-018-0063-6.

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Author contributions

D.K. and C.K. designed and directed the study, managed recruitment of study participants, obtained clinical samples, supervised B.M. and interpreted the data; B.M. conducted and analyzed functional assays on heterologous cellular models; D.M., E.F. and P.S. supervised T.B. and E.M.S., initiated genetic analysis and drafted the clinical report of P2 and P3, and provided critical revision of the manuscript; T.B. acquired and interpreted genetic data from P2 and P3; R.C. conducted immunophenotypic analysis of PBMCs; T.M. and A.S.L. performed functional immunological assays; S.M.W. performed CyTOF analysis; L.K. supervised S.M.W. and analyzed the CyTOF results; S.H. performed the bioinformatics analysis of sequencing data; K.-P.H. conducted structural analysis of protein variants encoded by the identified *TGFB1* mutations; W.S.L., I.B., F.H., P.B., E.M.S. and B.S.B. cared for the patients, collected patient samples and drafted clinical reports; C.W. examined histology; H.H.U. provided clinical information and a specimen from a patient with CED; A.M.M. and S.B.S. screened local cohorts of patients with very early-onset inflammatory bowel disease for mutations in *TGFB1* and were instrumental in the interpretation of the human data; C.K. provided laboratory resources; and D.K. and C.K. wrote the manuscript with help from B.M. The manuscript was reviewed and approved by all co-authors.

Competing interests

The authors declare no competing interests.

Additional information

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Methods

Patients. Patients were originally identified by the Departments of Pediatrics at the Faculty of Medicine, University Malaya, Kuala Lumpur, Malaysia (family A) or Oslo University Hospital, Norway (family B), and they were referred for further studies to the Dr. von Hauner Children's Hospital at the Ludwig-Maximilians-Universität (LMU) München, Germany. Peripheral blood samples and biopsies from patients and their unaffected first-degree relatives and from healthy volunteers for genetic and functional experiments, as well as photographs of patients for publication, were obtained upon written consent. The investigation was performed in accordance with current ethical and legal frameworks, and the study protocols were approved by the Institutional Review Boards at the LMU (#66-14) and by the Health South-East Regional Ethics Committee, Norway.

Whole-exome sequencing. Genomic DNA from patients and parents was isolated using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. After enrichment for all coding exons using the SureSelect Human All Exon Kit (Agilent Technologies), sequencing was performed on an Illumina Genome Analyzer II (family A) or Illumina HiSeq 2000 (family B). Short paired sequence reads were mapped to the human reference genome GRCh37 with BWA[28]. Genome Analysis Tool Kit (GATK)[29] was used to analyze the WES data, and functional annotation was performed with snpEff[30] and Variant Effect Predictor (VEP) using Ensembl[31] release 85 (family A) or 71 (family B). WES data were filtered and analyzed using an in-house SQL database (family A) or FILTUS v.0.99-934 (family B)[32]. Rare variants were distinguished by incorporating frequency information from the 1000 Genomes Project[33], NHLBI GO Exome Sequencing Project (ESP; http://evs.gs.washington.edu/EVS/) and/or ExAC[34]. Effects of filtered variants were predicted with a multitude of software, including snpEff[30], VEP[31], SIFT[35] and PolyPhen-2[36]. The remaining variants were compiled and filtered for rare homozygous and compound heterozygous mutations following a pattern of autosomal recessive inheritance.

DNA sequencing. Genomic DNA from patients, parents and healthy siblings was isolated using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. Segregation of identified mutations in *TGFB1* was confirmed in available family members in families A and B by DNA Sanger sequencing. Primer sequences are listed in Supplementary Table 1.

Sanger sequencing was done in house on a Hitachi 3130× genetic analyzer or by GATC Biotech, Konstanz, Germany. The sequence reads were analyzed using the DNASTAR Lasergene software.

Electroencephalography and magnetic resonance imaging. 24-channel EEG recordings using Xltek hardware and software equipment (Natus DBA, Excel-Tech Corp.) was performed using standard adjustments (0.5-Hz low-frequency filter, 70-Hz high-frequency filter, resistance 5–10 kΩ). MRI of the brain was obtained using a 3-Tesla high-resolution scanner (1.0- to 1.5-mm slices, T1 (longitudinal relaxation time) with and without gadolinium contrast enhancement, T2 (transverse relaxation time) and fluid attenuated inversion recovery techniques) in axial, sagittal and coronal planes (Philips Ingenia).

Structural analysis of TGF-β1 mutants. Structural visualization and modeling of the amino acids encoded by the identified *TGFB1* mutations was performed with PyMol (Schrödinger, LLC).

Construction of expression vectors, cell culture, transfection and lentiviral transduction. Human WT *TGFB1* was amplified from a Mammalian Gene Collection (MGC) sequence-verified cDNA clone (cat. no. MHS6278-202757887, accession: BC022242, Dharmacon GE Healthcare). Mutations in *TGFB1* (encoding Arg45Cys, Arg110Cys, Arg218Cys or Cys387Arg) were introduced by site-directed PCR mutagenesis using corresponding primer pairs. WT and mutated *TGFB1* cDNAs were cloned into the IRES-EGFP or IRES-RFP bicistronic lentiviral pRRL vectors.

Biochemical assays were performed on HEK293T cells (ATCC) that routinely tested negative for Mycoplasma contamination. For production of WT or mutant TGF-β1, HEK293T cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin–streptomycin and 200 mM L-glutamine (all from Gibco, Life Technologies) in a humidified incubator at 37 °C with 5% CO_2 and were transduced with lentiviruses according to previously published protocols[37]. Briefly, vesicular stomatitis virus G glycoprotein (VSV-g)-pseudotyped lentiviral particles were generated by transfection of HEK293T cells. Using polyethyleneimine (PEI; Polysciences) as a transfection agent, cells were incubated with 5 µg lentiviral vector, 12 µg pcDNA3.GP.4xCTE (which expresses HIV-1 gag–pol), 5 µg pRSV-Rev and 1.5 µg pMD.G (which encodes VSV-g) in the presence of 25 µM chloroquine (Sigma) for 12 h. Supernatants containing viral particles were collected every 24 h for 72 h and concentrated by ultracentrifugation. Viral titration was performed on HEK293T cells and viral concentrations were determined by flow cytometry. Next, HEK293T cells (at 60–80% confluency) were transduced with lentiviral particles in the presence of polybrene (8 µg/ml) for 6–12 h. To establish stable cell lines, transduced cells were sorted based on EGFP or RFP expression using a BD FACSAria cell sorter (BD Bioscience).

FACS and immunophenotyping. For immunophenotypic analysis, blood samples were washed with PBS and stained with monoclonal antibodies, as indicated in Supplementary Table 2. Red blood cells were lysed by 1× BD FACS Lysing Solution (BD Biosciences) according to the manufacturer's instructions. The samples were acquired using a LSRFortessa Flow Cytometer (BD Bioscience), and data were analyzed using FlowJo Software (TreeStar). Gating strategies are shown in Supplementary Fig. 5.

CyTOF analysis. Colonic tissue was digested overnight on a shaker at 37 °C in complete RPMI medium (Gibco, Life Technologies) with 2 µl of collagenase and 2 µl of DNase per 10 ml of medium. Undigested material was filtered out using a 10-µm filter. Single cells were resuspended in CyTOF staining buffer, and 1×10^6 to 2×10^6 cells/sample were prepared for CyTOF analysis according to the Fluidigm protocol with minor modifications. Briefly, cells were stained with Rh103 as a viability dye, washed, blocked with Fc Block and incubated with the cocktail of metal-coupled antibodies specific for surface molecules for 30 min. Next, cells were fixed in 1.6% formaldehyde and treated with isopropanol for detection by the phospho-specific antibodies or were permeabilized with the FOXP3/Transcription Factor Staining Buffer Set (eBioscience) for staining with a cocktail of intracellular antibodies. Cells were then re-fixed in 1.6% formaldehyde and stained with Ir-DNA intercalator solution (Fluidigm). Finally, cells were resuspended in water containing a 1:10 dilution of EQ beads and run on a Helios CyTOF machine, Fluidigm, at the Harvard Medical School (HMS) CyTOF Core. Antibodies used for CyTOF analysis are summarized in Supplementary Table 3. Antibodies not purchased from Fluidigm were conjugated at the HMS CyTOF core. Data were analyzed using the Premium CyTOBANK cloud-based software. Gating strategies are shown in Supplementary Fig. 6.

Protein blot analysis and ELISA. To study TGF-β1–LAP biosynthesis and secretion, cell lysates and supernatants of HEK293T cells overexpressing WT and mutant TGF-β1 were analyzed by immunoblotting and ELISA following standard protocols. Briefly, 0.5×10^6 HEK293T cells and their derivatives were cultured in 1 ml of FBS-containing DMEM. After incubation for 12 h, cell lysates or supernatants were fractionated under reducing conditions by SDS–PAGE. Proteins were blotted onto polyvinylidene difluoride membranes using the Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked in 5% skim milk before staining. Antibodies used for detection are indicated in Supplementary Table 4. Membranes were developed using a chemiluminescent substrate (Thermo Fisher Scientific). Images were captured using a ChemiDoc XRS+ System (Bio-Rad). Uncropped immunoblots are shown in Supplementary Fig. 7.

TGF-β1 levels in serum samples and cellular supernatants were measured by using the Human TGF-β1 ELISA DuoSet (DY240, R&D Systems) according to the manufacturer's instructions. To release the mature TGF-β1 from latent complexes, conditioned medium was treated with 1 N HCl for 10 min, followed by neutralization with a solution containing 1.2 N NaOH and 0.5 M HEPES. Supernatants were analyzed in duplicate by using a Synergy H1 microplate reader (BioTek Instruments).

TGF-β1-sensitive firefly luciferase reporter assays. A lentiviral TGF-β1-sensitive firefly luciferase reporter plasmid was designed by insertion of the SMAD response elements (CAGA)[38] into the pGreenFire1-mCMV vector (#TR010PA-1-SBI, Biocat) between the EcoRI and SpeI restriction sites. HEK293T cells were transduced with the reporter system as described above.

HEK293T cells overexpressing WT or mutant TGF-β1 were plated at a density of 1×10^6 cells per well of a 12-well plate in 1 ml of serum-supplemented DMEM. After 4–6 h of incubation, cells were rinsed with PBS, and medium was replaced with 1 ml of serum-free minimal essential medium (MEM; Gibco, Thermo Fisher) to avoid the potential influence of TGF-β1 contained in FBS. Conditioned medium from cultured cells was harvested after 12 h. To measure TGF-β1-mediated SMAD signaling activity of the identified mutants, 0.5×10^6 HEK293T cells encoding the firefly luciferase reporter were plated in 0.5 ml of serum-supplemented DMEM in each well of a 48-well plate. After 4–6 h of incubation, cells were rinsed with PBS, and medium was replaced with 1 ml of serum-free MEM. Conditioned medium was added to the reporter cell line in both the native and HCl-activated forms. Stimulated reporter cells were incubated for 12 h at 37 °C and subsequently lysed and assayed for firefly luciferase activity using the Firefly and *Renilla* Dual Luciferase Assay Kit (#30005, Biotium, USA) according to the manufacturer's instructions. Briefly, 45 µl of lysate from samples was transferred in duplicate to a 96-well luminometry plate (NUNC) and mixed with 80 µl of firefly working solution. Luminescence signals were measured for a period of 10 s.

TGF-β1–LAP re-association assays. To examine the stability of latent complexes for the proteins encoded by the identified *TGFB1* mutations, we assessed re-association of TGF-β1 and LAP after complex disruption in a time-dependent manner, as described previously[39]. To release the mature TGF-β1 from latent complexes, conditioned medium from transduced HEK293T cells was acidified with 1 M HCl for 10 min at room temperature and neutralized with 1.2 M NaOH and 0.5 M HEPES. After neutralization, samples were incubated at 37 °C for 5, 30, 60, 120 and 240 min, and levels of free TGF-β1 were analyzed by ELISA. These were plotted by applying a one-phase exponential decay data transformation using GraphPad Prism software (GraphPad Software).

Statistical analysis. Statistical evaluation of experimental data was performed using Prism version 6 (GraphPad Software). No method of randomization or blinding was used, and no samples were excluded from analysis. Data in Fig. 2e,f are reported as box-and-whisker plots, with the median (center line), upper and lower quartiles (box limits) and quartile range (whiskers) indicated. Data in Fig. 2g are means ± s.e.m. To analyze quantitative datasets, either a two-tailed unpaired *t* test with Welch's correction to account for unequal variances (Fig. 2e,f) or two-way repeated-measures ANOVA with Dunnett's correction for multiple comparisons (Fig. 2g) was performed. All tests were two-tailed, and *P* values <0.05 were considered to be statistically significant. Sample numbers are referred to as *n* unless indicated otherwise. Gaussian distribution of the data was confirmed by D'Agostino and Pearson's omnibus normality test. No statistical method was used to predetermine sample size for analyses.

Life Sciences Reporting Summary. Further information on experimental design is available in the Life Sciences Reporting Summary.

Data availability. The identified *TGFB1* mutations have been submitted to the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/) with accessions SCV000678250 [c.328C>T], SCV000678251 [c.1159T>C] and SCV000622112 [c.133C>T]. Information on the raw whole-exome sequencing data supporting the findings of this study are available from the corresponding author upon request. These data will not be publicly available as they contain information that could compromise research participant privacy.

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SUPPLEMENTARY INFORMATION

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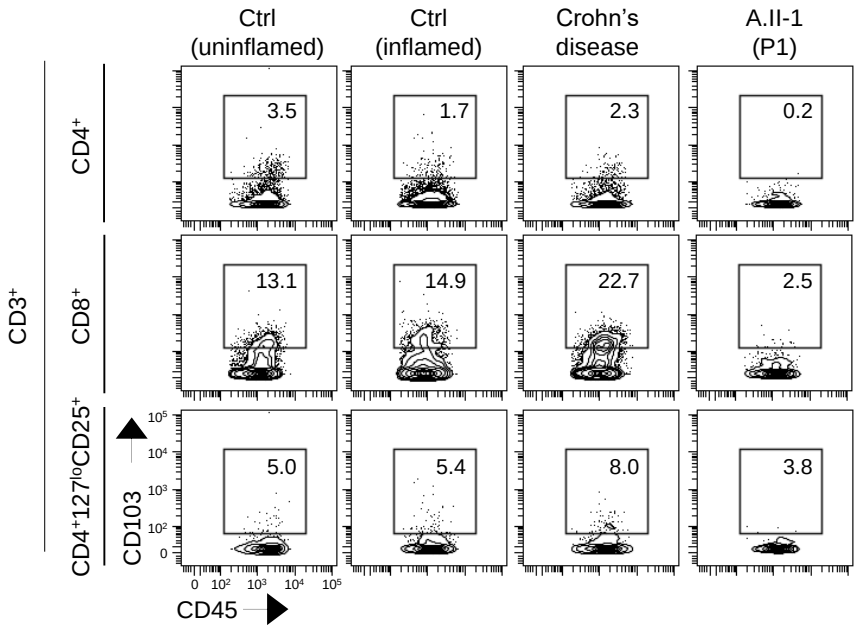
In the format provided by the authors and unedited.

Human TGF-β1 deficiency causes severe inflammatory bowel disease and encephalopathy

Daniel Kotlarz[1,17], Benjamin Marquardt[1,17], Tuva Barøy[2,3], Way S. Lee[4], Liza Konnikova[5,6,7], Sebastian Hollizeck[1], Thomas Magg[1], Anna S. Lehle[1], Christoph Walz[8], Ingo Borggraefe[1], Fabian Hauck[1], Philip Bufler[1], Raffaele Conca[1], Sarah M. Wall[5], Eva M. Schumacher[9], Doriana Misceo[2,3], Eirik Frengen[2,3], Beint S. Bentsen[9,10], Holm H. Uhlig[11], Karl-Peter Hopfner[12], Aleixo M. Muise[13,14,15], Scott B. Snapper[5,6,16], Petter Strømme[3,9] and Christoph Klein[1]*

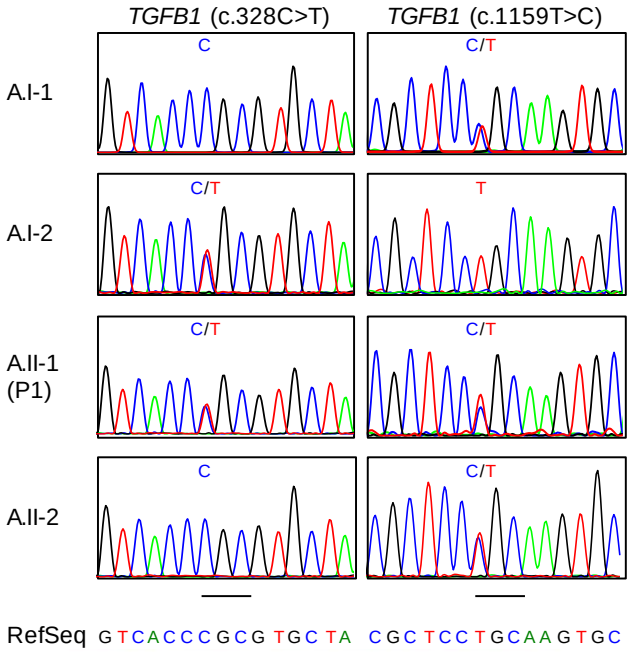
[1]Dr. von Hauner Children's Hospital, Department of Pediatrics, University Hospital LMU Munich, Munich, Germany. [2]Department of Medical Genetics, Oslo University Hospital, Oslo, Norway. [3]Faculty of Medicine, University of Oslo, Oslo, Norway. [4]Department of Pediatrics, University Malaya Medical Center, Kuala Lumpur, Malaysia. [5]Division of Gastroenterology, Hepatology and Nutrition, Boston Children's Hospital, Boston, MA, USA. [6]Harvard Medical School, Boston, MA, USA. [7]Department of Pediatric and Newborn Medicine, Brigham and Women's Hospital, Boston, MA, USA. [8]Institute of Pathology, Ludwig-Maximilians-Universität München, Munich, Germany. [9]Division of Pediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway. [10]Department of Pediatric Research, Pediatric Liver Kidney Alimentary Nutrition and Transplantation Research Group, Oslo University Hospital, Oslo, Norway. [11]Translational Gastroenterology Unit and Department of Pediatrics, University of Oxford, Oxford, UK. [12]Department of Biochemistry and Gene Center, Ludwig-Maximilians-Universität München, Munich, Germany. [13]SickKids Inflammatory Bowel Disease Center and Cell Biology Program, Research Institute, Hospital for Sick Children, Toronto, Ontario, Canada. [14]Division of Gastroenterology, Hepatology and Nutrition, Department of Pediatrics, University of Toronto, Hospital for Sick Children, Toronto, Ontario, Canada. [15]Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada. [16]Division of Gastroenterology, Brigham and Women's Hospital, Boston, MA, USA. [17]These authors contributed equally: Daniel Kotlarz and Benjamin Marquardt. *e-mail: christoph.klein@med.uni-muenchen.de



Supplementary Figure 1

CD103 expression in lamina propria T cells from patient 1

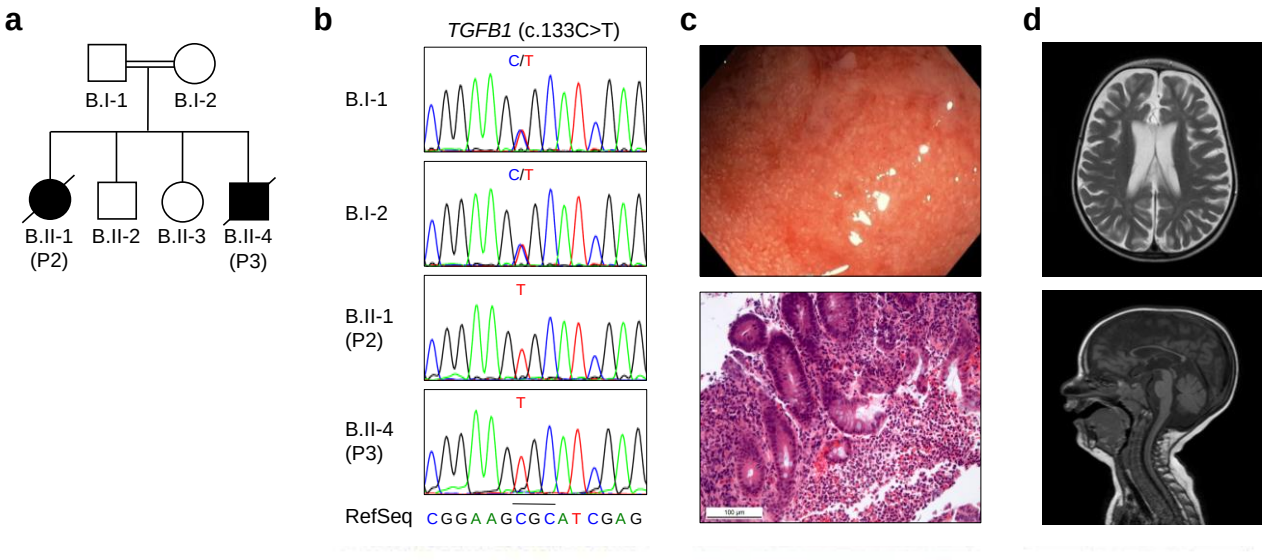
CyTOF analysis of CD103 expression in colonic lamina propria mononuclear cells derived from patients without IBD (controls: uninflamed; inflamed), a patient with Crohn's disease and P1. The percentage of CD103-expressing cells is shown for $CD3^+CD4^+$ (top), $CD3^+CD8^+$ (middle) and $CD127^{lo/-}CD25^+$ (bottom) T cells.



Supplementary Figure 2

Sanger sequencing of patient 1

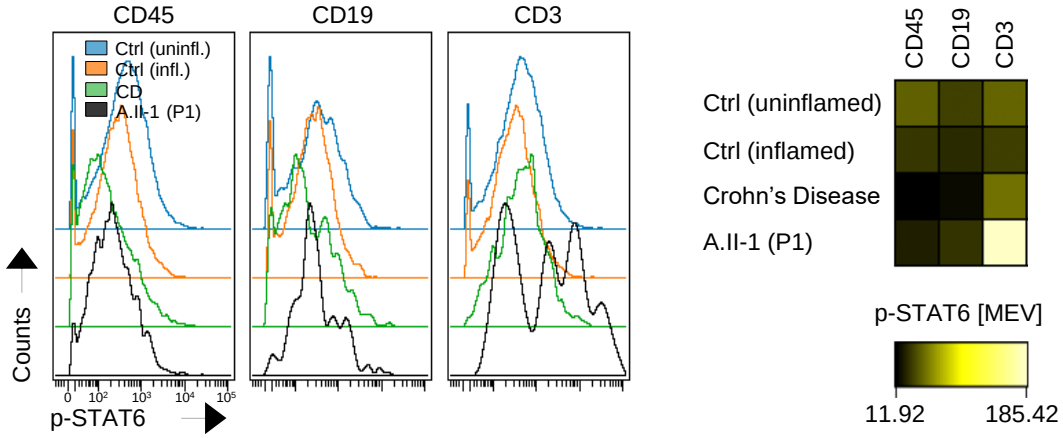
Chromatograms of DNA Sanger sequencing identifying a compound heterozygous mutation in *TGBF1* that segregates with the disease phenotype in P1.



Supplementary Figure 3

Clinical phenotype and mutational analysis of TGF-β1 deficiency in patients 2 and 3

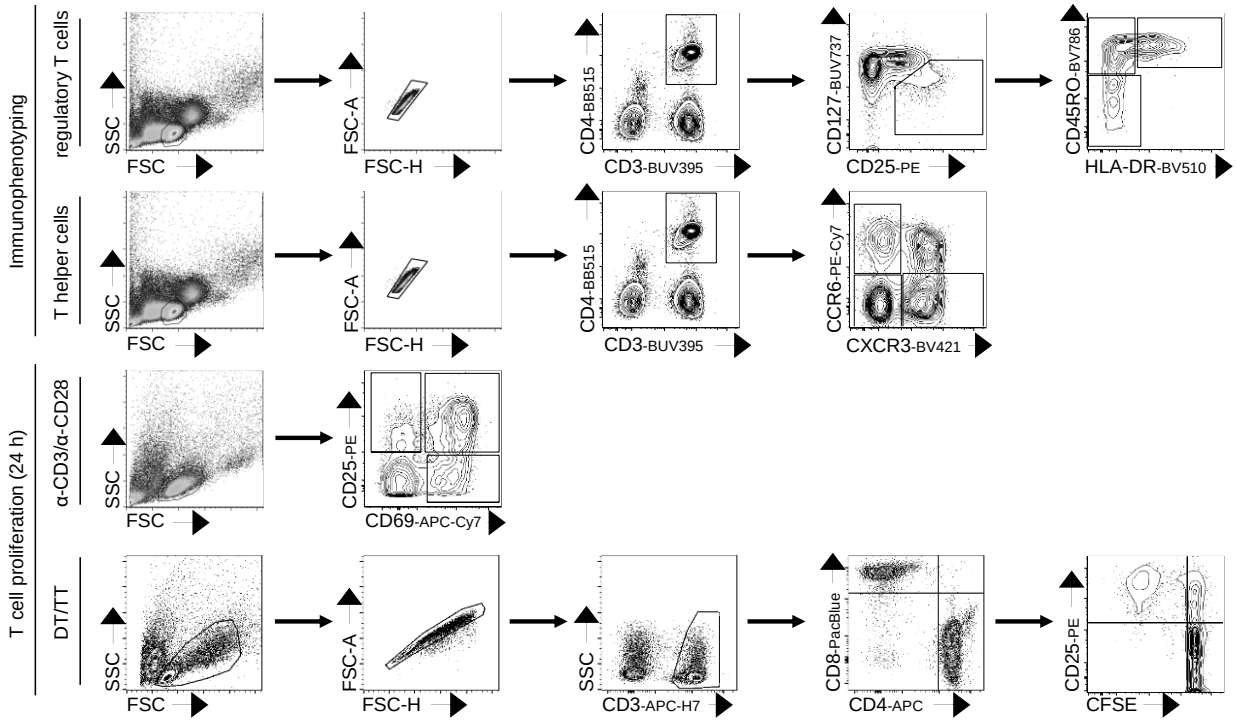
a, Pedigree of consanguineous Pakistani family B with two affected children. **b**, Sanger sequencing results confirming segregation of the identified biallelic *TGFB1* missense mutation with the disease phenotype in pedigree B. **c**, Gastrointestinal findings in P3. Colonoscopy (top) revealed extensive colitis, and histology on colonic biopsies (bottom) showed chronic active inflammation accompanied by abscesses and crypt branching. **d**, Cerebral MRI images of P3 at the age of 2 years displaying gross cortical atrophy with widening of the subarachnoid spaces, delayed myelination and marked thinning of the corpus callosum.



Supplementary Figure 4

Normal STAT6 activity in lamina propria immune cells from patient 1

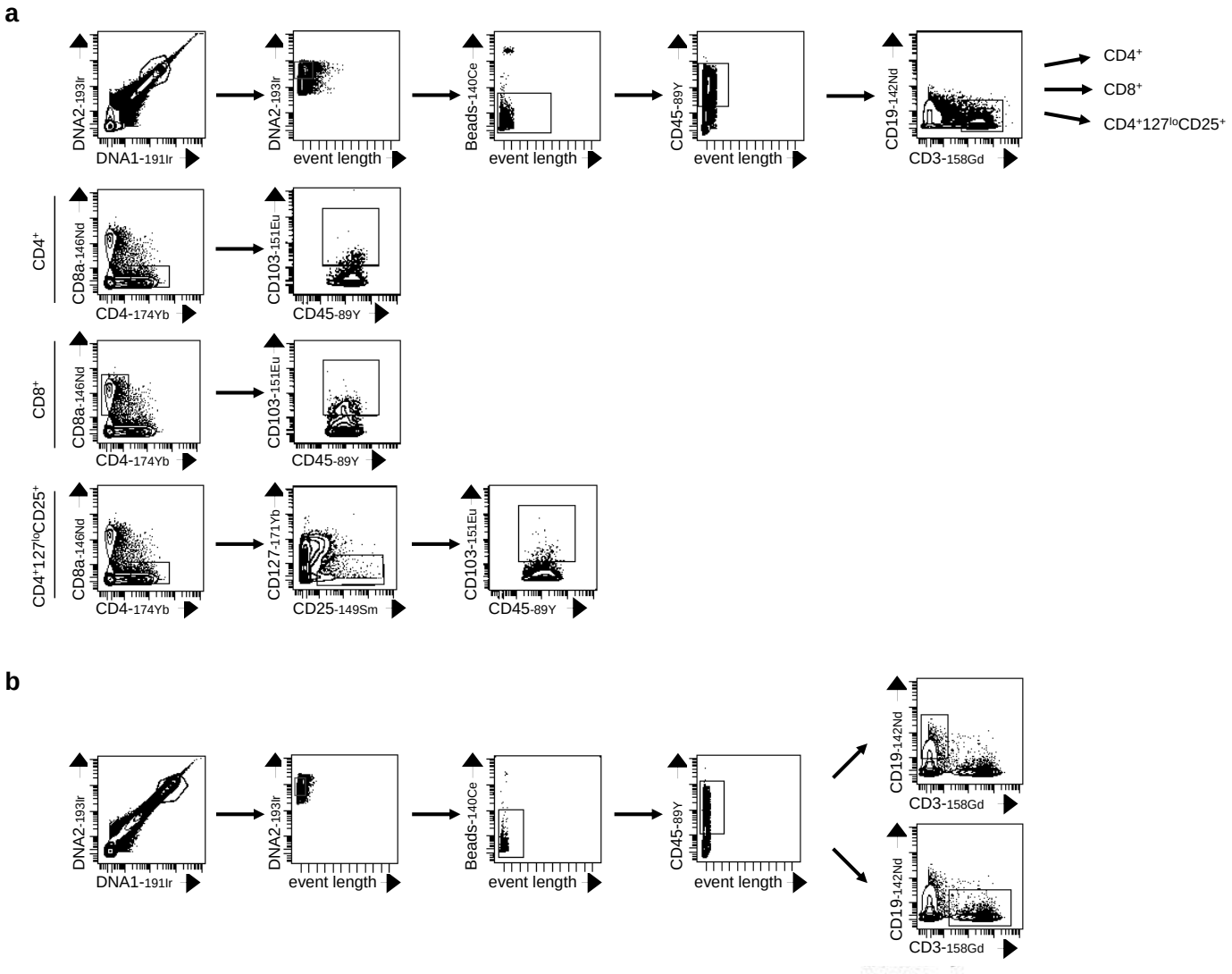
CyTOF analysis of STAT6 phosphorylation (Tyr641; p-STAT6) in lamina propria mononuclear cells derived from patients without IBD (controls: uninflamed, blue; inflamed, orange), a patient with Crohn's disease (CD) (green) and P1 (black). Histogram plots show baseline p-STAT6 in live cells that were gated on the indicated populations (left), and the heat map representation depicts the corresponding median expression values (MEV) for p-STAT6.



Supplementary Figure 5

Gating strategy for FACS analysis

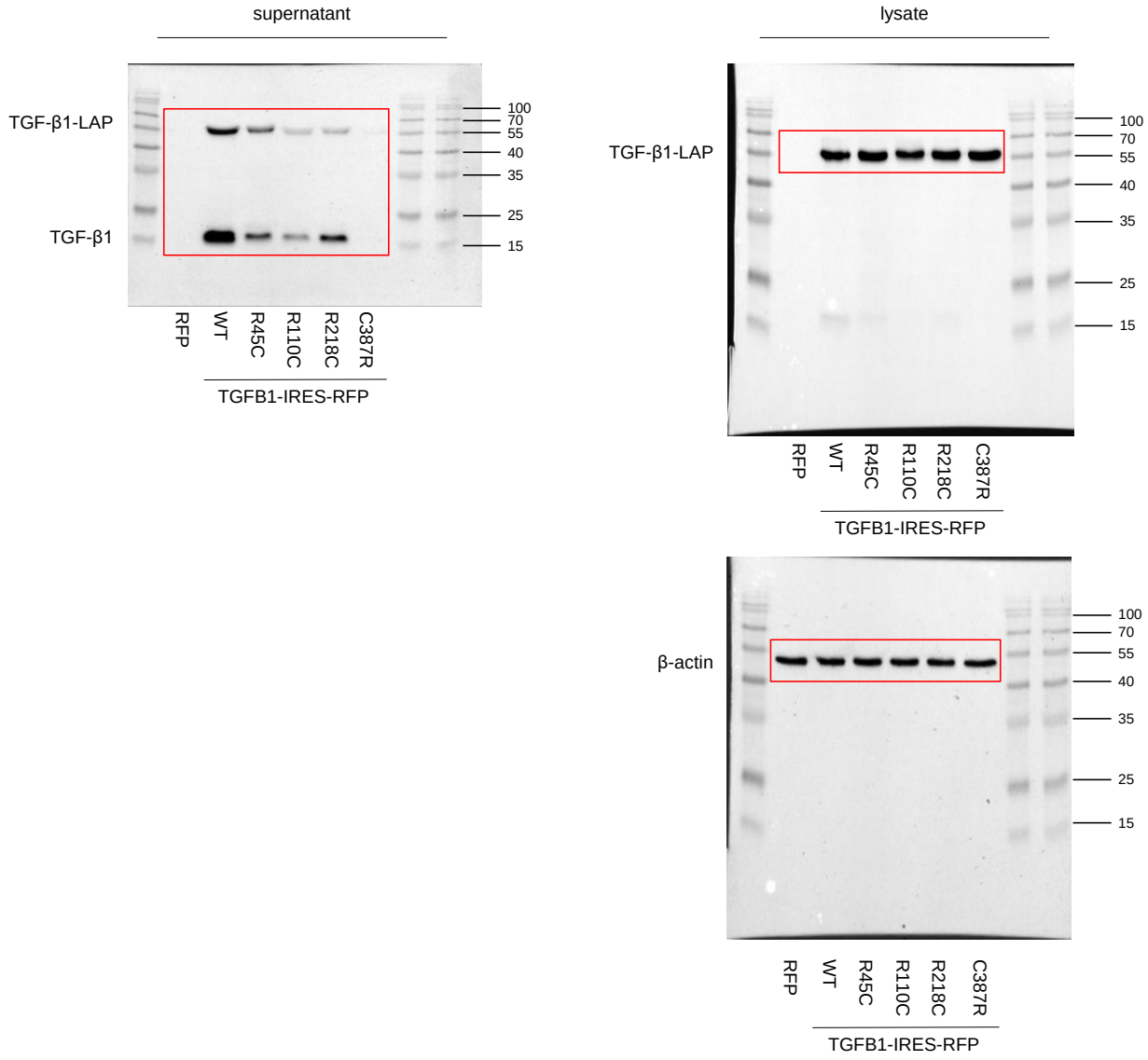
Gating strategy used in Fig. 1d.



Supplementary Figure 6

Gating strategy for CyTOF analysis

a,**b**, Gating strategies used in Supplementary Fig. 1 (**a**) and in Fig. 2h and Supplementary Fig. 3 (**b**).



Supplementary Figure 7

Uncropped immunoblots

Uncropped original immunoblots of Fig. 2d. The cropped areas are marked in red. Molecular weight markers are indicated in kDa.

Supplementary Tables

Supplementary Table 1: List of sequencing primers

Primer Name	Primer Sequence (5'-3')
TGFB1_c.133_F	ACCTGCCACAGATCCCCTAT
TGFB1_c.133_R	TCTGCCAGTCACTTCCTACC
TGFB1_c.328_F	TCGACATGGAGCTGGTGAA
TGFB1_c.328_R	GCACTCTAGAAGCGGTCCA
TGFB1_c.1159_F	TGGAGATGGGAAGAGGGGA
TGFB1_c.1159_R	GGAGAGAGAGGGAGTGGGA

Supplementary Table 2: List of antibodies used for flow cytometry

Antibody (anti-)	Clone	Cat.no.	Vendor
CCR6-PE-Cy7	11A9	560620	BD Biosciences
CCR7-BV421	150503	562555	BD Biosciences
CD3-APC-H7	SK7	560176	BD Biosciences
CD4-APC	SK3	345771	BD Biosciences
CD4-BB515	RPA-T4	564419	BD Biosciences
CD8-BUV737	SK1	564628	BD Biosciences
CD8-PacificBlue	RPA-T8	558207	BD Biosciences
CD10-PE	HI10a	340921	BD Biosciences
CD11c-BV421	B-Ly6	562561	BD Biosciences
CD14-BV650	M5E2	563420	BD Biosciences
CD16-APC	3G8	557758	BD Biosciences
CD19-BUV395	SJ25C1	563549	BD Biosciences
CD20-PE-Cy7	2H7	560735	BD Biosciences
CD21-BUV737	B-Ly4	564437	BD Biosciences
CD25-PE	M-A251	555432	BD Biosciences
CD27-BV786	L128	563328	BD Biosciences
CD28-BV510	CD28.2	566110	BD Biosciences
CD33-PE-Cy7	P67.6	333946	BD Biosciences
CD38-APC	HB7	340439	BD Biosciences
CD45RA-APC	HB7	550855	BD Biosciences
CD45RO-BV786	UCHL1	564290	BD Biosciences
CD56-PE-CF594	NCAM16.2	562289	BD Biosciences
CD57-BB515	NK1	560845	BD Biosciences
CD69-APC	FN50	555533	BD Biosciences
CD69-FITC	FN50	555530	BD Biosciences
CD123-BV786	7G3	564196	BD Biosciences
CD127-BUV737	HIL-7R-M21	564300	BD Biosciences
CXCR3-BV421	1C6	562558	BD Biosciences
HLA-DR-BV510	646-6	563083	BD Biosciences
IgD-BB515	IA6-2	565243	BD Biosciences
IgM-BV421	G20-127	562618	BD Biosciences
TCRαβ-PE	T10B9.1A31	555548	BD Biosciences
TCRγδ-APC-R700	11F2	657706	BD Biosciences

Supplementary Table 3: List of antibodies used for CyTOF

Antibody (anti-)	Clone	Cat.no.	Vendor
AHR (HMS: 161Dy)	FF3399	14-9854-82	eBioscience
CCR4 (HMS: 156Gd)	L291H4	359402	BioLegend
CCR6 (HMS: 168Er)	G034E3	353416	BioLegend
CCR7-159Tb	G043H7	3159003A	Fluidigm
CD3 (HMS: 158Gd)	UCHT1	300402	BioLegend
CD4-174Yb	SK3	3174004B	Fluidigm
CD8a (HMS: 146Nd)	RPA-T8	301002	BioLegend
CD11c (HMS: 115In)	Bu15	337202	BioLegend
CD14-148Nd	RM052	3148010B	Fluidigm
CD19 (HMS: 142Nd)	HIB19	302202	BioLegend
CD25-149Sm	2A3	3149010B	Fluidigm
CD38 (HMS: 154Sm)	HIT2	303502	BioLegend
CD39 (HMS: 144Nd)	A1	328202	BioLegend
CD45-89Y	HI30	3089003B	Fluidigm
CD45RA (HMS: 153Eu)	HI100	304102	BioLegend
CD45RO (HMS: 147Sm)	UCHL1	304202	BioLegend
CD103 (HMS: 151Eu)	Ber-ACT8	350216	BioLegend
CD117 (HMS: 141Pr)	104D2	313223	BioLegend
CD127 (HMS: 171Yb)	eBioRDR5	14-1278-82	eBioscience
CD152 (HMS: 152Sm)	L3D10	349902	BioLegend
CD161 (HMS: 164Dy)	HP-3G10	339902	BioLegend
CD223 (HMS: 167Er)	3DS223H	16-2231-85	eBioscience
CD294 (HMS: 145Nd)	BM16	350102	BioLegend
CD335 (HMS: 173Yb)	9E2	331902	BioLegend
CXCR3-163Dy	G025H7	3163004B	Fluidigm
FOXP3 (HMS: 165Ho)	PCH101	14-4776-82	eBioscience
HLA-DR-143Nd	L243	3143013B	Fluidigm
phospho-SMAD2/3 (S465/467)/(S423/425) (HMS: 156Gd)	D27F4	8828S	Cell Signaling
phospho-STAT6 (Tyr641) (HMS: 176Yb)	C11A12	9361	Cell Signaling
T-bet (HMS: 175Lu)	4B10	644802	BioLegend

Supplementary Table 4: List of antibodies used for Western blot analysis

Antibody (anti-)	Dilution	Clone	Cat.no.	Vendor
β-Actin-HRP	1:5000	C4	sc-47778	Santa Cruz
rabbit-IgG-HRP	1:3000		7074S	Cell Signaling
TGF-β1	1:1000	56E4	3709	Cell Signaling

3.2 Manuscript II: Novel Mutations in RASGRP1 are Associated with Immunodeficiency, Immune Dysregulation, and EBV-Induced Lymphoma

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ORIGINAL ARTICLE

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

Ido Somekh[1] · Benjamin Marquardt[1] · Yanshan Liu[1] · Meino Rohlfs[1] · Sebastian Hollizeck[1] · Musa Karakukcu[2] · Ekrem Unal[2] · Ebru Yilmaz[2] · Turkan Patiroglu[2] · Murat Cansever[2] · Shirly Frizinsky[3] · Vicktoria Vishnvenska-Dai[4] · Erez Rechavi[3] · Tali Stauber[3] · Amos J. Simon[3] · Atar Lev[3] · Christoph Klein[1] · Daniel Kotlarz[1] · Raz Somech[3]

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Abstract

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

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

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

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

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

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

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✉ Raz Somech
raz.somech@sheba.health.gov.il

[1] Dr. von Hauner Children's Hospital, University Hospital, Ludwig Maximilian University, Munich, Germany

[2] Department of Pediatrics, Division of Pediatric Hematology & Oncology, Erciyes University, Kayseri, Turkey

[3] Pediatric Department A and the Immunology Service, Jeffrey Modell Foundation Center; Edmond and Lily Safra Children's Hospital, Sheba Medical Center, Tel Hashomer, 52621, affiliated to the Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

[4] Ocular Oncology and Autoimmune center, The Goldschleger Eye Institute; Sheba Medical Center, affiliated to the Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

Introduction

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

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

Methods

Patients and Clinical Data

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

Immunological Evaluation

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

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

$$\text{And Gini-Simpson}'\text{s index of unevenness}:$$

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

Exome Sequencing Analysis and Sanger Sequencing

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

CRISPR-Cas9-Mediated Engineering of RASGRP1 Knockout Cell Lines

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

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

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

Table 1 (continued)

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

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

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

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

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

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

Immunoblotting and Active Ras Pull-Down Assay

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

Statistics

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

Results

Clinical Presentation of RASGRP1-Deficient Patients

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

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

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

Identification of Novel Biallelic *RASGRP1* Mutations

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

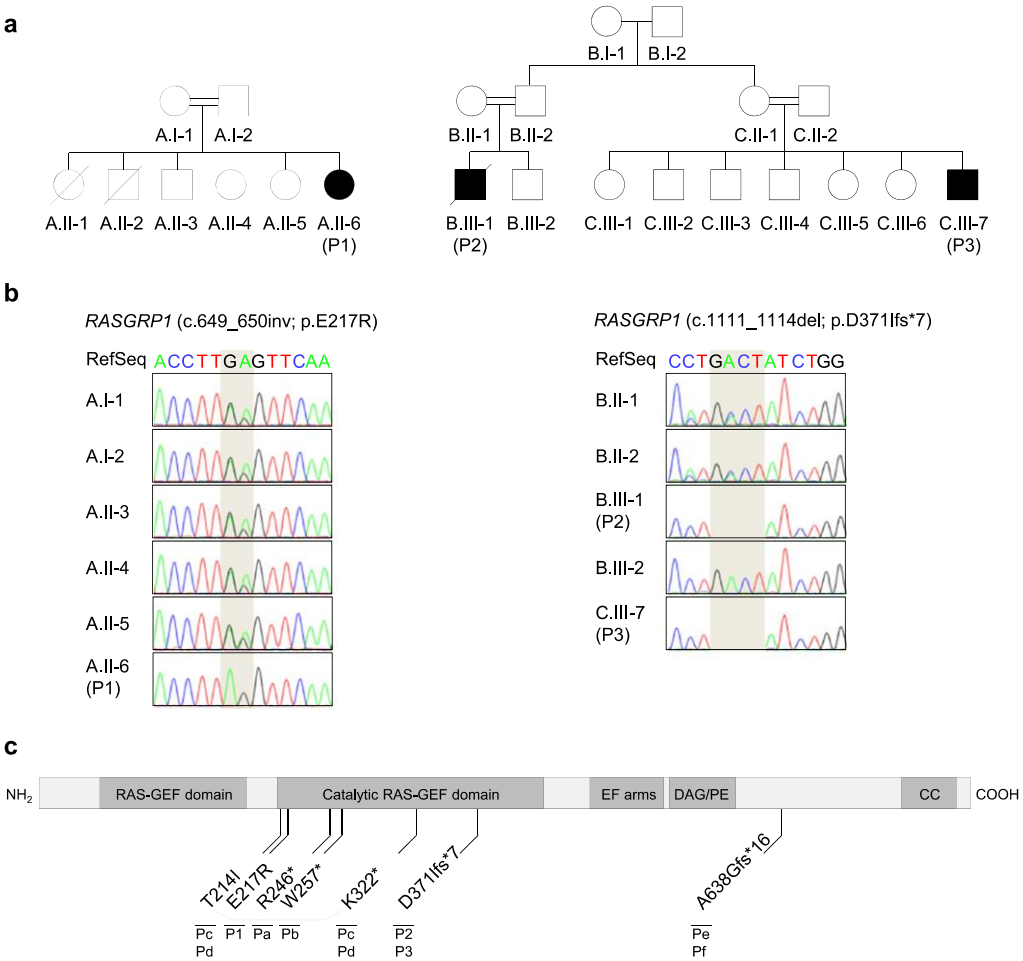

Fig. 1 Identification of patients with novel biallelic loss-of-function mutations in *RASGRP1*. **a** Pedigrees of two unrelated kindred with three patients: P1 (A.II-6), P2 (B.III-1), and P3 (C.III-7). Double lines indicate consanguinity (first-degree cousins); filled black circles or squares depict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

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

Analysis of RASGRP1 Protein Expression and Function

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

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

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

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

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

Discussion

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

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

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

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

Table 2 (continued)

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

Abnormal values in italics

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

*After rituximab treatment •Copies per 0.5 µg DNA

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

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

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

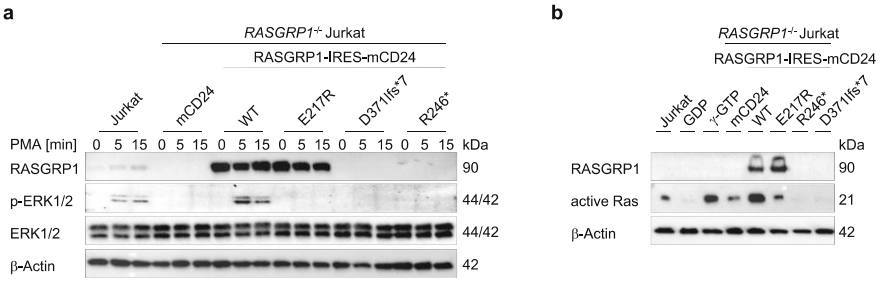

Fig. 2 Loss-of-function of mutated RASGRP1 in heterologous Jurkat T cell models. **a** Representative immunoblotting ($n = 3$) showed impaired phosphorylation of ERK1/2 (Thr202/Tyr204) upon treatment with PMA in Jurkat cells with *RASGRP1* knockout or ectopic expression of mutated RASGRP1 variants (E217R,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

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

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

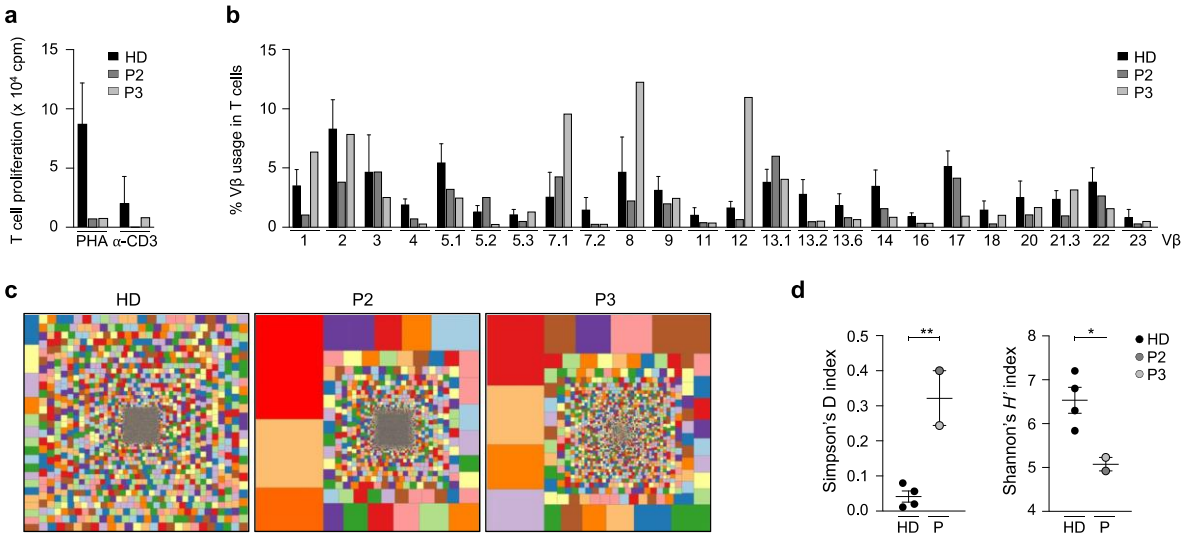

Fig. 3 RASGRP1 deficiency is associated with altered T cell clonal expansion and repertoire diversity. **a** Thymidine incorporation assays documented reduced T cell proliferation in P2 and P3 upon stimulation with (PHA) or anti-CD3, as compared to healthy donors (HD). **b** Flow cytometry-based TCR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

considered for patients with RASGRP1 deficiency as curative treatment.

Conclusions

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

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

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Compliance with Ethical Standards

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

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

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Table S1. Whole exome sequencing analysis, filtered for homozygous variants for P1, revealing 7 candidate genes including *RASGRP1*. Chromosomal location, gene description, nucleotide change, type of mutation, frequency in population and clinical relevance are presented.

Chr	Pos	Gene	Description	RefAlt	Mut	AvFreq	ClinVar
5	72873736	UTP15	Small subunit processome component	A/G	missense	0	No
7	78281029	ARSB	Aryl sulfatase B	C/G	missense	0.0001	No
8	41507672	NKX6-3	NK6 homebox 3	G/A	missense	0.0006	No
14	75520313	ACYP1	Acylphosphatase 1	G/A	missense	0.00002	No
15	**38808423**	**RASGRP1**	**RAS guanyl releasing protein 1**	**A/G**	**missense**	**0**	**Yes**
15	**38808424**	**RASGRP1**	**RAS guanyl releasing protein 1**	**G/A**	**missense**	**0**	**Yes**
15	40259734	EIF2AK4	Eukaryotic translation initiation factor 2 alpha kinase 4	C/T	missense	0.00002	No
15	45723028	C15orf48	Chromosome 15 open reading frame 48	GAAGT/G	Splice donor	0.00350	No

Table S2. Whole exome sequencing analysis, filtered for shared homozygous variants for P2 and P3, revealing the candidate gene *RASGRP1*. Chromosomal location, gene description, nucleotide change, type of mutation, frequency in population and clinical relevance are presented.

Chr	Pos	Gene	Description	RefAlt	Mut	AvFreq	ClinVar
15	**38800054**	**RASGRP1**	**RAS guanyl releasing protein 1**	**TGACT/T**	**frame shift**	**0**	**Yes**

Table S3. List of antibodies used for immunoblotting.

Antibody (anti-)	Clone	Working dilution	Cat.no.	Supplier
β-Actin-HRP	C4	1:5000	sc-47778	Santa Cruz
rabbit-IgG-HRP		1:3000	7074S	Cell Signaling
RasGRP1	H-120	1:1000	sc-28581	Santa Cruz
RasGRP1	10.1	1:1000	MABS146	Merck Millipore
Ras		1:2000	1862335	Thermo Scientific
p44/p42 MAPK (Erk1/2)		1:2000	9102S	Cell Signaling
phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	D13.14.4E	1:2000	4370S	Cell Signaling

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CORRECTION

Correction to: Novel Mutations in RASGRP1 Are Associated with Immunodeficiency, Immune Dysregulation, and EBV-Induced Lymphoma

Ido Somekh[1] · Benjamin Marquardt[1] · Yanshan Liu[1] · Meino Rohlfs[1] · Sebastian Hollizeck[1] · Musa Karakukcu[2] · Ekrem Unal[2] · Ebru Yilmaz[2] · Turkan Patiroglu[2] · Murat Cansever[2] · Shirly Frizinsky[3] · Vicktoria Vishnvenskia-Dai[4] · Erez Rechavi[3] · Tali Stauber[3] · Amos J. Simon[3] · Atar Lev[3] · Christoph Klein[1] · Daniel Kotlarz[1] · Raz Somech[3]

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The original version of this article unfortunately contained mistakes in Author's name, in Table 1 and in result section.

- The name of one of the authors in the paper "Vicktoria Vishnvenska-Dai" should have read as "Vicktoria Vishnvenskia-Dai"
- In Table 1, the word "Autologous" for P1 (column #8) should have read as "Allogeneic".
- In Results section, "Autologous" should have read as "Allogeneic".

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✉ Raz Somech
raz.somech@sheba.health.gov.il

[1] Dr. von Hauner Children's Hospital, University Hospital, Ludwig, Maximilian University, Munich, Germany

[2] Department of Pediatrics, Division of Pediatric Hematology & Oncology, Erciyes University, Kayseri, Turkey

[3] Pediatric Department A and the Immunology Service, JeffreyModell Foundation Center; Edmond and Lily Safra Children's Hospital, Sheba Medical Center, Tel Hashomer, 52621, affiliated to the Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

[4] Ocular Oncology and Autoimmune center, The Goldschleger Eye Institute; Sheba Medical Center, affiliated to the Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

4 DISCUSSION

4.1 Human TGF-β1 deficiency

Based on mouse studies and genetic associations in humans, TGF-β1 signaling has been suggested to be central in the pathogenesis of cancer, inflammatory disorders, and autoimmunity, particularly in diseases that are related to pathogenic expansion of Th1, Th2, and Th17 cells [71, 168, 169]. However, the exact mechanisms and relevance of altered TGF-β1 signaling are not completely understood. Dysregulated TGF-β1 expression has not only been reported in fibrosis, atherosclerosis, and osteoporosis [114-116], but was also repeatedly documented in IBD [117]. Genome-wide association studies have linked polymorphisms in *TGFB1* to an increased risk for schizophrenia [120] and cancer [121, 122]. Autosomal dominant gain-of-function mutations in TGF-β1 have been found to cause CED, a severe growth disorder characterized by abnormal bone formation, foremost confined to the skeleton, skull, and long bones [124]. Remarkably, nine distinct CED variants have been identified so far but were never linked to neurological defects in humans [170]. In addition to CED, few other diseases have also been related to defective TGF-β1 signaling such as Loeys-Dietz syndrome [126], hereditary hemorrhagic telangiectasia [127], Myhre syndrome [130] and Marfan syndrome [131]. Collectively, these TGF-β1-related disorders are primarily characterized by skeletal, (cardio-) vascular, and craniofacial developmental defects as well as deregulated tissue formation and homeostasis but do not affect the immune system or CNS development. In contrast, we described the first three patients with biallelic loss-of-function mutations in *TGFB1*, who mutually suffered from a severe immunodeficiency, intestinal inflammation and neurological abnormalities [171].

4.1.1 Human TGF-β1 deficiency affects T cell development and homeostasis

TGF-β1 is not only a key cytokine that orchestrates growth and development of various body tissues, but is also a master regulator in the immune system controlling maturation, proliferation, and activation of several immune cell types [107]. Experimental mouse models have clearly illustrated the fundamental role of TGF-β1 in the development of both myeloid and lymphoid lineages as well as maintaining immune homeostasis since abrogation of TGF-β1 signaling resulted in devastating phenotypes [105]. For instance, *Tgfb1* null mice developed a rapid wasting syndrome and succumbed to an extensive T cell-mediated inflammation within 3-4 weeks after birth [172]. The inflammatory phenotype was associated with a massive infiltration of lymphocytes and macrophages in many organs, especially heart and lungs.

Moreover, mice with T cell-specific disruption of TβR-I or TβR-II developed an acute systemic autoimmune disease with a profound decrease in forkhead box protein P3 (FOXP3)$^+$ Tregs which was accompanied by severe colitis at 3-4 months of age [173, 174]. Similarly, conditional deletion of *Tgfb1* in CD4$^+$ cells resulted in excessive T cell proliferation and activation, leading to a lethal immunopathology with multi-organ inflammation [175]. In line with these drastic phenotypical features seen in mice, all of our three patients presented with a pronounced gastrointestinal inflammatory disease with onset in the first months of life. Of note, all patients repeatedly suffered from bacterial, fungal, or viral infections. FACS- and CyTOF-based immunophenotypic evaluation of primary cells isolated from blood or intestinal tissues suggested an underlying primary immunodeficiency or immune dysfunction. Analysis of PBMC from P1 revealed decreased maturation of activated memory Tregs. Furthermore, CyTOF analysis showed reduced frequencies of CD4$^+$ and CD8$^+$ T cells as well as decreased numbers of CD45RA$^+$ and CD45RO$^+$ Tregs in colonic lamina propria mononuclear cells (LPMCs) of P1. Notably, previous studies have demonstrated that lymphocytes, in particular T cells, reside at mucosal surfaces and produce high amounts of active TGF-β1 which is essential for the development of peripheral Tregs via induction of the transcription factor FOXP3 [176, 177]. Since Tregs are highly immunosuppressive and downregulate the proliferation of effector T lymphocyte subsets, they are a central component in maintaining peripheral tolerance to self- and environmental antigens [178]. Accordingly, Treg deficiencies and dysfunctions can result in autoimmunity, inflammatory disorders, and allergies [179]. Based on these findings, it is tempting to speculate that the reduced Treg numbers seen in P1 may contribute to his gastrointestinal inflammation. Interestingly, similar observations have been made in mice with genetic knockout of *Tgfbr2* that showed significantly reduced numbers of peripheral FOXP3$^+$ Tregs and a lethal early-onset multiorgan autoimmune-associated inflammatory disorder [180]. Furthermore, we have found a considerable decrease in phosphorylated SMAD2/3 levels in both colonic B and T cells of P1, whereas STAT6 phosphorylation appeared normal. Because SMAD2/3 is known to drive FOXP3 expression in T cells [177], the defective Treg differentiation is likely to be linked to inadequate TGF-β1 signaling in our patients. Consequently, human TGF-β1 deficiency may resemble a phenotype overlapping with features seen in other disorders that are characterized by impaired Treg development. For example, immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome is caused by loss-of-function mutations in *FOXP3* and associated with defective Treg generation, resulting in a lethal autoimmune disease [93, 181].

In parallel to the important role for Treg development, TGF-β1 has been shown to control the differentiation of naïve $CD4^+$ T cells into specialized T helper (Th) subsets, which are key components of cellular and humoral immunity by producing Th-specific effector cytokines. Under normal conditions, TGF-β1 restricts the generation of Th1 and Th2 cells [112], whereas it promotes the generation of Th9 [93] and Th17 [113] lineages. We found significantly lower frequencies of Th1, Th2, and Th17 cells in P1 compared to healthy individuals and parents, indicating a disturbed Th cell homeostasis. Likewise, mice with a T cell-specific knockout of *Tgfbr2* showed abnormal differentiation of $CD4^+$ T cells into Th1 and Th2 lineages which was associated with reduced T cell activation and increased apoptosis [175]. While we could not directly link the defective Th differentiation to increased cell death, we observed that antigen-induced activation and proliferation of $CD4^+$ T cells was markedly attenuated in PBMC from patient P1. Of note, several inflammatory diseases, in particular IBD, are linked to perturbed $CD4^+$ T cell responses. Accordingly, CD has been postulated to be a Th1- and/or Th17-driven condition, while a Th2-mediated inflammation was associated with UC [182]. Th1 and Th2 cells are usually upregulated upon bacterial, viral or parasite infections and drive the expression of a number of cytokines that activate macrophages or $CD8^+$ T cells but also promote B cell proliferation and antibody production [183]. Th17 cells are critical in controlling the anti-microbial defense and maintenance of epithelial integrity at the mucosal gut barrier [184]. Collectively, each subset of $CD4^+$ Th cells secretes distinct cytokines which not only define the immune response but at the same time influence the development of other Th cell types. In consequence, a disturbance of the complex TGF-β1-dependent Th cell equilibrium may result in altered cytokine production and promote inflammation or autoimmunity, as seen in systemic lupus erythematosus (SLE) [185], Guillain-Barré syndrome [186], diabetes [187], and rheumatoid arthritis [188].

In summary, based on our clinical and laboratory observations, human TGF-β1 deficiency encompasses immune dysfunctions which overlap with features seen in murine models with absent or defective TGF-β1 signaling. Further studies on larger cohorts are needed to comprehensively analyze the intricate biology of TGF-β1 in humans, since the immune phenotype our our index patient might be influeced by the type of mutation, genetic background, microbiome, co-infections, medication etc.

4.1.2 Human TGF-β1 deficiency causes early onset encephalopathy

Inflammatory conditions with disruption of homeostatic pro- and anti-inflammatory cytokine networks in early childhood have been considered as causative events promoting neurodevelopmental disorders including autism, cognitive impairment, cerebral palsy, epilepsy, and schizophrenia [189]. Murine models highlighted the pivotal function of TGF-β1 signaling in terms of neuroinflammation, neurodegeneration and microgliosis although the underlying pathomechanisms still remain controversial [190-192]. Indeed, TGF-β1 is a potent anti-inflammatory cytokine which efficiently dampens the activity of immune cells [107], however, it also controls a large number of important developmental processes which are not exclusively linked to the immune system but orchestrate the formation of organs and other specialized body tissues [193]. In humans, TGF-β1 has been speculated to exert neuroprotective functions and thus was suggested to play a role in neurodegenerative disorders such as Alzheimer's disease (AD) or Parkinson's disease [194]. Of note, reduced neuronal TGF-β1 signaling correlated with increased formation of pathological ß-amyloid plaques in AD patients [195]. Therefore, drugs that promote TGF-β1 availability in the brain are considered as possible treatment for AD [196].

In addition to the pronounced immunodeficiency and intestinal inflammation, our patients presented with severe physical and cognitive deficits, characterized by atypical motion behavior, involuntary spontaneous movements, and seizures. Neurological examination highlighted a prominent cerebral atrophy in all three patients, encompassing severe myelination defects and loss of white brain matter. This observation is consistent with data from murine models, where loss of TGF-β1 signaling resulted in a remarkable reduction of brain mass due to neuronal cell death that was mainly driven by (auto-)inflammatory processes [190, 197]. Human brain tissue mainly consists of neurons, astrocytes, microglia, and vascular cells, which do not only actively produce high levels of TGF-β1 but are also extremely responsive to TGF-β1 signals [198]. In this regard, it is very likely that abnormal cytokine expression in combination with reduced TGF-β1 signaling in our patients has had a severe impact on their cerebral development and CNS function. In fact, murine models have underscored that TGF-β1 is critically involved in cerebral vasculogenesis and controls the formation of the blood-brain barrier [199, 200]. Similarly, blockade of TGF-β1 signaling in the mouse brain has been shown to promote rapid microglia-driven demyelination of neurons causing severe motor disease [201], which is reminiscent of the psychomotor deficits seen in our patients. Recent mouse studies confirmed that conditional deletion of integrin $\alpha_v\beta_8$ in the CNS (*Itgb8*$^{\Delta CNS}$ mice) fully blocked microglia maturation while resulting in microglia hyperactivation [202]. In

consequence, overactive microglia directly caused a unique neurodevelopmental syndrome which was characterized by oligodendrocyte maturational arrest, interneuron loss, and spastic neuromotor dysfunction. Because integrin $\alpha_v\beta_8$ is a physiological activator for latent TGF-β1 in the brain [203], the observations in *Itgb8*$^{\Delta CNS}$ mice are likely to resemble phenotypes seen in other models with defective TGF-β1 signaling. And in fact, microglia-specific deletion of *Tgfbr2* completely mirrored the hallmarks of the murine *Itgb8*$^{\Delta CNS}$ model [202].
Furthermore, the GARP homolog LRRC33 was recently shown to be essentially involved in brain-specific TGF-β1 activation in another animal study. In mice, LRRC33 on microglia presents latent TGF-β1 to astrocyte-bound integrin $\alpha_v\beta_8$, mediating the release of the growth factor. In the absence of LRRC33, animals developed normally up to 2 months of age, but then showed progressive neurodegeneration characterized by demyelination, loss of axons and neurons in the somatomotor cortex and spinal cord, and death by 5 months [204], underlining the crucial role of TGF-β1 signaling for maintaining CNS homeostasis in mice. As an alternative pathomechanism, Rojas *et al.* have shown that neuroinflammation in murine experimental autoimmune encephalomyelitis (EAE) can be ameliorated by IgA-secreting gut-derived plasma cells (PC) which migrate into the CNS and act as a significant source of anti-inflammatory IL-10 [205]. Since TGF-β1 is fundamental for the development of PC and stimulates IgA isotype switching in both mice and humans [206], defective TGF-β1 signaling may contribute to neuroinflammatory phenotypes via other non-cerebral pathways.

In aggregate, our findings confirmed that human TGF-β1 deficiency is associated with a distinct neuropathological phenotype, encompassing developmental and psychomotor defects, thus providing further validation for the neuroprotective features of TGF-β1. Unfortunately, our efforts to investigate the phenotype of human TGF-β1 deficiency in more detail was limited by restricted access to patient material. Additional studies employing appropriate human disease models such as iPSC-derived neural cells or cerebral organoids may be needed to outline the relevance of TGF-β1 deficiency in a neurodevelopmental context more comprehensively.

4.1.3 Mutations in TGF-β1 perturb protein structure and affect cytokine secretion

The amino acid sequence of TGF-β1 is well conserved among a wide number of species, indicating a strong connection between cytokine structure and biological function [70]. To assess whether the identified mutations have an impact on protein configuration, we performed a structural analysis based on the crystal structure of wild-type TGF-β1 (Protein Data Bank accession code: 3RJR). In brief, the monomeric precursor of TGF-β1 contains a N-terminal 249-residue prodomain, comprising a short signal peptide and LAP, which is linked with a furin-cleavable 112-residue growth factor domain at the C-terminus [77] (Fig. 8a).

The identified mutations in patient P1 are located in the central part of LAP (Arg110Cys) as well as the C-terminal section of the mature growth factor domain (Cys387Arg). The homozygous mutation found in P2 and P3 has been detected within the N-terminal LAP section (Arg45Cys) causing a replacement of a polar cationic arginine with a polar but neutral cysteine at amino acid position 45 in LAP. The TGF-β1 straitjacket domain that encircles each growth-factor monomer (Fig. 8b), extends from residues 1-45 and contains one α-helix followed by the latency lasso motif [77], where Arg45 is tightly conserved between many species and seems to be of equal importance in TGF-β2, TGF-β3 as well as other TGF-β family members [74]. Furthermore, there is a highly conserved stretch of 24 amino acids (Lys42-Gln65) in LAP, forming a contiguous epitope in the straitjacket α-helix, which is essential for the interaction with both mature TGF-β1 and LTBP1, but has been also shown to play a key role in biosynthesis and secretion of the cytokine [207]. Hence, the Arg45Cys mutation may perturb the N-terminal epitope by altered polarity and consequently affect growth factor assembly as well as secretion and latency of the TGF-β1-LAP complex. Accordingly, we could show that secretion and stability of the TGF-β1 variant Arg45Cys were significantly impaired, while intracellular protein expression was unaffected. In independent studies, Walton *et al.* showed misfolding of the precursor protein and substantially reduced secretion of latent TGF-β1, when Arg45 was changed to a nonpolar alanine (Arg45Ala) in a heterologous HEK293T cellular model because the hydrophobic motif at the beginning of LAP was disrupted [207]. Although we did not investigate protein misfolding of the mutated TGF-β1 variants in detail, the Arg45Cys variant seen in our patients P2/P3 may at least to some extent resemble the characteristics of the engineered Arg45Ala variant.

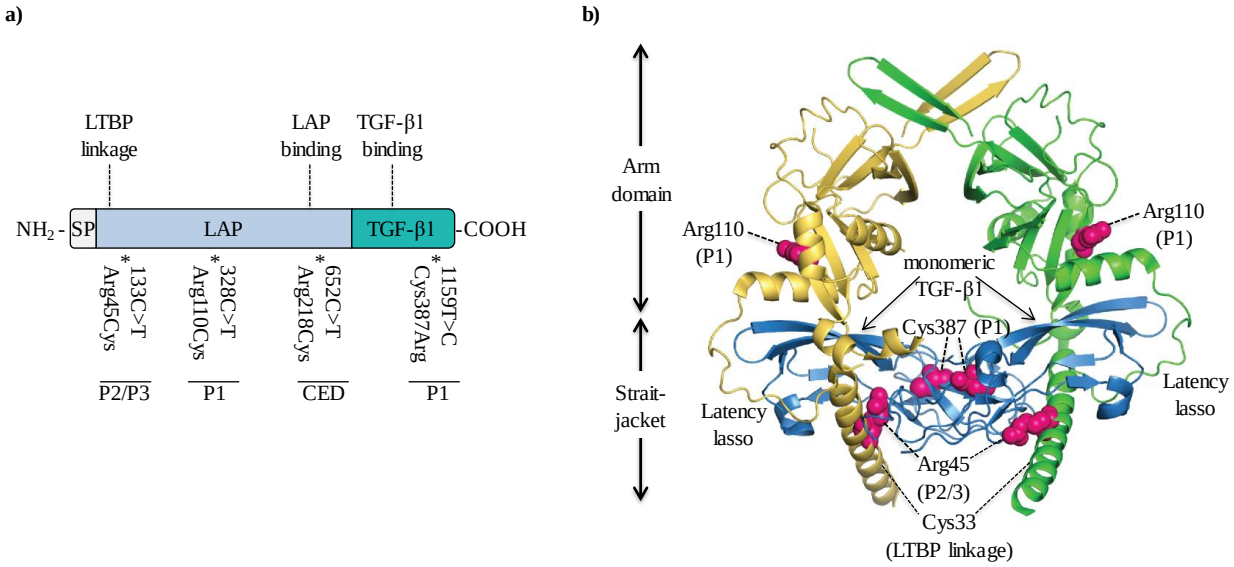

Figure 8: Schematic overview of the identified patient mutations in TGF-β1.
a) Derived crystal structure of the latent TGF-β1 complex. TGF-β1 monomers are indicated in blue, LAP molecules are colored in yellow and green, respectively. Amino acid exchanges found in patients are shown as purple spheres. **b**) Schematic illustration of the TGF-β1 precursor comprising a N-terminal signal peptide (SP), the latency-associated peptide (LAP) and the C-terminal mature growth factor (TGF-β1). The mutations identified in our patients, including a previously described gain-of-function mutation in *TGFB1* causing Camurati-Engelmann disease (CED), are asterisked (*) and specify variations in both cDNA and protein sequence.

In case of patient P1, the first mutation is localized within the LAP domain of TGF-β1, converting the arginine at position 110 into a cysteine. *In silico* structural analysis suggested that this amino acid substitution might damage the interaction between LAP and the mature growth factor. Biochemical assays revealed that the TGF-β1 mutant Arg110Cys showed a marked reduction of extracellular cytokine levels analogous to the Arg45Cys variant, while intracellular protein expression was comparable to wild-type TGF-β1. Correspondingly, we could observe an impaired reassociation of Arg110Cys LAP with mature TGF-β1, indicating a substantial change in favorable binding conditions. Although we did not specifically analyze intracellular protein processing, we noted a reduced secretion of the mutant cytokine which might be caused by the extra (unpaired) cysteine in the TGF-β1 Arg110Cys variant. In general, biosynthesis of secretory proteins involves a number of quality control steps during ER transition, including the formation of disulfide bonds [208]. If a protein is mispaired, misfolded, or aggregated, it is not further processed but redirected towards proteolysis. In the context of TGF-β1, the formation of correct disulfide bonds is of particular importance for biosynthesis, secretion, and latency. Especially, the amino acids 30-278 within the LAP domain of the pro-TGF-β1 precursor control activation and stability of the cytokine which is mediated by three cysteines [209]: Cys223/Cys225 crosslink with their equivalent in an adjacent LAP monomer to form a stable homodimer, while Cys33 is commonly linked to TGF-β1 binding proteins (e.g., LTBP1 [99] or GARP [101]). In case of P1, structural analysis suggested that the Arg45Cys variant may partially disrupt the interaction of the adjacent Cys33 with other binding partners

of TGF-β1. Moreover, the mutation gives rise to an extra (unpaired) cysteine which might compromise the correct folding of the LAP domain by random intra- or intermolecular cysteine-cysteine contacts. In that regard, previous studies have shown that genomic deviations within LAP can result in a pathologic setting such as CED, where heterozygous disease-causing mutations have been reported at positions 81, 156, 218, 222, and 225 [123, 210]. In contrast, the identified LAP mutations in our patients did not result in a gain-of-function phenotype as observed in CED, but caused a significant reduction or complete blockage of latent TGF-β1 secretion. Besides, the LAP domain is also important for stabilizing integrin-dependent conformational changes coming from the ECM, which then promote TGF-β1 activation by traction [68]. Consequently, the LAP mutations identified in our patients may weaken or abolish this process. Functional assays employing the mutant TGF-β1 complexes revealed impaired SMAD signaling in a heterologous reporter cell line, suggesting disturbances in cytokine activation. These observations were in accordance with CyTOF data from our index patient P1, confirming reduced SMAD phosphorylation in mucosal B and T cells.

The second mutation identified in P1 is located in the C-terminal part of the TGF-β1 precursor, which encodes for to the mature cytokine domain. Molecular modeling of the Cys387Arg variant indicated a serious structural deviation from the wild-type protein. In detail, replacement of Cys387 with a different amino acid is predicted to result in an unpaired Cys322 prohibiting the formation of an essential cystine knot which usually stabilizes the growth factor domain [211]. The C-terminal region of pro-TGF-β1 contains nine cysteines but only Cys356 forms an intermolecular S-S bond to another TGF-β1 precursor, while the remaining cysteines stabilize the protein by intramolecular disulfide bridges [74]. According to Brunner *et al.*, all nine cysteines are indispensable for an unimpeded secretion of latent TGF-β1, because site-specific mutagenesis of these cysteine residues blocked cytokine release up to 100% [75]. Correspondingly, we could demonstrate that the Cys387Arg mutation in P1 completely abrogated cytokine secretion in Cys378Arg-transduced 293T cells, while intracellular protein levels were comparable to wild-type TGF-β1. In addition, the unpaired Cys322 may undergo incorrect disulfide bond formation with other intra- or intermolecular cysteines, what may have unforeseen impacts on cell homeostasis.

The activation and bioavailability of latent TGF-β1 is controlled depending on the biological context. Collectively, all identified mutant TGF-β1's showed diminished cytokine secretion, TGF-β1-LAP complex reassociation, and SMAD signaling in comparison to the

wild-type protein. Based on the observed structural deviations, the release of mature, active TGF-β1 from latent complexes may be impaired due to several mechanisms. Numerous processes that activate latent TGF-β1 are facilitated through interactions with the ECM and integrins. In particular, integrins $\alpha_v\beta_6$ and $\alpha_v\beta_8$ forward physical traction forces via their connections with the actin cytoskeleton to induce conformational changes that allow binding of active TGF-β1 to its receptors [212]. Several mouse models could confirm the importance of integrins for the activation of latent TGF-β1, because knockout of both integrin $\alpha_v\beta_6$ and $\alpha_v\beta_8$ [213-215] or ablation of the RGD motif [216] resulted in severe inflammatory phenotypes similar to *Tgfb1* null mice. Furthermore, the conditional knockout of integrin $\alpha_v\beta_8$ on leukocytes caused severe colitis accompanied by low Treg numbers, which has been ascribed to defective T cell differentiation that is normally promoted by $\alpha_v\beta_8^+$ dendritic cells [215]. Another important link to impaired TGF-β1 bioavailability and activation seems to be associated with the lymphocyte-specific surface protein GARP [217]. Latent TGF-β1 has been shown to be presented by GARP on the surface of Tregs or B cells where the release of active TGF-β1 is triggered by integrins $\alpha_v\beta_6$ or $\alpha_v\beta_8$ [81, 218]. First and foremost the proper interaction of integrins and binding partners on Tregs seems to be critical for their immunosuppressive function and loss of these central cell-cell interactions can result in autoimmunity [219]. Recently, Liénart *et al.* have solved the crystal structure of GARP bound to latent TGF-β1 and documented a predominantly hydrophobic interaction between GARP and the N-terminus of LAP mediated by van der Waals contacts [101]. Interestingly, the Arg45Cys mutation seen in P1 is exactly located within this hydrophobic region relevant for the activation of latent TGF-β1 via GARP and may therefore negatively affect its bioavailability. Additionally, cryo-electron microscopy has revealed that integrin $\alpha_v\beta_8$ can directly initiate TGF-β1 signaling from LAP-bound mature TGF-β1 without the need of prior activation, thus deviations in the growth factors' protein sequence and structure are likely to disrupt such mechanisms *in vivo* [220]. To test whether the identified mutations impact the interaction between GARP and latent TGF-β1 we generated heterologous cell lines which coexpress GARP together with wild-type or mutant TGF-β1. Our studies revealed that the Cys387Arg variant failed to be displayed by GARP at the cell surface, whereas all other mutants showed a reduced membrane localization as compared to wild-type TGF-β1 based on mean fluorescence intensity (unpublished data). Since the secretion of Cys387Arg TGF-β1 was completely abrogated, we postulate that the mutation may affect protein trafficking to the cell membrane hampering cell surface presentation via GARP.

Taken together, human TGF-β1 deficiency displays a life-threatening medical condition with poor outcome for the affected individuals. Strikingly, the phenotypic presentation of our patients resembles many prominent features seen in *Tgfb1*$^{-/-}$, *Tgfbr1*$^{-/-}$, *Tgfbr2*$^{-/-}$, *Smad2*$^{-/-}$, *Smad3*$^{-/-}$, *Smad4*$^{-/-}$, *Itgb6*$^{-/-}$, and *Itgb8*$^{-/-}$ deficient mice [105, 214]. Even though we were not able to completely elucidate the exact pathomechanisms of the identified TGF-β1 mutations on a molecular basis, we could show that all mutants exhibit defective secretion and activation, leading to a substantially diminished bioavailability of TGF-β1. In conclusion, our study demonstrates a non-redundant role of TGF-β1-mediated signaling for intestinal immune homeostasis and neurological development in humans. Our results provide new insights into human TGF-β1 biology which may help to drive the development of novel personalized therapies for patients with VEO-IBD but might be also applicable to other common (auto-)immune-related disorders.

4.2 Human RASGRP1 deficiency

RASopathies encompass a distinct set of medical genetic syndromes caused by germline mutations in genes encoding components or regulators of the MAPK pathway [221]. Clinically, patients with RASopathies mainly present with developmental defects, neurologic anomalies, and cancers [135]. RASGRP1 is a potent Ras activator in humans and genetic variants of *RASGRP1* have been associated with a number of inflammatory disorders such as diabetes [222], IgA nephropathy [223], and rheumatoid arthritis (RA) [224]. Furthermore, dysregulated RASGRP1 expression has been observed in patients with systemic lupus erythematosus (SLE) [225] or acute T-cell lymphoblastic leukemia [226]. In 2016, Salzer *et. al* have reported the first case of human RASGRP1 deficiency which was associated with insufficient proliferation, activation and motility of B and T cells as well as impaired $CD8^+$ and NK cell cytotoxicity [227]. The affected individuals presented with an acute primary immunodeficiency and developed EBV-positive B cell lymphoma. On a molecular basis, loss of RASGRP1 expression was linked to defective ERK phosphorylation and decreased activation of the GTPase RhoA. Similarly, our patients with homozygous mutations in *RASGRP1* suffered from a severe immunodeficiency and were diagnosed with LD associated to an EBV-infection in patients P1 and P3 [228]. Whole exome sequencing revealed an inversion mutation (c.649_650inv; p.Glu217Arg) in patient P1, whereas siblings P2 and P3 had a 4-base pair deletion (c.1111_1114del; p.Asp371Ilefs*7) which caused a premature stop codon resulting in early protein truncation. Of note, all variants identified in our patients are located within the catalytic region of RASGRP1, spanning the CDC25 and REM domain, which has been shown to be critical for GTPase activity by controlling binding and interaction with Ras [133]. To date, five additional individuals with deleterious mutations in *RASGRP1* have been reported and patients presented with defects in lymphocyte proliferation and activation, autoimmunity, and LD [229-231].

4.2.1 Human RASGRP1 deficiency impairs lymphocyte development and immunity

RASGRP1 amplifies and promotes signals from extracellular surface receptors via Ras to the MAPK/ERK pathway [157]. Mice with specific knockout of *Rasgrp1* displayed impaired immune cell proliferation and presented with severe T cell-dependent immunodeficiency, including autoimmunity and LD [232]. Further murine models pointed out the indispensable role of RASGRP1 for the differentiation and development of T, B, and iNKT cells [160, 163, 165]. Since RASGRP1-deficient patients resemble the observations made in *Rasgrp1*$^{-/-}$ mice, it seems obvious that RASGRP1-mediated Ras activation must be of critical importance for

immunity in both species, particularly in controlling proliferation and lineage commitment of lymphocytes. Strikingly, all reported patients showed a pronounced $CD4^+$ T cell lymphopenia associated with defects in maturation, activation, and function of T, B, NK, and NKT cells [227, 229-231]. Our immunophenotypic analysis of patient-derived PBMC revealed low frequencies of $CD4^+$ T cells and increased proportions of TCR$\gamma\delta$ and $CD8^+$ T cells. However, in contrast to the reports of other groups, our RASGRP1-deficient patients showed normal numbers of B and NK cells.

Differentiation and cell fate decisions of T lymphocytes are highly dependent on signals mediated by the TCR-Ras-MAPK axis [233], however there are considerable differences in signaling activity between naïve and antigen-experienced (memory) T cells [234]. In T cells, the expression of three different types of Ras activators, comprising members of the RASGRF [235], RASGRP and SOS family [150], has been confirmed. Differential activation of these Ras GEF determines the quantity and quality of MAPK/ERK signaling strength, and there is accumulating evidence that minute variations in GEF-mediated Ras activation significantly influence T cell responses during distinct developmental stages, although it is not known how these nuances exactly adjust cellular activity [143]. Our functional studies revealed dysregulated TCR signaling indicated by delayed proliferation of TCR-stimulated T cells from P2 and P3. Moreover, we could prove a substantial defect in ERK1/2 activation and reduction of active GTP-bound Ras upon TCR-mediated signaling in cell lines overexpressing mutant RASGRP1, confirming a severe malfunction of the TCR-Ras-MAPK route. Murine knockout models have shown that MAPK signaling is central for thymic cell fate decisions and the intensity of ERK activation during T cell maturation significantly dictates development into $CD4^+$ or $CD8^+$ T cells. In particular, reduced ERK activation has been previously shown to favor differentiation of thymocytes into $CD8^+$ T cells [236]. Correspondingly, we found decreased frequencies of $CD4^+$ T cells in our patients, while TCR$\gamma\delta$ and $CD8^+$ T cells were above normal levels. Interestingly, patients with autoimmune diseases often present with defects in T cell differentiation, including $CD4^+$ and/or $CD8^+$ deficiency [237], what may explain the increased susceptibility for opportunistic infections observed in all RASGRP1-deficient patients. Furthermore, individuals with mutations in genes regulating TCR signaling, such as interleukin-2-inducible T cell kinase (ITK) [238], linker of activated T cells (LAT) [239], or lymphocyte-specific protein tyrosine kinase (LCK) [240], also present with low numbers of $CD4^+$ T cells and have an increased risk of developing lymphoproliferative and autoimmune diseases. Although the exact pathomechanisms remain largely unknown, improper

cellular responses upon TCR-antigen stimulation may negatively affect the development of functional T effector subsets and promote the generation of autoreactive T cell clones [241]. We could detect an abnormal, mainly decreased distribution of TCR Vβ chains in P2 and P3, suggesting an overt defect in the clonal expansion of TCRαβ T cells. TCR repertoire analysis is frequently used to identify and characterize autoreactive T cell clones in immunocompromised individuals, because immunocompetent children commonly show a diverse, but non-random TCR Vβ usage [242]. For instance, less diverse TCR repertoires have been documented in autoimmune diseases such as ankylosing spondylitis [243], juvenile RA [244], or SLE [245]. Interestingly, some of those patients presented with dysregulated RASGRP1 expression, providing further insights into the pivotal role of RASGRP1 in shaping the human T cell pool [224, 225]. In addition, we detected a low quantity of TCR excision circles in patients P2 and P3, a finding which is typically associated with autoimmunity and points to irregular TCR rearrangement and abnormal development of naïve T cells [246, 247]. The increased risk for autoimmunity may be linked to the involvement of RASGRP1 in positive selection of TCRαβ T cells, where it induces a low, but long-lived stimulus via the Ras-MAPK/ERK pathway leading to maturation and thymic egress [248]. Under physiological settings, conventional positively selected thymocytes do usually not show autoreactivity, since T cells expressing nonresponsive TCRs or binding with high affinity to MHC-self antigens will be deleted via apoptosis [249]. However, murine models confirmed that disruption of positive selection can allow the development of T cells which are highly responsive to self-antigens, promoting autoimmune diseases [250]. Therefore, it is tempting to speculate that human RASGRP1 deficiency may likewise result in the generation of a subset of defectively selected T cells, ultimately leading to autoimmunity. Taken together, our data confirmed that RASGRP1 is essential for the development and maintenance of distinct T effector lineages but also contributes to the establishment of a diverse TCR repertoire.

4.2.2 Human RASGRP1 deficiency promotes lymphoproliferative diseases

In mice, loss of *Rasgrp1* expression has been shown to result in acute autoimmune disease, including lymphoproliferation [153]. Similarly, all reported patients with RASGRP1 deficiency have developed LD [227, 229-231]. In line, we could also document the onset of an EBV-positive diffuse large B cell lymphoma in patients P1 and P3, whereas patient P2, who unfortunately died at the age of 4 years, only had elevated loads of both EBV and cytomegalovirus (CMV). PID patients are commonly diagnosed with abnormal lymphoproliferation during their disease course and in particular EBV seems to be a common

factor in the pathogenesis of LD [28]. EBV causes polyclonal activation and proliferation of infected B lymphocytes [30], which has been linked to certain pathologies such as infectious mononucleosis, Hodgkin's disease, and lymphoma [251]. In healthy individuals, mainly NK cells as well as $CD4^+$ and $CD8^+$ T cells confer immunity to viral infections, thereby limiting the expansion of malignant EBV-transformed B cells. In contrast, in immunodeficient patients, immune cells may fail to correctly respond to EBV invasion, what may result in the uncontrolled proliferation of infected cells. All our patients showed $CD4^+$ lymphopenia which might have contributed to the development of EBV-associated LD. Correspondingly, a higher risk for developing malignancy upon EBV infection has been documented in a number of primary immunodeficiencies caused by mutations in genes that regulate important pathways of innate and adaptive immunity such as *ITK* [238], SH2 domain-containing protein 1A (*SH2D1A*) [252], or serine/threonine kinase 4 (*STK4*) [253]. Interestingly, many PID associated with EBV-induced lymphoproliferation are linked to defects in growth, differentiation, or function of NK, B, or T cells, whereas PID related to complement deficiency, autoinflammatory disorders, perturbed phagocyte activation, or acquired immunodeficiencies do usually not involve malignancy [32]. Based on this knowledge, it seems reasonable that patients with dysregulated RASGRP1 expression may acquire LD. Remarkably, RASGRP1 is not only a key driver of lymphocyte development via induction of the TCR-Ras-MAPK pathway, but it is also critical for stimulating the transcription of genes which are needed for T cell homeostasis and define immunological responses, e.g. establishing effector functions or triggering the production of cytokines [163-165]. Previously reported RASGRP1-deficient patients showed decreased numbers and activation of B, NK, and iNKT cells [227, 229, 231]. In contrast, we documented normal numbers of B and NK cells in our patients. However, we could not perform functional assays on B or NK cells due to limited access to primary patient material. Taken together, these collective data suggest that loss of RASGRP1 expression results in impaired immunity towards viral infections and is linked to an increased risk for EBV-associated lymphoproliferation and malignancy.

4.3 Therapeutic approaches for TGF-β1 and RASGRP1 deficiency

PID are life-threatening diseases which usually manifest with a broad range of phenotypes and commonly involve secondary comorbidities. Interestingly, patients with PID background have an increased risk for developing cancer [27] or may present with IBD-like phenotypes [55]. Pediatric IBD have become more prevalent in recent years and consequently, therapeutic treatment strategies are of special clinical and socioeconomic interest [47]. Therefore, therapy regimens should ideally meet individual and disease-specific parameters. Specifically, genetic counseling and molecular diagnosis are central to decide on personalized therapeutic options. With growing knowledge of genes and pathways involved in the pathogenesis of IBD translational studies drive the evolution of new and innovative therapies. For instance, HSCT has proven as effective therapy to cure IBD with underlying PID [57]. Moreover, novel drugs which interfere with leucocyte trafficking [254], block pro-inflammatory cytokines [255], or reinforce the mucosal barrier [256] might optimize treatment of IBD in the near future. In addition to pharmacological approaches or HSCT, more unconventional methods such as fecal microbiota transplantation [257] or the use of probiotic bacteria [258] have been shown to be beneficial for IBD patients by modulating the intestinal microbial flora. Still, further studies focusing on understanding the molecular mechanisms of monogenic PID remain of paramount clinical importance for the development of modern and efficient therapies.

Our study showed that human TGF-β1 deficiency is a life-threatening disease which is refractory to symptomatic treatment. As an alternative therapy, external cytokine supplementation might be considered in patients with TGFB1 deficiency. In fact, there is evidence that pediatric IBD activity can be significantly reduced by a TGF-β2-enriched diet [259], although, oral TGF-β2 supplementation as a treatment for IBD remains controversially discussed [260]. Moreover, there is no formulation available to clinical care which is based on the delivery of TGF-β1. Although the external administration of TGF-β1 may seem practicable, the cytokine has a poor pharmacokinetic profile [261] which would require high dosage and repeated parenteral injections. In addition, in patients the systemic distribution of growth factors is not well controllable and may be influenced by unspecific or tissue-dependent TGF-β1 activation. Consequently, safety and efficiency of such a treatment are hardly to predict and could involve adverse reactions such as fibrosis [262] or cancer [263]. In 2011, the group of Simon Carding successfully developed a novel formulation for targeted intestinal delivery of recombinant human TGF-β1 using genetically engineered *Bacteroides ovatus*, which was able

to alleviate colitis in mice but had less systemic side effects [264]. However, many IBD patients showed increased intestinal TGF-β1 levels which are apparently not sufficient to resolve colitis [117]. In a murine *Tgfb1* knockout model, cytokine reconstitution did also not ameliorate the lethal phenotype of TGF-β1 deficiency, pointing to more intricate mechanisms controlling TGF-β1 bioavailability and function [106]. For that reason, the development of a TGF-β1-based pharmaceutical product that must not only meet GMP standards but also offers good efficiency in terms of bioavailability and low side-effects might take some more years. Despite attempts to compensate the lack of endogenous TGF-β1 via substitution therapies, allogeneic HSCT may present another curative approach for patients with TGF-β1 deficiency. Feasibility, safety, and therapeutic efficacy has been documented in PID patients suffering from VEO-IBD due to monogenic IL10 [265] or XIAP [266] deficiency, consequently HSCT might be considered for TGF-β1-deficient patients. However, before undergoing HSCT an intensive risk assessment is required and the treatment strongly depends on the patient's health condition. Since our patients showed debilitating phenotypes, including severe cerebral and developmental defects, which are most likely not reversible through neither HSCT or other therapeutic efforts, we did not pursue HSCT for P1. Still, early diagnosis will be critical for patients with TGF-β1 deficiency in order to decide whether HSCT should be performed. Further studies on the clinical spectrum and phenotype of TGF-β1 deficiency are needed to reason the best therapeutic options for the affected individuals.

In comparison to TGF-β1 deficiency, patients with loss-of-function mutations in *RASGRP1* did not present with chronic gut inflammation but showed aberrant T cell development and autoimmunity. However, RASGRP1-deficient patients presented with EBV-associated lympho-proliferation, which has been previously documented in other monogenic PID such as DOCK8 deficiency [267] or X-linked lymphoproliferative disease type 1 (XLP1) [268]. Conventionally, lymphoproliferation in PID patients can be addressed by chemotherapy and/or HSCT, but may be linked to increased mortality or the development of posttransplantational LD [269, 270]. Nevertheless, early HSCT has been shown to substantially raise life expectancy of transplanted individuals and warrants higher remission rates as compared to children or young adults who have undergone HSCT at later stages. For instance, patients with DOCK8 deficiency [267] or XLP1 [268], with early diagnosis and who timely received HSCT, showed overall survival rates of 84% and 93%, respectively. In view of the underlying PID in patients with RASGRP1 deficiency, HSCT should be considered as a curative treatment. Accordingly, our patient P1 successfully underwent HSCT at the age of 8

years whereas P3 is currently in stable condition and queued for transplantation. Although allogeneic HSTC has been efficiently used to cure immunodeficiencies, remission rates of pediatric PID patients who developed EBV-associated LD stagnate at around 60% [32]. Moreover, lacking an HLA-matched donor may completely prevent HSCT or at least significantly lower transplantation success in case of an HLA-mismatched donor [271]. Thus, there is still an urgent need for novel treatments. In particular, lentiviral gene therapy has raised clinical interest [23] and was successfully applied to treat PID such as X-linked severe combined immunodeficiency (X-SCID) [272], X-linked chronic granulomatous disease [273], or Wiskott-Aldrich syndrome [274]. Still, there is a certain risk that such a therapy may fail to fully reconstitute immunity or that some patients develop virus-induced leukemia over time [275], however, newer generations of self-inactivating lenti- and gammaretroviral vectors guarantee improved safety profiles [276]. Besides classic gene therapy, latest advances in genome editing using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 (CRISPR-associated protein 9)- or transcription activator-like effector nucleases (TALEN)-mediated approaches promise potential to repair a mutated gene or insert a healthy copy into the patient's genome *ex vivo* followed by reimplantation of the genetically corrected cells. For instance, defective T cell development in a humanized X-SCID mouse model could be successfully corrected by TALEN-based gene editing of embryonic stem cells [277]. Furthermore, in a preclinical study in 2016, scientists could successfully restore the expression of wild-type β-globin in hematopoietic stem cells from a sickle cell disease patient with more than 90% efficacy [278]. More recently, in a patient with HIV-1 infection and acute lymphoblastic leukemia the transplantation of CRISPR/Cas9-edited CCR5-ablated hematopoietic stem and progenitor cells resulted in complete remission with full donor chimerism without gene editing-related adverse events [279]. As another approach, the generation of various specialized cell lineages out of patient-derived (induced) pluripotent stem cells (iPSC) [24] may allow full recovery of immunity in PID. These state-of-the-art technologies hold great promises, however, genome editing technologies need to comply with moral, legal, and safety requirements to prevent unethical misuse. Governments, public authorities, clinicians as well as scientific investigators must work together to balance the risks and benefits for both patients and the environment and have to define to what extent clinical trials will be ethically and legally justifiable [280].

In conclusion, our studies on rare monogenic TGF-β1 and RASGRP1 deficiency have shed light on basic principles controlling human immunity. This knowledge provides critical insights to improve standard diagnostic tools or develop novel immunotherapies. Taken together, the identification of new PID genes and their underlying pathways is the driving force to advance customized and gene-specific cures which shall ultimately meet the worldwide medical needs of patients.

5 ABBREVIATIONS

AD	Alzheimer's disease
AKT	Ak strain thymoma (= Protein kinase B)
Ala	Alanine
ALK1	Activin receptor-like kinase 1
Arg	Arginine
Asp	Aspartic acid
BCR	B cell receptor
BMP	Bone morphogenetic protein
C	Cytosine / Carboxy
Cas9	CRISPR-associated protein 9
CC	Coiled coil
CCR5	C-C chemokine receptor type 5
CD	Crohn's disease / Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CED	Camurati-Engelmann disease
CMV	Cytomegalovirus
CNS	Central nervous system
CRISPR	Clustered regularly interspaced short palindromic repeats
Cys	Cysteine
CyTOF	Mass cytometry by time of flight
DAG	Diacylglycerol
DOCK8	Dedicator of cytokinesis 8
e.g.	Exempli gratia (= for example)
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ECM	Extracellular matrix
EGF	Epidermal growth factor
ENG	Endoglin
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FH	Follicular helper
FOXP3	Forkhead box protein P3
GARP	Glycoprotein A repetitions predominant

GDF	Growth and differentiation factor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
Gln	Glutamine
GTP	Guanosine triphosphate
HHT	Hereditary hemorrhagic telangiectasia
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
IBD	Inflammatory bowel disease
IEL	Intraepithelial lymphocyte
IFN-γ	Interferon gamma
IgA	Immunoglobulin A
IL	Interleukin
ILC	Innate lymphoid cell
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked (syndrome)
iPSC	Induced pluripotent stem cells
ITK	Interleukin-2-inducible T cell kinase
JNK	c-Jun N-terminal kinase
LAP	Latency-associated peptide
LAT	Linker of activated T cells
LCK	Lymphocyte-specific protein tyrosine kinase
LD	Lymphoproliferative disorder
LLC	Large latent complex
LPMC	Lamina propria mononuclear cell
LTBP	Latent TGF-β binding protein
Lys	Lysine
MAPK	Mitogen-activated protein kinase
mTOR	Mammalian target of rapamycin
N	Nitrogen
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Next-Generation Sequencing
NK	Natural killer (cell)
NKT	Natural Killer T (cell)
P	Patient
PBMC	Peripheral blood mononuclear cell(s)
PC	Plasma cell

pH	*Pondus hydrogenii*
PI3K	Phosphoinositide 3-kinase
PID	Primary immunodeficiency disorder
PLC-γ1	Phospholipase C gamma 1
PMA	Phorbol 12-myristate 13-acetate
RA	Retinoic acid / Rheumatoid arthritis
RASGRP1	Ras guanyl releasing protein 1
REM	Ras exchange motif
RGD	Arginine-glycine-aspartic acid
ROS	Reactive oxygen species
SH2D1A	SH2 domain-containing protein 1A
SLC	Small latent complex
SLE	Systemic lupus erythematosus
SLP-76	SH2 domain containing leukocyte protein of 76kDa
SMAD	Small mothers against decapentaplegic
SOS1	Son of sevenless 1
SP	Signal peptide
STK4	Serine/threonine kinase 4
T	Thymine
TALEN	Transcription activator-like effector nuclease
TCR	T cell receptor
TREC	T cell receptor excision circles
TEC	Thymic epithelial cell
TGFB1	Transforming growth factor beta 1 (gene)
TGF-α	Transforming growth factor alpha
TGF-β1	Transforming growth factor beta 1 (protein)
Th	T helper (cell)
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T (cell)
TβR	TGF-β receptor
UC	Ulcerative colitis
VEO-IBD	Very early onset inflammatory bowel disease
XLP1	X-linked lymphoproliferative disease type 1
X-SCID	X-linked severe combined immunodeficiency

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7 APPENDIX

7.1 Acknowledgement

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7.2 Statement of contributions

A) Contributions to Manuscript I: “Human TGF-β1 deficiency causes severe inflammatory bowel disease and encephalopathy”

For this study, I designed, conducted, and analyzed functional assays by employing primary patient cells as well as heterologous cellular models and performed the statistical analysis. Moreover, I cloned and validated the TGF-β1-sensitive luciferase reporter system and I contributed to the evaluation and interpretation of immunophenotypic data derived from FACS- and CyTOF-based assays. Finally, I participated in writing the manuscript and preparing the figures.

B) Contributions to Manuscript II: “Novel Mutations in RASGRP1 are Associated with Immunodeficiency, Immune Dysregulation, and EBV-Induced Lymphoma”

In this research project, I generated *RASGRP1*$^{-/-}$ cell lines using CRISPR/Cas9-mediated genome editing. Moreover, I (i) designed and cloned expression vectors encoding for RASGRP1 wild-type and patient-specific mutations, (ii) generated heterologous cellular models, and (iii) performed the corresponding *in vitro* experiments, including immunoblotting and pull-down assays. Lastly, I carried out statistical analysis for the obtained results and prepared the figures of the experiments. In cooperation with Ido Somekh and Daniel Kotlarz, I have analyzed and interpreted the results of the functional assays on patient-specific RASGRP1 mutants and we wrote the manuscript together.

We hereby confirm the above statements:

Munich, ……

……..	……..
Prof. Dr. Christoph Klein (PI)	Benjamin Marquardt

7.3 List of publications

1) Kotlarz D*, **Marquardt B***, Barøy T, Lee WS, Konnikova L, Hollizeck S, Magg T, Lehle AS, Walz C, Borggraefe I, Hauck F, Bufler P, Conca R, Wall SM, Schumacher EM, Misceo D, Frengen E, Bentsen BS, Uhlig HH, Hopfner KP, Muise AM, Snapper SB, Strømme P, and Klein C. Human TGF-β1 deficiency causes severe inflammatory bowel disease and encephalopathy. *Nature Genetics* 50, 344-348 (2018) DOI:10.1038/s41588-018-0063-6 * equal contribution

2) Somekh I*, **Marquardt B***, Liu Y, Rohlfs M, Karakukcu M, Unal E, Yilmaz E, Patiroglu T, Cansever M, Frizinsky S, Vishnvenskia-Dai V, Rechavi E, Stauber T, Simon AJ, Lev A, Klein C, Kotlarz D*, Somech R*. Novel mutations in *RASGRP1* are associated with immunodeficiency, immune dysregulation and EBV-induced lymphoma. *Journal of Clinical Immunology* 38(6), 699-710 (2018) DOI:10.1007/s10875-018-0533-8 * equal contribution

3) Lehle AS, Farin HF, **Marquardt B**, Michels BE, Magg T, Li Y, Liu Y, Ghalandary M, Lammens K, Hollizeck S, Rohlfs M, Hauck F, Conca R, Walz C, Weiss B, Lev A, Simon AJ, Groß O, Gaidt MM, Hornung V, Clevers H, Yazbeck N, Hanna-Wakim R, Shouval DS, Warner N, Somech R, Muise AM, Snapper SS, Bufler P, Koletzko S, Klein C, Kotlarz D. Intestinal Inflammation and Dysregulated Immunity in Patients with Inherited Caspase-8 Deficiency. *Gastroenterology* 156, 275-278 (2019) DOI:10.1053/j.gastro.2018.09.041

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