Human TGF-β1 deficiency causes severe inflammatory bowel disease and neurodevelopmental delay

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Introductory paragraph

Transforming growth factor beta-1 (TGF-β1) is the prototypic member of the TGF-β family of 33 proteins orchestrating embryogenesis, development, and tissue homeostasis[1,2]. Following its discovery[3], the TGF-β research field has evoked enormous interest and numerous controversies 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]. We here describe three patients from two pedigrees with biallelic loss-of-function mutations in the *TGFB1* gene presenting with severe infantile inflammatory bowel disease (IBD) and neurodevelopmental delay associated with epilepsy, brain atrophy, and posterior leukoencephalopathy. The proteins encoded by the mutant *TGFB1* alleles were characterized by impaired secretion, function, or stability of the TGF-β1-LAP complex, suggestive of perturbed bioavailability of TGF-β1. Our study highlights that TGF-β1 has a critical and non-redundant role for the development and homeostasis of the intestinal immunity and central nervous system in humans.

Results and Discussion

TGF-β1 is translated as a precursor protein consisting of an N-terminal signal peptide, the latency-associated peptide (LAP), and the C-terminal mature growth factor (TGF-β1). Upon proteolytic cleavage, LAP and TGF-β1 form the non-covalent 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 (LTBP), resulting in formation of large latent complexes (LLC)[9]. Multiple factors are known to control the release of active TGF-β1, e.g. proteases, reactive oxygen species, and integrins. Active TGF-β1 binds to a heterotetrameric transmembrane complex composed of TGF-β1 RI and TGF-β1 RII receptors, resulting in phosphorylation of 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 are associated with Camurati-Engelmann disease (CED) characterized by osteosclerotic lesions in the long bones and skull[11]. Increased TGF-β1-mediated signaling due to mutations in the TGF-β1 receptors (*TGFBR1* and *TGFBR2*) has been documented in patients with Loeys-Dietz syndrome, characterized by connective tissue disorders and arterial aneurysms[12]. In contrast to gain-of-function mutations, we report that biallelic loss-of-function mutations in *TGFB1* result in very early onset IBD and central nervous system dysfunction.

Patient 1 (P1, A.II-1), born to non-consanguineous 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 revealed crypt abscesses and inflammatory infiltrations of the epithelium with mucosal ulcerations (Fig. 1b). In addition, P1 showed eosinophilic esophagitis and esophageal candidiasis. The patient 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) showed a moderate global encephalopathic pattern lacking normal background activity and continuous mixed alpha and beta activity. No interictal epileptic discharges were recorded. Oligoclonal IgG bands and elevated IL-1β and IL-8 levels in the cerebrospinal fluid were suggestive of inflammatory processes.

P1 had a history of recurrent upper and lower respiratory tract infections and chronic CMV retinitis. Laboratory studies showed leukocytosis, thrombocytosis, and hypochromic anemia. Serum levels of IgG (4044 mg/dl, normal: 576-1507) and IgE (2665 IU/mL; normal: <90) were elevated, whereas IgA and IgM level were within normal ranges. Immunophenotypic analysis of peripheral blood mononuclear cells (PBMC) showed decreased proportions of memory activated regulatory T cells, as well as $CCR6^{-}CXCR3^{+}$ (Th1) and $CCR6^{+}CXCR3^{-}$ (Th17) T cells (Fig. 1d). T cell activation upon anti-CD3/anti-CD28 stimulation and T cell proliferation in response to specific antigens (diphtheria and tetanus toxoid) were reduced (Fig. 1d). CyTOF of colonic lamina propria mononuclear cells revealed a decreased frequency of $CD45RO^{+}$ and $CD45RA^{+}$ $FOXP3^{+}$, $CCR6^{-}CXCR3^{-}$, $CCR6^{+}CXCR3^{-}$, and $CD103^{+}$ T cells as compared to patients without IBD (uninflamed and inflamed controls) and a Crohn's disease (CD) patient (Fig. 1e, Supplementary Fig. 1). TGF-β1 exerts both stimulatory and inhibitory immunomodulatory effects[6], however we cannot exclude that some of the clinical and immunological features 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 the *TGFB1* gene (ENST00000221930.5) (c.[328C>T];[1159T>C], p.[R110C];[C387R]). Segregation of the sequence variant with the disease phenotype was confirmed by Sanger sequencing, indicating that the heterozygous mutation localized in the LAP domain was

inherited by the mother and the mutation in the mature TGF-β1 domain was inherited by the father (Supplementary Fig. 2 and Fig. 2a). In contrast to CED[11], radiographs of P1 were consistent with osteopenia (data not shown).

We also identified a homozygous missense mutation in the *TGFB1* gene (c.133C>T, p.R45C) in a second pedigree with two patients born by consanguineous parents from Pakistan (Supplementary Fig. 3a,b). The R45C mutation is localized in the LAP domain of the pre-pro TGF-β1 precursor (Fig. 2a). Patient 2 (P2, B.II-1) had a small head circumference (2.5th centile) and bloody diarrhea at three months of age. Neurological development reached a plateau at 9-10 months and subsequently regressed. At 19 months, she developed refractory complex partial and myoclonic seizures. EEG showed a pathological pattern similar to hypsarrhythmia. Cerebral MRI showed volume loss, cortical atrophy, and thinning of the corpus callosum. She lost her abilities to communicate and became increasingly spastic. Despite nutritional optimization she failed to thrive and died at the age of 25 months during hospitalization for suspected septicemia. Patient 3 (P3, B.II-4) had microcephaly at birth (head circumference <2.5th centile). At three months, he presented with failure to thrive and bloody diarrhea. Colonoscopy and histology revealed chronic active inflammation with abscesses and crypt branching (Supplementary Fig. 3c). Psychomotor regression, muscular hypotonia, and hyperreflexia was diagnosed at 12 months. At 25 months, he 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, lost visual and social contact, and the ability to perform voluntary movements. Moreover, he had fungal dermatitis, scabies skin infection, and an episode of severe varicella infection. An *Influenza A* virus infection triggered renal and subsequent multiorgan failure, causing his death at the age of 39 months. Immunophenotyping of PBMC revealed normal numbers and distributions for T, B, or NK cells, but a reduced proliferation of $CD4^+$ and $CD8^+$ T cells upon stimulation with anti-CD3 (data not shown).

We analyzed the mutation sites using the crystal structure of latent TGF-β1 (PDB accession number

3RJR)[15]. The identified mutations may perturb the interaction of TGF-β1 with the pro-domain or the TGF-β1 cysteine knot (Fig. 2b). R110 maps to a region denoted as the "fastener", which locks the interaction between the pro-domain and the growth factor domain (Fig. 2c, left panel). The beta-sheet harboring R110 forms a "super-beta-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. R45 maps to the interface between the TGF-β1 dimer and the pro-domain, suggesting that substitution R45C alters the interaction between these two functional elements (Fig. 2c, middle panel). Correct folding requires the presence of the pro-domain and may therefore be affected by the R45C mutation. The C387R mutation is predicted to perturb the correct formation of the cysteine knot in the growth factor domain, since the mutation will result in an unpaired cysteine and will prevent formation of the disulfide bond to C322 (Fig. 2c, right panel). Furthermore, the unpaired C322 might also affect correct formation of the other disulfide bonds as well by disulfide scrambling. Thus, C387R presumably affects fold or stability of the TGF-β1 growth factor domain dimer.

To validate the predicted consequences of the mutations on TGF-β1-LAP biosynthesis and function we used heterologous cellular models. We transduced HEK293T cells with lentiviral particles encoding wild-type or mutant TGF-β1 variants. The CED-causing TGF-β1 variant R218C was used as control[11]. Immunoblotting of cell lysates confirmed stable expression of the TGF-β1-LAP homodimers in cells transduced with either wild-type or mutant TGF-β1 (Fig. 2d). Latent and mature TGF-β1 could be detected in conditioned media from HEK293T cells overexpressing R45C, R110C, and R218C variants. The variants R45C and R110C showed reduced level of secreted TGF-β1. In contrast, the mutant C387R could not be detected in supernatants, suggesting that the abrogation of the disulfide bond prevents proper assembly and secretion. Correspondingly, ELISA showed that (i) only the variant R218C was detected as mature form under cell culture conditions (without HCl acidification) pointing to gain-of-function of this mutant[11], (ii) mature TGF-β1 can be released from the SLC upon HCl treatment in case of the R45C and R110C variants albeit at lower levels in comparison to wild-type, and (iii) secretion of the C387R variant was completely abrogated (Fig. 2e). To analyze downstream effects on TGF-β1-mediated signaling, we examined conditioned media in HEK293T

cells expressing a SMAD-sensitive luciferase reporter. The R45C and R110C variants exhibited reduced luciferase activity in comparison to wild-type TGF-β1, whereas no activity could be detected for the mutant C387R (Fig. 2f). To assess the stability of wild-type and mutant SLC, we monitored re-association of LAP-TGF-β1 over time. The SLC in supernatants from HEK293T cells was destabilized by HCl treatment and subsequently allowed to re-assemble upon neutralization with NaOH. In contrast to wild-type TGF-β1, all mutants showed compromised re-association capacity suggesting reduced stability of SLC (Fig. 2g). To assess TGF-β1 signaling in mucosal tissue we performed CyTOF analysis on colonic biopsies from P1. In comparison to patients without IBD (uninflamed and inflamed controls), the mean expression values of SMAD2/3 phosphorylation 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 p-SMAD2/3 was also seen in $CD45^+$ and $CD19^+$ cells from an unrelated CD patient, confirming impaired SMAD3 activity in mucosal tissue of IBD patients[16]. Taken together, all newly identified *TGFB1* mutant alleles appear 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 controversially discussed. While 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 highlighted a non-redundant role of TGF-β1 in controlling intestinal immune homeostasis and central nervous function, whereas other organ systems apparently were not affected. These findings are reminiscent of patients with IL-10 and IL-10R deficiency who present predominantly with infantile IBD[17,18], 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 been previously documented in experimental models. Mice with constitutive disruption of *Tgfb1*[19], T cell-specific deletion of *Tgfb1*[20], or expression of a dominant-

negative *TgfbRII*[21] develop a lethal wasting syndrome, including severe colitis. In CD patients, intestinal tissue or mucosal T cells are characterized by increased activation of SMAD7, an inhibitor of TGF-β1 signaling[16]. Treatment with SMAD7 antisense oligonucleotides holds promise to alleviate colitis in mice[22] and patients[23] 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 results in neuronal cell death and microgliosis[24]. Tissue-specific deletion of *Tgfb1* in the mouse central nervous system prevents lethal hyperinflammation, but leads to progressive defects in synaptic plasticity and loss of microglia[25]. Decreased TGF-β1 plasma levels[26] and reduced neuronal expression of TGFBR2[27] 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 Alzheimer's disease[28]. 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 the 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 co-morbidities. 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 central nervous system.

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

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Author Contributions

D.K. and C.K. have 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 of P2 and P3. T.M. and A.S.L. performed functional immunological assays. L.K. and S.M.W. conducted CyTOF analysis. S.H. performed the bioinformatics analysis of sequencing data and K.-P.H. conducted structural analysis of identified *TGFB1* mutations. W.S.L., I.B., F.H., P.B., E.M.S., and B.B. cared for the patients, collected patients' samples, and drafted clinical reports. C.W. examined histology. H.H.U. provided clinical information and specimen from a patient with CED. A.M.M. and S.B.S. screened local VEO-IBD cohorts for mutations in *TGFB1* and were instrumental in the interpretation of the human data. C.K. provided laboratory resources.

D.K. and C.K wrote the manuscript with the help from B.M. The manuscript was reviewed and approved by all co-authors.

Competing Financial Interests Statement

The authors declare that they have no competing financial interests.

References

1. Blobe, G.C., Schiemann, W.P. & Lodish, H.F. Role of transforming growth factor beta in human disease. *N Engl J Med* **342**, 1350-8 (2000).
2. Wu, M.Y. & Hill, C.S. Tgf-beta superfamily signaling in embryonic development and homeostasis. *Dev Cell* **16**, 329-43 (2009).
3. Derynck, R. *et al.* Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. *Nature* **316**, 701-5 (1985).
4. Principe, D.R. *et al.* TGF-beta: duality of function between tumor prevention and carcinogenesis. *J Natl Cancer Inst* **106**, djt369 (2014).
5. Silberstein, G.B. & Daniel, C.W. Reversible inhibition of mammary gland growth by transforming growth factor-beta. *Science* **237**, 291-3 (1987).
6. Li, M.O., Wan, Y.Y., Sanjabi, S., Robertson, A.K. & Flavell, R.A. Transforming growth factor-beta regulation of immune responses. *Annu Rev Immunol* **24**, 99-146 (2006).
7. Pohlers, D. *et al.* TGF-beta and fibrosis in different organs - molecular pathway imprints. *Biochim Biophys Acta* **1792**, 746-56 (2009).
8. Miyazono, K., Hellman, U., Wernstedt, C. & Heldin, C.H. Latent high molecular weight complex of transforming growth factor beta 1. Purification from human platelets and structural characterization. *J Biol Chem* **263**, 6407-15 (1988).
9. Rifkin, D.B. Latent transforming growth factor-beta (TGF-beta) binding proteins: orchestrators of TGF-beta availability. *J Biol Chem* **280**, 7409-12 (2005).
10. Li, M.O. & Flavell, R.A. TGF-beta: a master of all T cell trades. *Cell* **134**, 392-404 (2008).
11. Janssens, K. *et al.* Camurati-Engelmann disease: review of the clinical, radiological, and molecular data of 24 families and implications for diagnosis and treatment. *J Med Genet* **43**, 1-11 (2006).
12. Loeys, B.L. *et al.* A syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development caused by mutations in TGFBR1 or TGFBR2. *Nat Genet* **37**, 275-81 (2005).
13. Konkel, J.E. *et al.* Transforming Growth Factor-beta Signaling in Regulatory T Cells Controls T Helper-17 Cells and Tissue-Specific Immune Responses. *Immunity* **46**, 660-674 (2017).

14. Sharma, R. *et al.* IL-2 regulates CD103 expression on CD4+ T cells in Scurfy mice that display both CD103-dependent and independent inflammation. *J Immunol* **183**, 1065-73 (2009).
15. Shi, M. *et al.* Latent TGF-beta structure and activation. *Nature* **474**, 343-9 (2011).
16. Monteleone, G. *et al.* Blocking Smad7 restores TGF-beta1 signaling in chronic inflammatory bowel disease. *J Clin Invest* **108**, 601-9 (2001).
17. Glocker, E.O. *et al.* Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N Engl J Med* **361**, 2033-45 (2009).
18. Kotlarz, D. *et al.* Loss of interleukin-10 signaling and infantile inflammatory bowel disease: implications for diagnosis and therapy. *Gastroenterology* **143**, 347-55 (2012).
19. Shull, M.M. *et al.* Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* **359**, 693-9 (1992).
20. Li, M.O., Wan, Y.Y. & Flavell, R.A. T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. *Immunity* **26**, 579-91 (2007).
21. Gorelik, L. & Flavell, R.A. Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity* **12**, 171-81 (2000).
22. Boirivant, M. *et al.* Inhibition of Smad7 with a specific antisense oligonucleotide facilitates TGF-beta1-mediated suppression of colitis. *Gastroenterology* **131**, 1786-98 (2006).
23. Monteleone, G. & Pallone, F. Mongersen, an Oral SMAD7 Antisense Oligonucleotide, and Crohn's Disease. *N Engl J Med* **372**, 2461 (2015).
24. Brionne, T.C., Tesseur, I., Masliah, E. & Wyss-Coray, T. Loss of TGF-beta 1 leads to increased neuronal cell death and microgliosis in mouse brain. *Neuron* **40**, 1133-45 (2003).
25. Koeglsperger, T. *et al.* Impaired glutamate recycling and GluN2B-mediated neuronal calcium overload in mice lacking TGF-beta1 in the CNS. *Glia* **61**, 985-1002 (2013).
26. De Servi, B., La Porta, C.A., Bontempelli, M. & Comolli, R. Decrease of TGF-beta1 plasma levels and increase of nitric oxide synthase activity in leukocytes as potential biomarkers of Alzheimer's disease. *Exp Gerontol* **37**, 813-21 (2002).
27. Tesseur, I. *et al.* Deficiency in neuronal TGF-beta signaling promotes neurodegeneration and Alzheimer's pathology. *J Clin Invest* **116**, 3060-9 (2006).

28. Arosio, B. *et al.* +10 T/C polymorphisms in the gene of transforming growth factor-beta1 are associated with neurodegeneration and its clinical evolution. *Mech Ageing Dev* **128**, 553-7 (2007).

Figure Legends

Figure 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 non-consanguineous Malaysian family. (b) Gastrointestinal findings in P1: severe perianal disease with purulent discharge (far left panel); endoscopy revealed massive suppuration (left panel), superficial ulcerations and multiple pseudopolyps (right panel) in the colon; histology documented crypt abscesses and inflammatory infiltrations of the epithelium with mucosal ulceration (far right panel). (c) MRI of the brain indicating global brain atrophy and posterior periventricular leukencephalopathy in P1 (axial T2W, upper panel; coronar FLAIR, middle panel; ax3D_VISTA, lower panel). (d) Immunophenotypical analysis of regulatory T cells and T helper subsets as well as activation of $CD3^+CD4^+$ T cells upon stimulation with anti-CD3/anti-CD28 for 24h and proliferation of $CD3^+CD4^+$ T cells in response to specific antigens. Immunophenotyping was performed in two independent experiments. (e) CyTOF analysis of the composition of lamina propria mononuclear cells derived from two patients without IBD (uninflamed, inflamed), a CD patient, and P1. Clusters of $CD45^+$ viSNE plots were manually gated and color coded for various populations based on similar marker expression (left). Graphical representation depicts percentages of indicated immune cell populations (right). CyTOF analysis was performed once due to limited availability of patient material.

Figure 2. Effects of *TGFB1* mutations on the biosynthesis and bioavailability.

(a) Scheme of the distribution of identified mutations relative to the TGF-β1 structure depicting the N-terminal signal peptide, the latency-associated peptide (LAP), and the C-terminal mature growth factor (TGF-β1). The identified patients' mutations, including a previously described gain-of-function mutation in *TGFB1* causing CED[11], are depicted in DNA and protein sequence. (b) Structural visualization of the identified TGF-β1 mutations, using the crystal structure of latent TGF-β1 (PDB accession number 3RJR)[15]. The structure is depicted as ribbon model with highlighted secondary structure. Color code: yellow and green, pro-domain dimer; blue, TGF-β1 dimer; magenta spheres, mutation sites. (c) Detailed view of the mutation sites (stick model, with mutation side chains

highlighted in magenta). (d) Representative Immunoblotting of TGF-β1 expression in lysates and conditioned media from HEK293T stably overexpressing wild-type and mutant (R45C, R110C, R218C, C387R) TGF-β1 variants. Replicates: 3. (e) ELISA determining the TGF-β1 levels in conditioned media from HEK293T stably overexpressing wild-type and mutant TGF-β1 variants ± HCl treatment for release of mature growth factor from latent complexes. Samples of 10 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 were calculated using a two-tailed unpaired t test with Welch's correction. (f) SMAD luciferase reporter assay in HEK293T cells that were stimulated with conditioned medium from cells stably overexpressing wild-type or mutant TGF-β1. Samples of 9 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 mean of 11 independent cell culture experiments ± SEM. *P* values were calculated using a two-way repeated measures ANOVA with Dunnett's correction for multiple comparisons. (h) CyTOF analysis of SMAD2/3 phosphorylation in lamina propria mononuclear cells derived from patients without IBD (uninflamed, inflamed), a CD patient, and P1 (A.II-1). Histogram plots of baseline p-SMAD2/3 levels (left panel) and median expression values (MEV) of p-SMAD2/3 (right panel) are shown for indicated hematopoietic populations. CyTOF analysis was performed once (due to limited availability of patient material).

Online Methods

Patients

Patients have originally been identified by the Departments of Pediatrics, Faculty of Medicine, University Malaya, Kuala Lumpur, Malaysia (kindred I), or Oslo University Hospital, Norway (kindred II), and were referred for further studies to the Ludwig-Maximilians-Universität (LMU) München, Germany. Peripheral blood samples and biopsies from patients, their unaffected first-degree relatives, and 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, USA) according to the manufacturer's instructions. Upon enrichment for all coding exons using Agilent's SureSelect Human All Exon kit (Agilent Technologies, Canada), sequencing was performed on an Illumina Genome Analyzer II (pedigree A) or Illumina HiSeq2000 (pedigree B; Illumina Inc., USA). Short paired sequence reads were mapped to the human reference genome GRCh37 with BWA[1]. Genome Analysis Tool Kit (GATK)[2] was used to analyze the WES data and functional annotation was performed with snpEff[3] and Variant Effect Predictor (VEP), using Ensembl[4] release 85 (pedigree A) or 71 (pedigree B). WES data was filtered and analyzed using an in-house SQL-database (pedigree A) or FILTUS v.0.99-934 (pedigree B)[5]. Rare variants were distinguished by incorporating frequency information from the 1000 Genomes Project[6], NHLBI GO Exome Sequencing Project (ESP; evs.gs.washington.edu/EVS/), and/or ExAC[7]. Effects of filtered variants were predicted with a multitude of software, including snpEff[3], VEP[4], SIFT[8], and PolyPhen-2[9]. 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, USA) according to the manufacturer's instructions. Segregation of identified mutations in *TGFB1* was confirmed in available family members in pedigree A and B by DNA Sanger sequencing. Primer sequences are listed in Supplementary Table 1.

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

Electroencephalography and Magnetic Resonance Imaging

24-channel electroencephalography (EEG) recordings using Xltek hardware and software equipment (Natus DBA, Excel-Tech Corp., Canada) 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 with and without gadolinium contrast enhancement, T2 and fluid attenuated inversion recovery techniques) in axial, sagittal, and coronal planes (Philips Ingenia®, Netherlands).

Structural Analysis of TGF-β1 mutants

Structural visualization and modeling of the identified TGF-β1 mutations was performed with PyMol (Schrödinger, LLC).

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

Human wild-type *TGFB1* was amplified from a MGC sequence verified cDNA clone (MHS6278-202757887, Accession: BC022242, Dharmacon GE Healthcare, USA). Mutations in *TGFB1* (R45C, R110C, R218C, C387R) were introduced by site-directed PCR mutagenesis using corresponding primer pairs. Wild-type and mutant *TGFB1* cDNAs were cloned into IRES-EGFP or IRES-RFP bicistronic lentiviral pRRL vectors.

Biochemical assays were performed on HEK293T cells (ATCC, USA) that were routinely tested negative for Mycoplasma contaminations. For production of wild-type or mutant TGF-β1, HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 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 and lentivirally transduced according to previously published protocols[10]. Briefly, VSVg-pseudotyped lentiviral particles were generated by transfection of HEK293T cells. Using polyethyleneimine (PEI; Polysciences, USA) as transfection agent, cells were incubated with 5 µg lentiviral vector, 12 µg pcDNA3.GP.4xCTE (expressing HIV-1 gag/pol), 5 µg pRSV-Rev, and 1.5 µg pMD.G (encoding for VSV-g) in the presence of 25 µM chloroquine (Sigma, Germany) 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 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, USA).

Fluorescence-Activated Cell Sorting and Immunophenotyping

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

CyTOF analysis

Colonic tissue was digested overnight on a shaker at 37 ºC in complete RPMI media with 2 µL of collagenase and 2 µL of DNAse per 10 mL media. Undigested material was filtered out using a 10 µm filter. Single cells were resuspended in CyTOF staining buffer and 1-2x10^6 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 surface antibodies for 30 minutes. Next, cells were fixed in 1.6 % formaldehyde and treated with isopropanol for the detection of the phospho-antibodies or permeabilized with the FOXP3/Transcription Factor Staining Buffer Set (eBioscience) for staining with a cocktail of intracellular antibodies. Cells were then refixed in 1.6 % formaldehyde and stained with Ir-DNA intercalator solution. Finally, cells were resuspended in water containing 1:10 dilution of EQ beads and ran 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 was analyzed using the Premium CyTOBANK cloud-based software. Gating strategies are shown in Supplementary Fig. 6.

Western Blot Analysis and Enzyme-linked Immunosorbent Assay (ELISA)

To study TGF-β1-LAP biosynthesis and secretion, cell lysates and supernatants of HEK293T cells overexpressing wild-type and mutant TGF-β1 were analyzed by immunoblotting and ELISA following standard protocols. Briefly, 0.5 x 10^6 HEK293T cells and their derivates were cultured in 1 mL 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, Germany). Membranes were blocked in 5 % skim milk prior to staining. Antibodies used for detection are indicated in Supplementary Table 4. Membranes were developed using a chemiluminescent substrate (Thermo Fisher Scientific, USA). Images were captured using a ChemiDoc™ XRS+ System (Bio-Rad, Germany). Uncropped immunoblots are shown in Supplementary Fig. 7.

TGF-β1 levels in serum samples and cellular supernatants were measured by employing the Human TGF-β1 ELISA DuoSet (DY240, R&D Systems, USA) according to the manufacturer's instructions. To release the mature TGF-β1 from latent complexes, conditioned media were treated with 1 N HCl for 10 min, followed by neutralization with 1.2 N NaOH/0.5 M HEPES. Supernatants were analyzed

in duplicates using a Synergy™ H1 microplate reader (BioTek Instruments, USA).

TGF-β1-sensitive Firefly Luciferase Reporter Assay

A lentiviral TGF-β1-sensitive Firefly luciferase reporter plasmid was designed by insertion of the SMAD response elements (CAGA)[11] into the pGreenFire1-mCMV vector (#TR010PA-1-SBI, Biocat, Germany) between *EcoRI* and *SpeI* restriction sites. HEK293T cells were transduced with the reporter system as described above.

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

TGF-β1-LAP Re-association Assay

To examine the stability of latent complexes for the identified *TGFB1* mutations we assessed re-association of TGF-β1 and LAP upon complex disruption in a time-dependent manner, as described previously[12]. To release the mature TGF-β1 from latent complexes, conditioned media from transduced HEK293T cells were acidified with 1 M HCl for 10 min at room temperature and

neutralized with 1.2 M NaOH/0.5 M HEPES. After neutralization, samples were incubated at 37 °C for 5, 30, 60, 120, and 240 min and free TGF-β1 level were analyzed by ELISA. Free TGF-β1 levels were plotted by applying a one-phase exponential decay data transformation using the GraphPad Prism software (GraphPad Software, USA).

Statistics

Statistical evaluation of experimental data was performed using Prism version 6 (GraphPad Software, USA). No method of randomization or blinding was used and no samples were excluded from analysis. Data in Fig. 2e-f are reported as mean ± SEM. To analyze quantitative data sets either a two-tailed unpaired t test with Welch's correction to account for unequal variances (Fig. 2e and 2f) or a two-way repeated measures ANOVA with Dunnett's correction for multiple comparisons (Fig. 2g) were performed. All tests were two-tailed, and p values < 0.05 were considered statistically significant. Sample numbers are referred as n, unless indicated otherwise. Gaussian distribution of data was confirmed by D'Agostino & Pearsons omnibus normality test. No statistical method was used to predetermine sample size for analyses.

Data Availability and Accession Code Availability Statements

The identified *TGFB1* mutations have been submitted to the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/) with accession codes xxx (c.328C>T; p.R110C), yyy (c.1159T>C; p.C387R), and zzz (c.133C>T, p.R45C). 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.

Methods-only References

29. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**, 1754-60 (2009).

30. McKenna, A. *et al.* The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res* **20**, 1297-303 (2010).

31. Cingolani, P. *et al.* A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. *Fly (Austin)* **6**, 80-92 (2012).

32. McLaren, W. *et al.* Deriving the consequences of genomic variants with the Ensembl API and SNP Effect Predictor. *Bioinformatics* **26**, 2069-70 (2010).

33. Vigeland, M.D., Gjotterud, K.S. & Selmer, K.K. FILTUS: a desktop GUI for fast and efficient detection of disease-causing variants, including a novel autozygosity detector. *Bioinformatics* **32**, 1592-4 (2016).

34. Genomes Project, C. *et al.* A global reference for human genetic variation. *Nature* **526**, 68-74 (2015).

35. Lek, M. *et al.* Analysis of protein-coding genetic variation in 60,706 humans. *Nature* **536**, 285-91 (2016).

36. Kumar, P., Henikoff, S. & Ng, P.C. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc* **4**, 1073-81 (2009).

37. Adzhubei, I., Jordan, D.M. & Sunyaev, S.R. Predicting functional effect of human missense mutations using PolyPhen-2. *Curr Protoc Hum Genet* **Chapter 7**, Unit7 20 (2013).

38. Kotlarz, D. *et al.* Loss-of-function mutations in the IL-21 receptor gene cause a primary immunodeficiency syndrome. *J Exp Med* **210**, 433-43 (2013).

39. Dennler, S. *et al.* Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO J* **17**, 3091-100

(1998).

40. Walton, K.L. *et al.* Two distinct regions of latency-associated peptide coordinate stability of the latent transforming growth factor-beta1 complex. *J Biol Chem* **285**, 17029-37 (2010).