Immune responses need to be controlled tightly to prevent autoimmune diseases, yet underlying molecular mechanisms remain partially understood. Here, we identify biallelic mutations in three patients from two unrelated families in differentially expressed in FDCP6 homolog (DEF6) as the molecular cause of an inborn error of immunity with systemic autoimmunity. Patient T cells exhibit impaired regulation of CTLA-4 surface trafficking associated with reduced functional CTLA-4 availability, which is replicated in DEF6-knockout Jurkat cells. Mechanistically, we identify the small GTPase RAB11 as an interactor of the guanine nucleotide exchange factor DEF6, and find disrupted binding of mutant DEF6 to RAB11 as well as reduced RAB11\(^+\)CTLA-4\(^+\) vesicles in DEF6-mutated cells. One of the patients has been treated with CTLA-4-Ig and achieved sustained remission. Collectively, we uncover DEF6 as a player in immune homeostasis ensuring availability of the checkpoint protein CTLA-4 at T-cell surface, identifying a potential target for autoimmune and/or cancer therapy.
Although immune dysregulation and autoimmunity are hallmarks of multiple human diseases, their underlying molecular pathological mechanisms remain poorly understood. Studying monogenic disorders with predominant autoimmunity offers an attractive strategy to identify core regulators of immune homeostasis. Key regulatory components which help tune immune responses include regulatory T cells (Tregs) and the checkpoint protein CTLA-4. CTLA-4 is constitutively expressed on Tregs and upon activation on activated conventional helper-T cells (Tconv). CTLA-4 ligand engagement competes with the activating co-receptor CD28 for interaction with their shared ligands CD80/CD86 expressed on antigen-presenting cells (APCs), thereby inhibiting T-cell costimulation. Binding of CTLA-4 to CD80/CD86 results in ligand transendocytosis into the T cell, sequestering the costimulatory ligands from APCs. Inside the cell, CD80/CD86 are guided to lysosomal degradation, and shuttling to either lysosomes or RAB11+ recycling endosomes. Functionally, CTLA-4 competes with the activating co-receptor CD28 for interaction with their shared ligands CD80/CD86 expressed on antigen-presenting cells (APCs), thereby inhibiting T-cell costimulation. Binding of CTLA-4 to CD80/CD86 results in ligand transendocytosis into the T cells, sequestering the costimulatory ligands from APCs. Inside the T cell, CD80/CD86 are guided to lysosomal degradation.

The importance of CTLA-4 in regulating human immune tolerance is underlined by several SNPs conferring increased risk of autoimmunity and further solidified with the recent identification of patients with monoallelic mutations in CTLA4 or biallelic mutations in LRBA suffering from severe autoimmunity. Additional cellular regulators of CTLA-4 and their relevance to human disease remain to be investigated.

DEF6, also known as IRF4 binding protein (IBP) or SWAP-70-like adaptor of T cells (SLAT), is a unique guanine nucleotide exchange factor (GEF) which has an inverse conformation of the PH-DH domain compared to conventional GEFs. DEF6 acts downstream of the T-cell receptor (TCR) and can be phosphorylated by the tyrosine-protein kinases Lck and ITK. It can activate small GTPases of the RHOA and Ras family, promoting Ca2+ signaling, NFAT1 activation, and T-cell adhesion. Additionally, DEF6 binds and negatively regulates the transcription factor IRF4. Murine knockout studies have illustrated a role of Def6 in immunological synapse formation, Th1/Th2 lineage differentiation, IL17 and IL21 production, bacterial phagocytosis, T-cell proliferation, as well as a possible role in early-onset large vessel vasculitis and autoimmunity.

Interestingly, other studies of Def6-knockout mice contrarily revealed resistance to uveitis and experimental autoimmune encephalitis, and to date it remains unclear whether susceptibility to autoimmunity is dependent on the genetic background of the mice or other factors. Thus, the role of DEF6 in autoimmunity has remained controversial and partially enigmatic.

Here, we uncover an inborn error of immunity caused by biallelic mutations in DEF6 and characterized by early-onset systemic autoimmunity. We find impaired CTLA4 availability and trafficking, due to decreased interaction of mutated DEF6 with the small GTPase RAB11, as the mechanistic basis for the autoimmune manifestations.

Results

Systemic autoimmunity in three patients from two families. We studied three patients with severe autoimmune manifestations. Patient 1 is female (P1, Family A) born to consanguineous Pakistani parents (Fig. 1a) who presented with severe watery diarrhea at the age of 5 months (top) showed incomplete villous atrophy with villi focally reduced and plump (closed arrows). The inflammatory infiltrate contains clusters of eosinophilic granulocytes (lined arrows) and only few crypts with isolated apoptotic figures (asterisk). At the age of 16 months (bottom, 1 month of therapy with Abatacept, see Fig. 1e) duodenal biopsies showed presence of villi (closed arrows) and no signs of acute inflammation in the lamina propria (asterisk) of P1. Perianal fissures of P1 before (top) and after (bottom) therapy initiation present a marked improvement of patient quality of life (m - months).
diarrhea in the first month of life. Endoscopy revealed atrophy of gastric mucosa and villous atrophy with pronounced T- and eosinophilic cell infiltration in the colon and duodenum (Fig. 1b and Fig. S1a). Further disease features included hepatosplenomegaly, dilated cardiomyopathy, and increased susceptibility to viral and bacterial infections suggesting a primary immune defect (Tables 1 and 2). Immune phenotyping revealed reduced CD8+ T-cell numbers (Table 1) and slightly reduced percentages of CD25highCD127lowFoxP3+ Treg (Fig. S1b) in the circulation. Immunoglobulin levels were not consistently altered (Table 1), only few CD19+CD27–IgD– class-switched B cells were detected (Fig. S1c), and specific antibody responses were impaired (Table 2). Clinical signs of autoimmunity were paralleled by detectable anti-neutrophil cytoplasmatic antibodies (ANCA) and autoantibodies against cardiolipin, smooth muscle protein, and β2-glycoprotein 1 (Table 2). NK cells were in the normal range, and neutrophil function including oxidative burst as well as phagocytosis of opsonized bacteria was not impaired (Table 2). A serum cytokine/chemokine blot did not reveal elevation of pro-inflammatory cytokines but rather reduced levels of serum IL-12 and IL-6 compared to a healthy control (Fig. S1d). Upon clinical deterioration of symptoms, we initiated CTLA-4-Ig (Abatacept) treatment at 4-week intervals starting at 15 months of age (Fig. S1e). Consequently, bowel inflammation decreased markedly as reflected by fecal calprotectin values (Fig. 1c). Lymphocytic infiltration and complete villous atrophy of the duodenum improved within one month of treatment (Fig. 1d). In addition, persisting perianal lesions reversed and did not recur (Fig. 1e). P1 was consequently discharged and treated as an outpatient (Fig. S1e). To date, ~4 years after treatment initiation, no overt signs of autoimmunity have reoccurred, and cardiorespiratory fitness has been stable without arrhythmias or other overt pathology. Regular immunoglobulin treatment is given. Recurrent infections requiring antibiotic treatment have persisted (Fig. S1e). The female sibling of P1 (patient 2 or P2) had been diagnosed earlier with a systemic autoimmune/autoinflammatory disease that included bowel inflammation, hepatomegaly, cholestasis, and cardiac ventricular septal defect. P2 also presented with recurrent infections and exhibited reduced numbers of lymphoid cells (Table 1, Table 2), however immunological investigations could not be performed in-depth since P2 died at 10.5 months of age due to cardiomyopathy-associated cardiac and multi-organ failure.

A third patient (P3, family B), born to consanguineous Iraqi parents (Fig. 1a), presented at 7 months of age with hemolytic anemia in the context of a CMV infection which was successfully treated with corticosteroids/azathioprine and ganciclovir/valganciclovir as indicated by decreased CMV DNA levels. Direct Coombs test was positive and hemolytic anemia relapsed at the age of 27 months (Table 2) without detectable CMV DNA, prompting initiation of immunosuppressive treatment. Despite initiation, P3 developed transient thrombocytopenia (minimum 32 × 109/L) which resolved spontaneously at the age of 3.5 years. Blood counts revealed reduced lymphocyte numbers (0.9–2.5 × 109/L, Table 1) with low absolute numbers of T, B and NK cells, yet largely normal relative percentages of lymphocytes (Table 1). More in-depth immunophenotyping revealed slightly increased proportions of CD3highIgM+ transitional B cells (18.7%, reference 3.1–12.3% (ref. 32)) and CD3highIgM+ plasmablasts (7.7%, reference 4.0–4.0% (ref. 32)) but normal frequencies of

| Table 1 Immunological data on patients with DFE6 mutations |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Patient (age)** | **P1 (1–4 m)** | **P1 (5–8 m)** | **P2 (1–4 m)** | **P2 (5–6 m)** |
| **ALC (cells/mm³)** | 6040 (4054–7048) | 6250 (3320–7006) | 2230 (3320–7006) | 1450 (3320–7006) |
| **IgM (g/L)** | 0.17 (0.34–2.1) | 0.17 (0.34–2.1) | 0.17 (0.34–2.1) | 0.17 (0.34–2.1) |
| **CD3+ (%)** | 77 (62.7–81.6) | 77 (62.7–81.6) | 77 (62.7–81.6) | 77 (62.7–81.6) |
| **CD4+ (%)** | 34 (42.8–65.7) | 34 (42.8–65.7) | 34 (42.8–65.7) | 34 (42.8–65.7) |
| **CD8+ (%)** | 11 (15–23) | 11 (15–23) | 11 (15–23) | 11 (15–23) |
| **CD19+ (%)** | 8 (7.4–21.3) | 8 (7.4–21.3) | 8 (7.4–21.3) | 8 (7.4–21.3) |
| **CD6+56+ (%)** | 12 (4.2–14.8) | 12 (4.2–14.8) | 12 (4.2–14.8) | 12 (4.2–14.8) |
| **IgG (g/L)** | 1.60 (4.0–7.4) | 1.60 (4.0–7.4) | 1.60 (4.0–7.4) | 1.60 (4.0–7.4) |
| **IgA (g/L)** | 0.009 (0.017–0.049) | 0.009 (0.017–0.049) | 0.009 (0.017–0.049) | 0.009 (0.017–0.049) |
| **IgM (g/L)** | 0.17 (0.34–2.1) | 0.17 (0.34–2.1) | 0.17 (0.34–2.1) | 0.17 (0.34–2.1) |

Lymphocyte reference values (in brackets) were taken from ref. 68. Values outside reference range are marked in bold. Immunoglobulin (Ig) concentration was tested at least 4 weeks after the last intravenous Ig treatment. P1 was vaccinated three times with Prevenar 13® (Pfizer: pneumococcal polysaccharide conjugated vaccine) and INFANRIX hexa® (GSK: SmithKline: Cosynorbius diphtheriae, Clostridium tetani, Bordetella pertussis, Haemophilus influenzae type B, hepatitis B virus, poliovirus) at the age of 3, 4, and 10 months. The higher values might be caused by the presence of maternal antibodies.

m: months, y: years, ALC: absolute lymphocyte count, TCR: T-cell receptor, Ig: Immunoglobulin.
CD19<sup>+</sup>CD27<sup>+</sup>IgD<sup>−</sup> class-switched B cells (13.1%, reference 4.7–21.2% (ref. 32)), slightly reduced percentages of CD25<sup>high</sup>PD-1<sup>low</sup>FOXP3<sup>+</sup> T<sub>reg</sub> cells (Fig. S1f), and decreased mature CD56<sup>dim</sup>CD16<sup>+</sup>CD57<sup>+</sup> NK cell population (Fig. S1g). Distribution of T-helper cell subsets in PBMCs, after accounting for age-related high numbers of naïve T cells, did not reveal abnormal skewing (Fig. S1h and i). Immunoglobulin levels were in the normal range (Table 1).

**Germline mutations in DEF6 segregate in both families.** Exome sequencing was performed for P1 and P3 to identify the underlying molecular disease etiologies, and confirmed by Sanger sequencing in respective family members. Among the segregating variants, DEF6 was the single common gene affected in both pedigrees that segregated with the disease. Enlarged pedigrees were sequenced to confirm segregation of variants with disease (Fig. 2a, b). In family A, we identified a homozygous missense variant in DEF6 (c.G991A, p.E331K) affecting the highly conserved PH-DH domain in both affected siblings P1 and P2 (Fig. 2a, c), while exome sequencing in family B identified a second, more N-terminal homozygous missense variant in DEF6 (c.T628G, p.Y210D) in P3 (Fig. 2b, c). Genetic investigation was not performed for the newborn sister. Mutation Y210D affects a residue phosphorylated by ITK and necessary for interactions with the kinase<sup>22</sup>. This residue was previously shown to be phosphorylated by LCK as well and critical for induction of DEF6 activation<sup>25</sup>, however, these findings have not been corroborated by further studies. Both variants were predicted damaging by Polyphen-2, SIFT and CADD (Table S1). The mutated amino acids E331 and Y210 are conserved among vertebrates.

### Table 2 Clinical characteristics of patients with DEF6 mutations

| Patient (age) | P1 | P1 (1-4 m) | P1 (5-8 m) | P1 (9-10 m) | P1 (11-12 m) | P2 | P3 |
|--------------|----|------------|------------|-------------|-------------|----|----|
| Neutrophil function | normal | (E. coli opson., S. pneumoniae opson.) | normal | normal | normal | normal | normal |
| Phagocytosis | normal | (E. coli opson., S. pneumoniae opson.) | normal | normal | normal | normal | normal |
| Oxidative burst | normal | (E. coli opson., S. pneumoniae opson.) | normal | normal | normal | normal | normal |
| Hemoglobin | normal | (E. coli opson., S. pneumoniae opson.) | normal | normal | normal | normal | normal |
| Vaccination response | C. tetani | 0.73 IU/ml (≥0.4 IU/ml) | 0.66 IU/ml (≥0.4 IU/ml) | 0.51 IU/ml (≥0.4 IU/ml) | 0.51 IU/ml (≥0.4 IU/ml) | 0.51 IU/ml (≥0.4 IU/ml) | 0.51 IU/ml (≥0.4 IU/ml) |
| Bacteria | C. diphtheriae | 0.05 IU/ml (≥0.4 IU/ml) | 0.02 IU/ml (≥0.4 IU/ml) | 0.02 IU/ml (≥0.4 IU/ml) | 0.02 IU/ml (≥0.4 IU/ml) | 0.02 IU/ml (≥0.4 IU/ml) | 0.02 IU/ml (≥0.4 IU/ml) |
| S. pneumonia | 1:76 (≥1:200) | 1:20 (≥1:200) | 1:20 (≥1:200) | 1:20 (≥1:200) | 1:20 (≥1:200) | 1:20 (≥1:200) | 1:20 (≥1:200) |
| H. influenzae | 0.76 µg/ml (≥1 µg/ml) | 0.60 µg/ml (≥1 µg/ml) | 0.07 µg/ml (≥1 µg/ml) | 0.07 µg/ml (≥1 µg/ml) | 0.07 µg/ml (≥1 µg/ml) | 0.07 µg/ml (≥1 µg/ml) | 0.07 µg/ml (≥1 µg/ml) |
| B. pertussis | 0.6 VE (≥11 VE) | 0.6 VE (≥11 VE) | 0.6 VE (≥11 VE) | 0.6 VE (≥11 VE) | 0.6 VE (≥11 VE) | 0.6 VE (≥11 VE) | 0.6 VE (≥11 VE) |
| Autoantibodies | ANCA | Positive (1:160) | Positive (1:40) | Positive (12.1 U/ml) | Positive (12.1 U/ml) | Positive (12.1 U/ml) | Positive (12.1 U/ml) |
| Cardiolipin (IgG) | Elevated (10.5 U/ml) | Positive (28.8 U/ml) | Positive (28.8 U/ml) | Positive (28.8 U/ml) | Positive (28.8 U/ml) | Positive (28.8 U/ml) | Positive (28.8 U/ml) |
| Beta2-glycoprotein (IgM) | Normal (4.9 U/ml) | Positive (8 U/ml) | Positive (8 U/ml) | Positive (8 U/ml) | Positive (8 U/ml) | Positive (8 U/ml) | Positive (8 U/ml) |
| Direct Coombs test | Positive | Positive | Positive | Positive | Positive | Positive | Positive |
| Recurrent infections | Bacteria | S. pneumoniae, S. aureus, S. epidermis, E. aerogenes, E. cloacae, E. faecalis | E. aerogenes, K. oxytoca, S. epidermis, E. faecalis | E. aerogenes, K. oxytoca, S. epidermis, E. faecalis | E. aerogenes, K. oxytoca, S. epidermis, E. faecalis | E. aerogenes, K. oxytoca, S. epidermis, E. faecalis | E. aerogenes, K. oxytoca, S. epidermis, E. faecalis |
| Virus | Rhinovirus, influenza B, respiratory syncytial virus, rotavirus | Not specified | Not specified | Not specified | Not specified | Not specified | Not specified |
| Fungi | Not specified | Malassezia furfur | Malassezia furfur | Malassezia furfur | Malassezia furfur | Malassezia furfur | Malassezia furfur |

Reference values in brackets. Values outside reference range are marked in bold. Bacterial species are indicated in italic font.

m months, y years, n.d. not determined, opson. opsonized.
In summary, we identified three patients from two unrelated families presenting with features of systemic autoimmunity, bearing two distinct biallelic missense variants within the **DEF6** gene.

**DEF6** mutations affect **CTLA-4** cycling dynamics.** As DEF6 is predominantly expressed in T cells (Fig. S2b), we focused on investigating T-cell phenotypes. While calcium flux was found unaltered in feeder-expanded patient cells upon TCR stimulation (Fig. S2c and d), ERK phosphorylation and AKT phosphorylation were partially reduced but not abolished compared to healthy donor (Fig. S2e). Intriguingly, proliferation of PBMCs or feeder-expanded T cells was not compromised (Fig. S2f and g). DEF6 is also expressed, to a lesser extent, in NK cells (Fig. S2b). No defect in NK-cell immunological synapse formation could be detected (Fig. S2h). CTLA-4, similar to DEF6, is predominantly expressed in T cells. Given the marked response of P1 to Abatacept (CTLA-4-Ig) treatment enabling clinical disease remission (Fig. 1c–e), we hypothesized that autoimmunity in DEF6 deficiency may be linked to aberrant CTLA-4 regulation. Expression of CTLA-4 is predominantly regulated by FOXP3 (ref. 35) and calcium-dependent NFAT activation. We first analyzed CTLA-4
upregulation in stimulated memory-Treg cells that most robustly express CTLA-4, and normalized the expression to unstimulated, naïve conventional T cells as previously described. CD3/CD28 stimulation indeed showed significantly lower CTLA-4 expression in Treg cells of P3, while CTLA-4 levels in P1 were non-significantly decreased (Fig. 3a and Fig. S3a). P1 and P3 both showed slightly reduced FOXP3 levels in Treg (Fig. S1b and f). The checkpoint receptor CTLA-4 modulates T-cell responses through binding to and trans-endocytosis of the costimulatory molecules CD80/CD86 from APCs. While CD80/CD86 are degraded within T-cell lysosomes, CTLA-4 itself is recycled to the plasma membrane through the vesicular transport systems. Defective CTLA-4 lysosomal sorting has been described previously to underlie autoimmunity in LRBA deficiency. As DEF6 is a GEF for small GTPases, a protein class crucial for vesicular transports, we focused on studying DEF6 mutations affecting CD80 ligand uptake by CTLA-4.

**DEF6 mutations affect CD80 ligand uptake by CTLA-4.** We next investigated patient T cells for their ability to capture and trans-endocytose CTLA-4-ligands. We here investigated memory Treg as previous work has shown that this cell population most robustly reveals defects in CTLA-4 ligand binding. In accordance with our hypothesis that defective CTLA-4 cycling results in reduced surface availability of CTLA-4 and as a secondary consequence also in reduced relative ligand capture on the T-cell surface, we observed reduced uptake of CD80-Ig in P1 memory Treg (Fig. 4a, gating as in Fig. S3k), as indicated by the reduced slope of the best fit line when compared to healthy controls.
that CTLA-4 does not effectively reach surfaces in Fig. 4a, similar overall binding capabilities of the total expressed CTLA-4 cell permeabilization resulted in comparable slopes reflecting during stimulation. Anti-CTLA-4 blocked ligand uptake in both samples. For permeabilization control, CD80-Ig was added after T cells were stimulated with anti-CD3/anti-CD28 antibody-coated beads (16 h). CD80-Ig and anti-CTLA-4 (where applicable) antibodies were present for permeabilization control, CD80-Ig was added after T cells were stimulated with anti-CD3/anti-CD28 antibody-coated beads (16 h). CD80-Ig and anti-CTLA-4 (where applicable) antibodies were present during stimulation. Anti-CTLA-4 blocked ligand uptake in both samples. For permeabilization control, CD80-Ig was added after fixation/permeabilization, for binding all available CTLA-4. Data from flow cytometry were extracted, visualized and analyzed with Prism. Slopes were calculated with linear regression. Gating as in Fig. S3k. Representative of two independent experiments.

Fig. 4 DEF6 mutations affect CD80 ligand uptake by CTLA-4. a, ligand uptake assay of memory Treg reveals reduced uptake of CD80-Ig in P1 cells (bottom, orange lines) compared to HD control (top and bottom, black/grey lines), as depicted by the reduced slope of the best-fit lines. Purified CD4 T cells were stimulated with anti-CD3/anti-CD28 antibody-coated beads (16 h). CD80-Ig and anti-CTLA-4 (where applicable) antibodies were present during stimulation. Anti-CTLA-4 blocked ligand uptake in both samples. For permeabilization control, CD80-Ig was added after fixation/permeabilization, for binding all available CTLA-4. Data from flow cytometry were extracted, visualized and analyzed with Prism. Slopes were calculated with linear regression. Gating as in Fig. S3k. Representative of two independent experiments. b, Transendocytosis assay of CD4 T cells shows reduced CD80-GFP capture by P1 cells from CHO cells, indicated by reduced CTLA-4+CD80+ double-positive populations (orange numerical insert). Presence of anti-CTLA-4 blocked transendocytosis. Cells were stimulated for 16 h with anti-CD3 antibody. Co-stimulatory signal was provided by CD80-GFP expressing cells. Quantification as in Fig. S3j, gating as in Fig. S3l. Representative of two independent experiments. Source data of Fig. 4 are provided as a Supplementary Source Data file.

The observed differences are in line with previous reports on cells from patients with heterozygous CTLA4 mutations showing dysfunctional ligand capture. Preservation of CTLA-4-blocking antibody abolished ligand uptake (Fig. 4a, “CD80-Ig + anti-CTLA-4”). Finally, the addition of ligand after cell permeabilization resulted in comparable slopes reflecting similar overall binding capabilities of the total expressed CTLA-4 (Fig. 4a, “CD80-Ig (permeabilized)”)

The data demonstrate that CTLA-4 does not effectively reach surfaces in DEF6-mutated T cells, and as a result the surface-dependent function of CTLA-4 is disturbed. We also analyzed CTLA-4-dependent ligand transendocytosis from CD80-GFP expressing donor cells. In line with a reduced CTLA-4 surface abundance we found less CD80-GFP transendocytosed into CD4 T cells of P1 compared to healthy control (Fig. 4b and Fig. S3j, gating as in Fig. S3j). Presence of CTLA-4-directed antibody blocked transendocytosis (Fig. 4b and Fig. S3j). Altogether, due to impaired availability of the checkpoint protein CTLA-4 on T cell surfaces, the capturing and transendocytosis of ligands is consequently impaired.

**DEF6 knockout phenocopies CTLA-4 cycling defects.** To evaluate the causality of mutated DEF6 for aberrant regulation of CTLA-4 trafficking, we utilized several models. We performed CTLA-4 mobilization assays on CD4 T cells of P1 and a healthy donor to monitor CTLA-4 on cell surfaces after short stimulation, which effectively mobilizes CTLA-4 from internal stores. While total CTLA-4 expression was unaffected, mobilized CTLA-4 was reduced in patient T cells compared to HD (Fig. 5a, gating as in Fig. S4a). In a similar setup, we electroporated healthy control or DEF6E331K-mutated PBMCs with wild type DEF6-GFP (Fig. 5b) or mutated DEF6E331K-GFP (Fig. 5c). The observed mobilization defect of CTLA-4 in DEF6E331K-mutated CD4 T cells was reversed by wild-type DEF6 but not by DEF6E331K (Fig. 5b, c, gating as in Fig. S4a). To further prove causality, we generated CRISPR-mediated knockout clones of DEF6 and Renilla control in CTLA-4-mCherry transduced Jurkat cells (Fig. 5d).

Pronounced reduction of DEF6 expression resulted in defective CTLA-4 cycling, confirming the role of DEF6 in regulating CTLA-4 trafficking (Fig. 5e, gating as in Fig. S4b). These defects could be partly reconstituted by electrically reconstituting the wildtype by not mutant DEF6 (Fig. 5f, gating as in Fig. S4c), though slightly more mutant protein was expressed (Fig. S4d). DEF6 knockout cells furthermore showed reduced suppression tendency against CD4 target cells in presence of unlabeled PBMCs as APC source (Fig. S4e, gating as in Fig. S4f).

Our collective data on primary T cells reconstituted with wildtype DEF6 and on Jurkat knockout cells demonstrate that the decreased CTLA-4 availability in DEF6-mutated patient cells is caused by defective intracellular trafficking processes.

**DEF6 mutations affect RAB11 interactions.** Given our finding that the guanine nucleotide exchange factor DEF6 regulates CTLA-4 cycling processes, we hypothesized that DEF6 might regulate the small GTPase RAB11, a central protein for recycling endosomes that has been shown to co-localize to CTLA-4+ vesicles. We first assessed the localization of endogenous DEF6, RAB11 and CTLA-4 in activated patient-derived and control PBMCs. In line with previous studies, we observed prominent co-localization of CTLA-4 with RAB11 in the healthy control cells (Fig. 6a, b). In sharp contrast, RAB11/CTLA-4 co-localization was largely repressed in TCR/CD28-stimulated DEF6E331K-mutated cells of P1 (Fig. 6c). Line scans through CTLA-4+ vesicles confirmed the lack of co-localization with RAB11 in DEF6E331K-mutated cells (Fig. 6d).

To quantify RAB11 and CTLA-4 co-localization, defined regions of interest with high CTLA-4 expression and RAB11-positive vesicles were selected and analyzed for overlap coefficients. Quantitative analyses revealed a significant reduction of co-localization for P1 and P3 compared to respective healthy control cells (Fig. S5a-c), suggesting a negative impact of mutated/reduced DEF6 on CTLA-4 +RAB11+ recycling vesicles. Of note, RAB11 was expressed at similar levels in P1 and P3 as in healthy controls (Fig. S5d). To validate changes in interaction of wildtype or mutated DEF6 with
RAB11, we performed co-immunoprecipitation analyses in co-transfected HEK293T cells. While wildtype DEF6 co-immunoprecipitated with RAB11 (Fig. 6e), this interaction was reduced in the DEF6E331K-expressing cells (Fig. 6c). These results suggest a possible GEF activity of wildtype DEF6 for the small GTPase RAB11, and could hence play a causative role in reduced recycling of CTLA-4 in patient cells. Consistent with the fact that GEF proteins for small GTPases interact preferentially with the GDP-bound form of their target proteins, we found that DEF6 interacted strongly with dominant-negative (GDP-bound) RAB11Q70L in HEK293T cells (Fig. 5e). In contrast, DEF6E331K did not show relevant co-immunoprecipitation with wild-type, GDP- or GTP-locked RAB11. In Jurkat cells, endogenous DEF6 co-immunoprecipitated with Strep-HA-tagged RAB11 but not with the Strep-HA-GFP-expressing control (Fig. 6f), confirming a physical interaction in a T-cell model. The second identified mutation DEF6V210D was barely expressed in primary T cells of P3 (Fig. 2e), probably due to rapid degradation. We overexpressed GFP-tagged wildtype or Y210D-mutated DEF6 in Jurkat cells in presence or absence of proteasomal inhibitor...
Fig. 6 DEF6 mutations affect RAB11 interactions. a Representative images of endogenous CTLA-4, and RAB11 and DEF6 in TCR-stimulated healthy control (HD) PBMCs showing RAB11-CTLA-4 co-localization. b Line scans of images in (a) reveal high overlap of RAB11 and CTLA-4 signal in activated HD-PBMCs. Scale bar = 5 µm. c Representative images of endogenous CTLA-4, RAB11 and DEF6 in activated PBMCs of P1 reveal loss of RAB11-CTLA-4 co-localization in P1. d Line scans show reduced overlap of RAB11 and CTLA-4 signal in P1. Scale bar = 5 µm. (for a-d, representative images of 30–40 analyzed cells; cells were considered T cells through expression of CTLA-4 after TCR cross-linking). Quantification as in Fig. 5a. e MYC-tagged wildtype DEF6 co-immunoprecipitates with GFP-RAB11 from transfected HEK293T cells, revealing a hitherto unrecognized interaction of the GEF protein DEF6 with the small GTPase RAB11. Presence of mutation E331K abrogated this interaction. Samples were balanced on immunoprecipitated GFP-RAB11 fractions and blotted for interacting DEF6. Representative of three independent experiments. f Endogenous DEF6 co-immunoprecipitates from Jurkat lysates with overexpressed RAB11-Strep-HA, compared to GFP-Strep-HA control. Samples were balanced on immunoprecipitated HA-tag fractions. Western blots (e, f) were cropped for visualization. g, h Overexpressing inactive RAB11S25N in Jurkat-mCherry-CTLA-4 cells mimics DEF6-deficient defects in CTLA-4 cycling. Cells were electroporated with inactive RAB11S25N, constitutively active RAB11Q70L, wildtype RAB11 or empty vector (EV), stimulated with OKT3 and analyzed for cycling CTLA-4 after 10 min and 30 min, respectively. Cycling was reduced in RAB11S25N expressing cells (blue, blue numerical insert, g), while RAB11Q70L enhanced cycling at 30 min (h). Data are overlaid with mean ± SD. Representative of two individual experiments. Source data of Fig. 6 including uncropped immunoblots are provided as a Supplementary Source Data file.
MG132 and tracked GFP signals over time. Our data confirm loss of DEF610,12 two days after transfection which could be fully reverted by adding MG132 (Fig. S5g). Lastly, kinetic studies on purified proteins suggest that the PH-DH domain of DEF6 has GEF activity toward the small GTPase RAB11, while mutated PH-DH331K is inactive (Fig. S5h).

To confirm the link between RAB11 and CTLA-4 trafficking, we overexpressed inactive RAB11S25N and constitutively active RAB11Q70L in Jurkat-mCherry-CTLA-4 cells, and analyzed CTLA-4 cycling by counterstaining the cycling protein with BV421-coupled antibody. As shown in Fig. 6g, h (gating as in Fig. S5i), when compared to overexpressing wildtype RAB11 or empty vector, inactive RAB11S25N indeed blocked CTLA-4 cycling while presence of active RAB11Q70L contrarily enhanced this process at later time points.

In summary, our data reveal a previously unknown physical interaction and GEF activity of DEF6 toward the small GTPase RAB11, a recognized component of CTLA-4 recycling endosomes. Consistently, DEF6-mutated cells lacked this interaction and showed reduced RAB11+ CTLA-4+ double-positive vesicles, suggesting a direct link to the observed defect in CTLA-4 trafficking dynamics through RAB11 as demonstrated by inactive RAB11S25N compromising CTLA-4 cycling.

Discussion

The role of DEF6 in murine autoimmunity models has been controversial as the development of autoimmunity appears to depend on their genetic background. In humans, the intronic DEF6 SNP rs10807150 which alters gene expression, is associated with the onset of systemic lupus erythematosus. Here, we describe two unrelated families with three patients harboring two distinct biallelic missense mutations in DEF6. The patients present with immunodeficiency and systemic autoimmunity, thus indicating a critical role for DEF6 in preventing autoimmunity in humans. We uncover a role for DEF6 in regulating abundance and recycling of the T-cell cyclophilin protein CTLA-4, as the functional cause of the observed autoimmune manifestations in DEF6-mutated patients. We base our conclusions on the following observations and in line with previously outlined criteria: (i) we identified different biallelic mutations in DEF6 as the single common gene affected and segregating perfectly with the disease in two unrelated families; (ii) DEF6 has been previously shown to have a role in the immune system although its precise role in human immunity had not been determined; (iii) we identified a CTLA-4 trafficking defect amenable to rescue upon reconstitution of patient T cells with wildtype DEF6, explaining the predominant clinical presentation of autoimmunity; (iv) CRISPR-based DEF6 knockout in Jurkat cells recapitulates defective CTLA-4 cycling and could be reverted by reconstitution with the wildtype protein; (v) we provide a functional explanation involving compromised RAB11-DEF6 interaction affecting RAB11-dependent CTLA-4 shuttling; (vi) lastly, the successfully commenced CTLA-4-Ig therapy in P1 led to remission of symptoms.

CTLA-4 is a critical molecule in human immune homeostasis. A reduction of CTLA-4 levels by 50% as observed in CTLA-4 haploinsufficiency results in severe autoimmunity, while notably patients with biallelic loss-of-function germine mutations in CTLA4 have not been described and are potentially lethal. CTLA4+/- mice are viable, although they develop fatal autoimmunity early in life whereas their Cita4+/+ littermates are healthy. These studies suggest that humans appear to have a narrower window of tolerance regarding CTLA-4 critical abundance for the onset of disease. This assumption is further supported by genome-wide association studies which have identified SNPs affecting the relative cell surface expression of CTLA-4 associated with human autoimmune disease. Reduction of available CTLA-4 by enhanced lysosomal degradation is also the cause for severe autoimmunity in LRBA deficiency. Again, in contrast to the human phenotype, Lrba−/− mice do not develop overt autoimmunity, have a normal lifespan and also intriguingly exhibit an increased acceptance of allogeneic bone marrow grafts. Thus, Def6−/−, Cita4−/−, and Lrba−/− mice display inconsistent autoimmune manifestations or lack such. Our discovery of a mechanistic link between DEF6 mutations and CTLA-4 functional integrity offers insights to autoimmunity in humans. Clinical and immunological phenotypes in DEF6-mutated patients include T-cell lymphopenia, low class-switched B cells, hepatosplenomegaly, autoimmune hemolytic anemia and bowel inflammation, all of which are reminiscent of CTLA-4 haploinsufficiency and LRBA deficiency. In accordance with previous reports on genetically determined autoimmune diseases through compromised CTLA-4, clinical manifestations vary between patients due to the lowered thresholds of inhibitory T cell function, rather than through specific triggers. Still, they do represent the same disease. As for other newly described disease entities, larger patient cohorts in future studies will help to unravel the full phenotypic spectrum of disease due to functional DEF6 deficiency. It is impossible to dissect whether the strong immunosuppressive treatment in P1 may have contributed to the more pronounced B-cell deficiency including borderline-low frequencies of class-switched memory B cells and impaired vaccination titer generation, and also the persistent susceptibility to infections which has been described previously in individuals treated with abatacept. To date, P3 has exhibited less pronounced autoimmune manifestations. This could be due to a distinct mutation with distinct cellular effect, or possibly a different genetic or epigenetic background. Given the reduced CTLA-4 expression in P3 (Fig. 3a), it is possible that other autoimmune manifestations may present with time. Interestingly, in contrast to CTLA-4 and LRBA-mutated patients, DEF6-mutated patients do not show an obvious activation/exhaustion phenotype in peripheral blood T cells. This might be due to the fact that DEF6 is also involved in T-cell signaling. Def6−/− mice, for example, exhibit a reduced clonal expansion of CD8+ T cells. The interplay of DEF6 in T-cell signaling and regulation of CTLA-4 might result in a normal status of T cells derived from the blood, but increased activation in situ, where antigen is presented in higher concentrations as suggested by the massive T-cell infiltration in peripheral tissues. The homozygous frameshift mutation in SKIV2L that was additionally identified in P1 and the deceased sister P2 in family A (Table S1), could represent a disease-modifying factor potentially affecting cardiac function and/or bowel inflammation, but does not explain the autoimmune presentation observed in DEF6-mutated individuals from both families and our identified link to aberrant CTLA-4 shuttling. We proved causality by reconstitution of the CTLA-4 cycling defect in patient-derived cells through ectopic expression of wildtype DEF6, and a similar reconstitution of DEF6-knockout Jurkat models could revert the observed CTLA-4 cycling defect. Finally, the response of P1 to CTLA-4 replacement therapy suggests a T cell-mediated disease.

The co-localization, co-immunoprecipitation and overexpression data confirm that DEF6 regulates CTLA-4 vesicular trafficking via the small GTPase RAB11. RAB11 has previously been located at recycling vesicles containing CTLA-4. We identify a cellular regulation pathway of CTLA-4, which may involve direct activation of RAB11 by DEF6, a GEF protein that functions downstream of TCR engagement. RAB11 is a broadly expressed small GTPase and its deletion in a murine knockout model was found embryonically lethal. It is also considered a
crucial component of the so-called exoyct, which regulates late-endosomal trafficking. However, it is largely unknown which GEF proteins activate RAB11 to promote its multiple actions, and future studies are required to address this question. Our data reveal that RAB11 interacts with DEF6, and that DEF6 acts as GEF protein for RAB11 as suggested by a preferential interaction of DEF6 with the GDP-locked small GTPase, and further evidenced by kinetic GEF activity studies on purified protein domains. The phenocopy of defective CTLA-4 cycling by overexpressing inactive RAB11S251N in Jurkat cells further supports this theory. In patient-derived DEF6-mutant T cells, RAB11 recycling endosomes do not co-localize with CTLA-4 vesicles, suggesting that DEF6 promotes RAB11-mediated recycling of CTLA-4.

In conclusion, our work identifies a role for DEF6 in regulating CTLA-4 availability and trafficking to prevent autoimmunity, in line with CTLA-4 functioning both as immune rheostat and defining thresholds of immune activation for anti-cancer immunity. The work presented herein underlines the importance of RAB11 in the recycling of immune checkpoint proteins, allowing for a way to uncover immune regulatory pathways. The work presented herein underlines the importance of RAB11 in the recycling of immune checkpoint proteins, allowing for a way to uncover immune regulatory pathways. The work presented herein underlines the importance of RAB11 in the recycling of immune checkpoint proteins, allowing for a way to uncover immune regulatory pathways.

Methods

Results

Further clinical information on the patients. Patient 1 (P1, index patient of family A) presented oligohydramnion in prenatal ultrasounds and intrauterine growth retardation. A Caesarean section was performed at 38 weeks of gestation due to rupture of the membrane and pathological dopplersonographic measures. Weight and length at birth were below third percentile (1435 g; 39 cm; head circumference 25.5 cm). No abnormalities were observed during the perinatal period. She developed respiratory distress syndrome. P1 presented hyperbilirubinemia, inward mamillae, growth retardation (below third percentile) and abnormal fatty tissue distribution. At 23 days of life severe watery diarrhea was observed, associated with vomiting and electrolytes imbalances (hypernatremia and hyperchloremic acidosis; Na+: 159 mmol l\(^{-1}\); pH: 7.1; base excess: -18) and massive increase of inflammation markers (\(C\) reactive protein concentration: \(>20\) mg l\(^{-1}\)). Total parental nutrition was initiated with no obvious improvements of diarrhea. Hydrolyzed formula also did not improve P1’s health status. Viral, bacterial, parasitic or allergic causes of the diarrhea were excluded upon repeated testing. Massive bowel inflammation was suggested by increased stool calprotectin (Fig. 1d). At the age of 1 year, polyadenated atopy by IgE to milk, soy and eggs was diagnosed. Persistent vomiting ceased and stool consistency improved markedly. Inflammatory markers decreased and P1 tolerated a formula improved her clinical condition, necessitating parenteral nutrition.

Genetic analysis. DNA of P1 and P3 was extracted from whole blood with Genomic DNA Purification kits (Promega). DNA of P2 was extracted after death from stored histology slides. DNA of relatives was extracted either as described for P1 or from saliva samples with the QIAamp® DNA mini kit. For P1 (family A), homozygous intervals were determined applying Affymetrix® SNP-based homozygosity mapping and used as a filter for detected variants. Whole exome sequencing was performed on genomic DNA of P1 and analyzed for novel non-sense, missense and frameshift variants, as follows: After library prep with the Illumina True Seq and Exon Enrichment kit, the sample was multiplexed and loaded onto two lanes of one flow cell. DNA was sequenced on an Illumina HiSeq2000 and variant calling was performed with the Genome Analysis Toolkit (GATK) (v.3.3) and variant effector predictor (VEP) (version 73), respectively. Variant filtering was performed using GEMINI

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Assimilatory expression. Of RNA was used performing NEasy kit (Qiagen), first-strand cDNA DNA synthesis was used during Expand Reverse Transcriptase (Roche) using both oligo-dT and random hexamer primers. Intron-spanning primers were used for gene expression DEF6-forever: 5 ′-CATCGGAGGATGTTGCTCC-3 ′, DEF6 reverse: 5 ′-GAAGCTCATCTGG-TAAGGCT-3 ′, ACTB reverse: 5 ′-GACACAGACCTGCGCTT-3 ′.

Calcium flux. To assess calcium influx in P1, feeder-expanded T cells were harvested, washed with PBS and loaded with Calcium Sensor Dye eFluorTM 514 (ebioscience) for 30 min at 37 °C. After loading, cells were washed and resuspended in RPMI 10% FCS medium at 1 x 10^6 ml^-1. Anti-CD3 (OKT3, ebioscience) was added to the cells to final concentration of 0.5 μg ml^-1. After 5 min incubation at 37 °C, a baseline measurement of 30 sec was recorded, subsequently anti-Ig (IgG1 (Jackson ImmunoResearch) was added to a final concentration of 20 μg ml^-1 and measurement continued for 3 min. After stimulation, 1 μg ml^-1 of ionomycin was added to the cells and acquisition continued for 1 more min. To assess calcium influx in P3, cells were incubated in HBSS buffer containing 1 μM procyclin (Thermo Fisher) in the presence of CD3-biotin (OKT3, Biologend) and CD28-biotin (2B4, Biologend). Igg2a (MOPC-173, Biologend) antibodies for surface staining, and the calcium dye Fluo-8 (abcam). After 5 min incubation at 37 °C, a baseline measurement of 30 sec was recorded, streptavidin (abcam) was added and measurement continued. Following antibodies were used for surface staining: from Biologend: CD3-BV711 (RPA-T8), CD4-BV785 (OKT4), CD3A-PECy7 (HI100); from BD Biosciences: CD27-APC-M (M-T271); from Invitrogen: CD6q0605 (UCHT1).

Cell proliferation. Cells were stained for 10 min with CFSE or VPD-450 violet proliferation dye65, stained in PBS and cultured in growth media. After 4 days, dye dilution traces of proliferated cells were compared by flow cytometry.

Analysis of CTLA-4 mechanisms. For CTLA-4 cycling experiments (depicted in schematic Fig. 3b), CD T cells were isolated from PBMCs and left to recover at least 2 h in complete RPMI media at 37 °C in 5% CO2 atmosphere before proceeding. Cells were then seeded to wells in a density of 2 x 10^6 cells/ml and left unstimulated or stimulated with anti-CD3/CD28 coated dyna-beads. After 16 h incubation, antibodies were added to stain for cycling CTLA-4 according to the following procedure: Anti-CTLA-4-PE (14D3, ebioscience) was added at 37 °C for the indicated time points (60 min, 30 min or 10 min respectively). At time point zero, cells were placed immediately on ice and stained for surface CTLA-4. T-cell surface stains were added to all wells (anti-CD4-PerCpCy5.5 (RPA-T8), anti-CD8-PECy7 (OKT4), anti-CD3-PE-Cy7 (HI100, BD Pharmigen)) were carried out for 30 min on ice. Cells were then washed in PBS and fixed for 1 h using the FOXP3 fixation/permeabilization kit (ebioscience). After washing, stained cells were added for 1 h on ice. Cells were washed and analyzed by flow cytometry. Gating for naive and memory T neg, and Tcv cell was performed as shown in Fig. S3d. Using FlowJo software (10.4), percent-quantiles as well as geometric mean fluorescence intensity values for CTLA-4-positive and FOXP3-negative or -negative final gates were extracted and normalized sample-individually to respective total CTLA-4 stains. Experiments were performed at minimum two independent blood donations of both P1 and P3.

Re-cycling CTLA-4 (depicted in schematic Fig. S3g) was analyzed as follows: Isolated CD4 T cells were stimulated for 16 h as described. In step 1, unconjugated Isolated CD4 T cells were stimulated for 16 h as described. In step 1, unconjugated feeder cells were stained with anti-CD3 Alexa Fluor488 (BD-Biosciences) and anti-CD28 Alexa Fluor594 (Biolegend) and stained with Calcium Sensor Dye eFluorTM 514 (ebioscience) in the presence of CD3-biotin (OKT3, Biologend) and CD28-biotin (2B4, Biologend). Igg2a (MOPC-173, Biologend) antibodies for surface staining, and the calcium dye Fluo-8 (abcam). After 5 min incubation at 37 °C, a baseline measurement of 30 sec was recorded, streptavidin (abcam) was added and measurement continued. Following antibodies were used for surface staining: from Biologend: CD3-BV711 (RPA-T8), CD4-BV785 (OKT4), CD3A-PECy7 (HI100); from BD Biosciences: CD27-APC-M (M-T271); from Invitrogen: CD6q0605 (UCHT1).

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were seeded: Respective Jurkat: unlabeled PBMCs: VPD450-labelled CD4 T cells fractions. One fraction was used for CD4 T-cell isolation by negative selection.

above, by transfecting cells 24 h prior to analysis. Gating as in Fig. S4b.

incubating cells for 10 min at 37 °C, 5% CO2. Cells were immediately

antibodies against CD3/CD28 or left untreated. After a stimulation period of 48 h,

Confocal microscopy

Coverslips were mounted in Prolong

counterstained with 4

(A-21429, Life Technologies), and anti-goat (Life Technologies). Cells were

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Analysis of soluble ligand uptake by memory Treg was done as described previously87. In detail, CD4 T cells were isolated and stimulated with anti-CD3/CD28 coated dynabeads (see above), or left unstimulated. Where applicable, CD80-ligand was added and cells were incubated for 16 h. Subsequently, cells were labelled for T-cell surface markers, fixed and permeabilized as described above, and stained intracellularly with anti-CTLA-4-AP50 antibody (15162 S; Cell Signaling). CD80-ligand was visualized with anti-human IgC/PE antibody (6140–95, Southern Biotech). Where indicated, ligand binding was blocked with anti-CTLA-4 antibody (500405, BD Biosciences) during incubation. For total-uptake controls, respective samples were incubated with CD80-ligand after cell permeabilization. Using FlowJo software, cells were gated on memory Treg, and fluorescent values for CTLA-4 and CD80 were measured and quantified.

For suppression assays, PBMCs were transfected for 24 h with pcDNA-GFP-DEF6 or pcDNA-GFP-DEF6X338 using the AmoMax Nucleofector kit for primary human T cells, according to the manufacturer’s recommendations. Transfection efficiency was around 10% of GFP-expressing CD4+CD8+ T cells, and transfected and control cells were analyzed for the CTLA-4-positive portion.

For reconstitution experiments, PBMCs were transfected for 24 h with pcDNA-GFP-DEF6 or pcDNA-GFP-DEF6X338 using the AmoMax Nucleofector kit for primary human T cells, according to the manufacturer’s recommendations.

For Co-immunoprecipitation, cells were stimulated for 24 h with PMA (30 ng/ml) and ionomycin (1 µM) in the presence of CTLA-4 antibody (as above), before cycling CTLA-4-BV421 antibody was added for 10 or 30 min incubation, respectively. Cells were subsequently placed on ice, and then fixed with ICFix solution (eBioscience). Rescue and overexpression experiments were performed as above, by transfacing transfects 24 h prior to analysis. Gating as in Fig. 5a.

Statistical analysis. Data were analyzed with appropriate statistical tests as indicated in the results section. Unpaired t tests were two-sided, Welch’s correction was applied. Data are displayed as mean ± SD, with 95% confidence intervals (where applicable). Sample sizes and replicates are indicated in figure legends.

Data availability

The source data underlying subpanels of Figs. 2, 3, 5, and 6, and Supplementary Figs. S1–S5 are provided as a Supplementary Source Data file. Relevant data are available from the authors. Next generation sequencing data are deposited at the European Genome-phenome Archive (EGA) which is hosted by the EBI and the CRCG, under accession IDs EGAS00001003609 (P1) and EGAS00001003618 (P3 and mother). The data are not available publicly due to restrictions for controlled access, and may be accessible through the relevant Data Access Committee via formal application at the EGA (https://ega-archive.org).

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Author contributions

K.B. conceived and designed the study, and wrote the initial manuscript together with N.K.S. and B.H. N.K.S. and B.H. performed, analyzed and interpreted most of the experiments. R.C.A., S.V.S., Z.S., A. Krolo, O.Y.P., L.P., T.Z.H., N.H., E.S.-V., A. Kalinichenko, A. Kennedy, E.M.M., M. Mukherjee, B.T., A.S., W.F.P., J.H.L., J.-N.S., S.G., J.H., W.G., E.S., I.P., I.B., J.T. and F.P.B. performed and analyzed experiments. N.M., M. Meeths, R.K., B.B., F.P., J.F.-W. and W.-D.H., provided clinical care for the three patients and performed clinical routine investigations. J.S.O., E.M., D.S., Y.T.B., A.A. and K.B. provided extensive supervision of the performed experiments. N.K.S., B.H. and K.B. revised the manuscript, with input from D.M., Y.T.B., D.S. and A.A. All authors read the manuscript and agreed to this publication.

Additional information

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