Autosomal dominant immune dysregulation syndrome in humans with CTLA4 mutations

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The protein cytotoxic T lymphocyte antigen-4 (CTLA-4) is an essential negative regulator of immune responses, and its loss causes fatal autoimmunity in mice. We studied a large family in which five individuals presented with a complex, autosomal dominant immune dysregulation syndrome characterized by hypogammaglobulinemia, recurrent infections and multiple autoimmune clinical features. We identified a heterozygous nonsense mutation in exon 1 of CTLA4. Screening of 71 unrelated patients with comparable clinical phenotypes identified five additional families (nine individuals) with previously undescribed splice site and missense mutations in CTLA4. Clinical penetrance was incomplete (eight adults of a total of 19 genetically proven CTLA4 mutation carriers were considered unaffected). However, CTLA-4 protein expression was decreased in regulatory T cells (Treg cells) in both patients and carriers with CTLA4 mutations. Whereas Treg cells were generally present at elevated numbers in these individuals, their suppressive function, CTLA-4 ligand binding and transendocytosis of CD80 were impaired. Mutations in CTLA4 were also associated with decreased circulating B cell numbers. Taken together, mutations in CTLA4 resulting in CTLA-4 haploinsufficiency or impaired ligand binding result in disrupted T and B cell homeostasis and a complex immune dysregulation syndrome.

Adaptive immune responses must balance the response against foreign antigens with the need to avoid damage to host tissue. Inefficient activation of the immune response results in pathology due to infections, whereas overactivation may drive an autoimmune response. It might be expected that distinct genetic mutations underlie these apparently opposite outcomes, yet, paradoxically, it is well recognized that autoimmunity and immunodeficiency can manifest concurrently in the same individuals.

Common variable immunodeficiency (CVID) is the most frequent primary immunodeficiency in humans characterized by low immunoglobulin levels, recurrent upper respiratory tract infections and impaired vaccination responses. In many patients, CVID presents as an immune dysregulation syndrome with autoimmunity, granulomatous disease, enteropathy, and malignancy. The majority of familial CVID cases present an autosomal dominant pattern of inheritance, yet disease penetrance may appear incomplete owing to the late onset of symptoms. Dominant mutations causing CVID have been found in NFKB2 (ref.6), and some patients with activating PIK3CD mutations present with a CVID-like phenotype. Still, most autosomal dominant mutations causing CVID or increasing the disease risk remain to be identified.

The mammalian immune system contains self-reactive T cells, which are controlled by forkhead box P3-positive (FOXP3+) Treg cells. Accordingly, Treg deficiency caused by mutations in FOXP3 leads to an aggressive autoimmune syndrome termed IPEX (immune dysregulation polyendocrinopathy X-linked). In mice, deficiency of CTLA-4 results in a lethal autoimmune phe-
RESULTS

Identification of heterozygous mutations in CTLA4

We carried out whole-exome sequencing and genetic linkage analysis in 14 members of a large family with 39 individuals (family A, Fig. 1a and Supplementary Note 1). Five family members were diagnosed with CVID or selective IgA deficiency and presented with recurrent respiratory tract infections, hypogammaglobulinemia, autoimmune cytopenia, autoimmune enteropathy and granulomatous infiltrative lung disease. Because we could not identify any perfectly segregating novel mutations in these individuals, we performed an affected-only analysis to allow for reduced penetrance of the causative mutation. We identified 27 novel heterozygous mutations in 19 genes, which had not been listed in the dbSNP database (Supplementary Fig. 1), among them a nonsense mutation at position c.105 (C35*) in the first exon of CTLA4 that segregated with disease. This mutation also occurred in six members of family A who were so far considered healthy (I.2, II.2, II.3, II.10, III.5 and III.6) (Fig. 1a).

Screening of 71 unrelated patients with CVID and enteropathy or autoimmunity revealed five additional index patients with novel CTLA4 mutations. Working up the family histories revealed four more patients and two CTLA4 mutation carriers, yielding a total of six families (A–F) containing 14 patients (11 of them with a genetically proven heterozygous

notype with marked similarities to FOXP3 deficiency. CTLA-4 is an essential effector component of T<sub>reg</sub> cells that is required for their suppressive function in certain settings. The mechanism by which CTLA-4 functions is still debated; however, studies in chimeric mice containing a mixture of wild-type and C<sub>tna</sub>A<sup>−/−</sup> cells suggest that in vivo CTLA-4 primarily acts in a T cell–extrinsic manner. In keeping with a T cell–extrinsic mechanism of action, it has been recently shown that CTLA-4 can function by removing its ligands (CD80 and CD86) from antigen-presenting cells (APCs) via transendocytosis. These CTLA-4 ligands are shared with the stimulatory receptor CD28 (ref. 26), whose engagement drives T cell activation, cytokine production and memory T cell differentiation. Depletion of the co-stimulatory ligands CD80 and CD86 by CTLA-4 reduces APC-mediated activation of conventional T cells via CD28, resulting in dominant suppression of T cell activation. Thus, CTLA-4 and CD28 are linked to the control of both regulatory T cell suppression and effector T cell responses and sit at a nexus between autoimmunity and immunodeficiency. Following a hypothesis-free screening approach by next-generation sequencing, we identified CTLA4 mutations in humans resulting in impaired CTLA-4 function and a complex immune dysregulation syndrome.

**Figure 1** Genetics and pedigrees of families with CTLA4 mutations. (a) Pedigrees of families with CTLA4 mutations. Squares: male subjects; circles: female subjects; black filled symbols: patients with mutation; gray filled symbols: mutation carriers; crossed-out symbols: deceased subjects. CTLA4 was sequenced in all individuals with available genomic DNA (asterisks). Whole exome sequencing was carried out on subjects with a purple asterisk. (b) Confirmation of the mutations by Sanger sequencing showing cDNA (c) changes and their resulting amino acid (p) changes. WT, wild-type.
**Figure 2** Tissue infiltration and lymphadenopathy in patients with *CTLA4* mutations. (a,b) Duodenal biopsies from patient B.II.4 (a) and A.III.3 (b) stained for CD4. (c) High-resolution chest computed tomography scan of the lungs from patient E.II.3. Arrows point to granulomatous-lymphocytic infiltration in both lungs. (d) Pulmonary lymphoid fibrotic lesions stained for CD4 in pulmonary biopsy from patient E.II.3. (e) Magnetic resonance imaging (MRI) of the pelvic area of patient A.III.3 with two enlarged lymph nodes (arrows) measuring up to 4 cm. (f) Bone marrow biopsy from patient B.II.4 stained for CD4. (g) MRI of gadolinium-enhanced lesion (arrows) in the cerebellum of patient A.III.1. (h) Resected cerebellar lesion from patient A.III.1 stained for CD3. Scale bars, 50 µm (a,b,d,f,h), 20 mm (c) and 50 mm (e).

*CTLA4* mutation; for three patients (A.II.8, B.II.2 and B.III.2) no genomic DNA was available as they had died before this study) and eight carriers (Fig. 1a). Seven of the 14 patients fulfilled the diagnostic criteria of CVID. A splice-site mutation (family B) and a mutation in the start codon (family F), comparable to the nonsense mutation in family A, were predicted to result in haploinsufficiency due to a lack of CTLA-4 expression from one allele (Fig. 1b). Three distinct missense mutations (families C–E) affected conserved amino acids in the extracellular domain (Fig. 1b) and were predicted to interfere with ligand binding or CTLA-4 stability (Supplementary Fig. 2). Table 1 contains a summary of the clinical findings of all individuals with CTLA-4 deficiency, and details are given in Supplementary Note 2 and in Supplementary Table 1.

**Lymphocytic organ infiltration and lymphadenopathy**

*Ctla4*−/− mice die from CD4+ T cell–dependent organ infiltration. Investigating the clinical symptoms of patients with CTLA-4 mutations, we confirmed extensive CD4+ T cell infiltration in a number of organs including the intestines (Fig. 2a,b), lungs (Fig. 2c,d), bone marrow (Fig. 2f), central nervous system (Fig. 2g,h) and kidneys (Supplementary Note 2). We also found lymphadenopathy (Fig. 2e) and hepatosplenomegaly in these individuals (Supplementary Note 2).

**Activated T cells and reduced B cells in peripheral blood**

Where sufficient blood samples were available, we carried out detailed immunological investigations in families A–D. Consistent with the observed lymphoproliferation and lymphocytic tissue infiltration, analysis of peripheral blood revealed evidence of increased T cell activation in *CTLA4*+/− carriers and affected individuals, as assessed by reduced levels of CD4+CD45RA+ naive T cells (Fig. 3a). Although the affected individuals generally had lymphopenia in the periphery (Supplementary Table 2), the ratio of CD4+ to CD8+ T cells was in the normal range (Supplementary Fig. 3a). All symptomatic patients with *CTLA4* mutations except B.II.1 had reduced levels of at

**Table 1 Clinical phenotype of patients with *CTLA4* mutations**

| Clinical manifestations                  | Patients | Frequency |
|----------------------------------------|----------|-----------|
| Diarrhea/enteropathy                   | A.II.5, A.II.8, A.II.9, A.III.1, A.III.3, B.II.1, B.II.4, C.II.4, E.II.3, F.II.2 | 11/14 (78%) |
| Hypogammaglobulinemia                  | A.II.5, A.II.8, A.II.9, A.III.1, A.III.3, B.II.4, C.II.3, E.II.3, F.II.2 | 10/13 (76%) |
| Granulomatous lymphocytic interstitial lung disease | A.II.4, A.II.9, A.II.13, B.II.4, B.II.2, C.II.3, D.II.1, E.II.3 | 8/12 (66%) |
| Respiratory infections                 | A.II.5, A.II.8, A.II.9, B.II.4, B.II.2, C.II.3, D.II.1, E.II.3 | 8/14 (57%) |
| Organ infiltration (bone marrow, kidney, brain, liver) | A.II.5, A.II.9, A.II.3, A.III.3, B.II.2, C.II.3, D.II.1 | 7/14 (50%) |
| Splenomegaly                           | A.II.5, A.II.9, A.II.3, C.II.4, D.II.1, E.II.3 | 6/12 (50%) |
| Autoimmune thrombocytopenia            | A.II.1, A.II.3, C.II.3, E.II.3 | 5/14 (35%) |
| Autoimmune hemolytic anemia            | C.II.3, D.II.1, E.II.3, F.II.2 | 4/14 (28%) |
| Lymphadenopathy                        | A.III.3, C.II.3, D.II.1, E.II.3 | 4/14 (28%) |
| Psoriasis and other skin diseases      | A.II.1, B.II.1, B.II.2 | 3/14 (21%) |
| Autoimmune thyroiditis                 | A.II.5, D.II.1 | 2/13 (15%) |
| Autoimmune arthritic                   | A.II.5, A.III.1 | 2/14 (14%) |
| Solid cancer                           | B.II.4 | 1/14 (7%) |

Denominators vary between rows because some deceased patients had not been evaluated for all clinical manifestations. Details are shown in Supplementary Table 1.

*Upper and lower respiratory tract infections. Details in Supplementary Note 2.*
Figure 3 Impact of CTLA4 heterozygosity on T and B cells. (a) Percentage of naive CD4+CD45RA+ T cells, CD19+ B cells and CD19+IgM−CD27+ switched memory B cells in the peripheral blood of CTLA4+/+ carriers and patients. Gray background indicates normal range. (b) Proportion of IFN-γ-, IL-4- and IL-17-expressing CD3+CD4+CD45RO+ T cells after stimulation of PBMCs with phorbol 12-myristate 13-acetate and ionomycin from healthy CTLA4+/+ subjects, CTLA4+/− carriers and CTLA4−/− affected individuals. (c) Percentage of FOXP3+ Treg cells among CD4+ T cells in the peripheral blood under resting (ex vivo) conditions or following activation (with beads coated with CD3- and CD28-specific antibodies). (d) Representative (11 mutation carriers and 22 controls) flow cytometry plots (top) and quantification (bottom) of CTLA-4 expression in CD4+FOXP3+ cells under resting and activated conditions. Numbers in quadrants show percentage of CTLA-4-high (top), CTLA-4-intermediate (middle) and CTLA-4-low (bottom) cells within the FOXP3+ population. MFI, mean fluorescence intensity. Plots in b–d show the mean ± s.d.; each dot represents one individual. *P < 0.05; **P < 0.01; ***P < 0.001; ****P ≤ 0.0001. NS, not significant.

At least one immunoglobulin isotype (Supplementary Table 2); seven out of ten patients had low proportions of CD19+ B cells (Fig. 3a) and low numbers of switched (IgM−CD27+) memory B cells (Fig. 3a). Additional characterization of the lymphocyte compartment is shown in Supplementary Figure 3a and Supplementary Table 2. In five out of six patients who were monitored over at least 2 years, we observed a progressive loss of CD19+ B cells over time (Supplementary Fig. 4). In vitro re-stimulation of T cells from affected individuals did not suggest a bias toward T helper type 1, 2 or 17 differentiation (Fig. 3b). As some affected individuals showed T cell infiltrates into multiple organs, we were interested in whether their T cells had a polyclonal distribution of T cell receptors (TCRs). We observed that A.II.5 had an oligoclonally expressed T cell receptor β, γ and δ repertoire in the peripheral blood, whereas A.III.3 had a normal distribution (Supplementary Fig. 3b). The oligoclonal T cell repertoire of A.II.5 was confirmed by TCR spectratyping (Supplementary Fig. 3c).

CTLA-4 expression is reduced in Treg cells

Given the role of CTLA-4 in Treg cell function, we analyzed the Treg cell compartment in symptomatic individuals or healthy carriers bearing CTLA4 mutations. The proportion of FOXP3+ Treg cells within the CD4+ T cell compartment was higher in individuals with a heterozygous CTLA4 mutation compared to healthy CTLA4+/+ controls (Fig. 3c). Consistent with this, both homozygous and heterozygous loss of CTLA4 in mice (Supplementary Fig. 5) are associated with an increased frequency of Treg cells. To investigate the impact of the mutations on CTLA-4 protein expression, we carried out intracellular staining for CTLA-4. CTLA-4 expression was reduced in FOXP3+ T cells from all individuals with CTLA4 mutations compared with healthy CTLA4+/+ control cells (Fig. 3d), a deficit that was more pronounced following T cell activation. Thus, in healthy control subjects, activated Treg cells expressed levels of CTLA-4 in excess of those in the FOXP3+ conventional T cell population. In contrast, in individuals with CTLA4 mutations, the expression of CTLA-4 in activated Treg cells was similar to the expression in activated conventional T cells. Taken together, these data indicate that two functional CTLA4 alleles appear necessary to drive the high levels of protein required in activated Treg cells.

Ligand binding and capture is impaired by CTLA4 mutations

To investigate CTLA-4 function, we tested the ability of Treg cells to perform transendocytosis25 (see Supplementary Fig. 6 for assay design). We cocultured stimulated CD4+FOXP3+ T cells with CD80-GFP-expressing Chinese hamster ovary (CHO) cells and analyzed their ability to capture ligand by flow cytometry (Fig. 3a). Treg cells from healthy CTLA4+/+ individuals transendocytosed efficiently with 10–25% of CD80-GFP; this percentage was reduced to only 2–3% in individuals bearing CTLA4 mutations, indicating a deficit in ligand capture. Transendocytosis of CD80 was inhibited when a blocking CTLA-4-specific antibody was added to cell cultures, confirming that the ligand capture was CTLA-4 dependent (Fig. 3a).

To study the impact of CTLA4 point mutations in the absence of co-expressed wild-type protein, we cloned the CTLA4 mutants identified in our patients, expressed these in CHO cells and used these cells for soluble CD80 ligand uptake assays (Fig. 4b). We assessed protein expression by permeabilizing the cells and using an antibody that recognizes an epitope in the cytoplasmic domain of CTLA-4. This avoided antibody staining
being compromised by the mutations in the extracellular domain (Fig. 4b).

We detected full-length CTLA4 protein in cells transfected with the CTLA4 mutants R70W (family C) and T124P (family D; Fig. 4b). In contrast, we found no protein expression in cells transfected with the C35* (family A) mutant, ruling out the use of an alternative start codon at Met38 immediately downstream of the premature stop codon. Cells transfected with either the T124P or the R70W mutant were impaired in their ability to take up soluble CD80-immunoglobulin fusion protein (CD80-Ig) (Fig. 4b). Thus, although these mutations are not within the known MYPPPY ligand-binding motif of CTLA4 (Supplementary Fig. 2), they appear to impair ligand binding and uptake.

CTLA4 mutations impair Treg cell suppressor function

We tested the impact of CTLA4 heterozygosity on regulatory T cell function in vitro using T cells and dendritic cells from healthy donors as targets for suppression. Under control conditions, naive CD4+ cells proliferated in response to CD3-specific antibodies and cocultured dendritic cells (Fig. 4c). Proliferation was CD80 and CD86 ligand–dependent, as it was inhibited by the addition of CTLA-4–Ig (abatacept; Fig. 4c). Addition of control Treg cells from healthy donors efficiently suppressed CD4+ T cell proliferation, and this was reversed by a CTLA-4–specific blocking antibody, indicating that the suppressive function of the Treg cells in this assay is CTLA-4 dependent (Fig. 4c).

DISCUSSION

CD28 co-stimulation is required for T cell effector function and generation of T memory cells, and it also influences B cell class switching and Treg cell homeostasis32. These processes are negatively regulated by CTLA-4. Interfering with the CD28–CTLA-4 pathway can therefore have both immune-stimulatory and immune-inhibitory effects33–35. Here, we report the phenotype of patients with previously undescribed heterozygous CTLA4 mutations in CD80-CD25+ Treg cells, CTLA-4–Ig or CTLA-4–specific blocking antibodies. Quantification of total proliferating T cell numbers (top) and flow cytometry histograms depicting cell division and suppression assays (bottom), n = 4 CTLA4heterozygous, n = 4 CTLA4heterozygous. P values were determined by Student’s t-test. **P < 0.01, ***P < 0.001.
is important, especially for the function of regulatory T cells. In families C–E, the mutations affect the ligand binding of CTLA-4, impairing the interaction of CTLA-4 with CD80 and CD86. As CTLA-4 forms homodimers and clusters with its ligands, these mutants may exert a dominant-negative effect.

Of 19 individuals with a proven heterozygous mutation in CTLA4, 12 presented with severe clinical manifestations. The availability of samples from currently healthy family members carrying the CTLA4 mutation provided an opportunity to examine the consequences of this mutation in a setting uncoupled from illness or treatment. Notably, those individuals tested so far also exhibited a similar reduction in CTLA-4 expression, CTLA-4–dependent transendocytosis and Treg cell suppressive function. This suggests that additional modifiers, including genetic, epigenetic or environmental factors, may exist that influence the clinical outcome of CTLA-4 deficiency. As the age of disease onset for clinical symptoms associated with CTLA-4 deficiency ranges in our patient cohort from 7 to 40 years, currently healthy, young mutation carriers may develop disease later. Indeed, autoimmune features (for example, psoriasis, type 1 diabetes and prolonged episodes of diarrhea) are evident in carriers previously classified as healthy (Supplementary Table 1). The breadth of autoimmune targets in patients and carriers is consistent with the range of autoimmunity reported in the setting of Foxp3 deficiency.

One notable finding is that patients with defects in CTLA-4 expression and function present with hypogammaglobulinaemia in immunodeficiency clinics. Given that CTLA-4 inhibits the CD28 pathway, which plays a role in T cell help for B cell responses, deficiency in CTLA-4 might be expected to enhance CD28 function and promote humoral immunity. One possible explanation is that hyperactivation of T cells may result in infiltration and disruption of the bone marrow niche, impairing B cell development. This is consistent with the disruptions in B cell lymphopoiesis in Treg cell–deficient mice. Alternatively, increased CD28–dependent follicular helper T cell differentiation could result in chronic stimulation of B cells, leading to exhaustion. There may also be parallels with the phosophoinositide 3-kinase δ–activating mutations, which cause defects in B cell class switching despite hyperactivation of T cell responses. As CD28 is a major phosphoinositide 3-kinase activator in T cells, this link warrants future investigation.

Kuehn et al. recently reported a group of patients with heterozygous CTLA4 mutations. The clinical phenotype of their patients bears considerable similarity to those reported herein, and they also report incomplete penetrance of the disease phenotype, suggesting that CTLA-4 deficiency leads to a broad, yet well-defined, clinical syndrome. Our complete penetrance of the disease phenotype, suggesting that CTLA-4 structural stability is affected.

The binding of CTLA-4 to its ligands is closely coupled to its function as a competitor for CD28 co-stimulation. Accordingly, Treg cell function, which requires the ability of CTLA-4 to bind to and remove its ligands from APCs, is impaired in individuals bearing CTLA4 mutations. Given the key role of CTLA-4–ligand interactions, it is important in our view to study CTLA-4 in the context of CD80- or CD86-dependent T cell activation to probe its function. In this respect, although the defects in Treg cell suppressive function in individuals with CTLA4 mutations are consistent with those in Kuehn et al., we did not observe any obvious alterations in conventional T cell proliferation (data not shown). There are numerous possible explanations for such differences; however, we note their use of whole peripheral blood mononuclear cells (PBMCs) and stimulation with CD3-specific and CD28-specific antibodies. Given the enrichment of memory T cells in the patient samples, along with the presence of Treg cells in these assays, it is unclear whether the hyperproliferative T cell phenotype reported is necessarily due to loss of CTLA-4 function in conventional T cells. The relative role of CTLA-4 in Treg cells versus conventional T cells remains unclear, and additional work is needed to establish whether there is T cell–intrinsic hyperproliferation in patients bearing CTLA4 mutations. In addition, whereas Kuehn et al. report CTLA-4 expression on B cells, we were unable to detect CTLA-4 expression on B cells in the conditions we tested (Supplementary Fig. 7). Despite these differences in immunological detail, together our studies make a compelling argument that quantitative deficiencies in CTLA-4 protein expression predispose individuals to both autoimmunity and immunodeficiency.

Alterations in immune homeostasis are a feature of primary immunodeficiency, and organs with surfaces exposed to microbes, including the intestine, lungs and skin, seem to be particularly vulnerable to infections. The discovery of heterozygous loss-of-function mutations in CTLA4 suggests that the CD28 and CTLA-4 pathway may be therapeutically targeted in selected subsets of patients with inflammatory bowel disease, enteropathy and wasting disease, granulomatous lung disease and autoimmune cytopenias. Soluble CTLA-4 fusion proteins (abatacept and belatacept), which bind to CD80 and CD86 and inhibit immune activation, have proven beneficial for the treatment of autoimmune disease and prevention of organ rejection. Whether they could be beneficial in the context of CTLA-4 deficiency warrants investigation.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

D.S. conceived and performed experiments, analyzed and interpreted the data, and co-wrote the manuscript. C.B. performed experiments and analyzed the data. R.K. designed and performed CTLA-4 staining and transendocytosis experiments, and co-wrote the manuscript. C.B. performed experiments and analyzed the data. D.S. conceived and performed experiments, analyzed and interpreted the data. R.K. by Diabetes UK, J.B.W. by a Japan Society for the Promotion of Science Young Scientist B grant and S.S. by the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Japan Science and Technology Agency.
experiments. S.I. performed crystallographic modeling. A.F. and S. Sakaguchi conceived, designed and interpreted experiments. L.S.K.W. and D.M.S. conceived, designed and interpreted experiments and co-wrote the manuscript. B.G. managed patients, conceived and interpreted experiments, and co-wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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Online Methods

Ethics approval. All individuals donated samples following informed written consent under local ethics board–approved protocols 239/99_BG, 251/13_KW, and 282/11_SE version 140023 (Ethik Kommission der Albert-Ludwigs-Universität Freiburg) and protocols #04/Q5051/119_AM03 for affected individuals, #07/H0720/182 for family members and #08/H0720/46 for healthy controls (Royal Free Hospital & Medical School Research Ethics Committee, London).

Linkage analysis. Genotyping of microsatellite markers across the autosomes was done as described by Braig et al.66 in 2003. Of particular interest here, there were 28 markers genotyped across chromosome 2. The marker D2S1384 (Marshfield map 200.43cM, human genome build 37/hg19 205.2 Mbp) is close to CTLA4 (204.7 Mbp). The flanking markers genotyped were D2S1391 (186.21 cM, 185.0 Mbp) and D2S2944 (210.43 cM, 214.6 Mbp). At these markers, 15 family members were genotyped, including five individuals who were affected or obligate carriers. LOD scores were computed using FASTLINK47–49.

Whole-exome sequencing. Exome sequencing was performed for all 14 available individuals of the pedigree. The samples were enriched using the TrueSeq Exome Enrichment Kit (Illumina). Sequencing of 2 × 100 bp paired-end reads was performed for one quarter lane per sample on the Illumina HiSeq2000. The Exome Enrichment Kit (Illumina). Sequencing of 2 × 100 bp paired-end reads was able individuals of the pedigree. The samples were enriched using the TruSeq Whole-exome sequencing.

CTLA-4 staining in activated Treg cells. One million freshly isolated PBMCs were incubated overnight at 37 °C. 5% CO2 in Iscove's Modified Dulbecco's Medium (GIBCO) supplemented with 10% FCS (GIBCO) and 1% penicillin/streptomycin (GIBCO) in a 96-well plate. Cell were then treated with GolgiPlug (BD) to inhibit intracellular protein transport and stimulated with 50 units/ml IL-2 (Novartis), PMA (0.05µg/ml) and ionomycin (1µg/ml) for 4 h at 37 °C, 5% CO2. Subsequently, cells were stained for surface markers CD15 (SK7, BD), CD4 1:40 (SFC122D11, Beckman Coulter) and CD45RO 1:50 (UCHL1, BD) and intracellular markers IL-17 1:200 (eBioscience), IFN-γ 1:400 (B27, BD) and measured by flow cytometry. CD3+CD4+CD45RO+ cells were analyzed for cytokine expression levels.

TCR spectratyping and rearrangement studies. TCRγ, TCRδ and TCRα spectratyping was performed from RNA following synthesis of oligo dT–primed cDNA as described57. In order to study TCR rearrangements, DNA was amplified by PCR using the Biomed-2 primers and protocols58. All fluorescent fragments were analyzed on an ABI 3130-XL capillary sequencer (Life Technologies, Darmstadt, Germany).

CTLA-4-activated Treg cells. 200,000–300,000 PBMCs (freshly isolated or frozen samples) were incubated overnight and then cultured for 16 h in the presence of absence of CD3/CD28 beads (Dynabeads Human T-Activator CD3/CD28) at a concentration of 1:1 (beads to cells). Cells were then stained for surface markers CD4 1:100 (RPA-T4, BD Bioscience), CD45RA 1:100 (HI100, eBioscience), CD27 1:10 (HIL-7R-M21, BD Bioscience) and CD25 1:10 (2A3, BD Bioscience), fixed, permeabilized and stained for FOXP3 1:30 (236A/E7, eBioscience) and CD152/CTLA-4 1:30 (BN13, BD) before being assessed using a BD Canto II flow cytometer.

Mice. Heterozygous Ctla4-deficient (Ctla4+/−) mice and wild-type (WT) littermates on a C57BL/6 background were maintained under specific pathogen–free conditions and used in experiments between 6–10 weeks of age; matched numbers of male and female mice were used. Required sample sizes were estimated on the basis of prior experience of animal studies. Experiments were conducted in compliance with Osaka university regulations, under protocols approved by the Animal Experiment Committee of Osaka University. No formal randomization or blinding was used. Groups used in experiments were age and sex matched, and no exclusion criteria were used. C57BL/6 Ctla4−/− or WT littermates were vaccinated intraperitoneally with 100 µg 4-hydroxy-3-nitrophenylacetyl-ovalbumin (NP-OVA) at a molar ratio of 16 NP to 1 OVAin alun. Splenocytes were isolated by manual disaggregation with frosted slides and analyzed ex vivo on day 14 after immunization. Intracellular staining was carried out using the eBioscience FOXP3 staining buffer kit according to the manufacturer's instructions. Antibody clones were as follows: anti-B220: RA3-6B2, anti-CD4: RM4-5 (BD), anti-FOXp3: FJK-16s, anti-CD4+CD25+ T cells, activated with CD3/CD28 beads for 16 h in the presence of CHO cells expressing CD80-GFP. CHO-K1 cells were obtained European Collection of Cell Cultures (ECACC) and were tested negative for mycoplasma. After 16 h, CTLA-4 expression (anti-CTLA-4-PE, BN13, BD bioscience) was assessed by staining cells at 37 °C for the final 2 h. Subsequently, cells were stained for CD4 (RPA-T4, BD Bioscience, 1:50), CD45RA (HI100, eBioscience, 1:200), CD27 (HIL-7R-M21, BD Bioscience, 1:30) and CD25 (2A3, BD Bioscience, 1:50) fixed and permeabilized and then stained for FOXP3 1:30 (236A/E7, eBioscience, 1:50). Cells were gated on FOXP3+ cells and analyzed for GFP uptake.
**Human Treg suppression assays.** To study CTLA-4–dependent Treg suppression, conditions were established where stimulation of responder T cells (by DCs plus anti-CD3) was shown to be sensitive to blockade by abatacept (CTLA-4–Ig). This ensures that the response is sensitive to the presence of CD80 and CD86 ligands on the APC and thereby sensitive to ligand removal by CTLA-4–expressing Treg cells. In our experience, T cell responses that are not abatacept sensitive such as those stimulated using antibody-coated beads cannot be suppressed in a CTLA-4–dependent manner by Treg cells. To perform such assays, freshly isolated resting CD4+ naïve T cells were washed with PBS and incubated with CellTrace Violet according to the manufacturer’s instructions (Molecular Probes). The reaction was quenched with media containing serum followed by PBS wash, and cells were suspended at 1.8 × 10^6 cells/ml before use as responder T cells. T cell proliferation assays were performed in 250 µl RPMI 1640 culture media. Responder T cells (0.9 × 10^5) were stimulated with 0.5 µg/ml CD3-specific antibody (OKT3-ATCC). To provide co-stimulation, monocyte-derived DCs expressing CD80 and CD86 were used. To generate these, monocytes (2 × 10^6 cells/ml) were cultured in RPMI 1640 medium containing 10% FCS and antibiotics with GM-CSF (PeproTech, 800 U/ml) and IL-4 (PeproTech, 500 U/ml) for 5–7 d. DCs were present at a ratio of 1:10, DC to T cell. Cells were cultured for 5 d in the presence or absence of 10 µg/ml CTLA-4–Ig (abatacept) or anti–CTLA-4 (20 µg/ml). To measure Treg suppression, unlabeled negatively selected CD4+CD25+ Treg (2 Treg cells to 1 DC) were added. Division of responder T cells was measured by the dilution of violet dye using flow cytometry. Live proliferating T cell counts were performed using counting beads (Dako) and analyzed using FlowJo software.

**Statistical analyses.** Unless otherwise indicated, statistical analysis was performed using GraphPad Prism version 6, and P values were calculated by two-tailed unpaired Student’s t-test for the means with a 95% confidence interval. In Supplementary Figure 5, a two-sided Mann-Whitney U test was performed.

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