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Autosomal-dominant immune dysregulation syndrome in humans with *CTLA4* mutations

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Abstract

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 investigated a large autosomal-dominant family with five individuals presenting with a complex immune dysregulation syndrome characterized by hypogammaglobulinemia, recurrent infections and multiple autoimmune 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 novel splice site and missense mutations in *CTLA4*. While clinical penetrance was incomplete (eight adults of a total of 19 *CTLA4* mutation carriers were considered unaffected), CTLA-4 protein expression was decreased in regulatory T cells (T_{reg} cells) in patients and carriers with *CTLA4* mutations. Whilst T_{reg} cells were generally present at elevated numbers, 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 and antibody levels. Taken together, mutations in CTLA-4 resulting in CTLA-4 haploinsufficiency or impaired ligand binding results in a complex syndrome with features of both autoimmunity and immunodeficiency.

Adaptive immune responses must balance the response against foreign antigens with the need to avoid damage to self-antigens and host tissue. At one end of the spectrum, 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 (PID) in humans characterized by low immunoglobulin levels, recurrent upper respiratory tract infections and impaired vaccination responses[1,2]. In many patients, CVID presents as an immune dysregulation syndrome with autoimmunity, granulomatous disease, enteropathy, and malignancy[3]. The majority of familial CVID cases present an autosomal dominant (AD) pattern of inheritance, yet disease penetrance may appear incomplete due to the late onset of symptoms[4]. Dominant mutations causing CVID have been found in *NFKB2*[5] and some patients with activating *PI3Kδ* mutations present with a CVID-like phenotype[6]. 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 $FOXP3^+$ T_{reg} cells[7,8]. Accordingly, T_{reg} deficiency caused by mutations in *FOXP3* leads to an aggressive autoimmune syndrome termed IPEX (immune dysregulation polyendocrinopathy X-linked)[9]. In mice, deficiency of CTLA-4 results in a lethal autoimmune phenotype[10,11] with marked similarities to IPEX in humans[7,12,13]. CTLA-4 is an essential effector component of T_{reg} cells that is required for their suppressive function [14-18]. The mechanism whereby CTLA-4 controls T_{reg} cells is still debated[19-21], however studies in chimeric mice containing a mixture of wild type and $Ctla4^{-/-}$ cells suggest that *in vivo* CTLA-4 primarily acts in a T cell extrinsic manner[22,23]. In keeping with

a T cell extrinsic mechanism of action, it has been recently shown that CTLA-4 can function by removal of its ligands (CD80 and CD86) from antigen presenting cells via transendocytosis[24]. These CTLA-4 ligands are shared with the stimulatory receptor CD28[25], whose engagement drives T cell activation, cytokine production and memory T cell differentiation[26,27]. Depletion of the co-stimulatory ligands CD80 and CD86 by CTLA-4 reduces antigen presenting cell-mediated activation of conventional T cells via CD28, resulting in dominant suppression of T cell activation[20]. Thus, CTLA-4 and CD28 are linked to the control of 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 CTLA-4 mutations in humans resulting in CTLA-4 haploinsufficiency and impaired ligand binding and a complex immune dysregulation syndrome.

Results

Identification of heterozygous mutations in *CTLA4*

Whole exome sequencing (WES) and genetic linkage analysis were carried out in fourteen members of a large family with 39 individuals (Family A, Fig. 1a and Supplementary Notes). Five family members presented with recurrent respiratory tract infections, hypogammaglobulinemia, autoimmune cytopenia, autoimmune enteropathy and granulomatous infiltrative lung disease. Since no perfectly segregating novel mutations could be identified, we performed an affected-only analysis to allow for reduced penetrance of the causative mutation. Here, we identified 27 novel heterozygous mutations in 19 genes, which had not been listed in dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) (Supplementary Fig. 1). A nonsense mutation at position c.105 (C35*) was identified in the first exon of *CTLA4* which segregated with disease, which we also found 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, b).

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 three *CTLA4* mutation carriers, yielding a total of six families (A through F) containing 14 patients (11 of them with a proven heterozygous *CTLA4* mutation) and eight carriers. 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. 1a, b, Families B and F). Three distinct missense mutations (Families C, D, E) affected conserved amino acids in the extracellular domain (Fig. 1a, b) and were predicted to interfere with ligand binding or CTLA-4 stability (Supplementary Fig. 2). A summary of the clinical findings of all patients is displayed in Table 1 and details are given in the Supplementary Notes and in Supplementary Table 1.

Lymphocytic organ infiltration and lymphadenopathy

Ctla4$^{-/-}$ mice die from CD4^{+} T cell-dependent organ infiltration[10,11,28]. Investigating the clinical symptoms of our patients, we confirmed extensive CD4^{+} T cell infiltration in a number of organs including the intestine (Fig. 2a,b), lung (Fig. 2c,d), bone marrow (Fig. 2f),

central nervous system (Fig. 2g,h) and kidney (Supplementary Notes) of patients bearing *CTLA4* mutations. Lymphadenopathy (Fig. 2e) and hepatosplenomegaly were also found in patients (Supplementary Notes). 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 patients as assessed by reduced levels of CD4^{+}CD45RA^{+} naïve T cells (Fig. 3a). While patients were generally lymphopenic in the periphery (Supplementary Table 2), the ratio of CD4^{+} to CD8^{+} T cells was in the normal range (Supplementary Fig. 4a, normal range indicated by the grey background in Fig. 3a and Supplementary Fig. 4a).All symptomatic patients with *CTLA4* mutations had reduced immunoglobulin levels (Supplementary Table 2), seven out of ten patients had low proportions of CD19^{+} B cells (Fig. 3a) and reduced switched (IgM^{-} CD27^{+}) memory B cells (Fig. 3a). Additional characterization of the lymphocyte compartment are shown in Supplementary Fig. 4a and Supplementary Table 2. In five out of six patients who were monitored over at least two years, a progressive loss of CD19^{+} B cells was observed over time (Supplementary Fig. 3). *In vitro* re-stimulation of T cells did not suggest a bias towards Th1, Th2 or Th17 differentiation (Fig. 3b). Since patients suffered from T cell infiltrates into multiple organs, we were interested whether T cells had a polyclonal distribution of T cell receptors. 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 Figure 4b). The oligoclonal T cell repertoire of A.II.5 was confirmed by TCR spectratyping (Supplementary Figure 4c).

CTLA-4 expression is reduced in T_{reg} cells

Given the role of CTLA-4 in T_{reg} function[18] we analyzed the T_{reg} compartment in symptomatic individuals or healthy carriers bearing *CTLA4* mutations. The frequency of FOXP3^{+} T_{reg} cells within the CD4+ T cell compartment was increased in individuals with a heterozygous *CTLA4* mutation compared to healthy *CTLA4*$^{+/+}$ controls (Fig. 3c). Consistent with this, both knock-out[29] and heterozygous loss of *CTLA4* in mice (Supplementary Fig. 5) are associated with an increased frequency of T_{reg} 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 individuals with *CTLA4* mutations compared with healthy CTLA4$^{+/+}$ controls (Fig. 3d), a deficit that was more pronounced following T cell activation. Thus, in healthy controls, activated T_{reg} cells expressed levels of CTLA-4 in excess of those in the FOXP3-negative population. In contrast, in individuals with *CTLA4* mutations, the expression of CTLA-4 in activated T_{reg} cells was similar to 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 T_{reg} cells.

Ligand binding and capture is impaired by *CTLA4* mutations

To investigate CTLA-4 function, we tested the ability of T_{reg} cells to perform transendocytosis[24] (see Supplementary Fig. 6 for assay design). Stimulated CD4^{+}FOXP3^{+} T cells were co-cultured with CD80-GFP expressing CHO cells and analyzed for their ability to capture ligand by flow cytometry (Fig. 4a). T_{reg} cells from healthy CTLA4$^{+/+}$ individuals

transendocytosed efficiently with between 10–25% becoming positive for CD80-GFP, however this percentage was reduced to only 2–3% in both healthy and symptomatic individuals bearing *CTLA4* mutations, indicating a deficit in ligand capture. Transendocytosis of CD80 was inhibited when a blocking CTLA-4 antibody was added to cell cultures confirming ligand capture was CTLA-4-dependent (Fig 4a).

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). Protein expression was assessed 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 Left inset). Full-length CTLA4 protein was detected in cells transfected with the *CTLA4* mutants R70W (Family C) and T124P (Family D; Fig.4b Left inset). 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 methionine 38 immediately downstream of the premature stop codon. Cells transfected with either T124P and R70W mutants were impaired in their ability to take up soluble CD80-Ig (Fig. 4b,main panels). Thus, although these mutations are not within the known MYPPPY ligand binding motif of CTLA-4 (Supplementary Fig. 2)[30], they appear to impair ligand binding and uptake.

Impaired Treg suppression in *CTLA4* mutation carriers

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, naïve CD4 cells proliferated in response to CD3-specific antibodies and co-culture with dendritic cells (Fig. 4c –red bars). Proliferation was CD80 and CD86 ligand-dependent as it was inhibited by the addition of CTLA-4-Ig (Abatacept;Fig. 4c-orange bars)). Addition of control T_{reg} cells from healthy donors efficiently suppressed $CD4^+$ T cell proliferation (Fig. 4c-dark blue bar), which was reversed by a CTLA-4-specific blocking antibody, showing that suppressive function of the T_{reg} cells in this assay is CTLA-4-dependent (Fig 4c-cyan bar). T_{reg} cells from individuals with *CTLA4* heterozygous mutations were unable to suppress $CD4^+$ T cell proliferation as compared to healthy $CTLA4^{+/+}$ controls (Fig. 4c). These data reveal a defect in the CTLA-4-dependent suppressive activity of T_{reg} cells from individuals carrying heterozygous *CTLA4* mutations.

Discussion

CD28 co-stimulation is required for T cell effector function and generation of T memory cells and influences B cell class switching and T_{reg} homeostasis[31]. These processes are negatively regulated by CTLA-4. Interfering with the CD28/CTLA-4 pathway can therefore have both immune stimulatory and immune inhibitory effects[32-34]. Here, we describe the phenotype of patients with novel heterozygous mutations in *CTLA4* providing clear evidence of its importance in immune homeostasis and T_{reg} cell suppressive function.

In Families A, B and F, the *CTLA4* mutations most likely ablate CTLA-4 protein expression, rendering patients haploinsufficient for CTLA-4. From these families, we learn that the gene

dosage of *CTLA4* is important, especially for the function of regulatory T cells. In Families C, D and E, the mutations affect the ligand binding domain 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*, twelve 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 T_{reg} cell suppressive function. This suggests that additional modifiers, including genetic, epigenetic or environmental factors, may exist which influence the clinical outcome of CTLA-4 deficiency [35]. Since the age of disease onset for 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 (psoriasis, type 1 diabetes, 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 or with impaired T_{reg} cell function[36].

One notable finding is that patients with defects in CTLA-4 expression and function present with hypogammaglobulinemia in immunodeficiency clinics. Since 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 (Fig. 2f) impairing B cell development. This is consistent with the disruptions in B cell lymphopoiesis in T_{reg}-deficient mice[37]. Alternatively, increased CD28-dependent follicular helper T (T_{FH}) cell differentiation[38] could result in chronic stimulation of B cells leading to exhaustion. There may also be parallels with the PI3Kδ activating mutations[39,40] that cause defects in B cell class switching despite hyperactivation of T cell responses. Since CD28 is a major PI3K activator in T cells[41], this link warrants future investigation.

Kuehn *et al*. recently reported a group of patients with heterozygous CTLA-4 deficiency[42]. 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 patient cohort contains different mutations from those of Kuehn *et al*. suggesting there may be a spectrum of genetic alterations leading to defective CTLA-4 function. In our case we have identified several point mutations in the ectodomain of the expressed CTLA-4 protein. Whilst we show in cellular assays that ligand binding to these mutants is impaired, it remains to be formally determined whether this impairment is solely due to changes in CTLA-4 affinity for its ligands or if 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, T_{reg} 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, whilst the defects in T_{reg} cell suppressive function in individuals with *CTLA4* mutations are consistent with those in Kuehn *et al.*[42], 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 that whole PBMCs were stimulated using anti CD3/CD28 antibodies[42]. Given the enrichment of memory T cells in the patient samples, along with the presence of T_{reg} cells in these assays, it is unclear whether the hyperproliferative T cell phenotype reported is due to loss of CTLA-4 function in conventional T cells. The relative role of CTLA-4 in T_{reg} cells versus conventional T cells remains unclear and additional work is needed to conclude that there is T cell-intrinsic hyperproliferation in these patients bearing *CTLA4* mutations. In addition, whilst Kuehn *et al*. report CTLA-4 expression on B cells[42], 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 in T_{reg} cells predisposes individuals to both autoimmunity and immunodeficiency.

Alterations in immune homeostasis is a feature of PID 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 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[34,43,44]. Whether they could be beneficial in the context of CTLA-4 deficiency warrants investigation.

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 (Freiburg) and protocols #04/Q0501/119_AM03 for affected individuals, #07/H0720/182 for family members, and #08/H0720/46 for healthy controls (London).

Linkage analysis

Genotyping of microsatellite markers across the autosomes was done as described by Braig *et al*. in 2003[45]. Of particular interest here, there were 28 markers genotyped across chromosome 2. The marker D2S1384 (Marshfield map 200.43cM, human genome build 37/ hg19 205.2Mbp) is close to *CTLA4* (204.7Mbp). The flanking markers genotyped were D2S1391 (186.21cM, 185.0Mbp) and D2S2944 (210.43cM, 214.6Mbp). At these markers, 15 family members were genotyped including five individuals who were affected or obligate carriers. LOD scores were computed using FASTLINK[46-48].

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Whole exome sequencing

Exome sequencing was performed for all 14 available individuals of the pedigree. The samples were enriched using the TruSeq 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 reads were mapped against the human reference genome build hg19 using BWA[49] v0.7.9, sorted, converted to bam format and indexed with SAMtools[50] v0.1.17, followed by the removal of PCR duplicates with Picard v1.115 (http://picard.sourceforge.net). Local realignment around InDels and base quality score recalibration as well as variant calling and variant quality score recalibration were performed with the GATK[51] v2.8 according to their best practice recommendations. For annotation and predicting the effects of single nucleotide polymorphisms we applied SnpEff and SnpSift v3.6 (http://snpeff.sourceforge.net)[52]. Working with genetic variation data in the form of VCF files was conducted using VCFtools program package[53].

Genes were designated as related to the immune system using IRIS list[54] and GO list. The GO list was generated by composing the genes annotated in Gene Ontology[55] direct and indirect with the term “immune system process” (GO:0002376) defined as “Any process involved in the development or functioning of the immune system, an organismal system for calibrated responses to potential internal or invasive threats.” http://amigo.geneontology.org/amigo/term/GO:0002376

Immunohistochemistry

Bone marrow biopsies were decalcified in EDTA prior to paraffin embedding. 3 μm sections were cut from formalin-fixed, paraffin-embedded samples. Upper gastrointestinal biopsies were routinely stained with hematoxylin and eosin stain and periodic acid–Schiff stain. Bone marrow biopsies were routinely stained with naphthol AS-D chloroacetate esterase (NASDCL). Immunohistochemical staining was performed using a horseradish peroxidase catalyzed brown chromogen reaction together with ready-to-use antibodies in an automated staining system (Dako Autostainer Link®; Dako, Glostrup, Denmark), following the manufacturer's guidelines. Depending on the tissue section size, up to three droplet zones are stained with 100 μl antibody solution per droplet zone. The following antibodies were used: CD3 (rabbit polyclonal, Dako), CD4 (clone 4B12, Dako), CD8 (clone C8/144B, Dako), CD19 (clone LE-CD19, Dako), CD20 (clone L26, Dako), CD38 (clone SPC 32, Novocastra, Newcastle upon Tyne, UK), IgM, IgG, IgA (rabbit polyclonal antibodies; Dako). Photos were taken on an Olympus BX51 microscope (Olympus Germany, Hamburg, Germany) with the AxioCam MRc camera (Zeiss, Jena, Germany).

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated through a Ficoll step gradient and stained for cell surface markers with the following fluorochrome-conjugated antibodies: CD38 (HIT-2, BD), IgD (IA6-2, BD), IgM (MHM-88, BioLegend), CD10 (HI10a, BD), CD19 (SJ25C1, BD), CD21 (B-ly4, BD), CD27 (0323, eBiosience), CD138 (B-B4, AbD SeroTec), IgG (SouthernBioTech #2043-09), IgA (G20-359, BD), CD3 (SK7, BD), CD28 (CD28.2, BD), FOXP3 (PCH101, eBioscience), CD8 (SK1, BD), CD4 (RPA-T4, eBioscience), γδ-TCR (B1.1, eBioscience), αβ-TCR (IP26, eBioscience), CD45RO

(UCHL1, BD), CD45RA (HI100, BD), CD57 (NK-1, BD), CD31 (WM59, eBioscience), CD62L (DREG-56, BD), CD152/CTLA4 (BNI3, BD). Samples were acquired on a FACS-Canto II flow cytometer (BD) or on a Gallios flow cytometer (Beckman Coulter, Miami, FL) and analyzed using FlowJo version 7.6.5 analysis software (Treestar, Ashland, OR).

Cytokine expression by T cells

One million freshly isolated PBMCs were incubated overnight at 37°C, 5% CO_2 in Iscove's Modified Dulbecco's Medium (GIBCO) supplemented with 10 % fetal calf serum (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 IL-2, PMA (0.05μg/ml) and Ionomycin (1μg/ml) for 4 hours at 37°C, 5% CO_2. Subsequently, cells were stained for surface markers CD3 (SK7, BD), CD4 (SFCI12T4D11, Beckman Coulter) and CD45RO (UCHL1, BD) and intracellular markers IL17 (eBio64DEC17, eBioscience), IL-4 (8D4-8, eBioscience) and IFN-γ (B27, BD) and measured by flow cytometry. $CD3^+CD4^+CD45RO^+$ cells were analyzed for cytokine expression levels.

TCR spectratyping and rearrangement studies

TCR beta, gamma and delta spectratyping was performed from RNA following synthesis of oligo dT primed cDNA as described[56]. In order to study TCR rearrangements DNA was amplified by PCR using the Biomed-2 primers and protocols[57]. All fluorescent fragments were analyzed on an ABI 3130-XL capillary sequencer (Life Technologies, Darmstadt, Germany).

CTLA-4 staining in activated T_{reg} cells

200,000–300,000 PBMCs (freshly isolated or frozen samples) were incubated overnight then cultured for 16 hours in the presence or absence of CD3/CD28 beads (Dynabeads® Human T-Activator CD3/CD28) at a concentration of 1:1 (beads:cells). Cells were then stained for surface markers CD4 (RPA-T4, BD bioscience), CD45RA (HI100, Ebioscience), CD127 (HIL-7R-M21, BD bioscience) and CD25 (2A3, BD bioscience), fixed and permeabilized and stained for FOXP3 (236A/E7, Ebioscience) and CD152/CTLA-4 (BNI3, BD), before being assessed using a BD Canto II flow cytometer.

Mice

Heterozygous *Ctla4*-deficient (*Ctla4*$^{+/-}$) mice and wildtype (WT) littermates on a C57BL/6 background were maintained under specific pathogen free conditions and used in experiments between 6–10 weeks of age. Experiments were conducted in compliance with Osaka university regulations. C57BL/6 *Ctla4*$^{+/-}$ or WT littermates were vaccinated intraperitoneally with 100μg NP-ovalbumin in Alum. Splenocytes were manually disaggregated andanalyzed ex vivo on day 14 after immunisation. Intracellular staining was carried out using the eBioscence FOXP3 staining buffer kit according to manufacturer's instructions. Antibody clones were as follows. Anti-B220: RA3-6B2 (BD). GL7, anti-CD38: 90, anti-FOXP3: FJK-16s, anti-CD25: PC61.5 (eBioscience). anti-CD80: 16-10A1, anti-CD86: GL-1, (Biolegend). Dead cell discrimination was based on positivity for IR Live/Dead (Invitrogen).

Transendocytosis assays

Primary human $CD4^+$ T cells were purified from whole PBMCs using EasySep™ Human CD4+ T Cell Enrichment Kit (Stemcell Tech.) and activated with CD3/CD28 beads for 16 hours in the presence of CHO cells expressing CD80-GFP .. After 16 h CTLA-4 expression (anti-CTLA-4-PE, BNI3, BD bioscience) was assessed by staining cells at 37°C for the final 2 hours.. Subsequently cells were stained for CD4 (RPA-T4, BD bioscience, 1/50), , CD45RA (HI100, eBioscience, 1/200), CD127 (HIL-7R-M21, BD Bioscience, 1/50) and CD25 (2A3, BD bioscience. 1/50) then fixed and permeabilized and stained for FOXP3 (236A/E7, eBioscience, 1/50). Cells were gated on $FOXP3^+$ cells and analyzed for GFP uptake.

Human T_{reg} suppression assays

To study CTLA-4-dependent T_{reg} 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 T_{reg} cells. In our experience T cell responses which are not abatacept sensitive such as those stimulated using antibody coated beads cannot be suppressed in a CTLA-4 dependent manner by T_{reg} cells. To perform such assays, freshly isolated resting $CD4^+$ naïve T cells were washed with PBS and incubated with CellTrace Violet according to manufacturer's instructions (Molecular Probes). The reaction was quenched with media containing serum followed by PBS wash and cells 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 0.5μg/ml CD3-specific antibody (OKT3- ATCC). To provide costimulation, 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 (PeptroTech, 800 U/ml) and IL-4 (PeproTech, 500 U/ml) for 5-7 days. DC were present at a ratio of 1:10, DC:T cell. Cells were cultured for 5 days in the presence or absence of 10 μg/ml CTLA-4-Ig (Abatacept) or anti-CTLA-4 (20 μg/ml). To measure T_{reg} suppression, unlabeled negatively selected $CD4^+CD25^+$ T_{reg} (2 T_{reg} : 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Editorial summary

Mutations in the costimulatory molecule CTLA-4 in six families are associated with immune dysregulation.

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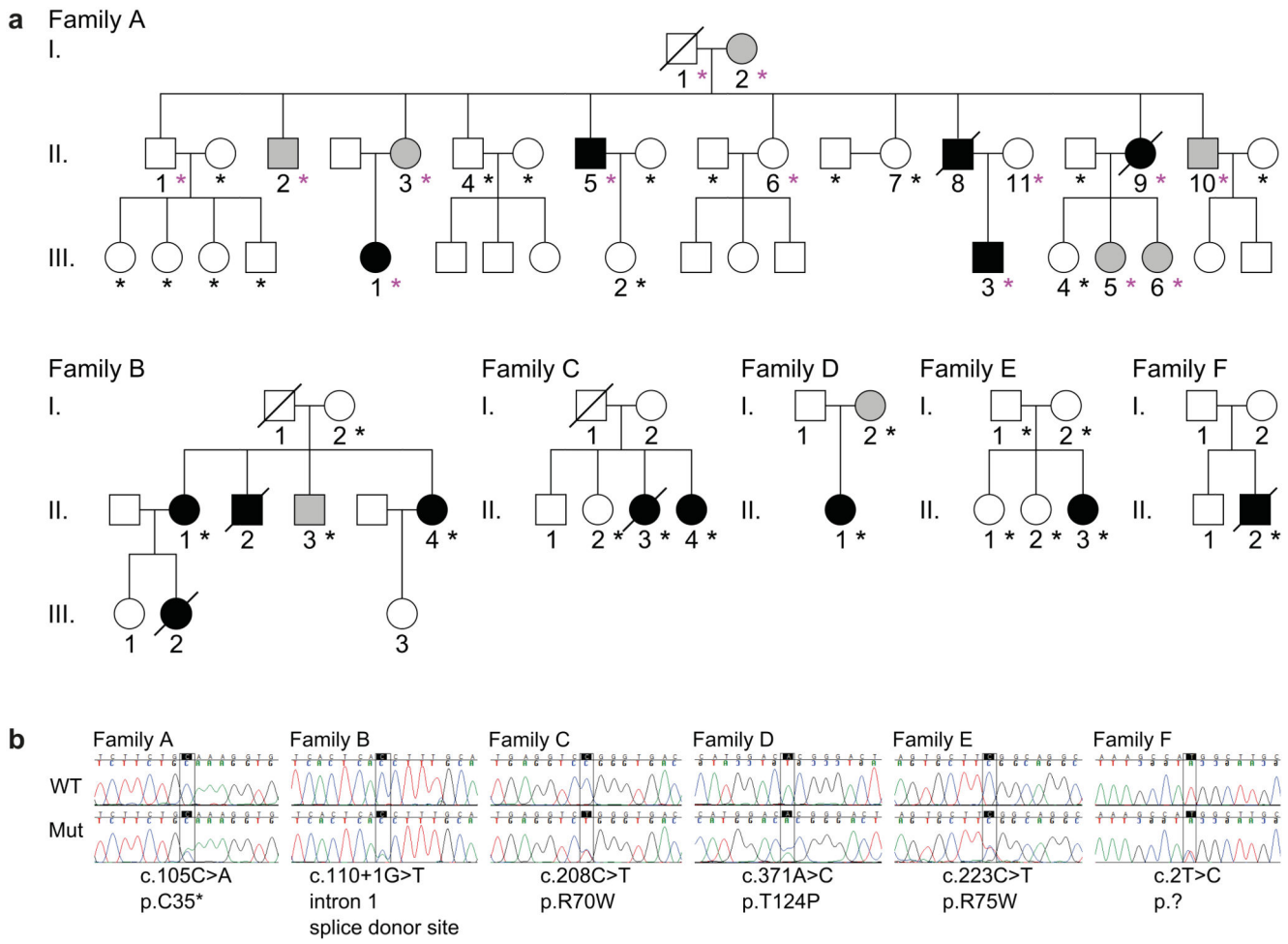

Figure 1. Genetics and pedigrees of families with *CTLA4* mutations

(**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 gDNA (asterisk). Whole exome sequencing was carried out on subjects with a pink asterisk. (**b**) Confirmation of the mutations by Sanger sequencing showing cDNA (c) changes and their resulting amino acid (p) changes.

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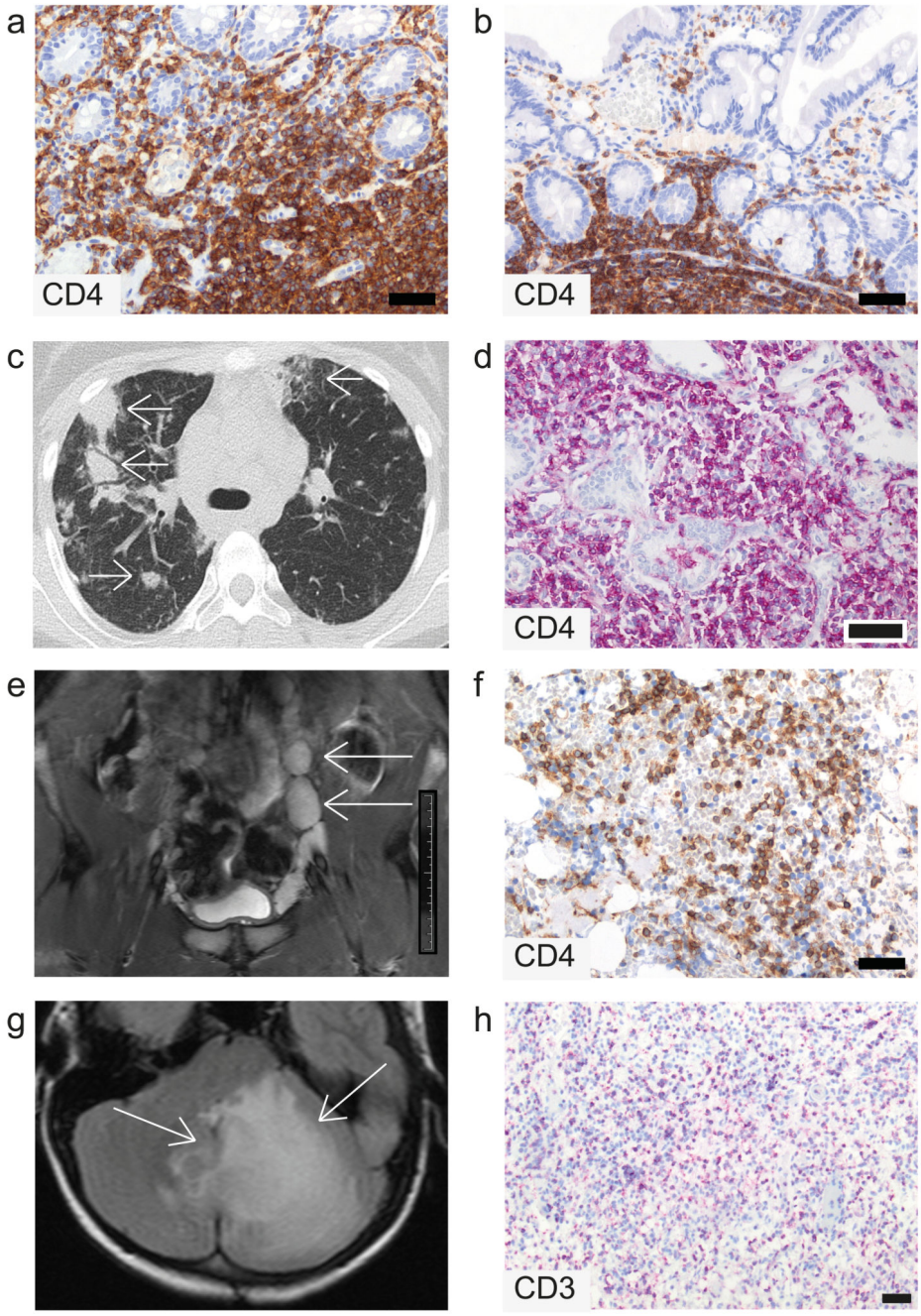

Figure 2. Tissue infiltration and lymphadenopathy in patients with *CTLA4* mutations

Duodenal biopsies stained for CD4 (patient B.II.4 (**a**) and A.III.3 (**b**)). (**c**) High resolution chest CT scan of the lungs (from patient E.II.3). (**d**) Pulmonary lymphoid fibrotic lesions stained for CD4 in pulmonary biopsies (from patient E.II.3). (**e**) Magnetic resonance imaging (MRI) of the pelvic area with two enlarged lymph nodes (arrows) measuring up to 5 cm (from patient A.III.3). (**f**) Bone marrow biopsy stained for CD4 (from patient B.II.4). (**g**) MRI of Gadolinium-enhanced lesions (arrows) in the cerebellum (from patient A.III.1) (**h**)

Resected cerebellar lesion stained for CD3 (from patient A.III.1). Scale bars, 50 μm (**a,b,d,f,h**).

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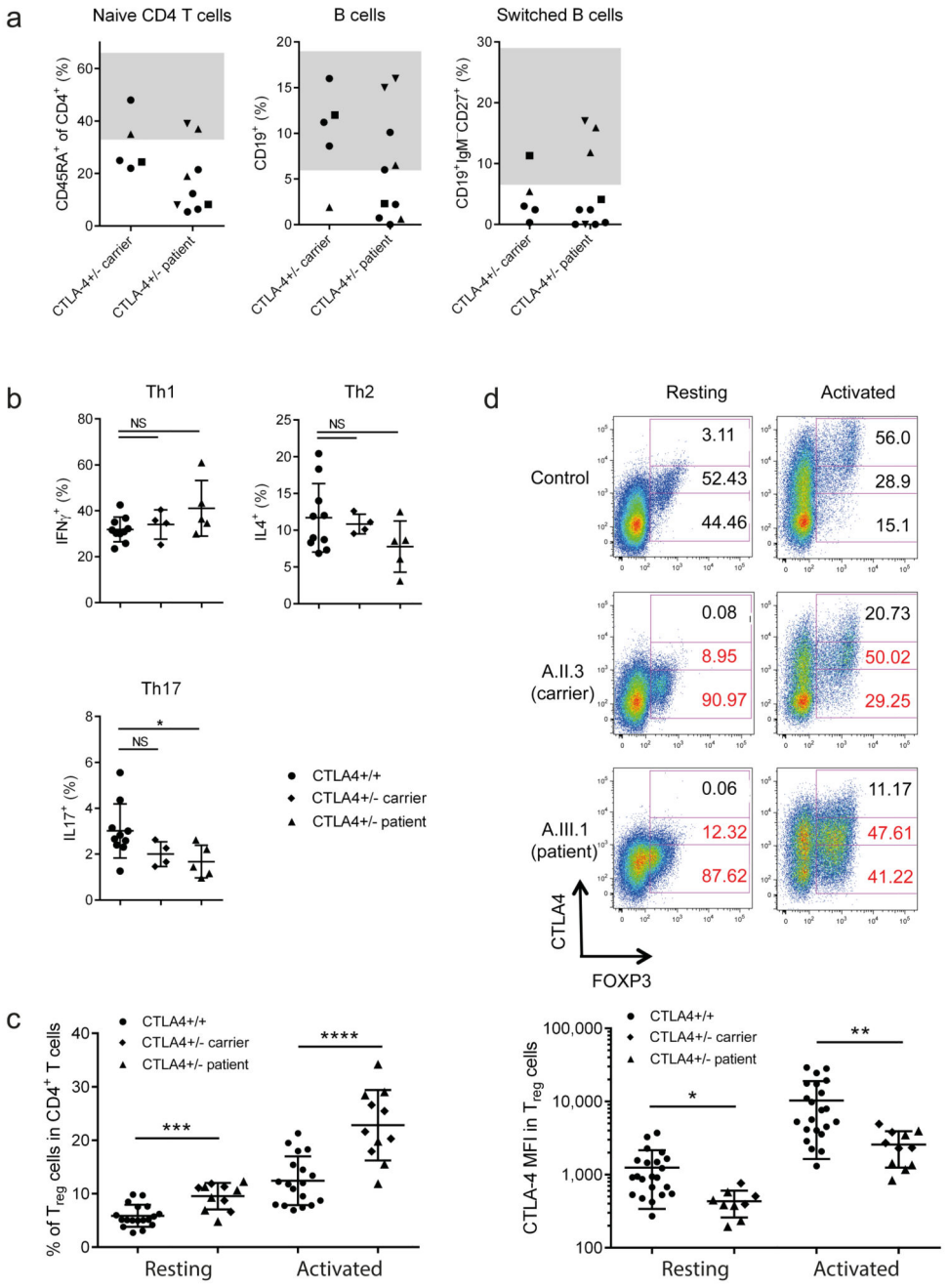

Figure 3. Impact of *CTLA4* heterozygosity on T and B cells

(**a**) Percentage of naïve $CD4^+CD45RA^+$ 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. ● Family A, ▲ Family B, ▼ Family C, ■ Family D. (**b**) Proportion of IFN-γ^+, IL-4^+ and and IL-17^+ expressing $CD3^+CD4^+CD45RO^+$ T cells after stimulation of PBMCs with PMA and Ionomycin in healthy $CTLA4^{+/+}$, $CTLA4^{+/-}$ carriers and $CTLA4^{+/-}$ patients. (**c**) Percentage of $FOXP3^+$ T_{reg} cells amongst CD4+ T cells in the peripheral blood under resting (*ex vivo*) conditions or following activation (with beads

containing CD3- and CD28-specific antibodies. (**d**) Representative flow cytometry plots (top) and quantification (bottom) of CTLA-4 expression in $CD4^+FOXP3^+$ cells under resting and activated conditions. (Resting: P=0.0130; Activated: P=0.0065). Numbers in quadrants show percentage of CTLA-4 high (top), intermediate (middle) and low (bottom) expressing cells within the $FOXP3^+$ population. Plots in **b**, **c** and **d** show the mean +/− SD; each dot represents one individual. P values were determined by Student's t test. *P≤0.05; **P≤0.01, ***P≤0.001, ****P≤0.0001.

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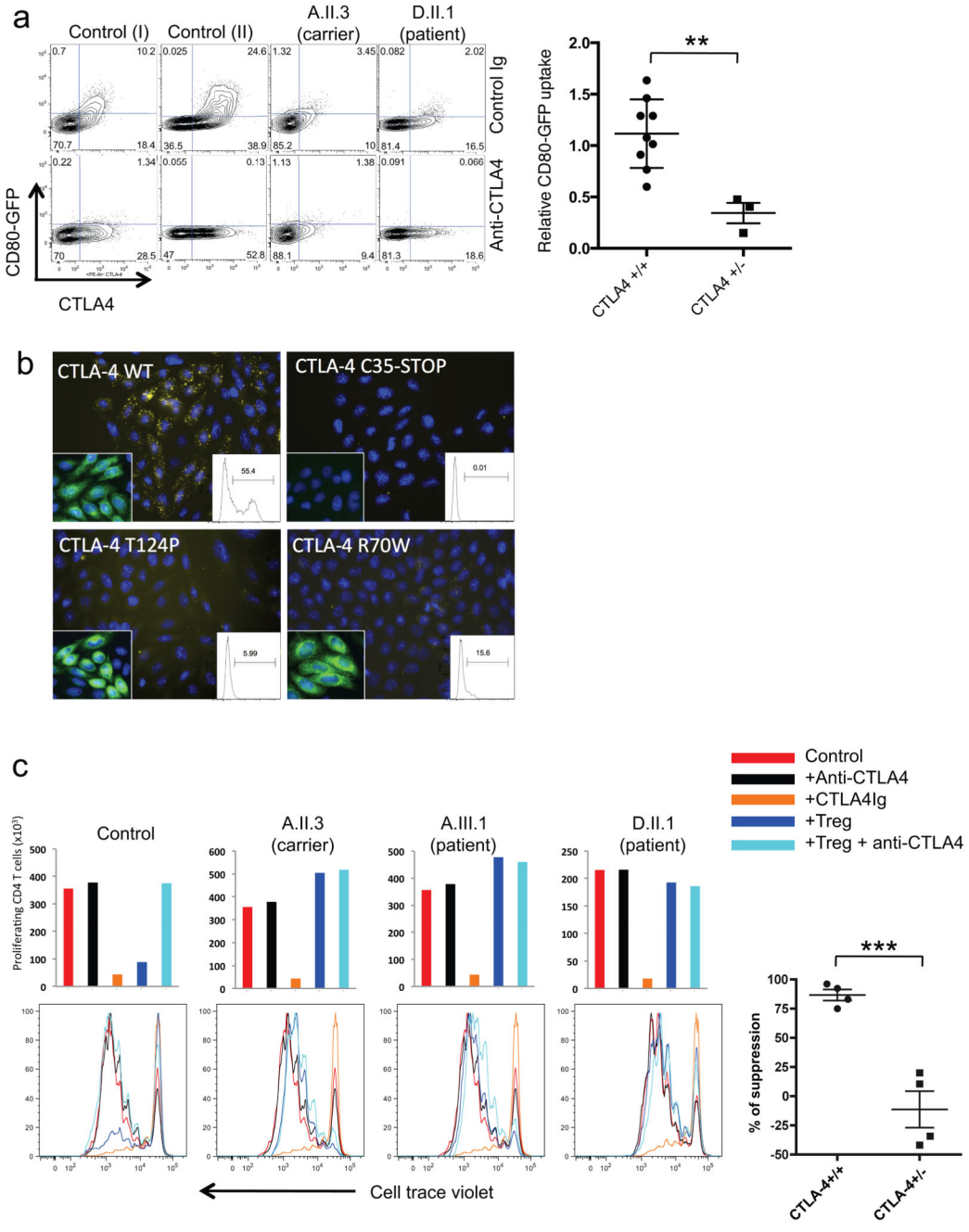

Figure 4. Impaired transendocytosis, ligand binding and T_{reg} suppressive activity in *CTLA4* heterozygotes

(**a**) Transendocytosis of CD80-GFP by stimulated primary $CD4^+FOXP3^+$ T_{reg} cells in the presence or absence of CTLA-4 blockade. Flow cytometry plots depict CD80-GFP uptake by T_{reg} cells in the absence (upper panels) and presence (lower panels) of CTLA-4 blockade. Dot plot shows the relative CD80-GFP uptake in homozygous versus heterozygous individuals $P = 0.0091$. (n=9 $CTLA4^{+/+}$, n=3 $CTLA4^{+/-}$). (**b**) (Main panel) Uptake of CD80-Ig (yellow) by CHO cells expressing wild-type and mutant *CTLA4*. (Right,

inset) Flow cytometric analysis of CD80-Ig staining in CHO cells (x axis, CD80-Ig staining; y axis, relative cell number). (Left, inset) CTLA-4 expression (green) in CHO cells, as assessed by staining with an antibody to the C-terminus of CTLA-4. Images are representative of 4 independent experiments. (**c**) Proliferation of cell trace-labeled $CD4^+$ responder T cells upon co-culture with monocyte-derived dendritic cells and CD3-specific antibodies with or without $CD4^+CD25^+$ T_{reg} 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 of responder T cells in suppression assays. *P* values were determined by Student's t test. ** P<0.01, *** P<0.001.

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Table 1

Clinical phenotype of patients and carriers 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.2, 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, C.II.3, B.III.2, D.II.1, E.II.3, F.II.2	10/13 (76%)
Granulomatous lymphocytic interstitial lung disease	A.II.8, A.II.9, A.III.3, B.II.4, B.III.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.III.2, C.II.3, E.II.3, F.II.2	8/14 (57%)
Organ infiltration (bone marrow, kidney, brain, liver)	A.II.9, A.III.1, A.III.3, B.II.2, B.II.4, C.II.3, D.II.1	7/14 (50%)
Splenomegaly	A.II.5, A.II.9, A.III.3, C.II.3, D.II.1, E.II.3	6/12 (50%)
autoimmune thrombocytopenia	A.III.1, A.III.3, C.II.3, E.II.3, F.II.2	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/other skin diseases[*]	A.III.1, B.II.1, B.II.2	3/14 (21%)
autoimmune thyroiditis	A.II.5, D.II.1	2/13 (15%)
autoimmune arthritis	A.II.5, A.III.1	2/14 (14%)
Solid cancer	B.II.4	1/14 (7%)

[#] Upper and lower respiratory tract infections;

[*] Details in the supplementary case reports; .

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