Phenotype, penetrance, and treatment of 133 CTLA-4-insufficient individuals

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Abstract

Background

Cytotoxic-T-lymphocyte-antigen-4 (CTLA-4) is a negative immune regulator. Heterozygous *CTLA4* germline mutations can cause a complex immune dysregulation syndrome in humans.

Objective

To characterize the penetrance, the clinical features and the best treatment options in 133 *CTLA4* mutation carriers.

Methods

Genetics, clinical features, laboratory values, and outcome of treatment options were assessed in a worldwide cohort of *CTLA4* mutation carriers.

Results

We identified 133 individuals from 54 unrelated families carrying 45 different heterozygous *CTLA4* mutations, including 28 previously undescribed mutations. Ninety mutation carriers were considered affected, suggesting the clinical penetrance of at least 67%; median age of onset was 11 years, and mortality rate within affected mutation carriers was 16% (n=15).

Main clinical manifestations included hypogammaglobulinemia (84%), lymphoproliferation (73%), autoimmune cytopenia (62%), respiratory- (68%), gastrointestinal- (59%), or neurological features (29%). Eight affected mutation carriers developed lymphoma, three gastric cancer. An EBV association was found in six malignancies. *CTLA4* mutations were associated with lymphopenia and decreased T-, B-, and NK-cell counts. Successful targeted therapies included the application of CTLA-4-fusion-proteins, mTOR-inhibitors, and hematopoietic stem cell transplantation. EBV reactivation occurred in two affected mutation carriers under immunosuppression.

Conclusions

Affected mutation carriers s with CTLA-4 insufficiency may present in any medical specialty. Family members should be counseled, as disease manifestation may occur as late as age 50. EBV- and CMV-associated complications must be closely monitored. Treatment interventions should be coordinated in clinical trials.

Clinical Implication

This large cohort of affected *CTLA4* mutation carriers gives first insights into different possible treatment options and presents available clinical information on treatment response and survival. With this knowledge, affected mutation carriers will benefit from an individualized management.

Capsule summary

We present the clinical spectrum, new mutations, and possible modifiers of the world-wide largest cohort of *CTLA4* mutation carriers. We encourage physicians to consider mutations in genes such as *CTLA4* as a monogenetic cause for complex disease presentations.

Key words

Cytotoxic T lymphocyte antigen 4, primary immunodeficiency, autoimmunity, hypogammaglobulinemia, hematopoietic stem cell transplantation, abatacept, sirolimus, immune dysregulation, common variable immunodeficiency

Abbreviations

alloHSCT, allogeneic hematopoietic stem cell transplantation

APC, antigen-presenting cells

CMV, cytomegalovirus

CTLA-4, cytotoxic T lymphocyte antigen 4

CVID, common variable immunodeficiency

EBV, Epstein-Barr virus

GLILD, granulomatous-lymphocytic interstitial lung disease

GvHD, graft-versus-host disease

PRCA, pure red cell aplasia

Treg, regulatory T cell

Introduction

Heterozygous germline mutations in cytotoxic T lymphocyte antigen 4 (*CTLA4*) can lead to haploinsufficiency, impaired CTLA-4 dimerization, or impaired ligand binding, and can cause an autosomal dominant immune dysregulation syndrome and immunodeficiency in humans.(1-3) CTLA-4 is a negative immune regulator essential for the function of regulatory T cells (Tregs), which are responsible for maintaining self-tolerance and immune homeostasis through the suppression of T cell proliferation and differentiation.(4-9) CTLA-4 competes with the costimulatory receptor CD28 for its ligands CD80 and CD86, expressed on antigen-presenting cells (APCs).(10, 11) CTLA-4 binds these ligands with a higher affinity and avidity than CD28 and removes them from the surface of APCs *via* transendocytosis, resulting in a reduction of APC-mediated activation of conventional T cells.(12, 13) *CTLA4* encodes for four exons; exon 1 encodes the signal peptide, exon 2 the ligand binding and dimerization domains, exon 3 the transmembrane domain, and exon 4 the cytoplasmic tail.(14)

The clinical diagnosis of CTLA-4 insufficiency is complicated by a highly variable phenotype including various organ-specific autoimmune diseases, hypogammaglobulinemia, recurrent infections, and malignancies; the natural history of this condition is largely unknown.(1, 3, 15-19) CTLA-4 insufficiency in humans was associated with incomplete penetrance.

Here, we describe the largest known cohort of *CTLA4* mutation carriers including 133 individuals to aid diagnosis in similar cases and give guidance for their treatment.

Methods

See Supplements.

Results

Age distribution and origin

We identified 133 individuals of 54 unrelated families (66 female, 67 male) from Europe (n=87), Asia (n=26), South America (n=7), and North America (n=13) (Table 1, Figure 1). Median age of onset was 11 (<1 to 59) years, median age at evaluation was 23 years in affected mutation carriers, and 46 years in unaffected carriers (Figure 2). Three-fourths of affected mutation carriers were under the age of 18 years when showing first symptoms; there was no significant difference in the age of onset between women and men.

Genetics and protein function

We identified 45 unique heterozygous *CTLA4* germline mutations including 28 missense mutations, ten deletions or insertions, and seven nonsense mutations (Table 1, Figure 3). Mutations in seven affected carriers had occurred *de novo.* Twenty-eight mutations were novel and seventeen have previously been described.(1, 3, 15, 17-21) Eight mutations were located in exon 1, 31 in exon 2 and six within exon 3. Mutations at seven loci were identified in multiple families (Table 2). CTLA-4 expression within stimulated Tregs was reduced in all tested *CTLA4* mutation carriers. CD4+ T cells were co-cultured with CD80-GFP-expressing CHO cells and GFP-uptake was measured within CTLA-4 positive cells to estimate the ability of cells to perform transendocytosis, which was reduced in all tested mutation carriers (Table 1, Figure S1).(13) An association between genotype and onset, penetrance, or disease phenotype was not observed. So far 115 exonic variants have been described within *CTLA4*; all but two variants have a minor allele frequency (MAF) <0.01, seven variants have been described to be disease causing or are part of our cohort (Table S3). (1, 2, 19)

Symptoms and signs at presentation

First symptoms included autoimmune cytopenia (33%), respiratory manifestations (21%), enteropathy (17%), type 1 diabetes (8%), neurological symptoms (seizures, headache, nausea) (6%),

thyroid disease (5%), arthritis (3%), growth retardation, fever or night sweats, atopic dermatitis, alopecia (2% each), and primary biliary cirrhosis, Addison's disease, or a wound healing disorder, in one affected mutation carrier each.

Main diagnoses

At the time of data collection, affected mutation carriers had diverse main diagnoses: Twenty-six (29%) had a diagnosis of cytopenia and 23 (26%) had common variable immunodeficiency (CVID). CVID was diagnosed according to the revised European society of immune deficiencies (ESID) registry.(22) Twenty affected mutation carrier (22%) suffered mainly from severe gastrointestinal symptoms such as enteropathy or inflammatory bowel disease (IBD) and ten (11%) from respiratory disease including infections (n=9), granulomatous lymphoproliferative interstitial lung disease (GLILD, n=9), bronchiectasis (n=9), and asthma (n=2). In seven affected mutation carriers (8%) lymphoma was the leading diagnosis, five (6%) had mainly endocrinopathies, and four (4%) had inflammatory CNS disease. Individual affected mutation carriers had widespread lymphadenopathy (n=3, 3%), an autoimmune lymphoproliferative syndrome (ALPS)-like phenotype (n=2, 2%), an immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX)-like phenotype (n=1, 1%), a primary biliary cirrhosis (n=1, 1%), liver cirrhosis of unknown etiology (n=1, 1%), rheumatoid arthritis (n=1, 1%), and psoriatic arthritis (n=1, 1%). Ten affected mutation carriers (11%) had several main diagnoses (Table S1). At the time of data collection 65 affected mutation carriers were under immunosuppression. Forty-three mutation carriers were considered unaffected (Table S1).

Clinical spectrum of CTLA-4 insufficiency

While *CTLA4* mutations were associated with autoimmunity and immune dysregulation in all affected mutation carriers, the affected organ systems varied substantially: hypogammaglobulinemia (84%), lymphoproliferation (73%), respiratory involvement (68%), gastrointestinal features (59%), autoimmune cytopenia (62%), dermatological involvement (56%, mainly atopic dermatitis), endocrinopathy (33%), and neurological features (29%) were often observed. Arthritis (14%), growth

retardation (14%), renal (12%) or liver (12%) involvement were less frequent (Figure 4, Table S1). One affected mutation carrier had severe psoriatic arthritis (T.II.1). In total, ninety of the 133 *CTLA4* mutation carriers (67.6%) were considered affected, as they had sought medical attention for disease-related symptoms. Case reports can be found in the Supplements.

Non-malignant lymphoproliferation

Sixty-two affected mutation carriers (73%) had non-malignant lymphoproliferation, including splenomegaly (n=51, Figure 3, Figure 5 Panel A), chronic lymphadenopathy (n=43), and hepatomegaly (n=17). Thirteen affected mutation carriers underwent splenectomy for severe cytopenia. Forty-three affected mutation carriers (50%) had lymphocytic infiltrations into lung (n=27), gastrointestinal tract (n=17), brain (n=12), bone marrow (n=6, Figure 6 Panel E), kidney (n=6), or retroperitoneal tissue (n=4). Upon biopsy, 21 affected mutation carriers had T cell infiltrations, both CD4+ (n=9) and CD8+ (n=8) infiltrations were observed. Twelve predominately had B cell infiltrations, four of them in the lung tissue as part of their GLILD. Ten out of 29 biopsied affected mutation carriers with non-malignant lymphoproliferation also had granulomas in at least two different organ systems upon biopsy; eight in the lung, two in the lymph nodes, and one each in kidney, brain, or gastrointestinal tract.

Respiratory tract involvement

Respiratory tract involvement was common (68%; 61/90; Figure 5 Panels C, D, E; Figure 6 Panel A, B) including recurrent lower (n=48) and upper (n=41) respiratory tract infections, granulomatous-lymphocytic interstitial lung disease (GLILD) (n=32), and bronchiectasis (n=20). Two affected mutation carriers underwent lung transplantation due to idiopathic lung fibrosis (B.III.2) or common variable immunodeficiency (CVID) (23) with recurrent infections, emphysema, and parenchymal lung damage (A.II.9); both died 12 and 15 months, respectively, after transplantation due to pulmonary demise following a relapse of disease.

Pathogens and infections

Sixty-one percent of affected mutation carriers (55/90) had respiratory tract infections including pneumonia, sinusitis, and otitis media. Isolated pathogens were *Haemophilus influenzae* (n=6) and *Streptococcus pneumoniae* (n=4). The most common enteritis pathogen was *Salmonella enteritidis* (6/7). *Staphylococcus aureus* was detected in various organs of eleven affected mutation carriers. Twenty-seven affected mutation carriers reactivated a Herpes virus infection: Epstein-Barr virus (EBV) led to clinically apparent infections in sixteen affected mutation carriers (Figure 6 Panel C), including EBV-induced hemophagocytic lymphohistiocytosis (B.II.3). Two affected mutation carriers developed EBV-associated lymphoid granulomatosis in lung or brain (H.II.2, N.III.2). Cytomegalovirus (CMV) reactivation was found in nine affected mutation carriers including CMV-associated diarrhea or gastritis (D.II.1, M.II.3, NN.II.1), chronic active CMV infection (LL.II.1), CMV lymphadenitis (K.II.1), bilateral parotid hypertrophy (O.II.1), and respiratory CMV infection (R.II.5); eight of them were on immunosuppressive treatment. *Mycobacterium tuberculosis* polymerase chain reaction was positive in four affected mutation carriers, with two of them developing pulmonary or esophageal tuberculosis (A.II.8, A.II.9).

Fungal infections were present in 15 affected mutation carriers with either *Candida species pluralis* infections (n=13) or *Aspergillus species pluralis* pneumonia (n=2); thirteen of them received immunosuppressive treatment at the time of data collection. Ten affected mutation carriers, of whom eight were immunosuppressed, developed sepsis due to bacterial or fungal pathogens leading to death in five. In one affected mutation carrier sepsis followed a perforation of the small bowel, and in one *Salmonella enteritidis* sepsis was the first manifestation of CTLA-4 insufficiency at the age of three months (UU.IV.12).

Gastrointestinal involvement

Gastrointestinal involvement across our cohort was frequent (59%; 53/90) and often severe. Nine of the 15 deceased affected mutation carriers had severe gastrointestinal features prior to their death. Diarrhea was frequent (n=51), ranging from mild to severe diarrhea with weight loss, wasting, and

total parenteral nutrition-dependency. Pathogens were rarely identified. Crohn disease (n=7), atrophic gastritis (n=8) (Figure 6 Panel D), coeliac disease (JJ.II.1), acute pancreatitis (M.II.3), and pancreatic insufficiency (N.III.2, QQ.II.1) were observed. In three affected mutation carriers, severe long-lasting CVID-gastroenteropathy preceded gastric cancer (B.II.4, G.III.2, M.II.3). Macroscopic findings ranged from normal appearing mucosa albeit histologically proven deep T cell infiltrations in the submucosa, to superficial ulcerative lesions or deep-seated inflammatory changes as seen in severe Crohn's disease. Despite decreased serum immunoglobulin levels, histology revealed increased numbers of plasma cells in the gastric (B.II.4, QQ.II.1), intestinal, and colonic (QQ.II.1) lamina propria. Further histology changes included severe lymphocytic infiltrates, and EBV-positive gastric cancer (Figure 6 Panel C). Median age of onset of gastrointestinal features was 15 (<1 to 51) years.

Cytopenia

Autoimmune cytopenia was often severe, life-threatening, and treatment-resistant and formed the main indication for allogeneic hematopoietic stem cell transplantation (alloHSCT) (7/12).

Sixty-two percent of affected mutation carriers (55/89) had autoimmune cytopenia, including immune thrombocytopenia (n=41), autoimmune hemolytic anemia (n=37), pure red cell aplasia (PRCA) (n=2) or autoimmune neutropenia (n=16). In 32 affected mutation carriers cytopenia affected more than one cell lineage, nineteen of those were diagnosed with Evans syndrome, and nine had a trilineage cytopenia. Median age of onset of cytopenia was 12 (1.3 to 48) years.

Neurological involvement

Twenty-eight percent of affected mutation carriers (28/90) presented with a broad spectrum of neurological features (Figure 5 Panel F, G, H, Figure 6 Panel F). Three had autoimmune encephalitis or encephalomyelitis with cerebral perivascular lymphocytic infiltrations leading to vomiting, headache or paraplegia with bladder dysfunction (N.III.2, P.II.2, GG.II.1). In four affected mutation carriers neurological features were attributed to cerebral infiltrations that were not biopsied: nausea

and headache (A.III.1), facial nerve paralysis (H.II.1), aphasia and paresis of the left arm (K.II.1), or a patchy inflammatory demyelinating process with twitching episodes of hands with normal electroencephalography (WW.II.1). Three affected mutation carriers had neurological features secondary to hematological causes: hemiplegia following brain ischemia during AIHA (DD.II.1), hemiparesis and aphasia due to cerebral arterial thrombosis (H.II.2), hemiparesis following cerebral bleeding due to thrombocytopenia (GG.II.1). In two affected mutation carriers clinical and radiological investigation could not identify an underlying cause for tonic-clonic seizures, or recurrent transient paralysis of the left leg respectively (A.II.8, EE.II.1). One affected mutation carrier had life-threatening HLH with increased cerebral pressure leading to cerebral herniation and seizures (J.II.1). Other diagnoses were stiff person syndrome (H.I.2), West-syndrome and developmental delay (UU.V.1), progressive memory loss starting age 57 years (UU.III.7), and chronic hydrocephalus (UU.III.4). Two affected mutation carriers suffered from optic neuritis (TT.II.4) and retinal tear due to lymphocytic infiltrations into the retina (SS.II.1). One had gliosis (ZZ.II.1) and one developed cognitive dysfunction, chorea, ataxia, and mood instability; biopsies revealed inflammation, lymphocytic infiltrations, and a demyelinating-like transformation, which was clinically responsive to steroid treatment (G.III.1). One affected mutation carrier was diagnosed with tuberous sclerosis with tonic-clonic seizures, right-sided hemiparesis, mental retardation, angiofibromas, angiomyolipomas, and a concurrent TSCA2 mutation (LL.II.1).

Malignancies

Eleven affected mutation carriers (12%) developed malignancies. Out of eight with lymphoma, EBV-positivity was found in five. Lymphoma in five affected mutation carriers was classified as Hodgkin lymphoma; one developed a relapsing EBV-associated diffuse large B cell lymphoma (K.II.1) and one a Burkitt lymphoma (FF.II.1) (Figure 6 Panel G, H). Four affected mutation carriers died due to complications of their lymphoma, two underwent successful alloHSCT.

Three affected mutation carriers developed a gastric adenocarcinoma, including one EBV-associated carcinoma (B.II.4, Figure 6 Panel C), and one CMV-associated carcinoma (M.II.3). Two affected

mutation carriers subsequently underwent total gastrectomy, one of whom died following bacterial sepsis (M.II.3) while the other one is alive and well (G.III.2).

Fatal Outcome

Sixteen percent of affected mutation carriers (15/90) died due to their clinical manifestations or resulting complications at a median age of 23 (14 to 60) years. Four died of sepsis on a background of wasting enteropathy, Evans syndrome, or CVID with infections (M.II.3, G.III.1, C.II.3, L.I.2). Three died due to complications of Non-Hodgkin lymphoma (K.II.1, FF.II.1, UU.III.3), one died during chemotherapy of his Hodgkin lymphoma due to septic multi-organ failure (H.II.1), and two following lung transplantation and relapse of disease (A.II.9, B.III.2). One affected mutation carrier died of acute liver failure following many years of gastrointestinal disease (B.II.2). Wasting enteropathy, respiratory insufficiency, and neurological features led to death in one affected mutation carrier (A.II.8). Another one suffered from severe enteropathy and cytopenia, and died following colectomy (F.II.1). Three affected mutation carriers died following alloHSCT due to GvHD (Q.II.1, LL.II.1) or due to diabetic ketoacidosis (S.II.1). There was a significant difference of the age of death between affected and the unaffected *CTLA4* mutation carriers (Figure 2 B).

Immunological phenotype

Thirty-nine percent (26/66) of affected mutation carriers with available immunological data had lymphopenia of which twenty-four were under immunosuppressive treatment at the time of data collection. The absolute CD3+ T cell count was reduced in 36% (16/44) of affected mutation carriers. The absolute CD3+CD4+ helper T cell count was reduced in 20% (13/62) of affected mutation carriers especially due to the noteworthy reduction of naïve CD4+ T cells. An elevated percentage of the activation marker HLA-DR+ was seen in one third of tested affected mutation carriers (11/31). Percentage of CD4+FoxP3+ Tregs was significantly increased in mutation carriers in comparison to healthy controls ($p=0.0034$). There was no significant difference in the Treg percentage between affected and unaffected mutation carriers ($p=0.3882$). Absolute CD3+CD8+ cytotoxic T cell count was

normal in 60% (35/58) of affected mutation carriers. Double-negative T cells were elevated up to 5.3% (median 2.2%; norm: 0.3-2.0%) in 53% of tested affected mutation carriers (9/17). Absolute CD19+ B cell counts were reduced in 41% (26/58) of affected mutation carriers. B cell subsets showed a decrease in switched memory B cells (23/30) and consecutively a relative increase in naïve B cells (14/29). CD21-low B cells were elevated in all affected mutation carriers tested. Five affected mutation carriers with no history of rituximab therapy had no measurable B cells. Hypogammaglobulinemia was present in 84% (65/77), with low IgM in 30, low IgG in 42, and low IgA in 53 affected mutation carriers (Figure 4). Absolute CD16+CD56+ NK cell counts were reduced in 52% (32/61). The percentage of CD3+ and CD3+CD4+ was increased in the majority of affected mutation carriers, as the overall lymphopenia affected CD3+CD8+, B, and NK cells more than the CD4 compartment (Figure S2). Antinuclear autoantibodies (ANA) and anti-neutrophil cytoplasmic antibodies (ANCA) were the most commonly measured autoantibodies; however, they were negative in most affected mutation carriers (ANA (4/51), ANCA (3/42)).

Treatment

CTLA-4 fusion proteins and mTOR inhibitors

CTLA-4 replacement by CTLA-4-Fc, or inhibition of the CD28 signaling pathway through mTOR inhibitors are potential targeted therapies to inhibit the underlying hyper-active signaling in *CTLA4* mutation carriers.

In total, fourteen affected mutation carriers received the CTLA-4 fusion proteins abatacept or belatacept; eleven of whom responded with an improvement of their clinical symptoms. In six of them enteropathy improved, leading to normal stool frequency and weight gain within three months (B.II.4, D.II.1, L.II.2, HH.II.1, SS.II.1, VV.II.1, Figure S3). In two affected mutation carriers primarily presenting with GLILD (RR.II.1, SS.II.1), CTLA4-Fc led to resolution of lymphoproliferation in the lung (SS.II.1), cough and sputum production decreased, and sIL2R concentration dropped from 1228 U/ml to 750 U/ml within five months (RR.II.1). Other observations were an improvement of

lymphadenopathy (G.III.2), stabilization of platelet counts, resolution of bleeding episodes, and regression of optic neuritis (TT.II.4). In two affected mutation carriers, additional systemic immunosuppressive medication could be reduced, as abatacept treatment led to inhibition of the disease progression (J.II.1) or to improvement of lung function and diarrhea (PP.II.1). In six affected mutation carriers treatment was discontinued: three underwent alloHSCT (L.II.2, VV.II.1, GG.II.1), two had an EBV reactivation (B.II.3, B.II.4), and one developed severe respiratory infections, neutropenia, and agranulocytosis (TT.II.4).

Thirteen affected mutation carriers were treated with the mTOR inhibitor sirolimus with a good response in eight (D.II.1, E.II.3, L.II.2, O.II.1, P.II.2, Z.III.1, TT.II.5, WW.II.1). Improvement of clinical features included resolution of transfusion-dependent PRCA (Z.III.1), regression of lymphadenopathy and splenomegaly, reduced IG consumption, and improved CMV viral load (O.II.1). Enteropathy improved in three affected mutation carriers following combination of sirolimus with either prednisolone (D.II.1), belatacept (L.II.2), or rituximab and steroids (WW.II.1). In one affected mutation carrier cytopenia stabilized on co-medication with rituximab, but neurological features and severe aphthae occurred (P.II.2). Sirolimus led to reduced spleen size (volume decreased from 5l to 2.8l) in one affected mutation carrier, who developed arthritis and erythema nodosum during the treatment (E.II.3). In two affected mutation carriers sirolimus treatment was discontinued due to ineffectiveness for cytopenia (GG.II.1), or due to increased blood pressure on the background of a renal impairment (B.II.4). In one affected mutation carrier CMV copies rose under sirolimus treatment in combination with methylprednisolone (DD.II.1), in one lymphopenia worsened (O.II.1), one died due to sepsis during sirolimus treatment (G.III.1), and in one sirolimus treatment was stopped due to serious respiratory infections (SS.II.1). Daily dosage ranged from 2 mg to 2.64 mg (n=5); trough levels were available for two affected mutation carriers (6,2 ng/ml and 8 ng/ml), for three affected mutation carriers target blood values were available (8-12 ng/ml (n=2); 12-15 ng/ml (n=1)).

Hematopoietic stem cell transplantation

Twelve affected mutation carriers underwent alloHSCT between 10 and 50 years of age.(15) Main indications for transplantation included treatment-resistant cytopenia, enteropathy, and Hodgkin lymphoma; often combined with other autoimmune manifestations, lymphoproliferation or severe infections. Nine of these affected mutation carriers are alive, of whom three are more than five years post-HSCT and currently well off all medication (L.II.1, T.II.1, Y.II.1), and six are between 100 days and 12 months post-transplant (B.II.3, L.II.2, P.II.2, W.II.2, GG.II.1, VV.II.1) (Table S2). In half of the affected mutation carriers the *CTLA4* mutation was known prior to transplantation (6/12), the other half was transplanted due to the severity of their symptoms and the *CTLA4* mutation was only identified after transplantation.

Immunoglobulin substitution

Sixty-three percent of affected mutation carriers (55/88) received immunoglobulin substitution either due to hypogammaglobulinemia or due to cytopenia. Twenty-eight affected mutation carriers had both diagnoses at the time of data collection and received immunoglobulin substitution due to both.

Additional treatment options can be found in the Supplements.

Chromosome 2 contiguous gene deletion involving CTLA4

Two unrelated individuals have a heterozygous 2q33.2-2q33.3 deletion involving *CTLA4* and present a CTLA-4 insufficiency-like phenotype, which is possibly influenced by the deletion of additional genes including *CD28* and *ICOS* (Supplements).

Mutation carriers who did not seek medical attention

We identified 43 unaffected family members carrying the same *CTLA4* mutation as their affected relatives. The treating physician of the affected mutation carrier classified family members as unaffected if they did not repeatedly seek medical care, were not under a long-term drug regimen

due to CTLA-4 insufficiency-related symptoms, or if they were not restricted in their health-related quality of life due to their symptoms. Their median age at evaluation was 46 (6 to 87) years, hence in most cases beyond the median age of manifestation. Upon thorough questioning and clinical investigation, seven carriers had diarrhea without weight loss, two had atrophic gastritis or pernicious anemia, and one had coeliac disease. Three carriers had respiratory infections and in one clinically unapparent pulmonary nodules were detected on a routine scan. Nine had dermatological involvement (psoriasis, eczema, vitiligo), and two hypothyroidism. One carrier developed colon cancer aged 78, which was successfully treated by surgery but is otherwise healthy at currently 87 years of age (A.I.2). Four carriers (without recurrent infections) had IgA-deficiency, one each had low IgG or IgM, and one had low IgA and IgM, possibly contributing to respiratory infections (R.III.1). Twenty-six carriers were reported to be clinically completely healthy.

Their immunological phenotyping revealed similarities to affected mutation carriers, including a decrease in NK and CD19+ B cells, but also differences, including significantly higher CD4+ T cells counts, and a higher percentage of switched memory B cells. There was no significant difference with regard to the Treg percentages in affected mutation carriers (Figure S2).

Discussion

In our work, we estimate the clinical penetrance of CTLA-4 insufficiency to be around 67%; however, as genetic analysis could not be performed in all healthy first degree family members, ascertainment was incomplete. Once symptoms have occurred, the clinical course can be severe and was fatal in 15 affected mutation carriers (16%).

The clinical phenotype was characterized by infections, autoimmunity, and lymphoproliferation, affecting various organ systems. Affected mutation carriers have an elevated risk to develop malignancies and for EBV reactivation highlighting the importance of monitoring EBV and possibly CMV viral load, especially under immunosuppressive treatment. Cytopenia and enteropathy were the most life-threatening and treatment-resistant manifestations. This is evidenced by the fact that cytopenia was one of the main indications for alloHSCT (7/12), and half of the deceased affected mutation carriers died following a history of enteropathy and associated complications. Initial symptoms were diverse, emphasizing the importance of raising awareness of this immunodeficiency not only among immunologists but also other specialists including hematologists, neurologists, gastroenterologists, pathologists, dermatologists, and chest physicians. As the age of onset in 75% of affected mutation carriers is under the age of 18 years, CTLA-4 insufficiency should be considered in children with severe immune dysregulation of unknown origin. Also in individuals being evaluated for IBD, CVID, and ALPS, CTLA-4 insufficiency should be considered.

To diagnose CTLA-4 insufficiency, we recommend sequencing the four exons of *CTLA4* and then testing the effect of identified mutations on the protein by measuring CTLA-4 expression or CTLA-4-mediated transendocytosis.(24) Both were reduced in all analyzed mutation carriers, but because this is also seen in individuals with mutations in other genes such as *LRBA*(25), these functional tests cannot be used as the only diagnostic tool to screen for *CTLA4* mutations. In addition to the clinical presentation, an autosomal dominant family history can hint towards CTLA-4 insufficiency.

589 The immunological phenotype revealed perturbed T and B cell homeostasis and significantly
590 increased Treg percentages within the CD4+ T cell compartment. The latter may be a compensatory
591 mechanism of the CTLA-4-deficient immune system to counteract the immune-activation. The
592 expanded and activated effector T cells may produce a cytokine profile leading to an increased Treg
593 cell polarization in order to counterbalance the accelerated immune activation.

594 We present first insights into targeted therapeutic strategies: Out of thirteen affected mutation
595 carriers treated with CTLA-4-Fc, eleven responded favorably, especially enteropathy improved.
596 Further clinical studies are necessary to determine the effectiveness and safety of CTLA-4-Fc
597 treatment for individual clinical manifestations. Out of twelve affected mutation carriers undergoing
598 alloHSCT, nine are alive and well (15); although long-term survival still has to be determined,
599 alloHSCT should be considered as a treatment option in carefully selected affected mutation carriers.

600 In individuals presenting with immunodeficiency, autoimmunity, and lymphoproliferation with
601 impaired Treg development or function, besides CTLA-4 insufficiency, also mutations in *FoxP3, LRBA,*
602 *IL2RA*, *FAS-L, FAS, PI3K, NFKB1 and 2, STAT3,* and *STAT5b* should be considered as a differential
603 diagnosis.(6)′(25-33) Mutations in *FOXP3* lead to a loss of Treg cells and cause IPEX which is an X-
604 linked condition and characterized by enteropathy, immune dysregulation, and polyendocrinopathy,
605 but has an earlier onset, and complete penetrance.(26, 33) Immunological findings in IPEX-syndrome
606 include normal lymphocyte counts and immunoglobulin levels in contrast to CTLA-4 insufficiency. In
607 LRBA deficiency lysosomal CTLA-4 degradation is accelerated and CTLA-4 trafficking to the cell
608 surface is disturbed; hence the inhibitory function of Treg cells is impaired. (25) Biallelic *LRBA*
609 mutations most often lead to complete absence of the LRBA protein; affected mutation carriers
610 present with a phenotype very similar to CTLA-4 insufficiency, characterized by various autoimmune
611 features, lymphoproliferation with dysregulated Treg function, and a defect in production cell
612 homeostasis,(27, 30, 31, 34-43) albeit with an earlier onset, complete penetrance and an autosomal
613 recessive inheritance. In addition, germline gain-of-function mutations in *STAT3* lead to a broad
614 range of autoimmune disorders such as autoimmune cytopenias and multiorgan autoimmunity (lung,

gastrointestinal, hepatic, and endocrine), in combination with an increased susceptibility to infections and a short stature. Further, *STAT3* gain-of-function mutations lead to secondary defects in STAT5 and STAT1 phosphorylation and impair the Treg compartment.(28, 32)

As our results were collected retrospectively, several limiting factors should be considered: affected mutation carriers were treated and evaluated by different physicians and medical departments worldwide. This can lead to an incomplete picture of the clinical phenotype. Also, data was collected at one time point, which often makes it difficult to reconstruct whether symptoms or the immunological phenotype are due to immunosuppressive treatment or the natural course of this immunodeficiency.

In LRBA deficiency, sIL2R, a biomarker for T cell-mediated inflammation, decreases on abatacept treatment.(25) In our cohort sIL2R was only sporadically measured; in one affected mutation carrier sIL2R levels dropped while being on abatacept treatment. Systematical measurement of sIL2R should be considered in all mutation carriers to see whether it indicates disease activity. Affected and unaffected mutation carriers both show impaired *in vitro* CTLA-4 function, indicating the presence of additional factors influencing the clinical phenotype and penetrance such as environmental, genetic, or epigenetic differences. Ethnicity and origin of the mutation carriers could influence age of onset, penetrance, and severity of disease-related symptoms. We cannot assess this, as the world-wide distribution in our study is not equal and the diverse countries of origin varied in diagnostic procedures and standards. In general, there could either be one single modifier, or multiple interacting factors influencing the clinical phenotype. The latter could explain the highly variable expressivity of the phenotype. Another hypothesis suggests an internal threshold within the immune system of CTLA-4-insufficient individuals. Once it is exhausted, immune dysregulation cannot be contained by the organism and individuals develop symptoms; this could explain why healthy mutation carriers may develop life-threatening symptoms late in life (e.g. patient B.II.3 developed hemophagocytic lymphohistiocytosis and Hodgkin lymphoma at the age of 50 years). These cases

teach us to carefully monitor all first-degree relatives for CTLA-4-associated disease activity, while the search for modifying factors in CTLA-4 insufficiency continues.

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Written consent was obtained from all individuals or their legal guardian(s).

Declaration of interest

The authors declare no competing financial or personal interests.

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Tables and figures

Table 1. Baseline description of CTLA-4-insufficient individuals

Subject No.	Case No.	Age of on-set	Age at Evaluation/ Death Δ	Sex	Country of origin	CTLA4-/+ cDNA position; Predicted Amino Acid change	Type of mutation	Reference
1	A.I.2	#	87	F	Germany ¶	c.105C>A; p.C35*; §	Nonsense	Schubert *et al.* (2)
2	A.II.2	#	60	M	Germany ¶	c.105C>A; p.C35*; §	Nonsense	Schubert *et al.* (2)
3	A.II.3	#	59	F	Germany ¶	c.105C>A; p.C35*; §	Nonsense	Schubert *et al.* (2)
4	A.II.5	41	56	M	Germany ¶	c.105C>A; p.C35*; §	Nonsense	Schubert *et al.* (2)
5	A.II.8	12	34 Δ	M	Germany ¶	Φ		Schubert *et al.* (2)
6	A.II.9	17	37 Δ	F	Germany ¶	c.105C>A; p.C35*; §	Nonsense	Schubert *et al.* (2)
7	A.II.10	#	49	M	Germany ¶	c.105C>A; p.C35*; §	Nonsense	Schubert *et al.* (2)
8	A.III.1	10	28	F	Germany ¶	c.105C>A; p.C35*; §	Nonsense	Schubert *et al.* (2)
9	A.III.3	15	20	M	Germany ¶	c.105C>A; p.C35*; §	Nonsense	Schubert *et al.* (2)
10	A.III.5	#	23	F	Germany ¶	c.105C>A; p.C35*; §	Nonsense	Schubert *et al.* (2)
11	A.III.6	#	20	F	Germany ¶	c.105C>A; p.C35*; §	Nonsense	Schubert *et al.* (2)
12	B.I.1	#	66 Δ	M	Germany ¶	Φ		Schubert *et al.* (2)
13	B.II.1	#	57	F	Germany ¶	c.109+1G>T; §	Splice-site	Schubert *et al.* (2)
14	B.II.2	15	23 Δ	M	Germany ¶	Φ		Schubert *et al.* (2)
15	B.II.3	50	51	M	Germany ¶	c.109+1G>T; §	Splice-site	Schubert *et al.* (2)
16	B.II.4	34	43	F	Germany ¶	c.109+1G>T; §	Splice-site	Schubert *et al.* (2)
17	B.III.2	10	16 Δ	F	Germany ¶	Φ		Schubert *et al.* (2)
18	B.III.3	#	17	F	Germany ¶	c.109+1G>T; §	Splice-site	Unpublished
19	C.II.3	7	20 Δ	F	Greece ¶	c.208C>T; p.R70W; §	Missense	Schubert *et al.* (2)
20	C.II.4	#	13	F	Greece ¶	c.208C>T; p.R70W; §	Missense	Schubert *et al.* (2)
21	D.I.2	#	43	F	India/UK †	c.371A>C; p.T124P; §	Missense	Schubert *et al.* (2)
22	D.II.1	10	22	F	India/UK †	c.371A>C; p.T124P; §	Missense	Schubert *et al.* (2)
23	E.II.3	10	22	F	Georgia ¶	c.223C>T; p.R75W; §	Missense	Schubert *et al.* (2)
24	F.II.1	8	23 Δ	M	Germany ¶	c.2T>C; p.?; §	Missense	Schubert *et al.* (2)
25	G.II.1	#	53	F	USA¶	c.179; A<G; p.Y60C	Missense	Zeissig *et al.* (3)
26	G.III.1	12	24 Δ	F	USA¶	c.179; A<G; p.Y60C	Missense	Zeissig *et al.* (3)
27	G.III.2	1.83	22	M	USA¶	c.179; A<G; p.Y60C	Missense	Zeissig *et al.* (3)
28	H.I.2	22	52	F	Germany ¶	c.407C>T; p.P136L	Missense	Unpublished
29	H.II.1	10	21 Δ	M	Germany ¶	Φ		Unpublished
30	H.II.2	7	26	M	Germany ¶	c.407C>T; p.P136L	Missense	Unpublished
31	J.I.2	#	50	F	Germany ¶	c.373G>A; p.G125R	Missense	Unpublished
32	J.II.1	11	22	M	Germany ¶	c.373G>A; p.G125R	Missense	Unpublished
33	K.II.1	26	53 Δ	F	Germany ¶	c.308G>C; p.C103S	Missense	Unpublished
34	L.I.2	20	40 Δ	F	UK ¶	c.437G>T; p.G146V	Missense	Slatter, *et al.* (15)
35	L.II.1	5	20	F	UK ¶	c.437G>T; p.G146V	Missense	Slatter, *et al.* (15)
36	L.II.2	14	16	M	UK ¶	c.437G>T; p.G146V	Missense	Slatter, *et al.* (15)
37	M.II.3	10	35 Δ	M	Japan †	c.76_77insT; p.F28Sfs*40	Frameshift	Hayakawa*et al.*(16)
38	N.I.2	#	71	F	Japan †	c.529_530insA; p.Y177*	Nonsense	Unpublished
39	N.II.1	#	47	F	Japan †	c.529_530insA; p.Y177*	Nonsense	Unpublished
40	N.II.3	#	42	M	Japan †	c.529_530insA; p.Y177*	Nonsense	Unpublished
41	N.III.2	10	10	M	Japan †	c.529_530insA; p.Y177*	Nonsense	Unpublished
42	O.II.1	8	13	M	Spain ¶	c.342_342delC; p.T115Lfs*5	Frameshift	Unpublished
43	P.II.2	2	13	M	Germany ¶	c.534C>G; p.S178R	Missense	Unpublished
44	Q.II.1	10	15 Δ	M	UK ¶	c.529T>G; p.Y177D	Missense	Slatter, *et al.* (15)
45	R.II.5	24	44	F	Italy ¶	c.410C>T; p.P137L	Missense	Unpublished
46	R.III.1	#	18	F	Italy ¶	c.410C>T; p.P137L	Missense	Unpublished
47	S.II.1	2	22	M	UK ¶	c.410C>G; p.P137R	Missense	Slatter, *et al.* (15)
48	T.II.1	1.5	21	M	UK ¶	c.518G>A; p.G173E	Missense	Slatter, *et al.* (15)
49	U.I.1	#	40	M	Japan †	c.494G>A; p.W165*	Nonsense	Unpublished
50	U.II.1	3.75	9	M	Japan †	c.494G>A; p.W165*	Nonsense	Unpublished
51	U.II.2	#	8	M	Japan †	c.494G>A; p.W165*	Nonsense	Unpublished
52	U.II.3	#	6	F	Japan †	c.494G>A; p.W165*	Nonsense	Unpublished
53	V.II.1	9	14	F	Japan †	c.436G>A; p.G146R	Missense	Unpublished
54	W.I.1	19	43	M	Japan †	c.34C>T; p.Q12*	Nonsense	Unpublished
55	W.II.1	#	16	M	Japan †	c.34C>T; p.Q12*	Nonsense	Unpublished
56	W.II.2	9	14	F	Japan †	c.34C>T; p.Q12*	Nonsense	Unpublished
57	W.II.3	4	6	F	Japan †	c.34C>T; p.Q12*	Nonsense	Unpublished
58	X.I.2	#	55	F	USA ‡	c.223C>T; p.R75W; §	Missense	Kucuk *et al.* (18)

59	X.II.1	6	15	F	USA ‡	c.223C>T; p.R75W; §	Missense	Kucuk *et al.* (18)
60	Y.I.1	uk	49	M	Germany ¶	c.226C>T; p.Q76*	Nonsense	Unpublished
61	Y.II.1	10	20	M	Germany ¶	c.226C>T; p.Q76*	Nonsense	Unpublished
62	Z.I.2	#	81	F	Norway ¶	c.94_101delinsTTCTCTTCATCA; p.P32Ffs*29	Frameshift	Unpublished
63	Z.II.1	#	50	F	Norway ¶	c.94_101delinsTTCTCTTCATCA; p.P32Ffs*29	Frameshift	Unpublished
64	Z.II.2	43	49	M	Norway ¶	c.94_101delinsTTCTCTTCATCA; p.P32Ffs*29	Frameshift	Unpublished
65	Z.II.3	#	uk	F	Norway ¶	c.94_101delinsTTCTCTTCATCA; p.P32Ffs*29	Frameshift	Unpublished
66	Z.II.6	#	uk	M	Norway ¶	c.94_101delinsTTCTCTTCATCA; p.P32Ffs*29	Frameshift	Unpublished
67	Z.III.1	16	21	F	Norway ¶	c.94_101delinsTTCTCTTCATCA; p.P32Ffs*29	Frameshift	Unpublished
68	AA.III.3	#	46	M	Japan †	c.155G>V; p.G52V	Missense	Unpublished
69	AA.IV.1	18	18	M	Japan †	c.155G>V; p.G52V	Missense	Unpublished
70	BB.I.2	uk	45	F	Japan †	c.119T>C; p.V40A	Missense	Unpublished
71	BB.II.1	#	20	F	Japan †	c.119T>C; p.V40A	Missense	Unpublished
72	BB.II.2	10	17	F	Japan †	c.119T>C; p.V40A	Missense	Unpublished
73	CC.II.1	10	43	F	Japan †	c.25_26insACAAGGCTCAGCTG; p.N14Tfs*5	Frameshift	Unpublished
74	DD.I.2	#	37	F	Japan †	c.232_232delG; p.D78Tfs*4	Frameshift	Unpublished
75	DD.II.1	13	15	M	Japan †	c.232_232delG; p.D78Tfs*4	Frameshift	Unpublished
76	EE.II.1	11	18	M	TheNetherlands¶	c.436G>T; p.G146*	Nonsense	Unpublished
77	FF.II.1	6	22 Δ	M	USA	c.208C>T; p.R70W; §	Missense	Unpublished
78	GG.I.1	#	47	M	Germany ¶	c.347T>C; p.I116T; §	Missense	Unpublished
79	GG.II.1	9	20	F	Germany ¶	c.347T>C; p.I116T; §	Missense	Unpublished
80	GG.II.2	#	18	M	Germany ¶	c.347T>C; p.I116T; §	Missense	Unpublished
81	GG.II.3	#	14	F	Germany ¶	c.347T>C; p.I116T; §	Missense	Unpublished
82	HH.II.1	2	28	F	USA ‡	c.254G>A; p.C85Y	Missense	Unpublished
83	JJ.II.1	11	28	M	Germany ¶	c.223C>T; p.R75W; §	Missense	Unpublished
84	KK.I.1	#	58	M	Czech Republic ¶	c.402_415del; p.M123Ifs*15	Frameshift	Unpublished
85	KK.II.1	21	36	F	Czech Republic ¶	c.402_415del; p.M123Ifs*15	Frameshift	Unpublished
86	LL.II.1	1	14 Δ	F	Czech Republic ¶	c.407C>T; p.P136L	Missense	Unpublished
87	MM.II.1	14	38	M	Germany¶	c.530_543del; p.F179Cfs*29	Framshift	Unpublished
88	NN.I.1	12	61	M	Uruguay¶	c.280G>T; p.E94*	Nonsense	Unpublished
89	NN.II.1	#	uk	F	Uruguay¶	c.280G>T; p.E94*	Nonsense	Unpublished
90	NN.II.6	23	29	F	Uruguay¶	c.280G>T; p.E94*	Nonsense	Unpublished
91	NN.II.8	13	20	F	Uruguay¶	c.280G>T; p.E94*	Nonsense	Unpublished
92	NN.II.9	18	23	M	Uruguay¶	c.280G>T; p.E94*	Nonsense	Unpublished
93	NN.II.10	#	17	F	Uruguay¶	c.280G>T; p.E94*	Nonsense	Unpublished
94	NN.II.11	6	21	M	Uruguay¶	c.280G>T; p.E94*	Nonsense	Unpublished
95	OO.II.1	18	24	M	Germany¶	c.224G>A; p.R75Q, §	Missense	Unpublished
96	PP.II.1	8	40	M	Canada¶	c.406C>T; p.P136S	Missense	Unpublished
97	QQ.II.1	13	31	M	Germany¶	c.410C>T; p.P137L	Missense	Unpublished
98	RR.II.1	14	16	F	USA¶	c.356T>G; p.L119R	Missense	Unpublished
99	SS.II.1	15	27	F	USA¶	c.436G>A; p.G146R	Missense	Unpublished
100	TT.I.1	5	50	M	Czech Republic¶	c.178T>A; p.Y60N	Missense	Unpublished
101	TT.II.2	21	26	M	Czech Republic¶	c.178T>A; p.Y60N	Missense	Unpublished
102	TT.II.3	11	24	F	Czech Republic¶	c.178T>A; p.Y60N	Missense	Unpublished
103	TT.II.4	4	10	M	Czech Republic¶	c.178T>A; p.Y60N	Missense	Unpublished
104	TT.II.5	1	6	M	Czech Republic¶	c.178T>A; p.Y60N	Missense	Unpublished
105	UU.II.1	#	86 Δ	F	Spain¶	Φ		Unpublished
106	UU.II.2	40	73	F	Spain¶	Φ		Unpublished
107	UU.III.2	6	65	F	Spain¶	c.223C>T; p.R75W; §	Missense	Hou *et al.* (17)
108	UU.III.3	59	60 Δ	F	Spain¶	Φ		Unpublished
109	UU.III.4	57	68	M	Spain¶	c.223C>T; p.R75W; §	Missense	Hou *et al.* (17)
110	UU.III.6	#	62	F	Spain¶	c.223C>T; p.R75W; §	Missense	Hou *et al.* (17)
111	UU.III.7	14	63	F	Spain¶	c.223C>T; p.R75W; §	Missense	Hou *et al.* (17)
112	UU.III.9	uk	55	M	Spain¶	c.223C>T; p.R75W; §	Missense	Hou *et al.* (17)
113	UU.III.10	#	53	F	Spain¶	c.223C>T; p.R75W; §	Missense	Hou *et al.* (17)
114	UU.IV.1	#	46	M	Spain¶	c.223C>T; p.R75W; §	Missense	Hou *et al.* (17)
115	UU.IV.2	31	42	M	Spain¶	c.223C>T; p.R75W; §	Missense	Hou *et al.* (17)
116	UU.IV.3	#	40	F	Spain¶	c.223C>T; p.R75W; §	Missense	Hou *et al.* (17)
117	UU.IV.4	#	33	M	Spain¶	c.223C>T; p.R75W; §	Missense	Hou *et al.* (17)
118	UU.IV.9	#	40	M	Spain¶	c.223C>T; p.R75W; §	Missense	Hou *et al.* (17)
119	UU.IV.10	uk	uk	M	Spain¶	c.223C>T; p.R75W; §	Missense	Hou *et al.* (17)
120	UU.IV.12	0.25	34	F	Spain¶	c.223C>T; p.R75W; §	Missense	Hou *et al.* (17)
121	UU.V.1	0.25	10	M	Spain¶	c.223C>T; p.R75W; §	Missense	Hou *et al.* (17)
122	UU.V.2	0.83	3	M	Spain¶	c.223C>T; p.R75W; §	Missense	Hou *et al.* (17)

123	VV.I.1	#	uk	M	Saudi Arabia¶	c.359_359delG; p.A121fs*23	Frameshift	Unpublished
124	VV.II.1	7	13	M	Saudi Arabia¶	c.359_359delG; p.A121fs*23	Frameshift	Unpublished
125	WW.II.1	8	12	M	UK¶	c.410C>G; p.P137R	Missense	Unpublished
126	XX.II.1	12	40	M	Belgium¶	c.407C>T; p.P136L	Missense	Unpublished
127	YY.II.1	3	14	F	Germany¶	c.326G>A; p.G109E; §	Missense	Unpublished
128	ZZ.I.2	uk	39	F	Germany¶	c.151C>T; p.R51*	Nonsense	Unpublished
129	ZZ.II.1	16	19	M	Germany¶	c.151C>T; p.R51*	Nonsense	Unpublished
130	AAA.II.1	23	46	M	Switzerland¶	c.257C>T; p.A86V; §	Missense	Navarini *et al.* (19)
131	BBB.II.1	1	17	F	USA¶	c.56_57insCTGG; p.T19Tfs*42	Frameshift	Unpublished
132	CCC.II.1	14	14	M	USA¶	c.406C>G;p.P136A	Missense	Unpublished
133	DDD.II.1	38	38	F	USA¶	c.173G>T; p.C58F	Missense	Unpublished
[Chr2_1	P1	5	37	F	Canada¶	2q33.2-2q33.3	Deletion	Unpuplished] Ω
[Chr2_2	P2	14	20	M	Australia¶	2q33.2-2q33.3	Deletion	Unpublished] Ω
Total no: 133	**54 different families**		**Penetrance** 90 affected mutation carriers 67.6%	**66 Female 67 Male**		**45 different mutations 28 novel mutations**		**82 unpublished mutation carriers**

= unaffected Mutation carrier; Φ = died prior to being genotyped; Δ = deceased due to disease associated manifestations or complications; age at death is shown; ¶= Caucasian; †= Asian; ‡ = African-American; § = disease causing effect of the mutation is functionally proven by transendocytosis assay (Figure S2); Ω = P1 and P2 with Chromosome 2 contiguous gene deletion involving *CTLA4* are not included within all calculations of the clinical spectrum. UK = United Kingdom, uk = unknown. F = female, M =male, USA = United States of America

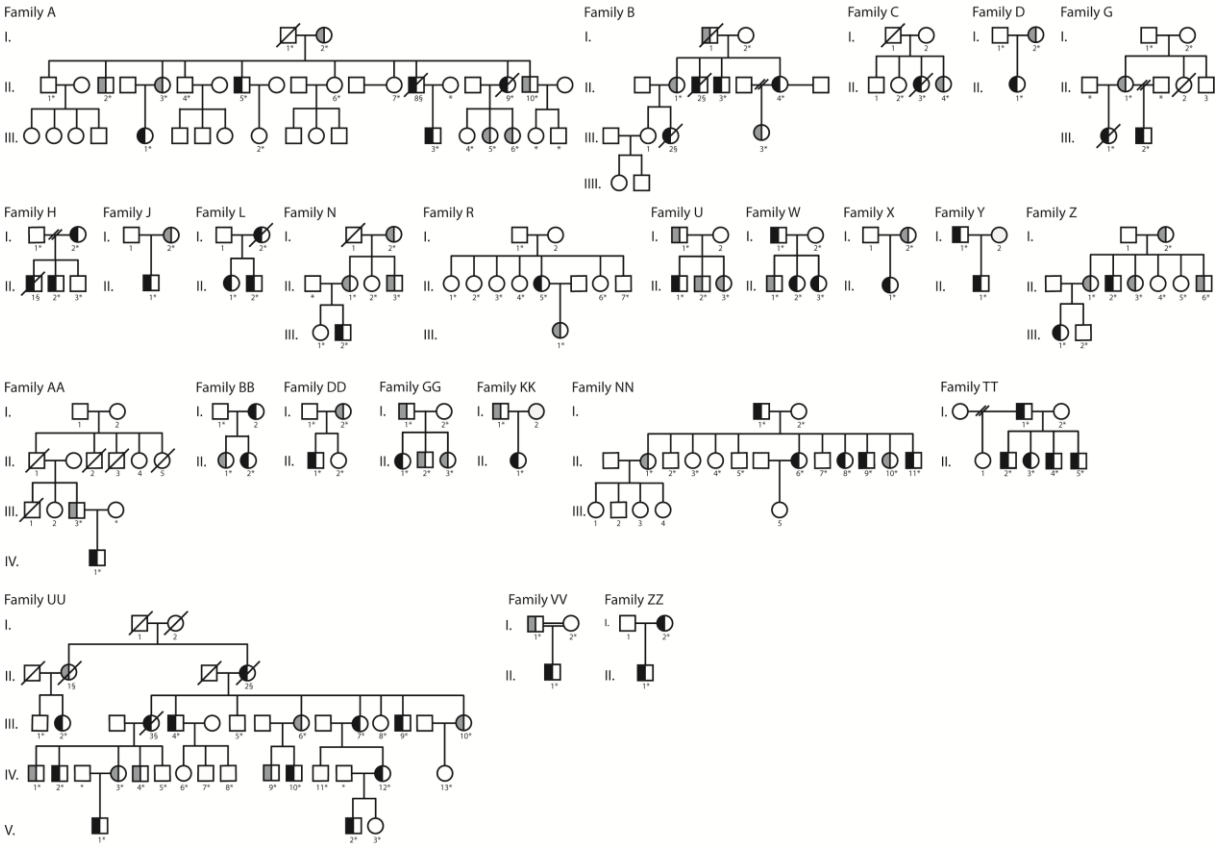
Table 2. Mutations identified in multiple families.

Exon	AA position	Mutations	Families
2	60	p.Y60C (c.179A>G); p.Y60N (c.178T>A)	Family G; Family TT
2	70	p.R70W (c.208C>T)	Family C, Family FF
2	75	p.R75W (c.223C>T); p.R75Q (c.224G>A)	Family E, Family X, Family JJ, Family UU; Family OO
2	136	p.P136L (c.407C>T); p.P136A (c.406C>G); p.P136S (c.406C>T)	Family H, Family LL, Family XX; Family CCC; Family PP
2	137	p.P137L (c.410C>T); p.P137R (c.410C>G)	Family R, Family QQ; Family S, Family WW
2	146	p.G146* (c.436G>T); p.G146R (c.436G>A); p.G146V (c.437G>T)	Family EE Family V, Family SS Family L
2	177	p.Y177* (c.529_530insA); p.Y177D (c.529T>G)	Family N; Family Q

At seven loci mutations were identified in multiple families.

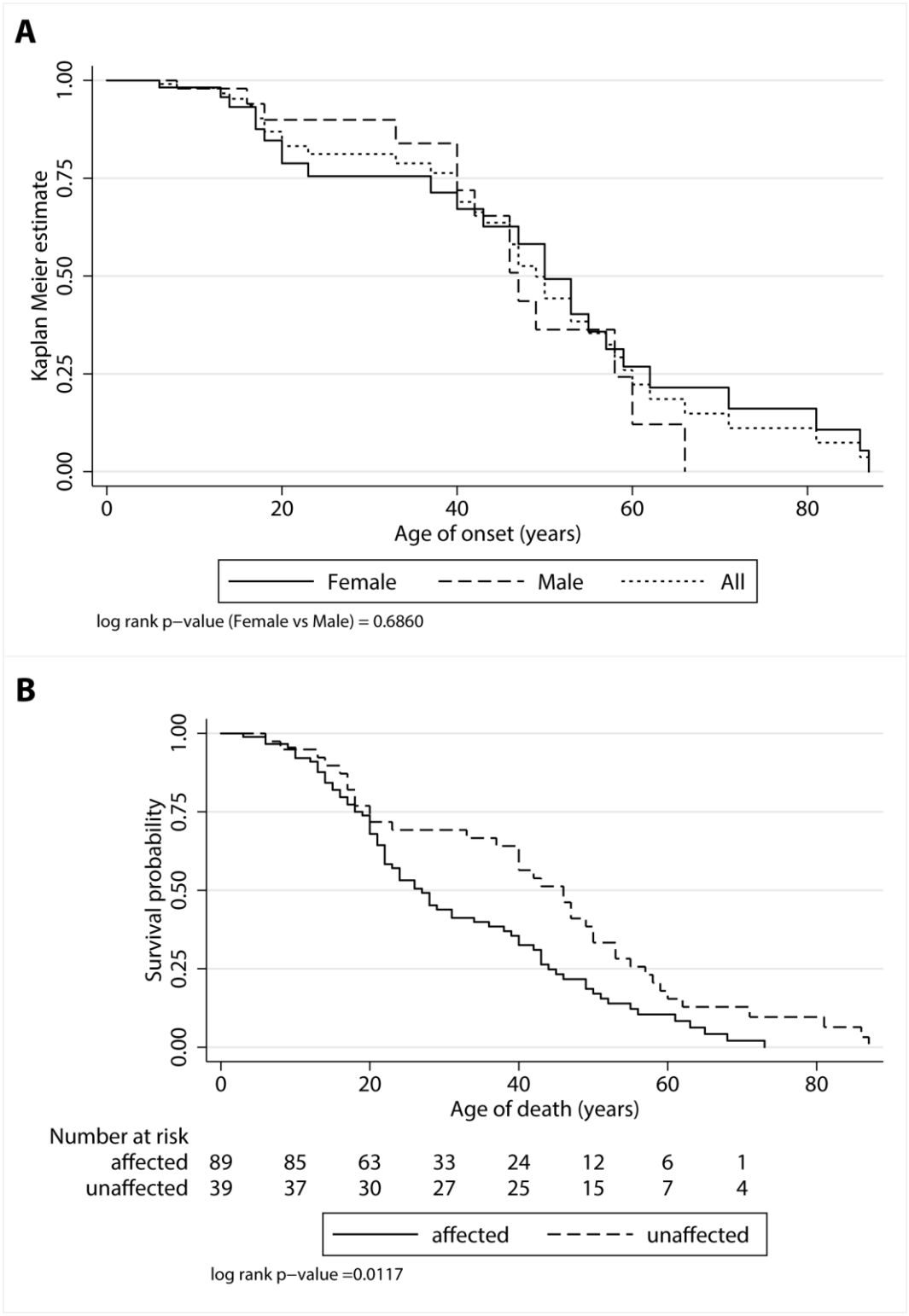
Figures

Figure 1. Pedigrees of families with CTLA-4 insufficiency



Pedigrees of all families with more than one *CTLA4* mutation carrier. Squares, male subjects; circles, female subjects; black filled symbols, mutation carriers classified as affected; gray filled symbols, mutation carriers classified as unaffected; slashed symbols, deceased subjects; *, sequencing of *CTLA4* was performed; §, genotype inferred from clinical symptoms.

Figure 2. Age of onset and age of death in CTLA-4 insufficient individuals



A. Kaplan Meier curve of age of onset of *CTLA4* mutation carriers (n=85).

B. Age of death in affected (n=86) versus unaffected mutation carriers (n=39).

Figure 3. Heterozygous germline mutations within the *CTLA4* gene are distributed throughout exon 1-3.

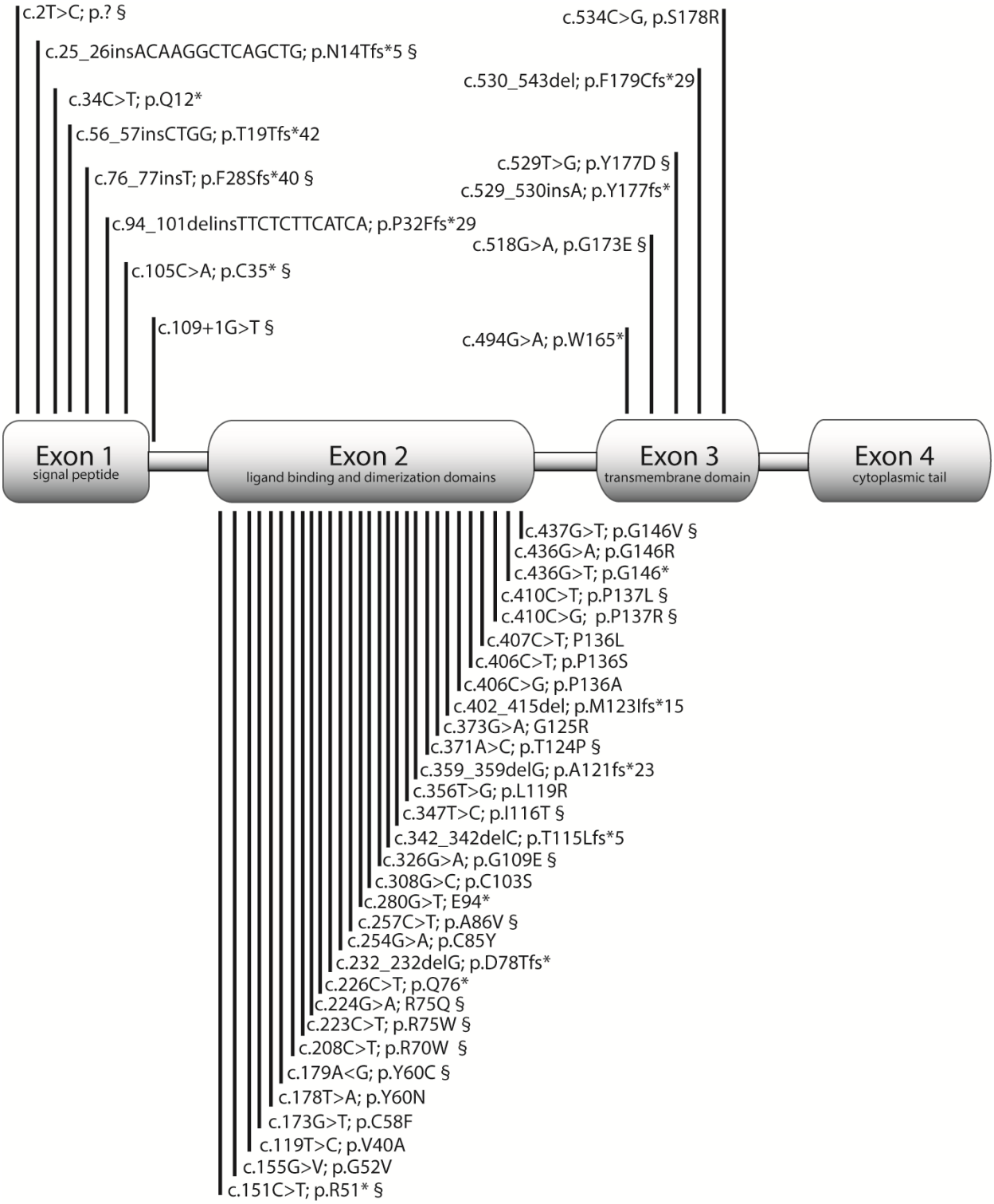
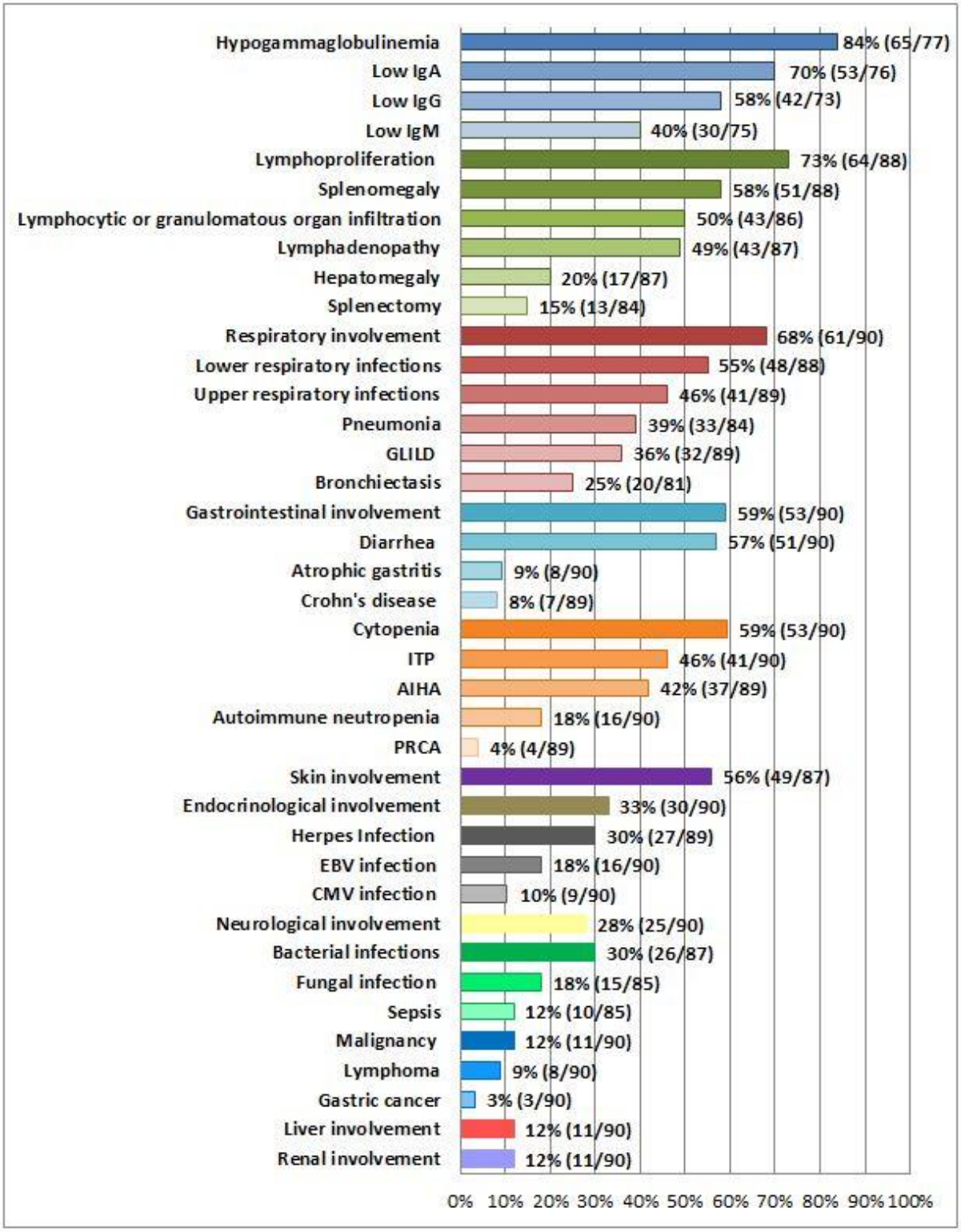


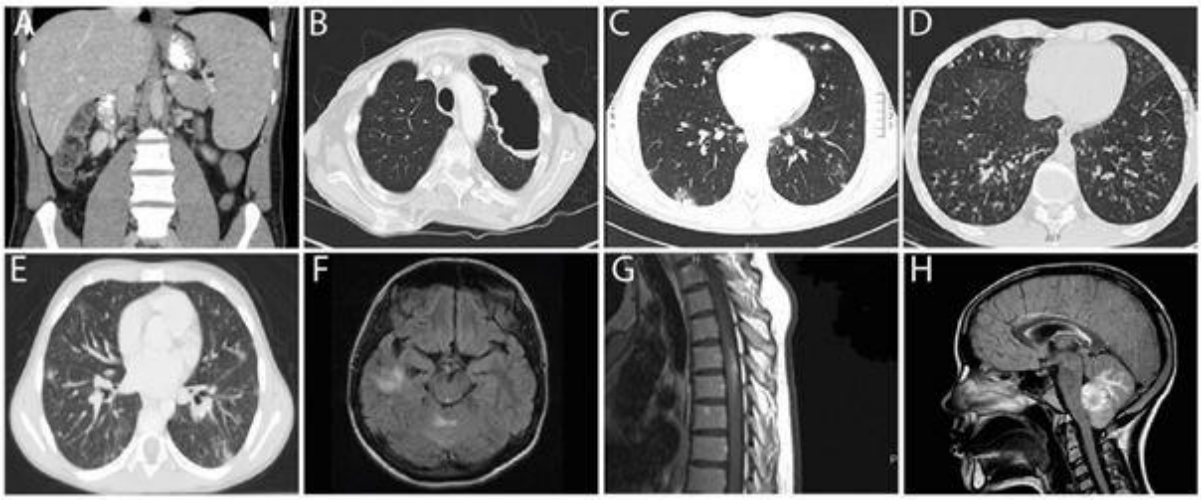
Figure 3 shows the distribution of the heterozygous germline mutations throughout the *CTLA4* gene. Eight mutations are located in exon 1, 31 are located in exon 2, and six are located in exon 3. §, mutation was functionally tested by transendocytosis assay.

Figure 4. Main clinical findings in CTLA-4 insufficiency



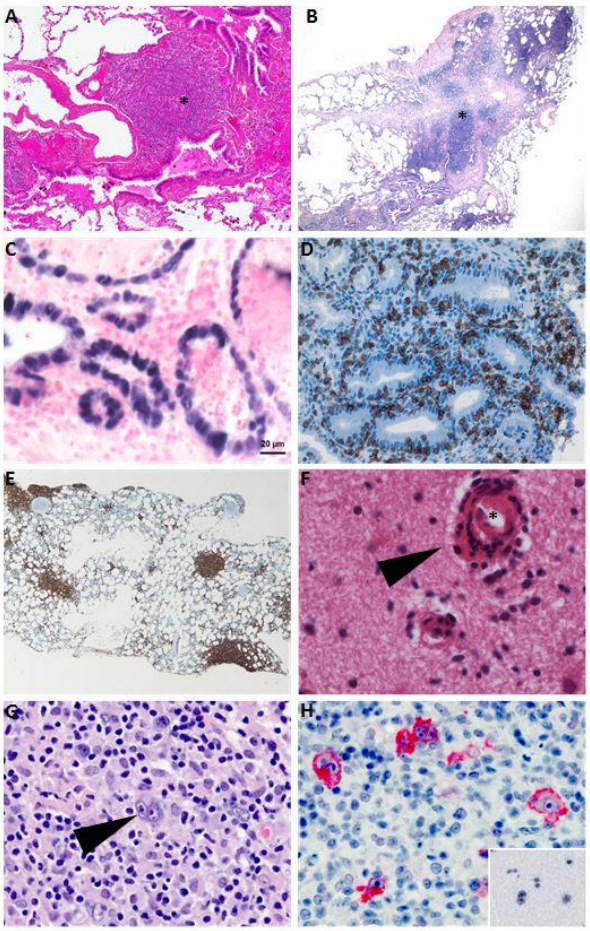
Percentage distribution of clinical manifestations within affected mutation carriers. Clinical data was available for 71 to 90 affected mutation carriers.

Figure 5. Exemplary findings upon CT and MRI in CTLA-4-insufficient individuals



Panel A: splenomegaly (17.5 cm in diameter) and lymphadenopathy in A.III.3. Panel B: large pneumatocele following necrotizing pneumonia in PP.II.1. Panel C: CT scan of ZZ.II.1 showing peripheral bronchiectasis with inflammatory nodules in all lobes of the lung. Panel D: bronchiectasis with peribronchial ground glass nodules in keeping with bronchiolitis in XX.II.1. Panel E: multiple inflammatory nodules in O.II.1. Panel F: signal change in the right temporal lobe and cerebellum consistent with inflammation in KK.II.1. Panel G: enhancement in the thoracic cord in keeping with inflammation in KK.II.1. Panel H: signal change and swelling in the cerebellum in keeping with inflammation in P.II.2.

Figure 6. Lymphocytic infiltrations and loss of EBV control define the spectrum of inflammatory and neoplastic lesions



Panel A and B: lung samples of PP.II.1 and KK.II.1 with follicular bronchitis/ bronchiolitis, respectively. Lymphoid follicles are marked by asterisks. In Panel A, the follicle contains a germinal center. Panel C: EBV-coded small RNAs (EBER) positive nuclei (dark blue staining) of an early invasive gastric adenocarcinoma of B.II.4. Panel D: autoimmune gastritis with severely atrophic mucosa of the stomach, antral metaplasia and numerous intraepithelial CD8+ T cells (brown staining) of B.II.4. Panel E: nodular T cell lymphocytosis (brown staining) in the bone marrow of Z.II.2. Panel F: perivascular lymphocytes in the brain tissue of KK.II.1 (arteriolar wall highlighted by arrowhead, lumen marked by asterisk). Panel G and H: Hodgkin lymphoma in a lymph node excision sample of MM.II.1. Reed-Sternberg cell is highlighted by an arrowhead (G) or CD30 immunohistochemistry (red staining in H). Nuclei of Hodgkin cells and Reed-Sternberg cells were positive for EBER (dark blue staining, inlet H).