

**Study of an extended family with CTLA-4 deficiency suggests a CD28/CTLA-4 independent mechanism responsible for differences in disease manifestations and severity**

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## Abstract

The CTLA-4 checkpoint regulates the activation of T cells. Individuals with heterozygous mutations in CTLA-4 have a complex phenotype typically characterized by antibody deficiency alongside variable autoimmunity. Despite severe disease in some individuals, others remain largely unaffected with reasons for this variation unknown. We studied a large family carrying a single point mutation in CTLA-4 leading to an amino acid change R75W and compared both unaffected with affected individuals. We measured a variety of features pertaining to T cell and CTLA-4 biology and observed that at the cellular level there was complete penetrance of CTLA-4 mutations. Accordingly, unaffected individuals were indistinguishable from those with disease in terms of level of CTLA-4 expression, percentage of Treg, upregulation of CTLA-4 upon stimulation and proliferation of CD4 T cells. We conclude that the wide variation in disease phenotype is influenced by immune variation outside of CTLA-4 biology.

## Key words:

CTLA-4; CD28; immunodeficiency; autoimmunity; mutation; regulatory T cells;

## 1. Introduction

The ability to regulate the activity of T lymphocytes is central to immune health and dysregulation of this process leads to a wide array of immune pathologies affecting both T and B cells. In particular, the decision to activate T cells is tightly regulated since the T cell repertoire contains significant reactivity to self-antigens[1] resulting from incomplete negative selection in the thymus[2, 3]. The peripheral tolerance mechanisms that mitigate this self-reactivity include the presence of specialized regulatory T cells[4] and the expression of proteins such as CTLA-4[5]. The fact that CTLA-4 is abundantly expressed by Treg and is required for their efficient function highlights the significant intersection of these pathways[6]. Consequently, defects in Treg development[7, 8], CTLA-4 expression[9] and pathways affecting CTLA-4 trafficking[10] all result in significant immune dysregulation and autoimmunity affecting a wide range of target organs. Recently, heterozygous mutations in CTLA-4 have been reported in individuals suffering from a form of common variable immunodeficiency (CVID)[11, 12] however, this disease is incompletely penetrant with ~70% of individuals carrying mutations being overtly affected. This raises the question of what additional factors affect disease manifestation?

CTLA-4 is a negative regulator that opposes a related receptor on T cells, CD28, which serves to reinforce or “co-stimulate” T cell activation. CD28 and CTLA-4 also share the same ligands, CD80 and CD86, expressed by antigen presenting cells and upregulation of these ligands in response to inflammatory signals during infection increases CD28 costimulation[13]. In contrast, binding of these same ligands by CTLA-4 down-regulates their expression thereby regulating T cell activation[14-16]. Ligand downregulation can be mediated by transendocytosis, whereby CTLA-4 physically removes its ligands from antigen presenting cells and degrades them in lysosomes[17]. In line with this concept, defects in the LPS-responsive and beige-like anchor (LRBA) gene, which affects CTLA-4 trafficking and degradation, result in low levels of CTLA-4 expression and defective Treg function[10, 18]. The fact that LRBA deficiency largely phenocopies CTLA-4 deficiency supports a general concept that the amount and correct trafficking of CTLA-4 is critical to maintaining its function in self- tolerance.

In addition to directly affecting Treg function, the CD28/CTLA-4 pathway also plays a significant role in maintaining Treg homeostasis. Accordingly, both CD28 and ligand-deficient mice have low numbers of Treg[19], whereas deficiency of CTLA-4 increases Treg numbers [20]. The latter effect is due to the fact that in the absence of CTLA-4, CD28 has increased access to its ligands thereby promoting Treg proliferation and survival. Somewhat surprisingly, LRBA deficient individuals (who have low CTLA-4) have

been reported to have lower numbers of Treg[21], although this was not seen in another study [22]. Nonetheless, it is clear that a delicate balance between CD28 and CTLA-4 biology plays a significant role in both Treg homeostasis and function, which is as yet poorly understood in humans. To better understand these aspects of CTLA-4 biology we took advantage of a large family carrying a heterozygous missense (R75W) mutation in CTLA-4 and looked for differences between affected and unaffected individuals carrying the same CTLA-4 mutation. The data presented here revealed no detectable differences in CTLA-4 expression, response to stimulation or Treg homeostasis, when comparing affected and unaffected individuals. Together this suggests that the difference in clinical status between affected and unaffected mutation carriers does not lie within the CD28/CTLA-4 pathway itself.

## 2. Materials and Methods

### 2.1 Clinical phenotype:

In absence of a CTLA-4 specific disease stratification system the study team decided to clinically stratify patients according to their symptoms: asymptomatic (0 symptoms), mild to moderate (1-5 symptoms) and severe (>5 symptoms) affected as detailed in **table 1**. All study participants were evaluated in a personal interview and a systematic chart review was performed in patients with clinical manifestations, adapted from [12]. In addition, the attending physician was contacted in order to complement missing details when appropriate.

2.2 Sequencing: For Whole Exome Sequencing (WES), extracted genomic DNA was randomly fragmented, amplified by ligation-mediated polymerase chain reaction (PCR) and captured and sequenced according to the protocol of the manufacturer as described previously [23]. Sequences were generated and aligned to the human genome reference (UCSC hg 19 version; build 37.1) using the SOAP aligner software (soap v.2.21). Duplicated reads were filtered out and only uniquely mapped reads were kept for subsequent analyses. The SOAPsnp software (v.1.03) was subsequently used with default parameters to assemble the consensus sequence and call genotypes in target regions [23]. Low-quality single nucleotide polymorphisms (SNP) that met one of the four following criteria were filtered out: a genotype quality of less than 20; a sequencing depth of less than 4; an estimated copy number of more than 2 and a distance from the adjacent SNPs of less than 5 bp. Small insertions/deletions (Indels) were detected using the Unified Genotype tool from GATK (v.1.0.4705) following the alignment of quality reads to the human reference genome using BWA (v.0.5.9-r16). As previously described, sequencing data analysis was performed focusing particularly on

known PID genes as well as their interacting genetic partners [23].

### 2.3 PBMC isolation

Patient samples were submitted for the purpose of diagnostic evaluation and processed under institutional approval for the investigation of immunodeficiency. Blood was diluted at 1/1 with phosphate-buffered saline, layered on Ficoll-Paque PLUS (GE Healthcare), and centrifuged at 1060g for 25 minutes. Peripheral blood mononuclear cells (PBMCs) were resuspended in phosphate-buffered saline containing 2 mM EDTA/0.5% bovine serum albumin for T-cell purification using a CD4+ T-cell enrichment kit (StemCell).

### 2.4 Flow cytometry:

For surface staining, cells were incubated with CD25 BV605 (clone 2A3; BD), CD4 Alexa Fluor 700 (clone RPA-T4; BD), CD45RA PerCP-Cy5.5 (clone HI100; eBioscience) at 4°C for 30 minutes. For analysis of total CTLA-4 and Foxp3 expression, cells were fixed and permeabilized with Foxp3 staining buffer (eBioscience) and incubated with Foxp3 allophycocyanin (clone 236A-E7; eBioscience) and CTLA-4 phycoerythrin (clone BNI3; BD). Cells were acquired on a BD LSRII cytometer and the data analyzed using FlowJo software (TreeStar). In some experiments relative expression of CTLA-4 or CD25 was calculated based on: level of expression (MFI) in memory Treg (CD45RA- Foxp3+)/ level of expression (MFI) in naïve conventional CD4 cells (CD4+CD45RA+ Foxp3-)

### 2.5 T-cell stimulation

CD4 T cells were resuspended at  $1 \times 10^6$ /mL in RPMI 1640 with 10% fetal bovine serum, 2 mM l-glutamine, 1% penicillin, and 1% streptomycin. 96,000 T cells were stimulated with 0.5µg/ml anti-CD3 plus 72,000 CHO-cells expressing the CD80 ligand as previously described[24] . Cells were cultured in a 96-well round-bottomed plate at 37°C, 95% humidity, and 5% CO<sub>2</sub>.

## 3. Results

### 3.1 Mutation identification and Clinical phenotype:

The detection of hypogammaglobulinemia in the context of recurrent respiratory and gastrointestinal infections in the son (V.1) of our index patient (IV.2) and hypogammaglobulinemia in her mother (III.2) resulted in a whole exome sequencing approach identifying a previously described mutation in CTLA4 (R75W) in all three patients. Sanger sequencing confirmed this finding and an extensive family screening

over four generations revealed a total of 15 mutation carriers (**Figure 1**). The spectrum and the severity of clinical manifestations were highly variable between the affected individuals (**Table 1**). Mild and variable skin disorders and recurrent non-infectious diarrhea/enteropathy were the most common clinical findings (60 and 53.3% respectively). Dyslipidemia requiring pharmacologic intervention was frequently reported (53.3%) and restricted to adult patients. Infectious manifestations ranged from recurrent upper and lower respiratory tract infections (20%), recurrent salmonellosis (20%), enterovirus (13.3%, poliomyelitis), persistent cutaneous papillomas (6.7%), recurrent vaginal candidiasis (6.7%) and post-infectious complications such as post-streptococcal nephritis (13.3%). Four patients (26.6%) showed neurologic symptoms consisting mainly of progressive memory loss, however one patient suffered from a severe developmental delay and seizures classified as West syndrome. Lymphoproliferation (splenomegaly and/or lymphadenopathy) was seen in 20% of patients. Autoimmune manifestations (thyroiditis, arthritis and gastritis) were reported in three patients, however none of the individuals had autoimmune cytopenias. One patient was successfully treated for breast cancer and one patient was recently diagnosed with a massive mesenteric panniculitis. Three patients reported non-infectious liver disease requiring organ transplantation in one patient who subsequently developed post-transplant lymphoproliferative disease. None of the patients suffered from recurrent or invasive CMV, EBV or VZV infection.

Three patients in our cohort had low IgG levels (mostly due to low IgG1 levels). Two of the three patients (III.2, IV2) are currently under immunoglobulin substitution. In addition in one patient (III.3) low IgM levels were detected.

### **3.2 Expression of CTLA-4 is not different between affected and unaffected individuals carrying CTLA-4 R75W.**

Given the variation in clinical picture, an outstanding question is the extent to which disease manifestation relates to the level of CTLA-4 deficit in each individual. Accordingly, it is possible that mutation carriers who do not develop symptoms are protected by higher CTLA-4 expression from the remaining wild-type allele or potentially other factors affecting CTLA-4 expression. The large family described here provided an excellent opportunity to test this hypothesis in individuals with the same mutation and we therefore stained blood samples for CTLA-4 and Foxp3 and measured CTLA-4 expression in gating on FoxP3+ Treg (**Figure 2A and B**). This revealed that CTLA-4 expression in all those carrying R75W mutation was low at ~45% of control values. Moreover, we did not detect differences in CTLA-4 expression between affected and unaffected individuals *ex-vivo*. Indeed, the absolute lowest expression level seen was in an unaffected individual. We also investigated the activation status of CD4 cells by

staining for CD25 expression (**Figure 2C and D**). Rather surprisingly CD25 expression on Treg was lower than controls, however all CTLA-4 mutation carriers had reduced levels irrespective of disease status. Moreover there was no evidence of generalised CD25 upregulation in Foxp3-ve populations. CTLA-4 expression was replicated and extended in a second series of experiments ~6 months later. As shown in **Figure 3A and B**, our initial observations were robust and no difference in the level of CTLA-4 expression was seen between affected individuals or unaffected individuals over time. Similarly, there was no correlation between CTLA-4 expression levels and disease severity (**Table1**). These experiments brought the total number of individuals studied to 10 family members including 2 unaffected carriers.

A frequent feature of CTLA-4 deficient patients is the enrichment of memory (CD45RA-ve) CD4 T cells, which accumulate possibly as a result of impaired Treg function and increased T cell stimulation. We therefore compared affected and unaffected individuals to address whether high numbers of memory T cells was also seen when stratified by age. As shown in **Figure 3C** both affected and unaffected individuals showed higher levels of memory T cells compared to age matched controls, consistent with the possibility that CTLA-4 deficiency was penetrant in this respect in both affected and unaffected individuals. Taken together we concluded that all individuals carrying the R75W mutation had impaired CTLA-4 expression ex-vivo and that the level of residual CTLA-4 expression was not different in individuals who remain unaffected. Furthermore, a tendency towards expansions of CD4 memory T cells was also noted in both affected and unaffected individuals as was lower CD25 expression on Treg.

### **3.3 Modest Treg expansions are seen in individuals with R75W mutation.**

Previously we have observed that some individuals with CTLA-4 mutations have expanded Treg numbers [12, 22]. However, the extent to which this occurs between different mutations and whether increased Treg numbers can compensate in unaffected individuals is not clear. We therefore investigated whether Treg percentage was different between affected and unaffected individuals. Staining for Foxp3 in the 10 individuals with R75W mutations *ex vivo* did not reveal a clear expansion of Treg relative to controls in the resting state *ex vivo* (**Figure 4A**). However, in addition, we also performed a brief stimulation (16h) with CD3/CD28 antibody-coated beads. This allows an additional estimate of Treg numbers to be made, as stimulation improves FoxP3 staining without inducing proliferation at this time point [22]. This provided a suggestion of increased Treg percentages in both affected and unaffected individuals (**Figure 4A and B**). However, these data did not suggest that compensatory expansions of Treg could account for differences in disease manifestation in this family.

### **3.4 CTLA-4 upregulation in response to T cell stimulation is similar between controls and R75W mutation carriers.**

Since CTLA-4 expression is strongly upregulated upon T cell stimulation, we also investigated whether CTLA-4 expression patterns were different between individuals following activation. We therefore stimulated T cells using anti-CD3/CD28 antibody coated beads. Importantly, stimulation with beads is not affected by CTLA-4 itself and therefore provides an equivalent stimulus in all mutation carriers and controls. The results of this stimulation assay (**Figure 4B**) revealed robust upregulation of CTLA-4 (compared with unstimulated cells shown in Figure 2A) in both Foxp3<sup>+</sup> and Foxp3<sup>-</sup> T cells, suggesting no defects in response to stimulation. Moreover upregulation of CTLA-4 was similar in all individuals with CTLA-4 mutations. In all cases the level of CTLA-4 expression in mutation carriers remained lower than in control individuals confirming defective expression, however, no difference in CTLA-4 induction or expression was apparent between affected and unaffected individuals (**Figure 4C**). Taken together these data indicated that differences in expression of CTLA-4 under either resting or stimulated conditions did not differentiate between affected and unaffected mutation carriers. Similarly, whilst the percentage of Foxp3<sup>+</sup> cells increased in response to stimulation, this increase again did not differentiate between affected and unaffected individuals.

### **3.5 No evidence of CD4 T cell hyper-proliferation associated with CTLA-4 deficiency.**

An issue in the CTLA-4 field is the extent to which CTLA-4 deficiency causes intrinsic hyper-proliferation of T cells. This is based on the concept that CTLA-4 expression intrinsically inhibits the T cells that express it: that is CTLA-4 generates an inhibitory signal [25, 26]. However there is now strong evidence for alternative mechanisms [27]. To provide further perspective on this issue and to investigate whether differences in the response of conventional T cells to stimulation might correlate with disease we tested responses of CD4<sup>+</sup> T cells from CTLA-4 deficient individuals to stimulation. In this assay we deliberately used the natural CTLA-4-biased ligand, CD80 in combination with a TCR stimulus (anti-CD3) to stimulate proliferation [24], since this should be sensitive to any CTLA-4 effects. As shown in **Figure 5A** CD4 T cells from blood ex vivo showed no obvious signs of hyperproliferation as measured by Ki67 analysis and in addition there was no difference between CTLA-4 mutation and healthy controls. Following stimulation (**Figure 5B**), T cell proliferation was very similar in all individuals tested. Neither comparison between healthy controls and mutation carriers, nor between affected and unaffected mutation carriers provided any evidence of hyper-proliferation in T cells CTLA-4 deficient individuals (**Figure 5C**). Thus, conventional CD4<sup>+</sup> T cells from clinically affected individuals were not overtly hyperproliferative ex vivo, nor more responsive to



stimulation using CD28/CTLA-4 ligands. Moreover, there was no correlation between response to stimulation and clinical severity.

#### **4. Discussion**

CTLA-4 is a critical regulator of immune tolerance and loss of expression in animal models is fatal shortly after birth due to extensive lymphoproliferation and autoimmunity[9]. The severity of this phenotype in animals, likely explains why only individuals with heterozygous mutations in CTLA-4 have been reported in humans. Whilst there are clear parallels between animal models of CTLA-4 deficiency and the human disorder, heterozygous individuals provide an opportunity to further understand the details of CTLA-4 biology in humans.

Similar to other CTLA-4 mutations, the clinical phenotype observed within this large family with R75W mutation was found to be highly variable and confirms the previously reported broad spectrum of clinical manifestations ranging from asymptomatic carriers to severely affected patients [11, 12]. Similarly, to the earlier reports we observed multiple organ involvement and patients showed raised infection susceptibility with mainly common pathogens affecting the respiratory tract. In addition, two patients in this family had a history of recurrent salmonellosis and poliomyelitis; not previously associated with CTLA-4 deficiency. Furthermore, we report here for the first time the occurrence of post-infectious complications (post-streptococcal nephritis) in two patients.

As previously described in patients with CTLA-4 mutations, several of our patients showed recurrent diarrhea/enteropathy. However, none of them suffered from extensive weight loss or required specific therapy other than gluten-free diet or avoiding lactose ingestion. Lymphoproliferation (> 6months of non-malignant/infectious lymphadenopathy or splenomegaly) was found in 20% in our cohort, which is in line with the previous reports [11, 12]. Neurologic symptoms in our cohort were generally unspecific and defined as progressive memory loss. However, one patient (V.3) was found to have recurrent and therapy refractory seizures (West syndrome). As brain tissue alterations have been reported in patients with CTLA-4 mutations, imaging was performed in patient V.3 as well as other symptomatic patients without evidence of granuloma formation. Autoimmunity was diagnosed in several patients but symptoms were mild and did not require long-term immunosuppression. Interestingly, whilst cytopenias are a commonly described (57% each) complication in CTLA-4 haploinsufficiency[11, 12]we did not observe this in our patients. Furthermore, we did

not identify patients with granulomatous lymphocytic interstitial lung disease as previously observed[11, 12].

Overall, comparing with the previous case series our cohort all of whom share the R75W mutation showed a milder clinical phenotype, although many of them require continuous medical care. In addition, one patient received a liver transplant for a non-infectious unspecified liver disease and our index patient is currently under evaluation for renal transplantation due to a chronic progressive renal insufficiency. To date, only patient (III.3) received immunomodulatory therapy including rituximab and sirolimus (mTor inhibitor) for EBV negative PTLD. Currently, none of the patients is receiving targeted therapy in form of abatacept, mimicking CTLA4 function.

Hypogammaglobulinemia was fairly common in the earlier cohorts (71% and 76%, respectively[11, 12]. In our family only three patients (33.3%) had low IgG levels and two of them are receiving IVIG substitution therapy. Patient V.I presented aged 16 months with recurrent infections and low IgG levels for age and received monthly intravenous immunoglobulin therapy. 12 months later IVIG substitution therapy was withdrawn and he maintained stable immunoglobulin levels over the last 24 months.

CTLA-4 deficiency is considered to be autosomal dominant disorder with incomplete penetrance[11, 12, 28, 29]. Accordingly, not all individuals carrying functionally significant mutations develop disease symptoms, raising the question of what dictates disease development. Whilst there are a number of genetic and environmental possibilities, one plausible explanation is that individuals who do not develop disease have higher residual CTLA-4 from the remaining functional allele. We have investigated this possibility here and shown that in all 10 related mutation carriers studied (8 affected and 2 unaffected) no clear difference in CTLA-4 expression was observed. Accordingly, all individuals tested who carried the CTLA-4 mutation had impaired levels of CTLA-4 expression strongly suggesting that the difference between affected and unaffected individuals does not relate to CTLA-4 expression itself. This result was the same under both resting and stimulated conditions, largely ruling out compensatory variation in CTLA-4 promoters, transcription factor binding and other factors that could affect CTLA-4 expression such as LRBA[10] in this family. Additional compensating possibilities affecting the CD28/CTLA-4 pathway could include weaker CD28 signaling in individuals who fail to develop disease. The fact that we observed similar fractions of Ki67+ T cells in response to a CD28-dependent stimulus, in both affected and unaffected individuals also argues against this possibility. Given that there does not appear to be an obvious difference in either the CD28 or CTLA-4 response between affected versus unaffected individuals, this argues in favour of alternative explanations.

Given the nature of CTLA-4 biology in controlling a system of T cells with highly variable TCRs there is ample opportunity for both genetic and environmental variation to influence disease outcome. As is evident in more complex autoimmune disorders, it may be that CTLA-4 heterozygosity itself is still insufficient to trigger disease but provides an increased (albeit extremely high) risk which needs additional genetic variation and or environmental influences to result in disease expression[30]. Thus the difference between disease development may depend on the presence of particular self-reactive T cell clones, additional genetic influences affecting the immune system[31], environmental stimuli such as infections, or host microbiota[32]. Interestingly it has been shown that the host microbiota can affect response to anti-CTLA-4 immunotherapy, which creates CTLA-4 deficiency with accompanying autoimmunity[33]. Clearly such factors are likely to vary considerably between individuals in the same family but will be challenging to elucidate.

Another interesting observation from this family is that we did not observe large Treg expansions. The expansion of Treg is observed in CTLA-4 deficient T cells or upon CTLA-4 blockade in mice and is dependent on CD28 function[20, 34, 35]. Moreover, there is a clear, but subtle, trend to increased Treg numbers in CTLA-4 heterozygote mice[12]. Accordingly, CTLA-4 deficiency should increase CD28 engagement by their shared ligands resulting in increased Treg numbers. The data here demonstrate that the picture in humans is also variable with some individuals carrying CTLA-4 mutations having significant Treg expansions and others less so. One possibility is that this depends on the nature of the mutation. For example, since CTLA-4 is dimeric there is the possibility for dominant interfering mutations in some cases. The R75W mutation is located a considerable distance from the ligand binding site[12] and may plausibly have a weaker effect than other CTLA-4 mutations. The extent of overall impaired CTLA-4 function may then be reflected in the level of Treg expansion.

Whilst we had insufficient material to carry out Treg suppression assays directly, we have previously shown that Treg from unaffected individuals carrying CTLA-4 mutations have compromised suppressive activity[12]. Moreover, the level of CTLA-4 expression directly relates to its ability to capture ligands and suppress T cell co-stimulation[22, 24]. It is therefore highly likely that Treg from unaffected mutation carriers will have defects in CTLA-4-dependent Treg function. It remains to be determined whether any Treg activities independent of CTLA-4 differ between affected and unaffected individuals.

We also addressed the question of whether proliferation of CD4 T cells was different between affected and unaffected individuals. In the experiments performed here we did

not observe any difference in proliferation. Importantly the experiments were performed using a natural ligand CD80, which engages both CD28 and CTLA-4. As we expected, no clear differences were observed between control T cell proliferation and those of CTLA-4 mutation carriers in response to stimulation. These results are consistent with the observation that CTLA-4 deficient cells are not themselves intrinsically defective and do not display a hyperproliferative phenotype[36, 37]. Perhaps surprisingly, we also found no evidence that conventional CD4 T cells in blood from patients were hyperproliferative *in vivo* with Ki67 staining *ex vivo* being in line with healthy controls. Similarly we did not observe high levels of CD25 expression on conventional cells as a marker of activation. Taken together this suggests that despite conferring susceptibility to autoimmune responses, a ~50% loss of CTLA-4 function does not appear to cause widespread activation of conventional CD4+ T cells. Considerable evidence now suggests that lymphoproliferation due to CTLA-4 deficiency *in vivo in mice* results from a lack of regulatory T cell function and not due to an intrinsic hyperproliferative capacity of CD4 T cells[34, 35, 38]. Thus whilst CTLA-4 can clearly influence conventional T cell function [39, 40] it does not appear to have major cell-intrinsic impacts on T cell proliferation to ligand stimulation[12, 24]. Thus in terms of detecting CTLA-4 defects in humans it is not clear that CD4 T cell proliferation assays are likely to be informative when assessing CTLA-4 deficiency. In contrast, assays that detect CTLA-4 expression, ligand binding and uptake may be a more reliable way to detect CTLA-4 defects [22].

## 5. Conclusion

In summary, we have investigated in detail some of the key immunological features of CTLA-4 deficiency as they relate to Treg and conventional CD4 T cell biology in the largest family reported so far with a CTLA-4 heterozygous mutation.

We were unable to detect differences in CTLA-4 expression, Treg expansion or T cell proliferation as a basis for discriminating clinical severity. Our observations provide new insights into the differences between affected and unaffected individuals and in this family largely rule out other compensatory changes in the CD28/CTLA-4 pathway as being an explanation for the prevention of disease development. Thus whilst the disease phenotype is incompletely penetrant, biological changes due to the CTLA-4 mutation itself appear completely penetrant at the cellular level, suggesting that variation in the downstream sequelae of T cell activation are likely to explain the disease outcome.

## Figure Legends:

**Figure 1. Pedigree of family with CTLA-4 R75W mutations.** Females are shown as circles and males as squares, with symptoms color coded as shown.

**Figure 2. CTLA-4 and CD25 expression levels are not different between asymptomatic and symptomatic mutation carriers. A).** Representative flow cytometry plots showing CTLA-4 expression in control and mutation carriers. Samples are shown from individuals with differing degrees of severity. Percentages are shown in quadrants and MFI of CTLA-4 in Foxp3 expressing cells is shown in large font. **B).** CTLA-4 expression in CD45RA<sup>-</sup>Foxp3<sup>+</sup> Treg is shown relative to naïve Tcon (CD4<sup>+</sup> CD45RA<sup>+</sup> Foxp3<sup>-</sup>). Asymptomatic carriers are shown as triangles. **C and D).** As above except for CD25 expression.

**Figure 3 The CTLA-4 deficient phenotype is stable over time. A).** Repeat samples from selected individuals taken approximately 6 months apart are shown (batch 1 vs batch 2). Individual samples are from individuals identified in Table I. **B).** Comparison of CTLA-4 expression in Foxp3<sup>+</sup> Treg between batches. **C).** Percentage memory CD4 (CD45RA<sup>-</sup>) cells in age adjusted patients, carriers and controls.

**Figure 4 Treg response to stimulation does not distinguish affected from unaffected mutation carriers. A).** Percentage Foxp3<sup>+</sup> cells as a fraction of CD4 cells in resting and stimulated conditions. Cells were stimulated with CD3/CD28 beads for 16h. **B).** Representative flow cytometry plots showing CTLA-4 expression in control and mutation carriers. CD4<sup>+</sup> T cells were stimulated for 16h with CD3/CD28 antibody coated beads and CTLA-4 expression measured. Samples are shown from individuals with differing degrees of severity as identified in Table I. Percentages are shown in quadrants and MFI of CTLA-4 in Foxp3 expressing cells is shown in large font. **C).** Relative CTLA-4 expression in Foxp3<sup>+</sup> Treg is shown following stimulation for all individuals tested plus healthy controls. Asymptomatic carriers are shown as triangles.

**Figure 5. Proliferative responses of CD4 T cells are similar between affected and unaffected individuals.** 90,000 CD4 T cells were stimulated with 0.5 µg/ml anti-CD3 and 72,000 CD80 expressing transfectants. At day 5 cells were gated on CD4<sup>+</sup> cells and proliferation determined by staining with Ki67. **A).** Representative flow cytometry plots showing Ki67 expression in control and mutation carriers with differing degrees of severity ex vivo before stimulation. Percentages are shown in quadrants. **B).**

Representative flow cytometry plots showing Ki67 expression in control and mutation carriers at day 5 after stimulation. Percentages are shown in quadrants. **C).** Ki67 expression in conventional CD4 cells shown for all individuals tested plus healthy controls. Asymptomatic carriers are shown as triangles.

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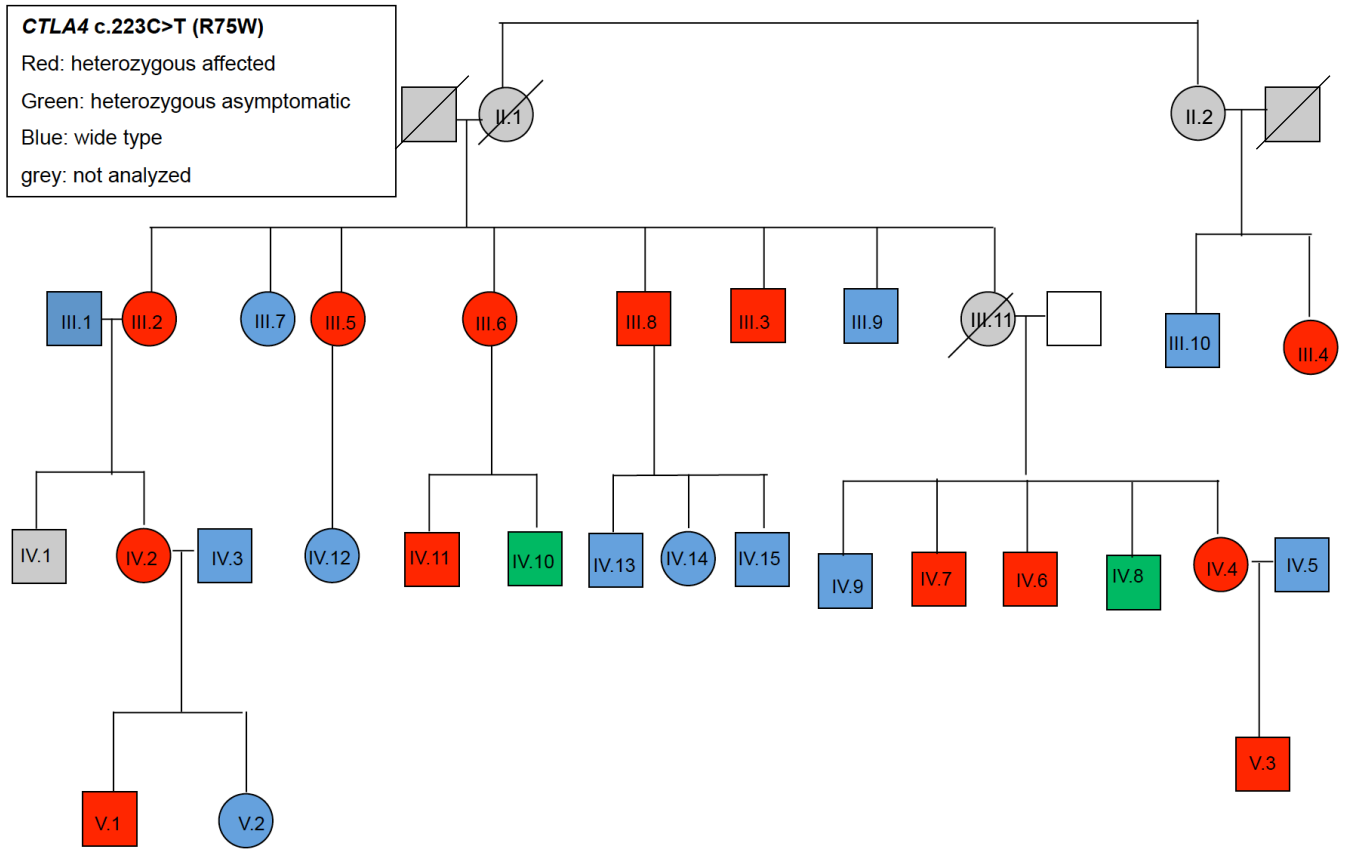
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# Fig 1



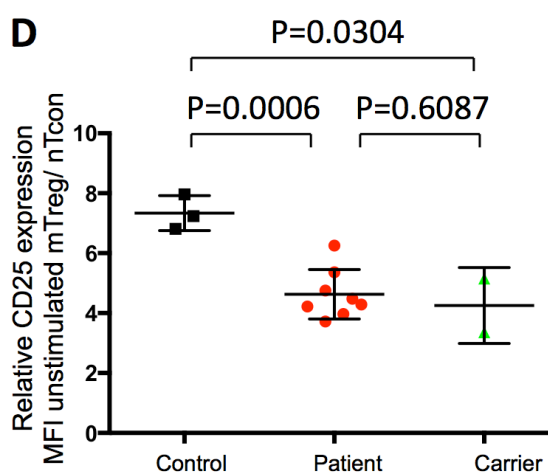
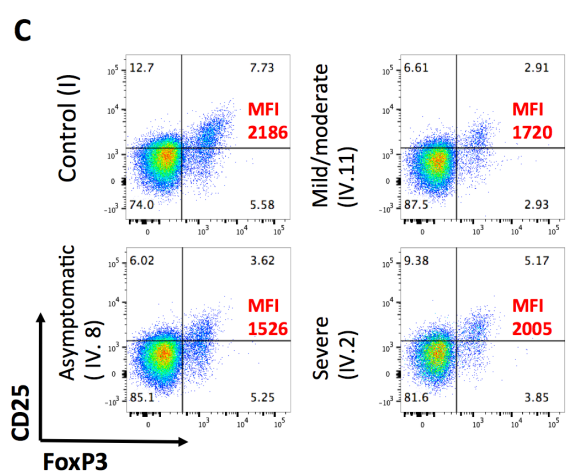
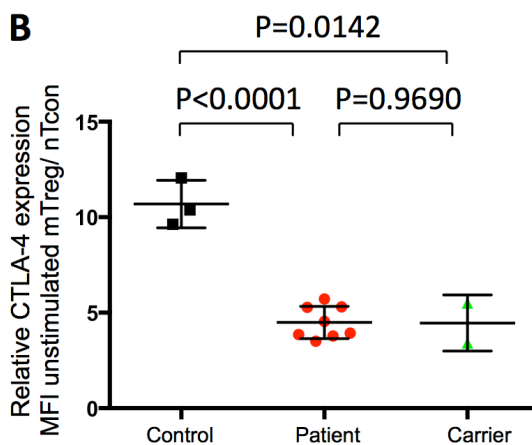
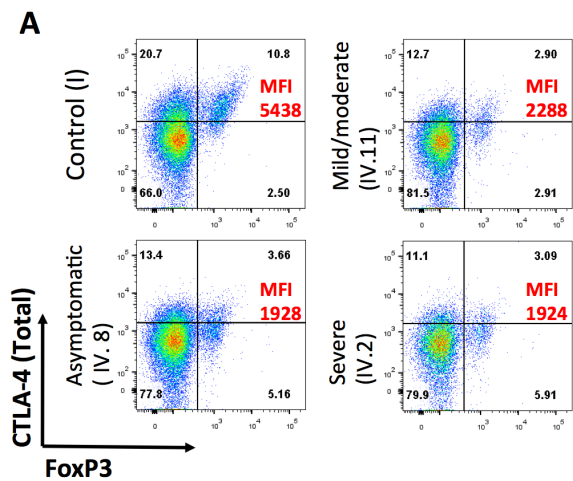


Fig 2

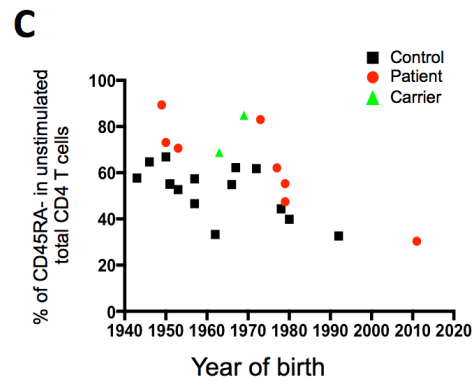
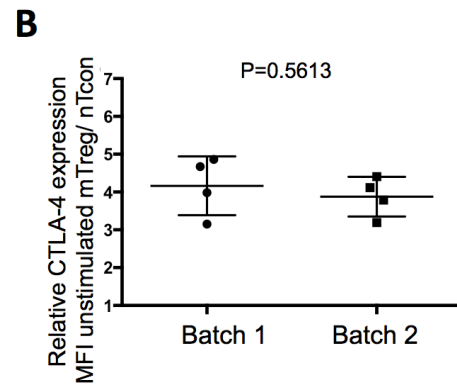
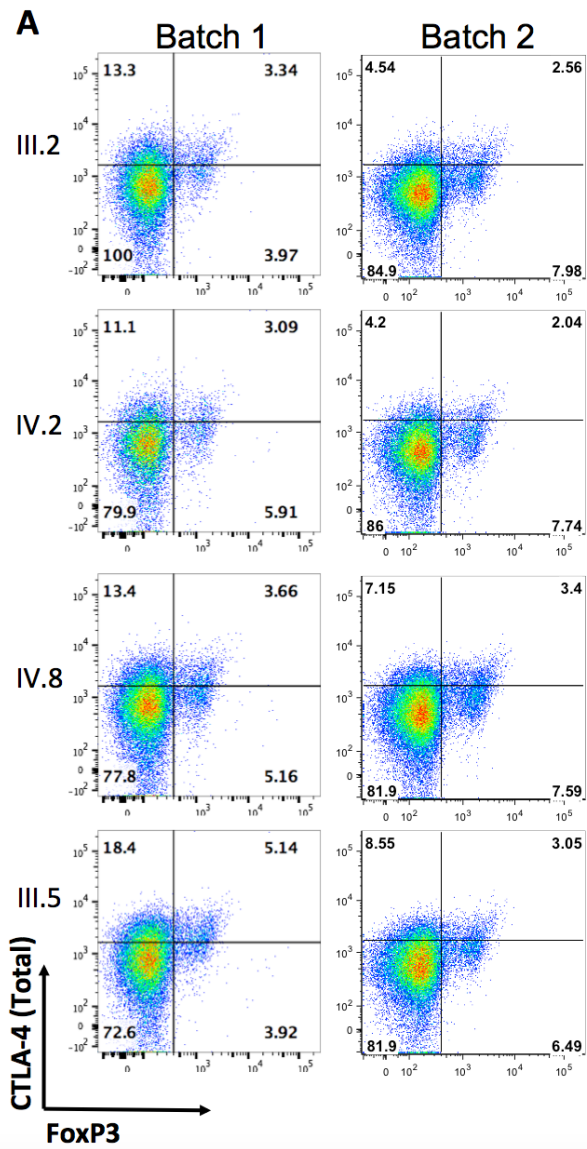
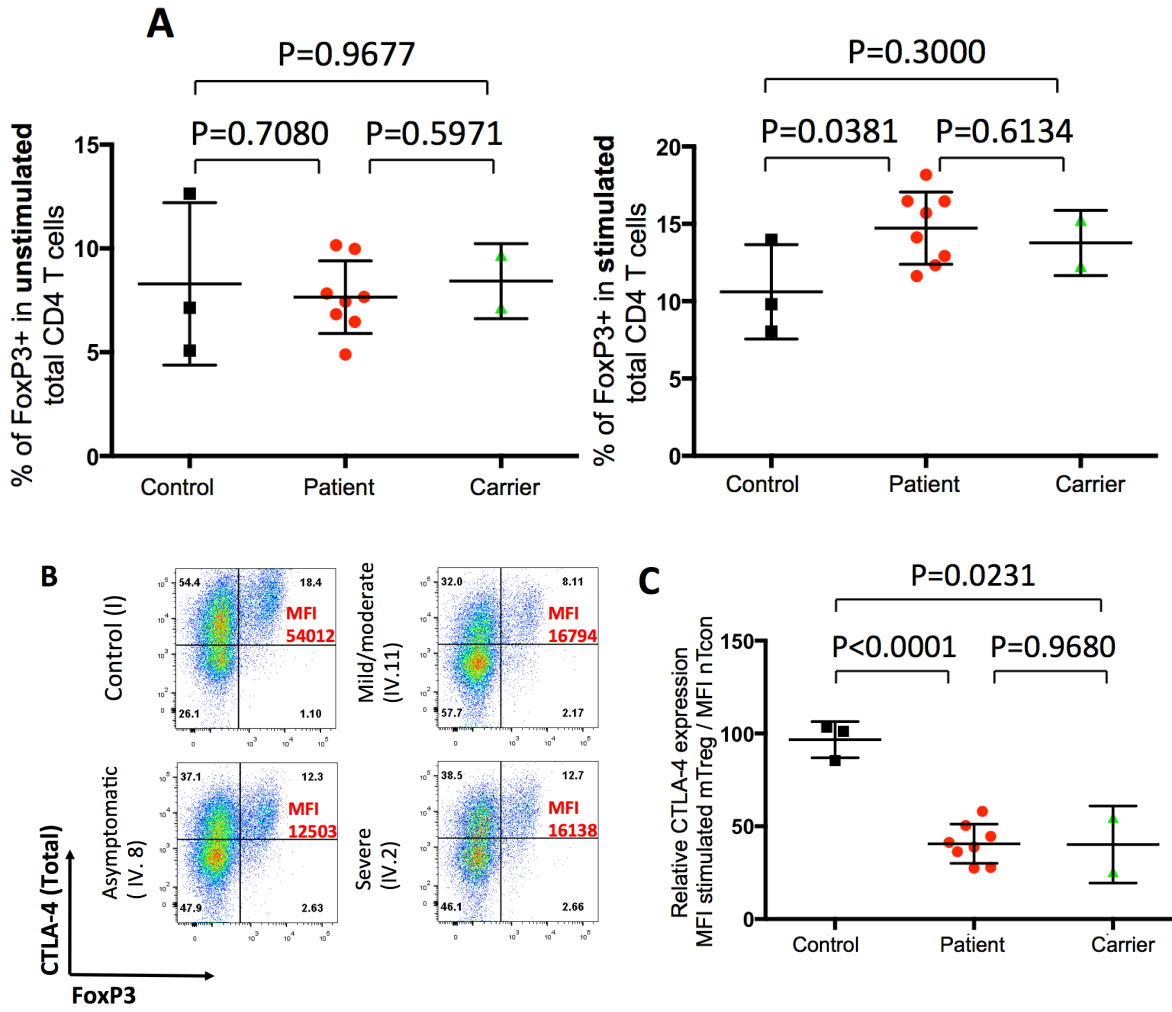
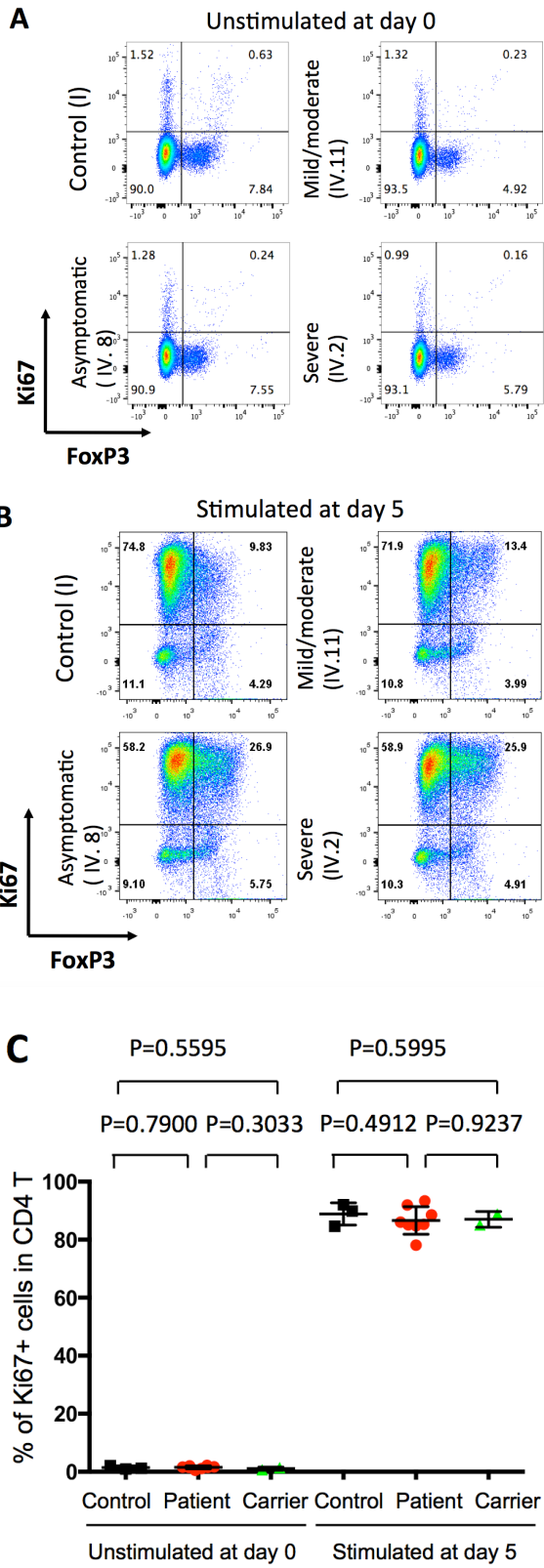


Fig. 3

Fig 4



# Fig 5





**Table 1: Clinical manifestations of CTLA4 c.223C>T mutation carriers**

	III.2	III.3	III.4	III.5	III.6	III.8	IV.2	IV.4	IV.6	IV.7	IV.8	IV.10	IV.11	V.1	V.3	
Clinical phenotype score: Aysymptomatic (A): 0 symptoms Mild-moderate (MM): 1-5 symptoms Severe (S): >5 symptoms	S	S	S	S	MM	S	S	MM	MM	MM	A	A	MM	MM	MM	Clinical manifestations (%)
Age at investigations (yrs), gender (F/M)	63, F	55, M	65, F	53, F	64, F	68, M	34, F	40, F	33, M	42, M	46, M	38, M	40, M	3, M	10, M	
Psoriasis / other skin disease *	Present	Not reported	Present	Present	Present	Present	Present	Not reported	Not reported	Present	Not reported	Not reported	Not reported	Present	Present	60,0
Diarrhea / enteropathy	Present	Present	Present	Present	Not reported	Present	Present	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Present	Present	53,3
Dyslipidemia	Present	Present	Present	Present	Present	Present	Present	Not reported	Not reported	Present	Not reported	Not reported	Not reported	Not reported	Not reported	53,3
Recurrent arthralgia	Not reported	Not reported	Present	Present	Not reported	Not reported	Not reported	Present	Not reported	Present	Not reported	Not reported	Not reported	Not reported	Not reported	26,7
Global neurodevelopmental delay/Progressive memory loss	Present	Not reported	Present	Present	Not reported	Present	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Present	26,7
Recurrent respiratory tract infections	Present	Not reported	Present	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Present	Not reported	20,0
Recurrent salmonella infection	Not reported	Not reported	Not reported	Not reported	Not reported	Present	Present	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Present	Not reported	20,0
Splenomegaly	Present	Present	Present	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	20,0
Liver disease	Not reported	Present	Present	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Present	Not reported	Not reported	Not reported	Not reported	Not reported	20,0
Autoimmune / atrophic gastritis	Present	Not reported	Not reported	Not reported	Not reported	Present	Not reported	Not reported	Present	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	20,0
Post-streptococcal nephritis	Present	Not reported	Not reported	Not reported	Not reported	Not reported	Present	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	13,3
Lymphadenopathy	Present	Not reported	Present	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	13,3
Malignancy **	Not reported	Present	Present	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	13,3
Gastrointestinal dysplasia (adenoma)	Present	Not reported	Not reported	Not reported	Present	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	13,3
Poliomyelitis infection	Not reported	Present	Not reported	Present	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	13,3
Recurrent cutaneous papilloma	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Present	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	6,7
AI thyroiditis	Not reported	Not reported	Present	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	6,7
Seizures	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Present	6,7
Growth retardation	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Present	Not reported	6,7
AI arthritis	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Present	Not reported	Not reported	Not reported	Not reported	Not reported	6,7
Recurrent vaginal candidiasis	Present	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	6,7
GLILD	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	0,0
AI hemolytic anemia	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	0,0
AI thrombocytopenia	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	0,0

Present or documented
Not reported
Not applicable

\* Granuloma annulare, chronic refractory urtikaria, atopic dermatitis; \*\* III.3 PTLD/B cell lymphoma, III.4 mamma carcinoma; GLILD: granulomatous-lymphocytic interstitial lung disease. III.8: Mesenteric panniculitis, Dupuytren's contracture, progressive hydrocephalus



