**Identifying functional defects in patients with immune dysregulation due to LRBA and CTLA-4 mutations.**

**Running head**: Characterisation of CTLA-4 and LRBA deficiency

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**Key points**

1. New approaches to identifying functionally relevant mutations in CTLA-4 deficiency syndromes.

2. Measuring responses to stimulation and degradation distinguishes between CTLA-4 and LRBA mutations

**Abstract**

Heterozygous CTLA-4 deficiency has been reported as a monogenic cause of common variable immune deficiency (CVID) with features of immune dysregulation. Direct mutation in CTLA-4 leads to defective regulatory T cell function associated with impaired ability to control levels of the CTLA-4 ligands, CD80 and CD86. However, additional mutations affecting the CTLA-4 pathway, such as those recently reported for LRBA, indirectly affect CTLA-4 expression resulting in clinically similar disorders. Robust phenotyping approaches sensitive to defects in the CTLA-4 pathway are therefore required to inform understanding of such immune dysregulation syndromes. Here we describe assays capable of distinguishing a variety of defects in the CTLA-4 pathway. Assessing total CTLA-4 expression levels was found to be optimal when restricting analysis to the CD45RA-negative Foxp3+ fraction. CTLA-4 induction following stimulation, and the use of lysosomal blocking compounds, distinguished CTLA-4 from LRBA mutations. Short term T cell stimulation improved the capacity for discriminating the Foxp3+ Treg compartment, clearly revealing Treg expansions in these disorders. Finally, we developed a functionally orientated assay to measure ligand uptake by CTLA-4, which is sensitive to ligand-binding or trafficking mutations, that would otherwise be difficult to detect and that is appropriate for testing novel mutations in CTLA-4 pathway genes. These approaches are likely to be of value in interpreting the functional significance of mutations in the CTLA-4 pathway identified by gene sequencing approaches.

**Introduction**

Common variable immune deficiency (CVID) is a heterogeneous group of primary immune deficiencies, containing of a number of different genetic aetiologies. Whilst diagnosis is characterised by low levels of immunoglobulins, a significant fraction of patients suffer from complications some of which are autoimmune in nature including enteropathy and cytopaenias[1,2]. The use of exome and genome sequencing has identified an increasing number of genes that are associated with CVID[3,4] however this raises the issue of determining whether individual mutations in such genes are functionally significant. Accordingly, functional dissection is required in order to validate the impact of gene mutations. Recently, heterozygous mutations in the CTLA-4 gene have been reported in humans with features of CVID with autoimmune complications[5,6]. In addition, biallelic mutations in a second gene LRBA, also affects the CTLA-4 pathway[7,8] resulting in a similar disease phenotype, which in contrast to CTLA-4 mutation, has nearly complete penetrance[9,10]. In both conditions insufficient functionally active CTLA-4 is produced to permit the proper functioning of regulatory T cells, giving rise to IPEX-like disorders. It is also likely that additional mutations affecting the function of the CTLA-4 pathway will be identified in the future, which will require robust functional assays. Treg testing *in vitro* is notoriously difficult and *in vitro* assays are frequently performed in ways that are uninformative for investigating CTLA-4 function[11].

Despite an understanding of the general principles of CD28 and CTLA-4 in T cell biology[12], the precise physiological mechanisms behind CTLA-4 function are still

debated[13-15], hampering the design of functional tests. Much of the biology of CTLA-4 concerns Foxp3+ regulatory T cells[16], although it is also induced upon activation of conventional Foxp3$^{-ve}$ T cells. Accordingly, mice completely deficient in CTLA-4 and those conditionally deficient only in Treg, develop wide-ranging and typically fatal autoimmunity[17-19] but with some variation[20,21]. We recently identified a mechanism of action whereby CTLA-4 acts to capture and remove its ligands from antigen presenting cells by a process known as transendocytosis[22]. Since T cell costimulation via CD28 is triggered by these same ligands (CD80 and CD86) CTLA-4 therefore acts to regulate CD28 stimulation. Accordingly uptake of ligands by CTLA-4 represents a measure of its functional capacity. Indeed the principle of controlling availability of CD28 ligands has been used to generate soluble forms of CTLA-4 (Abatacept and its high affinity derivative Belatacept) for use as immune suppressive agents[23], which are increasingly being evaluated in immune deficiencies with immune-dysregulation[7].

In addition to ligand binding, the cell biology of the CTLA-4 is unusual and requires consideration. Whilst approximately 10% of CTLA-4 protein is typically found at the plasma membrane, the majority of CTLA-4 is actually located intra-cellularly as a result of rapid internalization by clathrin-mediated endocytosis[24]. Subsequently, trafficking of CTLA-4-containing vesicles through the cell involves both re-cycling to the plasma membrane and degradation in lysosomes[25]. Accordingly, disturbances in trafficking can result in defective CTLA-4 expression. This issue has been recently highlighted by the discovery that LRBA affects CTLA-4 trafficking and lysosomal degradation. Consequently, individuals with defective LRBA have low levels of CTLA-4, but in the absence of CTLA-4 mutations[7].

Assessing CTLA-4 and LRBA mutations and the pathway in general therefore requires a number of approaches at the intersection of CTLA-4 and Treg biology to determine functional significance. Such methodologies should be capable of reliably detecting heterozygous (i.e. incomplete) loss of CTLA-4 expression in the presence of the remaining unaffected allele. Moreover, assays are needed that detect the impacts of different mutations as well as distinguishing between direct causes (eg. CTLA-4 mutation) and indirect causes (eg. LRBA mutation). Here we describe a number of approaches which when used together provide detailed assessment of the likely functional significance of mutations in this pathway as well as highlighting the differences between LRBA and CTLA-4 deficiency and their impact on CTLA-4 expression.

## Methods

PBMC isolation
Blood was diluted at 1:1 with PBS, layered on Ficoll-Paque PLUS (GE Healthcare) and centrifuged at 1060g for 25 minutes. PBMC were resuspended in PBS containing 2mM EDTA/0.5% BSA for T cell purification using a CD4$^+$ T cell Enrichment Kit (StemCell).

T cell stimulation
CD4 T cells were re-suspended at 1x10$^6$/ml in RPM1 with 10% FBS, 2mM L-glutamine, 1% penicillin and 1% streptomycin. Cells were stimulated with anti-CD3/CD28 T cell expander dynabeads (Invitrogen) at a ratio of 1 bead: 2 T cells for 16 hours. To inhibit lysosomal degradation, bafilomycin A (Sigma) was added at 50nM. Cells were cultured in 96 well round-bottomed plate at 37°C, 95% humidity and 5% $CO_2$.

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 permeabilised with Foxp3 staining buffer (eBioscience) and incubated with Foxp3 APC (clone 236A-E7) (eBioscience) and CTLA-4 PE (clone BNI3) (BD). Cells were acquired on a BD LSRII cytometer and the data analysed using FlowJo software (Tree Star).

Ligand uptake assay

CD4 T cells were incubated with recombinant human CD80-Ig (R&D) at 2μg/ml in the presence of CD3/CD28 bead stimulation for 16 hours. To block ligand uptake, abatacept (Bristol-Myers-Squibb) was added at 10μg/ml. Cells were then labeled for CD4, CD25 and CD45RA as above. For intracellular staining cells were fixed and permeabilised with Foxp3 staining buffer (eBioscience) and stained for Foxp3, total CTLA-4 and CD80-Ig uptake. For Foxp3, anti-Foxp3 eFluor 450 (clone 236A-E7) (eBioscience) was used. Total CTLA-4 was stained using a CTLA-4 C-terminal antibody (C-19) (SantaCruz) and detected with anti-goat IgG Alexa Fluor 647. CD80Ig was detected with rabbit anti-human IgG PE (SouthernBiotech). The efficiency of ligand uptake (ligand uptake/ CTLA-4) was calculated by extracting CD80-Ig and CTLA-4 MFI values and determining the slope of the line of best-fit using linear regression.

## Results

### CTLA-4 deficiency is most robustly detected in memory Treg cells

CTLA-4 is expressed in both activated conventional T cells and Foxp3+ Treg. We therefore performed flow cytometric staining using a multiplex panel to examine CTLA-4 and Foxp3 in both naïve and memory T cells. Total CTLA-4 stains where cells were fixed and permeabilised were used to determine overall deficits in expression. However, it should be appreciated that CTLA-4 trafficking is dynamic and can give rise to specific defects that are not detected in total stains. As shown in **Figure 1A**, analysis of peripheral blood CD4+ T cells revealed that Foxp3+ Treg cells expressed higher CTLA-4 compared to Foxp3-ve cells as expected. On average the MFI of Treg was ~5 fold brighter than Foxp3-ve T cells however this value was influenced by the numbers of naïve and memory T cells in the Foxp3-ve populations as well as their activation state. To account for variability in naïve and memory T cell fractions we analysed naïve and memory subsets in both Foxp3+ and Foxp3-ve compartments independently. This revealed a number of features. Firstly, as expected, the fraction of naïve or memory T cells varied considerably between individuals and we observed higher numbers of conventional CD4+ memory T cells in CTLA-4 deficiency **(Figure 1B).** Secondly, when gating on the naïve compartment it was more difficult to detect CTLA-4 deficiency even amongst Foxp3+ Treg cells as CTLA-4 had lower expression **(Figure 1C-upper panels).** In contrast, differences in CTLA-4 expression between individuals with CTLA-4 mutations and control individuals were readily detected in the memory ($CD45RA^{-ve}$ Foxp3+) Treg population **(Figure 1C-lower panels).** Therefore, analysing memory Treg was useful since it prevented incorrectly identifying low CTLA-4 expression simply due to high numbers of naïve Treg and instead focused analysis on cells expressing the highest levels of CTLA-4 thereby making detection of CTLA-4 deficiency more robust **(Figure 1C-lower panels).**

Since unstimulated conventional (CD45RA$^{+}$Foxp3$^{-ve}$) naïve CD4+ T cells (nTcon) do not express CTLA-4, we used this population as an internal control with which to compare CTLA-4 expression between individuals. Using this approach, memory Treg (mTreg) from healthy controls expressed on average 10-fold higher CTLA-4 (MFI) than naïve CD4 T cells **(Figure 1D)**. In contrast, patients with CTLA-4 deficiency generally had less than 5-fold increase **(Figure 1D).** Thus, the fold change in CTLA-4 MFI between naïve CD4 T cells and memory Treg is a robust indicator of CTLA-4 deficiency, which can be used to compare between individuals. Finally, since CTLA-4 affects Treg homeostasis we also examined the percentage Treg as a fraction of CD4+ cells in individuals with CTLA-4 deficiency **(Figure 1E).** This revealed some heterogeneity with marked expansions in some individuals but not others. Thus whilst expansions of Treg are a feature of CTLA-4 deficiency they are not observed in all individuals, suggesting they may be mutation specific.

**Defective CTLA-4 expression remains after T cell stimulation**

Given that CTLA-4 expression is induced upon activation of conventional T cells, we measured its induction in individuals with CTLA-4 mutations following stimulation. CTLA-4 expression was substantially increased upon stimulation in both Tcon as well as in Treg **(Figure 2A)** with approximately 10-fold increase in MFI over the unstimulated levels, in both Treg and non-Treg populations. This upregulation occurred in both healthy controls and in individuals carrying CTLA-4 mutations suggesting that mutation did not alter the response to stimulation. However, despite the ability to upregulate CTLA-4, the fold-change in CTLA-4 mutation carriers (relative to naïve T cells) remained approximately half that of healthy individuals (**Figure 2B**). Stimulation therefore provides important additional verification that reduced CTLA-4 expression due to genetic deficiency cannot be corrected by T cell activation. During the stimulation process we also noted that stimulation revealed increased percentages of Foxp3+ T cells which was particularly evident in individuals with CTLA-4 mutation. This suggested that brief T cell activation enhanced detection of Tregs that were otherwise missed, possibly due to low levels of Foxp3 expression in the ex vivo state (**Figure 2C**).

**T cell stimulation upregulates both CTLA-4 and Foxp3 expression**

Following stimulation, the increase in the percentage of Foxp3+ cells was accompanied by upregulation of CD25 and CTLA-4 but occurred in the absence of increased proliferation as measured by Ki67 upregulation (**Figure 3A**). This data along with the short time period of stimulation indicated the increase was not due to an outgrowth of induced Treg cells. The fold-increase in percentage Foxp3 expressing T cells was consistent between individuals and seen in both control and CTLA-4 mutation carriers (**Figure 3B**). Thus, we concluded that brief stimulation helped to enhance both Foxp3+ and CD25 staining and provided a more distinct population on which to base CTLA-4 analysis and to assess Treg percentages.

**Assessing functional capacity in CTLA-4 deficiency**

Whilst some mutations (e.g. stop mutations) may cause true haploinsufficiency, missense mutations in CTLA-4 can have a range of effects which require further dissection. For example, some mutations may result in proteins that do not bind CTLA-4 antibodies, whilst others may have a limited impact on antibody binding but still affect the ability to bind ligands. As shown in **Figure 4A** cells from an individual harboring a mutation in the CTLA-4 ligand-binding site revealed antibody staining similar to a healthy control. To account for such issues we established an assay that measures soluble ligand uptake by CTLA-4 as a surrogate for normal ligand capture and effector function. Previously, we have used assays, which rely on uptake of GFP-tagged ligands from transfected cells, however this requires specialized cellular

reagents and is strongly influenced by cell numbers and cell-cell contact. We therefore developed an assay monitoring the uptake of soluble ligands by stimulated Treg cells from patients carrying CTLA-4 mutations. Using this approach, ligand uptake at 37$^{o}$C can be compared to the total amount of CTLA-4 protein per cell. Importantly, ligand uptake requires both effective CTLA-4 trafficking to the cell surface as well as ligand binding capacity so the assay is therefore capable of probing a number of functional defects.

As shown in **Figure 4B**, in healthy controls the ability of CTLA-4 to capture ligands was proportional to its expression level. However, a much reduced slope was obtained when Treg from a patient with a known ligand binding defect (P137R). This indicated the presence of CTLA-4 protein that was impaired in its ligand capture ability. As a control, abatacept (CTLA-4-Ig) was used to block ligand uptake. Therefore, the decreased slope in these plots reflects lower ligand uptake per CTLA-4 molecule **(Figure 4B).** The quantification of this decrease in slope (CTLA-4 functional efficacy) is shown in **Figure 4C** providing an integrated assessment of level of expression, the ability of CTLA-4 to traffic to the membrane and to bind its ligands. Thus functionally significant mutations affecting the amount of CTLA-4, the quality of ligand binding or its trafficking can be detected using this assay.

**Distinguishing CTLA-4 mutations from LRBA deficiency**

Recently, mutations in the protein LRBA have been shown to impact on CTLA-4 expression. In LRBA deficiency CTLA-4 is synthesised normally, but appears aberrantly trafficked resulting in enhanced degradation in lysosomes. Since both CTLA-4 and LRBA mutations result in reduced CTLA-4 expression, we attempted to distinguish between these conditions.

As shown in **Figure 5 A and B** levels of CTLA-4 in LRBA-deficient mTreg cells were even lower than those bearing CTLA-4 mutations. However, in contrast to T cells from CTLA-4 deficient individuals (see Figure 2B), in response to stimulation the levels of CTLA-4 expression seen in stimulated LRBA T cells recovered to levels similar to controls **(Figure 5B)**, representing a 20-30-fold upregulation from baseline **(Figure 5C).** Thus following anti-CD3/anti-CD28 stimulation CTLA-4 gene expression in LRBA patients results in strong induction of CTLA-4 and a higher fold change from baseline levels.

In addition we also noted that whilst the percentage Foxp3+ as a fraction of CD4+ T cells was not obviously different between unstimulated LRBA samples and controls, brief stimulation revealed significantly higher Treg percentages in LRBA patients (**Figure 5D**) suggesting that stimulation preferentially helps detect Treg in conditions associated with CTLA-4 deficiency. This expanded Treg compartment, is highly consistent with the known impact of CTLA-4 deficiency on Treg homeostasis in mice[20,21,26].

Since in LRBA deficiency CTLA-4 protein is incorrectly trafficked to lysosomes we also assessed CTLA-4 expression in the presence of Bafilomycin A to prevent lysosomal degradation. As shown in **Figure 5E** both control individuals and those carrying CTLA-4 mutations showed a 1.5- 2-fold increase in CTLA-4 in response to BafA. In contrast, in patients with LRBA deficiency T cells stimulated in the presence of BafA displayed between 2-3 fold increase in CTLA-4 expression and recovered expression to levels similar to control values **(Figure 5F)**. Thus, whilst there was variation between individuals, increased responses to stimulation and enhanced Baf A sensitivity appears to be useful in distinguishing between low CTLA-4 expression due to genetic CTLA-4 deficiency and that as a result of aberrant handling of CTLA-4 due to LRBA deficiency.

Finally we also compared ligand uptake in patients with LRBA mutations, using CD80-Ig. In keeping with the fact that CTLA-4 expression is reasonably well corrected by transient stimulation and the CTLA-4 is qualitatively normal we observed that the slope of ligand binding against CTLA-4 expression in stimulated cells was very similar to controls **(Figure 6 A and B)**. Thus in patients with LRBA mutations, ligand uptake efficiency is much less affected in comparison to CTLA-4 mutations and may be useful in distinguishing LRBA from CTLA-4 defects **(Figure 6C)**.

## Discussion

CTLA-4 deficiency is a rare autosomal dominant disorder identified in patients with common variable immunodeficiency with a range of autoimmune complications[5,6]. In contrast, LRBA deficiency is a recessive disorder where biallelic mutations result in aberrant trafficking of proteins including CTLA-4[7], resulting in an earlier onset but phenotypically similar disease[9,27]. In order to understand the impact of different CTLA-4 and LRBA mutations we have probed a number of aspects of CTLA-4 biology. These include the level of detectable protein expression within T cell subsets and the assessment of protein trafficking coupled to the ability to interact with natural ligands. Together these approaches can be used to estimate the functional capacity of CTLA-4, without the need for specialized reagents or complex assays.

Using the above approaches, we identified characteristic features relating to both CTLA-4 and LRBA deficiency. The most robust estimate of CTLA-4 deficiency resulted from comparison of total CTLA-4 levels in memory Treg (mTreg) with CTLA-4 levels in naïve conventional CD4+ T cells (nTcon) in the same individual. Since nTcon express little or no CTLA-4 this provides a reliable internal control with which to compare Treg expression of CTLA-4. This reveals differences in level of expression in healthy mTreg, which on average are approximately 10-fold those of nTcon. In CTLA-4 haploinsufficient patients this difference is reduced to 5-fold or less and in LRBA patients approximately 3-fold. In general LRBA deficiency resulted in lower levels of CTLA-4 compared to CTLA-4 heterozygous mutations, which may contribute to its generally earlier disease onset. Whilst this approach to CTLA-4 staining is generally adequate, the extent of reduced CTLA-4 staining can be mutation dependent. Ultimately not all mutations in CTLA-4 will affect antibody staining and therefore be revealed by a simple staining approach. For example a mutation in the CTLA-4 ligand-binding site, gave limited differences in CTLA-4 antibody staining when compared to control. Therefore in cases where there is no obvious deficit in total CTLA-4 it is important to consider defects in CTLA-4 trafficking and ligand binding. Accordingly the P137R mutation, which occurs within the well-described CTLA-4 ligand binding site[28] displayed defects in soluble ligand uptake in our assays.

It is increasingly clear that a major aspect of CTLA-4 function relates to regulatory T cell biology and the ability of CTLA-4 to compete for CD28 ligands[16,29,30]. The ability of CTLA-4 to physically capture its ligands via transendocytosis[22] from APCs is predictive of CTLA-4 function on Treg[11]. Here we utilised a simplified ligand uptake assay, which uses soluble CD80-Ig, to test the key features of CTLA-4 function namely, ligand binding and CTLA-4 trafficking. We have previously shown that uptake of antibodies and ligands by CTLA-4 at 37°C is a convenient measure of CTLA-4 trafficking [25]. By gating on Foxp3+ cells, this provides an estimate of CTLA-4 function in Treg. Whilst direct studies of CTLA-4 dependent Treg suppression are functionally relevant, in reality accurate measurement is technically difficult, requiring

large numbers of T cells to generate meaningful data[5]. The popular surrogate of measuring Treg suppression using anti-CD3/anti-CD28 bead stimulation does not measure CTLA-4-dependent suppressive function in our view[11]. Accordingly, the assays outlined here represent a compromise, allowing testing of the largely agreed requirements for CTLA-4 function, i.e. level of expression, inducibility, trafficking and ligand binding. Importantly, all of these assays can be carried out using standard flow cytometric approaches, using commercially available reagents and can therefore be readily adopted.

Some studies have suggested that increased Tcon cell proliferation or inability to control IL-2 production may result from CTLA-4 mutation or deficiency[31,6,32]. We have been repeatedly unable to show any intrinsic effects of CTLA-4 deficiency on CD4 T cell responses in the absence of Treg[5] and are likewise unable to demonstrate an effect of anti-CTLA-4 blockade on proliferation of conventional CD4 T cells suggesting they are not subject to intrinsic CTLA-4 regulation[11]. We would urge caution in using CD4 T cell proliferation as a measure of CTLA-4 defects since there is abundant literature showing that CTLA-4 has little intrinsic ability to directly affect these aspects of T cell function[15,33]. In contrast, the cell-extrinsic (regulatory) function of CTLA-4 is borne out by numerous studies[34-36].

In the present study we did not identify deficits in Treg numbers associated with CTLA-4 or LRBA deficiency and observed that brief stimulation was a useful tool for confirming Foxp3 expression. Studies by Sakaguchi et.al, have shown that both Foxp3$^{hi}$ and Foxp3$^{lo}$ cells exist in human blood[37]. Since Foxp3 expression is influenced by levels of CD25 expression, factors such as IL-2 consumption CD4 lymphopenia and immunosuppressive treatments may all affect Foxp3. Thus whilst Foxp3 and CD25 expression may be decreased in CTLA-4 and LRBA deficiency, this may not indicate low Treg numbers *per se*. Indeed, it was reported in IPEX patients that numbers of natural Treg were underestimated due to decreased CD25 staining[38]. Charbonnier et.al,[39] also recently reported decreased Treg frequencies associated with LRBA deficiency. Whilst we did not observe this in our study, we noted that short *in vitro* stimulation increased the percentage of Foxp3+ cells without inducing their proliferation. This effect was particularly obvious in LRBA patients. Brief stimulation may therefore help to reveal Treg, which may otherwise have low expression of critical markers such as CD25 and Foxp3 resulting in underestimates. Our findings of increased Treg are consistent with the fact that in mice, CTLA-4 deficiency clearly promotes expansion of the Treg compartment[26]. Such an expansion might therefore be expected in LRBA deficiency where CTLA-4 levels are typically very low. Treg expansion is also seen in patients with CTLA-4 heterozygous mutations, however this occurs only in some individuals and may therefore be mutation specific. It is also important to note that the induction of Foxp3 can occur in conventional T cells[40] and therefore determining whether stimulated T cells expressing Foxp3 are natural Treg is complex. It is likely that analysis the methylation status of Foxp3 locus will be informative in this situation[41]. Nonetheless, from a functional perspective it is clear that expression of CTLA-4 itself is sufficient to confer suppressive activity in both Tcon as well as Treg[42-44], indicating the Foxp3+ CTLA-4+ T cells we observe after stimulation are nonetheless likely to be suppressive.

One feature that appears to distinguish LRBA from CTLA-4 deficiency is the upregulation of CTLA-4 in response to stimulation. Upregulation of CTLA-4 was higher in patients with LRBA mutations, consistent with the fact that there is no defect in CTLA-4 itself and synthesis is likely to be normal. Thus, during acute stimulation the ability to synthesise new CTLA-4 appears to outweigh any enhanced destruction due to the LRBA defect. This results in significant recovery of CTLA-4

from a very low baseline, providing a useful indicator of LRBA deficiency. In addition, the response of cells to Bafilomycin A, which inhibits lysosomal degradation gave a more significant enhancement of CTLA-4 staining in the case of LRBA mutations. This was clearer in some LRBA individuals than others and it will be interesting to determine whether the effect of BafA depends on particular LRBA mutations. Finally, functional efficacy of CTLA-4 proteins as measured by the slope of ligand uptake displayed very limited difference from controls, again showing that ligand capture is broadly unimpaired in LRBA deficiency. Taken together, the high fold-increase in response to stimulation, enhanced response to BafA and unimpaired ligand capture appear to be characteristics that distinguish LRBA from direct CTLA-4 mutations.

In summary, CTLA-4 expressed by Treg acts as a major mechanism to control self-reactive T cells, by regulating CD28 ligand availability. The approaches described here can be used to functional deficits in the CTLA-4 pathway.

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**Authorship contributions**:
T.Z.H. developed methods, performed experiments, analyzed data and helped write the manuscript. N.V. and J.W. performed experiments; B.S., A.K., D. J., N.H. and B.R. contributed to experimental design, developed methods and helped with data interpretation. A.W., W.Q., H.B., S.S., O.N., S.H. and P.A. provided clinical samples and contributed to data interpretation. H.S. L.S.K.W. and S.B. supervised the experiments and contributed to data analysis and co-wrote the paper. D.M.S. conceived of experiments, supervised the project, interpreted results, and wrote the paper.

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HS is a consultant and share holder in Cell Medica.The other authors declare no competing financial interests.

**Figure legends**

**Figure 1. Reduced CTLA-4 expression in memory Treg in individuals with CTLA-4 mutations.**
**A**. Expression of Foxp3 and total CTLA-4 in unstimulated CD4 T cells. CTLA-4 MFI (large font) is shown for the total Foxp3+ and Foxp3- populations. Percentage values are shown in quadrants. **B**. Comparison of percentage of memory CD4 T cells (CD45RA-ve) in Foxp3+ (Treg) and Foxp3- (Tcon) compartments in CTLA-4 deficient individuals (n=14) and controls (n=22). **C**. Representative expression of Foxp3 and total CTLA-4 in unstimulated CD4 T cells gated on CD45RA+ naïve (upper) or

CD45RA- memory subsets (lower). CTLA-4 MFI (large font) is shown for total Foxp3+ cells and Foxp3- cells. Percentages are shown in quadrants **D**. Relative CTLA-4 expression in healthy controls (n=33) and individuals with CTLA-4 heterozygous mutations (n=14). Relative expression is calculated as the fold CTLA-4 MFI change between of nTcon (naïve conventional T cells) and mTreg (memory regulatory T cells). **E**. Foxp3+ Treg percentage in unstimulated CD4 T cells comparing CTLA-4 mutation carriers and controls.

**Figure 2. CTLA-4 deficiency persists after stimulation**

**A.** CD4 T cells were stimulated with anti-CD3/ anti-CD28 beads for 16 hours to stimulate CTLA-4 expression. Foxp3 and total CTLA-4 (BN13) staining are compared between unstimulated (upper panels) or stimulated T cells (lower panels). Cells were gated on CD45RA-ve memory CD4 T cells. CTLA-4 MFI (large font) is shown for total Foxp3+ cells (right) and Foxp3-ve cells (left). Percentages are shown in quadrants and Foxp3 MFI on Treg (lower right). **B**. Relative CTLA-4 expression in healthy controls and individuals with CTLA-4 heterozygous mutations after stimulation. Relative expression is calculated as in figure 1. **C**. Foxp3+ percentage in stimulated CD4 T cells comparing CTLA-4 mutation (n=14) and control (n=22).

**Figure 3. T cell stimulation increases Treg detection by upregulating Foxp3 expression.**

**A**. CD4 T cells were analysed for Foxp3, CTLA-4 and CD25, Ki67 in a healthy control and a CTLA-4 deficient patient at 0h and 16 hours after CD3/28 bead stimulation. **B**. Percentage of Treg before or after bead stimulation in controls and individuals with CTLA-4 mutations.

**Figure 4. CTLA-4 ligand uptake reveals defects in patients with CTLA-4 deficiency.**

**A**. Expression of Foxp3 and total CTLA-4 (BN13) on unstimulated CD45RA- memory CD4 T cells were compared between a ligand-binding mutant (P137R) and healthy control. CTLA-4 MFI in Foxp3+ and Foxp3- populations is shown in large font. Percentages are shown in quadrants. **B**. Impaired ligand uptake by CTLA-4 deficient patient. CD4 T cells were stimulated with CD3/CD28 beads and gated on Foxp3+ cells. Total CTLA-4 staining (C19 antibody) is plotted against ligand uptake (CD80-Ig). Changes in slope reflect alterations in ligand uptake efficiency and are overlayed in control plot. CD80-Ig MFI (upper right) and CTLA-4 MFI (lower right) are shown in large font. **C**. Comparison of the slope of the line of best fit from the data in B.

**Figure 5. LRBA deficiency and CTLA-4 deficiency have different patterns of expression.**

**A**. Representative expression of Foxp3 and total CTLA-4 (BN13) on unstimulated and stimulated memory CD4 T cells from control, or LRBA mutations. CD4 T cells were stimulated with CD3/CD28 beads, CTLA-4 MFI is shown in large font. **B**. Relative expression of CTLA-4 in healthy controls and LRBA deficient patients (n=5) in unstimulated or stimulated conditions. Relative expression is calculated as the fold change in CTLA-4 MFI between of nTcon and mTreg **C**. Fold increase in CTLA-4 MFI between Foxp3+ memory CD4 T cells before and after stimulation with CD3/CD28 beads. **D**. Foxp3+ Treg percentage in unstimulated or stimulated LRBA deficient and control CD4 T cells. **E**. CD4 T cells were stimulated with CD3/CD28 beads in the presence of absence of Baf A and stained for Foxp3 and total CTLA-4 (BN13) expression. CTLA-4 MFI in Foxp3+ mTreg is shown in large font. **F**. Collated BafA data for healthy controls, CTLA-4 or LRBA mutations. Fold increase is the change in CTLA-4 MFI in mTreg before and after BafA treatment.

**Figure 6. Ligand uptake is relatively unaffected in LRBA deficient patients**.

**A**. CD4 T cells were stimulated with CD3/CD28 beads and total CTLA-4 (C19) plotted against CD80-Ig uptake gating on CD4+ memory Treg. Slope of the line represents efficiency of CD80 uptake. Dotted lines are overlayed in the control plot (top left) for comparison. CTLA-4-Ig treatment (lower panels) provides a negative control by blocking ligand uptake. CD80Ig MFI is shown in large font (upper right) and CTLA-4 MFI in large font (lower right). Percentages are shown in all quadrants. **B**. Graph is generated using the slope of the line of best fit from the data in A. **C.** Collated ligand uptake efficiency data is shown for CTLA-4 and LRBA mutations.

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Fig 1

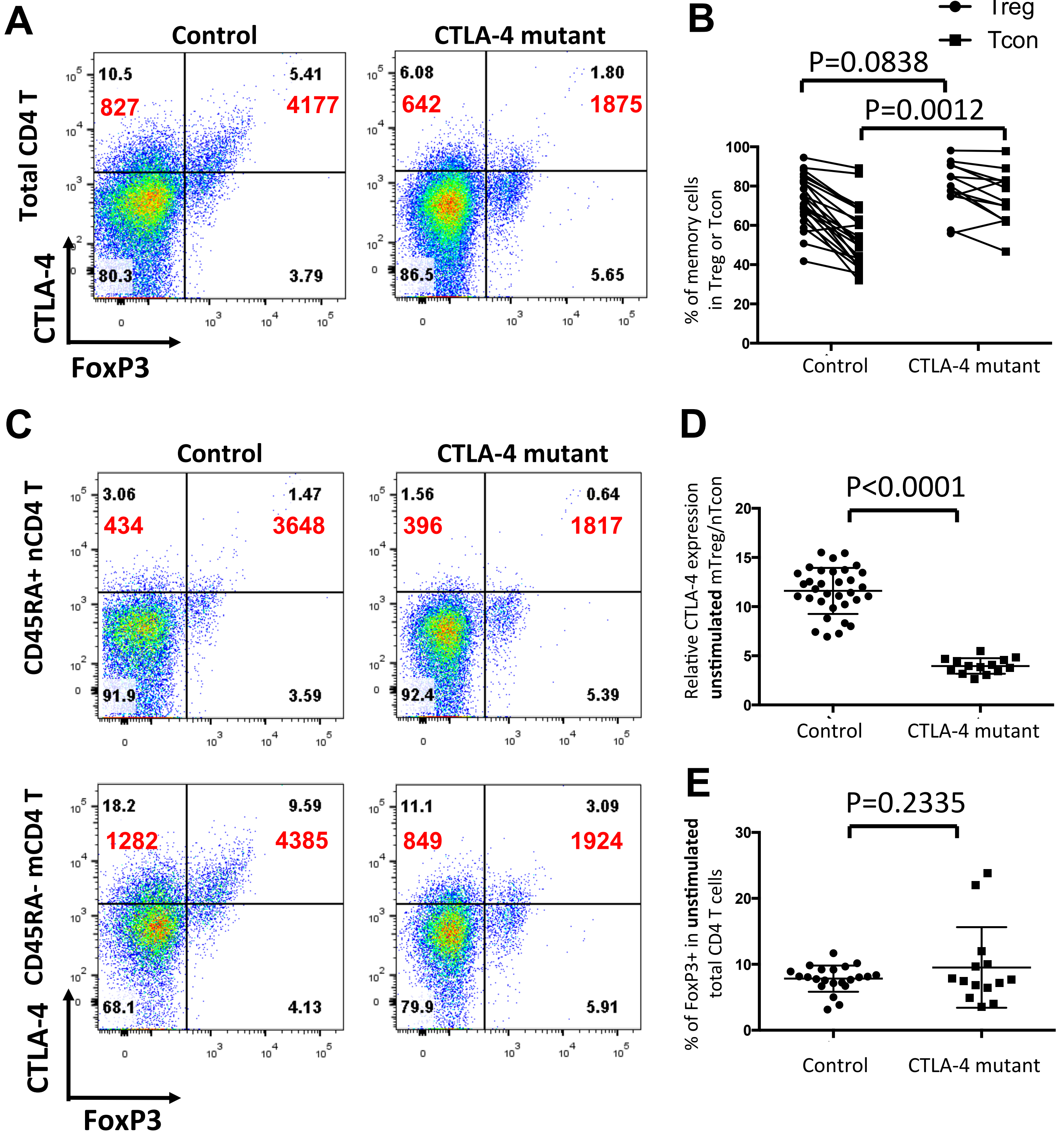
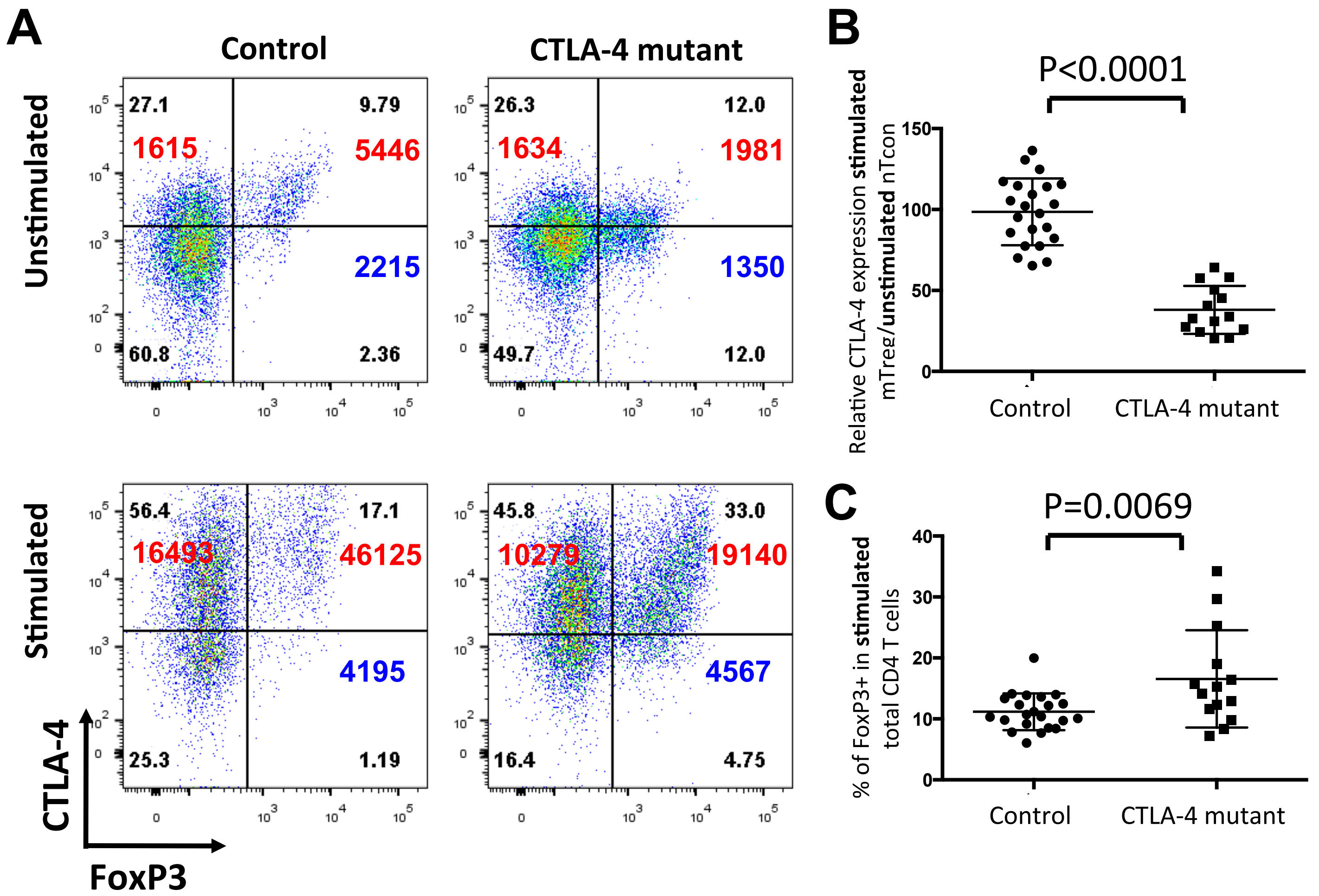


Fig 2



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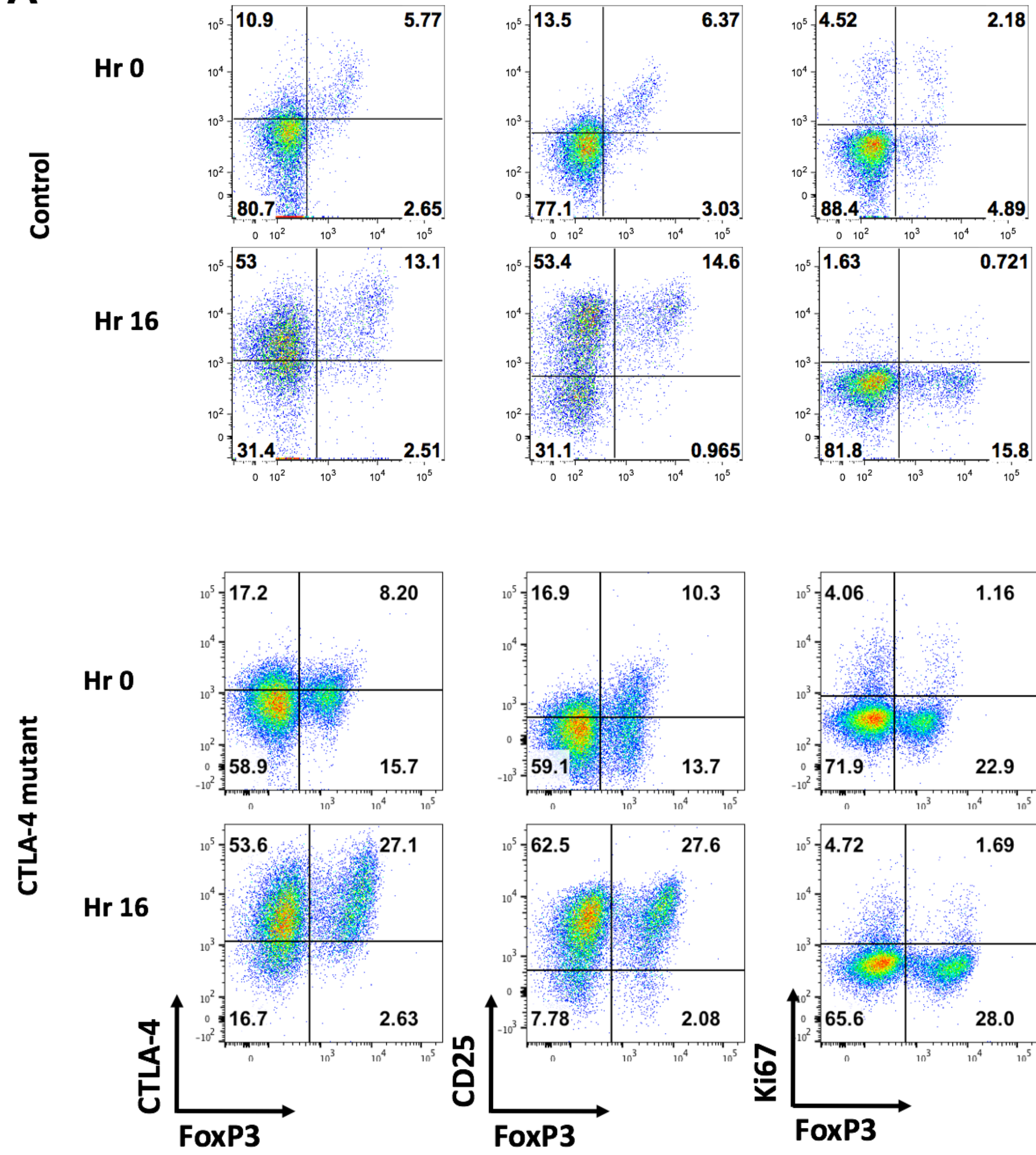


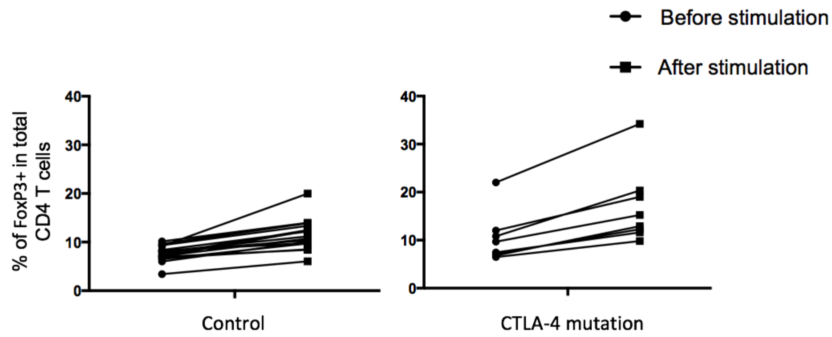

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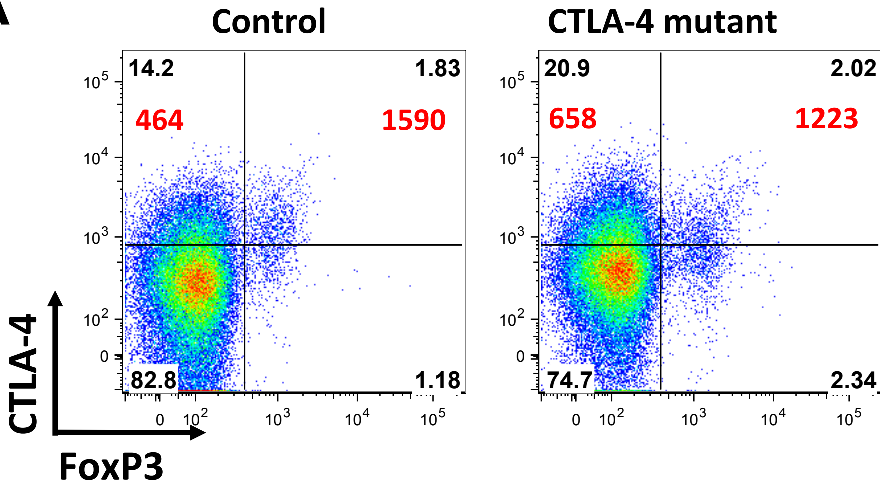
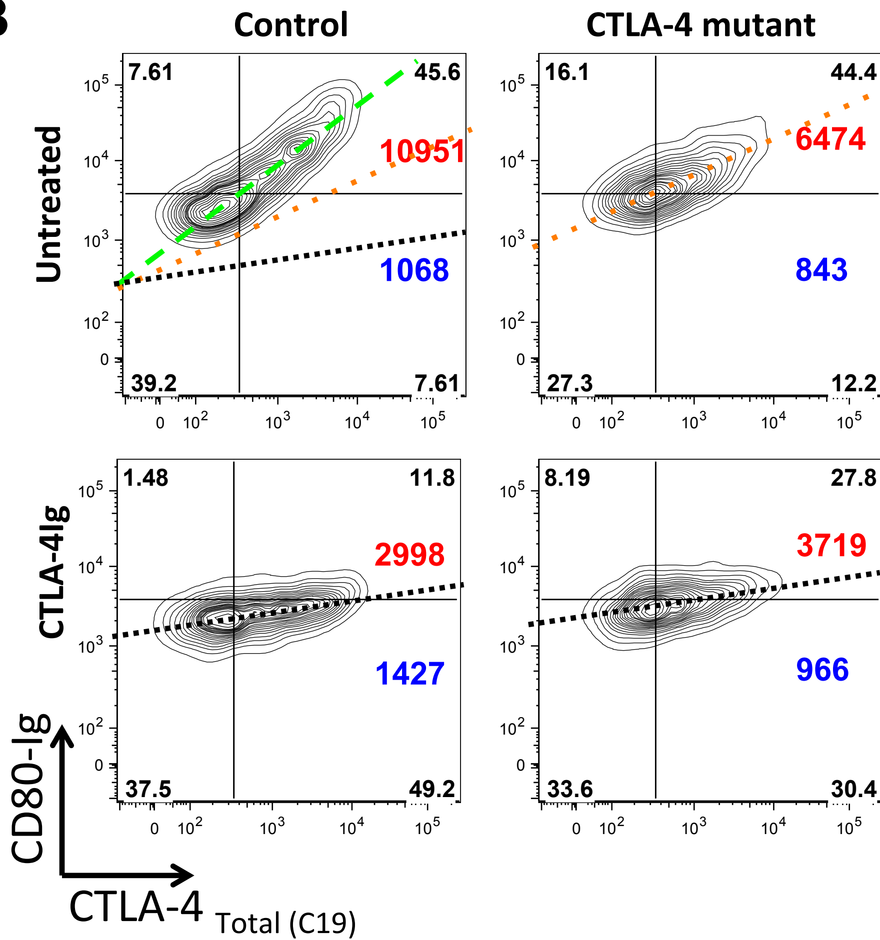
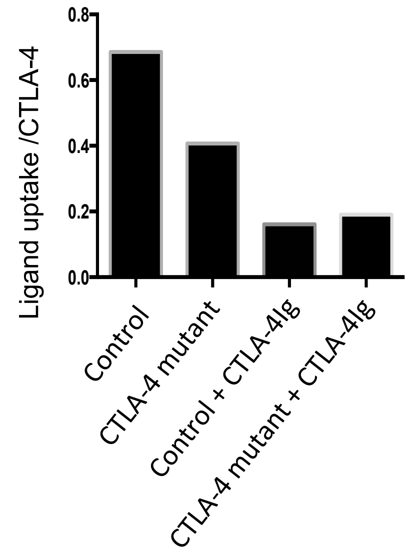
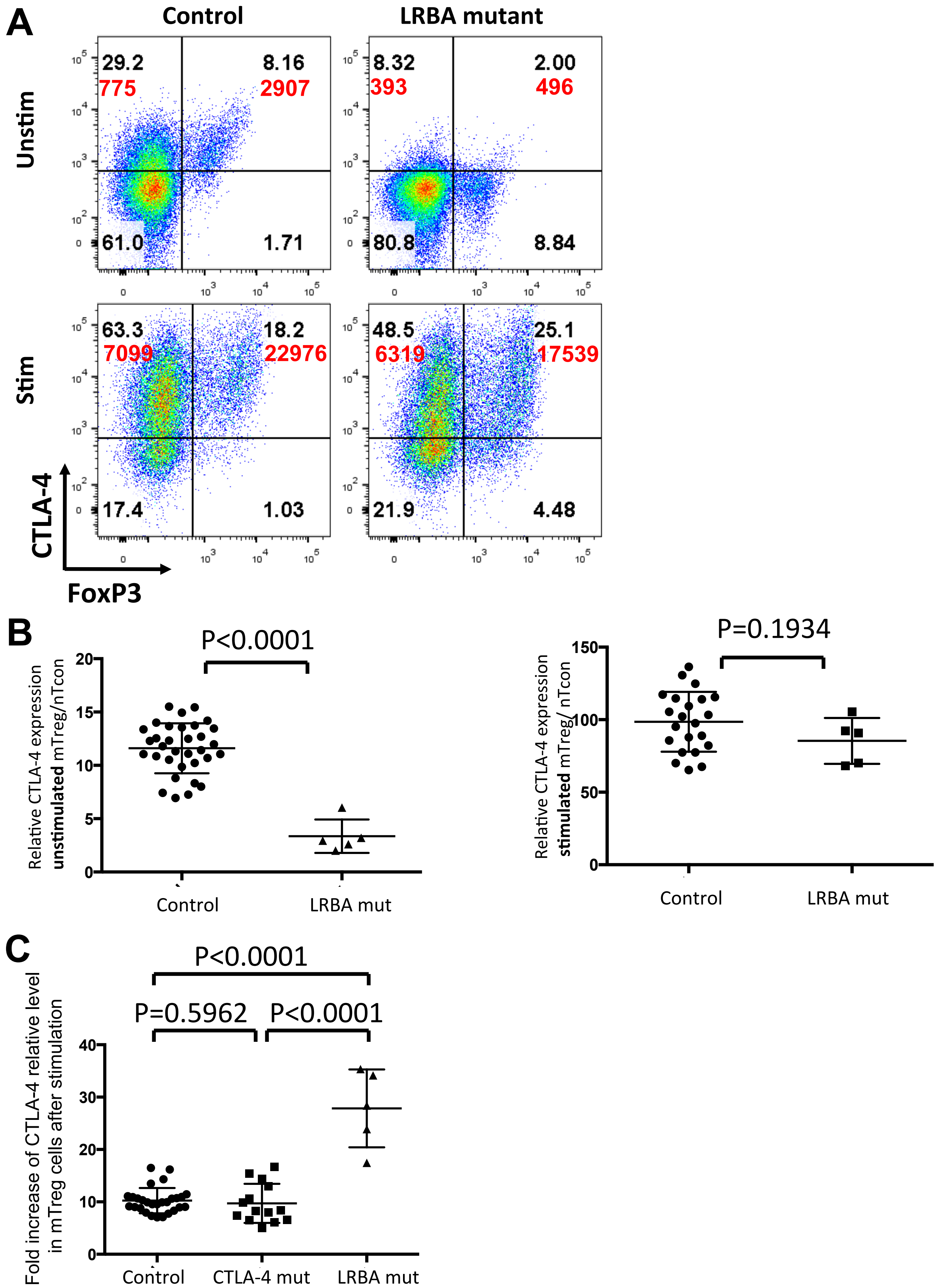


## Fig 5



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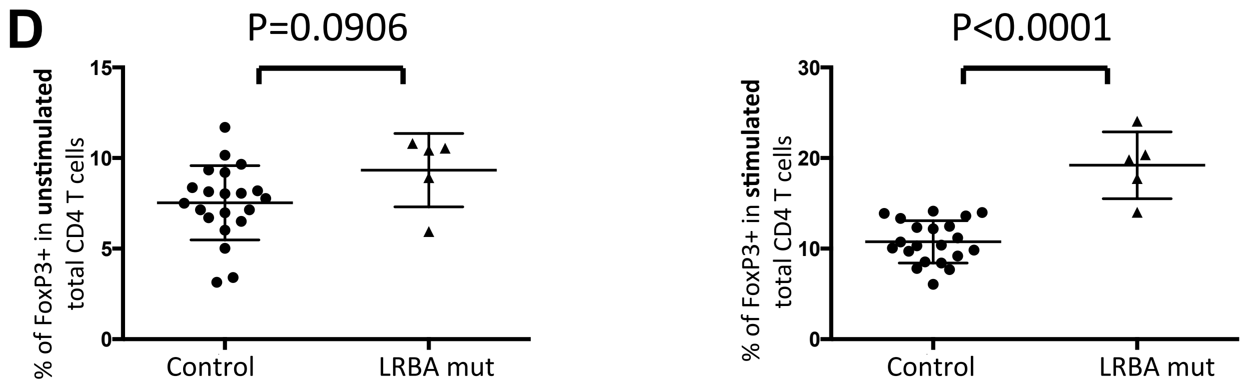


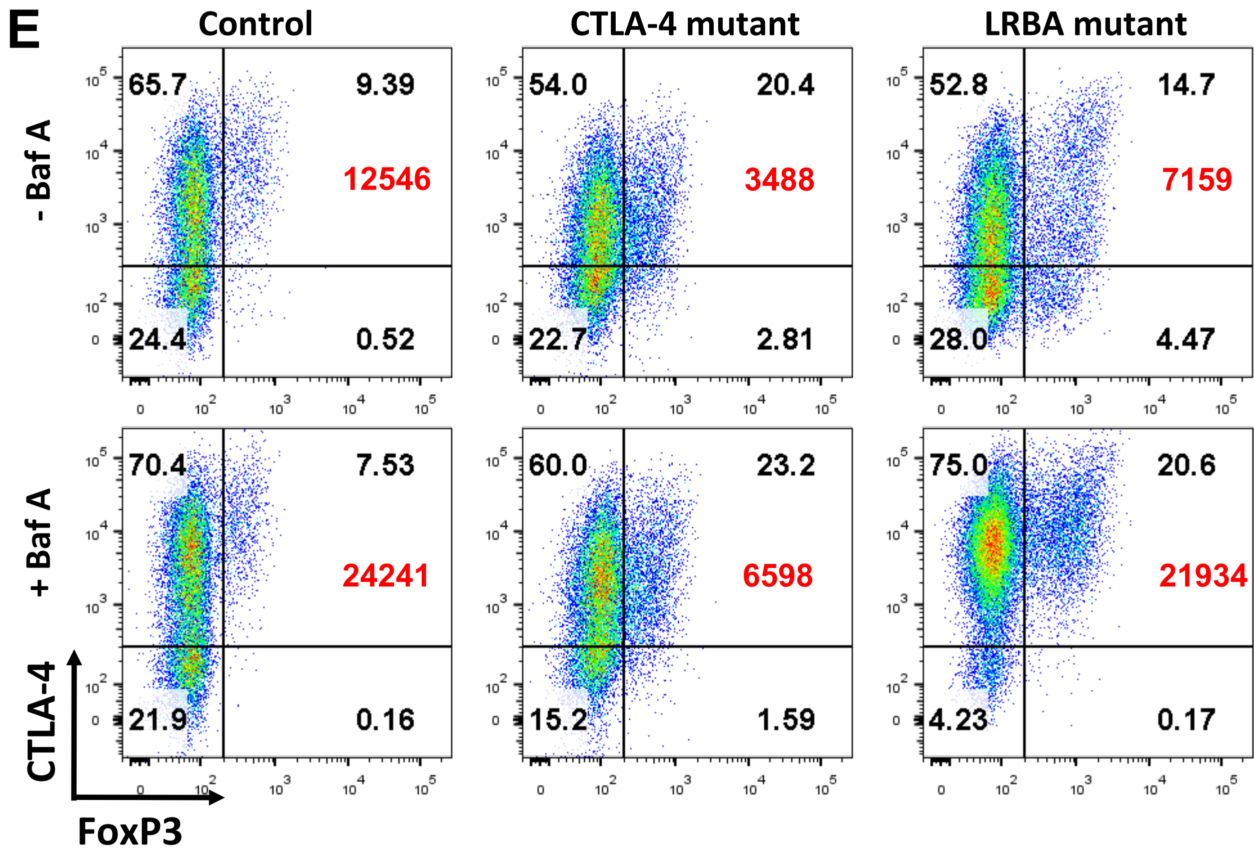


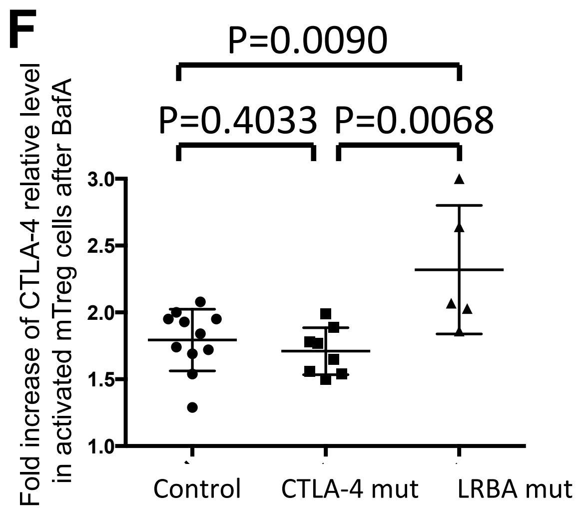

# Fig 6