Vedolizumab as a successful treatment of CTLA-4 associated autoimmune enterocolitis

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The authors declare no conflicts of interest

Capsule summary:

We report a case of a male patient with CTLA-4-deficiency presenting with pure red cell aplasia and severe autoimmune enterocolitis that was successfully treated with the $\alpha_4\beta_7$ integrin-blocking monoclonal antibody vedolizumab.

Abbreviations:

PID: primary immunodeficiency

IBD: inflammatory bowel disease

Treg: regulatory T cells

CVID: common variable immune deficiency

CFSE: carboxyfluorescein succinimidyl ester

CTLA-4: cytotoxic T-lymphocyte-associated Protein 4

APECED: autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy

PBMC: Peripheral blood mononuclear cells

MFI: Mean fluorescent intensity

GFP: green fluorescent protein

To the editor:

In 2007, a 39 year old Caucasian male presented with chronic, non-infectious diarrhea. The patients prior history was noticeable for adrenal insufficiency diagnosed in 1991.
In 2013 his diarrhea worsened, resulting in weight loss of >20 kg and severe dehydration. Prednisolone (1mg/kg of body weight given for several weeks) was entirely ineffective. Macroscopic enterocolitis was seen, corresponding histologically to extensive infiltration with $CD3^+$ T cells in cryptal areas (**Figure 1a**). Enterocytes showed enhanced positivity for Ki-67, indicating augmented proliferation (**Figure 1b**). Complete absence of mucus producing goblet cells was observed in colon and small intestine (data not shown). At that time, hypogammaglobulinemia (IgG 4.4g/l, normal: 7-16g/l; IgA 0.53g/l, normal: 0.7- 4g/l) was first noticed, while serum IgM was within normal range. On a CT scan no evidence for malignancy or lymphoproliferation was found, and lung morphology was normal. Intravenous immunoglobulin (IVIG) substitution (0.5g/kg body weight per month, given for 4 months) had no effect on diarrhea and the patient required i.v.-fluids repeatedly.
In 2014, the patient developed severe hypo-regenerative anemia. Bone marrow biopsy revealed isolated yet almost complete absence of erythropoietic cells (data not shown), and the diagnosis of pure red cell aplasia was established. Parvovirus was tested negative by PCR.
In May 2014, while the patient was still on IVIG treatment, an immunologic work-up was performed (**Table 1**). B cell counts were low (2% of lymphocytes, **Table 1**). Analysis of B cell subpopulations revealed normal relative differentiation into marginal zone-like (IgD^+CD27^+, 27% of al B cells) and class-switched memory (IgD^-CD27^+, 15% of all B cells) subsets. By contrast, the proportion of $CD21^{low}$ B cells was clearly elevated (28% of B cells) –a finding associated with granulomas and splenomegaly in patients with CVID[1]. Within the T cell fraction, regulatory T cells (both defined as $CD3^+CD4^+CD127^{low}CD25^{high}$ or $CD3^+CD4^+CD45RA^{neg}FOXP3^{high}$, **Figure 2a and 2c, respectively**) were normal or even enhanced in numbers, while the proportions of central- and effector-memory $CD4^+$ and $CD8^+$ T cells were comparable to healthy control (**Figure 2b**). T cell-mediated colitis has recently been described as a prominent feature in patients with heterozygous mutations in CTLA-4, a negative regulator of T cell-mediated immune responses[2,3]. Colitis is also commonly induced in melanoma patients treated with ipilimumab, an anti-CTLA-4 antibody[4,5]. The DNA of the patient was analyzed by whole exome sequencing which indeed identified a heterozygous missense mutation in the *CTLA4* gene at cDNA position 257 (c.C257T), resulting in an alanine

to valine substitution at position 86 (p.A86V) (a graphic representation of the mutation is shown in **supplemental Figure 1**). The alanine at this position is highly conserved across various species (**Table 2**) and the mutation was predicted to have a deleterious consequence (CADD score 24.2, PolyPhen 1 'probably damaging'). The other rare non-synonymous allelic variants found in PID genes (adapted from[6]) were unlikely to explain the patient's clinical phenotype (**Table 3**). At the protein level, expression of CTLA-4 expression on Treg was low compared to control, both in the absence or following *in vitro* stimulation of Treg (Figure 2c+d) with MFI reductions similar to what was published in patients with CTLA-4 deficiency[3]. To address CTLA-4 function, a previously published transendocytosis assay was performed measuring the CTLA-4 driven capacity to transendocytose a CD80-GFP fusion protein[3]. CTLA-4 mediated transendocytosis was clearly reduced in patient-derived $CD4^+$ T cells (Figure 2e). With the clinical condition of he patient unchanged, at this time, treatment with vedolizumab was started. Vedolizumab is an α4β7 integrin-specific humanized mAb that inhibits binding of this gut homing integrin to mucosal MAdCAM-1, while leaving the binding to the vascular adhesion protein VCAM-1 intact. Vedolizumab has recently been approved for the treatment of IBD refractory to TNF-α blockade[7].

After 3 infusions at standard dose, diarrhea was markedly reduced, and the patient gradually re-gained body weight. Diarrhea had completely resolved three months after start of vedolizumab. Currently, 18 months after initiating vedolizumab, the patient is back at work with no abdominal complaints. In a control endoscopy, normal colonic mucosa was seen. Vedolizumab was well tolerated and no infectious complications occurred. Vedolizumab had no impact on the pure red cell aplasia, and cyclosporine was started seven months after start of vedolizumab treatment at 2x100mg/d, and later reduced to 75mg/d. One and a half month later, hemoglobin raised from 78g/l to 127g/l coinciding with a 20-fold relative increase of reticulocytes.

The histopathology and the adult-onset of the colitis matches the description reported in other patients with CTLA-4 deficiency[2,3]. However, pure red cell aplasia, has not been previously linked to CTLA-4 deficiency. Cyclosporine A induces remission in roughly 70% of patients with acquired pure red cell aplasia[8]. We report here for the first time that it also can successfully induce remission in CTLA-4-associated pure red cell aplasia.

Adrenalitis resulting in adrenal insufficiency has rarely been described in ipilimumab treated patients while hypophysitis is a much more common side effect, occurring in 10-15% of patients treated with this monoclonal antibody[5]. The most important novelty of this case-study is the reporting of the efficacy of vedolizumab in the treatment of CTLA-4-associated colitis. Published evidence shows that vedolizumab has a good safety profile[9]. No cases of progressive multifocal leucencephalopathy, a major side-effect of other integrin-blocking antibodies such as natalizumab, have been reported in randomized clinical trials[9]. TNF-α blocking antibodies have been successfully used to treat anti-ipilimumab-induced colitis in melanoma patients[10]. However, avoiding TNF-α blockade in highly autoimmunity-prone PID patients –such as individuals with CTLA-4 deficiency– is desirable, since blocking TNF-α *per se* can promote autoimmunity[11]. Other immunosuppressive drugs may worsen hypogammaglobulinemia associated with CTLA-4 deficiency and, notably, high-dose prednisolone was ineffective in our patient. Steroid refractory colitis has also previously been described in CTLA-4 deficiency[2], underlining the need for effective therapies in this setting.

In summary, we describe a patient with a heterozygous *CTLA4* mutation, associated with low CTLA-4 expression and function of Treg, clinically associated with adrenal insufficiency, pure red cell aplasia and severe T cell mediated enterocolitis. The latter was successfully treated with vedolizumab, without apparent side effects. The clinical usefulness of vedolizumab should be assessed further in enterocolitis associated with genetic or drug-induced functional CTLA-4 deficiency.

Figure legends:

Figure 1: T cell mediated enterocolitis

Histology of CTLA-4 associated enterocolitis. (a) T cell mediated colitis: immunohistochemistry (brown, arrows)) for CD3 (T cells). (b) Ki-67 immuno-staining (detected by MIB-1 antibody) shows enlarged proliferative zones of enterocytes, even in the intercryptal epithelium (arrows).

Figure 2: Immunologic alterations in CTLA-4 deficiency

(a+b) Flow-cytometry for $CD25^{hi}CD127^{lo}$ Treg (a) and for naïve ($CD27^{+}CD45RO^{neg}$) central memory ($CD27^{+}CD45RO^{+}$) and effector memory ($CD27^{-}$) $CD4^{+}$ and $CD8^{+}$ T cells (b).

(c+d) CTLA-4 expression was measured by flow-cytometry on conventional $CD4^+$ T cells or $FOXP3^+$ Treg, in the absence (c) or following *in vitro* activation (d). Mean fluorescent intensity (MFI) of CTLA-4 fluorescence is indicated in red (in brackets the fold increase of CTLA-4 expression in $FOXP3^+$ Treg compared to naive FOXP3 negative $CD4^+$ T cells). The MFI of FOXP3 fluorescence is indicated in blue. CTLA-4 MFI of the patient was approximately 60% compared to the CTLA-4 MFI measured in the control sample.
(e) CTLA-4 function was measured by using a transendocytosis assay in which CTLA-4 mediates transendocytosis of CD80 from a green fluorescent protein (GFP) competent cell line. GFP positivity as a marker of acquisition of CD80 is measured by flow-cytometry in $CD4^+$, $CD45RO^+$, $FoxP3^+$ regulatory T-cells. Patients carrying the C35* or R70W *CTLA-4* alleles have previously been published[3]. The lower panel, designated "+Anti-CTLA-4" indicates control experiments where ipilimumab (a CTLA-4 blocking antibody) was co-incubated to block CTLA-4 mediated transendocytosis.

Supplementary Figure 1:

3D reconstruction of wild-type and A86V variant CTLA-4.

Table 1:

Immunologic parameters of the patient and lab reference values.

Table 2:

The alanine at position 86 of human CTLA-4 is highly conserved.

Table 3:

Next generation sequencing results from the patient derived DNA.

Keywords:

CTLA-4; regulatory T cell; Treg; autoimmune colitis; vedolizumab; α4β7 integrin; pure red cell aplasia; cyclosporine A, autoimmune adrenalitis; hypogammaglobulinemia

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Materials and methods:

Ethical approval:

Following informed consent, the patient was included into a prospective cohort of patients with primary immunodeficiency/immune-dysregulation that was ethically approved (EKNZ 2015-187) according to Swiss law.

Immunohistology:

Immunohistochemistry was performed using the avidin-biotin-peroxidase-complex (ABC) method. The antibodies employed were directed against CD3 (clone: PS1, Leica) and Ki67 (Clone: SP6, Cell Marque).

Immunophenotyping and flow-cytometry based proliferation assays:

The following antibodies from (Biolegend) were used for surface staining of specific lymphocyte subsets: CD27 (clone O323), CD25 (clone BC96), CD45RO (clone: UCHL1), CD4 (Clone: A161A1), CD3 (clone: UCHT1), CD8 (Clone: SK1), CD127 (clone: A019D5), CD19 (clone: HIB19), CTLA-4 (clone: L3D10).

Next generation sequencing:

Genetic sequencing was performed following informed consent. DNA was extracted from cultured T cell blasts and sheared, followed by pull-down of coding sequences, adapter ligation and massively parallel sequencing on Illumina HiSeq 2000 appliances at Functional Genomics Center Zurich. Read lengths of 2x100 bp were produced aiming for average target sequence coverage > 60x and generating > 20 reads for 90% of the Gencode exome. The raw sequence reads were quality controlled, aligned to the reference sequence, genotypes were called with Genome Analysis Toolkit (McKenna, Hanna et al. 2010, Genome Res) and variants annotated with the position of nucleotide change with respect of coding genes. Results were filtered according to a list of known PID genes (Picard, Al-Herz et al. 2015, J Clin Immunol). Alleles giving rise to non-synonymous amino acid substitutions, aberrant splicing or protein truncation events were filtered for functional impact based on PolyPhen2 (Adzhubei,

Schmidt et al. 2010, Nat Methods; Adzhubei, Jordan et al. 2013, Curr Protoc Hum Genet) and CADD (Kircher, Witten et al. 2014, Nat Genet) scores, on a minor allele frequency (MAF) of < 0.001 in public databases (1000 Genomes (Abecasis, Altshuler et al. 2010, Nature), NHLBI GO Exome Sequencing Project (Exome Variant Server, 2015), Exome Aggregation Consortium ExAC (Exome Aggregation Consortium (ExAC), 2015)) and our in-house database of >2'700 exomes.

Analysis of CTLA-4 expression by flow-cytometry

PBMCs were isolated from fresh blood of control or patient by density centrifugation. $CD4^+$ T cells were purified from PBMCs by negative selection using human $CD4^+$ T cell kit (Stemcell). $CD4^+$ T cells were cultured in the absence or presence of CD3/CD28 Beads (Invitrogen) in RMPI with 10% FBS culture media for 16 hours. Cells were then surface stained using anti-CD4 Alexa Fluor 700 (clone: RPA-T4, BD) and anti-CD45RA PerCP-Cy5.5 (clone: HI100, eBioscience) at 4°C for 30 mins. For intracellular staining, cells were then washed, fixed/ permeabilised using FoxP3 staining buffer (eBioscience) and stained by anti-CTLA-4 PE (clone: BN13, BD) and anti-FoxP3 APC (clone: 236A-E7, eBioscience). Cells were washed and analysed by BD FACS LSRII and FlowJo software.

Transendocytosis assay

The Transendocytosis assay was performed as previously published (Qureshi, O. S., et al. (2011). "Trans-Endocytosis of CD80 and CD86: A Molecular Basis for the Cell-Extrinsic Function of CTLA-4." Science **332**(6029): 600-603.). Briefly, $CD4^+$ T-cells were isolated from frozen PBMCs using CD4 T-cell isolation Kit (*Miltenyi Biotec GmbH*) and cutured 1:1 with CD80-GFP expressing CHO cells or control CHO cells upon stimulation with CD3/CD28 dynabeads (*Thermofisher*) for 16 hours at 37°C in RPMI containing 10%FCS and 1%PS. Stimulation was used in a ratio of 1:2 beads per T-cell. Bafilomycin was added to the co-culture (20nM). Anti-CTLA4 was used in 2.5µg per well as indicated. T-cells were labeled with anti-human CD4 PerCP-Cy5.5, CD45RO PE-Cy7, FoxP3 PE (*eBioscience*) and CTLA-4 BV

421 (*BD Bioscience*). Intracellular staining was performed after fixation and permeabilization using FoxP3 Fix/Perm Set (*eBioscience*).

CTLA-4 NMR structure

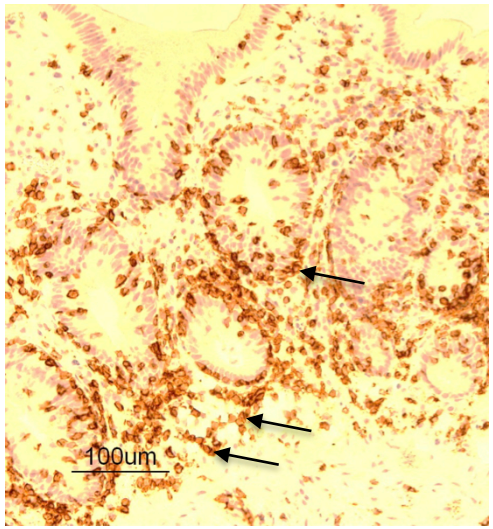
The NMR solution structure (PDB code 1AH1) {Basis for the ref: Solution structure of human CTLA-4 and delineation of a CD80/CD86 binding site conserved in CD28 Nature structural biology Violume 4 number 7, 1997} was used to construct the molecular representations, using the software VMD version 1.9.1, developped by the NIH center for biomolecular modelling and bioinformatics {ref: Humphrey, W., Dalke, A. and Schulten, K., "VMD - Visual Molecular Dynamics", J. Molec. Graphics, 1996, vol. 14, pp. 33-38}. The mutation was performed using the VMD plug in MUTATOR.

Acknowledgment:

This work was supported by a Swiss National Science Foundation grant to Mike Recher (PP00P3_144863) and Christoph T Berger (PZ00P3_148000). Mike Recher and Christoph Hess were supported by the Gebert Rüf foundation (GRS-058/14). Ipilimumab was a gift from Heinz Läubli.

Figure 1

a



b

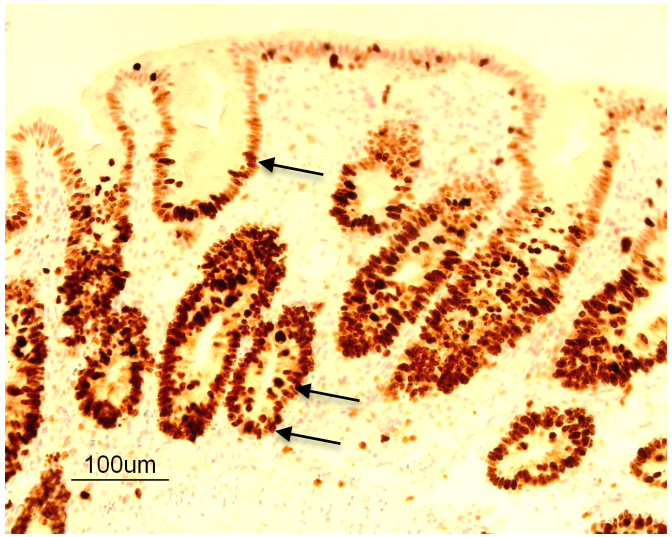


Figure 2

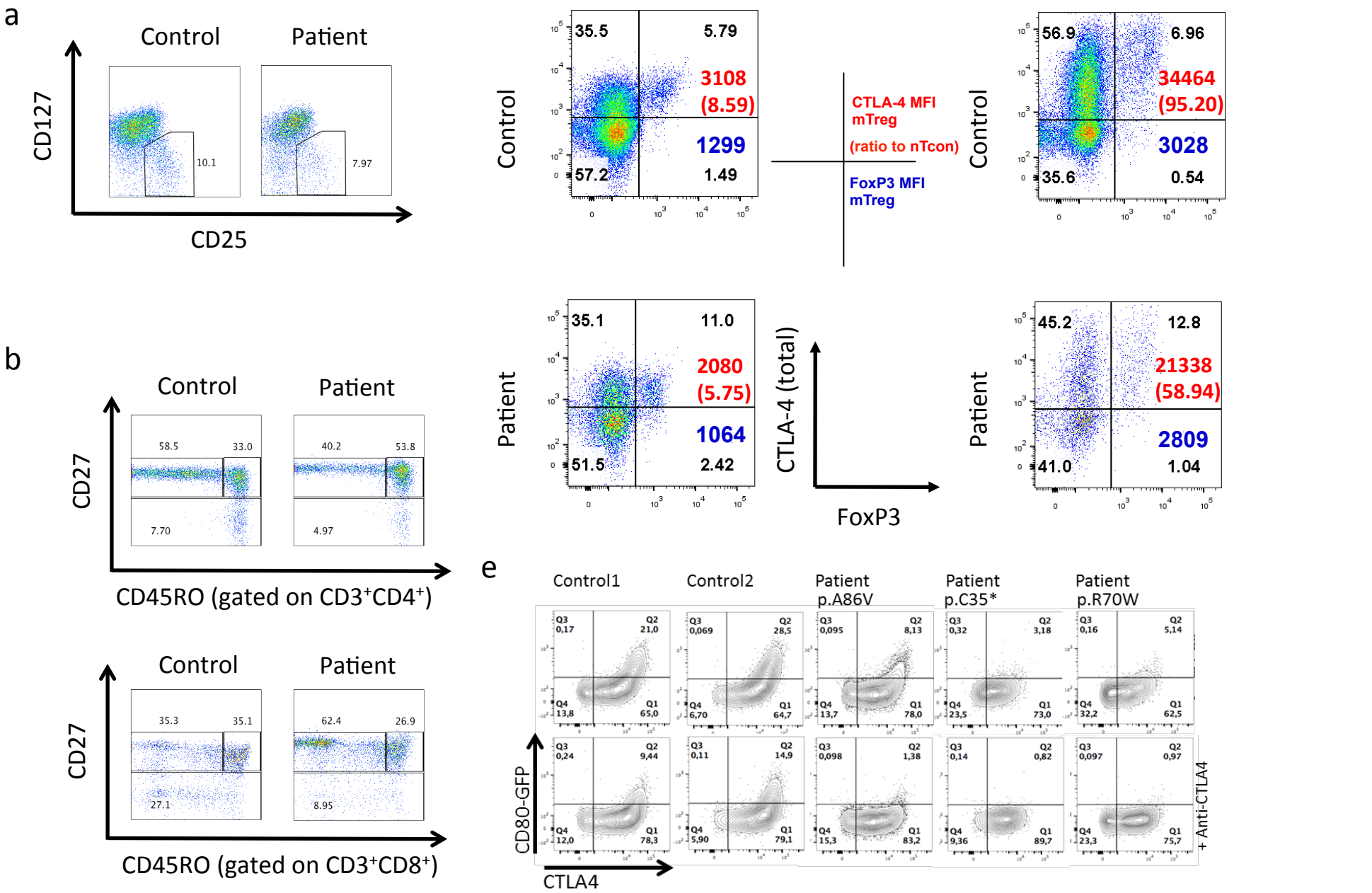

a
Control
Patient
CD127
CD25
10.1
7.97
b
Control
Patient
CD27
58.5
33.0
7.70
40.2
53.8
4.97
CD45RO (gated on CD3+CD4+)
Control
Patient
CD27
35.3
35.1
27.1
62.4
26.9
8.95
CD45RO (gated on CD3+CD8+)
c
Memory CD4+ T cells
(CD4+CD45RA-)
Control
35.5
5.79
3108
(8.59)
1299
57.2
1.49
CTLA-4 MFI
mTreg
(ratio to nTcon)
FoxP3 MFI
mTreg
Patient
35.1
11.0
2080
(5.75)
1064
51.5
2.42
CTLA-4 (total)
FoxP3
d
Memory CD4+ T cells
(CD4+CD45RA-)
Control
56.9
6.96
34464
(95.20)
3028
35.6
0.54
Patient
45.2
12.8
21338
(58.94)
2809
41.0
1.04
e
Control1
Control2
Patient
p.A86V
Patient
p.C35*
Patient
p.R70W
CD80-GFP
CTLA4
+ Anti-CTLA4

Table 1:

Cell Population	Patient value	Normal values
RBC [cells/µl]	4.34 G/l	4.5-6.3 G/l
WBC [cells/ml]	8.09 G/l	3.5-10 G/l
ANC [cells/ml]	5.8 G/l	1.3-6.7 G/l
Platelet count [cells/ml]	354 G/l	150-450 G/l
Lymphocytes absolute [cells/ml]	1.254 G/l	0.9-3.3 G/l
Lymphocyte subpopulations		
$CD3^+$ [cells/µl] and [%]	1331/µl (86%)	742-2750/µl (55-86%)
$CD3^+CD4^+$ T cells [cells/µl] and [%]	565/µl (36%)	404-1612/µl (33-58%)
$CD3^+CD8^+$ T cells [cells/µl] and [%]	728/µl (46%)	220-1129/µl (13-39%)
$CD19^+$ [cells/µl] and [%]	**24/µl (2%)**	**80-616/µl (5-22%)**
$CD56^+CD16^+$ [cells/µl] and [%]	190/µl (12%)	84-724/µl (5-26%)
B cell subpopulation		
IgD^+CD27^- [cells/µl] and [%] out of CD19+	7/µl (27.2% of CD19)	66-228/µl (25.1-92.4%)
IgD^-CD27^+ [cells/µl] and [%] out of CD19+	4/µl (15.2 % of CD19)	8-102/µl (2.4-32.6%)
$CD21^{low}CD38^-$ B cells [cells/µl] and [%]	**7/µl (28.6% of CD19)**	**1-12/µl (0.5-4.7%)**

Table 2:

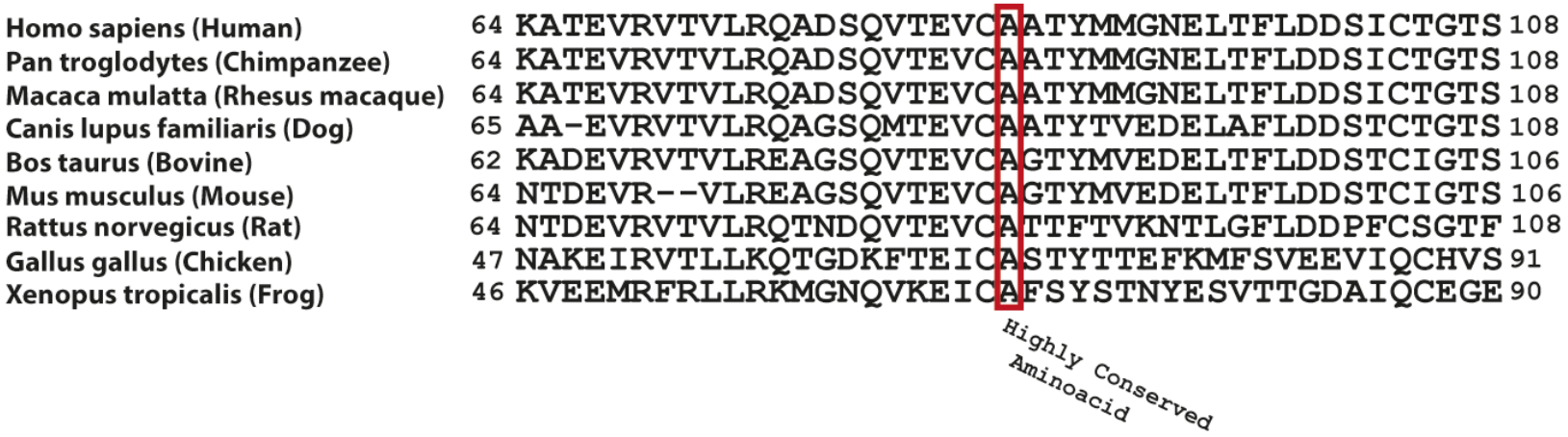
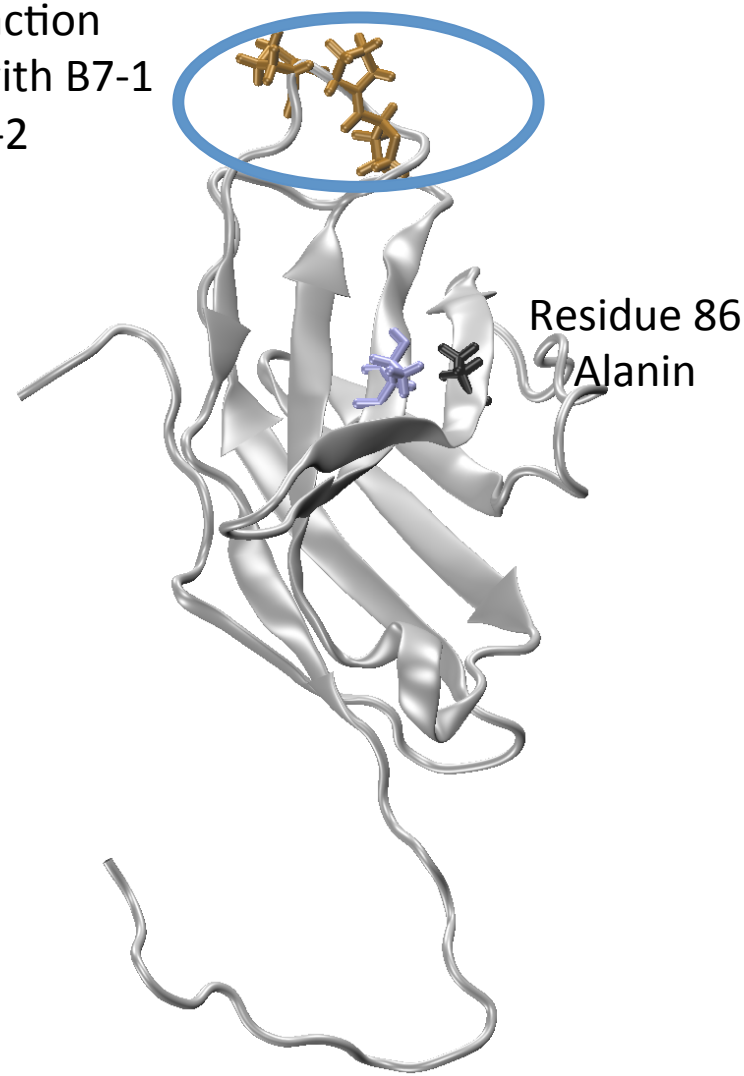

Homo sapiens (Human) 64 KATEVRVTVLRQADSQVTEVCAATYMMGNELTFLDDSICTGTS 108
Pan troglodytes (Chimpanzee) 64 KATEVRVTVLRQADSQVTEVCAATYMMGNELTFLDDSICTGTS 108
Macaca mulatta (Rhesus macaque) 64 KATEVRVTVLRQADSQVTEVCAATYMMGNELTFLDDSICTGTS 108
Canis lupus familiaris (Dog) 65 AA-EVRVTVLRQAGSQMTEVCAATYTVEDELAFLDDSTCTGTS 108
Bos taurus (Bovine) 62 KADEVRVTVLREAGSQVTEVCAGTYMVEDELTFLDDSTCIGTS 106
Mus musculus (Mouse) 64 NTDEVR--VLREAGSQVTEVCAGTYMVEDELTFLDDSTCIGTS 106
Rattus norvegicus (Rat) 64 NTDEVRVTVLRQTNDQVTEVCATTFTVKNTLGFLDDPFCSGTF 108
Gallus gallus (Chicken) 47 NAKEIRVTLLKQTGDKFTEICASTYTTEFKMFSVEEVIQCHVS 91
Xenopus tropicalis (Frog) 46 KVEEMRFRLLRKMGNQVKEICAFSYSTNYESVTTGDAIQCEGE 90
Highly Conserved Aminoacid

Table 3:

Chr.	*Basepair*	*Ref.*	*Alt.*	*Zygosity*	*Gene symbol*	*Nucleotide*	*Aminoacid*	*rsID*	*ESP*	*ExAC*	*1KG*	*CADD-PHRED*	*PolyPhen1*	*PolyPhen2*	*SIFT-Score*
2	**204735456**	**C**	**T**	**HET**	***CTLA4***	**c.C257T**	**p.A86V**	**RS376038796**	**0.0001**	**0.0000**	**0.0002**	**24.2**	**1**	**0.978**	**0.22**
2	47168856	C	G	HET	*TTC7A*	c.C176G	p.P59R	RS201805434	NA	0.0045	0.0010	NA	0.028	0.008	0.5
6	33281504	C	A	HET	*TAPBP*	c.G175T	p.D59Y	RS45583737	0.0055	0.0051	0.0034	26.2	1	0.996	0
6	109796653	G	A	HET	*ZBTB24*	c.C1237T	p.R413C	RS149690823	0.0005	0.0003	0.0002	35	0.999	0.828	0
9	311975	G	A	HET	*DOCK8*	c.G346A	p.V116M	RS143461644	0.0009	0.0009	0.0002	19.64	0.994	0.763	0.12

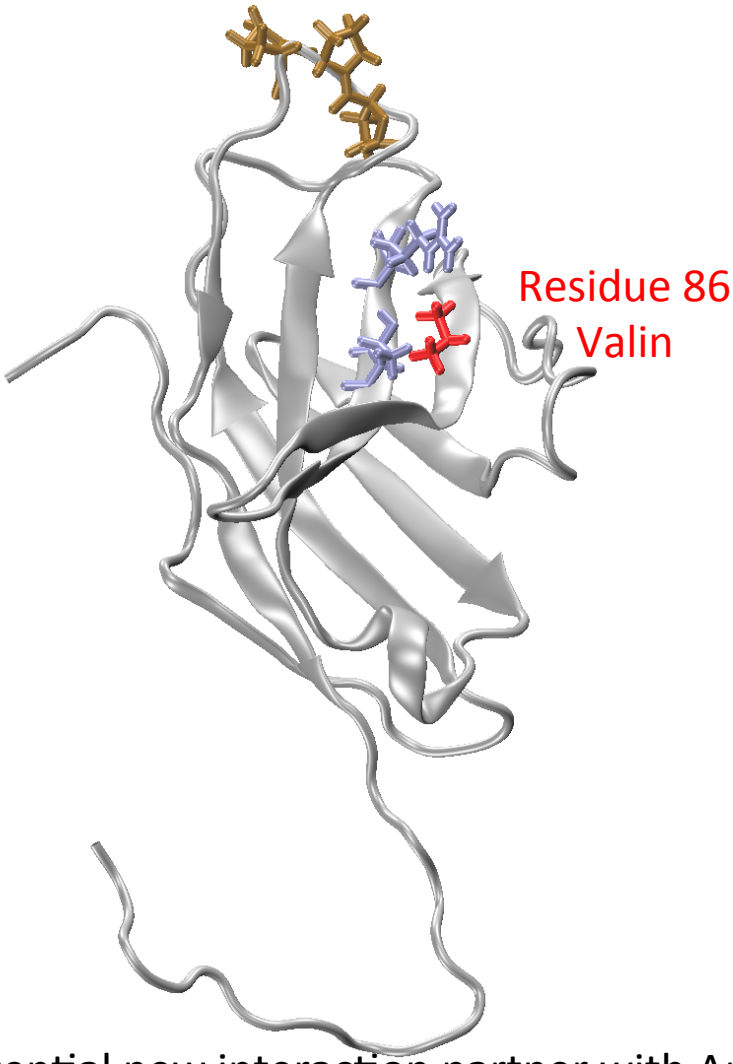
Supplemental Figure 1:

Wild type human CTLA-4



Side chain of Ala 86 interacts with Thr 35. Interaction defined as 2.5 Å.

CTLA-4 mutation p.A86V



Potential new interaction partner with Arg 33 (ice blue). Possible steric clash with Thr 35 (ice blue).