

Letter to the Editor

Abatacept alleviates severe autoimmune symptoms in a patient carrying a *de novo* variant in *CTLA-4*

To the Editor:

Inhibitory immune receptors presented on regulatory T (Treg) cells provide a critical balance in immune modulation by interacting with ligands expressed in antigen-presenting cells. Cytotoxic T-lymphocyte antigen 4 (CTLA-4) on Treg cells suppresses immune reactions by interacting with CD80 and CD86, which are displayed by antigen-presenting cells, making it a useful target for immune modulation. Haploinsufficiency of *CTLA-4* in human or knockout of the gene in mouse leads to immune dysregulation.¹⁻³ Here we describe a patient who presented with severe multiorgan autoimmune disorders. Genetic analysis of the patient genome revealed a functional mutation in *CTLA-4*, and treatment with CTLA-4-Ig agent, a CTLA-4 mimetic, reconstructed Treg-cell functionality and improved the patient's condition, suggesting CTLA-4-Ig agent as a potential therapeutic solution for autoimmune patients with *CTLA-4* mutation.

The patient is a 14-year-old Korean girl who has suffered from chronic diarrhea since age 3 months (Fig 1, A, and see Fig E1 in this article's Online Repository at www.jacionline.org). At the age of 4 years, esophagogastroduodenoscopy with biopsy revealed villi atrophy and heavy lymphoplasmic cell infiltration in the lamina propria of the duodenum, leading to the diagnosis of autoimmune enteropathy (see Fig E2 in this article's Online Repository at www.jacionline.org). At the age of 7 years, she developed anemia with vitamin B₁₂ deficiency. Autoimmune hepatitis was diagnosed at the age of 8 years with periodically high levels of AST (aspartate transaminase, >1000 IU/L) and ALT (alanine transaminase, >1000 IU/L) and liver biopsy. Antiplatelet antibody and smooth muscle antibody signal turned positive, and transiently elevated IgG level was noted (see Table E1 in this article's Online Repository at www.jacionline.org). At the age of 11 years, the patient was admitted with high fever (39°C) and jaundice accompanied by pancytopenia. Direct Coombs test result was positive. Bone marrow examination for the evaluation of pancytopenia revealed hemophagocytosis, and corticosteroids and antibiotics were applied. Subsequently, pulmonary aspergillosis and sepsis were observed. At age 12 years, she developed hematochezia and endoscopic evaluation revealed mixed tubular adenocarcinoma of the stomach (stage 1, T3, N0; see Fig E3 in this article's Online Repository at www.jacionline.org). She underwent a subtotal gastrectomy with surgical liver wedge biopsy. Hepatic pathology showed micronodular liver cirrhosis. Multiple areas of joint pain were noted before the surgery. She remained below the third percentile of Korean growth references for height and weight and virtually stopped growing after the surgery (see Fig E4 in this article's Online Repository at www.jacionline.org). Multiple rounds of immunosuppressants were administered during the course, but none achieved substantial improvements (see Fig E1). The patient does not carry any notable protein-altering variants in *FOXP3* or *AIRE* by Sanger sequencing, excluding

the possibility of suffering from these genetically predefined autoimmune syndromes.

Whole-exome sequencing of the patient and her healthy parents led to the discovery of a nonsynonymous *de novo* variant (p.Pro138Thr) in *CTLA-4* (Fig 1, B; see Tables E2, E3, and E5 and Figs E5-E8 in this article's Online Repository at www.jacionline.org). The point mutation lies in the extracellular MYPPPY ligand-binding motif (Fig 1, C).⁴ The motif is well conserved in most of the vertebrates, and the mutated residue Pro138 is completely conserved in all the vertebrate species examined. Structural analysis demonstrated that the motif forms the ligand interacting surface for CD80 and CD86 (Fig 1, D; see Fig E9 in this article's Online Repository at www.jacionline.org).^{5,6} Reconstructing T-cell stimulation *in vitro* demonstrated that the expression of wild-type CTLA-4 in Jurkat cells successfully implemented the immune modulation activity as assayed by IL-2 production while the introduction of the mutation into CTLA-4 eliminated the immune modulation activity (Fig 1, E).

Next, we tested whether controlling the CTLA-4-mediated signal through medication could improve the patient's condition. Abatacept (Orencia) is a fusion protein formed by an IgG₁ Fc region linked with the extracellular domain of CTLA-4 (CTLA-4-Ig), and is commonly used to alleviate rheumatoid arthritis symptoms. Notably, there was a recent case report on abatacept treatment in an adult with idiopathic autoimmune enteropathy.⁷ To investigate the molecular effect of the drug, we sampled the patient's blood at the trough level (ie, 0 day) and at 1, 4, 6, and 8 days (among these, samples from the trough level and days 4 and 8 were subjected to the fluorescence-activated cell sorting analysis) after the administration of the drug (Fig 2, A-D; for drug treatment details, see the Methods section in this article's Online Repository at www.jacionline.org). Notably, the proportion of CD25⁺ Treg cells (CD4⁺CD25⁺FOXP3⁺ or CD4⁺CD25^{high}CD127⁻ cells) was lower in the patient than in healthy donors while the proportion of CD4⁺FOXP3⁺ cells was not reduced in the patient when compared with healthy donors, as appeared in the previous report on patients with *CTLA-4* haploinsufficiency (Fig 2, A; see Fig E10 in this article's Online Repository at www.jacionline.org).¹ Moreover, administration of the drug actively reduced CD25 expression and increased FOXP3 expression as indicated by the proportion of CD4⁺FOXP3⁺ cells (Fig 2, A; see Fig E11 in this article's Online Repository at www.jacionline.org). This suggests a restoration of Treg-cell function, possibly through the wild-type allele of *CTLA-4*, as FOXP3 is a Treg-cell function regulator.⁸ Unlike the previous reports with patients having haploinsufficiency of CTLA-4 expression, our patient showed even higher CTLA-4 expression in Treg cells (CD4⁺CD25⁺FOXP3⁺) than did healthy donors (Fig 2, B).^{1,2} Remarkably, the drug treatment effectively reduced CTLA-4 expression in Treg cells, as demonstrated by fluorescence-activated cell sorting and quantitative RT-PCR analyses (Fig 2, B and C), indicating a reduced proliferation of Treg cells as a proliferating Treg-cell preferentially expresses CTLA-4.⁹ The drug treatment increased *TGF-β1* expression level, demonstrating that the drug successfully restores Treg-cell functionality (Fig 2, C; see Table E5).¹⁰ The drug treatment restored CD3⁺ T-cell level and attenuated inflammatory cytokine production such as IL-10, IL-17A, and IL-6 from patient-derived PBMCs

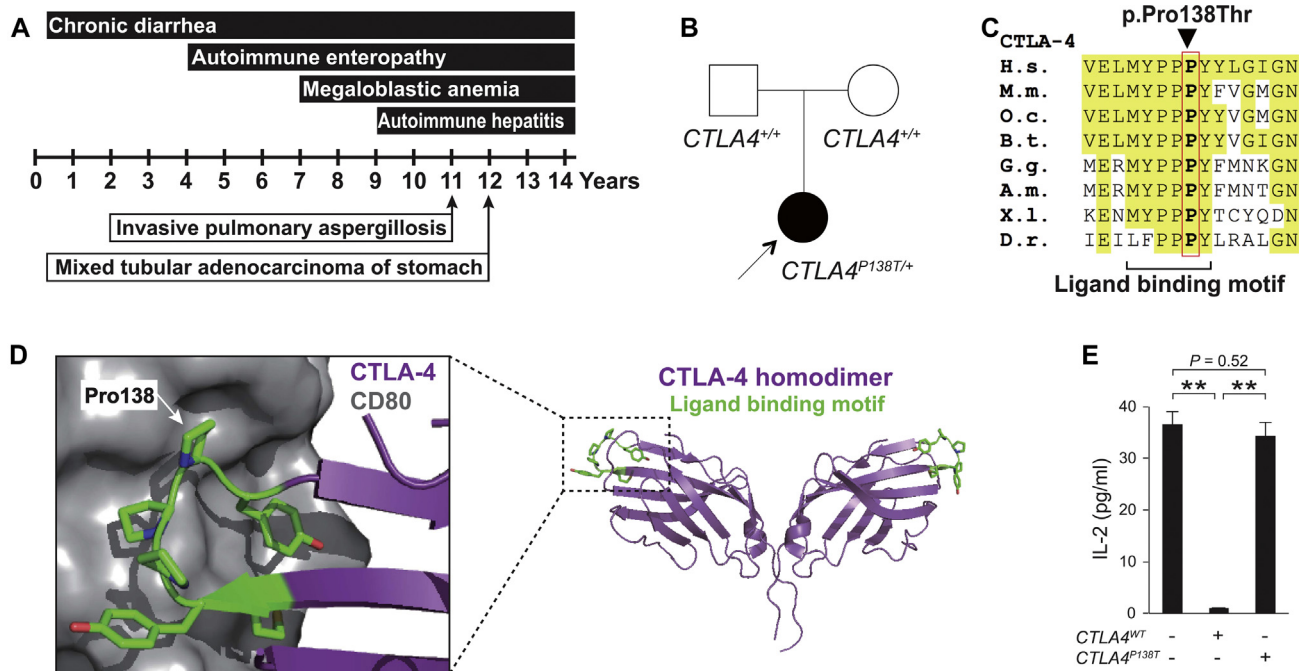


FIG 1. De novo CTLA-4 mutation in early-onset multiorgan autoimmune disease. **A** and **B**, Disease course and pedigree of the patient. **C**, The evolutionary conservation of the mutated residue. **D**, Structure of CTLA-4 homodimer (right)⁶ and interaction with CD80 (left).⁶ **E**, Overexpressed CTLA4^{P138T} lost the ability to suppress IL-2 production in Jurkat cells. A.m., Alligator mississippiensis; B.t., Bos taurus; D.r., Danio rerio; G.g., Gallus gallus; H.s., Homo sapiens; M.m., Mus musculus; O.c., Oryctolagus cuniculus; X.l., Xenopus laevis. ***P* < .005.

(Fig 2, D; see Table E4 in this article's Online Repository at www.jacionline.org). Initial serum levels of IL-10, IL-17A, and IL-6 were higher in the patient than in her healthy parents, and the increased serum cytokine levels were reduced in a similar fashion by the drug treatment (see Fig E12 in this article's Online Repository at www.jacionline.org).

Clinically, treatment with abatacept without other immunosuppressants reduced diarrhea output by about 67% (from 3 L/d to 1 L/d) and her stool consistency has recently improved from watery to partially formed status. Remarkably, the blood transfusion volume has dropped to zero (Fig 2, E and F), allowing the patient to be free of the other immunosuppressants. She was discharged to outpatient care with partial parenteral nutrition after 3 years of hospitalization. Her direct Coombs test results turned negative after 8 months of abatacept treatment. After cessation of the steroid, hyperglycemia and adrenal insufficiency were easily controlled.

Here we report a direct effect of CTLA-4 dysfunction in human; enhancing the CTLA-4-mediated signal with CTLA-4-Ig toned down immune responses in a patient with severe autoimmune features, eventually achieving substantial clinical improvements. Therefore, it would be worthwhile to investigate patients with idiopathic autoimmune features for additional CTLA-4 mutations that may affect proper protein function and use CTLA-4-Ig as a potential therapeutic solution.

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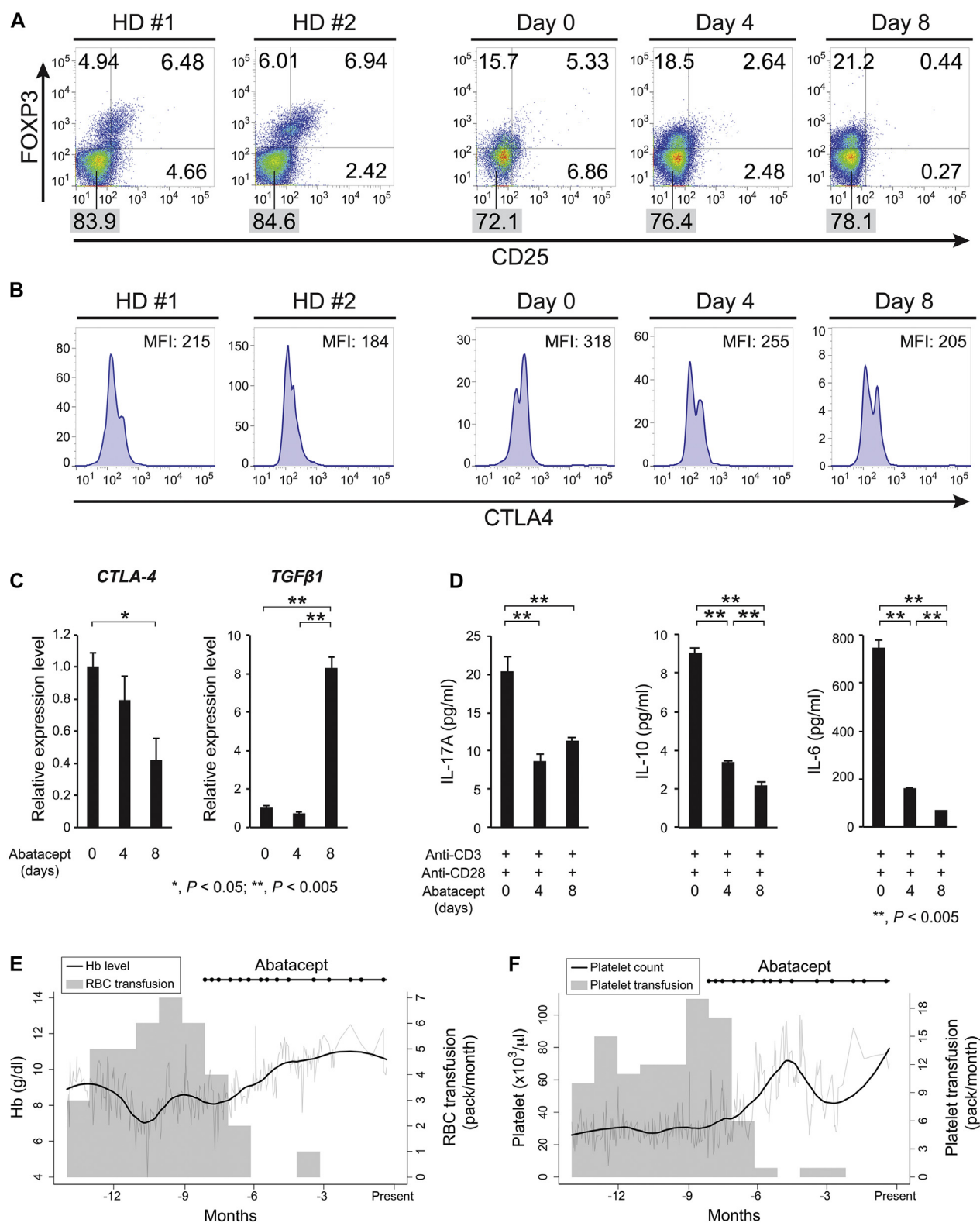


FIG 2. CTLA-4-Ig agent abatacept controls immune reactivity of the patient. **A**, Treg-cell ($CD4^+CD25^+FOXP3^+$) population in healthy donors (HD) and the patient. **B**, CTLA-4 expression level in $CD4^+CD25^+FOXP3^+$ cells. **C**, Quantitative RT-PCR of Treg-cell markers from the patient-isolated Treg cells. **D**, Concentration of cytokines from patient-derived PBMC culture supernatant. **E** and **F**, Patient blood levels of hemoglobin and platelet (gray and smoothed black lines) plotted against RBC and platelet transfusion rates (gray bars). Period and actual administration of abatacept treatment are depicted as a black line with dots. MFI, Mean fluorescence intensity. * $P < .05$ and ** $P < .005$.

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1 **Online repository**

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3 **Abatacept alleviates severe autoimmune symptoms in a patient carrying**
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22 **Table E1.** Serum immunoglobulin levels

	Age (year)	Level	Reference range
IgG	5.3	678 mg/dL	574-1,309
	7.0	770 mg/dL	501-1,459
	7.5	959 mg/dL	501-1,459
	8.3	1,585 mg/dL	501-1,459
	9.3	2,240 mg/dL	501-1,459
	11.7	857 mg/dL	599-1,590
	11.8	532 mg/dL	599-1,590
	11.9	1,302 mg/dL	599-1,590
	12.3	1,033 mg/dL	599-1,590
IgA	5.3	111 mg/dL	39-170
	7.0	102 mg/dL	34-181
	7.5	225 mg/dL	34-181
	8.3	286 mg/dL	34-181
	9.3	260 mg/dL	34-181
	11.7	243 mg/dL	61-198
	11.8	90 mg/dL	61-198
	11.9	153 mg/dL	61-198
	12.3	110 mg/dL	61-198
IgM	5.3	76 mg/dL	54-200
	7.0	58 mg/dL	42-181
	7.5	69 mg/dL	42-181
	8.3	115 mg/dL	42-181
	9.3	188 mg/dL	42-181
	11.7	134 mg/dL	54-228
	11.8	107 mg/dL	54-228
	11.9	142 mg/dL	54-228
	12.3	206 mg/dL	54-228
IgE	5.3	76 IU/ml	8-279
	7.5	27 IU/ml	8-279
IgD	12.8	<1.1 mg/dL	0-8

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24 **Table E2.** Whole exome sequencing run summary

Case	Proband	Mother	Father
Read length (bp)	2 x 74	2 x 74	2 x 74
Number of reads (M)	57.5	56.4	53.5
Median coverage depth (X)	46	51	48
Mean coverage depth (X)	57.5	58.9	54.8
% of reads on genome	90.3%	92.8%	92.7%
% of reads on target	66.2%	67.5%	66.3%
% of bases covered at least 4x	96.3%	97.0%	96.9%
% of bases covered at least 8x	93.1%	94.7%	94.4%
Per-base error rate	0.60%	0.33%	0.33%
% of PCR duplicate	4.4%	3.0%	3.0%

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Table E3. List of coding *de novo* variants

Gene	Chromosome: position (hg19)	Nucleotide substitution	Zygosity	Impact on protein	Amino acid change	Amino acid location/ protein length	Number of vertebrate species with different amino acid from human/Number of vertebrate species with ortholog	PhyloP	SIFT	PolyPhen-2	Coverage (alternative allele/total)		
											Proband	Mother	Father
<i>RAD54L</i>	1:46739123	A>G	Heterozygous	Missense	p.Glu491Gly	491/747	2/97	3.289	0.47	0.304	38/84 (45.2%)	0/97	0/93
<i>SARS</i>	1:109779127	G>A	Heterozygous	Missense	p.Arg405Gln	405/514	0/95	4.308	0.01	0.998	21/35 (60.0%)	0/58	0/42
<i>CTLA4</i>	2:204735611	C>A	Heterozygous	Missense	p.Pro138Thr	138/223	0/81	3.934	0.00	0.999	21/32 (65.6%)	0/35	0/56

Table E4. Peripheral blood lymphocyte counts

	Normal range ⁶		Healthy donor 1	Healthy donor 2	Patient		
	Adult	12-17 years			Day 0	Day 4	Day 8
T cell (CD3 ⁺)	527-2,846	1,000-2,200	1,329	863	33	264	286
B cell (CD19 ⁺)	78-899	110-570	272	405	262	206	127

Unit: number of cells/ μ l

Table E5. PCR primers used in this study

Purpose	Primer	Nucleotide sequences 5'-3'	Product length (bp)
<i>De novo validation</i>	<i>RAD54L</i> - F	GTG GAA GAG GAG GAT GGC TT	247
	<i>RAD54L</i> - R	TTA CCT GGG AAG AGC AGC AA	
	<i>SARS</i> - F	TCT TTG AAT CAT GCT GCC AGT	204
	<i>SARS</i> - R	GGC CGT GTA CTG AAG ACT CT	
	<i>CTLA4</i> - F	GGC AAC CTA CAT GAT GGG GA	363
	<i>CTLA4</i> - R	GTT GCC ACC CAC AAT AAG CA	
<i>qRT-PCR</i>	<i>CTLA4</i> - F	GAA GTC TGT GCG GCA ACC TA	201
	<i>CTLA4</i> - R	AAT CTG GGT TCC GTT GCC TA	
	<i>TGFβ1</i> - F	CCC AGC ATC TGC AAA GCT C	101
	<i>TGFβ1</i> - R	GTC AAT GTA CAG CTG CCG CA	
	<i>GAPDH</i> - F	GGA GCG AGA TCC CTC CAA AAT	197
	<i>GAPDH</i> - R	GGC TGT TGT CAT ACT TCT CAT GG	

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Abatacept alleviates severe autoimmune symptoms in a patient carrying a *de novo* variant in *CTLA-4*

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Text E1. Supplementary Methods

Subject

One subject with multi-organ autoimmune disease and her healthy parents were included in the study. Clinical features are summarized in FIG 1, A and Fig E1 in this article's Online Repository at www.jacionline.org. Genomic DNAs were extracted from peripheral leukocytes using standard procedures. The subject and her parents gave informed consent for DNA storage and genetic analysis. All samples had been collected for research purposes, after approval by institutional review board of Seoul National University Children's Hospital (1408-014-599).

Whole-exome sequencing and variant calling

DNA was prepared from venous blood samples of the proband and her parents and processed at Yale Center for Genome Analysis (YCGA). Genomic DNA was captured on the NimbleGen 2.1M human exome array and subjected to 74 base paired-end reads on the Illumina HiSeq instrument as described.¹ Sequence reads were mapped to the reference genome (hg19) using the ELAND program. Reads outside of the targeted sequences were discarded and statistics on coverage were collected from the remaining reads using Perl scripts. SAMtools was used to call variant bases appended with quality scores.² Among these, common variants that are listed in public databases (dbSNP build 137, 1000 Genomes Project release 10.31.2012 and NHLBI Exome Sequencing Project) were excluded and only rare variants were considered as potential causative

variants. Identified variants were annotated based on novelty, impact on the encoded protein, evolutionary conservation, and expression using an automated pipeline. PhyloP score and amino acid conservation are extracted from UCSC genome database.³

Identification and confirmation of *de novo* mutations

Identification of *de novo* mutations was performed as previously described.⁴

Briefly, heterozygous single nucleotide variants and short insertions and deletions (indels) in the proband that showed SAMtools QS ≥ 60 and 600, respectively, and rare non-reference calls in both parents were selected. A Bayesian algorithm was used to assist *de novo* mutation calls. Elements included probability of the proband being heterozygous at the test position; probability that parents are homozygous for the reference allele, given frequency of reference and non-reference reads and probability of heterozygosity in offspring; probability that a variant is *de novo* given its population frequency. Resulting Bayesian QSs were scaled from 0 to 100. All three *de novo* SNVs with Bayesian QS > 50 were validated by Sanger sequencing of the PCR fragments containing the variants.

The primer sequences for PCR amplifications are shown in Table E5 in this article's Online Repository at www.jacionline.org.

Orthologs

Proteins encoded by orthologs of CTLA-4 in vertebrate species were identified by a BLAST search. GenBank accessions for these included NP_005205.2 (*Homo*

sapiens), NP_033973.2 (*Mus musculus*), NP_001076154.1 (*Oryctolagus cuniculus*), NP_776722.1 (*Bos taurus*), NP_001035180.1 (*Gallus gallus*), XP_006267305.1 (*Alligator mississippiensis*), NP_001091201.1 (*Xenopus laevis*) and XP_005167576.1 (*Danio rerio*). Multiple sequences were aligned by ClustalW2 software.⁵

3D structure analysis

The crystal structure models of CTLA-4 homodimer (Protein Data Bank (PDB) ID: 3OSK), CTLA-4/CD80 complex (PDB ID: 1I8L), and CTLA-4/CD86 complex (PDB ID: 1I85) were downloaded from Protein Data Bank (<http://pdb.org>). PyMOL was used to visualize the structures (<http://www.pymol.org>).

Abatacept treatment

As an initial loading, 250 mg of abatacept (Orencia[®], Bristol-Myers Squibb, New York; 10 mg/kg every 2 weeks) was injected intravenously. After three injections, the patient is under a current maintenance dose of 125 mg (5 mg/kg in a month) via subcutaneous injections using prefilled syringes.

IL-2 production assay

Jurkat cells were transfected with plasmid DNA (*CTLA4^{WT}* or *CTLA4^{P138T}*) using Lipofectamine 2000 (Invitrogen) and the transfected cells (GFP⁺) were sorted by FACS Aria instrument (BD Biosciences). IL-2 release was detected by ELISA (eBioscience) according to the manufacture's instruction from the culture

supernatants of conjugates formed between SEE (Toxin Technology, Inc.) - loaded Raji cells and transfected Jurkat cells. Conjugates were formed and cultured in flat-bottom, 96-well plates. Culture supernatant was harvested after culture for 36 hours.

Complete blood cell count

Complete blood cell count and differential count of white blood cell are measured by ADVIA 2120i Hematology System (SIEMENS).

PBMCs isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from proband's peripheral venous blood, collected from Hickman catheter, by gravity gradient using Ficoll-PaqueTM PLUS (GE Healthcare Life Sciences).

Regulatory T cell analysis and sorting

For regulatory T cell population analysis and sorting, isolated PBMCs were stained with fluorochrome-conjugated anti-human CD4 (RPA-T4, BD Biosciences), anti-human CD25 (M-A251, BD Biosciences), and anti-human CD127 (eBioRDR5, eBioscience). Regulatory T cells (T_{reg} , $CD4^{+}CD25^{high}CD127^{-}$) were analyzed and sorted using FACS Aria III flow cytometer (BD Biosciences). The collected data were analyzed with FlowJo software (Treestar).

Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from isolated T_{reg} cells by RNA Cell Miniprep System (ReliaPrep™) and complementary DNAs were generated by reverse transcription. Quantitative RT-PCR analyses were performed using SYBR mix (Takara) and Mx3005p (Stratagene) with *CTLA-4* or *TGF- β 1* specific primer set (see Table E5 in this article's Online Repository at www.jacionline.org).

Cytokine measurement

To measure secreted IL-17A, IL-10 and IL-6 from activated PBMC, we isolated PBMC from patient before (day 0) and after (day 4 and 8) the drug treatment and the isolated 2×10^5 cells were activated with anti-human CD3 (UCHT1, eBioscience) and anti-human CD28 (CD28.2, eBioscience) antibodies at 2 μ g/mL each for 72 h. Cultured media and patient's serum were used for cytokine measurement. IL-17A, IL-10 and IL-6 were measured with Cytometric Bead Array (BD Bioscience) using Canto II flow cytometer (BD Bioscience).

Statistical analysis

The significance of mRNA level of regulatory T cells and cytokine production of PBMCs after activation differences between groups was tested with two-tailed Student's *t*-test. Changes in hemoglobin level and platelet count were smoothened by local polynomial regression fitting (loess function, span=0.3) in R software (<http://www.r-project.org>).

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Supplementary figure legends

FIG E1. Medical history of the patient depicting major autoimmune-related diagnoses and immunosuppressant treatments.

FIG E2. Microscopic view of endoscopic biopsy from colon epithelium at 12 years of age. **A**, Hematoxylin and eosin (H&E) staining of colon epithelium revealed diffuse lymphoplasmacytic infiltration in lamina propria. **B**, Immunohistochemical (IHC) staining with CD3 was strongly positive in lamina propria. **C**, CD20 was focally positive.

FIG E3. Pathology of gastric adenocarcinoma. **A**, H&E stained microscopy of gastric adenocarcinoma shows irregular glands and nuclear atypia which is consistent with mixed tubular adenocarcinoma of stomach. **B**, IHC staining with carcinoembryonic antigen 19 (CEA) revealed extensive expression in cytoplasm. **C**, p53 IHC was positive in majority of cells. **D**, Ki-67 was positive in about 45% of cells.

FIG E4. Growth curve of the patient (age 5-14). The times of subtotal gastrectomy and start of abatacept were indicated with arrow and arrowhead, respectively.

FIG E5. An extended pedigree of the studied family. No family history of autoimmune disease other than the proband was reported.

FIG E6. Genome-wide loss-of-heterozygosity (LOH) plot of the proband.

Alternative allele frequencies of dbSNP variants are plotted against chromosomal locations.

FIG E7. Direct Sanger sequencing traces for three de novo variants. The mutated nucleotides are indicated by asterisks, and the encoded amino acids are shown in single-letter code.

FIG E8. Direct Sanger sequencing traces for the *CTLA-4* mutation from genomic DNA samples obtained from patient's scalp hair, saliva, and urine.

FIG E9. Crystal structure of CTLA-4 interacting with CD86. CTLA-4 homodimer interacting with its ligand, CD86 (top) and its close-up view (bottom) (PDB ID: 1I85).⁷ Pro138 is in direct contact with CD86 surface.

FIG E10. Flow cytometric analysis of T_{reg} (CD4⁺CD25^{high}CD127⁻) population in healthy donors (HD) and the patient. Numbers in the plots denote percentages of T_{reg} among CD4⁺ cells.

FIG E11. Increased FOXP3 expression after abatacept treatment as measured by flow.

FIG E12. Serum cytokine level changes before and after abatacept treatment.

Significance of difference in cytokine level compared to that of day 0 is indicated by an asterisk ($P < 0.05$) or double asterisks ($P < 0.005$).

A, Serum IL-17A. **B,** Serum IL-10. **C,** Serum IL-6. F, father; M, mother.

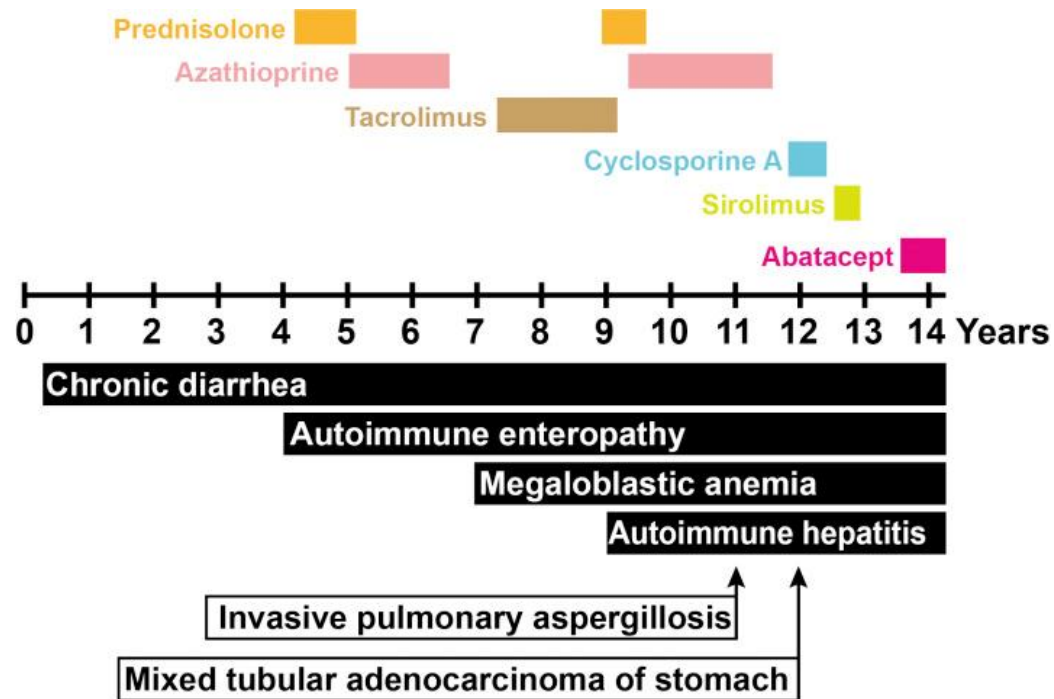


Fig E1.

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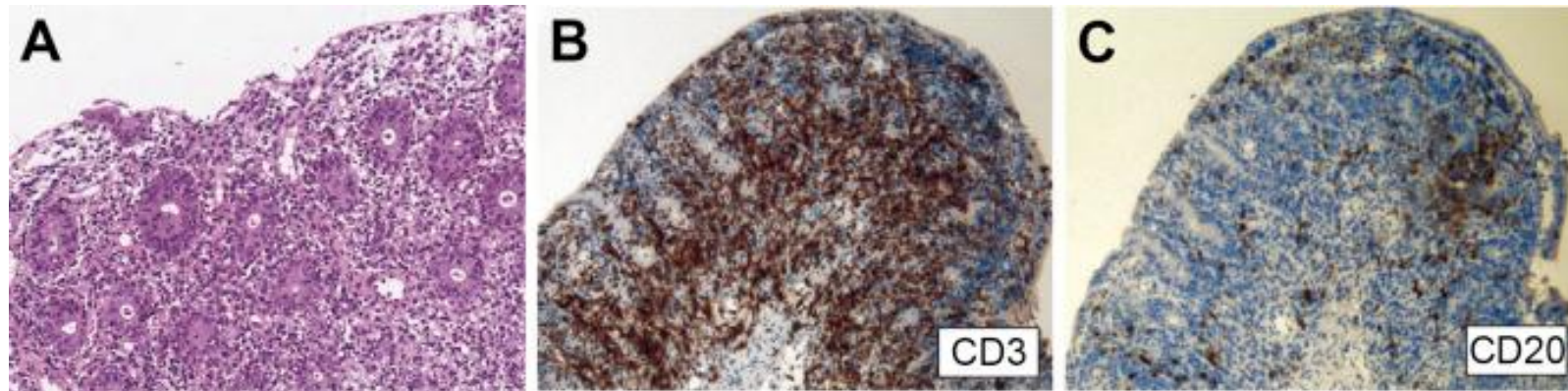


Fig E2.

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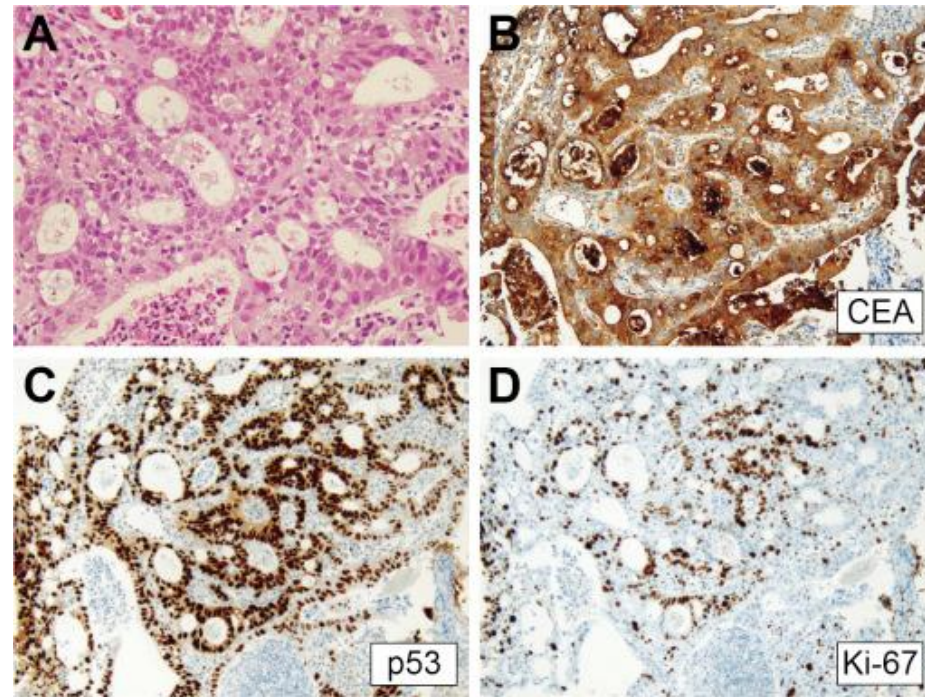


Fig E3.

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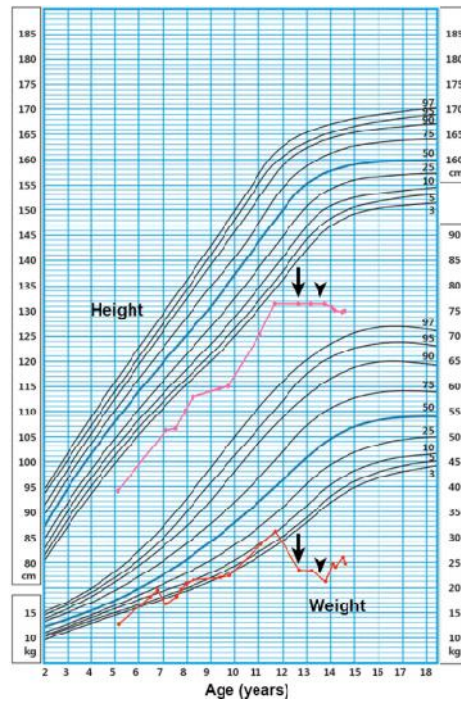


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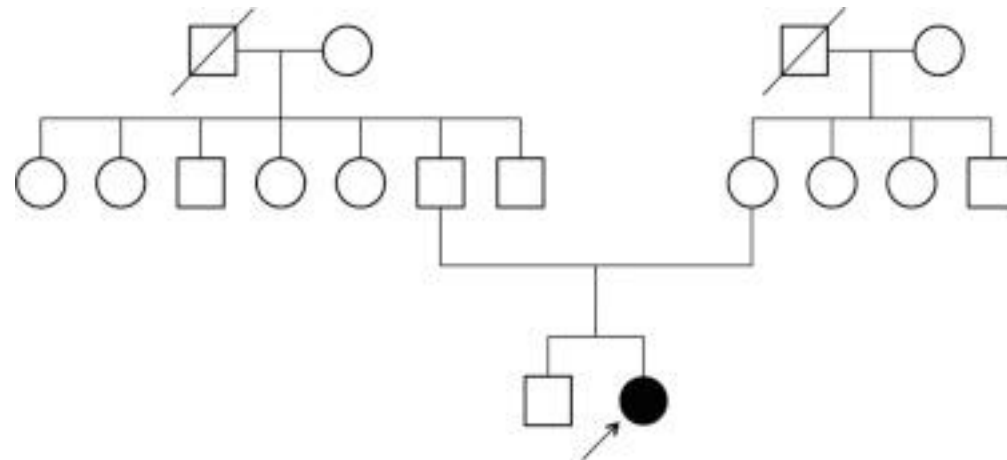


Fig E5.

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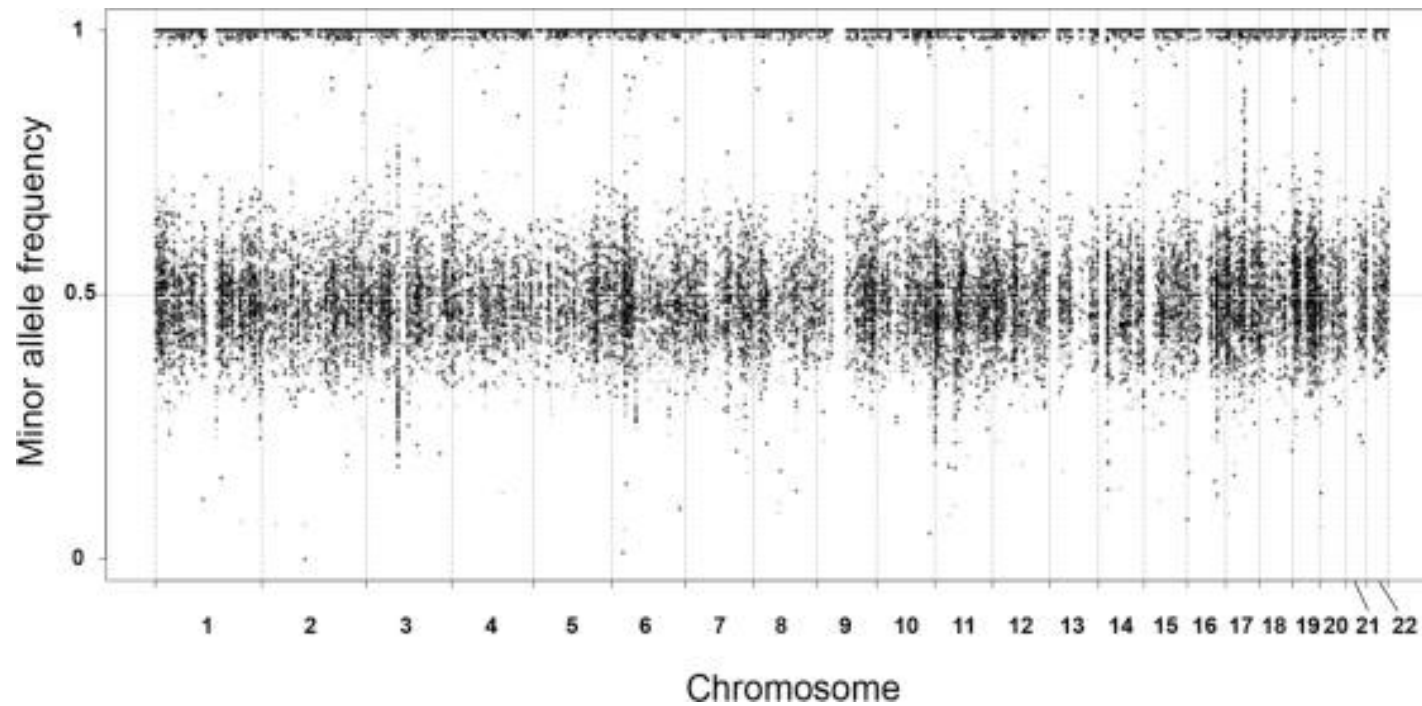


Fig E6.

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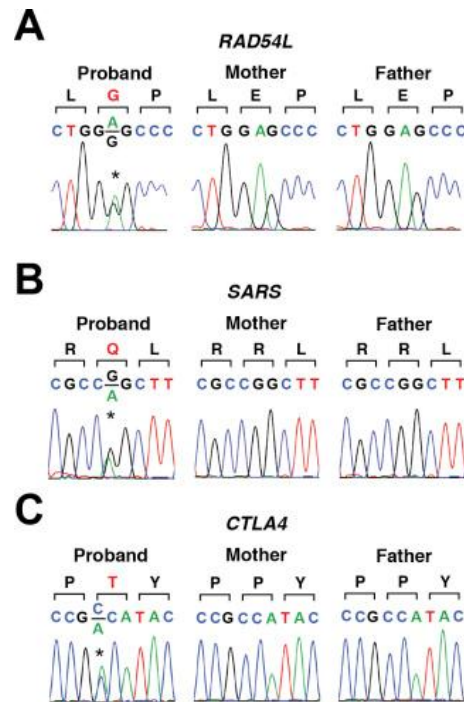


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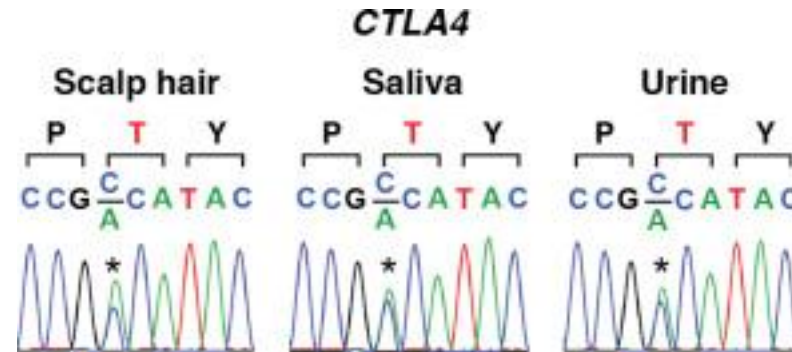


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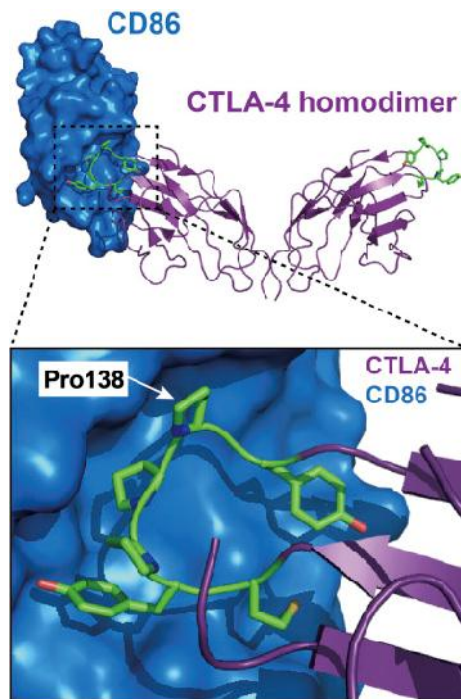


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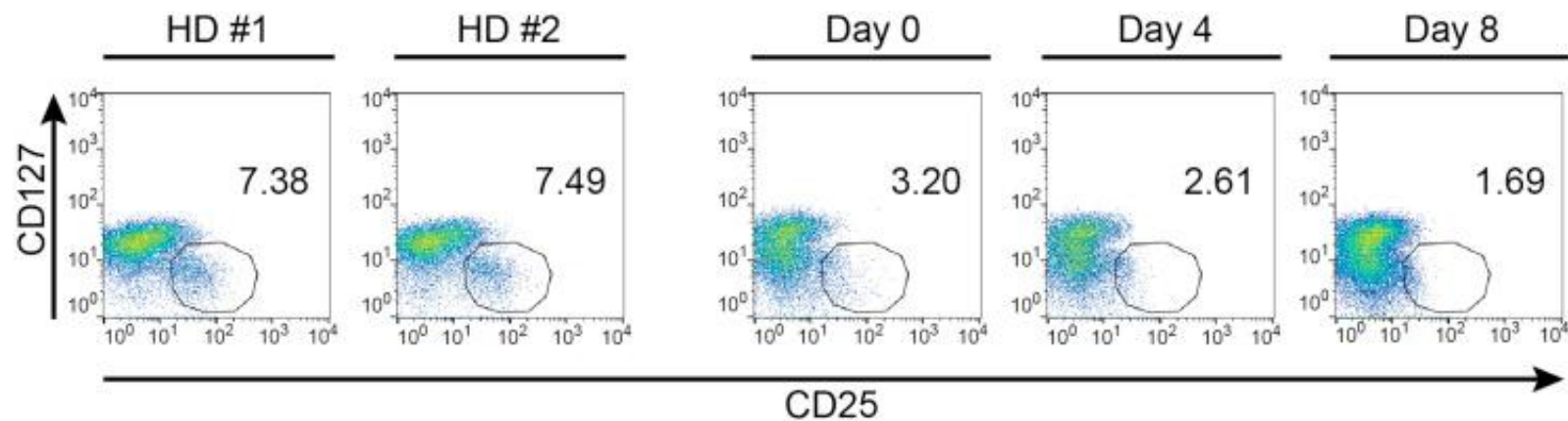


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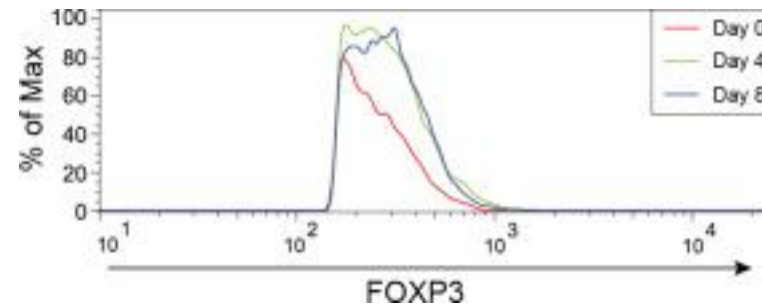


Fig E11.

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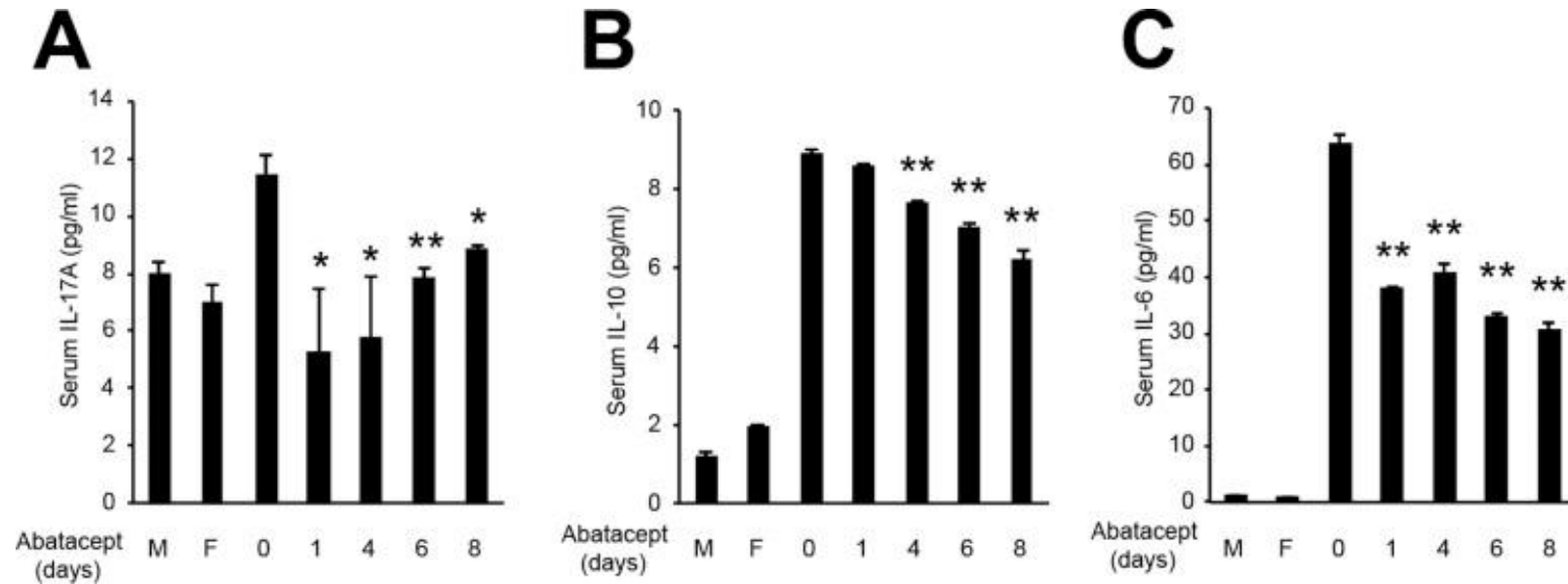


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