Immune dysregulation in human subjects with heterozygous germline mutations in CTLA4

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Cytotoxic T lymphocyte antigen–4 (CTLA-4) is an inhibitory receptor found on immune cells. The consequences of mutations in CTLA4 in humans are unknown. We identified germline heterozygous mutations in CTLA4 in subjects with severe immune dysregulation from four unrelated families. Whereas CTLA4 heterozygous mice have no obvious phenotype, human CTLA4 haploinsufficiency caused dysregulation of FoxP3+ regulatory T (Treg) cells, hyperactivation of effector T cells, and lymphocytic infiltration of target organs. Patients also exhibited progressive loss of circulating B cells, associated with an increase in predominantly autoreactive CD21+ B cells and accumulation of B cells in nonlymphoid organs. Inherited human CTLA4 haploinsufficiency demonstrates a critical quantitative role for CTLA-4 in governing T and B lymphocyte homeostasis.

REFERENCES AND NOTES


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SUPPLEMENTARY MATERIALS

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Immune tolerance is controlled by multiple mechanisms (I, 2), including regulatory T (Treg) cells (3-5) and inhibitory receptors (6, 7). Treg cells constitutively express the inhibitory receptor CTLA-4, which confers suppressive functions (8, 9). CTLA-4, also known as CD152, is also expressed by activated T cells and, upon ligation, inhibits their proliferation (10). Homozygous deficiency of Ctla4 in mice causes fatal multiorgan lymphocytic infiltration and destruction (11-13); hence, CTLA-4 functions at a key “checkpoint” in immune tolerance. CTLA-4–immunoglobulin (Ig) fusion protein and neutralizing CTLA-4 antibody are used to modulate immunity in autoimmune and cancer patients (14, 15), respectively. Studies have given conflicting results regarding the association of CTLA4 single-nucleotide variants (SNVs) with organ-specific autoimmunity (16). The consequences of genetic CTLA4 defecti...
Fig. 1. Clinical phenotype and pedigree of the patients. (A) Top: Computed tomography images of lung and brain from patient A.I.1. Bottom: Histological section (magnification 20×) from a duodenal biopsy from a healthy donor (HD) and patient A.II.1 stained for CD3 (brown cells), showing an increased number of transepithelial T cells within the villi. (B) Flow cytometric analyses of CD4+ cells or total lymphocytes stained for the indicated surface markers from a healthy donor and patient A.I.1. Data showing decreased CD45RA+CD62Lnaïve CD4+ T cells are representative of three patients (A.I.I, A.II.1, and B.I.I). Programmed cell death–1 (PD1) expression data shown are representative of five patients (A.I.I, A.II.1, B.I.I, C.I.I, and D.I.I) and three healthy donors. Data showing decreased circulating B cells are representative of two patients (A.I.I and A.II.1). (C) Mutations in patient alleles displayed on a schematic of the four exons of CTLA4, pedigrees, and phenotype summary highlighting organs (gray) with inflammatory infiltrates and autoimmune cytopenias for affected family members. TM, transmembrane domain. (D) Protein and mRNA expression of CTLA4 in Treg cells. Left: Levels of CTLA4 expression in Treg cells (CD4+CD25+FoxP3+) were assessed by intracellular staining. The numbers in the upper right corner depict mean fluorescence intensity (MFI) of anti-CD152 (CTLA-4) staining. Dotted line indicates the peak of CTLA-4 expression in a healthy donor. Data shown are representative of three experiments. Right: Levels of CTLA4 mRNA in Treg cells (CD4+CD25+CD127+) sorted from seven different healthy donors and four patients were measured by real-time PCR using the probe for CTLA4 transcript variant 1 (full length) and normalized to GAPDH. Data are means of replicates from six experiments. For relative gene expression, all data were normalized to the same HD. The horizontal lines indicate mean values from healthy donors or patients.

Fig. 2. Abnormal Treg cell phenotype and function in patients. (A) Flow cytometric analysis of FoxP3 and CD25 in CD4+ T cells from healthy donor (HD) and patients. (B) Mean fluorescence intensity of FoxP3 and CD25 in CD4+ FoxP3+ T cells from healthy donors and patients. Data are means ± SEM of replicates of indicated patient [A.I.I (N = 7), A.II.1 (N = 1), B.I.I (N = 2), C.II.1 (N = 2), D.I.I (N = 4)] and 10 healthy donors. The N values represent number of replicates from each patient. ***P = 0.0007 (Mann-Whitney test). Bar graph: Percentage of CD25+ and CD25– cells among CD4+FoxP3+ in 10 healthy donors and five patients. (C) Autologous and heterologous suppressive activities of Treg cells from five healthy donors and four patients (A.I.I, B.I.I, C.I.I, and D.I.I). The horizontal lines indicate the mean values. Data are from three experiments, with each indicated patient paired with one or two healthy donors. *P = 0.0239, **P = 0.0037 (paired t test).
CREATED EXPRESSION OF THE EXHAUSTION MARKER PD-1, IN MELANOMA PATIENTS (CAUSED BY CTLA-4 BLOCKING ANTIBODY TREATMENT). BIOPSY REVEALED HISTOPATHOLOGY SIMILAR TO THAT OF PHOCYTIC BRAIN INFECTION. IN MOST PATIENTS, GI HAD LYMPHADENOPATHY, SEVERE AIHA, AND LYMPHOCYTIC INfiltrATES. HIS 11-YEAR-OLD SON (D.III.1) was reportedly healthy but not clinically evaluated; however, his 11-YEAR-OLD SON (D.I.1) had lymphadenopathy, severe AIHA, and lymphocytic brain infiltration. IN most patients, GI biopsies revealed histopathology similar to that caused by CTLA-4 blocking antibody treatment in melanoma patients (17, 18).

both patients in family A had low CD4+ T cells with depleted CD45RA+CD62L+ naïve cells, increased expression of the exhaustion marker PD-1, and a progressive loss of circulating mature B cells (Fig. 1B and table S1). Similar and overlapping immune phenotypes were detected in the additional families (Fig. 1, B to D, and table S1).

We performed whole-exome sequencing using DNA from A.II.1 and identified a heterozygous, nonsense c.151C>T (p.R51X) mutation in CTLA4. This was confirmed by Sanger sequencing in the proband and A.I (Fig. 1C). cDNA analyses from A.I.T cells showed that the mutant allele mRNA was degraded >95%, consistent with nonsense-mediated decay (table S2). CTLA4 sequencing in B.I revealed a frameshift deletion (c.75delG; p.L28fs*44) (Fig. 1C) that introduced a stop codon in exon 2. Families C and D had mutations in introns 2 and 3 (458-1G>C and 567+6G>C) (Fig. 1C) disrupting the acceptor and donor sites of the second or third introns, respectively. these mutations generated a CTLA4 mRNA lacking the third exon, putatively encoding a soluble form of CTLA4 (fig. S2A). Full-length CTLA4 mRNA, encoding the membrane-bound form, was reduced. Serum-soluble CTLA-4 was comparable in patients and healthy individuals. IN an extended cohort, de novo mutants were not identified; however, because B.I.I's parental genotypes are unknown, his mutation could be de novo.

affected patients had reduced CTLA4 protein and mRNA expression in sorted Treg cells relative to healthy donors; this reduction persisted after activation (Fig. 1D and fig. S2, A and B). Deletion of Ctla4 in mice impairs Treg cell suppressive function, causing severe autoimmune disease and early lethality despite normal Foxp3 levels (11, 12). We found that patients had normal percentages of Foxp3+ Treg cells with a CD127+CD45R+B220+ Helios+ phenotype (fig. S3, A and B), but overall expressed significantly less Foxp3 and CD25 (interleukin-2 (IL-2) receptor α subunit and a marker of Treg cells) than Treg cells, from healthy donors, and a large proportion of their Treg cells were CD25+ (Fig. 2, A and B). Foxp3+ mRNA was also reduced in patient Treg cells (fig. S3C). Next, we tested the function of healthy donor or patient Treg cells and found that patient Treg cells poorly suppressed proliferation of cocultured
activated autologus or allogeneic T responder cells (Fig. 2C and fig. S3D).

Among the nine subjects harboring CTLA4 mutations, three relatives were reportedly healthy (C.I.1, D.I.1, and D.II.2). Only one of these unaffected healthy relatives (D.I.1) could be evaluated in detail, and she had no clinical findings similar to CTLA4–deficient patients. Her Treg cells showed higher expression of CTLA4 and normal levels of Foxp3 and CD25 (fig. S4, A and B), whereas her effector T cells displayed the same in vitro hyperproliferation observed in affected patients (fig. S4C); these findings suggest that Treg cell dysfunction might be essential for the full disease phenotype.

Consistent with the results of studies in mice (12–13), CTLA4–deficient patients with cells were hyperplorative, with an increased percentage expressing CD25 in response to T cell receptor stimulation (Fig. 3A and fig. S5A). To test whether patient T cell hyperproliferation resulted from reduced CTLA4 expression, we used small interfering RNA (siRNA) to inhibit CTLA4 expression in normal peripheral blood mononuclear cells (PBMCs). A factor of ~3 reduction in CTLA4 recapitulated the hyperproliferative T cell phenotype (Fig. 3B). Moreover, overexpression of wild-type CTLA4 in patient T cells suppressed the hyperproliferation (Fig. 3C and fig. S6, B and C), indicating that quantitative variations in CTLA4 controlled the proliferative potential of T cells. CTLA4–Ig fusion protein also suppressed patient T cell proliferation in vitro (Fig. 3D). Despite hyperproliferation of patient T cells in culture, the patients were lymphopenic. This may be explained by increased FAS (CD95) expression, caspase activity, and apoptosis of patient T cells, or by organ sequestration (fig. S6).

CTLA4 function in B cells has been investigated, but its role remains unclear (8, 12, 19). We found that CTLA4 expression was significantly reduced on activated B cells from patients (fig. S7A). The reduced frequencies of CD27+ class-switched memory B cells and progressive B cell lymphopenia (Fig. 4, A and B), together with known B cell abnormalities in human autoimmune diseases (20), prompted us to further investigate B cell maturation and function.

In inflammatory conditions such as systemic lupus erythematosus, rheumatoid arthritis, Sjogren's syndrome, CVID with autoimmunity and lymphoproliferation, and certain chronic infections, a population of B cells expressing reduced levels of CD21 (termed CD21lo B cells) has been identified (21–23). CD21lo B cells have been viewed as anergic or "exhausted" cells on the basis of observations such as enrichment of self-reactive B cell receptors (BCRs), an activated phenotype, reduced responsiveness to BCR engagement, and increased apoptosis (22–25). We found that the frequency of CD21lo B cells was greatly elevated in patients’ peripheral blood (Fig. 4B) (15 to 90% of B cells in CTLA4–deficient patients versus <5% in controls). This subset progressively accumulated in patient A.I.1 from 41.5% to >95% of peripheral blood B cells over 3 years. Consistent with the anergic/exhausted state of CD21lo B cells (22), we observed heightened apoptosis in patient B cells (fig. S7, B and C) and poor BCR-induced proliferation relative to controls (Fig. 4C). The CD21lo B cells in CTLA4–deficient patients were CD19+CD21loCD38hi, distinguishing them from transitional (CD19hiCD20loCD38lo) cells. Flow cytometric analysis revealed that these cells were phenotypically similar to the CD21lo B cell subset in other immune dysregulation disorders (fig. S8A) (21, 25). Accordingly, autoreactive IgG may be produced by the CD21lo B cells, as a greater proportion were IgG*CD27− cells, relative to the corresponding cells in healthy donors. Functional analysis in vitro indicated that naïve B cells from CTLA4–deficient patients secreted IgM and underwent class switching to secrete IgG and IgA robustly (fig. S8B). However, patient CD21hi B cells secreted less Ig than those of healthy donors (fig. S8B). The propensity of CD21lo B cells to exhibit more apoptosis ex vivo (fig. S7C) and their constitutive expression of CD95 (fig. S8A) could explain peripheral B cell lymphopenia and hypogammaglobulinemia in most CTLA4–deficient patients. Increased frequencies of autoreactive mature naïve B cells have been demonstrated in the blood of patients with immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), which is a primary Treg cell defect (26); this suggests a role for Treg cells in preventing the accumulation of autoreactive B cells in the periphery. Thus, CTLA4 haploinsufficiency decreases B cell tolerance and survival either intrinsically or extrinsically because of Treg dysfunction (26).

Our data indicate that germline CTLA4 haploinsufficiency causes lymphoproliferation, lymphocytic infiltration of nonlymphoid organs, autoimmune cytopenias, and B cell abnormalities with an accumulation of CD21lo B cells. These cells may account for antibody-mediated autoimmune in our patients. In contrast, heterozygous Ctla4 deficiency in mice shows no obvious phenotype (12, 13). Interestingly, patient D.I.1, who has a CTLA4 splicing mutation with no apparent somatic reversions or leakiness, is clinically healthy (table S2). This incomplete penetrance in disease may result from other genetic differences.

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**Fig. 4. Abnormal B cell phenotype in patients. (A) Percentages of B cells for patients A.I.1, A.II.1, B.I.1, C.I.1, D.I.1, and D.II.1: horizontal lines indicate means ± SD. **P = 0.0047 (Mann-Whitney test). (B) Absolute B cell numbers in blood for the indicated patients. Dotted lines indicate lower limit of normal range from the healthy donors. (C) B cells were labeled with antibodies to CD19, CD20, CD27, and CD10. Top panel: Proportions of B cells corresponding to transitional (CD10+CD27+), naïve (CD10−CD27−), or memory (CD10−CD27+) subsets. Middle and bottom panels: Abnormal accumulation of CD19+CD21lo B cells within the population of CD20+CD10+CD27+ B cells (middle) and CD20−CD27− B cells (bottom) in CTLA4–deficient patients compared to a healthy donor. (D) Enriched CD19+ cells (HD1 and A.I.1) or total PBMCs (HD2, HD3, A.II.1, C.II.1, and D.II.1) were stimulated with F(ab)2 specific for anti-IgM, IL-4, and CD40 ligand. CD19+ cell proliferation was assessed by dye dilution.
between family members. This is analogous to that in autoimmune lymphoproliferative syndrome, a human genetic immune disorder with only 60% penetrance among family members harboring the same heterozygous gene mutation (27). Contrary to genetic deficiencies in inbred strains of mice, phenotypic variability is commonly observed in human single-gene disorders (28). This may explain why D.I.1 has a CTLA4 mutation, yet is asymptomatic. C.I.1 and D.II.2 are apparently healthy because of incomplete penetrance; however, further immunological testing is required to confirm this assumption. We did not identify any common genetic modifiers in this study, as proven by our cohort analysis (see supplementary text). Also, our analysis of nonsynonymous SNVs in the CTLA4 coding region showed that CTLA-4 expression and T cell function are comparable to those of wild-type controls (table S3 and figs. S9 and S10).

Our results show the spectrum of clinical complications that can be anticipated from CTLA-4–blocking drugs. Consistent with these findings, treatment with the CTLA-4 mimetic, CTLA-4–Ig, suppressed patient T cell hyperproliferation in vitro (Fig. 3D) and could be a potential therapeutic intervention for CTLA-4–deficient patients. Taken together, our results show that heterozygous CTLA4 mutations in humans are associated with a severe immunoregulatory disorder, which we term CTLA-4 haploinsufficiency with autoimmune infiltration (CHAI) disease.

REFERENCES AND NOTES

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SUPPLEMENTARY MATERIALS
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Materials and Methods
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References (29–35)
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