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Tyrosine 201 of the cytoplasmic tail of CTLA-4 critically affects T regulatory cell suppressive function

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Cytotoxic T lymphocyte antigen-4 (CTLA-4) is a major negative regulatory molecule for T-cell activation with a complex biology and function. CTLA-4 is known to regulate homeostatic lymphoproliferation as well as tolerance induction and has been proposed to be an important effector molecule by which Treg cells suppress immunity. The immunoregulatory properties of CTLA-4 are primarily mediated by competition with the costimulator CD28 for ligand binding but also by delivering negative signals to T cells through its cytoplasmic tail. In this study, we addressed the effect of directly mutating the amino acid residue, Tyrosine 201 (Tyr201), of the intracellular domain of CTLA-4 in situ and its implications in T-cell function in the context of autoimmunity. Therefore, a novel CTLA-4 knock-in mouse (Y201V KI) was generated, in which Tyr201 was replaced by a valine that could not be phosphorylated. Mice expressing the CTLA-4 mutant molecule were generally healthy and did not show signs of disruption of T-cell homeostasis under steady-state conditions seen in CTLA-4 deficient mice. However, T cells isolated from Y201V KI mice expressed higher levels of CTLA-4 on the cell surface and displayed a Th2-biased phenotype following TCR stimulation. Furthermore, Y201V KI mice developed exacerbated disease as compared to wild-type upon antigen-specific T-cell activation in an in vivo model of EAE. Importantly, the Y201V mutation resulted in impaired suppressive activity of Treg cells while T effector function remained intact. These data suggest that effects associated with and mediated through Tyr201 of CTLA-4s intracellular domain are critical for Treg-cell function.

Keywords: Autoimmunity · CTLA-4 · Treg cells · EAE/MS

Additional supporting information may be found in the online version of this article at the publisher's web-site

Introduction

Cytotoxic T lymphocyte antigen-4 (CTLA-4) was the first negative checkpoint regulatory molecule identified on T cells [1–3]. The expression of CTLA-4 on the cell surface is essential for T-cell homeostasis in neonates, as genetic disruption leads to

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massive lymphoproliferative disease and death within 3–4 weeks after birth [4, 5]. Moreover, both functional and genetic data suggest that CTLA-4 plays a fundamental role in peripheral T-cell tolerance in the autoimmune setting [6]. CTLA-4 expression is induced on all activated T cells and constitutively expressed on Treg cells. CTLA-4 shares significant structural homology with the major costimulatory receptor CD28 [7–9] including sharing the same ligands B7-1 and B7-2. CTLA-4 binds the B7 molecules with a substantially higher avidity than CD28 [10–12], which has led to the suggestion that CTLA-4 functions as a checkpoint regulator by selectively competing with CD28 thus inhibiting costimulation. In fact, in the case of Treg cells, several groups have suggested that the major function of CTLA-4 on Treg cells is to downregulate or strip the CD28/CTLA-4 ligands from the cell surface of APCs leading to suppression of immune reactivity [13, 14]. However, in addition to the indirect role of CTLA-4 on T-cell activation, we and others have documented a direct role for CTLA-4 in modifying T-cell signaling through the T-cell receptor interactions with phosphatases bound to the intracellular domain of the coreceptor. The intracellular portion of the CTLA-4 molecule contains a lysine- and a proline-rich motif, as well as 2 tyrosine phosphorylation sites at position 201 and 218 [15, 16] that can function biochemically to directly regulate the activation of pathogenic T cells by modulating T-cell receptor signaling at a very proximal stage [17]. Moreover, subcellular localization of the CTLA-4 molecule is mediated by binding of the adaptor proteins AP-1 and AP-2 to the unphosphorylated Y201VKM motif in the cytoplasmic tail of CTLA-4 [18–20]. Upon T-cell activation, CTLA-4 is rapidly translocated to the site of TCR engagement and surface expression is stabilized by phosphorylation of the Y201VKM motif [7, 21, 22], therefore abolishing AP-2 binding and internalization [7, 23]. In the past, the generation of CD2-driven CTLA-4 transgenes which either lack the entire intracellular domain or carry a point-mutation within the Y201VKM motif [24, 25], are more effective in reaching the cell surface and localizing to appropriate immune synapses/lipid-rafts. The investigators observed a very limited direct effect on CTLA-4 function. The transgenic mice were largely normal suggesting that the intracellular functions of the molecule were secondary to their critical indirect role in blocking B7 engagement and subsequent CD28-mediated signaling. These results, however, might reflect the use of a constitutive promoter driven transgene resulting in nonphysiologic expression and high levels of constitutively expressed CTLA-4 that could alter the relevance of the tail in a natural setting [26, 27].

The aim of the present study was to explore the in vivo contribution of Tyrosine 201 (Tyr201), of the intracellular domain of CTLA-4 in the context of T-cell homeostasis and upon an immune response in the in situ setting at physiological levels. To achieve this goal, we generated a CTLA-4 knock-in mouse, (Y201V KI), in which the tyrosine residue at position 201 in the intracellular YVKM motif was replaced with a nonfunctional amino acid. This strategy assures the expression of the CTLA-4 Y201V mutant molecule at physiological levels. We observed that the Y201V mutation resulted in increased surface expression of CTLA-4 on T effector/memory cells as well as on activated T effector and Treg cells but had no effect on the overall T-cell phenotype in mutant mice under homeostatic conditions. However, mice expressing the Y201V mutant molecule develop exacerbated disease in a model of EAE due to impaired Treg-cell function rather than accelerated T effector function. Thus, these results demonstrate the importance of CTLA-4s intracellular domain in Treg-cell biology.

Results

Generation of Y201V KI mice

A genomic fragment containing the entire mouse CTLA-4 locus from a bacterial artificial chromosome (clone RP23-146J17: BACPAC) was obtained and the nucleotide sequence was modified to introduce an amino acid change from tyrosine (Y) to valine (V) at position 201 within Ex4 (Fig. 1A). This modified construct was used to target a B6 ES-cell line and selected clones were injected into BALB/c embryos. The chimeric mice were screened for germline transmission, and backcrossed onto the B6 background. The KI mice expressed the mutant form of the CTLA-4 protein, based on nucleotide sequence analysis (data not shown). Moreover, the Y201V KI CTLA-4 molecule was at least partially functional as it rescued the CTLA-4 KO lethal phenotype.

Similar levels of CTLA-4 isoforms but increased CTLA-4 surface expression in Y201V KI mice

Beside the full-length CTLA-4 molecule, two other splice variant isoforms of CTLA-4 have been described, including a ligand nonbinding (liCTLA-4) as well as a soluble, secreted variant (sCTLA-4) [28, 29]. Importantly, polymorphisms in the CTLA-4 gene, resulting in differential expression of the splice variants, have been associated with the susceptibility to multiple autoimmune diseases, including type 1 diabetes, multiple sclerosis, rheumatoid arthritis, Grave's disease, hypothyroidism, and systemic lupus erythematosus [29–31]. To examine whether the Y201V mutation altered overall CTLA-4 transcription, we examined mRNA levels of the full-length, ligand-independent, and soluble CTLA-4 isoforms in T naive and Treg cells isolated from lymph node and spleen of 8-week-old littermates. Consistent with previous observations, naive T cells only expressed the li-CTLA-4 form but Treg cells constitutively express all three isoforms. Of note, there were no differences in expression levels of any of the CTLA-4 isoforms when comparing WT and Y201V KI mice. These results demonstrated that the Y201V mutation did not affect relative CTLA-4 isoform expression patterns or mRNA levels (Fig. 1B). Next, we examined the protein expression of the full-length CTLA-4, both cell surface and intracellular staining. Surface protein expression of full-length CTLA-4 was significantly elevated on T conventional as well as Treg cells in Y201V KI mice (Fig. 1C, upper and Supporting Information Fig. 1A), whereas total CTLA-4 expression was unaltered (Fig. 1C, lower and Supporting Information Fig. 1B). This result is most likely a consequence of abolished adaptor protein (AP)-2 binding

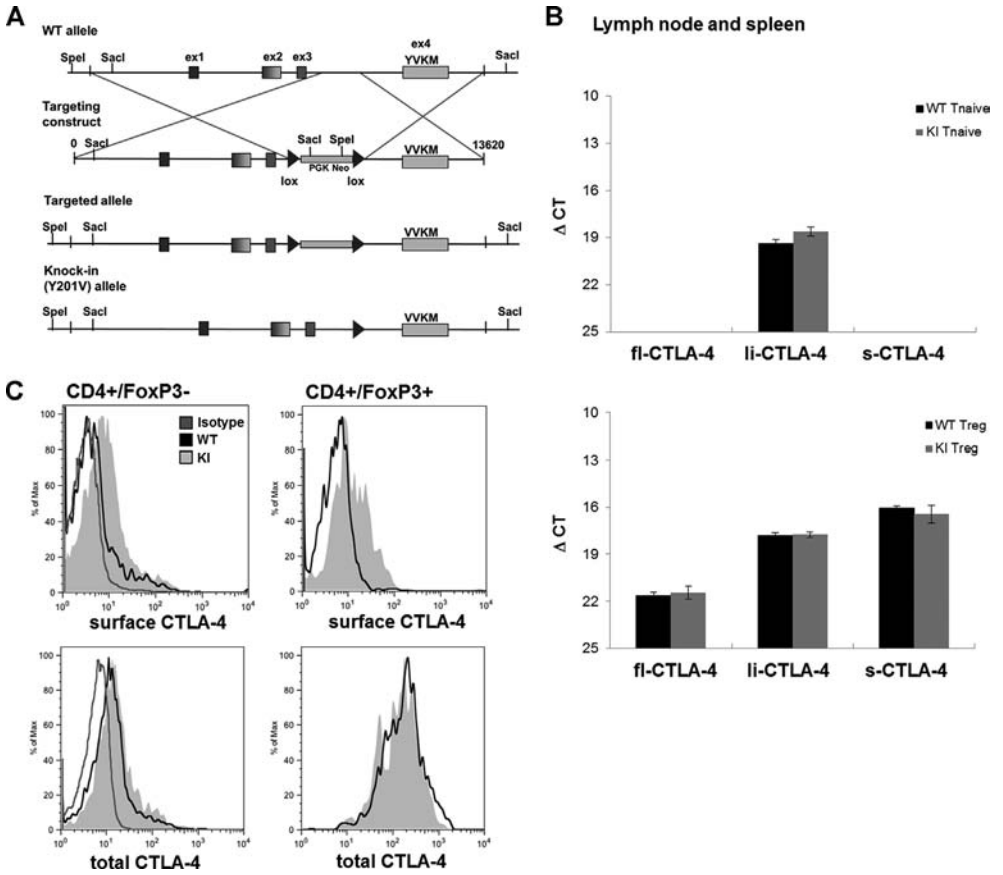

Figure 1. Higher surface levels of CTLA-4 in mice expressing the Y201V mutant molecule. (A) A 13.6 kb genomic fragment containing the entire mouse CTLA-4 locus was retrieved from a bacterial artificial chromosome (clone RP23-146J17: BACPAC). The nucleotide sequence was modified resulting in an amino acid change from Tyrosine (Y) to Valine (V) at position 201 within Exon4. Further, a LoxP-flanked PGK/em7-promotor driven neo cassette was inserted to allow selection of successful targeted B6 ES cell clones. Deletion of the NEO-cassette was achieved by breeding founder mice with OX40-Cre transgenic mice. (B) T naïve and Treg cells of CTLA-4 WT and Y201V knock-in mice were FACS sorted and mRNA expression levels of the different CTLA-4 isoforms were measured by quantitative real-time PCR. The ΔCt (threshold cycle) value was determined using the following formula: $Ct^{\text{test gene}} - Ct^{18S}$. Data are displayed as mean ΔCt ± SD of $n = 9$ samples per group and are pooled from three independent experiments. (C) Surface (upper) and total (lower) CTLA-4 expression in primary $CD4^+/FoxP3^-$ T conventional and $CD4^+$ $FoxP3^+$ Treg cells from WT and Y201V knock-in mice was assessed by flow cytometry. Data are representative of at least three independent experiments including a total of ≥10 mice per group.

to the mutated Y201VKM motif, which regulates internalization of the receptor from the surface [7, 23]. It is important to note that there were no differences in CTLA-4 cell surface expression levels, lymph node cellularity, and T-cell phenotype, even after activation between heterozygote and wild-type mice at 2–3 months of age.

Expression of the CTLA-4 Y201V mutant molecule alters the cytokine profile of activated T cells

Although KI mice did not appear to have gross defects in CTLA-4 function based on survival data, we examined whether the Y201V mutation might affect the activation state of T cells. There were comparable frequencies of various T-cell subsets $CD4^+/CD44^+/CD62L^-$ T effector, resting $CD4^+/CD44^-/CD62L^{hi}$ naive T cells (Fig. 2A) and $CD4^+/FoxP3^+$ Treg cells (Fig. 2B) harvested from lymph nodes of 8-week-old Y201V KI mice, and the total cellularity of axillary lymph nodes, mesenteric lymph nodes, and spleen, was unaltered as compared to wild-type animals (Supporting Information Fig. 2A). In addition, there were no detectable differences in absolute numbers of $CD4^+$ T conventional, as well as effector/memory and Treg cells (Supporting Information Fig. 2B) in young mice. However, the Y201V KI mice developed a mild form of lymphadenopathy by the age of 3 months. This phenotype of lymphoproliferation did not result in premature lethality but was accompanied by increased lymph node cellularity and an accumulation of $CD44^+/CD69^+$ activated T cells (Supporting Information Fig. 2C and D).

Importantly, there were significant changes in the cytokine production of mutant Y201V versus wild-type CTLA-4-expressing T cells. The proliferative response of the naive T cells was comparable in WT and Y201V T cells (Supporting Information Fig. 3). However, there was a significant increase in IL-4 production by activated Y201V T cells. IFN-γ and IL-17 production

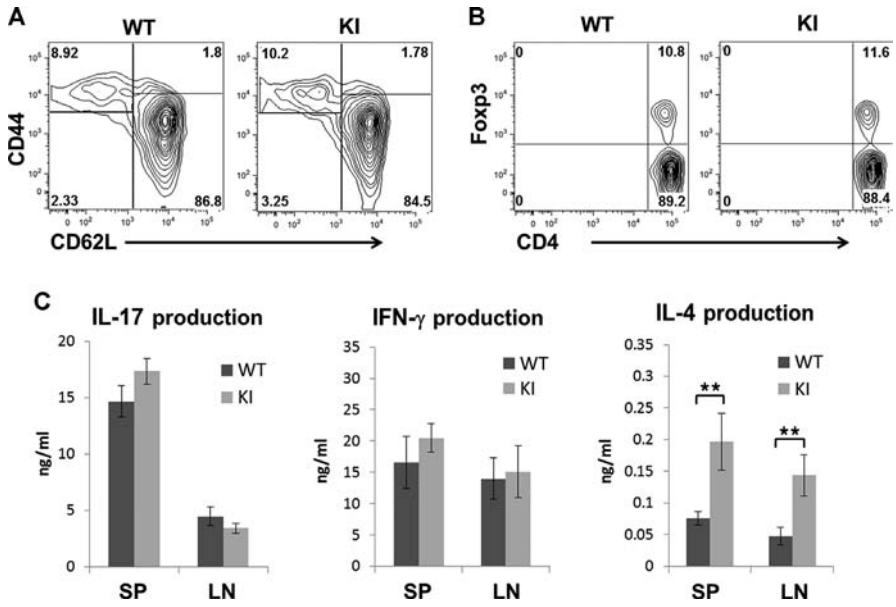

Figure 2. Expression of the Y201V CTLA-4 mutant results in a Th2-biased phenotype. (A, B) Representative flow plots of four independent experiments with a total of ≥12 mice per group, showing the frequencies of activated $CD4^+/CD44^+/CD62L^-$ T effector and resting $CD4^+/CD44^-/CD62L^{hi}$ naïve T cells (A) as well as $CD4^+/Foxp3^+$ Treg cells (B) in lymph node cells from 8-week-old WT and CTLA-4 Y201V knock-in mice. (C) $CD4^+/CD44^-/CD62L^{hi}$ naïve T cells from lymph nodes of 8-week-old WT and CTLA-4Y201V knock-in mice were stimulated with soluble anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL) in the presence of 200 U/mL IL2. At day 3, culture supernatants were assayed by ELISA for detection of IFN-γ, IL-17, and IL-4. Cytokine production data are displayed as mean ± SD of $n = 9$ samples per group and are pooled from three independent experiments. Statistical analysis was performed using an unpaired Student's t test (**, $p < 0.01$).

was unchanged (Fig. 2C). Thus, replacement of a single amino acid, in the Y201V CTLA-4 mutant results in a Th2-biased phenotype upon T-cell activation. The observed Th2 bias was consistent with previous studies demonstrating an altered T helper-cell subset differentiation in mice with altered CTLA-4 expression [24, 25, 32].

More severe EAE in Y201V KI mice is a consequence of impaired Treg-cell function

Next, we examined the effect of the mutation in the autoimmune setting of EAE that has been shown to be effected by T-cell cytokine balance. Y201V KI mice and wild-type littermate controls (5–7-week-old) were immunized with $MOG_{35–55}$ peptide emulsified in CFA and injected along with Pertussis toxin to induce this rapid and profound CNS autoimmune disease leading to paralysis. Y201V KI mice presented with exacerbated EAE as compared to wild-type (Fig. 3A). At initial stages of disease onset, clinical symptoms were similar to those observed in controls but were more severe at peak of disease and clinical scores remained high throughout remission phase. This was associated with an increase of MOG_{35-55} antigen specific but not total $CD4^+$ T cells infiltrating the CNS at peak disease (Fig. 3B). Overall, total cellularities of spleen and CNS as well as absolute numbers of $CD4^+$ T cells were unchanged comparing wild type and Y201V mutant mice (Supporting Information Fig. 4A and B). Nevertheless, $CD4^+$ conventional T cells as well as polyclonal and antigen-specific Treg cells isolated from the site of inflammation, the CNS, at peak disease displayed significantly higher CTLA-4 surface expression (Supporting Information Fig. 4C). Thus, the observation of exacerbated disease in Y201V KI mice was surprising, given that higher surface expression levels of CTLA-4 have been shown to sequester B7 ligands, thus diminishing CD28 costimulation [14]. Moreover, multiple studies report that a Th2-biased phenotype and or increased IL-4 expression ameliorates EAE [33–35]. Thus, we hypothesized that the mutant CTLA-4 KI molecule was not altering the development of effector T cells. To confirm this, $CD4^+/CD62L^{high}/CD25^-$ naive T cells were adoptively transferred into Rag-KO recipients followed by $MOG_{35–55}$/CFA immunization. The development of disease and its progression was comparable in mice receiving either Y201V KI or wild-type naive T cells (Fig. 3C), suggesting that the Y201V mutation does not promote accelerated T effector function and the Y201V KI did not affect the generation of effector Th1 or Th17 cells confirming the in vitro data.

Next, we tested whether the Y201V mutation might affect the suppressive activity of Treg cells. As seen in Figure 4A, Treg cells from Y201V KI mice expressed significantly less FoxP3 on a per cell basis as compared to wild-type Treg cells. The reduction in FoxP3 protein was most prominent in Treg cells isolated from spleen and CNS at peak disease (Fig. 4A). More importantly, we observed a significant decrease in antigen-specific Treg cells in the CNS of immunized Y201V KI mice as compared to wild-type mice (Fig. 4B) suggesting that the loss of FoxP3 expression might have led to the reduced number and function of Treg cells in this setting. Of note, Treg numbers in the thymus and periphery as well as FoxP3 expression in Treg cells in the thymus are unchanged under steady-state condition in Y201V mice (Supporting Information Fig. 2B and 5), suggesting that this phenotype is a result of T-cell activation in the autoimmune setting and not based on impaired Treg-cell development in the thymus.

To further test if the CTLA-4 Y201V mutation directly affects suppressive activity of Treg cells, we performed adoptive transfer experiments. Naive WT T cells alone or in combination with either WT or Y201V Treg cells were transferred into RAG-KO recipients at a ratio of 2:1 and mice were immunized to induce EAE as

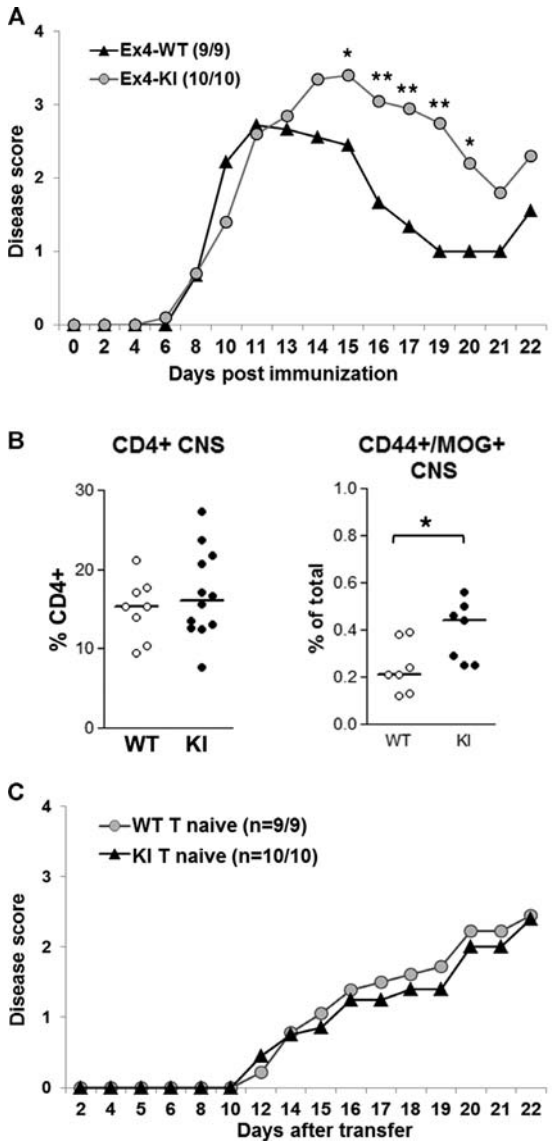

Figure 3. Exacerbated EAE in Y201V KI mice. (A) 5–7-week-old WT and CTLA-4Y201V knock-in mice were immunized with 200 μg MOG_{35-55} emulsified in CFA and injected with Pertussis toxin. Development of clinical symptoms of EAE was assessed over time. Data are displayed as mean clinical scores and statistical analyses were performed using an unpaired Student's *t* test (*, $p < 0.05$; **, $p < 0.01$). (B) Lymphoid cells were isolated from CNS at peak disease and the overall percentage of infiltrating $CD4^+$ (left) and $CD4^+/CD44^+/MOG^+$ antigen-specific T cells (right) was assessed by flow cytometry. Each dot represents a mouse. Statistical analysis was performed using an unpaired Student's *t* test (*, $p < 0.05$) (C) 3.5×10^5 purified $CD4^+/CD25^-/CD62L^{hi}$ naive T cells from WT and CTLA-4-Y201V knock-in mice were adoptively transferred into 5-week-old B6.Rag-KO's. Recipients were immunized with 200 μg MOG_{35-55} emulsified in CFA on day 2 posttransfer. Pertussis toxin was injected on day 2 and 4, respectively. Development of EAE was evaluated over time.

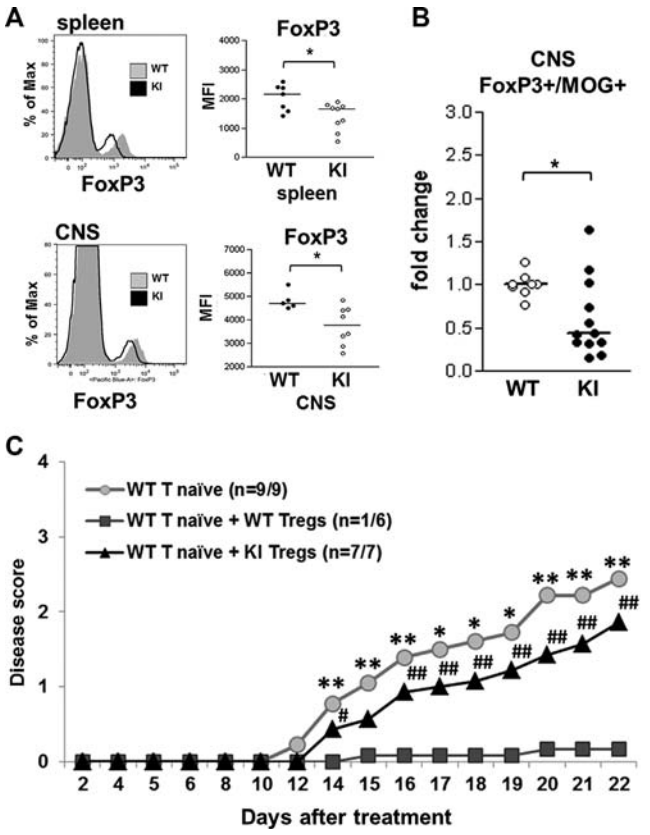

Figure 4. Reduced FoxP3 expression and impaired Treg cell function in Y201V KI mice. EAE was induced in 5–7-week-old WT and CTLA-4Y201V knock-in littermates with 200 μg MOG_{35-55} emulsified in CFA and injection with Pertussis toxin. Lymphoid cells from spleen and CNS were isolated at peak disease and assessed by flow cytometry. (A) Representative flow plots (left) and quantifications (right) displayed as MFI of FoxP3 protein expression on $CD4^+$ Treg cells. (B) The overall percentage of antigen-specific $CD4^+/FoxP3^+/MOG^+$ Treg cells in the CNS, shown as fold change compared to WT. Statistical analysis was performed using an unpaired Student's *t* test (*, $p < 0.05$). (C) 3.5×10^5 WT naive T cells were either transferred alone or together with 1.75×10^5 Treg cells from WT or CTLA-4Y201V knock-in mice into 5-week-old B6.Rag-KO's recipients were immunized with 200 μg MOG_{35-55} emulsified in CFA on day 2 posttransfer. Pertussis toxin was injected on day 2 and 4, respectively. Development of EAE was evaluated over time. Data are displayed as mean clinical scores and statistical analyses were performed using an unpaired Student's t test. *,# Mean disease score significantly higher than WT T naïve + WT Treg cells (*, $p < 0.05$; **, $p < 0.01$). There were no statistical differences between WT T naïve only and WT T naïve + KI Treg cells.

described above. As shown in Figure 4C, WT Treg cells efficiently suppress disease development in immunized recipients. Only one out of six mice developed clinical symptoms. In contrast, Treg cells derived from the Y201V KI mice were not able to control T effector functions, as all recipients presented with EAE and clinical scores were similar to those observed in mice not receiving any Treg cells. Taken together, the results suggest that Tyr201 in the intracellular domain of CTLA-4 is indispensable in the context of Treg-cell function.

Discussion

In the present study, we examined the importance of Tyr201 within the YVKM motif for CTLA-4 on its intrinsic function in vivo. Consistent with previous in vivo studies in mice expressing a

CD2-driven CTLA-4 Y201V-transgene in the CTLA-4 KO background [24, 25], we documented increased CTLA-4 surface expression (Fig. 1C) and a Th2-biased T-cell phenotype (Fig. 2C) in Y201V KI mice. Moreover, we observed that the Y201V mutation does not affect T-cell homeostasis in young mice up to 8 weeks of age (Fig. 2A and Supporting Information Fig. 1A and B) but results in a mild form of lymphadenopathy and an increase in activated T effector/memory cells as the mice grow older (Supporting Information Fig. 1C and D). Finally, we observed that Y201V KI mice immunized with $MOG_{35–55}$ peptide to induce EAE developed more severe disease compared to wild-type littermate controls (Fig. 3A). This outcome was unexpected; since we speculated that higher CTLA-4 surface expression and increased IL-4 production might confer a protective function, thus resulting in disease amelioration. The results were a consequence of defective Treg-cell function due to the Y201V mutation in the CTLA-4 gene (Fig. 4C). The single amino acid mutation led to a significant decrease of Treg cells in the CNS at peak disease and reduced FoxP3 expression within the antigen-specific cells. We cannot exclude that the reduction of Treg cells in the CNS is based on defects in cell survival, expansion, and or trafficking. However, we could not observe differences in the frequency of Treg cells in the thymus or the periphery under steady state conditions. Thus, the Treg-cell phenotype could as well be a direct consequence of impaired signal transduction, as it appears unlikely that the Y201V mutation affects Treg-cell development or homeostatic Treg-cell survival or expansion. Moreover, a recent study from our laboratory demonstrated that the loss of FoxP3 expression in a subset of Treg cells results in an exFoxP3 population, which acquires effector function [36]. It will be interesting to determine whether the altered CTLA-4 function not only increases the number of exFoxP3 cells but that those cells take on effector function that plays a role in the exacerbated disease observed in this setting.

Previous studies have shown that ERK1/2, CD3-ζ, and AKT phosphorylation is altered in Treg cells, in some instances as a direct result of CTLA-4 [37–40]. We hypothesize that the intracellular domain of CTLA-4 plays a role in controlling these TCR-mediated biochemical signals that has been shown to play a critical role for Treg cell development, homeostasis, and function. In this regard, the biochemical basis for cell intrinsic CTLA-4 function in T effector cells was described to be dependent on the association of the Y201VKM motif of the cytoplasmic domain with a variety of signaling molecules including the phosphatases, SHP-2 [41, 42], and PP2A [7, 16]. We, and others, have shown that this biochemical interaction promotes dephosphorylation of the TCRζ chain as well as other TCR complex components like LAT and ZAP70 [42–44]. Moreover, cross-linking of CTLA-4 in conjunction with costimulation, has been reported to inhibit ERK phosphorylation/activation as well as c-JNK and therefore differentially regulates members of the MAPK family [45]. In addition, multiple signaling pathways initiated by TCR/CD3, IL-2R/STAT, the PI3K/Akt/mTOR as well as the TGF-β/Smad and Notch signaling pathways have been implicated in FoxP3 transcriptional regulation [46, 47]. Specifically, recent work by Sauer et al. demonstrated that TCR signal deprivation as well as inhibition of PI3K-signaling promotes Treg-cell development and FoxP3 expression [48]. Although our study was not designed to address the specific signaling events downstream of Tyr201, we speculate that the above described studies together with our results and the fact that the Y201VKM motif of the CTLA-4 intracellular domain alters TCR and PI3K-signaling in T effector cells [17, 49] might provide a mechanism of action how the Y201V mutation could affect FoxP3 expression in Treg cells. In addition, Singer and colleagues reported that TCR-hyposignaling in Treg cells indeed requires the intracellular domain of CTLA-4 to control CD3-ζ phosphorylation as well as calcium mobilization suggests a potential link between the YVKM motif and Treg development and function [50]. Interestingly, unlike our work, recent studies observed that the cytoplasmic domain of CTLA-4 was dispensable for Treg function in vitro [51] and in a model of inflammatory bowel disease (IBD) [50]. This discrepancy could be based simply on the different disease models as there is a relatively higher ratio of Treg to Teff cells used in the IBD adoptive transfer experiments, compared to our study. Moreover, the critical function of the Treg cells to control EAE versus IBD, including the relative differences in the role of IL-10 in EAE regulation versus TGF-β and IL-10 as well as induced Treg cells in IBD may be significant. In this regard, it should be noted that the fundamental role of CTLA-4 on Treg cells appears to be different in different settings [13, 52] (Bluestone J.A. and Tang Q., unpublished observation). Further, one could speculate that Treg cells lacking a significant portion of the cytoplasmic domain as compared to the Y201V Treg cells that only carry a single point mutation may also be affecting T effector cells. Finally, mutating Tyr201 of the intracellular tail of CTLA-4 prevents AP-2 binding and consequently results in increased surface expression and decreased internalization of the molecule. This could negatively affect trans-endocytosis of CTLA-4's ligands B7-1 and B7-2 [14] leading to altered costimulation through CD28, which has been reported to be critical for induction of peripheral Treg cells from naïve T cells [53, 54]. In addition, increased CTLA-4 surface expression could modulate the TCR repertoire in the thymus [55], thus alter Treg cell generation. Of note, we did not observe differences in the frequency of Treg cells in the thymus nor the periphery under steady-state conditions, suggesting that CTLA-4 surface overexpression did not interfere with Treg-cell development or homeostasis.

Taken together, our data indicate that the intracellular domain and especially the Y201VKM motif of CTLA-4 influences Treg-cell biology, given the significant reduction of FoxP3 expression and the severely impaired suppressive activity of Y201V KI Treg cells in vivo.

Materials and methods

Generation of B6.CTLA-4 Y201V knock-in mice

Targeting strategy to generate CTLA-4 Y201V knock-in mice. A 13.6 kilobase genomic fragment containing the entire mouse

CTLA-4 locus was recovered from a bacterial artificial chromosome (clone RP23-146J17: BACPAC http://bacpac.chori.org). The fragment was cloned into the pBluescript II SK(-) vector (Stratagene, Santa Clara, CA, USA) using the RED-ET recombineering strategy [56] followed by insertion of a LoxP-flanked PGK/em7-promotor driven neo cassette. The nucleotide sequence was further modified, resulting in an amino acid change from Tyrosine (Y) to Valine (V) at position 201 within Ex4. This mutant construct was used to target B6-PRX mouse embryonic stem cells and selected clones were injected into BALB/c embryos. The chimeric mice were screened for germline transmission, and further crossed with OX40-Cre transgenic mice [57] to delete the selection cassette. Mice were housed in a specific pathogen-free facility at the University of California at San Francisco. All experiments complied with the Animal Welfare Act and the National Institutes of Health guidelines for the ethical care and use of animals in biomedical research and were approved by the Institutional Animal Care and Use Committee of the University of California at San Francisco.

Induction and clinical evaluation of EAE

Mice 5–7-week-old were immunized s.c. with an emulsion of Complete Freund's Adjuvant (containing 4 mg/mL heat inactivated and dried Mycobacterium tuberculosis H37 Ra; DIFCO laboratories, Franklin Lakes, NJ, USA) and 4 mg/mL $MOG_{35–55}$ peptide (Genemed Synthesis, INC; San Antonio, TX, USA). Hundred microliters of the emulsion were distributed over three spots on the flank. In addition, 200 ng of Pertussis toxin (List biological laboratories; Campbell, CA, USA) in 200 μL saline solution was injected i.p. on days 0 and 2 after immunization. Mice were evaluated daily and clinical development was assessed and rated according to the following criteria: 0 = no signs of disease, 1 = limp tail, 2 = limp tail, and hind limb weakness, 3 = partial hind limb paralysis, 4 = total hind limb paralysis.

Adoptive transfer experiments

$CD4^+/CD62L^{hi}/CD25^-$ naive T cells and $CD4^+/CD62L^{hi}/CD25^+$ Treg cells from 6–8–week-old WT and Y201V KI mice were purified by FACS-sorting. Either 3.5×10^5 naive T cells or 3.5×10^5 naive T cells together with 1.75×10^5 Treg cells were adoptively transferred into B6-RagKO mice. Mice were immunized with $MOG_{35–55}$ on day 2 posttransfer as described above. Pertussis toxin was injected on day 2 and 4 after transfer. Clinical evaluation of disease onset and progression was performed as stated above.

Isolation of lymphoid cells from the CNS and immunofluorescence

Spinal cord, cerebellum, and brain stem were collected in HBSS buffer after perfusing the mouse with 50 mL cold PBS. Organs were minced and digested with 300 U/mL Collagenase D in DMEM for 30 min at 37°C. Digestion was terminated by adding 10% FCS. Cells were filtered using a 40 μm nytex filter and washed with Ca^+/Mg^+ free HBSS, containing 2% FCS. Lymphocytes were separated from myelin using a percoll gradient. Cells were resuspended in 30% percoll (in PBS), then gently under-laid with 70% percoll (in PBS) and spun for 30 min at 2400 rpm at room temperature without braking. Mononuclear cells were collected from the interface and washed twice with Ca^+/Mg^+ free HBSS, containing 2% FCS. Centrifugation step in between washes was performed at 1400 rpm for 10 min. Cells were resuspended at 5×10^7/mL in tetramer staining buffer (DMEM, 25 mM Hepes, 2% FCS) supplemented with 5% rat serum (heat inactivated) and Fc Block (1:100 dilution) and incubated on ice for 10 min. Cells were washed and stained with APC-conjugated $MOG_{35–55}$-tetramer (NIH tetramer core facility, Atlanta, GA, USA) at a final dilution of 1:100 for 3 h in the dark at room temperature, followed by staining for surface and intracellular markers.

Flow cytometry and cell sorting

Labeled antibodies specific for CD4 (RM4-5), CD44 (IM7), CD25 (PC61), CD62L (MEL14), CTLA-4/CD152 (UC10-4F10), FoxP3 (FJK-16s), and Hamster IgG1,κ (A19-3) were purchased from BD Biosciences or eBioscience. Isolated cells from lymph nodes and spleen were stained with commercially available antibodies, listed above. Intracellular staining for FoxP3 and CTLA-4 (CD152) was performed using a FoxP3 staining buffer set, according to the manufacturers' instructions (eBioscience, San Diego, CA, USA). Stained single-cell suspensions were analyzed using a BD LSRII flow cytometer running FACSDiva software (BD Biosciences, San Jose, CA, USA). Cell sorting experiments were performed with a MoFlo cytometer high-speed cell sorter (Dako) or a FACSAria (BD Biosciences).

T-cell proliferation and cytokine assay

FACS-sorted $CD4^+/CD62L^{hi}/CD25^-$ naive T cells from spleen and lymph node were resuspended in PBS at a concentration of 1×10^8 cells/mL and labeled by adding an equal volume of 5 μM CFSE in PBS (Molecular probes, Grand Island, NY, USA) for 5 min at room temperature. Labeling was terminated by adding an equal volume of FBS for 1 min at room temperature. Cells were washed with PBS, resuspended at a concentration of 2×10^6/mL in complete culture media (DMEM/10% FCS supplemented with 50 μM 2-ME, 1 mM sodium pyruvate, nonessential amino acids, L-glutamine, and 100 U of penicillin/100 μg of streptomycin/mL). Cells were stimulated with soluble anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL) in the presence of 200 U/mL IL-2 and CFSE dilution upon T-cell proliferation was assessed by flow cytometry, at the indicated time points. To measure cytokine production, FACS-sorted $CD4^+/CD62L^{hi}/CD25^-$ T naive cells from lymph nodes were stimulated with soluble anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL) in the presence of 200 U/mL IL2. At day 3, culture

supernatants were collected and analyzed for IL-4, INF-γ, and IL-17 using standard ELISA methods (BD Biosciences, San Jose, CA, USA).

Real-time PCR analysis

RNA was isolated from sorted $CD4^+/CD62L^{hi}/CD25^-$ naive T cells and $CD4^+/CD62L^{hi}/CD25^+$ Treg cells from total lymph nodes, using the RNeasy Kit (Qiagen, Valencia, CA, USA). Reverse transcription into cDNA was performed using the Superscript kit (Invitrogen). The quantities of full-length CTLA-4 (fl-CTLA-4), ligand-independent CTLA-4 (li-CTLA-4), and soluble CTLA-4 (s-CTLA-4) cDNA were measured using quantitative real-time PCR analysis (GeneAmp 7900; Applied Biosystems, Carlsbad, CA, USA) and were normalized to 18S expression (Eukaryotic 18S rRNA). The TaqMan primer-probe for 18S was purchased from Applied Biosystems (ID: 4333760F). Primers and probes for fl-CTLA-4, li-CTLA-4, and sCTLA-4, according to Kissler et al. [58], were purchased from Integrated DNA Technologies.

Statistical analysis

Statistical analyses were performed using either an unpaired two-tailed Student's *t*-test or an unpaired two-tailed Mann–Whitney *U* test. Values of $p \leq 0.05$ were considered significant.

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Abbreviations: **CTLA-4**: cytotoxic T lymphocyte antigen-4 · **IBD**: inflammatory bowel disease

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