copy number in TG of GrB$^{-/-}$ or Pfn$^{-/-}$ mice. This mechanism might be particularly efficient during attempted HSV-1 reactivation events where ICP4 expression has escaped repression by viral miRNAs and host neuron epigenetic modifications. Thus, we propose a tripartite relation in which HSV-1 latency is maintained through the activity of the virus, host neuron, and contiguous CD8$^+$ T cells permitting viral persistence with neuronal survival (fig. S7).

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CTLA-4 Control over Foxp3$^+$ Regulatory T Cell Function

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Naturally occurring Foxp3$^+$CD4$^+$ regulatory T cells (Tregs) are essential for maintaining immunological self-tolerance and immune homeostasis. Here, we show that a specific deficiency of cytotoxic T lymphocyte antigen 4 (CTLA-4) in Tregs results in spontaneous development of systemic lymphoproliferation, fatal T cell–mediated autoimmune disease, and hyperproduction of immunoglobulin E in mice, and it also produces potent tumor immunity. Treg-specific CTLA-4 deficiency impairs in vivo and in vitro suppressive function of Tregs—in particular, Treg-mediated down-regulation of CD80 and CD86 expression on dendritic cells. Thus, natural Tregs may critically require CTLA-4 to suppress immune responses by affecting the potency of antigen-presenting cells to activate other T cells.

Naturally occurring CD25$^+$CD4$^+$ regulatory T cells (Tregs), which specifically express the transcription factor Foxp3, suppress aberrant immune responses, including autoimmune diseases and allergy (*1*). Furthermore, reduction or expansion of Tregs can be exploited to provoke effective tumor immunity or transplantation tolerance, respectively. Two cardinal features of Foxp3$^+$ Tregs are that they constitutively express cytotoxic T lymphocyte antigen 4 (CTLA-4), which only happens after activation in other T cell subsets (*2*–*4*), and that Foxp3 controls the expression of CTLA-4 in Tregs (*5*–*9*). CTLA-4 is a potent negative regulator of T cell immune responses, as illustrated by CTLA-4 knockout (KO) mice, which die prematurely from multiorgan inflammation (*10*, *11*). The polymorphism of the CTLA-4 gene contributes substantially to the genetic susceptibility to autoimmune diseases such as type 1 diabetes (*12*). Moreover, autoimmunity, inflammatory bowel disease, and tumor immunity can be elicited by blocking CTLA-4 with a specific antibody (*3*, *4*, *13*–*15*). Yet the manner in which CTLA-4 negatively controls immune responses is controversial (*16*). CTLA-4 expressed by activated effector T cells may mediate a negative signal that attenuates their activation. Alternatively, but not exclusively, Foxp3$^+$ Tregs may require CTLA-4 for their suppressive function. By specifically deleting the CTLA-4 gene in Foxp3$^+$ Tregs in mice, we have attempted to determine the role of CTLA-4 for the maintenance of self-tolerance and immune homeostasis.

We generated BALB/c mice expressing Cre under the control of the Foxp3 promoter—hereafter called FIC (Fox-IRES-Cre) mice—and BALB/c mice expressing a floxed CTLA-4 gene (CTLA-4$^{fl/fl}$) [supporting online material (SOM) text and fig. S1] (*17*). Compared with BALB/c wild-type (WT) mice, FIC mice expressed Foxp3 protein at slightly lower levels whereas CTLA-4$^{fl/fl}$ mice expressed equivalent levels of CTLA-4 (Fig. 1A). To assess the specificity of Cre expression, FIC mice were crossed with Cre reporter mice (CAG mice), which express enhanced green fluorescent protein (EGFP) only in Cre$^+$ cells (*18*). EGFP expression was confined to ~15% of CD4$^+$ T cells and ~1.5% of CD8$^+$ T cells (Fig. 1B). The vast majority of EGFP$^+$CD4$^+$ T cells in adult FIC$^{+/+}$ CAG mice were Foxp3$^+$ (97.1 ± 1.2%, $n = 4$ mice), indicating that Foxp3 expression is stable once the gene is turned on and Cre expression is not leaky in Foxp3$^-$ cells (Fig. 1C). On the basis of this specific expression of Cre in Foxp3$^+$ Tregs, we generated CTLA-4 conditional KO (CKO) mice by crossing FIC and CTLA-4$^{fl/fl}$ mice. CTLA-4 was specifically deleted in CD4$^+$Foxp3$^+$ T cells, as compared with FIC$^{+/+}$ WT or full CTLA-4 KO mice (Fig. 1D). CKO mice even harbored a higher frequency of CTLA-4–expressing CD4$^+$Foxp3$^-$ cells than did WT littermates (Fig. 1E). Whereas KO mice became moribund at ~20 days of age (*10*, *11*), CKO mice remained apparently unaffected until ~7 weeks of age, when they rapidly became inactive and began to develop general edema that was frequently accompanied by ascites (Fig. 1F). Thus, CTLA-4 deficiency in Tregs alone suffices to cause fatal disease, whereas the additional CTLA-4 deficiency in non-Treg cells enhances the disease. Yet, CTLA-4 expression in activated effector T cells per se is insufficient to prevent it.

Pathological analysis of CKO mice revealed splenomegaly and lymphadenopathy, which was reflected in increased cell numbers (Fig. 2, A and B). The proportion of CD4$^+$ T cells was unaltered, whereas CD8$^+$ T cells were decreased (Fig. 2C). Cardiomegaly and congestion of the liver was macroscopically evident in the terminal stage of every case. In affected hearts, mononuclear cells densely infiltrated into the myocardium and destroyed myocytes (Fig. 2, D to G), indicating that the plausible cause of sudden death in CKO mice is

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heart failure due to severe myocarditis (*19*). In addition, CKO mice possessed focal lymphocyte infiltrations in lung and salivary gland and suffered from gastritis with various degrees of destruction of gastric parietal cells and chief cells. Antiparietal autoantibodies were readily detected in the sera of CKO mice and a proportion of FIC$^{+/+}$ mice, in which the lower expression of Foxp3 in Tregs (Fig. 1A) might somehow affect Treg function (*20*) (Fig. 2, H to N, and SOM text). Myocarditis and gastritis in CKO mice (and gastritis in FIC mice) could be adoptively transferred with splenocytes and purified CD4$^+$ T cells into T cell–deficient BALB/c athymic nude (nu/nu) mice, indicating that these autoimmune conditions were both T cell–mediated (Fig. 2O and fig. S2). Furthermore, CKO mice developed several hundred–fold and threefold higher levels of serum immunoglobulin E (IgE) and immunoglobulin G (IgG), respectively, than the levels in FIC or WT mice (Fig. 2, P and Q).

Costaining of intracellular cytokines and Foxp3 revealed an increased frequency of interleukin-2 (IL-2)–, IL-4–, and IFN-γ–producing Foxp3$^-$CD4$^+$ cells in both the spleen and lymph node (LN) of diseased CKO and KO mice (Fig. 2R and fig. S3). IL-17–secreting (Th17) cells increased in KO but not CKO mice, suggesting that Th17 cells might contribute to the rapid disease progression in the former. Thus, CTLA-4–deficient Tregs fail to control the spontaneous activation of other T cells and their differentiation into Th1 and Th2 lineage cells that mediate autoimmune disease and allergy.

We next tested whether Treg-specific CTLA-4 deficiency also influenced the potency of tumor immunity. BALB/c nu/nu mice were reconstituted with splenocytes from CKO or control FIC mice containing equivalent numbers of T cells and inoculated with BALB/c-derived RL♂1 leukemia cells (*21*). All recipients of FIC splenocytes died of tumor progression within a month. In contrast, recipients of CKO splenocytes halted the tumor growth, with the majority surviving the 6-week observation period, during which 60% of them completely rejected the tumor (Fig. 3A). As previously shown (*21*), transfer of BALB/c splenocytes after depletion of CD25$^+$ T cells led to the rejection of RL♂1 leukemia cells in nu/nu mice. In this setting, FIC Tregs cotransferred with CD25$^-$ T cells suppressed tumor rejection, whereas CKO Tregs did not (Fig. 3B). Thus, Treg-specific CTLA-4 deficiency affects in vivo Treg suppressive function, leading to enhanced tumor immunity.

We next explored the possibility that CTLA-4 deficiency might impair the generation, survival, or suppressive function of Foxp3$^+$ Tregs. CKO mice exhibited no significant alteration in number or composition of CD4$^+$ and CD8$^+$ thymocytes (Fig. 4A). The majority of Foxp3$^+$ WT thymocytes expressed CTLA-4, whereas Foxp3$^+$ CKO thymocytes contained a mix of CTLA-4$^+$ and CTLA-4$^-$ cells in both the CD4–single positive and CD4/CD8–double positive compartments (Fig. 4A). Because the CTLA-4 gene is deleted only after Foxp3 is expressed, CTLA-4 is either up-regulated before Foxp3 expression in CKO mice or it may take some time for the Cre protein to accumulate in Foxp3$^+$ cells, meanwhile allowing the expression of CTLA-4. The frequency of Foxp3$^+$ thymocytes was not significantly changed between CKO and WT mice, whereas the number of Foxp3$^+$ and Foxp3$^-$ T cells in the spleen and LNs increased enormously by active proliferation (Fig. 4B, figs. S4 and S5, and SOM text). Thus, Foxp3-inducible CTLA-4 deficiency minimally alters thymic selection of Tregs and probably triggers immunological diseases through affecting Treg function in the periphery.

Because Foxp3 is encoded by the X chromosome, female nonautoimmune FIC$^{+/-}$CTLA-4$^{fl/fl}$ mice are a mosaic for CTLA-4–intact and –deficient Tregs. They harbored equal numbers of CTLA-4$^+$ and CTLA-4$^-$ Foxp3$^+$ T cells, indicating that both populations equally survive in physiological non-inflammatory conditions (Fig. 4C). Furthermore, when CTLA-4–deficient or –intact Tregs were transferred to nu/nu mice, both populations showed a similar degree of homeostatic proliferation, and neither one caused autoimmunity (fig. S6). CTLA-4–deficient Foxp3$^+$ Tregs were as poor at producing pro-inflammatory cytokines as were their WT or FIC counterparts (fig. S3). Taken together, CTLA-4 deficiency, per se, does not affect the survival of Tregs or render them pathogenic.

Phenotypically, CTLA-4–deficient naive Tregs in FIC$^{+/-}$CTLA-4$^{fl/fl}$ females normally expressed typical Treg markers including CD44, CD103, glucocorticoid-induced tumor necrosis factor receptor, latency-associated peptide, and intracellular IL-10 (Fig. 4D and fig. S7). The comparatively higher expression of these molecules by Tregs from CKO mice is presumably secondary to ongoing inflammation in CKO mice, as illustrated by an activated phenotype of their Foxp3$^-$ non-Treg cells.

CTLA-4–deficient Tregs, whether naive from FIC$^{+/-}$CTLA-4$^{fl/fl}$ CAG females or activated from CKO mice, had diminished suppressive capacity compared with CTLA-4–intact Tregs in cultures of carboxyfluorescein diacetate succinimidyl ester (CFSE)–labeled responder T cells (Tresp) in the presence of splenic CD11c$^+$ dendritic cells (DCs) and anti-CD3 monoclonal antibody (mAb), as assessed by the percentage and number of CFSE-diluting (i.e., divided) Tresp (Fig. 4E, figs. S8 and S9, and SOM text). Moreover, CKO Tregs clearly failed to suppress allo-reactive Tresp proliferation, even at high Treg/Tresp ratios (Fig. 4F). FIC or WT Tregs, whether cultured alone or together with Tresp cells, specifically hampered up-regulation of the expression of CD80 and CD86, but not CD40 and major histocompatability complex class II, in DCs (*22*–*26*). In contrast, CKO Tregs failed to exert this effect (Fig. 4G, figs. S10 to S13, and SOM text). Activated FIC Tregs (but

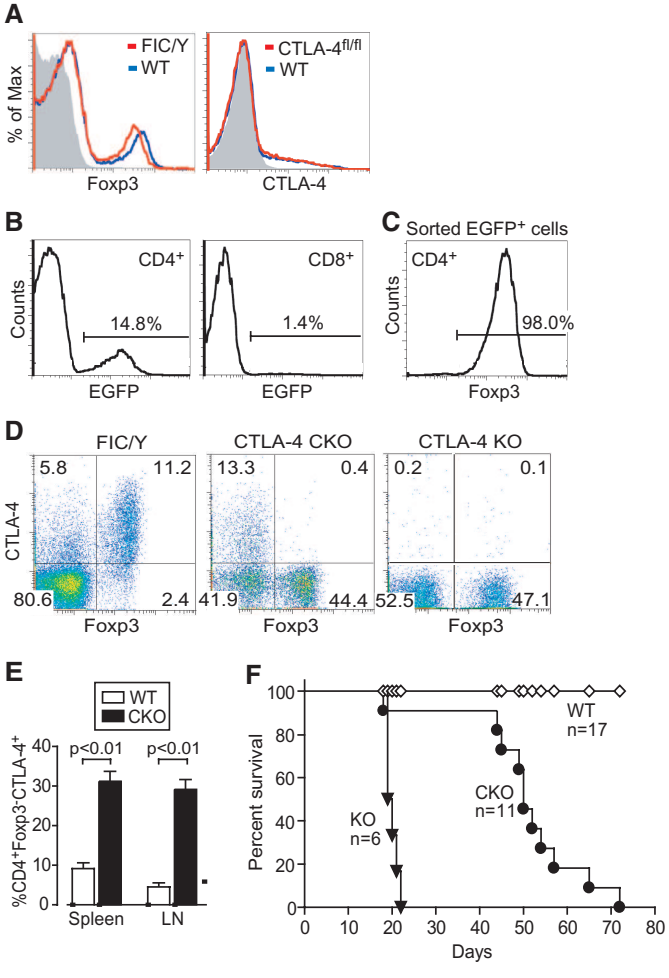

Fig. 1. Specific deletion of CTLA-4 expression in Foxp3$^+$ T cells results in fatal disease. (**A**) Flow cytometric analysis of intracellular Foxp3 (left) and CTLA-4 (right) in freshly isolated LN CD4$^+$ T cells from male FIC, CTLA-4$^{fl/fl}$, or BALB/c WT mice. (**B**) EGFP expression in CD4$^+$ or CD8$^+$ T cells derived from male FIC-CAG mice. (**C**) Sorted CD4$^+$EGFP$^+$ cells in FIC-CAG mice were stained for Foxp3. (**D**) CTLA-4 and Foxp3 expression in LN CD4$^+$ T cells from BALB/c WT, CKO, or KO mice. (**E**) Frequency of CTLA-4–expressing CD4$^+$Foxp3$^-$ T cells in CKO and normal littermates (n = 5). (**F**) Survival of KO and CKO mice as compared with normal littermates. Data represent three or more independent experiments. Vertical bars indicate SEM.

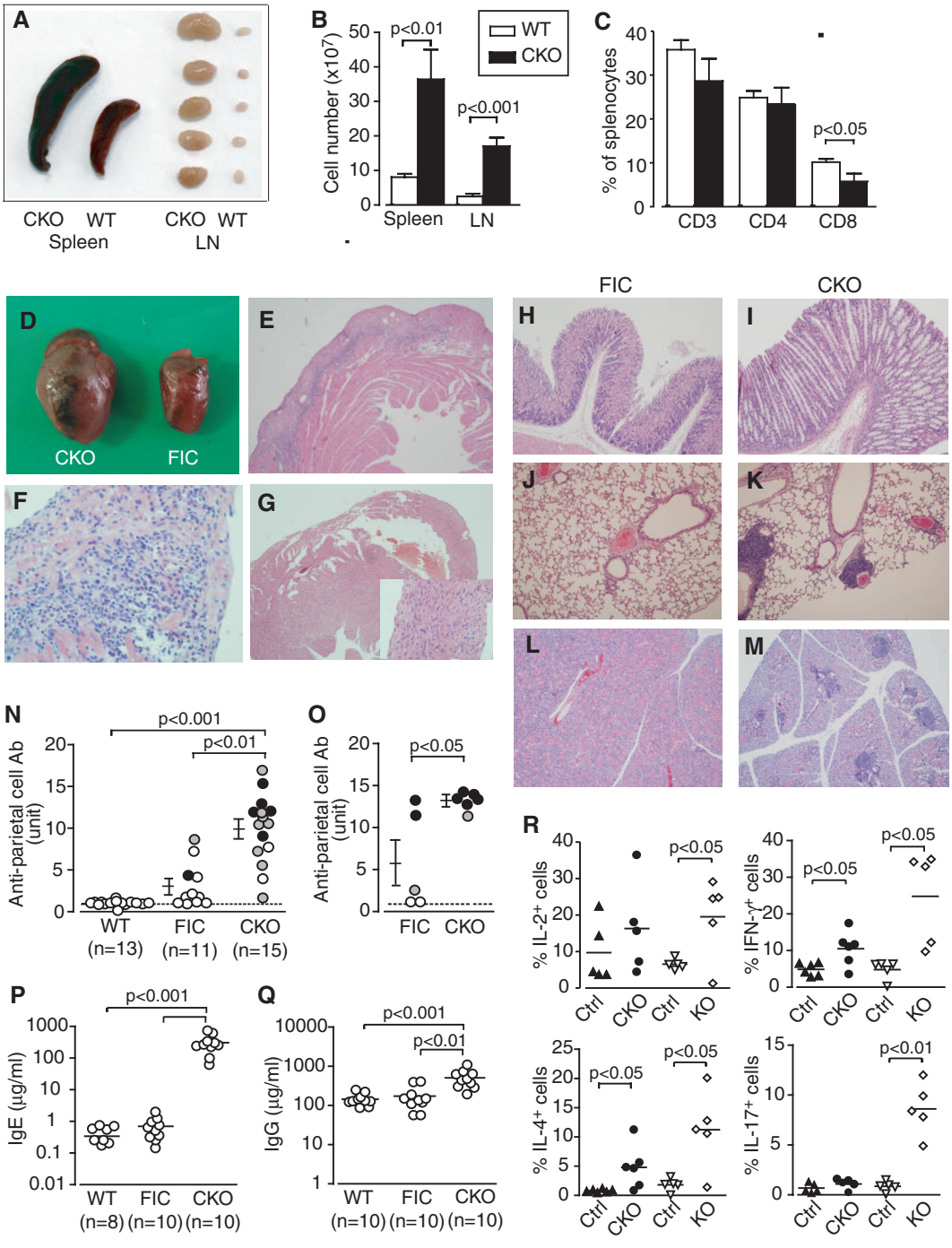

Fig. 2. Autoimmune disease and hyperproduction of IgE in CKO mice. (**A**) Splenomegaly and lymphadenopathy in a CKO and a WT littermate. Lymphocyte numbers (**B**) and frequencies of T cell subsets (**C**) in spleens of 6- to 10-week-old CKO and WT littermates (n = 11 to 13). (**D**) The heart of a CKO (left) and a FIC$^{+/-}$CTLA-4$^{fl/fl}$ mouse (right). Histology (hematoxylin and eosin staining) of the heart of a CKO [(**E** and **F**) ×50 and ×200, respectively] and a FIC mouse [(**G**) ×50; inset, ×200]. Histology of the stomach [(**H** and **I**) ×100], lung [(**J** and **K**) ×100], and salivary gland [(**L** and **M**) ×50] of a CKO [(H), (J), and (L)] and a FIC mouse [(I), (K), and (M)]. Serological and histological development of gastritis in WT, FIC$^{+/+}$, and CKO mice (**N**), and BALB/c nu/nu mice 7 weeks after cell transfer from CKO or FIC$^{+/+}$ mice (**O**). Gastric lesions were histologically graded as 2 (black circle), 1 (gray circle), and 0 (open circle) (*19*). Serum concentrations of IgE (**P**) and IgG (**Q**) in indicated groups of mice. (**R**) Frequencies of cytokine-producing cells among CD4$^+$Foxp3$^-$ splenocytes of 6- to 9-week-old CKO, 16- to 20-day-old KO, or normal littermates (n = 5 to 6). Error bars indicate SEM.

not activated CKO Tregs) also reduced the expression of CD86-GFP fusion protein retrovirally expressed in L-cells, a fibroblast cell line (Fig. 4H). This indicates that Treg-dependant modulation of CD86 expression on DCs is at least partly due to down-regulation of the expression and not masking of the molecule by soluble CTLA-4. Taken together, Treg-mediated CD80/CD86 down-regulation may limit the activation of naive T cells via CD28, resulting in specific immune suppression and tolerance.

Thus, CTLA-4 expressed in Foxp3$^+$ Tregs is critically required for their in vivo and in vitro suppression, which is mediated at least in part by

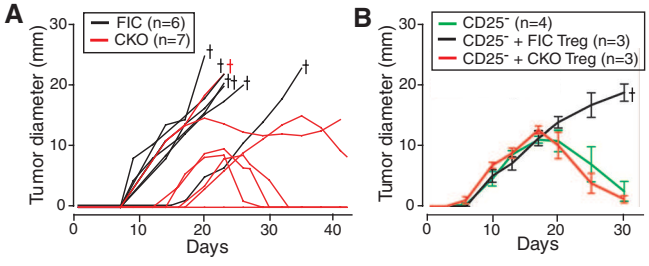

Fig. 3. Treg-specific CTLA-4 deficiency promotes tumor immunity. (**A**) BALB/c nu/nu mice received 3 × 10^7 splenocytes from FIC or CKO mice, followed by intradermal inoculation of 1.5 × 10^5 RL♂1 leukemia cells. Crosses indicate death due to tumor growth. (**B**) BALB/c CD25$^-$ cells (1.5 × 10^7) were cotransferred with 3.8 × 10^5 CD25^{high}CD4$^+$ T cells from CKO or FIC mice and inoculated with 1.5 × 10^5 RL♂1 cells (n = 3). Tumor diameters were measured every other day for 6 weeks. Mice were euthanized when tumor diameters exceeded 20 mm. Error bars indicate SEM.

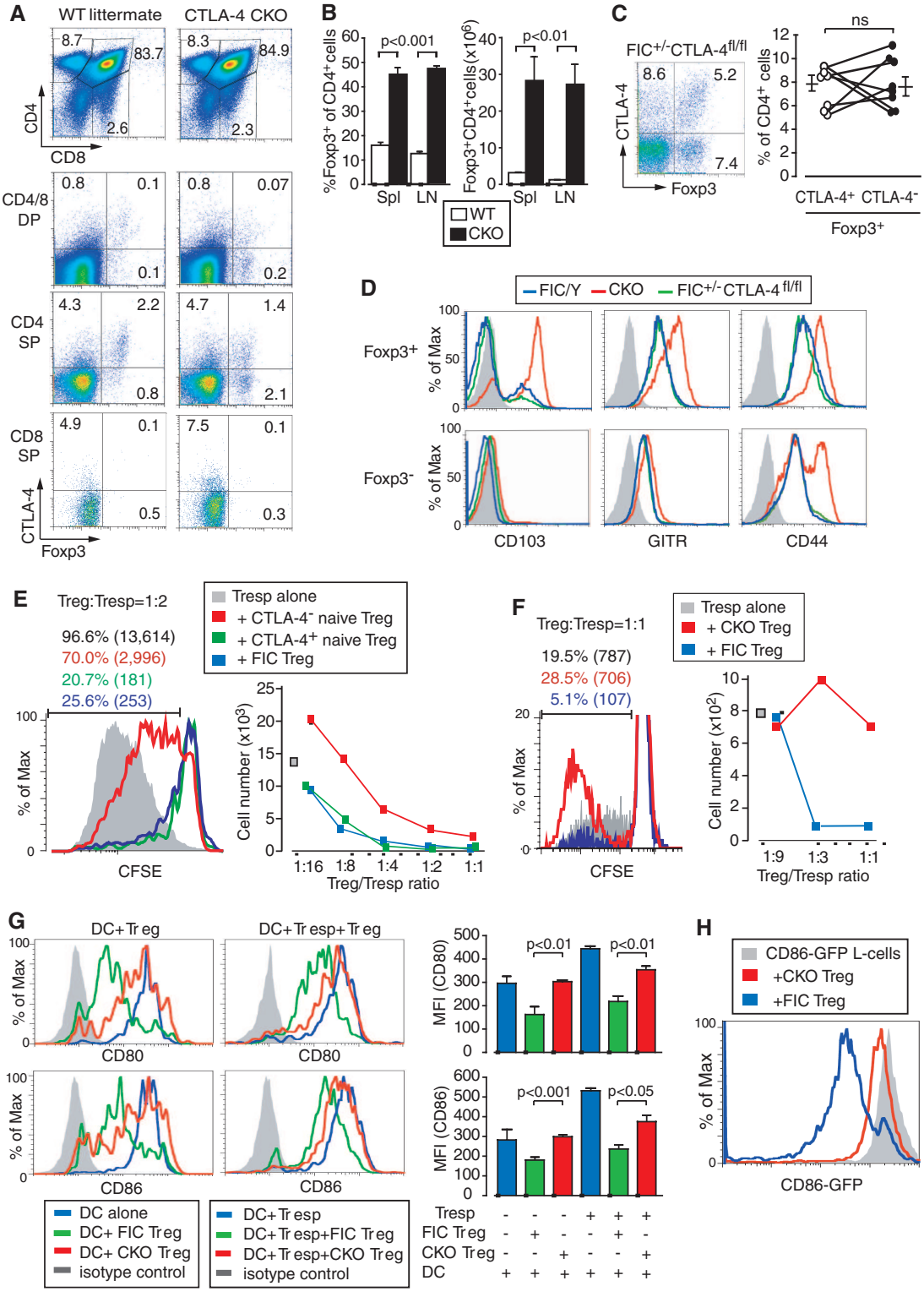

Fig. 4. CTLA-4–deficient Tregs develop and survive normally but have defective function. (**A**) Thymocyte expression of Foxp3 and CTLA-4 in a 2.5-week-old CKO or a WT littermate. SP, single positive; DP, double positive. (**B**) Frequency and number of $CD4^+Foxp3^+$ T cells in spleens and LNs of 6- to 8-week-old CKO or WT littermates (n = 7). (**C**) Foxp3 and CTLA-4 expression in splenic $CD4^+$ T cells from a $FIC^{+/-}CTLA\text{-}4^{fl/fl}$ female mouse (left). Percentages of $Foxp3^+CTLA\text{-}4^+$ and $Foxp3^+CTLA\text{-}4^-$ T cells in each mouse (5 to 8 weeks of age) are connected (n = 8) (right). ns, not significant. (**D**) Expression of cell surface molecules on $CD4^+$ LN cells from CKO, FIC, or $FIC^{+/-}CTLA\text{-}4^{fl/fl}$ mice. (**E**) $CD25^{high}EGFP^+$ cells (naive $CTLA\text{-}4^-$ Treg) and $CD25^{high}EGFP^-$ cells (naive $CTLA\text{-}4^+$ Treg) from $FIC^{+/-}CTLA\text{-}4^{fl/fl}CAG$ female mice and $CD25^{high}CD4^+$ T cells from FIC mice (FIC Treg) were cocultured with $CD25^-CD4^+$ T cells (Tresp), anti-CD3 mAb, and live splenic DCs for 3 days. Percentages and numbers (in parentheses) of CFSE-diluting Tresp cultured at a 1:2 Treg-to-Tresp ratio (left). Numbers of CFSE-diluting Tresp cultured at graded ratios of Treg:Tresp (right). (**F**) Percentage and numbers of CFSE-labeled BALB/c Tresp cocultured with CKO or FIC Tregs and X-irradiated C57BL/6 splenocytes for 4 days at 1:1 Treg:Tresp ratio (left) and numbers at graded ratios (right). (**G**) CD80 and CD86 expression of live splenic DCs after a 2-day culture with Tresp, $CD4^+EGFP^+$ Tregs, or a mix thereof, and anti-CD3 mAb. Histograms show mean fluorescence intensity (MFI). (**H**) L-cells, expressing the Fc receptor, were retrovirally transduced to express CD86-EGFP fusion protein, cocultured with indicated Tregs and anti-CD3 mAb for 2 days, and assessed for GFP level. Data in (A) and (D) to (H) represent three or more independent experiments. Error bars indicate SEM.

CTLA-4–dependent down-regulation of CD80 and CD86 on antigen presenting cells. Tregs probably use multiple suppressive mechanisms, and the importance of each one may vary depending on the environment and the context of immune responses (*1*). However, if the CTLA-4–mediated mechanism of suppression is defective, Tregs cannot sustain self-tolerance and immune homeostasis, even if other suppressive mechanisms become more active to compensate for the deficiency. Thus, CTLA-4 is a key molecular target for controlling Treg-suppressive function in both physiological and pathological immune responses including autoimmunity, allergy, and tumor immunity.

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Environmental Genomics Reveals a Single-Species Ecosystem Deep Within Earth

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DNA from low-biodiversity fracture water collected at 2.8-kilometer depth in a South African gold mine was sequenced and assembled into a single, complete genome. This bacterium, *Candidatus Desulforudis audaxviator*, composes >99.9% of the microorganisms inhabiting the fluid phase of this particular fracture. Its genome indicates a motile, sporulating, sulfate-reducing, chemoautotrophic thermophile that can fix its own nitrogen and carbon by using machinery shared with archaea. *Candidatus Desulforudis audaxviator* is capable of an independent life-style well suited to long-term isolation from the photosphere deep within Earth's crust and offers an example of a natural ecosystem that appears to have its biological component entirely encoded within a single genome.

A more complete picture of life on, and even in, Earth has recently become possible by extracting and sequencing DNA from an environmental sample, a process called environmental genomics or metagenomics (*1*–*8*). This approach allows us to identify members of microbial communities and to characterize the abilities of the dominant members even when isolation of those organisms has proven intractable. However, with a few exceptions (*5*, *7*), assembling complete or even near-complete genomes for a substantial portion of the member species is usually hampered by the complexity of natural microbial communities.

In addition to elevated temperatures and a lack of O_2, conditions within Earth's crust at depths >1 km are fundamentally different from those of the surface and deep ocean environments. Severe nutrient limitation is believed to result in cell doubling times ranging from 100s to 1000s of years (*9*–*11*), and as a result subsurface microorganisms might be expected to reduce their reproductive burden and exhibit the streamlined genomes of specialists or spend most of their time in a state of semi-senescence, waiting for the return of favorable conditions. Such microorganisms are of particular interest because they permit insight into a mode of life independent of the photosphere.

One bacterium belonging to the *Firmicutes* phylum (Fig. 1A), which we herein name *Candidatus Desulforudis audaxviator*, is prominent in small subunit (SSU or 16*S*) ribosomal RNA (rRNA) gene clone libraries (*11*–*14*) from almost all fracture fluids sampled to date from depths greater than 1.5 km across the Witwatersrand basin (covering 150 km by 300 km near Johannesburg, South Africa). This bacterium was shown in a previous geochemical and 16*S* rRNA gene study (*11*) to dominate the indigenous microorganisms found in a fracture zone at 2.8 km below land surface at level 104 of the Mponeng mine (MP104). Although Lin *et al.* (*11*) discovered that this fracture zone contained the least-diverse natural free-living microbial community reported at that time, exceeding the ~80% dominance by the methanogenic archaeon IUA5/6 of a comparatively shallow subsurface community in Idaho (*15*), we were nonetheless surprised when the current environmental genomics study revealed only one species was actually present within the fracture fluid. Furthermore, we found that the genome of this organism appeared to possess all of the metabolic capabilities necessary for an independent life-style. This gene complement was consistent with the previous geochemical and thermodynamic analyses at the ambient ~60°C temperature and pH of 9.3, which indicated radiolytically generated chemical species as providing the energy and nutrients to the system (*11*), with formate and H_2 as possessing the greatest potential among candidate electron donors, and sulfate (SO_4^{2-}) reduction as the dominant electron-accepting process (*11*).

DNA was extracted from ~5600 liters of filtered fracture water by using a protocol that has been demonstrated to be effective on a broad range of bacterial and archaeal species, including recalcitrant organisms (*16*). A single, complete, 2.35–megabase pair (Mbp) genome was assembled with a combination of shotgun Sanger sequencing and 454 pyrosequencing (*16*). Similar to other studies that obtained near-complete consensus genomes from environmental samples (*5*, *17*), heterogeneity in the population of the dominant species as measured with single-nucleotide polymorphisms (SNP) was quite low, showing only 32 positions with a SNP observed

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