We have shown that transcriptional noise is well predicted by molecularly detailed models for the two most common promoter architectures in *E. coli* as the various genetic knobs are tuned. This agreement is not the result of fitting theory curves to data, because the predicted curves are generated using physical parameter values reported elsewhere in the literature and in that sense are zero-parameter predictions. Earlier reports of "bursty" transcription (*5*, *21*) are based on the observation that the Fano factor is greater than 1 for constitutive mRNA production (as well as direct kinetic measurements). Various explanatory hypotheses have been proposed, including transcriptional silencing via DNA condensation by nucleoid proteins (*22*), negative supercoiling induced by transcription, or the formation of long-lived "dead-end" initiation complexes (*23*). Although our data do not rule out these hypotheses, we find that extrinsic noise is sufficient to explain the deviation from Fano = 1 in our constitutive expression data (Fig. 2B). Thus, we find no need to invoke alternative hypotheses to explain the observed "burstiness" of constitutive transcription.

Many interesting earlier experiments make it difficult to interpret differences between promoters and induction conditions in terms of distinct physical parameters because of the wide variety of promoter architectures in play as well as the diverse mechanisms of induction. We have instead taken a "synthetic biology" approach of building promoters from the ground up. By directly controlling aspects of the promoter architecture, our goal has been to directly relate changes in promoter architecture to changes in observed gene expression variability. We believe that this work has demonstrated that mutations in regulatory DNA can alter gene expression noise. This suggests that gene expression noise may be a tunable property subject to evolutionary selection pressure, as mutations in regulatory DNA could provide greater fitness by increasing (or decreasing) variability. Demonstrating the relevance of this hypothesis in natural environments remains an ongoing challenge.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/346/6216/1533/suppl/DC1
Materials and Methods
Supplementary Text
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IMMUNE TOLERANCE

Detection of self-reactive CD8$^+$ T cells with an anergic phenotype in healthy individuals

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Immunological tolerance to self requires naturally occurring regulatory T (T_{reg}) cells. Yet how they stably control autoimmune T cells remains obscure. Here, we show that T_{reg} cells can render self-reactive human CD8$^+$ T cells anergic (i.e., hypoproliferative and cytokine hypoproducing upon antigen restimulation) in vitro, likely by controlling the costimulatory function of antigen-presenting cells. Anergic T cells were naïve in phenotype, lower than activated T cells in T cell receptor affinity for cognate antigen, and expressed several coinhibitory molecules, including cytotoxic T lymphocyte–associated antigen-4 (CTLA-4). Using these criteria, we detected in healthy individuals anergic T cells reactive with a skin antigen targeted in the autoimmune disease vitiligo. Collectively, our results suggest that T_{reg} cell–mediated induction of anergy in autoimmune T cells is important for maintaining self-tolerance.

Naturally occurring CD25$^+$CD4$^+$ regulatory T (T_{reg}) cells, which specifically express the transcription factor FoxP3, actively maintain immunological self-tolerance and homeostasis (*1*). Developmental or functional anomalies of natural T_{reg} cells can cause autoimmune diseases (such as type I diabetes), allergy, and immunopathological diseases (such as inflammatory bowel disease) (*1*). How T_{reg} cells effectively control potentially hazardous self-reactive T cells in humans remains an open question. In particular, it is unknown whether T_{reg} cell–mediated suppression for a limited period has a critical long-lasting effect on cell fate and antigen reactivity of autoimmune T cells.

To address this issue, we examined proliferation, cytokine production, and cell fate of antigen-specific CD8$^+$ T cells in peripheral blood mononuclear cells (PBMCs) from healthy individuals stimulated in vitro with self-antigen peptide in the presence or absence of natural FoxP3$^+$CD25$^+$CD4$^+$ T_{reg} cells. Melan-A (also known as MART-1) used in the experiments is a self-antigen expressed by normal melanocytes and some melanoma cells and targeted in vitiligo vulgaris, an autoimmune disease of the skin (*2*–*5*). In the absence of T_{reg} cells, Melan-A–specific CD8$^+$ T cells [detectable by major histocompatibility complex (MHC) tetramers and peptide tetramers] expanded over 10 days from very few cells to a sizable fraction when cultured with peptide-pulsed autologous antigen-presenting cells (APCs) (Fig. 1A) (*6*). Natural T_{reg} cells, which appeared to be activated by endogenous self-peptides and class II MHC on autologous APCs (*7*–*9*), suppressed the expansion of Melan-A tetramer–positive (Tet$^+$) CD8$^+$ T cells in a dose-dependent manner. Similar stimulation with irrelevant peptide NY-ESO-1, another self- and tumor antigen, failed to induce Melan-ATet$^+$CD8$^+$ T cells. In cultures containing T_{reg} cells,

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we noted an accumulation of Tet$^+$CD8$^+$ T cells that had divided once and then stopped further proliferation. This proliferation-aborted population increased in ratio, whereas the population under multiple cell divisions reciprocally decreased, in proportion to the number of added T_{reg} cells. The proliferation-aborted cells had significantly lower tetramer staining intensity than the cells that had vigorously proliferated in the absence of T_{reg} cells (peak a versus b in Fig. 1A, Fig. 1B, and fig. S1). The staining intensity of T cell receptor–αβ (TCR–αβ) chains was equivalent in both populations, which indicated that the lower tetramer staining intensity was not due to down-modulation of TCR but to lower TCR affinity for the Melan-A peptide, as supported by significantly lower ratios of tetramer versus TCR-αβ staining intensities (Fig. 1C). Functionally, they produced reduced levels of cytokines such as interferon-γ (IFN-γ), tumor necrosis factor–α (TNF-α), and interleukin 2 (IL-2) (Fig. 1, D and E), despite the addition of exogenous IL-2 to maintain cultured T cells. Furthermore, upon secondary stimulation, they remained hypoproliferative and produced very low amounts of cytokines (fig. S2). Thus, antigenic stimulation under T_{reg} cell–mediated suppression allows responder T cells with relatively low affinity TCRs for a self-antigen to divide once but prevents their further proliferation, which drives them into a profoundly and stably hypoproliferative and cytokine-hypoproducing state, which can be immunologically defined as "anergy" (*10*–*13*).

In contrast with anti–Melan-A responses, CD8$^+$ T cells from the same donor, who had detectable serum anticytomegalovirus (CMV) immunoglobulin G (IgG) antibody, had CMV peptide–specific T cells with a memory phenotype (fig. S3, A to D). CMV-specific CD8$^+$ T cells, whether they were in a naïve or memory cell fraction, vigorously proliferated and produced inflammatory cytokines even at a high T_{reg}-to-responder T cell ratio, with no significant differences in CMV tetramer staining intensity among CD8$^+$ T cells proliferating in the presence or absence of T_{reg} cells (fig. S3, E to H). However, high numbers of T_{reg} cells completely inhibited the proliferation of polyclonally activated naïve CD8$^+$ T cells without allowing a single cell division (fig. S4). The nondividing CD8$^+$ T cells proliferated as actively as nonsuppressed cells upon restimulation after removal of T_{reg} cells.

Collectively, T_{reg}-cell dosage, the immunological states of responder T cells (e.g., in a naïve or memory state), and their TCR affinity for cognate antigen contribute to T_{reg} cell–mediated induction of anergy. This is an active process and differs from a mere naïve nonproliferative state.

Microarray gene expression analysis revealed that activated or anergic Tet$^+$CD8$^+$ T cells or Tet$^-$CD8$^+$ T cells obtained from T_{reg}-absent or -present cell cultures were substantially different in gene expression profiles (Fig. 2A). As the most striking differences, the transcription of *CTLA4*, encoding the coinhibitory molecule CTLA-4 (*14*), was significantly up-regulated, whereas *BCL2*, encoding the apoptosis-inhibiting molecule B cell lymphoma-2 (BCL-2) (*15*), was down-regulated in anergic CD8$^+$ T cells, as confirmed by quantitative reverse transcription polymerase chain reaction (RT-PCR) (Fig. 2B). There were no significant differences in the expression of *PDCD1* encoding the coinhibitory molecule PD-1; the genes encoding the anergy-related molecules GRAIL, CBL-B, and EGR-2 (*16*–*19*); BAT3, TBX21, and EOMES, putative markers for exhausted CD8$^+$ T cells (*20*, *21*); and p27KIP1, a cyclin-dependent kinase inhibitor. Anergic CD8$^+$ T cells did not express FoxP3 (Fig. 2B and fig. S5A). The majority (>90%) of anergic CD8$^+$ T cells expressed both CTLA-4 and the chemokine receptor CCR7, which differed from the phenotype of activated or naïve CD8$^+$ T cells (Fig. 2, C and D, and fig. S5, B to D) (*22*, *23*). Functionally, during secondary stimulation of anergic Tet$^+$CD8$^+$ T cells with Melan-A peptide–pulsed APCs after removal of T_{reg} cells, antibody blockade of CTLA-4 and PD-1 at doses enhancing cytokine production by activated conventional T cells failed to rescue proliferation resistance or cytokine hypoproduction of anergic CD8$^+$ T cells (fig. S5E) (*24*). Addition of a high dose of IL-2 induced apoptosis in restimulated Tet$^+$CD8$^+$ T cells rather than abrogating their hyporesponsiveness. Nevertheless, anergic CD8$^+$ T cells were not in the process of immediate apoptosis (fig. S6), despite their lower *BCL2* expression than activated T cells (Fig. 2B). Thus, anergic CD8$^+$ T cells induced by T_{reg} cell–mediated suppression are distinct from activated or naïve T cells in gene and protein expression profiles. They also appear to be different from "exhausted" CD8$^+$ T cells, which develop as PD-1$^+$ hypoproliferative and cytokine-hypoproducing cells

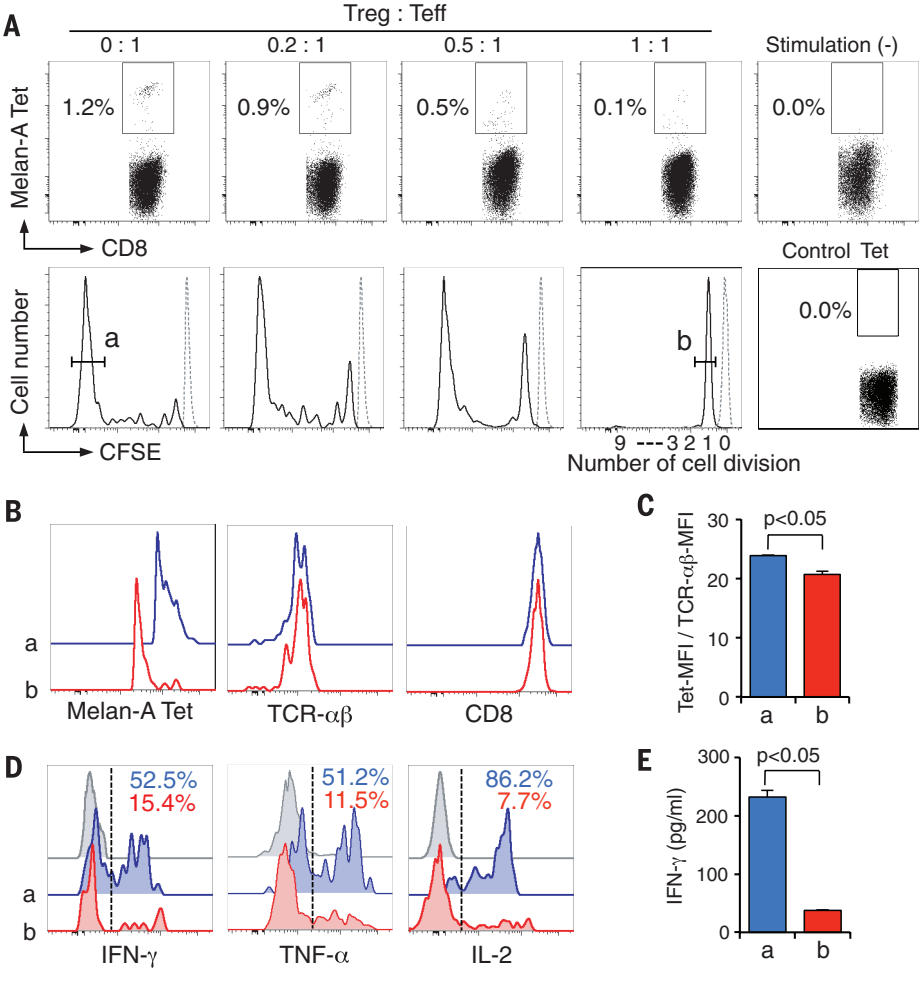

Fig. 1. Natural T_{reg} cells render low-affinity self-reactive CD8$^+$ T cells anergic upon antigen stimulation. (**A**) Melan-A–specific CD8$^+$ T cell induction. CFSE-labeled CD8$^+$ T cells of HLA-A*0201$^+$ healthy individuals were stimulated by T cell–depleted, γ-irradiated, and Melan-$A_{26\text{-}35}$ peptide–pulsed APCs with graded numbers of CD25^{high}CD4$^+$ T_{reg} cells for 10 days (*6*). Dotted lines mean Tet$^-$CD8$^+$ cells showing no CFSE dilution. Control tet: NY-ESO-$1_{157\text{-}165}$/HLA-A*0201 tetramer. T_{eff} refers to CD8$^+$ effector T cells. (**B**) Tet, TCR-αβ, and CD8 staining of Tet$^+$CD8$^+$ T cells. Results in (A) and (B) are representative of 10 independent experiments. (**C**) Relative tetramer staining intensities, calculated as mean fluorescence intensity (MFI) of Tet/MFI of TCR-αβ staining of Tet$^+$CD8$^+$ T cells (n = 5). (**D** and **E**) Cytokine production of Tet$^+$CD8$^+$ T cells by intracellular staining (D) and enzyme-linked immunosorbent assay (E) (*6*). Representative result of three independent experiments. The labels a and b in (B) to (E) mean the cell accumulations like a or b in (A). Error bars indicate means ± SEM. The significance was assessed by Student's two-tailed paired t test.

in chronic viral infections and in tumor tissues, because exhausted CD8$^+$ T cells are reportedly CCR7$^-$, CD45RA$^-$, and BAT3$^+$, and their exhaustion can be rescued by a PD-1–blocking antibody (*21*, *24*–*26*).

T_{reg} cells suppress the activation and/or proliferation of responder T cells (*27*), at least in part, by down-regulating the expression of the costimulatory molecules CD80 and CD86 on APCs (fig. S7A) (*28*, *29*). To determine whether low expression or down-modulation of CD80 and CD86 on dendritic cells (DCs) was responsible for the induction of antigen-specific anergic CD8$^+$ T cells, we stimulated carboxyfluorescein succinimidyl ester (CFSE)–labeled CD8$^+$ T cells with autologous immature or mature DCs pulsed with Melan-A peptide in the presence of graded amounts of CTLA-4–immunoglobulin (CTLA-4Ig), which blocked CD80 and CD86 (fig. S7B) (*30*). In contrast to the vigorous proliferation of Tet$^+$CD8$^+$ T cells cultured with mature DCs, the majority of Tet$^+$CD8$^+$ T cells generated with immature DCs, and some with mature DCs with a high dose (100 μg/ml) of CTLA-4Ig, were proliferation-aborted after one cell division (Fig. 3A). The proliferation-aborted T cells (peaks c and d in Fig. 3A) were lower than proliferating T cells in Melan-A tetramer staining intensity (Fig. 3B), and highly expressed CTLA-4 and CCR7 (fig. S7, C and D); they formed a discrete CTLA-4/CCR7 double-positive population (Fig. 3C and fig. S7E). They produced significantly lower amounts of IFN-γ, TNF-α, and IL-2 compared with Tet$^+$CD8$^+$ cells, having proliferated in culture with mature DCs (fig. S7F). Similar to peptide stimulation, polyclonal antibody against CD3-specific monoclonal antibody (mAb) stimulation of CTLA4$^-$ naïve CD8$^+$ T cells in the presence of CTLA-4Ig produced cells that were proliferation-aborted after one cell division (fig. S8A). Notably, increasing CTLA-4Ig dose proportionally intensified CTLA-4 expression by the aborted cells, while stably maintaining their high CCR7 expression (fig. S8, A and B). Taken together, antigen presentation with low CD80 and CD86 costimulation is able to drive CD8$^+$ T cells to differentiate into CTLA-4$^+$CCR7$^+$ anergic cells. DCs with moderate CD80 and CD86 reduction can concurrently generate both

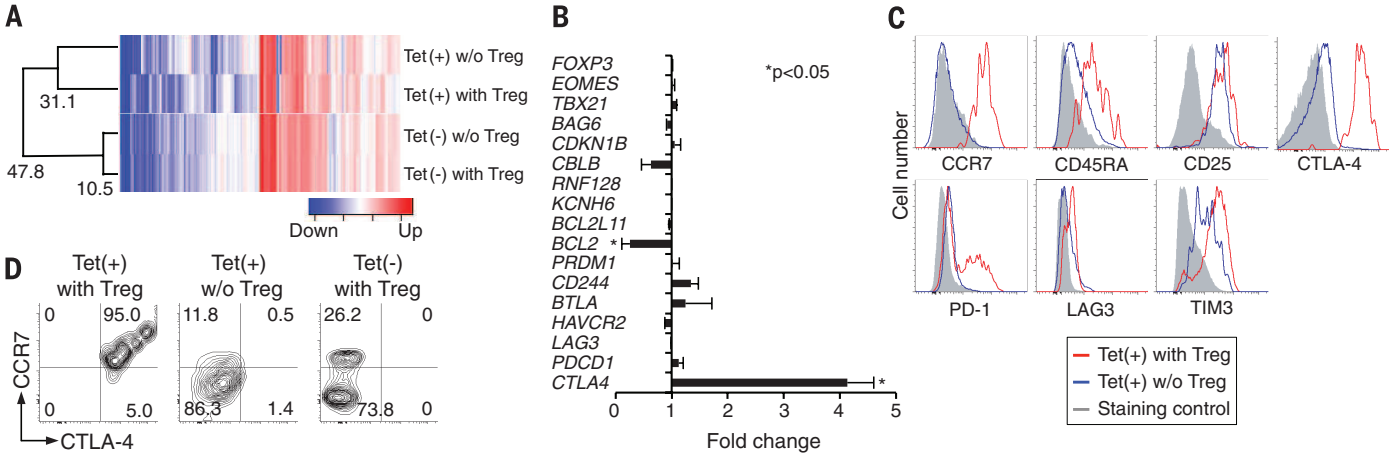

Fig. 2. Distinct phenotype and function of anergic CD8$^+$ T cells produced by T_{reg} cell suppression. (**A**) Global mRNA expression profile. Tet$^+$CD8$^+$ T cells induced at CD8$^+$ T cells: T_{reg} cell ratios of 1:0.5 and 1:0 were subjected to microarray analyses. Gene expression reportedly associated with CD8$^+$ T cell function was compared among the indicated four groups and expressed as a heat map. Correlation distances shown were calculated by h-clust (*6*). Representative of two independent experiments. (**B**) mRNA expression measured by quantitative real-time PCR. Fold changes of mRNA level as [Tet(+) with T_{reg}] versus [Tet(+) without T_{reg}] in five independent experiments are shown. Error bars indicate means ± SEM. (**C** and **D**) Expression of cell surface molecules by Tet$^+$CD8$^+$ T cells induced at CD8$^+$ T cells: T_{reg} cell ratios, 1:1 and 1:0. Representative histogram staining pattern (C) and contour plot staining pattern of CTLA-4 and CCR7 (D). Data are representative of five independent experiments (n = 10). The significance was assessed by Student's two-tailed paired t test.

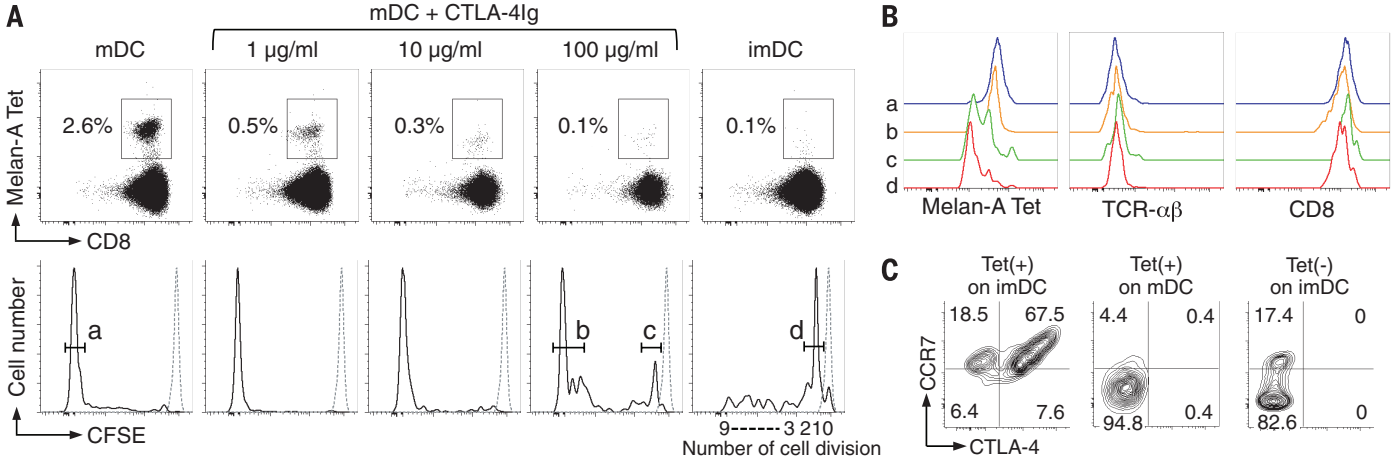

Fig. 3. DC expression of CD80 and CD86 controls the generation of CTLA-4$^+$CCR7$^+$ low-affinity anergic self-reactive T cells. (**A**) Melan-A–specific CD8$^+$ T cell induction. CFSE-labeled CD8$^+$ T cells of HLA-A*0201$^+$ healthy individuals were stimulated with γ-irradiated, Melan-$A_{26\text{-}35}$ peptide–pulsed monocyte-derived immature or mature DCs. CTLA-4Ig was added into mature DCs cultures at indicated concentrations (*6*). (**B**) Tet, TCR-αβ, and CD8 staining intensity of Tet$^+$CD8$^+$ T cells shown in (A). (**C**) Representative contour plot staining pattern of Tet$^+$ or Tet$^-$CD8$^+$ T cells shown in (A) for CTLA-4 and CCR7. Data in (A) to (C) are representative of five independent experiments.

activated T cells and anergic T cells, in part, depending on TCR affinity.

The above in vitro findings prompted us to ask whether healthy individuals harbored such anergic self-reactive $CD8^+$ T cells. Direct ex vivo staining of $CD8^+$ T cells in PBMCs of healthy donors ($n = 10$) for Melan-A peptide and MHC tetramer, with $CD8^+$ T cells from vitiligo patients ($n = 10$) as a positive control, revealed that a small number of Tet^+CD8^+ T cells were indeed present in healthy individuals and constituted ~0.03% of $CD8^+$ T cells in PBMCs, which contrasted with high percentages (~0.1%) in vitiligo patients (Fig. 4, A and B) (*6*). Two-thirds of the former had a naïve ($CCR7^+CD45RA^+$) phenotype, whereas the majority of the latter had an effector or memory phenotype (Fig. 4C and fig. S9A) (*4*, *5*). The Tet^+CD8^+ T cells from healthy individuals had significantly lower tetramer staining intensity than those from vitiligo patients (Fig. 4, D and E). They expressed CTLA-4 at higher levels than Tet^+CD8^+ T cells from vitiligo patients or Tet^-CD8^+ T cells from healthy individuals, or activated $CD8^+$ T or natural T_{reg} cells (Fig. 4F and fig. S9, B to D), and ~90% of the $Tet^+CD45RA^+CD8^+$ cells were double positive for CTLA-4 and CCR7 (Fig. 4G and fig. S9B). Functionally, Tet^+CD8^+ T cells directly prepared from healthy donors scarcely produced IFN-γ, TNF-α, or IL-2, contrasting with active cytokine production by naïve Tet^-CD8^+ T cells (Fig. 4H) or Melan-A–specific $CD8^+$ T cells from vitiligo patients (*4*, *31*).

To determine further the function of these anergic T cells, we cocultured $CTLA\text{-}4^+$ and $CTLA\text{-}4^-$ fractions of naïve ($CCR7^+CD45RA^+$) $CD8^+$ T cells from healthy individuals and assessed the proliferative activity of Tet^+CD8^+ T cells present in each fraction (Fig. 4I). The $CTLA\text{-}4^+$ fraction, which constituted less than 10% of naïve $CD8^+$ T cells in healthy donors, contained the majority (~95%) of Melan-A Tet^+CD8^+ T cells before stimulation (Fig. 4, I to K). These $CTLA\text{-}4^+Tet^+$ cells were

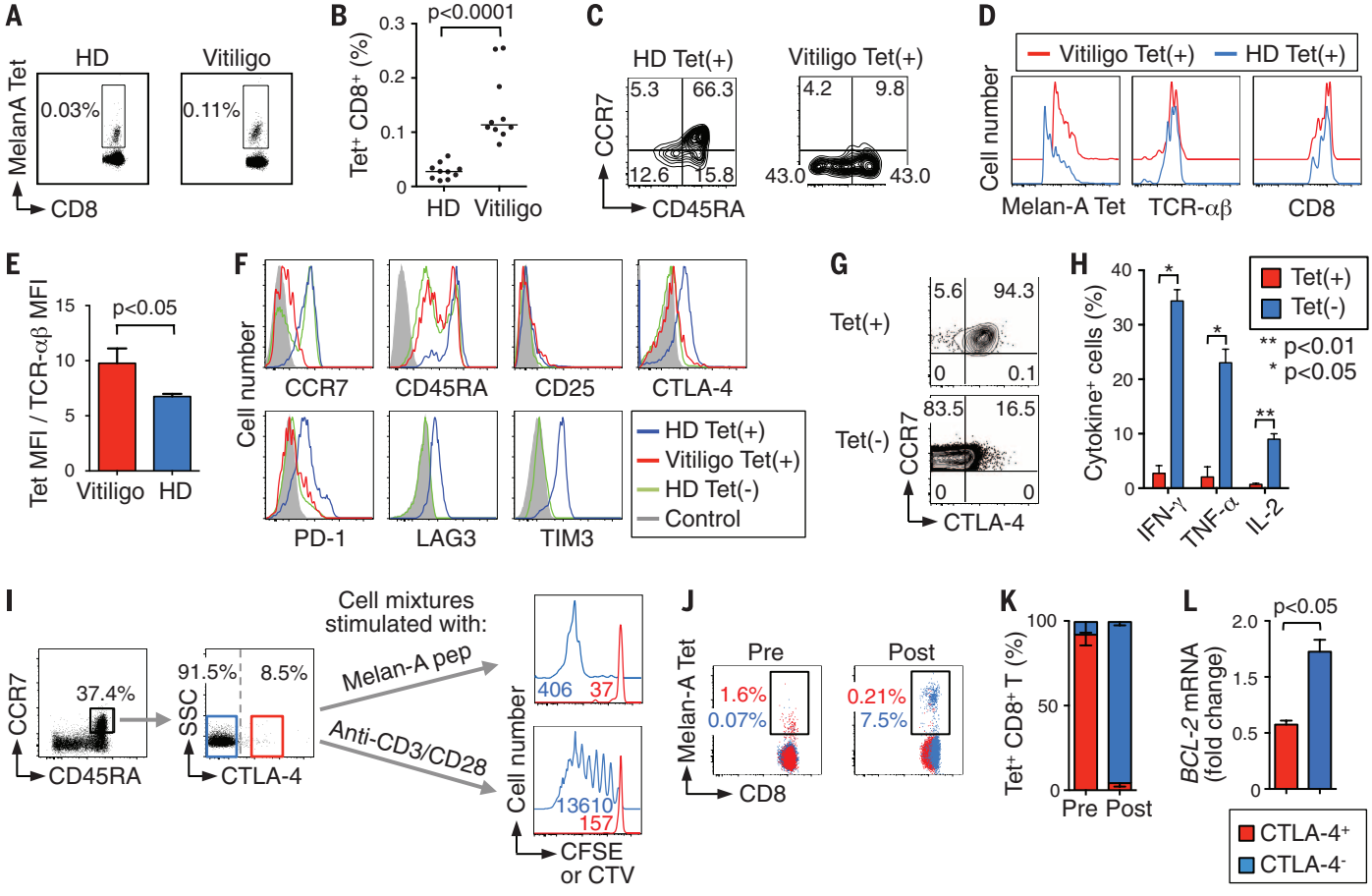

Fig. 4. Detection of low-affinity anergic self-reactive $CTLA\text{-}4^+CCR7^+CD8^+$ T cells in healthy individuals. (**A**) Melan-ATet^+CD8^+ T cells in PBMCs of a healthy donor (HD) and a vitiligo patient. (**B**) Percentages of Tet^+CD8^+ T cells in HDs and vitiligo patients ($n = 10$). (**C**) CCR7 and CD45RA expression by Tet^+CD8^+ T cells in an HD and a vitiligo patient. (**D**) Tet, TCR-αβ, and CD8 staining intensity of Tet^+CD8^+ T cells in an HD and a vitiligo patient. (**E**) Ratios of MFI of tetramer staining to MFI of TCR-αβ staining in Tet^+CD8^+ T cells in HDs and vitiligo patients ($n = 4$ each). (**F**) Expression of cell surface molecules by Tet^+ or Tet^-CD8^+ T cells in a representative HD and a vitiligo patient. (**G**) Representative staining for CTLA-4 and CCR7 of Tet^+ or Tet^- cells in $CD45RA^+CD8^+$ T cells of an HD. Data shown in (A), (C), (D), (F), and (G) are representative of four independent experiments. (**H**) Cytokine production by Tet^+CD8^+ T cells in HDs assessed by intracellular staining with $CCR7^+CD45RA^+Tet^-CD8^+$ T cells as control. Data summarize four independent experiments. (**I**) Proliferation and cytokine production of $CTLA\text{-}4^+$ or $CTLA\text{-}4^-$ naïve $CD8^+$ T cells in HDs. $CCR7^+CD45RA^+CD8^+$ T cells from HD PBMCs were further separated into $CTLA\text{-}4^+$ and $CTLA\text{-}4^-$ cells, labeled with Cell Trace Violet (CTV) or CFSE, respectively, mixed at a 1:1 ratio, stimulated with Melan-$A_{26\text{-}35}$ peptide–pulsed APCs for 10 days (top) or CD3/CD28-specific mAb for 5 days (bottom), and assessed for proliferation by CTV or CFSE dilution (red and blue, respectively) (*6*). Numbers in right two figures represent the numbers of cells in each cell fraction. SSC, side scatter. (**J**) Representative tetramer staining of the cell mixtures before (Pre) and after (Post) Melan-$A_{26\text{-}35}$ peptide stimulation for 10 days. Numbers represent percentages of Tet^+CD8^+ cells in the $CTLA\text{-}4^+$ or $CTLA\text{-}4^-$ fraction (red and blue, respectively). (**K**) Percentages of Tet^+CD8^+ T cells in the $CTLA\text{-}4^+$ (red) or $CTLA\text{-}4^-$ (blue) fraction in the cell mixtures before (Pre) and after (Post) cell culture as shown in (I) and (J). (**L**) *BCL2* mRNA expression of $CTLA\text{-}4^+CCR7^+CD45RA^+Tet^+CD8^+$ and $CTLA\text{-}4^-CCR7^+CD45RA^+Tet^+CD8^+$ T cells measured by quantitative real-time PCR. Data in (I) to (L) are representative of at least three independent experiments. Error bars indicate means ± SEM. The significance was assessed by Student's two-tailed paired *t* test.

hypoproliferative, low in *BCL2* expression, and prone to die upon Melan-A stimulation (Fig. 4, I to L). In contrast, the CTLA-4$^-$ fraction, which initially contained fewer than 5% of total Tet$^+$CD8$^+$ T cells, gave rise to proliferating Tet$^+$CD8$^+$ T cells, which made up ~95% of total Tet$^+$CD8$^+$ T cells after stimulation (Fig. 4, I to K). In addition, polyclonal stimulation of the cell mixtures with CD3-specific and CD28-specific mAb revealed that the CTLA-4$^+$ fraction as a whole was hypoproliferative (Fig. 4I) and cytokine hypoproducing (fig. S9E), in contrast with active proliferation and cytokine production of the CTLA-4$^-$ fraction.

These results collectively indicate that healthy individuals harbor at least two distinct populations of self-reactive CD8$^+$ T cells: one that is functionally anergic and expresses CTLA-4 and CCR7 and another that is CTLA-4$^-$ and naïve in function and phenotype. The latter, especially those with high-affinity TCRs, may become activated and expand upon self-antigen stimulation in the absence or reduction of natural T_{reg} cells, as shown in Fig. 1A.

Thus, anergic self-reactive T cells, which are phenotypically distinct from other T cells, are physiologically present in the immune system. They appear to be generated, at least in part, as a result of T_{reg}-mediated suppression, which can determine cell fate of responder T cells (i.e., activated, anergic, or ignorant) upon antigenic stimulation, depending on the number and suppressive activity of T_{reg} cells, the TCR affinity and differentiation states of responder T cells, and the condition of APCs. This T_{reg}-dependent switching of responder T cell fate can be a key target in controlling autoimmunity and tumor immunity, as illustrated by our analysis of Melan-A–specific immune responses, as well as a variety of other physiological and pathological immune responses.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Supplemental Text
Figs. S1 to S9
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Detection of self-reactive CD8$^+$ T cells with an anergic phenotype in healthy individuals

Yuka Maeda *et al.*

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