Deletion of CTLA-4 on regulatory T cells during adulthood leads to resistance to autoimmunity

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Cytotoxic T lymphocyte antigen-4 (CTLA-4) is an essential negative regulator of T cell responses. Germline Ctal4 deficiency is lethal, making investigation of the function of CTLA-4 on mature T cells challenging. To elucidate the function of CTLA-4 on mature T cells, we have conditionally ablated Ctal4 in adult mice. We show that, in contrast to germline knockout mice, deletion of Ctal4 during adulthood does not precipitate systemic autoimmunity, but surprisingly confers protection from experimental autoimmune encephalomyelitis (EAE) and does not lead to increased resistance to MC38 tumors. Deletion of Ctal4 during adulthood was accompanied by activation and expansion of both conventional CD4+Foxp3− (T conv) and regulatory Foxp3+ (T reg cells) T cell subsets; however, deletion of CTLA-4 on T reg cells was necessary and sufficient for protection from EAE. CTLA-4 deleted T reg cells remained functionally suppressive. Deletion of Ctal4 on T reg cells alone or on all adult T cells led to major changes in the Ctal4 sufficient T conv cell compartment, including up-regulation of immunoinhibitory molecules IL-10, LAG-3 and PD-1, thereby providing a compensatory immunosuppressive mechanism. Collectively, our findings point to a profound role for CTLA-4 on T reg cells in limiting their peripheral expansion and activation, thereby regulating the phenotype and function of T conv cells.

Although the specificity of T cell activation is determined by the interaction of antigenic peptide–MHC complex and the TCR, the functional outcome of the T cell response is profoundly influenced by co-stimulatory and co-inhibitory signals. The co-inhibitory receptor cytotoxic T lymphocyte antigen-4 (CTLA-4; CD152) is a potent negative regulator of T cell responses (Sharpe and Freeman, 2002; Fife and Bluestone, 2008). CTLA-4 is a structural homologue of the co-stimulatory receptor CD28, but binds with higher affinity to the same ligands, B7-1 (CD80) and B7-2 (CD86), which are primarily expressed by APCs (Freeman et al., 1991, 1993; Harper et al., 1991; Linsley et al., 1991). Whereas CD28 is constitutively expressed on most T cells, CTLA-4 is constitutively expressed on CD4+Foxp3+ regulatory T (T reg) cells (Metzler et al., 1999; Read et al., 2000; Takahashi et al., 2000), and appears on CD4+Foxp3− conventional T (T conv) cells after activation (Freeman et al., 1992; Linsley et al., 1992). Deletion of CTLA-4 on regulatory T cells during adulthood leads to resistance to autoimmunity.
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RESULTS

Deletion of Ctla4 in adult mice

Due to the limitations in using the germline Ctla4 knockout mouse to study CTLA-4 function, we generated a novel C57BL/6 strain harboring a conditional Ctla4 allele (Fig. 1 A). To validate this mouse strain, we crossed Ctla4fl/fl to LckCre mice to generate Lckcre+/Ctla4fl/+ mice, which would delete Ctla4 in T cells expressing Lck during thymic selection. These mice displayed a very similar phenotype to germline Ctla4 knockout mice, developing massive lymphadenopathy and dying by 28 d of age (unpublished data).
Although germline C\textit{tla4} knockout mice lack expression of full-length CTLA-4, they have increased mRNA expression of the 1/4CTLA-4 isoform, which lacks exons 2 and 3 (Liu et al., 2012). We therefore measured expression of this isoform in splenocytes from \textit{UBCCre/ERT2+Ctla4fl/fl} mice after treatment with tamoxifen and found it to be increased compared with controls, similar to germline \textit{Ctla4} knockout mice (Fig. 1 C). Deletion of CTLA-4 at the protein level was measured by co-staining with antibodies against Foxp3 and CTLA-4 11 d after initiation of tamoxifen treatment. Splenic Foxp3\textsuperscript{+} T reg cells, which are normally 91.24 ± 2.09% positive for CTLA-4 in \textit{UBCCre/ERT2-Ctla4fl/fl} mice were reduced.

To study the role of CTLA-4 during adulthood, we crossed the \textit{Ctla4fl/fl} strain to a tamoxifen-inducible Cre recombinase-expressing strain, \textit{UBC\textsuperscript{Cre/ERT2}}. The resulting \textit{UBC\textsuperscript{Cre/ERT2+Ctla4fl/fl}} mice were treated with tamoxifen for 5 consecutive days beginning at 7 wk of age. Within 7 d of initial tamoxifen administration, CTLA-4 mRNA expression in splenocytes from \textit{UBC\textsuperscript{Cre/ERT2+Ctla4fl/fl}} mice was substantially reduced compared with \textit{UBC\textsuperscript{Cre/ERT2}} mice as assessed by quantitative RT-PCR; CTLA-4 expression was reduced $\sim$10-fold in splenic Foxp3\textsuperscript{-} conventional T cells (T conv cells) and nearly 200-fold in splenic Foxp3\textsuperscript{+} regulatory T cells (T reg cells), in Cre\textsuperscript{+} mice as compared with Cre\textsuperscript{-} controls.

(Fig. 1 B).
to 7.00 ± 3.34% positive in UBCCre/ERT2+Ctla4fl/fl mice (Fig. 1 D). Whereas Foxp3+ T conv cells normally express lower levels of CTLA-4 than T reg cells, a reduction in CTLA-4 staining on this population was also observed. Deletion on this non-T reg cells subset was confirmed by staining cells after in vitro stimulation with anti-CD3 (unpublished data). We monitored adult mice treated with tamoxifen during the course of all experiments and observed no overt disease in UBCCre/ERT2+Ctla4fl/fl mice (unpublished data). Thus, in contrast to germ-line Cita4 knockout mice, inducible deletion of Cita4 in adult mice does not result in a spontaneous phenotype of lethal inflammation.

**Deletion of Cita4 in adult mice induces resistance to EAE**

We investigated the consequences of inducing autoimmunity in adult UBCCre/ERT2-Ctla4fl/fl mice treated with tamoxifen by immunization with the self-peptide MOG35-55 to induce EAE. In contrast to UBCCre/ERT2-Ctla4fl/fl littermate controls, which developed disease, UBCCre/ERT2+Ctla4fl/fl mice were markedly resistant to EAE (Fig. 2 A and Table 1). Consistent with clinical disease score, UBCCre/ERT2+Ctla4fl/fl mice had ~10-fold fewer inflammatory foci in the brains and spinal cords than their littermate controls (Fig. 2 C and Table 1).

We next compared the phenotype of T cells in the cervical LNs (cILNs; draining the CNS) of control and UBCCre/ERT2+Ctla4fl/fl mice 14 d after immunization with MOG35-55. Within the CD4+ T cell subset, both Foxp3+ T reg cells and Foxp3− T conv cells were expanded in UBCCre/ERT2+Ctla4fl/fl mice when compared with UBCCre/ERT2+Ctla4fl/fl mice, with the proportional increase of T reg being greater (Fig. 2 B and D). Intracellular Ki-67 staining was increased in both subsets, suggesting increased peripheral expansion of these cells (Fig. 2 E). Analysis of CD62L and CD44 surface staining revealed T conv cells, and to a lesser extent T reg cells, from the UBCCre/ERT2+Ctla4fl/fl mice to be more activated than those from control mice (Fig. 2 F). In the CNS, at the onset of disease, we found a similar percentage of total CD4+ T cells in both groups (not depicted) and an increase in the frequency of T reg cells in UBCCre/ERT2+Ctla4fl/fl mice (Fig. 2 G), similar to that observed in the peripheral T reg cells. In the CNS of UBCCre/ERT2+Ctla4fl/fl mice were profoundly deleted for CTLA-4, as in the periphery. Thus, deletion of CTLA-4 in adult mice is accompanied by resistance to autoimmunity and is characterized by expansion and increased activation of T conv cells. This is counterbalanced by a proportionally greater expansion of T reg cells both in the periphery and in the target tissue.

**Resistance to autoimmunity is dependent on CTLA-4 deletion on T reg cells**

To determine if resistance to EAE is caused by the absence of Cita4 during thymic selection, we adoptively transferred mature CD4+ T cells from either UBCCre/ERT2+Ctla4fl/fl or UBCCre/ERT2+Ctla4fl/fl adult mice into TCRα−/− recipients, which were then treated with tamoxifen and immunized with MOG35-55 to induce EAE. This approach deletes Cita4 in mature T cells and eliminates possible effects of Cita4 deficiency in the thymus. Recipients of UBCCre/ERT2+Ctla4fl/fl T cells were protected from EAE, recapitulating the protection seen in tamoxifen-treated, intact UBCCre/ERT2+Ctla4fl/fl mice (Fig. 3 A). In this setting, we again observed expansion of both T reg cells and T conv cells when Cita4 was deleted (unpublished data). Thus, deletion of Cita4 on mature CD4+ T cells can lead to EAE resistance.

To investigate the impact of Cita4 deletion on T conv cells versus T reg cells, we first assessed whether an antigen-specific T conv cell population could transfer disease when Cita4 is deleted. We bred UBCCre/ERT2+Ctla4fl/fl mice to mice bearing the 2D2 transgenic TCR, specific for MOG35-55, and transferred 2D2+CD4+ T cells from UBCCre/ERT2+Ctla4fl/fl or UBCCre/ERT2+Ctla4fl/fl mice to Rag1−/− recipients, and then immunized with MOG35-55 concurrently with tamoxifen treatment. Recipients of 2D2+UBCCre/ERT2+Ctla4fl/fl and 2D2+UBCCre/ERT2+Ctla4fl/fl T cells developed EAE with similar onset and severity (Fig. 3 B). Additionally, we sorted CD4+Foxp3− T cells from non-TCR transgenic UBCCre/ERT2+Ctla4fl/fl and UBCCre/ERT2+Ctla4fl/fl mice and transferred these to TCRα−/− recipients, which were then treated with tamoxifen and immunized with MOG35-55. UBCCre/ERT2+Ctla4fl/fl and UBCCre/ERT2+Ctla4fl/fl mice displayed identical disease susceptibility to their littermate controls (unpublished data). Thus, effector CD4+ T cells that lack Cita4 can become pathogenic and do not appear to be responsible for the EAE resistance of mice in which Cita4 is deleted during adulthood.

We next asked whether deletion of Cita4 only in the T reg cell compartment would recapitulate resistance to EAE. We bred the Cita4fl/fl mouse to a Foxp3GFP/Cre/ERT2 mouse (Rubtsov et al., 2010) to enable tamoxifen-inducible deletion of Cita4 specifically on T reg cells. Tamoxifen treatment of adult mice resulted in deletion of Cita4 on Foxp3+ T reg cells to a similar degree to that achieved in the UBCCre/ERT2+Ctla4fl/fl mice, whereas Cita4 expression on CD4+Foxp3− cells was unchanged (Fig. 3 C). After tamoxifen treatment, mice were immunized with MOG35-55 to induce EAE. EAE developed in control mice (either non-Cre-expressing Cita4fl/fl, Foxp3GFP/Cre/ERT2+Ctla4fl/fl, or Foxp3GFP/Cre/ERT2+Ctla4fl/fl mice), but not in Foxp3GFP/Cre/ERT2+Ctla4fl/fl mice (Fig. 3 D and Table 1). The degree of protection was profound and comparable to that observed in UBCCre/ERT2+Ctla4fl/fl mice. Thus, deletion of Cita4 specifically on T reg cells, during adulthood, leads to protection from induced autoimmunity.

**Inducible Cita4 deletion leads to cell intrinsic changes in T conv cells**

Because Cita4 deletion on T conv cells alone failed to confer protection from EAE, we questioned whether there might be a functional role for Cita4 deletion on T conv cells in a different setting. We targeted deletion of Cita4 specifically to T conv cells by transferring T conv cells from either UBCCre/ERT2+Ctla4fl/fl or UBCCre/ERT2+Ctla4fl/fl mice,
Figure 2. Deletion of Ctla4 leads to EAE resistance and expanded, apparently activated, T cell populations. Ctla4fl/fl mice that were either UBCCreERT2- Cre− or UBCCreERT2+ Cre+ were treated with tamoxifen and immunized with MOG35-55 to induce EAE. Clinical scores are shown in A and representative CNS pathology is shown in C. CD4+ cells from cervical LNs of mice immunized with MOG35-55 to induce EAE were analyzed at the peak of disease for (B) the number of CD4+Foxp3− and CD4+Foxp3+ cells, (D) the percentage of Foxp3+ cells, (E) Ki-67 expression by intracellular staining, and (F) CD62L and CD44 expression by surface staining within the cervical LN. (E, right) Representative dot plots gated on CD4+, for which mean data are plotted on the left. (G) Brains and spinal cords from MOG35-55 immunized mice were analyzed at the onset of disease for percentage of Foxp3+ cells and CTLA-4 expression. Data are gated on CD4+ T cells. Data in each panel are representative of at least two independent experiments (mean and SEM for at least 7 mice per group). Bars: 200 µm (100x magnification); 50 µm (400x magnification). *, P < 0.05; ***, P < 0.001.
Data in Clinical EAE are pooled from seven independent experiments (four with UBC-CreERT2 mice and three with Foxp3-CreERT2 mice) in which EAE was induced by immunization with 150 µg MOG35-55 subcutaneously, and administration of 250 ng pertussis toxin intraperitoneally on the day of immunization and two days later. Mice with no disease were included in calculations of maximal score, but excluded from calculations of day of disease onset.

*aAll p-values are compared with Cre− control.

Together with T reg cells from UBC-CreERT2 mice into TCRα−/− recipients that were subsequently treated with tamoxifen. Input T conv cells were distinguished from T reg cells by the expression of a Cre reporter (ROSA26–tdTomato). We found that targeted deletion of CTLA-4 on T conv cells led to expansion of T conv but not T reg cells (Fig. 4 A), suggesting that CTLA-4 expression on T conv cells, while insufficient to confer disease protection, does restrain the homeostatic expansion of T conv cells.

We also compared the proliferation response of sorted CD4+ Foxp3-GFP− cells from tamoxifen-treated TCR transgenic 2D2+UBC-CreERT2+Ctla4fl/fl or 2D2+UBC-CreERT2-Ctla4+/+ mice to a range of MOG35-55 peptide concentrations in vitro and observed similar proliferation profiles between the two groups (Fig. 4 B). Similarly, we found that non-TCR transgenic cells with inducible loss of CTLA-4 showed equal proliferation to controls in response to an anti-CD3 stimulus (unpublished data).

Furthermore, we analyzed the propensity of T conv cells that do or do not express CTLA-4 to differentiate into various T helper (Th) subsets, independently of T reg cells. We sorted naïve, CD4+Foxp3− T cells from UBC-CreERT2+Ctla4fl/fl or UBC-CreERT2+Ctla4+/+ mice that had undergone a truncated in vivo tamoxifen treatment regimen (days −4 and −3 only; to minimize the indirect effects of Ctla4 deletion on T reg cells) and cultured them under various Th differentiation conditions in the presence of 4-hydroxy tamoxifen, to ensure complete deletion. We found no differences in the percentages of either Foxp3-GFP−, IFN-γ−, or IL-17A− expressing populations under conditions of T reg, Th1, or Th17 cell differentiation, respectively (Fig. 4 C). In summary, although we observed a T conv cell–intrinsic role for CTLA-4 in regulating in vivo homeostatic expansion, in vitro measures of proliferation and differentiation were equal between CTLA-4–expressing versus −deleted T conv cells.

T reg cells deleted for CTLA-4 during adulthood are functional

We next assessed the function of T reg cells in which Cita4 was inducibly deleted in adult mice using in vitro and in vivo assays. We first co-cultured wild-type versus CTLA-4–deleted T reg cells (sorted from tamoxifen–treated UBC-CreERT2+Ctla4+/+ or UBC-CreERT2+Ctla4fl/fl mice) in the presence of wild-type T conv cells and APC (live dendritic cells), and stimulated with anti-CD3. In this assay, T reg cells lacking CTLA-4 were able to suppress T conv cell proliferation in vitro equally as well as T reg cells expressing CTLA-4 (Fig. 5 A).

To assess T reg cell function in vivo, we developed an assay for suppression of homeostatic proliferation in lymphopenic mice. Wild-type T conv cells were transferred into Rag1−/− hosts in the presence or absence of cotransferred T reg cells from UBC-CreERT2+Ctla4+/+ or UBC-CreERT2+Ctla4fl/fl mice. Recipient mice were treated with tamoxifen to induce CTLA-4 deletion in T reg cells, and homeostatic proliferation of the transferred T conv cells was quantitated 10 d after initial tamoxifen administration (on the day of transfer). We found that CTLA-4–deleted T reg cells were equivalent, if not better, than control T reg cells in suppressing homeostatic proliferation in vivo (Fig. 5 B). The number of T reg cells obtained at the end of the assay was not significantly different between groups, suggesting that, on a per cell basis, CTLA-4−deficient T reg cells have suppressive capacity equal to that of control T reg cells.
Collectively, these data indicate that T reg cells lacking CTLA-4 maintain their ability to suppress T cell proliferation in vitro and in vivo.

**T reg cells lacking CTLA-4 impose an overabundance of IL-10 and increased expression of co-inhibitory receptors**

Collectively, our data suggest that whereas deletion of CTLA-4 in mature T cells leads to increased activation of T conv cells, these cells are rendered functionally impotent by the presence of T reg cells lacking CTLA-4, resulting in protection from EAE. To test this hypothesis, we further characterized the phenotype of T conv and T reg cells from UBC^Cre/ERT2^Ctla4^fl/fl^ mice by performing a focused transcriptional analysis of CTLA-4–deleted T conv and T reg cells using a pathogenic Th17 NanoString codeset (Lee et al., 2012; Yosef et al., 2013). UBC^Cre/ERT2^Ctla4^fl/fl^ and UBC^Cre/ERT2^Ctla4^fl/fl^ mice were treated with tamoxifen and immunized with MOG 35-55. At the peak of disease, RNA was isolated from T conv cells and T reg cells that had been sorted from spleen and cLNs. We found that both T reg and T conv cell populations adopt a gene expression signature associated with IL-10 production, which has been shown to have a protective role in EAE (Bettelli et al., 1998; Fig. 6 A). Among the most up-regulated genes in UBC^Cre/ERT2^Ctla4^fl/fl^ relative to UBC^Cre/ERT2^Ctla4^fl/fl^ T conv cells were Ahr (aryl-hydrocarbon receptor), EBi3 (shared IL27/IL35 subunit), and Icos; each of these genes is involved in promoting the biology of IL-10 production and of type 1 regulatory-1 cells, an IL-10–producing, Foxp3-negative regulatory T cell population. When T reg cells were compared in a similar fashion, Il10, Prdm1 (Blimp-1), and Icos were up-regulated in UBC^Cre/ERT2^Ctla4^fl/fl^ samples, also suggesting that IL-10 production plays a significant functional role in conferring protection from EAE upon CTLA-4 deletion. Consistent with this hypothesis, we observed increased production of IL-10 by T conv cells and T reg cells from CTLA-4–deleted mice, relative to control mice, during EAE (Fig. 6 B). Although UBC^Cre/ERT2^Ctla4^fl/fl^ mice had a higher frequency of CD4^+^ T cells from the cLN that produced the pathogenic cytokines IFN-γ and IL-17 compared with littermate controls (Fig. 6 C), a significantly larger proportion of each of these subsets additionally produced IL-10, relative to the corresponding cells from control mice (Fig. 6 D). CD4^+^ T cells lacking CTLA-4 also had increased surface expression of the co-inhibitory receptors Lag3 and PD-1 on both T conv and T reg cells in the cLN (Fig. 6, E and F), as well as in the spleen (not depicted). Collectively, these data indicate that although CTLA-4 inhibits expansion of both T conv cells and T reg cells, it also acts to inhibit IL-10 production and the expression of co-inhibitory receptors.

Next, we asked whether loss of CTLA-4 on T reg cells alone was sufficient to induce the phenotypic changes in T conv cells observed in UBC^Cre/ERT2^Ctla4^fl/fl^ mice. T reg cells from Foxp3^GFP/Cre/ERT2^Ctla4^fl/fl^ mice treated with tamoxifen and immunized with MOG^35-55^ showed strikingly similar cellular phenotypes to those observed in UBC^Cre/ERT2^Ctla4^fl/fl^ mice. At time points at which control mice had ongoing disease,
the CD4+ population of the cLN of Foxp3+/Cre/ERT2 Cta4fl/fl mice was as high as 60% Foxp3+ (Fig. 7 A). Moreover, both T reg cells and T conv cells were increased in number in these mice, although T reg cells were proportionately more so (approximately twofold increase in T conv cells vs. fivefold in T reg cells; Fig. 7 B). The CTLA-4–sufficient T conv cell population from Foxp3+/Cre/ERT2 Cta4fl/fl mice displayed increased activation, as measured by the percentage of CD62LloCD44hi cells (Fig. 7 C), whereas proliferation as assessed by Ki-67 staining was increased in both T conv cells and T reg cells (Fig. 7 D). Furthermore, expression of IL-10, Lag3, and PD-1 was increased in both T cell subsets (Fig. 7 E–G). These findings confirm that the phenotype of T conv cell activation accompanied by increased expression of inhibitory molecules that we observe in UBC Cre/ERT2 Cta4fl/fl mice, is driven by deletion of CTLA-4 specifically on T reg cells and is independent of CTLA-4 expressed by T conv cells.

There were some differences, however, among T conv cells from UBC Cre/ERT2 Cta4fl/fl compared with Foxp3+/Cre/ERT2 Cta4fl/fl mice after tamoxifen treatment and EAE induction with MOG35-55 peptide. In contrast to UBC Cre/ERT2 Cta4fl/fl mice (Fig. 6 C), mice with specific, inducible T reg cell deletion of CTLA-4 did not display increased production of IFN-γ and IL-17 among peripheral T conv cells (Fig. 7 H). This is an interesting difference between these two models, as it suggests that control of T conv cell production of these inflammatory cytokines is intrinsic to the T conv cells themselves and not an indirect effect of CTLA-4 deletion on T reg cells.

Within the CNS, on day 12 after immunization (time of disease onset in controls), we observed significantly fewer CD45+CD4+ T cells in Foxp3+/Cre/ERT2 Cta4fl/fl mice, compared with Foxp3+/Cre/ERT2 Cta4fl/fl controls (Fig. 7 I). However, the proportion of these cells that were Foxp3+ is higher (Fig. 7 J), consistent with what we have observed in the periphery. We also observed significantly fewer IFN-γ–producing CD4+ cells within the CNS of T reg cells—specific CTLA-4–deleted mice, whereas there are no significant differences in the production of IL-17 (Fig. 7 K).

Increased B7 and MHC class II expression on peripheral, but not CNS, APCs of CTLA-4–deleted mice

Because CTLA-4 may exert some of its effects by modulating B7 expression, we compared B7-1 and B7-2 expression on APCs in the periphery and CNS of mice that are inducibly deleted for CTLA-4 on T reg cells with those from control mice. Our group has previously shown that survival of T cells within the CNS during EAE is dependent on B7 expression (Chang et al., 2003). We observed increases in B7-1 and B7-2 expression on APCs from mice that are inducibly deleted for CD4+ cells within the CNS of T reg cell–specific CTLA-4–deleted mice, whereas there are no significant differences in the production of IL-17 (Fig. 7 K).

Figure 4. T conv cell–intrinsic effects of inducible deletion of Cta4. (A) CD4+Foxp3–GFP+ cells from either UBC Cre/ERT2 Cta4fl/fl (WT) or UBC Cre/ERT2 Cta4fl/fl (DEL) mice to TCRα−/− recipients, which were subsequently treated with tamoxifen and immunized with MOG35-55. T reg and T conv cells were quantified in the spleen 26 d after immunization. n ≥ 4 mice per group. (B) Sorted CD4+Foxp3–GFP+ cells from tamoxifen-treated TCR transgenic 2D2+UBC Cre/ERT2 Cta4fl/fl (Cre−) or 2D2+UBC Cre/ERT2 Cta4fl/fl (Cre+) mice were cultured with splenocytes from TCRα−/− mice, 4-hydroxy tamoxifen and a dose titration of MOG35-55 peptide for 72 h. Proliferation was assessed by quantitation of CellTrace Violet dilution. n = 3 mice per group. (C) Naive, CD4+CD62LloFoxp3–GFP+ splenocytes were sorted from UBC Cre/ERT2 Cta4fl/fl (Cre−) or UBC Cre/ERT2 Cta4fl/fl (Cre+) mice that had been treated with tamoxifen on days −4 and −3. Cells were cultured in the presence of irradiated TCRα−/− splenocytes, soluble anti-CD3, 4-hydroxy tamoxifen, and the indicated cytokines for 72 h. Cells were then analyzed by flow cytometry for expression of Foxp3–GFP or intracellular cytokines. For analysis of cytokines, cells were stimulated first with PMA/Ionomycin and GolgiStop for 4 h. Gray histograms represent data from cells grown without addition of cytokine for TGF–β condition and cells stimulated with only GolgiStop (no PMA/Ionomycin) for intracellular cytokine stains. n ≥ 2 mice/group. Data in all panels are representative of at least two independent experiments. *, P < 0.05.
Both the CD8/T reg cells and CD4+ T conv cells/T reg cells ratios were dramatically decreased in the draining (inguinal, proximal to tumor; Fig. 9, B and C) and nondraining (cervical) LNs (not depicted). In contrast to the periphery, the CD8/T reg cells ratio within the tumor was not significantly different between deleted and control mice and the CD4+ T conv/T reg cell ratio was higher in deleted versus control tumors (Fig. 9, B and C). Ki-67 expression is consistent with this finding; CD8+ T cell, CD4+ T conv cell, and T reg cell Ki-67 expression is increased in the dLN of UBC Cre/ERT2+Ctla4 fl/fl mice relative to controls, whereas among tumor-infiltrating lymphocytes (TILs), only CD4+ T conv cells, but not CD8+ T cells or T reg cells, have increased Ki-67 upon CTLA-4 deletion (Fig. 9, D–F). Thus, there is massive peripheral expansion of T reg cells upon Ctla4 deletion, similar to our findings in EAE; however, this expansion is not seen at the site of the tumor.

The function of CD8+ TILs in this model appeared to be altered by Ctla4 deletion. Fewer CD8+ TILs from CTLA-4–deleted mice were CD44 hi and produced IFN-γ upon re-stimulation (Fig. 9, G and H), but more CD8+ TILs produced Granzyme B in CTLA-4 deleted mice than controls (Fig. 9 I). In the dLN, however, there were more CD8+ T cells that were CD44hi and produced IFN-γ. Based on our previous findings of increased IL-10 production in both T conv cells and T reg cells after Ctla4 deletion during EAE, we examined IL-10 production in the dLN and tumor. We found that we also observed highly significant increases in the level of MHC class II expression on APC populations (Fig. 8, C, F, and I), indicating that their overall maturation/activation level is enhanced. Therefore, it is unclear whether the changes in B7 expression are caused by the direct effect of absence of CTLA-4–B7 interactions that lead to their down-regulation (either by transendocytosis or back-signaling) or by indirect effects of other cellular changes. Importantly, we did not find increased B7 levels on the CD45hiMHC class II+ APCs in the CNS of mice immunized for EAE (Fig. 8, J–L), although we did observe increased percentages of T reg cells in the CNS of CTLA-4–deleted mice (Fig. 7 J). These findings suggest that CTLA-4–deficient T reg cells are not causing loss of control of B7 expression on CNS APCs.

**Conditional deletion of Ctla4 does not impede tumor growth**

We next examined the consequences of Ctla4 deletion in adult mice on responses to an implanted tumor. We used the subcutaneous tumor model, MC38 colon adenocarcinoma because anti–CTLA-4 mAb leads to modestly enhanced antitumor immunity in this model (Kocak et al., 2006; Allard et al., 2013). We administered tamoxifen to induce deletion of Ctla4 in UBC Cre/ERT2 mice before tumor implantation. CTLA-4 deletion did not slow or diminish tumor growth curves. Rather, UBC Cre/ERT2+Ctla4β/β mice had slightly larger tumors compared with UBC Cre/ERT2-Ctla4β/β littermate controls (Fig. 9 A). Both the CD8/T reg cells and CD4+ T conv cells/T reg cells ratios were dramatically decreased in the draining (inguinal, proximal to tumor; Fig. 9, B and C) and nondraining (cervical) LNs (not depicted). In contrast to the periphery, the CD8/T reg cells ratio within the tumor was not significantly different between deleted and control mice and the CD4+ T conv/T reg cell ratio was higher in deleted versus control tumors (Fig. 9, B and C). Ki-67 expression is consistent with this finding; CD8+ T cell, CD4+ T conv cell, and T reg cell Ki-67 expression is increased in the dLN of UBC Cre/ERT2+Ctla4 β/β mice relative to controls, whereas among tumor-infiltrating lymphocytes (TILs), only CD4+ T conv cells, but not CD8+ T cells or T reg cells, have increased Ki-67 upon CTLA-4 deletion (Fig. 9, D–F). Thus, there is massive peripheral expansion of T reg cells upon Ctla4 deletion, similar to our findings in EAE; however, this expansion is not seen at the site of the tumor.

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in both sites, higher percentages of cells in all subsets (CD8+, CD4+ T conv cells, and T reg cells) produced IL-10 (Fig. 9, J–L). This increase in IL-10 may predominate and dampen antitumor immunity in the CTLA-4 deleted mice.

**DISCUSSION**

The dramatic, lethal phenotype of the germline CItla4 knockout mouse demonstrates the potency of this molecule as an inhibitory receptor (Tivol et al., 1995; Waterhouse et al., 1995). Because CTLA-4 is largely T cell specific and constitutively expressed on T reg cells, but is also inducibly expressed on T conv cells upon activation, the question of the relative contribution of CTLA-4 expression on T reg cells versus T conv cells has been of great interest. Here, we have developed genetic approaches to address this question, uncoupling the role of CTLA-4 during development and in the adult immune system by using inducible Cre systems that delete CTLA-4 either ubiquitously or specifically on Foxp3+ T reg cells. By deleting CTLA-4 during adulthood, either on all cells or specifically on T reg cells, we have uncovered a surprising phenotype of resistance to induced autoimmunity. The underlying phenotype of T cells in these mice is one of apparent...
with this, when we crossed this conditional CTLA-4 knockout allele to either LckCre or noninducible Foxp3Cre mice the lethal phenotype of germline Ctla4−/− mice was recapitulated (unpublished data). This indicates that there is a critical window of time between when Lck or Foxp3 genes are turned on in the thymus and adulthood, during which CTLA-4 activation, coupled with T reg cell expansion and increased production of anti-inflammatory molecules. The distinct phenotype seen in our model, in contrast to that seen with germline deletion of Ctla4, may reflect necessary roles for CTLA-4 during the neonatal period, at which time T reg cells develop in and exit from the thymus (Asano et al., 1996). Consistent

Figure 7. Cellular phenotypes of mice lacking CTLA-4 specifically on T reg cells. Foxp3GFP/Cre/ERT2Ctla4 fl/fl and Foxp3GFP/Cre/ERT2Ctla4 fl/+ littermates were treated with tamoxifen and immunized with MOG35-55 to induce EAE. Cervical LNs were analyzed at peak of disease for (A) the percentage of Foxp3+ cells, (B) the number of Foxp3− and Foxp3+ cells, (C) surface staining for CD62L and CD44, (D) intracellular staining for Ki-67, (E) intracellular staining for IL-10, (F) surface staining for PD-1, (G) surface staining for Lag-3, and (H) intracellular staining for IFN-γ and IL-17A. On day 12 after immunization (onset of disease in controls) leukocytes were isolated from CNS for frequency of (I) CD45+CD4+ cells, (J) Foxp3+ cells (of CD4+ population), or (K) IFN-γ− and IL-17A−producing CD4+ cells. For cytokine analyses, cells were stimulated with PMA/Ionomycin/GolgiStop and analyzed for intracellular expression of IL-10, IFN-γ, or IL-17A. Data in each panel are representative of at least two independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
in the neonate, loss of control on T reg cell proliferation may lead to abolished T reg cells suppressive function.

The EAE resistance of adult mice in which CTLA-4 is inducibly deleted ubiquitously or selectively on Foxp3+ T reg cells contrasts with published studies showing that anti–CTLA-4 administration exacerbated EAE in various non-B6 models (Karandikar et al., 1996; Perrin et al., 1996). Karandikar et al. reported worsening of relapsing/remitting PLP139-151-induced EAE on an SJL background upon anti–CTLA-4 administration, and Perrin et al. reported exacerbation of EAE induced by MBP protein in (PL x SJL)F1 mice. To determine if there are differences in the effects of CTLA-4 in the C57BL/6 MOG-induced EAE and other models, we immunized wild-type C57BL/6 mice with MOG35-55 to induce EAE and administered anti–CTLA-4 mAb either before or after immunization. We observed a slight worsening of disease (unpublished data).

Recent studies indicate that anti–CTLA-4 treatment in cancer needed to prevent systemic autoimmunity. The perinatal period, in which T reg cells seed the periphery, is appreciated to be critical for the establishment of peripheral tolerance (Asano et al., 1996; Dujardin et al., 2004; Samy et al., 2008). It has recently been shown that T reg cells that emerge from the thymus during this period are dependent on Aire for their generation and have a distinct transcriptional profile from T reg cells generated in adult mice (Yang et al., 2015). CTLA-4 (whose expression in T reg cells is regulated by Foxp3) may be essential for selecting the T reg cell repertoire in the thymus and protecting them from thymic deletion during development. However, once selected and seeded to the peripheral immune compartment, CTLA-4 may serve to restrain T reg cell expansion (as it does for T conv cells). In addition, the relationship between preservation of T reg cell function and control of expansion may be very different in the relatively lymphopenic neonatal environment compared with that of the adult;
CTLA-4 is deleted on the vast majority of cells, a small percentage of CTLA-4–expressing cells persists. We treated UBC Cre-ERT2+Ctla4 fl/fl mice with anti–CTLA-4 mAb after tamoxifen treatment in an effort to neutralize the effect of any remaining CTLA-4+ cells, and did not observe development of spontaneous autoimmunity or abrogation of EAE protection in these experiments (unpublished data). Furthermore, we observed similar frequencies of CTLA-4–deleted T cells in lymphoid organs and in the CNS during EAE progression (Figs. 1 D and 2 G), arguing against a competitive advantage models can lead to T reg cell depletion by FcR-dependent mechanisms (Bulliard et al., 2013; Selby et al., 2013; Simpson et al., 2013), suggesting that effects other than pure blockade may explain the difference in EAE phenotypes in anti–CTLA-4–treated mice and inducible CTLA-4–deficient mice.

Several groups have demonstrated that mice chimeric for wild-type and CTLA-4–deleted cells, either through adoptive transfer or bone marrow chimerism, are spared the lethal phenotype of germline CTLA-4 knockout mice (Bachmann et al., 1999; Friedline et al., 2009). In our system, although CTLA-4 is deleted on the vast majority of cells, a small percentage of CTLA-4–expressing cells persists. We treated UBC Cre-ERT2+Ctla4 fl/fl mice with anti–CTLA-4 mAb after tamoxifen treatment in an effort to neutralize the effect of any remaining CTLA-4+ cells, and did not observe development of spontaneous autoimmunity or abrogation of EAE protection in these experiments (unpublished data). Furthermore, we observed similar frequencies of CTLA-4–deleted T cells in lymphoid organs and in the CNS during EAE progression (Figs. 1 D and 2 G), arguing against a competitive advantage
for CTLA-4–sufficient cells in accessing or accumulating in the target organ.

We find that T reg cells from mice in which CTLA-4 was deleted in adulthood are able to suppress T conv cell proliferation both in vitro and in vivo. We observed massive accumulation of T reg cells in the dLN of CTLA-4–deleted mice, and an increased proportion of T reg cells in the CNS at early time points of EAE. To explain the observed resistance to EAE in this setting, we propose that CTLA-4 on peripheral T reg cells acts as a brake to inhibit T reg cell proliferation. When CTLA-4 is absent, this constraint on T reg cell expansion is removed, and T reg cells accumulate dramatically, while remaining functionally competent. It has been shown that CD28 regulates peripheral homeostasis of T reg cells (Tang et al., 2003), and that T reg cell–specific deletion of CD28 leads to the generation of peripheral T reg cells that are unable to prevent autoimmunity (Zhang et al., 2013). Our findings likely reflect the converse situation; that is, loss of CTLA-4 leads to unrestrained CD28 signaling in T reg cells, resulting in T reg cell expansion and ultimately in EAE resistance.

Importantly, the proposed role for CTLA-4 in inhibiting peripheral T reg cell expansion does not preclude it also functioning as a mediator of T reg cells suppression, as has been demonstrated by many groups. Our data suggest that CTLA-4 is not required for the ability of T reg cells to suppress T conv cell proliferation and that the net result of CTLA-4 loss in vivo leads to protection from EAE. It may be that increased proliferation of T reg cells, coupled with their increased production of antiinflammatory cytokines, may compensate for a per-cell T reg cells dysfunction that would become apparent in other settings. CNS inflammation and antitumor immunity may be suppressible through CTLA-4–independent T reg cell mechanisms, such as IL-10 production, when T reg cells are present in sufficient numbers, which could explain why CTLA-4–deleted T reg cells are competent to suppress EAE and prevent tumor regression. However, in other models, the trans-acting function of CTLA-4 in modulating B7-expressing cells may be more important. Indeed, our data suggest that this trans-acting role is important in regulating B7 expression in the periphery, but that it may not be critical in the CNS (Fig. 8). Thus, we propose that there are opposing roles for CTLA-4 on T reg cells: CTLA-4 has an intrinsic function that acts as an inhibitor of T reg cell proliferation, which, by virtue of the suppressive nature of T reg cells, counters its extrinsic function as a modulator of B7-expressing cells. Consistent with this, we have recently shown that CTLA-4 has dual roles on T follicular regulatory (Tfr) cells, an effector T reg cell subset that specifically suppresses B cell responses in the germinal center (GC) of the LN. We found that CTLA-4 inhibited differentiation and expansion of Tfr cells and that it was required for their suppressive capacity (Sage et al., 2014). Differences between the dependence on CTLA-4 for suppressive function of Tfr cells in the GC versus effector T reg cells in the context of EAE could be a result of different suppressive mechanisms. Effector T reg cells in the CNS may have multiple, redundant suppressive mechanisms, and any defect in suppression by loss of CTLA-4 may be compensated for by other mechanisms (e.g., IL-10 production) as well as by expansion of T reg cells. Tfr cells, however, may have fewer redundant mechanisms to compensate for the lack of CTLA-4, resulting in severely reduced suppression even with expansion of cells.

Our work does not reveal a necessary role for Ctla4 deletion on T conv cells for the EAE resistance that we have observed; rather, loss of CTLA-4 on T reg cells alone, is sufficient. We do, however, observe profound changes in the T conv cell compartment when Ctla4 is specifically deleted on T reg cells (Fig. 7), consistent with the idea that the expanded CTLA-4–deficient T reg cells are impinging on T conv cells due to the extrinsic nature of T reg cell function. In addition, we do observe a T conv cell–intrinsic role for CTLA-4 in preventing T cell expansion in our adoptive transfer experiments, in which we target Ctc4 deletion only to Foxp3+ populations in a lymphopenic environment (Fig. 4 A). We did not, however, find an in vitro role for Ctc4 in T conv cell proliferation or T helper cell differentiation (Fig. 4, B and C). Because Ctc4 deletion on T cells is likely to have its earliest effects on T reg cells (which constitutively express CTLA-4), the timing of tamoxifen administration relative to disease onset may be relevant.

Given the importance of CTLA-4 as an immunotherapeutic target in cancer, we also investigated the consequences of CTLA-4 deletion in adult mice on antitumor immunity. We used a tumor model (MC38 colon adenocarcinoma) in which antibody targeting of CTLA-4 promotes antitumor immunity (Kocak et al., 2006; Allard et al., 2013), albeit likely due to deletion of T reg cells (Selby et al., 2013). In adult mice deficient for Ctc4, MC38 tumors grew slightly faster than in control mice, with indications of diminished antitumor immunity (Fig. 9 A). These findings show that CTLA-4 is not required for induction of T cell dysfunction in tumors. There are several parallels between these findings and our findings in EAE. In both cases, Ctc4 deficiency leads to a massive peripheral expansion of T reg cells. Similarly to the CLN of mice with EAE, in the dLN of tumor-bearing mice, there was increased proliferation of both effector cells (CD8+ T cells and CD4+ T conv cells) and T reg cells in deleted mice; however, the ratios greatly favor T reg cells. In contrast to EAE, at the effector site in the tumor model (the tumor itself), the ratio of effector cells to T reg cells did not shift to favor T reg cells in deleted mice. In the tumor, CD8+ TIL (the dominant antitumor effector cells) in CTLA-4–deleted mice appeared to be incompletely activated based on lower surface expression of CD44 and less production of IFN-γ, similar to the CD4+ T conv cells in the CNS of deleted mice with EAE, and a greater percentage of these cells produced Granzyme B, a key cytotoxic molecule in antitumor immunity, in deleted mice. The phenotype of these CD8+ TILs is reminiscent of terminally differentiated exhausted CD8+ T cells seen during chronic LCMV infection (Odorizzi et al., 2015). Recent work has shown that there is a critical proliferative hierarchy between Tbet+ and Eomes+ subsets of exhausted CD8+ T cells that is
needed to maintain exhausted T cells long-term. Analogous to recent studies in the chronic LCMV model in which the absence of PD-1 led to the accumulation of more cytotoxic but terminally differentiated exhausted CD8+ T cells, CTLA-4 inhibitory signals may be needed to prevent overstimulation, excessive proliferation and terminal differentiation of exhausted T cells. The modest effects on tumor growth may reflect effects of Cda4 deletion on development of dysfunctional CD8+ TILs, as well as on T reg cells. Increased IL-10 in TILs in CTLA-deleted mice also may dampen antitumor immunity and promote effector T cell dysfunction.

Our data point broadly toward the concept that co-inhibitory receptors expressed by T reg cells have inhibitory effects on the T reg cells themselves, with complex and, occasionally, paradoxical impact on the immune response in certain settings. The use of anti–CTLA-4 mAb to unleash an effective immune response against cancer is already an FDA-approved approach. However, the mechanisms by which these antibodies promote antitumor immunity are not yet fully understood, and some data suggest that the therapeutic effects of anti–CTLA-4 are being achieved through depletion of T reg cells (Bulliard et al., 2013; Simpson et al., 2013). Our data suggest that the use of a nondepleting anti–CTLA-4 antibody may, in fact, have undesired effects of promoting T reg cell proliferation and production of anti-inflammatory molecules. Such considerations will become increasingly important with use of anti–CTLA-4 in combination with other cancer therapies.

MATERIALS AND METHODS

Mice. Mice 6–10 wk of age were used for all experiments. Wild-type C57BL/6 mice, B6.Cg-Tg(UBC-cre/ERT2)1Ejb/J (UBCCreERT2), B6.129S-Rag1tm1Mom/J, B6.SJL-Pep7tm1Sjb/J, and Foxp3tm1dc-R2/J mice were purchased from The Jackson Laboratory. 2D2 TCR transgenic mice (Bettelli et al., 2003) and Foxp3-GFP reporter mice (Bettelli et al., 2006) were generated as previously described. The wild-type B6.129S7-Rag1tm1Mom/J (Bettelli et al., 2003) and Foxp3-GFP reporter mice (Bettelli et al., 2006) were generated as previously described. The UBCCreERT2 transgene was maintained in a hemizygous x wild-type breeding strategy, such that UBCCreERT2 littermates could be used as controls. All experimental mice were housed in specific pathogen-free conditions and used in accordance with animal care guidelines from the Harvard Medical School Standing Committee on Animals and the National Institutes of Health. Animal protocols were approved by the Harvard Medical School Standing Committee on Animals.

Generation of conditional CTLA-4 knockout mouse. A conditional knockout targeting vector for the Cda4 gene was generated to contain three loxp sites: The first and second loxp sites flank a selection cassette consisting of a neomycin resistance gene (neo), under the control of the phosphoglycerate kinase (PGK) promoter and thymidine kinase under the control of the herpes simplex virus (HSV) promoter. This construct also contains diphtheria toxin under the control of the PGK promoter, which allows for selection of animal prototoxin-expressing cells. This construct also contains diphtheria toxin under the control of the HSV promoter. This construct also contains diphtheria toxin under the control of the PGK promoter, which allows for selection of animal prototoxin-expressing cells.

Gene expression quantitation by real-time quantitative PCR. Total RNA was isolated using the RNeasy Mini Plus kit (Qiagen) and quantitated using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized by reverse transcription with random hexamer primers using the High Capacity cDNA Synthesis Kit (Applied Biosystems). Real-time qPCR was performed either using SYBR Green chemistry (Roche) on a LightCycler 480 instrument or TaqMan chemistry (Applied Biosystems) on an ABI 7500 instrument, as indicated. All samples were run at least in triplicate, and no-template control samples were included for each gene to exclude DNA contamination. Primer sequences for the CTLA-4(1,4) isoform were as in Liu et al. (2012; forward: 5'-TGGCTCTTACGCTTGGCCCT-3'; reverse: 5'-GAGGACTCTTTTTTCTTATTGCTACAGA-3'; probe: 5'-ACCGCTGCCACTCTTTTCTTATCCCA-3'). All other primer sequences (CTLA-4 forward: 5'-GAAGACCATCGCCGTAATTCTGC-3'; CTLA-4 reverse: 5'-CATCCTGTGCTAAGACAAACACAG-3'; B2m forward: 5'-GCTCGGTCACCCGTGTTCTCCTT-3'; B2m reverse: 5'-TGTGTCGCTCCATTCCCTCC-3') were obtained from PrimerBank (Wang et al., 2012).

Tamoxifen-induced conditional deletion in UBCCreERT2 mice. To prepare tamoxifen solution for injection, tamoxifen (Sigma-Aldrich) was dissolved in ethanol, diluted 1:20 in sunflower oil, and sonicated for 5 min at 37°C. Mice were injected daily with 1 mg tamoxifen intraperitoneally for 5 consecutive days, then rest for a minimum of 72 h before any experimental manipulation.

EAE. MOG35-55 peptide (MEVGWYRSPFSRVVHLVRNYGK) was synthesized at the Biopolymer Laboratory (David Geffen School of Medicine, University of California, Los Angeles, CA), purity (>95%) was verified by HPLC. Amounts used were corrected for purity. To induce EAE, groups of 8–12-wk-old mice were immunized with 150 µg of MOG35-55 emulsified 1:1 in CFA with 400 µg of Mycobacterium tuberculosis H37RA (Difco Laboratories) in the two flanks subcutaneously. 250 ng of pertussis toxin (List Biological Laboratories) was injected intraperitoneally on the day of immunization and 2 d later. Mice were observed daily for clinical signs of EAE up to 30 d after immunization, and scored on a scale of 0–5: 0, no disease; 1, limp tail; 2, hind limb weakness; 3, hind limb paralysis; 4, hind and fore limb paralysis; and 5, moribund state. Mean clinical score was calculated by averaging the scores of all mice in each group, including animals that did not develop EAE.

Histopathology. Tissues were fixed in 10% formalin, and paraffin-embedded sections were stained with hematoxylin and eosin (H&E) or Luxol fast blue/periodic acid-Schiff for light microscopic analysis. CNS pathology was scored blindly by a neuropathologist. Inflammatory foci in meninges and parenchyma were counted as described previously (Sobel et al., 1990). Photomicrographs were acquired on an Olympus DP71 camera and software provided by the manufacturer. Horizontal bars represent 200 µm for 100x images and 50 µm for 400x images.

Analysis of CNS-infiltrating mononuclear cells. Before dissection, mice were perfused through the left ventricle with cold PBS. The brain and the spinal cord isolated, and CNS tissue was minced with a sharp razor blade and digested for 20 min at 37°C with collagenase D (2.5 mg/ml; Roche) and DNaseI (1 mg/ml; Sigma-Aldrich). Mononuclear cells were isolated by passage of the tissue through a cell strainer (70 µm), followed by centrifugation.
through a Percoll gradient (30% and 70%). Mononuclear cells in the interface were removed, washed, and resuspended in culture medium for analysis.

**Intracellular cytokine staining.** Cells from lymphoid organs were isolated and restimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin (both from Sigma-Aldrich) in the presence of Golgistop (BD) for 4–5 h, then processed for flow cytometric analysis as described below.

**Antibodies and flow cytometry.** Cells from lymphoid organs, CNS, or in vitro cultures were isolated and resuspended in staining buffer (PBS containing 1% FCS and 2 mM EDTA) and were stained with the following directly labeled antibodies: anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD8α (53–6.7), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD62L (MEL-14), anti-IL–10 (JES5-16E3), anti–IL-17F (9D3.1C8), anti–PD-1 (RMP1-30), anti-Lag3 (CB97W), and anti–IFN-γ (XM1G1.2, all from BioLegend). 7-aminoactinomycin D (7-AAD; BioLegend) was used for exclusion of nonviable cells. For intracellular staining (anti-CTLA-4 [UC10–4B9, BD]; anti–Foxp3 [FJK-16, e Bioscience]; anti–K–Ig (BS6, BD)), cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining kit (eBioscience) after surface staining, and LIVE/DEAD–Violet reagent (Life Technologies) was used for exclusion of nonviable cells. All flow cytometry data were acquired on an LSR II cytometer with standard filter sets using FACSDiva software (BD), and were further analyzed with FlowJo software (Tree Star).

**Adaptive transfers.** Bulk CD4+ (isolated from spleens by CD4 MicroBeads; Miltenyi Biotech) or CD4+Foxp3-GFP+ (isolated from spleens by fluorescence-activated cell sorting; FACS) were transferred to TCRα−/− or Rag1−/− recipients that were then treated with tamoxifen and immunized with MOG65–72 to induce EAE.

**T helper differentiation assays.** CD4+CD62L−Foxp3-GFP+ cells were sorted by fluorescence-activated cell sorting (purity >99%) and placed in culture in 24-well plates with TCRα−− splenocytes (1:4 T cell/splenocyte ratio), 2 µg/ml anti-CD3 (clone 2C11), 1 µM 4-hydroxytamoxifen and either 5 ng/ml TGF-β (T reg cell conditions), 5 ng/ml TGF-β 30 ng/ml IL-6 (Th17 conditions), or 10 ng/ml IL-12 + 10 µg/ml anti-IL-4 (Th1 conditions; all cytokines were obtained from BioLegend; anti–IL–4 clone 1B11 was purchased from BioXCell). Cells were harvested on day 4 of culture and analyzed by flow cytometry. For Th1 and Th17 conditions, cells were stimulated with PMA and ionomycin, as described above, before analysis.

**In vitro T reg cells suppression assay.** Responder T conv cells were purified by sorting of CD4+CD62L−CD25− T cells from CD45.1+ congenic splenocytes and labeling with CellTrace Violet Cell Proliferation kit (Molecular Probes) according to the manufacturer’s instructions. CD4+Foxp3−GFP−tuTomato+ T reg cells were purified by sorting from splenocytes from tamoxifen-treated UBCCreERT2+;Col4α1−/−Foxp3−GFPPKIKROS426StaSTOPfoxP3floxGFP− mice. DCs were isolated from collagenase-treated, wild-type C57BL/6 spleens using CD11c MicroBeads and MACS columns (Miltenyi Biotech); T conv and T reg cells were co-cultured at varying ratios in the presence of 10% DCs and 2 µg/ml anti-CD3 for 3 d. Co-cultures were then harvested and analyzed by flow cytometry. Responder T conv cells were gated on CD4 and CD45.1 (7–AAD+ cells were excluded), and dilution of CellTrace Violet was assessed using the proliferation platform of FlowJo.

**In vivo T reg cell suppression assay.** This assay was adapted from Workman et al. (2011). CD4+Foxp3−GFP− cells were purified by sorting from lymphoid organs of Foxp3−GFPPKIK mice that were either UBCCreERT2+;Col4α1−/− or UBCCreERT2+;Col4α1−/−;uba−/−, CD4+CD62L−CD25− T conv cells were purified by sorting from lymphoid organs of CD45.1 congenic mice. T reg and T conv cells were adoptively cotransferred into Rag1−− hosts, such that T reg cells comprised 25% of the total transferred cell population; a separate group of mice received T conv cells alone (positive control). Recipients were treated with tamoxifen on days 0–4, 10 d after transfer, mice were sacrificed and CD45.1+ CD4+ T conv cells and CD45.1+ CD4+Foxp3−GFP+ T reg cells were quantitated by cell counting and flow cytometric analysis of splenocytes.

**Gene expression quantification by NanoString analysis.** Gene expression in sorted T conv cells and T reg cell populations was quantified using a custom nCounter probe set containing 301 probe pairs, including additional probes for positive and negative controls and housekeeping genes (NanoString Technologies; Yosef et al., 2013). In brief, total cellular RNA was isolated and quantitated as above, and 100 ng was hybridized with customized Reporter CodeSet and Capture ProbeSet (NanoString Technologies) by overnight incubation in a thermal cycler at 65°C. The next day, flow cell preparation and scanning were performed using an nCounter instrument (NanoString Technologies) according to the manufacturer’s instructions. Raw data were normalized using nSolver software (NanoString Technologies) and exported as raw transcript counts for presentation. To normalize data, average background was subtracted from raw count, and normalization was performed using the geometric mean count from four housekeeping genes (Gapdh, Hprt, Acoh, and Tubb5). Heat maps were generated using GENE-E software.

**MC38 tumor model.** Mice were treated with tamoxifen on days −7 through −3 as described above. On day 0, 100,000 MC38 adenocarcinoma cells (a gift from D. Vignali, University of Pittsburgh School of Medicine, Pittsburgh, PA) were injected s.c. on the flank. Tumor measurement was performed on day 7 after tumor injection and every other day thereafter. Tumor volume was calculated as 1/2 × D × d², where D is the major axis and d the minor axis. On the day of harvest, tumors were dissected, disaggregated, and subjected to collagenase type I (400 U/ml; Worthington Biochemical) digestion for 30 min at 37°C, after which they were passaged through a cell strainer (70 µm). Mononuclear cells were isolated by centrifugation through a Percoll gradient (30 and 70%). The interface was removed, washed and resuspended in culture medium for analysis.

**Statistical analysis.** Prism software (GraphPad) was used for statistical analysis. Student’s two-tailed t test was used for all pairwise comparisons. Differences were considered statistically significant with a p-value of <0.05 (*), <0.01 (**), or <0.001 (***)

**The authors thank Baolin Chang, Xiaohui He, Vition Mbrica, Milagros Ávalo, and Louise Barias for assistance with mouse genotyping and colony maintenance, and Sarah Hillman for administrative support. This work was supported by grants from the National Institutes of Health (AI38310 and AI40614 to A.H. Sharpe; P01 AI39671 to A.H. Sharpe and V.K. Kuchroo; P01 AI56298 to A.H. Sharpe, G.J. Freeman, and V.K. Kuchroo) and the National Multiple Sclerosis Society (FG-1916-A-1 to S.B. Lovitch). The authors declare no competing financial interests.**

**Submitted: 30 May 2014 Accepted: 11 August 2015**

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JEM Vol. 212, No. 10

Published September 14, 2015

Downloaded from on April 1, 2017


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