Immunity
Article

Dendritic Cells Ameliorate Autoimmunity in the CNS by Controlling the Homeostasis of PD-1 Receptor$^+$ Regulatory T Cells

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SUMMARY

Mature dendritic cells (DCs) are established as unrivaled antigen-presenting cells (APCs) in the initiation of immune responses, whereas steady-state DCs induce peripheral T cell tolerance. Using various genetic approaches, we depleted CD11c$^+$ DCs in mice and induced autoimmune CNS inflammation. Unexpectedly, mice lacking DCs developed aggravated disease compared to control mice. Furthermore, when we engineered DCs to present a CNS-associated autoantigen in an induced manner, we found robust tolerance that prevented disease, which coincided with an upregulation of the PD-1 receptor on antigen-specific T cells. Additionally, we showed that PD-1 was necessary for DC-mediated induction of regulatory T cells. Our results show that a reduction of DCs interferes with tolerance, resulting in a stronger inflammatory response, and that other APC populations could compensate for the loss of immunogenic APC function in DC-depleted mice.

INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE) is a T cell-mediated autoimmune disease of the CNS and is widely used as a multiple sclerosis model (Gold et al., 2006). EAE is induced by priming and expanding CD4$^+$ T cells using CNS-derived myelin antigens, which are normally sequestered behind the blood-brain barrier (Zamvil and Steinman, 1990). It is not known exactly which antigen-presenting cells (APCs) are responsible for activating these autoreactive T cells. Dendritic cells (DCs) are considered prototypic APCs (Banchereau and Steinman, 1998; Théry and Amigorena, 2001). In the absence of inflammation or infection, most DCs in peripheral tissues and lymphoid organs have a resting, immature phenotype, characterized by high endocytic capacity and low surface expression of major histocompatibility complex (MHC) and costimulatory molecules. However, upon interaction with microbial ligands, proinflammatory cytokines, or CD40 ligand, DCs rapidly acquire an activated phenotype and become proficient in activating naive T cells (Steinman et al., 2003).

DCs are sparse in a healthy CNS and are primarily found in vessel-rich areas, such as the meninges and choroid plexus (Anandasabapathy et al., 2011; Greter et al., 2005). CNS inflammation induced by an autoimmune response or infection is accompanied by entry of DCs into the CNS (Matyszak and Perry, 1996; Serafini et al., 2000). These CNS DCs, but not resident microglia or infiltrating macrophages, are the most efficient APCs in driving the reactivation of transferred myelin-specific CD4$^+$ T cells (Greter et al., 2005).

In addition to their proinflammatory role, DCs also promote immune homeostasis by inducing and maintaining peripheral T cell tolerance (Steinman and Nussenzweig, 2002). In contrast to the naturally occurring regulatory T (nTreg) cells developing in the thymus, induced Treg (iTreg) cells are generated in the periphery, and DCs appear to play an essential role in their development and maintenance (Darrasse-Jèze et al., 2009; Kretschmer et al., 2005; Yamazaki et al., 2008).

We recently established that animals that constitutively lack classical CD11c^{hi} DCs display unimpaired thymic negative selection, but that the absence of DCs triggers a progressive myeloproliferative disorder (Birnberg et al., 2008). As a result of this disorder, DC-less mice develop signs of lymphocyte hyperactivation. Moreover, Ohnmacht et al. (2009) reported that animals lacking DCs succumb to a spontaneous fatal autoimmune disorder. In line with the above, a recent report uncovered a direct correlation between DC numbers and Treg cells as part of a feedback-control mechanism (Darrasse-Jèze et al., 2009).

In this study, we show that depletion of DCs, either from birth or later in adulthood, did not prevent EAE induction, but instead led to a lower state of tolerance and stronger inflammatory responses. We also show that DCs are responsible for the upregulation of PD-1 on antigen-specific T cells and subsequently induce the de novo conversion of Treg cells from naive T cells during immune responses.

RESULTS

Targeted Expression of MOG to DCs Results in Peripheral Tolerance and Prevention of CNS Inflammation

In order to elucidate the role of DCs in maintaining and controlling autoimmune CNS inflammation, we opted for a system that allows us the inducible and specific expression of an autoantigen by DCs. We therefore crossed the tamoxifen (TAM)-inducible CD11c-CreER$_T$ mice (Probst et al., 2003) to the IiMOG mice (Frommer et al., 2008), resulting in animals in which the MOG$_{35-55}$ peptide is expressed as a CLIP replacement peptide upon injection with TAM (iDC^{MOG} mice, Figure S1A available online). Upon TAM treatment, about 5% of all CD11c$^+$ cells present the MOG peptide all over the body (Probst et al., 2003). As seen in Figure 1A, iDC^{MOG} mice were resistant to EAE induction when DCs were forced to express the MOG peptide following TAM injection 2 days prior to active immunization, confirming previous studies that showed that steady-state DCs induce tolerance (Bonifaz et al., 2002; Jiang et al., 1995; Probst et al., 2003). Importantly, we discovered that the tolerogenic effect was maintained even when TAM was administered 2 days after disease induction (Figure 1B), a time point at which strong DC activating and maturation signals as well as the first T cell priming had already taken place. At the same time, TAM injection of wild-type (WT) animals had no adverse effect on EAE development (Figure 1B). These findings show that MOG-presenting DCs can downmodulate EAE even after the initial priming phase of the disease. Furthermore, we investigated whether EAE can be moderate if peptide presentation by DCs is induced at a later time point. Remarkably, we found that TAM injection 7 days after disease induction completely blocked any signs of EAE (Figure 1C). To study whether the presentation of MOG by DCs directly influences effector T (Teff) cells, we induced EAE in mice, treated them with TAM after 7 days, and isolated their splenocytes and reactivated them in vitro with MOG 14 days later. Then, we gated on CD40 ligand$^+$ (CD40L) Teff cells. CD40L is rapidly expressed after activation and can therefore serve as a marker for MOG-specific Teff cells (Figure 1D, left) (Chattopadhyay et al., 2005). As seen in Figure 1D, the percentage of interleukin-17A (IL-17A)-producing cells and cells that coproduce IL-17A and interferon-γ (IFNγ) was significantly reduced in TAM-treated iDC^{MOG} mice compared to control mice. Thus, our findings are consistent with the emerging evidence that antigen-expressing DCs can inactivate memory T and Teff cells (Kenna et al., 2008).

To elucidate the molecular mechanism of DC-induced tolerance, we transferred carboxyfluorescein succinimidyl ester (CFSE)-labeled Thy1.1$^+$ MOG$_{35-55}$-specific CD4$^+$ 2D2 T cells into Thy1.2$^+$ iDC^{MOG} mice and analyzed their proliferation and expression of tolerance-associated cell surface molecules. Although the 2D2 T cells proliferated when injected into TAM-treated iDC^{MOG} mice (Figure 1E), we found a strong upregulation of PD-1 and a moderate elevation of BTLA on these cells compared to 2D2 T cells transferred to control mice (Figure 1F).

Injection of anti-CD40 was previously shown to activate DCs and shift the balance from tolerance to immunity (Probst et al., 2003). We found that 14 and 21 days after transfer, 2D2 T cells were still present in control mice injected with CD40-specific antibodies, but we could not detect these cells in the iDC^{MOG} mice (Figure S1B). To investigate whether we could force DCs to become immunogenic, we injected iDC^{MOG} mice with TAM, anti-CD40, complete Freund's adjuvant (CFA), pertussis toxin (PTx), or various combinations of these stimuli. As seen in Figure 1G, only when mice were injected with a combination of CFA, anti-CD40, and PTx in addition to TAM treatment did half of them develop signs of EAE (maximal score of 2.5). The latter shows that once DCs are properly activated, they can revert from inducing T cell tolerance to driving pathogenic T cell responses. To test whether the low EAE incidence results from Treg cell induction, iDC^{MOG} mice were treated with TAM, injected with isotype control (HRPN) or anti-CD25 antibody (PC61) 7 days later, and immunized with MOG-CFA after an additional 7 days. Although PC61 treatment could restore EAE induction, the clinical score remained moderate (Figure 1H).

Next, we transferred naive 2D2 (eGFP$^-$) cells isolated from 2D2-FoxP3-eGFP reporter mice and analyzed their BTLA and PD-1 expression by FoxP3$^-$ or FoxP3$^+$ T cells. We found that FoxP3$^+$ cells exhibit elevated expression levels of BTLA and PD-1 regardless of the host mice. However, in FoxP3$^-$ cells, these molecules were upregulated only when 2D2 cells were transferred into iDC^{MOG} mice (Figures S1C and S1D).

The observation that presentation of autoantigen by steady-state DCs could completely prevent EAE, whereas disease induction could occur solely when activated DCs express the self-peptide, suggests that a physiological function of steady-state DCs is crucial for the induction and/or maintenance of self-tolerance.

Conventional DCs Are Dispensable for the Induction of CNS Inflammation

To further analyze the role of DCs in EAE induction, we utilized our previously described inducible diphtheria toxin receptor (iDTR) model, which, when crossed to the CD11c-Cre line (Buch et al., 2005; Caton et al., 2007), allows the ablation of DCs. Depletion of DCs in CD11c-iDTR mice is achieved by injection of diphtheria toxin (DT, Figure S2A). Following 3 DT injections, we reached a prolonged DC reduction of more than 94% in the spleen and about 80% in the lymph nodes (LNs) (Figure S2B), including both CD8α^+ and CD8α^- DCs (Figure S2C),

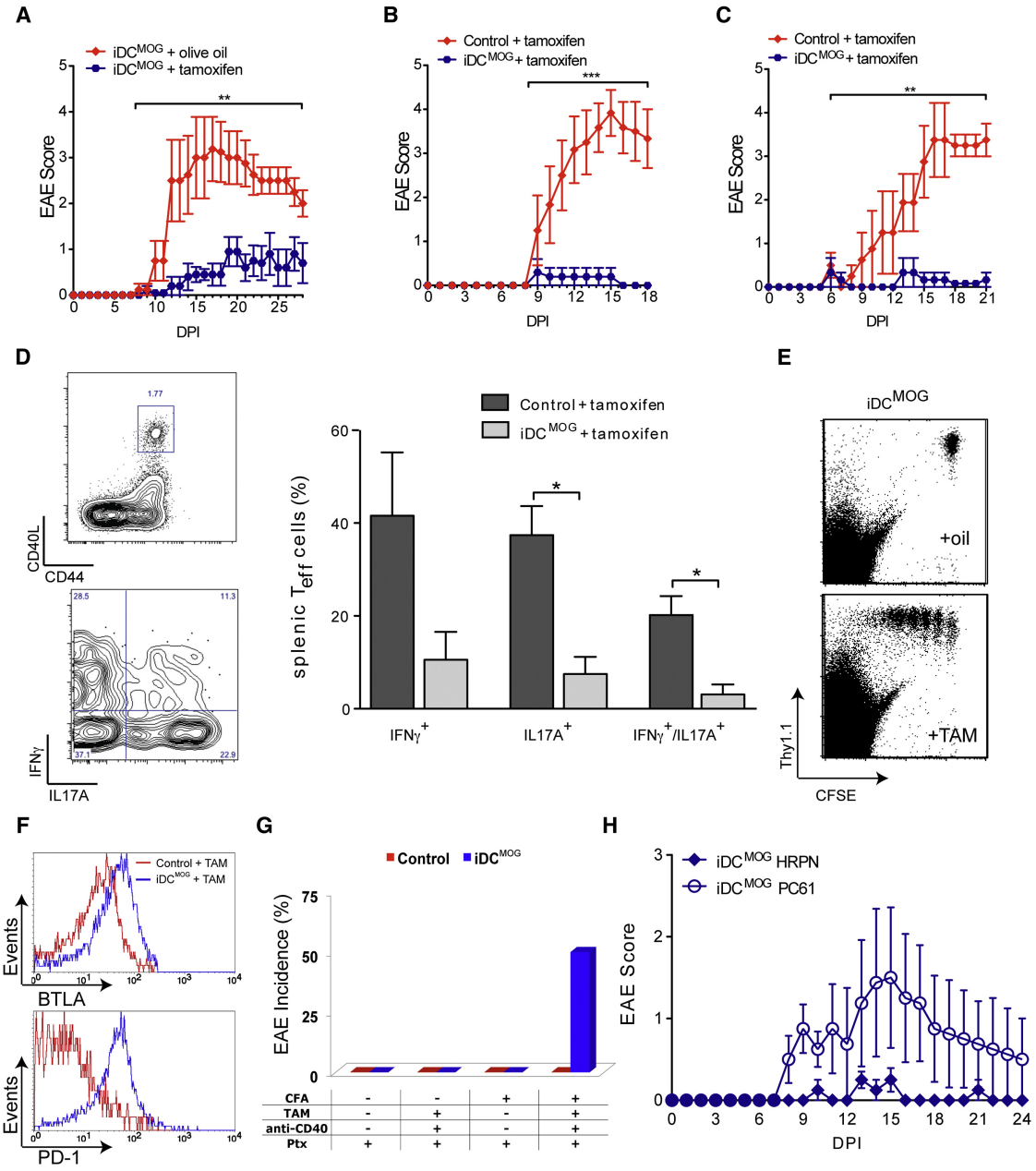

Figure 1. Antigen Presentation by DCs Induces Tolerance to EAE and Expression of Coinhibitory Molecules on T Cells

(A–C) EAE was actively induced by immunization with MOG_{35-55}-CFA in iDC^{MOG} or control mice. TAM was injected 2 days before (A), 2 days after (B), or 7 days after (C) EAE induction.

(D) Splenocytes taken from EAE-afflicted mice (shown in C) were cultured in the presence of MOG_{35-55} for 6 hr. Three hr after MOG stimulation, Brefeldin A was added. $CD90.2^+CD4^+CD44^+CD40L^{hi}$-gated cells (Teff cells) were then analyzed for their expression of IFNγ and IL17A (left), and percentages of $IFN\gamma^+$, $IL17A^+$ or $IFN\gamma^+IL17^+$ Teff cells were calculated (right).

(E and F) Naive CFSE-labeled $Thy1.1^+$ 2D2 $CD4^+$ T cells were adoptively transferred to $Thy1.2^+$ control or iDC^{MOG} mice. Four days after transfer, mice were injected with TAM, and 5 days later, cells were monitored for proliferation or for the expression of coinhibitory molecules. Shown are $Thy1.1^+$-gated cells.

(G) iDC^{MOG} and control mice were treated with CFA, TAM, and/or anti-CD40 (50 μg/mouse), as indicated in the table below. TAM and anti-CD40 were injected i.p. 2 days before subcutaneous injection of CFA (day 0). PTx was given to all mice on days 0 and 2. Mice that were not treated with TAM or anti-CD40 received oil or PBS, respectively. Mice were observed daily for clinical signs of EAE. Data are representative of two or more independent experiments ($n \geq 4$ mice per group, maximal clinical score 2.5).

(H) iDC-MOG mice were treated with TAM to induce MOG presentation by immature DCs. Seven days later, the mice were injected with either PC61 or HRPN. Following another 7 days, the mice were immunized with MOG-CFA and disease was monitored for an additional 24 days (n = 5 mice/treatment).

EAE area under curve (AUC) was used to determine statistical significance via Student's t test. Statistical analysis of splenic T cells was assessed via one-way ANOVA; Newman-Keuls test. *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$; n.s., not significant. Error bars represent the mean ± SEM.

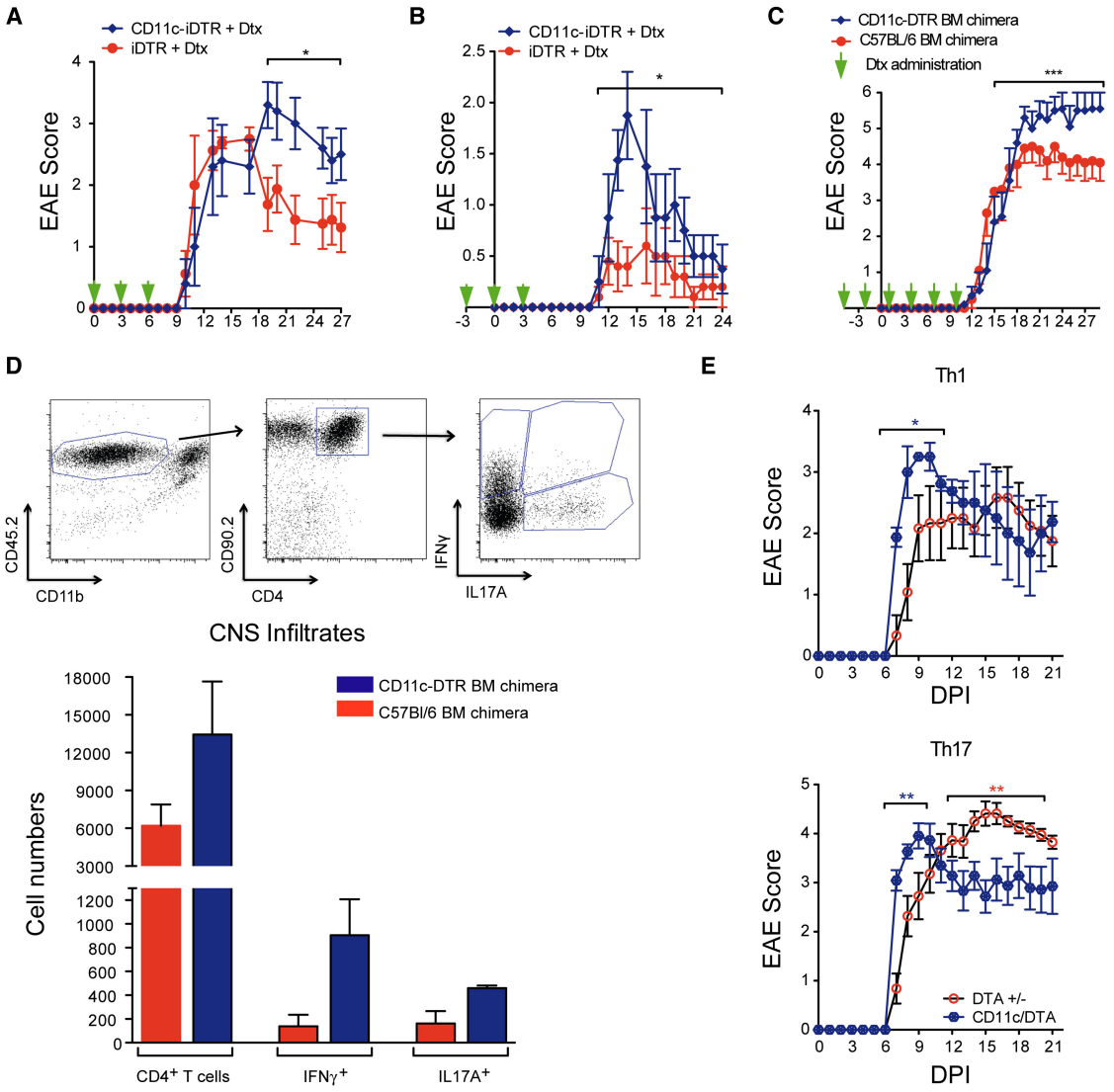

Figure 2. Absence of DCs Is Permissive to EAE Induction and Enhances EAE Severity

(A–C) EAE was induced by immunization of MOG_{35-55} emulsified in CFA in CD11c-iDTR or iDTR mice (A and B), as well as in C57BL/6→C57BL/6 or CD11c-DTR→C57BL/6 BM chimeric mice (C). Mice were subjected to DT injection (green arrows) at days 0, 3, and 6 (A), −3, 0, and 3 (B), or −5, −2, 1, 4, 7, and 10 (C) of EAE induction (5–9 mice per group).

(D) CNS-infiltrating cells were isolated using a Percoll gradient. Shown is a representative dot plot of CNS-infiltrating IFNγ- and IL17A-producing $CD4^+$ T cells, as well as the calculated cell numbers (shown is a representative experiment [of four] with $n \geq 7$ mice/group).

(E) Passive EAE. WT mice were immunized with MOG-CFA, and 10 days later, splenocytes were isolated and cultured in the presence of MOG peptide under conditions favoring Th1 or Th17 cell differentiation. Four days later, 5×10^6 blasting T cells were adoptively transferred i.v. into the indicated mice, and disease progression was monitored for an additional 22 days. (Shown is a representative EAE graph of two individual experiments. Th1 cell $n \geq 5$ mice/group, Th17 cell $n \geq 11$ mice/group.)

EAE AUC was used to determine statistical significance via Student's t test. *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$; n.s., not significant. Error bars represent the mean ± SEM.

whereas plasmacytoid DCs (pDCs) essentially remained unaffected (Figure S2D). Additionally, staining of epidermal sheets for MHC class II showed effective depletion of Langerhans cells (Figure S2E).

Surprisingly, when EAE was induced in DC-depleted CD11c-iDTR mice, disease severity increased in comparison to control littermates (Figure 2A). This difference became even more apparent in experiments wherein control mice developed only mild EAE (Figure 2B). When we analyzed the inflammatory CNS infiltrates, we found no major differences in $CD4^+$ T cell frequencies (CD11c-iDTR 23.0% versus iDTR 21.1%). DCs returned to 25% of their numbers in the control group 72 hr after the last DT injection. Due to adverse toxic side effects of using CFA, PTx, and DT together (Meyer Zu Hörste et al., 2010), which impaired our scoring, we had to discontinue DT injections 6 days post immunization (dpi). As a result, the amounts of DCs in the

CNS were similar in both sets of mice at 14 dpi (CD11c-iDTR 8.8% and iDTR 10.7%).

To compare the effect of DC ablation on EAE using a more established system, we turned to the CD11c-DTR mice (Jung et al., 2002). As these mice die upon repetitive DT injections (Zaft et al., 2005), we prepared bone marrow (BM) chimeric mice wherein the host was CD45.1 WT and the donor BM was either CD45.2 WT or CD45.2-CD11c-DTR. Similarly to the findings using CD11c-iDTR mice, we found that DC ablation before and after immunization resulted in enhanced EAE (Figure 2C). In line with the elevated disease score, analysis of CNS infiltrates confirmed higher numbers of $CD4^+$ T cells infiltrating the inflamed CD11c-DTR CNS, as well as higher numbers of both single-positive $IFN\gamma^+$ and $IL\text{-}17A^+CD4^+$ T cells (Figure 2D). Importantly, these elevated total numbers of T helper 1 (Th1) and Th17 cells reflect the higher infiltrates, and not a change in ratio of these cells among the $CD4^+$ T cells. To exclude the potential toxicity of DT and the role of CFA as a potent adjuvant, we performed adoptive-transfer EAE using WT T cells (Figure 2E). In accordance with our previous results, MOG-primed Th1 cells induced a stronger disease when transferred into DC-less mice (Figure 2E, top). Similarly, the transfer of MOG-primed Th17 cells into DC-less mice also resulted in a stronger disease following the first days of disease onset. Interestingly, 6 days after the first signs of EAE, the DC-less mice showed reduced clinical scores as compared with control mice (Figure 2E, bottom). This reverted EAE may reflect the previously reported function of DCs as indispensable APCs in the CNS during EAE (Greter et al., 2005).

To circumvent the inherent problem with using DT in combination with active immunization, we used a third system in which DCs are continuously ablated by crossing the CD11c-Cre mice to Rosa-DTA mice (Birnberg et al., 2008; Brockschnieder et al., 2004). In these animals, termed CD11c-DTA, DT is expressed specifically in DCs, leading to constitutive ablation of $CD11c^+$ cells as of birth (Figure S3A). In accordance with our previous results, we found that these mice exhibited somewhat higher disease scores compared to control littermates (Figure S3B). This difference was not statistically significant, possibly due to the high individual variability in these mice. The clinical EAE scores of the CD11c-DTA mice tie in well with the histological analyses, showing similar degrees of demyelination, APP, and infiltration/activation of macrophages/microglia as well as T and B cells in DC-proficient mice compared with DC-less mice (Figures S3C–S3E). Taken together, our data demonstrate that DCs are dispensable for the induction of EAE, and their function can be carried out by other MHC class II^+, $CD11c^-$ APCs.

Neither Plasmacytoid DCs nor B Cells Overtake the Role of DCs in the Induction of Autoimmune Response

pDCs can replace the priming function of DCs in LNs after ablation of conventional DCs, and they have been shown to contribute to EAE development (Sapoznikov et al., 2007) (Isaksson et al., 2009). Given that pDCs were only partially affected in the CD11c-iDTR (Figure S2D) and CD11c-DTA mice (Birnberg et al., 2008), we combined the CD11c-iDTR system with a depleting antibody specific to pDCs (anti-mPDCA-1). When we injected the mice with the antibody, they were devoid of pDCs and DCs (Figure S4A) but still developed EAE similar to mice that lacked only DCs (Figure S4B).

Recently, it has been reported that granulocyte macrophage colony-stimulating factor (GM-CSF)-dependent $CD103^+$ $CD207^+$ dermal DCs are crucial for EAE induction (King et al., 2010). These cells are $MHC\text{-}II^{hi}$ and $CD11c^+$ and are present in both the dermis and draining LNs. Our peripheral lymphoid staining showed near-complete absence of $MHC\text{-}II^{hi}CD11c^+$ cells in DC-less mice (Figure S2B). A careful examination of skin (including both epidermis and dermis) from the CD11c-DTR BM chimeras revealed nearly complete loss of these dermal $CD103^+CD207^+$ DCs, which did not influence the host-derived radio-resistant epidermal Langerhans cells (Figure S5A), indicating that although these cells are sufficient for EAE induction, they are not needed for it and can be replaced by other APCs.

Apart from DCs, macrophages and B cells are also considered professional APCs. To test which cells may present antigen in the absence of DCs, we immunized the mice with fluorescent microbeads emulsified with CFA. We then analyzed which cells took up the beads and migrated to the draining LNs. In the absence of DCs, the beads are confined to B cells and macrophages (Figure S5B). As complete macrophage ablation is not possible, because it leads to death of the mice (Heppner et al., 2005), we were not able to perform such an experiment. B cells, on the other hand, can be ablated using anti-CD20 antibodies (Pöllinger et al., 2009). We could not find any effect of the B cell depletion on the course of EAE when DCs were also ablated (Figure S5C). In contrast, in DT-treated control mice, depletion of B cells enhanced the disease, as shown previously for B cell-deficient mice (Figure S5D) (Fillatreau et al., 2002). These experiments demonstrate that in the absence of DCs, antigen presentation is performed by macrophages or other $CD11c^{low/-}$ cells.

DCs Control T Cell Proliferation and Expression of BTLA and PD-1 in Response to Antigen

We have shown that the interaction of T cells with tolerizing DCs results in upregulation of both PD-1 and BTLA expression (Figure 1F). As PD-1 expression is strongly associated with tolerance (Fife and Bluestone, 2008; Probst et al., 2005; Waisman and Yogev, 2009) and BTLA is a negative costimulatory molecule (Han et al., 2004; Watanabe et al., 2003), we tested whether the expression of these molecules upon immunization would be affected by DC ablation. To this end, we transferred $Thy1.1^+$ CFSE-labeled 2D2 $CD4^+$ T cells into MOG-immunized, DT-treated $Thy1.2^+$ CD11c-iDTR or CD11c-DTA mice and analyzed cell proliferation and surface expression of BTLA and PD-1 5 days later. Remarkably, 2D2 T cell proliferation was enhanced in mice in which DCs were conditionally depleted or missing from birth (Figures 3A and 3B, respectively). Importantly, 2D2 cells upregulated PD-1 and BTLA only when transferred to DC-proficient animals (Figures 3C and 3D). Because the proliferation of 2D2 cells was even stronger in the absence of DCs, the upregulated PD-1 and BTLA expression is not a mere sign of activation, but indeed a clear indication of a specific interaction of DCs with $CD4^+$ T cells that leads to the upregulation of these tolerance-associated molecules.

Steady-State DCs Exhibit their Suppressive Capacity by Promoting the Development of iTreg Cells

Because our data indicated that the tolerogenic effect of DCs is present in the priming phase, we analyzed the differentiation of

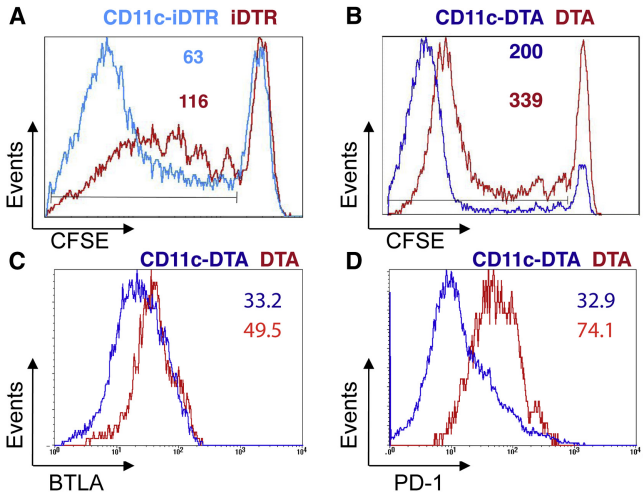

Figure 3. Proliferation of 2D2 Transgenic T cells Is Enhanced in the Absence of DCs In Vivo

(A and B) Naive CFSE-labeled 2D2 Thy1.1$^+$ T cells were transferred i.v. into MOG_{35-55}-CFA-immunized iDTR mice and compared to CD11c-iDTR mice (A), or into DTA mice compared to CD11c-DTA littermates (B–D) (all recipient mice were Thy1.2$^+$). Five days later, cells were isolated from the draining LNs and proliferation was assessed by flow cytometry.

(C and D) Surface expression levels of the inhibitory molecules BTLA (C) and PD-1 (D) on the transferred Thy1.1$^+$CD4$^+$ 2D2 T cells. Data are representative of three independent experiments with no fewer than three mice per group. The respective mean fluorescence intensity (MFI) values in the marked region are indicated by color.

Error bars represent the mean ± SEM.

CD4$^+$ T cells 8 days after immunization. Despite the fact that CD11c-DTA mice lacked DCs (Figure 4A), we did not find discrepancies in Th1 or Th17 cell differentiation at this time point (Figure 4B). However, we observed a significantly lower number of Foxp3$^+$ T cells in spleens of DC-depleted animals (Figure 4C). Given that this finding may explain the higher degree of EAE severity, we analyzed Treg cells from CD11c-DTR BM chimeras 12 days after EAE induction, and, once more, we found a significant reduction in the number of Treg cells in the spleen, but not in the LNs (Figure 4D, left). Moreover, we observed that Treg cells in these animals expressed significantly lower Foxp3 levels compared to those of control mice (Figure 4D, middle). Nonimmunized, DT-treated BM chimeras showed comparable numbers of Treg cells (Figure 4D, right). Additionally, messenger RNA (mRNA) expression for both Foxp3 and transforming growth factor β (TGF-β) were strongly reduced (Figure 4E). To assess the induction of bona fide Treg cells by DCs in vivo, naive CD45.1$^+$GFP$^-$ T cells were isolated from FoxP3-eGFP mice (Korn et al., 2007) and transferred into either CD45.2$^+$ WT or CD11c-DTR BM reconstituted mice. Prior to T cell transfer, the BM chimeric mice were subjected to DT injection. A day later, the mice were immunized, and 7 days later, the newly generated iTreg cells were assessed (Figure 4F). In line with the previous data, iTreg cells differentiated much more efficiently when DCs were present, showing that, indeed, de novo Treg cell differentiation is disturbed in the absence of DCs during immunization. Numerous publications had shown that DCs are responsible for both nTreg and iTreg cell differentiation (for a review, see Kushwah and Hu, 2011). To assess whether, in our model, Treg cells expand in the presence of or contract in the absence of DCs, we transferred naive Thy1.1 2D2-Foxp3(eGFP$^-$) T cells into Thy1.2 host CD11c-DTA or DTA mice and analyzed the ratio of endogenous and transferred Treg cells. As expected, following immunization, we found increasing numbers of host-derived (i+n) Treg cells in the presence of DCs (Figure 5). Nevertheless, DC-proficient mice displayed higher percentages and total (i+n) Treg cell numbers compared with DC-deficient mice (Figures 5A, 5B, 5D, and 5E). When gating on the transferred Thy1.1$^+$ cells and analyzing GFP$^+$ cells, we noticed that DC-deficient mice had far less iTreg cell conversion than control mice (Figures 5A, 5C, 5D, and 5F). Similar results were obtained when the cells were transferred into the CD11c-DTR BM system (Figure 5G). Taken together, our results demonstrate that steady-state DCs are essential in the conversion of naive T cells to iTreg cells in the process of immune responses.

DCs Guide the Development of Treg Cells via PD Ligand Interaction with PD-1 Expressed by T Cells

Naive CD11c-DTA mice harbor normal numbers of Treg cells (Birnberg et al., 2008). We assumed that the lower number of Treg cells found in immunized CD11c-DTA and CD11c-DTR animals are due to insufficient induction of Treg cells after EAE induction, shown previously (Korn et al., 2008). To test this hypothesis, we coincubated WT splenic APCs, which were depleted of DCs, with naive 2D2 cells, MOG peptide, and TGF-β. In agreement with our finding of lower Treg cell numbers in DC-depleted immunized mice, in vitro DC-depleted APCs were considerably less efficient in inducing Foxp3$^+$ Treg cells compared to DC-containing APCs (Figures 6A and 6B). Not only were the Treg cell numbers reduced, but we also found decreased Foxp3 protein expression in the absence of DCs (Figure 6C). When coculturing Treg cells isolated from either DC-proficient or DC-deficient mice with 2D2 cells and MOG-pulsed BMDCs, we noticed that the 2D2 cells showed reduced proliferation in the presence of DC-proficient derived Treg cells (Figure 6D). Hence, not only does the loss of DCs results in reduced Treg cell conversion and lower expression levels of Foxp3, it also translates into reduced suppression.

As PD-1 signaling was recently demonstrated to favor the development of iTreg cells (Francisco et al., 2009), we hypothesized that the higher PD-1 expression by T cells in the presence of DCs may favor iTreg cell development, leading to reduced EAE. We therefore tested whether PD-1 expression on T cells was necessary for DC-mediated Treg cell induction. Thus, we used naive 2D2-PD-1^{ko} T cells and performed a similar experiment, as described in Figure 6. Interestingly, we found that PD-1^{ko} T cells did not respond to the presence of DCs with higher iTreg cell differentiation, whereas PD-1-sufficient T cells did (Figure 7A). In line with these experiments, we found that DCs are a major population of PD-L1 expression, which is lost upon depletion of DCs in BM chimeric CD11c-DTR mice and in the other DC-less models (Figure S6). To assess the functional implication of the lower expression of the PD-1 ligands, we tested whether PD-1 ligands were able to restore the iTreg cell induction. Indeed, the combination of PD-L1 and PD-L2, when added as recombinant proteins linked to Fc, fully complemented the lacking DCs in inducing Treg cells (Figure 7B). These findings

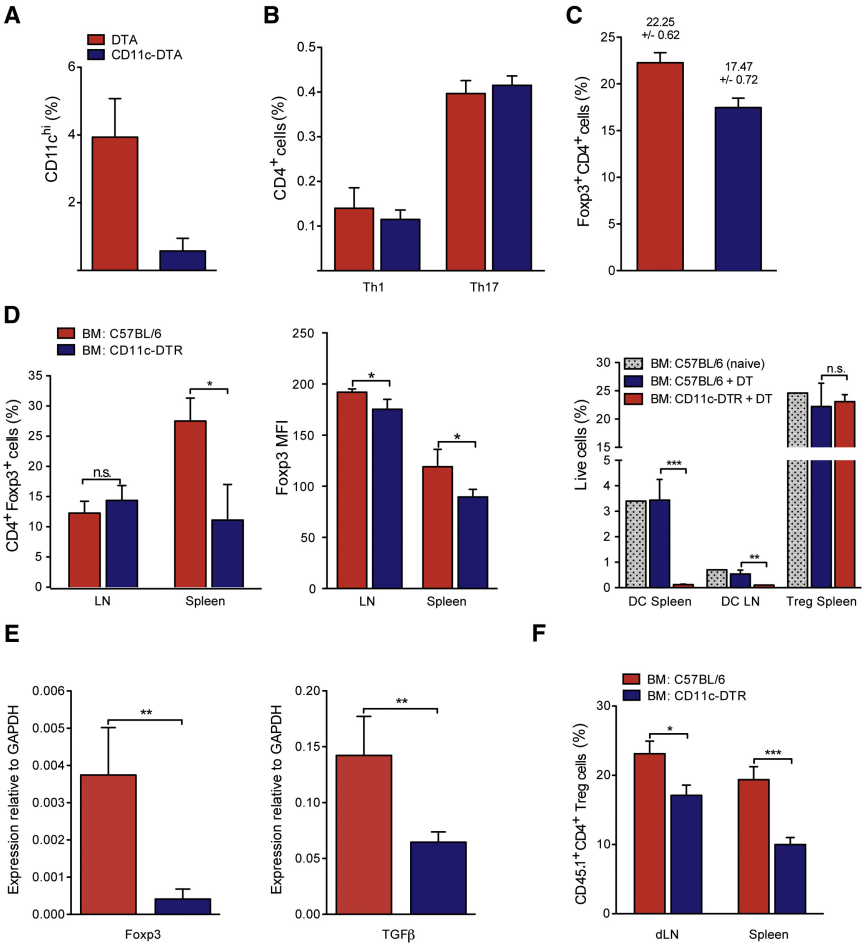

Figure 4. Immunization of Mice Lacking DCs Results in Reduced Treg Cell Number

(A–C) The indicated mice were immunized with MOG_{35-55}-CFA and analyzed 8 days later for the presence of DCs, Foxp3$^+$, IL-17A, and IFNγ-producing T cells.

(A) Analysis showing the absence of splenic DCs ($CD11c^{hi}$) in the DTA system.

(B) Percentages of IFNγ single-producing or IL-17A single-producing CD4$^+$ cells (designated Th1 and Th17, respectively) in the draining LNs.

(C) Percentages of CD4$^+$ Foxp3$^+$ Treg cells in the spleen of MOG_{35-55}-CFA-immunized mice. ($DTA^{+/-}$, n = 3; $CD11c/DTA^{+/-}$, n = 3.) Data are representative of at least three independent experiments.

(D) Percentage of Treg cells as found in the spleen or LNs of immunized BM chimeric CD11c-DTR mice on day 12 after EAE induction (BM: C57BL/6, n = 4; BM: CD11c-DTR, n = 3) (left). Foxp3 expression as measured by MFI in Treg cells of LNs and spleens of the latter mice (middle). Percentage of Treg cells or DCs found in spleen or LNs of DT-treated BM chimeric CD11c-DTR or C57BL/6 mice 8 days after first DT treatment (BM: C57BL/6, n = 5; BM: CD11c-DTR, n = 5) (right).

(E) Real-time mRNA analysis of splenocytes from DT-treated BM chimeric animals 5 days after immunization (BM: C57BL/6, n = 5; BM: CD11c-DTR, n = 4). Data are representative of three or more independent experiments.

(F) MACS-purified CD45.1$^+$GFP$^-$ naive CD4$^+$ T cells were transferred into CD45.2$^+$ BM chimeric mice previously injected with DT (BM: C57BL/6, n = 9; BM: CD11c-DTR, n = 9). DT was applied every second day, starting from day −6 until day +4 post immunization. dLN, draining LN.

Statistical analysis was assessed using Student's t test or one-way ANOVA; Newman-Keuls test. $^*p \leq 0.05$, $^{**}p \leq 0.005$, $^{***}p \leq 0.0005$; n.s., not significant. Error bars represent the mean ± SEM.

suggest that DCs induce Treg cells via a PD-1-dependent mechanism, and thereby contribute to tolerance. Next, we cotransferred 2D2 and 2D2-PD-1^{ko} T cells into iDC^{MOG} (Figure 7C) or CD11c-DTA mice (Figure 7D) and assessed the role of PD-1 in Treg cell differentiation in vivo. Consistent with our previous observations, we found higher percentages of 2D2 Treg cells in iDC^{MOG} mice compared to control mice. Similarly, 2D2-PD-1^{ko} Treg cells were increased in the iDC^{MOG} mice, though to a lesser degree (Figure 7C). A further dissection revealed higher numbers of both 2D2 Foxp3$^+$ and IFNγ$^+$ cells, but not IL-17A$^+$ cells (Figures 7E–H). When CD11c-DTA mice were used, we saw that the lack of DCs resulted in lower percentages of 2D2 Treg cells (Figure 7D). Moreover, Treg cell percentages were comparable between 2D2 Treg cells in CD11c-DTA mice and 2D2-PD-1^{ko} Treg cells in DTA mice (Figure 7D), thus confirming our in vitro data.

Treg cell suppression needs to be tightly controlled and, therefore, believed to take place in the target tissue. Because we found an enhanced autoimmune response that was associated with reduced numbers of Treg cells, we tested whether Treg cell numbers in the CNS were also reduced. As seen in Figure S7, the numbers of CNS-infiltrating Treg cells were indeed diminished in the CNS of DC-depleted mice compared with control mice.

DISCUSSION

In this paper, we demonstrated that induction of EAE through immunization with MOG_{35-55} in CFA was possible in mice that possessed less than 5% of the normal number of DCs. Remarkably, DC-less mice developed more severe EAE compared to mice with normal DC numbers, suggesting that during immunization, DCs reduce the responsiveness of CD4$^+$ T cells to self-antigens, including those sequestered in the CNS. We identified PD-1-dependent induction of MOG_{35-55}-specific Treg cells by antigen-presenting DCs as a crucial mechanism underlying reduced T cell responsiveness and susceptibility to EAE. Furthermore, our results suggest that either the number of mature DCs required for priming is extremely low, or other APC types, most likely CD11c$^-$, are sufficient for EAE induction. Interestingly, we could show that even when T cells were adoptively transferred to DC-less mice, disease was enhanced in the first few days, although at the later stage of the disease it became milder. These findings have two conclusions: first, that even without active immunization, Treg cells (that develop from the transferred cells or host cells) play an important role in disease development, and second, that once disease is in its peak, CNS DCs are important for maintenance of the

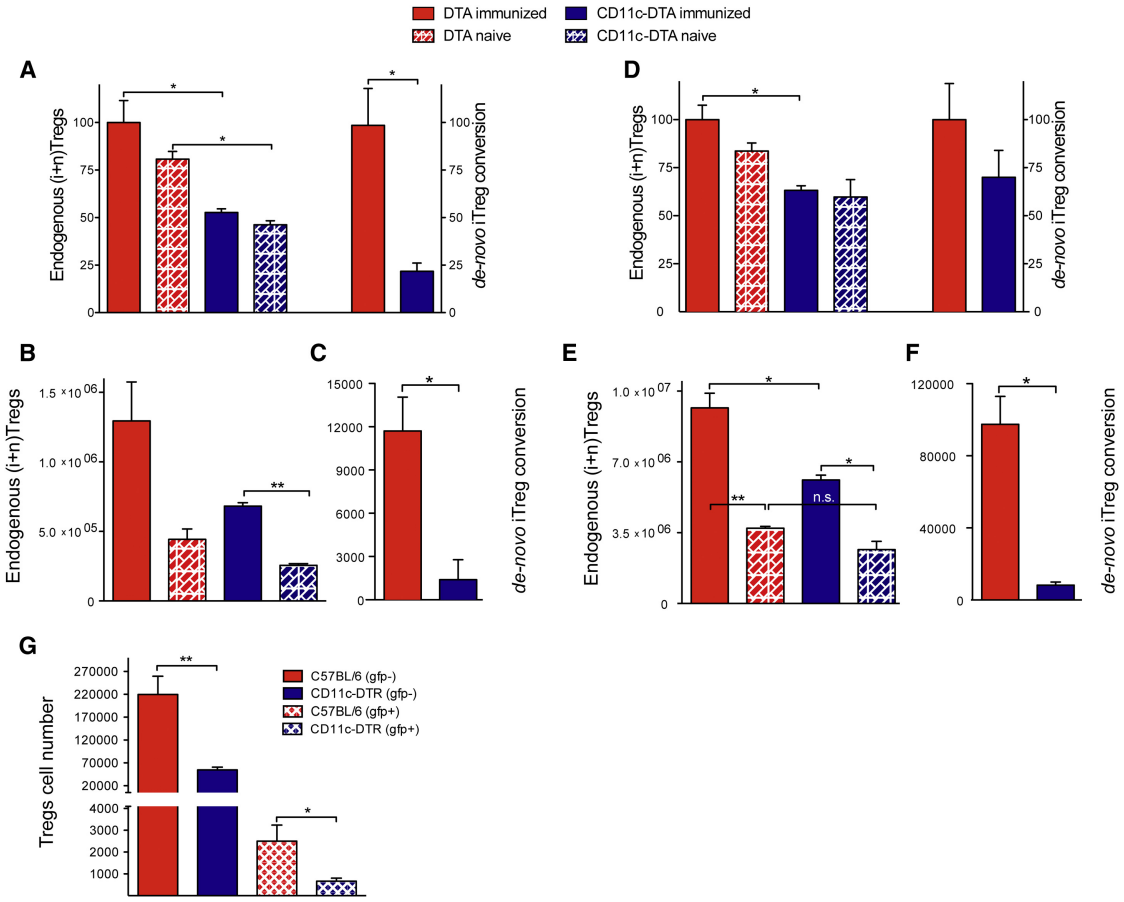

Figure 5. Reduced Treg Cell Numbers in the Absence of DCs

(A–F) 5 × 10^6 naive Thy1.1$^+$ 2D2-Foxp3-eGFP$^-$ transgenic T cells were transferred into the indicated mice and one day later the mice were immunized with MOG-CFA. Eight days after cell transfer, inguinal LN and splenic Treg cells (A–C and D–F, respectively) were assessed based on the expression of congenic markers, eGFP, and Foxp3 expression. Endogenous (i+n) Treg cells = host-derived Thy1.2$^+$Foxp3$^+$ cells, whereas de novo iTreg cells = Thy1.1$^+$eGFP$^+$ cells. Treg cell conversion was set to maximal based on the immunized DTA mice, and all other groups were calculated relative to that (A and D); absolute cell numbers are shown in (B), (C), (E), and (F). Data are representative of two independent experiments; n = 3 mice per group.

(G) Similar experiment to that described above (A–F), except that the recipient mice in this case are the CD11c-DTR or C57BL/6 BM chimeric mice.

Error bars represent the mean ± SEM.

full-blown clinical manifestations, as shown before (Greter et al., 2005).

CD11c-positive DCs are bona fide APCs inducing CD4 and CD8 T cell responses upon immunization (Sapoznikov et al., 2007) and have been implicated in thymic negative selection (Brocker et al., 1997). However, recent studies using DC-less mice showed that both thymic and peripheral T cell repertoire are not affected by the loss of DCs (Birnberg et al., 2008). Nevertheless, these experiments confirmed the importance of splenic and thymic DCs for inducing mixed lymphocyte alloreactions and the formation of antiviral T cell immune responses (Birnberg et al., 2008). Indeed, ovalbumin-specific T cells did not react to systemic antigen in spleens upon depletion of DCs, but, surprisingly, these T cells reacted strongly in the LNs to subcutaneous immunizations with antigen, despite a lack of DCs (Birnberg et al., 2008). This DC-independent T cell activation in the LNs may explain the efficient MOG peptide-specific T cell activation in DC-less mice reported in this paper. Our experiments, in which both DCs and pDCs were depleted, imply the possibility that cells other than DCs act as primary APCs in the LNs when DCs are absent. B cells and macrophages are both potential candidates. In the case of B cells, it has been shown that they are not mandatory for EAE induction with the MOG peptide, as B cell-deficient animals develop a severe, chronic form of EAE (Fillatreau et al., 2002; Wolf et al., 1996). In addition, a role for B cells was recently shown in multiple sclerosis, as it was found that B cell depletion with anti-CD20 has a profound positive effect in patients with multiple sclerosis (Hauser et al., 2008). We have shown that ablation of both DCs and B cells does not prevent the disease induction, which suggests that a possible non-DC myeloid population is sufficient for T cell priming. These data either point to a strong redundancy between the distinct DC populations or the presence of another (as yet) undiscovered CD11c$^-$ APC as the main priming cell type in EAE. However, we cannot exclude the possibility that the residual numbers of DCs are sufficient to prime T cells in LNs.

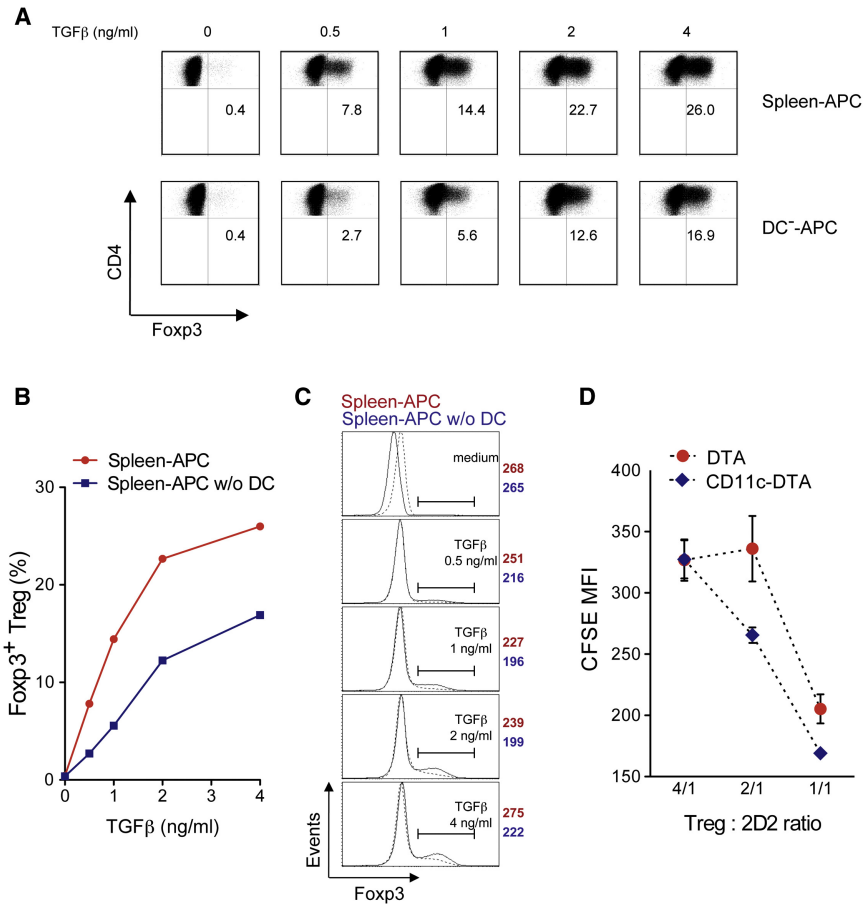

Figure 6. Impaired Induced Treg Cell Differentiation and Suppression in the Absence of DCs

(A and B) MACS-purified naive CD4$^+$ 2D2 transgenic T cells were differentiated using TGF-β at indicated concentrations in the presence of MOG_{35-55} and cocultured with either WT total splenic APCs or WT splenic APCs depleted of DCs, using MACS beads. Cells were FACS-analyzed by CD4 and Foxp3 staining. Percentages of CD4$^+$Foxp3$^+$ cells are indicated.

(B) Percentages of Foxp3$^+$ of total live CD4$^+$ cells are summarized in the diagram.

(C) Differences in the MFI of CD4$^+$Foxp3$^+$ T cells are shown by histograms. The respective MFI values of cells in the marked region are indicated by color. Data are representative of four independent experiments.

(D) MFI of the CFSE-labeled 2D2 cells upon coculturing with MOG-pulsed BM DCs and Treg cells isolated from either MOG-immunized DC-less or control mice 10 days post immunization.

Error bars represent the mean ± SEM.

A recent paper showed that CD103$^+$CD207(langerin)$^+$ dermal DCs are crucial for EAE induction (King et al., 2010). These cells are MHC-II^{hi} and CD11c$^+$ and are present in both the dermis and in the draining LNs. Our staining showed nearly complete absence of MHC-II^{hi}CD11c$^+$ cells in DC-less mice. Moreover, a careful examination of skin (including both epidermis and dermis) from the CD11c-DTR BM chimeras revealed nearly complete loss of these dermal CD103$^+$CD207$^+$ DCs without influence on the host-derived radio-resistant epidermal Langerhans cells. The disparity of EAE development in the two models could be explained by the concurrent depletion of tolerogenic steady-state DCs in our system, which may lower the threshold for EAE induction and therefore circumvent the need for CD103$^+$CD207$^+$ dermal DCs in EAE induction. In line with this, our DC-less CD11c-iDTR mice showed an absence of CD8$^+$ DCs, which are most notably implicated in tolerance induction (Yamazaki et al., 2008).

Similarly, as described previously (Birnberg et al., 2008), we found enlargements of the spleen and LNs in aged CD11c-DTA mice (data not shown), but not a strong autoimmune lymphoproliferative infiltration with a fatal autoimmune syndrome, as reported by others (Ohnmacht et al., 2009). This distinct outcome may be explained by different housing hygienic conditions, as our data point to a strong role of DCs in the formation of adaptive Treg cells, which may be crucial in controlling the immune response against viral or bacterial infections. Naive, nonimmunized CD11c-DTA mice harbor an unimpaired thymic Treg cell compartment and numbers of splenic Treg cells similar to those of WT animals. Here, we show that, upon immunization, CD11c-DTA and DT-treated BM chimeric CD11c-DTR mice display a reduced number of Treg cells in the spleen in comparison to WT animals. We think that this finding indicates a role of DCs in the induction of Treg cells. This is in line with previous findings that immunization induces a substantial amount of novel antigen-specific Treg cells (Korn et al., 2008; Kretschmer et al., 2005). Indeed, when we transferred naive T cells before immunization, we observed a strong reduction of iTreg cell differentiation in the absence of DCs. Our in vitro Treg cell induction experiments and in vivo analysis of Foxp3 expression fully support this role of DCs, because naive T cells differentiated considerably better into Foxp3$^+$ cells in the presence of DCs. The iTreg cells that developed in the presence of DCs also expressed higher Foxp3 levels than those that developed in the absence of DCs. High expression of Foxp3 is shown to promote a full Treg cell program and an enhanced suppression profile by Treg cells (Williams and Rudensky, 2007). In addition, we found that Treg cells isolated from DC-less mice were less efficient in T cell suppression compared to those isolated from WT mice. Together, these data suggest that Treg cells depend on DCs for full phenotypic and functional development.

When we transferred naive antigen-specific T cell receptor (TCR)-transgenic T cells to immunized CD11c-DTA mice, we discovered that they did not upregulate PD-1 expression when compared with cells transferred to control mice, in spite of a strong and even enhanced proliferative response. These findings suggest that DCs are involved in the programming of gene expression in T cells in a unique manner, i.e., not shared by other APCs. Although we do not fully understand the precise

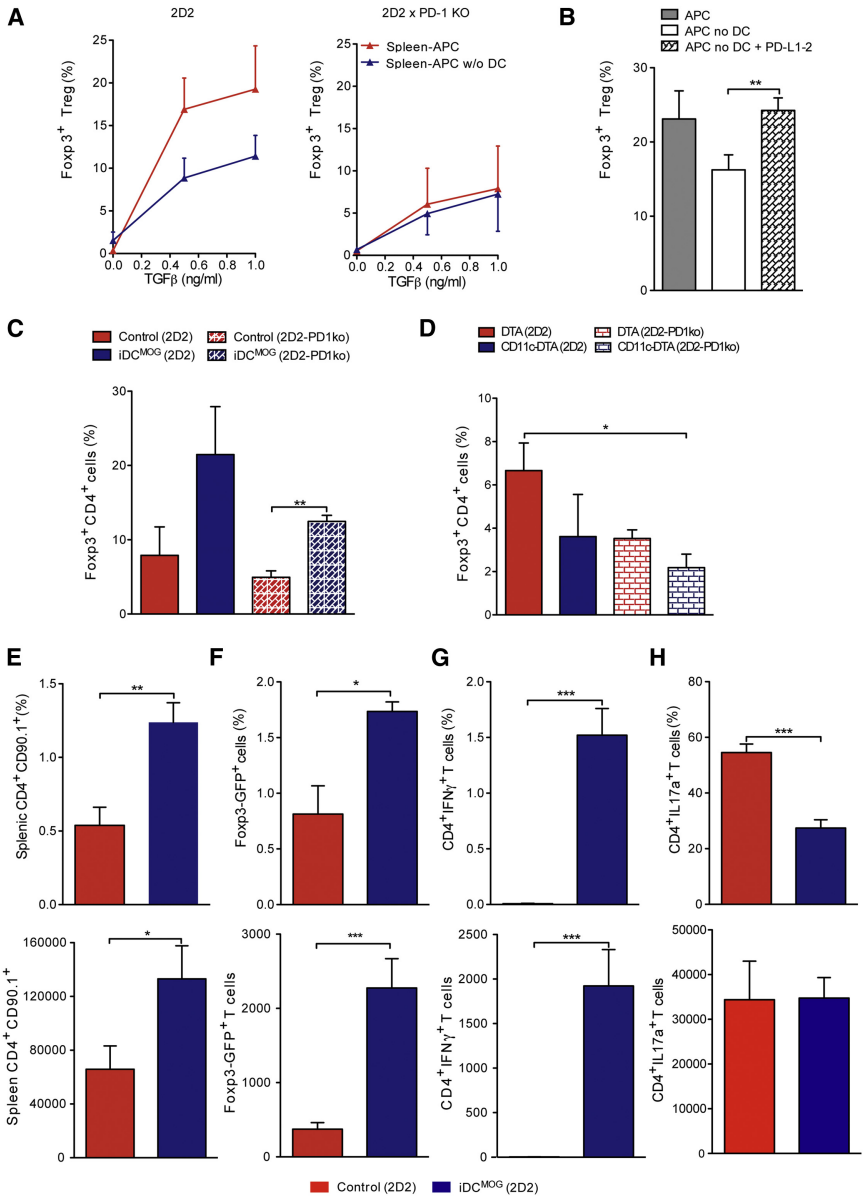

Figure 7. PD-1 Expression on T Cells Is Required for Induced Treg Cell Differentiation by DCs

(A) MACS-purified naive $CD4^+$ T cells from either 2D2 or 2D2-$PD\text{-}1^{ko}$ transgenic mice were differentiated using TGF-β at the indicated concentrations in the presence of $MOG_{35\text{-}55}$ and cocultured with WT T cell-depleted total splenic APCs or APCs additionally depleted for DCs.

(B) Recombinant PD-L1 and PD-L2 Fc-fusion proteins complement the lack of DCs for iTreg cell development in vitro. Naive $CD4^+$ 2D2 T cells were cultured under iTreg cell-polarizing conditions (TGF-β = 1 ng/ml) together with total splenic APCs, DC-depleted splenic APCs, or DC-depleted splenic APCs supplemented with recombinant PD-L1 (5 μg/ml) and PD-L2 (10 μg/ml). Data are representative of three independent experiments.

(C) 2D2 and 2D2-$PD\text{-}1^{ko}$ cells were cotransferred into TAM-treated iDC^{MOG} or control mice, and comparative in vivo Treg cell conversion was assessed.

(D) Similar experiment to (C), performed in CD11c-DTA or DTA mice.

(E–H) Transferred cells, as shown in (C), were analyzed for total cell numbers; Treg, Th1, and Th17 cell subsets (C–H, n ≥ 3 mice per group). Error bars represent the mean ± SEM.

mechanism by which DCs induce tolerance, prime candidates are distinct costimulatory pathways or cytokine milieus in the presence of DCs. We propose that, upon PD-1 cell surface expression, signaling via PD-L1 and PD-L2 is enhanced, leading to a primary tolerogenic program and differentiation of antigen-specific T cells to $Foxp3^+$ regulatory cells. The latter is in line with our findings that Treg cell development is dependent on PD-1 expression by T cells, as seen in experiments wherein PD-1-deficient T cells were transferred to WT or DC-less mice. Furthermore, we found that DCs express high levels of PD-L1, indicating that one major PD-L1-expressing population vanishes upon depletion of DCs in immunized animals. A recent publication (Amarnath et al., 2010) shows in a xenogenic transfer system that the transfer of human DCs, which were preincubated with Treg cells, induced the upregulation of PD-1 on Teff cells. Due to the fact that this depended on PD-L1 being expressed on these tolerogenic DCs, it is conceivable that PD-L1 signaling in a tolerogenic positive feedback loop leads to PD-1 upregulation on T cells.

Here, we show that EAE can be induced in the absence of classical DCs. Furthermore, our data confirm that DCs are important tolerance mediators for $CD4^+$ T cell responses. Given that substantial numbers of $PD\text{-}L1^{hi}$ DCs are found in the spleen, we favor the following scenario: T cells are first primed by DCs or other cells in the draining LNs. After priming, T cells migrate to the spleen and are modulated there by DCs. Depending on the initial priming strengths, this second phase of modulation dictates whether immunity or tolerance prevails.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice were obtained from Jackson Laboratories. CD11c-Cre (Caton et al., 2007), DTA (Brockschnieder et al., 2004), CD11c-$CreER_T$ (Probst et al., 2003), liMOG (Frommer et al., 2008), iDTR (Buch et al., 2005), $PD\text{-}1^{ko}$ (Nishimura et al., 1999), and 2D2 (Bettelli et al., 2003) mice have been previously described. All mice were housed in specific pathogen-free conditions and used in accordance with the guidelines of the Central Animal Facility Institution of the University of Mainz.

Depletion of Dendritic Cells and B cells

For in vivo DC depletion, mice were injected intraperitoneally (i.p.) with 25 ng/g body weight DT. DC depletion during EAE was carried out following repetitive DT injections. Generally, DT was applied every third day. To achieve in vivo

pDC depletion, mice were injected with 0.5 mg followed by three consecutive injections of 0.25 mg anti-mPDC-1 every 2 days. In vitro DC depletion: total splenocytes were depleted of T cells and DCs (Thy1.2 and CD11c beads) using MACS depletion columns. In vivo B cell depletion: mice were injected i.p. with 250 μg anti-CD20 (clone 18B12; IgG2a; Biogen Idec), kindly provided by R. Dunn, or control IgG2a antibodies weekly, starting 14 days prior to MOG immunization, and continuing until 6 dpi.

Induction and Assessment of EAE

EAE (active and passive) was induced and assessed as previously described (Frommer et al., 2008).

Flow Cytometry Staining and Acquisition

Fluorescence-activated cell sorting (FACS) surface and intracellular antibodies were purchased from eBioscience or BD Pharmingen and used in accordance with the manufacturer's protocol. Samples were acquired with FACS-Scan or FACS Canto II.

Isolation of Splenic DCs and CNS Infiltrates

Spleens were incubated with 2 mg/ml Collagenase D for 30 min and then mashed through a 70 μm cell strainer. CNS infiltrates were isolated as previously described (Greter et al., 2005). In brief, mice were perfused using ice-cold PBS. The brain and spinal cords were removed and incubated in PBS containing collagenase type II (2 mg/ml, GIBCO) and DNase (20 units, Sigma-Aldrich). The tissue was then homogenized and loaded on a 30:37:70% Percoll gradient for enrichment of CNS infiltrates.

Tamoxifen Preparation and Administration

TAM (ICN Biomedicals, Aurora, OH, USA) was suspended in 96% EtOH, diluted in olive oil (1:10 ratio; ICN Biomedicals). Prior to injection, the mixture was further dissolved in 37°C. Cre-recombinase activity of CD11c-CreER$_T$ mice was induced in vivo by twice injecting i.p. 2 mg of TAM in 100 μl.

Adoptive T Cell Transfer and CFSE Labeling

Freshly isolated CD4$^+$ T cells were washed twice with PBS (pH 7.4) and then incubated with 0.5 mM CFSE (Molecular Probes, Eugene, OR, USA), as previously described (Frommer et al., 2008). For proliferation studies, enriched CD4 2D2 transgenic (Thy1.1$^+$) T cells were CFSE-labeled, counted, and 7 × 10^6 cells-injected intravenously (i.v.) in PBS into MOG-CFA-immunized (Thy1.2$^+$) recipient mice. The mice were sacrificed 5 days after T cell transfer, and cells were analyzed by flow cytometry.

In Vitro iTreg Cell Differentiation

1.5 × 10^5 MACS-purified naive CD4$^+$ 2D2 transgenic T cells were cocultured with 5 × 10^4 splenic APCs, 20 μg/ml MOG peptide, and TGF-β at the indicated concentration. For intracellular Foxp3 detection, cells were stained with a commercially available kit (eBioscience). Intracellular cytokine analysis was performed after stimulation of T cells for 4 hr with phorbol 12-myristate 13-acetate (PMA, 100 ng/ml), Ionomycin (200 ng/ml), and Brefeldin A (1 μg/ml). A commercially available kit was used for intracellular cytokine staining in accordance with the manufacturer's protocol (BD Biosciences).

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.05.025.

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