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Although mature dendritic cells (DCs) are potent initiators of adaptive immune response, immature steady-state DCs contribute to immune tolerance. In this study, we show that *ex vivo* splenic DCs are capable of inducing conversion of naïve CD4$^+$ T cells to adaptive Foxp3$^+$CD4$^+$ regulatory T cells (aTreg) in the presence of TGF-β. In particular, when compared with splenic CD8α^- DCs, the CD8α^+ DC subset were superior in inducing higher frequencies of conversion. This was not attributable to the difference in basal level of costimulation, because deficiency of CD40 or CD80/86 signaling did not diminish the differential induction of Foxp3. Conversion was regulated by DC maturation status. Further insights into the molecular mechanisms of conversion were gained by analyzing the contribution of several costimulatory and coinhibitory receptors. Costimulatory signals through GITR suppressed conversion, whereas coinhibitory signaling via programmed death 1 ligand (PD-L1) but not PD-L2 was required for conversion. *Ex vivo* PD-L1$^{-/-}$ DCs failed to support Foxp3 induction in the presence of TGF-β. *In vivo* blocking PD-L1 signaling abolished conversion in a tumor-induced aTreg conversion model. Collectively, this study highlights the cellular and molecular parameters that might be exploited to control the *de novo* generation of aTregs and peripheral tolerance.

dendritic cells | immune suppression | costimulation

Naturally occurring CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (nTregs) represent 5–10% of peripheral CD4 T cells, and are critical regulators of immune tolerance (1). The transcription factor Foxp3 is a specific lineage marker for nTregs and is both necessary and sufficient for Treg function (2).

It is established that naïve CD4$^+$CD25$^-$Foxp3$^-$ T cells can convert into Foxp3$^+$ regulatory T cells (aTregs). *In vitro* conversion occurs in the presence of TGF-β (3), typically under conditions of low costimulation (4, 5). This process requires cytotoxic T lymphocyte antigen (CTLA)-4-mediated negative costimulation (6). The aTregs resemble nTregs both phenotypically and functionally (7–9). *In vivo*, the extrathymic induction of Foxp3$^+$ aTregs from naïve CD4$^+$ T cells occurs upon subimmunogenic antigen stimulation (10–13). Consistent with *in vitro* studies, TGF-β signaling and B7 costimulation are required for peripheral conversion (13, 14).

The Foxp3GFP reporter mice allow the isolation of naive CD4$^+$Foxp3GFP$^-$T cells at high purities (2). By breeding onto a T cell antigen receptor (TCR) Tg background, one can quantify the differentiation of antigen-specific effector T cells to Foxp3GFP$^+$ aTregs and monitor the steady-state conversion in response to soluble antigen, antigen derived under inflammatory conditions, or pathological conditions, tumor-derived antigens, etc. Indeed, previous studies have suggested that tumors could induce CD25$^+$Foxp3$^+$ aTregs from naïve CD4 T cells in the absence of thymus (15, 16). The cellular and molecular basis for tumor-induced conversion, however, is not well understood.

Because resting DCs are constantly presenting tissue or tumor antigens under subimmunogenic conditions, it is imperative to understand their potential roles in the peripheral tolerance as well as tumor-induced tolerance. One of the key questions is whether and how DCs regulate the *de novo* induction of Foxp3$^+$ aTregs. To this end, we examined the capacity of *ex vivo* splenic DC subsets to induce Foxp3 expression in the presence of TGF-β. Our results show that among the splenic DC subsets, the CD8α^+ DCs exhibit a superior capacity to drive conversion. Multiple costimulatory and coinhibitory molecules have been identified to nonredundantly regulate this process. In particular, programmed death 1 ligand (PD-L1) expression on DCs is required for conversion not only *in vitro* but also in a tumor-induced *in vivo* conversion model. Collectively, this study has illuminated the cellular and molecular parameters that regulate the *de novo* generation of Foxp3+ aTregs, which might be exploited to prevent tumor-induced immune tolerance.

Results

***Ex Vivo* Splenic CD8α^+ DCs Are Superior to CD8α^- DCs for the Induction of Antigen-Specific Foxp3$^+$ Adaptive Tregs.** Although it has recently been reported that splenic DCs as a whole population can differentiate Foxp3$^+$ aTregs in the presence of TGF-β and that the induced aTregs could suppress autoimmune rejection or antitumor immunity (17, 18), the efficacy of different DC subsets in this process has not been evaluated.

To determine the influence of splenic DC subsets on aTreg differentiation, purified DC subsets, namely the CD8α^+ or CD8α^- CD11c^{high} DCs, were tested for their capacity to induce Foxp3 expression in naïve CD4$^+$ T cells *in vitro*. OTII TCR transgenic mice were bred onto the Foxp3GFP knockin mice (2). Naïve OTII CD4$^+$Foxp3GFP$^-$ T cells were electronically sorted to >95% homogeneity and used *in vitro* and *in vivo* to quantify the conditions that control their conversion to Foxp3GFP$^+$ cells.

After *in vitro* culture for 5 days with either the CD8α^+ or CD8α^- CD11c^{high} splenic DCs in the presence of antigenic ovalbumin (OVA) peptide and TGF-β, the induction of Foxp3 in OTII CD4$^+$ T cells was measured by GFP expression by using flow cytometry (Fig. 1*A*). When compared to CD8α^- DCs, CD8α^+ DCs were superior inducers of Foxp3 expression in the presence of TGF-β (Fig. 1*b*). This more efficient induction was also seen when the whole OVA protein was provided as antigen (data not shown). The induction of Foxp3 expression by both DC subsets requires TGF-β. To compare

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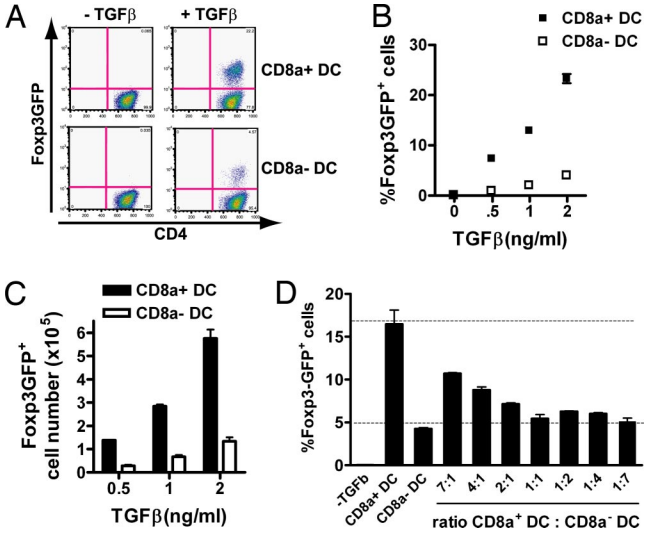

Fig. 1. *Ex vivo* splenic CD8α^+ DCs induce Foxp3 expression more efficiently than CD8α^- DCs in the presence of TGF-β. Freshly isolated splenic DC subsets (CD8α^+ CD11c^{high} and CD8α^- CD11c^{high}) were cocultured with naïve OTII CD4$^+$ T cells (CD25$^-$Foxp3GFP$^-$) in the presence of OVA$_{323–339}$ peptide (500 ng/ml) and increasing amounts of TGF-β1. Foxp3GFP expression was analyzed on day 5 by flow cytometry. The total cell number was counted, and the number of Foxp3$^+$ cells was calculated and plotted. (*A*) Representative FACS plots showing Foxp3GFP induction among OTII CD4$^+$ T cells. (*B–C*) The percentage and absolute number of Foxp3GFP$^+$ cells per well after 5 day culture. (*D*) CD8α^- DCs were mixed with CD8α^+ DCs at the indicated ratios to stimulate OTII CD4 T cells in the presence of TGF-β (2 ng/ml). The total number of DCs per well was kept constant. Foxp3GFP expression was analyzed as above. All conditions were performed in duplicate wells and reported as means ± SEM. Shown are representative results of three independent experiments.

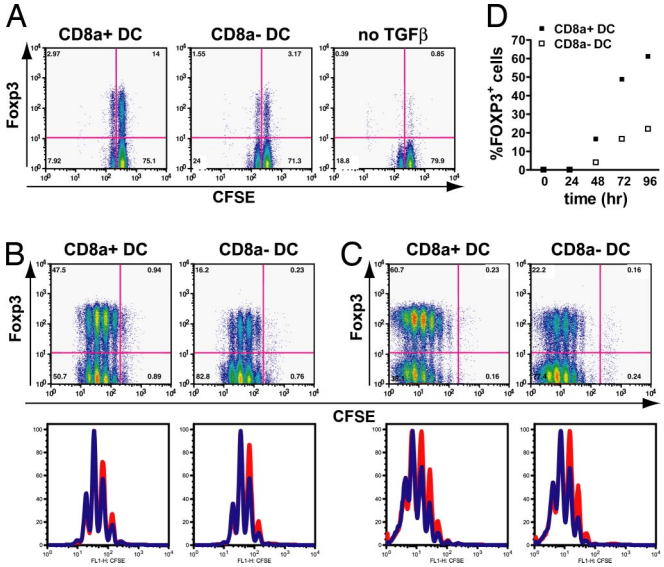

Fig. 2. Kinetics of Foxp3 induction and proliferation of DC-induced Foxp3$^+$ cells. Naïve OTII CD4 T cells from nonreporter background were sorted as Vβ5^{high}CD25$^-$, labeled with 5 μM CFSE, and cultured with splenic DC subsets in the presence of OVA$_{323–339}$ peptide (500 ng/ml), IL-2 (50 units/ml), and TGF-β (2 ng/ml). Foxp3 expression was analyzed at 48 h (*A*), 72 h (*B*), and 96 h (*C*). The CFSE dilution profiles for Foxp3$^+$ (blue) and Foxp3$^-$ (red) cells were overlaid. The percentage of Foxp3$^+$ cells at each time point was plotted (*D*). Shown are representative results of two independent experiments.

the ability for each DC subset to induce clonal expansion, the number of OTII cells was quantified at the end of the assay. Although CD8α^- DCs induced modestly better proliferation of total CD4$^+$ T cells (unpublished data), the number of induced Foxp3$^+$ cells was still greater with the CD8α^+ DC culture (Fig. 1*C*). Because only 20–25% splenic DCs are CD8α^+, we evaluated the impact of CD8α^- DCs on conversion when mixed with CD8α^+ DCs at different ratios (Fig. 1*D*). At a given TGF-β concentration (2 ng/ml), conversion induced by CD8α^+ DCs was 16.45% (±1.65), whereas by CD8α^- DCs was 4.25% (±0.13). The presence of CD8α^- DCs, even as low as 12.5% (7:1 ratio of CD8α^+/CD8α^- DCs), reduced conversion significantly to 10.7% (±0.10). Increasing the amount of CD8α^- DCs continued to reduce conversion. Therefore, CD8α^- DCs actively interfered with conversion, and their effect was dominant when present at physiological percentages (≈1:4 ratio of CD8α^+/CD8α^- DCs).

To assess the kinetics of Foxp3 induction with cell cycle progression, we labeled OTII CD4$^+$ T cells from the nonreporter background with 5(6)-carboxyfluorescein diacetate, succinimidyl-ester (CFSE) and tracked their proliferation and Foxp3 expression over time (Fig. 2*A*). More efficient Foxp3 induction by CD8α^+ DCs than by CD8α^- DCs was seen as early as 48 h (14% vs. 3.17% before cell division, 2.97% vs. 1.55% during first division). At later time points (48 h-96 h), both DC subsets were able to drive cell division at similar rates, as shown by the similar CFSE peak profile (Fig. 2 *B* and *C*), but the CD8α^+ DCs continued to induce a higher frequency of Foxp3$^+$ T cells than CD8α^- DCs (Fig. 2*D*). The converted Foxp3$^+$ cells divided at similar rate as Foxp3$^-$ cells, as judged by their overlapping CFSE dilution profiles (Fig. 2 *B* and *C*). Thus, splenic DCs, especially the CD8α^+ DCs, cannot only efficiently drive the conversion of naïve CD4$^+$ T cells to Foxp3$^+$ aTregs but also induce their efficient expansion.

To confirm and compare the suppressive function of induced Foxp3$^+$ CD4$^+$ T cells from DC subsets coculture, we performed *in vitro* suppression assays by using induced Foxp3$^+$ OTII cells that were sorted based on Foxp3GFP expression. We routinely obtained ≥95% Foxp3$^+$ purity after sorting. CFSE-labeled, congenically mismatched naïve OTII CD4 T cells were used as responder T cells and were stimulated with splenic antigen-presenting cell (APCs) and antigenic peptide (supporting information (SI) Fig. S1). At higher suppressor:effector ratios, the proliferative response of naive OTII T cells were equivalently suppressed by induced Foxp3$^+$ OTII cells from both CD8α^+ and CD8α^- DC coculture. This suppression diminished at lower number of Foxp3$^+$ cells.

Studies using an APC-free *in vitro* system have suggested that strong costimulation provided by extensive CD28 signaling inhibits Foxp3 induction (5). Similarly, when *ex vivo* splenic DCs were activated with an agonistic anti-CD40 antibody or the TLR4 ligand LPS, Foxp3 induction was diminished [i.e., from 11.10% (±1.00) to 6.95% (±0.74) and 4.15% (±0.35), respectively, with the CD8α^+ DC culture] (Fig. 3*A*). Interestingly, a synergistic effect can be seen between anti-CD40 and LPS, both of which were thought to independently induce DC maturation and up-regulate costimulatory molecules. On the other hand, the neutralizing antibody against CD40 ligand (CD154) enhanced Foxp3 expression significantly, indicating that certain degree of DC maturation occurred, presumably through the interaction with CD154 expressed on activated T cells.

To further determine how DC maturation and costimulation during *in vitro* culture could regulate the induction of Foxp3 expression in T cells, we examined DC subsets from CD40$^{-/-}$ mice and CD80/86$^{-/-}$ mice (Fig. 3 *B* and *C*). Consistent with the inhibitory role of costimulation on conversion, both CD40$^{-/-}$ and CD80/86$^{-/-}$ DCs induced greater frequencies of Foxp3$^+$ T cells than WT DCs. However, the CD8α^+ and

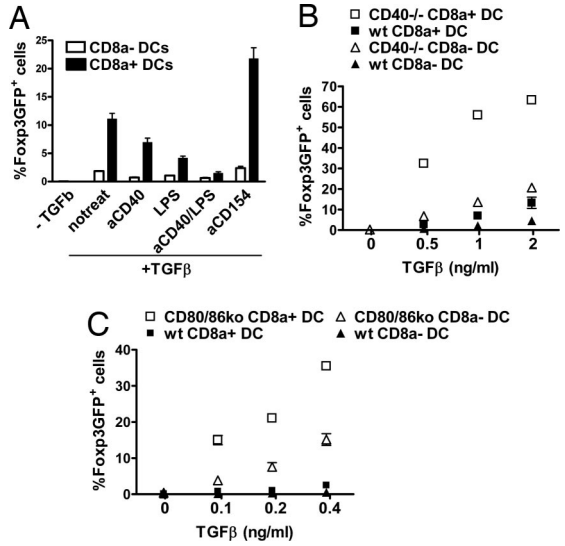

Fig. 3. Conversion is regulated by DC maturation status. (*A*) Conversion cultures with naïve OTII CD4 T cells ($CD25^{-}Foxp3GFP^{-}$) and splenic DC subsets were set up as before in the presence of TGF-β (2 ng/ml) and IL-2 (50 units/ml). Antibodies (αCD40 or αCD154, 5 ng/ml) or LPS (5 ng/ml) were added as indicated. (*B* and *C*) DC subsets were purified from $CD40^{-/-}$ mice (*B*) or $CD80/86^{-/-}$ mice (*C*) and used in the conversion assay. The percentage of $Foxp3^{+}$ cells was analyzed on day 5 and plotted. All conditions were performed in duplicate wells and reported as means ± SEM. Shown are representative results of three independent experiments.

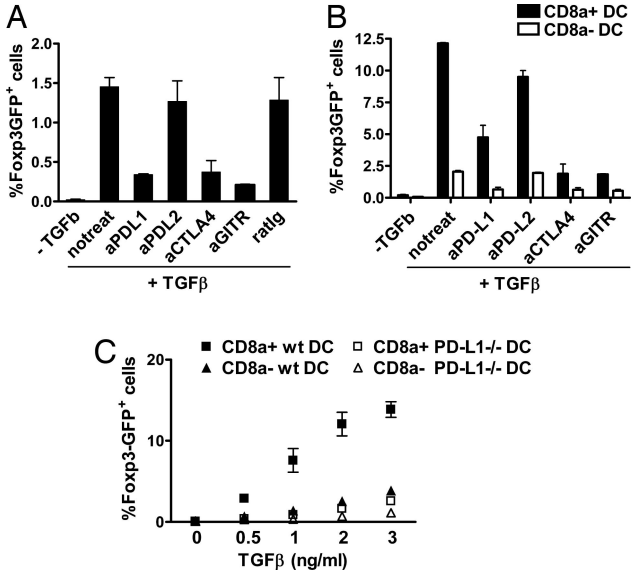

Fig. 4. Multiple costimulatory and coinhibitory pathways regulate conversion *in vitro*. (*A*) Unfractionated splenic DCs were cultured with naive OTII CD4 T cells ($CD25^{-}Foxp3GFP^{-}$) in the presence of TGF-β (2 ng/ml). Antibodies (5 ng/ml) against PD-L1, PD-L2, CTLA-4, GITR, or control rat Ig were added in the beginning of the culture. (*B*) Conversion cultures were set up as in *A* but with the use of purified $CD8\alpha^{+}$ and $CD8\alpha^{-}$ DC subsets. (*C*) PD-L1 expression on DCs is required for the induction of Foxp3. WT or $PD\text{-}L1^{-/-}$ splenic DC subsets were used as APCs in conversion cultures. The percentages of $Foxp3^{+}$ cells were analyzed on day 5 and plotted. All conditions were performed in duplicate wells and reported as means ± SEM. Shown are representative results of three to five independent experiments.

$CD8\alpha^{-}$ DC subsets maintained their differential capacity to induce conversion.

Multiple Coinhibitory Pathways Regulate DC-Mediated Foxp3 Induction in Naïve $CD4^{+}$ T Cells *in Vitro*. To gain additional insights into the molecular regulation of DC-mediated aTreg differentiation, we evaluated the roles of multiple costimulatory and coinhibitory molecules. These include B7/CD28 superfamily members CTLA-4 and programmed cell death (PD)-1, as well as the TNF/TNFR superfamily member glucocorticoid-induced TNF receptor (GITR).

The functional involvement of these costimulatory molecules was studied by using blocking antibodies to ligands or agonistic antibodies to receptors. Both PD-L1 and PD-L2 are ligands for PD-1 (19, 20). When unfractionated splenic DCs were used as APCs, blocking antibodies against both CTLA-4 and PD-L1 inhibited Foxp3 induction (from 1.46 ± 0.11% to 0.37 ± 0.14% and 0.34 ± 0.01%, respectively), whereas αPD-L2 antibody was without effect (1.27 ± 0.26%) (Fig. 4*A*). The agonistic GITR antibody also abolished conversion (0.21 ± 0.01%). Similar results were obtained when purified $CD8\alpha^{+}$ and $CD8\alpha^{-}$ DC subsets were used (Fig. 4*B*). These data confirmed previous studies regarding the role of B7/CTLA-4 axis in conversion (6, 14) but also indicated the involvement of additional molecules, namely PD-L1-mediated coinhibitory signals and GITR-mediated costimulatory signals in Foxp3 induction. Antibodies against other TNF/TNFR family receptor ligands (i.e., 4-1BB and CD30) were also tested. but were without any effect (unpublished data).

PD-L1 is broadly expressed on many cell types (19), as well as on both $CD8\alpha^{+}$ and $CD8\alpha^{-}$ DC subsets (ref. 21 and data not shown). Unlike the $PD\text{-}1^{-/-}$ mice, the $PD\text{-}L1^{-/-}$ mice did not develop overt spontaneous autoimmune diseases, except a phenotype of compromised fetal–maternal tolerance (22, 23). In addition, no intrinsic defect in DC maturation or function has been found in the absence of PD-L1 (24). We also analyzed DC maturation/activation status from the knockout mice but did not find significant changes in the expression of CD80, CD86, class II MHC, or CD40 when compared to age-matched WT mice (data not shown). To confirm that PD-L1 expression on DCs but not on activated CD4 T cells is required for conversion, we isolated $PD\text{-}L1^{-/-}$ DCs and examined their ability to induce conversion *in vitro*. Consistent with the results from antibody treatment, $PD\text{-}L1^{-/-}$ DC subsets were severely impaired in their ability to induce conversion in the presence of TGF-β (Fig. 4*C*). These data suggest that PD-L1-mediated coinhibitory signals are critical for the induction of $Foxp3^{+}$ aTregs.

PD-L1 Signaling Is Required for Tumor-Induced Conversion in the Periphery. Given the pronounced involvement of PD-L1 in aTreg conversion *in vitro*, its role *in vivo* was addressed. We have established a tumor system to examine the molecular and cellular mechanisms of tumor-induced conversion. We used a B16 melanoma tumor line that over-expresses chicken OVA as a surrogate tumor antigen. Naive $Foxp3^{-}$ OTII CD4 T cells were isolated from the Foxp3GFP reporter mice and adoptively transferred into lightly irradiated, tumor-bearing mice. Phenotypes of these cells in the tumor draining and contralateral nondraining lymph node (dLN and ndLN), spleen, and within the tumor infiltrating population (TILs) were analyzed over time. When analyzed ≈3 weeks after tumor challenge, transferred OTII CD4 cells were detected mostly in the tumor dLN, among which ≈5–10% (8.09 ± 1.36%, $n = 12$) converted into $Foxp3^{+}$ cells (Fig. 5 *A* and *B*). Similar percentage of conversion was also detected in the spleen (6.93 ± 0.96%, $n = 12$). Interestingly, significantly higher percentages of conversion (68.65 ± 5.59%, $n = 11$) were found within the TILs. This indicated that the tumor microenvironment provided a favorable milieu that enhanced conversion. Very few OTII cells (typically <0.001% of gated total CD4 T cells) were detected

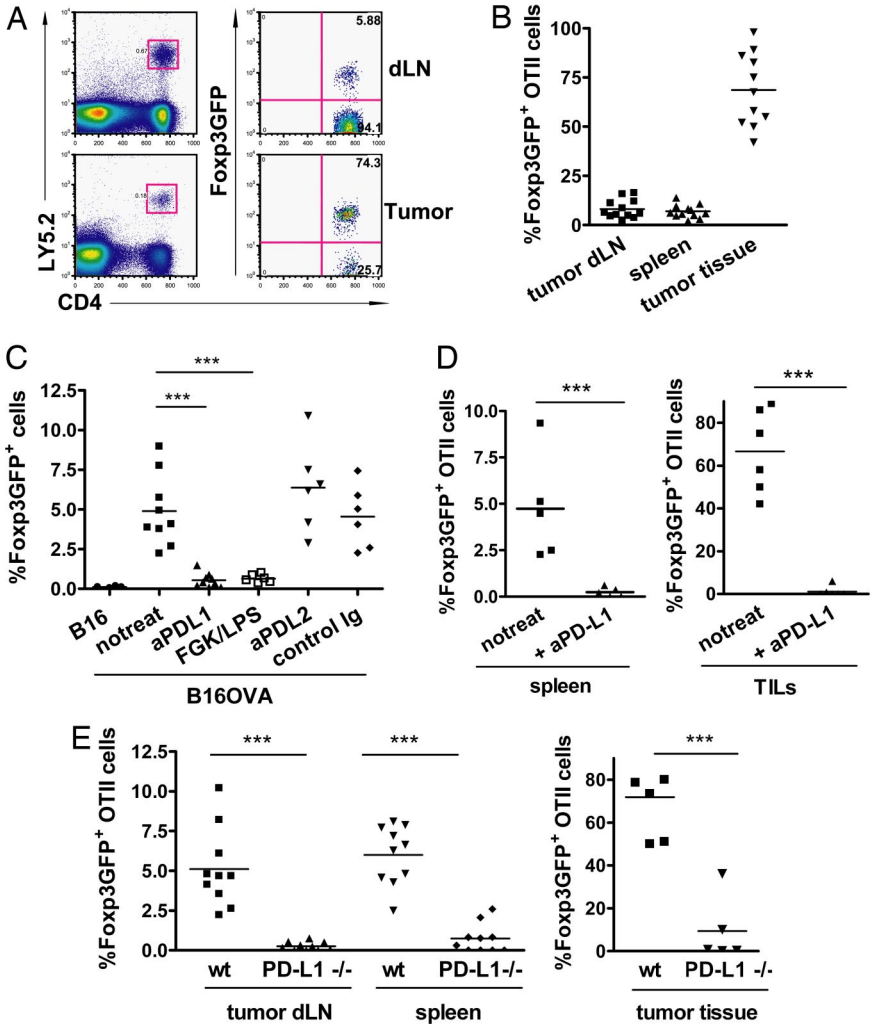

Fig. 5. Tumor-induced conversion depends upon PD-L1 signaling. B16OVA tumor cells (200,000) were inoculated on the right flank of irradiated mice. Naïve OTII CD4$^+$ T cells (1×10^6) were adoptively transferred next day. Mice were analyzed when tumors reached ≥100 mm^2. Cells from tumor dLN, spleen, and tumor site were analyzed for Foxp3GFP expression among transferred OTII cells. Representative FACS plots for detecting OTII CD4$^+$ T cells from tumor dLN and tumor tissues were illustrated in *A*. Percentages of conversion were summarized in *B*. The data were combined from four experiments. The average conversion efficiency (mean ± SEM) in tumor dLN was 8.09 ± 1.36% ($n = 12$); in spleen, 6.93 ± 0.96% ($n = 12$); and within tumor tissues, 68.65 ± 5.59% ($n = 11$). (*C*) Conversion of OTII CD4$^+$ T cells in tumor dLN was inhibited by αCD40/LPS (0.64 ± 0.10%, $n = 6$) and αPD-L1 treatment (0.53 ± 0.15%, $n = 9$) but not by αPD-L2 treatment (6.37 ± 1.13%, $n = 6$). The conversion in the control tumor B16 group was 0.11 ± 0.03% ($n = 4$); in the no-treatment group, 4.90 ± 0.75% ($n = 9$); and in the control rat Ig-treated group, 4.55 ± 0.81% ($n = 6$). (*D*) The conversion in the spleen and TILs was blocked by αPD-L1 treatment. The conversion in the spleen ($n = 5$) was 4.74 ± 1.27% (notreat) and 0.23 ± 0.11% (αPD-L1-treated); and in TILs ($n = 6$), 66.67 ± 7.96% (notreat) and 1.12 ± 0.98% (αPD-L1 treated). (*E*) Conversion in the PD-L1$^{-/-}$ mice was significantly reduced. The conversion in the tumor dLN was 5.123 ± 0.78% (WT, $n = 10$) and 0.2585 ± 0.083% (KO, $n = 10$); in the spleen, 6.01 ± 0.59% (WT, $n = 10$) and 0.74 ± 0.29% (KO, $n = 10$); and at the tumor site, 71.83 ± 7.551% (WT, $n = 6$) and 9.414 ± 6.905% (KO, $n = 5$). Unpaired Student's *t* tests was performed to obtain a *P* value. ***, $P \leq 0.001$.

in the tumor ndLN (unpublished data). Conversion required the expression of OVA antigen, because control tumors that did not express OVA failed to induce conversion (Fig. 5*C*).

CD8α^+ and CD8α^- DC subsets were also isolated from tumor-bearing mice and tested in the *in vitro* conversion assay (data not shown). Similar level of conversion was shown when comparing naïve and tumor DC cultures. Thus tumor development did not abolish the differential ability of DC subsets to induce Foxp3.

Surface phenotypes of converted cells were examined (Fig. S2). Converted Foxp3$^+$ cells had a bimodal expression pattern of CD25 and CD62L and expressed higher level of GITR than their Foxp3$^-$ counterparts. Relatively more converted aTregs within tumor tissues were CD25^{high} and CD62L^{low}, indicating a more effector cell phenotype than naïve phenotype at the tissue sites.

Next, we sought to determine the molecular mechanisms in tumor-induced conversion. First, we treated tumor-bearing mice with αCD40/LPS, which induced robust DC maturation. Conversion at tumor dLN was abolished (0.64 ± 0.10%, $n = 6$) (Fig. 5*C*). These data are in agreement with the *in vitro* results that DC maturation abolishes their ability to support conversion; the data are also consistent with the previous report in which conversion induced by antigen targeting via DEC205 antibody is inhibited by αCD40 treatment (13). The combined treatment of αCD40 and TLR agonist has been shown previously to induce potent antitumor immune response in a B16 melanoma lung metastasis model (25). Under the s.c. B16 model, however, such treatment only marginally slowed down but did not prevent tumor growth (data not shown).

The role of PD-L1 in tumor-mediated conversion was also evaluated. By using the neutralizing antibody to block PD-1 signaling in tumor-bearing mice, we found that αPD-L1 sig-

nificantly delayed tumor growth, which is consistent with previous studies showing the inhibitory role of PD-L1 in tumor immunity (26–28) (Fig. S3). This inhibitory effect could also be reversed by adoptive transfer of *in vitro* generated OTII aTregs. To exclude the effect of tumor size on conversion, we chose the antibody dose that allowed tumor to grow to the comparable size as untreated control group and analyzed conversion when tumors reached ≥100 mm^2. Conversion was blocked in the tumor dLN upon αPD-L1 (0.53 ± 0.15%, n = 9) but not αPD-L2 (6.37 ± 1.13%, n = 6) treatment (Fig. 5*C*). Similar reduction was seen in the spleen and TILs (Fig. 5*D*). Consistent with the antibody blocking data, conversion was significantly inhibited in PD-L1$^{-/-}$ mice (Fig. 5*E*).

In conclusion, we have examined the cellular and molecular mechanisms that regulate the *de novo* induction of Foxp3$^+$ aTregs from naïve CD4$^+$ T cells. We discovered the superior ability of splenic CD8α$^+$ DCs to differentiate Foxp3$^+$ aTregs. DC-induced conversion requires TGF-β and the PD-L1 signaling pathway and is regulated by DC maturation status.

Discussion

Although mature DCs are potent antigen presenting cells that initiate primary immune responses, steady-state lymphoid tissue DCs contribute to the peripheral tolerance (29). Recently, it has been reported that splenic DCs are capable of differentiating Foxp3$^+$ aTregs from Foxp3$^-$ precursors, along with TGF-β signaling (18). However, the contribution of DC subsets, namely the CD8α$^+$ and CD8α$^-$ DCs, has not been determined. Resting splenic CD8α$^+$ DCs tolerize self-reactive CD8 T cells via continuous cross-presentation in the absence of inflammation (30, 31). On the other hand, antigen targeting to this DC subset by using DEC205 antibody resulted in the antigen-specific CD4 T cell tolerance, manifested as deletion and anergy, as well as induction of Foxp3$^+$ aTregs (13, 32, 33). Because antigen targeting did not address whether CD8α$^+$ DCs are not only sufficient but also necessary for the induction of Foxp3$^+$ aTregs, we analyzed both CD8α$^+$ and CD8α$^-$ DC subsets *ex vivo* for their capacity to induce Foxp3 expression. Our study has demonstrated that CD8α$^+$ DCs are superior to the CD8α$^-$ DCs for inducing Foxp3 in the presence of TGF-β, whereas CD8α$^-$ DCs not only are poor inducers for Foxp3 but dominantly inhibit conversion when present together with the CD8α$^+$ DCs.

Further analysis on the molecular determinants for DC-induced conversion revealed the critical role of PD-L1 signaling. Because it has been shown that the major coinhibitory CTLA-4/B7 axis is required for conversion (6, 14), it is surprising to see another coinhibitory pathway playing a nonredundant role. Our result, however, is consistent with the role of its receptor PD-1 in peripheral tolerance (34). It has been established that PD-L1 signaling negatively regulate T cell response (35) plays essential roles in peripheral tolerance (22, 24, 40) and tumor-mediated immune suppression (26). We have now extended the mechanisms of how PD-L1 contributes to peripheral tolerance. By using the *in vitro* culture system, we provide evidence that signaling through PD-L1 expressed on DCs is required for the induction of Foxp3$^+$ aTregs. Furthermore, by using a tumor-induced *in vivo* conversion system, we confirmed the requirement of PD-L1 on conversion of adoptively transferred naïve CD4 T cells. Because PD-L1 is expressed by many cell types in addition to DCs, further studies are needed to determine whether DCs are the critical antigen presenting cells that are required for tumor-induced conversion and whether non-DC expressing PD-L1 also contributes to this process.

In addition to PD-1, PD-L1 also binds B7-1 (36). The specific contribution of both receptors in PD-L1-mediated conversion remains to be determined. Although overlapping roles for PD-L1 and PD-L2 in limiting CD4 T cell activation have been indicated (24), we did not find the involvement of PD-L2 in conversion process.

DC maturation leads to IL-6 secretion, which inhibits TGF-β-induced Foxp3 expression (37). In addition, Th1/2 cytokines IL-4 and IFN-γ inhibit conversion (38). We, thus, examined the potential involvement of these cytokines in DC-mediated Foxp3 induction. Similar to the WT DCs, IL-6$^{-/-}$ CD8α$^+$ DCs are superior inducers for Foxp3 than IL-6$^{-/-}$ CD8α$^-$ DCs (Fig. S4*A*). On the other hand, neutralizing antibodies of IL-4 and IFNr enhanced conversion of both DC subsets, either from WT or PD-L1$^{-/-}$ background (Fig. S4*B*). Thus, IL-6 and Th1/2 cytokines do not appear to be the effector molecules that account for the differential induction of Foxp3 by DC subsets.

Previous studies by using a lymphoma model (15) and a colon cancer model (16) have implicated natural and/or adaptive Tregs in tumor-mediated immune suppression. Our results now have clearly implicated the role of PD-L1 in the differentiation of aTregs. New strategies, thus, are emerging to allow the selective manipulation of aTreg development *in vivo*. As such, this study should provide useful insights for understanding tumor-mediated immune evasion and provide strategies to enhance anti-tumor immunity.

Materials and Methods

Mice. WT or CD40$^{-/-}$, CD80/86$^{-/-}$, and IL-6$^{-/-}$ C57BL/6 mice were purchased from The Jackson Laboratory. Foxp3GFP reporter mice were previously described (2) and were provided by Alexander Rudensky (University of Washington School of Medicine, Seattle, WA). Foxp3GFP mice were bred onto OTII CD4-Tg mice specific for chicken OVA peptide 323–339. PD-L1$^{-/-}$ mice were as described (22). All animals were maintained in a pathogen-free facility at Dartmouth Medical School and were used between 6–8 weeks of age.

Abs and Reagents. Antibodies αCD40 (FGK-45), αCD154 (MR1), αCD28 (PV-1), and αCTLA-4 (UC10-4F10-11) were purchased from Bioexpress. αPD-L1 (MIH6) mAb was generously provided by Miyuki Azuma (Tokyo Medical and Dental University, Tokyo, Japan) (39). αPD-L2 (TY25) antibody was generously provided by Mohamed H. Sayegh (Harvard Medical School). LPS (Sigma), recombinant human TGF-β1 (R&D Systems), and human IL-2 (Peprotech) were used at indicated concentrations.

Flow Cytometry. Flow cytometric analysis was performed on FACScan by using CellQuest software (BD Bioscience). Data analysis was performed by using FlowJo software (Treestar).

Cell Preparation. Total CD4 T cells were isolated from OT-II TCR-Tg transgenic mice bred onto the Foxp3GFP reporter background, following instructions in the CD4 T cell isolation kit (Miltenyi). Naïve OTII CD4$^+$ T cells were obtained by FACS sorting gated on Vβ5^{high}CD25$^-$FoxP3$^-$ (BD FACSAria). Purity typically exceeded 95%. In some cases, naïve OT-II CD4 T cells were sorted from nonreporter background by gating on Vβ5^{high}CD25$^-$CD62L^{high}, labeled with 5 μM CFSE (Molecular Probes) for 10 min at 37°C, washed twice before being used *in vitro*. Spleen DCs were purified from digested spleens [50 μg/ml DNase I (Sigma) and 250 μg/ml Liberase (Roche) at 37° for 30 min] by negative enrichment with CD19 microbeads (Miltenyi) and anti-biotin microbeads bound with biotin-conjugated TCR antibody. CD8α$^+$ and CD8α$^-$ CD11c^{high} conventional DC subsets were obtained by FACS sorting based on CD8α expression.

***In Vitro* Conversion Assay.** A total of 50,000 naïve OT-II CD4 T cells were cultured in 96-well plates with 30,000 purified splenic DCs. Replicate cultures were in RPMI medium 1640 supplemented with 10% FBS, 10 mM Hepes, 50 μM 2-mercaptoethanol, penicillin/streptomycin/L-glutamine, 50 units/ml human IL-2 (PeproTech), 250 ng/ml synthetic OVA$_{323–339}$ peptide (Anaspec), and the indicated concentration of human recombinant TGF-β1 (R&D Systems). Antibodies against CTLA-4, PD-L1, PD-L2, GITR, CD40, and CD154 were added at 5 ng/ml when indicated. Cultures were analyzed on day 5 or according to a time course.

Tumor-Induced Conversion. On day 0, mice were irradiated with 280 rad before tumor inoculation and cell transfer. B16 or B16OVA tumor cells (200,000) were resuspended in HBSS and inoculated s.c. on the right flank. Sorted congenically marked naïve OTII CD4 T cells (1 × 10^6) were adoptively

transferred intravenously on the same day. Tumor growth was monitored every 3–4 days. Mice were killed when tumors reached 100–150 mm^2. To examine conversion, single-cell suspensions from tumor dLN (the inguinal LN on the right flank), contralateral ndLN (the inguinal LN from left flank), and spleen were obtained by mechanical dissociation and surface stained for congenic marker and CD4. Live cell dye 7AAD (eBioscience) was used to exclude dead cells that gave autofluorescence. To examine the tumor infiltrating cells, single-cell suspensions of tumors were fractionated on a 40/80% Percoll gradient (420 × *g* for 20 min). Infiltrating lymphocytes were collected from the gradient interface and analyzed by flow cytometry. To block conversion, intraperitoneal injection of αCD40 (100 μg) and LPS (50 μg) 1 day after tumor inoculation, or 100 μg antibodies (αPD-L1 or αPD-L2 or control Ig) every other day was administered until tumors reached ≥100 mm^2.

Statistical Analysis. Means ± SEM are shown. For comparison of groups, the two-tailed Student's *t* test was performed, and $P \leq 0.05$ was considered significant (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$).

SI. Fig. S1 shows the suppressive function of DC-induced Foxp3$^+$ OTII aTregs. Fig. S2 depicts surface expression of CD25, CD62L, and GITR on converted Foxp3$^+$ OTII CD4$^+$ T cells in B16OVA tumor-bearing mice. Fig. S3 shows the inhibitory effect of PD-L1 antibody on tumor growth. Fig. S4 shows the roles of IL6 and Th1/2 cytokines in conversion.

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