# Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3<sup>+</sup>CD4<sup>+</sup> regulatory T cells

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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<sup>+</sup> T cells to adaptive Foxp3<sup>+</sup>CD4<sup>+</sup> regulatory T cells (aTreg) in the presence of TGF- $\beta$ . In particular, when compared with splenic CD8 $\alpha^-$  DCs, the CD8 $\alpha^+$  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<sup>-/-</sup> 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

**N** aturally occurring CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> 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<sup>+</sup> regulatory T cells (aTregs). *In vitro* conversion occurs in the presence of TGF- $\beta$  (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<sup>+</sup> aTregs from naïve CD4<sup>+</sup> T cells occurs upon subimmunogenic antigen stimulation (10–13). Consistent with *in vitro* studies, TGF- $\beta$  signaling and B7 costimulation are required for peripheral conversion (13, 14).

The Foxp3GFP reporter mice allow the isolation of naive CD4<sup>+</sup>Foxp3GFP<sup>-</sup>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<sup>+</sup> 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<sup>+</sup>Foxp3<sup>+</sup> 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<sup>+</sup> aTregs. To this end, we examined the capacity of ex vivo splenic DC subsets to induce Foxp3 expression in the presence of TGF- $\beta$ . Our results show that among the splenic DC subsets, the  $CD8\alpha^+$  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 tumorinduced immune tolerance.

#### Results

Ex Vivo Splenic CD8 $\alpha^+$  DCs Are Superior to CD8 $\alpha^-$  DCs for the Induction of Antigen-Specific Foxp3<sup>+</sup> Adaptive Tregs. Although it has recently been reported that splenic DCs as a whole population can differentiate Foxp3<sup>+</sup> aTregs in the presence of TGF- $\beta$ 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  $CD8\alpha^+$  or  $CD8\alpha^-$  CD11c<sup>high</sup> DCs, were tested for their capacity to induce Foxp3 expression in naïve CD4<sup>+</sup> T cells *in vitro*. OTII TCR transgenic mice were bred onto the Foxp3GFP knockin mice (2). Naïve OTII CD4<sup>+</sup>Foxp3GFP<sup>-</sup> 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<sup>+</sup> cells.

After *in vitro* culture for 5 days with either the  $CD8\alpha^+$  or  $CD8\alpha^-$  CD11c<sup>high</sup> splenic DCs in the presence of antigenic ovalbumin (OVA) peptide and TGF- $\beta$ , the induction of Foxp3 in OTII CD4<sup>+</sup> T cells was measured by GFP expression by using flow cytometry (Fig. 1*A*). When compared to CD8 $\alpha^-$  DCs, CD8 $\alpha^+$  DCs were superior inducers of Foxp3 expression in the presence of TGF- $\beta$  (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- $\beta$ . To compare

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**Fig. 1.** *Ex vivo* splenic CD8 $\alpha^+$  DCs induce Foxp3 expression more efficiently than CD8 $\alpha^-$  DCs in the presence of TGF- $\beta$ . Freshly isolated splenic DC subsets (CD8 $\alpha^+$  CD11c<sup>high</sup> and CD8 $\alpha^-$  CD11c<sup>high</sup>) were cocultured with naïve OTII CD4+ T cells (CD25<sup>-</sup>Foxp3GFP<sup>-</sup>) in the presence of OVA<sub>323-339</sub> peptide (500 ng/ml) and increasing amounts of TGF- $\beta$ 1. Foxp3GFP expression was analyzed on day 5 by flow cytometry. The total cell number was counted, and the number of Foxp3<sup>+</sup> 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<sup>+</sup> cells per well after 5 day culture. (*D*) CD8 $\alpha^-$  DCs were mixed with CD8 $\alpha^+$  DCs at the indicated ratios to stimulate OTII CD4 T cells in the presence of TGF- $\beta$  (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.

the ability for each DC subset to induce clonal expansion, the number of OTII cells was quantified at the end of the assay. Although CD8 $\alpha^{-}$  DCs induced modestly better proliferation of total CD4<sup>+</sup> T cells (unpublished data), the number of induced Foxp3<sup>+</sup> cells was still greater with the  $CD8\alpha^+$  DC culture (Fig. 1C). Because only 20-25% splenic DCs are  $CD8\alpha^+$ , we evaluated the impact of  $CD8\alpha^-$  DCs on conversion when mixed with  $CD8\alpha^+$  DCs at different ratios (Fig. 1D). At a given TGF- $\beta$  concentration (2 ng/ml), conversion induced by  $CD8\alpha^+$  DCs was 16.45% (±1.65), whereas by  $CD8\alpha^-$  DCs was 4.25% (±0.13). The presence of CD8 $\alpha^{-}$  DCs, even as low as 12.5% (7:1 ratio of  $CD8\alpha^+/CD8\alpha^-$  DCs), reduced conversion significantly to 10.7% (±0.10). Increasing the amount of  $CD8\alpha^{-}$  DCs continued to reduce conversion. Therefore,  $CD8\alpha^{-}$  DCs actively interfered with conversion, and their effect was dominant when present at physiological percentages ( $\approx$ 1:4 ratio of CD8 $\alpha^+$ /CD8 $\alpha^-$  DCs).

To assess the kinetics of Foxp3 induction with cell cycle progression, we labeled OTII CD4<sup>+</sup> 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 CD8 $\alpha^+$  DCs than by CD8 $\alpha^-$  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 CD8 $\alpha^+$  DCs continued to induce a higher frequency of Foxp3<sup>+</sup> T cells than CD8 $\alpha^-$  DCs (Fig. 2*D*). The converted Foxp3<sup>+</sup> cells divided at similar rate as Foxp3<sup>-</sup> cells, as judged by their overlapping CFSE dilution profiles (Fig. 2 *B* and *C*). Thus, splenic DCs, especially the CD8 $\alpha^+$  DCs, cannot only efficiently drive the conversion of



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

naïve  $CD4^+$  T cells to Foxp3<sup>+</sup> aTregs but also induce their efficient expansion.

To confirm and compare the suppressive function of induced Foxp3<sup>+</sup> CD4<sup>+</sup> T cells from DC subsets coculture, we performed *in vitro* suppression assays by using induced Foxp3<sup>+</sup> OTII cells that were sorted based on Foxp3GFP expression. We routinely obtained  $\geq$ 95% Foxp3<sup>+</sup> purity after sorting. CFSE-labeled, congenically mismatched naïve OTII CD4 T cells were used as responder T cells and were stimulated with splenic antigenpresenting 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<sup>+</sup> OTII cells from both CD8 $\alpha^+$  and CD8 $\alpha^-$  DC coculture. This suppression diminished at lower number of Foxp3<sup>+</sup> 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% ( $\pm$ 1.00) to 6.95% ( $\pm$ 0.74) and 4.15% ( $\pm$ 0.35), respectively, with the CD8 $\alpha^+$  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<sup>+</sup> T cells than WT DCs. However, the  $CD8\alpha^+$  and



**Fig. 3.** Conversion is regulated by DC maturation status. (A) Conversion cultures with naïve OTII CD4 T cells (CD25<sup>-</sup>Foxp3GFP<sup>-</sup>) and splenic DC subsets were set up as before in the presence of TGF- $\beta$  (2 ng/ml) and IL-2 (50 units/ml). Antibodies ( $\alpha$ CD40 or  $\alpha$ CD154, 5 ng/ml) or LPS (5 ng/ml) were added as indicated. (B and C) DC subsets were purified from CD40<sup>-/-</sup> mice (B) or CD80/86<sup>-/-</sup> mice (C) and used in the conversion assay. The percentage of Foxp3<sup>+</sup> 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.

 $CD8\alpha^{-}$  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  $1.46 \pm 0.11\%$  to  $0.37 \pm 0.14\%$  and  $0.34 \pm 0.01\%$ , respectively), whereas  $\alpha$ PD-L2 antibody was without effect (1.27  $\pm$  0.26%) (Fig. 4A). The agonistic GITR antibody also abolished conversion  $(0.21 \pm 0.01\%)$ . Similar results were obtained when purified  $CD8\alpha^+$  and  $CD8\alpha^-$  DC subsets were used (Fig. 4B). 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-1<sup>-/-</sup> mice, the PD-L1<sup>-/-</sup> 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



**Fig. 4.** Multiple costimulatory and coinhibitory pathways regulate conversion *in vitro*. (*A*) Unfractionated splenic DCs were cultured with naive OTII CD4 T cells (CD25<sup>-</sup>Foxp3GFP<sup>-</sup>) in the presence of TGF- $\beta$  (2 ng/ml). Antibodies (5 ng/ml) against PD-L1, PD-L2, CTLA-4, GITR, or control rat lg 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-L1<sup>-/-</sup> splenic DC subsets were used as APCs in conversion cultures. The percentages of Foxp3<sup>+</sup> 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.

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-L1<sup>-/-</sup> DCs and examined their ability to induce conversion *in vitro*. Consistent with the results from antibody treatment, PD-L1<sup>-/-</sup> DC subsets were severely impaired in their ability to induce conversion in the presence of TGF- $\beta$  (Fig. 4C). These data suggest that PD-L1-mediated coinhibitory signals are critical for the induction of Foxp3<sup>+</sup> 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<sup>-</sup> 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  $\approx 3$  weeks after tumor challenge, transferred OTII CD4 cells were detected mostly in the tumor dLN, among which  $\approx 5-10\%$  (8.09  $\pm 1.36\%$ , n = 12) converted into  $Foxp3^+$  cells (Fig. 5 A and B). Similar percentage of conversion was also detected in the spleen (6.93  $\pm$  0.96%, n =12). Interestingly, significantly higher percentages of conversion (68.65  $\pm$  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<sup>+</sup> T cells (1 × 10<sup>6</sup>) were adoptively transferred next day. Mice were analyzed when tumors reached  $\geq$ 100 mm<sup>2</sup>. Cells from tumor dLN, spleen, and tumor site were analyzed for Foxp3GFP expression among transferred OTII cells. Representative FACS plots for detecting OTII CD4<sup>+</sup> 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  $\pm$  SEM) in tumor dLN was 8.09  $\pm$  1.36% (*n* = 12); in spleen, 6.93  $\pm$  0.96% (*n* = 12); and within tumor tissues, 68.65  $\pm$  5.59% (*n* = 11). (*C*) Conversion of OTII CD4<sup>+</sup> T cells in tumor dLN was inhibited by  $\alpha$ CD40/LPS (0.64  $\pm$  0.10%, *n* = 6) and  $\alpha$ PD-L1 treatment (0.53  $\pm$  0.15%, *n* = 9) but not by  $\alpha$ PD-L2 treatment (6.37  $\pm$  1.13%, *n* = 6). The conversion in the control tumor B16 group was 0.11  $\pm$  0.03% (*n* = 4); in the no-treatment group, 4.90  $\pm$  0.75% (*n* = 9); and in the control rat lg-treated group, 4.55  $\pm$  0.81% (*n* = 6). (*D*) The conversion in the spleen and TILs was blocked by  $\alpha$ PD-L1 treatment. The conversion in the spleen (*n* = 5) was 4.74  $\pm$  1.27% (notreat) and 0.23  $\pm$  0.11% ( $\alpha$ PD-L1-treated); and in TILs (*n* = 6), 66.67  $\pm$  7.96% (WT, *n* = 10) and 0.2585  $\pm$  0.083% (KO, *n* = 10); in the spleen, 6.01  $\pm$  0.59% (WT, *n* = 10) and 0.74  $\pm$  0.29% (KO, *n* = 10); and at the tumor site, 71.83  $\pm$  7.551% (WT, *n* = 6) and 9.414  $\pm$  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*).

 $CD8\alpha^+$  and  $CD8\alpha^-$  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<sup>+</sup> cells had a bimodal expression pattern of CD25 and CD62L and expressed higher level of GITR than their Foxp3<sup>-</sup> counterparts. Relatively more converted aTregs within tumor tissues were CD25<sup>high</sup> and CD62L<sup>low</sup>, indicating a more effector cell phenotype than naïve phenotype at the tissue sites.

Next, we sought to determine the molecular mechanisms in

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tumor-induced conversion. First, we treated tumor-bearing mice with  $\alpha$ CD40/LPS, which induced robust DC maturation. Conversion at tumor dLN was abolished (0.64 ± 0.10%, n = 6) (Fig. 5C). 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  $\alpha$ CD40 treatment (13). The combined treatment of  $\alpha$ CD40 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  $\alpha$ PD-L1 significantly 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  $\geq 100 \text{ mm}^2$ . Conversion was blocked in the tumor dLN upon  $\alpha$ PD-L1 (0.53  $\pm$  0.15%, n =9) but not  $\alpha$ PD-L2 (6.37  $\pm$  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<sup>-/-</sup> mice (Fig. 5*E*).

In conclusion, we have examined the cellular and molecular mechanisms that regulate the *de novo* induction of Foxp3<sup>+</sup> aTregs from naïve CD4<sup>+</sup> T cells. We discovered the superior ability of splenic CD8 $\alpha^+$  DCs to differentiate Foxp3<sup>+</sup> aTregs. DC-induced conversion requires TGF- $\beta$  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<sup>+</sup> aTregs from Foxp3<sup>-</sup> precursors, along with TGF- $\beta$  signaling (18). However, the contribution of DC subsets, namely the  $CD8\alpha^+$  and  $CD8\alpha^-$  DCs, has not been determined. Resting splenic CD8 $\alpha^+$  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  $CD8\alpha^+$ DCs are not only sufficient but also necessary for the induction of Foxp3<sup>+</sup> aTregs, we analyzed both  $CD8\alpha^+$  and  $CD8\alpha^-$  DC subsets *ex vivo* for their capacity to induce Foxp3 expression. Our study has demonstrated that  $CD8\alpha^+$  DCs are superior to the CD8 $\alpha^{-}$  DCs for inducing Foxp3 in the presence of TGF- $\beta$ , whereas CD8 $\alpha^{-}$  DCs not only are poor inducers for Foxp3 but dominantly inhibit conversion when present together with the  $CD8\alpha^+$  DCs.

Further analysis on the molecular determinants for DCinduced 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<sup>+</sup> 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- $\beta$ -induced Foxp3 expression (37). In addition, Th1/2 cytokines IL-4 and IFN- $\gamma$  inhibit conversion (38). We, thus, examined the potential involvement of these cytokines in DC-mediated Foxp3 induction. Similar to the WT DCs, IL-6<sup>-/-</sup> CD8 $\alpha^+$  DCs are superior inducers for Foxp3 than IL-6<sup>-/-</sup> CD8 $\alpha^-$  DCs (Fig. S44). On the other hand, neutralizing antibodies of IL-4 and IFNr enhanced conversion of both DC subsets, either from WT or PD-L1<sup>-/-</sup> background (Fig. S4B). 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<sup>-/-</sup>, CD80/86<sup>-/-</sup>, and IL-6<sup>-/-</sup> 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<sup>-/-</sup> 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  $\alpha$ CD40 (FGK-45),  $\alpha$ CD154 (MR1),  $\alpha$ CD28 (PV-1), and  $\alpha$ CTLA-4 (UC10-4F10-11) were purchased from Bioexpress.  $\alpha$ PD-L1 (MIH6) mAb was generously provided by Miyuki Azuma (Tokyo Medical and Dental University, Tokyo, Japan) (39).  $\alpha$ PD-L2 (TY25) antibody was generously provided by Mohamed H. Sayegh (Harvard Medical School). LPS (Sigma), recombinant human TGF- $\beta$ 1 (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<sup>+</sup> T cells were obtained by FACS sorting gated on V $\beta$ 5<sup>high</sup>CD25<sup>-</sup>FoxP3<sup>-</sup> (BD FACSAria). Purity typically exceeded 95%. In some cases, naïve OT-II CD4 T cells were sorted from nonreporter background by gating on V $\beta$ 5<sup>high</sup>CD25<sup>-</sup>CD62L<sup>high</sup>, labeled with 5  $\mu$ M CFSE (Molecular Probes) for 10 min at 37°C, washed twice before being used *in vitro*. Spleen DCs were purified from digested spleens [50  $\mu$ g/ml DNase I (Sigma) and 250  $\mu$ g/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. CD8 $\alpha^+$  and CD8 $\alpha^-$  CD11 $c^{high}$  conventional DC subsets were obtained by FACS sorting based on CD8 $\alpha$  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  $\mu$ M 2-mercaptoethanol, penicillin/streptomycin/L-glutamine, 50 units/ml human IL-2 (PeproTech), 250 ng/ml synthetic OVA<sub>322-339</sub> peptide (Anaspec), and the indicated concentration of human recombinant TGF- $\beta$ 1 (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 ( $1 \times 10^6$ ) 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<sup>2</sup>. 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 ( $420 \times g$  for 20 min). Infiltrating lymphocytes were collected from the gradient interface and analyzed by flow cytometry. To block conversion, intraperitoneal injection of  $\alpha$ CD40 (100  $\mu$ g) and LPS (50  $\mu$ g) 1 day after tumor inoculation, or 100  $\mu$ g antibodies ( $\alpha$ PD-L1 or  $\alpha$ PD-L2 or control Ig) every other day was administered until tumors reached  $\geq$ 100 mm<sup>2</sup>.

- Sakaguchi S (2004) Naturally arising CD4+ regulatory t cells for immunologic selftolerance and negative control of immune responses. Annu Rev Immunol 22:531–562.
- Fontenot JD, et al. (2005) Regulatory T cell lineage specification by the forkhead transcription factor foxp3. Immunity 22:329–341.
- Chen W, et al. (2003) Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med 198:1875–1886.
- Kim JM, Rudensky A (2006) The role of the transcription factor Foxp3 in the development of regulatory T cells. *Immunol Rev* 212:86–98.
- Benson MJ, Pino-Lagos K, Rosemblatt M, Noelle RJ (2007) All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. J Exp Med 204:1765–1774.
- Zheng SG, et al. (2006) TGF-beta requires CTLA-4 early after T cell activation to induce FoxP3 and generate adaptive CD4+CD25+ regulatory cells. J Immunol 176:3321–3329.
- Horwitz DA, et al. (2004) Regulatory T cells generated ex vivo as an approach for the therapy of autoimmune disease. Semin Immunol 16:135–143.
- 8. Fantini MC, et al. (2006) Transforming growth factor beta induced FoxP3+ regulatory T cells suppress Th1 mediated experimental colitis. *Gut* 55:671–680.
- Ochando JC, et al. (2006) Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. Nat Immunol 7:652–662.
- 10. Cobbold SP, et al. (2004) Induction of foxP3 + regulatory T cells in the periphery of T cell receptor transgenic mice tolerized to transplants. J Immunol 172:6003–6010.
- Karim M, Kingsley CI, Bushell AR, Sawitzki BS, Wood KJ. (2004) Alloantigen-induced CD25+CD4+ regulatory T cells can develop in vivo from CD25-CD4+ precursors in a thymus-independent process. J Immunol 172:923–928.
- Apostolou I, von Boehmer H (2004) In vivo instruction of suppressor commitment in naive T cells. J Exp Med 199:1401–1408.
- Kretschmer K, et al. (2005) Inducing and expanding regulatory T cell populations by foreign antigen. Nat Immunol 6:1219–1227.
- Liang S, et al. (2005) Conversion of CD4+ CD25- cells into CD4+ CD25+ regulatory T cells in vivo requires B7 costimulation, but not the thymus. J Exp Med 201:127–137.
- Zhou G, Drake CG, Levitsky HI (2006) Amplification of tumor-specific regulatory T cells following therapeutic cancer vaccines. *Blood* 107:628–636.
- Valzasina B, Piconese S, Guiducci C, Colombo MP (2006) Tumor-induced expansion of regulatory T cells by conversion of CD4+CD25– lymphocytes is thymus and proliferation independent. *Cancer Res* 66:4488–4495.
- Luo X, et al. (2007) Dendritic cells with TGF-beta1 differentiate naive CD4+CD25-T cells into islet-protective Foxp3+ regulatory T cells. Proc Natl Acad Sci USA 104:2821– 2826.
- Yamazaki S, et al. (2007) Dendritic cells are specialized accessory cells along with TGF-β for the differentiation of Foxp3+ CD4+ regulatory T cells from peripheral Foxp3precursors. Blood 110:4293–4302.
- Freeman GJ, et al. (2000) Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 192:1027–1034.
- Latchman Y, et al. (2001) PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat Immunol 2:261–268.

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

**5I.** Fig. S1 shows the suppressive function of DC-induced Foxp3<sup>+</sup> OTII aTregs. Fig. S2 depicts surface expression of CD25, CD62L, and GITR on converted Foxp3<sup>+</sup> OTII CD4<sup>+</sup> T cells in B160VA 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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- Yamazaki T, et al. (2002) Expression of programmed death 1 ligands by murine T cells and APC. J Immunol 169:5538–5545.
- 22. Guleria I, et al. (2005) A critical role for the programmed death ligand 1 in fetomaternal tolerance. J Exp Med 202:231–237.
- 23. Dong H, et al. (2004) B7–H1 determines accumulation and deletion of intrahepatic CD8(+) T lymphocytes. *Immunity* 20:327–336.
- 24. Keir ME, et al. (2006) Tissue expression of PD-L1 mediates peripheral T cell tolerance. J Exp Med 203:883–895.
- 25. Ahonen CL, et al. (2008) Enhanced efficacy and reduced toxicity of multifactorial adjuvants compared to unitary adjuvants as cancer vaccines. Blood.
- Dong H, Chen L (2003) B7–H1 pathway and its role in the evasion of tumor immunity. J Mol Med 81:281–287.
- 27. Subudhi SK, Alegre ML, Fu YX (2005) The balance of immune responses: Costimulation verse coinhibition. J Mol Med 83:193–202.
- Blank C, et al. (2004) PD-L1/B7H-1 inhibits the effector phase of tumor rejection by T cell receptor (TCR) transgenic CD8+ T cells. Cancer Res 64:1140–1145.
- Steinman RM, Turley S, Mellman I, Inaba K (2000) The induction of tolerance by dendritic cells that have captured apoptotic cells. J Exp Med 191:411–416.
- Bonifaz L, et al. (2002) Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. J Exp Med 196:1627–1638.
- Liu K, et al. (2002) Immune tolerance after delivery of dying cells to dendritic cells in situ. J Exp Med 196:1091–1097.
- Mahnke K, Qian Y, Knop J, Enk AH (2003) Induction of CD4+/CD25+ regulatory T cells by targeting of antigens to immature dendritic cells. *Blood* 101:4862–4869.
- Hawiger D, et al. (2001) Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. J Exp Med 194:769–779.
- Nishimura H, Nose M, Hiai H, Minato N, Honjo T (1999) Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* 11:141–151.
- Latchman YE, et al. (2004) PD-L1-deficient mice show that PD-L1 on T cells, antigenpresenting cells, and host tissues negatively regulates T cells. Proc Natl Acad Sci USA 101:10691–10696.
- Butte MJ, Keir ME, Phamduy TB, Sharpe AH, Freeman GJ (2007) Programmed death-1 ligand 1 interacts specifically with the b7–1 costimulatory molecule to inhibit T cell responses. *Immunity* 27:111–122.
- Bettelli E, et al. (2006) Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 441:235–238.
- Wei J, et al. (2007) Antagonistic nature of T helper 1/2 developmental programs in opposing peripheral induction of Foxp3+ regulatory T cells. Proc Natl Acad Sci USA 104:18169–18174.
- Tsushima F, et al. (2003) Preferential contribution of B7–H1 to programmed death-1mediated regulation of hapten-specific allergic inflammatory responses. Eur J Immunol 33:2773–2782.
- Habicht A, et al. (2007) A link between PDL1 and T regulatory cells in fetomaternal tolerance. J Immunol 179:5211–5219.

## **Supporting Information**

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**Fig. S1.** Induced Foxp3<sup>+</sup> OTII cells from DC subset coculture are suppressive *in vitro*. Foxp3GFP<sup>-</sup> OTII CD4 T cells were cultured for 5 days with CD8 $\alpha^+$  and CD8 $\alpha^-$  splenic DC subsets in the presence of TGF- $\beta$  (10 ng/ml). Induced Foxp3<sup>+</sup> OTII cells, designated as CD8 $\alpha^+$  DC aTreg and CD8 $\alpha^-$  DC aTreg, were then sorted based on GFP expression. Congenically mismatched naïve CD25<sup>-</sup> CD4 OTII responder T cells (50,000) were CFSE-labeled and stimulated with spleen APCs (150,000) and antigenic peptide ISQ (1  $\mu$ g/ml), in the presence of graded numbers of Foxp3GFP<sup>+</sup> aTregs. CFSE dilution profile was analyzed after 3 days. Representative flow plots from duplicate wells are shown. Similar results were obtained from two independent experiments.



**Fig. S2.** Surface expression of CD25, CD62L, and GITR on converted Foxp3<sup>+</sup> OTII cells in tumor-bearing mice. Irradiated B16OVA-bearing mice were adoptively transferred with sorted naïve OTII CD4 T cells (CD25<sup>-</sup>Foxp3GFP<sup>-</sup>). Surface phenotypes of these cells, including CD25 (*A*), CD62L (*B*), and GITR (*C*) were analyzed when tumors reached  $\geq$ 100 mm<sup>2</sup>. Histograms of Foxp3<sup>-</sup> (blue line) and Foxp3<sup>+</sup> (red line) cells were overlaid. Representative results from at least three individual mice are shown.



**Fig. S3.** PD-L1 blockade inhibits tumor growth, and this effect is reversed by adoptive transferred OTII-Tregs. Mice were irradiated (200 rad) on day 0, inoculated with B16OVA tumor (100,000 cells), and adoptively transferred with sorted naïve OTII CD4 T cells (1 million). For antibody treatment, 300  $\mu$ g of  $\alpha$ PD-L1 were injected i.p. every other day from day 0 until the end of the experiment. OTII-Tregs were *in vitro* generated by culturing OTII CD4 T cells in the presence of plate-bound  $\alpha$ CD3 (10  $\mu$ g/ml), TGF- $\beta$  (5 ng/ml), and retinoic acid (10 nM) for 4 days. The resulting Foxp3<sup>+</sup> OTII-Tregs (purity  $\geq$ 90%) were injected i.v. into mice (10 million per mouse) on day 1. A 2D measurement of tumor size is shown as mean  $\pm$  SEM. The number of mice per group is also shown.



**Fig. 54.** Effects of IL6 and Th1/Th2 cytokines on DC-mediated conversion. DC subsets, either from WT or PD-L1<sup>-/-</sup> mice (*A*) or from IL6<sup>-/-</sup> mice (*B*), were cocultured with naïve OTII T cells in the presence of TGF- $\beta$ . In *A*, neutralizing antibodies against IL-4 and IFN- $\gamma$  (10 µg/ml) (Peprotech) were added as designated. Foxp3 expression was analyzed on day 4. The percentage of Foxp3<sup>+</sup> OTII cells was plotted. All conditions were performed in duplicate wells and are reported as means  $\pm$  SEM. Shown are representative results of two independent experiments.

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