Foxp3$^+$ natural regulatory T cells preferentially form aggregates on dendritic cells *in vitro* and actively inhibit their maturation

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Naturally occurring CD4$^+$CD25$^+$ regulatory T cells (Treg) suppress *in vitro* the proliferation of other T cells in a cell-contact-dependent manner. Dendritic cells (DCs) appear to be a target of Treg-mediated immune suppression. We show here that, in coculture of dye-labeled Treg cells and CD4$^+$CD25$^-$ naïve T cells in the presence of T cell receptor stimulation, Treg cells, which are more mobile than naïve T cells *in vitro*, out-compete the latter in aggregating around DCs. Deficiency or blockade of leukocyte function-associated antigen-1 (LFA-1) (CD11a/CD18) abrogates Treg aggregation, whereas that of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (CD152) does not. After forming aggregates, Treg cells specifically down-regulate the expression of CD80/86, but not CD40 or class II MHC, on DCs in both a CTLA-4- and LFA-1-dependent manner. Notably, Treg exerts this CD80/86-down-modulating effect even in the presence of strong DC-maturating stimuli, such as GM-CSF, TNF-α, IFN-γ, type I IFN, and lipopolysaccharide. Taken together, as a possible mechanism of *in vitro* Treg-mediated cell contact-dependent suppression, we propose that antigen-activated Treg cells exert suppression by two distinct steps: initial LFA-1-dependent formation of Treg aggregates on immature DCs and subsequent LFA-1- and CTLA-4-dependent active down-modulation of CD80/86 expression on DCs. Both steps prevent antigen-reactive naïve T cells from being activated by antigen-presenting DCs, resulting in specific immune suppression and tolerance.

CD80 | CD86 | CTLA-4 | LFA-1 | LPS

Naturally occurring CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (Treg) actively engage in the maintenance of immunological self-tolerance and immune homeostasis (1). A well known characteristic of natural Treg is that they suppress *in vitro* the proliferation of other T cells in a cell contact-dependent manner (2, 3). A substantial number of reports have suggested that dendritic cells (DCs), which play a central role in the initiation of immune reactions by activating naïve T cells (4), can be an important target of Treg-mediated suppression in humans and mice (5–15). Yet it remains to be determined whether an antigen-presenting DC merely provides a platform for the recruitment of antigen-reactive Treg and effector T cells and for their interaction, or whether Treg actively control DC function and thereby inhibit the activation/proliferation of other T cells.

A prominent phenotypic feature of natural Treg cells is that they constitutively and highly express CTLA-4, which interacts with CD80/86 more strongly than the alternate ligand CD28 (16–18). Deficiency or blockade of CTLA-4 abrogated *in vitro* Treg suppression and cancelled Treg-mediated inhibition of experimentally induced colitis *in vivo* (16, 17). Although it remains unclear how CTLA-4 is involved in the physical interaction between Treg and DCs, it is of note that Treg cells continuously express both LFA-1 and CTLA-4 at higher levels than naïve T cells (Tn) (16–19), and that CTLA-4 up-regulates LFA-1-mediated cell adhesion and clustering (20). This suggests that CTLA-4 and LFA-1 expressed by Treg cells might contribute to Treg-mediated suppression via interacting with CD80/86 and ICAM-1 expressed on DCs.

In this report, we have analyzed the cellular and molecular basis of the *in vitro* physical interaction between Treg, Tn, and DC by dye-labeling each T cell population. We propose a possible mechanism underlying Treg-mediated suppression.

Results

Tregs Form Aggregates Around Splenic DCs on TCR Stimulation in a CTLA-4-Independent but LFA-1-Dependent Manner. To analyze the mode of physical interaction of Treg and Tn cells with DCs, we set up an *in vitro* imaging assay in which PKH-67 (green)-labeled Tn or PKH-26 (red)-labeled Treg, or a mixture of the two populations, were cocultured with freshly isolated splenic DCs. Both Tn and Treg cells from DO11.10 (DO) transgenic mice expressing a transgenic T cell receptor (TCR) specific for an OVA peptide readily formed aggregates around DCs but only in the presence of the OVA peptide [supporting information (SI) Fig. S1]. Importantly, when a mixture of Tn and Treg cells at a 1:1 ratio was cocultured with DCs in the presence of the peptide, Treg cells formed aggregates but Tn cells did not, indicating that the former out-competed the latter for space on the surface of DCs (Fig. 1*A*). The addition of LPS to the culture at various concentrations or preincubation of DCs with LPS for 1 day failed to alter this preferential Treg aggregation or their out-competition over Tn cells on DCs (Fig. S2).

When Tregs prepared from CTLA-4$^{-/-}$ DO mice (Fig. S3) were used in the assay, CTLA4$^{-/-}$ DO Tregs similarly out-competed DO Tn cells, forming aggregates around DCs to a degree comparable with wild-type Treg cells (Fig. 1*A*). The superior aggregate formation by DO Tregs over DO Tn cells, especially Treg occupancy of the center of aggregates around the body of DC, was not inhibited by adding blocking Fab fragments of anti-CTLA-4 mAb at a concentration capable of abrogating *in vitro* Treg-mediated suppression (100 μg/ml) (16) (Fig. 1*B*). This indicates that the expression of CTLA-4 on Tregs is not required for their ability to edge out Tn cells and preferentially interact with DCs to form aggregates.

To assess a possible contribution of LFA-1 to the preferential Treg aggregation on DCs, we cultured either Tn or Treg cells from LFA-1$^{-/-}$ mice with LFA-1-intact splenic DCs. T cell–DC aggregates were formed with LFA-1$^{-/-}$ Treg or Tn cells after TCR stimulation with anti-CD3 mAb (Fig. 1*C*). However, in contrast to what was observed with CTLA-4$^{-/-}$ Tregs, LFA-1$^{-/-}$ Treg cells

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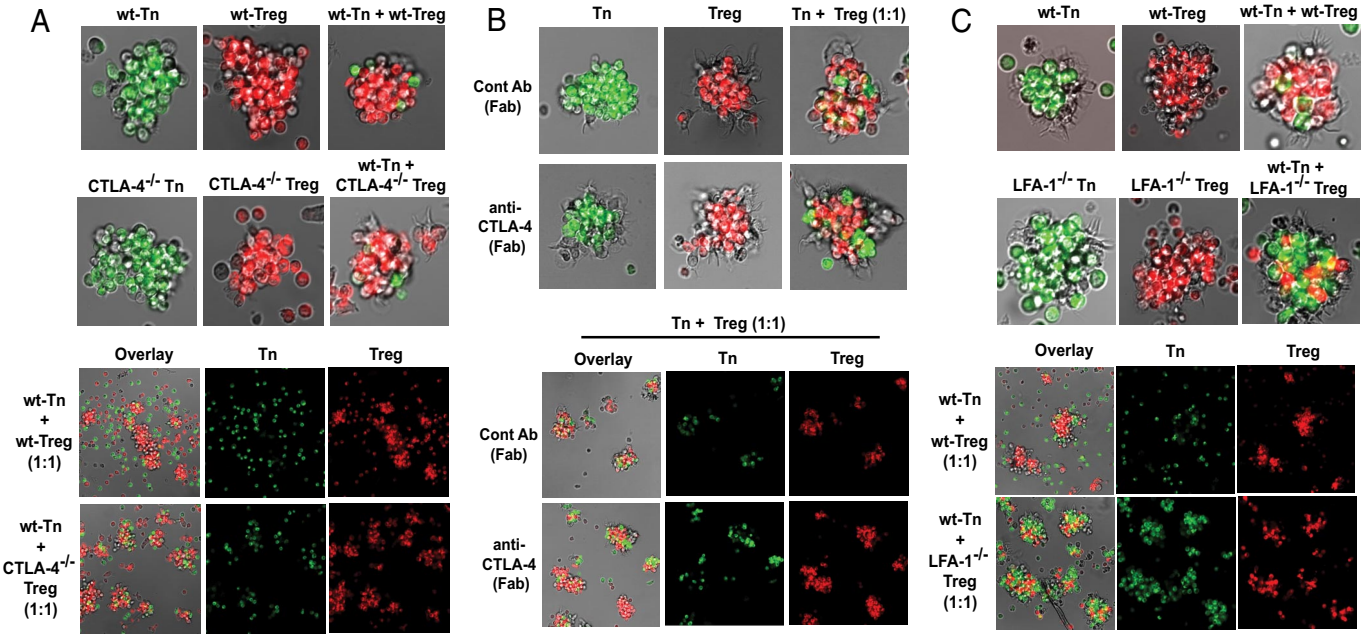

Fig. 1. CTLA-4-independent and LFA-1-dependent aggregation of activated Treg cells on DCs. Green-dye-labeled $CD4^+CD25^-$ naïve T (Tn) cells or red-dye-labeled $CD4^+CD25^+$ Treg cells or two populations mixed at a 1:1 ratio were cultured for 12 h with BALB/c splenic DCs. (*A*) Tn and Treg cells from DO11.10 or CTLA-$4^{-/-}$ DO11.10 mice were cultured with 1 μM $OVA_{323-339}$. (*B*) Tn and Treg cells from DO11.10 mice were cultured with 100 μg/ml anti-CTLA-4 mAb (Fab) or control Ab (Fab). (*C*) Tn and Treg cells from wild-type or LFA-$1^{-/-}$ mice were cultured with 0.1 μg/ml anti-CD3 mAb. Results represent three independent experiments.

were unable to out-compete wild-type Tn cells at a 1:1 ratio; i.e., the latter predominantly formed aggregates in the presence of the former (Fig. 1*C*). This finding suggests that the advantage of Tregs over Tn cells in the physical interaction with DCs can be attributed at least in part to the expression of LFA-1 by Tregs (19) (Fig. S4).

Tracing DO Treg and Tn cells, cultured with immature bone marrow-derived DCs (BMDCs) in the presence of OVA peptide, revealed that Tregs were more mobile than Tn cells around DCs in an early phase (within 12 h) of culture (Fig. 2). Within 2 h, Tregs moved around DCs with changing their cell shape from small and round to large and elongated, and more Tregs had contacts with DCs than Tn cells. We obtained a similar result with splenic DCs as well, although they were more mobile than immature BMDCs and made the tracing of Treg and Tn cells rather difficult (data not shown).

Taken together, antigen-stimulated Tregs, which are highly mobile around antigen-presenting DCs, out-compete Tn cells to form aggregates on DCs in an LFA-1-dependent but CTLA-4-independent manner.

TCR-Stimulated Tregs Down-Regulate the Expression of CD80 and CD86, But Not CD40 or Class II MHC, on Splenic DCs. To examine whether Tregs modulate the expression of functional molecules on DCs after interaction, we analyzed DC expression of CD80, CD86, CD40, and MHC class II ($I\text{-}A^d$), which are all associated with the maturation status of DCs (4), 1 or 2 days after coculture of BALB/c DCs with DO Tregs in the presence of OVA peptide. Notably, DCs cocultured with Tregs showed a decrease in their CD80 and CD86 expression when compared with control DCs cultured in medium alone (Fig. 3). The decrease contrasted with an increase in CD80 and CD86 expression on DCs cocultured with Tn cells. The difference in CD80/86 expression between DCs cultured with Tn or Treg cells was more evident on day 2 than on day 1. In the absence of OVA peptide, there was no difference in CD80/86 expression by DCs under any of the culture conditions, indicating that the up- and down-regulation of CD80/86 on DCs by Tn and Treg cells, respectively, depends on TCR stimulation. However, the expression of CD40 and $I\text{-}A^d$ on DCs cultured with Tregs was not significantly different from those with Tn cells either on day 1 or day 2 (Fig. 3). In addition, compared with Treg-cocultured DCs, which showed 5- to 8-fold less expression of CD86 than Tn-cocultured DCs, B cells

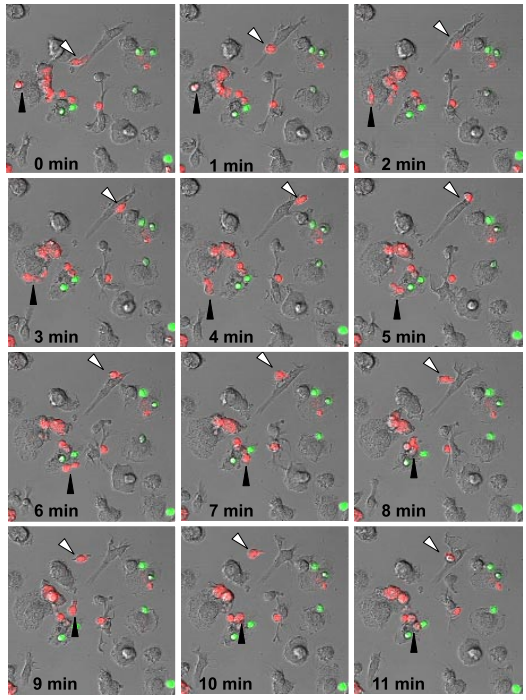

Fig. 2. High mobility of Tregs interacting with DC. A mixture of green-dye-labeled Tn cells and red-dye-labeled Treg cells at 1:1 ratio from DO11.10 were cultured with immature bone-marrow-derived DCs and stimulated with 1 μM $OVA_{323-339}$. A series of images were taken at intervals of 1 min starting 12 h after culture. Black and white arrows point at two Treg cells and trace them.

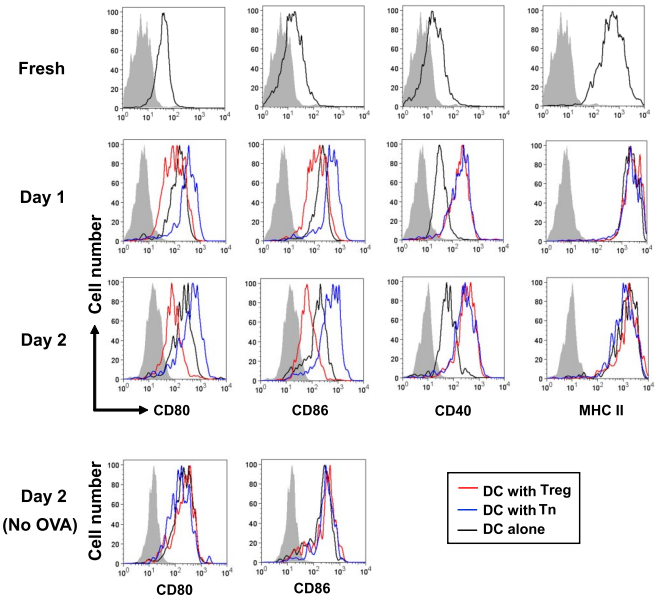

Fig. 3. Treg-mediated selective down-regulation of CD80 and CD86 expression on splenic DCs. CD80, CD86, CD40, and MHC class II expression on splenic DCs were determined by flow cytometry before and 1 or 2 days after the culture with Treg or Tn cells. Splenic DCs were isolated from BALB/c mice. Treg and Tn cells were purified from DO11.10 mice and stimulated with 1 μM $OVA_{323–339}$. Results represent three independent experiments.

similarly cultured with Tregs decreased the expression to a lesser degree (2-fold) compared with Tn-cocultured B cells (Figs. 3 and 4, Fig. S5). Thus, Treg–DC interaction specifically down-regulates CD80 and CD86 expression on antigen-presenting cells (APCs), in particular DCs.

Tregs Down-Regulate CD80/86 Expression on Splenic DCs in a CTLA-4- and LFA-1-Dependent Manner. We then examined expression of CD80 and CD86 on DCs 2 days after coculture with a mix of Tn and Treg cells at a 1:1 ratio and compared with DCs cocultured with either Tn or Treg cells, or medium alone. Interestingly, as assessed by staining profiles and mean fluorescence intensity (MFI) of each staining, the level of CD80/86 expression on DCs was comparable between the cultures of a mix of Treg/Tn cells and Treg cells alone, being much lower than the level of DCs cultured with Tn cells or medium alone (Fig. 4*A* and Fig. S6*A*). This result indicates that antigen-activated Tregs actively down-regulate CD80/86 expression on DCs in the presence of antigen-specific Tn cells and suggests that this may contribute to *in vitro* Treg-mediated suppression.

To determine the molecular basis of the above finding, we first examined the possible contribution of CTLA-4. When DCs were cocultured with CTLA-$4^{-/-}$ Tregs, their CD80/CD86 expression was not down-modulated but was higher than that of control DCs incubated in medium alone (Fig. 4*A* and Fig. S6*A*). Addition of Fab anti-CTLA-4 mAb into the coculture of CTLA-4-intact Treg and DCs, at a concentration of 100 μg/ml to block CTLA-4 (16), inhibited the Treg-mediated CD80/86 down-regulation, recapitulating the results with CTLA-$4^{-/-}$ Tregs (Fig. 4*B* and Fig. S6*B*).

Similar to CTLA-$4^{-/-}$ Tregs, LFA-$1^{-/-}$ Tregs also failed to down-regulate CD80/86 on DCs (Figs. 4*C* and S6*C*). However, LFA-$1^{-/-}$ Tn cells up-regulated CD80/86 on DCs, which expressed CD80/86 at slightly higher levels than DCs cultured with wild-type Tn cells. The use of blocking anti-LFA-1mAb abrogated Treg-mediated CD80/86 down-regulation (Figs. 4*D* and S6*D*).

These findings thus indicate that Treg cells actively down-regulate DC expression of CD80 and CD86 in both a CTLA-4- and LFA-1-dependent manner.

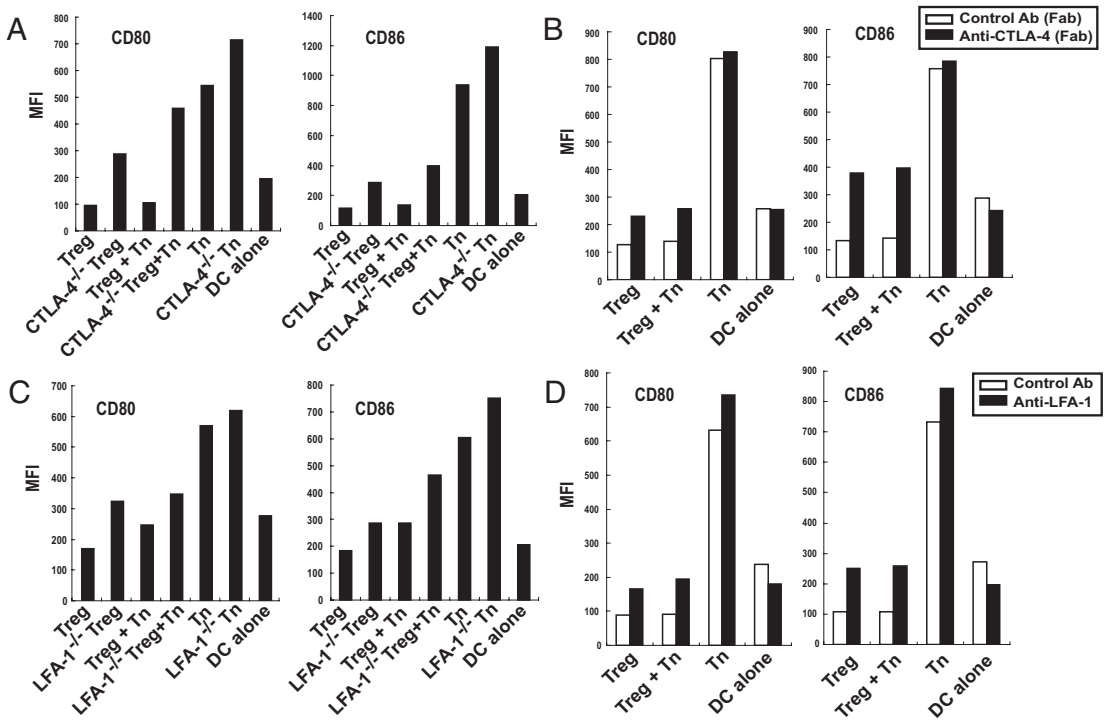

Fig. 4. Treg-mediated, CTLA-4-, and LFA-1-dependent down-regulation of CD80 and CD86 expression on splenic DCs. Splenic DCs from BALB/c mice were cultured 2 days with Tn or Treg cells or a mix of both populations at a 1:1 ratio, and then CD80 and CD86 expression on splenic DCs was determined. (*A*) Tn and Treg cells from DO11.10 or CTLA-$4^{-/-}$ DO11.10 mice were cultured with 1 μM OVA $_{323–339}$. (*B*) Tn and Treg cells from DO11.10 mice were cultured with 100 μg/ml anti-CTLA-4 mAb (Fab) or control Ab (Fab). (*C*) Tn and Treg cells from wild-type or LFA-$1^{-/-}$ mice were cultured with 0.1 μg/ml anti-CD3 mAb. (*D*) Tn and Treg cells from DO11.10 mice were cultured with 2 μg/ml anti-LFA-1 mAb or control Ab. Results represent three independent experiments.

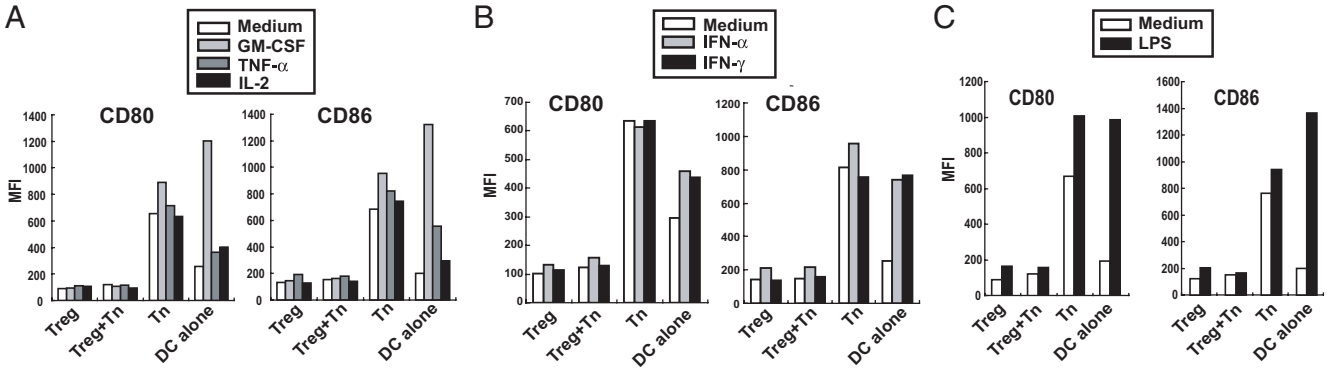

Fig. 5. Effects of proinflammatory cytokines and LPS on Treg-mediated down-regulation of CD80 and CD86 expression on splenic DCs. GM-CSF (100 ng/ml), TNF-α (20 ng/ml), or IL-2 (200 units/ml) (*A*); IFNα (1,000 units/ml) or IFNγ (100 ng/ml) (*B*); LPS (1 μg/ml) (*C*). See Fig. S7 for staining profile of each experiment. Results represent three independent experiments.

Effects of DC-Maturating Stimuli on Treg-Mediated Down-Regulation of CD80/86 on Splenic DCs. We then assessed another possibility that Tregs might suppress DC maturation by inhibiting the production of cytokines, such as GM-CSF, TNF-α, and IL-2, by Tn cells during the culture. Both GM-CSF and TNF-α, which stimulate DC maturation (4), were indeed able to up-regulate CD80/86 expression on DCs (Fig. 5*A*, Fig. S7*A*). However, strikingly, Tregs or a mix of Treg and Tn cells suppressed the up-regulation even in the presence of GM-CSF, TNF-α, or IL-2 (Figs. 5*A* and S7*A*). It is known that Type I IFN (IFN-α and -β), IFN-γ, LPS, and Zymosan activate DCs (21–24). Addition of these cytokines or microbial substances, including Zymosan (Fig. S8), to the culture of DCs indeed increased their CD80/86 expression but failed to exert this effect on DCs in the presence of Tregs or a mix of Treg and Tn cells (Figs. 5 *B* and *C* and S7 *B* and *C*).

DC-Dependent Treg-Mediated Suppression of Tn Cell Proliferation. We next examined whether the findings on the roles of LFA-1 and CTLA-4 in Treg aggregation and CD80/86 down-modulation (Figs. 1 and 4) should correlate with Treg-mediated suppression of Tn proliferation assessed by ^{3}H-thymidine incorporation or carboxyfluorescein-succinimidyl ester (CFSE) dilution of labeled Tn cells. LFA-1$^{-/-}$ Tregs were indeed unable to suppress Tn proliferation in the coculture with wild-type Tn cells even at a very high Treg-DC ratio of 10:1 with the use of nonirradiated splenic DCs (Fig. 6*A*). Blockade of CTLA-4 with Fab anti-CTLA-4 mAb abrogated Treg suppression and allowed Tn cell proliferation in the setting using splenic DCs, as shown with splenic B cells (16), in both ^{3}H-thymidine incorporation and CFSE dilution assays (Fig. 6 *B* and *D*).

The addition of LPS only marginally increased the proliferation of Tregs (Fig. 6*C*) and also Tn cells cocultured with Tregs (Fig. 6 *C* and *D*) in the presence of splenic DCs, consistent with the inability of LPS to inhibit the formation of Treg aggregates or abrogate the Treg-mediated CD80/86 down-regulation on DCs.

Discussion

We have shown in this study that antigen-activated Tregs outcompete Tn cells in forming aggregates around DCs *in vitro*. Tregs also actively and specifically down-regulate the expression of CD80/86 on DCs. This aggregate formation and CD80/86 down-regulation occur even in the presence of strong DC-activating stimuli such as LPS, Zymosan, and type I IFN. LFA-1 and CTLA-4 distinctly contribute to these processes and hence Treg-mediated suppression of Tn proliferation, at least in an *in vitro* culture system using splenic DCs as APCs. This also provides a coherent interpretation to the previous findings by us and others that LFA-1 deficiency or CTLA-4 blockade attenuates Treg suppression *in vitro* (10, 16, 25).

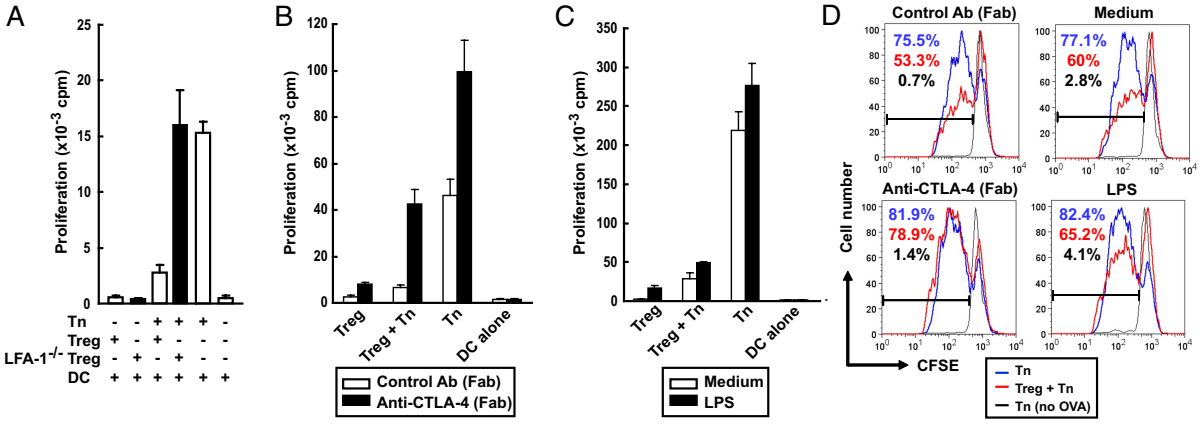

Fig. 6. Treg-mediated suppression of Tn cell proliferation on DC. (*A*) Wild-type Tn cells, Treg cells from LFA-1$^{-/-}$ or wild-type mice, or a mix of Tn and Treg cells at a 1:1 ratio were cultured for 3 days with anti-CD3 mAb, along with nonirradiated BALB/c splenic DCs. (*B*) Anti-CTLA-4 mAb (Fab) or control Ab (Fab) were added to the culture of anti-CD3-stimulated Tn or Treg cells, or the mixture of these at an equal ratio. (*C*) Tn or Treg cells, or a mixture of these, were cultured with or without LPS. (*D*) In the presence of anti-CTLA-4 mAb (Fab) or control Ab (Fab) or LPS, CFSE-labeled DO Tn cells were stimulated with 1 μM OVA$_{323–33}$ peptide for 3 days with or without DO Tregs, and the degree of CFSE dilution in Tn cells was assessed by cytofluorometry. Percentages of divided Tn cells are also shown. Results represent three independent experiments.

The roles of CTLA-4 for Treg function have been controversial. We and others reported that CTLA-4$^{-/-}$ Treg cells were able to suppress *in vitro* T cell proliferation, whereas blockade of CTLA-4 solely expressed by Tregs can abrogate the suppression (16, 26, 27). This apparent contradiction can be resolved, at least in part, by the fact that CTLA-4$^{-/-}$ Tregs, which are highly activated in CTLA-4$^{-/-}$ mice presumably as a result of systemic inflammation, can efficiently out-compete Tn cells in forming aggregates on DCs. It was also shown *in vivo* in a colitis model that anti-CTLA-4 mAb inhibited suppression via direct effects on Tregs, and not via hyperactivation of effector T cells (28). Interestingly, however, accumulation of Tregs in the model was not inhibited by the presence of anti-CTLA-4 mAb; their absolute number even increased. This suggests that CTLA-4 blockade may inhibit *in vivo* suppression not by impairing accumulation of Tregs or their conjugation with APCs but by affecting their other functions, such as their ability to down-regulate CD80/86 expression on DCs. It remains to be determined whether the development of a fatal lymphoproliferative disease in CTLA-4$^{-/-}$ mice might be due to inefficiency of CTLA-4$^{-/-}$ Tregs to down-regulate CD80/86 expression on DCs *in vivo* (29, 30).

Suppressive cytokines such as TGF-β and IL-10, indoleamine 2, 3-dioxygenase, Lymphocyte activation gene 3 (CD223), and IL-2 have been implicatd in Treg-mediated suppression (reviewed in ref. 31). However, neutralization or inhibition of any of those factors did not affect the CD80/86 down-regulation by Treg (Fig. S9 *A*–*C*). Further, the addition of TGF-β and IL-10 failed to enhance the Treg-mediated CD80/86 down-regulation (Fig. S10).

We and others have reported that LPS-matured BMDCs, which express high levels of CD80, CD86, and MHC class II, are able to abrogate Treg suppressive function (7, 8). Isolated splenic DCs expressed high levels of MHC class II but low levels of CD80 and CD86 (Fig. 3). It is therefore likely that, in contrast with mature BMDCs, the low expression of CD80/86 on splenic DCs at the start of culture is critical for Treg-mediated suppression. Tregs then sustain the level of CD80/86 expression below the level required for activation and expansion of Tn cells. The ability of immature DCs to induce antigen-specific immunologic tolerance could be attributed in part to their low CD80/86 expression and active maintenance of this state by Tregs, which can be antigen-activated even by immature DCs and form aggregates on them (Fig. 1). Tregs exert suppression on plasmacytoid dendritic cells (pDCs) as well (Fig. S11*A*), which expresses low to undetectable levels of CD80/86 (Fig. S11*B*) (32). When a mixture of Tn and Treg cells was cocultured with freshly isolated pDCs from the spleen in the presence of antigen, Tregs predominantly formed aggregates, but Tn cells did not (Fig. S11*C*). Thus, Tregs control not only conventional DCs but also pDCs, thereby suppressing a variety of immune responses and maintaining immunologic tolerance.

The resistance of Treg-mediated CD80/86 down-regulation to DC-maturating microbial stimuli, such as LPS and Zymosan, could be important for Tregs to tune the intensity of antimicrobial immune responses (33). For example, LPS elicits via TLR4 the production of inflammatory cytokines, including IL-6, and the up-regulation of costimulatory molecules in DCs. The former effect depends on MyD88, but the latter does not (34). In the present study, the addition of LPS increased IL-6 production in the culture of DCs alone or the coculture of DCs with Treg, Tn cells, or a mix of both populations (Fig. S12). These findings, when taken together, indicate that Treg suppress LPS-induced MyD88-independent CD80/86 up-regulation in DCs but not their MyD88-dependent IL-6 production by LPS. It remains to be determined how this apparently differential effect of Tregs on DC functions controls antimicrobial immune responses. Although IL-6 produced by LPS stimulation only marginally reduced the Treg-mediated suppression (Figs. 6 and S12), IL-6 may render responder T cells resistant to Treg suppression depending on the amount of IL-6 produced by DCs, and also facilitate the differentiation of naïve T cells to Th17 cells (35, 36).

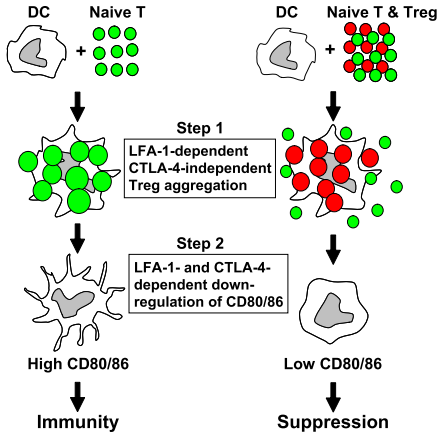

Fig. 7. Two-step model of Treg-mediated suppression (see text).

In contrast to the CD80/86 down-regulation, activated Tregs fail to suppress the expression of CD40 and class II MHC on splenic DCs, allowing the expression levels of these molecules to increase to a comparative level as in the coculture with naïve T cells (Fig. 3). This dissociation between CD80/86 and CD40/class II MHC expression on Treg-cultured DCs suggests that there may be a mechanism by which Tregs specifically and actively down-regulate CD80/86 expression. This mechanism is currently under investigation.

It is likely that Tregs, naturally produced by the thymus or induced in the periphery from naïve T cells, suppress the activation and proliferation of other T cells by more than one mechanism and in a context-dependent manner (1). Based on our findings in this report, we here propose a "two-step model" as a possible mechanism of Treg-mediated contact-dependent suppression on DCs (Fig. 7). The model consists of (*i*) the LFA-1-dependent initial formation of Treg aggregates on DCs and (*ii*) LFA-1- and CTLA-4-dependent active down-modulation of CD80/86 expression on DCs; that is, Treg-mediated active sustenance of CD80/86 expression at low levels on immature DCs. Both steps are required to stably keep antigen-reactive Tn cells from being activated by antigen-presenting DCs, hence to suppress immune responses. This possible mechanism of suppression is in accord with the *in vivo* finding using intravital two-photon microscopy that Tregs apparently inhibited stable contacts between antigen-activated T cells and DCs (13, 14). In addition to this two-step cell contact-dependent suppression, Tregs might further differentiate *in vivo* as the third step to exert other suppressive activities, such as secretion of IL-10 in a particular local milieu and granzyme-B-dependent cytotoxicity (1, 28, 37, 38). Multiple mechanisms involving cell contact-dependent and -independent ones might also operate in synergy to suppress a particular *in vivo* immune response. Further study is required to elucidate the molecular basis of Treg-mediated cell contact suppression, in particular how CTLA-4 and LFA-1 contribute to the Treg-mediated specific down-regulation of CD80/86 on DCs. Whether augmentation or attenuation of each step of Treg-mediated suppression can control *in vivo* immune responses also need to be investigated.

Materials and Methods

Mice. Female BALB/c and C57BL/6 mice 6–8 wk of age were purchased from Japan SLC. DO11.10 TCR transgenic mice and C57BL/6 LFA-1$^{-/-}$ mice, which were purchased from The Jackson Laboratory, were bred in our animal facility. BALB/c CTLA-4$^{-/-}$ mice were backcrossed onto DO11.10 TCR transgenic mice (16). All mice were maintained in our animal facility and treated in accordance with the guidelines for animal care approved by the Institute for Frontier Medical Sciences, Kyoto University.

Antibody. The following reagents were purchased from BD Biosciences: anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD11a (M17/4), anti-CD11c (HL3), anti-CD16/CD32 (2.4G2), anti-CD25 (PC61), anti-CD40 (3/23), anti-CD80 (16-10A1), anti-CD86 (GL1), anti-I-A^d (AMS-32.1), anti-IL2 (S4B6), anti-CD210 (1B1.3a), anti-CD223 (C9B7W), and isotype control IgG. Anti-TGF-$\beta_{1,2,3}$ (1D11) was purchased from R&D Systems. Purified rat-IgG and hamster IgG were purchased from Sigma and Cappel, respectively. Anti-CTLA-4 (UC10–4F10–11) and anti-LFA-1 (FD441.8) were purified from culture supernatant in our laboratory.

Cell Sorting. Spleen and lymph node cell suspensions prepared from 6- to 10-wk-old DO11.10, C57BL/6, or LFA-$1^{-/-}$ mice or 3- to 4-wk-old CTLA-$4^{-/-}$ DO11.10 mice were stained with FITC- or PE-Cy5-labeled anti-CD4 mAb and PE-anti-CD25 mAb and sorted using a MoFlo (Dako Cytomation). FITC-labeled anti-CD4 mAb was used for the FACS analysis, and PE-Cy5-labeled anti-CD4 mAb was for imaging analysis. Purity of sorted $CD4^+CD25^+$ Treg cells or $CD4^+CD25^-$ Tn cells were >96% or >99%, respectively.

Splenic DC Preparation. $CD11c^+$ splenic DCs were isolated by using MACS (Miltenyi Biotec) from spleens treated with Librase Blendzyme II (Roche Diagnostics).

Preparation of BMDCs. BM-derived DCs were prepared according to Inaba *et al.* (39). Briefly, BM cells from BALB/c mice were grown in Iscove's Modified Dulbecco's Medium containing 10% FCS with the addition of 10 ng/ml GM-CSF and 10 ng/ml IL-4 (PeproTech). Immature BMDC were harvested on day 5.

***In Vitro* Proliferation Assay.** Lymph node and spleen T cells (2.5×10^4)., sorted as described above, and splenic DCs (2.5×10^3) were cultured for 3 d in 96-well round-bottomed plates (Costar) in RPMI medium 1640 supplemented with 10% FCS, penicillin (100 units/ml), streptomycin (100 μg/ml), and 50 μM 2-ME. OVA peptide (amino acids 323–339) for the DO11.10 strain at a final concentration of 1 μM or anti-CD3 mAb (145-2C11) at a final concentration of 0.1 μg/ml was added to the culture for stimulation. [^{3}H]thymidine (1 μCi per well; Du Pont/New England Nuclear) was added during the last 6 h of culture. Fab fragments of anti-CTLA-4 mAb or normal hamster IgG, prepared by digesting these Abs with immobilized papain (Pierce), was used at 100 μg/ml. LPS (Sigma) was used at 1 μg/ml in cell culture.

Lymphocyte Labeling with CFSE. Tn cells were labeled with 1 μM CFSE (Dojindo), and Tn cells (5×10^4) were cultured with splenic DCs (5×10^3) for 3 d as described above and served to FACS analysis of their proliferation. CFSE dilution was used to determine the degree of proliferation of Tn cells.

Flow-Cytometric Analysis of DC Phenotypic Changes. Treg or Tn cells ($6–8 \times 10^4$) or a mix of two populations at a 1:1 ratio were cultured with splenic DCs ($3–4 \times 10^4$) in 96-well round-bottomed plates. After 18- or 42-h culture, cells were collected, treated with 5 mM EDTA, stained with biotin-anti-CD11c, FITC-coupled Abs specific for CD80, CD86, CD40, and I-A^d, and then APC-streptavidin and 7-amino-actinomycin D. Cells were analyzed on FACSCalibur (BD Biosciences). GM-CSF, TNFα, and IL-2 were purchased from PeproTech. 1MT and Zymosan were purchased from Sigma-Aldrich. IFN-α and -γ were purchased from PBL Biomedical Laboratories.

Confocal Microscopy. Treg and Tn cells were labeled with PKH-26 and PKH-67 (Sigma), respectively, according to the manufacturer's instruction. PKH-labeled Treg and Tn cells (5×10^4) were cultured with nonlabeled splenic DCs (5×10^3) in 96-well round-bottomed plates. After 12-h culture, cells were gently transferred to glass-bottomed dish (as a drop of 100 μl). Glass dishes coated with fibronectin were used from the start of the culture for analysis of mobility of T cells on immature BM-DC. Images were taken by using a Zeiss LSM 510 confocal microscope eppuipped with a ×20 objective. Acquisition was performed by using Zeiss LSM 510 (version 3.0) software.

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1. Sakaguchi S, Powrie F (2007) Emerging challenges in regulatory T cell function and biology. *Science* 317:627–629.
2. Takahashi T, *et al.* (1998) Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int Immunol* 10:1969–1980.
3. Thornton AM, Shevach EM (1998) CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation *in vitro* by inhibiting interleukin 2 production. *J Exp Med* 188:287–296.
4. Banchereau J, Steinman RM (1998) Dendritic cells and the control of immunity. *Nature* 392:245–252.
5. Cederbom L, Hall H, Ivars F (2000) CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. *Eur J Immunol* 30:1538–1543.
6. Serra P, *et al.* (2003) CD40 ligation releases immature dendritic cells from the control of regulatory CD4+CD25+ T cells. *Immunity* 19:877–889.
7. Yamazaki S, *et al.* (2003) Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. *J Exp Med* 198:235–247.
8. Fehervari Z, Sakaguchi S (2004) Control of Foxp3+CD25+CD4+ regulatory cell activation and function by dendritic cells. *Int Immunol* 16:1769–1780.
9. Misra N, Bayry J, Lacroix-Desmazes S, Kazatchkine MD, Kaveri SV (2004) Cutting edge: Human CD4+CD25+ T cells restrain the maturation and antigen-presenting function of dendritic cells. *J Immunol* 172:4676–4680.
10. Oderup C, Cederbom L, Makowska A, Cilio CM, Ivars F (2006) Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression. *Immunology* 118:240–249.
11. Houot R, Perrot I, Garcia E, Durand I, Lebecque S (2006) Human CD4+CD25high regulatory T cells modulate myeloid but not plasmacytoid dendritic cells activation. *J Immunol* 176:5293–5298.
12. Veldhoen M, Moncrieffe H, Hocking RJ, Atkins CJ, Stockinger B (2006) Modulation of dendritic cell function by naive and regulatory CD4+ T cells. *J Immunol* 176:6202–6210.
13. Tadokoro CE, *et al.* (2006) Regulatory T cells inhibit stable contacts between CD4+ T cells and dendritic cells *in vivo*. *J Exp Med* 203:505–511.
14. Tang Q, *et al.* (2006) Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. *Nat Immunol* 7:83–92.
15. Yamazaki S, Inaba K, Tarbell KV, Steinman RM (2006) Dendritic cells expand antigen-specific Foxp3+ CD25+ CD4+ regulatory T cells including suppressors of alloreactivity. *Immunol Rev* 212:314–329.
16. Takahashi T, *et al.* (2000) Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* 192:303–310.
17. Read S, Malmstrom V, Powrie F (2000) Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med* 192:295–302.
18. Salomon B, *et al.* (2000) B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 12:431–440.
19. Itoh M, *et al.* (1999) Thymus and autoimmunity: Production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* 162:5317–5326.
20. Schneider H, Valk E, da Rocha Dias S, Wei B, Rudd CE (2005) CTLA-4 up-regulation of lymphocyte function-associated antigen 1 adhesion and clustering as an alternate basis for coreceptor function. *Proc Natl Acad Sci USA* 102:12861–12866.
21. Ito T, *et al.* (2001) Differential regulation of human blood dendritic cell subsets by IFNs. *J Immunol* 166:2961–2969.
22. Luft T, *et al.* (1998) Type I IFNs enhance the terminal differentiation of dendritic cells. *J Immunol* 161:1947–1953.
23. Gallucci S, Lolkema M, Matzinger P (1999) Natural adjuvants: endogenous activators of dendritic cells. *Nat Med* 5:1249–1255.
24. Akira S, Takeda K, Kaisho T (2001) Toll-like receptors: Critical proteins linking innate and acquired immunity. *Nat Immunol* 2:675–680.
25. Marski M, Kandula S, Turner JR, Abraham C (2005) CD18 is required for optimal development and function of CD4+CD25+ T regulatory cells. *J Immunol* 175:7889–7897.
26. Kataoka H, *et al.* (2005) CD25(+)CD4(+) regulatory T cells exert *in vitro* suppressive activity independent of CTLA-4. *Int Immunol* 17:421–427.
27. Birebent B, *et al.* (2004) Suppressive properties of human CD4+CD25+ regulatory T cells are dependent on CTLA-4 expression. *Eur J Immunol* 34:3485–3496.
28. Read S, *et al.* (2006) Blockade of CTLA-4 on CD4+CD25+ regulatory T cells abrogates their function *in vivo*. *J Immunol* 177:4376–4383.
29. Tivol EA, *et al.* (1995) Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3:541–547.
30. Waterhouse P, *et al.* (1995) Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science* 270:985–988.
31. Sakaguchi S, Yamaguchi T, Nomura T, Ono M (2008) Regulatory T cells and immune tolerance. *Cell* 133:775-787.
32. Liu YJ (2005) IPC: Professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol* 23:275–306.
33. Belkaid Y, Rouse BT (2005) Natural regulatory T cells in infectious disease. *Nat Immunol* 6:353–360.
34. Kaisho T, Takeuchi O, Kawai T, Hoshino K, Akira S (2001) Endotoxin-induced maturation of MyD88-deficient dendritic cells. *J Immunol* 166:5688–5694.
35. Pasare C, Medzhitov R (2003) Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science* 299:1033–1036.
36. Bettelli E, Oukka M, Kuchroo VK (2007) T(H)-17 cells in the circle of immunity and autoimmunity. *Nat Immunol* 8:345–350.
37. Gondek DC, Lu LF, Quezada SA, Sakaguchi S, Noelle RJ (2005) Cutting edge: Contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol* 174:1783–1786.
38. Cao X, *et al.* (2007) Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity* 27:635–646.
39. Inaba K, *et al.* (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 176:1693–1702.