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Dendritic Cells Induce Regulatory T Cell Proliferation through Antigen-Dependent and -Independent Interactions

Tao Zou,*,† Andrew J. Caton,‡ Gary A. Koretzky,*,†§ and Taku Kambayashi†

Regulatory T cells (Tregs) are a subset of T cells with suppressive function that protect the host from autoimmunity and prevent excessive immunopathology. Functional Tregs must be present throughout life to provide continuous protection for the host. Despite the intense study of this lineage, the mechanisms by which Tregs are maintained in the steady-state remain incompletely understood. In this study, we investigated the role of dendritic cells (DCs) in the control of Treg proliferation. In the absence of overt TCR stimulation, we found that DCs induce polyclonal Treg division in murine splenocyte cultures. In vivo expansion of DCs also correlated with polyclonal Treg expansion. DC-induced Treg division required IL-2, which was provided by conventional CD4+ T cells through an MHC class II (MHC II)-dependent interaction with DCs. Provision of exogenous IL-2 obviated the need for conventional CD4+ T cells in the induction of Treg proliferation, but this process still required a contact-dependent but MHC II-independent interaction between DCs and Tregs. Although Treg division could occur in the absence of MHC II expression by DCs, direct stimulation of Tregs by cognate Ag/MHC II complexes enhanced IL-2-induced Treg proliferation. These data demonstrate that DCs coordinate the interactions that are necessary to initiate polyclonal Treg proliferation. The Journal of Immunology, 2010, 185: 2790–2799.

R egulatory T cells (Tregs) are a subset of CD4+ T cells crucial for protecting the host from autoimmunity by suppressing self-reactive T cells and preventing immunopathology by restraining immune responses directed against foreign Ags (1, 2). The peripheral Treg pool is comprised of naturally arising Tregs (nTregs) that develop in the thymus and inducible Tregs (iTregs) that are converted from conventional CD4+ T cells (Tconvs) in the periphery (1–4). Tregs constitute 5–15% of the total T cell population in the periphery (5–9). The importance of Tregs is illustrated by deficiencies in their number or function, which lead to widespread autoimmune disease (5, 10, 11). An inadequate number of Tregs can result either from the inability to produce nTregs in the thymus or from a defect in maintaining their survival and/or proliferation in the periphery (10). For example, mice or humans lacking a functional form of the transcription factor forkhead box P3 (Foxp3) are devoid of Tregs (10–12). As Foxp3 is necessary for Treg lineage specification and maintenance of Treg fate (11–15), its absence results in failed generation of Tregs and development of a fatal autoimmune syndrome (11, 12, 14). In addition, mice deficient in several proteins, including CD28, TGF-β, and IL-2, also display autoimmune manifestations due to an inability to maintain their peripheral Treg pool in the steady-state (10, 16).

Despite the importance of Tregs, the mechanisms that regulate peripheral Treg homeostasis remain incompletely defined. Using Ag-specific TCR transgenic Tregs, previous work has suggested a connection between dendritic cells (DCs) and the homeostasis of peripheral Tregs. These studies have shown that Ag-specific Tregs depend on cognate Ag/MHC class II (MHC II) and costimulatory interactions with DCs to proliferate (17–20). Furthermore, targeting Ag to CD8α+ DCs are able to induce Ag-specific Treg proliferation in vivo (19). Of note, in addition to direct Ag stimulation of Tregs, MHC II expressing DCs can induce Ag-specific Treg proliferation in the presence of only exogenous IL-2 (17). Although the use of TCR transgenic mice has facilitated the study of Tregs, cells from these mice may not completely recapitulate the biology of Tregs that develop in the setting of a T cell compartment possessing diverse antigenic reactivity. This fact is of particular concern, as the antigenic specificity of polyclonal Tregs remains controversial (21–23). Studies examining polyclonal Tregs have demonstrated that Tregs undergo a physiologic rate of proliferation in specific pathogen-free mice in the absence of overt antigen stimulation (8). This physiologic Treg proliferation and Treg homeostasis is dependent on IL-2, which likely derives from Tconvs activated by self-Ags or commensal organisms (24). Recent work has identified the importance of DCs and their expression of MHC II in the maintenance of both physiological proliferation and homeostasis of polyclonal Tregs (25). From these studies, it is presumed that polyclonal Tregs are induced to proliferate by self-Ags presented on MHC II-expressing DCs. However, the cellular and molecular interactions that underlie DC induction of polyclonal Treg proliferation and maintain Treg homeostasis remain incompletely defined.

Several reports investigating the mechanisms regulating the expansion and maintenance of Tregs have focused on the ability of the cytokine GM-CSF to expand this population of T cells (26–29).
Although GM-CSF can act as a proinflammatory cytokine with biologically relevant roles in both antitumor immunity and as a promotor of autoimmune processes (30), combined deficiency of GM-CSF and the related cytokine IL-3 results in the development of autoimmune (31). Moreover, in vivo administration of GM-CSF prevents or attenuates autoimmune disease in a variety of mouse models (26, 28, 29). The finding that GM-CSF treatment results in DC expansion and an accumulation of Tregs suggests a potential mechanism for its function in immune tolerance (26, 28, 29).

In this study, we show that CD8α+ DCs are uniquely able to induce polyclonal Tregs to undergo proliferation in splenocyte cultures through both MHC II-dependent and -independent interactions. Molecules capable of activating or expanding DCs, including GM-CSF and TLR ligands, enhanced this Treg division through direct effects on DCs. In vivo expansion of DCs also correlated with an increase in the frequency and proliferation of polyclonal Tregs. Treg proliferation depended strictly on IL-2, which Tconvs produced upon receiving MHC II signals and B7 costimulation from DCs. Unlike Tconvs, Tregs need only IL-2, B7 costimulation, and unidentified interactions with DCs to proliferate. However, stimulation of Tregs through the TCR further augmented Treg division in the presence of IL-2 production by Tconvs. These data confirm a role for DCs in Treg homeostasis and provide a model in which the interactions between DCs, Tconvs, and Tregs are required to support polyclonal Treg proliferation.

Materials and Methods

Mice

Wild-type (WT) C57BL/6 (B6), WT BALB/c, and IL-3β/β double knockout (IL-3β/βKO) (31) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). I-Aβ (MHC II) knockout (KO) and BALB/c RAG2 KO mice were purchased from Taconic Farms (Germantown, NY). D011.10, MyD88 KO, and Foxp3 GFP-reporter mice were gifts from Drs. David Artis, David LaRosa, and Vijay Kuchroo, respectively. TSI×HA28 mice have been previously described (7) and were also bred onto a BALB/c Foxp3 block (2.4G2), anti–I-Ab–biotin (KH74), anti-CD86 (GL1) FITC, rat control PE, rat IgG1 isotype control PE, rat IgG2b isotype control allophycocyanin; or Molecular Probes, Invitrogen (Carlsbad, CA): Fc block (2.4G2), anti–I-Aβ–biotin (KH74), anti-CD6 (GL1) FITC, rat IgG2a κ isotype control FITC, anti-CD25 PE (PC61), anti–CTLA-4 PE (UC10-4F10-11), anti-Thy1.2 PE (53-2.1), Armenian hamster IgG isotype control PE, rat IgG1 isotype control PE, rat IgG2b κ isotype control PE, anti-CD4 PerCP-Cy5.5 (RM4-5), anti–B220 PerCP-Cy5.5 (RA3-6B2), anti-Brdu allophycocyanin, anti-CD25 allophycocyanin-Cy7 (PC61), and streptavidin-PE-Cy7; Biolegend (San Diego, CA); anti-CD80 PE (16-10A1), anti-folate receptor 4 (FR4) PE (TH6), anti–glucocorticoid-induced TNF (GITR) PE (DTA-1), anti–CD80 Alexa Fluor700 (53-6.7), anti–CD11c allophycocyanin (N418), anti–CD3 Pacific Blue (17A2), and anti–CD19 Pacific Blue (6D5); eBiosciences (San Diego, CA); anti-Brdu FITC (PB2-1), anti–Foxp3 allophycocyanin (FJK-16s), and rat IgG2a κ isotype control allophycocyanin; or Molecular Probes, Invitrogen (Carlsbad, CA): CFSE, LIVE/DEAD Fixable Red Dead Cell Stain Kit. The 6.5 clonotype Ab was previously described (7).

Flow cytometry, cell sorting, and data analysis

Cells were stained in PBS with Dead Cell Stain and blocked with either 2.4G2 or mouse and rat IgG, followed by staining with Abs against surface Ags in staining buffer (2% FBS [HyClone], 0.05% sodium azide in PBS) on ice for 25–30 min, ending with two washes. Isotype control staining was performed where appropriate. For cell sorting, T cells and DCs were purified with Thy1.2 and CD11c magnetic beads using MACS columns (Miltenyi Biotec, Auburn, CA) prior to cell surface staining. Intracellular Foxp3 (eBioscience) and BrdU (BD Pharmingen) staining were performed according to manufacturer’s protocol. Flow cytometry of cells was performed with an LSR II or a FACSCalibur (BD Biosciences, San Jose, CA). Fluorescence activated cell sorting (FACS) was performed with a FACSAria cell sorter (BD Biosciences) at the University of Pennsylvania Flow Cytometry and Cell Sorting Core. FACS-sorted populations were typically of >90–95% purity. Data were analyzed with FlowJo software (TreeStar, Ashland, OR). Dead cells were excluded from analysis with LIVE/DEAD Fixable Red Dead Cell staining. Doubles were excluded using forward scatter height by forward scatter width and side scatter height by side scatter width parameters.

Isolation of unfractionated splenocytes and splenic DCs

Spleens were harvested from mice and processed in single-cell suspension. Spleens were lysed with 0.83% ammonium chloride. For unfractionated splenocyte cultures, spleens were not treated with collagenase. B16 melanoma cells expressing FLT3L were a gift from Dr. Terri M. Lauber. The 5 × 10^6 B16 melanoma FLT3L cells (B16-FLT3L) (32) were injected s.c. into mice. After 2 wk, spleens were harvested and injected with cell number-matched MEM-a with 10% FBS containing 1 mg/ml collagenase D (Sigma-Aldrich) and 50 μg/ml DNase I (Sigma-Aldrich), cut into small pieces, and incubated for 30 min at 37˚C prior to manual vortexing. In some experiments, spleens from untreated WT mice were used for isolation of DCs.

In vitro Treg proliferation assay

Unfractionated splenocytes, FACS-sorted Thy1.2 CD8α–CD25+ or Foxp3–GFP+ Tregs, or Thy1.2 CD8α–CD25+ Tconv cells were labeled with CFSE by washing with room temperature PBS, resuspending at 10^5 cells/ml PBS, and mixing with an equal volume of CFSE containing 750 nM final concentration. Cells were continuously shaken for 9 min with intermittent vortexing. The reaction was quenched with 100% FBS and washed with T cell media (MEM-a [Invitrogen] with 10% FBS, 1% penicillin/streptomycin, 10 mM HEPES, and 1 × 10^-5 M 2-ME) before use. CFSE-labeled unfractionated splenocytes were plated at 5 × 10^6 cells/well in 200 μl T cell media in 96-well flat bottom plates. When using FACS-sorted cells, varying numbers of T cells and DCs or T cell-depleted splenocytes were cocultured depending on the experimental setup, although the ratio of each T cell subset to DCs was kept constant, except where indicated. T cell-depleted splenocytes were obtained from the Thy1.2 flow-through fraction of Thy1.2 MACS purification. In transwell experiments, cell populations were cocultured in 24-well transwell plates (Corning Glass, Corning, NY) containing a 0.4-μm porous membrane insert. Cells were placed either in the insert in 100 μl or in the well beneath in 600 μl media. Cell numbers were scaled up accordingly. Cells were cultured untreated in T cell media or treated with the indicated factors in a 37˚C tissue culture incubator. On the fourth day, cells were harvested, stained, and analyzed by flow cytometry. Multiple cultured wells were combined for staining. Live cell counts were performed using a hemocytometer and Trypan Blue dead cell exclusion.

In vivo BrdU incorporation

WT B6 mice were untreated or injected s.c. with B16 or B16-FLT3L melanoma. Nine days later, a 3-d pulse of BrdU was initiated with 1 mg i.p. injection of BrdU, followed by feeding of BrdU in the drinking water at 1 mg/ml until time of sacrifice on day 12. Intracellular Foxp3 and BrdU staining were performed sequentially according to each manufacturer’s protocol.
Statistical analyses

Data from independent repeats of experiments were graphed as individual data points with a mean ± SEM for each group. Proliferation data from Treg CFSE dilution profiles, gated on live CD4+Foxp3+ cells, were transformed into “Division Index” and “% Divided” data using Flowjo. The “Division Index” measures the average number of divisions a cell that was present in the input population has undergone, whereas the “% Division” measures the percentage of cells in the input population that has undergone at least one cell division. These measurements do not take into account death of the input cell population. These values were graphed and analyzed for statistical significance using Prism (GraphPad, San Diego, CA). The statistical test used to calculate each p value is indicated in the figure legends. The p values <0.05 were considered significant.

Results

GM-CSF selectively promotes the proliferation of Treg in vitro

T cells proliferate when stimulated through their TCR on encounter with their cognate Ag presented by MHC molecules on APCs. Because most T cells that react strongly against self-derived peptides are deleted in the thymus, peripheral T cells would not be expected to proliferate when cultured with syngeneic APCs. However, on culture of both B6 and BALB/c splenocytes in cell culture media alone, we unexpectedly but consistently observed the selective proliferation of a modest fraction of CD4+Foxp3+ Tregs (Fig. 1A), suggesting that Tregs proliferate at a basal level in splenocyte cultures. This preferential Treg division increased significantly with the addition of GM-CSF (Fig. 1B, 1C), in a dose-dependent manner (Fig. 1D). Neither CD4+Foxp3– Tconvs nor CD8α+ cytotoxic T cells proliferated to the same extent under these cell culture conditions (Fig. 1A, 1B), even though all T cell subsets proliferated vigorously when stimulated with anti-CD3 Ab (Fig. 1B). Furthermore, we observed no synergy between GM-CSF and anti-CD3 on Treg proliferation (Fig. 1B). Although we consistently observed increased division of Tregs in B6 and BALB/c splenocytes, there was variability with regards to total Treg numbers. Total cell counts and total Tregs numbers were modestly increased in BALB/c but not B6 splenocytes cultured with GM-CSF compared with media alone (Fig. 1E, 1F). Thus, the proliferation that is observed in GM-CSF–treated splenocyte cultures may model Treg

![Image](https://www.jimmunol.org/content/205/5/2792/F1.jpg)

**FIGURE 1.** CD4+Foxp3+ cells in splenocyte cultures selectively undergo basal proliferation that is enhanced by GM-CSF. A. Splenocytes from BALB/c mice were labeled with CFSE and cultured in T cell media only or (B) in the presence of anti-CD3 (0.5 μg/ml), GM-CSF (10 ng/ml), both, or (G) anti–GM-CSF Ab (20 ng/ml). Four days later, cell division of various T cell subsets was analyzed by flow cytometry. C, Treg Division Index and % Divided data were compiled from 22 independent experiments with B6 or BALB/c splenocytes cultured with or without GM-CSF. D, A GM-CSF dose response was performed with B6 or BALB/c splenocytes and shown as Treg Division index and % Divided data compiled from five independent experiments. E, Live cell count of B6 and BALB/c splenocytes cultured for 4 d with or without GM-CSF from three (B6) and seven (BALB/c) independent experiments. F, Cultures were stained and analyzed by flow cytometry to determine the frequency of live, CD4+Foxp3+ cells after 4 d in culture. From these frequencies, the total number of Tregs after culture was determined for each condition. Data in (E, F) are shown as fold change in GM-CSF–treated compared with untreated control cultures. Histograms in (G) are gated on CD4+Foxp3+ cells and Treg Division Index and % Divided data (G) summarize three independent experiments. In all graphs, individual data points are shown with the mean ± SEM for each group. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 by paired, two-tailed Student t test (C), Wilcoxon’s signed-rank test (E, F), or one-way repeated measures ANOVA and Tukey’s post test (G).
proliferation for homeostatic maintenance. These results show that Tregs display basal proliferation in the absence of overt TCR stimulation in splenocyte cultures, which is enhanced by GM-CSF. GM-CSF belongs to the common β-chain (βc) cytokine family, which also includes IL-3 and IL-5 (33). Both IL-3 and IL-5 enhanced basal Treg division, albeit to a lesser extent than GM-CSF (data not shown), suggesting that the augmentation of basal Treg proliferation extended to other βc cytokines. Because multiple splenic cell types can produce βc cytokines (33), we next tested whether endogenous production of βc cytokines was responsible for the basal level of Treg proliferation. The addition of neutralizing anti–GM-CSF Ab did not affect this basal amount of Treg division (Fig. 1G). Furthermore, Tregs in splenocyte cultures from WT mice and mice doubly deficient in both the IL-3β and the βc signaling chains, IL-3β/βc DKO, exhibited a similar amount of basal proliferation (data not shown), suggesting that endogenous production of βc cytokines was not responsible for basal Treg proliferation. These results indicate that Tregs in cultured splenocytes display a basal level of proliferation that does not depend on, but can be augmented by, βc cytokine signaling.

GM-CSF-expanded CD4+Foxp3+ cells do not arise from Tconvs

The proliferating Tregs in the GM-CSF–treated cultures may have arisen from pre-existing Tregs or Tconvs that had converted to iTregs in our culture conditions. To identify the origin of the proliferating Tregs, we cultured either FACS-sorted Tregs or Tconvs with T cell-depleted splenocytes with or without GM-CSF. We found proliferating CD4+ T cells and CD4+Foxp3+ cells almost exclusively in cultures initially receiving CD4+CD25+ T cells (Fig. 2A), demonstrating that the proliferating Tregs derived predominantly from a CD4+CD25+ population enriched in Tregs rather than the conversion of Tconvs to iTregs during culture.

GM-CSF–expanded CD4+Foxp3+ cells are bona fide Tregs with potent suppressive function

Although Foxp3 is considered a specific marker for Tregs, we wished to determine whether the proliferating CD4+Foxp3+ T cells possessed phenotypic and functional properties of Tregs. Similar to freshly isolated Tregs, the proliferating Tregs in our cultures expressed the IL-2R α-chain (CD25), CTLA-4, FR4, and GITR (Fig. 2B). To test their regulatory function, we cocultured GM-CSF–expanded Tregs and freshly isolated Tregs with Tconv effectors at various Treg/Tconv ratios. Compared with freshly isolated Tregs, Tregs cultured in GM-CSF displayed potent suppressive function toward Tconvs stimulated with T cell-depleted splenocytes or with without GM-CSF for 4 d, followed by analysis of cell division by flow cytometry. The contour plots and histograms are gated on CD4+ cells and are representative of two independent experiments. B, CFSE-labeled BALB/c splenocytes were cultured in the presence of GM-CSF for 4 d and analyzed for Treg markers by flow cytometry. Representative histograms are gated on CD4+ T cells for Foxp3 expression or on CD4+Foxp3+ Tregs for CD25, GITR, CTLA-4, and FR4 expression and show expression levels for the indicated proteins or isotype control (top), on freshly isolated (middle), and cultured GM-CSF-treated cells (bottom). One representative of three independent experiments is shown. C, Freshly isolated BALB/c Tregs (●) and Tregs from 4-d GM-CSF–treated BALB/c splenocyte cultures (▲) were FACS-sorted and cocultured with FACS-sorted freshly isolated BALB/c Tconv effectors and BALB/c irradiated feeder cells at various Treg/Teff ratios for 3 d in the presence of anti–CD3 Ab (0.5 μg/ml). Cultures were pulsed with [3H]thymidine and uptake of irradiated feeders alone was shown (▼). The data are represented as mean ± SEM of triplicate determinations and is representative of four independent experiments.

**FIGURE 2.** Cultured CD4+Foxp3+ cells derive predominantly from pre-existing Treg and exhibit potent suppressive function. A, 1 × 10^6 CFSE-labeled, FACS-sorted BALB/c Tregs (CD4+CD25+) or Tconvs (CD4+CD25−) were cocultured with 4 × 10^5 BALB/c T cell-depleted splenocytes with or without GM-CSF for 4 d, followed by analysis of cell division by flow cytometry. The contour plots and histograms are gated on CD4+CD25+ T cells and are representative of two independent experiments. B, CFSE-labeled BALB/c splenocytes were cultured in the presence of GM-CSF for 4 d and analyzed for Treg markers by flow cytometry. Representative histograms are gated on CD4+ T cells for Foxp3 expression or on CD4+Foxp3+ Tregs for CD25, GITR, CTLA-4, and FR4 expression and show expression levels for the indicated proteins or isotype control (top), on freshly isolated (middle), and cultured GM-CSF-treated cells (bottom). One representative of three independent experiments is shown. C, Freshly isolated BALB/c Tregs (●) and Tregs from 4-d GM-CSF–treated BALB/c splenocyte cultures (▲) were FACS-sorted and cocultured with FACS-sorted freshly isolated BALB/c Tconv effectors and BALB/c irradiated feeder cells at various Treg/Teff ratios for 3 d in the presence of anti–CD3 Ab (0.5 μg/ml). Cultures were pulsed with [3H]thymidine and uptake of irradiated feeders alone was shown (▼). The data are represented as mean ± SEM of triplicate determinations and is representative of four independent experiments.

**DCs induce polyclonal Treg proliferation**

We subsequently investigated whether the effect of GM-CSF on enhancing Treg proliferation was purely cell-intrinsic or whether another splenic cell type was required for this process. To address this question, we cultured FACS-sorted T cells either alone or together with T cell-depleted splenocytes in the presence of GM-CSF. Tregs failed to divide when T cells were cultured alone but proliferated robustly in the presence of T cell-depleted splenocytes (Fig. 3A). These results suggest that Treg proliferation required a non-T cell, accessory splenic cell type. To determine which cell population(s) in the spleen was responsible for inducing Treg proliferation, we cultured FACS-sorted T cells with equal numbers of FACS-sorted CD11c+ DCs, CD19+ B220+ B cells, or F4/80+CD11b+ macrophages in the presence of GM-CSF. The cultures containing DCs stimulated Tregs to proliferate more robustly than those containing

B cells or macrophages (Fig. 3B). In fact, we observed that as few as 25,000 DCs were able to induce robust Treg proliferation in the presence of GM-CSF (Fig. 3C). Thus, these data suggest that DCs are sufficient to support Treg proliferation in culture.

Although GM-CSF is known to act on DCs, it has also been shown to augment TCR-induced Treg proliferation through direct effects on Tregs (27). To distinguish whether GM-CSF acted on DCs and/or T cells in enhancing Treg proliferation, we used IL-3β/βc DKO mice, which are unable to signal through the GM-CSFR. To obtain sufficient numbers of DCs for our experiments, DCs were isolated from mice treated with FLT3L (34). CD4+ T cells and DCs
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We next tested whether other modulators of DC phenotype or function, such as the TLR ligands LPS and CpG DNA, could also promote Treg proliferation. Coculture of total CD4+ T cells with DCs in the presence of LPS and CpG also significantly enhanced Treg proliferation compared with untreated controls (Fig. 3E). To test whether the effects of TLR signaling were on DCs or on T cells, we cocultured CD4+ T cells and DCs from WT B6 and MyD88 KO mice in all possible combinations in the presence or absence of CpG. We observed a trend suggesting that the effect of CpG on Treg proliferation occurred in a MyD88-dependent manner and relied on signaling through DCs but not through T cells (Supplemental Fig. 1). Thus, multiple factors that promote DC activation, survival and/or proliferation augmented DC-induced Treg proliferation.

Having established that DCs promote polyclonal Treg proliferation in vitro, we sought to investigate whether an increase in DCs may lead to enhanced polyclonal Treg proliferation in vivo. We first attempted to increase DC numbers by treating with GM-CSF in vivo, but the administration of GM-CSF to WT B6 or BALB/c mice did not increase the frequency of DCs or Tregs and caused only a modest increase in the frequency of Tregs incorporating BrdU over the short half-life of GM-CSF in vivo (35). Thus, as an alternative approach, we expanded DCs in WT B6 mice using B16-FLT3L and observed an increase in the frequency and total number of DCs in the spleens of inoculated mice compared with untreated mice or those injected with B16 cells that do not express FLT3L (Fig. 4A, data not shown). Concomitant with an increase in DC number, B16-FLT3L–treated mice displayed an increased percentage of Tregs in the spleen (Fig. 4B). Moreover, BrdU incorporation studies revealed that Tregs in the B16-FLT3L–treated mice exhibited increased cell division compared with Tregs from control mice (Fig. 4C). These data demonstrate that expansion of DCs in vivo correlates with expansion of Tregs and an increased rate of Treg proliferation, suggesting that DC interactions with Tregs may regulate Treg homeostasis in vivo.

**FIGURE 3.** DCs preferentially induce Treg proliferation. A, CFSE-labeled unfractionated BALB/c splenocytes or 1 x 10^5 FACS-sorted Thy1.2+ BALB/c T cells cultured alone or with 4 x 10^5 BALB/c T cell-depleted splenocytes were treated with GM-CSF. B, The 1 x 10^5 FACS-sorted B6 CD11c+ DCs, CD19+B220+ B cells, or F4/80+CD11b+ macrophages were cocultured with 1 x 10^7 CFSE-labeled, FACS-sorted Thy1.2+ T cells in the presence of GM-CSF. C, Decreasing numbers of FACS-sorted BALB/c DCs were cocultured with 1 x 10^5 CFSE-labeled, FACS-sorted BALB/c Thy1.2+ T cells in the presence of GM-CSF. Data are representative of four independent experiments. D, The 7 x 10^5 CFSE-labeled, FACS-sorted CD4+ T cells from WT B6 or IL-3β/βc DKO mice were cocultured with 1 x 10^5 FACS-sorted WT B6 or IL-3β/βc DKO DCs with or without GM-CSF. To normalize Treg numbers from WT B6 and IL-3β/βc DKO mice, CD4+CD25+ Tconvs were FACS-sorted separately, CFSE-labeled, and cocultured at a 1:6 ratio. Graphs show fold changes in division index comparing GM-CSF–treated to untreated controls. E, 7 x 10^5 CFSE-labeled, FACS-sorted CD4+ T cells from B6 mice were cocultured with 1 x 10^5 B6 DCs with media alone, LPS (100 ng/ml), or CpG (100 ng/ml). All cell cultures were incubated for 4 d and analyzed for cell division by flow cytometry. Representative histograms are gated on CD4+Foxp3+ cells (B, D, E). Compiled proliferation data are shown as Treg Division Index and % Divided data using B6 and BALB/c cells (B) or B6 cells only (D, E) from four (B), three (D), and five (E) independent experiments and represented as individual data points with the mean ± SEM. *p < 0.05; **p < 0.01 by one-way, repeated measures ANOVA with Dunnett’s (B, E) or Tukey’s (D) post tests.
CD8α− DCs stimulate Treg proliferation through cell-to-cell contact

In the spleen, conventional DCs can be divided broadly into the CD80+ and the CD80−B220− DC subpopulations (36, 37). To determine which subset could support Treg proliferation, we cocultured FACS-sorted CD4+ T cells with each population of FACS-sorted DCs in the presence of GM-CSF. CD80+B220− DCs promoted significantly more Treg proliferation compared with CD80+ DCs (Fig. 5A). Next, we asked whether DC-induced Treg proliferation was contact dependent. We cultured FACS-sorted CD4+ T cells with FACS-sorted CD80+ DCs and FACS-sorted CD80−DCs either in the same chamber or across separate chambers of a 24-well transwell plate and cultured alone or with either FACS-sorted WT or MHC II KO DCs. We observed Treg proliferation only when CD4+ T cells either in the same chamber or across separate chambers of a 24-well transwell plate in the presence of GM-CSF for 4 d. The contents of each chamber of the transwell were analyzed separately by flow cytometry. All histograms are gated on CD4+Foxp3+ events and are representative of four independent experiments.

CD8α+ DCs were cocultured with FACS-sorted WT or MHC II KO DCs, and the frequency of DCs in the presence of GM-CSF is represented as individual data points with the mean ± SEM of three or four mice. Contour plots are gated on live, CD19−CD25−B220−DCs from these mice were measured by flow cytometry. Representative contour plots are gated on CD4+ cells and representative histograms are gated on CD4+Foxp3+ cells. The graphs show compiled data of Treg frequency as a percentage of CD4+ T cells and BrdU incorporation by Tregs (n = 3–7 mice/group combined from two independent experiments) and individual mice are represented along with the mean ± SEM. *p < 0.05; **p < 0.01; and ***p < 0.001 by one-way, repeated measures ANOVA with Tukey’s post test.

FIGURE 4. Expansion of DCs in vivo correlates with increased Treg frequency and proliferation. WT B6 Mice were injected s.c. with or without B16 or B16-FLT3L tumor cells and 9 d later administered BrdU for 3 d. A, Splenic DCs from these mice were analyzed by flow cytometry and the frequency of DCs is represented as individual data points with the mean ± SEM of three or four mice. Contour plots are gated on live, CD19−CD3-negative events. B and C, The frequency (B) and BrdU incorporation (C) of splenic Tregs from these mice were measured by flow cytometry. Representative contour plots are gated on CD4+ cells and representative histograms are gated on CD4+Foxp3+ cells. The graphs show compiled data of Treg frequency as a percentage of CD4+ T cells and BrdU incorporation by Tregs (n = 3–7 mice/group combined from two independent experiments) and individual mice are represented along with the mean ± SEM. *p < 0.05; **p < 0.01; and ***p < 0.001 by one-way, repeated measures ANOVA with Tukey’s post test.

FIGURE 5. CD8α− DCs support Treg division in a cell-contact dependent manner. A, Top panel, 1 × 10^5 FACS-sorted B6 CD8α−B220− or CD8α+ DCs were cocultured with 7 × 10^5 CFSE-labeled, FACS-sorted B6 CD4+ T cells with the presence of GM-CSF for 4 d and CFSE dilution of CD4+Foxp3+ DCs was analyzed by flow cytometry. The Treg Division Index and % Divided are represented as individual data points with the mean ± SEM of four independent experiments. *p < 0.01; **p < 0.001 by paired, two-tailed Student t test. B, The 7 × 10^5 CD11c+ DCs were FACS-sorted and cocultured with 5 × 10^5 CFSE-labeled, FACS-sorted B6 CD4+ T cells either in the same chamber or across separate chambers of a 24-well transwell plate in the presence of GM-CSF for 4 d. The contents of each chamber of the transwell were analyzed separately by flow cytometry. All histograms are gated on CD4+Foxp3+ events and are representative of four independent experiments.

IL-2 production by Tconvs is necessary for Treg proliferation

The coculture experiments described thus far used populations of total T cells or CD4+ T cells rather than purified Tregs. Thus, it remained unclear whether Tconvs were necessary for Treg proliferation or whether DCs stimulated Tregs directly to induce their proliferation. We cultured FACS-sorted Tregs with FACS-sorted DCs alone or in the presence of Tconvs. Only a modest fraction of Tregs proliferated when cultured alone with DCs, whereas Treg division was robust when Tconvs were added back to the cultures (Fig. 6A), suggesting that Tconvs were required for optimal Treg proliferation. Because of the limitations of FACS, our sorted Treg populations usually contained ∼5% contaminating Tconvs, which may explain the modest Treg division in cocultures of Tregs and DCs.

Because cell contact between DCs and T cells appeared critical and cognate Ag has been implicated in promoting Treg proliferation (2, 17), we next tested whether MHC II expression by DCs was required to support Treg proliferation by coculturing FACS-sorted CD4+ T cells with either FACS-sorted WT or MHC II KO DCs. We observed Treg proliferation only in cocultures of CD4+ T cells and MHC II-expressing DCs (Fig. 6B). However, it was unclear whether MHC II interactions were necessary between DCs and Tregs, between DCs and Tconvs, or both. To address this issue, FACS-sorted Tregs and Tconvs were separated by a cell-impermeable membrane in a transwell plate and cultured alone or with either FACS-sorted WT or MHC II KO DCs. We observed Treg proliferation only when Tregs and DCs were in direct contact and when Tconvs were cocultured with MHC II-expressing DCs across the transwell (Fig. 6C). These data suggest that DC-induced Treg proliferation depended on a soluble factor produced from Tconv interactions with MHC II-expressing DCs. Surprisingly, MHC II expression was not required by DCs that were in contact with Tregs (Fig. 6C). Thus, Tregs required an MHC II-independent, cell contact-dependent interaction with DCs to divide.

We next sought to identify the soluble factor produced by Tconv–WT DC interactions that was required for Treg proliferation. A logical candidate was IL-2, because of its central role in Treg survival and proliferation (1, 16, 24). Indeed, IL-2 was detected in supernatants of cocultures of CD4+ T cells with WT DCs, but was barely detectable in cocultures of CD4+ T cells and MHC II KO DCs (Fig. 6D). Moreover, the addition of anti–IL-2 neutralizing Abs to
unfractionated splenocyte cultures decreased both the frequency of Tregs and their proliferation significantly (Fig. 6E). These data suggested that MHC II-independent signals from DCs and a source of IL-2 might be sufficient to stimulate Treg proliferation. In support of this notion, although IL-2 had no effect on Treg proliferation when Tregs were cultured alone, coculture of Tregs with MHC II KO DCs in the presence of exogenous IL-2 was sufficient to induce Tregs to divide (Fig. 6F). These data demonstrated that MHC II expression by DCs is required for stimulation of Tconvs to produce IL-2. This paracrine IL-2 cooperated with contact-dependent but MHC II-independent interactions between DCs and Tregs to induce Treg proliferation.

**TCR stimulation and B7 costimulation cooperate with IL-2 to stimulate Treg proliferation**

To determine which molecules on DCs might provide the MHC II-independent signals to Tregs, we examined the surface phenotype of FACS-sorted DCs cultured with GM-CSF and TLR ligands. We first observed that only GM-CSF treatment, but not LPS or CpG treatment, increased the number of DCs in culture (Fig. 7A), suggesting that increased DC numbers alone could not explain the effects of enhanced Treg proliferation. In addition, cultured DCs upregulated the expression of CD80, CD86, and MHC II in the presence of GM-CSF, LPS, and CpG compared with untreated media alone (Fig. 7B). Blockade of B7 signals from DCs with CTLA-4–Ig abrogated much of the Treg proliferation observed in cocultures of FACS-sorted CD4+ T cells and WT DCs (Fig. 7C), likely resulting from the failure of Tconvs in these cultures to produce IL-2 (Fig. 6D). In addition, CTLA-4–Ig partially but significantly attenuated the proliferation of FACS-sorted Tregs cocultured with MHC II KO DCs in the presence of IL-2 (Fig. 7D). These data suggest that DC-derived B7 signals were important for both the production of IL-2 by Tconvs and also as a direct signal to Tregs that contributes to their MHC II-independent proliferation.

Although MHC II expression on DCs was not necessary for Treg proliferation, we consistently observed increased Treg division when Tregs were cocultured with WT DCs compared with MHC II KO DCs in the presence of IL-2 (Fig. 8A). These data implied that MHC II/TCR interactions might augment Treg division. To further investigate the role of TCR stimulation by cognate Ag, we used the TS1×HA28 mouse, which possesses a transgenic TCR specific for the S1 peptide of hemagglutinin and a second transgene expressing hemagglutinin protein, resulting in the development of S1-specific Tregs. To dissociate paracrine IL-2 from TCR signals delivered to Tregs, TS1×HA28 Tregs were cocultured with Tconvs from the sorted B6 WT or MHC II KO DCs in the indicated combinations in separate chambers of transwell plates. D, IL-2 content of supernatants from cocultures of GM-CSF-treated B6 T cells and WT or MHC II KO DCs were detected by ELISA. Results are expressed as mean IL-2 concentration ± SEM of triplicate determinations. E, CFSE-labeled BALB/c splenocytes were cultured in GM-CSF in the presence or absence of anti–IL-2 neutralizing Ab (20 μg/ml). F, 1 × 10^5 CFSE-labeled FACS-sorted B6 Tregs were cocultured with IL-2 (50 U/ml) or cocultured with 1 × 10^5 FACS-sorted B6 MHC II KO DCs in the presence or absence of IL-2. All cell cultures contained GM-CSF (10 ng/ml) and were incubated for 4 d. Representative histograms are gated on CD4+Foxp3+ cells and were shown in the top panels of (A, B, E), whereas compiled proliferation data are shown as Treg Division Index and % Divided data using B6 and BALB/c cells (A) or B6 cells only (B, E) from eight (A), five or eight (B), and three (E) independent experiments. In all graphs, individual data points are shown with the mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 by paired, two-tailed Student t test (A, B) or by one-way, repeated measures ANOVA with Dunnett’s (E) post test.
DO11.10 TCR transgenic mouse, whose Tcons respond not to S1 but instead to an OVA-derived peptide. Thus, stimulation with OVA peptide would result in TCR-mediated activation of DO11.10 Tcons but not TS13 HA28 Tregs, providing a source of IL-2 but not direct TCR signals to the TS13 HA28 Tregs. We cultured FACS-sorted TS13 HA28 Tregs and DO11.10 Tcons in the presence of irradiated splenocyte feeder cells and stimulated the cultures with S1 peptide, OVA peptide, or both peptides. We observed TS13 HA28 Treg proliferation in the presence of either S1 or OVA peptide, suggesting either TCR or IL-2 signals separately could induce Treg proliferation (Fig. 8B). However, the presence of both S1 and OVA peptide further enhanced TS13 HA28 Treg proliferation (Fig. 8B).

FIGURE 7. DCs deliver B7 costimulatory signals to promote Treg proliferation in conjunction with IL-2. A, 1 × 10⁵ FACS-sorted B6 DCs were cultured with media alone, GM-CSF (10 ng/ml), LPS (100 ng/ml), or CpG (100 ng/ml) for 4 d. CD11c+MHC II+ DCs were analyzed by flow cytometry. DC numbers were calculated based on the number of CD11c+MHC II+ cells per 1 × 10⁵ acquired events and are represented as individual data points with the mean ± SEM from three independent experiments. B, Representative histograms of cell surface marker expression for each treatment condition are shown along with appropriate isotype controls or staining of KO cells. The vertical lines in each histogram delineate the threshold used to calculate the percentage of DCs that express high levels of each cell marker analyzed. Cumulative data from three independent experiments are represented as individual data points with the mean ± SEM in the bottom panel.

GM-CSF with or without CTLA-4-Ig (20 μg/ml) for 4 d. Representative histograms in top panels of C and D are gated on CD4+Foxp3+ cells or (B) CD4+Foxp3+6.5+ cells. The 6.5 is an anti-clonotypic TS1 TCR Ab. Compiled data from five (A) or four (B) independent experiments are shown as individual data points and with the mean ± SEM of Treg Division Index or % Divided in the bottom panels. *p < 0.05; **p < 0.001 by paired, two-tailed Student t test (A) or one-way, repeated measures ANOVA with Dunnnett’s post test (B).

FIGURE 8. TCR and IL-2 signals cooperate to induce optimal Treg proliferation. A, 1 × 10⁵ CFSE-labeled, FACS-sorted B6 Tregs (CD4+ CD25+) and 1 × 10⁵ FACS-sorted B6 WT or MHC II KO DCs were cocultured in the presence of GM-CSF with or without IL-2 (50 U/ml) for 4 d. B, 2 × 10⁵ CFSE-labeled, FACS-sorted TS1×HA28 Tregs (CD4+ CD25+) and DO11.10 Tcons (CD4+CD25−) were cocultured with 1 × 10⁵ irradiated BALB/c or BALB/c Rag2 KO feeder splenocytes in media alone, with S1 peptide (1 μM), OVA323–339 peptide (1 μM), or both peptides together for 4 d. Cell division was analyzed by flow cytometry. Representative histograms in top panels are gated on (A) CD4+Foxp3+ cells or (B) CD4+Foxp3+6.5+ cells. The 6.5 is an anti-clonotypic TS1 TCR Ab. Compiled data from five (A) or four (B) independent experiments are shown as individual data points and with the mean ± SEM of Treg Division Index or % Divided in the bottom panels. *p < 0.05; **p < 0.001 by paired, two-tailed Student t test (A) or one-way, repeated measures ANOVA with Dunnnett’s post test (B).
These results demonstrate that although Tconv-derived IL-2 can support Treg proliferation independent of MHC II signals, the combination of cognate Ag and IL-2 signals result in optimal stimulation of Treg proliferation (Fig. 9).

Discussion

Despite the large body of literature on Tregs, the process of Treg homeostasis remains incompletely understood. In this study, we demonstrated that CD8α− DCs are crucial in the initiation and coordination of polyclonal Treg proliferation. DCs are involved in this process in multiple ways, requiring direct interactions with both Tconvs and Tregs. DCs stimulated Tconvs to produce IL-2 in an MHC II and B7-dependent manner. This IL-2, in conjunction with MHC II-independent costimulatory signals from DCs, was sufficient to induce Treg proliferation. Although dispensable, TCR stimulation of Ag-specific Tregs by cognate Ag combined with paracrine IL-2 to promote optimal Treg division. Based on these data, we propose a model to describe the interactions among DCS, Tconvs, and Tregs that are required for optimal Treg proliferation. This model may provide insight into the mechanisms underlying the physiologic proliferation and homeostasis of polyclonal Tregs (Fig. 9).

By culturing unfractionated splenocytes with GM-CSF, we observed the induction of a selective proliferation of bona fide polyclonal Tregs. To our knowledge, our report is the first to demonstrate the ability of Tregs to proliferate in splenocyte cultures without overt TCR stimulation or exogenous IL-2. This system allowed us to dissect the cellular and molecular interactions underlying the proliferation of polyclonal Tregs. Several groups have reported that distinct subsets of DCs have roles in inducing Ag-specific Treg expansion (17–19) or conversion of Tconvs into iTregs (38–40). Our data add to these findings and demonstrate that DCs are crucial regulators of polyclonal Treg proliferation. However, some have also argued that DCs may be dispensable for Treg generation and maintenance, because Treg frequency is normal in CD11c-BTmice that constitutively lack DCs (41). In contrast, a recent report demonstrated that acute depletion of DCs in vivo results in decreased Treg numbers and proliferation, which leads to an increase in T cells that are competent to produce inflammatory cytokines (25). This apparent discrepancy may be explained by compensatory mechanisms that may be initiated in the constitutive absence of DCs, which allows a different cell type to substitute for DCs to maintain Treg homeostasis. Moreover, the physiologic proliferation of Tregs was not measured in the context of the constitutive absence of DCs (41).

The finding that GM-CSF can enhance a basal level of Treg proliferation in vitro fits with its role in increasing the survival and proliferation of monocyte lineage cells (30). Unlike GM-CSF and TLR ligands, FLT3L did not enhance DC-induced Treg proliferation in unfractonated splenocyte cultures or cocultures of CD4+ T cells and DCs, despite promoting DC survival and/or proliferation in vitro (data not shown). Conversely, FLT3L has clear effects on DC expansion and Treg proliferation in vivo (Fig. 4) (25, 42). A potential explanation is that FLT3L treatment of DCs in vitro prevents their maturation, as these DCs do not upregulate B7 costimulation molecules (data not shown), and thus FLT3L-treated DCs may be inefficient simulators of Treg proliferation. Perhaps in vivo administration of FLT3L facilitates differentiation of DCs from hematopoietic progenitors and these DCs can mature in the appropriate microenvironment, away from FLT3L signals.

Although unmodified recombinant GM-CSF was ineffective at expanding DCs or Tregs when administered in vivo, formulations of polyethylene glycol-modified GM-CSF with enhanced t1/2 may hold promise for this purpose. In support of its use, numerous mouse models of autoimmunity, including autoimmune diabetes and myasthenia gravis, can be ameliorated through administration of GM-CSF (26, 28, 29). Furthermore, GM-CSF could be potentially used to expand autologous Tregs for therapy in vitro. However, as an increase in the number of Tregs was observed only with GM-CSF–treated BALB/c splenocytes (Fig. 1F), differences in genetic background will likely contribute to whether Tregs expand in response to exogenous GM-CSF. Nevertheless, GM-CSF–induced Treg proliferation requires only autologous DCs and T cells, without the addition of supraphysiologic doses of TCR stimuli or IL-2. Therefore, Tregs generated in this manner may more closely resemble physiologically derived Tregs and be more appropriate for therapy.

Two recent publications (25, 42) demonstrated a role for DCs in polyclonal Treg proliferation, but they presented conflicting data on the requirement of MHC II expression by DCs in this process. Although one study indicated that DC-induced Treg proliferation is MHC II-independent (42), the other argued that MHC II expression by DCs is required for Treg homeostasis (25). Our data support the conclusions of both studies, as although DC expression of MHC II is not required for Treg proliferation in our culture system, stimulation of the TCR through cognate Ag/MHC II can supplement IL-2 production by Tconvs to enhance Treg division further. Although the precise requirements for Ag/MHC II-independent proliferation of Tregs is unclear at this time, we showed that DC-derived B7 costimulation plays a partial role in supporting Treg proliferation both in the presence and absence of MHC II. The identification of additional DC-derived molecules involved in inducing Treg proliferation and promoting Treg homeostasis warrants further study.

Our work has potential bearing on the controversial question of the composition of the Treg TCR repertoire. Studies on nTreg development suggest a model in which strong TCR signals against self-Ags specify developing thymocytes to the Treg lineage (7, 43). According to this model, interaction with self-Ags in the periphery allows Tregs to proliferate and maintain their numbers in the steady-state (8, 21, 23, 25). Although studies demonstrating a bias of polyclonal Treg TCRs toward self-reactivity support this notion (21, 23), others have argued that the Treg TCR repertoire does not display such skewing (22). Our data suggest the possibility that both Ag-dependent and -independent pathways exist to induce Tregs to proliferate and maintain their homeostasis. An Ag-independent mode of Treg proliferation might ensure survival of Tregs against Ags that are sequestered or are in inaccessible compartments, so that a diverse repertoire of Tregs can be continuously maintained. Furthermore, the ability of Tregs to divide in an Ag-independent but IL-2 dependent fashion may represent an inherent inhibitory feedback mechanism
couples T cell activation to immunosuppression to restrain immune responses.

It has been suggested that Treg homeostasis is maintained, at least in part, by proliferation of peripheral Tregs to balance losses of this important T cell subset. We have identified the DC as a key cell type that initiates and coordinates this Treg division. Further studies are required to address the relevance of these DC/Treg/Tconv interactions in the steady-state and during immune responses in vivo.

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Disclosures
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