Dendritic cells induce antigen-specific regulatory T cells that prevent graft versus host disease and persist in mice

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Regulatory T cells (T reg cells) that express the Foxp3 transcription factor and suppress immunity are generated in the thymus and are called nT reg cells (Marie et al., 2005; Sakaguchi, 2005). CD4+Foxp3+ T reg cells are also induced (iT reg cells) in the periphery upon TCR stimulation in the presence of TGF-β (Shevach et al., 2008) with either anti-CD3 (Chen et al., 2003) or antigen-presenting DCs (Luo et al., 2007; Yamazaki et al., 2007). The vitamin A metabolite all-trans retinoic acid (ATRA) enhances iT reg cell formation along with TGF-β (Benson et al., 2007; Coombes et al., 2007; Mucida et al., 2007; Sun et al., 2007; von Boehmer, 2007; Hill et al., 2008). Antigen-specific iT reg cells have the potential to suppress autoimmunity, allergy, and transplantation (Horwitz et al., 2004). However, a major gap, which limits their therapeutic use, is to sustain Foxp3 expression after adoptive transfer in vivo (Sakaguchi et al., 2008; Edinger, 2009).

In the case of nT reg cells, adoptively transferred polyclonal populations can ameliorate immune pathology (Roncarolo and Battaglia, 2007) and recently were found to exhibit stability under physiological and inflammatory conditions (Rubtsov et al., 2010). Nevertheless, there are several studies reporting that T reg cells lower their expression of the essential transcription factor Foxp3 in the context of inflammation (Wan and Flavell, 2007; Tang et al., 2008; Murai et al., 2009; Zhou et al., 2009). Less certain, however, is whether iT reg cells, especially antigen-specific iT reg cells induced in vitro, can sustain Foxp3+ T reg cells, after proliferating and differentiating into antigen-specific suppressive T cells, can persist for long periods while suppressing a powerful inflammatory disease.
In this paper, we will show that induced Foxp3+ T reg cells can have many features of an adaptive immune response. We find that mouse spleen DCs are specialized inducers of antigen-specific and suppressive iT reg cells from the alloreactive T cell repertoire, in the presence of exogenous TGF-β and ATRA. Although prior studies either failed to resealuate iT reg cells (Floess et al., 2007) or required exogenous addition of the suppressive cytokine TGF-β (Polansky et al., 2008; Koenencke et al., 2009), a cytokine which might not be available under inflammatory condition in vivo, we observe that iT reg cells can be restimulated specifically by DCs in vitro in the absence of TGF-β. Importantly, the DC-induced Foxp3+ iT reg cells suppress graft versus host disease (GVHD), maintaining foxp3 expression for 6 mo and acquiring a demethylated foxp3 CNS2 sequence comparable to nT reg cells.

RESULTS

DCs induce antigen-specific suppressive iT reg cells from a polyclonal T cell repertoire in the presence of added TGF-β and ATRA

To induce iT reg cells from the polyclonal repertoire, we tested splenic BALB/c DCs as stimulators of C57BL/6 T cells in a mixed leucocyte reaction (MLR) together with TGF-β, a known co-factor for iT reg cell development, according to Chen et al. (2003). To deplete the starting B6 CD4+ responding T cells of preexisting T reg cells, we always used B6.FIR mice in which the Foxp3 promoter drives RFP expression (Wan and Flavell, 2005). CD4+CD25+Foxp3+ (RFP+) cells were isolated by FACS sorting and stimulated with DCs plus 20 ng/ml TGF-β alone or with ATRA, a recently described co-factor for T reg cell development in the periphery (see Introduction). Without TGF-β, DCs induced a vigorous MLR, but only 1% of proliferating CFSE-low cells was Foxp3+. Addition of TGF-β or TGF-β plus ATRA greatly increased the induction of CD4+CD25+Foxp3+ (RFP+) cells to ~20 and 70%, respectively, of the viable cells (Fig. 1 A, left FACS). At an optimal dose of 20 ng/ml TGF-β, increasing doses of ATRA up to 10 nM increased the frequency and absolute number of induced CD4+CD25+Foxp3+ cells (Fig. 1 A, right), with no induction by ATRA alone (not depicted). Only the CD11c+ spleen cells induced T reg cells (Fig. 1 B). Splenocytes selected by anti-CD11c beads are not pure DCs, so we further separated them into CD11c high, intermediate, and low fractions. The CD11c+ high DCs, which are also MHC II high, were the main active cells, whereas CD11c-intermediate cells that include macrophages and plasmacytoid DCs had much lower activity, and CD11c−low lymphocytes were almost inactive (Fig. S1 A).

To test if the Foxp3+ iT reg cells exhibited suppressive activity, 5-d MLR cultures were sorted into dominant RFP+ fractions that were then added in graded doses to a fresh MLR between CFSE-labeled C57BL/6 CD45.1+ T cells and BALB/c DCs to detect suppressive capacity (reduced CFSE dilution). We also tested C57BL/6 Foxp3+ nT reg cells expanded in MLR with IL-2 (Fig. 1 D). As in C, but the suppressive activity of sorted iT reg cells induced with 20 ng/ml TGF-β alone or together with ATRA were compared. (E) iT reg cells generated as in A were added to cultures comprised of BALB/c DCs and either CFSE-labeled naïve C57BL/6 CD45.1+ CD4+ T cells (1° MLR) or sorted activated allospecific CD4+CD25+ cells generated from CD4+CD25− cells in a 1° MLR (2° MLR). 5 d after addition of sorted iT reg cells, suppression of either 1 or 2° MLR was compared by calculating the percentage (% of MLR) of CFSE-diluted cells out of total proliferating cells in MLR (see Fig. S1 C) or percentage of CFSE-diluted cells. One representative experiment of three is shown. Error bars denote mean ± SD. * P < 0.05.

Figure 1. DCs induce suppressive CD4+CD25+Foxp3+ (RFP+) cells from a polyclonal T cell repertoire. (A) Sorted C57BL/6 CD4+CD25+ Foxp3+ (RFP+) cells were cultured 5 d with BALB/c splenic CD11c+ DCs alone (MLR), or with 20 ng/ml TGF-β with or without graded doses of ATRA. Typical FACS plots (top) and data on the frequency (bottom, blue) and absolute number (bottom, red) of CD4+CD25+Foxp3+ (RFP+) iT reg cells at day 5 (mean ± SD). (B) As in A, but T reg cell induction (dashed and solid lines indicate frequency and absolute number, respectively) in an MLR with CD11c− DC-enriched (CD11c Pos) and CD11c− DC-depleted (CD11c Neg) BALB/c spleen cells with 20 ng/ml TGF-β and 10 nM ATRA. (C) iT reg cells, as well as CD25− Foxp3+ (RFP−; n T reg) cells, were isolated from a day-5 MLR as in A. Cells were then added to a fresh 5-d MLR between CFSE-labeled C57BL/6 CD45.1+ T cells and BALB/c DCs to detect suppressive capacity (reduced CFSE dilution). We also tested C57BL/6 Foxp3+ nT reg cells expanded in MLR with IL-2. (D) As in C, but the suppressive activity of sorted iT reg cells induced with 20 ng/ml TGF-β alone or together with ATRA were compared. (E) iT reg cells generated as in A were added to cultures comprised
and 2° MLR whether we enumerated the Foxp3− response as frequency (Fig. 1 E) or total numbers (Fig. S1 D). Therefore, DC-induced iT reg cells suppress secondary or activated T cells at least as well as a primary response.

Polyclonal CD4+CD25+Foxp3+ iT reg cells are specific for antigens on the inducing DC

To evaluate the antigenic specificity of suppression, we first induced C57BL/6 CD45.2+CD4+CD25−Foxp3− (RFP−) cells to become iT reg cells with either BALB/c or SJL DCs. Then we purified the RFP+ iT reg cells (suppressors) to test suppression of fresh C57BL/6 CD45.1+CD4+ cells in a primary MLR stimulated with either BALB/c or SJL DCs (stimulators; Fig. S2). When iT reg cells had been induced by BALB/c DCs, they more efficiently suppressed an MLR stimulated by Balb DCs than iT reg cells induced by SJL DCs (Fig. 2 A and Fig. S2), whereas the reverse was observed with an MLR stimulated with SJL DCs (stimulators; Fig. S2). When iT reg cells had been induced by BALB/c DCs, they more efficiently suppressed an MLR stimulated by Balb DCs than iT reg cells induced by SJL DCs (Fig. 2 A and Fig. S2), whereas the reverse was observed with an MLR stimulated with SJL DCs (stimulators; Fig. S2).

Figure 2. Polyclonal CD4+CD25+Foxp3+ iT reg cells are specific for antigens on the inducing DC. Sorted C57BL/6 CD4+CD25−Foxp3− (RFP−) cells were incubated with either BALB/c (green) or SJL (gray) splenic CD11c+ DCs with TGF-β and ATRA. After 5 d, the iT reg cells (CD4+CD25+Foxp3+RFP+) were sorted and compared for suppression (as in Fig. 1 C) of an MLR (blue) stimulated with either BALB/c (A) or SJL (B) CD11c+ DCs. One representative experiment of four is shown. Error bars denote mean ± SD. *, P < 0.05 comparing suppression between the two iT reg cells sources; **, P < 0.05 comparing suppression to MLR.

To find out if iT reg cells could suppress an MLR when the responding T cells had been preactivated, we isolated activated CD4+CD25−Foxp3− cells at day 3–4 of an MLR culture. When CFSE-labeled activated and naive C57BL/6 T cells were each challenged with allogeneic BALB/c DCs, the proliferation of activated T cells in a 2° MLR was four to five times stronger than the 1° MLR (Fig. S1 D, left data points), as expected. Nevertheless, iT reg cells suppressed both 1° and 2° MLR whether we enumerated the Foxp3− response as frequency (Fig. 1 E) or total numbers (Fig. S1 D). Therefore, DC-induced iT reg cells suppress secondary or activated T cells at least as well as a primary response.

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Figure 3. Splenic DCs specifically restimulate allo-iT reg cells expansion. (A) Sorted iT reg cells induced with either BALB/c or SJL CD11c+ DCs were CFSE labeled and stimulated in various ratios with either BALB/c (left) or SJL (right) CD11c+ DCs. (B) As in A, but the restimulation cultures contained either control or anti–TGF-β mAb (left) or anti–IL-2 mAb (right). 3 d later, live proliferating (CFSE diluted) CD3+CD4+Foxp3+ (RFP+) cells were analyzed by FACS. (C) Proliferating iT reg cells numbers with or without restimulation with BALB/c DCs over 3 d. (D) As in C, but after 3 d of DC restimulation, the CFSE-diluted CD3+CD4+Foxp3+ (RFP+) iT reg cells and CD3+CD4+ Foxp3− (RFP−) ex-Foxp3 cells were sorted and incubated for 2 d, and IL-2 in the medium was measured by ELISA. One representative experiment of three is shown. Error bars denote mean ± SD.
Splenic DCs specifically restimulate allo-iT reg cells but independently of TGF-β
To determine if iT reg cells could undergo restimulation, and to further assess their specificity, we set up secondary DC restimulation cultures. Successful restimulation would also indicate that iT reg cells had a measure of stability, which to our knowledge has not been established for antigen-specific iT reg cells from the polyclonal repertoire. When B6 iT reg cells were induced by BALB/c DCs, they were restimulated more vigorously by BALB/c than SJL DCs (Fig. 3 A, left, blue vs. pink), whereas iT reg cells induced by SJL DCs responded better to SJL versus BALB/c (Fig. 3 A, right, pink vs. blue). In the suppression cultures, simultaneously with suppression of T cell proliferation, the iT reg cells proliferated achieving a high ratio of CFSE-diluted iT reg cells to suppressed CFSE-diluted T cells (Fig. S3 A).

To begin to understand mechanisms for restimulation by DCs, we used blocking mAbs. Surprisingly restimulation of iT reg cells no longer required TGF-β (Fig. 3 B, left) but was totally dependent on IL-2 (Fig. 3 B, right). Kinetic studies showed that restimulated iT reg cells were already expanding at day 2 of the secondary cultures (Fig. 3 C, left). Approximately 25–35% of the restimulated iT reg cells lost Foxp3 on day 2–3 (Fig. 3 C bottom right; and Fig. S3 B), whereas in the absence of DCs, iT reg cells did not proliferate (Fig. 3 C top right) and only ~5% lost Foxp3 (Fig. S3 B). When we sorted Foxp3+ and Foxp3− cells from the restimulation cultures, only Foxp3− or “ex− Foxp3” were producing IL-2 (Fig. 3 D). Also, the sorted restimulated IL-2−dependent daughter Foxp3+ cells were functional and able to suppress fresh T cell proliferation (Fig. S3 C). These data indicate that polyclonal iT reg cells induced by DCs are able to be restimulated upon contact with specific antigen and IL-2, and that most continue to express Foxp3. Given these properties, we decided to proceed with in vivo experiments to determine if iT reg cells could persist under inflammatory conditions.

iT reg cells induced by DCs suppress GVHD.
To study the persistence and function of allospecific iT reg cells in vivo, we created a GVHD model based on prior work (Taylor et al., 2002). We transferred 10^6 C57BL/6 CD4+CD45RBhiCD25−CD45.1+ cells into 4–5-wk non-irradiated BALB/c scid (C.B-17) mice lacking lymphocytes. GVHD gradually developed over 1.5–2 mo with weight loss, diarrhea, and skin lesions, including alopecia and eruptions, starting around the eyes and nose and extending to the back (Fig. S4 and Table I). Splenomegaly and lymphadenopathy developed (Fig. S4), as well as systemic multiorgan inflammation mainly in the bowel, liver, skin, and lung, whereas the disease-inducing C57BL/6 CD4+CD45RBhiCD25−CD45.1+ cells into 4–5-wk non-irradiated BALB/c scid (C.B-17) mice lacking lymphocytes. GVHD gradually developed over 1.5–2 mo with weight loss, diarrhea, and skin lesions, including alopecia and eruptions, starting around the eyes and nose and extending to the back (Fig. S4 and Table I). 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eliminate disease-inducing cells but rather suppressed them. When we followed the mice for longer periods, iT reg cells were found in lymphoid and other organs 6 mo after adoptive transfer (Fig. 5 A), although the mice were starting to have signs of GVHD (Table I and Fig. S4 D).

Because previous studies attributed therapeutic failures with iT reg cells to Foxp3 gene instability (Edinger, 2009), we studied CpG methylation status in CNS2 (Fig. 5 B), a region of the Foxp3 gene linked to maintenance and stability of this transcription factor (Zheng et al., 2010). Immediately after induction, iT reg cells had almost fully methylated CpG in CNS2 (Fig. 5 B) compared with demethylation in nT reg cells. However, CNS2 demethylation in iT reg cells increased significantly 3 d after restimulation in vitro but, more strikingly, 1.5 mo after transfer of the iT reg cells in vivo, when demethylation matched nT reg cells (Fig. 5 B). Thus iT reg cells stability and Foxp3 CNS2 demethylation are both associated with prolonged iT reg cells survival and GVHD remission in vivo.

iT reg cells induced by DCs can treat active GVHD
To verify that iT reg cells can persist and function when adoptively transferred under ongoing conditions of inflammation and active GVHD, we conducted a treatment experiment. The mice were treated with iT reg cells at 2 wk, after the onset of weight loss (Fig. 5 C) and up-regulation in IFN-γ (not depicted), and compared with control and prevention groups (iT reg cell treatment at time 0). After treatment with iT reg cells, the mice were monitored for an additional 7 wk. The iT reg cell–treated sick mice regained weight to an almost equivalent level to that of healthy control mice and length of the remission brought about with iT reg cells, we followed the mice for 6 mo. In three experiments, mice treated with iT reg cells survived for 6 mo after disease induction (Fig. 4 A), whereas none of the mice induced with CD4+CD45RBhi cells and treated with PBS survived 2.5 mo. This was associated with a significantly better H&E inflammation score of several organs (Fig. 4 B). Thus, DC–induced antigen–specific iT reg cells can prevent GVHD, extending prior evidence that adoptively transferred nT reg cells also can prevent GVHD (Joffre et al., 2008).

Alloreactive DC-induced T reg cells remain stable for months in vivo and acquire highly demethylated foxp3 CNS2 gene sequences
To determine if the transferred iT reg cells persisted in these recipients, we looked for CD45.2+ cells 2.5 mo after adoptive transfer. Many CD45.2+CD45RBhi cells were observed in all organs tested, including lymphoid tissues (Fig. S5 A), liver, and lung (not depicted). Although these mice were clinically healthy, we could find disease-inducing CD45.1+ cells in all these sites (Fig. S5), suggesting that iT reg cells did not
equivalent to mice that were given iT reg cells as prevention. This is in contrast to untreated mice that continued to lose weight (Fig. 5 C), developed skin manifestation and diarrhea, and died early (not depicted). This significant improvement with treatment was accompanied by the persistence of iT reg cells in secondary lymphoid tissues and organs 7 wk after iT reg cell adoptive transfer to sick mice (Fig. 5 D).

**DISCUSSION**

We have chosen the MLR to generate antigen-specific iT reg cells from a polyclonal repertoire because many T cells are allospecific (Steinman and Witmer, 1978), and there are excellent models of GVHD to study the in vivo function of the iT reg cells. It is known that DCs induce Foxp3+ T reg cells through endogenous (Coombes et al., 2007; Sun et al., 2007; Yamazaki et al., 2007) or exogenous (Luo et al., 2007) TGF-β, using stimulation of bulk populations with anti-CD3 or TCR transgenic T cells with antigen. In this study, we used BALB/c DCs to present antigens to polyclonal allogeneic C57BL/6 Foxp3− CD4+ T cells in the presence of TGF-β and ATRA (although the iT reg cells were also induced when the MLR was run in the reverse direction; unpublished data). We found that the DCs induced iT reg cells efficiently because most of the MLR proliferating cells became Foxp3+ at a DC/T cell ratio of 1:10. These iT reg cells specifically suppressed alloimmunity in vitro, both by naive and previously activated T cells (primary and secondary MLR). Importantly, the iT reg cells also suppressed active GVHD and persisted in vivo under conditions of inflammation, although in vivo conversion to ex-Foxp3 cells was observed (25–30%; Fig. S5 B).

The development of clinical manifestations of GVHD after 6 mo suggests that treatment with iT reg cells in our experiments does not bring to cure, as although they persisted, the iT reg cells did not eliminate the disease-inducing cells (2.5 and 6 mo after adoptive transfer) but rather induced prolonged remission. If treatment with iT reg cells will come to patients in the future, this may suggest that at this point when there is relapse in clinical manifestations, a boost with iT reg cells might be given to renew the remission. In addition, regarding the possibility that iT reg cells converted to ex-Foxp3 will mediate development of GVHD, we injected iT reg cells alone and monitored the mice for 2 mo. During this time, we did not observe any clinical change comparing to healthy mice (unpublished data).

The latter findings were surprising because prior publications questioned the stability of Foxp3+ expression by in vitro generated iT reg cells (Floess et al., 2007). Because of this instability, the therapeutic use of iT reg cells was questioned (Sakaguchi et al., 2008; Edinger, 2009). However, iT reg cells were not previously shown to undergo restimulation in vitro with DCs from lymphoid tissues, which are known to induce T reg cells from antigen-specific TCR transgenic T cells (Kretschmer et al., 2005; Luo et al., 2007; Yamazaki et al., 2007).

We found, in contrast to previous publication (Floess et al., 2007; Polansky et al., 2008; Koenecke et al., 2009), that fresh DCs alone can restimulate iT reg cells that maintain a high rate of Foxp3 expression without the need for exogenous cytokine supplementation. Perhaps our results reflect the very high frequency of iT reg cells in our primary MLR cultures. Nevertheless, not all Foxp3+ iT reg cells induced in a primary MLR were stable. A minority lost Foxp3 expression when rechallenged with DCs, and began to make IL-2 (most of the ex-Foxp3 cells were described to be methylated; Zhou et al., 2009). The population of iT reg cells that maintained Foxp3 exhibited higher levels of CNS2 demethylation in the Foxp3 gene, a marker for stability. IL-2 is known to maintain Foxp3+ nT reg cells in vivo (Oldenhove et al., 2009; Grinberg-Bleyer et al., 2010), and we found that IL-2 generated by ex-Foxp3 cells, but not TGF-β, was required for DCs to restimulate iT reg cells. Several recent publications (Wan and Flavell, 2005, 2007; Tang et al., 2008; Yang et al., 2008; Murai et al., 2009; Zhou et al., 2009) have reported that T reg cells in the context of inflammation lower Foxp3 expression. In contrast, the DC-induced iT reg cells we studied were specifically suppressive and a significant fraction was able to persist and function in vivo and under inflammatory conditions. This encourages the use of DCs to generate antigen-specific iT reg cells for therapeutic use.

**MATERIALS AND METHODS**

**Mice.** We purchased 6–8-wk-old female BALB/c H-2d, C57BL/6 CD45.1 H-2b, and SJL H-2s mice from Taconic and 3–4-wk-old C.B-17 scid (BALB/c background) mice from The Jackson Laboratory. Foxp3-IRES-RFP (FIR) knock-in mice were a gift from R. Flavell (Yale University, New Haven, CT; Wan and Flavell, 2005). We followed guidelines of the institutional animal care and use committee of the Rockefeller University.

**Antibodies and reagents.** We purchased from BD APC conjugated anti-mouse CD25, -CD4, -CD45.1, and -CD11c; Alexa Fluor 700–conjugated anti-CD3, -CD4, and -CD11c; PE-conjugated anti-CD3, -CD19, and -CD49b; FITC-conjugated anti-CD3, -CD19, -CD49b, and isotype control; biotin anti-CD4, -CD8, -DX5, -B220, -CD3, -CD11b, -Ly-6G, and -Ter119 and purified anti-CD16/CD32 (2.4G2). We purchased CD11c and streptavidin beads (SA) from Miltenyi Biotec; CFSE and live dead fixable aqua from Invitrogen; ATRA from Sigma-Aldrich; human TGF-β1, anti-mouse TGF-β (1D11), anti–IL-2, and Ig isotype control from R&D Systems; and IL-2 from Novartis or PeproTech.

**T cells and DCs.** Non-CD4+ lymph node and spleen T cells were removed by MACS beads (Miltenyi Biotec) after coating with biotin anti-CD8α, DX5, B220, CD3, CD11b, Ly-6G, and Ter119 and were further purified with a FACSARia 2 sorter (BD) to >97%. Spleen CD11c+ DCs were partially enriched with anti-CD11c beads (Miltenyi Biotec) and, where indicated, enriched with a FACSARia 2 or FACSVantage (BD) cell sorter as CD11c+CD19−CD24+CD123+ cells (≥95%).

**De novo in vitro induction of T reg cells in the allo-MLR.** CD4+ T cells from C57BL/6 Foxp3− RFP mice were sorted as CD4+CD25+ RFP− cells. These T cells were then co-cultured for 5 d with fresh splenic BALB/c DCs. Induction of CD4+CD25+ RFP− cells was analyzed by FACS (LSR-II; BD) and FlowJo software (Tree Star) and sorted (FACSARia 2) as needed for functional assays.

**In vitro suppression assay.** iT reg cells or activated nT reg cells (CD4+CD25+RFP− from naive mice activated for 5 d with allo-DCs) were co-cultured in round-bottom 96-well plates with CFSE-labeled fresh
CD45.1+CD4+ T cells at the indicated ratios plus allo-DCs (ratio of 1 DC to 3 CD45.1+ CD4+ T cells). After 5 d, proliferation was assessed by FACS (LSR-II; BD) to determine CFSE diluted, live CD45.1+ T cells. Statistical data (Student’s t test) were analyzed using Excel (Microsoft).

Restimulation of iT reg cells. Sorted DC-induced T reg cells were labeled with CFSE and recultured with fresh allo-DCs for several days as indicated, after which CFSE dilution as a marker for iT reg cells proliferation was analyzed by FACS (LSR-II).

GVHD induction, prevention, and treatment. To induce GVHD, 105 sorted (FACS Aria II) CD45.1+CD4+CD45RBlowCD25+CD11c+ cells were injected i.v. into 4–5-wk-old C.B-17 scid (BALB/c background) mice. For prevention, the mice were concomitantly injected with BALB/c DC-induced T reg cells in five separate injections (0.8 x 106 every 3 d over 12 d) to a total of 4 x 106 cells per mouse. The mice were monitored weekly for skin manifestations, diarrhea, weight, and survival. Tissues were stained with H&E to evaluate pathology and scoring was done as previously described (Blazar et al., 1998). CD3+ T cells in different tissues were analyzed for CD45.1 disease-inducing T cells and CD45.2 disease-suppressing iT reg cells by FACS. For treatment, after injection of CD45.1+CD45RBlowCD25+ cells, the mice were monitored for weight loss and up-regulation of IFN-γ and then treatment with iT reg cells was started. Statistical data were analyzed with Prism (GraphPad Software) for nonparametric Mann-Whitney U test and for survival.

Methylation analysis. CpG dinucleotide methylation analysis of the Foxp3 CNS2 (Zheng et al., 2010) sequence was determined by bisulfite treatment of RNase-treated genomic DNA, followed by PCR amplification and pyrosequencing. Pyro Q–CpG was performed by EpigenDX.

Online supplemental material. Fig. S1 shows that DCs generate de novo suppressive CD4+CD25+Foxp3+ (RFP+) from a polyclonal T cell population by foreign antigen. Pyro Q–CpG was performed by EpigenDX.

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Figure S1. DCs generate de novo suppressive CD4+CD25+Foxp3+ (RFP+) from a polyclonal T cell repertoire in the MLR. (A) Sorted C57BL/6 CD4+CD25−Foxp3− (RFP−) spleen cells were cultured 5 d with BALB/c splenic CD11c+ DCs that had been sorted into CD11c-high, -intermediate, and -low fractions, together with 20 ng/ml TGF-β and ATRA. The frequency (left) and total numbers (right) of induced CD4+CD25−Foxp3− (RFP+) iT reg cells by FACS analysis are shown on day 5. (B) iT reg cells were induced as in A, and CD25−Foxp3− (RFP−; double negative [DN]) cells were isolated from a separate MLR with BALB/c splenic CD11c+ DCs at day 5. These were added to a fresh MLR that contained CFSE-labeled CD45.1 C57BL/6 CD4+ T cells and BALB/c DCs to detect MLR suppression (reduced CFSE dilution) 5 d later. We also compared foxp3+ nT reg cells from B6 mice that had been expanded in MLR with IL-2 to iT reg cells induced as in A. (C) FACS plots (top row) of CFSE-labeled naive B6 CD45.1+CD4+ T cells (1° MLR) or sorted activated/effector allospecific CD4+CD25− cells generated from CD4+CD25− cells in 1° MLR (2° MLR) that were incubated with BALB/c DCs with or without sorted B6 anti-BALB/c iT reg cells at the indicated ratios of iT reg cells to CD4+ T cells. 5 d after addition of the FACS-sorted iT reg cells, the suppression of either primary MLR (naive CD4+, top row) or secondary MLR (1° MLR activated/effector, bottom row) was compared by analyzing dilution of CFSE-labeled cells (FACS plot in C and enumeration of absolute proliferating cell number in graph D). Results are each one representative experiment of three. Error bars denote mean ± SD.
Figure S2. Polyclonal CD4+CD25+Foxp3+ iT reg cells are specific for antigens on the inducing DC. Sorted B6 CD4+CD25−Foxp3− (RFP−) cells were incubated 5 d with either BALB/c or SJL, and splenic CD11c+ DCs along with TGF-β and ATRA to induce B6 iT reg cells that were either anti-BALB/c or anti-SJL. Then the two different iT reg cells (CD4+CD25+Foxp3+RFP+; Suppressors) were sorted and compared for their suppressive activity of an MLR that was stimulated with either BALB/c (left) or SJL (right) CD11c+ DCs (Stimulators). Results are one representative experiment of four.

Figure S3. Splenic DCs efficiently restimulate allo-iT reg cells that maintain their suppressive activity. (A) CFSE-labeled iT reg cells were induced with either BALB/c (anti-BALB/c, line 4) or SJL (anti-SJL, line 5) DCs and were restimulated with BALB/c DCs. CFSE-labeled CD45.1+ C57BL/6 CD4+ T cells were incubated with BALB/c DCs alone (line 1) or with either anti-Balb (line 2) or anti-SJL (line 3) iT reg cells. The dilution of CFSE was measured with FACS for 5 d. (B) CFSE-labeled iT reg cells were incubated either alone (top) or restimulated in a secondary culture with DCs (bottom), and the percentage of Foxp3+ cells was measured over 3 d (as in Fig. 3 C). (C) Fresh BALB/c DCs and CFSE-labeled CD45.1 C57BL/6 CD4+ T cells were added to a 3-d culture of iT reg cells restimulated with BALB/c DCs to show that the restimulated iT reg cells retained suppressive activity (reduced CFSE dilution). Results are one representative experiment of three. Error bars denote mean ± SD.
Figure S4. iT reg cells induced by DCs suppress GVHD in vivo. (A) C.B-17 scid (BALB/c) mice were adoptively transferred with allogeneic (B6) CD4^+CD45RB^hiCD25^-CD45.1^+ cells along with PBS and, 2 mo later, the mice were evaluated for skin manifestations of GVHD. (B) The group described in A (left) was compared with control (PBS only) mice (right) for changes in the size of the spleen (top) and lymph nodes (bottom). (C) H&E staining of various organs 2 mo after adoptive transfer with CD4^+CD45RB^hiCD25^- cells + PBS to induce GVHD. (D) C.B-17 scid (BALB/c) mice were treated with PBS only (left) or adoptively transferred with allogeneic C57Bl/6 CD4^+CD45RB^hiCD25^-CD45.1^+ cells to induce GVHD along with iT reg cells (CD4^+CD25^-Foxp3^+) to suppress GVHD (middle). Skin manifestations (left and middle) or weight (right) of mice was measured 6 mo later. Results are one representative experiment of four. Error bars denote mean ± SD.

Figure S5. iT reg cells induced by DCs persist in vivo. (A) C.B-17 scid (BALB/c) mice were adoptively transferred with B6 CD45.1^+CD4^+CD45RB^hiCD25^- T cells (to induce GVHD) or B6 CD45.1^+CD4^+CD45RB^hiCD25^-CD45.1^+ cells and iT reg cells, induced by BALB/c DCs in a 5-d MLR supplemented with TGF-β and ATRA. The indicated lymphoid tissues were compared 2.5 mo later for expression of Foxp3 in CD45.1-positive or -negative cells. (B) As in A, but 1.5 mo after adoptive transfer, iT reg cells were evaluated by FACS for rate of conversion to CD4^+Foxp3^- (RFP^-). The dot plot is gated on live CD3^+ cells after exclusion of CD45.1 (disease inducing) cells. Results are one representative experiment of three.