

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

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Foxp3⁺ regulatory T cells (T reg cells) effectively suppress immunity, but it is not determined if antigen-induced T reg cells (iT reg cells) are able to persist under conditions of inflammation and to stably express the transcription factor Foxp3. We used spleen cells to stimulate the mixed leukocyte reaction (MLR) in the presence of transforming growth factor β (TGF- β) and retinoic acid. We found that the CD11c^{high} dendritic cell fraction was the most potent at inducing high numbers of alloreactive Foxp3⁺ cells. The induced CD4⁺CD25⁺Foxp3⁺ cells appeared after extensive proliferation. When purified from the MLR, iT reg cells suppressed both primary and secondary MLR in vitro in an antigen-specific manner. After transfer into allogeneic mice, iT reg cells persisted for 6 mo and prevented graft versus host disease (GVHD) caused by co-transferred CD45RB^{hi} T cells. Similar findings were made when iT reg cells were transferred after onset of GVHD. The CNS2 intronic sequence of the Foxp3 gene in the persisting iT reg cells was as demethylated as the corresponding sequence of naturally occurring T reg cells. These results indicate that induced 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.

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Abbreviations used: ATRA, all-trans retinoic acid; CNS, conserved noncoding sequence; GVHD, graft versus host disease; MLR, mixed leukocyte reaction.

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 expression and suppressive function upon reengagement with antigen in vivo (Sakaguchi et al., 2008; Edinger, 2009). The instability of iT reg cells is attributed at least in part to a low demethylation status of specific conserved noncoding sequences (CNSs) in the Foxp3 gene, which are fully demethylated in nT reg cells (Floess et al., 2007; Kim and Leonard, 2007; Polansky et al., 2008; Zheng et al., 2010).

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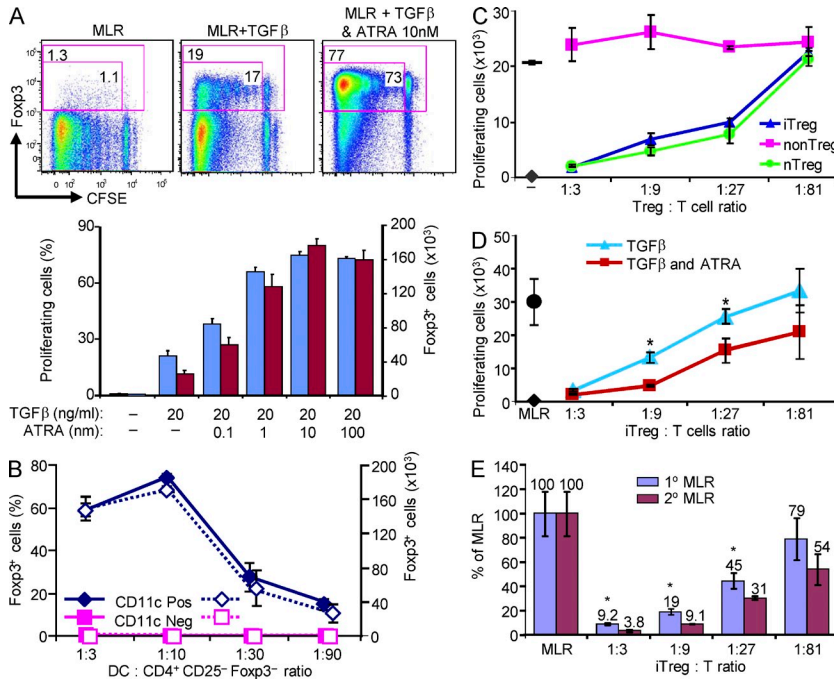


Figure 1. DCs induce suppressive CD4⁺CD25⁺Foxp3⁺ (RFP⁺) cells from a polyclonal T cell repertoire. (A) Sorted C57BL/6 CD4⁺CD25⁻Foxp3⁻ (RFP⁻) cells were cultured 5 d with BALB/c splenic CD11c⁺ DCs alone (MLR), or with 20 ng/ml TGF-β with or without graded doses of ATRA. Typical FACS plots (top) and data 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⁻; non 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 CD4⁺ 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

of BALB/c DCs and either CFSE-labeled naive 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.

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 restimulate iT reg cells (Floess et al., 2007) or required exogenous addition of the suppressive cytokine TGF-β (Polansky et al., 2008; Koenecke 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 leukocyte 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⁺ and minor CD4⁺CD25⁻Foxp3⁻ fractions that were then added in graded doses to a fresh MLR between BALB/c DCs and CD45.1⁺ CFSE-labeled CD4⁺ naive B6 T cells. At ratios of 1:3 to 1:27 iT reg cells/fresh T cells, iT reg cells (Fig. 1 C, blue triangles; and Fig. S1 B), but not Foxp3⁻ cells (pink squares), significantly suppressed the MLR. The suppression by iT reg cells was equivalent to nT reg cells generated in the presence of IL-2 as described previously (Yamazaki et al., 2006;

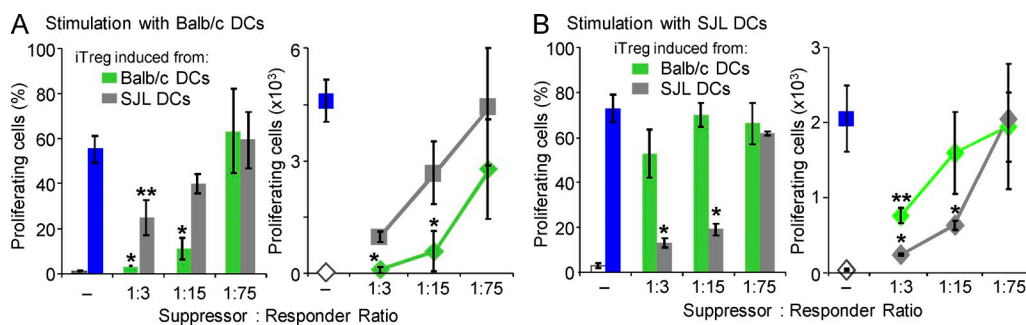


Figure 2. Polyclonal CD4⁺CD25⁺Foxp3⁺ iT reg cells are specific for antigens on the inducing DC. Sorted C57BL/6 CD4⁺CD25⁻Foxp3⁻ (RFP⁻) cells were incubated with either BALB/c (green) or SJL (gray) splenic CD11c⁺ DCs with TGF- β and ATRA. After 5 d, the iT reg cells (CD4⁺CD25⁺Foxp3⁺RFP⁺) were sorted and 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 \pm SD. *, P < 0.05 comparing suppression between the two iT reg cells sources; **, P < 0.05 comparing suppression to MLR.

Fig. 1 C, green circles). iT reg cells generated with ATRA were two- to threefold more effective on a cell-dose basis, indicating that ATRA improved iT reg cell induction both in quantitative (Fig. 1 A) and qualitative (Fig. 1 D) terms. Given these findings, we used T reg cells that were induced with both ATRA and TGF- β in all of the subsequent experiments.

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^o MLR was four to five times stronger than the 1^o MLR (Fig. S1 D, left data points), as expected. Nevertheless, iT reg cells suppressed both

1^o and 2^o 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 (Fig. 2 B and Fig. S2). These “criss-cross” experiments showed that DC-induced polyclonal CD4⁺CD25⁺Foxp3⁺ iT reg cells specifically suppress MLR alloreactivity.

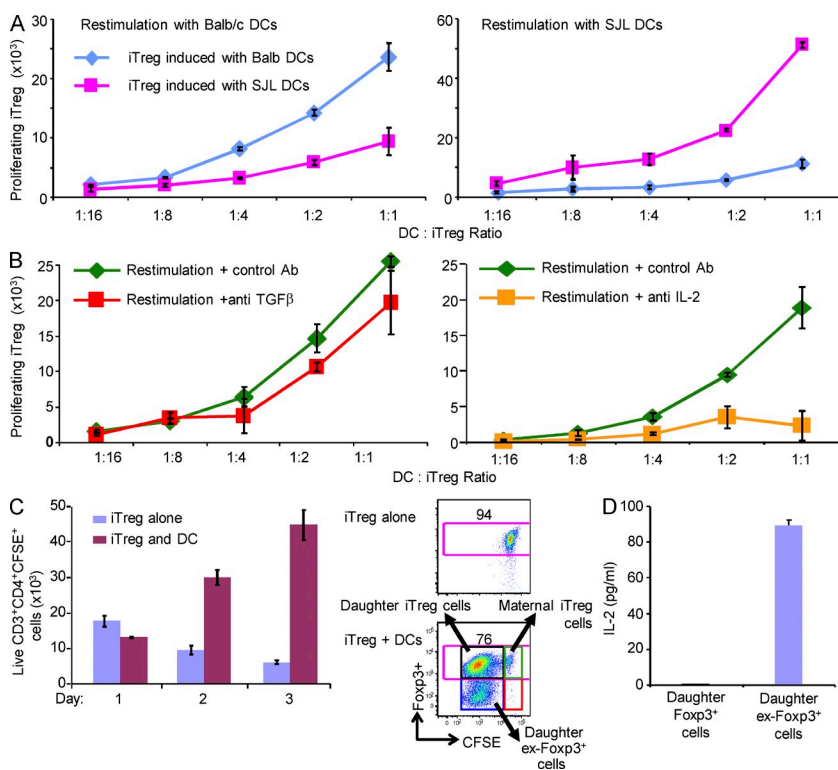


Figure 3. Splenic DCs specifically restimulate allo-iT reg cells expansion. (A) Sorted iT reg cells induced with either BALB/c or SJL CD11c⁺ DCs were CFSE labeled and stimulated in various ratios with either BALB/c (left) or SJL (right) CD11c⁺ DCs. (B) As in A, but the restimulation cultures contained 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 \pm SD.

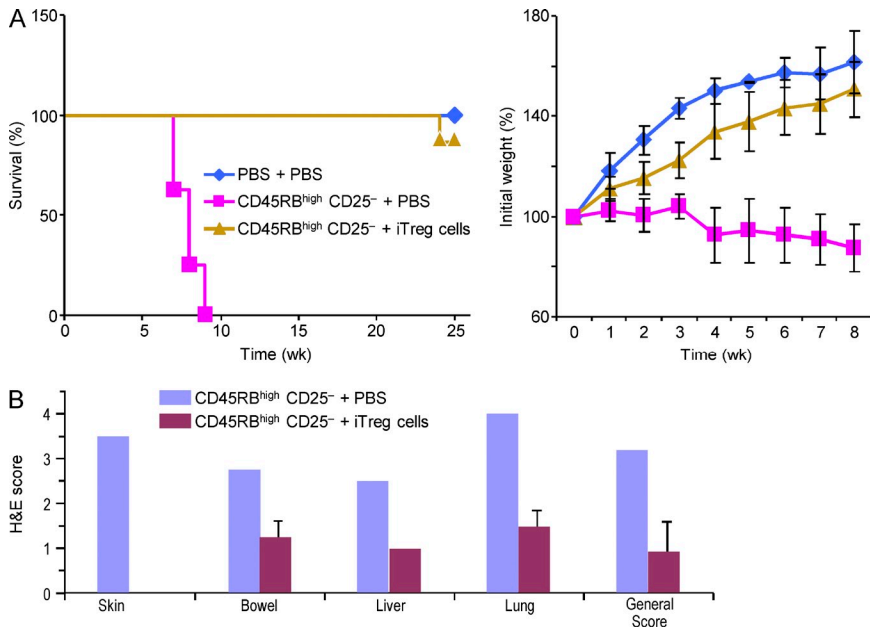


Figure 4. iT reg cells induced by DCs suppress GVHD. (A) C.B-17 scid (BALB/c) mice were transferred with allogeneic C57BL/6 CD4⁺CD45RB^{hi}CD25⁻ CD45.1 (disease inducing) cells along with PBS or anti-Balb iT reg cells. In parallel, control mice were injected twice with PBS (PBS+PBS). Animal survival (left) and weight (right) were followed. (B) H&E inflammation score of tissues 2–2.5 mo after disease induction with CD4⁺ CD45.1 T cells, with or without prevention with CD4⁺ CD45.2 Foxp3⁺(RFP⁺) cells. 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 10⁶ C57BL/6 CD4⁺CD45RB^{hi}CD25⁻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⁺CD45RB^{hi} cells infiltrated these sites (not depicted). The mice lost weight progressively and survived 2 mo on average (Fig. 4 A, right). Life spans were shorter if more CD4⁺CD45RB^{hi} cells were injected (not depicted). When we simultaneously transferred C57BL/6 anti-BALB/c CD45.2⁺ iT reg cells, GVHD was markedly attenuated, and the recipients of iT reg cells were almost free of disease manifestations (Fig. 4 A, right; and Table I), for example, weight loss, skin involvement, and diarrhea, at a time when most PBS-treated mice had died. To evaluate the

Table I. Clinical manifestations

Induction + Prevention	Alopecia 2 mo	Alopecia 6 mo	Diarrhea 2 mo	Diarrhea 6 mo
PBS + PBS	A	A	No	No
CD4 + CD45RB ^{hi} + PBS	C	–	Yes	–
CD4 + CD45RB ^{hi} + iT reg cells	A	B–C	No	No

C.B-17 scid (BALB/c) mice were transferred with allogeneic C57BL/6 CD4⁺CD45RB^{hi}CD25⁻CD45.1⁺ cells (induction) along with PBS or anti-Balb iT reg cells (prevention). Control mice were injected twice with PBS (PBS + PBS). Skin manifestations and diarrhea were evaluated 2 and 6 mo later. Grading of skin involvement: A, no involvement; B, involvement of mouth or nose; C, involvement of back. Diarrhea was graded as yes or no. – denotes that this mouse group did not survive.

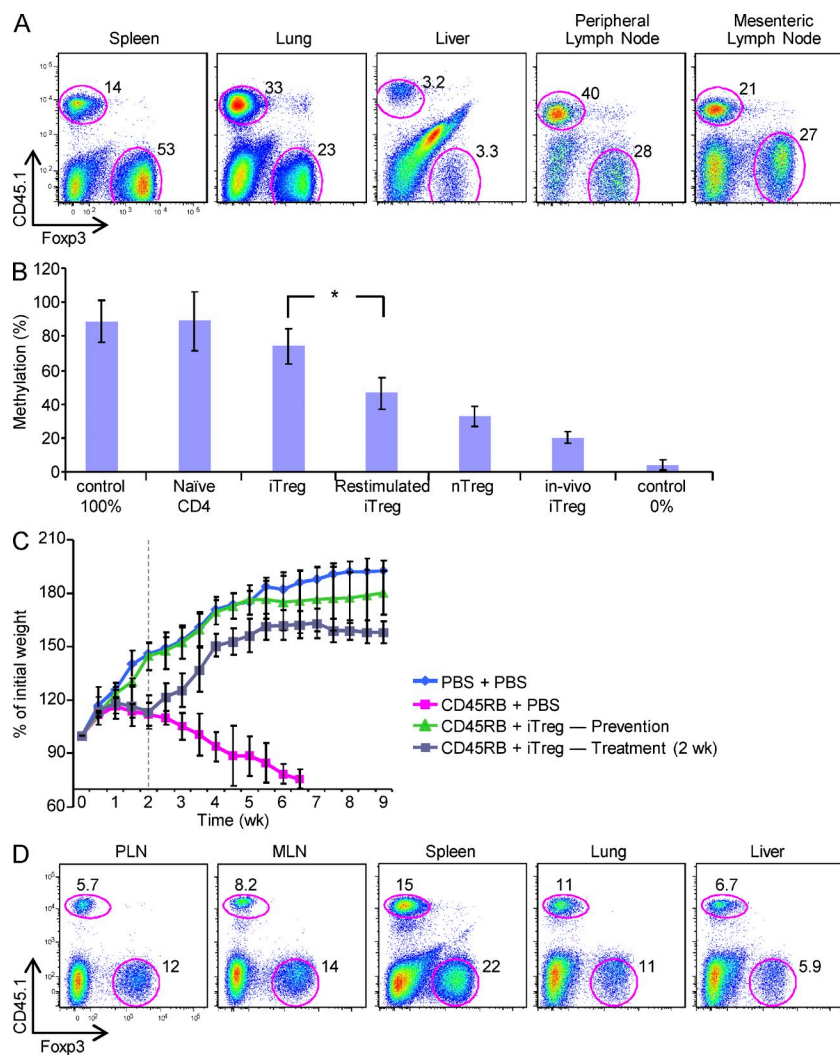


Figure 5. iT reg cells induced by DCs persist in vivo and remain stable. (A) C.B-17 scid (BALB/c) mice were transferred with allogeneic (C57BL/6) CD4⁺CD45RB^{hi}CD25⁻ CD45.1 disease-inducing cells along with either PBS or with anti-Balb iT reg cells. Infiltration of secondary lymphoid organs and tissues with CD4⁺ CD45.1 disease-inducing cells and CD4⁺CD45.2 Foxp3⁺ (RFP⁺) disease protective cells was analyzed by FACS 6 mo after GVHD induction. (B) iT reg cells from a primary MLR (iT reg cells) or restimulated (3 d) with allo DC (restimulated iT reg cells), or iT reg cells 1.5 mo after adoptive transfer (in vivo iT reg cells), were compared with naive CD4⁺ cells or nT reg cells for CpG dinucleotide methylation at CNS2 of the Foxp3 gene by Bisulfite pyrosequencing. (C) As in Fig. 4 A, but with an additional group of mice that were injected with iT reg cells as treatment only after the appearance of weight loss at 2 wk. (D) Infiltration of secondary lymphoid organs and tissues with CD4⁺ CD45.1 and CD4⁺ CD45.2 Foxp3⁺ (RFP⁺) of sick mice that were treated with iT reg cells. One representative experiment of three is shown. Error bars denote mean \pm SD. *, $P < 0.05$.

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⁺CD45RB^{hi} 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⁺CD4⁺Foxp3⁺ 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-2^d, C57BL/6 CD45.1 H-2^b, and SJL H-2^k 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- β 1, 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-CD8 α , 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, purified with a FACSAria 2 or FACSVantage (BD) cell sorter as CD11c^{high}CD19⁻CD3⁻DX5⁻ DCs (>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, 10⁶ sorted (FACSAria 2) CD45.1⁺CD4⁺CD45Rb^{hi}CD25⁻ cells were injected i.v. into 4–5-wk-old C.B-17 *said* (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 × 10⁶ every 3 d over 12 d) to a total of 4 × 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⁺CD4⁺CD45Rb^{hi}CD25⁻ 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 repertoire in the MLR. Fig. S2 shows that polyclonal CD4⁺CD25⁺Foxp3⁺ iT reg cells are specific for antigens on the inducing DC. Fig. S3 shows that splenic DCs efficiently restimulate allo-iT reg cells that maintain their suppressive activity. Fig. S4 shows that iT reg cells induced by DCs suppress GVHD in vivo. Fig. S5 shows that iT reg cells induced by DCs persist in vivo. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20110466/DC1>.

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