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# Kinetics of Alloantigen-Specific Regulatory CD4 T Cell Development and Tissue Distribution After Donor-Specific Transfusion and Costimulatory Blockade

Yusuke Tomita, MD,<sup>1</sup> Miwa Satomi, MD,<sup>1</sup> William Bracamonte Baran, MD, PhD,<sup>1</sup> Ewa Jankowska Gan, MS,<sup>1</sup> Andrea Szymczak Workman, PhD,<sup>2</sup> Creg J. Workman, PhD,<sup>2</sup> Dario Angelo Alberto Vignali, PhD,<sup>2</sup> William J. Burlingham, PhD<sup>1</sup>

**Background.** The influence of donor-side regulation toward recipient antigens on graft outcome is poorly understood. **Methods.** Because this influence might be due in part to the accumulation of tissue-resident memory T cells in the donor organ, we used a standard murine tolerization model (donor-specific transfusion plus CD40L blockade) to determine the kinetics of development and peripheralization of allospecific regulatory T cell in lymphoid tissues and liver, a secondary lymphoid organ used in transplantation. **Results.** We found that donor-specific transfusion and CD40L blockade leads to a progressive and sustained T regulatory allospecific response. The cytokines IL10, TGF $\beta$ , and IL35 all contributed to the regulatory phenomenon as determined by trans vivo delayed hypersensitivity assay. Unexpectedly, an early and transient self-specific regulatory response was found as well. Using double reporter mice (forkhead box p 3 [Foxp3]-yellow fluorescent protein, Epstein-Barr virus-induced gene 3 [Ebi3]-TdTomRed), we found an increase in Foxp3+CD25+ regulatory T (Treg) cells paralleling the regulatory response. The Ebi3+ CD4 T cells (IL35-producing) were mainly classic Treg cells (Foxp3+CD25+), whereas TGF $\beta$ + CD4 T cells are mostly Foxp3-negative, suggesting 2 different CD4 Treg cell subsets. Liver-resident TGF $\beta$ + CD4 T cells appeared more rapidly than Ebi3-producing T cells, whereas at later timepoints, the Ebi3 response predominated both in lymphoid tissues and liver. **Conclusions.** The timing of appearance of donor organ resident Treg cell subsets should be considered in experiments testing the role of bidirectional regulation in transplant tolerance.

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The adaptive alloimmune response of the host direct, semidirect, and indirect allorecognition by T cells, and allospecific B cells is thought to be responsible for rejection of organ transplants.<sup>1-3</sup> Several therapeutic treatments are needed to control the immune response and prolong graft survival. Nevertheless, in current clinical practice, most of these treatments generate global immune suppression and as consequence increase the risk of severe and even life-threatening opportunistic infections. Furthermore, most of these drugs have significant side effects affecting the cardiovascular, endocrine, and hematologic systems.<sup>4,5</sup>

In contrast, the best possible outcome after transplantation would be the development of graft-specific tolerance, in such a way that the immune system becomes unresponsive to graft-derived antigens without the need of immunosuppressive drugs.<sup>6</sup> A still suboptimal but desirable condition is the development of donor-specific regulation in the recipient immune system, which is not sufficient to achieve complete tolerance, but is associated with longer graft survival and increased likelihood of successful withdrawal of immunosuppressive drugs.<sup>7,8</sup> Notwithstanding, the induction of allospecific tolerance or regulation is still challenging, partially because some physiologic aspects of regulation development, particularly on the donor side, are still unknown.<sup>6</sup>

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<sup>1</sup> Division of Transplantation, Department of Surgery, University of Wisconsin-Madison, WI.

<sup>2</sup> Department of Immunology, University of Pittsburgh, Pittsburgh, PA.

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Y.T. and M.S. contributed equally to this work.

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Correspondence: William J Burlingham, Division of Transplantation, Department of Surgery, University of Wisconsin, Madison, WI 53792. (burlingham@surgery.wisc.edu).

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To analyze the role of donor-side regulation toward recipient antigens, we chose to study a mouse model, including lymphoid tissues and a secondary lymphoid organ typically used in vascularized organ transplantation, where donor-specific transfusion (DST) and anti-CD40L monoclonal antibody are a well-known protocol to induce allospecific tolerance. Such treatment, when applied to a future transplant donor, could give us insights as to the type of tissue-resident lymphocytes that give rise to bidirectional regulation in organ transplantation. It is known that DST and anti-CD40L treatment can induce forkhead box p 3 [Foxp3]<sup>+</sup> regulatory T (Treg) cells, and IL10 as well as and TGF $\beta$  secretion by allospecific Treg cells.<sup>9,10</sup> Far less is known about the induction of IL35-secreting T cells. Meanwhile, the alloreactive effector T cell clones are depleted, anergized, or shifted to regulatory functions by the CD40L blockade during the alloresponse induced by DST, whereas other T-cell clones are minimally affected.<sup>11,12</sup>

One way that host-based regulation approaches may fail to achieve long-term tolerance is the absence of a regulatory response on donor side. For example, the highest level of pretransplant regulation, both in rhesus macaques and in human, was directed to noninherited maternal antigen (NIMA).<sup>13</sup> Yet analysis of more than 10 000 live-related kidney transplants in the United States has shown that transplants from a maternal kidney donor fare worse than any other type of 1 HLA haplotype-mismatched graft within a family.<sup>14</sup> In contrast, grafts from a sibling kidney transplant donor fared much better if the mismatched HLA haplotype was the NIMA, as compared with the noninherited paternal, or noninherited paternal antigen, mismatched siblings.<sup>15</sup> One possible explanation for this, so-called NIMA paradox, is that the sibling donor's immune response to the host is also a NIMA response, that is, conditioned by microchimerism and development in the same mother as the host. The maternal donor's response to the offspring as host is a memory response to inherited paternal antigens of her child and thus may be a sensitized response, subverting tolerance induction.<sup>16</sup> Analysis of graft outcomes in a group of 18, 1 HLA haplotype-mismatched donor-recipient pairs enrolled in a depletion protocol showed poor graft survival in cases where the recipient strongly regulated to donor antigens before transplant, whereas the donor did not regulate to the recipient. In contrast, excellent long-term outcome was seen in cases where both donor and recipient regulated to each other's antigen before transplantation.<sup>17</sup>

IL35, a member of the IL12 family of cytokines, is a heterodimeric cytokine comprising the p35 ( $\alpha$ -chain) shared by IL12 and Epstein-Barr virus-induced gene 3 (Ebi3) ( $\beta$ -chain) shared by IL27. The IL35 receptor is composed of gp130, the receptor for IL6 and the IL12 receptor  $\beta$ 2. IL35 was first reported by Vignali et al,<sup>18</sup> and subsequently was shown to play a critical role in the regulation of various immune responses. It is produced by Foxp3-positive T cells and causes suppression of the proliferation of Th1 and Th17 effector T cells, and expansion of non-Foxp3 Treg and Breg cells. Besides these, activated dendritic cells, macrophages, endothelial cells, and smooth muscle cells can also express IL35. IL35 has also been identified as a novel cytokine in infectious tolerance, because it can convert CD4<sup>+</sup> Foxp3-negative T cells into a nonconventional Treg cell, called iT<sub>r</sub>35, which produces IL35, but does not express Foxp3, IL10, or TGF $\beta$ .<sup>18</sup>

We hypothesized that Treg cells developed in response to DST and MR-1 will exhibit alloantigen specificity, appear to other tissues besides the lymph nodes and spleen, where they produce immunosuppressive cytokines in response to specific tolerogen, and thereby mediate allospecific linked suppression of delayed type hypersensitivity (DTH), a pre-condition for bidirectional regulation after transplant of that organ.<sup>17</sup> In this study, we investigated the characteristics of the peripheral Treg cells induced by DST and anti-CD40L treatment. We were particularly interested in the kinetics of their development, and their migration into secondary lymphoid (transplantable) tissues, where they could potentially serve as facilitators of a robust tolerance via bidirectional regulation.

## MATERIALS AND METHODS

### Mice

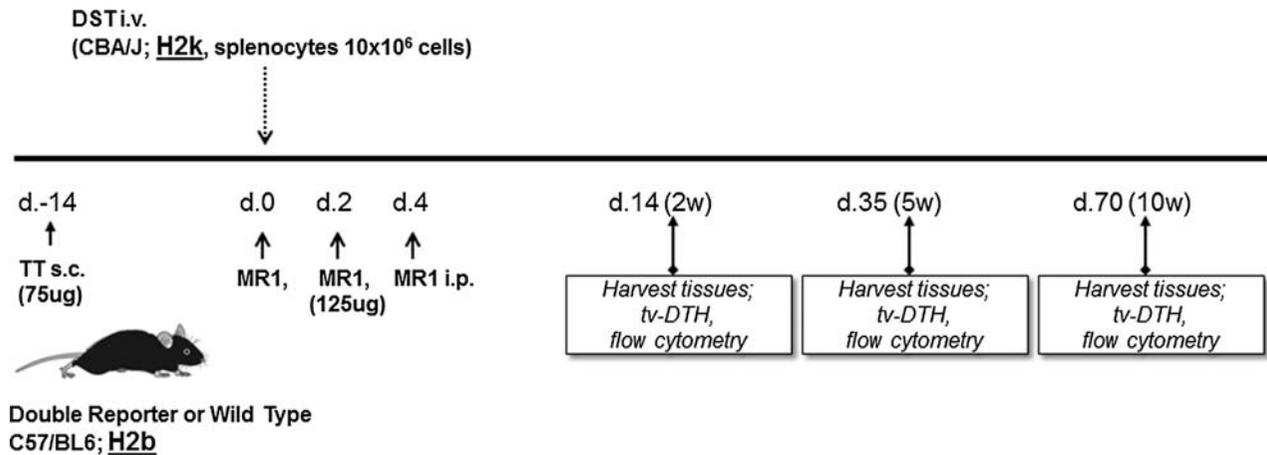
Mice were bred and maintained at University of Wisconsin-Madison Hospital animal facilities according to specific pathogen-free conditions. All animal care and handling were performed under institutional guidelines. C57 BL/6 (B6) [H2b], CBA/J (CBA) [H2k], and DBA/2 [H2d] were purchased from Harlan Sprague Dawley (Indianapolis, IN). In addition, it was used a specific strain of double reporter transgenic mice expressed YFP under the Foxp3 promoter and Td-Tomato-Red (TdTomRed) under the Ebi3 promoter (C57BL/6 background). In addition, we used a specific strain of double reporter transgenic mice expressed YFP under the Foxp3 promoter and Td-Tomato-Red (TdTomRed) under the Ebi3 promoter (C57BL/6 background).<sup>19</sup> Homozygous males and females were used to establish breeding pairs and maintain the colony in a BSL-2 facility (Medical Science Center, University of Wisconsin-Madison). Reporter expression was confirmed by flow cytometry. We chose to use tolerance induction by DST and anti-CD40L monoclonal antibody (MR-1), as a means to analyze our hypothesis.

### Study Design

Figure 1 shows the experimental timeline, including the time points when tetanus toxoid (TT)/diphtheria toxoid (DT) immunization, DST and MR-1 treatment, euthanized for trans vivo DTH (tv-DTH) and flow cytometry assays were performed. We immunized wild-type and double reporter B6 mice with 70  $\mu$ g TT/DT pediatric vaccine by subcutaneous injection in their inguinal pouch 2 weeks before initiation the tolerance protocol. We tolerized those mice with  $1.0 \times 10^7$  CBA spleen cells (200  $\mu$ L) by retro orbital intravenous injection (DST) on day 0 and MR-1 (Bio X Cell) by intraperitoneal injection of 125  $\mu$ g/dose on days 0, 2, and 4. In this model the CBA haplotype [H2k] is considered the "donor." Mice were euthanized on days 14, 35, and 70, spleen and lymph nodes (inguinal, axially, and surficial cervical) were harvested. Trans vivo DTH was performed using splenocytes, whereas flow cytometry was performed with lymph nodes, splenocytes, and liver cells.

### Tissues Processing

Spleen tissue was mechanically separated with micro-forceps and 100- $\mu$ m nylon strainer. After washing on



**FIGURE 1.** Study design. All wild type B6 and B6 Double Reporter (YFP-Foxp3/TdTomRed-Ebi3) mice were immunized s.c. with 70  $\mu$ g TT/DT pediatric vaccine by subcutaneous injection in their inguinal pouch 2 weeks before initiation of the tolerance protocol. Then, these mice were tolerized with  $1.0 \times 10^7$  CBA spleen cells by retro orbital intravenous injection (DST) on day 0, and anti-CD40L IgG (MR-1) by intraperitoneal injection of 125  $\mu$ g dose on days 0, 2, and 4. Mice were euthanized on days 14, 35, and 70. Spleen, lymph nodes (inguinal, axial, and cervical), and liver were harvested for tv-DTH and flow cytometry assay.

phosphate-buffered saline, red blood cells and platelets were lysated with ammonium-chloride-potassium lysing buffer and then washed. After cell counting, splenocytes were resuspended in desired volume. Lymph nodes were just mechanically separated with microforceps and 100- $\mu$ m cell strainer, and then washed in PBS. To generate single-cell suspensions from liver tissues, it was mechanically separated with microforceps and a 100- $\mu$ m cell strainer. Then, suspension was incubated with collagenase IV (2 mg/mL) and DNase I (0.2 mg/mL) at 37°C for 60 minutes. After washing with PBS, cells were mononuclear cells enriched using Percoll buffer (35%). Red blood cells were lysated with ammonium-chloride-potassium lysing buffer, and finally the cell suspension washed with PBS.

### Tv-DTH Assay

We used the mouse-to-mouse DTH transfer assay protocol described previously.<sup>17,20,21</sup> Briefly,  $1.0 \times 10^7$  splenocytes of B6, CBA, or DBA/2 were washed twice in sterile PBS, and resuspended in 0.1 mL PBS containing 10  $\mu$ g/mL phenylmethylsulfonyl fluoride (Sigma-Aldrich). The cells were then sonicated using a VR50 sonicator fitted with a 2-mm probe (Sonics). The disrupted cells were centrifuged for 30 min at 14 000g at 4°C to remove debris. The protein content of the supernatant was determined using a micro BCA Protein Assay kit (Pierce). A total of 20  $\mu$ g of protein was used for each injection in the DTH assays and referred to as either B6 [syngeneic/self], CBA [allogeneic/donor], or DBA/2 [3rd party] Ag. At the time points indicated in Figure 1, we euthanized and harvested a spleen, recovered splenocytes, and performed a tv-DTH assay. Specifically, we injected  $1.0 \times 10^7$  splenocytes from treated mice into syngeneic naïve mice footpads plus either: PBS [negative control], TT/DT recall antigen (25  $\mu$ g) [positive control], test antigen (20  $\mu$ g) [B6, CBA, or DBA/2 cell lysate], or a combination of TT/DT and test Ag [for linked suppression determination]. It is essential to notice that spleen cells DST is more efficient than bone marrow cells DST in regulation induction.<sup>22</sup> Before injection and after 24 hours, we used a dial-thickness gauge to measure footpad thickness; change in thickness, after subtraction of PBS value [net swelling] was expressed in

units of  $1.0 \times 10^{-4}$  inches. To determine the extent of “linked suppression” we used the following formula:

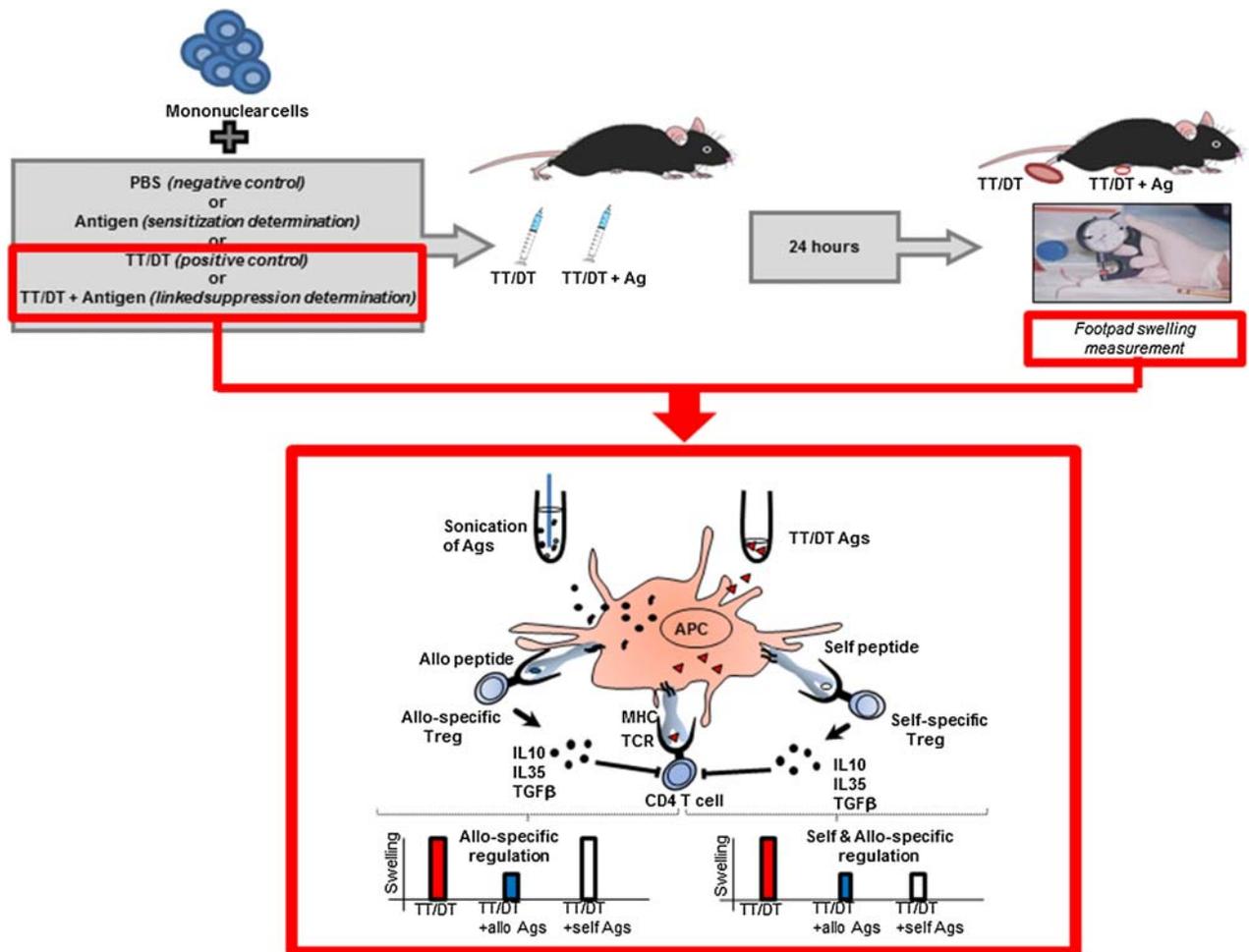
$$\% \text{Inhibition} = \left( 1 - \frac{(\text{TT plus antigen}) \text{ response}}{\text{TT response}} \right) \times 100$$

The linked suppression was determined by comparing the footpad swelling response to TT/DT recall antigen alone, with the response to the combination of TT/DT and test antigen. The results were expressed as % inhibition of the recall response. When the % inhibition value with syngeneic Ag was equal or greater than the % inhibition value with allogeneic-Ag, we considered the regulation in the mouse to be entirely nonspecific. When the % inhibition value with syngeneic Ag was less than the % inhibition value with allogeneic-Ag, we considered this to be evidence of allospecific regulation. All values represent the observed tv-DTH response for the respective group minus the PBS control. Coinjected blocking antibodies were:  $\alpha$ TGF $\beta$  (BD Pharmingen) (10  $\mu$ g),  $\alpha$ IL10 (R&D Systems) (10  $\mu$ g), a combination of  $\alpha$ p35 (R&D Systems) +  $\alpha$ Ebi3 (provided by Dr. Vignali, University of Pittsburgh) (1  $\mu$ g of each), and  $\alpha$ IL4 (BD Bioscience) (10  $\mu$ g).

A scheme of how this assay might detect linked suppression caused by self- or allogeneic-Ag, is shown in Figure 2. The functionality of memory allo-specific Treg cells was analyzed using tv-DTH assay. In this “single APC” model, suppression of the footpad swelling to TT/DT is “linked” when antigen sonicate is coinjected, because both the effector T-cell recognizing TT/DT peptide, and Treg cells recognizing either self-peptide or allopeptides are seeing their p/MHC ligand on the same APC. If the APC is a dendritic cell, there is the possibility of thrombospondin- and indoleamine 2, 3-dioxygenase-mediated “trans” suppression effects.<sup>11</sup> The APC-Treg cell interactions also result in production of regulatory cytokines IL10, TGF $\beta$ , or IL35.

### Flow Cytometry Assay

Fluorochrome-labeled monoclonal antimouse antibodies were used at proper concentrations according to vendor technical sheets and/or titration experiments in our laboratory.



**FIGURE 2.** Trans vivo DTH bioassay. When naive B6 mice are used as recipients of footpad injection for the adoptive transfer of cells from TT-immunized, CBA-tolerized B6 hosts, cotransfer of spleen cells plus TT/DT results in activation of specific effector T cells, and a footpad swelling response relative to PBS control challenge is measured at 24 hrs (red bars in graph below diagram). When the splenocytes are coinjected with alloantigen (crude CBA spleen cell lysate, 14 000g supernatant) as well as TT, indirect allopresentation will activate the allopeptide-specific memory regulatory T cells developed during DST and MR-1 treatment. Those cells will then release anti-inflammatory cytokines that can counterbalance the TT-induced response (linked suppression). The lower left depicts the results of an assay in which only donor-specific Treg cells are present in the transferred responder cells. The lower right depicts the results of an assay in which both self-antigen specific (white bars) and donor-specific (blue bars) Treg cells are present. In either case, the regulatory phenomenon may be probed to see if coinjected blocking antibodies neutralize any cytokines produced by the regulatory T cells and restore the TT/DT response.

Cells from spleen and lymph nodes were harvested and stained with CD3 (145-2C11), CD4 (RM4-5), CD25 (PC61), TGF $\beta$ /LAP (TW7-16B4) for 30 minutes at 4°C in FACS buffer. Depending on the panel, the following fluorochromes were used: eFlour450, BV421, APC, Alexa700, APC-eFlour780. Antibodies were purchased either from eBioscience, BD Bioscience, or Biolegend. Fc blocking was made with anti-CD16/32 Ab. Fluorescein isothiocyanate (or equivalents) and PE (or equivalents) were avoided because they overlap the emission range of YFP and TdTomRed, respectively. YFP has an excitation peak at 514 nm and the emission peak is 527 nm (capture by 488B standard channel in flow cytometry). TdTomRed has an excitation peak of 557 nm and emission peak of 592 nm (captured by 561E standard channel). Flow cytometric analysis was performed on a BD FACS LSR II bench-top analyzer (BD Bioscience, San Jose, CA) and analyzed using FlowJo version 10 (Tree Star, Ashland, OR).

### Statistical Analysis

Statistical analyses were performed by GraphPad Prism (GraphPad Software v5.01, San Diego, CA). The *P* values were calculated using Student *t* tests and with 95% confidence interval set as the measure of statistical significance (\**P*  $\leq$  0.05, \*\**P* < 0.01, \*\*\**P* < 0.001).

## RESULTS

### DST + CD40L Blockade Induced a Transient Regulatory Response to Self-Antigens But Persistent Allo-specific Immunoregulation

First, we determined the time course of allospecific regulation as a measure of the immune regulatory response. The timeline of treatment of the mice and harvest of spleen, lymph nodes, and liver is shown in Figure 1. The functionality of memory allospecific Treg cells was analyzed at various times after the DST and anti-CD40L blockade treatment using

the tv-DTH-linked suppression assay. Figure 2 shows 1 view of how linked suppression of tv-DTH may occur in this assay. Regulatory T cells recognizing an allopeptide or a self-peptide may be activated to release IL10, TGF $\beta$ , or IL35, suppressing the TT/DT-specific recall response.

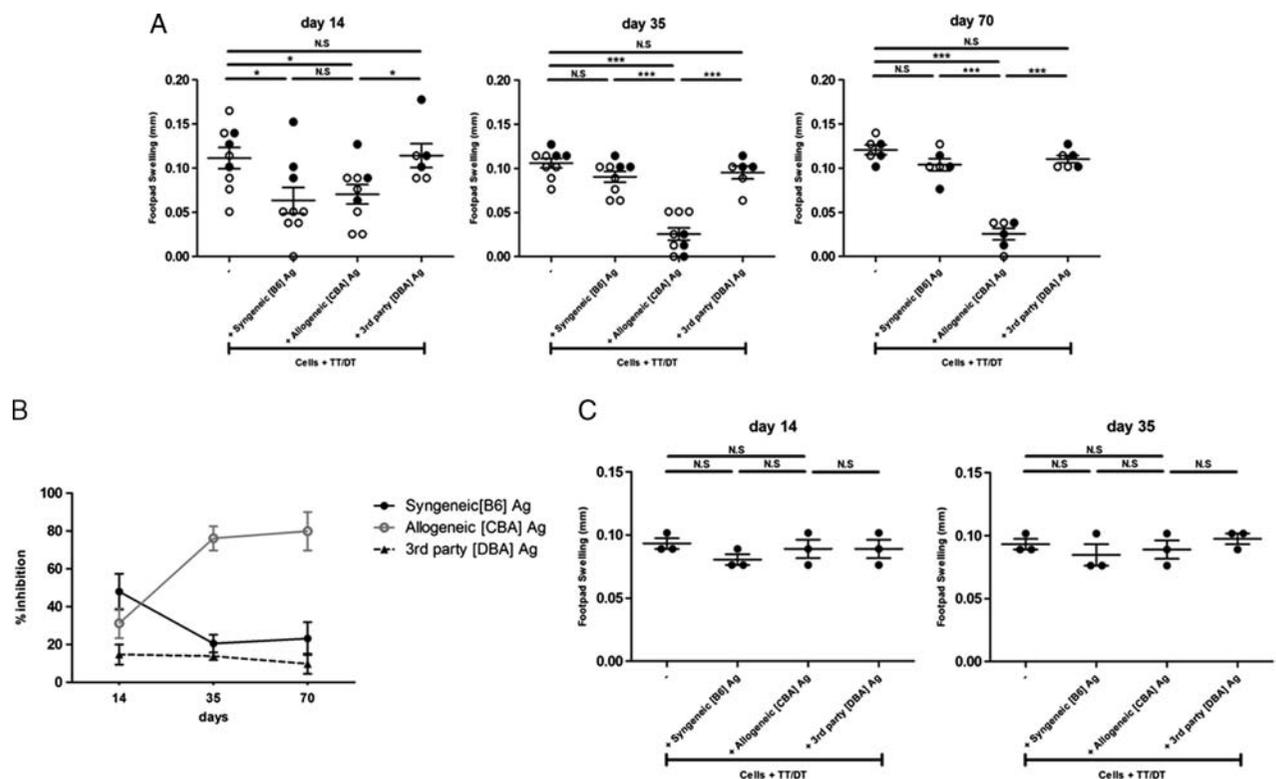
We found that DST and anti-CD40L blockade induced a sustained allospecific regulation toward CBA antigen (H2k). This regulatory effect was already evident on day 14 after treatment and persisted for up to 70 days after treatment ( $P < 0.001$  on day 35 and  $P < 0.001$  on day 70, respectively) (Figure 3A). Also, the magnitude of the allospecific regulation increased between days 14 and 35 (Figures 3A, B). As expected, we found no regulation toward third-party antigens (DBA/2; H2d). Unexpectedly, we observed linked suppression induced by self-antigens (B6; H2b) on day 14 ( $P \leq 0.05$ ). This regulation occurred only at this early time point and disappeared by day 35 ( $P = \text{N.S.}$ , % linked suppression with B6 antigen vs TT alone). On days 35 and 70 neither self H2b nor third-party H2d antigens elicited linked suppression of tv-DTH, suggesting that self-reactive Treg cells developed quickly, but subsided quickly in response to tolerization treatment (Figure 3B).

To determine if the DST was required or if MR-1 alone was sufficient to cause linked suppression, similar experiments

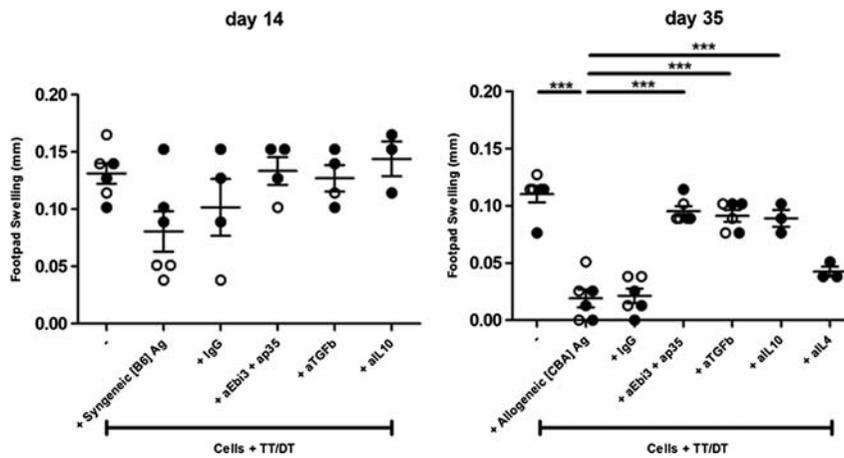
were performed on mice that received just MR-1 injections (Figure 3C). In this case, no linked suppression was seen, either on day 14 or day 35, consistent with the known mechanism of action for costimulatory blockade treatments, in which specific antigen challenge, and subsequent T cell-APC engagement are required to achieve the tolerogenic effect.

### Immune-Suppressive Cytokines Are Required for DST + CD40L Blockade-Induced Regulation

Following the same experimental protocol, the tv-DTH assay was used to determine the regulatory cytokines that mediate linked suppression. In this case, splenocytes from mice treated with DST and MR-1 were transferred into footpads of naive syngeneic B6 mice and coinjected with TT/DT recall antigens, alloantigens, and blocking antibodies: (a)  $\alpha$ IL10, (b)  $\alpha$ TGF $\beta$ , or (c)  $\alpha$ Ebi3+ $\alpha$ IL12p35 ( $\alpha$ IL35). On day 35, all 3 blocking antibodies uncovered the alloresponse, abrogating the regulatory phenomenon, suggesting that all 3 cytokines are involved in the allospecific regulation developed after DST and MR-1 treatment (Figure 4). The coinjection of IgG isotype control did not elicit a significant effect, whereas  $\alpha$ IL4 had only a marginal impact in linked-suppression. This profile was similar on days 35 and 70 after DST and



**FIGURE 3.** Time course of regulation response after DST and MR-1 tolerization. A, The tv-DTH linked suppression response to syngeneic [B6], allogeneic [CBA], and third-party [DBA/2] antigen preps on days 14, 35, and 70 after the DST and MR-1 treatment. Splenocytes from tolerized B6 mice at each time-point were tested in tv-DTH assay. The TT/DT response represents the positive control. Responses to TT/DT recall antigen (25  $\mu$ g) were shown with each test antigen (20  $\mu$ g) and analyzed by Student *t* test ( $*P \leq 0.05$ ;  $***P < 0.001$ ). Data were presented as mean with SEM from 6 to 9 mice in each group (open symbols; wild type B6 mice, close symbols; Foxp3/YFP, TdTomRed/Ebi3 reporter B6 mice). B, Timeline of the linked suppression. Here is plotted in the Y axis the “% of inhibition,” that is, percentage of decrease in the TT response in response to the coinjection of each antigen [self B6, donor CBA, and third-party DBA/2]. Although self plus allospecific regulation was observed on day 14 after DST and MR-1 treatment, allospecific regulation had developed in the central lymphoid tissue by day 35. There was no regulation at any time toward third-party Ag. Self-regulation was transient at early time-points, whereas allospecific regulation increased over-time, reaching a plateau on day 70. C, Isolated impact of CD40L blockade. The footpad swellings to TT/DT challenge were not inhibited by any of the 3 antigens on days 14 and 35. Data were presented as mean with SEM from 3 mice in each group and analyzed by Student *t* test.



**FIGURE 4.** Abrogation of linked suppression by co-injection of blocking antibodies. Self-B6 regulation responses were variable, as measured by linked suppression of recall tv-DTH response on day 14. No significant differences in recovery of TT/DT response from linked suppression by self (B6) antigens were observed on day 14 with IL10, TGF $\beta$  or IL35 neutralization, although a trend ( $P = 0.06$ ,  $P = 0.09$ , and  $P = 0.06$ , respectively) was observed. However, allo (CBA)-specific linked suppression responses on day 35 were clearly reversed by the IL10, TGF $\beta$  or IL35 cytokine-neutralizing antibodies (all  $P < 0.001$ ). The co-injection of aIL4 antibody did not elicit a significant LS reversal effect relative to IgG isotype control. Data were presented as mean with SEM from 3 to 6 mice in each group (Open symbols, wild type B6 mice; closed symbols, Foxp3/YFP, TdTomRed/Ebi3 reporter B6 mice), and analyzed by Student *t* test ( $***P < 0.001$ ).

MR-1 treatment ( $P < 0.001$  for all 3, IL10, TGF $\beta$ , and IL35 anticytokines versus IgG control on day 35 and on day 70) (day 70 data not shown). These results indicate that the allospecific regulatory phenomenon in the spleen is mediated by IL10, TGF $\beta$ , and IL35.

In contrast, self-specific responses were uncovered only in half of the mice by IL10, TGF $\beta$ , and IL35 neutralization on day 14. There was no statistically significant abrogation of self-induced linked suppression, although a trend was observed ( $P = 0.06$ ,  $P = 0.09$ , and  $P = 0.06$ , respectively).

#### Ebi3 Producer and TGF $\beta$ Producer Treg Cells Belong to Different Subsets

The mice used in the first set of experiments were double reporters (Foxp3/YFP and Ebi3/TdTomRed). The expression of the TdTomRed reporter indicates an active transcription of the Ebi3 gene. Epstein-Barr virus-induced gene 3 transcription and surface TGF $\beta$ /LAP expression in CD4 T cells of tolerized mice were analyzed with flow cytometry to determine the phenotype of the CD4 T cells producing 2 of the cytokines involved in the alloregulation (Figures 5A, B).

Analyzing lymph nodes harvested on day 35 after treatment, we confirmed that approximately 50% of T cells actively transcribing Ebi3 (TdTomRed) were Foxp3+CD25+ Treg cells. We also found that CD4 T cells which actively transcribed Ebi3 and the ones expressing surface TGF $\beta$ /LAP were different cells (Figures 5B, C). One third of TGF $\beta$ + CD4 T cells showed the typical phenotype of Treg cells (Foxp3+CD25+), but the majority were Foxp3neg, and within those TGF $\beta$ +Foxp3neg cells, only half were CD25+. No significant double positive Ebi3/TGF $\beta$  proportion was found either in lymph nodes or spleen on day 14; on day 35, this proportion was only slightly increased. Therefore, we conclude that the induced IL35-producing and TGF $\beta$ -producing CD4 T cells belong to mostly nonoverlapping subsets.

#### Peripheralization of Treg Cells

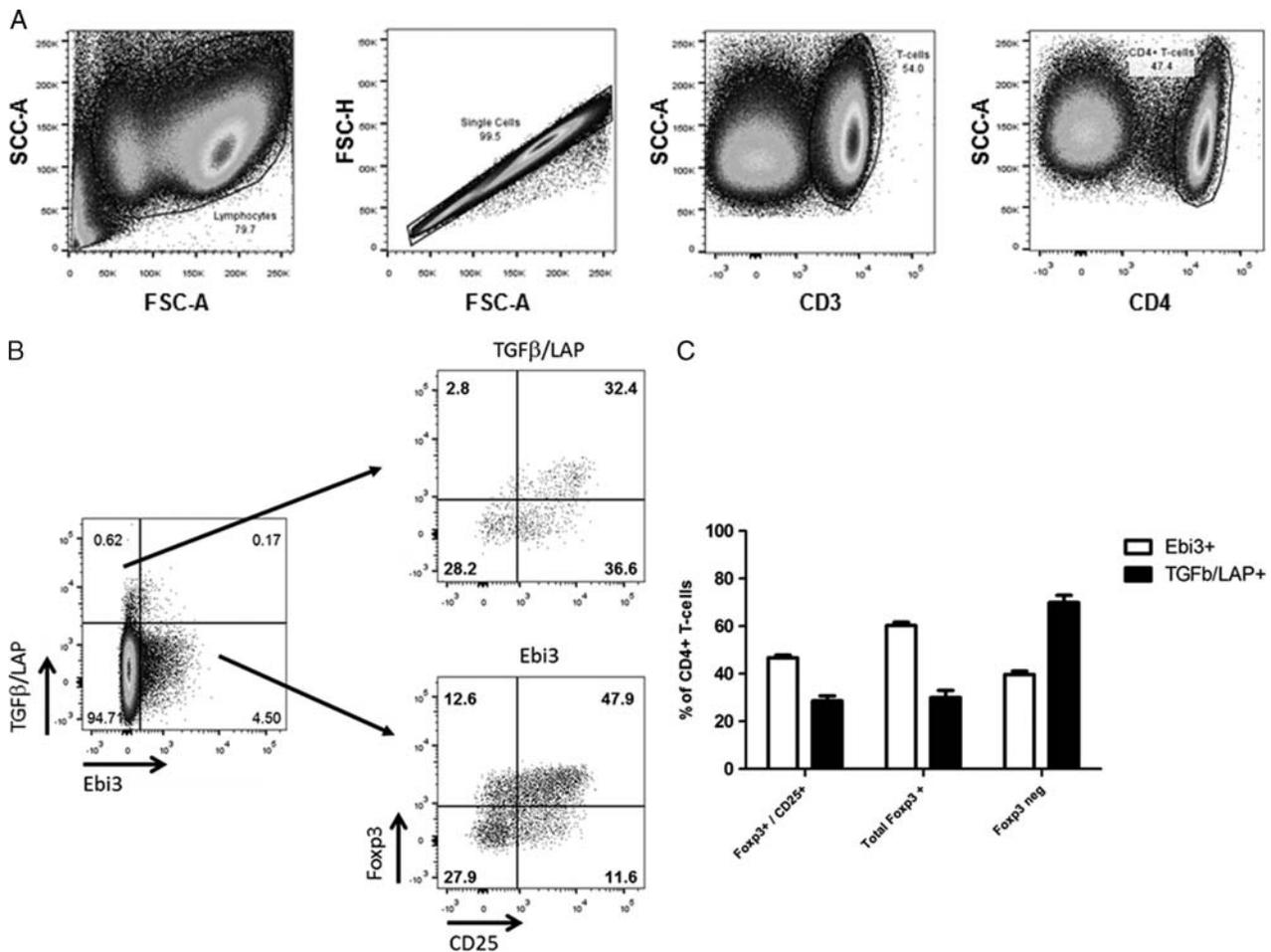
To determine the dynamics of regulatory cells development and homing in peripheral tissues, lymph nodes, spleen,

and liver were harvested from naive animals, and from mice treated with DST and MR-1, at days 14 and 35.

The Foxp3+CD25+ cells showed an increase over time in primary lymphoid tissues (spleen and lymph nodes) and liver ( $P < 0.01$  in lymphoid tissue and  $P < 0.001$  in liver on day 35, respectively) (Figures 6B, 7B). However, in liver, the proportion and the absolute number of Foxp3+CD25+ CD4 T cells were lower than that of lymphoid tissues (compare Figure 7B with Figure 6B). The IL35-producing cells (Ebi3/TdTomRed+) had a frequency and an absolute number that significantly increased over time after tolerization treatment in lymphoid tissues and liver, with a similar pattern ( $P < 0.001$ ) in lymphoid tissue and in liver on day 35, respectively (Figures 6D, 7D). The trend of these IL-35+ cells was consistent with that of the Foxp3+CD25+ conventional Treg cells. Interestingly, the kinetics of these cells was different from the TGF $\beta$  producer CD4 T cells. TGF $\beta$ + CD4 T cells had a similar baseline in spleen and lymph nodes. The proportion and the absolute number of those cells increased at day 35 after DST and MR-1 treatment in a similar magnitude in both lymphoid tissues (Figure 6E). Remarkably, TGF $\beta$ -producer cells were nearly absent in the liver at day 0, but its proportion and the absolute number increased significantly on day 14 after treatment ( $P < 0.01$  and  $P \leq 0.05$ , respectively), with further increase at day 35 ( $P < 0.001$  and  $P < 0.01$ , respectively) (Figure 7E). This suggests a migratory process of the Treg cells into specific peripheral niches. It is interesting that after tolerization treatment, the proportion of TGF $\beta$ + cells among CD4 lymphocytes was significantly higher in the liver as compared with lymphoid tissues. These results indicate that IL35-producing and TGF $\beta$ -producing cells may have different migratory kinetics in lymphoid tissues and liver.

#### Treg Cells in Tolerized Wild-Type B6 Mice

To rule out the possibility that the dual-marker B6 mice had unusual Treg cell response kinetics, similar experiments were performed to assess the kinetics of the regulatory CD4



**FIGURE 5.** Immunophenotype of TGF $\beta$  and IL35 cytokine-producer CD4 T cells. **A**, Gating strategy: live cells gated on FSC-A/SSC-A  $\rightarrow$  singlets based on deviation from the linear function in FSC-A/FSC-H  $\rightarrow$  T cells on CD3/SSC-A  $\rightarrow$  CD4 T cells on CD4/SSC-A. Foxp3+ and Internal Ebi3+ cells are those lymphocytes expressing the YFP and the TdTomRed reporter (under YFP and Ebi3 promoter). **B**, Cytokine profile of gating CD4 T cells in lymph nodes, which were harvested on day 35 after DST and MR-1 treatment, were analyzed by flow cytometry. Approximately 60% of the lymphocytes actively producing Ebi3 (TdTomRed) were Foxp3+ (classic Treg cells). Approximately 65% of TGF $\beta$ + cells are Foxp3 negative. The population of Ebi3/TGF $\beta$  double positives is around 0.2%. **C**, Summary; phenotype of CD4 T lymphocytes producing Ebi3/IL-35 or TGF $\beta$  cytokines. IL35-producing and TGF $\beta$ -producing cells were mostly non-overlapping subsets differing in phenotype and cytokine profile. Data were from 7 mice in each group.

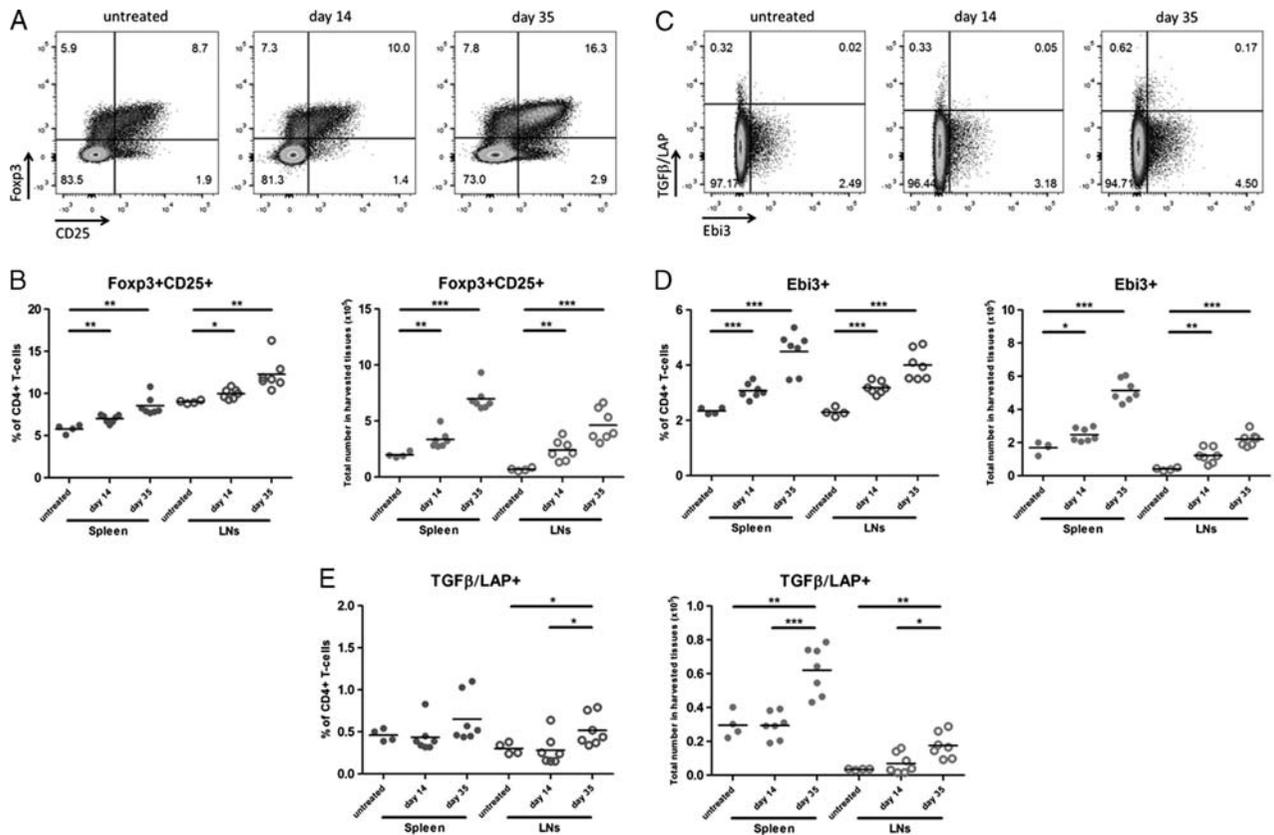
T-cell development in wild-type B6 mice. The spleen, lymph nodes, and liver were harvested from wild-type B6 mice treated with DST and MR-1 at days 14, 35, and 70, and immunostained for CD3, CD4, CD25, and TGF $\beta$ /LAP.

As with the double reporter mice, DST and MR-1 treatment induced an increased frequency of CD4+CD25+ cells in lymphoid tissues (Figure 8A). Trends, but no significant differences between nontreated mice and tolerized mice were seen in spleen ( $P = 0.2$  on day 14,  $P = 0.07$  on day 35, and  $P = 0.06$  on day 70, respectively). However, there were significant differences in lymph nodes ( $P \leq 0.05$  on day 14,  $P < 0.001$  on day 35, and  $P < 0.001$  on day 70, respectively). In liver, CD25+ cells had a very low baseline frequency on day 14 (1.0% of CD4 T cells). These cells increased until day 35 after tolerization, but the proportion was lower than that of lymphoid tissues ( $P \leq 0.05$  on day 35, and  $P \leq 0.05$  on day 70, respectively). This trend was consistent with that of the YFP-labeled Foxp3+ subpopulation in double reporter mice (Figures 6B, 7B). Interestingly, the proportion of TGF $\beta$ + cells in CD4 T cells of wild-type B6 mice was lower than that in

the double reporter mice (compare Figure 6E and 7E); however, the kinetics of appearance of these cells was similar.

## DISCUSSION

Pretransplant regulation on the donor side (toward the recipient), as well as the recipient side (toward the donor), led to long-term graft survival of a kidney allograft in a depletion protocol.<sup>17</sup> Donor-derived hematopoietic cells are transferred to the host in organ transplantation, creating a risk of graft versus host disease,<sup>23</sup> whereas stable microchimerism in the recipient was associated with improved long-term graft outcome.<sup>24–26</sup> Conlon et al<sup>27</sup> showed that host CD4 T cells trafficking into germinal centers provided help for activated B cells to produce IgG alloantibody via the indirect allorecognition pathway, whereas donor CD4 T cells via direct recognition pathway were able to activate host B cells to get them into germinal centers, but were unable to induce class-switched alloantibody. In rat heart transplantation, Ko et al<sup>28</sup> reported that selective depletion of donor leukocytes



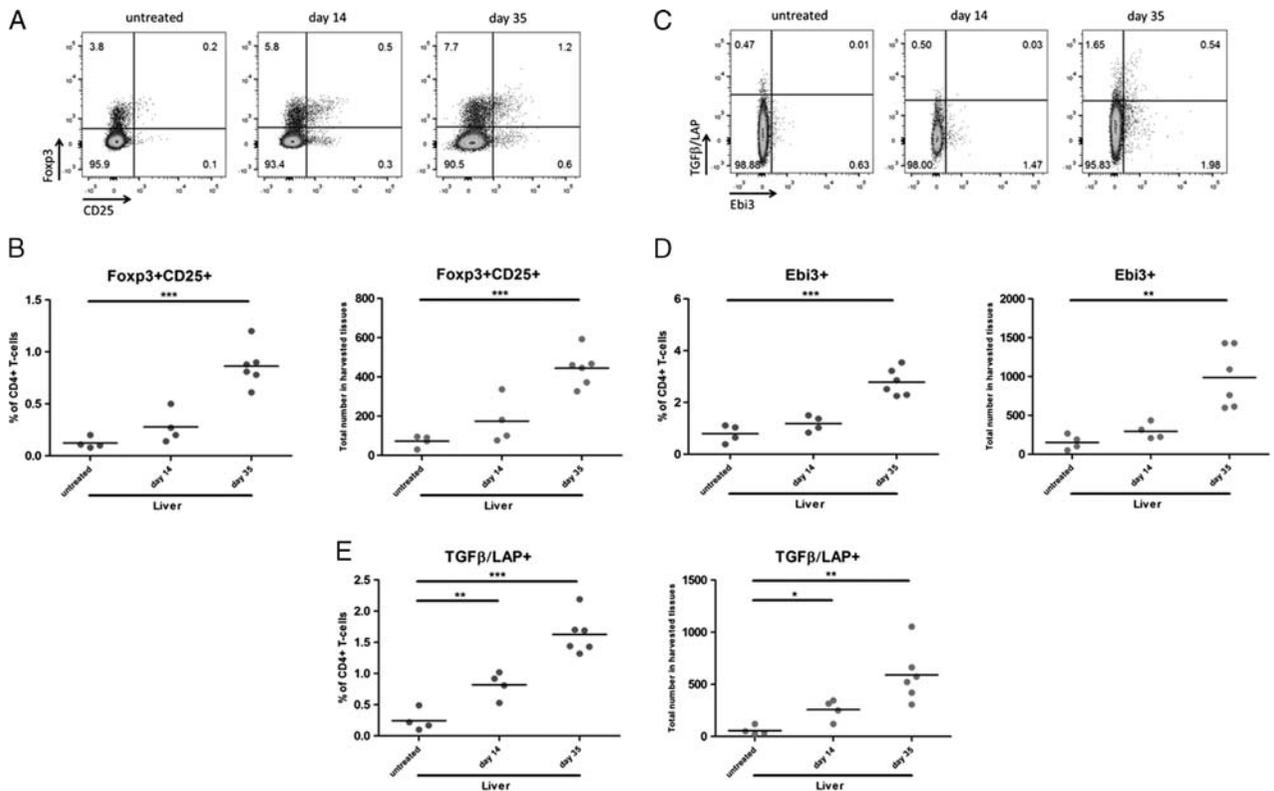
**FIGURE 6.** Time course of increase in CD4 regulatory T cells in tolerized double reporter B6 mice—lymph nodes. A. Gated CD4 T cells in lymph nodes, which were harvested on days 0 (untreated), 14, and 35 after DST and MR-1 treatment, were analyzed for Fopx3<sup>+</sup> (reporter) and CD25<sup>+</sup> by flow cytometry. B, Comparison of the frequency and the total number of Fopx3<sup>+</sup>CD25<sup>+</sup> cells in spleen and lymph nodes cells on day 0, 14, and 35 after the tolerized treatment. Total cell number means the absolute Fopx3<sup>+</sup>CD25<sup>+</sup> CD4 T cells recovered from the same spleen and lymph nodes. C, Gated CD4 T cells in lymph nodes over time after DST and MR-1 treatment were analyzed for Ebi3<sup>+</sup> (reporter) and TGFβ/LAP<sup>+</sup> by flow cytometry. D, E, Comparison of the frequency and the total number of Ebi3<sup>+</sup> (D) and TGFβ/LAP<sup>+</sup> (E) cells in lymphoid tissues on day 0, 14, and 35 after the tolerized treatment. Data were presented as mean from 4–7 mice in each group and analyzed by Student *t* test (\**P* ≤ 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001, Close symbols; spleen, Open symbols; lymph nodes).

by antibody 2 weeks after engraftment had no effect upon long-term allograft function. However, passenger leukocyte depletion at the time of transplant was associated with development of severe chronic allograft vasculopathy. Josien et al<sup>29</sup> have also shown that the presence of interstitial allospecific dendritic cells in heart graft can elicit tolerance in CD4 T cells from rats after DST treatment. These results suggested that donor-derived leukocytes in graft might play a role in either undermining, or facilitating successful engraftment both within the local environment of the transplant, and by their impact on distal immune sites.

Our study showed data about the progression of tolerance after DST and anti-CD40L monoclonal antibody treatment. Importantly, while self-specific and allospecific regulation was observed 2 weeks after tolerization, allospecific regulation took approximately 5 weeks to develop in the lymphoid tissue (Figure 3B). In regards to this point, concerning the timing of graft placement in relation to the development of tolerance, we found that 5 weeks after the tolerization treatment was sufficient for allospecific regulation to manifest itself in both the lymphoid tissue and liver. Rosenblum et al<sup>30</sup> reported that there were many tissue resident T cells in transplanted organs, including inflammatory memory T cells and Treg cells. On days 30 to 40 after resolution of an inflammatory response, activated T cells which had

appeared from central lymphoid tissue were maintained in the target organ, thus developing “Treg memory” to that organ. This time frame corresponds to the kinetics of development of allospecific-linked suppression responses. Several studies have demonstrated that DST and anti-CD40L monoclonal antibody treatment induced tolerance, as evidenced by long-term tissue or solid organ allograft survival in mice.<sup>31–34</sup> In these studies, the transplantation was performed early after treatment (usually <7 days). In fact, in some cases, DST was not performed, but anti-CD40L monoclonal antibody was injected the same day of transplantation, in such a way that the graft itself provided the allogeneic challenge needed for the costimulatory blockade to work.<sup>11,35,36</sup> Honey et al<sup>36</sup> reported that anti-CD40L monoclonal antibody induced Ag-specific tolerance and linked suppression in CD4 T cells, but not CD8 T cells, suggesting that additional therapies to control aggressive CD8<sup>+</sup> T cells may augment the tolerance state. Primarily vascularized skin allografts behaved similarly to heart allografts, whereas conventional skin grafts were not prolonged by anti-CD40L, suggesting that rapid escape of “passenger leukocytes” provides the DST-like stimulus for tolerance induction.<sup>37</sup>

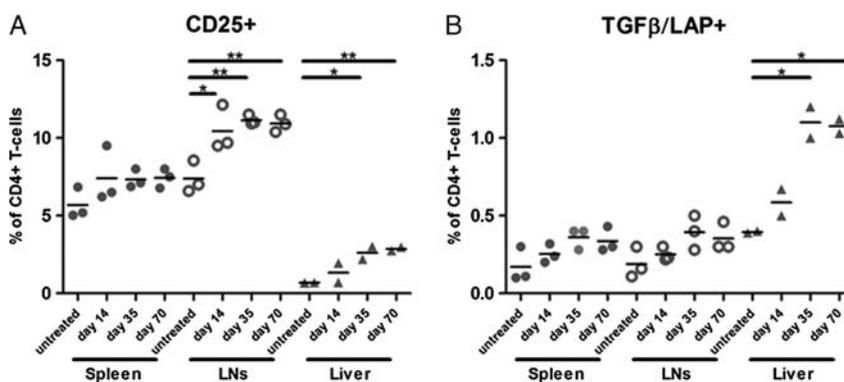
Regulatory T cell development in response to alloantigen stimulation by DST appears to rely on host antigen-presenting cells, or the indirect pathway of allorecognition,



**FIGURE 7.** Time course of increase in CD4 regulatory T cells in tolerized double reporter B6 mice—liver, A, Gated CD4 T cells in liver, which were harvested on day 0 (untreated), 14, and 35 after DST and MR-1 treatment, were analyzed for Fopx3+ (reporter) and CD25+ by flow cytometry. B, Comparison of the frequency and the total number of Fopx3+CD25+ cells in liver on day 0, 14, and 35 after the tolerized treatment. C, Gated CD4 T cells in liver over time after DST and MR-1 treatment were analyzed for Ebi3+ (reporter) and TGFβ/LAP+ by flow cytometry. D, E, Comparison of the frequency and the total number of Ebi3+ (D) and TGFβ/LAP+ (E) cells in liver on days 0, 14, and 35 after the tolerized treatment. Data were presented as mean from 4 to 6 mice in each group and analyzed by Student *t* test (\**P* < 0.01; \*\*\**P* < 0.001).

as early studies showed a requirement for MHC antigen sharing between host and donor in order for graft prolongation by F<sub>1</sub> DST alone.<sup>38</sup> Fully allogeneic DST did not prolong heart allograft survival. Costimulation blockade with anti-CD40L monoclonal antibody permits tolerization with a fully allogeneic DST. However, few studies have addressed the kinetics of allospecific Treg cell induction on the key indirect allorecognition pathway. Burrell and Bromberg<sup>12</sup> showed an early phase burst of proliferation and Fopx3

conversion of transferred allospecific “indirect pathway” T cells in the first 72 hours after transfusion of BALB/c cells into B6 mice treated with anti-CD40L monoclonal antibody. This induced Treg (iTreg) induction occurred mainly in the lymph nodes. Follow-up studies by the same group found that rapid matrix remodeling of the lymphoid vessels occurring within hours after DST and anti-CD40L monoclonal antibody treatment was essential for the early burst of T-cell proliferation resulting in allospecific iTreg,



**FIGURE 8.** Organ distribution patterns in spleen, lymph nodes, and liver of freshly harvested regulatory CD4 T lymphocytes of tolerized wild-type B6 mice. A, Gated CD4 T cells in lymph nodes, which were harvested on day 0 (untreated), 14, 35, and 70 after DST and MR-1 treatment, were analyzed for CD25+ and TGFβ/LAP+ by flow cytometry. Comparison of the frequency of CD4+CD25+ cells in spleen, lymph nodes, and liver over time after the treatment. B, Comparison of the frequency of CD4+TGFβ/LAP+ cells in lymphoid and secondary lymphoid tissues on days 0, 14, 35, and 70 after the tolerized treatment. Data were presented as mean from 2 to 3 mice in each group and analyzed by Student *t* test (\**P* ≤ 0.05; \*\**P* < 0.01, close symbols; spleen, open symbols; lymph nodes, and triangle symbols; liver).

whereas by day 7 transfer of the same TcR transgenic T cell resulted in anergy and apoptosis.<sup>39</sup> This suggests a 2-phase model of naive conventional T to Foxp3<sup>+</sup> Treg cell conversion, followed by a phase in which tolerance may be based on allospecific T-cell anergy. Our studies have identified yet a third phase: the evolution of a robust, donor-specific immunoregulatory response, involving both Foxp3<sup>+</sup> and Foxp3neg CD4 T cells.

The proportion of regulatory Foxp3<sup>+</sup>, TGFβ<sup>+</sup>, and Ebi3-producing CD4 T cells increased in lymphoid and peripheral tissues over time. The flow cytometry results corroborated the functional analysis of the regulatory function, that is, the increase in regulatory CD4 T cells paralleled the increase of allospecific-linked suppression in tv-DTH assay. We found that the allospecific regulatory phenomenon in the spleen on day 35 was mediated by IL10, TGFβ, and IL35. It is important to note that, in previous studies of mouse<sup>40</sup> and human,<sup>41</sup> the regulation detected in the linked suppression tv-DTH assay was reversed by anti-Ebi3 and by anti-p35 tested separately, whereas anti-p28, the subunit of IL27 associated with Ebi3, had no effect. This suggests that the effects we are seeing are indeed due to the action of IL35. In a previous study by Maynard et al,<sup>42</sup> it was found using a IL10 and Foxp3 dual reporter mouse that leukocytes distributed to lung and liver were enriched in Foxp3+IL10neg Treg cells, whereas the gut-homing Treg cells included both Foxp3+IL10+ and Foxp3neg IL10+ subsets. Thus, the tissue specificity of different types of Treg cells makes it important to consider the organ that is being transplanted, as far as bidirectional regulation is concerned. Interestingly, the TGFβ-producing and IL35-producing cells were different in Foxp3 phenotype (Figures 5B, C). This was compatible with previous studies showing that, within the CD4 T cells, IL35 was secreted by classic Foxp3+ Treg cells but not by Th0 or activated T effector cells.<sup>18</sup> Induction of allospecific IL35-producing Treg cells is implied by the abolition of tv-DTH-linked suppression. Because this induction occurs after allogeneic challenge, the Ag-specific Treg cells were most likely a peripheral (or inducible) type. Nevertheless, the possibility that DST-derived migratory dendritic cells can influence thymic Treg cell development cannot be entirely ruled out. In contrast to the Ebi3-producing Treg cells, the majority of cells producing TGFβ were Foxp3neg (Figure 5C), as previously reported.<sup>43,44</sup> The precise identity of the TGFβ+ CD4 T cells has yet to be determined—they might be either Tr1 cells or, alternatively, “Th3” CD4 T effector cells<sup>45,46</sup> that synthesize the cytokine as part of their effector program.

We also found that TGFβ-producing and IL35-producing cells both appeared in the liver, but with different kinetics. Remarkably, TGFβ-producing cells appeared into peripheral tissues (liver) significantly faster than did IL35-producing cells. TGFβ-producing cells appeared in liver at day 14 after treatment, whereas IL35-producing CD4 T cells reached a maximum at day 35 (Figure 7D vs Figure 7E). Also, the fold-increase compared with baseline status was higher for TGFβ-producing CD4 T cells.

Bidirectional regulation implies a pretransplant induced unresponsiveness of the recipient immune system to donor antigens, and vice versa. The beneficial effects of this condition have been described in leukodepleted posttransplant kidney patients.<sup>17</sup> Regarding the application of our data to the study of bidirectional regulation in primarily vascularized

transplant models, the results suggest that mice to be used as transplant donors should receive tolerization treatment at least 35 days before transplantation to allow regulatory cells to home to the organ.

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