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Induction of transplantation tolerance by allogeneic donor-derived $CD4^+CD25^+Foxp3^+$ regulatory T cells

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Abstract

Several studies have shown that recipient-derived $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) are involved in transplantation tolerance. However, it is not clear whether allogeneic donor-derived Tregs are able to regulate T cell alloreactivity after solid organ allograft transplantation. Related studies in experimental bone marrow transplantation have shown that allogeneic donor-derived Tregs are capable of promoting early and long-term allogeneic hematopoietic engraftment, accompanied by tolerance to donor and recipient antigens. However, in these models, donor-derived Tregs are syngeneic with respect to the T responder cells. The role of Tregs in solid organ transplantation models where recipient-derived T responder and donor-derived Tregs are allogeneic has been scarcely studied.

In order to determine whether allogeneic Tregs were able to regulate T cell alloreactivity, $CD4^+CD25^-$ and $CD8^+$ T responder cells were cultured with stimulator dendritic cells in several responder–stimulator strain combinations (C57BL/6→BALB/c, BALB/c→C57BL/6 and C3H→BALB/c) in the presence of responder-derived, stimulator-derived or 3rd-party-derived Tregs. Then, the frequency of IFN-γ+ alloreactive T cells was determined by means of ELISPOT assay. The results of this study demonstrate that, regardless of the responder–stimulator strain combination, both responder-derived and stimulator-derived Tregs, but not 3rd-party-derived Tregs, significantly inhibited $CD4^+$ and $CD8^+$ T cell alloreactivity. The effect of allogeneic stimulator-derived Tregs was dependent on IL-10 and TGF-β and reversed by exogenous IL-2.

In vivo experiments in *nu/nu* recipients reconstituted with $CD4^+CD25^-$ T responder and Tregs showed that recipient and donor-derived, but not 3rd-party-derived Tregs, significantly enhanced skin allograft survival. Importantly, T cells from both recipient-derived and donor-derived Treg-reconstituted *nu/nu* recipients exhibited donor-specific unresponsiveness *in vitro*. These results show that allogeneic donor-derived Tregs significantly inhibit T cell alloreactivity and suggest their potential use in the induction of transplantation tolerance.

Keywords: $CD4^+CD25^+Foxp3^+$ regulatory T cells; Transplantation; Tolerance; Mice

1. Introduction

Several T cell populations with regulatory properties have been described exhibiting different phenotypes and mechanisms of action [1,2]. Among these cells are the natural $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) which constitute 5–10% of mature $CD4^+CD8^-$ thymocytes and about 10% of peripheral $CD4^+$ T cells [3–6]. Tregs are thought to be essential for self and allogeneic tolerance [3–6]. Experimental evidence suggests that Tregs inhibit T cell proliferative responses through a cell–cell contact-dependent mechanism [4–6]. However, other studies have shown that murine and human Tregs can exert their regulatory effect through IL-10- and/or TGF-β-dependent

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mechanisms [4,7–12]. Thus, it is possible that cell–cell contact and cytokine-dependent mechanisms act coordinately during different phases of the immune response. [4,7–13]. Although Tregs require antigen exposure to initiate their inhibitory activity, *in vitro* studies revealed that once activated, Tregs inhibit immune responses in a nonspecific manner through bystander suppression during the effector phase [8,13]. However, the precise mechanism by which Tregs exert their regulatory effect may vary depending on the model and the nature of the antigenic stimulus [13].

The role of Tregs in transplantation tolerance has been extensively documented [1,14–16]. Studies in bone marrow and solid organ transplantation have shown that Tregs have the ability to regulate the rejection of minor- or MHC-mismatched allografts by $CD4^+$ and $CD8^+$ alloreactive T cells [9,16]. Thus, Tregs have been proposed as a major contributing factor for the maintenance of allograft tolerance [14–16]. Most of the evidence for the role of Tregs in bone marrow transplantation tolerance has been obtained with donor-derived Tregs that are syngeneic in respect to the T responder cells inducing graft-versus-host disease [1,9,17,18]. These studies have shown that donor-derived Tregs suppress lethal acute graft-versus-host disease after experimental bone marrow transplantation [17,18]. On the contrary, most of the evidence for the role of Tregs in solid organ transplantation tolerance has been obtained with syngeneic recipient-derived Tregs [1,14,15]. In this regard, a previous study by Mathew et al. [19] demonstrated that donor-derived Tregs can also participate in the induction and maintenance of transplantation tolerance in humans. This study showed that patients who received bone marrow infusions after kidney transplantation presented peripheral donor-derived chimerism and T cells with potent donor-specific regulatory activity. Although these findings may have a great impact in the development of strategies for the induction of transplantation tolerance, the effect of allogeneic donor-derived Tregs has not been extensively studied.

2. Objective

This study was designed to determine the regulatory effect of allogeneic donor-derived Tregs on T cell alloreactivity and allograft survival in the context of solid organ transplantation. We show herein that allogeneic simulator/donor-derived Tregs inhibit $CD4^+CD25^-$ and $CD8^+$ T cell alloreactivity *in vitro* and induce donor-specific tolerance *in vivo*. These results strongly indicate the potential role of allogeneic donor-derived Tregs in the induction of tolerance in solid organ transplantation.

3. Materials and methods

3.1. Animals

C57BL/6 ($H2^b$), BALB/c ($H2^d$), C3H ($H2^k$) and BALB/c *nu/nu* male mice of 6–12 wk of age were purchased from the National Cancer Institute (Bethesda, MD) and the Jackson Laboratories (Ann Harbor, MI) and maintained under pathogen-free conditions. This study was approved by the Institutional Animal Care and Use Committees of Rush University Medical Center and the Universidad de Antioquia.

3.2. $CD4^+$ and $CD8^+$ T cell isolation

Spleen $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were isolated by magnetic selection using a Treg isolation kit (Miltenyi Biotech, Auburn, CA). Intracellular staining for Foxp3 was performed using a Treg identification kit (eBioscience, San Diego, CA). Purified cells were >95% $CD4^+CD25^+Foxp3^+$ and >99.5% $CD4^+CD25^-Foxp3^-$ as determined by flow cytometric analysis. Spleen $CD8^+$ T cells were isolated by negative selection using a $CD8^+$ T cell isolation kit (Miltenyi Biotech). Purified cells were >95% $CD8^+$ as determined by flow cytometric analysis.

3.3. Dendritic cell isolation

Spleens were injected with collagenase D (5 mg/ml, Boehringer Mannheim, Indianapolis, IN). After 60 min, dendritic cells (DC) were isolated by positive selection using a DC isolation kit (Miltenyi Biotech). DCs were allowed to undergo maturation for 18–24 h and irradiated (2000 rads). Purified cells were >90% $CD11c^+$ as determined by flow cytometric analysis.

3.4. Mixed leukocyte culture and enzyme-linked immunospot (ELISPOT) assay

MultiScreen 96-well plates (Millipore, Bedford, MA) were coated with anti-IFN-γ mAbs (BD Biosciences) and blocked with 1% BSA. Different combinations of C57BL/6, BALB/c and C3H cells were used as responder–stimulator pairs. $CD4^+CD25^-$ and $CD8^+$ T cells (1×10^5/well) were cultured in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with FBS (10%, Hyclone, Logan, UT), HEPES (25 mM), penicillin (100 U/ml), streptomycin (100 mg/ml), and 2-ME (50 mM) in the presence of irradiated stimulator DCs (24×10^3/well). Responder-derived, stimulator-derived or 3rd-party-derived $CD4^+CD25^+$ Tregs were added to the cultures at Treg:T responder ratios of 2:1 and 1:1 for $CD4^+CD25^-$ and $CD8^+$ T cell cultures, respectively (previously determined optimal ratios for Treg function, data not shown). After 48 h, biotin-conjugated anti-IFN-γ mAb (BD Biosciences) was added for 2 h. Afterwards, HRP-labeled streptavidin (BD Biosciences) was added for 2 h. Subsequently, AEC substrate (BD Biosciences) was added for 5 min. The plates were washed and analyzed in an ImmunoSpot Series I analyzer (Cellular Technology, Cleveland, OH).

3.5. Cytokine modulation of Treg activity

C57BL/6 $CD4^+CD25^+$ T cells (3×10/well) were cultured in the presence of irradiated BALB/c spleen cells ($3_{\times}10^5$/well). C57BL/6 (responder-derived) or BALB/c (stimulator-derived) Tregs were added to the cultures at a Treg:T responder ratio of 2:1. The cultures were then treated with anti-IL-10 (20 μg/ml, clone JES052A5, RD Systems, Minneapolis, MN) and/or anti-TGF-β (4 μg/ml, clone 1D11, RD Systems) mAbs or rIL-2 (5 ng/ml, RD Systems) [20,21]. Control cultures were treated with equal concentrations of control mAbs (clones 43414 and 11711, respectively, RD Systems) or left untreated. After 48 h, T cell alloreactivity was determined by IFN-γ ELISPOT assay.

3.6. Skin transplantation and adoptive cell transfer

Full-thickness C57BL/6 skin tail allografts were transplanted onto the dorsum of BALB/c *nu/nu* mice. Within 18 h, 1×10^6 BALB/c $CD4^+CD25^-$ T cells were adoptively-transferred (i.p.) into the BALB/c *nu/nu* recipients in the presence or absence of 3×10^6 BALB/c (recipient-derived), C57BL/6 (donor-derived) or C3H (3rd-party-derived) Tregs (Treg:T responder ratio: 3:1). Allografts were examined daily and were considered rejected when >80% of necrosis was detected. Rejection was confirmed histologically by H&E staining [22].

3.7. Donor-specific tolerance evaluation

Recipients that displayed skin allograft rejection were euthanized at the time of rejection to harvest their spleen cells. Recipients that did not display skin allograft rejection were euthanized at >80 days after transplantation to harvest their spleen cells. Total spleen T cells (3×10^5/well) were co-cultured with irradiated BALB/c (recipient-derived), C57BL/6 (donor-derived) or C3H (3rd-

party-derived) DCs (24×10^3/well). After 48 h, T cell alloreactivity was determined by IFN-γ ELISPOT assay.

3.8. Statistical analysis

Differences in T cell responses were assessed by means of 2-way ANOVA with Bonferroni´s post-test corrections. Differences in graft survival were assessed by means of Log-rank (Mantel–Haenszel) test. The Prism 5.0 software (GraphPad, San Diego, CA) was used for all analyses with the α set *a priori* at $p < 0.05$.

4. Results

4.1. Inhibition of T cell alloreactivity by allogeneic stimulator-derived Tregs

To analyze the effect of allogeneic Tregs on T cells alloreactivity we used an *in vitro* model of T cell alloreactivity using different responder–stimulator strain combinations (C57BL/6→BALB/c,

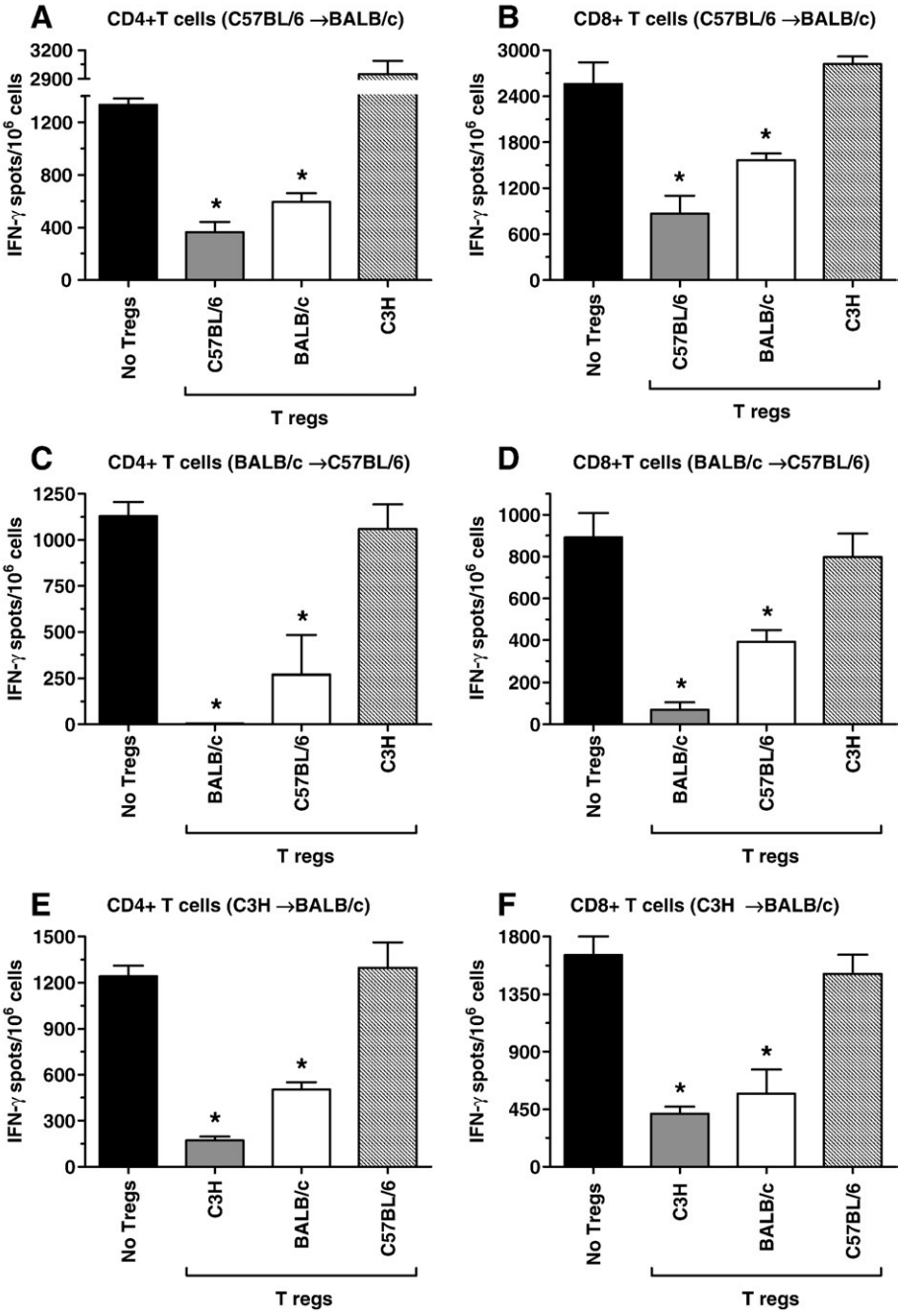

Fig. 1. Inhibition of T cell alloreactivity by allogeneic stimulator-derived Tregs. C57BL/6 $CD4^+CD25^-$ (A) and $CD8^+$ (B) T cells were cultured with BALB/c DCs in the absence (no Tregs) or presence of C57BL/6 (responder-derived), BALB/c (stimulator-derived) or C3H (3rd-party-derived) Tregs. Also, BALB/c $CD4^+CD25^-$ (C) and $CD8^+$ (D) T cells were culture with irradiated C57BL/6 DCs in the absence (no Tregs) or presence of BALB/c (responder-derived), C57BL/6 (stimulator-derived) or C3H (3rd-party-derived) Tregs. In parallel experiments, C3H $CD4^+CD25^-$ (E) and $CD8^+$ (F) T cells were cultured with BALB/c DCs in the absence (no Tregs) or presence of C3H (responder-derived), BALB/c (stimulator-derived) or C57BL/6 (3rd-party-derived) Tregs. After 48 h, T cell alloreactivity was determined by IFN-γ ELISPOT assay. Results are expressed as the mean ± SEM of triplicate cultures and are representative of 3 different experiments. No Tregs vs. Tregs: $*p < 0.001$ (ANOVA).

BALB/c→C57BL/6 and C3H→BALB/c). Toward this, $CD4^+CD25^-$ (Fig. 1A, C and E) and $CD8^+$ (Fig. 1B, D and F) T cells were stimulated with DCs in the presence of responder-derived, stimulator-derived or 3rd-party-derived Tregs. After 48 h, T cell alloreactivity was determined by IFN-γ ELISPOT assay. In all the responder–stimulator combinations tested, both responder-derived and stimulator-derived Tregs induced a significant inhibition of $CD4^+$ and $CD8^+$ T cell alloreactivity (Fig. 1A–F, $p<0.001$). On the contrary, 3rd-party-derived Tregs did not show any regulatory effect on $CD4^+$ or $CD8^+$ T cell alloreactivity (Fig. 1A–F). These results indicate that allogeneic stimulator-derived Tregs have MHC-restricted regulatory effect on both $CD4^+$ and $CD8^+$ T cell alloreactivity [23,24]. It is noteworthy that comparable results were also obtained with human Tregs (see supplementary material).

4.2. Inhibition of T cell alloreactivity by allogeneic stimulator-derived Tregs is dependent on IL-10 and TGF-β and reversible by exogenous IL-2

To determine whether the regulatory effect of allogeneic stimulator-derived Tregs on T cell alloreactivity was cytokine-dependent, C57BL/6 $CD4^+CD25^-$ T cells were cultured with BALB/c spleen cells in the presence of C57BL/6 (responder-derived) or BALB/c (stimulator-derived) Tregs and treated with anti-IL-10 and/or anti-TGF-β mAbs. After 48 h, T cell alloreactivity was determined by IFN-γ ELISPOT assay. Treatment with anti-IL-10 and/or anti-TGF-β mAbs significantly reversed the regulatory effect of both C57BL/6 (responder-derived) and BALB/c (stimulator-derived) Tregs (Fig. 2A and B, respectively, $p<0.05$). Isotype control mAbs did not have any effect on Treg-mediated inhibition of T cell alloreactivity (Fig. 2A and B). These results indicate that both IL-10 and TGF-β are involved in the regulatory effect exerted by allogeneic stimulator-derived Tregs [7–12].

It has been previously shown that exogenous IL-2 reverses the regulatory effect of Tregs [21,25]. Thus, C57BL/6 $CD4^+CD25^-$ T cells were cultured with BALB/c spleen cells in the presence of C57BL/6 (responder-derived) or BALB/c (simulator-derived) Tregs and treated with exogenous IL-2. Treatment with exogenous IL-2 reversed the regulatory effect of both C57BL/6 (responder-derived) and BALB/c (stimulator-derived) Tregs as T cell response observed in the IL-2-treated cultures was comparable to that observed in cultures without

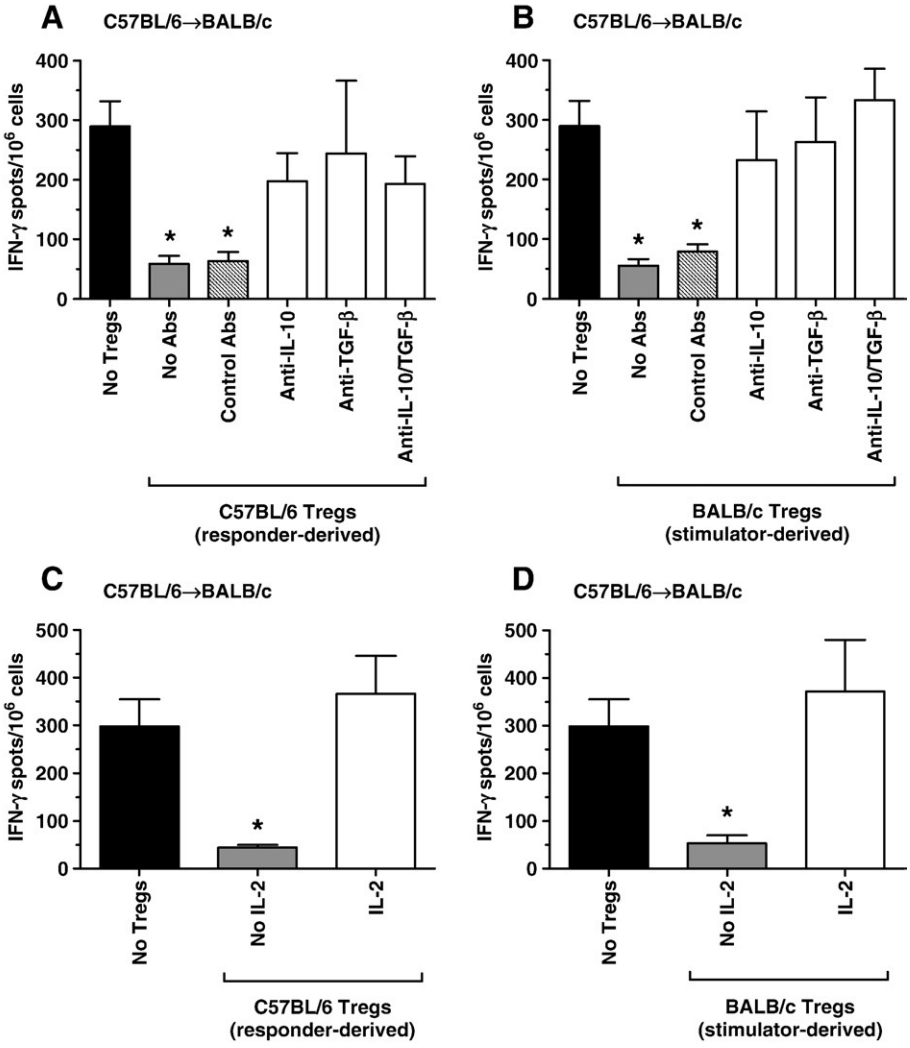

Fig. 2. Inhibition of T cell alloreactivity by allogeneic stimulator-derived Tregs is dependent on IL-10 and TGF-β and reversible by exogenous IL-2. C57BL/6 $CD4^+CD25^-$ T cells were cultured with BALB/c spleen cells in the absence (no Tregs) or presence of C57BL/6 (responder-derived) (A, C) or BALB/c (stimulator-derived) Tregs (B, D). Selected cultures were treated with anti-IL-10 and/or anti-TGF-β mAbs (20 μg/ml and 4 μg/ml, respectively) (A, B) or exogenous rIL-2 (5 ng/ml) (C, D). After 48 h, T cell alloreactivity was determined by IFN-γ ELISPOT assay. Results are expressed as the mean±SEM of 4 different experiments. No Tregs vs. Tregs±treatment: *$p<0.05$ (ANOVA).

Tregs (no Tregs) (Fig. 2C and D, respectively). These results suggest that the regulatory effect exerted by allogeneic stimulator-derived Tregs is associated with induction of anergy of responder T cells.

4.3. Allogeneic donor-derived Tregs enhance skin allograft survival

To evaluate the capacity of allogeneic donor-derived Tregs to mediate alloantigen-specific regulation *in vivo*, C57BL/6 skin allografts were transplanted onto BALB/c *nu/nu* recipients reconstituted with $CD4^+D25^-$BALB/c T cells in the presence of BALB/c (recipient-derived), C57BL/6 (donor-derived) or C3H (3rd-party-derived) Tregs. Allografts on recipients that did not receive Tregs (no Tregs) were readily rejected (MST = 11.1 ± 1.2 days, Fig. 3A). Similarly, allografts on recipients treated with C3H (3rd-party-derived) Tregs were also readily rejected with comparable kinetics (MST = 14.0 ± 3.6 days, Fig. 3A). Histological analysis of these allografts showed extensive inflammatory cellular infiltration, arteritis and focal epidermal necrosis (Fig. 3B and C, respectively). In contrast, allografts transplanted onto recipients treated with BALB/c (recipient-derived) or C57BL/6 (donor-derived) Tregs showed a significant enhancement of allograft survival (Fig. 3A, $p < 0.05$). At >80 days after transplantation, 66% and 50% of the C57BL/6 allografts transplanted onto BALB/c *nu/nu* recipients reconstituted with BALB/c (recipient-derived) or C57BL/6 (donor-derived) Tregs, respectively, failed to show any macroscopic signs of rejection. Histological analysis of these allografts showed minimal cellular infiltration and normal tissue architecture without necrosis (Fig. 3D and E, respectively). Taken together, these results demonstrate that allogeneic donor-derived Tregs are able to induce long-term survival of fully-mismatched allografts comparable to the effect exerted by recipient-derived Tregs observed herein as well as in previous studies [26,27].

4.4. Donor-specific tolerance induced by allogeneic donor-derived Tregs

To determine whether enhancement of allograft survival observed in recipients of allogeneic donor-derived Tregs was due to the induction of donor-specific tolerance, total spleen T cells were harvested at the time of allograft rejection from recipients that did not receive Tregs (no Tregs) and from recipients reconstituted with BALB/c (recipient-derived) or C57BL/6 (donor-derived) Tregs with non-rejected allografts at >80 days after transplantation. Then, total spleen T cells were cultured in the presence of BALB/c (recipient-derived), C57BL/6 (donor-derived) or C3H (3rd-party-derived) DCs. After 48 h, T cell alloreactivity was determined by IFN-γ ELISPOT assays. Recipients that did not received Tregs (no Tregs) showed a significantly higher alloreactivity against both C57BL/6 (donor-derived) and C3H (3rd-party-derived) DCs as compared to the reactivity observed against syngeneic BALB/c (recipient-derived) DCs (Fig. 4A, $p < 0.001$). As expected, the alloreactivity observed in these recipients against C57BL/6 (donor-derived) DCs was higher than that observed against C3H (3rd-party-derived) DCs (Fig. 4A). In contrast, recipients that received BALB/c (recipient-derived) or C57BL/6 (donor-derived) Tregs did not show any reactivity to C57BL/6 (donor-derived) DCs as compared to the reactivity observed against syngeneic BALB/c (recipient-derived)

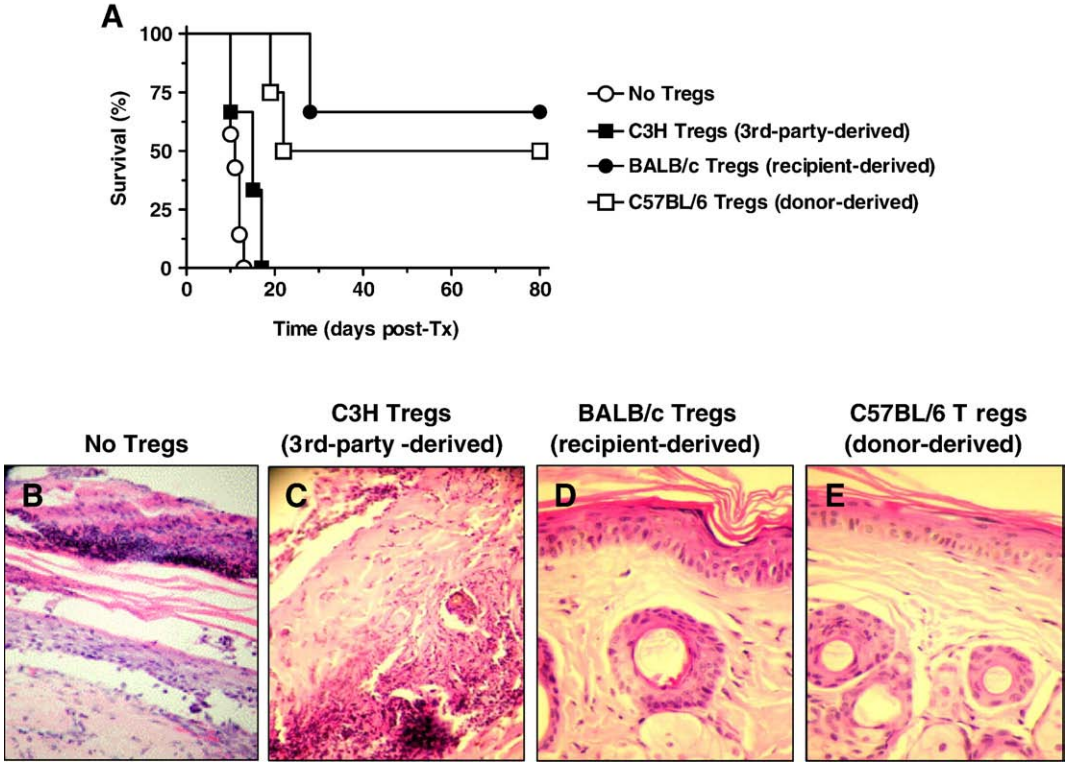

Fig. 3. Allogeneic donor-derived Tregs enhance skin allograft survival. Full-thickness C57BL/6 skin allografts were transplanted onto the dorsum of BALB/c *nu/nu* mice. Within 18 h, $CD4^+CD25^-$ BALB/c T cells were adoptively-transferred (i.p.) into the BALB/c *nu/nu* recipients without Tregs (no Tregs, $n = 7$) or with BALB/c (recipient-derived, $n = 3$), C57BL/6 (donor-derived, $n = 4$) or C3H (3rd-party-derived, $n = 3$) Tregs. The allografts were examined daily and were considered rejected when >80% necrosis was detected (A). Allografts from recipients reconstituted with no Tregs (B) and C3H (3rd-party-derived) (C) Tregs were harvested at the time of rejection. Functioning allografts from recipients reconstituted with BALB/c (recipient-derived) (D) and C57BL/6 (donor-derived) (E) Tregs were harvested at >80 days after transplantation. Harvested allografts were analyzed histologically by H&E staining. Original magnification: ×400. Allograft survival: No Tregs vs. BALB/c (recipient-derived) or C57BL/6 (donor-derived) Tregs: $p < 0.05$ (Log-rank test).

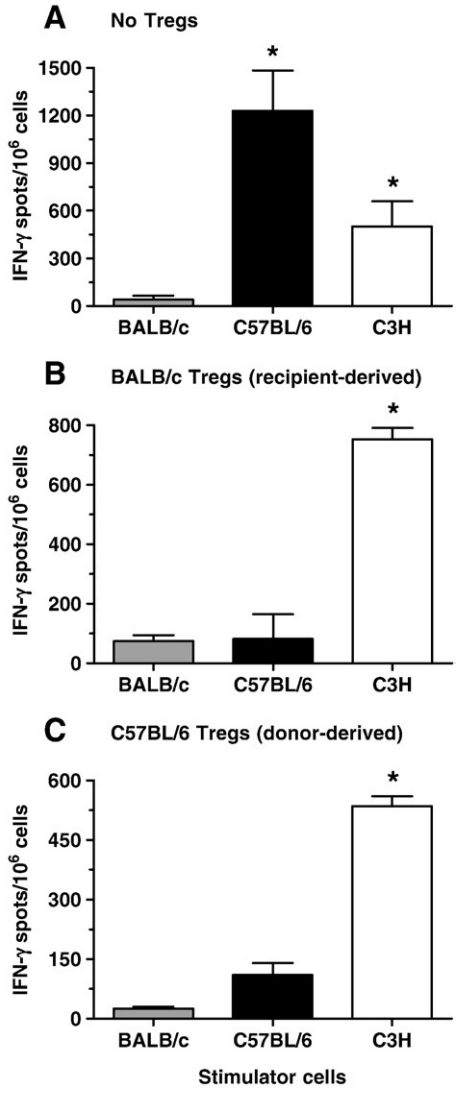

Fig. 4. Donor-specific tolerance induced by allogeneic donor-derived Tregs. Skin allografts from C57BL/6 mice were transplanted heterotopically onto BALB/c *nu/nu* recipients. Within 18 h, $CD4^+CD25^-$ BALB/c T cells were adoptively-transferred (i.p.) into the BALB/c *nu/nu* recipients without Tregs (no Tregs) or with BALB/c (recipient-derived) or C57BL/6 (donor-derived) Tregs. Total spleen T cells were harvested from recipients reconstituted with no Tregs at the time of allograft rejection (A) and from recipients reconstituted with BALB/c (recipient-derived) (B) or C57BL/6 (donor-derived) (C) Tregs with non-rejected allografts at >80 days after transplantation. Then, the T cells were cultured with BALB/c (recipient-derived), C57BL/6 (donor-derived) or C3H (3rd-party-derived) DCs. After 48 h, T cell alloreactivity was determined by IFN-γ ELISPOT assay. Results show the mean±SEM of triplicate cultures and are representative of 2 different experiments. BALB/c reactivity (syngeneic) vs. C57BL/6 or C3H reactivity (allogeneic): *$p<0.001$ (ANOVA).

DCs (Fig. 4B and C, respectively). The donor-specific tolerance induced by both BALB/c (recipient-derived) and C57BL/6 (donor-derived) Tregs was confirmed by the significantly higher T cell alloreactivity observed in these recipients against C3H (3rd-party-derived) DCs as compared to the reactivity observed against syngeneic BALB/c (recipient-derived) DCs (Fig. 4B and C, respectively, $p<0.001$). These results suggest that enhancement of allograft survival induced by allogeneic donor-derived Tregs is due to the induction of donor-specific tolerance.

5. Discussion

There is extensive evidence that different populations of T cells are involved in the regulation of T-cell mediated allogeneic immune responses [28,29]. Among these, Tregs have been the most extensively studied [30–33]. However, most of the experimental evidence has been developed in models of transplantation tolerance mediated by syngeneic Tregs. In this regard, in experimental bone marrow transplantation models, it has been previously demonstrated that donor-derived Tregs can suppress acute graft-versus-host disease. However, in these studies of haematopoietic cell transplantation, T responder and Tregs are syngeneic since both are donor-derived [17,18]. Although, in solid organ transplantation the possibility that T cell alloreactivity can be modulated by allogeneic donor-derived Tregs has been less studied, the seminal studies by Mathew et al. [19] demonstrated that donor-derived T cells with highly specific and potent regulatory activity are present in kidney graft recipients that received donor bone marrow infusion and had stable graft function 6 months to 4 years post-transplantation. Adeegbe et al. [34] showed that adoptive transfer of allogeneic Tregs into IL-2Rβ-deficient mice resulted in long-term engraftment of these cells, prevented autoimmunity and induced donor-specific tolerance to skin allografts. More recently, Demirkiran et al. [35] reported that high amounts of donor-derived Tregs detach from the liver graft into circulation where they can be detected even after 6 months post-transplantation although in lower numbers than in the first weeks post-transplantation. In their model, donor-derived Tregs were able to control the recipient-derived alloreactive T cells *in vitro*. These authors proposed that donor-derived Tregs contributed to chimerism-associated tolerance after liver transplantation [35]. Evidence that donor-derived Tregs may control alloreactivity was also provided by Benghiat [36], who also reported that donor-derived Tregs are able to control both Th1 and Th2 alloreactive responses and proliferate *in vitro* due to self-antigen recognition between donor Tregs and donor-derived immature DCs.

Our results show that allogeneic stimulator-derived Tregs have a regulatory effect on both $CD4^+$ and $CD8^+$ T cell alloreactivity. In addition, this study shows that the regulatory effect of allogeneic Tregs is not restricted to a specific genetic background since comparable results were observed in mice with different allogeneic strain combinations. Our results also show that the regulatory effect of the allogeneic stimulator-derived Tregs observed in our model is IL-10- and TGF-β-dependent. These findings are in agreement with previous reports that Tregs can exert their regulatory effect on T cell alloreactivity through mechanisms that required IL-10 and TGF-β production [7–13]. Although our *in vitro* studies were performed in short term cultures (48 h), it is possible that adaptive T helper type 3 cells (Th3) and type-1 regulatory T cells (Tr1) may be mediating the regulatory effect observed in our experiments. Tr1 and Th3 cells are adaptive regulatory T cells that inhibit T cell-mediated alloresponses through IL-10 and TGF-β production [13,37]. Similarly, it is possible that in our *in vivo* model adaptive Tr1 and Th3 cells were induced in the course of the allogeneic response, contributing to the regulatory effect of Tregs and the induction of

tolerance. Future studies in our laboratory will be conducted to clarify the possible generation of adaptive Tregs in our experimental conditions, and also to evaluate the possibility that stimulator/donor-derived Tregs that were added in our *in vitro* and *in vivo* experiments proliferate during the allogeneic reaction, as previously described [36].

Our finding that exogenous IL-2 restores T cell alloreactivity and reverses the regulatory effect of allogeneic Tregs indicates that they exert their activity by the induction of anergy in T responder cells. These findings are in agreement with previous studies showing that exogenous IL-2 reverses the regulatory effect of syngeneic Tregs by reversion of anergy [21,25]. In this regard, it has been reported that IL-10 and TGF-β induce T cell anergy by inhibition of IL-2 transcription [38].

The observation that allogeneic stimulator/donor-derived Tregs, but not 3rd-party-derived Tregs, were able to inhibit T cell alloreactivity *in vitro* and induce donor-specific tolerance *in vivo*, respectively, supports the notion that the effect of these cells could be MHC-restricted [23,24]. These data suggest that allogeneic stimulator/donor-derived Tregs may be activated by recognition of self-MHC molecules present on the stimulator cells and the skin grafts, respectively. This recognition did not occur with the 3rd-party-derived Tregs. Nevertheless, the question remains of why 3rd-party-derived Treg cells do not display a regulatory effect on allogeneic T responder cells given that they also have the ability to directly recognize allo-MHC molecules [17,29]. It may be possible that 3rd-party-derived Tregs do not have a regulatory effect due to an insufficient activation since, in our model, there were no 3rd-party-derived antigen-presenting cells capable of inducing activation through indirect allorecognition [39–42]. Our results are in agreement with those reported by Sanchez-Fueyo et al. [43] who demonstrated that indirect allorecognition leads to a more potent Treg activation than the direct pathway. This possibility is also supported by previous studies showing that allogeneic 3rd-party-derived Tregs have a regulatory effect on T cell alloreactivity *in vivo* only when co-administered with rapamycin which is known to selectively promote the expansion of functional adaptative Tregs [42,44].

The role of allogeneic donor-derived Tregs was also demonstrated *in vivo* by skin allograft transplantation on BALB/c *nu/nu* mice reconstituted with $CD4^+CD25^-$ T cells. BALB/c *nu/nu* recipients that did not receive Tregs or were treated with 3rd-party-derived Tregs readily rejected skin allografts within 10–15 days. In contrast, reconstitution with either recipient- or donor-derived Tregs induced a significant enhancement of allograft survival. The lack of a regulatory effect by 3rd-party-derived Tregs could be also due to the absence of self-antigen recognition in graft APCs by these cells. Benghiat reported that recognition of self-antigens by donor-derived Tregs in stimulator cells (*in vitro*) allows their permanence, regulatory effect and proliferation [36].

Importantly, in our experiments, enhancement of allograft survival by allogeneic donor-derived Tregs was associated with donor-specific tolerance, demonstrated by the lack of response of T cells from Treg-reconstituted recipients stimulated with donor-derived cells. This finding is in agreement with previous studies showing MHC-specificity of the regulatory effect of allogeneic Tregs *in vivo* [34].

It has been previously shown that Tregs have the capacity to convey IL-10-dependent regulatory activity to conventional $CD4^+CD25^-$ naive T cells, a phenomenon consequently called "infectious tolerance" [45–48]. There is a possibility that enhancement of allograft survival and induction of systemic donor-specific tolerance by allogeneic donor-derived Tregs could have been mediated by the induction of regulatory T cells in the recipient through a similar mechanism and/or by engraftment and expansion of allogeneic donor-derived Tregs *in vivo*. In this regard, previous studies have shown that Tregs successfully expand and migrate to peripheral lymphoid tissues and cardiac allografts in reconstituted Rag-deficient mice [49]. Interestingly, minimal cellular infiltration was observed in functioning allografts harvested from recipients reconstituted with syngeneic recipient-derived or allogeneic donor-derived Tregs in our model. It remains to be demonstrated whether the scarce graft-infiltrating cells observed in tolerant animals display a Treg phenotype as shown in previous studies [16,49].

Altogether, our results provide the proof of principle for the use of allogeneic donor-derived Tregs as potential therapeutic reagents for the induction of clinical transplantation tolerance. Although, the number of donor-derived Tregs available from blood or tissues could be a limiting factor for such treatment, new methodologies for Treg expansion *in vitro* are currently being explored by different investigators to overcome such limitation [39,40,49,50]. However, these options are technically difficult and costly methodologies that are not currently feasible for implementation in the clinical setting. Thus, the potential use of donor-derived Tregs from lymphoid organs (i.e. spleen and lymph nodes from deceased donors) as well as donor/recipient-matched 3rd-party-derived Tregs for improving the yield of functional Tregs is of paramount practical importance in clinical transplantation. This would significantly increase the number of available donor-derived Tregs as well as the donor pool for adoptive Treg immunotherapy, respectively.

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