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Donor-derived, tolerogenic dendritic cells suppress immune rejection in the indirect allosensitization-dominant setting of corneal transplantation

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ABSTRACT

Significant interest has been focused on the use of ex vivo-manipulated DCs to optimally induce transplant tolerance and promote allograft survival. Although it is understood that donor-derived, tolerogenic DCs suppress the direct pathway of allosensitization, whether such DCs can similarly suppress the indirect pathway remains unclear. We therefore used the murine model of corneal transplantation to address this, as these allografts are rejected in an indirect pathway-dominant manner. Interestingly, recipients administered with donor bone marrow-derived DCregs, generated via culturing with GM-CSF, IL-10, and TGF-β1, significantly prolonged survival of corneal allografts. Correspondingly, these recipients demonstrated a potent reduction in the frequency of indirectly allosensitized T cells, as determined by ELISPOT. Examination of DCregs relative to mDCs or iDCs showed a resistance to up-regulation of MHC-II and costimulatory molecules, as well as an impaired capacity to stimulate MLRs. In vivo, DCreg administration in corneal-allografted recipients led to inhibition of $CD4^+IFN\text{-}\gamma^+$ T cell frequencies and an associated increase in Foxp3 expression in the Treg compartment. We conclude that donor-derived, tolerogenic DCs significantly suppress the indirect pathway, thereby identifying a novel regulatory mechanism for these cells in transplantation. ***J. Leukoc. Biol.*** **91: 621–627; 2012.**

Introduction

The induction of donor-specific tolerance in organ transplantation would avoid graft rejection and in theory, limit patient dependency on lifelong immunosuppressive drug therapy, which is fraught with toxic side-effects [1]. There is currently considerable interest in use of cell-based therapies to promote transplant tolerance, such as administration of immunoregulatory (or tolerogenic) DCs that can suppress immunity in an antigen-specific manner. Administration of tolerogenic DCs generated from bone marrow cells in the presence of various pharmacological mediators (e.g., rapamycin, cyclosporine, and corticosteroids) or cytokines (e.g., IL-10, TGF-β) has been shown to be able to protect transplants from deleterious alloimmunity in various models (e.g., GVHD, heart, skin, and islets) [2–17]. However, the mechanisms by which such tolerogenic DCs suppress alloimmunity and promote graft survival remain incompletely understood.

Alloreactive T cells, which cause immune rejection, are primed or "allosensitized" by at least two pathways, which include direct and indirect allosensitization [18]. Direct pathway involves T cells directly responding to foreign MHC molecules expressed by donor-derived DCs. In contrast, in the indirect pathway, recipient DCs present donor-derived allopeptides in the context of self-MHC to allosensitize recipient T cells. In recent years, there has been accumulating evidence that the indirect pathway represents an essential component of the allograft-rejection process [19], particularly in chronic forms of immune rejection. Although previous studies have shown that administration of donor-derived, tolerogenic DCs to allografted recipients decreases anti-donor T cell responses induced by the direct pathway [3], whether tolerogenic DCs suppress the indirect pathway remains unclear.

We used the corneal transplant model to address this, as immune rejection of corneal allografts occurs, by in large, via indirect allosensitization [20–25]. We used the $CD200R3^+$ DCregs, as described previously by Sato et al. [15, 26], to determine the mechanisms by which these cells suppress alloreactivity and immune rejection. We found that donor-derived DCregs significantly prolonged cornea allograft survival and did so via maximal suppression of indirect

Abbreviations: DCregs=regulatory DCs, DLN=draining LN, Foxp3=forkhead box p3, GVHD=graft-versus-host disease, iDC=immature DC, mDC=mature DC, Treg=regulatory T cell

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allosensitization, as determined by ELISPOT assays. This was associated with increased Foxp3 expression and concomitant impairment of IFN-γ+ T cell frequencies in DLNs. Taken together, our results indicate a novel, regulatory mechanism used by donor-derived DCreg administration in transplantation.

MATERIALS AND METHODS

Mice

Eight- to 10-week-old BALB/c (H-2^d) and C57BL/6 (H-2^b) male mice were purchased from Taconic Farms (Hudson, NY, USA) and housed in the specific pathogen-free animal facility of Schepens Eye Research Institute (Boston, MA, USA). Moreover, all animals were treated according to guidelines established by the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and the Public Health Policy on Humane Care and Use of Laboratory Animals (U.S. Public Health Review). All procedures were approved by the Institutional Animal Care and Use Committee. Anesthesia was used for all surgical procedures with i.p.-administered ketamine/xylazine suspensions (120 and 20 mg/kg, respectively).

Corneal transplantation

Murine orthotopic corneal transplantation was used as described previously [27]. In brief, donor corneas (2-mm diameter) were excised by vannas scissors from C57BL/6 mice. Donor corneas were placed onto the recipient graft beds prepared by excising a 1.5-mm site in the central cornea of BALB/c mice using 11-0 nylon suture. The corneal sutures were removed 7 days after surgery. All grafts were examined by slit-lamp microscopy at weekly intervals. At each time-point, the grafts were scored for opacity and neovascularization by using the scoring system as described previously [27]. For the infusion of DCs into the recipients, 0.2 or 1.0 × 10^6 iDCs, mDCs, and DCregs were injected i.v. via the lateral vein of the tail, 7 days before transplantation.

Generation of DC subsets

iDCs, mDCs, and DCregs were generated as described previously by Sato et al. [26]. C57BL/6 mice bone marrow cells were harvested from femur and tibia. After RBC lysis (Sigma-Aldrich, St. Louis, MO, USA), cells were plated at a density of 0.2 × 10^6/ml in petri dishes (BD Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) in RPMI 10% FCS medium. iDCs were generated by culturing with GM-CSF (20 ng/ml; BioLegend, San Diego, CA, USA) for 7 days. For the preparation of mDCs, iDCs were subsequently stimulated with LPS (1 μg/ml; Sigma-Aldrich) for 24 h. DCregs were generated by culturing freshly harvested bone marrow cells with GM-CSF, IL-10 (20 ng/ml; BioLegend), and TGF-β (20 ng/ml; BioLegend) for 7 days. After stimulating with LPS (100 μg/ml) for 24 h, $CD40^+$, $CD80^+$, $CD86^+$ cells were depleted by magnetic sorting (Miltenyi Biotec, Auburn, CA, USA) using biotinylated mAb to CD40, CD80, CD86 (BioLegend), and antibiotin microbeads (Miltenyi Biotec). DCs were stained with PE anti-CD11c, PE anti-CD14, Alexa647 anti-CD80, Alexa647 anti-CD86, Alexa647 anti-CD40, and PE anti-IA^b (all antibodies are from BioLegend) and analyzed by flow cytometry (LSR II, BD Biosciences, San Jose, CA, USA).

Real-time PCR

RNA was isolated with the RNeasy Micro kit (Qiagen, Valencia, CA, USA) and reverse-transcribed using the Superscript III kit (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed using TaqMan Universal PCR Mastermix (Applied Biosystems, Foster City, CA, USA) and preformulated primers for CD200R3 (assay ID Mm00512443_m1), IL-10 (Mm00439616-m1), IL-12p35 (Mm00434165_m1), Foxp3 (Mm00475156_ml), and GAPDH (Mm99999915_gl). The results were analyzed by the comparative threshold cycle method and normalized by GAPDH as an internal control.

MLR

Purified, allogeneic T cells (2×10^5) were prepared from BALB/c splenocytes. T cells were MACS-sorted for CD90.2 (Miltenyi Biotec), according to the manufacturer's instructions. Isolated T cells were cocultured with mytomicin C (50 μg/ml; 37°C for 20 min; Sigma-Aldrich)-treated C57BL/6 iDCs, mDCs, or DCregs for 72 h in 96-well, round-bottom plates. BrdU reagent (Sigma-Aldrich) was added to each well, 16 h before collecting the cells. The proliferation of T cells was measured by using the BrdU incorporation assay kit (Millipore, Billerica, MA, USA), according to the manufacturer's instructions. Flow cytometry was also used for detection of $BrdU^+$ cells in $CD4^+$ T cells, as described previously [28]. Briefly, after cell-surface staining with PECy5 anti-CD4 antibody was performed, cells were then fixed and permeabilized, and intracellular staining was performed using FastImmune anti-BrdU FITC with DNase (Becton Dickinson). Stained cells were analyzed by flow cytometry.

ELISPOT assay

ELISPOT assay was used to analyze a direct pathway and an indirect pathway response, as described previously [21, 29–31]. In brief, 96-well ELISPOT plates (Whatman Polyfiltronics, Rockland, MA, USA) were coated with anti-IFN-γ antibody (BD PharMingen, San Diego, CA, USA). After plates were blocked with 1% BSA, purified T cells ($CD90.2^+$ MACS-sorted) from allografted BALB/c mice (3 weeks post-transplantation of C57BL/6 corneas) were incubated with C57BL/6 APCs (CD90.2-negative, MACS-sorted splenocytes) for 48 h to quantify frequencies of directly allosensitized T cells or with syngeneic APCs pulsed with sonicated donor antigen (2×10^7 C57BL/6 APCs/ml) to quantify frequencies of indirectly allosensitized T cells.

Detection of $CD4^+$ $Foxp3^+$ Tregs

Ipsilateral draining submandibular and cervical LNs were harvested, and single-cell suspensions were prepared. The isolated LN cells were fixed/permeablilized and stained with the following antibodies: anti-CD4 FITC, anti-CD25 PE, and anti-Foxp3 PECy5 (eBioscience, San Diego, CA, USA), as described previously [32], and analyzed by a flow cytometer.

Intracellular staining of cytokines

Purified T cells, stimulated with PMA/ionomycin for 6 h in the presence of Golgi block (Becton Dickinson), were stained with FITC anti-CD4 antibody, and then, intracellular staining of cytokines (APC anti-IFN-γ antibody or APC anti-IL-10 antibody; BioLegend) was performed using Fix buffer and permeabilization buffer (eBioscience), according to the manufacturer's instructions. Fluorescence staining was analyzed with flow cytometry.

Statistical analyses

Statistical analyses for comparison of difference were performed using Student's *t* test. Error bars in figures were calculated from the ± SEM, and *P* values <0.05 were considered significant. Kaplan-Meier survival curves and respective log-rank tests were used to compare corneal graft survival.

RESULTS

DCregs are phenotypically and functionally resistant to maturation

One important feature of tolerogenic DCs is their capacity to resist phenotypic/functional maturation in response to inflammatory/immunologic stimuli [16, 33], and we thus tested whether our cultivated DCregs are resistant to maturation in response to LPS stimulation. To assess this, we examined the

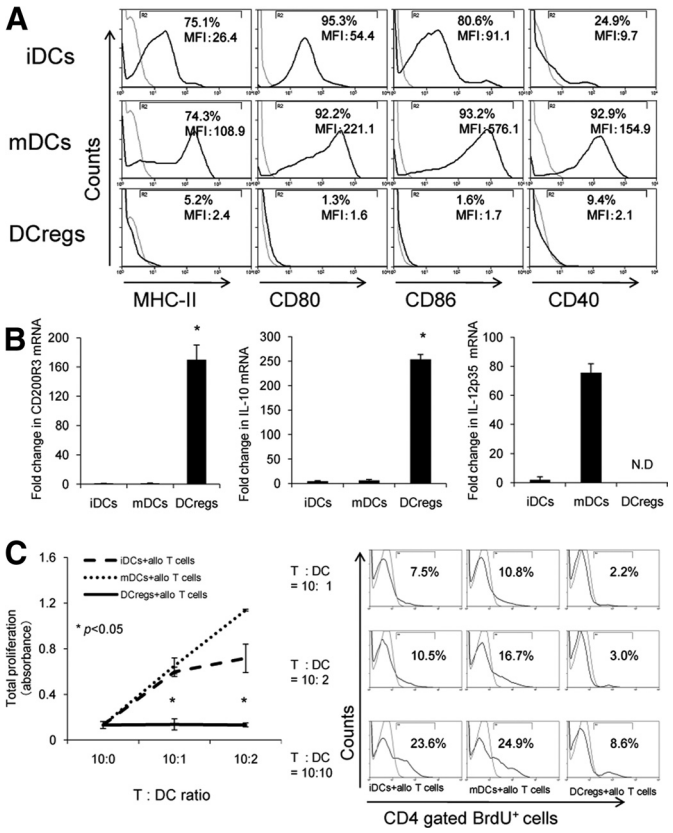

Figure 1. DCregs are phenotypically and functionally resistant to maturation. (A) Expression of maturation markers (MHC-II and costimulatory molecules) on DCregs versus iDCs or mDCs was analyzed by flow cytometry. Gray lines shown are isotype control, and percent-positive cells and mean fluorescence intensity (MFI) are indicated. (B) Expression of CD200R3, IL-12, and IL-10 in DCregs versus iDCs or mDCs was examined by quantitative real-time PCR. $*P < 0.05$. N.D., mRNA not detected. C57BL/6 iDCs, mDCs, or DCregs were cocultured with naïve BALB/c T cells at indicated ratios. (C) T cell proliferation was measured via spectrometric analysis of BrdU incorporation assay. $*P < 0.05$. (D) Proliferation of $CD4^+$ T cells was measured via flow cytometry as well. Histograms shown are gated on a $CD4^+$ population, and percent-positive cells are indicated. Gray lines shown in each histogram are $CD4^+$-gated $BrdU^+$ T cells in cultured, naïve T cells. Data are representative of three experiments.

expression of maturation markers, including MHC-II and costimulatory molecules CD80, CD86, and CD40, by flow cytometry. We found that iDCs, which were not subjected to LPS stimulation, expressed low levels of MHC-II and costimulatory molecules (**Fig. 1A**). In contrast, subjection to LPS stimulation led to a mDC phenotype, defined by high expression of MHC-II and costimulatory molecules (Fig. 1A). However, DCregs, despite undergoing LPS stimulation, exhibited much lower MHC-II and costimulatory levels compared with mDCs, as well as iDCs, thus indicating a resistance of DCregs to phenotypic maturation.

We further characterized DCregs phenotypically by examining mRNA levels of CD200R3 by quantitative real-time PCR. As shown in Fig. 1B, mRNA levels of CD200R3 in DCregs were significantly higher than in iDCs and mDCs. We also analyzed the cytokine expression of IL-10 and IL-12 in DCregs versus iDCs and mDCs by quantitative real-time PCR. This analysis revealed that DCregs expressed significantly lower levels of IL-12p35, and this was similarly observed in iDCs. In contrast, mRNA levels of IL-10 in DCregs were significantly higher than those of iDCs and mDCs (Fig. 1B), thus indicating that DCregs exhibit an immunodulatory phenotype.

We next examined the functional allostimulatory capacity of DCregs in vitro by coculturing naïve, allogeneic T cells with iDCs, mDCs, or DCregs. All three types of DCs were generated from C57BL/6 mice and cocultured with naïve BALB/c T cells for 72 h at different T cell:DC ratios as indicated in Fig. 1C. We found that DCregs resulted in much weaker T cell-proliferation responses, as indicated by the BrdU incorporation assay (Fig. 1C). To confirm that this impairment in T cell proliferation was consistent in the $CD4^+$ T cells (the compartment critical for corneal allograft rejection [20, 34]), we measured $CD4^+BrdU^+$ T cells by flow cytometry following MLR stimulation. As shown in Fig. 1D, we found that the frequency of $CD4^+BrdU^+$ T cells stimulated by DCregs (2.2%) was strongly reduced, relative to those stimulated by mDCs (10.8%) or iDCs (7.5%). This reduction was observed in all different T cell:DC ratios (Fig. 1D). Hence, these aggregate data suggest that DCregs have an impaired stimulatory activity for naïve, allogeneic T cells in vitro.

Donor-derived DCregs impair T cell alloreactivity to orthotopic corneal transplants

Corneal transplantation is a model of indirect allosensitization-dominant-mediated rejection [20–24], and thus, we tested whether donor-derived DCregs could inhibit alloreactivity in this model. We analyzed T cells from DLNs obtained postcorneal transplantation. Donor-derived mDCs or DCregs (1×10^6) were infused 7 days before corneal transplantation, and T cells were harvested 3 weeks after transplantation from recipient DLNs. First, IFN-γ secretion from T cells harvested from DLNs was analyzed by flow cytometry, and to do this, T cells were stimulated in culture with PMA/ionomycin (in the presence of brefeldin A), and CD4 and IFN-γ antibody staining was performed. As shown in **Fig. 2**, we observed a frequency of ~1.0% $CD4^+IFN\text{-}\gamma^+$ T cells in mDC-infused recipients, and this was similarly found in untreated recipients. In contrast,

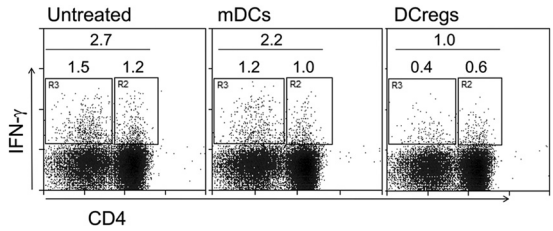

Figure 2. Administration of donor-derived DCregs impairs T cell alloreactivity to transplanted corneal allografts. Isolated T cells obtained from DCreg, mDC, or uninfused (i.e., Untreated) recipients or untreated recipients were stimulated with PMA/ionomycin, and IFN-γ^+ ($CD4^+$ and $CD4^-$ fractions) was subsequently measured by flow cytometry.

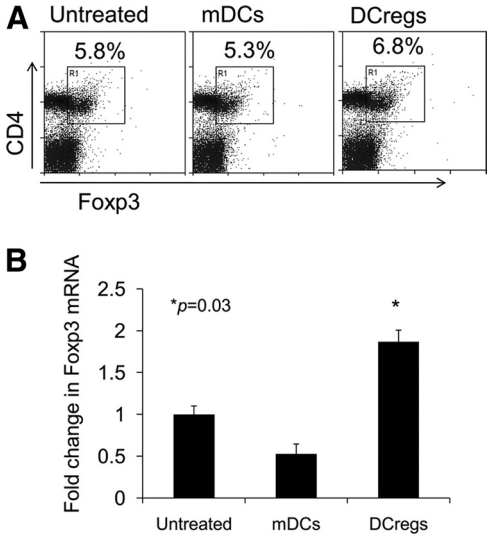

Figure 3. Donor-derived DCregs lead to increased Foxp3 expression in corneal allografted recipients. (A) The frequencies of $CD4^+Foxp3^+$ cells in DLN cell postcorneal transplantation were measured by flow cytometry. (B) Expression levels of Foxp3 in DLNs post-transplantation were analyzed by quantitative real-time PCR. $*P = 0.03$. Data are representative of two experiments.

$CD4^+IFN\text{-}\gamma^+$ T cell frequencies in DCreg-infused recipients were 0.6% (an approximate twofold decrease). The results also showed that not only in the $CD4^+$ fraction but also in the $CD4^-$ fraction, the $IFN\text{-}\gamma^+$ frequencies were reduced in DCreg-infused recipients compared with all other groups. However, we did not observe any difference in $CD4^+IL\text{-}10^+$ frequencies among these groups (data not shown). These data indicate that DCregs inhibited Th1 responses to corneal allografts and thus, suggest that alloreactivity was suppressed.

DCregs lead to increased Foxp3 expression and suppressor function of Tregs following orthotopic transplantation of corneal allografts

We have previously implicated a role for increased Foxp3 expression of Tregs in inhibiting indirect allosensitization and corneal transplant rejection [32]. We therefore examined the effect of DCreg administration on the Treg compartment of corneal-allografted recipients. To address this, $CD4^+Foxp3^+$ Tregs in DLNs post-transplantation were enumerated by flow cytometry and performed quantitative real-time PCR of Foxp3 mRNA levels [32]. We found that the frequency of $CD4^+Foxp3^+$ cells in DCreg-infused recipients (6.8%) was slightly higher than in other groups (untreated: 5.8%; mDC: 5.2%; **Fig. 3A**). Moreover, when mRNA levels for Foxp3 were compared, there was an approximate fourfold increase of Foxp3 mRNA expression in the DLNs of DCreg-infused recipients compared with those of mDC-infused recipients, as well as a twofold increase compared with untreated recipients (Fig. 3B). Thus, these data suggest that DCregs increased Foxp3 expression in recipients.

Promotion of orthotopic corneal transplantation with administration of donor-derived DCregs

Donor-derived DCregs were capable of inhibiting alloreactivity and enhancing increased Foxp3 expression, and thus, we next tested whether corneal transplants would enjoy enhanced survival with administration of DCregs. We infused DCregs i.v. into recipients (BALB/c), 7 days before transplantation, with C57BL/6 corneas. Donor-derived mDCs were also infused in separate animals as a positive control, or animals were left untreated as an additional control. As shown in **Fig. 4**, infusion of 1×10^6 mDCs led to universal rejection of corneal allografts within 2 weeks post-transplantation. Infusion of even fewer mDCs (0.2×10^6) still resulted in universal rejection of transplanted corneas within 2 weeks also (data not shown). In contrast, infusion of as few as 0.2×10^6 DCregs prolonged graft survival, although not in a statistically significant manner, relative to untreated recipients (50%; P=0.17). However, infusion of 1×10^6 DCregs prolonged allograft survival and did so in a statistically significant manner (83%; P=0.043), thus indicating that donor-derived DCregs prolong graft survival in a corneal transplantation model.

Donor-derived DCregs suppress the indirect pathway of allosensitization

We next evaluated whether donor-derived DCregs suppress the indirect pathway of allosensitization in graft recipients. The ELISPOT assay (IFN-γ) was used to measure the alloreactive T cell frequencies post-transplantation of untreated versus DCreg-infused recipients. Donor-derived DCregs (C57BL/6) were infused into recipients (BALB/c), 7 days before corneal transplantation. Purified recipient T cells were stimulated with allogeneic APCs (C57BL/6) to measure the direct pathway-type response [29, 31]. To measure the indirect pathway, puri-

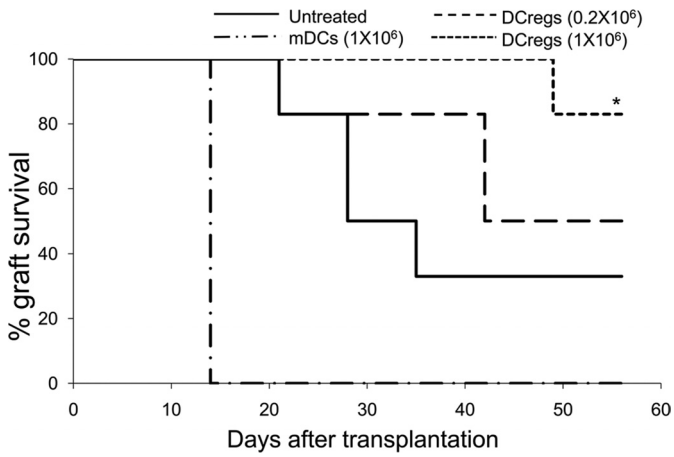

Figure 4. Donor-derived DCregs prolong corneal graft survival. C57BL/6-derived mDC (1×10^6)- or DCreg (0.2×10^6 or 1×10^6)-infused BALB/c mice or untreated mice were transplanted with C57BL/6 corneas, and graft survival was followed biomicroscopically for 8 weeks (n=6 in each group). Kaplan-Meier survival curves indicate that infusion of 1×10^6 donor-derived DCregs prolonged graft survival. This is representative of multiple experiments. $*P = 0.043$.

fied recipient T cells were stimulated with syngeneic APCs (BALB/c) with sonicated alloantigens [29, 31]. We found that in untreated recipients, there is significant increase in the direct and indirect pathway-sensitized T cells, relative to naïve mice (**Fig. 5A** and **B**). However, the magnitude of the increase is much higher in the indirect pathway (28-fold increase) than in direct pathway (1.4-fold increase), further confirming that corneal graft rejection was primarily mediated by the indirect pathway, as reported previously [20–25]. Strikingly, although DCreg-infused recipients showed decreases in the direct (P=0.007) and indirect pathways (P=0.0007) of allosensitization, relative to untreated recipients, the relative magnitude of the reduction is markedly higher for the indirect pathway (30-fold reduction) than the direct pathway (1.5-fold reduction; Fig. 5A and B). Thus, taken together, these data suggest that donor-derived DCregs suppress not only the direct but also the indirect pathway of allosensitization in corneal transplant recipients.

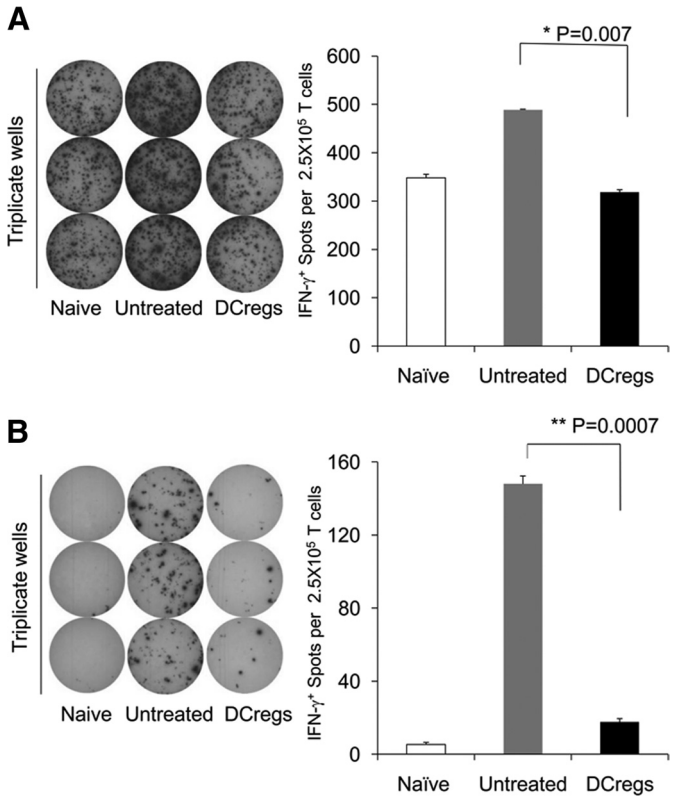

Figure 5. Donor-derived DCregs suppress indirect pathway in corneal-transplanted recipients. T cells were isolated from DLNs of untreated and DCreg-infused recipients (n=4/group), 3 weeks post-transplantation and tested in an IFN-γ ELISPOT assay. (A) Donor-derived DCregs reduced frequency of direct pathway-type alloreactivity. Isolated T cells were cocultured with allogeneic APCs to measure direct pathway. (B) Donor-derived DCregs also reduced frequency of indirect pathway-type alloreactivity, relative to untreated recipients, and the reduction (30.2-fold reduction) is much higher than the direct pathway (1.5-fold reduction). Isolated T cells were cocultured with syngeneic APCs in the presence of allogeneic peptide to measure indirect pathway. Data are representative of two experiments.

DISCUSSION

It is known that donor-derived, tolerogenic DCs suppress the direct pathway of allosensitization [3]; however, the effect of these cells on the indirect pathway has not been defined [16]. To address this question, we used the murine model of corneal transplantation, as it is a well-characterized model of indirect, pathway-dominant rejection [20–24], and found that infusion of donor-derived, tolerogenic DCs prolongs the survival of these allografts. Corresponding with this, using the ELISPOT assay to measure the frequencies of indirect- and direct-allosensitized T cells [29, 31], we observed that donor-derived DCregs suppress primarily by the indirect pathway of allosensitization (albeit, a lesser, although statistically significant, suppression of the direct pathway was also observed). Hence, these data strongly indicate that donor-derived DCregs suppress the indirect pathway of allosensitization and thus, highlights a novel, immunosuppressive mechanism for DCregs in transplantation.

The suppressive mechanisms by which DCregs down-regulate alloimmunity are likely explained, in part, by the phenotypic and functional characteristics reported here. For example, stimulation of iDCs with LPS led to a very high frequency of mDCs; in contrast, DCregs showed an immature phenotype (i.e, MHC-II^{low} CD80^{low}/CD86^{low}/CD40^{low}), despite LPS stimulation, in accord with observations reported by Sato et al. [15] and Lan et al. [8]. Such an impairment in maturation of DCregs correlates with their poor capacity to stimulate a naïve, allogeneic T cell proliferation, as demonstrated here. Furthermore, DCregs express exponentially higher mRNA levels of IL-10 relative to iDCs and mDCs, further indicating their immunosuppressive phenotype.

Analysis of the T cell phenotype in DLNs after corneal transplantation confirmed the suppressive effect of DCregs in vivo. When corneal transplant recipients were administrated with donor-derived DCregs, the frequency of IFN-γ^+ T cells was twofold lower than in untreated or mDC-treated recipients. Relatedly, Chauhan et al. [32] reported that increased Foxp3 mRNA levels are tightly associated with Treg suppressor function and correlate with survival of corneal allografts. In line with this finding, the decreased IFN-γ^+ T cell frequency in DCreg-administrated recipients of corneal allografts in our study was also associated with a twofold increase in Foxp3 levels in DLNs. Furthermore, Fujita et al. [35] and Lan et al. [8] demonstrated independently that DCregs suppress Th1 responses and concomitantly expand Tregs in GVHD and heart-transplant models and thus, are in accord with our findings.

There are several possibilities that could explain the effect of donor DCregs on indirect allosensitization. First, it is known that Tregs (i.e., natural or induced) can suppress proliferation of effector T cells in a bystander or nonspecific manner [36, 37]. This is thought to be possible, as Tregs secrete immunomodulatory cytokines that impact effector T cells in a noncontact-dependent manner. It is conceivable that such a nonspecific suppression mechanism will have a greater impact on higher frequency of T cell responses, which in the case of corneal allografts, represents

the indirectly allosensitized host T cells. Second, donor-derived DCregs may also modulate allosensitization through the impairment of recipient APC function. Divito et al. [17] have shown that donor-derived, maturation-resistant DCs do not contact host T cells but rather, are processed by recipient APCs in vivo. This suggests that modulation of antidonor response by donor-derived, tolerogenic DC therapy is through recipient APCs and perhaps explains how donor-specific transfusion suppresses alloreactivity. However, although there is strong evidence to support the relevance of this, it does not explain completely why donor-derived mDCs do not promote survival in our study, as shown here and by others [8, 15]. Alternatively, another possibility for how donor-derived DCregs suppress the indirect pathway-type allosensitization is via immunosuppressive molecules (e.g., programmed cell death ligand 1 and CTLA-4), which are known to be expressed highly by tolerogenic DCs [8, 15]. These molecules induce hyporesponsiveness or apoptosis of effector T cells and expansion of Tregs, as shown by Wang et al. [38] and Chambers et al. [39]. Further studies are ongoing in our laboratory to explore these potential mechanisms in the prolongation of corneal allograft survival by donor-derived DCregs.

In conclusion, we show a therapeutic effect of donor-derived DCregs in corneal transplant survival, a potentially important observation, given that corneal grafts represent the most common form of allotransplantation. This is explained by their resistance to maturation, poor capacity to stimulate alloreactive effector T cells, and increase of Foxp3 levels in vivo. Moreover, our data clearly show that donor-derived DCregs suppress the indirect pathway of allosensitization, in addition to the direct pathway. This novel, immunoregulatory mechanism of donor-derived, tolerogenic DCs may potentially yield strong therapeutic value, given that ideally, direct and indirect pathways inhibition will have a maximal effect on promotion of long-term transplant survival.

AUTHORSHIP

T.H. and D.R.S. designed the study, performed the experiments, and wrote the paper. S.K.C. designed the study. P.E., T.F., and H.U. performed the experiments. R.D. designed the study and wrote the paper.

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