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Levels of Foxp3 in Regulatory T Cells Reflect Their Functional Status in Transplantation

Sunil K. Chauhan,* Daniel R. Saban,* Hyung K. Lee,* and Reza Dana2*†

Foxp3 expressing CD4+/CD25+ regulatory T cells (Tregs) have been shown to prevent allograft rejection in clinical and animal models of transplantation. However, the role of Foxp3 in regulating Treg function, and the kinetics and mechanism of action of Tregs in inducing allograft tolerance in transplantation, are not yet fully understood. Thus, we investigated the kinetics and function of Tregs in a mouse model of orthotopic corneal transplantation, the most common form of tissue grafting worldwide. In this study, using in vitro functional assays and in vivo adoptive transfer assays, we show that far more relevant than Treg frequency is their level of Foxp3 expression, which is directly associated with the potential of Tregs to prevent allograft rejection by producing regulatory cytokines and suppressing effector T cell activation. In addition, our data clearly demonstrate that Tregs primarily suppress the induction of alloimmunity in regional draining lymph nodes rather than suppressing the effector phase of the immune response in the periphery. These findings provide new insights on Treg dynamics in transplantation, which are crucial for designing therapeutic strategies to modulate Treg function and to optimize Treg-based cell therapies for clinical translation. The Journal of Immunology, 2009, 182: 148–153.

Immune-mediated rejection remains the single most important cause of graft failure in all forms of tissue transplantation. However, spontaneous development of transplant tolerance in some recipients indicates that, just as rejection is mediated by active immune-mediated events, graft acceptance is also sustained by active immunoregulatory mechanisms. One such immune mechanism is mediated by regulatory T cells (Tregs), that have the capacity to suppress host immunity against transplants. Among the various forms of Tregs, CD4+/CD25+ Tregs, which constitute 5–10% of CD4+ T cells, play a central role in inducing and maintaining tolerance to both self- and alloantigens (1–3).

Tregs are exemplified by their expression of transcription factor Foxp3 and have been reported in a number of clinical and animal models of transplantation (5–8). Despite significant advances in understanding Treg biology, several aspects relevant to transplant settings remain unclear. The majority of previous reports have associated the outcome of the allograft primarily with changes in the frequency of Tregs, either in the lymphoid compartment or at the graft site (9, 10). Moreover, levels of Foxp3 expression have been interpreted primarily to reflect Treg frequency rather than defining the regulatory functions of these cells (11–15), although it has been shown that Foxp3 is critically important for both the development of Tregs as well as their suppressor function (16, 17). Thus, understanding the significance of Foxp3 expression per se to analyze Treg function and the dynamics of Treg-mediated tolerance in transplantation may afford new opportunities to harness the potential of these cells to promote tolerance.

We herein investigated the kinetics and function of Tregs in allograft acceptors and rejectors using a well-characterized mouse model of corneal transplantation (18–20). In this model, ~50% of allografts are rejected within 3 wk of transplantation while the remaining half enjoy indefinite survival, indicating involvement of active regulatory mechanisms that promote allograft longevity. Compared with other transplant systems (e.g., skin, heart, kidney, etc.) where a 100% graft rejection rate is observed in unmanipulated recipients, this model provides an exceptional opportunity to study why some of the allografts are spontaneously accepted and to evaluate the role of Tregs in this high frequency of transplant survival.

In the present study, we clearly demonstrate that contrary to previous reports the functional status of Tregs is more reflective of allograft outcome than their numbers. Moreover, the levels of Foxp3 expression in Tregs are directly associated with their potential to suppress T cell activation and prevent allograft rejections.

Materials and Methods

Mice

The 8- to 10-wk-old BALB/c (H-2d), C57BL/6 (H-2b), and C3H (H-2h) male mice were purchased from Taconic Farms (and used as recipients, donors, and third-party controls, respectively. The study was approved by the Institutional Animal Care and Use Committee, and the mice were treated according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Corneal transplantation

Standard protocol for murine orthotopic corneal transplantation was used, as described previously (18, 20). Briefly, donor center corneas (2-mm diameter) were excised from C57BL/6 mice and sutured on to the recipient graft beds prepared by excising a 1.5-mm site in the central cornea of BALB/c mice. Simultaneously, some BALB/c mice received syngeneic (BALB/c) grafts to control for the non-allospecific effects of surgery. The corneal sutures were removed 7 days after surgery. All grafts were evaluated using slit-lamp biomicroscopy at weekly intervals. Grafts were

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*Abbreviations used in this paper: Treg, regulatory T cell; LN, lymph node; Teff, T effector; MFI, mean fluorescence intensity.

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defined as rejected when they became opaque and the iris details could not be recognized clearly using a standardized opacity grading (ranges, 0–5) scheme. This model renders an easy and confirmatory method to directly evaluate graft endpoints in vivo at different time points without sacrificing the animals, which makes it an outstanding system to study immune responses in the transplant setting.

Flow cytometry
Ipsilateral draining submandibular and cervical lymph nodes (LNs) as well as grafted corneas were harvested and single-cell suspensions were prepared. Single-cell suspensions of corneal samples were prepared by collagenase digestion, as previously described (19). The isolated cells were stained with the following Abs: Anti-CD4/CD3 FITC, anti-CD25 PE, and anti-Foxp3 PE/Cy5 (eBioscience). Stained cells were analyzed by FACSCalibur and FlowJo.

Cell sorting
CD4+ T cells and CD4+CD25+ Tregs from the LN of naïve and/or different graft recipients were isolated by magnetic separation using CD4+ T cell and Treg isolation kits (Miltenyi Biotec). Purity of sorted cells was >97%, and 95% of CD4+CD25+ Tregs were Foxp3+ in all the groups as confirmed by flow cytometry.

Real-time PCR
RNA was isolated with RNaseasy Micro Kit (Qiagen) and reverse transcribed using Superscript III Kit (Invitrogen). Real-time PCR was performed using TaqMan Universal PCR Mastermix and preformulated primers for Foxp3 (assay ID Mm00475156_m1) and GAPDH (assay ID Mm99999915_g1) (Applied Biosystems). The results were analyzed by the comparative threshold cycle method and normalized by GAPDH as an internal control.

Western blot
Tregs (n = 5 × 10⁴) isolated from the LN of allograft acceptors and rejectors were lysed in lysis buffer. The protein concentration in cell lysates was determined using BCA reagent (Thermo Scientific). Equal amounts of protein were boiled in an equal volume of Laemmli sample buffer and resolved by 10% SDS-PAGE. Proteins were then transferred to polyvinylidene difluoride membrane (Immobilon; Millipore) and probed overnight with primary anti-Foxp3 Ab (Santa Cruz Biotechnology) or anti-β-actin Ab (Abcam). Immunoreactive bands were detected with HRP-conjugated secondary Abs and visualized by ECL. The intensities of immunoreactive bands were analyzed using GelPro software.

Suppression assay
For all the suppression assays, including cytokine and Transwell assays, CD4+ T effectors (Teff, total CD4+ cells without depleting the CD25+ population) and Tregs were isolated from the draining LNs. T cell effector population from different groups of allograft recipients at 18 h postsurgery. This time point for Treg transfer was selected on the basis of our previous observation of detecting considerable numbers of graft-derived APCs in the draining LNs of recipients at 24 h posttransplantation (18). Allograft survival rate in each group (n = 6 per group) was monitored up to 8 wk posttransplantation.

Statistical analysis
Student’s t test was used for comparison of mean between the groups. Kaplan-Meier analysis was adopted to construct survival curves, and the log-rank test was used to compare the rates of corneal graft survival. Data are presented as mean ± SEM and considered significant at p < 0.05.

Results
Tregs in the draining LN express different levels of Foxp3
Regional draining LN and grafted corneas were harvested from graft recipients at weekly intervals up to wk 4 posttransplantation. At wk 3 posttransplantation, allograft recipients were separated into acceptors and rejectors. The flow cytometric analysis of draining LN cell suspensions showed no difference in the frequencies of CD4+CD25+Foxp3+ Tregs, either as a proportion of the total CD4+ T cells (range: 8.5–10.5%) or of the total LN cells (range: 4–5%) among syngeneic recipients, acceptors, and rejectors at any of the time points (Fig. 1, a and b). Interestingly, in contrast to the Treg frequencies, we observed considerable differences in the levels (mean fluorescence intensity: MFI) of Foxp3 by the Tregs of different groups (Fig. 1c). The MFI of Foxp3 in Tregs of allograft acceptors was ~50% higher compared with those in the Tregs of allograft rejectors and syngeneic recipients. Additionally, to confirm these differences in the Foxp3 expression levels, we investigated Foxp3 protein levels by Western blot in sorted Tregs and mRNA levels by real-time PCR in the draining LN as well as in equal number of sorted Tregs of different graft recipient groups. The densities of immunoreactive Foxp3 bands (normalized to densities of β-actin show that acceptor-Tregs express 2-fold higher Foxp3 protein compared with those by rejector-Tregs (Fig. 1c). Similarly, there was a ~2-fold increase in Foxp3 mRNA expression in the LN of acceptors as compared with those of rejectors at wk 3 posttransplantation and thereafter. In addition, the levels of Foxp3 mRNA expression in the LN of rejectors were lower than those of syngeneic recipients, which nearly remained unchanged from baseline levels (Fig. 1d). Similar to the Foxp3 mRNA expression in total LN, the isolated Tregs from the LN of acceptors expressed 2-fold higher levels of Foxp3 mRNA compared with those from the LN of rejectors (Fig. 1e) confirming that Tregs in allograft acceptor express high levels of Foxp3.

In addition, we wanted to determine whether Tregs migrate to the graft site and, similar to LN-Tregs, express different levels of Foxp3 in accepted vs rejected allografts. The relative frequencies of Foxp3+ Tregs (11–13%) in the LN of rejectors was ~2-fold higher in corneas with rejected grafts than those with accepted grafts (Fig. 1f). This higher expression of Foxp3 in rejected grafts was primarily due to the nearly ~2-fold increase in the frequencies of infiltrating T cells (Fig. 1g) as well as expression levels of Foxp3 (MFI: 33–36) in Tregs (Fig. 1g) remained the same among the three groups. However, the expression of Foxp3 mRNA was ~2-fold higher in corneas with rejected grafts than those with accepted grafts (Fig. 1h). This higher expression of Foxp3 in rejected grafts was principally due to the nearly ~2-fold increase in the frequencies of infiltrating T cells (and hence also Tregs) in the corneas with rejected as compared with accepted allografts (Fig. 1f).

Tregs of allograft acceptors are highly effective in suppressing naive, but not allos primed, T cell activation
Using a coculture suppression assay system, we investigated the regulatory potential of Tregs to suppress the proliferation of CD3 Ab-stimulated naive T cells (isolated from the LN of naive BALB/c mice) and allo-primed T cells (isolated from the LN of allograft rejectors). Tregs isolated from the LN of acceptors (acceptor-Tregs) were significantly more effective in suppressing the proliferation of naive T cells compared with those isolated from...
the LN of rejectors (rejector-Tregs) and syngeneic recipients (syngeneic-Tregs). Rejector-Tregs were even less effective in suppressing the T cell proliferation than syngeneic-Tregs (Fig. 2a). However, in contrast to suppression of naive T cell activation, Tregs of all the groups failed to efficiently suppress the proliferation of alloprimed effector T cells (Fig. 2b).

Tregs of allograft acceptors produce higher levels of regulatory cytokines and use both contact-dependent and -independent mechanisms of suppression

To delineate the mechanisms by which Tregs suppress the activation of T cells, we measured the cytokine levels in the supernatant of suppression assays and investigated the mechanisms of suppression using ELISA and Transwell assays, respectively. The levels of regulatory cytokines, TGFβ1 and IL-10, were significantly higher (~2- to 3-fold) in the supernatant of suppression assays with acceptor-Tregs compared with those with rejector-Tregs and syngeneic-Tregs (Fig. 3a). In Transwell assays, only acceptor-Tregs were capable of significantly suppressing the T cell proliferation, suggesting that these Tregs can use both cell-cell contact-dependent and -independent (cytokine-mediated) mechanisms of suppression.

FIGURE 1. Kinetics of Treg frequency and Foxp3 expression in syngeneically grafted recipients (BALB/c→BALB/c) and fully disparate allograft (C57BL/6→BALB/c) acceptors and rejectors. a, FACS analysis of draining LN cell suspension showing frequencies of CD4+ CD25+Foxp3+ Tregs at wk 3 post-transplantation. b, Frequencies of Tregs of CD4+ and total LN cells at weekly intervals from day 0 to wk 4 post-transplantation. c, MFI and Western blot analyses showing expression levels of Foxp3 protein in purified Tregs isolated from the LN at wk 3 post-transplantation (*, p = 0.036). d, Real-time PCR analysis of Foxp3 mRNA expression levels in total LN cells at weekly intervals from day 0 to wk 4 post-transplantation (acceptor vs syngeneic and rejector at wk 3, *, p = 0.034 and 0.027; and at wk 4, ‡, p = 0.01 and 0.001); and (e) in equivalent numbers of purified Tregs sorted from the LN at wk 3 post-transplantation (*, p = 0.026). f, Frequencies of corneal graft-infiltrating Foxp3+ Tregs and total CD3+ T cells and (g) MFI of Foxp3 levels in graft-infiltrating Tregs. h, Real-time PCR analysis of Foxp3 mRNA expression in the grafted corneas of syngeneically graft recipients, allograft acceptors, and allograft rejectors at wk 3 post-transplantation (+, p = 0.037). Each transplant group consists of 4–6 recipient mice and data present the mean ± SEM of three independent experiments. Statistical significance for mRNA and MFI levels is calculated on fold-change values over those of syngeneic or naive Tregs. p values are calculated using Student’s t test.

FIGURE 2. Suppressor potential of Tregs. a, Naive CD4+ T cells isolated from the LN of unprimed BALB/c recipients and (b) allo-primed CD4+ T cells isolated from the LN of allograft rejectors were stimulated with CD3-Ab for 3 days in the presence of different Tregs isolated from syngeneically grafted recipients, allograft acceptors, and allograft rejectors at wk 3 post-transplantation (+, p = 0.037). The activity of Tregs is measured at Treg:Teff cell ratio of 1:2. Proliferation was measured using the BrdU incorporation assay and compared with the proliferative responses of respective CD3-stimulated naive or alloprimed effector T cells in the absence of Tregs (*, p = 0.018; ‡, p = 0.0002).
were placed in Transwell inserts of 1.0-

were either added directly to the culture or

unprimed BALB/c recipients were stimulated with anti-CD3 Ab, and the

expression of Tregs and % suppression was calculated as described in

with the proliferative responses of CD3-stimulated naive T cell in the ab-

liferation was measured using the BrdU incorporation assay and compared

sent SEM.

Each transplant group consists of 4–6 recipient mice. Data from a repre-

plantation. The activity of Tregs is measured at Treg:Teff cell ratio of 1:2.

recipients, allograft acceptors, and allograft rejectors at wk 3 posttrans-

Thus, we performed a Treg adoptive transfer experiment in which

vivo by demonstrating there ability to prevent graft rejection.

Our in vitro Treg functional data on T cell suppression and cyto-

expression clearly demonstrated that Foxp3 high-expressing

allograft survival

Effect of Treg adoptive transfer on allograft survival. Tregs

were isolated from the LN of syngeneically grafted recipients (syngeneic Treg), allograft acceptors (acceptor Treg) and allograft rejectors (rejector Treg) at wk 3 posttransplantation; 1 × 10^5 Tregs per recipients were i.v. transferred to three different groups of allograft (C57BL6→BALB/c) re-

FIGURE 5. Effect of Treg adoptive transfer on allograft survival. Tregs were isolated from the LN of syngeneically grafted recipients (syngeneic Treg), allograft acceptors (acceptor Treg) and allograft rejectors (rejector Treg) at wk 3 posttransplantation; 1 × 10^5 Tregs per recipients were i.v. transferred to three different groups of allograft (C57BL6→BALB/c) re-

in the LN and grafts. However, Tregs isolated from the LN of

Discussion

In this study, we show that the frequencies of Tregs remain

unchanged in allograft-acceptors vs allograft-rejectors in both the LN and grafts. However, Tregs isolated from the LN of

allograft acceptors express significantly higher levels of Foxp3

Tregs of allograft acceptors show allospecificity

Increased production of TGFβ1 and IL-10 by acceptor-Tregs point toward a subset of induced Tregs which may be alloan-

tigen-specific. To characterize this specificity further, we analy-

alyzed the suppression potential of Tregs of the different groups

against proliferation of recipient naive T cells (BALB/c) to do-

nor (C57BL6)-specific APCs and third party (C3H) APC, allo-

disparate to both recipients and donors (Fig. 4). Similar to sup-

pression of CD3 Ab-stimulated T cells, acceptor-Tregs were

more effective in suppressing donor-specific Ag (C57BL6-

-APC)-stimulated T cells compared with syngeneic-Tregs. In

contrast, no difference was observed in the potential of accep-

tor-Tregs and syngeneic-Tregs in suppressing the activation of

nonspecific third party Ag (C3H-APC)-stimulated T cells; in-

deed, this suppression was equal to the suppression of donor-

specific Ag-stimulated T cells by syngeneic-Tregs. However,

rejucer-Tregs showed little to no suppression of any of the

Ag-stimulated T cells. These results clearly suggest induction of

allograft-specific Tregs only in acceptors.

Tregs of allograft acceptors on adoptive transfer prevent

allograft survival

Our in vitro Treg functional data on T cell suppression and cyto-

ekine expression clearly demonstrated that Foxp3 high-expressing

Tregs from the LN of allograft acceptors are highly functionally
effective compared with those of rejectors. Finally, we wanted to

confirm this functional efficacy of Foxp3 high-expressing Tregs in vivo by demonstrating there ability to prevent graft rejection.

Thus, we performed a Treg adoptive transfer experiment in which

Tregs isolated from the LN of syngeneically grafted recipients,

allograft acceptors, and rejectors at wk 3 posttransplantation were

transferred i.v. to three different groups of allograft recipients (Fig. 5).

Only the group that was adoptively transferred with acceptor-

Tregs showed significant increase in the allograft survival rate

(83%, p = 0.035, Kaplan-Meier analysis); no improvement in graft

survival was observed in the other groups of recipients which re-

survived. Controls recipients were syngeneically grafted

BALB/c and received no Tregs (n = 6 per group; *, p = 0.035, Kaplan-Meier survival analysis).

FIGURE 4. Alloantigen-specificity of Tregs. The suppression potential of Tregs was evaluated against proliferation of recipient’s naive T cells to
donor-specific and third-party (allodisparrate to both recipient and donor)
Ags. Naive T cells from the LN of unprimed BALB/c mice (graft recipients) were cocultured with APCs of C57BL6 mouse (graft donors) or C3H mouse (third-party) in the presence of Tregs isolated form the draining LNs of syngeneic recipients, allograft acceptors, and allograft rejectors at wk 3 posttransplantation (*, p = 0.012, Acceptor-Tregs: C57BL6-APC vs C3H-

-APC). Proliferation was measured using the BrdU incorporation assay and compared with the proliferative responses of respective APC stimulated

naive T cell in the absence of Tregs and % suppression was calculated as

described in Materials and Methods. Each transplant group consists of 4–6 recipient mice. Data from a representative experiment of three performed

is shown. p values are calculated using student’s t test and error bars represent SEM.
compared with those of allograft rejectors. In addition, these Foxp3\textsuperscript{high}-expressing Tregs are functionally highly effective in preventing graft rejection as demonstrated by adoptive transfer experiments.

Despite strong evidence about the association of Foxp3 primarily with Treg development and function, care must be taken with any simple interpretation of Foxp3 analysis. Recent human studies have shown that most T cells transiently turn on Foxp3 during early activation stages (21, 22). Thus, it is imperative to characterize Tregs at both the phenotypic and functional levels, as most transplant studies are now using Foxp3 as a marker to define Tregs. Moreover, these reports are associating allograft outcome with Foxp3 PCR analysis or Treg frequencies (9–15). Our data clearly suggest that Foxp3 PCR analysis does not simply reflect Treg frequencies. Increased Foxp3 expression in the LN of allograft acceptors was mainly due to the high level of Foxp3 expression in Tregs themselves and not simply due to their increased numbers. In contrast to the LN, we did not observe differences either in Foxp3 levels in graft-infiltrating Tregs or their frequencies in relation to total infiltrating T cells in accepted vs rejected grafts. However, the absolute numbers of Tregs in rejected grafts were higher than those in accepted grafts, which is the main reason for the increased Foxp3 mRNA expression in rejected grafts. In addition to Tregs homing into grafts, these findings suggest that evaluation of only a single T cell subset (Tregs or Teffs) either at the graft site or in the lymphoid compartment may potentially lead to incomplete conclusions. Treg migration to the graft site appears to be either a response to down-regulate the ongoing inflammation or nonspecific homing due to a greater propensity of T cells to be recruited to sites of inflammation in the periphery. These findings are in accord with several previous studies demonstrating increased Foxp3 expression in the biopsies of rejected kidney grafts and suggest the presence of intragraft Tregs as a consequence of the normal immune response during rejection (10, 23).

The essential role of Foxp3 in regulating the suppressor function of Tregs has been well documented (16, 17). Our observations of increased Foxp3 levels in the LN-Tregs of allograft acceptors prompted us to hypothesize that higher Foxp3 levels in Tregs are associated with their enhanced regulatory functions. Thus to validate our hypothesis, we analyzed the function of Tregs isolated from the LN of different transplant groups. Our data demonstrate that Foxp3\textsuperscript{high} Tregs isolated from the LN of allograft acceptors were more effective in suppressing naive T cell proliferation and in producing higher levels of TGF-\(\beta\) and IL-10 compared with those isolated from allograft rejectors and syngeneic recipients. However, Tregs of all the transplant groups failed to efficiently suppress the proliferation of allo-primed effector T cells. These findings suggest that Tregs may be more effective in regulating allograft response at the induction phase than at the effector phase. Our previous findings of more numbers of Tregs in rejected corneas with their inability to prevent rejection also lend support to this notion. The inefficiency of Tregs to suppress the allo-primed effector T cells may be due to the intrinsic resistance of Teff cells to suppression, potentially due to Teff cells’ increased capacity to produce cytokines (e.g., IL-6, TNF-\(\alpha\), and IFN-\(\gamma\)), which can inhibit and antagonize Treg function (24). Although, peripherally induced CD4\textsuperscript{CD25} Tregs are phenotypically indistinguishable from thymus-derived CD4\textsuperscript{CD25} natural Tregs, higher levels of TGF-\(\beta\) and IL-10 secretion by the acceptor-Tregs point toward the development of induced-Tregs in allograft acceptors. In addition, our data demonstrate the induction of alloantigen-specific Tregs only in the LN of allograft acceptors, which corroborates with previous reports suggesting that peripheral development of alloantigen-specific Tregs occurs in the LNs (25) and constitutively high expression of Foxp3 is required for Treg differentiation and function (16, 17). Finally, our findings of Treg adoptive transfer experiment clearly show that only Tregs isolated from the LN of allograft acceptors are capable of preventing allograft rejection.

In summary, these findings provide new insights into the role of Tregs in transplantation immunity. For the first time, the present study reports that the functional status of Tregs is more reflective of the allograft outcome than their absolute frequencies, and that the levels of Foxp3 expression are directly associated with the Treg potential in preventing graft rejection. In addition, this is the first description of Treg localization and function in corneal transplantation, the most common form of tissue grafting. Our data also demonstrate the induction of allospecific Tregs in graft recipients, which clearly are able to suppress the induction of allograft immunity in regional draining LNs but are not equally effective in suppressing the effector phase of the immune response. These observations have at least two important implications in regulating the immune responses to tissue transplants beyond the obvious applications to corneal allograft immunity. First, predicting and/or correlating allograft survival, or studying the effect of therapeutic agents on Treg biology in transplantation, exclusively on the basis of Treg frequencies (either in lymphoid tissues or graft site) may potentially lead to inaccurate conclusions. Second, potentiating Treg function to suppress allograft immunity, particularly at the induction phase, may be more helpful in promoting transplant longevity than at the effector phase.

Disclosures
The authors have no financial conflict of interest.

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