A Key Role for TGF-β Signaling to T Cells in the Long-Term Acceptance of Allografts[1]

Stephen R. Daley,[2] Jianbo Ma, Elizabeth Adams, Stephen P. Cobbold,[3] and Herman Waldmann[3,4]

TGF-β is a key immunoregulatory cytokine which supports self-tolerance by signaling to T cells. In this report, we show a crucial role for TGF-β signaling to T cells in enabling the long-term acceptance of allografts, whether natural or induced therapeutically by coreceptor and costimulation blockade. The requirement for TGF-β appears most pronounced during the initial exposure to alloantigens. We demonstrate the ability of TGF-β to direct the development in vitro of regulatory cells that suppress graft rejection in vivo. Such suppression was not affected by anti-TGF-β treatment of the recipient mice. Despite this, TGF-β may still have a role in CD4$^+$ cell-mediated suppression of antiallograft responses in vivo, since its neutralization can, in some cases, abrogate suppression. These results show that TGF-β signaling to T cells is dispensable for mounting destructive responses against skin allografts while appearing to be an essential intermediary in establishing long-term tolerance. ***The Journal of Immunology,*** **2007, 179: 3648–3654.**

Transforming growth factor β has been shown to have a crucial role in immunological self-tolerance (1, 2). Recently, a major checkpoint for its protective activity has been narrowed to that of T cell signaling, since ablation of the TGF-β receptor type II (*Tgfbr2*)[5] gene in T cells produces catastrophic autoimmunity (3, 4) associated with increased turnover and maturation of CD4$^+$ and CD8$^+$ T cells and decreased "competitive fitness" of Foxp3$^+$ regulatory T cells (Treg) (3). Ablation of the TGFβRII gene in B cells heightens the responsiveness of IgM$^+$ and IgG$^+$ B cells and blocks isotype switching to IgA (5), suggesting that TGF-β serves parallel purposes in controlling the differentiation of both T and B cells. Interference with TGF-β signaling in CD11c$^+$ cells using a dominant-negative *Tgfbr2* transgene resulted in the accumulation of an abnormally high frequency of NK cells, most of which synthesized IFN-γ in response to stimulation, relative to wild type (6). Thus, a common thread is that TGF-β regulates the homeostasis of several leukocyte subsets in vivo and it clearly has the capacity to regulate immunity.

TGF-β is not, however, an obligate anti-inflammatory cytokine, because TGF-β was identified as a component of in vitro milieu that favor Th17 commitment in CD4$^+$ T cells (7–9). Its activities for good or bad must then be contextual and cannot always be predicted.

The first indication that TGF-β may have a role in transplantation tolerance came from studies examining tolerance induced by donor-specific transfusions (10). More recently, a role for TGF-β has been highlighted in the induction of tolerance by coreceptor blockade with a CD4-specific mAb. This study used a TCR- transgenic mouse (A1.RAG1$^{-/-}$) where all T cells bear a TCR specific for a male transplantation Ag (Dby presented by H2-E^k) (11). Female A1.RAG1$^{-/-}$ mice normally reject male skin grafts, but nondepleting anti-CD4 treatment permits life-long graft survival (11). We showed that TGF-β, in conjunction with coreceptor blockade, had a clear role in conversion of naive CD4$^+$ T cells to Foxp3$^+$ Treg (12). We showed that neutralization of TGF-β prevented tolerance induction to male grafts by CD4 coreceptor blockade (12). These, along with the finding that transplanted tumors survived in wild-type hosts but were rejected in hosts where CD8$^+$ T cells were deficient in TGF-β signaling (13), all argue for TGF-β as a protective factor against allograft rejection.

Building on our previous studies using systemic TGF-β neutralization, we use here recipient mice that express a T cell-specific dominant-negative TGF-β receptor type II (dn*Tgfbr2*). By introducing this transgene into the A1.RAG1$^{-/-}$ model, we examine whether one activity of TGF-β in supporting allograft tolerance is by signaling to T cells. We use dn*Tgfbr2*$^+$ mice as a tool to seek tolerance checkpoints bypassed by dn*Tgfbr2*$^+$ T cells. We also study the interval during which TGF-β is required to act: before exposure, during the initial exposure, or after prolonged exposure to alloantigens shed by skin grafts.

Materials and Methods

Mice

CBA/Ca (CBA), B10.BR, BALB/k, hCD52-Tg (14), A1.RAG1$^{-/-}$ (11), and CD4dn*Tgfbr2* (dn*Tgfbr2*$^+$) (15) mice have been described. dn*Tgfbr2*$^+$ mice had been backcrossed to C57BL/6 (B6) for >10 generations. Wild-type and dn*Tgfbr2*$^+$ mice were offspring of dn*Tgfbr2*$^+$ males hemizygous for this transgene. We crossed dn*Tgfbr2*$^+$ mice with B6.RAG1$^{-/-}$ mice for two generations to produce dn*Tgfbr2*$^+$RAG1$^{-/-}$ animals that were then crossed with A1.RAG1$^{-/-}$ to introduce the Dby-specific TCR transgene. Genotyping of dn*Tgfbr2*$^+$ vs wild-type mice used PCR on genomic DNA

Therapeutic Immunology Group, Sir William Dunn School of Pathology, Oxford, United Kingdom

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[2] Current address: John Curtin School of Medical Research, Building 54, ANU, Mills Road, Acton, Australia.

[3] S.C. and H.W. contributed equally as joint senior authors.

[4] Address correspondence and reprint requests to Prof. Herman Waldmann, Sir William Dunn School of Pathology, South Parks Road, Oxford, U.K. E-mail address: herman.waldmann@path.ox.ac.uk

[5] Abbreviations used in this paper: *Tgfbr2*, TGF-β receptor type II; dn*Tgfbr2*, dominant-negative TGF-β receptor type II; Treg, regulatory T cell.

Table I. *Forward and reverse primer sequences and TaqMan probes*

Gene Transcript	Forward Primer; Reverse Primer (5′–3′)	TaqMan Probe
CD3γ	TTACAGAATGTGTGAAAACTGCATTG; CACCAAGAGCAAGGAAGAAGATG	VIC-5′-ACATAGGCACCATATCCGGC TTTATCTTCG-3′-TAMRA
Foxp3	CCCAGGAAAGACAGCAACCTT; TTCTCACAACCAGGCCTCTTG	FAM-5′-CTACCCACTGCTGGCAAATG GAGTC-3′-TAMRA
GAPDH	TCACTGCCACCCAGAAGACTG; TCAGATCCACGACGGACACAT	FAM-5′-TGGCATGGCCTTCCGTGTTC C-3′-TAMRA

extracted using the DNEasy Tissue kit (Qiagen); primers: 5′-CCCAACCAACAAGAGCTCAAG-3′ and 5′-TTG GGGTCATGGCAAACTGTCTC-3′. The dn*Tgfbr2* transgene generates an ~350-bp PCR product. All mice were bred and housed in the specific pathogen-free facility at the Sir William Dunn School of Pathology (Oxford, U.K.) and were first used at the age of 8–12 wk. All procedures were conducted in accordance with the Home Office Animals (Scientific Procedures) Act of 1986.

Antibodies

The mAbs anti-CD4 (YTS 177) (16), anti-CD8 (YTS 105) (16) anti-CD40L (MR1) (17), Campath-1H (CP-1H) (18), anti-TGF-β (1D11) (19), anti-IL-10R (1B1.2) (20), anti-IFN-γ (XMG1.2) (21), or OX21 (22) were administered i.p. on the days described in the figure legends. All mAbs were concentrated by ammonium sulfate precipitation from cultures grown in hollow-fiber bioreactors. IgG concentration was determined by spectrophotometry and PAGE for all mAbs except anti-TGF-β, for which a mouse IgG-specific ELISA was used.

Skin grafting

Full-thickness tail skin (~1 cm × ~1 cm) was transplanted onto a vascularized graft bed on the lateral thoracic flank as previously described (16). Skin grafts were considered rejected when no viable donor tissue was visible.

Establishment of TGF-β-converted cells

Splenocytes (5×10^5 cells/well) from female A1.RAG1$^{-/-}$ mice were cultured with female CBA bone marrow-derived dendritic cells (23) (10^5 cells/well) in 24-well plates for 7 days. Dby peptide (REEALHQFRS GRKPI) was added at a final concentration of 100 nM and rTGF-β (R&D Systems) at 2 ng/ml. Control cells were activated with peptide only. Viable cells were separated over Histopaque gradients, resuspended in PBS, and either 10^7 or 2×10^7 cells were injected i.v. into female A1.RAG1$^{-/-}$ mice. The following day the recipient mice were grafted with male CBA.RAG1$^{-/-}$ tail skin. The percentage of Foxp3$^+$ cells was checked by flow cytometry: TGF-β-converted and peptide-only suspensions were typically 15–25% and 0.2% Foxp3$^+$, respectively.

ELISA for rat IgG2a and hamster IgG

Flat-bottom 96-well microtiter plates were coated with anti-rat IgG2a (MARG2a-1; Serotec) or goat anti-hamster IgG (Jackson ImmunoResearch Laboratories) diluted in PBS. Nonspecific binding was blocked using 30 g/L BSA in PBS. Bound Ig was detected using biotinylated MARK-1/MARL-15 (anti-rat κλ L chains; Serotec) or rabbit anti-hamster IgG detection Abs (The Jackson Laboratory). In both cases, the assay was developed by extravidin peroxidase followed by *o*-phenylenediamine substrate. The absorbance at 492 nm was read using a microplate reader.

Estimation of serum IgG concentrations

Sigmoidal dose-response (variable slope) nonlinear regression was performed on dose-response (serum volume-OD_{492}) curves using GraphPad Prism version 4.00 for Windows (GraphPad Software). Values for top and bottom were constrained to the plate maximum and minimum readings. Hill slope was calculated from those samples that had extreme OD_{492} values close to the top and bottom values; this constant for hill slope was then applied to all samples on the plate. From each fitted dose-response curve, Prism computed the point of inflection (LOGEC50), which is proportional to the IgG concentration, as well as 95% confidence intervals. Limit of detection was estimated using the mean plus two SDs of OD_{492} values generated by serially diluted normal mouse serum spiked with "off-target" mAbs that were given to the mice but were not a target for detection on that plate.

Real-time RT-PCR

DNase-treated total RNA from tissues was prepared using the SV Total RNA isolation system (Promega). Reverse transcription was performed using the StrataScript kit (Stratagene). Real-time quantification was performed using gene-specific, fluorogenic probes (Table I) and a Universal Mastermix kit (PE Applied Biosystems) in a final volume of 25 μl. The reaction mixture contains all primers at a concentration of 300 nM and the probe at 200 nM. A hot start, two-step PCR (15 s at 95°C and 60 s at 60°C) was applied for 50 cycles. PCR and TaqMan analysis were performed using the Applied Biosystems PRISM 7700 sequence detector system (PE Applied Biosystems). Geometric means of the ratio: transcript of interest: normalizer transcript for triplicate reactions is given. Each spot on the graphs represents an individual mouse, while bars show the group mean.

Adoptive transfer

Splenocytes were passed through a 70-μm filter (BD Biosciences) and washed in RPMI 1640 (Invitrogen Life Technologies). RBCs were removed by osmotic lysis. Cell numbers were adjusted to 2.5×10^8 cells/ml in PBS and 0.2 ml/mouse was injected i.v.

Statistics

Statistical differences in skin graft survival were analyzed using the log rank method, which is equivalent to the Mantel-Haenszel test. Statistical differences in mRNA levels were analyzed using the Student *t* test (unpaired, two tailed).

Results

Natural tolerance to H-Y minor Ags requires TGF-β signaling to host T cells

In B6 mice, proteins encoded by the Y chromosome (H-Y) are sufficiently immunogenic to elicit rejection of male skin grafts by female littermates, whereas in CBA mice H-Y Ags do not usually elicit rejection. We grafted female F_1 hybrids of these two strains with skin grafts collected from male littermates and found that seven of eight recipients accepted the grafts (Fig. 1*A*). By contrast, a group of eight dn*Tgfbr2*$^+$ female F_1 hybrids, in which transgene expression limits TGF-β signaling specifically in T cells, all rejected their grafts within 53 days ($p = 0.0005$). No rejection was observed in control male dn*Tgfbr2*$^+$ F_1 hybrids, showing that foreignness was necessary to elicit rejection. This shows that TGF-β signaling to T cells contributes to raising the threshold for allograft rejection.

Tolerance induction through coreceptor blockade in a monospecific TCR-transgenic mouse requires TGF-β signaling in host T cells

We have previously shown that systemic anti-TGF-β abrogates anti-CD4-induced tolerance in the A1.RAG1$^{-/-}$ monospecific TCR-transgenic mouse (12). To examine whether TGF-β signaling to T cells was critical for tolerance, we followed graft survival in monospecific TCR-transgenic littermates with or without the dn*Tgfbr2* transgene. Whereas anti-CD4 induced long-term graft survival or tolerance in females with intact TGF-β signaling in T cells, it failed in the dn*Tgfbr2*$^+$ group (Fig. 1*B*). Importantly, because the conversion to a memory phenotype in T cells deficient in TGF-β signaling (15) is TCR activation dependent (4), it would

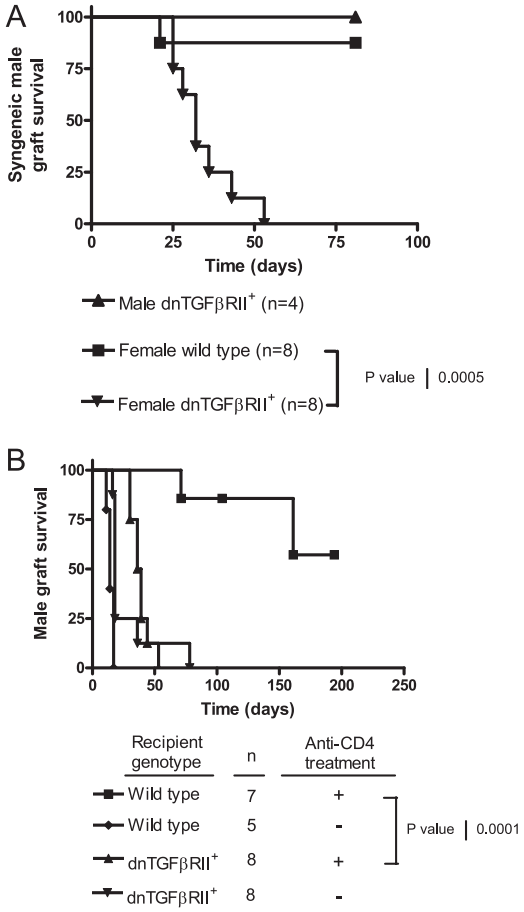

FIGURE 1. T cell TGF-β signaling is necessary for "natural" and induced tolerance to skin grafts expressing male (H-Y- encoded) transplantation Ags. *A,* Wild-type or dn*Tgfbr2*$^+$ (CBA $\times$ B6)F_1 or (hCD52-Tg $\times$ B6)F_1 mice received skin grafts collected from male littermates in the absence of any other treatment. The percentage of surviving grafts is represented on the *y*-axis. *B,* Wild-type or dn*Tgfbr2*$^+$ (A1.RAG1$^{-/-}$ $\times$ B6.RAG1$^{-/-}$)F_1 mice received male CBA.RAG1$^{-/-}$ skin grafts with or without anti-CD4 (1 mg i.p. on day 0). The percentage of surviving grafts is represented on the *y*-axis.

seem unlikely that immune responsiveness is hyperactive before exposure to male Ag in these monospecific TCR-transgenic mice. This suggests that one critical activity for TGF-β in this form of therapeutic tolerance is signaling to T cells.

*Tolerance induction fails in dn*Tgfbr2$^+$ *recipients with a diverse TCR repertoire*

We then devised a tolerization model that accommodated mice with a diverse TCR repertoire to allow us to elucidate tolerance checkpoints bypassed by dn*Tgfbr2*$^+$ cells. We used (CBA $\times$ B6)F_1 (H-2^{k+b}) mice as recipients of minor histocompatibility Ag-mismatched BALB/k (H-2^k) skin allografts. Untreated mice rejected their grafts within 1 mo, whereas the combination of anti-CD4 and anti-CD8 plus anti-CD40L enabled life-long allograft survival in wild-type recipients (Fig. 2*A*). In contrast, the same therapeutic Ab protocol failed to induce tolerance in dn*Tgfbr2*$^+$ recipients. Nevertheless, the treatment significantly prolonged allograft survival in dn*Tgfbr2*$^+$ recipients when compared with untreated dn*Tgfbr2*$^+$ recipients ($p = 0.0003$).

ELISA was used to determine whether the administered therapeutic Abs persisted equivalently in treated mice. Sera of dn*Tgfbr2*$^+$ recipients contained lower and more variable levels of administered Abs than sera of wild-type mice 65 days after grafting (Fig. 2*B*). However, there was no correlation between day 65 serum concentrations and graft survival in treated dn*Tgfbr2*$^+$ recipients, suggesting that factors other than simple Ab persistence determined the tempo of allograft rejections in dn*Tgfbr2*$^+$ recipients.

*Combined coreceptor and costimulation blockade delays intragraft T cell accumulation, but less so in dn*Tgfbr2$^+$ *mice*

We examined intragraft T cell accumulation soon after transplantation using the model above. This was done by quantifying intragraft levels of (T cell-restricted) CD3γ mRNA normalized to constitutively expressed GAPDH mRNA. CD3γ mRNA levels were readily detectable and similar in grafts collected on day 14 from untreated recipients. Relative to these levels, there was a marked ($\sim$100-fold) reduction in the level of CD3γ mRNA in day 14 grafts collected from Ab-treated wild-type recipients (Fig. 2*C*; $p < 0.0001$). Untreated and Ab-treated recipients had similar frequencies of CD3$^+$ cells among splenocytes (data not shown), excluding a systemic decrease in T cells as a possible reason for this contrast. In day 14 grafts collected from Ab-treated dn*Tgfbr2*$^+$ recipients, CD3γ levels were significantly higher than the levels in grafts from Ab-treated wild-type littermates ($p = 0.0031$). However, these increased levels were still significantly lower than those in allografts from untreated dn*Tgfbr2*$^+$ controls ($p = 0.0115$), indicating that intragraft T cell accumulation in dn*Tgfbr2*$^+$ mice was somewhat inhibited by the Ab mixture (Fig. 2*C*). However, by day 25, CD3γ levels were equivalent in both wild-type and dn*Tgfbr2*$^+$ Ab-treated groups.

Ab blockade would appear to inhibit intragraft accumulation of Foxp3$^+$ *and Foxp3*$^-$ *cells equally*

We examined whether our tolerization protocol resulted in a marked accumulation of Foxp3 mRNA inside the graft, as has been described for tolerization via donor-specific transfusion plus anti-CD40L (24), and whether dn*Tgfbr2* expression has any effect on this parameter. The levels of Foxp3 mRNA (normalized to CD3γ mRNA) appeared similar regardless of both treatment and dn*Tgfbr2* expression (Fig. 2*D*). Thus, we found no evidence of enrichment of Foxp3 mRNA in grafts bound for tolerance vs those bound for rejection.

Systemic anti-TGF-β at the time of transplantation prevents long-term allograft survival in mice with normal TCR repertoire

The previous experiments used mice with a life-long deficiency in T cell TGF-β signaling. To investigate whether TGFβ is required during the initial exposure to alloantigens, we gave a TGF-β-neutralizing Ab at the time of transplantation. Control mice given B10.BR allografts alone rejected their skin grafts within 3 wk, whereas recipients treated with coreceptor blockade, with or without anti-IL-10R treatment, maintained their grafts indefinitely (Fig. 3*A*). In contrast, anti-TGF-β treatment resulted in rejection in all allograft recipients (Fig. 3*A*). Similar results were seen with a combination of anti-CD40L plus anti-CD8 as inductive therapy (Fig. 3*B*). In contrast to the variable persistence of administered Abs in dn*Tgfbr2*$^+$ recipients, the levels of administered Abs were similar in the sera of test mice, whether they were treated with anti-TGF-β or not (Fig. 3*C*). Thus, the failure to induce tolerance in recipients of anti-TGF-β was not due to accelerated clearance of the therapeutic Abs and, importantly, there is a clear requirement for TGF-β to act during the period of initial exposure to alloantigens.

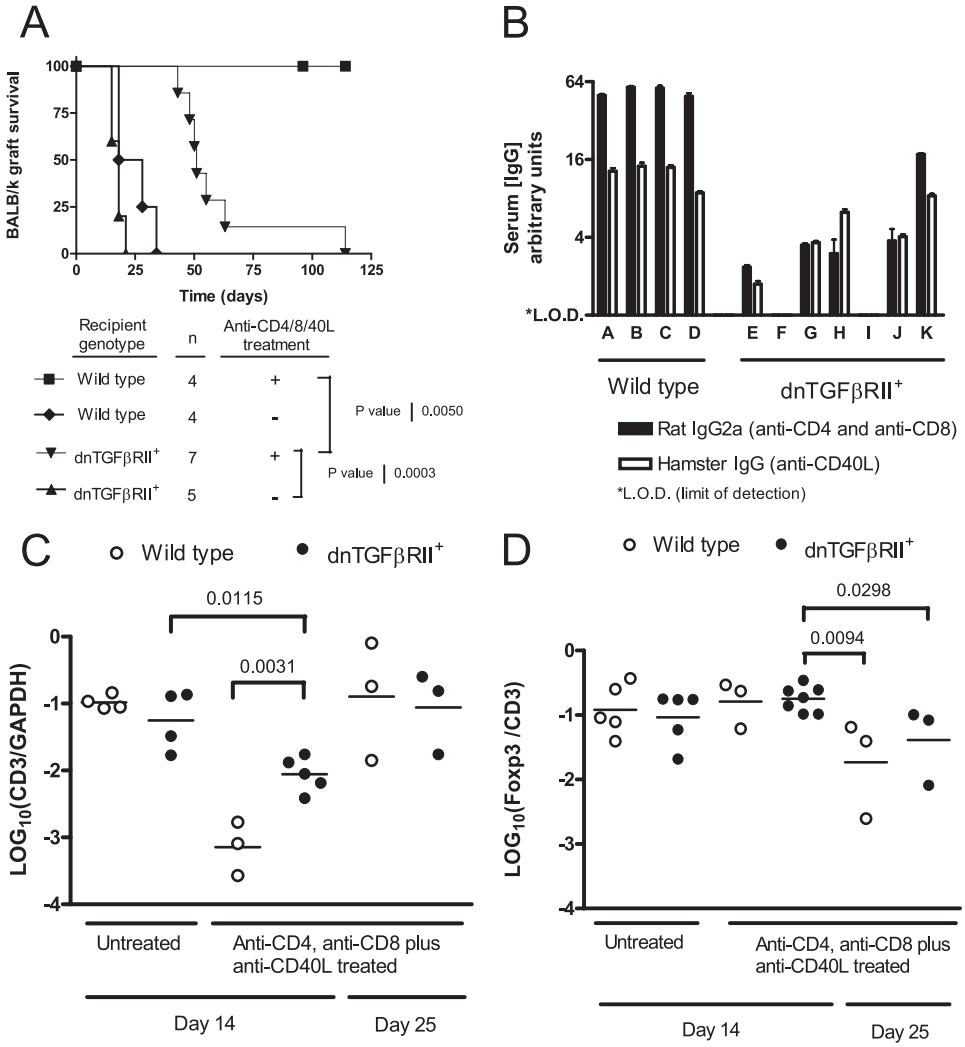

FIGURE 2. Graft rejection in Ab-treated dn*Tgfbr2*$^+$ recipients is preceded by an abnormally early intragraft T cell accumulation. *A,* Male wild-type or dn*Tgfbr2*$^+$ (CBA × B6)F_1 mice received BALB/k skin grafts on day 0 with or without anti-CD4 and anti-CD8 plus anti-CD40L (3 × 1 mg each i.p. on days 0, 2, and 4). The percentage of surviving grafts is represented on the *y*-axis. *B,* Serum samples were collected from the mice described in *A* on day 65 and rat IgG2a (■, anti-CD4 and anti-CD8) or hamster IgG (□, anti-CD40L) concentrations were measured by ELISA. Individual mice are denoted by the letters A–K and error bars show 95% confidence intervals. *C,* Male wild-type or dn*Tgfbr2*$^+$ (CBA × B6)F_1 mice received male BALB/k skin grafts on day 0 with or without anti-CD4 and anti-CD8 plus anti-CD40L (3 × 1 mg i.p. each on days 0, 2 and 4). Skin grafts were collected on day 14 or 25 and were processed for real-time RT-PCR assaying CD3γ as the transcript of interest and GAPDH as the normalizing transcript. cDNA prepared from CBA splenocytes was given a value of 0 on the *y*-axis. *D,* The cDNA samples prepared in *C* were analyzed again using Foxp3 as the transcript of interest and CD3γ as the normalizing transcript. cDNA prepared from FACS-sorted CD4$^+$ CD25$^+$ CBA splenocytes is given a value of 0 on the *y*-axis.

TGF-β-converted cells are able to suppress graft rejection and Foxp3$^+$ *cells accumulate in the tolerated graft*

The results from the monospecific TCR-transgenic model above provide evidence that the T cell is one target cell type for TGF-β in establishing allograft tolerance. In vitro, TGF-β combined with TCR ligation can induce naive T cells to express Foxp3 and develop suppressive properties (25), and there is evidence that TGF-β facilitates the extrathymic conversion of naive T cells into Foxp3$^+$ Treg in vivo (26). To examine whether TGF-β-converted cells can suppress allograft rejection in vivo, we exposed monospecific CD4$^+$ T cells from female A1.RAG1$^{-/-}$ mice to Dby peptide with or without TGF-β in vitro and expanded these cells to achieve suitable numbers for in vivo administration. Splenocytes expanded in the presence of Dby peptide without TGF-β did not suppress rejection in vivo, whereas TGF-β-converted cells suppressed male graft rejection by A1.RAG1$^{-/-}$ females, and this suppression was not prevented by neutralization of any of TGF-β, IL-10, or IFN-γ (Fig. 4*A*). Real-time RT-PCR analysis of spleens and tolerated grafts of mice receiving the TGF-β-converted cells detected substantial levels of Foxp3 mRNA in the grafts (Fig. 4*B*), consistent with all our previous findings where dominant tolerance was accompanied by Treg infiltrates in grafted tissue (27).

Does regulation that has developed in vivo require TGF-β signaling?

Although we found that TGF-β-converted cells can suppress graft rejection in vivo in a mechanism unaffected by anti-TGF-β, it remains open whether TGF-β is required for full regulation of the alloimmune response by a diverse T cell repertoire. We asked whether T cell suppression in the maintenance phase of tolerance would be similarly TGF-β independent. To test this, we attempted to exploit the phenomenon of "resistance" (16, 28), whereby regulatory CD4$^+$ T cells (29) in tolerant hosts suppress the anti-allogeneic response of infused immunocompetent T cells.

Recipient male (CP-1 × B6)F_1 mice were thymectomized at 4 wk of age to prevent the export of T cells from the thymus later in the experiment. At 2–3 mo of age, these mice were tolerized to a primary BALB/k skin graft using CD4, CD8, and CD40L specific Abs. After 100 days, the mice were tested for resistance to infusion with 5 × 10^7 naive (CBA × B6)F_1, or dn*Tgfbr2*$^+$ splenocytes, or left untreated. All recipients were then rechallenged with a fresh BALB/k skin graft. None of the infused mice rejected its primary or secondary BALB/k grafts (data not shown). However, transfer of the same cells into lymphocyte-depleted secondary hosts indicated that dn*Tgfbr2*$^+$, unlike control (CBA × B6)F_1 cells, could neither produce sustained repopulation of the hosts or elicit allograft rejection (data not shown). This meant that we simply could not use transfer of dn*Tgfbr2*$^+$ splenocytes to examine suppression by host T cells. Nor could we contemplate transfer of Treg into dn*Tgfbr2*$^+$ recipients, as this would require transfer of unwieldy numbers of Treg.

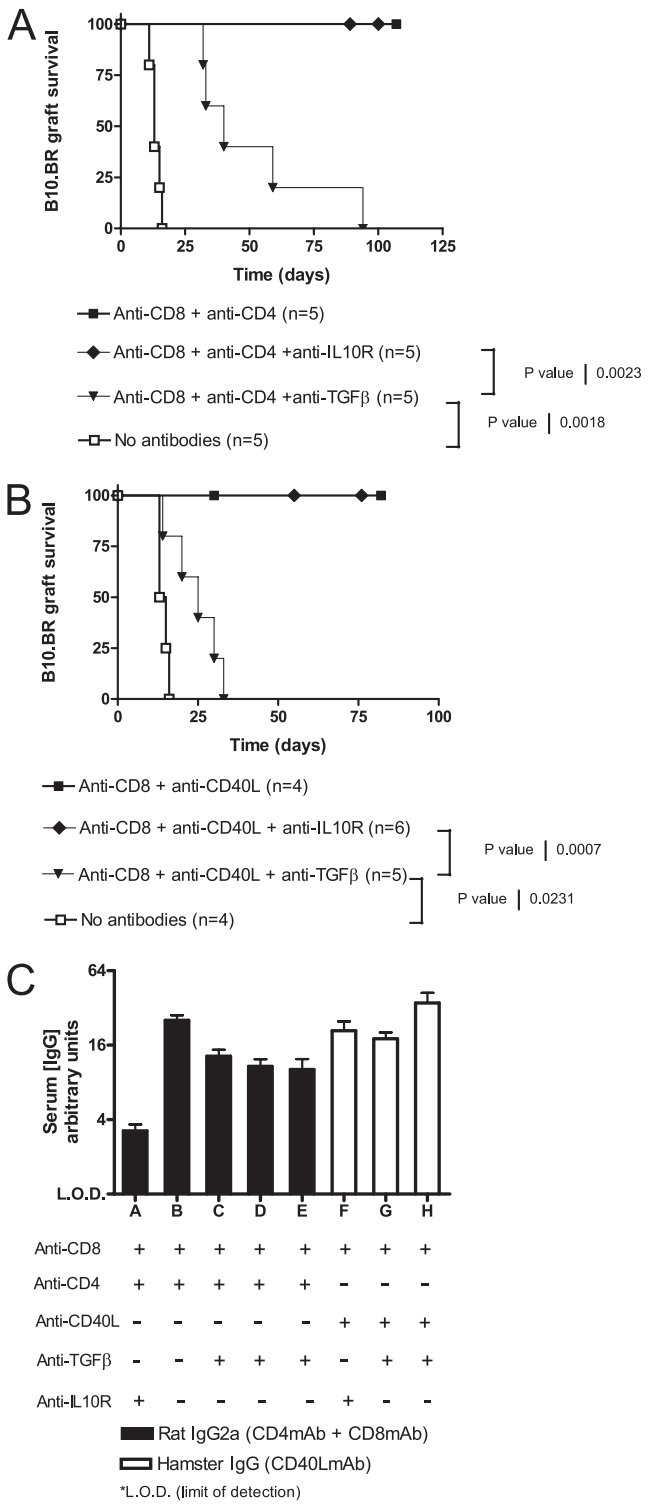

FIGURE 3. Long-term allograft survival is abrogated by anti-TGF-β and tolerance-inducing Abs persist normally in this setting. *A,* Male hCD52-Tg mice were given B10.BR skin grafts on day 0 with or without anti-CD8 plus anti-CD4 (3 $\times$ 1 mg each i.p. on days 0, 2, and 4). Some mice also received anti-TGF-β or anti-IL-10R (10 $\times$ 2 mg i.p. over 3 wk starting on day -1). The percentage of surviving grafts is represented on the *y*-axis. *B,* Experimental design as in *A*; however, anti-CD40L (3 $\times$ 0.67 mg i.p. on days 0, 2, and 4) was given instead of anti-CD4. The percentage of surviving grafts is represented on the *y*-axis. *C,* The Abs administered to eight mice denoted by the letters A–H are shown in the key. Serum samples collected on days 49–51 were analyzed in ELISA detecting either rat IgG2a (■) or hamster IgG (□). Error bars, 95% confidence intervals.

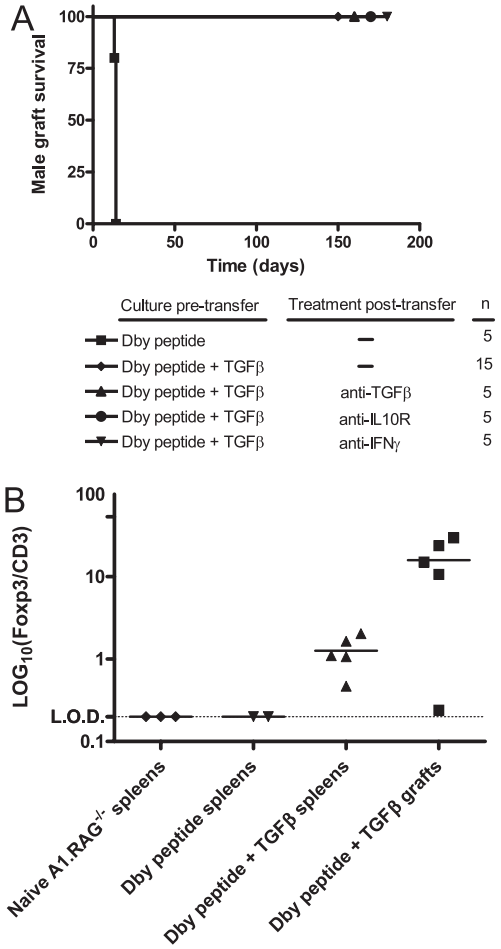

FIGURE 4. TGF-β-converted cells suppress graft rejection and Foxp3$^+$ cells accumulate in the tolerated graft. *A,* Female A1.RAG1$^{-/-}$ splenocytes were cultured for 7 days in the presence of syngeneic female bone marrow-derived dendritic cells and cognate peptide (100 nM) with or without recombinant human TGF-β (2 ng/ml). In brief, 1.0 $\times$ 10^7 or 2.0 $\times$ 10^7 viable cells were injected i.v. into female A1.RAG1$^{-/-}$ recipients of a male CBA.RAG1$^{-/-}$ skin graft placed the following day. Some mice that received TGF-β-converted cells received cytokine-neutralizing Abs indicated in the key (10 injections each of 1 mg given over 3 wk for anti-IL-10R and anti-TGF-β, or six injections each of 0.5 mg over 2 wk for anti-IFN-γ, starting on the day of grafting). *B,* Spleens and tolerated skin grafts from mice described in *A* were collected at the end of the experiment and processed for real-time RT-PCR assaying Foxp3 as the transcript of interest and CD3γ as the normalizing transcript.

We opted instead to examine whether Ab neutralization of TGF-β in the maintenance phase would abrogate suppression. Adult-thymectomized male (hCD52-Tg $\times$ B6)F$_1$ mice were rendered tolerant to BALB/k skin allografts as above. After 100 days, a course of anti-TGF-β or mouse IgG1 isotype control was initiated. The next day, all mice received 5 $\times$ 10^7 naive (CBA $\times$ B6)F$_1$ splenocytes, and on the day following, all mice received a fresh BALB/k skin graft (day 0 on the graph; Fig. 5). Flow cytometry conducted on peripheral blood samples confirmed the presence of adoptively transferred CD3$^+$humanCD52$^-$ cells in all recipients (data not shown). Although none of the mice treated with the mouse IgG1 control Ab rejected its BALB/k graft during the observation period, three of five mice treated with anti-TGF-β rejected their grafts (p = 0.03), while two of the five mice did not.

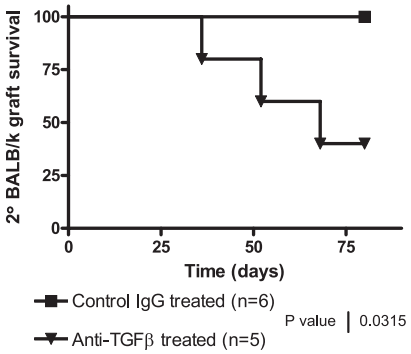

FIGURE 5. Resistance is abrogated by anti-TGF-β in a proportion of mice. To generate tolerant mice, adult-thymectomized (CD52-Tg $\times$ B6)F_1 mice received a BALB/k skin graft and anti-CD4 and anti-CD8 plus anti-CD40L (1 mg each i.p. on the day of grafting, repeated 2 and 4 days later). The initial tolerization step occurred in several cohorts. One hundred fifteen to 217 days later, all mice received 5×10^7 naive (CBA $\times$ B6)F_1 splenocytes and then a fresh BALB/k skin graft the next day. One day before the transfer of splenocytes, a course of either anti-TGF-β or mouse IgG1 control Ab (OX21; 10 $\times$ 1 mg i.p. over 3 wk) was initiated. The percentage of surviving secondary BALB/k grafts is represented on the *y*-axis.

Discussion

This study examined the influence of signals mediated by TGF-β on the survival of skin allografts. Unlike one experimental autoimmune encephalomyelitis model in which T cell infiltration and destruction of nervous tissue requires TGF-β (30), we show here that TGF-β acts to prevent destruction of skin allografts. Acceptance of allografts was TGF-β dependent whether the alloantigen load was restricted to one Ag, a set of H-Y-encoded Ags, or genome-wide minor transplantation Ags. Our preliminary investigations have not revealed an obligatory role for TGF-β in resistance, a measure of host T cell suppression in the maintenance phase. We have seen similar data in another readout of suppression; so-called "linked suppression". CBA/Ca hosts rendered tolerant of B10.BR skin (coreceptor blockade) were able to accept subsequent BALB/k grafts that share some "minors" and differ in others. Anti-TGF-β neutralization was able to abrogate linked suppression in only two of six tolerant hosts (S. Daley, S. Cobbold, and H. Waldmann, unpublished data). In explaining the failure to influence resistance or "linked suppression" in the remaining mice, we speculate that either TGF-β can be dispensable in the effector phase of suppression or that the neutralizing Ab had failed to inactivate all pockets of TGF-β. The inability of dn*Tgfbr2*$^+$ T cells to repopulate on adoptive transfer left us unable to directly test whether TGF-β signaling to T cells was critical to sustained regulation in tolerant hosts.

dn*Tgfbr2*$^+$ mice have a known immunopathology and autoimmunity in diverse tissues (15). Thus, the skin allograft rejections that occurred in dn*Tgfbr2*$^+$ mice may be due, at least in part, to a hyperactive state of the host immune system before transplantation. The importance of TGF-β signaling to T cells in therapeutic tolerance induction is nevertheless clear in the failure to induce tolerance in monospecific dn*Tgfbr2*$^+$ recipients. Given that the dysregulation of TGFβ-insensitive T cells is TCR activation dependent (4), this monospecific mouse should not have the repertoire to have its immune system preactivated.

We used dn*Tgfbr2*$^+$ mice to examine the mechanism underlying the failure of therapeutic Abs to induce tolerance, finding that T cells accumulate abnormally early inside skin grafts in Ab-treated dn*Tgfbr2*$^+$ recipients. To our surprise, there was no difference in normalized intragraft Foxp3 mRNA levels in grafts bound for tolerance vs rejection. At face value, this latter result argues against the idea of a simple Treg vs T effector cell imbalance determining whether grafts are bound for rejection. This still leaves open several explanations for graft rejection in this model, including accelerated T cell differentiation, enhanced effector function, and resistance of T cells to suppression. One potential explanation for our findings and many in the literature is that limitation of TGF-β signaling to T cells eliminates a key controller of how T cells perceive calcium influx (31), a pivotal variable in determining T cell responses that is still incompletely understood.

An obvious and important issue in all such experiments is the source of TGF-β that is responsible for preventing transplant rejection. Given the ubiquitous distribution of this cytokine, it has been a common limitation of publications of this type that the exact source cannot be easily identified. The recent demonstration that mice with a T cell-specific deletion of the TGF-β1 gene developed lethal and diverse immunopathology, associated with enhanced T cell activation and polarization into Th1 and Th2 cells (32), suggests that T cells may be a significant source of the TGF-β we are implicating. Indeed, TGF-β1 produced by Foxp3-expressing Treg was, in the same study, required to inhibit Th1 cell differentiation and inflammatory bowel disease in a transfer model. Studies in these mice would be very valuable in determining whether T cells can be implicated as the TGF-β source in the models of dominant transplantation tolerance above.

TGF-β is clearly a key cytokine in determining the success of allograft survival, although the challenge remains to define the entire TGF-β pathway and its influence on immune responses in detail. Understanding the TGF-β pathway may lead to the development of new biomarkers for the diagnosis of tolerance vs immunity in human transplant recipients. Furthermore, genetic profiling focusing on the TGF-β pathway in transplant recipients may one day allow better combinations and doses of immunosuppressive agents to be prescribed on a personalized basis, including perhaps the therapeutic Abs that are shown here to depend on TGF-β for their efficacy in vivo.

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Disclosures

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