

Induction of *foxP3*⁺ Regulatory T Cells in the Periphery of T Cell Receptor Transgenic Mice Tolerized to Transplants¹

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Transplantation tolerance can be induced in mice by grafting under the cover of nondepleting CD4 plus CD8 or CD154 mAbs. This tolerance is donor Ag specific and depends on a population of CD4⁺ regulatory T cells that, as yet, remain poorly defined in terms of their specificity, origin, and phenotype. Blocking of the Ag-specific response in vitro with an anti-CD4 mAb allowed T cells from monospecific female TCR-transgenic mice against the male Ag *Dby*, presented by H-2E^k, to express high levels of *foxP3* mRNA. *foxP3* induction was dependent on TGF- β . The nondepleting anti-CD4 mAb was also able to induce tolerance in vivo in such monospecific TCR-transgenic mice, and this too was dependent on TGF- β . As in conventional mice, acquired tolerance was dominant, such that naive monospecific T cells were not able to override tolerance. Splenic T cells from tolerant mice proliferated normally in response to Ag, and secreted IFN- γ and some IL-4, similar to control mice undergoing primary or secondary graft rejection. High levels of *foxP3* mRNA, and glucocorticoid-induced TNFR superfamily member 18 (GITR)⁺ CD25⁺ T cells were found within the tolerated skin grafts of long-term tolerant recipients. These data suggest that regulatory T cells maintaining transplantation tolerance after CD4 Ab blockade can be induced de novo through a TGF- β -dependent mechanism, and come to accumulate in tolerated grafts. *The Journal of Immunology*, 2004, 172: 6003–6010.

A short treatment of mice with saturating amounts of nondepleting anti-CD4 mAbs can induce tolerance to foreign protein Ags, and enables long-term survival of minor-Ag-mismatched skin or MHC-mismatched cardiac grafts (1, 2). The addition of anti-CD8 and -CD154 Abs can extend this effect to full donor-specific tolerance such that second donor grafts are accepted at any later time, while third party grafts are rapidly rejected (3). The maintenance of tolerance is dependent on CD4⁺ regulatory T cells, characterized by their ability to suppress graft rejection by naive T cells (both CD4⁺ and CD8⁺) after adoptive transfer into secondary recipients (4), or in situ as revealed by the phenomenon of linked suppression of (donor \times third party)F₁ skin grafts (5, 6). In the continued presence of donor Ag, CD4⁺ regulatory T cells are further able to convert naive, potentially aggressive CD4⁺ T cells into fresh cohorts of regulatory cells, a process known as “infectious tolerance” (7, 8). Little is known about the molecular mechanisms by which these regulatory T cells are induced in vivo, whether they act systemically or within the graft, and whether they are related to the “natural” CD4⁺CD25⁺ regulatory T cells characterized in various autoimmune disease models (9–11).

Natural CD4⁺CD25⁺ regulatory T cells are thought to play an important role in controlling responses to self Ags, nonpathogenic organisms within the gut, lymphocyte homeostasis, and the innate response, particularly in lymphopenic conditions (12). They are generated within the thymus, possibly via high avidity recognition of self peptides (13), and have recently been shown to express the transcription factor *foxP3*. Ectopic expression of *foxP3* can also convert naive CD4⁺ T cells to a regulatory phenotype (14–16). The mechanisms by which natural CD4⁺CD25⁺ regulatory T cells act in the different autoimmune disease models seem to involve a discretionary requirement for the cytokines IL-10 and TGF- β (17), and may depend on signals through CTLA-4 (18–20), as well as regulation coordinated by glucocorticoid-induced TNFR superfamily member 18 (GITR)⁵ (21, 22). A role for both CD4⁺CD25⁺ and CD4⁺CD25⁻ cell populations has been demonstrated in transplantation tolerance after adoptive transfer to lymphopenic mice, but involvement of IL-10 or CTLA-4 remains controversial in that context (23). Regulatory T cells have also been described within the tolerated graft itself, their presence detected following transfer and expansion within recombination-activating gene-1^{-/-} (RAG-1^{-/-}) recipient mice, although most expanded cells exhibited a CD4⁺CD25⁻ phenotype (24).

An experimentally induced population of cytokine-polarized T cells, known as Tr1 cells, are also able to suppress autoimmune diseases after adoptive transfer in vivo (25, 26). Tr1 cells are thought to be dependent on IL-10 for both their generation and suppressive activity, but it is still unclear how, or if, they may relate to CD4⁺CD25⁺ regulatory populations. Gene expression analyses have shown that CTLA-4, GITR, CD25, CD103, and OX40 are all constitutively expressed on both Tr1-like clones and CD4⁺CD25⁺ cells from the spleen, but there are many other differences to suggest the populations are quite distinct (27). Tr1-like

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⁵ Abbreviations used in this paper: GITR, glucocorticoid-induced TNFR superfamily member 18; 7-AAD, 7-aminoactinomycin D; bmDC, bone marrow-derived dendritic cell; HPRT, hypoxanthine phosphoribosyltransferase; RAG-1^{-/-}, recombination-activating gene-1^{-/-}; ROG, repressor of GATA.

cells seem to be related to Th2 cells, with the former expressing very few unique genes, but rather expressing only a subset of the Th2-associated transcripts (28). We have found that neither Tr1-like nor Th2 clones express *foxP3* by RT-PCR, even after activation with CD3 cross-linking, although activated Tr1-like cells overexpress the repressor of GATA (ROG) (28).

It has previously been shown that Th2 (and Th1) clones with identical specificity can cause rapid, and Ag-specific, rejection of skin grafts after adoptive transfer to lymphocyte-deficient mice (29). Two different Tr1-like clones from such mice were unable to reject male skin, yet were instead able to suppress the subsequent rejection of fresh male grafts by either Th1 or Th2 cells from the same source (26, 27). Although such experiments confirm that Tr1-like cells are capable of suppressing graft rejection *in vivo*, it also remains a critical issue whether dominant tolerance is a result of the selection of a pre-existing population of natural regulatory T cells that cross-react with graft alloantigens, or the differentiation or polarization of graft-specific peripheral T cells toward a regulatory function.

In this study, we use female transgenic mice expressing a single TCR specific for the male transplantation Ag (*Dby* presented by H-2E^k) on a RAG-1^{-/-} background (29) that have no detectable pre-existing CD4⁺CD25⁺*foxP3*⁺ regulatory T cells in the thymus or periphery. We show that Ag stimulation in the presence of saturating nondepleting CD4 Abs induces *foxP3* expression in a TGF- β -dependent manner, both *in vitro* and *in vivo*, and that this correlates with lifelong acceptance of male skin grafts. This long-term acceptance is shown to be tolerance as such mice accepted further male skin grafts at any time thereafter, while mice receiving Ab without the tolerizing graft rejected subsequent male skin grafts. Evidence for the presence of induced regulatory T cells resides in the property of tolerant mice to resist the rejection by infused naive, male-specific, monospecific T cells and the simultaneous expression of *foxP3*, GITR, and CD25 within tolerated skin grafts. This suggests that *foxP3*⁺ regulatory T cells can be induced *de novo* in the periphery by appropriate Ag presentation in the presence of TGF- β .

Materials and Methods

Mice, surgery, and tolerance induction

A1(M).RAG-1^{-/-} TCR-transgenic mice (29), CBA/Ca.RAG-1^{-/-}, C57/B10, and CBA/Ca mice were bred and maintained in specific pathogen-free conditions at the Sir William Dunn School of Pathology (Oxford, U.K.). Skin grafting was conducted by grafting full thickness tail skin (1 × 1 cm) on the lateral flank as previously described (1). Grafts were observed on alternate days after the removal of the bandage at day 8 and considered rejected when no viable donor skin was present. Statistical analysis of graft survival was made by the log rank method. Tolerance to H-Y was induced at the time of grafting female A1(M).RAG-1^{-/-} mice with male CBA.RAG-1^{-/-} skin by a single injection on day 0 of 1 mg of anti-CD4 mAb (YTS 177.9 (1)). Tolerance was induced in CBA/Ca mice at the time of grafting C57/B10 skin by three doses of 1 mg of the anti-CD4 (YTS 177.9), anti-CD8 (YTS 105.18), and anti-CD154 (MR1 (30)) nondepleting mAbs over 1 wk. In some experiments, neutralizing Abs to TGF- β (1D11 (31)) or IL-10R (1B1.2 (32)) were given at doses indicated. All procedures were conducted in accordance with the Home Office Animals (Scientific Procedures) Act of 1986.

Immunofluorescence analysis and Abs

Spleen cells were either labeled directly or after stimulation with PMA (50 ng/ml) plus ionomycin (500 ng/ml) for 4 h at 37°C in the presence of brefeldin A. FACS staining: CD3-FITC (clone KT3; produced in-house), CD25-PE or CD25-allophycocyanin (clone PC61; BD PharMingen, San Diego, CA), CD4-CyChrome (CD4-PerCP; BD PharMingen), and biotin conjugated anti-GITR mAb (YGITR 765 (33)) were used to label live T cells in PBS containing 0.1% NaN₃, 1% BSA, 10 μ g/ml Fc Block (BD PharMingen), and 5% heat-inactivated normal rabbit serum at 4°C. When required, the cells were then washed, fixed in 2% paraformaldehyde, and permeabilized in PBS containing 0.5% saponin and stained with anti-IFN- γ -PE (BD PharMingen) in the dark at 4°C. Streptavidin-allophycocyanin (BD PharMingen) was also added to detect biotinylated first-step Abs. Four-color analysis was performed using a FACScalibur (BD Biosciences, Oxford, U.K.) with dual laser (488 nm and 633 nm) excitation. The analysis gate was set on the forward and side scatters to eliminate cell debris and dead cells.

Regulatory T cells from tolerated skin grafts

T cells were prepared from tolerated skin grafts by cutting them into small pieces, digesting with trypsin at 37°C for 1 h, and removing dead cells on nylon wool. Viable lymphocytes were enriched by Histopaque-1083 centrifugation followed by AutoMACS (Miltenyi Biotec, Auburn, CA) positive selection of CD4⁺ T cells according to the manufacturer's instructions. These T cells were tested either by real-time RT-PCR or immunofluorescence and FACS analysis.

Real-time quantitative RT-PCR

DNase-I-treated total RNA from tissues was prepared using the SV Total RNA isolation system (Promega, Madison, WI). Reverse transcription was performed using the proStar kit with random hexamers (Stratagene, Cedar Creek, TX). Real-time quantification was performed using gene-specific, fluorogenic probes and the Universal MasterMix kit (PE Applied Biosystems, Warrington, U.K.) in a final volume of 25 μ l. The reaction mixture contains all primers at 300 nM and the probe at 200 nM. The enzyme was heat activated 10 min at 95°C. A two-step PCR procedure of 15 s at 95°C and 60 s at 60°C was applied for 50 cycles. PCR and TaqMan analysis were performed using the ABI/PRISM 7700 sequence detector system (PE Applied Biosystems). Multiplex PCRs were performed using appropriate gene-specific primers and VIC-labeled CD3 γ or hypoxanthine phosphoribosyltransferase (HPRT) probes and FAM-labeled test probes as shown in Table I. Standard curves of cDNAs from normal spleen or Tr1D1 clone were used to calibrate threshold cycles to amounts of test and normalizing cDNAs on each 96-well plate run. Normalized values for mRNA expression were calculated as test mean/normalizer mean. All samples were run in triplicate.

T cell clones

The production and maintenance of the male-specific Th1, Th2, Tr1D1, and SKA T cell clones from A1(M).RAG-1^{-/-} TCR female transgenic mice has been described elsewhere (26, 29). The clones were harvested by Histopaque-1083 centrifugation and in some cases then stimulated with anti-CD3 (100 ng/ml; 145.2C11 absorbed to 24-well plates) at 37°C overnight.

Proliferation assays

To assess T cell responses from A1(M).RAG-1^{-/-} groups, spleen cell suspensions were depleted of RBC by water lysis, washed, and set up at 5 × 10⁴ cells/200 μ l well in RPMI 1640 medium containing 10% FCS. Stimulator cells were mitomycin C-treated (30 min at 37°C) male or female CBA/Ca spleen cells (2 × 10⁵/well), or male or female bone marrow-derived dendritic cells (bmDCs; 1 × 10⁴/well) generated by 7 days culture in recombinant mouse GM-CSF (34). Where required, H-Y peptide (*Dby*-E^k: REEALHQFRSGRKPI) was added at 10 or 100 nM as specific Ag.

Table I. Real-time RT-PCR primers and TaqMan probes

Gene Transcript	Forward Primer	Reverse Primer	TaqMan Probe
HPRT	5'-gaccggtcccgctatgc-3'	5'-tcataacctgttcatcatcgc-3'	VIC-5'-accgcagtcaccgagctgctg-3'-TAMRA
CD3 γ	5'-ttacagaatgtgaaactgcattg-3'	5'-caccaagagcaaggaagaatg-3'	VIC-5'-acataggaccatatacggctttcttcg-3'-TAMRA
ROG	5'-cttctcccctagtagcccccata-3'	5'-agagggtaaaggagggtttggt-3'	FAM-5'-ctggcaggtccgcctcaagatca-3'-TAMRA
<i>foxP3</i>	5'-cccaggaaagacagcaacctt-3'	5'-ttctcaaccaggccacttg-3'	FAM-5'-ctaccactgctggcaaatggagtc-3'-TAMRA

After 72 h culture at 37°C, 100 μ l of supernatants were taken from each well for cytokine ELISAs, and the volume replaced with medium containing 20 μ Ci/ml [³H]thymidine for further culture overnight. Plates were harvested onto glass fiber filters and [³H]thymidine incorporation was counted in a Betaplate (Pharmacia, Gaithersburg, MD) scintillation counter. All cultures were tested in triplicate, presented as geometric means and SDs, and significance was calculated by Student's *t* test.

Alternatively, CD4⁺ T cells were enriched from responder spleen cell suspensions using the "CD4 untouched" MACS kit (Miltenyi Biotec) according to manufacturer's instructions, and labeled with 5 μ M CFSE (Molecular Probes, Eugene, OR) at room temperature for 10 min followed by washing in FCS-containing medium. The CFSE-labeled CD4⁺ T cells (10⁵/well) were incubated for 96 h with female CBA/Ca bmDCs (10⁵/well) alone or with 10 nM DBY-E^k peptide, in duplicate. Cultures were harvested, stained with anti-CD4-biotin, anti-CD25-PE, and 100 μ M 7-aminoninocin D (7-AAD) to label dead cells, followed by streptavidin-allophycocyanin (BD PharMingen). Cells were fixed in 1% formaldehyde and analyzed using a FACSCalibur (BD Biosciences).

Cytokine detection in culture supernatants

Specific cytokines were assayed by ELISA according to the manufacturer's instructions (BD PharMingen) with the following Abs: IL-4 was captured by 11B11 and detected by biotin-BVD6.24G2, IL-10 was captured by JESS2A5 and detected by biotin-JESS16E3, and IFN- γ was captured by R4-6A2 and detected by biotin-XMG1.2, and in each case the assay developed by streptavidin peroxidase followed by *o*-phenylenediamine substrate. Each ELISA was calibrated against a standard dilution series of the appropriate recombinant mouse cytokine.

Serum CD4 Ab levels

Sera from Ab-treated mice were taken at -1, 63, 94, and 132 days from the time of grafting and Ab injection, heat inactivated at 56°C for 30 min, and incubated with CBA/Ca thymocytes for 1 h at 4°C at 1:2 or 1:10 dilutions. After washing, bound CD4 Ab was detected using FITC anti-rat IgG (Sigma-Aldrich, St. Louis, MO) and flow cytometry (FACSCalibur; BD Biosciences), and calibrated against a standard dilution series of YTS 177.9 in heat inactivated normal mouse serum. The limit of detection of this assay was equivalent to 100 ng of YTS 177.9 per milliliter of neat serum, while saturation was achieved above 2 μ g/ml.

Results

Anti-CD4 blockade in vitro induces foxP3 in a TGF- β -dependent manner

With the recent discovery that the transcription factor *foxP3* programs T cells to develop regulatory functions (14–16, 35), it has become possible to use *foxP3* induction as a surrogate marker to identify conditions that may favor regulatory T cell differentiation. We have found that stimulation of naive T cells from female A1(M).RAG-1^{-/-} (monospecific) mice, when stimulated in vitro with their cognate Ag (*Dby*-E^k peptide; 100 nM) in the presence of the nondepleting anti-CD4 mAb (YTS177.9), were not only inhibited in their proliferation (Fig. 1*a*), but also started to express *foxP3* mRNA by day 3 (data not shown). By day 6 (Fig. 1*b*), there were levels of *foxP3* transcripts similar to that of CD4⁺CD25⁺ regulatory T cells from normal mice (i.e., 10–100 U of *foxP3*/CD3 γ). This induction of *foxP3* was dependent on TGF- β , as it was completely inhibited by including the neutralizing Ab 1D11 in the culture (Fig. 1*b*). The proliferation block at 100 μ g/ml anti-CD4 was also partially reversed by anti-TGF- β , but only to levels similar to 10 μ g/ml anti-CD4 alone where considerable *foxP3* mRNA was still induced (Fig. 1*a*). An isotype-matched anti-IL-10 receptor Ab had no effect on either proliferation or *foxP3* induction (data not shown).

Tolerance induction by treatment with anti-CD4 in vivo

We next wished to test whether the anti-CD4 mAb could induce dominant tolerance in mice with no endogenous CD4⁺CD25⁺ regulatory T cells or *foxP3* expression. Female A1(M).RAG-1^{-/-} TCR-transgenic mice normally reject male skin grafts acutely, within 15–20 days (Fig. 2). A single injection of the nondepleting

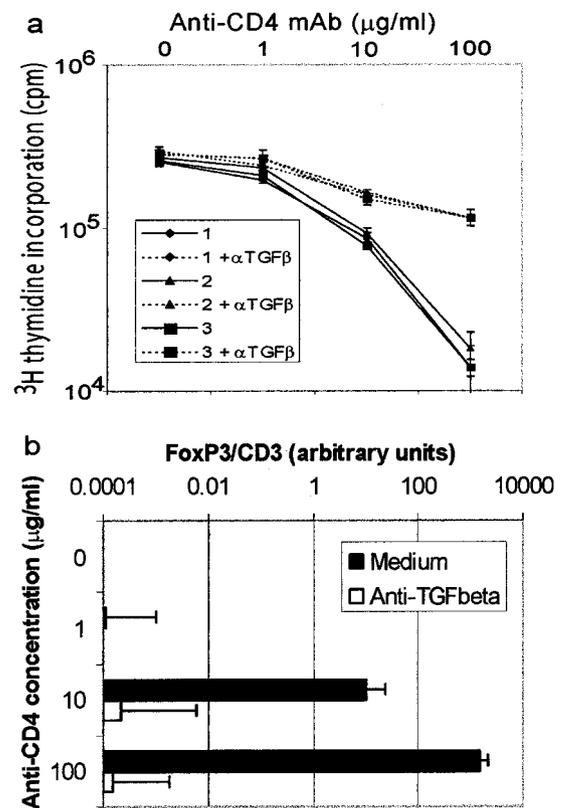


FIGURE 1. Anti-CD4 blockade induces *foxP3* in vitro in a TGF- β -dependent manner. Spleen cells from female A1(M).RAG-1^{-/-} mice (5 × 10⁴/well) were stimulated with female bmDCs (10⁴/well) together with 100 nM *Dby*-E^k peptide and varying concentrations of anti-CD4 mAb (YTS 177.9; 0–100 μ g/ml), either with or without the anti-TGF- β neutralizing mAb (1D11; 100 μ g/ml). *a*, Proliferation was measured on day 6 by [³H]thymidine incorporation; means \pm SD of triplicate values from three individual spleen donors are shown with or without anti-TGF- β . *b*, Triplicate wells were pooled and harvested and mRNA prepared for the Taq-Man RT-PCR assay. *foxP3* transcripts were quantified by comparison to normal CBA/Ca spleen cells, and normalized to CD3 γ , as described in *Materials and Methods*.

anti-CD4 Ab was able to prolong skin graft survival reliably and indefinitely, at doses of 0.5 mg (data not shown) and 1 mg (Fig. 2). Anti-CD4 Ab was still detectable in the serum (up to 3 μ g/ml) at day 63 after grafting, but had been eliminated from the circulation (<100 ng/ml) by day 94 (data not shown). Treated mice carrying male skin grafts for >100 days were able to accept fresh second male grafts indefinitely, demonstrating immune tolerance, while the group initially given Ab without a tolerizing graft rejected their male grafts (Fig. 2). Mice that had initially been allowed to reject a first male graft without Ab cover also rejected fresh second grafts at a similar rate to the first skin.

Tolerance does not require systemic clonal deletion nor ignorance of Ag

Tolerance to graft Ags can be achieved by a number of mechanisms, including clonal elimination, immune regulation, or ignorance of the graft Ag. To determine which of these mechanisms might play a role in the TCR-transgenic system, where all the T cells have the potential to recognize donor Ag, we could determine the number and phenotype of the T cells in each animal. First, it was possible to rule out T cell clonal elimination, as the absolute numbers of CD4⁺ T cells in the spleen (Fig. 3*a*), blood, and lymph nodes (data not shown) were not significantly different between

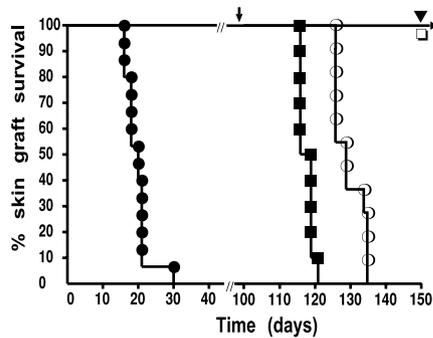


FIGURE 2. Induction of tolerance by coreceptor blockade. Female A1(M).RAG-1^{-/-} mice were given male CBA/Ca skin grafts (rejecting group, $n = 15$, ●), 1 mg of YTS 177.9 anti-CD4 mAb (no skin graft, $n = 11$), or both anti-CD4 mAb and skin grafts together (tolerized group, $n = 9$, ▼). After >100 days, each group was then given second challenge male CBA/Ca skin grafts (rejecting group, $n = 9$, ■; mAb control group, $n = 11$, ○; tolerized group, $n = 9$, □). Only in the tolerized group did both first and second grafts survive indefinitely (>200 and >100 days, respectively; $p < 0.001$ for first or second accepted grafts compared with anti-CD4 mAb-only control second grafts).

mice that were naive, anti-CD4-treated, skin grafted without treatment, or tolerized with a graft plus anti-CD4, even after second graft challenges.

Second, the T cells in tolerant recipients were clearly not ignorant of Ag, as they were activated by a second male skin graft, and able to express activation markers such as CD25 (32% in the example of Fig. 3*b*) and CD44 (data not shown) on a considerable proportion of their splenic cells 7 days after graft challenge, even though they failed to reject such grafts. To our surprise, the splenic T cells from tolerant mice could also be stained for IFN- γ after a brief activation *in vitro* with PMA plus ionomycin (Fig. 3*b*). Furthermore, although the proportions of cells staining were quite variable between individual tolerant mice, they were generally very similar to primed (rather than naive) mice in the expression of a large number of immune markers (including CD152, CD45RB, CD69, CD103, IL-4, IL-10, CD62L, CD154, CD30, and LAG-3; data not shown).

CD25 is considered a marker for both activated T cells as well as for CD4⁺ regulatory T cells. Although CD25 was highly expressed 7 days after a graft challenge in all groups, indicating recent T cell activation, there was evidence of only a small excess of CD4⁺CD25⁺ T cells in the spleens of tolerant mice at >100 days after the second graft challenge (Fig. 3*c*).

Tolerant mice still make a proliferative T cell response to male Ag

In vitro assays of T cell responsiveness to donor Ag after the induction of peripheral tolerance in normal mice with a full T cell repertoire have not generally shown any consistent correlation with the immune status of the recipient (36). We have now observed a similar outcome in the H-Y Ag monospecific TCR-transgenic system consistent with no substantive deletion to achieve this form of tolerance. All the groups, whether tolerant or immune to male skin grafts, were able to make similar dose-dependent, proliferative (Fig. 4*a*), and cytokine (>100 ng/ml IFN- γ , 0.5–5 ng/ml IL-4, <1 ng/ml IL-10; data not shown) responses *in vitro* when female spleen cells or bmDCs were used to present varying concentrations (1–1000 nM) of the *Dby*-E^k peptide. However, the tolerant group did seem to respond poorly to native male APCs (that in naive mice is equivalent to 10 nM *Dby*-E^k peptide), raising

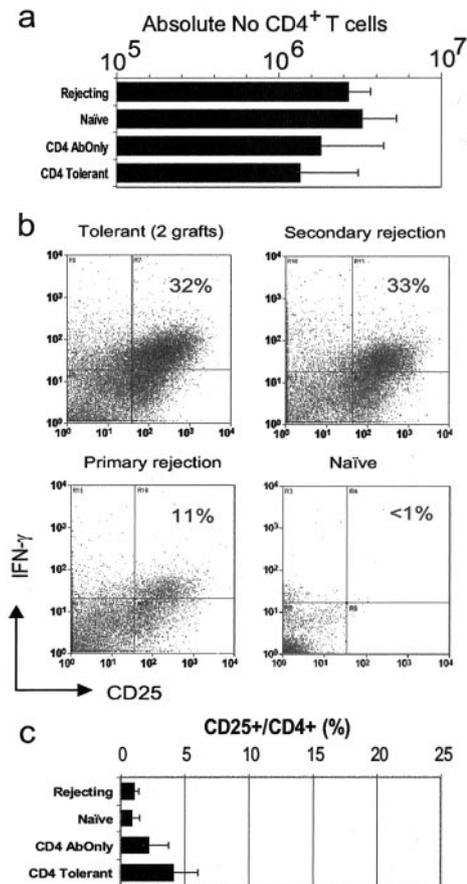


FIGURE 3. Phenotype of CD4⁺ T cells from tolerant, rejecting, or control mice. *a*, Absolute numbers of CD4⁺ T cells (plotted as mean \pm SD of log₁₀ transformed values) in the spleens of CD4 mAb-treated and male skin-grafted female A1(M).RAG-1^{-/-} mice, >100 days after challenge male skin grafts, as measured by flow cytometry. *b*, Two parameter histograms of surface CD25 expression and intracytoplasmic IFN- γ staining, as measured by flow cytometry, on the spleen CD3⁺ T cells of tolerant, previously rejecting, and naive female A1(M).RAG-1^{-/-} mice, 7 days after being given a fresh male CBA/Ca skin graft. Each plot is representative of three different mice in each group that were analyzed. *c*, Proportion of CD4⁺ splenic T cells from female A1(M).RAG-1^{-/-} mice treated with anti-CD4 mAb that expressed CD25 on their surface >100 days after challenge male skin grafts (plotted as mean \pm SD).

the possibility that tolerant T cells may in some way down-regulate presentation or processing of the endogenous *Dby* Ag. Prior removal of the small numbers of CD4⁺CD25⁺ T cells from the tolerant spleen cell populations had no effect on the proliferation of any population *in vitro*, whether stimulated with *Dby*-E^k peptide, native male Ag, or anti-CD3 (data not shown).

One possibility for the similar gross [³H]thymidine uptake *in vitro* was that there were more subtle differences in the proportions of T cells entering the cell cycle or dying in culture. This was assessed by labeling the responder CD4⁺ T cells with CFSE to track the number of cell divisions, and by labeling the dead cells with 7-AAD at the end of the culture period. This confirmed that while there were subtle differences, particularly between naive and primed mice, with the latter showing an increase in both survival and number of cell divisions, tolerized recipients were generally intermediate in their response (Fig. 4*b*), and certainly did not show any reduction in the proportion of cells dividing or surviving after exposure to donor Ag when compared with naive mice.

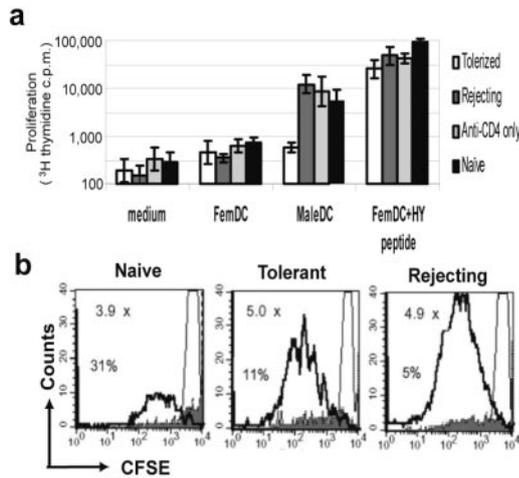


FIGURE 4. Tolerant mice can still respond to Ag in vitro. *a*, T cell proliferation, as measured by [^3H]thymidine incorporation, in spleen cells from CD4 mAb-tolerized ($n = 10$), rejecting ($n = 10$), CD4 mAb only ($n = 8$), and naive ($n = 8$) female A1(M).RAG-1 $^{-/-}$ mice, >100 days after any challenge skin grafts (plotted as mean \pm SD of \log_{10} transformed values). Cultures included female or male CBA/Ca bmDC, no APCs (medium control), or female DC plus 100 nM Dby-E k peptide. Tolerant group compared with naive group stimulated with male bmDCs, $p < 0.05$. *b*, CD4 $^+$ T cell proliferation and survival, as measured by CFSE dilution and 7-AAD exclusion, in spleen cells from naive, CD4 mAb-tolerized, or rejecting female A1(M).RAG-1 $^{-/-}$ mice, at least 100 days after any skin grafting. Representative histograms shown are from pooled triplicate cultures at 72 h of an individual mouse from each group, and show CFSE fluorescence of CD3 $^+$ 7-AAD $^-$ live T cells with female DC (no Ag; thin lines) compared with female DC plus 10 nM Dby-E k peptide (H-Y Ag; thick lines). The average number of cell divisions calculated by comparing the median fluorescence of the cells with and without Ag is indicated (e.g., 3.9 \times). Also shown is the CFSE profile of 7-AAD $^+$ (i.e., dead) cells in the cultures with Ag (filled curves) and the proportion (%) of CD3 $^+$ T cells that were 7-AAD $^+$ is indicated.

Tolerance is associated with foxP3 $^+$, GITR $^+$ CD25 $^+$ T cells in the graft

We have previously found that two of the major regulatory T cell populations, the natural CD4 $^+$ CD25 $^+$ cells and the “induced” Tr1-like cells, are associated with the expression of two different transcription factors, respectively foxP3 (14–16) and ROG (28, 37). As expected for TCR-transgenic mice on a RAG-1 $^{-/-}$ background (38), there was no significant expression of mRNA by RT-PCR for either transcript in the thymus or spleens of naive male or female animals (Fig. 5*a*). High levels of foxP3 were found in the original, tolerizing, and secondary challenge skin grafts of the tolerized groups, but not in rejecting or syngeneic (i.e., female) skin grafts, or normal skin from any of the groups, at >100 days from first grafting. The level of foxP3 mRNA detected was similar to that seen in purified CD4 $^+$ CD25 $^+$ T cells from normal CBA/Ca mice, and to that seen in the accepted skin grafts of C57/B10-tolerized nontransgenic CBA/Ca mice, when normalized to the amount of CD3 mRNA in each sample (Fig. 5*a*). Analysis of tissues from anti-CD4-treated female A1(M).RAG-1 $^{-/-}$ mice at various times after grafting indicated that foxP3 message could first be detected in male, but not female, skin grafts at day 21, and at this time there was also a weak signal for foxP3 in the spleens of male tolerized mice that disappeared by day 28 (Fig. 5*b*). foxP3 has not been detected in any effector Th1, Th2, or Tr1-like cell clones from female A1(M).RAG-1 $^{-/-}$ mice, whether resting or activated (28), suggesting that a regulatory T cell phenotype may have been induced specifically in those T cells colonizing a tolerated skin graft.

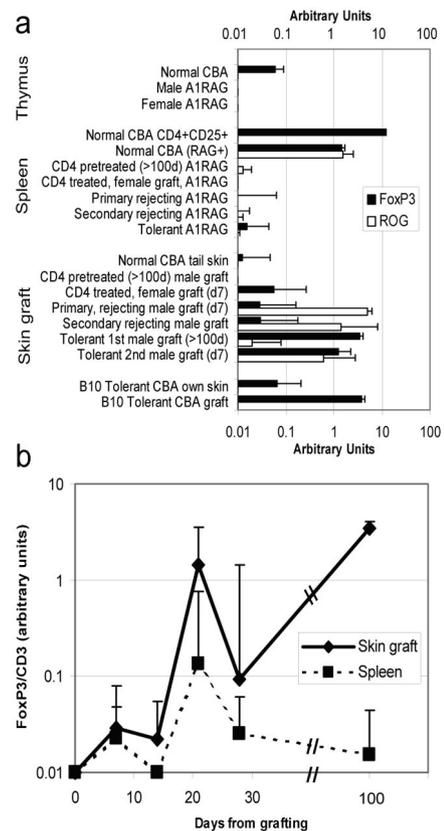


FIGURE 5. foxP3 mRNA is expressed in tolerated skin grafts. Real-time quantitative RT-PCR was performed on the indicated sources of RNA as described in *Materials and Methods*. All tolerized female A1(M).RAG-1 $^{-/-}$ mice were given CBA.RAG-1 $^{-/-}$ skin grafts to ensure any infiltrating T cells were derived only from the recipients. Skin graft and spleen samples from rejecting or tolerized mice were taken 7 days after either the initial grafting or 7 days after a second challenge graft (>100 days after the initial tolerizing graft). *a*, Samples were tested using a multiplex format for foxP3 or ROG and CD3 γ with FAM- and VIC-labeled-specific TaqMan probes (Table I). Threshold cycles of foxP3 or ROG mRNA were calibrated using a dilution series of normal spleen cell RNA, normalized to CD3 values, and normal spleen was defined as having a single arbitrary unit of foxP3/CD3. Samples were tested in triplicate and plotted as the mean \pm SD of \log_{10} transformed data. *b*, Male skin grafts or recipient spleen cells were taken from anti-CD4-tolerized female A1(M).RAG-1 $^{-/-}$ mice at various times after grafting and tested for foxP3 and CD3 γ as above. Each data point represents the mean \pm SD (\log_{10} transformed) of at least five individual mice. The last time point shown is for the original graft, but 7 days after grafting a second male skin challenge graft.

In contrast, ROG was detected, but was not differentially expressed, in tolerated and rejecting skin grafts (Fig. 5*a*).

Therefore, we extracted the infiltrating CD4 $^+$ T cells from tolerated male skin grafts and found that they expressed abundant foxP3 mRNA (Fig. 6*a*) and \sim 50% coexpressed CD3 $^+$ CD4 $^+$ GITR $^+$ CD25 $^+$ (Fig. 6*b*), supporting a regulatory phenotype for these cells (14–16, 22). These T cells must have been derived from the naive CD3 $^+$ CD4 $^+$ GITR $^-$ CD25 $^-$ foxP3 $^-$ population of the monospecific mice, as the male skin grafts were derived from T cell-deficient RAG-1 $^{-/-}$ donors.

Tolerance is associated with immune regulation in vivo

The presence of regulatory T cells and peripheral tolerance has generally been associated with a resistance to the rejection of established or fresh grafts by the infusion of immunocompetent lymphocytes (39). The male tolerized, monospecific mice also showed

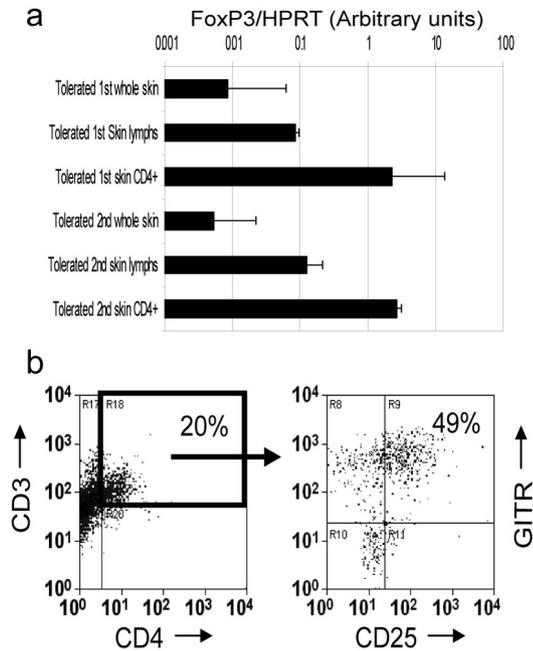


FIGURE 6. *foxP3*⁺, CD25⁺GITR⁺ T cells are found within tolerated skin grafts. *a*, Total RNA samples from original or second challenge skin grafts, the pooled intraepithelial lymphocytes fractions extracted from the grafts, and the purified CD4⁺ T cells from these fractions, from five tolerated female A1(M).RAG-1^{-/-} mice, were analyzed using a multiplex format for *foxP3* and normalized to HPRT with FAM- and VIC-labeled-specific TaqMan probes (Table I). Normal spleen was defined as having a single arbitrary unit of *foxP3*/HPRT. Samples were tested in triplicate and plotted as the mean \pm SD of log transformed data. *b*, The phenotype of CD4⁺ T cells infiltrating tolerated grafts: CD4 mAb-treated female A1(M).RAG-1^{-/-} mice tolerant of male CBA.RAG-1^{-/-} skin grafts for >100 days were given second challenge CBA.RAG-1^{-/-} skin grafts. Seven days after regrafting, the second grafts were removed and CD4⁺ T cells were extracted and MACS purified as described in *Materials and Methods*. The yield was $\sim 10^4$ CD3⁺CD4⁺ cells per graft. Flow cytometric histogram plots are shown for CD3 vs CD4 staining (20% were double positive), and these were gated and indicated to be 49% CD25⁺GITR⁺.

evidence of such regulatory activity, as the infusion of sufficient naive male-specific T cells to cause rapid graft rejection in empty RAG-1^{-/-} recipients was unable to break the tolerant state (Fig. 7*a*). Giving sufficient Ag-primed cells, in this case a male-specific Th1 clone (29), was able to override tolerance in monospecific mice in a fashion similar to CD4 mAb-induced tolerance in normal CBA/Ca mice (1), leading to graft rejection, albeit with a small, but statistically significant, delay (Fig. 7*b*).

Tolerance induction by anti-CD4 is dependent on TGF- β

Having demonstrated that anti-CD4 treatment was able to induce tolerance and regulatory T cells associated with the expression of *foxP3*, we wished to determine whether this would be TGF- β dependent. We found that coadministration of a neutralizing anti-TGF- β mAb (1D11) blocked the ability of the anti-CD4 to induce tolerance in the majority of female A1(M).RAG-1^{-/-} mice (Fig. 8), while an Ab of the same isotype against the IL-10 receptor (1B1.2) had no effect, with all but one of the grafts surviving indefinitely.

Discussion

Donor-specific transplantation tolerance, in the absence of hemopoietic chimerism, can be induced by a number of different regimens and is almost always associated with CD4⁺ regulatory T

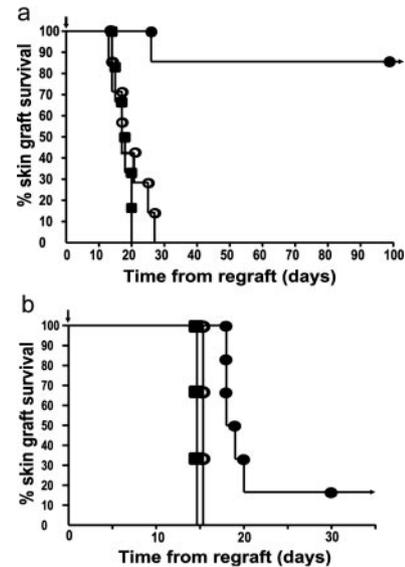


FIGURE 7. Tolerant mice resist graft rejection by immunocompetent T cells. *a*, CD4 mAb-treated female A1(M).RAG-1^{-/-} mice that had accepted male CBA/Ca skin grafts for >100 days ($n = 7$, ●), similar mice given CD4 mAb without a graft ($n = 7$, ○), or CBA.RAG-1^{-/-} empty mice ($n = 6$, ■) were given 2×10^7 naive A1(M).RAG-1^{-/-} spleen cells together with a fresh male CBA/Ca skin graft. Only the tolerized group was able to resist rejection of the fresh (and original, data not shown) male skin grafts ($p < 0.001$). *b*, CD4 mAb-treated female A1(M).RAG-1^{-/-} mice that had accepted male CBA/Ca skin grafts for >100 days ($n = 6$, ●), similar mice given CD4 mAb without a graft ($n = 3$, ○), or CBA.RAG-1^{-/-} empty mice ($n = 3$, ■), were given 5×10^6 male-specific Th1 clone (R2.2 (29)) together with a fresh male CBA/Ca skin graft. The tolerized group showed delayed graft rejection compared with the combination of the two control groups ($p < 0.01$).

cells (39). The two main hypotheses to explain the origin of these regulatory T cells are that T cell blockade either 1) converts (deviates) naive or effector, Ag-specific T cells to a regulatory phenotype, or 2) favors selection of a pre-existing, "self"-reactive population of regulatory T cells that may cross-react with donor Ag.

The natural CD4⁺CD25⁺ regulatory T cell population is thought to be primarily specific for self Ags and to originate from selection events during T cell development within the thymus (13, 40–43). Regulatory T cells that control mucosal inflammation are also thought to have some specificity for Ags expressed by commensal flora (44, 45), although it is not clear whether these are selected by cross-reaction from the thymic self-reactive population, whether they are generated in the periphery de novo (46), or whether they act primarily to control innate mechanisms of inflammation (47) and T cell homeostasis (48, 49).

The monospecific mice used in this study were found not to contain measurable *foxP3*⁺, CD4⁺CD25⁺ natural regulatory T cells in the thymus or periphery, as expected (9, 38), and all their T cells have identical specificity for the male (*Dby*) transplantation Ag (29) and are therefore very unlikely to have any significant cross-reaction with self Ags or normal gut flora. When taken together, the induction of tolerance, expression of *foxP3*, GITR, and CD25 on T cells within the tolerated graft, and evidence of regulatory activity as shown by the resistance to breaking tolerance by naive T cells in vivo, strongly support the hypothesis that donor-specific peripheral T cells are being converted to a regulatory phenotype by exposure to graft-derived Ag under the cover of coreceptor blockade. This is further supported by the observation that anti-CD4 blockade in vitro also leads to the induction of *foxP3* and that this, like the tolerance induction, is dependent on TGF- β . This

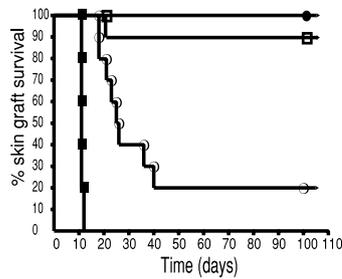


FIGURE 8. Tolerance induction by anti-CD4 is TGF- β dependent. Female A1(M).RAG-1^{-/-} mice were given male skin grafts on day 0. The untreated group (■) rejected within 12 days ($n = 5$). Mice given 1 mg of anti-CD4 (YTS 177.9; rat IgG2a) on day 0 (●) all accepted their grafts indefinitely ($n = 9$). The group given anti-CD4 as above, plus anti-TGF- β (1D11; rat IgG1, 2 mg \times 7 over 2 wk), rejected their grafts (○) with a median of 25 days ($n = 10$), while a separate group given anti-CD4 plus anti-IL-10R (1B1.2; rat IgG1, 2 mg \times 7 over 2 wk) kept their grafts (□) indefinitely ($n = 10$). Comparing anti-TGF- β (○) and anti-IL-10R (□) groups, $p < 0.003$.

is consistent with recent data by Chen et al. (35) who have shown that CD4⁺CD25⁻ cells can be converted to CD4⁺CD25⁺ regulatory cells due to a TGF- β -dependent induction of *foxP3*.

The fact that *foxP3* is highly enriched in the tolerated grafts and is only transiently detected in the spleen suggests that such regulatory T cells are accumulating and perhaps acting locally within the tolerated tissue. This result is particularly intriguing with respect to how *foxP3*⁺ regulators are found within the secondary challenge skin grafts given to already tolerant recipients. It remains to be determined whether these *foxP3*⁺ regulators are recruited from the original tolerizing graft, or some lymphoid reservoir of tolerant *foxP3*⁺ T cells outside of the spleen or draining lymph nodes, or whether they derive from a *foxP3*⁻ T cell, perhaps via the influence of modulated APCs (50, 51) within the tolerant mouse. Others have also shown that regulatory T cell activity can be generated in the periphery of Ag-specific TCR-transgenic mice (46), but these were CD25⁻ and of unknown *foxP3* status.

Although the expression of *foxP3*, CD25, and GITR suggests that the maintenance of tolerance in this model may involve mechanisms related to the natural CD4⁺CD25⁺*foxP3*⁺ regulatory cell population, we cannot exclude a role for Tr1-like or CD25⁻ cells, as we do not know the proportion of T cells in the tolerated grafts that expressed *foxP3*, and only about half of the CD3⁺CD4⁺ T cells coexpressed CD25 and GITR. In addition, we did detect the presence of ROG mRNA, a marker we have found previously associated particularly with Tr1-like cells (28), but this was not differential between tolerant and rejecting grafts. It may be that tolerance is maintained by a combination of different regulatory T cells, including CD25⁺, *foxP3*⁺, CD25⁻, or ROG⁺ populations, even in a monospecific TCR-transgenic mouse.

The ability to block CD4 mAb-induced tolerance by neutralizing TGF- β , but not by blocking the IL-10R, is of considerable interest, as we have previously been unable to break established tolerance or linked suppression by neutralizing either TGF- β (52) or IL-10 (23). This would suggest that TGF- β plays a specific role in inducing *foxP3*-mediated differentiation to regulatory cells (35), but that neither TGF- β nor IL-10 may be a critical component of their mechanism of action. There has been some debate over the role of TGF- β in CD4⁺CD25⁺ regulatory T cells, with evidence from in vitro suppression assays both for (53) and against any such requirement (54). However, TGF- β has been found essential to induce tolerance to allografts in rats by donor-specific blood transfusions (55), and in the induction of regulatory T cells by anti-CD3

treatment of either IL-2-deficient (56) or diabetic nonobese diabetic (57) mice.

In summary, peripheral tolerance can be induced in TCR-transgenic mice where every T cell is CD4⁺ and directed to the donor transplantation Ag. A nondepleting CD4 mAb was able to induce *foxP3* during Ag stimulation in vitro and tolerance to skin grafts in vivo, and in both cases this was dependent on TGF- β . CD4⁺ T cells from tolerant mice still made proliferative and cytokine-producing responses to donor Ag in vitro, and systemically to fresh donor-type skin grafts, even though these were not rejected. Tolerance was associated with the presence of regulatory T cells that were able to resist the rejection normally caused by the infusion of donor-specific naive T cells. In the TCR-transgenic mice, putative regulatory T cells were concentrated in tolerated skin and expressed CD4, CD25, GITR, and *foxP3*, and would appear to have arisen de novo in the periphery.

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