Combined donor-specific transfusion and anti-CD154 therapy achieves airway allograft tolerance


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Abstract

Background: The state of tolerance allows long-term graft survival without immunosuppressants. Lung transplantation tolerance has not been consistently achieved in either small or large animal models.

Methods: We studied the mechanisms and effectiveness of a tolerance induction protocol consisting of donor-specific transfusion (DST; day 0) and a short course of costimulatory blockade (anti-CD154 antibody) (days -7, -4, 0 and +4) in the mouse heterotopic tracheal transplant model of chronic lung rejection. C57BL/6 mice received BALB/c tracheal grafts (day 0) and were treated with DST alone, anti-CD154 alone, the combination (DST/anti-CD154), or no treatment. No nonspecific immunosuppressants were used.

Results: DST/anti-CD154, but neither treatment alone, markedly prolonged the lumen patency and survival (>100 days) of fully-histoincompatible allografts (P < 0.05 versus control allografts at every time point studied up to 16 weeks) without immunosuppression. This protocol was donor antigen-specific as third party grafts (C3H) were promptly rejected. In addition, DST/anti-CD154 did not result in mixed chimerism but induced transplantation tolerance via a peripheral mechanism(s), which included significantly reduced cytotoxic T cell activity (P < 0.001) and a significantly increased percentage of CD4+CD25+ cells (P = 0.03).

Conclusions: In summary, the DST/anti-CD154 protocol successfully induced and maintained long-term, donor-specific tolerance in the mouse heterotopic airway graft model of chronic lung rejection. This finding may lead us closer to successful tolerance induction in the lung transplantation arena.

ABBREVIATIONS

APCs: antigen presenting cells
CFSE: Carboxy-fluorescein diacetate, succinimidyl ester
CTL: cytotoxic lymphocyte
DST: Donor specific transfusion
MHC: major histocompatibility complex
OB: Obliterative bronchiolitis
TCR: T cell receptor
T_R: Regulatory T cells
INTRODUCTION

Lung transplantation has become an established therapeutic option for patients with end-stage pulmonary diseases. Its success is markedly limited by chronic lung rejection or obliterative bronchiolitis (OB), manifested clinically as progressive airway obstruction. Obliterative bronchiolitis is the leading cause of late death after lung transplantation and is the principle reason that the 5 (~45%) and 10 (~20%) year survival rates are 20-30% lower, on an absolute basis, than those after heart, kidney or liver transplantation. Currently available immunosuppressants, which must be maintained for life, are neither transplant-antigen specific nor effective in treating or preventing chronic graft rejection. In addition, they possess many serious adverse effects. A more attractive method of inducing graft acceptance is through selectively coaxing the recipient’s immune response into accepting the transplanted organ as self, while maintaining normal immune reactivity against non-graft foreign antigens. This approach, called tolerance induction, has become the ultimate goal of transplant immunology. Tolerance research in the arena of lung transplantation has been very limited and has lagged behind that in other organs. In fact, tolerance of lung allografts has not been consistently achieved in either small or large animal models (1).

While allore cognition, through engagement of the TCR with the donor-derived MHC-peptide complex, is the mandatory event for allospecific T cells to recognize donor antigens (or signal 1), it is the presence or absence of the “costimulation signal” (or signal 2) that educates allospecific T cells to either become fully activated, proliferate, and develop into effector cells or to become anergic or tolerogenic, respectively (2). The two most extensively studied costimulatory pathways include the B7/CD28 and CD40/CD154 (CD40L) (3). Through competitive binding to B7 molecules on APCs, CTLA-4 Ig (a soluble form of the high-affinity receptor to B7 molecules) blocks CD28 signaling to the T cells, therefore limiting the activation of the alloreactive T cells. Treatment with CTLA4-Ig alone supports long-term allograft survival of islet cells (4) and hearts in animal models (5). However, since a CD28-independent CTLA4 signal might deliver a strong negative signal to CD4+ T cells and, thus, depress the CTLA4/B7 interaction, alloreactive T cells are not effectively shut down (6). Other means by which to disrupt T cell activation includes the blockage of the CD40/CD154 costimulatory pathway, which, in turns, limits the maturation of APCs and down-modulates the B7/CD28 interaction (7). In the mouse model of chronic lung rejection, the greater importance of CD40/CD154, in comparison to B7/CD28, has been demonstrated by the finding that recipient mice deficient in CD154, but not CD28, expression on T cells reject tracheal allografts more slowly than wild-type recipient mice (8).

Translating tolerance protocols from one organ to another has been elusive. Thus, the success of a tolerance induction protocol in one organ may not necessarily ensure the success in others (1). We hypothesize that selective blockade of immune responses that allows allore cognition of donor antigen by allospecific T lymphocytes through specific TCRs (signal 1), but prevents costimulatory signaling (signal 2), may prevent the initiation of rejection in an antigen-specific manner. One tolerance induction protocol, namely combined donor-specific transfusion and anti-CD154 monoclonal antibody (DST/anti-CD154), has emerged as a robust tolerance induction protocol in non-lung transplant models, including islet (9), heart (10), and skin (11) allografts. Its effectiveness in lung transplantation has not been tested. In this study using the mouse model of chronic lung rejection (or obliterative bronchiolitis), which most faithfully models chronic airway rejection (12, 13), we tested the hypothesis that DST/ anti-CD154 therapy induces lung transplantation tolerance and explored the immunological mechanisms of action.
METHODS

Mice

Female BALB/c (H-2^d) or third-party C3H (H-2^k) donors and C57BL/6 (H-2^b) recipient mice (8 to 12 weeks old) were used (Harlan Labs; Indianapolis, IN). Mice were maintained following guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals under an approved IACUC protocol at the University of North Carolina-Chapel Hill.

Transplantation

Heterotopic tracheal transplantation surgery, tissue processing and staining, image acquisition, and the measurement of lumen fibroproliferation, the determinant of airway rejection, were performed as previously described (14-17).

Treatment Protocols

DST consisted of a single dose of 10^7 freshly isolated donor splenocytes given to recipients on day -7 relative to transplantation (18). Briefly, single cell suspensions were obtained by passing splenocytes through a 70 micron nylon filter (BD Biosciences, San Jose, CA), subjected to hypotonic erythrocyte lysis, counted, diluted in 200 µl of PBS, and transfused into the tail vein of recipient mice. The hamster anti-mouse CD154 monoclonal Ab (MR-1, Bioexpress, West Lebanon, NH) was administered i.p. at a dose of 0.5 mg/mouse on days -7, -4, 0, and +4. Recipient mice received either treatment, or both combined, or none (figure 1). No immunosuppressants were used. Tolerance was defined as survival > 100 days as previously described (17, 18).

CTL Assay

As previously described (19), splenocytes from transplanted or naïve C57BL/6 mice were restimulated with gamma-irradiated (20Gy) donor (BALB/c) or control syngeneic (C57BL/6) stimulator splenocytes at a 1:1 ratio for 6 days before testing their efficacy as effector cells. At 37°C in 5% CO_2_, effector cells were then co-cultured in triplicates for 4 h with 5000 concanavalin-A stimulated, [^3H]-thymidine labeled target (allogeneic or self) blast cells, with effector to target cell ratios ranging from 100:1 to 12.5:1 in 96 well plates. The cells were harvested and the specific [^3H] release was determined using liquid scintillation counting on a Microtiter Plate Reader (Packard Instrument, Meriden, CT). Percent cytotoxicity was calculated as the difference in spontaneous lysis and targeted release over spontaneous lysis.

CFSE Staining

As previously described (19), single-cell suspensions of freshly isolated splenocytes (10^7/ml in PBS) were incubated with an equal volume of 10 µM of CFSE (Molecular Probes, Eugene, OR) in the dark with periodic agitation (37°C, 10 min). Unbound CFSE was quenched with an equal volume of fetal bovine serum (Sigma-Aldrich, St Louis, MO). In order to determine the fate of transfused donor splenocytes in vivo, CFSE-labeled cells were washed three times and transfused into the tail vein of recipient mice. After 12 h, the fluorescence intensities of single-cell suspensions obtained from the recipient’s spleen, axillary lymph nodes, and the thymi were analyzed by flow cytometry using a FACScan® (Becton Dickinson, San Jose, CA) and analyzed with FlowJo™ software (Tree Star, San Carlos, CA).

Immunohistochemistry
Whole spleen, lymph node and thymus were embedded in Tissue-Tek™ OTC (Sakura Finetek, Torrance, CA) and stored at -20°C until use. Frozen sections were air dried, fixed in chilled acetone, and blocked with 5% normal goat serum (Jackson ImmunoResearch, West Grove, PA). The sections were incubated with HRP-conjugated rabbit monoclonal anti-FITC antibody (DakoCytomation, Carpinteria, CA). Optimal dilutions and incubation periods were determined empirically. Duplicate sections on the same slide were incubated with rabbit IgG (Jackson ImmunoResearch) as a negative isotype control. Diaminobenzidine (DAB) (Sigma-Aldrich) was utilized as the substrate and the sections were counterstained with Light Green™ (Fisher Scientific Company, Pittsburgh, PA).

**Immunofluorocytochemistry for Regulatory T cells**

Whole spleen and lymph nodes were incubated with RPMI medium containing 10% FCS and collagenase IV (1mg/ml) (Sigma-Aldrich, St. Louis, MO) at 37°C for 15 min. Single cell suspensions were obtained by passing splenocytes through a 70-µm nylon filter (BD Biosciences), subjected to hypotonic erythrocyte lysis, and collected into cold Dulbecco’s PBS plus 2% dialyzed serum albumin (Sigma-Aldrich). After blocking with purified rat anti-mouse CD16/32 (III/II FcR, 2.4G2, IgG2b) and 10% v/v mouse IgG (15 min; 4°C), cells (5 x 10^6) were incubated on ice with FITC-conjugated rat anti-CD4 (RM4-5, IgG2a) and PE-conjugated rat anti-CD25 (PC61, IgG1) or PE-conjugated rat anti-CD45RB (anti-B220, clone RA3-6B2, IgG2a), or appropriate isotype-matched Ig controls (30 min; 4°C) (all from BD Pharmingen, San Diego, CA). Data were acquired on a FACScan® (Becton Dickinson, San Jose, CA) and analyzed with FlowJo™ software (Tree Star, San Carlos, CA).

**Statistics**

Analyses were performed using the student’s t test (data presented as mean ± SD) or, if the data were not normally distributed, nonparametric tests with the data presented as medians and 25-75% confidence intervals, (SigmaStat software, SPSS Inc., Chicago, IL) as previously described (14-17). To test the efficacy of the DST/antiCD154 protocol different endpoints in different experimental arms were compared with T tests at each time point and also with ANOVAs, which reflected a comparison across all time points.

**RESULTS**

**Combined DST and anti-CD154, but not either treatment alone, induces and maintains transplantation tolerance of fully-histoincompatible tracheal allografts**

Without treatment, tracheal allografts (n = 6 to 9, at each time point) were rapidly rejected resulting in nearly complete airway obliteration at 4 and 5 weeks and complete airway obliteration within 6 weeks (Figures 1 and 2 a-c). Treatment with DST alone resulted in allograft rejection similar to control allografts (n = 5 to 8, at each time point; P >0.2 at 3, 4, 5, and 6 weeks) (Figures 1 and 2 d-f). Treatment with anti-CD154 alone modestly prolonged allograft survival up to 5 weeks (n = 7 to 8, at each time point; P ≤ 0.03, at 3, 4, and 5 weeks). However, the grafts underwent nearly complete lumen obliteration at 6 weeks (n=6; P = 0.38) and complete lumen obliteration at 8 weeks (n=7; P = 0.40) similar to control allografts (Figures 1 and 2 g-i). In sharp contrast, combined treatment with DST/ anti-CD154 resulted in markedly preserved allograft lumen patency and prolonged survival (>100 days) (n = 6 to 11, P < 0.05 versus control allografts at each time point from 3-16 weeks and P < 0.001 using an ANOVA) (Figures 1 and 2 j-n). In addition, with DST/ anti-CD154 treatment, tracheal grafts maintained normal epithelialization for up to 8-10 weeks and cuboidal epithelialization for up to 14 weeks (Figures 2 o-s), but interestingly had varying amounts of lymphocytic infiltrates.
**DST/anti-D154 inhibits donor-specific T lymphocyte cytotoxicity**

The capability of allograft recipients to generate donor-specific immune responses was tested by examining CTL activity. At 2 weeks post-transplant, T lymphocytes from recipient mice that received combination treatment of DST/ anti-CD154 showed a significant (P < 0.001) decrease in cytotoxicity against donor-specific (BALB/c) target cells when compared with those from recipients who received DST alone, anti-CD154 alone, or no treatment (Figure 3 A). Similar results were observed at 4 weeks post-transplantation (data not shown).

**DST/anti-CD154 induces donor-specific transplantation tolerance**

Third party allografts or third-party DST were used to examine the donor-specificity of the DST/ anti-CD154 protocol. While treatment with BALB/c DST/ anti-CD154 in the C57BL/6 recipients markedly prolonged BALB/c tracheal allograft survival, third party C3H allografts, under BALB/c DST/ anti-CD154 treatment, were rejected similar to C3H allografts under anti-CD154 treatment alone at 6 weeks (n=6, median patency of 15, 6-22 (25-75% confidence interval) versus 0 (0.22-0.85); P > 0.2), with complete lumen obliteration at 8 weeks (n=4, median patency of 0 (25-75% CI = 0-0) versus 0.0 (0-0), P > 0.2). In addition, BALB/c allografts transplanted in the C57BL/6 recipients under C3H DST/ anti-CD154 treatment were rejected similar to BALB/c allografts under anti-CD154 treatment alone (n=4, P > 0.2 both at 6 weeks and 8 weeks). Last, C3H allografts (n = 5) transplanted at week 4 into C57BL/6 that had already received a BALB/c allograft at day 0 under cover of BALB/c DST/anti-CD154 were fully rejected (0.0 ± 0.0 % lumen patency) at 5 weeks not significantly different (p > 0.2) than C3H allografts (n = 5) placed in C57BL6 animals without treatment, but significantly different (p = 0.01 by the Wilcoxon Rank Sum Test) than similarly treated C57BL6 animals that received a second BALB/c graft (100 ± 0.0 % at 5 weeks).

**Transfused donor splenocytes migrate to and rapidly disappear from the recipient’s secondary lymphoid organs**

In order to determine the fate and kinetics of transfused donor cells in vivo, donor (BALB/c) splenocytes were labeled with immunofluorescence dye prior to the intravenous injection into naïve recipient (C57BL/6) mice (Figure 4 A-C). The frequencies of the transfused CFSE+ve donor cells within the recipient’s primary (thymus) and secondary [spleen and transplant-draining (axillary) lymph nodes] lymphoid organs at 12, 24, 48 and 72 h after transfusion are shown in Figure 4 D. At 24 h after transfusion, donor splenocytes were detected within the white pulp areas of the spleen (~0.35% of the resident splenocytes) (Figures 4 D and 5 A-C), and, to a lesser extent, within the non-follicular areas of the axillary lymph nodes (~0.15% of the resident lymphocytes) (Figures 4 D and 5 D-F). These cells could not be detected within these lymphoid organs at 72 h after transfusion (Figure 4 D). No significant donor cells were detected within the thymus at any time point (< 0.001%) (Figure 4 D). When anti-CD154 was co-administered, the kinetics of transfused donor splenocytes was not altered (data not shown).

**The frequencies of CD4+CD25+ or CD4+CD45RBlo regulatory-suppressor T cells were not increased during the induction phase of tolerance by the DST/ anti-CD154 protocol**

Using flow cytometry, CD4+ T cells derived from recipient’s secondary lymphoid organs at 2 and 3 weeks after transplantation were gated according to the surface expression of CD25 or CD45RB (Figure 6 A-B). The percentage of splenic CD4+CD25+ cells was significantly higher in the DST/ anti-CD154 group in comparison to the no treatment transplant control group (p = 0.03), but only trended toward being higher in comparison to the anti-CD154 alone group (P = 0.19) (2 week data; Figure 6 C). Similar findings were found in both the CD4+CD25+ cells derived from recipient’s draining lymph nodes and splenic CD4+CD45RBlo cells at both 2 and 4 weeks (data not shown).
DISCUSSION

We report herein the long-term survival of fully-histoincompatible airway allografts without the use of immunosuppressants in the mouse model of chronic lung rejection. This is one of the first reports where tolerance has been reliably achieved in any lung rejection model and, in addition, in a animal model of chronic graft rejection. Donor-specific transplantation tolerance was successfully induced and maintained by a single treatment of donor splenocytes in combination with a short course of antagonistic, non-T cell-depleting anti-CD154 monoclonal antibody (DST/anti-CD154). By providing donor antigen-specific signal while blocking the costimulation to the recipient’s T cells within the secondary lymphoid organs, this combination protocol markedly suppressed alloreactive T-cell effector response and achieved donor-specific transplantation tolerance. This protocol clearly induced transplantation tolerance via a peripheral mechanism(s) as it did not result in mixed chimerism or central deletion. In addition, we found that during the induction phase of transplantation tolerance, the percentages of \( CD4^+CD25^+\) or \( CD4^+CD45RB^{lo}\) regulatory/suppressor T cells within the spleen and draining lymph nodes of the recipient with the DST/anti-CD154 treatment were significantly increased compared to the no treatment group.

Alloimmunity is initiated by the recognition of alloantigen (allorecognition) present on the surface of donor (direct pathway) and/or recipient (indirect pathway) APCs to recipient T cells (signal 1) (2). With the help of costimulatory signals (signal 2), allospecific T cells then become activated, proliferate, and differentiate into effector cells that, through powerful, complex, and well-orchestrated immune cascades, ultimately destroy the graft. In this study, using the mouse heterotopic tracheal transplant model for chronic lung rejection, we evaluated the effectiveness of a combination DST/anti-CD154 protocol by exposing the recipient to donor antigens (signal 1) concomitant with costimulation (signal 2) blockade prior to transplantation. We found that the DST/anti-CD154 protocol was very effective in inducing and maintaining airway allograft tolerance, in keeping with studies in other transplant mouse models, showing improved islet, heart and skin allograft survival in euthymic and thymectomized recipients (9-11). In the lung transplantation arena, several studies have reported the prolongation of murine heterotopic tracheal allograft survival without the use of immunosuppressants (21-25). However, those studies demonstrated significant improvement of endpoints of allograft survival at no more than 42 days after the last treatment. In addition, when lymphocyte proliferation (MLR) was reported, the treatment did not result in donor-specific suppression (22, 23). In sharp contrast, we demonstrated that, first, the DST/anti-CD154 protocol maintained nearly complete airway patency beyond 100 days after the final anti-CD154 administration. Thus, the DST/anti-CD154 protocol is by far the most effective protocol to induce tolerance in murine models of chronic lung rejection to date. Second, in vivo, we showed that this state of transplantation tolerance was donor-specific since the (BALB/c) DST/anti-CD154 did not prolong survival of the fully-histo-incompatible third-party (C3H) grafts nor did the third-party (C3H) DST/anti-CD154 prolong survival of BALB/c allografts in the C57BL/6 recipients, and nor could third party (C3H) grafts be successfully engrafted into C57BL/6 mice who had received a BALB/c graft under cover of (BALB/c) DST/antiCD154. Donor-specific signal provided by the DST/anti-CD154 protocol may selectively capture allospecific T cell repertoires and be responsible for this distinction.

The mechanism(s) by which the DST/anti-CD154 protocol induces tolerance has not been fully elucidated but has been studied in non-lung transplantation models. DST/anti-CD154 induces Th2 polarization (10) and the deletion of the alloreactive CD8+ cells (26). These effects appear to be CTLA4-dependent (11) and require the presence of a subset of CD4+ T cells, presumably regulatory/suppressor cells (11, 27). In this study, first, we showed that each component was mandatory since treatment with DST alone or anti-CD154 alone was far less effective than the combination. This finding is consistent with previous findings showing that
donor whole blood transfusion modestly prolonged the longevity of human renal allografts (28) and that anti-CD154 used as monotherapy did not induce mouse cardiac allograft tolerance (29) due to its inability to block rejection elicited by CD8+ or CD4+ effector T cells. Second, while it remains controversial (30), it is generally believed that antigen recognition occurs in the secondary lymphoid organs (31, 32). In this study, we demonstrated that DST cells homed to the recipient’s secondary lymphoid organs, primarily to the white pulp of the spleen or nonfollicular areas of the lymph nodes, respectively, extending a previous observation in the spleen after allogeneic B cell transfusion (33). In addition, the rapid disappearance of transfused donor cells from these lymphoid organs (within 72 h), in the presence or absence of anti-CD154, suggests that mixed chimerism was not achieved, corroborating and extending the findings of others (18, 26). Third, transfused donor cells were undetectable in the recipient’s thymi at any study time, suggesting that central (intrathymic) deletion is less likely to account for tolerance induction by this protocol. Last, varying numbers of lymphocytes infiltrated the tolerized allografts, but did not lead to tissue rejection, suggesting either nonspecific inflammation (i.e., T cells without donor specificities) or the recruitment of anergic T cells. Taken together, our data are in accord with the hypothesis that the DST/anti-CD154 protocol induces transplantation tolerance by a peripheral mechanism(s) leading to, at an effector function level, a suppressed donor-specific CTL response and, although not studied herein, the possibility of generating anergic T cells that traffic to the graft.

Regulatory T (TR) cells, including naturally-arising TR cells (i.e. CD4+CD25+ or CD4+CD45RBlo) have been evident with many tolerance-inducing protocols in many transplant and autoimmunity models and may be responsible for tolerance induction. While others have suggested that, under the DST/anti-CD154 protocol, the presence of a subset of CD4+ cells is critical for prolonged allograft survival (11, 18, 27, 34), they have not identified this subset of CD4+. In this study, we found a significant increase in the percentage of CD4+CD25+ and CD4+CD45RBlo TR cells within the spleen and lymph nodes of recipient animals treated with this protocol at 2 and 3 weeks after transplantation, suggesting a role for these cells in tolerance induction. However, since the functions of these cells were not evaluated in vitro or in vivo and we did not measure FoxP3 expression, we cannot prove that these cells were responsible for tolerance induction. The precise mechanisms by which DST/anti-CD154 protocol induces and maintains lung transplant tolerance, including the involvement of TR cells, have yet to be elucidated and are under current investigation in our laboratory.

This study has several potential limitations. First, the mouse model of chronic lung rejection has limitations as previously described (14-17), but it remains one of the best, if not the best, model for chronic rejection since orthotopic models do not characteristically reproduce human obliterative bronchiolitis (35, 36). Nonetheless, this is the first study to demonstrate the use of this model of chronic lung rejection and tolerance induction protocol to accomplish interventional lung transplantation tolerance. Therefore the merits of this model argue for further testing of lung transplant tolerance-inducing protocols using this and other models, including those that primarily model acute rejection (e.g. the rat orthotopic model). Second, the brevity and simplicity of DST/anti-CD154 protocol obviates the need for nonspecific immunosuppression. However, in an effort to facilitate preemptive tolerance induction of the allospecific T cell compartment, DST/anti-CD154 must be introduced prior to transplant. Therefore application of this protocol would be restricted from the cadaveric donor transplant setting in clinical medicine. Evaluation of the effectiveness of this type of protocol, when DST is given at the time of, or a few hours prior to, transplantation (i.e. at -1h to -6h), are being investigated currently, with the goal of improving its clinical application. Thirdly, although the operational criteria defining indefinite graft tolerance is graft survival greater than 100 days, tracheal allografts under DST/anti-CD154 protocol may not truly maintain indefinite tracheal graft survival as they displayed a gradual, progressive loss of differentiated epithelium over time and completely lost epithelialization...
between 14 and 16 weeks. The loss of the epithelial lining of the murine tracheal allograft lumen has been consistently observed preceding lumen obliteration (12-15, 37, 38), suggesting that these grafts may be rejected eventually. The potential benefits of additional DST and/or anti-CD154 treatment(s) used at later time points after transplantation to suppress new allospecific thymic emigrants remains to be determined.

In conclusion, long-term donor-specific airway allograft tolerance in the stringent, fully-histoincompatible, heterotopic tracheal transplant model of chronic lung rejection can be induced and maintained by the combination treatment of a single donor-specific transfusion and a short course of the monoclonal anti-CD154 antibody around the time of transplantation. This finding has propelled us a significant step closer to successful tolerance induction in the lung transplantation arena. Further studies are needed to identify the mechanisms by which this protocol achieves tolerance and to modify this protocol to become more clinically applicable, thereby positioning clinical lung transplant tolerance trials into arenas currently occupied only by other organ transplants.

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Figure Legends

Figure 1

**DST/anti-CD154 induces and maintains long-term airway allograft tolerance.** Without the use of immunosuppressants, treatment with DST/αCD154, but not either DST or anti-CD154 alone, maintained lumen patency and prolonged tracheal allografts survivals indefinitely (>100 days). The freedom from airway rejection in allografts transplanted into recipients under treatments of DST, anti-CD154, DST/anti-CD154, or no treatment is shown as mean percentage of airway lumen patency (n=5 to 11 and SDs ≤ 18%, for each treatment arm at each time point). The * denotes comparisons (P<0.05) to the control allografts at the same time point.

Figure 2

**Representative histology of tracheal allografts treated with DST/anti-CD154.** Tracheal allografts transplanted into recipients who received DST/anti-CD154 (J-N) displayed a complete or nearly complete patent airway lumen for up to 16 weeks (H&E, 10x). This was not the case for those allografts transplanted into recipients who received DST alone (D-F), anti-CD154 alone (G-I), or no treatment (A-C). Of note, under DST/anti-CD154 treatment, tracheal allografts maintained epithelialization for up to 16 weeks and displayed a gradual, progressive loss of differentiated epithelium over time (o-s, magnification: 40x). Ep = Epithelium.

Figure 3

**Recipients treated with DST/anti-CD154 exhibited reduced donor-specific CTL.** The secondary CTL assays were performed at 2 weeks after transplantation on splenocytes derived from recipient mice, who received different treatment groups (n =3 for each group). Isolated splenocytes were re-stimulated with irradiated spleen cells from mice of the same strain as donor grafts. After 6 days in culture, the restimulated recipient cells were harvested and assayed for the ability to lyse radioactive-labeled target cells. Under different treatments, CTLs from recipients of H2<sup>d</sup> grafts variably lysed allogeneic H2<sup>d</sup> targets (A), but did not lyse syngeneic H2<sup>b</sup> control targets (B). The X axis shows effector to target cell ratios. Data are shown as the mean (± 1 SD) percent lysis in triplicate cultures from three animals and are representative of two separate experiments. The SD bars on the no treatment group are too small to be seen on this graph.

Figure 4

**Flow Cytometry demonstrates that donor splenocytes home to secondary and primary lymphoid organs.** Transfused donor cells were traced in vivo using CFSE labeling. Freshly isolated splenocytes readily acquired intracellular CFSE label (A, light microscopy; B fluorescence microscopy; and C, flow cytometer). As an internal control, unlabeled splenocytes were mixed with labeled splenocytes (C). (D) A small number of transfused cells migrated to, and then rapidly disappeared from the recipient lymphoid organs. Naïve C57BL/6 mice were transfused with 10<sup>7</sup> freshly isolated, CFSE-labeled BALB/c splenocytes. The frequencies of CFSE<sup>+</sup> cells within lymphoid organs (spleen, transplant-draining lymph nodes, and thymus) were determined at 12, 24, 48 and 72 hours after transfusion (n=4, at each time point). The Y Axis depicts the percentages of cells within the recipient primary (thymus) and secondary (spleen and transplant-draining lymph nodes) lymphoid organs. The fluorescence intensities of single-cell suspensions from primary and secondary lymphoid organs were acquired by FACScan<sup>®</sup> and analyzed with FlowJo™ with a minimum of 10<sup>6</sup> events for each analysis. The data are shown as mean (± SD) percentages of CFSE<sup>+</sup> cells within the whole respective lymphoid organs and are representative of three separate experiments.
**Figure 5**

**Immunocytochemistry localizes donor splenocytes in secondary and primary lymphoid organs.** Representative histology of recipient’s primary and secondary lymphoid organs at 12 hours after donor-specific transfusion. Sections of spleen were examined by immunohistochemistry staining for CFSE label. The section was incubated with HRP-conjugated rabbit anti-FITC antibody, and the color was developed with DAB. Transfused donor CFSE-labeled T cells (dark brown) were localized primarily in the white pulp of the spleen (A-C) or non-follicular areas of the draining lymph nodes (D-F). Comparison H&E stained sections are shown in A and D. WP = white pulp, Fo = follicular area.

**Figure 6**

**DST/anti-CD154 increased the percentage of CD4⁺CD25⁺ regulatory T (T_R) cells.** Splenocytes were obtained from recipient mice at 2 weeks post-transplant. Representative flow cytometric histograms of CD4⁺CD25⁺ and CD4⁺CD45RBlo cells are shown in (A) and (B), respectively. CD4⁺ T cells were evaluated according to the presence of surface expression of CD25 or CD45RB marker (in boxes). A minimum of 10⁵ events was acquired for each analysis. (C) The mean percentages of CD4⁺CD25⁺ cells were significantly increased in the DST/anti-CD154 treatment group compared to the no treatment transplant group ((5.2 ± 1.3 vs. 4.4 ± 0.8%, P = 0.03). The results are representative of two separate experiments performed at 2 and 4 weeks post transplant. Lymph node analyses gave similar results (data not shown).

**REFERENCES**
