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Long-Term Survival of Skin Allografts Induced by Donor Splenocytes and Anti-CD154 Antibody in Thymectomized Mice Requires CD4+ T Cells, Interferon-γ, and CTLA4

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Abstract

Treatment of C57BL/6 mice with one transfusion of BALB/c spleen cells and anti-CD154 (anti–CD40-ligand) antibody permits BALB/c islet grafts to survive indefinitely and BALB/c skin grafts to survive for ∼ 50 d without further intervention. The protocol induces long-term allograft survival, but the mechanism is unknown. We now report: (*a*) addition of thymectomy to the protocol permitted skin allografts to survive for > 100 d, suggesting that graft rejection in euthymic mice results from thymic export of alloreactive T cells. (*b*) Clonal deletion is not the mechanism of underlying long-term graft survival, as recipient thymectomized mice were immunocompetent and harbor alloreactive T cells. (*c*) Induction of skin allograft acceptance initially depended on the presence of IFN-γ, CTLA4, and CD4+ T cells. Addition of anti-CTLA4 or anti–IFN-γ mAb to the protocol was associated with prompt graft rejection, whereas anti–IL-4 mAb had no effect. The role of IFN-γ was confirmed using knockout mice. (*d*) Graft survival was associated with the absence of IFN-γ in the graft. (*e*) Long-term graft maintenance required the continued presence of CD4+ T cells. The results suggest that, with modification, our short-term protocol may yield a procedure for the induction of long-term graft survival without prolonged immunosuppression. (*J. Clin. Invest.* 1998. 101:2446–2455.) Key words: transplantation • tolerance • CD154 • skin • allograft

Introduction

We have previously reported that treatment of C57BL/6 mice with a single transfusion of BALB/c donor spleen cells (donor-specific transfusion, DST)[1] and a brief course of anti-CD154 mAb permits BALB/c islet grafts to survive indefinitely (1, 2) and BALB/c skin grafts to survive for ∼ 50 d (3). The protocol is based on the observation that presentation of antigen in the absence of costimulation leads to T cell nonresponsiveness (4–10). We hypothesize that when graft recipients are treated with DST and anti-CD154 mAb: (*a*) alloantigen is presented by transfused splenocytes, (*b*) costimulation is prevented by blocking CD40–CD154 interaction and subsequent upregulation of B7 (11, 12), and (*c*) unresponsiveness to the alloantigens present in the transfusion is permissive to continued acceptance of the tissue allograft. The mechanism by which this protocol induces long-term allograft survival and the mechanism by which long-term skin allografts are ultimately rejected are not known.

The experiments reported here were designed to determine if late rejection of successful allografts in mice treated with DST and anti-CD154 mAb is mediated by alloreactive thymic emigrants. We also sought to identify cell types and soluble mediators required for the initial acceptance and subsequent maintenance of skin allografts, and to determine if the protocol leads to a state of microchimerism in recipient mice. In this report, we define tolerance functionally as a state of long-term graft acceptance in the absence of immunosuppression. This functional definition, it should be noted, is different from the "immunological" definition of tolerance as the absence of any detectable immune response to a graft in the absence of immunosuppression.

We hypothesized that treatment with DST and anti-CD154 mAb would impede the priming of alloreactive CD4+ and CD8+ T cells and skew the balance of Th1- and Th2-type cytokines. This hypothesis was based on studies demonstrating that treatment of bone marrow allograft recipients with anti-CD154 mAb (*a*) blocks acute and chronic graft vs host disease (13) and (*b*) decreases both the number of alloreactive CD4+ thoracic duct lymphocytes and the expression of IFN-γ, IL-2, and perforin mRNA (14). It was also based on reports that heart allograft survival in animals treated with anti-CD154 mAb and donor spleen cells is associated with inhibition of intragraft Th1-type cytokines and reciprocal up-regulation of Th2-type cytokines (15).

We observed that induction of tolerance to skin allografts with DST and anti-CD154 mAb depends on the presence of CD4+ T cells, IFN-γ, and CTLA4, suggesting, surprisingly, that allograft tolerance induction may not be an exclusive function of Th2-type CD4+ T cells. In contrast, long-term graft maintenance appeared to require the presence of peripheral CD4+ T cells, and was associated with the absence of IFN-γ in the graft 7 d after surgery. Finally, we found that eventual rejection of skin allografts initially accepted by euthymic mice results in part from continuing export of alloreactive T cells by the thymus, suggesting that our protocol had blocked the priming of peripheral alloreactive T cells. The results identify mechanisms by which transplantation in the absence of continuous immunosuppression can be achieved.

1. *Abbreviations used in this paper:* D-PBS, Dulbecco's PBS; DST, donor-specific transfusion; MST, median survival time; RT-PCR, reverse transcriptase PCR; VRC, vanadyl ribonucleoside complex.

Methods

Animals. C57BL/6 (H-2^b), BALB/c (H-2^d), and C3H/HeJ (H-2^k) mice were obtained from the National Cancer Institute (Frederick, MD). C57BL/6-*Prkdc^{scid}/Prkdc^{scid}* mice (H-2^b, referred to as C57BL/6-*scid* mice) and C57BL/6 mice in which the IL-4 (16), IL-10 (17), or IFN-γ (18) genes were disrupted by homologous recombination were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were certified to be specific pathogen-free, housed in microisolator cages, and given ad libitum access to autoclaved food and water. They were maintained in accordance with local and federal guidelines (Department of Health, Education, and Welfare Publication National Institutes of Health [NIH] 78-23, 1985).

Skin transplantation. Skin graft recipients were male C57BL/6 mice or male C57BL/6-*scid/scid* mice 6–8 wk of age. Cells used for DST and full-thickness skin specimens for transplantation were obtained from female BALB/c mice 4–6 mo of age. Donated skin was prepared and transplanted as described (3). Skin graft survival was assessed three times weekly. Time of rejection was defined as the first day on which the entire epidermal surface of the graft was necrotic. This typically occurred within a few days after the first sign of rejection in grafts lost soon after transplantation and within 2 wk in the case of grafts lost months after transplantation.

Treatment of skin allograft recipients with donor-specific transfusion and anti-CD154 mAb. BALB/c mice were killed in 100% CO_2. Spleens were removed, dispersed in sterile medium (RPMI-1640), washed, and counted. Cell viability was assayed by Trypan blue exclusion, and was > 90% in all cases. The MR1 hamster anti–mouse CD154 mAb was produced as ascites in *scid* mice (19, 20). Antibody concentration in ascites was determined by ELISA. Anti-CD154 ascites was administered at doses of 0.25 mg/mouse. Skin allograft recipients received either no treatment, a single transfusion of donor spleen cells, four doses of anti-CD154 mAb during a 2-wk interval, or combined therapy with both reagents. Each donor-specific transfusion consisted of 10×10^6 viable spleen cells in a volume of 0.5 ml given via the tail vein 7 d before grafting. Four 0.25-mg doses of anti-CD154 mAb were given intraperitoneally twice weekly beginning on the day of spleen cell injection (i.e., standard protocol).

Thymectomy. In certain experiments, anesthetized C57BL/6 mice 4–6 wk of age were thymectomized as previously described (21). Completeness of thymectomy was assessed at the conclusion of experiments by visual inspection.

T cell subset depletion. In vivo depletion of CD4$^+$ and CD8$^+$ T cell subsets used mAbs produced by the rat anti–mouse hybridomas GK1.5 (anti-CD4) and 2.43 (anti-CD8) obtained from the American Type Culture Collection (Rockville, MD) as described (22). Antibody was produced as ascites in *scid* mice and immunoglobulin concentration was measured by radial immunodiffusion (The Binding Site, San Diego, CA). To deplete CD4$^+$ or CD8$^+$ cells, mice were given 0.5 mg of the appropriate mAb intraperitoneally for three consecutive days. This protocol was documented by flow microfluorimetry to deplete > 90% of CD4$^+$ and > 95% of CD8$^+$ T cells 48 h after the last dose of mAb.

Treatment with antibodies against IL-4, IFN-γ, and CTLA4. Monoclonal rat IgG1 antibodies that react with mouse IL-4 and IFN-γ were obtained from 11B11 and R4-6A2 hybridomas, respectively (American Type Culture Collection). Antibodies were produced in *scid* mice. Antibody concentration was measured as described above. Mice received 1 mg of the appropriate mAb intraperitoneally every other day for 14 d beginning 1 wk before grafting (on the same day as treatment with DST).

Hamster monoclonal antibody directed against CTLA4 (UC10-4F10-11) was obtained from PharMingen (San Diego, CA). Mice were given 0.075 mg in a volume of 0.2 ml intraperitoneally on days −7, −6, and −5 before the placement of skin grafts on day 0. Control hamster immunoglobulin was purchased from Sigma Chemical Co. (St. Louis, MO) and administered at the same dose and concentration on the same schedule.

Mixed lymphocyte reactions (MLRs). Responder cells for cytokine assays were negatively selected from spleen and lymph node cells by incubation with anti-HSA (J11d), anti-FcγR (2.462), and anti-B220 (RA3.6B2) mAb followed by mouse anti–rat kappa- (MAR 18.5) conjugated MACS® beads (Miltenyi Biotec, Auburn, CA) and passage over a MIDI MACS column. Cells were 95% CD3$^+$ by flow cytometry. Culture supernatants were harvested at times indicated and assayed for the presence of immunoreactive IL-4 and IFN-γ. Stimulator cells were T cell depleted and irradiated as described (1).

Measurement of IL-4 and IFN-γ concentration. Antibodies directed against mouse IL-4 (11B11) and mouse IFN-γ (R4-6A2) were used at concentrations of 1 and 3 μg/ml, respectively. ELISA plates (Corning Glass, Corning, NY) were coated overnight at 4°C with 50 μl/well of antibody diluted in 0.1M Na_2HPO_4 (pH 9.0) and washed twice with Dulbecco's PBS (D-PBS). Nonspecific binding was blocked using 10% horse serum in D-PBS for 30 min. After four washes with D-PBS containing 0.05% Tween-20 (D-PBS/Tween), standards and experimental samples were added to plates (100 μl/well) for a second overnight incubation at 4°C. Standards were diluted in the same culture medium used for the MLRs. Standards ranged from 7.8 to 2,000 U/ml for IL-4 and 7.8 to 2,000 pg/ml for IFN-γ. Plates were washed six times with D-PBS/Tween and then reacted with biotinylated secondary antibodies (100 μl) for IL-4 (BVD6-24G, 0.5 μg/ml; PharMingen) and for IFN-γ (XMG1.2, 0.25 μg/ml; PharMingen). After 1 h of incubation at 25°C, plates were washed eight times with D-PBS/Tween and reacted with 100 μl of avidin-horse radish peroxidase conjugate (Vector Laboratories, Burlingame, CA) for 45 min at 25°C. Plates were then washed eight times with D-PBS/Tween, washed twice with D-PBS, and then reacted with *o*-phenylenediamine (100 μl, 1 mg/ml in pH 5.0 0.05M Na_2HPO_4, 0.025M $Na_3C_6H_5O_7$). The reaction was stopped after 10–20 min by the addition of 25 μl of 3 N HCl. Optical density was measured spectrophotometrically at a wavelength of 490 nm.

Detection of donor-origin DNA by PCR. The PCR assay for the presence of donor class I MHC DNA has been described (3). This nested PCR technique was used to test for donor class I in DNA obtained from spleen, lymph nodes, liver, and lung. This method was documented to detect 0.01% BALB/c spleen cells in a mixture containing 99.99% C57BL/6 spleen cells.

Detection of cytokine mRNA by reverse-transcriptase (RT)-PCR. To detect mRNA encoding IL-4, IFN-γ, IL-12Rβ2, and TCRCβ, total RNA was extracted from skin grafts or normal BALB/c skin using a modification of a standard guanidinium thiocyanate procedure (23). Briefly, 20 mM vanadyl ribonucleoside complexes (VRC; GIBCO BRL, Bethesda, MD) were added to the denaturing solution and the RNA pellet was dissolved in H_2O containing 10 mM EDTA to remove trace VRCs, reprecipitated, and dissolved in H_2O. RT-PCR was performed as described (24) using published primer sequences for rat TCRCβ (24), mouse IL-4, and IFN-γ (25). The primer sequences for IL-12Rβ2 were designed using GCG prime software (Genetics Computer Group) and the published sequence for IL-12Rβ2 (26). The sequences are: forward primer 5′-TCT TCT TCA CTT CCG CAT AC, and reverse primer 5′-CTC CAA TTA CTC CAA CTT CC (predicted product size 302 bp). PCR buffer containing 10 mM Tris-HCl, 50 μM KCl, pH 8.3, and either 1.5 mM $MgCL_2$ (TCRCβ) or 2.5 mM $MgCL_2$ (IL-4, IFN-γ, IL12Rβ2) and was performed for 30 cycles using threefold dilutions of cDNA. Each cycle consisted of 94°C, 30 s; 55°C, 30 s; 72°C, 60 s. Positive controls for each primer were run using either mRNA from C57BL/6 spleen cells stimulated with concanavalin A (TCRCβ, IL-12Rβ2, IFN-γ) or mRNA from EL-4 cells stimulated with phorbol myristate and ionomycin (mouse control RNA; PharMingen).

Adoptive transfer protocols. Spleen cells were obtained from donor C57BL/6 mice that had received the pretreatments listed in Table I. Cells were washed in RPMI-1640, counted, and assessed for viability by the method of Trypan blue exclusion. Varying doses of viable cells in a volume of 0.5 ml were injected into recipients intravenously via the tail vein. Recipients were C57BL/6-*scid* mice that had borne

Table I. Survival of Skin Allografts on scid Mice after Adoptive Transfer of Spleen Cells

Treatment of C57BL/6 spleen cell donors	Median survival time of BALB/c skin grafts on C57BL/6-*scid* mice after cell transfer	Survival of individual BALB/c and C3H/HeJ grafts (d) after transfer for each cell dose		
		Cell number	BALB/c grafts	C3H/HeJ grafts
	d			
Thymectomy; DST + anti-CD154 mAb; BALB/c skin graft for > 100 d	41*	15×10^6	41, 43	ND
		20×10^6	41, 43, 49	ND
		25×10^6	38, 42, 52	10, 10
		75×10^6	39, 40, 41	ND
No treatment; no thymectomy; no graft	9‡	15×10^6	7, 10	ND
		20×10^6	8, 8, 8	ND
		25×10^6	8, 8, 9, 9, 11, 13, 14,	8, 13
		75×10^6	10	ND
No thymectomy; DST + anti-CD154 mAb; BALB/c skin graft > 100 d	11‡	25×10^6	8, 8, 10, 11, 13, 18, 18	6, 13
Thymectomy; no other treatment	10‡	25×10^6	9, 10, 10, 12	8, 9, 12, 13
No treatment; no thymectomy; no graft	8‡	20×10^6	6, 8, 8	ND
Thymectomy, DST, anti-CD154 mAb, BALB/c skin graft for > 100 d		+		
		20×10^6		

Spleen cells from four groups of C57BL/6 mice were transfused at the doses indicated into C57BL/6-*scid/scid* mice bearing a completely healed BALB/c or C3H/HeJ skin graft. All donor mice that had long-term BALB/c skin allografts in place had been treated with anti-CD154 mAb on days −7, −4, 0, and +3, and BALB/c DST on day −7 relative to skin grafting on day 0 in conformity with our standard protocol. *‡ *P* < 0.001. Within each group of cell donors, there were no statistically significant effects of cell dose. ND, not done; no graft, no primary sensitization.

BALB/c skin grafts for ∼ 10 d before spleen cell transfusion. Grafts were fully healed at the time of transfusion. Duration of graft survival was calculated from the day of transfer (day 0).

Statistics. Average duration of graft survival is presented as the median. Graft survival among groups was compared using the method of Kaplan and Meier (27); the equality of allograft survival distributions for animals in different treatment groups was tested using the log rank statistic (28). *P* values < 0.05 were considered statistically significant.

Results

Prolonged survival of skin allografts in mice treated with DST, anti-CD154 mAb, and thymectomy

We first tested the hypothesis that delayed rejection of skin allografts in euthymic mice treated with DST and anti-CD154 mAb is due to alloreactive T cells that emigrate from the thymus after completion of treatment. C57BL/6 (H-2^b) mice were thymectomized and then given anti-CD154 mAb on days −7, −4, 0, and +4, and BALB/c (H-2^d) DST on day −7 relative to BALB/c skin grafting (day 0) in conformity with our standard protocol. Nearly all (*n* =23/24) thymectomized mice treated in this way retained their grafts for > 100 d (Fig. 1). In contrast, ∼ 80% of identically treated euthymic mice (with or without sham thymectomy) rejected grafts within 100 d (*P* < 0.001), an observation consistent with previous results (3). The median survival times (MST) of BALB/c skin allografts on thymectomized C57BL/6 mice given no treatment, DST alone, or anti-CD154 mAb alone were 8, 7, and 13 d, respectively.

Thymectomized mice given DST, anti-CD154 mAb, and skin allografts are immunocompetent and harbor alloreactive T cells

Adoptive transfer of spleen cells from euthymic mice given DST, anti-CD154 mAb, and skin allografts leads to rejection

of identical allografts present on C57BL/6-*scid/scid* mice (*scid* mice) (3). Based on our first result, we hypothesized that adoptive transfer of spleen cells from thymectomized mice given DST, anti-CD154 mAb, and skin allografts would not lead to rejection of MHC-identical allografts on *scid* mice. This hypothesis proved incorrect.

Spleen cells were obtained from thymectomized C57BL/6 mice that had been given DST and anti-CD154 mAb and had borne BALB/c skin allografts for > 100 d. Adoptive transfer of these spleen cells to C57BL/6-*scid* mice bearing BALB/c (H-2^d) or third party C3H/HeJ (H-2^k) skin grafts led to rejection of both types of grafts. Rejection of the C3H/HeJ grafts occurred promptly after transfer (MST = 10 d). Rejection of the BALB/c skin allografts occurred later (MST = 41 d, *P* < 0.001, Table I). Adoptive transfer of spleen cells from control C57BL/6 mice that had been thymectomized up to 100 d previously but not otherwise treated led to prompt rejection of both C3H/HeJ and BALB/c skin allografts present on C57BL/6-*scid* mice (MST = 10 d in both cases, Table I). Consistent with previous observations (3), spleen cells from euthymic C57BL/6 mice treated with DST, anti-CD154 mAb, and bearing long-term grafts promptly rejected BALB/c skin grafts in C57BL/6-*scid* mice (MST = 11 d, Table I).

We also tested mixtures of spleen cells from euthymic control C57BL/6 mice and spleen cells prepared from thymectomized C57BL/6 mice that had been given DST and anti-CD154 mAb and had borne BALB/c skin allografts for > 100 d. Adoptive transfer of these spleen cell mixtures led to prompt rejection of BALB/c skin grafts present on C57BL/6-*scid* mice (MST = 8 d, Table I).

Treatment with anti-CD4 but not anti-CD8 mAb for 3 d leads to rejection of successful skin allografts in thymectomized mice
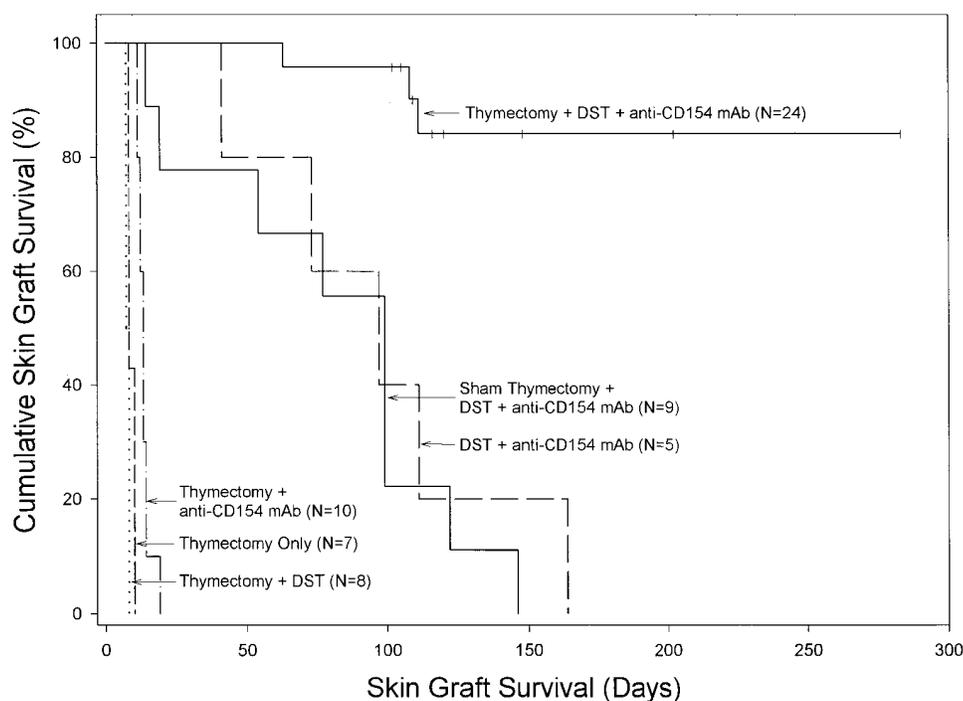
Figure 1. Survival of skin allografts. Groups of 4–6-wk-old C57BL/6 mice were randomized to undergo thymectomy, sham-thymectomy, or no surgery. After recovery, thymectomized animals were further randomized and given either no treatment, DST alone, anti-CD154 mAb alone, or the combination of DST and mAb. Sham-thymectomized and nonoperated animals were given DST plus anti-CD154 mAb. All animals also received a BALB/c skin allograft. DST (10×10^6 BALB/c spleen cells) was given on day -7 relative to transplantation, and anti-CD154 mAb was given as four doses of 0.25 mg each on days -7, -4, 0, and $+4$. Results are shown as the Kaplan-Meier probability of survival. Censored data (animals still alive with successful grafts at the termination of the study) are indicated by vertical bars. The probability of graft survival in the group treated with thymectomy, DST, and mAb is statistically greater than that in any other group ($P < 0.001$).

that had received DST and anti-CD154 mAb either 2 wk or 3 mo earlier

Because thymectomized mice given DST, anti-CD154 mAb, and skin allografts were found to be immunocompetent and to harbor alloreactive T cells, we hypothesized that graft survival was dependent on an active mechanism rather than permanent deletion of all alloreactive cells. We tested this hypothesis in thymectomized C57BL/6 mice given DST, anti-CD154 mAb, and a BALB/c skin graft (Table II). Recipient mice were then given depleting doses of either anti-CD4 or anti-CD8 mAb

Table II. Survival of BALB/c Skin Allografts on C57BL/6 Mice

Experiment	Recipient treatments				
	Thymectomy	DST + α-CD154	mAb treatment schedule relative to grafting	Skin allograft survival	MST
				d	*d*
1	−	+	−	16, 18, 29, 32, 46, 63, 66, 66, 68	46*
	−	+	α-CD4 (−11 to −9)	11, 11, 12, 13, 18, 18, 21, 21, 21, 22	18‡
	−	−	α-CD4 (−11 to −9)	11, 12, 12, 12, 15, 15, 18, 18, 18	15§
2	−	+	−	17, 17, 24, 52, 52, 63, 66, 87, 101, 117	58‖
	−	+	α-CD8 (−11 to −9)	17, 17, 17, 28, 34, 38, 38, 40	31¶
	−	−	α-CD8 (−11 to −9)	10, 10, 10, 10, 10	10**
3	+	+	−	63, 74, > 102, > 102, 108, > 168	> 100‡‡
	+	+	α-CD4 (−11 to −9)	12, 12, 14, 17, 17, 19, 31, 34, 34, 40	18§§
	+	+	α-CD8 (−11 to −9)	17, 28, 33, 38, 47, 63, 66, 77, 90, 122	55‖‖
	+	+	α-CD4 (+14 to +16)	17, 19, 21, 24, 26, 28, 31, 31, 45	26¶¶
	+	+	α-CD8 (+14 to +16)	17, 124, 133, 142, 147, > 168, 184, > 200	> 138***
4	+	+	α-CD4 (+107 to +109)	123, 133, 133, 135	133‡‡‡
	+	+	α-CD8 (+107 to +109)	> 290, > 290, > 290	> 290§§§

Four groups of C57BL/6 mice were given BALB/c skin allografts on day 0. Some of the allograft recipients in each group were also given anti-CD154 mAb on days -7, -4, 0, and $+3$, and BALB/c DST on day -7 relative to skin grafting in conformity with our standard protocol. Some mice in each category were also treated with a depleting mAb directed against either CD4 or CD8 on the days indicated. All times are relative to placement of the skin graft on day 0. Statistical comparisons: * vs ‡, $P < 0.005$; ‖ vs ¶, $P < 0.025$; § vs **, $P < 0.001$; ‡‡ vs §§ and ¶¶, $P < 0.001$; §§ vs ‖‖, $P < 0.01$; ‡‡ vs ‖‖, $P < 0.05$; ¶¶ vs ***, $P < 0.001$; ‡‡ vs ***, $P = $ NS; ‡‡‡ vs §§§, $P < 0.025$.

daily on days 14–16 (experiment 3) or 107–109 (experiment 4) after grafting. At both time points, anti-CD4 mAb treatment was associated with prompt rejection of the graft, whereas anti-CD8 mAb treatment had no effect on graft survival (Table II, experiments 3 and 4). In the case of the mice treated with mAb on days 14–16 after transplantation, the MST of grafts after giving the first dose of antibody was 12 d in the anti-CD4 group but > 126 d in the anti-CD8 group ($P < 0.001$, Table II, experiment 3). In the case of mice treated with mAb on days 107–109 after successful grafting, MST after the first dose of antibody was 26 d in the anti-CD4 group and > 183 d in the anti-CD8 group ($P < 0.025$, Table II, experiment 4).

Pretreatment with either anti-CD4 or anti-CD8 mAb shortens allograft survival in C57BL/6 mice subsequently given DST, anti-CD154 mAb, and BALB/c skin allografts

These data suggested that maintenance of permanent skin allografts on thymectomized mice after treatment with DST and anti-CD154 mAb is an active process that depends on CD4$^+$ T cell populations. We therefore hypothesized that the presence of CD4$^+$ cells would be required at the time DST and anti-CD154 mAb were being administered.

We first tested this hypothesis in thymectomized C57BL/6 mice given either anti-CD4 or anti-CD8 mAb daily on days 11, 10, and 9 before grafting (Table II). The recipients then received anti-CD154 mAb on days -7, -4, 0, and $+4$, and BALB/c DST on day -7 relative to BALB/c skin grafting on day 0 in conformity with our standard protocol. Pretreatment with anti-CD4 mAb reduced allograft survival from a median of > 100 d in controls to only 18 d ($P < 0.001$, Table II, experiment 3). Pretreatment with anti-CD8 mAb also reduced allograft survival, but to a lesser extent (55 d, $P < 0.05$, Table II, experiment 3).

Experiments were also performed in euthymic recipients using the same treatment protocols. Pretreatment with anti-CD4 mAb reduced allograft survival from a median of 46 d in controls to 18 d ($P < 0.005$, Table II, experiment 1). Pretreatment with anti-CD8 mAb also reduced allograft survival from a median of 58 d in controls to 31 d ($P < 0.025$, Table II, experiment 2).

We also tested the effect of administration of anti-CD4 and anti-CD8 mAbs on allograft survival in euthymic animals that received no DST or anti-CD154 mAb before grafting. Consistent with previous reports (29), graft survival in mice treated with anti-CD4 mAb was statistically superior to that observed in mice treated with anti-CD8 mAb ($P < 0.001$, Table II). However, in the absence of DST and anti-CD154 mAb, neither anti-CD4 nor anti-CD8 mAb treatment was associated with clinically significant prolongation of graft survival (MST = 15 and 10 d, Table II, experiments 1 and 2, respectively).

BALB/c skin allograft survival is reduced in thymectomized C57BL/6 mice given anti-CTLA4 mAb in addition to DST and anti-CD154 mAb

These data suggested that both initial acceptance and maintenance of skin allografts in mice treated with DST and anti-CD154 mAb involve an active T cell–dependent process. Because this process appeared to involve the down-regulation of alloreactivity, we hypothesized that signaling via the B7-CTLA4 receptor pathway might be required.

To test this hypothesis, thymectomized C57BL/6 mice were randomized into two groups. The first group received anti-CTLA4 mAb daily on days 7, 6, and 5 before grafting; the sec-

Table III. Skin Allograft Survival in Mice Treated with Donor Specific Transfusion and Anti-CD154 mAb with and without Intercurrent Anti-CTLA4 mAb

Donor treatment	n	MST	Survival of individual grafts
		d	
Anti-CTLA4	5	8*	7, 8, 8, 8, 8
Hamster IgG	6	$> 22^{\ddagger}$	8, > 22, > 22, > 22, > 22, > 22,

C57BL/6 mice were given anti-CD154 mAb on days -7, -4, 0, and $+4$, and BALB/c DST on day -7 relative to BALB/c skin grafting on day 0 in conformity with our standard protocol. Treated mice were randomized into two groups, one of which was given hamster IgG on days -7, -6, and -5 relative to grafting, and the other anti-CTLA4 mAb on the same schedule. * vs ‡, $P < 0.01$.

ond group received control hamster Ig on the same schedule. Both groups of recipients also received anti-CD154 mAb on days -7, -4, 0, and $+4$, and BALB/c DST on day -7 relative to BALB/c skin grafting on day 0. All recipients treated with anti-CTLA4 mAb rapidly rejected their skin grafts (MST = 8 d, Table III). In contrast, graft survival in recipients of hamster Ig was > 22 d ($P < 0.01$, Table III).

IFN-γ but not IL-4 or IL-10 is required for the initial acceptance of skin allografts by mice treated with DST and anti-CD154 mAb

Studies in knockout mice. We hypothesized that the active process involved in the acceptance and maintenance of skin allografts in mice treated with DST and anti-CD154 mAb depends on cytokines that modulate T cell function. We studied the possible role of Th1- and Th2-type cytokines using C57BL/6 mice in which the IFN-γ, IL-4, or IL-10 gene had been disrupted by homologous recombination (knockout mice). Euthymic knockout recipient mice were given BALB/c DST, anti-CD154 mAb, and BALB/c skin allografts in conformity with our standard protocol. We observed comparable survival of BALB/c skin allografts in normal, IL-4 knockout, and IL-10 knockout C57BL/6 mice (MST = 51, 22, and 39 d, respectively, Table IV, $P = NS$). In contrast, median survival of allografts in IFN-γ knockout mice was reduced to 15 d ($P < 0.001$ vs all other groups).

In vivo neutralization of IFN-γ and IL-4. We also studied the possible role of Th1- and Th2-type cytokines in the acceptance of skin allografts using neutralizing mAbs directed against IFN-γ and IL-4. Thymectomized C57BL/6 mice were randomized to three groups. Two groups were given neutralizing doses of either anti–IFN-γ or anti-IL-4 mAb on days -7, -5, -3, -1, $+1$, $+3$, and $+5$ relative to grafting on day 0. All recipients in each group were treated with anti-CD154 mAb on days -7, -4, 0, and $+4$, and with BALB/c DST on day -7 relative to BALB/c skin grafting in conformity with our standard protocol. Consistent with the observations made in knockout mice, skin allograft survival in recipients treated with anti–IFN-γ mAb (MST = 15 d) was shorter than that in controls (> 72 d $P < 0.01$) or in recipients treated with anti–IL-4 mAb (> 72 d, $P < 0.01$, Table IV).

Reduced production of IFN-γ by spleen cells from mice given anti-CD154 mAb and skin allografts with or without DST

Because IFN-γ but not IL-4 appeared to be important in the initial acceptance of skin allografts by mice given DST and

Table IV. Survival of Skin Allografts on Mice Deficient in IFN-γ, IL-4, or IL-10

Recipients	mAb	n	MST	Survival of individual grafts
			d	
Euthymic C57BL/6	—	15	51*	11, 18, 21, 25, 39, 43, 43, 51, 53, 56, > 82, > 82, > 82, > 100, > 100
Euthymic C57BL/6-IL-4 knockout	—	14	22*	11, 13, 13, 13, 18, 20, 21, 23, 74, > 82, > 82, > 100, > 100, > 100
Euthymic C57BL/6-IFN-γ knockout	—	9	15‡	13, 13, 13, 13, 15, 18, 20, 20, 28
Euthymic C57BL/6-IL-10 knockout	—	10	39*	11, 11, 13, 13, 37, 40, 47, 47, 70, 75
Thymectomized C57BL/6	—	5	> 72§	56, > 59, > 72, > 72, > 72
Thymectomized C57BL/6	Anti–IL-4	5	> 72§	> 72, > 72, > 72 > 72, > 72
Thymectomized C57BL/6	Anti–IFN-γ	5	15‖	13, 13, 15, 22, > 72

Recipient mice were given anti-CD154 mAb on days −7, −4, 0, and +4, and BALB/c DST on day −7 relative to BALB/c skin grafting on day 0 in conformity with our standard protocol. Comparisons of graft survival on normal and congenic cytokine knockout C57BL/6 mice were performed using euthymic recipients. Comparisons of graft survival in the presence or absence of anticytokine neutralizing antibodies were performed in thymectomized animals. The 11B11 anti–IL-4 and R4-6A2 anti–IFN-γ mAbs were given intraperitoneally at a dose of 1 mg on days −7, −5, −3, −1, 1, 3, and 5 relative to grafting. Statistical comparisons: * vs ‡, $P < 0.001$; § vs ‖, $P < 0.01$.

anti-CD154 mAb, we assayed for their production by spleen and lymph node cells in MLRs. In these experiments, responder cells were obtained from mice 7 d after skin grafting.

As expected, production of IFN-γ by cells from euthymic C57BL/6 mice given only BALB/c skin allografts was high (> 2,000 pg/ml) in the presence of BALB/c stimulator cells. In contrast, its production by cells from mice given DST plus anti-CD154 mAb in addition to the skin allograft was low to undetectable (< 18 pg/ml).

Similarly, production of IFN-γ by cells from thymectomized C57BL/6 mice given only BALB/c skin allografts was also high, and its production by cells from thymectomized mice given DST plus anti-CD154 mAb in addition to the allograft was low to undetectable. This result was obtained when either BALB/c or third party (C3H/HeJ) cells were used as stimulators. Interestingly, production of IFN-γ by cells from thymectomized, allografted mice given anti-CD154 mAb alone was also low to undetectable in the presence of either BALB/c or C3H/HeJ stimulator cells.

Production of IL-4 by cells obtained from each group of thymectomized mice was low under all experimental conditions. The highest value was 180 U/ml but most values were less than background (< 61 U/ml). Production of both IFN-γ and IL-4 by cells from thymectomized mice that received no treatment and no graft was minimal to undetectable.

Detection of cytokines in skin allografts by PCR

Skin specimens from normal, untreated BALB/c mice and from euthymic C57BL/6 mice bearing BALB/c allografts were assayed for the presence of T cell receptor Cβ (TCRCβ), IL-12 receptor β2 (IL-12Rβ2), IL-4, and IFN-γ mRNA by RT-PCR assay. Allograft skin specimens were obtained 7 d after grafting onto mice that had received either DST plus anti-CD154 mAb or no treatment. TCRCβ and IL-12Rβ2 mRNA, but neither IL-4 nor IFN-γ mRNA could be detected in normal BALB/c skin (data not shown). In contrast, high levels of IFN-γ, TCRCβ, and IL-12Rβ2 mRNAs but no IL-4 mRNA could be detected in skin allograft specimens from both untreated and DST plus anti-CD154 mAb treated euthymic recipients (data not shown).

It was possible that the IFN-γ mRNA observed in the allografts obtained from DST plus anti-CD154 mAb-treated eu-

thymic recipients was present in T cells that had recently emigrated from the thymus. To exclude this possibility, we tested skin allografts obtained from three groups of thymectomized recipients 7 d after graft placement. The first group of C57BL/6 recipients received DST and anti-CD154 mAb in conformity with our standard protocol; the second group received the anti-CD154 mAb component alone; the third group was untreated.

In contrast to the results obtained using euthymic recipient mice, little to no IFN-γ mRNA was detectable in grafted skin from thymectomized recipients that had been given both anti-CD154 mAb and DST ($n = 6$, Fig. 2). IFN-γ mRNA was, however, detected in grafted skin from both the untreated group ($n = 6$) and the anti-CD154 mAb-only ($n = 3$) groups. TCRCβ and IL-12Rβ2 mRNAs but no IL-4 mRNA could be detected in skin allograft specimens from any group.

No evidence of lymphohemopoietic chimerism in thymectomized C57BL/6 mice given BALB/c DST, anti-CD154 mAb, and BALB/c skin allografts

It has been hypothesized that the maintenance of allograft survival requires, or is facilitated by, the presence of donor-origin lymphohemopoietic cells in graft recipients (30). We used a PCR assay to determine if lymphohemopoietic chimerism were present in C57BL/6 mice that had been given BALB/c DST, anti-CD154 mAb, and BALB/c skin allografts. The PCR assay detects donor MHC (H2-K^d) class I DNA and has been documented to be capable of detecting BALB/c DNA in 1:10^4 mixtures of BALB/c and C57BL/6 spleen cells (3). No BALB/c DNA was detected in spleen, lymph nodes (axillary, brachial, and inguinal), liver, or lung taken from any of six thymectomized C57BL/6 mice treated with BALB/c DST and anti-CD154 mAb, and that had borne BALB/c skin allografts for > 100 d (data not shown).

Discussion

We report five principal findings. (*a*) Thymectomy prevents late rejection of successful skin allografts in mice treated with DST and anti-CD154 mAb. (*b*) The induction of tolerance to skin allografts by treatment with DST and anti-CD154 mAb depends on the presence of IFN-γ, CTLA4, and CD4$^+$ and (to
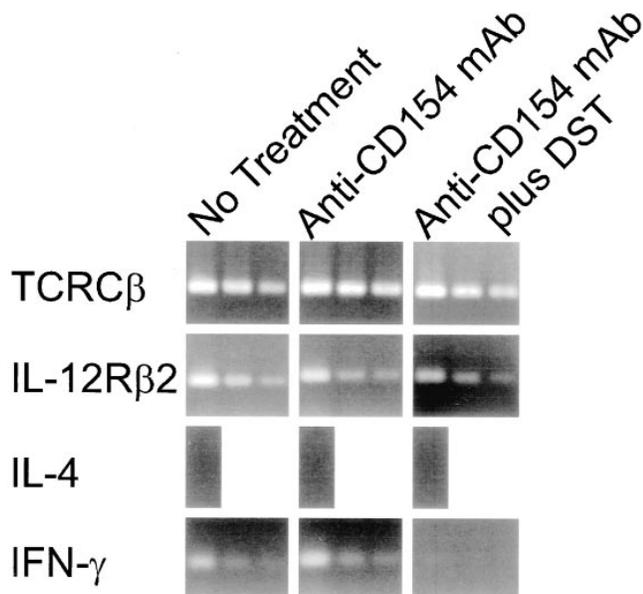
Figure 2. Expression of cytokine mRNA in skin grafts. Cytokine mRNA expression in BALB/c skin allografts removed from thymectomized C57BL/6 mice 7 d after graft placement. Recipient mice had received in addition to the graft either no treatment (*left*), anti-CD154 mAb alone (*center*), or both DST and anti-CD154 mAb (*right*). After reverse transcription, 4 µl of cDNA product were serially diluted threefold, and amplified. Aliquots of 20 µl of PCR products were electrophoresed on 1.5% agarose gels and then stained with ethidium bromide. All PCR products are of the expected size. Each column shows results for each dilution from a single representative skin graft from the indicated group. A total of six grafts in the no treatment group, six from the DST plus anti-CD154 mAb group, and three from the anti-CD154 mAb alone group were analyzed for each cytokine, and in all cases comparable results were obtained.

a lesser extent) CD8^{+} T cells. (*c*) Long-term maintenance of skin allografts requires the presence of CD4^{+} T cells. (*d*) Treatment of thymectomized skin allograft recipients with both DST and anti-CD154 mAb (but not with mAb alone) is associated with absence of IFN-γ in the graft 7 d after surgery. (*e*) Finally, the protocol appears not to lead to a state of microchimerism.

We hypothesize that long-term allograft survival in protocol-treated mice involves DST-induced activation of allorsponsive T cells, possibly leading to deletion and/or anergy of Th1 alloreactive cells. Our data also suggest that induction of graft survival involves CD4^{+} T cell activation, the presence of IFN-γ, and functional availability of CTLA4 receptors. A role for CD4^{+} regulatory cells in allograft responsiveness has been suggested in other experimental models (29, 31–33). Our data do not identify the source of the IFN-γ required for initial graft acceptance. IFN-γ is, however, known to be produced by Th1-type CD4^{+} T cells, CD8^{+} T cells, and natural killer (NK) cells (34). The critical role for CD4^{+} cells in the initial acceptance of skin allografts suggests that Th1-type CD4^{+} cells or CD4^{+} NK T cells could have a regulatory function in the induction of allotolerance. The presence of IFN-γ suggests that the induction of allotolerance by DST and anti-CD154 mAb, is not simply a function of the regulation of Th1-type effector T cell activity by Th2-type cytokines. The absence of IL-4 in

grafts destined for long-term survival, our observation of long-term graft survival in IL-4 knockout mice, and previous reports of enhance graft survival in IL-4 knockout mice (35, 36) are consistent with this view.

Our data also demonstrate that anti-CTLA4 mAb interferes with the induction of graft survival. Interaction of B7 with CTLA4 is hypothesized to lead to down-regulation of activated T cells (37), and it may be speculated that graft survival induced by DST and anti-CD154 mAb requires ligation of CTLA4 receptor on alloreactive effector T cells. Alternatively, CTLA4 ligation may be required to generate regulatory cells. CTLA4 engagement is reportedly required for the induction of unresponsiveness (38).

Our data generated using anti-CTLA4 mAb contrast with the recent report that combination treatment with anti-CD154 mAb and CTLA4-Ig prolongs heart and skin allograft survival (39). CTLA4-Ig is known, however, to block not only the CTLA4 receptor, but also B7-CD28 mediated costimulation (40, 41). CTLA4-Ig blockade of CD28 may circumvent the requirement for CTLA4-dependent down-regulatory activity in the early stages of graft survival documented in the present study. Because the earlier report using combined treatment with anti-CD154 mAb and CTLA4-Ig monitored the survival of skin allografts for only 50 d, it cannot be determined if its tolerizing effect is as durable as that achieved with DST and anti-CD154 mAb.

We have previously shown that treatment of euthymic C57BL/6 mice with a single transfusion of BALB/c donor spleen cells and a brief course of anti-CD154 mAb extends BALB/c skin graft survival to a median of ∼ 50 d (3). Approximately 20% of the allografts in that study were observed to survive for > 100 d, a level of durability that was remarkable given that all tolerance induction procedures were completed within 5 d of transplantation and that no form of chronic immunosuppression was administered.

The late failure of skin allografts in euthymic recipients treated with DST and anti-CD154 mAb in the earlier studies could have been due, in part to, the emergence of new alloreactive thymic emigrants after the conclusion of the treatment. The present thymectomy data support this view, although they do not formally exclude the possibility that the thymus plays some other, as yet unknown, role in the maintenance of peripheral T cell alloreactivity. The addition of thymectomy to our basic short-term protocol enabled us to achieve nearly 100% survival of skin allografts for > 100 d. Several grafts survived for > 330 d. We conclude that the combination of DST and anti-CD154 mAb induces peripheral but not central allograft tolerance.

We can also conclude that the long-term survival of allografts observed in thymectomized recipients treated with DST and anti-CD154 mAb is not due to microchimerism (30, 42–47). Our PCR data suggest that few (< 0.01%) if any lymphohemopoietic cells of BALB/c origin were present in thymectomized C57BL/6 animals given DST and anti-CD154 mAb, and bearing long-term BALB/c skin grafts.

The extension of skin allograft survival by thymectomy could be interpreted to suggest that all peripheral alloreactive T cells undergo deletion or anergy during treatment with DST and anti-CD154 mAb. If this interpretation were correct, adoptively transferred spleen cells from treated animals would not lead to rejection of MHC-identical allografts on *scid* mice. This proved not to be the case. Spleen cells obtained from

thymectomized C57BL/6 mice that had been given DST and anti-CD154 mAb and had borne BALB/c skin allografts for > 100 d were found to be capable of rejecting BALB/c skin grafts on C57BL/6-*scid* recipients. It does remain possible, however, that the process of adoptive transfer could nonspecifically reverse the anergic state.

The suggestion that residual alloreactive cells were present in tolerant mice was further supported by challenge graft experiments. Challenge of thymectomized, graft-bearing recipients led to rejection of both BALB/c and third party challenge grafts (Markees, T.G., unpublished observations). The results of the adoptive transfer and challenge graft experiments suggest that that peripheral tolerance induction by DST and anti-CD154 mAb represents a state of split tolerance (48) that depends on additional processes for its maintenance.

This additional process does not appear to be infectious tolerance, as it cannot be transferred to adoptive recipients (31). Our observation of long-term skin allograft survival in thymectomized recipients also contrasts with recent observations made in a swine model of transplantation (49). In that model, allotolerance to kidneys was induced by a short course of cyclosporin A, but only in euthymic recipients. It was postulated that thymic emigrants with regulatory ability prevented rejection (49). In this model, however, donors and recipients were matched for MHC class II and mismatched only for MHC class I and minor antigens. Our data indicate that CD4+ regulatory cells are required for maintenance of graft survival, and we suggest that this regulatory function in recipients mismatched at both class I and class II can be overwhelmed by the activity of alloreactive thymic emigrants.

We hypothesize that the maintenance of allografts in animals treated with DST and anti-CD154 mAb is due to a deficiency of intragraft Th1-type cytokines. Our study of skin grafts in thymectomized animals treated with DST and anti-CD154 mAb demonstrated the total, or near total, absence of IFN-γ message by postoperative day 7. In contrast, IFN-γ mRNA was detected in grafts placed on mice treated with anti-CD154 mAb alone; our data show that such grafts are destined for rapid rejection. The possibility that the absence of IFN-γ is due to immune deviation is, however, weakened by our observation of IL-12Rβ2 mRNA in grafts destined for long-term survival. IL-12Rβ2 expression stops when CD4+ T cells commit to the Th2-type pathway, and the expression of IL-12Rβ2 mRNA has been associated with the absence of IL-4 production (50). The absence of IFN-γ suggests, however, that the cells expressing IL-12Rβ2 mRNA are anergic.

Parenthetically, it is of interest to note that the Th1-type cytokine IFN-γ appears to play a dual role in allotolerance induction by DST and anti-CD154 mAb. IFN-γ is required for the induction of tolerance, but it is absent in grafts that are destined for long-term survival. These observations are consistent with reports that IFN-γ can both suppress and activate T cells (51, 52). This could represent an example of the compartmentalization of the immune response as a function of expression of IFN-γ in the graft (activation) vs regional lymph nodes (suppression).

Our data show that (*a*) thymectomy is associated with very long-term skin graft survival in animals treated with DST and anti-CD154 mAb, and (*b*) that CD4+ but not CD8+ T cells are required for long-term maintenance of skin allografts. The importance of CD4+ T cells is consistent with previous studies (29, 31–33). These observations suggest that the fate of long-

term skin allografts is a function of the relative balance between tolerized CD4+ cells with regulatory activity and alloreactive T cells in peripheral tissues. This interpretation is supported by our observation of a high frequency of late (> 50 d) skin allograft rejection in euthymic mice treated with DST and anti-CD154 mAb. We favor the hypothesis of Sprent et al. (48) that, during the induction of tolerance, high affinity alloreactive T cells are deleted or anergized. As a corollary, it could be speculated that residual low affinity alloreactive T cells are polarized towards a Th2-type cell, and that graft failure is ultimately due to the release of new populations of high affinity alloreactive T cells. If this last speculation is correct, it would imply that not only thymectomy, but any procedure capable of deleting or anergizing such cells would prolong graft survival.

It can be argued that anti-CD154 mAb is an immunosuppressive agent. Anti-CD154 mAb can prolong the survival of mouse pancreatic islet allografts (1). In the present study, however, treatment of thymectomized mice with anti-CD154 mAb alone proved incapable of prolonging allograft survival as would be expected of a conventional immunosuppressive pharmaceutical.

Induction of unresponsiveness to skin allografts is a noteworthy achievement (53, 54), given the presence of large numbers of Langerhans cells in this tissue (55). Until recently, prolonged skin graft survival has typically required extraordinary interventions such as the use of neonatal recipients (56), radiation (57, 58), or other forms of generalized immunosuppression (31, 59, 60). We have now achieved long-term (> 100 d) survival of nearly all fully allogeneic skin grafts that are placed on adult thymectomized mice treated with a donor-specific transfusion and a short-term course of anti-CD154 mAb. The result suggests that modifications of our short-term tolerance-induction protocol, originally developed for the transplantation of pancreatic islets (1), may yield a clinically applicable procedure for the induction of durable allotolerance without prolonged immunosuppression.

Acknowledgments

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