Skin Allograft Maintenance in a New Synchimeric Model System of Tolerance

Neal N. Iwakoshi, Thomas G. Markees, Nicole Turgeon, Thomas Thornley, Amy Cuthbert, Jean Leif, Nancy E. Phillips, John P. Mordés, Dale L. Greiner and Aldo A. Rossini

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Skin Allograft Maintenance in a New Synchimeric Model System of Tolerance

Neal N. Iwakoshi, Thomas C. Markees, Nicole Turgeon, Thomas Thornley, Amy Cuthbert, Jean Leif, Nancy E. Phillips, John P. Mordes, Dale L. Greiner, and Aldo A. Rossini

Treatment of mice with a single donor-specific transfusion plus a brief course of anti-CD154 mAb uniformly induces donor-specific transplantation tolerance characterized by the deletion of alloreactive CD8⁺ T cells. Survival of islet allografts in treated mice is permanent, but skin grafts eventually fail unless recipients are thymectomized. To analyze the mechanisms underlying tolerance induction, maintenance, and failure in euthymic mice we created a new analytical system based on allo-TCR-transgenic hemopoietic chimeric graft recipients. Chimeras were CBA (H-2b) mice engrafted with small numbers of syngeneic TCR-transgenic KB5 bone marrow cells. These mice subsequently circulated a self-renewing trace population of anti-H-2b alloreactive CD8⁺ T cells maturing in a normal microenvironment. With this system, we studied the maintenance of H-2b allografts in tolerated mice. We documented that alloreactive CD8⁺ T cells deleted during tolerance induction slowly returned toward pretreatment levels. Skin allograft rejection in this system occurred in the context of increasing numbers of alloreactive CD8⁺ T cells; 2) a decline in anti-CD154 mAb concentration to levels too low to inhibit costimulatory functions; and 3) activation of the alloreactive CD8⁺ T cells during graft rejection following deliberate depletion of regulatory CD4⁺ T cells. Rejection of healed-in allografts in tolerated mice appears to be a dynamic process dependent on the level of residual costimulation blockade, CD4⁺ regulatory cells, and activated alloreactive CD8⁺ thymic emigrants that have repopulated the periphery after tolerization. The Journal of Immunology, 2001, 167: 6623–6630.

The CD40-CD154 interaction is a major costimulatory pathway involved in T cell activation (1, 2). Blockade of this pathway with mAb specific for CD154 greatly prolongs the survival of allografts in several species. In mice, it prolongs islet (3–5) and cardiac (6) allograft survival. In rats, it prolongs survival of islet allografts in autoimmune diabetic recipients (7). In monkeys, it prolongs survival of islet (8), skin (9), and kidney (10) allografts. When applied to stem cell transplantation, anti-CD154 mAb treatment in combination with sublethal conditioning permits the generation of allogeneic hemopoietic chimeraism and permanent transplantation tolerance (11–14).

Combined therapy consisting of anti-CD154 mAb plus a single donor-specific transfusion (DST) in mice is even more effective than anti-CD154 mAb monotherapy in prolonging the survival of islet, skin, and heart allografts (5, 6, 15–19). The mechanism by which DST enhances graft survival appears in part to involve the deletion of recipient CD8⁺ alloreactive T cells (15). Combined therapy consisting of anti-CD154 mAb plus CTLA4-Ig also leads to deletion of alloreactive CD8⁺ T cells and prolonged allograft survival (20–22).

Most skin allografts placed on mice treated with any of these protocols are eventually rejected (23) unless recipients are thymectomized (18). Skin allografts can actually survive indefinitely on thymectomized mice treated with DST and anti-CD154 mAb (18, 24). These observations have led us to hypothesize that allograft rejection in tolerated mice is due to the emergence of alloreactive thymic emigrants in a milieu in which declining levels of anti-CD154 mAb preclude the blockade of costimulation (5, 18). In support of this hypothesis, we (25) and others (26, 27) have shown that hemopoietic stem cell reconstitution of mice with successful intact allografts will lead to the rejection of these grafts in the absence of surgical trauma or other forms of activation, but direct evidence that this is due to newly developed T cells is lacking.

To test this hypothesis more directly and to analyze the underlying mechanisms in detail, we established a new analytical system based on allo-TCR-transgenic hemopoietic chimeric graft recipients. Chimeras were normal CBA (H-2b) mice that were irradiated and given small numbers of syngeneic TCR-transgenic KB5 bone marrow cells. These mice subsequently circulated a self-renewing trace population of anti-H-2b alloreactive CD8⁺ T cells that matured in a normal microenvironment. With this system, we studied the immune response to H-2b allografts in tolerated mice. We documented that alloreactive CD8⁺ T cells were deleted during tolerance induction, but slowly returned toward pretreatment levels in euthymic mice. Skin allograft rejection in this system occurred in the context of increasing numbers of alloreactive CD8⁺ cells.
Using this new model system, we also tested the hypotheses that 1) declining concentrations of anti-CD154 mAb in the circulation, 2) deletion of CD4⁺ regulatory cells, and 3) activation of alloreactive CD8⁺ cells would correlate with eventual graft rejection. Each of these hypotheses was confirmed.

Materials and Methods

Animals

CBA/JCr (H-2b), C57BL/6 (H-2b), and BALB/c (H-2b) mice were obtained from the National Cancer Institute (Frederick, MD). (C57 × CBA/J)CrF₁, TCR-transgenic mice were obtained from a colony maintained in our facility (28). The founders were the generous gift of Dr. J. Iacomini (Harvard Medical School, Boston, MA), who obtained the mouse from the original developer, Dr. A. Mellor (Medical College of Georgia, Augusta, GA). The TCR transgene is expressed in CBA (H-2b) mice by CD8⁺ cells, and the transgenic TCR has specificity for native H-2K⁺ (29).

All animals were certified to be free of Sendai virus, pneumonia virus of mice, murine hepatitis virus, minute virus of mice, ectromelia, lactate dehydrogenase-elevating virus, mouse poliovirus (G7), Reo-3 virus, mouse adenovirus, lymphocytic choriomeningitis virus, polyoma, Mycoplasma pulmonis, and encephalitozoon cuniculi. All animals were housed in microisolator cages, given ad libitum access to autoclaved food, and maintained in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996) and the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Generation of KB5 TCR-transgenic hemopoietic CBA chimeras

To examine the fate of both mature and developing alloreactive CD8⁺ T cells in a normal microenvironment, we used TCR-transgenic hemopoietic chimeras (30) generated by injecting small numbers of bone marrow cells from KB5-transgenic donors into sublethally irradiated syngeneic CBA-nontransgenic hosts. We refer to these as syngeneic mice. Marrow donors were male and female (KB5 × CBA/JCrF₁ mice, 8–12 wk of age). Donor femurs and tibias were flushed with RPMI 1640 medium using a syringe with a 24-gauge needle. Recovered cells were filtered through sterile nylon mesh (70 μm; BD Biosciences, Franklin Lakes, NJ), counted, and resuspended in RPMI 1640. Recipient mice were male CBA/JCrF₁ mice 4–7 wk of age. Recipient mice were treated with whole body gamma irradiation using a 137Cs source (GammaCell 40; Atomic Energy of Canada, Ottawa, Ontario, Canada). They were then given a single i.v. injection of KB5 bone marrow cells in a volume of 0.5–1 ml via the lateral tail vein within 2–5 h of irradiation. The transgenic T cells that develop from KB5 bone marrow express an anti-H-2K⁺-specific TCR that is recognized by the clonotypic mAb DES (29). In preliminary dosing experiments, it was determined that 2 Gy radiation and 0.5 × 10⁶ KB5 bone marrow cells were optimal for the generation of hemopoietic chimeras circulating DES⁺ CD8⁺ T cells (see Results), and these parameters were used in all experiments reported in this work.

Flow microfluorometry

A mouse hybridoma cell line secreting the KB5-specific clonotypic DES Ab (29) was a gift from Dr. J. Iacomini (Harvard Medical School). FITC-conjugated anti-mouse IgG2a developing reagent for DES (clone R19-15), PerCP-conjugated and CyChrome-conjugated anti-mouse CD8α mAbs (both clone 53-6-7), and PE-conjugated anti-mouse mAb directed against the transferrin receptor (clone 2FL2) (Mel-14) were obtained from BD PharMingen (San Diego, CA). Isotype control mAbs including the PE-conjugated rat IgG2b κ (clone A95-1) for CD62L, rat PerCP-conjugated and CyChrome-conjugated IgG2a κ (clone R35-95) for CD8, and mouse IgG2a κ anti-tryptophanophenol (clone G155-178) for DES were purchased from BD PharMingen.

The use of three-color flow microfluorometry analyses of lymph node and spleen cells were performed, as previously described (15). Briefly, 1 × 10⁶ viable cells were reacted with anti-DES Ab for 20 min at 4°C. Cells were then washed and reacted with FITC-conjugated anti-mouse IgG2a mAb (to develop the DES Ab) plus a mixture of conjugated mAbs for 20 min. Whole blood was processed using FACS lysing solution (BD Biosciences) in accordance with the protocol supplied by the manufacturer. Labeled cells were washed, fixed with 1% paraformaldehyde-PBS, and analyzed using a FACSscan instrument (BD Biosciences). Lymphoid cells were gated according to their light-scattering properties, and 30–50 × 10⁶ events were acquired for each analysis.

Transplantation procedures

Male CBA/JCr and KB5 synchromic CBA/JCr mice were treated with DST and anti-CD154 mAb and transplanted with skin allografts, as described elsewhere (15, 18, 19). Briefly, the DST consisted of 10⁴ C57BL/6 splenocytes from 5–10-wk-old female donors injected i.v. in a volume of 0.5 ml. DST was given on day −7 relative to skin transplantation. MR1 hemopoietic anti-mouse CD154 mAb was produced as ascites in SCID mice and purified by affinity chromatography (31, 32). Ab concentration was determined by measurement of OD and confirmed by ELISA (see below). The concentration of contaminating endotoxin was determined commercially (Charles River Endosafe, Charleston, SC) and was uniformly <10 U/ml mAb (31). Mice were injected i.p. with anti-CD154 mAb (0.5 mg/ dose) on days −7, −4, 0, and +4 relative to skin transplantation. Full-thickness skin grafts (5 × 5 cm) were obtained from the flank of donor mice and transplanted onto the dorsal flanks of recipients, as described previously (18). Graft rejection was defined as the first day on which the entire graft was necrotic (18, 19).

The KB5 synchromics received skin allografts when 11–17 wk of age, 7–12 wk after irradiation and bone marrow injection, to establish hemopoietic chimeras. Wild-type CBA/JCr mice received skin grafts when 6–14 wk of age.

Concentration of hamster anti-mouse CD154 mAb

To measure the concentration of anti-CD154 mAb in serum and in preparations of purified mAb, we developed an ELISA specific for hamster IgG. Briefly, high protein-binding affinity 96-well ELISA plates (Costar, Cambridge, MA) were incubated overnight at 4°C with affinity-purified goat anti-hamster Ig Ab (Boehringer Mannheim, Indianapolis, IN) in 0.1 M NaH₂PO₄ (pH 9), washed, and blocked with 0.1% BSA in PBS. Plates were washed with PBS containing 0.05% Tween 20 (PBS-Tween). Samples and standards prepared in our laboratory were diluted as required in PBS-Tween, incubated overnight at 4°C, washed with PBS-Tween, and incubated with excess biotinylated goat anti-hamster IgG Ab (Vector Laboratories, Burlingame, CA), avidin-HRP conjugate (Vector Laboratories), and the peroxidase substrate o-phenylene diamine (OPD; Sigma, St. Louis, MO). Plates were washed between incubations with PBS-Tween. Before the addition of OPD, the plates were washed in PBS alone. OPD (final concentration 1 mg/ml) and H₂O₂ (0.003%) were added to sterile substrate buffer (0.05 M NaH₂PO₄, 0.025 M sodium citrate, pH 5) just before use. The reaction was stopped by addition of 3 M HCl, and absorbance was read at 490 nm using a plate-reading spectrophotometer (Bio-Rad model 3550; Bio-Rad, Hercules, CA).

Concentration of mouse anti-hamster Ig

To measure the serum concentration of mouse anti-hamster Ig Ab produced in response to the injection of anti-CD154 mAb, 96-well ELISA plates (Costar) were coated with 50 μl of a 5 μg/ml solution of MR1 Ab in PBS. Plates were washed and blocked as described above. A standard mouse serum was produced by immunization of a pool of C57BL/6 mice with hamster Ig (20 μg; Jackson ImmunoResearch Laboratories, West Grove, Pa) in incomplete Freund’s adjuvant. After 2 wk, mice were bled, and serum was recovered. Serum was recovered 7 days later, divided into aliquots, and stored at −70°C until used. This standard positive control serum was assigned an arbitrary value of 100,000 U/ml anti-hamster IgG-binding activity. MR1 hamster Ig-coated plates were incubated with dilutions of the standard serum and serum from experimental animals. After overnight incubation, bound mouse Ab was detected with affinity-purified biotinylated goat anti-mouse IgG (Vector Laboratories), avidin-HRP, and OPD, as described above.

Hemagglutination titers in mice immunized with SRBC

Anti-CD154 mAb prevents T-dependent Ab responses both in vivo and in vitro (1, 2, 33). To determine the serum concentration of anti-CD154 mAb required to achieve this effect, we used an SRBC hemagglutination assay. C57BL/6 mice were given a single i.v. injection of 0.2 ml of 1% SRBC (Colorado Serum, Denver, CO). Immediately thereafter, the mice were randomized into six groups. One group received no further treatment; the others were treated with a single injection of anti-CD154 mAb at five dosage levels that ranged from 0.016 to 0.5 mg. Serum samples were collected from control and experimental mice immediately before and 2, 7, and 14 days after treatment. SRBC hemagglutination titers were assayed as described previously (34). Briefly, serial 2-fold dilutions of heat-inactivated mouse sera were placed in 96-well round-bottom plates (50 μl/well). Each assay included dilutions of serum obtained from control mice that received SRBC only and control wells containing PBS alone. To each well was added 50 μl of 1% SRBC in PBS (washed packed cells/total volume).
The plates were covered and the cells were allowed to settle undisturbed overnight at room temperature. The SRBC hemagglutination titer was defined as the highest dilution that produced visible agglutination.

**Statistics**

Average duration of graft survival is presented as the median. Graft survival among groups was compared using the method of Kaplan and Meier (35). The equality of allograft survival distributions for animals in different treatment groups was tested using the log rank statistic (35). The \( p \) values <0.05 were considered to be statistically significant. Comparisons of two means used unpaired \( t \) tests (36). Regression curves were fitted using SigmaPlot software (version 6.0; SPSS, Chicago, IL).

**Results**

**Persistence of DES\(^+\)CD8\(^-\) cells in CBA synchimeras**

To generate KB5 TCR-transgenic hemopoietic chimeras, we first performed preliminary experiments to determine appropriate doses of radiation and bone marrow. These revealed that the combination of 2 Gy radiation and \( 0.5 \times 10^7 \) KB5 bone marrow cells generated chimeras that circulated 2–8% DES\(^+\)CD8\(^-\)-transgenic T cells in the peripheral blood within 6–8 wk (data not shown). These parameters were used in all subsequent experiments. Overall, ~90% of mice treated when 4–7 wk of age became chimeric, defined as the presence of \( \geq 2\% \) DES\(^+\)CD8\(^-\)-transgenic T cells 8 wk after bone marrow injection.

We then determined the stability of the chimeric state in CBA recipients that were not otherwise manipulated. As shown in Fig. 1A, the percentage of DES\(^+\)CD8\(^-\)-transgenic T cells was similar in all mice and remained quite stable between 50 and 197 days after bone marrow injection. The data were fitted by linear regression, and the slope of the regression line is statistically indistinguishable from 0, indicating that the average number of circulating DES\(^+\)CD8\(^-\) cells is statistically less than that in the untreated, ungrafted controls (A), the percentage of DES\(^+\)CD8\(^-\) cells in CBA synchimeras thereafter rose slowly, and on day 140 at the end of the experiment was 5.4 \( \pm \) 3.2%. At the time points marked by *, the percentage of DES\(^+\)CD8\(^-\) cells is statistically less than that in the untreated, ungrafted controls (A). The number of mice tested is given in parentheses. One mouse with an intact graft was removed from the study for use in another experiment. The dotted line is the same as the solid regression line in A.

**FIGURE 1.** Percentage of DES\(^+\)CD8\(^-\) PBMC in synchimeric mice. Male CBA mice 4–7 wk of age were irradiated and injected with syngeneic KB5 bone marrow, as described in Materials and Methods. On days 50–84 after bone marrow injection, the mice were randomized into four groups. Group 1 received no further treatment (A). Groups 2–4 all received a C57BL/6 skin allograft 57 days after bone marrow injection (day 0). Mice in group 2 (B) received no other treatment. Mice in group 3 (C) received four doses of MR1 anti-CD154 mAb beginning on day −7 relative to skin transplantation. Mice in group 4 (D) received a single DST on day −7, followed immediately by the first of four injections of anti-CD154 mAb. The percentage of DES\(^+\)CD8\(^-\) PBMC was measured through day 197 after the injection of bone marrow (day 140 after skin grafting). The upper \( x \)-axes show time relative to bone marrow injection; the lower \( x \)-axes show time relative to skin transplantation. A–C, each data point gives the result from an individual mouse, and the solid lines were fitted by linear regression. A, The percentage of DES\(^+\)CD8\(^-\) PBMC in untreated control chimeras was nearly constant (\( y = 7.46 - 0.001 \cdot x, p = \text{NS} \)). One mouse died on day 155. The lower \( x \)-axis shows time relative to skin transplantation, which was performed on the experimental mice in B–D. B, The percentage in otherwise untreated chimeras that received skin allografts was also nearly constant (\( y = 7.5 + 0.004 \cdot x, p = \text{NS} \)). C, The average percentage in skin graft recipients treated with anti-CD154 mAb alone tended to rise slightly after grafting (\( y = 6.4 + 0.008 \cdot x, p < 0.05 \)). D, Mice in this group represent a pool of three cohorts that received skin grafts 50–84 days after bone marrow injection. The average percentage in chimeric mice that received a skin allograft plus anti-CD154 mAb plus a DST fell nearly to 0 (0.65 ± 0.4%) between day −7 and day 0, the day of skin graft transplantation. The percentage of DES\(^+\)CD8\(^-\)-transgenic T cells thereafter rose slowly, and on day 140 at the end of the experiment was 5.4 ± 3.2%. At the time points marked by *, the percentage of DES\(^+\)CD8\(^-\) cells is statistically less than that in the untreated, ungrafted controls (A). The number of mice tested is given in parentheses. One mouse with an intact graft was removed from the study for use in another experiment. The dotted line is the same as the solid regression line in A.
Treatment of KB5 TCR-transgenic hemopoietic CBA chimeras with DST and anti-CD154 mAb results in near disappearance, followed by slow recovery of peripheral blood DES⁺ CD8⁺ T cells

We first demonstrated that the KB5 TCR-transgenic hemopoietic CBA chimeras respond to treatment with DST plus anti-CD154 mAb by reducing existing DES⁺ CD8⁺ alloreactive T cells in peripheral blood. Chimeras received a single transfusion of C57BL/6 spleen cells, a 2-wk course of anti-CD154 mAb, and a C57BL/6 skin allograft, as described in Materials and Methods. As expected (15), the percentage of circulating DES⁺ CD8⁺ alloreactive T cells fell dramatically early in the course of treatment (Fig. 1D). The percentage before treatment was 5.8 ± 0.9%; this fell to 0.6 ± 0.4% (range, 0.2–1.4%) on the day of skin grafting. In contrast, levels of DES⁺ CD8⁺ T cells remained essentially unchanged in untreated chimeras given skin allografts (Fig. 1B) and in chimeras given skin grafts and anti-CD154 mAb monotherapy (Fig. 1C).

In mice treated with DST and anti-CD154 mAb, the percentage of transgenic alloreactive DES⁺ CD8⁺ T cells appeared to recover slowly over time (Fig. 1D). Compared with the untreated control mice (Fig. 1A), levels of peripheral blood DES⁺ CD8⁺ T cells remained statistically significantly lower in tolerized mice immediately after the start of treatment and through 98 days (p < 0.001 at each time point). After day 98, levels of DES⁺ CD8⁺ T cells remained somewhat lower than, but statistically similar to, levels in untreated, grafted controls.

Skin allografts in KB5 hemopoietic CBA chimeras tolerized with DST and anti-CD154 mAb survive uniformly until shortly after levels of DES⁺ CD8⁺ T cells begin to recover

The median survival time (MST) of skin allografts on KB5 hemopoietic chimeras that were otherwise untreated (13 days, n = 7) or treated only with a brief course of anti-CD154 mAb monotherapy (15 days, n = 7) was uniformly brief (Fig. 2A). In contrast, MST of skin allografts in KB5 hemopoietic chimeras that were treated with DST plus anti-CD154 mAb was substantially prolonged (169 days, n = 12, p < 0.001). Nearly half of the grafts survived for 200 days.

To determine whether the procedures used to create the chimeric mice affected graft survival, wild-type CBA mice were transplanted with C57BL/6 skin allografts. As was true for the KB5 chimeric recipients, the MST of skin allografts on CBA mice that were otherwise untreated (14 days, n = 25) or treated with anti-CD154 mAb monotherapy (17 days, n = 10) was uniformly brief (Fig. 2B). Survival of skin allografts on wild-type CBA mice that were treated with DST plus anti-CD154 mAb was substantially prolonged (125 days, n = 31, p < 0.001) in comparison to CBA controls, and was statistically similar to that of the KB5 chimeric recipients treated in the same way (169 days, p = NS; Fig. 2A).

Inspection of Fig. 2 reveals that the rejection of skin allografts in mice treated with DST and anti-CD154 mAb tended to occur largely between ~100 and 150 days after transplantation. Comparison with Fig. 1D reveals that this time frame coincides with the time period during which levels of alloreactive DES⁺ CD8⁺ T cells in treated mice began to approach, and were statistically indistinguishable from, pretransplant levels.

The reappearance of DES⁺ CD8⁺ T cells in tolerized mice could result from the expansion of DES⁺ CD8⁺ T cells that had escaped deletion or from colonization of the periphery by new thymic emigrants. As a first step toward analyzing these possibilities, thymocytes were obtained from a separate cohort of otherwise untreated KB5 TCR-transgenic mice 79 days after bone marrow injection and analyzed for the presence of DES⁺ cells with an immature, i.e., CD4⁻CD8⁻, phenotype. We observed that of total CD4⁻CD8⁻ thymocytes, 2.3 ± 1.9% were also DES⁺ (n = 5).
Depletion of CD4+ T cells is associated with activation of allograft rejection in tolerant mice

Depletion of CD4+ T cells is known to cause rapid rejection of successful healed-in skin allografts in thymectomized mice treated with DST and anti-CD154 mAb (18). We tested the hypothesis that depletion of CD4+ T cells in graft-bearing animals leads to activation of allograft rejection. KB5 hemopoietic CBA chimeras were treated with DST and anti-CD154 mAb and given a C57BL/6 skin allograft. A cohort of seven mice bearing healed-in skin allografts were treated with DST and anti-CD154 mAb and given an additional dose of anti-CD4 mAb (0.5 mg/dose). Consistent with our previous report (18), all treated mice rejected their skin grafts within 40 days (Fig. 2A). In contrast, only 12 (8%) of control mice not given anti-CD4 mAb rejected its graft during that same interval (Fig. 2A). In contrast, only 12 (8%) of control mice rejected its graft during that same interval (Fig. 2A). In contrast, only 12 (8%) of control mice not given anti-CD4 mAb rejected its graft during that same interval (Fig. 2A). In contrast, only 12 (8%) of control mice not given anti-CD4 mAb rejected its graft during that same interval (Fig. 2A).

Graft rejection occurs when residual concentrations of anti-CD154 mAb have declined to levels too low to prevent T-dependent Ab responses

We next assessed the role of anti-CD154 mAb concentration in the mechanism of allograft survival in mice treated with our standard protocol. To do so, we performed several analyses of Ab concentration and correlation with two functional assays of costimulatory blockade and with skin graft survival. We tested the hypothesis that graft rejection occurs when residual concentrations of anti-CD154 mAb are too low to prevent costimulation and the generation of T-dependent Ab responses. As shown in Fig. 2A, all treated mice rejected their skin grafts within 40 days (Fig. 2A). In contrast, only 12 (8%) of control mice not given anti-CD4 mAb rejected its graft during that same interval (Fig. 2A).

Normal C57BL/6 mice were selected at random and either left untreated or given a single i.p. injection of the MR1 hamster anti-mouse CD154 mAb at concentrations ranging from 0.016 to 0.5 mg. All mice also received a single injection of SRBC, as described in Materials and Methods. The concentration of hamster Ig was measured before and 2, 7, and 14 days after injection. As shown in Fig. 3A, the concentration of Ab from the peritoneal space occurred slowly and was maximal by day 7.

As a functional index of costimulatory blockade, SRBC hemagglutination titers were measured in the same mice at the same time points. As shown in Fig. 3B, the titer in control mice that did not receive anti-CD154 mAb became positive and maximal by day 7; the titer was 1:1280 in each of the three tested mice. Increasing doses of anti-CD154 mAb were associated with decreasing hemagglutination titers. The three mice treated with a single injection of 0.5 mg anti-CD154 mAb generated only weakly positive titers. Comparison of the data in Fig. 3A and B, suggests that substantial inhibition of anti-SRBC Ab production required the presence of less than 0.01 mg/ml or more than 0.5 mg/ml anti-CD154 mAb in the circulation.

As an additional functional index of the inhibition of costimulatory blockade, we also measured the concentration of mouse anti-hamster Ig. Anti-CD154 mAb was detectable when the concentration of anti-CD154 mAb present was greater than ~50 μg/ml.

Taken together, the data in Fig. 3, A—C, suggest that concentrations of MR1 anti-CD154 mAb that are in the range of ~50–300 μg/ml in vivo are required to block costimulation and prevent T-dependent Ab responses.

We next determined the concentration of anti-CD154 mAb in synchimeric mice that were actually treated with DST, four 0.5-mg injections of anti-CD154 mAb, and a skin allograft. AB concentrations were determined serially beginning on day 14 after grafting, which was day 10 after the final injection of Ab. As shown in Fig. 3D, the concentration of Ab at this first time point was ~200 μg/ml and only declined to 50 μg/ml ~50 days after grafting. Comparison with Fig. 2A suggests that concentrations of Ab insufficient to block costimulation occur at about the time skin allografts begin to reject.

Discussion

It is now well documented that various methods of peripheral tolerance induction can lead to prolonged survival of tissue and organ grafts in rodents (5, 37). With few exceptions, however (38, 39), peripheral tolerance induction protocols do not lead to permanent graft survival when translated to primates, reflecting the fact that the mechanisms responsible for allograft maintenance in tolerant recipients are not well understood. Reports of kidney allografts that continue to function long after discontinuation of systemic immunosuppression (40) make it clear, however, that functional transplantation tolerance is achievable in humans. Understanding the mechanisms of tolerance that maintain healed-in allografts in the absence of immunosuppression was the goal of the experiments reported in this work.

In those experiments, we analyzed mechanisms underlying not only the induction, but also the maintenance or failure of peripheral tolerance in euthymic mice treated with DST and anti-CD154 mAb. We have previously documented that treatment with this protocol leads to functional tolerance in TCR αβ T cells and rejection of healed-in skin grafts in tolerant mice.

Table I. Percentage of CD62Llow DES CD8+ T cells

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<th>Treatment</th>
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<tr>
<td></td>
<td>PBMC</td>
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<td>DST + anti-CD154 mAb</td>
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*Percentage of DES CD8+ T cells in PBMC, skin graft-draining lymph node cells, and spleen cells expressing CD62Llow. All mice were taken from among the synchimeric recipients of C57BL/6 skin grafts shown in Fig. 2A, and all samples were obtained on day 85 irrespective of the status of the graft.

*One mouse was killed with an intact graft on day 85 after transplantation to obtain lymph node cells and splenocyte samples.

*p < 0.05 vs DST plus anti-CD154 mAb plus anti-CD4 mAb treatment group. Numbers in parentheses indicate the number of mice tested.
FIGURE 3. Concentration of anti-CD154 mAb over time and in relation to inhibition of T-dependent Ab responses. A–C, C57BL/6 mice were selected at random and either left untreated or given a single i.p. injection of MR1 hamster anti-mouse CD154 mAb at doses of 0.016, 0.032, 0.063, 0.125, 0.25, or 0.5 mg. All mice also received a single injection of SRBC, as described in Materials and Methods. Each mouse was bled before and then 2, 7, and 14 days after treatment. Each data point represents the mean value determined in three mice. A, Mean serum hamster IgG concentration is plotted against time and injected Ab dose. The lower limit of detection in the assay was 5 μg/ml. B, Mean serum hemagglutination titer in the presence of SRBC was measured as described in Materials and Methods and is plotted against time and injected Ab dose. The maximum variability at any data point was a 4-fold dilution; 1/1280 was the maximal dilution assayed. Serum samples were the same as those in A, and comparison of the two data sets suggests that the generation of anti-SRBC Ab was largely inhibited when the concentration of anti-CD154 mAb was in excess of 100 μg/ml. C, Mean serum concentration of mouse anti-hamster IgG in arbitrary units was measured as described in Materials and Methods and is plotted against time and injected Ab dose. The lower limit of detection was 25 U/ml. No Ab was detectable before day 14. Comparison with the data in A indicates that no anti-hamster IgG Ab response was detectable when the concentration of anti-CD154 mAb present was greater than −50 μg/ml. D, A group of seven syngeneic KB5 TCR-transgenic CBA mice was selected at random and transplanted with C57BL/6 skin allografts on day 0. In addition to the grafts, mice received a C57BL/6 transfusion on day −7 and four 0.5-mg doses of MR1 anti-CD154 mAb on days −7, −4, 0, and +4 relative to skin transplantation. Each data point represents the mean hamster IgG concentration ± 1 SD. The solid line shows the data fitted by first-order nonlinear regression (\( y = y_0 + ae^{-kt} \)), as described in Materials and Methods. The fitted equation is: \( y = 0.13 + 331 \times e^{-0.0419t} \) \((p < 0.0001)\) and yields a calculated \( t_{1/2} \) \((t_{1/2} = \ln 2/k)\) of 16.5 days.

protocol reproducibly prolongs skin allograft survival in euthymic mice (5). Tolerization is dependent on the presence of CD4+ cells and the deletion of CD8+ cells (15, 18). Although relatively non-antigenic islets generally survive indefinitely, only ~20% of skin allografts survive >100 days (19). Permanent skin allograft survival in a majority of recipients is achieved only if the graft recipients are thymectomized (18), and even in thymectomized mice deletion of CD4+ cells can induce graft rejection. Given these data, we hypothesized that long-term maintenance of allografts in tolerized recipients is a dynamic process that is dependent on the number and activation state of alloreactive and regulatory cells.

To analyze this dynamic process quantitatively, we determined levels of therapeutic anti-CD154 mAb concentrations and correlated these levels with graft survival and circulating alloreactive CD8+ T cells in a new analytical system based on allo-TCR-transgenic hemopoietic chimeric graft recipients. We developed this model because it permitted the study of a trace population of high-affinity alloreactive T cells that not only circulated (28, 30), but also developed in a normal microenvironment. One might argue that these analyses could have been conducted using TCR-transgenic mice as recipients, but it is important to recall that TCR-transgenic mice do not generate normal immune responses due to severe skewing of their T cell repertoire (28, 30). One might also argue that the standard Jenkins single-transfusion chimera could have been used (30). This system has successfully been used to study the induction of transplantation tolerance (15), but again it should be recalled that standard Jenkins chimeras cannot be used to study the maintenance of tolerance in the context of newly released thymic emigrants. We designed our TCR-transgenic hemopoietic chimera to respond to tolerance induction in a manner similar to that observed in normal mice and to overcome the limitations inherent in both alternative model systems.

To validate our model system, we first documented that control KB5 TCR-transgenic hemopoietic chimeras reject allografts with normal kinetics. More importantly, we then documented that the median survival of skin allografts in KB5 chimeras tolerized with DST and anti-CD154 mAb was similar to that observed in tolerized normal CBA mice. As expected (15), the alloreactive
DES^CD8^+ T cells were deleted in the chimeras during tolerance induction. We also documented that TCR-transgenic T cells developed in the thymus and, over time, were able to repopulate the allograft. This TCR-transgenic hemopoietic chimera model permitted us to examine the relationships among 1) newly released allograft CD8^+ T cells in peripheral tissues, 2) levels of anti-CD154 mAb, and 3) regulation of allograft CD8^+ T cell activation in the presence or absence of CD4^+ cells in tolerized mice bearing healed-in skin allografts. Our findings in this system provide the first direct evidence that 1) allograft CD8^+ T cells reappear in tolerant euthymic mice bearing intact allografts, and 2) graft rejection occurs in the context of increasing numbers of these allograft CD8^+ T cells in the absence of costimulation blockade.

We suggest specifically that the initiation of allograft rejection correlates with a decline in anti-CD154 mAb concentration to levels that are no longer sufficient to prevent costimulation. Concentrations less than 50 μg/ml were insufficient to prevent the generation of T-dependent Ab responses. This observation is of particular interest in light of our data demonstrating that graft rejection is also associated with the activation of CD8^+ allograft cells. We (25) and others (26, 27) have shown that some of the T cells that develop in the thymus of mice bearing a healed-in allograft have not beentolerized to that graft. It has also been reported in nonhuman primates tolerized with anti-CD154 mAb that islet allograft maintenance requires periodic retreatment with the Ab (41). We interpret this set of observations to suggest that maintenance of allografts in tolerized recipients is stable or declines over time. We believe this to be unlikely, however, because the phenotype of most DES^+ cells in tolerized chimeras was that of naive cells rather than activated or memory cells. Only after CD4^+ T cells had been deliberately deleted and skin allografts were undergoing rejection did we detect activated DES^CD8^+ T cells.

Our data demonstrate that graft rejection was accompanied by activation of allograft T cells, but we were unable to correlate percentages of DES^CD8^+CD62Llow peripheral blood cells with impending graft rejection (data not shown). We speculated that the lack of predictive power associated with this phenotype is due to rapid emigration from the blood of activated cells to nonlymphoid tissues (42), presumably including the graft itself. Another alternate explanation for the reappearance of allograft CD8^+ T cells could be the redistribution of DES^+ cells to tissues that we did not analyze; it could be argued that over time such cells then reverted to a naive phenotype (43). If this were the case, however, the DES^CD8^+ T cells that had migrated to the lymphoid compartment from sites of sequestration would again be expected to exhibit an activated phenotype. The present data do not exclude this possibility, but in a preliminary experiment we attempted to detect DES^CD8^+ cells in KB5 hemopoietic chimeras that had been thymectomized just before tolerance induction and deletion of preexisting alloreactive CD8^+ T cells; no DES^CD8^+ cells were detectable for up to 97 days (T. G. Markees, unpublished observations).

Although the majority of high-affinity alloreactive DES^CD8^+ T cells seem to disappear from the circulation within 3 days (15), we are still left to account for the rejection of healed-in grafts in thymectomized recipients treated with anti-CD4 mAb (18). To account for this observation, we would point out that our data document the deletion of a clonal, high-affinity trace cell population, specifically the DES^CD8^+ cells. Allograft rejection, however, represents a polyclonal immune response that also involves lower affinity cells, some of which are likely to escape deletion. Our documentation that the DES^CD8^+ cells are deleted allows us to hypothesize that residual low-affinity alloreactive cells are the agents of graft rejection in thymectomized mice treated with anti-CD4 mAb. In this regard, it will be of interest to determine i

References


