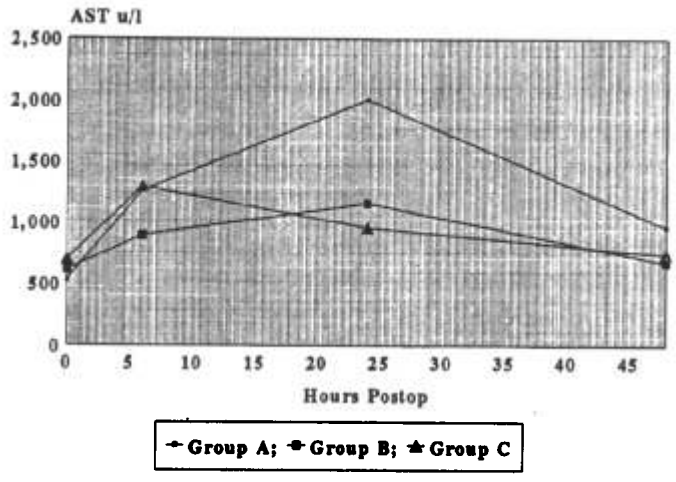

FIGURE 1. Mean AST levels 0, 6 h, 24 h and 48 h postop.

significant differences were found at any time point. The technical failure rate was similar in all 3 experimental groups.

The presented test model can demonstrate the increased injury sustained by fatty livers after transplantation in a reproducible way. The results suggest that PGE_1 treatment is helpful in protecting fatty livers from ischemic injury associated with transplantation. An infusion of PGE_1 into a mesentery vein branch might be preferable in view of the significant first pass clearance of PGE_1 by the lungs. However, this could not be carried out successfully in our rat model. Liver transplantation without arterial anastomosis was attempted initially but yielded highly variable and nonreproducible results. ALT measurements might be preferable theoretically as this enzyme is more liver specific than AST. Again reproducible differences and results were not obtained.

Several studies have shown that PGE_1 treatment can protect organ transplants (liver, heart, kidney) from damage created by warm ischemia and cold storage preservation (*4–6*). Prostaglandins have also been shown to possess an immunosuppressive effect of their own (*7*). The study presented here shows that PGE_1 can, at least in rats, protect livers that are more susceptible to transplant-related ischemia because of fatty metamorphosis. Studies are now underway in our laboratory to test other agents that could potentially protect fatty liver grafts from transplant-related ischemic damage.

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REFERENCES

1. Todo S, Demetris AJ, Makowka L, et al. Primary nonfunction of hepatic allografts with preexisting fatty infiltration. Transplantation 1989; 47: 903.
2. Mor E, Klintmalm GB, Gonwa TA, et al. The use of marginal donors for liver transplantation. A retrospective study of 365 liver donors. Transplantation 1992; 53: 383.
3. Kamada N, Calne RY. A surgical experience with five hundred thirty liver transplants in the rat. Surgery 1983; 93: 64.
4. Crafa F, Gugenheim J, Saint-Paul MC, et al. Protective effects of prostaglandin E_1 on normothermic liver ischemia. Eur Surg Res 1991; 23: 278.
5. Ueno T, Horita K, Okazaki Y, Itoh T, Natsuaki M. The effect of prostaglandin E_1 on heart preservation. Transplantation 1992; 52: 418.
6. Tojimbara S, Ohsaki S, Teraoka S, et al. Effects of prostaglandin E_1 on transplanted kidneys in dogs by laser Doppler blood perfusion monitor. Transplant Proc 1992; 24: 1349.
7. Fabrega AJ, Blanchard J, Rivas PA, Moran SM, Pollak R. Prolongation of rat heart allograft survival using cyclosporine and enisoprost, a prostaglandin E_1 analog. Transplantation 1992; 53: 1363.

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SINGLE AS WELL AS PAIRS OF SYNERGISTIC ANTI-CD4+CD8 ANTIBODIES PREVENT GRAFT-VERSUS-HOST DISEASE IN FULLY MISMATCHED MICE[1]

Marrow engraftment at reduced doses of total body irradiation together with prevention of graft-versus-host disease (GVHD)* in homozygous fully mismatched radiation chimeras had been observed after a single injection of rat IgG2b or mouse IgG2a anti-Thy-1 mAb (*1*). Similar data were obtained after pairs of noncompetitive rat IgG2b antibodies against epitopes on the murine L3T4 (CD4) and Lyt-2 (CD8) T cell antigens had been injected in prospective recipients of marrow and spleen cells from thymectomized donors predepleted by the same synergistic mAb pairs (*2*). In further studies, single or synergistic anti-CD4 and/or anti-CD8 were not compared as to their anti-GVHD effects, but prevention of GVHD in homozygous 2-haplotype-mismatched irradiated mice, such as found for anti-Thy-1, was not reported (*3, 4*). A formal comparison between synergistic and single anti-$CD4^+CD8$ mAb in euthymic radiation chimeras therefore appeared desirable, given the remarkable immunosuppressive effects of synergistic as well as of single anti-CD4 mAb in delaying skin and vascularized organ graft rejection with and without coinjection of donor cell antigen (*5–8*).

Interest in the degree of GVHD suppression that is possible with mAb against the CD4 and –CD8 T cell subsets also lies, of course, in the comparable distribution of the CD4 and CD8 antigens in mice and humans. We therefore generated a number of rat IgG2a and IgG2b mAb and selected single and synergistic IgG2b anti-CD4 (RmCD4–1, RmCD4–2) and anti-CD8 (RmCD8–1, RmCD8–2) mAb for their potent effect on suppression of GVHD in the difficult homozygous 2-haplo-

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* Abbreviations: GVHD, graft-versus-host disease.

type MHC and minor antigens mismatched donor-recipient combination where irradiated mice received bone marrow and up to 5×10^7 spleen cells. Here we show that synergistic mAb were not required, because already single IgG2b anti-CD4 (RmCD4–2) mAb prevented GVHD completely and induced full chimerism and specific skin graft tolerance, but only if combined with anti-CD8 (RmCD8–2) mAb.

In contrast anti-CD4 (RmCD4–4) and anti-CD8 (RmCD8–5) mAb of the rat IgG2a isotype were much less immunosuppressive (Fig. 1). mAb (400 μg) were injected in CBA(H-2^k) mice 4 hr after 600 cGy and 20 hr before transfer of bone marrow (2×10^7) and spleen (5×10^7) cells from C57BL/6(H-2^b) donors. Rat antibodies against CD4 and CD8 were produced (*9*) after immunization with AKR/J thymocytes using either the rat myeloma line Y3/Ag 1.2.3 (*10*) for RmCD8–1 and RmCD8–2 or the mouse myeloma line P3X63 Ag 8–653 (*11*) for the RmCD4–1,-2,-4 and RmCD8–5 mAb. Antibody specificity was defined by FACS and confirmed by intercalation of C1q with published synergistic antibodies. For cross-inhibition with published anti-CD4 and -CD8 antibodies (*12*, *13*), see Table 1.

In vitro, the synergistic anti-CD4 and -CD8 mAb pairs showed an antibody-coating density on T lymphocytes similar to that of RmT1, an anti-Thy-1 mAb of the same rat IgG2b isotype (*14*). It allowed intercalation of C1q, the subunit of the first component of the C cascade (Fig. 2), as could be expected from the basic studies of Hughes-Jones et al. (*15*) with synergistic alloantibodies to MHC antigens on rat red cells.

Antibody-coating density and isotype-dependent affinity for C1q leading to high in vitro uptake of C1q had been found to correlate with immunosuppression in vivo in the case of anti-Thy-1 antibodies (*1*, *14*, *16–18*). The synergistic IgG2b anti-CD4+CD8 mAb pairs were very immunosuppressive. They prevented GVHD altogether. Rat IgG2a RmCD4–4+RmCD8–5 mAb were relatively ineffectual in delaying GVHD (Fig. 1) and, in vitro, negative in C1q uptake and C-dependent cell lysis (data not shown). Interestingly, our single IgG2b anti-CD4 (RmCD4–2) mAb, if combined with anti-CD8 (RmCD8–2) mAb, prevented GVHD as completely as the synergistic mAb pairs, although in vitro C1q uptake and C-dependent cell lysis were low or negative (Fig. 2 and Table 1).

TABLE 1. Coating density and C-mediated cell lysis after incubation of T lymphocytes with single or synergistic anti-T cell mAb

Antigen	mAb	Ab-molecules[a]/ cell × 10^3	Cell lysis[b] (%)
Thy-1	RmT1	60	49
CD8	RmCD8–1*	26	13
CD8	RmCD8–2	34	14
CD4	RmCD4–1**	27	12
CD4	RmCD4–2***	24	12
CD4	RmCD4–1 + 2	53	27
CD8	RmCD8–1 + 2	62	28
CD4/CD8	RmCD4–1 + 2 +RmCD8–1 + 2	—	47

[a] After calibrating the FACScan with quantitative fluorescein microbead standards, the amount of mAb-molecules/cell was determined with fluoresceinated, purified anti-T cell antibodies by FACS analysis. The mean fluorescence of the mAbs was converted to fluorescein equivalents (20).

[b] Percentage of dead lymphoid cells was measured in a FACScan using C57BL/6 lymph node cells incubated at 37°C with purified mAb in saturated concentration and undiluted syngeneic mouse serum as C source. Dead cells were labeled with propidium iodide.

* Inhibiting YTS 169.4; ** inhibiting YTS 156.7.7; *** inhibiting YTA 3.1.

Since affinity for C1q is isotype related, the in vitro findings with the less C1q-affine rat IgG2a mAb corelate with their poor suppression of GVHD in vivo. The finding of single RmCD4–2+RmCD8–2 mAb preventing GVHD in a stringent radiation chimera model could be reproduced in 3 separate experiments, each with groups of 6–9 mice accompanied also by positive controls (i.e., mice injected with the rat IgG2b anti-Thy-1.2 mAb RmT1). Their T lymphocytes with an anti-Thy-1.2 antibody-coating density of around 60,000 molecules/cell (*14*) compare with the 53 and 62×10^6 molecules/cell of the RmCD4 and RmCD8 mAb pairs in Table 1. However, if donor cells express only half of the Thy-1 antigen dose in heterozygous (Thy-1.1×Thy-1.2)F1 strains, the resulting reduction of antibody cell coating no longer allowed prevention

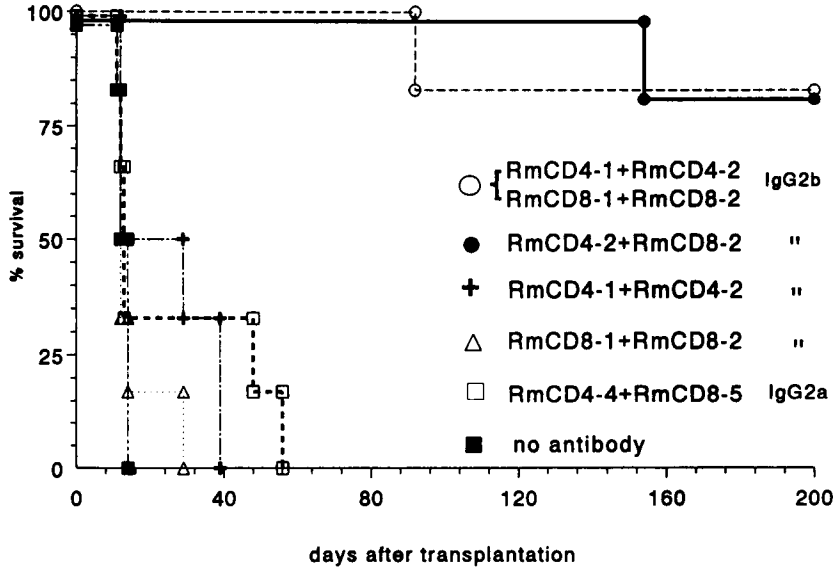

FIGURE 1. Suppression of GVHD in fully mismatched radiation chimeras conditioned with single or synergistic pairs of anti-CD4 and -CD8 (RmCD4+RmCD8) mAb. GVHD mortality was little delayed after transfer of C57Bl/6J bone marrow and spleen cells to irradiated CBA/J mice preinjected with either rat IgG2a anti-CD4+8 mAb or pairs of synergistic rat IgG2b CD4 or CD8 mAb. In contrast GVHD was equally prevented with single or pairs of synergistic rat IgG2b anti-CD4+CD8 mAb.

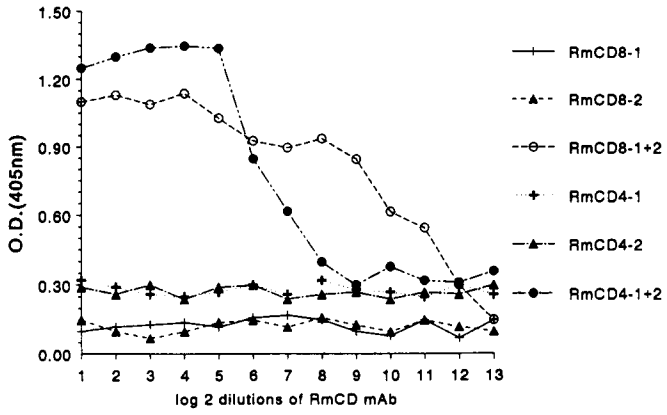

FIGURE 2. C1q uptake by C57BL/6 thymocytes coated with diluted single or pairs of synergistic rat IgG2b anti-CD4 (RmCD4) or anti-CD8 (RmCD8) mAb. It was measured in ELISA after incubation of the antibody-coated cells with 2% human serum as C source. C1q binding was detected using a polyclonal POX-labeled anti-human C1q antibody (*1*).

of GVHD (*14*). Since our single RmCD4–2 and RmCD8–2 mAb show a similarly low antibody coating between 24 and 34×10^6 molecules/cell, their continually successful prevention of GVHD in this donor-recipient combination indicates an additional immunosuppressive mechanism, e.g., interference of anti-CD4 and -CD8 mAb with functions of the neighboring TCR complex (*19*).

Thus our approach of conditioning marrow recipients for both prevention of GVHD and engraftment at reduced radiation doses with only 1 dose of anti-Thy-1 mAb (*1*) also proved applicable to a cocktail containing single anti-CD4+CD8 mAb. Neither reduction of antibody dose nor increasing the number of spleen cells to up to 10^8—approximately the maximally tolerable cell dose of murine marrow recipients—allowed the documentation of a survival bonus of pairs of synergistic versus single anti-CD4+CD8 mAb. Complete long-term chimerism both after 6 or 8 Gy and specific tolerance of marrow donor strain skin grafts up to an observation time of over 200 days are encouraging. It is to be hoped that conditioning of marrow recipients with a high dose of anti-CD4+CD8 mAb will help to overcome GVHD and graft failure, the stumbling blocks in clinical transplantation of bone marrow from mismatched donors.

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REFERENCES

1. Thierfelder S, Kummer U, Schuh R, Mysliwietz J. Antilymphocytic antibodies and marrow transplantation. VIII. Recipient conditioning with Clq-affine monoclonal anti-pan T antibodies prevents GVHD in homozygous fully mismatched mice. Blood 1986; 68: 818.
2. Cobbold SP, Martin G, Qin S, Waldmann H. Monoclonal antibodies to promote marrow engraftment and tissue graft tolerance. Nature 1986; 323: 164.
3. Cobbold SP, Martin G, Waldmann H. Monoclonal antibodies for the prevention of graft-versus-host disease and marrow graft rejection. The depletion of T cell subsets in vitro and in vivo. Transplantation 1986; 42: 239.
4. Knulst AC, Bril Bazuin C, Benner R. Prevention of lethal graft-vs.-host disease by a single low dose injection of anti-T cell monoclonal antibody to the allograft recipients. Eur J Immunol 1991; 21: 103.
5. Cobbold SP, Waldmann H. Skin allograft rejection by L3/T4+ and Lyt-2+ T cell subsets. Transplantation 1986; 41: 634.
6. Qin SX, Cobbold SP, Benjamin R, Waldmann H. Induction of classical transplantation tolerance in the adult. J Exp Med 1989; 169: 779.
7. Pearson TC, Madsen JC, Larsen CP, Morris PJ, Wood KJ. Induction of transplantation tolerance in adults using donor antigen and anti-CD4 monoclonal antibody. Transplantation 1992; 54: 475.
8. Shizuru JA, Seydel KB, Flavin TF, et al. Induction of donor-specific unresponsiveness to cardiac allografts in rats by pretransplant anti-CD4 monoclonal antibody therapy. Transplantation 1990; 50: 366.
9. Reinecke K. Production and characterization of C1q-binding synergistic monoclonal antibodies against murine T-cell antigens. Thesis, LM University, Munich 1990.
10. Galfre G, Howe SC, Milstein C, Butcher GW, Howard JC. Antibodies to major histocompatibility antigens produced by hybrid cell lines. Nature 1977; 266: 550.
11. Kearney JF, Radbruch A, Liesegang B, Rajewsky K. A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. J Immunol 1979; 123: 1548.
12. Cobbold SP, Jayasuriya A, Nash A, Prospero TD, Waldmann H. Therapy with monoclonal antibodies by elimination of T-cell subsets in vivo. Nature 1984; 312: 548.
13. Qin S, Cobbold SP, Tighe H, Benjamin R, Waldmann H. CD4 monoclonal antibody pairs for immunosuppression and tolerance induction. Eur J Immunol 1987; 17: 1159.
14. Kummer U, Thierfelder S, Mysliwietz J. Antigen density on target cells determines the immunosuppressive potential of rat IgG2b monoclonal antibodies. Eur J Immunol 1990; 20: 107.
15. Hughes-Jones NC, Gorick BD, Howard JC. The mechanism of synergistic complement-mediated lysis of rat red cells by monoclonal IgG antibodies. Eur J Immunol 1983; 13: 635.
16. Kummer U, Thierfelder S, Hoffmann-Fezer G, Schuh R. In vivo immunosuppression by pan-T cell antibodies relates to their isotype and to their C1q uptake. J Immunol 1987; 138: 4069.
17. Mysliwietz J, Thierfelder S, Hoffmann-Fezer G, Kummer U. Antilymphocytic antibodies and bone marrow transplantation. XI. Evidence that reduced Thy-1 expression in Thy-1.1 mice prevents suppression of graft-versus-host disease with anti-Thy-1 monoclonal antibodies. Transplantation 1990; 49: 749.
18. Thierfelder S, Mysliwietz J, Hoffmann-Fezer G, Kummer U. Antilymphocytic antibodies and marrow transplantation. XIV. Antibody-induced suppression of graft-versus-host disease in C3-decomplemented mice differentiates two T-cell-depletion pathways. Blood 1991; 77: 2285.
19. Mysliwietz J, Thierfelder S. Antilymphocytic antibodies and marrow transplantation. XII. Suppression of graft-versus-host

disease by T-cell-modulating and depleting antimouse CD3 antibody is most effective when preinjected in the marrow recipient. Blood 1992; 80: 2661.

20. Ledbetter JA, Roise RV, Micklem HS, Herzenberg LA. T cell subsets defined by expression of Lyt-1,2,3 anti Thy-1 antigen. Two parameter immunofluorescence and cytotoxicity analysis with monoclonal antibodies modified current views. J Exp Med 1980; 152: 280.

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EVIDENCE THAT DONOR-SPECIFIC SUPPRESSOR CELLS CAN BE PRODUCED INDEPENDENTLY OF THE THYMUS[1]

We have described previously a method for inducing tolerance using cyclophosphamide (CP)* in a murine model, in which 150–200 mg/kg of CP is administered intraperitoneally 2 days after intravenous priming with 10^8 donor spleen cells (SC) (*1*). In donor-recipient combinations disparate only at minor histocompatibility Ag, this procedure induces specific tolerance to donor skin grafts in association with the establishment of long-lasting mixed chimerism both in the thymus and the periphery (*2*, *3*). In addition, we have shown that Ts cells of CD8 phenotype contribute to the maintenance of this type of tolerance at a late stage when mixed chimerism has waned in some strain combinations (*4*, *5*).

Although numerous reports have demonstrated the existence of Ts, it is not yet clear whether the thymic environment is required for generating Ts, especially in a system in which intrathymic chimerism can be induced as in our model using CP. It has been shown that suppressor inducer T cells (Tsi) can be detected early after (usually within a week) the induction of various types of tolerance (*6*, *7*), suggesting that Tsi can be produced independently of the thymus. In contrast, more direct executor, suppressor effector T cells (Tse) have been shown to take time to become detectable in vivo (*6*, *7*). It has been speculated that a relatively long time is required for Tse to be activated by Tsi and to expand sufficiently to exert effective immune suppression. However, another explanation might be that most Ts are produced in the thymus, and therefore suppressor activity in the periphery takes time to become detectable. In fact, Hendry et al. (*8*) and Hutchinson et al. (*9*) reported, using an adoptive transfer system, that T cells responsible for donor-specific immune suppression exist in the thymus. Alternatively, naive T cells generated in the thymus might be preferentially affected by Tsi. In the present report, to investigate the role of the thymus in inducing Ts, thymectomized mice were used as recipients for tolerance induction using CP, and adoptive transfer assays were performed at the late stage of tolerance.

Female mice of BALB/cCrSlc (BALB/c), DBA/2CrSlc (DBA/2), and B10.D2 nSnSlc (B10.D2) strains (all H-2^d) were obtained from the Japan SLC (Hamamatsu, Shizuoka, Japan). Thymectomy or a sham operation was carried out on recipient BALB/c mice 10 days before intravenous priming with 10^8 donor DBA/2 SC. CP (Shionogi, Japan) was given intraperitoneally at a dose of 200 mg/kg 2 days after the priming. Skin grafting was carried out 14 days after CP treatment. For the adoptive transfer experiments, 10^8 SC from BALB/c mice made tolerant of DBA/2 14–20 weeks earlier were transferred into syngeneic BALB/c mice that had been irradiated with 300 rad on the same day. One day after irradiation and cell transfer, grafting with DBA/2 skin was performed. In some experiments, the transferred SC were pretreated with anti-CD4 mAb (30% culture supernatant of RL172.4 hybridoma), anti-CD8 mAb (×500; anti-Lyt-2.2; Meiji, Tokyo, Japan), or anti-Thy1.2 mAb (×100; Meiji) plus C′. After these treatments, percentages of each subset decreased to less than 2%.

As shown in Figure 1, thymectomized recipients given donor SC and CP acquired comparable levels of tolerance to DBA/2 skin grafts as compared with euthymic tolerant mice, and rejected third-party skin grafts (B10.D2) promptly. SC from these tolerant mice were obtained about 100 days after CP injection, and were transferred into lightly irradiated secondary recipients. The results of the adoptive experiments are shown in Figures 2, 3, and 4. As has been reported previously (*4*, *5*), when unfractionated SC from tolerant euthymic mice were transferred, significant prolongation of skin graft survival could be obtained in the secondary recipients compared with the controls receiving irradiation alone or irradiation plus SC from untreated BALB/c mice (Fig. 2).

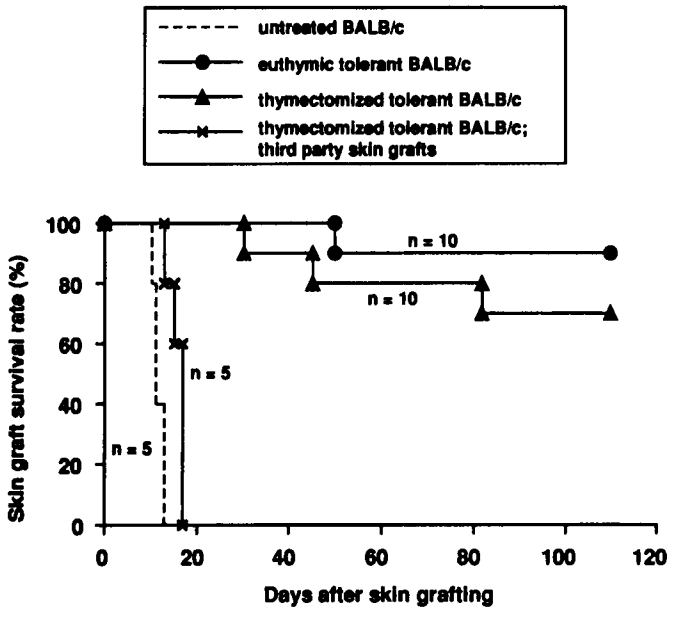

FIGURE 1. Skin graft tolerance could be induced both in euthymic (sham) and thymectomized recipients. Euthymic and thymectomized BALB/c mice were injected intravenously with DBA/2 SC on day −2 and subsequently treated with CP on day 0. Grafting with DBA/2 or B10.D2 (third party) skin was performed on day 14.

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* Abbreviations: CP, cyclophosphamide; SC, spleen cells; Tse, suppressor effector T cell; Tsi, suppressor inducer T cell.