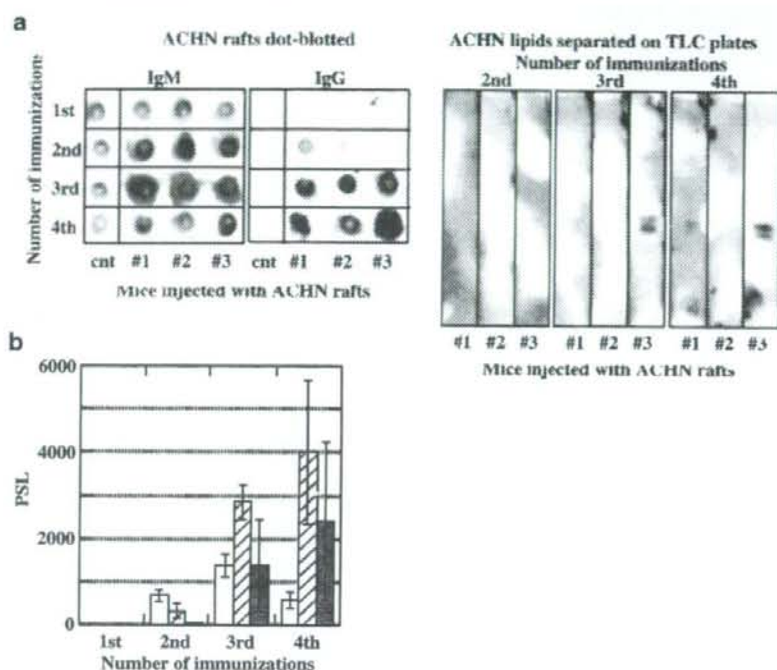

Fig. 3 Kinetics of production of antibody against ACHN rafts. Balb/c mice were injected with an ACHN raft suspension in triplicate (*#1, #2, #3*) or PBS (*cnt*) four times at 7 day intervals, and the sera were obtained 5 days after each immunization. The rafts dot-blotted on PVDF membranes were probed with each antiserum, and then probed with the HRP-conjugated anti-mouse IgM μ chain-specific antibodies or IgG γ chain-specific antibodies as secondary antibody. The lipids separated on the TLC plate were probed with each antiserum, and then with the HRP-conjugated anti-mouse IgG+M antibodies. **a** The images of dot-blot immunostaining of ACHN rafts (*left*) and TLC immunostaining of ACHN lipids (*right*) with the antisera. **b** Measurement of anti-raft IgM antibodies (*open column*), the anti-raft IgG antibodies (*striped column*), and anti-sialylGb5 antibodies (*shaded column*)

(column 1 in Fig. 6) and 0.240±0.043 (column 2 in Fig. 6), respectively, and the difference between the two groups was not significant. The A_{450} for anti-ssDNA IgM in the serum of NZB/WF1, which are well known to spontaneously develop autoimmune disease, was 0.325. No elevation of IgG class anti-DNA antibodies or anti-dsDNA antibodies was observed in the sera of either the immunized mice or NZB/WF1 mice (data not shown). No anti-DNA antibody production or other diagnostic signs of autoimmune disease were observed in these mice. These results show that the development of antibodies against syngeneic rafts components by the mice was not due to the development of an autoimmune disease.

The results of this study show that subcutaneous injection of mice with rafts prepared from specific cell lines induces production of antibodies that recognize single glycolipids, namely monoglycolipid-specific antibodies. For example, rafts prepared from ACHN cells and Vero

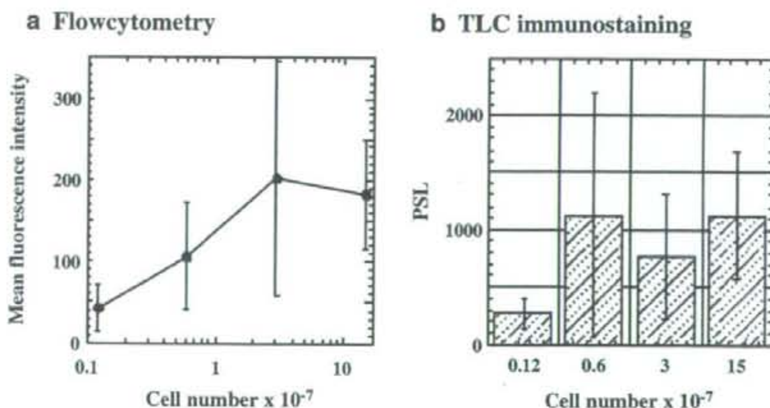

Fig. 4 Reactivity of mouse sera after immunization with the rafts prepared from various numbers of EL4 cells. The sera were obtained from C57BL/6 mice immunized with rafts prepared from 0.12, 0.6, 3 and 15×10^7 EL4 cells. The experiments were performed in triplicate. **a** Evaluation of antibody reactivity to EL4 cells by flowcytometry. **b** Evaluation of antibody reactivity to GD2 by TLC immunostaining

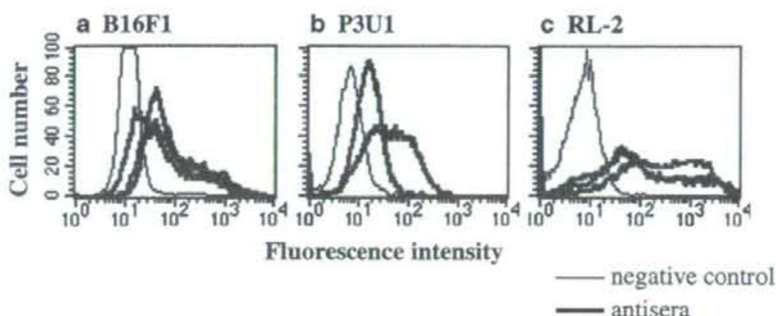

Fig. 5 Flow cytometric analysis of the antisera of mice immunized with syngeneic rafts. Cells were stained with the antisera of C57BL/6 mice immunized with B16F1 rafts (**a**), Balb/c mice immunized with P3U1 rafts (**b**) and Balb/c mice immunized with RL-2 rafts (**c**), and analyzed by flow cytometry (*bold line*). The sera of each mouse injected with PBS were used as a negative control (*thin line*)

cells strongly induced the production of mono-specific antibodies against sialyGb5. However, since sialyGb5 is not the quantitatively predominant glycolipid in ACHN cells or Vero cells, quantitative lipid dominance may not be necessary for monospecific antibody production. Since all four glycolipids to which specific antibodies were produced shown in Fig. 1b were sialylated, sialylation is thought to be the most important factor for inducing monospecific antibody production. However, the rafts from other cell lines gave no production of such antibodies. For example, although B16 melanoma cells are known to highly express GM3 [8], injection of the B16 melanoma rafts did not induce monoglycolipid-specfic antibody. Since Kawashima *et al.* [9] reported that when they intravenously injected ten strains of inbred mice with 100 μg of gangliosides adsorbed to *Salmonella minnesota*, gangliosides such as GD3, GD2, GD1b, GT1a, and GQ1b that have a trisaccharide sequence of NeuAcα2,8NeuAα2,3Gal induced high-titer antibody responses, whereas gangliosides such as GM4, GM3, GM2, GM1, GD1a, and GT1b that have a disaccharide sequence of NeuAcα2,3Gal induced low-titer antibody responses, the diversity of immunogenicity among the glycolipids should be present. Since SSEA-4, an epitope carried by sialyGb5 has been well known highly immunogenic, a saccharide sequence of sailylGb5 can be thought to induce high-titer antibody production. Therefore, if the cells contain highly immunogenic glycolipids such as sialyGb5 and GD2 in lipid rafts, these glycolipids may be effectively presented as immunological targets for antibody production, whereas the rafts containing only low immunogenic glycolipids may be insufficient for antigen presentation to produce anti-glycolipids antibodies. Yamazaki Y. *et al.* [10] obtained several monoclonal antibodies by injecting mice with HL60 cell lipid rafts. One of the antibodies reacted with both GM1a and GD1b, and another reacted with phosphatidylglucoside. HL60 cells, however, mainly express glycolipids of the neolactoseries, not the ganglioseries [11], suggesting that raft immunization enables antibody production against such an extremely minor glycolipid. In order to induce effective

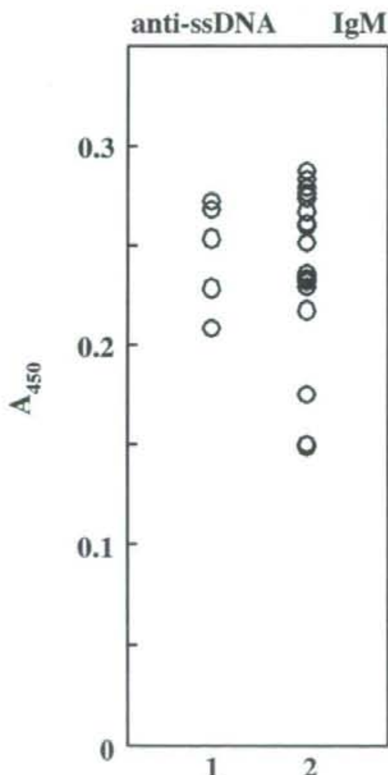

Fig. 6 ELISA of anti-ssDNA antibodies in the sera of mice immunized with syngeneic rafts. Calf thymus ss-DNA was coated and probed with the serum of C57BL/6 or Balb/c mice injected with PBS (*column 1*) and C57BL/6 mice injected with EL4 rafts or Balb/c mice injected with P3U1 rafts (*column 2*). The mean values of triplicate experiments are shown

immune responses against glycolipids in mice, a large amount of purified antigen usually must be immobilized by adsorbing it to the cell walls of bacteria, such as *Salmonella minnesota*, or by incorporating it into liposomes [12], whereas rafts themselves are insoluble and do not need to be immobilized. Furthermore, without mixing with Freund's adjuvant, rafts may retain adjuvant effects and be capable of inducing an immune response even in syngeneic mice.

It still remains unclear how monoglycolipid-specific antibodies are produced, which cells should be used for raft preparation, and to which glycolipids antibodies are predominantly produced. Although further experiments are certainly needed to answer these questions, raft immunization can be used as an effective method of producing monoclonal antibodies against glycolipids and can be applied as new approach in many fields.

Acknowledgments We thank Ms. S. Yamauchi for her excellent secretarial work. This work was supported in part by Health and Labour Sciences Research Grants and Grant for Child Health and Development from the Ministry of Health, Labour and Welfare of Japan, a grant from the Japan Health Sciences Foundation for Research on Health Sciences Focusing on Drug Innovation, JSPS. KAKENHI 18790263, the Budget for Nuclear Research of the Ministry of Education, Culture, Sports, Science and Technology, based on screening and counseling by the Atomic Energy Commission. This work was also supported by CREST, JST, and a grant from Charitable Trust Japan Leukemia Research Fund.

References

1. Katagiri, Y.U., Mori, T., Nakajima, H., Katagiri, C., Taguchi, T., Takeda, T., Kiyokawa, N., Fujimoto, J.: Activation of Src family kinase yes induced by Shiga toxin binding to globotriaosyl ceramide (Gb3/CD77) in low density, detergent-insoluble microdomains. J. Biol. Chem. **274**, 35278–35282 (1999)
2. Katagiri, Y.U., Ohmi, K., Katagiri, C., Sekino, T., Nakajima, H., Ebata, T., Kiyokawa, N., Fujimoto, J.: Prominent immunogenicity of monosialosyl galactosylgloboside, carrying a stage-specific embryonic antigen-4 (SSEA-4) epitope in the ACHN human renal tubular cell line—a simple method for producing monoclonal antibodies against detergent-insoluble microdomains/raft. Glycoconj. J. **18**, 347–353 (2001)
3. Asanuma, H., Takahashi, S., Ishikawa, M., Kamiguchi, K., Sato, N., Poppema, S., Fujimoto, J., Kikuchi, K.: A monoclonal antibody, 3G12, reacts with a novel surface molecule, Hal-1, with high expression in CD30-positive anaplastic large cell lymphomas. Br. J. Haematol. **106**, 55–63 (1999)
4. Katagiri, Y.U., Kiyokawa, N., Nakamura, K., Takenouchi, H., Taguchi, T., Okita, H., Umezawa, A., Fujimoto, J.: Laminin binding protein, 34/67 laminin receptor, carries stage-specific embryonic antigen-4 epitope defined by monoclonal antibody Raft.2. Biochem. Biophys. Res. Commun. **332**, 1004–1011 (2005)
5. Iizuka, J., Katagiri, Y., Tada, N., Murakami, M., Ikeda, T., Sato, M., Hirokawa, K., Okada, S., Hatano, M., Tokuhisa, T., Uede, T.: Introduction of an osteopontin gene confers the increase in B1 cell population and the production of anti-DNA autoantibodies. Lab. Invest. **78**, 1523–1533 (1998)
6. Jacob, F.: Mouse teratocarcinoma and embryonic antigens. Immunol. Rev. **33**, 3–32 (1977)
7. Tang, W.R., Kiyokawa, N., Eguchi, T., Matsui, J., Takenouchi, H., Honma, D., Yasue, H., Enosawa, S., Mimori, K., Itagaki, M., Taguchi, T., Katagiri, Y.U., Okita, H., Amemiya, H., Fujimoto, J.: Development of novel monoclonal antibody 4G8 against swine leukocyte antigen class I alpha chain. Hybridoma Hybridomics. **23**, 187–91 (2004)
8. Sawada, M., Moriya, S., Shinehara, R., Satomi, S., Miyagi, T.: Comparative study of sialidase activity and GM3 content in B16 melanoma variants with different metastatic potential. Acta. Biochim. Pol. **45**, 343–349 (1998)
9. Kawashima, I., Nakamura, O., Tai, T.: Antibody responses to gangliosides-series gangliosides in diffeent strains of inbred mice. Mol. Immunol. **29**, 625–632 (1992)
10. Yamazaki, Y., Nagatsuka, Y., Oshima, E., Suzuki, Y., Hirabayashi, Y., Hashikawa, T.: Comprehensive analysis of monoclonal antibodies against detergent-insoluble membrane/lipid rafts of HL60 cells. J. Immunol. Methods **311**, 106–116 (2006)
11. Stroud, M.R., Handa, K., Salyan, M.E., Ito, K., Levery, S.B., Hakomori, S., Reinhold, B.B., Reinhold, W.N.: Monosialogangliosides of human myelogenous leukemia HL60 cells and normal human leukocytes. 1. Separation of E-selectin binding from nonbinding gangliosides, and absence of sialosyl-Le(x) having tetraosyl to octaosyl core. Biochemistry **35**, 758–769 (1996)
12. Kannagi, R.: Monoclonal anti-glycosphingolipid antibodies. Methods Enzymol. **312**, 160–179 (2000)

Foxp3-expressing Regulatory T Cells Expanded With CD28 Superagonist Antibody Can Prevent Rat Cardiac Allograft Rejection

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Background: It is well known that $CD4^+CD25^+$ regulatory T (Treg) cells play a central role in the suppression of autoimmunity, inflammation and allograft rejection. Therefore, therapeutic agents that capable of enhancing the number and activity of this T-cell subset are highly desirable.

Methods: The present study was designed to investigate the effects of superagonistic CD28-specific monoclonal antibody (supCD28 MAb) on preferentially expanded rat naturally occurring $CD4^+CD25^+$ Treg (nTreg) cells and its applicability in cardiac transplantation.

Results: A single administration of supCD28 MAb preferentially proliferated nTreg cells. The increase of Foxp3 expression and polarization toward a Th2 cytokine profile correlated with decreased production of interferon-γ and increased production of interleukin-4 and -10 in the expanded $CD4^+CD25^+$ Treg subset, which was capable of suppressing $CD4^+CD25^-$ T-cell proliferation after purification. Furthermore, supCD28 MAb administration revealed that nTreg cells were preferentially proliferating in vivo and recruited into the grafts, resulting in significant prolongation of full MHC-mismatch cardiac graft survival.

Conclusions: Our data demonstrate that supCD28 MAb targets expansion of nTreg cells in vivo and maintains and enhances their regulatory functions, which represents a major advance toward the therapeutic use of polyclonally activated Treg cells as cellular therapy for treatment of allograft rejection. J Heart Lung Transplant 2008;27:362–71. Copyright © 2008 by the International Society for Heart and Lung Transplantation.

Regulatory lymphocytes play a pivotal role in preventing organ-specific autoimmune disease[1] and in induction and maintenance of tolerance in various experimental transplantation models.[2-4] Enhancement of the number and activity of peripheral $CD4^+CD25^+$ Treg cells is an obvious goal in the treatment of autoimmunity and for the suppression of allograft rejection. However, the normal physiology of this population of suppressor T cells is still incompletely understood and the molecular basis for the anergic state of $CD25^+$ T cells remains only partially known. A crucial area for future study is the identification of drugs, cytokines or co-stimulatory molecules that reverse anergy and enhance growth while preserving the suppressor function of the $CD4^+CD25^+$ Treg cell population.

A unique superagonistic CD28-specific monoclonal antibody (supCD28 MAb) was developed to induce proliferation of all primary resting T cells without T-cell receptor (TCR) engagement, which potently stimulates both in vivo and vitro the expansion of $CD4^+CD25^+$ Treg cells.[5,6] T-cell activation by supCD28 MAb occurs without an increase in ZAP-70 and TCR-γ phosphorylation, and hence without stimulation of the TCR complex.[7] In keeping with the Th2-promoting effect of CD28 signals in co-stimulation, it was shown that T-cell activation with supCD28 primed $CD4^+$ T cells for Th2 differentiation in vitro, and induced interleukin (IL)-4 and IL-10 expression along with Th2-dependent immunoglobulin (Ig) isotypes in vivo.[8] Recently, studies in

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rodent autoimmune disease models have shown efficacy of supCD28 MAb treatment in the prevention of experimental autoimmune neuritis and experimental autoimmune encephalomyelitis with marked reduction of infiltrating cells and interferon (IFN)-γ production,[9] adjuvant arthritis with amelioration of Th1/Th2 cell balance and induction of high IL-10 expression in synovia,[10] and in the Type 1 diabetes BB rat model with an increase of $CD4^+CD25^+$ T-cell frequency.[11]

In this study we show that in vivo application of supCD28 MAb to Lewis rats causes preferential expansion of Foxp3-expressing Treg cells over conventional T cells, and maintains not only their phenotype but also their potent regulatory functions against T-cell proliferation in response to mitogens, antibodies and allostimulation in vitro. Furthermore, using a full MHC-mismatch rat heterotopic heart transplantation model, we found that supCD28 MAb preferentially expands naturally occurring Treg (nTreg) cells and recruits these nTreg cells to the grafts, resulting in significant prolongation of cardiac graft survival. Thus, it was shown that this therapeutic effect is mediated by the in vivo expansion of nTreg cells.

METHODS

Animals

Adult male Lewis (*RT-1^l^*) and DA (*RT-1^a^*) rats were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan) and used at 4 to 8 weeks of age. The animals were maintained under standard conditions and fed rodent food and water according to laboratory animal care principles and the guide for the care and use of laboratory animals at our institution. This investigation complied with the *Guide for the Care and Use of Laboratory Animals*, published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

T-cell Proliferation Assays

The preparation of purified spleen T cells and $CD4^+CD25^+$ and $CD4^+CD25^-$ T-cell purification were carried out as described previously.[4] Carboxyfluorescein diacetate-succinimidyl ester (CFSE; Molecular Probes, Inc., Eugene, OR)-labeled quantitative estimation of the mitotic assay was used according to a modified method described previously.[12] $CD4^+CD25^+$ T cells from supCD28 MAb-treated or mIgG-treated control Lewis rat spleens were prepared by serial dilution (1:0.25, 1:0.5 and 1:1), mixed with $CD4^+CD25^-$ T cells (1×10^5), and cultured with 20 Gy of gamma-irradiated DA splenocytes (APC, 1×10^5) in a 96-well plate at 37°C for 5 days. The proliferation of T cells was measured using cell-proliferation enzyme-linked immunoassay (ELISA) kits (Roche Diagnostics GmbH, Penzberg, Germany).[13]

Flow Cytometry Analysis

$CD4^+$ T cells from rat spleens were collected and suspended in phosphate-buffered saline (PBS) and then incubated with an optimal concentration of Cy-Chrome-conjugated anti-rat CD4 antibody (OX35; BD Pharmingen) in combination with fluorescein isothiocyanate (FITC)-conjugated anti-rat CD25 antibody (OX39; BD Pharmingen) diluted with PBS. Intracellular staining was performed using a BD Cytofix/Cytoperm kit according to the manufacturer's instruction. Phycoerythrin (PE)-conjugated anti-rat IL-4 and IL-10 antibody (BD Pharmingen) and PE-conjugated anti-Foxp3 antibody (FJK-16s; eBioscience, San Diego, CA) were used. The stained cells were analyzed by flow cytometry (FACScan; Becton Dickinson).

RNA Preparation and Semi-quantitative RT-PCR

Total cellular RNA isolation and semi-quantitative reverse transcript-polymerase chain reaction (RT-PCR) was carried out as described previously.[14] PCR primers were designed on the basis of the reported cDNA sequences; the expected fragment sizes for these genes are specified in Table 1.

Cytokine Assay

Supernatants and serum were collected and quantified by BD Cytometric Bead Array Rat IL-4, IL-10 Flex Kit or IL-2, IL-4, IL-10 and IFN-γ rat ELISA kit (BioSource, Camarillo, CA) according to the manufacturer's protocol.

Heterotopic Cardiac Transplantation

Heterotopic cardiac transplantation at the cervix was performed between donor DA and recipient Lewis rats as described previously.[15] supCD28 MAb was adminis-

Table 1. Primers Used in this Study

Genes	Forward (5′-3′)	Reverse (5′-3′)	Cycle	Product (bp)
GAPDH	CGGCAAGTTCAACGGCACA	AAGACGCCAGTAGACTCCACGA	24	147
FoxP3	CAGCTGCCTACAGTGCCCCTAG	CATTTGCCAGCAGTGGGTAG	21	382
IL-2	CAGCTGTTGCTGGACTTACAGG	CACAGTTGCTGGCTCATCATCG	33	300
IL-4	GGGTGCTTCGCAAATTTTAC	CTTTCAGTGTTGTGAGCGTGG	48	159
IL-10	CTCAGCACTGCTATGTTGCCTGCT	GCAAGGCAGTGGAGCAGGTGAAGA	27	422
IFN-γ	GGATATCTGGAGGAACTGGCAAAAG	CACCAGCTGTCACCAGAATCTAGC	29	286
TGF-β	TGGAAGTGGATCCACGAGCCCAAGG	GTCCAACATGATCGTGCGCTCCTGC	27	240

tered to the recipient Lewis rats via tail veins at various time-points before and after grafting (Day −3, Day 0 and Day −3, 0 and 7 after grafting).

Cardiac Graft Pathology and Immunohistology Studies

Formalin-fixed samples were embedded in paraffin and cut into 4-μm-thick sections. Tissues were stained with hematoxylin and eosin for assessing tissue damage and inflammation.[16] For immunohistochemistry, monoclonal anti-CD4 (W3/25; Serotec Co. Ltd., Oxford, UK), anti-CD25 (OX-39, Serotec) and biotoin-anti-Foxp3 antibody were used to evaluate infiltrated T cells. Color development was performed with Levamisole solution (Invitrogen), an alkaline phosphatase substrate kit and Ni^{2+} + Co^{2+} DAB solution.

Statistical Analysis

Student's *t*-tests were used to compare the paired and unpaired analyses. A statistical evaluation for graft survival was performed using the Kaplan-Meier test. $p < 0.05$ was considered statistically significant. All in vitro experimental data were representative of three independent experiments and expressed as mean ± standard deviation (SD).

RESULTS

SupCD28 MAb–expanded T Cells Suppress Naive T-cell Proliferation in Alloantigen- and ConA–induced Stimulation

A single injection of supCD28 MAb JJ316 led to a significant increase in the $CD4^+CD25^+$ cell population in the spleen, peripheral blood and lymph nodes, from an initial 3% to 5% to about 15% at 72 hours after stimulation (Figure 1A, top). Furthermore, supCD28 treatment dramatically increased the representation of the $CD4^+CD25^+$ cell population among CD4 cells of the spleen (4.5-fold), peripheral blood lymphocytes (PBL; 5.18-fold) and lymph nodes (LN; 8.15-fold) (Figure 1A, bottom). Furthermore, lymphocytes from the supCD28-treated groups revealed significantly higher expression of IL-2, IL-4 and IL-10 (Figure 1B and C). Lymphocytes from CD28-stimulated rats reduced mitotic events from 2,520 to 1,403 (56%) and from 4,422 to 1,930 (46%) in the alloantigen-presenting-cell (allo-APC) and Concanavalin A (ConA)-induced responses, respectively (Figure 2, left). Moreover, the bivariate dot plots show a significant suppressive effect on the division of the CD4 T-cell subset of the CFSE-labeled lymphocytes by the same stimuli (Figure 2, right).

SupCD28-expanded $CD4^+CD25^+$ Treg Cells Have Regulatory Activity

To specifically examine the suppressor cell activity of supCD28-expanded $CD4^+CD25^+$ cells, $CD4^+CD25^+$ T cells were isolated from rats at Day 3 of supCD28 stimulation as described earlier and suppression assays were performed using anti-CD3, anti-CD3/CD28 or ConA along with autologous feeder APCs. Both $CD4^+CD25^+$ T cells derived from naive rats and from supCD28 MAb-stimulated rats were able to suppress proliferation of $CD4^+CD25^-$ T cells in a dose-dependent manner (Figure 3A–C). Figure 3 also shows that, at a ratio of 1:1, $CD4^+CD25^+$ T cells inhibited the proliferation of naive $CD4^+CD25^-$ T cells stimulated with alloantigen by allogeneic APCs (75.0 ± 2.9%). These data demonstrate that supCD28 MAb-expanded $CD4^+CD25^+$ T cells are even more suppressive than

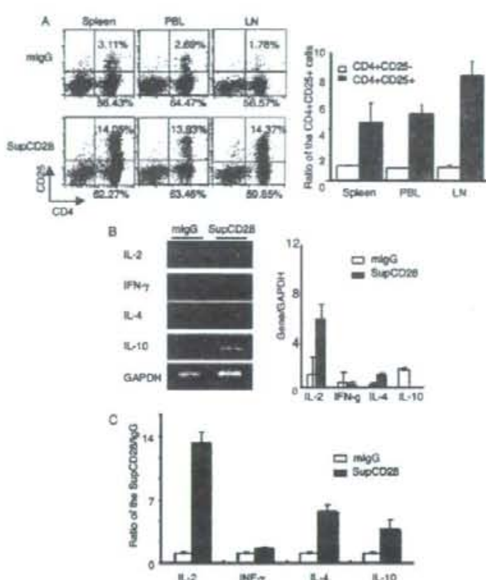

Figure 1. SupCD28 MAb induced $CD4^+CD25^+$ expansion of T cells, and increased expression of IL-2, IL-4 and IL-10. (A) The proportion of $CD4^+CD25^+$ T cells increased to 14% in the spleen, PBL and LN cells in the supCD28 MAb–treated groups, which was significantly greater than in the mIgG-treated control groups (upper panels). SupCD28-only treatment dramatically increased $CD4^+CD25^+$ populations of the spleen (4.5-fold), PBL (5-fold) and LN (8-fold). The population of $CD4^+CD25^-$ cells was unchanged in these two groups after supCD28 MAb administration (lower panels). (B) Compared with the control mIgG groups, lymphocytes from the supCD28-treated groups revealed significantly higher expression of IL-2 and IL-10 mRNA by semi-quantitative RT-PCR assay. The relative quantities of the genes are presented as the ratios of the intensities of the IL-2, IFN-γ, IL-4 and IL-10 bands against those of the housekeeping gene, GAPDH. (C) The serum of the supCD28 MAb–treated rats also showed higher levels of IL-2, IL-4 and IL-10, as determined by flow cytometry analysis (bead array assay). Data are representative of three independent experiments and indicate the mean ratio of triplicate results in each experiment.

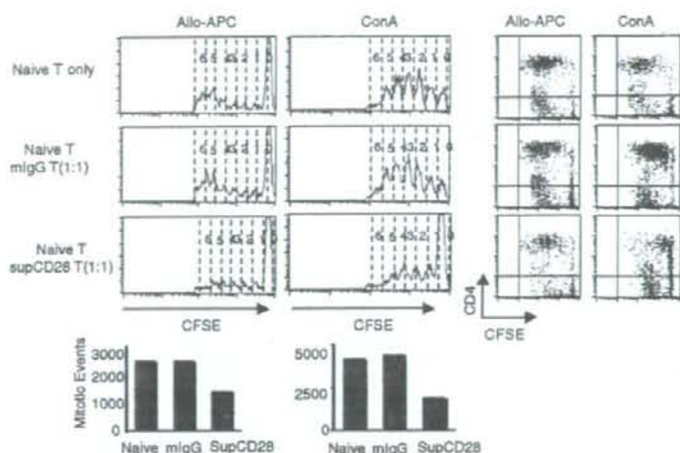

Figure 2. SupCD28 MAb expanded lymphocytes suppressed naive T-cell proliferation induced by allostimulation and ConA stimulation. CFSE-labeled naive Lewis lymphocytes were stimulated with allogeneic APCs or 2 μg/ml ConA. The CFSE fluorescence profiles of the live lymphocytes from each culture at 72 hours and quantitative estimation of mitotic events represented by each histogram are shown (left panels). Bivariate dot plots show the cell division on the CD4 T-cell subset of CFSE-labeled lymphocytes stimulated with APC and ConA (right panels). The data clearly show that the division of the naive lymphocytes was suppressed when mixed at an equal ratio of supCD28 MAb–expanded lymphocytes compared with naive and mIgG-treated groups. Data are representative of three independent experiments.

$CD4^+CD25^+$ nTreg cell populations from normal rats.

SupCD28-expanded $CD4^+CD25^+$ T Cells Express Higher Levels of IL-2, -4, -10 and Foxp3 than Naive nTreg Cells

The expanded $CD4^+CD25^+$ T cells expressed much higher levels of IL-4 and IL-10 compared not only to $CD4^+CD25^-$ T cells but also to nTreg cells (Figure 4A and B). With regard to IL-2 mRNA, there was an increased expression compared with nTreg cells from unstimulated animals and no significant difference in IL-2 mRNA expression between the $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from the supCD28 MAb-stimulated rats. Because $CD4^+CD25^+$ cells from supCD28-stimulated animals contain a small but significant fraction of Foxp3-negative, activated effector CD4 T cells, these represent the likely source of IL-2 mRNA detected in this population. In addition, purified $CD4^+CD25^-$ T cells had no expression of Foxp3 after in vivo supCD28 MAb stimulation, and both naive and supCD28 MAb–expanded $CD4^+CD25^+$ cells exhibited high levels of Foxp3 mRNA (Figure 4C).

Expansion of nTreg Cells by Stimulation of SupCD28 MAb Without Conversion from $CD4^+CD25^-$ Cells

As shown in Figure 5, in vitro supCD28 MAb stimulation induced expression of CD25 in $CD4^+CD25^-$ T cells, which, however, contained only a few Foxp3-positive cells (Figure 5A, upper right), whereas all supCD28 MAb–expanded rat $CD4^+CD25^+$ Treg cells remained positive for not only CD25 but also highly expressed Foxp3 (Figure 5A, lower right). Furthermore, bivariate dot plots show that both the Foxp3-positive and -negative T-cell subset of CFSE-labeled sorted $CD4^+CD25^+$ and $CD25^-$ T cells stimulated with plate-bound supCD28 MAb had undergone cell division (Figure 5B, left). The CFSE fluorescence profiles of the live lymphocytes showed that both the $CD25^+$ and $CD25^-$ T cells had also proliferated (Figure 5B, right).

In Vivo Stimulation of SupCD28 MAb Preferentially Expands nTreg Cells, Which Were Not Derived from $CD4^+CD25^-$ Cells

To demonstrate that the suppressive capacity of expanded $CD4^+CD25^+$ Treg cells is an intrinsic property of T cells constitutively expressing Foxp3 in vivo, purified $CD4^+CD25^-$ T cells were labeled with CFSE, injected into naive rats, and stimulated with supCD28 MAb (Figure 6A). All of the CFSE-labeled transferred cells had undergone one or several cell divisions, but virtually none of them expressed CD25 (Figure 6B). Accordingly, $CD4^+CD25^-$ T cells that had responded to supCD28 MAb stimulation by proliferation did not contribute to the elevated frequency of $CD25^+$ CD4 cells observed in spleen, LN and PB cells (12.6%, 14.5% and 26.7% of CFSE $CD4^+$ cells, respectively); instead, they were derived from pre-existing endogenous $CD25^+$ cells. This contrasts with the result seen in vitro, in which T cells became $CD25^+$ after in vitro activation

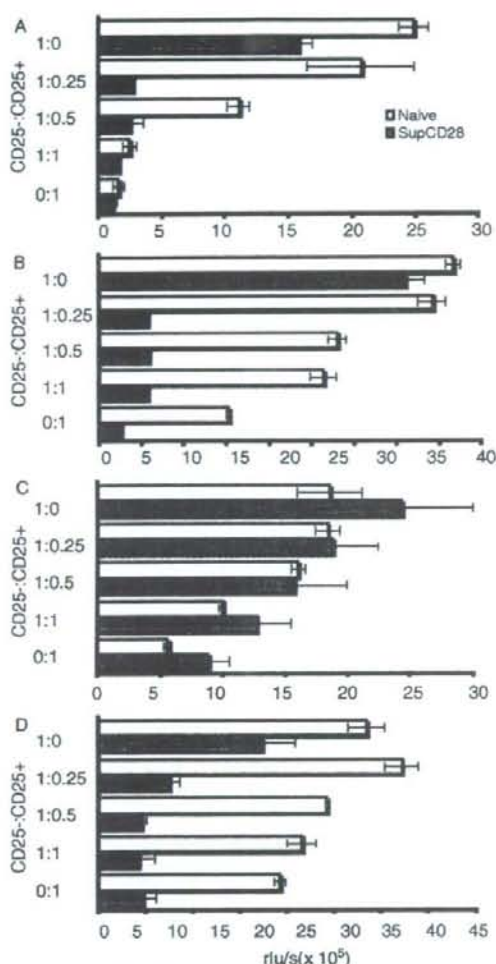

Figure 3. SupCD28 MAb–expanded $CD4^+CD25^+$ T cells are superior to naive Treg cells in inhibiting $CD25^-$ T-cell proliferation induced by various stimuli. SupCD28 MAb–expanded $CD4^+CD25^+$ T cells inhibit $CD25^-$ T-cell proliferation induced by anti-CD3 (A), anti-CD3+CD28 (B), ConA (C) and allogeneic stimulator (D) cells in a dose-dependent manner and more potently than naive Treg cells. Filled bars: sorted in vivo–expanded $CD4^+CD25^+$ T cells; open bars: naive $CD4^+CD25^+$ isolated T cells. Data are representative of three independent experiments and indicate the mean ratio of triplicate results in each experiment.

and proliferation in response to supCD28, yet they did not express Foxp3.

SupCD28 MAb Treatment Prolongs Rat Cardiac Allograft Survival

Using a heterotopic cardiac transplantation model, a comparison of graft survival was made between recipients that underwent various protocols of supCD28 MAb treatment (Figure 7A). Recipients that received supCD28 MAb at Day −3 or Day 0 had prolonged graft survival, from 5.3 ± 0.5 (control groups) to 12.9 ± 2.5

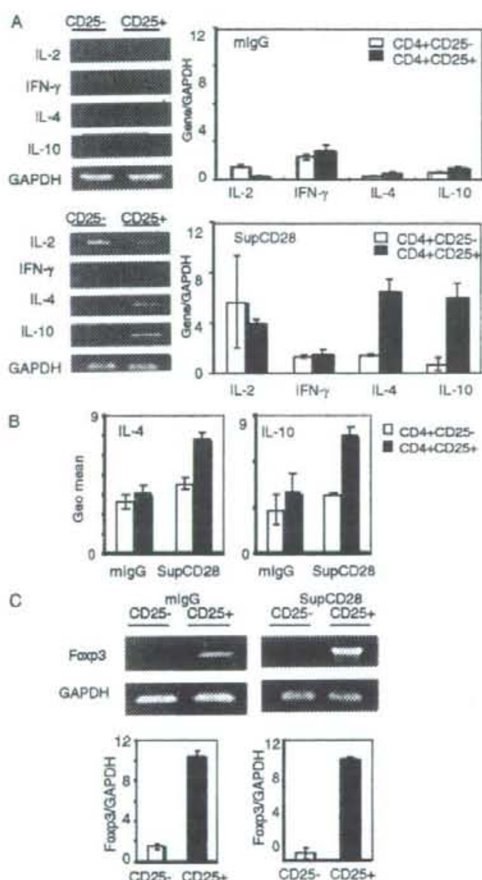

Figure 4. Cytokine and Foxp3 expression of the supCD28 MAb–expanded $CD4^+CD25^+$ and $CD25^-$ T cells. (A) Compared with mIgG control (upper panel), supCD28 MAb–expanded $CD4^+CD25^-$ and $CD25^+$ T cells expressed significant amounts of IL-2, whereas $CD25^+$ cells expressed high levels of IL-4 and IL-10 mRNA by semi-quantitative RT-PCR assay. The relative quantities of the genes are presented as ratios of the intensities of IL-2, IFN-γ, IL-4 and IL-10 bands against those of the housekeeping gene, GAPDH (right panel). (B) SupCD28 MAb–expanded $CD4^+CD25^+$ T cells revealed high levels of intracellular IL-4 and IL-10 molecule, as confirmed by FCM analysis. (C) Purified $CD4^+CD25^-$ T cells did not express Foxp3 after supCD28 MAb stimulation, and both naive and supCD28 MAb–expanded $CD4^+CD25^+$ T cells exhibited high levels of Foxp3 mRNA expression. The relative quantities of the genes are presented as ratios of the intensities of Foxp3 bands against those of GAPDH (lower panel). Data are representative of three independent experiments and indicate the mean ratio of triplicate results in each experiment.

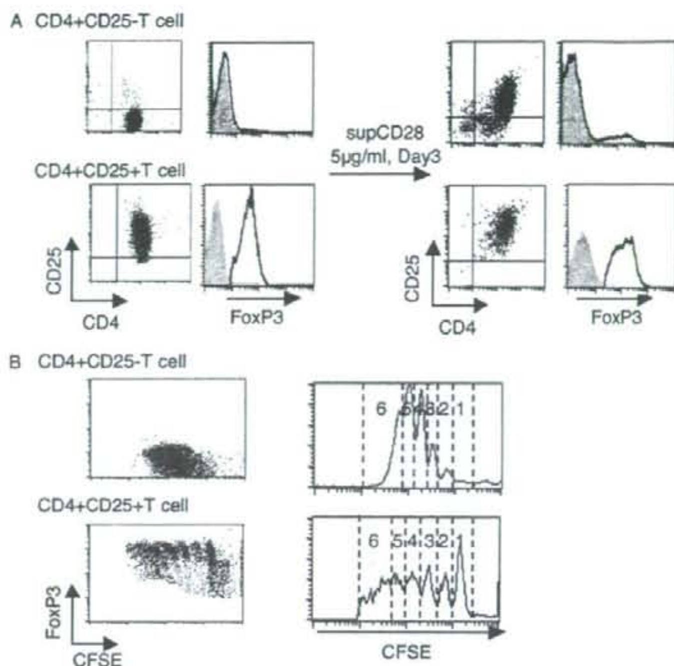

Figure 5. $CD4^+CD25^+Foxp3^+$ Treg cells could be expanded by activation of $CD4^+CD25^+$ nTreg cells via supCD28 MAb, but not be converted from $CD4^+CD25^-Foxp3^-$ T cells in vitro. (A) FCM plots showing Foxp3 expression of the sorted cells in each population before activating (left) and in in vitro–stimulated plate-bound supCD28 MAb for 72 hours. In vitro supCD28-expanded rat $CD4^+CD25^+$ Treg cells remained positive for not only CD25 but also for high expression of Foxp3. In contrast, all $CD4^+CD25^-$ T cells expressed CD25 after activation, but Foxp3 expression was rare. Data are representative of three independent experiments. (B) Bivariate dot plots show the cell division on both $Foxp3^+$ or $Foxp3^-$ T-cell subsets of CFSE-labeled sorted $CD4^+CD25^+$ and $CD25^-$ T cells stimulated with plate-bound supCD28 MAb (left panel). The CFSE fluorescence profiles of live lymphocytes, represented in each histogram, showed no difference between $CD25^+$ and $CD25^-$ T cells (right panel).

or 7.3 ± 0.8 days (mean) after grafting (Figure 7B). Recipients that received supCD28 MAb at Days −3, 0 and 7 exhibited significantly prolonged graft survival (13.5 ± 1.8 days). Moreover, extensive perivascular infiltration of mononuclear cells and myocardial structure damage were seen on histologic studies of cardiac grafts from mIgG-treated control rats (Figure 7C, top). In contrast, grafts from supCD28 MAb-treated rats revealed relatively little infiltration regarding mononuclear cells, and nearly normal tissue architecture was observed (Figure 7C, bottom). These results demonstrate that supCD28 MAb might expand $CD4^+CD25^+$ Treg cells, which have a potential immunoregulatory function to inhibit graft rejection and prolong allograft survival.

High Level of Foxp3-expressing Cells in Peripheral Blood and Intragraft After SupCD28 MAb Therapy

A kinetic analysis of $Foxp3^+$ T cells in peripheral blood and intragraft on Day 5 after a full MHC mismatch cardiac grafting showed a higher population of $Foxp3^+$ T cells in $CD4^+CD25^+$ T cells and total T cells of the recipient peripheral blood after supCD28 MAb injection, and this population progressively decreased on Day 5 after cardiac grafting (Figure 8, middle and bottom). However, the $Foxp3^+$ population of allograft-infiltrating cells in supCD28 MAb-treated recipients at Day 5 after transplantation was increased compared with the control group (Figure 8Ac–f). Foxp3 protein was localized to a subset of infiltrating $CD4^+$ and $CD25^+$ cells in allografts of rats treated with supCD28 MAb (Figure 8Ab and f). On the other hand, an increase of $CD4^+CD25^+$ T cells was observed after grafting in peripheral blood of the control group, and these cells were activated $Foxp3^-$ T cells (Figure 8B, top). The population of $Foxp3^+$ cells in $CD4^+CD25^+$ T cells and total T cells was decreased or unchanged, respectively (Figure 8B, middle and bottom). These data indicate that supCD28 MAb-expanded $CD4^+CD25^+Foxp3^+$ cells traffic to the graft, a site of active immunity, to inhibit alloimmune responses, thereby leading to prolonged graft survival.

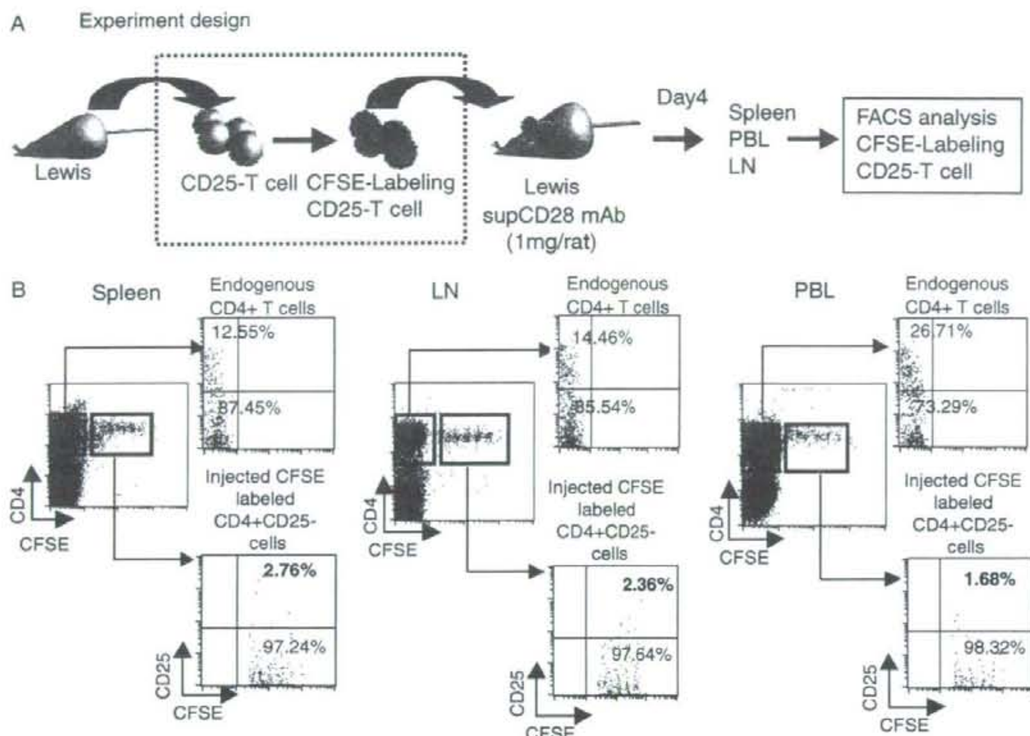

Figure 6. SupCD28 MAb selectively expands nTreg cells in vivo. (A) Experimental design. (B) Schematic for in vivo expansion and generation of $CD4^+CD25^+$ Treg cells. FCM plot showing the percentage of $CD25^+$ cells of the transferred, CFSE-labeled $CD4^+CD25^-$ T cells (5×10^7 cells/rat) and endogenous $CD4^+CD25^+$ T cells in each population at 48 hours after injection of supCD28 MAb from spleen (left panels), lymph node (middle panels) and peripheral blood (right panels). Data are representative of three independent experiments.

DISCUSSION

This study has documented for the first time that supCD28 MAb could expand Foxp3-expressing Treg cells to inhibit cardiac allograft rejection. nTreg cells specifically express the transcription factor *Foxp3*, which appears to act as a master control gene for their development and function. Forced expression of the Foxp3 gene can convert mouse naive T cells to Treg cells that phenotypically and functionally resemble nTreg cells.[17] In addition to being a marker for Treg cells, CD25 expression on $CD4^+$ T cells is an indicator of cell activation. The stimulation of $CD4^+CD25^-$ T cells leads to induction of cell-surface expression of CD25. Therefore, it may be possible that some or all of the $CD4^+CD25^+$ T cells are the result of recent activation, whereas our data, in agreement with previous reports,[6,18] demonstrate that supCD28 MAb has a unique capacity to expand intrinsic constitutive $CD25^-$ and Foxp3-expressing nTreg cells, which are not activated $CD25^+$ effector T cells (see Figure 5).

We showed that in vivo–expanded $CD4^+CD25^+$ T cells with suppressive capacity were derived from pre-existing nTreg cells constitutively expressing CD25 and Foxp3 in vivo (Figure 6), in contrast to results seen with in vitro stimulation where T cells became $CD25^+$ after in vitro activation in response to supCD28 but did not express Foxp3. From these data, which can be correlated with those of Lin and Hunig,[6] we concluded that, unlike what has been described in mice for antigen-driven conversion of $CD4^+25^-$ to Treg cells,[19] stimulation of $CD4^+CD25^-$ T cells through supCD28 MAb stimulation results in the expansion of pre-existing $CD4^+CD25^+$ Treg cells.

IL-2 is a cytokine indispensable for the survival of nTreg cells in the peripheral immune system; CD25 is also indispensable, as it is a constituent of the high-affinity IL-2 receptor.[20] Thus, IL-2 signaling seems to be critical for maintaining the homeostasis and competitive fitness of Treg cells in vivo. In the present study, we demonstrated that IL-2 production was increased after

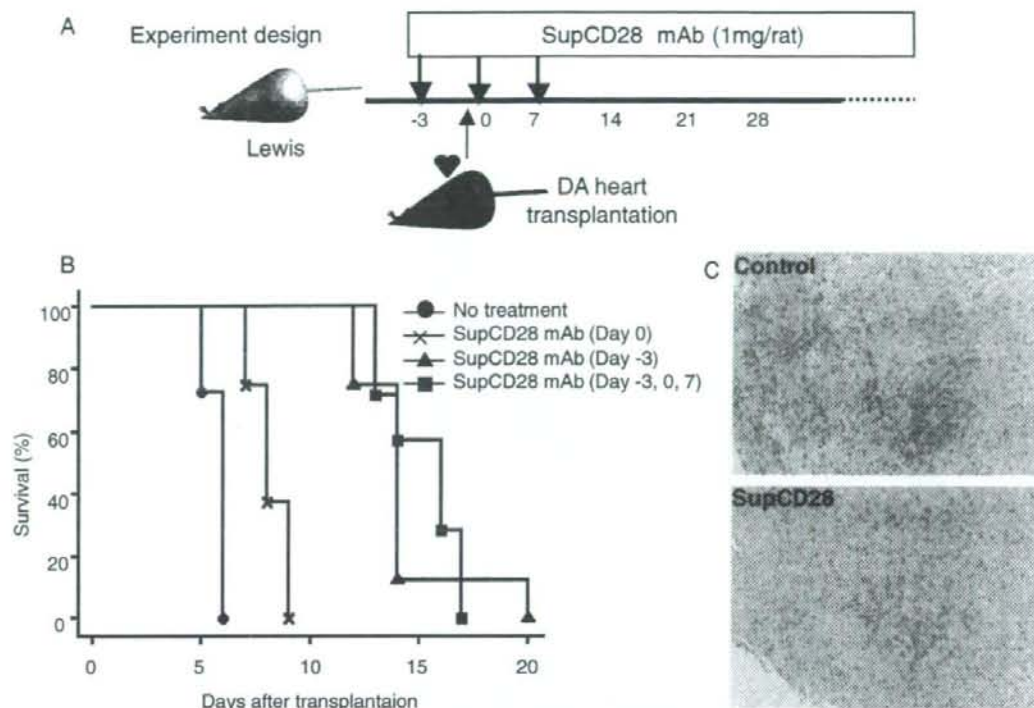

Figure 7. SupCD28 MAb therapy prolongs rat cardiac allograft survival. (A) Experimental design. (B) Lewis rats treated with supCD28 MAb at Day −3 (triangles [mean ± SD]: 12.9 ± 2.5 days; $n = 8$; $p = 0.0001$), Day 0 (×: 7.3 ± 0.8 days; $n = 8$; $p = 0.0012$) or Days −3, 0 and 7 (squares: 13.5 ± 1.8 days; $n = 6$; $p = 0.0012$) exhibited significantly prolonged survival of MHC-mismatched DA heart allografts as compared with control rats (circles: 5.3 ± 0.5 days; $n = 11$). The p-value was compared with mIgG control group rats and determined by Kaplan–Meier test. (C). Representative photomicrographs reveal mononuclear cells infiltrated with myocyte necrosis in the control grafts (top), and less infiltration of mononuclear cells and almost normal tissue architecture in the supCD28 MAb–treated group (lower) at Day 5 after transplantation. Original magnification: ×100.

supCD28 stimulation in vivo (see Figures 1 and 4). Recent studies have concluded that the functional significance of CD25 expression on Treg cells is a requirement for IL-2 in their generation or survival.[21,22] Furthermore, in vitro studies have shown that Treg cells rapidly lose viability in the absence of IL-2. Thus, the present data suggest that IL-2 may play an important role in supCD28 MAb–mediated Treg expansion in vivo (Figure 4A).

The number of Treg cells is important to maintain transplantation tolerance.[4,15,23-25] We expected that systemic injection of supCD28 MAb would expand and activate nTreg cells to prolong cardiac allograft survival. We found that the recipients receiving supCD28 MAb exhibited significant prolongation of graft survival (Figure 7B) while mononuclear cell infiltratration into the grafts occurred, although without tissue injury (Figure 8A), suggesting a potentially active process of regulation within the graft. Furthermore, our results show Foxp3 expression within cardiac allografts and that this expression is markedly enhanced when supCD28 MAb treatment is used to prolong graft survival. These findings are consistent with those of other studies describing that both the presence of $Foxp3^+$ cells in tolerized grafts in skin and heart allograft and their homing to allografts are required for allograft tolerance.[26,27] However, in the present study, such inhibition of graft rejection was not very strong (7 days longer than the control), presumably because the available duration and dynamics of the expanded Treg cells in the recipient were limited (Figure 8B).

In conclusion, our results confirm that supCD28 MAb expands rat Foxp3-expressing $CD4^+CD25^+$ nTreg cells in vivo, which maintain their unique cell-surface marker profile and inhibit alloreactive T-cell proliferation. Moreover, these expanded $CD4^+CD25^+$ Treg cells potently inhibit alloreactivity and prolong cardiac allograft survival. Thus, our findings illustrate again, and in a new

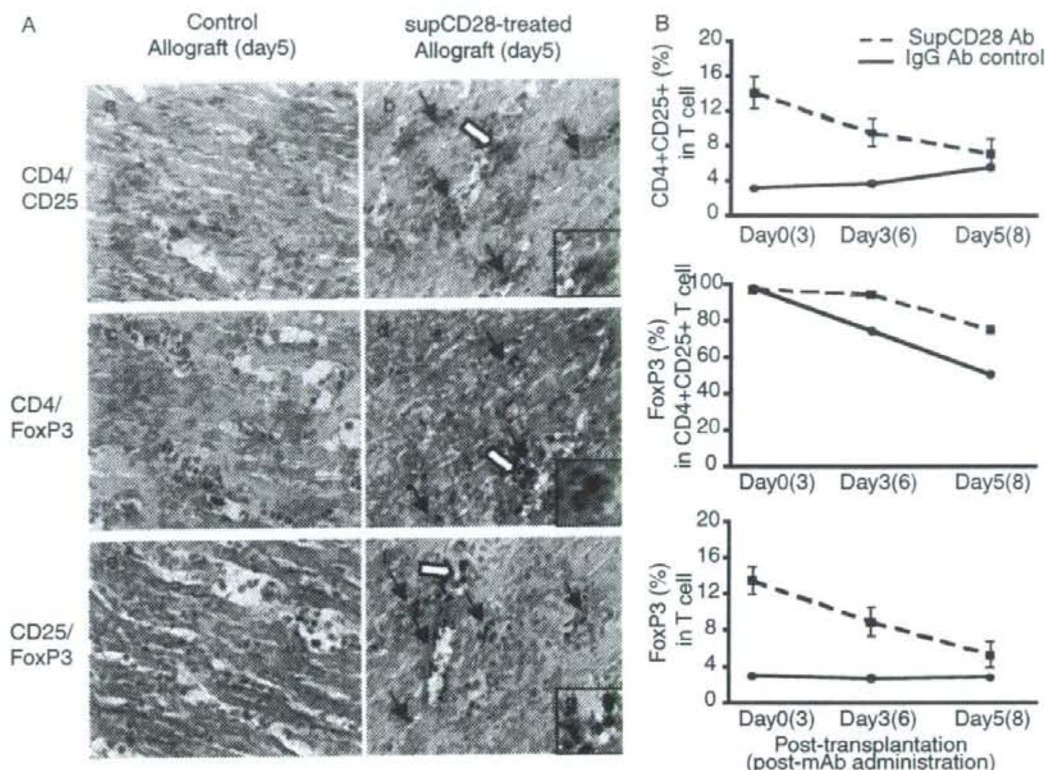

Figure 8. Immunohistologic analysis of cell infiltration in allografts and kinetic changes in the Foxp3 Treg cell population in recipient PB. (A) Cryosection of heart allografts from the groups (control and supCD28-treated) at 5 days after transplantation were stained for infiltration of $CD4^+$ (black)/$CD25^+$ (red) (a, b), $CD4^+$ (black)/$Foxp3^+$ (red) (c, d) and $CD25^+$ (black)/$Foxp3^+$ (red) (e, f). Foxp3 staining was exceedingly rare in allografts undergoing acute rejection (left), but readily detected as Foxp3 mononuclear cells in rats treated with supCD28 MAb. Original magnification: ×100 (inset has a higher original magnification of ×200, presented to show nuclear staining of the Foxp3 protein). (B) FCM analysis shows a higher population of $CD4^+CD25^+Foxp3^+$ T cells in recipient peripheral blood after supCD28 MAb injection, and a progressive decrease of this population at Day 5 after cardiac grafting. Although an increase in $CD4^+CD25^+$ cells can be observed after grafting in the control group, these cells are $Foxp3^-$, indicating activated T cells. The population of $Foxp3^+$ cells in the $CD4^+CD25^+$ T cells and total T cells decreased or was unchanged, respectively.

setting, the therapeutic power of transient polyclonal activation of Treg cells in vivo. Although a recent attempt to translate supCD28-mediated nTreg stimulation failed because of the unexpected induction of a life-threatening cytokine release syndrome,[28] transient polyclonal Treg activation as such, regardless of the tool used for its induction, still represents an attractive therapeutic option for autoimmunity and transplantation tolerance. Regardless of the feasibility of direct in vivo expansion, the ability to isolate and expand human $CD25^+CD4^+$ nTreg cells in vitro for re-infusion will allow the further biologic and biochemical characterization of this unique T-cell subset and expedite progress toward clinical use of Treg cells as a cellular therapy in the treatment of allograft rejection.

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REFERENCES

1. Asano M, Toda M, Sakaguchi N, Sakaguchi S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. J Exp Med 1996;184:387-96.
2. Hara M, Kingsley CI, Niimi M, et al. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. J Immunol 2001;166:3789-96.
3. Chiffoleau E, Beriou G, Dutartre P, et al. Role for thymic and splenic regulatory CD4+ T cells induced by donor dendritic cells in allograft tolerance by LF15-0195 treatment. J Immunol 2002; 168:5058-69.
4. Guo L, Fujino M, Kimura H, et al. Simultaneous blockade of co-stimulatory signals, CD28 and ICOS, induced a stable toler-

ance in rat heart transplantation. Transplant Immunol 2003; 12:41–8.
5. Tacke M, Hanke G, Hanke T, Hunig T. CD28-mediated induction of proliferation in resting T cells in vitro and in vivo without engagement of the T cell receptor: evidence for functionally distinct forms of CD28. Eur J Immunol 1997;27: 239–47.
6. Lin CH, Hunig T. Efficient expansion of regulatory T cells in vitro and in vivo with a CD28 superagonist. Eur J Immunol 2003;33: 626–38.
7. Luhder F, Huang Y, Dennehy KM, et al. Topological requirements and signaling properties of T cell-activating, anti-CD28 antibody superagonists. J Exp Med 2003;197:955–66.
8. Rodriguez-Palmero M, Hara T, Thumbs A, Hunig T. Triggering of T cell proliferation through CD28 induces GATA-3 and promotes T helper type 2 differentiation in vitro and in vivo. Eur J Immunol 1999;29:3914–24.
9. Schmidt J, Elflein K, Stienekemeier M, et al. Treatment and prevention of experimental autoimmune neuritis with superagonistic CD28-specific monoclonal antibodies. J Neuroimmunol 2003;140:143–52.
10. Rodriguez-Palmero M, Franch A, Castell M, et al. Effective treatment of adjuvant arthritis with a stimulatory CD28-specific monoclonal antibody. J Rheumatol 2006;33:110–8.
11. Beaudette-Zlatanova BC, Whalen B, Zipris D, et al. Costimulation and autoimmune diabetes in BB rats. Am J Transplant 2006;6: 894–902.
12. Wells AD, Gudmundsdottir H, Turka LA. Following the fate of individual T cells throughout activation and clonal expansion. Signals from T cell receptor and CD28 differentially regulate the induction and duration of a proliferative response. J Clin Invest 1997;100:3173–83.
13. Funeshima N, Fujino M, Kitazawa Y, et al. Inhibition of allogeneic T-cell responses by dendritic cells expressing transduced indoleamine 2,3-dioxygenase. J Gene Med 2005;7: 565–75.
14. Kitazawa Y, Fujino M, Wang Q, et al. Involvement of the programmed death-1/programmed death-1 ligand pathway in CD4+CD25+ regulatory T-cell activity to suppress alloimmune responses. Transplantation 2007;83:774–82.
15. Fujino M, Kitazawa Y, Kawasaki M, et al. Differences in lymphocyte gene expression between tolerant and syngeneic liver grafted rats. Liver Transplant 2004;10:379–91.
16. Fujino M, Li XK, Funeshima N, et al. Exogenous gene expression by Cre/loxP-mediated switching system containing adenovirus vector in mouse [in Japanese with English abstract]. Organ Biol 1998;5:45–52.
17. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003; 299:1057–61.
18. Beyersdorf N, Balbach K, Hunig T, Kerkau T. Large-scale expansion of rat CD+ CD25+ T(reg) cells in the absence of T-cell receptor stimulation. Immunology 2006;119:441–50.
19. Kretschmer K, Apostolou I, Hawiger D, et al. Inducing and expanding regulatory T cell populations by foreign antigen. Nat Immunol 2005;6:1219–27.
20. Malek TR, Bayer AL. Tolerance, not immunity, crucially depends on IL-2. Nat Rev Immunol 2004;4:665–74.
21. Zhang H, Chua KS, Guimond M, et al. Lymphopenia and interleukin-2 therapy alter homeostasis of CD4+CD25+ regulatory T cells. Nat Med 2005;11:1238–43.
22. Setoguchi R, Hori S, Takahashi T, Sakaguchi S. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. J Exp Med 2005;201:723–35.
23. Guo L, Fujino M, Kimura H, et al. AdCTLA-4Ig combined with donor splenocytes, bone marrow cells and anti-ICOS antibody treatment induce tolerance in a rat heart transplantation model. Transplant Int 2004;17:15–21.
24. Hofmann B, Tao K, Mai L, West LJ. Acceptance of related and unrelated cardiac allografts in neonatally tolerized mice is cardio-specific and transferable by regulatory CD4+ T cells. J Heart Lung Transplant 2004;23:1069–76.
25. Watanabe M, Mencel RL, Cramer DV, Starnes VA, Barr ML. Transforming growth factor-beta/interleukin-2-induced regulatory CD4+ T cells prolong cardiac allograft survival in rats. J Heart Lung Transplant 2005;24:2153–9.
26. Graca L, Cobbold SP, Waldmann H. Identification of regulatory T cells in tolerated allografts. J Exp Med 2002;195:1641–6.
27. Lee I, Wang L, Wells AD, Dorf ME, Ozkaynak E, Hancock WW. Recruitment of Foxp3+ T regulatory cells mediating allograft tolerance depends on the CCR4 chemokine receptor. J Exp Med 2005;201:1037–44.
28. Suntharalingam G, Perry MR, Ward S, et al. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. N Engl J Med 2006;355:1018–28.

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Survival of skin allografts is prolonged in mice with a dominant-negative H-Ras

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Abstract

Ras is a guanine nucleotide-binding protein that plays a major role in regulating the proliferation of T cells. To investigate the mechanism of the Ras/mitogen-activated protein kinase pathway, one of the downstream signal-transduction pathways of T-cell receptors, in the response to alloantigen, we performed full-thickness skin grafting in the major histocompatibility complex (MHC) incompatible strain BALB/c (H-2K[d]) (donor) and T-cell-specific H-Ras dominant-negative (dnRas) transgenic (tg) C57BL/6 (H-2K[b]) (recipient) male mice. *In vitro* and *in vivo* dnRas tg mouse T-cell proliferation and cytotoxic T lymphocyte (CTL) activity assay were also performed. The median graft survival time in control B6/wild type (wt) mouse allografts was seven days. Conversely, the dnRas tg mouse group exhibited a significant ($p<0.01$) prolongation of graft survival to 15 days. However, all grafts were eventually rejected after one month. Mixed lymphocyte reaction and popliteal lymph node assay revealed that T-cell proliferation was decreased in response to alloantigen, but CTL activity was not changed in the dnRas tg mice. These results suggested that Ras is essential for peripheral T lymphocytes to respond to allo-MHC antigens, and Ras may be a molecular target for controlling transplant rejection.
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Keywords: Dominant negative; Ras; Transgenic mouse; Skin graft

1. Introduction

Ras is a 21-kDa small guanosine triphosphate-binding protein and mediates important cellular signaling events that regulate activation, proliferation, motility, and other key cellular functions [1–3]. The localization of Ras to lipid bilayers facilitates its activation and subsequent activation of effector molecules Raf-1 serine/threonine kinase [4]. Raf-1 activates mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinases (ERK) kinase (MEK), which regulate ERK activity. Ras is widely known to play an important role in T-cell activation and function. The importance of Ras in immune cell physiology became evident when it was discovered that ligation of the T-cell receptor (TCR) led to Ras activation in T cells [5]. Subsequent studies demonstrated that Ras, along with calcineurin, mediates signals from the TCR to activate the nuclear factor of activated T cells (NFAT) [6]. Ras was also found to have an important role in T-cell interleukin (IL)-2 production [7]. Early work suggested that Ras had a necessary role in T-cell activation, while it was later shown that in some situations T cells could undergo Ras-independent activation [8].

Given the importance of Ras in T-cell activation and function, we tried to investigate the mechanism of the Ras/MAPK pathway, a downstream signal-transduction pathway of TCR/Lck, in the

Abbreviations: tg, transgenic; MHC, major histocompatibility antigen complex; TCR, T-cell receptor; Tregs, regulatory T cells; wt, wild type; IL, interleukin; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinases.

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response of the T cell to alloantigen. In the present study, we demonstrated that the skin allograft survival was prolonged, and mixed lymphocyte reaction and popliteal lymph node assay revealed a decrease in the T-cell proliferation response to alloantigen, but CTL activity was not changed in the mice with a dominant-negative Ras.

2. Objective

The lymphocyte-specific protein tyrosine kinase Lck plays a crucial role for TCR-mediated antigen-specific signal transduction of T lymphocyte. Little is known about the effects of its downstream such as the Ras/MAPK pathway on T lymphocyte mediated immune response to alloantigen. Here, we designed an experiment to investigate the involvement of the Ras/MAPK pathway by allogenic skin transplantation model with T-cell-specific dominant-negative Ras transgenic mice that are block from TCR stimulation.

3. Materials and methods

3.1. Animals

MHC incompatible strain BALB/c (H-2K^d) and C57BL/6 (B6) (H-2K^b) male mice, at an age of 8–12 weeks were obtained from Shizuoka, Laboratory Animal Center (Shizuoka, Japan). A T-cell-specific H-Ras dominant-negative (dnRas) tg B6 mice with the *lck* proximal promoter has been described [9]. All mice used in this study were maintained in our animal facility under specific pathogen-free conditions in accordance with institutional animal care policies.

3.2. Skin grafting

Full thickness skin grafting was performed with an adaptation of the method of Miyamoto et al. [10]. Briefly, skin was removed from the ventral wall of the donor mice and trimmed free of underlying fat, muscle, and panniculus. A graft bed of approximately 1.5×2 cm with an intact panniculus was prepared on the lateral thoracic wall of recipient mice. The donor skin was placed on this bed, anchored with silk sutures, and covered with a sterile surgical gauze dressing that was held in place with an adhesive elastic bandage. Dressings were removed on day 5–6 after grafting and the grafts were examined daily for evidence of rejection. Rejection was defined as the loss beyond 80% of the surface of the transplanted tissue.

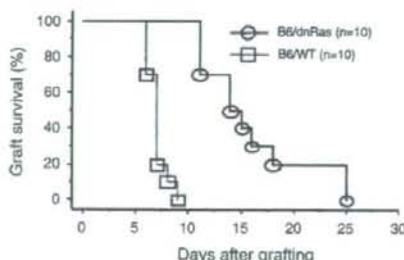

Fig. 1. Survival of allogeneic skin graft in B6/dnRas and B6/wt mice. The trunk skin grafts from BALB/c (H-2K^d) mice were transplanted to B6/dnRas (○; H-2K^b; n=10) mice and B6/wt (□; H-2K^b; n=10) mice. The survival of the skin grafts was observed daily. The median graft survival in control B6/wt allografts was seven days. In contrast, B6/dnRas tg mice exhibited a significant (p<0.01) prolongation of graft survival to 15 days. However, all grafts were eventually rejected after one month.

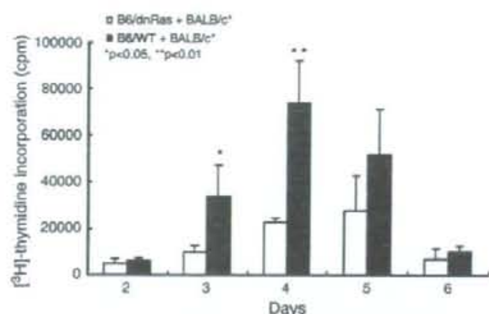

Fig. 2. Inhibition of T-cell response by alloantigen in splenic T cells from B6/dnRas tg mice *in vitro*. Splenic lymphocytes were harvested from naïve B6/wt or from B6/dnRas mice. Cells were then co-cultured with irradiated BALB/c splenocytes for 96 h. Proliferation was assessed by uptake of [^{3}H] thymidine for 4 h at the end of the culture period. Results are the mean± SD of three to five mice per group.

3.3. In vitro T-cell proliferation assay

In order to assay the allo-reactivities, the responder cells were purified and enriched with nylon wool column T cells from spleens of naïve and BALB/c skin grafted B6/dnRas tg mice or B6/wild type (wt) mice, respectively. Responder cells (2×10^5 T cells) were cultured with irradiated (20 Gy x-ray, MBR-1520A-TWZ, Hitachi, Tokyo, Japan) stimulators (4×10^5 spleen cells from BALB/c) in triplicate in the wells of 96-well V-bottom microtiter plates (Nunc, Wiesbaden, Germany) in a volume of 200 µl. RPMI-1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated FCS, 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin was used as culture medium. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air for 2–6 days. Controls included responder or stimulator alone. At the indicated time, individual micro-cultures were pulsed with 1 µCi ^{3}H-thymidine (Amersham, Buckinghamshire, UK) per well and incubated for another 18 h. Thereafter, cultures were harvested onto glass fibers and counted for beta emission using a trace-96 automatic filter counting system (Berthold, Inotech, Lansing, MI).

3.4. In vivo T-cell proliferation assay

The popliteal lymph node assay was used according to the modified method as described previously [11]. In brief, B6/dnRas or B6/wt mice were immunized with 5×10^6 BALB/c splenocytes in 50 µl PBS in their food pads. Seven days after immunization, the draining popliteal lymph node cells were removed. The weight of each node was determined and number of the lymph node was counted.

3.5. Generation of alloantigen specific CTL and cytotoxic assay

The cytotoxic assay was performed by a standard chromium ^{51}Cr-release assay. To generate allo-specific CTL, purified enriched with nylon wool column splenic T cells (2×10^6) from BALB/c skin grafted B6/dnRas or B6/wt recipient mice on 30 days after transplanted, were co-cultured with an equal number of irradiated (20 Gy) BALB/c splenocytes in the presence of 40 U/ml mouse recombinant IL-2 in 24-well plates. After 6 days of culture, viable cells were isolated by Ficoll-Hypaque (lympholyte-M, Cedarlane, Ontario) density gradient centrifugation and used as effector cells in cytotoxic assays. The ^{51}Cr-labelled A20 (H-2K^d) target cells were incubated with the effector cells at the various effector: target (E:T) ratios (40:1–5:1) for 4 h in a final volume of 200 µl. Cytotoxicity was estimated as a percentage of specific lysis and calculated as: specific lysis (%)=(experimental release−spontaneous release)×100/(maximum release−spontaneous release). The maximal or spontaneous ^{51}Cr release was obtained by the incubation of target cells with 1% Toriton X (Wako Pure Chemical Industries Ltd. Osaka, Japan) or medium only, respectively. The data were then expressed as the mean value of triplicate samples±SD.

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References

[1] Downward J. Targeting RAS signalling pathways in cancer therapy. Nat Rev Cancer 2003;3:11.
[2] Hancock JF. Ras proteins: different signals from different locations. Nat Rev Mol Cell Biol 2003;4:373.
[3] Oxford G, Theodorescu D. Ras superfamily monomeric G proteins in carcinoma cell motility. Cancer Lett 2003;189:117.
[4] Kikuchi A, Williams LT. The post-translational modification of ras p21 is important for Raf-1 activation. J Biol Chem 1994;269:20054.
[5] Downward J, Graves JD, Warne PH, Rayter S, Cantrell DA. Stimulation of p21ras upon T-cell activation. Nature 1990;346:719.
[6] Woodrow M, Clipstone NA, Cantrell D. p21ras and calcineurin synergize to regulate the nuclear factor of activated T cells. J Exp Med 1993;178: 1517.
[7] Rayter SI, Woodrow M, Lucas SC, Cantrell DA, Downward J. p21ras mediates control of IL-2 gene promoter function in T cell activation. Embo J 1992;11:4549.
[8] Lafont V, Ottones F, Liautard J, Favero J. Evidence for a p21(ras)/Raf-1/MEK-1/ERK-2-independent pathway in stimulation of IL-2 gene transcription in human primary T lymphocytes. J Biol Chem 1999;274:25743.
[9] Swan KA, Alberola-Ila J, Gross JA, Appleby MW, Forbush KA, Thomas JF, et al. Involvement of p21ras distinguishes positive and negative selection in thymocytes. Embo J 1995;14(2):276–85.
[10] Miyamoto M, Furui K, Kimura H. Male skin isografts can induce unresponsiveness in female rats. Transplantation 1980;30:180.
[11] Funeshima N, Fujino M, Kitazawa Y, Hara Y, Hara Y, Hayakawa K, et al. Inhibition of allogeneic T-cell responses by dendritic cells expressing transduced indoleamine 2,3-dioxygenase. J Gene Med 2005;7(5):565–75.
[12] Cantrell DA. GTPases and T cell activation. Immunol Rev 2003;192:122.
[13] Si MS, Ji P, Lee M, Kwok J, Kusumoto J, Naasz E, et al. Potent farnesyltransferase inhibitor ABT-100 abrogates acute allograft rejection. J Heart Lung Transplant 2005;24(9):1403–9.
[14] Rapoport MJ, Lazarus AH, Jaramillo A, Speck E, Delovitch TL. Thymic T cell anergy in autoimmune nonobese diabetic mice is mediated by deficient T cell receptor regulation of the pathway of p21ras activation. J Exp Med 1993;177:1221.
[15] Fields PE, Gajewski TF, Fitch FW. Blocked Ras activation in anergic CD4+ T cells. Science 1996;271:1276.
[16] Yamashita M, Kimura M, Kubo M, Shimizu C, Tada T, Perlmutter RM, et al. T cell antigen receptor-mediated activation of the Ras/mitogen-activated protein kinase pathway controls interleukin 4 receptor function and type-2 helper T cell differentiation. Proc Natl Acad Sci U S A 1999;96(3):1024–9.
[17] Li W, Whaley CD, Mondino A, Mueller DL. Blocked signal transduction to the ERK and JNK protein kinases in anergic CD4+ T cells. Science 1996;271:1272.
[18] Kang SM, Beverly B, Tran AC, Brorson K, Schwartz RH, Lenardo MJ. Transactivation by AP-1 is a molecular target of T cell clonal anergy. Science 1992;257:1134.
[19] Zha Y, Marks R, Ho AW, Peterson AC, Janardhan S, Brown I, et al. T cell anergy is reversed by active Ras and is regulated by diacylglycerol kinase-alpha. Nat Immunol 2006;7(11):1166–73 (Epub 2006 Oct 8).
[20] Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J Exp Med 1998;188:287.
[21] Li L, Godfrey WR, Porter SB, et al. CD4+CD25+regulatory T-cell lines from human cord blood have functional and molecular properties of T-cell anergy. Blood 2005;106:3068.
[22] Hickman SP, Yang J, Thomas RM, Wells AD, Turka LA. Defective activation of protein kinase C and Ras-ERK pathways limits IL-2 production and proliferation by CD4+CD25+regulatory T cells. J Immunol 2006;177:2186.
[23] Berg NN, Puente LG, Dawicki W, Ostergaard HL. Sustained TCR signaling is required for mitogen-activated protein kinase activation and degranulation by cytotoxic T lymphocytes. J Immunol 1998;161:2919.
[24] Puente LG, He JS, Ostergaard HL. A novel PKC regulates ERK activation and degranulation of cytotoxic T lymphocytes: plasticity in PKC regulation of ERK. Eur J Immunol 2006;36:1009.

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allograft survival. As depicted in Fig. 4, however, there was no significant difference between B6/wt and B6/dnRas tg mice in CTL activities in response to allo-MHC antigens.

5. Discussion

Current immunosuppressive anti-rejection therapies, which include calcineurin inhibitors, anti-proliferative agents, and corticosteroids, have significant side effects. There is thus a need to discover and develop new agents to be used as monotherapy or as an adjunct in a strategy to reduce the dosage of current immunosuppressive agents needed to prevent rejection. Ras, as well as calcineurin, is well known to have essential functions during T-cell activation [5]. A previous report demonstrated that interfering with the Ras function *in vitro* by transfection of T cells with dnRas genes blocked IL-2 gene induction after T-cell receptor activation, resulting in abrogation of T-cell activation and function [7]. Furthermore, *in vitro* studies have demonstrated that Ras and calcineurin synergize to activate NFAT in T cells [6]. Because conventional immunosuppressants such as cyclosporin A or Tacrolimus/FK506 are calcineurin inhibitors, inhibition of Ras was expected to produce synergistic activity in inhibition of T-cell proliferation and function. Therefore, we explored our hypothesis that inhibiting Ras function by transgenes with dnRas would abrogate allograft rejection [12].

The dnRas tg mice in this study that expressed relatively low copy numbers of the transgene, and only a weak inhibition of positive selection of T cells in the thymus was observed. In addition, slightly decreased but substantial numbers of splenic CD4 T cells were detected in the dnRas tg mice. In the spleen of dnRas tg mice, naive (CD44low) CD41 T cells show a normal range of surface phenotypes, such as usage of the variable region of the TCR β chain and expression levels of TCRαβ, CD3ε CD4, CD25, and CD69. The TCR-mediated signal-transduction pathways in T cells from dnRas tg mice have been shown that the freshly isolated splenic CD4 T cells responded normally to TCR crosslinking and mobilized intracellular calcium. Anti-TCR-mAb-induced TCR-phosphorylation was not impaired. In marked contrast, the activation of MAPK kinase (MAPKK) after anti-TCR stimulation, as determined by ERK phosphorylation, was compromised severely. Anti-TCR-induced proliferative responses and IL-2 production of naïve CD4 T cells of dnRas tg mice were decreased slightly, whereas the levels of IL-4 and interferon production were not decreased but increased [16].

First, in this study, we determined here for the first time that allograft rejection is delayed and survival of skin allograft is prolonged in dnRas tg mice, although the skin allografts did not survive indefinitely. One cause of this result may be that the inhibition of Ras function by dnRas is not complete in our dnRas tg mice. In addition, this result is partly in accord with previous reports indicating that, although the farnesyltransferase inhibitor which inhibits the function of Ras was able to abrogate acute allograft rejection using the rat cardiac transplantation model [13], increasing the inhibitor dose did not result in significant gains in anti-rejection activity. Furthermore, the farnesyltransferase inhibitor could not completely suppress immune cell activation and function, even at high drug exposure *in vitro*. Our results and those of others revealed that the Ras-independent pathway is due to the activation of immune cells or the presence of a population of lymphocytes that do not rely on Ras for robust activation [14,15].

T-cell anergy is one strategy for obtaining transplantation tolerance. Previous reports demonstrated that anergic T cells exhibit defective activation of Ras, which is associated with diminished activation of ERK and c-Jun N-terminal Kinase (JNK) [15,17], as well as blunted transactivation of AP-1 transcription factor [18]. In addition, a recent study demonstrated that constitutively active Ras restored cytokine production in anergic T cells [19]. Therefore, regulation of Ras may not only down-regulate T-cell function but may also down-regulate induction of anergy. Indeed, CD4+CD25+regulatory T cells (Tregs), which play an important role in maintenance of self tolerance, are characteristically anergic to TCR stimuli mediated by APC and fail to expand [20]. Further, recent analyses demonstrated that Tregs uncovered defects in Ras, MEK, and ERK activation as well as the delay and reduced appearance of NFAT in the nucleus, resulting from impaired calcium flux [21,22].

We next demonstrated that the alloantigen-induced proliferative response of splenic T cells from dnRas tg mice was significantly decreased *in vitro*, which is similar to anti-TCR monoclonal antibody stimulation [16]. Furthermore, popliteal lymph node assay revealed that *in vivo* T-cell response was also more inhibited in B6/dnRas tg mice than in B6/wt mice. These *in vitro* and *in vivo* data demonstrated that Ras is involved in allograft rejection via the T lymphocyte proliferation response to alloantigen. Further, previous studies indicated the relationship between Ras defective and anergy induction [15,17–22], and suggested that T lymphocytes from dnRas tg mice partially may retain features of anergy.

Lastly, in contrast with the results of the *in vitro* and *in vivo* T-cell proliferation response, CTL activity was not changed in CTL generated from B6/dnRas tg mice. A previous study demonstrated that ERK activation is required for TCR-triggered CTL activity [23]. This result suggested that CLT activity might be independent of Ras. Puente et al. [24] indicated that activation of ERK through a conventional protein kinase C (PKC) pathway acting downstream or independent of Ras leads to ERK activation, which is consistent with our data. In our alloantigen-stimulated CTL generation, CTL activity was probably the same or similar to that of ERK activation through PKC in response to TCR ligation.

In conclusion, we demonstrated here for the first time that skin allograft rejection and allo-immune response *in vitro* and *in vivo* are prevented in T-cell specific dnRas tg mice. These results suggested that blockading Ras signaling in T cells might effectively inhibit allograft rejection via T-cell proliferation response to alloantigen, and may provide a new immunomodulatory strategy to prevent the allo-rejection of organ transplants.

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References

[1] Downward J. Targeting RAS signalling pathways in cancer therapy. Nat Rev Cancer 2003;3:11.

[2] Hancock JF. Ras proteins: different signals from different locations. Nat Rev Mol Cell Biol 2003;4:373.

[3] Oxford G, Theodorescu D. Ras superfamily monomeric G proteins in carcinoma cell motility. Cancer Lett 2003;189:117.

[4] Kikuchi A, Williams LT. The post-translational modification of ras p21 is important for Raf-1 activation. J Biol Chem 1994;269:20054.

[5] Downward J, Graves JD, Warne PH, Rayter S, Cantrell DA. Stimulation of p21ras upon T-cell activation. Nature 1990;346:719.

[6] Woodrow M, Clipstone NA, Cantrell D. p21ras and calcineurin synergize to regulate the nuclear factor of activated T cells. J Exp Med 1993;178: 1517.

[7] Rayter SI, Woodrow M, Lucas SC, Cantrell DA, Downward J. p21ras mediates control of IL-2 gene promoter function in T cell activation. Embo J 1992;11:4549.

[8] Lafont V, Ottones F, Liautard J, Favero J. Evidence for a p21(ras)/Raf-1/MEK-1/ERK-2-independent pathway in stimulation of IL-2 gene transcription in human primary T lymphocytes. J Biol Chem 1999;274:25743.

[9] Swan KA, Alberola-Ila J, Gross JA, Appleby MW, Forbush KA, Thomas JF, et al. Involvement of p21ras distinguishes positive and negative selection in thymocytes. Embo J 1995;14(2):276–85.

[10] Miyamoto M, Furui K, Kimura H. Male skin isografts can induce unresponsiveness in female rats. Transplantation 1980;30:180.

[11] Funeshima N, Fujino M, Kitazawa Y, Hara Y, Hara Y, Hayakawa K, et al. Inhibition of allogeneic T-cell responses by dendritic cells expressing transduced indoleamine 2,3-dioxygenase. J Gene Med 2005;7(5):565–75.

[12] Cantrell DA. GTPases and T cell activation. Immunol Rev 2003;192:122.

[13] Si MS, Ji P, Lee M, Kwok J, Kusumoto J, Naasz E, et al. Potent farnesyltransferase inhibitor ABT-100 abrogates acute allograft rejection. J Heart Lung Transplant 2005;24(9):1403–9.

[14] Rapoport MJ, Lazarus AH, Jaramillo A, Speck E, Delovitch TL. Thymic T cell anergy in autoimmune nonobese diabetic mice is mediated by deficient T cell receptor regulation of the pathway of p21ras activation. J Exp Med 1993;177:1221.

[15] Fields PE, Gajewski TF, Fitch FW. Blocked Ras activation in anergic CD4+ T cells. Science 1996;271:1276.

[16] Yamashita M, Kimura M, Kubo M, Shimizu C, Tada T, Perlmutter RM, et al. T cell antigen receptor-mediated activation of the Ras/mitogen-activated protein kinase pathway controls interleukin 4 receptor function and type-2 helper T cell differentiation. Proc Natl Acad Sci U S A 1999;96(3):1024–9.

[17] Li W, Whaley CD, Mondino A, Mueller DL. Blocked signal transduction to the ERK and JNK protein kinases in anergic CD4+ T cells. Science 1996;271:1272.

[18] Kang SM, Beverly B, Tran AC, Brorson K, Schwartz RH, Lenardo MJ. Transactivation by AP-1 is a molecular target of T cell clonal anergy. Science 1992;257:1134.

[19] Zha Y, Marks R, Ho AW, Peterson AC, Janardhan S, Brown I, et al. T cell anergy is reversed by active Ras and is regulated by diacylglycerol kinase-alpha. Nat Immunol 2006;7(11):1166–73 (Epub 2006 Oct 8).

[20] Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J Exp Med 1998;188:287.

[21] Li L, Godfrey WR, Porter SB, et al. CD4+CD25+regulatory T-cell lines from human cord blood have functional and molecular properties of T-cell anergy. Blood 2005;106:3068.

[22] Hickman SP, Yang J, Thomas RM, Wells AD, Turka LA. Defective activation of protein kinase C and Ras-ERK pathways limits IL-2 production and proliferation by CD4+CD25+regulatory T cells. J Immunol 2006;177:2186.

[23] Berg NN, Puente LG, Dawicki W, Ostergaard HL. Sustained TCR signaling is required for mitogen-activated protein kinase activation and degranulation by cytotoxic T lymphocytes. J Immunol 1998;161:2919.

[24] Puente LG, He JS, Ostergaard HL. A novel PKC regulates ERK activation and degranulation of cytotoxic T lymphocytes: plasticity in PKC regulation of ERK. Eur J Immunol 2006;36:1009.

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Superagonistic CD28 Antibody Induces Donor-Specific Tolerance in Rat Renal Allografts

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The ultimate goal of organ transplantation is to establish graft tolerance where CD4+CD25+FOXP3+ regulatory T (Treg) cells play an important role. We examined whether a superagonistic monoclonal antibody specific for CD28 (CD28 SA), which expands Treg cells *in vivo*, would prevent acute rejection and induce tolerance using our established rat acute renal allograft model (Wistar to Lewis). In the untreated or mouse IgG-treated recipients, graft function significantly deteriorated with marked destruction of renal tissue, and all rats died by 13 days with severe azotemia. In contrast, 90% of recipients treated with CD28 SA survived over 100 days, and 70% survived with well-preserved graft function until graft recovery at 180 days. Analysis by flow cytometry and immunohistochemistry demonstrated that CD28 SA induced marked infiltration of FOXP3+ Treg cells into the allografts. Furthermore, these long-surviving recipients showed donor-specific tolerance, accepting secondary (donor-matched) Wistar cardiac allografts, but acutely rejecting third-party BN allografts. We further demonstrated that adoptive transfer of CD4+CD25+ Treg cells, purified from CD28 SA-treated Lewis rats, significantly prolonged allograft survival and succeeded in inducing donor-specific tolerance. In conclusion, CD28 SA treatment successfully induces donor-specific tolerance with the involvement of Treg cells, and thus the therapeutic value of this approach warrants further investigation and preclinical studies.

Key words: CD28, regulatory T cell, tolerance, transplantation

Abbreviations: APCs, antigen-presenting cells; CD28 SA, superagonistic monoclonal antibody specific for CD28; FACS, fluorescence-activated cell sorting; HE, hematoxylin and eosin; INF, infinity; MHC, major histocompatibility complex; PAS, periodic acid Schiff; PI3 K, phosphatidylinositol 3-kinase; Qu, quarter; TCR, T-cell receptor; Treg, regulatory T.

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Introduction

Renal transplantation has become a common procedure for end-stage renal diseases that are otherwise uncontrollable by medical treatment. The allograft rejection by host's immune system can be ameliorated but not eliminated by introduction of various immunosuppressive drugs. In addition, sustained immunosuppression leaves the recipients susceptible to infectious complications. Therefore, the development of donor-specific tolerance without the requirement for lifelong immunosuppression has been the goal for organ transplantation. One of the most promising strategies for maintaining tolerance is the development of naturally arising CD4+CD25+FOXP3+ regulatory T (Treg) cells (1). These T cells have been suggested to alter the actions of both antigen-presenting cells (APCs) and effector T cells.

T cells mediate the allograft response, and naïve T cells require two distinct signals for activation. The first signal is provided by engagement of the T-cell receptor (TCR) with the major histocompatibility complex (MHC) molecule plus peptide complex on the APCs. The second signal, the so-called costimulatory signal, is provided by engagement of T-cell surface receptors with their ligands on the APCs. Signaling through the TCR alone without a constimulatory signal leads to a prolonged state of T-cell anergy (2). Among the multiple constimulatory pathways identified interaction of CD28 on T cells with either of two ligands, B7-1 or B7-2,

on the APC is the most important constimulatory pathway involved in the response to alloantigens (3).

Recently, the possibility of targeting CD28 as a therapeutic approach has been highlighted by several findings. One anti-rat CD28 antibody (JJ319) has been shown to induce CD28 internalization *in vivo* (4). While this antibody, JJ319, may transiently activate T cells after receptor engagement, its effect appears to be equivalent to functional CD28 blockade in that it inhibits the alloreactive response of T cells stimulated by allogeneic APCs without causing T-cell depletion (5). Although this antibody prevented allograft vasculopathy in a rat chronic kidney rejection model, it was shown to delay heart allograft rejection without tolerance induction (5).

More recently, Hünig et al. reported a novel class of CD28 antibody, JJ316, the so-called superagonistic CD28 antibody (CD28 SA), which can induce the *in vitro* and *in vivo* expansion of T cells without any need for TCR engagement (4,6,7). Of interest is that CD28 SA can expand CD4+CD25+ Treg cells (8). While the conventional CD28 antibody binds to a membrane-distal part of CD28, which is close to the B7 binding site, CD28 SA binds to a lateral, membrane-proximal loop of the CD28 (6,9,10). While conventional CD28 antibodies can bind CD28 and form a tangled structure, interaction of CD28 SA with the extracellular domain of CD28 leads to the formation of a linear complex (6), suggesting that this event results in strong activation due to aggregation of stimulatory signaling components such as phosphatidylinositol 3-kinase (PI3 K). As administration of CD28 SA *in vivo* leads to the preferential expansion and strong activation of naturally occurring CD4+CD25+FOXP3+ Treg cells over conventional T cells, CD28 SA therapy may be markedly effective for induction of tolerance in transplant recipients.

In the present study, we demonstrated that treatment with CD28 SA markedly increased infiltration of CD4+CD25+FOXP3+ Treg cells into allogeneic renal allografts as well as their increment in the spleen and peripheral blood. We also found that CD28 SA afforded effective allograft protection with good preservation of graft function and histological structure. Furthermore, examination of secondary cardiac bi-allografts of both donor-matched Wistar heart and third-party BN heart 120 days after kidney transplantation proved that CD28 SA could develop donor-specific tolerance. In addition, adoptive transfer of Treg cells collected from CD28 SA-treated rats induced donor-specific tolerance, suggesting that CD28 SA-induced Treg cells were able to induce donor-specific tolerance in transplantation.

Materials and Methods

Acute renal transplantation rejection model

Inbred 200–250 g male Lewis rats (SLC Inc., Hamamatsu, Japan) were used as graft recipients, and MHC-incompatible male Wistar rats (SLC Inc.) served as donors. The left kidney was removed from a Wistar rat after perfusing it with ice-cold heparinized saline (1 U/mL) through the renal artery. The kidney was transplanted orthotopically into a bilaterally nephrectomized Lewis recipient by end-to-end anastomosis of the left renal vessels and the left ureter using microsurgical techniques (11). During the procedure, the donor kidney was subjected to 30 min of cold ischemic injury. All experimental protocols were conducted in accordance with the policies of the Animal Ethics Committee of our institution. In this model, animals typically suffer acute rejection, become oligo- or anuric and die due to uremia within 10 days. Only animals dying due to surgical technical failure within the first 24 h after transplantation were excluded from the analysis.

Effect of CD28 SA on the acute rejection model

The generation of CD28 SA has been described previously (4). Lewis recipients received triple intravenous injection of CD28 SA (0.5 mg) 3 days before, on the day of and 3 days after transplantation ($n = 8$; CD28 SA group). We employed disease control recipient rats untreated ($n = 10$; untreated group) or treated with triple intravenous injection of mouse IgG (0.5 mg) 3 days before, on the day of and 3 days after transplantation ($n = 8$; mIgG group). Age-matched bilaterally nephrectomized isografts recipients (Lewis to Lewis, $n = 8$) were used as another control (isograft group). In order to investigate the effect of CD28 SA on acute rejection, three animals from each group were randomly selected; their donor kidneys were recovered on day 6 and prepared for histological analysis, immunohistochemical study, RT-PCR analysis and flow cytometric analysis. The remaining animals from each group were subjected to the evaluation of graft survival. The survival time of the renal allograft was defined as the time from transplantation to the time of death.

To examine whether CD28 SA can induce donor-specific 'tolerance' or nonspecific immunosuppression, all CD28 SA-treated recipients, which accepted allograft kidney for 120 days, received bilateral cardiac allografts from both donor-matched Wistar and third-party BN (SLC Inc.) in each animal on day 120 (12). Age-matched untreated Lewis rats also received cardiac allograft from Wistar rats as acute cardiac rejection model.

At recovery of organ, blood samples and spleen were collected for flow cytometric analysis. The kidneys were perfused with cold autoclaved phosphate-buffered saline (PBS), and samples of the cortex were taken for flow cytometric analysis and histology.

Graft function and morphology

Twenty-four-hour urine was collected on alternate days after transplantation. Urine volume was measured serially, and urinary sediment was examined in each sample. We then assessed graft function more quantitatively by measuring serial serum creatinine levels in the recipients. Serum was obtained on alternate days by tail vein puncture, and serum creatinine was measured by the Jaffe reaction method.

Renal tissues were fixed in 4% paraformaldehyde in PBS. Paraffin sections were stained with hematoxylin and eosin (HE), periodic acid Schiff (PAS) and Mallory Azan and then assessed by light microscopy. The animals were coded so that analysis was performed without any knowledge of which treatment each individual animal had received. Histological evaluation was performed on the basis of the Banff criteria.

Staining for FOXP3 (FJK-16s, eBioscience, Inc., San Diego, CA), vimentin (Dako Cytomation, Glostrup, Denmark), αSMA (Immunotech, Marseilles, France) and ED1 (Serotec, Raleigh, NC) was performed by the immunoperoxidase method. ED1- and FOXP3-positive cells were counted in the cortex at ×200 magnification in a minimum of 10 fields.