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AdCTLA-4Ig combined with donor splenocytes, bone marrow cells and anti-ICOS antibody treatment induce tolerance in a rat heart transplantation model

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Abstract It is difficult to induce rat cardiac allograft tolerance by co-stimulator blockade of the B7-CD28 pathway with CTLA-4Ig monotherapy alone. However, combined therapies of AdCTLA-4Ig plus donor-specific spleen-cell infusion, bone marrow cell infusion, and anti–ICOS antibody have been demonstrated to effectively induce indefinite acceptance of rat cardiac allografts. In this report, we compared the tolerance of cardiac allograft tolerant recipients induced by the above three strategies. The results show that treating Lewis recipients of a DA cardiac allograft with a combination of AdCTLA4-Ig and anti-ICOS antibody, donor splenocytes or bone marrow cells produced indefinite graft survival. Second transplantation of donor type skin or heart grafts could not affect the survival of primary heart graft in anti-ICOS treated groups, but results in rejection of primary heart grafts in other two groups, and that co-stimulator blockade, CD28 and ICOS simultaneously with CTLA-4Ig and anti-ICOS antibody, facilitates the development of CD25+ CD4+ regulatory T cells and induces stable transplantation tolerance in the rat cardiac allograft model. This also provides an effective therapy in clinical transplantation for promoting permanent graft survival by generating regulatory T cells.

Keywords Allograft · Gene therapy · Co-stimulator molecular · Regulatory T cell · Tolerance

Abbreviations *BMC* bone marrow cells · *ICOS* inducible co-stimulator · *pfu* plaque-forming units · *SPC* spleen cell

Introduction

Co-stimulatory signals between antigen-presenting and responding cells are essential for a normal alloimmune response. Blocking these pathways during initial graft-host interaction has been used to ameliorate destructive responses or prevent them from continuing. Co-stimulatory blockade thus has significant potential as a future immune-modulating mechanism for use in clinical transplantation.

The CD80-CD86 pathway is a critical co-stimulatory pathway required to fully activate T cells and IL-2 production during alloimmune responses. Blocking this pathway with a recombinant CTLA-4Ig protein has been reported to prolong allograft survival in experimental transplantation models and to induce tolerance in some instances. Our previous study also demonstrated that an adenovirus vector containing the CTLA-4Ig gene effectively prolonged heart allograft similarly to recombinant CTLA-4Ig protein (our unpublished data). However, neither monotherapy with recombinant CTLA-4Ig protein nor adenovirus vector could induce stable heart allograft tolerance to a strong response combination of DA to Lewis strain rats. Therefore,

combined therapies of CTLA-4Ig plus donor-specific transfusion [1], bone marrow infusion [2], or CD154 blockade by anti-CD154 antibody have been tried [3] and demonstrated to effectively induce long-term acceptance of heart allografts. Some studies, including ours, have indicated that treatment against an inducible co-stimulator (ICOS) with an antibody significantly prolonged survival of liver allografts [4] and combined therapy with co-stimulator blockade, for instance with CTLA-4Ig or antibody to CD154, simultaneously induced an indefinite acceptance of heart allografts [5].

These studies suggest that co-stimulatory blockade is very important in inducing and maintaining tolerance. However, the tolerance state induced by different strategies has not been studied yet. In this study, we compared the tolerant state of recipients induced by three different strategies in rat heart transplantation models. Our result indicated that 1) different treatments produce different tolerances, 2) combined treatment of CTLA-4Ig and anti-ICOS antibody resulted in the development of CD25+CD4+ regulatory T cells and induced a stable donor-specific tolerance, and 3) the maintenance of donor-specific tolerance in combined treatment of CTLA-4Ig and anti-ICOS antibody was an active immunological process in which CD25+CD4+ regulatory T cells may play an important role in maintaining heart-allograft tolerance.

Materials and methods

Adenoviral vector

An adenovirous containing the expression cassette for the human CTLA-4Ig gene or Escherichia coli β-galactosidase gene (LacZ) was constructed by homologous recombination of the expression cosmid cassette (pAdex/CAhCTLA4Ig) [6] and the parental virus genome [7]. The recombinant viruses were subsequently propagated with 293 cells, and the prepared vector solutions were stored at –80°C. The adenovirus containing the CTLA-4Ig gene was termed Ad CTLA-4Ig, and that containing AdLacZ was termed LacZ.

Animals and antibody

We used adult male Lewis (RT1 l) rats as recipients, and DA (RT1a) or BN (RT1n) rats as donors. The animals weighed 210 to 250 g and were maintained under standard conditions of feeding with rodent food and water, according to the principles of laboratory animal care and guide for the use of laboratory animals in our institution. Mouse monoclonal IgG1 to rat ICOS (JTT.1) (anti-ICOS mAb) was generated by a method described previously [8].

Heart and skin transplant model

For primary heart transplantation, DA hearts were transplanted into the abdominal cavity of Lewis recipients by the method reported by Ono and Lindsey [9]. The recipients received an intravenous single dose of anti-ICOS mAb at 1 mg/kg and /or Ad CTLA-4Ig at 10^9 plaque-forming units (pfu) immediately after transplantation. Spleen cells from donor rats were obtained by grinding with frosted objective slides in PBS and subsequently overlaid on Ficoll Isopaque (Lympholyt-Rat). After centrifugation at 3000 g for 20 min, the cells of the interface layer were harvested and washed twice with PBS. Single-cell suspensions of bone marrow were obtained by flushing donor tibias and femurs with 20 ml of PBS and washed twice with PBS. Viability of the cells in suspension was estimated by trypan blue dye exclusion and consistently exceeded 90%. Cell suspensions were adjusted to 5×10^7/ ml in 1 ml PBS and injected via the tail vein after transplantation. The recipients without any treatment and those administered AdLacZ served as controls. Graft rejection was defined as a cessation of graft palpitation and was confirmed histologically by mononuclear cell infiltration and myocyte necrosis in the graft sections with hematoxylin and eosin staining.

Some recipients with a graft survival over 100 days received a transplanted full-thickness donor-type skin graft to the lateral thoracic wall. No further immunosuppression was attempted after skin grafting. The final day of skin graft survival was determined as the time when the viable area was reduced to less than 10% of the graft. In some recipients, secondary hearts from specific donor (DA) or third party (BN) rats were grafted into the cervical location using the cuff technique, essentially as described previously [10]. No further immunosuppression was attempted after skin, secondary and third party heart grafting.

Histological studies

Heart grafts were harvested on rejection of the primary heart graft after donor skin grafting and secondary heart grafting from tolerant recipients. The samples were formalin-fixed and embedded in paraffin for hematoxylin and eosin (HE) staining.

Lymphocyte preparation and adoptive transfer

Spleen and mesenteric lymph nudes were harvested from long-term accepted recipients when the allograft survival reached more than 100 days, and from naïve Lewis rats. We gently grounded these spleens and mesenteric lymph nudes with frosted objective slides in PBS for single cell-suspension preparation. After lysing erythrocytes with RBC lysis buffer (Sigma, St. Louis Mo.), the mixture of lymphocytes were washed three times. For adoptive transfer study, the lymphocytes were intravenously administered at 5×10^7 cells into 7.5 Gy-irradiated Lewis rats (secondary recipients) and, thereafter, heart transplantation from specific donors was performed in the abdominal cavity. For sorting CD4+CD25+ T cell fraction, the lymphocytes were stained with PE-conjugated anti-CD25 antibody (αchain of IL-2 receptor, Pharmingen) for 15 min, and then washed and re-suspended in 80 µl of sorting buffer per 10^7 total cells. We added 20 µl of MACS-anti-PE micro-beads in this cell suspension and incubated for 15 min in refrigerator at 4°C. For cell separation, 10^8 cells were suspended in 500 µl of buffer, and applied onto the column of Auto-MACS. After passage of the negative cells through the column, we collected CD25+ cells and stained the cells with FITC-conjugated anti-CD4 antibody (Pharmingen) for FACS sorting. Viability of the cells was estimated by trypan blue dye exclusion and consistently exceeded 90%. The cells without CD4+CD25+ T cell fraction were collected and subjected to the same process as described above. Lymphocytes from naïve Lewis rat were subjected to the same protocol using as controls.

Flow cytometric analysis

The peripheral blood from all long-term accepted recipients (over 100 days) before re-transplantation or adoptive transfer and that from syngeneic recipients was collected and overlaid on Ficoll

Isopaque (Lympholyt-rat, CEDARLANE, Ontario). After centrifugation at 3000 g for 20 min, the cells of the interface layer were harvested and suspended at 2×10^6/ ml in PBS. The cells (2×10^6) were incubated at 4°C for 30 min with a saturating concentration of PE-conjugated anti-CD25 (αchain of IL-2 receptor, OX-39, Pharmingen) in combination with FITC-conjugated anti-CD4 antibody (Pharmingen) diluted with PBS containing 1% fetal calf serum. The cells were washed and resuspended in 1 ml of PBS, followed by analysis with flow cytometry (FACScn, Becton Dickinson). The dead cells were excluded from the analysis using propidium iodide fluorescence.

Statistics

We statistically evaluated graft survival using Kaplan-Meier's test. The difference in the population of CD25+CD4+ T-cell subsets was analyzed for significance using the Student's *t* test.

Results

Primary heart allograft survival

As shown in Table 1, AdCTLA-4Ig treatment combined with transfusion of donor SPC or BMC simultaneously after transplantation induced long-term survival (more than 100 days) (Groups 4 and 5). Combination of AdCTLA-4Ig and simultaneous anti-ICOS antibody treatment results in indefinite acceptance of heart allografts (exceeding 300 days except the animals subjected to histological analysis) (Group 6).

Skin graft in long-term accepting recipients

Naïve Lewis rat and three long-term graft-accepting recipients from Groups 4, 5 and 6 received the donor strain skin grafts after the primary heart graft had been accepted for over 100 days. As shown in Table 2, skin grafts in Group 4 and 5 were rejected completely at 11.7 days (mean) 12 days (mean), similar to the control group 12 days (mean). However, the rejection of skin grafts in Group 6 was little delayed (15.7 days). In accordance with skin rejection, the primary heart grafts in Group 4 and 5 were also rejected at 14.7 days (mean) and 12.7 days (mean) respectively. After skin grafting, unlike those Groups, the recipients in Group 6 accepted the primary heart graft indefinitely, despite the complete rejection of donor skin grafts.

Table 2 Survival of primary heart grafts and skin grafts after donor-strain skin grafting in tolerant recipients: *: survival of primary heart grafts were calculated from the time point of grafting. [a] $P<0.05$ compared with Groups 4, 5 and Control group [b]: $P<0.001$ compared with Group 3 and 4

Group	*n*	Skin graft (days)	Mean	Primary heart grafts* (days)	Mean
Naïve Lewis	5	11×2, 12×3	12	ND	
Group 4	3	11, 12×2	11.7	12, 14, 18	14.7
Group 5	3	12×3	12	11, 12, 15	12.7
Group 6	3	15, 16×2	15.7[a]	>200×3	>200[b]

Secondary heart graft in long-term accepting recipients

Three recipients with long-term accepted primary heart grafts (over 100 days) from Groups 4, 5, and 6 were challenged with a secondary donor strain heart graft. The secondary heart grafts were rejected when transplanted into Groups 4 and 5. The secondary heart graft rejection in Group 4 (4 days) and 5 (4.3 days) was a little faster than in the Control group (5 days), but no significant differences were seen. Only secondary heart graft acceptance was observed in Group 6 (more than 100 days). In accordance with secondary heart graft rejection, the primary heart grafts were rejected by 20 days in Groups 4 and 5 after secondary heart grafting. Re-challenge of secondary heart grafts into the recipients from Group 6 did not affect the survival of primary heart grafts (Table 3).

Third-party heart grafts in long-term accepting recipients

BN hearts were transplanted as a third-party graft into the recipients with long-term accepted primary heart grafts in Groups 4, 5, and 6. These recipients rejected the BN heart following a similar time course as naïve Lewis

Table 1 Survival of primary heart grafts. [a] recipients used for donor-strain skin grafting, [b] and histological study, [c] secondary donor-strain heart grafting, [d], third party (BN) heart grafting, and [e], adoptive transfer study. *P**: compared with Group 2 and 3. All the long-term survival recipients were used for FACS study before subsequently treatment

Group	Treatment	*n*	Survival	Median	*P**
1	Syngenic	3	>100×3	>100	
2	No-treatment	10	5×3, 6×7	6	
3	AdLacZ	3	5×2, 6	5	
4	AdCTLA-4Ig+SPC	11	>100×3[a,b] >100×3[c], >100×3[d] >100×2[e]	>100	<0.001
5	AdCTLA-4Ig+BMC	11	>100×3[a,b] >100×3[c], >100×3[d] >100×2[e]	>100	<0.001
6	AdCTLA-4Ig+anti-ICOS	14	>100×3[a,b], >100×3[c], >100×3[d], >100×5[e]	>100	<0.001

Table 3 Survival of primary and secondary heart grafts after secondary heart grafting in tolerant recipients: * survival of primary heart grafts were calculated from the time point of secondary heart grafting. $^{ab}P < 0.05$ compared with Group 3 and 4

Group	*n*	Secondary heart graft (days)	Mean	Primary heart graft* (days)	Mean
Group 4	3	3,4, 5	4	13, 14, 28	18.3
Group 5	3	4×2,5	4.3	13, 15, 30	19.3
Group 6	3	>100×3	>100[a]	>200×3	>200[b]

Table 4 Survival of primary and third-party heart grafts after third-party heart grafting: * survival of primary heart graft were calculated from the time point of third-party heart grafting

Group	*n*	Third-party heart graft (days)	Mean	Primary heart graft* (days)	Mean
BN to Naïve LEW	5	5×3, 6×2	5.4		
Group 4	3	6×3	6	>50	>50
Group 5	3	4, 5×2	4.7	>50	>50
Group 6	3	5×3	5	>50	>50

rats. However, the primary heart grafts beat normally (more than 50 days) (Table 4).

Adoptive transfer

Some recipients with long-term accepted primary heart grafts (over 100 days) from Groups 4, 5, and 6 were used for adoptive transfer study. After whole body irradiation (7.5 Gy) of Lewis rats (secondary recipients), prepared lymphocytes (5×10^7) from long-term survival recipients and naive Lewis rats with or without subtraction of CD4+CD25+ T cell fraction were transferred and, thereafter, donor-strain hearts were transplanted. As showed in Table 4, the recipients received lymphocytes from naive Lewis rats, Group 4 and 5 rejected donor-strain hearts on 13.6, 14.5 and 14.8 days (mean) after grafting respectively. However, secondary recipients received lymphocytes from Group 6 significantly prolonged the survival of heart graft to 25 days (mean). Subtraction of CD4+CD25+ T cells from naïve Lewis lymphocytes did not significantly affect the survival of heart graft (mean 15 days), and those received the cells from Group 6 without CD4+CD25+ T cell fraction resulted in no significant prolongation of graft survival (mean 16.7 days) (Table 5).

Histological studies after skin grafting and secondary heart grafting

The heart grafts from Groups 4, 5, and 6 were harvested whether donor-specific skin grafts were rejected or not.

Table 5 Graft survival in irradiated Lewis rats transferred with lymphocytes from long-term survival recipients and naïve Lewis rats: * compared with other groups Subtraction of CD4+CD25+ T cell fraction were performed by Auto-MACS with anti-CD25 antibody and sorted by FACS with anti-CD4 antibody

Transfer cells	*n*	Survival (days)	Mean	*P**
Naïve Lewis	3	13, 14×2	13.6	
Group 4	4	14×2, 15×2	14.5	
Group 5	5	12, 15×2, 16×2	14.8	<0.001
Group 6	4	21, 25×2, 29,	25	
Na Lewis without CD4+CD25+	3	14×2, 17	15	
Group 6 without CD4+CD25+	4	15×3, 18	15.7	

An extensive perivascular accumulation of mononuclear cells was seen in Groups 4 and 5, and myocyte necrosis and bleeding were also noted after skin grafting (Fig. 1A, B). A slight perivascular accumulation of mononuclear cells with normal tissue architecture was seen in Group 6 on day 50 after skin grafting (Fig. 1C). The primary heart grafts (Fig. 1D, E) and secondary heart grafts (Fig. 1G, H) from Groups 4 and 5 were also harvested after rejection. Extensive perivascular accumulations of mononuclear cells with architecture damage of myocytes were seen in both primary and secondary heart grafts. An almost normal histological finding was observed in both primary and secondary heart grafts in Group 6 on day 50 after secondary heart grafting (Fig. 1F, I).

Population CD25+CD4+ T cells in peripheral lymphocytes

We performed FACS analysis using peripheral lymphocytes prepared from long-term accepting recipients (Groups 4, 5, 6 and syngenic recipients) before retransplantation or adoptive transfer study. The proportion of CD25+CD4+ T cells was $11.39\pm1.34\%$ (mean) in Group 6, which was significantly higher than proportions in Group 4 ($6.11\pm1.84\%$), Group 5 ($6.53\pm1.59\%$) and syngenic recipients ($7.13\pm0.23\%$) ($P<0.001$) (Fig. 2).

Discussion

The ultimate goal of organ transplantation is modulation of the immune response to induce tolerance without immunosuppression. Thus, various strategies have been sought to induce tolerance and prevent acute and chronic rejection. CTLA-4Ig combined with donor-specific transfusion, donor bone marrow infusion [1, 2] and anti-ICOS antibody treatment (in pressed) during rat heart transplantation has been reported to facilitate

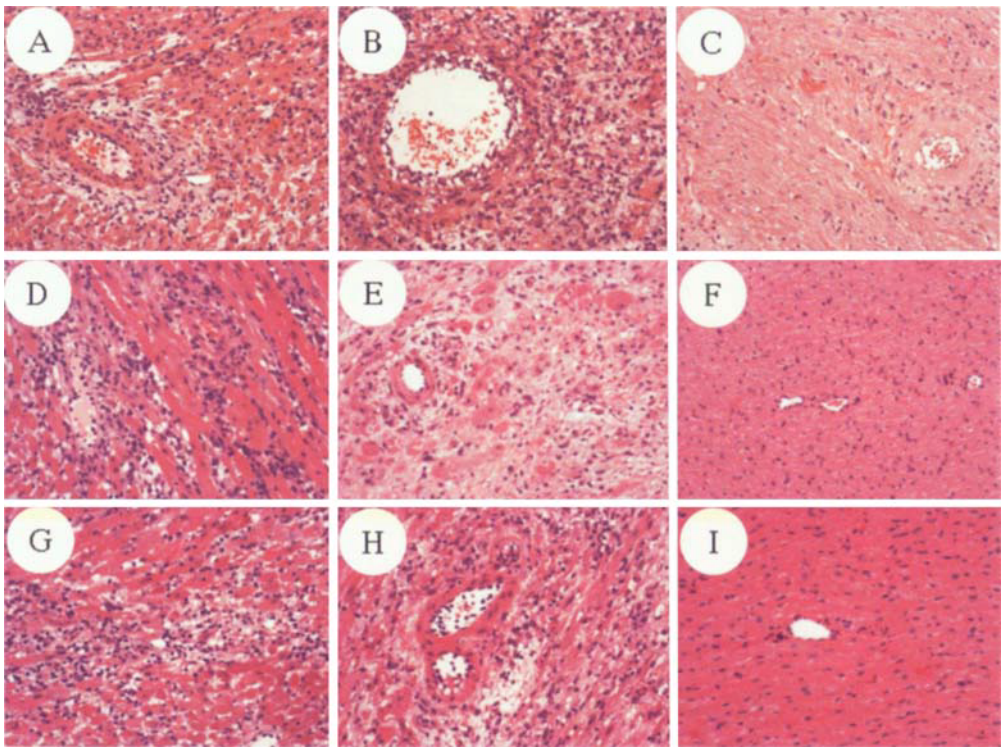

Fig. 1A–I Histological findings in heart grafts after rejection of skin graft and in secondary heart graft after secondary heart transplant. Extensive perivascular accumulations of mononuclear cells with clear evidence of myocytes necrosis and bleeding were seen in Groups 3 and 4 after donor strain skin grafting **A**, **B**. A normal tissue architecture with little infiltration of mononuclear cells was observed after the recipient had rejected the skin graft **C**. In Groups 3 and 4, the primary heart grafts **D**, **E**) and secondary heart grafts **G**, **H**) were harvested after rejection. Extensive perivascular accumulations of mononuclear cells with architecture damage of myocytes were seen in both primary and secondary heart grafts. An almost normal histological finding was observed in both primary and secondary heart grafts in Group 5 **F**, **I**

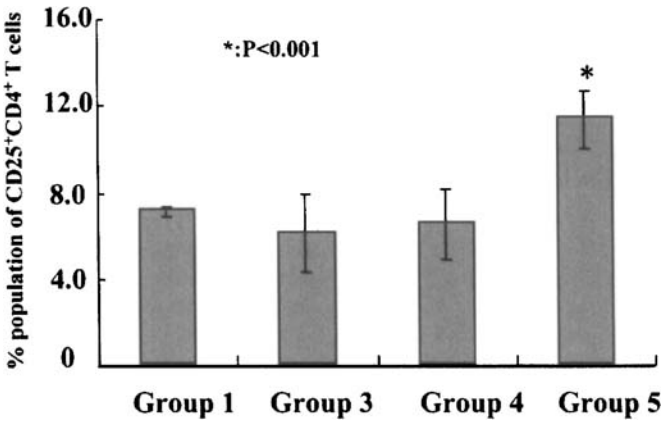

Fig. 2 FACS analysis in peripheral lymphocytes from long-term accepted recipients. The population of CD25+CD4+ T cells was 11.39±1.34% (mean) in Group 5, which was significantly higher than that of Group 3 (6.11±1.84%), Group 4 (6.53±1.59%) and syngenic recipients (7.13±0.23%). *: $P<0.001$ compared to the other three groups

development of tolerance and abrogate acute and chronic rejection. However, the tolerant state has not yet been studied. To this end, we produced tolerant rats with long-term acceptance of heart allografts and challenged them with donor-strain skin and secondary heart grafts. Pearson et al. reported that treatment with fusion protein of CTLA4-Ig produced indefinite survival of vascularized cardiac allografts in mouse models and that these recipients also accepted donor strain skin grafting without evoking the rejection of primary heart grafts [11]. However, this is not the case in our rat model. All the recipients from three groups rejected skin grafts, and rejection of skin grafts in Groups 4 and 5 restored the primary heart-graft rejection. In contrast, despite the rejection of skin grafts in Group 6, the recipients did not reject primary heart grafts.

To exclude the possibility that the rejection of skin graft is due to a skin-specific antigen, we transplanted a secondary heart graft into long-term accepted recipients. The results showed that recipients from Groups 4 and 5 also rejected secondary heart grafts and simultaneously rejected primary heart grafts. In contrast, recipients treated with AdCTLA-4Ig plus anti-ICOS mAb (Group 6) accepted secondary heart grafts and primary heart grafts beat normally throughout the experiment. We thus concluded that tolerant induction with different strategies resulted in different tolerances. Combined treatment with AdCTLA-4Ig and anti-ICOS mAb induced a more stable tolerance than the other two groups.

A great deal has been learned about CD25+CD4+ regulatory T cells, their functions and suppressive properties, both in vivo and in vitro, since they were first reported by Sakaguchi [12] and Taguchi [13]. Shevach's group found that CD25+CD4+ regulatory T cells represent a unique lineage of immunoregulatory cells [14] that can polyclonally suppress T-cell activation in vitro by inhibiting IL-2 production [15]. CD25+CD4+ regulatory T cells have also been demonstrated

to play a significant role in preventing the development of autoimmune disease and in their dual properties as anergic and suppressive cells [16, 17, 18, 19, 20].

Induced production of CD25+CD4+ regulatory T cells has been studied because of their significant immunomodulating function. Taylor et al. reported that murine CD4+ T cells were tolerized to alloantigen via ex vivo CD40 ligand/ CD40 or CD28/CTLA-4/B7 blockade resulting in secondary mixed leukocyte reaction hyporesponsiveness and tolerance to alloantigen in vivo, and suggested that CD25+CD4+ T cell are essential for inducing tolerance to alloantigen [21]. In vivo studies had also shown that donor-specific regulatory cells could be generated during the induction phase of unresponsiveness by anti-CD4 Ab plus a donor-specific transfusion [22], oral antigen administration [23, 24]. Other reports showed that 1 alpha, 25-dihydroxyvitamin D3 and mycophenolate mofetil treatment [25], in vitro repetitive stimulation with immature dendritic cells [26] and CD154 blockade in vivo [27]. Our finding demonstrated that AdCTLA-4Ig plus anti-ICOS antibody also induced development of CD25+CD4+ T cells when compared with other two groups, and these CD25+CD4+ T cells possessed potent regulatory properties because adoptive transfer lymphocytes from AdCTLA-4Ig plus anti-ICOS antibody treated recipients resulted in a significant prolongation of heart grafts in irradiated secondary recipients, and subtracting CD25+CD4+ T cells from these lymphocytes accelerates heart-graft rejection. However, induced development of CD25+CD4+ T cells was not observed in the other two groups, and transferred lymphocytes from these groups could not prolong the survival of heart grafts in irradiated secondary recipients. These results suggest that tolerances induced in the three different ways described in this study operate through different mechanisms.

Other investigations of immunomodulating and tolerizing mechanisms of bone marrow infusion have suggested that they include induction of anergy, clonal deletion, promotion of microchimerism, or combinations of these [28]. Our study with a DA-to-Lewis rat heart transplantation model did not support these observations. The recipients from Groups 4 and 5 rejected skin and secondary heart grafts, which indicated that these recipients are immunologically competent to reject donor-strain grafts, and suggested presence of donor-reactive lymphocytes. We also assayed the microchimerism in the peripheral blood from Groups 4, 5 and 6 by transplanting male heart grafts into female recipients. We could not find male cells in the female recipient's peripheral blood by PCR assay over two months after primary heart grafting (unpublished data). We thus concluded that induction of anergy, clonal deletion, and promotion of peripheral microchimerism are not the mechanisms in our present rat heart transplantation model.

To investigate whether the suppressor effector function of CD25+CD4+ regulatory T cells is antigen nonspecific or not, we transplanted a third-party (BN) heart graft into recipients from Groups 3, 4 and 5. The results showed that the third-party heart grafts were rejected, and primary heart grafts beat normally, suggesting that the suppressor effector function of regulatory T cells is antigen-specific. Similar results have also been observed in vitro and in vivo [29].

Accumulating evidence suggests that regulatory T cells play a significant role in maintaining transplantation tolerance. In this study, we demonstrated that blockade of the B7:CD28 and ICOSL:ICOS co-stimulatory pathway simultaneously facilitated the development of CD25+CD4+ regulatory T cells and was responsible for maintaining allograft tolerance. Compared with donor-cell infusion, simultaneous co-stimulator signal blockade with AdCTLA-4Ig and anti-ICOS antibodies induced stable donor-specific tolerance. This also provides an effective therapy for clinical transplantation to promote permanent graft survival by generating regulatory T cells.

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