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ORIGINAL ARTICLE

Induction of regulatory T cells from mature T cells by allogeneic thymic epithelial cells *in vitro*

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Summary

The ability of thymic epithelial cells (TEC) to re-educate mature T cells to be regulatory T cells has not been addressed. In the present study, this issue was directly investigated by co-culturing of mature T cells and allo-TECs. B6 macrophage cell line 1C21-cultured BALB/c splenocytes responded to B6 antigens *in vitro*. However, BALB/c splenocytes precultured with B6-derived TECs 1-4C18 or 1C6 did not proliferate to B6 antigens, but responded to rat antigens. Exogenous interleukin-2 (IL-2) failed to revise the unresponsiveness of these T cells. Allo-TEC-cultured T cells predominantly expressed Th2 cytokines (IL-4 and IL-10). B6 TEC-cultured BALB/c splenocytes markedly inhibited the immune responses of naïve BALB/c splenocytes to B6 antigens, but not to rat or the third-party mouse antigens. BALB/c nude mice that received naïve syngeneic splenocytes rejected B6 or rat skin grafts by 17 days postskin grafting; however, co-injection of B6 TEC-cultured BALB/c splenocytes significantly delayed B6 skin graft rejection ($P < 0.01$), with the unchanged rejection of rat skin grafts. These studies demonstrate that allo-TECs are able to 'educate' mature T cells to be regulatory cells, and suggest that regulatory cells derived from mature T cells by TECs may play an important role in T cell tolerance to allo- and auto-antigens.

Introduction

It has been well defined that thymus plays a crucial role in T-cell tolerance to auto- and allo-antigens during T-cell development [1,2–4]. T-cell tolerance to allo- and xenogeneic antigens can be induced by grafting donor thymus or thymic epithelium into T-cell-depleted thymectomized or genetically athymic animals [2,5–9]. In addition to T-cell clonal deletion and anergy, regulator T cells may play an important role in T-cell tolerance in these models [2,5–10]. However, whether host regulatory cells were derived from host bone marrow stem cells or from nondepleted mature host T cells in the allo- or xeno-thymus grafts was not clear.

Accumulating evidence also showed that thymus plays an important role in self-tolerance, 'infectious tolerance' and other allogeneic tolerance-inducing models [5,10–19]. Neonatal thymectomy increases the incidence of spontaneous and methylcholanthrene-enhanced thyroiditis in rats [20], indicating thymus-produced regulatory cells may play a role in autoantigen tolerance [19,21]. T-cell tolerance to renal allografts in miniature swine is induced across a two-haplotype class I plus minor histocompatibility antigen disparity by a 12-day course of cyclosporine A, in contrast to irreversible rejection observed in recipients without cyclosporine A treatment [22]. However, a complete thymectomy of the recipients 21 days before renal transplantation led to the acute cellular rejection of

kidney grafts even though the recipients were treated similarly as nonthymectomized and sham thymectomized animals, which accepted kidney grafts for long term [22]. Mice treated with nondepleting anti-CD4 and -CD8 monoclonal antibodies accepted allogeneic heart grafts for long term, but similarly treated thymectomized mice rejected allogeneic heart grafts [12]. These results collectively indicate that the thymus is essential for induction of immune tolerance in these models and that regulatory cells play an important role in these allogeneic transplant tolerance models. The product of regulatory cells may be dependent on the host thymus, although it is not clear that these regulatory T cells are derived from immature T cells or peripheral mature T cells that circulate back to the thymus.

Indeed, it has been reported that a proportion of mature thymocytes might represent T cells that re-entered the thymus from the peripheral lymphoid tissues and the entry of mature T cells to the adult thymus may be restricted to the activated T cells [23,24]. Re-entry of allo-major histocompatibility complete (MHC) peptide-activated T cells to the thymus may be related with the induction and maintenance of acquired antigen-specific tolerance [25]. The interaction of thymic dendritic cells with activated T cells that circulate through thymus may induce T-cell tolerance through physical elimination, inactivation or activation-induced cell death of alloreactive T cells [26]. It has been suggested that mature T cells activated by an allograft could be deleted upon re-entry into the thymus [27]. Whether mature T cells can become regulatory cells after they re-enter the thymus has not been evaluated.

In the present study, we investigated whether or not allogeneic thymic epithelial cells (TEC) have the ability to render mature T cells to become regulatory cells using an *in vitro* culture model. The ability of allogeneic TECs to induce regulatory cells from mature T cells opens the possibility that mature allo-reactive T cells may re-enter to the thymus and become regulatory cells *in vivo*. In addition, the induction of recipient regulatory cells by culturing recipient T cells with donor TECs may offer a new approach to developing host regulatory cells, which may be used to specifically prevent organ graft rejection. Furthermore, host self-antigen-specific regulatory cells induced by host TECs *in vitro* may be used to prevent the occurrence of autoimmune diseases, which are caused by auto-reactive T cells.

Materials and methods

Animals

Five-to-seven week-old BALB/c (H-2^d), BALB/c nude, C57BL/6 (B6, H-2^b) and C3H (H-2^k) mice were purchased from Taconic (Germantown, NY, USA) or from Beijing University Experimental Animal Center (Beijing, China). All mice were maintained in a specific pathogen-free facility and were housed in microisolator cages containing autoclaved feed, bedding, and water. Lewis rats were purchased from Charles River Laboratories (Wilmington, MA, USA). Animal care was in accordance with the American Association for the Accreditation of Laboratory Animal Care and institutional guidelines.

Monoclonal antibodies (mAbs)

The following mAbs were purchased from PharMingen (San Diego, CA, USA). Fluorescein isothiocyanate (FITC)-conjugated rat antimouse CD40 mAb (3/32; IgG2a), FITC-labeled hamster antimouse CD54 (ICAM-1) mAb (3E2; IgG), FITC-labeled hamster antimouse CD80 (B7–1) mAb (16–10A1; IgG), FITC-conjugated rat antimouse CD86 (B7-2) mAb (GL1, IgG2a), FITC-labeled mouse antimouse H-$2K^b$ mAb (AF6–88.5; IgG2a), FITC-labeled mouse antimouse I-A^b mAb (AF6–120.1; IgG2a), FITC-labeled hamster antimouse TCR β-chain mAb (H57–597, IgG), phycoerythrin (PE)-labeled rat antimouse CD4 mAb (RM4–5; IgG2a), PE-labeled rat antimouse CD8α mAb (53–6.7; IgG2a), and rat antimouse FcR mAb 2.4G2 (IgG2b) were produced by 2.4G2 hybridoma *in vitro* (ATCC, Rockville, MD, USA).

Cell staining and flow cytometry (FCM)

To determine the levels of costimulatory receptor expression on B6-derived TEC cell lines 1–4C18, 1C6, and macrophage cell line 1C21 (from ATCC, Manassas, VA, USA), 5×10^5 of these cells were stained with FITC-labeled anti-CD40, CD54, CD80, CD86 mAb, or the nonspecific staining control mAb, respectively. Nonspecific FcR binding was blocked by antimouse FcR mAb 2.4G2. Ten thousand cells were assayed by one- or two-color FCM using a FACScan FCM (Becton Dickinson, Mountain View, CA, USA).

Mouse splenocytes were prepared, and red blood cells were lyzed with ACK Lysis buffer (Invitrogen, San Diego, CA, USA) as described before [28]. Peripheral blood mononuclear cells were prepared with Histopaque-1077 (Sigma Chemical Co., St Louis, MO, USA). Cells were stained with PE-labeled anti-CD4 or CD8 mAb versus FITC-labeled anti-TCRβ mAb. Nonspecific FcR binding was blocked by antimouse FcR mAb 2.4G2. Cells were analyzed by two-color FCM. Nonviable cells were excluded using the vital nucleic acid stain propidium iodide. The percentage of cells staining with a particular reagent or reagents was determined by subtracting the percentage of cells staining nonspecifically with the negative control

mAb from staining in the same dot-plot region with the antimouse mAbs.

Mixed lymphocyte reactions (MLR)

Murine splenocytes were prepared using sterile technique as described before [29]. Red blood cells were lyzed using ACK Lysis buffer. Cells were suspended in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (GibcolBrl, Grand Island, NY, USA), 2 mM L-glutamine, 0.1 mM nonessential amino acids (GibcolBrl), 1 mM sodium pyruvate, 10 U/ml penicillin and 10 μg/ml streptomycin, 10 mM HEPES buffer (GibcolBrl), and 10 μM 2-mercaptoethanol. Triplicate wells containing 4×10^5 responders with 4×10^5 stimulators (30 Gy irradiated) in a total volume of 0.2 ml of medium were incubated at 37 °C in 5% CO_2. Duplicate plates were pulsed with 0.5 μCi of ^{3}H-labeled thymidine (Perking Elmer-Life Sciences, Foster City, CA, USA) per well on days 3 and 4 and, after 18 h further incubation, were harvested with a cell harvester (Skatron Instruments, Lier, Norway). Samples were assayed in a Liquid Scintillation Analyzer, Tri-Carb 2100TR (Packard, Meriden, CT, USA). Stimulation index (SI) was calculated as follows: SI = c.p.m. of responders in wells with BALB/c or Lewis rat stimulators/ c.p.m. of the same responders in wells with medium or syngeneic stimulator cells.

TEC and macrophage cell lines

B6-derived TEC cell lines 1–4C18 and 1C6 were cultured in S-MEM medium with 10 mM HEPES, 50 μg/ml Gentamicin, 10 μM 2-mercaptoethanol, 0.5 μg/ml hydrocortisone, and 10% FCS at 37 °C in a 5% CO_2 incubator, as previously described [30,31]. B6-derived macrophage cell line 1C21 [32], which was received from ATCC, was cultured in RPMI 1640 medium with 10 mM HEPES, 10 μM 2-mercaptoethanol, 10% FCS, 10 U/ml penicillin, and 10 μg/ml streptomycin at 37 °C in 5% CO_2.

Labeling of splenocytes with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) and cell division assay

Splenocytes were labeled with CFSE with minor modification [33]. Single-cell suspensions of splenocytes were suspended in PBS at a concentration of 5×10^7 cells/ml and labeled with the tracking fluorochrome CFSE (Molecular Probes, Eugene, OR, USA). Splenocytes were incubated with CFSE at a final concentration of 5 μM in PBS for 5 min, and labeling was terminated by the addition of FCS (10% of the total volume). Splenocytes were washed twice in complete RPMI 1640 medium and resuspended for cell culture. Labeled cells were cultured with stimulator cells, similarly as performed in MLR assay described above. After culture, cells were stained with PE-antimouse CD4 or CD8 mAb, and assayed by FCM, with gating $CD4^+$ or $CD8^+$ cell population.

Adoptive transfer animal model

Six-to-eight week-old BALB/c nude mice received i.v. injections of 1×10^7 naïve BALB/c splenocytes with or without 2×10^7 BALB/c splenocytes precultured with irradiated B6-derived TECs 1–4C18 or 1C6 for 3 days on days −1. B6 and Lewis rat tail skin tissues were grafted on day 0.

Skin grafting

Graft beds were prepared on the posterolateral thorax of recipient BALB/c mice under Ketamine/Xylazine anesthesia (Fort Dodge, Iowa, IW, USA; Bayer, Kansas, KS, USA, respectively). Full thickness B6 and Lewis rat tail skin were grafted onto the lateral thoracic wall with 5–0 silk sutures and bandaids as described [34]. Skin grafts were evaluated daily from day 7 onward.

Statistical analysis

All data are reported as the mean ± SD. Student's *t*-test for comparison of means was used to compare groups. A *P*-value <0.05 was considered to be statistically significant.

Results

Phenotypes of TEC cell lines 1-4C18 and 1C6

It has been reported that TECs express low levels of MHC molecules and co-receptors, and their phenotypes are very heterogeneic [30,35–39]. To confirm the phenotypes of B6-derived TEC lines, 1-4C18, and 1C6, the expression of CD40, CD80 (B7-1), CD86 (B7-2), and ICAM-1 molecules was detected by FCM. The positive control B6-derived macrophage cell line 1C21, which shares many properties with normal mouse macrophages and displays macrophage-specific antigens [40], expressed high levels of CD40, B7-1, B7-2, and ICAM-1 (data not shown). However, B6 mouse cortical TEC 1-4C18 did not express CD40, B7-2, and ICAM-1 molecules, and expressed low levels of B7-1 (data not shown). B6 mouse medullary TEC 1C6 did not express detectable CD40, B7-2, and ICAM-1 molecules, and expressed high levels of B7-1 as determined by FCM (data not shown). 1-4C18 and IC6 cells expressed low levels of MHC class I and class II molecules as detected by FCM (data not shown)

[30,35,41]. Culturing with IFN-γ for 24 or 48 h, the expressions of CD40, B7-1, B7-2, and ICAM-1 molecules on 1-4C18 and 1C6 cells were slightly increased, the increased expression of MHC molecules on 1-4C18 and 1C6 was also observed as reported (data not shown) [30,35].

Low allogeneic immunogenicity of TECs

It has been reported that TECs lack full capacity for antigen presentation to both Th1 and Th2 cells [42]. To evaluate the immunogenicity of TECs to allogeneic T cells, the ability of TECs to stimulate allogeneic T-cell proliferation was detected in MLR assays. As is shown in Fig. 1, allogeneic BALB/c splenocytes showed strong proliferative reaction to irradiated B6-derived macrophage cell line 1C21, but failed to proliferate to irradiated B6-derived TEC cell lines 1-4C18 or 1C6. In addition, the poor responses of naïve BALB/c splenocytes to allogeneic B6 TECs were observed when cells in MLR were cultured for different days (data not shown). These results indicate that TECs have the low allogeneic immunogenicity.

Unresponsiveness of splenocytes induced by allogeneic TECs

We investigated whether T cells will be specifically tolerant to TEC-expressed alloantigens *in vitro* after culture with allogeneic TECs. After culturing with 30 Gy-irradiated B6 TEC cell line 1-4C18 or 1C6 or B6 macrophage cell line 1C21 for 3 days, BALB/c splenocytes were harvested and re-cultured with 30 Gy-irradiated B6 splenocytes, rat splenocytes, or Con A for 3 days. Cell proliferation was detected by 3H-thymidine incorporation. As is shown in Fig. 2, 1C21-precultured BALB/c splenocytes responded strongly to B6 or rat antigens and Con A stimulation. However, BALB/c splenocytes precultured with B6 TECs 1-4C18 or 1C6 did not proliferate to B6 antigens presented by 30 Gy-irradiated B6 splenocytes, whereas they proliferated to rat antigens and Con A stimulation. These studies suggest that TECs are able to directly induce the unresponsiveness of naïve allogeneic mature T cells *in vitro*.

IL-2 did not improve the poor proliferative reaction of splenocytes to allogeneic TECs

It has been reported that provision of signal 1 (TCR signal) in the absence of signal 2 (costimulatory signal) can lead to T-cell unresponsiveness or anergy [43–45]. Because of the low levels of co-receptors expressed on TECs, T cells may become anergic after culturing with allogeneic TECs. T-cell nonreactivity because of anergy can be reversed *in vitro* when IL-2 is exogenously provided. The ability of IL-2 to revise the poor proliferative reaction to allogeneic TECs of T cells was evaluated. As is shown in Fig. 3, 50 or 300 pg/ml mouse IL-2 slightly enhanced the spontaneous proliferation of BALB/c T cells. However, exogenous mouse IL-2 did not improve the poor proliferative reaction of BALB/c T cells to B6-derived TECs 1-4C18 and 1C6. Thus, even with the

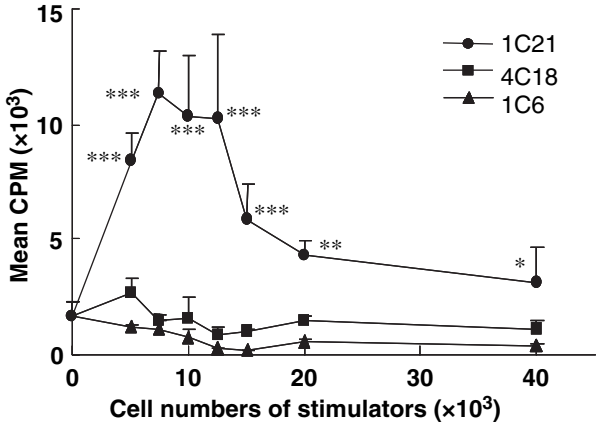

Figure 1 Allogeneic thymic epithelial cells (TECs) failed to induce T-cell proliferation *in vitro*. Freshly separated naïve BALB/c splenocytes were cultured with different doses of irradiated B6-derived TECs 1-4C18, 1C6, or macrophage cell line 1C21 for 4 days. The cell proliferation was detected by 3H-thymidine incorporation. Data are presented as mean ± SD. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$ compared with BALB/c splenocytes cultured with TECs. Four independent experiments were performed with similar results.

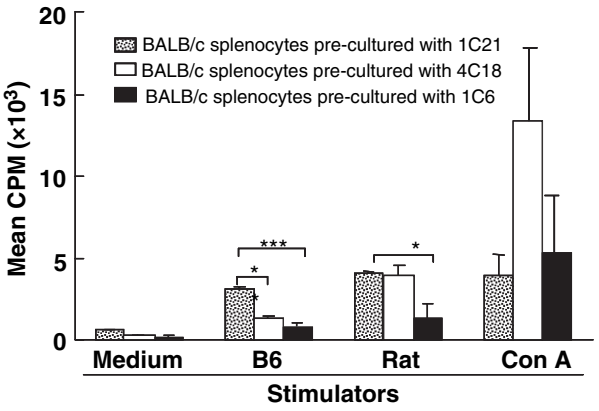

Figure 2 Thymic epithelial cells induced the unresponsiveness of allogeneic T cells. Freshly separated naïve BALB/c splenocytes were cultured with irradiated B6-derived TECs 1-4C18, 1C6, or macrophage cell line 1C21 for 3 days. Alive cells were separated and further cultured with irradiated B6 splenocytes, rat splenocytes, or mitogen Con A (3 μg/ml) for 4 days. Data are presented as mean ± SD. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$ compared among the indicated groups. One representative of three independent experiments was shown.

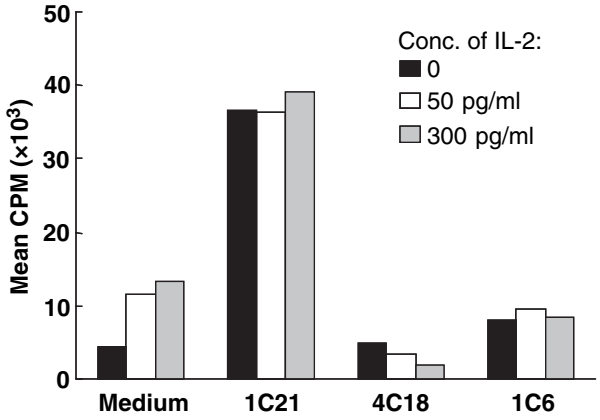

Figure 3 IL-2 failed to revise the unresponsiveness of T cells induced by allogeneic TECs. Naïve BALB/c splenocytes were cultured with irradiated B6-derived TECs 1-4C18, 1C6, or macrophage cell line 1C21 in the presence of 0, 50, or 300 pg/ml of IL-2 for 4 days. One representative of two independent experiments with similar results was shown.

low levels of co-receptor expressions, TECs failed to induce anergy of allogeneic T cells *in vitro*.

Specific immune suppressive effects of allogeneic TEC-cultured T cells *in vitro*

To determine the immunosuppressive function of T cells precultured with allogeneic TECs, the specific inhibiting effect of these T cells on allogeneic MLR of naïve syngeneic T cells was detected. Naïve BALB/c splenocytes were cultured with irradiated B6 or rat splenocytes in the presence of TECs 1-4C18 or 1C6 precultured BALB/c splenocytes, whereas naïve BALB/c splenocytes cultured with irradiated B6 or rat splenocytes with or without 1C21 precultured BALB/c splenocytes were used as control. Cell proliferation was detected by 3H-thymidine incorporation.

As is shown in Fig. 4a, BALB/c splenocytes that precultured with B6-derived TECs 1-4C18 and 1C6 significantly inhibited the proliferation of naïve BALB/c splenocytes to B6 antigens but not to rat antigens ($P < 0.01$, compared with BALB/c splenocytes alone without precultured cells). In contrast, BALB/c splenocytes that precultured with B6-derived macrophage 1C21 showed enhanced proliferative reaction of naïve BALB/c splenocytes to B6 antigens. Similarly, BALB/c splenocytes that precultured with B6-derived TECs 1-4C18 and 1C6 significantly inhibited the proliferation of naïve BALB/c splenocytes to B6 antigens but not to the third-party allogeneic C3H antigens ($P < 0.01$, compared with BALB/c splenocytes alone without precultured cells; Fig. 4b).

The cell division of naïve BALB/c $CD4^+$ splenocytes in allogeneic MLR was determined in the presence or absence of TEC-precultured BALB/c splenocytes. Naïve splenocytes were labeled with CFSE and cultured with irradiated 1C21 for 3 days in the presence or absence of 1-4C18, 1C6, or 1C21 precultured BALB/c splenocytes. Alive cells were harvested and stained with PE-antimouse CD4 mAb. 1×10^4 gated $CD4^+$ cells were assayed by FCM. As is shown in Fig. 4c, naïve BALB/c $CD4^+$ cells divided markedly when cultured with lethally irradiated 1C21, regardless of the presence of BALB/c splenocytes precultured with 1C21. However, in the presence of BALB/c splenocytes precultured with TECs 1-4C18 or 1C6, naïve BALB/c $CD4^+$ cells showed significantly decreased cell division after the stimulation of allogeneic antigens (Fig. 4c).

In order to confirm whether T cells were involved in the immunosuppressive function of splenocytes precultured with allogeneic TECs in this systems, the separated BALB/c T cells using MACS kit were precultured with 4C18 or 1C6, their immunosuppressive function was evaluated using the *in vitro* cell culture assay. As is shown in Fig. 4d, the purified BALB/c T cells precultured with allogeneic B6 TECs, 4C18, or 1C6 significantly inhibited the proliferation of naïve T cells stimulated by B6 antigens, but not by the third-party allogeneic C3H antigens. Thus, these results collectively indicate that allogeneic TECs-cultured T cells have the ability to inhibit the proliferative response of naïve syngeneic T cells to TECs-expressed antigens, even when they were expressed on professional antigen presenting cells (APCs).

Furthermore, the immunosuppressive function of T cells precultured with allogeneic TECs on the cytotoxic function of naïve syngeneic cytotoxic T lymphocytes was detected in cytotoxic T lymphocyte (CTL) assays. As is shown in Fig. 5, naïve BALB/c splenocytes significantly killed B6 or rat target cells. The presence of BALB/c splenocytes precultured with TECs 1-4C18 or 1C6 markedly inhibited the CTL function of naïve BALB/c splenocytes against B6 target, but not against rat targets, whereas BALB/c splenocytes precultured with 1C21 did not prevent the cytotoxicity of naïve BALB/c splenocytes. The immunosuppressive effect of TECs-cultured BALB/c splenocytes on naïve syngeneic CTL function is dose-dependent (Fig. 5a).

Suppressor cells induced by allogeneic donor TECs significantly delayed donor skin graft rejection

The specific immune suppressive function of T cells cultured with allogeneic TECs was evaluated *in vivo* using an adoptive transfer animal model. BALB/c nude mice received an i.v. injection of 1×10^7 naïve syngeneic BALB/c splenocytes rejected B6 or rat skin grafts by 17 days postskin grafting. However, co-injection of 2×10^7 1-4C18- or 1C6-precultured BALB/c splenocytes

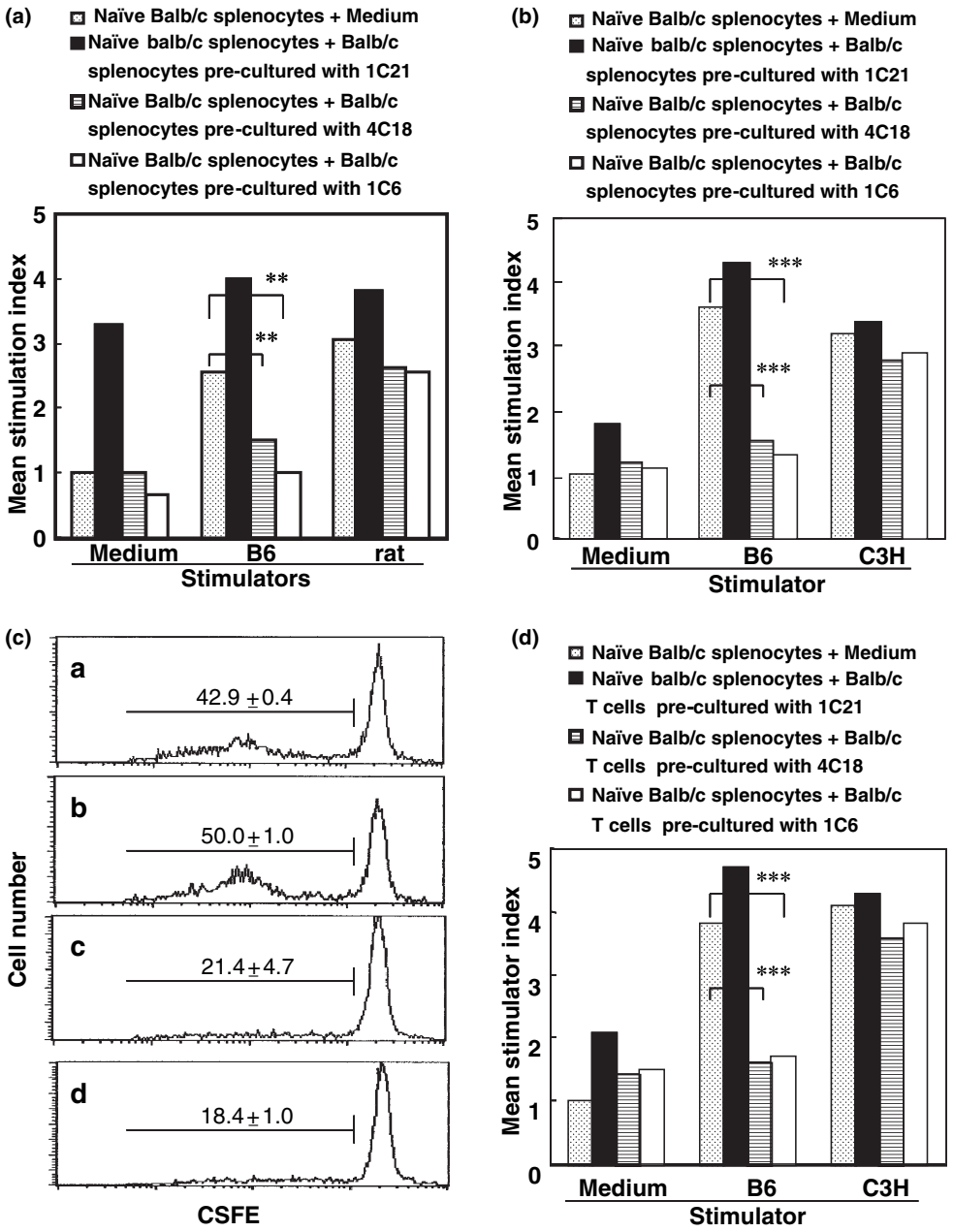

Figure 4 Specific immune suppression of T cells cultured with allogeneic TECs. (a) Significant inhibition of the proliferation of naïve BALB/c splenocytes to B6 antigens by B6 TECs-treated BALB/c splenocytes. Naïve BALB/c splenocytes were cultured with irradiated B6-derived TECs 1-4C18, 1C6, or macrophage cell line 1C21 for 3 days. Alive cells were separated and used as regulatory cells. Freshly separated naïve BALB/c splenocytes were cultured with irradiated B6 or rat splenocytes in the presence or absence of TECs or 1C21-precultured BALB/c cells for 4 days as described in the Materials and methods. $*P < 0.05$; $**P < 0.01$ compared among the indicated groups. (b) Significant inhibition of the proliferation of naïve BALB/c splenocytes to B6 antigens, but not to the third-party allogeneic C3H antigens, by B6 TECs-treated BALB/c splenocytes. (c) Significant inhibition of B6 antigen-induced BALB/c $CD4^+$ T-cell division by B6 TECs-treated BALB/c splenocytes. The cell division was assayed by flow cytometry (FCM) with gate on $CD4^+$ cells. Significantly decreased $CD4^+$ cells division (c and d) was observed when TECs-treated BALB/c splenocytes presented ($P < 0.01$ compared with group which no TEC-treated BALB/c splenocytes). Three independent experiments were performed with similar results. (d) The proliferative reaction of naïve BALB/c splenocytes to B6 antigens, but not to the third-party allogeneic C3H antigens, was significantly inhibited by separated BALB/c T cells that precultured with B6 TECs, 4C18, or 1C6. $***P < 0.001$ compared among the indicated groups.

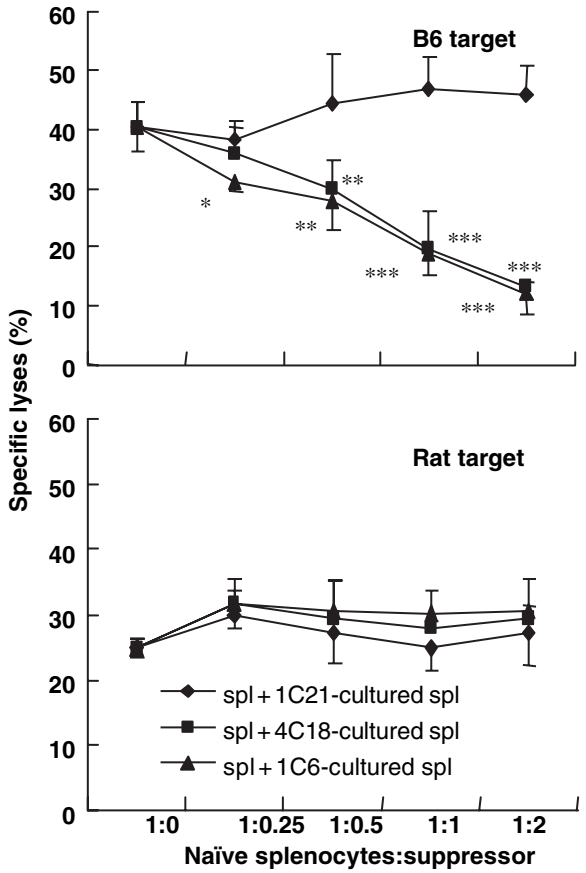

Figure 5 Specific inhibition of CTL function of naïve syngeneic T cells by T cells precultured with allogeneic TECs. Naïve BALB/c splenocytes were cultured with irradiated B6-derived TECs 1-4C18, 1C6, or macrophage cell line 1C21 for 3 days. Alive cells were separated and used as regulatory cells. Freshly separated naïve BALB/c splenocytes were used as effector cells. CTL was performed as described in the Materials and methods in the presence of different doses of suppressor cells. Data are presented as mean ± SD. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$ compared with groups in which there were effector cells alone without suppressor cells. Three independent experiments with similar results were performed.

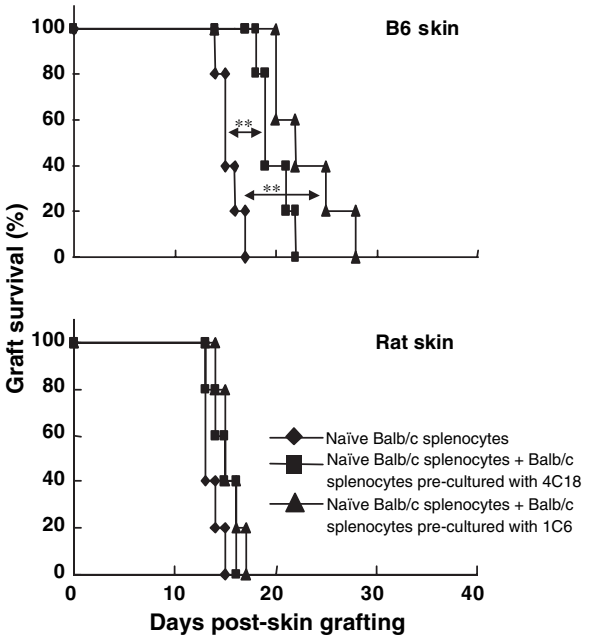

Figure 6 T cells precultured with allogeneic donor TECs specifically delayed donor skin graft rejection of naïve syngeneic T cells. Naïve BALB/c splenocytes were cultured with irradiated B6-derived TECs 1-4C18, 1C6, or macrophage cell line 1C21 for 3 days. Alive cells were separated and used as regulatory cells. Naïve BALB/c splenocytes were injected i.v. into syngeneic BALB/c nude mice with or without suppressor cells on day −1. B6 and rat tail skin were grafted on day 0. $**P < 0.01$; $***P < 0.005$ compared among the indicated groups. Data from two separated experiments are summarized. Ten mice in each group were performed.

markedly delayed B6 skin graft rejection of naïve BALB/c splenocytes in BALB/c nude recipients (by 28 days post-skin grafting, $P < 0.01$ versus control group), whereas the rejection of the third-party rat skin grafts in these mice was not significantly changed, compared with the control group which received naïve BALB/c splenocytes alone (Fig. 6). These results suggest that regulatory cells induced by donor TECs are able to significantly prevent donor allografts from cellular immune rejection.

Discussion

Earlier studies of murine radiation chimeras showed that functional unresponsiveness, rather than clonal deletion, to antigens expressed by radioresistant thymic epithelium was achieved [46]. Transplantation tolerance to allo- and xeno-antigens can be induced by thymic epithelium or thymic tissues in T-cell-depleted thymectomized or genetically athymic animal models [2,5,47–53]. It has been demonstrated that allogeneic TEC-selected T regulatory cells can inhibit effector activities of graft-reactive cells and play an important role in T-cell tolerance to donor antigens [2,7–9,52,54]. It has been generally recognized that some of the immature T cells could become T regulator cells after their interaction with TECs. However, whether regulatory cells could be derived from re-entered mature T cells in the allo- or xeno-thymus grafts has not been addressed. This possibility was indirectly supported by the presence of re-entered mature T cells in the thymus [23,24]. In the present report, we directly investigated the ability of allogeneic TECs to render mature T cells to become T suppressor cells after culturing mature T cells with allogeneic TEC cell lines *in vitro*.

Compared with the syngeneic macrophage cell line, TECs did not express or expressed much lower levels of

costimulatory molecules. TECs have significantly lower immunogenic ability to allogeneic T cells, as indicated by the poor proliferative reaction to B6 TECs of BALB/c splenocytes. T cells cultured with allogeneic TECs became unresponsive to TEC-expressed antigens that expressed on professional APCs (Fig. 3). The poor proliferative response to allogeneic TECs of T cells was further confirmed using CFSE-labeled T cells (data not shown). The unresponsiveness of allogeneic TEC-cultured T cells could not be revised by additional supply of exogenous IL-2, indicating that these T cells are not in a state of anergy.

The activation of naïve T cells has been proposed to require both signals 1 (T-cell antigen receptor signal) and 2 (costimulatory signal). Provision of signal 1 in the absence of signal 2 can lead to T-cell unresponsiveness or anergy [43–45]. Donor-specific transplantation tolerance can be induced in adult rodents using nondepleting mAbs against T-cell co-receptor and co-stimulation molecules or by immunization with tolerogenic APCs. It is generally accepted that regulatory $CD4^+$ T cells are the principal mediators in all these models of peripheral tolerance [55]. It has been reported that culture with parenchymal or endothelial cells lacking costimulatory molecules induces T-cell allo-specific unresponsiveness [56–58]. Interestingly, it has been reported that an indirect pathway of alloantigen presentation via liver sinusoidal endothelial cells is involved in alloreactive T-cell tolerance induced by portal venous injection of donor cells [59,60]. However, T cells cultured with allogeneic TECs did not become anergic. It has been reported that a T-cell clone did not respond to its antigens presented by TECs, and that the poor response could not be revised by addition of exogenous IL-2, indicating that anergy was not involved in the negligible responses [61]. The reasons for the failure of allogenic TECs to induce anergy of mature T cells *in vitro* are not clear. The nonanergic unresponsive state of T cells induced by allogeneic TECs may be related with the unique APC functions of TECs. With the highly diversity of TECs in mind, the defective APC functions of certain types of TECs may be attributed to the lack of co-receptor/stimulatory molecules, low levels of MHC expression, and/or deficiency of the antigen-processing/presenting pathways [30,35,42,62–64]. Furthermore, certain type of TECs may have different ability to present different types of antigens. It has been demonstrated that mTECs and cTECs have different antigen processing pathways and presenting ability, and that mTECs but not cTECs are able to present soluble antigen to helper T cells [30,31,35].

Importantly, after culturing with allogeneic TECs, T cells become regulatory cells. TECs-precultured T cells had specific immunosuppressive function on the immune responses of naïve syngeneic T cells to TEC-expressed antigens, even when these antigens were presented by professional APCs. Co-injection of donor TECs-precultured host T cells significantly delayed the donor but not the third-party skin graft rejection of naïve syngeneic T cells in adoptive transfer animal models. It should be noted that the prolongation of the donor skin graft survival is very modest, the suppressive efficiency of regulatory T cells induced by this approach and its potential clinical applications need to be further studied.

Our preliminary data on the cytokine expression in allogeneic TECs-precultured T cells showed that these T cells favored to express Th2 cytokines, whereas allogeneic macrophage cell line-stimulated T cells predominantly express Th1 cytokines. BALB/c T cells cultured with allogeneic macrophage cells 1C21 expressed high levels of Th1 cytokine (IFN-γ and IL-2), whereas BALB/c T cells cultured with allogeneic TECs expressed high levels of Th2 (IL-4 and IL-10) mRNA and lower levels of Th1 cytokine mRNA. Balb/c T cells expressed similar levels of TGF-β mRNA when they were cultured with 1C21 or TECs (data not shown). The unchanged TGFβ mRNA expression and the enhanced IL-4 expression on T cells cultured with allogeneic TECs suggest that allogeneic TEC-induced regulatory T cells may not belong to the Tr1 cells, which predominately produce IL-10 and TGF-β, but not IL-4 [65]. Differential cytokine production within Th1 and Th2 cells is controlled at the levels of gene transcription. IL-2 and IFN-γ mRNA are induced within 6 h in G1 to S phase, while IL-4 mRNA is induced after 48 h [66], indicating that cell division might be needed to induce Th2 cytokine transcription. However, other reports using a cell cycle blocker and IL-4 withdrawal to control early IL-4 receptor signaling have shown that IL-4 instructed Th2 cytokine production in the first S phase [67,68]. These data suggested that Th2 cytokine productions may not be critically dependent on cell division. Our preliminary results were in accordance with the last possibility. Whether the increased Th2 cytokine products are related to the immunosuppression mediated by the TECs-treated T cells is not determined in the present studies.

It has been reported that different types of TECs have a unique ability to present antigens and mediate positive/negative selection of thymocytes [6,30,31,35,42,69]. However, in the present studies, allogeneic regulatory T cells could be induced by both mTECs and cTECs, no obvious difference for mTECs and cTECs to induce allogeneic regulatory T cells was observed. The inconsistency between the present studies and the previous reports regarding the different antigen presenting ability of mTECs and cTECs may be explained by the different types of antigens, which were used in these studies. We used allogeneic antigens expressed on TECs to induced regulatory T cells, whereas

the previous studies used soluble antigens [30,31,35]. It will be interesting to investigate whether or not mTECs and cTECs have different ability to induce regulatory T cells against soluble antigens.

In conclusion, our studies demonstrate for the first time that TECs have the ability to render mature allogeneic T cells to become regulatory cells. Regulatory cells derived from mature T cells by TECs may play an important role in T cell tolerance to transplantation and self-antigens. In addition, host regulatory cells, produced by culturing host mature T cells with donor TECs, may be used to prevent graft rejection.

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