ORIGINAL ARTICLE

Evaluation of the impact of conventional immunosuppressant on the establishment of murine transplantation tolerance – an experimental study

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SUMMARY

Regulatory T cells (Tregs) play a significant role in immune tolerance. Since Treg function deeply depends on Interleukin-2 signaling, calcineurin inhibitors could affect their suppressive potentials, whereas mammalian target of rapamycin (mTOR) inhibitors may have less impact, as mTOR signaling is not fundamental to Treg proliferation. We previously reported a novel mixed hematopoietic chimerism induction regimen that promotes Treg proliferation by stimulating invariant natural killer T cells under CD40 blockade. Here, we use a mouse model to show the impact of tacrolimus (TAC) or everolimus (EVL) on the establishment of chimerism and Treg proliferation in the regimen. In the immunosuppressive drug-dosing phase, peripheral blood chimerism was comparably enhanced by both TAC and EVL. After dosing was discontinued, TAC-treated mice showed gradual graft rejection, whereas EVL-treated mice sustained long-term robust chimerism. Tregs of TAC-treated mice showed lower expression of both Ki67 and cytotoxic T lymphocyte antigen-4 (CTLA-4), and lower suppressive activity *in vitro* than those of EVL-treated mice, indicating that TAC negatively impacted the regimen by interfering with Treg proliferation and activation. Our results suggest that the usage of calcineurin inhibitors should be avoided if utilizing the regimen to induce Tregs *in vivo* for the establishment of mixed hematopoietic chimerism.

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Key words
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Introduction

While current regimens for immunosuppressive treatment have significantly improved outcomes of organ transplantation, long-term usage of immunosuppressants has several drawbacks, such as increases in infectious [1] and noninfectious complications [2], high medical costs [3], and nonadherence [4]. Several regimens for acquiring transplant tolerance and aimed at overcoming those issues have been reported in both preclinical animal studies and clinical trials [5]. One of the most promising approaches involves transplantation of bone marrow or hematopoietic stem cells together with organ allograft from the same donor. Seminal clinical trials involving kidney transplantation that used this strategy have been published from three institutions [6–9]. Although the induction method was different in each of these studies, a strategy common to all of them was the maintenance of the transplanted kidney graft by a conventional immunosuppressive regimen, including calcineurin inhibitor (CNI) and tapering the drugs according to the status of blood cell chimerism and kidney graft function. However, the impact of conventional immunosuppressants on the establishment of chimerism has remained elusive.

The role of regulatory T cells (Tregs) in transplant tolerance has recently attracted great interest because tolerance was associated with accumulation of Tregs in the lymphoid tissues and transplanted organs [10,11]. Therapeutic effects for *ex vivo*-expanded Tregs have been reported in murine models, in which their adoptive transfer facilitated the establishment of mixed chimerism, resulting in promotion of engraftment [12,13]. These data collectively suggest the importance of Tregs in tolerance induction.

Tacrolimus (TAC), one of the most commonly-used CNIs for maintenance of immunosuppression after solid organ transplantation, inhibits immune responses by suppressing T-cell receptor (TCR) signaling and downstream expression of interleukin (IL)-2, which is an essential cytokine for maintenance of Tregs [14]. Therefore, TAC administration impairs Treg activation and generation. On the other hand, an inhibitor of the mammalian target of rapamycin (mTOR-I) such as everolimus (EVL), another type of commonly used immunosuppressant for solid organ transplantation [15], shows immunosuppressive function by inhibiting phosphatidylinositol 3-kinase (PI3K) and the Akt pathway [16]. Since Tregs preferentially utilize the STAT-5 phosphorylation pathway over the mTOR signaling pathway after IL-2 stimulation, it is assumed that they are resistant to mTOR-I [17].

We previously reported a novel approach to induce mixed chimerism by stimulating invariant natural killer T (iNKT) cells with liposomal formation of alpha-galactosylceramide (lipo-αGalCer) plus anti-CD40 ligand monoclonal antibody (anti-CD40L mAb). Activated iNKT cells produce enormous amounts of cytokines including IL-4, IL-10, and IL-2, and resulted in an increase in Tregs in the spleen. Treg activation seems to be fundamental for this protocol because when Tregs were deleted with anti-CD25 mAbs, selective deletion of the donor-reactive T cell repertoire was abrogated, resulting in loss of chimerism [18,19]. Here, we explore the difference between TAC and EVL in terms of their impact on chimerism induction and the function of Tregs following iNKT cells stimulation.

Materials and methods

Animals

BALB/c (H-2^d), C57Bl/6 (B6; H-2^b) and AKR (H-2^k) mice were purchased from Japan SLC., Inc. (Hamamatsu, Japan). All mice were maintained in pathogen-free animal facilities in our institution. An internal committee on the use and care of laboratory animals approved all experiments (AE17-104-B).

Reagents

A liposome formulation of α-GalCer, RGI-2001 was provided by REGiMMUNE (Tokyo, Japan) and diluted in Hanks' balanced salt solution (HBSS; Life Technologies, Carlsbad, CA, USA) for intravenous injection. An anti-CD40L mAb (MR-1) was purchased from Bio X Cell (West Lebanon, NH, USA) and was diluted in Dulbecco's phosphate-buffered saline (Life Technologies, Carlsbad, CA, USA) for intraperitoneal administration. Recombinant mouse IL-2 (Carrier-free) was purchased from Tonbo Bioscience (San Diego, CA, USA).

Hematopoietic chimerism-induction protocol and immunosuppressive therapy

Eight- to twelve-week old BALB/c recipient mice underwent 3 Gy of total body irradiation (TBI) prior to intravenous injection of 20–25 × 10^6 whole bone marrow cells (BMCs) donated from age-matched B6 or AKR

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mice, followed by a single-dose intravenous injection of 0.2 μg RGI-2001 and a single-dose intraperitoneal injection of 0.5 mg anti-CD40L mAb. Mice received daily injections of TAC (Prograf; Astellas Pharma Incorporated, Japan) intramuscularly or EVL (LC Laboratories, MA, USA) intraperitoneally from 1 to 14 days after bone marrow transplantation (BMT).

Cardiac transplantation

Murine cardiac transplantation was performed as described by Niimi *et al.* [20]. Cardiac allograft donated from B6 mice was transplanted on abdominal aorta/vena cava of BALB/c mice and underwent reperfusion immediately after transplantation. Graft survival was monitored daily by direct abdominal palpitation, and graft rejection was defined as complete cessation of contraction by direct visualization with laparotomy.

Flow cytometric analysis

Blood or spleen samples were hemolyzed with ACK Lysing Buffer (Lonza, Basel, Switzerland) and were incubated with purified anti-mouse CD16/32 (BD Pharmingen, San Jose, CA, USA) to block nonspecific staining, followed by monoclonal antibodies mixtures: anti-H-2K^{b}, H-2D^{d}, CTLA-4, CD4, TCR-β, and CD25. For intracellular staining of Foxp3 and Ki67, an Intracellular Fixation & Permeabilization Buffer Set (eBioscience, San Diego, CA, USA) was used as instructed. These antibodies were purchased from TONBO Bioscience (San Diego, CA, USA), eBioscience, or BD Pharmingen. Samples were acquired on FACS Canto™ II (BD Biosciences, San Jose, CA, USA) and analyzed by FlowJo software (Tree Star Inc., Ashland, OR, USA).

Mixed lymphoid reaction (MLR)

1×10^5 CD4^{+}CD25^{-} enriched T cells isolated from BALB/c splenocytes were co-cultured with 1×10^5 30 Gy-irradiated antigen presenting cells (APCs) of BALB/c (auto) or B6 (allo) mice in 100 μl of RPMI 1640, containing 10% FCS, HEPES, MEM Non-Essential Amino Acids Solution, sodium pyruvate, 2-mercaptoethanol, and Antibiotic-Antimycotic (Gibco) in a 96-well plate. Recombinant mouse IL-2 was added at the concentration indicated. Responder cells were labeled with carboxyfluorescein succinimidyl ester (CSFE) before culture, and proliferation of responder cells was analyzed after 4 days.

Tregs isolation and suppression MLR assay

Isolation of CD4^{+}CD25^{hi} cells from recipient splenocytes at 7 days after BMT was performed using a FACS Aria cell sorter (BD Bioscience). Isolated Tregs (5×10^4) were co-cultured for 96 h with the mixture of CFSE-labeled host T cells (5×10^4) and T-activator CD3/CD28 beads. Responder cells were labeled with CSFE before culture. After 4-day culture, the proliferation of responder cells was analyzed using flow cytometry. CD4^{+}CD25^{hi} cells were also labeled with eFluor before culture, and the population was gated out in this analysis.

Statistical analysis

One-way ANOVA was performed to determine statistical significance among the groups, and Tukey's multiple comparison test was applied as a post-hoc test. Allograft survival analysis was performed using Kaplan–Meier methods, and log-rank tests were used for comparisons between the groups. All *P*-values were two sided, and *P*-values <0.05 were considered to indicate statistical significance. All statistical analyses were performed using Prism 7 software (GraphPad software, Inc., San Diego, CA, USA).

Results

Both TAC and EVL prevented rejection of cardiac allograft without BMT as long as they were administered

To determine the optimal dose of conventional immunosuppressant for maintenance of organ grafts, we used cardiac transplantation model mice without BMT. BALB/c mice were transplanted with cardiac allograft derived from B6 donor mice, and administered either two dosages of TAC or one dosage of EVL, daily for 14 days respectively. As shown in Fig. 1, grafts of both the untreated ($N = 6$) and low-dosage (1 mg/kg/day, $N = 4$) TAC-treated mice were completely rejected at 20 days after transplantation. The graft survival time of high-dosage (5 mg/kg/day, $N = 6$) TAC-treated mice was significantly longer than that of low-dosage TAC-treated mice ($P = 0.024$). On the other hand, the graft survival rate of EVL-treated mice was not significantly higher than that of high dosage of TAC-treated mice ($P = 0.067$). All allografts were eventually rejected following discontinuation of drug administration on day 14. These results suggested that the high dose of TAC

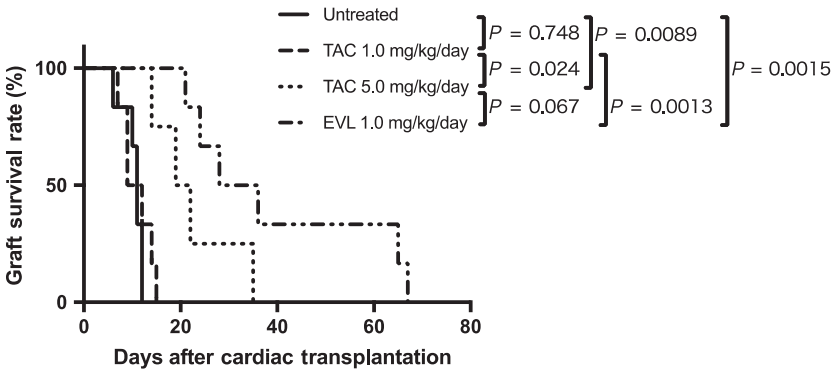

Figure 1 TAC and EVL prevented cardiac allograft rejection while they were administered. BALB/c mice were transplanted with cardiac allografts from B6 donor mice without treatment (untreated; $N = 6$), with TAC administration at 1.0 mg/kg/day ($N = 6$) or at 5.0 mg/kg/day ($N = 4$), or EVL administration at 1.0 mg/kg/day ($N = 6$). TAC/EVL was discontinued on day 14. Kaplan–Meier analysis is shown. *P* values were calculated by log-rank test. EVL, everolimus; TAC, tacrolimus.

would be optimum for the prevention of allograft rejection in this model.

Short-term TAC administration abrogates maintenance of hematopoietic cell engraftment

To evaluate the impact of immunosuppressants on the establishment of mixed hematopoietic chimerism by our original regimen, recipients were treated with a high dose of either TAC or EVL from day 1 to day 14 after BMT, in addition to the regimen using sublethal irradiation, and a single injection of RGI-2001 and anti-CD40L antibodies. Then, we analyzed the proportion of donor cell engraftment in PBMCs by using flow cytometry on day 14. Consistent with our previous reports, 75% of mice without any additional immunosuppressants showed robust establishment of mixed chimerism on day 14 ("Control" in Fig. 2). In all mice treated with either TAC or EVL, mixed chimerism was established on day 14 ("TAC "and "EVL" in Fig. 2). Since the proportion of $CD4^+CD25^-$, $CD4^+CD25^+$, $CD8^+$, and iNKT cells within host T cell lineages were not significantly different among the three treatment groups, TAC and EVL did not appear to have much effect on host immune systems (Fig. S1). The majority of engrafted donor cells at this time point were not significantly different among the groups, suggesting that TAC and EVL also did not affect donor cell reconstruction (data not shown). After discontinuing treatment, TAC-treated mice showed a gradual decrease in the proportion of donor cells, and eventually all except one had rejected the donor cells by day 56 (Fig. 2b). The same trend was also shown even when the mice were treated with low doses of TAC (data not shown). In EVL-treated mice, donor cells were not rejected, or were present in a higher proportion than in the control, even 56 days after transplantation.

Neither TAC nor EVL had an impact on peripheral deletion

To clarify the negative impact of TAC treatment, we next tested anti-donor antigen reactivity, as CNIs are well known to interrupt activation-induced cells death (AICD) by down-modulating CD95L [21]. Splenic $CD4^+CD25^-$ T cells were prepared from chimeras that were not treated with immunosuppressants (chimera control), TAC-treated chimeras, or EVL-treated chimeras on day 7 after BMT. $CD4^+CD25^-$ T cells from the mice that did not receive RGI-2001 and anti-CD40L mAb were prepared as a "rejection control". All chimeras had already shown 20–50% donor cell chimerism on day 7, although T cell lineages were not included in the donor cell fraction at this early time point. Consistent with this result, no proliferation was observed against BALB/c APCs in T cells from chimeras (data not shown). As with the $CD4^+CD25^-$ T cells of the chimera control mice, those from TAC- or EVL-treated mice also showed hyporesponsiveness against donor antigen-bearing cells (Fig. 3). As hyporesponsiveness in these cells was not restored despite additional IL-2 stimulation, it was presumed that peripheral deletion/anergy of donor-reactive T cells could be established even in the presence of conventional immunosuppressants. The presence of peripheral deletion in the mice that were treated with TAC was also confirmed by reduction in $\%V\beta6^+$ T cells in BALB/c recipients that were tolerized against AKR mice (Fig. S2). Consistent with delayed donor cell rejection in TAC-treated mice, recovery of the T cell response

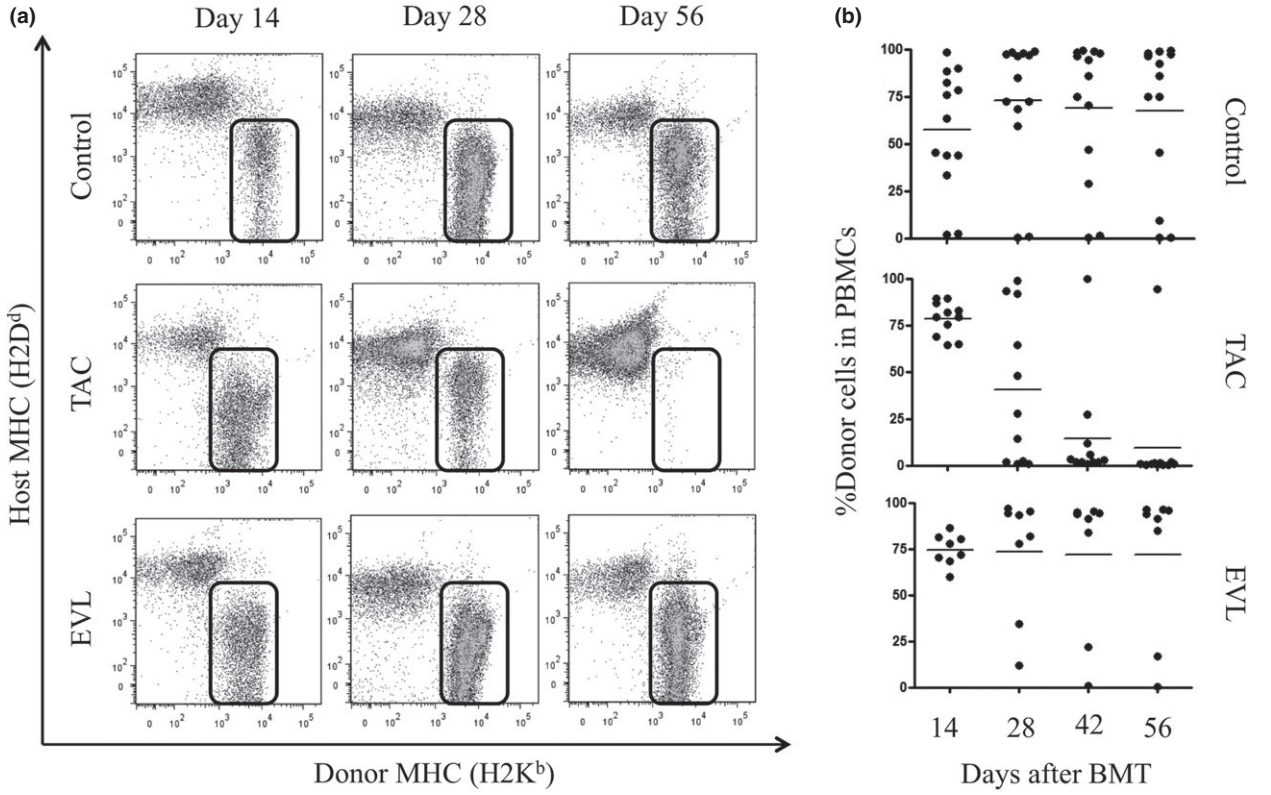

Figure 2 The proportion of donor cells in peripheral blood mononuclear cells. BALB/c mice ($H2^{Dd}$) were transferred with 20–25 × 10^6 whole BMCs from B6 donor mice ($H2^{Kb}$) after 3 Gy TBI. Immediately after cell transfer, RGI-2001 (10 μg/kg) plus anti-CD40L mAb (0.5 mg) were injected intravenously and intraperitoneally respectively. Recipient mice were injected TAC (5.0 mg/kg/day, $N = 11$) or with EVL (1.0 mg/kg/day, $N = 8$) from day 1 to day 14. Control mice were not injected with immunosuppressants (control, $N = 13$). The proportion of donor cells in the peripheral blood of host mice in each group was analyzed by flow cytometry on 14, 28, and 56 days after BMT. (a) Representative dot plots of PBMC gated cells. (b) Summary of the proportion of donor cells in each treatment group. Each plot represents the data from individual mice, pooled from two individual experiments. BMT, bone marrow transplantation; EVL, everolimus; PBMCs, peripheral blood mononuclear cells; TAC, tacrolimus.

against donor APC was found in TAC-treated mice on day 28 after BMT (Fig. S3).

TAC administration interfered with Treg proliferation whereas EVL did not

As we had excluded any remaining possibility of anti-donor-reactive T cells in TAC-treated mice, we next wondered if TAC treatment might negatively impact Treg functions, resulting in donor cell rejection. To clarify this possibility, we analyzed the proportion of $CD4^+CD25^+Foxp3^+$ Tregs among splenic $CD4^+$ T cells on day 7 after transplantation. As shown in Fig. 4a and b, the proportion of Tregs decreased in mice that received TAC treatment compared with that in control or EVL-treated mice. Both control and EVL-treated mice showed high expression levels of Ki67 and CTLA-4 on Tregs, while TAC-treated mice displayed lower expression levels of both molecules (Fig. 4c-f). As Ki67 expression in $CD4^+CD25^-Foxp3^-$ conventional T cells (Tconvs) showed significant decreases in both TAC- and EVL-treated mice (Fig. 4c and d), it was presumed that TAC could suppress the proliferation of both Tconvs and Tregs whereas EVL preferentially suppressed that of Tconvs without interrupting Treg proliferation. The absence of negative impact of TAC on early iNKT cell proliferation after αGalCer stimulation was also confirmed in the spleen (Fig. S4).

TAC decreased the suppressive function of Tregs

In addition to the inhibition of Treg proliferation, we tested the possibility that TAC treatment might be involved in impairing the function of Tregs. $CD4^+CD25^{hi}$ T cells were isolated from the spleens of the mice treated with RGI-2001 and anti-CD40LmAb without immunosuppressants (control), and those with TAC or EVL treatment 7 days after BMT (Fig. 5a).

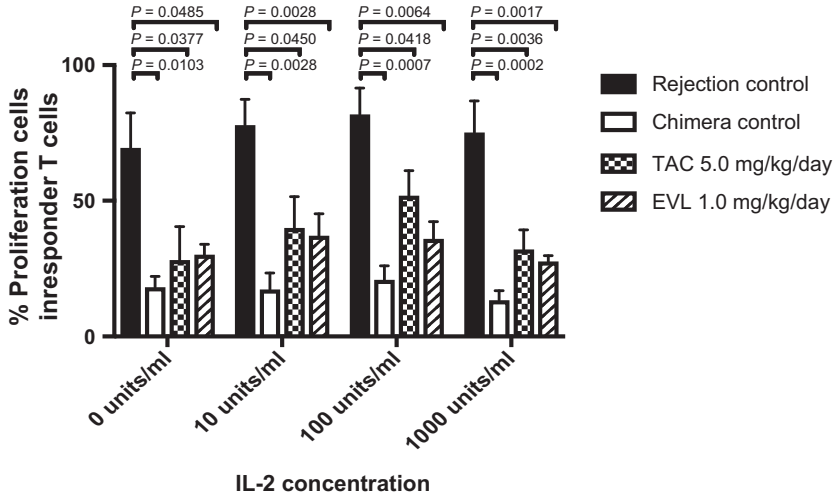

Figure 3 Host T cells acquired hyporesponsiveness against donor antigen on day 7 after BMT regardless of treatment regimen. BALB/c mice irradiated with 3 Gy were transplanted with B6 BMCs, with (open bar; chimera control, $N = 4$) or without (closed bar; rejection control, $N = 4$) RGI-2001 plus anti-CD40L mAb. Additional immunosuppressants, 5.0 mg/kg/day (checkered; $N = 4$) TAC, or 1.0 mg/kg/day EVL (slashed; $N = 4$), were administered consecutively after RGI-2001 plus anti-CD40L therapy. Seven days later, splenic $CD4^+CD25^-$ T cells were recovered from each mouse and labeled with CFSE. 1×10^5 splenic $CD4^+CD25^-$ T cells were co-cultured with 30 Gy irradiated APCs obtained from B6 (donor) mice in a 1:1 ratio, with or without the presence of recombinant mouse IL-2 at the indicated concentration. Four days later, cells were recovered and the proportion of CFSE-diluted cells was analyzed as proliferated responder T cells by flow cytometry. Data presented are the mean ± SD of each treatment group. Data presented is the mean ± SD of each treatment group. *P* values calculated by one-way ANOVA Tukey multiple comparison are shown. *P* values were not reported when there was no statistical difference. APC, antigen presenting cell; BMT, bone marrow transplantation; CFSE, carboxyfluorescein succinimidyl ester; EVL, everolimus; TAC, tacrolimus.

Since donor T cells were not observed in the chimeras at the early time point, we assumed that isolated $CD4^+CD25^{hi}$ T cells should have $H2K^{d+}$ restriction. Hence, we added the $CD4^+CD25^{hi}$ T cells into the primary culture of T cells freshly isolated from naïve $H2K^{d+}$ BALB/c mice that were stimulated with anti-CD3/CD28 beads to evaluate the suppressive functions of isolated cells. In primary culture without $CD4^+CD25^{hi}$ T cells, the population of total T cells that remained undivided was less than 10% (Fig. 5b and c). In the wells that were co-cultured with $CD4^+CD25^{hi}$ T cells obtained from control or EVL-treated mice, appropriately 40% of T cells remained unproliferated. On the other hand, the proliferation rate of T cells that were co-cultured with the $CD4^+CD25^{hi}$ T cells from TAC-treated chimeras was comparable with that of the primary culture, suggesting that $CD4^+CD25^{hi}$ cells in TAC-treated mice did not the ability to prevent T cell proliferation.

Discussion

Since Medawar reported in the 1950s that transplantation tolerance could be acquired by inducing donor mixed chimerism, a growing number of protocols for establishing chimerism have been invented in animal models [22]. This has made it feasible to attempt operational tolerance trials under clinical conditions [6–9]. However, in clinical settings, any induction regimen should include standard immunosuppressants to avoid organ rejection before tolerance is established. The impact of immunosuppressants should be tested for each protocol in preclinical animal experiments because the mechanisms underlying the establishment of chimerism differ between protocols. We previously reported a novel regimen for inducing mixed chimerism by using lipo-αGalCer plus a suboptimal dose of anti-CD40L mAb. This strategy is mildly preconditioning as the regimen relies on the endogenous regulatory immune system, amplified by iNKT cell activation. As a result of weak immunosuppression, this approach has a high risk of rejection before establishment of tolerance. Therefore, induction of initial immunosuppression would be mandatory.

Here, we tested the impact of TAC and EVL, popular immunosuppressants in clinical solid transplantation, on our regimen. Both TAC and EVL treatment provided additional inhibition of T cell alloreactivity, reduced Tconv proliferation, and promoted donor cell engraftment before drug cessation, suggesting that both

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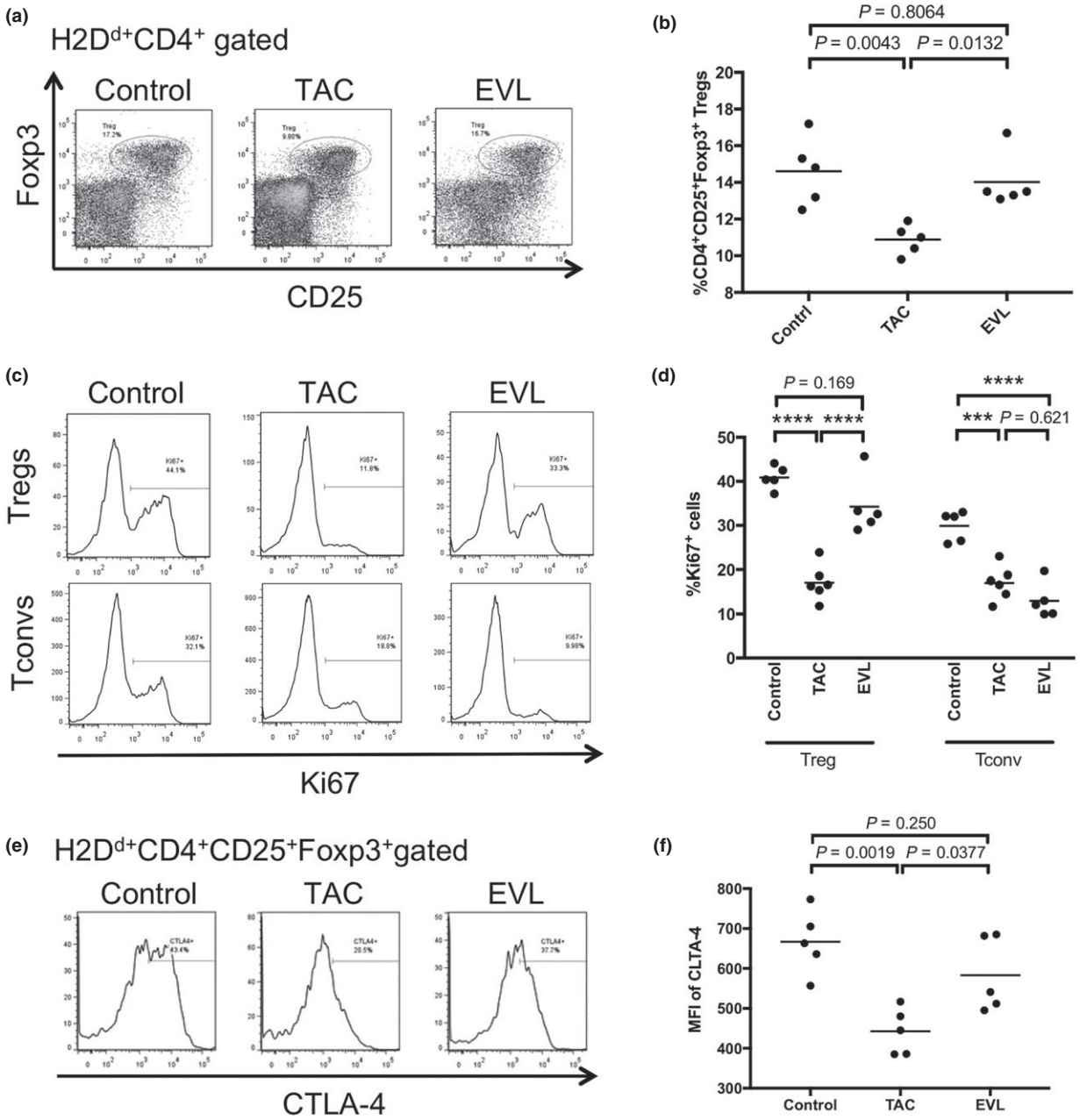

Figure 4 Tacrolimus administration abrogated Treg proliferation and activation. Splenocytes were recovered from recipient mice that were treated with RGI-2001 plus anti-CD40L mAb without immunosuppressant ($N = 5$), with 5.0 mg/kg/day TAC ($N = 5$), or with 1.0 mg/kg/day EVL ($N = 5$) on day 7 after BMT, and analyzed by flow cytometry. (a) Representative dot plots of H2D^{d+}CD4^{+} gated cells in mice with each treatment. (b) Summary of the proportion of CD4^{+}CD25^{hi}Foxp3^{+} Tregs in CD4^{+}T cells. (c) Representative histogram of Ki67 expression on H2D^{d+}CD4^{+}CD25^{hi}Foxp3^{+} Tregs and H2D^{d+}CD4^{+}CD25^{-}Foxp3^{-} Tconvs in each group. (d) Summary of the proportion of Ki67 expression of Tregs (left) and on Tconvs (right). (e) Representative histogram of the expression level of CTLA-4 on H2D^{d+}CD4^{+}CD25^{hi}Foxp3^{+} host Tregs. (f) Summary of the MFI level of CTLA4 in CD4^{+}CD25^{hi}Foxp3^{+} Tregs each treatment groups. Data are representative of two individual experiments. *P* value was calculated by one way ANOVA with Tukey multiple comparison test. ***$P < 0.001$; ****$P < 0.0001$; BMT, bone marrow transplantation; CTLA-4, cytotoxic T lymphocyte associated protein 4; EVL, everolimus; TAC, tacrolimus; Tregs, regulatory T cells. Tconv, conventional T cells; Tregs, regulatory T cells.

drugs have temporal graft-facilitating effects. However, whereas EVL did not have detrimental effects on establishment of long-term mixed chimerism, TAC treatment impeded persistence of donor cell engraftment over the long-term. Therefore, we supposed that TAC might have negative impact on the tolerance induction.

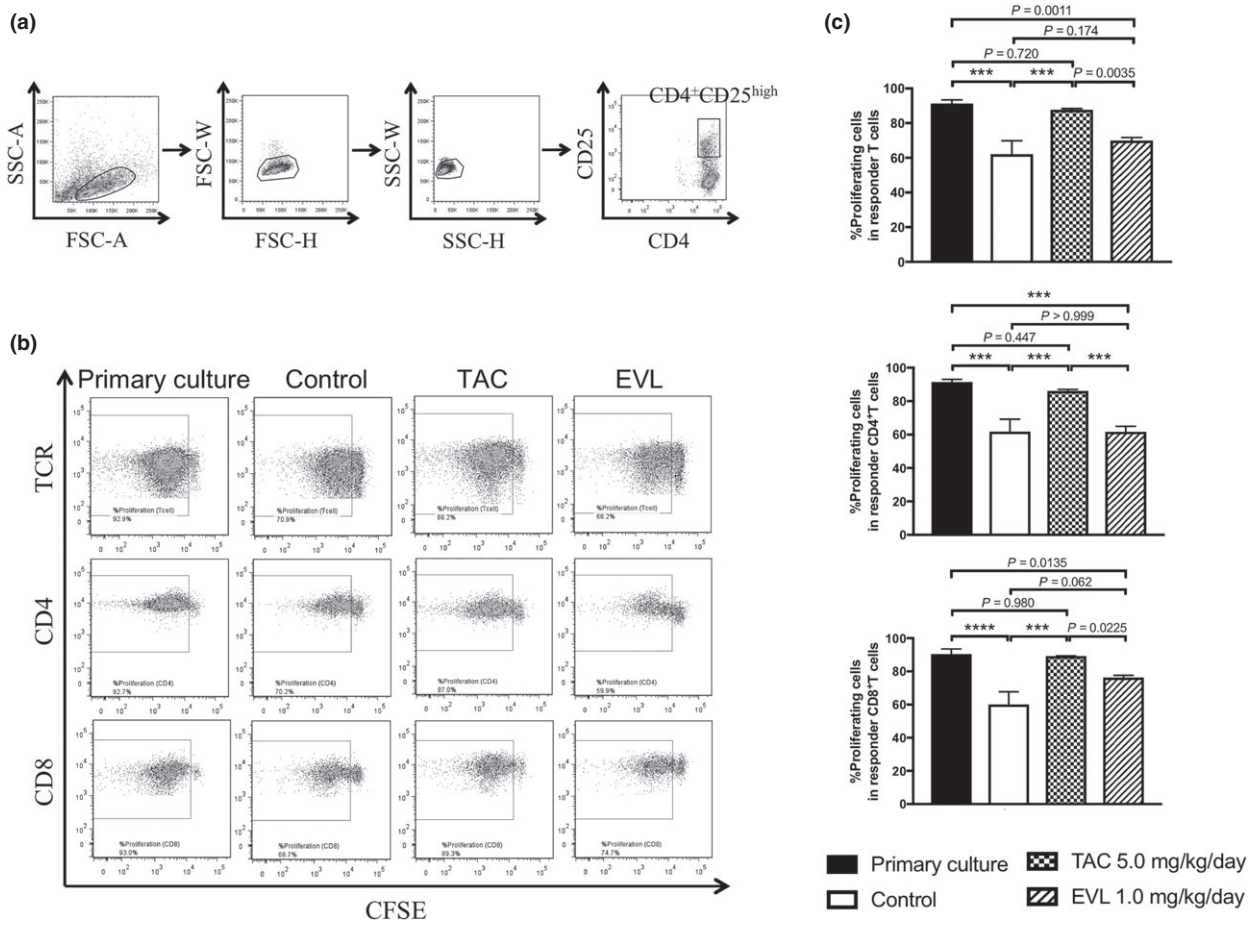

Figure 5 Tacrolimus administration abrogated the suppressive function of Tregs. Three-Gy-irradiated BALB/c mice were transplanted with B6-BMCs and treated with RGI-2001 plus anti-CD40L (control, $N = 3$), RGI-2001 plus anti-CD40L and 5.0 mg/kg/day TAC ($N = 4$), or RGI-2001 plus anti-CD40L and 1.0 mg/kg/day EVL ($N = 3$). Seven days later, splenocytes were recovered and pooled from each experimental group, following which the $CD4^+CD25^{hi}$ phenotype were isolated by flow cell sorting. Primary T cells that were freshly isolated from naïve BALB/c mice were labeled with CFSE and stimulated with CD3/CD28 beads at cells-to-beads ratio of 2:1 (primary culture). Sorted $CD4^+CD25^{hi}$ cells from each group were labeled with eFluor, then added into primary culture at 1:1 ratio (5×10^4/well). Cells were recovered on day 4 and the percentage of CFSE diluted cells were assessed as proliferating cells by flow cytometry. (a) Sorting strategy for $CD4^+CD25^{hi}$ cells. Splenocytes were enriched in $CD4^+$ cells. These cells were stained with FITC-conjugated anti-CD25 and PECy7-conjugated anti-CD4 mAb, and the $CD4^+CD25^{hi}$ fraction was sorted. (b) Representative dot plots of $CFSE^+eFluor^-$ cells gated on TCR-β^+ (top), $CD4^+$ (middle), or $CD8^+$ (bottom) T cells. (c) The mean (SD) of the percentage of proliferating cells in whole (top), $CD4^+$ (middle), or $CD8^+$ T cells (bottom) of triplicated wells of primary culture (closed bar), those from the co-culture with $CD4^+CD25^{hi}$ cells isolated from control (open bar), TAC- (checkered), or EVL-treated mice (slashed). *P* values calculated by one way ANOVA Tukey multiple comparison are shown. ***$P < 0.001$; ****$P < 0.0001$. Results are representative of two independent experiments. BMT, bone marrow transplantation; CFSE, carboxyfluorescein succinimidyl ester; EVL, everolimus; TAC, tacrolimus; Tregs, regulatory T cells.

The detrimental effects of CNIs on a tolerance induction protocol which used a co-stimulatory blockade were reported previously. Blaha *et al.* [23] evaluated the effects of immunosuppressive drugs on the development of long-term chimerism in their protocol consisting of anti-CD40L mAb and CTLA-4 Ig combined with 3 Gy TBI. When recipient mice were injected with TAC for four consecutive weeks, chimerism started declining from 5 weeks after transplantation. On the other hand, some reports showed that CNIs did not diminish the chimerism. Taylor *et al.* [24] demonstrated that cyclosporine (CsA) did not abrogate donor cell engraftment in their regimen, which consisted of anti-CD40L mAb without CTLA4 Ig. This discrepancy can be potentially explained by differences in the number of transferred BMCs (40×10^6 is twice that in our protocol). Kurtz *et al.* [25] also reported that CsA did not impede long-term chimerism in a model that uses anti-CD40L mAb combined with pretransplant $CD8^+$ T cell depletion, though CsA

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treatment weakened peripheral deletion of the donor antigen-specific repertoire in $CD4^+$ T cells. Wekerle *et al.* [26] reported that deletion of donor-reactive T cells by AICD was inhibited by CsA one week after BMT. We also reported that $V\beta6^+$ T cells, which are eliminated by negative selection during differentiation in AKR mice, were deleted in BALB/c mice that were tolerized to AKR donors by our regimen. In contrast with Kurtz's and Wekerle's reports, however, the reduction in $V\beta6^+$ T cell repertoire was not significantly different between chimeric mice without immunosuppressants and TAC-treated mice. *In vitro* hyporesponsiveness against donor antigen shown in T cells that were isolated from TAC-treated mice also suggested that the deletion of donor-reactive T cells occurred in the presence of TAC in the current study.

The other mechanism recently focused on as a fundamental for tolerance is the peripheral regulation by Tregs. We previously reported that Tregs played a key role in our tolerance induction regimen because Tregs were increased within a week after BMT and chimerism was lost when Tregs were depleted within 7 days after BMT [18,19]. Similarly, it has been reported that Tregs required for maintenance of transplant tolerance in other rodent models [27,28]. It has been reported that CNIs inhibit the intracellular phosphatase calcineurin, which dephosphorylates cytosolic nuclear factor of activated T cells (NFAT) and leads its nuclear translocation and transcriptional activation of the IL-2 gene [29,30]. Treg homeostasis especially depends on IL-2 from other T cells and APCs because of higher expression of CD25, known as IL-2 receptor α-chain. Therefore, CNIs inhibit Treg proliferation and maintenance indirectly by suppressing the production of IL-2 [14,31,32]. Miroux *et al.* [33] reported CNIs inhibit Treg proliferation in a dose-dependent manner under *in vitro* culture conditions. This negative effect of CNIs on Tregs occurred in both experimental animal models and transplant patients to whom CNIs were administered orally [34–37]. Consistent with these findings, flow cytometry analysis 7 days after BMT in the current study showed a lower proportion of, and lower expression level of Ki67 in, $CD4^+CD25^+Foxp3^+$ Tregs of TAC-treated mice than those of control or EVL-treated mice.

The expression of CTLA-4 in $CD4^+CD25^+Foxp3^+$ Tregs was significantly lower in the TAC-treated mice. Furthermore, the suppression MLR assay showed that TAC treatment impaired the immunoregulatory function of Tregs. It is believed that expression of CTLA-4, which has higher affinity to B7-1 (CD80) and B7-2 (CD86) expression on APCs and inhibits T cell activation by competing with CD28 [38–40], is fundamental for Tregs immune-regulatory potential because a deficiency and inhibition of CTLA-4 on Tregs impaired their immunosuppressive function [41,42]. It has been reported that the transcription factor NFAT regulates the expression level of CTLA-4 by cooperating with Foxp3, or by directly binding to the promoter of CTLA-4 [43,44]. It is presumed that TAC treatment reduced CTLA-4 expression through the inhibition of NFAT dephosphorylation and resulted in impeding the suppressive potential of Tregs against CD3/CD28 stimulation.

Our results suggested that TAC-treatment could interfere not only with Treg proliferation but also with their immunosuppressive functions, resulting in abrogation of the establishment of mixed chimerism. Contrary with TAC treatment, EVL did not diminish the establishment of mixed chimerism in this study. EVL treatment combined with lipo-αGalCer and anti-CD40L mAb promoted engraftment of donor cells on day 14 after transplantation and sustained long-term, robust mixed chimerism after cessation of the drug. The synergic effect of mTOR-I and anti-CD40L mAb was reported in a murine BMT and organ transplantation model [23,45]. We also showed that EVL did not impair the proliferation and function of Tregs. It has been reported that mTOR-I, which suppress downstream PI3K/Akt signaling, has a beneficial effect on Tregs expansion [16,34,46]. Gao *et al.* [47] reported that a combination of mTOR-I (3.0 mg/kg rapamycin, RPM) and anti-CD40L mA increased Tregs in the spleen. Others reported that, in comparison with CsA-treated mice, the Tregs population was preserved after BMT in mice treated with low doses of RPM [48]. The EVL dose used in the current study was decided according to the minimum requirement for cardiac allograft acceptance. This therapeutic dose did not affect Treg proliferation and function. Taken together, it is supposed that EVL treatment prevents donor cells rejection without interfering with Tregs expansion and activation, thus resulting in better engraftment.

In summary, TAC inhibited the long-term establishment of mixed chimerism and impaired Treg proliferation and suppressive function, whereas EVL did not, when administered after combination therapy of lipo-αGalCer plus anti-CD40L mAb. These results suggest that CNIs could introduce risk when used in an initial treatment regimen for mixed chimerism induction, especially regimens that are highly dependent on the regulatory immune system, such as Tregs cell therapy, low-dose IL-2 therapy, and αGalCer therapy. The mTOR-I could benefit Tregs, and is thought to be a

better choice for the maintenance of immunosuppression, though mTOR-I has not been associated with improvement of allograft survival in organ transplant recipients [48,49]. Further studies are needed to seek appropriate immunosuppressants that can be combined with tolerance induction regimen.

Authorship

HK: performed research, analyzed data and wrote the paper. SM: performed research and analyzed data. TH: designed study. MI, HF, TK, RI, and KO: performed research. KS, MO, TY, and KT: supported experiment planning and supervised the study. YI: provided reagents (RGI-2001) and supported experiment planning. All authors participated in manuscript editing and provided final approval of the text of the manuscript.

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Conflict of interest

The authors of this manuscript have conflicts of interest to disclose as described by *Transplant International*. Y. Ishii is the executive chairman of REGiMMUNE Corp. The other authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. The frequencies of host immune cells 14 days after bone marrow transplantation.

Figure S2. Tacrolimus did not impaired peripheral tolerance in the early phase after bone marrow transplantation.

Figure S3. T cell response against donor antigen was recovered in tacrolimus-treated mice on day 28 after bone marrow transplantation.

Figure S4. Tacrolimus did not affect lipo-αGalCer mediated iNKT cell activation.

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