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ORIGINAL ARTICLE

Improving allogeneic islet transplantation by suppressing Th17 and enhancing Treg with histone deacetylase inhibitors

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Summary

Islet transplantation is a new treatment for achieving insulin independence for patients with severe diabetes. However, major drawbacks of this treatment are the long graft survival, the necessity for immunosuppressive drugs, and the efficacy of transplantation. Donor-specific transfusion (DST) has been shown to reduce rejection after organ transplantation, potentially through enhanced regulatory T-cell (Treg) activity. However, recent findings have shown that activated Treg can be converted into Th17 cells. We focused on histone deacetylase inhibitors (HDACi) because it was reported that inhibition of HDAC activity prevented Treg differentiation into IL17-producing cells. We therefore sought to enhance Treg while suppressing Th17 cells using DST with HDACi to prolong graft survival. To stimulate Treg by DST, we used donor splenocytes. In DST with HDACi group, Foxp3 mRNA expression and Treg population increased in the thymus and spleen, whereas Th17 population decreased. qPCR analysis of lymphocyte mRNA indicated that Foxp3, IL-10, and TGF-b expression increased. However, interleukin 17a, Stat3 (Th17), and IFN-g expression decreased in DST + HDACi group, relative to DST alone. Moreover, DST treated with HDACi prolonged graft survival relative to controls in mice islet transplantation. DST with HDACi may therefore have utility in islet transplantation.

Introduction

Beneficial effects and unsolved issues of islet transplantation

The Edmonton protocol of 2000 opened a new age of clinical islet transplantation research for the treatment of type 1 diabetes. In their report, seven patients with type 1 diabetes became insulin independent after islet transplantation with glucocorticoid-free immunosuppression [1]. Approximately 80% of study subjects had islet function as indicated by the presence of C-peptide at 5-year follow-up, although only 10% of patients maintained insulin independence [2]. That study demonstrated that while there are several issues to be solved, islet transplantation holds promise as a treatment for severe diabetes.

Islet transplantation still faces several challenges, including the requirement for immunosuppressants to prevent rejection. Immunosuppressants cause side effects and hinder beta cell regeneration, and incomplete immunosuppression can lead to autoimmune recurrence or allorejection [3]. Eliminating the need for immunosuppressants is therefore a major goal for islet transplantation and would significantly improve its efficacy.

Donor-specific blood transfusion for immunological tolerance

Donor-specific blood transfusion (DST) has been shown to reduce rejection after organ transplantation [4]. A possible mechanism of DST is stimulation of regulatory T cells that have potent immunosuppressive effects. It has also been shown that the simultaneous infusion of islets and regulatory T cells reduces the rejection and prolongs islet survival in a mouse model [5].

Histone deacetylase inhibitors (HDACi) for enhancing Treg

We focused on HDACi for promoting the generation of Treg [6–8]. Histone deacetylases (HDACs), in conjunction with histone acetyltransferases, control the level of acetylation on lysine residues in histones. Treatment of cells with HDACi, such as trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA), leads to hyperacetylation of histones, resulting in a more open chromatin architecture and increased access for transcription factors [9]. HDACi regulates gene expression as well as the functions of more than 50 transcription factors and nonhistone proteins [10]. Treg is a target of HDACi. Studies on Foxp3, a key gene of Treg, reveal that histone H4 is hyperacetylated when the gene is activated [11]. Another study revealed that acetylated Foxp3 is upregulated in $CD4^+$ $CD25^+$ Treg cells [12]. Moreover, TSA induced Treg production from naïve $CD4^+$ CD25-T-cell populations following epigenetic modification [13]. These results suggest that HDACi treatments altered CpG island methylation sites that allow FOXP3 to enter the space between DNA and histone proteins, allowing transcription. Both methylated and acetylated FOXP3 within $CD4^+$ CD25-T cells induced Treg phenotypes *in vitro*. To summarize, HDACi treatment may enhance Treg expression by methylation and acetylation of Foxp3.

HDACi for anti-Th17 effect

Studies suggest that activated Treg promotes Th17 cell differentiation from CD4 T cells, through production of TGF-b. In addition, transfer of Treg enhanced IL17 production in a mouse model, and enhanced IL17 is associated with systemic autoimmune disease [14]. Therefore, expansion of Th17 cells may disrupt Treg and immunological tolerance.

Importantly, treatment of HDACi may help address this problem. Interestingly, differentiation of Treg into IL17-producing cells depended on HDAC activity, and inhibition of HDAC activity prevented differentiation into IL17-producing cells, yet sustained Foxp3 expression [15]. Based on these data, we hypothesized that HDACi could be critical for increasing Treg growth and preventing Treg from becoming Th17 cells.

Materials and method

In vitro experiments

Balb/c mice were used as donors, and C57BL/6 mice were used as recipients. To stimulate Treg by DST, we used donor splenocytes. Splenocytes (1.0×10^8 cells) derived from Balb/c mice were injected into C57BL/6 mice (day 0, i.v.). TSA, a HDACi, was also injected (1.0 mg/kg/day, day 0–6, i.p.). On day 7, thymic and splenic lymphocytes were isolated and analyzed by flow cytometry (CD4, CD25, Foxp3, and IL17a) as *in vitro* experiments. In addition, mRNA expressions in thymic and splenic lymphocytes were analyzed by qPCR (Foxp3, TGF-b, IL-6, IL-10, IL-17a, IL-21, Stat3, and IFN-g) (SABiosciences, Frederick, MD, USA) as *in vitro* experiments.

In vivo experiments

Streptozotocin (180 mg/kg, i.v.)-induced diabetic C57BL/6 mice were used as recipients. Donor splenocytes (1.0×10^8 cells, day 0, i.v.) and TSA (1.0 mg/kg/day, day 0–6, i.p.) were injected, and on day 7, 400 islets from donor mice were transplanted into the left renal capsule of recipient mice as *in vivo* experiments. Graft survival was observed by checking the blood glucose level three times a week.

Immunohistochemistry

Frozen sections were made with Cryostat (CM 3050S, Leica, Wetzlar, Germany) from the left renal capsule of DST + TSA recipient mice 60 days after islet transplantation and control recipient mice after graft rejection. Sections were stained with anti-mouse insulin antibody (Abcam, Cambridge, MA, USA).

Statistics

Statistical significance was determined by one-way ANOVA and Tukey/Kramer *post hoc* test. All statistical analyses were performed using STATVIEW 5.0 (SAS Institute Inc, Cary, NC, USA). Differences were considered significant if $P < 0.05$.

Results

DST and HDACi induced Treg expression

In *in vitro* and *in vivo* models, mice were divided into four groups ($n = 5$): (i) control, (ii) DST, (iii) TSA (HDACi), and (iv) DST + TSA. In preliminary data, we observed that Treg did not significantly increase on day 3 after DST. However, Treg significantly increased on day 7 after DST.

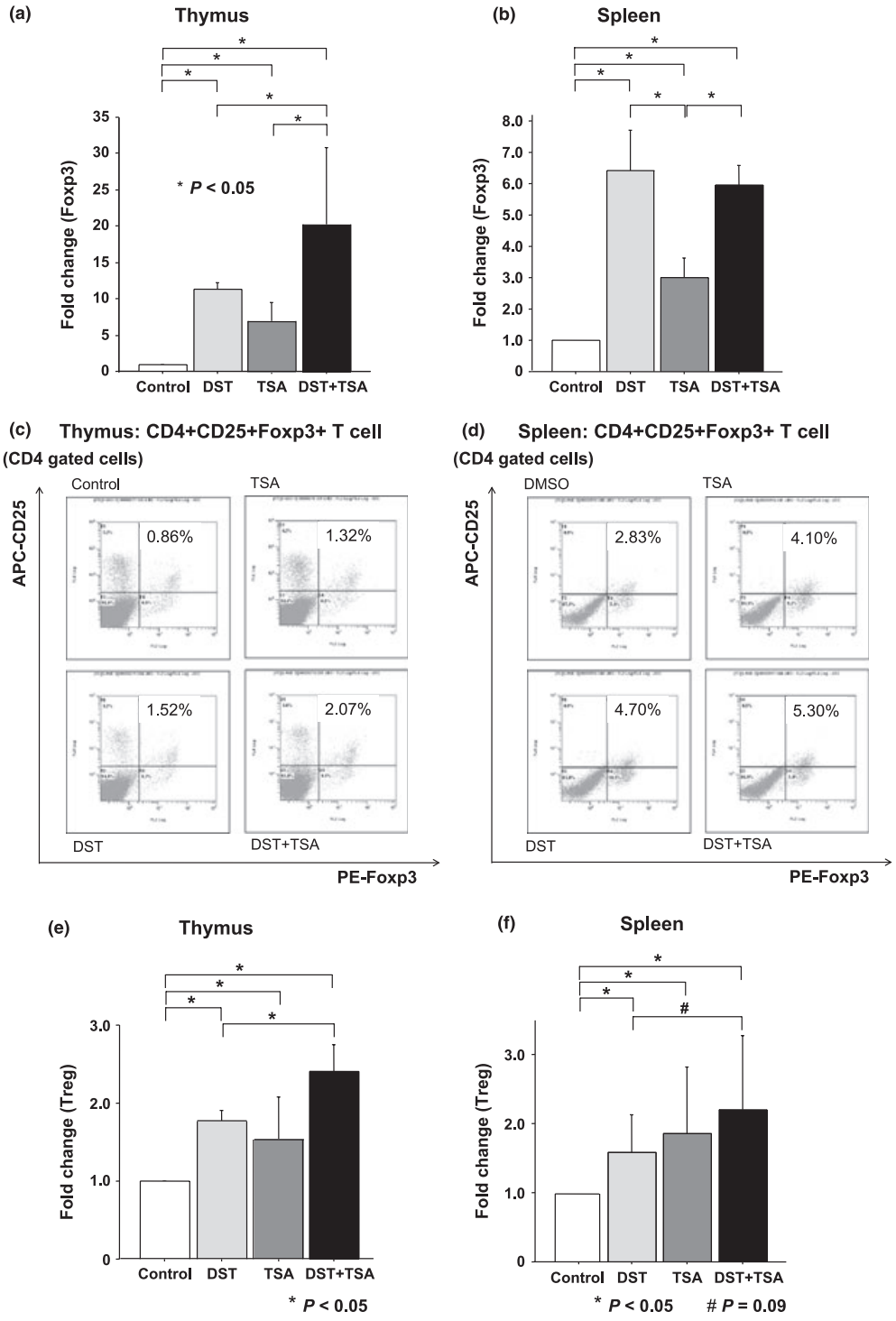

Figure 1 Donor-specific transfusion (DST) and HDACi induced Treg expression. (a) Foxp3 mRNA expression in thymus (day 7). Foxp3 mRNA expression following DST + TSA treatment is significantly higher than with DST or TSA alone ($P < 0.05$). (b) Foxp3 mRNA expression in spleen (day 7). There is no significant difference between DST and DST + TSA groups. (c) Flow cytometry chart of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells in thymus (day 7). The representative data of FACS analysis in thymus were shown. (d) Flow cytometry chart of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells in spleen (day 7). The representative data of FACS analysis in spleen were shown. (e) FACS analysis of the fold change in Treg in thymus. The fold change of Treg in DST + TSA was significantly higher than that in the DST group ($P < 0.05$). (f) FACS analysis of the fold change of Treg in spleen. Treg increase observed for DST + TSA tended to be higher than that in the DST group ($P = 0.09$). TSA, trichostatin A.

So we analyzed the immune response on day 7. In *in vitro* model, we made the four groups, and on day 7, Foxp3 mRNA expression in thymus was significantly increased in DST, TSA, and DST + TSA, relative to controls. Moreover, Foxp3 mRNA expression in DST + TSA was significantly higher than DST or TSA alone (Fig. 1a). On the other hand, splenic Foxp3 mRNA expression was significantly increased in DST, TSA, and DST + TSA relative to controls. However, there was no significant difference between the DST and DST + TSA groups (Fig. 1b). As determined by FACS analysis, the fold change of Treg in thymus was significantly higher in DST, TSA, and DST + TSA than in controls. Moreover, the Treg increase observed for DST + TSA treatment was significantly higher than that observed in DST-only treatment (Fig. 1e). In spleen, the fold increase in Treg was significantly higher for DST, TSA, and DST + TSA against controls. However, the Treg increase in DST + TSA group tended to be higher than that for DST treatment ($P = 0.09$) (Fig. 1f). The representative data of FACS analysis of lymphocytes in thymus and spleen are shown in Fig. 1c and d.

HDACi decreased Th17 expression

We next focused on Th17 expression. In *in vitro* model, we used four groups ($n = 5$): (i) control, (ii) DST, (iii) TSA (HDACi), and (iv) DST + TSA. We made the four groups, and on day 7, splenocytes were taken and analyzed for mRNA expression and flow cytometry. IL-17, a major Th17 cytokine, increased (not significantly) mRNA expression when treated with DST. However, IL-17 mRNA expression decreased significantly in DST + TSA group in comparison with DST-alone group (Fig. 2a). FACS analysis indicated that the population of $CD4^+$ $IL17^+$ cells in DST + TSA treatment group was significantly lower than that in DST group (Fig. 2c). The representative data of FACS analysis of $CD4^+$ $IL17^+$ cells in spleen are shown in Fig. 2b.

DST and HDACi changed mRNA expression associated with Treg and Th17

We measured various mRNAs associated with Treg and Th17 from splenocytes of each of the four groups (Fig. 3). Treg secretes IL-10 and TGF-b, and they suppress the immunological reaction. We measured IL-10 and Tgfb1, mRNA of IL-10 and TGF-b, and the expression of those cytokines in recipient splenocytes was significantly higher in DST + TSA treatment relative to TSA only, or control (Fig. 3a and b). Th17 secretes IL-17 family, and they involve in inducing and mediating proinflammatory responses. We measured IL-17a, a member of IL-17 family, and IL-17a mRNA expression in recipient splenocytes was significantly

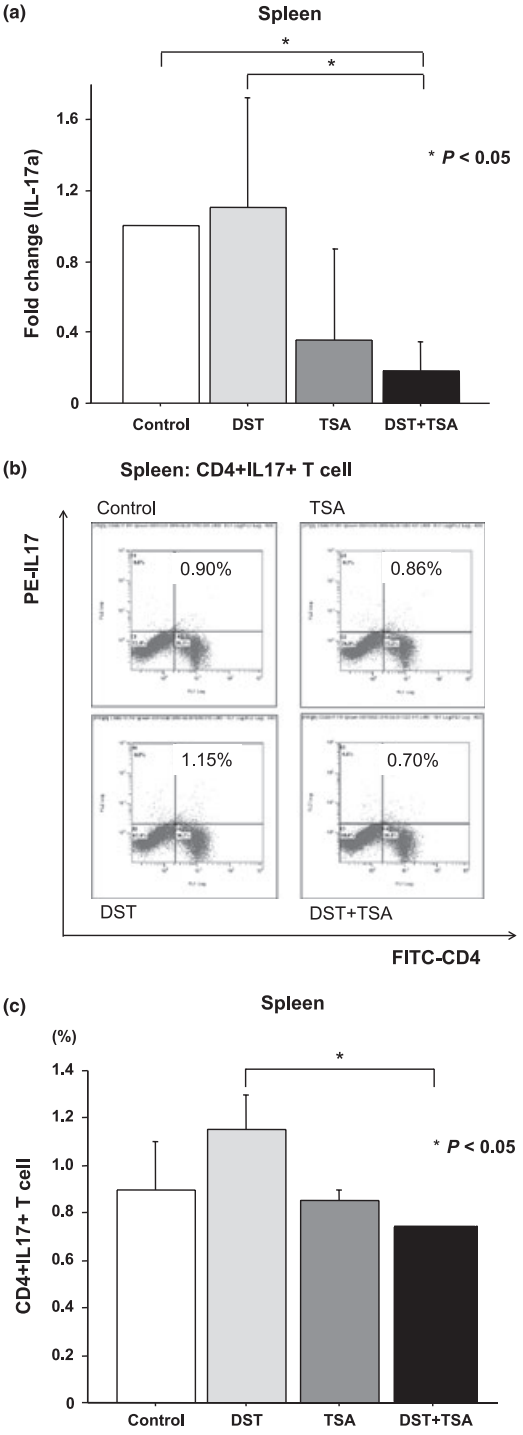

Figure 2 Histone deacetylase inhibitors (HDACis) decreased Il-17 expression. (a) IL-17 mRNA expression decreased significantly in DST + TSA group ($P < 0.05$). (b) Flow cytometry chart of $CD4^+$ $IL17^+$ T cells in spleen (day 7). The representative data of FACS analysis in spleen were shown. (c) FACS analysis of $CD4^+$ $IL17^+$ cells following DST + TSA treatment IL-17 was significantly lower than that of DST only ($P < 0.05$). DSA, donor-specific transfusion; TSA, trichostatin A.

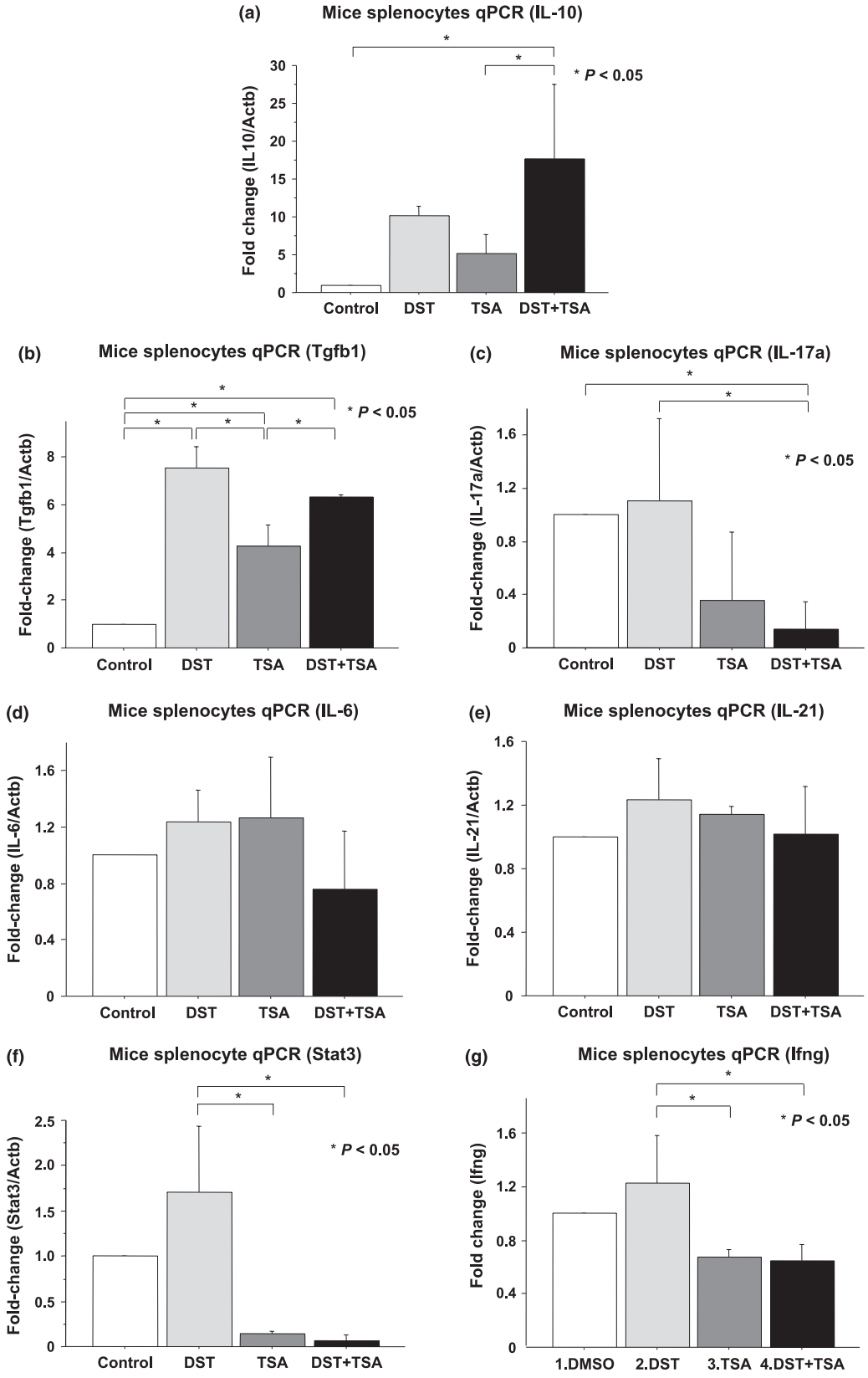
(a)
Mice splenocytes qPCR (IL-10)
Fold change (IL10/Actb)
* P < 0.05
Control
DST
TSA
DST+TSA
(b)
Mice splenocytes qPCR (Tgfb1)
Fold-change (Tgfb1/Actb)
* P < 0.05
Control
DST
TSA
DST+TSA
(c)
Mice splenocytes qPCR (IL-17a)
Fold-change (IL-17a/Actb)
* P < 0.05
Control
DST
TSA
DST+TSA
(d)
Mice splenocytes qPCR (IL-6)
Fold-change (IL-6/Actb)
Control
DST
TSA
DST+TSA
(e)
Mice splenocytes qPCR (IL-21)
Fold-change (IL-21/Actb)
Control
DST
TSA
DST+TSA
(f)
Mice splenocyte qPCR (Stat3)
Fold-change (Stat3/Actb)
* P < 0.05
Control
DST
TSA
DST+TSA
(g)
Mice splenocytes qPCR (Ifng)
Fold change (Ifng)
* P < 0.05
1.DMSO
2.DST
3.TSA
4.DST+TSA

Figure 3 Donor-specific transfusion (DST) and HDACi changed mRNA expression associated with Treg and Th17. Various mRNAs associated with Treg and Th17 from lymphocytes of the four groups were measured by qPCR. (a) IL-10 mRNA expression in spleen. IL-10 mRNA expression following DST + TSA treatment is significantly higher than following control or TSA alone. (b) Tgfb1 mRNA expression in spleen. Tgfb1 mRNA expressions following DST + TSA treatment or DST only are significantly higher than following control or TSA alone. (c) IL-17 mRNA expression in spleen. IL-17 mRNA expression following DST + TSA treatment is significantly lower than following DST only. (d) IL-6 mRNA expression in spleen. There is no significant difference in IL-6 mRNA expression. (e) IL-21 mRNA expression in spleen. There is no significant difference in IL-21 mRNA expression. (f) Stat3 mRNA expression in spleen. Stat3 mRNA expression following DST + TSA treatment is significantly lower than following DST only. (g) Ifng mRNA expression in spleen. Ifng mRNA expression following DST + TSA treatment is significantly lower than following DST only. TSA, trichostatin A.

decreased in DST + TSA treatment group in comparison with control and DST-alone groups (Fig. 3c). However, IL-6 and IL-21 mRNA expression was not significantly different between groups (Fig. 3d and e). Moreover, we measured Stat3, a major Th17 transcription factor, to evaluate the activity of Th17 cells. Stat3 was expressed significantly higher following DST treatment relative to control. However, Stat3 expression did not differ between DST + TSA and control. In addition to Treg and Th17 system, IFN-g was measured because it was critical for innate and adaptive immunity and produced from natural killer cells, natural killer T cells, CD4 Th1 cells, and CD8 cytotoxic T cells. Ifng mRNA expression in TSA and DST + TSA was significantly lower than that following DST only.

DST + TSA improved the graft survival in mouse islet transplantation

We examined islet graft survival in a mouse model (n = 5). There was no difference in graft survival between mice in the control, DST, and TSA groups. However, with DST + TSA treatment, graft survival was significantly improved. Moreover, we observed extended survival (over 60 days) in the DST + TSA group (Fig. 4). Insulin staining indicated that islets secrete insulin in the transplantation site 60 days after transplantation (Fig. 5a and b). On the other hand, in control group there was no islet secreting insulin, and we recognized the fibrillization and inflammation cells under the renal capsule after rejection (Fig. 5c and d). We observed the similar changes in DST or TSA group.

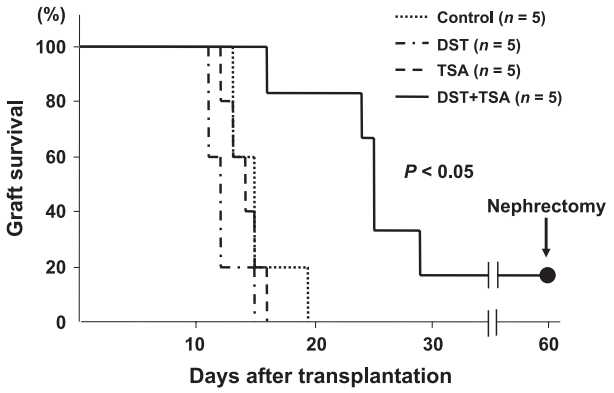

Figure 4 DST + TSA improved graft survival in a mouse islet transplantation model. A total of 400 islets from donor mice were transplanted into the left renal capsule of recipient mice, and graft survival was observed by checking blood glucose level three times a week. Following DST + TSA treatment, graft survival was significantly improved ($P < 0.05$). DSA, donor-specific transfusion; TSA, trichostatin A.

Discussion

Islet transplantation is a promising treatment for diabetes. However, there are several problems to be solved. These problems include length of graft survival, the reduction of immunosuppressive drugs, and transplantation efficacy. Treg is likely a key regulatory cell type that needs to be managed to solve these problems. Sakaguchi *et al.* originally identified this cell population as a regulator for autoreactive T cells [16]. Treg can strongly regulate other T cells depending on cell-associated molecules such as CTLA-4 and GITR, as well as soluble mediators including IL-10 or TGF-b, and cytotoxic $CD8^+$ T cells. Graft

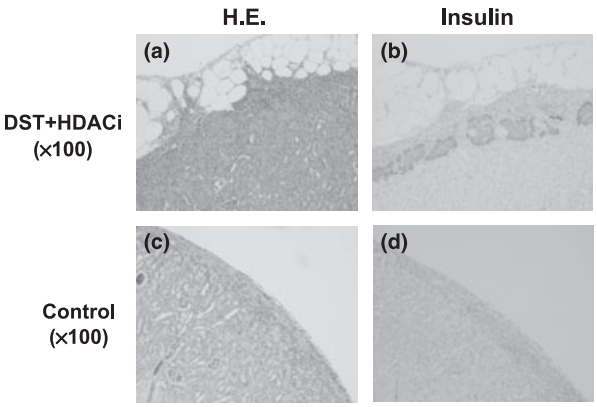

Figure 5 Insulin staining of transplantation site in a mouse islet transplantation model. (a) H.E. staining (×100). H.E. staining identified islets under the renal capsule of recipient mice. (b) Insulin staining (×100). Insulin staining identified islets that secrete insulin in the transplantation site 60 days following transplantation. (c) H.E. staining (×100). H.E. staining identified no islets under the renal capsule of recipient mice after rejection. We recognize the fibrillization and inflammation cells under the renal capsule after rejection. (d) Insulin staining (×100). Insulin staining identified no islets that secrete insulin in the transplantation site.

rejections are T-cell-mediated immunoreactions, making Tregs a natural target for researchers to consider in controlling graft rejection. Indeed, it is known that Treg increases in patients with immunological tolerance [17,18] and that increasing the number of Tregs in recipients prevents both acute and chronic rejection in several animal models [19]. Treg can be increased in several experimental models, such as multiple blood transplantations [20], blocking of CD40-CD154 or CD80/CD86-CD28 costimulatory interactions [21–23], anti-CD28 antagonist [24,25], and ex vivo Treg expansion [26]. In this study, we used donor-specific transfusion (DST). DST is a classic and empirical method. However, we still sought to evaluate the utility of this approach. It is reported that anergy [27,28], clonal deletion [29,30], regulation of cytokine production [31,32], microchimerism [33,34], generation of soluble MHC antigen [35], or a combination of these mechanisms may mediate DST. However, the specific mechanism of DST is still unknown [36,37].

Our data show that DST increased the Treg population and increased Foxp3, IL-10, and Tgfb1 mRNA expression. In this respect, it may be thought that DST has utility by itself. However, other data suggest that DST is not effective for graft survival in an islet transplantation model [38], indicating that DST has limits.

We focused on Th17 cells because previous studies showed that Treg enhancement also increases Th17 cells via TGF-b and IL-6 induction. Treg differentiates into Th17 cells. A GAD vaccination study showed that GAD vaccination enhanced not only Treg but also Th1 and Th17 cells, which failed to prevent type 1 diabetes. Moreover, in a mouse syngeneic islet transplantation model, blockade of IL-17 resulted in extended graft survival [39]. Therefore, we hypothesized that DST with blockage of anti-inflammatory drugs could improve graft survival.

We therefore used HDACi to inhibit inflammatory cytokines (Th1 and Th17). HDACi was also known to increase Treg and decrease Th17 differentiation by sustaining Foxp3 expression and inhibiting IL-6. Moreover, it has been shown that HDACi blocks IL-23 production and inhibits Th17 differentiation [40]. Our study showed that TSA increased Treg expression and regulatory cytokines (IL-10 and Tgfb1). Moreover, DST + TSA induced expansion of Tregs and IL-10 and significantly decreased Th17 (IL-17a) and Th1 (Ifng), compared with DST only. In the mouse islet transplantation model, DST + TSA improved graft survival, and we observed extended survival (over 60 days after transplantation).

Cytokine analysis indicated that IL-10 and Tgfb1 mRNA were significantly increased by DST + TSA and improving, in principle, Treg function. IL17a and Ifng mRNA expression was decreased by TSA. However, IL-6 and IL-21 expression was not significantly decreased.

In conclusion, HDACi increased Treg expression and inhibited Th17 differentiation, accompanied with Treg induction. These results suggest certain therapeutic strategies that may be useful for improving graft survival.

Authorship

KS: designed and performed research, analyzed data, wrote paper. TI: performed research, analyzed data. MT: performed research, analyzed data. MS: analyzed data. DC: analyzed data. JASR: analyzed data. BN: analyzed data. MFL: analyzed data. MS: analyzed data. SM: designed research, analyzed data.

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