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Original Article

Induction of antigen-specific immune tolerance by TGF-β-induced CD4+Foxp3+ regulatory T cells

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Abstract: Like natural $CD4^+CD25^+$ Treg cells, TGF-β-induced Treg cells also prevent allograft rejection in MHC-mismatched organ transplantation models. In analyzing this effect with greater detail, we determined that injection of TGF-β-induced, alloactivated $CD4^+CD25^+$ cells induces antigen-specific immune tolerance *in vivo*. Increased $CD4^+CD25^+$ cells in recipients contribute to this immune tolerance. In addition, adoptive transfer of TGF-β-induced $CD4^+CD25^+$ cells did not result in significant toxic and side effects in recipients. These results indicate that TGF-β-induced, alloactivated $CD4^+CD25^+$ cells may provide a safe and effective approach to protect MHC-mismatched organ grafts from rejection in a clinical setting.

Key words: TGF-β, Foxp3, regulatory T cells, Immunoregulation, transplant tolerance

Introduction

A thymus-derived, naturally-occurring, regulatory/suppressor cell subset of $CD4^+$ cells that express the IL-2 receptor alpha chain (CD25) and the transcription factor Foxp3 (nTregs), plays an important role in maintaining immunologic tolerance and controlling T-cell homeostasis [1, 2]. In addition to their role in controlling autoimmune diseases, nTregs cells are also able to prevent acute and chronic graft rejection. The absence or reduction of $CD4^+CD25^+Foxp3^+$ nTregs within the grafted kidney and peripheral blood appears to be associated with irreversible acute rejection (AR), indicating their role in systemic and local immunoregulation [3]. Dysfunction of nTregs has also been associated with transplant rejection. Dijke *et al* reported that nTregs in heart transplant patients who experienced acute rejection had an up-regulated CD127 expression and an inadequate regulatory function compared with those of non-rejecting patients [4].

Several lines of evidence indicate that $CD4^+CD25^+Foxp3^+$ nTregs induce immune or transplantation tolerance. Adoptive transfer of expanded nTregs resulted in the efficient and durable control of GVHD and prevented the allograft rejection in skin and liver transplantation model [5-9]. Others also provide indirect evidence that nTregs induce immune tolerance. Trani et al reported that inoculation of donor Ags into the recipient thymus can promote donor-specific tolerance through up-regulation of $CD4^+CD25^+Foxp3^+$ cells [10]. Maurik et al also observed that the generation of donor alloantigen-specific $CD4^+CD25^+$ cells is responsible for long-term allograft acceptance by blockade of CD40-CD154 interactions [10].

It is notable that the frequency of $CD4^+CD25^+$-$Foxp3^+$ nTregs is very low and that the phenotypic and functional activity varies following expansion. In addition, nTregs are unstable in the inflammatory milieu and their functional activity decreases in the presence of IL-6 or TNF-α [11, 12]. They began to lose Foxp3 expression and functional activity when stimulated with IL-6 [13, 14]. However, another type of Tregs, TGF-β-induced $CD4^+$ Treg cells (iTregs), may have an advantage in an

inflammatory milieu [2, 14]. TGF-β-iTregs share many similar phenotypic and functional characteristics with nTregs [15-17]. They do not proliferate when stimulated with anti-CD3 but can do so when in the presence of IL-2. Addition of iTregs to $CD4^+CD25^-$ responder T cells significantly suppresses the activation, proliferation and cytokine production of the latter [18]. Adoptive transfer of these cells prevents allergic pathology and inflammation in the lungs [16], spontaneous development of type 1 diabetes [19], autoimmune gastritis [20], colitis [21] and lupus [22].

We previously reported that adoptive transfer of TGF-β-primed alloactivated T cells signify-cantly prevented allograft rejection in MHC-mismatched heart transplantation [23]. Herein we demonstrate that injection of TGF-β-primed alloactivated $CD4^+CD25^+$ cells results in antigen-specific T cell non-response *in vivo*. Increased $CD4^+CD25^+Foxp3^+$ Tregs in recipients contribute to this T cell non-responsiveness. This antigen-specific T cell non-response might have an important clinical implication on transplantation tolerance.

Materials and methods

Animals

Male C57BL/6 (B6, H-2^b), DBA/2 (D2, H-2^d), C3H (H-2^k) and TGF-β RII KO (C57BL/6 strain) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and Shanghai laboratory animal center. Animals 8–10 weeks of age were used as recipients and cells were isolated from H-2^d mice and allostimulated with antigens from H-2^b or H-2^k mice. All mice were housed in conventional facilities at the University of Southern California or at Tongji University using animal care protocols approved by the Institutional Animal Care and Use Committee of the University of Southern California or Tongji University.

Antibodies and reagents

The following antibodies were obtained from eBioscience (San Diego, CA, USA): Anti-CD3–PE (145-2011), anti-CD4–FITC (RM4-5), anti-CD4–PE (GK1.5), anti-CD8–PE (53-6.7), anti-CD25–PE (PC61), anti-Foxp3 (FJK-16S), The anti-H-2^d–FITC (SF1-1.1) and anti-H-2^b (AF6-88.5) came from BD Pharmingen (San Diego, CA, USA). Isotype control antibodies were also obtained from eBioscience and BD Pharmingen. RhIL-2 and rhTGF-β were obtained from R&D Systems (Minneapolis, MN, USA).

Cell preparation and adoptive transfer

T cells were prepared from spleen cells by collecting nylon wool column nonadherent cells as described previously [18]. $CD4^+$ T cells were isolated by negative selection. Briefly, T cells were labeled with PE-conjugated anti-CD8, anti-CD11b, and anti-B220 mAbs, incubated with anti-PE magnetic beads, and loaded onto MACS separation columns (Miltenyi Biotec). The $CD4^+$ cells were further labeled with FITC-conjugated anti-CD25 mAb, and $CD4^+CD25^-$ and $CD4^+CD25^+$ cells were obtained by cell sorting (purity >98%). To prepare naive $CD4^+CD25^-$ cells, $CD4^+CD25^-$ cells were labeled with PE-conjugated anti-CD62L and positively selected by anti-PE magnetic beads ($CD4^+CD62L^+CD25^-$ cells) [18]. Naïve CD4+ cells were stimulated with similar numbers of irradiated (2000-rad) B6 nylon adherent, non-T cells for 5–6 days in 24-well plates (2 ml per well) (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in AIM V (Invitrogen, Carlsbad, CA, USA) serum-free medium with additives. Some wells contained TGF-β1 (2 ng ml^{-1}) and rhuIL-2 (40 units ml^{-1}) (CD4reg) (R&D Systems) or IL-2 only (CD4con). $CD4^+CD25^+$ cells were positively selected using autoMACS technologies (Miltenyibiotec, Germany) from $CD4^+$reg or $CD4^+$con cells. 10 million of $CD4^+CD25^+$ cells were intravenously injected to syngeneic mice. In some groups, H-2^d mice also received H-2^b or H-2^k irradiated (2000-rad) non-T cells for antigen boost.

Assessment of Treg cell activity in vitro

The suppressive activity of these cells generated as above was assessed with a standard assay. The proliferative activity of T cells to alloantigens was measured using a one-way mixed-lymphocyte culture with 2 x 10^5 T cells and an equal number of irradiated allogeneic non-T cells in a 96-well flat-bottom plate using RPMI 1640 culture medium and 10% FCS with additives as described previously [18]. Proliferation was measured after 4–5 days as uptake of [3H]-thymidine in triplicate cultures. In some experiments, T responder cells were labeled with CFSE and the proliferative degree was assessed by inhibition of percentages or total cycling $CD8^+$

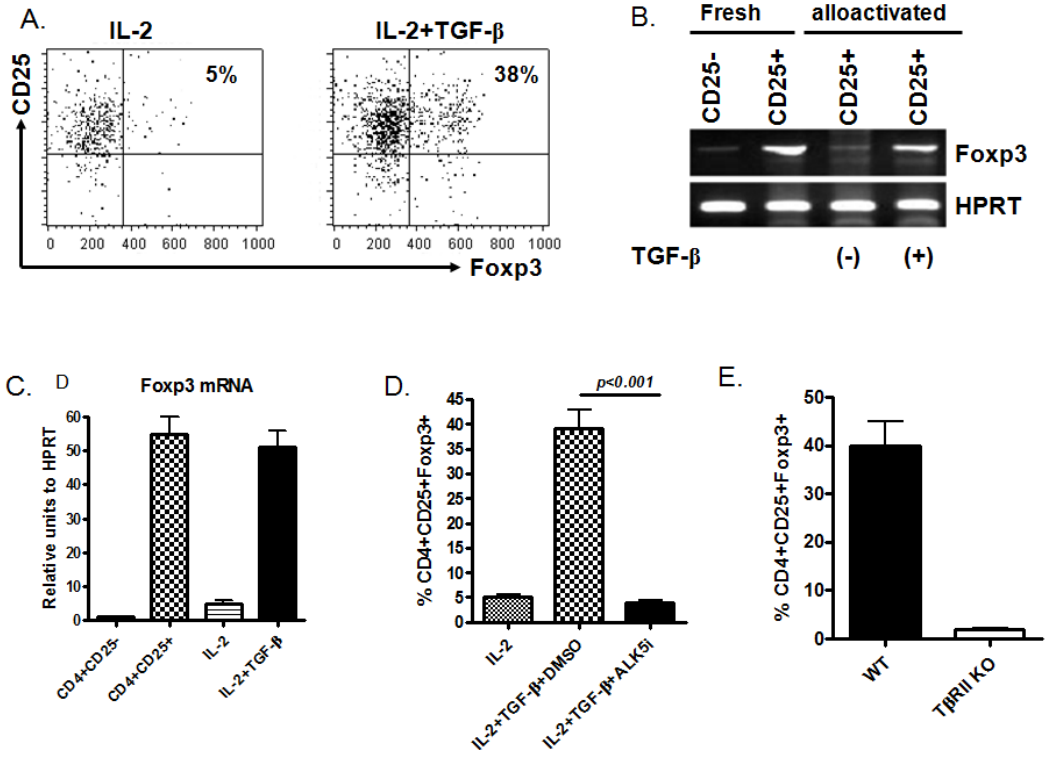

Figure 1. TGF-β is able to induce alloactivated CD4+ cells to express Foxp3. Naïve CD4+CD25- cells isolated from DBA/2 mice were stimulated with γ–irradiated (2000-rad) non-T cells from C57BL/6 mice in the presence of IL-2 (40 units/ml) with (CD4reg) or without TGF-β (2 ng/ml) (CD4con) for 5 days. Foxp3 and CD25 expression on CD4+ cells were determined by flow cytometry (A), Foxp3 mRNA were determined by RT-PCR and quantitative RT-PCR (B &C). Effect of TGF-β receptor I inhibitor on Foxp3 expression induced by TGF-β (D). TGF-β-induced Foxp3 production in wild type and TGF-β receptor II Knock out mice was determined by flow cytometry (E). Results were representative of three independent experiments (A-C). The values indicate the mean ± SEM of three separate experiments (D-E).

or $CD4^+$ T cells as shown with the dilution of CFSE in the $CD4^+$ and $CD8^+$ gates as described previously [18]. In cultures used to assess the suppressive activity of $CD4^+CD25^+$ cells, the ratio of these cells to T responder cells was 1:4.

Foxp3 expression by flow cytometry and real-time–PCR

For intracellular staining of Foxp3, following various days of culture, $CD4^+$ cells from the different treatment groups were harvested and stained according to the manufacturer's protocol. To determine the Foxp3 mRNA levels, total RNA was prepared with TRIzol LS reagent (Invitrogen). First-strand cDNA was synthesized using Omniscript TR kit (Qiagen, Valencia, CA, USA) with random hexamer primers (Invitrogen). Real-time PCR was performed with a LightCycler (Roche, Mannheim, Germany), and message levels were quantified using the LightCycler Fast Start DNA Master SYBR Green I Kit (Roche), according to the manufacturer's instructions. Amplification was conducted for 45 cycles. The recovered PCR product and amplicon were checked by agarose gel electrophoresis for a single band of the expected size. The samples were run in triplicate and the relative expression of Foxp3 was determined by normalizing the expression of each target to hypoxanthine guanine phosphoribosyl transferase (HPRT). Primer sequences were as follows: HPRT 5'-TGA AGA GCT ACT GTA ATG ATC AGT CAA C-3' and 5'-AGC AAG CTT GCA ACC TTA ACC A-3'; Foxp3 primers: 5'-CCC AGG AAA GAC AGC AAC CTT-3' and 5'-TTC TCA CAA CCA GGC CAC TTG-3' [24].

activities [15-18]. Here we demonstrate that

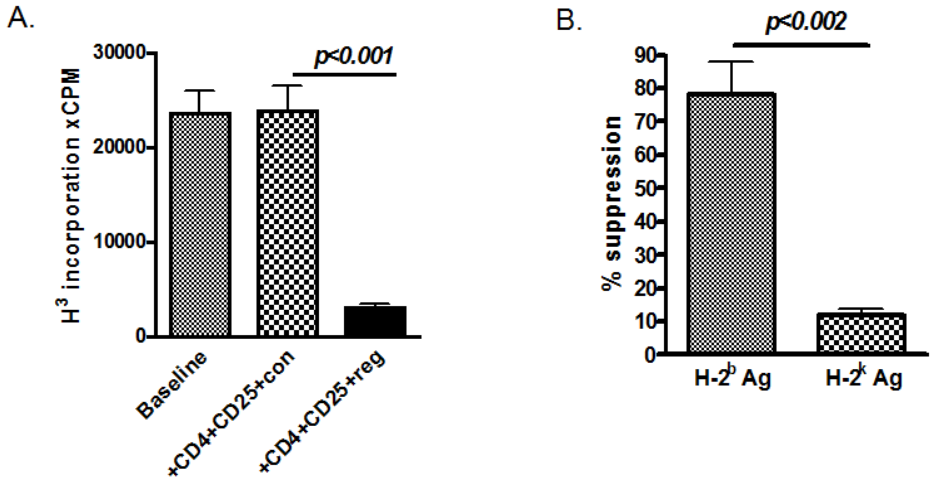

Figure 2. TGF-β-primed, alloactivated CD4+CD25+ cells display potent antigen-specific suppression. CD4con or CD4reg cells were generated as above and CD4+CD25+ subset was positively selected by AutoMACS separator. 20% of these CD4+CD25+ cells were added to syngeneic T cells and stimulated with γ–irradiated (2000-rad) non-T cells from C57BL/6 mice (H-2^b, specific antigen) or C3H (H-2^k, third party antigen) mice for three days. Proliferation was measured as uptake of H^3-thymidine in triplicate cultures. Values indicate the mean ± SEM of three separate experiments.

Pathological examination

Spleen, Lung, Kidneys, liver and brain were fixed in 10% buffered Formalin in three weeks following injection of 10 million of $CD4^+CD25^+$ Tregs or PBS. H&E slides were prepared using standard techniques. The slides were examined by a pathologist who was unaware of the experimental data.

Statistical analysis

Analysis for statistically significant differences between groups of mice was performed by *t* test and Wilcoxon test survival curves with the log rank test using GraphPad PRISM software (GraphPad, San Diego, CA, USA).

Results

TGF-β induces alloactivated mouse $CD4^+CD25^-$ cells to express Foxp3 and develop antigen-specific suppressive activity

We previously reported that culture of alloactivated human $CD4^+$ and mouse T cells, or polyclonally activated mouse $CD4^+$ cells with IL-2 and TGF-β resulted in both the up-regulation expression of CD25, CTLA-4 and Foxp3 and the development of suppressive IL-2 and TGF-β are also able to induce alloactivated mouse $CD4^+$ cells to express Foxp3. As shown in **Figure 1**, naïve $CD4^+CD25^-$ $Foxp3^-$ cells from DBA/2 mouse stimulated with γ–irradiated non-T cells from C57BL/6 mice in the presence of TGF-β for five days resulted in their expression of CD25 and Foxp3 (**Figure 1A**) and Foxp3 mRNA (**Figure 1B & C**). TGF-β/TGF-β receptor signaling plays a crucial role in the Foxp3 induction since addition of ALK5 (TGF-β receptor I) inhibitor completely inhibited Foxp3 induction (**Figure 1D**), and TGF-β failed to induce Foxp3 expression on CD4+ cells isolated from TGF-β receptor II knock out mice (**Figure 1E**). In contrast, Foxp3 expression was not induced in the absence of TGF-β (**Figure 1A-C**).

In addition to Foxp3 induction, TGF-β-treated $CD4^+CD25^+$ cells exhibited suppressive activity against T cell proliferation. As shown in Fig 2, T cells from DBA/2 mouse proliferated when stimulated with alloantigen from C57BL/6 mouse. Proliferation levels were identified by ^{3}H-thymidine incorporation or by dilution of Carboxyfluorescein Succinimidyl Ester (CFSE) in T responder cells. Addition of TGF-β-treated $CD4^+CD25^+$ cells (1:5 ratio of conditioned $CD4^+$ cells to T responder) significantly suppressed T cell proliferation (**Figure 2A**).

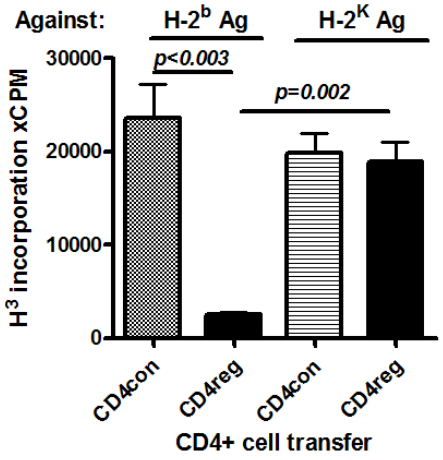

Figure 3. Adoptive transfer of TGF-β-primed, alloactivated CD4+CD25+ cells induces immune tolerance *in vivo*. 5 million of CD4+CD25+ subset from CD4con or CD4reg cells was intravenously injected to syngeneic DBA/2 mice. After three weeks, T cells isolated from mice received CD4con or CD4reg cells were stimulated with γ–irradiated (2000-rad) non-T cells from C57BL/6 mice (H-2^b, specific antigen) or C3H (H-2^k, third party antigen) mice for three days and T cell proliferation was determined as above. Values indicate the mean ± SEM of three mice. The experiment was repeated with similar results.

Conversely, addition of a similar dose of $CD4^+$ control cells (treated without TGF-β) displayed no suppressive activity (**Figure 2A**).

We also observed that the suppressive activity of TGF-β-treated $CD4^+CD25^+$ cells is antigen-specific. While TGF-β-treated, C57BL/6 (H-2^b) alloantigen stimulated $CD4^+CD25^+$ cells suppressed the proliferation of T cells that had been stimulated with H-2^b alloantigen, these cells were unable to suppress the proliferation of T cells that had been stimulated with alloantigen from C3H (H-2^k) mouse antigen (**Figure 2B**). These results indicate TGF-β is capable of inducing antigen-specific $CD4^+$-$Foxp3^+$ regulatory T cells.

Adoptive transfer of TGF-β-treated $CD4^+CD25^+$ cells induce antigen-specific immune tolerance in vivo

We next tested whether adoptive transfer of TGF-β-treated $CD4^+CD25^+$ cells from DBA/2 mouse (H-2^d) stimulated with H-2^b alloantigen could lead to immune tolerance *in vivo*. $5x10^6$ TGF-β-treated $CD4^+CD25^+$ or control $CD4^+CD25^+$ cells were adoptively transferred to syngeneic DBA/2 mice. Mice were also given 10^7 of H-2^b alloantigen cells on day 0 and day 14. Three weeks later, the recipient mice were sacrificed and T cell proliferation against H-2^b alloantigen was tested. As shown in **Figure 3**, T cells from mice given $CD4^+CD25^+$ control cells proliferated vigorously to H-2^b alloantigen. In contrast, T cells from mice receiving TGF-β-treated $CD4^+CD25^+$ cells almost completely lost their immune response to H-2^b alloantigen. In addition, this T cell non-responsiveness was antigen-specific since they responded normally to a third party alloantigen (C3H mouse cell, H-2^k).

Enhanced $CD4^+CD25^+Foxp3^+$ cells in recipient mice contribute to antigen-specific immune tolerance in vivo

We next tried to determine the mechanism(s) by which adoptive transfer of TGF-β-treated $CD4^+CD25^+$ cells induces immune tolerance *in vivo*. $5x10^6$ TGF-β-treated $CD4^+CD25^+$ or control $CD4^+CD25^+$ cells that had been labeled with CFSE were adoptively transferred to syngeneic mice. One month following transfer, Foxp3 expression levels in donor ($CFSE^+$) cells was comparable to that measured one week after injection although total numbers of donor cells were slightly decreased (data not shown). Interestingly, the frequency of $Foxp3^+$ cells in spleen (**Figure 4A**) and lymph nodes (not shown) in recipient mice was significantly increased in mice receiving TGF-β-treated $CD4^+CD25^+$ cells than in those given $CD4^+CD25^+$ control cells.

The increased numbers of $CD4^+CD25^+Foxp3^+$ cells might possibly be responsible for the observed T cell non-responsiveness to specific alloantigen. T cells from mice given TGF-β-treated $CD4^+CD25^+$ cells proliferated strongly when stimulated with specific alloantigen if the $CD4^+CD25^+$ subset was depleted (**Figure 4B**). Restoration of $CD4^+CD25^+$ cells back to the pool of T cells that had been depleted of this $CD4^+CD25^+$ subset also restored the tolerogenic effect of T cell non-responsiveness (**Figure 4B**). We did not observe any significant alteration in $CD8^+Foxp3^+$ cells or NKT cells in mice given TGF-β-treated $CD4^+CD25^+$ cells (data not shown). These results suggest that increased $CD4^+CD25^+Foxp3^+$ cells in recipient mice mostly contributed to immune tolerance *in vivo*.

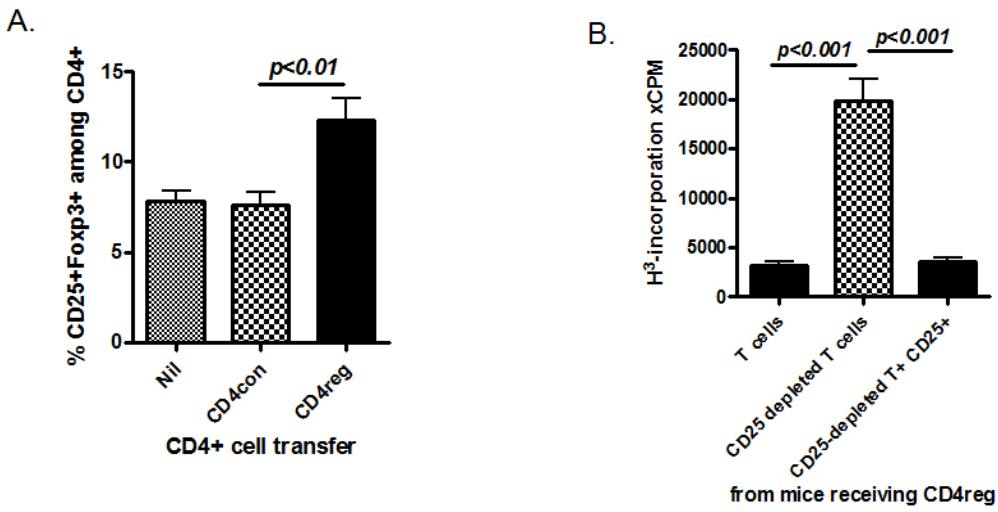

Figure 4. Increased CD4+CD25+ cells in recipients contribute to immune tolerance *in vivo*. Experiments were similarly conducted as Fig. 3 and CD4+CD25+Foxp3+ cells on the gate of CFSE- cells were counted in mice received from CD4con or CD4reg cells. T cells in these mice were depleted CD25+ subset and their proliferation against alloantigen was determined as Fig. 2. In some experiments, 10% of CD4+CD25+ subset was added to back and T cell proliferation was determined as above. Values indicate the mean ± SEM of four mice. The experiment was repeated with similar results.

Adoptive transfer of TGF-induced CD4⁺CD25⁺ cells to syngeneic mice does not result in pathological alteration in important organs

We next evaluated whether there might be any toxic side effects upon adoptive transfer of TGF-β-induced CD4⁺CD25⁺ cells *in vivo*. As shown in **Figure 5**, when 10 million CD4⁺CD25⁺ Treg cells were adoptively transferred to syngeneic DBA/2 mice, there were no evident pathological alterations in the spleens, kidneys or lungs of mice receiving TGF-β-induced CD4⁺CD25⁺ cells compared to those mice receiving PBS in three weeks after cell injection. We also examined the levels of creatinine, urea nitrogen, alanine aminotransferase and aspartate aminotransferase in blood at three weeks following cell transfer and observed these lab parameters were comparable in mice received TGF-β-induced CD4⁺CD25⁺ Treg cells to the mice received PBS (data not shown). These results suggest that injection of autologous human TGF-β-induced CD4⁺CD25⁺ cells provides a feasible approach in human clinical cell therapy in protection of solid transplantation rejection.

Discussion

In this study we observed that adoptive transfer of CD4⁺CD25⁺Foxp3⁺ Tregs to syngeneic mice induces antigen-specific T-cell non-responsiveness. This effect may account for the protective effect of graft survival in heart transplantation models [23]. Others have reported that adoptive transfer of nTregs also induces immune tolerance and prevents allograft rejection [5-8]. Salama et al also revealed that successful human kidney transplant recipients had an increased number of CD4⁺CD25⁺ cells and that these cells were responsible for the immune non-responsiveness to HLA-DR allopeptides [25].

Antigen-specific immune tolerance provides a unique advantage in protection of allograft rejection without the severe global immune suppression resulting from the use of Corticoidsteroids and other immune-suppressive agents. Our data reveal that T cells from mice received Tregs respond normally to third-party antigens. This is consistent with Long's finding where they found Treg cells induced immune tolerance in mice but maintained immune responsiveness to pathogens and malignant cells [26]. In addition, injection of these cells to syngeneic mice has no evident toxic side effects. Conversely, Corticoidsteroids and immune-suppressive agents not only cause global immune suppression, but also fail to induce long-term immune tolerance.

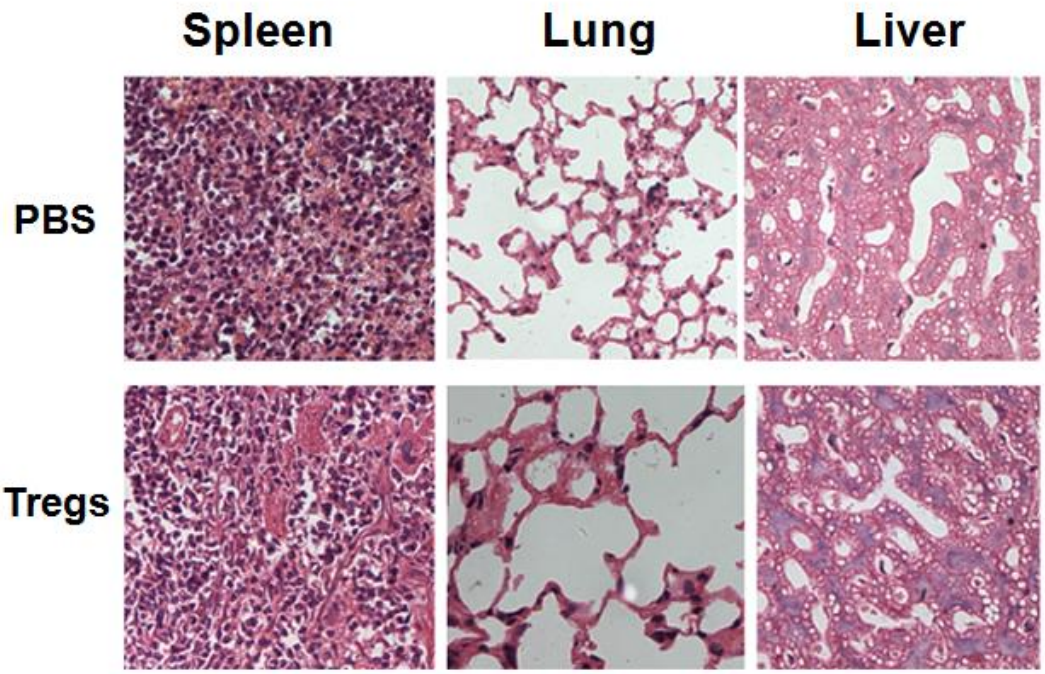

Figure 5. Adoptive transfer of TGF-β-primed, alloactivated CD4+CD25+ cells to syngeneic mice does not lead to evident toxic effects in important organs. 10 million of CD4reg cells were intravenously injected to syngeneic mice and pathological characteristics were evaluated by H&E staining in three weeks after cell injection. Results were representative of two similar experiments.

As in other approaches, the induction of immune tolerance by adoptive transfer of TGF-β-induced Tregs appeared to be also secondary. Other approaches, such as blocking CD40-CD40L interaction, infusion of donor antigen and anti-CD4 antibody therapy, induced immune tolerance via induction of new Tregs in the host [10]. Since both nTregs and iTreg can induce non-Tregs to become Tregs by a mechanism called "infectious tolerance" [27, 28], we believe that adoptive transfer of TGF-β-induced iTregs can trigger conventional T cells to become antigen-specific iTreg cells when recipients were boosted by specific antigen. This helps explain why single injection of TGF-β-induced iTregs can sustain long-term protective effects in heart transplantation and autoimmune disease models [23]. In this way, TGF-β-induced iTregs can act like a vaccine, which has an important clinical implication for solid organ transplantation and treatment of autoimmune diseases.

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