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Adoptive transfer of FTY720-treated immature BMDCs significantly prolonged cardiac allograft survival

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Summary

A sphingosine 1 phosphate receptor modulator, FTY720, has been used to alleviate symptoms in allotransplantation and autoimmune disease models with impressive efficacy, while it only achieved moderate success in clinical trials. Infusion of immature bone marrow-derived dendritic cell (BMDC) progenitors before transplantation could induce donor specific tolerance. In this study, we investigated the possibility of using FTY720-DCs (FTY720-treated immature BMDCs) to prevent severe alloimmune response. Our results indicate that FTY720-DCs could markedly prolong graft survival compared with Ctrl-DCs (nonconditioned immature BMDCs) as manifested by reduced inflammatory infiltration into the graft. IFN-γ production by $CD4^+$ and $CD8^+$ T cells were significantly reduced, while $FoxP3^+$ regulatory T cells among $CD4^+$ T cells were upregulated. Although FTY720 seldom altered the phenotype or the phagocytosis of BMDCs *in vitro*, it severely hampered their capability to trigger antigen-specific and allogeneic T-cell response. When splenic T cells were co-cultured with FTY720-DCs, the proportion of regulatory T cells increased, accompanied by elevated IL-10 production. Consistently, infusion of FTY720-DCs could preferentially promote Treg proliferation and upregulate PD-1 expression on conventional T cells in allogeneic mature BMDC priming experiment. These results suggest that infusion of FTY720-DCs before cardiac transplantation could significantly prolong functional graft survival by acting as a balancer of alloimmune response.

Introduction

FTY720, a sphingosine 1 phosphate (S1P) receptor modulator, could induce long-term graft acceptance [1,2], prevent the development of graft-versus-host disease, autoimmune type I diabetes, ischemia stock [3,4], multiple sclerosis [5] and rheumatoid arthritis [6] with potent efficacy. It was thought to act through triggering apoptotic or necrotic cell death at high concentration, rapidly inducing systemic lymphopenia, altering lymphocyte trafficking/homing patterns and therefore leading to decreased T-cell infiltration into inflammation site [7–9]. It is a matter of debate as to whether FTY720 acts as an agonist or antagonist on S1P receptors. FTY720 might represent a super agonist eliciting a short burst of S1P receptor signalling but also causing sustained receptor internalization, thereby generating an antagonistic phenotype in the long run [10]. Clinical trials have targeted S1P1 receptor modulators for autoimmune diseases, particularly for the potential treatment of multiple sclerosis and the prevention of transplant rejection [11]. Nevertheless, a phase III study of 696 *de novo* renal transplant

patients suggested that FTY720 combined with cyclosporine provided no benefit over standard care [12].

Systemic injection of FTY720 could modify the function of immune cells. In a Th1-mediated TNBS colitis model, FTY720 treatment significantly attenuated the disease by upregulation of FoxP3, IL-10, TGF-β and CTLA4 expression and induction of the functional activity of $CD4^+$ $CD25^+$ Treg [13]. Interestingly, FTY720 differentially affects the homing properties of Treg cells compared with other T-cell subsets, resulting in an increased number of Treg cells in the blood and spleens of FTY720-treated mice [14]. Furthermore, FTY720 could effectively induce Foxp3-expressing cells from Foxp3 negative cells *in vitro* and *in vivo* [15], while some studies also suggest that FTY720 was potent to inhibit Treg proliferation *in vitro* and *in vivo* without affecting their viability or phenotype while abrogating their *in vivo* immunosuppressive potential by blocking IL-2 induced expansion [16]. Local treatment with FTY720 could inhibit the migration of lung dendritic cells(DCs) to mediastinal lymph nodes, which in turn inhibited the formation of allergen-specific Th2 cells [17]. In allotransplantation models, FTY720 prolonged allograft survival for reasons attributable to DCs with enhanced retention in the skin, impaired migration to the secondary lymphoid organ (SLO) and reduced T-cell stimulatory activity [18,19].

New directions in inducing immunosuppression during transplantation include co-stimulation-based therapy, mixed chimerism and adoptive cellular transfer. Each of these modalities targets specific elements of the alloimmune response with the hope of achieving the elusive tolerogenic state [20]. In particular, infusion of tolerogenic DCs and Treg demonstrated impressive experimental results. Positioned at the interface between innate and adaptive immunity and the upstream of immune response, DCs represent a promising therapeutic target towards transplant tolerance. Bone marrow-derived dendritic cells (BMDCs) cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) can induce alloantigen-specific hyporesponsiveness *in vitro* and prolong cardiac allograft survival in nonimmunosuppressed recipients *in vivo* [21]. Interestingly, DCs, especially tolerogenic DCs, promote Treg generation and vice versa [22–24]. Importantly, the infusion of DCs can be resistant to activating inflammatory stimuli by 'conditioning' through exposure to immunosuppressants such as rapamycin (RAPA)[25]. Here we report that the infusion of FTY720-treated immature BMDCs before heart transplantation could notably prolong graft survival. It may provide an alternative FTY720 therapeutic method based on allogeneic dendritic cells vaccination in the future.

Materials and methods

Animals and reagents

BALB/c ($H\text{-}2^d$) mice and C57Bl/6 ($H\text{-}2^b$) were obtained from the Animal Facility of Wuhan University, China. DO11.10 OVA-specific TCR transgenic mice ($H\text{-}2^d$) were from the Jackson Laboratory. Female mice, 8–12 weeks old (body weight 20–25 g), used for this study were housed in a specific pathogen-free facility (SPF) in microisolator cages and supplied with autoclaved food and acidified water. All of the studies were approved by the Institutional Animal Care and Use Committee at Tongji Medical College. Fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or Allophycocyanin (APC)-conjugated monoclonal antibodies (mAbs) for CD11c(HL3), CD80(16-10A1), CD86(GL1), I-Ad (39-10-8), B7-DC(TY25), CD4 (GK1.5), CD8(53-6.7), CD25(PC61), IFN-γ(XMG1.2), Ki-67(35) and IgG isotype controls were from BD Bioscience. Anti B7-H3(M3.2D7), B7-H4(188), PDCA-1(129c) and Foxp3 staining kit were from eBioscience (San Diego, CA , USA). Rabbit anti-mouse polyclonal S1P1 antibody was from Abcam. FITC-conjugated anti-rabbit IgG secondary antibody was from Pierce (Rockford, IL, USA). ELISA kits for IL-10 and IIFN-γ were from eBioscience.

Generation of bone marrow-derived dendritic cells

Bone marrow-deried dendritic cells were generated from the bone marrow cells as described previously [26]. In brief, BMDCs were propagated from naive BALB/c or C57Bl/6 mouse at 5×10^6 cells per 100-mm dish in IMDM (Sigma-Aldrich, Shanghai, China) supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml), L-glutamine (2 mM), 2-mercaptoethanol (50 mM) and 10% fetal bovine serum (FBS) in the presence of GM-CSF (10 ng/ml; PeproTech, Rocky Hill, NJ, USA) and IL-4 (2 ng/ml; PeproTech). The same volume of fresh medium was added to the original culture at day 2. Half of the supernatant was collected at day 6 and the resulting cell pellets were resuspended in 10 ml of fresh medium supplemented with GM-CSF and IL-4 and added back to the original dishes. FTY720 (Novartis, Basel, Switzerland) dissolved in DMSO at 10 μg/μl was used at a concentration of 10 ng/ml and maintained in the medium throughout the culture period. At day 8, immature BMDCs were harvested, and washed thoroughly before use. The purity of DCs was around 70% normally based on CD11c staining. Immature BMDCs were stimulated with 100 ng/ml lipopolysaccharide (LPS) (*E. coli* 0111:B4; Sigma-Aldrich) for 24 h to trigger maturation in some experiments. S1P1 staining was performed with fixed and permeabilized BMDCs using BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD

Pharmingen Franklin Lakes, NJ, USA). For adoptive infusion experiments, DCs were purified using anti-CD11c-FITC antibody and EasyStep FITC Selection kit (STEMCELL Vancouver, BC, Canada).

Cardiac transplantation and adoptive transfer of imBMDCs

For heterotopic cardiac transplantation, C57Bl/6 (H-2^b) mice served as recipients, while BALB/c (H-2^d) mice were used as donors. To reduce the rejection of allogeneic BMDCs, recipients received 3.5 Gy whole-body irradiation 24 h before infusion of BMDCs. In total, 5×10^6 FTY720-DCs or Ctrl-DCs were resuspended in 150 μl of PBS and then injected i.v. into recipient mice 24 h before cardiac transplantation. Abdominal heterotopic cardiac transplantation was performed according to the protocol described by Corry *et al.* [27] without administering immunosuppressive drugs. Age-matched naive BALB/c mice that received syngeneic grafts were used as controls. Daily abdominal palpation was done to check graft survival. Rejection was defined as the absence of detectable heartbeat and was verified by direct visualization and histologic examination.

Histologic analysis

Cardiac grafts were fixed in 4% paraformaldehyde and embedded in paraffin. Eight-micrometre tissue sections were prepared and stained with hematoxylin and eosin.

Antigen uptake assay

The mannose receptor-mediated endocytosis of BMDCs was measured using FITC-conjugated dextran particles (FITC-Dx; Sigma-Aldrich) diluted in 1 × PBS at 10 mg/ml and used at a final concentration of 0.5 mg/ml in the culture medium. FTY720-DCs or Ctrl-DCs (with or without LPS stimulation) were seeded in the culture medium at 1×10^6 cells/ml and incubated with FITC-Dx at 37 °C for 30 min. Negative control was kept at 4 °C. Cells were washed three times with cold PBS and analyzed immediately by flow cytometry. Cells were prestained with CD11c-PE mAbs before the uptake assay and gated on $CD11c^+$ cells for analysis.

Cytolytic activity assay

Cytolytic activity of FTY720-DCs and Ctrl-DCs was measured using FCM as reported previously [28]. Briefly, 2×10^5 CMAC (7-amino-4-chloromethylcoumarin; Invitrogen, Shanghai, China)-prestained Jurkat cells (ATCC) was used as targets and incubated with 8×10^5 or 1.6×10^6 BMDCs. After 4 h of incubation, mixed cells were stained with PI to check the percentage of PI^+ cells in total $CMAC^+$ Jurkat cells.

Tracking CFSE-labeled allogeneic BMDCs *in vivo*

Bone marrow-derived dendritic cells from BALB/c mice were washed twice with PBS and then stained with 1 μM 5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester [CFSE; Sigma-Aldrich] in PBS. After 10 min of incubation at 37 °C, the same volume of FBS was added to stop CFSE staining and the stained cells were washed three times with 1 × PBS before intravenous administration (5 million cells/mouse) into C57Bl/6 recipients. Spleen, lymph nodes (LNs) and blood from the recipients were collected 24 h later to check CFSE-labeled CD11c+ cells in each compartment. CD86, MHCII and CD8α expression were also analyzed by FCM.

Antigen presentation capacity

Splenocytes from naive C57bl/6 or DO11.10 mice were stained with CD3-FITC or CD4-FITC and total T cells or $CD4^+$ T cells were purified using EasyStep FITC Selection kit (stem cell) after RBC lysis. The purity was normally above 90%. For proliferation test, purified T cells were stained with 1 μM CFSE as described above. BMDCs from BALB/c were mixed with splenic C57Bl/6 T cells in a round bottom 96-well plate at 1:5 or 1:10 ratio for 3 days to test allogeneic T-cell response. To detect OVA antigen presenting capacity of FTY720-DC, FTY720-DCs or Ctrl-DCs were loaded with 500 μg/ml OVA, incubated at 37 °C for 5 h and washed twice with PBS before being mixed with DO11.10 $CD4^+$ T cells. At the end of the culture period, cells and supernatant were collected for FCM and ELISA assay.

Intracellular IFN-γ detection by flow cytometry

Splenocytes from C57Bl/6 recipient mice were collected at day 7 after transplantation. The cells were stimulated with 20 ng/ml PMA, 1 μg/ml ionomycin (Sigma-Aldrich) plus 2 μM of Monesin (Sigma-Aldrich) for 4 h, followed by intracellular staining of IFN-γ using a Cytofix/Cytoperm kit (BD Pharmingen, Franklin Lakes, NJ, USA) as instructed. Isotype-matched control antibodies were used as negative controls.

In vivo priming experiment with allogeneic matured dendritic cells

Naïve C57Bl/6 mice were injected i.v. with 5 million FTY720-DCs or Ctrl-DCs from BABL/c mice or the same

volume of PBS. Twenty hours later, 2×10^6 matured BALB/c BMDCs stimulated with 100 ng/ml LPS were injected into their footpad subcutaneously. Draining LNs (popliteal LN) were harvested 5 days later to make single cell suspensions. Total cell numbers as well as Ki-67 expression were determined. Foxp3 and Ki-67 co-staining were carried out using Foxp3 staining kit.

Statics

Statistical analysis was performed using GRAPHPAD PRISM 5 (GraphPad Software, La Jolla, CA, USA). Graft survival curves were generated by the Kaplan and Meier method and log-rank (Mantel–Cox) test was used to compare the differences between each groups. Results from flow cytometry and ELISA are shown as mean ± SD from three replications and Student's *t*-test was applied with statistical significance assumed to be at $P < 0.05$.

Results

Infusion of FTY720-treated imBMDCs notably prolonged cardiac allograft survival

We first investigated whether FTY720-DCs could modulate allograft rejection in an abdominal heterotopic cardiac transplantation model. To prevent acute rejection of infused allogeneic DCs, some recipient C57BL/c mice received a sublethal dose of irradiation at 3.5 Gy. Purified FTY720-DCs or Ctrl-DCs (>90% $CD11c^+$) were adoptively transferred to recipients 24 h before transplantation. We used the following settings as controls: (i) C57Bl/6 → non-irradiated C57Bl/6 with PBS infusion; (ii) BALB/c → non-irradiated C57Bl/6 with PBS infusion; (iii) BALB/c → irradiated (3.5 Gy) C57Bl/6 with PBS infusion. We monitored survival of cardiac allograft as well as recipient mice everyday. Recipient mice died within 12 h after surgery (as a result of severe surgical damage) were excluded from later observation. As expected, the syngeneic cardiac grafts were accepted, while allografts were rejected from nonirradiated recipients within 9 days (MST, median graft survival time = 7 days, SD = 0.75, $n = 6$). Allografts were rejected from irradiated recipients within 12 days (MST = 9 days, SD = 1.46, $n = 8$). Thus, total body irradiation could prolong allografts survival around 2 days but this improvement is not statistically significant ($P = 0.172$). Survival of allografts in Ctrl-DCs group was significantly prolonged (MST = 25.5 days, SD = 1.9, $n = 8$, $P = 0.006$). Interestingly, MST in FTY720-DC group was remarkably prolonged to 48 days with one of nine allografts surviving up to 65 days (SD = 5.9, $n = 9$, $P = 0.002$) (Fig. 1a). We further performed histologic analysis with the grafts. Consistently, severe rejection characterized by allograft destruction and rigorous inflammatory infiltration were found in allografts from nonirradiated recipients compared with syngeneic cardiac grafts 8 days after transplantation. Twenty-four days after transplantation, allografts from recipients treated with FTY720-DCs only had mild inflammatory infiltration with well-preserved myocardium compared with that from recipients treated with Ctrl-DCs (Fig. 1b). These results suggest that FTY720-DCs possess the notable advantage over Ctrl-DCs in prolonging cardiac allograft survival.

FTY720-DCs hampered IFN-γ-based Th1 response and increased the proportion of regulatory T cells among $CD4^+$ T cells

Proinflammatory cytokines, such as IFN-γ, IL-2, IL-6 and IL-10, are increased during graft rejection [29–31] and cardiac graft rejection is strongly attenuated in IFN-$\gamma^{-/-}$ mice [32]. Moreover, cardiac allograft vasculopathy is mediated mainly by alloreactive T cell and vascular infiltrated memory Th1 cell-derived IFN-γ [33–35]. Therefore, we next tested IFN-γ-based response at day 7 after transplantation. Allogeneic transplantation induced copious amount of IFN-γ production by both $CD4^+$ and $CD8^+$ T cells in the spleen. In the spleen of Ctrl-DC infused recipients, IFN-γ production by $CD8^+$ and in particular by $CD4^+$ T cells was reduced, along with a substantial increment in $FoxP3^+$ cells among $CD4^+$ T cells. Of note, infusion of FTY720-DCs could further weaken Th1 and CTL response and augment regulatory T-cell population, thus tuning the balance to attenuate allograft rejection (Fig. 1c).

Phenotype and phagocytosis of FTY720-DCs

We further examined the phenotype of FTY720-DCs. Compared with Ctrl-DCs, FTY720-DCs did not show altered CD80, CD86, CD40 and MHCII expression (Fig. 3a). We also analyzed the expression of B7H3(CD276), B7H4 (V-set domain containing T-cell activation inhibitor 1) and B7-DC (programmed cell death 1 ligand 2), which have been reported as negative regulators of T-cell activation and function [36,37]. We also tested the expression of PDCA-1, a specific marker for plasmacytoid DCs (Fig. 2a). FTY720 could slightly upregulate B7H4 expression on BMDCs (Fig. 2a,b) and significantly downregulate S1P1 expression after 7 days of culture. Upon LPS stimulation, the expression of S1P1 on FTY720-DCs remained lower compared with that of Ctrl-DCs (Fig. 2c).

Immature DCs is highly phagocytic, a function required for antigen uptake. FTY720-DCs and control BMDCs showed similar potency of phagocytosis as determined by Dextran-FITC uptake assay. After LPS stimula-

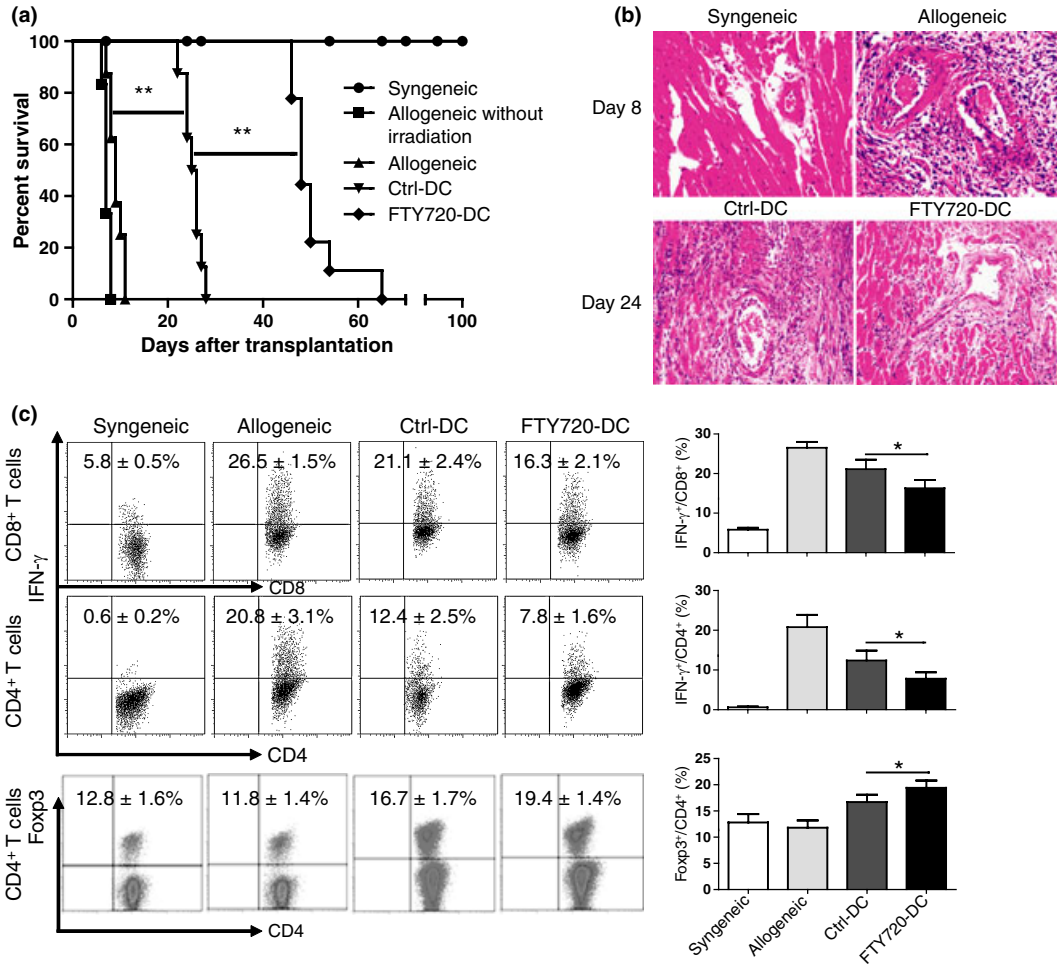

Figure 1 Infusion of FTY720-DCs significantly prolonged murine cardiac allograft survival and reduced inflammatory infiltration. (a). Survival curve of cardiac grafts from each group. (b). Histologic analysis of cardiac allograft after transplantation. HE staining was performed for allografts per group. One sample representative is shown here. Cardiac allografts and syngeneic grafts from nonirradiated recipients treated with PBS were examined 8 days after transplantation. Allografts from irradiated recipients treated with Ctrl-DCs or FTY720-DCs were examined 24 days after transplantation. (c). FTY-DCs infusion regulated splenic T-cell subpopulation. Splenic T cells of recipients infused with PBS, Ctrl-DCs and FTY720-DCs were harvested after day 7 of transplantation and subjected to intracellular IFN-γ and Foxp3 Staining. Syngeneic recipients were used as a control. CD8 and CD4 T cells were gated to evaluate CTL and Th1 response. And $CD4^+$ cells were gated to calculate $Foxp3^+$ Treg proportion among helper T cells. The data obtained from four mice of each study group are presented as mean ± SD. $^*P < 0.05$.

tion, both FTY720-DCs and Ctrl-DCs showed reduced antigen uptake ability (Fig. 2d).

In addition to acting as professional APCs and regulators of immune response, DCs have also been reported to act as 'killers' [38]. We also tested their killing efficacy and Jurkat T cells were used as target cells as reported previously [39]. BMDCs were able to kill Jurkat T cells, but FTY720 treatment did not enhance their killing capability (Fig. 2e). These results show that FTY720 did not modify the maturation state, phagocytosis or cytotoxic function of BMDCs, but it could upregulate the surface expression of co-inhibitory molecule B7H4 and downregulate S1P1, which is involved in S1P-mediated chemotaxis.

Distribution of infused allogeneic FTY720-DCs

To further dissect the underlying mechanisms, which endow FTY720-DCs with the capability to regulate allogeneic reaction, we analyzed the distribution of infused BMDCs. CFSE-labeled BMDCs (day 7 culture, >70% purity) were infused into nonirradiated recipients. Spleens, peripheral LNs and blood were collected from C57Bl/6 mice 24 h later to examine the percentage of $CFSE^+$ $CD11c^+$ cells. Interestingly, $CD11c^{high}$ BMDCs preferentially migrated into the spleens and LNs, while $CD11c^{int\ or\ low}$ DCs, as well as $CD11b^+$ $CD11c^-$ BMDC progenitors (<30% in BMDCs culture) were enriched in

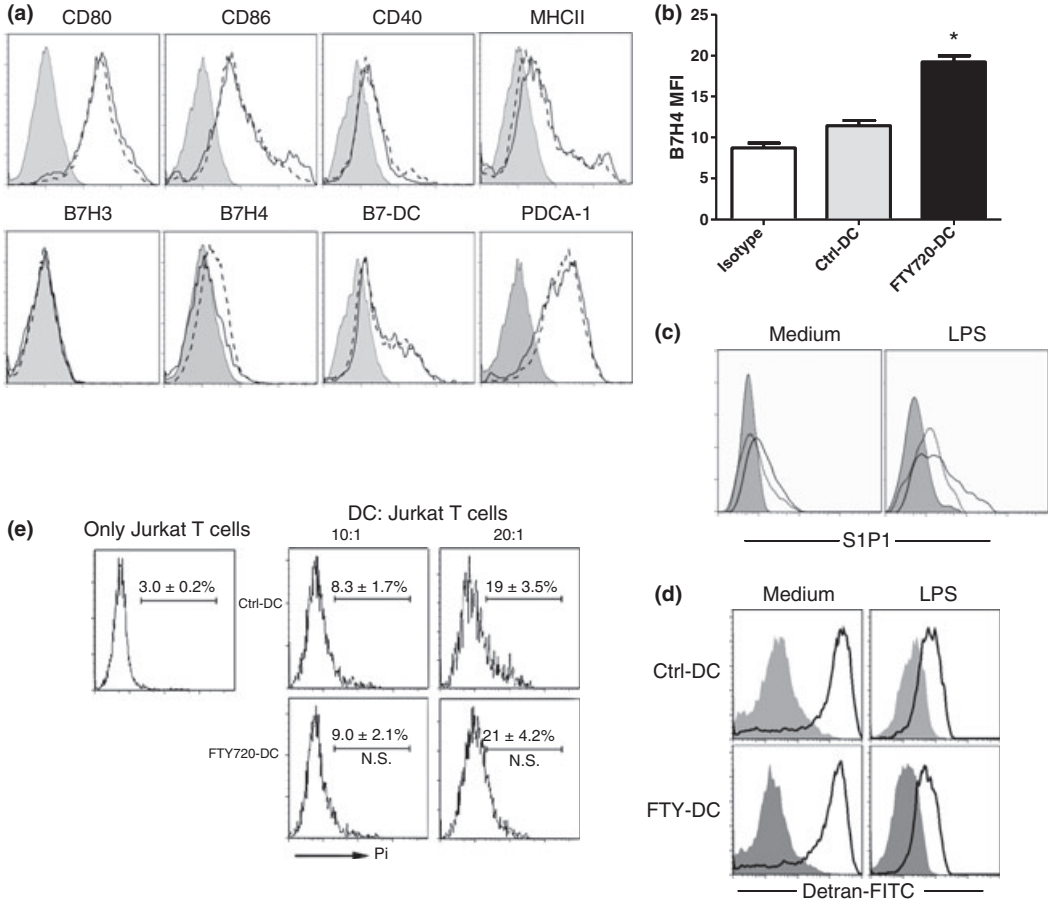

Figure 2 Phenotype, phagocytosis and cytotoxic capacity of FTY720-DCs. (a) Ctrl-DCs or FTY720-DCs were stained for analyzing the expression of different surface molecules. Shaded curves and continuous lines indicate staining with isotype controls and Ctrl-DCs, while broken lines resemble FTY720-DCs staining. (b) B7-H4 expression on BMDCs was compared according to the mean fluorescent index (MFI). *$P < 0.05$. (c) FTY720 treatment decreased S1P1 expression. Shaded curves and continuous lines indicate staining with isotype controls and Ctrl-DCs, while dotted line resembles FTY720-DC staining. (d) Dextran-FITC uptake assay at 37 °C indicates phagocytosis of FTY-DCs (continuous lines) and cells were incubated at 4 °C as a negative control (shaded lines). (e) Ctrl-DCs or FTY720-DCs were incubated with CMAC-stained Jurkat T cells with different ratios for 4 h. These experiments were performed three times independently.

the blood. The infused BMDCs were found to be MHC $II^{low}CD86^{int}$ in the spleen, MHC $II^{high}CD86^{low}$ in blood and MHC $II^{high}CD86^{high}$ in LNs. $CD8\alpha^+$ BMDCs, which are critical for cross-presentation, were preferentially enriched in the spleen and LN while not in the blood. We found that the percentage of infused FTY720-DCs was slightly lower in the blood but not in the spleen or LNs compared with that of Ctrl-DCs (Fig. 3).

FTY720-DCs are less potent in triggering antigen-specific and allogeneic T-cell response

To test the ability of BMDCs to trigger antigen-specific response, ovalbumin (OVA) protein-loaded Ctrl-DCs or FTY720-DCs from BABL/c mice were used to stimulate the proliferation of OVA-specific T cells from DO11.10 mice at the ratio of 1:5 and 1:10. CFSE intensity was analyzed by flow cytometry at day 3 and culture supernatants were collected for dosing of IFN-γ and IL-10. Compared with Ctrl-DCs, FTY720-DCs showed reduced potency to induce the division of DO11.10 T cells ($P < 0.01$), while it significantly increased the proportion of $FoxP3^+$ cells among DO11.10 T cells ($P < 0.05$, Fig. 4a,b). Consistently, there was also less IFN-γ within the supernatant of FTY720-DCs plus DO11.10 T cells co-culture (Fig. 4c).

We further checked the ability of BMDCs to induce allogeneic response *in vitro* using the splenic T cells from naive C57Bl/6 mice as responsor cells. FTY720-DCs induced weakened proliferation of both $CD4^+$ T cells ($P < 0.01$) and $CD8^+$ T cells ($P < 0.01$) along with less IFN-γ secretion ($P < 0.05$) and more IL-10 production ($P < 0.05$, Fig. 5a,c). Interestingly, $FoxP3^+$ cells among

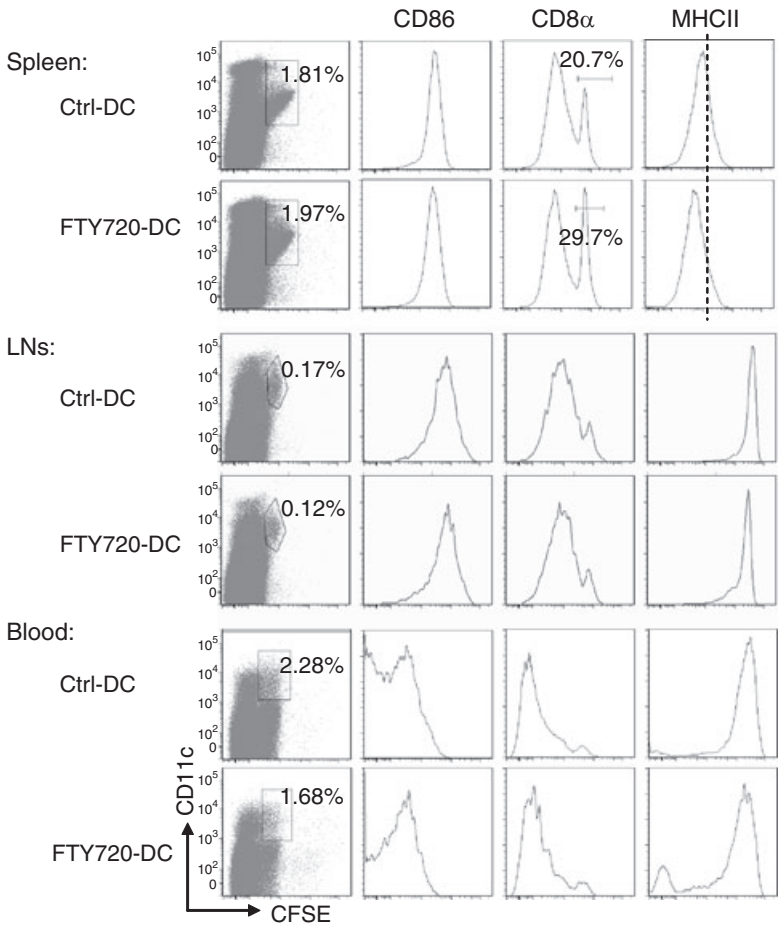

Figure 3 Distribution of allogeneic infused immature dendritic cells *in vivo*. Five million CFSE-labeled Ctrl-DCs or FTY720-DCs from BABL/c were injected intravenously into naïve C57bl/6 recipients via the lateral tail vein. Twenty-four hours after injection, blood samples, spleens and LNs (Axillary, Inguinal, cervical and brachial) were collected from Ctrl-DC- and FTY720-DC-infused recipients. Cells were stained with anti-CD11c, CD86, CD8 and MHC II for subsequent flow cytometric analysis. Data are from one experiment representative of three performed.

$CD4^+$ T cells were significantly increased after 3 days of co-culture with FTY720-DCs ($P < 0.05$, Fig. 5b). These results suggest that FTY720-DCs are less potent to induce antigen-specific and allogeneic T-cell response compared with Ctrl-DCs. Instead, it may preferentially promote regulatory T-cell enrichment.

Infusion of FTY720-DCs hampered allogeneic T-cell response *in vivo*.

Ctrl-DCs or FTY720-DCs from BABL/c mice were infused i.v. into C57Bl/6 mice followed by challenging with mature BMDCs of BABL/c mice through subcutaneous injection in the footpad. After 5 days, we found that infusion of FTY720-DCs notably attenuated total cell proliferation from the draining popliteal LNs as manifested by the reduced LN volume and total cell number (Fig. 6a). Interestingly, Ki67, a marker for cell proliferation, was particularly upregulated in $FoxP3^+$ regulatory T cells but not in the conventional T cells ($P < 0.05$). When we monitor PD1 expression, which is responsible for anergy induction in many disease models, we found that this molecule was upregulated on proliferating conventional T cells ($P < 0.05$, Fig. 6b). These results suggested that infusion of FTY720-DCs could inhibit allogeneic T-cell response by directly enhancing the proliferation of Treg cell while inducing anergy of conventional T cells *in vivo*.

Discussion

Immunosuppressive drugs can deplete donor-specific lymphocytes as well as other cell populations and the remaining lymphocytes reveal hampered cytotoxicity. This leads to increased chronic viral, fungal and bacterial infections and poses a challenging mission to induce donor-specific tolerance for transplant immunotherapy. Clinical

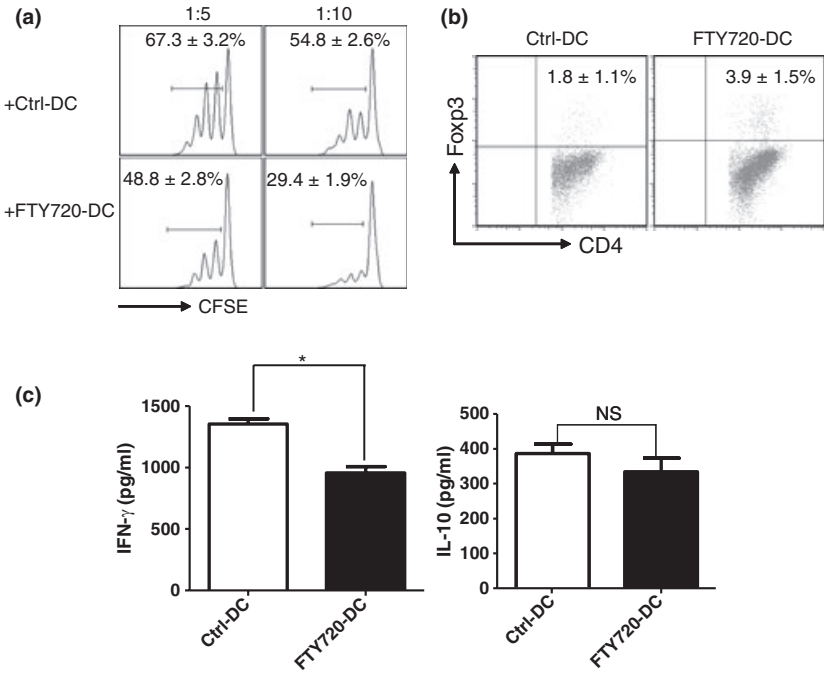

Figure 4 FTY720-DCs are less potent in triggering antigen-specific T-cell response. BABL/c OVA-loaded Ctrl-DCs and FTY720-DCs were co-cultured with CFSE-labeled DO11.10 DO11.10T cells at DC:T = 1:5 and 1:10. After 3 days of culture, the cells were detected by FACS to evaluate proliferation of DO11.10T cells (a) and Treg proportion in $CD4^+$ T cells (b). The supernatant was collected for the detection of IFN-γ and IL-10 and the result is shown in (c). $*P < 0.05$; NS, not significant. This experiment was performed three times independently yielding comparable results.

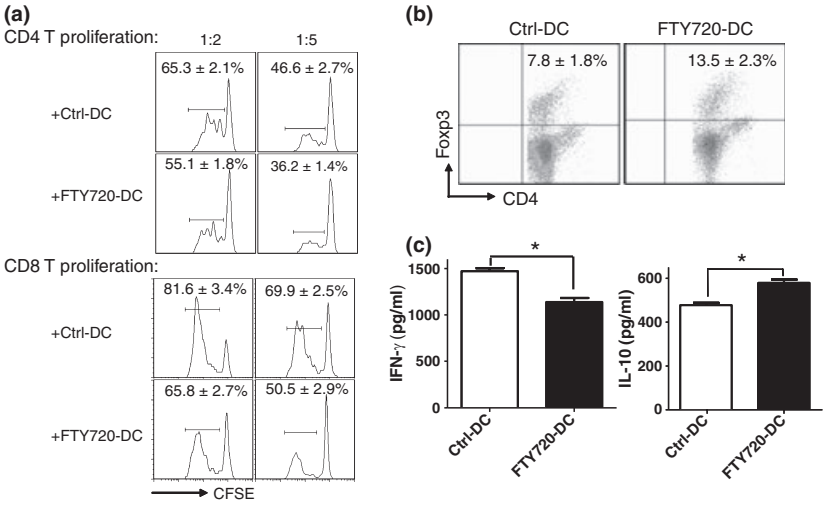

Figure 5 FTY720-DCs are less potent in triggering allogeneic T-cell response. BABL/c-derived Ctrl-DCs and FTY720- DCs were co-cultured with varying number of CFSE-labeled C57Bl/6 splenic $CD3^+$ T cells for 3 days. $CD4^+$, $CD8^+$ T-cell proliferation (a) and Treg proportion in $CD4^+$ cells (b) were detected by FACS. IFN-γ and IL-10 in the supernatant were analyzed as well ($*P < 0.05$) (c). These experiments were performed three times independently yielding comparable results.

studies showed that infusion of donor bone marrow cells followed by cocktail of immunosuppressive drugs could prolong graft survival after kidney, liver, heart, lung and pancreas transplantation [40–43]. There is a large body of evidence indicating that DCs play a certain regulatory role in the process of inducing tolerance. Possible mechanisms include allogeneic microchimerism, immature DCs or specialized tolerogenic DCs subsets inducing apoptosis or anergy of recipient T cells, while shifting naive T cells towards a regulatory phenotype and expanding existing

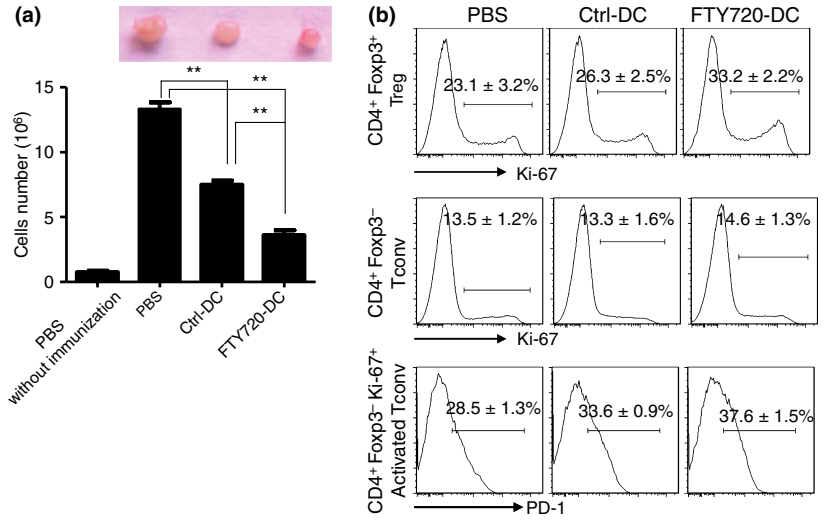

Figure 6 Infusion of FTY720-DCs hampered allogeneic T-cell response *in vivo*. Five million Ctrl-DCs or FTY720-DCs from BABL/c mice were infused i.v. into C57Bl/6 mice. These mice were challenged with mature BMDCs from BABL/c mice through footpad injection. PBS-infused mice were used as a control. After 5 days of allogeneic immunization, the draining popliteal LNs were collected for counting total cell number and performing FACS analysis. Photographs of the popliteal LNs and total cell number are shown in (a). (b) Intracellular staining of Ki67 and surface staining of PD-1 were performed comparing Treg ($CD4^+$ $Foxp3^+$) and Tconv ($CD4^+$ $Foxp3^-$). The data are shown as mean ± SEM with four mice per group. $^{**}P < 0.01$.

Treg [44,45]. Some pioneered research tried to reinforce tolerance by conditioning DCs through exposure to defined pharmacologic agents [25]. It has been reported that RAPA-conditioned BMDCs show resistance to maturation even when exposed to inflammatory stimuli. These cells could significantly prolong graft survival by inducing hyporesponsiveness and apoptosis in alloreactive T cells, while retaining their ability to stimulate and enrich Treg [46–48]. Compared with RAPA-conditioned BMDCs (MST = 45 days) [47], FTY720-DCs also show potent capacity to prolong allograft survival although they respond to LPS-triggered maturation similar to Ctrl-DCs.

Immature DCs express low level of MHC II and co-stimulate molecules and therefore show stunted capacity to prime immune response. Our study showed that compared with infusion of Ctrl-DCs, systemic administration of donor-derived FTY720-DCs could significantly improve graft survival. FTY720-DCs infusion could upregulate proportion of Treg by enhancing Treg proliferation and therefore inhibit Th1 and Tc1 response. Although FTY720 did not change maturation state of DCs, phagocytosis and killing efficacy *in vitro*, it could upregulate B7H4 expression and decrease S1P1 expression. To analyze trafficking properties and functional characteristics of infused DCs, we prelabeled Ctrl-DCs and FTY720-DCs before infusion. We found that both type of DCs trafficked effectively to the spleen and to peripheral lymph nodes as well (Fig. 3). Interestingly, infused BMDCs remained $CD86^{int}$ MHC II^{low} in the spleen of recipients. As priming of naive T cells occurs largely in specialized SLOs such as the spleen and peripheral lymph nodes, Ctrl-DCs and FTY720-DCs trafficking to spleen prior to allograft transplantation could downregulate allogeneic T-cell activity. BMDCs have also been reported to migrate to the thymus, suggesting the possibility that transferred donor DCs may be capable of affecting the repertoire and/or function of recipient T cells by central as well as peripheral mechanisms [49]. There were less FTY720-DCs than Ctrl-DCs circulating in the blood and these circulating DCs were $MHC^{high}CD86^{low}$, rendering their lacking of co-stimulatory signal to fully activate allogeneic T cells. In this sense, when infused systematically, FTY720-DCs have advantages over the drug FTY720 because of their potential to migrate and function locally to induce donor-specific hyperactivity. Tolerogenic DCs isolated from tolerant mice can transfer graft-specific tolerance to naive mice. It has been reported that splenocytes from tolerant recipients, but not purified splenic T cells, transfer donor-specific infectious tolerance without chronic rejection, after infusion into naive recipients [50]. However, purification of splenic DCs induces maturation and capacity to stimulate Th1 response *in vivo* [51]. BMDCs are less mature and much more potent than splenic DCs to expand Treg and maintain FoxP3 expression at high level *in vitro* [52]. Furthermore, these expanded Treg could act back on DCs to prevent their maturation [53]. We found that FTY720-DCs could increase proportion of Treg more potently than BMDCs *in vivo*. In line with this

result, co-culture of FTY720-DCs with DO11.10 T cells as well as allogeneic splenic T cells led to a substantial increment of Treg among activated T cells. This is due to inhibited proliferation of the bulk of $CD4^+$ and $CD8^+$ T as well as the preferential proliferation of either naturally occurring or induced Treg. Therefore, FTY720-DCs could balance the switch between 'on' and 'off' of acute allogeneic response.

We found that IFN-γ production by $CD4^+$ T cells was preferentially inhibited after FTY720-DC transfer. $CD4^+$ help T cells are critical for alloreactive CD8 T-cell activation, function and memory formation [54]. CD4 T cells can also serve as effector cells for primary acute cardiac allograft rejection [55] and also contribute to vasculopathy and hinder long-term graft acceptance [32]. Importantly, after re-challenge of mature allogeneic DCs, PD-1 expression on proliferating conventional CD4 T cells was upregulated in FTY720-DC group. There are two known binding partners for PD-1. PD-L1 can be highly expressed by tissue cells [56] and contribute to immune privilege [57], while PD-L2 (B7DC) expression is more restricted and is expressed mainly by DCs and a few tumor lines. We found that BMDCs express PD-L1 (data not shown) and PD-L2, while FTY720 treatment did not interfere with their expression. In resting mice, PD-L1 mRNA can be detected in the heart [58]. It was reported that PD-1 engagement could enhance T-cell motility and reduce their chance to engage antigen-bearing DCs [59]. Interestingly, FTY720-treated DCs were reported to have intrinsically less potential in activating naïve and effector Th2 cells because of a reduced capacity to stably interact with T cells through forming immunologic synapses [17]. It is conceivable that enhanced PD-1 expression on conventional T cells could reduce DC-T-cell interaction opportunity, and PD-1 engagement could abolish DC-T-cell long-lasting interaction. Thus, priming of T helper cells in the draining lymphoid organ could be hampered and anergy could be induced.

Treg could exert their suppressive function through Ganzyme B and perforin-mediated cytotoxicity, interrupting metabolisms such as tryptophan catabolism, those of regulatory cytokines such as IL-10, IL-35 and TGF-β, which could inhibit maturation and antigen presenting function of DCs, etc. [60,61]. Manipulating Treg during immunotherapy has been confronted with one major obstacle of determining the specificity of Treg. Infusion of FTY720-DCs renders a simple and efficient method to maintain specific hyporesponse to allografts by enhancing tolerogenic function of Treg.

Although FTY720 treatment *in vitro* was reported to reduce human monocyte-derived DC chemotaxis [17,62], infused FTY720-DCs showed similar distribution pattern as Ctrl-DCs (Fig. 3). Thus, the induction of allograft tolerance by FTY720-DCs could be mediated by either soluble factors such as IL-10 or regulatory surface molecules such as B7H4 and B7DC. Further study should address the contributing factors either from FTY720-DCs or from Treg cells expanded by FTY720-DCs to help us better understand how to improve and apply this cell-based anti-alloreaction vaccine before transplantation.

Authorship

FG, FZ and YC: participated in the research design. HY and YM participated in the writing of the manuscript and transfer of BMDCs. HY: was responsible for the mice cardiac transplantation. LD and YX: performed the flow cytometric analysis. PX, WF, MF and ZT: participated in the performance of other research and data analysis. All authors have no financial conflict of interest.

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