Original Paper

Interleukin-10 Gene-Modified Dendritic Cell-Induced Type 1 Regulatory T Cells Induce Transplant-Tolerance and Impede Graft Versus Host Disease After Allogeneic Stem Cell Transplantation

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Key Words

Interleukin (IL)-10 • Dendritic cells (DCs) • Type 1 T regulatory (Tr1) cells • Immune tolerance • Graft versus host disease (GVHD)

Abstract

Background/Aims: Tr1 cells can induce peripheral tolerance to self- and foreign antigens, and have been developed as a therapeutic tool for the induction of tolerance to transplanted tissue. We explored the feasibility of generating Tr1 cells by using IL-10 gene-modified recipient DCs (DC_{LV-IL-10}) to stimulate donor naive CD4⁺ T cells. We also investigated some biological properties of Tr1 cells. **Methods:** DC_{LV-IL-10} were generated through DCs transduced with a lentivirus vector carrying the IL-10 gene, and Tr1 cells were produced by using DC_{LV-IL-10} to stimulate naive CD4⁺ T cells. The effects of Tr1 cells on T-cell proliferation and the occurrence of graft versus host disease (GVHD) following allogeneic stem-cell transplantation (allo-HSCT) were investigated. **Results:** The DC_{LV-IL-10}-induced Tr1 cells co-expressed LAG-3 and CD49b. Moreover, they also expressed CD4, CD25, and IL-10, but not Foxp3, and secreted significantly higher levels of IL-10 ($1,729.36 \pm 185.79$ pg/mL; $P < 0.001$) and INF- γ ($1,524.48 \pm 168.65$ pg/mL; $P < 0.01$) than the control T cells upon the stimulation by allogeneic DCs. Tr1 cells markedly suppressed T-lymphocyte proliferation and the mixed lymphocytic response (MLR) *in vitro*. The mice used in the allo-HSCT model had longer survival times and lower clinical and pathological GVHD scores than the control mice. **Conclusion:** IL-10 gene-modified DC-induced Tr1 cells may be used as a potent cellular therapy for the prevention of GVHD after allo-HSCT.

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Introduction

T regulatory cells (Tregs) play a crucial role in the induction of peripheral tolerance to self- and foreign antigens. Numerous studies have demonstrated that Tregs are involved in the immunological dysregulation that underlies autoimmune diseases, chronic inflammatory diseases, and certain immunological complications of transplantation. Tregs are known to suppress the proliferation and effector functions of several different cell types, including T cells, B cells, natural killer (NK) cells, and antigen (Ag)-presenting cells (APCs) [1]. Tregs are also involved in immune homeostasis, regulation of inflammation, establishment and maintenance of immune tolerance, and prevention of graft versus host disease (GVHD) following an allogeneic hematopoietic stem cell transplant (allo-HSCT) [2, 3]. Tregs are divided into two categories: i) natural Tregs (nTregs), derived from the thymus and ii) adaptive or induced Tregs (aTregs or iTregs) induced outside the thymus in the presence of an antigen (Ag) and tolerogenic conditions. Tr1 cells comprise the main subset of Tregs.

The nTregs constitutively express CD4, CD25 and Foxp3, protect self-tolerance, and prevent autoimmunity [4]. However, the paucity of nTregs tends to impede investigations into their applications in both animal models and clinical studies. There are also some obstacles to expanding nTregs *in vitro*. First, other types of T cells expand preferentially in culture [5]. Second, the immunosuppressive function of nTregs is not Ag-specific [6], and high nTreg to effector CD4⁺ T cell ratios (usually > 1:1) are needed to prevent GVHD [7]. In some cases, the large number of nTregs required to prevent GVHD can be impossible to achieve in humans [8]. Third, human nTregs often display unstable inhibitory activity in an ex-vivo environment, which makes them unsuitable for use in some preclinical models [9].

In contrast to nTregs, Tr1 cells specifically co-express CD49b and LAG-3 [10]. Moreover, they also express CD4 and CD25, but not Foxp3. Upon activation, Tr1 cells modulate immune suppression mainly by secreting high levels of interleukin (IL)-10 and transforming growth factor (TGF)- β [11-13]. A previous study showed that Tr1 cells can mediate immune suppression by killing antigen presenting cells (APCs) via a perforin/granzyme-dependent process that requires major histocompatibility complex (MHC) class I recognition [14]. Because Tr1 cells provide tolerance to foreign antigens, they also play an important role in organ transplantation.

Many protocols for the *in vitro* induction of Tr1 cells describe a process in which the Tr1 cells are induced in the presence of either recombinant IL-10 and IFN- γ [15] or recombinant TGF- β and IL-27 [16]. Tr1 cells can also be generated by a single stimulation with IL-10-treated tolerogenic DCs [17]. However, only a minority of the Tr1 cells generated with most protocols produce IL-10. The main impediment to obtaining Tr1 cells for use in clinical applications such as cell therapy is the presence of contaminating effector T-cells in the resulting cell population. As an alternative method for obtaining a large and homogeneous population of Tr1 cells, lentivirus-mediated human *IL-10* gene transfer has been used to convert conventional T cells into Tr1-like cells, which mirror the phenotype and function of Tr1 cells, and can suppress or prevent transplant rejection and GVHD. However, an important limitation of this method is that the generated Tr1-like cells cannot acquire autoantigen specificity [18].

We previously induced Tr1 cells by stimulating transgenic OT II mouse-derived naive CD4⁺ T cells with IL-10-expressing adenovirus (AdV_{IL-10})-transfected and ovalbumin (OVA)-pulsed dendritic cells (DC_{OVA/IL-10}). That study demonstrated that both *in vitro* and *in vivo*, DC_{OVA/IL-10}-stimulated CD4⁺ Tr1 cells acquired OVA peptide MHC class I (pMHC I), which targeted the suppressive effect of CD4⁺ Tr1 cells [12]. This targeting led to an ~ 700% enhancement in the suppression of DC_{OVA}-induced CD8⁺ T cell responses and the immune response against OVA-expressing murine B16 melanoma cells, when compared with the responses shown by analogous CD4⁺ Tr1 cells without acquired pMHC I [12].

Therefore, we hypothesized that Tr1 cells could be generated through *IL-10* gene-modified recipient DCs by stimulating donor naive CD4⁺ T cells. Moreover, we also hypothesized that the presence of recipient pMHC I on Tr1 cells should enhance their

suppressive effect towards recipient cells. If so, an infusion of these Tr1 cells might induce transplant tolerance and prevent GVHD after allogeneic transplantation. In this study, we designed an experiment to explore the feasibility of using *IL-10* gene-modified DCs to induce and expand Tr1 cells *in vitro*. We also assessed the immunosuppressive effects of the newly expanded Tr1 cells both *in vitro* and *in vivo*. Indeed, for the first time, we investigated the effects of *in vitro*-generated Tr1 cells on the incidence and severity of GVHD after an allo-HSCT. Our data suggest that Tr1 cells generated with *IL-10* gene-modified DCs can effectively inhibit T cell proliferation and the mixed lymphocyte reaction (MLR) *in vitro*, and also reduce the occurrence of GVHD in mice after a transplant.

Materials and Methods

Animal studies

C57BL/6 (H-2kb) and BALB/c (H-2kd) mice (age, 8–10 weeks) were purchased from Slac Laboratory Animal Co, Ltd. (Shanghai, China), and housed under specific pathogen-free conditions. All animal experiments were conducted according to guidelines published by The Ethics Committee of Xinhua Hospital Affiliated with the Shanghai Jiao Tong University School of Medicine. The protocols used in this study were approved by the Ethics Committee of Xinhua Hospital affiliated with Shanghai Jiao Tong University School of Medicine, Shanghai China.

Cell culture and reagents

RPMI-1640 medium, fetal bovine serum (FBS), recombinant mouse granulocyte- macrophage colony-stimulating factor (rmGM-CSF), recombinant mouse interleukin (rmIL)-4 and rmIL-2 were purchased from PeproTech (PeproTech; Shanghai, China).

Lentiviral *IL-10* vector construction and transduction into dendritic cells

The murine *IL-10* gene was amplified by the polymerase chain reaction (PCR) performed with the following primers: forward: 5'-ACACCTCGAGATGCCTGGCTCAGCACTGC-3' and reverse: 5'-ATTAGCGGCCGCTTATTTGTCTCATCATCCTT-3'. The purified *IL-10* gene fragment was cloned into the pHLV-CMV-IRES-ZS Green plasmid (Hanbio Co., Ltd; Shanghai, China) using *XhoI* and *NotI* restriction sites. Lipofilter™ (Hanbio; Shanghai, China) was used to co-transfect the recombinant plasmid containing the *IL-10* gene and *green fluorescent protein (GFP)* gene into 293T cells with a packaging plasmid (pSPAX2) and pMD2G envelop plasmid. Next, the virus particles were harvested from the cell supernatant and designated as LV-IL-10. Additionally, a control vector expressing GFP was constructed and designated as LV-GFP.

Bone marrow (BM)-derived DCs were generated as previously described [12]. Briefly, BM cells were isolated from the femora and tibiae of normal BALB/c mice, and the red blood cells were removed with 0.84% ammonium chloride. The remaining cells were plated using RPMI-1640 medium containing 10% FBS, 20 ng/mL rmGM-CSF, and 20 ng/mL rmIL-4 (Day 0). On day 3, the non-adherent granulocytes and T and B cells were gently dislodged and fresh medium was added. On day 5, the loosely adherent proliferating DC aggregates were removed and re-plated using the previously described culture medium containing 1 µg/mL lipopolysaccharides (LPS) (Sigma; Shanghai, China) and cultured until day 6, when the non-adherent DCs were collected. The collected DCs were then transduced with LV-GFP or LV-IL-10 overnight at 37°C with a multiplicity of infection of 100. GFP expression in the transduced DCs was detected by fluorescence microscopy at 24 hours after transduction, and the GFP-positive cells were sorted by flow cytometry. The purified GFP-positive DCs transduced with LV-GFP or LV-IL-10 were designated as DC_{GFP} or DC_{LV-IL-10}, respectively.

Preparation of DC_{LV-IL-10}-induced Tr1 cells

Naive CD4⁺ T cells were isolated from the spleens of C57BL/6 mice using a Mouse Naive CD4⁺ T Cell Isolation Kit (StemCell Technologies; Shanghai, China) according to the manufacturer's protocol. The purity of the isolated naive CD4⁺ T cell populations routinely exceeded 98%. For the generation of Tr1 cells, naive CD4⁺ T cells (2 × 10⁵ cells/mL) were stimulated for 3 days with irradiated (40 Gy) BM-derived DCs, DC_{GFP} or DC_{LV-IL-10} (1 × 10⁵ cells/mL), respectively, in the presence of 20 U/mL rmIL-2, and then purified using a

Mouse CD4⁺ T cell Selection Kit (StemCell Technologies; Shanghai, China). The purified CD4⁺ T cells were designated as T_{DC}, T_{GFP} or Tr1 cells.

Flow cytometry

Expression of CD11c and CD83 on DC_{GFP} and DC_{LV-IL-10}, and expression of CD49b, LAG3, CD4, CD25, Foxp3 and IL-10 on T_{DC}, T_{GFP} or Tr1 cells were detected by flow cytometry. Staining for CD11c, CD83, CD49b, LAG3, CD4, and CD25 on the cell surface was accomplished by incubating individual aliquots of cells on ice with fluorochrome-conjugated antibodies (eBioscience; Shanghai, China) for 30 min. For intracellular staining of Foxp3, Tr1 cells were fixed and permeabilized, and then stained with FITC-conjugated anti-Foxp3 monoclonal antibodies (eBioscience; Shanghai, China). For assays of the intracellular cytokines IL-10, Tr1 cells were treated with Cell Stimulation Cocktail (2 µL/mL) for 5 h at 37°C in the presence of 2 µL/mL Protein Transport Inhibitor Cocktail, and then permeabilized prior to staining with fluorescent dye-labeled anti-cytokine antibodies (eBioscience; Shanghai, China). Fluorescence-activated cell sorting (FACS) analyses for detection of various cell markers and cytokines were performed with a BD Canto II flow cytometer (Becton-Dickinson Biosciences, Shanghai, China).

RNA isolation and real-time PCR

To examine the levels of IL-10 mRNA expression in DC_{LV-IL-10}, total RNA was extracted from DCs, DC_{GFP} and DC_{LV-IL-10} using TRIzol reagent (Takara; Liaoning, China); after which, a PrimeScript RT Master Mix kit (Takara Liaoning, China) was used to reverse transcribe 1 µg of total RNA into cDNA. Real-time PCR array analysis was performed with a SYBR Premix Ex Taq™ Kit (Takara; Liaoning, China) in a total volume of 20 µL. Each reaction mixture contained 2 µL of cDNA primers (0.2 mM each), 10 µL of SYBR Green, and 0.4 µL of Rox Dye II. The PCR conditions were 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s, with a final dissociation stage. The PCR samples were then analyzed using an ABI 7500 detector (Applied Biosystems; Foster City, CA, USA). The numbers of target genes were determined and normalized to the amount of GAPDH cDNA, which served as an internal standard (Sangon Biotech; Shanghai, China). The primers used for IL-10 were: forward: 5-GGACAACATACTGCTAACCGACTC-3 and reverse: 5-TGGGGCATCACTTCTACC-3 (Sangon Biotech; Shanghai, China).

ELISA

To detect IL-10, IL-6, TNF-α, and IL-12p70 levels in the DCs, DC_{GFP} and DC_{LV-IL-10}, these cells were adjusted to concentrations 1 × 10⁶ cells/mL and activated with 200 ng/mL of LPS plus 50 ng/mL of rmIFN-γ (Sigma; Shanghai, China) for 48 h. The supernatants were then collected for analysis. The levels of IL-10, IL-6, TNF-α, and IL-12p70 were measured with an ELISA kit (R&D Systems; Shanghai, China) according to the manufacturer's instructions.

To detect IL-10, INF-γ, IL-2, and IL-4 secreted by T_{DC}, T_{GFP} and Tr1 cells, these cells (1 × 10⁶ cells/well) were stimulated with irradiated (40 Gy) BM-derived DCs (5 × 10⁵ cells/well) obtained from BALB/c mice for 48 h. Next, the supernatants were harvested and the levels of IL-10, INF-γ, IL-2, and IL-4 were measured with an ELISA kit (R&D Systems; Shanghai, China) according to the manufacturer's instructions.

Briefly, microtiter plates were coated overnight at room temperature with mouse monoclonal antibodies (the capture antibody); after which, the wells were blocked for 1 h with PBS containing 1% FBS and then washed four times with 0.05% Tween-20 in PBS. Triplicate standards and samples were added to adjacent wells and incubated for 2 h at room temperature. After incubation, the wells were washed and the detection antibodies were added. After 2 h, the wells were washed again, and incubated with a horse radish peroxidase (HRP)-conjugated antibody in 1% FBS/PBS. Next, the plates were washed four times and incubated for 30 min with the substrate (0.0125% tetramethylbenzidine/0.008% H₂O₂ in citrate buffer, pH 5). Color development was terminated by addition of 2 M H₂SO₄. The optical density of each well was measured with an ELISA reader, and the cytokine concentration in each well was calculated using a double standard curve (R&D System, Shanghai, China).

Proliferation and suppression of T cells

To investigate the proliferative capacity of Tr1 cells induced by DC_{LV-IL-10} in response to the antigen used for their generation, T_{DC}, T_{GFP} and Tr1 cells (2 × 10⁵ cells/well) were stimulated for 2 days with irradiated (40 Gy) BM-derived DCs obtained from BALB/c mice (1 × 10⁵ cells/well). A total of 1 µCi of ³H-TdR was added

to each well at the start of the final 16 h of incubation time. Following incubation, the cells were harvested for measurements of incorporated ^3H -thymidine with a beta-counter. Percent inhibition was calculated with the following equation: $1 - \text{experimental c.p.m.} / (\text{c.p.m. of responder only} + \text{c.p.m. of inhibitor only})$ [17].

CD8^+ and CD4^+ T cells proliferation tests were performed to investigate the Ag-specific immunosuppressive function of $\text{DC}_{\text{LV-IL-10}}$ -induced Tr1 cells. Naive CD8^+ T cells or naive CD4^+ T cells as effector cells were isolated from the spleens of C57BL/6 mice using a Mouse Naive CD8^+ T Cell or Naive CD4^+ T Cell Isolation Kit (StemCell Technologies; Shanghai, China) according to the manufacturer's instructions. Irradiated (40 Gy) BM-derived DCs obtained from BALB/c mice (0.1×10^5 cells/well) were incubated with naive CD8^+ T cells (0.5×10^5 cells/well) in the presence of irradiated (40 Gy) T_{DC} , T_{GFP} or Tr1 cells (2×10^5 cells/well) and 2-fold dilutions thereof in 96-well U-bottom plates for 2 days. In the same way, irradiated (40 Gy) BM-derived DCs obtained from BALB/c mice (0.1×10^5 cells/well) were incubated with naive CD4^+ T cells (0.5×10^5 cells/well) in the presence of irradiated (40 Gy) T_{DC} , T_{GFP} or Tr1 cells (2×10^5 cells/well) and 2-fold dilutions thereof in 96-well U-bottom plates for 2 days. When the ratio of T_{DC} , T_{GFP} or Tr1 cells to effector cells reached 4:1, neutralizing anti-IL-10 (10 $\mu\text{g/mL}$) or anti-IFN- γ antibodies (10 $\mu\text{g/mL}$) were added to some of the cultures. A total of 1 μCi of ^3H -TdR was added to each well at the start of the final 16 h of incubation time. Cell proliferation was then assessed by measuring ^3H -thymidine incorporation as previously described.

Mixed lymphocyte reaction assays

Splenocytes (SCs) from BALB/c and C57BL/6 mice were used as stimulator and effector cells, respectively. Irradiated (40 Gy) SCs from BALB/c mice (4×10^5 cells/well) were cultured with SCs from C57BL/6 mice (4×10^5 cells/well) in the presence of irradiated (40 Gy) T_{DC} , T_{GFP} or Tr1 cells (1.6×10^6 cells/well) and 2-fold dilutions thereof for 2 d in 96-well U-bottom plates maintained at 37°C in a 5% CO_2 atmosphere. A total of 1 μCi of ^3H -TdR was added to each well at the start of the final 16 h of incubation time. Cell proliferation was then assessed by measuring ^3H -thymidine incorporation as previously described.

Allogeneic bone marrow transplantation (BMT) animal study

For allogeneic BMTs, bone marrow cells (BMCs) and SCs were harvested from donor mice (C57BL/6). The harvested were then resuspended in RPMI-1640 medium containing 10% FBS and 1% penicillin/streptomycin and adjusted to suitable cell concentrations. Recipient mice (BALB/c) were given a lethal dose of radiation (8 Gy) [18] and then intravenously transplanted with donor BMCs (1×10^7) and SCs (4×10^7) [19]. The recipients were randomly assigned to the following groups based on their number of injected Tr1 cells; control group: BM + T; BM + T + Tr1 cells (2×10^6); BM + T + Tr1 cells (4×10^6). Survival of the mice after BMT was monitored daily for 50 days. Individual clinical indicators of GVHD, including each animal's weight, posture, activity, fur texture, and skin integrity, received a weekly score ranging from 0 to 2 [20, 21]. On day 14 after BMT, the mice were sacrificed for a histopathology evaluation of GVHD. Samples of skin, liver, intestine, and lung tissue were fixed in 10% formalin and embedded in paraffin. Sections for histology (5 μm) were stained with hematoxylin and eosin and scored for GVHD [22].

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp. All results are expressed as the mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) was performed to assess the effects of a single factor on multiple groups, and the Tukey test was used for post hoc testing. Survival curves were plotted using Kaplan-Meier estimates. A P -value ≤ 0.05 was considered statistically significant.

Results

Characteristics of DCs transduced with the lentivirus IL-10 vector

To generate IL-10 gene modified tolerogenic DCs, we transduced DCs with LV-GFP or LV-IL-10 overnight at 37°C at a multiplicity of infection of 100 to obtain DC_{GFP} and $\text{DC}_{\text{LV-IL-10}}$. Examinations by fluorescence microscopy showed that the transduced DCs expressed GFP (Fig. 1A). Our observations indicated that $49.61 \pm 5.23\%$ of DC_{GFP} and $52.16 \pm 6.24\%$ of $\text{DC}_{\text{LV-IL-10}}$ expressed GFP ($P > 0.05$) (Fig. 1B). Moreover, FACS sorting results showed that 76.26

Fig. 1. LV-GFP and LV-IL-10 efficiently transduced DCs without changing their phenotype. (A) Expression of GFP and the morphologic characteristics of DC_{GFP} and DC_{LV-IL-10} were observed under a fluorescence microscope (x100 magnification). (B) Transduced DCs were analyzed by FACS. The frequency of GFP positive cells was indicated. (C) Expression of the typical DC markers CD83 and CD11c in GFP positive DCs. Results obtained from at least three independent experiments.

Fig. 2. DC_{LV-IL-10} secreted high levels of IL-10 and IL-6 but low amounts of TNF- α and IL-12. (A) IL-10 mRNA levels were measured by Real-time PCR; (B) After DCs, DC_{GFP} and DC_{LV-IL-10} were activated with LPS and IFN- γ for 48 h, the levels of IL-10, IL-6, TNF- α , IL-12 were measured by ELISA. Results represent the mean $\pm$ SD of data obtained from at least three independent experiments (** $P < 0.01$, *** $P < 0.001$).

$\pm 9.18\%$ and $78.39 \pm 8.67\%$ of GFP positive DC_{GFP} and DC_{LV-IL-10}, respectively, expressed CD11c and CD83 ($P > 0.05$) (Fig. 1C), which are markers of mature DCs.

Fig. 3. The characteristics of Tr1 cells which were induced by IL-10 gene-modified DCs. Naive CD4⁺ T cells were co-cultured with DCs, DC_{GFP} and DC_{LV-IL-10} for 3 days in the presence of IL-2. The cells were then purified using a CD4⁺ T cell positive enrichment kit, and designated as T_{DC}, T_{GFP} and Tr1 cells, respectively. (A) The expression of LAG3 and CD49b, which are the typical markers of Tr1 cells were analyzed by FACS in DC_{LV-IL-10}-induced Tr1 cells. (B) CD4, CD25, IL-10 and Foxp3 expression in DC_{LV-IL-10}-induced Tr1 cells were evaluated by FACS. (C) After stimulated with irradiated (40 Gy) BM-derived DCs from BALB/c mice for 48 h, the cytokine profile of DC_{LV-IL-10}-induced Tr1 cells were measured by ELISA. Results represent the mean ± SD of data obtained from at least three independent experiments (**P<0.01, ***P<0.001).

IL-10 mRNA expression on DC_{LV-IL-10} was significantly higher than that on DCs and DC_{GFP} ($P < 0.001$) (Fig. 2A). We also used ELISA to detect the levels of cytokines secreted by DCs, DC_{GFP} and DC_{LV-IL-10} that had been activated with LPS and IFN- γ . As shown in Fig. 2B, the levels of IL-10 secreted by DC_{LV-IL-10} (923.65 ± 85.37 pg/mL) were significantly higher than the levels secreted by DCs (84.76 ± 7.19 pg/mL) and DC_{GFP} (95.33 ± 9.17 pg/mL) ($P < 0.01$). However, there were no significant differences between the levels IL-6, TNF- α and IL-12p70 secreted by DC_{LV-IL-10} when compared with those levels secreted by DCs and DC_{GFP} ($P > 0.05$).

Taken together, our data suggest that DCs can be successfully transduced with LV-IL-10 and maintain the morphology and phenotype of mature DCs; and at the same time, express high levels of IL-10. They therefore possess the properties needed to induce a population of Tr1 cells.

Induction of Tr1 cells by IL-10 gene modified DCs

To determine whether or not DC_{LV-IL-10} could induce Tr1 cells, naive CD4⁺ T cells were co-cultured with DCs, DC_{GFP} or DC_{LV-IL-10} for 3 days in the presence of rmIL-2. The cells were then purified using a CD4⁺ T cell positive enrichment kit, and designated as T_{DC}, T_{GFP} or Tr1 cells respectively. We next analyzed whether DC_{LV-IL-10}-induced Tr1 cells express Tr1 cell-associated surface markers [10] and/or transcription factors [12]. As shown in Figs. 3A and 3B, $51.27 \pm 3.86\%$ of DC_{LV-IL-10}-induced Tr1 cells co-expressed CD49b and LAG-3. Moreover, they also expressed CD4, CD25, and IL-10, but not Foxp3. Next, the cytokines secreted by DC_{LV-IL-10}-induced T cells were analyzed by ELISA, and our results showed that Tr1 cells secreted significantly higher levels of IL-10 ($1,729.36 \pm 185.79$ pg/mL) than T_{DC} (115.82 ± 13.29 pg/mL) and T_{GFP} did (90.34 ± 11.83 pg/mL) ($P < 0.001$). Similarly, the levels of INF- γ secreted by Tr1 cells ($1,524.48 \pm 168.65$ pg/mL) were significantly higher than those

Fig. 4. Tr1 cells were anergic and inhibited cell proliferation in vitro. (A) T_{DC} , T_{GFP} and Tr1 cells were stimulated with DCs from BALB/c mice. Naive $CD8^+$ T cells (B) and naive $CD4^+$ T cells (C) from C57BL/6 mice were stimulated with DCs from BALB/c mice alone or in the presence of T_{DC} , T_{GFP} and Tr1 cells at different ratios. When the ratio of T_{DC} , T_{GFP} or Tr1 cells to effector cells reached 4:1, neutralizing anti-IL-10 or anti-IFN- γ antibodies were added to the cultures. (D) SCs from C57BL/6 mice were stimulated with irradiated SCs from BALB/c mice alone or in the presence of T_{DC} , T_{GFP} and Tr1 cells at different ratios. After two days of culture, [3H]-thymidine was incubated with the cells for 16 hours. Results represent the mean $\pm$ SD of data obtained from least three independent experiments (* $P < 0.01$, ** $P < 0.001$).

secreted by T_{DC} (496 ± 72.56 pg/mL) and T_{GFP} (368 ± 65.79 pg/mL) ($P < 0.01$). However, the IL-2 and IL-4 levels secreted by Tr1 cells were not significantly different from those secreted by T_{DC} and T_{GFP} ($P > 0.05$) (Fig. 3C).

These data showed that DC_{LV-IL-10} co-cultured with naive $CD4^+$ T cells could induce Tr1 cells that secrete high levels of IL-10 and IFN- γ , which are the main cytokines required for Tr1 cells to exert an immunosuppressive effect.

Tr1 cells were capable of inhibiting T-cell proliferation in vitro

To determine whether our induced Tr1 cells were anergic, T_{DC} , T_{GFP} or Tr1 cells were re-stimulated with DCs obtained from BALB/c mice. Our results showed that the Tr1 cells exhibited a significantly lower proliferative response when compared with T_{DC} and T_{GFP} ($P < 0.01$) (Fig. 4A). This indicated that the Tr1 cells were Ag-specific anergic toward the antigen used for their generation.

Fig. 5. Effect of a Tr1 cell infusion on graft versus host disease (GVHD) in BALB/c mice. (A) Clinical GVHD scores of the transplanted mice. Results represent the mean $\pm$ SD of data gathered from one of three independent experiments ($n = 12$ mice per group). (B) Histopathology results are shown for samples of skin, small intestine, liver, and lung tissue. The sections were stained with H&E on day 14 after BMT. Results obtained from a least three independent experiments ($n = 5$ mice per group); (original magnification $\times 100$). (C) Histopathology scores for samples of skin, small intestine, liver, and lung tissue on day 14 after BMT. Results represent the mean $\pm$ SD of data obtained from one of three independent experiments ($n = 5$ per group) (* $P < 0.05$, ** $P < 0.01$).

The suppressive effect of Tr1 cells was examined in a CD8⁺/CD4⁺T cell proliferation assay. Irradiated (40 Gy) T_{DC}, T_{GFP} or Tr1 cells were added to cultures containing naive CD8⁺ T cells or naive CD4⁺ T cells obtained from C57BL/6 mice and also irradiated (40 Gy) DCs obtained from BALB/c mice at different ratios of T_{DC}, T_{GFP} or Tr1 : effector cells (4:1, 2:1, 1:1, and 1:2). As shown in Figs. 4B and 4C, the Tr1 cells markedly inhibited CD8⁺ and CD4⁺ T cell proliferation when the Tr1/effector cell ratio was 4:1 ($P < 0.01$), 2:1 ($P < 0.01$) or 1:1 ($P < 0.05$). The strongest suppressive effect was obtained when the ratio was 4:1, and no suppressive effect was observed when the Tr1 to effector cell ratio was 1:2, indicating that Tr1 cells inhibited CD8⁺ and CD4⁺ T cell proliferation in a dose-dependent manner. To determine the role played by IL-10 in the suppressive effect of Tr1 cells, neutralizing anti-IL-10 and IFN- γ antibodies were added to some of the cultures. When the ratio of T_{DC}, T_{GFP} or Tr1 cells to effector cells reached 4:1, the inhibitory effect of Tr1 cells was decreased > 90% by anti-IL-10 antibodies ($P < 0.01$), but not by anti-IFN- γ antibodies ($P > 0.05$), indicating that IL-10 plays an important role in the suppressive activity of regulatory T cells.

MLR assays were performed to further confirm the immunosuppressive effect of DC_{LV-IL-10}-induced Tr1 cells. Irradiated (40 Gy) T_{DC}, T_{GFP} or Tr1 cells accompanied by effector

Fig. 6. Effect of a Tr1 cell infusion on the survival of transplanted mice. BALB/c mice were infused with BM + T, BM + T + Tr1 cells (2×10^6) or BM + T + Tr1 cells (4×10^6), and their survival times were recorded over a period of 50 days. Results obtained from one of three independent experiments ($n = 12$ per group); (* $P < 0.05$, ** $P < 0.01$).

cells at different ratios of T_{DC} , T_{GFP} or Tr1 to effector cells (4:1, 2:1, 1:1, and 1:2) were added to cultures containing SCs obtained from BALB/c and C57BL/6 mice. As shown in Fig. 4D, Tr1 cells inhibited the MLR in a dose-dependent manner. The strongest suppressive effect was obtained when the ratio of Tr1 to effector cells was 4:1 ($P < 0.01$), and no suppressive effect was found when the Tr1 to effector cell ratio was 1:2 ($P > 0.05$).

Taken together, our data suggest that the $DC_{LV-IL-10}$ -induced Tr1 cells were Ag-specific anergic, and capable of inhibiting $CD8^+$ and $CD4^+$ T cell proliferation and the MLR *in vitro*.

$DC_{LV-IL-10}$ -induced Tr1 cells induced transplant tolerance after an allogeneic bone marrow transplantation

We next investigated whether $DC_{LV-IL-10}$ -induced Tr1 cells could inhibit alloreactivity and induce transplant-tolerance *in vivo*. Recipient BALB/c mice were given a lethal dose of radiation, and then infused with BMCs and SCs obtained from donor C57BL/6 mice with or without accompanying $DC_{LV-IL-10}$ -induced Tr1 cells. The transplanted mice were monitored for signs and symptoms of GVHD. As shown in Fig. 5A, the GVHD scores of mice which received BM + T + Tr1 cells were significantly lower from day 6 onwards when compared with the GVHD scores of the control mice ($P < 0.01$).

Histopathological analyses of tissue sections revealed that the control mice displayed more obvious signs of GVHD in specimens of their skin, intestine, liver, and lung tissue on day 14 after the BMT, when compared with mice in the Tr1-treated groups (Fig. 5B). We also used pathology scores to confirm the effect of a Tr1 cell infusion on GVHD. As shown in Fig. 5C, the histopathological GVHD scores of mice which received BM + T + Tr1 cells were significantly lower than those of the control mice with regards to skin, small intestine, liver, and lung damage (all P -values < 0.05). Furthermore, it appeared that the GVHD scores of mice which received a high dose of Tr1 cells (4×10^6) were lower than those of mice which received a low dose (2×10^6) of Tr1 cells. However, this difference was not statistically significant ($P > 0.05$).

Our data also suggest that an infusion of Tr1 cells prolonged the mean survival time of the transplanted mice. As shown in Fig. 6, the mean survival time of mice infused with BM + T was 28.7 ± 1.02 d, which was significantly less than the mean survival time of mice which received BM + T + Tr1 cells (2×10^6) (39.5 ± 3.02 d) ($P < 0.05$), as well as the mice which received BM + T + Tr1 cells (4×10^6) (mean survival time point not reached) ($P < 0.01$). At 50 days post-transplantation, 75% of the BALB/c mice that received BM + T + Tr1 cells (4×10^6) remained alive, whereas only 40% of the mice that received BM + T + Tr1 cells (2×10^6) were still alive ($P < 0.05$), indicating that infusing greater numbers of Tr1 cells could further increase the survival time of BMC-transplanted mice.

Discussion

Tregs play a crucial role in the induction of peripheral tolerance to self and foreign antigens, and can potentially be developed as a therapeutic tool to promote or restore immune tolerance in patients who have received a transplant or have inflammatory or

autoimmune diseases. The major obstacles to developing Tregs as therapeutic agents have been an inability to efficiently expand pure cultures of polyclonal Tregs and generate Ag-specific Tregs. Despite recent advances in the methods used to generate antigen Ag-specific Tr1 cells *in vitro*, the resulting populations still contain contaminants that may limit the *in vivo* efficacy of the cultured Tr1 cells [17, 23].

Adaptive Tr1 cells are generated peripherally when dendritic cells present antigens to naive T cells; after which, the resultant adaptive Tr1 cells can assume a variety of phenotypes depending upon the conditions under which they are induced. It was previously shown that IL-10 and TGF- β -secreting Tr1 cells are generated upon Ag-presentation by immature DCs and also DCs that express IL-10 or TGF- β [24, 25].

In this study, $78.39 \pm 8.67\%$ of DC_{LV-IL-10} sorted by FACS displayed morphologic and phenotypic characteristics typical of mature DCs. The IL-10 levels secreted by DC_{LV-IL-10} were higher than those secreted by DCs and DC_{GFP}, and were satisfactory to induce a population of Tr1 cells [17]. Subsequently, we incubated naive CD4⁺ T cells with irradiated DC_{LV-IL-10} for 3 days, and found that $51.27 \pm 3.86\%$ of the purified T cells co-expressed CD49b and LAG-3, which are Tr1-related phenotypic markers [10]. Moreover, the Tr1 cells also expressed CD4, CD25 and IL-10, but not Foxp3 [12]. After re-stimulation *in vitro*, the Tr1 cells secreted high levels of IL-10 and IFN- γ , but only low levels IL-4 and IL-2. Taken together, our data suggest that LV-IL-10-transduced DCs are capable of stimulating the differentiation of naive CD4⁺ T cells into Tr1 cells that secrete the characteristic cytokines, IL-10 and IFN- γ [10, 26, 27]. Notably, when Tr1 cells were re-stimulated with the antigen used for their generation, the cells exhibited a significant hyporesponsiveness to re-stimulation. That result indicated that the Tr1 cells were Ag-specific anergic toward the antigen used for their priming [13].

Tregs can suppress the proliferation of CD8⁺ and CD4⁺ T cells in part, by inhibiting IL-2 production, or reducing the effector function of T cells [5, 28, 29]. They can also transfer their suppressive properties to other CD4⁺ T cells, resulting in new CD4 suppressive T cells or T cell anergy [30-32]. Additionally, Tregs can downregulate MHC II, CD80, and CD86 expression on DCs and induce indoleamine 2, 3-dioxygenase (IDO) expression [33, 34].

We performed Tr1-mediated T cell proliferation and MLR inhibition assays to determine whether the Tr1 cells suppressed CD8⁺ or CD4⁺ T cells. Our results showed that DC_{LV-IL-10} induced Tr1 cells could significantly suppress both CD8⁺ T and CD4⁺ T cell proliferation and the MLR *in vitro* in dose-dependent manners. Furthermore, addition of anti-IL-10, but not anti-IFN- γ antibodies to the CD8⁺ T and CD4⁺ T cell cultures assays caused Tr1 cell proliferation to be inhibited by > 90%, suggesting that IL-10 is crucial for the activity of these regulatory T cells.

IL-10 is an immune regulatory cytokine that is indispensable for controlling inflammation, downregulating immune responses, and inducing tolerance [35]. Importantly, IL-10 induces the differentiation of Tr1 cells and long-lasting Ag-specific T-cell anergy in both humans [36] and mice [37]. Although IL-10 is essential for inducing Tr1 cells, when used *in vitro*, its function requires the presence of APCs [15, 38]. Some studies have also shown that T cell priming drives the differentiation of Tr1 cells *in vitro* by immature DC or DCs rendered tolerogenic by biologic or pharmacologic agents [38].

The suppressive function of natural regulatory T cells is Ag-nonspecific [39]. However, we previously demonstrated that CD4⁺CD25⁺ Tregs can acquire Ag-specificity via the uptake of Ag-specific DC-released exosomes (EXO) [12]. In a previous study, CD4⁺CD25⁺ Tregs with acquired pMHC I complexes (Ag-specificity) displayed a greater ability to inhibit Ag-specific immunity than Tregs without pMHC I [12]. Yamazaki et al [40]. demonstrated that under *in vitro* conditions, either Ag-specific DCs or non-specific DCs administered in combination with IL-2 could stimulate the proliferation of fully functional CD4⁺CD25⁺ Tregs. This suggests that cognate Ag-unrelated CD4⁺CD25⁺ Tregs in peripheral blood can also be expanded by incubation with DCs presenting cognate Ag, and that the pMHC I and pMHC II complexes acquired from those DCs by the CD4⁺ Tregs may produce some degree of Ag-specificity.

Sanchez-Fueyo et al [25]. demonstrated that Ag-specific Tregs derived from T cell receptor (TCR)-transgenic mice inhibit immune responses more effectively than do polyclonal Tregs

obtained from non-transgenic mice. Furthermore, Ag-specific Tregs have been shown to be more effective than polyclonal Tregs in preventing graft rejection in humanized pre-clinical models [41, 42]. Therefore, we speculated that during induction of Tr1 cells, DC_{LV-IL-10} derived from recipient mice might present the pMHC I and pMHC II complexes of recipient mice to Tr1 cells derived from donor mice. These Tr1 cells might then suppress the response of donor T cells towards their specific Ag, and thereby induce immune tolerance of donor T cells after an allogeneic BMT. In our study, the recipient mice were infused with BMCs and SCs obtained from donor mice to induce GVHD after a lethal dose of radiation. Infusing recipient mice with DC_{LV-IL-10}-induced donor mouse Tr1 cells remarkably reduced the severity of GVHD. This was shown by the significantly lower histopathological and clinical GVHD scores among the mice treated with Tr1 cells rather than control cells. Furthermore, the transplanted mice in the Tr1-infused groups survived significantly longer than mice in the control group. Additionally, mice infused with 4×10^6 Tr1 cells survived longer than mice infused with 2×10^6 Tr1 cells, indicating that the suppressive effect of Tr1 cells was dose-dependent manner. In conclusion, *ex vivo* induction and expansion of Tr1 cells achieved by co-culturing IL-10 gene-modified DCs with naive CD4⁺ T cells is a promising approach for inducing Tr1 cells, and may be used to facilitate the development of cellular therapies for graft versus-host disease and other autoimmune diseases.

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Disclosure Statement

The authors have no competing interests to disclose.

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