Clinical Immunology (2008) **127**, 313–321

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/yclim

Generation of therapeutic dendritic cells and regulatory T cells for preventing allogeneic cardiac graft rejection

Xusheng Zhang [a,b,c,1], Mu Li [a,b,c,1], Dameng Lian [a,b,c], Xiufen Zheng [a,b,c], Zhu-Xu Zhang [a,b,c], Thomas E. Ichim [a,b,c,d], Xiaoping Xia [a,b,c], Xuyan Huang [a,b,c], Costin Vladau [a,b,c], Motohiko Suzuki [a,b,c], Bertha Garcia [a,b,c,e], Anthony M. Jevnikar [a,b,c,e,f], Wei-Ping Min [a,b,c,e,f,*]

[a] *Department of Surgery, University of Western Ontario, Canada*
[b] *Department of Pathology, University of Western Ontario, Canada*
[c] *Department of Microbiology and Immunology, University of Western Ontario, Canada*
[d] *Medistem Laboratories, San Diego, CA, USA*
[e] *Multi-Organ Transplant Program, London Health Sciences Centre, Canada*
[f] *Lawson Health Research Institute, London, Ontario, Canada*

Received 27 July 2007; accepted with revision 16 January 2008
Available online 20 March 2008

KEYWORDS
Dendritic cells;
T regulatory cells;
Transplant tolerance;
Immune modulation

Abstract Tolerogenic dendritic cells (Tol-DCs) and regulatory T cells (Treg) are key factors in the induction and maintenance of transplantation tolerance. We previously demonstrated that *ex vivo*-isolated Tol-DCs promote Treg generation, and *vice versa*, in an *in vitro* co-culture system. Here we demonstrate the occurrence of such an immune regulatory feedback loop *in vivo*. Tol-DC generated *in vitro* by treatment with LF 15-0195 exhibited features of immature DC and express low levels of MHC class II, CD86 and CD40. These Tol-DCs were capable of augmenting $CD4^+CD25^+CTLA4^+$ and $FoxP3^+$ Treg cell numbers and activity in cardiac allograft recipients. On the other hand, Tol-DCs possessed an ability to generate Treg cells *in vitro*. The adoptive transfer of these *in vitro*-generated Treg cells resulted in an increase of Tol-DC *in vivo*, suggesting that an immune regulatory feedback loop, between Tol-DC and Treg, exists *in vivo*. Furthermore, the administration of *in vitro*-generated Tol-DCs or Treg cells prevented rejection of allografts. Co-administration of Tol-DC and Treg synergized efficacy of promoting allograft survival heart transplantation. The present study highlights the therapeutic potential of preventing allograft rejection using *in vitro*-generated Tol-DCs and Treg.

Abbreviations: DCs, dendritic cells; Tol-DC, tolerogenic T cells; iDC, immature dendritic cells; Treg, regulatory T cells; LF, LF15-0195, an analogue of 15-deoxyspergualine (DSG).

* Corresponding author. 339 Windermere Road, University Hospital C9-136, London, Ontario, Canada N6A 5A5. Fax: +519 663 3295.
E-mail address: weiping.min@uwo.ca (W.-P. Min).
[1] These authors equally contributed to this study.

1521-6616/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.clim.2008.01.013

Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cell, having the unique ability to activate or suppress adaptive immune responses depending on maturation status, phenotype, or tissue of origin [1,2]. "Tolerogenic DC" (Tol-DC) has been assigned as a blanket description of immune regulatory DC that are usually immature [3], plasmacytoid in morphology [4], or alternatively-activated [5,6]. Previous studies have conclusively demonstrated that donor-specific, allogeneic Tol-DCs can enhance the survival of transplanted grafts. Experimental generation of Tol-DC has been accomplished through treatment with maturation-inhibiting agents [2], blockade of costimulatory molecules either with antibodies[7] or antisense oligonucleotides [8], as well as pretreatment with and chemical immunosuppressants [9].

Additionally, expression of the T cell inhibitory molecules PD-1 and PD-2 ligands has also been demonstrated selectively on iDC [10]. The possibility of selectively generating Tol-DC by *in vitro* blockade of DC maturation would allow for the exciting prospect of donor-specific Tol-DC therapy in organ transplantation, as well as antigen-specific tolerance induction for autoimmune diseases.

LF 15-0195 (LF) is a chemically-synthesized analogue of the immune suppressant 15-deoxyspergualin which possesses higher immunosuppressive activity and less *in vivo* degradation than its parent compound [11]. It has been demonstrated that part of the immune suppressive effects of LF are due to activation of caspases in activated T cells [12]. Additionally, our laboratory reported that LF inhibits DC maturation through downregulating NF-κB activity by the selective targeting of the upstream effector molecule I Kappa B kinase (IKK) [13]. To our knowledge, LF is the only immunosuppressive drug that specifically blocks IKK activity. Therefore, the unique ability of LF to target IKK in DCs suggests that it may possess distinctive properties that allow for the generation of immature tolerogenic DCs. We have successfully induced tolerance in transplantation via systemic LF administration [14]. However, it is important to note that LF has significant cytotoxic side effects *in vivo*, thus emphasizing the possible deleterious effects of LF therapy [11]. To avoid such negative side effects, we chose to generate Tol-DC *in vitro*, by treatment with LF, which represents a safer and more clinically-applicable alternative to LF systemic administration.

Currently, it is believed that T regulatory cells (Treg) are a key mechanism of tolerance to self antigens and alloantigens [15–17]. Therefore, the *in vitro* production of Treg is a highly desirable and promising strategy for the treatment of autoimmune diseases and the prevention of transplant rejection. Unfortunately, cellular therapy with $CD4^+CD25^+$ Treg is hampered by the inability to consistently generate and expand antigen-specific suppressors *in vitro* and, subsequently, *in vivo*. Several strategies have been attempted for *ex vivo* propagation of Treg, but a significant pitfall has been that large-scale expansion is associated with a loss of suppressor function [18].

We have previously defined an "immune regulatory feedback loop", in which we observed *in vivo*-isolated Tol-DCs inducing the generation of Treg *in vitro* [19]. Therefore, it appears that the generation of stable and reproducible Tol-DCs could be crucial for the efficient expansion of functional Treg. In the present study, we developed a protocol for generating Tol-DC by treatment of LF and have utilized these Tol-DCs to induce Treg expansion *in vitro*. Tol-DC and Treg generated *in vitro* were capable of significantly prolonging allograft survival in a murine heart transplantation model. We further demonstrated that the *in vitro*-generated Tol-DC reconstitute a regulatory feedback loop *in vivo*, through augmentation of Treg formation. Furthermore, a synergistic effect of promoting allograft survival was achieved by co-administration of these *in vitro*-generated Tol-DC and Treg cells.

Materials and methods

DC cultures and generation of Tol-DC *in vitro*

Bone marrow (BM) cells were flushed from the femurs and tibias of C57/BL6 mice; washed and cultured in 6-well plates (Corning) at 4×10^6 cells/well in 4 ml of a complete medium (RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml of streptomycin, 50 μM 2-ME, and 10% FCS (all from Life Technologies, Canada), supplemented with recombinant GM-CSF (10 ng/ml) and recombinant mouse IL-4 (10 ng/ml) (both from PeproTech, USA). Cultures were incubated at 37 °C in 5% humidified CO_2. Non-adherent cells were removed (Day 2) and fresh medium was added. At Day 4, DCs were treated either with LF (10 μg/ml) or PBS, and fresh medium was added every 24 h At Day 7, DCs were purified using directly conjugated CD11c MACS magnetic beads and passed over a MACS column (Miltenyi Biotec). Purity was checked by FACS and was found to be >90%. Then Purified DCs were activated with LPS (10 ng/ml; Sigma) and TNFα (10 ng/ml; PeproTech) for 24 h, washed extensively, and used for subsequent transfer experiments.

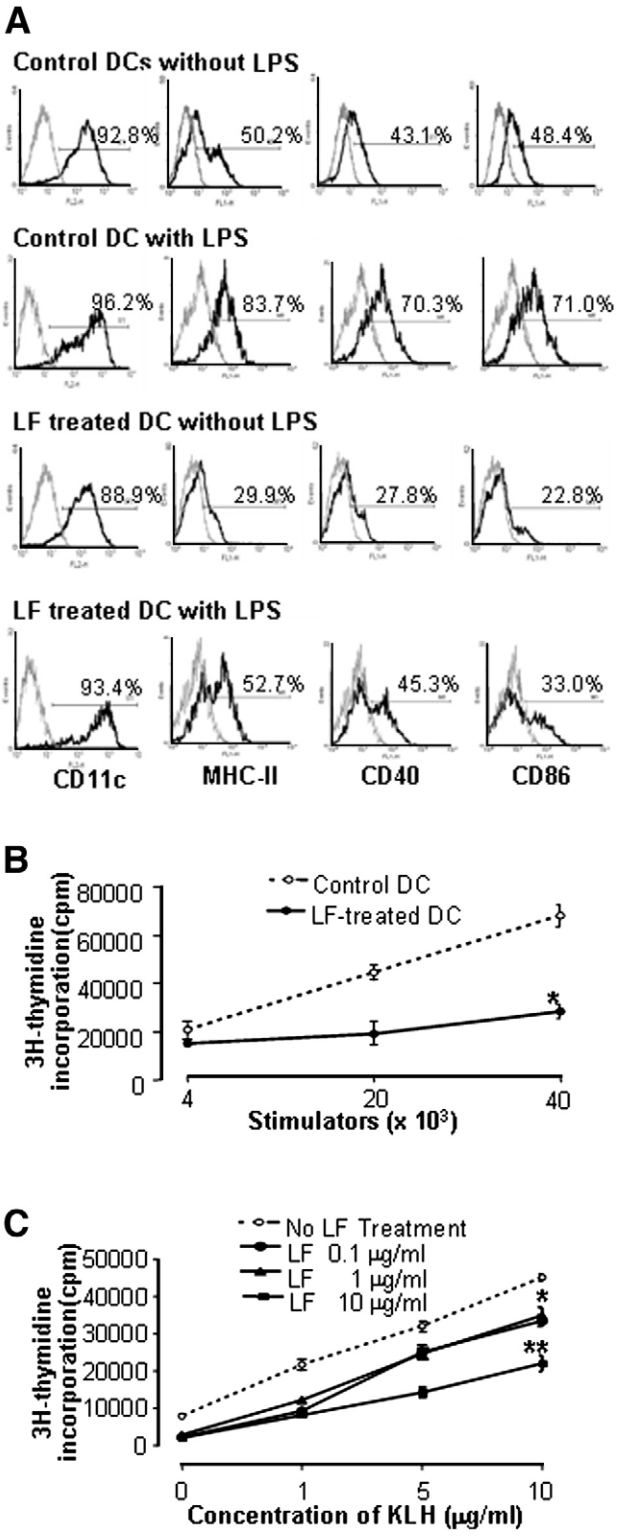
Adoptive transfer of Tol-DCs and heterotopic cardiac transplantation

8–12 week old male C57/BL6 (H-2^b) and BALB/c (H-2^d) mice were purchased from the Jackson Laboratory (Bar Harbour, ME), and used as donors and recipients, respectively. Recipient mice (BALB/c) were treated intravenously (i.v.) with LF-treated or non-treated DCs (5×10^6 cells), 7 days before transplantation. Except for a low dose (2 Gy) of total body irradiation, no immunosuppressants were administered before or after transplantation. Treated and untreated BALB/c mice were subjected to allogeneic cardiac transplantation, using organs from C57/BL6 donors. Direct abdominal palpation was used to assess graft viability. Heterotopic heart transplantation was performed, according to our laboratory's routine procedures [20]. Pulsation of heart grafts was monitored daily by two independent observers who were blinded to the treatment protocol. Grafted recipients that survived more than 100 days were classified as tolerant and were used for *in vitro* experiments. Untreated recipients (BALB/c) were used as rejecting controls.

Mixed leukocyte reaction (MLR)

Splenic DCs isolated from tolerant or rejecting recipients (BALB/c) were irradiated at 30 Gy. T cells (2×10^5/well) from C57/ BL6 mice were added to the DC cultures, with the final MLR taking place in 200 μl of complete RPMI 1640 (Life

Technologies). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 for 3 days, and pulsed with 1 μCi of [3H]thymidine (Amersham Pharmacia Biotech) for the last 16 h of culture. Cells were harvested onto glass fiber filters, and the radioactivity incorporated was quantified using a Wallac Betaplate liquid scintillation counter. Results were expressed as the mean cpm of triplicate cultures ± SEM.



To determine the ability of Treg cells to inhibit a MLR, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were added to an MLR using normal BALB/c $CD4^+$ T cells as responders (2×10^5/well). The irradiated (30 Gy) spleen cells (2×10^5/well) from C57/ BL6 or C3H mice were used as donor-specific and third party stimulators, respectively. The experimental procedure of [3H]thymidine incorporation was the same as described above.

Immunization of mice with peptide-pulsed DCs and antigen-specific T cell response

7-Day cultured DCs were treated with LF or PBS, as described above, and then pulsed with 10 μg/ml of keyhole limpet hemocyanin (KLH) (Sigma-Aldrich Rockford IL) for 24 h. DCs then were activated with LPS+ TNFα for 24 h. Antigen-pulsed DCs (5×10^5 cells/mouse) were injected subcutaneously into syngeneic mice. Mice were sacrificed after 10 days, and cell suspensions were prepared from the draining lymph nodes. These cells were cultured in 96-well plates at a concentration of 2×10^5 cells/well, in the presence or absence of KLH antigen for 3 days. An [^{3}H]thymidine incorporation assay was performed, as described for the MLR.

Flow cytometry

Phenotypic analysis of isolated or cultured DC was performed on a FACScan (Becton Dickinson, San Jose, CA). All antibodies were purchased from BD PharMingen (San Diego, CA), unless

Figure 1 LF prevents DC maturation. A, Phenotypic analysis of LF-treated DCs. DCs were cultured from C57/BL6 bone marrow progenitors in the presence of GM-CSF (10 ng/ml) and IL-4 (10 ng/ml) for 7 days. DCs were treated by the addition of LF (10 μg/ml) in the culture medium from Day 4 onwards, and fresh medium was added every 24 h (LF-treated DC, lower panel) or treated with PBS (control DC, upper panel). DCs were purified by CD11c MACS beads. Control DCs and LF-treated DCs were activated using TNF-α (10 ng/ml) and LPS (10 ng/ml) during the last 24 h of culture. Control DCs and LF-treated DCs and activated control or LF-DCs were stained with FITC or PE-conjugated mAbs and analyzed by flow cytometry. B, LF inhibits DC's allostimulatory capacity in MLR. LF-treated DCs and untreated control DCs were used as stimulators, and were seeded into a 96-well plate. Responders consisted of 2×10^5/well of BALB/c $CD4^+$T cells purified by MACS beads. Stimulators and responders were co-cultured, and proliferation was assessed, as described in Materials and methods. Data shown are the mean ± SEM of three independent experiments. C. LF-treated DCs inhibit anti-KLH T cell responses. DCs were cultured and treated with LF at indicated concentrations (0.1, 1, and 10 μg/ml) or with PBS alone. On Day 7 of culture, 10 μg/ml of KLH was added to the cells for 24 h, and then cells were activated with TNFα/LPS (10 ng/ml). On Day 9 of DC culture, 5×10^5 cells/ mouse were injected *s.c.* into syngeneic C57/BL6 mice. After 10 d, the mice were sacrificed and T cells from lymph nodes were isolated. A KLH-specific recall response was determined by the degree of proliferation, as described in Materials and methods. Lymphocytes were re-stimulated *in vitro* with different concentrations of KLH (1, 5, and 10 μg/ml) or with PBS alone. Data represent mean ± SEM and are representative of three experiments. (*, $p<0.05$; **, $p<0.01$ by one-way ANOVA and Newman–Keuls test).

otherwise indicated. For T cells, we used FITC, PE, or CyChrome conjugated anti-mouse CD4, CD152 (CTLA-4), FoxP3 and CD25 (eBiosciences, San Diego, CA). For DCs, we used FITC, or PE conjugated anti-mouse I-$A^{b,d}$, CD11c, CD40, and CD86 mAb. CD152 expression was assessed by intracellular staining, using a cell permeabilization kit (Caltag Laboratories, Burlingame, CA). Foxp3 expression was assessed by intracellular staining, using Foxp3 Staining Buffer Set (eBiosciences, San Diego, CA). T cell and DC subsets were analyzed by 2 or 3-color staining with various combinations of mAbs. All flow cytometric analyses were performed using appropriate isotype controls (Cedarlane Laboratories, Hornby, Ontario, Canada).

Statistical analysis

Graft survival was compared among experimental groups, using the rank-log test. MLR data were analyzed using one-way ANOVA, followed by the Newman–Keuls Test. Differences with *p*-values less than 0.05 were considered statistically-significant.

Results

Generating Tol-DC *in vitro* by treatment with LF

We have previously demonstrated that LF treatment of isolated DCs *in vitro* is capable of inhibiting the maturation-inducing kinase, IKK, as well as the downstream transcription factor NF-κB [19]. We therefore investigated the potential of LF to generate immature tolerogenic DCs that could be used for *in vivo* antigen-specific immunotherapy. BM-derived DCs were generated using a standard 7-day culture in GM-CSF/IL-4. LF was added at Day 4 of culture, whereas control DCs were treated with PBS instead of LF. Activation of control and LF-treated DCs was performed via the addition of TNFα/LPS for 24 h Assessment of MHC class II, CD40, and CD86 expression by flow cytometry revealed that control DCs underwent marked maturation, whereas LF-treated DC did not up-regulate maturation markers (Fig. 1A). Additional LPS could make the DC more matured (Fig. 1A). However, DC generated in the presence of LF, treated with or without LPS, expressed less maturation marker on the surface compared to control DC. Functional assessment of LF-treated DCs was performed by using these cells as allogeneic stimulators in MLR. In contrast to control DCs that expressed potent allostimulatory activity, LF-treated DCs evoked a much weaker T cell proliferative response (Fig. 1B).

It has previously been reported that antigen-pulsed DCs with a blocked NF-κB pathway can induce specific hyporesponsiveness in an antigen-specific manner [21]. Since we have recently demonstrated that LF blocks NF-κB translocation [13], and have shown here that LF treatment inhibits DC maturation, we sought to assess whether LF-treated DCs could induce tolerance to a nominal antigen, such as KLH. On Day 4 of culture, DCs were treated with 0.1, 1, and 10 μg/ml of LF, and control DCs were treated with PBS. KLH was added to DC at Day 7 for 24 h; subsequently, cells were activated with TNFα/LPS. On Day 9, 5×10^5 DCs were injected subcutaneously into C57/BL6 mice. To test antigen-specific T cell activation, recall response to KLH was assessed *in vitro* 10 days after the administration of KLH-pulsed control and LF-treated DCs. KLH-specific T cell responses were suppressed at all KLH concentrations used, in an LF dose-dependent manner (Fig. 1C), suggesting that LF-treated DCs play regulatory roles *in vivo*.

Preventing allograft rejection using *in vitro*-generated Tol-DC

We previously demonstrated that tolerance induced by LF treatment is associated with the generation of Tol-DCs [22]. However, clinical uses of LF are limited by its toxicity. Above, we have shown that LF-treated DCs can suppress T cell responses *in vitro*. Next, we determined whether LF-treated DCs might have a therapeutic function preventing the rejection of allocardiac grafts. When donor-derived and LF-treated DC were administered to naive recipients 7 days before heart transplantation, survival of the allogeneic heart graft was significantly prolonged, in the absence of any immunosuppressive treatment, as comparing with control DC-treated and untreated groups (Fig. 2). These data imply a potential of preventing graft rejection through DC-based cellular therapy, using *in vitro*-generated Tol-DCs.

Generating $CD4^+CD25^+$ Treg *in vitro* through interaction with Tol-DCs

According to our previous study, *ex vivo*-isolated Tol-DCs are capable of inducing expansion of $CD4^+CD25^+$ Treg cells *in vitro* [19]. Here we aimed to generate Treg using *in vitro*-generated Tol-DCs. When we co-cultured LF-generated Tol-DCs with naive allogeneic CD4T cells, the percentage of $CD4^+CD25^+CTLA4^+$ and $CD4^+CD25^+FoxP3^+$ T cells was significantly increased (Fig. 3A). The regulatory function of these Tol-DC-generated Treg was shown in an inhibitory MLR, in which $CD4^+CD25^+CTLA4^+$ Treg inhibited T cell proliferation against allogeneic stimulation by C57/BL6 cells, whereas no inhibition of T cell proliferation in response to stimulation by

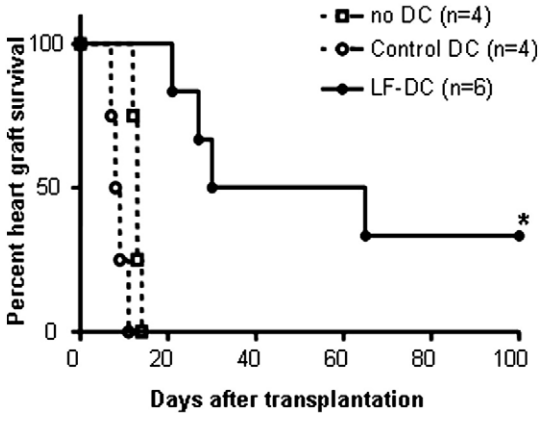


Figure 2 LF-treated DCs prevent allograft rejection. Donor (C57/BL6) derived DCs were cultured and treated with LF, as described in Figure 1. 5×10^6 LF-treated DCs (LF-DC) and PBS-treated DCs (control DC) were injected i.v. through the tail vein of recipients (BALB/c) at Day 7 before transplantation. An allogeneic (C57/BL6 to BALB/c) heart transplantation then was performed. The percentage of grafts surviving after transplantation was displayed (**, $p<0.01$).

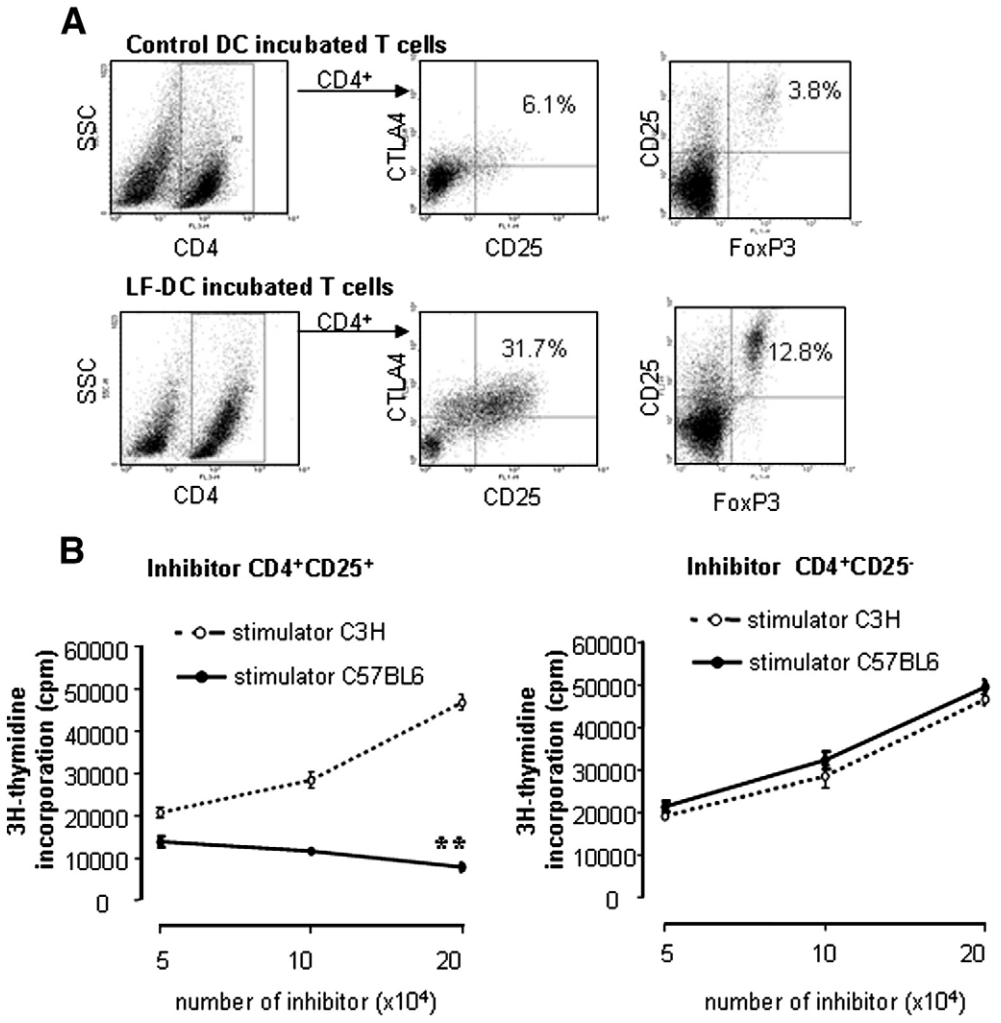


Figure 3 LF-treated DCs generate Treg *in vitro*. A, Phenotypic analysis of *in vitro*-generated Treg. DCs were generated, as described in Figure 1. Donor (C57/BL6) derived LF-treated DCs and control DCs (1×10^6) were incubated with BALB/c T cells (10×10^6) for 7 days. T cells were enriched and stained with Cy5-CD4, PE-CTLA4, and FITC or PE-CD25 and FITC-FoxP3 mAbs. Phenotypes of T cells were analyzed by flow cytometry. B, Assessment of inhibitory function of Treg. LF-treated and untreated C57/BL6 DCs were co-cultured with BALB/c T cells for 7 days. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were isolated, as described in Materials and methods, and added to an ongoing MLR at indicated numbers as described in material and methods. The MLR setting was the same as described in Figure 1B. Data represent mean ± SEM and are representative of three experiments. (**, $p<0.01$ by one-way ANOVA and Newman–Keuls test).

third party (C3H). As a control, $CD4^+CD25^-$ T cells did not inhibit T cell proliferation elicited by C57/BL6 or C3H stimulators (Fig. 3B). These data imply that Treg were generated possessing what appears to be alloantigen specificity.

Preventing allograft rejection using *in vitro*-generated Treg

To verify the *in vivo* protective function of such *in vitro*-generated alloantigen-specific Treg, the cells were administered intravenously into syngeneic BALB/c recipients, following an allogeneic (C57/BL6 to BALB/c) heart transplantation. Treg-treated recipients exhibited significantly prolonged allograft survival (Fig. 4). These data suggest that *in vitro*-generated Treg cells might be an alternative means of cellular therapy by which to prevent graft rejection post transplantation.

Reconstituting an *in vivo* immune regulatory loop using *in vitro*-generated Tol-DCs and Treg

Even though the 'regulatory feedback loop' between Tol-DCs and Treg has been addressed in an *in vitro* co-culture system [19], the *in vivo* interaction between these two types of regulatory cell has not been demonstrated. In order to dissect the mechanism of tolerance is induced by LF-treated Tol-DCs, we examined whether these Tol-DCs augment Treg *in vivo*. In agreement with the *in vitro* experiments [19], we found that adoptively transferring Tol-DCs augmented Treg populations *in vivo* (Fig. 5A).

Additionally, in order to verify the *in vitro* findings that Treg augment the levels of Tol-DC via a suppressive "licensing" event [23], we generated Treg *in vitro*, by means of LF-treated DCs, which resulted in 31.7% $CD4^+CD25^+CTLA4^+$ and 12.8% $CD4^+CD25^+FoxP3^+$ Treg cells (Fig. 3A). These alloantigen-experienced *in vitro*-generated Treg then were

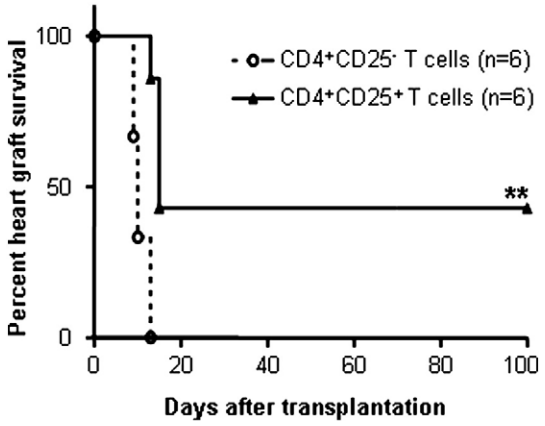


Figure 4 Preventing allograft rejection using *in vitro*-generated Treg cells. $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were generated and isolated, as described above. 2×10^6 $CD4^+CD25^+$ or $CD4^+CD25^-$ cells were injected i.v. through the tail vein of recipients (BALB/c) at Day +1 after transplantation. An allogeneic (C57/BL6 to BALB/c) heart transplant then was performed, as described in Materials and methods. Data demonstrate the percentage of grafts surviving after transplantation (**, $p<0.01$).

transferred to naive syngeneic recipients, followed by an allogeneic cardiac transplantation. Splenic DCs of recipients were assessed for maturation, and a comparison was made between Treg- ($CD4^+CD25^+CTLA4^+$) and control T cell- ($CD4^+CD25^-CTLA4^-$) transferred recipients. After Treg administration, an increased number of DCs expressing the MHC II^{lo}, $CD40^{lo}$ phenotype was observed (Fig. 5B). Furthermore, the purified DCs with low-level expression of MHC-II and CD40 induced weak T cell proliferation; in contrast, normal mature DCs purified from graft-rejected mice elicited a high degree of T cell proliferation (Fig. 5C). These findings support the notions that Tol-DCs can generate Treg, and that the *in vivo* administration of Treg can lead to increased Tol-DC numbers, specifically via the feedback loop we have proposed [19].

Synergistic effects in promoting allograft survival by co-administration of Tol-DCs and Treg

As we previously demonstrated, synergistic transplant tolerance can be achieved by simultaneously targeting DCs and T cells using LF and anti-CD45RB, in a murine allogeneic heart transplantation model [22]. We accordingly explored whether synergistic tolerance can be achieved using *in vitro*-generated Tol-DCs and Treg. We co-administrated Tol-DCs and $CD4^+CD25^+$ Treg cells which had been generated *in vitro*, as described above. With the combination of Tol-DCs and $CD4^+CD25^+$ Treg (Fig. 6), about 70% of the recipients achieved tolerance with allograft survival in excess of 100 days. In contrast, the adoptive transfer of Tol-DCs alone or $CD4^+CD25^+$ Treg alone only induced tolerance of 20% and 40%, respectively (Figs. 2 and 4). These findings are in agreement with the bi-directional regulation model that we demonstrated previously, between Treg and Tol-DCs [19]. Therefore, the interaction between Treg and Tol-DCs, in fact, may enhance the efficacy of tolerance transfer.

Discussion

We have previously demonstrated that subpopulations of Tol-DC exist in transplant tolerant recipients that are capable of inducing *in vitro* generation of Treg, and conversely that freshly isolated Treg induce generation of Tol-DC [19]. Here we have expanded on these experiments by demonstrating that: 1) *in vitro*-generated Tol-DC are capable of prolonging

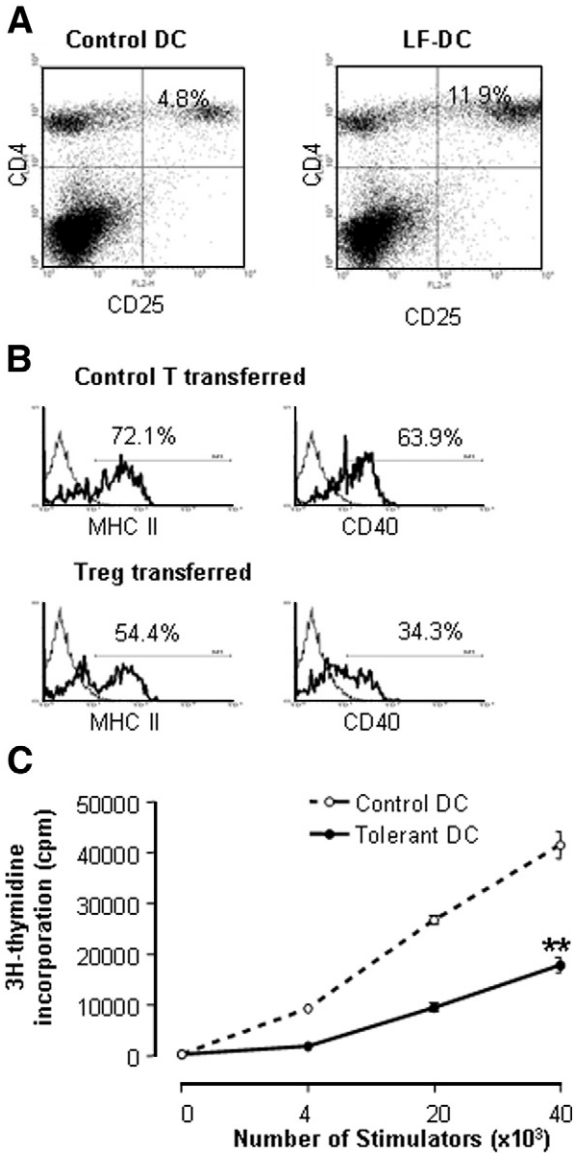


Figure 5 Treg induce Tol-DCs *in vivo*. (A) Adoptive transfer of Tol-DCs augmented Treg populations *in vivo*. DCs were cultured and treated with LF as described in Figure 1, and then adoptively transfer to the recipients as described in Figure 2. After transferred LF-treated Tol-DCs and control DCs to mice, spleen cells will be isolated to assess $CD4^+CD25^+$ Treg population. (B and C) Adoptive transfer of Treg induced Tol-DC *in vivo*. DCs from tolerant recipients, recovered from Figure 4, were isolated using $CD11^+$ MACS beads. The phenotypic analysis of DCs and functional assessment, using flow cytometry (B) and MLR (C), respectively, were performed, as described in Figure 1 (**, $p<0.01$).

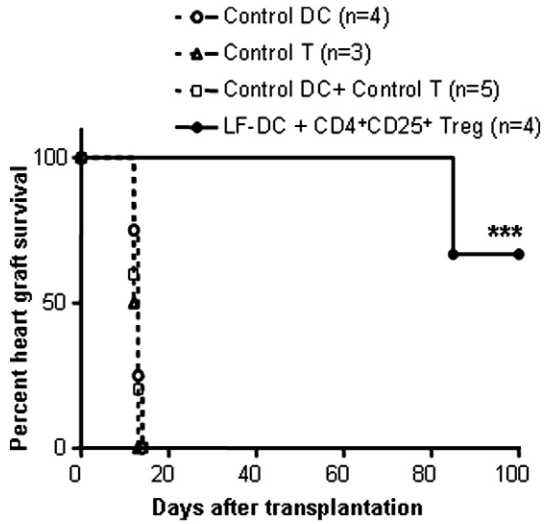


Figure 6 Synergistic tolerance induced by *in vitro*-generated LF-DCs and Treg. LF-DCs and Treg were generated, as described in Figures 1 and 3, respectively. LF-treated DCs (5×10^6 cells, i.v., day −7 and day +1) were co-administered to recipients with *in vitro*-generated $CD4^+CD25^+$ Treg cells (2×10^6, i.v., Day +1), followed by allogeneic (C57/BL6 to BALB/c) heart transplantation. Control DCs were non-LF treated DCs. Control T cells were LF-DC incubated $CD4^+CD25^-$ T cells. Data demonstrate the percentage of grafts surviving after transplantation (***, $p<0.001$).

allograft survival and augmenting *in vivo* numbers of Treg cells; 2) Treg cells can be generated *in vitro* by Tol-DC co-culture and adoptive transfer of these Treg cells prolong allograft survival; and 3) Co-administration of *in vitro*-generated Tol-DC and Treg synergistically enhance allograft survival.

Transplantation tolerance is viewed as an active process that is dependent upon functional Treg cells. The main subset of $CD4^+$ Treg cells is comprised of $CD4^+CD25^+$ $Foxp3^+$ T cells and type 1 regulatory (Tr1) cells, both of which are essential for the maintenance of peripheral tolerance [24]. Previous studies have demonstrated that functional inactivation of $CD4^+CD25^+$ cell suppressive activity by GITR-ligand administration [25], or antibody mediated depletion [26] of these cells leads to an abrogation of transplant tolerance. Furthermore, administration of *ex vivo* expanded Treg specific to allogeneic MHC peptides has been demonstrated to result in prolonged allograft survival [27]. Unfortunately, to date, the wide-scale expansion of antigen-specific Tregs has not been performed in a scenario that can be clinically translated. In our previous studies we have observed that the Tol-DC isolated from tolerant recipients possessed a low expression of MHC, as well as costimulatory molecules, features that mimicked artificially-generated Tol-DC using LF treatment [22,28]. Since our data, and that of other groups [24,29], suggested that Tol-DC have the ability to promote Treg generation, we therefore questioned whether it may be possible to substitute *in vivo* derived Tol-DC with "artificial" LF-generated Tol-DC population. Indeed we observed that co-culture of LF-generated Tol-DC with naive T cells was capable of expanding a population of $CD4^+CD25^+$ $FoxP3^+$, $CTLA\text{-}4^+$ cells (Fig. 3) that exerted T cell suppressing activity (Fig. 3B) and that administration of these Tol-DC cells *in vivo* also was expanded a similar Treg cell population. Functionally, the administration of Treg generated *in vitro* to transplant recipients was able to prolong allograft survival after adoptive transfer (Fig. 4).

The generation of Treg using Tol-DC has previously been performed by other groups, for example, rapamycin-generated Tol-DC were able to induce a Treg population that prolonged allograft survival [30]. Further, Treg generated by auto-antigen-pulsed Tol-DC were capable of reversing hyperglycemia in an animal model of diabetes [31]. These studies, together with our current data, support the existence of immunoregulatory feedback loop from the sense that Tol-DC can effectively generated Tregs. The converse situation of Treg generating Tol-DC, is seen in our previous *in vitro* data [19], as well as in our current data in which *in vivo* Tol-DC generation *were* observed following Treg administration (Fig. 5).

While T cell regulation by DC is widely established, regulation of DC by T cells is an area of intense investigation. From the stimulatory perspective, T cells elaborate various membrane bound and soluble signals that induce DC maturation such as CD40 ligand and various cytokines, respectively [32,33]. The *in vivo* control of DC maturation by T cells is supported by studies showing that T cell deficient animals possess a relatively immature DC compartment [34,35]. Evidence exists that Treg cells possess inhibitory activity on DC maturation *in vivo*. For example, in a highly-defined antigen-specific system, Bluestone's group utilized two-photon laser-scanning microscopy to analyze lymph node priming of diabetogenic T cells *in vivo*. They reported that Treg inhibition of islet antigen reactive cells occurred not through direct contact between Treg and T helper cells, but by Treg "swarming" DC in the lymph node and inhibiting ability to prime the T helper cell [36]. These data support the concept that in general, Treg cells inhibit effector T cell function not through direct contact, but through inhibition of DC function in a highly localized environment. Further support for Treg modulation of DC function *in vivo* are experiments demonstrating that $CD4^+CD25^+$ Treg act as a 'brake' to DC maturation, whose inhibitory influence can only be released by potent innate activators, such as the CpG stimulation of TLR-9 [37]. Furthermore, such inhibitory "licensing" of DCs by T cells was demonstrated in an oral tolerance model by Matzinger's group. They showed that TGF-β-expressing T cells were capable of bestowing upon DCs the ability to generate new antigen-specific inhibitory T cells from naive T cells [23]. Accordingly, our current data support the possibility that Treg cells maintain a suppressive effect on DC maturation and in some conditions actually promote Tol-DC generation.

In summary, we report a practical method of generating donor-derived Tol-DC *in vitro*. The administration of *in vitro*-generated Tol-DCs prolonged allograft survival in a murine heart transplant model. Furthermore, the co-administration of *in vitro*-generated Tol-DCs and Treg synergized promotion of allograft survival. Our findings that Tol-DCs augmented Treg cells *in vivo*, and that adoptively transferring Treg can increase Tol-DC numbers *in vivo*, suggest that an immune feedback loop is formed. These findings support the development of therapeutic approaches concurrently promoting Tol-DC and Treg generation for clinical conditions ranging from organ transplantation to autoimmunity.

Acknowledgments

We thank Dr. Gill Strejan for his critical comments and suggestions. This study was supported by the Heart and Stroke Foundation of Canada and by a grant from the Multi-Organ Transplant Program at the London Health Sciences Centre.

References

[1] R. Lechler, W.F. Ng, R.M. Steinman, Dendritic cells in transplantation--friend or foe? Immunity 14 (2001) 357–368.
[2] R.M. Steinman, D. Hawiger, M.C. Nussenzweig, Tolerogenic dendritic cells, Annu. Rev. Immunol. 21 (2003) 685–711.
[3] K. Mahnke, E. Schmitt, L. Bonifaz, A.H. Enk, H. Jonuleit, Immature, but not inactive: the tolerogenic function of immature dendritic cells, Immunol. Cell Biol. 80 (2002) 477–483.
[4] M. Gilliet, Y.J. Liu, Human plasmacytoid-derived dendritic cells and the induction of T-regulatory cells, Hum. Immunol. 63 (2002) 1149–1155.
[5] D.L. Roelen, D.H. Schuurhuis, D.E. van den Boogaardt, K. Koekkoek, P.P. van Miert, J.J. van Schip, S. Laban, D. Rea, C.J. Melief, R. Offringa, F. Ossendorp, F.H. Claas, Prolongation of skin graft survival by modulation of the alloimmune response with alternatively activated dendritic cells, Transplantation 76 (2003) 1608–1615.
[6] H. Hackstein, C. Steinschulte, S. Fiedel, A. Eisele, V. Rathke, T. Stadlbauer, T. Taner, A.W. Thomson, H. Tillmanns, G. Bein, H. Holschermann, Sanglifehrin a blocks key dendritic cell functions *in vivo* and promotes long-term allograft survival together with low-dose CsA, Am. J. Transplant. 7 (2007) 789–798.
[7] D. Homann, A. Jahreis, T. Wolfe, A. Hughes, B. Coon, M.J. van Stipdonk, K.R. Prilliman, S.P. Schoenberger, M.G. von Herrath, CD40L blockade prevents autoimmune diabetes by induction of bitypic NK/DC regulatory cells, Immunity 16 (2002) 403–415.
[8] X. Liang, L. Lu, H. Zhang, T. Vickers, J.J. Fung, S. Qian, Phenotype and allostimulatory function of dendritic cells treated with antisense oligodeoxyribonucleotides targeting CD80 or CD86 mRNA, Transplant. Proc. 33 (2001) 235.
[9] C. Lagaraine, Y. Lebranchu, Effects of immunosuppressive drugs on dendritic cells and tolerance induction, Transplantation 75 (2003) 37S–42S.
[10] C. Chen, Q.X. Qu, J.A. Huang, Y.B. Zhu, Y. Ge, Q. Wang, X.G. Zhang, Expression of programmed-death receptor ligands 1 and 2 may contribute to the poor stimulatory potential of murine immature dendritic cells, Immunobiology 212 (2007) 159–165.
[11] D. Zhou, C. O'Brien, J. Shum, B. Garcia, W. Min, A.M. Jevnikar, P. Dutartre, R. Zhong, LF 15-0195, a novel immunosuppressive agent prevents rejection and induces operational tolerance in a mouse cardiac allograft model, Transplantation 76 (2003) 644–650.
[12] P. Ducoroy, O. Micheau, S. Perruche, L. Dubrez-Daloz, D. de Fornel, P. Dutartre, P. Saas, E. Solary, LF 15-0195 immunosuppressive agent enhances activation-induced T-cell death by facilitating caspase-8 and caspase-10 activation at the DISC level, Blood 101 (2003) 194–201.
[13] J. Yang, S.M. Bernier, T.E. Ichim, M. Li, X. Xia, D. Zhou, X. Huang, G.H. Strejan, D.J. White, R. Zhong, W.P. Min, LF15-0195 generates tolerogenic dendritic cells by suppression of NF-kappaB signaling through inhibition of IKK activity, J. Leukoc. Biol. 74 (2003) 438–447.
[14] T.E. Ichim, R. Zhong, W.P. Min, Prevention of allograft rejection by *in vitro*-generated tolerogenic dendritic cells, Transpl. Immunol. 11 (2003) 295–306.
[15] R.S. McHugh, E.M. Shevach, The role of suppressor T cells in regulation of immune responses, J. Allergy Clin. Immunol. 110 (2002) 693–702.
[16] K.J. Wood, S. Sakaguchi, Regulatory T cells in transplantation tolerance, Nat. Rev., Immunol. 3 (2003) 199–210.
[17] S. Sakaguchi, Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self, Nat. Immunol. 6 (2005) 345–352.
[18] P.A. Taylor, C.J. Lees, B.R. Blazar, The infusion of *ex vivo* activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality, Blood 99 (2002) 3493–3499.
[19] W.P. Min, D. Zhou, T.E. Ichim, G.H. Strejan, X. Xia, J. Yang, X. Huang, B. Garcia, D. White, P. Dutartre, A.M. Jevnikar, R. Zhong, Inhibitory feedback loop between tolerogenic dendritic cells and regulatory T cells in transplant tolerance, J. Immunol. 170 (2003) 1304–1312.
[20] N. Parry, A.I. Lazarovits, J. Wang, B. Garcia, P. Luke, S. Poppema, R. Zhong, Cyclosporine inhibits long-term survival in cardiac allografts treated with monoclonal antibody against CD45RB, J. Heart Lung Transplant. 18 (1999) 441–447.
[21] M. Nouri-Shirazi, E. Guinet, Direct and indirect cross-tolerance of alloreactive T cells by dendritic cells retained in the immature stage, Transplantation 74 (2002) 1035–1044.
[22] W.P. Min, D. Zhou, T.E. Ichim, X. Xia, X. Zhang, J. Yang, X. Huang, B. Garcia, P. Dutartre, A.M. Jevnikar, G.H. Strejan, R. Zhong, Synergistic tolerance induced by LF15-0195 and anti-CD45RB monoclonal antibody through suppressive dendritic cells, Transplantation 75 (2003) 1160–1165.
[23] O. Alpan, E. Bachelder, E. Isil, H. Arnheiter, P. Matzinger, "Educated" dendritic cells act as messengers from memory to naive T helper cells, Nat. Immunol. 5 (2004) 615–622.
[24] R. Bacchetta, S. Gregori, M.G. Roncarolo, CD4+ regulatory T cells: mechanisms of induction and effector function, Autoimmun. Rev. 4 (2005) 491–496.
[25] A. Bushell, K. Wood, GITR ligation blocks allograft protection by induced CD25+CD4+ regulatory T cells without enhancing effector T-cell function, Am. J. Transplant 7 (2007) 759–768.
[26] W. Li, K. Carper, Y. Liang, X.X. Zheng, C.S. Kuhr, J.D. Reyes, D.L. Perkins, A.W. Thomson, J.D. Perkins, Anti-CD25 mAb administration prevents spontaneous liver transplant tolerance, Transplant. Proc. 38 (2006) 3207–3208.
[27] D. Golshayan, S. Jiang, J. Tsang, M.I. Garin, C. Mottet, R.I. Lechler, *In vitro*-expanded donor alloantigen-specific CD4+CD25+ regulatory T cells promote experimental transplantation tolerance, Blood 109 (2007) 827–835.
[28] X. Xia, X. Zhang, X. Huang, M. Li, L. Zhao, D. Tian, R. Zhong, W.P. Min, LF 15-0195 generates synergistic tolerance by promoting formation of CD4+CD25+CTLA4+ T cells, J. Immunother. (1997) 28 (2005) 560–563.
[29] S.F. Yates, A.M. Paterson, K.F. Nolan, S.P. Cobbold, N.J. Saunders, H. Waldmann, P.J. Fairchild, Induction of regulatory T cells and dominant tolerance by dendritic cells incapable of full activation, J. Immunol. 179 (2007) 967–976.
[30] H.R. Turnquist, G. Raimondi, A.F. Zahorchak, R.T. Fischer, Z. Wang, A.W. Thomson, Rapamycin-conditioned dendritic cells are poor stimulators of allogeneic CD4+ T cells, but enrich for antigen-specific Foxp3+ T regulatory cells and promote organ transplant tolerance, J. Immunol. 178 (2007) 7018–7031.
[31] K.V. Tarbell, L. Petit, X. Zuo, P. Toy, X. Luo, A. Mqadmi, H. Yang, M. Suthanthiran, S. Mojsov, R.M. Steinman, Dendritic cell-expanded, islet-specific CD4+ CD25+ CD62L+ regulatory T cells restore normoglycemia in diabetic NOD mice, J. Exp. Med. 204 (2007) 191–201.
[32] A. Volkmann, J. Neefjes, B. Stockinger, A conditionally immortalized dendritic cell line which differentiates in contact with T cells or T cell-derived cytokines, Eur. J. Immunol. 26 (1996) 2565–2572.
[33] T. Kitajima, G. Caceres-Dittmar, F.J. Tapia, J. Jester, P.R. Bergstresser, A. Takashima, T cell-mediated terminal maturation of dendritic cells: loss of adhesive and phagocytotic capacities, J. Immunol. 157 (1996) 2340–2347.

[34] V. Shreedhar, A.M. Moodycliffe, S.E. Ullrich, C. Bucana, M.L. Kripke, L. Flores-Romo, Dendritic cells require T cells for functional maturation *in vivo*, Immunity 11 (1999) 625–636.
[35] S. Grabbe, R.L. Gallo, A. Lindgren, R.D. Granstein, Deficient antigen presentation by Langerhans cells from athymic (nu/nu) mice. Restoration with thymic transplantation or administration of cytokines, J. Immunol. 151 (1993) 3430–3439.
[36] Q. Tang, J.Y. Adams, A.J. Tooley, M. Bi, B.T. Fife, P. Serra, P. Santamaria, R.M. Locksley, M.F. Krummel, J.A. Bluestone, Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice, Nat. Immunol. 7 (2006) 83–92.
[37] P. Serra, A. Amrani, J. Yamanouchi, B. Han, S. Thiessen, T. Utsugi, J. Verdaguer, P. Santamaria, CD40 ligation releases immature dendritic cells from the control of regulatory CD4+ CD25+ T cells, Immunity 19 (2003) 877–889.