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ORIGINAL ARTICLE

Diltiazem induces regulatory T cells *in vitro* by modulating human dendritic cell maturation

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Summary

Diltiazem is a calcium channel antagonist that has been commonly associated with currently used immunosuppressants to prevent acute graft rejection in humans. In this study, we examined the possibility that diltiazem may affect human dendritic cell (DC) functions in response to lipopolysaccharide (LPS) stimulation and may induce the generation of DC with the capacity to generate $CD4^+$ regulatory T cells (Tregs). Blood monocytes were cultured in the presence of diltiazem at the beginning of their differentiation process into DC. Monocyte-derived DCs were stimulated with LPS, and DCs differentiated in the presence of diltiazem showed a decreased interleukin (IL)-12 production and an enhanced IL-10 production. When cultured with $CD4^+CD45RA^+$ they were able to enhance the $CD4^+Foxp3^+$ T-cell population and to induce slowly proliferating T cells, which showed a significant increase of transforming growth factor (TGF)-β production. These T cells suppress proliferation of activated autologous T cells, and we show that this effect is attributable to soluble factors, primarily to TGF-β. Blockade of TGF-β by specific monoclonal antibodies reversed this inhibitory effect. Herein, we provide new evidence that diltiazem-conditioned monocyte-derived DC induce T cells which acquire a regulatory phenotype and activity similar to those described for Tregs.

Introduction

Diltiazem is a calcium channel antagonist widely used in clinical practice. Many of its effects make this drug particularly useful in organ transplantation. In fact, it is relatively safe, has a useful antihypertensive action, gives protection against cyclosporine nephrotoxicity and may even confer a survival advantage [1]. For these reasons, it has been used as the cyclosporine-sparing agent in renal and cardiac transplant patients [2]. More recently, it has been shown that diltiazem shows a similar sparing effect with tacrolimus administration also [3].

In addition to its vasodilatory effects on blood vessels, various biological and metabolic actions of diltiazem have been reported recently. They include protection of the myocardium and liver against ischaemia–reperfusion (I/R) injury in animal models [4–6], anti-inflammatory actions [7,8], and neurological protection during I/R of the spinal cord in experimental animals [9].

Numerous investigators have demonstrated that diltiazem inhibits lymphocyte functions and depresses the immune response [10–13]. We have previously shown that diltiazem affects maturation and functions of human monocyte-derived dendritic cells (MDDC), and decreases their interleukin (IL)-12 production [14]. We also demonstrated that the inhibition of IL-12 production in diltiazem-treated MDDC is caused by the reduction of nuclear factor-kB transcriptional activity [15].

Dendritic cells (DC) are professional antigen-presenting cells, which in their immature form continuously patrol their microenvironment in peripheral tissues for invading pathogens. On pathogen encounter, immature DCs

develop into mature, immunostimulatory DCs. In addition to the polarization of conventional Th1 and Th2 cells, DC is able to generate adaptive regulatory T cells (Tregs). A growing body of evidence indicates that adaptive Tregs can develop in the periphery from uncommitted naïve or memory T cells triggered by DCs [16–21]. In particular, $CD4^+$ Tregs differentiate in the periphery from naïve precursors stimulated by DCs in the presence of IL-10 and ultimately regulate T-cell response via their ability to produce IL-10 [22,23]. A series of studies have shown that transforming growth factor (TGF)-β also is critical in generating, maintaining, and expanding the *in vivo* pool of $CD4^+CD25^+$ Treg cells via Foxp3 modulation and/or induction, as well as in mediating *in vivo* suppressive function [24–27]. Thus, TGF-β seems to be critical both in the maintenance of the function of naturally occurring Treg derived from thymus (natural Treg), and in the induction of Treg induced from Foxp3 $CD4^+$ T cells in the periphery (induced Treg) [28].

It is not yet established whether it is the natural Tregs or the induced Tregs that are more important in transplantation tolerance. Nonetheless, Treg cells, which are easily enriched on the basis of their cell surface markers, have been shown to suppress allospecific effector T cells and prevent allograft rejection [29–32]. Arising out of the issues contained in these observations, the manipulation of Treg cells has lately attracted considerable attention with respect to transplantation.

The aim of this study was to investigate whether diltiazem-treated MDDC might induce Treg cells. Our findings show that diltiazem-treated MDDC are capable of promoting the induction of slowly proliferating T cells, which show a significantly enhanced production of TGF-β, a slight increase of IL-10, and which have the ability to suppress T-cell proliferation of autologous T cells. Thus, T cells induced by diltiazem–conditioned MDDC acquire a regulatory phenotype and a functional behaviour similar to those described for Treg cells.

Materials and methods

Chemical and reagent

Diltiazem hydrochloride (Dilzene; Sigma-tau, Rome, Italy) was supplied as a powder, dissolved in culture medium and used at 10^{-4} M concentration. A possible cytotoxic effect of the drug was excluded using evaluation of cell viability with trypan blue and propidium iodide exclusion.

Human peripheral blood mononuclear cell isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated by the Ficoll–Hypaque method from buffy-coats from healthy voluntary blood donors, courtesy of the Transfusional Medicine Department, Policlinico Umberto I, University 'La Sapienza', Rome.

All human studies were approved by the appropriate ethics committee and were performed in accordance with the ethical standards set down in the 1964 declaration of Helsinki. All participants gave their informed consent prior to their inclusion in the study.

Generation of MDDC from peripheral blood of healthy donors

The method for *in vitro* culture of human DCs has been already described [33]. Briefly, monocytes were isolated from PBMCs by magnetic activated cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) using anti-CD14 microbeads. The recovered monocytes, that were 95% pure as shown by flow cytometry with an anti-CD14 fluorescent antibody (Fig. 1a), were cultured at 5×10^5/ml in RPMI 1640 (Bio Whittaker, Walkersville, MD, USA), supplemented with 15% FCS (Hyclone, South Logan, UT, USA), 1% L-glutamine, and 1% penicillin/streptomycin (SIGMA Aldrich, St. Louis, MO, USA) containing 50 ng/ml of GM-CSF (Peprotech Inc, Rocky Hill, NY, USA) and 1000 U/ml of recombinant human IL-4 (R&D System Inc., Minneapolis, MN, USA) at 37 °C and 5% CO_2 for 5 days. Diltiazem at 10^{-4} M was added at day 0 of culture. At day 5, after monocyte differentiation in immature DC (MDDC) (>90% $CD1a^+CD14^-$ cells, analysed by flow cytometry) (Fig. 1a), cells, treated or not with diltiazem, were stimulated with 1 μg/ml lipopolysaccharide (LPS) from *Escherichia coli* (SIGMA Aldrich) (LPS-MDDC and dil-LPS-MDDC respectively) for additional 24 h to induce their maturation. The phenotype of LPS-MDDC and dil-LPS-MDDC was analysed for CD40, HLA-A,-B-C, HLA-DR, CD80, CD86, and CD83 expression by flow cytometry (Fig. 1b). At the end of the culture period, cell supernatants were collected and analysed for IL-10 and IL-12p70 production.

T-cell culture

$CD4^+CD45RA^+$ lymphocytes were positively selected from human peripheral blood of healthy donors using the $CD4^+$ isolation kit II followed by CD45RA magnetic beads (MACS; Miltenyi Biotec) according to the manufacturer's instructions. Recovered $CD4^+CD45RA^+$ cells were more than 95% pure as indicated by flow cytometry (Fig. 1c). Purified T cells were resuspended at the concentration of 1×10^6/ml in RPMI 1640, supplemented with 10% of FCS, 1% L-glutamine, and 1% penicillin/streptomycin (SIGMA Aldrich) (complete medium) and cultured for 7 days with allogeneic LPS-MDDC or dil-LPS-MDDC at the ratio of 10:1. After this period, T cells stimulated

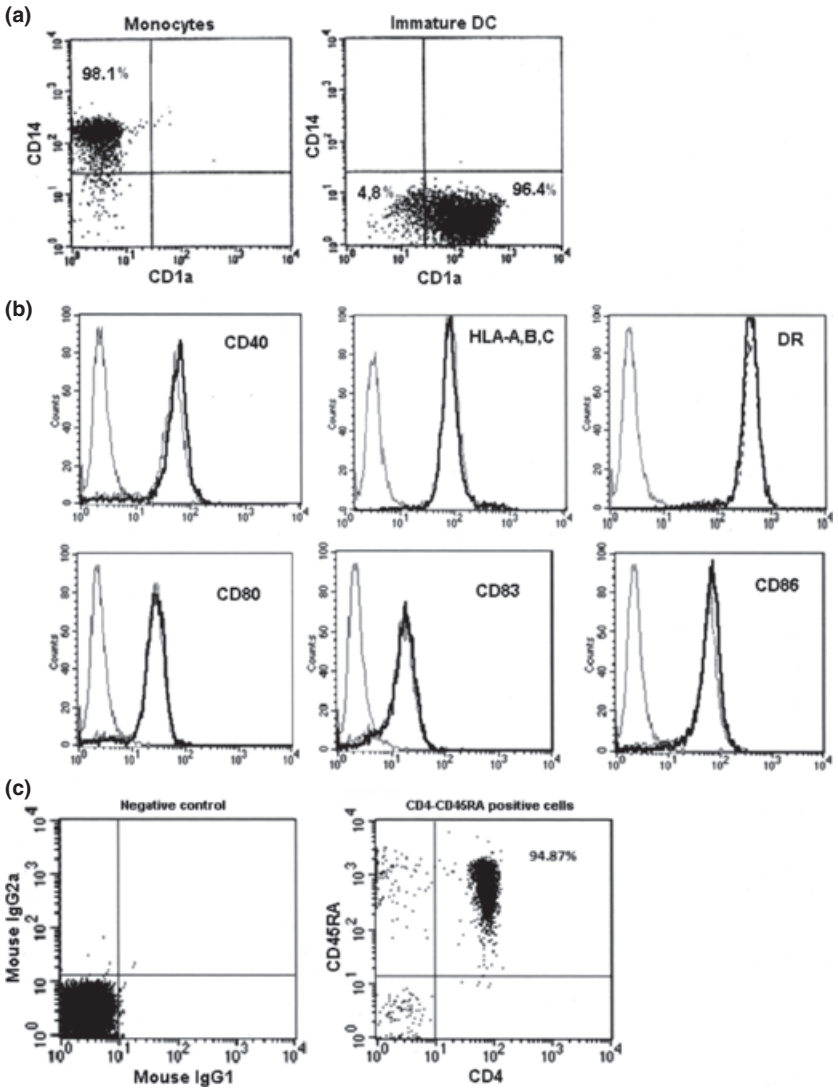

Figure 1 (a) Percentages of CD14 and CD1a expression on monocytes freshly isolated from PBMC (left) and on immature DC analysed after 5 days of culture (right). (b) Expression of surface molecules on LPS-MDDC: black line; and on dil-LPS-MDDC: dotted line. The grey line represents the negative control. (c) Cell purity of $CD4^+CD45RA^+$ T cells after magnetic beads purification.

with LPS-MDDC ($T_{LPS\text{-}MDDC}$) and T cells stimulated with dil-LPS-MDDC ($T_{dil\text{-}LPS\text{-}MDDC}$) were treated with 10 U\ml IL-2 (Roche Diagnostic GmbH, Germany), and left in culture for a further week. At day 14, cells were collected, washed and used for different assays.

Analysis of T-cell function (suppression assay)

To investigate $T_{dil\text{-}LPS\text{-}MDDC}$ suppressive capacity, $T_{LPS\text{-}MDDC}$ cells were cultured in a 96-well plate, alone or in the presence of 25%, 50% or 75% of $T_{dil\text{-}LPS\text{-}MDDC}$. The total number of T cells per well was 2×10^5. To induce activation, T cells were stimulated with LPS-MDDC from the same donor used for the $CD4^+CD45RA^+$ T-cell priming at 10:1 ratio for 4 days. In some experiments, 50 μg/ml of anti-TGF-β1, -β2, -β3 monoclonal antibodies (mAbs) (R&D System Inc.) were added at the beginning of the culture period. Cells were pulsed with methyl ^{3}H-thymidine (Amersham-GE Healthcare, Milano, Italy), 18 h before the end of the culture period. ^{3}H-thymidine incorporation was measured in a LKB Beta-plate liquid scintillation counter (Wallac- Perkin Helmer, Waltham, MA, USA). Assays were performed in triplicate and the results were expressed as mean counts per minute (c.p.m.) ± standard error of the mean (SEM).

In order to characterize $T_{dil\text{-}LPS\text{-}MDDC}$ cells, an intracytoplasmic staining for Foxp3 was performed according to the manufacturer's instructions.

Supernatant collection and assay to verify their suppressive capacity

$T_{LPS\text{-}MDDC}$ and $T_{Dil\text{-}LPS\text{-}MDDC}$, at day 14 of culture, were harvested, washed, and stimulated with LPS-MDDC from the same donor used for the $CD4^+CD45RA^+$ T-cell priming at 10:1 ratio. After 6, 12, 24, 48 or 72 h, supernatants were collected and tested in ELISA for IL-10 and TGF-β1 presence. To assess whether the cytokines present in the culture were responsible for the suppressive capacity of $T_{dil\text{-}LPS\text{-}MDDC}$, 50 μl of collected supernatants were added to CD45RA purified third party T cells stimulated with plate-bound anti-human CD3ε (10 μg/ml) (R&D Systems Inc.) and soluble anti-human CD28 mAbs (2 μg/ml) (R&D Systems Inc.). As most cultured cells secrete TGF-β in an inactive form [34], supernatants underwent an acid treatment (1 N HCl for 10 min and then neutralized by 1.2 N NaOH/0.5 mol/l HEPES) in order to release active TGF-β. After 48 h of culture, the proliferative capacity was assessed by ^3H thymidine incorporation.

Staining with mAbs

Cell staining was performed using the following mouse anti-human mAbs: HLA-DR, HLA-A,B,C, CD80, CD86, CD83, CD4, CD45 RA (Pharmingen, Becton-Dikinson Italia, Milano, Italy) and Foxp3 (eBioscience, Dan Diego, CA, USA). Percentages of positive cells for the specific molecules were analysed using a Bioscience FACScan flow cytometer (FACS) and CellQuest software (Pharmigen, Becton–Dickinson). Propidium iodide staining was used to exclude dead cells.

Cytokine assays

Cytokine content in the culture supernatants was measured by sandwich ELISA using commercially available kits: IL-10, IL-12p70, and TGF-β1 ELISA kits (R&D Systems Inc.). Optical densities were measured on a Bio-Rad Novapath ELISA reader and the results were expressed as picograms per millilitre ± SEM.

Statistical analysis

Results were expressed as mean ± SEM. Statistical analysis was performed using Student's *t*-test for paired data. *P*-values < 0.05 were considered significant.

Results

Diltiazem treatment affects maturation of human MDDC

In this study, we confirmed our previous findings on the ability of diltiazem to affect DC maturation, by reducing significantly their IL-12 production in response to LPS-induced maturation [14]. At the same time, a consistent increase of IL-10 production has been observed (Fig. 2). No statistically significant differences were found for MHC class I and II molecules, CD80, CD86, CD40, and CD83 expression in DC treated with diltiazem, when compared with untreated DC (Fig. 1b). These results suggest that diltiazem is able to prevent the LPS-induced maturation of MDDC, which acquire characteristics that resemble those of tolerogenic DC [35].

Diltiazem-treated MDDCs induce the generation of $CD4^+$ T cells with regulatory properties

To investigate the effect of dil-LPS-MDDC on T cells, allogeneic $CD4^+CD45RA^+$ T cells were cultured with dil-LPS-MDDC ($T_{dil\text{-}LPS\text{-}MDDC}$) or LPS-MDDC ($T_{LPS\text{-}MDDC}$) for 1 week. IL-2 was then added and cells cultured for another week. Cells were collected, washed, and the expression of Foxp3 was investigated. As shown in Fig. 3, treatment with diltiazem increases the $CD4^+Foxp3^+$ population.

Cells were then stimulated with LPS-MDDC, and analysed for proliferative capacity. $T_{dil\text{-}LPS\text{-}MDDC}$ showed a significantly reduced proliferative capacity when compared with $T_{LPS\text{-}MDDC}$ (Fig. 4a). To test the regulatory activity of $T_{dil\text{-}LPS\text{-}MDDC}$, the two populations were mixed at 25%, 50% and 75% ratios of $T_{dil\text{-}LPS\text{-}MDDC}$. We observed a

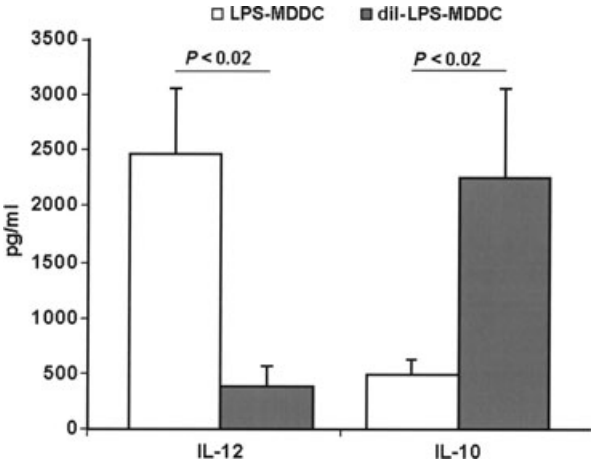

Figure 2 Diltiazem consistently inhibits IL-12p70 production by LPS-MDDC, whereas increases IL-10 production. Monocytes isolated from PBMC were cultured in the presence of GM-CSF and recombinant human IL-4 for 5 days. Diltiazem at 10^{-4} M was added at day 0 of culture. At day 5, monocytes were stimulated with 1 μg/ml LPS for additional 24 h. At the end of the culture period, cell supernatants were collected and analysed for IL-10 and IL-12p70 production by a specific sandwich ELISA. Data represent mean ± SEM of five independent experiments performed.

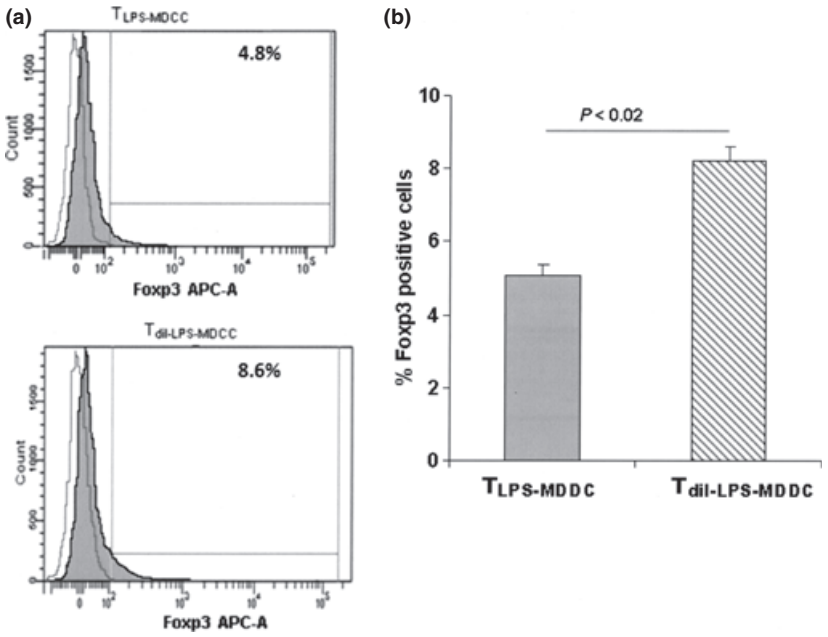

Figure 3 Effect of diltiazem on the $CD4^+Foxp3^+$ population. Allogeneic $CD4^+CD45RA^+$ T cells were cultured with dil-LPS-MDDC ($T_{dil-LPS-MDDC}$) or LPS-MDDC ($T_{LPS-MDDC}$) for 1 week. IL-2 was then added and cells cultured for another week. Cells were collected, washed, and analysed by FACS for the expression of Foxp3. (a) FACS analysis of $CD4^+Foxp3^+$ population from a representative experiment. The proportion of $Foxp3^+$ T cells prior to co-culture with LPS-MDDC or dil-LPS-MDDC was 0.2%. (b) Data represent mean ± SEM of three independent experiments performed.

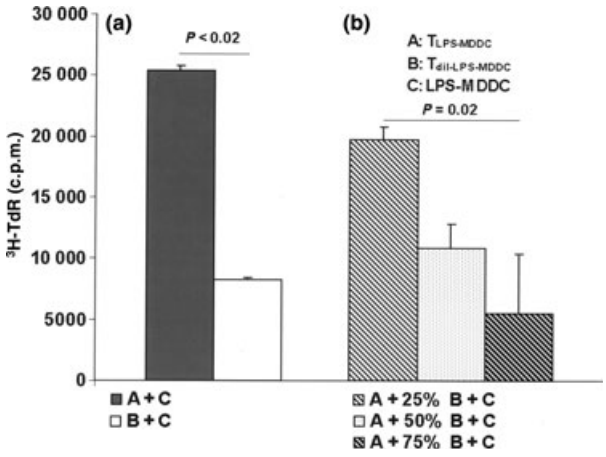

Figure 4 Dil-LPS-MDDC induce a slowly proliferating T-cell population able to suppress proliferation of autologous T cells. Allogeneic $CD4^+CD45RA^+$ T cells were cultured with dil-LPS-MDDC ($T_{dil-LPS-MDDC}$) or LPS-MDDC ($T_{LPS-MDDC}$) for 1 week. IL-2 was then added and cells cultured for another week. Cells were collected, washed, stimulated with LPS-MDDC, and analysed for proliferative capacity (a). The two populations were also mixed at 25%, 50% and 75% ratios of $T_{dil-LPS-MDDC}$ and analysed for proliferative capacity (b). Control response in (b) is the same as shown in (a). Data represent mean ± SEM of three independent experiments performed.

dose-dependent reduction of $T_{LPS-MDDC}$ proliferation with statistical significance at the 75% ratio (Fig. 4b). Thus, $T_{dil-LPS-MDDC}$ are able to promote the induction of slowly proliferating T cells, which have the ability to suppress proliferation of autologous T cells.

Diltiazem-treated MDDC exert their suppressor activity through soluble factors

To investigate whether the $T_{dil-LPS-MDDC}$ inhibitory effect could depend on soluble factors, supernatants from $T_{dil-LPS-MDDC}$ and $T_{LPS-MDDC}$ cultures stimulated with autologous LPS-MDDC were collected at different time points during the culture period (6, 12, 24, 48 and 72 h). Supernatants collected after 6 and 12 h were ineffective, while supernatants collected after 24, 48 and 72 h were able to induce a significant suppression of proliferation when added to the purified $CD45RA^+$ T cells stimulated with anti-human CD3/CD28 mAbs. The suppression observed was around 30% without significant differences among the 24-, 48- and 72-h supernatants (Fig. 5).

Supernatants were then analysed for their cytokine content. The analysis showed that, when compared with $T_{LPS-MDDC}$, $T_{dil-LPS-MDDC}$ produce a significantly higher amount of TGF-β (Fig. 6a), and a slightly nonsignificant increased level of IL-10 (data not shown).

To ascertain the role of TGF-β in exerting the regulatory activity of $T_{dil-LPS-MDDC}$, we performed experiments in the presence of TGF-β–blocking mAbs. Allogeneic $CD4^+CD45RA^+$ T cells were cultured with dil-LPS-MDDC

($T_{dil\text{-}LPS\text{-}MDDC}$) or LPS-MDDC ($T_{LPS\text{-}MDDC}$) for 1 week. IL-2 was then added and cells cultured for another week. Cells were collected, washed, and stimulated with LPS-MDDC for proliferation. $T_{dil\text{-}LPS\text{-}MDDC}$ and $T_{LPS\text{-}MDDC}$ were mixed at the 75% ratio of $T_{dil\text{-}LPS\text{-}MDDC}$ with or without TGF-β–blocking mAbs. As shown in Fig. 6b, the presence of anti-TGF-β mAbs in the cultures consistently reversed the inhibitory effect. In previous studies, we observed that the addition of anti-TGF-β mAbs on $CD4^+CD45RA^+$ T cells cultured with LPS-MDDC did not significantly influence their proliferation. However, to evaluate whether the impact by anti-TGF-β might be significant on the above listed condition, we performed an additional experiment that indeed confirms our previous finding (A+C: 26,436 ± 1,708 c.p.m.; A+C+ α-TGF-β mAbs: 28,341 ± 1,466 c.p.m.; A+B+C: 14,697 ± 895 c.p.m.; A+B+C+ anti-TGF-β mAbs: 22,787 ± 782 c.p.m. A: $T_{LPS\text{-}MDDC}$; B: $T_{dil\text{-}LPS\text{-}MDDC}$; C: LPS-MDDC).

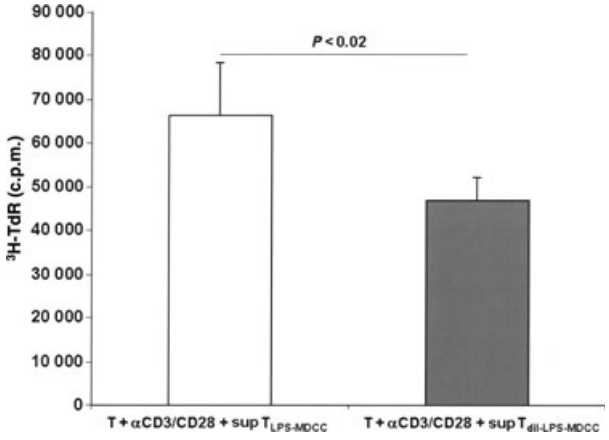

Figure 5 Supernatants from $T_{dil\text{-}LPS\text{-}MDDC}$ and $T_{LPS\text{-}MDDC}$ cultures stimulated with LPS-MDDC from the same donor used for the $CD4^+CD45RA^+$ T-cell priming, were collected at different periods of time of culture (6, 12, 24, 48 and 72 h). Supernatants collected after 24, 48 and 72 h showed a significantly comparable suppressive activity when added to purified $CD45RA^+$ T cells stimulated with anti-human CD3/CD28 mAbs. The response of T cells to CD3/CD28 stimulation in the absence of added supernatants is 81 750 ± 13 671 c.p.m. Data represent mean ± SEM of four independent experiments performed.

These results indicate that Foxp3's increased expression on $T_{dil\text{-}LPS\text{-}MDDC}$ is associated with regulatory activity. Taken together, data indicate that $T_{dil\text{-}LPS\text{-}MDDC}$ display the properties described for Treg cells.

Discussion

As DCs play a pivotal role in organ transplantation tolerance and immunity, any factor that regulates DC growth, maturation and function will affect allograft acceptance or rejection.

The induction of immune tolerance is one of the final therapeutic goals in clinical transplantation. At present, transplanted organs are rapidly rejected by the recipient's

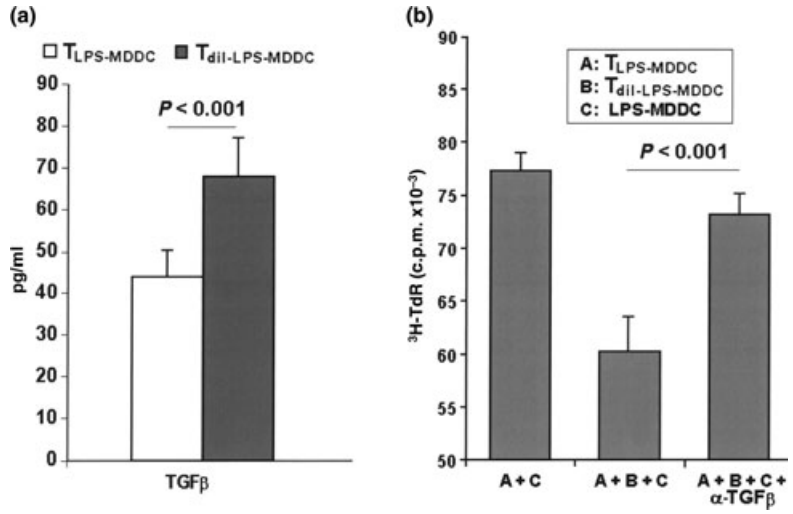

Figure 6 $T_{dil\text{-}LPS\text{-}MDDC}$ produce an increased amount of TGF-β (a). Allogeneic $CD4^+CD45RA^+$ T cells were cultured with dil-LPS-MDDC ($T_{dil\text{-}LPS\text{-}MDDC}$) or LPS-MDDC ($T_{LPS\text{-}MDDC}$) for 1 week. IL-2 was then added and cells cultured for another week. Cells were collected, washed, stimulated with LPS-MDDC, and supernatants analysed for cytokine production. Data represent mean ± SEM of nine independent experiments performed. TGFβ production by MDDC and naïve T cells alone is 9.7 and 7.8 pg/ml respectively. $T_{dil\text{-}LPS\text{-}MDDC}$ exert regulatory activity on $T_{LPS\text{-}MDDC}$ via TGF-β production (b). Blocking experiments were set up as described in Fig. 4. In addition, anti-TGF-β mAbs were added to the $T_{LPS\text{-}MDDC}$ cultures with 75% $T_{dil\text{-}LPS\text{-}MDDC}$, where the maximum suppression activity was observed. Data represent mean ± SEM of three independent experiments performed.

immune system in the absence of any immunosuppressive therapy. The current immunosuppressive drugs effectively reduce the immune response to allo-antigens, resulting in a relatively low incidence of acute rejection [36,37]. Unfortunately, long-term administration of conventional immunosuppressants leads to many detrimental side-effects, reducing patient and graft survival [38]. Therefore, it is essential to restrain the use of immunosuppression and achieve alternative strategies to induce and maintain transplant tolerance. A possible strategy that is now extensively investigated is the induction and maintenance of transplant tolerance by Treg. As immunosuppressive drugs affect effector T cells, they may also affect Treg. This notion has raised concerns about the influence of immunosuppressive drugs on Treg-mediated tolerance and triggered intensive research on this issue.

This study provides the first evidence that diltiazem, a calcium channel blocker, induces Treg cells *in vitro* by influencing DC maturation and function. Our data show that diltiazem-treated MDDC, when cultured with naïve $CD4^+$ T cells, promote the induction of a slowly proliferating cell population with an increased number of $Foxp3^+$ T cells which has the ability to suppress the proliferation of autologous T cells. We show that the regulation depends on the highly enhanced production of TGF-β–associated with an increased level of IL-10, and that it is abrogated by the addition of anti-TGF-β antibodies in the culture. Recent studies suggest that TGF-β production by $Foxp3^+$ Treg cells plays a unique role in the maintenance of self-tolerance [39]. Several studies strongly suggest that TGF-β is synthesized during Treg cell activation, complexed to LAP, and maintained on the surface of the Treg cells as a LAP-TGF-β complex [40–42]. The contribution of Treg cell-produced TGF-β to the inhibition of the proliferation of $CD4^+$ T cells in co-cultures *in vitro* has remained controversial [41–43]. Some studies have previously demonstrated that $CD4^+$ $CD25^+$ T cells from TGF-$\beta^{-/-}$ mice or mice with a T-cell-specific deletion of TGF-β are fully competent suppressors *in vitro* [33,42], whereas others have claimed that Treg cell suppression *in vitro* can be reversed by anti-TGF-β [42] or LAP [41]. Thus, in agreement with the latter studies, we demonstrate that T cells induced by diltiazem-treated MDDC acquire a regulatory phenotype and a functional behaviour similar to those already described for Treg.

Many investigators have reported so far the capacity of different immunosuppressants to induce Treg in animal or human studies [44–52]. Conventional drugs used in transplantation were studied in experimental and clinical protocols with contrasting effects on Treg cells according to their mode of action. In recent years, many investigators have performed studies on a single drug [44–47], while others have performed studies in which the effects of drugs with different mode of action had been compared [48–52]. Among the latter, Lim *et al.* [50] show that co-stimulatory blockers such as CTLA4Ig and anti-CD40L, and mycophenolate mofetil can be utilized therapeutically in the induction of immune tolerance in mice. In a comparative study Gao *et al.* [49] demonstrated that rapamycin and cyclosporine A differentially affect T-cell regulation. Rapamycin induces *de novo* generation of biologically active Treg cells that mediate graft protection. Also corticosteroids have a beneficial effect on the induction of the Treg population in contrast to their negative effect on natural Treg [45]. Further studies [44,52] provided the evidence that rapamycin and mycophenolate mofetil rather than cyclosporine A preserve murine Treg function and expansion, and Foxp3 expression levels when administered after allogeneic bone-marrow transplantation. Finally, Demirkiran *et al.* [51] reviewed experimental as well as clinical data. They speculated that the best therapy to promote Treg-mediated transplant tolerance may be treatment with T-cell–depleting antibodies before transplantation, followed by rapamycin treatment possibly combined with corticosteroids. The data indicate that the use of specific immunosuppression can be an essential component of combined therapeutic strategies that might overcome the limits of the sole use of immunosuppressive drugs to prevent rejection, despite their capacity to induce Treg cells.

$Foxp3^+$ T cells play a role in the suppression of donor-activated effector T cells and in tolerance induction in transplantation. In a recent study on renal transplant patients [53], investigation of Foxp3 expression in the cells revealed that chronic rejection is associated with a decreased number of $CD4^+$CD25high $Foxp3^+$ T cells with normal regulatory profile, whereas graft acceptance is associated with $CD4^+$CD25high $Foxp3^+$ T-cell numbers similar to healthy volunteers. Similar reports came from operationally tolerant liver transplant patients: $CD4^+CD25high^+$ T-cell ratios were increased in the tolerant patients' peripheral blood, and Foxp3-expressing T cells were also present in the tolerant liver [54]. The presence of Treg also seems to correlate with graft acceptance in living donor liver transplantation [55].

In conclusion, our study reports the first evidence that diltiazem is able to generate human Treg cells *in vitro* by affecting DC maturation. As a further step, diltiazem has to be used in an animal model of transplantation to confirm Treg development and to evaluate rejection. Experimental mouse models have provided valuable insights into the effects of immunosuppressive reagents on Treg, and facilitated strategies to expand or induce alloantigen reactive Treg either *in vivo* or *in vitro*. These techniques facilitate the translation of strategies designed to promote transplantation tolerance to the clinic [31]. Therefore, clinical studies will be necessary to evaluate the efficacy of

diltiazem in transplanted patients. As diltiazem has been already used in transplantation as an agent capable of reducing detrimental side-effects induced by conventional therapies, as also in exerting anti-inflammatory and immunosuppressive effects, its capacity to promote Treg expansion should qualify it for inclusion among the drugs eligible for the induction of tolerance in transplantation.

Authorship

OP: designed and performed the research, collected and analysed the data, wrote the paper. AD'A: designed and performed the research, analysed the data. DC: performed the research. FQ: designed the research, analysed the data and wrote the paper.

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