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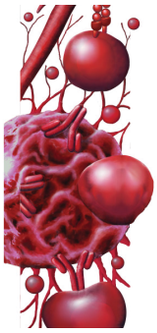

Rapamycin-conditioned dendritic cells activated with monophosphoryl lipid A promote allograft acceptance *in vivo*

Aim: To date, there is no human dendritic cell (DC) based therapy to prevent allograft rejection in transplanted patients. Here, we evaluate a potential protocol using a murine *in vivo* transplant model. **Materials & methods**: We generated murine bone marrow-derived DCs (BM-DCs), modulated with rapamycin (Rapa) and activated with monophosphoryl lipid A (Rapamycin-treated and monophosphoryl lipid A-matured DCs [Rapa-mDCs]). DCs phenotype was evaluated by flow cytometry, cytokine production by ELISA and their T-cell stimulatory ability was tested in co-cultures with $CD4^+$ T cells. Using an *in vivo* skin graft model, we evaluated DCs tolerogenicity. **Results**: *In vitro*, Rapa-mDCs exhibit a semi-mature phenotype given by intermediate levels of co-stimulatory molecules and cytokines, and inhibit $CD4^+$ T-cell proliferation. *In vivo*, skin-grafted mice treated with Rapa-mDCs show high allograft survival, accumulation of Foxp3+Tregs and cytokine pattern modification. **Conclusion**: Rapa-mDCs re-educate the inflammatory microenvironment, promoting skin-allograft survival.

Keywords: cellular therapy • dendritic cells • regulatory T cells • tolerance

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The development of cellular therapy to control autoimmune diseases and prevent allograft rejection is still the quest of many immunologists, being the manipulation of dendritic cells (DCs) one of the main strategies. DCs are professional APC with the capacity to: trigger an inflammatory response, a task performed by mature DCs which are characterized by the elevated expression of MHC and co-stimulatory molecules, in addition to high secretion of proinflammatory cytokines such as IL-6, IL-12, TNF, among others [1–3]; and mitigate self-directed immune responses as a protective mechanism [4]. In other words, DCs are also the main players in inducing and maintaining tolerance. In this case, tolerogenic DCs are characterized by the low expression of MHC and co-stimulatory molecules, reduced production of proinflammatory cytokines, but elevated amounts of anti-inflammatory ones, such as IL-10 and TGFβ [5–7].

With respect to clinical applications of mature DCs, several efforts involving DC vaccines have been applied for cancer therapy, and some of them are currently in clinical trials [8,9]. On the other hand, only one study, done in autoimmune diabetes, has been reported regarding therapies using tolerogenic DCs [10]; therefore, the need for protocols applicable to other conditions and the knowledge of how these DCs would behave *in vivo* is indispensable. Thus, many approaches have been developed to endow DCs with tolerogenic properties, including treatment with anti-inflammatory cytokines, the modulation of DCs biology by genetic engineering or the use of immunosuppressive compounds [11–19]. For instance, the conditioning of murine DCs using rapamycin, a macrolide widely used in transplantation, induce them to display: resistance to phenotypic maturation induced by proinflammatory stimuli; the ability to propagate Tregs able to produce IL-10; and the capability to migrate to the lymph nodes [20].

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Very recently, our laboratory developed a protocol to generate modulated human DCs that display tolerogenic properties, since these DCs produce low levels of proinflammatory cytokines such as IL-12, IL-6 and TNF, suppress $CD4^+$ T-cell proliferation and IFN-γ production and, importantly, maintain the expression of migratory molecules that confer trafficking properties to lymphoid tissues *in vivo* [21]. Because DC-based cellular therapy is proposed to be used in pathologies with inflammatory environments, such as autoimmune diseases or allograft rejection, the utilization of an activating stimulus such as monophosphoryl lipid A (MPLA), a nontoxic analog of lipopolysaccharide, is relevant to confer stability to tolerogenic DCs.

In this study, we demonstrate that rapamycin-conditioned DCs and activated with MPLA (Rapamycin-treated and monophosphoryl lipid A-matured DCs [Rapa-mDCs]) show a tolerogenic profile in which low expression of co-stimulatory molecules such as CD40 and CD86, but high levels of the migration molecule CCR7 are displayed. Rapa-mDCs produce markedly diminished levels of both IL-6 and IL-12 with no differences in IL-10 secretion among all groups. In addition, Rapa-mDCs exhibited reduced capabilities to stimulate effector $CD4^+$ T-cell proliferation *in vitro*. Finally, in *in vivo* transplantation experiments, Rapa-mDCs-treated RAG-/- mice show high survival of skin allografts, mainly by accumulating $Foxp3^+$ T cells in the graft draining lymph nodes (dLN) and diminishing IFN-γ and IL-17 secretion, together with an up-regulation on IL-10 production in the spleen, possibly indicating a re-education via suppressive DCs to allow the induction and/or proliferation, or both, of Tregs. Our results demonstrate that Rapa-mDCs show essential features, allowing them to be used as therapeutic agents for inflammatory responses, autoimmunity and the prevention of graft rejection.

Materials & methods

Media & reagents

RPMI-1640 (Gibco BRL, CA, USA) supplemented with 10 mM HEPES, 100 IU/ml penicillin/streptomycin, 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL, CA, USA) and 2000 U/ml rmGM-CSF (eBiosciences, CA, USA) is referred to as lymphocyte media. Iscove's Modified Dulbecco's Medium (IMDM; Gibco BRL) supplemented with 100 IU/ml penicillin/streptomycin, 10% heat-inactivated fetal bovine serum (FBS), 50 µM 2β-mercaptoethanol and 2000 U/ml rmGM-CSF was used as complete medium and is referred to as DC media.

Mice

Six to 8 week old wild-type C57BL/6, C57BL/6 Foxp3-GFP reporter mice, C57BL/6 × BALB/c F1 and RAG-/- were used in this study. C57BL/6 Foxp3-GFP reporter mice were a generous gift from Dr Rodrigo Mora. Mice were maintained in accordance with the Bioethical Committee guidelines from the Facultad de Medicina, Universidad de Chile.

Generation, purification & stimulation of bone marrow derived DC

Bone marrow (BM) was prepared from femurs and tibias of C57BL/6 or F1 mice, and red blood cells were eliminated using Tris-buffered ammonium chloride (ACT) lysis buffer. On day 0, BM cells were seeded at 3×10^6 per well in 6-well culture plates (Corning Plastic, Amsterdam, The Netherlands) in a total of 3 ml of DC media. On days 3 and 5, half of the culture supernatant was collected, centrifuged and the cells pellet were resuspended in fresh DC media and added back to the wells. On day 7, the cells were washed thoroughly and $CD11c^+$ DCs were purified to more than 90% of purity using anti-CD11c immunomagnetic beads (STEMCELL Technologies, Canada). The $CD11c^+$ cells were used in four different conditions: control-DCs (Ctrl-DCs), rapamycin-DCs (Rapa-DCs), MPLA-DCs (mDCs) and rapamycin-MPLA-DCs (Rapa-mDCs). Rapamycin was added at 10 ng/ml (Sigma-Aldrich, MO, USA) for 48 h and the current Good Manufacturing Practices grade MPLA (Avanti Polar Lipids, Inc., AL, USA) was used at 1 µg/ml to activate DCs for the last 24 h.

Proliferation assay *in vitro*

This assay mimics, although not identical, a mixed leukocyte reaction, which is known for cultivating cells from allo-mismatched donors. Bone marrow-derived DCs (BM-DCs) from C57Bl/6 × Balb/c F1 mice were generated and differentially modulated as explained above. Splenic $CD4^+$ T cells were isolated from C57Bl/6 mouse using magnetic beads (STEMCELL Technologies) and labeled with carboxyfluorescein succinimidyl ester (CFSE; 5 µM; Molecular Probes, NY, USA). Co-cultures between DCs (ratio 2:1 [DC:T], 1×10^5 and 5×10^4, respectively) or T cells alone (5×10^4 cells, parental peak control) were performed in U-bottom 96-well plates, with no additional stimulation, and maintained for 72 h at 37°C and 5% CO_2. After this time, cells were harvested and stained with anti-CD11c and anti-CD4 antibodies (BioLegend, CA, USA) to analyze CFSE dilution (on $CD4^+$ T cells) by flow cytometry.

Flow cytometry analysis

The following antibodies were used for cell surface molecule expression analysis: CD11c APC, CD40 FITC, CD86 PerCP, MHC-II PE, MHC-I FITC, PD-L1

PE/Cy7, CCR7 PE, CD4 APC, CD25 FITC, FoxP3 PE, all from BioLegend. Cells were re-suspended in PBS + 10% FBS and incubated with antibodies (1:400) for 15 min, then washed twice with phosphate buffer saline (PBS) + 5% FBS and fixed with IC Fixation Buffer (eBioscience, CA, USA). Foxp3 intracellular staining was performed following the provider's instructions (eBioscience). Data were collected on a FACSCalibur (Beckton Dickinson, CA, USA), and analyzed using the Flowjo software (version 10.0.5).

ELISA test

BM-DCs were harvested on day 7, washed and seeded in 96-well U-bottom plates (BD Biosciences, CA, USA) at a concentration of 1×10^5 cells/100 µl/well in DC media under different conditions: Ctr-DCs, Rapa-DCs, mDCs and Rapa-mDCs, for 2 days. Supernatants were collected and stored at -80°C and later analyzed for cytokine quantification by ELISA (sandwich) test using anti-IL-6, anti-IL-10, anti-IL-12 and anti-IL-4 antibodies (eBioscience).

Skin transplantation

Full thickness tail skin was collected from C57BL/6 × BALB/c F1 mice. Skin grafts (1 × 1 cm in size) were transplanted onto the dorsal area of age-matched RAG-/- mice as described before [22]. Skin graft survival was monitored two-times per week and rejection was defined as complete necrosis and loss of the skin grafts. At day 25 post transplantation, spleen and dLN were removed and cell suspensions prepared to first analyze cellular phenotype (by flow cytometry), and secondly to culture them at 1×10^6 cells/ml in lymphocyte media for 3 days under polyclonal re-stimulation with anti-CD3 (5 µg/ml, clone 2c11, BioLegend). Secreted IFN-γ, IL-17 and IL-10 levels were measured via ELISA (all antibodies from eBiosciences).

Statistical analysis

All data are presented as the mean ± SD. One-way ANOVA for repeated measures and Tukey post-tests analyses were done using Prism 5.01 GraphPad Software (CA, USA). A value of $p < 0.05$ was considered to be statistically significant.

Results

Rapamycin imprints a semi-activated phenotype on mDCs

Recently, our laboratory reported that human monocytes can be differentiated into tolerogenic DCs in the presence of GM-CSF, IL-4 and the corticosteroid dexamethasone (Dex-DCs), and that activation with MPLA generates Dex-DCs with a semi-activated phenotype (MHC-II^{high}, CD40^{low}, CD80^{low}, CD83^{low}, CD86^{low}), able to produce reduced levels of proinflammatory cytokines and inhibit CD4$^+$ T-cell proliferation [23]. In this study, we generated murine BM-DCs, which were incubated with rapamycin and activated with MPLA (Rapa-mDCs) for the last 48 h of culture, and their phenotype was studied evaluating the expression of canonical surface markers. The control conditions corresponded to untreated DCs, DCs modulated with rapamycin alone (Rapa-DCs) and DCs activated with MPLA alone (mDCs). In all conditions ≥60% of DCs express MHC-I and MHC-II, but only for MHC-I we observed an inhibitory effect, at frequency (%) and not mean fluorescence intensity, due to rapamycin treatment (Supplementary Figure 1; see online at: www.futuremedicine.com/doi/suppl/10.2217/imt.14.116). In the case of MHC-II, we did not obtain significant differences between conditions (Supplementary Figure 1). Regarding co-stimulatory molecules, upon MPLA activation, mDCs upregulate CD40 (~70% in mDCs vs ~15% in Ctrl-DCs) and CD86 (~40% in mDCs vs ~15% in Ctrl-DCs), but the pretreatment with rapamycin prevents this maturation resulting in a semi-mature phenotype (~40% for CD40 and ~20% for CD86). Interestingly, the modulation of DCs with rapamycin slightly induces the expression of the migratory molecule CCR7 (~5% of CD11c$^+$ cells), effect that is not altered after MPLA stimulation (Figure 1).

Regarding cytokine production, mDCs produce high levels of the proinflammatory cytokines IL-6 (~40 ng/ml vs ≤5 ng/ml in Ctrl-DCs) and IL-12 (~50 ng/ml vs ≤5 ng/ml in Ctrl-DCs), but the treatment with rapamycin downregulates their production in both Rapa-DCs (similar amounts as Ctrl-DCs) and Rapa-mDCs (~15 ng/ml and 30 ng/ml for IL-6 and IL-12, respectively; Figure 2), implying that MPLA activation does not revert completely the rapamycin tolerogenic effect. For the case of the anti-inflammatory cytokine IL-10, we did not observe any differences in its production regardless the conditioning and/or activation treatment (Supplementary Figure 2). IL-4 was not detectable in any condition.

Taken together, our data suggest that rapamycin induces a semi-mature phenotype on MPLA-activated DCs based on the intermediate expression levels of CD40 and CD86 and secretion of IL-6 and IL-12.

Rapamycin-modulated DCs suppress CD4$^+$ T-cell proliferation *in vitro*

Since Rapa-mDCs display a semi-mature phenotype, we wanted to address the potential effect of these cells on CD4$^+$ T-cell proliferation *in vitro*. For this purpose, we performed proliferation assays *in vitro*, in which all different DCs populations were first generated from C57Bl/6 × Balb/c F1 mice and differentially activated

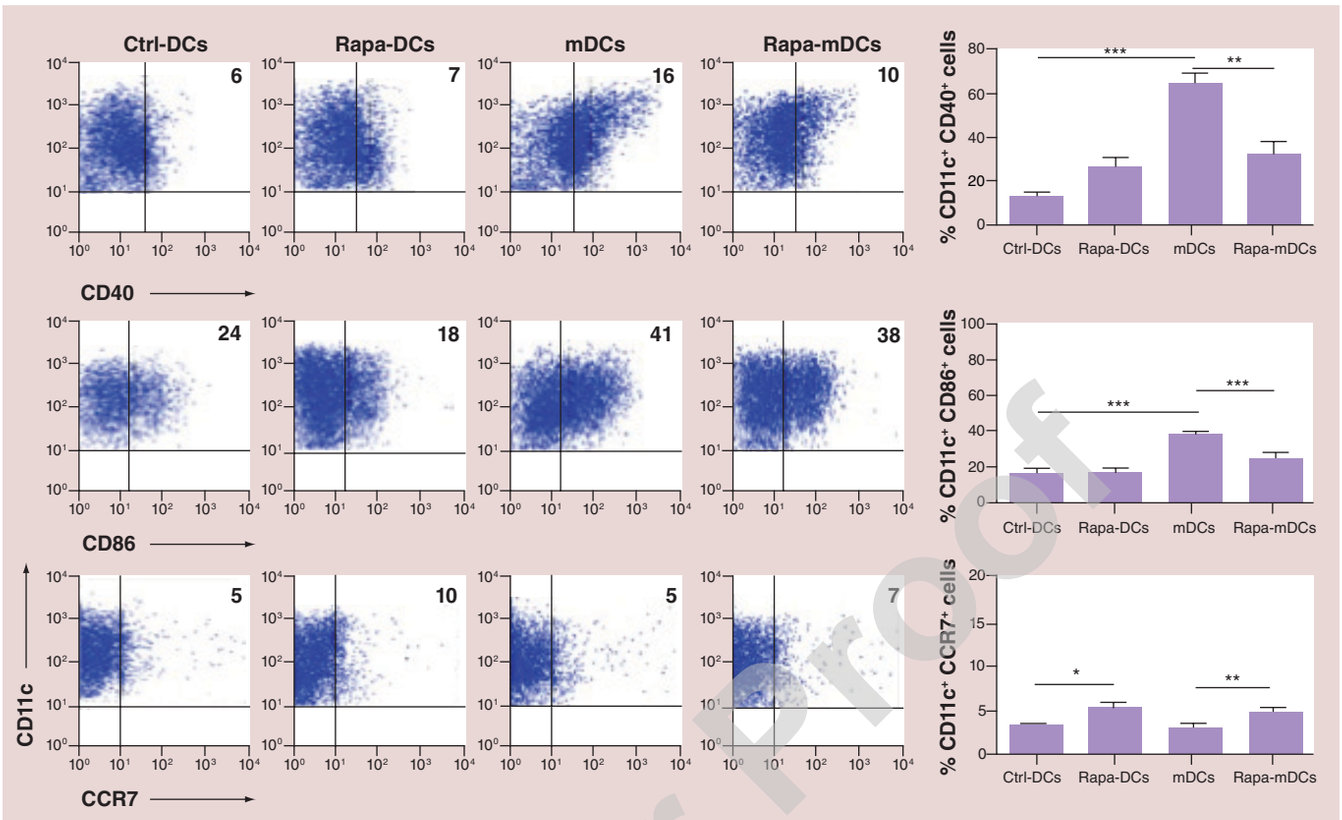

Figure 1. Rapamycin-treated and monophosphoryl lipid A-matured DCs display a semi-mature phenotype. Bone marrow-derived dendritic cells (DCs) were generated from 6–8 weeks old C57Bl/6 mice. Bone marrow progenitors were cultured for 7 days in the presence of granulocyte-macrophage-colony stimulating factor. At day 7, the cells were harvested and CD11c+ DCs were magnetically isolated using anti-CD11c beads, and 10^6 DCs were seeded in 24-well plates, and treated with rapamycin (10 ng/ml) and/or activated with MPLA (1 μg/ml). After the mentioned treatment, cells were recovered to study their phenotype by flow cytometry. Bars correspond to the standard deviation, and the statistical significance was assessed by analysis of variance.
*p < 0.05; **p < 0.01; ***p < 0.001; n = 8 independent experiments.
Ctrl-DC: Control dendritic cell; mDC: Mature dendritic cell; Rapa-DC: Rapamycin dendritic cell; Rapa-mDC: Rapamycin-treated and monophosphoryl lipid A-matured dendritic cell.

to further be co-cultivated with splenic CFSE-labeled CD4+ T cells isolated from C57BL/6 mice, mimicking a mixed leukocyte reaction assay, Figure 3. As expected, our results show that mDCs induce the highest proliferation rate, followed by Ctrl-DCs (~80% and ~65% of CD4+ T cells, respectively). On the other hand, Rapa reduces the capacity of DCs to promote CD4+ T-cell proliferation, on both Rapa-DCs and Rapa-mDCs (~%40), suggesting that the modulatory effect of Rapa can be maintained or fixed regarding the activating effect of MPLA on DCs.

Rapamycin-modulated mDCs prevent skin allograft rejection, promote the accumulation of Foxp3+ T cells, & modulate the production of CD4+ T cell-derived IFN-γ, IL-17 & IL-10

To evaluate the potential suppressive function of the Rapa-mDCs *in vivo*, we tested their effectiveness in a murine skin transplant model in which RAG-/- recipient mice received C57Bl/6 × Balb/c F1 skin grafts after the transfer of CD4+ T cells. In Figure 4A, it is depicted a scheme of the *in vivo* experiment, with the corresponding timing for CD4+ T cell transfer, surgery and Eα-pulsed DCs treatment injection (Eα peptide corresponds to the positions 52 to 68 from the α-chain of I-E class II molecules and is expressed in all APCs from H-2^b/I-E+ strains (in the present work, in APCs from C57Bl/6 × Balb/c or F1 mice). As observed in Figure 4B, the administration of Rapa-mDCs prevents allograft rejection (~90% survival), in comparison with the groups treated with mDCs (all rejected), and Rapa-DCs (~50% survival).

In order to analyze skin graft dLNs, we stopped the experiment at day 25, removed dLNs and spleen and studied cells phenotype and cytokine production. As shown in Figure 5, we did not observe differences in the frequencies of CD4+Foxp3+ T cells from mice treated with the different DCs groups (Figure 5A, top graph)

but when $CD4^+Foxp3^+$ T cell absolute number was evaluated, we found that mice treated with Rapa-mDCs have slightly more $CD4^+Foxp3^+$ T cells in the skin graft dLNs (~40,000 cells vs ~30,000 cells in mDCs-treated group, and ≤1000 cells in Rapa-DCs-treated group (Figure 5A, bottom graph). On the other side, when we polyclonally re-stimulated spleen cells from all groups and measure cytokine production, we detected comparable levels of IFN-γ, IL-17 and IL-10 (Figure 5B, top, middle and bottom graphs, respectively). Interestingly, and according with the $CD4^+Foxp3^+$ T cell data, splenic cells from Rapa-mDCs treated mice produced less IFN-γ and IL-17 than mDCs-treated group (~7000 pg/ml vs ~11,000 pg/ml for IFN-γ and 1000 pg/ml vs ~4000 pg/ml for IL-17), but similar to Rapa-DCs. Interestingly, Rapa-mDCs-treated mice produce slightly more IL-10 than Rapa-DCs (~600 pg/ml vs 100 pg/ml), Figure 5B, bottom graph, but with no statistical significance. Regarding this, our data suggest that Rapa-mDCs promotes either the differentiation or expansion, or both, of $CD4^+Foxp3^+$ T cells, reducing the production of inflammatory cytokines such as IFN-γ and IL-17, and maintaining comparable levels of IL-10 with effector $CD4^+$ T cells- or mDCs-treated mice. Focusing on the impact of Rapa-mDCs treatment and considering the same or reduced production of proinflammatory cytokines (vs $CD4^+$ T cells alone or mDCs) and same or augmented levels of IL-10 (vs $CD4^+$ T cells and mDCs, compared with Rapa-DCs), our data suggest the importance of the balance on the inflammatory milieu plus the potential interaction of the administrated DCs with dLNs-resident or -infiltrating cells, which may influence the proliferation, viability and/or migration of the present cells. All these variables, as a whole, have a net impact on the immune response against the allograft.

Discussion

Recently, our group demonstrated that human monocyte-derived DCs treated with dexamethasone maintained their tolerogenic properties after an inflammatory stimulus such as MPLA, the nontoxic lipopolysaccharide derivative developed to activate human cells (under current Good Manufacturing Practices conditions) for clinical purposes [21]. In the mentioned study, these alternatively-activated DCs show intermediate levels of MHC-II, CD40, CD83, CD80/86 and PD-L1, low production of proinflammatory cytokines such as IL-12, TNF and IL-23, upregulation of anti-inflammatory cytokines like IL-10 and TGFβ, in addition to a low T-cell allostimulatory capacity and reduced stimulation of IFN-γ expression by $CD4^+$ T cells. Importantly, the alternatively-activated DCs keep their migratory properties to inflamed environments; altogether the data suggest that the protocol described to generate human DCs could be suitable for therapeutic purposes. Regardless of these findings, we still do not know the mechanisms by which DCs modulated with an immunosuppressive drug, and subsequently activated with MPLA, could induce tolerance in an *in vivo* setting.

In the present report, we described that murine BM-DCs modulated with rapamycin and activated with MPLA express intermediate levels of CD40, CD86 and MHC-I, similar to our previously published work on human DCs [21]. The expression levels of MHC-II, at the frequency and mean fluorescence intensity levels, were not affected (Supplementary Figure 1). In addition, our Rapa-mDCs produce intermediate amounts of IL-6 and IL-12, with no significant differences in the amount of IL-10 secreted (Supplementary Figure 2). The modulation of DCs phenotype by rapamycin has

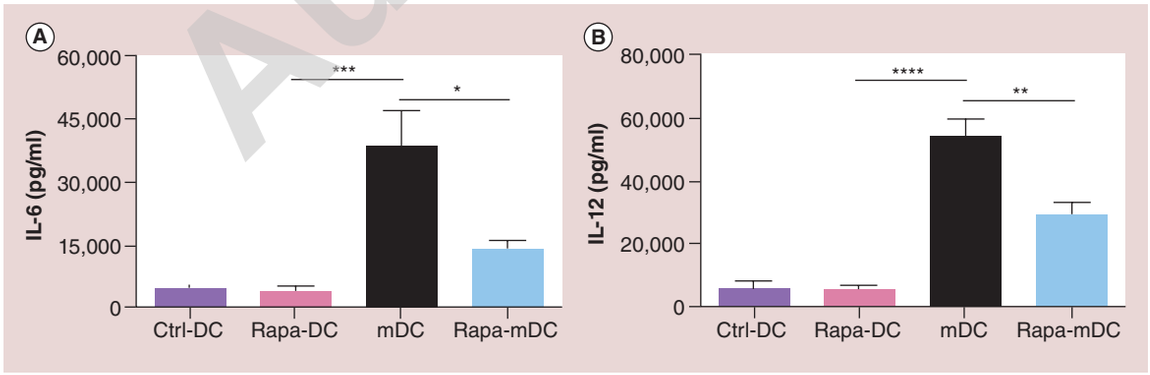

Figure 2. Rapamycin-treated mature dendritic cells secrete low amounts of the proinflammatory cytokines IL-12 and IL-6. Bone marrow-derived dendritic cells were generated, rapamycin-treated and activated as described earlier. After 24 h postactivation, the supernatants were collected and tested for IL-6 **(A)** and IL-12 **(B)** using ELISA. Bars correspond to the standard deviation, and the statistical significance was assessed by analysis of variance. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; n = 5 independent experiments.
Ctrl-DC: Control dendritic cell; mDC: Mature dendritic cell; Rapa-DC: Rapamycin dendritic cell; Rapa-mDC: Rapamycin-treated monophosphoryl lipid A-matured dendritic cell.

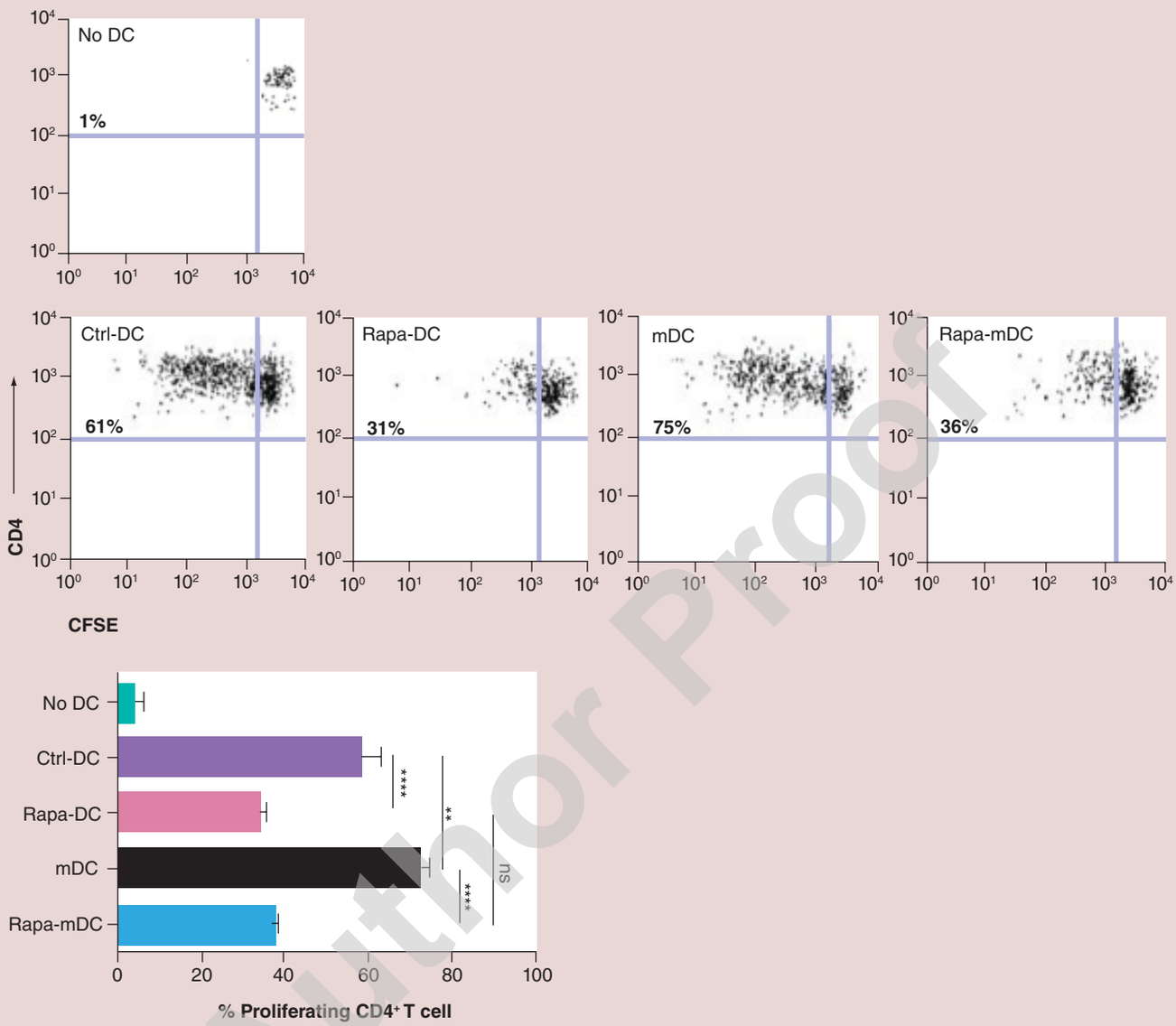

Figure 3. Rapamycin-treated dendritic cells suppress effector CD4⁺ T-cell proliferation *in vitro*. Bone marrow-derived DCs were generated from Balb/c × C57Bl/6 F1 mice, rapamycin-treated and activated as described earlier. Total CD4⁺ T cells were isolated from the spleen of C57Bl/6 mice, stained with carboxyfluorescein succinimidyl ester and added to the wells with DCs (ratio 2:1 [DC:T], 1 × 10^5 and 5 × 10^4, respectively) or left alone (5 × 10^4 cells, parental peak control). Co-cultures were maintained for 3 days. CD4⁺ T-cell proliferation was assayed by gating on live leukocytes (FSC/SSC) and on CD4⁺ T cells. CFSE dilution on CD4⁺ T cells was analyzed by flow cytometry. Bars correspond to the standard deviation, and the statistical significance was assessed by analysis of variance. **p < 0.01; ****p < 0.0001; n = 1 experiment performed in triplicate.
Ctrl-DC: Control dendritic cell; CFSE: Carboxyfluorescein succinimidyl ester; DC: Dendritic cell; mDC: Mature dendritic cell; Rapa-DC: Rapamycin dendritic cell; ns: Nonsignificant; Rapa-mDC: Rapamycin-treated and monophosphoryl lipid A-matured dendritic cell.

been extensively studied, indicating that this drug inhibits DCs differentiation and induces cell apoptosis when added during the full length of the DCs generation protocol [23,24]. Alternatively, if rapamycin is used to treat differentiated DCs, it targets DCs phenotype by inducing the expression of CD80, CD86 and CCR7 [24], promoting IL-12 and inhibiting IL-10 production [24–27], all these changes depending on the subset of DCs studied. Therefore, our data are partially in agreement with what has been published, and more studies are needed to conclude the direct effect of rapamycin on murine DCs.

In vivo data indicate that rapamycin affects the biology of CD4⁺ T cells as well, including regulatory and effector functions. For example, it is now known that rapamycin promotes tolerance not by inducing Tregs

de novo, but by promoting the apoptosis of CD4+ T effector cells, resulting in an enrichment of Tregs populations [28–30]. We can presume that these *in vivo* observations are partially mediated by the direct effect of rapamycin on DCs, since our *in vitro* proliferation assays data indicate that Rapa-mDCs block CD4+ T-cell proliferation (Figure 3). Furthermore, the results obtained from our skin graft transplantation model support previous studies [31,32], supporting that rapamycin could mediate allograft acceptance by enriching functional Tregs in skin graft-dLNs and by skewing CD4+ T-cell responses, modulating pro- and anti-inflammatory cytokines secretion.

It is interesting to point out that the accumulation of Tregs only occurred in skin graft dLNs compared with the spleen, supporting the idea that the residence of Tregs in close proximity to the skin graft mediates acceptance [33]. Supporting this notion, we are able to find Rapa-mDCs at much higher frequencies that the other groups, in the skin graft-dLNs after 15 days post last DCs treatment (Supplementary Figure 3).

Furthermore, and since we did not directly determine if the accumulation of Tregs observed in the skin graft-dLNs correspond to natural or induced Tregs, one could suggest that Tr1 cells could be generated because we did not detect differences in the production of BM-DCs-derived IL-10 (regardless the treatments, Supplementary Figure 2), but an upregulation in the secretion of IL-10 was found after stimulation of skin graft-dLNs cells from mice treated with Rapa-mDCs. On the other hand, the moderate production of IFN-γ and IL-17 found after stimulation of skin graft dLNs cells, compared with the control treatment with mDCs, suggests an impaired CD4+ T-cell stimulation to drive inflammatory Th1 and Th17 lymphocyte subset differentiation. Taking in consideration the enhanced production of IL-10 by graft dLNs cells in Rapa-mDCs-treated mice, together with the other results generated, we can suggest that Rapa-mDCs are able to modulate the immunological balance inducing a tolerogenic response by re-educating T-cell populations in an inflammatory environment.

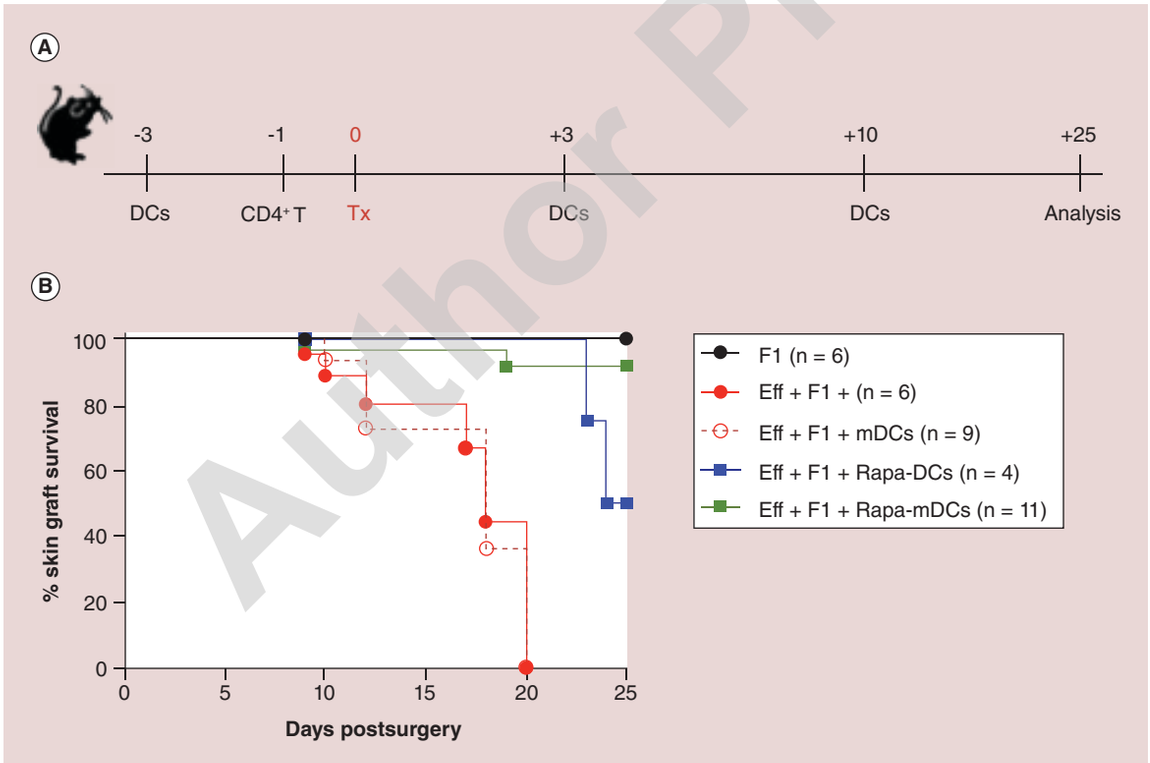

Figure 4. Rapamycin-treated mature dendritic cells promote allograft survival. (A) Bone marrow-derived DCs were generated, rapamycin treated and activated as described earlier. A total of 24 h postactivation (corresponding to day -3 of the experiment), mDCs, Rapa-DCs and Rapa-mDCs were incubated with Eα peptide, recovered and injected into RAG-/- recipient mice. At day -1 these mice received total CD4+ T cells (as eff) from C57Bl/6 mice, and at day 0 mice were transplanted with allogeneic (C57Bl/6 × Balb/c, F1) skin grafts (Tx). DCs treatment was repeated on day +3 and +10, as shown in the scheme. **(B)** Skin graft survival was monitored two-times per week up to day 25 postsurgery. Pooled data of two independent experiments, with four to eight mice per group, are shown. DC: Dendritic cell; Eff: Effectors; mDC: Mature dendritic cell; Rapa-DC: Rapamycin dendritic cell; Rapa-mDC: Rapamycin-treated and monophosphoryl lipid A-matured dendritic cell; Tx: Transplant.

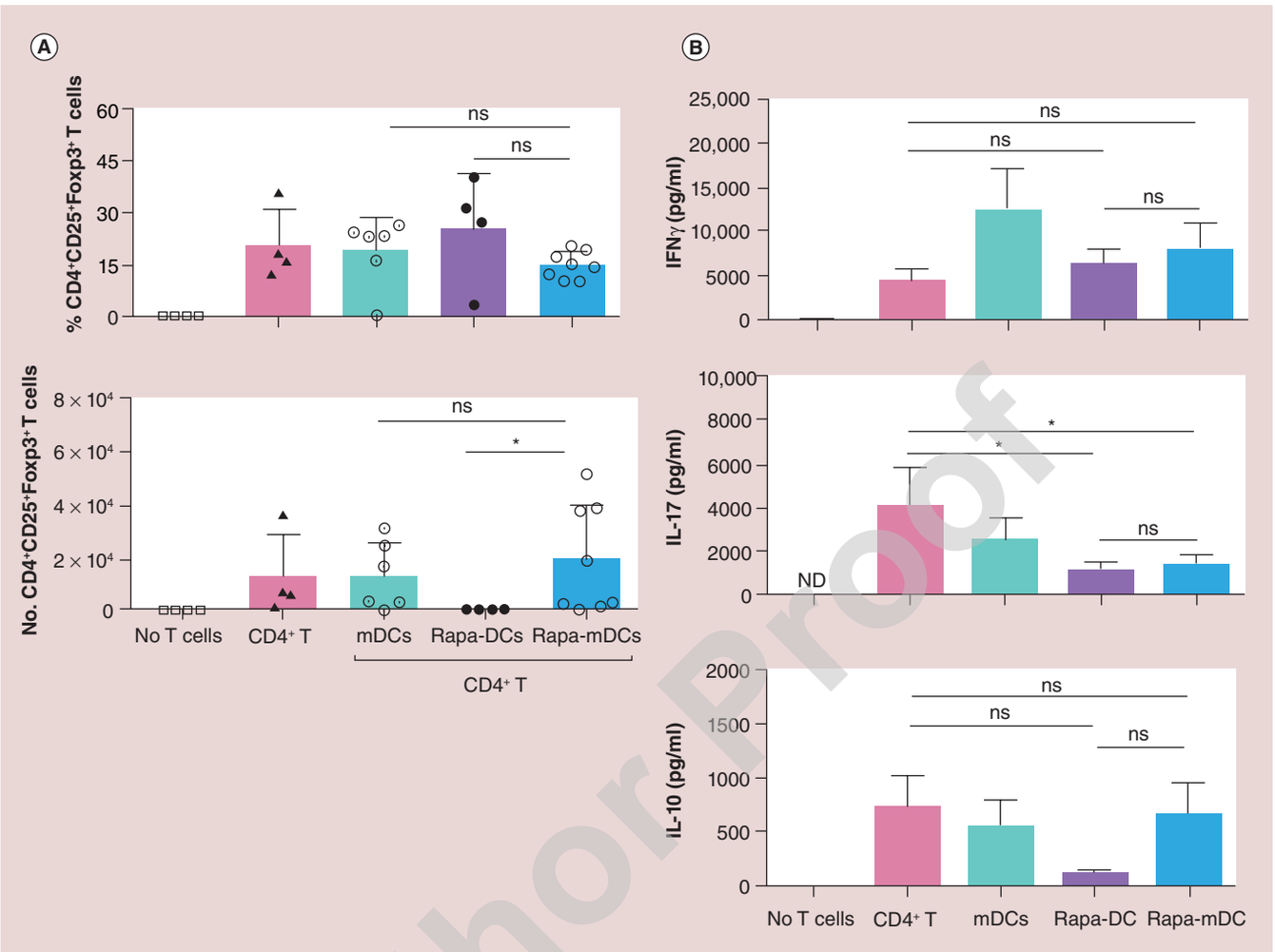

Figure 5. Rapamycin-treated mature dendritic cells allow the accumulation of Tregs and modulate CD4⁺ T cell-derived cytokine pattern. (A) Dendritic cells were generated, and mice treated, as described above. At day 25 postsurgery, draining lymph nodes and spleens were removed and cell suspensions prepared to analyze T-cell phenotype and cytokine secretion. Graphs show pooled data depicting the frequencies (top) and cell number (bottom) of Tregs (CD4⁺CD25⁺Foxp3⁺ T cells) in each experimental group, where NT group corresponds to RAG-/- mice grafted with F1 skin, with no transfer of CD4⁺ T cells or dendritic cells. **(B)** To quantify cytokine production, cell suspensions from spleens were plated and polyclonally activated *in vitro* for 72 h, to further collect the supernatants and measure cytokine release by ELISA. Bars correspond to the standard deviation, and the statistical significance was assessed by analysis of variance.

*$p < 0.05$; n = 2 independent experiments, with four to eight mice per group.

mDC: Mature dendritic cell; ND: Not detected; ns: Nonsignificant; Rapa-DC: Rapamycin dendritic cell; Rapa-mDC: Rapamycin and activated with monophosphoryl lipid A dendritic cell.

To our knowledge, this is the first report in which MPLA, a reagent to stimulate human cells, is used in murine DCs with the purpose to evaluate its effect *in vivo*. Taken together, this report contributes to unveil the possible mechanisms by which DCs treated with rapamycin and activated with MPLA could modulate the immune response during inflammation-driven process.

Conclusion

Similar to the protocol to produce human DCs, we are able to mimic the generation of tolerogenic murine DCs after the modulation with rapamycin and activation with MPLA. Our results show that the use of Rapa-mDCs as a potential cellular therapy is able to induce transplant acceptance, mainly by maintaining Tregs and promoting an anti-inflammatory environment, as demonstrated in an *in vivo* skin graft murine model.

Future perspective

The field of clinical transplantology has had enormous advances in the last decades. However, there are still some problems regarding tissue availability and graft

rejection that need to be addressed in order to fulfill patient's expectation and correct treatment. The results of this work show a promissory therapy that could be applied complementing current procedures for the treatment of organ rejection if it proves to be safe and efficient in clinical trials. However, more studies are necessary to completely elucidate the mechanism of Rapa-mDCs during an inflammatory reaction in both mice and human patients.

Authors' contributions

J Campos-Acuña and E Narváez, participated in performing the experiments, data analysis and writing the paper; F Pérez, M Campos-Mora and T Gajardo participated in performing the experiments; D Catalán and JC Aguillón participated in writing the paper; K Pino-Lagos, participated in the research design, data analysis and writing the paper.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Dendritic cells (DCs) treated with rapamycin acquire a low level of co-stimulatory molecules expression and proinflammatory cytokines production. Upon maturation with monophosphoryl lipid A, they upregulate these features, resulting with an intermediate maturation/activation phenotype.
- Rapamycin increases the expression of CCR7, suggesting the ability to migrate to lymph nodes.
- Rapa-mDCs inhibits the proliferation of $CD4^+$ T cells.
- Administration of Rapa-mDCs promotes skin allograft acceptance *in vivo*, and the accumulation of $CD4^+Foxp3^+$ T cells in the skin graft draining lymph nodes.
- Polyclonal restimulation of spleen cells show that cells from Rapa-mDCs-treated mice produce less proinflammatory cytokines, such as IFN-γ and IL-17, and more of the anti-inflammatory cytokine IL-10.

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