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Abstract:

Background: Immunosuppressive-drugs are needed after solid organ transplantation to prevent allograft rejection but induce severe side effects. Understanding the alloimmune response is critical to modulate it and to achieve graft operational tolerance. The role of regulatory T cells and tolerogenic dendritic cells (Tol-DCs) is undoubtedly essential in tolerance induction. **Tacrolimus is considered as the cornerstone of immunosuppression in solid organ transplantation. mTOR inhibitor such as rapamycin are thought to induce tolerance and are used as anticancer drugs in several cancers.** The aim of this study was to better understand the effect of **these** immunosuppressive drugs on the differentiation, maturation and function of human monocyte derived dendritic cells (DCs).

Material and methods: DCs were differentiated from monocytes of healthy donors with either rapamycin (Rapa-DCs) or tacrolimus (Tac-DCs). The phenotype was evaluated by flow cytometry analysis. The production of pro- and anti-inflammatory cytokines was assessed by ELISA. The mRNA expression level of IDO and PD-L1 was assessed by RTqPCR. Mixed leukocytes reactions were performed to analyse suppressive activity of DCs.

Results: Rapa-DC were characterised by a lower expression of the co-stimulatory molecules and CD83 than control-DCs (CTR-DC) ($p < 0.05$). In contrast, tacrolimus had no effect on the expression of surface markers compared to CTR-DCs. Rapamycin reduced both IL-12 and IL-10 secretions ($p<0.05$). Rapa-DCs had a suppressive effect on $CD4^+$ allogenic T cells compared to CTR-DCs ($p<0.05$). However, neither Rapa-DCs nor Tac-DCs favoured the emergence of a $CD4^+CD25^{high}Foxp3^+$ population compared to CTR-DCs. Surprisingly, Rapa-DCs had a reduced expression of IDO and PD-L1 compared to Tac-DCs and CTR-DCs.

Conclusion: **Rapa-DCs exhibit an incomplete phenotypic tolerogenic profile**. To our knowledge this is the first paper showing a reduction of expression of pro-tolerogenic enzyme IDO in DCs. **Tacrolimus does not change the phenotypical or functional characteristics of moDCs.**

Keywords: Tolerance, IDO, immunosuppressive drugs

Introduction:

Dendritic cells (DCs) are bone marrow-derived professional antigen presenting cells (APCs) that play a critical role in regulating both adaptive and innate immune responses. They promote self-tolerance in healthy steady-state and are important in many disease states including rejection after solid organ transplantation 1-3. DCs as well as other mononuclear phagocytes are major actors of the innate immunity. Beside this role, they are efficient stimulators of B and T lymphocytes. Their migration capacities to secondary lymphoid organs, their cytokine secretion profile, the presentation of antigen on the Major Histocompatibility Complex (MHC) molecules and the surface expression of costimulatory molecules are all part of this process. Therefore, they undergo a specific differentiation and maturation process that can be modulated by different drugs 1.

In the context of solid organ transplantation, alloantigens from donor cells can be presented to recipient T cells in at least three different pathways: direct, indirect and semi-direct. As professional APCs, DCs are central in these processes. In the direct pathway, donor DCs process and present alloantigens from the donor to recipient T cells **as well as secrete pro-inflammatory cytokines which favours graft rejection**. The indirect pathway involves recipient DCs presenting donor antigens to recipient T cells. Eventually, donor MHC complexes can be transferred from donor DCs to recipient DCs through exosomes and can present donor antigens through a semi-direct pathway 4 .

Tacrolimus is considered as the cornerstone in the immunosuppression therapy in solid organ transplantation 5,6. The mammalian target of rapamycin (mTOR) signaling pathway is a master regulator of cell growth and metabolism. Rapamycin is an mTOR inhibitor used in the treatment of several pathological conditions such as cancer and organ transplantation 7-9. It has been considered

by many as a potentially tolerogenic drug in the latter situation especially through the promotion of regulatory T cells (Tregs) [10].

A good knowledge of the effect of immunosuppressive drugs on DCs during their differentiation and maturation process raises the opportunity to better understand how these drugs act in the particular immune context of organ transplantation [11,12]. Human DCs development has been modelled *in vitro* using monocyte differentiation protocols with GM-CSF and IL-4 to obtain monocyte-derived dendritic cells (moDCs). Tolerogenic DCs (Tol-DCs) can be obtained by cultivating monocytes with tolerance-inducing agents during the differentiation step[13]. **Human moDCs generated in the presence of GM-CSF without IL-4 have been described as a good tolerogenic DC model.**[14,15] ENREF 14

Usually, Tol-DCs are defined as DCs with reduced surface expression of MHC molecules and costimulatory molecules, decreased production of IL-12 and increased production of IL-10, limited induction of T cell proliferation and increased regulatory T cells (Treg) induction compared to mature DCs[16,17]. Immature DCs (iDCs) exhibit these tolerogenic characteristics. As the immature state is relatively unstable, especially under pro-inflammatory conditions, several pharmaceutical agents have been proposed to enhance and stabilise their tolerogenic properties. There is to date no consensus on the optimal drug to use to induce Tol-DCs. Dexamethasone, vitamin D, retinoic acid, minocycline and rapamycin have all been studied -and may stabilise a tolerogenic state [13,18]. **Given the central place of tacrolimus in the immunosuppressive regimen after solid organ transplantation, the aim of this work was to assess the *in vitro* effect of tacrolimus on DC differentiation and maturation, compared to rapamycin, an mTOR inhibitor which is considered a tolerogenic drug** [19]. We also evaluated the impact of treated DCs on T cell alloactivation to assess their functional capacities.

Results

Rapamycin but not Tacrolimus influences monocyte derived dendritic cells phenotype *in vitro*.

To better characterize the effect of rapamycin and tacrolimus on DCs, we treated human CD14+ monocytes with rapamycin (Rapa-DCs) at a physiological concentration of 5 ng/mL or tacrolimus (Tac-DCs) at a supraphysiological concentration of 20ng/mL. It is known that physiological concentration of tacrolimus does not induce phenotypical modifications of DCs [20]. A tolerogenic control group (Tol-DCs) was differentiated in the presence **of GM-CSF alone** [14,15]. iDCs, Rapa-iDCs, Tac-iDCs and Tol-iDCs are immature DCs after 5 days of culture. CTR-DCs, Rapa-DCs, Tac-DCs and Tol-DCs undergo a 2 day maturation after LPS stimulation [21].

First as rapamycin is known to induce apoptosis of immune cells[22,23], this was confirmed in our study (Figure 1 A-B) [22,23]. Indeed, when looking at a 20-fold magnification of the cell culture-wells, we observe a lot of debris in the rapamycin treated cells (Figure 1 A). To confirm this first observation, flow cytometry assessment of viability showed a reduction of 52 ± 19.8% (mean +/- SD) in the Rapa-DCs after LPS stimulation compared to CTR-DCs 71.5 ± 8.7% (mean ± SD) (p=0.0015; n=7). Moreover, there was no significant difference in terms of viability between Tac-DCs compared to control cells.

Next, the effect of immunosuppressive drugs on DC differentiation and maturation was determined by the analysis of maturation markers (CD80, CD83 and CD86) as well as an iDC marker (CD209) by flow cytometry. As expected, Tol-DCs showed reduced

expression of most maturation markers except CD80 after LPS stimulation (Figure 1 C-D). Rapa-DCs were characterised by an immature phenotype compared to untreated DCs (Figure 1D). They showed significantly lower expression of costimulatory molecules such as CD80 and CD86 than CTR-DCs. Indeed, it was significantly different for the costimulatory molecule CD80 after LPS stimulation (p=0.0292; n=7). The expression of CD86 was also reduced in Rapa-DC compared to CTR-DCs (p <0.001, n=7) (Figure 1 C-D). Tacrolimus had no significant effect on the expression of surface markers CD80, CD83, CD86 and CD209 compared to CTR-DCs after LPS stimulation (Figure 1 D).

Rapa-DCs were resistant to LPS maturation compared to CTR-DCs and TAC-DCs. (Figure 2A.)

The expression of CD80, CD86 and CD83 was significantly increased in CTR-DCs after LPS stimulation (one-way ANOVA, p = 0.0021, Bonferroni's multiple comparison tests) unlike

Rapa-DCs after LPS-stimulation.

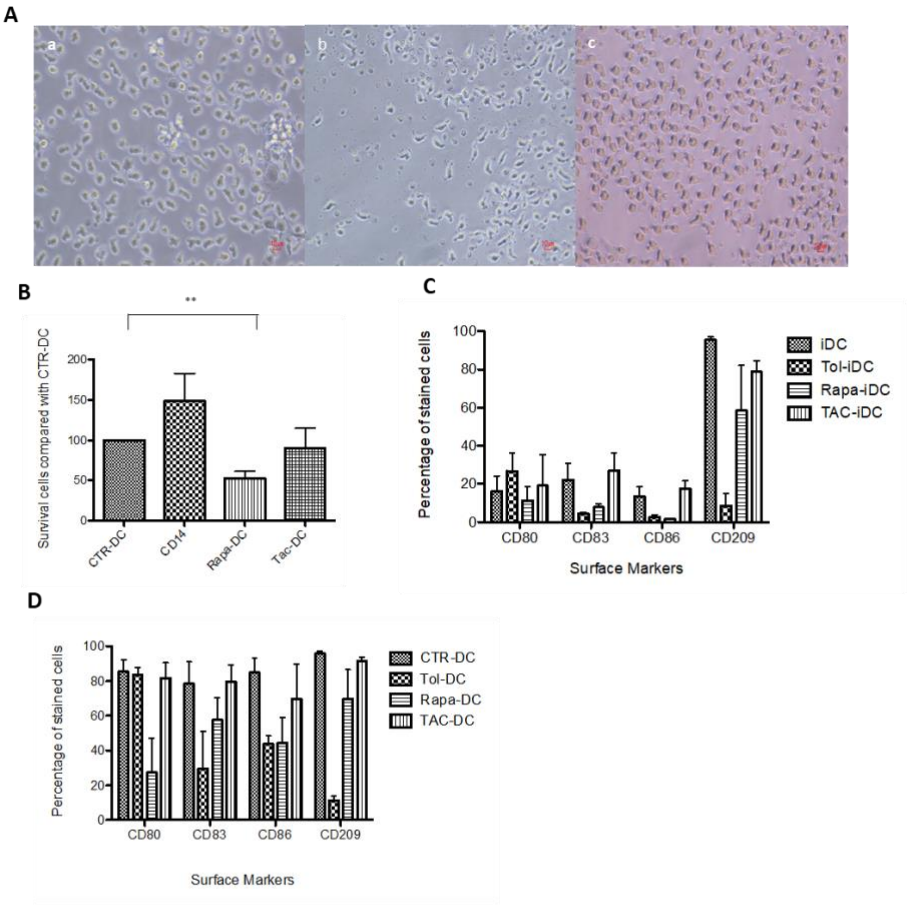

Figure 1. Viability and phenotype characterisation of moDCs after treatment with either rapamycin or tacrolimus. A. Microscopic image (20x) at day 7 of culture of (a) tolerogenic DCs, (b) Rapamycin-DCs and (c) Tacrolimus-DCs; showing cellular debris in Rapa-DCs. **B.** Relative cell viability measured by annexin V and propidium iodide staining compared to the control group (CTR-DCs)(n=7; p=0.0015 between CTR-DCs and Rapa-DCs). **C.** Percentage of stained cells for costimulatory surface markers CD80, CD83, CD86 and CD20 iDC marker after differentiation at day 5 **D.** and after LPS stimulation at day 7 (mean ± SEM of n=8; one-way ANOVA p <0.01). *(iDC = immature dendritic cells; CTR-DC= control mature dendritic cells; Tol-DC = tolerogenic dendritic cells; Rapa-DC= rapamycin-treated dendritic cells; Tac-DCs= tacrolimus-treated dendritic cells).*

Rapamycin treated DCs do not have a tolerogenic cytokine secretion profile.

To better characterize their ability to modulate their environment we performed ELISA experiments on the supernatants of DC cultures to determine IL-10 and IL-12p70 secretion capacity of Rapa-DCs, Tac-DCs, Tol-DCs and CTR-DCs (Figure 2 B). **We observe a strong blockade of IL-10 secretion in Rapa-DCs (1.03 ±1.068 (mean ± SD), p<0.001) compared to control Tol-DC (35.07 ± 41.18 fold). IL-10 concentrations were 666.3 ± 819.6 pg/mL (mean ± SD) in CTR-DC, 7.6 ± 4.871 pg/mL in Rapa-DCs and 791.9 ± 890.5 pg/mL in Tac-DCs (Figure 2 B a). As expected, IL-12p70 secretion is the highest for CTR-DCs and very low for Tol-DCs after LPS stimulation. Rapa-DCs (1.35 ± 1 fold p<0.05) and Tac-DCs (11.86 ± 16.77 ns) exhibit a major hindering of IL-12p70 secretion compared to mature CTR-DCs (Figure 2 B b)**. Interestingly, rapamycin significantly reduced the release of both pro-inflammatory and anti-inflammatory cytokines in the supernatant whereas tacrolimus does not have any significant effect. It seems rapamycin might have inhibited the capacity of DCs to respond to LPS stimulation. Thus, the IL-10/IL-12p70 ratio was largely in favour of IL-10 secretion for Tol-DCs compared to CTR-DCs and Rapa-DCs (p=0.01) but it was not significantly different for Tac-DCs (Figure 2 B c).

A reduced TNF alpha secretion in Rapa-DCs after LPS stimulation was also observed (data not shown).

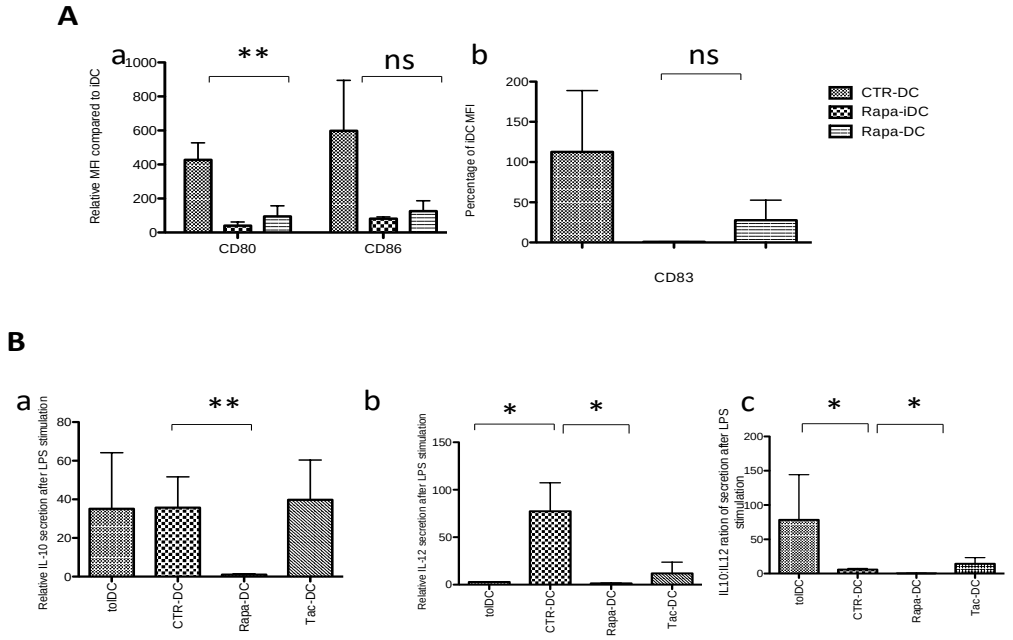

Figure 2. Phenotype and cytokine secretion characterisation of treated MoDCs following LPS maturation. Relative Mean Fluorescence Intensity (MFI) of maturation surface markers CD80, CD83 and CD86 of rapamycin-treated MoDCs **A.** after differentiation (Rapa-iDC) and after LPS maturation (Rapa-DC). Results are standardized to the MFI of the isotype control staining of iDCs (mean ± SEM; one-way ANOVA and Bonferroni post-test **p< 0.01; ns= non significant). **B.** Fold induction of IL-10 **(a)** and IL-12 **(b)** after LPS stimulation in supernatant. **(c)** Ratio of IL-10/IL-12 secretion after LPS stimulation (mean ± SEM; n=5; one-way Anova and Dunnet post-test compared to the CTR-DC group * p<0.05; ** p<0.01). (*CTR-DC= control mature dendritic cells; Tol-DC = tolerogenic dendritic cells; Rapa-DC= rapamycin-treated dendritic cells; Tac-DCs= tacrolimus-treated dendritic cells)*

Rapa-DCs inhibit suppressive markers compared to CTR-DCs and Tac-DCs. [24]

Indoleamine 2,3-dioxygenase (IDO) is a potent immunosuppressive enzyme of 45-kDa expressed in DCs. This monomeric heme-containing protein is the rate-limiting enzyme of the kynurenine pathway which degrades the essential amino acid tryptophan (Trp)[25,26]. Through degradation of the neighbouring Trp, IDO inhibits T-cell proliferation and accelerates T-cell apoptosis. IDO is often associated with tolerogenic DCs although it is not a bona fide Tol-DC marker. Moreover, PD-L1 is an immune-

suppressive protein also associated to a tolerogenic state. Thus, their expression could give insight into the function of DCs treated with immunosuppressive drugs. For this RTqPCR analysis and western-blot were performed following LPS stimulation. Relative transcriptomic expression profiles of IDO and PD-L1 follow the same tendency, Rapa-DCs and iDCs showed a significantly reduced expression of IDO compared to CTR-DCs. No significant change in IDO expression was observed in Tac-DC. However, PD-L1 is significantly overexpressed when compared to control mature DCs (CTR-DCs) and control tolerogenic DCs (Tol-DCs) (Figure 3 Aa - Ab). Moreover, the Western blot confirms the IDO expression pattern found by RTqPCR assay with a strongly hindered production of IDO in Rapa-DCs (Figure 3 B).

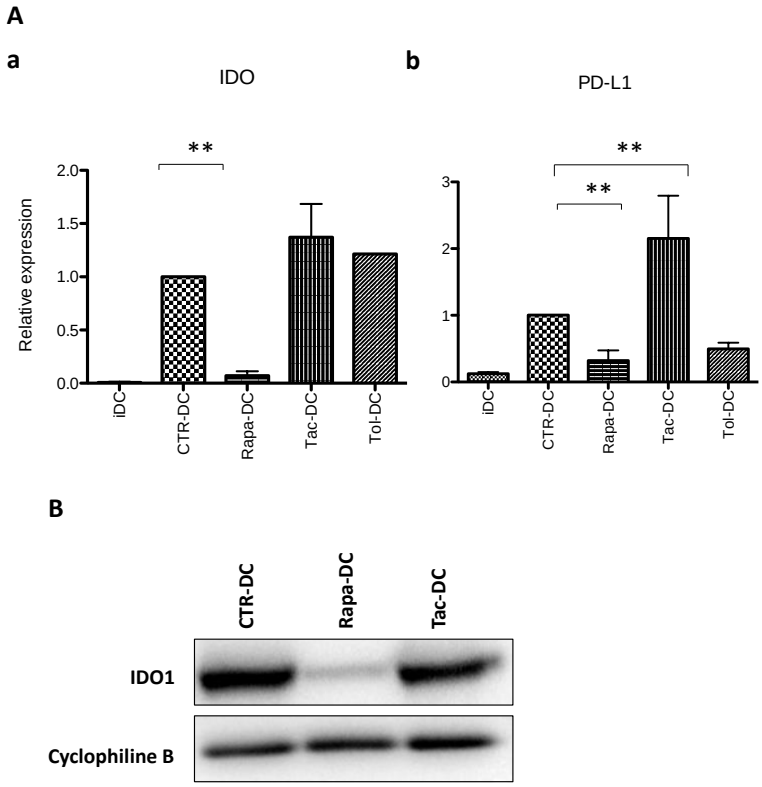

Figure 3. Transcriptomic and protein expression of suppressive markers IDO and PD-L1 on treated MoDCs. A. Relative gene expression of **(a)** IDO1 and **(b)** PD-L1 (one-way ANOVA + Bonferroni's test; ($p<0.002$, n=3) in ether rapamycin or tacrolimus treated-MoDCs. **B.** Western-blot analysis for IDO protein expression following treatment with either immunosuppressive drug rapamycin or tacrolimus (one representative experiment, n=3). *(iDC= immature dendritic cells; CTR-DC= control mature dendritic cells; Tol-DC = tolerogenic dendritic cells; Rapa-DC= rapamycin-treated dendritic cells; Tac-DCs= tacrolimus-treated dendritic cells)*

Rapa-DCs have a reduced capacity to stimulate CD4⁺ T cell proliferation compared to Tac-DCs, Tol-DCs and immature-DCs.

To assess the inhibitory capacity of treated moDCs on PBMC and CD4 T-cell proliferation, we labelled CD4⁺ cells with CFSE and cultured the cells for 7 days with moDCs treated with either tacrolimus or rapamycin.

To assess the suppressive capacity of treated DCs on PBMCs a mixed leukocyte reaction with irradiated DCs and PBMCs was performed (ratio 1:10). Proliferation was assessed after 72 hours of coculture using radioactivity reading of [^{3}H] thymidine incorporation. **Results showed that Rapa-DCs significantly suppress PBMC proliferation in comparison to CTR-DCs (one-way ANOVA and Bonferroni post-test: p<0.0001 and 0.001 respectively, Figure 4 A). However, Tac-DCs do not seem to have the same suppressive effect on PBMCs.**

We compared those co-cultures with activated CD4⁺ T cells using anti-CD3 and anti-CD28 antibodies. Interestingly, Rapa-DCs showed a significantly lower stimulation capacity of CD4⁺ T cells than CTR-DC (p=0.05, Figure 4 B). **No significant difference in CD4⁺ proliferation capacity was seen between Tol-DCs and CTR-DCs probably due to the low number of experiments.**

Moreover, following the 7 days DC/CD4⁺ T cell (1/10 ratio) MLR assay, T cells were harvested and studied by flow cytometry to determine the percentage of natural regulatory T cells (nTregs) CD4⁺CD25^{high}Foxp3⁺CD127⁻. No significant change in nTreg prevalence was observe in T cells cultured with either Rapa-DCs or Tac-DCs compared the control condition (Figure 4 C).

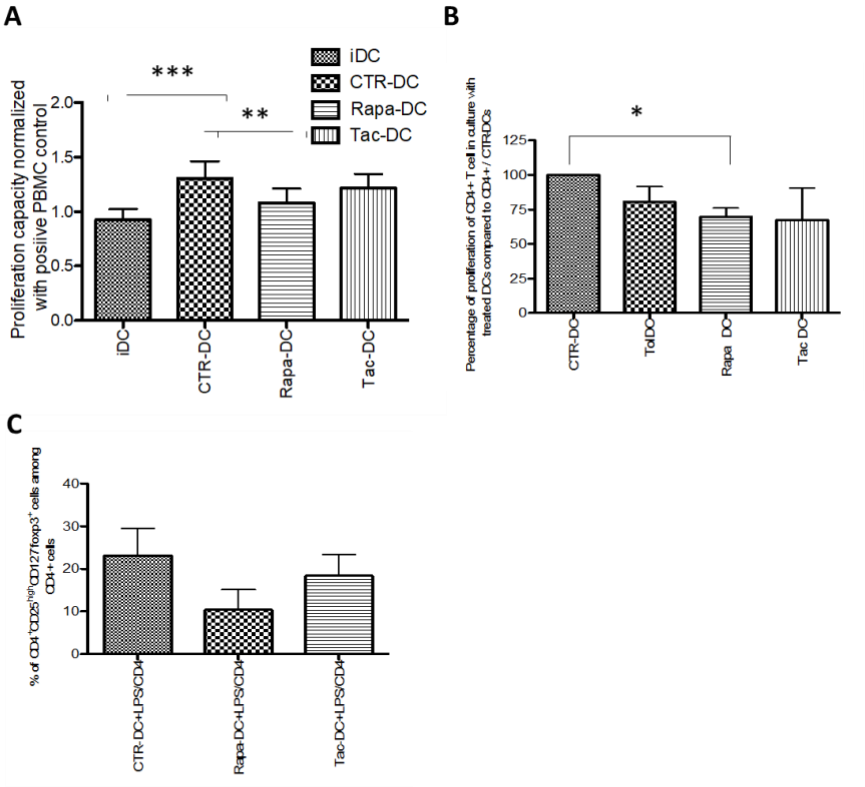

Figure 4. Functional characteristics of treated moDCs on co-cultured PBMCs and T cells. A. Mixed leukocyte reaction with DCs and PBMCs (ratio 1:10). Proliferation was measured after [^{3}H] thymidine incubation. iDC and Rapa-DCs showed PBMC suppression compared to CTR-DCs (*** p<0.0001; **p<0.001). **B.** Percentage of proliferation of CD4+ T cells after co-culture with MoDCs, treated or not, expressed in relative CFSE positivity compared to control. (Mean ± SEM; p=0.037*) **C.** Percentage of nTregs in CD4+ cells with $CD4^+CD25^{high}Foxp3^+$ staining after 6 days of coculture with either mature CTR-DC or treated DCs. (mean ± SEM; n=4, one-way ANOVA NS).
(iDC= immatude dendritic cells; CTR-DC= control mature dendritic cells; Tol-DC = tolerogenic dendritic cells; Rapa-DC= rapamycin-treated dendritic cells; Tac-DCs= tacrolimus-treated dendritic cells)

Discussion

Dendritic cells are professional antigen presenting cells that play a critical role in the initiation and regulation of the adaptive immune responses. The quest to immune tolerance leads to the development of different immunosuppressive strategies. The use

of tolerogenic DCs is a promising therapeutic strategy 13 that could be coupled to other treatments such as a progressive reduction in calcineurin inhibitors (CNI) long after transplantation 27 or the switch from CNI to mTOR inhibitors28.

mTOR inhibitors, such as rapamycin or everolimus inhibit the mammalian target of rapamycin (mTOR), a serine/threonine kinase that regulates protein synthesis and cell growth in response to different factors. It plays a crucial role in T cell activation and differentiation 8. There are several reports showing the capacity of mTOR to control differentiation, cytokine production in DCs. The reports are somehow controversial depending on the dendritic cell subset and culture conditions 29,30.

Rapamycin, as well as other mTOR inhibitors, is often mentioned as a pro-tolerogenic drug through its capacity to induce Tregs in contrast with tacrolimus 31-36. In murine models, Taner et al. have shown prolonged graft survival when recipient derived rapamycin-treated DCs were infused prior to transplantation followed with a low dose of tacrolimus 37,38. *In vivo*, Levitsky et al. recently suggested that a switch from a CNI to a mTOR inhibitor seems to help maintaining tolerance in very few patients with immunological tolerogenic signature 10. In contrast, in a randomised trial in liver transplant recipients, De Simone et al. observed that the incidence of acute rejection was higher in patients receiving mTOR inhibitors alone compared to the groups receiving CNIs or mTOR inhibitors plus CNIs 39.

The effects of CNIs and mTOR inhibitors on $CD4^+$Tcells, the main targets of immunosuppressive (IS) drugs, have been largely investigated *in vitro* and *in vivo* 40,41. However, reports investigating DC function in the presence of rapamycin display controversial results and unanswered questions remain regarding the possibility of using Rapa-DCs as a tolerance inducing strategy.

As previously described, rapamycin has shown capacity to modify the differentiation process of moDCs [29,38]. This is related to the major role of mTOR on moDCs survival and differentiation as this last process is dependent on GM-CSF activation of the mTOR pathway. This explains how rapamycin induces apoptosis in moDCs but not in macrophages nor monocytes. Hence, this explains the immunosuppressive properties of this drug compared to calcineurin inhibitors [22].

In our study we also confirm that rapamycin but not tacrolimus induces tolerogenic phenotype in DCs when added during the differentiation process of monocytes. Tolerogenic DCs are phenotypically characterised as immature or semi-mature DCs. Indeed, compared to CTR-DCs and Tac-DCs, Rapa-DCs exhibit a more immature phenotype with a lower expression of co-stimulatory molecules, notably CD80 and CD86. It is well known that CD80 and CD86 expression on DCs constitutes the most important first step in the co-stimulatory pathway of T cell activation [42]. By reducing their expression on the cell surface, rapamycin seems to induce a tolerogenic profile in moDCs by diminishing their T cell activation capacity. In contrast, tacrolimus does not induce apoptosis in moDCs and does not affect DC phenotype, even at supra-therapeutic concentrations [20,43]. Thus, in line with those findings we have shown that Rapa-DCs were resistant to maturation after LPS stimulation.

To further assess DCs treated with either rapamycin or tacrolimus, their environment was studied by establishing the cytokine secretion profile evaluation before and after maturation. IL-10 is a key cytokine promoter of tolerance which acts by blocking IL-12 synthesis in DCs, downregulating co-stimulatory molecules thus potentiating their tolerogenicity [29,44-46]. Interestingly, rapamycin reduced IL-10 and IL-12p70 secretion compared to CTR-DCs as previously described [29]. **The capacity of secreting pro-inflammatory cytokine IL-12p70 after LPS stimulation was reduced in control Tol-DCs and**

in Rapa-DCs supporting the idea of maturation blockade and therefore a tolerogenic function. Moreover, Rapa-DCs' IL-10 secretion was considerably hindered, even in comparison to control Tol-DCs. This observation is most likely linked to rapamycin blockade of the mTOR pathway, a known regulator of IL-10 in dendritic cells and mononuclear cells [47,48]. Finally, Tac-DCs did not exhibit such a clear-cut overall effect on DC secretion.

This illustrates the essential role played by mTOR in cytokine secretion through NF-kB regulation and STAT3. There were no significant differences in cell surface markers of Tac-DCs nor in the cytokine secretion profile (IL-10, IL-12p40, TNF-α, TGF-β) compared to CTR-DCs but the IL-10/IL-12 ratio was in favour of pro-tolerogenic cytokines. Some experiments in mice suggest that tacrolimus-treated DCs could suppress $CD4^+$ T-cell proliferation and enhance IL-10 secretion [49].

Rapa-DCs exhibit an immature phenotype as observed in Tol-DCs, block secretion of the pro-inflammatory cytokine IL-12p70 but do not increase the suppressive cytokine IL-10. Contrary to Rapa-DCs, Tol-DCs have a higher IL-10 secretion capacity and have the ability to generate regulatory T cells as already described in other studies [13,18].

Navarro-Barriuso et al. have described the transcriptomic profile of different types of Tol-DCs. They have shown that many pathways related to inflammation and chemotaxis were downregulated in rapamycin-tolerogenic DCs in contrast with vitamin-D3 and dexamethasone tolerogenic DCs [50-52].

For the first time to our knowledge, we have shown reduced Indoleamine 2,3-dioxygenase (IDO) mRNA and protein expression in Rapa-DCs compared to the other moDCs subsets. Tryptophan metabolism through IDO activity is important in the development of a tolerogenic state [53]. IDO acts by depleting the cellular

microenvironment of tryptophan leading to a blockage of T cell activation and a promotion of suppressive $CD4^{+}CD25^{high}Foxp3^{+}$ regulatory T cells. Surprisingly, we observed here that rapamycin did not enhance IDO secretion in DCs whereas IDO secretion is maintained in Tac-DCs.

A link between the GM-CSF signalling pathways, AKT/mTOR signalling and IDO has been suggested by Ribechini et al. They have shown that the IDO protein expression and suppressor functions of human suppressor monocytes (monocytes cultured in the presence of GM-CSF and activated by IFN-γ) were impaired by rapamycin treatment 54.

In several animal models, IDO has been shown to be induced by LPS 55,56. The inhibition of IDO mRNA and protein expressions by rapamycin in our study is consistent with the blockade of LPS-dependent maturation of moDCs. As this inhibition of IDO is only observed in the rapamycin treated cells, it suggests a regulatory role of mTOR in IDO protein synthesis. These findings open perspectives for future cellular and animal studies to better understand the link between IDO and mTOR and have important implications for the therapeutic use of mTOR inhibitors in cancer or transplantation.

It has been shown that inhibiting IDO activity in a rodent model of graft tolerance rapidly led to rejection. Using an *in vitro* mesangial cell injury model mimicking chronic allograft nephropathy, Liang et al. demonstrated that tacrolimus, cyclosporine A and mycophenolate mofetil (MMF) upregulated the expression of IDO mRNA and protein in mesangial cell compared to untreated cell whereas rapamycin had no effect 25.
Bracho-Sanchez et al. recently reported that exogeneous supply of IDO maintains immunoregulatory functions in a murine model of DCs suggesting a potential important role of IDO in immune tolerance 57.

We also observed a significantly lower expression of PD-L1 mRNA in Rapa-DCs compared to CTR-DCs or Tac-DCs. In the presence of rapamycin, Haidinger et al. had already shown a reduced surface expression of B7-H1 (also known as PD-L1), a PD-1 ligand that contributes to the negative regulation of T lymphocyte activation[29]. Tacrolimus treatment enhances the PD-L1 mRNA expression in moDCs.

The inhibition of mTOR by rapamycin during the differentiation and maturation process of moDC enhanced some pro-inflammatory molecules with a low IL-10:IL-12p-40 ratio and inhibits anti-inflammatory molecules such as IDO, PD-L1 and IL10. Tacrolimus did exert its immunosuppressive capacity through the expression of anti-inflammatory molecules, with IDO expression being similar to CTR-DCs and showing increased PD-L1 expression. Immature and mature DCs are known to induce the emergence of regulatory T cells in a $CD4^+$ population *in vitro* [58] [59]. Tregs exert a suppressive activity on DCs and help to maintain their tolerogenic steady state in a non-inflammatory environment.

Rapa-DCs and Tac-DCs in co-culture with $CD4^+$ T-cells did not show an increased capacity of inducing Tregs compared to CTR-DCs. Tacrolimus did not reduce the capacity of CTR-DCs to induce Tregs. Mixed leucocyte reaction with DCs and PBMCs showed a significantly reduced proliferation of PBMCs in the presence of Rapa-DCs or iDCs compared to CTR-DCs or Tac-DCs. Rapamycin exerted its immunosuppressive effect on moDCs through phenotypical modifications and global reduction in cytokine secretion rather than through tryptophan metabolism or pro-tolerogenic cytokine secretion. The reduction of surface costimulatory molecules reduced interaction with T cells and T cell activation. Although tacrolimus treatment of moDCs did not reduce PBMC or T cell proliferation in our experiments, it has been shown under other experimental conditions that tacrolimus-treated DCs retained tacrolimus in the cell

cytoplasm and continuously released the drug in the supernatant having an indirect immunosuppressive effect on T cells 19.

In conclusion, we show here the phenotypical and functional changes in moDCs in presence of rapamycin or tacrolimus during the whole differentiation process compared to control iDCs or Tol-DCs. **We conclude that rapamycin treated moDCs exhibit some phenotypical characteristics of tolerogenic DCs that suppress PBMC proliferation but are not fully tolerogenic. Indeed, rapamycin reduces the expression of pro-tolerogenic proteins such as PD-L1, IDO and IL-10 and do not induce Tregs.** This probably explains a part of the anti-tumoral effect of rapamycin and inflammatory effect triggering Th1 response in some patients (interstitial pneumonitis, glomerulonephritis) 60. Tacrolimus does not change the phenotypical or functional characteristics of moDCs at supratherapeutic concentration but interestingly enhances anti-inflammatory proteins such as PD-L1 and IL-10 that could lead to allograft acceptance.

Materials and methods:

CD14+ monocytes magnetic sorting

Buffy coats were obtained from healthy donors, according to the approval by the Ethics Committee (CPP) for Ile de France III. PBMCs were isolated from buffy coats from healthy donors using Ficoll-Hypaque density gradient centrifugation. $CD14^+$ monocytes were isolated by positive selection using micro-magnetic beads (Miltenyi Biotec) according to the manufacturer's protocol.

moDC generation and maturation

$CD14^+$ monocytes were cultured for 7 days in 6-wells plate in 3ml RPMI supplemented with 10%FCS, sodium pyruvate (1mM), HEPES Buffer Solution 1%, minimum essential medium 1% and penicillin and streptomycin 0,1%. DCs were differentiated using GM-

CSF (25ng/mL) and IL-4 (10ng/mL). Rapamycin treated moDC (Rapa-DC) were generated by adding rapamycin to the culture from day 0 at two different doses (1 ng/mL and 5 ng/mL) and tacrolimus-treated moDC (Tac-DC) were generated by adding tacrolimus from day 0 at the dose of 20 ng/mL. Tol-DC were obtained by adding GM-CSF alone without IL-4.

Maturation of DC was induced at day 5 by adding LPS (100ng/mL) for 48 hours. The cells were harvested on day 5 and 7.

Flow cytometry analysis

The following antibodies were used for cell-surface molecule expression analysis: CD14-FITC, CD80-PE-Vio770, CD86-VIoblue, CD83-APC and CD209-PE or the corresponding isotype (Miltenyi Biotech).

Cell viability was analysed using Annexin V FITC staining in combination with propidium iodide. Cells were harvested, washed and labelled with Annexin V FITC for 15 minutes at 4°C then washed again and stained with propidium iodide. Flow cytometry analysis was performed on Beckmann-Coulter Gallios 8 and the data was analysed using Kaluza and Flowjo software.

ELISA

At day 5 and 7, supernatants of moDC cultures were analysed for IL-10, TGF-β, TNF- α and IL-12-p70 concentration.

$CD4^+$ magnetic sorting

PBMC were isolated from blood from healthy donors using Ficoll-Hypaque density gradient centrifugation. Total $CD4^+$ cells were isolated by negative selection using micro-magnetic beads (Miltenyi Biotec) according to the manufacturer's protocol.

moDCs and $CD4^+$ co-culture

moDCs were cultured for 6 days with $CD4^+$ T cells (ratio 1 :10) in a 12 or 24-wells plate in RPMI complete medium.

At the end of the treatment the cells were harvested and analysed by flow cytometry.

T cell proliferation assay

Purified $CD4^+$ T cells (Miltenyi $CD4^+$ T cell isolation kit) were CFSE-Labeled (Invitrogen CellTrace cell proliferation kit) according to the manufacturer's instructions. $2x10^5$ $CD4^+$ T cells were cocultured with $2x10^4$ allogeneic DCs for 7 days. DCs were Tol-DCs, Rapa-DCs, Tac-DCs or immature DCs (iDC).

Mixed Leukocyte Reaction (MLR) - suppression assay

Suppressive activity of DCs was measured by their ability to inhibit the proliferative response of allogenic PBMCs in a MLR. Assays were set up with a mixture of irradiated DCs and PBMCs (1:10) in a round bottom 96-well plate (Corning Costar) and cultured for 48 and 72 hours. Cells were activated with plate-bound anti-CD3 (1 µg/mL), incubated at 37°C for 2 hours before the culture and soluble anti-CD28 (10 ng/mL) (Clinisciences, Montrouge, France) was added at the time of the culture. There are three types of DCs are: iDC, CTR-DC, Rapa-DC and Tac-DC. Proliferation was measured after [^{3}H] thymidine (1µCi/well) (PerkinElmer, Courtaboeuf, France) incubation for the last 18 hours before harvesting. Radioactivity was determined using a β-counter (1450 Trilux, Wallac, Finland). Each proliferation assay was carried out in triplicate and estimated in count per minute (cpm) and results were normalized compared with the positive control.

Western Blot analysis

Cells were lysed (10 minutes on ice) in PY buffer consisting of 20 mM Tris-HCl, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.02% sodium azide, and a cocktail of proteases inhibitors (Roche, Basel, Switzerland) during 10 min on ice. After centrifugation

(14 000rpm, 30 minutes, +4°C), cell debris were removed, and supernatants were collected. Protein concentrations were measured using Bio-Rad Protein Assay according to manufacturer's instructions (Bio-Rad, Marnes la Coquette, France). Total cell extracts were then analyzed by western blotting.

Briefly, proteins were separated by SDS-PAGE electrophoresis using gradient pre-casts gels (4-12% gradient, Bis-Tris, Invitrogen) in standard conditions. Then proteins were transferred on PVDF membranes (Hybon d^{TM}-C Extra, Amersham Biosciences, UK). The latter was blocked for 2 hours at room temperature in blocking buffer containing 2% casein, 0.1% Tween20 (Sigma-Aldrich) and PBS (1X), and then incubated overnight at 4°C with primary antibodies anti-IDO (Cell Signaling Technology, USA) and anti-cyclophilin B 1:400 (Cell Signaling Technology, USA).

Membranes were washed with blocking buffer, then incubated for 1 hour at room temperature with peroxydase-conjugated secondary antibodies (anti-mouse or anti-rabbit, 1:10000) (GE Healthcare, Wauwatosa, USA) and washed again with blocking buffer. Specific protein signals were visualized using Western Lightning® Plus-ECL, Enhanced Chemiluminescence Substrate kit (PerkinElmer, Boston, MA, USA) and signal was detected using BioRAd ChemiDoc XRS+ machine with the ImageLab software.

RTqPCR analysis

Total RNA was extracted using RNeasy minikit according to the manufacturer's instructions (Qiagen SA, Courtaboeuf, France.) Single strand cDNA was synthesized, and real-time quantitative PCR was done using LightCycler 480 SYBR Green I Master Kit (Roche Diagnostics, Meylan, France). We used the following primers:

- IDO sens 5'-CGCTGTTGGAAATAGCTTCTTGC-3', anti-sens 5'-CTTCCCAGAACCCTTCATACACC-3'
- PD-L1 [61] , sens 5'-GGTGGTGCCGACTACAAG-3' , anti-sens 5'-ATTGGTGGTGGTGGTCTTAC-3'

Flow cytometry analysis of CD4⁺ T Cells

PBMCs from patients (EDTA tubes) were isolated from whole blood using Ficoll gradient centrifugation. Cells were stained with anti-CD4, anti-CD25 and anti-CD127 (Miltenyi Biotech) for 30 min at 4°C. After washing, cells were fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences) then stained with anti-FoxP3 (eBiosciences) for 1h at 4°C. Flow cytometry data was acquired with Gallios Flow Cytometer (Beckman Coulter) and results were analysed using Kaluza software (Beckman Coulter). nTreg population was defined as $CD4^+$ $CD25^+$ $CD127^{-/low}$ $FoxP3^+$ lymphocytes and expressed as percentages of $CD4^+$ lymphocytes.

Statistical analysis

Statistical analysis was performed using Prism 5 software for Mac OS X. One way ANOVA, Bonferroni post-tests and unpaired t-tests were performed with p value <0.05 considered as significant. The RTqPCR results were analysed using the "Relative Gene Expression Method" as described by Livak et al [62]. Briefly, individual CT values were normalised using the average CT values for housekeeping genes ($\Delta CT = CT - CT_{HKG}$). Average ΔCT values for each group were then compared with the ΔCT values of the control group (mild recurrence group) ($\Delta\Delta CD = \Delta CT - \Delta CT_{MRgroup}$). An evaluation of $2^{-\Delta\Delta CT}$ then indicated the fold change in gene expression relative to the control group. P-value are noted (*) for $p<0.05$ and (**) $p<0.01$.

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