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Pharmacological induction of tolerogenic dendritic cells and regulatory T cells

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Abstract

Immunosuppressive and anti-inflammatory agents are able to generate tolerogenic DCs, leading, in some cases, to induction or enhancement of regulatory T cells with suppressive activity. This novel mechanism of action, shared by several immunosuppressive and anti-inflammatory agents, is becoming firmly established and contributes to explain their functional properties. The possibility to manipulate DCs in vivo using more or less conventional low molecular weight drugs, enabling them to exert tolerogenic activities, could be exploited to better control a variety of chronic inflammatory conditions, from autoimmune diseases to allograft rejection.

Keywords: Tolerogenic dentritic cell; Pharmacological induction; Regulatory T cell

1. Introduction

Dendritic cells (DCs), a highly specialized antigen-presenting cell (APC) system critical for the initiation of $CD4^+$ T-cell responses are present, in different stages of maturation, in the circulation as well as in lymphoid and non-lymphoid organs, where they exert a sentinel function. After antigen uptake, DCs migrate through the afferent lymph to T-dependent areas of secondary lymphoid organs where they can prime naive T cells. During migration to lymphoid organs, DCs mature into potent APCs by increasing their immunostimulatory properties while decreasing antigen-capturing capacity [1].

It is now clear that DCs can be not only immunogenic but also tolerogenic, both intrathymically and in the periphery [2]. In particular, immature DCs have been found to have tolerogenic properties, and to induce T cells with suppressive activity [3]. Interest in the role of regulatory/suppressor T cells (Treg) cells has recently resurged and, among the various populations of Treg cells described, naturally occurring thymic and peripheral $CD4^+$ T cells that co-express CD25 are currently the most actively investigated [4]. Because DCs are pleiotropic modulators of T-cell activity, pharmacological agents that manipulate DC function to favor the induction of DCs with tolerogenic properties leading to the development of Treg cells could be exploited to inhibit immune responses, and be applied clinically in the treatment of autoimmune diseases and graft rejection.

2. Pharmacological induction of tolerogenic dendritic cells

A variety of immunosuppressive agents are currently used in the treatment of autoimmune diseases and some of them have been instrumental in the control of allograft rejection, giving a decisive impulse to clinical transplantation in the late 1970s. Interestingly, the mechanism of action of major immunosuppressive drugs, like the calcineurin inhibitors cyclosporine A and tacrolimus, has been only understood after almost 20 years of clinical use [5]. Thus, it is perhaps not surprising that a novel mechanism of action shared by many immunosuppressive and anti-inflammatory agents, based on the induction of DCs with tolerogenic properties, has only recently emerged.

Indeed, several immunosuppressive agents currently used to treat allograft rejection and autoimmune diseases have been shown to induce DCs with tolerogenic phenotype and function (Table 1). Notable examples are glucocorticoids [6–9], mycophenolate mofetil (MMF) [10,11], and sirolimus [12,13]. These agents impair DC maturation and inhibit upregulation of costimulatory molecules, secretion of proinflammatory cytokines, in particular IL-12, and allostimulatory capacity. Conversely, controversial effects of calcineurin inhibitors, like cyclosporine A and tacrolimus,

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Table 1
Effects of pharmacologic agents on dendritic cells

Agent	Effects on							References
	Differentiation	Maturation	Costimulatory molecules	IL-12 production	IL-10 production	Allostimulatory capacity	Inhibition of NF-κB	
Acetylsalicylic acid	Enhanced	Inhibited	Reduced	Reduced	Unmodified	Reduced	Yes	[19,20]
Butyric acid	Inhibited	Inhibited	Reduced	n.t.	n.t.	Reduced	n.t.	[21]
Calcineurin inhibitors	Unmodified	Reduced/ unmodified	Reduced/ unmodified	Reduced	Enhanced/ unmodified	Reduced	Yes	[9,14,15,66]
Deoxyspergualin	n.t.	Inhibited		Reduced		Reduced	Yes	[16–18]
Glucocorticoids	Inhibited	Inhibited	Reduced	Reduced	Unmodified	Reduced	Yes	[6–9]
N-acetyl-L-cysteine	n.t.	Inhibited	Reduced	Reduced	n.t.	Reduced	Yes	[22]
Mycophenolate mofetil	Inhibited	Inhibited	Reduced	Reduced	n.t.	Reduced	n.t.	[10,11,42]
Sirolimus	Inhibited	Inhibited/ unmodfied	Reduced	Reduced	n.t.	Reduced	n.t.	[12,13]
Vitamin D3 analogs	Inhibited	Inhibited	Reduced	Reduced	Enhanced	Reduced	Yes	[32,34–39]

have been reported on DC maturation, although these drugs have a clear inhibitory effect on DC, decreasing their cytokine production and allostimulatory capacity [9,14,15]. Other immunosuppressive agents, like desoxyspergualin, also inhibit the allostimulatory capacity of DCs, impairing their maturation and IL-12 production as well [16–18]. Similar effects are exerted on DCs by anti-inflammatory agents, such as acetylsalicylic acid [19,20], butyric acid [21], and *N*-acetyl-L-cysteine [22]. Finally, as detailed in the following, the activated form of Vitamin D, $1,25(OH)_2D_3$, and its analogues have been found to inhibit DC maturation, leading to reduced expression of costimulatory moleules and alloreactive capacity.

As summarized in Table 1, a common feature of drugs targeting DCs is their capacity to inhibit NF-κB, a signal transduction pathway crucially involved in the inflammatory response. The NF-κB family member RelB is required for myeloid DC differentiation [23], and controls APC function via regulation of CD40 and MHC class II molecule expression [24]. Interestingly, antigen-pulsed DCs in which RelB function is inhibited can induce regulatory $CD4^+$ T cells able to transfer tolerance to primed recipients in an IL-10-dependent fashion [25]. Another common feature of DC-targeting drugs is the inhibition of IL-12, a cytokine critically involved in the development of Th1-dependent autoimmune diseases [26]. In contrast, only $1,25(OH)_2D_3$ and its analogues, among the pharmacological agents tested, are able to enhance the secretion by DCs of IL-10, a potent anti-inflammatory cytokine (Table 1). Although it is somewhat difficult to directly translate induction of tolerogenic DCs into establishment of T-cell tolerance [27], calcineurin inhibitors tend to inhibit tolerance induction, whereas this is favored by MMF, sirolimus, deoxyspergualin, and $1,25(OH)_2D_3$. This relationship suggests a link between arrest of DC maturation and tolerance induction, consistent with the capacity of immature DCs to induce regulatory T cells [2].

3. Tolerogenic dendritic cells induced by Vitamin D receptor ligands lead to enhancement of regulatory T cells

The activated form of Vitamin D, $1,25(OH)_2D_3$, is a secosteroid hormone that has, in addition to its central function in calcium and bone metabolism, important effects on the growth and differentiation of many cell types, and pronounced immunoregulatory properties [28–32]. The biological effects of $1,25(OH)_2D_3$ are mediated by the Vitamin D receptor (VDR), a member of the superfamily of nuclear hormone receptors functioning as a ligand-activated transcription factor that binds to specific DNA sequence elements, Vitamin D responsive elements, in Vitamin D responsive genes and ultimately influences their rate of RNA polymerase II-mediated transcription [33].

APCs, and notably DCs, express the VDR and are key targets of VDR ligands, both in vitro and in vivo. A number of studies has clearly demonstrated that $1,25(OH)_2D_3$ and its analogues inhibit the differentiation and maturation of DCs [34–39]. These studies, performed either on monocyte-derived DCs from human peripheral blood or on bone-marrow derived mouse DCs, have consistenly shown that in vitro treatment of DCs with $1,25(OH)_2D_3$ and its analogues leads to downregulated expression of the costimulatory molecules CD40, CD80, CD86, and to decreased IL-12 and enhanced IL-10 production, resulting in decreased T-cell activation. The block of maturation, coupled with abrogation of IL-12 and strongly enhanced production of IL-10, highlight the important functional effects of $1,25(OH)_2D_3$ and its analogues on DCs and are, at least in part, responsible for the induction of DCs with tolerogenic properties. The combination of these effects can explain the capacity of VDR ligands to induce DCs with tolerogenic properties that favor suppressor T-cell enhancement. DCs are able to synthesize $1,25(OH)_2D_3$ in vitro as a consequence of increased 1α-hydroxylase expression [40], and this could also con-

tribute to promote regulatory T-cell induction. It is also possible that $1,25(OH)_2D_3$ may contribute to the physiological control of immune responses, and possibly be also involved in maintaining tolerance to self antigens, as suggested by the enlarged lymph nodes containing a higher frequency of mature DCs in VDR-deficient mice [41].

The prevention of DC differentiation and maturation as well as the modulation of their activation and survival leading to DCs with tolerogenic phenotype and function play an important role in the immunoregulatory activity of $1,25(OH)_2D_3$. These effects are not limited to in vitro activity: $1,25(OH)_2D_3$ and its analogues can also induce DCs with tolerogenic properties in vivo, as demonstrated in models of allograft rejection by oral administration directly to the recipient [42] or by adoptive transfer of in vitro-treated DCs [41]. Tolerogenic DCs induced by a short treatment with $1,25(OH)_2D_3$ are probably responsible for the capacity of this hormone to induce $CD4^+CD25^+$ suppressor T cells ($CD25^+$Treg) that are able to mediate transplantation tolerance [42].

VDR ligands are interesting immunomodulators, as shown by their capacity to enhance $CD25^+$Treg cells and promote tolerance induction in transplantation [42] and autoimmune disease [43] models. A short treatment with $1,25(OH)_2D_3$ and mycophenolate mofetil, a selective inhibitor of T and B cell proliferations [44] that also modulates APCs [10], induces tolerance to islet allografts associated with an increased frequency of $CD4^+CD25^+$ regulatory T cells able to adoptively transfer transplantation tolerance (Fig. 1). The induction of tolerogenic DCs could indeed represent a therapeutic strategy promoting tolerance to allografts [31,45] and the observation that immature myeloid DCs can induce T-cell tolerance to specific antigens in human volunteers represents an important proof of concept for this approach [46]. Also the direct effects of VDR ligands on T cells could play a role in inhibiting graft rejection, in particular the inhibition of IL-2, by impairing the formation of the transcription factor complex NF-AT [47,48], and IFN-γ production, through interaction of the ligand-bound VDR complex with a VDRE in the promoter region of the cytokine [49].

$CD25^+$Treg cells able to inhibit the T-cell response to a pancreatic autoantigen and to significantly delay disease transfer by pathogenic $CD4^+CD25^-$. T cells are also induced by treatment of adult non-obese diabetic (NOD) mice with a VDR ligand (Fig. 2). This treatment arrests insulitis, blocks the progression of Th1 cell infiltration into the pancreatic islets, and inhibits type 1 diabetes development at non-hypercalcemic doses [43]. Although the type 1 diabetes and islet transplantation models are quite different, in both cases administration of VDR ligands doubles the number of $CD25^+$Treg cells, in the spleen and pancreatic lymph nodes, respectively (Figs. 1 and 2).

In both islet transplantation and type 1 diabetes models, treatment with VDR ligands has a profound effect on the migration of effector T cells, preventing their entry into the pancreatic islets [42,43]. It remains to be seen if VDR ligands can also affect the migration of $CD25^+$Treg cells by regulating their chemokine receptor expression, or by modulating chemokine production in target tissues such as pancreatic islets. Our preliminary experiments show evidence for the latter possibility.

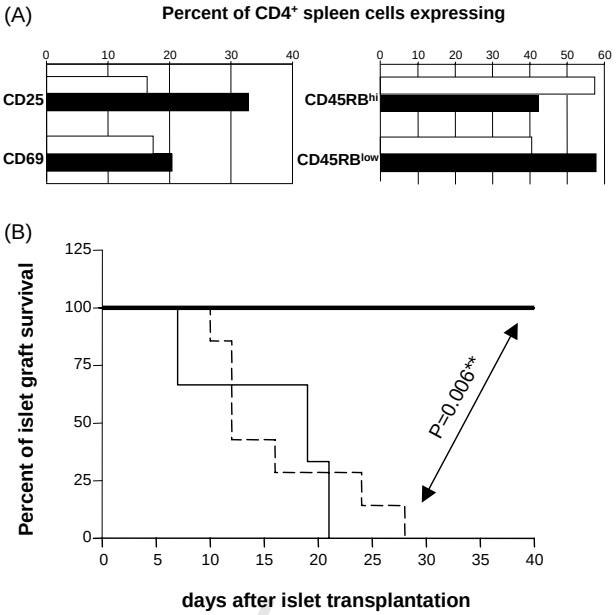

Fig. 1. Treatment with $1,25(OH)_2D_3$ and MMF enhances the frequency of $CD4^+CD25^+$ T cells able to transfer transplantation tolerance. (A) Spleen cells pooled from untreated, acutely rejecting (30 days after transplantation, open bars) and tolerant (180 days after transplantation, filled bars) mice treated from day −1 to day 30 with MMF (100 mg/kg p.o., daily) and $1,25(OH)_2D_3$ (5 μg/kg p.o., 3 times weekly) were stained with mAbs specific for the indicated surface molecules, and analyzed by flow cytometry. Acquisition was performed on $CD4^+$ cells. (B) Naïve BALB/c mice rendered diabetic by a single injection of streptozotocin were transferred with 0.5×10^6 $CD4^+CD25^+$ (thick line) or 4×10^6 $CD4^+CD25^-$ (thin line) T cells isolated from tolerant mice upon $CD4^+$ T cells transfer. Two days after they were transplanted with B6 islets. As controls, naive BALB/c mice were transplanted with B6 islets (broken line). The function of islet allografts was monitored two times weekly by blood glucose measurement. The *P*-value was determined by Fisher's exact test. See ref. [42] for further details.

Regulatory $CD4^+$ T cells express CCR4, CCR8, and CCR5, displaying a rather unique chemokine receptor profile [50]. We have recently documented that, in contrast to the high production by circulating human myeloid DCs (M-DCs), the CCR4 ligands CCL17 and CCL22 are poorly produced by plasmacytoid DCs (P-DCs) [51]. It is noteworthy that blood-borne M-DCs, in contrast to P-DCs, constitutively produce CCL17 and CCL22 ex vivo [51]. This selective constitutive production of CCR4 ligands by immature M-DCs could lead to the preferential attraction of $CD25^+$Treg cells, a mechanism expected to favor tolerance induction. Intriguingly, the production of CCL22, a CCR4 ligand, is markedly enhanced by $1,25(OH)_2D_3$ in blood M-DCs but not P-DCs (Penna et al., manuscript in preparation). Besides maintaining peripheral immunolog-

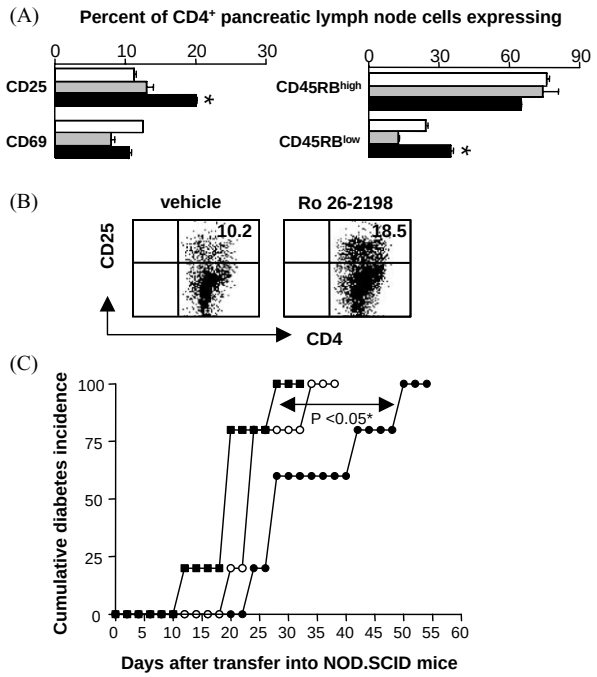

Fig. 2. Treatment of adult NOD mice with the VDR ligand Ro 26-2198 enhances the frequency of $CD4^+CD25^+$ cells able to control the diabetogenic potential of $CD4^+CD25^-$ cells. (A) Positively selected $CD4^+$ T cells were stained with mAbs specific for the indicated surface molecules and analyzed by flow cytometry. Acquisition was performed on $CD4^+$ cells. Bars represent the percent of lymph node $CD4^+$ T cells expressing the indicated surface molecules from untreated 8-week-old (open bars) or 20-week-old mice treated five times weekly from 8 to 16 weeks of age with vehicle (grey bars) or with 0.03 μg/kg Ro 26-2198 (filled bars). Data are presented as mean ± S.E. of three separate experiments. The *P*-values were calculated by Mann–Whitney *U* test (*$P < 0.05$ vs. 8-week-old NOD mice). (B) Positively selected $CD4^+$ pancreatic lymph node cells isolated from 20-week-old NOD mice treated five times weekly from 8 to 16 weeks of age with vehicle or with 0.03 μg/kg Ro 26-2198 were stained with mAbs specific for the indicated surface molecules and analyzed by flow cytometry. Acquisition was performed on $CD4^+$ cells. (C) Eight-week-old NOD-SCID mice (five mice/group) were injected with 4×10^6 CD25-depleted splenocytes from newly diabetic NOD mice alone (filled squares) or together with 2×10^6 $CD4^+$ cells isolated from 20-week-old NOD mice treated five times weekly from 8 to 16 weeks of age with vehicle (open circles) or with 0.03 μg/kg Ro 26-2198 (filled circles). Diabetes development was monitored twice weekly by measurement of blood glucose levels. The *P*-value was calculated by Mann–Whitney *U* test. See ref. [43] for further details.

ical tolerance in homeostatic conditions, Treg cells could turn-off and limit ongoing inflammatory responses. Inflammatory signals strongly induce maturation and influx of both M-DCs and P-DCs to secondary lymphoid tissues [52], and maturation of M-DCs and P-DCs enhances their production of several proinflammatory chemokines that can potentially attract different T-cell subsets. Interestingly, maturing P-DCs, similarly to activated B cells, produce large quantities of the CCR5 ligand CCL4 [51]. Thus, in analogy with the proposed role for CCL4 in $CD25^+$Treg-cell attraction by activated B cells, mature P-DCs could recruit these cells to limit ongoing inflammatory responses.

However, tolerogenic DCs may not always be necessarily involved in the generation of Treg cells by VDR ligands. A combination of $1,25(OH)_2D_3$ and dexamethasone has been shown to induce human and mouse naive $CD4^+$ T cells to differentiate in vitro into Treg cells, even in the absence of APCs [53]. These Treg cells produced IL-10, but no IL-5 nor IFN-γ, thus distinguishing them from the previously described Tr1 cells [54]. Upon transfer, the IL-10-producing Treg cells could prevent central nervous system inflammation, indicating their capacity to exert a suppressive function in vivo [53]. Thus, although DCs appear to be primary targets for the immunomodulatory activities of VDR ligands, they can also act directly on T cells, as expected by VDR expression in both cell types and by the presence of common targets in their signal transduction pathways, such as the nuclear factor NF-κB that is down-regulated in APCs [55] and in T cells [53].

4. Upregulation of inibitory receptor expression in dendritic cells by VDR ligands

To further characterize mechanisms accounting for the induction of DCs with tolerogenic properties by VDR ligands, we have examined the expression of immunoglobulin-like transcripts (ILT), receptors structurally and functionally related to killer cell inhibitory receptors (KIR) [56], by $1,25(OH)_2D_3$-treated DCs. ILT family members possess a long cytoplasmic tail containing immunoreceptor tyrosine-based activatory (ILT1) or inhibitory (ILT2–ILT10) motifs [57]. The high homology between ILTs and KIRs suggests that ILTs can also interact with class I MHC molecules, but this has been confirmed only for ILT2 and ILT4 [58]. A connection between ILTs and tolerance induction has been established by the observation that $CD8^+CD28^-$ suppressor T cells upregulate ILT3 and ILT4 expression on DCs, rendering them tolerogenic [59]. Such tolerogenic DCs have been reported to anergize alloreactive $CD4^+$ $CD45RO^+$ $CD25^+$ T cells converting them into regulatory T cells which, in turn, continue the cascade of suppression by tolerizing other DCs [60]. We have found that incubation of monocyte-derived human DCs, either immature or during maturation, with $1,25(OH)_2D_3$ leads to a selective upregulation of ILT3 (three- to six-fold increase in MFI), but not of ILT1 or ILT4 (Fig. 3), nor ILT2 or ILT5 (data not shown). Analysis of DC subsets revealed a higher ILT3 expression on P-DCs compared to M-DCs [61,62]. CD40 ligation reduced ILT3 expression on M-DCs but had little effect on P-DCs [63]. Maintaining high ILT3 expression on P-DCs matured via CD40 ligation is of interest, because this cell population has been shown to induce $CD8^+$ regulatory T cells able to suppress the proliferation of naïve $CD8^+$ cells through an IL-10-dependent pathway [64]. While incubation with $1,25(OH)_2D_3$ did not affect the already high ILT3 expression by P-DCs, it increased its expression on M-DCs considerably [63]. The down-regulation

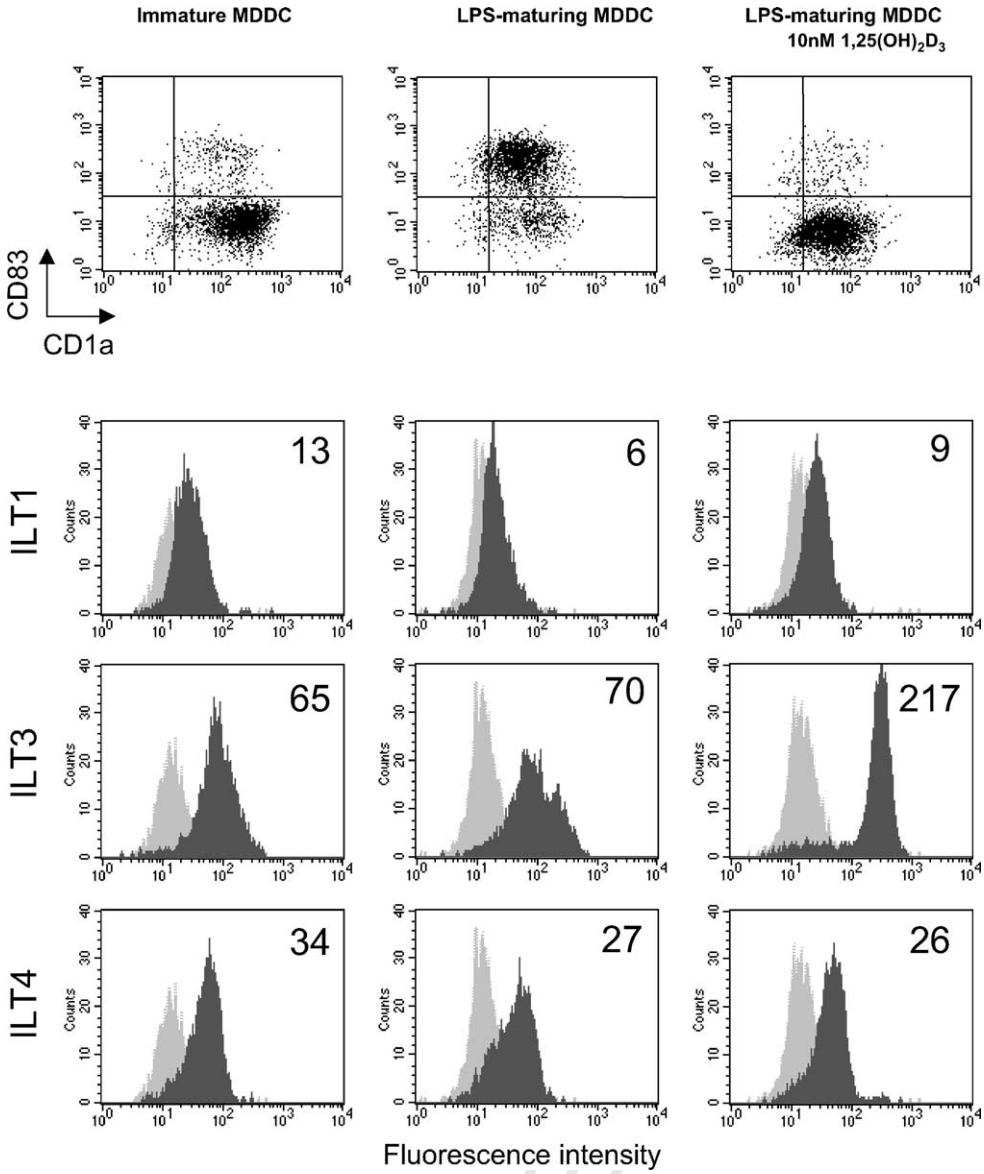

Fig. 3. $1,25(OH)_2D_3$ enhances ILT3 expression on DCs. Monocyte-derived human DCs, obtained as previously described [34], were incubated for 48 h with 10 nM $1,25(OH)_2D_3$, either when immature (iMDDC) or during LPS-induced maturation (mMDDC). Surface expression of the indicated ILT molecules was determined by cytofluorimetry, as described [62]. The upper panels show the degree of maturation, as indicated by CD83 expression. In the lower panels, light grey histograms represent staining with an isotype control, and the dark grey histograms staining with the indicated anti-ILT mAbs. Geometric mean fluorescence intensity (MFI) values are shown in the upper right corner.

of ILT3 on M-DCs by T-cell-dependent signals, and the up-regulation of this inhibitory receptor by $1,25(OH)_2D_3$ in DCs suggests a novel mechanism for the immunomodulatory properties of this hormone that could play a role in the control of T-cell responses.

As tolerogenic DCs induced by different pharmacological agents share several properties (Table 1), we analyzed up-regulation of ILT3 expression in immature and mature DCs by selected immunomodulatory agents. Results in Fig. 4 demonstrate that $1,25(OH)_2D_3$ markedly upregulates ILT3 expression on both immature and mature DCs, whereas IL-10 has a much less pronounced effect, and dexamethasone no observable activity. In the same experiment, all the three agents inhibited DC maturation, as shown by decreased CD83 expression. These results indicate that drug-induced ILT3 upregulation is not a general feature of tolerogenic DCs, as proposed by a recent study [60], and are consistent with the view that VDR ligands and glucocorticoids modulate DCs using distinctive pathways [65]. In any case, our preliminary results show that $1,25(OH)_2D_3$-induced up-regulation of ILT3 on DCs is involved in the inhibition of T-cell responsiveness, further supporting an important role of this inhibitory receptor in the tolerogenic function of DCs.

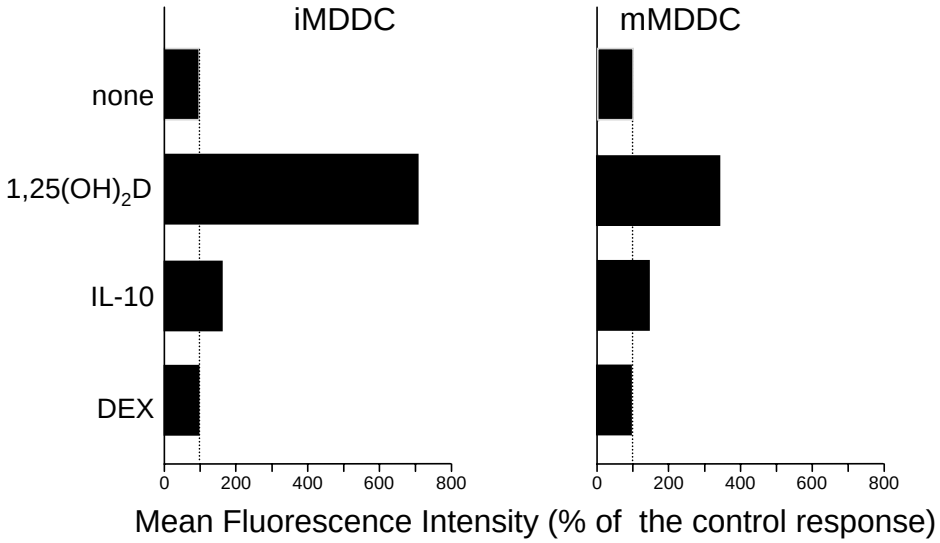

Fig. 4. Dexamethasone fails to enhance ILT3 expression on DCs. Monocyte-derived human DCs, obtained as previously described [34], were incubated for 48 h with 10 nM $1,25(OH)_2D_3$, 10 ng/ml IL-10, or 100 nM dexamethasone (DEX), either when immature (iMDDC) or during LPS-induced maturation (mMDDC). Surface expression of ILT molecules was determined by cytofluorimetry, as described [62]. Results are expressed as percent of the control geometric MFI.

5. Conclusions

Most immunosuppressive and anti-inflammatory drugs share the capacity to target DCs, rendering them tolerogenic and, in some cases, fostering the induction of regulatory T cells. Among immunosuppressive and anti-inflammatory drugs, VDR ligands are particularly interesting agents able to directly target DCs and T cells, leading to the inhibition of pathogenic effector T cells and enhancing the frequency of T cells with suppressive properties, effects largely mediated via induction of tolerogenic DCs. Multiple mechanisms probably contribute to induction of DC tolerogenicity by VDR ligands, and a potentially important one we are actively exploring is based on their capacity to upregulate the inhibitory receptor ILT3 expression. Analysis of inhibitory receptor expression by tolerogenic DCs may indeed represent a fruitful area for further research. VDR ligands can also modulate chemokine secretion, enhancing the production of chemokines able to recruit regulatory/suppressor T cells. It remains to be seen if VDR ligands can also directly affect chemokine production by the target organ in inflammatory conditions. The immunoregulatory activities of VDR ligands, coupled with the absence of major side effects once calcemia is under control, have been translated into effective immunointervention in a variety of autoimmune disease [30] and graft rejection [31] models, highlighting their potential applicability in chronic inflammatory conditions sustained by autoreactive or alloreactive immune responses. In addition to the topical treatment of psoriasis, a Th1-mediated autoimmune disease of the skin where VDR ligands are the most used drugs, these agents may find a broader application in the treatment of inflammatory conditions, where their effects on DCs and Treg cells could turn out to be highly beneficial.

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