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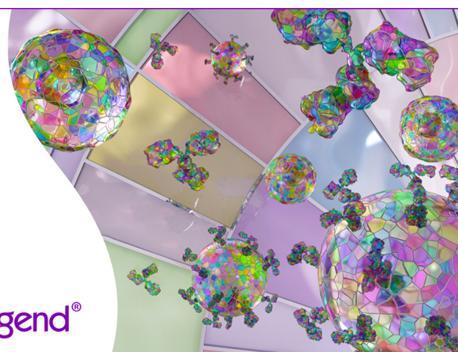

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1,25-Dihydroxyvitamin D_3 Selectively Modulates Tolerogenic Properties in Myeloid but Not Plasmacytoid Dendritic Cells[1]

Giuseppe Penna,* Susana Amuchastegui,* Nadia Giarratana,* Kenn C. Daniel,* Marisa Vulcano,† Silvano Sozzani,† and Luciano Adorini[2]*

1,25-Dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) is an immunomodulatory agent inducing dendritic cells (DCs) to become tolerogenic. To further understand its mechanisms of action, we have examined the effects of 1,25$(OH)_2D_3$ on tolerogenic properties of blood myeloid (M-DCs) and plasmacytoid (P-DCs) human DC subsets. Exposure of M-DCs to 1,25$(OH)_2D_3$ up-regulated production of CCL22, a chemokine attracting regulatory T cells, whereas production of CCL17, the other CCR4 ligand, was reduced. 1,25$(OH)_2D_3$ also decreased IL-12p75 production by M-DCs, as expected, and inhibited CCR7 expression. 1,25$(OH)_2D_3$ treatment markedly increased $CD4^+$ suppressor T cell activity while decreasing the capacity of M-DCs to induce Th1 cell development. Surprisingly, 1,25$(OH)_2D_3$ did not exert any discernible effect on tolerogenic properties of P-DCs, and even their high production of IFN-α was not modulated. In particular, the intrinsically high capacity of P-DCs to induce $CD4^+$ suppressor T cells was unaffected by 1,25$(OH)_2D_3$. Both DC subsets expressed similar levels of the vitamin D receptor, and its ligation by 1,25$(OH)_2D_3$ similarly activated the primary response gene *cyp24*. Interestingly, 1,25$(OH)_2D_3$ inhibited NF-κB p65 phosphorylation and nuclear translocation in M-DCs but not P-DCs, suggesting a mechanism for the inability of 1,25$(OH)_2D_3$ to modulate tolerogenic properties in P-DCs. ***The Journal of Immunology,*** **2007, 178: 145–153.**

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The activated form of vitamin D, 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$),[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 (1–4). 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 agonist-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 (5).

APCs, and notably dendritic cells (DCs), express the VDR and are key targets of VDR agonists, both in vitro and in vivo. A number of studies has clearly demonstrated that 1,25$(OH)_2D_3$ and its analogs markedly modulate DC phenotype and function (6–11). These studies, performed either on monocyte-derived DCs from human peripheral blood or on bone marrow-derived mouse DCs, have consistently shown that in vitro treatment of DCs with 1,25$(OH)_2D_3$ and its analogs leads to down-regulated 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 analogs on DCs and are, at least in part, responsible for the induction of DCs with tolerogenic properties (12).

DCs are heterogeneous in terms of origin, morphology, phenotype, and function. Two distinct DC subsets, defined as myeloid DCs (M-DCs) and plasmacytoid DCs (P-DCs), have been identified in human and mouse blood (13–15). Human and mouse DCs of lymphoid and myeloid origin express a different repertoire of pattern recognition receptors and are characterized by a different cytokine production profile. P-DCs produce large amounts of type I IFNs in response to viruses and other pathogens, whereas M-DCs are the main IL-12 producers (16). Likewise, P-DCs and M-DCs produce a different pattern of chemokines (17) and exhibit a different migratory response upon exposure to a variety of ligands (18). The different properties of DC subsets highlight their complementary roles in the induction and regulation of innate and adaptive immune responses. M-DCs are the most efficient APCs directly able to prime naive T cells and can become, under different conditions, immunogenic or tolerogenic (19). P-DCs, under steady-state conditions, appear to play a key role in maintaining peripheral immune tolerance, and may be considered naturally occurring tolerogenic DCs (16).

Although the immunomodulatory effects of 1,25$(OH)_2D_3$ on DCs are well established, the capacity of this hormone to modulate DC subsets has not yet been addressed. In the present study, we have analyzed the immunomodulatory effects of 1,25$(OH)_2D_3$ on human blood M-DCs and P-DCs. The results demonstrate a differential capacity of 1,25$(OH)_2D_3$ to modulate cytokine and chemokine production in DC subsets, showing marked effects in M-DCs and negligible ones in P-DCs. Also inhibition of Th1 development and enhancement of $CD4^+$ suppressor T cell activity are selectively induced by 1,25$(OH)_2D_3$ in M-DCs but not P-DCs. This different capacity of DC subsets to respond to 1,25$(OH)_2D_3$ is

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[3] Abbreviations used in this paper: 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; DC, dendritic cell; M-DC, myeloid DC; P-DC, plasmacytoid DC; VDR, vitamin D receptor; BDCA, blood DC Ag; ILT, Ig-like transcript.

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not due to a diverse VDR expression or to VDR-dependent signal transduction, but is associated with differential effects of this hormone on NF-κB p65 phosphorylation and nuclear translocation in DC subsets. The selective modulation of tolerogenic properties in DC subsets has important implications for understanding the immunoregulatory role of $1,25(OH)_2D_3$.

Materials and Methods

Peripheral blood DC purification and culture

PBMCs were isolated from buffy coats by Ficoll gradient (Pharmacia Biotech), and peripheral blood M-DCs and P-DCs were magnetically sorted with blood DC Ag (BDCA)-1 and BDCA-4 cell isolation kits (Miltenyi Biotec), respectively, as described (20), to a purity of 95–98% in both cases. Blood M-DCs and P-DCs (2×10^4 cells/well) were cultured as described (18) in RPMI 1640 culture medium supplemented with 10% FCS (HyClone), 2 mM L-glutamine, 50 μg/ml gentamicin, 1 mM sodium pyruvate, and 1% nonessential amino acids (complete medium) containing 10 ng/ml recombinant human GM-CSF (BD Pharmingen) or 20 ng/ml IL-3 (BD Pharmingen), respectively. DC subset cells were stimulated with 1 μg/ml LPS (*Escherichia coli* O111:B4; Sigma-Aldrich), 6 μg/ml CpG oligonucleotide 2006 (MWG Biotec), CD40L (CD154)-transfected J558L cells at a ratio of 4:1, or PMA (100 ng/ml) plus ionomycin (1 μg/ml), both from Sigma-Aldrich. Crystalline $1,25(OH)_2D_3$ was a gift of M. Uskokovic (BioXell), dexamethasone was purchased from Sigma-Aldrich, and recombinant human IL-10 from BD Pharmingen.

$CD4^+$ T cell purification and culture

$CD4^+$ T cells were purified from PBMC by negative selection with CD4 T cell isolation kit (Miltenyi Biotec), and $CD4^+CD45RA^+$ T cells were subsequently negative selected with CD4CD45RO beads (Miltenyi Biotec). $CD4^+$ T cell were cocultured with allogenic ex vivo purified blood M-DC or P-DC in complete medium with or without 10 nM $1,25(OH)_2D_3$. After 6 days of culture, $CD4^+$ T cells were stained for the detection of intracellular IFN-γ, IL-4, and IL-2 as previously described (21).

Assay for suppressive activity

Peripheral blood naive $CD4^+$ T cells, purified as previously described from donor C, were cultured in the presence of purified DC subsets from donor D (ratio 1:10) with or without 10 nM $1,25(OH)_2D_3$. At 7–10 days after primary stimulation, cells were restimulated under the same conditions. After two rounds of restimulation, $CD4^+$ T cells were tested for suppressive activity. The read-out system was composed of $CD4^+CD25^-$ cells from donor A PBMCs cultured in the presence of 1:10 LPS-matured monocyte-derived DCs from donor B. To evaluate the induction of regulatory T cells, graded amounts of $CD4^+$ T cells from donor C, generated by three rounds of restimulation with allogeneic DC subsets from donor D, were added to the MLR read-out culture system. The suppression assay was performed in the presence of 1 μg/ml anti-human CD3 mAb. After 72 h of culture, IFN-γ production in culture supernatants was quantified by two-site ELISA.

Chemokine and cytokine quantification

Concentrations of CCL17/TARC (R&D Systems), CCL22/MDC (R&D Systems), IL-12p75 (BD Pharmingen), IFN-α (Bender MedSystem) in DC subset culture supernatants were evaluated by sandwich ELISA according to the manufacturer's instructions. IFN-γ concentration was determined using paired mAb, as described previously (6). Detection limits were 5–15 pg/ml.

Flow cytometric analysis

Flow cytometric analysis was performed as previously described (17), in the presence of 200 μg/ml mouse IgG, using the mAbs anti-CD1c (BDCA-1) FITC or PE, anti-BDCA-2 FITC, or PE (Miltenyi Biotec), anti-HLA-DR (BD Pharmingen), and anti-CCR7 PE (BD Pharmingen). Analysis of NF-κB p65 phosphorylation was performed using the K10-895.12.50 PE mAb recognizing the phosphorylated S529 in the transactivation domain of NF-κB p65 (BD Biosciences), according to the manufacturer's protocol. Cells were analyzed with a LSR flow cytometer (BD Biosciences) using CellQuest software.

Real-time quantitative RT-PCR

RNA was extracted using TRIzol (Invitrogen Life Technologies) according to the manufacturer's instruction, followed by a cleanup with the RNeasy kit (Qiagen). Reverse transcription was performed, and real-time quantitative RT-PCR of total cDNA using specific primers was conducted using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) and TaqMan chemistry. The primers used are commercially available from Applied Biosystems as assays-on-demand. Relative quantification of target cDNA was determined by arbitrarily setting the control value to 1 and changes in cDNA content of a sample were expressed as a multiple thereof. Differences in cDNA input were corrected by normalizing to β-actin or GAPDH signals. To exclude amplification of genomic DNA, RNA samples were treated with DNase (Sigma-Aldrich).

NF-κB nuclear translocation

Cells placed on poly-L-lysine-coated glass slides were permeabilized with 0.1% Triton X-100 for 4 min at 4°C and stained with rabbit anti-NF-κB p65 Ab (Santa Cruz Biotechnology), followed by polyclonal anti-rabbit FITC (Sigma-Aldrich). After washing, cells were stained with 2.5 μg/ml propidium iodide. Negative controls were performed by incubation with appropriate isotype-matched primary Abs. The slides were then washed again and mounted with 90% glycerol/PBS. Slides were analyzed with an MRC-1024 confocal microscope (Bio-Rad). Images were acquired and processed with Laser Sharp 3.2 software (Bio-Rad).

Results

$1,25(OH)_2D_3$ differentially modulates CCR4 ligand production in M-DCs without affecting it in P-DCs

We have previously shown that production of the homeostatic chemokines CCL17 and CCL22 is strictly compartmentalized in human DC subsets, and is a prerogative of M-DCs (17). Data in Fig. 1 confirm the selective capacity of M-DCs but not P-DCs to constitutively produce CCL17 and CCL22. In addition, they clearly show an opposite effect of $1,25(OH)_2D_3$ on the production of these CCR4 ligands by M-DCs. CCL17 production is inhibited dose-dependently by $1,25(OH)_2D_3$ in culture supernatants of unstimulated M-DCs after both 24 and 48 h of culture, whereas a marked up-regulation of CCL22 production is observed in the same culture supernatants. Conversely, neither CCL22 nor CCL17 production was induced in P-DCs by $1,25(OH)_2D_3$.

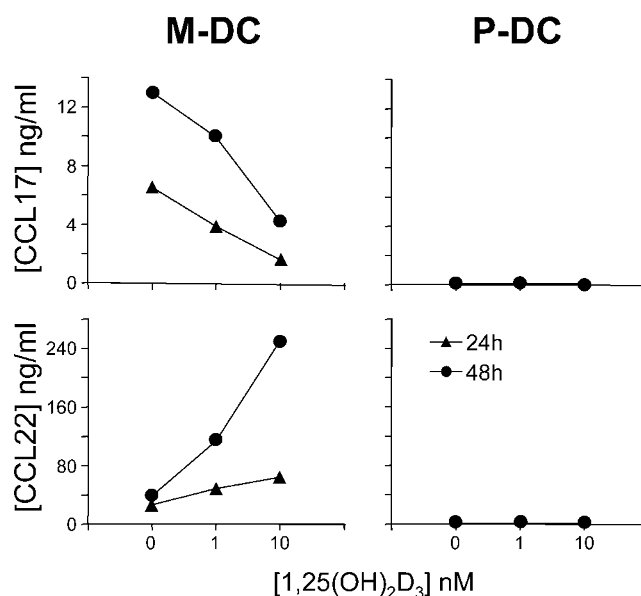

FIGURE 1. $1,25(OH)_2D_3$ modulates constitutive production of CCR4 ligands in M-DCs but not in P-DCs. Magnetically purified blood M-DCs and P-DCs (2×10^4 cells/well) were cultured in GM-CSF or IL-3, respectively, in the presence of the indicated concentrations of $1,25(OH)_2D_3$. Secreted chemokines (ng/ml) were detected 24 h (▲) and 48 h later (●) by two-site ELISA. The data are from one representative experiment of five performed.

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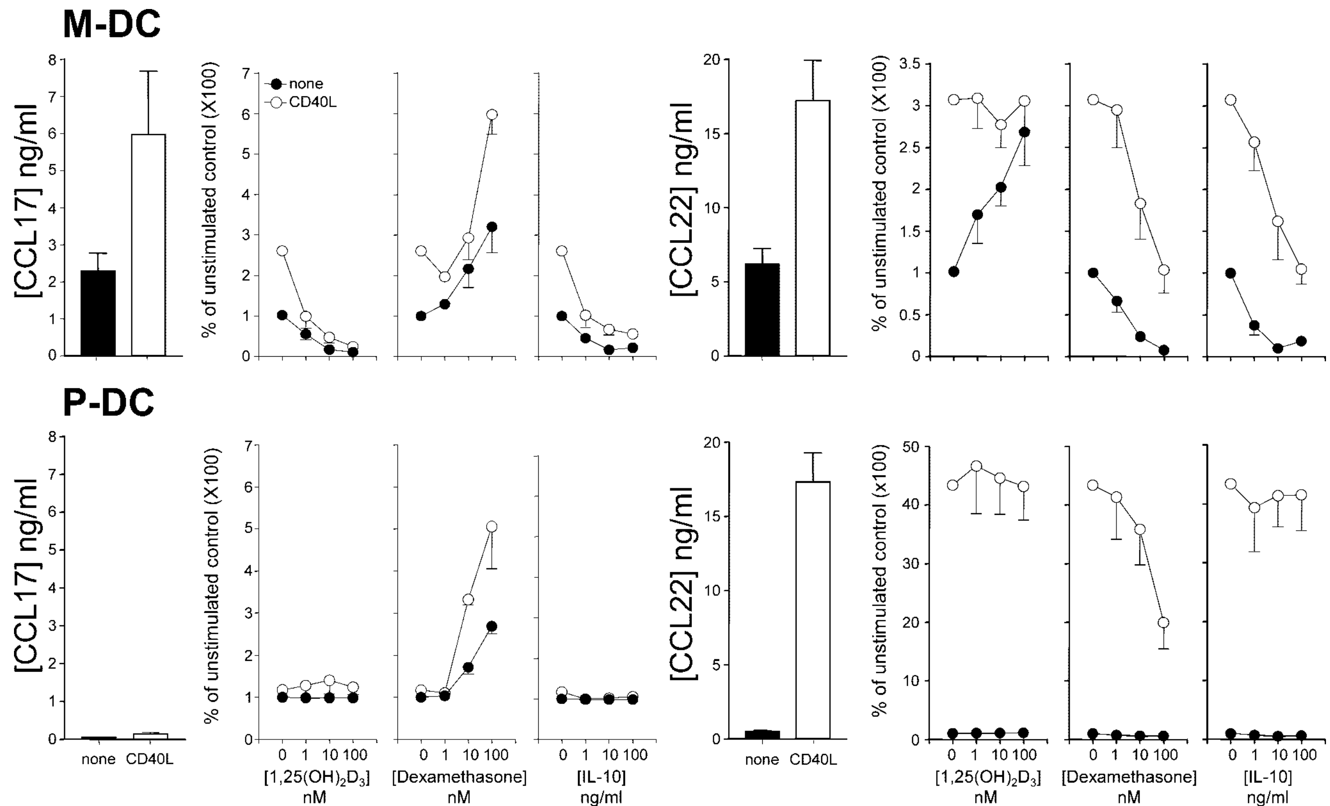

FIGURE 2. 1,25(OH)$_2$D$_3$, dexamethasone, and IL-10 modulate basal and CD40L-induced CCL17 and CCL22 production by M-DCs and P-DCs. Magnetically purified M-DCs and P-DCs (2×10^4 cells/well) were cultured in GM-CSF or IL-3, respectively, with or without CD40L-transfected J558 cells (CD40L). The indicated concentrations of 1,25(OH)$_2$D$_3$, dexamethasone, or IL-10 were added at culture initiation. Mean and SE of secreted chemokines (ng/ml) detected by two-site ELISA from 7 to 10 experiments are indicated. Data are representative of absolute values of chemokine production after 48 h of culture in basal conditions and following CD40 ligation. The effects of immunomodulatory agents tested are expressed as a percentage of the chemokine production in unstimulated control cultures.

Next, the effect of 1,25(OH)$_2$D$_3$ on CCR4 ligand production by DC subsets unstimulated or stimulated via CD40 ligation was compared (Fig. 2). CD40 ligation up-regulated CCL17 production in M-DCs, but failed to induce it in P-DCs (Fig. 2, see graphs), as previously reported (17). 1,25(OH)$_2$D$_3$ inhibited constitutive and induced CCL17 production by M-DCs equally well, with negligible effects on its production by P-DCs (Fig. 2). Two other anti-inflammatory agents targeting DCs, the glucocorticoid dexamethasone and IL-10, were also tested. Opposite to 1,25(OH)$_2$D$_3$, dexamethasone markedly up-regulated CCL17 production by M-DCs, whereas this production was inhibited by IL-10 similarly to 1,25(OH)$_2$D$_3$. A strong dose-dependent up-regulation of CCL17 was also induced by dexamethasone in unstimulated and CD40L-stimulated P-DCs, whereas no effect was induced by IL-10 (Fig. 2).

CCL22 production was up-regulated by CD40 ligation both in M-DCs and P-DCs, as expected (17), and was further enhanced in unstimulated but not stimulated M-DCs by 1,25(OH)$_2$D$_3$, whereas a profound inhibition was induced by dexamethasone and IL-10 in both unstimulated and stimulated M-DCs. In CD40L-stimulated P-DCs, CCL22 production was again not substantially modulated by 1,25(OH)$_2$D$_3$, but was decreased markedly by dexamethasone and only slightly by IL-10.

1,25(OH)$_2$D$_3$ inhibits the Th1 cell-inducing capacity of M-DCs but not P-DCs

1,25(OH)$_2$D$_3$ selectively affects DC subsets also in their capacity to direct T cell development. Results in Fig. 3*A* show that both M-DC and P-DC induce naive alloreactive CD4$^+$ T cells to differentiate mainly into Th1 cells, as defined by intracytoplasmic staining for IFN-γ and IL-4. M-DCs compared with P-DCs activate a larger proportion of CD4$^+$ T cells characterized by production of both IFN-γ and IL-2. Incubation with 1,25(OH)$_2$D$_3$ inhibits the Th1-inducing capacity of M-DCs, as indicated by the selective inhibition of IFN-γ-producing CD4$^+$ T cells, *w*ith a modest effect on IL-2-producing CD4$^+$ T cells (Fig. 3*A*). Conversely, no effect on the Th1-inducing capacity of P-DCs is exerted by 1,25(OH)$_2$D$_3$ (Fig. 3*A*). No observable skewing to the Th2 phenotype was induced by 1,25(OH)$_2$D$_3$ treatment in cultures containing either M-DCs or P-DCs. To analyze mechanisms potentially accounting for these differential effects, we have determined the capacity of 1,25(OH)$_2$D$_3$ to modulate IL-12p75 and IFN-α signature Th1-inducing cytokines selectively produced by M-DCs and P-DCs, respectively (16). 1,25(OH)$_2$D$_3$ reduces IL-12p75 production by CD40L-stimulated M-DCs, whereas IFN-α production by P-DC is not affected, thus suggesting an explanation for the selectivity of this agent in the inhibition of Th1 cell development (Fig. 3*B*). In contrast to 1,25(OH)$_2$D$_3$, other DC-targeting immunomodulatory agents, as dexamethasone and IL-10, inhibit constitutive and CD40L-induced IFN-α production by P-DCs (Fig. 3*C*).

1,25(OH)$_2$D$_3$ enhances the capacity of M-DCs but not P-DCs to induce CD4$^+$ suppressor T cell activity

To evaluate the capacity of 1,25(OH)$_2$D$_3$ to modulate tolerogenic properties of DC subsets, purified M-DCs and P-DCs were cultured with allogenic naive CD4$^+$ cells for three rounds of restimulation in the presence or absence of 1,25(OH)$_2$D$_3$. The marked IFN-γ production of CD4$^+$CD25$^-$ cells (5853 pg/ml) is completely inhibited by addition of blood CD4$^+$CD25$^+$ cells (Fig. 4*A*). M-DCs give rise to CD4$^+$ cells with suppressive activity, which at a 1:1 cell ratio reduces

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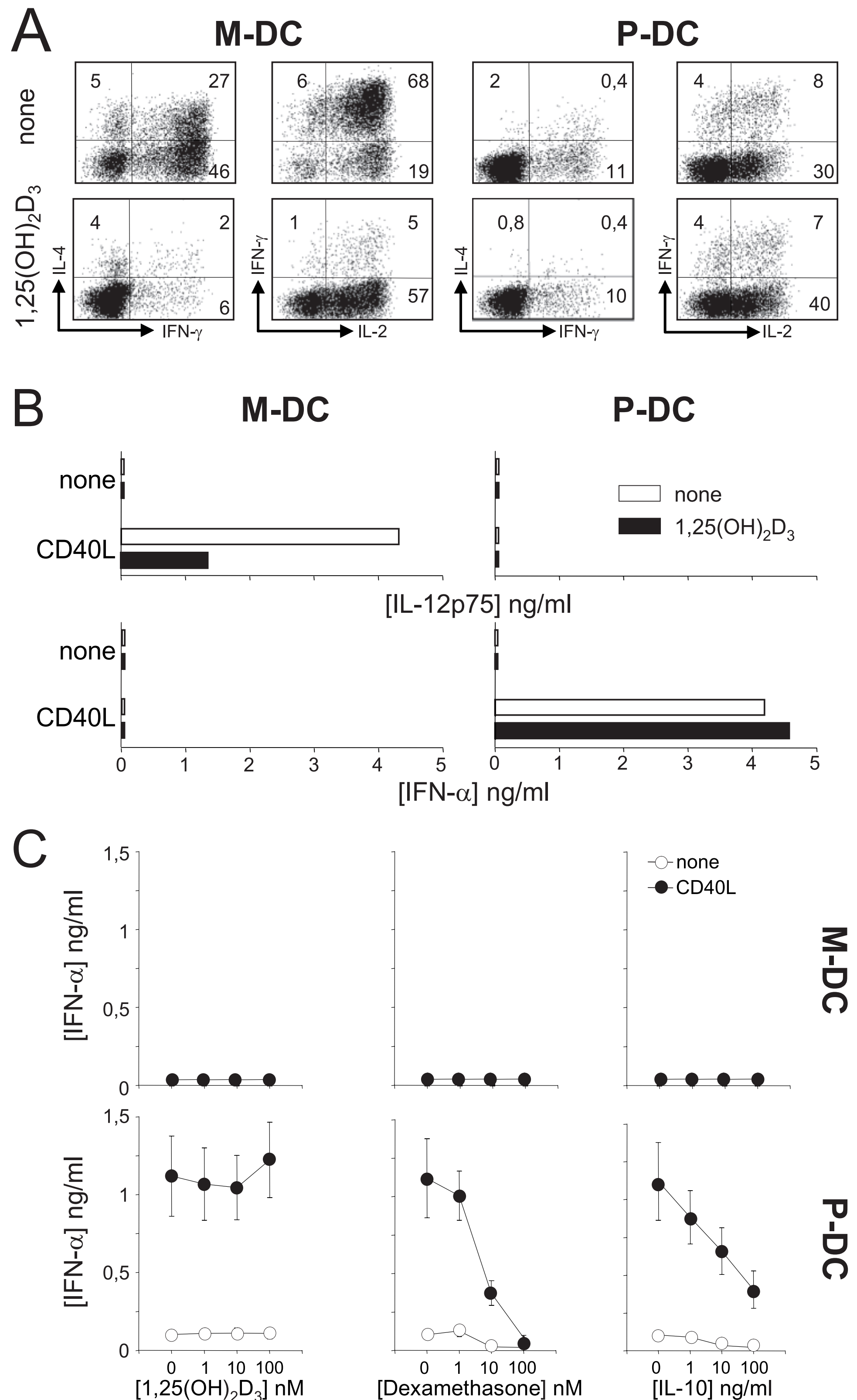

FIGURE 3. 1,25(OH)$_2$D$_3$ inhibits the Th1 cell-inducing capacity of M-DCs but not P-DCs. Magnetically purified blood DC subsets were cocultured with allogeneic blood naive CD4$^+$ cells for 7 days with or without 10 nM 1,25(OH)$_2$D$_3$. *A*, Percentage of positive cells evaluated by cytofluorimetry following intracellular staining for IFN-γ, IL-2, and IL-4. A representative experiment of three performed is shown. *B*, IL-12p75 and IFN-α production by magnetically purified blood DC subsets cultured in GM-CSF or IL-3, respectively. After 48 h of culture, DC subsets were stimulated with CD40L-transfected J558 cells (CD40L) for additional 24 h with or without 10 nM 1,25(OH)$_2$D$_3$. Secreted IL-12p75 and IFN-α (ng/ml) were detected by two-site ELISA. *C*, 1,25(OH)$_2$D$_3$, unlike dexamethasone or IL-10, fails to inhibit IFN-α production by P-DCs. Magnetically purified M-DCs and P-DCs (2×10^4 cells/well) were cultured for 24 h in GM-CSF or IL-3, respectively, with medium alone or containing CD40L-transfected J558 cells (CD40L). The indicated concentrations of 1,25(OH)$_2$D$_3$, dexamethasone, or IL-10 were added at culture initiation. Mean and SE of secreted IFN-α detected by two-site ELISA from three independent experiments are indicated.

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IFN-γ production by ~60% from 5853 to 2418 pg/ml. This suppressive activity is greatly enhanced by addition of 1,25(OH)$_2$D$_3$, leading to near abrogation of IFN-γ production at 1:1 suppressor to target cell ratio (Fig. 4*B*). P-DCs also induce CD4$^+$ cells with suppressive activity, which appears to be slightly more potent compared with M-DCs, inducing 84% suppression at 1:1 cell ratio, but in this case no effect of 1,25(OH)$_2$D$_3$ can be observed (Fig. 4*C*).

1,25(OH)$_2$D$_3$ modulates MHC class II, Ig-like transcript (ILT)3, and costimulatory molecule expression selectively in M-DCs

The selective capacity of 1,25(OH)$_2$D$_3$ to modulate cytokine and chemokine production in M-DCs but not P-DCs, prompted us to examine whether surface molecules crucial for DC activity were differentially affected in DC subsets. To this end, we analyzed the

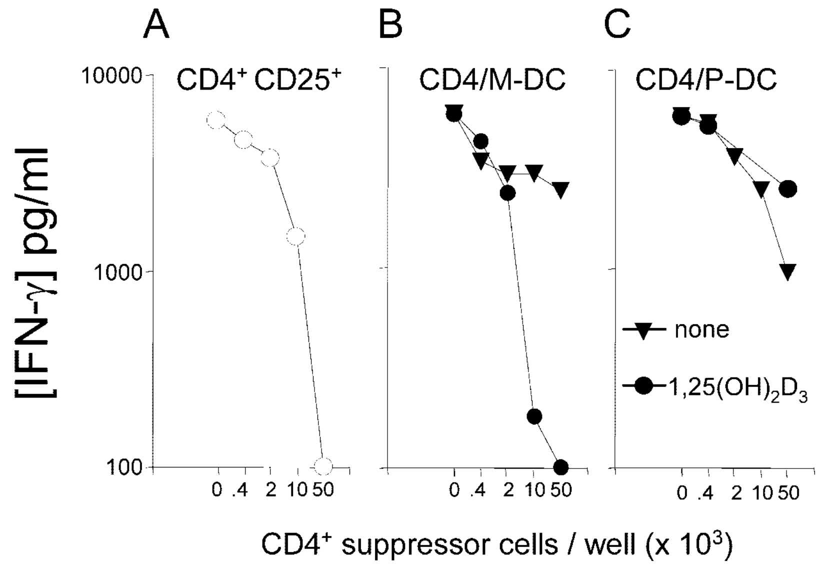

FIGURE 4. $1,25(OH)_2D_3$ enhances the capacity of M-DCs but not P-DCs to induce $CD4^+$ suppressor T cell activity. The read-out system to test for suppressive activity was composed of $CD4^+CD25^-$ cells from donor A PBMCs cultured with 1:10 DC subset cells from donor B in the presence of 1 μg/ml anti-human CD3 mAb. To evaluate the induction of T cells with suppressive activity, $CD4^+$ T cells from donor C were generated by three rounds of restimulation with allogeneic DC subsets from donor D cultured with or without 10 nM $1,25(OH)_2D_3$. IFN-γ production was quantified in culture supernatants by two-site ELISA. *A*, IFN-γ production by $CD4^+CD25^-$ cells (5×10^4/well) cultured alone or with the indicated number of purified blood $CD4^+CD25^+$ T cells. *B*, Suppressive activity of $CD4^+$ cells induced by allogenic M-DCs cultured with (circles) or without (triangles) 10 nM $1,25(OH)_2D_3$. *C*, Suppressive activity of $CD4^+$ cells induced by allogenic P-DCs cultured with (circles) or without (triangles) 10 nM $1,25(OH)_2D_3$.

effects of $1,25(OH)_2D_3$ on MHC class II and CD40 molecules, key players in $CD4^+$ T cell responses, and expression of ILT3, an inhibitory receptor expressed by tolerogenic DCs (11, 22). Incubation with $1,25(OH)_2D_3$ inhibited basal levels of MHC class II and CD40 molecule expression in M-DCs but not P-DCs (Fig. 5*A*). ILT3 expression was selectively up-regulated in M-DCs, while it remained unaffected in P-DCs, following treatment with $1,25(OH)_2D_3$ (Fig. 5*A*). In addition, no modulation of CD80 and CD86 expression was induced by $1,25(OH)_2D_3$ in P-DCs unstimulated or stimulated with CD40L-transfected cells. In contrast, a marked inhibition of CD80 and CD86 expression was induced by $1,25(OH)_2D_3$ in M-DCs, either unstimulated or stimulated with CD40L-transfected cells (Fig. 5*B*). CD40 ligation enhanced expression of costimulatory molecules in both DC subsets, up-regulating

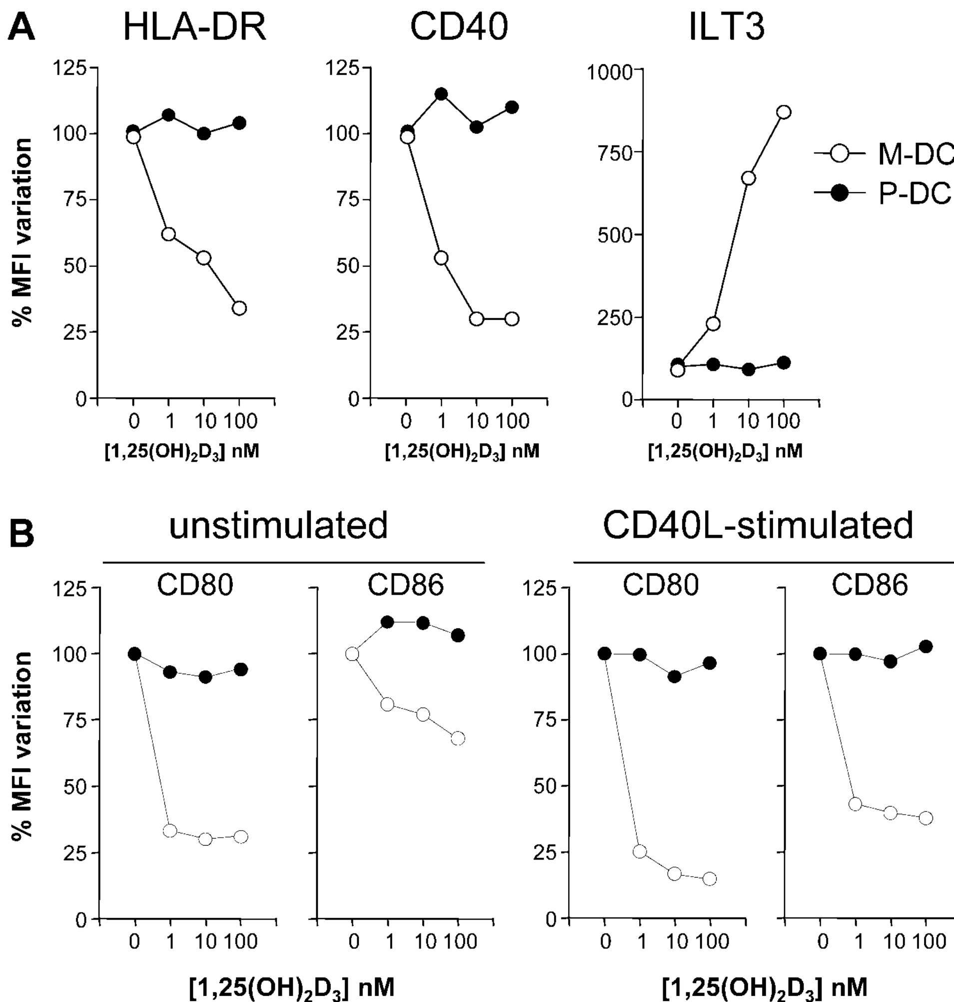

FIGURE 5. $1,25(OH)_2D_3$ modulates MHC class II, ILT3, and costimulatory molecule expression selectively in M-DCs. Magnetically purified blood M-DCs and P-DCs (2×10^4 cells/well) were cultured in GM-CSF or IL-3, respectively. *A*, Surface expression of HLA-DR, CD40, and ILT3 in unstimulated DC subpopulations from a representative donor cultured for 48 h in medium alone or containing the indicated concentrations of $1,25(OH)_2D_3$. *B*, Surface expression of CD80 and CD86 in DC subsets unstimulated or stimulated with CD40L-transfected J558 cells (CD40L) cultured for 48 h in medium alone or containing the indicated concentrations of $1,25(OH)_2D_3$. Mean data from two individual donors are shown. The geometric mean fluorescence intensity (MFI) data represent the percentage variation compared with the marker expression in M-DCs (○) or P-DCs (●) cultured without $1,25(OH)_2D_3$.

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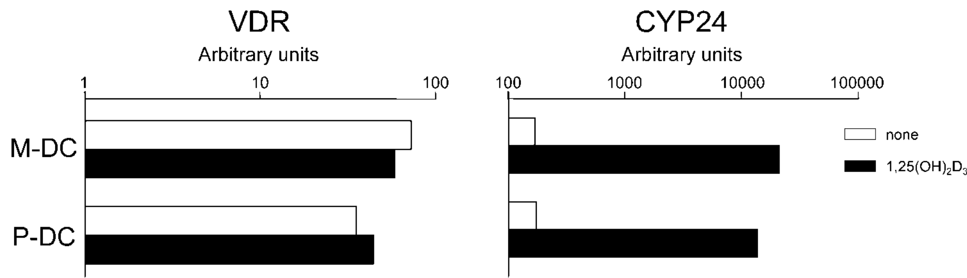

FIGURE 6. Gene modulation induced by 1,25(OH)$_2$D$_3$ in M-DCs and P-DCs. M-DCs and P-DCs express similar levels of VDR and up-regulate equally well primary VDR response genes following stimulation with 1,25(OH)$_2$D$_3$. Magnetically purified blood M-DCs and P-DCs (2×10^4 cells/well) were cultured with or without 10 nM 1,25(OH)$_2$D$_3$. After 2 h, RNA was extracted and expression of VDR and *cyp24* transcripts evaluated by real-time RT-PCR. Data are representative of arbitrary units normalized to GAPDH signals and are from one representative experiment of five conducted.

CD80 mean fluorescence intensity from 438 to 1647 in M-DCs and from 673 to 2981 in P-DCs, whereas CD86 mean fluorescence intensity increased from 1352 to 6051 in M-DCs and from 426 to 3922 in P-DCs.

Gene modulation induced by 1,25(OH)$_2$D$_3$ in M-DCs and P-DCs

Given the apparent lack of P-DC responsiveness to 1,25(OH)$_2$D$_3$, we investigated VDR expression and response to 1,25(OH)$_2$D$_3$ in DC subsets. A comparably high VDR expression was observed in ex vivo purified M-DCs and P-DCs (Fig. 6), which was similar to VDR expression detected in immature monocyte-derived DC and monocytes (data not shown). VDR signal transduction was also comparable in both DC subsets, as shown (Fig. 6) by the marked up-regulation of *cyp24*, a primary VDR response gene encoding the 24-hydroxylase enzyme catalyzing 1,25(OH)$_2$D$_3$ metabolism, which is rapidly induced following exposure to 1,25(OH)$_2$D$_3$ (5). Thus, both M-DCs and P-DCs express similar VDR levels, and respond equally well to VDR ligation by up-regulating primary response genes.

1,25(OH)$_2$D$_3$ inhibits NF-κB phosphorylation and nuclear translocation in M-DCs but not P-DCs

The transcription factor NF-κB regulates in DCs expression of genes encoding several cytokines and chemokines. NF-κB family proteins, c-Rel, RelA (p65), RelB, NF-κB1 (p50), and NF-κB2 (p52) are present in the cytoplasm in an inactive form as a result of their association with the inhibitory proteins IκBα, IκBβ, and

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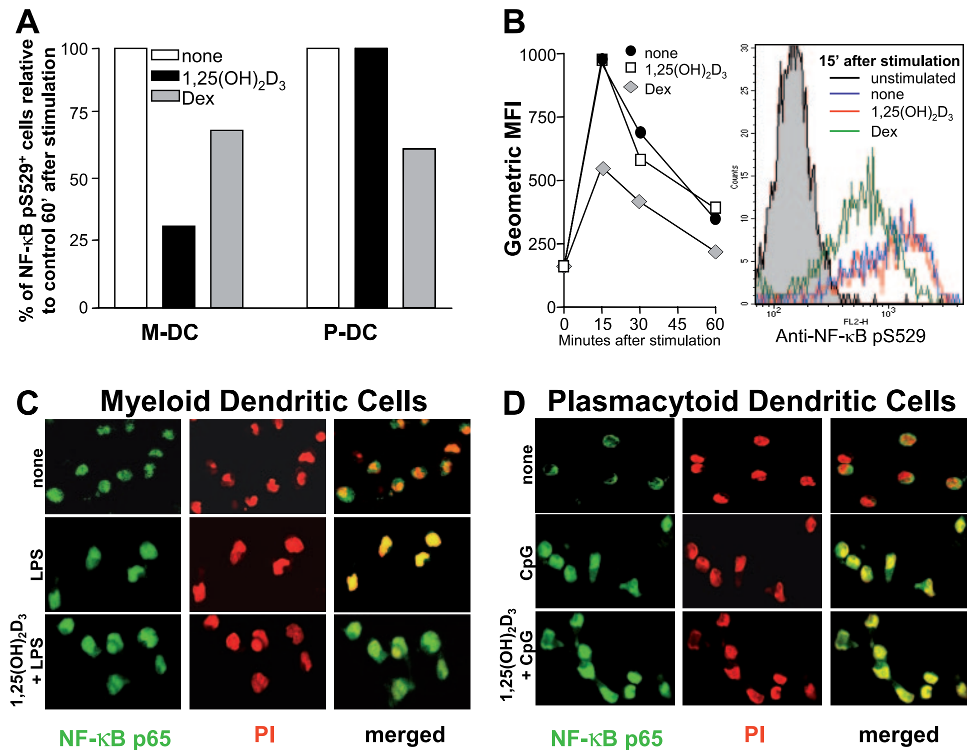

FIGURE 7. 1,25(OH)$_2$D$_3$ inhibits NF-κB p65 phosphorylation and traps it into the cytoplasm in M-DCs but not P-DCs. Magnetically purified M-DCs and P-DCs were cultured in GM-CSF or IL-3, respectively, with medium alone or containing 100 nM 1,25(OH)$_2$D$_3$ or 100 nM dexamethasone (Dex). After an 18-h culture, M-DCs and P-DCs were incubated with medium only (unstimulated), or containing 100 ng/ml PMA plus 1 μg/ml ionomycin (*A* and *B*), 1 μg/ml LPS (*C*), or 6 μg/ml CpG 2006 (*D*), respectively, for 45 min or as indicated. NF-κB p65 phosphorylation was determined by cytofluorimetry. *A*, The percentage of cells positive for phosphorylated S529 in the transactivation domain of NF-κB p65, relative to controls, for 60 min after stimulation. *B*, The geometric mean fluorescence intensity (MFI) of P-DCs positive for phosphorylated NF-κB p65 at the indicated times after PMA-ionomycin stimulation. Histogram analysis of positive cells is shown (*right*) 15 min after stimulation. *C* and *D*, Cells were fixed and stained with anti-NF-κB p65 mAb and propidium iodide (PI). Individual and merged stainings are shown. Original magnifications is ×63.

IκBε, and must be phosphorylated and translated to the nucleus to become activated (23). To test whether $1,25(OH)_2D_3$ could reduce phosphorylation of NF-κB p65, untreated and $1,25(OH)_2D_3$-treated M-DCs and P-DCs were stimulated with PMA with ionomycin, a nonspecific stimulus activating both subsets, and stained with a mAb recognizing the phosphorylated S529 in the transactivation domain of NF-κB p65. Results in Fig. 7*A* show that phosphorylation of NF-κB p65, detected by cytofluorimetry, was reduced by $1,25(OH)_2D_3$ in M-DCs but not in P-DCs, whereas dexamethasone was able to inhibit it in both DC subsets. Inhibition of NF-κB p65 phosphorylation in P-DCs by dexamethasone but not by $1,25(OH)_2D_3$ was confirmed in a time-course experiment (Fig. 7*B*). In this experiment, the area under the curve for control P-DCs was 34,913, which was reduced by ~40% in dexamethasone-treated cells (area under the curve was 22,065), but was unaffected following treatment of P-DCs with $1,25(OH)_2D_3$ (area under the curve was 36,270). Supporting these data, $1,25(OH)_2D_3$ could reduce translocation of NF-κB p65 to the nucleus in M-DCs but not P-DCs. M-DCs and P-DCs were cultured for 18 h with or without $1,25(OH)_2D_3$ followed by stimulation for 45 min with LPS or CpG, respectively, before staining with anti-NF-κB p65 mAb and propidium iodide (Fig. 7, *C* and *D*). Individual and merged staining obtained by confocal microscopic analysis clearly show translocation of NF-κB p65 to the nucleus in stimulated M-DCs, whereas NF-κB p65 is mostly retained in the cytoplasm in cells treated with $1,25(OH)_2D_3$ (Fig. 7*C*). Conversely, the nuclear translocation induced in P-DCs by CpG stimulation is not affected by $1,25(OH)_2D_3$ treatment (Fig. 7*D*).

Discussion

VDR agonists share with other immunomodulatory agents, and in particular with immunosuppressive and anti-inflammatory drugs, the capacity to target DCs, rendering them tolerogenic and fostering the induction of regulatory rather than effector T cells (12, 24). Multiple mechanisms contribute to induction of DC tolerogenicity by VDR agonists, from down-regulation of costimulatory molecules, both membrane-bound as CD40, CD80, CD86 and secreted as IL-12, to up-regulation of anti-inflammatory molecules like IL-10 (4, 25). Additional mechanisms involve modulation of chemokine secretion, enhancing the production of chemokines able to recruit regulatory/suppressor T cells, and inhibiting chemokine production by target organs in autoimmune diseases (26, 27). These effects are not limited to in vitro activity: $1,25(OH)_2D_3$ and its analogs can also induce DCs with tolerogenic properties in vivo, as demonstrated in models of allograft rejection (28, 29). Tolerogenic DCs induced by a short treatment with $1,25(OH)_2D_3$ are probably responsible for the capacity of this hormone to induce $CD4^+CD25^+$ regulatory T cells that are able to mediate transplantation tolerance (28) and arrest the development of type 1 diabetes (30).

CCL22 and CCL17 are chemokines able to recruit activated T cells, and in particular Th2 cells, via CCR4 (31, 32). In addition, these chemokines can recruit $CD4^+CD25^+$ regulatory T cells (33). CCL22, a chemokine mostly produced by DCs (34), has been found to selectively recruit, in ovarian carcinoma patients, $Foxp3^+CCR4^+CD4^+CD25^+$ regulatory T cells able to suppress antitumor responses, leading to reduced patient survival (35). Similarly, CCL22 secreted by lymphoma B cells attracts $Foxp3^+$ $CCR4^+CD4^+CD25^+$ regulatory T cells able to suppress proliferation and cytokine production by tumor-infiltrating $CD4^+CD25^-$ T cells (36). We have found that, in contrast to the high production by circulating M-DCs, the CCR4 agonists CCL17 and CCL22 are very poorly produced by unstimulated P-DCs and only CCL22 is secreted at high levels by this DC subset following CD40 ligation, as previously reported (17). Blood-borne M-DCs, in contrast to P-DCs, constitutively produce high levels of CCL17 and CCL22 ex vivo, which are further enhanced by CD40 stimulation, confirming previous data (17). This high constitutive and inducible production of CCR4 agonists by immature M-DCs could lead to the preferential attraction of $CD4^+CD25^+$ regulatory T cells. Intriguingly, the production of CCL22 is markedly enhanced by $1,25(OH)_2D_3$ in blood M-DCs, indicating that VDR agonists may favor the recruitment of regulatory T cells by this DC subset. $1,25(OH)_2D_3$ not only regulates in M-DCs production of chemokines potentially able to recruit regulatory T cells, but also enhances their induction, a novel finding consistent with previous in vivo data (28, 30). Conversely, neither CCL22 nor CCL17 are modulated by $1,25(OH)_2D_3$ in P-DCs, and although this DC subset induces regulatory T cells, consistent with previous reports (37, 38), this property is not modified by $1,25(OH)_2D_3$. However, other chemokines may be involved in regulatory T cell recruitment by P-DCs, which produce large quantities of the CCR5 agonist CCL4 (17). Thus, in analogy with the proposed role for CCL4 in $CD4^+CD25^+$ regulatory T cell attraction by activated B cells (39), P-DCs could recruit them via secretion of this chemokine.

In addition to CCL22 and CCL17, neither IFN-α, the signature cytokine produced by P-DCs (16), nor expression of MHC class II molecules, CD40, CD80, CD86 costimulatory molecules, or ILT3 inhibitory molecule are affected in P-DCs by $1,25(OH)_2D_3$ treatment. Conversely, production of IL-12, the M-DC signature cytokine (16), as well as MHC class II and costimulatory molecule expression are markedly inhibited by $1,25(OH)_2D_3$ in M-DCs, while the inhibitory molecule ILT3 is strongly up-regulated. Interestingly, these novel findings confirm in blood M-DCs the capacity of $1,25(OH)_2D_3$, previously observed in monocyte-derived DCs (6, 11), to inhibit MHC class II and costimulatory molecule expression while up-regulating the inhibitory molecule ILT3. All these molecules are controlled by NF-κB, a signal transduction pathway crucially involved in the inflammatory response (23). In particular, production of IL-12 (40, 41), type I IFNs (42), CCL17 (43, 44), CCL22 (44–46), and expression of MHC class II molecules (47), CD40 (48), CD80 (49), CD86 (50), and ILT3 (22) have been shown to be regulated by NF-κB activity. Our data showing inhibition of NF-κB p65 phosphorylation and nuclear translocation by $1,25(OH)_2D_3$ in M-DCs but not P-DCs demonstrate a mechanism of action selectively targeting NF-κB in DC subpopulations. The selective targeting of NF-κB components by VDR agonists in M-DCs could thus contribute to explain the lack of activity of these agents on cytokine and chemokine production by P-DCs, which nevertheless respond to $1,25(OH)_2D_3$, as shown by *cyp24* up-regulation.

NF-κB is a target for many anti-inflammatory and immunosuppressive agents (12), including glucocorticoids, anti-inflammatory drugs that bind to a nuclear receptor in the same superfamily as the VDR, as shown by the observation that dexamethasone up-regulates the transcription of *Nfkbia*, resulting in increased rate of IκBα synthesis and in reduced NF-κB p65 phosphorylation and translocation to the nucleus (51, 52). Our results show that $1,25(OH)_2D_3$ and dexamethasone exert opposite effects on CCL17 and CCL22 regulation in both M-DCs and P-DCs. Strikingly, dexamethasone markedly enhances constitutive and inducible CCL17 production in M-DCs, and induces it in P-DCs. Differential effects of these anti-inflammatory agents on chemokine production by bone marrow-derived DCs have been reported, possibly due to differential regulation of NF-κB family members by the two steroid pathways (53, 54). Additional levels of NF-κB regulation by $1,25(OH)_2D_3$ and dexamethasone have also been reported in other immune and nonimmune cell types, including up-regulation of

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IκBα, direct intranuclear binding of NF-κB proteins, and direct negative regulation of NF-κB-induced genes (26, 40, 51, 52, 55, 56). This complex regulation could also explain the increased CCL22 and decreased CCL17 production induced by $1,25(OH)_2D_3$ in M-DCs.

DCs are able to synthesize $1,25(OH)_2D_3$ in vitro as a consequence of increased 1α-hydroxylase expression (57), and this could also contribute to promote regulatory T cell induction and recruitment. 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 Ags, as suggested by the enlarged lymph nodes containing a higher frequency of mature DCs in VDR-deficient mice (29). Our results showing increased induction of suppressor $CD4^+$ T cell activity by M-DCs in the presence of $1,25(OH)_2D_3$ support this possibility. Conversely, $1,25(OH)_2D_3$ does not modulate the intrinsic tolerogenic capacity of P-DCs, consistent with earlier observations showing selective up-regulation of the inhibitory receptor ILT3 on M-DCs, without affecting its high expression on P-DC (11). Because P-DCs are key cells linking innate and adaptive immunity (16), the lack of up-regulation by $1,25(OH)_2D_3$ of their constitutive tolerogenic function may contribute to explain the intact host resistance against infectious agents following $1,25(OH)_2D_3$ administration (58).

In conclusion, $1,25(OH)_2D_3$ appears to up-regulate tolerogenic properties in M-DCs, down-regulating IL-12 and Th1 cell development, while promoting $CD4^+$ suppressor T cell activity and enhancing the production of CCL22, a chemokine able to recruit regulatory T cells. In contrast, no immunomodulatory effects induced by $1,25(OH)_2D_3$ are observed in P-DCs, a DC subset prone to favor tolerance under steady-state conditions (16) or during an immune response (59). Indeed, P-DCs, characterized by an intrinsic ability to prime naive $CD4^+$ T cells to differentiate into IL-10-producing T cells (60–63) and $CD4^+CD25^+$ regulatory T cells (38), and to suppress immune responses (64, 65), may represent naturally occurring regulatory DCs (16). The lack of immunoregulatory feature modulation by $1,25(OH)_2D_3$ in P-DCs would thus leave their tolerogenic potential unmodified.

Disclosures

G. Penna, S. Amuchastegui, and L. Adorini own stock or equity interests in BioXell.

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