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Suppressive Effect of 1α,25-Dihydroxyvitamin D_3 on Type I IFN-Mediated Monocyte Differentiation into Dendritic Cells: Impairment of Functional Activities and Chemotaxis[1]

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Dendritic cells (DCs) generated by a single-step exposure of human monocytes to type I IFN and GM-CSF (IFN-DCs) are endowed with potent immunostimulatory activities and a distinctive migratory response to specific chemokines. In this study, we evaluated the effects of 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), the biologically active metabolite of vitamin D_3, on the DC differentiation/activation induced by type I IFN. We found that 1,25$(OH)_2D_3$ prevented the generation of IFN-DCs when added to freshly isolated monocytes, and was capable of redirecting already differentiated IFN-DCs toward a more immature stage, as revealed by their immunophenotype, reduced allostimulatory activity, and impaired LPS-induced production of Th1-polarizing cytokines. Control and 1,25$(OH)_2D_3$-treated IFN-DCs exhibited a similar expression of vitamin D receptor, as well as comparable cell death rates. Furthermore, the chemotactic response of IFN-DCs to CCL4 and CCL19 was markedly reduced or completely abrogated by 1,25$(OH)_2D_3$. Despite these changes in the IFN-DC migratory behavior, the expression of CCR5 and CCR7 and the calcium fluxes triggered by CCL4 and CCL19 were not affected. These findings indicate that, in this innovative single-step DC generation model from monocytes, the suppressive effect of 1,25$(OH)_2D_3$ is associated with a potent impairment of DC migration in response to inflammatory and lymph node-homing chemokines, thus unraveling a novel mechanism involved in 1,25$(OH)_2D_3$-mediated immunomodulation. *The Journal of Immunology,* **2005, 174: 270–276.**

Dendritic cells (DCs)[3] are potent APCs that play a pivotal role in the immune response, by linking innate and adaptive immune responses (1). These cells are well-recognized inducers and modulators of T cell immunity and increasingly viewed as mediators of T cell tolerance (2, 3). Integral to their APC function is the DC migratory behavior, as these cells continuously traffic between peripheral tissues and secondary lymphoid organs, performing specific functions at each site (4). In peripheral tissues, resting immature DCs exert a sentinel function, taking up and processing available Ags. Upon activation, they migrate to draining lymph nodes and home to T cell-rich areas, where they interact with T cells to initiate immune responses. A spontaneous migration of DCs is also observed under healthy steady state conditions, and increasing evidence demonstrates that these steady state migrating DCs, loaded with tissue self Ags, contribute to the maintenance of peripheral tolerance (2). Each step of DC trafficking is likely to be controlled, at least in part, by chemokines. Immature, tissue resident DCs respond to many inflammatory/inducible chemokines (CCL3, CCL4, CCL5, and CCL20), while maturing DCs lose responsiveness to most of these chemokines, as a result of down-regulation of cognate receptor expression or function. In a coordinated process, maturing DCs up-regulate CCR7 and gain responsiveness to lymph node-homing chemokines (CCL19 and CCL21). Increasing evidence indicates that soluble factors present in the DC microenvironment are instrumental in determining the outcome of DC differentiation (immunostimulatory vs tolerogenic) and, as a direct consequence, the quality of the T cell response generated, in particular tolerance, memory, and polarized Th1/Th2 differentiation (1–3).

Type I IFNs are cytokines spontaneously expressed at low levels under physiologic conditions whose expression is highly enhanced soon after cell exposure to viruses or other stimuli. Although first characterized as potent antiviral molecules, type I IFNs are also endowed with immunoregulatory activities and serve as important links between innate and adaptive immune responses (5, 6). DCs and their precursors are important cellular targets of type I IFN immunomodulatory activities, as demonstrated by the profound effects of these cytokines on DC activation, maturation, and survival (7). Although apparently contrasting results have been reported on the effects of type I IFNs on monocyte differentiation into DCs in different experimental models (8–12), we have previously shown that a single-step treatment of freshly isolated monocytes with type I IFN, together with GM-CSF added as survival factor, results in the rapid generation of DCs (IFN-DCs) endowed with important immune priming activities (13). IFN-DCs potently stimulate the in vitro proliferative response of allogeneic T cells, and, after viral priming, of autologous PBL, inducing a strongly Th1-polarized response. In vivo studies have shown that virus-pulsed IFN-DCs are potent inducers of both humoral and CD8-mediated immune responses in the human PBL-SCID mouse

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[3] Abbreviations used in this paper: DC, dendritic cell; 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; CYP24, 25-hydroxyvitamin D-24-hydoxylase; D_3 IFN-DC, IFN-DC generated in the continuous presence of 1,25$(OH)_2D_3$; VDR, vitamin D receptor.

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model (13, 14). Of note, IFN-DCs show a unique pattern of expression of chemokine receptors and migratory response to proinflammatory and lymph node-homing chemokines. Compared with GM-CSF/IL-4-generated monocyte-derived DCs (IL-4-DCs), IFN-DCs express higher levels of CCR5 associated with a stronger chemotactic response to β-chemokines, express CCR7, and are able to migrate in response to its ligand CCL19 without the need for any further maturation stimuli (15).

The biologically active metabolite of vitamin D_3, 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), is a secosteroid hormone with pleiotropic actions, including maintenance of bone and calcium/phosphate homeostasis, regulation of growth and differentiation of cancer cells, and modulation of the immune response (16, 17). The immunoregulatory properties of $1,25(OH)_2D_3$ and its analogues have been demonstrated in different models of autoimmune diseases and in experimental organ transplantation (18–20). DCs are both primary cellular targets and mediators of the immunomodulatory effects of $1,25(OH)_2D_3$. Studies performed either on human monocyte-derived DCs or on bone marrow mouse DCs have clearly demonstrated that $1,25(OH)_2D_3$ inhibits their in vitro differentiation and maturation (21–23). Moreover, vitamin D receptor (VDR)-deficient mice have increased numbers of DCs in lymph nodes, suggesting a physiologically relevant inhibition of DC differentiation by $1,25(OH)_2D_3$ in vivo (24). By modulating DC activation and survival, $1,25(OH)_2D_3$ also profoundly affects the phenotype and function of interacting T cells, ultimately leading to the induction of T cell hyporesponsiveness in vitro (21) and of regulatory T cells mediating transplantation tolerance (25) and arrest of autoimmune diseases in vivo (26).

The aim of this study was to investigate the effects of $1,25(OH)_2D_3$ on the type I IFN-mediated pathway for the differentiation of monocytes into DCs. We found that $1,25(OH)_2D_3$ prevents the generation of IFN-DCs when added to freshly isolated monocytes, and is capable of redirecting already differentiated IFN-DCs toward a more immature stage. The suppression of IFN-DC differentiation/activation by $1,25(OH)_2D_3$ is associated with disruption of their capability to migrate in response to inflammatory and lymph node-homing chemokines, although the expression of the cognate receptors and the calcium response are unaffected.

Materials and Methods

Cell separation and culture

Peripheral blood monocytes from healthy donors were isolated by counterflow centrifugal elutriation and further purified by depleting the nonmonocytic population by immunomagnetic beads selection (MACS monocyte isolation kit from Miltenyi Biotec) using the manufacturer's instructions. To obtain IFN-DCs, monocytes were seeded at 1×10^6 cells/ml and cultured for 3–5 days in RPMI 1640 (Invitrogen Life Technologies) medium containing 10% FBS, supplemented with 50 ng/ml human rGM-CSF (a generous gift from Schering-Plough) and 1000 U/ml human rIFN-β (a generous gift from Serono). Depending on the experimental setting, 10 nM $1,25(OH)_2D_3$ (BioXell) was added at seeding or at day 3.

Real-time quantitative RT-PCR

RNA was extracted using TRIzol (Invitrogen Life Technologies), according to the manufacturer's instructions. 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 either Taqman or SIBRgreen chemistry. The primer sequences used are available on request. Relative quantification of target cDNA was determined by calculating the difference in cross-threshold (*CT*) values after normalization to hypoxanthine phosphoribosyltransferase or GAPDH signals, according to the CT $2^{-\Delta CT}$. To exclude amplification of genomic DNA, RNA samples were treated with DNase (Sigma-Aldrich).

Flow cytometric analysis

For the analysis of the surface immunophenotype and of CCR5, cells were stained with FITC-conjugated mAbs (BD Pharmingen). The following mAbs were used: CD14 (M5E2), CD1a (HI149), CD80 (L307.4), CD83 (HB15e), HLA-DR (G46-6), CD40 (5C3), CCR5 (2D7), and CD54. Abs directed against α and β catenins (BD Transduction Laboratories), VCAM (Chemicon International) E-cadherin, and CD44 (Santa Cruz Biotechnology) were used for indirect fluorescence studies by using as secondary Ab the R-PE $F(ab')_2$ goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). Briefly, $1–2 \times 10^5$ cells were preincubated for 30 min on ice with PBS 10% human AB serum to block nonspecific Ig binding, and then incubated for an additional 30 min with the appropriate mAb. Cells were washed in PBS 10% human serum and fixed in 1% formaldehyde. For detection of apoptosis/cell death, IFN-DCs were stained with Annexin V^{FITC} and propidium iodide (BD Pharmingen). Cells were analyzed with a FACS flow cytometer (BD Biosciences) using the CellQuest software.

Cytokine analysis

IL-12 and IL-10 secreted in the culture supernatant were quantified using commercial ELISA kits (R&D Systems and Pierce; detection limit of 7.8 and 32.5 pg/ml, respectively). Type I IFNs secreted in culture supernatants were measured by using a cytopathic effect reduction assay with HeLa cells (1×10^4 cells/well in 96-well microplates) and vesicular stomatitis virus, as challenge virus, at the multiplicity of infection of 0.1 PFU/cell. Human IFN-α reference standard (National Institutes of Health; Ga23-902-530) was used at the dilution of 500 IU/ml. The sensitivity of the assay was in the range of 2–8 IU/ml.

Mixed leukocyte reaction

The MLR was performed in RPMI 1640 culture medium supplemented with 5% heat-inactivated normal human AB serum. Allogeneic T cells ($1–2 \times 10^5$/well) were cultured for 5 days in 96-well culture microplates as responder cells with $1–20 \times 10^3$ stimulatory cells (DCs). [^{3}H]Thymidine incorporation (sp. act., 5 Ci/mmol; Amersham Bioscience Europe) was measured after a 16-h pulse with 0.5 μCi/well. Results are shown as mean cpm of triplicate samples. [^{3}H]Thymidine incorporation in negative control wells, with responder T cells or stimulator DCs alone, was always $<$800 cpm.

Chemotaxis assay

Blind well chemotaxis chambers (NeuroProbe) with a lower well volume of 200 μl were used. A maximum volume of 200 μl of RPMI 1640 containing 1% BSA was placed in the lower well in the presence or absence of chemokines. Cells (2×10^5) were placed in the upper compartments of Boyden chambers above the filters. The chambers were incubated for 3 h at 37°C in a 5% CO_2 incubator. The filters were then removed, dehydrated, stained with 15% Giemsa for 7 min, and then mounted on glass slides. Cells in 10 high power fields from four to six filters were counted and averaged for each sample. Migration index was calculated as the number of cells migrating toward the concentration gradient of chemokines divided by the number of cells migrating toward medium only.

Measurement of intracellular calcium

Cells (1×10^7/ml) were resuspended in a buffer containing 0.25% BSA, 145 mM NaCl, 5 mM KCl, 10 mM sodium/MOPS, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4, and incubated with 2 μM fura 2-AM (Molecular Probes) for 40 min at room temperature. The cells were washed once, resuspended in the buffer containing 0.25% BSA, and kept at room temperature in the dark. Just before use, cell aliquots (1×10^6) were washed and resuspended in 2 ml of buffer containing 0.005% BSA in a stirred cuvette at 37°C. Measurement of Ca^{2+} was performed, as previously described (27).

Results

The $1,25(OH)_2D_3$ strongly impairs differentiation, maturation, and function of IFN-DCs

In a first set of experiments, we investigated the effect of $1,25(OH)_2D_3$ on the in vitro generation of IFN-DCs. To this aim, freshly isolated monocytes were cultured with GM-CSF and IFN-β in the continuous presence of $1,25(OH)_2D_3$, and their immunophenotype and functional activities were compared with control IFN-DCs. IFN-DCs generated in the presence of $1,25(OH)_2D_3$ (D_3 IFN-DCs) failed to up-regulate the CD1a differentiation

marker as well as the CD83 maturation marker, while retaining high levels of CD14 (Fig. 1*A*). Moreover, expression of CD40, CD80, and MHC class II molecules was significantly reduced in D_3 IFN-DCs, compared with untreated IFN-DCs. In addition, the expression of a panel of adhesion molecules, including CD54, CD44, VCAM, E-cadherin, and α and β catenins, has been analyzed. None of these molecules, with the only exception of CD54, was found to be modulated by 1,25(OH)$_2$D$_3$ treatment, with respect to control cells. CD54 was abundantly expressed by 99% of both control and 1,25(OH)$_2$D$_3$-treated cells. However, a 50% reduction in the mean fluorescence intensity was consistently observed in these latter (2611 $\pm$ 256 SE in control cells vs 1154 $\pm$ 84 SE in 1,25(OH)$_2$D$_3$-treated cells). Consistently with their altered immunophenotype, D_3 IFN-DCs also displayed strongly impaired functional activities. IFN-DCs exhibited a potent allostimulatory capacity, while cells generated in the presence of 1,25(OH)$_2$D$_3$ were almost unable to stimulate T cell proliferation (Fig. 1*B*). In addition, while IFN-DCs produced high levels of IL-12p70 upon LPS stimulation, no detectable LPS-induced IL-12 secretion was found in D_3 IFN-DC cultures (Fig. 1*C*). Thus, similar inhibitory effects are induced by 1,25(OH)$_2$D$_3$ on the differentiation and maturation of IFN-DCs and IL-4-DCs.

1,25(OH)$_2$D$_3$ partially reverts IFN-DC differentiation/activation

Next, we evaluated the effect of 1,25(OH)$_2$D$_3$ on already differentiated IFN-DCs. As previously described (13), after a 3-day culture, IFN-DCs are irreversibly committed to DC differentiation/maturation and retain a DC phenotype upon cytokine removal, while IL-4-DCs readily reacquire macrophage features. Thus, 3-day IFN-DCs were exposed to 1,25(OH)$_2$D$_3$ or left untreated, upon removal of GM-CSF and IFN-β, and their immunophenotype and functional properties were analyzed 48 h later. As shown in Fig. 2*A*, in the presence of 1,25(OH)$_2$D$_3$, IFN-DCs regained high levels of CD14 expression, while the expression of CD83 was markedly down-modulated. No significant differences were observed for CD80, CD86, CD40, and HLA-DR (data not shown). Likewise, the extent of apoptosis/cell death in control and 1,25(OH)$_2$D$_3$-treated IFN-DCs was comparable (Fig. 2*B*). The allostimulatory activity (Fig. 2*C*) and LPS-induced IL-12 production (Fig. 2*D*) were also significantly reduced, although not completely abolished, as observed when the immunomodulatory agent was added from the beginning of the culture (Fig. 1). These results indicate that 1,25(OH)$_2$D$_3$ can also revert differentiated IFN-DCs toward a more immature phenotype and function.

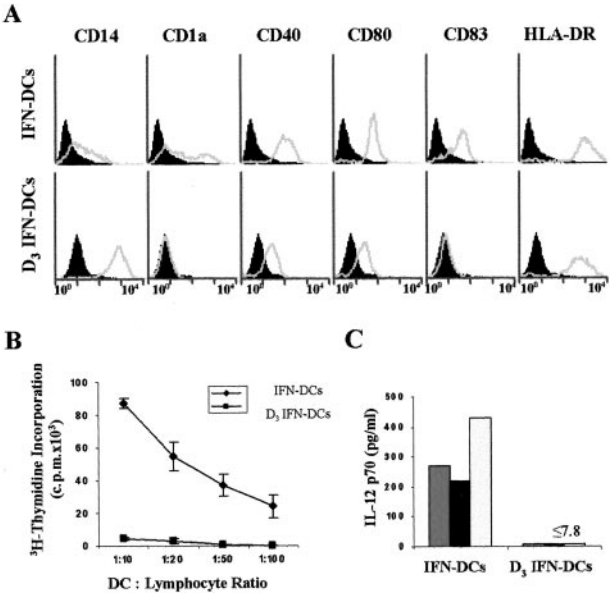

FIGURE 1. Effect of 1,25(OH)$_2$D$_3$ on the generation of IFN-DCs. Negatively selected peripheral blood monocytes were cultured for 4 days in the presence of GM-CSF (50 ng/ml) and IFN-β (1000 IU/ml). The 1,25(OH)$_2$D$_3$ (10 nM) was added soon after cell seeding. *A*, Immunophenotype. Cytofluorimetric analysis was performed at day 4. Cells were stained with FITC-conjugated mAbs specific for the indicated markers. Filled histograms represent the background staining of isotype-matched control Ab. A representative experiment of eight performed is shown. *B*, MLR assay. Purified lymphocytes were seeded into 96-well microplates at the concentration of 1×10^5 cells/well. Allogeneic IFN-DCs were added at graded doses to lymphocytes. After 5 days, [^{3}H]thymidine incorporation was measured. A representative experiment of three performed is shown. *C*, LPS-induced IL-12 secretion. IFN-DCs or D_3 IFN-DCs were treated with LPS (200 ng/ml) at day 3, and culture supernatants were collected 24 h later. IL-12p70 secreted in culture supernatants was quantified by ELISA. Each bar represents the value obtained with cells from a different donor.

Effect of 1,25(OH)$_2$D$_3$ on LPS-induced type I IFN production in response to activating stimuli

It is well recognized that, upon exposure to a variety of maturation-inducing stimuli, IL-4-DCs secrete type I IFNs (28–30). Thus, we investigated whether production of type I IFNs in response to LPS (a prototypic type I IFN inducer in DCs) also occurred in IFN-DC cultures and whether it was affected by 1,25(OH)$_2$D$_3$. As shown in Fig. 3, IFN-DCs produced significant amounts of biologically active type I IFNs following LPS stimulation (ranging between 100 and 1000 IU/ml, with the majority of donors producing 250–500 IU/ml). This response to LPS stimulation was 10-fold reduced when cells were generated in the continuous presence of 1,25(OH)$_2$D$_3$. On the contrary, exposure of already differentiated IFN-DCs to 1,25(OH)$_2$D$_3$ did not affect their ability to produce type I IFNs in response to LPS. Likewise, the production of LPS-induced IL-10 by IFN-DCs (238.9 pg/ml $\pm$ 160.7) was not significantly modified when these cells were cultured in the continuous presence of 1,25(OH)$_2$D$_3$ (210.6 pg/ml $\pm$ 161.9) or treated at day 3 for 48 h (384.7 pg/ml $\pm$ 81.5).

VDR and 25-hydroxyvitamin D-24-hydoxylase (CYP24) mRNA expression during in vitro differentiation/maturation of IFN-DCs

The biological effects of 1,25(OH)$_2$D$_3$ are mediated by the VDR, a member of the superfamily of nuclear hormone receptors, and the enzyme CYP24 has been described as a primary vitamin D-responsive gene (31). Therefore, we investigated, by real-time RT-PCR analysis, whether expression of these genes could be modulated during the course of the type I IFN-driven in vitro differentiation of monocytes into DCs. As shown in Fig. 4, time course experiments indicated that VDR mRNA expression did not change significantly, neither during the in vitro differentiation process, nor after a short (2-h) or prolonged treatment with 1,25(OH)$_2$D$_3$ of both monocytes and IFN-DCs. Conversely, the basal levels of CYP24 transcripts observed in monocytes as well as in 3- and 5-day cultured IFN-DCs, were strongly up-regulated upon 1,25(OH)$_2$D$_3$ exposure. In particular, a 2-h treatment with 1,25(OH)$_2$D$_3$ resulted in a 1,000-fold increase of the expression level of CYP24 mRNA in monocytes, and in a 100-fold in IFN-DCs. Upon longer treatments, as routinely performed in this study, the increase in the levels of CYP24 was even stronger, reaching 100,000-fold in D_3 IFN-DCs and 10,000-fold in IFN-DCs D_3 48 h, compared with control IFN-DCs.

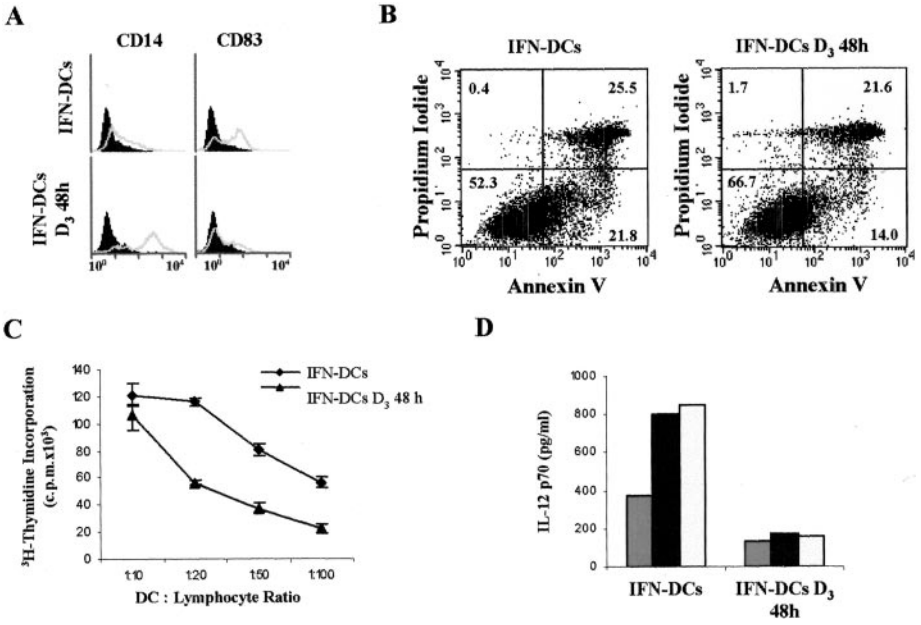

FIGURE 2. Effect of $1,25(OH)_2D_3$ on differentiated IFN-DCs. After 3 days of culture, IFN-DCs were washed and reseeded without GM-CSF and IFN-β, in the presence or in the absence of $1,25(OH)_2D_3$. Phenotype and functional activities were analyzed 48 h later. *A*, Immunophenotype. Cytofluorimetric analysis was performed, as described in Fig. 1. Filled histograms represent the background staining of isotype-matched control Ab. A representative experiment of four performed is shown. *B*, Cell death rates. IFN-DC apoptosis/necrosis was quantified by double staining with Annexin V^{FITC} and propidium iodide 48 h upon removal of GM-CSF/IFN-β in the presence or in the absence of $1,25(OH)_2D_3$. A representative experiment of three performed is shown. *C*, MLR assay. The assay was performed, as described in the legend to Fig. 1. A representative experiment of three performed is shown. *D*, LPS-induced IL-12 secretion. LPS (100 ng/ml) was added at day 3, 1 h after reseeding in the presence or in the absence of 1,25 $(OH)_2D_3$. Supernatants were collected 48 h later, and secreted IL-12p70 was quantified by ELISA, as described in the legend to Fig. 1. Each bar represents IL-12 production from a different donor.

Migration of IFN-DCs in response to CCL4 and CCL19 is abolished in the presence of 1,25(OH)$_2$D$_3$

Because IFN-DCs display a distinctive pattern of chemokine receptor expression and migratory response to specific chemokines (15), we investigated the effect of $1,25(OH)_2D_3$ on the expression and function of CCR5 and CCR7 in IFN-DCs. As shown in Fig. 5, CCR5 was expressed at the surface of control IFN-DCs (Fig. 5*A*), and its triggering by CCL4 induced a significant elevation in intracellular Ca^{2+} (Fig. 5*B*) as well as a marked chemotactic response (Fig. 5*C*). The $1,25(OH)_2D_3$ neither decreased receptor expression nor impaired CCL4-induced raise in intracellular Ca^{2+}. In contrast, the chemotactic response to CCL4 (Fig. 5*C*) was nearly abolished in the presence of $1,25(OH)_2D_3$. Although CCR7 expression was barely detectable at the surface of control IFN-DCs, as assessed by using the currently available Abs to CCR7 (data not shown), CCL19 addition to these cultures induced a rapid rise in intracellular Ca^{2+}, which was not significantly modified in the presence of $1,25(OH)_2D_3$ (Fig. 6*A*). In contrast, the migratory response to CCL19 was almost completely abolished by the addition

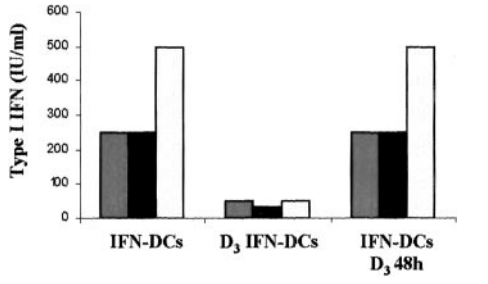

FIGURE 3. Effect of $1,25(OH)_2D_3$ on type I IFN production in response to LPS. LPS stimulation (100 ng/ml; 48 h) was performed at day 3, after extensive washing and reseeding without GM-CSF and IFN-β, to ensure removal of the exogenous IFN. The $1,25(OH)_2D_3$ has been added at culture initiation (D_3 IFN-DCs) or at day 3, 1 h before LPS addition (IFN-DCs D_3 48 h). The presence of biologically active type I IFN in culture supernatants collected 48 h after LPS stimulation was assessed in a vesicular stomatitis virus-cytopathic reduction assay. Each bar represents type I IFN production from a different donor.

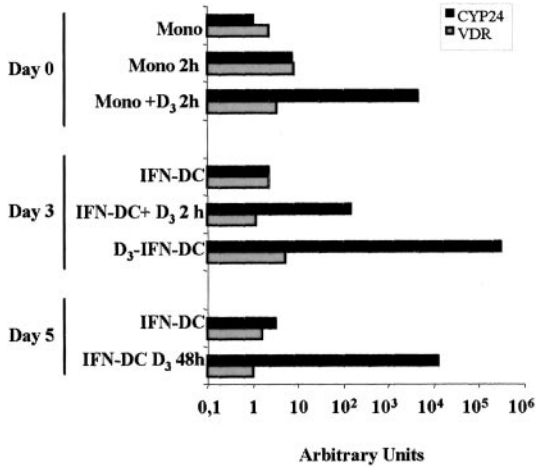

FIGURE 4. Effect of $1,25(OH)_2D_3$ on VDR and CYP24 mRNA expression. RNA samples were obtained at the indicated time points by freshly isolated monocytes and fully differentiated IFN-DCs treated with $1,25(OH)_2D_3$ or left untreated. Quantification of VDR and CYP24 transcripts by real-time RT-PCR was performed, as described in *Materials and Methods*. The levels of mRNA are shown as arbitrary units normalized to GAPDH expression. Data are from one representative experiment of three performed.

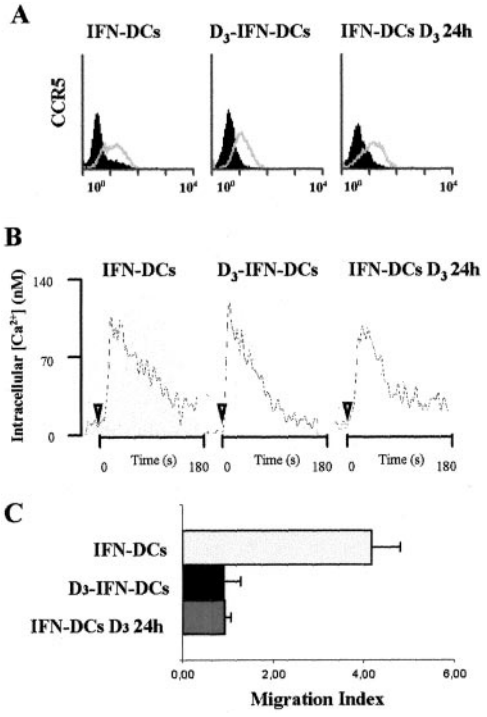

FIGURE 5. Effect of $1,25(OH)_2D_3$ on CCR5 expression and response to CCL4. *A*, CCR5 expression. Cytofluorimetric profiles of CCR5 surface staining on IFN-DCs, D_3 IFN-DCs, or IFN-DCs treated for 24 h with $1,25(OH)_2D_3$. Filled histograms represent the background staining of isotype-matched control Ab. A representative experiment of three performed is shown. *B*, Intracellular calcium levels. IFN-DCs, D_3 IFN-DCs, or IFN-DCs treated for 24 h with $1,25(OH)_2D_3$ were labeled with fura 2-AM and stimulated with 1 μg/ml CCL4. Arrows indicate the time of chemokine addition. Similar results have been obtained with 100 ng/ml (data not shown). *C*, Migratory response. Chemotaxis assay was performed on IFN-DCs, D_3 IFN-DCs, or IFN-DCs treated for 24 h with $1,25(OH)_2D_3$, in the presence of 50 ng/ml CCL4. Similar results were obtained at the CCL4 concentration of 5 ng/ml (data not shown).

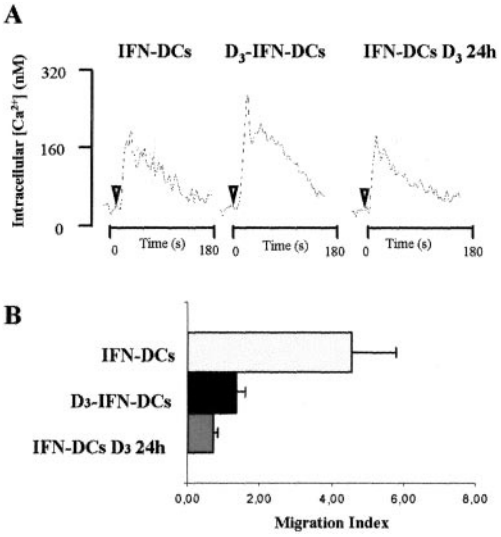

FIGURE 6. Effect of $1,25(OH)_2D_3$ on the response to CCL19. *A*, Intracellular calcium levels. IFN-DCs, D_3 IFN-DCs, or IFN-DCs treated for 24 h with $1,25(OH)_2D_3$ were stimulated with 40 ng/ml CCL4. Similar results were obtained with 100 ng/ml (data not shown). *B*, Migratory response. Chemotaxis assay was performed on IFN-DCs, D_3 IFN-DCs, or IFN-DCs treated for 24 h with $1,25(OH)_2D_3$ in the presence of 50 pg/ml CCL19. Similar results were obtained with 500 pg/ml CCL19 (data not shown).

of $1,25(OH)_2D_3$ (Fig. 6*B*). Thus, in $1,25(OH)_2D_3$-treated IFN-DCs, CCR5 and CCR7 expression and CCL4- and CCL19-triggered calcium mobilization were uncoupled from chemotaxis.

Discussion

In this study, we demonstrate that $1,25(OH)_2D_3$ potently inhibits the type I IFN-mediated pathway of monocyte differentiation into DCs. These results are in agreement with previous reports on the inhibitory effect of $1,25(OH)_2D_3$ on differentiation and maturation of IL-4-DCs (21, 22), further highlighting the role of $1,25(OH)_2D_3$ as a DC modulator. Even though the IL-4/GM-CSF culture method has allowed the performance of many important studies on DC biology, this differentiation pathway may not truly reflect the physiologic process by which monocytes differentiate in vivo into DCs, as high levels of IL-4 are unlikely to be present, for example, during the immune response to infections. Conversely, concentrations of type I IFNs similar to those used in our in vitro model are likely to be transiently present in the microenvironment of circulating or tissue monocytes during viral infections or in other pathological conditions. Indeed, it has been reported that IFN-α in sera from patients with systemic lupus erythematosus induced normal monocytes to differentiate into DCs (32). Thus, it is reasonable to hypothesize that exposure of monocytes to type I IFNs can represent at least one of the early mechanisms involved in the in vivo generation of immunostimulatory DCs in response to pathogen invasion or in the pathogenesis of autoimmune diseases. The observation that $1,25(OH)_2D_3$ potently inhibits this DC differentiation pathway could be important both in light of DC manipulation for therapeutic intervention, and in consideration of recent evidence pointing to $1,25(OH)_2D_3$ as a physiological regulator of DC homeostasis (33). VDR-deficient mice display hypertrophy of s.c. lymph nodes and an increase in mature DCs in lymph nodes, indicating that the $1,25(OH)_2D_3$/VDR complex can mediate a physiologically relevant inhibition of DC maturation in vivo (24). Furthermore, two very recent studies have demonstrated that monocyte-derived DCs are able to synthesize $1,25(OH)_2D_3$. This capability is up-regulated upon induction of terminal maturation, while a concomitant down-modulation of the receptor is observed, suggesting a potential autocrine/paracrine loop in the regulation of DC differentiation and function by $1,25(OH)_2D_3$ (34, 35).

In our in vitro model of DC differentiation/activation, the addition of the immunomodulatory agent to freshly isolated monocytes completely abrogated the generation of IFN-DCs. Cells cultured in the continuous presence of $1,25(OH)_2D_3$ retained high levels of CD14 and did not gain expression of CD1a and CD83. Moreover, the expression of costimulatory molecules was significantly lower than in control IFN-DCs. Conversely, $1,25(OH)_2D_3$ treatment did not modify the expression of the mannose receptor, thus suggesting that the immunomodulatory agent does not affect the phagocytic activity of IFN-DCs. Similarly, we observed that both subunits of the type I IFN receptor were expressed on IFN-DCs, and were not modulated in the presence of $1,25(OH)_2D_3$ (data not shown). Notably, D_3 IFN-DCs were unable to stimulate allogeneic T cell proliferation and to produce IL-12 and type I IFNs in response to further stimulation with LPS. When the immunomodulatory agent was added to already differentiated IFN-DCs, a qualitatively similar effect was observed, although less dramatic. Interestingly, although no significant changes were observed in the expression of VDR under all experimental conditions tested, a clear-cut up-modulation of CYP24 was detected in IFN-DCs treated with $1,25(OH)_2D_3$ for 48 h, and to a higher extent in

IFN-DCs generated in the continuous presence of $1,25(OH)_2D_3$. Similarly, we observed a higher up-modulation of CYP24 upon a 2-h treatment with $1,25(OH)_2D_3$ in monocytes than in IFN-DCs. These results indicate that freshly harvested monocytes exhibit a higher responsiveness to $1,25(OH)_2D_3$ compared with IFN-DCs, as assessed by CYP24 mRNA induction. This higher responsiveness is most likely correlated with the stronger inhibitory activity of $1,25(OH)_2D_3$ on the IFN-mediated DC differentiation/maturation pathway observed when it is added to monocytes at seeding, compared with its activity on already differentiated IFN-DCs.

Despite the fact that $1,25(OH)_2D_3$ induces overall similar effects on the differentiation/maturation and functional activities of DCs generated under classical conditions or in the presence of IFN, some differences were observed. We have previously reported that $1,25(OH)_2D_3$ promotes the spontaneous apoptosis of mature IL-4 DCs (21). In the present study, the already high levels of spontaneous apoptosis detected in DC generated in the presence of IFN, in agreement with previous data (13), were not significantly modified in IFN-DCs treated with $1,25(OH)_2D_3$ for 48 h. This excludes that the inhibitory effect of $1,25(OH)_2D_3$ is due to a selective loss of mature DCs. In addition, while a significant increase in IL-10 production was observed in $1,25(OH)_2D_3$-treated IL-4-DCs in response to maturation stimuli (21, 36, 37), the secretion of this cytokine was not significantly modified in IFN-DCs exposed to $1,25(OH)_2D_3$ as compared with control cultures.

In contrast with the results on IL-12 production, the type I IFN response appears to depend on the timing of $1,25(OH)_2D_3$ addition. Although IFN production was strongly reduced when $1,25(OH)_2D_3$ was added to monocytes at seeding, it was not affected when $1,25(OH)_2D_3$ treatment was performed on already differentiated IFN-DCs. With the exception of a study reporting an inhibitory effect of $1,25(OH)_2D_3$ on IFN-α production by PBMCs of psoriatic patients (38), no data are available on the effects of this immunomodulatory agent on the type I IFN induction pathway. Although the main type I IFN-producing cell is the plasmacytoid DC (39), both mouse myeloid DCs (40) and human monocyte-derived DCs (28–30) can also produce high amounts of type I IFNs when appropriately activated. This ability of nonplasmacytoid DCs to produce type I IFNs may be of biological importance because, at variance with plasmacytoid DCs (41), they are present at sites of pathogen entry. The IFNs produced by nonplasmacytoid DCs in response to danger signals, besides their direct antiviral effect, could contribute to the differentiation/activation of DCs and then promote protective immune responses. In addition, once DCs have migrated into the lymph node, type I IFNs could induce, in synergy with IL-12, Th1 responses (42).

Our data show that chemotaxis of IFN-DCs in response to both CCL4 and CCL19 is strongly impaired in the presence of $1,25(OH)_2D_3$. To the best of our knowledge, this is the first report showing an inhibitory effect of $1,25(OH)_2D_3$ on DC migration. However, despite the dramatic changes in the IFN-DC migratory behavior, the expression of CCR5 and CCR7 and the calcium fluxes triggered by CCL4 and CCL19 were not affected. Uncoupling of inflammatory chemokine receptor expression and migratory responses has been described in human DCs and monocytes exposed to IL-10 (43) as well as in blood plasmacytoid DCs (44), most likely representing a strategy to block excessive DC recruitment and activation upon inflammation. Although the immunological consequences of DC migration impairment by IL-10 in IL-4-DCs and by $1,25(OH)_2D_3$ in IFN-DCs could be similar, the molecular mechanisms involved appear to be different. Indeed, no downstream signaling event was activated after receptor engagement in IL-4-DCs treated with IL-10 (43), whereas in IFN-DCs treated with $1,25(OH)_2D_3$ the calcium mobilization in response to both CCL4 and CCL19 was unaffected. The results that we obtained in IFN-DCs are in keeping with the previously observed dissociation between chemotaxis and calcium mobilization (45) and suggest that other intracellular pathways required for migration, downstream or parallel to calcium mobilization, could be affected by $1,25(OH)_2D_3$. Although adhesion molecules play a key role in regulating cell migration, they do not seem to be the main targets of the $1,25(OH)_2D_3$ inhibitory activity on IFN-DC migration. Indeed, FACS analysis of a panel of these molecules, including CD44, VCAM, E-cadherin, and α and β catenins, did not reveal differences in their expression in control and $1,25(OH)_2D_3$-treated IFN-DCs, while a reduction in the amount of CD54 expressed per cell was observed in the presence of $1,25(OH)_2D_3$. It remains to be determined whether integrin expression is affected under these experimental conditions.

Engagement of chemokine receptors by their cognate ligands induces an elaborate biochemical program, ultimately leading to the induction of a variety of functions, including cell migration and proliferation (46). The biochemical events include G protein-coupled receptor-dependent calcium flux, as well as changes in intracellular cAMP levels and phosphoinositol lipid metabolism (46). Chemokine receptors and their downstream signaling cascades, including activation of c-Src, $p125^{FAK}$, PyK2, paxillin, and MAPK, are known to be involved in the regulation of chemotactic migratory properties of DCs (47). Interestingly, some of these signaling pathway components (i.e., c-Src family proteins and MAPK) have been reported to be modulated by $1,25(OH)_2D_3$ in other cell types (48–50).

Localization in tissues and migration to lymphoid organs are essential steps in DC immunobiology that are linked to the differentiation and T cell stimulatory function of these cells. Thus, our finding that $1,25(OH)_2D_3$ markedly impairs chemotactic responses of IFN-DCs to both proinflammatory and lymph node-homing chemokines unravels a novel mechanism potentially contributing to the multiple immunomodulatory properties of this hormone.

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