1α,25-Dihydroxyvitamin D_3 Inhibits Differentiation, Maturation, Activation, and Survival of Dendritic Cells Leading to Impaired Alloreactive T Cell Activation

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1α,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active form of vitamin D_3, is a potent immunomodulatory agent. Here we show that dendritic cells (DCs) are major targets of $1,25(OH)_2D_3$-induced immunosuppressive activity. $1,25(OH)_2D_3$ prevents the differentiation in immature DCs of human monocytes cultured with GM-CSF and IL-4. Addition of $1,25(OH)_2D_3$ during LPS-induced maturation maintains the immature DC phenotype characterized by high mannose receptor and low CD83 expression and markedly inhibits up-regulation of the costimulatory molecules CD40, CD80, and CD86 and of class II MHC molecules. This is associated with a reduced capacity of DCs to activate alloreactive T cells, as determined by decreased proliferation and IFN-γ secretion in mixed leukocyte cultures. $1,25(OH)_2D_3$ also affects maturing DCs, leading to inhibition of IL-12p75 and enhanced IL-10 secretion upon activation by CD40 ligation. In addition, $1,25(OH)_2D_3$ promotes the spontaneous apoptosis of mature DCs. The modulation of phenotype and function of DCs matured in the presence of $1,25(OH)_2D_3$ induces cocultured alloreactive $CD4^+$ cells to secrete less IFN-γ upon restimulation, up-regulate CD152, and down-regulate CD154 molecules. The inhibition of DC differentiation and maturation as well as modulation of their activation and survival leading to T cell hyporesponsiveness may explain the immunosuppressive activity of $1,25(OH)_2D_3$. *The Journal of Immunology,* **2000, 164: 2405–2411.**

Dendritic cells (DC)[2], a highly specialized 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 nonlymphoid organs (1). Immature DCs, such as Langerhans cells in the skin, are found in nonlymphoid tissues, where they exert a sentinel function. After Ag uptake, they migrate through the afferent lymph to T-dependent areas of secondary lymphoid organs where priming of naive T cells may occur (1, 2). During migration to lymphoid organs, DCs mature into potent APCs by increasing their immunostimulatory properties while decreasing Ag-capturing capacity (3). Recently, it has become clear that DCs can be not only immunogenic but also tolerogenic, both intrathymically (4, 5) and in the periphery (6).

Induction of T cell responses requires T cell receptor activation and costimulatory interactions between DCs and T cells (1); in the absence of costimulation, T cells become anergic (7). The two major costimulatory pathways for T cell activation depend on engagement of CD28 and CD154 on T cells by CD80/CD86 and CD40 on DCs, respectively (8, 9). Once activated, T cells also express CD152, a CD28 homologue that binds to CD80/CD86 with higher affinity than CD28 itself and inhibits IL-2 production, IL-2 receptor expression, and cell cycle progression in activated T cells (10).

Disruption of these costimulatory pathways by biological agents such as CD152-Ig and anti-CD154 mAb has been shown to be beneficial in autoimmune diseases and allograft rejection (8, 9). Interestingly, a short treatment with CD152-Ig and anti-CD154 mAb can induce tolerance to allografts in mice (11) and, possibly, also in nonhuman primates (12, 13). This has stimulated the search for low m.w. compounds able to disrupt costimulatory pathways for T cell activation. The unique capacity of DCs to activate naive T cells correlates with elevated expression of MHC Ags and costimulatory molecules (1), rendering them attractive targets for costimulation blockade.

1α,25-Dixydroxyvitamin D_3 ($1,25(OH)_2D_3$), the biologically active metabolite of vitamin D_3, is a secosteroid hormone that not only regulates bone and calcium/phosphate metabolism but exerts a number of other biological activities, including modulation of the immune response via specific receptors expressed in APC and activated T cells. Immunosuppression by $1,25(OH)_2D_3$ and its analogues has been demonstrated in different models of autoimmune diseases and in experimental organ transplantation (14). $1,25(OH)_2D_3$ and its analogues can prevent systemic lupus erythematosus in *lpr/lpr* mice (15), experimental allergic encephalomyelitis (16–18), and autoimmune diabetes in nonobese diabetic mice (19). In addition, $1,25(OH)_2D_3$ and its analogues prolong the survival of heart (20, 21) and small bowel allografts (22) and have been reported to inhibit, in association with cyclosporin A (CsA), not only acute but also chronic allograft rejection (23).

Although $1,25(OH)_2D_3$ and its analogues clearly inhibit T cell proliferation and cytokine production (24, 25), it is not yet clear whether these inhibitory effects are exerted directly on T cells (26–29) or via inhibition of APC activity (30). In the present study, we demonstrate that DCs are primary targets for the immunosuppressive effects of $1,25(OH)_2D_3$ on T cell activation. $1,25(OH)_2D_3$ inhibits differentiation and maturation of DCs and modulates their activation and survival, leading to a profound modulation of T cell phenotype and function.

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[2] Abbreviations used in this paper: DC, dendritic cell; $1,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; CsA, cyclosporin A; MMF, mycophenolate mofetil; PI, propidium iodide; NAC, *N*-acetyl-L-cysteine; TRANCE, TNF-related activation-induced cytokine.

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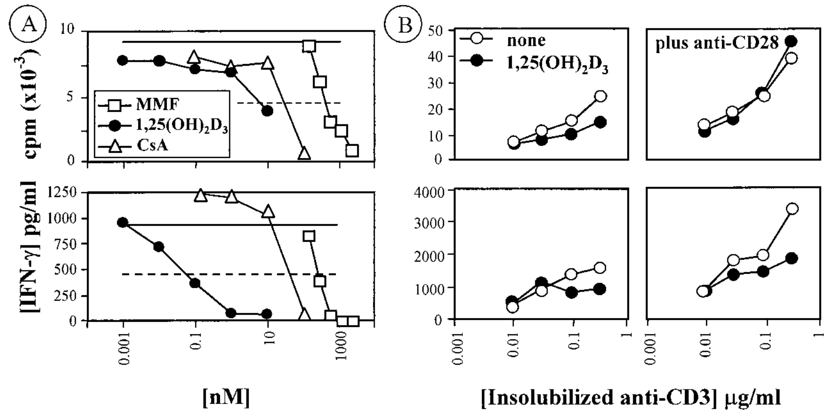

FIGURE 1. Inhibition of APC function by 1,25 $(OH)_2D_3$. *A*, Allogeneic PBMCs from two different donors (3×10^5 cells/well each) were cocultured in 96-well flat-bottom plates in the presence of the indicated concentrations of MMF, CsA, or 1,25$(OH)_2D_3$. After 5 days, proliferation and IFN-γ secretion were measured. The solid line indicates the control response obtained in the absence of immunosuppressive drugs, and the stippled line 50% of the control response. *B*, Purified T cells (10^5/well) were cultured in the presence of 10 nM 1,25$(OH)_2D_3$, with or without 1 μg/ml soluble anti-CD28, in 96-well round-bottom plates precoated with the indicated concentrations of anti-CD3 mAb. After 72 h, proliferation and IFN-γ secretion were measured. The data are from a representative experiment out of two to eight performed.

Materials and Methods

Cell lines and cell culture reagents

Human monocytes were cultured in RPMI 1640 medium supplemented with 10% FCS (HyClone Laboratories, Logan, UT), 2 mM glutamine, 50 μg/ml gentamicin, 1 mM sodium pyruvate, and 1% nonessential amino acids (complete medium). J558L cells expressing CD154 (31) (a gift of Dr. Peter Lane, Basel Institute for Immunology, Basel, Switzerland) were grown in complete medium supplemented with 5 mM L-histidinol dihydrochloride (Sigma, St. Louis, MO).

1,25$(OH)_2D_3$

Crystalline 1,25$(OH)_2D_3$ was a gift of Dr. Milan Uskokovic (Hoffmann-La Roche, Nutley, NJ). 1,25$(OH)_2D_3$ was reconstituted in ethanol and stored in concentrated solutions at −80°C. 1,25$(OH)_2D_3$ was freshly diluted before each experiment, and the ethanol concentration in the test conditions did not exceed 0.00025%.

Human DC cultures

Immature DCs were prepared as described (32). Briefly, PBMC were isolated from a buffy coat by Ficoll gradient (Pharmacia Biotec AB, Uppsala, Sweden), and monocytes, obtained from PBMC by negative selection with monocyte isolation kit (Milteny Biotec, Bergish Gladblach, Germany), were grown for 6–7 days in medium containing 800 U/ml GM-CSF (Mielogen, Schering-Plough) and 1000 U/ml IL-4 (PharMingen, San Diego, CA). DC maturation was induced by stimulation of immature DCs for 48 h with 200 ng/ml LPS (*Escherichia coli* 0111:B4; Sigma), 20 ng/ml TNF-α (PharMingen), or 1:5000 SAC (Pansorbin cells; Calbiochem, San Diego, CA). For activation, DCs were incubated with CD154-J558L cells at a ratio of 4:1. After 24 h, supernatants were collected, and IL-12 p75 and IL-10 concentrations were measured by ELISA.

Allogeneic and anti-CD3-induced T cell activation

PBMC were separated from buffy coats by Ficoll gradient. For the bidirectional MLR, the same number (3×10^5) of allogeneic PBMC from two different donors were cocultured in 96-well flat-bottom plates. After 5 days, proliferation and cytokine production were measured. For anti-CD3-induced T cell activation, total T cells were purified from PBMC using Pan T Cell isolation kit (Miltenyi Biotec). T cells (10^5/well) were cultured in 96-well round-bottom plates precoated by overnight incubation with anti-human CD3 mAb (clone TR66) (33) with or without 1 μg/ml soluble anti-human CD28 mAb (CD28.2; PharMingen). After 72 h, proliferation and cytokine production were measured. For the primary T cell response, $CD4^+$ cells were purified from PBMC using $CD4^+$ T cell isolation kit. $CD4^+$ cells (2×10^5/well) were cultured with graded amounts of DCs (300–10,000) in 96-well flat-bottom plates. After 5 days, proliferation and cytokine production were measured. T cell costimulatory molecules were analyzed 72 h after culture initiation.

Secondary MLR

$CD4^+$ T cells (2×10^5/well) were cocultured during the primary stimulation with 10^3 DCs. T cells were separated 36 h later by Ficoll gradient, and DCs were removed using FITC anti-human CD1a, anti-human CD14, anti-human CD40, anti-human CD86 (all from PharMingen), followed by anti-FITC microbeads (Miltenyi Biotec). T cells were rested for 2–4 days in complete medium supplemented with 2 U/ml hIL-2 and then restimulated with mature DCs generated from the same donor used for the primary culture. Proliferation and cytokine production were measured 48 h later. T cell costimulatory molecules were analyzed 24 h and 48 h after the beginning of culture.

Flow cytometric analysis

Flow cytometric analysis was performed in the presence of 100 μg/ml mouse IgG using the following mAbs, all from PharMingen except when indicated: anti-CD1a FITC/PE (HI149), anti-CD14 FITC (M5E2), anti-CD25 FITC (M-A251), anti-CD28 PE (CD28.2), anti-CD40 FITC (5C3), anti-CD58 FITC (1C3), anti-CD80 FITC (BB1), anti-CD83 PE (HB15A; Immunotech, Marseille, France), anti-CD86 PE (IT2.2), anti HLA-DR FITC/PE (G46-6), anti-Mannose Receptor PE (19), anti-CD152 PE (BNI3), anti-CD154 PE (TRAP1). For detection of apoptosis, DCs were stained with Annexin-V FITC (PharMingen) and propidium iodide (50 μg/ml, Sigma). Cells were analyzed with a FACScan flow cytometer using CellQuest software (Becton Dickinson, Mountain View, CA).

Cytokine analysis

ELISA for IL-12p75 was performed as described (34). Human recombinant IL-12 and anti IL-12 mAbs were a gift from Dr. Maurice Gately (Hoffman-La Roche, Nutley, NJ). ELISA for human IL-10 was performed using commercially available mAbs and standard IL-10 (PharMingen) according to the manufacturer's instructions. ELISA for human IFN-γ was performed as described (35). Detection limits were 15 pg/ml for IL-12p75, 10 pg/ml for IL-10, and 50 pg/ml for IFN-γ.

Results

Inhibition of APC function by 1,25$(OH)_2D_3$

1,25$(OH)_2D_3$ inhibits alloreactive T cell activation, as shown by the inhibition of proliferation and IFN-γ production in a bidirectional human MLR (Fig. 1*A*). Compared with other immunosuppressive agents targeting T cells, like mycophenolate mofetil (MMF) or CsA, 1,25$(OH)_2D_3$ was more potent in the inhibition of IFN-γ production (IC_{50} 0.04 nM vs 214 nM for MMF and 30 nM for CsA), but it did not inhibit cell proliferation completely. The inhibitory effect on T cell responses appeared to be indirect because T cell activation by plate-bound anti-CD3, with or without costimulation by anti-CD28, was scarcely affected by 1,25$(OH)_2D_3$, as determined by cell proliferation or IFN-γ secretion (Fig. 1*B*). This indicates that 1,25$(OH)_2D_3$ inhibits the ability of APCs to induce alloreactive T cell activation, rather than directly inhibiting T cells.

Inhibition of DC differentiation by 1,25$(OH)_2D_3$

To determine whether the inhibitory effect of 1,25$(OH)_2D_3$ on APCs might involve DCs, we have first analyzed its effect on DC differentiation (Fig. 2*A*). Human peripheral blood monocytes can be differentiated into immature DCs by culture with GM-CSF and IL-4 (36). During this process, they down-regulate the monocyte

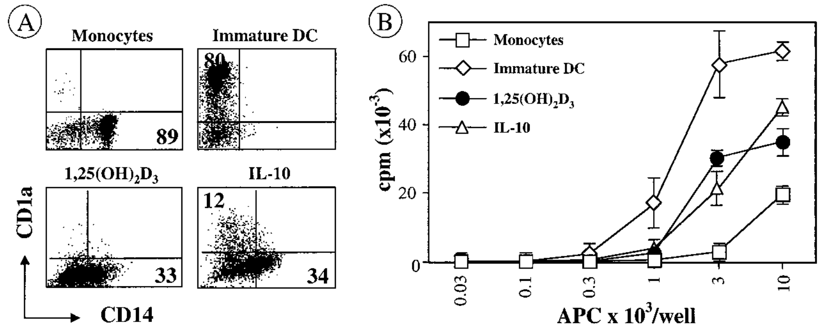

FIGURE 2. Inhibition of DC differentiation by $1,25(OH)_2D_3$. Negatively selected $CD14^+$ monocytes were cultured in medium supplemented with 800 U/ml GM-CSF and 1000 U/ml IL-4 to generate immature DCs. Cells were fed fresh medium every 2–3 days. $1,25(OH)_2D_3$ (10 nM) or IL-10 (10 ng/ml) were added at culture initiation. *A,* Six days after culture initiation, cells were double stained with anti-CD14 and anti-CD1a mAbs and analyzed by flow cytometry. Values indicate the percentage of positive cells. *B,* After extensive washing, the APCs described above were cocultured with $CD4^+$ T cells (2×10^5/well) from a different donor. After 5 days, proliferation was measured by [^{3}H]thymidine incorporation. Proliferative responses are shown as mean (±SE) from triplicate cultures. The data are from a representative experiment out of four performed.

marker CD14 and express the DC marker CD1a. Addition of 10 nM $1,25(OH)_2D_3$ to $CD14^+$ human monocytes cultured for 7 days in the presence of GM-CSF and IL-4 completely inhibited the differentiation of $CD1a^+$ DCs. IL-10, a cytokine that inhibits APCs at different levels, did not completely prevent DC differentiation when added at 10 ng/ml. Both agents gave rise to cells with down-regulated CD14, compared with freshly isolated monocytes. Monocytes differentiated in the presence of $1,25(OH)_2D_3$ or IL-10 yielded APCs with a similarly reduced capacity to stimulate $CD4^+$ cell proliferation in a primary MLR assay (Fig. 2*B*). Thus, $1,25(OH)_2D_3$ inhibits phenotypically and functionally the differentiation of peripheral blood monocytes into immature DCs.

Inhibition of DC maturation by $1,25(OH)_2D_3$

Immature DCs obtained by a 7-day culture with GM-CSF and IL-4 can be induced to mature by incubation with LPS (37). DC maturation is accompanied by slight down-regulation of CD1a, decreased expression of the mannose receptor, induction of the maturation marker CD83, and up-regulation of HLA-DR, CD40, and CD86 molecules (Fig. 3*A*). Addition of $1,25(OH)_2D_3$ prevented the LPS-induced maturation of immature DCs, maintaining DCs at the immature stage characterized by high mannose receptor and low CD83 expression (Fig. 3*B*). Compared with control mature DCs, addition of $1,25(OH)_2D_3$ during maturation inhibited by about 50%, as determined by median fluorescence intensity, the expression of MHC class II, CD58, CD40, CD80, and CD86 molecules. A less pronounced effect was observed when the differentiating DCs were exposed to $1,25(OH)_2D_3$ or IL-10 for 24 h before LPS-induced maturation (data not shown). The inhibitory effect of $1,25(OH)_2D_3$ on DC maturation was comparable to that induced by IL-10, with two exceptions: IL-10 was less efficient in inhibiting CD40 but more efficient in inhibiting CD86 expression. Mature DCs, compared with immature DCs, induced a higher proliferation (Fig. 3*C*) and IFN-γ secretion (Fig. 3*D*) by alloreactive $CD4^+$ cells, due to their increased expression of class II and costimulatory molecules. Immature DCs, matured in the presence of either agent, displayed a reduced Ag-presenting capacity in the activation of alloreactive $CD4^+$ cells, as determined by the decreased proliferation (Fig. 3*C*) and IFN-γ secretion (Fig. 3*D*) in MLR assays. This reduction was more evident for cells treated with $1,25(OH)_2D_3$. Indeed, DCs matured in the presence of $1,25(OH)_2D_3$ showed a capacity to activate alloreactive $CD4^+$ cell as low as that of immature DCs. Similar results were obtained using negatively selected total T cells, indicating that the presence of $CD8^+$ cells in the responding T cell

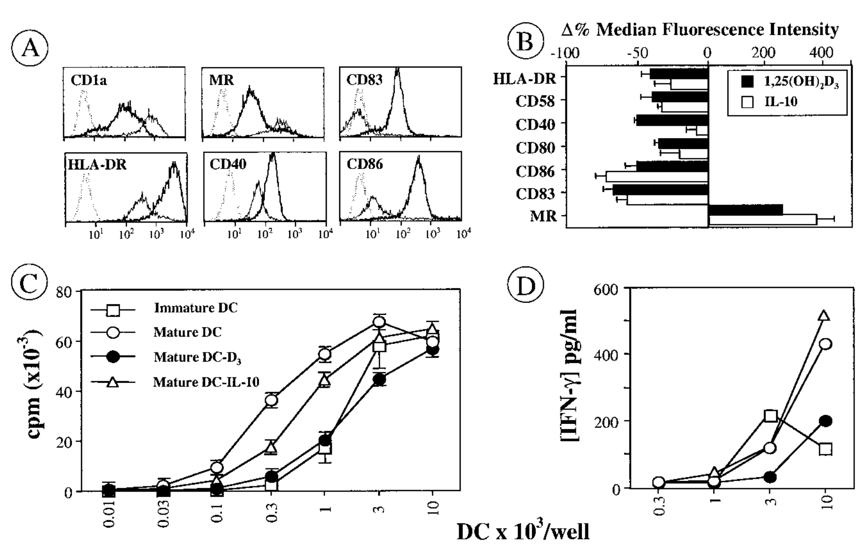

FIGURE 3. Inhibition of DC maturation by $1,25(OH)_2D_3$. Maturation was induced by incubation of immature DCs with LPS (200 ng/ml) for 48 h. *A,* Induction of maturation in control immature DCs, as determined by surface marker expression. Stippled lines refer to isotype controls. The staining profile of immature (solid thin line) and mature (thick line) DCs for the indicated surface molecules is shown. MR, mannose receptor. *B,* Percentage variation of median fluorescence intensity in DCs matured in the presence of 10 nM $1,25(OH)_2D_3$ or 10 ng/ml IL-10. *C* and *D,* After extensive washing, the indicated numbers of DCs, generated as described above, were cocultured with $CD4^+$ T cells (2×10^5/well) from a different donor. After 5 days, proliferation (*C*) and IFN-γ secretion (*D*) were measured. Proliferative responses are shown as mean (±SE) from triplicate cultures. The data are from a representative experiment out of four performed.

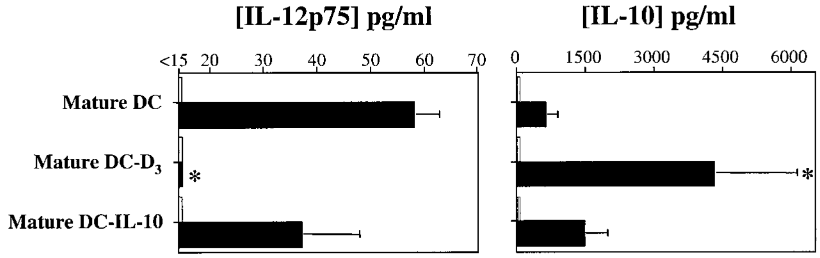

FIGURE 4. Abrogation of IL-12 and enhancement of IL-10 secretion upon activation of $1,25(OH)_2D_3$-treated DCs. Maturation of immature DCs was induced by culture for 48h in the presence of 200 ng/ml LPS with or without 10 nM $1,25(OH)_2D_3$ or 10 ng/ml IL-10. After washing, DCs were cultured with (filled bars) or without (open bars) CD154-transfected J558L cells at a ratio of 1:4. After 24 h, IL-12p75 and IL-10 secretion were measured. Bars represent the mean (±SE) from three separate experiments. *, $p < 0.05$ by Mann-Whitney U test compared with cytokine levels induced by mature DCs.

population does not affect the inhibitory capacity of $1,25(OH)_2D_3$ (data not shown).

Abrogation of IL-12 and enhancement of IL-10 secretion upon activation of $1,25(OH)_2D_3$-treated DCs

Mature DCs can be activated by CD40 cross-linking (38). Activation of mature DCs by incubation with J558L cells transfected with the gene encoding CD154 resulted in IL-12p75 and IL-10 secretion (Fig. 4). Addition of $1,25(OH)_2D_3$ during LPS-induced maturation gave rise to DCs unable to secrete IL-12p75 upon CD40 ligation (Fig. 4*A*) but secreting 7-fold higher levels of IL-10 (Fig. 4*B*). Conversely, addition of IL-10 during DC maturation up-regulated IL-10 production by only 2-fold and did not significantly inhibit IL-12p75 secretion upon activation by CD154-transduced J558L cells, consistent with the resistance of differentiated DCs to the effects of IL-10 (39, 40). Thus, $1,25(OH)_2D_3$ modulates maturing DCs, leading to abrogation of IL-12 but higher IL-10 secretion upon activation.

$1,25(OH)_2D_3$ enhances DC apoptosis following maturation

Apoptosis of mature DCs is strongly enhanced by IL-10 (41). To determine the effect of $1,25(OH)_2D_3$ on DC apoptosis, DCs matured in the presence of LPS with or without $1,25(OH)_2D_3$ or IL-10 were examined immediately after maturation or following a 24-h culture in complete medium. DC apoptosis was quantified by staining with annexin V, which detects changes in the asymmetry of phosphatidylserine in the cell membrane, an early apoptotic marker. Simultaneous staining of cells with FITC-annexin V and with the non-vital dye propidium iodide (PI) allows the discrimination of intact cells ($FITC^-PI^-$), early apoptotic ($FITC^+PI^-$), and late apoptotic or necrotic cells ($FITC^+PI^+$) (42). As shown in Fig. 5, the apoptosis of DCs immediately after maturation (2% $FITC^+PI^-$, 4% $FITC^+PI^+$) was enhanced by addition of $1,25(OH)_2D_3$ (8% $FITC^+PI^-$, 10% $FITC^+PI^+$) or IL-10 (10% $FITC^+PI^-$, 27% $FITC^+PI^+$), confirming the proapoptotic effects of IL-10 and indicating a similar activity of $1,25(OH)_2D_3$. The apoptosis of DCs matured in the presence of $1,25(OH)_2D_3$ was 6-fold enhanced, compared with controls, following a 24-h incubation in plain culture medium. Thus, $1,25(OH)_2D_3$ promotes, when present during DC maturation, DC apoptosis.

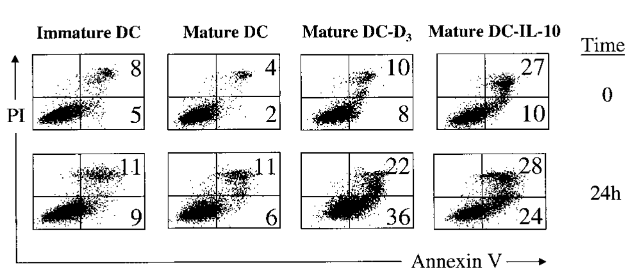

FIGURE 5. $1,25(OH)_2D_3$ enhances DC apoptosis following maturation. Maturation of immature DCs was induced by culture for 48 h in the presence of 200 ng/ml LPS with or without 10 nM $1,25(OH)_2D_3$ or 10 ng/ml IL-10. DC apoptosis was quantified by double staining with FITC-annexin V and PI immediately after maturation or after a 24-h culture period. The data are from a representative experiment out of three performed.

Induction of T cell hyporesponsiveness by $1,25(OH)_2D_3$-treated DCs

These results suggest that the ability of $1,25(OH)_2D_3$ to prevent differentiation, decrease expression of costimulatory molecules during maturation, and modulate cytokine production upon activation of human DCs might contribute to its inhibitory effect on APC-dependent T cell activation. Thus, we examined whether DCs matured in the presence of $1,25(OH)_2D_3$ could modulate T cell responsiveness. Coculture of alloreactive $CD4^+$ T cells with $1,25(OH)_2D_3$-treated DCs resulted in T cell hyporesponsiveness, as demonstrated by their reduced IFN-γ secretion upon restimulation by untreated, mature DCs in a secondary MLR assay (Fig. 6). In contrast, T cell proliferation was only slightly inhibited (data not shown). The IFN-γ secreted, upon restimulation, by $CD4^+$ T cells preincubated with immature or $1,25(OH)_2D_3$-treated mature DCs was similarly reduced, compared with untreated mature DCs.

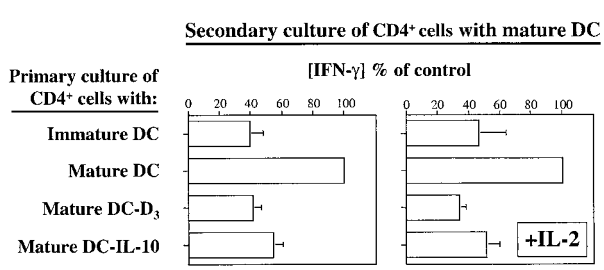

FIGURE 6. Induction of T cell hyporesponsiveness by $1,25(OH)_2D_3$-treated DCs. Allogeneic $CD4^+$ T cells (2×10^5/well) were cocultured with different DC populations (10^3 cells/well). Immature DCs were generated as described in Fig. 2. Maturation of immature DCs was induced by culture for 48 h in the presence of 200 ng/ml LPS with or without 10 nM $1,25(OH)_2D_3$ or 10 ng/ml IL-10. T cells were recovered 36 h after culture initiation, rested for 2 to 4 days in complete medium supplemented with 2 U/ml IL-2, and restimulated with mature untreated DCs, generated from the same donor used as a DC source for the first culture, with (*right panel*) or without (*left panel*) 100 U/ml IL-2. IFN-γ secretion was measured 48 h after restimulation. Bars represent the mean (±SE) percent variation (n = 3) of IFN-γ secretion compared with restimulation with untreated mature DCs. IFN-γ secretion induced by untreated mature DCs in the secondary culture was, in the three individual experiments, 210/2140, 1748/4424, and 345/1038 pg/ml, in the absence or presence of 100 U/ml IL-2, respectively.

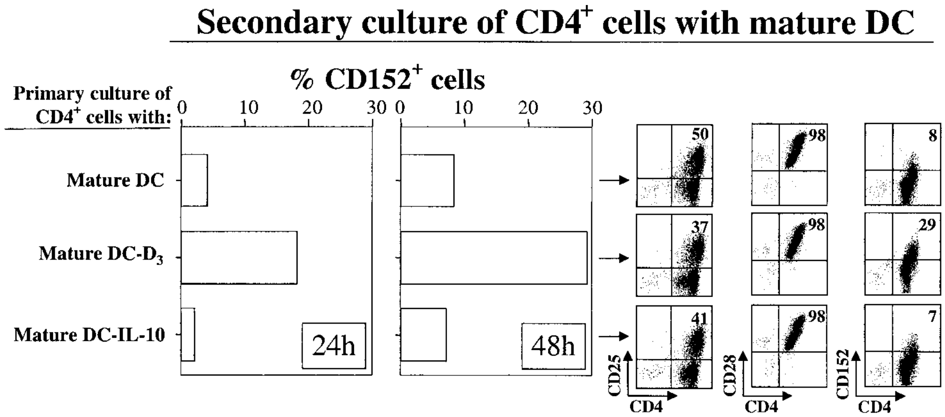

FIGURE 7. Interaction with 1,25(OH)$_2$D$_3$-treated DCs up-regulates CD152 on CD4$^+$ alloreactive T cells. Allogeneic CD4$^+$ T cells (2 $\times$ 10^5/well) were cocultured with DCs (10^3/well) generated as described in Fig. 3. T cells were recovered 36 h after culture initiation, rested for 2 to 4 days in complete medium supplemented with 2 U/ml IL-2, and restimulated with mature untreated DCs, generated from the same donor used as a DC source for the first culture. After 24 h and 48 h, CD152 expression on CD4$^+$ cells was analyzed by flow cytometry. Values refer to the percentage of CD152$^+$ cells. Double staining for CD4 and CD25, CD28, or CD152 expression by T cells analyzed after 48 h of culture with mature DCs is also shown. The data are from a representative experiment out of two performed.

Induction of T cell hyporesponsiveness by DCs matured in the presence of IL-10 was slightly less pronounced. Addition to cultures of exogenous IL-2 could not reverse the inhibition of IFN-γ secretion (Fig. 6, *right panel*).

Interaction with 1,25(OH)$_2$D$_3$-treated DCs up-regulates CD152 on CD4$^+$ alloreactive T cells

To explore possible mechanisms leading to T cell unresponsiveness, we examined whether 1,25(OH)$_2$D$_3$-treated DCs could modulate T cell expression of inhibitory molecules such as CD152 or stimulatory ligands like CD154. The percentage of T cells expressing CD152 was reduced by >50% in a primary coculture of alloreactive CD4$^+$ cells with mature compared with immature DCs or DCs matured in the presence of 1,25(OH)$_2$D$_3$ as well as IL-10. In contrast, CD154 was similarly expressed on T cells stimulated by immature or mature DCs, but CD154-expressing T cells were reduced by >40% in the presence of 1,25(OH)$_2$D$_3$ whereas they were unaffected by IL-10 (data not shown). A time course analysis revealed an early up-regulation of CD152, already expressed on 20% of alloreactive CD4$^+$ cells following a 24-h interaction with 1,25(OH)$_2$D$_3$-treated DCs (Fig. 7). This up-regulation was selective for CD152, because its homologue CD28 was not affected whereas expression of the T cell activation marker CD25 was slightly down-regulated (Fig. 7).

Discussion

The present study demonstrates that 1,25(OH)$_2$D$_3$, the active metabolite of vitamin D$_3$, inhibits the differentiation and maturation of human DCs in vitro. In addition, it abrogates the capacity of mature DCs to secrete IL-12 upon activation, while strongly enhancing IL-10 production. 1,25(OH)$_2$D$_3$ also promotes DC apoptosis. These effects result in inhibition of alloreactive T cell activation and induction of T cell hyporesponsiveness.

Our results clearly establish that DCs are a primary target of the immunosuppressive activity of 1,25(OH)$_2$D$_3$, which affects all major stages of the DC life cycle: differentiation, maturation, activation, and survival. CD14$^+$ peripheral blood monocytes cultured with GM-CSF and IL-4 develop into a homogeneous population of immature DCs, characterized by high capacity for Ag capture but low T cell stimulatory activity (36). Immature DCs can be induced to mature by inflammatory stimuli such as LPS, a process associated with loss of Ag-capturing ability but increased costimulatory capacity for T cell activation (37). T cell-derived signals, such as CD40 ligation by CD154-expressing T cells, can not only induce DC maturation but also activate them to secrete IL-12, a key cytokine for Th1 cell development (43). Finally, mature DCs are programmed to die unless they receive a survival signal from T cells (44). Strikingly, 1,25(OH)$_2$D$_3$ profoundly affects all stages of the DC life cycle, inhibiting their differentiation and maturation as well as modulating their activation and survival.

Monocytes (45) and DCs (46) express vitamin D$_3$ receptors constitutively. Incubation of normal human monocytes with 1,25(OH)$_2$D$_3$ has been previously shown to decrease accessory cell function with down-regulation of Ag-presenting capacity (30), but this did not appear to correlate with decreased expression of CD18, CD44, CD54, CD58, or HLA-DR molecules (47), suggesting the involvement of other costimulatory molecules. Decreased CD86 but not CD80 expression was found to be induced by 1,25(OH)$_2$D$_3$ on human resting monocytes stimulated with IL-10, IFN-γ, or TNF-α, but not with LPS (48). Indeed, as shown in the present study, CD40, CD58, CD80, and CD86 expression is clearly inhibited by addition of 1,25(OH)$_2$D$_3$ during LPS-induced DC maturation.

The inhibition of DC costimulatory molecule expression by 1,25(OH)$_2$D$_3$ is intriguing, given the critical role of costimulatory signals delivered by CD40, CD80, and CD86 for optimal T cell activation (8, 9). Administration of anti-CD154 mAb disrupting the CD40-CD154 pathway (9) and/or CD152-Ig blocking the CD80/CD86-CD28 pathway (8, 11) have been shown to be effective in several models of autoimmune diseases and allograft rejection. This suggests that at least part of the immunosuppressive activity exerted by 1,25(OH)$_2$D$_3$ in similar models (14) may be due to down-regulation of costimulatory molecule expression. Our results also demonstrate that T cells cultured with 1,25(OH)$_2$D$_3$-treated DCs up-regulate CD152, a ligand that inhibits both early and late T cell activation (49), and down-regulate CD154, further contributing to the disruption of costimulatory pathways. The early up-regulation of CD152 coupled with the down-regulation of CD154 may be particularly relevant, considering the capacity of CD152-Ig to synergize with anti-CD154 in costimulation blockade (11). Costimulation blockade has also been shown, in some cases, to induce transplantation tolerance (11). Stimulation by

$1,25(OH)_2D_3$-treated DCs leads to T cell unresponsiveness, suggesting a similar mechanism of action. Inactivation of host DCs has been recently proposed to prevent graft-vs-host disease in allogeneic bone marrow transplantation (50), and a similar strategy is applicable to solid organ allografts (51). We are currently examining whether a short-term administration of $1,25(OH)_2D_3$ to islet allograft recipients may be able, via inhibition of host DCs, to induce long-lasting graft acceptance and transplantation tolerance. If this is the case, a $1,25(OH)_2D_3$ analogue with enhanced immunosuppressive activity and reduced effects on the calcium/phosphate metabolism may lead to an effective prevention of human allograft rejection. Efforts to reach this goal are already ongoing (52).

Several agents have been shown to inhibit, at different levels, DCs, including glucocorticoids (53, 54), PGE_2 (55), *N*-acetyl-L-cysteine (NAC) (56), and IL-10. IL-10 decreases Ag-presenting capacity (57) and IL-12 production (58, 59) by DCs, as well as promoting their apoptosis (41). In addition, IL-10-treated DCs can induce anergy in $CD4^+$ cells (39). In the present study, IL-10 has been routinely compared with $1,25(OH)_2D_3$, and, overall, their activity is quite similar. However, $1,25(OH)_2D_3$ is very effective in inhibiting IL-12 production by mature DCs whereas IL-10 is not, consistent with the resistance of mature DCs to IL-10 effects (39, 40, 60). Moreover, the inhibition of CD40 expression is more evident in DCs matured in the presence of $1,25(OH)_2D_3$ rather than IL-10, whereas CD80 and CD86 are similarly inhibited. $1,25(OH)_2D_3$ or IL-10-treated DCs induce a similar hyporesponsiveness in alloreactive $CD4^+$ cells, as judged by their impairment in IFN-γ secretion upon restimulation. However, T cells are differentially modulated, as shown by the up-regulation of CD152 expression induced on alloreactive $CD4^+$ cells by DCs treated with $1,25(OH)_2D_3$ but not IL-10. In addition to glucocorticoids (54), PGE_2 (55), like other cAMP-raising agents (61), and NAC (56) also inhibit IL-12 production by DCs. The inhibition of IL-12 production induced by $1,25(OH)_2D_3$ in mature DCs is mediated by inhibition of the transcription factor NF-κB (62) and, interestingly, inhibition of IL-12 production by NAC has also been found to be associated with NF-κB inhibition (56).

Several TNF family members contribute to DC survival. In addition to TNF-α itself (41), and CD154 (38), TNF-related activation-induced cytokine (TRANCE) is a DC-specific survival factor that regulates the expression of the anti-apoptotic molecule Bcl-x_L (63). TRANCE is expressed in activated T cells and promotes, in cooperation with TNF-α and CD154, the survival of mature DCs, which selectively express high levels of TRANCE receptor (64). It would be of interest to analyze TRANCE receptor expression in $1,25(OH)_2D_3$-treated DCs, in light of our results showing their enhanced apoptosis, given the emerging role of TRANCE and its receptors in the control of DC and bone homeostasis (65). A proapoptotic activity of $1,25(OH)_2D_3$ has been described in tumor cells (66) and may also take place in vivo, as suggested by its apoptosis-enhancing effects on diabetogenic Th1 cells (67).

In conclusion, DCs are primary targets for the immunosuppressive activity of $1,25(OH)_2D_3$, as indicated by its capacity to inhibit DC differentiation and maturation, leading to marked down-regulation of MHC class II and costimulatory molecules. In addition, $1,25(OH)_2D_3$ inhibits IL-12, while enhancing IL-10 production, and promotes DC apoptosis. These effects could contribute substantially to decrease DC-dependent T cell activation and could largely account for the immunosuppressive properties of $1,25(OH)_2D_3$. The use of a $1,25(OH)_2D_3$ analogue with immunosuppressive activity at nonhypercalcemic doses may permit the exploitation of the modulation of DC activity in the treatment of autoimmune diseases and allograft rejection.

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