# TGF-$\beta$ and Vitamin $D_3$ Utilize Distinct Pathways to Suppress IL-12 Production and Modulate Rapid Differentiation of Human Monocytes into $CD83^+$ Dendritic Cells

Lyudmila A. Lyakh,* Michael Sanford,† Sebel Chekol,* Howard A. Young,† and Anita B. Roberts[1]*

**We previously demonstrated that agents known to signal infection or inflammation can rapidly and directly drive differentiation of human $CD14^+$ monocytes into $CD83^+$ dendritic cells (DCs) when introduced to cells under serum-free conditions. In this study, we evaluated the effects of TGF-$\beta$ and vitamin $D_3$ ($VitD_3$) on the proportion and function of monocytes that adopt DC characteristics. TGF-$\beta$ significantly decreased the proportion of cells that rapidly adopted stable DC characteristics in response to LPS, but had little or no effect on calcium ionophore-induced differentiation. In contrast, $VitD_3$ showed no such pathway specificity and dramatically suppressed differentiation of monocytes into DCs in response to these agents. Both TGF-$\beta$ and $VitD_3$ altered cytokine and chemokine production in LPS-treated monocytes, inhibited IL-12 and IL-10 secretion, and decreased the functional capacity of DCs. Despite the similar effects of TGF-$\beta$ and $VitD_3$, there are significant differences in the signaling pathways used by these agents, as evidenced by their distinct effects on LPS- and calcium ionophore-induced DC differentiation, on LPS-induced secretion of IL-10, and on two members of the NF-$\kappa$B family of transcription factors, RelB and cRel. These studies identify TGF-$\beta$ and $VitD_3$ as potent regulatory factors that use distinct pathways to suppress both the differentiation of DCs as well as their capacity to secrete the Th1-polarizing cytokine IL-12. Because these agents are present in serum and negatively affect DC differentiation at physiological concentrations, our findings are likely to have significance regarding the in vivo role of TGF-$\beta$ and $VitD_3$ in determining the type of immune responses.** ***The Journal of Immunology,* 2005, 174: 2061–2070.**

Dendritic cells (DCs)[2] are professional APCs that initiate and mediate immune responses. They are equipped with the biochemical machinery for processing and presenting peptide fragments of protein Ags on major MHC molecules. DCs sense and process Ags in the periphery (immature stage), migrate to lymphoid tissues, and present antigenic peptides to naive T cells (mature stage) (1). Both the activation status of DCs and the local cytokine milieu appear to play important roles in the outcome of T cell responses and in determining the balance between immunity and tolerance induction (2, 3). Both myelomonocytic precursor cells and differentiated monocytes have been shown to be capable of differentiating to peripheral DCs.

Blood monocytes can give rise to macrophages, but they also develop into DCs under specific conditions (3, 4). An estimated 25% of the circulating inflammatory monocytes will differentiate into migrating DCs, whereas the others will give rise to resident macrophages (4, 5). It is not yet completely understood which factors and/or conditions regulate differentiation along the DC pathway. It is conceivable that the differentiation of monocytes into DCs or macrophages in tissues is shaped by exposure to local environmental signals encountered during their transit, and that there can be conditions that favor the development of one or the other cell type.

We described previously a serum-free system whereby human $CD14^+$ peripheral blood monocytes can be induced to rapidly differentiate into mature $CD83^+$ DCs in response to LPS, TNF-$\alpha$, or calcium ionophore (CI), without the need for a lengthy predifferentiation step (6, 7). We demonstrated that half of $CD14^+$ monocytes cultured under serum-free conditions and treated with GM-CSF and LPS rapidly adopted this DC phenotype. The remaining cells retained the $CD14^+/CD83^-$ monocyte/macrophage phenotype. Using this system, we identified IFN-$\gamma$ as a DC differentiation cofactor and delineated a role for IL-10 as an autocrine/paracrine regulatory factor that suppresses IL-12 production and the proportion of monocytes that differentiate into DCs in response to LPS (8). We also suggested that serum may actually contain components that retard the generation of DCs under the conditions tested.

To identify additional factors that influence whether monocytes adopt a DC immunophenotype, we have now examined the effects of TGF-$\beta$ and an active form of vitamin $D_3$, 1$\alpha$,25-dihydroxyvitamin $D_3$ ($VitD_3$). TGF-$\beta$ is a member of a large family of evolutionary conserved proteins with highly pleiotropic properties. One or more of the TGF-$\beta$ family receptors and ligands are expressed in every tissue in the body. A loss-of-function mutation in TGF-$\beta$1 in mice results in a severe, multifocal inflammatory response and death within 3–4 wk, which demonstrates its essential role in maintenance of immune homeostasis (9–12). The deregulation of multiple and interconnected immune cell compartments in TGF-$\beta$1-deficient mice, including $CD8^+$ T cells, $CD4^+$ T cells,

*Laboratory of Cell Regulation and Carcinogenesis, National Cancer Institute, Bethesda, MD 20892; and †Laboratory of Experimental Immunology, National Cancer Institute, Frederick, MD 21702

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[1] Address correspondence and reprint requests to Dr. Anita B. Roberts, National Cancer Institute, 41 Library Drive, MSC 5055, Bethesda, MD 20892-5055. E-mail address: Robertsa@dce41.nci.nih.gov

[2] Abbreviations used in this paper: DC, dendritic cell; CI, calcium ionophore; LC, Langerhans cell; RPA, RNase protection assay; SFM, serum-free medium; SFM/G, macrophage SFM supplemented with human rGM-CSF; TGF-$\beta$ receptor, T$\beta$R; VDR, vitamin D receptor; $VitD_3$, vitamin $D_3$.

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B cells, macrophages, and DCs, has complicated the elucidation of the role of TGF-β in the regulation of each cell type. An essential function of TGF-β in the regulation of T cell homeostasis was further demonstrated in various genetically modified mice in which TGF-β signaling is blunted in T cells specifically. The inhibitory effects of TGF-β on T cells include most, if not all, effector functions of naive T cells (13).

In addition to its direct inhibitory effects on T cells, TGF-β might also hamper T cell activation and differentiation indirectly through APCs. Studies in TGF-β1-deficient mice have identified an essential role for TGF-β1 in differentiation of specific DC subsets in vivo (9–12). Moreover, TGF-β has been shown to prevent maturation of DCs in vitro, inhibiting the up-regulation of critical costimulatory molecules on the surface of DCs and reducing their Ag-presenting capacity (14). Molecular targets of inhibitory effects of TGF-β on DCs are not yet clear, making it important to study its role in regulation of these cells.

$VitD_3$ and its analogues also have important effects on regulation of T cells and APCs, including DCs, both in vivo and in vitro (15), in which they have been shown to modulate DC differentiation and maturation in both human and murine culture systems (16, 17). $VitD_3$ acts at two different steps, by inhibiting their differentiation from monocyte precursors, thus impairing the normal turnover of DCs in tissues, and by inhibiting the terminal differentiation of DCs into potent APCs (18–20). $VitD_3$ can also modulate TGF-β production and TGF-β receptor expression. In several cell types (osteoblasts, breast cancer cells, prostatic epithelial cells, and human promyelocytic leukemia HL-60 cells), $VitD_3$ inhibits cellular growth by increasing secretion of TGF-β and up-regulating expression of TGF-β receptors, type I and II (TβRI and TβRII) (21–23). However, other investigators have reported an absence of increased expression of the TβRI and TβRII caused by $VitD_3$ in particular cell types, such as human myelomonocytic U937 leukemia cells (24). The complexity of interactions between the TGF-β signaling system and $VitD_3$ is further highlighted by the observation that Smad proteins, downstream mediators of TGF-β signaling, can act both as positive and negative modulators of vitamin D receptor (VDR). Moreover, the cooperative actions of $VitD_3$ and TGF-β can be synergistic or antagonistic in a cell-specific manner (23, 25, 26), or in other contexts, they can act independently (16).

In the studies presented in this work, we have tested the ability of TGF-β and $VitD_3$, both present in serum, to influence the differentiation of human normal peripheral blood monocytes into DCs, cultured in vitro under serum-free conditions. These studies identify that TGF-β and $VitD_3$ act through distinct mechanisms to suppress both the differentiation of $CD14^+$ monocytes into $CD83^+$ DCs as well as their capacity to secrete the Th1-polarizing cytokine IL-12.

## Materials and Methods

### *mAbs and FACS analysis*

FACS protocols including mAbs specific for human CD14, CD83, CD80, CD40, CD33, and HLA-DR, as well as isotype-matched control mAbs were identical with those used in previously published studies (27, 28). Anti-human TLR2 and TLR4 mAbs were purchased from eBioscience, and anti-human TβRII mAbs were purchased from R&D Systems. A gate (R1) was defined in all FACS analysis to exclude all nonviable cells and debris, based on size and propidium iodine staining. The instrumentation used in the flow cytometry studies was a FACSCalibur flow cytometer (BD Biosciences) running CellQuest analysis software.

### *Human peripheral blood monocytes and allogenic mixed lymphocyte culture*

Human $CD14^+$ peripheral blood monocytes (90–95% purity) were obtained from healthy donors by leukapheresis and countercurrent centrifugal elutriation according to National Institutes of Health guidelines for human subjects (7). Lymphocyte-rich fractions were also collected and cryopreserved. T cells for allosensitization studies were purified using T cell isolation columns (R&D Systems). Monocytes were further purified using MACS CD14 microbeads (Miltenyi Biotec). Cells were plated at a density of $2.5 \times 10^6$ cells/well in 24-well tissue culture plates (Costar) in 2 ml/well macrophage serum-free medium (SFM) (Invitrogen Life Technologies) supplemented with 50 ng/ml human rGM-CSF (Amgen), which is necessary for maintaining cell viability, as described previously (6). This combination of SFM and GM-CSF, which constitutes basal culture medium for all monocytes and DCs used in these studies, is henceforth referred to in the text simply as SFM/G. In some experimental groups, cultures were supplemented at time zero with 0.25–10 ng/ml human rTGF-β1 (a kind gift of R&D Systems) or 0.01–100 nM $1\alpha,25(OH)_2$-$VitD_3$ (Roche). Cells cultured overnight were induced to differentiate by addition of 50 ng/ml LPS derived from *Escherichia coli* strain O26:B6 (Sigma-Aldrich) or 188–225 ng/ml calcium ionophore A23187 (Sigma-Aldrich). Anti-human IL-10-neutralizing mAbs (MAB217; R&D Systems) or isotype-matched negative control mAbs were added before the addition of LPS. For allosensitization studies, T cells ($2 \times 10^5$ per well) were plated in 96-well plates in RPMI 1640 medium, as described previously (6, 27). The gamma-irradiated (30 Gy) monocytes or monocyte-derived DCs were added at varying T cell-to-APC ratios and cocultured for 96 h at 37°C in 5% $CO_2$. Cells were then pulsed with 1 μCi (.038 MBq) per well [$^3$H]thymidine ([$^3$H]TdR, 87 Ci/mmol; Amersham Biosciences) and harvested 18 h later. The [$^3$H]TdR incorporation was assessed by liquid scintillation spectroscopy.

### *Preparation of cellular extracts and immunoblotting*

Cells were rinsed and resuspended in ice-cold hypotonic buffer (25 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$, 5 mM KCl) with protease inhibitor mixture (Roche) at a concentration of $1–2 \times 10^7$ cells/ml, incubated on ice for 15 min, and lysed with an equal volume of hypotonic buffer containing 0.3% Nonidet P-40, additional protease inhibitor $\alpha_2$-macroglobulin (2 U/ml; Roche), and phosphatase inhibitors (1 mM sodium vanadate (Sigma-Aldrich) and 1 μM okadaic acid (Alexis Biochemicals)). The lysate was centrifuged at $500 \times g$ for 5 min; the supernatant constitutes the cytoplasmic fraction. The pelleted nuclei were extracted with 30–50 μl of a solution containing 10 mM Tris (pH 7.4), 220 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 5 μM $ZnCl_2$, 1% Triton X-100, and inhibitors at 4°C for 20 min with agitation. The extract was centrifuged at $15{,}000 \times g$ for 20 min at 4°C; the supernatant constitutes the nuclear extract.

For whole cell extracts, cells were pelleted and lysed in a buffer containing 10 mM Tris (pH 7.4), 220 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 5 μM $ZnCl_2$, 1% Triton X-100, and inhibitors, as described previously (7). A total of 10–30 μg of protein was resolved by 10% Tricine SDS-PAGE (Invitrogen Life Technologies) and transferred to Immunobilon-P (Millipore) or nitrocellulose (Schleicher & Schuell Microscience). The membrane was probed with specific Abs. Immunoreactive proteins were revealed with an ECL reagent (Amersham Biosciences).

Rabbit antisera that recognize human cRel (serum 265), human RelB (serum 1319), human p105/p50 (serum 1157), human p100/p52 (serum 1267), and human p65 (serum 1226) have been described previously (6, 29, 30). Anti-phosphorylated Smad2, anti-phosphorylated p38, and anti-total p38 were purchased from Cell Signaling Technology; anti-total Smad 2/3 mAb from BD Biosciences; anti-Oct-1 rabbit polyclonal Abs from Santa Cruz Biotechnology; and anti-actin mAb from Chemicon International.

### *Cytokine and chemokine quantitation*

ELISA kits (R&D Systems) were used to quantify several cytokines and chemokines in 48- to 72-h culture supernatants of monocytes exposed to $VitD_3$ and/or various cytokine combinations with or without LPS. Detection limits were as follows: for TNF-α and RANTES, 16 pg/ml; for IL-10, 47 pg/ml; for IL-12 p70, 15 pg/ml. Assays were performed by the Lymphokine Testing Laboratory, Science Applications International, National Cancer Institute.

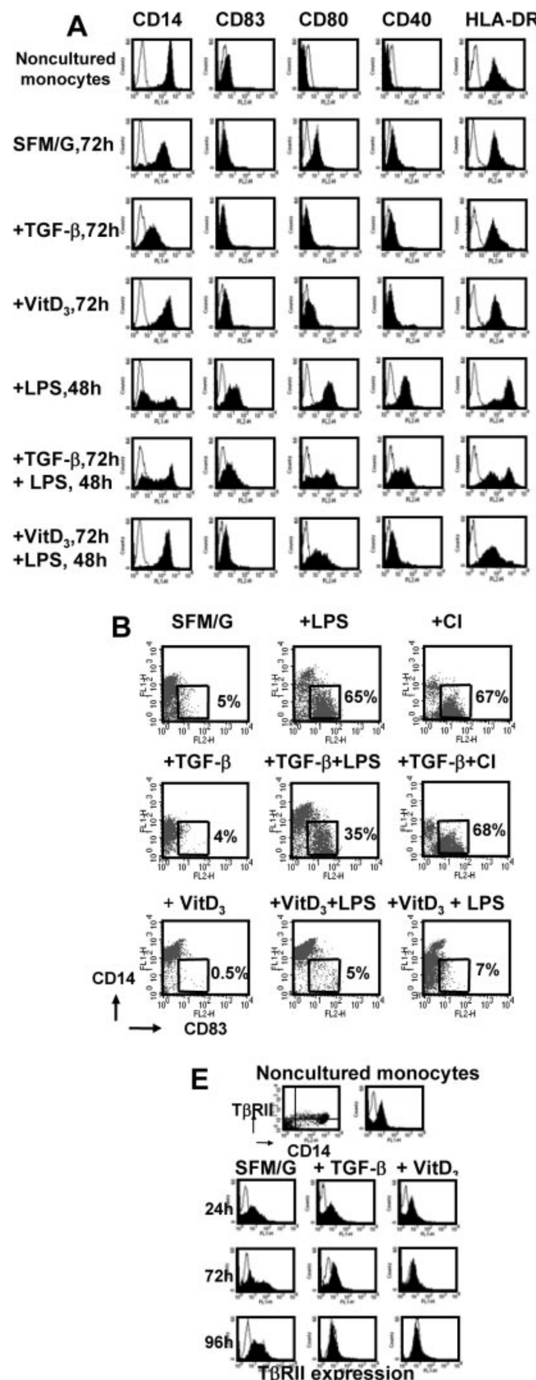
### *RNase protection assay (RPA)*

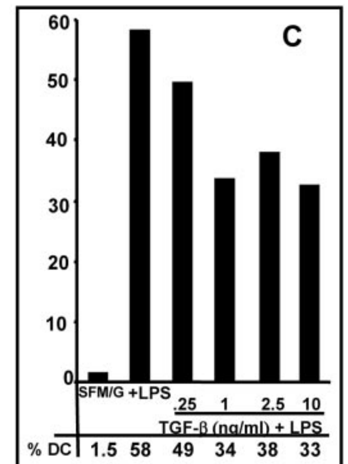
The multiprobe RNase protection assay (RPA) was performed, as described in detail elsewhere (31).

## Results

### *TGF-β and $VitD_3$ selectively modulate the rapid acquisition of a DC immunophenotype by human monocytes under serum-free conditions*

Peripheral DC differentiation pathway derives from myelomonocytic precursor cells or differentiated monocytes. Because the net outcome of T cell immune responses seems to be significantly influenced by the activation stage of DCs, we evaluated the effects of TGF-β and $VitD_3$, both present in serum, on rapid LPS- or CI-induced differentiation of human $CD14^+$ peripheral blood monocytes into DCs, using the serum-free system that we have previously described (6). In this system, human monocytes treated with inducing agents in the absence of serum and IL-4 can proceed directly to DCs without a defined intermediate stage. As detailed in Fig. 1, *A* and *B*, noncultured, elutriated human monocytes almost uniformly expressed CD14 and HLA-DR, but were largely low/negative for the expression of the mature DC marker CD83, or of costimulatory molecules CD80 and CD40. As reported (6), the culture of monocytes for 3 days under such conditions led to a small down-regulation of CD14 and virtually no up-regulation of CD83. In addition, a moderate up-regulation of CD80 and CD40, with little or no change in HLA-DR, was observed. The addition of TGF-β further decreased CD14 expression and prevented the modest up-regulation of CD80 observed under SFM/G conditions. When monocytes were treated with $VitD_3$, cells retained a high level of CD14 expression and were negative for the expression of CD83, CD80, and CD40. There was little effect on HLA-DR expression.



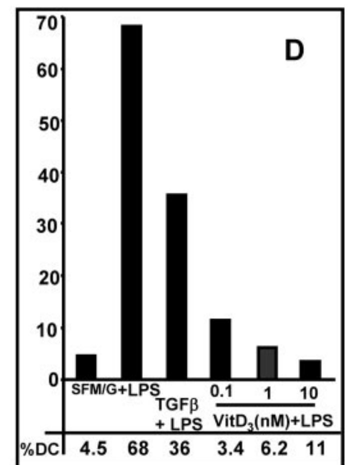




**FIGURE 1.** Both TGF-β and $VitD_3$ suppress the acquisition of the DC immunophenotype by human $CD14^+$ monocytes under serum-free conditions. *A*, Multipanel histogram analysis of human monocytes that were treated in one of the following ways: not cultured, cultured in SFM/G only, cultured in SFM/G plus TGF-β (2 ng/ml), in SFM/G plus $VitD_3$ (1 nM), cultured overnight in SFM/G with or without TGF-β or $VitD_3$, as indicated, and then exposed to LPS for additional 48 h. Open traces represent isotype-matched control Ab staining; filled traces indicate a marker-specific Ab. *B*, Cells were cultured overnight in SFM/G with or without TGF-β or $VitD_3$ and then exposed or not to LPS (50 ng/ml) for 2 additional days or CI (188 ng/ml) for 1 additional day. Percentages indicate proportion of cells adopting DC immunophenotype ($CD14^-/CD83^+$). *A* and *B*, Results are representative of 35 experiments with 30 different donors. *C* and *D*, Cells were cultured overnight in SFM/G with or without TGF-β and $VitD_3$ and then exposed or not to LPS (50 ng/ml) for 2 additional days, harvested, and subjected to FACS analysis. Percentages indicate proportion of cells adopting DC immunophenotype ($CD14^-/CD83^+$). *C* and *D*, Representative results of single donor. *E*, Histogram analysis of human monocytes that were treated in one of the following ways: not cultured, cultured in SFM/G only, cultured in SFM/G plus TGF-β (2 ng/ml), and in SFM/G plus $VitD_3$ (1 nM), as indicated. Open traces represent isotype-matched control Ab staining; filled traces indicate a marker-specific Ab. Results are representative of five experiments with five different donors.

Based on the above data, we predicted that both TGF-β and $VitD_3$ might suppress the rapid adoption of a $CD14^-/CD83^+$ DC immunophenotype induced by LPS and CI under serum-free conditions. As reported previously (6), $CD14^+$ monocytes cultured overnight in SFM/G and then exposed to LPS for 2 additional days of culture commonly presented two well-defined subpopulations: the first retained a $CD14^+/CD83^-$ monocyte/macrophage phenotype, and the other acquired a $CD14^-/CD83^+$ DC phenotype (~65% of cells in this case; Fig. 1*B*). Treatment with CI induced an even more rapid (24–48 h) acquisition of DC phenotype (in this case, 67% of cells). When cells were cultured in SFM/G plus TGF-β (2 ng/ml) for 16–18 h before the addition of LPS, the proportion of monocytes that adopted stable DC characteristics in response to LPS was significantly decreased (only 35% of cells were $CD14^-/CD83^+$). At the same time, TGF-β had no inhibitory effect on CI-induced differentiation (67% of $CD14^-/CD83^+$ cells for CI alone vs 68% for TGF-β plus CI). Dose-response studies showed that the inhibitory effect of exogenously added TGF-β on LPS-induced DC differentiation was detectable at 0.25 ng/ml and reached a maximum by 1 ng/ml TGF-β (Fig. 1*C*).

In contrast to TGF-β, overnight treatment of monocytes with $VitD_3$ completely prevented the acquisition of a $CD14^-/CD83^+$ DC immunophenotype in response to both agents (Fig. 1, *A* and *B*). Dose-response studies indicated that monocytes cultured in SFM/G were very sensitive to $VitD_3$ over a range of normal physiological concentrations. Plateau responses were seen in the dose range of 0.1–100 nM (Fig. 1*D*), although an inhibitory effect was still evident at concentrations as low as 0.01 nM when cells were exposed to $VitD_3$ for 16–18 h before LPS addition. $VitD_3$ was also able to significantly inhibit the acquisition of the $CD14^-/CD83^+$ phenotype when cells were treated simultaneously with $VitD_3$ and LPS, or even when $VitD_3$ was added 2 h after LPS treatment (data not shown).

Next, we tested the effects of TGF-β and $VitD_3$ on LPS-induced surface expression of costimulatory molecules (Fig. 1*A*). In addition to changes in CD14 and CD83, treatment with LPS greatly enhanced expression of CD80, CD40, and HLA-DR compared with that of controls cultured in SFM/G only (Fig. 1*A*). In cells cultured with TGF-β before the addition of LPS, the expression of CD83, CD80, CD40, and HLA-DR was lower than in cells treated with LPS alone, but still considerably higher than in cells treated with SFM/G alone. In cells treated with $VitD_3$ plus LPS, the expression of CD80, CD40, and HLA-DR was dramatically reduced, nearly to control levels. Neither TGF-β nor $VitD_3$ treatment had a significant effect on cell viability over the 96-h culture period. In most experiments, viability ranged from 92 to 99% for LPS treatment and from 85 to 90% for CI treatment.

TGF-β exerts its biological effects by interacting with specific cell surface receptors. TβRII is essential for the binding of TGF-β to the receptor complex, and TβRI is necessary for downstream signal transduction (32). We tested whether TGF-β or $VitD_3$ can modulate the expression of TβRII on human monocytes. As detailed in Fig. 1*E*, monocytes cultured in SFM/G were able to maintain the expression of TβRII up to 96 h. At the time of treatment with inducing agents, human monocytes expressed TβRII on the cell surface at all conditions tested. Addition of $VitD_3$ or TGF-β resulted in down-regulation of TβRII expression at later time points. However, it remains to be proven whether the TβRII expression level defines the ability of monocytes to maintain the monocyte/macrophage immunophenotype in response to LPS.

TGF-β and $VitD_3$ also dramatically affected the appearance and behavior of cells in culture. Monocytes cultured with SFM/G were moderately adherent with little tendency to aggregate. LPS-treated cells formed large, loosely adherent clusters (Fig. 2*A*), which are typical of DCs matured in vitro (33, 34). In contrast, monocytes treated with TGF-β or $VitD_3$ and LPS formed much smaller clusters. When fixed and stained cells were examined at higher magnifications, morphological changes of individual cells induced by LPS became apparent. Cells from LPS-treated cultures displayed elaborate cellular processes consistent with DCs (Fig. 2*B*). In contrast, LPS-treated cells cultured for the same length of time in SFM/G supplemented with TGF-β or $VitD_3$ almost completely lacked these distinctive elaborate processes. Together, these results demonstrate that under serum-free conditions, TGF-β and $VitD_3$ act as potent regulatory factors that suppress the rapid differentiation of $CD14^+$ monocytes into $CD83^+$ DCs induced by LPS or CI.

### *Both $VitD_3$ and TGF-β decrease APC function in the allogenic MLR*

Given the dramatic effect of $VitD_3$ and TGF-β on expression of LPS-induced markers, we decided to test its effects on the functional capacity of the treated cells as APCs in the allogenic MLR. Uncultured monocytes and monocytes cultured in SFM/G plus $VitD_3$ had the poorest capacity to stimulate proliferation of T cells (Fig. 3). Culture in SFM/G resulted in enhanced function, consistent with the up-regulation of expression of CD80 and CD40. Cells treated with LPS were the most efficient at stimulating T cell proliferation. A profound weakening in MLR-inducing activity was seen when cells were cultured in SFM/G with $VitD_3$ before LPS

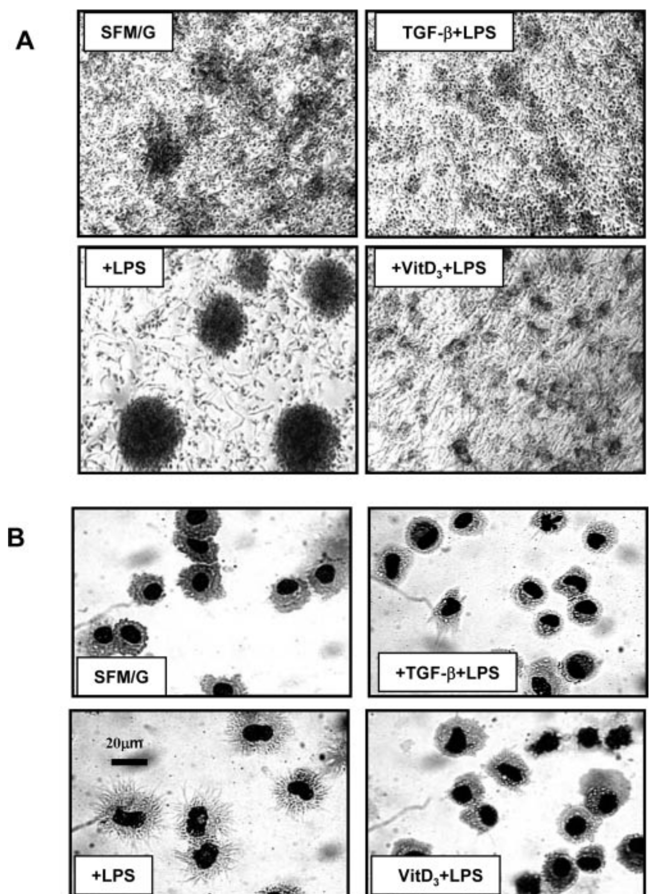



**FIGURE 2.** Effect of TGF-β and $VitD_3$ on microscopic appearance of LPS-treated monocytes undergoing DC differentiation under serum-free conditions. Cells treated as in Fig. 1 were harvested after 5-day culture. Live cells (*A*) and cytospin preparations made from these cells (*B*) were subjected to photomicroscopy.

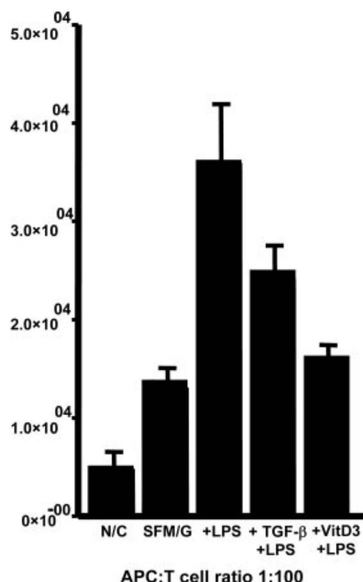



**FIGURE 3.** $VitD_3$ and TGF-$\beta$ decrease APC potency of LPS-differentiated DCs. Human monocytes were cultured in SFM/G with or without $VitD_3$, and TGF-$\beta$ and then exposed or not to LPS. Seventy-two hours postaddition of LPS, cultured APCs were harvested and gamma-irradiated (30 Gy). Irradiated monocytes or monocyte-derived DCs were added to 96-well tissue culture plates with purified allogenic T lymphocytes ($2 \times 10^5$ per well). Cells were cocultured for 96 h, pulsed with 1 $\mu$Ci (0.038 MBq) of [$^3$H]TdR for additional 18 h, and then harvested, and the total incorporation was assessed by liquid scintillation spectrometry. Results are representative of two experiments with three different donors. Results are expressed as the average ($\pm$SEM) of triplicate wells.

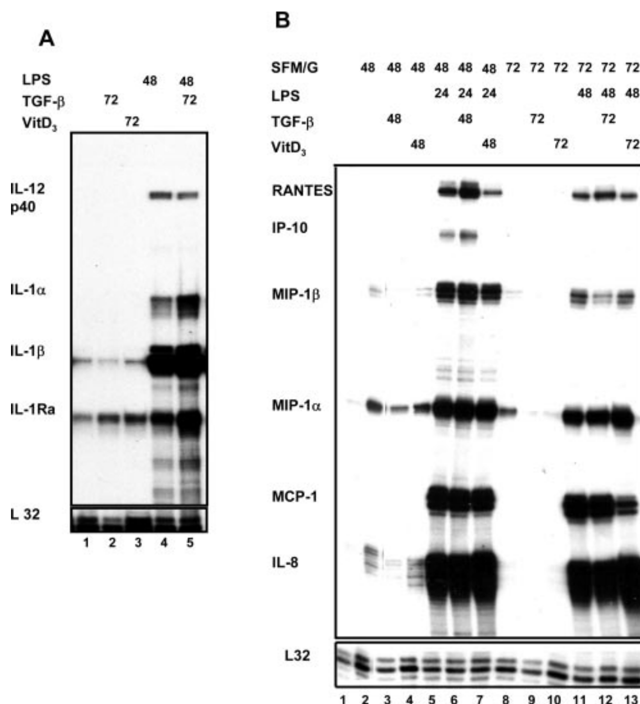



**FIGURE 4.** TGF-$\beta$ and $VitD_3$ alter cytokine and chemokine mRNA expression in human monocytes treated with LPS. RPA analysis was conducted, as described in *Materials and Methods*, using commercially available probe sets (hck-2 (*A*) and hck-5 (*B*); BD Pharmingen). Presented results are representative of multiple monocyte preparations.

treatment (Fig. 3). This dramatically diminished APC function was consistent with demonstrated lack of expression of CD83, the marker of activated DCs, and the low level of expression of costimulatory molecules (CD80, CD40, and HLA-DR) together with the inability of cells to down-regulate CD14 in response to LPS. Incubation of monocytes with TGF-$\beta$ also reduced the functional capacity of LPS-treated cells, but the effect was more modest than that of $VitD_3$, in keeping with the more limited ability of TGF-$\beta$ to suppress LPS-induced DC differentiation.

### *Both TGF-$\beta$ and $VitD_3$ alter cytokine production in LPS-treated monocytes*

DCs synthesize a large number of cytokines and chemokines that exert profound autocrine and paracrine effects (35). It has been demonstrated previously that calcium-activated DCs secrete no TNF-$\alpha$ (6), IL-10 (our unpublished data), IL-12, or IL-6, and minimal MIP-1$\beta$, and show preferential ability to promote Th2 characteristics (36). To test whether TGF-$\beta$ or $VitD_3$ treatment alters the pattern of LPS-induced cytokine/chemokine production, we performed RPA. Housekeeping genes L32 and GAPDH (data not shown) provided internal controls. Previous studies indicated that $VitD_3$ specifically inhibits mRNA expression for both IL-12 p35 and p40 subunits in human monocytes (37). In contrast to $VitD_3$, we found that TGF-$\beta$ did not block IL-12 p40 mRNA expression in cells treated with LPS (Fig. 4*A*, compare *lane 5* with *lane 4*). In addition, TGF-$\beta$ caused slight increases in the LPS-induced expression of mRNA for IL-1$\alpha$ and IL-1 receptor antagonist (*lane 5*).

Chemokine genes, which are up-regulated by LPS, include RANTES, MIP-1$\alpha$, MIP-1$\beta$, MCP-1, IL-8, and IFN-induced protein 10, all of which are commonly expressed by DCs (38–40). TGF-$\beta$ did not significantly affect LPS-induced expression of any of these chemokine mRNAs (Fig. 4*B*, *lanes 6* and *12*). In contrast, $VitD_3$ blocked up-regulation of IFN-induced protein 10 mRNA, a Th1-type chemokine, and reduced expression of RANTES mRNA, when measured 24 h after addition of LPS (*lane 7*). By 48 h after treatment with LPS, $VitD_3$ partially inhibited MCP-1 mRNA expression (*lane 13*). In cells, cultured in SFM/G only, both TGF-$\beta$ and $VitD_3$ inhibited expression mRNA for MIP-1$\alpha$ and IL-8 (*lanes 3* and *4*).

We also monitored the secretion of several cytokines and chemokines (Fig. 5). Neither IL-12 nor IL-10 was detectable in supernatants of cells treated with $VitD_3$ or TGF-$\beta$ alone, but cells treated with LPS produce low to moderate amounts of both cytokines (Fig. 5, *A* and *B*). This LPS-induced secretion of IL-12 was completely inhibited (>50-fold) by coincubation with either $VitD_3$ or TGF-$\beta$. The observation that TGF-$\beta$ does not block IL-12 p40 mRNA suggests either differences in the sensitivities of the RPA vs the ELISA or may possibly indicate a posttranscriptional effect of TGF-$\beta$ on IL-12 expression. This latter hypothesis awaits further testing. $VitD_3$ completely blocked (>100-fold) IL-10 secretion, while TGF-$\beta$ was only partially inhibitory (3-fold). Dose-response studies indicated that these modulatory effects of TGF-$\beta$ on IL-12 and IL-10 secretion were evident even at extremely low concentrations (as low as 0.25 ng/ml or 10 pM) (Fig. 5, *E* and *F*). In contrast to their dramatic effects on IL-10 and IL-12 secretion, TGF-$\beta$ and $VitD_3$ had more modest suppressive effects on both RANTES and TNF-$\alpha$ production (Fig. 5, *C* and *D*). Because IL-10 has been shown to inhibit DC differentiation (8, 41), TGF-$\beta$- or $VitD_3$-induced secretion of IL-10 was one hypothetical explanation for their inhibitory effects. However, the facts that both $VitD_3$ and TGF-$\beta$ actually suppress IL-10 production and that IL-10-neutralizing Abs do not influence the inhibitory effects of $VitD_3$ on DC differentiation (data not shown) eliminate this possibility.

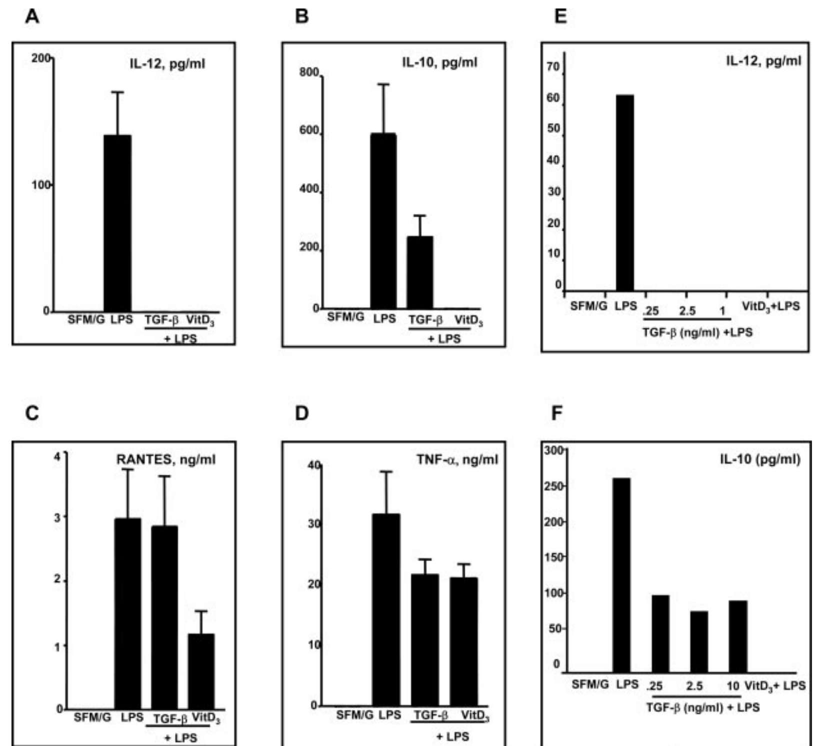



**FIGURE 5.** TGF-$\beta$ and $VitD_3$ alter cytokine and chemokine secretion in cells undergoing DC differentiation induced by LPS. Monocytes were cultured, as described in Fig. 1*A*. Culture supernatants were collected 2 days after the addition of LPS and subjected to ELISA analysis for the presence of heterodimeric IL-12, IL-10, RANTES, and TNF-$\alpha$ (*A–D*), respectively. Results are the average ($\pm$SEM) of three separate experiments with five different donors. *E* and *F*, Representative results of single donor.

*Mechanism of modulatory effects of TGF-$\beta$ and $VitD_3$ on LPS-induced DC differentiation under serum-free conditions*

***TLR expression in human monocytes treated with TGF-$\beta$ and $VitD_3$.*** Because uncontrolled activation of the innate immune response and hyperresponsiveness to LPS in TGF-$\beta$-deficient mice may suggest cross talk between the TGF-$\beta$ and TLR signaling pathways (42), we further focused our attention on inhibitory effects of TGF-$\beta$ and $VitD_3$ on LPS-induced DC differentiation. TLR4 is a critical receptor and signal transducer for LPS (43). Commercial preparations of LPS are often contaminated with TLR2 agonists (44). Therefore, we tested whether TGF-$\beta$ and $VitD_3$ are modulators of TLR2 and TLR4 expression in human monocytes. As detailed in Fig. 6*A*, elutriated monocytes expressed both receptors: low levels of surface TLR4 and higher levels of TLR2. Expression of TLRs was almost unaffected by treatment of cells with TGF-$\beta$ or $VitD_3$ for 24 h of culture. Because TGF-$\beta$ and $VitD_3$ did not significantly influence the TLR expression levels before exposure of monocytes to LPS, we examined whether these

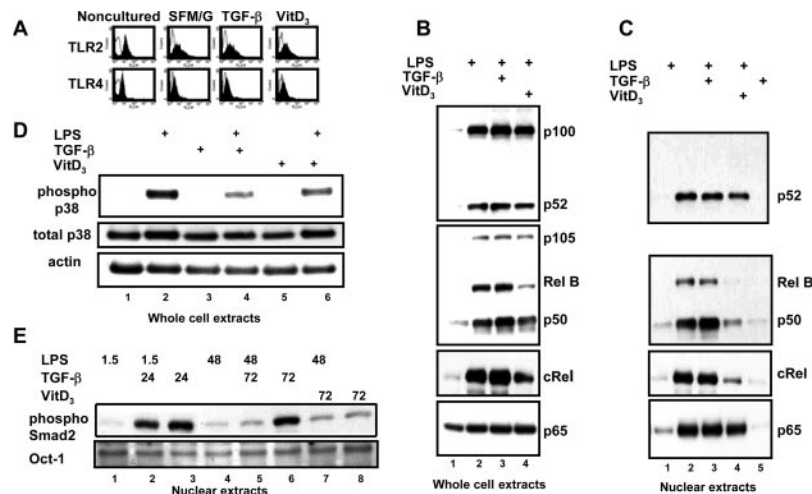



**FIGURE 6.** Mechanism of modulatory effects of TGF-$\beta$ and $VitD_3$ on LPS-induced differentiaton of human monocytes into DCs under serum-free conditions. *A*, Histogram analysis of human monocytes that were treated in one of the following ways: not cultured or cultured overnight in SFM/G with or without TGF-$\beta$ (2 ng/ml) or $VitD_3$ (1 nM). Open traces represent isotype-matched control Ab staining; filled traces indicate a marker-specific Ab. *B*, Human monocytes were cultured in SFM/G with or without TGF-$\beta$ or $VitD_3$, and then exposed to LPS for additional 48 h. Whole cell extracts were separated by SDS-PAGE, and immunoblots were probed with anti-RelB, c-Rel, p65, p105/p50, and p100/p52 Abs. *C*, Cells were treated as described in *B*; nuclear extracts were analyzed by Western blot analysis with Abs specific to different NF-$\kappa$B proteins. *D*, Human monocytes were cultured overnight in SFM/G with or without TGF-$\beta$ or $VitD_3$, and then exposed or not to LPS for additional 24 h. Whole cell extracts were separated by SDS-PAGE, and immunoblots were probed with Abs recognizing the phosphorylated form or total p38. *E*, Cells were treated as described in *B*, nuclear extracts were separated by SDS-PAGE, and immunoblots were probed with anti-phospho Smad2 Abs. Results are representative of three experiments.

agents might instead modulate downstream signal transduction induced by ligand binding to TLR.

***TGF-β and VitD$_3$ only partially block NF-κB activation in monocytes undergoing DC differentiation.*** Previous studies have identified essential, subunit-specific functions for NF-κB transcription factors in regulating DC differentiation, cytokine production, and cell survival (45). Expression of RelB and p52 appears to be necessary for the development of DCs (46–48). Another family member, cRel, is involved in regulation of IL-12 production (49).

To test whether the pretreatment of monocytes with TGF-β or VitD$_3$ under serum-free conditions inhibited LPS-induced NF-κB activation, immunoblots from whole cell extracts were probed with specific Abs (Fig. 6*B*). Treatment of cells with LPS for 48 h resulted in dramatic up-regulation of RelB, cRel, p100/p52, and p105/p50 (compare *lane 2* with *lane 1*). There was little or no change of p65/RelA expression, as expected. However, TGF-β, which partially blocked LPS-induced differentiation, had no inhibitory effect on LPS-induced NF-κB up-regulation (*lane 3*). In addition, VitD$_3$, which was able to almost completely suppress LPS-induced differentiation, had only a modest effect on NF-κB up-regulation: the synthesis of RelB and cRel was somewhat inhibited, but that of p100/p52 and p105/p50 was unaffected (*lane 4*). Neither TGF-β nor VitD$_3$ was able to completely block the up-regulation of RelB and cRel in CI-treated monocytes (data not shown).

In unstimulated cells, NF-κB is usually found in the cytoplasm. Treatment of cells with an appropriate activating agent leads both to increased synthesis of family members and to nuclear translocation (50). We therefore tested whether the up-regulated NF-κB could be found in the nucleus. Low levels of p65, cRel, and p50 were detected in nuclear extracts from monocytes cultured in SFM/G for 72 h (Fig. 6*C*, *lane 1*). LPS induced a high level of nuclear RelB, cRel, p65, p50, and p52, as expected (*lane 2*). Once again, TGF-β had no discernible inhibitory effect on NF-κB activation induced by LPS, as shown by the similar levels of nuclear NF-κB in cells treated with TGF-β plus LPS, as in cells treated with LPS alone (*lanes 2* and *3*). In contrast, nuclear levels of at least some family members were suppressed by VitD$_3$ (*lane 4*) in keeping with the reduced synthesis seen in the whole cell extracts. In particular, nuclear RelB, cRel, and p50 were reduced to nearly the control levels. However, it is clear that VitD$_3$ did not block nuclear translocation per se, because levels of nuclear p52 and p65 were similar to that seen in cells treated with LPS alone. These data demonstrate that, unlike *N*-acetyl cysteine, neither TGF-β nor VitD$_3$ was able to completely inhibit NF-κB activation. However, VitD$_3$ selectively targeted LPS-induced up-regulation and nuclear translocation of two members of NF-κB family proteins, associated with mature DCs: RelB and cRel.

***Both TGF-β and VitD$_3$ inhibit LPS-induced p38 MAPK activation.*** Several reports indicated that the p38 pathway is involved in the functional maturation of DCs induced by LPS (51–53) and that p38 appears to be constitutively activated in mature murine DCs (54). Based on this, we tested whether the p38 MAPK kinase signaling pathway is activated by LPS in cells undergoing DC differentiation under serum-free conditions. Activation of p38 results in phosphorylation, which can be detected by Western blotting using phosphorylation-specific Abs. We found that LPS induced a high level of p38 phosphorylation that was sustained for 24 h (Fig. 6*D*, *lane 2*). Phosphorylated p38 was below the detection limit in extracts of cells cultured in SFM/G, with or without TGF-β or VitD$_3$ (*lanes 1*, *3*, and *5*). Incubation of monocytes with TGF-β or VitD$_3$ significantly reduced activation of p38 induced by LPS (*lanes 4* and *6*), consistent with their ability to suppress DC differentiation.

***Treatment of human monocytes with TGF-β results in nuclear translocation and sustained activation of Smad proteins.*** Smad2 and Smad3 are direct signaling effectors of TGF-β. Receptor-activated phosphorylated Smad2 and Smad3 combine in a heteromeric complex with the common Smad4 and translocate into the nucleus, where they regulate gene expression by interacting with various cofactors. Smads' access to target genes and the recruitment of transcriptional coactivators or corepressors of these genes depend on cell type-specific cofactors (55, 56).

To determine whether Smad proteins are activated in the SFM/G medium, monocytes were treated with TGF-β or VitD$_3$ with or without LPS, and nuclear proteins were analyzed by Western blot analysis with total and phospho-specific anti-Smad2 and anti-Smad3 Abs. Extracts from cells cultured in SFM/G plus LPS had a low level of nuclear phosphorylated Smad2 (Fig. 6*E*, *lanes 1* and *4*). Phosphorylation and nuclear translocation were significantly increased following treatment of cells with TGF-β for 24 h (*lanes 2* and *3*). Elevated levels of nuclear Smad2 expression were observed up to 72 h after treatment (*lane 6*). Treatment with LPS for 48 h inhibited Smad2 activation induced by TGF-β (compare *lane 6* with *lane 5*). The effect of VitD$_3$ treatment on activation of Smad2 was minimal (*lanes 7* and *8*). Similar results were obtained with anti-total Smad2/3-specific Abs (data not shown). Thus, our data indicate that the TGF-β signaling pathway is functional in CD14$^+$ human monocytes, because TGF-β treatment of monocytes under serum-free conditions led to sustained activation of Smad proteins. Our results also suggest that treatment of monocytes with LPS shortens the duration of Smad activation.

## Discussion

CD14$^+$ monocytes are nonproliferating cells that differentiate into macrophages unless redirected to a DC phenotype under specific conditions (3–5). Several recent studies have identified potential physiological conditions that support the very rapid (in a period of 3–4 days or less) development of DCs from monocytic precursors (4, 5). These studies are of critical importance because they not only demonstrate the rapid acquisition of DC characteristics, but they also link this rapid DC development to plausible physiologic behavior of monocytes. Our previous work has defined serum-free culture conditions that support the 2- to 4-day differentiation of human CD14$^+$ peripheral blood monocytes into CD83$^+$ DCs (6, 7, 28). In the current study, we investigated the effects of TGF-β and VitD$_3$, two agents that negatively affect differentiation of monocytes into DCs. We demonstrated that TGF-β significantly decreased the proportion of monocytes that adopted stable DC characteristics in response to LPS, but had little or no inhibitory effect on another DC differentiation pathway induced by CI. In contrast, VitD$_3$ dramatically suppressed differentiation of monocytes to DCs in response to either agent. Both TGF-β and VitD$_3$ altered cytokine and chemokine production by LPS-treated monocytes, inhibiting secretion of IL-12 and IL-10, and both decreased the functional capacity of DCs. Our data indicate that in the presence of TGF-β or VitD$_3$, a subset of partially mature DCs with impaired costimulatory capacity and low IL-12 production is generated that potentially could give rise to tolerogenic T lymphocytes. Because these agents are present in serum and negatively affect DC differentiation at physiological concentrations, our findings are likely to have significance regarding the in vivo role of TGF-β and VitD$_3$ as important regulatory factors in determining the type of responses and altering immune function.

The pathway-specific effects of TGF-β in DC development are illustrated by studies in *Tgf-β1* knockout mice in which there is a

specific absence of epithelial Langerhans cells (LCs), despite apparently normal numbers of CD11c$^+$ myeloid DCs (9). In addition, it has been established that TGF-$\beta$1 is absolutely required for the development of LCs from hemopoietic CD34$^+$ progenitor cells in vitro in the absence of serum supplementation (57). These positive regulatory effects of TGF-$\beta$1 on immature LCs are in contrast to its inhibitory effects on the Ag-presenting function and DC maturation. DCs generated in cultures of human CD34$^+$ cells in the presence of TGF-$\beta$1 are arrested in their maturation at an immature differentiation stage resembling highly specialized epidermal LCs in vivo (58). These observations are in line with murine studies that show that TGF-$\beta$1 addition to GM-CSF-supplemented cultures of murine bone marrow cells did not negatively affect the generation of immature DCs, but almost totally blocked DC maturation (59, 60). Our results further demonstrate that effects of TGF-$\beta$ on differentiation of human monocytes into DCs are highly dependent on the inducing agent and the signaling pathway. Whereas LPS-induced differentiation of monocytes to DCs is partially inhibited by TGF-$\beta$, another pathway to DCs induced by CI remains largely unaffected. These data are consistent with the observation that CD40 ligand-induced maturation of LCs was not influenced by TGF-$\beta$, while LPS-induced maturation was strongly blocked (61). We propose that by differentially regulating both the differentiation and function of DC subsets, TGF-$\beta$ can fine-tune the immune response mediated by DCs. This assumption is supported by findings revealing that APCs, generated in vitro and exposed to TGF-$\beta$, are capable of exerting a tolerizing function in vivo (59, 62, 63). Moreover, it has been demonstrated that TGF-$\beta$ inhibits the Ag-presenting functions and antitumor activity of DC vaccines (64, 65).

The other agent we tested in our system, 1$\alpha$,25(OH)$_2$D$_3$, the active metabolite of VitD$_3$, is a steroid hormone that regulates calcium/phosphate metabolism and has marked immunomodulatory effects on both T cells and APCs (17). VitD$_3$ may play an important role in determining whether monocytes will acquire DC or macrophage characteristics and functions, by inhibiting DC differentiation (19). Recent studies show that VitD$_3$ has complex effects on DCs in vitro. Addition of VitD$_3$ at the beginning of the culture partially blocks the GM-CSF- and IL-4-driven differentiation of monocytes to immature DCs, and addition of VitD$_3$ to cultures of differentiated immature DCs inhibits maturation and can partially reverse DC differentiation (18, 20). Furthermore, increased numbers of mature DCs in lymph nodes of VDR-deficient mice suggest that physiological VitD$_3$ levels are involved in the control of DC maturation in vivo (16). Our novel observation that VitD$_3$ inhibits not only LPS-induced, but also CI-induced DC differentiation was unexpected. Thus, in contrast to TGF-$\beta$, VitD$_3$ appears to interfere with multiple signaling pathways that induce cytokine production and full maturation of DCs, promoting the generation of semimature DCs.

Our data also demonstrate that human monocytes are extremely sensitive to VitD$_3$ in vitro. It is known that monocytes and monocyte-derived DCs express the VDR as well as 25-hydroxyvitaminD$_3$-1$\alpha$-hydroxylase, required for activation of VitD$_3$. Coexpression of these components indicates autocrine and paracrine regulation of DC function by VitD$_3$ (20, 66). The secretory role of macrophages may be central to the production of localized concentrations of VitD$_3$ within immune microenvironments. In granulomatous disorders such as sarcoidosis and tuberculosis, macrophages are able to produce VitD$_3$ and appear to be insensitive to feedback control by VitD$_3$ itself as well as to other regulators, such as calcium and parathyroid hormone. It is tempting to speculate that, by inhibiting differentiation and function of DCs, 1$\alpha$,25-(OH)$_2$D$_3$ may contribute to the peripheral anergy and to the persistence of granulomatous lesions (19, 67).

VitD$_3$ has been reported to act via induction of an autocrine TGF-$\beta$ loop in several different hemopoietic cell lines (21, 68), such that antagonists of TGF-$\beta$ completely block the effect of VitD$_3$ on these cells. Although the mechanisms of the modulatory effects of TGF$\beta$ and VitD$_3$ on differentiation of human monocytes to DCs are still largely unknown, it is clear that they are distinct. Unlike the situation in HL-60 cells, VitD$_3$ was unable to induce secretion of TGF-$\beta$1 (data not shown) or significant phosphorylation of the key TGF-$\beta$ signaling intermediate Smad2. This observation is also supported by different effects of these two agents on LPS- and CI-induced DC differentiation, on LPS-induced secretion of IL-10, and on two members of NF-$\kappa$B family of transcription factors, RelB and cRel.

Although we had previously shown a role for IL-10 as an autocrine/paracrine regulatory factor that suppresses LPS-induced DC differentiation and IL-12 production and the proportion of monocytes that differentiate into DCs in response to LPS (8), IL-10 clearly is not important in the effects of VitD$_3$ on DC differentiation. VitD$_3$ completely inhibited IL-10 production, and TGF-$\beta$ inhibited it partially. Moreover, we found that suppression of monocyte differentiation into DCs is not due to failure to activate NF-$\kappa$B: VitD$_3$ only partially inhibited NF-$\kappa$B activation, and TGF-$\beta$ inhibited not at all. VitD$_3$, in contrast, selectively targeted LPS-induced up-regulation and nuclear translocation of two members of the NF-$\kappa$B family proteins: cRel, involved in transcriptional control of IL-12 p40 gene expression, and RelB, associated with mature DCs. It is possible that VitD$_3$ inhibits the transcription of both p35 and p40 IL-12 subunits in macrophages and DCs, at least partially through interference with NF-$\kappa$B transcriptional activation (37). Our results are consistent with the recently identified negative transcriptional regulation of RelB expression via a VDR-dependent process (69). Several lines of evidence have implicated RelB as a critical regulator of the differentiation and maturation of DCs. RelB-deficient mice lack mature myeloid DCs (46, 48). Moreover, DCs in which RelB expression is inhibited retain an immature phenotype and are associated with induction of immune tolerance in vivo (70–72). Although our data with VitD$_3$ are consistent with an important role for RelB in the DC differentiation process, the absence of modulation of this pathway by TGF-$\beta$ shows that other pathways may also mediate suppressive effects, independent of inhibition of NF-$\kappa$B activation. Moreover, our data indicate that suppression of proinflammatory cytokine production by TGF-$\beta$ may be mediated not through NF-$\kappa$B regulation, but rather through posttranscriptional mechanisms.

In contrast, our data indicate that both TGF-$\beta$ and VitD$_3$ modulate LPS-induced p38 MAPK activation and that the stronger effects of VitD$_3$ on DC maturation may result from its effects on both NF-$\kappa$B and p38 MAPK. The relative levels of activation of these two pathways might modulate the capacity of DCs to secrete IL-12 and/or up-regulate expression of costimulatory molecules and initiate Th1 or Th2 polarization. Inhibition of the p38 MAPK pathway with the chemical inhibitor SB203580 was found to significantly reduce the LPS-induced up-regulation of CD80, CD83, CD86, and HLA-DR, and expression of several cytokine and chemokine mRNAs (39, 53). Thus, TGF-$\beta$ or VitD$_3$ may represent physiological regulators of this signaling pathway.

In light of our findings, we speculate that TGF-$\beta$ and VitD$_3$, both of which are secreted by tumor cells (73–77), may participate in the state of apparent immunologic hyporesponsiveness displayed by many cancer patients toward bacterial, viral, and tumor-associated Ags through the negative regulation of myeloid DC differentiation and function. Several reports have recently shown

that antitumor immunity benefits when TGF-$\beta$ is ablated by neutralizing Abs (65), soluble T$\beta$RII (78), or dominant-negative T$\beta$RII (79). The development of antitumor responses can be dramatically influenced by the cytokine and chemokine secretion by DCs, and DC gene expression should be an important consideration in the development of cancer vaccines.

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## References

1. Sundquist, M., C. Johansson, and M. J. Wick. 2003. Dendritic cells as inducers of antimicrobial immunity in vivo. *APMIS 111:715*.
2. Gad, M., M. H. Claesson, and A. E. Pedersen. 2003. Dendritic cells in peripheral tolerance and immunity. *APMIS 111:766*.
3. Steinman, R. M. 2003. Some interfaces of dendritic cell biology. *APMIS 111:675*.
4. Randolph, G. J., K. Inaba, D. F. Robbiani, R. M. Steinman, and W. A. Muller. 1999. Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo. *Immunity 11:753*.
5. Randolph, G. J., S. Beaulieu, S. Lebecque, R. M. Steinman, and W. A. Muller. 1998. Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking. *Science 282:480*.
6. Lyakh, L. A., G. K. Koski, W. Telford, R. E. Gress, P. A. Cohen, and N. R. Rice. 2000. Bacterial lipopolysaccharide, TNF-$\alpha$, and calcium ionophore under serum-free conditions promote rapid dendritic cell-like differentiation in CD14$^+$ monocytes through distinct pathways that activate NK-$\kappa$B. *J. Immunol. 165:3647*.
7. Lyakh, L. A., G. K. Koski, H. A. Young, S. E. Spence, P. A. Cohen, and N. R. Rice. 2002. Adenovirus type 5 vectors induce dendritic cell differentiation in human CD14$^+$ monocytes cultured under serum-free conditions. *Blood 99:600*.
8. Koski, G. K., L. A. Lyakh, and N. R. Rice. 2001. Rapid lipopolysaccharide-induced differentiation of CD14$^+$ monocytes into CD83$^+$ dendritic cells is modulated under serum-free conditions by exogenously added IFN-$\gamma$ and endogenously produced IL-10. *Eur. J. Immunol. 31:3773*.
9. Borkowski, T. A., J. J. Letterio, A. G. Farr, and M. C. Udey. 1996. A role for endogenous transforming growth factor $\beta$1 in Langerhans cell biology: the skin of transforming growth factor $\beta$1 null mice is devoid of epidermal Langerhans cells. *J. Exp. Med. 184:2417*.
10. Borkowski, T. A., J. J. Letterio, C. L. Mackall, A. Saitoh, X. J. Wang, D. R. Roop, R. E. Gress, and M. C. Udey. 1997. A role for TGF$\beta$1 in Langerhans cell biology: further characterization of the epidermal Langerhans cell defect in TGF$\beta$1 null mice. *J. Clin. Invest. 100:575*.
11. Borkowski, T. A., J. J. Letterio, C. L. Mackall, A. Saitoh, A. G. Farr, X. J. Wang, D. R. Roop, R. E. Gress, and M. C. Udey. 1997. Langerhans cells in the TGF $\beta$1 null mouse. *Adv. Exp. Med. Biol. 417:307*.
12. Hacker, C., R. D. Kirsch, X. S. Ju, T. Hieronymus, T. C. Gust, C. Kuhl, T. Jorgas, S. M. Kurz, S. Rose-John, Y. Yokota, and M. Zenke. 2003. Transcriptional profiling identifies Id2 function in dendritic cell development. *Nat. Immunol. 4:380*.
13. Gorelik, L., and R. A. Flavell. 2002. Transforming growth factor-$\beta$ in T-cell biology. *Nat. Rev. Immunol. 2:46*.
14. Strobl, H., and W. Knapp. 1999. TGF-$\beta$1 regulation of dendritic cells. *Microbes Infect. 1:1283*.
15. Mathieu, C., and L. Adorini. 2002. The coming of age of 1,25-dihydroxyvitamin D(3) analogs as immunomodulatory agents. *Trends Mol. Med. 8:174*.
16. Griffin, M. D., W. Lutz, V. A. Phan, L. A. Bachman, D. J. McKean, and R. Kumar. 2001. Dendritic cell modulation by 1$\alpha$,25 dihydroxyvitamin D3 and its analogs: a vitamin D receptor-dependent pathway that promotes a persistent state of immaturity in vitro and in vivo. *Proc. Natl. Acad. Sci. USA 98:6800*.
17. Hackstein, H., and A. W. Thomson. 2004. Dendritic cells: emerging pharmacological targets of immunosuppressive drugs. *Nat. Rev. Immunol. 4:24*.
18. Penna, G., and L. Adorini. 2000. 1$\alpha$,25-Dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. *J. Immunol. 164:2405*.
19. Piemonti, L., P. Monti, M. Sironi, P. Fraticelli, B. E. Leone, E. Dal Cin, P. Allavena, and V. DiCarlo. V. 2000. Vitamin D3 affects differentiation, maturation, and function of human monocyte-derived dendritic cells. *J. Immunol. 164:4443*.
20. Hewison, M., L. Freeman, S. V. Hughes, K. N. Evans, R. Bland, A. G. Eliopoulos, M. D. Kilby, P. A. Moss, and R. Chakraverty. 2003. Differential regulation of vitamin D receptor and its ligand in human monocyte-derived dendritic cells. *J. Immunol. 170:5382*.
21. Cao, Z., K. C. Flanders, D. Bertolette, L. A. Lyakh, J. U. Wurthner, W. T. Parks, J. J. Letterio, F. W. Ruscetti, and A. B. Roberts. 2003. Levels of phospho-Smad2/3 are sensors of the interplay between effects of TGF-$\beta$ and retinoic acid on monocytic and granulocytic differentiation of HL-60 cells. *Blood 101:498*.
22. Danielpour, D. 1996. Induction of transforming growth factor-$\beta$ autocrine activity by all-trans-retinoic acid and 1$\alpha$,25-dihydroxyvitamin D3 in NRP-152 rat prostatic epithelial cells. *J. Cell. Physiol. 166:231*.
23. Nagel, D., and R. Kumar. 2002. 1$\alpha$,25-Dihydroxyvitamin D3 increases TGF $\beta$1 binding to human osteoblasts. *Biochem. Biophys. Res. Commun. 290:1558*.
24. Defacque, H., D. Piquemal, A. Basset, J. Marti, and T. Commes. 1999. Transforming growth factor-$\beta$1 is an autocrine mediator of U937 cell growth arrest and differentiation induced by vitamin D3 and retinoids. *J. Cell. Physiol. 178:109*.
25. Subramaniam, N., G. M. Leong, T. A. Cock, J. L. Flanagan, C. Fong, J. A. Eisman, and A. P. Kouzmenko. 2001. Cross-talk between 1,25-dihydroxyvitamin D3 and transforming growth factor-$\beta$ signaling requires binding of VDR and Smad3 proteins to their cognate DNA recognition elements. *J. Biol. Chem. 276:15741*.
26. Yanagi, Y., M. Suzawa, M. Kawabata, K. Miyazono, J. Yanagisawa, and S. Kato. 1999. Positive and negative modulation of vitamin D receptor function by transforming growth factor-$\beta$ signaling through Smad proteins. *J. Biol. Chem. 274:12971*.
27. Czerniecki, B. J., C. Carter, L. Rivoltini, G. K. Koski, H. I. Kim, D. E. Weng, J. G. Roros, Y. M. Hijazi, S. Xu, S. A. Rosenberg, and P. A. Cohen. 1997. Calcium ionophore-treated peripheral blood monocytes and dendritic cells rapidly display characteristics of activated dendritic cells. *J. Immunol. 159:3823*.
28. Koski, G. K., G. N. Schwartz, D. E. Weng, R. E. Gress, F. H. Engels, M. Tsokos, B. J. Czerniecki, and P. A. Cohen. 1999. Calcium ionophore-treated myeloid cells acquire many dendritic cell characteristics independent of prior differentiation state, transformation status, or sensitivity to biologic agents. *Blood 94:1359*.
29. Rice, N. R., M. L. MacKichan, and A. Israel. 1992. The precursor of NF-$\kappa$B p50 has I$\kappa$B-like functions. *Cell 71:243*.
30. Whiteside, S. T., J. C. Epinat, N. R. Rice, and A. Israel. 1997. I$\kappa$B$\epsilon$, a novel member of the I$\kappa$B family, controls RelA and cRel NF-$\kappa$B activity. *EMBO J. 16:1413*.
31. Young, H. A., J. J. Subleski, and S. M. Krebs. 2003. Multiprobe ribonuclease protection assay for simultaneous measurement of mRNA expression of 8–12 genes. In *Current Protocols in Immunology*. John Wiley and Sons, Hoboken, pp. 10.29.1–10.29.15.
32. Wrana, J. L., L. Attisano, R. Wieser, F. Ventura, and J. Massague. 1994. Mechanism of activation of the TGF-$\beta$ receptor. *Nature 370:341*.
33. Winzler, C., P. Rovere, M. Rescigno, F. Granucci, G. Penna, L. Adorini, V. S. Zimmermann, J. Davoust, and P. Ricciardi-Castagnoli. 1997. Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. *J. Exp. Med. 185:317*.
34. Zhou, L. J., and T. F. Tedder. 1996. CD14$^+$ blood monocytes can differentiate into functionally mature CD83$^+$ dendritic cells. *Proc. Natl. Acad. Sci. USA 93:2588*.
35. Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y. J. Liu, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. *Annu. Rev. Immunol. 18:767*.
36. Faries, M. B., I. Bedrosian, S. Xu, G. Koski, J. G. Roros, M. A. Moise, H. Q. Nguyen, F. H. Engels, P. A. Cohen, and B. J. Czerniecki. 2001. Calcium signaling inhibits interleukin-12 production and activates CD83$^+$ dendritic cells that induce Th2 cell development. *Blood 98:2489*.
37. D'Ambrosio, D., M. Cippitelli, M. G. Cocciolo, D. Mazzeo, P. Di Lucia, R. Lang, F. Sinigaglia, and P. Panina-Bordignon. 1998. Inhibition of IL-12 production by 1,25-dihydroxyvitamin D3: involvement of NF-$\kappa$B down-regulation in transcriptional repression of the p40 gene. *J. Clin. Invest. 101:252*.
38. Lore, K., A. Sonnerborg, A. L. Spetz, U. Andersson, and J. Andersson. 1998. Immunocytochemical detection of cytokines and chemokines in Langerhans cells and in vitro derived dendritic cells. *J. Immunol. Methods 214:97*.
39. Re, F., and J. L. Strominger. 2001. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells. *J. Biol. Chem. 276:37692*.
40. Sallusto, F., B. Palermo, D. Lenig, M. Miettinen, S. Matikainen, I. Julkunen, R. Forster, R. Burgstahler, M. Lipp, and A. Lanzavecchia. 1999. Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *Eur. J. Immunol. 29:1617*.
41. Moore, K. W., M. R. de Waal, R. L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol. 19:683*.
42. McCartney-Francis, N., W. Jin, and S. M. Wahl. 2004. Aberrant Toll receptor expression and endotoxin hypersensitivity in mice lacking a functional TGF-$\beta$1 signaling pathway. *J. Immunol. 172:3814*.
43. Akira, S., and K. Takeda. 2004. Toll-like receptor signalling. *Nat. Rev. Immunol. 4:499*.
44. Hirschfeld, M., Y. Ma, J. H. Weis, S. N. Vogel, and J. J. Weis. 2000. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine Toll-like receptor 2. *J. Immunol. 165:618*.
45. Ouaaz, F., J. Arron, Y. Zheng, Y. Choi, and A. A. Beg. 2002. Dendritic cell development and survival require distinct NF-$\kappa$B subunits. *Immunity 16:257*.
46. Burkly, L., C. Hession, L. Ogata, C. Reilly, L. A. Marconi, D. Olson, R. Tizard, R. Cate, and D. Lo. 1995. Expression of relB is required for the development of thymic medulla and dendritic cells. *Nature 373:531*.
47. Poljak, L., L. Carlson, K. Cunningham, M. H. Kosco-Vilbois, and U. Siebenlist. 1999. Distinct activities of p52/NF-$\kappa$B required for proper secondary lymphoid organ microarchitecture: functions enhanced by Bcl-3. *J. Immunol. 163:6581*.
48. Weih, F., D. Carrasco, S. K. Durham, D. S. Barton, C. A. Rizzo, R. P. Ryseck, S. A. Lira, and R. Bravo. 1995. Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-$\kappa$B/Rel family. *Cell 80:331*.
49. Sanjabi, S., A. Hoffmann, H. C. Liou, D. Baltimore, and S. T. Smale. 2000. Selective requirement for c-Rel during IL-12 P40 gene induction in macrophages. *Proc. Natl. Acad. Sci. USA 97:12705*.
50. Stancovski, I., and D. Baltimore. 1997. NF-$\kappa$B activation: the I$\kappa$B kinase revealed? *Cell 91:299*.

51. Agrawal, S., A. Agrawal, B. Doughty, A. Gerwitz, J. Blenis, T. Van Dyke, and B. Pulendran. 2003. Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. *J. Immunol. 171:4984*.
52. Ardeshna, K. M., A. R. Pizzey, S. Devereux, and A. Khwaja. 2000. The PI3 kinase, p38 SAP kinase, and NF-$\kappa$B signal transduction pathways are involved in the survival and maturation of lipopolysaccharide-stimulated human monocyte-derived dendritic cells. *Blood 96:1039*.
53. Arrighi, J. F., M. Rebsamen, F. Rousset, V. Kindler, and C. Hauser. 2001. A critical role for p38 mitogen-activated protein kinase in the maturation of human blood-derived dendritic cells induced by lipopolysaccharide, TNF-$\alpha$, and contact sensitizers. *J. Immunol. 166:3837*.
54. Fukao, T., S. Matsuda, and S. Koyasu. 2000. Synergistic effects of IL-4 and IL-18 on IL-12-dependent IFN-$\gamma$ production by dendritic cells. *J. Immunol. 164:64*.
55. Roberts, A. B., and R. Derynck. 2001. Meeting report: signaling schemes for TGF-$\beta$. *Sci. STKE 2001:E43*.
56. Shi, Y., and J. Massague. 2003. Mechanisms of TGF-$\beta$ signaling from cell membrane to the nucleus. *Cell 113:685*.
57. Strobl, H., E. Riedl, C. Scheinecker, C. Bello-Fernandez, W. F. Pickl, K. Rappersberger, O. Majdic, and W. Knapp. 1996. TGF-$\beta$1 promotes in vitro development of dendritic cells from CD34$^+$ hemopoietic progenitors. *J. Immunol. 157:1499*.
58. Strobl, H., C. Bello-Fernandez, E. Riedl, W. F. Pickl, O. Majdic, S. D. Lyman, and W. Knapp. 1997. *flt3* ligand in cooperation with transforming growth factor-$\beta$1 potentiates in vitro development of Langerhans-type dendritic cells and allows single-cell dendritic cell cluster formation under serum-free conditions. *Blood 90:1425*.
59. Lu, L., W. Li, F. Fu, F. G. Chambers, S. Qian, J. J. Fung, and A. W. Thomson. 1997. Blockade of the CD40-CD40 ligand pathway potentiates the capacity of donor-derived dendritic cell progenitors to induce long-term cardiac allograft survival. *Transplantation 64:1808*.
60. Yamaguchi, Y., H. Tsumura, M. Miwa, and K. Inaba. 1997. Contrasting effects of TGF-$\beta$1 and TNF-$\alpha$ on the development of dendritic cells from progenitors in mouse bone marrow. *Stem Cells 15:144*.
61. Geissmann, F., P. Revy, A. Regnault, Y. Lepelletier, M. Dy, N. Brousse, S. Amigorena, O. Hermine, and A. Durandy. 1999. TGF-$\beta$1 prevents the noncognate maturation of human dendritic Langerhans cells. *J. Immunol. 162:4567*.
62. Faunce, D. E., A. Terajewicz, and J. Stein-Streilein. 2004. Cutting edge: in vitro-generated tolerogenic APC induce CD8$^+$ T regulatory cells that can suppress ongoing experimental autoimmune encephalomyelitis. *J. Immunol. 172:1991*.
63. Alard, P., S. L. Clark, and M. M. Kosiewicz. 2004. Mechanisms of tolerance induced by TGF$\beta$-treated APC: CD4 regulatory T cells prevent the induction of the immune response possibly through a mechanism involving TGF$\beta$. *Eur. J. Immunol. 34:1021*.
64. Kao, J. Y., Y. Gong, C. M. Chen, Q. D. Zheng, and J. J. Chen. 2003. Tumor-derived TGF-$\beta$ reduces the efficacy of dendritic cell/tumor fusion vaccine. *J. Immunol. 170:3806*.
65. Kobie, J. J., R. S. Wu, R. A. Kurt, S. Lou, M. K. Adelman, L. J. Whitesell, L. V. Ramanathapuram, C. L. Arteaga, and E. T. Akporiaye. 2003. Transforming growth factor $\beta$ inhibits the antigen-presenting functions and antitumor activity of dendritic cell vaccines. *Cancer Res. 63:1860*.
66. Fritsche, J., K. Mondal, A. Ehrnsperger, R. Andreesen, and M. Kreutz. 2003. Regulation of 25-hydroxyvitamin D3–1$\alpha$-hydroxylase and production of 1$\alpha$,25-dihydroxyvitamin D3 by human dendritic cells. *Blood 102:3314*.
67. Ota, M., R. Amakawa, K. Uehira, T. Ito, Y. Yagi, A. Oshiro, Y. Date, H. Oyaizu, T. Shigeki, Y. Ozaki, et al. 2004. Involvement of dendritic cells in sarcoidosis. *Thorax 59:408*.
68. Defacque, H., T. Commes, V. Contet, C. Sevilla, and J. Marti. 1995. Differentiation of U937 myelomonocytic cell line by all-trans retinoic acid and 1,25-dihydroxyvitamin D3: synergistic effects on tissue transglutaminase. *Leukemia 9:1762*.
69. Dong, X., T. Craig, N. Xing, L. A. Bachman, C. V. Paya, F. Weih, D. J. McKean, R. Kumar, and M. D. Griffin. 2003. Direct transcriptional regulation of RelB by 1$\alpha$,25-dihydroxyvitamin D3 and its analogs: physiologic and therapeutic implications for dendritic cell function. *J. Biol. Chem. 278:49378*.
70. Martin, E., B. O'Sullivan, P. Low, and R. Thomas. 2003. Antigen-specific suppression of a primed immune response by dendritic cells mediated by regulatory T cells secreting interleukin-10. *Immunity 18:155*.
71. Hutchings, A., W. J. Hubbard, F. T. Thomas, and J. M. Thomas. 2002. STEALTH in transplantation tolerance. *Immunol. Res. 26:143*.
72. Hutchings, A., J. Wu, C. Asiedu, W. Hubbard, D. Eckhoff, J. Contreras, F. T. Thomas, D. Neville, and J. M. Thomas. 2003. The immune decision toward allograft tolerance in non-human primates requires early inhibition of innate immunity and induction of immune regulation. *Transplant Immunol. 11:335*.
73. Adams, J. S., and V. Kantorovich. 1999. Inability of short-term, low-dose hydroxychloroquine to resolve vitamin D-mediated hypercalcemia in patients with B-cell lymphoma. *J. Clin. Endocrinol. Metab. 84:799*.
74. Akhurst, R. J., and R. Derynck. 2001. TGF-$\beta$ signaling in cancer: a double-edged sword. *Trends Cell Biol. 11:S44*.
75. Brown, R. D., B. Pope, A. Murray, W. Esdale, D. M. Sze, J. Gibson, P. J. Ho, D. Hart, and D. Joshua. 2001. Dendritic cells from patients with myeloma are numerically normal but functionally defective as they fail to up-regulate CD80 (B7-1) expression after huCD40LT stimulation because of inhibition by transforming growth factor-$\beta$1 and interleukin-10. *Blood 98:2992*.
76. Pasche, B. 2001. Role of transforming growth factor $\beta$ in cancer. *J. Cell. Physiol. 186:153*.
77. Engleman, E. G., J. Brody, and L. Soares. 2004. Using signaling pathways to overcome immune tolerance to tumors. *Sci. STKE 2004:e28*.
78. Won, J., H. Kim, E. J. Park, Y. Hong, S. J. Kim, and Y. Yun. 1999. Tumorigenicity of mouse thymoma is suppressed by soluble type II transforming growth factor $\beta$ receptor therapy. *Cancer Res. 59:1273*.
79. Gorelik, L., and R. A. Flavell. 2001. Immune-mediated eradication of tumors through the blockade of transforming growth factor-$\beta$ signaling in T cells. *Nat. Med. 7:1118*.