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Vitamin D Receptor-Mediated Suppression of RelB in Antigen Presenting Cells: A Paradigm for Ligand-Augmented Negative Transcriptional Regulation ¹

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Abstract

The immunological effects of vitamin D receptor (VDR) ligands include inhibition of dendritic cell (DC) maturation, suppression of T-helper type 1 (Th1) T-cell responses and facilitation of antigen-specific immune tolerance *in vivo*. While studying the molecular profile of DCs cultured in the presence of 1 α ,25(OH)D₃ or synthetic D₃ analogs we observed that expression of the NF- κ B family member RelB, which plays an essential role in DC differentiation and maturation, is selectively suppressed by VDR ligands. Further *in vitro* and *in vivo* studies of VDR-mediated RelB suppression indicated that the mechanism for this effect involves direct binding of VDR/RXR α to a defined region of the *relB* promoter and assembly of a negative regulatory complex containing HDAC3, HDAC1, SMRT and, most likely, other factors. Interestingly, promoter engagement by VDR and HDAC3, but not the other identified components, is enhanced by addition of a VDR ligands and inhibited by a pro-maturation stimulus (LPS) that results in RelB upregulation. Promoter assays in a panel of cell lines suggest that the VDR ligand-dependent component of *relB* suppression may occur selectively in antigen presenting cells. Cell type-specific, ligand-enhanced negative transcriptional regulation represents a potentially novel paradigm for VDR-controlled genes. In this report we review the experimental data to support such a mechanism for *relB* regulation in DCs and present a model for the process.

Introduction

During the past several years substantial interest has been generated in the use of vitamin D receptor (VDR) agonists for the treatment or prevention of autoimmune diseases and transplant rejection [1–5]. Much of this enthusiasm has arisen from a growing body of literature indicating VDR-mediated *in vivo* effects on T-cells and dendritic cells (DCs) that specifically inhibit T-helper type 1 (Th1) T-cell responses and may also facilitate antigen-specific tolerance [2–10]. Given the narrow therapeutic window for the exogenously administered native VDR ligand (1 α ,25-dihydroxyvitaminD₃ (1 α ,25(OH)D₃)) [2,11], the eventual translation of this

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work from bench to bedside will depend upon the successful pursuit of one or more additional goals: (a) Identification of synthetic VDR agonists for which the *in vivo* potency of immune modulatory effects is uncoupled from that of other biological effects such as induction of hypercalcemia [1,5,6,11]. (b) Harnessing of the synergistic properties of VDR agonists when combined with other immunosuppressive agents [6,12]. (c) Elucidation of the cell-specific molecular mechanisms underlying VDR-mediated inhibition of T-cell and DC functions [13–22].

In the case of this latter goal, it is noteworthy that analyses of individual candidate genes and microarray profiles have identified a range of important immune mediators that are expressed by T-cells and DCs and are modulated by exposure to $1\alpha,25(\text{OH})\text{D}_3$ and synthetic VDR agonists [6,15,16,17,21,22,23]. Many of these VDR-modified gene products, which include cytokines, chemokines, growth and differentiation factors, signaling pathway components and activation-related surface receptors, are suppressed by VDR ligand exposure and are clearly linked with the observed functional effects of VDR ligands on *in vitro* or *in vivo* immunological phenomena [2,4,24]. Despite this, the current level of mechanistic insight into the effects of the $1\alpha,25(\text{OH})\text{D}_3$ /VDR system on the expression of key gene products in T-cells and DCs is relatively poor. Evidence is available for multiple mechanisms of VDR-mediated negative gene regulation in these cells including cross-talk with other intracellular signaling pathways, competition for transcription factor binding sites within individual gene promoters and altered expression levels of the signaling pathway components that are required for cellular differentiation and maturation [6,15–17,21–23]. Thus, the phenotypic features of VDR ligand-conditioned T-cells and DCs almost certainly represent the result of a complex, multi-faceted alteration to the intracellular signaling and transcriptional profile that involves genes with and without VDR binding sites [25]. Complete mapping these protean events is unlikely to be feasible but further characterization of the mechanisms underlying transcriptional suppression of individual functionally-relevant genes may prove useful in designing more discrete interventional strategies for modulating DC/T-cell interactions *in vivo* or for revealing novel aspects of gene regulation during immune responses. Of high interest in this regard are genes for which VDR ligand-dependent negative transcriptional suppression can be shown to occur in a cell-type specific manner and to result directly from DNA binding of VDR/RXR heterodimers. Recently described examples of such “active suppression” mechanisms for $1\alpha,25(\text{OH})\text{D}_3$ /VDR and other ligand-dependent nuclear receptor (NR) systems provide evidence for the involvement of potent negative regulatory complexes that incorporate specific co-repressors and histone modifying enzymes [20,26–30]. We have published a series of findings indicating that transcription of the gene encoding RelB, a nuclear factor kappa B (NF- κ B) family member that is essential for DC differentiation and maturation [31–34], is suppressed in these cells by the $1\alpha,25(\text{OH})\text{D}_3$ /VDR system through a mechanism that involves recruitment of VDR/RXR and other components of a negatively regulatory complex to distinct binding sites on the promoter and is enhanced by exposure to a VDR agonist [18,35,36]. In the current report we review our published findings regarding this active mechanism of *relB* transcriptional suppression in bone marrow-derived DCs, a DC-like cell line and DCs extracted directly from lymphoid organs. We also provide experimental data from additional cell lines which suggest that ligand-enhanced transcriptional suppression of *relB* may be a specific feature of antigen presenting cells (APCs). Mechanistic models for this novel form of VDR-mediated transcriptional regulation are discussed.

Review of Published Experimental Findings

RelB is depleted in a VDR-dependent manner in DCs following exposure to $1\alpha,25(\text{OH})_2\text{D}_3$ and D_3 analogs

We were initially drawn to examine intracellular levels of members of the NF- κB family of signaling proteins in DCs by observing phenotypic changes of mouse BMDCs cultured in the presence of $1\alpha,25(\text{OH})_2\text{D}_3$ and synthetic D_3 analogs that were consistent with a failure of these cells to undergo effective maturation - a complex genetic program that arms the DC to potentially activate T-cells in response to microbial infection and is heavily dependent upon NF- κB signaling [9,25,35,37–41]. Similar cultures of bone marrow cells from VDR-deficient mice were unaffected essentially ruling out non-VDR-dependent phenomena [9,37]. Interestingly, we observed that addition of a D_3 analog to 7-day BMDC cultures was associated with substantially reduced levels of two major NF- κB components - RelB and c-Rel - but not of a third - RelA (p65). In keeping with a putative transcriptional effect, both protein and mRNA levels were suppressed [35]. Similar *in vitro* observations were made in the DC-like cell line D2SC1 ruling out an indirect effect of VDR ligands occurring through modulation of stromal or other non-DC elements of the bone marrow cultures. Subsequently, we have been able to demonstrate potent selective suppression of intracellular RelB in DCs purified directly from the spleens of mice treated 24 hours previously with a single intraperitoneal dose of a D_3 analog [18]. These basic observations suggested that the gene encoding *relB* is subject to VDR-mediated negative regulation in a ligand-dependent manner in DCs through a mechanism that is not shared by other NF- κB family members. The potential significance of this mechanism was well supported by an existing body of literature indicating an essential, non-redundant role for RelB in DC differentiation and maturation [31–33] as well as the potential for RelB-deficient DCs to mediate immune tolerance *in vivo* [34].

The mouse *relB* promoter contains a VDR/RXR α binding site and is negatively regulated in a DC-like cell line by VDR over-expression or by exposure to VDR ligand

Sequence analysis of approximately 1000 bp of chromosomal DNA proximal to the start site of the mouse *relB* gene identified a potential non-canonical VDRE. This motif was confirmed, by EMSA, to bind recombinant VDR and RXR α *in vitro* and, by luciferase reporter assay in D2SC1 cells, to be essential for VDR-mediated negative transcriptional regulation [36]. Additional reporter assays demonstrated that *relB* transcriptional suppression could be augmented in dose-dependent fashion by VDR over-expression (in the absence of exogenous VDR ligand) as well as by addition of $1\alpha,25(\text{OH})_2\text{D}_3$ or D_3 analogs. Importantly, very similar phenomena were detected for the human *relB* promoter with the exception that two rather than one functionally active VDREs were identified [36].

The *relB* promoter VDRE is constitutively occupied by VDR and RXR in DCs but only VDR binding is dynamically regulated

In order to more directly detect the binding of VDR/RXR α heterodimers to the VDRE-containing region of the mouse *relB* promoter we have employed ChIP assays of mouse BMDCs, D2SC1 cells and purified splenic DCs under different experimental conditions. A basic but important observation is illustrated in Figure 1. In the absence of exogenous VDR ligand, immunoprecipitates of both VDR and RXR α from VDR wild-type (WT) BMDC cultures can be readily shown to contain a 178 bp chromosomal DNA fragment incorporating the *relB* VDRE but not a fragment located proximally to this region (data not shown for negative control PCR). Chromatin immunoprecipitation from similarly cultured VDR deficient (VDR KO) BMDCs demonstrate, as expected, lack of the *relB* VDRE-containing fragment in anti-VDR precipitates but persistence of this amplicon in anti-RXR α precipitates. In addition, exposure of VDR WT BMDCs to the pro-maturation stimulus LPS is associated with apparent shedding of VDR but not RXR α from this region of the *relB* promoter (Figure 1,

lower panel). This latter observation cannot be explained by reduced VDR expression following LPS exposure [18]. Conversely, addition of a D₃ analog to VDR WT (but not VDR KO) BMDCs, results in an increased amount of the *relB* VDRE-containing DNA fragment in anti-VDR precipitates indicating additional ligand-induced recruitment of VDR to the promoter [18]. In this case also, the apparent binding of RXR α to the same region of the promoter is unchanged. These same observations were made in ChIP assays carried out from D2SC1 cultures and from splenic DCs purified from the spleens of mice treated with LPS or D₃ analog [18]. In addition to confirming that VDR and RXR α are constitutively associated with the predicted VDRE-containing region of the *relB* promoter in DCs, these observations indicate that VDR, but not RXR α , binding to this region is reciprocally regulated by pro-maturation intracellular signaling and by availability of VDR ligand. The relatively invariable RXR α association in these ChIP assays under different conditions contrasts with the observed lack of binding of recombinant RXR α alone to the *relB* VDRE motif in EMSA experiments [36] and suggests that additional DNA binding proteins serve to stabilize RXR α binding.

Histone deacetylation and HDAC3 are necessary for VDR-dependent suppression of *relB* in vitro and in vivo

Our most recently published study provides strong evidence that VDR-mediated recruitment of histone deacetylase (HDAC) activity to the VDRE-containing region of the *relB* promoter is an essential functional component of both ligand-independent and ligand-dependent negative transcriptional regulation of this gene [18]. In both *in vitro* and *in vivo* experimental systems, non-specific HDAC blockade resulted in attenuation or elimination of the *relB* suppression associated with VDR over-expression and D₃ analog exposure. Although ChIP assays identified two HDACs (HDAC1 and 3) and one co-repressor (SMRT) that were associated with the *relB* promoter in DCs, only one of these proteins - HDAC3 - exhibited modified binding following LPS and D₃ analog treatment in similar fashion to VDR itself. Strikingly, over-expression of HDAC3 was associated with a suppressive effect on *relB* promoter activity in reporter assays that was additive to the effects of D₃ analog exposure and/or VDR over-expression and was eliminated by mutation of the *relB* VDRE [18]. The overall suppressive potential of the putative VDR/HDAC3-containing negative regulatory complex could be appreciated from the observation that combined over-expression of VDR and HDAC3 resulted in abolition of the otherwise robust augmentation of *relB* reporter construct activity following LPS exposure in culture [18]. The results of these experiments clearly implicate HDAC3 in a VDR ligand-augmented negative regulatory mechanism that is centered upon the VDRE-containing region of the *relB* promoter but do not rule out involvement of additional histone modifying enzymes.

New Experimental Findings: Materials and Methods

Reagents, antibodies, cell lines and plasmid constructs

Crystalline preparations of 1 α ,25(OH)₂D₃ and of the vitamin D₃ analog 1 α ,25(OH)₂-16-ene-23-yne-26,27-hexafluoro-19-nor-D₃ (subsequently referred to as D₃ analog) were provided by Dr. Milan Uskokovic (Hoffman La-Roche, Nutley, NJ) and stored under nitrogen at -80°C as stock solutions in absolute alcohol. Anti-mouse VDR polyclonal antibody and anti-mouse RXR α antibody were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. All oligonucleotides were synthesized by Integrated DNA Technologies, Coralville, IA. Lipopolysaccharide (LPS) was purchased from Sigma Aldrich, St. Louis, MO. Murine bone marrow-derived DCs (BMDCs) were prepared from bone marrow of adult female C57BL/6 mice as previously described [37]. Mouse D2SC1 cells were cultured in Iscove's Modified Dulbecco's Medium containing L-glutamine, penicillin/streptomycin, and 5% fetal bovine serum. HEK293 ROS17/28, SW480 and RAW cells were cultured in DMEM medium containing 10% fetal bovine serum. Methods for the generation of a mouse VDR expression

construct and mouse *relB* promoter-driven luciferase constructs have been described previously [36]. An additional mutated mouse *relB* promoter construct was generated by replacing the wild type VDRE sequence with the sequence of a positive regulatory VDRE in promoter of the mouse osteopontin (OPN) gene [38] using a mutagenesis kit from Stratagene, La Jolla, CA.

Transient transfection, luciferase reporter assays and immunoblotting of D2SC1 cells

Cell lines were lifted from stock cultures and were seeded in 6-well plates at a density of 5×10^5 cells/well. Twenty four hours later, the cells were transfected as previously described with 1 g of plasmid-encoded mouse *relB* promoter construct with 10 ng of pRL-TK plasmid encoding renilla luciferase under the thymidine kinase (TK) promoter as an internal control [18,36]. In some experiments, the cells were co-transfected with 0.5 g of mouse VDR expression construct or with an equivalent amount of the empty expression vector. Ten hours later, the medium was removed and replaced with control medium or medium containing 10^{-10} M D₃ analog. After a further 24 hours, the cell lysates were assayed for both the firefly and renilla luciferase using the Dual-Luciferase Assay Kit (Promega). Final results for each sample were expressed as renilla-adjusted relative light units (RLU) or as percent of the relevant control sample. Between 2 and 4 replicates of each experimental condition were carried out and between-sample differences were tested for statistical significance by unpaired, two-tailed Student's t-test with significance assigned to $p < 0.05$. Preparation of D2SC1 cell lysates and immunoblotting for VDR and β -actin were carried out as previously described [36].

Chromatin Immunoprecipitation (ChIP) assay of BMDCs

Culture additions to day 6 mouse BMDCs consisted of D₃ analog to a final concentration of 10^{-10} M, LPS to a final concentration of 50 ng/ml or appropriate vehicle controls. Twenty four hours later, aliquots of 10^6 cells each were treated with 1% formaldehyde for 10 minutes at 37°C followed by addition of glycine to final concentration of 0.125 M for 5 minutes. Subsequent lysis steps were carried out in the presence of protease inhibitors (1 mM PMSF, 1 g/ml aprotinin, and 1 g/ml pepstatin A). Cells were rinsed twice with ice-cold PBS, scraped in 1 ml of PBS, pelleted, resuspended in 200 l of lysis buffer, incubated on ice for 10 minutes, and then sonicated to produce DNA fragments of 300-1,000 bp. Debris was cleared by centrifugation and supernatants were removed and diluted 10-fold in ChIP dilution buffer (0.01% SDS/1.1% Triton X-100/1.2 mM EDTA/16.7 mM Tris-HCl, pH 8.1/167.0 mM NaCl). Diluted supernatants were pre-cleared with 80 l of salmon sperm DNA/protein A Sepharose-50% slurry for 30 minutes at 4°C. The agarose was pelleted and supernatants were divided into two fractions: one retained for no antibody control ("total input") and the second incubated with 5 g of appropriate antibody overnight at 4°C. Sepharose protein A beads (50 l) were added and allowed to interact with the mixture at 4°C for 1 hour and then pelleted. Supernatants were discarded, and the beads were sequentially washed for 5 minutes using 1 ml each of the following buffers: low salt buffer (0.1% SDS/1.0% Triton X-100/2.0 mM EDTA/20.0 mM Tris-HCl, pH 8.1/150 mM NaCl), high salt buffer (0.1% SDS/1.0% Triton X-100/2.0 mM EDTA/20.0 mM Tris-HCl, pH 8.1/500 mM NaCl), LiCl buffer (0.25 M LiCl/1.0% Nonidet P-40/1.0% deoxycholate/1.0 mM EDTA/10.0 mM Tris-HCl, pH 8.1), and TE buffer. Protein/DNA complexes were eluted twice by incubation at room temperature for 15 minutes in 250 l of elution buffer (1% SDS/0.1 M NaHCO₃). The eluates were combined, 20 l 5 M NaCl was added, and protein/DNA cross-linking was reversed by 4 hours of incubation at 65°C. Finally, 10 l of 0.5 M EDTA, 20 l of 1 M Tris HCl (pH 6.5), and 2 l of 10 g/ml proteinase K were added for 1 hour at 45°C. Samples were extracted with phenol/chloroform/isoamyl alcohol and precipitated overnight at -20°C with ethanol/20 g of glycogen. Precipitated DNA was subjected to PCR using primers flanking the VDRE motif in the mouse *relB* promoter as well as control primers from a proximal promoter region. PCR reactions were separated on agarose gels and bands were imaged using a UVP Gel Documentation System (UVP, Upland, CA).

Primer sequences for amplification of the VDRE-containing region of the mouse *relB* promoter were as follows: Forward, 5'-CTCAGTGCTGCATAGACTAGGT-3'; Reverse, 5'-GGAAGTTAGCAGCCATTGATAA-3'. Primer sequences for amplification of a proximal non-VDRE-containing region of the mouse *relB* promoter were as follows: Forward, 5'-TTAAGCTCAACCACCGCACC-3'; Reverse, 5'-CCTCTGCCATCTAAGTTAGCTC-3'.

New Experimental Findings: Results

Negative transcriptional regulation of the *relB* promoter is not dependent upon a specific VDR binding domain sequence

It is possible that ligand-dependent VDR-mediated HDAC recruitment and negative transcriptional regulation requires distinct VDRE binding characteristics or, as recently reported by Fujiki et al, represents indirect association of VDR with promoter DNA via an intermediary protein [42]. The role of the mouse *relB* VDR binding domain sequence itself in determining the nature of transcriptional regulation was examined by mutating this sequence to that of a VDRE associated with classical ligand-dependent transcriptional up-regulation. As shown in Figure 2A, transcriptional activity of a luciferase expression construct placed 3' to the mouse osteopontin (mOPN) promoter [38] was strongly induced in the osteoblast-derived cell line ROS17/2.8 by addition of a D₃ analog. In contrast, the mouse *relB* promoter was unaffected by D₃ analog in this cell line. Mutation of the *relB* VDRE sequence to that of the known mOPN VDRE (see Figure 2B) was carried out for the purpose of determining the effect of this alteration on VDR ligand-dependent and VDR ligand-independent modulation of *relB* promoter activity in the DC-like cell line D2SC1. As shown in Figure 2C, the mOPN VDRE mutant construct exhibited similar negative regulation to the wild-type construct in D2SC1 cells following D₃ analog exposure or VDR over-expression alone as well as a similar combined effect of the two interventions. Of interest the degree of suppression associated with VDR over-expression, but not D₃ ligand exposure, was greater for the mutant construct. These results support three additional conclusions regarding the mechanism of VDR-mediated transcriptional suppression of *relB*: (a) Recruitment of VDR and HDAC activity to the promoter represents classical direct binding of VDR/RXR α to a VDRE rather than an indirect association. (b) Ligand-enhancement of the suppression is not simply a manifestation of limited VDR availability and low VDR affinity for the DNA binding domain as it occurs in the context of VDR over-expression and a high-affinity VDRE. (c) Ligand-dependent suppression of *relB* may not be manifest in all cell types.

VDR ligand-dependent augmented suppression of the *relB* promoter occurs specifically in antigen presenting cells

Combined with the published findings, the above results raised the possibility that ligand-dependent *relB* suppression requires expression of one or more co-factors by DCs and DC-like cells. The potential role of cell-type-specific factors in VDR-dependent negative regulation of *relB* was investigated by performing luciferase reporter assays in a diverse panel of cell lines. In each cell line, the transcriptional activity of the wild-type mouse *relB* promoter construct was compared under control conditions, D₃ analog exposure, VDR over-expression or combined D₃ analog/VDR over-expression (Figure 3A). The cell lines used included D2SC1 (mouse DC), HEK293 (human embryonic kidney), ROS17/2.8 (rat osteoblast), SW480 (human colonic carcinoma) and RAW (mouse macrophage). As shown, significant promoter suppression by D₃ analog exposure alone and augmented suppression by combined VDR over-expression and D₃ analog exposure were observed only in D2SC1 and RAW cells. In contrast, VDR over-expression alone was associated with reduced *relB* promoter activity in all cell lines except ROS17/2.8. The possibility that the difference observed between D2SC1 and ROS17/2.8 cells could be explained entirely by differences in VDR expression under control and D₃ analog-treated conditions was examined by Western blot of the two cell lines. As

shown in Figure 3B, while basal VDR level was moderately higher in ROS17/2.8 cells, VDR was also readily detectable in D2SC1 cells under control conditions and was not strongly induced following a 24 hour exposure to VDR ligand. In this experiment cells were cultured in medium containing charcoal-adsorbed fetal calf serum to eliminate potential effects of residual $1\alpha,25(\text{OH})_2\text{D}_3$.

Discussion

Our results to date provide an incomplete but potentially interesting profile of how the $1\alpha,25(\text{OH})_2\text{D}_3$ /VDR system regulates transcription of a gene that is central to the role of DCs in orchestrating innate and adaptive immunity. Novel aspects of this profile include the definitive localization of transcriptional suppression to a clearly-identified VDRE in the *relB* promoter, the clear augmentation of negative transcriptional regulation by *in vitro* or *in vivo* exposure to VDR ligands, the regulated association of VDR and HDAC3 but not RXR α and HDAC1 with the promoter, and the apparent restriction of the ligand-dependent component to certain cell types.

In Figure 4A we present a model to explain experimental results we have obtained thus far in DCs and DC-like cells at steady-state (comparable to immature or partially mature DCs *in vivo*) and following delivery of a pro-maturational stimulus (e.g. LPS). As indicated, the evidence favors constitutive binding of RXR α , HDAC1 and SMRT to the VDRE-containing region of the *relB* promoter. Binding of these components is not dependent on the presence of VDR and is not displaced by pro-maturational stimuli or further enhanced by exposure to exogenous VDR ligand. Under steady-state conditions the association of VDR and HDAC3 with the promoter appears to be less stable and is amenable to displacement by intracellular signaling events associated with pro-maturational stimuli with resulting increased *relB* transcription. Additional experimental results indicate that this displacement is not associated with reduced expression or nuclear export of VDR or HDAC3 and may be overcome by increased expression of one or both proteins [18]. Importantly, steady-state binding of VDR/HDAC3 to the *relB* promoter, while clearly present on the basis of ChIP analysis of freshly isolated splenic DCs [18], does not appear to be inherently suppressive of *relB* transcription *in vivo* as evidenced by the fact that we have not observed increased intracellular RelB mRNA or protein levels in DCs derived from VDR KO mice. This may explain why animals deficient in VDR have normal development of the immune system, normal differentiation and maturation of DCs from bone marrow and are not inherently prone to autoimmunity [[9,43, 44], and M. Griffin, X. Dong unpublished observations].

In contrast, *in vitro* or *in vivo* exposure to VDR ligand clearly results in suppression of intracellular RelB in VDR WT but not VDR KO DCs [18,35]. Furthermore, there is a wide-ranging literature demonstrating that immunological events involving DC/T-cell interactions are significantly modified by administration of $1\alpha,25(\text{OH})_2\text{D}_3$ and synthetic VDR agonists [2, 4,6,8,9,12,13,35,45,46]. Mechanistically, our experiments indicate that VDR ligand-dependent suppression of RelB expression in DCs is linked with histone deacetylation and with increased recruitment of VDR and HDAC3 to the promoter. Based on the absence of a ligand-dependent suppression in osteoblast and other non-APC-derived cell lines, it is possible that this effect is dependent upon the presence of one or more cell type-specific co-factors. Alternatively, some experimental results could be explained by low basal level of VDR in DCs with ligand-induced up-regulation of this protein and resulting increased competitive binding of VDR/HDAC3 to the *relB* promoter. These two alternative models to explain the ligand-dependent component of RelB suppression in DCs are illustrated in Figure 4B. For a number of reasons, we favor a model in which additional co-factors are recruited to the complex in DCs following exposure to $1\alpha,25(\text{OH})_2\text{D}_3$ or D_3 analogs: (a) VDR is readily detectable in DCs and DC-like cell lines in the absence of VDR ligand and is not strikingly induced prior

suppression of *relB* transcription. (b) The ligand-associated component of *relB* suppression in D2SC1 cells remains clearly demonstrable in the context of transfection-based VDR over-expression. (c) We find an absence of ligand-augmented transcriptional suppression in multiple non-immunogenic cell lines in which VDR is over-expressed while *relB* suppression in a macrophage-derived cell-line (RAW) was weakly augmented by VDR ligand. (d) Selective expression of co-repressor proteins has been described for a variety of cell types including DCs and may play an important role in cell-specific gene regulation [47]. From a physiological perspective, selective susceptibility of activated DCs or other APC populations to the suppressive effects of VDR ligands would be consistent with a paracrine model of immune regulation in which inducible localized production of $1\alpha,25(\text{OH})_2\text{D}_3$ serves to limit systemic T-cell responses while preserving or even augmenting protective innate immunity [35,48]. Modulated expression of VDR and 1α -hydroxylase in DCs and macrophages in response to pro-inflammatory stimuli as well as high localized concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$ at sites of inflammation have also been well demonstrated [48] indicating that the influence of the vitamin D system on these cell types is regulated at multiple levels and is most likely to be exerted during the course of active immune responses.

For further elucidation of the models we propose here, it will be important to examine VDR-mediated suppression of *relB* in a wider range of cell lines and primary cell-types. Specifically, the possibility that results may be skewed by altered expression of gene regulatory components including nuclear receptors, co-repressors and co-activators in transformed or tumor-derived cell lines must be borne in mind. Nonetheless, the results to date lend further support to a mechanism of transcriptional suppression comprising a ligand-independent component that can be augmented, in most cell types, by increasing the availability of VDR for DNA binding as well as a ligand-dependent component involving expression of additional co-factors that may be restricted to antigen presenting cell populations such as DCs and macrophages. Ongoing characterization of this pathway of negative regulation may shed further light on the mechanisms underlying VDR-mediated immune modulation *in vivo* as well as providing more general insights into DC-specific gene regulation and nuclear receptor ligand-dependent transcriptional suppression.

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Figure 1.

Chromatin immunoprecipitation (ChIP) assay results are shown for day 7 bone marrow-derived DCs (BMDCs) from untreated VDR wild-type (WT) and VDR knockout (KO) mice and from VDR WT BMDCs treated overnight with 50 ng/ml LPS. Amplification of a 178 bp chromosomal DNA fragment incorporating the mouse *relB* promoter VDRE are shown for total genomic DNA and for protein/DNA complexes precipitated with Rabbit IgG (Control IgG) or with polyclonal rabbit antibodies against VDR (Anti-VDR) and RXRα (Anti-RXRα).

Figure 2.

A. Results are shown for an experiment in which the effect of D₃ analog treatment on transcriptional activity of a mouse osteopontin (mOPN) promoter construct was compared, by luciferase reporter assay, with that of a mouse *relB* promoter construct (mRelB) in an osteoblast cell line (ROS17/2.8) known to express VDR. Aliquots of transfected cells were treated with vehicle (Control) or with 10⁻¹⁰ M D₃ analog. The results are expressed as mean ± SD luciferase activity from triplicate samples for each condition. † = *p* < 0.05 for D₃ analog-treated vs. control-treated. **B.** The position and sequences are shown for the wild type VDRE within the mouse *relB* promoter and the mOPN VDRE generated within the *relB* promoter by site-directed mutagenesis. **C.** The effect of 10⁻¹⁰ M D₃ analog treatment, VDR over-expression, or both on

transcriptional activity of the wild-type mouse *relB* promoter was compared, by luciferase reporter assay in D2SC1 cells, with that of a mouse *relB* promoter construct in which the VDRE was mutated to the sequence of the mOPN promoter VDRE. The result is expressed as mean $\pm$ SD luciferase activity from triplicate samples for each condition. $\dagger = P < 0.05$ compared to untreated control; $\ddagger = p < 0.05$ compared to D₃ analog alone and VDR alone.

Figure 3.

A. The results of luciferase reporter assays in a panel of cell lines are shown. The effect of 10^{-10} M D₃ analog treatment, VDR over-expression (VDR), or both (D3 Analog + VDR) on promoter activity of the wild-type mouse *relB* promoter was compared in the following cell lines: (i) D2SC1 (mouse DC), (ii) HEK293 (human embryonic kidney), (iii) ROS17/2.8 (rat osteoblast), (iv) SW480 (human colonic carcinoma), and (v) RAW (mouse macrophage). The results for each cell line are expressed as the % of untreated (control) cells for luciferase activity (mean $\pm$ SD of triplicate samples for each condition). † = $P < 0.05$ compared to untreated control; ‡ = $p < 0.05$ compared to D₃ analog alone and VDR alone. **B.** Results of a Western blot for VDR and β -actin are shown for D2SC1 and ROS17.2.8 (ROS) cells following 24 hour

culture in the absence (Ctrl) or presence (D3) of 10^{-7} M $1\alpha,25(\text{OH})_2\text{D}_3$. Cultures were carried out using medium supplemented by charcoal adsorbed fetal calf serum to remove the potential influence of residual $1\alpha,25(\text{OH})_2\text{D}_3$ in control conditions.

A**Steady-State Promoter Status** **Maturation Stimulus**

Figure 4.

A. Schematic representations of a negative regulatory complex centered on the identified VDRE with the mouse *relB* promoter are shown for steady state (immature) DCs and DCs subject to a maturational stimulus. The components that have been shown experimentally to be constitutively or variably present by ChIP assay are VDR, RXR α , SMRT, HDAC1 and HDAC3. **B.** Schematic representations of two models for *relB* transcriptional suppression in DCs exposed to exogenous VDR ligand ($1\alpha,25(\text{OH})_2\text{D}_3$ or D_3 analog).