Distinctive dendritic cell modulation by vitamin D3 and glucocorticoid pathways

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Abstract

Dendritic cell (DC) maturation plays a central role in regulating immunity. We show that glucocorticoid and 1α,25(OH)2D3 agonists modulate DCs via distinct and additive signaling pathways. Phenotypic and functional indices were examined in DCs treated with dexamethasone (DEX) and/or a 1α,25(OH)2D3 analog (D3 analog). DEX potently attenuated pro-inflammatory cytokines and chemokines but had modest, reversible effects on T-cell stimulatory capacity. D3 analog produced significantly greater inhibition of T-cell stimulation in vitro and in vivo and, unlike DEX, increased expression of the chemokines MCP-1 and MIP-1α. Both DEX and D3 analog were associated with reduced expression of the NF-κB proteins c-Rel and Rel B but not Rel A. Combined DEX and D3 analog treatment of DCs resulted in significant additive inhibition of pro-inflammatory cytokines, T-cell stimulation, chemokines, chemokine receptors, and NF-κB components. Additive inhibition was most striking for RANTES, CCR5, CCR7, and Rel B. The combined effects of the two hormonal pathways on DCs have unique immunomodulatory potential.

Keywords: Dendritic cells; Antigen presentation; Steroid hormones; Vitamin D; Glucocorticoid; Chemokines; Nuclear factor κB; Interleukin 12; T lymphocytes; Immune responses

Dendritic cells (DCs) occupy a unique role in mediating cross talk between innate and cognate immunity [1]. Migration of DC precursors to inflamed tissue is controlled by factors produced during the innate response to disease. The process by which DCs become competent to activate cognate immunity is termed “maturation” and is also intimately linked with exposure to inflammatory products. Mature DCs are characterized by the ability to migrate to secondary lymphoid organs and to potently stimulate T and B-cells through the abundant surface expression of antigen and accessory ligands [1,2]. Evidence has also accumulated for the role of DCs in the maintenance of immune tolerance to non-threatening antigens and a plausible model has emerged whereby trafficking of antigens from tissue to lymphoid organ by immature DCs results in responses such as clonal deletion and regulatory T-cell recruitment [3,4]. Thus, regulation of DC maturity is central to maintenance of an appropriate balance between immune activation and tolerance.

Among the substances reported to inhibit DC maturation are steroids such as glucocorticoids and the active form of vitamin D (1α,25-dihydroxyvitamin D3), subsequently referred to as 1α,25(OH)2D3 [5–12]. There is widespread expression of glucocorticoid and vitamin D receptors (VDR) within the immune system [13,14] and both glucocorticoid and 1α,25(OH)2D3 agonists are known to exert in vivo suppression of immune-mediated disease [14–19]. In this study, we present novel evidence that the modulatory effects of the glucocorticoids and 1α,25(OH)2D3 agonists on DCs differ significantly and, when combined, are predominantly additive.
Methods

Experimental animals and reagents. C57BL/6 (B6) and BALB/C mice (Jackson Laboratories, Bar Harbor, ME) and DO11.10 TCR transgenic mice were maintained in a specific-pathogen-free facility. Cultures were carried out in DMEM (Life Technologies, Grand Island, NY, USA) with 10% FCS. The vitamin D₃ analog 1,25(OH)₂D₃ (Afrez, Lilly, Indianapolis, IN) was provided by Dr. Milan Uskokovic (Hoffman La-Roche, Nutley, NJ). Monoclonal antibodies used in the study were: biotinylated and FITC-coupled anti-I-Aβ (AF6-120.1), anti-murine CD4-FTTC (RM4-4), biotinylated and FITC-coupled anti-murine CD11c (HL3), streptavidin-PE, anti-murine ICAM1-PE (3E3), anti-murine CD40-FTTC (3/23), purified anti-murine CD40 (HM40-3) (BD Pharmingen, San Diego, CA, USA), biotinylated anti-DO11.10 TCR (KJ1-26, provided by Dr. Larry R. Pease, Dept. of Immunology, Mayo Foundation), and biotinylated mCTLA-4Ig. Rabbit polyclonal antibodies against RelA (p65), RelB, and c-Rel were purchased from Santa Cruz Biotechnology, Santa Cruz, CA.

Preparation of murine DCs. Murine DC cultures were prepared from bone marrow as previously described [7, 8]. DEX (Sigma–Aldrich, St. Louis, MO) and D₃ analog were added on days 2, 4, and 6. Flow cytometry was carried out on a FACScan Flow Cytometer (Becton–Dickinson) and analyzed with CellQuest software. For experiments involving the addition of maturing stimuli, DCs were re-plated in equal numbers on day 6 in fresh cytokine-containing medium for 48 h in 24-well plates with or without anti-CD40 (2.5 μg/ml), or LPS (Sigma–Aldrich, 10 ng/ml).

Mixed lymphocyte reaction (MLR) and Ag presentation assays. Lymph node T-cells from B6 or DO11.10 mice were purified and labeled with CFSE (Molecular Probes, Eugene, OR) as previously described [6]. DCs were irradiated (1500 rad) and mixed at a ratio of 1:6 with CFSE-labeled T-cells in 96-well round-bottomed plates. For DO11.10 experiments, OVAα was added at 0.01–10 μg/ml. Three to six identical wells were prepared for each experimental condition. Following 72 h of culture, cells from individual wells were stained with fluorochrome-coupled mAbs and analyzed immediately by flow cytometry. Relative quantification of cell numbers between samples was achieved by timed accumulation of data. Viability was assessed by exclusion of propidium iodide (PI) and cell division by analysis of CFSE-staining intensities was compared to control conditions [7, 8].

Adoptive transfer of TCR transgenic T-cells and DC inoculations. Pooled splenic and lymph node cells from female DO11.10 mice were depleted of CD8⁺⁺ cells, suspended in PBS, and injected intravenously into female BALB/C (8 × 10⁶ cells/animal). Dendritic cell cultures were lifted on day 7, re-plated overnight in the presence of OVAα, 100 μg/ml with GM-CSF/IL-4, then washed, and injected intravenously (0.3 × 10⁶ cells/animal). Three days later, spleens were harvested from individual animals, depleted of erythrocytes, and analyzed for the presence of DO11.10 T-cells by three-color flow cytometry using anti-CD4-FTTC, anti-idiotypic antibody (biotinylated KJ1-26, followed by streptavidin-PE), and PI. The proportion of KJ1-26⁺⁺ cells within the total CD4⁺⁺ cells was calculated for each animal and results are expressed as means ± SD for each experimental group. Equal numbers of splenocytes from individual animals were cultured in triplicate with graded concentrations of OVA peptide 72 h, analyzed by the same flow cytometric methods and the results were again expressed as means ± SD for each experimental group.

Cytokine and chemokine assays. ELISAs for murine IL-12 p70, IFNγ (optEIA, BD Pharmingen), MIP-1α, RANTES, and MCP-1 (R&D systems, Minneapolis, MN) were carried out according to manufacturer’s instructions.

Reverse transcription and PCR for chemokine and chemokine receptors. Total RNA was extracted from day-7 DCs using Trizol reagent (Invitrogen). Reverse transcription with oligo-dT priming was carried out using 1 μg RNA by Superscript II First Strand Synthesis kit (Invitrogen). Polymerase chain reaction using β-actin-specific primers was first carried out at variable cycle numbers and template amounts to achieve equal loading of first strand cDNA from each sample in subsequent PCR. Primers pairs were designed to amplify regions of the cDNAs for the following chemokines and chemokine receptors: MIP-1α (GenBank Accession No. NM011337); MIP-1β (NM013652); MCP-1 (AF065931); RANTES (AF065947); CCR1 (mmn296787); CCR2 (MMU56819); CCR5 (AF022990); and CCR7 (MUSEB1CDN). In each case, a single band of the expected size was amplified. For each primer set, a sub-maximal cycle number was identified and used to compare relative amplification of each product from different samples.

Western Blot Analysis. Whole cell lysates were prepared as previously described [20] and protein was quantified using Bio-Rad protein assay (Bio-Rad, Hercules, CA). Aliquots of 20 μg were separated on 10% precast Tris HCl gels (Bio-Rad), transferred to an Immobilon membrane (Millipore, Bedford, MA), blotted sequentially with primary antibodies and protein A HRP (Kirkegaard and Perry Laboratories, Gaithersburg, MD), and visualized by ECL (Amersham Pharmacia Biotech, Piscataway, NJ). Western blot exposures were digitized on a flattened scanner with transparency capabilities (ScanMaker III, Microtek Lab, Redondo Beach, CA) and band intensities were quantified using NIH Image software (National Institute of Health, Bethesda, MD).

Northern blotting. DNA fragments corresponding to non-homologous portions of the cDNAs of murine RelA, RelB, and c-Rel were generated by PCR using DC-derived, oligo-dT primed, first strand DNA as a template and primer pairs were derived from published sequences: Rel A (GenBank Accession No: AF199371); Rel B (M33380); c-Rel (X15842). Total RNA was isolated from DC populations using the RNeasy Mini Kit (Qiagen, Valencia, CA). Aliquots of 5 μg were separated on 1.2% agarose, 2.2 M formaldehyde gels and transferred to Nytran membranes (Schleicher & Schuell, Keene, NH) by TurboBlotter (Schleicher & Schuell). Membranes were probed sequentially with α³²P-dATP and α³²P-dCTP-labeled probes (NEN Life Science, Boston, MA and Radprime, Invitrogen, Carlsbad, CA). Normalization of RNA content was achieved by probing for the acidic ribosomal phosphoprotein 36B4 (probe provided by Dr. Yi Chen from Department of Ophthalmology, Mayo Foundation). Northern blots were subjected to quantitative imaging densitometry as described for Western blot analysis.

Statistical Analysis. Data are expressed as means ± standard deviation (SD) for each condition. Differences between groups were analyzed by two-sided, non-paired t test with significance assigned to differences of p < 0.05. All experiments were carried out with consistent results a minimum of three times.

Results and discussion

Separate and combined conditioning effects of D₃ analog and dexamethasone on surface markers of dendritic cell maturation and on pro-inflammatory cytokines

Although multiple studies have documented the capacity for glucocorticoids and 1α,25(OH)₂D₃ to inhibit DC immunostimulatory function [5–12], the separate and combined effects of the two steroids on DC differentiation and maturation have not been closely compared. Our previously reported results [7, 8] as well as preliminary experiments for the current study (data not shown) established optimized concentrations of DEX (10⁻⁷ M) and D₃ analog (10⁻¹⁰ M) at which maximal
inhibition of DC surface levels of MHC II and the co-stimulatory ligands CD80/CD86 occurred without inhibition of DC yield in GM-CSF/IL-4-stimulated murine bone marrow cultures. As shown in Fig. 1a, combined DC conditioning with $10^{-7}$ M DEX and $10^{-10}$ M D$_3$ analog was associated with additive inhibition of multiple maturational markers (MHC II, CD80/CD86, CD40, and ICAM-1) compared with either agent alone. While CD40 and ICAM-1 were not significantly reduced by DEX, an additive effect of D$_3$ analog and DEX was consistently observed. Thus, it was clear that the 1α,25(OH)$_2$D$_3$ and glucocorticoid pathways were capable of additively inhibiting DC maturational markers without aborting DC differentiation. In subsequent text and figures, the terms unconditioned DCs, Dex-DCs, D3-DCs, and Dex/D3-DCs are used in reference to DC populations generated using the optimized concentrations of the two agonists.

Secretion of cytokines by the conditioned DC populations was measured in the absence or presence of maturational stimuli (LPS + CD40 ligation). The conditioning agents (DEX and/or D$_3$ analog) were removed for the period of stimulation. Results for IL-12 p70 and IFN$\gamma$ are shown (Fig. 1b). For IL-12, basal secretion was low for all DC populations. Dex-DCs failed to respond to LPS alone but responded robustly to anti-CD40. The IL-12 response of D3-DCs to LPS was blunted and was not significantly enhanced by additional CD40 ligation. Dex/D3-DCs exhibited significantly reduced IL-12 response to both LPS and LPS + anti-CD40. Secretion of IFN$\gamma$ exhibited a similar pattern with the exception that Dex-DCs remained profoundly deficient in the ability to produce this cytokine even with combined LPS and CD40 stimuli. For D3-DCs, IFN$\gamma$ secretion was similar to that of IL-12 with a blunted response to LPS and no augmentation by CD40 ligation. Dex/D3-DCs did not secrete IFN$\gamma$ under any conditions. We concluded that the 1α,25(OH)$_2$D$_3$ and glucocorticoid agonists induced discreet defects in the capacity of DCs to secrete immunomodulatory cytokines following maturational stimuli and that DCs conditioned by a combination of the two steroid pathways were profoundly resistant to induced secretion.

Fig. 1. Effects of optimized concentrations of DEX and D$_3$ analog on DC surface marker expression and cytokine secretion. (a) Results of surface staining for MHC II, CD80/CD86, CD40, and ICAM 1 are shown for DCs cultured in the presence of: no addition (UnTx-DCs), $10^{-7}$ M DEX (Dex-DCs), $10^{-10}$ M D$_3$ analog (D3-DCs), $10^{-7}$ M DEX, and $10^{-10}$ M D$_3$ analog (Dex/D3-DCs). Results are expressed as MFI of CD11c-gated cells (means ± SD of triplicate wells for each concentration). For all markers combined D$_3$ analog and DEX conditioning resulted in significantly reduced surface expression when compared to conditioning with D$_3$ analog alone. * = $p < 0.05$ for values lower than corresponding results for unconditioned cells, † = $p < 0.05$ for values lower than corresponding results for D$_3$ analog-alone conditioned cells. (b) Effects of D$_3$ analog and DEX on DC secretion of IL-12 p70 and IFN$\gamma$ following stimulation by LPS with or without CD40 ligation. Day-7 DC populations were re-plated and stimulated for 48 h following removal of conditioning agents. Supernatants were assayed by ELISA for IL-12 p70 and IFN$\gamma$. Results are expressed as pg/ml (means ± SD of triplicate wells for each condition). Dex-DCs demonstrated reduced basal secretion of IFN$\gamma$, unresponsiveness to LPS for both cytokines, and retained responsiveness to LPS + anti-CD40 for IL-12 p70. D3-DCs demonstrated reduced basal secretion of IFN$\gamma$ and blunted responses to LPS and LPS + anti-CD40 for both IL-12 p70 and IFN$\gamma$. Dex/D3-DCs demonstrated additional blunting of IL-12 p70 responses and an absence of detectable IFN$\gamma$ secretion. * = $p < 0.05$ for values lower than corresponding results for unconditioned cells, † = $p < 0.05$ for values lower than corresponding results for D$_3$ analog-alone conditioned cells.
Combined conditioning of dendritic cells with D₃ analog and DEX additively impairs peptide-specific T-cell responses in vitro and in vivo

The capacity of each DC population to stimulate T-cell proliferation in vitro was examined using a peptide-specific system (BALB/C DCs + DO11.10 T-cells, Fig. 2). T-cell proliferation to a graded concentration of ovalbumin-derived antigenic peptide was measured by timed flow cytometric analysis of CFSE-labeled purified T-cells. To determine whether the immuno-stimulatory phenotypes were retained following withdrawal of the conditioning agents, T-cell proliferation was compared using DCs harvested directly from culture (Day-7 DCs) or following an additional 48 h in the absence of conditioning agents (Day-9 DCs). For Day-7 DCs, a clear hierarchy was present for immuno-stimulatory potency: unconditioned DCs > Dex-DCs > D3-DCs > D3 = Dex-DCs. Dex/D3-DCs were profoundly impaired in the ability to activate resting T-cells. The results for Day-9 DCs showed that Dex-DCs reverted to full (unconditioned) potency upon withdrawal of DEX while D3-DCs and Dex/D3-DCs retained significantly reduced immuno-stimulatory capacity. Dex/D3-DCs remained significantly poorer than D3-DCs at initiating T-cell proliferation. An identical result was obtained using alloantigen-stimulated murine T-cells (data not shown).

To demonstrate that the T-cell stimulatory effects observed in vitro were also present in vivo, an adoptive transfer model was used. Following adoptive transfer of
DO11.10 T-cells, groups of BALB/C mice were inoculated with peptide-pulsed DC populations. A control group received non-peptide pulsed, unconditioned DCs. Three days later, expansion of DO11.10 T-cells within the spleen was determined by flow cytometric analysis (Fig. 3a) and secondary responses to graded concentrations of antigen were examined in vitro (Fig. 3b). DC-stimulated T-cell expansion in vivo followed the same pattern as for in vitro responses resembling most closely the pattern observed using day-9 DCs, suggesting that conditioning of DCs by DEX alone is reversible upon transfer to an in vivo environment. The results of secondary in vitro stimulation underscored the pattern observed for experimental groups. This experiment also demonstrated that the peak response following inoculation with peptide-pulsed unconditioned DCs, Dex-DCs, and D3-DCs occurred at a peptide concentration (0.1 μg/ml) lower than that of cells from the unstimulated control group (1.0 μg/ml). In contrast, prior inoculation with peptide-pulsed Dex/D3-DCs resulted in a flat response to peptide titration. Repeated experiments were carried out with very similar results.

We concluded from our in vitro and in vivo T-cell stimulation assays that: (a) glucocorticoid conditioning of DCs results in moderately inhibited capacity to stimulate T-cell proliferation that is reversible following glucocorticoid withdrawal; (b) DC conditioning by D3 analog results in a significantly greater inhibition of the ability to induce T-cell proliferation that is not rapidly reversed; (c) in combination glucocorticoid and 1α,25(OH)2D3 induce a profound and prolonged attenuation of DC T-cell stimulatory mechanisms.

Chemokine and chemokine receptor expression by dendritic cells are regulated in two characteristic patterns by D3 analog and dexamethasone

The importance of regulated trafficking of DCs has been highlighted by studies of the role of chemokines and chemokine receptors in sequentially guiding the DC from circulation to peripheral tissues to secondary lymphoid organs [21,22]. Furthermore, DCs contribute directly to the orchestration of inflammatory responses by releasing chemokines capable of recruiting additional cellular immune effectors [21,22]. The effects of DEX and D3 analog on DC RNA levels of a panel of chemokines and chemokine receptors were examined by semi-quantitative RT-PCR (Fig. 4a). Two patterns of modulated expression were observed. Pattern 1 (opposing regulation) consisted of strongly reduced levels of transcript in Dex-DCs compared with unconditioned DCs, unchanged or increased levels in D3-DCs, and an intermediate level in Dex/D3-DCs (lower than that of D3-DCs but higher than that of Dex-DCs). This pattern was observed for the chemokines MIP-1α, MIP-1β, and MCP-1, and for the chemokine receptor CCR1. The

![Fig. 4. Distinct regulation patterns of chemokines and chemokine receptors by D3 analog and DEX. (a) Total RNA from four populations of day-7 DCs was analyzed by RT-PCR for the presence and relative amounts of transcript for chemokines and chemokine receptors. Equal loading of RNA was tested by amplification of a β-actin-specific product at two sub-optimal cycle numbers (upper panels: 25 and 30 cycles). For MIP-1α, MIP-1β, MCP-1, and CCR1 strikingly reduced transcript was observed in Dex-DCs compared with unconditioned DCs, with increased or unchanged transcript in D3-DCs, and an intermediate level in Dex/D3-DCs (pattern 1: opposing regulation). For RANTES, CCR2, CCR5, and CCR7, modest reductions in transcript were observed in both Dex-DCs and D3-DCs compared to unconditioned DCs while Dex/D3-DCs demonstrated strikingly reduced transcript levels (pattern 2: additive regulation). (b) Four populations of day-7 DCs were re-plated in equal numbers for 48 h in the absence of conditioning agents and supernatants assayed 48 h later by ELISA for MIP-1α, RANTES, and MCP-1. Results are expressed as ng/ml (means ± SD for triplicate wells for each condition). Readily detectable levels of each chemokine were secreted by UnTx DCs. Dex-DCs secreted significantly lower amounts of all three. D3-DCs secreted significantly lower amounts of RANTES but significantly higher levels of MIP-1α and MCP-1. Dex/D3-DCs secreted significantly less RANTES than all other DC populations but also secreted significantly greater amounts of MIP-1α and MCP-1 than unconditioned cells. * = p < 0.05 for values lower than corresponding results for unconditioned DCs, † = p < 0.05 for values higher than corresponding results for unconditioned DCs, ‡ = p < 0.05 for values lower than corresponding results for Dex-DCs and D3-DCs.
inhibition of these factors by DEX is consistent with reported effects on other cell types [14,15,23]. The physiological and therapeutic significance of their induction by D3 analog might imply a pro-inflammatory component to the phenotype of DCs modulated by D3 analog- has been shown to protect against LPS-induced sepsis, suggesting a counter-regulatory role [24]. Pattern 2 (additive regulation) consisted of moderately reduced transcript levels in Dex-DCs and D3-DCs and a clearly greater reduction in Dex/D3-DCs. This pattern was observed for RANTES, CCR5, and CCR7. The inhibition of RANTES/CCR5 expression is of specific interest in regard to allograft rejection as expression and genetic variability of these factors has been closely linked with acute and chronic graft injury [25,26]. The inhibition of CCR7, a receptor involved in trafficking of DCs to lymphoid organs, represents another potentially novel mechanism for attenuating immune-mediated injury in the setting of transplantation or autoimmunity [21,22,30]. Importantly, ELISA of DC culture supernatants for MIP-1α, RANTES, and MCP-1 yielded results that correlated with those of RNA analysis (Fig. 4b).

**Intracellular levels of NF-κB components in DCs are inhibited in distinct patterns by separate and combined D3 analog and DEX conditioning**

Dendritic cell differentiation and maturation are intimately linked to activity of NF-κB intracellular signaling and individual NF-κB components have been shown to play specialized functional roles and to be separately regulated in DCs [27–30]. We hypothesized that conditioning of DCs during differentiation results in distinct patterns of expression of NF-κB components. To begin to address this issue, Western blot analysis of total cell lysates (Fig. 5a) and Northern blot analysis of RNA (Fig. 5b) were carried out on the four DC populations. For Western blots, DCs were analyzed on day 7 and after an additional 48 h of culture in the absence of conditioning agents. For three NF-κB components—Rel A, Rel B, and c-Rel—distinct expression profiles were observed. Rel A protein expression was unaffected by DEX and D3 analog and Rel A RNA was minimally reduced. Rel B protein and RNA were moderately reduced by both D3 analog and DEX (D3 analog inhibition > DEX) but greatly reduced by combined DEX and D3 analog. The expression of c-Rel protein was slightly increased by combined DEX and D3 analog, while DEX alone had no effect on c-Rel expression. The expression of NF-κB components in DCs was also assessed by Northern blotting. The results showed that DEX conditioning was associated primarily with reduced c-Rel transcript. D3 analog conditioning was associated with reduced levels of both Rel B and c-Rel. Combined DEX and D3 analog conditioning resulted in further reductions in Rel B and c-Rel transcript levels.

![Fig. 5. Modified DC expression of components of the NF-κB pathway by D3 analog and DEX. (a) Total cell lysates from four populations of DCs were prepared at day 7 or following an additional 48 h of culture in the absence of conditioning agents (day-9 DCs) and analyzed by immunoblotting for the NF-κB proteins Rel-A, Rel-B, c-Rel, and the control protein IP-90. The populations were: (A) unconditioned, (B) Dex-DCs, (C) D3-DCs, and (D) Dex/D3-DCs. Individual immunoblots (upper panels) and the results of densitometric analysis (lower panel: expressed for each band as ratio of the IP-90-adjusted density to that of day-7 unconditioned DCs) are shown. For day-7 samples, Rel A levels were comparable among all samples, Rel B levels were reduced in both Dex-DCs and D3-DCs with additional reduction in Dex/D3-DCs. c-Rel levels were reduced to a greater degree in Dex-DCs than in D3-DCs and Dex/D3-DCs. For day-9 samples, there was an increase in levels of all three proteins in unconditioned DCs compared with day 7. The effect of DEX on Rel B and c-Rel levels was less apparent while the effects of D3 and Dex/D3 were maintained. (b) Total RNA from four populations of day-7 DCs was analyzed by Northern blotting using probes specific for Rel A, Rel B, c-Rel, and the control transcript 36B4. Samples are labeled as for (a). Individual blots (upper panel) and the results of densitometric analysis (lower panel: expressed for each band as ratio of the 36B4-adjusted density to that of unconditioned DCs) are shown. The Rel B specific band is indicated by an arrow, with the activity above this band representing residual presence of labeled Rel A transcript. DEX conditioning was associated primarily with reduced c-Rel transcript. D3 analog conditioning was associated with reduced levels of both Rel B and c-Rel. Combined DEX and D3 analog conditioning resulted in further reductions in Rel B and c-Rel transcript levels.](image-url)
reduced by both DEX and D₃ analog (DEX inhibition > D₃). An additive effect of combined DEX and D₃ analog conditioning on c-Rel expression was observed for RNA but not protein. The effect of DEX on Rel B and c-Rel protein levels was found to be predominantly reversed upon DEX withdrawal while the effects of D₃ analog and combined conditioning were persistent. We concluded that the two steroid pathways separately and (for Rel B) additively regulate DC expression of individual NF-κB family members. It is important to stress that these observations do not preclude additional levels of regulation of NF-κB as have been reported both for glucocorticoids and for 1α,25(OH)₂D₃ in other immune and non-immune cell types including upregulation of IκBα, direct intranuclear binding of NF-κB proteins, and direct negative regulation of NF-κB-induced genes [31–35]. Furthermore, although the functional alterations attributable to glucocorticoid and 1α,25(OH)₂D₃ in this report could directly or indirectly stem from NF-κB modulation, it is likely that modification of additional signaling cascades and direct regulation of individual genes may also be involved.

The experimental results described here reveal considerable differences in the influence of glucocorticoid and 1α,25(OH)₂D₃ pathway agonists on maturationally regulated DC functions. They also show that, in combination, the two steroid pathways potently attenuate multiple DC contributions to innate and cognate immune responses. Our results have significant implications for the clinical use of steroid agonists in immunomodulatory therapy. The spectrum of DC effects we have observed with DEX alone underscores the strength of glucocorticoid therapy as a broad “anti-adjuvant” that targets primarily pro-inflammatory cytokines and chemokines [14,15]. The relatively mild inhibition of MHC and co-stimulatory ligands (particularly at lower concentrations) and the tendency for rebound DC maturation to occur upon withdrawal are also compatible with recognized limitations of glucocorticoid immunotherapy—the need for large dosages to achieve control of T-cell-mediated diseases and the frequency of disease relapse upon dose reduction. In contrast, the profile of D₃ analog-mediated DC modulation would predict the capacity to potently attenuate antigen-specific T-cell activation at relatively low concentrations with weaker or even paradoxical effects on non-specific inflammatory mediators. Although the therapeutic use of 1α,25(OH)₂D₃ itself is limited by hypercalcemia, structural analogs of 1α,25(OH)₂D₃ with significantly reduced calcemic effects but preserved immunosuppressive properties offers the prospect of harnessing this pathway for treatment or prevention of autoimmune disease and allograft rejection [36,37]. As the majority of such disease processes involve an ongoing interplay between innate mechanisms of inflammation and cognate, antigen-specific responses our results would predict that the combination of glucocorticoid and 1α,25(OH)₂D₃ agonists offers a unique therapeutic profile. The recent demonstration by Barat et al. [38] that the same combination exerts striking direct effects on antigen-stimulated T-cells to promote a regulatory (IL-10-secreting) phenotype lends an additional strong incentive to explore the therapeutic and physiological roles of these steroid hormone pathways.

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