Mechanism of 1α,25-dihydroxyvitamin D3-dependent repression of interleukin-12B

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Interleukin 12 (IL-12) is a heterodimeric, pro-inflammatory cytokine that plays a central role in activation and differentiation of CD4+ T cells into interferon-γ secreting T-helper type 1 cells. IL-12B, a gene encoding the larger subunit of active IL-12, has been reported to be down-regulated by the nuclear hormone 1α,25-dihydroxyvitamin D3 (1α,25(OH)2D3), but the mechanism of the regulation is unknown. In this study, we have examined the molecular mechanism of transcriptional regulation of the IL-12B gene by 1α,25(OH)2D3 in lipopolysaccharide (LPS)-treated human monocytes (THP-1). Quantitative RT-PCR showed that IL-12B mRNA displays a cyclical expression profile and is down-regulated 2.8-fold during the first 8 h and even 12.1-fold 24 h after exposure to 1α,25(OH)2D3. Gel shift and quantitative chromatin immunoprecipitation (ChIP) assays demonstrated vitamin D receptor (VDR) binding to genomic regions 480 and 6300 bp upstream of the IL-12B transcription start site (TSS). Quantitative ChIP assays also revealed that together with VDR and its partner RXR the above regions recruited the co-repressor NCOR2/SMRT and histone deacetylase 3 leading to a repression of IL-12B transcription. We suggest that these repressive epigenetic changes eventually cause down-regulation of IL-12B expression. This article is part of a Special Issue entitled: 11th European Symposium on Calcium.

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1. Introduction

Interleukin 12 (IL-12) is a heterodimeric, pro-inflammatory cytokine that consists of two protein subunits, p40 and p35, that are encoded by distinct genes and together form the biologically active IL-12 cytokine, IL-12p70 [1]. Interestingly, p40 is also used as a subunit of IL-23, which is another pro-inflammatory cytokine [2]. Both IL-12 and IL-23 are produced predominantly by local antigen-presenting cells, such as activated monocytes, macrophages and dendritic cells, in response to bacterial antigens and intracellular pathogens via toll-like receptor 4 (TLR-4) signaling [2,3]. IL-12 plays a central role in the activation and differentiation of CD4+ T cells into interferon-γ secreting T-helper (Th) type 1 cells, while IL-23 is essential for the survival and proliferation of IL-17 producing CD4+ memory Th17 cells [4,31,32].

Although induction of IL-12 and IL-23 by intracellular pathogens is indispensable to an efficient immune response against microbial infections, over-expression of IL-12 and/or IL-23 may lead to the development and perpetuation of chronic inflammatory and autoimmune diseases, such as rheumatoid arthritis, psoriasis, multiple sclerosis, Crohn’s disease and inflammatory bowel disease [5,6,33,34]. To prevent the development of these serious diseases, the production of IL-12 and IL-23 is normally tightly controlled by multiple negative regulatory mechanisms. There are over 50 substances with reported IL-12B inhibitory effects [4], one of which is 1α,25-dihydroxyvitamin D3 (1α,25(OH)2D3) that has been reported to inhibit IL-12 production in activated macrophages [7-9].

The secosteroid hormone 1α,25(OH)2D3 is the biologically most active metabolite of vitamin D3 that regulates calcium and bone metabolism, controls growth and differentiation of multiple cell types and plays an important role as an immuno-regulatory and anti-inflammatory agent [10,11]. 1α,25(OH)2D3 mediates its effects via the vitamin D receptor (VDR), a ligand-activated transcription factor that belongs to the superfamily of nuclear receptors [35]. VDR is expressed in a number of different immune cells including dendritic cells and
macrophages [12]. In addition, activated macrophages are able to synthesize and secrete 1α,25(OH)2D3, which further underlines the potential of 1α,25(OH)2D3 in the regulation of immune responses [13].

Binding of 1α,25(OH)2D3 to the VDR promotes heterodimerization with the retinoid X receptor (RXR). This leads to the association of VDR–RXR heterodimeric complex with vitamin D response elements (VDREs) within the regulatory regions of primary VDR target genes and subsequently results in activation or repression of these genes. VDREs are formed by direct repeats (DRs) of the hexameric consensus sequence RGKTCR (R=A or G, K=G or T) with three or four intervening nucleotides (DR3 and DR4) or by reverted repeats (ERs) with six to nine spacing nucleotides (ER6 to ER9) [14–16]. Although a suppressive effect of 1α,25(OH)2D3 on IL-12 expression has been reported [9,17], no functional VDREs have been found within the genes IL-12B or IL-12A. Instead, binding sites for NF-κB, C/EBP and AP-1 have been identified at the proximal promoter of IL-12B, via which the activation of IL-12 appears to happen [18–20]. The repressive effect of 1α,25(OH)2D3 has been associated with the proximal IL-12B promoter, but no direct binding of VDR to this region has been reported [9].

In this study, we aimed to clarify the molecular mechanism of 1α,25(OH)2D3-dependent IL-12 repression in lipopolysaccharide (LPS)-activated THP-1 human monocyteic cells. Our data suggest that the 1α,25(OH)2D3-dependent repression is mediated via VDREs that locate upstream of the binding sites of NF-κB, C/EBP and AP-1.

Interestingly, the IL-12B repression involves cyclical recruitment of a repressive protein complex that leads to a non-permissive chromatin environment at the IL-12B promoter and TSS and ultimately to the inhibition of IL-12 expression.

2. Materials and methods

2.1. Cell culture

THP-1 human acute monocytic leukemia cells were maintained in RPMI-1640 medium (Sigma-Aldrich) and SW-480 human colon adenocarcinoma cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM). Both media contained 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin and culturing was in a humidified 95% air/5% CO2 incubator. Before use in experimental procedures, FBS was stripped from lipophilic compounds, such as endogenous nuclear receptor ligands, by stirring it with 5% activated charcoal (Sigma-Aldrich) for 3 h at room temperature. Charcoal was then removed by centrifugation, and the medium was sterilized by filtration (0.2 μm pore size). THP-1 and SW-480 cells were maintained for experiments in phenol red-free DMEM, supplemented with 5% charcoal-stripped FBS. Liposomes containing control or VDR siRNA were formed by incubating 100 pmol of each siRNA duplex with 5 μl of RNAiMAX for 20 min at room temperature in a total volume of 500 μl of phenol red-free DMEM without antibiotics.

The liposomes were added to the cells and siRNA treatment was continued for 48 h, then the cells were treated with 100 ng/ml LPS for 24 h and finally exposed to either solvent (ethanol, 0.1% final concentration) or 10 nM 1α,25(OH)2D3 for indicated time periods. Silencing of VDR at the protein level was verified by Western blotting using 25 μg of whole-cell extract from THP-1 cells and anti-VDR antibody (sc-1008, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cellular proteins were separated and translated proteins were incubated for 15 min in a total volume of 5 μl of poly(dI-C), 5% glycerol. 1 ng of [32P]-labeled DNA complexes were resolved by gel electrophoresis and autoradiography on X-ray films (Eastman Kodak, Rochester, NY, USA). The positions of protein/DNA complexes were determined by a phosphor imager (Amersham-Searle, Arlington Heights, IL, USA).

2.2. Human monocyte isolation

The isolation of human primary monocytes was essentially done as indicated before [21]. In short, the peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare) and Leucospex tubes (Greiner bio-one, Wendel, Germany). The number of PBMCs was determined using a hematocytometer. The CD14+ cells were magnetically labeled and the cell suspension was loaded onto a MACS LS separation column (Miltenyi Biotech, Utrecht, The Netherlands) in a magnetic field and eluted with MACS buffer from the column after washing and removal of the column from the magnetic field. The purity of the CD14+ cells was evaluated by flow cytometry using anti-CD14-FITC and anti-IgG2a-FITC antibodies (ImmunoTools, Friesoythe, Germany) and a FACSCantoll Flow Cytometry System (Becton Dickinson, Franklin Lakes, NJ, USA). The number of the primary monocytes was determined after which the cells were resuspended in pre-warmed RPMI-1640 medium containing 5% human serum minus 2 mM L-glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin to a density of 2–3 × 106 cells/ml and incubated overnight in a humidified 95% air/5% CO2 incubator. Then, ligand treatments were performed followed by total RNA extraction.

2.3. Total RNA extraction, cDNA synthesis and real-time quantitative PCR

Total RNA was extracted using High Pure RNA Isolation Kit (Roche) and cDNA synthesis was performed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche) according to the manufacturer’s instructions. Real-time quantitative PCR was performed with a LightCycler 480 apparatus (Roche) using TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA, USA) for IL-12B (Hs00233688_m1), TLR-4 (Hs00152939_m1) and RPLP0 (4333.761 F) and Maxima™ Probe qPCR Master Mix (Fermentas, Vilnius, Lithuania). PCR cycling conditions were: 10 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and 60 s at 60 °C. Fold changes were calculated using the formula 2−ΔΔCt, where ΔΔCt = ΔCt(1α,25(OH)2D3) − ΔCt(EtOH), and ΔCt = Ct(Δ – Ct(RPLP0). Ct is the cycle at which the threshold line is crossed. For statistical analysis, fold changes were log2-transformed and two-tailed Student’s t-tests were performed to calculate statistical significance between solvent treated and ligand-treated samples.

2.4. siRNA silencing

THP-1 cells were transfected with either non-specific siRNA oligomers or Stealth™ siRNAs targeting VDR mRNA (Invitrogen, Carlsbad, CA, USA) by using RNAiMAX reagent (Invitrogen) according to the manufacturer’s instructions. The cells were seeded into 6-well plates and grown in phenol red-free DMEM supplemented with 5% charcoal-stripped FBS. Liposomes containing control or VDR siRNA were formed by incubating 100 pmol of each siRNA duplex with 5 μl of RNAiMAX for 20 min at room temperature in a total volume of 500 μl of phenol red-free DMEM without antibiotics.

2.5. Gel shift assay

VDR and RXR proteins were generated by coupled in vitro transcription/translation reaction using full-length cDNAs subcloned into the T7/SV40 promoter-driven pSG5 expression vector (Stratagene, LaJolla, CA, USA) [34] and TNT Quick Coupled Transcription/Translation Systems (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Oligonucleotides were labeled with Klenow fragment DNA polymerase (Fermentas) in the presence of a nucleotide mixture containing [32P]α-dCTP. In gel shift assays, 10 ng of the appropriate in vitro translated proteins were incubated for 15 min in a total volume of 20 μl of binding buffer (10 mM Hepes, pH 7.9, 150 mM KCl, 1 mM dithiothreitol, 0.2 μg/μl of poly(dI-dC), 5% glycerol). 1 ng of [32P]-labeled double-stranded oligonucleotides were then added and incubation was continued at room temperature for 20 min. Protein–DNA complexes were resolved by electrophoresis through non-denaturing 5% (w/v) polyacrylamide gels in 0.5 x TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) and quantified on a FLA-3000 reader (Fuji, Tokyo, Japan) using Image Gauge software (Fuji).
2.6. Cell transfections and luciferase reporter gene assays

SW-480 cells were transfected with the pGL4 containing minimal promoter of thymidine kinase (tk) gene and the upstream regions of the human IL-12B gene. A construct containing the proximal promoter of the human CYP24 gene with a cluster of DR3-type VDREs was used as a positive control. SW-480 cells were transfected using DOTAP transfection protocol with a mixture of 1 μg of different reporter constructs and 1 μg of expression vector for human VDR gene (pSG5-hVDR) in a 6-well plate format. After 5 h, 1α,25(OH)2D3 was added to 100 nM and luciferase reporter assay was performed 16 h post-treatment with BriteLite luciferase substrate (Perkin Elmer, Waltham, MA, USA) in a Victor3 multilabel counter (Perkin Elmer) equipped with automatic dispenser. Luciferase activities were normalized with respect to total protein concentration.

2.7. ELISA assays

For protein expression analysis, THP-1 cells (2×10^6 cells/ml) were stimulated with 1 μg/ml LPS for 24 h prior to treatment with either solvent.
(ethanol, 0.1% final concentration) or 100 nM 1α,25(OH)2D3 for 48 and 72 h, after which the cell culture supernatants containing secreted IL-12B protein were collected. The effect of 1α,25(OH)2D3 on the expression of IL-12B at protein level was determined using Human IL-12/IL-23 p40 Dimer Quantikine ELISA Kit (R&D Systems, NE, USA) according to the manufacturer’s instructions.

2.8. ChIP assays

For ChIP assays, nuclear proteins were cross-linked to DNA by adding formaldehyde directly to the medium to a final concentration of 1% and incubating at room temperature for 10 min on a rocking platform. Cross-linking was stopped by adding glycine to a final concentration of 0.125 M and incubating at room temperature for 5 min on a rocking platform. The medium was removed and the cells were washed twice with ice-cold PBS. The cells were collected and resuspended in lysis buffer containing protease inhibitors. The chromatin was fragmented by sonication using Bioruptor (Diagenode, Liège, Belgium) with adaptors for 15 min at 4 °C to result in DNA fragments of 300 to 500 bp and non-specific background was removed using salmon sperm DNA/protein A agarose slurry (Millipore, Temecula, CA, USA) at 4 °C for 1 h. The recovered chromatin solutions were diluted 1:10 (v/v) in ChIP dilution buffer and incubated with 1 μg of indicated antibodies at 4 °C overnight. Antibodies against histone H4 acetylated at K5, K8, K12 and K16 (H4Ac, 06–598) and antibodies against histone H3 trimethylated at K27 (H3K27me3, 17–622) were from Upstate Biotechnology (Lake Placid, NY, USA), the antibodies against VDR (sc-1008), RXRα (sc-553), NCOR2/SMRT (sc-1610), SRC1 (sc-8995) and HDAC3 (sc-11417) from Santa Cruz Biotechnologies. The immuno-complexes were collected using protein A agarose slurry (Millipore) and reverse cross-linked in the presence of 2 μl of proteinase K (18.9–20.1 mg/ml) (Fermentas) at 64 °C overnight, after which phenol:chloroform extraction and ethanol precipitation were performed. ChIP samples were analyzed with quantitative PCR using BHQ1-FAM6 hydrolysis probes (Eurogentec, Liège, Belgium) and Maxima™ Probe qPCR Master Mix. The sequences of the primers and the hydrolysis probes are listed in Tables S1 and S2, respectively. The qPCR reaction was performed with a LightCycler 480 apparatus using the same PCR profile as with cDNA samples in an appropriate annealing temperature. The results were normalized with respect to input. Fold changes were calculated by using the formula 

$$2^{-\Delta\Delta Ct} = \frac{Ct_{\text{immunoprecipitated DNA}} - Ct_{\text{input}}}{Ct_{\text{non-specific IgG}} - Ct_{\text{input}}}$$

where ΔCt is Ct_{immunoprecipitated DNA} − Ct_{input} and Ct is the cycle at which the threshold line is crossed.
3. Results

3.1. Expression profiling of IL-12B and TLR-4 in THP-1 cells

The effect of 1α,25(OH)2D3 on the expression of IL-12B mRNA was studied in THP-1 human acute monocytic leukemia cells using TaqMan gene expression assays. Because the basal expression of IL-12B mRNA was very low, the cells were primed using LPS for 24 h before ligand treatments. This increased the basal expression and emphasized the ligand effects (Fig. S1A). The IL-12B mRNA expression was measured every 15 min up to 8 h and again 24 h after onset of 1α,25(OH)2D3 treatment (Fig. 1A). According to our data, 1α,25(OH)2D3 treatment caused a significant, up to 2.5-fold down-regulation of IL-12B mRNA levels within the first 6 h. Interestingly, the expression profile showed a cyclical behavior, having minima at time points 45 min, 105 min, 195 min, 270 min, 315 min, 360 min and 435 min. However, the lowest expression was reached after 24 h with a 12.2-fold repression of basal expression levels. The effect of 1α,25(OH)2D3 on IL-12B expression after 6 h was also examined in primary human monocytes (Fig. 1B). A significant 1.5-fold repression was observed, suggesting that the 1α,25(OH)2D3-dependent repression of IL-12B is not only related to cultured cells but also observable in primary cells.

Because LPS induces IL-12B expression via TLR-4, we wanted to confirm that the observed repression of IL-12B did not result from changes in TLR-4 signaling and therefore examined the effect of 1α,25(OH)2D3 treatment to the TLR-4 expression using hormone treatment times 270 min, 360 min and 450 min that resulted in strongest repression of IL-12B. According to our data, the expression of TLR-4 did not change markedly due to 1α,25(OH)2D3 treatment although a slight decrease could be observed at time point 450 min (Fig. S1B).

We repeated the expression assays using opposite order of treatments to find out, if the hormone treatment affected to the LPS induction of IL-12B expression. Our data suggest that, when the cells were treated with 1α,25(OH)2D3 prior to LPS treatment, TLR-4 expression did not change markedly (Fig. S1C). Instead, IL-12B repression was even more prominent than with LPS pre-treatment (Fig. S1D). Similar results were obtained when the experiment was repeated with hormone treatment times of 270, 360 and 450 min prior to LPS treatments (Fig. S1E). Thus, the 1α,25(OH)2D3 pre-treatment appeared to suppress the induction of IL-12B by LPS.

To further verify that the observed repression of IL-12B mRNA expression was dependent on VDR, we silenced VDR using siRNA before measuring IL-12B expression. Silencing of VDR at protein level was confirmed by immuno-blotting (Fig. 1C). Non-specific control
siRNA did not affect the repressive effect of 1α,25(OH)2D3 on IL-12B expression, whereas VDR-specific siRNA completely abolished it (Fig. 1D).

The effect of 1α,25(OH)2D3 on the expression of IL-12B at protein level was determined using ELISA. According to our data, 1α,25(OH)2D3 treatment caused small but significant decrease in the amount of secreted IL-12B when compared to vehicle treatment (Fig. 1E). However, it should be noted that although IL-12B levels slowly decreased, there was still a reasonable amount of secreted IL-12B present in the medium after hormone treatments of 48 h and 72 h suggesting that clearing of IL-12B from the medium is relatively slow.

In summary, in THP-1 cells IL-12B mRNA expression is down-regulated in a cyclical fashion after short-term 1α,25(OH)2D3 treatment and the down-regulation can be observed also at protein level suggesting that the IL-12B gene is a primary VDR target.

3.2. 1α,25(OH)2D3 treatment induces association of VDR with several genomic regions of the IL-12B gene

In order to locate putative VDREs within the IL-12B gene, we performed a ChIP scanning analysis with antibody against VDR (Fig. 2A). In total, 22 overlapping PCR primer pairs were used that covered the genomic region from −10,010 bp to +1938 bp relative to the IL-12B TSS. Our data suggest that VDR was recruited ligand-dependently to regions 5, 13, 17 and 20. The weaker VDR binding at adjacent regions is likely due to a flanking effect from active regions. Although all four regions recruited VDR ligand-dependently, their binding profiles were slightly different. Association with regions 5 and 17 reached a maximum 30 min after onset of ligand treatment, whereas strongest binding of VDR to regions 13 and 20 could be observed at time point 90 min.

An in silico analysis for hexameric nuclear receptor binding sites using the net-based program NUBIScan [22] revealed that region 5 contains a DR3- and a DR4-type element (REs 1 and 2), region 13 an ER8-type element (RE3), regions 16 and 17 two ER6-type elements (REs 4 and 5) and region 20 a DR3-type element RE6 (Fig. 2B). A sequence comparison between human, rhesus monkey, mouse and dog shows that RE2 is highly conserved across different species, REs 1, 3 and 6 are conserved excluding the mouse and REs 4 and 5 are specific for primates.

Taken together, within 10 kbp of the IL-12B promoter four VDR binding regions (5, 13, 17 and 20) were identified, which together contain six putative VDREs (REs 1–6).

3.3. Functionality of the putative VDREs of the IL-12B promoter

In order to determine, whether the VDR associated genomic regions of the IL-12B promoter contain any functional VDREs, we cloned them in front of the luciferase gene and performed reporter gene analysis (Fig. S3). Due to very low transfection efficiency of THP-1 cells, we had to shift the cellular system to SW-480 human colon adenocarcinoma cells. Although SW-480 cells also express TLR-4 (as a mediator of LPS effects), the basal IL-12B mRNA expression in these cells was clearly lower than in THP-1 cells (Fig. S2A). However, also in SW-480 cells 1α,25(OH)2D3 treatment caused cyclical repression of IL-12B mRNA expression (Fig. S2B). This suggests that SW-480 cells are suitable in depicting the functionality of putative VDREs in the regulation of the IL-12B gene.

The positive control of the reporter gene analysis, the proximal promoter of human CYP24 gene, was 7.4-fold inducible by 1α,25(OH)2D3 (Fig. S3). In contrast, the basal activities of reporter constructs containing REs 1 and 2 (region 5) or REs 4 and 5 (region Fig. 4. Ligand treatment leads to cyclical recruitment of repressive protein complexes to the IL-12B TSS and promoter. ChIP assays were performed using antibodies against VDR, RXR, HDAC3 or NCOR2/SMRT with 10 min intervals of 10 nM 1α,25(OH)2D3 treatment up to 100 min in LPS-treated THP-1 cells. The IL-12B TSS is indicated in black and the genomic regions containing RE2 and RE5 in red and blue, respectively. Two-tailed Student’s t-tests were performed to calculate p-values in reference to time point 0 ( *p<0.05, **p<0.01, ***p<0.001). In each panel, n is at least 3. Error bars indicate S.D.
were omitted from further investigations. The ability of the putative VDREs 1–5 (for sequences see Figs. 3A and S4A) within regions 5, 13 and 17 to associate with in vitro translated VDR–RXR heterodimers was examined using gel shift analysis. Compared with the positive control, the established VDRE of the rat Pit-1 gene (gaAGTTTCatgagAGTTCA) [15] (Fig. 3B), RE2 was able to bind VDR–RXR heterodimers even more efficiently (Fig. 3C), while the binding to RE5 was significantly weaker (Fig. 3D). Moreover, the binding of VDR–RXR heterodimers to the reference RE and REs 2 and 5 increased significantly in the presence of 1α,25(OH)2D3, but not statistically significantly in case of RE5 (Fig. 3E). REs 1, 3 and 4 showed no detectable binding of VDR–RXR heterodimers (Fig. S4B) and thus were omitted from further investigations.

In summary, in reporter gene analysis only the IL-12B promoter regions 5 and 17 showed significant, 1α,25(OH)2D3-dependent down-regulation. Only RE2 (region 5) and RE5 (region 17) showed in vitro VDR–RXR heterodimer binding.

3.4. Cyclical association of repressive protein complexes to 1α,25(OH)2D3 responsive regions of the IL-12B gene

To further investigate the role of REs 2 and 5 in the repression of IL-12B, we performed in THP-1 cells quantitative ChIP analysis of the effect of 1α,25(OH)2D3 on the recruitment of VDR, RXR, HDAC3 and NCOR2/SMRT to regions 5 and 17 in 10 min intervals over 80 min (Fig. 4). NCOR2/SMRT was chosen, since VDR–RXR heterodimers have been reported to associate preferably with NCOR2/SMRT than with NCOR1 [37,38]. The TSS region of the IL-12B gene served as a reference. VDR and RXR were enriched at all three regions at time periods 20–30 min and 60–80 min, which to a great extent was followed by the recruitment of HDAC3 and NCOR2/SMRT. Instead, no significant association of co-activator SRC1 could be observed within any regions in the absence or in the presence of hormone suggesting that the RE2 and RE5 mediated only repressive effects (Fig. S5).

Taken together, within a measuring period of 100 min two 1α,25(OH)2D3-dependent cycles of recruitment of VDR, RXR and repressive proteins HDAC and NCOR2/SMRT to regions 5 (RE2) and 17 (RE5) and the TSS were observed.

3.5. 1α,25(OH)2D3-dependent epigenetic changes within the IL-12B gene

The 1α,25(OH)2D3-dependent recruitment of repressive proteins, such as HDAC3 and NCOR2/SMRT, to regulatory regions of the IL-12B gene suggested that 1α,25(OH)2D3 treatment may cause a shift from a permissive to a less permissive chromatin status at these regions. In order to challenge this hypothesis, we performed quantitative ChIP analysis with antibodies against H4ac and H3K27me3 (Fig. 5). In a similar time course as in the previous experiment, the changes in chromatin status were assessed every 10 min after onset of ligand treatment. H4ac levels, which are marks of active chromatin, decreased significantly at region 5 and the TSS within the first 30–40 min and remained at low level for the remaining measuring period. In contrast, for the repressive mark, H3K27me3, the levels went up on the same two regions with a maximum reached after 70–90 min. The effects observed on region 17 showed for both epigenetic marks the same tendency as for region 5 and the TSS, but they were less prominent.

In summary, 1α,25(OH)2D3 treatment resulted in significant epigenetic changes leading to chromatin repression on region 5 (containing RE2) and the TSS, which are to a lower extent followed by that on region 17 (containing RE5).

4. Discussion

IL-12 is a pro-inflammatory cytokine that connects innate and adaptive immune responses by stimulating the development of Th1 cells and thus enhancing the immune response against intracellular pathogens. Therefore, IL-12 expression is vital for functional immune system and survival in vertebrates, but over-expression of IL-12 can also lead to serious diseases [5,6]. Another cytokine, IL-23, having IL-12 as a common subunit with IL-12, is necessary for T cell-dependent inflammation, and similarly the over-expression of IL-23 has been associated with several autoimmune diseases [33]. The mechanism of transcriptional activation of IL-12B has been extensively studied and a number of up-regulating elements have been located at the proximal promoter of IL-12B, but the molecular mechanisms of transcriptional repression of this gene are still unclear.

Inhibition of IL-12B mRNA expression by 1α,25(OH)2D3 treatment is well documented [9,23,24], but functional VDREs within IL-12B gene has not been reported before. In this study, we examined a genomic region from −10,010 bp to +1938 bp relative to IL-12B TSS using ChIP and in
silico analysis in order to locate the putative VDREs, via which the repressive effects of 1α,25(OH)2D3 are mediated. We found that the IL-12B promoter contains a DR3-type VDRE at position −488 to −460 bp (RE2) and an ER6-type element at position −6303 to −6274 bp (RE5). Both elements appeared to be functional in vitro and in vivo, although the more proximal element (RE2) was clearly more potent. Interestingly, Weinmann and co-workers [25] reported that murine IL-12B promoter contains positioned nucleosomes approximately 350 and 550 bp upstream of the TSS. We observed that this region is highly acetylated in the absence of 1α,25(OH)2D3, indicating that at this region chromatin is in the "open" form. Together these data suggest that the actively transcribed IL-12B gene, RE2 should be readily accessible to VDR binding.

Both RE2 and RE5 recruited ligand-dependently HDAC3 and NCoR2/SMRT, which are components of a large repressive protein complex. The activity of this complex leads to decreased histone acetylation (i.e. lower H4ac levels) and increased histone methylation (i.e. higher H3K9me3 levels) at different regions of the IL-12B gene including the TSS and regions 5 and 17 containing functional VDREs. H3K27me3 modification is linked to permanently silenced genes, for example those related to early phases of development. Interestingly, Roh and co-workers reported recently a group of gene promoters that have this modification simultaneously with activating histone modifications H3K9acK14ac and H3K4me3 in primary human T cells [39]. The activity of genes within above group appeared to be dependent on absolute and relative levels of activating and repressive histone modifications. According to our data, IL-12B belongs to the above group of genes and we assume that the level of activating versus repressing histone modifications is a possible explanation for the observed decrease in IL-12B mRNA synthesis. 1α,25(OH)2D3-dependent IL-12B repression has been suggested to be an indirect effect resulting in VDR association with NF-κB binding sites that would interfere with the binding of NF-κB components to proximal IL-12B promoter [9]. However, our data suggest a slightly different mechanism where repressive complexes that are recruited to VDREs upstream of the NF-κB site change the chromatin environment at proximal promoter to non-permissive form. This may prevent recruitment of NF-κB components to proximal IL-12B promoter and via that cause the repression.

The recruitment of VDR, RXR, HDAC3 and NCoR2/SMRT to the regions of RE2 and RE5 showed a cyclical pattern. In this respect, the mechanism of transcriptional regulation of IL-12B had similar features as transcriptional mechanisms of a number of both positively regulated VDR target genes, such as CSDKNA [26], CYP24 [27], and IGFBP3 [36], but also of negatively regulated genes, such as MYC [28] and IL-10 [21]. The cyclic behavior at the IL-12B promoter is mirrored by the mRNA levels, which also showed a clear periodicity.

Importantly, the 1α,25(OH)2D3-dependent repression of IL-12B expression could also be observed in primary monocytes. The repression of the IL-12B gene was clearly due to 1α,25(OH)2D3 treatment, since silencing of VDR by siRNA prevented the repression. In order to model inflammation, THP-1 cells were primed with LPS before 1α,25(OH)2D3 treatment and LPS was also present throughout expression profiling. Thus, the IL-12B gene was constantly activated and 1α,25(OH)2D3-dependent, cyclic recruitment of repressive protein complexes resulted in temporal stalls in mRNA synthesis. This could be observed as periodic decreases of IL-12B mRNA levels due to degradation of mRNA. This suggests that at early state of inflammation, 1α,25(OH)2D3 appears to control IL-12B mRNA synthesis to prevent over-expression of IL-12. Interestingly, the main down-regulating agent for IL-12B expression, the cytokine IL-10, is also under direct control of 1α,25(OH)2D3 [21]. However, the 1α,25(OH)2D3-dependent up-regulation of IL-10 takes place after longer ligand treatment times, such as 24 h [17,21,25,30]. Thus, 1α,25(OH)2D3 appears to have dual effect on the expression of IL-12B. At the beginning of inflammation, the primary effect of 1α,25(OH)2D3 is cyclical down-regulation of IL-12B expression, in order to keep the magnitude of immune response on acceptable level, and later a secondary effect via IL-10 occurs that turns the IL-12B gene off. This would prevent tissue damage resulting from prolonged inflammation.

In conclusion, this study reveals two VDREs at position −488 to −460 bp and −6303 to −6274 bp upstream of the IL-12B TSS, via which the primary, repressive effects of 1α,25(OH)2D3 are mediated during the first hours of inflammation. The primary effects consist of cyclic recruitment of repressive protein complexes to the above elements, which cause repressive bursts that slow down IL-12B mRNA synthesis thus preventing over-expression of IL-12 and IL-23.

Acknowledgements

We would like to thank Mrs. Hanna Eskelin for her technical assistance and help in cell culture and Martine Schmitz for her assistance in human monocye isolation during the execution of this work. The buffy coats were provided by Blood Transfer Centre, Luxembourgish Red Cross. This work was supported by the Academy of Finland, the Biocenter Finland and the Nordic Centre of Excellence in Systems Biology of Diet Intervention (SYSDIET).

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbamcr.2011.01.037.

References


### SUPPLEMENTARY TABLES

**Table S1.** PCR primers used in ChIP scanning analysis. Sequences and location relative to the *IL-12p40* TSS (+1) are shown.

<table>
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<tr>
<th>Location of the amplicon</th>
<th>Primer sequences (5’-3’)</th>
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<td>1 1468 to 1938</td>
<td>TAATCATCAAGACTCTTCAGAC</td>
</tr>
<tr>
<td></td>
<td>AGGAAGAGTGAAGGGAACAAT</td>
</tr>
<tr>
<td>2 1011 to 1488</td>
<td>GAAGGAGGGGAGCAATAGTF</td>
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<tr>
<td></td>
<td>GTCTGAGAGCTTGTGATGATTA</td>
</tr>
<tr>
<td>3 508 to 1030</td>
<td>TTGTGATGCTTGTGGTGCTC</td>
</tr>
<tr>
<td></td>
<td>ACTATTTTGCTCCCCCTCCTTC</td>
</tr>
<tr>
<td>4 -36 to 527</td>
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<td></td>
<td>GACGACCAAGACCACATCACA</td>
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<tr>
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Table S2. PCR primers and hydrolysis probes used in quantitative ChIP assays. Sequences and location relative to the IL-12 p40 TSS (+1) are shown.

<table>
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<tr>
<th>Location of the amplicon</th>
<th>Primer sequences (5’-3’)</th>
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<table>
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<td>RE2 (-4343 to -4319)</td>
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<td>RE3 (-6387 to -6360)</td>
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Supplementary figure legends

**Fig. S1.** Basal mRNA expression of *IL-12B* in the presence and absence of LPS in THP-1 cells as measured by quantitative PCR (A). Relative mRNA expression of *TLR-4* was measured by quantitative PCR in LPS pre-treated THP-1 cells after stimulation with 10 nM 1α,25(OH)2D₃ for indicated time periods (B). Relative mRNA expression of *TLR-4* in THP-1 cells pre-treated with 10 nM 1α,25(OH)2D₃ for indicated time periods prior to LPS treatment (C). Relative mRNA expression of *IL-12B* in THP-1 cells treated for 6 h with 10 nM 1α,25(OH)2D₃ before or after LPS treatments (D). Relative mRNA expression of *IL-12B* in THP-1 cells pre-treated for 270 min, 360 min or 450 min with 10 nM 1α,25(OH)2D₃ before LPS treatments (E). The results were normalized to the housekeeping gene *RPLP0*. Two-tailed Student's t-tests were performed to calculate p-values in reference to solvent treated samples (* p < 0.05). Error bars indicate S.D.

**Fig. S2.** Basal mRNA expression of *IL-12B* in SW-480 and THP-1 cells as measured by quantitative PCR (A). Relative mRNA expression of *IL-12B* was measured by quantitative PCR in SW-480 cells for indicated time periods after stimulation with 10 nM 1α,25(OH)2D₃ (B). The results were normalized to the housekeeping gene *RPLP0*. Two-tailed Student's t-tests were performed to calculate p-values in reference to solvent treated samples (* p < 0.05, *** p < 0.001). Error bars indicate S.D.

**Fig. S3.** Functionality of putative VDREs at the *IL-12B* promoter. The upstream regions of the human *IL-12B* gene were cloned in front of thymidine kinase (*tk*) promoter driving the luciferase gene. The proximal promoter of the human *CYP24* gene containing a cluster of
DR3-type VDREs was used as a positive control and the empty reporter vector as a negative control. Luciferase activity was determined from SW-480 cells after 16 h 1α,25(OH)2D3 treatment. The fold repressions or induction are indicated above the columns. Two-tailed Student's t-tests were performed to calculate p-values in reference to solvent treated samples (* p < 0.05, ** p < 0.01, *** p < 0.001). In each bar, n is at least 3. Error bars indicate S.D.

**Fig. S4.** VDR-RXR heterodimer association with the putative VDREs at the *IL-12B* promoter. Schematic presentation of locations and sequences of RE1, RE3 and RE4 (A). Gel shift analysis was used to study the binding of *in vitro* translated VDR and RXR to RE1, RE3 or RE4 in the presence and absence of 1α,25(OH)2D3 (B). Representative gels are shown. NS indicates non-specific complexes.

**Fig. S5.** Putative VDREs do not recruit co-activator SRC-1. ChIP assays were performed using antibody against SRC-1 with 20 min intervals of 10 nM 1α,25(OH)2D3 treatment up to 100 min in LPS-treated THP-1 cells. The *IL-12B* TSS is indicated in black and the genomic regions containing RE2 and RE5 in red and blue, respectively. Two-tailed Student's t-tests were performed to calculate p-values in reference to time point 0 (* p < 0.05, ** p < 0.01, *** p < 0.001). In each panel, n is at least 3. Error bars indicate S.D.
Figure S1. Expression assays

A

B

C

D

E

Basal IL-12B mRNA expression

1α,25(OH)₂D₃ treatment time (min)

Relative TLR-4 mRNA expression (fold)

1α,25(OH)₂D₃ treatment time (min)

Relative TLR-4 mRNA expression (fold)

1α,25(OH)₂D₃ treatment time (min)

Relative IL-12B mRNA expression (fold)

1α,25(OH)₂D₃ treatment time (min)
Figure S2. Expression assays

A

mRNA expression levels relative to RPLP0

SW-480  THP-1

B

Relative mRNA expression

1α,25(OH)2D3 treatment time (min)
Figure S3. Reporter gene analysis
Figure S4. Gel shift assay

A

-375\text{CCTCCGACTCAAGTACAGCCCTACCTTGGT}^{404}

-6227\text{CAGTCCCTCTGAGAAGTCCCATCCAGTTCTTT}^{656}

\text{IL-12p40}

B

<table>
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<tr>
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<th>RE4</th>
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<td>- - - - - - +</td>
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VDR-RXR

NS

NS

Free probe
Figure S5. ChIP assays