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Mechanism of 1α,25-dihydroxyvitamin D_3-dependent repression of *interleukin-12B*

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ABSTRACT

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 D_3 ($1\alpha,25(OH)_2D_3$), 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 $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$. 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 decreased histone 4 acetylation and increased histone 3 trimethylation at the *IL-12B* promoter and its TSS. We suggest that these repressive epigenetic changes eventually cause down-regulation of *IL-12* expression.

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 D_3 ($1\alpha,25(OH)_2D_3$) that has been reported to inhibit IL-12 production in activated macrophages [7–9].

The seco-steroid hormone $1\alpha,25(OH)_2D_3$ is the biologically most active metabolite of vitamin D_3 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]. $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$, which further underlines the potential of $1\alpha,25(OH)_2D_3$ in the regulation of immune responses [13].

Abbreviations: $1\alpha,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; ChIP, chromatin immunoprecipitation; DR, direct repeat; ER, everted repeat; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; HDAC, histone deacetylase; H3K27me3, histone 3 trimethylated at lysine 27; H4ac, acetylated histone 4; IL, interleukin; LPS, lipopolysaccharide; NCOR, nuclear receptor co-repressor; RXR, retinoid X receptor; SMRT, silencing mediator for retinoid and thyroid hormone receptors; Th, T-helper; TLR, toll-like receptor; VDR, vitamin D receptor; VDRE, vitamin D response element

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Binding of 1α,25(OH)$_2$D$_3$ 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 RGKTCA (R = A or G, K = G or T) with three or four intervening nucleotides (DR3 and DR4) or by everted repeats (ERs) with six to nine spacing nucleotides (ER6 to ER9) [14–16]. Although a suppressing effect of 1α,25(OH)$_2$D$_3$ 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)$_2$D$_3$ 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)$_2$D$_3$-dependent *IL-12B* repression in lipopolysaccharide (LPS)-activated THP-1 human monocytic cells. Our data suggest that the 1α,25(OH)$_2$D$_3$-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 cyclic 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% CO_2 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, 2 mM L-glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin. Prior to RNA or chromatin extraction, the cells were treated with 100 ng/ml LPS for 24 h and then exposed to either solvent (ethanol, 0.1% final concentration) or 10 nM 1α,25(OH)$_2$D$_3$ (Sigma-Aldrich).

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 Leucosep tubes (Greiner bio-one, Wemmel, Belgium). The number of PBMC 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 FACSCantoII 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 × 10^6 cells/ml and incubated over night in a humidified 95% air/5% CO_2 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* (4,333,761 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 $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct = \Delta Ct_{(1\alpha,25(OH)2D3)} - \Delta Ct_{(EtOH)}$, and $\Delta Ct = Ct_{(IL-12B)} - Ct_{(RPLP0)}$. 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. 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)$_2$D$_3$ 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 using 12% SDS polyacrylamide gel electrophoresis. The blotted proteins were detected by using IR800 fluorescence labeled secondary antibodies (Thermo Fisher Scientific, Waltham, MA, USA) and Odyssey reader (Li-Cor Biosciences, Lincoln, NE, USA).

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 T$_7$/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 [^{32}P] 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-C), 5% glycerol). 1 ng of [^{32}P]-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 × 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).

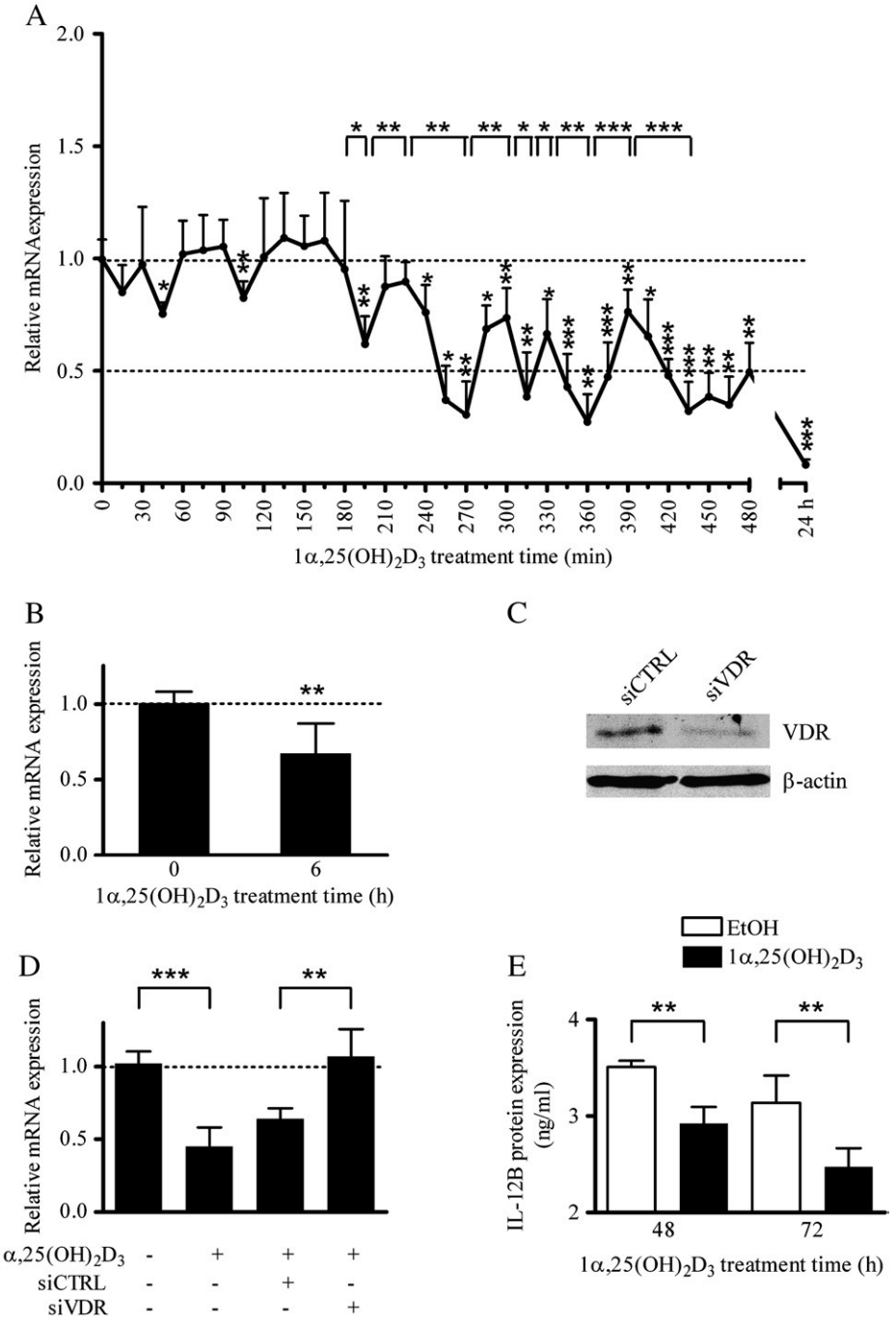

Fig. 1. Expression profiling of the human *IL-12B* gene. Relative mRNA expression of *IL-12B* was measured by quantitative PCR in LPS-treated THP-1 cells after stimulation with 10 nM $1\alpha,25(OH)_2D_3$ for indicated time periods (A) and for primary monocytes after 6 h treatment with 100 nM $1\alpha,25(OH)_2D_3$ (B). (C) siRNA silencing of VDR at protein level was verified by Western blotting using whole-cell extracts from LPS-treated THP-1 cells. (D) Relative mRNA expression of *IL-12B* was measured after 6 h of 10 nM $1\alpha,25(OH)_2D_3$ treatment after silencing of VDR. The results were normalized to the housekeeping gene *RPLP0*. (E) *IL-12B* expression at protein level was determined from cell culturing medium using ELISA. Two-tailed Student's t-tests were performed to calculate p-values in reference to vehicle treatment (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). In each panel, *n* is at least 3. Error bars indicate S.D.

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, $1\alpha,25(OH)_2D_3$ 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

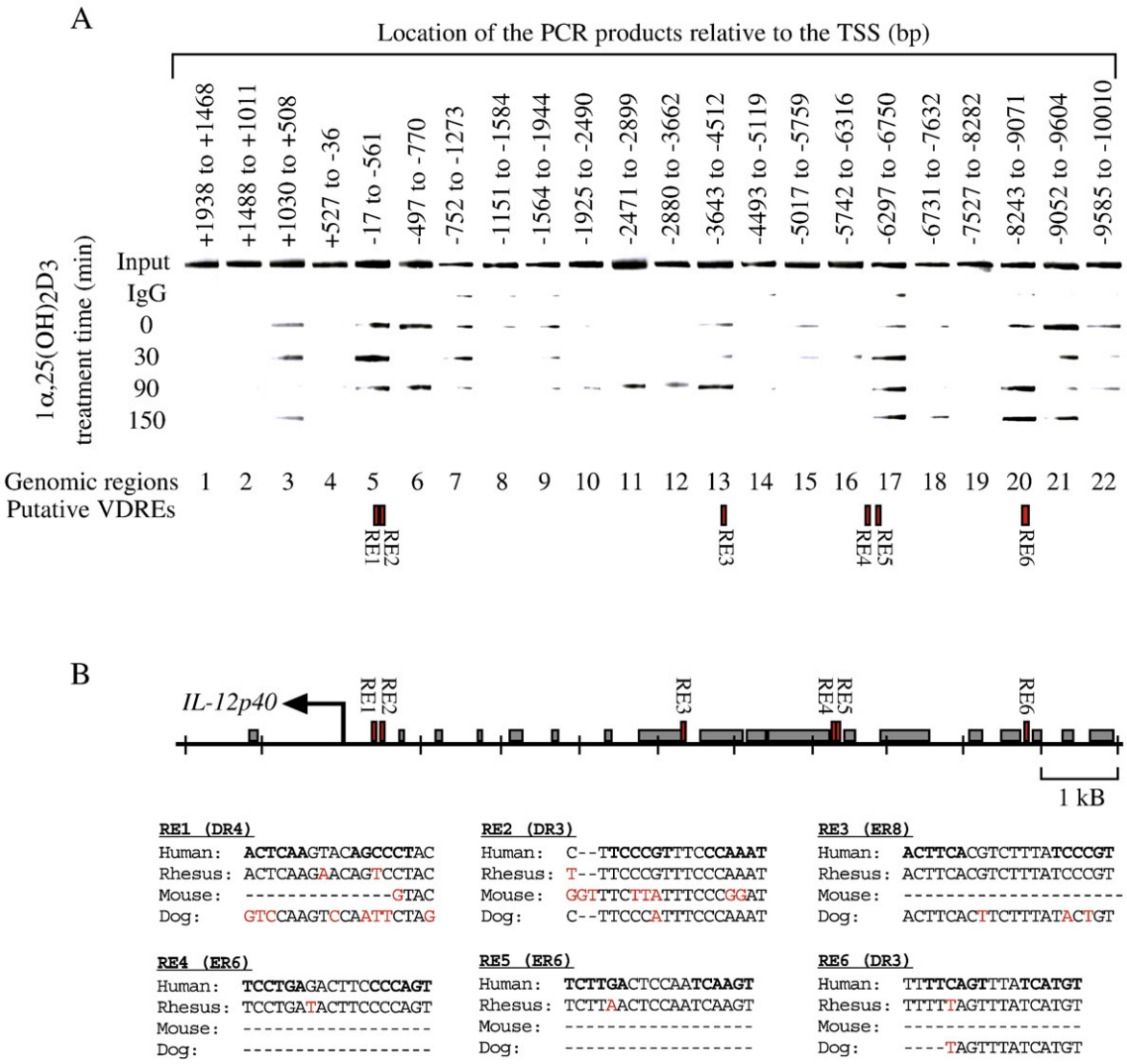

Fig. 2. ChIP scanning and *in silico* analysis of the *IL-12B* promoter. The region spanning the genomic region from −10,010 to +1938 bp relative to the *IL-12B* TSS was studied using both ChIP with antibody against VDR (A) and *in silico* analysis (B) to reveal putative VDREs. Repetitive sequences are indicated by grey boxes and putative VDREs (RE1–RE6) by red boxes. A detailed sequence comparison between human, rhesus monkey, mouse and dog sequences is shown for the studied elements.

(ethanol, 0.1% final concentration) or 100 nM 1α,25(OH)$_2$D$_3$ for 48 and 72 h, after which the cell culture supernatants containing secreted IL-12B protein were collected. The effect of 1α,25(OH)$_2$D$_3$ 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. Non-specific IgG (12–370) and antibodies against histone H4 acetylated at K5, K8, K12 and K16 (H4Ac, 06–598) and 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, Liege, 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 $2^{-(\Delta Ct)} * 100_{(\text{specific antibody})} / 2^{-(\Delta Ct)} * 100_{(\text{non-specific IgG})}$, where ΔCt is $Ct_{(\text{immunoprecipitated DNA})} - Ct_{(\text{input})}$ and Ct is the cycle at which the threshold line is crossed.

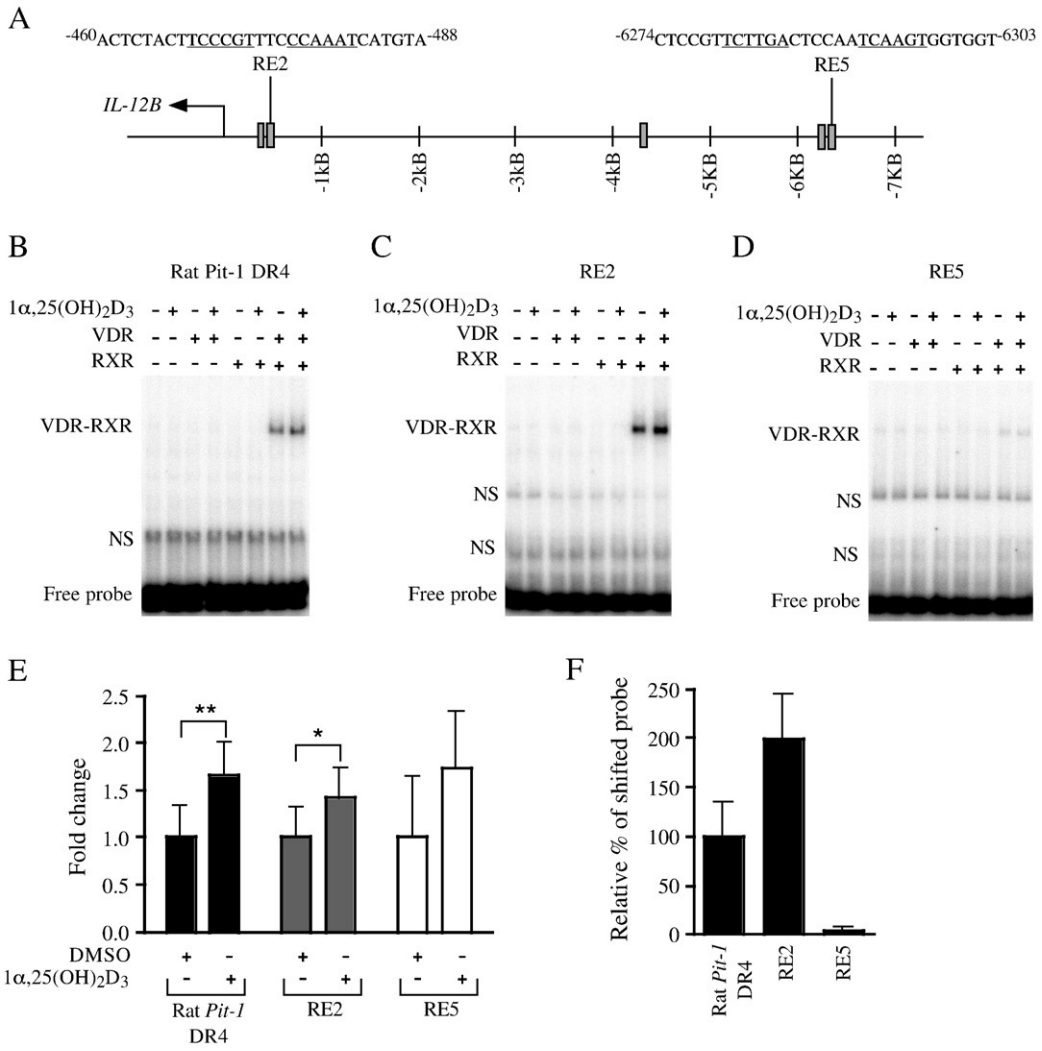

Fig. 3. The ability of VDR–RXR heterodimer to associate with the putative VDREs. (A) Schematic presentation of locations and sequences of RE2 and RE5. Gel shift analysis was used to study the binding of *in vitro* translated VDR and RXR to DR4-type element of rat *Pit-1* (B) and RE2 (C) or RE5 (D) in the presence and absence of $1\alpha,25(OH)_2D_3$. NS indicates non-specific complexes. Representative gels are shown. (E) $1\alpha,25(OH)_2D_3$ treatment enhanced the binding of VDR–RXR to the putative VDREs. (F) Percentage of protein-complexed DNA. Two-tailed Student's t-tests were performed to calculate p-values in reference to solvent treated samples (* $p<0.05$, ** $p<0.01$). In each bar, *n* is at least 3. Error bars indicate S.D.

3. Results

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

The effect of $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ treatment (Fig. 1A). According to our data, $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$-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 $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ 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 to the repressive effect of 1α,25(OH)$_2$D$_3$ on *IL-12B* expression, whereas VDR-specific siRNA completely abolished it (Fig. 1D).

The effect of 1α,25(OH)$_2$D$_3$ on the expression of *IL-12B* at protein level was determined using ELISA. According to our data, 1α,25(OH)$_2$D$_3$ 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)$_2$D$_3$ treatment and the down-regulation can be observed also at protein level suggesting that the *IL-12B* gene is a primary VDR target.

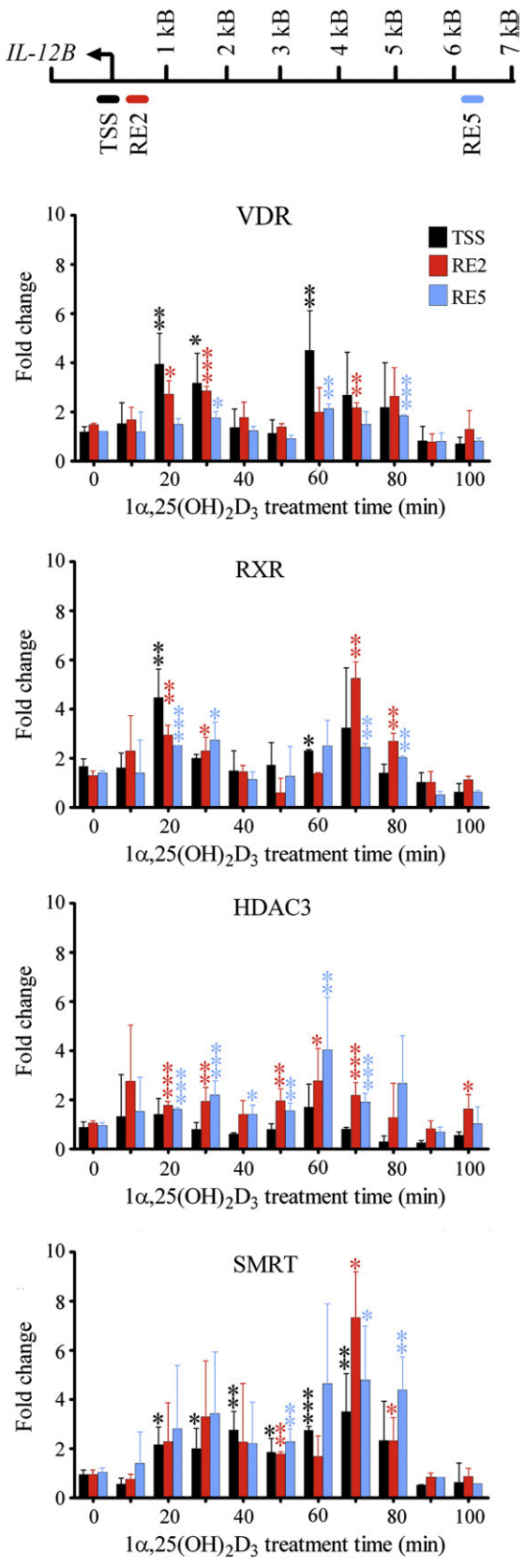

Fig. 4. Ligand treatment leads to cyclical recruitment of repressive protein complexes to the *IL-12B* TSS and promoter. ChIP assays were performed using antibodies against VDR, RXR, HDAC3 or NCOR2/SMRT with 10 min intervals of 10 nM 1α,25(OH)$_2$D$_3$ treatment up to 100 min in LPS-treated THP-1 cells. 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.

3.2. 1α,25(OH)$_2$D$_3$ 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 recptor 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 kB 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)$_2$D$_3$ 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)$_2$D$_3$ (Fig. S3). In contrast, the basal activities of reporter constructs containing REs 1 and 2 (region 5) or REs 4 and 5 (region

17) were reduced by 1.4- and 1.5-fold, respectively. Interestingly, region 17 mediated a 3-fold higher basal activity than the empty cloning vector. This could be a sign that in the absence of 1α,25$(OH)_2D_3$ this genomic region may have an activating effect on *IL-12B* expression. Region 13 (containing RE3) also caused repression in response to 1α,25$(OH)_2D_3$ treatment although not significant. The construct containing region 20 showed no significant response to 1α,25$(OH)_2D_3$ and thus the putative element RE6 was omitted from further studies.

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 (gaAGTTCAtgagAGTTCA) [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$(OH)_2D_3$, 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$(OH)_2D_3$-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$(OH)_2D_3$ 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$(OH)_2D_3$ on the recruitment of VDR, RXR, HDAC3 and NCOR2/SMRT to regions 5 and 17 in 10 min intervals over 100 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$(OH)_2D_3$-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$(OH)_2D_3$-dependent epigenetic changes within the IL-12B gene

The 1α,25$(OH)_2D_3$-dependent recruitment of repressive proteins, such as HDAC3 and NCOR2/SMRT, to regulatory regions of the *IL-12B* gene suggested that 1α,25$(OH)_2D_3$ 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.

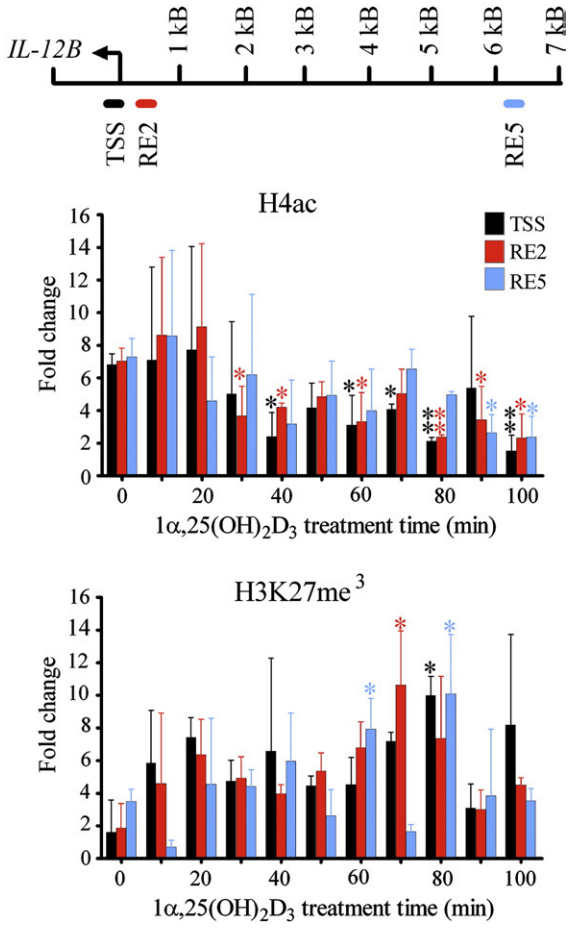

Fig. 5. Ligand-dependent epigenetic changes at the *IL-12B* promoter and the TSS. ChIP assays were performed using antibodies against H3K27me3 and H4ac with 10 min intervals of 10 nM 1α,25$(OH)_2D_3$ treatment up to 100 min in LPS-treated THP-1 cells. The *IL-12B* TSS is indicated in black and the genomic 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 (* $p<0.05$, ** $p<0.01$). In each panel, *n* is at least 3. Error bars indicate S.D.

In summary, 1α,25$(OH)_2D_3$ 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-12B 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$(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ indicating that at this region chromatin is in the "open" form. Together these data suggest that in 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 H3K27me3 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.

$1\alpha,25(OH)_2D_3$-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 *CDKN1A* [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 $1\alpha,25(OH)_2D_3$-dependent repression of *IL-12B* expression could also be observed in primary monocytes. The repression of the *IL-12B* gene was clearly due to $1\alpha,25(OH)_2D_3$ treatment, since silencing of VDR by siRNA prevented the repression. In order to model inflammation, THP-1 cells were primed with LPS before $1\alpha,25(OH)_2D_3$ treatment and LPS was also present throughout expression profiling. Thus, the *IL-12B* gene was constantly activated and $1\alpha,25(OH)_2D_3$-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, $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ [21]. However, the $1\alpha,25(OH)_2D_3$-dependent up-regulation of *IL-10* takes place after longer ligand treatment times, such as 24 h [17,21,29,30]. Thus, $1\alpha,25(OH)_2D_3$ appears to have dual effect on the expression of *IL-12B*. At the beginning of inflammation, the primary effect of $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ 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.

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Appendix A. Supplementary data

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

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