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Research Article

The Orosomucoid 1 protein is involved in the vitamin D – mediated macrophage de-activation process ☆

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ABSTRACT

Orosomucoid 1 (ORM1), also named Alpha 1 acid glycoprotein A (AGP-A), is an abundant plasma protein characterized by anti-inflammatory and immune-modulating properties. The present study was designed to identify a possible correlation between ORM1 and Vitamin D3 (1,25(OH)2D3), a hormone exerting a widespread effect on cell proliferation, differentiation and regulation of the immune system. In particular, the data described here indicated that ORM1 is a 1,25(OH)2D3 primary response gene, characterized by the presence of a VDRE element inside the 1 kb sequence of its proximal promoter region. This finding was demonstrated with gene expression studies, Chromatin Immunoprecipitation and luciferase transactivation experiments and confirmed by VDR full length and dominant negative over-expression. In addition, several experiments carried out in human normal monocytes demonstrated that the 1,25(OH)2D3 – VDR – ORM1 pathway plays a functional role inside the macrophage de-activation process and that ORM1 may be considered as a signaling molecule involved in the maintenance of tissue homeostasis and remodeling.

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Introduction

1α, 25 dihydroxyvitamin D3 is the metabolic active form of Vitamin D_3 [1,2]. Initially synthesized in the skin from 7-dehydrocholesterol, it is subsequently processed in the liver and then in the kidneys in order to obtain the most physiologically active VD_3 molecule (1,25(OH)2D3) [1,2]. The biological activity of 1,25(OH)2D3 is mediated by its receptor VDR, belonging to the

Abbreviations: AF2, Activation function domain 2; AGP-A, Alpha 1 acid glycoprotein A; AKNA1, AT-Hook Transcription Factor 1; CAMP, Cathelicidin Antimicrobial Peptide; CB, Cord Blood; COL27A1, Collagen, Type XXVII, alpha-1; MMP9, Matrix Metalloproteinase 9; MRC1, Mannose Receptor, C-type1; ORM1, Orosomucoid 1; ORM2, Orosomucoid 2; QRT-PCR, Quantitative Real Time PCR; RT, Room Temperature; siRNA, small interference RNA; SLR, Signal Log Ratio; TF, Transcription Factor; TSS, Transcriptional Start Site; 1,25(OH)2D3, 1α, 25 dihydroxyvitamin D3; VDRE, Vitamin D Responsive Element

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nuclear hormone receptor family [2,3]. Accordingly, the protein structure of VDR is composed of different domains exhibiting specific functions, such as DNA binding (DBD), ligand binding (LBD) and trans-activation activity (AF2) [3]. VDR is able to regulate gene expression as a ligand-activated transcription factor, i.e. upon 1,25(OH)2D3 binding and RXR dimerization events [3,4]. The VDR/RXR complex recognizes specific 1,25(OH)2D3 Responsive Elements (VDREs) inside the promoter regions of primary response genes, resulting in either gene activation or repression [3–5]. VDRE elements are composed of tandem motifs having the consensus PuG(G/T)TCA, which are often arranged as direct repeats separated by 3 nucleotides (DR3-Type) [1,3]. However, other types of VDREs, such as direct repeats with 4 spacing nucleotides (DR4-Type) or everted repeats with a spacer of 6–9 nucleotides (ER6-9) have been described [3,6,7]. This process is normally indicated as the "VDR genomic pathway", in order to discern it from the "non genomic pathway" that is based on a putative 1,25(OH)2D3 surface receptor, followed by the activation of several intracellular transduction signaling cascades [3,8–10]. As many other vitamins, 1,25(OH)2D3 is involved in the regulation of a large number of cellular processes, including hematopoietic differentiation and immune response [11,12]. In particular, on the basis of experimental data also obtained in our laboratory, it is well-known that 1,25(OH)2D3 is able to promote monocyte differentiation both in leukemic myeloid cell lines, such as HL60, U937 and THP1 cells [13–15], and in human CD34+ hematopoietic progenitors [11]. Further investigations performed in these cellular contexts demonstrated the presence of VDRE elements in the 5′ flanking region of several genes related to monocyte differentiation and biological functions, providing the molecular mechanism underlying 1,25(OH)2D3 cellular effects [5,16–19]. In this regard, our research group reported that *Hox-A10*, a well known 1,25(OH)2D3 primary response gene [17], is in turn able to induce the expression of a number of monocyte differentiation and activation markers [15], including in this gene list also the *MafB* transcription factor, already indicated in an our previous paper as a key regulator of human normal monocytopoiesis [20].

1,25(OH)2D3 is also an important modulator of the immune response since it exerts its effects on several cell types involved in the regulation of innate and acquired immunity, respectively macrophage/dendritic cells and T/B lymphocytes [12,21]. In particular, it has been demonstrated, both in murine and in human macrophages, that 1,25(OH)2D3 displays a strong anti-inflammatory properties, being able to inhibit the production of several cytokines and interleukins (ILs), as TNFa, IL-10, IL-2, IL-12B [22,23]. This 1,25(OH)2D3 ability is the result of an indirect regulation mechanisms, mediated by NFKB and other transcription factors [24,25], but is also due to a direct 1,25(OH)2D3 transcriptional control through the binding to VDRE elements contained in the promoter regions of the mentioned genes [22,23]. Globally, this background supports the important role played by 1,25(OH)2D3 both in monocyte differentiation and in macrophage functional polarization, and, more precisely, in the anti-inflammatory and de-activating pathway mediated by resident macrophages. Inside these processes, we are investigating the biological function of Orosomucoid 1 (ORM1) protein, also named alpha 1 acid glycoprotein A (AGPA), and its relationship with the nuclear hormone under consideration.

ORM1 is an abundant plasma protein, composed of a single polypeptide chain characterized by a high level of glycosylation [26,27]. In human, two isoforms have been described, named *ORM1* and *ORM2*, differing each other for 22 base substitutions [26,27]. ORM1 is implicated in two main different functions: (1) as a lipocalin family member, it exerts a carrier activity especially for drug and lipid molecules; (2) being an acute phase protein, ORM1 is also expressed and secreted in the plasma under stressful signals, such as tissue injury, infection or inflammation, displaying an immune-modulatory activity [28]. In fact, ORM1 is considered a natural anti-inflammatory agent since it is able to inhibit neutrophils activation and complement activity, to promote IL1RA secretion by murine macrophages, and to modulate pro-inflammatory cytokines production from human monocytes [28]. In addition, extra-hepatic *ORM1* expression has been described, demonstrating that THP1 monoblastic cells and monocytes [26] as well as alveolar macrophages [29] are able to synthesize and secrete ORM1 protein. Based on this premise, we propose to investigate the existence of a transcriptional and functional correlation between 1,25(OH)2D3 nuclear hormone, its receptor VDR and orosomucoid proteins. To this aim, through transcriptome analysis, over-expression and silencing experiments, ChIP and luciferase assays, our study was able to demonstrate that *ORM1* is a 1,25(OH)2D3 primary response gene and that this transcriptional regulation is functionally relevant in the anti-inflammatory response mediated by Vitamin D.

Materials and methods

Hematopoietic cell lines and primary cells

HL60 and U937 cell lines were obtained from ATCC (Rockville, MD) and cultured in RPMI1640 medium (Euroclone, Devon, UK), supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) (Biowhittaker, Walkersville, MD) and 1 mM L-Glutamine (Euroclone). Human CD34+ hematopoietic stem/progenitor cells, myeloblast and monoblast precursors, and terminally differentiated monocytes were obtained and maintained in liquid culture as already described [11,15,20,30]. Treatment with 1,25(OH)2D3 (Hoffman-Laroche, Basel, Switzerland) was carried out at various times and concentrations depending on the experimental settings. In details, hematopoietic cell lines were stimulated with the high concentration of 10^{-7} M Vitamin D3, due to their low responsivity to compounds inducing monocyte-macrophage differentiation; on the contrary, primary cell populations were treated with a lower dose of the nuclear hormone, equal to 5×10^{-8} M. In a set of time-course experiments, normal human monocytes were exposed to the physiological dose of 250 μg/ml [27,31] of ORM1 protein, purified from human plasma and provided by Sigma-Aldrich (St. Louise, MO, USA).

RNA purification and quantitative RT-PCR

Total cellular RNA was isolated by means of Qiagen total RNA purification kits as recommended by the manufacturer (Qiagen, Valencia, CA). RNA integrity and concentration were then verified by using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Total RNAs (100 ng) were reverse transcribed using High Capacity cDNA Archive kit (Applied Biosystems) and Quantitative Real Time PCR (QRT-PCR) was performed with an ABI PRISM 7900 sequence detection system

(Applied Biosystems) using primers and probes provided by Applied Biosystems. Each cDNA samples was run in triplicate for gene targets and *GAPDH*, used as endogenous control, following cycle conditions already described [30]. Statistical analysis of QRT-PCR results was obtained using the ($2^{-\Delta\Delta Ct}$) method which calculates relative changes in gene expression of the target gene normalized to the endogenous control and relative to a calibrator sample [32].

Biotin labeled transcription, Genechip hybridization and microarray data analysis

Total cellular RNAs were collected, as described above, from 5×10^6 of U937 cells untreated or stimulated with a 10^{-7} M concentration of 1,25(OH)2D3 for 72 h. To give reliability to the data, three independent experiments were performed. RNA pools of primary myeloblasts and monoblastst precursors, untreated or stimulated with a 5×10^{-8} M 1,25(OH)2D3 concentration for 7 days, were obtained from four independent experiments. Biotin-labeled target sequences were synthesized according to the manufacturer guidelines (Affymetrix, Santa Clara, CA) and used to hybridize Affymetrix HG-U133A GeneChip arrays. Images obtained by scanning chips were processed using the GeneChip Operating Software (GCOS) as already described [15,20,30,33].

Retroviral vector construction and Packaging

A VDR full length and a truncated version lacking the transactivation domain AF2, named respectively VDR_{FL} and VDRΔAF2 cDNAs, were generated by RT-PCR performed on total RNA extracted from human normal monocytes and using a proof-reading thermostable DNA polymerase and the following primers: VDR_{FL} DP, 5′CAGAAGCCTTTGGGTCTGAAGTGTC-3′; VDR_{FL} RP, 5′–ACGTGGCCCTGGAGGAGCAGCC-3′; VDRΔAF2 DP: 5′–GCCCCCTGCTC CTTCAGCCATGGAGGCAATGGCGGC-3′; VDRΔAF2 RP: 5′–GCACTCAGGCTGGGCGGCCGCTCAGCGGTACTGCTT-3′. The amplified fragments were then inserted in the pCR3.1 T/A cloning vector (Invitrogen, Carlsbad, CA, USA) generating the pCR3.1VDR_{FL} and pCR3.1VDRΔAF2 plasmids, where the investigated transgenes were fully sequenced to exclude polymerase-induced mutations. Subsequently, VDR_{FL} and VDRΔAF2 cDNAs were EcoRI excised and cloned in the EcoRI site of LXIΔN retroviral vector resulting in the construction of LVDR_{FL}IΔN and LVDRΔAF2IΔN retroviral vectors respectively. The LXIΔN basal backbone used to obtain the mentioned constructs is a bicistronic vector assuring the cloned cDNAs expression under the control of the viral LTR. A truncated version of the Nerve Growth Factor receptor (NGFr) is used as immunophenotypic marker in order to monitor the transfection efficiency and to purify the transduced cells.

Packaging lines for all the described constructs were generated by transinfection in the ecotropic GP+E-86 and amphotropic GP+envAm12 cells, as described. Viral titers were assessed by flow cytometry analysis of ΔLNGFR expression percentage upon infection of U937 cells.

Cell transduction and immune-phenotypic analysis

U937 monoblastic cells were transduced by 2–3 cycles of infection (4 h each) with viral supernatant in the presence of polybrene (8 μg/ml). Transduction efficiency of U937 cells was monitored by flow cytometry analysis (Epics XL, Coulter) of ΔLNGFR expression using a murine anti-human p75-NGFR monoclonal antibody (moAb) conjugated with biotin (BD Biosciences, San Diego, CA) and streptavidin conjugated to R – Phycoerythrin – Cy5 (RPE-Cy5) (DAKO A/S, Copenhagen, Denmark). Transduced U937 cells were then sorted by an immunomagnetic procedure using mouse anti-human p75 – NGFR moAb and tiny FACS-compatible magnetic nanoparticles in a column-free magnetic system [EasySep™ "Do-It-Your Self" Selection Kit; (Stem cells Technologies, Vancouver, BC, Canada)], following the manufacturer guidelines. The extent of monocyte differentiation was monitored using a mouse anti-CD14 moAb conjugated to fluorescein isothiocyanate and a mouse PE – conjugated anti-CD11b moAb (both from BD Biosciences). Antibodies incubations were carried out as described [11]. Morphological analysis of transduced and differentiated cells was performed through May Grunwal-Giemsa staining of cytocentrifuged speciments.

Protein extract preparation and western blot analysis

Nuclear and cytoplasmic protein extracts were obtained as previously reported [14] with minor modifications. To evaluate VDR_{FL} and VDRΔAF2 protein expression in transduced U937 cells, 30 μg of nuclear extracts of the analyzed cell populations were loaded onto 12.5% SDS-polyacrylamide gel electrophoresis and blotted as described. Membranes were pre-blocked in blocking solution, supplemented with 4 mg/ml normal serum and 10% blocking reagent (Roche Diagnostics, Mannheim, Germany), for 1 h at room temperature (RT) and then incubated with a 1: 500 dilution of the primary 9A7γ rat anti-VDR moAb (Enzo Life Science), followed by a 1 h incubation at RT with a secondary goat anti-rat immunoglobulin antibody conjugated to horse-radish peroxidise (HRP) at 1: 5000 concentration (Santa Cruz Biotechnology, Inc., Santa Cruz, California). To analyze ORM1 and ORM2 proteins expression in 1,25(OH)2D3 treated U937 and HL60 cells, 30 μg of cytoplasmic extracts were loaded on 10% SDS-polyacrylamide gel electrophoresis, followed by electroblotting to nitrocellulose sheets. Blotted membranes were pre-blocked for 1 h at RT in blocking solution, composed of 5% nonfat milk (Regilat, Saint-Martin-Belle-Roche, France) in 0.1% TBST. Mouse moAb to ORM1 (Abcam, Cambridge, UK) was incubated in blocking solution diluted 1:100 for 1 h at RT whereas ORM2 protein detection was obtained by using primary mouse moAb (Abcam) diluted in 0.1% TBST containing 3% bovine serum albumin (Sigma-Aldrich) and 1% nonfat milk (Regilat). Finally, membranes were incubated, for 1 h at RT, with a secondary goat anti mouse-HRP Ab (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at concentration of 1:10000. To normalize the analyzed protein samples, a mouse anti-human pan actin moAb (Sigma Aldrich) was used. Detection was carried out by means of the BM Chemiluminescence Blotting Substrate (Roche Diagnostics).

Plasmid expression vectors and luciferase assay

The following single strand oligomers and their complement, corresponding to putative VDR binding site and its mutated form, were used in luciferase assay: VDRE: 5′-GATCGGCAGG**AGAA-TC**GCT**TGAACC**CAGGAGG-3′, VDREMutant(m): 5′-GATCGGCAGG**A-GAGTC**GCT**TGAAAC**CAGGAGG-3′. Mutated oligonucleotide was generated, according to Wang et al. 2005 [5], by replacing one nucleotide (underlined font) in each halfsite of the considered

VDRE. Complementary oligonucleotides, modified at the 5′ end with the *Bam*HI and *Sal*I overhang restriction sites, were annealed and inserted into the *Bam*HI/*Sal*I digested pT81Luc vector [34] generating the pT81LucVDRE and pT81LucVDREm constructs. Nucleotide sequence analysis of the plasmids demonstrated that VDRE sequences had been inserted as single-copy oligomers. VDR cDNA fragment, obtained as described above, was cloned in the *Eco*RI site of pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA, USA) resulting in pcDNA3.1VDR construct. Luciferase reporter assays were carried out in two different cell lines, i.e. HEK293 and COS-1. Transient transfection of HEK293 was conducted in 24-well plate with Lipofectamin 2000 (Invitrogen), using 200 ng of pT81Luc reporter plasmids, 50 ng of pcDNA3.1VDR expression vector, 200 ng of pCMVβ-galactosidase (Clontech Laboratories, Inc. Mountain View, CA, USA) to normalize for transfection efficiency, and a carrier plasmid to maintain a total DNA concentration of 800 ng. On the contrary, luciferase experiments performed in COS-1 cells did not require pcDNA3.1VDR transfection since these cells endogenously express *VDR* gene. After 24 h, 1,25(OH)2D3 was added to the transfected cells at 10^{-7} M concentration for an additional 24 h period.

Chromatin Immunoprecipitation

20×10^7 U937 cells were treated with 10^{-7} M concentration of VD for 18 h and the same amount of cells were maintained in liquid culture and used as control. According to standard protocols, these cells were cross-linked, lysed, wash, and sonicated in order to better lysed the cells and shear DNA to 100–1000 bp fragments. Chromatin was immune-precipitated by using 5 μg of VDR antibody (Santa Cruz) or FLAG tag antibody (Sigma-Aldrich), used as control. The enrichment for VDR-bound sequence in ChIPs was verified by semiquantitative PCR, using the following primers amplifying the genomic region spanning the VDRE element: DP: 5′-CGAGACGGGTGAGTCACCTG-3′; RP: 5′-GGAATGAGTGATTCCGTGGC-3′. PCR reaction was performed using 1.5 μl of immunoprecipitated DNA with 0.3 mM dNTPs, 150 ng of both primers, 5 × Go-*Taq* buffer, and 2 units of Go*Taq* (Promega) in a 50 μl total volume. In order to further validate the specificity of VDR binding on ORM1 VDRE element, PCR amplifications were carried out on two different genomic sites selected as negative or positive control, that are, respectively, the microsatellite region located on chromosome 3 (DP: 5′-CACATCACGAAGCAGTTTAACATA-3′; RP: 5′-CTTTGAGGCCTATCATGAAAAAGG -3′) and the promoter region of *CAMP* gene (DP: 5′-ACCGTGCCCTGCCTCATTC-3′; RP: 5′-TGGTCCCCATGTCTGCCTC-3′). The ethidium bromide gel images were analyzed by ImageJ software (National Institute of Health) and, following normalization on the negative control genomic site, the percentage of enrichment was determined from the ratio between the amount of PCR-amplified VDR-bound DNA in ChIP samples and in input chromatin. To give reliability to the results obtained, three different ChIP experiments were performed.

Statistical analysis

All experiments were repeated at least three times and results were presented as mean ± S.E.M. values. Multiple comparisons were performed by one way analysis of variance (ANOVA) whereas Pairwise comparisons were carried out using student's *t*-test procedure. Results of statistical analysis were considered significant when exhibiting *p*-values ≤ 0.05 and are indicated by asterisks. *, $p < 0.05$; **, $p < 0.001$

Results

Activation of ORM1 and ORM2 gene expression in response to 1,25(OH)2D3 treatment in myeloid cells

To investigate the genetic program activated by VD, we analyzed the mRNA expression profile induced by this differentiation inducer through the Affymetrix microarray methodology. Firstly, three independent experiments were carried out in U937 monoblastic cell line and untreated cells were used as control. A preliminary analysis, based on the Signal Log Ratio value obtained by the comparison between 1,25(OH)2D3 stimulated and normal cells, demonstrated that *CD14 Antigen (CD14)*, *Orosomucoid 1* (ORM1), *Secreted Phosphoprotein 1* (*SPP1*) and *Cathelicidin Antimicrobial Peptide* (*CAMP*) genes disclosed the highest level of induction (Table 1). Among them, *ORM1* had never been related to 1,25(OH)2D3 biological function whereas *CD14*, *SPP1* and *CAMP* were already known targets of 1,25(OH)2D3 [5,16,18,35,36]. To better characterize the expression levels of all members of Orosomucoid gene family, an additional analysis was performed in primary myeloid precursors, i.e. myeloblasts and monoblasts by considering the absolute Signal values. These cells, achieved from human normal CD34+ hematopoietic progenitors after a 7 day period of in-vitro liquid culture [30], were exposed to 1,25(OH)2D3 for further 7 days. Control samples were represented by the cells maintained in the same culture conditions without the differentiation inducer. The expression profile of the two ORM members highlighted that 1,25(OH)2D3 is able to promote a clear up-regulation of *ORM1* gene in both precursor populations, whereas *ORM2* transcript showed increased expression levels only in myeloblast cells (Table 2). In order to validate microarray data, *Orosomucoid* genes expression was also investigated by the more sensitive quantitative real time PCR (QRT-PCR) technique. Taken together, the data, reported in Fig. 1, showed the capacity of 1,25(OH)2D3 to induce both *ORM1* and *ORM2* gene expression

Table 1 – List of probe sets showing the highest expression levels in the comparison between 1,25(OH)2D3 treated and control cells.

PROBESET ID	GENE TITLE	GENE SYMBOL	AV. SLR
201743_at	CD14 antigen	CD14	10.95556
205041_s_at	orosomucoid 1	ORM1	9.711111
205040_at	orosomucoid 1	ORM1	8.188889
209875_s_at	secreted phosphoprotein 1	SPP1	7.344444
210244_at	cathelicidin antimicrobial peptide	CAMP	6.477778

Gene expression data obtained by Microarray experiments performed on 1,25(OH)2D3 treated and unstimulated U937 cells. The table reports the five probe-sets disclosing the major level of induction, expressed as Average Signal Log Ratio (AV. SLR), in VD stimulated as compared to untreated cells. Gray bars highlight the two different ORM1 probesets localized on HG_U133A genechip array.

Table 2 – Table summarizing *ORM1* and *ORM2* gene expression data obtained by microarray methodology in VD treated myeloid precursors.

SAMPLE TYPE	PROBESET ID	GENE SYMBOL	ABSOLUTE SIGNAL VALUE		CHANGE
			CONT.	*VD*	
PRIMARY MYEOLOBLASTS	205040_at	ORM1	1007	4222.8	I
	205041_s_at	ORM1	831.5	4069.8	I
	214465_at	ORM2	98.8	448.6	I
PRIMARY MONOBLASTS	205040_at	ORM1	3.2	531.6	I
	205041_s_at	ORM1	7.2	522.8	I
	214465_at	ORM2	5.5	40.5	NC

Table displaying absolute Signal values and "Change" parameters regarding *ORM1* and *ORM2* genes. These data were obtained, by Gene Chip Affymetrix platform, in unstimulated (*CONT.*) and 1,25(OH)2D3 treated (*VD*) normal myeloblasts and monoblasts precursors.

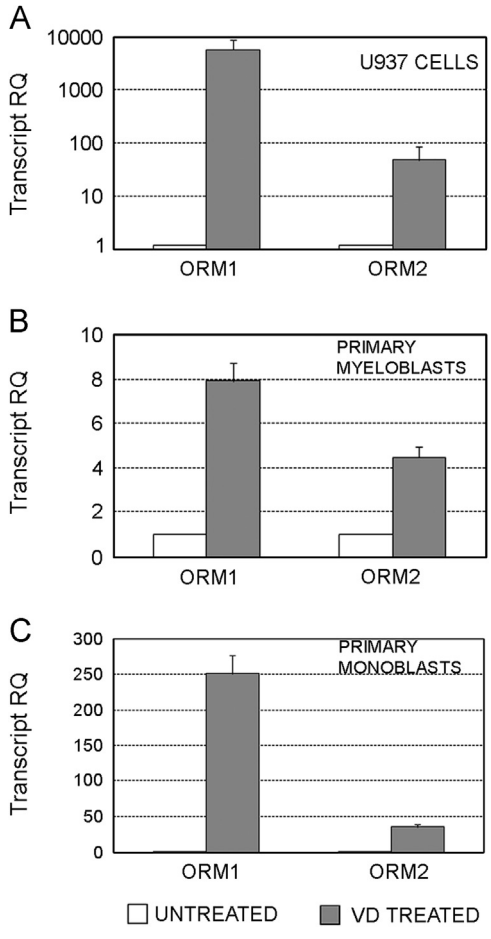


Fig. 1 – Validation of transcriptomic data obtained by cDNA Genechip Affymetrix platform. The histograms show the results of QRT-PCR analysis performed in 1,25(OH)2D3 stimulated U937 cells (A), normal myeloblasts (B) and monoblasts (C) as compared to untreated cells, used as calibrator sample. Treatment conditions are clearly described in the "Material and Method" section. On x-axis, gene symbols are indicated whereas on y-axis the relative quantity of mRNA transcripts are reported. The data are indicated as mean ± S.E. M. of three independent experiments.

suggesting the existence of a transcriptional correlation between 1,25(OH)2D3 and the investigated genes.

Analysis of ORM1 and ORM2 expression in response to 1,25 (OH)2D3 in time course experiments

To further verify the ability of 1,25(OH)2D3 to promote the up-regulation of *ORM* genes, we carried out a set of time-course experiments in which two different cell lines, i.e. HL60 and U937 cells, were treated with a 10^{-7} M concentration of 1,25(OH)2D3 for up to 72 or 96 h respectively. Subsequently, the expression of ORM1 and ORM2 transcripts was estimated by QRT-PCR at the indicated treatment times. As positive control for 1,25(OH)2D3 stimulation efficiency, the mRNA of 1,25-dihydroxyvitamin D3 24-hydroxylase (CYP24A1), a well-known 1,25(OH)2D3 direct target [3], was also monitored. The results are reported in Fig. 2, panels A-C, as mean ± s.e.m. of three independent stimulations and demonstrated the capacity of the investigated nuclear hormone to induce both *ORM1* and *ORM2* expression (see Table S1 and Table S2 for statistical analysis). In addition, they also underlined that 1,25(OH)2D3 is able to promote the induction of the investigated genes depending on different levels and kinetic modalities in the biological cell contexts analyzed, highlighting a more pronounced increase in U937 cells as compared to HL60 promyelocytic cell line. This observation may be probably ascribed to the different basal level of expression of the analyzed genes observed by QRT-PCR, showing highest mRNAs levels in HL60 as compared to U937 cells (data not shown). In agreement with the transcriptomic data, the results of Western blot analysis, reported in Fig. 2, Panel D, display a high capacity of 1,25(OH)2D3 to induce both ORM1 and ORM2 proteins. Western blot autoradiograms showed two bands, probably due to the different glycosylated forms characterizing the considered proteins.

Effect of VDR full length and VDR dominant negative on ORM1 and ORM2 expression

In order to verify the involvement of the VDR genomic pathway in the transcriptional control of *ORMs* gene, we constructed two retroviral vectors expressing the full length (FL) and a truncated (ΔAF2) form of VDR that were named, respectively, LVDRFLIΔN

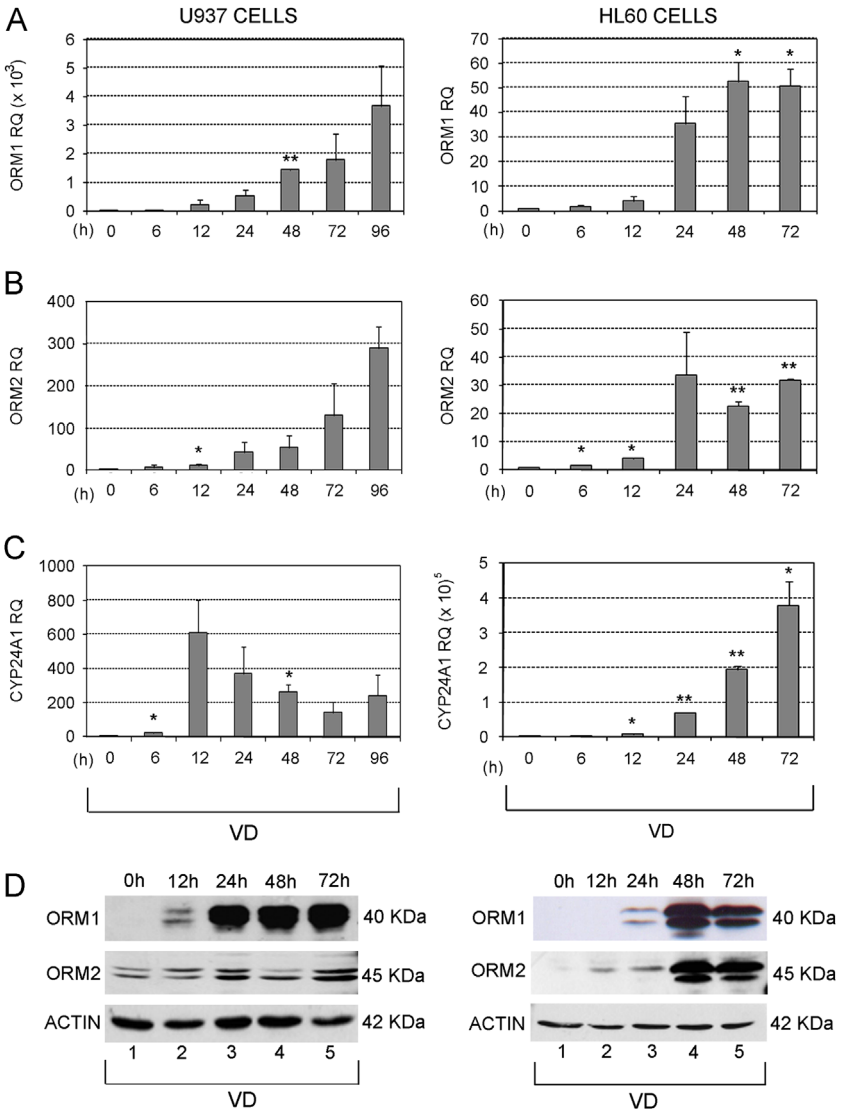


Fig. 2 – Time-course experiments to analyze *ORM1* and *ORM2* gene and protein expression in two different myeloid cell lines upon 1,25(OH)2D3 stimulation. Left and right column reported the data achieved in U937 and HL60 cells, respectively. The histograms describe the *ORM1* (A), *ORM2* (B) and *CYP24A1* (C) gene expression data obtained by QRT-PCR by using "T0 sample" as calibrator. *x-axis*, treatment times; *y-axis*, mRNA Relative Quantity. Data are indicated as mean ± S.E.M. of at least three independent experiments. (D) Western blot analysis of ORM1 and ORM2 protein expression in 1,25(OH)2D3 time course experiment. Treatment times, gene symbol and the molecular weights are indicated, respectively, at the top, on the right and on the left of each panel. To normalize the amounts of total extracts loaded in the various lanes, expression of β-actin was also included in this assay. The images showed are representative of one out of the three experiments performed.

and LVDRΔAF2IΔN. In particular, the VDRΔAF2 version was obtained by deleting the transactivation domain (AF2) and was expected to compete with the VDR endogenous protein in the dimerization with RXR and in the DNA binding capacity.

U937 cells were retrovirally transduced with LXIΔN empty vector (CONT.), with the full length (VDRFL) and the deleted (VDRΔAF2) forms of VDR and then purified as described in the "Material and Methods" section. Subsequently, 1,25(OH)2D3 treatment with 10^{-7} M concentration for 72 h was carried out on the homogeneous cell populations and the biological effects were firstly monitored by immune-phenotypic and morphological examinations. Flow cytometry analysis of CD14 and CD11b antigens, selected as monocyte differentiation markers, clearly demonstrated the ability of VDRFL to up-regulate CD14 and CD11b expression as compared to control cells (CD14, 90.3 ± 2.2% vs. 51.6 ± 9.5%; CD11b, 89.8 ± 2.3% vs. 70.2 ± 4.1%; mean ± S.E.M. of three independent experiments) and the capacity of VDRΔAF2 to strongly down-regulate the investigated antigens to expression levels lower than those observed in the control cells (CD14, 27.8 ± 17.1%; CD11b, 40.6 ± 14.4%; mean ± S.E.M. of three independent experiments) (Fig. 3, Panel A). Morphological examination was performed on May Grunwald-Giemsa speciments obtained from the same cell populations upon five days of 1,25(OH)2D3 stimulation. The images, reported in Fig. 3, Panel B, display a remarkable macrophage differentiation effect promoted by VDRFL whereas the deleted form do not modify the

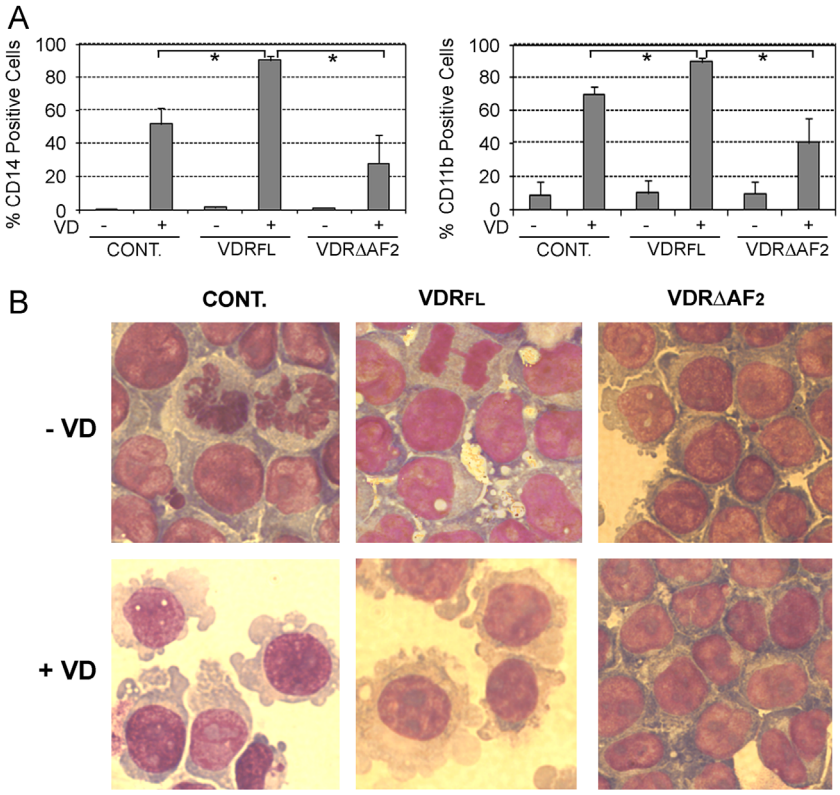


Fig. 3 – Evaluation of immunophenotypic and morphological effects induced by VDR full length and dominant negative retroviral transduction in U937 cells. (A) Histograms reporting the cytofluorimetric analysis of CD14 (right panel) and CD11b (left panel) antigens achieved in normal (CONT.), LVDRFLIΔN (VDRFL) and LVDRΔAF2IΔN (VDRΔAF2) transduced U937 cells upon 1,25(OH)2D3 treatment. *y-axis*, percentage of positive cells; *x-axis*, samples and treatment conditions. Data are indicated as mean ± S.E.M. of at least three independent experiments. (B) Morphological analysis obtained by May Grunwald – Giemsa staining of cytocentrifuged speciments. Sample name and treatment conditions are indicated, respectively, at the top and on the right.

blast cell morphology characterizing the unstimulated or untransduced cells. Globally, these results allowed us (i) to define the VDRΔAF2 as a VDR dominant negative and (ii) to provide an efficient experimental model to investigate the involvement of the VDR genomic pathway in the identification of novel 1,25(OH)2D3 response genes.

On these bases, we used U937 cells expressing the mentioned retroviral constructs to analyze the gene expression of the putative VDR response genes. Fig. 4, panels A and B, reports the results of QRT-PCR assay concerning the amount of *ORM1* and *ORM2* transcripts in transduced and 1,25(OH)2D3 treated U937 cells. The histograms show that VDRFL is able to promote a remarkable increase of the investigated genes whereas, as expected, the dominant negative form counteracted the capacity of 1,25(OH)2D3 to induce the *ORM1* and *ORM2* transcription, leading the expression levels of these genes to those observed in control untreated cells. *CYP24A1* gene, included in this set of experiment as positive control, displays the same expression pattern, supporting the involvement of the signaling genomic pathway in the transcriptional regulation exerted by VDR on the analyzed genes (Fig. 4, Panel C). Finally, panel D displays the Western blot analysis performed on U937 cells transduced with the retroviral constructs. Lane 1 reports a mild band corresponding to VDR endogenous form, that is strongly incremented in lane 3, corresponding to the full length over-expression. In lane 2, the deleted form VDRΔAF2 is detected and exhibits a lower molecular weight than the full length form.

Identification of a VDR binding site in ORM1 promoter region

To further demonstrate the involvement of the VDR genomic pathway in the control of *Orosomucoid* genes expression, we inspected the 1 Kb proximal promoter region upstream to the *ORM1* Transcriptional Start Site (TSS) in order to identify a VDRE response element. To this aim, the UCSC Genome Browser [37] was used and according to the interspecies sequence conservation and to high level of similarity with the canonical VDRE element, we were able to recognize a putative VDRE motif in the promoter region of *ORM1* gene (Fig. 5, Panel A). This element, localized at –865 bp from the TSS, is composed of the following sequence AGAATCgctTGAACC and it was firstly investigated for in-vivo VDR binding through Chromatin Immunoprecipitation (ChIP) experiments. Formaldehyde-cross-linked chromatin was extracted from normal and 1,25(OH)2D3 stimulated U937 cells and then immunoprecipitated with a specific anti-VDR or with an anti-Flag antibody, used as control. The enrichment for VDR-bound sequence in ChIPs was verified by semiquantitative PCR, using primers amplifying the genomic region spanning the VDRE element. The histogram showed in Fig. 5, Panel B, reports the

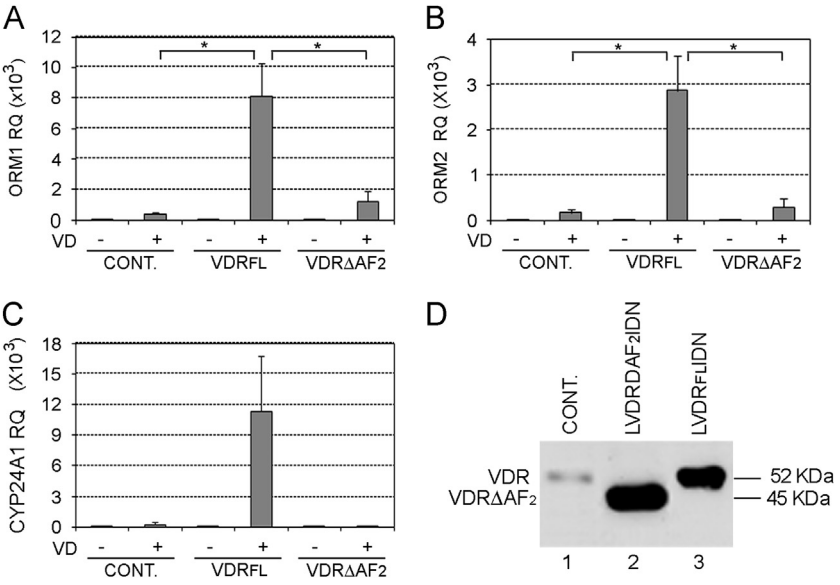


Fig. 4 – Analysis of the molecular effect promoted by VDR full length and dominant negative retroviral transduction in U937 cells. The histograms describe the QRT-PCR analysis performed on LXIΔN (CONT.), LVDRFLIΔN (VDRFL) and LVDRΔAF2IΔN (VDRΔAF2) transduced U937 cells upon 1,25(OH)2D3 treatment in order to evaluate the mRNA levels of *ORM1* (A), *ORM2* (B) and *CYP24A1* (C). The results are reported as Relative quantity (mean ± S.E.M.) of at least three independent experiments. (D) Western blot analysis to evaluate VDR protein (endogenous, lane 1; ectopic expression, lane 3) and VDRΔAF2 dominant negative (lane 2) in transduced U937 cells. Sample name, protein name and the molecular weight are indicated, respectively, at the top, on the right and on the left of the panel.

results (mean ± S.E.M) achieved from three independent ChIPs, demonstrating a clear and specific enrichment of VDR-bound DNA in 1,25(OH)2D3 treated cells as compared to unstimulated control sample. Finally, to evaluate the trans-activation capacity of the investigated VDRE sequence, luciferase transactivation experiments were performed in two different cell lines, i.e. HEK293 and COS-1, the latter characterized by an endogenous VDR expression. The results of these experiments demonstrated, in both cell contexts, the capacity of VDR to determine a significant three-fold induction in luciferase activity upon VDRE binding whereas this ability was inhibited in the presence of the mutated sequence (pT81VDREm Samples) (Fig. 6, panel A and B). Altogether, these results allowed us to conclude that ORM1 is transcriptionally regulated by the VDR nuclear receptor through its genomic pathway, defining it a 1,25(OH)2D3 primary response gene. Since *ORM1* and *ORM2* genes are localized adjacent to each other on the same chromosome, we also screened their 3 Kb intergenic area in order to identify a VDRE element regulating *ORM2* gene expression. Unfortunately, this inspection did not reveal any functional VDRE motif and, as a consequence, it is therefore possible to suppose that *ORM2* is a secondary 1,25(OH)2D3 target gene or that the previously isolated VDRE is involved in the regulation of the neighboring *ORM2* gene. This kind of transcriptional regulation is not surprising since it has been already described inside the IGFBP family members [38].

It is worth to consider that a number of recent reports based on VDR ChIP-Seq analysis [39–42] apparently failed to detect the ORM1 VDRE element that we have described here. This discrepancy is in our opinion not surprising and, at least for those experiments that were carried out in a monocytic cell context [39,40], it is probably related to the different 1,25(OH)2D3 treatment times (up to 18 h in our experimental settings vs. <1 h of other reports). These results clearly indicate that, as many other differentiation markers, ORM1 is a late target gene of the investigated compound, thus requiring a longer exposure time to undergo the expected up-regulation. To support this statement, we performed a ChIP assay on the *CAMP* 1,25(OH)2D3 early response gene [16], demonstrating that the VDRE element localized in its promoter region is strongly bound by VDR, under our experimental conditions (data not shown). An additional and relevant issue raised by the above mentioned ChIP-Seq data deals with the presence of other VDRE elements inside the considered genomic region [40]. To address this possibility, we extended our investigation to the complete 9q32 genomic locus, analyzing the capacity of 1,25(OH)2D3 to up-regulate the expression of ORM1 neighboring genes, such as *Collagen Type XXVII alpha-1* (*COL27A1*) and *AT-Hook Transcription Factor 1* (*AKNA*). The results obtained, achieved by QRT-PCR, showed that 1,25(OH)2D3 was unable to determine this effect (data not shown), leading to the conclusion that the analyzed genomic region lacks additional functional VDR binding sites.

Involvement of ORM1 in 1,25(OH)2D3-induced monocyte polarization

In order to examine the role played by 1,25(OH)2D3-VDR – ORM1 pathway in the immune system regulation, we initially performed time course experiments in which human primary monocytes were treated with a 5×10^{-8} M concentration of 1,25(OH)2D3. In this cellular context, we firstly analyzed the gene expression of

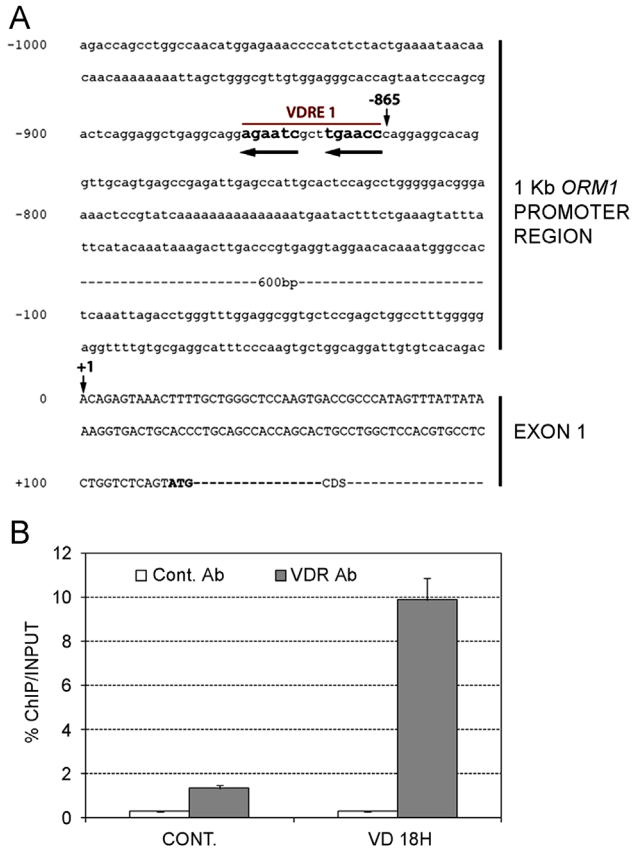


Fig. 5 – Identification and in-vivo characterization of VDRE element inside the promoter region of *ORM1* gene. (A) 1 Kb proximal promoter sequence of *ORM1* gene. Transcriptional start site and the translation start site (ATG) are indicated by +1 and by bold letters, respectively. The location of the DR3-type VDRE element, positioned at −865 bp from +1, is highlighted by arrows. (B) ChIP experiments to detect in-vivo VDR binding to the putative VDRE element. The histogram shows the results of three independent ChIPs performed on U937 cells stimulated with a concentration of 10^{-7} M 1,25(OH)2D3 for 18 h; unstimulated cells were used as control (CONT.). Immunoprecipitation was conducted with an anti-FLAG control Ab (white bars) and a specific VDR Ab (gray bars). The chromatin enrichment is indicated as the ratio between the amount of PCR-amplified VDR-bound DNA in ChIP samples and in input chromatin (% ChIP/INPUT).

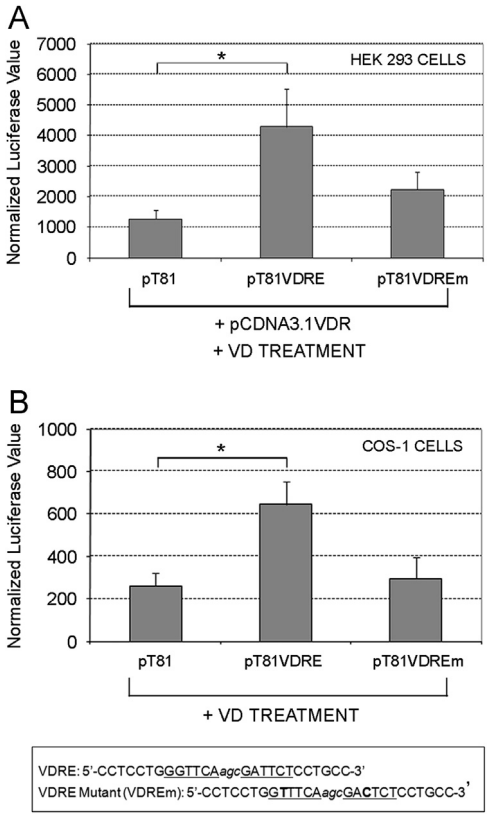


Fig. 6 – Analysis of Luciferase transactivation ability of VDR protein upon binding to VDRE element. The histograms describe the results of luciferase assays performed in HEK 293 (A) and in COS-1 (B) cells, upon 1,25(OH)2D3 treatment for 24 h at 10^{-7} M concentration. HEK 293 cells were transfected with the pT81Luc reporter plasmids and pcDNA3.1VDR expression vector, whereas COS-1 were transfected with reporter plasmids alone. *x-axis*, combination of transfected plasmids; *y-axis*, β-galactosidase normalized luciferase value. Bars represent the mean luciferase activity ± s.e.m. of at least three independent experiments. (C) Box reporting the oligonucleotide sequences of VDRE element (VDRE) and the corresponding mutated form (VDREm). Underlined font indicates the two half site of the investigated VDRE motifs whereas the mutated nucleotides are indicated in bold characters.

the following well-known macrophage polarization markers: (i) *IL-6* and *TNFα*, typically induced by classical activation stimuli, (ii) *MRC1*, characterizing the alternative polarization, (iii) *CD163* and *MMP9*, selected as de-activating markers [43]. As expected, the results demonstrated the capacity of 1,25(OH)2D3 to down-regulate the pro-inflammatory cytokines whereas *MRC1*, *CD163* and *MMP9* displayed a strong mRNA induction (Fig. 7, Panel A). Interestingly, the gene expression analysis carried out on *ORM1* highlighted a similar profile, showing a progressive increase of transcript amounts reaching the maximum level at 72 h upon 1,25 (OH)2D3 stimulation (Fig. 7, Panel A, see Table S3 for statistical analysis). These preliminary data obtained in the time-course experiments provided additional evidence on the role played by 1,25(OH)2D3 in the regulation of the anti-inflammatory process exerted by human macrophages. Furthermore, they allowed us to hypothesize that *ORM1* might be involved in this regulatory function of the considered nuclear hormone. To elucidate this issue, *ORM1* silencing experiments were carried out in 1,25(OH) 2D3 treated human primary monocytes and the effects of this inhibition were estimated by analyzing, through QRT-PCR assay, the mRNA expression of the selected macrophage polarization markers. Interestingly, the results of this investigation demonstrated (i) the induction of pro-inflammatory cytokines (*IL-6* and *TNFa*), (ii) the significant down-regulation of *CD163* surface antigen and (iii) a similar trend for *MMP9* and *MRC1* antigens (Fig. 7, Panel B, see Table S4 for statistical analysis). This behavior is exactly opposed to that observed under 1,25(OH)2D3 treatment, further confirming that the anti-inflammatory effect exhibited by Vitamin D3 on human macrophages is partially mediated by the transcriptional control exerted on *ORM1* gene.

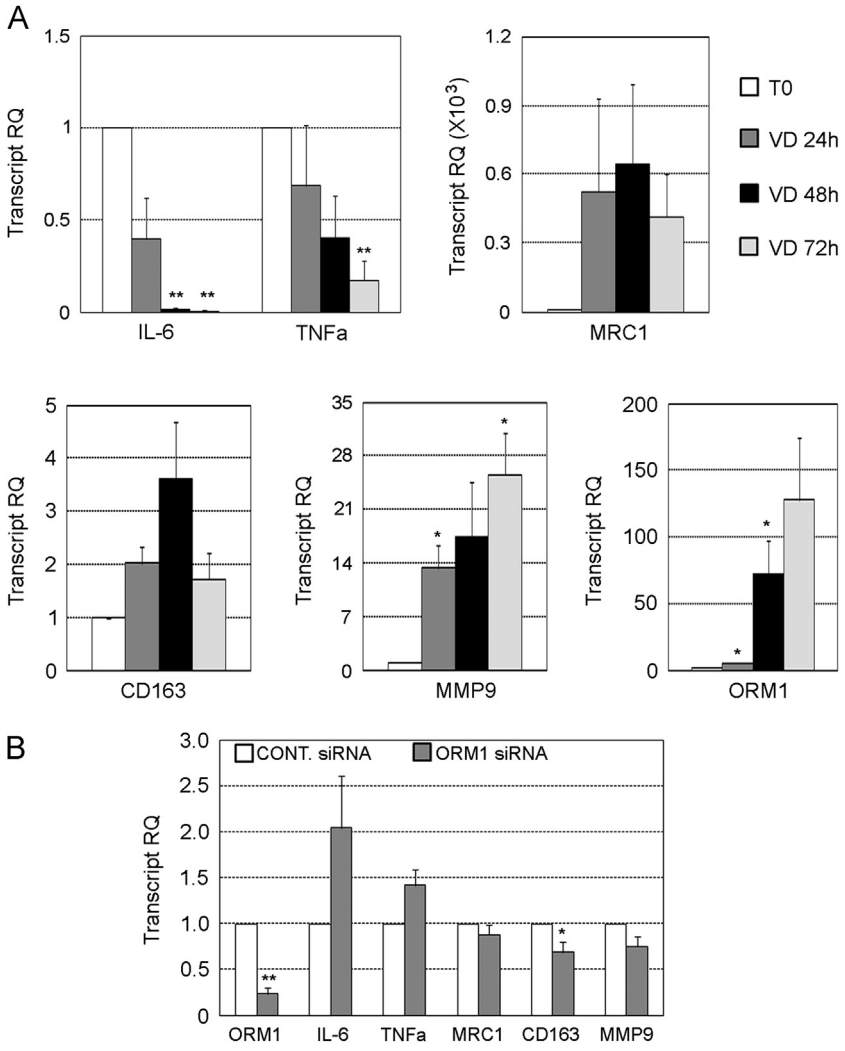


Fig. 7 – Gene expression analysis of macrophage polarization markers and *ORM1* gene in 1,25(OH)2D3 – and *ORM1* siRNA – treated human monocytes. (A) Histograms showing the QRT-PCR results obtained in time course experiments performed in human monocytes under 1,25(OH)2D3 stimulations. Gene symbols of the investigated macrophage polarization markers are reported on x-axis whereas Relative Quantity amount of mRNAs are indicated on y-axis. Treatment times are indicated by different colored bars and explained in the legend. T0 was used as calibrator sample and the data are represented by mean ± S.E.M of three independent experiments. (B) ORM1 silencing effect on macrophage polarization markers. The histogram reports the gene expression analysis obtained by QRT-PCR in human monocytes under ORM1 silencing conditions (gray bars) as compared to control siRNA (white bars). All samples were exposed to 1,25(OH)2D3 for 24 h at 5×10^{-8} M concentration. *x-axis*, gene symbol of the analyzed genes; *y-axis*, mRNA Relative Quantity. The data are represented by mean ± S.E.M of three independent experiments.

Effects of ORM1 treatment on human macrophage polarization

The data described above also suggested that ORM1 protein might be able to regulate, by itself, the immune response of human macrophages, acting as a signaling molecule able to switch off an inflammatory condition. To verify this hypothesis, we adopted a similar experimental design in which human monocytes were exposed to ORM1 protein at 250 μg/ml concentration for up to 72 h. This amount corresponds to a physiological plasma concentration, ranging from 0.2 to 1 mg/ml whereas, during an acute phase response, this levels is three to four fold increased with respect to the normal range [31]. Macrophage polarization markers were then assessed by means of the QRT-PCR reaction performed upon ORM1 stimulation. Interestingly, the data achieved by this assay demonstrated that acting alone, i.e. without 1,25(OH)2D3 stimulation, ORM1 is able to inhibit pro-inflammatory cytokine expression and, at the same time, to promote the mRNA up-regulation of *MMP9, MRC1* and *CD163* antigens (Fig. 8, see Table S5 for statistical analysis), evoking the same macrophage immune response observed following 1,25(OH)2D3 stimulation (see Fig. 7A). In addition, the transcripts variations observed in response to 1,25(OH)2D3 or ORM1 treatment were included in the same range, further confirming the direct involvement of ORM1 protein in the immune role of the nuclear hormone.

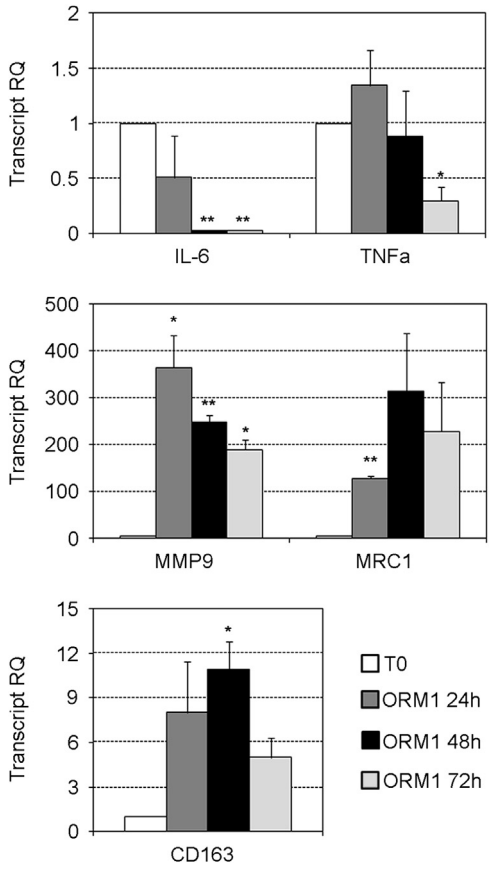


Fig. 8 – Gene expression analysis of macrophage activation molecules upon ORM1 stimulation of human monocytes. The histograms report the mRNA amount, indicated as transcript RQ (y-axis), of *IL-6* and *TNFa* inflammatory cytokines (upper panel), *MMP9* and *MRC1* markers (middle panel) and *CD163* scavenger receptor (lower panel), as compared to T0 sample, selected as calibrator. Gene symbols are indicated on the *x*-axis and treatment times are showed with different colored bars explained in the legend. The data are represented by mean ± S.E.M of three independent experiments.

Discussion

1α, 25 dihydroxy Vitamin D3, the metabolic active form of Vitamin D, was firstly characterized as a molecule having a restricted role in calcium homeostasis and bone mineralization. In spite of this, a large body of evidence has highlighted, during the last two decades, a pleiotropic action of this compound, indicating its involvement in several physiological processes, including growth and differentiation of many cell types [2,11,44]. In our laboratory, we have previously investigated the biological effects exerted by vitamin D on myeloid differentiation, demonstrating its capacity to promote a monocyte maturation program in M2/M3 – and M5 – type leukemic blast cells [13,14], as well as in CD34+ hematopoietic stem / progenitor cells [11,15]. Additional data, obtained in other laboratories, also suggested that 1,25(OH)2D3 is involved in the regulation of the immune system, acting on several cell types belonging both to acquired and innate immunity [4,12,16,45–47]. Recent papers, carried out by means of the Chip-seq high-throughput technology, has recently provided further support to this finding, demonstrating that several genes associated with autoimmune and neoplastic diseases are 1,25(OH)2D3 direct targets [39–42,48]. Moreover, a growing body of literature underlined the relationship existing between 1,25(OH)2D3 deficiency and an increased risk to develop immune disorders and autoimmune diseases, such as Tubercolosis, Type 1 Diabetes (T1D), Systemic Lupus Erythematosus (SLE) and Inflammation Bowel Diseases (IBD) [45]. Starting from this premise, we tried to identify a direct correlation between 1,25(OH)2D3 and ORM1 protein, based on the natural immuno-modulator and anti-inflammatory properties of these molecules [28]. The experiments described in this study took place from the analysis of the gene expression profile promoted by 1,25(OH)2D3 treatment on U937 monoblastic cells, indicating *ORM1* as one of the most up-regulated genes. The inspection of the *ORM1* promoter region, followed by ChIP and Luciferase assays were then able to demonstrate, for the first time, that *ORM1* is a 1,25(OH)2D3 primary response gene, characterized by the presence of a functional VDRE element inside the 1 kb region placed upstream its TSS. A similar experimental approach failed to identify a VDRE motif in the *ORM2* promoter region, corresponding to the 3 kb intergenic area spacing the two considered ORM family members, in spite of the capacity of 1,25(OH)2D3 to strongly up-regulate the expression of this gene. Two different hypotheses might, in our opinion, explain this result, i.e.: *ORM2* might be a secondary response gene of 1,25(OH)2D3 or, alternatively, the VDRE element isolated inside the *ORM1* promoter region might also be involved in the transcriptional control of *ORM2* gene, as already described for IGFBP family members [38]. In light of the results obtained, we focused our attention on the possible functional correlation existing between 1,25(OH)2D3 and ORM1, in relation to the biological role that these signaling molecules could exert on human monocytes and macrophages, i.e. the main cellular components of the innate immune system. In this regard, we demonstrated that treatment of these cells with 1,25(OH)2D3 is able to elicit a strong up-regulation of the CD163 and MMP9 phenotypic markers that is accompanied by a parallel inhibition of pro-inflammatory cytokine expression, suggesting that the considered vitamin is able to control the resolution phase of inflammation. Inside this process, we also observed that *ORM1* gene is induced at high levels upon 1,25(OH)2D3 stimulation whereas *ORM1* silencing experiments, carried out in 1,25(OH)2D3 treated monocytes, caused an inverse effect on macrophage marker expression, represented by an up-regulation of IL-6 and TNF-α pro-inflammatory cytokines and a down-regulation of CD163 and MMP9 de-activation molecules. Altogether, these data allowed us to conclude that *ORM1* is a primary response gene of the Vitamin D3 receptor and this pathway is involved in the anti-inflammatory activities of human macrophages aimed to limit tissue damage and restore its integrity.

Several papers have indicated that ORM1 protein does not bind selectively to a specific receptor but it is rather able to interact with different serum molecules as well as with promiscuous cell surface receptors. In particular, among the former it is possible to mention C3, sCD14 and IgG proteins [31] whereas, the latter category included CCR5 [49], SIGLEC 5 and TLR4/CD14 complex [50]. In this regard, a recent report has demonstrated that, during the hemolysis process, ORM1 protein is able to enhance the expression of the CD163 scavenger receptor by binding to the CD14

receptor and activating the TLR4 dependent intra-cellular pathway [50]. In our opinion, this molecular mechanism might be invoked to explain the similar results emerging from our experiments and involving the CD163 antigen. Moreover, the same consideration could be extended to the other macrophage markers analyzed in our report, such as the MMP9 and MRC1 proteins. It is also worth mentioning that, as demonstrated by our previous research activity, exposure of human hematopoietic cells to 1,25(OH)2D3 determines a remarkable up-regulation of the CD14 surface antigen [11,15], bringing further support to our data. Taken together, these considerations allow us to hypothesize the existence, inside the immune system, of a feedback regulatory loop explaining all the described effects. According to this mechanism, in presence of an inflammatory stimulus, 1,25(OH)2D3 promotes the release of ORM1 protein that, in turn, activates the TLR4/CD14 signaling pathway, leading to an up-regulated expression of the CD163, MMP9 and MRC1 de-activating molecules. These last proteins are then responsible for a compensatory inhibition of the inflammatory process. The last experiments presented in our report, and designed to elucidate the role of ORM1 protein in macrophage polarization, revealed that an endogenous concentration of this molecule is indeed sufficient to promote a de-activating response. This finding is, in our opinion, of great relevance because it suggests that ORM1 is not only implicated in the resolution of an acute inflammatory process but it might also be considered as a physiological signaling molecule involved in the maintenance of tissue homeostasis and remodeling, i.e. the main functions exerted by resident macrophages.

Conflict of Interest

Any financial/commercial conflict of interests have been disclosed.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2013.08.017.

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