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RESEARCH PAPER

Vitamin D boosts immune response of macrophages through a regulatory network of microRNAs and mRNAs

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Abstract

Vitamin D is associated with the stimulation of innate immunity, inflammation, and host defense against pathogens. Macrophages express receptors of Vitamin D, regulating the transcription of genes related to immune processes. However, the transcriptional and post-transcriptional strategies controlling gene expression in differentiated macrophages, and how they are influenced by Vitamin D are not well understood. We studied whether Vitamin D enhances immune response by regulating the expression of microRNAs and mRNAs. Analysis of the transcriptome showed differences in expression of 199 genes, of which 68% were up-regulated, revealing the cell state of monocyte-derived macrophages differentiated with Vitamin D (D3-MDMs) as compared to monocyte-derived macrophages (MDMs). The differentially expressed genes appear to be associated with pathophysiological processes, including inflammatory responses, and cellular stress. Transcriptional motifs in promoter regions of up- or down-regulated genes showed enrichment of VDR motifs, suggesting possible roles of transcriptional activator or repressor in gene expression. Further, the microRNA-Seq analysis indicated that there were 17 differentially expressed miRNAs, of which, seven were up-regulated and 10 down-regulated, suggesting that Vitamin D plays a critical role in the regulation of miRNA expression during macrophages differentiation. The miR-6501-3p, miR-1273h-5p, miR-665, miR-1972, miR-1183, miR-619-5p were down-regulated in D3-MDMs compared to MDMs. The integrative analysis of miRNA and mRNA expression profiles predicts that miR-1972, miR-1273h-5p, and miR-665 regulate genes *PDCD1LG2, IL-1B*, and *CD274*, which are related to the inflammatory response. Results suggest an essential role of Vitamin D in macrophage differentiation that modulates host response against pathogens, inflammation, and cellular stress.
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Keywords: microRNAs; mRNA; Transcriptome; Vitamin D; immunomodulation; macrophages.

1. Introduction

Vitamin D is a (pro)-hormone, which has been traditionally considered a key regulator in calcium, phosphorus, and bone homeostasis [1]. Nevertheless, Vitamin D has a variety of roles outside the regulation of calcium levels, and it plays a fundamental role in modulating the immune response [2,3]. Several studies have suggested that Vitamin D insufficiency and deficiency are associated with an increased risk of bacterial, and viral infections, and chronic inflammatory diseases including but not limited to rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, cardiovascular diseases, diabetes, and obesity [4–6].

Vitamin D mediates its biological effects through binding to the Vitamin D receptor (VDR) which binds calcitriol (1,25-$(OH)_2D_3$), the active form of Vitamin D, to induce both non-genomic as well as genomic responses acting as a transcription factor [7]. The presence of VDR and Vitamin D–metabolizing enzymes in immune cells, monocytes, macrophages, dendritic cells (DCs), and activated B and T cells, indicate that Vitamin D exerts immunoregulatory effects on innate and adaptive immune response [8–11].

Macrophages are key specialized cells of innate immune defense against pathogens [12]. Notably, key roles of Vitamin D in macrophages include enhancing differentiation, activation, autophagy, and phagosome maturation [13,14]. Other biological effects of Vitamin D in macrophages include, (1) Induction of expression of antimicrobial peptides such as cathelicidin antimicrobial protein (CAMP) [15] and defensin [16] (2) T-cell stimulatory capacity of the macrophage by the downregulation of CD80, CD86, and *IL-12* [17], (3) Induction of tissue adhesion by upregulation of adhesion molecules, including P-selectin glycoprotein ligand 1 (SELPLG) and Integrin Beta-1 (ITGB1), encoded by genes *SELPLG* and *ITGB1* [18], respectively, and (4) Inflammation modulation by regulation expression of genes encoding *IL-1β, IL-6, IL-12, TNF-α, CXCL9, CXCL10, CXCL11, CCL2,* and miRNA-155 [16,17,19–21].

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However, the mechanisms underlying Vitamin D modulated functions of macrophages remain poorly understood.

Considering the complexity of the regulatory mechanisms of Vitamin D, we deemed it necessary to examine gene expression profiles of MDMs that mediate the biological effect of Vitamin D. Previous studies on gene expression in immune cells provided insights on molecular changes underlying Vitamin D signaling [22–30]. These studies, however, used cell lineages instead of primary cells, and importantly, none examined the effects of Vitamin D on gene expression in macrophages. Further, different levels of regulation of gene expression induced by Vitamin D through an integrative transcriptomic profile of mRNAs and miRNAs have not been examined. The integration of mRNA and miRNA profiles offers an insight into the transcriptional and post-transcriptional regulatory network of Vitamin D signaling in macrophages, which will greatly improve the design and outcome of clinical trials.

Here, we examined integrated miRNA and mRNA expression profiles that mediate the biological effects of Vitamin D during macrophage differentiation. Such a comprehensive analysis allowed us to uncover transcriptional and post-transcriptional regulatory strategies controlling gene expression in Vitamin D differentiated monocyte-derived macrophages (D3-MDMs). We identified differentially expressed genes related to inflammation, host defense against pathogens, cellular stress through respiratory burst, and T-cell activation. We explore the relationships of molecular pathways, including miRNAs targeting inflammation and respiratory burst-associated genes. Together, our results show for the first time, that Vitamin D regulates a set of genes that boost the immune response of macrophages through the establishment of a network of transcriptional and posttranscriptional events.

2. Material and methods

2.1. Culturing of human monocytes and differentiation into monocytes-derived macrophages (MDMs)

Human peripheral blood was mixed with EDTA 4% v/v and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Histopaque (Sigma-Aldrich) gradient at 650 x g for 30 min. Platelet depletion was performed by washing with PBS 1X (Sigma-Aldrich) three times at 250 x g for 10 min. The percentage of CD14+ cells was determined by staining 5×10^5 cells with 1 μL of anti-CD14 (eBiosciences, San Diego, CA, USA) for 30 min. Monocytes were obtained from total PBMCs by plastic adherence procedure as described before [31]. Non-adhering cells were removed by washing twice with PBS. Differentiation of monocytes into macrophages was allowed to occur until day 6 in RPMI 1,640 medium supplemented with 10% autologous serum at 37°C and 5% CO_2. Monocytes-derived macrophages (MDMs) were differentiated in presence of 1 nM of 1,25 di-hydroxyvitamin D3 (D3-MDMs; Sigma-Aldrich) and replenished with fresh medium every 24 h until day 6 of differentiation [32]. The control group (MDMs) was treated with vehicle [0.1% ethanol (EtOH)].

2.2. Total RNA isolation, library preparation, and RNA-Seq

Total RNA was extracted using the Direct-zol RNA MiniPrep kit (Zymo Research) with on-column DNase treatment, according to the manufacturer's instructions. The RNA integrity was ensured by obtaining RNA Integrity Number (RIN) > 8 (Agilent 2100 Bioanalyzer; Agilent Technologies, Germany). For the mRNA sequencing library, four MDMs samples and four D3-MDMs were constructed with TruSeq Stranded mRNA Sample Prep Kits (Illumina, USA) using 1,000 ng of total RNA. Samples were indexed with adaptors and submitted for single-end sequencing using a HiSeq 4,000 instrument (Illumina, USA). mRNA-Seq experiments have been sequenced with a sequencing depth between 20 M to 50 M reads per sample. miRNA sequencing (miRNA-Seq) libraries, 2 MDMs (controls), and 2 D3-MDMs were constructed using a single adapter and circularization approach [33]. miRNA libraries were 50 bp single-end sequenced by a HiSeq 4,000 instrument (Illumina, USA). miRNA-Seq experiments have been sequenced with a sequencing depth between 7 M to 12 M reads per sample.

2.3. Read Alignment and differential gene expression analysis

The data output in the FASTQ file format contained sequence information, including the sequencing quality (Phred quality score). Average Phred scores of ≥ 20 per position were used for alignment. Single-end reads for mRNA were mapped to the human genome version 38 (GRCh38) using TopHat2 [34] with the following options: –mate-inner-dist 200 –mate-std-dev 100 –no-novel-juncs –min-intron-length 40. Single-end reads for miRNA were mapped to the miRBase version 22 using Bowtie [35] with the following options: -n 0 -l 8 -a –best –strata –phred33-quals. Counts for RefSeq genes were obtained using Feature Counts [36] with the default settings, and DESeq2 (version 1.32) [37] was used to normalize expression counts. Genes were considered differentially expressed if |fold change| (FC) ≥ 1.5, and the FDR ≤ 0.05. For assessing mRNA and miRNA transcript abundance, the reads were converted to transcripts per million (TPM).

2.4. Motif analysis

Homer [38] was used to detect de novo transcription factor motifs overrepresented in the promoter of the differentially expressed genes. Gene promoters were considered between nucleotides −300 and +50 relative to the Transcription Start Site (TSS). Significance was tested against CpG-content-matched promoters as background. Binding sites were considered significantly overrepresented when *P*-value < .01. In addition, we explored the binding of the transcription factor VDR to the promoters of differentially expressed genes. For this, we use ChIP-Seq for VDR in the macrophages cell line THP-1 after stimulation with the $1,25(OH)_2D_3$, publicly available at NCBI GEO under the accession GSE89431 [39]. The mapped reads of ChIP-Seq for VDR were visualized with the Integrated Genome Viewer (IGV-v2.3.90) [40].

2.5. Gene enrichment analysis

Enrichr was used for the gene enrichment analysis with the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and Biological Process (Gene Ontology; GO) as databases. The enrichment result is represented as a combined score computed by Enrichr [41]. The KEGG pathway and Biological Process terms were included in an integrative analysis using the criterion of over-representation (log2 combined score > 2).

2.6. miRNA target prediction

Candidate miRNA-mRNA targets relationships were predicted by TargetScan 5.1 (conservation and non-conservation sites) [42]. We also filtered our data using differentially expressed genes (mRNA and miRNA) identified by RNA-Seq, considering that mRNA and miRNA expression levels should be inversely correlated. Jaccard index was used to determine similarity in targets between differentially expressed genes.

2.7. Interaction network

Based on the differentially expressed genes, protein-protein interaction networks were generated via the STRING database [43,44] (http://string-db.org/), which also detects functional interactions among the corresponding genes. Network visualization was performed using the open-source software platform Cytoscape [45] v. 3.6.1 (https://cytoscape.org/).

3. Results

3.1. Vitamin D differentiated macrophages (D3-MDMs) show differentially expressed mRNA as compared to MDMs

Principal Component Analysis (PCA) was used to discriminate MDMs and D3-MDMs (Fig. 1A). PCA showed high heterogeneity of mRNAs in D3-MDMs as suggested by spatial dispersion of samples showing intragroup variability. Out of approximately 12,979 mRNA transcripts, we filtered mRNAs of log2 fold change ≥ 1 (FDR < 0.05), showing that Vitamin D treatment significantly affected expression of 199 genes, of which 136 (68%) and 63 (33%), respectively were up-or down-regulated, in D3-MDMs (*Table S1*). Heat map shows different gene expression profiles of MDMs and D3-MDMs (Fig. 1B). Our transcriptomic analysis indicates that Vitamin D treatment reprograms gene expression during macrophage differentiation. Changes in differentially expressed genes showed 136 up-regulated genes, 46 (33.8 %) by at least 5-fold (Fig. 1C). On the other hand, down-regulated genes were not as marked as the up-regulated genes (only 11 or 15.5% were repressed above the cut-off used for induction) (Fig. 1D).

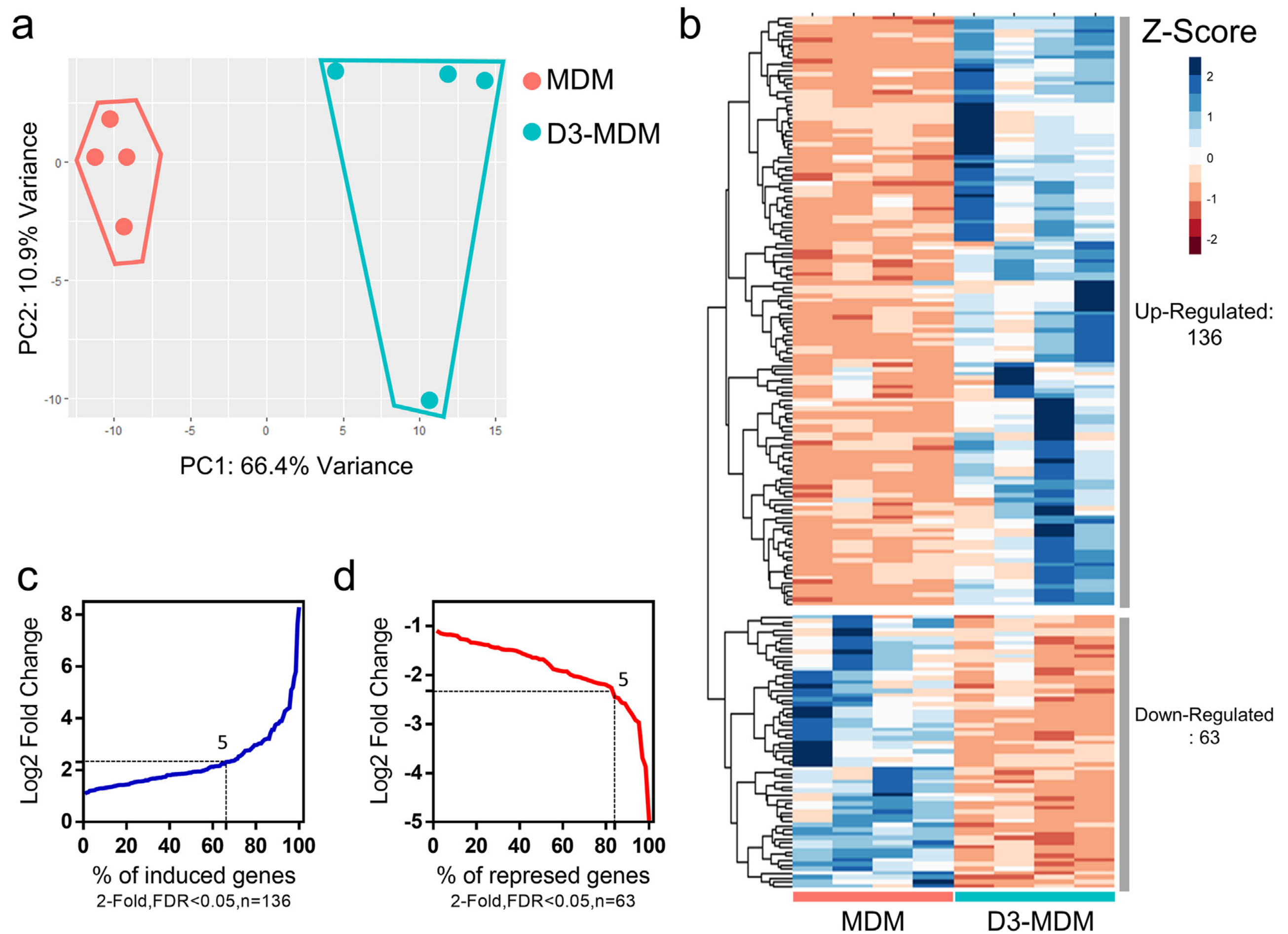


Fig. 1. Transcriptome characterization of Vitamin D differentiated macrophages. (A) Principal component analysis of mRNA data of MDMs and D3-MDMs. Each principal component (PC1 and PC2) is showed the percentage of the variance. (B) Heatmap of Z-score normalized differentially expressed genes of MDMs and D3-MDMs by unsupervised hierarchical clustering analysis. Down-regulated and up-regulated genes with absolute values of fold-change > 2 and FDR < 0.05 are shown in red and blue, respectively. Cumulative frequency distribution, indicated as a percentage (%, x-axis), of the (C) up-regulated and (D) down-regulated differentially expressed genes (log2-fold change, y-axis).

3.2. Vitamin D differentiated macrophages show enhanced expression of genes related to pathogenic response, inflammation, and cellular stress

We analyzed gene expression in D3-MDMs, focusing on the highest degree of regulation. A (scatter) plot that integrated the degree of regulation (fold change; FC) and the number of transcripts in control (Transcripts Per Million; TPM), showed that genes with a high number of transcripts, compared with a low number of transcripts, are associated with small changes in gene expression (Fig. 2A). We note the up-regulation of the *CYP24A1* gene that encodes an enzyme involved in Vitamin D metabolism which was undetected in control macrophages (MDMs) (Fig. 2A).

We examined the expression of genes related to antiviral response, inflammation, and cellular stress (Fig. 2B). Pathogen response genes up-regulated in D3-MDMs included cathelicidin (*CAMP*), serglycin (*SRGN*), lysozyme (*LYZ), CD14,* and myeloid cell receptor *1 (TREM1)* (Fig. 2B). Moreover, we noted up-regulation of *IL1B, CXCl8 (IL-8), ALOX5, VSIG4, NINJ4,* and *THBD,* key inflammatory genes (Fig. 2B). Interestingly, we identified a down-regulation of *CXCR1* gene, also known as *CXCL8 (IL-8)* receptor (Fig. 2B). Up-regulation of cytokine *CXCL8* and down-regulation of the receptor *CXCR1*, suggests that IL-8 signaling in Vitamin D differentiated macrophages does not occur in an autocrine manner. Finally, we observed that D3-MDMs expressed members of the metallothionein genes, which have been implicated in cellular stress processes (Fig. 2B). Besides metallothionein genes, D3-MDMs expressed high levels of *NOD2*, a pattern recognition receptor (PRR) involved in the activation of transcription factors, such as *NFκB*, activator protein-1 (*AP-1*), and interferon regulator factor 5 (*IRF5*), as well as expression of antimicrobial and pro-inflammatory molecules.

To understand the biological processes implicated we performed an enrichment analysis. The analysis showed 19 gene ontology groups clustered in five modules related to immune response, metabolism, cellular stress, cell transport, and other biological process (Fig. 2C and *Table S2*). Notably, in the immune response group, we observed that macrophages differentiated with Vitamin D tend to be more pro-inflammatory, except for Interleukin-10 (*IL-10*) production, an anti-inflammatory cytokine with a role in infection by limiting the immune response (Fig. 2C). In addition, in terms of metabolism, the Vitamin D macrophages are more glycolytic (e.g., down-regulation of glyceraldehyde-3-phosphate metabolic process) (Fig. 2C). Taken together, the results suggest that Vitamin D potentiates a set of genes in macrophages with key roles in directing host defense against pathogens.

Finally, we performed an integrated protein-protein interaction (PPI) network of the differentially expressed genes that

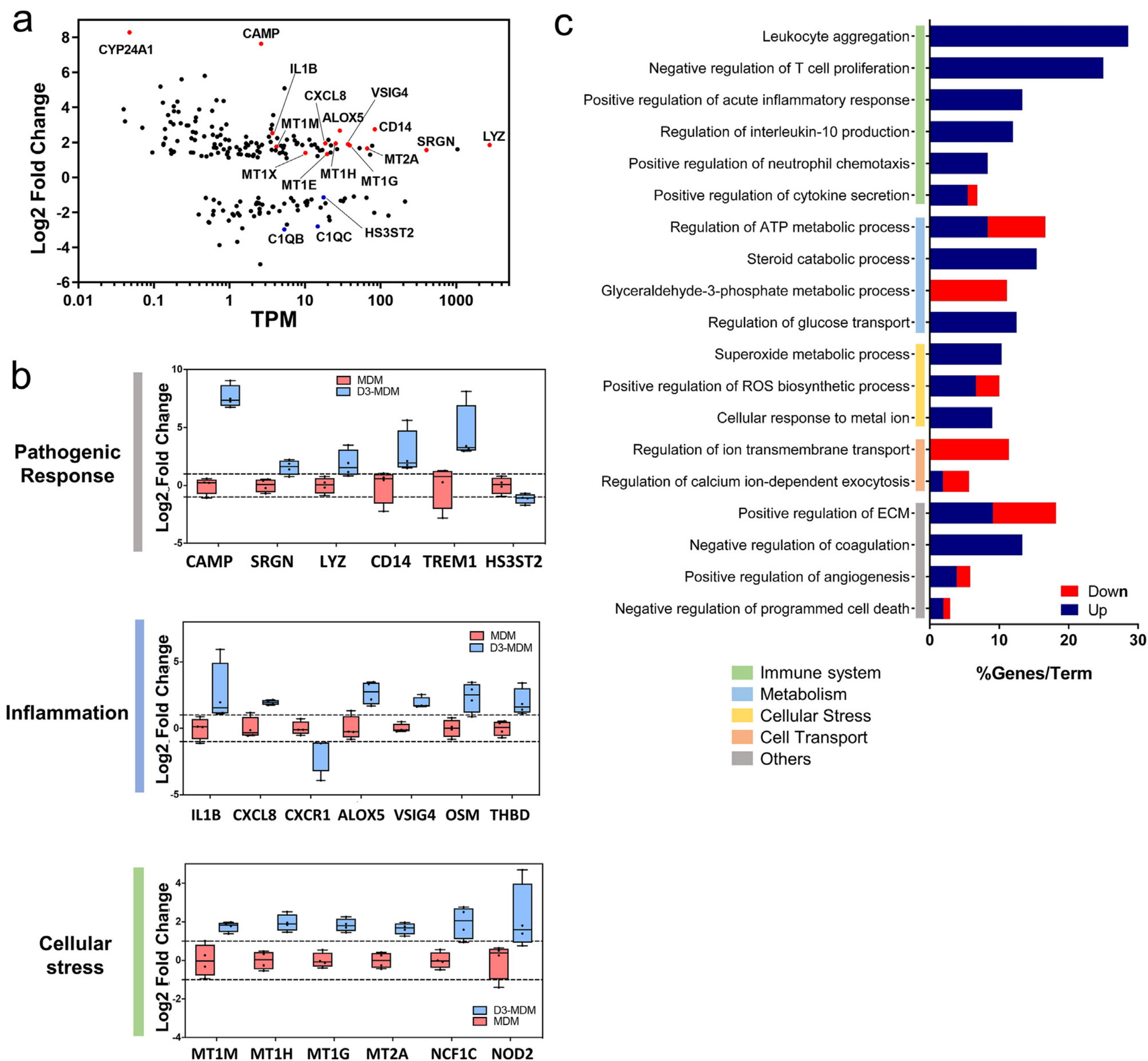


Fig. 2. Genes and regulatory pathways related to Vitamin D differentiated macrophages. (A) Scatterplot comparing abundance (TPM, x-axis) and their degree of expression (log2 fold change, y-axis). Each dot represents differentially expressed genes (DEG; fold-change > 2 and FDR < 0.05;) relevant to macrophage biological function are highlighted (red and blue dots represent up-and down-regulated genes, respectively) (B) Boxplots of normalized differentially expressed genes with a key function on pathogenic response, inflammation, and cellular stress for MDMs and D3-MDMs. The threshold for up-and down-regulated (|fold change| ≥ 2) is indicated by dashed lines. (C) Gene-term enrichment analysis of DEG in D3-MDMs. The colored horizontal bars represent the percentage of genes present in the data set compared to the total number of genes in each term (% Genes/Term). The fraction of up-and down-regulated genes (horizontal bars) in each term are shown in red and blue, respectively. The vertical colored bars (y-axis) represent major gene terms modules.

showed genes *CXCL8*, *IL1β*, and Kinase Insert Domain Receptor (*KDR*) present in the highest number of interactions, suggesting a prominent role of Vitamin D in transcriptional reprogramming of macrophages (Fig. 3).

3.3. Transcriptional regulation in Vitamin D differentiated macrophages

Based on our results we hypothesize that Vitamin D-induced changes in gene expression in macrophages may be associated with transcriptional control. To address it, we performed an analysis of transcription factors (TF). Venn diagram revealed that MDM and D3-MDM expressed 895 TF out of the nearly 1500 reported in humans (Fig. 4A). Out of 895 expressed TF, D3-MDM affected the expression of 10 TF resulting in over two-fold changes. Of these genes, 5 were up-regulated, including *MYCL*, *TBX21*, and *HOPX* (Fig. 4B). Remarkably, *MLXIPL* and *EPAS1* have been shown to be associated with immune suppression and metabolism, respectively [46].

Additionally, we analyzed the enrichment of transcriptional motifs in the promoter sequences of differentially expressed genes. Remarkably, promoters of the up-and down-regulated genes revealed enrichment of *VDR* (Figs. 4C and D), implying a dual role of *VDR* acting as a transcriptional activator or repressor of gene expression. We also noted enrichment of other TF; among these are TF related to cellular proliferation and differentiation (*ESR1* and *FOXP1*), cell growth (*CEBP: AP1* and *PLAGL1*), and TF involved in immune response (*IRF1*) (Fig. 4C).

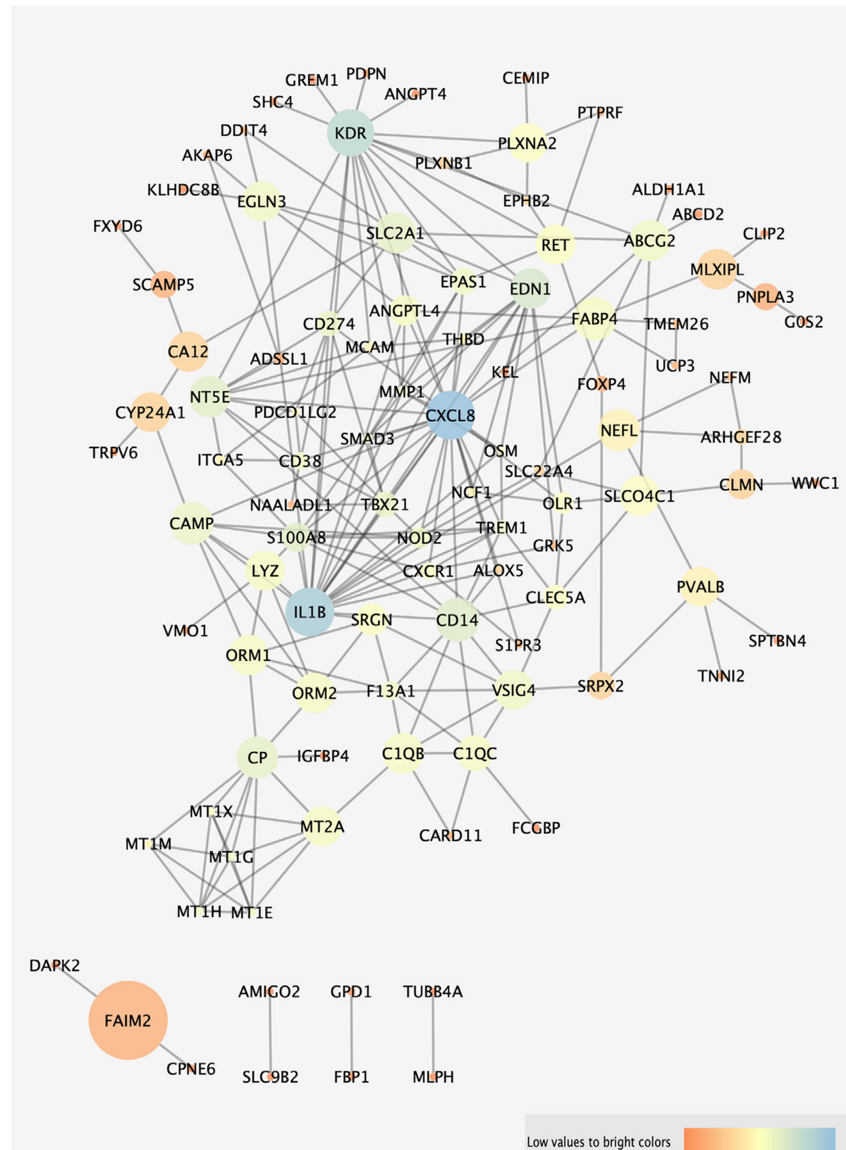


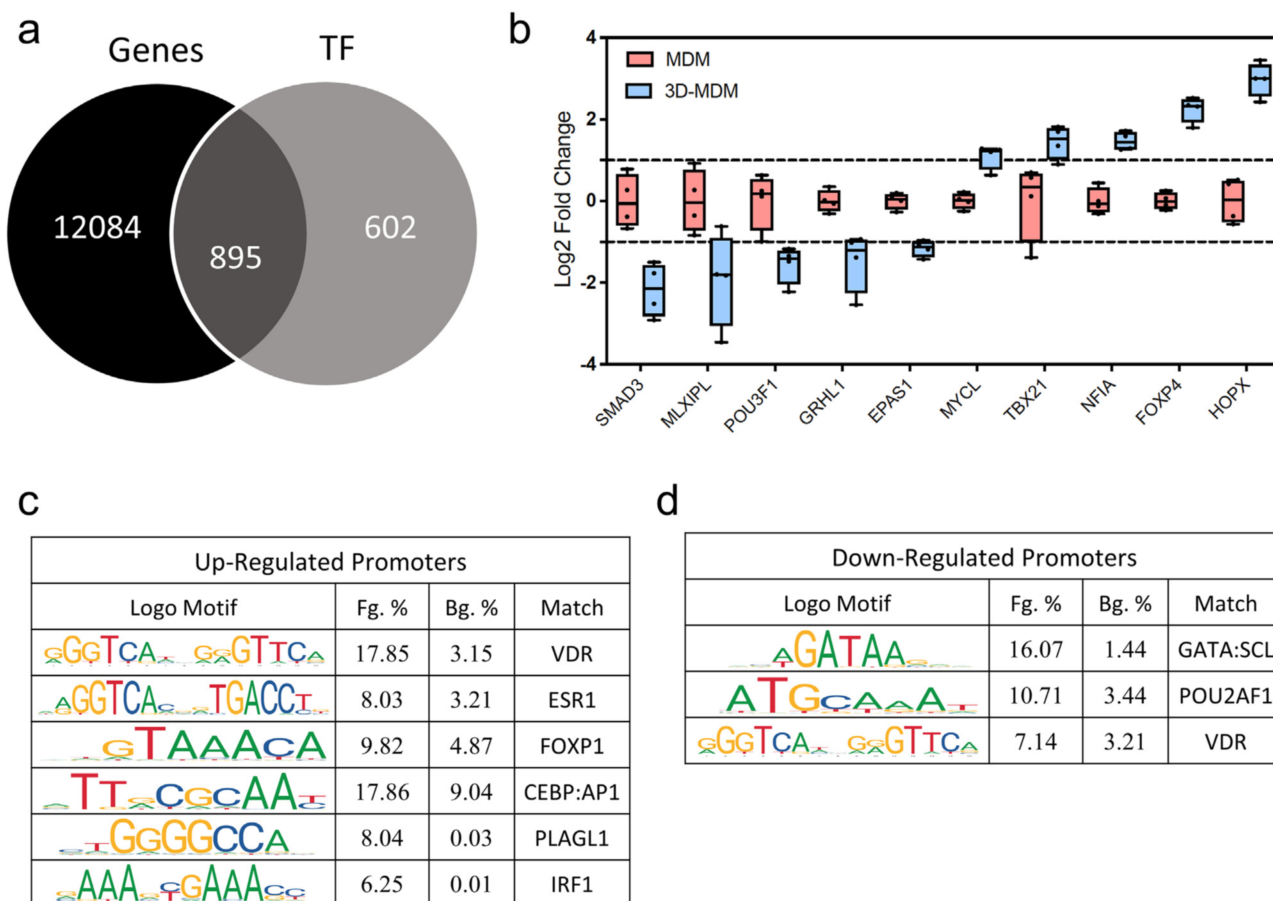
Fig. 3. Protein-protein interaction (PPI) network in D3-MDMs. Colors highlight the betweenness centrality of the node. The larger the node, the higher the number of interactions identified. STRING v10.5.1 was used to generate protein interactions, and the resulting network was visualized using Cytoscape v3.4.0.

3.4. Vitamin D regulates differential expression of miRNA during macrophage differentiation

Next, we asked if Vitamin D modifies miRNA expression levels during macrophage differentiation. We found that out of 2,654 human mature miRNAs, 364 were expressed in D3-MDMs. Vitamin D differentiated macrophages significantly affected the expression of 17 miRNAs (FDR $\leq$ 0.05 and |fold change| $\geq$ 1.5), of which 7 were up-regulated and 10 were down-regulated (Fig. 5A and *Table S3*). Principal component analysis (PCA) showed that expression profile of miRNAs is distinct in MDMs and D3-MDMs (Fig. 5B). PCA and heatmap showed variability within experimental groups (Figs. 5A and B). Furthermore, miRNAs that were differentially expressed exhibited low number of transcripts in the cell (TPM between 10 and 1.000) (Fig. 5C). Of these miRNAs, miR-6501-3p, miR-665, miR-1972, miR-1183, and miR-619-5p were down-regulated less than four times in D3-MDMs, compared to MDMs (Fig. 5C). We also explored whether the differential expression of miRNAs was dependent on transcription factor *VDR*. For this, we reanalyzed the ChIP-seq data performed on THP1 macrophages treated with vitamin D for 24 h [38]. The analysis showed that *VDR* is only associated with the gene locus for miR-619-5p in the intronic region of the gene *SSH1* (Fig. 5D). This implies that among the miRNAs whose expression is regulated in Vitamin D differentiated macrophages, only miR-619-5p is a direct *VDR* target. Results imply substantial differences in post-transcriptional regulation of miRNA, among MDMs and D3-MDMs.

3.5. miRNAs modulate host-defense genes in Vitamin D differentiated macrophages

To understand the impact of miRNAs modulated in Vitamin D differentiated macrophages, we considered contrasting effects of deregulated expression between miRNA and target mRNAs in the same samples. This approach showed that out of 17 differentially expressed miRNAs, 13 miRNAs have predicted targets in mRNA (*Table S4*). We observed 147 miRNA-mRNA interactions for those 13 miRNAs (*Table S4*) that imply target multiplicity. The analysis re-



Up-Regulated Promoters

Logo Motif	Fg. %	Bg. %	Match
	17.85	3.15	VDR
	8.03	3.21	ESR1
	9.82	4.87	FOXP1
	17.86	9.04	CEBP:AP1
	8.04	0.03	PLAGL1
	6.25	0.01	IRF1

Down-Regulated Promoters

Logo Motif	Fg. %	Bg. %	Match
	16.07	1.44	GATA:SCL
	10.71	3.44	POU2AF1
	7.14	3.21	VDR

Fig. 4. Transcription factors mediating gene expression in Vitamin D differentiated macrophages. (A) Venn diagram of total genes expressed in macrophages and total transcription factors annotated in the human genome. (B) Box plots of differentially expressed transcription factors. Pink and light blue colors represent MDMs and D3-MDMs, respectively. The threshold for up-and down-regulated (|fold change| ≥ 2 and FDR <0.05) are indicated by dashed lines. De novo motif analysis was performed on promoters (-300 and +50 relative to Transcription Start Site, TSS) of up- (C) and down-regulated (D) genes. Motifs were compared by using the transcription factor HOMER database to determine the closest annotated matches. Percentage (%) represents a fraction of foreground (Fg) and background (Bg) sequences that contain at least the occurrence of one motif.

vealed that among the down-regulated miRNA, miR-1273h-5p has a higher number of target sites in mRNAs ($n = 25$) (Fig. 6A). Target genes of this miRNA were related to inflammation such as *ALOX5, ANGPTL4, CD274, CD38,* and *IL1β* (Fig. 6B).

Based on the integrative miRNA-mRNA analysis, we identified pathways enriched for deregulated genes targeted by differentially expressed miRNAs (Fig. 6C). Gene-enrichment analysis revealed miRNA interactions affecting mRNAs mainly related to the inflammatory and cell stress phenotype. To elucidate the functions of mRNAs and miRNAs interactions, we integrated Gene-enrichment analysis with an alluvial plot. Furthermore, we noted that miR-1972, miR-1273h-5p, and miR-665 regulate genes *PDCD1LG2, IL1β,* and *CD274* related to an inflammatory response (Fig. 6C).

4. Discussion

Despite numerous studies on the effect of Vitamin D on innate immune system, its overall effect on genomic and non-genomic targets in immune cell types is still not fully understood [4,47–49]. It seemed essential to characterize transcriptional and post-transcriptional regulatory events that underlie the effects of Vitamin D in macrophages, one of the main cell types of innate immune system. We interrogated miRNA-Seq and mRNA-Seq data to determine the extent of Vitamin D-regulated transcription of macrophages. Our analysis indicates that genes associated with host response to pathogens, including inflammation, and respiratory burst events are primarily regulated by miRNAs, whose expression is regulated by Vitamin D during monocyte-derived macrophage differentiation. Thus, in addition to the regulation of genes of innate immune system, such as cathelicidin and defensins, genes that encode proteins related to inflammation and respiration burst may also be regulated by miRNAs and contribute to the Vitamin D effect.

Our RNA-Seq data expands on prior genome-wide studies of the effects of Vitamin D on gene expression in innate immune cell [29,39,50–56], on two levels: (1) Previous studies focused on human monocytic cell line THP-1. Our studies rely on interrogating gen expression in human macrophages as model primary cells and (2) We integrated mRNA and microRNA transcriptome profiling from the same set of samples. The experimental design allowed us to distinguish miRNA targets with higher accuracy and sensitivity for the identification of transcriptional and post-transcriptional events modulated by Vitamin D in macrophage differentiation. mRNA and miRNA expression profiles distinguished MDMs from D3-MDMs. In particular, results show that Vitamin D can reprogram cellular fate of macrophages. It is known that macrophages exhibit great phenotypic variability, the two states have been described as M1 and M2 [57]. However, single-cell RNA-Seq data has identified a spectrum of macrophage subsets characterized by heterogeneous transcriptional signatures [58,59]. Our results show

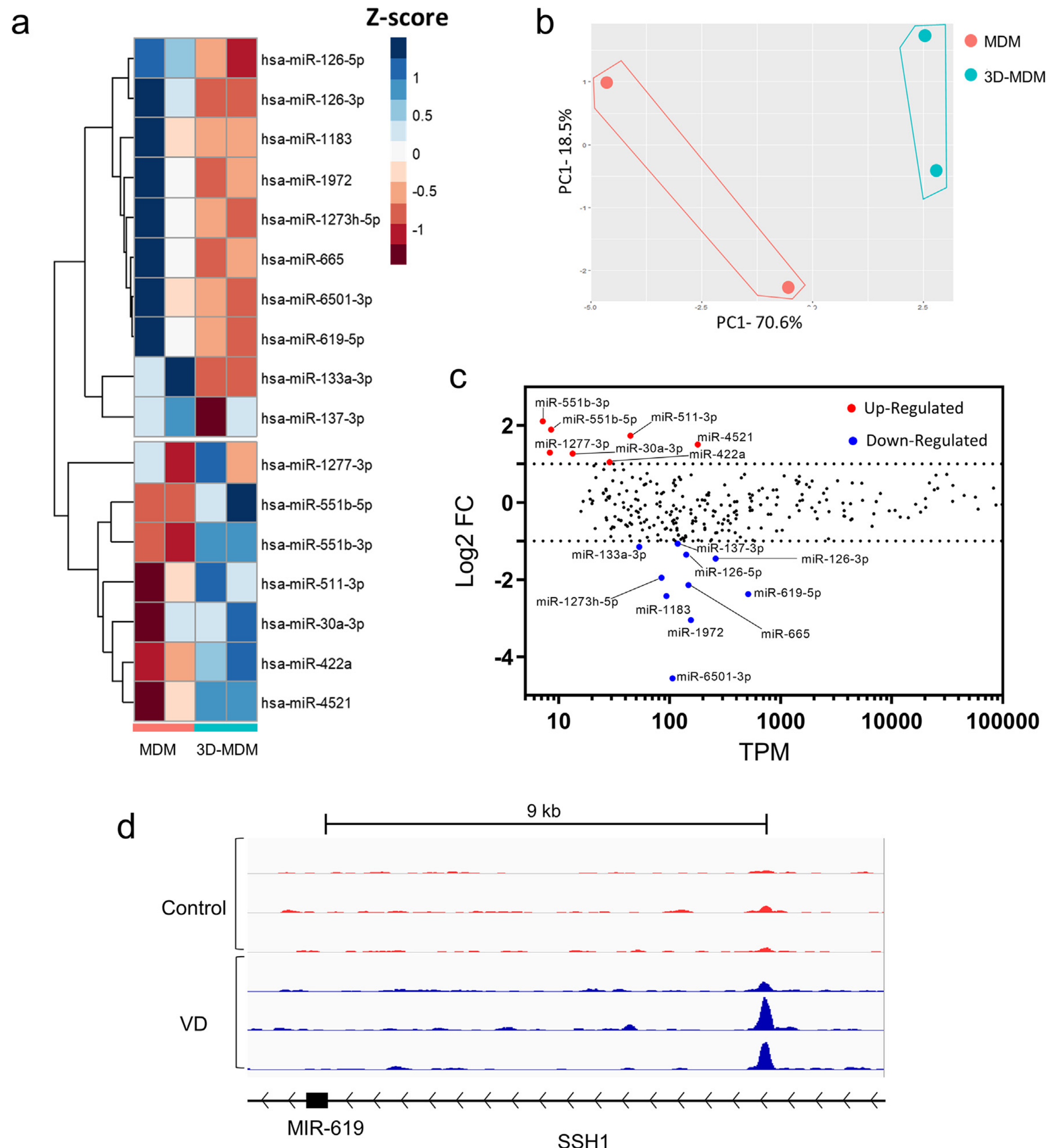


Fig. 5. miRNA profile of Vitamin D differentiated macrophages. (A) Heatmap of 17 Z-score normalized differentially expressed miRNAs of MDMsand D3-MDMsby unsupervised hierarchical clustering analysis. Down-regulated and up-regulated miRNAs with absolute values of fold-change > 2 and FDR < 0.05 are shown in red and blue, respectively. (B) Principal component analysis of miRNA data of MDMs and D3-MDMs. Each principal component (PC1 and PC2) is showed the percentage of the variance. (C) Scatterplot comparing abundance (Transcripts per Million, TPM; x-axis) and their degree of expression (log2 fold change, y-axis). Each dot represents differentially expressed miRNAs (fold-change > 1.5 and FDR < 0.05), and red and blue dots (up-and down-regulated miRNAs, respectively). The threshold for up-and down-regulated (|fold change| ≥ 1.5) is indicated by dashed lines. (D) Genomic tracks display VDR ChIP-seq data. Shown are enrichment profiles for VDR on THP1 control macrophages (Red) and treated with vitamin D (blue), across a 9 kb region where is found the miR-619 inside the intron of the gene SSH1.

that macrophages are transcriptionally reprogramed in response to Vitamin D, implying a new functional state. Moreover, it is evident from the PCA analysis that there is an inter-individual variation in mRNA and miRNA expression in Vitamin D differentiated macrophages, (perhaps related to individual genetic diversity). In fact, GWAS and eQTL studies have linked Vitamin D deficiency with several gene polymorphisms including but not limited to *CYP2R1* (cytochrome P450 Family 2 Subfamily R Member 1), *CYP24A1* (Cytochrome P450 Family 24 Subfamily A Member 1), *GC* (GC Vitamin D Binding Protein), *DHCR7* (7-Dehydrocholesterol Reductase), *CCL28* (C-C Motif Chemokine Ligand 28), *PAIP1* (Polyadenylate-binding protein-interacting protein 1), *ETV3L* (ETS variant transcription factor 3 like), and *FRMD6* (FERM domain-containing protein 6) [22,60,61]. As well, transcriptome studies in human peripheral blood mononuclear cells show changes in gene expression in response to Vitamin D supplementation [62,63].

Here, we have shown that Vitamin D transcriptional reprogramming boost some of the biological functions of the macrophages such as host-pathogen response, inflammation, and respiratory burst. As expected of host-pathogen response, we noted up-

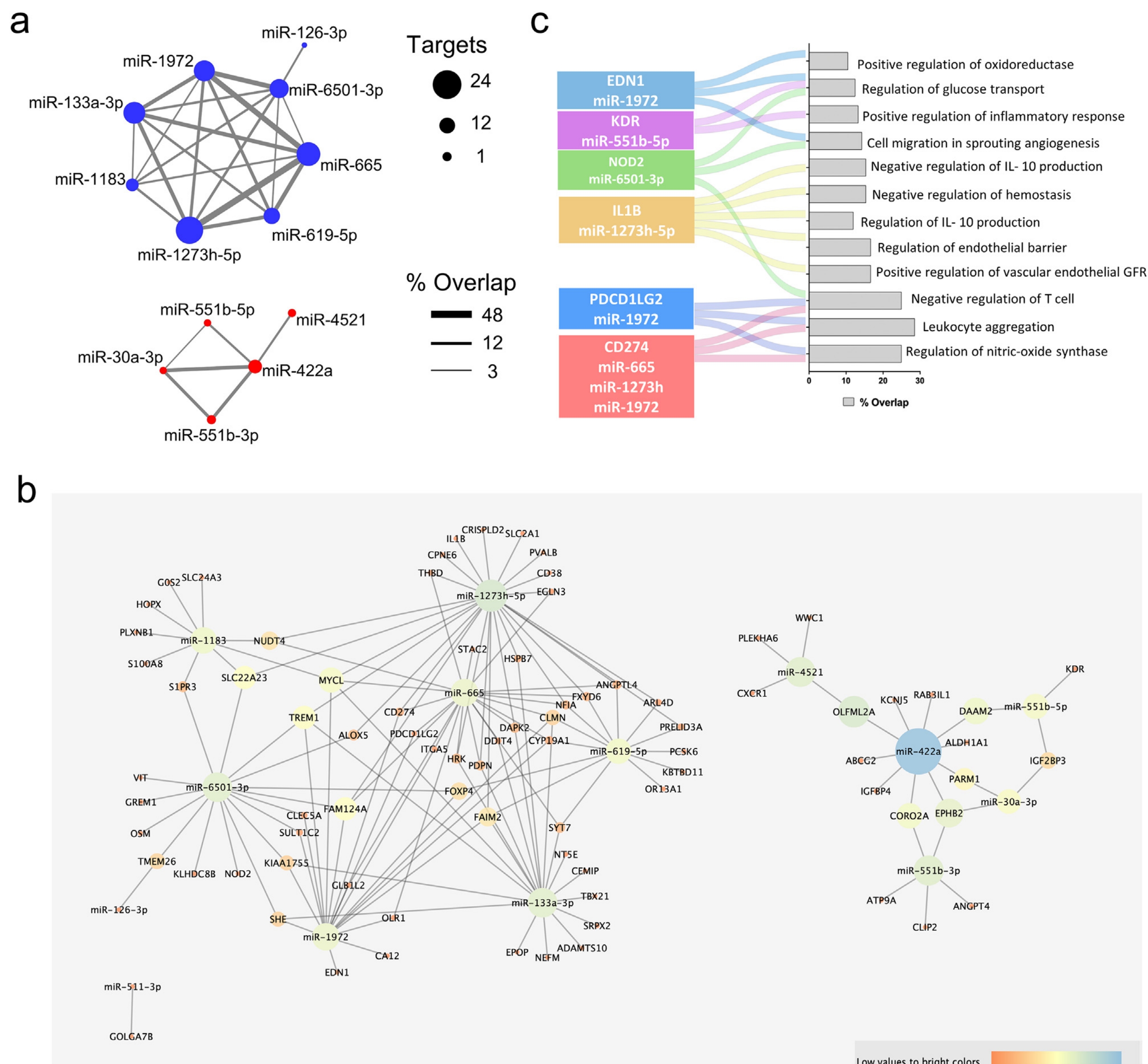


Fig. 6. The miRNA–target gene interactions of Vitamin D differentiated macrophages. (A) The network generated is consisted of 147 interactions between 13 miRNAs and 81 unique target genes. Up-and down-regulated miRNAs (fold-change > 1.5 and FDR < 0.05) are represented by red and blue nodes, respectively. Node size indicates the number of miRNA-target gene transcripts, and gray edge width denotes overlapping miRNA-target gene transcripts measured by the Jaccard coefficient (JC). (B) The miRNA–mRNA target interaction network in D3-MDMs. The larger the circles, the higher the number of interactions identified. (C) Gene-Ontology analysis of the mRNAs predicted and validated as regulated by microRNAs. Each horizontal black bar represents the ontology term enrichment.

regulation of antimicrobial peptides such as CAMP (LL-37) and LYZ (lysozyme) that function in defense of invasive bacterial and virus infections [64–67]. In fact, Vitamin D response elements (VDREs) were found adjacent to the transcription start sites of *CAMP* gene [16]. Antiviral mechanisms related to LL-37 implicate viral dsRNA interaction, which facilitates its recognition by the TLR3 [68]. As well, LL-37 peptide binds directly to SARS-CoV-2 Spike protein and prevents binding to the ACE2 receptor, and inhibits the virus entry into host cells [69].

Similarly, our data revealed that Vitamin D3 treatment up-regulated *SRGN* gene (Serglycin) transcription that has been reported to act in the formation of storage granules and secretory vesicles in some antimicrobial peptides [70,71]. In addition to antimicrobial peptides, Vitamin D up-regulates genes that enhance recognition of pathogens, such as *CD14* and *TREM1*, the last one known as a Vitamin D target gene [72], both involved in TLR1-4, 6, 7, and 9 signalings pathway in pathogen recognition [73–75].

Moreover, our results suggest that D3-MDMs represent a cell population with high plasticity with diverse functions including pro- and anti-inflammatory responses. Differentiation of macrophages in the presence of Vitamin D enhances the rate of transcription of genes involved in the inflammatory response, a key feature against pathogens [76]. Expression of genes such as *IL1β*, *CXCL8*, *AlOX5* and *NINJ4*, were up-regulated. *IL1β* [77,78], *CXCL8* [78–80], *CXCR1* [81], and *OSM* [82,83] and leukotrienes Synthase (ALOX5 [84]), are involved in the up-regulation of pro-inflammatory signals. *IL1β* is one of the first cytokines triggered in response to an infection via pathogen-directed activation of the inflammasome [85]. Vitamin D induces *IL1β* expression in the control of *Mycobacterium tuberculosis* infection [86]. On the other

hand, we observed an up-regulation of the *THBD* gene, which encodes thrombomodulin (TM) protein. Our finding is in agreement with previous report that *THBD* is a Vitamin D target in both monocytes and PBMCs [62,87]. TM functions as a cofactor in the thrombin-induced activation of protein C in the anticoagulant pathway [88]. Since TM can bind LPS and trigger the CD14 /TLR4/MD-2 complex, and thus may prevent pro-inflammatory consequences of NF-kB signaling [88].

Our results showing up-regulation of *VSIG4* gene, which encodes V-set immunoglobulin domain containing 4 (VSIG4) is interesting since it has been reported that VSIG4 inhibits proinflammatory macrophage activation by reprogramming mitochondrial pyruvate metabolism [89]. Also, VSIG4 is a strong negative regulator of murine and human T cell proliferation and IL-2 production [90]. Since our results show an increase in the rate transcription of the VSIG4 gene in D3-MDMs, we propose that the expression of this gene is regulated directly or indirectly, by Vitamin D. Our hypothesis is supported by the presence of VDRE in the gene promoter. To the best of our knowledge, this is the first study on the relationship between VSIG4 and Vitamin D.

In addition, our findings suggest that Vitamin D enhances the activation of macrophages respiratory burst by up-regulation of the *NCF1* gene, which encodes neutrophil cytosolic factor 1 (NCF1, earlier denoted $p47^{phox}$), a cytosolic component of the NADPH oxidase 2 complex [91]. It has been reported that *NCF1* is required for the NOX2 complex to trigger oxidative burst, i.e., increases ROS production [92], and contributes to the removal of phagocyted pathogens [93]. Moreover, it has been reported that Vitamin D promotes ROS *via* activating NADPH oxidase complexes, NOX4, $p22^{phox}$, and NFC1 [94]. Cells protect themselves by the up-regulation of some members of the metallothionein [95–97]. Here, we found up-regulation of many isoforms of the *MT* family. *MT* has been linked to a variety of functions such as protection from oxidative damage, angiogenesis, suppression of cytotoxic T cells, promotion of regulatory T cells, polarization in macrophages, and NK cell activity [98–101]. Our data suggest regulation of *MT* expression by Vitamin D on macrophages, as has been reported for keratinocytes in vivo [102]. Nucleotide-binding and oligomerization domain-containing protein 2 (NOD2) is a member of the NOD-like receptor (NLR) family. NOD2 played an important role in reactive oxygen species generation [103]. We found that *NOD2* was up-regulated in D3-MDMs and one of the genes involved in cellular stress. Noteworthy transcription of the NOD2 gene has been related to Vitamin D treatment in many cells [29,104]. In fact, Vitamin D upregulated *NOD2*, and induced the antimicrobial human neutrophil peptides (HNP1-3) and LL-37, resulting in increased killing of pneumococci in a VDR-dependent manner [105].

To explore a whole image of the regulatory mechanisms behind the Vitamin D transcriptional reprogramming, we focus to identify transcriptional regulation. Our motif analysis for up-regulated genes identified enrichment for several TF, including *MYCL, TBX21, NFIA, FOXP, HOPX*, and *IRF1*, among others. These factors have already been described in the regulation of macrophage function by activating genes related to the immune response against pathogens (*VDR* [49]), differentiation and macrophage function (*FOXP1* [106–108]), host inflammatory, antimicrobial defenses (*IRF1* [109] and *NFIA* [110]), and T-cell activation (*MYCL* [111]). Also, we found down-regulation transcription factors that control the expression of genes related to the suppression of an anti-inflammatory phenotype (*SMAD3* [112]) and genes linked to shifting to an oxidative metabolism (*MLXIPL* [113] and *EPAS1* [114]). Thus, transcriptional control analysis suggests that Vitamin D transcriptional reprogramming of macrophages can only be understood in the context of simultaneous transcription factors as opposed to the idea that the VDR is a single master regulator [30].

In addition to transcriptional control, identifying miRNA-mediated post-transcriptional regulations is also crucial to understanding the genetic regulation of Vitamin D. To have better predictions of miRNA-mRNA interactions, we measure the expression profiles in the same set of samples. This approach allowed us to identify biological processes such as inflammatory phenotype, respiratory burst, and T-cell activation as regulated by miRNAs in Vitamin D differentiated macrophages. Within these interactions, we highlight the up-regulation of the miR-1972, which regulates many targets such as *EDN1, CD274,* and *PDCD1LG2* that are related to respiratory burst and T-cell activation, respectively. Also, we found some differentially expressed miRNAs with key immune functions as listed below: (1) miR-655 facilitates cell proliferation by targeting Wnt5a/β-Catenin and represses cell apoptosis by targeting TRIM8 [115]; (2) miR-511-3 up-regulation has been associated to an M2 phenotype in macrophages [116] and the control of pathogen responses through TLR4 signaling inflammation [117], and (3) miR-551b-5p was reported involved in the regulation of inflammatory response [118]. On the other hand, miR-422a has been reported to target SMAD transcription factors family expression [119]. Although our data show an up-regulation of miR-422a and a down-regulation of SMAD3, the bioinformatic analysis does not highlight it.

Regarding the link between miRNA expression and Vitamin D, it has been reported that intake of Vitamin D negatively correlated with the expression of the highest number of circulating miRNAs, particularly miR-1277-5p, which was also down-regulated in our data [120]. The fact that we observed high mRNA expression and downregulation in the miR-1273h-5p suggests that *IL1*β might be the target gene of miR-1273h-5p. In the same way, our RNA-Seq profiling revealed an increased level of NOD2 mRNA and down-regulation of miR-6501-3p, suggesting *NOD2* expression might be regulated by miRNA, being miR-6501-3p one candidate. Taken together, we suggest that this can be one mechanism by which vitamin D regulated inflammatory response. Also, it has been described as an increased anti-inflammatory property by Vitamin D through the promotion of *VDR* and miR-126-3p expression in endothelial cells [121]. On the other hand, the *VDR* ChIP-seq data suggest regulation of miR-619 by Vitamin D, but so far no experiment has shown a causal relationship. The above suggests that there is not enough experimental evidence to suggest that Vitamin D directly regulates the expression of miRNAs on macrophages.

While our data points out key genes that boost defense against pathogens and identified transcriptional and post-transcriptional gene expression control, our in-silico approach is restricted in the lack of experiments to quantify transcription factors sequence-specific DNA-binding activity. In the same way, we predicted miRNA-target interactions that deserve experimental verification. While mRNA levels explain ~84% of protein levels [122], our RNAseq data could be validated at the protein level to confirm the expression of key genes. Moreover, we hypothesize that Vitamin D may participate in regulating the pathogen response, inflammation, and cellular stress, in Vitamin D differentiated macrophages, which requires further validation. In the same way, the expression profiles of RNAs (mRNAs and miRNAs) were discussed in this study, it still needs more validated evidence to support the results.

5. Limitations of the study

A major limitation of this work was that the results were obtained thanks to the transcriptome analysis of RNA-Seq but no real protein change was determined. Nevertheless, our experimental approach allowed us to dissect an alternate mechanism by which

Vitamin D controlling gene expression in D3-MDMs is linked with inflammation, host defense against pathogens, and cellular stress. Further developments and validations may provide a reference for in-depth analysis of the molecular mechanism through which vitamin D regulated gene expression.

Another limitation of this study is the vitamin D concentration used, which is > 10 times of serum concentration that adult humans received with vitamin D supplements. However, the selection of the concentration of vitamin D was based on two parameters: (1) In our laboratory doing experiments treating MDMs with 0.1nM and 1nM of 1,25-(OH)2D3, only the concentration of 1nM has an effect in decreasing viral infection (manuscript in preparation). (2) 1nM concentration has been used in the literature in experiments using in vitro cell culture.

6. Conclusion

This study revealed that the monocytic-derived macrophages differentiated in the presence of Vitamin D3, pointed out an enhanced inflammatory response, host-pathogen response, and respiratory burst determined through mRNA-Seq and miRNA-Seq data.

Author statement

Thank you for providing us with the opportunity to resubmit the revised version of our manuscript entitled "Vitamin D boosts immune response of macrophages through a regulatory network of microRNAs and mRNAs." Based on the constructive criticism from the reviewers, we believe the corrections and alterations we have done significantly improved the manuscript.

A point-by-point response to the remarks and suggestions by the reviewers was submitted according to the journal instructions. In the revised version of our manuscript, we made modifications based on the reviewer's suggestions. We hope that after our thorough revision, the manuscript can be considered for publication in The Journal of Nutritional Biochemistry

Author contributions

Conceptualization (GJF and SUI); Data curation (GJF, JMRM, and SUI); Formal analysis (GJF and JMRM); Funding acquisition (GJF and SUI); Investigation (GJF and JMRM); Methodology (GJF and SUI); Project administration (GJF and SUI); Resources (SUI); Software (GJF); Supervision (SUI); Visualization (GJF); Writing – original draft (GJF); Writing – review & editing (GJF, JMRM, and SUI). All the authors have read the paper and have agreed to be co-authors data Availability Statement

The datasets generated for this study can be found in the Gene Expression Omnibus (GEO) DataSets (https://www.ncbi.nlm.nih.gov/gds) under the accession numbers:GSE209698

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Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2022.109105.

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