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1,25-Dihydroxyvitamin D₃ Does Not Affect MicroRNA Expression When Suppressing Human Th17 Differentiation

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Background: Vitamin D is an import regulator of T helper 17 (Th17) differentiation, but our understanding of the underlying mechanisms remains limited. In the present study, we aimed to detect the expression levels of microRNAs (miRNAs) during human Th17 differentiation and evaluate the effects of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), the bioactive form of vitamin D, on Th17 differentiation and miRNA expression.

Material/Methods: We cultured human peripheral blood mononuclear cells (PBMC) *in vitro* and activated them with anti-CD3 and anti-CD28 antibodies in the presence of Th17-promoting cytokines interleukin (IL)-23, IL-1β, TGF-β1, and IL-6 for 72 hours. 1,25(OH)₂D₃ was added to the medium at a final concentration of 100 nM on day 0. The production of IL-17A in culture medium was detected by enzyme-linked immunosorbent assay (ELISA). The expression levels of miRNAs during Th17 differentiation were determined by quantitative polymerase chain reaction (qPCR).

Results: Six miRNAs were found to be dysregulated during human Th17 differentiation. Of these miRNAs, hsa-miR-155 was significantly up-regulated (median fold change: 3.61, *P*<0.05), whereas hsa-miR-20b, hsa-miR-21, hsa-miR-181a, hsa-miR-210, and hsa-miR-301a were significantly down-regulated (median fold change: 0.44, 0.37, 0.18, 0.15, and 0.26, respectively, *P*<0.05). 1,25(OH)₂D₃ treatment significantly decreased IL-17A production (median [interquartile range], 745.7 [473.5] pg/mL vs. 2535.4 [2153.3] pg/mL, *P*<0.05). However, expression of these miRNAs was not changed after 1,25(OH)₂D₃ treatment.

Conclusions: 1,25(OH)₂D₃ suppressed human Th17 differentiation without affecting miRNA expression.

MeSH Keywords: Calcitriol • MicroRNAs • Th17 Cells

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Background

T helper 17 (Th17) cells are a subset of CD4⁺ Th cells characterized by the production of high levels of interleukin (IL)-17A (also known commonly as IL-17) [1]. Besides IL-17A, Th17 cells secrete other pro-inflammatory cytokines, such as IL-21, IL-22, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor. Differentiation of naïve CD4⁺ T cells into Th17 cells requires the coordinated activities of several microenvironmental cues. The cytokines IL-6 and TGF- β induce Th17 cell differentiation by activating retinoid-related orphan receptor (ROR)- γ t, a master transcription factor required for the generation of Th17 cells [2]. IL-1 β and IL-23 cytokines can promote and stabilize previously differentiated Th17 cells [3]. Accumulating evidence suggests that Th17 cells are major drivers of inflammation and have been implicated in the pathogenesis of multiple autoimmune and immune-mediated inflammatory diseases, including rheumatoid arthritis, multiple sclerosis, asthma, and Behçet's disease [4–8]. Given the important role of IL-17 cells in regulating autoimmunity and inflammation, elaborating the factors and processes that govern the differentiation of Th17 cells are necessary for developing Th17-targeted therapies.

Vitamin D is an important regulator of the immune system. Almost every immune cell type has been found to express vitamin D receptor (VDR) and/or the enzyme CYP27B1 that is required for the generation of the bioactive form of vitamin D, 1,25(OH)₂D₃ (also known as calcitriol) [9]. The action of 1,25(OH)₂D₃ is based on its binding to VDR, which is a ligand-dependent transcription factor, belonging to the steroid receptor superfamily. Upon ligand binding, VDR undergoes heterodimerization with the retinoid X receptor (RXR) and binds to hormone response elements on DNA, resulting in expression or transrepression of specific gene products [10]. Both *in vitro* and *in vivo* studies have found that 1,25(OH)₂D₃ treatment inhibits T cell commitment to the Th17 lineage as well as Th17 production of IL-17A. 1,25(OH)₂D₃ directly inhibits Th17 cell differentiation through the VDR signal in CD4⁺ T cells, independent of the IL-2, IL-10, and STAT1 signals [11]. In addition, 1,25(OH)₂D₃ suppresses cytokines in Th17 cells by inducing the C/EBP homologous protein (CHOP), a molecule involved in endoplasmic reticulum stress and translational inhibition [12]. Furthermore, 1,25(OH)₂D₃ inhibits production of IL-17A by blocking nuclear factor for activated T cells (NFAT), recruitment of histone deacetylase (HDAC), sequestration of Runt-related transcription factor 1 (Runx1), and induction of forkhead box P3 (Foxp3), a transcription factor associating with NFAT and Runx1 for transcriptional repression [13]. Although previous studies have obtained some encouraging evidence, our understanding of the mechanisms by which 1,25(OH)₂D₃ regulates Th17 differentiation remains limited.

MicroRNAs (miRNAs) are endogenous, small, non-coding RNAs approximately 22 nucleotides (nt) in length that are highly conserved across various species of eukaryotes. miRNAs post-transcriptionally repress gene expression by binding to complementary sequences in the 3' untranslated region (UTR) of target messenger RNA (mRNA) [14]. miRNAs are vital for controlling many processes within the immune system including the development and differentiation of lymphocytes, secretion of cytokines and chemokines, antibody switching, and regulation of immune tolerance [15]. Recently, several miRNAs have been implicated in Th17 cell differentiation. miR-155 promotes Th17 cell differentiation and IL-17A production by targeting suppressor of cytokine signaling 1 (SOCS-1), which is a molecule responsible for the negative regulation of the JAK-STAT pathway [16,17] and suppressing the inhibitory effects of the DNA-binding protein Jarid2 [18]. miR-155-deficient mice show a defect in Th17 differentiation and strong resistance to experimental autoimmune encephalomyelitis (EAE), an experimental animal model for central nervous system demyelinating disease [18,19]. miR-20b expression is down-regulated in Th17 cells during mouse EAE, and over-expression of miR-20b inhibits Th17 differentiation and ameliorates EAE by targeting ROR- γ t and STAT3 [20]. In addition, miR-21, miR-181a, miR-210, and miR-301a are found to be involved in the development of Th17 cells [21–25].

Since both miRNAs and 1,25(OH)₂D₃ are crucial regulators of Th17 differentiation, it is of great interest to investigate their interaction in regulating Th17 differentiation. In the present study, we aim to evaluate the expression of several candidate miRNAs during Th17 differentiation and the effects of 1,25(OH)₂D₃ on Th17 differentiation and miRNA expression.

Material and Methods

Peripheral blood mononuclear cells (PBMC) isolation

Human peripheral blood was obtained by venipuncture from 3 young healthy subjects (LZB, KY, and JFJ) and was collected into K2-ethylene diamine tetraacetic acid (K2-EDTA) vacutainer tubes (Shenzhen Medrey Medical Technology, China). Whole blood (4 mL) was mixed with 4 mL of phosphate-buffered saline and layered gently on 4 mL of lymphocyte separation medium (Dakewe Biotech Company, China) in a 15-mL centrifugate tube. PBMC were separated by using density centrifugation in a 25-min centrifugation step at 2500 rpm at room temperature. The mononuclear layer was collected from the interface of the blood plasma and the separation medium. After separation, PBMC were washed and cultivated at a density of 1.0×10⁶ cells/mL in RPMI 1640 (KeyGEN Biotech, China) containing 10% fetal bovine serum, L-glutamine, NaHCO₃, penicillin,

Table 1. qPCR primers for miRNAs.

miRNA	Accession number in miR base	miRNA sequence	5' primer
hsa-miR-20b	MIMAT0001413	CAAAGUGCUCAUAGUGCAGGUAG	GGCTCATAGTGCAGGTAGAAA
hsa-miR-21	MIMAT0000076	UAGCUUAUCAGACUGAUGUUGA	TCGTGGCAGACTGATGTTGAAAA
hsa-miR-155	MIMAT0000646	UUAUUGCUAAUCGUGAUAGGGGU	CAATGCTAATCGTGATAGGGGT
hsa-miR-181a	MIMAT0000256	AACAUUCAACGCUGUCGGUGAGU	CCTTCGCTGTCGGTGAGTAAA
hsa-miR-210	MIMAT0026475	AGCCCCUGCCACCGCACACUG	CGTTCACCGCACACTGAAA
hsa-miR-301a	MIMAT0022696	GCUCUGACUUUAUUGCACUACU	GCTCTGACTTATTGCACTACT

and streptomycin. Cells were incubated in a humidified atmosphere (37°C and 5% CO₂).

Th17 cell generation

To generate Th17 cells in PBMCs, anti-CD3 mAb (0.5 ng/L; BioLegend, USA), anti-CD28 mAb (0.5 ng/L; BioLegend, USA), recombinant human IL-1β (rhIL-1β; 100 ng/mL), rhIL-23 (100 ng/mL), rhIL-6 (100 ng/mL), and rhTGF-β1 (25 ng/mL) (all cytokines from PeproTech, USA) were added to the medium on day 0. When 1,25(OH)₂D₃ (Selleck Chemicals, USA) was used to treat the cells, a final concentration of 100 nM was employed according to the results of our pre-experiments (not shown). After 72 hours of culture, supernatants were collected, and IL-17A production in culture supernatants was analyzed by enzyme-linked immunosorbent assay (ELISA) (Dakewe Biotech Company, China) according to the manufacturer's instructions.

Quantitative PCR (qPCR)

After 72 hours of culture, total RNA containing small-size RNA was isolated from PBMCs using the miRCURY™ RNA Isolation Kit (Exiqon, Denmark). The concentration of RNA was quantified using the NanoDrop 2000 spectrophotometer (Thermo, USA). First strand cDNA synthesis of miRNA was performed with the Mir-X miRNA First-Strand Synthesis Kit (Clontech, Japan). For generating cDNA, 5 μL of 2× mRQ buffer, 1.25 μL of mRQ enzyme, and 3.75 μL of RNA were used, for a total volume of 10 μL. The synthesis reaction was carried out at 37°C for 60 min, followed by 85°C for 5 min. cDNA was diluted with 90 μL of ddH₂O to bring the total volume to 100 μL after reaction. For miRNA quantification, qPCR analysis was performed using the miR-X miRNA qRT-PCR SYBR Kit (Clontech, Japan) and the CFX96 qPCR system (BioRad, USA). The delta-delta C_t method was used to determine the expression of hsa-miR-20b, hsa-miR-21, hsa-miR-155, hsa-miR-181a, hsa-miR-210, and hsa-miR-301a normalized against that of U6 snRNA. The 3' primer for qPCR was the mRQ 3' primer supplied with the kit, whereas the 5' primers for miRNAs were designed and synthesized by Takara Biotechnology (Dalian) (Table 1). The forward and

reverse primers of U6 were provided with the kit. Reactions were incubated at 95°C for 10 seconds, followed by 40 cycles at 95°C for 5 seconds and 60°C for 20 seconds. Each sample was run in duplicate for analysis. At the end of the PCR cycles, melting curve analysis was performed to ensure the specificity of PCR products. Amplicons were also analyzed by agarose gel electrophoresis and visualized with ethidium bromide under UV transillumination to validate the specific generation of the expected PCR.

Statistical analysis

Data are presented as median with interquartile range (IRQ) and were analyzed by nonparametric statistics with Stata version 11.0. A *P* value of <0.05 was considered significant.

Results

1,25(OH)₂D₃ suppressed *in vitro* differentiation of Th17 cells

To generate Th17 cells, we isolated PBMC from healthy donors and activated them *in vitro* with anti-CD3 and anti-CD28 antibodies in the presence of polarizing cytokines IL-23, IL-1β, TGF-β1, and IL-6. After 72 hours of culture, we collected the culture medium and assessed IL-17A production by ELISA. As shown in Figure 1, IL-17A was undetectable when cells received no stimulation, whereas a high level of IL-17A was detected under Th17-polarizing conditions (median [IRQ], 2535.4 [2153.3] pg/mL, *P*<0.05) (Figure 1), indicating a substantial increase in the number of IL-17A-expressing T cells. We tested 1,25(OH)₂D₃ for its capacity to inhibit Th17 differentiation by adding 1,25(OH)₂D₃ to the medium at a final concentration of 100 nM. After 72 hours of culture, IL-17A production was significantly decreased by 1,25(OH)₂D₃ under Th17-polarizing conditions (median [IRQ], 745.7 [473.5] pg/mL vs. 2535.4 [2153.3] pg/mL, *P*<0.05) (Figure 1).

Figure 1. Suppression of IL-17A expression by 1,25(OH)₂D₃. No stimulation: peripheral blood mononuclear cells (PBMC) were cultured for 72 hours without any stimulation. Th17: PBMC were cultured for 72 hours under Th17-polarizing conditions. Th17 + Vit D: PBMC were cultured for 72 hours under Th17-polarizing conditions, and 1,25(OH)₂D₃ was added to the medium at a final concentration of 100 nM on day 0. Data are presented as median with interquartile range. * *P*<0.05.

Detection of miRNA expression by qPCR

The expression of miRNAs hsa-miR-20b, hsa-miR-21, hsa-miR-155, hsa-miR-181a, hsa-miR-210, and hsa-miR-301a was found to be changed under Th17-polarizing conditions. Of these miRNAs, hsa-miR-155 was significantly up-regulated (median fold change: 3.61, *P*<0.05), whereas hsa-miR-20b, hsa-miR-21, hsa-miR-181a, hsa-miR-210, and hsa-miR-301a were significantly down-regulated (median fold change: 0.44, 0.37, 0.18, 0.15, and 0.26, respectively; *P*<0.05) (Table 2, Figure 2). Although 1,25(OH)₂D₃ significantly suppressed human Th17 differentiation, there was no change of miRNA expression after 1,25(OH)₂D₃ treatment (Table 2, Figure 2). Melting curve analysis and agarose gel electrophoresis of qPCR products confirmed that the amplicons were specific (not shown).

Discussion

The main findings of the present study are as follows: (1) we observed increased expression of hsa-miR-155 and decreased expression of hsa-miR-20b, hsa-miR-21, hsa-miR-181a, hsa-miR-210, and hsa-miR-301a during human Th17 differentiation; and (2) 1,25(OH)₂D₃ significantly suppressed human Th17 differentiation, but did not affect miRNA expression during this process. As far as we know, we provide the first evidence that 1,25(OH)₂D₃ suppresses human Th17 differentiation without influencing miRNA expression.

The classical function of 1,25(OH)₂D₃ is to maintain calcium-phosphate homeostasis and skeletal function. However, its role in modulation of immune responses has received considerable attention. Several previous studies have found that 1,25(OH)₂D₃ has the capacity to inhibit Th17 differentiation. The study by Ikeda et al. demonstrated that 1,25(OH)₂D₃ significantly inhibited Th17 differentiation by down-regulating the mRNA expression levels of RORC and aryl hydrocarbon receptor [26]. In addition, the inhibitory effect of 1,25(OH)₂D₃ on Th17 differentiation was found to be via the VDR signal in CD4+ T cells, but independent of IL-2, IL-10, and STAT1 signals [11]. Furthermore, it was shown that 1,25(OH)₂D₃ suppressed Th17 differentiation by blocking NFAT, recruitment of HDAC, sequestration of Runx1, and inducing CHOP and Foxp3 [12,13]. Recently, Nanduri et al. reported that 1,25(OH)₂D₃ repressed Th17-specific genes by the Smad pathway and extracellular signal-regulated kinase activation [27]. Despite of these findings, our understanding of the mechanisms whereby 1,25(OH)₂D₃ regulates Th17 differentiation remains limited.

miRNAs represent a large family of about 22 nt long, noncoding, single-stranded RNA molecules. In mammals, miRNAs predominantly regulate gene expression by induction of mRNA degradation, and they play an important role in a variety of physiological and pathological processes. An involvement in

Table 2. Fold change of miRNA expression.

miRNA	Fold change		
	No stimulation ¹	Th17-polarizing conditions ²	Th17-polarizing conditions + 1,25(OH) ₂ D ₃ treatment ³
hsa-miR-155	1	3.61*	2.04 ^Δ
hsa-miR-20b	1	0.44*	0.34 ^Δ
hsa-miR-21	1	0.37*	0.70 ^Δ
hsa-miR-181a	1	0.18*	0.37 ^Δ
hsa-miR-210	1	0.15*	0.26 ^Δ
hsa-miR-301a	1	0.26*	0.39 ^Δ

* ¹ vs. ², *P*<0.05; ^Δ ³ vs. ², *P*>0.05.

Figure 2. Detection of miRNA expression by real time PCR. No stimulation: peripheral blood mononuclear cells (PBMC) were cultured for 72 hours without any stimulation. Th17: PBMC were cultured for 72 hours under Th17-polarizing conditions. Th17 + Vit D: PBMC were cultured for 72 hours under Th17-polarizing conditions, and 1,25(OH)₂D₃ was added to the medium at a final concentration of 100 nM on day 0. Data are presented as median with interquartile range. * $P < 0.05$, Δ $P > 0.05$.

the regulation of Th17 differentiation has recently been proposed for several miRNAs, including miR-20b, miR-21, miR-155, miR-181a, miR-210, and miR-301a [20–25]. Given that both 1,25(OH)₂D₃ and miRNAs are important regulators of Th17 differentiation, it is necessary to evaluate whether 1,25(OH)₂D₃ affects miRNA expression when exerting its inhibitory effects on Th17 differentiation.

In this study, we focused on six candidate miRNAs, including miR-20b, miR-21, miR-155, miR-181a, miR-210, and miR-301a, based on existing evidence from previous studies showing their relation with Th17 differentiation, rather than performing

miRNA microarray analysis to identify candidates, because the former method was more efficient and targeted. In addition, we did not identify candidate miRNAs using in silico analysis because this method was relatively unreliable. The change of miRNA expression during Th17 differentiation was evaluated using qPCR. In agreement with the results from previous studies, all six miRNAs were found to be significantly dysregulated in the development of Th17 cells, with five down-regulated (hsa-miR-20b, hsa-miR-21, hsa-miR-181a, hsa-miR-210, and hsa-miR-301a) and one up-regulated (hsa-miR-155). It was noteworthy that most evidence on expression of miRNAs during Th17 differentiation to date has been derived from results

using animal cell cultures [20–23,25], whereas we evaluated these miRNAs using human cell cultures, which could provide more valuable evidence. In addition, most previous studies concentrated on a single miRNA, but we evaluated several miRNAs simultaneously in this study, which would provide more detailed information on change of miRNA expression during Th17 cell development.

We evaluated the effects of 1,25(OH)₂D₃ on Th17 differentiation and miRNA expression. 1,25(OH)₂D₃ significantly suppressed Th17 differentiation, but did not affect miRNA expression during this process, suggesting that 1,25(OH)₂D₃-mediated Th17 cell suppression was not dependent on regulation of miRNA expression. We noticed that our results were distinct from those on endothelial tissues and cancer cells, which showed that vitamin D regulated miRNA expression and was involved in miRNA-directed posttranscriptional mechanisms [28,29]. The study by Zitman-Gal et al. found that miR-20b expression was up-regulated by 1,25(OH)₂D₃ in endothelial cells exposed to a diabetic-like environment [28], and this change of expression played a role in the modulation of endothelial function. Another study by Sheane et al. demonstrated that vitamin D deficiency up-regulated miR-21 expression in the aorta, which contributed to the development of atherosclerosis [29].

In addition, cancer studies also showed that 1,25(OH)₂D₃ regulated the expression of a subset of miRNAs with potential regulatory functions in cancer pathways [30–32]. However, we did not find miRNA involvement in 1,25(OH)₂D₃-mediated suppression of Th17 differentiation in this study. Our results indicate that 1,25(OH)₂D₃ exerts its functions via distinct mechanisms across different cell types, not through miRNAs in Th17 cells.

Conclusions

Our study showed that six miRNAs were dysregulated during human Th17 differentiation, with one significantly up-regulated (hsa-miR-155) and five significantly down-regulated (hsa-miR-20b, hsa-miR-21, hsa-miR-181a, hsa-miR-210, and hsa-miR-301a). 1,25(OH)₂D₃ did not affect the expression of these miRNAs when suppressing Th17 differentiation.

Disclosures

The authors declare no conflicts of interest. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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