1,25-Dihydroxy-Vitamin D3 induces macrophage polarization to M2 by upregulating T-cell Ig-mucin-3 expression

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Abstract. Macrophage polarization serves an important role in immune regulation that is regulated by T-cell immunoglobulin-mucin-3 (Tim-3). The objective of the present study was to explore the role of 1,25-dihydroxy-vitamin D3 [$1,25(OH)_2D3$] in macrophage polarization. Plasmid transfection techniques were applied to prepare RAW264.7 cells with silenced or overexpressed Tim-3 gene. ELISAs were used to examine the level of inflammatory factors secreted by macrophages. Proteins levels were determined by western blot analysis. mRNAs expression levels were assessed using reverse transcription quantitative polymerase chain reaction. It was identified that $1,25(OH)_2D3$ upregulated Tim-3 levels and promoted the secretion of interleukin (IL)-10. $1,25(OH)_2D3$ was also observed to increase the level of transforming growth factor-β and to inhibit tumor necrosis factor-α and IL-6. The results also suggested that Tim-3 gene silencing induced macrophages polarization to classically activated macrophages (M1), and that overexpression of the Tim-3 gene induced macrophage polarization to alternatively activated macrophages (M2). $1,25(OH)_2D3$ treatment upregulated the expression level of Tim-3 in macrophages, which promoted cell polarization to M2 and inhibited polarization to M1. The data from the present study indicated that Tim-3 may induce macrophage polarization to M2, and that $1,25(OH)_2D3$ produced immunosuppressive effects by upregulating Tim-3.

Introduction

Autoimmune disorders are mediated by self-cells; that is, induced by the immune response to autoantigens (1). Though common autoimmune disorders have high incidence rates, the pathogeneses of these diseases remain incompletely characterized (2). At present, to the best of our knowledge, no drug has been identified to be sufficiently effective for the treatment of autoimmune diseases, and clinical therapy is largely based on symptomatic treatment and targeting the control of disease progression (3-5). Therefore, exploring the pathogenesis of these diseases is of great significance in developing effective drugs and clinical strategies for the treatment of autoimmune diseases.

Vitamin D3 is a derivative of cholesterol and the most active form of Vitamin D3 is 1,25-dihydroxy-vitamin D3 [$1,25(OH)_2D3$], formed by the action of hepatic microsomal 25-hydroxylase and kidney mitochondrial 1α hydroxylase (6). Previous studies have demonstrated that abnormal levels of $1,25(OH)_2D3$ are closely associated with various autoimmune diseases (7,8). At present, studies investigating $1,25(OH)_2D3$ and immune function have attracted much attention. It has been identified that $1,25(OH)_2D3$ blocked the differentiation and maturation of dendritic cells (DCs) to promote T cell responses and differentiation (9,10). $1,25(OH)_2D3$ also promotes B lymphocyte-programmed death and inhibits the differentiation of activated B cells into plasma cells and memory B lymphocytes, which secrete immunoglobulins (11,12). However, only a limited number of studies have examined the macrophage polarization promoted by $1,25(OH)_2D3$.

It has been identified that $1,25(OH)_2D3$ promotes macrophage polarization: Macrophages are induced by tumor necrosis factor α (TNF-α) or interleukin-10 (IL-10) (13,14), and macrophage polarization to classically activated macrophages (M1) may assist in virus clearance and participate in inflammatory reactions; however, polarization of macrophages to alternatively activated macrophages (M2) may promote tumor development and inhibit inflammatory responses (15,16).

The polarization mechanism of macrophages has been studied extensively. T-cell Immunoglobulin-mucin-3 (Tim-3) was identified as a novel immunomodulatory protein (17). Tim-3 has the function of regulating natural immune cells (18). At present, previous studies have identified that Tim-3 may promote macrophage polarization to M2; however, its mechanism has not been fully described (19,20). Tim-3 belongs to the Tim protein family (21). Previous studies have suggested that Tim-3 is significantly upregulated in chronic infectious diseases, including chronic Hepatitis C virus infection (22), Human immunodeficiency virus infection (23) and tuberculosis (24) and downregulated in autoimmune diseases, including multiple sclerosis (25) and rheumatoid arthritis (17).

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Therefore, Tim-3 is considered to be included in a novel generation of immunomodulatory targets.

In the present study, genetic interference techniques were applied to explore the effect of 1,25(OH)$_2$D3 on macrophages polarization, and its mechanism. The expression levels of polarization-associated factors with or without Tim-3 expression were detected. The results of the present study enrich the current understanding of the mechanisms underlying autoimmune diseases, and suggest alternative approaches for disease treatment.

Materials and methods

Cells culture. The mouse macrophage RAW264.7 cell line was purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) that contained 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) with penicillin (100 U/ml) and streptomycin sulfate (100 μg/ml) at 37˚C in an incubator with 5% CO$_2$. Culture reagents were purchased from Gibco (Thermo Fisher Scientific, Inc.).

Cell transfection. The recombinant Tim-3 overexpression plasmid (pTim-3), empty vector (pScramble) and small interfering (si)Tim-3 were purchased from Promega Corporation (Madison, WI, USA). The sequences of siTim-3 were Sense 5':GGUGUCUUAAUCCUUAAAU, and Antisense 3':CCA CAGAAUUAGGAAUUUA. The cells were seeded at a density of 1x10^4 cells/well into a 24-well plate. The cells were then starved overnight by incubating them in DMEM without FBS prior to cell transfection. When the confluence reached 60-70%, the cells were transfected with pTim-3 or siTim-3 (50 pmol) using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the protocol of the manufacturer. The time interval between transfection and subsequent experimentation was 24 h. For screening the positive clones, the RAW264.7 cell line with the stable knockdown or Tim-3 overexpression plasmid was cultured in the aforementioned complete medium with 200 and 700 μg/ml G418 respectively, at 37˚C for 24 h. According to data from previous studies (26-28), 10^{-8} or 10^{-7} M 1,25(OH)$_2$D3 was used to treat the RAW264.7 cells for 3 days. Cell morphology was observed using a laser confocal microscope, with magnifications x100 and x200 (Leica TCS SP8; Leica Microsystems, Wetzlar, Germany).

ELISAs. ELISAs were used to measure the concentrations of TNF-α, IL-6, IL-10 and transforming growth factor-β (TGF-β). The kits were Mouse TNF-α Quantikine ELISA Kit (MTA00B, R&D Systems, Inc., Minneapolis, MN, USA), Mouse IL-6 Quantikine ELISA Kit (M6000B, R&D), Mouse IL-10 Quantikine ELISA Kit (M1000B, R&D), and Mouse/Rat/Porcine/Canine TGF-beta 1 Quantikine ELISA Kit (MB100B, R&D). Following washing, blocking solution was added at 4˚C and incubated for 2 h. Next, the primary antibody was added at 4˚C and incubated overnight. The secondary antibody was then added and incubated for 1 h at room temperature. Horseradish peroxidase was added dropwise for 30 min at room temperature, and then tetramethylbenzidine

was added for 10 min. The absorbance value was measured at 450 nm using a microplate reader (ELX 800; Bio-Tek Instruments, Winooski, VT, USA) and the concentration was calculated following the standard curve.

Western blot analysis. The expression levels of Tim-3, nitric oxide synthase, inducible (NOS2) and macrophage mannose receptor 1 (COD206) proteins were detected using western blot analysis. Cells were lysed with liquid nitrogen and radio-immunoprecipitation assay lysis buffer (Abmole Bioscience Inc., Houston, TX, USA). Next, the cells were cleaved with 1% phenylmethyl sulfonyl fluoride mixed with phosphatase inhibitors (Abmole Bioscience Inc) for 30 min at 4˚C. The supernatant was collected using centrifugation at 13,000 x g at 4˚C for 15 min. A standard curve was drawn using the BCA method to determine the protein concentration. Then, proteins (20 μg/lane) were separated using 10% SDS-PAGE gels, and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using a Trans-Blot Transfer Slot (Bio-Rad Laboratories, Inc.). The PVDF membranes were then blocked with 5% skimmed milk for 2 h at room temperature. Primary antibodies against Tim-3 (cat. no. ab185703; 1:800; Abcam, Cambridge, MA, USA), NOS2 (cat. no. 14-5920-80; 1:800; eBioscience; Thermo Fisher Scientific, Inc.), CD206 (cat. no. 141703; 1:600; BioLegend, Inc., San Diego, CA, USA) were then added according to the manufacturers' protocols. The samples were agitated at room temperature for 2 h and then incubated at 4˚C for 12 h. Rabbit anti-mouse IgG (cat. no. ab99697; 1:9,000 Abcam), mouse anti-rabbit IgG (cat. no. BA1034; 1:1,000; Invitrogen; Thermo Fisher Scientific, Inc.) and goat anti-mouse IgG (cat. no. ab6785; 1:8,000; Abcam) HRP-conjugated secondary antibodies were added and incubated at room temperature for 1.5 h. Chemiluminescence detection was performed using ECL reagent (EMD Millipore, Billerica, MA, USA). The densitometric analysis was performed using Bio-Rad ChemiDoc™ XRS+ System with Image Lab™ Software version 4.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis. RT-qPCR was performed to determine the mRNA levels of Tim-3, NOS2 and COD206. The cells were triturated and lysed by TRIzol® (Thermo Fisher Scientific, Inc.) at 0˚C for 5 min. RNA was then extracted using CCl$_3$ (Shanghai Aladdin Biochemical Technology Co., Ltd., Shanghai, China) and dissolved in diethyl pyrocarbonate-treated water (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). RNA concentration was measured using a UV NanoDrop One Microvolume UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). Reverse transcription assays were performed on RNA samples to synthesize cDNA using a reverse transcription kit (Takara Biotechnology, Co., Ltd., Dalian, China). The samples were incubated at 37˚C for 15 min, followed by heat inactivation at 85˚C for 15 sec. RT-qPCR experiments were performed using the SYBR Prellix Ex Taq™ Real-Time PCR kit (Takara Biotechnology, Co., Ltd.). qPCR was performed by activating the DNA polymerase at 95˚C for 5 min, which was followed by 40 cycles of a two-step PCR (at 95˚C for 10 sec and at 60˚C for 30 sec) with a final extension step at 75˚C for 10 min. Samples were then held

Table I. Sequences of primers.

Primer name	Sequence (5'-3')	Product size, bp
Tim-3-forward	CTCCAAGAACCCTAACCACG	
Tim-3-reverse	AGCCCATGTGGAAATTTTTG	288
NOS2-forward	CAGCTGGGCTGTACAAACCTT	
NOS2-reverse	CATTGGAAGTGAAGCGTTTCG	245
CD206-forward	TTCGGACACCCATC-GGAATTT	
CD206-reverse	CACAAGCGCTGCGTGGAT	262
β-actin-forward	GCTGCGTGTGGCCCCTGAG	
β-actin-reverse	ACGCAGGATGGCATGAGGGA	252

Tim-3, T-cell Immunoglobulin-mucin-3; NOS2, nitric oxide synthase, inducible; CD206, macrophage mannose receptor 1.

at 4°C. β-actin was used as an internal control. All primers were purchased from Genewiz, Inc., (Suzhou, Jiangsu China), and are summarized in Table I. The $2^{-\Delta\Delta Cq}$ method was used to quantify the gene expression levels (29).

Statistical analysis. All experimental data are presented as means ± standard deviation. Statistical analysis was performed using SPSS 20 (IBM Corp., Armonk, NY, USA). The one-way analysis of variance followed by Tukey's multiple comparison post-hoc test was performed to analyze the differences among experimental groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Confocal microscopy. The viable RAW264.7 macrophages were normal at magnifications, x100 and x200 (Fig. 1).

$1,25(OH)_2D3$ regulates the secretion of inflammatory cytokines in RAW264.7 cell. It was identified that the levels of TNF-α and IL-6 secreted by RAW264.7 cells were decreased in the presence of $1,25(OH)_2D3$. However, the levels of IL-10 and TGF-β were increased ($P < 0.01$; Fig. 2A-D). These differences were the most significant at concentrations of 10^{-7} M ($P < 0.05$).

$1,25(OH)_2D3$ upregulates Tim-3 expression. To investigate the mechanism of how $1,25(OH)_2D3$ affects the expression levels of inflammatory factors, levels of Tim-3, and NOS2 and COD206, the markers of M1 and M2, were examined. The results indicated that the expression level of Tim-3 was gradually increased in the 10^{-8} M and 10^{-7} M groups. However, whilst $1,25(OH)_2D3$ did not affect the expressions of the NOS2 and COD206, it decreased NOS2 mRNA levels ($P < 0.05$; Fig. 2E-K). This observation indicated that $1,25(OH)_2D3$ promoted Tim-3 expression in a dose-dependent manner.

Effects of Tim-3 gene silencing on cytokines. Following silencing of the Tim-3 gene, the levels of TNF-α and IL-6 secreted by RAW264.7 cells were upregulated, while the levels of IL-10 and TGF-β were downregulated ($P < 0.01$; Fig. 3A-D). Following the decrease of the Tim-3 gene expression, the mRNA and protein expression levels of NOS2 were observed increased compared with that of the NC and control groups, while the expression levels of CD206 mRNA and protein were significantly decreased ($P < 0.01$; Fig. 3E-K). This phenomenon suggested that silence of Tim-3 gene promoted the polarization of macrophages to M1.

Effects of overexpression of Tim-3 on cytokines. The effects of the overexpression of Tim-3 on cytokines were also examined. It was identified that the levels of TNF-α and IL-6 in the pTim-3 group were decreased compared with those in the NC group, and that the levels of IL-10 and TGF-β were increased compared with those in the NC group ($P < 0.01$; Fig. 4A-D). When Tim-3 was overexpressed, the mRNA and protein expression levels of NOS2 were decreased, while the expression levels of CD206 mRNA and protein were increased ($P < 0.01$; Fig. 4E-K). This suggested that the overexpression of Tim-3 promoted the polarization of macrophages to M2.

Effects of $1,25(OH)_2D3$ on cytokine under Tim-3 silencing or overexpression conditions. To additionally explore the effect of $1,25(OH)2D3$ on the polarization of macrophages, RAW264.7 cells were cultured in the presence of 10^{-7} M $1,25(OH)_2D3$. The expression levels of inflammatory factors, and M1 and M2 marker proteins NOS2 and CD206, were detected.

It was identified that $1,25(OH)2D3$ downregulated the levels of TNF-α and IL-6 and upregulated the level of TGF-β in siTim-3 cells. In addition, $1,25(OH)_2D3$ promoted the expression of Tim-3 and increased the expression levels of CD206 protein in siTim-3 cells. However, $1,25(OH)_2D3$ decreased the expression levels of NOS2. For RAW264.7 cells with stable overexpression of Tim-3, $1,25(OH)2D3$ was observed to additionally decrease TNF-α and IL-6 levels and increase IL-10 and TGF-β levels, compared to the Tim-3 overexpression group. In addition, $1,25(OH)_2D3$ increased the level of Tim-3 and the expression levels of CD206 in RAW264.7 cell with stable expression of Tim-3 gene (Fig. 5A-K). This indicated that $1,25(OH)_2D3$ promoted the polarization of macrophages to M2 and inhibited polarization of macrophages to M1 via upregulating the expression of Tim-3.

Discussion

Macrophages may be induced to polarize to M1 or M2 subtypes by different cytokines, and also secrete different cytokines to
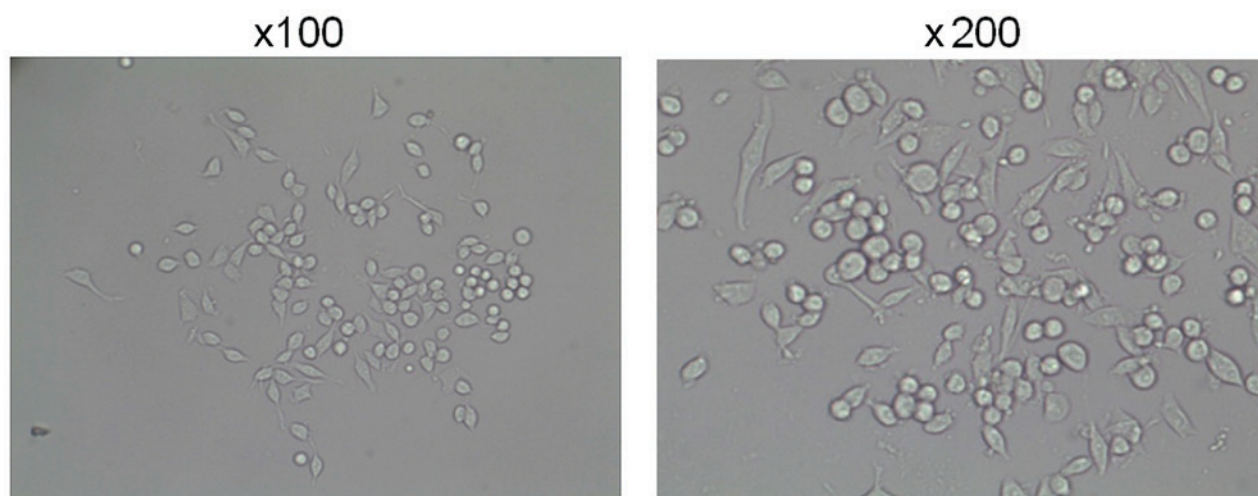
Figure 1. Confocal microscopy imaging. The morphology of live RAW264.7 cells at magnifications, x100 and x200 were observed using confocal microscopy.
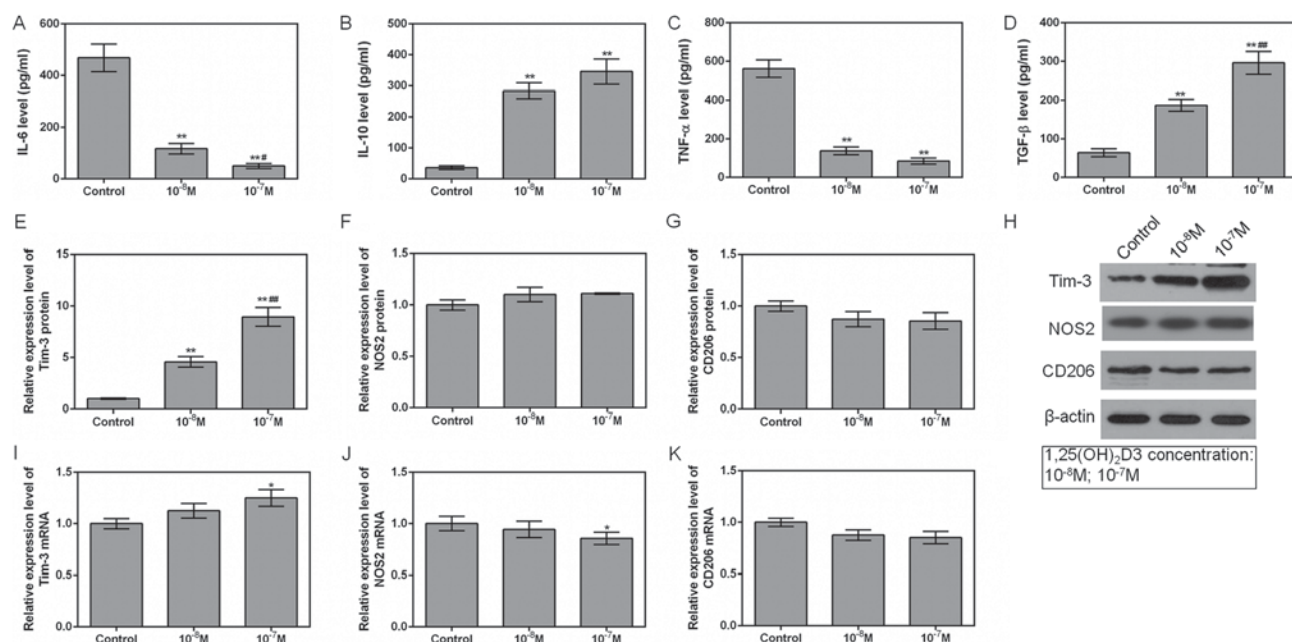

Figure 2. Effects of 1,25(OH)₂D3 on macrophage at 10^{-8} and 10^{-7} M concentrations. RAW264.7 cells were cultured at different 1,25(OH)₂D3 concentrations (0, 10^{-8} and 10^{-7} M). ELISAs were used to detect the (A) IL-6, (B) IL-10, (C) TNF-α and (D) TGF-β protein expression levels among different groups. (E) Tim-3, (F) NOS2 and (G) CD206 protein levels were assessed by western blot analysis. (H) Representative western blot image. (I) Tim-3, (J) NOS2 and (K) CD206 mRNA levels were analyzed by reverse transcription quantitative polymerase chain reaction assays. $^*P<0.05$ and $^{**}P<0.01$ vs. control group; $^#P<0.05$ and $^{##}P<0.01$ vs. 10^{-8} M group. Data are presented as mean ± standard deviation (n=5). 1,25(OH)₂D3, 1,25-dihydroxy-vitamin D3; IL, interleukin; TNF-α, tumor necrosis factor α; TGF-β, transforming growth factor; Tim-3, T-cell Immunoglobulin-mucin-3; NOS2, nitric oxide synthase, inducible; CD206, macrophage mannose receptor 1.

promote or suppress the immune response (30). Macrophage cells may also maintain the homeostasis and serve an important role in the formation and treatment of diseases (31).

It has been identified that Vitamin D3 has immunomodulatory effects on chicken macrophages (32). Yin *et al* (33) also revealed that Vitamin D may induce macrophage polarization by activating CYP27A1 to prevent atherosclerosis in hypercholesterolemic animals. The present study demonstrated that 1,25(OH)₂D3 inhibited TNF-α and IL-6 and promoted the secretion of IL-10 and TGF-β. TNF-α induces macrophage polarization to M1 and the secretion of TNF-α and IL-6. IL-10 induces macrophage polarization to M2 and secretion of IL-10

and TGF-β (13,14). The results of the present study indicated that 1,25(OH)₂D3 decreased the level of NOS2 mRNA, but did not produce a significant effect on NOS2 protein expression or CD206 protein and mRNA expression. However, the expression of Tim-3 was increased by 1,25(OH)₂D3. Tim-3 serves a role in modulating immunity (34), through inducing macrophage polarization. This suggests that 1,25(OH)₂D3 may regulate macrophage polarization through Tim-3. Therefore, the effect of 1,25(OH)₂D3 on macrophages was investigated through silencing or overexpressing Tim-3. The results demonstrate that silencing of the Tim-3 gene may promote the secretion of TNF-α and IL-6 but inhibit the secretion of IL-10
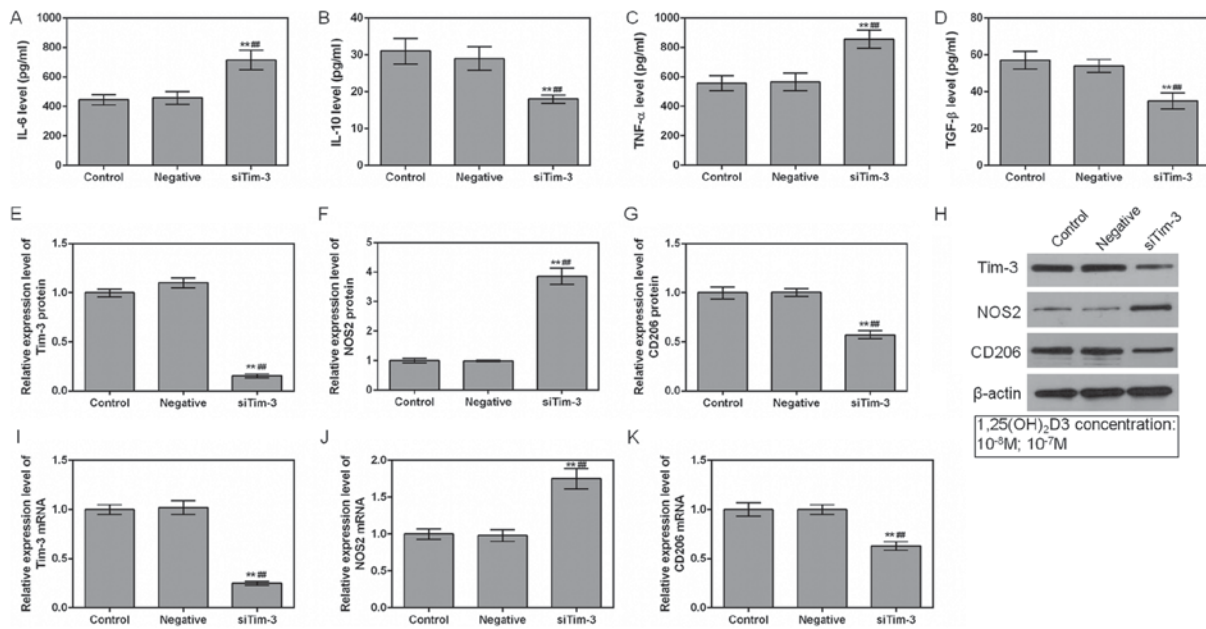
Figure 3. Effects of Tim-3 gene silencing on macrophages. ELISAs were applied to detect the (A) IL-6, (B) IL-10, (C) TNF-α and (D) TGF-β levels in the control, NC and siTim-3 groups. (E) Tim-3, (F) NOS2 and (G) CD206 protein levels were assessed by western blot analysis. (H) Representative western blot image. (I) Tim-3, (J) NOS2 and (K) CD206 mRNA levels were analyzed by reverse transcription quantitative polymerase chain reaction assays. **P<0.01 vs. control group; ##P<0.01 vs. NC group. Data are presented as mean ± standard deviation (n=5). IL, interleukin; TNF-α, tumor necrosis factor α; TGF-β, transforming growth factor; Tim-3, T-cell Immunoglobulin-mucin-3; NOS2, nitric oxide synthase, inducible; CD206, macrophage mannose receptor 1; si, small interfering.
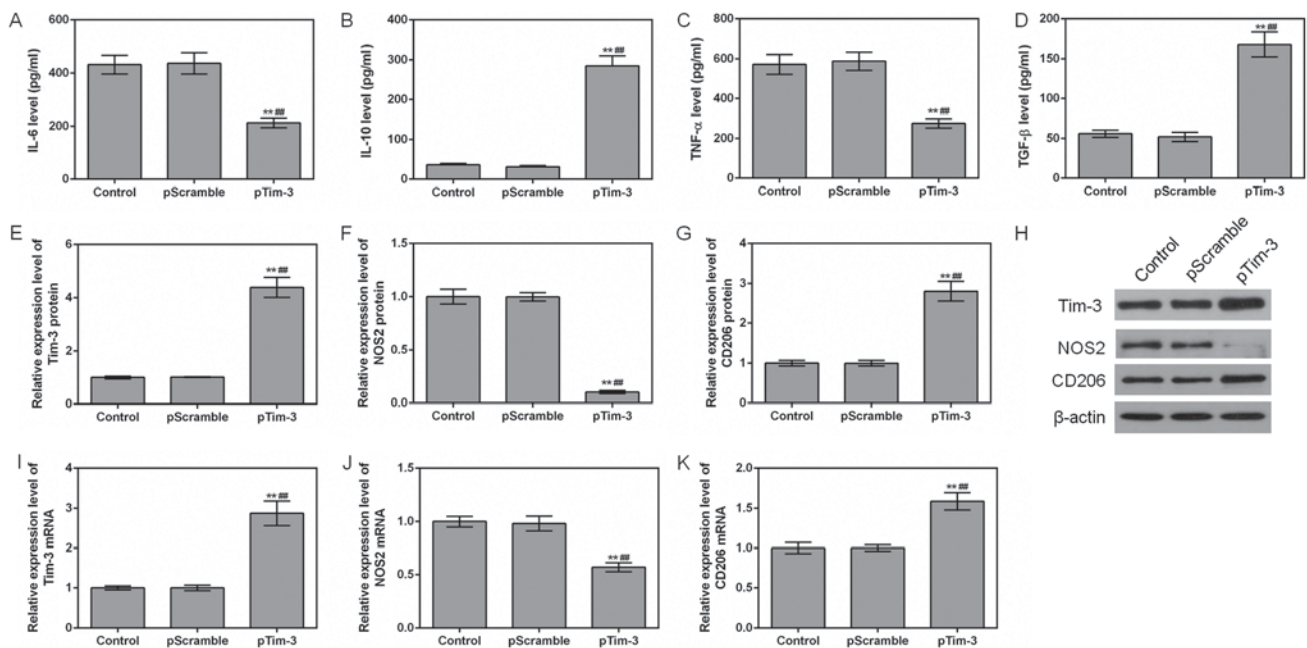

Figure 4. Effects of Tim-3 gene overexpression on macrophages. The levels of (A) IL-6, (B) IL-10, (C) TNF-α and (D) TGF-β in the control, NC and pTim-3 groups were detected using ELISAs. (E) Tim-3, (F) NOS2 and (G) CD206 protein levels were assessed by western blot analysis. (H) Representative western blot image. (I) Tim-3, (J) NOS2 and (K) CD206 mRNA levels were analyzed by reverse transcription quantitative polymerase chain reaction assays. **P<0.01 vs. control group; ##P<0.01 vs. NC group. Data are presented as mean ± standard deviation (n=5). IL, interleukin; TNF-α, tumor necrosis factor α; TGF-β, transforming growth factor; Tim-3, T-cell Immunoglobulin-mucin-3; NOS2, nitric oxide synthase, inducible; CD206, macrophage mannose receptor 1; pTim-3, overexpressing Tim-3 plasmid.

and TGF-β. In addition, downregulation of Tim-3 increased the protein and mRNA levels of NOS2 and decreased the protein and mRNA levels of the M2 marker CD206. By contrast, overexpression of the Tim-3 gene exerted the opposite effects

This is consistent with previous data (17,22-25), indicating that a high expression level of Tim-3 gene promotes macrophage polarization to M2 and produces immunosuppressive effects. Therefore, we hypothesized that 1,25(OH)₂D3 may induce
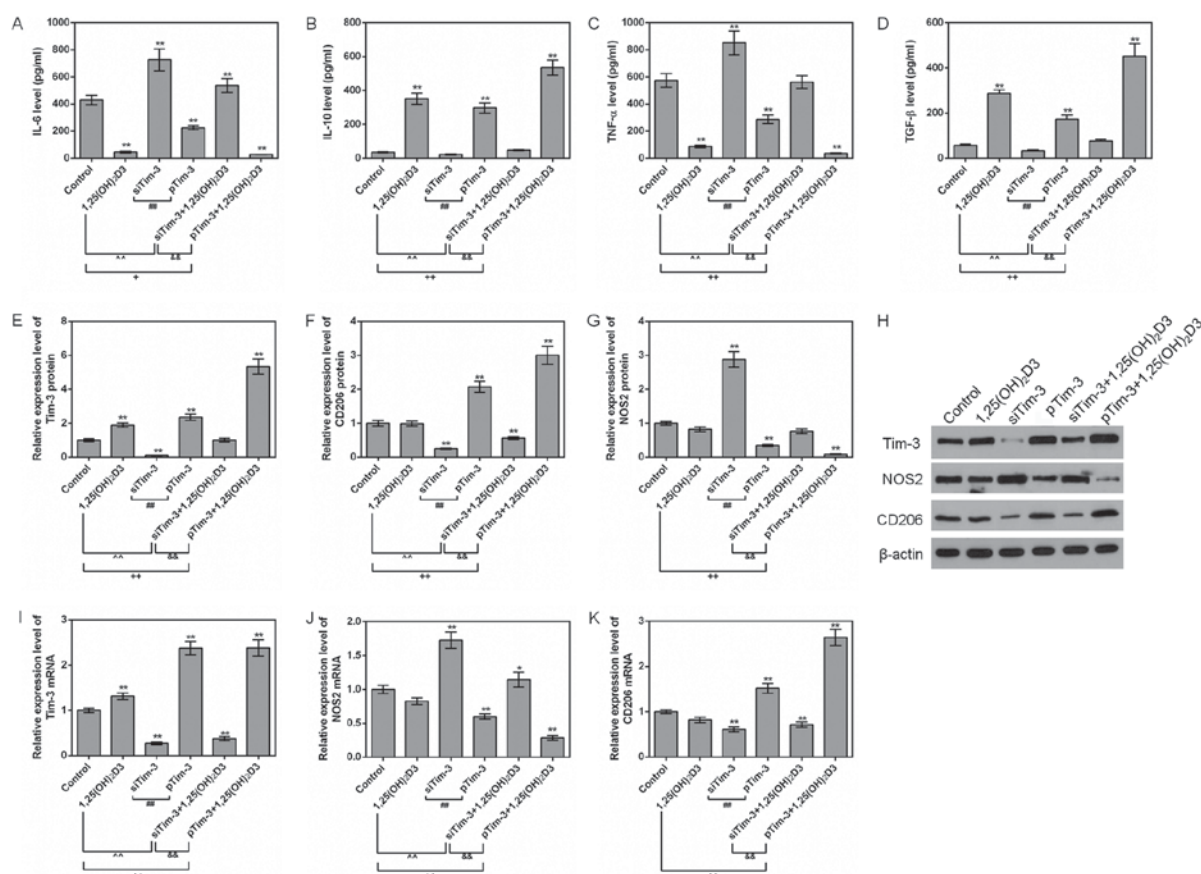
Figure 5. Effects of 1,25(OH)₂D3 on macrophages with different Tim-3 expression levels. RAW264.7 macrophages with silenced Tim-3 expression and Tim-3 overexpression were cultured with 10^{-7} M 1,25(OH)₂D3. ELISAs were used to assess the levels of (A) IL-6, (B) IL-10, (C) TNF-α and (D) TGF-β in the control group. (E) Tim-3, (F) CD206 and (G) NOS2 protein levels were assessed by western blot analysis. (H) Representative western blot image. (I) Tim-3, (J) NOS2 and (K) CD206 mRNA levels were analyzed by reverse transcription quantitative polymerase chain reaction assays. *P<0.05 and **P<0.01 vs. control group; ⁺P<0.05; ⁺⁺P<0.01; ##P<0.01; &&P<0.01. Data are presented as mean ± standard deviation (n=5). 1,25(OH)₂D3, 1,25-dihydroxy-vitamin D3; IL, interleukin; TNF-α, tumor necrosis factor α; TGF-β, transforming growth factor; Tim-3, T-cell Immunoglobulin-mucin-3; NOS2, nitric oxide synthase, inducible; CD206, macrophage mannose receptor 1; pTim-3, overexpressing Tim-3 plasmid; si, small interfering.

cell polarization to M2 by upregulating Tim-3 expression and exerting immunosuppressive effects.

The role of 1,25(OH)₂D3 in macrophages polarization was also explored. The data indicated that the decreased levels of IL-6 and TNF-α and the increased IL-10 and TGF-β were reversed in the presence of siTim-3. However, the effect of 1,25(OH)₂D3 was enhanced by the overexpression of Tim-3. This suggested that 1,25(OH)₂D3 produced an immunosuppressive effect by promoting Tim-3 protein. It was consistent with a previous study, in which 1,25(OH)2D3 was identified to induce macrophage polarization to M2, leading to immunosuppression (33).

However, the mechanism underlying the regulation of Tim-3 protein levels by 1,25(OH)₂D3 remains unclear. An additional limitation of the present study is that RAW264.7 is a cancerous macrophage cell line, as opposed to a primary cell line. Therefore, additional investigation in primary cells or *in vivo* experiments would strengthen the results of the present study. Altogether, the mechanism for the regulation of M1/M2 conversion and inflammation by Tim-3 remains to be determined.

In summary, Tim-3 induces macrophage polarization to M2. 1,25(OH)₂D3 exhibited immunosuppressive effects by upregulating Tim-3 protein levels. The present study suggested that 1,25(OH)₂D3 may serve as an immunosuppressive drug and that Tim-3 may be a potential target in treating autoimmune diseases.

Acknowledgements

Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

SL and JC did all of the experiments, YL analyzed the data, RY designed the study, and SL wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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