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1,25-Dihydroxyvitamin D regulates macrophage polarization and ameliorates experimental inflammatory bowel disease by suppressing miR-125b

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ABSTRACT

Macrophages are highly plastic cells. Depending on stimulation, macrophages rapidly polarize to functionally distinct phenotypes that are involved in the pathogenesis of inflammatory bowel disease (IBD). 1,25-Dihydroxyvitamin D ($1,25(OH)_2D_3$) has immunomodulatory activity, and $1,25(OH)_2D_3$ deficiency is correlated with autoimmune diseases, especially IBD. This study aimed to explore whether $1,25(OH)_2D_3$ modulates macrophage polarization in inflammation. Peripheral blood mononuclear cells and colitis mice were treated with $1,25(OH)_2D_3$. Macrophages were transfected with siRNA-vitamin D receptor (VDR) or miR-125b mimic or inhibitor, and $1,25(OH)_2D_3$-pretreated colitis mice were injected with a miR-125b agomir. The distribution of macrophage subsets and macrophage subtype characteristics was analyzed. As expected, $1,25(OH)_2D_3$ transformed lipopolysaccharide-induced M1 macrophages to the M2 subset, downregulated tumor necrosis factor-α and interleukin (IL)-6 expression and interferon regulatory factor 5 (IRF5) phosphorylation, and upregulated IL-10, arginase-1, VDR, and IRF4 expression. SiRNA-VDR and miR-125b mimic significantly impaired $1,25(OH)_2D_3$ activity. In colitis mice, $1,25(OH)_2D_3$ pretreatment ameliorated disease activity, converted M1 macrophages to the M2 subtype, suppressed IRF5 phosphorylation, and increased IRF4 expression in lamina propria mononuclear cells (LPMC). miR-125b agomir injections reversed $1,25(OH)_2D_3$ action. Collectively, the results demonstrate that $1,25(OH)_2D_3$ downregulates miR-125b expression and promotes M1 macrophage polarization to the M2 subtype. $1,25(OH)_2D_3$ pretreatment ameliorated colitis by restoring the LPMC macrophage subtype balance.

1. Introduction

Inflammatory bowel diseases (IBDs) are chronic inflammatory disorders of the intestinal mucosa, which are subdivided into two forms, Crohn's disease (CD) and ulcerative colitis (UC). Several factors can trigger IBD. One well-recognized trigger is an abnormal immune response against microorganisms. The immune cells involved in IBD pathogenesis include innate-arm dendritic cells, macrophages, and neutrophils, and adaptive-arm Treg and Th17 cells. Macrophages are the most abundant phagocytic cells in the lamina propria, and they are one of the most important cells in the development of IBD [1]. Macrophages phagocytose microbes, secrete cytokines, modulate T-cell subtype polarization, and contribute to intestinal homeostasis. High plasticity is a characteristic feature of macrophages. Depending on the microenvironment, macrophages can acquire distinct functional phenotypes. Classically activated (M1) macrophages are induced by interferon (IFN)-γ and lipopolysaccharide (LPS) and produce large amounts of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-12, and IL-23, and low amounts of the anti-inflammatory cytokine IL-10. M1 macrophages are engaged in inflammatory and tumoricidal activities. In contrast, IL-4, IL-13, and macrophage colony-stimulating factor (M-CSF) promote the generation

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of alternatively activated (M2) macrophages, which produce high levels of IL-10 and express arginase-1 (Arg-1) and inflammatory zone 1. M2 macrophages dampen inflammation and promote tumor progression [2]. Macrophage phenotype polarization mainly relies on cytokine production in the surrounding tissue. Therefore, macrophage phenotype polarization plays a key role in the inflammation process [3]. Our previous study demonstrated that macrophage subtype disequilibrium occurred during the development of dextran sulfate sodium (DSS)-induced colitis, and transfer of M2 macrophages ameliorated colitis severity by regulating Treg cell polarization [4]. Further, we demonstrated that baicalin modulates macrophage polarization to the M2 phenotype and alleviates DSS-induced colitis by upregulating interferon regulatory factor 4 (IRF4) expression [5]. Other studies have also demonstrated that regulating macrophage polarization to the M2 subset could alleviate DSS-induced colitis [6,7].

Vitamin D, a fat-soluble vitamin, is primarily synthesized from 7-dehydrocholesterol in the skin upon exposure to sunlight and, alternatively, can be ingested as a dietary supplement. 1,25-Dihydroxyvitamin D (1,25(OH)$_2$D$_3$) is the active form of vitamin D. 1,25(OH)$_2$D$_3$ activities are regulated by binding to the vitamin D receptor (VDR), which then forms a transcription factor complex with the retinoid X receptor (RXR) that interacts with the vitamin D-responsive element in the promoter regions of target genes. More than 1,000 genes are VDR target genes. Therefore, 1,25(OH)$_2$D$_3$ has broad physiological functions, and vitamin D deficiency has been correlated with many diseases [8]. Toll-like receptors (TLRs) are pattern recognition receptors expressed on professional phagocytes that mediate the response to infection and subsequently trigger adaptive immune responses. 1,25(OH)$_2$D$_3$ negatively regulates TLR2, 4, and 5 surface expression, whereas it positively regulates TLR10 surface expression in macrophages [9]. In addition, 1,25(OH)$_2$D$_3$ decreases LPS-induced TNF-α, IL-1β, and cyclo-oxygenase-2 production and increases IL-10 production in macrophages [10,11]. Loss of the VDR in macrophages positively affects the development of colitis and is accompanied by increased pro-inflammatory cytokine expression in the inflammatory colon. This suggests that vitamin D signaling in innate cells plays a crucial role in controlling colon inflammation [12]. Vitamin D deficiency (< 20 ng/mL) has been correlated with the prevalence and severity of IBD [13]. Ananthakrishnan et al. [14] investigated the relationship between vitamin D status and the risk of IBD in 72,719 women over 12 years. The authors found that women with low 25(OH)D level developed IBD, whereas a high level of 25(OH)D reduced the incidence of CD. These researchers also found that sufficient vitamin D levels are important to maintain immune system homeostasis, and insufficient vitamin D promotes proinflammatory conditions. Zai et al. [15] used a nanostructured lipid carrier to encapsulate 1,25(OH)$_2$D$_3$ for the treatment of DSS-induced colitis. The authors demonstrated that 1,25(OH)$_2$D$_3$ suppressed colitis symptoms through expansion of the anti-inflammatory CX3CR1^{high} macrophage population and a reduction in the colonic tissue concentration of inflammatory cytokines. Mice with VDR deletion in colon non-intestinal epithelial cells (NEC-VDRKO) are more susceptible to DSS-induced colitis and show more extreme weight loss and strong M1 macrophage-characteristic cytokine expression under colitis [16]. Although various studies have demonstrated that vitamin D modulates macrophage activity and is correlated with IBD, the underlying mechanism remains unclear.

In this study, we hypothesized that vitamin D modulates macrophage polarization, and thus aids in ameliorating IBD. THP-1 cells, owing to their high responsiveness to LPS, are a meaningful model to investigate the mechanism of vitamin D-regulated innate immune activation [17–19]. Therefore, after demonstrating that 1,25(OH)$_2$D$_3$ regulates macrophage subtype redistribution in healthy volunteers, we used THP-1 cells to investigate the mechanisms of 1,25(OH)$_2$D$_3$-induced macrophage subtype distribution.

2. Materials and methods

2.1. Animals

Male 6–8-week-old C57BL/6 (B6) mice were obtained from Vital River Laboratory Animal Technology (Beijing, China). The mice were maintained under specific-pathogen-free conditions. All animal experiments were performed in accordance with the protocols for the Care and Use of Laboratory Animals published by China National Institute of Health.

2.2. Cell culture and treatments

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood samples from healthy volunteers. Briefly, the blood samples were layered onto 1.077 g/mL Ficoll-Hypaque (final volume ratio, 1:2) and the tubes were centrifuged at 366 ×g for 20 min to obtain the PBMCs. The PBMCs were cultured in Dulbecco's modified Eagle's medium (DMEM) for 6 h and then, non-adherent cells were discarded. The adherent PBMCs were cultured with phorbol 12-myristate 13-acetate (PMA; 50 ng/mL) for 24 h to induce macrophage differentiation [20]. PMA-induced macrophages were cultured with LPS (1 μg/mL) alone or with different concentrations of 1,25(OH)$_2$D$_3$ (99% purity, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) (2, 20, and 50 nM) for 24 or 48 h for subsequent analyses.

THP-1 cells were purchased from National Infrastructure of Cell Line Resource (Shanghai, China). The cells were grown in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin. For transfection assays, cells were transiently transfected with miR-125b mimic (miR10004731-1-5, RIBOBIO, Guangzhou, China), miR-125b mimic negative control (mimic NC; miR01201-1-5, RIBOBIO), miR-125b inhibitor (miR20004731-1-5, RIBOBIO), miR-125b inhibitor negative control (inhibitor NC; miR02201-1-5, RIBOBIO), or siR-VDR (GenePharma Co., Ltd., Suzhou, China) using Lipofectamine 2000 (Invitrogen, USA). Six hours later, the supernatants were discarded, and the transfected cells were cultured with LPS (1 μg/mL) plus 1,25(OH)$_2$D$_3$ (20 nM) for 24 or 48 h for subsequent analyses.

2.3. Animal experiments and treatment

Mice were randomly divided into seven treatment groups (n = 6 mice/group): vehicle (sterile drinking water, control), DSS (2% DSS-induced colitis), 2% DSS-induced colitis with 1,25(OH)$_2$D$_3$ (at three doses in three treatment groups), 2% DSS-induced colitis with 1,25(OH)$_2$D$_3$ 0.1 μg/mouse, and miRNA agomir (miR-125b agomir (miR40004731-1-10, RIBOBIO) and miRNA-125b agomir negative control (agomir NC; miR04201-1-10, RIBOBIO), in two treatment groups). Colitis was induced by 2% (w/v) DSS (MP Biomedicals, USA) in the sterile drinking water for 7 days followed by provision of normal drinking water [21]. For 1,25(OH)$_2$D$_3$ treatment, 1,25(OH)$_2$D$_3$ was dissolved in sterile corn oil and was injected intraperitoneally every other day for 4 days (days 1–4) prior to DSS administration. On days 5–11, the mice were administered 2% DSS dissolved in sterile drinking water for another 7 days. 1,25(OH)$_2$D$_3$ administration was continued until the end of the DSS treatment. The three doses tested were 0.025, 0.05, and 0.1 μg/mouse. For miR-125b agomir treatment, 1,25(OH)$_2$D$_3$-treated colitis mice were injected with miR-125b agomir or miRNA agomir NC via the tail vein every 2 days on days 5–11. Disease severity was evaluated based on the disease activity index (DAI) consisting of loss of body weight, stool consistency, and hemoccult, and the maximum DAI score was 12 (severe colitis) [21]. The animals were sacrificed on day 12, and colons and lamina propria mononuclear cells (LPMCs) were harvested for analyses.

For hematoxylin and eosin (HE) staining, formalin-fixed colons were embedded in paraffin, cut into 5-μm sections, and stained with

HE. Cell infiltration and tissue damage were assessed to evaluate the inflammation degree.

2.4. Chromatin immunoprecipitation (ChIP)

ChIP assays were conducted with the SimpleChIP® Enzymatic Chromatin IP Kit (Cell Signaling Technology, Danvers, MA, USA) following the manufacturer's protocol. Briefly, THP-1 cells were cultured in the presence of $1,25(OH)_2D_3$ (20 nM) for 48 h, and then, the cells were fixed with formaldehyde for 10 min to crosslink proteins to DNA. The chromatin was digested into 150–900-bp DNA/protein fragments using micrococcal nuclease. After centrifugation, 10 μL of the supernatant was removed to serve as the 2% input control. The remainder of the supernatant was incubated with anti-VDR or normal control immunoglobulin G (IgG) antibody (Cell Signaling Technology). The complex co-precipitates were captured using Protein G magnetic beads. After elution of the chromatin from the magnetic beads, the DNA was purified and used as a template for real-time (q)PCR targeting a fragment of the IL-10 promoter containing the VDR-binding side, with 40 cycles of amplification. The primer sequences were: forward 5′-AAGCTGAGAACCAAGACCCA-3′ and reverse 5′-AGATGGTATTTTG GGGGCAGC-3′. The following formula was used to analyze the ChIP results: percent input = $2\% \times 2^{(C_T\ 2\%\text{Input Sample} - C_T\ \text{IP Sample})}$.

2.5. Isolation of LPMCs

LPMCs were isolated as described previously [22]. Briefly, colons were cut into pieces and were washed with phosphate-buffered saline to remove debris and blood. The colon tissues were incubated with 5 nM EDTA at 37 °C for 30 min under vigorous shaking to separate the epithelial cells from the mucosa. After several washes with Hank's balanced salt solution, the tissues were digested with collagenase IV (1 mg/mL; Sigma-Aldrich) and DNase (40 μg/mL; Roche, Basel, Switzerland) at 37 °C for 2 h, with gentle shaking. The digestion solutions were supplemented with 5% FBS and 50 μg/mL gentamicin. LPMCs were collected by centrifugation with 40/70% Percoll–DMEM solution. Cells at the interface were used for subsequent assays.

2.6. RNA extraction and quantitative reverse transcription (RT-q)PCR

Total RNA was extracted using an RNA Kit (Omega, USA) following the manufacturer's instruction. First-strand cDNA was generated from 1 μg of total RNA using a reverse transcription kit (Roche). PCRs were conducted using SYBR Green mix (Roche) on an ABI Step-One Real-Time PCR System. The primers were purchased from Shenggong and RIBOBIO, and the sequence of the primer was as follows: human TNF-α, forward (F) 5′-TGGCGTGGAGCTGAGAGATA-3′, reverse (R) 5′-CTGGT AGGAGACGGCGATG-3′; human IL-6-F 5′-TTCGGTCCAGTTGCCT TCT-3′, IL-6-R 5′-GGTGAGTGGCTGTCTGTGTG-3′; human IL-10-F 5′-CTTGCTGGAGGACTTTAAGGGTTACC-3′, Il-10-R 5′-GCCTTGATGTC TGGGTCTTGGTTC-3′; human Arg-1-F 5′-GCCAAGTCCAGAACCAT AGG-3′, Arg-1-R 5′-CTCAAGCAGACCAGCCTTTC-3′; mouse TNFα-F 5′-ATGTCTCAGCCTCTTCTCATTC-3′, TNFα-R 5′-GCTTGTCACTCGAAT TTTGAGA-′; mouse IL-6-F 5′-CTCCCAACAGACCTGTCTATAC-3′, Il-6-R 5′-CCATTGCACAACTCTTTTCTCA-3′; mouse IL-10-F 5′-TTCTTTCAAAC AAAGGACCAGC-3′, IL-10-R 5′-GCAACCCAAGTAACCCTTAAAG-3′; mouse Arg-1-F 5′-CATATCTGCCAAAGACATCGTG-3′, Arg-1-R 5′-GAC ATCAAAGCTCAGGTGAATC-3′. Relative mRNA and miRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method. GAPDH or U6 small nuclear RNA (RNU6) served as endogenous controls for normalization.

2.7. Western blotting

Total protein of cells was extracted with RIPA buffer containing protease inhibitor. Equal amounts of protein were separated on 10% Bis-Tris gels and were transferred onto polyvinylidene fluoride membranes. After blocking with 5% non-fat milk at room temperature for 1 h, the membranes were incubated with primary antibodies at 4 °C overnight, with gentle shaking. Then, the membranes were incubated with horse radish peroxidase-conjugated secondary antibodies at room temperature for 1.5 h, with gentle shaking. Immunoreactive bands were visualized using a chemiluminescence kit. Target protein expression was quantified by densitometry and was normalized to the levels of IRF5 and GAPDH.

2.8. Flow cytometry

Single-cell suspensions from human PMA-induced macrophages or mouse LPMCs were stained with fluorochrome-conjugated CD68 or F4/80 (BD Biosciences or BioLegend, USA) or their isotype control antibodies at 4 °C for 1 h. After fixation for 30 min and permeabilization for 10 min, human macrophages or mouse LPMCs were treated with fluorochrome-labeled anti-HLA-DR and CD163 (BD Biosciences) or anti-iNOS and CD206 (BD Biosciences, BioLegend), respectively, at room temperature for 30 min. Fluorochrome-stained cells were analyzed by flow cytometry (BD Biosciences). $CD68^+HLA\text{-}DR^+$ and $F4/80^+iNOS^+$ cells were defined as human or mouse M1 macrophages, whereas $CD68^+CD163^+$ and $F4/80^+CD206^+$ cells were defined as human or mouse M2 macrophages.

2.9. Statistical analysis

All results are expressed as means ± standard deviations. The two-tailed Student's *t*-test was used to analyze differences between two groups, and analysis of variance was applied for more than two groups. $P < 0.05$ was considered significant.

3. Results

3.1. $1,25(OH)_2D_3$ polarizes macrophages to the M2 phenotype and alters subtype-related mRNA expression

PMA-induced macrophages from healthy volunteers were cultured with LPS (1 μg/mL) alone or in the presence of $1,25(OH)_2D_3$ at different concentrations for 48 h. Fluorescence-activated cell sorting (FACS) results showed that LPS promoted the M1 subtype, whereas $1,25(OH)_2D_3$ (20 nM or 50 nM) prevented this polarization to M1 (Fig. 1C). The ratio of M1 to M2 macrophages was 2.696 ± 0.346 in cultures with LPS alone, but was only 0.64 ± 0.19 or 0.44 ± 0.14 in the presence of 20 nM or 50 nM $1,25(OH)_2D_3$, respectively. Consistent with the macrophage subtype redistribution, $1,25(OH)_2D_3$ at 20 nM and 50 nM downregulated the expression of LPS-induced M1 macrophage-characteristic cytokines, TNF-α and IL-6, and upregulated that of the M2 subtype-associated factors IL-10 and Arg-1 (Fig. 1D). The results indicated that 20 nM $1,25(OH)_2D_3$ was the most effective concentration; therefore, this concentration was used for further experiments.

$1,25(OH)_2D_3$ is inactive when it is not bound to the VDR. In humans, cells express a basal level of VDR. $1,25(OH)_2D_3$ (20 nM) upregulated VDR expression, which was inhibited after siR-VDR transfection (Fig. 2A). In addition, siR-VDR transfection affected $1,25(OH)_2D_3$-induced macrophage polarization in PBMCs. The M1/M2 ratio was 0.592 ± 0.23 upon treatment with 20 nM $1,25(OH)_2D_3$ and increased to 1.475 ± 0.505 after siR-VDR transfection (Fig. 2D). The cytokine production levels were also changed upon siR-VDR transfection (Fig. 2E). These findings confirmed that VDR is involved in macrophage polarization induced by 20 nM $1,25(OH)_2D_3$.

3.2. $1,25(OH)_2D_3$ promotes IL-10 expression by affecting IL-10 promoter activity

Bioinformatics analysis of data in the transcription factor database JASPAR identified *IL-10* as a VDR target gene. $1,25(OH)_2D_3$ (20 nM)

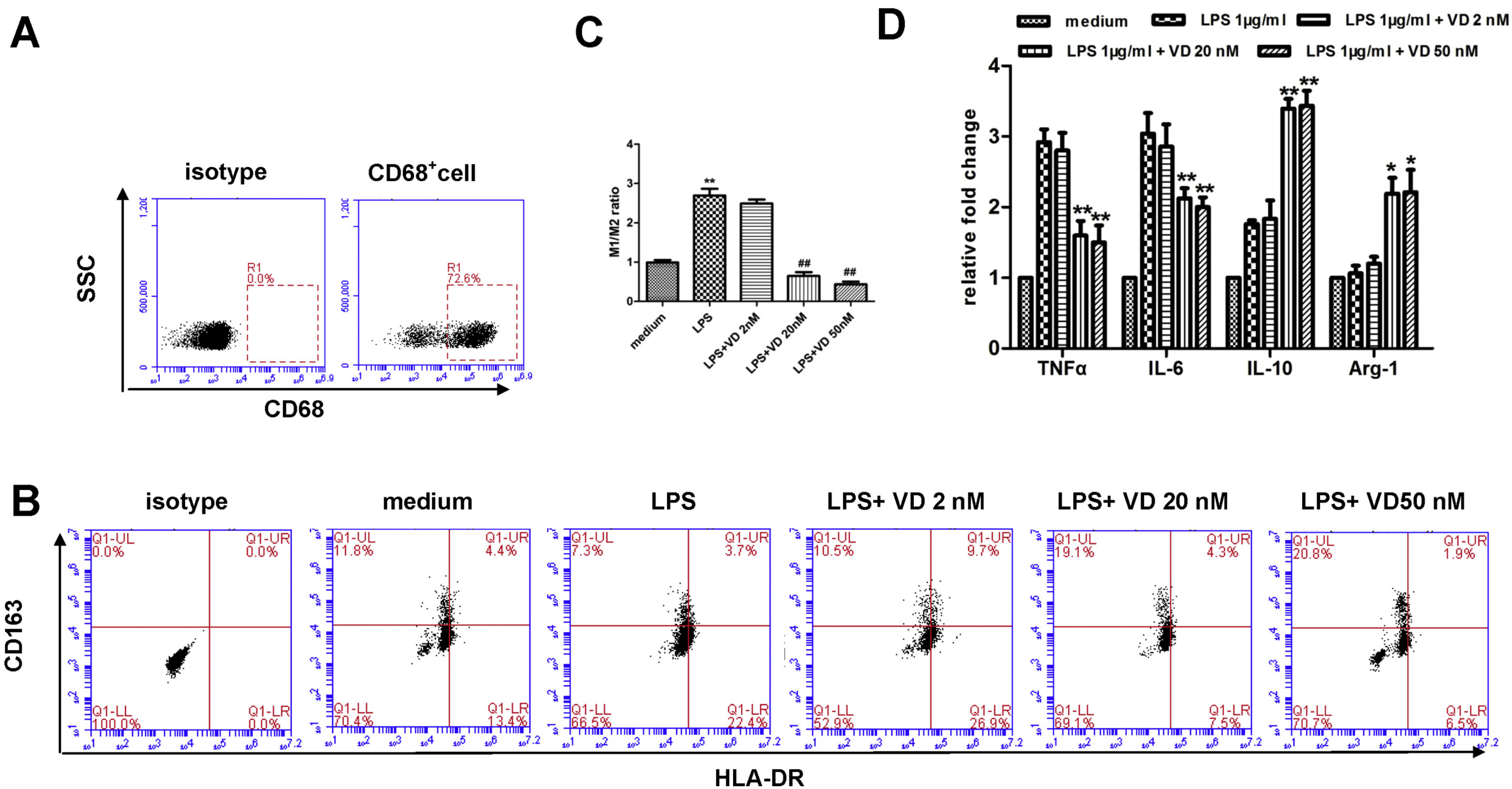

Fig. 1. 1,25$(OH)_2D_3$ alters LPS-induced macrophage subtype distribution and subtype-relevant cytokine expression. PBMCs from healthy volunteers were cultured with PMA to induce macrophages and were then treated with LPS (1 μg/mL) alone or in combination with 1,25$(OH)_2D_3$ at different concentrations for 24 or 48 h. (A) $CD68^+$ cells were gated. (B) $CD68^+$ cells. HLA-DR and CD163 served as M1 and M2 phenotype markers, respectively. (C) Quantitative data of (B). $^{**}P < 0.01$ versus medium (DMEM); $^{\#\#}P < 0.01$ versus LPS. n = 4. (D) Macrophage subtype-relevant cytokine expression. $^{*}P < 0.05$, $^{**}P < 0.01$ versus LPS. n = 4. VD: 1,25$(OH)_2D_3$.

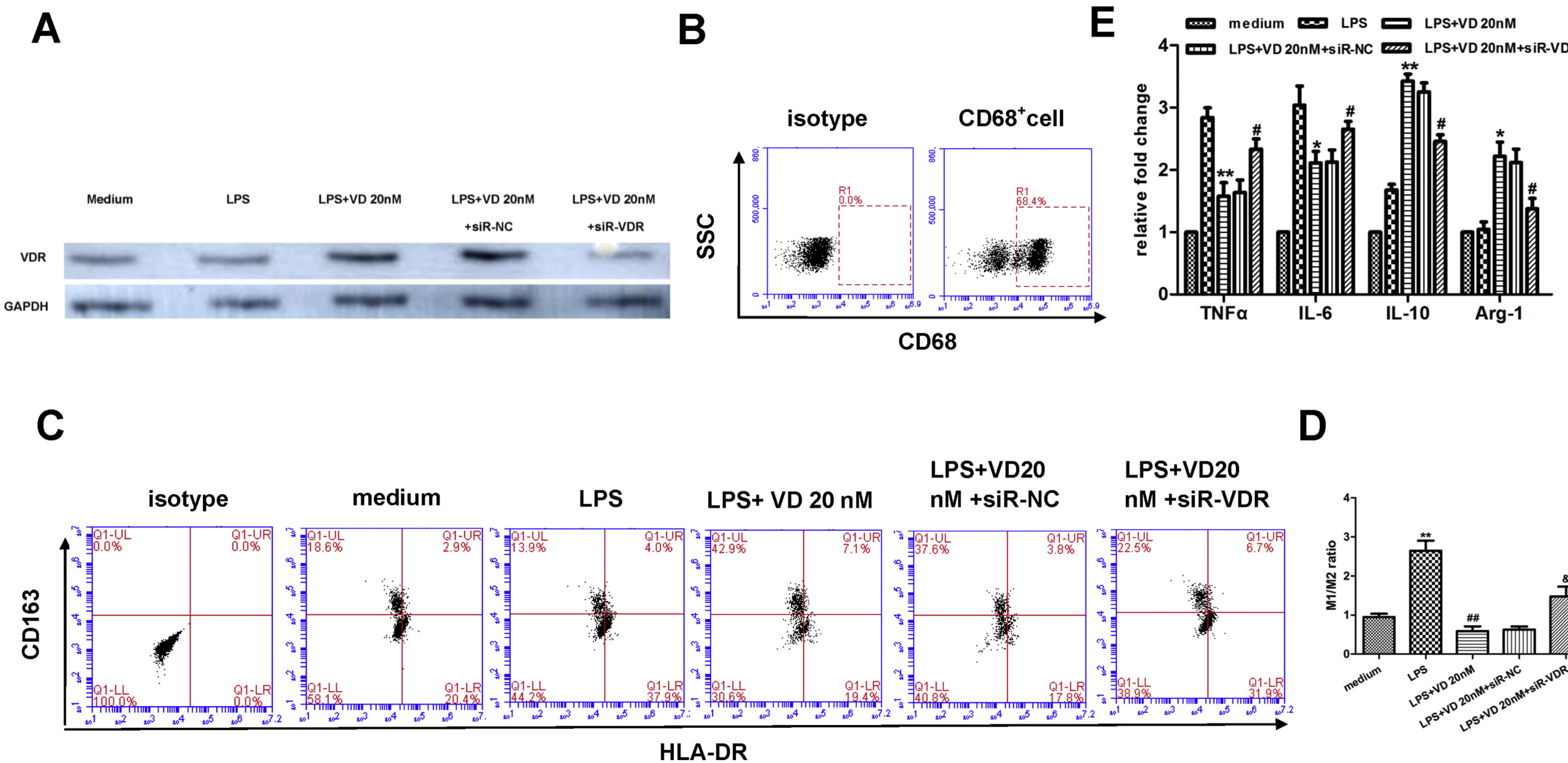

Fig. 2. VDR is involved in 1,25(OH)$_2$D$_3$-induced macrophage polarization. PBMCs were transfected with siR-VDR for 6 h and were then cultured with LPS (1 μg/mL) plus 1,25(OH)$_2$D$_3$ (20 nM) for 24 h or 48 h. (A) VDR protein expression. (B) CD68$^+$ cell gate. (C) CD68$^+$ cells. HLA-DR and CD163 served as M1 and M2 phenotype markers, respectively. (D) Quantitative data of (C). $^{**}P < 0.01$ versus medium (DMEM); $^{\#\#}P < 0.01$ versus LPS; $^{\&}P < 0.05$ versus LPS + VD 20 nM. n = 4. (E) Macrophage subtype-relevant cytokine expression. $^{*}P < 0.05$, $^{**}P < 0.01$ versus LPS. $^{\#}P < 0.05$ versus LPS + VD 20 nM. n = 4. VD: 1,25(OH)$_2$D$_3$.

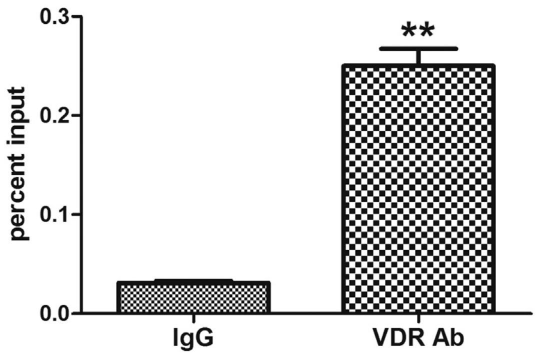

Fig. 3. $1,25(OH)_2D_3$ promotes VDR recruitment to the *IL-10* promoter. A chip assay was conducted using digested chromatin from THP-1 cells and VDR (and IgG control) antibodies. The amount of immunoprecipitated DNA in each sample was normalized to the total amount of chromatin. $^{**}P < 0.01$ versus IgG. n = 4.

significantly upregulated relative IL-10 mRNA expression from 1.762 ± 0.093 to 3.396 ± 0.236 (Fig. 1D). Therefore, a ChIP assay was used to evaluate whether $1,25(OH)_2D_3$ treatment could induce VDR recruitment to the *IL-10* promoter. As shown in Fig. 3, compared with IgG, VDR antibody induced an increase in IL-10 expression. Thus, $1,25(OH)_2D_3$ upregulates IL-10 expression through direct interaction with the IL-10 promoter.

3.3. $1,25(OH)_2D_3$ alters LPS-induced IRF4 expression and IRF5 phosphorylation in macrophages

IRFs are transcription factors that regulate type I IFN gene expression. Previous studies have shown that IRFs also regulate macrophage polarization. IRF4 specifically modulates macrophage polarization to the M2 phenotype in response to chitin and helminth infection [23]. IRF5 is characteristic for M1 macrophages, and it not only activates M1 genes, but also represses M2 genes by binding to their promoters [24]. To determine whether IRFs are associated with $1,25(OH)_2D_3$-induced macrophage polarization, we detected IRF4 and phosphorylated IRF5 protein levels and transfected THP-1 cells with siR-VDR to examine the role of VDR in $1,25(OH)_2D_3$-induced IRF4 expression and IRF5 phosphorylation. $1,25(OH)_2D_3$ (20 nM) promoted IRF4 and VDR protein expression and inhibited IRF5 phosphorylation in LPS-treated cells (Fig. 4A). The VDR protein level decreased by 58.39% after siR-VDR transfection. IRF4 and phosphorylated IRF5 protein levels were 0.607 ± 0.114 and 0.267 ± 0.083 in LPS plus $1,25(OH)_2D_3$-treated cells, respectively. However, after siR-VDR transfection, these levels decreased to 0.291 ± 0.065 and increased to 0.734 ± 0.144, respectively (Fig. 4B). These results showed that $1,25(OH)_2D_3$ upregulates IRF4 expression and downregulates IRF5 phosphorylation through the VDR.

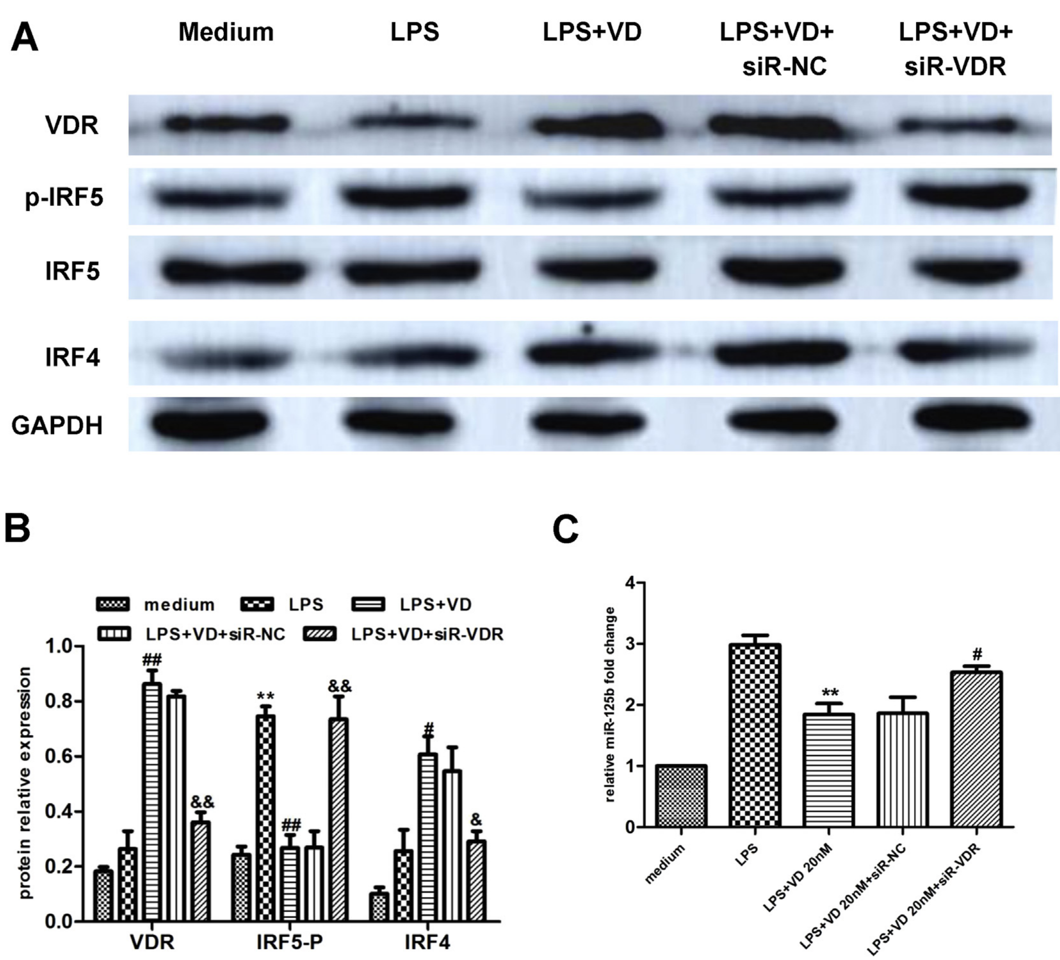

Fig. 4. $1,25(OH)_2D_3$ regulates LPS-induced VDR, IRF4, IRF5, and miR-125b expression, and VDR is involved in the regulation. THP-1 cells were transfected with siR-VDR for 6 h and were then cultured with LPS (1 μg/mL) plus $1,25(OH)_2D_3$ (20 nM) for 24 or 48 h. (A) Western blots showing VDR, IRF4, and phosphorylated (p-)IRF5 protein levels. (B) Quantitative data of (A). $^{**}P < 0.01$ versus medium. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus LPS. $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ versus LPS + VD 20 nM. n = 4. (C) MiR-125b expression as assessed by qPCR. $^{**}P < 0.01$ versus LPS; $^{\#}P < 0.05$ versus LPS + VD 20 nM. n = 4. VD: $1,25(OH)_2D_3$.

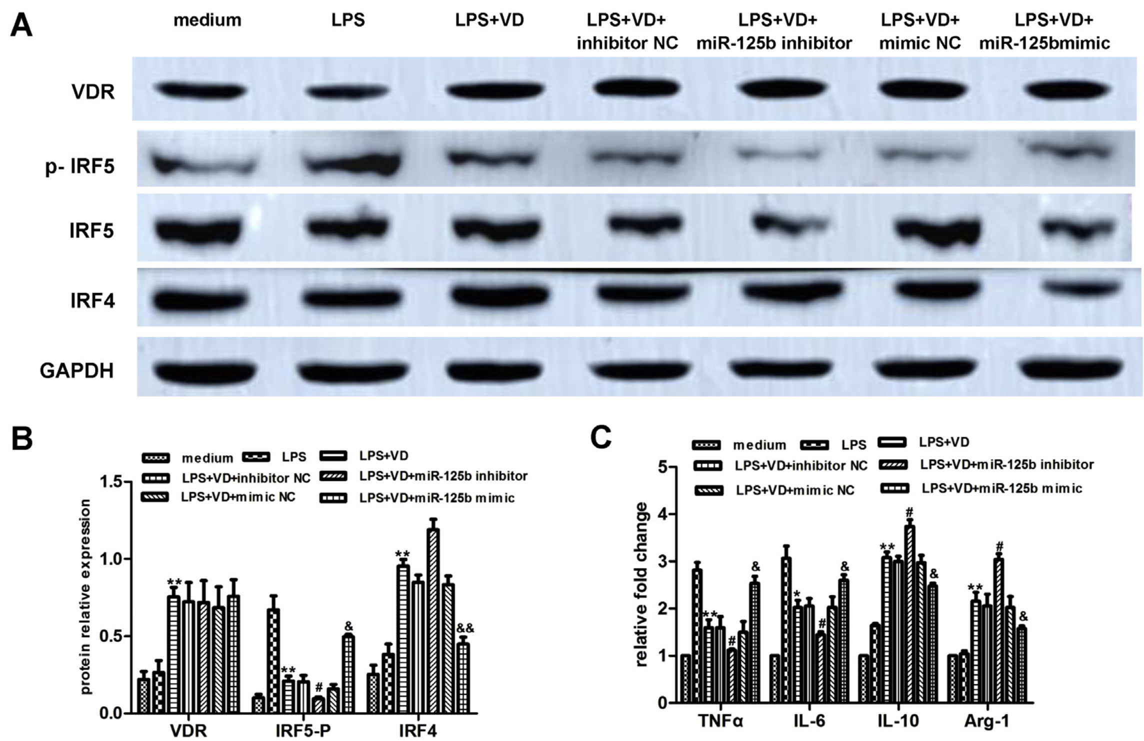

Fig. 5. MiR-125b affects 1,25(OH)$_2$D$_3$-induced IRF4, IRF5, and macrophage subtype cytokine expression. THP-1 cells were transfected with miR-125b mimic or inhibitor for 6 h and were then cultured with LPS (1 μg/mL) plus 1,25(OH)$_2$D$_3$ (20 nM) for 24 or 48 h. (A) Western blots showing VDR, IRF4, and p-IRF5 protein levels. $^{**}P < 0.01$ versus LPS. $^{\#}P < 0.05$, $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ versus LPS + V D 20 nM. n = 4. (B) Quantitative data of (A). (C) Macrophage subtype-relevant cytokine expression. $^{*}P < 0.05$, $^{**}P < 0.01$ versus LPS. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ versus LPS + VD 20 nM. n = 4. VD: 1,25(OH)$_2$D$_3$.

3.4. MiR-125b affects 1,25(OH)$_2$D$_3$-induced IRF expression and macrophage polarization

MicroRNAs are important mediators of the macrophage activation process. Recent studies have demonstrated that miR-125b can directly target IRF4 and promote macrophage-mediated inflammation [25]. 1,25(OH)$_2$D$_3$ decreased LPS-induced miR-125b expression through the VDR (Fig. 4C). Thus, we transfected macrophages with a miR-125b mimic or inhibitor to detect whether 1,25(OH)$_2$D$_3$ induces macrophage polarization via miR-125b. As shown in Fig. 5B, compared with LPS treatment, VDR and IRF4 expression was increased by 1.88- and 1.8-fold, respectively, and phosphorylated IRF5 was decreased by 0.68-fold in 1,25(OH)$_2$D$_3$-exposed cells. MiR-125b inhibitor and mimic did not affect VDR expression in 1,25(OH)$_2$D$_3$-treated cells. MiR-125b mimic suppressed 1,25(OH)$_2$D$_3$-induced IRF4 expression from 0.954 ± 0.072 to 0.449 ± 0.078 ($P < 0.01$). 1,25(OH)$_2$D$_3$-induced IRF5 phosphorylation decreased from 0.21 ± 0.05 to 0.09 ± 0.018 and increased from 0.21 ± 0.05 to 0.496 ± 0.027 in inhibitor- and mimic-treated cells, respectively ($P < 0.05$). Moreover, the expression of the M1 macrophage-related cytokines TNF and IL-6 was increased, and that of the M2 macrophage-related cytokines IL-10 and Arg-1 was decreased in miR-125b mimic-treated cells, whereas miR-125b inhibitor had opposite effects (Fig. 5C).

3.5. 1,25(OH)$_2$D$_3$ plays a protective role in murine experimental colitis

DSS-induced colitis is a well-established model for investigating the pathogenesis of IBD. DSS treatment impairs the intestinal epithelial barrier, exposes the submucosa to luminal bacteria, and induces inflammatory cell infiltration and innate immunity. 1,25(OH)$_2$D$_3$ pretreatment ameliorated DSS-induced colitis severity; the DAI of mice pretreated with 0.1 μg 1,25(OH)$_2$D$_3$ (3 ± 0.89) was significantly lower ($P < 0.05$) than that of the group treated with DSS alone (4.5 ± 1.22) (Fig. 6A). Histological assessment of the colitis-affected tissues revealed that 1,25(OH)$_2$D$_3$ pretreatment lowered cell infiltration and crypt damage (Fig. 6B).

3.6. 1,25(OH)$_2$D$_3$ alters the macrophage subset distribution and related factor expression in colonic LPMCs from colitis mice

As 1,25(OH)$_2$D$_3$ suppressed the LPS-induced switch to the M1 phenotype in vitro, we investigated whether the suppression of intestinal inflammation by 1,25(OH)$_2$D$_3$ was correlated with macrophage subset redistribution. After administration of DSS through drinking water for 7 days, mouse colonic LPMCs were isolated to analyze macrophage polarization and the expression of related factors. In the vehicle-treated group, the M1 and M2 macrophage populations were equally large as indicated by FACS analysis. DSS treatment induced macrophage polarization to the M1 phenotype; however, 1,25(OH)$_2$D$_3$ (0.1 μg) reverted the DSS-induced M1 macrophages to the M2 phenotype. The M1/M2 ratio was 8.11 ± 1.81 in the DSS-treated group and 0.71 ± 0.24 in the group pretreated with 0.1 μg 1,25(OH)$_2$D$_3$ (Fig. 7C).

Next, we evaluated macrophage subset-characteristic cytokine expression. TNF-α and IL-6 expression was increased significantly in the DSS group, corroborating that DSS treatment induces macrophage polarization to the M1 phenotype. IL-10 and Arg1 mRNA levels were increased in the 1,25(OH)$_2$D$_3$ (0.1 μg) pretreatment group compared with the DSS-only group ($P < 0.01$, Fig. 7D). These findings suggested that 1,25(OH)$_2$D$_3$ promoted M1 macrophages to switch to the M2 phenotype, and the mobilization of M2 macrophages ameliorated DSS-induced colitis severity. Consistent with the macrophage subtype redistribution, 1,25(OH)$_2$D$_3$ (0.1 μg) pretreatment decreased the phosphorylated IRF5 protein level from 0.93 ± 0.25 to 0.56 ± 0.12 ($P < 0.05$) and increased IRF4 expression from 0.18 ± 0.08 to 0.61 ± 0.12 ($P < 0.01$) in LPMCs from colitis mice (Fig. 7F).

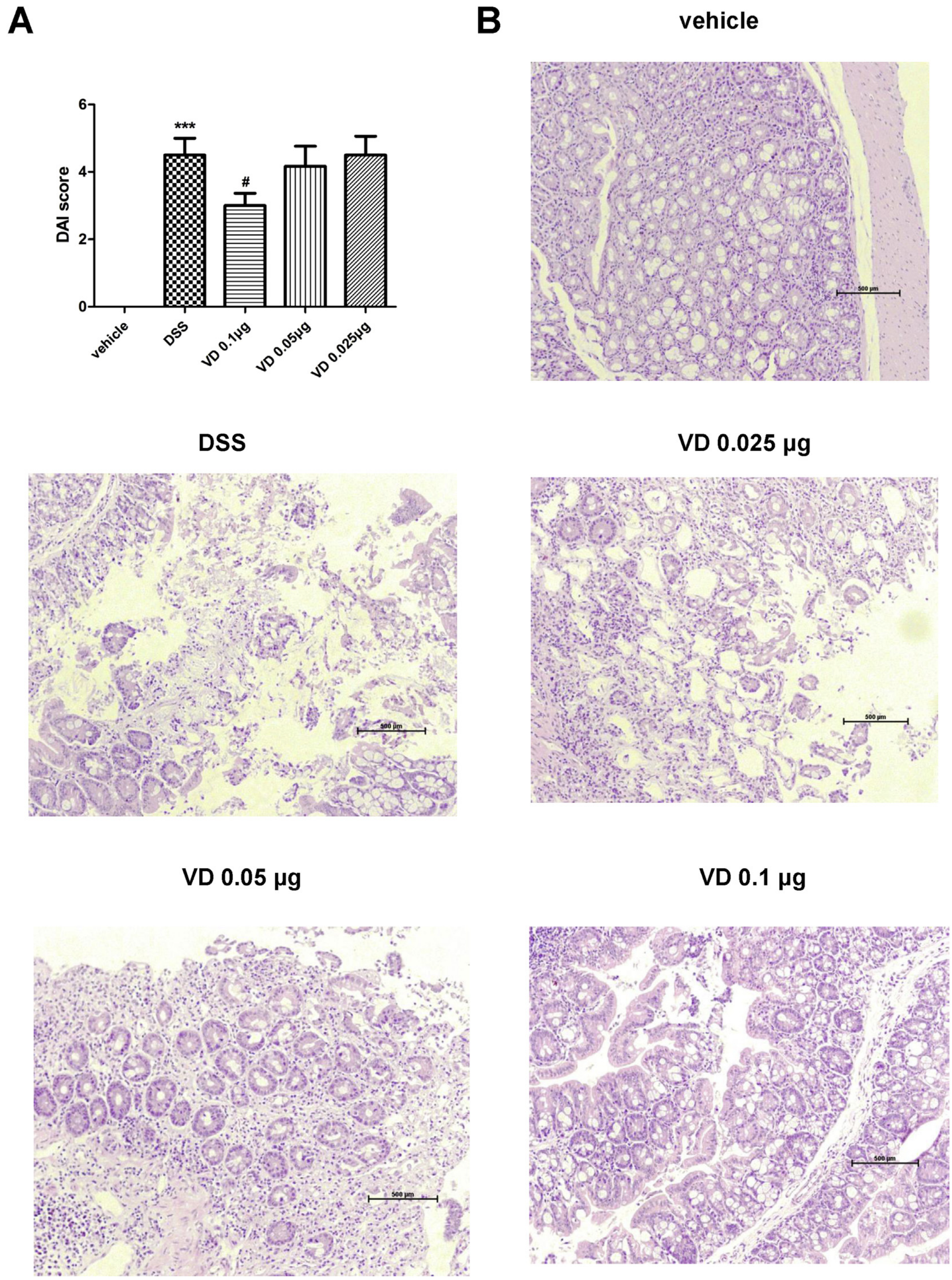

Fig. 6. $1,25(OH)_2D_3$ ameliorates the severity of DSS-induced colitis. Mice were pretreated with $1,25(OH)_2D_3$ for 4 days. On day 5, the mice were given 2% (w/v) DSS in drinking water for another 7 days (days 5–11). (A) DAI scores determined on day 12. $^{***}P < 0.001$ versus vehicle. $^{\#}P < 0.05$ versus DSS. n = 6 mice/group. (B) On day 12, distal colons were collected and stained with HE. VD: $1,25(OH)_2D_3$.

3.7. MiR-125b interferes with the severity of $1,25(OH)_2D_3$-related amelioration of experimental colitis

As we had observed that miR-125b regulated $1,25(OH)_2D_3$-induced macrophage polarization in vitro, we injected miR-125b agomir into $1,25(OH)_2D_3$-treated colitis mice and analyzed the macrophage subtype distribution and related factors to determine whether miR-125b correlated with the $1,25(OH)_2D_3$ amelioration of colitis severity. The results showed that miR-125b expression was indeed correlated with colitis severity and that miR-125b agomir accelerated the development of colitis. Further, miR-125b agomir promoted $1,25(OH)_2D_3$-induced macrophage polarization to the M1 subtype as well as TNF-α and IL-6 expression and IRF5 phosphorylation, whereas it suppressed IL-10, Arg-1, and IRF4 expression (Fig. 8).

4. Discussion

The incidence and prevalence of IBD are increasing worldwide, particularly in Europe, North America, and Australia [26]. IBD has recently become one of the most prevalent intestinal diseases globally.

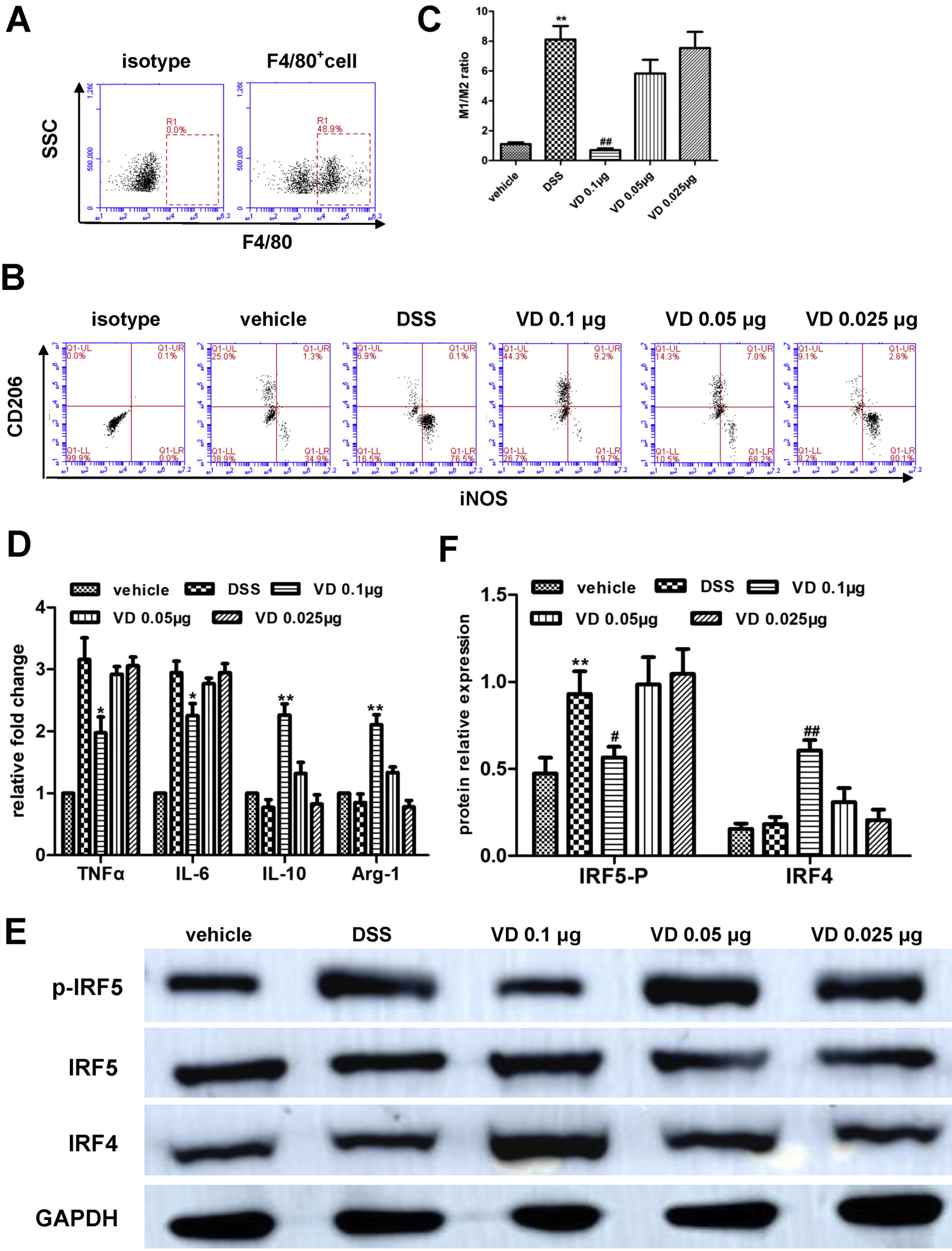

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Fig. 7. $1,25(OH)_2D_3$ alters the macrophage subtype distribution in mice DSS-colitis colonic LPMCs. Mice were pretreated with $1,25(OH)_2D_3$ for 4 days. On day 5, the mice were given 2% (w/v) DSS in drinking water for another 7 days. $1,25(OH)_2D_3$ administration was continued until the end of the DSS treatment. LPMCs were isolated on day 12. (A) F4/80$^+$ cell gate. (B) F4/80$^+$ cells. iNOS and CD206 served as M1 and M2 phenotype markers, respectively. (C) Quantitative data of (B). $^{**}P < 0.01$ versus vehicle. $^{\#\#}P < 0.01$ versus DSS. n = 6 mice/group. (D) Macrophage subtype-relevant cytokine expression. $^{*}P < 0.05$, $^{**}P < 0.01$ versus DSS. n = 6 mice/group. (E) Western blots showing IRF4 and p-IRF5 protein levels. (F) Quantitative data of (E). $^{**}P < 0.01$ versus vehicle. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus DSS. n = 6 mice/group. VD: $1,25(OH)_2D_3$.

Although the etiology of IBD remains to be fully elucidated, a number of studies have indicated that deregulated immunological responses are responsible for the pathogenesis of IBD. Vitamin D is well known to have important roles in calcium homeostasis and bone mineralization, increasing evidence indicates that several immunological cells express the VDR and that vitamin D plays a key role in the maintenance of intestinal barrier integrity and immune regulation. Therefore, the effects of vitamin D on immune-related diseases have gained increasing research attention. Myeloid-specific, but not intestinal-specific VDR knock-out mice showed aggravated colitis symptoms, which suggested that vitamin D-mediated immune regulation might be related to IBD [12]. Moreover, epidemiological studies have shown that individuals with low serum vitamin D have an increased risk of IBD, and serum vitamin D levels are associated with disease activity. Recent studies have revealed that robust vitamin D supplementation is beneficial for IBD patients [27,28]. Thus, vitamin D deficiency may play a role in the development of IBD.

Macrophages are the major population of mononuclear phagocytes in the lamina propria and play a crucial role in the maintenance of intestinal homeostasis by recognizing and eliminating bacteria and regulating T-cell activity. M1 and M2 macrophages have opposite effects on inflammation: M1 macrophages promote inflammation, whereas M2 macrophages suppress inflammation. In response to a stimulus, macrophages can switch their phenotype. Regulating macrophage polarization to the M2 subtype might protect against inflammation in colitis. Numerous studies have demonstrated that vitamin D lowers the expression of pro-inflammatory cytokines, such as IL-1β, IL-12, and TNF-α, and increases IL-10 expression in macrophages. Our current study indicated that $1,25(OH)_2D_3$ promotes M2 polarization in LPS-induced macrophages and colitis-associated LPMCs. Further, our findings demonstrated that $1,25(OH)_2D_3$ regulates macrophage subtype-associated factor expression. Hayashi et al. [29] demonstrated that macrophages from p85α hetero-deficient mice suppress DSS-induced colitis development by producing high levels of IL-10. IL-10 is an essential regulator of intestinal homeostasis maintenance. IL-10 knockout mice exhibit normal lymphocyte development, aberrant cytokine production of macrophages and T cells, and extensive inflammatory reactions in the intestine [30,31]. Daily dietary vitamin D supplementation ameliorated colitis symptoms in these IL-10 knockout mice [32,33]. Vitamin D promotes IL-10 production, creating a feedback loop via suppression of gene expression of cytokine signaling 3 (SOCS3), which downregulates LPS-activated microglia and the expression of pro-inflammatory cytokines [10]. IL-10 is a target gene of VDR, and Matilainen et al. [34] demonstrated that VDR binds to a promoter region upstream of the *IL-10* transcription start site in $1,25(OH)_2D_3$-treated macrophages. In this study, $1,25(OH)_2D_3$ treatment induced VDR recruitment to the *IL-10* promoter. Our findings suggest that $1,25(OH)_2D_3$ induces *IL-10* expression by directly affecting promoter activity. Our previous study showed that IL-10, a key immune regulator factor produced mainly by M2 macrophages, ameliorates IBDs by promoting Treg generation [4]. Korf et al. [35] demonstrated that $1,25(OH)_2D_3$ curtailed the inflammatory and T-cell stimulatory capacity of macrophages through an IL-10-dependent mechanism. In this study, we demonstrated that $1,25(OH)_2D_3$ treatment suppresses the development of DSS-induced colitis by modulating M2 macrophage polarization. The effect of $1,25(OH)_2D_3$-induced M2 macrophages on T-cell activity requires further investigation.

The IRF transcription factor family regulates immune cell activity and has therefore gained increasing attention. IRF5 directly induces the transcription of M1 macrophage-characteristic genes, such as *TNFα*, *IL-12*, and *IL-23*, whereas it represses *IL-10* expression, by binding to the promoters of these genes [24]. IRF5-deficient mice show impaired TH1- and TH17-specific cytokine production and are resistant to lethal shock induced by LPS [24,36]. IRF4 is a characteristic of M2 macrophages and induces specific M2 marker expression. Interestingly, IRF4 and IRF5 have the same binding site on MyD88 and compete with each other for activation, and the IRF4-IRF5 axis is crucial in modulating macrophage polarization [37]. NF-κB is another important regulator of macrophages and inflammation. It is activated by IκB kinase, which also phosphorylates and activates IRF5. Vitamin D suppresses LPS-induced inflammation in macrophages by promoting VDR interaction with the NF-κB p50 subunit, which attenuates LPS-induced Krüppel-like zinc-finger transcription factor 5 (KLF5) interaction with the p50 subunit [38]. In this study, consistent with modulating LPS-induced macrophage subtype redistribution, $1,25(OH)_2D_3$ upregulated IRF4 expression and decreased IRF5 phosphorylation in macrophages, both in vitro and in DSS-induced colitis mice. IRF5 contains two nuclear localization signals (NLSs). Upon activation by microbial stimuli, IRF5 phosphorylation results in the NLSs becoming exposed and mediating the nuclear translocation and retention of IRF5 in activated cells. Ren et al. [39] demonstrated that IKKβ phosphorylates IRF5 at serine residues, and this phosphorylation is important for inflammatory cytokine induction. The current study demonstrated that $1,25(OH)_2D_3$ decreased LPS-induced IKKβ expression, and IKKβ expression in LPMCs was correlated with the severity of DSS-induced colitis (data not shown). $1,25(OH)_2D_3$ decreased LPS-induced IRF5 phosphorylation, which may be associated with the suppression of IKKβ activity. However, this requires further investigation.

MiRNAs are a class of small, non-coding RNAs of 18 to 22 nucleotides that control gene expression by post-transcriptional regulation, and are involved in the regulation of cell functions. Different miRNAs are expressed in different macrophage subsets [40]. MiR-125b targets IRF4, increases the surface expression of MHC II, CD40, CD86, CD80, and IFN-γ receptor, and inhibits proinflammatory pathways in macrophages. Increasing evidence suggests that aberrant miRNA expression is correlated with IBD development. Valmiki et al. [41] took colonic mucosal pinch biopsies from UC patients and analyzed miRNA expression in inflamed and non-inflamed regions of the same patient. The authors reported that, compared with the non-inflamed regions, miR-125b and miR-155 expression was increased by 2.56- and 2.33-fold, respectively, in inflamed regions of the colon [41]. MiRNAs also regulate several genes involved in the vitamin D pathway, such as *VDR*, *CYP24A1*, *CYP27B1*, and *RXRα*. MiR-125b was the first miRNA identified that targets the VDR and post-transcriptionally regulates *CYP24A1* expression [42]. In this study, $1,25(OH)_2D_3$ decreased miR-125b expression in LPS-treated macrophages. SiR-VDR transfection promoted miR-125b expression and suppressed $1,25(OH)_2D_3$-induced miR-125b downregulation. When we used miR-125b mimic and inhibitor to investigate whether $1,25(OH)_2D_3$ regulates IRF4 expression and IRF5 phosphorylation through miR-125b, we found that the mimic and inhibitor did affect $1,25(OH)_2D_3$-induced IRF5 phosphorylation and subtype-associated cytokine expression in macrophages. However, the miR-125b inhibitor did not significantly increase $1,25(OH)_2D_3$-induced IRF4 expression. Thus, $1,25(OH)_2D_3$-induced macrophage activities do not directly target IRF4. Although *VDR* is a target of miR-125b, miR-125b mimic and inhibitor did not alter $1,25(OH)_2D_3$-

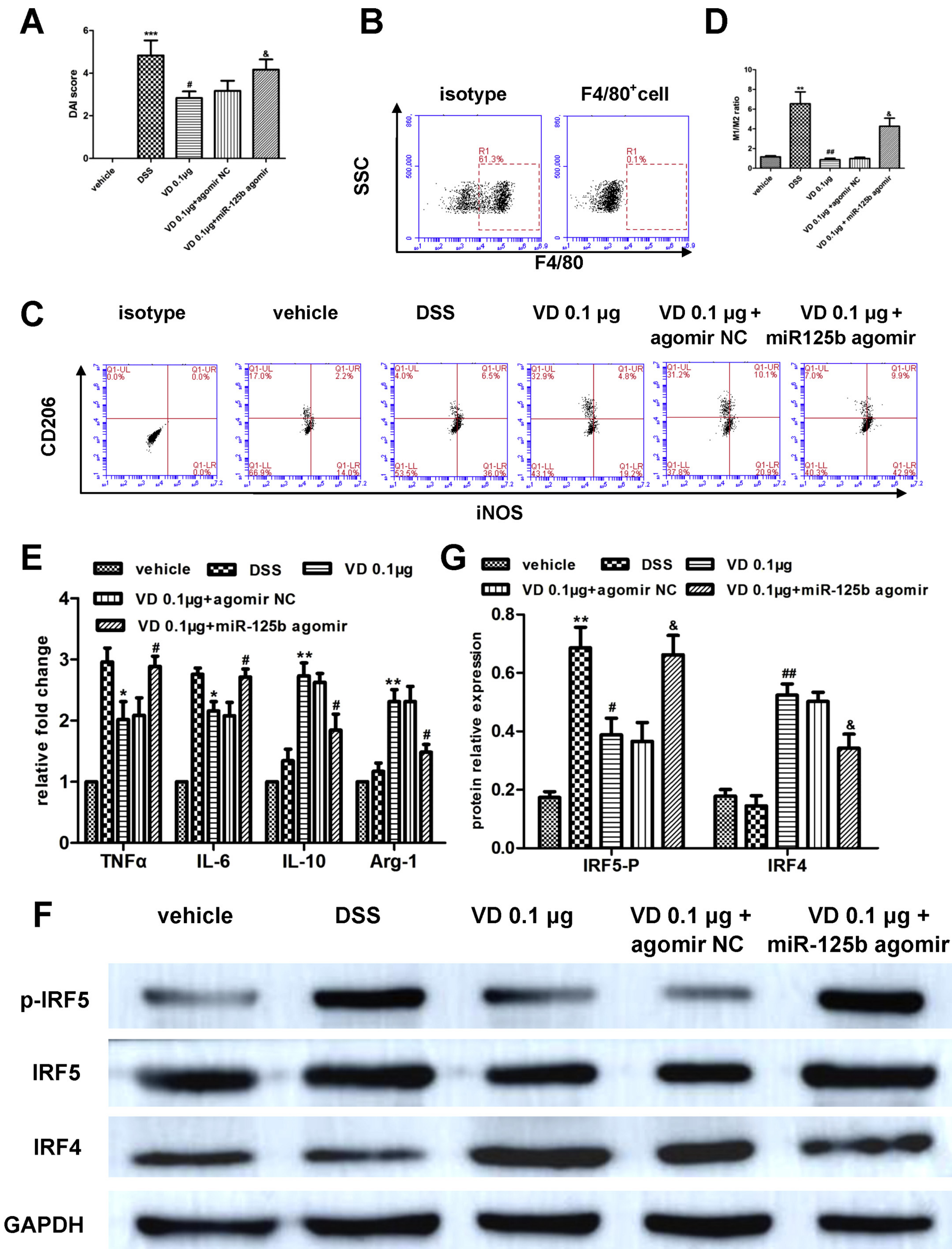

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Fig. 8. MiR-125b interferes with 1,25(OH)$_2$D$_3$ amelioration of experimental colitis severity. 1,25(OH)$_2$D$_3$-treated DSS mice were injected with miR-125b agomir via the tail vein every 2 days from day 5 to day 11. On day 12, LPMCs were isolated. (A) DAI scores determined on day 12. $^{***}P < 0.001$ versus vehicle. $^{\#}P < 0.05$ versus DSS. $^{\&}P < 0.05$ versus VD 0.1 μg. n = 6 mice/group. (B) F4/80$^+$ cell gate. (C) F4/80$^+$ cells. iNOS and CD206 served as M1 and M2 phenotype markers, respectively. (D) Quantitative data of (C). $^{**}P < 0.01$ versus vehicle. $^{\#\#}P < 0.01$ versus DSS. $^{\&}P < 0.05$ versus VD 0.1 μg. n = 6 mice/group. (E) Macrophage subtype-relevant cytokine expression. $^{*}P < 0.05$, $^{**}P < 0.01$ versus DSS. $^{\#}P < 0.05$ versus VD 0.1 μg. n = 6 mice/group. (F) Western blots showing IRF4 and p-IRF5 protein levels. (G) Quantitative data of (F). $^{**}P < 0.01$ versus vehicle. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus DSS. $^{\&}P < 0.05$ versus VD 0.1 μg. n = 6 mice/group. VD: 1,25(OH)$_2$D$_3$.

induced VDR expression. 1,25(OH)$_2$D$_3$-suppressed miR-125b expression could not create a feedback loop via VDR to downregulate pro-inflammatory macrophage activity. Moreover, 1,25(OH)$_2$D$_3$ pretreatment ameliorated the DAI of colitis and, along with M2 macrophage polarization, decreased IRF5 phosphorylation and IL-6 and TNF-α expression, and increased IRF4, IL-10, and Arg-1 expression. MiR-125b agomir injections caused 1,25(OH)$_2$D$_3$-induced M2 macrophages to polarize to the M1 subtype and increased IRF5 phosphorylation and subtype-related cytokine expression. We demonstrated that 1,25(OH)$_2$D$_3$ modulates macrophage polarization by regulating miR-125b expression; however, the mechanism by which miR-125b regulates IRF4 expression and IRF5 phosphorylation requires further investigation.

In summary, the current study demonstrated that 1,25(OH)$_2$D$_3$ promotes macrophage polarization to the M2 subset by downregulating miR-125b expression. Furthermore, 1,25(OH)$_2$D$_3$ pretreatment ameliorated colitis by restoring the LPMC macrophage subtype balance.

Conflict of interest

The authors report no conflicts of interest.

Acknowledgments

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