VDR-deficient keratinocytes-derived exosomal miR-4505 promoted the macrophage M1 polarization

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Background. Vitamin D receptor (VDR) is low expressed in keratinocytes of patients with psoriasis, which is participated in the occurrence and development of psoriasis. Increasing evidence uncovered that the crosstalk between macrophages and psoriatic keratinocytes-derived exosomes displayed the vital function in psoriasis progression. However, the roles and molecular mechanisms of VDR-deficient keratinocytes-derived exosomes (Exos-shVDR) in macrophages remain largely unknown.

Methods. The VDR-deficient keratinocytes were constructed by infecting HaCaT cells with a VDR-interfering lentivirus to mimic the clinical VDR-deficient psoriatic keratinocytes model. Exosomes were evaluated by transmission electron microscopy assay (TEM), nanoparticle tracking analysis (NTA), and western blot. Then, the effect of Exos-shVDR on proliferation, apoptosis and M1/M2 polarization in macrophages was measured by CCK-8 assay, flow cytometer, RT-PCR, and ELISA. Finally, the underlying mechanism of Exos-shVDR on the function of macrophages was elucidated by data mining, bioinformatics, RT-PCR and rescue experiments.

Results. Our results showed that both Exos-shVDR and Exos-shNC had typical exosome characteristics, such as a hemispheroid shape with a concave side and particle size between 50 and 100nm. Besides, the VDR levels in Exos-shVDR were dramatically lower than that in Exos-shNC. Function experiments demonstrated that Exos-shVDR significantly promoted proliferation, anti-apoptosis and M1 polarization in macrophage. Moreover, we found that miR-4505 is highly expressed in skin tissue of patients with psoriasis, and overexpression of miR-4505 obviously increased macrophage proliferation, anti-apoptosis and M1 polarization. Importantly, we uncovered that Exos-shVDR outstandingly enhanced macrophage proliferation, anti-apoptosis and M1 polarization by delivering miR-4505.

Conclusions. Our data implied that Exos-shVDR aggravated macrophage proliferation, anti-apoptosis, and M1 polarization by delivering high abundance of miR-4505, which will provide a theoretical basis for the intervention of macrophage as a target for the treatment of psoriasis.

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Abstract

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Conclusions. Our data implied that Exos-shVDR aggravated macrophage proliferation, anti-apoptosis, and M1 polarization by delivering high abundance of miR-4505, which will provide a theoretical basis for the intervention of macrophage as a target for the treatment of psoriasis.

Introduction

Psoriasis is a prevalent chronic inflammatory skin disease with a long course and high relapse rate. Epidemiologic studies indicated that the incidence of psoriasis was approximately 0.4% in China and 2%~3% in Europe and America[22]. Psoriasis frequently occurs in young adults and always caused hyperkeratosis of epidermal tissue, disappearance of granular layer, epidermal abscess and even serious complications such as arthritis, lymph node inflammation, immunity dysfunction and cardiovascular diseases, leading to a substantial burden for individuals and society[10]. However, psoriasis had a complex pathogenesis and more researches were needed to uncover the potential mechanism.

Emerging evidences showed that macrophage was polarized into M1 phenotype, which was contributed to psoriasis via activating inflammatory response and releasing a large number of inflammatory factors. For example, Lu at et. reported that blocking M1 macrophage polarization can attenuated psoriatic inflammation which could be a strategy for psoriasis treatment[17]. Zhang et al. reported that IL-35 retarded the inflammatory process in Psoriasis via decreasing the M1/M2 Macrophage Ratio[29]. Furthermore, exosomes from psoriatic keratinocytes were frequently reported as a key regulator of immune system in patients with psoriasis. For example, Jiang et al. found that exosomes derived from psoriatic keratinocytes activated neutrophils and enhanced skin inflammation[12]. Lv et al. reported that Luteolin attenuated the lesions and symptoms of psoriasis in HaCaT cells through suppressing the expression of HSP90 and exosome secretion, and regulating the proportion of immunocytes[18]. Moreover, Zhou et al. indicated that exosome derived from wound-edge keratinocytes was outstandingly enhanced after injury and then was selectively engulfed by wound macrophages[34]. These studies

uncovered that the crosstalk between immunocytes and psoriatic keratinocytes-derived exosomes displayed the vital function in psoriasis progression.

Vitamin D receptor (VDR), a member of the nuclear receptor superfamily, is a nucleophilic protein that mediates the biological effects of Vitamin D3 [1,25(OH)2D3][3, 11, 28]. Studies have shown that VDR is abnormally low expressed in keratinocytes of patients with psoriasis, and the expression levels of VDR are significantly negatively correlated with the severity of psoriasis[4]. It has also been shown that VDR inhibits psoriasis-like skin inflammation by inhibiting STAT signaling pathway[8]. Additionally, it has been proved that VDR is taken part in the anti-proliferation and pro-differentiation of macrophages[20]. However, whether VDR-deficient keratinocytes exosomes (Exos-shVDR) affect the biological functions of macrophages was still unclear. Herein, we found that VDR-deficient keratinocytes exosomes (Exos-shVDR) promoted macrophage proliferation, anti-apoptosis and M1 polarization. Mechanically, Exos-shVDR promoted proliferation, anti-apoptosis and M1 polarization of macrophages by transmitting miR-4505, thereby promoting the development of psoriasis. Our study would offer a theoretical basis for exploring new diagnostic and therapeutic targets for psoriasis.

Materials and Methods

Cell culture

The spontaneously immortalized HaCaT cell line (HaCaT cells, #h066) and the THP-1 human monocytic leukemia cell line (THP-1 cells) were purchase from iCell Bioscience Inc (Shanghai, China). The cells were maintained in DMEM medium (Procell #PM150210) with 10% fetal bovin serum (Gibco, 42G3279K) and 100U/mL penicillin/streptomycin (Gibco#15140122) and cultured in an incubator at 37℃ with 5% CO_2. Before exosomes uptake, THP-1 cells were induced into macrophage by treating with 100 ng/mL Phorbol 12-myristate 13-acetate (PMA) for 48h.

Transfection

The short hairpin RNA (shRNA) target VDR (shVDR#1, shVDR#2, shVDR#3), miR-4505 mimics, miR-4505 inhibitor and their negative control (shNC, NC mimics, and NC inhibitor) were obtained from RiboBio Corporation (Guangzhou, China). The transient transfection was implemented with Lipofectamine 8000™ (Beyotime#C0533) according to the product's protocol. Additionally, miR-4505 inhibitor was transfected into exosomes using Exo-Fect™ Exosome Transfection Kit (systembio, USA). Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was applied to verify the transfection effect. The sequences of shRNA, mimics and inhibitors were provided as follows:

shVDR#1:

CCGGTCGAAGTGTTTGGCAATGAGATCTCGAGATCTCATTGCCAAACACTTCGTTTT

TGAATT;
shVDR#2:
CCGGTCCTCCAGTTCGTGTGAATGATCTCGAGATCATTCACACGAACTGGAGGTTTT
TGAATT;
shVDR#3:
CCGGTGTCATCATGTTGCGCTCCAATCTCGAGATTGGAGCGCAACATGATGACTTTT
TGAATT;
scramble:
CCGGTCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGTTTT
TGAATT;
miR-4505 mimics,
Sense:5'-AGGCUGGGCUGGGACGGA-3', Antisense: 5'- CGUCCCAGCCCAGCCUUU -3';
NC mimics,
Sense: 5'- UUGUACUACACAAAAGUACUG-3', Antisense: 5'-
GUACUUUUGUGUAGUACAAUU-3';
miR-4505 inhibitor: TCCGUCCCAGCCCAGCCT;
NC inhibitor: CAGUACUUUUGUGUAGUACAA.

Extraction and characterization of exosomes from HaCaT cells

The exosomes were derived from HaCaT cells using Total Exosome Isolation Reagent (Invitrogen, USA) following the instruction procedures. Briefly, culture supernatants of HaCaT cells with stable transfected sh-VDR or sh-NC were collected and then was used for centrifuged at 2000g for 20 min to remove cells and debris. Then, this supernatants were filtered through the 0.22 μm filtering membrane to remove residual impurities and collected in ultrafiltration cube. Next, equal volume of Total Exosome Isolation Reagent (Invitrogen, USA) was added into ultrafiltration cube. Finally, the mixture in ultrafiltration cube was centrifuged at 10000g for 1h and exosomes were collected from the sediment. All operating procedures were carried out at 4 °C. The concentration of exosomes was detected by the BCA protein assay (Pierce, T6hermo Scientific). The characterization of exosomes was evaluated by transmission electron microscopy assay (TEM), nanoparticle tracking analysis (NTA), and protein expression of exosomal markers (CD63, TSG101). For TEM, the exosomes were mixed with 10μL Uranyl acetate for 1min and then photographed in a transmission electron microscope (Hitachi, Japan) after a few minutes' dry. For NTA, particle size analyzer (NanoFCM, China) was apllied to tracked the particle size distribution and concentration of the exosomes. Additionally, the protein expression of exosomal markers was measured by western blot.

Exosomes uptake assay

The PKH26 Green Fluorescent Cell Linker Kit (#NA.32, Sigma) was apllied to label the indicated exosomes. Briefly, the indicated exosomes were cultured with diluent C and PKH26

dye for 5 min, and then the exosomes spin columns were used to remove unmerged dye. Subsequently, the macrophages were incubated in medium containing PKH26 labeled exosomes. Finally, the treated macrophages were stained by DAPI and photographed under a fluorescence microscope (Mshot, MF52).

RT-qPCR

Total RNA was extracted by RNA exaction kits (Invitrogen, USA), and then reverse transcribed into cDNA by PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, #RR047A). RT-qPCR amplification was performed on ABI7500 quantitative PCR instrument using the SYBR Green qPCR Mix (Beyotime, #D7260). U6 and GAPDH was used as housekeeping gene for miRNAs and other mRNAs, respectively. Relative expression levels of miRNAs and mRNAs were calculated by $2^{-\Delta\Delta Ct}$ method, and all of the RT-qPCR reactions were performed in triplicate. The primers used in the present study were listed as follows:

VDR,
F: 5’- GTGAGCTGAGATCGTGCCGTTA -3’,
R: 5’- GGTCCTGTCCTGGTCCACTTCT -3’;
GAPDH,
F: 5’- AAGTATGACAACAGCCTCAAG -3’,
R: 5’- TCCACGATACCAAAGTTGTC -3’;
miR-4505:
F: 5’- TTATCTTTAGGCTGGGCTGG -3’,
R: 5’-GTCGTATCCAGTGCGTGTC -3’,
RT: 5’- GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGAC GGA -3’;
miR-4507:
F: 5’- AACTAAACTGGGTTGGGCTGG -3’,
R: 5’-GTCGTATCCAGTGCGTGTC -3’,
RT: 5’- GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGCT GGG -3’;
miR-4563:
F: 5’- CGTGGAGTTAAGGGTTGCT -3’,
R: 5’-GTCGTATCCAGTGCGTGTC-3’,
RT: 5’- GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTGG AGA -3’.
U6:
F: 5’- CTCGCTTCGGCAGCACATATACT -3’,
R: 5’- ACGCTTCACGAATTTGCGTGTC-3’,
RT: 5’- AAAATATGGAACGCTTCACGAATTTG -3’.

Western blot

The total protein from cells or exosomes was isolated using the RIPA lysis buffer (Solarbio, China) and was quantified by bicinchoninic acid (BCA) detection kit (Beyotime, China) according to the instruction procedures. And then the proteins with suitable concentration were separated by SDS-PAGE gels and then transferred into PVDF membranes (#WGPVDF22, Servicebio). The membranes were incubated with anti-CD63(#ab134045, Abcam), anti-TSG101(#ab30871, Abcam), anti-VDR (Invitrogen#PA5-109276) , GAPDH (#GB11002, Servicebio) overnight at 4 ℃ after blocking with 5% blocking solution. Next, the membranes were incubated with secondary antibody (#IH-0011, DINGGUO, China) at room temperature for 2h. Finally, the bands and gray values of protein were visualized and quantified by the Gel imaging system (GE Healthcare) and the Image J software (NIH, USA), respectively.

Cell Counting Kit-8 (CCK-8) assay

Cell viability of THP-1 cells was determined by the CCK-8 kit (Beyotime, China) according to the operating Instructions. Briefly, the THP-1 cells were inoculated in a 96-well plate. CCK-8 solution was appended to per well and incubated in dark for 2h. The absorbance values at 450 nm was evaluated in a spectrophotometer (Tecan, Switzerland).

Flow cytometry

Flow cytometry was used to evaluate the cells apoptosis and the macrophage polarization. For the cell apoptosis, the treated cells were incubated with Annexin V-FITC reagent and PI reagent at room temperature in the dark for 20 min. Finally, flow cytometry (BD, USA) was applied to evaluated cell apoptosis. For the macrophage polarization, the treated cells were fixed with 1% paraformaldehyde (PFA) overnight at 4 °C, and then incubated with CD86 (ab239075, Abcam) and CD206 (ab300621, Abcam) antibodies for 30 min at 20℃. Finally, the cells were analyzed by flow cytometry (BD, USA).

Data Collection and Analysis

The miRNAs expression matrix from patients with or without psoriasis was derived from Gene Expression Omnibus (GEO) database (No, GSE115293). The dataset contains four psoriasis skin-samples and four normal skin samples. The filter criteria for the differential expressed miRNAs between psoriasis patients and normal control was $|\log(\text{fold change, FC})| > 1.5$ and adj. $P < 0.05$ using the “edgeR” package. Finally, we applied the volcano plots to show differential expressed miRNAs.

Statistical analysis

All statistical analyses and image export were conducted by GraphPad Prism 9.0 (GraphPad

Software, USA). The Student's t-test and one-way ANOVA assay were used for comparison differences between groups. $P < 0.05$ was considered as statistically significant.

Results

Characterization of Exos-shNC and Exos-shVDR

In order to silence VDR expression in HaCaT cells, three shRNAs of VDR (shVDR#1, shVDR#2, shVDR#3) and negative control (shNC) was constructed and transfected into HaCaT cells. The expression levels of VDR were detected after transfection by RT-qPCR and western blot. The result showed that three shVDRs significantly inhibited the VDR expression in HaCaT cells, among which shVDR#1 (shVDR) showed the most significant inhibitory effect and was used for subsequent study (**Figure 1A**). Subsequently, HaCaT cells with stable transfected shNC and shVDR were constructed by infecting HaCaT cells with lentiviral packaging of shVDR and shNC, respectively. Then exosomes from the above stable cells were extracted using Total Exosome Isolation Reagent, named Exos-shVDR and Exos-shNC. Finally, the exosomes were identified by western blot, transmission electron microscopy, and NTA. Western blot was applied to measure the expression levels of exosome specific markers (CD63 and TSG101), and the results indicated that the expression of CD63 and TSG101 were obviously enhanced in Exos-shVDR and Exos-shNC, but were almost not expressed in the supernatant of HaCaT cells with stable transfected shVDR or shNC treated with exosome inhibitor GW4869 (**Figure 1B**). The morphological characteristics of exosomes was evaluated by transmission electron microscopy, and found that the Exos-shVDR and Exos-shNC displayed typical double membrane structure with a particle size of 100 nm (**Figure 1C**). Further, NTA analysis demonstrated that the particle size of Exos-shVDR and Exos-shNC ranged from 50 nm to150 nm (**Figure 1D**). In addition, to determine that VDR was silenced in exosomes derived from HaCaT cells with stable transfected shVDR, the expression levels of VDR in Exos-shNC and Exos-shVDR were measured by RT-qPCR and western bolt. The results uncovered that VDR expression was significantly suppressed in Exos-shVDR compared with Exos-shNC (**Figure 1E**). Taken together, these results indicated that exosomes with VDR knocked down and its negative control were successfully extracted and meet the needs of subsequent experiments.

Exos-shVDR promoted proliferation, anti-apoptotsis, and M1 polarization in macrophages

To explore the function of Exos-shVDR *in* macrophage, THP-1 cells were first differentiated into macrophages induced by 100 ng/mL PMA for 48h and then co-cultured with PKH26-labeled Exos-shVDR or PKH26-labeled Exos-shNC. The result showed that both Exos-shVDR and Exos-shNC were uptake into macrophages after co-culture for 24h (**Figure 2A**). CCK-8 assay was used to cells proliferation, and found that Exos-shVDR could outstandingly enhance macrophages proliferation compared with Exos-shNC (**Figure 2B**). Apoptosis was evaluated by

flow cytometry with AV-FITC/PI staining, and uncovered that Exos-shVDR obviously inhibited macrophages apoptosis compared with Exos-shNC (**Figure 2C**). To further investigate the effect of Exos-shVDR on macrophage M1/M2 polarization, we used flow cytometry, RT-qPCR and ELISA assay to detect the expression levels of macrophage polarizations related markers. The results of flow cytometry indicated that Exos-shVDR significantly promoted the expression levels of macrophage M1 polarization marker CD86 but had no influence on the expression levels of macrophage M2 polarization marker CD206 (**Figure 3A**). Consistent with the result of flow cytometry, the results of RT-PCR showed that Exos-shVDR dramatically increased the mRNA expression levels of macrophage M1 polarization markers (IL-1β, TNFα, and IL-6) but had no significant influence on the mRNA expression of macrophage M2 polarization markers IL-10 (**Figure 3B**). Meanwhile, ELISA assay was used to detected the expression levels of macrophage M1 polarization markers (IL-1β, TNFα, and IL-6) and M2 polarization markers IL-10 in the macrophage supernatant. The results demonstrated that Exos-shVDR dramatically enhanced the content of macrophage M1 polarization markers (IL-1β, TNFα, and IL-6) in the macrophage supernatant, but had no significant influence on the content of macrophage M2 polarization markers IL-10 (**Figure 3C**). Additionally, our study revealed that Exos-shNC had no effect on macrophage proliferation, apoptosis and M1/2 polarization (**Figure 2 and Figure 3**). In all, our results demonstrated that Exos-shVDR aggravated macrophage proliferation, anti-apoptosis and M1 polarization.

The high expression of miR-4505 in Exos-shVDR promoted macrophage proliferation and M1 polarization as well as inhibited macrophages apoptotsis

Emerging evidenced indicated that miRNAs carried by exosomes were involved in the process of many dieases such as cancers[27, 33], stroke[5, 32], diabetes[6, 25] and etc. To determine the intrinsic molecular mechanism by which Exos-shVDR promotes macrophage M1 polarization, we downloaded the miRNA expression matrix (GSE115293) associated with psoriasis from the GEO database and performed miRNA differential expression profiling. Volcano Plot showed that 26 dysregulated miRNAs were identified in the skin tissue of the patient with psoriasis compared with in the normal skin tissue, including 12 upregulated and 14 downregulated (**Figure 4A**). Then, we chose three of significantly upregulated miRNAs (miR-4507, miR-4653, miR-4505) to determined their expression levels in Exos-shVDR and Exos-shNC. The results of RT-qPCR showed that miR-4507 was significantly inhibited while miR-4653 and miR-4505 was dramatically increased in Exos-shVDR compared with Exos-shNC (**Figure 4B**). Since the degree of miR-4505 difference between Exos-shVDR and Exos-shNC was significantly higher than that of miR-4653-3p between Exos-shVDR and Exos-shNC, miR-4505 was selected for further study. We used Exos-shVDR and Exos-shNC to treat macrophages for 24h, and found that the expression level of miR-4050 in Exos-shVDR-treated macrophages was significantly higher than that in Exos-shVDR-treated macrophages (**Figure 4C**). These results revealed that miR-4505 was highly expressed in Exos-shVDR, which might be a key molecule in the regulation of macrophage function.

To further explore the function of miR-4505 in macrophage, miR-4505 mimic or NC mimic was transfected into macrophage, respectively. The results showed that the miR-4505 expression was significantly enhanced in macrophage transfected with miR-4505 mimic compared with macrophage transfected with NC mimic (**Figure 4D**). CCK-8 assay disclosed that the overexpression of miR-4505 obviously enhanced macrophage proliferation (**Figure 4E**). AV-FITC/PI staining attested that the overexpression of miR-4505 dramatically reduced macrophage apoptosis (**Figure 4F**). To further explore the effect of miR-4505 on macrophage M1/M2 polarization, the RT-qPCR and ELISA assay were used to detect the expression levels of macrophage polarizations related markers. The results of RT-PCR showed that the upregulation of miR-4505 dramatically enhanced the mRNA expression levels of macrophage M1 polarization markers (IL-1β, TNFα, and IL-6) but had no obvious influence on the mRNA expression of macrophage M2 polarization markers IL-10 (**Figure 4G**). Consistent with the above result, the results of ELISA assay showed that the upregulated miR-4505 dramatically enhanced the content of macrophage M1 polarization markers (IL-1β, TNFα, and IL-6) in the macrophage supernatant, but had no significant influence on the content of macrophage M2 polarization markers IL-10 (**Figure 4H**). taken together, our results disclosed that the overexpression of miR-4505 promoted macrophages proliferation and M1 polarization as well as inhibited macrophages apoptosis.

Exos-shVDR promoted macrophages proliferation and M1 polarization as well as inhibited macrophages apoptosis via transferring miR-4505

To determine whether Exos-shVDR regulates macrophage function by delivering miR-4505, Exos-shVDR was transfected with NC inhibitor and miR-4505 inhibitor using the Exo-Fect™ Exosome Transfection kit, respectively. The results disclosed that the expression level of miR-4505 in Exos-shVDR transfected with miR-4505 inhibitor (Exos-shVDR-miR-4505 inhibitor) was significantly lower than that in Exos-shVDR transfected with NC inhibitor (Exos-shVDR-NC inhibitor), suggesting that miR-4505 was significantly inhibited in Exos-shVDR transfected with miR-4505 inhibitor (**Figure 5A**). Subsequently, we used Exos-shVDR-miR-4505 inhibitor and Exos-shVDR-NC inhibitor to dealt with macrophage, and RT-qPCR was applied to measure the expression level of miR-4505 in macrophages. It was found that the expression level of miR-4505 in macrophages treated with Exos-shVDR-miR-4505 inhibitor was dramatically lower than that in macrophages treated with Exos-shVDR-NC inhibitor (**Figure 5B**). CCK-8 assay proved that the Exos-shVDR-miR-4505 inhibitor obviously reduced macrophage proliferation compared with Exos-shVDR-NC inhibitor (**Figure 5C**). AV-FITC/PI staining attested that the Exos-shVDR-miR-4505 inhibitor dramatically increased macrophage apoptosis compared with Exos-shVDR-NC inhibitor (**Figure 5D**). To further confirm the Exos-shVDR regulates macrophage M1 polarization by delivering miR-4505, the RT-qPCR and ELISA assay were used to detect the expression levels of macrophage polarizations related markers. The results of RT-PCR revealed that the Exos-shVDR-miR-4505 inhibitor dramatically decreased the mRNA expression levels of macrophage M1 polarization markers (IL-1β, TNFα, and IL-6) in macrophage compared with

Exos-shVDR-NC inhibitor (**Figure 5E**). Consistent with the above results, the results of ELISA assay demonstrated that Exos-shVDR-miR-4505 inhibitor dramatically decreased the content of macrophage M1 polarization markers (IL-1β, TNFα, and IL-6) in the macrophage supernatant compared with Exos-shVDR-NC inhibitor (**Figure 5F**). taken together, our results indicated that Exos-shVDR promoted macrophages proliferation and M1 polarization as well as inhibited macrophages apoptosis via transferring miR-4505.

Discussion

Psoriasis is a common hereditary skin disease which is characterized by excessive growth and disordered differentiation of keratinocytes, expansion of microvessels in the superficial dermis and infiltration of inflammatory cells[16]. However, the pathogenesis of psoriasis has not been fully elucidated, but the abnormal immune function especially excessive secrete of inflammatory factors (such as, IL-1β and IL-6) contributed a lot to the progression of psoriasis[1]. It's well known that the infiltration of macrophages at the dermal epidermal junction and the release of inflammatory factors is a typically characteristic in psoriasis, indicating that macrophages exerted indispensable function in the development of psoriasis[24, 26]. Additionally, macrophages were the key source of TNF-α in psoriatic lesions, thus anti-TNF-α agents were proved to have therapeutic effect on psoriasis[14]. Lu et al. reported that inhibiting macrophage polarization into the M1 phenotype inhibited the endosomal TLR-activated psoriatic inflammation[17]. Li et al. found that PSORI-CM02 formula reduce macrophage infiltration and inhibited M1 polarization in mice induced by imiquimod, which may possess therapeutic action in psoriasis treatment[15]. Furthermore, keratinocytes, as the main components of the epidermis, maintain a mechanical barrier during the pathogenesis of psoriasis, participate in the initiation and maintenance of skin immune responses, and interact with immune cells. Jiang at el. found that keratinocyte exosomes from patients with psoriasis activated neutrophils and aggravated skin inflammation in psoriasis[12]. Lv at el. reported that Luteolin attenuated the lesions and symptoms of psoriasis through inhibiting the expression and exosome secretion of HSP90, and regulating the proportion of immunocytes[18]. These studies demonstrated that exosomes derived from keratinocytes exerted an key role in psoriasis progression. However, the underlying mechanism by which keratinocytes-derived exosomes mediate immunocytes involved in psoriasis remains unclear.

The skin is the only organ that can synthesize, activate and degrade vitamin D independently, but some investigation showed that patients with psoriasis generally had insufficient serum 25 $(OH)_2D_3$[2]. VDR is a key element that is indispensable for the normal biological function of vitamin D3, and lack of VDR will aggravate the inflammation, increase cells proliferation and promote the psoriasis initiation and progression. German et al. found that the vitamin D3 analog calcipotriol inhibited the pivotal IL-23/IL-17 axis and neutrophil infiltration via VDR signaling pathway, thus attenuating the inflammation in psoriatic skin[9]. In the present study, exosomes released from VDR knockdown HaCaT cells promoted proliferation, anti-apoptosis, and M1 polarization in THP-1 cells.

MicroRNAs (miRNAs) are a class of endogenous small noncoding RNAs with a length of 18-24

nucleotides, which display an crucial role in the occurrence and development of a variety of diseases, including psoriasis[19]. Karabacak et al. reported that decreased level of miR-1910-3p abolishes the suppressive effect of its target gene IL-17A, accelerating the enhancement of pro-inflammatory molecules in the pathogenesis of psoriasis and lead to the promotion of keratinocyte proliferation[13]. Qu et al. reported that miR-125a-5p was downregulated in psoriasis and its upregulation increased the TGFβ/SMAD pathway activation and aggravated the development of psoriasis[21]. Su et al. supposed that miR-125a suppressed proliferation and promoted apoptosis via negatively regulated the IL-23R/JAK2/STAT3 pathway in HaCaT cells[23]. In recent years, exosomal miRNAs have been identified and were transferred into the recipient cells to play its biological function. In gastric cancer, the exosomal transfer of macrophage-derived miR-223 conferred doxorubicin resistance[7]. miRNA-29a-3p delivery by exosomes derived from engineered human mesenchymal stem cells enhanced tumor form by inhibiting migration and vasculogenic mimicry in glioma[31]. Based on GEO data mining and experimental verification, we uncovered that miR-4505 was highly expressed in keratinocytes of patients with psoriasis. More importantly, we also suggested that the expression level of miR-4505 in exosomes derived from VDR knockdown HaCaT cells were outstandingly higher than that from control HaCaT cells. At present, the role of miR-4505 in the physiology and pathology of the body is still unexplored. Zhang at el. found that miR-4505 aggravated lipopolysaccharide-induced vascular endothelial damage by inhibiting heat shock protein A12B[30]. However, miR-4505 was hardly reported in the regulation of psoriasis. Our data uncovered that miR-4505 promoted macrophage proliferation and M1 polarization, as well as inhibited macrophage apoptosis. Furthermore, our results revealed that keratinocytes derived exosomes with VDR knockdown promoted macrophage proliferation, anti-apoptosis and M1 polarization by delivering miR-4505. Our studies indicated that miR-4505 might be a new potential therapeutic target for psoriasis.

Conclusion

Our study demonstrated that Exos-shVDR promoted macrophage proliferation, anti-apoptosis, and M1 polarization. Mechanically, VDR knockdown resulted in high abundance of miR-4505 in the exosomes secreted by keratinocytes, which carried miR-4505 to macrophages, leading to macrophage proliferation, anti-apoptosis and M1 polarization. This study would offer a theoretical basis for exploring new diagnostic and therapeutic targets for psoriasis.

Acknowledgments

Not applicable.

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Figure 1

Characterization of exosomes derived from HaCaT cells with stable transfected shVDR and shNC.

(**A and B**) The expression levels of VDR were detected by RT-PCR and western blot in HaCaT cells with transfected shVDR #1, shVDR #2, shVDR #3, and shNC. (**B**) Western blot was applied to evaluate the protein expression levels of exosome specific markers (CD63, TSG101) in exosome and supernatants. (**C**) The morphological characteristics of exosomes was evaluated by transmission electron microscopy. (**D**) The partical size of exosomes was evaluated by transmission electron microscopy Nanoparticle Tracking Analysis (NTA). (**E**) The expression of VDR in exosomes derived from HaCaT cells with or without VDR knockdown was measured by qRT-PCR and western blot. shNC, shRNA of negative control; shVDR, the short hairpin RNA target VDR; Exos-shNC, exosomes derived from HaCaT cells with stable transfected shNC; Exos-shVDR, exosomes derived from HaCaT cells with stable transfected shVDR. *P < 0.05, **P<0.01, and ***P < 0.001.

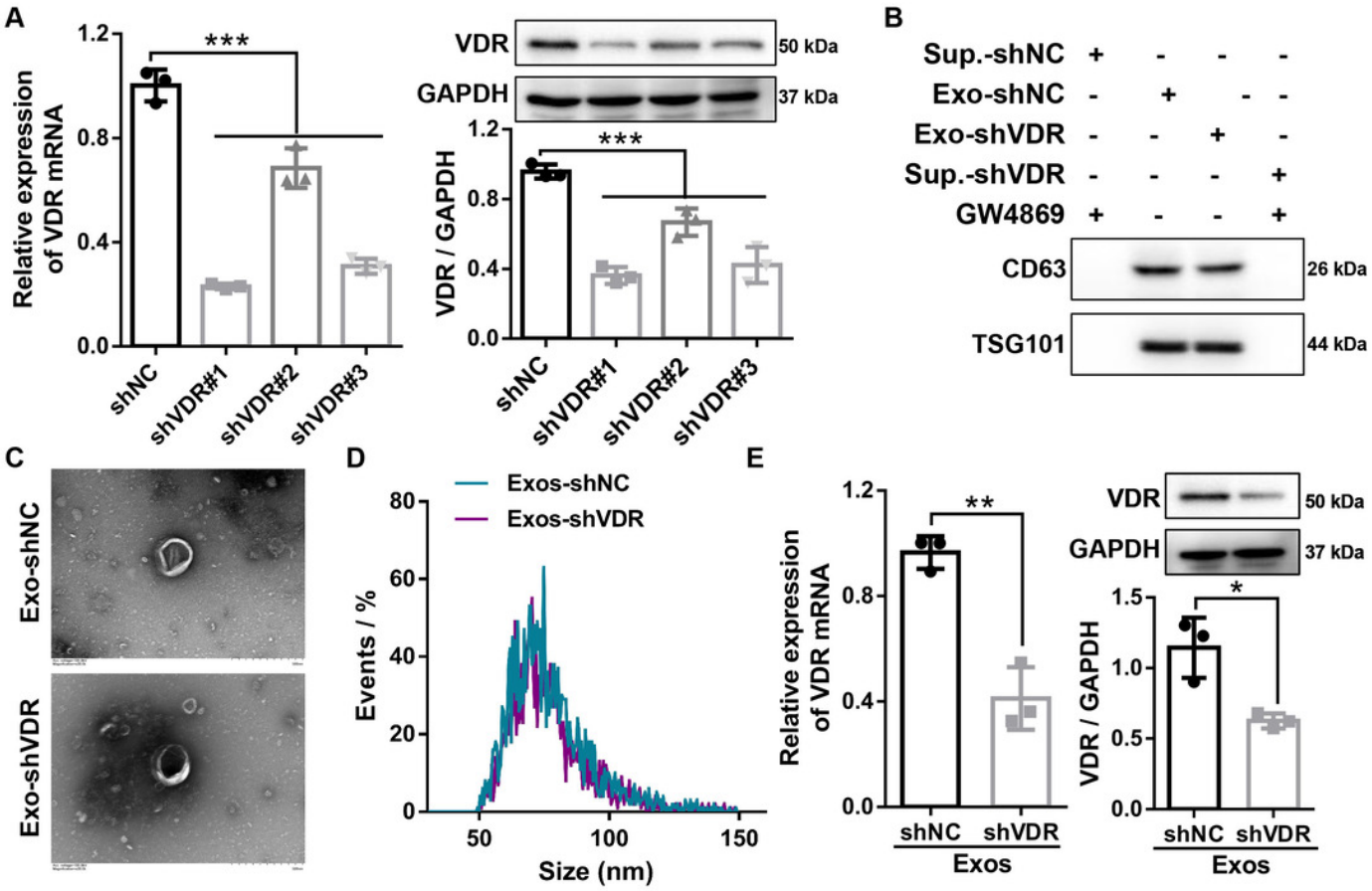

A
Relative expression of VDR mRNA
1.2
0.8
0.4
0.0
***
shNC
shVDR#1
shVDR#2
shVDR#3
VDR
GAPDH
50 kDa
37 kDa
VDR / GAPDH
1.2
0.8
0.4
0.0
***
shNC
shVDR#1
shVDR#2
shVDR#3
B
Sup.-shNC + - - -
Exo-shNC - + - -
Exo-shVDR - - + -
Sup.-shVDR - - - +
GW4869 + - - +
CD63
26 kDa
TSG101
44 kDa
C
Exo-shNC
Exo-shVDR
D
Exos-shNC
Exos-shVDR
Events / %
80
60
40
20
0
50
100
150
Size (nm)
E
Relative expression of VDR mRNA
1.2
0.8
0.4
0.0
**
shNC
shVDR
Exos
VDR
GAPDH
50 kDa
37 kDa
VDR / GAPDH
1.5
1.0
0.5
0.0
*
shNC
shVDR
Exos

Figure 2

Exosomes derived from HaCaT cells with stable transfected shVDR (Exos-shVDR) display pro-proliferative and anti-apoptotic effects in macrophage.

(**A**) The uptake degree of exosomes by macrophage was tracked by PKH26 staining. (**B**) Proliferation ability was evaluated by the CCK-8 assay. (**C**) Apoptosis ratio was detected by flow cytometry with AV-FITC/PI staining. Exos-shNC, exosomes derived from HaCaT cells with stable transfected shNC; Exos-shVDR, exosomes derived from HaCaT cells with stable transfected shVDR. $^{n.s.}$ P> 0.05, ***P < 0.001.

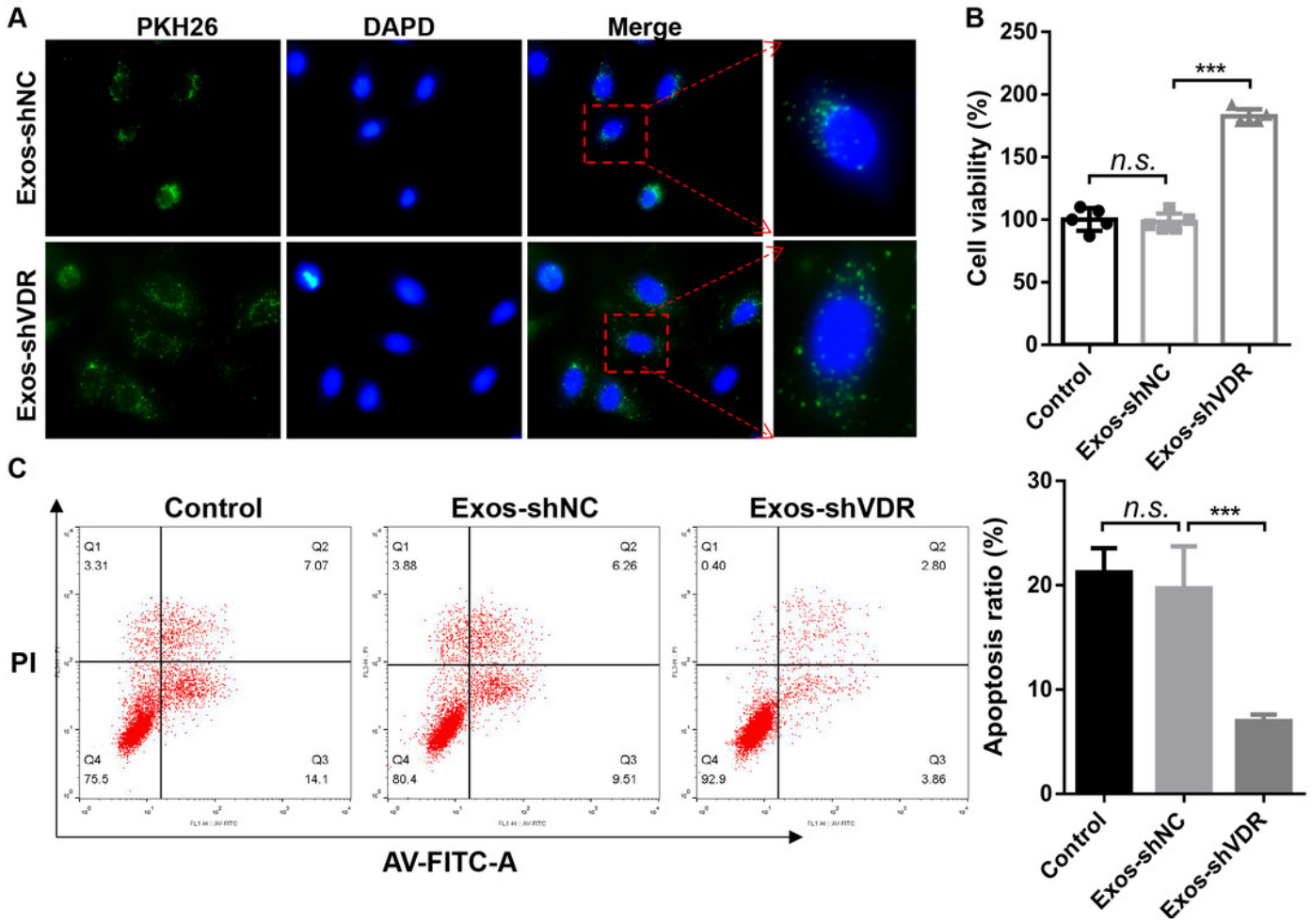


Figure 3

Exos-shVDR promoted macrophage M1 polarization.

(**A**) The expression levels of macrophage M1 polarization markers (CD86) and M2 polarization marker (CD206) in macrophages were measured by flow cytometry. (**B**) The mRNA expression levels of macrophage M1 polarization markers (IL-1β, TNF-α, IL-6) and M2 polarization marker (IL-10) in macrophages were detected by RT-PCR (**C**) ELISA assay was applied to detect the content of macrophage M1 polarization markers (IL-1β, TNF-α, IL-6) and M2 polarization marker (IL-10) in supernatants of macrophages. Exos-shNC, exosomes derived from HaCaT cells with stable transfected shNC; Exos-shVDR, exosomes derived from HaCaT cells with stable transfected shVDR. $^{n.s.}P > 0.05$, $^{***}P < 0.001$.

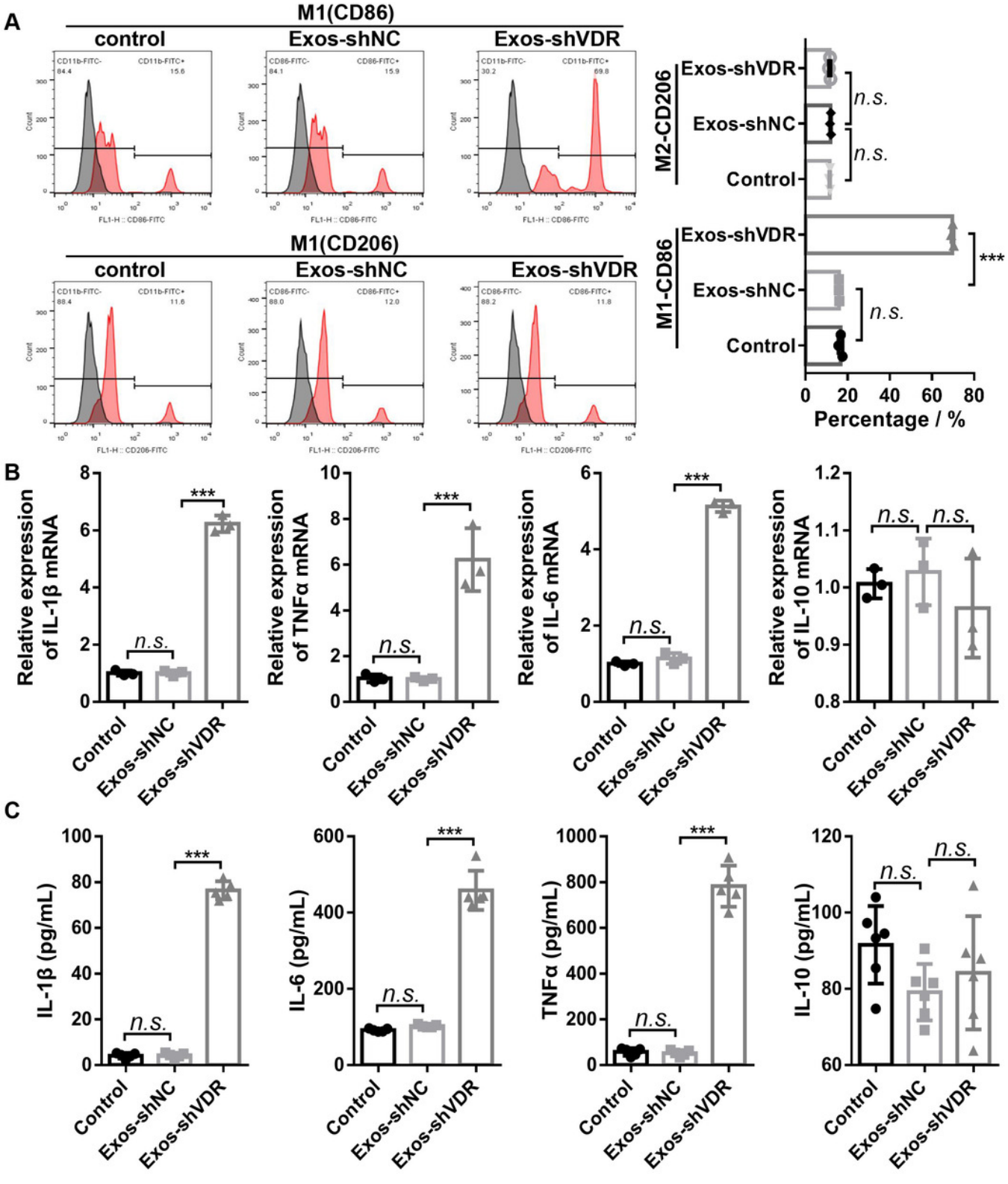

A
M1(CD86)
control
Exos-shNC
Exos-shVDR
M1(CD206)
M2-CD206
M1-CD86
Exos-shVDR
Exos-shNC
Control
n.s.
***
Percentage / %
B
Relative expression of IL-1β mRNA
Relative expression of TNFα mRNA
Relative expression of IL-6 mRNA
Relative expression of IL-10 mRNA
C
IL-1β (pg/mL)
IL-6 (pg/mL)
TNFα (pg/mL)
IL-10 (pg/mL)

Figure 4

The high expression of miR-4505 in Exos-shVDR promoted macrophage proliferation and M1 polarization as well as inhibited macrophages apoptotsis.

(**A**) The volcano plot shows the miRNA expression profile between the psoriatic skin tissues and the normal skin tissues in the GSE115293 expression matrix, and bar graphs showed the expression levels of 12 abnormally upregulated miRNAs in normal skin tissue and psoriatic skin tissue. (**B**) RT-qPCR was performed to evaluated the expression of miR-4507, miR-4653 and miR-4505 between Exos-shVDR and Exos-shNC. (**C**) The expression of miR-4505 in macrophages treated with Exos-shVDR or Exos-shNC was detected by RT-qPCR. (**D**) The expression of miR-4505 in macrophages transfected with miR-4505 mimics or NC mimics was assessed by RT-qPCR. (**E**) Macrophages proliferation ability was detected by the CCK-8 assay. (**F**) Macrophages apoptosis was assessed by flow cytometry with AV-FITC/PI staining. (**G**) The expression levels of M1 macrophage markers (IL-1β, TNF-α, and IL-6) and M2 macrophage markers (IL-10) in macrophage were assessed by RT-qPCR. (**H**) ELISA was performed to evaluate the content of M1 macrophage markers (IL-1β, TNF-α, and IL-6) and M2 macrophage markers (IL-10) in the supernatant of macrophages. Exos-shNC, exosomes derived from HaCaT cells with stable transfected shNC; Exos-shVDR, exosomes derived from HaCaT cells with stable transfected shVDR; NC mim., negative control mimics; miR-4505 mim., miR-4505 mimics. $^{n.s.}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.051$, and $^{***}P < 0.001$.

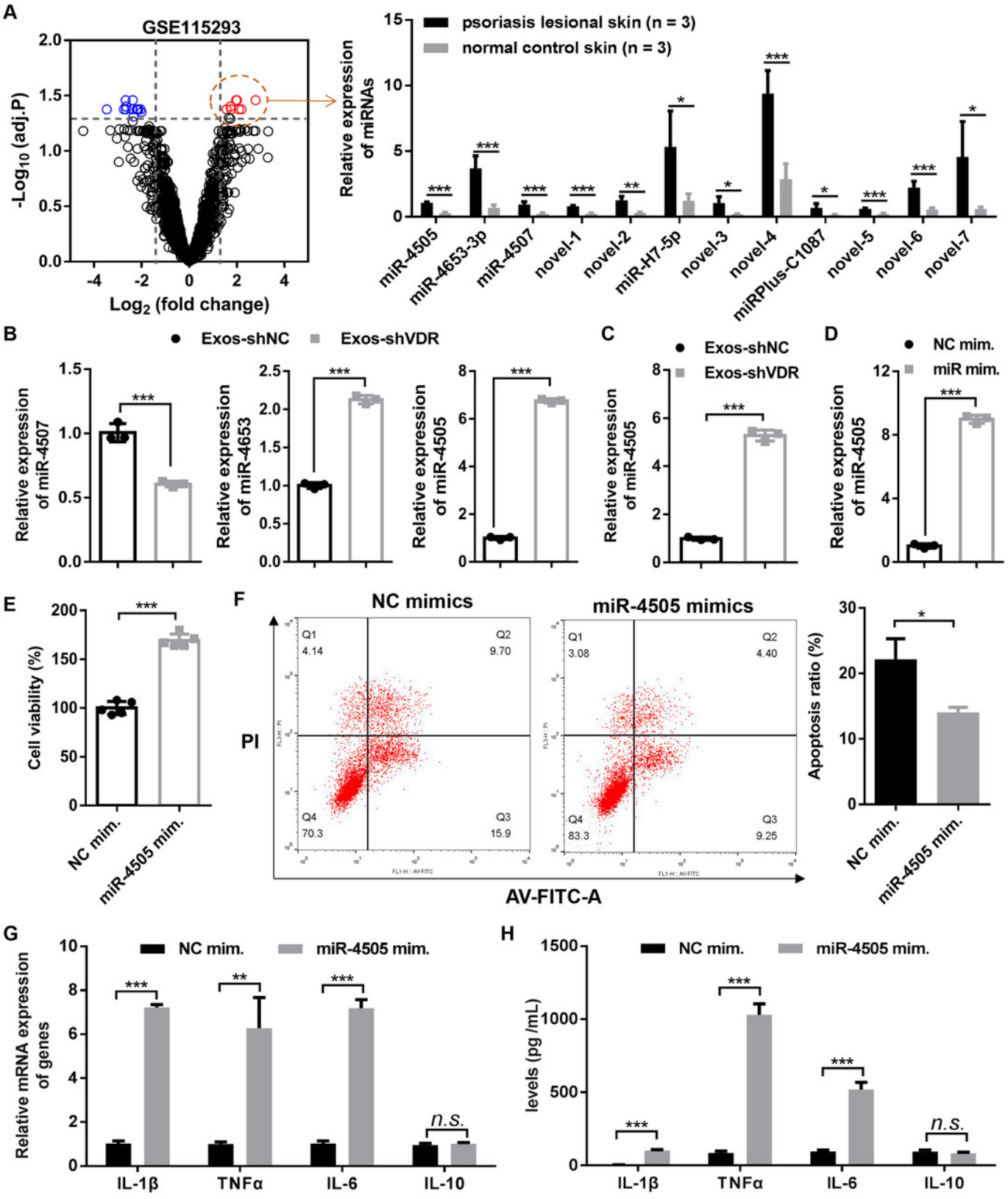
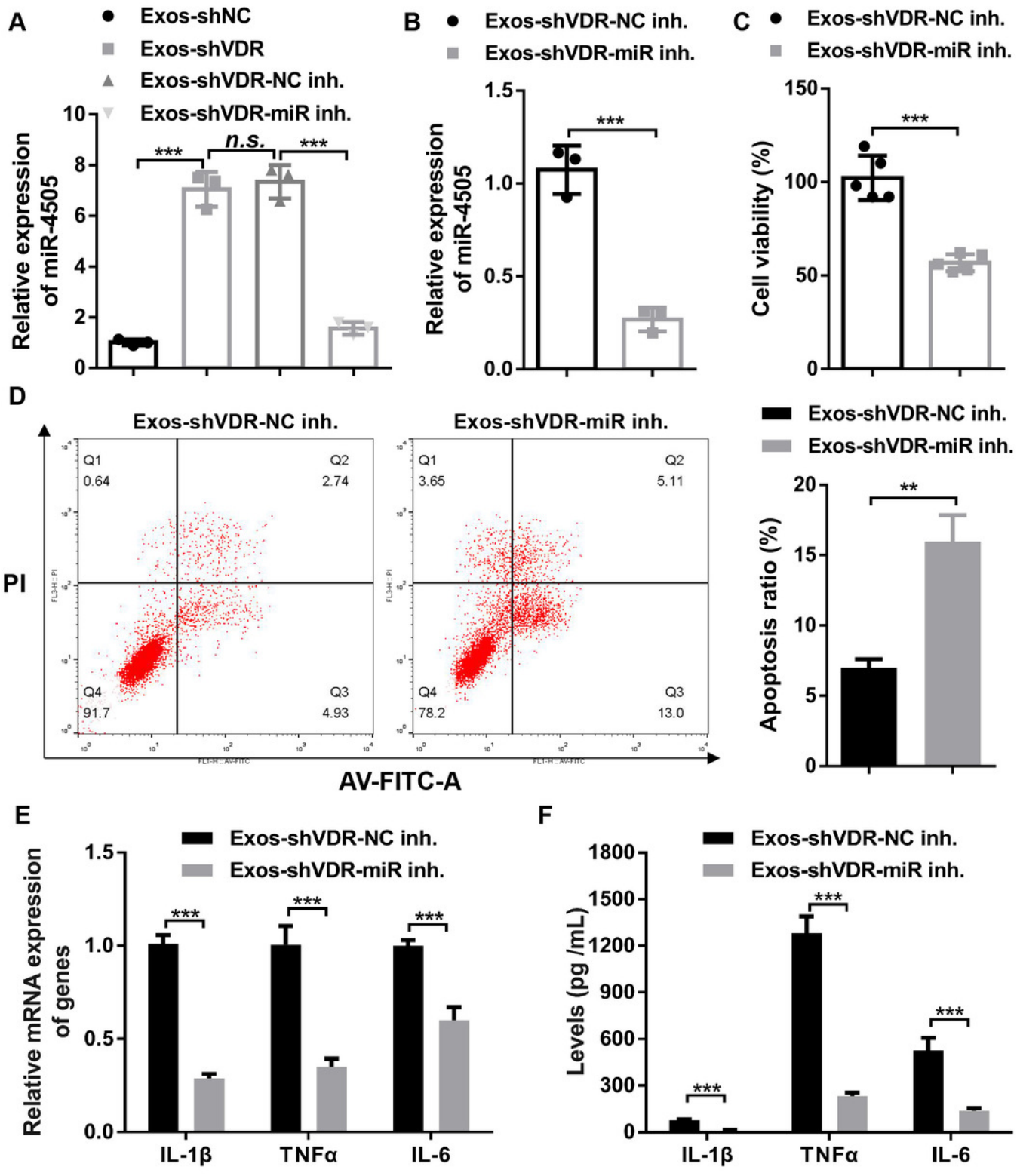

A
GSE115293
-Log10 (adj.P)
Log2 (fold change)
psoriasis lesional skin (n = 3)
normal control skin (n = 3)
Relative expression of miRNAs
miR-4505
miR-4653-3p
miR-4507
novel-1
novel-2
miR-H7-5p
novel-3
novel-4
miRPlus-C1087
novel-5
novel-6
novel-7
B
Exos-shNC
Exos-shVDR
Relative expression of miR-4507
Relative expression of miR-4653
Relative expression of miR-4505
C
Exos-shNC
Exos-shVDR
Relative expression of miR-4505
D
NC mim.
miR mim.
Relative expression of miR-4505
E
Cell viability (%)
NC mim.
miR-4505 mim.
F
NC mimics
miR-4505 mimics
PI
AV-FITC-A
Q1 4.14
Q2 9.70
Q4 70.3
Q3 15.9
Q1 3.08
Q2 4.40
Q4 83.3
Q3 9.25
Apoptosis ratio (%)
NC mim.
miR-4505 mim.
G
NC mim.
miR-4505 mim.
Relative mRNA expression of genes
IL-1β
TNFα
IL-6
IL-10
n.s.
H
NC mim.
miR-4505 mim.
levels (pg /mL)
IL-1β
TNFα
IL-6
IL-10
n.s.

Figure 5

Exos-shVDR promoted macrophages proliferation and M1 polarization as well as inhibited macrophages apoptosis via transferring miR-4505.

(**A**) Exos-shVDR was transfected with NC inhibitor and miR-4505 inhibitor using the Exo-Fect™ Exosome Transfection kit, and RT-qPCR was applied to assess the expression of miR-4505. (**B**) Exos-shVDR-miR-4505 inhibitor and Exos-shVDR-NC inhibitor was applied to treat marcophages, and the expression of miR-4505 in macrophage was detected by RT-qPCR. (**C**) Macrophages proliferation was measured by the CCK-8 assay. (**D**) Macrophages apoptosis was assessed by flow cytometry with AV-FITC/PI staining. (**E**) The expression levels of M1 macrophage markers (IL-1β, TNF-α, and IL-6) in macrophage were evaluated by RT-qPCR. (**F**) ELISA was applied to evaluate the content of M1 macrophage markers (IL-1β, TNF-α, and IL-6) in the supernatant of macrophages. Exos-shVDR-NC inhibitor, Exos-shVDR transfected with NC inhibitor; Exos-shVDR-miR-4505 inhibitor, Exos-shVDR transfected with miR-4505 inhibitor. $^{n.s.}P > 0.05$, $^{**}P < 0.051$, and $^{***}P < 0.001$.


A
Exos-shNC
Exos-shVDR
Exos-shVDR-NC inh.
Exos-shVDR-miR inh.
Relative expression of miR-4505
***
n.s.
***
B
Exos-shVDR-NC inh.
Exos-shVDR-miR inh.
Relative expression of miR-4505
***
C
Exos-shVDR-NC inh.
Exos-shVDR-miR inh.
Cell viability (%)
***
D
Exos-shVDR-NC inh.
Exos-shVDR-miR inh.
PI
AV-FITC-A
Q1
0.64
Q2
2.74
Q4
91.7
Q3
4.93
Q1
3.65
Q2
5.11
Q4
78.2
Q3
13.0
Exos-shVDR-NC inh.
Exos-shVDR-miR inh.
Apoptosis ratio (%)
**
E
Exos-shVDR-NC inh.
Exos-shVDR-miR inh.
Relative mRNA expression of genes
***
IL-1β
TNFα
IL-6
F
Exos-shVDR-NC inh.
Exos-shVDR-miR inh.
Levels (pg /mL)
***
IL-1β
TNFα
IL-6