ORIGINAL RESEARCH

Regulation of M1/M2 Polarization in LPS-Stimulated Macrophages by 1,25(OH)$_2$D3

Hong Zhen, MM; Hongbo Hu, MM; Chang Tan, MM; Xinyuan Yu, MM; Xinglin Gan, MM; Xiuxiu Huang, MM

ABSTRACT

Objective • This study aims to investigate the impact of 1,25(OH)$_2$D3 on the polarization of LPS-stimulated macrophages and the underlying regulatory mechanisms.

Methods • Primary macrophages were isolated and identified using immunofluorescence assays to detect macrophage biomarker expression levels. RT-PCR was employed to measure the expression of Arginase 1 (Arg-1), Interleukin-10 (IL-10), Inducible isoform of nitric oxide synthase (iNOS), and Tumor necrosis factor-α (TNF-α) in macrophages treated with various strategies. Western blotting assessed the protein expression levels of AKT1, p-AKT1, NF-κB p65, p-NF-κB p65, STAT3, and p-STAT3 in LPS-stimulated macrophages exposed to different concentrations of 1,25(OH)$_2$D3.

Results • As the LPS concentration increased from 0 to 0.5 mg/L, Arg-1, IL-10, iNOS, and TNF-α expression levels significantly increased. However, at LPS concentrations ranging from 1 mg/L to 10 mg/L, the expression of Arg-1, IL-10, iNOS, and TNF-α displayed a trend from increase to decline. The highest M2 polarization (Arg-1 and IL-10) was observed in macrophages stimulated with 0.5 mg/L LPS among the lower concentrations, while the highest M1 polarization (iNOS and TNF-α) was observed in macrophages stimulated with 5 mg/L LPS among the higher concentrations. Subsequent experiments utilized 0.5 mg/L and 5 mg/L LPS as incubation concentrations. Under LPS stimulation, iNOS was significantly upregulated, surpassing the expression level of IL-10, a marker of M2 macrophages. The introduction of 1,25(OH)$_2$D3 facilitated M2 polarization, with 50 nM as the incubation concentration of 1,25(OH)$_2$D3. Furthermore, 1,25(OH)$_2$D3 reversed the elevated expression of p-AKT1, p-NF-κB p65, and p-STAT3 in macrophages stimulated with 5 mg/L LPS.

Conclusions • 1,25(OH)$_2$D3 effectively regulates the M1/M2 polarization in LPS-stimulated macrophages. (*Altern Ther Health Med.* 2023;29(8):501-505).

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INTRODUCTION

Acute respiratory distress syndrome (ARDS) is a clinical syndrome characterized by extensive inflammation in lung tissues, often arising due to conditions such as pneumonia, sepsis, and trauma.[1] In cases of acute pulmonary inflammation, approximately 90% of the total cells in bronchoalveolar lavage fluid are occupied by neutrophils and alveolar macrophages. Both neutrophils and alveolar macrophages have been involved in the pathological mechanisms underlying ARDS.[2,3]

In the general population, macrophages constitute the predominant leukocyte phenotype within lung tissues, accounting for more than 89% of leukocytes. These macrophages typically exist in an inactive state, known as M0 macrophages. When activated, macrophages differentiate into two distinct subtypes: M1 macrophages and M2 macrophages.[4] M1 macrophages promote inflammatory responses by releasing pro-inflammatory factors, including IL-1β, IL-12, TNF-α, IL-6, and inducible nitric oxide synthase (iNOS).[5] On the other hand, M2 macrophages play an important role in reducing inflammation. They achieve this by producing Th2 cytokines, such as IL-4 and IL-10, which contribute to tissue repair processes.[6]

The activation of neutrophilic granulocytes can be initiated by the secretion of TNF-α and IL-1β by macrophages. This activation, in turn, leads to the upregulation of adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), on both immune cells and endothelial cells.[7,8] During the initial stages of ARDS, the pulmonary alveolar macrophages become activated, producing excessive pro-inflammatory factors and chemokines.[9] Consequently, this activation triggers the accumulation of neutrophils and monocytes while

disrupting the delicate balance of M1/M2 polarization. This imbalance further exacerbates inflammation and tissue injury.

Studies have highlighted that macrophages exhibit a propensity for activation under physiological conditions, and various subtypes of macrophages are broadly distributed throughout the body.[10] Presently, two primary pathways are widely recognized as the main mechanisms for macrophage activation: the classical activation pathway and the alternative activation pathway. Activation of macrophages through LPS stimulation is associated with the classical activation pathway, and LPS-activated macrophages can enhance immune function. Research indicates that the polarization of macrophages can be modulated by varying concentrations of LPS. Lower concentrations of LPS induce the differentiation of M2 macrophages,[11] whereas higher concentrations of LPS promote the development of M1 macrophages.[12]

Furthermore, 1,25-dihydroxyvitamin D3 (1,25$(OH)_2$D3) within the macrophage system promotes the differentiation of monocytes into macrophages in peripheral blood and enhances the immune capabilities of macrophages. Activated vitamin D plays a crucial role in regulating cellular processes such as growth, proliferation, differentiation, and apoptosis by binding to the vitamin D receptor (VDR).[13] Notably, macrophages are characterized by a high expression of VDR.[14]

Presently, the relationship between macrophage polarization and 1,25$(OH)_2$D3 and its underlying mechanism remains unclear. This study aims to explain the regulatory impact of 1,25$(OH)_2$D3 on macrophage polarization following stimulation with varying concentrations of LPS. The objective is to uncover the potential mechanisms involved in this regulation.

MATERIALS AND METHODS

Study Design

This research employed a controlled laboratory study design. Rats obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd acclimated for two weeks before undergoing anesthesia and surgical procedures for primary macrophage isolation from pulmonary alveoli. The study involved the manipulation of LPS concentrations *in vitro* and adding 1,25$(OH)_2$D3 to investigate its regulatory effects on macrophage polarization. Various analytical techniques, including immunofluorescence assays, RT-PCR, and Western blotting, were utilized to assess biomarker expression levels and protein activation.

Isolation of Rat Pulmonary Alveolar Macrophages

Anesthesia was first administered by intraperitoneal injection of 1% sodium pentobarbital (dose 45 mg/kg). After disinfecting for 1-3 min using 75% ethyl alcohol on rats, animals lay on their backs against an ice board under the sterile environment, then opened the thorax with a scissor to reach the laryngeal trachea. The vessels surrounding the lung and heart tissues were excised, and the trachea was removed to access the lung tissues. These tissues were then immersed in pre-chilled PBS buffer to eliminate residual blood clots and vessels.

Subsequently, 10 mL of PBS buffer was added to facilitate the collection of bronchoalveolar lavage fluids (BALF). After repeating this lavage process 5-8 times, the BALF was subjected to centrifugation at 500 g for 10 minutes to separate the red blood cell mass. To further process the sample, 5 mL of red blood cell lysis buffer was added and incubated for 10 minutes. The white cell mass was collected following centrifugation, and a cell suspension was obtained by resuspending the cell mass in a DMEM medium.

Immunofluorescence Assay

Macrophages isolated in this study were characterized using an immunofluorescence assay. In brief, cells underwent three washes with PBS buffer and were subsequently fixed in 4% paraformaldehyde for 15 minutes. Following this, cells were treated with 0.5% Triton X-100 in PBS buffer for 20 minutes at room temperature. Cells were incubated with 5% BSA for 30 minutes to block non-specific binding. Next, the primary antibody against CD68 (1:200, ab201340, Abcam, Cambridge, UK) was applied and incubated at 37°C for 3 hours.

Subsequently, cells were exposed to a Cy3-conjugated secondary antibody (1:200, Lot01321, CWBIO, Beijing, China) at 37°C for 45 minutes. After three PBS buffer washes, samples were stained with DAPI and incubated in the dark for 5 minutes, followed by blocking with 50% glycerin. Finally, fluorescence images were captured using a microscope (CKX53, OLYMPUS, Tokyo, Japan).

Real-Time PCR

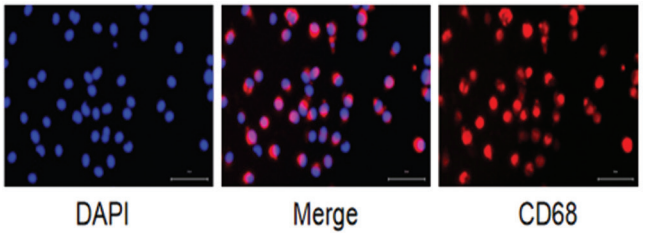
Total RNAs were extracted from macrophages using TRIZOL© reagent (CW0580S, CWBIO, Beijing, China) and the Ultrapure RNA extraction kit (CW0581M, CWBIO, Beijing, China). These RNAs were then reverse transcribed into cDNA using the TaqMan miRNA reverse transcription kit (Invitrogen, California, USA). The real-time PCR (RT-PCR) was performed using HiScript II Q RT SuperMix (R223-01, Vazyme, Nanjing, China) with 2 × SYBR Green PCR Master Mix (A4004M, Lifeint, Xiamen, China). The thermal cycling conditions comprised an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 58-61°C for 30 seconds, and extension at 72°C for 2 minutes. GAPDH served as the internal control for normalizing the relative expression of target genes, and the $2^{-\Delta\Delta Ct}$ method was employed for calculation.

Western Blotting Assay

Total proteins were extracted from the treated macrophages using lysis buffer. The isolated proteins were quantified using a BCA kit (Cwbiotech, Beijing, China), and approximately 40 μg of proteins were loaded and separated on a 12% SDS-PAGE gel. Following protein transfer to the PVDF membrane (Millipore, Massachusetts, USA), the membrane was incubated in 5% BSA (Solarbio, Beijing, China).

Afterwards, it was probed with primary antibodies against AKT1 (1:1000, Abcam, Cambridge, UK), p-AKT1 (1:1000, Abcam, Cambridge, UK), NF-κB p65 (1:1000,

Figure 1. Macrophage Identification via CD68 Immunofluorescence (400×)



Note: Macrophages isolated from the study were identified through immunofluorescence staining of CD68 expression. Microscopic observation was conducted at a magnification of 400×.

Abcam, Cambridge, UK), p-NF-κB p65 (1:1000, Abcam, Cambridge, UK), STAT3 (1:1000, Abcam, Cambridge, UK), p-STAT3 (1:1000, Abcam, Cambridge, UK), and GAPDH (1:1000, Abcam, Cambridge, UK). Subsequently, the membrane was incubated with a secondary antibody (1:2000, Abcam, Cambridge, UK) at room temperature for 1.5 hours. Finally, protein bands were visualized using an ECL solution, and the relative expression levels of target proteins were quantified using Image J software.

Statistical Analysis

Data analysis was performed using GraphPad software, and the results were presented as mean ± SD ($\bar{x} \pm s$). To assess differences between two independent datasets, a t test was employed. For comparisons involving multiple groups, the one-way ANOVA method was utilized, with statistical significance defined as $P < .05$. These rigorous statistical analyses ensured the robustness and reliability of our study findings.

RESULTS

Alveolar Macrophage Identification

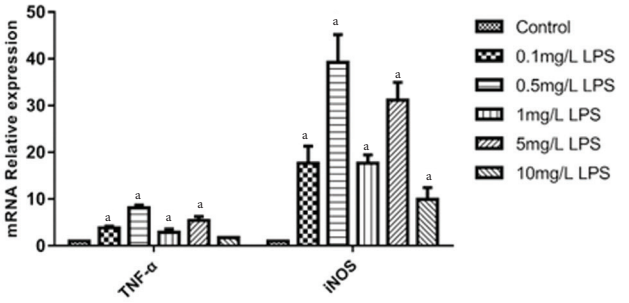
The isolated macrophages derived from the tissue samples underwent thorough identification through immunofluorescence analysis, specifically by evaluating the expression of CD68. As depicted in Figure 1, a striking presence of CD68-positive cells was evident across the visual field.

Determination of LPS Incubation Concentration

The impact of LPS concentration on macrophage polarization was assessed using the RT-PCR assay. As depicted in Figure 2, an increase in LPS concentration from 0 to 0.5 mg/L resulted in a significant elevation in the expression levels of Arg-1, IL-10, iNOS, and TNF-α ($P < .05$ vs. control). However, as the LPS concentration was raised from 1 mg/L to 10 mg/L, the expression of Arg-1, IL-10, iNOS, and TNF-α exhibited a trend from an increase to a decline ($P < .05$ vs. control).

Among the lower concentrations, the highest expression levels of Arg-1 and IL-10 were observed in macrophages stimulated with 0.5 mg/L LPS, while among the higher concentrations, the highest expression levels of iNOS and TNF-α were seen in macrophages stimulated with 5 mg/L

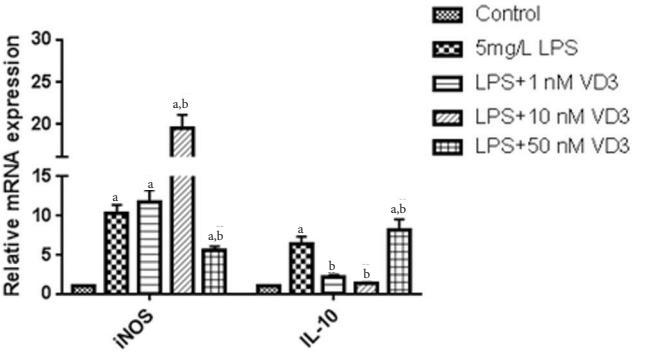
Figure 2. Influence of LPS Concentration on Macrophage Polarization



[a]$P < .05$ vs. control

Note: The expression levels of Arg-1, IL-10, iNOS, and TNF-α were assessed using RT-PCR following macrophage incubation with varying LPS concentrations.

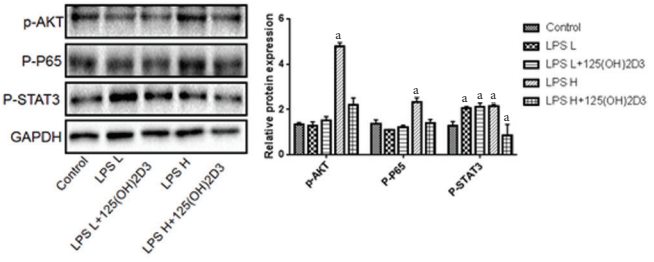
Figure 3. Optimization of $1,25(OH)_2D3$ Concentration in Macrophage Polarization



[a]$P < .05$ vs. control
[b]$P < .05$ vs. Low LPS

Note: The expression levels of IL-10 and iNOS were assessed via RT-PCR in macrophages stimulated with 5 mg/L LPS and incubated with varying concentrations of $1,25(OH)_2D3$.

Figure 4. Modulation of p-AKT1, p-NF-κB p65, and p-STAT3 by $1,25(OH)_2D3$



[a]$P < .05$ vs. control

Note: The influence of $1,25(OH)_2D3$ on the expression levels of p-AKT1, p-NF-κB p65, and p-STAT3 was assessed through Western blotting following treatment with $1,25(OH)_2D3$.

LPS ($P < .05$ vs. control). Consequently, 0.5 mg/L and 5 mg/L LPS concentrations were selected as the incubation concentrations for subsequent experiments.

Determination of 1,25(OH)$_2$D3 Incubation Concentration

The expression of polarization-related genes was monitored to identify the optimal concentration of 1,25(OH)$_2$D3 using the RT-PCR assay. As depicted in Figure 3, under LPS stimulation, the expression level of iNOS significantly increased, surpassing the expression level of IL-10. After introducing a high concentration of 50 nM 1,25(OH)$_2$D3, M2 polarization was notably enhanced. Hence, 50 nM was chosen as the incubation concentration for subsequent experiments.

Effects of 1,25(OH)$_2$D3 on p-AKT1, p-NF-κB p65, and p-STST3 Expression

After treatment with 1,25(OH)$_2$D3, the expression levels of NF-κB p65, p-NF-κB p65, AKT1, p-AKT1, STAT3, and p-STAT3 were assessed using Western blotting. As illustrated in Figure 4, when compared to the control, the expression level of p-STST3 was significantly increased upon stimulation with 0.5 mg/L LPS, while the expression levels of p-AKT1, p-NF-κB p65, and p-STAT3 were notably elevated upon stimulation with 5 mg/L LPS ($P < .05$ vs. control).

In 0.5 mg/L LPS-stimulated macrophages, treatment with 1,25(OH)$_2$D3 did not significantly change the expression levels of p-AKT1, p-NF-κB p65, and p-STAT3. However, in macrophages stimulated with 5 mg/L LPS, the introduction of 1,25(OH)$_2$D3 led to a significant downregulation of p-AKT1, p-NF-κB p65, and p-STAT3 ($P < .05$ vs. LPS H). These findings suggest that 1,25(OH)$_2$D3 helps maintain immune homeostasis by balancing M1/M2 polarization in macrophages under the influence of high LPS concentrations.

DISCUSSION

The diversity of alveolar macrophages has been recognized as a pivotal regulator in the development and repair processes of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), contributing differently during distinct phases of these conditions.[3,15] Alveolar macrophages are classified into M1 and M2 subtypes based on their membrane biomarker expression and secretion profiles of chemokines, cytokines, and transcription factors.[15] M1 macrophages initiate inflammatory reactions by secreting pro-inflammatory factors like TNF-α and IL-6, and they exhibit high expression of iNOS, CD54, or CD11C. Conversely, M2 macrophages dampen pro-inflammatory factor production and display high expression of Arg1, CD206, and CD71.[3] The balance of M1/M2 polarization within macrophages determines the inflammatory state of tissues.

LPS acts as the stimulant for the classical activation pathway of macrophages, binding to receptors on the cell membrane to form an LPS-protein complex.[16] However, the polarization of macrophages induced by varying LPS concentrations does not consistently follow the same pattern. In our study, we observed that as the LPS concentration increased, there was a shift from an initial rise to a subsequent decline in the population of both M1 and M2 macrophages.

Following stimulation, there was a significant increase in the proportion of M2 macrophages after exposure to 0.5 mg/L LPS, whereas the proportion of M1 macrophages was markedly elevated upon exposure to 5 mg/L LPS. Consequently, we adopted 0.5 mg/L and 5 mg/L as the low and high LPS concentrations for subsequent experiments. These findings suggest that the low LPS concentration may induce a negative regulation of immunity, contributing to the polarization of macrophages towards the M2 phenotype. In contrast, when exposed to a high concentration of LPS, macrophages were significantly induced towards an M1 polarization, leading to the creation of an inflammatory environment.

Vitamin D, a fat-soluble vitamin primarily synthesized from 7-dehydrocholesterol in the skin through sunlight exposure or obtained as a dietary supplement, plays a vital role in various physiological functions. The active form of vitamin D, 1,25-dihydroxyvitamin D (1,25(OH)$_2$D3), is associated with a wide range of physiological processes, while vitamin D deficiency is linked to numerous diseases.[17]

Toll-like receptors (TLRs) are pattern recognition receptors expressed on specialized phagocytes responsible for mediating response to infections and initiating adaptive immune responses. Within macrophages, 1,25(OH)$_2$D3 has been observed to have regulatory effects. It negatively regulates the expression of TLR2, TLR4, and TLR5 while enhancing the expression of TLR10.[18] Furthermore, 1,25(OH)$_2$D3 has been shown to repress the LPS-induced production of TNF-α, IL-1β, and cyclooxygenase-2 while concurrently increasing IL-10 production in macrophages.[19,20]

The absence of VDR (Vitamin D receptor) in macrophages is considered to have a favorable impact on colitis development. This effect is accompanied by a heightened expression of pro-inflammatory cytokines within the inflamed colon, underscoring the significance of vitamin D signaling in innate cells for regulating colon inflammation.[21] Our current study observed that 1,25(OH)$_2$D3 played a role in restoring balance to the M1/M2 polarization within LPS-induced cellular inflammation.

LPS can influence several inflammatory signaling pathways, with the NF-κB pathway being one of the most crucial. The phosphorylation level of NF-κB p65 serves as an indicator of the intensity of the inflammatory response.[22] Studies have reported that LPS triggers the secretion of TNF-α, IL-6, and IL-1β by modulating the NF-κB pathway.[23]

Our results align with previous findings as we observed that the expression level of p-NF-κB p65 increased upon LPS stimulation. Interestingly, the combination of 1,25(OH)$_2$D3 with 0.5 mg/L LPS further upregulated p-NF-κB p65, while co-incubation of 1,25(OH)$_2$D3 with 5 mg/L LPS resulted in its downregulation. These findings suggest that 1,25(OH)$_2$D3 has the capacity to suppress the activated NF-κB pathway induced by high LPS concentrations. Furthermore, the AKT

and STAT3 pathways have emerged as pivotal players in regulating inflammatory reactions.[24,25] Our data demonstrate that the activation of the AKT and STAT3 pathways induced by high LPS concentrations can be substantially suppressed by $1,25(OH)_2D3$.

Study Limitations

While this study provides valuable insights into the regulatory effects of $1,25(OH)_2D3$ on macrophage polarization and inflammatory signaling, there are certain limitations to consider. First, the study primarily relied on *in vitro* experiments and translating these findings to *in vivo* settings may require further investigation. Additionally, the specific mechanisms underlying the observed effects of $1,25(OH)_2D3$ on AKT, STAT3, and NF-κB pathways warrant deeper exploration. Furthermore, the study focused on a specific concentration range of LPS, and investigating the effects of a broader range of LPS concentrations could offer a more comprehensive understanding of the relationship between $1,25(OH)_2D3$ and macrophage polarization. Lastly, while our findings suggest the potential therapeutic implications of vitamin D in immune-related conditions, clinical studies are needed to validate these effects in human subjects.

CONCLUSION

In summary, our study highlights the dynamic nature of macrophage polarization, which responds differently to varying concentrations of LPS. Lower LPS concentrations favor M2 polarization, while higher concentrations promote M1 polarization. Notably, we have identified 1,25(OH)2D3 as a potential regulator of this polarization balance, possibly acting through the inhibition of NF-κB, AKT, and STAT3 signaling pathways. These insights into the intricate control of macrophage polarization shed light on potential avenues for therapeutic intervention in immune-related disorders. Further research, including in vivo studies and clinical trials, is warranted to harness the full therapeutic potential of these findings.

DATA AVAILABILITY

The data used to support this study is available from the corresponding author upon request.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHORS' CONTRIBUTIONS

All authors made equal contributions to this study, participated in data analysis and interpretation, and have read and agreed to the publication of the manuscript.

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