Article

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Roles of 630 nm Red Light-Emitting Diode in Inhibition of RhoA Signal Transduction Pathway via Reducing PLEKHG5 Expression and Alleviation of Inflammatory Response in Macrophages

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Background: The joint erosion occurs due to the classically activated macrophages (M1) that mainly secrete pro-inflammatory cytokines. Existing research results show the significant efficacy of LED (light emitting diode) in promoting cell proliferation, relieving oxidative stress, and inhibiting inflammatory factors in macrophage-like cells.
Methods: In this study, we performed transcriptomic analysis of the control group (Lipopolysaccharide (LPS)-treated M0 cell and M1 cell) and the experimental group, to find the role of 630 nm red LED irradiation on macrophages and their inflammatory response. To investigate the molecular mechanisms that underline the LED light radiation efficacy in the treatment of hyper-immune response of macrophages, we employed efficient molecular techniques such as western blot and qRT-PCR (Real-Time Quantitative Reverse Transcription PCR) analysis.
Results: These results showed that the 630 nm red LED irradiation, reduced the inflammatory response in macrophage-like cells after inducing the inflammatory response with LPS by suppressing TNF-α (Tumor necrosis factor α), IL-1β (interleukin-1 β), IL-6 (Interleukin-6) and CCL8 (Chemokine (C-C motif) ligand 8). The 630 nm red LED irradiation reduced the expression of NF-κB (nuclear factor kappa B) p65 protein and reduced the cell stress of macrophage-like cells after treatment with LPS. Moreover, 630 nm red LED light suppressed the illicit of PLEKHG5 (Pleckstrin Homology and Rho GEF Domain Containing G5) in macrophage-like cells induced by LPS treatment and subsequently, the activation of the RhoA exchange factor and NF-κB signaling pathway.
Conclusions: This study demonstrated that red LED may inhibit the hyperimmune response in macrophages via inhibiting the RhoA signaling pathway via PLEKHG5 and subsequently suppresses the illicit of proinflammatory cytokines. These findings showed that red LED light irradiation could be used to alleviate the macrophage's immune response in RA (rheumatoid arthritis) patients.

Keywords: 630 nm light-emitting diode; LED; inflammation; macrophages rheumatoid arthritis; physiotherapy; RhoA; PLEKHG5

Introduction

RA (rheumatoid arthritis) is a chronic inflammatory disease that caused synovium inflammation that affects 1% of the world's population and is marked by the presence of macrophage-derived proinflammatory cytokines [1]. RA is characterized by symmetrical inflammation of the affected joints, with consequent complications that may lead to cartilage destruction, bone erosion, and even disability [2]. Arthritis is caused and maintained by complex interactions between different dendritic cell (DC) subtypes, T cells, macrophages, B cells, neutrophils, fibroblasts, and osteoclasts [3]. Since the ubiquitous RA-specific autoantigens are not completely cleared, this persistent immune cell activation leads to a chronic inflammatory state and synovial swelling of the joints [4]. In RA patients Classically activated macrophages (M1) and M2 cells in inflamed synovial tissue are primarily activated and considered the main source of cytokines. Reports are showing RA patients' joint slip membrane exists in liquid macrophages with M1 type-based subpopulation imbalance [1]. M1 causes joint erosion, mainly secreting pro-inflammatory cytokines, which are also considered to be an innate immune response that causes inflammation via macrophages [5]. The RA treatment strategies are based on drug therapy and non-drug therapy. Drug therapy is mainly non-

steroidal anti-inflammatory drugs, opioid analgesics, antirheumatic drugs and disease-modifying anti-rheumatic drugs (DMARDs). Non-drug therapy includes physical therapy, lifestyle change, also known as therapeutic patient education (TPE), and surgery to remove and/or replace the affected joint and bone area [6]. Due to the cost of these treatment strategies, physiotherapy for RA has emerged as a potential alternative therapy. Therefore, developing a therapy strategy that targets macrophage-derived cytokines pathogenesis emerged to improve RA clinical symptoms. Certain physiotherapies have been practiced to reduce pain and stiffness, increase flexibility and restore function such as cold, heat, electricity, light and other physiotherapies [7]. For instance, photobiomodulation (PBM) therapy emerged as important supplementary and adjuvant therapy, which is cost-effective, faster response, has more positive treatment and has no side effects [8].

Currently, light sources like PBM, and LEDs (light emitting diode) are used in biomedicine, due to their advantages of narrow-spectrum incoherent light sources and have received enough attention in the mitigation and treatment of various diseases in the past two decades. Particularly, red-lighted LED has better advantages, due to its efficient tissue penetration among the visible wavelengths and showed significant effects such as pain relief, inflammation reduction, and promote wound healing [9,10]. Existing research results show that the efficacy of LED is related to promoting cell proliferation, relieving oxidative stress, and inhibiting inflammatory factors [11–13]. However, the molecular mechanisms underlying the LED light radiation efficacy in the treatment of cell proliferation and others need to be further studied. The PLEKHG5 (Pleckstrin Homology and Rho GEF Domain Containing G5) gene encodes a protein that activates the nuclear factor kappa B (NF-κB) signaling pathway [14].

In this study, we aimed to study the therapeutic effects of the 630 nm red LED on THP-1-derived (a human leukemia monocytic cell line) macrophages induced with LPS (Lipopolysaccharide). Moreover, we have to study the downstream molecular mechanisms that are related to inflammatory responses.

Materials and Methods

Cell Culture and Preparation

Human THP-1 cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco, Rockville, MD, USA) and incubated at 37 °C and 5% CO_2 incubator. THP-1 monocytes were seeded at 1×10^6/in a 6-well plate and incubated at 37 °C for 16–18 hours and treated with (100 ng/mL) phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, St. Louis, MO, USA), for 48 hours to induce the differentiation of THP-1 into macrophages. The medium was replaced with a serum-free medium to synchronize the cell's growth phase. Subsequently, cells were treated with (500 ng/mL) lipopolysaccharide (LPS) (Sigma Aldrich, St. Louis, MO, USA), for 4 hours to simulate the polarization of THP-1-derived macrophages into M1 type. Gene Set Enrichment Analysis (GSEA) was performed to determine whether the macrophages was polarized. The experimental group cells were set as PMA-induced macrophage, LED irradiated macrophage, LPS stimulated activated and LED-LPS irradiated macrophage-like cells.

Table 1. The experimental parameters setting of the LED in this study.

Parameter	Experimental value
Wavelength	630 nm
Optical power density	50.98 mW/cm^2
Irradiation time	10 minutes
Energy density	30.6 J/cm^2
Irradiation times	2 times

Red LED Light Treatment

Previously it has been studied the effects of the 630 nm red LED light radiation on the THP-1 monocytes/macrophages. The 630 nm red LED light source was procured from (Beijing Chuangwen Optoelectronics Medical Co., Ltd., Beijing, China) and the irradiation parameters were set according to (Table 1). To explore the mechanism of red-light LED on macrophages and their inflammatory responses, we divided the treatment of the cells into four groups; Macrophages (group A), LED-treated macrophages (group B), LPS-induced macrophage inflammation model (group C) and LED-LPS treated macrophage inflammation model (group D) underwent transcriptome sequencing and downstream experiments. The macrophage cell groups were exposed to the light radiation macrophages-like and LPS-induced inflammatory model macrophages twice within four hours at a dose of the optical power density of 50.98 mW/cm^2 and energy density of 30.6 J/cm^2. The LED light source is placed at the bottom of the orifice plate, and the LED is illuminated (no incandescent light interference). The control group and the experimental group were wrapped in aluminum foil to protect them from light.

The irradiation time and cell and processing were done according to the flow work (Fig. 1), and inside laminar flow hood level-II (Thermo Scientific, Waltham, MA, USA). The temperature does not exceed 37 °C. Before the start of the experiment and the power of the LED device was checked with a power and energy meter PM100D (Thorlabs, USA).

Bioinformatics Analysis

To investigate the effects of 630 nm red LED light irradiation on the macrophage's genes expression we have treated the macrophages as mentioned above four groups

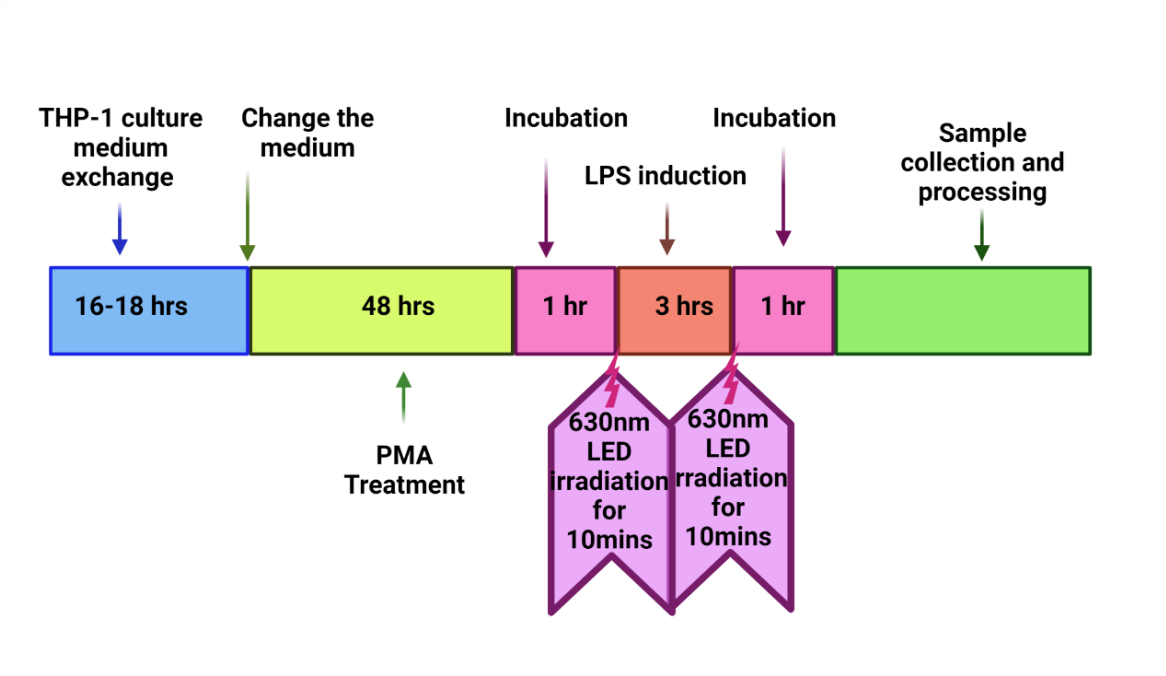

Fig. 1. Descriptive graph shows macrophage-like processing ant treatment using red LED light.

of cells were collected, and three biological replicate samples for each group were sent to Tiansheng Biotechnology Co., Ltd. (Shanghai, China). Briefly, total RNA (Ribonucleic Acid) was extracted and the RNA quality was qualified. Second-generation high-throughput sequencing technology was used for transcriptome sequencing and Deseq2 software (version 4.2; https://bioconductor.org/packages/release/bioc/html/DESeq2.html) was used to analyze the differentially expressed genes among the four groups [15].

Real Time-PCR Analysis

Total RNA was extracted from the treated and untreated macrophages using TRIzol (Invitrogen, Carlsbad, CA, USA) method as per the manufacturer's instructions. Using 1 μg of total RNA from each sample was reserve transcribed using PrimeScript^{TM} RT with gDNA (genomic DNA) Eraser (TaKaRa Bio, Tokyo, Japan). The expression of TNF-α (Tumor necrosis factor α), IL-1β (interleukin-1 β), IL-6 (Interleukin-6), CCL8 (Chemokine (C-C motif) ligand 8) and GAPDH as an internal control were quantified using TB Green^{TM} Premix Ex Taq^{TM} II (TaKaRa Bio, Tokyo, Japan) and the corresponding pairs of primer were purchased from GENEWIZ (Suzhou, China) (**Supplementary Table 1**). Data were analyzed using $\Delta\Delta$CT value method.

Western Blot Analysis

To detect the expression of the proteins in macrophages exposed to the red LED irradiation we analyzed the expression of proteins expression using western blot analysis. Briefly, the LED exposed macrophages and control were lysed using ice-cold RIPA buffer for 10 minutes on ice and centrifuged at 12,000 g for 15 minutes at 4 °C. The nuclear and cytoplasmic and membrane proteins were extracted using NE-PER (Nucleus-plasma-cell membrane preparation kit) nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, Waltham, MA, USA) as per the manufacturer's instructions. Proteins were quantified using the bicinchoninic acid (BCA) method (Thermo Scientific, Waltham, MA, USA). Samples were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene fluoride (PVDF) and subsequently, the membrane was blocked with 5% skimmed milk and incubated with primary antibodies overnight at 4 °C (anti-PLEKHG5 (1:800, #19830-1-Proteintech company, USA), anti-NF-κB P65 phosphor Ser536 (Immunoway Biotechnology, USA) (1:1000, #YP0191) and anti-GAPDH (1:500, #AC027, anti-RhoA, Wuhan Newster Biotechnology Co., Ltd., Wuhan, China)). The membrane was washed thrice with washing buffer and incubated with either horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (1:5000 #ZDR-5306, #ZDR-5307, Zhongshan Jinqiao, Zhongshan, China) for 2 hours at room temperature. To detect the bands, we used an enhanced chemiluminescence system (ECL) kit (Pierce, Rockford, IL, USA) and ImageQuant^{TM} LAS 4000 (GE Healthcare Bio-Sciences, Sweden). The band density were anlaysed using ImagJ software (1.8.0; LOCI, University of Wisconsin, Madison, WI, USA).

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Table 2. Number of significantly differentially expressed genes by transcriptome sequencing.

Groups	Total	Up	Down
PMA+LED/PMA	88	41	47
PMA+LPS/PMA	2737	1416	1321
PMA+LPS+LED/PMA	2909	1484	1452
PMA+LPS+LED/PMA+LPS	74	33	41

Statistical Analysis

Data were analyzed using GraphPad Prism8 (GraphPad Software Inc., La Jolla, CA, USA). We employed paired *t*-test was used to compare the test set to the control, while we used Two-way ANOVA (Analysis of Variance) followed by Tukey's test for comparison between control and treatment groups. $p < 0.05$ considered as a significant value. The data are presented as mean $\pm$ the standard error (of mean) of triplicate independent experiments.

Results

Transcriptomic Analysis Reveals that RhoA Pathway-Related Genes were Suppressed

To preliminary check, the effects of red LED light we have performed transcriptomics analyses on treated macrophages. The genes (*DEGs*) (differentially expressed genes) with p- < 0.05 and $|\log_2\text{-fold change}| > 1$ were regarded as differentially expressed genes and those that do not meet the above conditions were not considered as *DEGs*. The results showed that in LPS-induced macrophage inflammatory response the *DEGs* got upregulated were 1484 and the downregulated were 1452 *DEGs*, while, in response to treatment with LPS+LED compared to LPS 33 *DEGs* got upregulated (Up) and 41 *DEGs* were downregulated (Table 2). Preliminary analysis for the results showed that 630 nm red light LED may involve in the mechanism of macrophage inflammatory response using the Venn diagram (**Supplementary Fig. 1**). Interestingly, we found 15 *DEGs*, which were involved in the inflammatory response and got changed after exposure to 630 nm red light LED. Results showed that MS4A2, AMN, PLEKHG5, TSPYL6 and ANGPTL6 expression level was increased after treatment with LPS and got suppressed after got exposed to red LED (Fig. 2A,B, **Supplementary Fig. 2**). On the other hand, the expression of C15orf38-AP3S2, SLC2A14, NR1I2, WDR93, GCNT2, NR3C2, DLG2, CHTF8, RGPD3, HSPA4L was increased after exposure to 630 nm red light LED compared to the LPS induction (Fig. 2C,D, **Supplementary Fig. 2**). Form these gene ontology (GO) annotations showed that the signal transducer activity such as RhoA-related genes like PLEKHG5 was differentially expressed. These results revealed that irradiation of macrophages using 630 nm red light LED may reduce inflammation response related *DEGs* compared to the same induced using LPS.

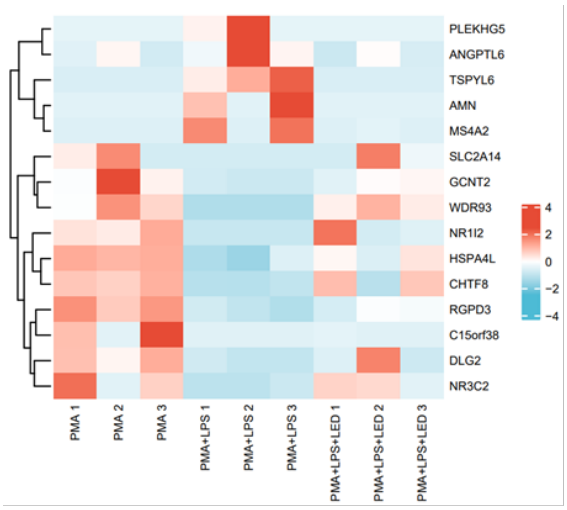

Fig. 2. Heatmap represents 15 *DEGs*, which were involved in the inflammatory response and got suppressed after exposure to 630 nm red light LED.

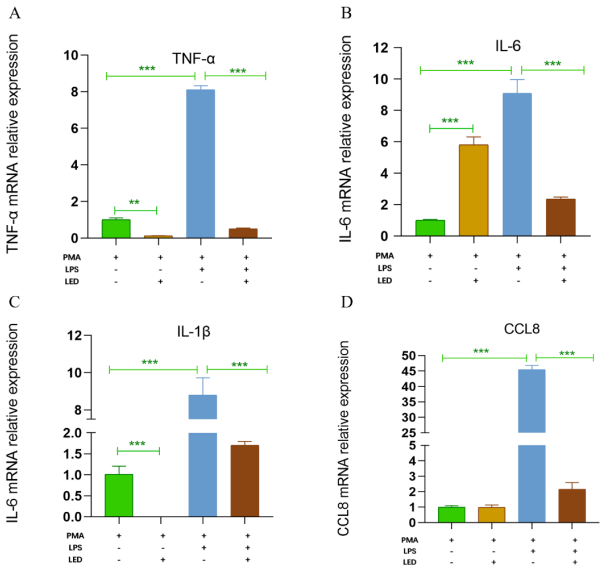

Fig. 3. The effects of 630 nm red LED irradiation on macrophages-like cells inflammatory cytokines TNF-α, IL-1β, IL-6, and CCL8 mRNA expression levels. Macrophage-like cells were induced to adhere by PMA for 48 hours and treated with LPS induces the inflammatory response, or 630 nm red LED after exposing it 10 minutes and 3-hour time gap followed by the second dose of exposure LED exposure for 10 minutes. The qRT-PCR analysis showed that the expression of IL-1β (A), CCL8 (B), TNF-α (C) and IL-6 (D) was high in LPS-treated cells and the red LED radiation suppressed the cytokine mRNA expression levels. $^{**}p < 0.01$ $^{***}p < 0.001$.

Red LED Radiation Suppresses the Inflammatory Response Factors to Stress

To study the effect of red LED light irradiation on the inflammatory response to the expression of macrophage inflammatory factors we irradiated macrophage-like cells with 630 nm red LED light. Subsequently, we analyzed the mRNA expression levels of macrophage inflammatory cytokines TNF-α, IL-1β, IL-6 and CCL8 using qRT-PCR (Real-Time Quantitative Reverse Transcription PCR). The results showed that the red LED radiation significantly suppressed the mRNA expression levels of TNF-α, IL-1β, IL-6 and CCL8 compared to their expression levels when the macrophage-like cells were treated with LPS. Moreover, compared with unirradiated M0 cells, the mRNA expression levels of cytokines IL-β and TNF-α in M0 cells irradiated with 630 nm red LED decreased, and the expression levels of cytokine IL-6 increased, while CCL8 did not change (Fig. 3A–D). Overall, these results have shown that the red LED light with 630 nm remarkably reduced inflammatory response in macrophage-like cells after inducing the inflammatory response with LPS.

Red LED Radiation Reduces Cell Stress via the NF-κB Pathway

To study the effect of red LED light radiation on cell stress, particularly the NF-κB signaling pathway have studied NF-κB p65 protein expression in red LED radiated macrophage-like cells. The results showed that the red LED radiation significantly suppressed the NF-κB p65 protein expression level compared to its expression level when the macrophage-like cells were treated with LPS (Fig. 4A,B). Overall, these results have shown that the red LED light with 630 nm reduced the expression of NF-κB p65 protein and reduce the cell stress of macrophages-like cells after treatment with LPS.

Red LED Radiation Abrogates the Expression of PLEKHG5 upon Induction with LPS

To study the effect of red LED light radiation on the expression of PLEKHG5 macrophage-like cells with 630 nm red LED light. Subsequently, we analyzed the mRNA expression levels of PLEKHG5 using qRT-PCR and western blot. The results showed that LPS significantly induced the expression of PLEKHG5 expression at mRNA and protein levels. Interestingly, the red LED radiation significantly counteracts the induction of the expression of PLEKHG5 in macrophage-like cells treated with LPS (Fig. 5A–C). These results showed that the red LED light with 630 nm suppressed the illicit of PLEKHG5 in macrophages-like cells, which controlled the expression of PLEKHG5 induced by LPS treatment and subsequently may activation of the RhoA exchange factor and NF-κB signaling pathway.

Red LED Radiation Inhibits the Immune Response Signal Transduction via RhoA

Marion De Toledo *et al*. [16], reported for the first time that GEF720, also known as PLEKHG5, tech, SYX, is a member of the Dbl (The diffuse B cell lymphoma) family. The guanine nucleotide exchange factor of the Dbl family is a proto-oncoprotein that activate the small GTPase of the Rho family, specifically activating RhoA.

To investigate the role of red LED on the regulation of the RhoA transduction pathway, the expression of total and membrane RhoA protein was analyzed by western blot. We observed that exposing macrophage-like cells to the 630 nm red LED irradiation after treatment with LPS the cells significantly reduced the expression of total RhoA. Consequently, the RhoA localized at membrane presence was reduced. Overall, 630 nm red LED irradiation reduced the counteract of the LPS effect on RhoA induction and significantly reduced it is the expression (Fig. 6A–C). These results showed that red LED may inhibit the hyperimmune response via inhibiting the RhoA signaling pathway.

Discussion

The increased number of macrophages in the synovial tissue of RA patients correlates with the degree of joint erosion and leads to hyperplasia of the intimal layer [17]. The presence of macrophages in the synovium during inflammation is a key factor in the severity of RA [18] and is considered the major source of cytokines such as TNF-α that significantly contribute to RA pathogenesis [19]. Previously, it has been reported that irradiation of normal macrophages using 630 nm red LED could regulate the expression of nuclear transcription factor-like nuclear factor-erythroid factor 2-related factor 2 (Nrf2) protein levels and reduce cytokine, which induces an inflammatory response TNF-α, IL-1β, IL-6 secretion, meanwhile, by changing ROS expression levels alleviate oxidative stress [20]. However, how the light signal affects the inflammatory response of macrophages, and its upstream mechanism is still unclear. In this study, we used 630 nm red LED to irradiate THP-1-derived macrophages that were induced using LPS-induced to trigger the inflammatory responses. These results demonstrated that 630 nm red LEDs could inhibit LPS-induced inflammatory response in macrophages and the mRNA levels of inflammatory cytokines (TNF-α, IL-1β, IL-6 and CCL8) in macrophages. The LPS significantly induced macrophages' inflammatory response through PLEKHG5 at mRNA and protein levels. Interestingly, treatment of LPS-induced macrophages using 630 nm red LEDs abrogated the elevation of PLEKHG5 expression. PLEKHG5 is a RhoA-specific activator [16] and Rho GTPases are cytoskeletal regulatory proteins that mediate the formation of intercellular junctions. Rho proteins control the actin cytoskeleton during cell extravasation, migration, and phagocytosis, and directly participate in in-

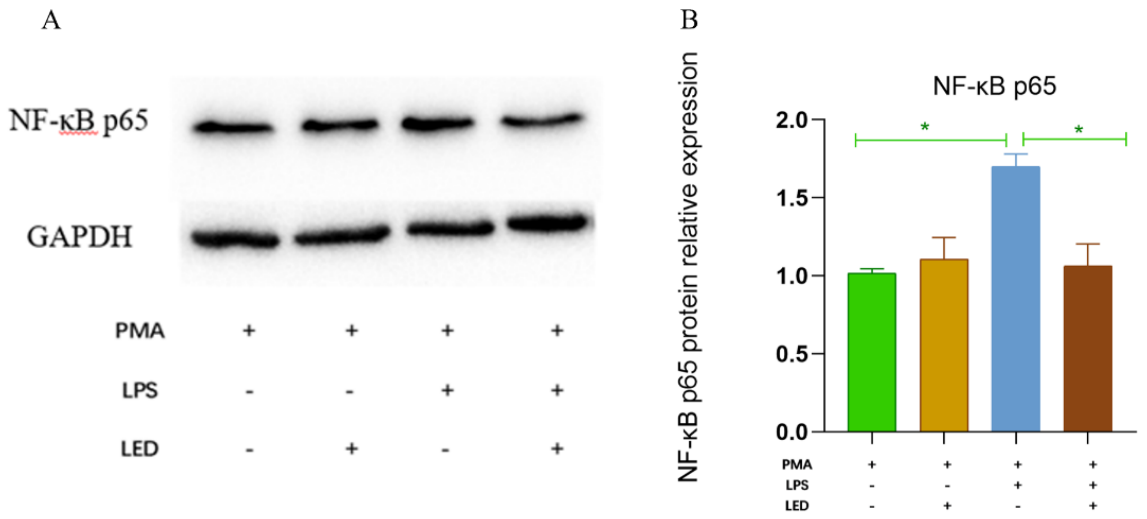

Fig. 4. The effect of 630 nm red LED irradiation on the expression levels of NF-κB p65 total protein in macrophage-like cells after induction with LPS. Macrophage-like cells were irradiated using 630 nm red LED radiation followed by a western blot analysis of NF-κB protein expression. (A) The western blot shows the downregulation of NF-κB after exposing it to the red LED. (B) The density of the band was analyzed using ImageJ software. $^{*}p < 0.05$.

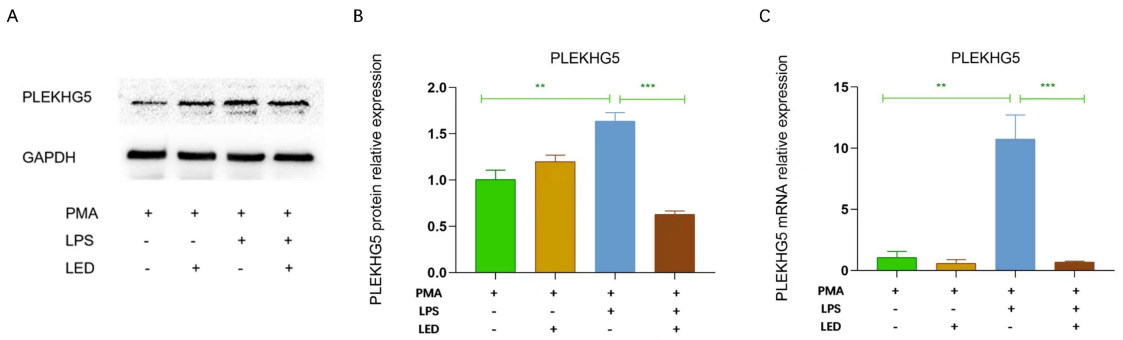

Fig. 5. Effect of 630 nm red LED irradiation on the expression of PLEKHG5 mRNA and protein expression levels in macrophage-like cells. (A) The relative expression of PLEKHG5 mRNA induced by the LPS was suppressed by the red LED radiation treatment. (B,C) Similarly, with protein expression of PLEKHG5 ($^{**}p < 0.01$; $^{***}p < 0.001$).

tracellular signaling pathways. Rho proteins interact with and activate downstream effector proteins when bound to GTP, thereby stimulating a variety of processes, including morphogenesis, migration, neuronal development, cell division, and adhesion [21]. Furthermore, they regulate vesicular transport, microtubule dynamics, cell cycle progression and gene expression [22]. These activities are important for the normal function of macrophages and other inflammatory cells. Inhibition of GTPases to block Rho protein activity has been proposed as a strategy to reduce inflammation and treat arthritis, atherosclerosis, and other inflammatory diseases [23]. The 630 nm LED light alleviates the induction of LPS to the proinflammatory response in macrophages via the RhoA signaling pathway

PLEKHG5 is a RhoA-specific guanine nucleotide exchange factor, that passes through the DH domain and binds RhoA thereby promoting the release of GDP and binding to GTP to activate Rho GTPases [24]. Macrophages in LPS induced inflammation model when receivingTLR4 the transmitted signal, Rho GTPases are activated and translocate to the membrane as RhoA-GTP. The red LED could reduce the PLEKHG5 expression levels, thereby downregulating activity protein expression levels of RhoA and membrane RhoA. Cut off the downstream signal of RhoA and shut down the cellular NF-κB signaling pathway. Therefore, we infer 630 nm red light led is by lowering PLEKHG5 expression level, reducing the protein expression level of total RhoA while promoting membrane RhoA transfer film, changing the activation state of RhoA, thereby regulating NF-κB Cell signal transduction pathway, alleviating the inflammatory response of macrophages. In addition, we also noticed that in M0 macrophages not stimu-

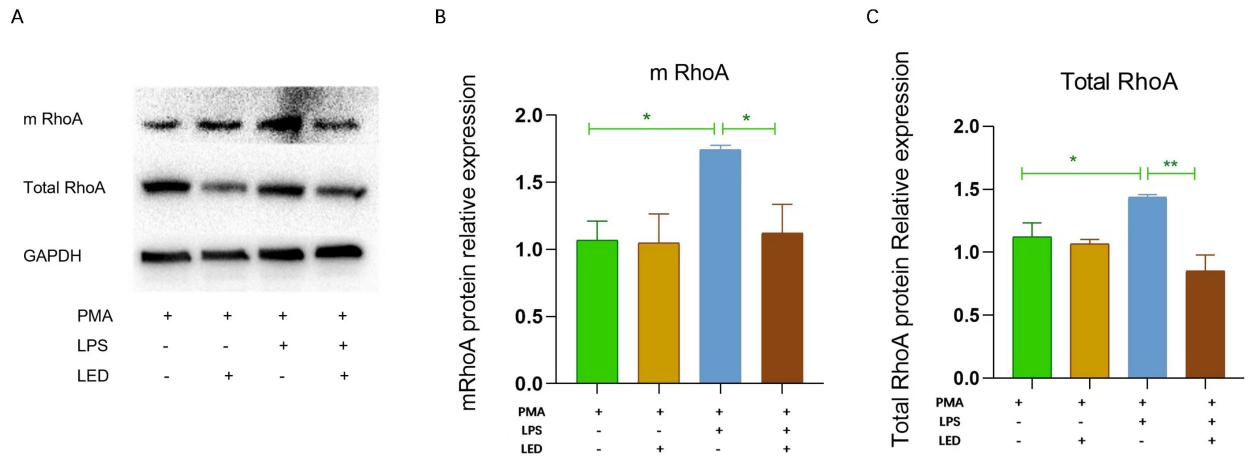

Fig. 6. Effect of 630 nm red LED irradiation on the expression of RhoA protein expression in macrophage-like cells. (A) The relative expression of membrane and total RhoA induced by the LPS was suppressed by a red LED radiation treatment. (B) Membrane and (C) total protein. Similarly, with protein expression of PLEKHG5 ($^{*}p < 0.05$; $^{**}p < 0.01$).

lated by LPS, 630 nm red LED also affected the expression of their cytokines, compared with M0 macrophages not irradiated with red LED. The mRNA expression levels of cytokines IL-1β and TNF-α in M0-type cells irradiated with 630 nm red LEDs decreased, and the expression levels of cytokine il-6 increased, while CCL8 did not change. However, PLEKHG5 and RhoA did not undergo similar changes, which suggests that the effect of 630 nm red LED on the secretion of cytokines by M0 macrophages may be through other pathways.

Photobiomodulation with wavelengths ranging from 630 to 1100 nm has demonstrated positive effects in animal models of neurodegenerative diseases such as Alzheimer's [25]. In humans, photobiomodulation has been reported to be effective against a variety of pain conditions including mucositis [26], and carpel tunnel syndrome [27,28].

Conclusions

In conclusion, 630 nm red light LED inhibits M1 cells IL-1β and TNF-α CCL8 and IL-6 mRNA expression levels. 630 nm red light LED may effectively suppress M1 cytokines via lowering the NF-κB level of p65 that regulates the expression levels of inflammatory factors. Similarly, 630 nm red light LED downregulated PLEKHG5 protein and mRNA level expression. Consequently, 630 nm red light LED ameliorated the LPS induction of total RhoA and membrane RhoA protein expression. Henceforth, this study demonstrated that 630 nm red light LED lower PLEKHG5 that could influence NF-κB RhoA cell signaling pathways induction, by suppressing NF-κB cell signal transduction, reducing the expression level of inflammatory cytokines and inhibiting the inflammatory response of macrophages.

Author Contributions

KZ, FL and FZ—designed the research study; KZ, FL, MQ, HW and ML—performed the research; MQ, HW, ML and FZ—analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.23812/j.biol.regul.homeost.agents.20223605.152.

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