Negative Modulation of Mir-21-Mediated Inflammatory Response by Retinoic Acid

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UNIVERSITY OF MIYAZAKI

JAPAN

2021

DECLARATION OF AUTHORSHIP

I declare that this dissertation titled, "Negative Modulation of Mir-21-Mediated Inflammatory Response by Retinoic Acid" and the work presented in it are my own. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this university.
- Where any part of this dissertation has previously been submitted for a degree or any other qualification at this university or any other institution, this has been clearly stated.
- Where I have consulted the published work of others, this is always clearly attributed.
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this dissertation is entirely my own work.
- I have acknowledged all main sources of help.
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Miyazaki, September 2021

Queen Intan Nurrahmah

Student ID J1711003

SUMMARY

QUEEN INTAN NURRAHMAH. Negative Modulation of Mir-21-Mediated Inflammatory Response by Retinoic Acid. Supervised by Prof. Masugi Maruyama.

Introduction

Macrophages are essential components of the immune system, specialized in either removing pathogens as part of innate immunity or contributing to adaptive immunity through antigen presentation. They can either induce inflammation or repair damaged tissues by secreting different signaling proteins. The dual role of macrophages occurs because macrophages are not a single homogeneous population. Different phenotypical and functional subpopulations exist due to their activation status, influenced by microenvironmental stimuli. The two generally recognized phenotypes are classically activated (M1) and the alternatively activated (M2) macrophages. M1-type macrophages are characterized by the production of pro-inflammatory cytokines and antimicrobial activity. M1 macrophages can be activated under the influence of interferon-γ (IFN-γ), lipopolysaccharide (LPS), or pro-inflammatory cytokines (e.g., tumor necrosis factor (TNF). In contrast, M2 macrophage are linked to immunosuppression and wound repair, with low levels of pro-inflammatory cytokine secretion. M2 polarization is induced by exposure to Th2 cytokines (IL-4, IL-10, and IL-13) and glucocorticoid. M1 and M2 phenotypes play distinctive roles in the progression and regression of inflammation-related diseases.

MicroRNA (miRNA) are short (21-26 nucleotide) eucaryotic RNAs produced by the processing of specialized RNA transcripts coded in the genome that regulate gene expression through complementary base-pairing with target mRNA. Depending on the extent of base pairing, mRNA can suppress gene expression by causing target mRNA degradation or blocking its translation. MiRNA has been found to influence macrophage polarization with consequent influence on inflammation. MicroRNA-21 (miR-21) is one of the miRNAs that have a role in the pathophysiology of conditions such as cancer, cardiac injury, and inflammation. In this study we investigated the molecular factors that modulate macrophage polarization during inflammatory conditions. We analyzed the role of

miR-21 in lipopolysaccharide (LPS)-induced inflammatory response in macrophages.

Retinoic acid (RA), an active metabolite form of vitamin A, plays a positive role in a broad range of biological processes, including differentiation proliferation, apoptosis, and morphogenesis. It also has a vital role in the maintenance of immune homeostasis during inflammatory responses.

Objective

The current study aimed to elucidate the molecular mechanisms by which Retinoic acid (RA), a potent immunomodulator, suppresses LPS-induced inflammatory response in macrophages.

Method

RAW 264.7 macrophages were treated with RA and/or LPS, and analyzed for inflammatory genes and miR-21 by PCR. The roles of miR-21 and NF-OEB signaling pathway were also assessed by knock-down experiments, immunofluorescence, and ChIP assays.

Result

Pretreatment with RA quenched the LPS-induced inflammatory responses, including phagocytosis, ROS generation, and NO production. RA shifted the polarization away from the M1 state by negative regulation of IKKa/b, p65, and miR-21. RA hindered the phosphorylation of IKKa/b, translocation of p65 into the nucleus, and the subsequent upregulation of miR-21. Knock-in and knock-down experiments showed that miR-21 is central for the polarization shift toward the pro-inflammatory M1 state.

Conclusion

miR-21 is involved in the LPS-induced pro-inflammatory profile of macrophages and that RA negatively regulates the inflammatory response by targeting NF-κB/miR-21 signaling. Our data exposes RA's potential as a pharmacological agent to manipulate miR-21 and counteract hyperinflammatory response.

Keywords: Macrophage polarization; retinoic acid; NF-κB; miR-21; pro-inflammatory

Negative Modulation of Mir-21-Mediated Inflammatory Response by Retinoic Acid

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Queen Intan Nurrahmah

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1. INTRODUCTION

Immune system's that have response to against any harmful stimuli, such as damaged cells, microbial invasion, infected cells, and toxic compounds by removal of injurious stimulus and initiating the healing of damaged tissue is well known as inflammation [1,2]. Inflammation is a first line defense mechanism for cellular physiology to avoid any escalation of its unfavorable circumstances. Based on the duration and pathological characteristics, inflammation can be classified into two main categories: acute and chronic. Acute inflammation is typically of relatively short duration (hours to days), early responses to an injurious agent and is quickly resolved. Inflammatory response is normally terminated once the disturbance is eliminated, the infection is cleared, and damage tissue is repaired, followed by resolution—the restoration of affected tissues to their normal structural and functional state in acute inflammation [3,4]. Inflammation is normally activated within minutes in any host with a functional innate immune system. The primary cellular component of the innate immune system are macrophages, dendritic cells, natural killer (NK) cell and neutrophils [5,6].

Macrophages are essential components of the mononuclear phagocyte system, and they play crucial roles in the initiation, maintenance, and resolution of inflammation. Macrophages are mature forms of mononuclear phagocytes derived from the yolk sac or fetal liver and bone marrow [7,8]. From there, they enter the peripheral bloodstream and continually migrate into all tissues and organs. They are widely distributed throughout the body and exhibit significant variations in functional behavior, not only as a result of the maturation/differentiation processes but also due to local environmental conditions [9]. They can be programmed to distinct functional phenotypes: the pro-inflammatory M1 type that functions as the first line of defence in innate immunity by phagocytizing and killing invading pathogens; and the anti-inflammatory M2 phenotype that resolves inflammation and promotes tissue restoration [10,11]. During the inflammatory phase following tissue injury, the innate immune system is activated, and pro-inflammatory mediators are released. On exposure to inflammatory mediators such as lipopolysaccharides (LPS), and other microbial products, macrophages may

undergo reprogramming to become the classical M1 phenotype. M1 phenotype produces proinflammatory cytokines such as TNF-a, IL-1b, IL-6, and IL-12 [6,7]. M1 macrophages also increase the generation of reactive oxygen species (ROS) and inducible nitric oxide synthase (iNOS) [8]. These macrophages possess cytotoxic activity capable of killing pathogens and tumor cells but can also destroy normal tissue. On the contrary, M2 macrophages are induced by factors such as IL-4 and glucocorticoids. They are characterized by high expression of anti-inflammatory proteins such as IL-10, TGF-b, and Arginase-1 (Arg-1), with immune-regulatory functions that can promote immunity against parasites and repair damaged tissue [12,15,16].

MicroRNAs (miRNAs) are small non-coding single-stranded RNAs comprising 19–22 nucleotides and function as posttranscriptional regulators of gene expression. Recent studies show evidence that miRNA can regulate cellular processes such as cell proliferation, differentiation, and apoptosis [17,18]. MiRNAs are known to regulate macrophage polarization and inflammation [19]. MiR-21 is one of the most highly studied miRNAs in mammalian cells due to its role in pathophysiological conditions. A few studies have reported the role of miR-21 in modulating inflammation [20,21]. Our previous studies on osteoclasts and macrophages indicated the pro-inflammatory nature of miR-21 [22,23].

Retinoic acid (RA), an active form of vitamin A, is a known antioxidant with a positive role in a broad range of biological processes, including cell proliferation, differentiation, morphogenesis, and cell apoptosis [24-28]. In this study, we analyzed the effect of RA on LPS-induced inflammation in macrophages and the role of miR-21. Our data provide evidence of inhibition of IKKa/b activation by RA and that it can antagonize inflammation by down-regulating NF-κB/miR-21 signaling. We propound that RA's potential as a miRNA modulator to alleviate excessive inflammation is worthy of further investigation.

2. MATERIALS AND METHODS

2.1 Reagents

All-trans-Retinoic acid (RA) (≥97%) was purchased from Fujifilm Wako Pure Chemicals Co., Ltd (Tokyo, Japan). Dimethyl sulfoxide (DMSO), LPS (from *Escherichia coli*), and methyl thiazol tetrazolium (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The antibodies against IRAK 1 (4504), IRAK 4 (4363), TRAF 6 (8028), TAK 1 (4505), phospho- TAK 1 (4508), IKKα (2682), IKKβ (8943), phospho IKKα/β (2697), IκBα (4814), phospho- IκBα (2859), NF-κB p65 (8242), phospho- NF-κB p65 (3033), β-actin (4970), and PCNA (2586) were purchased from Cell Signaling Technology (Danvers, MA, USA). MiRNeasy kit for miRNA isolation, pre miR-21, and anti miR-21 were from Qiagen (Valencia, CA, USA). ELISA kits targeting mouse TNF-α, IL-1β, IL-6, IL-10, and IL-4 were from Invitrogen (Thermo Fisher Scientific, Vienna, Austria).

2.2 Cell culture and RA/LPS treatment

RAW 264.7 macrophages (RAW cells) were purchased from RIKEN Cell Bank (Tsukuba, Japan). Cells in early passages 4 -7 were cultured in Dulbecco's Modified Eagle low glucose medium (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (Funakoshi, Tokyo, Japan), and antibiotics (5 mg/ml of Penicillin, 5 mg/ml of Streptomycin, and 10 mg/ml Neomycin) (Gibco, Tokyo, Japan) under standard culture conditions of 37°C, 5% CO_2 and 95% humidity. Semi-confluent cells were pre-treated with 50 μM RA for 2h, followed by 4h exposure to 1 μg/ml LPS. The cells were processed for studies as explained below.

2.3 MiRNA transfection

RAW cells cultured in 12-well plates were transfected with pre miR-21 or anti miR-21 in OptiMEM medium (Invitrogen, NY, USA) using HiPerfect transfection reagent (Qiagen) as per manufacturer's protocol. At 48 h post-transfection, transfected cells were used for PCR, ELISA, and phagocytosis studies.

2.4 Cytotoxicity Assay

Cytotoxicity was determined by MTT assay. RAW cells were seeded in a 96-well plate at a density of $1x10^4$ cells/well and maintained at 37°C for 24 h. The cells were pretreated with 50 µM RA for 2 h before treatment with 1 µg/ml LPS for 4 h. The treated cells were cultured in fresh DMEM medium for 24 h, before processing as per standard MTT protocol. The intracellular purple formazan was quantified with a UV-Vis spectrophotometer at an absorbance of 570 nm (Multiskan FC, Thermo Scientific, Pittsburgh PK, United States).

2.5 ROS generation assay

Intracellular ROS was studied by fluorescence microscopy and spectrofluorometric methods, using OxiSelectTM Intracellular ROS Assay Kit (Cell Biolabs, Inc., San Diego, CA, USA). Cells were seeded on coverslips in a 12-well plate at a density of $1x10^5$ cells/well (for fluorescence microscopy analysis) or a 96-well plate at a density of $1x10^4$ cells/well (for spectrofluorometric analysis). Following RA/LPS treatment, as mentioned above, 10 mM 20,70-Dichlorofluorescin diacetate (DCFH-DA) was added to cells at 37 ºC for 1 h in the dark. Cells on coverslips were fixed in 4% PFA, stained with 40,6-diamidino-2-phenylindole (DAPI), and mounted on slides. Fluorescence was observed under a

confocal laser scanning microscope TCS SP8 (Leica, Wetzlar, Germany). The fluorescence signal from cells in the 96-well plate was quantified using a spectrofluorometer (DTX800, Beckman Coulter Inc., CA, USA) at excitation and emission wavelengths 485 and 530 nm, respectively.

2.6 Detection of secreted proteins

RAW cells were seeded in a 96-well microplate at a density of $1x10^4$ cells/well. Following RA/LPS treatment, as mentioned above, the conditioned medium from treated cells was centrifuged at 15,000 rpm, and the cleared supernatant was used to analyze secreted proteins. ELISA was used for quantitative detection of secreted forms of TNF-a, IL-1b, IL-6, IL-4, and IL-10. Nitrite (NO_2) accumulation in the conditioned medium was measured as an indicator of NO production and detected using the Griess Reagent system kit (Promega, USA), according to the manufacturer's instructions. Arginase activity was assessed by QuantiChrom^{TM} Arginase Assay kit (BioAssay Systems, CA, USA), following the kit protocol.

2.7 MiRNA isolation and RT-PCR

MiRNA-enriched total RNA was isolated using the miRNeasy kit (Qiagen). RNA concentration and quality were assessed by measuring absorbances A260, A280, and A230 using UV/VIS Biophotometer Plus (Eppendorf AG, Hamburg, Germany). One mg total RNA was reverse transcribed to cDNA using the miScript II RT kit (Qiagen), according to the manufacturer's instructions. The first-strand cDNA was used with miScript primer assays and SYBR green polymerase chain reaction (PCR) kit (Qiagen) to analyze precursor and mature forms of miR-21 using StepOne Plus Real-Time PCR system (Applied Biosystems, CA, USA). Small non-coding RNA RnU6 was used as a housekeeping gene to normalize the expressions of precursor and mature forms of miR-21. Primary miR-21, pro-inflammatory (TNF-a, IL-1b, IL-6, and iNOS), and anti-inflammatory (Arg-1, IL-10, and IL-4) genes were analyzed by semi-quantitative PCR using DreamTaq Green PCR Mastermix (Thermo Fisher Scientific, Tokyo, Japan) in Gene Atlas thermocycler

(Astec, Tokyo, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene to normalize primary miR-21 and the inflammation response genes. The PCR products were electrophoresed on 1% agarose gel, and resulting bands were detected using the LAS 4000 digital imaging system (Fujifilm, Tokyo, Japan) and analyzed by Image Quant TL software (GE Healthcare Life science, Tokyo, Japan).

2.8 Chromatin immunoprecipitation (cHIP) assay

ChIP assay was performed as per standard protocols. Briefly, RA/LPS treated cells were cross-linked with 1% formaldehyde, and chromatin was isolated using PierceTM Chromatin Prep module (Thermo Scientific). The chromatin was immunoprecipitated with anti-NF-OEB p65 antibody or IgG (negative control), using PierceTM agarose ChIP kit (Thermo Scientific). After stringent washing, DNA was eluted and subjected to PCR using miR-21 primers encompassing NF-OEB binding sites (set A: Forward 50-GGAGTGGATGGGTTCTGCCTTA-30 and Reverse 50-CAAGGTGGATTGCATCGAGG-30; set B: Forward 50-TGCAACAGACTGGCCTTC-30 and Reverse 50-CATGCAAGACTGTTATCCAATCT-30).

2.9 Western Blot

RA/LPS treated cells were lysed in ice-cold N-PER reagent (Pierce, USA) supplemented with protease inhibitors cocktail (Nacalai Tesque, Inc., Kyoto, Japan). Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific) following the manufacturer's protocol. Ten micrograms of cytoplasmic or nuclear extracts were resolved over 10% SDS-PAGE gels and transferred to PVDF membranes (Immuno-blot, Bio-Rad, California, USA) using a Bio-Rad Semi-Dry Electrophoretic Transfer Cell. The blotted membranes were blocked with EzBlock Chemi (Atto, Tokyo, Japan) for 1 h at room temperature and incubated overnight with primary antibodies at 4 _C, followed by HRP conjugated IgG antibody for 1 h at room temperature. β-actin or PCNA were

used as loading controls for cytoplasmic or nuclear extracts, respectively. The enhanced chemiluminescence ECL reagent (GE Healthcare) was used to detect immune reactivity. Images were obtained with the LAS 4000 digital imaging system (Fujifilm). Band intensities were quantified by using Image Quant TL software (GE Healthcare).

2.10 Immunofluorescence staining

RAW cells were cultured overnight on coverslips in 6-well plates at a density of $3x10^5$ cells/well. Following RA/LPS treatment, cells were fixed and permeabilized using the Image-IT® Fix-Perm kit (Molecular Probes, USA). Following fixing and blocking, the coverslips were incubated overnight with anti-p65 rabbit antibody at 4°C and then incubated with goat anti-rabbit AlexaFluor 488 (Life Technologies, Carlsbad CA, USA) secondary antibody for 1 h at room temperature (protected from light). Nuclei were stained with DAPI. Fluorescence images were obtained using a confocal laser scanning microscope TCS SP8 (Leica).

2.11 Phagocytosis Assay

Phagocytic activity was assessed using an IgG FITC phagocytosis assay kit (Cayman Chemical, Ann Arbor, MI, USA). Cells seeded in 24-well plates at a density of $1x10^5$ cells/well were treated with RA for 2 h followed by LPS for 4 h. Post LPS treatment, cells were cultured in standard DMEM medium for 24 h. After 24 h, the cells were washed with PBS and incubated with rabbit IgG-FITC conjugates latex beads for 3 h. Nuclei were counterstained with DAPI, and fluorescence images were obtained using confocal laser scanning microscope TCS SP8 (Leica).

2.12 Statistical analysis

Data are expressed as a mean ± standard deviation of three independent experiments. Data were analyzed by SPSS Statistics 20 software using one-way analysis of variance (ANOVA) with LSD post-tests for multiple comparisons or by unpaired, two-tailed t-test for single measurements. Differences were considered statistically significant at $p<0.05$, and are represented with asterisks as follows: # p < .05 vs. control; *p < .05 vs. LPS group; ##, p < .001 vs. control; **p < .001 vs. LPS group.

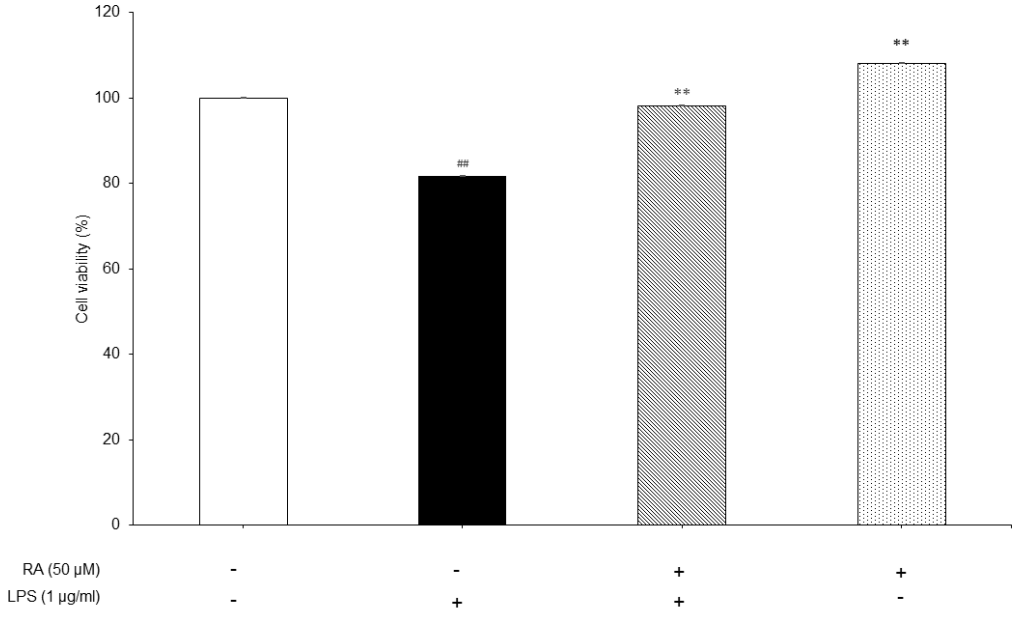
3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 RA inhibits LPS-induced cytotoxicity and ROS generation in macrophages

RAW macrophages were conditioned with 50 mM RA for 2 h before treatment with 1 mg/ml LPS for 4 h and analyzed for cytotoxicity. The dose and periods were based on our preliminary data on RA's ROS prevention (data not shown). MTT cytotoxicity assay showed that a 4 h treatment with LPS resulted in a reduction in the number of viable cells by 20%. Treatment with RA alone did not show a cytotoxic effect. Besides, it prevented the cytotoxicity induced by LPS (Figure 1(A)). ROS generation was measured by using the cell-permeable ROS indicator, DCFH-DA. The non-fluorescent DCFH-DA is transformed into highly fluorescent 20,70-Dichlorofluorescin (DCF) in the presence of ROS. LPS treatment enhanced the DCF fluorescence generation significantly compared to control cells (Figure 1(B)). On the other hand, RA treatment completely suppressed the intracellular fluorescence, indicating the total abolishment of ROS generation.

(A)



(B)

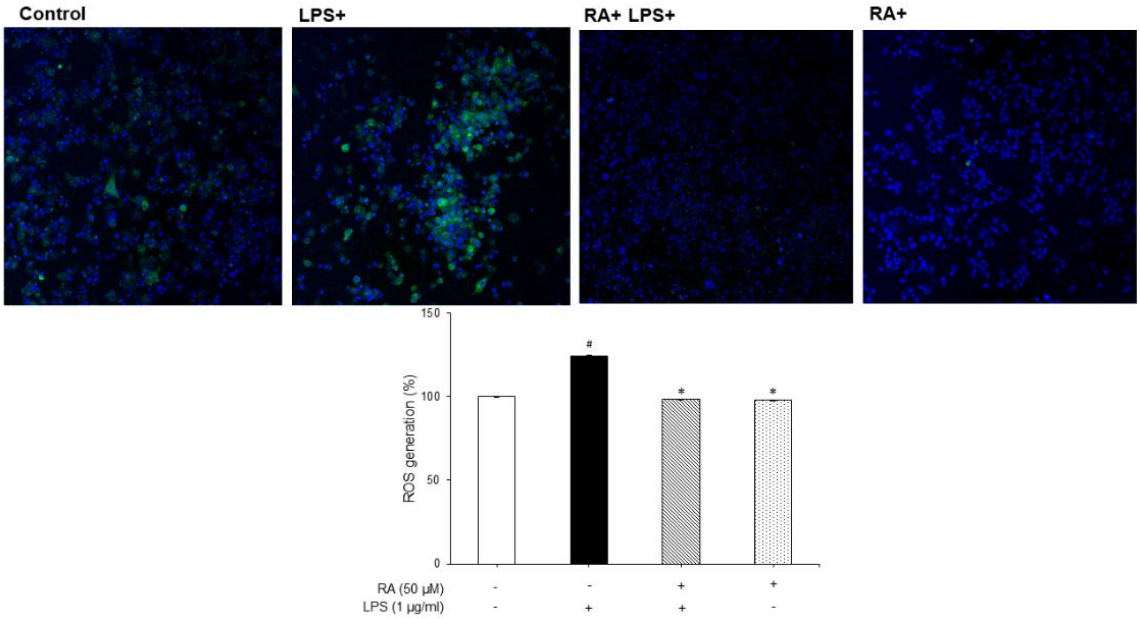


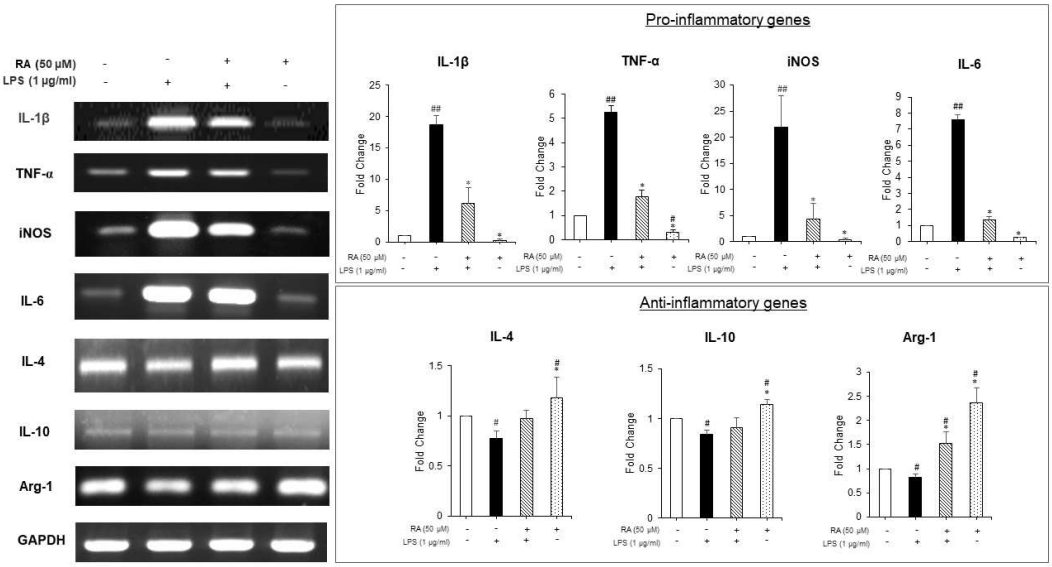
Figure 1. RA arrests LPS-induced toxicity, and ROS generation in RAW 267.4 macrophages. RAW cells treated with 1 mg/ml LPS, with or without 50 mM RA pretreatment were analyzed for (A) cytotoxicity, and (B) ROS generation by MTT, and DCFH-DA assays respectively. Data is representative of 3 independent experiments. ##, $p < .001$ vs. control; **, $p < .001$ vs. LPS group; # $p < .05$ vs. control; *$p < .05$ vs. LPS group.

3.1.2 RA decreases LPS-induced expression of pro-inflammatory cytokines

The mRNA expression levels of genes related to pro-inflammation (TNF-α, IL-1β, IL-6, and iNOS) and anti-inflammation (IL-4, IL-10, and Arg-1) were analyzed by semi-quantitative PCR. LPS caused significant increases in expression levels of IL-1β (20-fold), TNF-α (5-fold), iNOS (20-fold), and IL-6 (7-fold), and moderately decreased the levels of IL-4 and Arg-1 (Fig. 2a). RA drastically reduced the expression levels of pro-inflammatory genes and increased the expression of IL-4 (1.5 fold) and Arg-1 (2.5 fold). RA also suppressed the LPS-induced overexpression of pro-inflammatory genes. Analysis of secreted proteins by ELISA revealed a similar pattern, i.e., LPS increased the secretion of IL-1β, TNF-α, and IL-6 (Fig. 2b). Nitric oxide was analyzed by Greiss assay, which utilizes nitrite levels in the culture medium as an index of NO production by cells. Basal NO

production by RAW cells was at the lower limit of the sensitivity of the nitrite assay. Nitrite levels increased 2-fold in cells treated with LPS, whereas it was below detection in cells treated with RA. LPS effect on pro-inflammatory cytokines and NO production was counteracted by RA (Fig. 2b). Arginase activity, an index of secreted arginase, could not be detected in any of the groups.

(A)



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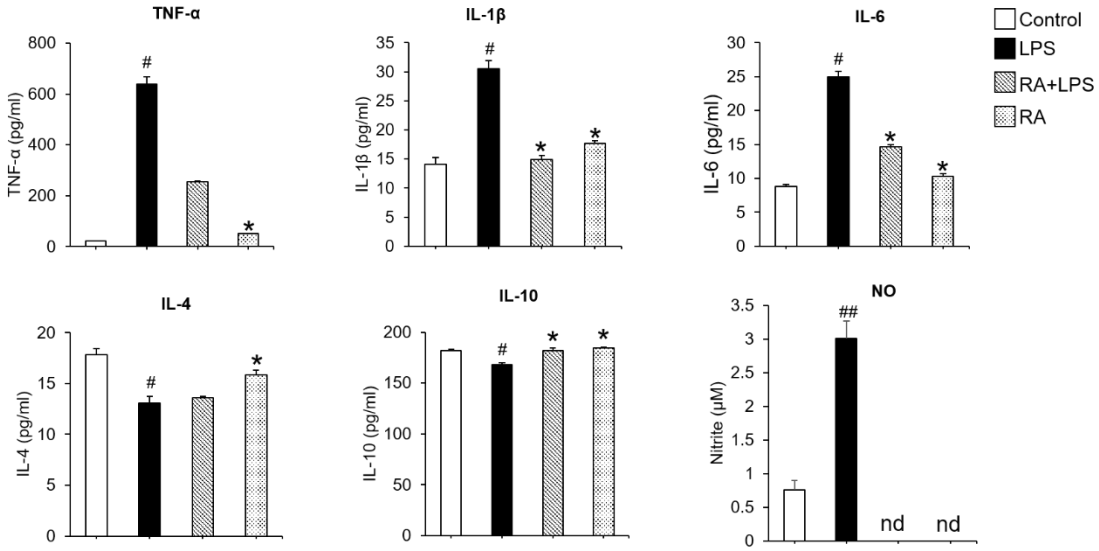


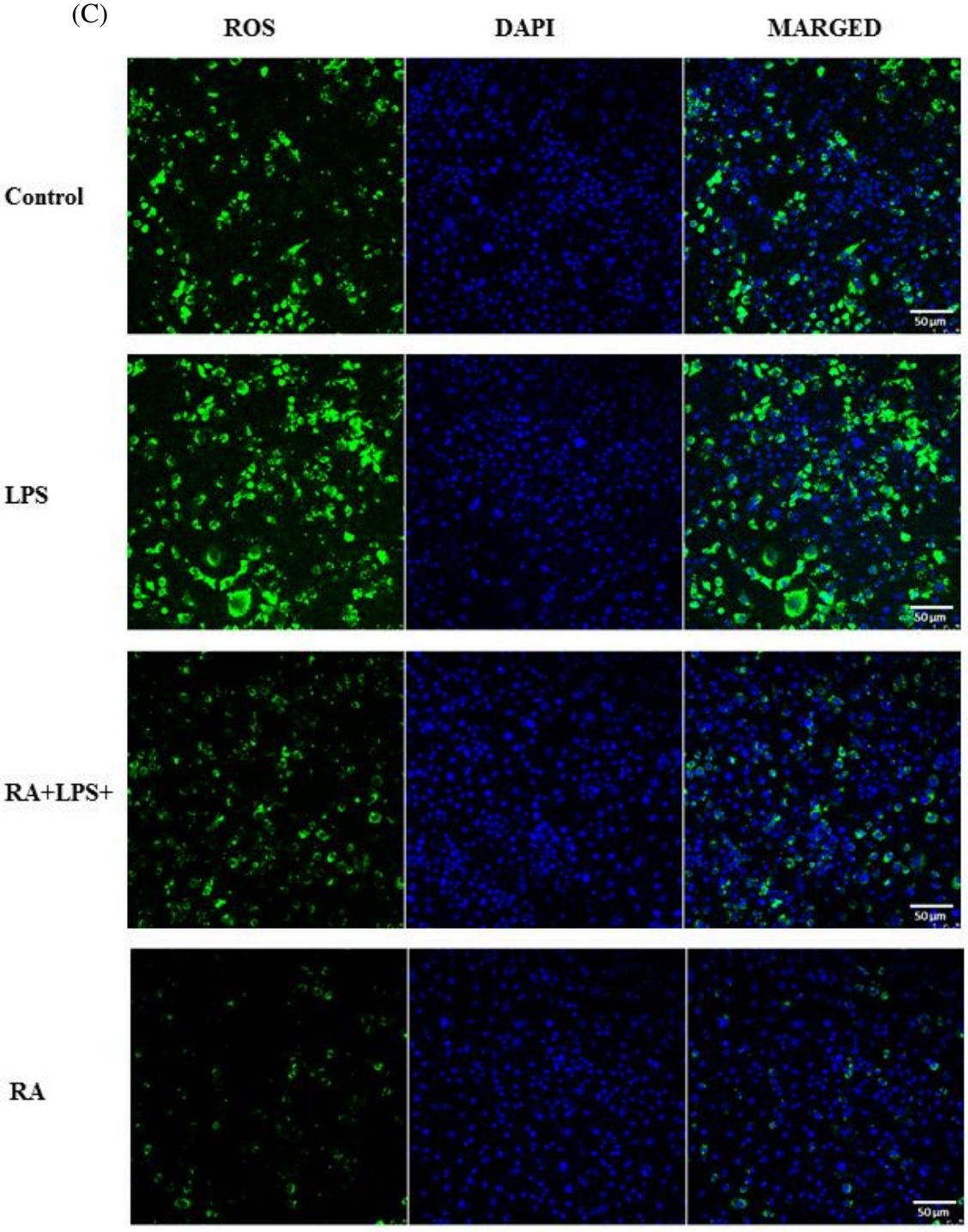


Figure 2. RA decreases LPS-induced pro-inflammatory response of macrophages. RAW cells treated with 1 mg/ml LPS, with or without 50 mM RA pretreatment were analyzed for (A) cytokines expression by semi-quantitative PCR, (B) secreted proteins by ELISA and (C) phagocytic activity. Data is representative of 3 independent experiments. ##, $p < .001$ vs. control; **, $p < .001$ vs. LPS group; # $p < .05$ vs. control; *$p < .05$ vs. LPS group. Cell images were captured at 40x magnification. Scale bar denotes 50µm.

3.1.3 RA abrogates phagocytic activity of macrophages

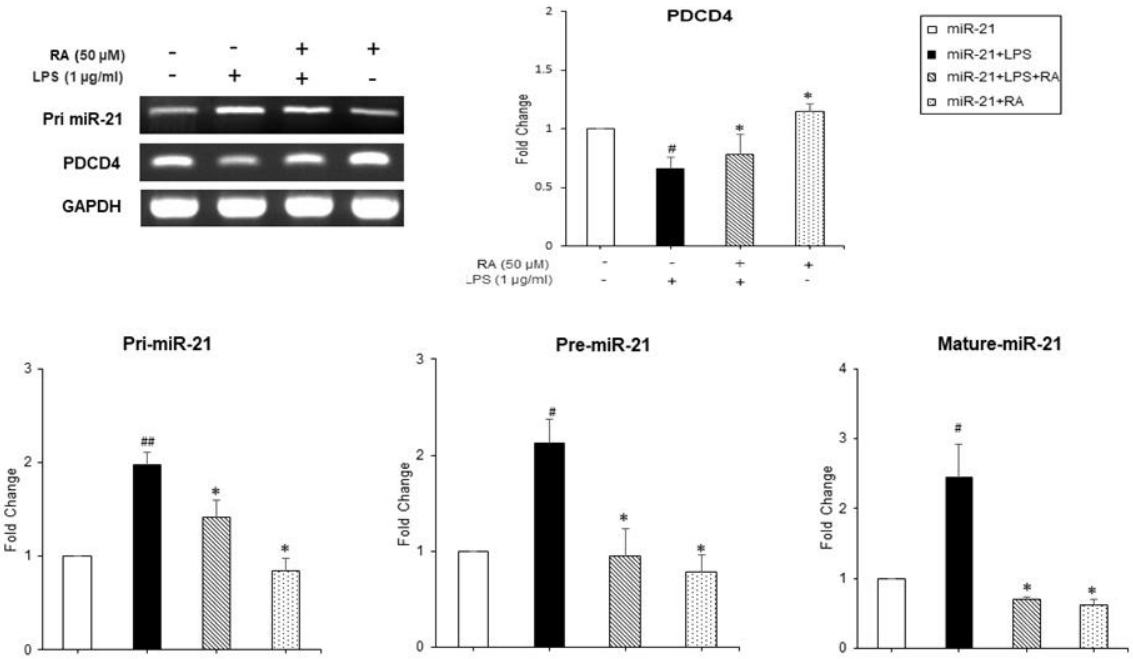
Phagocytosis, the process of recognizing and engulfing foreign bodies and tissue debris, is another crucial role of macrophages in inflammation modulation. We proceeded to study the effect on the phagocytic property by using latex beads coated with fluorescently-labeled rabbit IgG as a probe. RAW cells treated with LPS showed a high intensity of fluorescence, implying a high rate of phagocytosis. Pretreatment with RA resulted in a significant reduction in the number of fluorescent cells. In other words, RA nullified the phagocytic activity induced by LPS (Figure 2(C)).

3.1.4 RA decrease miR-21 expression

Our previous study using diabetic mice showed that miR-21 was dysregulated in diabetic wounds [22]. Besides, in vitro experiments using RAW cells indicated that miR-21 was proinflammatory [16,17]. Hence, we studied the expression pattern of miR-21 in RA/LPS treated cells. LPS indeed upregulated miR-21 at all levels (primary, precursor, and mature forms) (Figure 3(A)), with concomitant downregulation of PDCD4, a well-known target of miR-21. LPS-induced miR-21 upregulation and PDCD4 downregulation were attenuated by RA pretreatment. To test if miR-21 was instrumental to the pro-inflammatory profile, we performed miRNA transfection experiments to overexpress or subdue the expression of miR-21 in the cells, using pre miR-21 or anti-miR-21, respectively. PDCD4 was used as an indicator of successful transfection. Cells transfected with pre-miR-21 showed reduced PDCD4 expression whereas those transfected with anti-miR-21 showed an increase in PDCD4 expression, indicating successful overexpression or knockdown of miR-21, respectively. MiR-21 overexpression caused a shift toward the M1 phenotype, with higher expression of pro-inflammatory cytokines at the levels of gene (Figure 3(B)) and secreted proteins (Figure 3(C)). On the other hand, miR-21 knockdown resulted in reduced expression of the pro-inflammatory cytokines. Phagocytosis assay also revealed a similar pattern; phagocytosis of fluorescent beads was dramatically increased in pre miR-21 transfected cells, and almost abolished in anti-miR-21 transfects (Figure 3(D)). We examined if RA could modulate LPS-induced pro-inflammatory cytokines in miR-21 overexpressing cells.

Treatment of LPS to the miR-21 overexpressing cells further induced a significant increase in the expressions of IL-1b, TNF-a, and iNOS (Figure 3(E)). There was no further increase in IL-6. Pretreatment with RA led to a decrease in the expression of the inflammatory genes.

(A)



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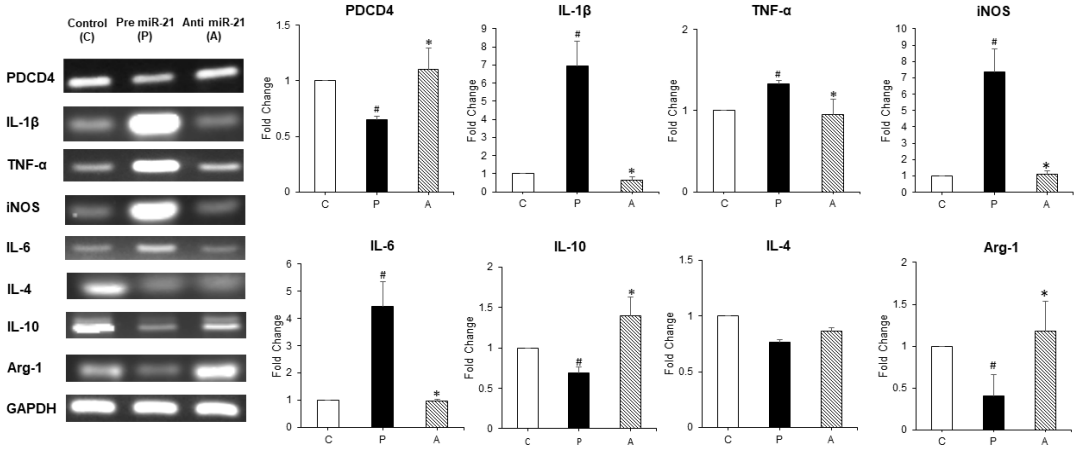

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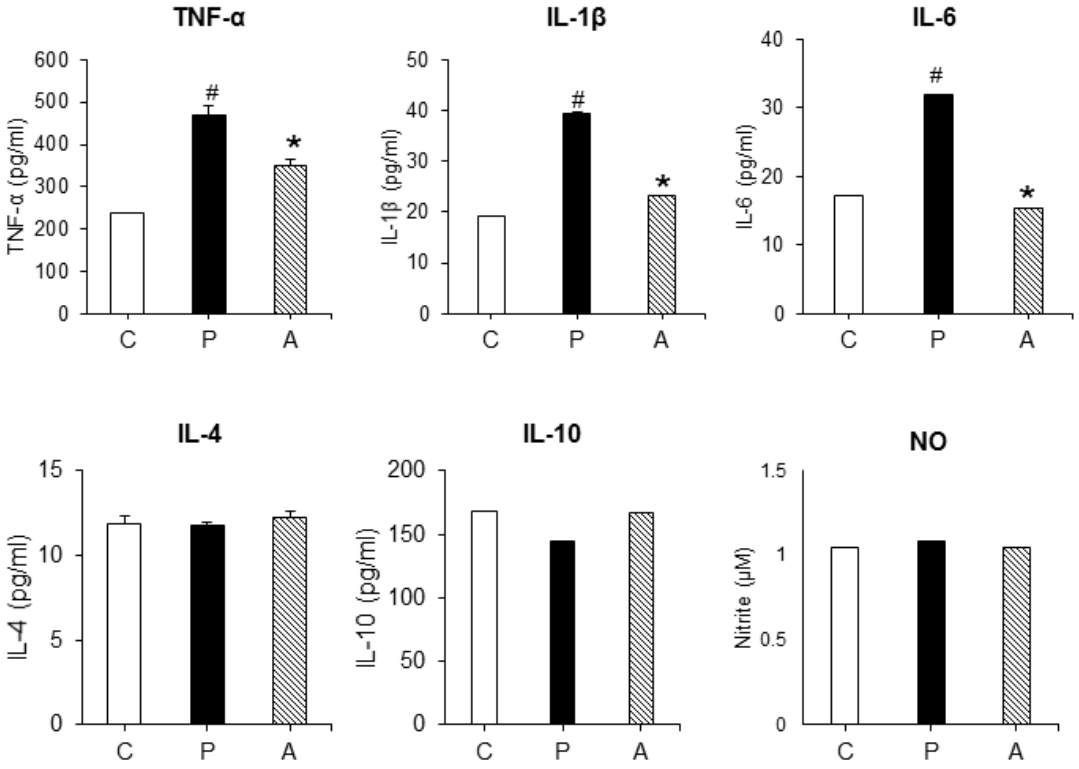
TNF-α
TNF-α (pg/ml)
600
500
400
300
200
100
0
#
*
C
P
A
IL-1β
IL-1β (pg/ml)
50
40
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IL-6
IL-6 (pg/ml)
40
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20
10
0
#
*
C
P
A
IL-4
IL-4 (pg/ml)
15
10
5
0
C
P
A
IL-10
IL-10 (pg/ml)
200
150
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50
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Nitrite (μM)
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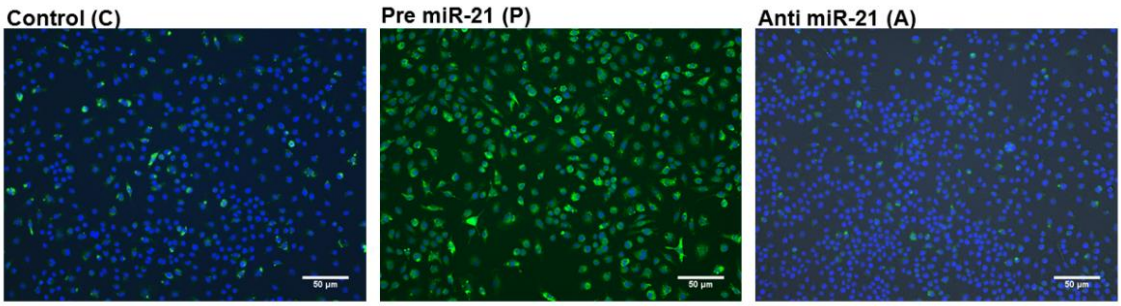
(D)


Control (C)
Pre miR-21 (P)
Anti miR-21 (A)

(E)

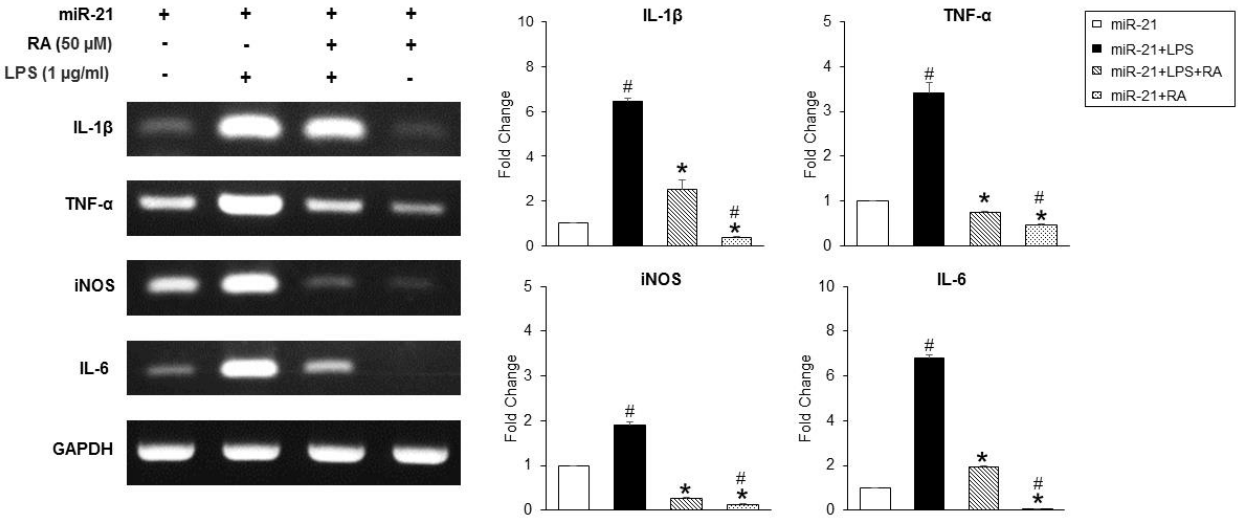


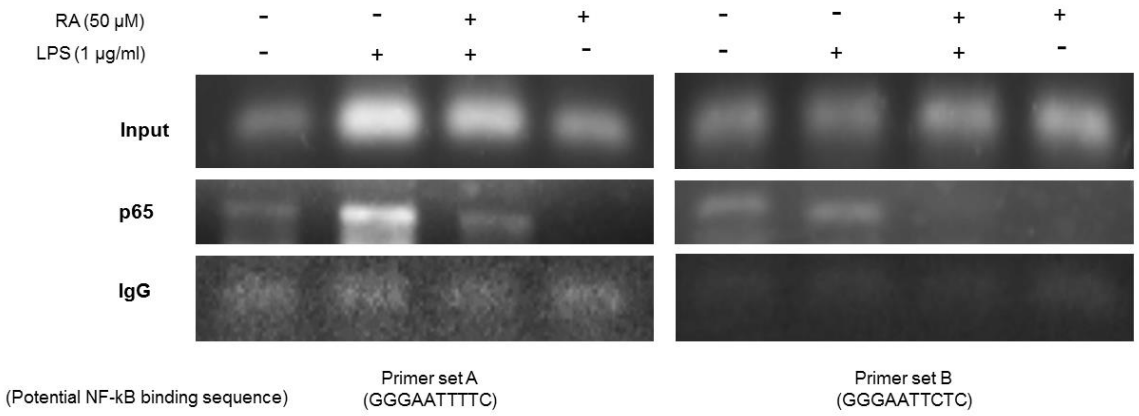
Figure 3. RA decreases LPS-induced expression of miR-21. RAW cells treated with 1 mg/ml LPS, with or without 50 mM RA pretreatment were analyzed for miR-21 expression by PCR. (A) Primary form of miR-21 (Pri-miR-21) and PDCD4 were analyzed by semi quantitative RT-PCR, and normalized to GAPDH housekeeping gene. Precursor (Pre-miR-21) and mature (Mature-miR-21) forms were analyzed by Real time PCR, and normalized to RnU6 housekeeping gene. Pre miR-21 or anti miR-21 transfect cells were analyzed for (B) inflammatory cytokines expression by semi-quantitative PCR, (C) secreted proteins by ELISA, and (D) phagocytic activity. (E) MiR-21 overexpressing cells were subjected to RA/LPS treatment, and analyzed for inflammatory cytokines expression by RT-PCR. Data is representative of 3 independent experiments. ##, $p < .001$ vs. control; **, $p < .001$ vs. LPS group; # $p < .05$ vs. control; *$p < .05$ vs. LPS group. Cell images were captured at 40x magnification. Scale bar denotes 50µm.

3.1.5 RA inhibit NF-κB activity

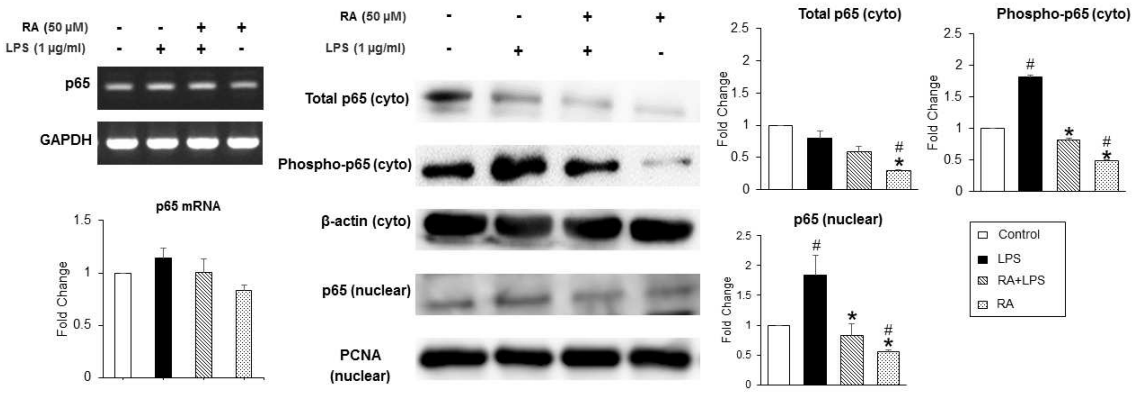
The study so far confirmed that RA abolished LPS's proinflammatory activity. It also indicated that miR-21 was responsible for the pro-inflammatory profile and that RA nullified the shift by targeting miR-21 expression. We proceeded to investigate the molecular mechanism of miR-21 regulation by RA. Since our previous studies showed that miR-21 is strongly regulated by NF-κB [23,28], we asked if NF-κB was instrumental in this scenario. As miR-21's promoter region contains two potential binding sites for NF-κB p65 subunit [28–30], we analyzed

the direct interaction of p65 with the miR-21 promoter by ChIP analysis. LPS induced an increased affinity of p65 to the miR-21 promoter; the association was abolished by RA (Figure 4(A)). RNA and protein analyses revealed that LPS did not have a significant effect on p65 at the RNA level, but induced the phosphorylation of p65 and its nuclear localization (Figure 4(B)). Accumulation of p65 in the nucleus was confirmed by immunofluorescence studies (Figure 4(C)). RA significantly reduced the basal levels of p65 and suppressed the LPS-induced translocation. Since PCR analysis of p65 showed a marginal inhibitory effect of RA on p65 mRNA, it implied that RA regulated the NF-κB pathway at the protein level. We proceeded to study the activation of the NF-κB family. LPS induced an increase in the expressions of IRAK 4, IKKb, and phosphorylated forms of TAK 1 (phospho-TAK1), IKKa/b (phospho-IKKa/b), and IκBa (phospho-IκBa) (Figure 4(D)).

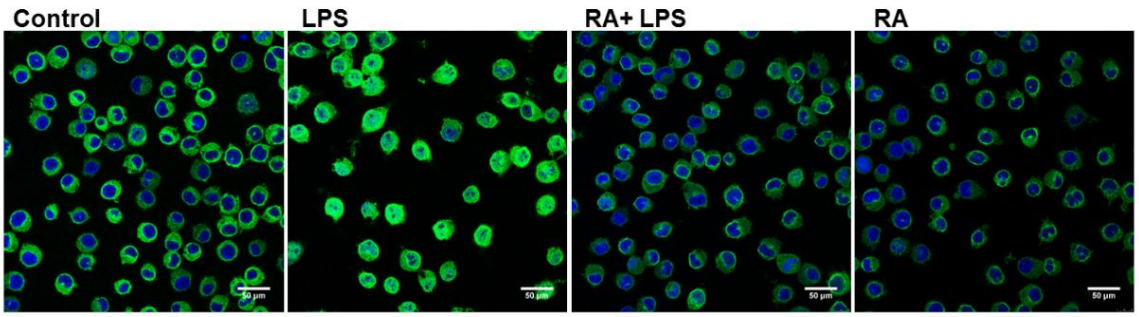
(A)



(B)



(C)



(D)

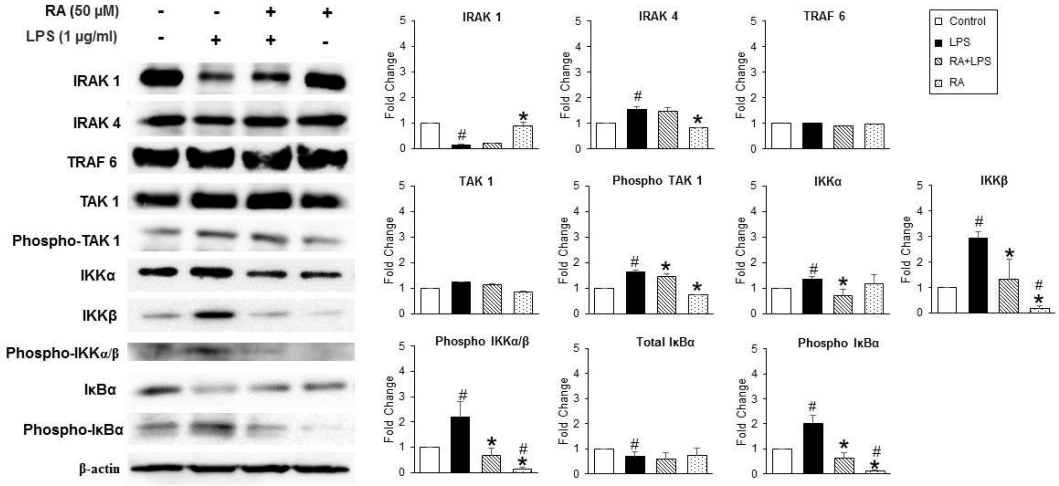


Figure 4. RA inhibits NF-κB activation. RAW cells treated with LPS and RA were subjected to (A) ChIP assay with anti p65 antibody or nonspecific IgG (control), followed by PCR amplification with two sets of miR-21 primers encompassing NF-κB p65 binding sites. (B) RT-PCR and Immunoblot of NF-κB p65 in cytoplasmic and nuclear extracts. GAPDH was used as house-keeping gene for PCR; b-actin and PCNA were used as loading controls for cytoplasmic and nuclear extracts, respectively. (C) Immunofluorescence of p65 subunit. Cell images were captured at 63x magnification. Scale bar denotes 50mm. (D) Immunoblot of NF-κB family in cytoplasmic extracts. b-actin was used as loading control.Data is representative of 3 independent experiments. ##, $p < .001$ vs. control; **, $p < .001$ vs. LPS group; # $p < .05$ vs. control; *$p < .05$ vs. LPS group.

IκBa is the NF-κB family's inhibitory protein, binding to and preventing the nuclear localization of p65. Phosphorylation of IKKa/b causes the activation of the IKK complex and phosphorylation of IκBa by the complex. Phosphorylated IκBa is ubiquitinated and degraded. p65 is released from the inhibitory complex and subsequently translocates into the nucleus, where it binds to target genes and functions as a transcription factor [25]. LPS-induced activation of the NF-κB pathway was antagonized by pretreatment with RA. These results imply that RA attenuates the pro-inflammatory profile of macrophages through negative regulation of IKKa/b, p65, and miR-21 (Figure 5).

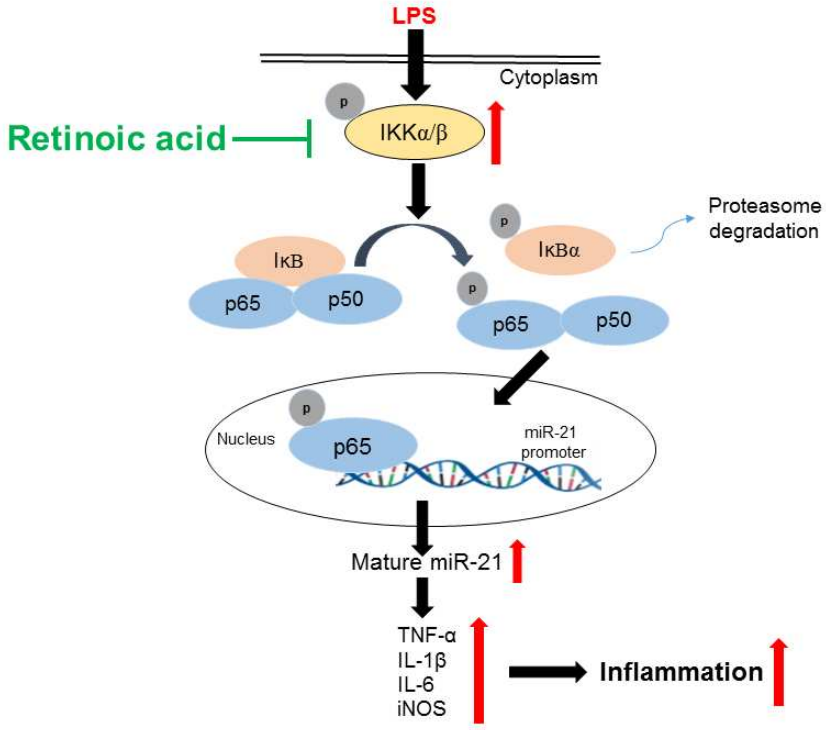


Figure 5. Schematic illustration of negative influence of RA on LPS induced inflammatory response via NF-κB/miR-21 signaling. LPS activates NF-κB through induction and activation of IKK complex, which phosphorylates IκBa, resulting in phosphorylation and nuclear translocation of p65. Binding of p65 to miR-21 promoter leads to increased transcription of miR-21, which in turn upregulates the expression of pro-inflammatory genes TNF-a, IL-1b, IL-6, and iNOS. Retinoic acid (RA) abrogates this pro-inflammatory response by negative regulation of the NF-κB signaling pathway via inhibition of IKK a/b activation.

3.2 Discussion

Retinoic acid (RA), an active form of vitamin A, plays an essential role in controlling inflammatory disease [32–34]. Studies indicate that RA mediates its functions partly through miRNA regulation [29]. RA downregulates miRNAs involved in inflammatory responses, such as miR-17, miR-20, and miR-106 [36]. Our previous studies on miR-21 implicated it as having a pro-inflammatory function [22,23]. Furthermore, in vivo and in vitro experiments by several other researchers revealed the upregulation of miR-21 by LPS stimulation [29,30,37]. MiR-21 shifts the macrophage polarization toward a proinflammatory phenotype by activating NF-κB, and upregulating inflammatory genes [23]. In this study, we show that RA modulates inflammatory response through negative regulation of miR-21. The pro-inflammatory effect of miR-21 could be attributed to the downregulation of its target gene, PDCD4. PDCD4 can drive alveolar macrophages toward the anti-inflammatory M2 phenotype. Overexpression of PDCD4 increases the expression of M2 markers Arg-1 and TGF-b1 [38]. It can also directly bind to NF-κB p65 and prevent its nuclear translocation, thus inhibiting NF-κB-dependent transcription of target genes [39]. NF-κB is a critical transcription factor involved in the macrophage activation and inflammation process. Studies have shown that NF-κB can be modulated by miRNAs. MicroRNAs miR-15, miR-16, and miR-223 can down-regulate NF-κB by binding to IKKa 30-UTR [40,41]. MiR-22 negatively regulated LPS-induced inflammatory response by preventing p65 phosphorylation [42]. We presume that miR-21 could be upregulating the NF-κB signaling pathway by targeting PDCD4. Notably, miR-21 itself is upregulated by NF-κB. The promoter region of the mir-21 gene has two potential NF-OEB responsive sites to which the NF-κB p65 subunit can bind [22,28,30]. We observed that LPS induced the direct binding of p65 to one of these binding sites and that the association was attenuated in the presence of RA. RA was also effective in preventing IκBa degradation by inhibiting phosphorylation and thus the activation of the upstream molecules IKKa/b. Our data is consistent with other studies conducted in myeloblastic cells, adipocytes, and mouse models [33,43,44]. Considering that RA drastically reduces total p65 per se, we believe that RA could also be

downregulating p65 protein through another mechanism and are currently attempting to elucidate the same. Although miR-21 plays a dynamic role in modulating inflammatory responses [7], its role in macrophage polarization is controversial. Some studies show that miR-21 induces inflammation by upregulating pro-inflammatory genes [21,23,37,45–47]. MiR-21 deficiency inhibited M1 markers and enhanced M2 markers in peritoneal macrophages [21]. Dysregulation of miR-21 induces a chronic inflammatory state [48]. On the contrary, a few others reveal its anti-inflammatory profile, through suppression of M1 phenotype [7,20,49,50]. MiR-21 appears to be involved in a number of positive and negative feedback loops [51], and signaling pathways such as NF-κB, STAT3, and PI3K could be major controlling factors determining miR-21's role in inflammatory responses [23,52]. It is suggested that several factors such as the healing phase of the diseased tissue or the target mRNAs engaged in the specific disease condition could be deciding the functional consequences of miR-21 upregulation [19,29]. Albeit, rapid induction of miR-21 following pathogenic infection [53] and its general recognition as a prognostic biomarker [29] make it an ideal candidate for therapeutic intervention.

4. CONCLUSION

Our current study suggests that miR-21 has a significant role in the pro-inflammatory profile of macrophages and that RA negatively regulates the inflammatory response by targeting NF-κB/miR-21 signaling. RA, thus, displays good potential for use as a pharmacological agent to manipulate miR-21 and counteract chronic inflammation.

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