Lipoxin A4 Regulates M1/M2 Macrophage Polarization via FPR2-IRF Pathway

Jixiang Yuan
Wenzhou Medical University First Affiliated Hospital: The First Affiliated Hospital of Wenzhou Medical University https://orcid.org/0000-0002-8555-9158

Feihong Lin
The First Affiliated Hospital of Wenzhou Medical University

Lichen Chen
The First Affiliated Hospital of Wenzhou Medical University

Weikang Chen
The First Affiliated Hospital of Wenzhou Medical University

Xiaodong Pan
Wenzhou Medical University First Affiliated Hospital: The First Affiliated Hospital of Wenzhou Medical University

Yongheng Bai
The First Affiliated Hospital of Wenzhou Medical University

Yong Cai
The First Affiliated Hospital of Wenzhou Medical University

Hong Lu (luhonglisa@163.com)
The First Affiliated Hospital of Wenzhou Medical University https://orcid.org/0000-0002-4171-016X

Research Article

Keywords: Lipoxin A4, Macrophage polarization, Inflammation, FPR2-IRF

Posted Date: December 3rd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1122685/v1

Version of Record: A version of this preprint was published at Inflammopharmacology on March 2nd, 2022. See the published version at https://doi.org/10.1007/s10787-022-00942-y.

Abstract

Lipoxin A4 (LXA4) has been shown to have anti-inflammatory activity, but its underlying molecular mechanisms are not clear. Herein, our team investigated the potential role of LXA4 in the macrophage polarisation and elucidated its possible molecular mechanism. The RAW264.7 macrophage cell line was subjected to pre-treatment with LXA4 with or without lipopolysaccharides (LPS) and interleukin-4 (IL-4). In cultured macrophages, LXA4 inhibits LPS-induced inflammatory polarization, thereby decreasing the release of proinflammation cell factors (IL-1β, IL-6, TNF-α) and increasing the release of antiinflammation cytokines (IL-4 and IL-10). Notably, the inhibitory effect of LXA4 on inflammation macrophage polarisation was related to the downregulation of p-NF-κB p65 and IRF5 activity, thereby downregulating LPS-induced phenotypic and functional polarization of macrophage M1 via the FPR2/IRF5 signaling pathway. Moreover, LXA4 also promotes the IL-4-induced polarization of M2 macrophages by promoting the FPR2/IRF4 signaling pathway. Therefore, Lipoxin A4 regulates M1/M2 polarization of macrophages via FPR2-IRF pathway.

1. Background

Macrophages participate in the onset, progression and digestion of inflammatory events and other phases.At diverse phases of the inflammation reaction, macrophages display diverse phenotypes : classically activated inflammation (M1) or alternatively activated (M2) (Huen and Cantley 2017). Activated by TLRs and CKs like IFN-γ, M1 macrophages excrete IL-12 and iNOS, which have pro-inflammatory, chemotactic, and matrix degradation-inducing effects (Mosser and Edwards 2008). By comparison, M2 macrophages express arginase-1 (Arg-1) and IL-10, which play an antiinflammation, tissue repairment and angiogenic role (Meng et al. 2019). In the early stages of the inflammatory response, the polarisation of M1 macrophages and following inflammation factors releasing might be crucial factors for inducing inflammation damage (Jo et al. 2006; Tian et al. 2015). Therefore, diverse polarization phenotypes of macrophages decide the pivotal effects on the onset and progression of inflammatory events and sustaining of homoiostasis.

Lipoxygenins (LXs) are an endogenic lipidic mediator generated by arachidonic acid under the continuous catalysis of diverse lipoxidase, which could be classified into four types as per the molecule conformation (Levy and Serhan 2014), among which lipoxygenin A4 (LXA4) is produced by sequential oxidative catalysis. As a negative modulator of inflammatory events, LXA4 is an antiinflammation agent, facilitating the regression of inflammatory events, modulating immunofunction, and stimulating tissular and cellular injury repairment (Karra et al. 2015; Romano et al. 2015). In a variety of inflammation-associated illnesses, LXA4 suppresses the releasing of proinflammation factors and infiltrative ability of inflammation cells and facilitates the chemotaxis and recruiting of macrophages, thereby reinforcing the non-inflammation phagocytosis role (Zhang et al. 2007). Moreover, LXA4 suppresses ROS generation and prevents oxidation stress-mediated damage to tissues or macrophages (Zong et al. 2016). LXA4 is discovered to elevate the antioxygen enzyme activities in a variety of organs and to exert a protection effect on the restoration of the oxidant/antioxygen equilibrium (Chen et al. 2013).

Herein, we explored the protection roles of LXA4 in LPS or IL-4-stimulated macrophages (RAW264.7), and found that LXA4 may inhibit IRF5 activity, promote IRF4 activation, and down-regulate p-NF-κB p65 activity through FPR2-mediated inhibition. Thus, the polarisation of M1 macrophages was inhibited and the polarisation of M2 macrophages was promoted. This finding may shed light on the cellular and molecular mechanisms of relating diseases.

2. Materials And Methods

2.1 Cellular cultivation and therapy

The murine macrophages line (RAW264.7) was bought from the Cellular Bank of the CAS (China).RAW264.7 cells were kept in DMEM (Invitrogen, USA) added with 5% FBS (Invitrogen), 100 U/ml PNC and 100 μg/ml kyowamycin (Invitrogen). The RAW264.7 cells were inoculated into the maximaum medium involving 5% FBS at about 70% confluence in six-well cultivation dishes. Posterior to 24 hours, the maximaum medium was substituted by the intermediary without sera for 24 hours prior to the exposure to 1 μg/ml recombination LPS (Lot No. 5164948, Lianke Biotechnology, China), 20 ng/ml IL-4 (Lot No. 081449, PeproTech, USA) or LXA4 (10, 100 μmol/ml).

2.2 Cellular activity analysis and morphology analyses

Cellular activities were evaluated via the CCK-8 analysis through a 96-well cultivation dish. Briefly, RAW264.7 cells were inoculated and exposed to diverse levels of LXA4 with or without LPS for 24 hours. CCK-8 liquor (10 μl) was supplemented into all wells. Posterior to the cultivation for 2 hours under 37°C, the absorption at 450 nm was identified via the micro plate reading device (Thermo Fisher Scientic, MA, USA). For morphology analyses, the images of cells were captured via an Olympus microscopic system (Japan) under 20× magnification.

2.3 Flow cell technique

Cells were inoculated into 6-well plates at 1×10^6 each well and cultivated as previously described for 24 hours. Posterior to the supplementation of LXA4 for 0.5 hour, LPS was supplemented into all wells and the cells were cultivated for an extra 24 hours. The cells within the cultivation plate were subjected to digestion via trypsinase, and they were cleaned and subjected to resuspension within cold PBS at 1×10^6 cells/ml. The membranous protein CD86 and CD206 was identified via straight immunofluorescence dyeing. The cells were cultivated via APC-conjugated monoclone murine CD86 antisubstances or PE-conjugated monoclone murine CD206 antisubstances under RT without light for 0.5 hour. The cells were cleaned in PBS for 2 times and subjected to resuspension within 500uL of 1×PBS liquor. APC and PE-conjugated monoclone antisubstances with unrelated specificity utilized as NCs. Light scattering features of every specimen (10^5 cells) were studied via flow Dell technique (CytoFlex, USA).

2.4 ELISA for the identification of CK(cytokine) levels

A cellular suspension was accomplished via the inoculation of RAW264.7 cells in the log-growth stage into a 6-well cultivation dish. Posterior to 24 hours, cells were subjected to pre-treatment with diverse levels of LXA4 for 0.5 hour, and cultivated with/with no LPS (1 μg/ml) for 24 hours. The supernate was harvested, and the levels of IL-6、IL-10、TNF-α, and iNOS were identified via ELISA as per the supplier's specification. OD(optical delnsity) was measured at 450 nm with the micro plate reading device (Thermo Scientific).

2.5 qPCR analysis

Overall RNA was abstracted via the RNAeasy^{TM} animal RNA separation kit with a spin column as per the supplier's specification. The separated RNAs were converted to cDNA via reverse transcription via the PrimeScript^{TM} RT Master Mix (Perfect Real Time) as per the supplier's specification. The qPCR analyses were finished via TB Green^{TM} Premix Ex Taq^{TM} II (Tli RNaseH Plus) with the Applied Biosystems 7500 RealTime PCR System (USA). The amplification parametric results were 95°C for 0.5 minute, before 40 cycles of 95°C for 5 seconds and 60°C for 34 seconds, 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds. All specimens were studied independently for three times, and the comparative expression of mRNA was computed posterior to standardization to β-actin. All primer sequences used were presented by Table 1. The eventual outcomes were standardized and were described as the fold variation in contrast to the targeted gene/Actin.

Table 1
Primers for qPCR were used in this study

Gene	Sense (5′-3′)	Anti-sense (5′-3′)
IL-1β	TTTGAAGTTGACGGACCCCAA	CACAGCTTCTCCACAGCCACA
IL-4	ATCGGCATTTTGAACGAGGTCACA	CGAAGCACCTTGGAAGCCCTA
IL-6	TTCTTGGGACTGATGCTGGTG	CACAACTCTTTTCTCATTTCCACGA
IL-10	TTACCTGGTAGAAGTGATGCCC	GACACCTTGGTCTTGGAGCTTA
Arg-1	AGGAAAGCTGGTCTGCTGGAA	AGATGCTTCCAACTGCCAGAC
iNOS	GGGCTGTCACGGAGATCAATG	GCCCGGTACTCATTCTGCATG
Actin	CTGAGAGGGAAATCGTGCGT	CCACAGGATTCCATACCCAAGA

2.6 Western blot analysis

The overall protein from cultivated cells was harvested, and the protein level was identified via the BCA protein analysis kit (Thermo Fisher). The protein (20ug) from all specimens was isolated via SDS-PAGE and translocated onto a PVDF film (Bio-Rad, Hercules, CA, USA). Following blocking in 5% skim milk at room temperature for 1 h, the film was incubated with primary antibodies at 4℃ nightlong, and afterwards cultivated with the suitable HRP-conjugated second antisubstance (Thermo Fisher) for 1 h at room temperature. The visualization of the bound antisubstances were realized via chemiluminescent

identification on autoradiographic film. For macrophages biomarkers, anti-iNOS (1:1000, Cat# ab178945, Abcam, USA), anti-CD86 (1:1000, Cat# 76755, Cell Signaling Technology (CST), MA, USA), anti-Arginase-1 (1:1000, Cat# 93668, CST), anti-CD206 (1:1000, Cat# 24595, CST) antibodies were utilized. For NF-κB signal path, anti-NF-κB p65 (1:1000, Cat# 8242, CST), anti-p-NF-κB p65 (1:1000, Cat# 3033, CST) antibodies were utilized. For IRF signal path, anti-IRF5 (1:1000, Cat# BS60674, Bioworld, USA), anti-IRF4 (1:1000, Cat# BS2659, Bioworld), anti-FPR2 (1:1000, Cat# DF2719, Affinity, USA) antibodies were utilized. The bands were subjected to quantification via identifying the signal intensity through Image-Pro Plus 6.0 and standardizing them to the signal for the GAPDH (1:5000, Cat# AP0063, Bioworld) antisubstance.

2.7 Immunofluorescent dyeing

Macrophages were cultivated with/without treating in the six-well dishes involving glass slides and were afterwards cleaned in PBS and subjected to fixation in 4% PFA (Sigma-Aldrich, USA) under 4°C for 0.5 hour. Posterior to permeabilisation via 0.1% Triton X-100 for 600 seconds, the sample was cleaned in PBS and afterwards subjected to blockade in 10% FBS to realize the elimination of the nonspecificfluorescence.Immunofluorescent dyeing was completed via p-NF-κB p65 (1:1600), IRF4 (1:100), IRF5 (1:100), FPR2 (1:200), iNOS (1:500), CD206 (1:200), and F4/80 (1:200, Cat# ab6640, Abcam) as the first antibodies, and the cellular sample was cultivated via DyLight 488/594 labeled second antisubstances (Abcam). Immunocytochemistry specimens were evaluated in a semi-quantitative or quantitative manner by two separate researchers in a blinded way.

2.8. Statistics

Data were described as average ± SD. The entire statistic assay was finished via SPSS 16.0 (USA). A two-sided Student'st-test was employed to study the diversity between these 2 groups. One-way ANOVA and Bonferroni's posttest would be employed if there were over 2 groups. The P <0.05 had significance on statistics.

3. Results

3.1 Roles of LXA4 in the activation of RAW264.7 cells

Figure 1A displays the molecular structural formula of LXA4. For the purpose of identifying if LXA4 affects the activity of RAW264.7 cells, a CCK-8 analysis was completed after 24 hours of LXA4 action at different concentrations (10 nM to 500 nM), and we found that merely LXA4 failed to trigger any identifiable cell toxicity (Fig. 1B). Therefore, in subsequent experiments, RAW264.7 cells were pretreated with LXA4 levels between 10 and 500 nM for 0.5 hour and then cultivated via LPS (1 μg/ml) for 24 hours. We found that the inhibitory roles of LPS in RAW264.7 cellular activity could be alleviated to the greatest extent when the concentration of LXA4 was 200nM, and the results of IL-4 incubation (20 ng/ml) were consistent with this. In order to study the roles of LXA4 in macrophages cell stimulation, RAW264.7 cells were challenged with and without LXA4 at 200nM concentration, respectively, and morphological

changes were observed. As presented by Figure 1C, no remarkable variation existed in cell morphology in the two groups, so we finally selected LXA4 at 200nM concentration for further experiments.

To explore the roles of LXA4 in LPS-induced M1 macrophages polarisation in vitro and IL-4-induced polarization of M2 macrophages in vitro, we observed the morphological changes of macrophages RAW264.7 after the combined effect of LXA4 (200 nM) and LPS. The results of light microscopy and immunofluorescence chemical staining (Fig. 1F) showed that resting macrophages RAW264.7 cells were round or oval, densely packed, well adhered to the wall, with few protrusions. However, the LPS-triggered morphology variations of RAW264.7 cells were diminished posterior to the exposure to 200 nM LXA4. In contrast, the morphology of IL-4-induced RAW264.7 cells was further rounded after administration of LXA4. These results suggest that 200 nM LXA4 can inhibit LPS-induced morphological changes in macrophages and promote IL-4-induced morphological changes in macrophages.

3.2 Effect of LXA4 on LPS-triggered inflammation cell factor generation in RAW264.7 cells

RAW264.7 macrophages were pretreated with LXA4 for 0.5 hour and then co-cultivated via LPS for 24 hours. To determine the roles of LXA4 in LPS-triggered inflammatory mediators, the effect of LXA4 on inflammatory cytokine expression was identified via ELISA and qRT-PCR. The production of IL-1β, IL-6, TNF-α, IL-4 and IL-10 induced by LXA4 was identified by qRT-PCR. The results revealed that M1 pro-inflammatory cytokine levels were remarkably elevated posterior to LPS exposure, as evidenced by the generation of IL-1β and TNF-α (Fig. 2A-2C), whereas the content of antiinflammation cell factors (IL-10 and IL-4) was remarkably reduced (Fig. 2D-2E). Consistent with the quantitative reverse transcription-polymerase chain reaction results, ELISA results revealed that the content of pro-inflammatory cytokines (IL-6 and TNF-α) was significantly elevated, while the content of anti-inflammatory cytokines (IL-10) was significantly reduced in the LPS-induced cells (Fig. 2F-2H). These results suggest that LXA4 plays an antiinflammation role in LPS-exposed RAW264.7 macrophages.

3.3 LXA4 inhibits M1 macrophage polarization in vitro

CD86 is an M1 subtype macrophage-specific membrane surface protein that serves as a marker of M1 polarization. To investigate the effect of LXA4 on RAW246.7 macrophage polarization, we detected the expression of CD86 by flow cytometry. CD86 protein expression was significantly up-regulated after LPS administration, while CD86 protein expression was significantly down-regulated after LXA4 pretreatment (Fig. 3E). To further clarify whether LXA4 could inhibit the shift of RAW264.7 cells to M1 phenotype, we examined the expression of iNOS by ELISA and qRT-PCR, and the outcomes revealed that iNOS was significantly increased in the LPS-treated group, while the expression of iNOS was inhibited after LXA4 administration (Fig. 3A-3B). Western blot results revealed that after the administration of LPS, the expressing of iNOS and CD86 expression were remarkably elevated, while in the LXA4 pre-treating group, the expressing of iNOS and CD86 was inhibited (Fig. 3C). Later, we detected iNOS expression using immunofluorescence. Compared with the control group, iNOS immunoreactivity was enhanced after LPS

administration, while iNOS immunoreactivity was reduced after LXA4 pretreatment (Fig. 3D). The above results suggest that LXA4 can inhibit the LPS-induced shift of RAW264.7 macrophages to M1 phenotype.

3.4 LXA4 downregulated the activity of NF-KB 65 and IRF5 in vitro

Subsequently, our team explored the mechanism where LXA4 suppresses tthe polarisation of M1 macrophages in vitro. In LPS-exposed macrophages, western blot and immunocytochemistry dyeing revealed that LXA4 decreased the expressing of FPR2 and IRF5 in LPS-challenged macrophages (Fig. 4A-4C), unveiling that IRF5 was involved in LXA4-mediated suppression of polarized M1 macrophages. In addition, increased p-P65 expression was observed by western blot results (Fig. 4D). Immunocytochemistry dyeing revealed that the expression of p-NF-κB p65 was remarkable within macrophage nuclei (Fig. 4E). After lipoxin treatment, p-NF-κB p65 expression was decreased and intranuclear localization was decreased. Thus, LXA4 acts as an inhibitor of the polarisation of M1 macrophages via targeting the typical NF-κB signal. Collectively, those discoveries reveal that LXA4 suppresses the polarizing effects of p-NF-κB p65 and IRF5 on M1 macrophages in vitro.

3.5 LXA4 promote the polarisation of M2 macrophage in vitro

As aforesaid, macrophages are classified into M1 and M2. Given that LXA4 plays a suppressive role in M1 macrophages, our team afterwards explored whether LXA4 could affect the M2 macrophages polarisation. Our team therefore induced M2 macrophages via IL-4 (20 ng/ml) for 48 hours and examined the expressing of the specific phenotype biomarkers Arg-1 and CD206 in M2 macrophages. We detected the expression of CD206 via flow cell technique to identify the roles of LXA4 in M2 macrophages. As presented by Fig. 5A, in contrast to the controls, CD206 protein content was elevated within the IL-4 group, and the expressing of CD206 was remarkably greater in contrast to the IL-4 group posterior to LXA4 pretreatment. In addition, the mRNA expressing of the M2 biomarker (Arg-1) was also enhanced in macrophages treated with IL-4 in contrast to the controls, and the results of ELISA were consistent with it (Fig. 5B-5C). To further clarify whether LXA4 facilitated the M2 macrophages polarisation, our team detected the expression of CD206 by immunofluorescence, and the immune response to CD206 was enhanced in the IL-4 group in contrast to the controls, and LXA4 pretreatment further enhanced the immune response to CD206. Also, the Western blot results verified the above findings (Fig. 5D-5E).

To further elucidate the mechanism of LXA4-induced polarization of M2 macrophages, our team explored the activity of FPR2 and IRF4, which were found to be involved in the modulation of M2 macrophages (Satoh et al. 2010). We found that the expression of FPR2 was increased in IL-4-exposed macrophages, as was the expressing of IRF4 (Fig. 6A-6C). Thus, those discoveries reveal that FPR2 and IRF4 might be vital for promoting LXA4-mediated polarization of M2 macrophages. LXA4 might have an effect on

promoting the polarisation of M2 macrophages by targeting enhanced IRF4 activity through FPR2-mediated enhancement.

4. Disscusion

Macrophages are pivotal inflammation cells during inflammation damage (Jang and Rabb 2015; Humphreys 2018; Calle and Hotter 2020). Early assays on animal models of M1 depletion and replenishment showed that macrophages are vital for inflammation damage (Jang and Rabb 2015). Interestingly, it was subsequently shown that macrophages not only cause inflammatory damage but promote the repairment and mitigation of nephropathy (Holdsworth and Tipping 1985). Those biology roles of macrophages in inflammatory damage are associated with the polarisation of proinflammation M1 or antiinflammation M2 (Ricardo et al. 2008; Mily et al. 2020). The polarisation of those type 2 macrophages participates in the inflammation damage and repairment processes of kidney tissue, separately (Dellepiane et al. 2020). On the foundation of those results, our team hypothesized that the protection roles of LXA4 in renal damage might be related to the modulation of polarized macrophages. In the present research, M1 macrophages were the predominant inflammation cells facilitating the production of inflammation factors (Ko et al. 2008). LXA4 exhibits antiinflammation activities via suppressing the polarisation of M1 macrophages and inflammatory factor releasing. IL-4 or IL-13 stimulates the formation of M2 macrophages, which mainly exert anti-inflammatory effects, while our study also showed that LXA4 promotes the production of M2 macrophages even more. Previous studies have demonstrated the protective effect of LXA4 against inflammation in certain diseases, such as neuromyelitis optica spectral diseases (Wang et al. 2019), obesity adipose inflammation and cerebrovascular endothelial cell dysfunction (Börgeson et al. 2015; Liu et al. 2019). Therefore, the above results support that the antiinflammation potency of LXA4 might be related to the suppression of the polarisation of M1 macrophages and the promotion of M2 macrophages production.

M1/M2 represents only a simplification model of the 2 inflammation reactions (Tang and Le 2016). Representatively, the stimulated M1 macrophages could be triggered in vitro via LPS to express a variety of proinflammation factors like IL-1β, iNOS, and CD16. By comparison, IL-4 is discovered to be able to trigger a stimulated M2 macrophages featured by the expressing of antiinflammation and neurotrophy mediating factors like IL-10, Arg-1, and CD206 (Wu et al. 2018; Zhang et al. 2018). Therefore, timely conversion of the M1-type macrophages to the M2-type macrophages is considered a promising therapeutic regimen of related diseases.

The present research highlighted the exploration of the effects of LXA4 on regulating macrophage polarization and its possible mechanisms. Herein, our team discovered that LPS-triggered M1 polarization resulted in increased IL-1β, IL-6, CD86, iNOS mRNA and protein expressing and strong iNOS dyeing, whereas LXA4 reversed the polarisation of M1 macrophages and potently elevated the expressing of M2 macrophage biomarkers, such as IL-4, IL-10, CD206 and Arg-1. In conclusion, those outcomes suggest that LXA4 inhibit the shift of LPS-stimulated RAW264.7 macrophages to M1-type polarization

and further promote the shift of IL-4-activated macrophages to M2-type, which correlates with enhanced anti-inflammatory cytokine production.

The molecule-level causal link of the polarisation of macrophages remains elusive. Studies recently have revealed that the NF-κB is a pivotal modulator of inflammatory events and resolution via modulating the polarisation of macrophages (Zheng et al. 2020). NF-κB is a pleiotropy TF comprising 5 sub-groups: RelA (p65), NF-κB1 (p105/p50), NF-κB2 (p100/p52), c-Rel, and RelB (Holdsworth and Neale 1984). Canonical signal transmission is stimulated via the p65, p50 and c-Rel sub-groups, while the stimulation of the non-canonical signal path is under the mediation of relB and p100/p52. Researches have revealed that the p-NF-κB p65 participates in the polarisation of M1 macrophages (Lowe et al. 2014). Our results also support the involvement of p-NF-κB p65 in the polarisation of M1 macrophages, which is related to the inhibition of LXA4-induced M1 macrophage polarization.

Apart from NF-κB signal transmission, IRF5 is found to be related to the M1 macrophages polarisation (Krausgruber et al. 2011). IRF5, a component of the IFN-regulation factor family, is able to trigger the stimulation genes that encode type I IFN and inflammation cell factors, such as TNF-α, IL-6 and IL-12 (Krausgruber et al. 2011). Those Cytokines participate in the modulation of immunocytes and are responsible for the mediation of inflammation damage in various tissue, such as renal tissues (Awad et al. 2015). Herein, elevated IRF5 expressing in inflammation renal tissue and LPS-exposed macrophages revealed an underlying effect on the triggering of the polarisation of M1 macrophages. Posterior to LXA4 exposure, the IRF5 activities were regulated downward and hence induced the suppression of the polarisation of M1 macrophages and the decrease of inflammation damage. For that reason, such proof revealed that IRF5 might be a pivotal modulator of the polarisation of M1 macrophages and might be involved in the antiinflammation roles of LXA4 in inflammatory damage.

Interestingly, unlike the polarisation of M1 macrophages, LXA4 has a promotional effect on M2 macrophage polarization, as evidenced by an increase in the proportion of M2 cells and Arg-1 levels in vitro. In addition, upregulating the expressing of IRF4 might be one of the causal links where LXA4 promotes M2 macrophage polarization. Previous studies found that IRF4 was found to induce the polarisation of M2 macrophages with JMJD3 treatment (Gaikwad and Heneka 2013). Our results also support that IRF4 is involved in the triggering of the polarisation of M2 macrophages and that LXA4-mediated promotion might be achieved via upregulation of IRF4 activity.

In vivo, the biological effects of LXA4 are mediated through its receptor FPR2, a G-protein-coupled membrane receptor that is highly expressed on macrophages and can bind to its ligand LXA4 through the lipid-binding domain and then transfer extracellular signals into effector cells through the protein-binding domain to elicit the corresponding biological effects (Pirault and Bäck 2018). It was shown that LXA4 mediates the M2-type macrophage transformation and promotes connective tissue repair through FPR2 (Dakin et al. 2012).We showed that LXA4 may be mediated through the FPR2 pathway to inhibit M1-type macrophage polarization and reduce inflammatory factor synthesis and release, while enhancing macrophage polarization toward the M2 type and producing anti-inflammatory effects.

Holistically, the present research is the first to show that LXA4 may be mediated via FPR2 to inhibit IRF5 activity, promote IRF4 activation, and lead to down-regulation of p-NF-κB p65 activity, thereby suppressing the polarisation of M1 macrophages and further facilitating the polarisation of M2 macrophages, which may, to some extent, elucidate the cellular and molecular mechanisms of organ damage and repair, and offer a novel strategy for the prevention and therapy of inflammatory injury in the future. This may, to some extent, elucidate the cellular and molecular mechanisms of inflammatory injury and repair, and offer a novel strategy for the prevention and therapy of inflammatory injury in the future, and offer a theory basis for drug development and therapy of organ protection during inflammatory injury.

Abbreviations

arginase-1 (Arg-1); bicinchoninic acid (BCA); cell counting kit-8 (CCK-8); Dulbecco's Modified Eagle Medium (DMEM); fetal bovine serum (FBS); horseradish peroxidase (HRP); interferon-γ (IFN-γ); inducible nitric oxide synthase (iNOS); interleukin-4 (IL-4); lipoxin A4 (LXA4); lipopolysaccharides (LPS); Lipoxygenins (LXs); polyvinylidene difluoride (PVDF); reactive oxygen species (ROS); Toll-like receptor (TLR)

Declarations

Funding

This study was sponsored by the Natural Science Foundation of Zhejiang Province, China (LY21H050005) and the Natural Science Foundation of China (81772264). Additionally, this study was also supported by the Wenzhou Municipal Science and Technology Project (Y2020025) and the Wenzhou Municipal Science and Technology Bureau (Y2020274).

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Jixiang Yuan], [Feihong Lin] and [Lichen Chen]. The first draft of the manuscript was written by [Jixiang Yuan] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

All data is available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

This study was sponsored by the Natural Science Foundation of Zhejiang Province, China (LY21H050005) and the Natural Science Foundation of China (81772264). Additionally, this study was also supported by the Wenzhou Municipal Science and Technology Project (Y2020025) and the Wenzhou Municipal Science and Technology Bureau (Y2020274).

References

Awad AS, You H, Gao T et al (2015) Macrophage-derived tumor necrosis factor-α mediates diabetic renal injury. Kidney Int 88:722-733. doi:10.1038/ki.2015.162

Börgeson E, Johnson AM, Lee YS et al (2015) Lipoxin A4 Attenuates Obesity-Induced Adipose Inflammation and Associated Liver and Kidney Disease. Cell Metab 22:125-137. doi:10.1016/j.cmet.2015.05.003

Calle P, Hotter G (2020) Macrophage Phenotype and Fibrosis in Diabetic Nephropathy. Int J Mol Sci 21. doi:10.3390/ijms21082806

Chen XQ, Wu SH, Zhou Y, Tang YR (2013) Lipoxin A4-induced heme oxygenase-1 protects cardiomyocytes against hypoxia/reoxygenation injury via p38 MAPK activation and Nrf2/ARE complex. PLoS One 8:e67120. doi:10.1371/journal.pone.0067120

Dakin SG, Werling D, Hibbert A et al (2012) Macrophage sub-populations and the lipoxin A4 receptor implicate active inflammation during equine tendon repair. PLoS One 7:e32333. doi:10.1371/journal.pone.0032333

Dellepiane S, Leventhal JS, Cravedi P (2020) T Cells and Acute Kidney Injury: A Two-Way Relationship. Front Immunol 11:1546. doi:10.3389/fimmu.2020.01546

Gaikwad SM, Heneka MT (2013) Studying M1 and M2 states in adult microglia. Methods Mol Biol 1041:185-197. doi:10.1007/978-1-62703-520-0_18

Holdsworth SR, Neale TJ (1984) Macrophage-induced glomerular injury. Cell transfer studies in passive autologous antiglomerular basement membrane antibody-initiated experimental glomerulonephritis. Lab Invest 51:172-180.

Holdsworth SR, Tipping PG (1985) Macrophage-induced glomerular fibrin deposition in experimental glomerulonephritis in the rabbit. J Clin Invest 76:1367-1374. doi:10.1172/JCI112112

Huen SC, Cantley LG (2017) Macrophages in Renal Injury and Repair. Annu Rev Physiol 79:449-469. doi:10.1146/annurev-physiol-022516-034219

Humphreys BD (2018) Mechanisms of Renal Fibrosis. Annu Rev Physiol 80:309-326. doi:10.1146/annurev-physiol-022516-034227

Italiani P, Boraschi D (2014) From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation. Front Immunol 5:514. doi:10.3389/fimmu.2014.00514

Jang HR, Rabb H (2015) Immune cells in experimental acute kidney injury. Nat Rev Nephrol 11:88-101. doi:10.1038/nrneph.2014.180

Jo SK, Sung SA, Cho WY, Go KJ, Kim HK (2006) Macrophages contribute to the initiation of ischaemic acute renal failure in rats. Nephrol Dial Transplant 21:1231-1239. doi:10.1093/ndt/gfk047

Karra L, Haworth O, Priluck R, Levy BD, Levi-Schaffer F (2015) Lipoxin B promotes the resolution of allergic inflammation in the upper and lower airways of mice. Mucosal Immunol 8:852-862. doi:10.1038/mi.2014.116

Ko GJ, Boo CS, Jo SK, Cho WY, Kim HK (2008) Macrophages contribute to the development of renal fibrosis following ischaemia/reperfusion-induced acute kidney injury. Nephrol Dial Transplant 23:842-852. doi:10.1093/ndt/gfm694

Krausgruber T, Blazek K, Smallie T et al (2011) IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. Nat Immunol 12:231-238. doi:10.1038/ni.1990

Levy BD, Serhan CN (2014) Resolution of acute inflammation in the lung. Annu Rev Physiol 76:467-492. doi:10.1146/annurev-physiol-021113-170408

Liu L, Zhang P, Zhang Z et al (2019) LXA4 ameliorates cerebrovascular endothelial dysfunction by reducing acute inflammation after subarachnoid hemorrhage in rats. Neuroscience 408:105-114. doi:10.1016/j.neuroscience.2019.03.038

Lowe JM, Menendez D, Bushel PR et al (2014) p53 and NF-κB coregulate proinflammatory gene responses in human macrophages. Cancer Res 74:2182-2192. doi:10.1158/0008-5472.CAN-13-1070

Meng XM, Mak TS, Lan HY (2019) Macrophages in Renal Fibrosis. Adv Exp Med Biol 1165:285-303. doi:10.1007/978-981-13-8871-2_13

Mily A, Kalsum S, Loreti MG et al (2020) Polarization of M1 and M2 Human Monocyte-Derived Cells and Analysis with Flow Cytometry upon Mycobacterium tuberculosis Infection. J Vis Exp . doi:10.3791/61807

Mosser DM, Edwards JP (2008) Exploring the full spectrum of macrophage activation. Nat Rev Immunol 8:958-969. doi:10.1038/nri2448

Pirault J, Bäck M (2018) Lipoxin and Resolvin Receptors Transducing the Resolution of Inflammation in Cardiovascular Disease. Front Pharmacol 9:1273. doi:10.3389/fphar.2018.01273

Ricardo SD, van Goor H, Eddy AA (2008) Macrophage diversity in renal injury and repair. J Clin Invest 118:3522-3530. doi:10.1172/JCI36150

Romano M, Cianci E, Simiele F, Recchiuti A (2015) Lipoxins and aspirin-triggered lipoxins in resolution of inflammation. Eur J Pharmacol 760:49-63. doi:10.1016/j.ejphar.2015.03.083

Satoh T, Takeuchi O, Vandenbon A et al (2010) The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. Nat Immunol 11:936-944. doi:10.1038/ni.1920

Tang Y, Le W (2016) Differential Roles of M1 and M2 Microglia in Neurodegenerative Diseases. Mol Neurobiol 53:1181-1194. doi:10.1007/s12035-014-9070-5

Tian S, Zhang L, Tang J, Guo X, Dong K, Chen SY (2015) HMGB1 exacerbates renal tubulointerstitial fibrosis through facilitating M1 macrophage phenotype at the early stage of obstructive injury. Am J Physiol Renal Physiol 308:F69-75. doi:10.1152/ajprenal.00484.2014

Wang X, Jiao W, Lin M et al (2019) Resolution of inflammation in neuromyelitis optica spectrum disorders. Mult Scler Relat Disord 27:34-41. doi:10.1016/j.msard.2018.09.040

Wang Y, Harris DC (2011) Macrophages in renal disease. J Am Soc Nephrol 22:21-27. doi:10.1681/ASN.2010030269

Wu F, Luo T, Mei Y et al (2018) Simvastatin alters M1/M2 polarization of murine BV2 microglia via Notch signaling. J Neuroimmunol 316:56-64. doi:10.1016/j.jneuroim.2017.12.010

Wu L, Li HH, Wu Q et al (2015) Lipoxin A4 Activates Nrf2 Pathway and Ameliorates Cell Damage in Cultured Cortical Astrocytes Exposed to Oxygen-Glucose Deprivation/Reperfusion Insults. J Mol Neurosci 56:848-857. doi:10.1007/s12031-015-0525-6

Zhang B, Wei YZ, Wang GQ, Li DD, Shi JS, Zhang F (2018) Targeting MAPK Pathways by Naringenin Modulates Microglia M1/M2 Polarization in Lipopolysaccharide-Stimulated Cultures. Front Cell Neurosci 12:531. doi:10.3389/fncel.2018.00531

Zhang L, Wu P, Jin SW et al (2007) Lipoxin A4 negatively regulates lipopolysaccharide-induced differentiation of RAW264.7 murine macrophages into dendritic-like cells. Chin Med J (Engl) 120:981-987.

Zhao Q, Shao L, Hu X et al (2013) Lipoxin a4 preconditioning and postconditioning protect myocardial ischemia/reperfusion injury in rats. Mediators Inflamm 2013:231351. doi:10.1155/2013/231351

Zheng ZC, Zhu W, Lei L, Liu XQ, Wu YG (2020) Wogonin Ameliorates Renal Inflammation and Fibrosis by Inhibiting NF-κB and TGF-β1/Smad3 Signaling Pathways in Diabetic Nephropathy. Drug Des Devel Ther 14:4135-4148. doi:10.2147/DDDT.S274256

Zhou XY, Wu P, Zhang L et al (2007) Effects of lipoxin A(4) on lipopolysaccharide induced proliferation and reactive oxygen species production in RAW264.7 macrophages through modulation of G-CSF secretion. Inflamm Res 56:324-333. doi:10.1007/s00011-007-7012-7

Zhu JJ, Yu BY, Fu CC et al (2020) LXA4 protects against hypoxic-ischemic damage in neonatal rats by reducing the inflammatory response via the IκB/NF-κB pathway. Int Immunopharmacol 89:107095. doi:10.1016/j.intimp.2020.107095

Zong H, Li X, Lin H, Hou C, Ma F (2017) Lipoxin A4 pretreatment mitigates skeletal muscle ischemia-reperfusion injury in rats. Am J Transl Res 9:1139-1150.

Zong L, Li J, Chen X et al (2016) Lipoxin A4 Attenuates Cell Invasion by Inhibiting ROS/ERK/MMP Pathway in Pancreatic Cancer. Oxid Med Cell Longev 2016:6815727. doi:10.1155/2016/6815727

Figures

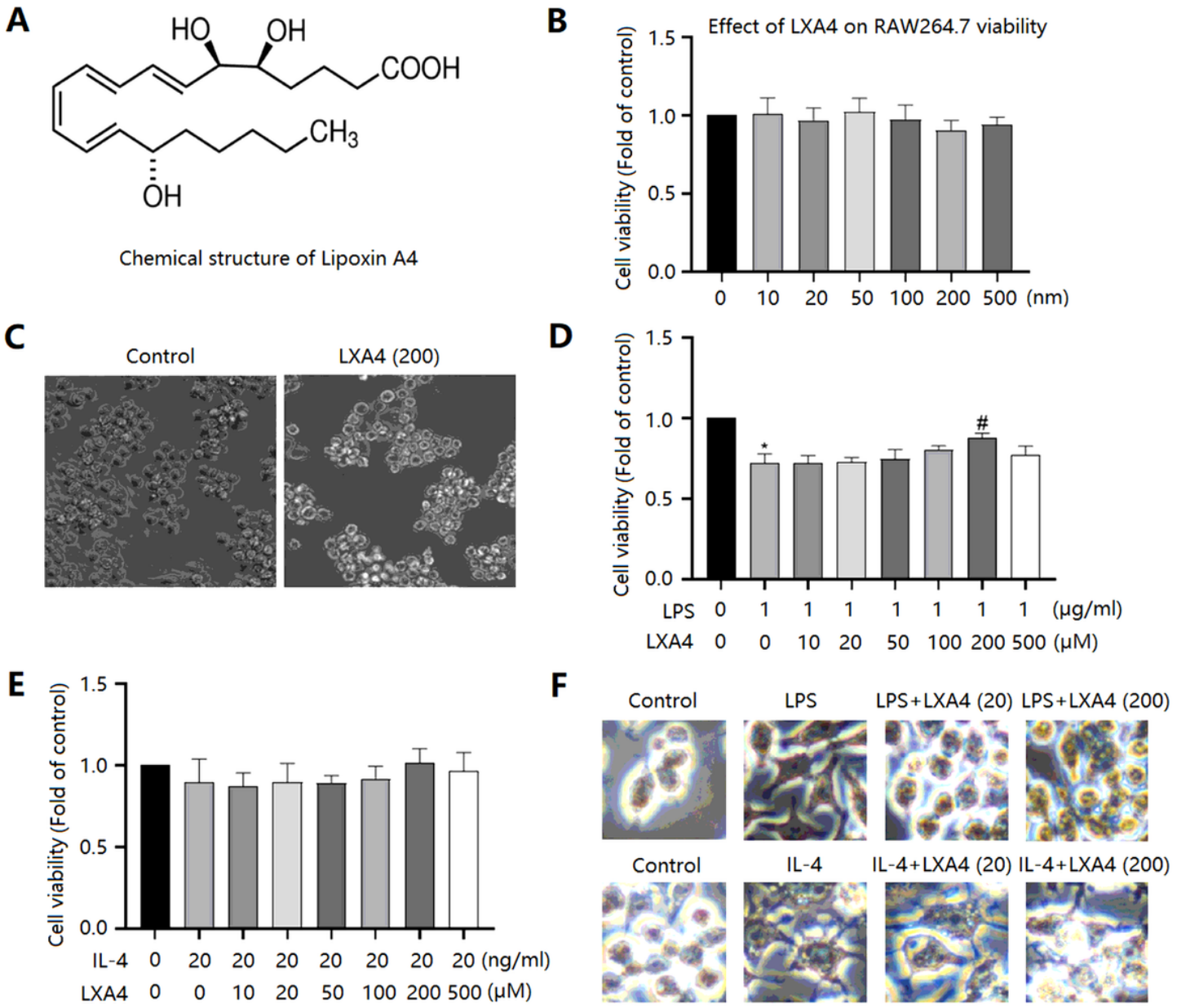

Figure 1

Effect of LXA4 on the activation of RAW264.7 macrophages stimulated by LPS or IL-4. (A) Molecular structural formula of LXA4; (B) Effect of different concentrations of LXA4 alone on cell viability; (C) Light microscopic observation of cell morphology; (D) RAW264.7 macrophages were pretreated with different concentrations of LXA4 for 30 min, incubated with or without the addition of LPS (1 μg/ml) for 24 h, and cell viability was determined by the CCK-8 method; (E) The cell viability was determined by pretreatment of RAW264.7 macrophages with different concentrations of LXA4 for 30 min, incubated with or without IL-4 (20 ng/ml) for 48 h, and cell survival was determined by the CCK-8 method; (F) Cell morphology was observed by light microscopy under different modes of intervention. Data are presented as the means ± SEM from three independent experiments performed in triplicate. #P < 0.05 compared with the control group.

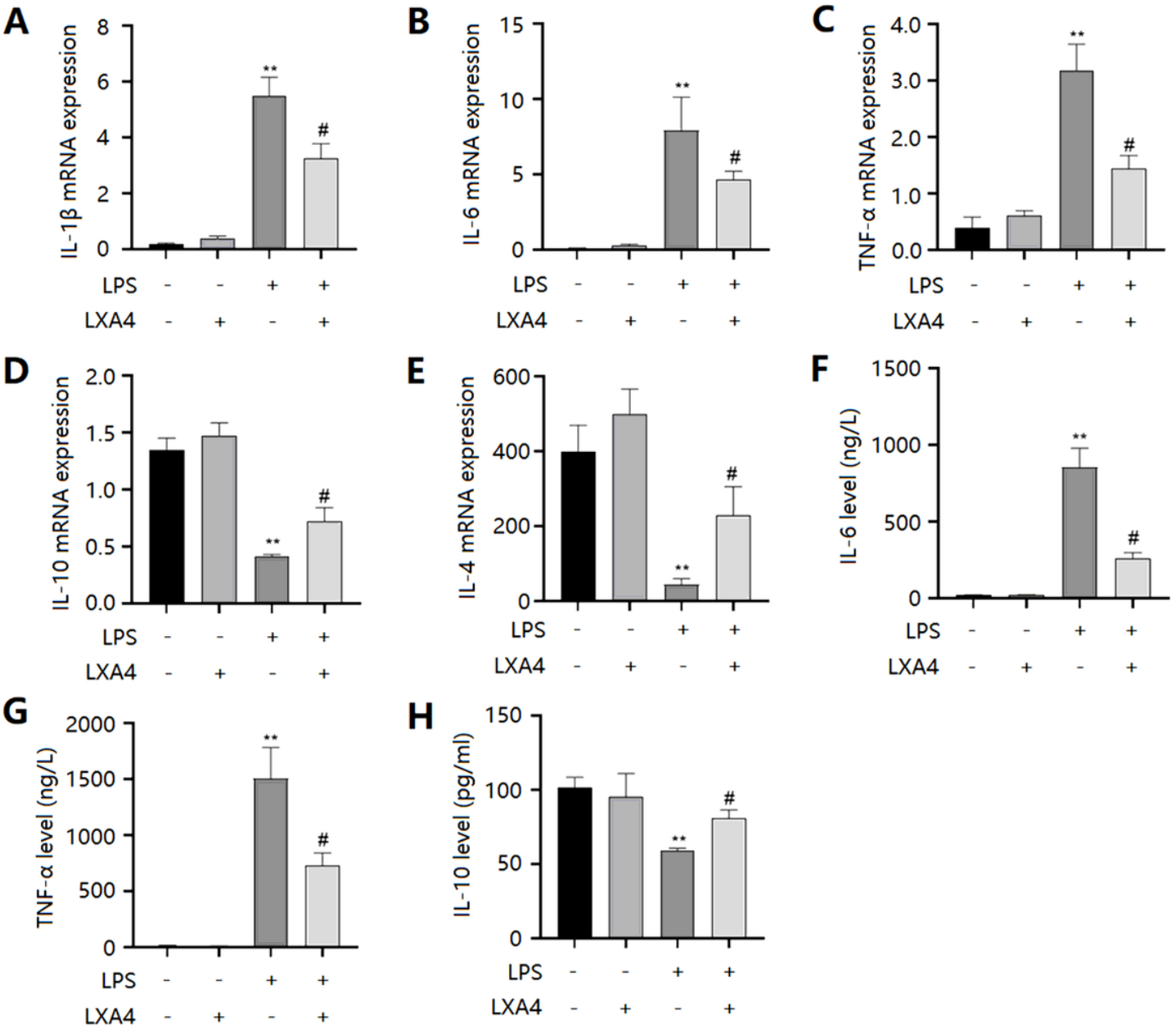

Figure 2

Effect of LXA4 on LPS-induced inflammatory cytokine production. RAW264.7 macrophages were pretreated with LXA4 for 30 min and incubated with or without LPS (1 μg/ml) for 24 h. (A-E) Cells were collected and real-time RT-PCR was performed to detect IL-1β, IL-6, TNF-α, IL-10 and IL-4 gene expression; (F-H) ELISA was performed to detect IL-6, TNF-α and IL-10 levels in the supernatant. Data are presented as the means SEM of three independent experiments. **P < 0.05 compared with the control group; #P < 0.05 compared with the LPS-treated group.

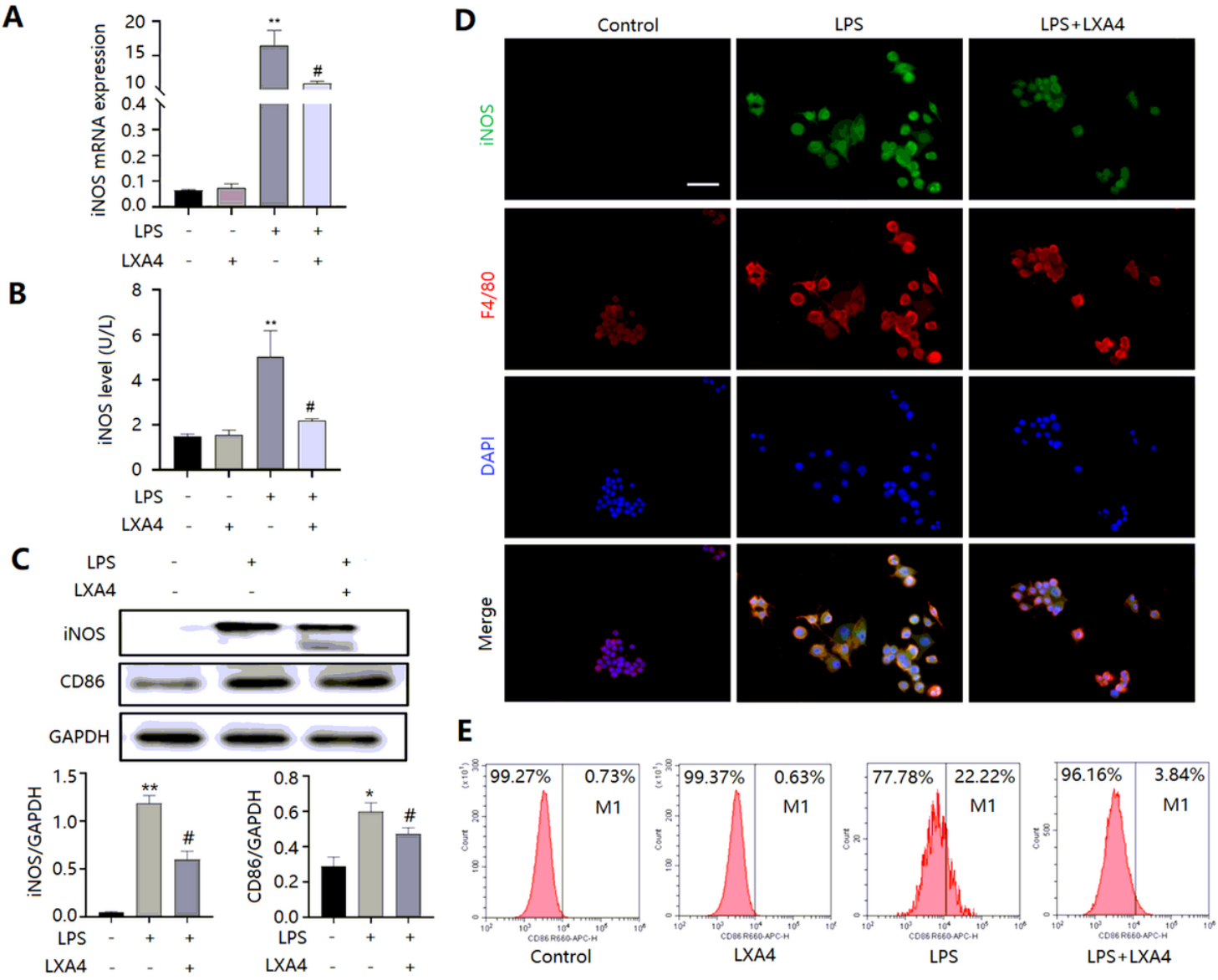

Figure 3

LXA4 inhibits M1 macrophage polarization in vitro. (A) Cells were collected and real-time RT-PCR was performed to detect iNOS gene expression; (B) ELISA was performed to detect iNOS levels in the supernatant; (C) Cultured cells were collected and detected by western blot for CD86, iNOS protein expression; (D) observation of cultured cells by immunostaining with anti-iNOS (M1 marker, green) and anti-F4/80 (Macrophage marker, red) antibodies. Nuclei were stained for DAPI (blue); Bar = 50 μm. (E) Study of the effect of LXA4 on LPS-induced CD86 protein expression in macrophages. Cells were pretreated with 200 nM LXA4 for 30 min and incubated with or without the addition of LPS (1 μg/ml) for 24 h. CD86 (M1) protein expression was detected by flow cytometry. Data are presented as the means SEM of three independent experiments. *P < 0.05, **P < 0.05 compared with the control group; #P< 0.05 compared with the LPS-treated group.

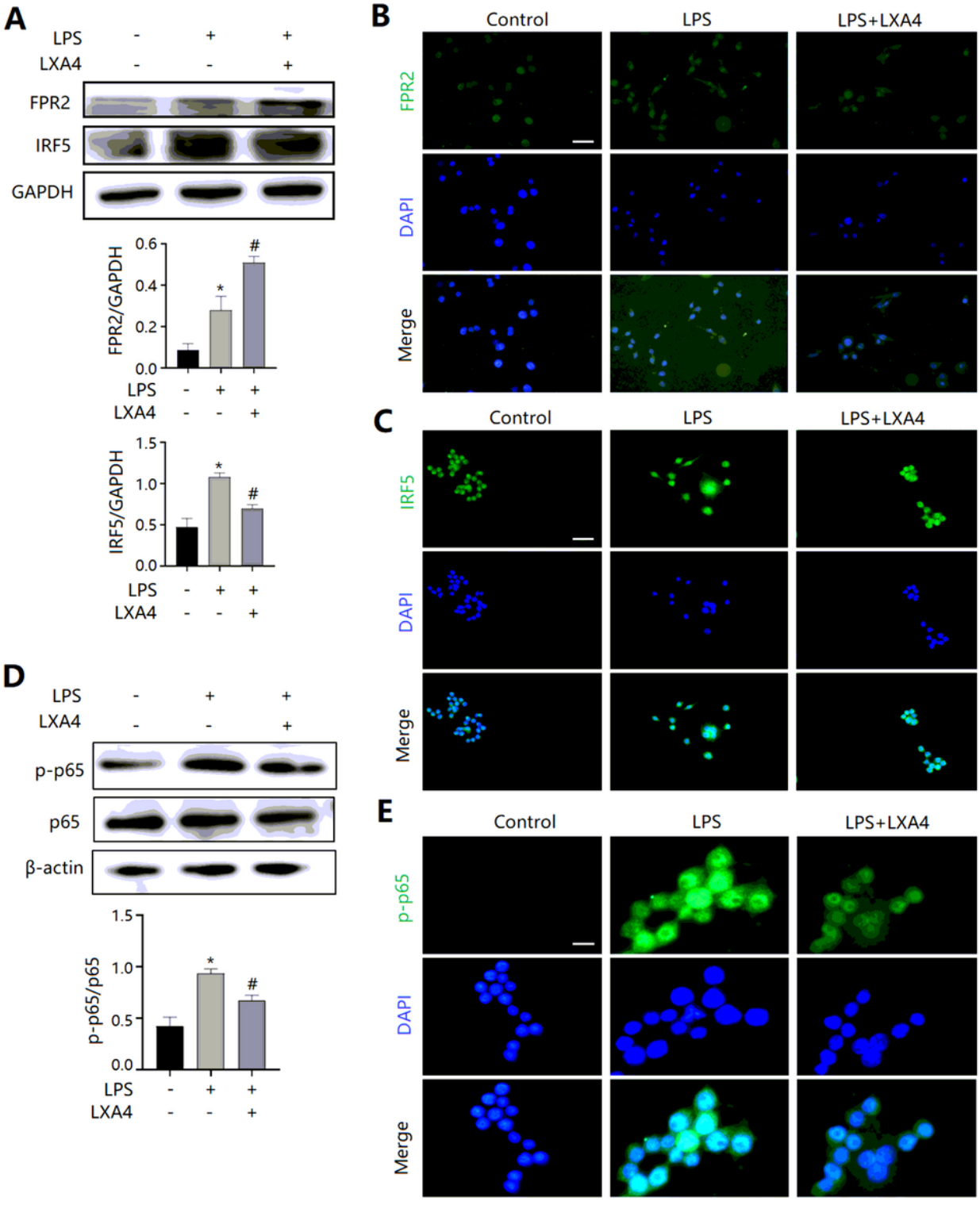

Figure 4

LXA4 down-regulates the activities of IRF5 and NF-κB 65 in vitro; (A) Cells were pretreated with LXA4 for 30 min with or without LPS (1 μg/ml) for 24 h. Cultured cells were collected and detected by western blot for FPR2, and protein expression of IRF5; (B) Immunofluorescence staining showed upregulation of macrophage FPR2 expression after LPS treatment. LXA4 promotes LPS-mediated induction. Bar = 50 μm. (C) LXA4 decreased LPS-induced IRF5 overexpression in macrophages; Bar = 50 μm. (D) Western blot

results showed that LXA4 inhibited LPS-induced increase in macrophage p-NF-κB p65 expression level without significant change in p65 level; (E) Immunofluorescence staining showed upregulation of macrophage p-NF-κB p65 expression after LPS treatment. Bar = 50 μm. Data are presented as the means ± SEM of three independent experiments. *P < 0.05 compared with the control group; #P < 0.05 compared with the LPS treated group.

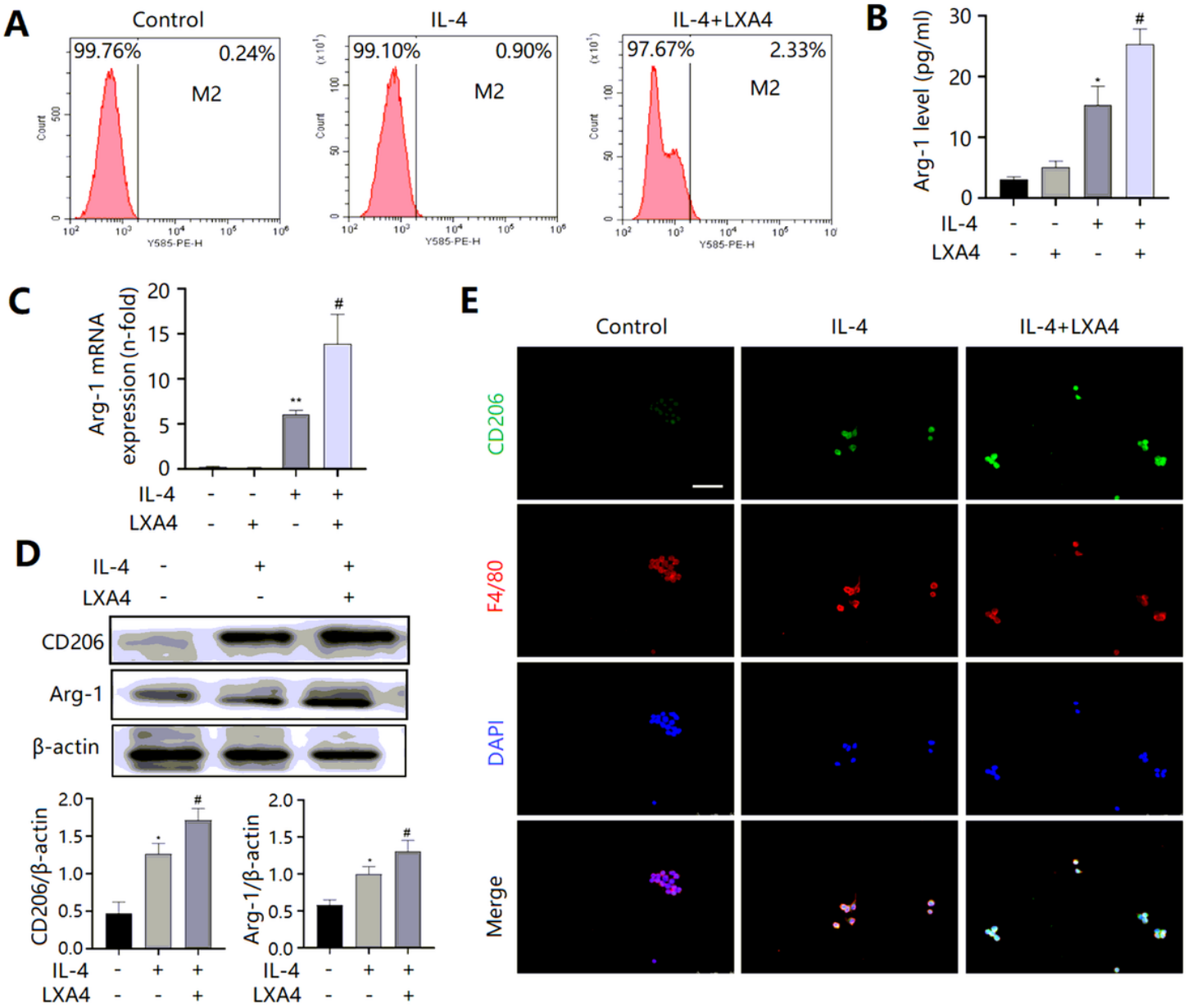

Figure 5

LXA4 promotes M2 macrophage polarization in vivo. (A) The effect of LXA4 on IL-4-induced CD206 protein expression in macrophages was investigated. The cells were pretreated with 200 nM LXA4 for 30 min and incubated with or without IL-4 (20 ng/ml) for 48 h. CD206 (M2) protein expression was detected by flow cytometry; (B) Real-time RT-PCR for Arg-1 gene expression; (C) ELISA for Arg-1 levels in supernatants; (D) Cultured cells were collected and protein expression of CD206, Arg-1, FPR2, and IRF4 was detected by western blot; (E) Cultured cells were visualized by immunostaining with anti-CD206 (M2

marker, green) and anti-F4/80 (macrophage marker, red) antibodies. Nuclei were stained with DAPI (blue); Immunofluorescence staining showed that CD206 expression was upregulated in macrophages after IL-4 treatment, and further enhanced after administration of LXA4. Bar = 50 μm. Data are presented as the means SEM of three independent experiments. *P < 0.05, **P < 0.05 compared with the control group; #P < 0.05 compared with the LPS-treated group.

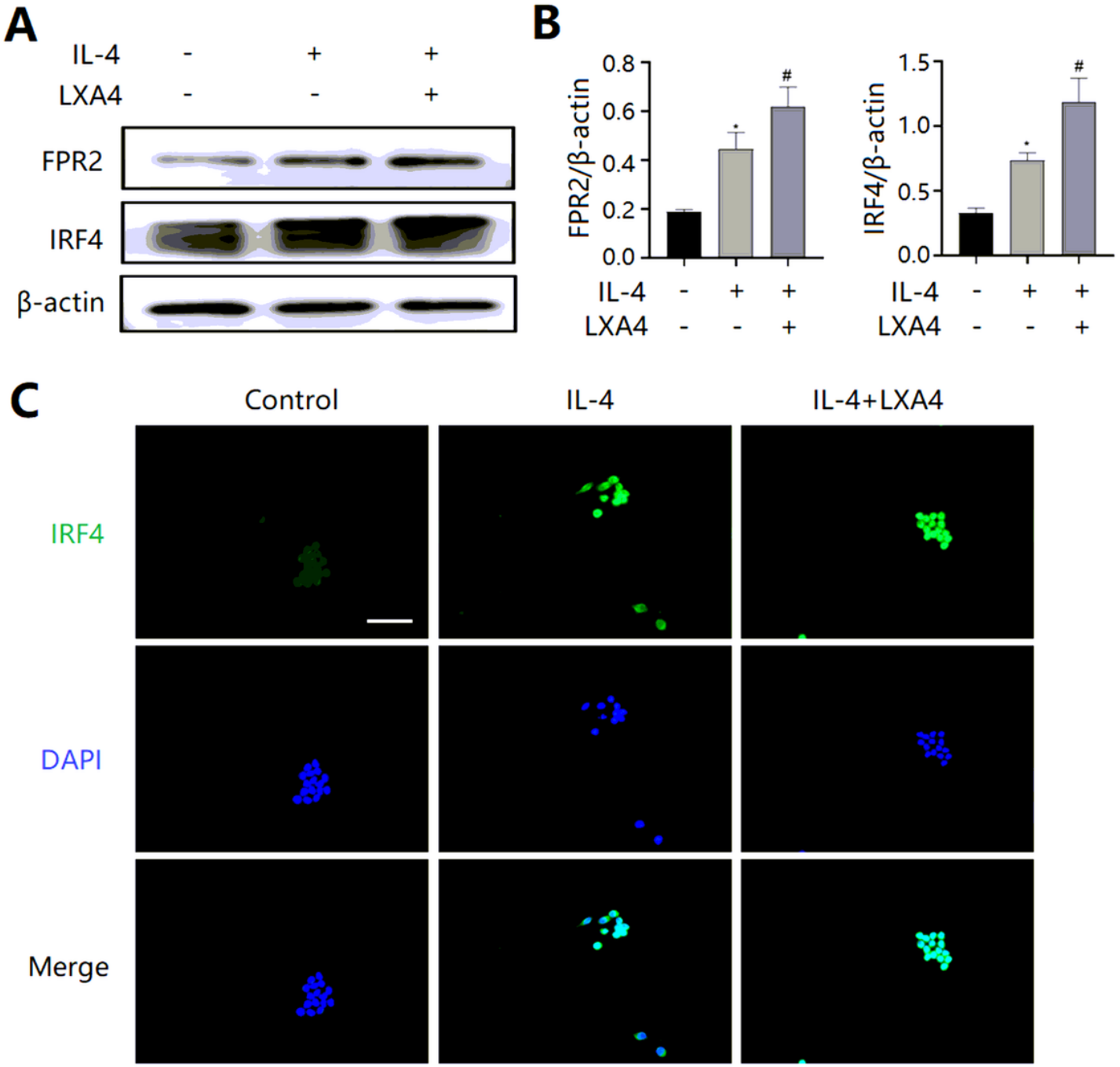

Figure 6

LXA4 promotes M2 macrophage polarization by targeting enhanced IRF4 activity through FPR2-mediated enhancement. (A, B) Western blot and semiquantitative analysis of FPR2/IRF4 proteins expression in RAW264.7 cells. (C) Observation of cultured cells by immunostaining with anti-IRF4 (green) and Nuclei

were stained for DAPI (blue). Bar = 50 μm. Data are presented as the means SEM of three independent experiments. *P < 0.05, **P < 0.05 compared with the control group; #P < 0.05 compared with the IL4-treated group.