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Resolvin D1 promotes the interleukin-4-induced alternative activation in BV-2 microglial cells

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Abstract

Background: Microglia play key roles in innate immunity, homeostasis, and neurotropic support in the central nervous system. Similar to macrophages, microglia adopt two different activation phenotypes, the classical and alternative activation. Resolvin D1 (RvD1) is considered to display potent anti-inflammatory and pro-resolving actions in inflammatory models. In this present study, we investigate the effect of RvD1 on IL-4-induced alternative activation in murine BV-2 microglial cells.

Methods: BV-2 cells were incubated with RvD1 alone, IL-4 alone, or the combination of RvD1 and IL-4. Western blot and immunofluorescence were performed to detect protein levels of alternative activation markers arginase 1 (Arg1), chitinase 3-like 3 (Ym1). Moreover, we investigated the effects of RvD1 on IL-4-induced activation of signal transducer and activators of transcription 6 (STAT6) and peroxisome proliferator-activated receptor gamma (PPAR γ).

Results: RvD1 promoted IL-4-induced microglia alternative activation by increasing the expression of Arg1 and Ym1. RvD1 also enhanced phosphorylation of STAT6, nuclear translocation of PPAR γ and the DNA binding activity of STAT6 and PPAR γ . These effects were reversed by butyloxycarbonyl-Phe-Leu-Phe-Leu-Phe (a formyl peptide receptor 2 antagonist). Further, the effects of RvD1 and IL-4 on Arg1 and Ym1 were blocked by the application of leflunomide (a STAT6 inhibitor) or GW9662 (a PPAR γ antagonist).

Conclusions: Our studies demonstrate that RvD1 promotes IL-4-induced alternative activation via STAT6 and PPAR γ signaling pathways in microglia. These findings suggest that RvD1 may have therapeutic potential for neuroinflammatory diseases.

Keyword: Resolvin D1, Microglia, Alternative activation, STAT6, PPAR γ

Background

Microglia are considered to be the resident immune cells of the central nervous system (CNS). In the normal CNS, microglia survey their surrounding microenvironment [1]. However, in a pathological state, microglia respond to environmental changes with a variety of activation states, changing both morphology and phenotype [2].

It is becoming increasingly clear that microglia, like macrophages, have at least two polarized activation states:

classical and alternative activation [3-6]. Classical activation (M1) induced by lipopolysaccharide (LPS) or Th1 cytokines is associated with elevations of pro-inflammation cytokines such as IL-1, IL-6 and TNF- α , nitric oxide (NO) and reactive oxygen species (ROS), which are involved in pathogen destruction and cytotoxicity [7,8]. Alternative activation (M2) induced by Th2 cytokines IL-4, IL-13 or IL-10 is associated with elevation of arginase 1 (Arg1), chitinase 3-like 3 (Ym1), mannose receptor and found in inflammatory zone 1 (FIZZ1), which is primarily involved in tissue remodeling and healing [7,8]. Therefore, modulating microglia activation states may be a potential therapeutic targeting of neuroinflammatory diseases.

Signal transducer and activators of transcription 6 (STAT6) is activated by IL-4 and IL-13 and plays an important role in alternative activation. STAT6 is able to

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regulate transcription of genes typical of M2 activation [9,10]. Downstream of STAT6, peroxisome proliferator-activated receptor gamma (PPAR γ) is activated in a STAT6-dependent manner [11]. PPAR γ is a member of the nuclear hormone receptor superfamily and is considered to associate with lipid metabolism, oxidative metabolism, macrophage and microglia M2 polarization [12-14].

Resolvins are a family of bioactive metabolites derived from omega-3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) and display potent anti-inflammatory and pro-resolving actions in inflammatory models [15]. Resolvin D1 (7S,8R,17S-tri-hydroxy-4Z,9E,11E,13Z,15E,19Z-DHA, RvD1) is produced physiologically from the sequential oxygenation of DHA by 15-lipoxygenase and 5-lipoxygenase [16]. RvD1 directly activates lipoxin A4 receptor/formyl peptide receptor 2 (ALX/FPR2) and orphan receptor G protein coupling receptor 32 (GPR32), limits excessive leukocyte infiltration, attenuates the production of pro-inflammatory cytokines [17], regulates macrophage phagocytosis [18] and up-regulates pro-resolving miRNAs [19]. RvD1 can also elicit macrophage polarization toward an M2-like phenotype [20]. Previous research has indicated that another ALX/FPR2 activator, aspirin-triggered lipoxin A4, can down-regulate the LPS-induced expression of M1 markers in microglia [21-23]. However, the effect of RvD1 on microglia M2 polarization and molecular mechanisms is still unknown.

Here, we investigated the impact of RvD1 on IL-4-induced expression of M2 markers in BV-2 microglial cells and the signaling pathways involving in these processes. Our data show that RvD1 up-regulates expression of Arg1 and Ym1 in IL-4-treated microglial cells depending on STAT6 and PPAR γ signaling pathways.

Methods

Cell culture

The immortalized murine microglia cell line BV-2 was purchased from the Cell Resource Center of Peking Union Medical College (Beijing, China) and maintained in DMEM with F12 supplement (DMEM/F12, Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Gibco, Grand Island, NY, USA), 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO $_2$. Before each experiment, cells were serum-starved for 12 hours. BV-2 cells were incubated with different concentrations (1 nM, 10 nM or 100 nM) of RvD1 (Cayman Chemical, Ann Arbor, MI, USA) or vehicle (0.038% ethanol) for 30 minutes before addition of 10 ng/ml murine IL-4 (PeproTech, Hamburg, Germany) under serum-free conditions. To investigate the involvement of ALX, STAT6 or PPAR γ , the cells were treated with 10 μ M butyloxycarbonyl-Phe-Leu-Phe-Leu-Phe (Boc-2) (GenScript Corporation,

Piscataway, NJ, USA), 100 μ M leflunomide (Sigma-Aldrich, St. Louis, MO, USA) or 1 μ M GW9662 (Sigma-Aldrich, St. Louis, MO, USA) prior to the treatment with RvD1 for 30 minutes.

Immunocytochemistry

BV-2 cells were cultured on sterile glass cover slips and treated according to the experimental design. Afterward, cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS. After rinsing, cells were blocked with 3% BSA in PBS for one hour and incubated with primary antibodies overnight at 4°C. The primary antibodies used were as follows: rabbit anti-arginase-1 (1:100, Santa Cruz, Heidelberg, Germany), Ym1 (1:50, Stem Cell Technologies, Vancouver, Canada), PPAR γ (1:100, Santa Cruz, Heidelberg, Germany). After washing, cells were incubated with FITC-conjugated goat anti-rabbit IgG (1:400, Jackson Immuno Research Laboratories, West Grove, PA, USA) for one hour and counterstained with 4, 6-diamidino-2-phenylindole (DAPI, Roche, Shanghai, China) for the identification of nuclei. After washing with PBS, the cover slips were mounted with antifade mounting medium (Beyotime, Shanghai, China) on slides, and the cells were observed with an Olympus immunofluorescence microscope (Olympus, Tokyo, Japan).

Protein extraction

For making whole cell lysates, the cells were lysed in radio-immune precipitation assay (RIPA) buffer supplemented with protease inhibitor cocktail (Roche, Shanghai, China). Nuclear and cytoplasmic fractionations were performed with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL, USA) according to manufacturer's protocol.

Western blot analysis

Equal amounts of nuclear or whole cell extracts were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels, and then transferred onto a polyvinylidene difluoride membrane (Millipore, Schwalbach, Germany). The transformed membrane was blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween-20 (TBST) for one hour and incubated with primary antibodies overnight at 4°C. The primary antibodies used were as follows: rabbit anti-arginase-1 (1:500), Ym1 (1:1,000), PPAR γ (1:500), phospho-Stat6 (1:500, Santa Cruz, Heidelberg, Germany), β -actin (1:1,000, Santa Cruz, Heidelberg, Germany), lamin B1 (1:1,000, Santa Cruz, Heidelberg, Germany). The membrane was washed three times with TBST for ten minutes and incubated with anti-rabbit IgG-horseradish peroxidase (1:5,000, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at room temperature for one hour. The Supersignal West Pico chemiluminescent substrate system

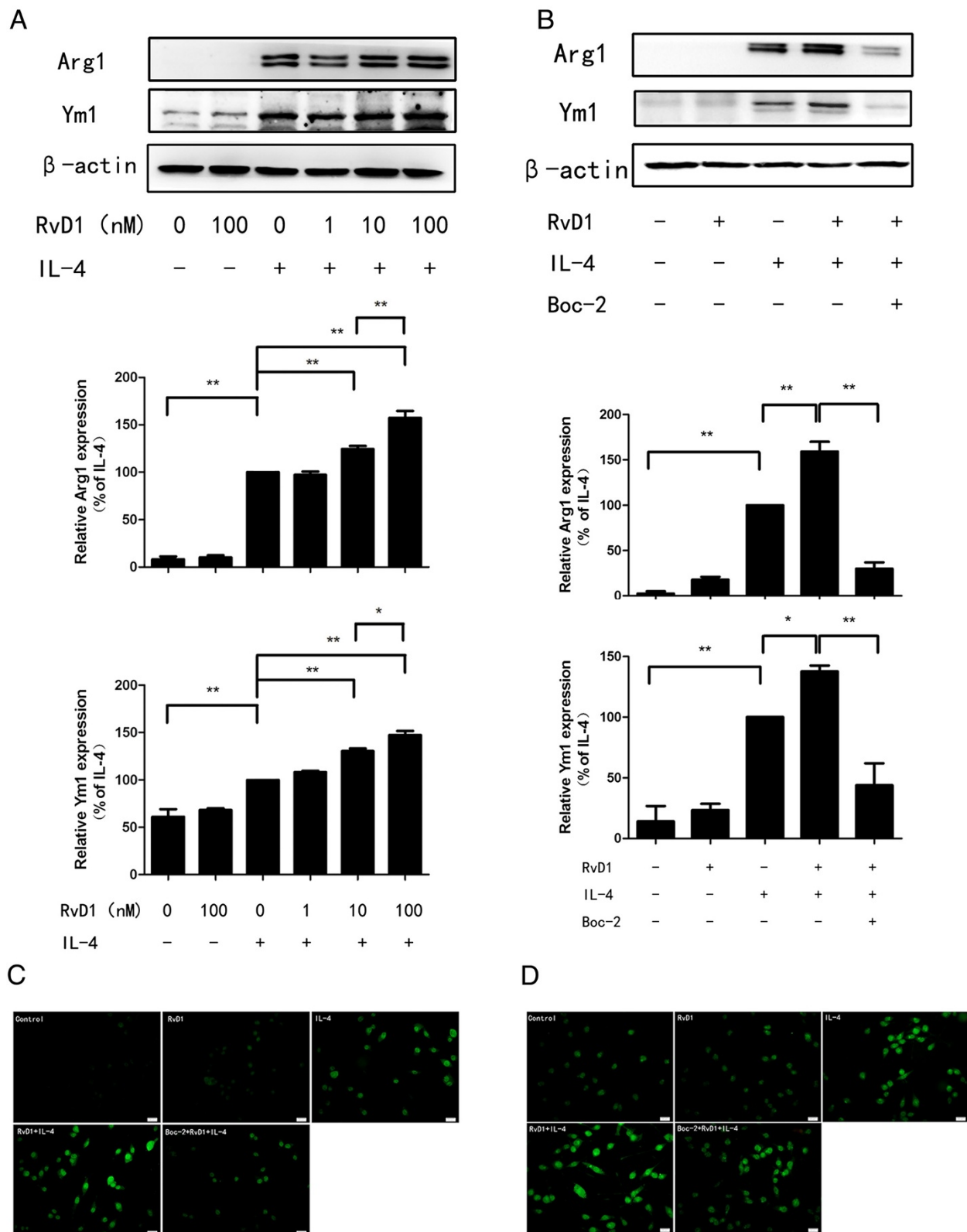


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Figure 1 RvD1 promotes the IL-4-induced alternative activation of BV-2 cells. BV-2 cells were treated with vehicle (0.038% ethanol) or different concentrations of RvD1 (1 nM, 10 nM and 100 nM) for 30 minutes in the absence or presence of Boc-2 (10 μ M), and then stimulated by IL-4 (10 ng/ml) for 24 hours. **(A, B)** The expression of Arg1 and Ym1 proteins was assessed by Western blot. A representative result from three independent experiments is shown. Quantification for Arg1 and Ym1 was normalized by β -actin. Data are presented as mean \pm SEM for three independent experiments. Asterisks indicate statistically significant difference (* P < 0.05, ** P < 0.01). **(C, D)** Representative images of BV-2 cells with positive immunofluorescence staining for Arg1 **(C)** and Ym1 **(D)**. Scale bars indicate 20 μ m.

(Millipore, Schwalbach, Germany) was used to detect immunoreactive bands. The intensity of protein bands after Western blot were quantitated by using Quantity One Version 4.6.3 Image software (Bio-Rad, Hercules, CA, USA) and normalized against proper loading controls.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described above. Oligonucleotides corresponding to the STAT6 (5'-TGCCTTAGTCAACTTCCCAAGAACAAGA-3') and PPAR γ (5'-GGAAGTCTAGGTCAAAGGTCATCCCT-3') binding site consensus sequences were synthesized and end-labeled with biotin by Invitrogen (Invitrogen, Shanghai, China). EMSAs were performed using the LightShift chemiluminescent EMSA kit (Thermo Scientific, Rockford, IL, USA). Briefly, 10 fmol of biotin-labeled, double strand probe were incubated for 20 minutes at room temperature in 20 μ l of EMSA binding buffer containing 2.5% glycerol, 5 mM MgCl₂, 50 ng/ μ l poly (dI-dC), 0.05% Nonidet P-40, and 6 μ g of nuclear proteins. For competition EMSA, 200-fold (2 pmol) excess unlabeled, double strand probe was added to the binding reaction. The DNA-nuclear protein complexes were resolved by electrophoresis in 6% nondenaturing polyacrylamide gel in 0.5 \times Tris-borate-EDTA (TBE) buffer at 100 V. Gels were then electroblotted onto Hybond nylon membranes (GE Healthcare, Freiburg, Germany) at 380 mA for 50 minutes. The membranes were then cross-linked for 15 minutes with the membrane face down on a transilluminator at 312 nm, and the biotinylated protein-DNA bands were detected with HRP-conjugated streptavidin using the chemiluminescent nucleic acid detection system.

Statistical analysis

Data are expressed as means \pm SEM of the indicated number of independent experiments. Statistical significance between multiple groups was analyzed by one-way ANOVA. Least significant difference (LSD) *post hoc* test was used for multiple comparisons. Statistical analysis was performed using the SPSS software version 13.0 (SPSS Inc., Chicago, IL, USA). P < 0.05 was considered statistically significant.

Results

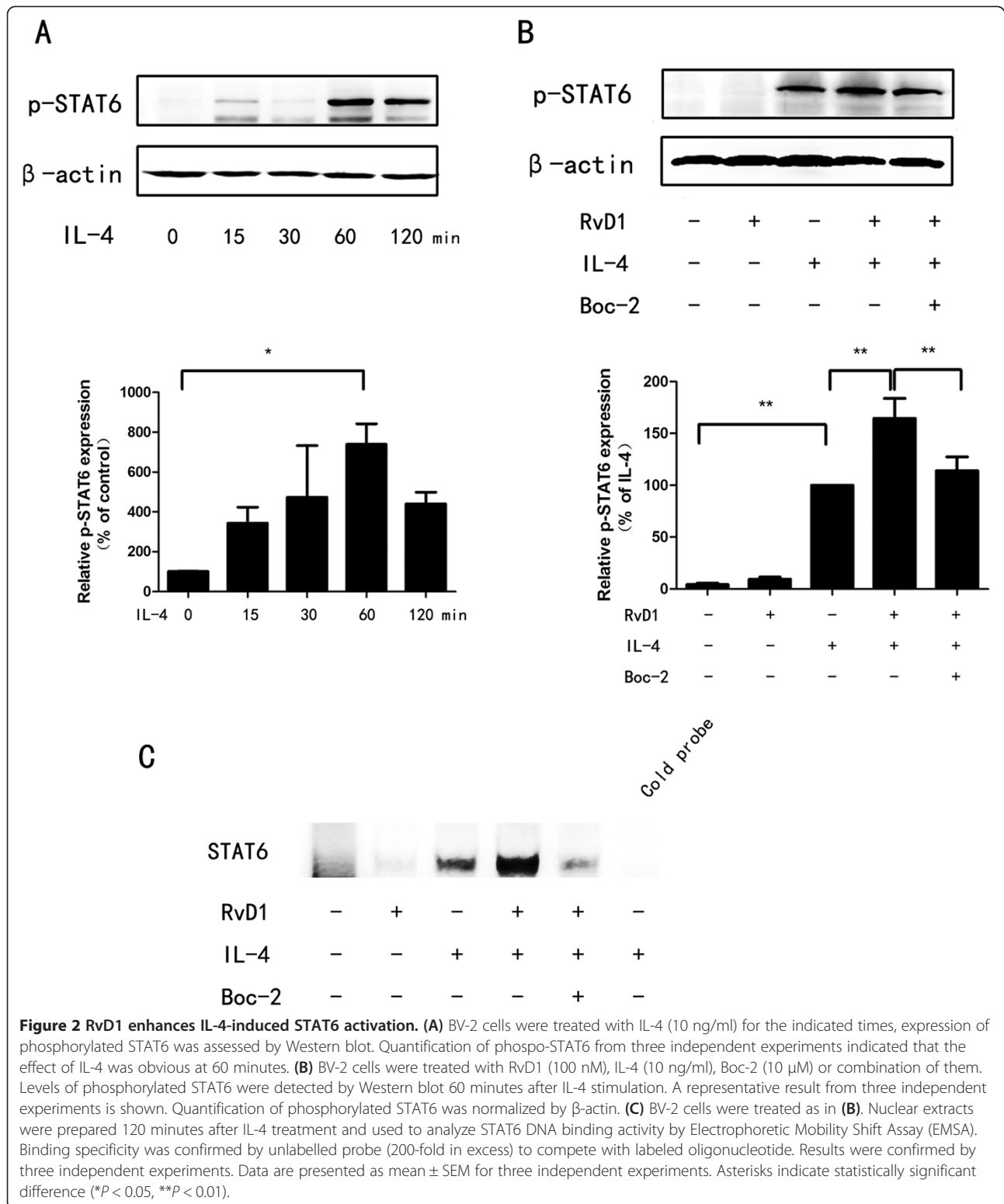
RvD1 promotes IL-4-induced alternative activation of BV-2 microglial cells

Initially, we evaluated the effects of RvD1 on IL-4-induced microglia alternative activation; BV-2 cells were treated with vehicle or different concentrations of RvD1 (1 nM, 10 nM and 100 nM) for 30 minutes and stimulated with IL-4 (10 ng/ml) for 24 hours. As the assessment of the alternative activation relied on the molecule markers such as Arg1 and Ym1, the expression of Arg1 and Ym1 were analyzed. We tested the protein levels of Arg1 and Ym1 using Western blot analysis. As shown in Figure 1A, IL-4 stimulation significantly enhanced Arg1 and Ym1 protein levels in BV-2 cells (P < 0.01). Treatment with RvD1 alone did not affect Arg1 and Ym1 protein levels (P > 0.05); while combination of RvD1 and IL-4 markedly increased expression of Arg1 and Ym1 in BV-2 cells compared to IL-4 treatment alone, the effects of RvD1 were concentration dependent. Immunofluorescence staining demonstrated that Arg1 and Ym1 staining intensity increased after IL-4 treatment, pretreatment with RvD1 further increased the staining intensity (Figure 1C and D).

To evaluate the role of ALX/FPR2 in the effects of RvD1, an ALX antagonist Boc-2 (10 μ M) was administered 30 minutes prior to treatment with RvD1. The effects in response to RvD1 were reversed by Boc-2 (Figure 1B-D).

RvD1 promotes IL-4-induced STAT6 phosphorylation and activation

STAT6 plays a critical role in IL-4-dependent induction of Arg1 and Ym1 [24,25]. As RvD1 promotes IL-4-induced expression of Arg1 and Ym1 (Figure 1), we investigated the involvement of STAT6 activation in these effects. STAT6 phosphorylated at 60 minutes after IL-4 treated (Figure 2A), the 60 minute treatment of IL-4 was determined to examine the effect of RvD1 on STAT6 phosphorylation. RvD1 markedly enhanced IL-4-induced phosphorylation of STAT6 (P < 0.01), the effect of RvD1 was reversed by Boc-2 (P < 0.05, Figure 2B). Further, we performed EMSA to determine the effects RvD1 and IL-4 on STAT6 DNA binding activity in BV-2 cells. IL-4



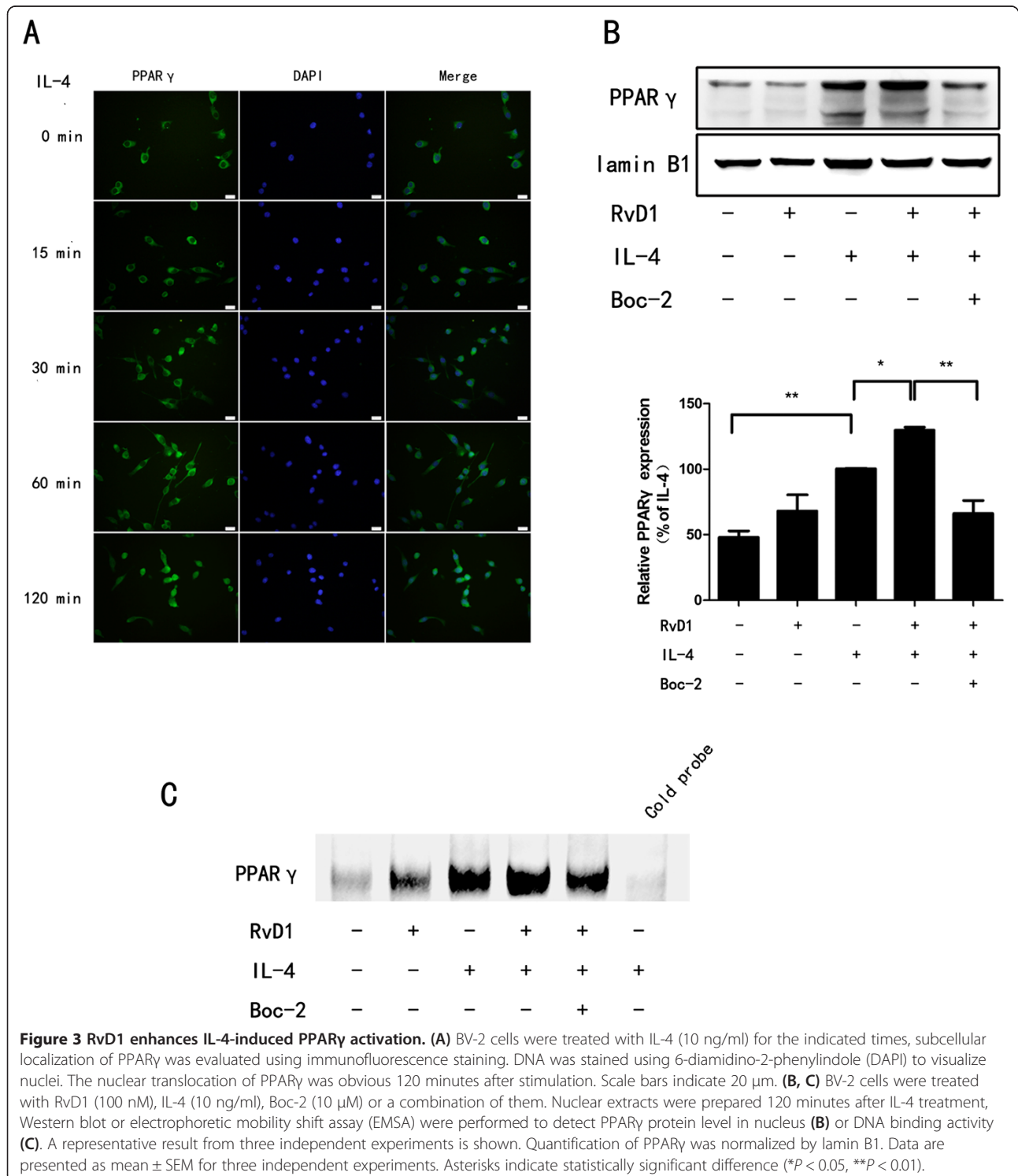
treatment increased STAT6 binding activity, combination of RvD1 and IL-4 further increased the activity. Moreover, treatment with Boc-2 reversed the effect of RvD1 (Figure 2C).

RvD1 promotes IL-4-induced PPARγ nuclear translocation and activation

Along with STAT6, PPARγ is known to control genes associated with alternatively activation [12,26]. Therefore, we

examined the effects of RvD1 on IL-4-stimulated PPAR γ nuclear translocation and DNA binding activity in BV-2 cells. Immunofluorescence staining indicated that PPAR γ translocated from the cytoplasm into the nucleus obviously 120 minutes after IL-4 stimulation (Figure 3A). Then we tested whether IL-4 and RvD1 affected PPAR γ protein

levels in nucleus and DNA binding activity. As shown in Figure 3B and C, IL-4 increased PPAR γ protein levels in nucleus and DNA binding activity. RvD1 alone failed to activate PPAR γ ($P > 0.05$). However, combination of RvD1 and IL-4 further increased PPAR γ protein levels in nucleus and DNA binding activity compared to treatment with IL-4



alone, the effects of RvD1 were abolished by pretreatment with Boc-2.

STAT6 is required for PPAR γ activation induced by IL-4 and RvD1

STAT6 is a regulator of PPAR γ response; IL-4 induces augmented PPAR γ activity through a STAT6-dependent mechanism [11,27]. To identify the role of STAT6 on PPAR γ activation induced by IL-4 and RvD1, we used leflunomide (an inhibitor suppressing tyrosine phosphorylation of STAT6 and preventing subsequent DNA binding) and GW9662 (a PPAR γ antagonist) [26,28]. One hundred micromoles of leflunomide or 1 μ M GW9662 was administrated 30 minutes prior to treatment with RvD1, PPAR γ expression and DNA binding activity were analyzed. Consistent with GW9662, leflunomide decreased PPAR γ protein levels in nucleus and DNA binding activity in IL-4 and RvD1 treated BV-2 cells (Figure 4).

STAT6 and PPAR γ are essential for induction of Arg1 and Ym1 by IL-4 and RvD1

RvD1 promotes IL-4-induced STAT6 and PPAR γ activation, these facts suggest, but do not prove, that

STAT6 and PPAR γ are required for the effects of RvD1 and IL-4 on Arg1 and Ym1 induction. To directly test the effects of STAT6 and PPAR γ , BV-2 cells were pretreated with 100 μ M leflunomide or 1 μ M GW9662 for 30 minutes to inhibit STAT6 or PPAR γ signaling pathway and stimulated with RvD1 and IL-4 for 24 hours. Immunofluorescence and Western blot were performed to determine Arg1 and Ym1 expression. As expected, Arg1 and Ym1 induction by RvD1 and IL-4 was markedly blunted by inhibition of STAT6 and PPAR γ (Figure 5).

Discussion

In this study, we provide the first evidence that RvD1 enhances the IL-4-induced M2 polarization in BV-2 microglial cells. Pretreatment with RvD1 was able to increase IL-4-induced expression of two major alternative activation markers, Arg1 and Ym1. In addition, RvD1 potentiated the effects of IL-4 on STAT6 and PPAR γ signaling pathways, administering leflunomide or GW9662 before RvD1 and IL-4 blunted the induction of Arg1 and Ym1. These results suggest that RvD1 can promote IL-4-induced M2 polarization in microglial cells via STAT6 and PPAR γ signaling pathways.

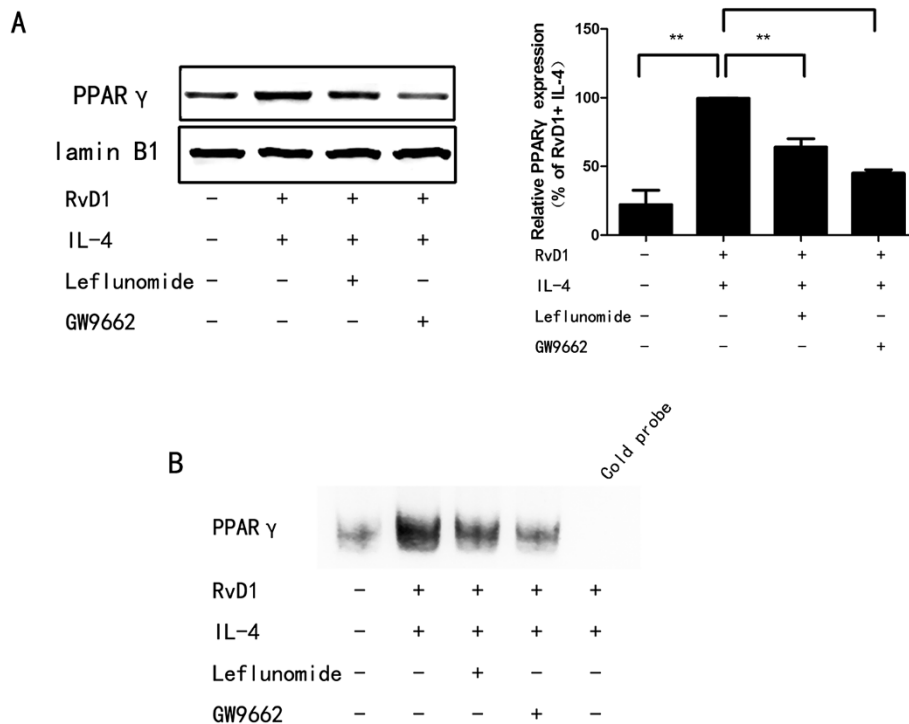
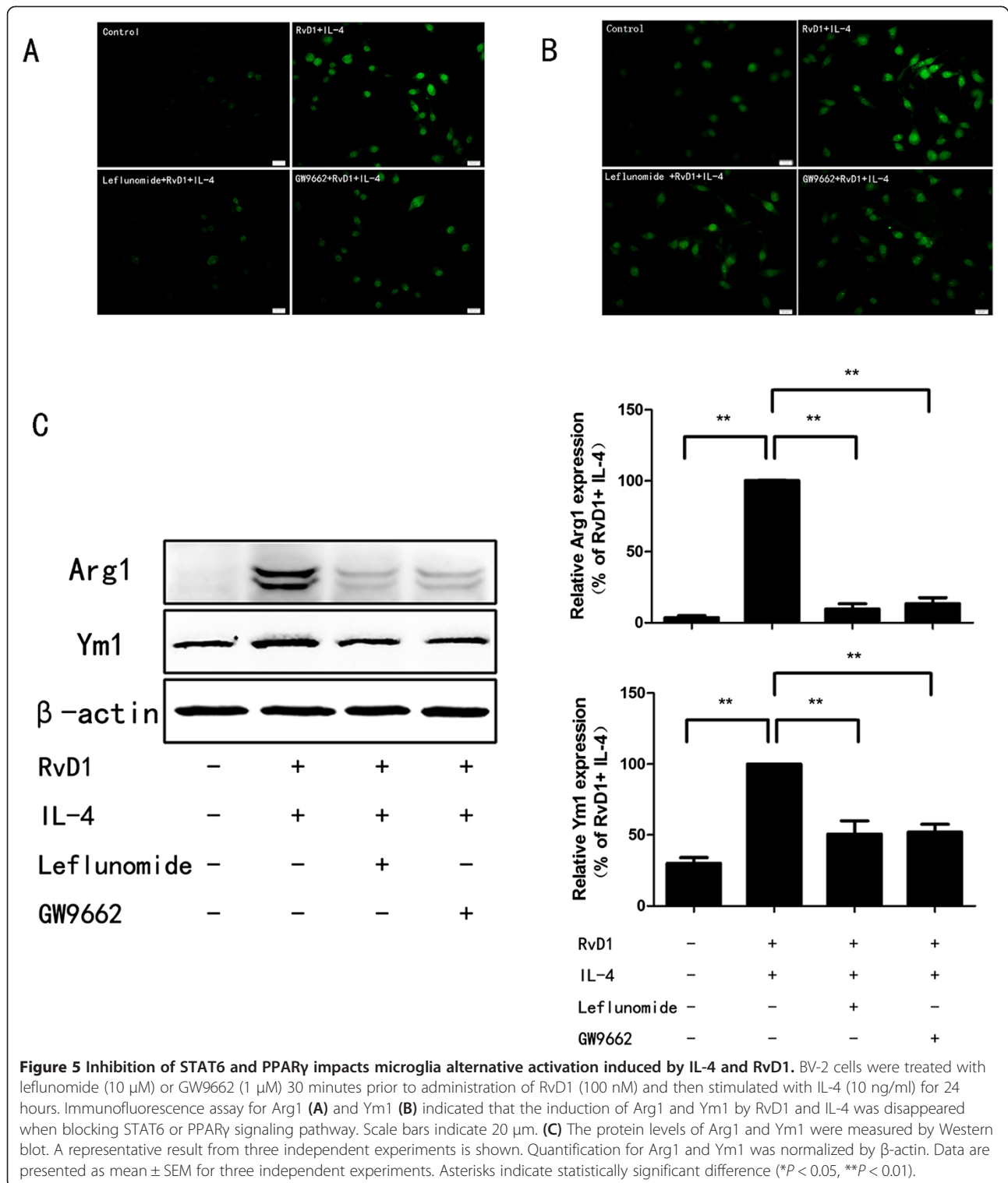


Figure 4 STAT6 is required for PPAR γ activation. BV-2 cells were treated with leflunomide (10 μ M) or GW9662 (1 μ M) 30 minutes prior to administration of RvD1 (100 nM), followed by incubation with IL-4 (10 ng/ml) for 120 minutes. PPAR γ protein level in nucleus (**A**) and DNA binding activity (**B**) were analyzed using Western blot or electrophoretic mobility shift assay (EMSA). A representative result from three independent experiments is shown. Quantification of PPAR γ was normalized by lamin B1. Data are presented as mean \pm SEM for three independent experiments. Asterisks indicate statistically significant difference (* P < 0.05, ** P < 0.01).



So far, two receptors for RvD1 have been identified, ALX/FPR2 and an orphan, GPR32. RvD1 activates ALX and GPR32 with high affinity (EC_{50} approximately 1.2 pM for ALX/FPR2 and 8.8 pM for GPR32) [18]. Interestingly, on human polymorphonuclear leukocytes, low

concentration of RvD1 (1 nM) appear sensitive to GPR32 blockade, while responses to high concentration (10 nM) are ALX-dependent [29]. Our findings demonstrated that RvD1 promoted IL-4-induced microglia alternative activation with high concentrations (10 nM

and 100 nM). Moreover, in murine physiology, we did not identify the murine orthologs of human GPR32, while mouse ALX has been confirmed to express in neuron, astrocyte, microglia, neutrophils, macrophage and monocyte, as well as BV-2 cells [21,30]. Boc-2 is an ALX/FPR2 antagonist, and can block the ability of RvD1 [31]. Thus, we investigated the receptor dependency of RvD1 in microglia M2 polarization by using Boc-2. Previous studies have shown that activation of ALX by lipoxin A4, RvD1, BML-111 or other agonists have potential anti-inflammatory and pro-resolving effects in a rat brain ischemia-reperfusion injury model [32,33] and other inflammatory disease models [34-37]. These findings suggest that RvD1 could modulate the inflammatory reaction in CNS diseases.

Microglia exhibit various functions at different stages in life or in response to different pathological situations [6]. The activation states of microglia are plastic and flexible, both blunting classical activation and promoting alternative activation are involving in anti-inflammatory and pro-resolving process. Here, we used Arg1 and Ym1 as markers for microglia alternative activation. Expression of Arg1 in microglia is induced by Th2 cytokines [38]. Arg1 can reduce NO production, and it is also involved in wound healing, fibrosis and supporting neuron

survival [39-41]. Apart from Arg1, Ym1 is another marker for alternative activation [42]. Ym1 is considered to be associated with tissue remodeling and regulating inflammation [43]. First, we examined the effect of IL-4 and RvD1 on Arg1 and Ym1 expression. According with previous studies, our results showed that expression of Arg1 and Ym1 was low in unstimulated BV-2 microglial cells, which was enhanced by stimulation with 10ng/ml IL-4 [7,44]. Titos *et al.* reported that RvD1 and its precursor DHA promoted resolution by eliciting macrophage M2 polarization [20]. In our study, treatment with RvD1 alone had no effect on Arg1 and Ym1 expression in microglia, while pretreatment with RvD1 enhanced the effect of IL-4 on Arg1 and Ym1 expression. This indicated that RvD1 may be possible to promote resolution of neuroinflammation by regulating microglia polarization. The effect of RvD1 was receptor-dependent as it was reversed by the use of ALX antagonist Boc-2.

The synergistic effect of RvD1 and IL-4 on Arg1 and Ym1 expression might be explained by RvD1 potentiating IL-4-induced STAT6 and PPAR γ activation. IL-4-induced expression of Arg1 and Ym1 depends on activation of STAT6. In response to IL-4, STAT6 is phosphorylated, translates into the nucleus and binds to consensus sites at

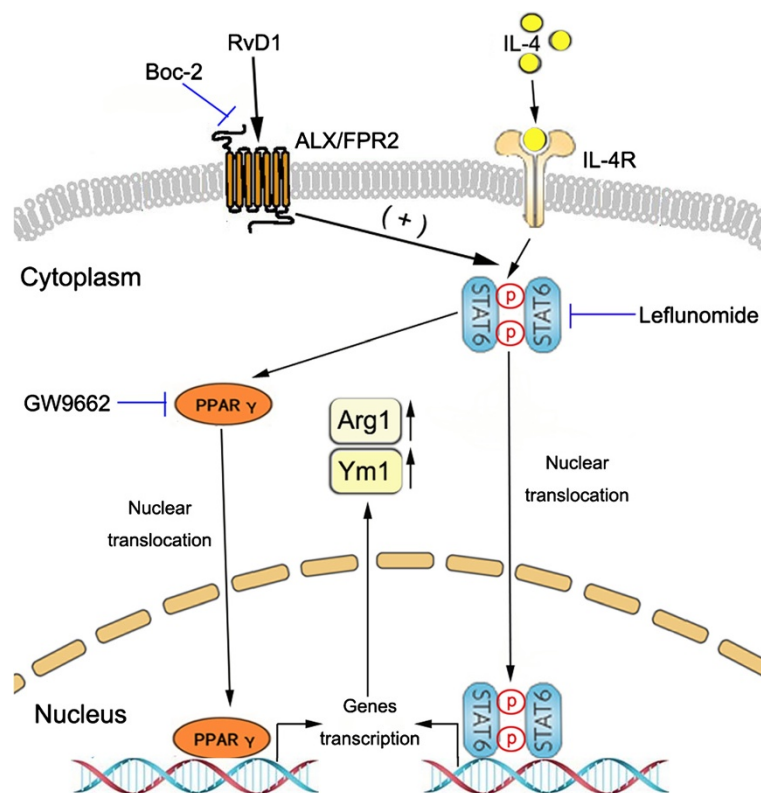


Figure 6 Schematic representation of the RvD1 contribution to microglia alternative activation. RvD1 promotes IL-4-induced microglia alternative activation by increasing Arg1 and Ym1 expression via STAT6/PPAR γ signaling pathway.

the *Jmjd3* promoter, then regulates transcriptional activation of M2 marker genes [45]. STAT6 can also modulate the activity of nuclear receptor PPAR γ [11]. PPAR γ plays an important role in macrophage/microglia alternative activation; the addition of a PPAR γ agonist directly regulates the Arg1 and Ym1 expression [12,14,46,47]. In this study, RvD1 was able to enhance the phosphorylation and DNA binding activity of STAT6 induced by IL-4. Inhibiting STAT6 by leflunomide abolished the induction of Arg1 and Ym1. Moreover, leflunomide inhibited PPAR γ translocating into the nucleus and binding to DNA sites, which suggested that STAT6 was required for PPAR γ activation induced by RvD1 and IL-4. Consistent with STAT6, the activity of PPAR γ induced by RvD1 and IL-4 was greater than stimulating with IL-4 alone. A previous study reported that activation of PPAR γ by pioglitazone promoted microglia M2 polarization by increasing Arg1 and Ym1 expression [14]. In our research, the effects of RvD1 and IL-4 on Arg1 and Ym1 were impaired by using PPAR γ antagonist GW9662, which proved PPAR γ was essential for RvD1 and IL-4-induced microglia alternative activation. These findings suggested that RvD1 enhanced IL-4-induced microglia alternative activation depending on the STAT6/PPAR γ signaling pathway (Figure 6).

Conclusions

Our results show that RvD1 promotes IL-4-induced microglia alternative activation. A possible mechanism relates to activation of STAT6 and PPAR γ signaling pathways. Given the anti-inflammatory and pro-resolving effects of RvD1, it may be a potential treatment for CNS diseases involving neuroinflammation.

Abbreviations

ALX: lipoxin A4 receptor; Arg1: arginase 1; Boc-2: butyloxycarbonyl-Phe-Leu-Phe-Leu-Phe; BSA: bovine serum albumin; CNS: central nervous system; DAPI: 6-diamidino-2-phenylindole; DHA: docosahexaenoic acid; DMEM: Dulbecco's modified Eagle's medium; EMSA: electrophoretic Mobility Shift Assay; EPA: eicosapentaenoic acid; Fizz1: found in inflammatory zone 1; FPR2: formyl peptide receptor 2; GPR 32: G protein coupling receptor 32; IL: interleukin; LPS: lipopolysaccharide; NO: nitric oxide; PBS: phosphate-buffered saline; RvD1: resolvin D1; PPAR γ : peroxisome proliferator-activated receptor gamma; RIPA: radioimmune precipitation assay buffer; ROS: reactive oxygen species; STAT6: signal transducer and activators of transcription 6; TBE: Tris-borate-EDTA; TBST: Tris-buffered saline containing 0.05% Tween-20; TNF: tumour necrosis factor; Ym1: chitinase 3-like 3.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LYL and YW designed the experiments. LYL, YW, WJX and LMS performed the experiments. LYL and YW analyzed the data. YPW, JW and LP provided useful advice and reviewed the manuscript. SY and YS supervised the experimental work. YS conceived the study, participated in its design and coordination, and wrote the manuscript. All authors of this paper have read and approved the final version of the manuscript.

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