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Research Article

Stimulation of Formyl Peptide Receptor-2 by the New Agonist CMC23 Protects against Endotoxin-Induced Neuroinflammatory Response: A Study in Organotypic Hippocampal Cultures

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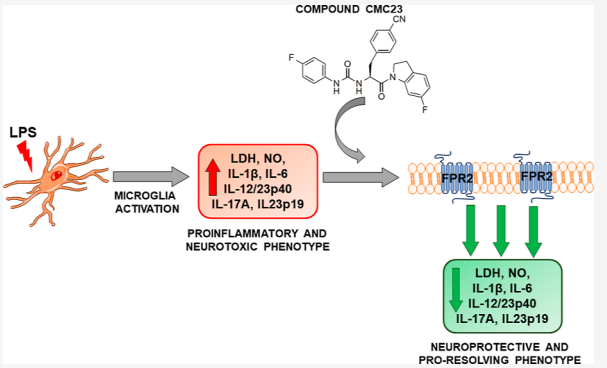
ABSTRACT: A substantial body of evidence demonstrates an association between a malfunction in the resolution of acute inflammation and the development of chronic inflammation. Recently, in this context, the importance of formyl peptide receptor 2 (FPR2) has been underlined. FPR2 activity is modulated by a wide range of endogenous ligands, including specialized pro-resolving mediators (SPMs) (e.g., LXA4 and AT-LXA4) and synthetic ligands. Since SPMs have unfavorable pharmacokinetic properties, we aimed to evaluate the protective and pro-resolving effects of a new potent FPR2 agonist, compound CMC23, in organotypic hippocampal cultures (OHCs) stimulated with lipopolysaccharide (LPS). The protective activity of CMC23 limited the lactate dehydrogenase release in LPS-stimulated cultures. This activity was mediated by the interaction with FPR2 as pretreatment with the FPR2 selective antagonist WRW4 abolished CMC23-induced protection. Furthermore, decreased levels of pro-inflammatory IL-1β and IL-6 were observed after CMC23 administration in LPS-treated OHCs. CMC23 also diminished the LPS-induced increase in IL-17A and both IL-23 subunits p19 and p40 in OHCs. Finally, we demonstrated that CMC23 exerts its beneficial impact via the STAT3/SOCS3 signaling pathway since it attenuated the level of phospho-STAT3 and maintained the LPS-induced SOCS3 levels in OHCs. Collectively, our research implies that the new FPR2 agonist CMC23 has beneficial protective and anti-inflammatory properties in nanomolar doses and FPR2 represents a promising target for the enhancement of inflammation resolution.



KEYWORDS: *formyl peptide receptor 2, ureidopropanamide agonist, neuroinflammation, lipopolysaccharide, intracellular pathways, hippocampus*

1. INTRODUCTION

A growing body of evidence indicates that uncontrolled inflammation is a prominent component of many pathological events including psychiatric and neurodegenerative disorders. The inflammatory response is a complex process that involves molecular, cellular, and physiological reactions in its initiation, execution, and resolution. Recent data underlined that the regulation of the inflammatory response is multidirectional as the different steps do not occur sequentially but overlap.[1] Regulation dysfunction offsets the beneficial effect of an acute inflammatory reaction and causes chronic inflammation with adverse consequences, which is associated with the aberrantly increased activation of microglia and the elevated production of various proinflammatory and harmful factors.[2−4] The resolution of acute inflammation (RoI) refers to the integration of multiple biological processes involved in the resolution of physiological inflammation. The onset-to-peak phase of the response to injury/infection is followed by temporally appropriate and controlled resolution, leading to homeostasis and normal function.[5,6] Hence, it is essential to identify mediators and their central targets that promote active resolution.

RoI has been shown to be regulated by specialized pro-resolving mediators (SPMs),[1,7,8] which are a group of fatty acid metabolites and include, among others, lipoxin A4 (LXA4) and its analogue aspirin-triggered lipoxin (AT-LXA4).[6,9] They act as immuno-resolvents by combining anti-inflammatory and pro-resolving activities.[6,10] In addition, they attenuate oxidative stress by reducing oxygen and/or nitrogen reactive species production and by potentiating several natural antioxidant

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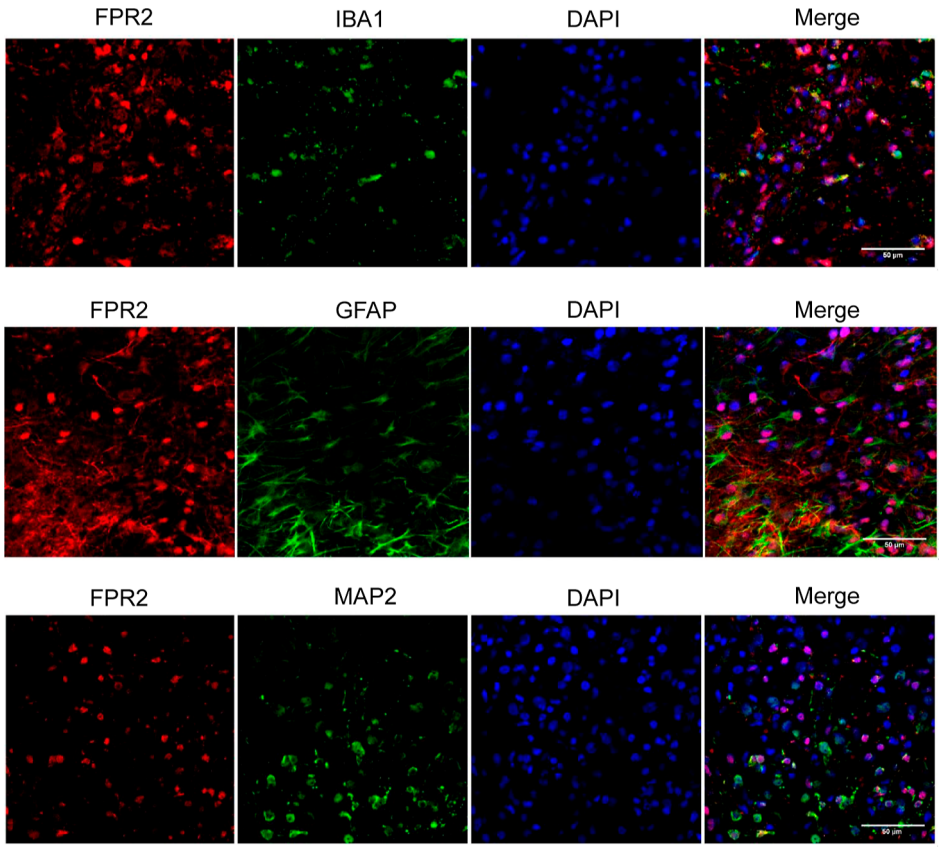

Figure 1. Representative fluorescence images of control (vehicle-treated) OHCs were obtained from the CA1 area of the OHCs using confocal microscopy. Nuclei appear in blue due to Hoechst 33342 staining, FPR2 was labeled in red using Alexa Fluor 647, IBA1 was labeled using Alexa Fluor 555, and GFAP and MAP2 were both labeled in green using Alexa Fluor 488. Scale bar: 50 μm is located in the bottom right corner of each image.

systems.[11] However, there are substantial limitations for the in vivo evaluation of LXA4 and AT-LXA4, including the poor pharmacokinetic properties and rapid chemical inactivation by dehydrogenation, which often occurs in microglial cells.[12]

The biological actions of LXA4 and AT-LXA4 are exerted by interactions with specific formyl peptide receptors (FPRs),[13] among which formyl peptide receptor 2 (FPR2) is the most intriguing.[14] The expression of FPR2 was demonstrated in several immune cells, including neutrophils and monocytes/macrophages.[15−17] In the brain, FPR2 is expressed mainly in microglia, astrocytes, and neuronal cells.[18−20] Interestingly, FPR2 is a highly variable receptor because it can interact with chemically diverse ligands and with several classes of synthetic ligands (e.g., substituted quinazolinone Quin-C1, ureidopropanamide MR-39), which can activate various signal transduction pathways, depending on the ligand's structure, concentration, and the cell type involved.[21] Generally, its activation plays an immunomodulatory role by regulating pro-resolving, anti-inflammatory, and proinflammatory activities; thus, activating this receptor might have complex consequences. Therefore, FPR2 may represent a unique target for balancing inflammatory processes and, consequently, for developing new therapeutic strategies.

We have identified a series of ureidopropanamide-based FPR2 agonists that can reduce the intracellular levels of proinflammatory mediators in rat primary microglial cell cultures stimulated with lipopolysaccharide (LPS).[22,23] Furthermore, the compounds are stable to oxidative metabolism and have reasonable permeation rates in hCMEC/D3 cells, which are used as an *in vitro* blood−brain barrier model.[22] The most active and promising ligand, MR-39, exerts beneficial in vitro effects in the micromolar range. Thus, it is not ideal for in vivo preclinical studies because a high dosage would imply the risk of unpredictable and confounding off-target effects. In a subsequent study, the FPR2 agonist potency of the ureidopropanamide derivatives was improved, leading to the identification of compound CMC23 [(*S*)-1-(3-(4-cyanophenyl)-1-(6-fluoroindolin-1-yl)-1-oxopropan2-yl)-3-(4-fluorophenyl)urea], which was able to permeate the blood−brain barrier *in vivo* and accumulate into the brain.[11]

In the present study, we studied the anti-inflammatory and pro-resolving profiles of CMC23 to explore the role of FPR2 in neuroinflammation and propose this compound as a new tool in the treatment of inflammation. For this purpose, considering the interplay between neuronal cells and glia, we performed *ex vivo* studies in organotypic hippocampal cultures (OHCs). First, we visualized the localization of FPR2 in hippocampal cultures using MAP2, IBA1, and GFAP antibodies. Then, to compare the dose-dependent effects of CMC23 on lactate dehydrogenase (LDH) and nitric oxide (NO) release under basal conditions and after immune activation, we used a bacterial endotoxin (LPS). Moreover, the FPR2 specificity of the effect of CMC23 was verified using selective antagonist WRW4. Furthermore, considering that FPR2 activation is crucial for the modulation of microglial reactivity, we explored the expression of microglial markers (*Cd40*, *Cd68*, *Arg-1*, and *Igf-1*) and the profile of cytokines (IL-1β, IL-6, IL-10, IL-12/23p40, IL-17A, IL-23p19, and TGF-β) released in the hippocampus under basal conditions and after LPS stimulation. Last, for further characterization of the CMC23 pro-resolving activity in

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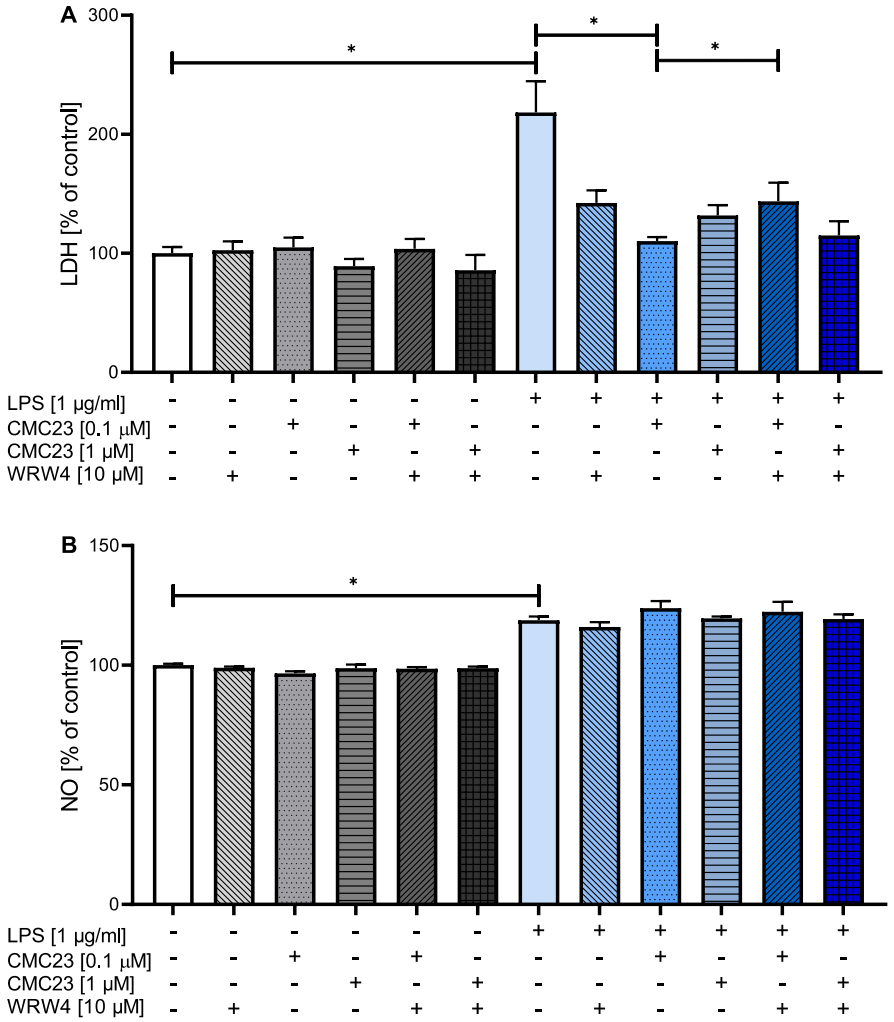

Figure 2. Impact of CMC23 and WRW4 treatment on the LPS-stimulated LDH (A) and NO (B) release. OHCs were pretreated with the FPR2 antagonist WRW4 (10 μM) for 30 min. Subsequently, OHCs were treated with CMC23 (0.1 or 1 μM) for 1 h and then stimulated with lipopolysaccharide (LPS; 1 μg/mL) for 24 h. Control slices were treated with an appropriate vehicle. The data are presented as the mean ± SEM percentage of control (vehicle-treated OHCs) of independent experiments, $n = 4-8$ in each experiment. Statistical analysis was performed using two-way analysis of variance (ANOVA) with the Duncan post hoc test to assess the differences between the treatment groups. Significant differences are indicated by $^*p < 0.05$. LDH—lactate dehydrogenase; NO—nitric oxide; LPS—lipopolysaccharide.

OHCs, we assessed the molecular signaling pathways by which CMC23 alleviates LPS-evoked immune challenge.

2. RESULTS

2.1. Immunofluorescence Staining of the Formyl Peptide Receptor 2 in OHCs. The presence of FPR2 in various brain cells was described previously.[19,23−25] However, for the first time, we visualized FPR2 in OHCs. In the present study, we confirmed that FPR2 colocalized with microglia (IBA1), astrocytes (GFAP), and neurons (MAP2) in vehicle-treated OHCs (Figure 1).

2.2. Impact of CMC23 Treatment on Lactate Dehydrogenase and Nitric Oxide Release in OHCs. In the preliminary experiments, we evaluated the effect of the FPR2 agonist CMC23 at two doses, 0.1 and 1 μM, on the release of LDH, which is a well-known marker of cell death after the damage of the plasma membrane, and on NO release using the Griess reaction. Treatment of the OHCs with CMC23 at both doses and with the FPR2 selective antagonist WRW4 (10 μM) did not significantly change the levels of NO and LDH in control OHCs. Stimulation of OHCs with LPS (1 μg/mL) for 24 h increased the level of both LDH ($p = 0.000018$) (Figure 2A) and NO ($p = 0.000019$) (Figure 2B). Furthermore, CMC23 decreased the harmful LPS-induced effect on LDH release at 0.1 μM ($p = 0.000056$) (Figure 2A) but not at 1 μM (Figure 2A). Significantly, pretreatment of the OHCs with the FPR2 antagonist WRW4 attenuated the protective activity of CMC23 (0.1 μM) ($p = 0.047104$) (Figure 2A). This outcome suggests that the observed effect of CMC23 is mediated via FPR2. However, we did not observe a favorable effect of CMC23 administration on LPS-induced NO release. Considering the obtained results, we selected the dose of 0.1 μM CMC23 for further research.

2.3. Impact of CMC23 Treatment on the mRNA Expression of Microglial Markers in OHCs. In the brain, microglia are the primary resident immune cells, constituting approximately 10−15% of the total number of cells. Under physiological conditions, microglia remain in a "surveillance state" with the constant expression of various markers. However, after activation, the expression of microglial markers dramatically changed. Therefore, we determined the mRNA expression of proinflammatory (*Cd40*, *Cd68*, *Ccl2*, *Il-6*, *Il-12*, and *Il-23*) and

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anti-inflammatory (*Arg1*, *Igf-1*, *Tgf-β*, and *Il-10*) factors that are considered crucial microglial markers under basal conditions and after LPS challenge in OHCs. Moreover, the impact of CMC23 treatment was evaluated. Under control conditions, CMC23 treatment did not show any effect on the expression of the assessed markers (Table 1A,B). The statistical analysis revealed that LPS significantly upregulated the expression of the following genes: *Cd40* ($p = 0.00397$), *Ccl2* ($p = 0.007797$), *Il-6* ($p = 0.038869$), *Arg-1* ($p = 0.000502$), and *Il-10* ($p = 0.000069$). In contrast, LPS stimulation led to the downregulation of *Il-12* ($p = 0.045665$), *Igf-1* ($p = 0.001205$), and *Tgf-β* ($p = 0.001440$) expression. The administration of CMC23 in LPS-stimulated OHCs significantly reduced the expression level of proinflammatory factors, namely, *Cd40* ($p = 0.005027$) and *Cd68* ($p = 0.039337$) and slightly *Ccl2* as well as anti-inflammatory *Arg-1* ($p = 0.005561$) and *Il-10* ($p = 0.012514$). In contrast, we observed that CMC23 increased the level of LPS-induced upregulation of *Il-6* ($p = 0.27809$).

Table 1. Impact of CMC23 on the mRNA Expression of Proinflammatory (A) (*Cd40*, *Cd68*, *Ccl2*, *Il-6*, *Il-12*, and *Il-23*) and Anti-Inflammatory (B) (*Arg-1*, *Igf-1*, *Tgf-β*, and *Il-10*) Factors in Control and LPS-Stimulated OHCs[a]

A				
factors	gene expression			
	control	LPS	CMC23	CMC23 + LPS
	proinflammatory factors			
Cd40	1.01 ± 0.07	1.59 ± 0.18*	0.82 ± 0.09	1.01 ± 0.13#
Cd68	1.03 ± 0.10	1.00 ± 0.17	0.86 ± 0.15	0.57 ± 0.07#
Cc12	1.05 ± 0.19	2.94 ± 0.62*	1.81 ± 0.42	1.93 ± 0.13
Il-6	1.07 ± 0.18	8.42 ± 1.90*	1.63 ± 0.51	18.71 ± 4.57#
Il-12	1.02 ± 0.09	0.60 ± 0.13*	1.05 ± 0.13	0.85 ± 0.17
Il-23	1.02 ± 0.10	1.24 ± 0.11	1.54 ± 0.74	1.05 ± 0.14
B				
factors	gene expression			
	control	LPS	CMC23	CMC23 + LPS
	anti-inflammatory factors			
Arg-1	1.09 ± 0.19	6.48 ± 1.33*	1.66 ± 0.49	2.71 ± 0.74#
Igf-1	1.07 ± 0.17	0.25 ± 0.08*	1.06 ± 0.20	0.36 ± 0.06
Tgf-β	1.00 ± 0.05	0.54 ± 0.06*	0.92 ± 0.17	0.49 ± 0.03
Il-10	1.06 ± 0.18	24.43 ± 3.55*	1.31 ± 0.32	14.50 ± 2.31#

[a]OHCs were treated with CMC23 (0.1 μM) for 1 h and then with lipopolysaccharide (LPS; 1 μg/mL) for 24 h. Control slices were treated with the appropriate vehicle. The data are presented as the average fold change ± SEM of independent experiments, $n = 4–6$ in each experiment. Statistical analysis was performed using two-way analysis of variance (ANOVA) with the Duncan post hoc test to assess the differences between the treatment groups. Significant differences are indicated by *$p < 0.05$ control compared to that by the LPS group; #$p < 0.05$ LPS compared to that by the CMC23 + LPS group.

2.4. Impact of CMC23 and WRW4 on the Release of Proinflammatory Cytokines IL-1β, IL-6, IL-17A, and IL-23 Subunits p19 and p40 in OHCs. To establish the anti-inflammatory and pro-resolving properties of CMC23 (0.1 μM), we first measured the protein level of proinflammatory IL-1β and IL-6 in control and LPS-stimulated OHCs using enzyme-linked immunosorbent assay (ELISA). Moreover, to determine whether the obtained results were mediated by FPR2, we pretreated cultures with the FPR2 antagonist WRW4 (10 μM). Our findings indicate that CMC23 did not change the level of either cytokine under basal conditions. As expected, exposure of the OHCs to LPS (1 μg/mL) markedly increased the levels of both factors: IL-1β ($p = 0.00027$) (Figure 3A) and IL-6 ($p = 0.000019$) (Figure 3B). Importantly, CMC23 diminished the LPS-evoked increase in the levels of IL-1β ($p = 0.01813$) (Figure 3A) and IL-6 ($p = 0.000113$) (Figure 3B) release in OHCs. Moreover, pretreatment with FPR2 antagonist WRW4 significantly attenuated the CMC23-reduced protein level of IL-1β ($p = 0.032668$) (Figure 3A) and IL-6 ($p = 0.041436$) (Figure 3B) in LPS-treated OHCs.

Next, we assessed the protein levels of IL-17A and both IL-23 subunits p19 and p40. As shown in Figure 4, none of the tested compounds affected the levels of the mentioned cytokines under basal conditions. However, stimulation with LPS elevated the release of IL-17A ($p = 0.000023$) (Figure 4A) and both IL-23 subunits: p40 ($p = 0.000023$) (Figure 4B) and p19 ($p = 0.001729$) (Figure 4C). In addition, CMC23 attenuated the LPS-induced production of IL-17A ($p = 0.00428$) (Figure 4A) and p40 ($p = 0.000496$) (Figure 4B) and p19 ($p = 0.024013$) (Figure 4C) subunits in OHCs. Nevertheless, this anti-inflammatory effect of CMC23 only tended to be mediated by FPR2 since WRW4 pretreatment did not significantly modulate the levels of p19 and p40 in LPS- and CMC23-treated OHCs.

2.5. Impact of CMC23 on the Release of Anti-inflammatory Cytokines TGF-β and IL-10 in OHCs. In the next set of experiments, we examined the pro-resolving properties of the new FPR2 agonist by assessing whether CMC23 (0.1 μM) affects the protein level of the anti-inflammatory cytokines TGF-β and IL-10 in control and LPS-treated (1 μg/mL) OHCs. As revealed in Figure 5, CMC23 administration under basal conditions did not modulate the level of anti-inflammatory cytokines. However, exposure to LPS increased the levels of TGF-β ($p = 0.032104$) (Figure 5A) and IL-10 ($p = 0.11957$) (Figure 5B). In the control groups treated with WRW4 and CMC23, we did not observe crucial changes. The release of both cytokines was elevated in LPS-stimulated OHCs, and we observed that CMC23 treatment slightly increased TGF-β ($p = 0.00064$) (Figure 5A) and IL-10 ($p = 0.005894$) (Figure 5B) release.

2.6. Impact of CMC23 Treatment on the mRNA Expression and Protein Level of JAK/STAT3/SOCS3 Pathway-Related Factors in OHCs. The JAK/STAT3 signaling pathway is an extensively established mediator of the signal transduction of many growth factors, hormones, and cytokines. In the canonical pathway, SOCS3, a negative inhibitor of the JAK/STAT3 pathway, is activated in response to the release of several cytokines.[26] Therefore, to elucidate the intracellular mechanism of the anti-inflammatory and pro-resolving ability of CMC23 (0.1 μM), we carried out a set of experiments to examine the gene expression and protein levels of factors related to the JAK/STAT3 pathway in OHCs. As shown in Figure 6A, the mRNA expression of *Jak1*, *Jak2*, and *Stat3* was slightly upregulated after LPS (1 μg/mL) and CMC23 treatment; however, these changes were not significant. Nevertheless, Western blot studies demonstrated that LPS stimulation increased the protein levels of phospho-STAT3 ($p = 0.000441$) (Figure 6B) and phospho-STAT3/STAT3 ($p = 0.000095$) (Figure 6B). Importantly, this effect was abolished after CMC23 administration (phospho-STAT3 $p = 0.00436$; phospho-STAT3/STAT3 $p = 0.012604$) (Figure 6B). Although WRW4 (10 μM) reversed the favorable effect of CMC23 in LPS-evoked OHCs, these changes were not statistically significant. Finally, since SOCS3 is a well-known inhibitor of

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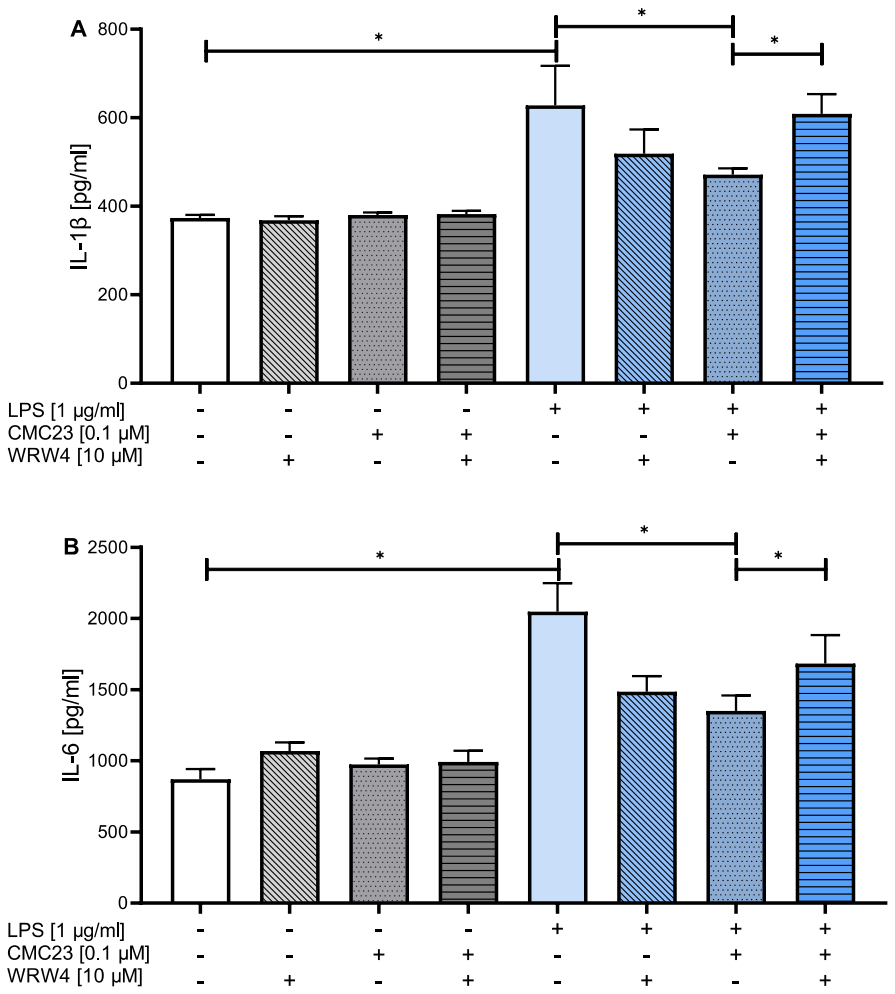

Figure 3. Impact of CMC23 and WRW4 on the release of proinflammatory cytokines IL-1β (A) and IL-6 (B) in LPS-stimulated OHCs. OHCs were pretreated with the FPR2 antagonist WRW4 (10 μM) for 30 min. Subsequently, OHCs were treated with CMC23 (0.1 μM) for 1 h and then stimulated with lipopolysaccharide (LPS; 1 μg/mL) for 24 h. Control slices were treated with the appropriate vehicle. The data are presented as the mean ± SEM of independent experiments, n = 5−8 in each experiment. Statistical analysis was performed using two-way analysis of variance (ANOVA) with the Duncan post hoc test to assess the differences between the treatment groups. Significant differences are indicated by *$p < 0.05$. IL-1β—interleukin 1β; IL-6—interleukin 6; LPS—lipopolysaccharide.

the Jak1/2/STAT3 activation pathway, we assessed the level of SOCS3 using ELISA. Twenty-four hours after LPS treatment, the protein level of SOCS3 was increased ($p = 0.03649$) (Figure 6C), and CMC23 maintained the LPS-evoked effect ($p = 0.038734$) (Figure 6C) in OHCs.

3. DISCUSSION

The present study provides, for the first time, evidence that FPR2 activation by the compound CMC23 limits cell death and inhibits the proinflammatory status of OHCs stimulated by bacterial endotoxin administration. Moreover, our results strongly supported the importance of the STAT3 pathway in the molecular anti-inflammatory mechanism of CMC23 activity, identified by the suppression of proinflammatory cytokines IL-6, IL-17A, and IL-23 during LPS-induced neuroinflammation in OHCs.

We have been studying the role of FPR2 in neuroinflammation for several years. Using the agonist MR-39,[22] we have demonstrated that the activation of FPR2 can attenuate the proinflammatory response in primary microglial cells, and this effect is long-lasting compared to that of LXA4, the endogenous FPR2 pro-resolving ligand.[23] In addition, *in vivo* administration of MR-39 (10 mg/kg) was able to improve the neuroinflammation status in APP/PS1, a mice model of Alzheimer's disease,[27] and in BTBR mice and mice prenatally exposed to valproic acid, which are two animal models of autism spectrum disorders.[28] However, MR-39 elicited *in vitro* neuroprotective and anti-inflammatory effects at micromolar doses, translating into high in vivo dosages that can lead to unwanted side effects. Therefore, in a subsequent study, we identified a new series of ureidopropanamide FPR2 agonists that are highly potent and have suitable pharmacokinetic properties.[11] Within this series, CMC23 [compound (*S*)-11l][11] emerged as a promising pharmacological tool for *in vivo* studies because it activated FPR2 in the submicromolar range (EC_{50} = 0.13 μM), elicited neuroprotective and anti-inflammatory effects in primary microglial cells at nanomolar concentrations, had acceptable metabolic stability ($t_{1/2}$ = 44 min), was rapidly distributed after i.p. administration (plasma t_{max} = 30 min and C_{max} = 192 ng/mL), and accumulated in the brain with a brain-to-plasma ratio of 0.378.

Hence, in this study, we studied the neuroprotective and anti-inflammatory profile of CMC23 in OHCs and the molecular pathways related to the observed anti-inflammatory effects. The

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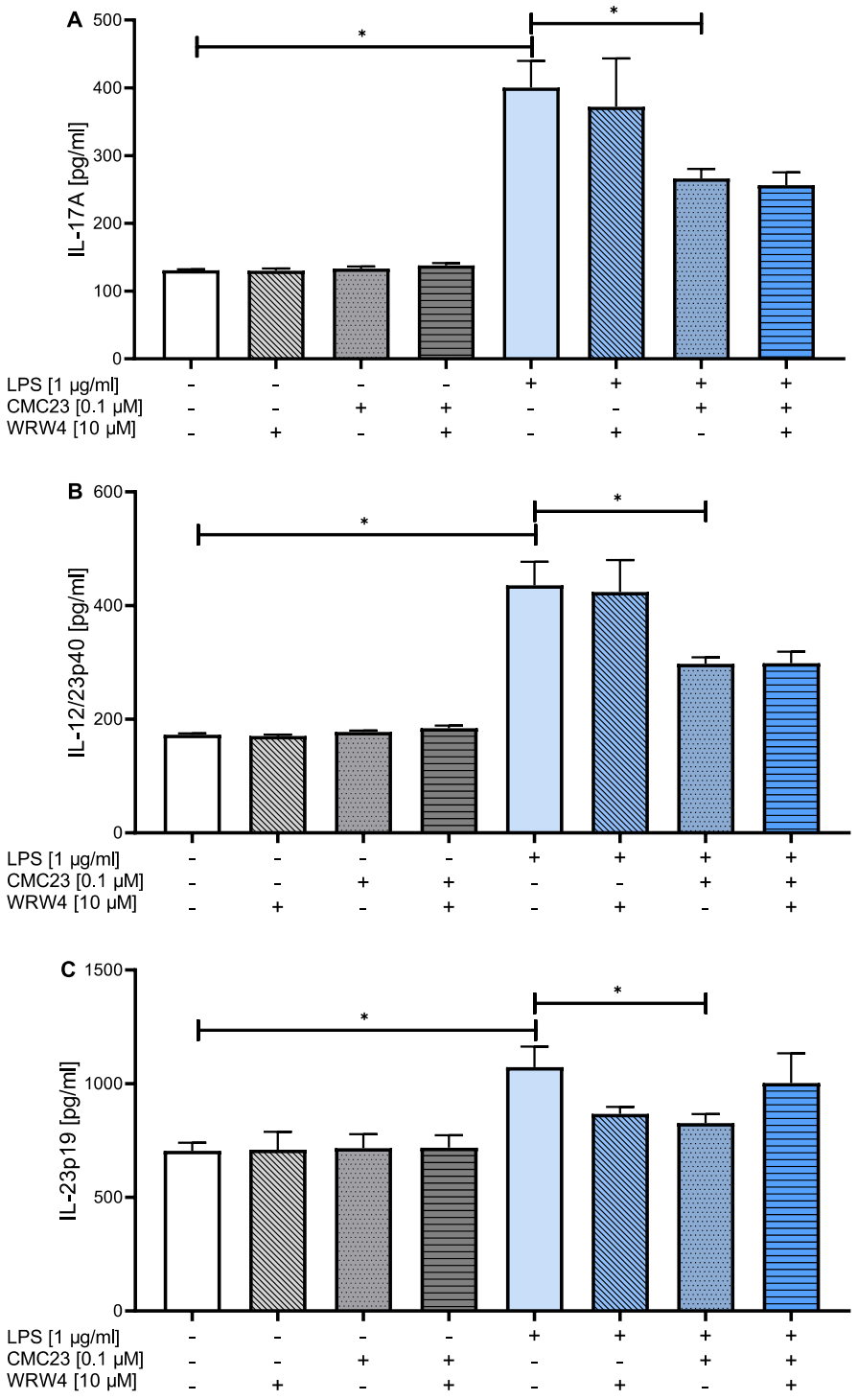

Figure 4. Impact of CMC23 and WRW4 on the release of IL-17A (A), IL-23 subunits IL-12/23p40 (B), and IL-23p19 (C) in LPS-stimulated OHCs. OHCs were pretreated with the FPR2 antagonist WRW4 (10 μM) for 30 min. Subsequently, OHCs were treated with CMC23 (0.1 μM) for 1 h and finally with lipopolysaccharide (LPS; 1 μg/mL) for 24 h. Control slices were treated with the appropriate vehicle. The data are presented as the mean ± SEM of independent experiments, $n = 4–9$ in each experiment. Statistical analysis was performed using two-way analysis of variance (ANOVA) with the Duncan post hoc test to assess the differences between the treatment groups. Significant differences are indicated by $^*p < 0.05$. IL-17A—interleukin 17A; IL-12/23p40—interleukin 12/23p40; IL-23p19—interleukin 23p19; LPS—lipopolysaccharide.

primary dissociated cultures permit a single homogeneous cell population to be studied. However, there is a clear need to explore the function of brain cells in a 3D system where the prominent architecture of the cells is preserved. Considering past observations and data, which postulate the presence of FPR2 in other cell types besides microglial cells in the brain, we introduced an OHC model in the present research. This experimental design maintains the functional interaction between several cell types and the neuroimmune and endocrine systems in a complex network. Thus, OHCs are an exciting tool for investigating the neuroimmune processes of the brain *ex vivo*.[27,29] To induce the neuroinflammatory condition, we used a

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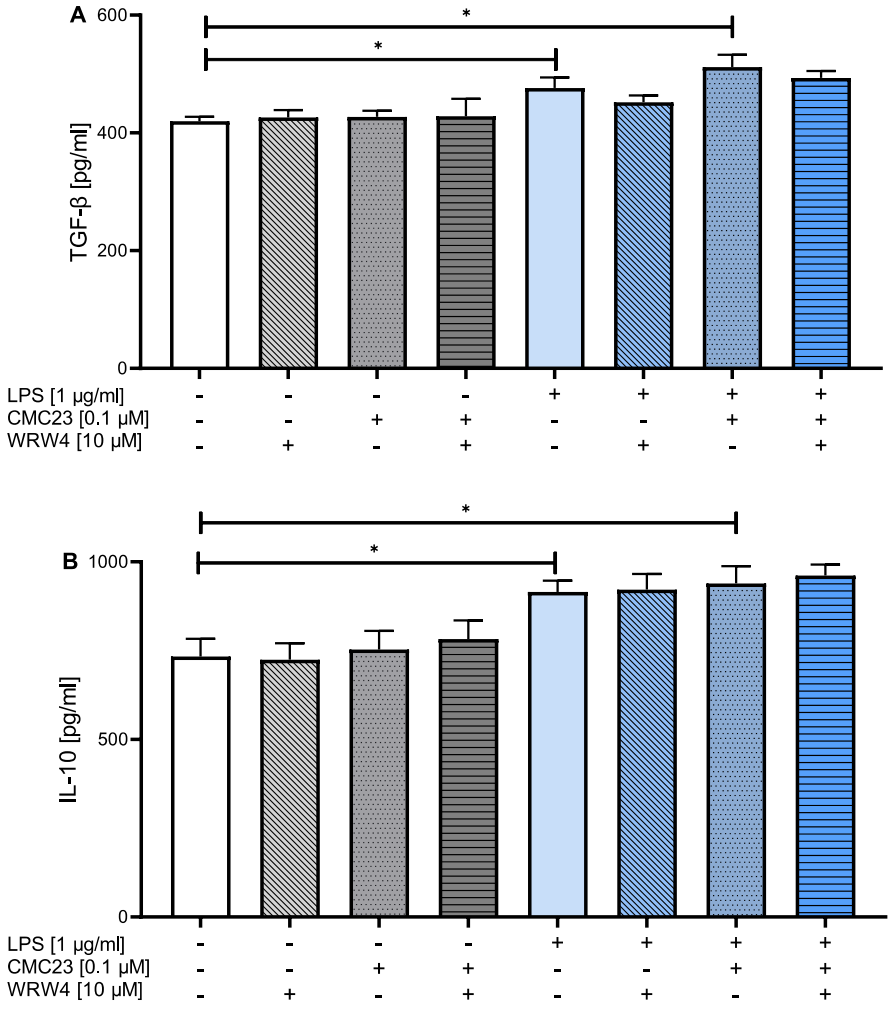

Figure 5. Impact of CMC23 and WRW4 on the release of anti-inflammatory cytokines TGF-β (A) and IL-10 (B) in LPS-stimulated OHCs. OHCs were pretreated with the FPR2 antagonist WRW4 (10 μM) for 30 min. Subsequently, the OHCs were treated with CMC23 (0.1 μM) for 1 h and then with lipopolysaccharide (LPS; 1 μg/mL) for 24 h. Control slices were treated with the appropriate vehicle. The data are presented as the mean ± SEM of independent experiments, $n = 6–8$ in each experiment. Statistical analysis was performed using two-way analysis of variance (ANOVA) with the Duncan post hoc test to assess the differences between the treatment groups. Significant differences are indicated by $^*p < 0.05$. TGF-β − transforming growth factor − β; IL-10—interleukin 10; LPS—lipopolysaccharide.

nonspecific activator of bacterial origin—LPS—a commonly accepted approach, especially *in vitro*, to study the modulatory mechanisms of proinflammatory processes.[30] This model of immunostimulation was also used in our previously published studies to evaluate the pro-resolving ability of the MR-39 agonist in OHCs. Therefore, in this paper, we continued the research using this endotoxin in the context of the potential of CMC23 to a potential resolution of inflammation (RoI), the endogenous deficits of which are increasingly described in the context of various brain diseases, including neurodegenerative or psychiatric ones.[31]

In this study, we reported the colocalization of FPR2 with microglial cells, astrocytes, and neurons in OHCs. These data complement and extend previous observations made in microglial cultures, including the finding that 24 h after stimulation with LPS, FPR2 expression is upregulated, as shown by fluorescence intensity.[23] Moreover, these data are in agreement with the results published by other authors who used different models, also in *in vivo* studies, which showed the expression of FPR2 in various cell types in the hippocampus.[32,33] Hence, the effect of CMC23 described in this paper should be widespread and extended to FPR2 expressed in microglial cells, astrocytes, and neurons.

We demonstrated that CMC23 attenuated the LPS-induced LDH release. This effect was mediated by FPR2, as it disappeared when the OHCs were pretreated with the FPR2 antagonist WRW4. Since the protective activity of CMC23 was dose-dependent and was observed at nanomolar concentrations, it could be proposed as a valuable pharmacological tool for in vivo studies. The impact on NO production was also assessed to complete the characterization of the neuroprotective properties of CMC23. NO is a cellular messenger essential in many physiological processes in the brain, but excessive NO synthesis leads to neuronal cell death. Although we observed that LPS stimulation increases the NO level, neither CMC23 nor WRW4 modulated NO release both under resting conditions and after stimulation. Therefore, CMC23 has limited antioxidant potential compared to that of MR-39.[23] In our earlier studies in microglial cultures, we did not observe any protective effect of LXA4 and AT-LXA4 on LPS-induced NO potentiation, which shows that some endogenous FPR2 ligands do not have both antioxidant and pro-resolving effects.[23]

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A

Factor	Gene expression			
	Control	LPS	CMC23	CMC23+LPS
Jak1	1,16 ± 0,34	1,84 ±1,16	1,52 ± 0,52	1,79 ± 0,34
Jak2	1,14 ± 0,30	2,84 ± 1,51	2,11 ± 0,57	3,00 ± 0,49
Stat3	1,08 ± 0,17	1,89 ± 0,97	2,39 ± 0,73	3,15 ± 0,78

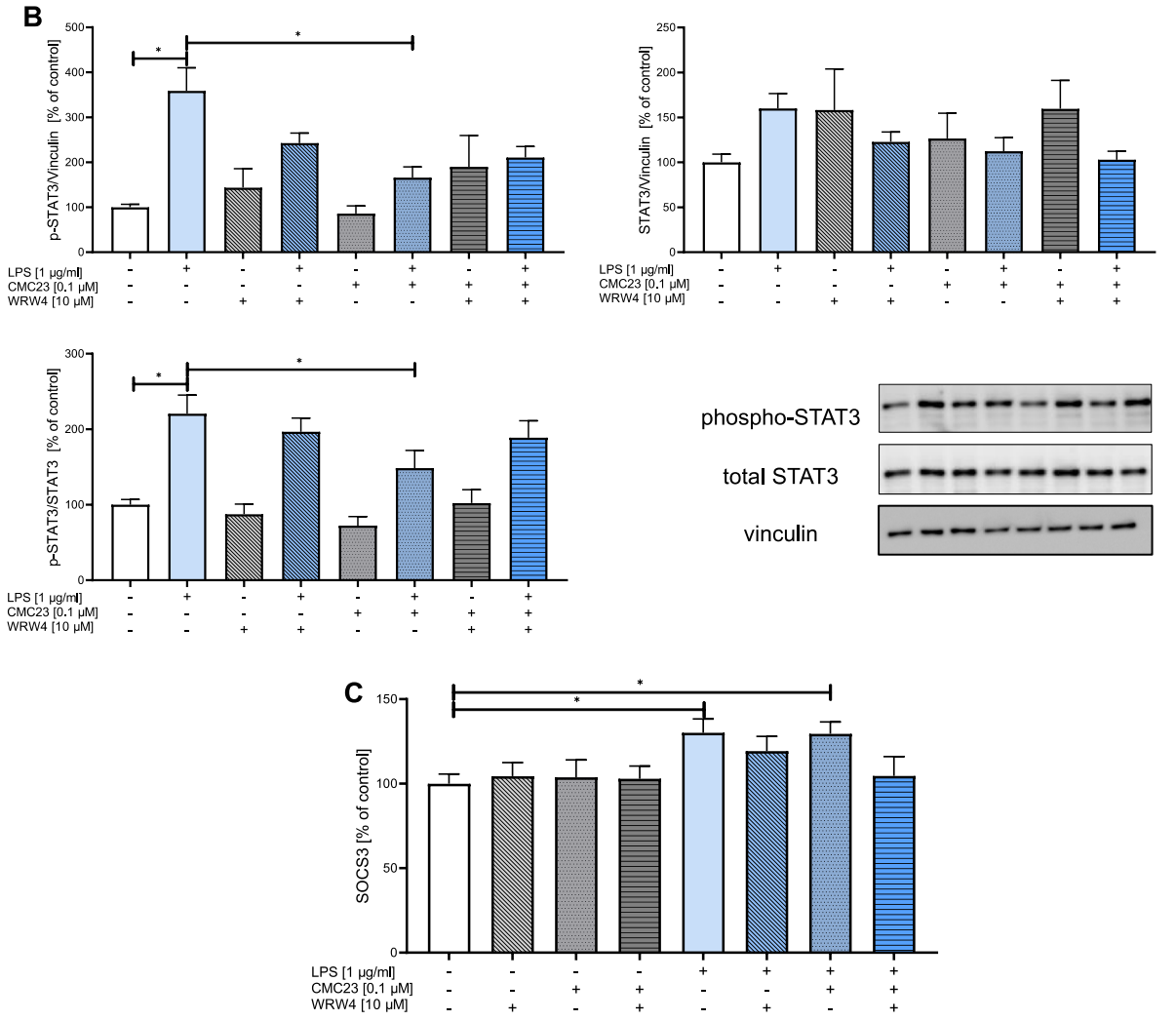

Figure 6. Impact of CMC23 and WRW4 on the gene expression of *Jak1*, *Jak2*, and *Stat3* (A) and protein level of phospho-STAT3, STAT3 (B), and SOCS3 (C) in LPS-stimulated OHCs. OHCs were pretreated with the FPR2 antagonist WRW4 (10 μM) for 30 min. Subsequently, OHCs were treated with CMC23 (0.1 μM) for 1 h and then stimulated with lipopolysaccharide (LPS; 1 μg/mL) for 24 h. Control slices were treated with the appropriate vehicle. The data are presented as the average fold change ± SEM (A) and mean ± SEM percentage of control (vehicle-treated OHCs) of independent experiments (B), (C) $n = 3-8$ in each experiment. Statistical analysis was performed using two-way analysis of variance (ANOVA) with the Duncan post hoc test to assess the differences between the treatment groups. Significant differences are indicated by $^*p < 0.05$. JAK1—Janus kinase 1; JAK2—Janus kinase 2; STAT3—signal transducer and activator of transcription 3; SOCS3—suppressor of cytokine signaling 3; LPS—lipopolysaccharide.

In the brain, microglia are the crucial targets in response to the immune challenge evoked by LPS administration. Therefore, we assessed the impact of CMC23 on the microglial trajectory in OHCs. We observed that CMC23 attenuated the upregulation of *Cd40* and *Cd68* expression. Since CD40 activation must be the first step of microglial cell activation along with microglial cell proliferation and upregulation of other markers (e.g., MHC class II and CD86), CMC23 can inhibit the inflammatory response in the initiation phase.[34,35] Additionally, the effect of CMC23 on the gene expression of *Cd68* suggests that it influences the elimination of cellular debris via the phagocytic activity of microglia after LPS stimulation. Moreover, CMC23 treatment tends to downregulate LPS-evoked *Ccl2* expression. CCL2 participates in multiple neuroinflammatory processes, mainly through the recruitment of glial cells. However, CCL2 has also been proven to exert different actions on these cells, including modifying their response to inflammatory stimuli. Interestingly the genetic removal of CCL2 increases the

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expression of the enzymes responsible for the synthesis of SPMs, including arachidonate 15-lipoxygenase and arachidonate 5-lipoxygenase in the brain cortex of 5xFAD mice.[36] The expression of the FPR2, known to mediate the activity of pro-resolving mediators, was also increased in mice lacking CCL2. Therefore, it may be proposed that CMC23-induced suppression of *Ccl2* may be at least in part crucial in enhancing RoI.[37]

In contrast, the LPS-evoked downregulation of *Igf-1* was not affected, while *Arg-1* upregulation was even slightly suppressed by CMC23 administration. The potential beneficial role of ARG-1 in inflammation involves competition with iNOS, as both of these factors utilize L-arginine as a substrate.[23,38,39] Thus, the decrease in *Arg-1* expression may come from *iNOS* activation and related NO production in OHCs. Nevertheless, the potential compensatory participation of ARG-1 in repairing microglial damage after LPS stimulation is also noted.

As a follow-up, in this study, we investigated the anti-inflammatory and pro-resolving ability of CMC23 in OHCs during neuroinflammation. Regarding the anti-inflammatory factors, CMC23 did not affect the LPS-induced increase of TGF-β and IL-10 expression. Unexpectedly, in the case of IL-10, discrepancies were observed between the expression and protein levels.

Moreover, a differential effect of CMC23 on gene and protein expression levels was also observed in the case of proinflammatory cytokine IL-6. These contrasting effects might emerge from alterations in the regulation of various stages of mRNA expression, starting with changes in the chromatin conformation, gene activation in response to external stimuli, and control of the transcription process.[40,41] In the case of IL-6, the disturbances in the mRNA's nuclear retention, an essential mechanism for maintaining the dynamic balance between de novo transcription and protein translation, should be considered.[42] Generally, transcript levels alone are not sufficient to predict cytokine protein levels in many scenarios, and the genotype−phenotype patterns may be modulated by various processes, which may reduce the correlation of protein and respective mRNA levels at the cellular level.[43]

Nevertheless, in the case of IL-10, some compensatory mechanisms leading to the maintenance and enhancement of the proper level of biologically active IL-10 in the OHCs might be stressed.

LPS is one of the most potent bacterial inducers of cytokine release, including TNF-α, IL-1β, and IL-6, and anti-inflammatory factors, such as IL-10. In fact, LPS triggers the induction of IL-10 secretion, efficiently preventing pro-IL-1β expression. Thus, the balance between IL-10 induction and the level of pro-IL1β potentially determines the final level of IL-1β,[44] which in our study was reduced by CMC23 treatment. Our previous studies showed that the FPR2 agonist MR-39 suppresses LPS-evoked IL-1β release by inhibiting the NLRP3/NF-κB pathway.[29] Therefore, it may be suggested that the same mechanism may also be considered in the anti-inflammatory effect of CMC23. Nevertheless, as we demonstrated, the strong pro-resolving effect of CMC23 was also reflected in the LPS-induced reduction in IL-6 release. Notably, the beneficial impact of CMC23 on the protein levels of IL-1β and IL-6 was FPR2-mediated, as the FPR2 antagonist WRW4 weakened this pro-resolving effect.

Since the growing body of evidence points to the importance of IL-23, which is secreted in the brain by astrocytes and infiltrating macrophages under inflammatory conditions[45,46] in parallel in the defense mechanism in bacterial infections,[47−49] we investigated the potential ability of CMC23 to modulate this proinflammatory factor. IL-23 consists of p19 and a common p40 subunit shared by the structurally related IL-12.[50−52] Although the heterodimeric molecule is the bioactive cytokine and both subunits p19/p40 for IL-23 and p35/p40 for IL-12 must be coexpressed in the same cell to generate the bioactive form, an effector function of p40 alone by microglia upon immune stimulation was postulated.[45] Our study demonstrated that LPS-induced IL-12/23p40 and IL-23p19 release was strongly inhibited by CMC23 treatment. FPR2 partially mediated this effect, as it tended to be attenuated by pretreatment with WRW4 in OHCs. It is well-known that after binding to the IL-23 receptor, IL-23 leads to conformational changes and, subsequently, to the phosphorylation of STAT3. Paradoxically, the STAT3 pathway can also be activated by IL-6 and IL-10.[44,53] In addition, STAT3 activation in response to IL-6 in combination with TGF-β and IL-23 leads to the activation of brain immunocompetent cells.[54−56] Thus, the role of IL-23 as a broad regulator of late-stage inflammatory processes should be postulated.[45]

An intriguing observation in this study was that CMC23 strongly inhibited STAT3 phosphorylation induced by LPS, and this effect tended to be mediated by FPR2 activation. Recently, data showed that the activation of FPR2 by ANAX1 inhibited STAT3 phosphorylation.[57] ANAX1 also inhibited IL-23 and IL-17A release in this experimental system. These findings may be interesting in the context of the pro-resolving effects induced by CMC23 since we observed that this ligand inhibited the LPS-induced increase in IL-1β, IL-6, and IL-17A levels that were most likely released mainly by activating glial cells (especially microglia),[58,59] which prolonged the inflammatory response. Therefore, the inhibitory potential of CMC23 demonstrated in this research may be crucial in limiting chronic inflammatory processes and supporting disturbed endogenous RoI mechanisms in OHCs.

The JAK/STAT3/SOCS3 signaling pathway actively participates in cytokine signaling regulation. Therefore, we also evaluated the impact of CMC23 on the SOCS3 level. In our study, none of the compounds affected the SOCS3 level under the resting conditions. However, after immune challenge, CMC23 (and LPS alone) maintained an increased level of this protein in OHCs. Therefore, we suggest that inflammatory activation induces regulatory mechanisms in OHCs that are supposed to prevent excessive inflammatory responses by limiting STAT3 activation. Qin et al.[60] demonstrated the prominent role of SOCS3 in suppressing IL-6 signaling by inhibiting STAT3 and IL-23 release. Moreover, the ability of IL-10 to induce *de novo* synthesis of SOCS3 was correlated with its ability to modulate the trajectory of microglia and inhibit proinflammatory genes, including LPS-inducible IL-1β.[61,62] Hence, it is possible to hypothesize that the lack of effect of CMC23 on the elevated level of SOCS3 in OHCs allows for the proper regulation of the JAK/STAT3 pathway and, consequently, modulation of LPS-induced RoI activation, at least in part by changing the polarization of microglia.[63] A similar trend as that in the current study has also been observed in microglial cell cultures, where the activity of the JAK2/STAT3 signal transduction pathway rapidly increased in response to LPS stimulation. Simultaneously, this effect was related to SOCS3 upregulation which, in a time-dependent manner, efficiently blocked STAT3 function, regulating the secretion of inflammatory cytokines.[64−66] The relationship between the

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inflammatory response in the brain and STAT3 and SOCS2/3 levels was also observed in our previous research in an *in vivo* model.[67]

We are aware that our study has some limitations. The proposed pro-resolving mechanisms of CMC23 action in the applied *ex vivo* model undoubtedly require further verification. The use of siRNA, currently unavailable to our research team, may be particularly important in the introduced paradigm. The effectiveness of these techniques in organotypic cultures has recently been the subject of many intensive studies. Undoubtedly, their optimization will allow their implementation in the future also in the study of the mechanisms of action of new agonists in the RoI processes.

4. CONCLUSIONS

The results reported herein provide a new approach to understanding the role of FPR2 activation and downstream signaling modulation in the mechanisms that lead to the limitation of inflammatory response. The newly identified FPR2 agonist CMC23 inhibits LDH release and modulates microglial trajectory and proinflammatory cytokine production in OHCs stimulated with bacterial endotoxin. Moreover, our data provide the first evidence that points to the crucial role of the STAT3-regulated pathway in the molecular anti-inflammatory mechanism of CMC23.

The strong protective and pro-resolving effect of CMC23 suggests that this compound is a valuable tool to support endogenous deficits in suppressing inflammatory reactions. Furthermore, since neuroinflammatory activation contributes to the pathogenesis of various brain disorders, CMC23 may be considered a potential therapeutic agent for inflammatory-based pathologies.

5. MATERIALS AND METHODS

5.1. Animals. Sprague−Dawley rats (Charles River, Sulzfeld, Germany) were housed under standard conditions at room temperature (23 °C) under a 12/12 h light/dark cycle beginning at 8:00 and given ad libitum access to food and water. Animals were kept for 1 week for acclimation, and the proestrus phase of the menstrual cycle was identified by taking vaginal smears daily. Once the proestrus phase was detected, females were paired with males for 12 h, and afterward, the presence of sperm in the vaginal smear was checked. During pregnancy, females were kept undisturbed under standard conditions in home cages. The experiments were approved by the Committee for Laboratory Animal Welfare and Ethics of the Maj Institute of Pharmacology, Polish Academy of Sciences, Cracow, Poland (approval no. 204/2018, 28.06.2018).

5.2. Chemicals. The FPR2 agonist compound CMC23 [(*S*)-1-(3-(4-cyanophenyl)-1-(6-fluoroindolin-1-yl)-1-oxopropan2-yl)-3-(4-fluorophenyl)urea] was synthesized at the Department of Pharmacy, University of Bari, according to the procedure previously described.[11] The spectroscopic properties were in agreement with those previously reported.[11] The FPR2 antagonist WRW4 was obtained from Alomone Laboratories, Israel. The bacterial endotoxin LPS (*Escherichia coli* 0111:B4) was purchased from Sigma-Aldrich, St. Louis, MO, USA.

5.3. Establishment of Organotypic Hippocampal Cultures. Six- to seven-day-old Sprague−Dawley rat pups were used to establish OHCs. Cultures were prepared following the Stoppini et al.[68] method with our slight modifications. Briefly, after decapitation, brains were placed in a sterile ice-cold working buffer (96% HBSS, 3.5% glucose, and 0.5% penicillin/streptomycin; all reagents were obtained from Gibco, Waltham, MA, USA). Then, isolated hippocampi were placed on Teflon discs and cut into 350 μm slices with a McIlwain tissue chopper. Selected sections were placed on ThinCerts-TC Inserts with 0.4 μm pore size membranes (Greiner bioone, Kremsmünster, Austria) and cultured in 1 mL of medium containing 25% horse serum (50% DMEM + GlutaMax-I, pH 7.4; 20.5% HBSS; 25% horse serum; 0.1 mg/mL glucose; 1% amphotericin B; 0.4% penicillin and streptomycin; 1% B-27 supplement) and HEPES (all reagents were obtained from Gibco, UK). OHCs were grown in 6-well plates for 7 days (DIV) in an incubator (37 °C) with an adjustable CO_2 flow (5%). The initial medium was changed 24 h later (0.5 mL of medium) and then every 48 h (1 mL of medium). On the fifth DIV, the concentration of horse serum in the medium was tapered down to 10% (50% DMEM + GlutaMax-I, pH 7.4; 35.5% HBSS; 10% horse serum; 0.1 mg/mL glucose; 1% amphotericin B; 0.4% penicillin and streptomycin; 1% B-27 supplement; and HEPES) (all reagents were obtained from Gibco, UK), and finally, on the seventh DIV, the medium was changed to serum-free medium (containing 50% DMEM F-12, pH 7.4; 44% HBSS; 0.1 mg/mL glucose; 1% amphotericin B; 0.4% penicillin and streptomycin, 1% B-27, 1% N-2; and HEPES) (to maintain the pH).

5.4. Treatment. On the seventh DIV, OHCs were pretreated for 30 min with the FPR2 antagonist WRW4 (10 μM). Then, a new FPR2 agonist, compound CMC23 (in two doses: 0.1 or 1 μM), was added to the culture medium for 1 h, and OHCs were stimulated with 1 μg/mL LPS for 24 h. Control groups were subjected to the appropriate vehicle in the corresponding volume and regimen. Stock solutions of the examined chemicals were prepared as follows: compound CMC23 in DMSO to 1 mM concentration, LPS in PBS to 1 mg/mL concentration, and WRW4 in distilled water to 1 mM concentration. The final concentrations of used compounds were in distilled water.

5.5. Lactate Dehydrogenase Release Assay. Twenty-four hours after LPS administration, LDH release into the culture medium was measured using the colorimetric method as described previously[69] according to the manufacturer's instructions (Cytotoxicity Detection Kit, Roche Diagnostic, Mannheim, Germany). The data were normalized to the LDH release values of the control groups (100%; vehicle-treated WT OHCs) and presented as a percentage of the control ± the standard error of the mean (SEM).

5.6. Nitric Oxide Release Assay. To determine the quantitative release of NO into the culture medium, the Griess reaction was performed as previously described.[70] Briefly, 24 h after LPS administration, 50 μL of collected supernatants and an equal volume of Griess A (0.1% *N*-1-naphthyl ethylenediamine dihydrochloride) and Griess B (1% sulfanilamide in 5% phosphoric acid) were mixed in a 96-well plate. After 10 min of incubation, the absorbance was measured at 540 nm wavelength using an Infinite M200PRO microplate reader (TECAN, Männedorf, Switzerland). The data were normalized to the NO release of the control groups (100%; vehicle-treated OHCs) and presented as a percentage of the control ± SEM.

5.7. Quantitative Real-Time Polymerase Chain Reaction. Organotypic hippocampal slices were lysed using 200 μL of TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA) 24 h after LPS administration. The samples were stored at −20 °C until isolation. Total RNA was extracted according to the manufacturer's instructions (TRIzol Reagent User Guide Instructions; Thermo Fisher Scientific, Waltham, MA, USA) based on the Chomczynski[71] method. Immediately after isolation, the RNA concentration was assessed with a NanoDrop spectrophotometer (ND/1000 UV/vis, Thermo Fisher NanoDrop, Waltham, MA, USA). Then, cDNA synthesis was conducted via reverse transcription using an NG dART RT kit (EURx, Gdansk, Poland) following the manufacturer's instructions. Obtained cDNA was amplified using a FastStart Universal Probe Master (Rox) kit (Roche, Basel, Switzerland) and TaqMan probes (Thermo Fisher Scientific, Waltham, MA, USA) for the following genes: *Arg1* (*arginase 1*; Rn00691090_m1), *Ccl2* (*C−C motif chemokine ligand 2*; Rn00580555_m1), *Cd40* (*cluster of differentiation 40*; Rn01423590_m1), *Cd68* (*cluster of differentiation 68*; Rn01495634_g1), *Igf-1* (*insulin-like growth factor 1*; Rn00710306_m1), *Il-6* (*interleukin 6*; Rn01410330_m1), *Il-10* (*interleukin 10*; Rn01644839_m1), *Il-12* (*interleukin 12a*; Rn00584538_m1), *Il-23* (*interleukin 23a*; Rn00590334_g1), *Jak1* (*Janus kinase 1*; Rn01763899_m1), *Jak2* (*Janus kinase 2*; Rn00676341_m1), *Stat3* (*signal transducer and activator of transcription 3*; Rn00680715_m1), and *Tgf-β1* (*transforming growth factor β1*; Rn00572010_m1) (all obtained from Thermo Fisher Scientific,

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Waltham, MA, USA). Furthermore, β2 microglobulin (*B2M*; Rn00560865_m1) was used as a normalizing control. The samples were run on a CFX96 Real-Time System (BIO-RAD, Hercules, CA, USA). The threshold value (Ct) for each sample was set in the exponential phase of PCR, and the ΔΔCt method was used for data analysis.

5.8. Enzyme-Linked Immunosorbent Assays. Twenty-four hours after LPS administration, culture medium from OHCs was collected to measure the levels of the following cytokines: interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 10 (IL-10), interleukin 12p40 (IL-12p40), interleukin 17A (IL-17A), interleukin 23p19 (IL-23p19), and transforming growth factor beta (TGF-β). Furthermore, OHCs were lysed in 160 μL of RIPA buffer with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA, USA). Then, the level of suppressor of cytokine signaling 3 (SOCS3) was measured in lysed OHCs. The cytokine levels were measured using commercially available ELISA kits obtained from Wuhan Fine Biotech Co., Ltd. Wuhan, China (IL-1β, IL-12p40, IL-17A, IL-23p19, and TGF-β); BD Biosciences, Franklin Lakes, NJ, USA (IL-6 and IL-10); and ELK Biotechnology, Wuhan, China (SOCS3). All assays were conducted according to the manufacturer's instructions. The detection limits were as follows: IL-1β < 18.75 pg/mL, IL-6 < 78 pg/mL, IL-10 < 15.6 pg/mL, IL-12p40 < 7.031 pg/mL, IL-17A < 9.375 pg/mL, IL-23p19 < 9.375 pg/mL, TGF-β < 18.75 pg/mL, and SOCS3 < 0.059 ng/mL. The interassay precision of all ELISA kits was CV % < 10%. The intra-assay precision of all ELISA kits was CV % < 8%.

5.9. Western Blot Analyses. Western blot analyses were performed as previously described.[72,73] Briefly, 24 h after treatment, OHCs were lysed in RIPA buffer with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA, USA). Protein concentration analysis of the samples was conducted using a BCA protein assay kit (Sigma-Aldrich, St. Louis, MO, USA) according to the supplier's instructions, and the optical density was measured using an Infinite M200PRO microplate reader (TECAN, Männedorf, Switzerland). Then, lysates (equal protein concentration) in 4× Laemmli buffer (Roche, Basel, Switzerland) were heated in an Eppendorf thermomixer comfort (Sigma-Aldrich, St. Louis, MO, USA) for 8 min at 95 °C, separated by SDS−PAGE (4−20% Criterion TGX Precast Gels, with 26 Well Comb, Bio-Rad, Hercules, CA, USA) and transferred to polyvinylidene fluoride (PVDF) membranes (Trans-Blot Turbo, Bio-Rad, Hercules, CA, USA). Next, the membranes were washed with Tris-buffered saline [(TBS) pH = 7.5], blocked in 5% bovine serum albumin for 1 h at RT, and incubated overnight at 4 °C with the primary antibodies, antiphospho-STAT3 (1:1000, #9145, Cell Signaling, Danvers, MA, USA), and antivinculin (1:15000, V9264, Sigma-Aldrich, St. Louis, MO, USA), diluted in a Signal Boost Immunoreaction Enhancer kit buffer (Millipore, Warsaw, Poland). Afterward, membranes were washed in TBS containing 0.1% Tween-20 (TBST) three times for 10 min and incubated with the horse antimouse immunoglobulin G (IgG, 1:10,000, PI-2000 Vector Laboratories) and goat-antirabbit IgG (1:10,000, PI-1000, Vector Laboratories) secondary antibodies for 1 h. After the second incubation, the blots were washed again with TBST three times for 10 min, detected using Pierce ECL Western blotting substrate (Thermo Fisher, Waltham, MA, USA) and visualized by a Fujifilm LAS1000 system (Fuji Film, Tokyo, Japan). After visualization, the membranes were stripped with stripping buffer containing 100 μL of Tris−HCl (pH = 6.7), 2% SDS, and 700 μL of 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA). Then, membranes were reprobed with antibodies against the unphosphorylated protein STAT3 (1:500, #9139, Cell Signaling, Danvers, MA, USA) diluted in a Signal Boost Immunoreaction Enhancer kit buffer (Millipore, Warsaw, Poland). Detection and visualization were carried out as previously described. The relative levels of immunoreactivity were quantified by using Fujifilm Multi Gauge software (Fuji Film, Tokyo, Japan).

5.10. Immunofluorescence Staining of Organotypic Hippocampal Cultures. Immunofluorescence staining of OHCs was performed according to the protocol reported in Gogolla et al.[74] with slight modifications. Briefly, the OHCs were fixed with 4% paraformaldehyde (PFA, ChemCruz, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h, washed with PBS, and kept at 4 °C until experiments. Next, before staining, hippocampal slices were cut off from the inserts, transferred to a 12-well plate, and permeabilized in 0.5% Triton X-100 in PBS for up to 18 h at 4 °C. The next day, the sections were kept in blocking solution (20% bovine serum albumin, Sigma-Aldrich, St. Louis, MO, USA) overnight at 4 °C. OHCs were incubated with an anti-FPR2 rabbit polyclonal antibody (Huabio, Greater Boston, MA, USA; 1:50) and then with a goat antirabbit antibody conjugated with the fluorescent dye Alexa Fluor 647 (Abcam, Cambridge, UK; 1:300) overnight at 4 °C in a closed wet chamber. Secondary staining was performed using one of the primary antibodies: anti-IBA1 goat polyclonal antibody, anti-MAP2 chicken polyclonal antibody, or anti-GFAP chicken polyclonal antibody (all obtained from Abcam Cambridge, UK; 1:50). Subsequently, one of the following secondary antibodies was used: donkey antigoat conjugated with the fluorescent dye Alexa Fluor 555 or goat antichicken conjugated with the fluorescent dye Alexa Fluor 488 (both Abcam, Cambridge, UK; 1:300). Finally, after washing in 5% BSA in PBS, sections were incubated in Hoechst 33342 (Invitrogen, Waltham, MA, USA; 1:5000) for 15 min at RT in the dark to stain the nuclei. Next, the sections were briefly washed and placed onto glass microscope slides, mounted using ProLong Glass Antifade Mountant (Invitrogen, Waltham, MA, USA), covered with cover glass, and kept at 4 °C until imaging with a Leica TCS SP8 X confocal laser-scanning microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) using a 63x HC PL APO CS2 1.40 NA oil immersion objective. The images were reconstructed using ImageJ 1.53n (Wayne Rasband, National Institute of Health, Bethesda, MD, USA).

5.11. Statistical Analyses. Statistical analyses were performed using Statistica 13.3 software (Stat Soft, Tulsa, Tulsa, Tulsa, USA). All biochemical experiments were carried out under the same conditions for all samples, regardless of the type of treatment. The presented results were obtained from three independent experiments conducted under the same conditions, and "*n*" for each culture was 2−4. All data are presented as the mean ± SEM. The results of the LDH and NO release assays, Western blotting, confocal fluorescence image analysis, and SOCS3 ELISA are presented as the mean percentage ± SEM of the control. The data obtained from the other ELISAs are presented as the mean ± SEM, and those for qRT-PCR are presented as the average fold change ± SEM. All groups were compared using factorial analysis of variance (ANOVA) to determine the effects of the factors, followed, when appropriate, by Duncan's post hoc test. A p value of less than 0.05 was considered to be statistically significant. All graphs were prepared using GraphPad Prism 9.

■ ASSOCIATED CONTENT

Data Availability Statement

All data supporting the conclusions of this manuscript are provided in the text, figures, and tables.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschemneuro.3c00525.

Western blot analysis of lysates from organotypic hippocampal cultures demonstrating the protein level of phospho-STAT3 and total STAT3 protein and schematic representation of the hippocampus (PDF)

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Author Contributions

#K.T. and E.T. contributed equally to this work. Conceptualization: E.T., K.T., and A.B.-K.; methodology: E.T., K.T., M.L., and F.F.; formal analysis: E.T. and K.T.; investigation: M.L., E.T., and, K.T.; resources: A.B.-K.; data curation: M.L., K.T., and E.T. with supervision from A.B.-K.; writing—original draft preparation: E.T., K.T., and A.B.-K.; writing—review and editing: E.T., K.T., M.L., E.L., M.L., and A.B.-K.; supervision: A.B.-K.; project administration: A.B.-K.; and funding acquisition: E.L. and A.B.-K. All authors have read and agreed to the published version of the manuscript.

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Notes

The authors declare no competing financial interest.

Institutional Review Board Statement: All procedures were approved by the Animal Care Committee of the Maj Institute of Pharmacology, Polish Academy of Sciences, Cracow, and met the criteria of the International Council for Laboratory Animals and Guide for the Care and Use of Laboratory Animals (approval no. 204/2018, 28.06.2018).

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