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# Ocena roli ligandów receptora ALX/FPR2 w wyciszaniu procesów zapalnych w ośrodkowym układzie nerwowym: badania w doświadczalnych modelach immunoaktywacji

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*Dla Mamy i Taty…*

## Spis artykułów naukowych stanowiących podstawę rozprawy doktorskiej

1. **Tylek K.,** Trojan E., Regulska M., Lacivita E., Leopoldo M., Basta-Kaim A. (2021) Formyl peptide receptor 2, as an important target for ligands triggering the inflammatory response regulation: a link to brain pathology. *Pharmacological Reports.* 73(4):1004-1019. doi: 10.1007/s43440-021-00271-x. 5-letni współczynnik wpływu czasopisma: 3.472; Punkty MNiSW: 140

2. **Tylek K.,** Trojan E., Leśkiewicz M., Regulska M., Bryniarska N., Curzytek K., Lacivita E., Leopoldo M, Basta-Kaim A. (2021) Time-Dependent Protective and Pro-Resolving Effects of FPR2 Agonists on Lipopolysaccharide–Exposed Microglia Cells Involve Inhibition of NF-κB and MAPKs Pathways. *Cells.* 2021 9;10(9):2373. doi: 10.3390/cells10092373. 5-letni współczynnik wpływu czasopisma: 7.677; Punkty MNiSW: 140

3. **Tylek K.,** Trojan E., Leśkiewicz M., Francavilla F., Lacivita E., Leopoldo M., Basta-Kaim A. (2023) Stimulation of Formyl Peptide Receptor-2 by the New Agonist CMC23 Protects against Endotoxin-Induced Neuroinflammatory Response: A Study in Organotypic Hippocampal Cultures. *ACS Chemical Neuroscience,* 2023 Oct 18;14(20):3869-3882. doi: 10.1021/acschemneuro.3c00525. 5-letni współczynnik wpływu czasopisma: 4.8; Punkty MNiSW: 140

4. **Tylek, K.;** Trojan, E.; Leśkiewicz, M.; Ghafir El Idrissi, I.; Lacivita, E.; Leopoldo, M.; Basta-Kaim, A. (2023) Microglia Depletion Attenuates the Pro-Resolving Activity of the Formyl Peptide Receptor 2 Agonist AMS21 Related to Inhibition of Inflammasome NLRP3 Signalling Pathway: A Study of Organotypic Hippocampal Cultures. *Cells.* 2023 Nov 3;12(21):2570. doi: https://doi.org/10.3390/cells12212570 5-letni współczynnik wpływu czasopisma: 7.677; Punkty MNiSW: 140

5. W hybrydowej wersji rozprawy zamieszczono także wyniki nieopublikowanych badań *in vivo* przeprowadzone w zwierzęcym modelu uogólnionej immunoaktywacji indukowanej podaniem endotoksyny bakteryjnej – lipopolisacharydu (LPS).

# Spis treści

# 1. Wykaz najważniejszych skrótów

**Aβ** – Amyloid β (ang. *Amyloid-β*)

**AD** – Choroba Alzheimera (ang. *Alzheimer's disease*)

**ALX/FPR2** – Receptor formylowy 2 (ang. *Formyl peptide receptor 2*)

**ANXA1** – Aneksyna A1(ang. *Annexin-A1*)

**ARG-1** – Arginaza 1 (ang. *Arginase-1*)

**AT-LXA4** – Epilipoksyna (ang. *Aspirin-triggered lipoxin A4*)

**BBB** – Bariera krew-mózg (ang. *Blood-brain barrier*)

**CASP1** – Kaspaza 1 (ang. *Caspase-1*)

**CCL** – Białko chemotaktyczne monocytów (ang. *(C-C motif) chemokine ligand 2*)

**CD –** Antygen różnicowania komórkowego (ang. *Cluster of differentiation*)

**COX-2** – Cyklooksygenaza 2 (ang. *Cyclooxygenase-2*)

**CX** – Kora czołowa (ang. *Prefrontal cortex*)

**DAMP** – Wzorce molekularne związane z uszkodzeniami (ang. *Danger/Damage associated molecular patterns*)

**ERK** – Kinazy regulowane zewnątrzkomórkowo (ang. *Extracellular signal-regulated kinases*)

***f*MLP** – N-formylometionylo-leucylo-fenyloalanina (ang. *N-formyl-methionine-leucine-phenylalanine*)

**FPR** – Receptor formylowy (ang. *Formyl peptide receptor)*

**FST** – Test wymuszonego pływania (ang. *Forced swim test*)

**GPCR** – Receptor sprzężony z białkiem G (ang. *G protein-coupled receptors*)

**HP** – Hipokamp (ang. *Hippocampus*)

**IGF-1** – Insulinopodobny czynnik wzrostu 1 (ang. *Insulin-like growth factor 1*)

**IL** – Interleukina (ang. *Interleukin*)

**JAK –** Kinaza janusowa (ang. *c-Jun N-terminal kinases*)

**LDH** – Dehydrogenaza mleczanowa (ang. *Lactate dehydrogenase*)

**LPS** – Lipopolisacharyd (ang. *Lipopolysaccharide*)

**LXA4** – Lipoksyna A4 (ang. *Lipoxin A4*)

**MAPK** – Kinazy aktywowane mitogenami (ang. *Mitogen-activated protein kinases*)

**MARCO** – Receptor makrofagów o strukturze kolagenowej (ang. *Macrophage receptor with collagenous structure*)

**NF-κB** – Jądrowy czynnik transkrypcyjny (ang. *Nuclear factor kappa-light-chain-enhancer of activated B cells*)

**NLRP3 –** Receptor Nod-podobny 3, (ang. *Nod-like receptor protein 3*)

**NO** – Tlenek azotu (ang. *Nitric oxide*)

**OHC** – Hodowla organotypowa hipokampa (ang. *Organotypic hippocampal culture*)

**OUN** – Ośrodkowy układ nerwowy (ang. *Central nervous system*)

**PAMP** – Wzorce molekularne związane z patogenami (ang. *Pathogen-associated molecular pattern*)

**PrPc** – Białko prionowe (ang. *Prion protein*)

**PYCARD (ASC)** – Białko adaptorowe ASC (ang. *Apoptosis-associated speck-like protein containing a CARD*)

**ROS** – Reaktywne formy tlenu (ang. *Reactive oxygen species*)

**RoI** – Wyciszanie stanu zapalnego (ang. *Resolution of Inflammation*)

**RvD1** – Resolwina D1 (ang. *Resolvin D1*)

**SAA** – Surowiczy amyloid A (ang. *Serum amyloid A*)

**SOCS3 –** Supresor sygnalizacji cytokin 3 (ang. *Suppressor of cytokine signaling 3*)

**SPM** – Endogenne molekuły (ang. *Specialized pro-resolving mediators*)

**STAT3** – Przekaźnik sygnału i aktywator transkrypcji 3 (ang. *Signal transducer and activator of transcription 3*)

**7TM** – Receptor 7 transmembranowy (ang. *7-Transmembrane receptor*)

**TGF-β** – Transformujący czynnik wzrostu β (ang. *Transforming growth factor β*)

**TLR4 -** Receptor Toll-podobny 4 (ang. *Toll- Like Receptor 4*)

**TNF-α** – Czynnik martwicy nowotworów α (ang. *Tumor necrosis factor α*)

# 2. Streszczenie w języku polskim i angielskim

## 2.1.Streszczenie

Podłoże zmian prowadzących do rozwoju chorób ośrodkowego układu nerwowego (OUN) w dalszym pozostaje przedmiotem wieloośrodkowych analiz. Szczególne miejsce w badaniach ostatnich lat zajmuje poznanie przebiegu procesu zapalnego w tym mechanizmów jego prawidłowego wygaszania (ang. *resolution of inflammation,* RoI). Krótkotrwała reakcja zapalna jest procesem korzystnym, pozwalającym na eliminację patogenów, zwalczenie infekcji, regenerację oraz szybki powrót do homeostazy. Natomiast długotrwały proces zapalny ma działanie niekorzystne, które determinowane jest między innymi przez dysfunkcję endogennych procesów kontrolujących wyciszenie ostrego procesu zapalnego.

Dlatego celem badań przedstawionych w niniejszej rozprawie było określenie czy wzmocnienie RoI, poprzez zastosowanie nowych egzogennych ligandów receptora ALX/FPR2 o obiecujących właściwościach farmakokinetycznych oraz lepszej od endogennych ligandów, biodostępności może stanowić nową strategię ograniczającą proces zapalny. Badania prowadzono wieloetapowo, w warunkach *in vitro* hodowli pierwotnych mikrogleju, *ex vivo* hodowli organotypowych hipokampa, a następnie dokonano weryfikacji uzyskanych wyników w warunkach immunoaktywacji *in vivo* w wybranych strukturach mózgu. Zastosowano techniki kolorymetryczne, immunofluorescencyjne, służące do oceny ekspresji genów, poziomu całkowitych i ufosforylowanych form białek (Western blot, ELISA) oraz metody wyciszania komórek mikroglejowych (klodronat).

W pierwszej części rozprawy zaprezentowano badania przeprowadzone *in vitro* z wykorzystaniem pierwotnych hodowli mikrogleju stymulowanych lipopolisacharydem (LPS; niespecyficznym immunoaktywatorem pochodzenia bakteryjnego) uzyskanych od 0-2 dniowego potomstwa szczurów Spraque-Dawley. Wyniki pokazały zależne od czasu działanie ochronne i przeciwzapalne wszystkich badanych agonistów receptora ALX/FPR2: lipoksyny A4 (LXA4), jej analogu AT-LXA4 (ang. *aspirin-triggered lipoxin A4*) oraz nowego, egzogennego liganda związku MR-39. Tym niemniej w przypadku związku MR-39 spektrum korzystnego działania było najszersze i najdłużej zaznaczone, bowiem obserwowane także po 24 godzinach od jego dodania do hodowli mikrogleju. Co istotne, wykazano także, że w mediowaniu korzystnych efektów nowego mocznikopochodnego

związku MR-39 zaangażowane są analogiczne szlaki wewnątrzkomórkowego przekazu sygnału jak aktywowane po interakcji ligandów endogennych (LXA4, AT-LXA4) z receptorem ALX/FPR2 (w tym ERK1/2, kinaza p38, czynnik transkrypcyjny NF-κB).

W kolejnym etapie badania prowadzono w układzie *ex vivo* stosując hodowle organotypowe hipokampa (OHC). Model ten stanowi przydatne narzędzie do analiz nie tylko zależności pomiędzy układem nerwowym, immunologicznym oraz endokrynnym, ale umożliwia także analizę fizjologicznych interakcji między komórkami mózgu dzięki zachowaniu funkcjonalnych połączeń neuronalno-glejowych. Ma zatem kluczowe znaczenie dla oceny działania związków o wnioskowanym potencjale przeciwzapalnym i pro-wyciszeniowym w przebiegu procesów zapalnych (w tym indukowanych LPS). W modelu tym przeprowadzono analizę kolejnych uzyskanych od grupy badawczej z Uniwersytu im. Aldo Moro w Bari (Włochy) nowych agonistów receptora ALX/FPR2 – AMS21 oraz CMC23. Zastosowane modyfikacje w strukturze tych związków (w porównaniu do związku referencyjnego MR-39) sugerowały ich lepszy potencjał pro-wyciszeniowy oraz immunomodulujący. Dlatego ocena ich działania neuroprotekcyjnego, przeciwzapalnego oraz pro-wyciszeniowego w OHC przeprowadzona została w stężeniach nanomolarnych. Wykazano, że nowe związki CMC23 i AMS21 w sposób zależny od receptora ALX/FPR2 normalizowały podwyższone w wyniku działania LPS poziomy cytokin prozapalnych (IL-1β, IL-6). Aktywność CMC23 związana była z wpływem na szlak sygnalizacyjny STAT3/SOCS3, w tym hamowanie stymulowanej podaniem LPS aktywnej, ufosforylowanej formy białka STAT3. Natomiast w przypadku liganda AMS21, wspierający RoI efekt polegał na ograniczaniu nadmiernej aktywacji kompleksu inflammasomu NLRP3. Co więcej, doświadczenia z zastosowaniem OHC pozwoliły po raz pierwszy na wykazanie kluczowej roli receptora ALX/FPR2 obecnego na komórkach mikrogleju w modulacji korzystnych efektów AMS21, gdyż w OHC pozbawionych mikrogleju poprzez zastosowanie klodronatu, korzystne działanie tego liganda nie było obserwowane.

W ostatnim etapie badań, w zwierzęcym modelu immunoaktywacji *in vivo* tzw. „*sickness behavior*" opartym o jednorazowe dootrzewnowe podanie LPS dorosłym szczurom, przeprowadzono ocenę wpływu nowych egzogennych agonistów ALX/FPR2 oraz liganda endogennego LXA4 na zaburzenia behawioralne oraz parametry immunoaktywacji w homogenatach kory czołowej i hipokampa dorosłych, 3-miesięcznych samców. Zaobserwowano, że jednorazowe dootrzewnowe podanie LPS powoduje deficyty

w zachowaniu zwierząt, wyrażone jako wydłużenie czasu bezruchu, przy jednoczesnym skróceniu czasu pływania i wspinania (test wymuszonego pływania, tzw. test Porsolta). Normalizujący wpływ dokomorowego podania LXA4 na zmiany opisane w teście Porsolta, był krótkotrwały, natomiast związek CMC23 wykazywał dłuższą aktywność biologiczną w tym układzie doświadczalnym. Jednocześnie przeprowadzone badania biochemiczne wykazały przeciwzapalne działanie LXA4 polegające na obniżaniu poziomu cytokin prozapalnych (IL-1β, TNF-α) tylko 1 godzinę po podaniach, natomiast działanie związku CMC23 było przedłużone i obecne także 4 godziny po jego podaniu. Wyniki te wskazują na obniżanie aktywacji prozapalnej przez badane ligandy w modelu immunoaktywacji *in vivo,* a dłuższy okres działania nowych ligandów pozwala na potencjalnie bardziej efektywne stosowanie ich w strategii RoI.

Zaprezentowane w hybrydowej wersji niniejszej rozprawy wyniki pozytywnie weryfikują cele podjętych badań. Co więcej, w oparciu o przeprowadzone wielopłaszczyznowe badania można sugerować, że nowatorskie podejście do wzmacniania endogennych mechanizmów regulacji RoI stanowić może w przyszłości o rozwoju nowej ścieżki modulacji procesów zapalnych w przebiegu wielu chorób OUN w oparciu o farmakoterapię ich wygaszania (ang. *pharmacotherapy of resolution).*

## 2.2.Summary

Despite many years of research, the background of alterations leading to the development of central nervous system (CNS) diseases still remains not fully understood. Recently, the mechanisms involved in the course of inflammatory processes, and the resolution of inflammation (RoI) have become a major focus of research. The short-term inflammatory response is a beneficial process that leads to the elimination of pathogens, combating infections, regeneration, and in consequence, restoring homeostasis. However, the long-term inflammation has an unfavorable effect, which results from the dysfunction of endogenous processes that control the resolution of the acute inflammatory process.

Therefore, the aim of the research presented in this thesis was to determine whether enhancing RoI, through the activity of new exogenous ALX/FPR2 ligands with plausible pharmacokinetic properties and superior bioavailability than endogenous ligands, may provide a new strategy for alleviating the inflammatory process. The research was conducted in several stages: *in vitro* using primary microglia cultures, *ex vivo* with organotypic hippocampal cultures (OHC), and *in vivo* to verify the obtained results in the immunoactivation model in selected brain structures.

In the first part of the presented thesis, the *in vitro* research was conducted using primary microglia cultures stimulated with lipopolysaccharide (LPS; a non-specific immunoactivator with bacterial origin), that were obtained from 0-2-day-old offspring of Spraque-Dawley rats. The results have shown the time-dependent protective and anti-inflammatory effects of all tested ALX/FPR2 receptor agonists lipoxin A4 (LXA4), its analog aspirin-triggered lipoxin A4 (AT-LXA4), and the new exogenous ligand, the compound MR-39. Noteworthy, MR-39 revealed the broadest spectrum of beneficial effects and the longest activity, which lasted 24 hours after microglia culture treatment. Importantly, the observed effects of the new ureidopropanamide ligand MR-39 were mediated by the same intracellular signaling pathways as endogenous ALX/FPR2 ligands (including ERK1/2, kinase p38, and NF-κB).

In the next stage, the research was carried out using organotypic hippocampal cultures (OHC) which constitute an excellent *ex vivo* model for analyzing not only the correlation between the nervous, immune, and endocrine systems but also enabling analysis of physiological interactions between brain cells because they maintain functional neuronal-glial connections. Therefore, OHC model is crucial for assessing the effects of compounds

with anti-inflammatory and pro-resolving potential in the course of inflammatory processes (including LPS-induced ones). This model was used as a tool for analysis of new ALX/FPR2 agonists - AMS21 and CMC23 designed and synthesized by the research group from the University of Bari (Italy). The adjustments made in the structure of these compounds (compared to the reference compound MR-39) suggested their better pro-resolving and immunomodulatory potential. Therefore, the assessment of their neuroprotective, anti-inflammatory, and pro-resolving effects in OHC was carried out at nanomolar concentrations.

The new compounds CMC23 and AMS21 normalized the increased levels of proinflammatory cytokines (IL-1β, IL-6) in LPS-stimulated OHC in an ALX/FPR2 dependent manner. The beneficial effect of CMC23 was conveyed via the STAT3/SOCS3 signaling pathway since the inhibition of the active phosphorylated form of STAT3 protein stimulated by LPS administration, was inhibited. However, AMS21 enhanced RoI disturbed by LPS via limiting excessive activation of the NLRP3 inflammasome complex. Moreover, using the OHC model the key role of the ALX/FPR2 receptor present on microglial cells in the modulation of the beneficial effects of AMS21 was demonstrated for the first time because in microglia-depleted OHCs the beneficial effect of this ligand was not observed.

In the final phase of research, the assessment of the impact of new synthetic ALX/FPR2 agonists and the endogenous ligand LXA4 on behavioral disturbances and immunoactivation factors was assessed in homogenates of the frontal cortex and hippocampus of adult 3-month-old males using an *in vivo* model of sickness behavior (a single intraperitoneal (i.p.) administration of LPS). The study carried out using the forced swimming test revealed deficits in the animals' behavior, manifested as an increase in the immobility time with a simultaneous decrease in the swimming and climbing time after a single administration of LPS. The normalizing effect of intraventricular (*icv*) administration of LXA4 on the changes described in the Porsolt test was observed only 1 hour after the treatment, while CMC23 has shown a longer biological activity. At the same time, the biochemical analysis indicated the anti-inflammatory effect of LXA4 manifested as a reduced level of proinflammatory cytokines (IL-1β, TNF-α) only 1 hour after administration, while the effect of CMC23 was prolonged and also present 4 hours after *icv* drug injection. The obtained data indicate a time-dependent modulation of behavioral deficits and the reduction of proinflammatory activation by the tested ligands in the *in vivo*

immunoactivation model. Long-lasting effects of new ligands allow for their potentially more effective use in RoI-targeting strategies.

The results presented in the thesis positively verified the objectives of the undertaken research. Moreover, according to the presented multi-level studies, it can be suggested that an innovative approach to enhancing endogenous mechanisms of RoI regulation, in the future may lead to the development of a new path for modulating inflammatory processes in the course of many CNS diseases based on pharmacotherapy of resolution.

# 3. Wprowadzenie[1]

Wśród wielu hipotez próbujących wyjaśnić przyczyny chorób ośrodkowego układu nerwowego (OUN) istotne znaczenie ma teoria dotycząca dysfunkcji układu odpornościowego. Uważa się, że procesy zapalne w mózgu, zwane stanem zapalnym układu nerwowego, mogą prowadzić do rozwoju wielu chorób w tym neurodegeneracyjnych (np. choroby Alzheimera i Parkinsona) oraz zaburzeń psychicznych (np. depresji i schizofrenii) (Chen *i in.*, 2016).

Jednocześnie panuje pogląd, że krótkotrwały stan zapalny jest procesem korzystnym, umożliwiającym eliminację czynników go indukujących, a następnie powrót do homeostazy (Medzhitov, 2008; Soehnlein i Lindbom, 2010). Natomiast zaburzenie lub deficyty wyciszania/terminacji procesu zapalnego (ang. *resolution of inflammation*, RoI) prowadzą do rozwoju przewlekłego procesu zapalnego, którego efekty są niekorzystne i stanowią podłoże rozwoju patologii (Serhan *i in.*, 2008; Sugimoto *i in.*, 2016).

Biorąc pod uwagę fakt, iż fazy procesu zapalnego, a mianowicie jego inicjacja, rozprzestrzenianie się oraz jego ustąpienie (wygaszenie) nie zachodzą sekwencyjnie jedna po drugiej, lecz współistnieją w czasie trwania jego trwania, (Ortega-Gómez *i in.*, 2013; Schwartz i Baruch, 2014) ostatnie lata przyniosły nieco inne spojrzenie na farmakoterapię tych procesów. Coraz częściej proponuje się w miejsce terapii immunosupresyjnych, tłumiących odpowiedź układu odpornościowego, strategie oparte na poszukiwaniu punktów uchwytu dla nowych związków o potencjale wspierającym i zwiększającym efektywność endogennych mechanizmów RoI.

W tym kontekście kluczowego znaczenia nabierają ligandy i ich receptory, które ze względu na stronniczy agonizm (ang. *biased agonism*) mogłyby modulować odpowiedź prozapalną w kierunku pro-wyciszeniowej wspierając jednocześnie deficyty endogenne w ograniczaniu reakcji zapalnych. W wyciszaniu reakcji zapalnych duże znaczenie mają endogenne molekuły (ang. *specialized pro-resolving mediators,* SPM), które poprzez interakcje ze specyficznymi receptorami formylowymi, głównie ALX/FPR2, regulują procesy RoI (Recchiuti *i in.,* 2020). Niestety ich niekorzystna farmakokinetyka

[1] Rozdział „Wprowadzenie" powstał w oparciu o pracę przeglądową pt. *Formyl peptide receptor 2, as an important target for ligands triggering the inflammatory response regulation: a link to brain pathology.* (punkt 1. w spisie artykułów stanowiących podstawę rozprawy doktorskiej), a także najnowszą literaturę przedmiotu.

i biodostępność sprawiają, że bardzo szybko ulegają inaktywacji przez aktywność enzymatyczną komórek w mózgu.

Dlatego w niniejszej rozprawie przeprowadzono analizę przeciwzapalnych i pro-wyciszeniowych właściwości nowych, mocznikopochodnych agonistów receptora ALX/FPR2, których syntezę przeprowadzono w ramach współpracy w grupie badawczej prof. Lacivita na Uniwersytecie im. Aldo Moro w Bari (Włochy). Potencjał nowych ligandów tego receptora porównywano z efektywnością modulacji zaburzonego RoI przez znane powszechnie ligandy endogenne, w doświadczalnych modelach immunoaktywacji.

### 3.1.Proces zapalny – rys ogólny

Stan zapalny jest fizjologicznym procesem zachodzącym w organizmie, mającym na celu natychmiastowe usunięcie, bądź odizolowanie jego źródła, a tym samym zaburzenia równowagi organizmu i umożliwienie przywrócenia homeostazy (Serhan, 2014a; Panigrahy *i in.,* 2021). Jest to proces gwałtowny i samoograniczający się, którego szeroko pojęta intensywność zależy w dużej mierze od powstałych w tkankach uszkodzeń.

Ostry stan zapalny jest procesem, który zwykle trwa krótko i ustępuje po usunięciu jego przyczyny, bez znaczących uszkodzeń organizmu. Prawidłowo kontrolowany stan zapalny zapewnia ochronę przed rozprzestrzenianiem się infekcji lub uszkodzeń, a po jego ustąpieniu dotknięte tym procesem tkanki powracają do pierwotnego stanu strukturalnego i funkcjonalnego (Soliman i Barreda, 2022). W trakcie procesu zapalnego obserwuje się szybką migrację neutrofili, do miejsca urazu lub zakażenia, a także rekrutację monocytów, które poprzez uwalnianie czynników prozapalnych umożliwiają neutralizację czynnika indukującego ten proces (Jones *i in.,* 2016). W transporcie i zasiedlaniu neutrofili pośredniczą głównie receptory sprzężone z białkami G (ang. *G protein-coupled receptor*, GPCR) Tym niemniej, chociaż neutrofile są komórkami niezbędnymi do prawidłowej eliminacji patogenów, nadmierny ich napływ może być niebezpieczny dla organizmu i dlatego po spełnieniu swojej funkcji kierowane są na drogę apoptozy oraz eliminowane przez makrofagi (Fox *i in.,* 2010).

Wygaszanie reakcji zapalnej jest procesem kompleksowym i kontrolowanym na wielu płaszczyznach. Do głównych objawów ustąpienia ostrego stanu zapalnego należą: ograniczenie lub ustanie napływu komórek krwiopochodnych, ograniczenie udziału chemokin oraz cytokin prozapalnych, a także proces ich apoptozy przez makrofagi, zmiana

fenotypu makrofagów i wreszcie inicjacja procesów gojenia i/lub rekonwalescencja (Schett i Neurath, 2018). Należy podkreślić, że jakiekolwiek dysfunkcje w przebiegu tego procesu mogą prowadzić do przedłużenia się procesu zapalnego głównie na skutek ciągłej stymulacji układu odpornościowego do: nadprodukcji czynników prozapalnych jak cytokiny np. interleukina 1β (IL-1β), IL-6, czynnik martwicy nowotworu α (TNF-α), reaktywne formy tlenu (ROS), niszczenia tkanek w miejscu zapalenia i w konsekwencji zaburzenia powrotu do homeostazy (Schwartz i Baruch, 2014).

Przebieg procesu zapalnego w OUN ma swoją specyfikę i różni się od reakcji zapalnych mediowanych w tkankach obwodowych. Na jego obraz składają się dwie składowe: infiltracja immunokompetentnych komórek z obwodu oraz miejscowa reakcja zapalna będąca wynikiem zbiorowego działania różnych komórek mózgowych (mikrogleju, astrocytów, oligodendrocytów i glejów NG2). Co ciekawe, proces zapalny może pełnić dwojaką rolę w OUN, zależną od przebiegu choroby i środowiska zapalnego (Perry i Teeling, 2013). Ostry proces zapalny o krótkotrwałym przebiegu, najczęściej pełni funkcje neuroprotekcyjną pomagającą w przywróceniu i utrzymywaniu homeostazy. Z drugiej jednak strony, powszechnie przyjmuje się, że przewlekły proces zapalny układu nerwowego jest dominującą cechą postępującej neuropatologii oraz neurodegeneracji (Graeber, 2014; Leszek *i in.*, 2016). Obecnie wiadomo, że zaburzona homeostaza układu nerwowego spowodowana: nadmiernym i przewlekłym uwalnianiem mediatorów prozapalnych, nieprawidłową pracą mitochondriów, degeneracją białek, a w konsekwencji dysfunkcją lub śmiercią komórek OUN należą do niezwykle istotnych przyczyn leżących u podłoża rozwoju chorób takich jak: choroba Alzheimera (AD), stwardnienie rozsiane (SM), choroba Parkinsona (PD) czy stwardnienie zanikowe boczne (ALS) (Amor *i in.*, 2014; Chen *i in.*, 2016; Stephenson *i in.*, 2018; Tansey *i in.*, 2022; Zhang *i in.*, 2023).

### 3.2.Rola komórek glejowych w procesie zapalnym

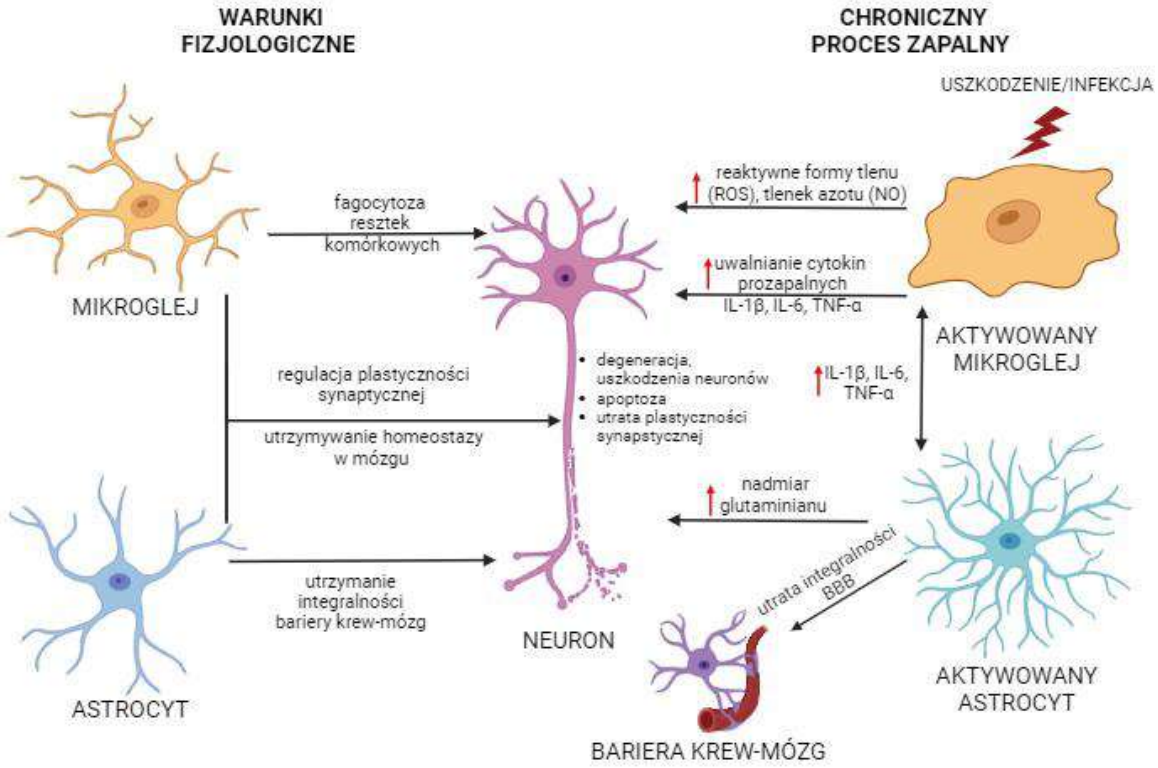
W mózgu komórki glejowe odgrywają kluczową rolę w przebiegu reakcji zapalnej. Komórki glejowe poprzez interakcje z neuronami i komórkami układu odpornościowego monitorują środowisko w warunkach fizjologicznych oraz stanowią pierwszą linię obrony w momencie narażenia mózgu na niekorzystne bodźce (Sousa *i in.*, 2017). Uczestniczą one w aktywacji, rozpoznawaniu oraz modulacji reakcji immunologicznych, a także uwalnianiu wielu mediatorów wspomagających obronę immunologiczną (Paolicelli *i in.*, 2022).

Wśród komórek glejowych najistotniejszą rolą w procesach zapalnych w OUN odgrywa mikroglej. Są to niewielkie fagocytarne komórki pochodzenia mieloidalnego, których populacja w mózgu szacowana jest na ok. 10% wszystkich komórek układu nerwowego (Katsumoto *i in.*, 2014; Sousa *i in.*, 2017). Warto podkreślić, że rola mikrogleju określana jest często mianem „miecza obosiecznego" (ang. *double-edged sword*), wskazując na możliwy przeciwstawny efekt biologiczny aktywacji tych komórek, a mianowicie neuroproteokcyjny lub neurotoksyczny (Ransohoff, 2016a; Wang *i in.*, 2023). Ostatnie lata przynoszą coraz więcej dowodów wskazujących na różnorodny i złożony fenotyp komórek mikrogleju, a badania z użyciem mikroskopii dwufotonowej pokazały także możliwość zmian fenotypu pojedynczych komórek w zależności od środowiska (Ransohoff, 2016a), co może w przyszłości zmienić pogląd na panującą dotychczas tradycyjną interpretację dotyczącą ich udziału w procesach zapalnych.

W stanie spoczynkowym mikroglej cechuje się rozgałęzioną budową morfologiczną, która ułatwia tym komórkom monitorowanie środowiska w mózgu (Trojan *i in.*, 2019). Jednak w wyniku aktywacji, będącej następstwem pojawienia się czynników zaburzających homeostazę (np. endotoksyna bakteryjna – lipopolisacharyd, LPS), komórki mikrogleju zmieniają swoją morfologię zwykle przyjmując postać ameboidalną. Jednocześnie po aktywacji mikrogleju następuje wzrost ekspresji markerów powierzchniowych w tym głównego układu zgodności tkankowej (MHC) klasy I oraz II (Rock *i in.*, 2004) oraz antygenów różnicowania komórkowego (CD) np. CD11b i CD14. Stan aktywacji mikrogleju, pozwala komórkom na uczestnictwo w wielu procesach zachodzących w mózgu zarówno w warunkach fizjologicznych jak i patologicznych. Najistotniejszymi z tych procesów są: przeprowadzanie programowej śmierci neuronów (Marín-Teva *i in.*, 2004; Schafer i Stevens, 2015), regulacja plastyczności synaptycznej, fagocytoza obumierających komórek (Michell-Robinson *i in.*, 2015), czy też uwalnianie spektrum czynników przeciwzapalnych i neurotroficznych (Perego *i in.*, 2011; Zhang *i in.*, 2021). Chociaż aktywacja mikrogleju konieczna jest do monitorowania inicjacji, a następnie przebiegu RoI, to jednak przedłużenie aktywacji prozapalnej tych komórek ma działanie niekorzystne i prowadzi do zmiany ich fenotypu regulatorowego na neurotoksyczny. Przeprowadzone badania wykazały, że mediatory uwalniane przez mikroglej w tym cytokiny prozapalne (IL-1β, IL-6, IL-18, TNF-α), chemokiny, prostaglandyna E2, aminokwasy pobudzające, tlenek azotu (NO) czy reaktywne formy tlenu to czynniki neurotoksyczne, zwłaszcza jeśli

są rezultatami długotrwałej aktywacji mikrogleju (Block *i in.*, 2007; Guzman-Martinez *i in.*, 2019).

Astrocyty, stanowią drugą populację komórek gleju, która ma istotny udział w przebiegu oraz regulacji procesów zapalnych w OUN. Komórki te odpowiedzialne są za utrzymanie homeostazy w mózgu oraz dostarczanie metabolitów i czynników wzrostu neuronom (Siracusa *i in.*, 2019). Badania wykazały także udział astrocytów w tworzeniu synaps, plastyczności synaptycznej, a także w utrzymaniu właściwej przepuszczalności bariery krew-mózg (ang. *blood-brain barier*, BBB) (Sofroniew, 2009; Singh, 2022). Ciekawą obserwacją jest fakt, że chociaż astrocyty ulegają aktywacji w odpowiedzi na bodźce zewnętrzne, to jednak ich efekt biologiczny zwykle jest wynikiem ich złożonej interakcji (Bylicky *i in.*, 2018). Pokazano, że po kontakcie z czynnikiem inicjującym proces zapalny np. IL-1β uwalnianą przez mikroglej, następuje akywacja kaskady wewnątrzkomórkowych ścieżek przekazu sygnału w komórkach astrocytów, co prowadzi do zwiększenia wydzielania innych cytokin, w tym IL-6 nasilając tym samym proces zapalny (Matejuk i Ransohoff, 2020). IL-1β może także zmieniać efektywność ponownego wchłaniania kwasu glutaminowego przez astrocyty oraz nasilać uwalnianie wolnych rodników (Hu *i in.*, 2000; Sama *i in.*, 2008; Rindflesch *i in.*, 2018). Wydaje się, zatem, że mikroglej poprzez uwalniane mediatory reguluje fenotyp oraz funkcje astrocytów. Pokazano, że antagonista receptora IL-1 (ang. *interleukin 1 receptor antagonist*, IL-1RA) ogranicza aktywację astrocytów i w konsekwencji zapobiega powstawaniu patologii mediowanych przez te komórki w mózgu (Huang *i in.*, 2023). Co więcej, aktywacja mikrogleju może stymulować uwalnianie przez astrocyty przeciwzapalnych cytokin jak TGF-β (ang. *transforming growth factor β*) czy IL-10, których wysokie poziomy są czynnikiem inicjującym wyciszenie stanu zapalnego (Matejuk i Ransohoff, 2020). Dane doświadczalne pokazały, że interakcje astrocyt-mikroglej są również niezwykle ważne w kontekście integralności BBB i w konsekwencji regulacji napływu komórek immunokompetentnych z obwodu podczas stanu zapalnego do mózgu (Ronaldson i Davis, 2020; Cruz *i in.*, 2023). Ich dysfunkcja jest głównym czynnikiem powodującym uszkodzenia BBB i nadmierną jej przepuszczalność, co powoduje zwiększony i niekontrolowany napływ leukocytów obwodowych, dodatkowo aktywując komórki glejowe do wytwarzania prozapalnych cytokin, chemokin oraz ROS. Jest zatem dodatkowym źródłem mediatorów prozapalnych – podtrzymujących proces zapalny w mózgu, ograniczając jego efektywne wygaszenie (Rys. 1).




***Rys. 1 Rola komórek glejowych w warunkach fizjologicznych i długotrwałej aktywacji zapalnej w mózgu.*** *Komórki mikrogleju oraz astrocyty uczestniczą między innymi w regulacji plastyczności synaptycznej, procesach apoptozy i fagocytozy oraz w utrzymywaniu homeostazy w mózgu. Długotrwała aktywacja zapalna prowadzi do nadmiernej aktywacji mikrogleju oraz uwalniania czynników prozapalnych takich jak: ROS, NO, IL-1β, IL-6. Mediatory te, szczególnie IL-1β powodują silną aktywację astrocytów oraz nasiloną syntezę czynników zapalnych np. IL-6, przy ograniczonej zdolności do syntezy czynników przeciwzapalnych. Przedłużona aktywacja komórek glejowych jest przyczyną rozszczelnienia bariery krew-mózg, co powoduje napływ obwodowych komórek immunokompetentnych np. leukocytów do mózgu, nasilając odpowiedź prozapalną. W wyniku chronicznego procesu zapalnego dochodzi do zjawisk deficytowych w mózgu w tym: degeneracji neuronów, apoptozy oraz utraty plastyczności synaptycznej. Autorstwo własne na podstawie Leng i Edison (2021); Wykonano w programie BioRender.*

### 3.3.Wyciszanie procesu zapalnego (ang. *resolution of inflammation*, RoI)

W wielu badaniach wykazano, że u podstaw patomechanizmów chorób OUN leży występowanie długotrwałego procesu zapalnego w mózgu z nasiloną aktywacją komórek

glejowych, szczególnie mikrogleju oraz zwiększoną produkcją czynników prozapalnych (Kwon i Koh, 2020). Dlatego też, oprócz tradycyjnej, celowanej farmakoterapii tych chorób stosuje się często terapie uzupełniające, których celem jest ograniczenie stanu zapalnego w mózgu. Jednak biorąc pod uwagę fakt, iż strategie mogą zaburzać także endogenne procesy wyciszania stanu zapalnego, w ostatnich latach zwrócono uwagę na farmakoterapie oparte o RoI, których celem jest poszukiwanie metod wspomagania endogennych procesów przeciwzapalnych przy jednoczesnym wyciszeniu, ale co istotne nie całkowitym stłumieniu aktywności prozapalnej układu odpornościowego (Serhan *i in.*, 2008; Sugimoto *i in.*, 2016).

Prawidłowy przebieg wyciszania stanu zapalnego jest procesem aktywnym, który wymaga przekierowania endogennej odpowiedzi i uwalniania mediatorów prozapalnych w kierunku przeciwzapalnym i pro-wyciszeniowym (Neurath, 2019). Wykazano, że kluczową rolę w tym procesie pełnią endogenne mediatory (SPM). Warto wspomnieć, że SPM nie blokują przekazywania sygnału w obrębie kaskady zapalnej, natomiast sprzyjają procesom, które zmniejszają ekspresję cząsteczek prozapalnych i w ten sposób ograniczają ten proces (Trojan *i in.*, 2019; Recchiuti *i in.*, 2020).

Opierając się na dotychczasowych wynikach badań panuje pogląd, że spektrum działania biologicznego SPM w procesach zapalnych jest bardzo szerokie. Mediatory te wspomagają hamowanie rekrutacji komórek zapalnych, regulacją wydzielanie cytokin i chemokin oraz zmianę fenotypu makrofagów/mikrogleju w kierunku przeciwzapalnym. Ponadto, coraz więcej danych mówi o ich zdolności do: induckji eferocytozy apoptotycznych neutrofili (przez makrofagi), modulowania odpowiedzi immunologiczną poprzez pobudzenie supresyjnych komórek odpornościowych i nasilania nabytej odpowiedzi immunologicznej (Tiberi i Chiurchiù, 2021; Valente *i in.*, 2022). Taka specyfika modulacji procesu RoI może być jednak realizowana tylko przez współdziałające ze sobą SPM, dlatego do grupy tej zalicza się wiele cząsteczek, żeby wymienić najważniejsze z nich, a mianowicie lipoksyny (LX), resolwiny, protektyny oraz marezyny (Serhan, 2014a, 2014b).

W kontekście niniejszej rozprawy szczególne znaczenie mają lipoksyny (Rys. 2), dlatego też, tej grupie poświęcono nieco więcej uwagi. Już w roku 1984 endogenne lipoksyny jako pierwsze uznano za „sygnały hamujące" w stanach zapalnych (Serhan *i in.*, 1984). Należą one do grupy eikozanoidów, które są pochodnymi kwasu arachidonowego (AA). Wskazuje się na trzy szlaki biosyntezy lipoksyn, które są katalizowane przez: 5-lipooksygenazę (5-LOX), 15-lipooksygenzaę (15-LOX) oraz

acetylowaną aspiryną cyklooksygenazę 2 (ASA-COX2). Klasyczna droga biosyntezy lipoksyn obejmuje podwójne utlenianie AA katalizowane przez 15-LOX w leukocytach i komórkach nabłonkowych, a następnie przez 5-LOX. Powstają wówczas produkty ostateczne lipoksyna A4 (LXA4) oraz lipoksyna B4 (LXB4) (Bennett i Gilroy, 2016). Synteza może także przebiegać z utlenianiem kwasu arachidonowego, co prowadzi do powstania leukotrieny A4, która ulega następnie konwersji do LXA4 i LXB4, a reakcja ta katalizowana przez jest przez 12-lipooksygenazę (12-LOX) (Freire i Van Dyke, 2013). Ponadto, w wyniku acetylacji ASA-COX2 powstaje produkt pośredni, który następnie w wyniku działania 5-LOX jest metabolizowany i tworzone są epimery lipoksyny zależne od aspiryny – AT-LXA4 oraz AT-LXB4 (Hawkins *i in.*, 2017).

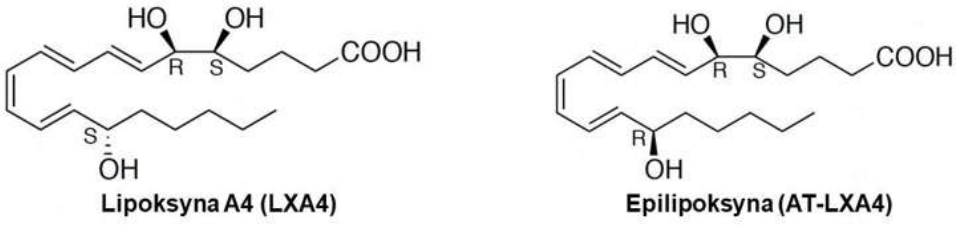
Wszystkie związki z grupy lipoksyn mają działanie immunomodulacyjne w tym przeciwzapalne i pro-wyciszeniowe, głównie poprzez ograniczanie wydzielania mediatorów prozapalnych oraz redukcję stresu oksydacyjnego (Ariel *i in.*, 2006; Leuti *i in.*, 2019; Kooij *i in.*, 2020). Wykazano, że związki te zmniejszają produkcję mediatorów prozapalnych, w tym IL-1β, IL-6 oraz TNF-α, a jednocześnie ułatwiają uwalnianie mediatorów przeciwzapalnych (TGF-β, IL-10, prostaglandyna E2) (Zhang *i in.*, 2022). Kolejne obserwacje pokazały, że ich działanie związane jest także z: ograniczaniem chemotaksji i infiltracji neutrofilii, nasilaniem fagocytozy makrofagów, modulacją fenotypu makrofagów/mikrogleju (Lawrence *i in.*, 2002; Recchiuti *i in.*, 2020; Beaino *i in.*, 2021). Takie działanie sugeruje, że lipoksyny wykazują właściwości przeciwutleniające, antyapoptotycznye i modulujące autofagię (Wu *i in.*, 2012a; Jia *i in.*, 2015). Działanie przeciwzapalne LXA4 jest efektem jej wpływu na wiele wewnątrzkomórkowych szlaków sygnałowych w komórkach, w tym hamowania czynników transkrypcyjnych NF-κB oraz AP-1 i aktywacji jądrowego czynnika erytroidalnego 2 (Nrf2). Sugeruje się również udział tych mechanizmów w protekcyjnym, antyapoptotycznym i przeciwutleniajacym działaniu LXA4 na komórki neuronalne w modelach z zastosowaniem staurosporyny, glutaminianu, parakwatu, czy deprywacji tlenu i glukozy (Zhu *i in.*, 2016, 2020; Livne-Bar *i in.*, 2017).

Niedobór LXA4 zwiększa odpowiedź prozapalną mikrogleju w warunkach aktywacji pogłębiając brak równowagi pomiędzy działaniem prozapalnym i przeciwzapalnym (Feng *i in.*, 2017). Wykazano także, że AT-LXA4 osłabia wywołaną endotoksyną bakteryjną odpowiedź zapalną poprzez hamowanie aktywacji NFκB i MAPK (kinazy aktywowane mitogenami) w komórkach mikrogleju BV-2 (Wang *i in.*, 2011) oraz ogranicza wytwarzanie ROS za pośrednictwem modulacji aktywności oksydazy NADPH (Taetzsch *i in.*, 2015).

Coraz częściej sugeruje się udział LXA4 i AT-LXA4 w modulacji aktywności astrocytów m.in. poprzez hamowanie wytwarzania NO, a także indukowanie ekspresji oksygenazy hemowej-1 (HO-1) czy uwalnianie glutationu (GSH) (Yao *i in.*, 2014; Cekanaviciute i Buckwalter, 2016; Wu *i in.*, 2019a).

Obserwacje te stały się podstawą do zwrócenia uwagi na potencjalną przydatność zastosowania tej grupy związków w przebiegu chorób neurologicznych jak udar czy urazy oraz neurodegeneracyjnych (Ransohoff, 2016b; Devanney *i in.*, 2020; Mészáros *i in.*, 2020). Dla przykładu wspomnieć można badania Dunn *i in.*, (2014), którzy pokazali, że zastosowanie LXA4 powodowało obniżenie poziomu mediatorów prozapalnych, zmniejszenie ilości płytek amyloidowych oraz ilości ufosforylowanego białka tau, a także prowadziło do istotnej poprawy funkcji poznawczych w mysim modelu choroby Alzheimera 3xTg-AD.

Warto podkreślić, że większość efektów biologicznych LXA4 i jej analogów opisanych powyżej determinuje ich wiązanie się ze specyficznymi receptorami GPCR. Pierwsze badania wskazujące specyficzne miejsce rozpoznawania LXA4 na leukocytach (ang. *polymorphonuclear leukocytes*, PMN) przeprowadzono przy użyciu LXA4 znakowanej radioaktywnie trytem w 1994 roku. Zidentyfikowano wówczas cDNA ludzkiego, 7-transbłonowego receptora (pINF114) o wysokim powinowactwie dla LXA4. Receptor ten nazywany jest obecnie receptorem formylowym 2 (ALX/FPR2) i należy do rodziny receptorów formylowych (FPR) (Fiore *i in.*, 1994). Należy jednak wspomnieć, że LXA4 i AT-LXA4 mogą również w niektórych warunkach wiązać się z innymi receptorami. LXA4 może hamować receptor leukotrieny cysteinylowej w naczyniach komórek śródbłonka (Norel i Brink, 2004) i aktywować grupę arylową receptora węglowodorów w komórkach dendrytycznych (Schaldach *i in.*, 1999; Machado *i in.*, 2006), pośrednicząc w ten sposób w działaniu przeciwzapalnym. W mózgu natomiast w działaniu LXA4 niekiedy pośredniczy receptor GPR32 (Zhu *i in.*, 2016). Co więcej, LXA4 jest także endogennym allosterycznym ligandem receptora kannabinoidowego 1 (CB1), wywierając tą drogą działanie kannabimimetyczne w mózgu (Pamplona *i in.*, 2012).




***Rys. 2*** *Wzory strukturalne wybranych, endogennych cząsteczek regulujących wyciszenie reakcji zapalnej (ang. specialized pro-resolving mediators, SPM).*

### 3.4.Rodzina receptorów formylowych (FPR)

Różnorodność wiązania ligandów przez receptory należące do rodziny receptorów formylowych (ang. *formyl peptide receptors*, FPR) przez wiele lat stwarzała problemy z ich nomenklaturą. W celu ujednolicenia nazewnictwa tej rodziny receptorów Międzynarodowa Unia Farmakologii Podstawowej i Klinicznej (ang. *International Union of Basic and Clinical Pharmacology*; IUPHAR) wprowadziła rekomendacje ich nazewnictwa (Ye *i in.*, 2009). Uwzględniając strukturalne zróżnicowanie agonistów receptorów formylowych IUPHAR rekomenduje stosowanie nazw: FPR1, ALX/FPR2 oraz FPR3 opierając ten wybór na wspólnej zdolności wszystkich receptorów formylowych do wiązania peptydów N-formylowanych, a także o zdolność receptorów ALX/FPR2 do interakcji z LXA4 i AT-LXA4 (Fiore *i in.*, 1994; Hanson *i in.*, 2013). Wszystkie ludzkie receptory FPR kodowane są przez geny znajdujące się na chromosomie 19q13.3 i cechują się wysoką homologią (Le, *i in.*, 2001a).

Receptory rodziny FPR zostały zidentyfikowane także u wielu innych ssaków m.in. świnek morskich, naczelnych, królików, myszy oraz szczurów (Ye *i in.*, 2009). Generalnie najlepiej przebadanymi zwierzęcymi receptorami FPR są te występujące u gryzoni, w szczególności u myszy (Boillat *i in.*, 2021). Mysi Fpr2 wykazuje podobny zakres wiązania ligandów co jego ludzki odpowiednik, gdyż wchodzi on w interakcje zarówno z LXA4 (Takano *i in.*, 1997; Vaughn *i in.*, 2002), jak i silnie prozapalnymi białkami surowiczego amyloidu A (SAA) (Liang *i in.*, 2000), oraz amyloidu $\beta_{1\text{-}42}$ ($A\beta_{1\text{-}42}$) (Le *i in.*, 2001b). Pozostali członkowie rodziny mysich receptorów formylowych zdają się nie wykazywać tak znaczących i charakterystycznych cech oraz powinowactwa z receptorami rodziny FPR obecnymi u ludzi.

### 3.4.1. Lokalizacja i funkcje receptora ALX/FPR2

Rodzina receptorów FPR ma zdolność do rozpoznawania wzorców molekularnych związanych z patogenami (PAMP) oraz uszkodzeniami (DAMP). Nadrzędną funkcją wszystkich receptorów FPR jest ich udział w nieswoistej odpowiedzi odpornościowej, dlatego też występują bardzo licznie na komórkach układu immunologicznego . Ponadto, są one zaangażowane w chemotaksję, mobilizację $Ca^{2+}$ oraz rekrutację komórek odpornościowych (Weiß i Kretschmer, 2018). U ludzi receptory rodziny FPR obecne są na monocytach oraz komórkach dendrytycznych (Becker *i in.*, 1998; Devosse *i in.*, 2009). Ich obecność pokazano także na komórkach nabłonka, limfocytach T oraz neutrofilach (Boulay *i in.*, 1990; Bao *i in.*, 1992; Prossnitz i Ye, 1997).

Co istotne, doniesienia ostatnich lat sugerują obecność receptorów rodziny FPR na komórkach ośrodkowego układu nerwowego, ze szczególnym uwzględnieniem silnej ekspresji receptora ALX/FPR2 w neuronach, mikrogleju i astrocytach (Liu *i in.*, 2014; Wu *i in.*, 2019b). Obecność receptorów ALX/FPR2 w OUN jest bezpośrednio związana z pełnionymi przez nie korzystnymi funkcjami takimi jak: modulacja procesów zapalnych, monitorowanie aktywacji gleju (Han *i in.*, 2020), przyspieszanie różnicowania neuronów (Urbán *i in.*, 2019) oraz regulacja przepuszczalności BBB (Liu *i in.*, 2021). Ich funkcja jest jednak silnie związana ze zdolnością receptorów FPR do wiązania różnorodnych ligandów. Tym niemniej, jedynym receptorem z rodziny FPR, który posiada miejsce wiązania zarówno dla peptydów, jak i endogennych lipidów jest receptor ALX/FPR2 (Dufton i Perretti, 2010; Kretschmer *i in.*, 2015).

### 3.4.2. Zmiany konformacyjne oraz stronniczy agonizm

Receptory FPR należą do rodziny siedem transmembranowych (7TM) receptorów sprzężonych z białkami G (GPCR) (Fredriksson *i in.*, 2003; Hilger *i in.*, 2018). Są one zbudowane z siedmiu domen transmembranowych (TM1-7) połączonych ze sobą za pomocą trzech pętli zewnątrzkomórkowych (EL1-3) i trzech pętli wewnątrzkomórkowych (IL1-3). N-koniec receptora zlokalizowany jest zewnątrzkomórkowo, zaś C-koniec znajduje się po stronie cytoplazmatycznej komórki (Venkatakrishnan *i in.*, 2013; Sensoy i Weinstein, 2015). Domeny znajdujące się na pętlach IL1-3 odpowiedzialne są za m.in. za wiązanie z arestynami, białkami czy kinazami receptorowymi (Zhang *i in.*, 2015), natomiast zewnątrzkomórkowe EL1-3 pełnią funkcję w detekcji ligandów receptora (Skvortsov i Gabdoulkhakova, 2017). Transmembranowe domeny TM1-7 biorą udział w wiązaniu ligandów oraz przekazywaniu sygnału do wnętrza komórki poprzez kluczowe dla

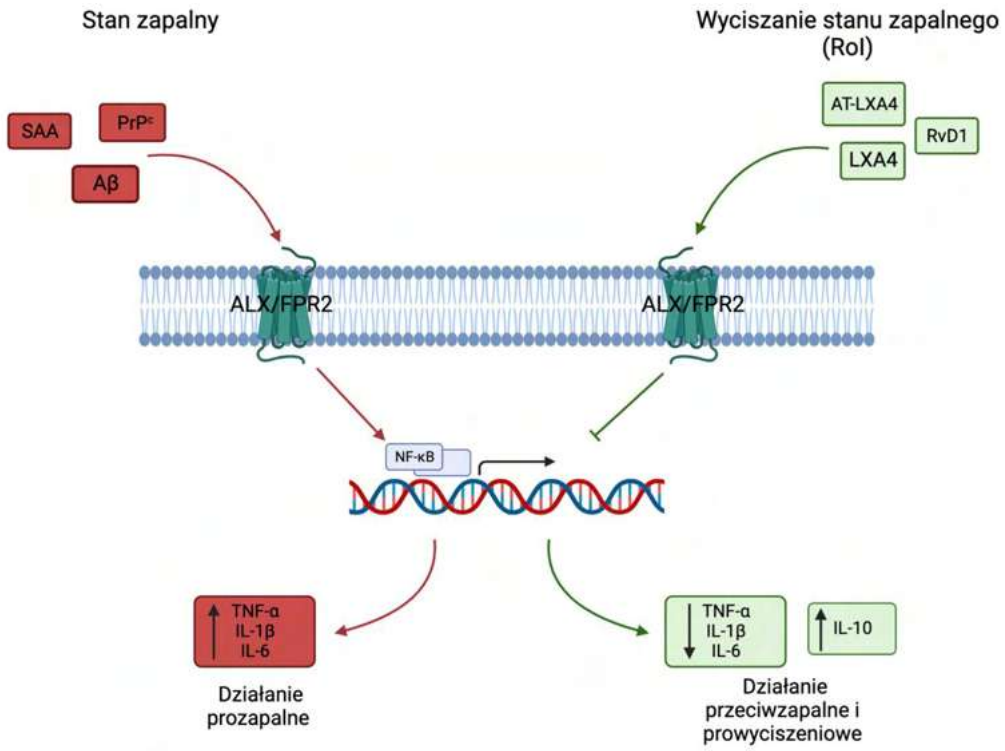
aktywności receptora zmiany konformacyjne (Skvortsov i Gabdoulkhakova, 2017). Wśród motywów receptorowych na uwagę zasługują dwa konserwatywne, kluczowe dla zmian konformacyjnych, a więc również sygnalizacji komórkowej całej rodziny FPR: NPXXY w TM7, który jest odpowiedzialny za aktywację receptora oraz E/DRY (połączenie TM3 i TM6), który stabilizuje działanie receptora będąc swoistym „blokerem jonów" (Bennett *i in.*, 2000; He *i in.*, 2001).

Efekty biologiczne aktywacji receptorów FPR, a szczególnie receptora ALX/FPR2 determinowane są nie tylko budową chemiczną, lecz także zdolnością do tworzenia struktur wyższego rzędu. W badaniach pokazano, że receptory omawianej rodziny mogą tworzyć homodimery FPR1-FPR1 czy ALX/FPR2-ALX/FPR2, a także heterodimery jak FPR1-ALX/FPR2. Zjawisko dimeryzacji receptorów formylowych prowadzi do zmian konformacyjnych i w konsekwencji wpływa na zmianę specyficzności powstałego dimeru dla poszczególnych agonistów oraz prowadzi często do uruchomienia innych szlaków sygnalizacji komórkowej (Sodin-Semrl *i in.*, 2004; Lohse, 2010). Co ciekawe, wydaje się, że ligandy receptorów FPR determinować mogą rodzaj tworzonego dimeru. Przykładem takiego działania jest fakt, że LXA4 i aneksyna (ANXA1) promują homodimeryzację ALX/FPR2-ALX/FPR2, zaś peptyd $Ac_{2–26}$ tworzenie heterodimerów FPR1-ALX/FPR2 (Cooray *i in.*, 2013). Badania grupy Brandenburg *i in.* (2010) pokazały, że ALX/FPR2 może tworzyć także oligomery z receptorami MARCO, co ma istotne znaczenie w transdukcji sygnału indukowanej obecnością $A\beta_{1\text{-}42}$.

Chociaż większość receptorów FPR wykazuje zdolność do interakcji z wieloma różnymi ligandami, to jednak receptor ALX/FPR2 wiąże ich największy zakres o zróżnicowanej strukturalnie i chemicznie budowie (Cattaneo *i in.*, 2013; Corminboeuf i Leroy, 2015; He i Ye, 2017). W zależności od przyłączonego liganda, a także, co istotne od typu komórki wykazującej ekspresję receptora ALX/FPR2 aktywowane są szlaki transdukcji sygnału poprzez podjednostki $G_{\alpha i1}$, $G_{\alpha i2}$ oraz $G_{\alpha i3}$ białka G (Ye *i in.*, 2009). Efektem takiego działania jest indukcja odpowiedzi, która może prowadzić do dwóch przeciwstawnych procesów, a mianowicie promowania odpowiedzi prozapalnej lub przeciwzapalnej sprzyjającej RoI. Dla przykładu można w tym miejscu wymienić ligand $PrP_{106\text{-}126}$ (ang. *prion protein*) , który po interakcji z ALX/FPR2 indukuje wzrost uwalniania cytokin prozapalnych w tym IL-6, TNF-α, a także nasila chemotaksję oraz zwiększa moblizację $Ca^{2+}$ w komórkach glejowych (Le *i in.*, 2001c). Silnie prozapalnym ligandem receptora ALX/FPR2 jest również SAA (ang. *serum amyloid A*), które promuje wiązanie do

DNA czynników transkrypcyjnych takich jak NF-κB oraz AP-1 w konsekwencji nasilając uwalnianie prozapalnych mediatorów np. IL-1β. Co ciekawe, efekt ten osłabiany jest przez interakcję LXA4 z receptorem ALX/FPR2 (Cattaneo *i in.*, 2013). Wykazano, również, że przyłączenie $A\beta_{1-42}$ uruchamia wewnątrzkomórkowe szlaki sygnałowe, które w efekcie prowadzą do: fosforylacji kinaz w tym ERK (ang. *extracellular signal-regulated kinases*), w komórkach glejowych (Brandenburg *i in.*, 2008, 2010), nasilenia chemotaksji, mobilizacji $Ca^{2+}$ oraz uwalniania anionorodników ponadtlenkowych ($O_2^{\bullet -}$) (Le *i in.*, 2001b; Tiffany *i in.*, 2001). Dane te pokazują, że interakcja wspomnianych ligandów z receptorem ALX/FPR2 poprzez aktywację ścieżek prozapalnych w komórkach nasila proces zapalny. Jednocześnie należy podkreślić, że receptor ALX/FPR2 jest głównym punktem uchwytu dla indukcji przeciwzapalnego i pro-wyciszeniowego działania LXA4, a także epimeru AT-LXA4. Fakt ten potwierdzają odkrycia wskazujące na zdolność receptora ALX/FPR2 do wiązania ligandów w dwóch różnych miejscach (Ge *i in.*, 2020). Udowodniono, że ALX/FPR2 posiada dwa miejsca wiązania dla AT-LXA4. Jedno z tych miejsc jest również częściowo miejscem interakcji innego liganda – WKYMVm i dlatego też AT-LXA4 wiąże się w nim z mniejszym powinowactwem (Ge *i in.*, 2020). Co więcej, wykazano także, że peptydowi agoniści ALX/FPR2: $Ac_{2-26}$ oraz $A\beta_{1-42}$ posiadają dwa miejsca wiązania do receptora (Zhang *i in.*, 2020). W kontekście takich interakcji ligand-receptor cząsteczki te nazywane są stronniczymi modulatorami allosterycznymi (ang. *biased allosteric modulators*).

Jak dotychczas klasyczne podejście do wzajemnych interakcji ligand-receptor wskazywało, że receptory aktywowane są przez ligandy o agonistycznym potencjale, natomiast antagonista tą aktywację hamuje (Kenakin i Williams, 2014; Raabe *i in.*, 2019). Tym niemniej, wykazanie zróżnicowanych efektów aktywacji receptorów ALX/FPR2 przez różne związki o potencjale agonistycznycm zmieniło nieco to podejście. Zjawisko to nazywane stronniczym agonizmem (ang. *biased agonism*) (Rys. 3) pozwala na wyjaśnienie w jaki sposób różne ligandy receptora ALX/FPR2 prowadzą do skrajnie różnych efektów, w tym odgrywają kluczową rolę zarówno w inicjacji, jak i wygaszaniu procesów zapalnych. Jednocześnie daje ono podstawy do zrozumienia „podwójnej" roli receptorów ALX/FPR2, która oprócz promowania odpowiedzi przeciwzapalnych jednocześnie utrzymuje aktywność prozapalną na niskim poziomie, co ma kluczowe znaczenie w warunkach uogólnionych reakcji zapalnych (Schepetkin *i in.*, 2014).




***Rys. 3 Stronniczy agonizm (ang. biased agonism).*** *Aktywacja receptora ALX/FPR2 w zależności od liganda prowadzi do powstania odmiennych, zróżnicowanych efektów biologicznych. Przyłączenie ligandów takich jak: SAA, PrP$^c$, Aβ determinuje rozwój stanu zapalnego, gdyż ligandy te łącząc się z receptorem ALX/FPR2 aktywują szlakói sygnalizacji komórkowej, których efektem jest uwalnianie cytokin prozapalnych np. TNF-α, IL-1β, IL-6. Natomiast interakcja receptora z ligandami: AT-LXA4, LXA4, RvD1 ma działanie odmienne. Obserwuje się hamowanie uwalniania czynników prozapalnych i jednocześnie zwiększenie uwalniania mediatorów przeciwzapalnych jak IL-10, czyli wyciszanie reakcji zapalnej (RoI). Schemat z Tylek i in. (2021a); Wykonano w programie BioRender.*

### 3.5.Ligandy syntetyczne receptora ALX/FPR2

Jak wspomniano powyżej, zarówno LXA4 jak i AT-LXA4 w wielu modelach oraz badaniach przedklinicznych wykazują działanie przeciwzapalne i pro-wyciszeniowe (Perretti i Godson, 2020; Regulska *i in.*, 2020). Jednocześnie ich właściwości farmakokinetyczne, szybki metabolizm *in vivo* oraz niska stabilność metaboliczna znacząco ograniczają ich biodostępność, a tym samym czas ich korzystnego działania, co ogranicza możliwość stosowania ich we wspomaganiu farmakoterapii chronicznego procesu zapalnego. Dlatego też prowadzone są poszukiwania nowych egzogennych analogów, wykorzystując w ich modelowaniu szkielet budowy chemicznej LXA4 z zachowaną

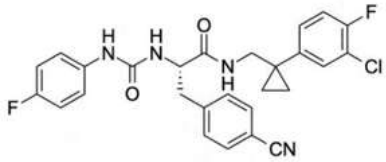
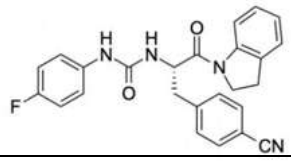
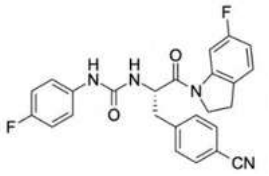
specyfiką interakcji z receptorem ALX/FPR2, ale charakteryzujących się lepszymi parametrami farmakokinetycznymi oraz lepszą bioaktywnością w OUN.

Wśród pierwszych syntetycznych ligandów receptora ALX/FPR2, należy wymienić pochodne LXA4, w tym związek Compound 1, który hamował zdolność transmisji ludzkich neutrofilli w stężeniach nanomolarnych, a więc porównywalnych z efektywnością LXA4. Potencjał terapeutyczny tego związku *in vivo* okazał się jednak niewystraczający, ponieważ po podaniu dożylnym bądź doustnym był on bardzo szybko usuwany z ustroju (Rabiet *i in.*, 2005). Większym potencjałem terapeutycznym w modelach modulacji reakcji zapalnych charakteryzowały się analogi II generacji, których przykładem jest Compound 2. W tym przypadku jednak wysoka lipofilność stanowiła ograniczenie ich stosowania zwłaszcza w modelach procesów zapalnych w OUN. Kolejna generacja mimetyków LXA4, omijała te ograniczenia (Brandenburg *i in.*, 2010; Cooray *i in.*, 2013) i wykazywała silny potencjał przeciwzapalny *in vitro* w tym zdolność do hamowania czynnika transkrypcyjnego NF-κB (Bena *i in.*, 2012; Filep, 2013).

Inną klasą syntetycznych ligandów receptora ALX/FPR2 o potencjale agonistycznym są związki drobnocząsteczkowe. Jednym z najczęściej badanych ligandów z tej grupy jest Quin-C1. Związek ten jest pochodną chinazoliny, a jednocześnie selektywnym agonistą receptora ALX/FR2. Quin-C1 indukuje m.in. wewnątrzkomórkową mobilizację jonów $Ca^{2+}$, ma działanie przeciwzapalne poprzez zmniejszenie poziomu cytokin takich jak IL-1β oraz TNF-α między innymi w mysim modelu uszkodzenia płuc bleomycyną (He *i in.*, 2011). Przykładem małocząsteczkowych agonistów receptora ALX/FPR2 jest także pochodna chloropirazonolu Compound 43, który jak wykazano zwiększa zdolność do mobilizacji jonów $Ca^{2+}$, a także hamuje migrację neutrofili PMN stymulowanych przez fMLP oraz IL-8 (Dufton i Perretti, 2010).

Badania prowadzone we współpracy z grupą badawczą prof. Lacivita z Katedry Farmacji Uniwersytetu im. Aldo Moro w Bari (Włochy) oraz w oparciu o projekty naukowe realizowane wspólnie z innymi zespołami w Niemczech oraz Stanach Zjednoczonych, zaprojektowano i przeprowadzono syntezę chemiczną nowej grupy mocznikopochodnych związków o zadawalających parametrach fizykochemicznych oraz potencjale agonistycznym względem receptora ALX/FPR2 (Tabela 1.).

***Tabela 1.*** *Budowa strukturalna nowych mocznikopochodnych agonistów receptora ALX/FPR2 I i II generacji. Autorstwo własne za zgodą i w oparciu o Stama i in. (2017) oraz Mastromarino i in. (2022).*

| **Związek** | **Wzór strukturalny** | **Nazwa chemiczna** |
|---|---|---|
| MR-39 |  | (S)-3-(4-Cyanophenyl)-N-[[1-(3-chloro-4-fluorophenyl)cyclopropyl]methyl]-2-[3-(4-fluorophenyl)ureido]propanamide |
| AMS21 |  | (S)-1-(3-(4-Cyanophenyl)-1-(indolin-1-yl)-1-oxopropan-2-yl)-3-(4-fluorophenyl)urea |
| CMC23 |  | (S)-1-(3-(4-Cyanophenyl)-1-(6-fluoroindolin-1-yl)-1-oxopropan-2-yl)-3-(4-fluorophenyl)urea |

Związek MR-39, który jest mocznikopochodnym agonistą receptora ALX/FPR2 pierwszej generacji jest jak dotychczas związkiem referencyjnym najlepiej przebadanym w tej grupie. Aktywuje on receptor ALX/FPR2 w stężeniach submikromolowych ($EC_{50}$ = 5,2 μM), a także wykazuje dobrą stabilność metaboliczną w szczurzych mikrosomach ($t_{1/2}$ = 48 min) oraz przepuszczalność pasywną przez warstwę komórek hCMEC/D3 (stanowiącą model *in vitro* bariery krew-mózg) o współczynniku wypływu BA/AB niższym niż 3, który sugeruje możliwość jego penetracji do mózgu (Stama *i in.*, 2017). Co więcej, badania pokazały, że MR-39 ma potencjał przeciwzapalny, gdyż zmniejsza uwalnianie mediatorów prozapalnych (IL-1β i TNF-α) (Stama *i in.*, 2017). Tym niemniej, pomimo obiecujących wyników jak dotychczas działanie MR-39 obserwowane było jedynie w stężeniach mikromolowych, a więc wyższych niż w przypadku ligandów endogennych: LXA4 i AT-LXA4. Prawdopodobne więc jest, że korzystna efektywność tego agonisty w warunkach *in vivo* może być obserwowana jedynie przy zastosowaniu wysokich dawek.

Obserwacje te były przesłankami do syntezy nowych mocznikopochodnych związków II generacji. Ich synteza jest przedmiotem przygotowywanego międzynarodowego wniosku patentowego. Polegała ona na wprowadzeniu modyfikacji,

poprzez zmianę molekularnego rusztowania związku MR-39, w celu połączenia wysokiej siły działania i stabilności metabolicznej. Poprzednie badania zależności struktura-aktywność wykazały, że objętość podstawnika związanego z funkcją amidową jest kluczowa zarówno dla aktywności receptora ALX/FPR2, jak i stabilności metabolicznej, niemniej jednak, w trakcie syntezy związków II generacji ugrupowanie fenylocyklopropylowe MR-39 zastąpiono ugrupowaniami o innych wymiarach, co doprowadziło do identyfikacji związku AMS21. Związek ten aktywuje receptor ALX/FPR2 w stężeniu nanomolowym ($EC_{50}$= 26 nM), chociaż jest bardziej podatny na metabolizm oksydacyjny w porównaniu z MR-39 ($t_{1/2}$ = 21 min w szczurzych mikrosomach) (Mastromarino *i in.*, 2022).

Dalsze modyfikacje strukturalne doprowadziły do identyfikacji kolejnego związku CMC23, wykazującego najbardziej zrównoważoną kombinację siły działania na receptor ALX/FPR2 oraz stabilności metabolicznej ($EC_{50}$= 130 nM; $t_{1/2}$ = 44 min w mikrosomach wątroby szczura). Co więcej, analiza biodyspozycji *in vivo* wykazało, że CMC23 penetruje przez barierę krew-mózgu i może gromadzić się w mózgu myszy (Mastromarino *i in.*, 2022).

Jak dotychczas potencjał przeciwzapalny i pro-wyciszeniowy mocznikopochodnych zwiazkow II generacji w porównaniu ze związkiem MR-39 oraz ligandami endogennymi nie był badany w doświadczalnych modelach immunoaktywacji, dlatego analiza ta stanowi przedmiot niniejszej rozprawy doktorskiej.

### 3.6.Doświadczalne modele immunoaktywacji

Poznanie mechanizmów patofizjologii i możliwości modulacji procesu zapalnego wymaga zastosowania odpowiedniego modelu doświadczalnego. Reakcję tą wywołać można poprzez zastosowanie wielu immunoaktywatorów, wśród których powszechnie stosowanym jest model immunoaktywacji z użyciem lipopolisacharydu (LPS), który jest endotoksyną bakteryjną znajdującą się w ścianach komórkowych bakterii Gram-ujemnych (Tucureanu *i in.*, 2017). Pierwotnie LPS został zidentyfikowany jako ligand dla receptora Toll-podobnego 4 (TLR4), jednak obecnie wiadomo, że jego efekty mediowane są także przez inne receptory (Meseguer *i in.*, 2014; Alpizar *i in.*, 2017; Boonen *i in.*, 2018). LPS aktywując receptor TLR4, którego ekspresja w OUN jest obecna głównie na komórkach mikrogleju, powoduje dimeryzację i rekrutację białek adaptorowych, takich jak białko pierwotnej odpowiedzi różnicowania mieloidalnego 88 (MyD88), białko indukujące zawierające domenę TIR interferon-β (TRIF) i cząsteczka adaptorowa związana z TRIF

(TRAM) (Batista *i in.*, 2019). Rekrutacja białek adaptorowych aktywuje dalsze szlaki przekazu sygnału w komórce i w konsekwencji prowadzi do aktywacji czynników transkrypcyjnych jak NF-κB oraz syntezy czynników prozapalnych jak: TNF-α, IL-1β, PGE2 czy NO (Lu *i in.*, 2008; Gong *i in.*, 2019).

LPS jest najczęściej wykorzystywany jako substancja modelowa do aktywacji komórek glejowych, głównie mikrogleju (Cianciulli *i in.*, 2015; Cunha *i in.*, 2016). Ekspresję TLR4 wykazano także na neuronach, dlatego też model immunoaktywacji z użyciem LPS z powodzeniem stosowany jest także w badaniach *ex vivo* np. hodowli organotypowych hipokampa (OHC), gdzie zachowane interakcje glejowo-neuronalne, dają możliwość szerszej i funkcjonalnej analizy udziału wielu komórek w mechanizmach procesu zapalnego (Trojan *i in.*, 2021a).

Co więcej, obwodowe podawanie LPS jest najpowszechniej stosowanym modelem doświadczalnym wywołującym uogólniony stan zapalny i zachowania chorobowe (ang. *sickness behaviour*). Warto zaznaczyć, że obecnie protokoły stosowania LPS niejednokrotnie bardzo różnią się od siebie np. ilością (jednokrotne bądź wielokrotne) czy czasem podań, co wpływa na nieco odmienny obraz działania LPS w modelach immunoaktywacji. Tym niemniej, model ten zweryfikowano pod kątem kryteriów zaproponowanych modelom zwierzęcym, a mianowicie trafności, spójności predykcyjnej oraz konstruktu (Lasselin *i in.*, 2020). W dotychczasowych badaniach pokazano między innymi, że iniekcja LPS naśladuje ważne aspekty infekcji bakteriami Gram-ujemnymi (Suffredini i Noveck, 2014). Ponadto jedną z najbardziej wiarygodnych odpowiedzi organizmu na infekcję bakterią Gram-ujemną, jest wzrost stężenia białka ostrej fazy białka C-reaktywnego, które obserwuje się także po obwodowym wstrzyknięciu LPS (Iraz *i in.*, 2015; Engler *i in.*, 2017; Kyvelidou *i in.*, 2018). Niezwykle istotny jest fakt, że podanie LPS prowadzi do zmian w zachowaniu zwierząt. Obserwuje się zmniejszenie spożycia wody i pokarmu, upośledzenie funkcji poznawczych, utraty masy ciała, czy zwiększony niepokój i lękliwość (Schaedler i Dubos, 1961; Moore *i in.*, 1977; Lagerspetz i Väätäinen, 1987). Niektóre z tych zmian, a w szczególności zmniejszona ogólna aktywność i spożycie pokarmu oraz interakcje społeczne wydają się, podobnie jak gorączka, zachowane ewolucyjnie i przypominają zmiany powszechnie obserwowane u chorych ludzi. Dlatego uznano, że model ten wiarygodnie odzwierciedla deficyty i zmiany, które rozwijają się u chorych podczas uogólnionego stanu zapalenia wywołanego infekcją (Dantzer, 2001).

# 4. Cel pracy

Patogeneza chorób ośrodkowego układu nerwowego (OUN) w dalszym ciągu pozostaje niejednoznacznie wyjaśniona, głównie ze względu na jej złożony i różnorodny charakter. Coraz więcej danych wskazuje, że u podłoża tych patologii istotną rolę odgrywa proces zapalny, zwłaszcza przedłużony do którego dochodzi w wyniku deficytów wygaszania tej reakcji (RoI). Proces RoI regulowany jest przez endogenne molekuły (SPM), które balansują reakcję zapalną w kierunku przeciwzapalnym, prowadząc w konsekwencji do jej prawidłowego zakończenia. Niestety korzystne właściwości endogennych SPM, które swój pro-wyciszeniowy efekt biologiczny realizują głównie poprzez interakcję z receptorami formylowymi (FPR), są często ograniczone ze względu na ich niekorzystny profil farmakologiczny i ograniczoną biodostępność.

Ogólnym celem przedstawionych w niniejszej rozprawie badań była weryfikacja hipotezy, że wzmocnienie RoI, poprzez zastosowanie nowych egzogennych ligandów receptora ALX/FPR2 o lepszych parametrach fizykochemicznych oraz efektywnej biodostępności i zadawalającej biodystrybucji może stanowić nową interesującą strategię ograniczającą proces zapalny, przydatną w modulacji deficytów układu odpornościowego w przebiegu wielu chorób OUN. W oparciu o międzynarodową współpracę z prof. Marcello Leopoldo oraz prof. Enzą Lacivita z Uniwersytetu w Bari, przeprowadzono ocenę przeciwzapalnego i pro-wyciszeniowego potencjału nowych zsyntetyzowanych przez ten ośrodek mocznikopochodnych agonistów receptora ALX/FPR2 w warunkach *in vitro* hodowli pierwotnych mikrogleju, *ex vivo* hodowli organotypowych hipokampa, a następnie dokonano weryfikacji uzyskanych wyników w warunkach immunoaktywacji *in vivo* w wybranych strukturach mózgu.

We wszystkich modelach do indukcji reakcji zapalnej wykorzystano lipopolisacharyd (LPS), który jest uznanym niespecyficznym aktywatorem układu odpornościowego i powszechnie stosowanym doświadczalnym narzędziem w badaniach molekularnych podstaw regulacji procesów zapalnych.

Szczegółowe cele rozprawy obejmowały:

1. W hodowlach pierwotnych mikrogleju porównawczą ocenę wpływu nowego mocznikopochodnego agonisty ALX/FPR2 związku MR-39 z agonistami endogennymi: LXA4 i jej analogiem AT-LXA4 na procesy śmierci/przeżycia komórek, ekspresję genów i poziomy wybranych cytokin o profilu prozapalnym oraz przeciwzapalnym.

2. W hodowlach organotypowych hipokampa ocenę pro-wyciszeniowego potencjału nowych II generacji, mocznikopochodnych agonistów ALX/FPR2 (związków AMS21, CMC23), w tym ich wpływu na ekspresję i poziomy wybranych czynników o profilu prozapalnym i przeciwzapalnym. Określenie roli receptorów ALX/FPR2 na komórkach mikrogleju w obserwowanych efektach poprzez zastosowanie procedury eliminacji tych komórek z hodowli organotypowych hipokampa.

3. W zwierzęcym modelu immunoaktywacji *in vivo* tzw. „*sickness behaviour*" opartym o jednorazowe dootrzewnowe podanie lipopolisacharydu dorosłym szczurom, ocenę wpływu nowych mocznikopochodnych agonistów ALX/FPR2 oraz LXA4 na zaburzenia behawioralne w teście wymuszonego pływania (test Porsolta) oraz parametry immunologiczne w korze czołowej i hipokampie.

4. Wyjaśnienie potencjalnych molekularnych mechanizmów działania endogennych oraz nowych mocznikopochodnych agonistów ALX/FPR2 poprzez ocenę ich wpływu na poziom:

   - białek związanych z wewnątrzkomórkowymi szlakami sygnałowymi: ERK1/2 (ang. *extracellular signal-regulated kinases 1/2*), p38 (ang. *p38 mitogen-activated protein kinase*) oraz czynnikiem transkrypcyjnym NF-κB (ang. *nuclear factor kappa-light-chain-enhancer of activated B cells*)

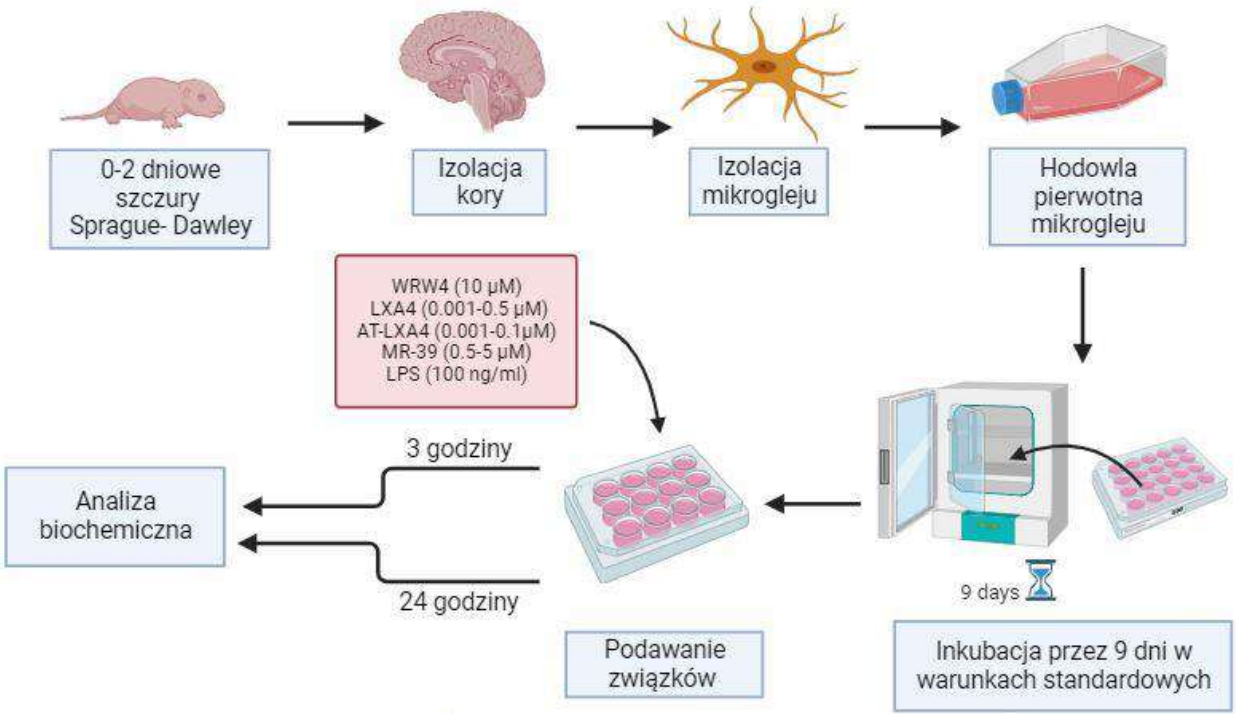
   - białek związanych ze ścieżką STAT3/SOCS3: STAT3 (ang. *signal transducer and activator of transcription 3*) oraz białka SOCS3 (ang. *suppressor of cytokine signaling 3*)

   - białek kompleksu NLRP3: NLRP3 (ang. *nucleotide-binding oligomerization domain-like (NOD-like) receptor pyrin-containing 3 inflammasome*), kaspazy 1 oraz białka ASC (PYCARD) (ang. *apoptosis-associated speck-like protein containing a caspase recruitment domain*).

## 5. Zarys metod i technik badawczych zastosowanych w pracach opublikowanych stanowiących podstawę rozprawy doktorskiej

**Praca nr. 2**

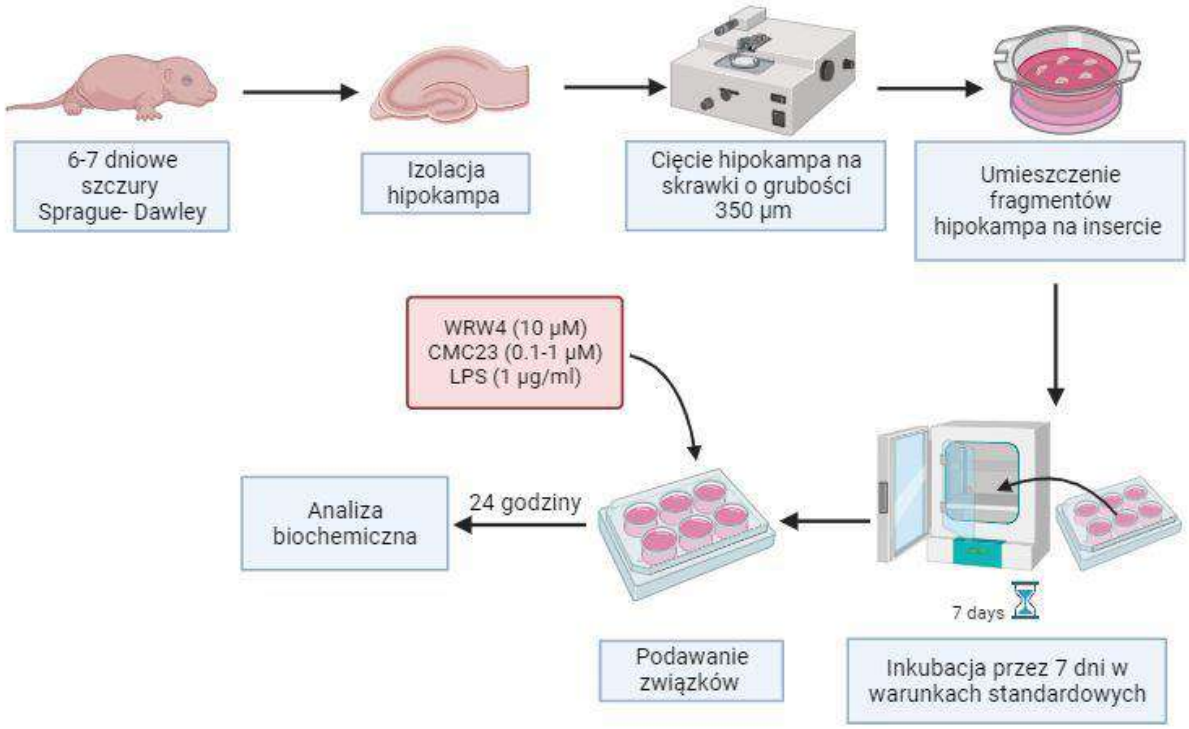
**Hodowla pierwotna mikrogleju**



| **Technika** | **Oceniany parametr** |
|---|---|
| Test uwalniania dehydrogenazy mleczanowej (LDH) | Śmiertelność, procesy nekrotyczne, toksyczność substancji |
| Reakcja Griess'a | Wydzielanie tlenku azotu (NO) |
| Test JC-1 | Analiza potencjału błony mitochondrialnej ($\Delta\psi m$) |
| Test DCFH-DA (ang. *2,7-dichlorofluorescein diacetate*) | Poziom reaktywnych form tlenu – ROS (ang. *reactive oxygen species*) |
| Test aktywności kaspazy 3 | Poziom kaspazy 3 |
| Immunohistochemia | Obecność i poziom receptora ALX/FPR2 w mikrogleju |
| qRT-PCR | Ekspresja genów: *Cd40, Cd68, Cd206, Arg1, Igf-1, Il-1β, Il-10, Tnf-α* |
| ELISA | Poziom białka cytokin: IL-1β, IL-6, IL-10, TNF-α |
| Western blot | Poziom białka: ERK1/2, NF-κB, p38 – formy całkowite i ufosforylowane |

**Praca nr. 3**

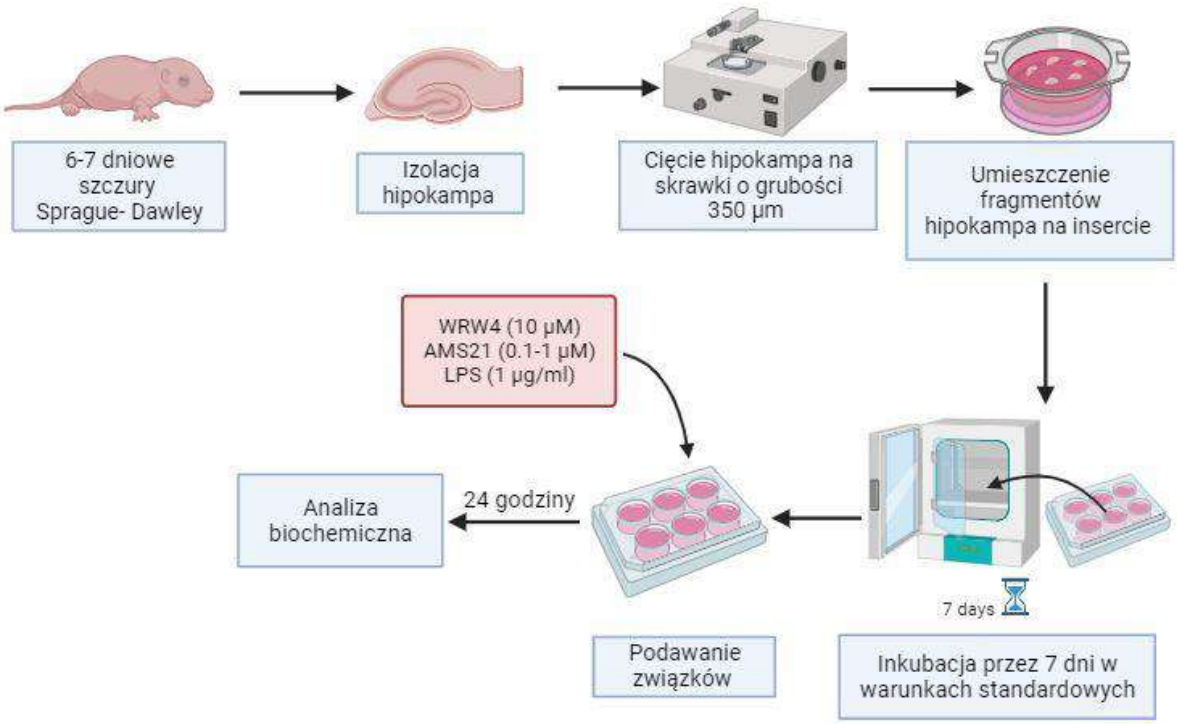
## Hodowla organotypowe hipokampa (OHC)



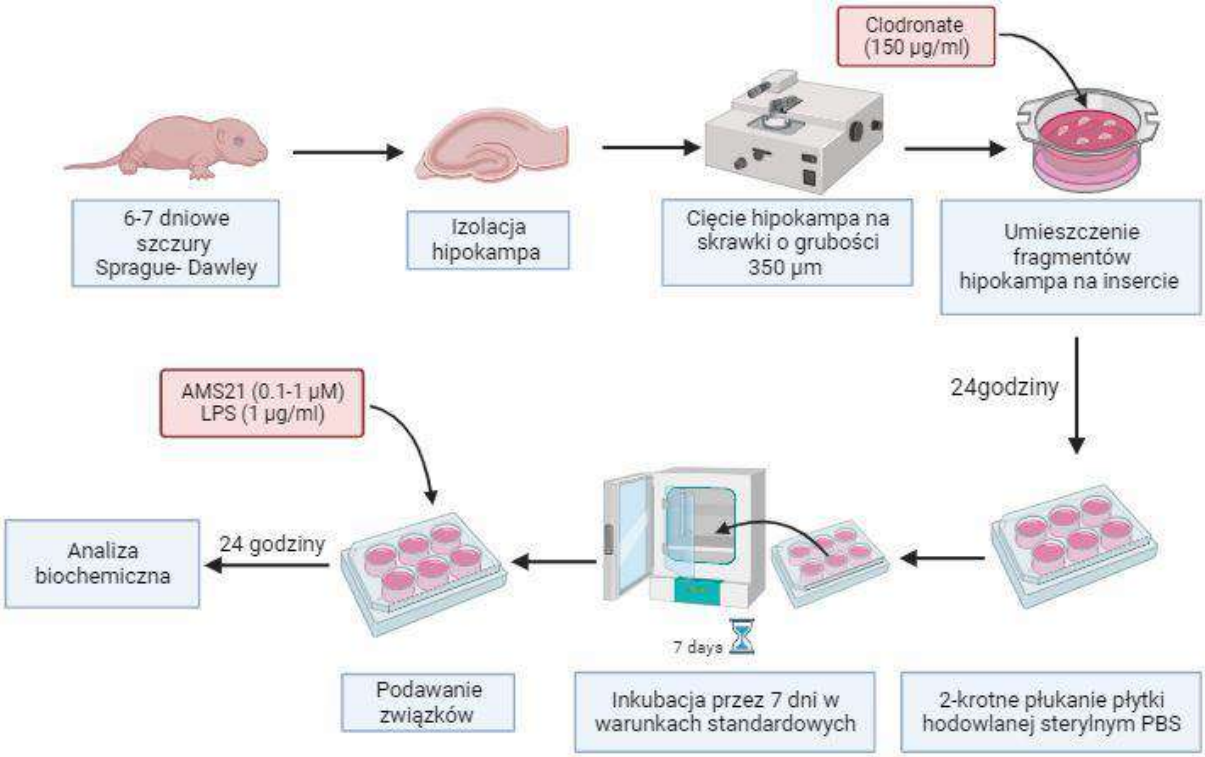
| **Technika** | **Oceniany parametr** |
|---|---|
| Test uwalniania dehydrogenazy mleczanowej (LDH) | Śmiertelność, procesy nekrotyczne, toksyczność substancji |
| Reakcja Griess'a | Wydzielanie tlenku azotu (NO) |
| Immunohistochemia | Obecność markera IBA1 i receptora ALX/FPR2 w hodowlach organotypowych hipokampa |
| qRT-PCR | Poziom ekspresji genów: *Arg-1*, *Ccl2*, *Cd40*, *Cd68*, *Igf-1*, *Il-6*, *Il-10*, *Il-12*, *Il-23*, *Jak1*, *Jak2*, *Stat3 Tgf-β* |
| ELISA | Poziom białka cytokin: IL-1β, IL-6, IL-10, IL-12/23p40, IL-17A, IL-23p19 TGF-β oraz czynnika SOCS3 |
| Western blot | Poziom białka: STAT3 – forma całkowita i ufosforylowana |

**Praca nr. 4**

## Hodowla organotypowa hipokampa (OHC)




## Hodowla organotypowa hipokampa (OHC) z procedurą eliminowania mikrogleju



| **Technika** | **Oceniany parametr** |
|---|---|
| Test uwalniania dehydrogenazy mleczanowej (LDH) | Śmiertelność, procesy nekrotyczne, toksyczność substancji |
| Reakcja Griess'a | Wydzielanie tlenku azotu (NO) |
| Immunohistochemia | Poziom markera IBA1 i receptora ALX/FPR2 w hodowlach organotypowych hipokampa |
| qRT-PCR | Ekspresja genów: *Casp1, Cd40, Cd68, Igf-1,Il-1β, Il-1Ra, Il-6, Il-18, Nlrp3, Pycard, Tgf-β* |
| ELISA | Poziom białka: CASP1, IL-1β, IL-6, IL-10, NLRP3, PYCARD, TGF-β |
| Western blot | Poziom białka: IBA1 |

# 6. Metody i techniki badawcze zastosowane w dodatkowych nieopublikowanych badaniach włączonych do hybrydowej wersji rozprawy doktorskiej

## 6.1.Zwierzęta

Do badań wykorzystano 3-miesięczne szczury szczepu Sprague-Dawley (Charles River, Sulzfeld, Niemcy). Zwierzęta hodowano w warunkach standardowych: temperaturze pokojowej (23°C), 12/12 godzinnym cyklu światło/ciemność rozpoczynającym się o godzinie 8:00 rano, z nieograniczonym dostępem do wody oraz pożywienia (*ad libitum*). Doświadczenia przeprowadzono zgodnie z akceptacją II Lokalnej Komisji Etycznej do Spraw Doświadczeń na Zwierzętach Instytutu Farmakologii im. Jerzego Maja PAN w Krakowie (nr zgody 204/2018 z dnia 28.06.2018).

## 6.2.Procedura operacyjna

Samce Sprague-Dawley wprowadzono w stan głębokiej narkozy poprzez domięśniowe podanie mieszaniny chlorowodorku ketaminy (75 mg/kg, Bioketan; Biowet, Puławy, Polska) i ksylazyny (5 mg/kg, Sedazin; Biowet, Puławy, Polska), a następnie umieszczono w aparacie stereotaktycznym (David Kopf Instruments, Tujunga, Kalifornia, USA). Implantację prowadnicy kaniuli (*ang. guide cannula*) ze stali nierdzewnej (26 mm, długość 8 mm, Plastic One, Roanoke, VA, USA) wykonano do prawej komory mózgu (współrzędne od Bregma: AP: -0,8 mm; ML: -1,5 mm i -2,0 mm) według atlasu Paxinos i Watson (1998). Podstawę kaniuli mocowano do czaszki dentystycznym cementem akrylowym i dwiema miniaturowymi śrubami ze stali nierdzewnej (AgnTho's, Sztokholm, Szwecja). Obturator ze stali nierdzewnej wprowadzano do prowadnicy kaniuli w celu utrzymania drożności. Po operacji wszystkie zwierzęta przetrzymywano indywidualnie w standardowych plastikowych klatkach (39 cm × 28 cm × 28 cm) przez cały okres rekonwalescencji (5-7 dni).

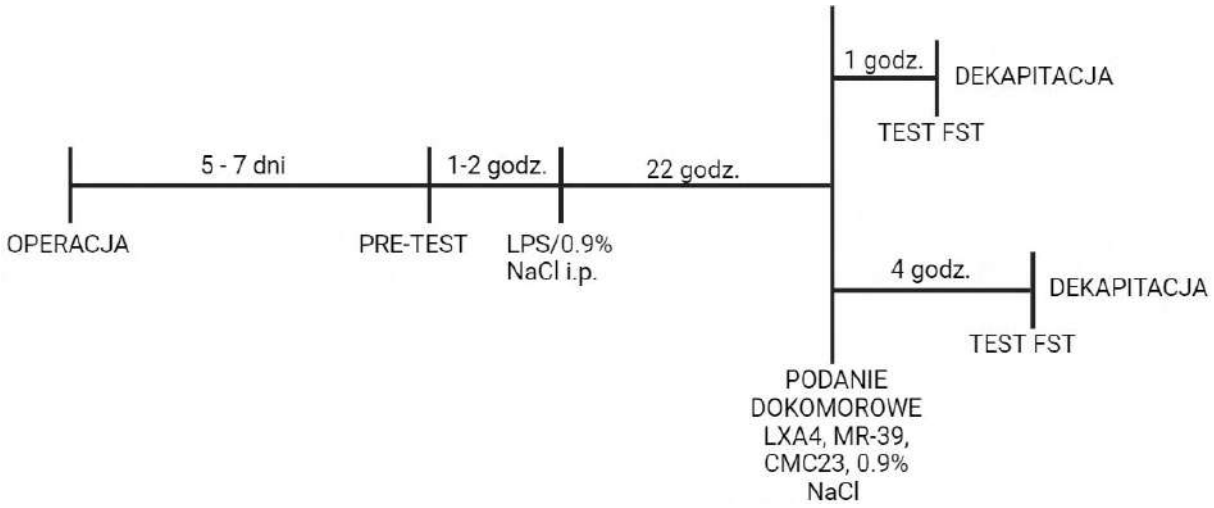
## 6.3.Związki wykorzystane do badań *in vivo*

Wszystkie związki przygotowano bezpośrednio przed ich wykorzystaniem. Bakteryjną endotoksynę (lipopolisacharyd; LPS; *Escherichia coli* 026:B6; Sigma-Aldrich, St. Louis,MO, USA) rozpuszczono w soli fizjologicznej (0,9% NaCl). Lipoksynę A4

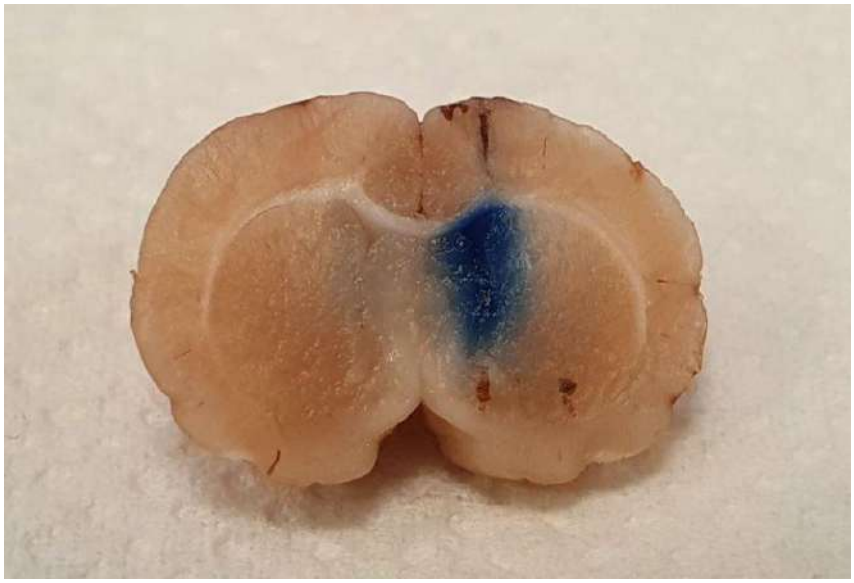
(LXA4, Cayman Chemical Compan, Ann Arbor, USA) rozpuszczono w roztworze etanolu z 0.9% NaCl (w stosunku 1:4). Związki MR-39 (S)-3-(4-Cyanophenyl)-N-[[1-(3-chloro-4-fluorophenyl)cyclopropyl]methyl]-2-[3-(4-fluorophenyl)ureido]propanamide oraz CMC23 ((S)-1-(3-(4-cyanophenyl)-1-(6-fluoroindolin-1-yl)-1-oxopropan2-yl)-3-(4-fluorophenyl)urea) zsyntetyzowane w Katedrze Farmacji Uniwersytetu w Bari, rozpuszczono w roztworze DMSO (dimetylosulfotlenek; Gibco, Waltham, MA, USA) oraz 0.9% NaCl (w stosunku 1:10). Po okresie rekonwalescencji zwierzęta losowo dzielono na dwie grupy doświadczalne: kontrolną oraz traktowaną LPS (po 20 zwierząt na grupę). Odpowiednio, LPS w dawce 250 μg/kg masy ciała (grupa LPS) lub iniekcje z soli fizjologicznej (0,9% NaCl) (grupa kontrolna) wykonano 1-2 godziny po wstępnej weryfikacji behawioralnej (tzw. pre-test, test Porsolta), a tym samym 22-godziny przed dokomorowymi podaniami poszczególnych agonistów.

### 6.4.Podania dokomorowe (ang. *intracerebroventricular, icv*)

Jednostronne podanie *icv:* badanych agonistów receptora ALX/FPR2 lub 0.9% NaCl przeprowadzono przy użyciu mikro strzykawki Hamiltona połączonej drenem polietylenowym z kaniulą podającą (rozmiar 33, długość 10 mm, Plastic One, Roanoke, VA, USA), dłuższą o 2 mm niż kaniula prowadząca. Świeżo przygotowany roztwór: LXA4 (5 μl; 0.1 μg/5 μl), MR-39 (5 μl; 1 μg/5 μl) oraz CMC23 (5 μl; 1 μg/5 μl) lub sterylną sól fizjologiczną podawano pojedynczo z prędkością 2 μl/min. Podawanie poszczególnych związków (LXA4, MR-39 lub CMC23) prowadzono zgodnie ze schematem przedstawionym poniżej (Rys.4). Kaniulę podającą pozostawiono na miejscu na 1 minutę, aby umożliwić dyfuzję, a następnie zastąpiono ją obturatorem. Prawidłowe umiejscowienie podania *icv* sprawdzono w pilotażowym zabiegu chirurgicznym z użyciem fioletu krezylu (Rys.5).




***Rys. 4*** *Plan układu eksperymentalnego w części badań in vivo. Autorstwo własne; Schemat wykonano w BioRender.*



***Rys. 5*** *Pilotażowe operacje stereotaktyczne – podanie fioletu krezylu do prawej komory mózgu szczura.*

### 6.5.Test Porsolta (ang. *forced swimming test,* FST)

Test wymuszonego pływania (FST, test Porsolta) został przeprowadzony zgodnie z metodyką opisaną przez Porsolt *i in.* (1978) z późniejszymi modyfikacjami Detke *i in.* (1995) oraz zgodnie z naszymi wcześniej opublikowanymi badaniami (Basta-Kaim *i in.*, 2014; Szczesny *i in.*, 2014; Detka *i in.*, 2015). Doświadczenia wykonywano w plastikowym cylindrze (wysokość 50 cm, średnica 18 cm) wypełnionym wodą (23°C) do wysokości 35 cm. Po 5-7-dniowym okresie rekonwalescencji przeprowadzono trening (tzw. pre-test), który trwał 15 min. Test właściwy w trakcie, którego przez 5 min mierzono całkowity czas

bezruchu (ang. *immobility*), poruszania się/pływania (ang. *swimming*) oraz wspinania się (ang. *climbing*) zwierząt, wykonano 1 lub 4 godziny po dokomorowym podaniu badanych agonistów receptora ALX/FPR2 (opis grup doświadczalnych przedstawiono poniżej; Tabela 2.). Następnie po przeprowadzeniu testu zwierzęta poddano dekapitacji w celu pobrania struktur do oznaczeń biochemicznych.

***Tabela 2**. Podział grup doświadczalnych*

| **L.P.** | **Grupa** | **Podanie dokomorowe (*icv*)** | **Liczba osobników** |
|---|---|---|---|
| **1.** | grupa kontrolna | podawano 0.9% NaCl | n = 5 |
| **2.** | grupa kontrolna | podawano LXA4 | n = 5 |
| **3.** | grupa kontrolna | podawano MR-39 | n = 5 |
| **4.** | grupa kontrolna | podawano CMC23 | n = 5 |
| **5.** | grupa LPS | podawano NaCl | n = 5 |
| **6.** | grupa LPS | podawano LXA4 | n = 5 |
| **7.** | grupa LPS | podawano MR-39 | n = 5 |
| **8.** | grupa LPS | podawano CMC23 | n = 5 |

### 6.6.Pobieranie tkanek

Wyizolowane struktury mózgu, tj. korę czołową (*Cx*) i hipokamp (*Hp*), umieszczano na suchym lodzie, a następnie przechowywano w temperaturze -80°C do czasu przeprowadzenia homogenizacji i wykonywania właściwych oznaczeń immunoenzymatycznych metodą ELISA (ang. *enzyme-linked immunosorbent assay*).

### 6.7.Izolacja białka

Homogenizację tkanek (kory czołowej oraz hipokampa) przeprowadzano w buforze RIPA, który zawierał koktajl inhibitorów Halt™ proteazy i fosfatazy (Thermo Fisher Scientific, Waltham, MA, USA) przy użyciu Tissue Lyser II (Qiagen Inc, Valencia, Kalifornia, USA). Stężenie białka w analizowanych próbkach oznaczono przy użyciu kwasu bicinchoninowego metodą BCA (Sigma-Aldrich, St. Louis, MO, USA) i mierzono przy długości fali 562 nm w spektrofotometrze Tecan Infinite 200 Pro (Tecan, Mannedorf, Niemcy).

### 6.8.Test ELISA

Do oznaczeń białek metodą ELISA wykorzystano komercyjnie dostępne zestawy analityczne. Poziom interleukiny 1β (IL-1β) oraz czynnika martwicy nowotworów α (TNF-α) w homogenatach kory czołowej oraz hipokampa oznaczano zgodnie z instrukcjami dołączonymi przez producenta (Wuhan Fine Biotech Co., Wuhan, Chiny). Stężenie próbek dobierano indywidualnie dla każdego oznaczenia w oparciu o wyniki próby przeprowadzonej dla różnych rozcieńczeń. Błędy wewnątrz seryjne przeprowadzonych oznaczeń wynosiły mniej niż 7,5%, zaś między seryjne mniej niż 10%.

# 7. Wyniki z dodatkowych, nieopublikowanych badań *in vivo*

## 7.1. Badanie wpływu dokomorowych (*icv*) podań agonistów receptora ALX/FPR2 w modelu LPS na parametry testu wymuszonego pływania

Wszystkie badania *in vivo* prowadzono porównawczo u samców szczurów szczepu Sprague-Dawley kontrolnych (zwierzęta otrzymały iniekcje z 0.9% NaCl) oraz traktowanych LPS (zwierzęcy model immunoaktywacji). Zastosowany w niniejszej pracy test wymuszonego pływania (test Porsolta) wykorzystywany jest powszechnie do oceny zachowań behawioralnych oraz weryfikacji efektywności badanych związków (Lucki, 1997; Pollak *i in.*, 2010; Szczesny *i in.*, 2014).

Test ten przeprowadzono zgodnie ze schematem opracowanym przez Porsolt *i in.* (1978) z modyfikacją Detke *i in.* (1995). Wykonanie pomiaru czasu bezruchu (ang. *immobility*) pozwoliło na obserwację objawu jakim jest stan rezygnacji, przejawiający się tym, że zwierzęta wykonują jedynie ruchy niezbędne do utrzymania głowy na powierzchni wody. Kolejnym mierzonym parametrem był czas pływania (ang. *swimming*), czyli czas aktywnego ruchu zwierząt. Przeprowadzono także ocenę czasu wspinania się (ang. *climbing*) mierząc czas trwania prób wydostania się zwierząt z cylindra.

Wykazano, że w porównaniu ze zwierzętami kontrolnymi jednorazowe dootrzewnowe podanie lipopolisacharydu prowadzi do zmian behawioralnych u samców szczurów. Obserwowano istotne skrócenie czasu pływania, i wspinania przy jednoczesnym wydłużeniu czasu bezruchu (Ryc. 1).

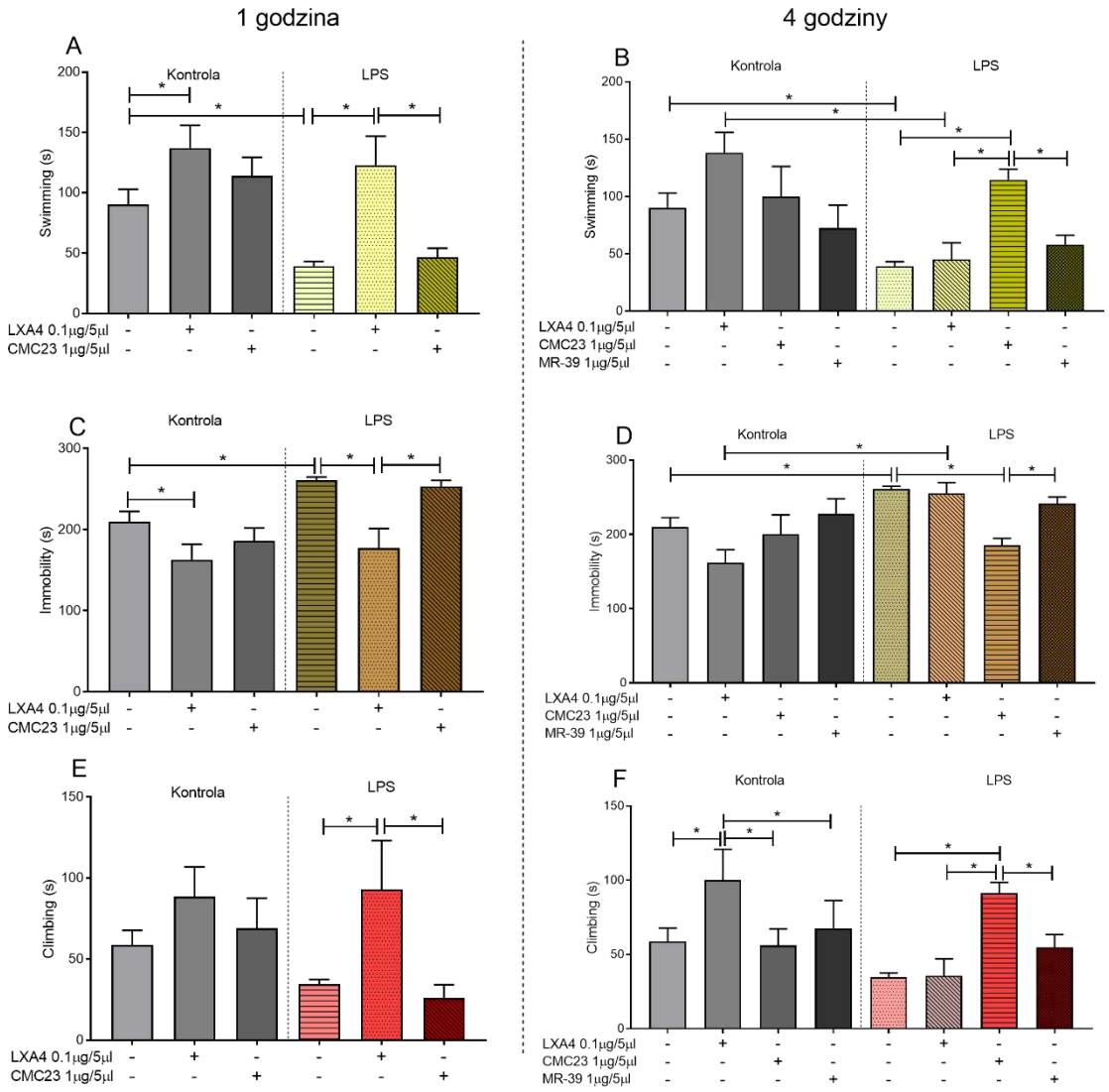
Wpływ badanych agonistów receptora ALX/FPR2 na zachowania zwierząt zarówno kontrolnych, jak i w modelu immunoaktywacji LPS oceniano w dwóch punktach czasowych tj. 1 godzinę oraz 4 godziny po dokomorowych podaniach poszczególnych związków, a uzyskane wyniki przedstawiono porównawczo na kolejnych panelach Ryciny 1.

Zaobserwowano, że 1 godzinę po dokomorowym podaniu endogennego liganda LXA4 zarówno u zwierząt kontrolnych, jak i traktowanych LPS wydłużeniu uległ czas pływania (Ryc. 1A), natomiast skróceniu czasu bezruchu (Ryc. 1C). U zwierząt po podaniu LPS wykazano także istotny wpływ LXA4 na wydłużenie czasu wspinania (Ryc. 1E). Analiza tych samych parametrów w teście Porsolta 4 godziny po podaniu LXA4 nie

wykazała jednak wpływu tego liganda zarówno na parametry u zwierząt kontrolnych jak i po podaniach LPS-u.

W przeprowadzonych w pracy badaniach oceniono także wpływ CMC23 na analogiczne parametry testu wymuszonego pływania. Wykazano, że związek ten nie wpłynął na czas bezruchu, pływania i wspinania u zwierząt kontrolnych. Niemniej jednak, u zwierząt z uogólnioną reakcja zapalną, wywołaną podaniem LPS, CMC23 4 godziny po podaniu istotnie skracał czas bezruchu oraz wydłużał czas pływania i wspinania (Ryc. 1B, 1D, 1F), co świadczy o jego potencjale normalizującym deficyty behawioralne wywołane immunoaktywacją.

W przypadku zastosowania agonisty receptora ALX/FPR2 związku MR-39 mając na uwadze wcześniejsze wyniki zaprezentowane w opublikowanej pracy nr 2 ocenę jego wpływu na parametry w teście Porsolta przeprowadzono jedynie 4 godziny po podaniu. Przeprowadzona analiza statystyczna nie wykazała istotnego wpływu tego agonisty na oceniane parametry zarówno w grupie kontrolnej jak i poddanej uogólnionej immunoaktywacji.




***Ryc. 1*** *Wpływ podań dokomorowych agonistów receptora ALX/FPR2: LXA4 (0.1 µg/5 µl), MR-39 (1 µg/5 µl) oraz CMC23 (1 µg/5 µl) na czas ruchu (Ryc.**1A-B**), bezruchu (Ryc.**1C-D**) oraz wspinania (Ryc. **1E-F**) u zwierząt kontrolnych oraz traktowanych LPS (250 µg/kg) oceniany w teście wymuszonego pływania (FST). Parametry w teście FST były mierzone odpowiednio 1 godzinę oraz 4 godziny po dokomorowym podaniu poszczególnych ligandów. Grupy kontrolne traktowane były 0.9% roztworem NaCl. Dane przedstawiono jako średnią arytmetyczną ± SEM, n=5 w grupie. Różnice istotne statystycznie oznaczono *p<0.05. LXA4 – Lipoksyna A4; LPS – Lipopolisacharyd.*

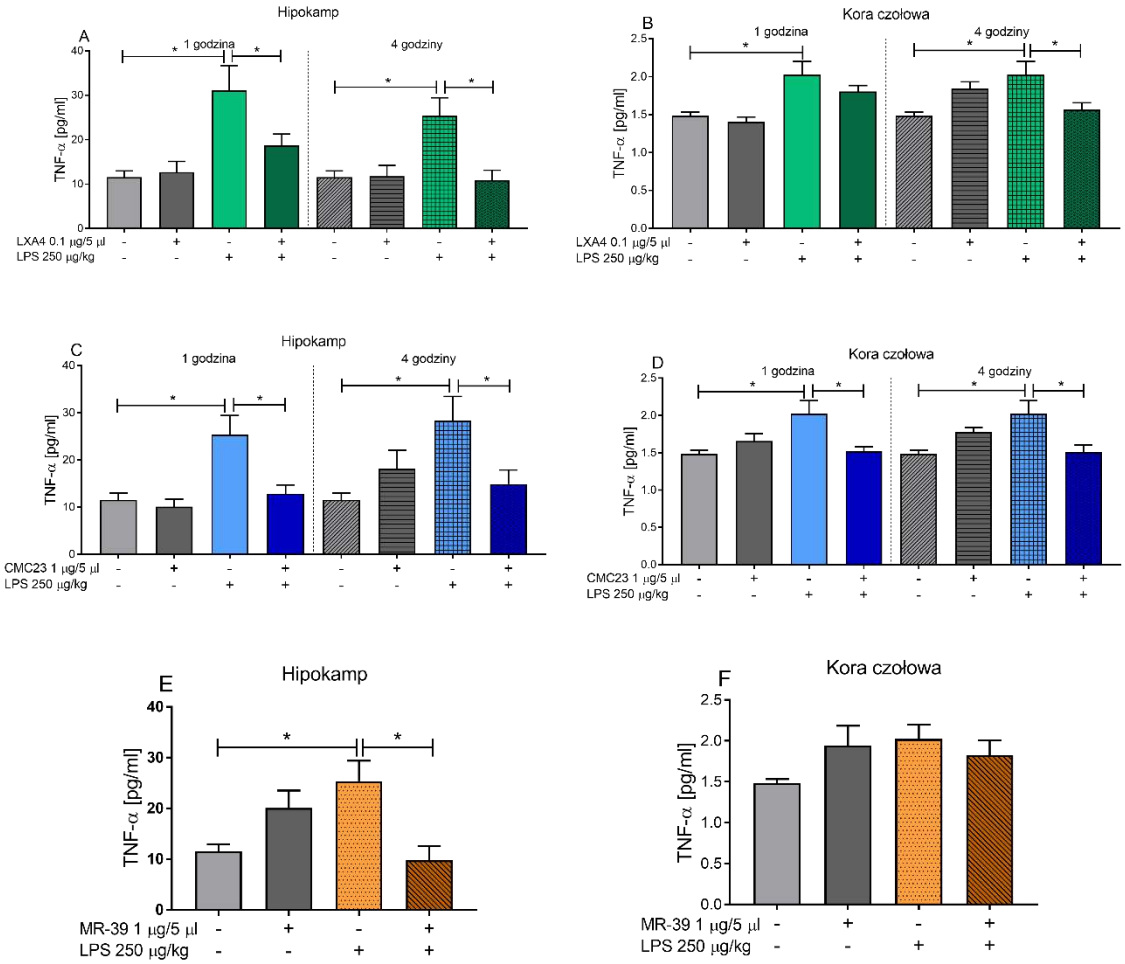
## 7.2. Badanie wpływu dokomorowych (*icv*) podań agonistów receptora ALX/FPR2 w modelu LPS na poziom białka TNF-α

Zaprezentowane w pracy wyniki analiz biochemicznych przeprowadzono także porównawczo u samców szczurów szczepu Sprague-Dawley kontrolnych (zwierzęta otrzymały iniekcje z soli fizjologicznej) oraz traktowanych LPS (zwierzęcy model immunoaktywacji). Wykazano, że podanie LPS-u powoduje istotny wzrost aktywacji zapalnej w hipokampie oraz korze czołowej u badanych zwierząt, wyrażony jako wzrost poziomu cytokiny prozapalnej TNF-α w homogenatach tych struktur.

Natomiast przeprowadzona analiza w hipokampie zarówno 1 godzinę jak i 4 godziny po podaniu LXA4 wykazała normalizujący wpływ tego endogennego związku na wywołany podaniem endotoksyny wzrost poziomu TNF-α. (Ryc.2A) W przypadku kory czołowej korzystne normalizujące działanie LXA4 na poziom tej cytokiny obserwowano jedynie 4 godziny po podaniu tego liganda (Ryc.2B).

Analiza statystyczna uzyskanych wyników wykazała, że związek CMC23 obniżał wywołany podaniem LPS wzrost poziomu TNF-α zarówno w korze czołowej (Ryc. 2D) jak i hipokampie (Ryc. 2C). Efekt ten był obserwowano w obu pubktach czasowych: 1 godzinę i 4 godziny po podaniu nowego agonisty receptora ALX/FPR2.

Ocenę wpływu agonisty MR-39 podobnie jak w badaniach behawioralnych przeprowadzono jedynie 4 godziny po jego podaniu dokomorowym. Wykazano, normalizujące działanie tego liganda na podwyższony w wyniku immunoaktywacji poziom cytokiny TNF-α, ale tylko w hipokampie (Ryc. 2E).



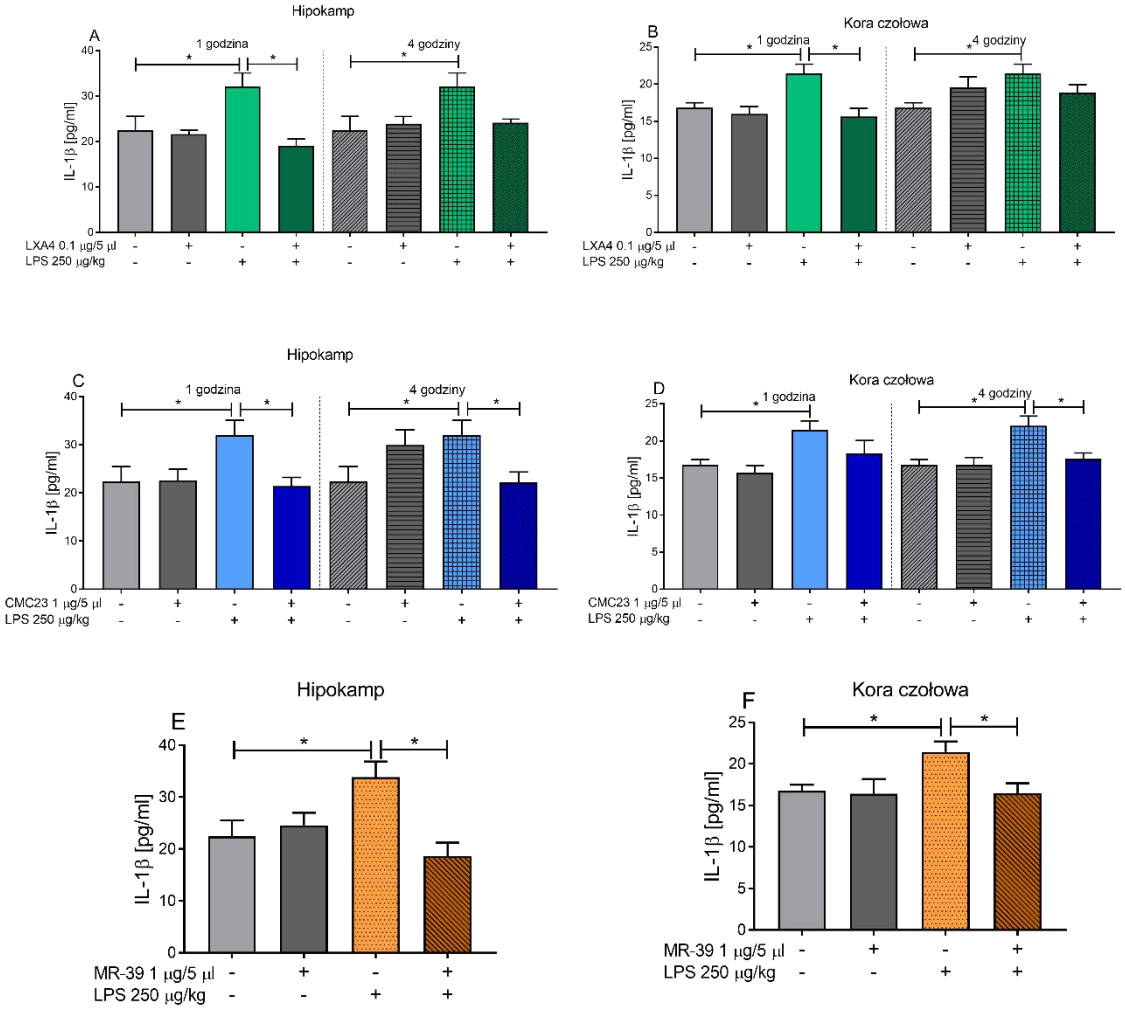

***Ryc. 2*** *Wpływ podań dokomorowych agonistów receptora ALX/FPR2: LXA4 (0.1 μg/5 μl), MR-39 (1 μg/5 μl) oraz CMC23 (1 μg/5 μl) na poziom białka TNF-α u zwierząt kontrolnych oraz traktowanych LPS (250 μg/kg). Oznaczenie stężenia białka wykonano odpowiednio 1 godzinę oraz 4 godziny po dokomorowym podaniu poszczególnych ligandów w hipokampie (Ryc.**2A, 2C, 2E**) oraz korze czołowej* ***(Ryc.2B, 2D, 2F)****. Dane przedstawiono jako średnią arytmetyczną ± SEM, n=5 w grupie. Różnice istotne statystycznie oznaczono *p<0.05. LXA4 – Lipoksyna A4; LPS – Lipopolisacharyd; TNF-α – Czynnik martwicy nowotworów α.*

### 7.3. Badanie wpływu dokomorowych (*icv*) podań agonistów receptora ALX/FPR2 w modelu LPS na poziom białka IL-1β

Potencjał przeciwzapalny i pro-wyciszeniowy badanych agonistów receptora ALX/FPR2 oceniano także poprzez pomiar modulacji poziomu innej cytokiny prozapalnej – interleukiny 1β. Jak pokazano na rycinie 3, zaobserwowano istotne podwyższenie poziomu IL-1β zarówno w hipokampie, jak i w korze czołowej u zwierząt po dootrzewnowym podaniu LPS.

Zaobserwowano, że 1 godzinę po dokomorowym podaniu LXA4, wywołany podaniem endotoksyny bakteryjnej wzrost poziomu IL-1β zarówno w hipokampie, jak i korze czołowej, ulegał normalizacji (Ryc. 3A-B). Natomiast związek CMC23 obniżał hipokampalny wzrost poziomu tej cytokiny zaobserwowany po uogólnionej immunoaktywacji w obu badanych punktach czasowych po dokomorowej iniekcji tego liganda (Ryc. 3C), a w korze czołowej jedynie po 4 godzinach od podania CMC23 (Ryc. 3D). Wykazano, także korzystne działanie polegające na normalizacji aktywacji zapalnej wyrażonej wzrostem poziomu IL-1β w obu badanych strukturach mózgu 4 godziny po dokomorowym zastosowaniu agonisty MR-39 (Ryc. 3E-F).

Wyniki badań biochemicznych wskazują, że ligandy receptora ALX/FPR2 wykazują działanie przeciwzapalne i/lub pro-wyciszeniowe, chociaż ich potencjał jest zróżnicowany i zależny nie tylko od budowy chemicznej liganda, czasu działania, ale także od struktury mózgu.




***Ryc. 3*** *Wpływ podań dokomorowych agonistów receptora ALX/FPR2: LXA4 (0.1 μg/5 μl), MR-39 (1 μg/5 μl) oraz CMC23 (1 μg/5 μl) na poziom białka IL-1β u zwierząt kontrolnych oraz traktowanych LPS (250 μg/kg). Oznaczenie stężenia białka wykonano odpowiednio 1 godzinę oraz 4 godziny po dokomorowym podaniu poszczególnych ligandów w* hipokampie *(Ryc.**3A, 3C, 3E**) oraz korze czołowej **(Ryc.3B, 3D, 3F)**. Dane przedstawiono jako średnią arytmetyczną ± SEM, n=5 w grupie. Różnice istotne statystycznie oznaczono *p<0.05. LXA4 – Lipoksyna A4; LPS – Lipopolisacharyd; IL-1β – Interleukina 1β.*

# 8. Dyskusja

W badaniach mechanizmów biologicznych procesów zapalnych jednym z najistotniejszych elementów jest wybór odpowiedniego modelu doświadczalnego. W trzech pracach oryginalnych wchodzących w skład rozprawy doktorskiej przedstawiono wyniki wielopłaszczyznowych badań *in vitro* z wykorzystaniem hodowli pierwotnych mikrogleju oraz *ex vivo* hodowli organotypowych hipokampa aktywowanych lipopolisacharydem, a następnie przeprowadzono weryfikację uzyskanych wyników w zwierzęcym modelu uogólnionej reakcji zapalnej. Zastosowana w badaniach endotoksyna bakteryjna jest niespecyficznym immunostymulatorem, którego użycie prowadzi do aktywacji komórek immunokompetentnych i w efekcie uwalniania mediatorów procesu zapalnego w tym cytokin, chemokin oraz ROS, zmiany fenotypów komórek w mózgu, a poprzez interakcje pomiędzy układem neuroendokrynnym i immunologicznym także do deficytów zachowań w warunkach *in vivo.* Zastosowane modele doświadczalne pozwoliły zatem na kompleksową ocenę potencjału badanych ligandów receptora ALX/FPR2, w patomechanizmach procesów zapalnych, a szczególnie ich wyciszania w warunkach zakłóconej przez LPS homeostazy.

***Badania in vitro***

W pierwszym etapie badań prezentowanych w rozprawie dokonano zależnej od czasu oceny właściwości dwóch endogennych ligandów receptora ALX/FPR2, czyli LXA4 oraz AT-LXA4, a także nowego, I generacji mocznikopochodnego agonisty, związku MR-39 w hodowlach pierwotnych mikrogleju. Mikroglej to główne komórki immunokompetentne w mózgu, które ulegają silnej aktywacji w wyniku stymulacji LPS (Helmut *i in.,* 2011; Du *i in.,* 2017). W badaniach własnych potwierdzono te obserwacje i wykazano, że LPS zwiększa poziom uwalnianej dehydrogenazy mleczanowej (LDH) oraz tlenku azotu (NO), a także reaktywnych form tlenu (ROS) oraz nasila aktywność kaspazy 3. Należy także wspomnieć, o utracie potencjału błony mitochondrialnej ($\Delta\psi m$) w wyniku stymulacji LPS, co wykazano jako obniżenie poziomu barwnika karbocyjnaninowego (JC-1) kumulującego się w komórkach (Zorova *i in.,* 2018; Sivandzade *i in.,* 2019). W omawianych badaniach w pracy Tylek *i in.,* (2021b) wykazano protekcyjne, chociaż zróżnicowane działanie wszystkich badanych ligandów. Endogenny ligand LXA4 obniżał podwyższony przez LPS poziom uwalnianego LDH, jedynie po 3 godzinach; natomiast jego

epimer AT-LXA4 oraz MR-39 w stężeniu mikromolarnym skutecznie normalizowały poziom LDH 24 godziny po stymulacji LPS. Ciekawą obserwacją jest wykazanie, że efekty te były w większości zależne od receptora ALX/FPR2, ponieważ antagonista tego receptora związek WRW4 blokował protekcyjne działanie badanych ligandów. Zwiększone uwalnianie LDH sugeruje utratę potencjału mitochondrialnego mikroglejowych komórek oraz nasilenie procesów nekrotycznej śmierci komórkowej, ale także aktywację szlaku apoptozy w komórce (Tiefenthaler *i in.,* 2001). Istotne znaczenie ma zatem obserwacja, że spośród badanych agonistów utrzymujące się do 24 godzin działanie antyapoptotyczne wykazał jedynie związek MR-39, a podniesienie potencjału błony mitochondrialnej przez ten ligand korelowało z jego korzystnym wpływem na aktywność kaspazy 3, która jak wskazują badania innych autorów pełni kluczową rolę w regulacji procesów apoptotycznych (Burguillos *i in.,* 2011; Venero *i in.,* 2011; Nelson *i in.,* 2014).

Kolejną obserwacją jest wykazany korzystny antyoksydacyjny potencjał ligandów receptora ALX/FPR2 w hodowlach mikrogleju. LXA4 modulowała podniesiony przez 3-godzinną stymulację LPS poziom ROS, natomiast MR-39 wykazał podobne działanie, lecz po 24 godzinach od podania endotoksyny bakteryjnej, a dodatkowo modulował także poziom tlenku azotu. Wydaje się, że skoro podniesiony poziom ROS nasila procesy zapalne i apoptotyczne (Allen i Tresini, 2000), a modulacja poziomu NO w mózgu ma kluczowe znaczenie w utrzymaniu balansu pomiędzy procesami neurotoksycznymi i neuroprotekcyjnymi (Araújo i Carvalho, 2005; Pacher *i in.,* 2007), zdolność ich modulacji przez ligandy receptora ALX/FPR2, a szczególnie długotrwały efekt wykazany w przypadku MR-39 może stanowić o przydatności tych związków w regulacji zaburzonej przez czynniki zapalne homeostazy.

W prezentowanych w pracy Tylek *i in.* (2021b) badaniach *in vitro* pokazano także, że endotoksyna bakteryjna podnosi ekspresję genów (*Il-1β, Tnf-α)* oraz poziom czynników prozapalnych (IL-1β, TNF-α, IL-6) będących markerami aktywacji mikrogleju w wyniku zainicjowania procesu zapalnego. Zatem zaproponowany model stymulacji mikrogleju LPS ma swoje odzwierciedlenie w silnej immunoaktywacji (Minogue *i in.*, 2012; Ślusarczyk *i in.,* 2018; Porro *i in.,* 2019).

Uzyskane rezultaty pokazały obniżenie ekspresji genu *Tnf-α* przez wszystkich badanych agonistów receptora ALX/FPR2. Co więcej, LXA4 obniżała poziom tej cytokiny prozapalnej po 3-godzinnej stymulacji LPS, a przeciwzapalne działanie MR-39

obserwowano także po 24-godzinnej stymulacji. Przeciwzapalne właściwości związku MR-39 wykazano także w przypadku modulacji poziomu ekspresji genu *Il-1β* i jego transkryptu. Warto podkreślić, że korzystne działanie mediowane było przez receptor ALX/FPR2. Zaskakująca pozostaje także obserwacja, że komórki mikrogleju stymulowane LPS w obecności ligandów endogennych (LXA4 oraz AT-LXA4) wykazały zwiększoną ekspresję genu *Il-1β* .Wydaje się, że u podstaw ograniczonego działania przeciwzapalnego tych agonistów leżeć może wykazany w mikrogleju proces ich enzymatycznej dehydrogenacji (Romano, 2010).

Dalsze badania pokazały natomiast, że sytuacji takiej nie obserwuje się w przypadku normalizacji poziomu białka IL-6 przez LXA4 i AT-LXA4 (3 godziny), jak i MR-39 (24 godziny) po indukcji procesu zapalnego. Warto jednak wspomnieć, że w prezentowanych badaniach metodą immunofluorescencji wykazano, że poziom receptora ALX/FPR2 w komórkach mikrogleju znacząco zwiększa się, ale dopiero po 24-godzinnej stymulacji endotoksyną, co może przynajmniej częściowo tłumaczyć obserwowane zależne od czasu różnice w potencjale przeciwzapalnym badanych związków (Romano, 2010; Stama *i in.*, 2017).

Wiele danych wskazuje, że w procesach wyciszania reakcji zapalnych duże znaczenie ma zmiana (ang. *shift*) fenotypu mikrogleju (Guo *i in.*, 2022). Przeprowadzona analiza wykazała, że zastosowanie LPS obniża ekspresję markerów przeciwzapalnych *Igf-1* oraz *Cd206*, przy jednoczesnym podniesieniu ekspresji genów pro-wyciszeniowych *Il-10* oraz *Arg-1*. Co więcej, zaobserwowano, że wysoki poziom ekspresji *Il-10* indukowany przez LPS został dodatkowo wzmocniony poprzez działanie badanych ligandów receptora ALX/FPR2. Obserwacje te pozostają zatem w zgodzie z doniesieniami innych autorów, które potwierdzają korzystną rolę LXA4 oraz AT-LXA4 w procesach proliferacji i różnicowania komórek neuronalnych w stanach uszkodzeń i po aktywacji zapalnej np. w przebiegu udaru (Wu *i in.*, 2012b; Wu *i in.*, 2019b).

Liczne dane pokazują, korelację pomiędzy poziomami aktywacji kinaz białkowych aktywowanych mitogenami (MAPK) oraz czynnika transkrypcyjnego NF-κB, a działaniem prozapalnym endotoksyny bakteryjnej (Bachstetter i Van Eldik, 2010; Ślusarczyk *i in.*, 2018; Falcicchia *i in.*, 2020). Prezentowane w rozprawie wyniki pozostają w zgodzie z tymi doniesieniami pokazując wzrost poziomu ufosforylowanych białek: ERK1/2, p38 oraz czynnika NF-κB w hodowlach mikrogleju po zastosowaniu LPS. NF-κB jest powszechnie

znanym czynnikiem regulującym odpowiedź zapalną w organizmie poprzez indukcję ekspresji genów prozapalnych takich cytokin jak TNF-α i IL-6 (Majumder *i in.*, 1998; Hyam *i in.*, 2013). Można zatem wnioskować, że zmniejszenie poziomu fosforylacji czynnika transkrypcyjnego NF-κB przez AT-LXA4 oraz MR-39 ma istotne znaczenie w przeciwzapalnej aktywności tych agonistów. Co więcej, LXA4 i MR-39 istotnie zmniejszyła poziom fosforylacji kinazy ERK1/2, zaś związek AT-LXA4 ograniczał fosforylację kinazy p38. Dane te sugerują, że przeciwzapalne efekty agonistów receptora ALX/FPR2 związane są z hamowaniem fosforylacji kluczowych ścieżek (ERK1/2 i p38) przekazu sygnału od receptora ALX/FPR2. Jednocześnie pokazują, że nowy, mocznikopochodny agonista MR-39 w sposób analogiczny do ligandów endogennych moduluje przekaz sygnału w komórce.

W oparciu o przedstawione wyniki można zatem postulować, że badane związki wykazują zróżnicowane w czasie, ale korzystne: przeciwzapalne, antyoksydacyjne oraz pro-wyciszeniowe działanie poprzez indukcję hamowania ścieżek sygnałowych ERK1/2, p38 oraz NF-κB w modelu immunoaktywacji w hodowlach mikrogleju. Działanie związku MR-39 obserwowane było dłużej niż agonistów endogennych tym niemniej korzystny efekt zauważalny był dopiero w dawkach mikromolarnych.

***Badania ex vivo***

Działanie agonistów receptora ALX/FPR2 omawiane w cyklu publikacji włączonych do rozprawy oceniano także w modelu *ex vivo* - hodowli organotypowych hipokampa (OHC) (Tylek, *i in.*, 2023a; Tylek *i in.*, 2023b). Technika ta jest przydatnym narzędziem dającym możliwość analizy i badań podstaw wielu zjawisk w warunkach zachowanej cytoarchitektury hipokampa oraz w obecności funkcjonalnych interakcji pomiędzy komórkami tej struktury mózgu. Należy podkreślić, że w warunkach hodowli OHC zachowana jest także wzajemna wymiana i interakcje pomiędzy mediatorami układów immunologicznego, nerwowego oraz endokrynnego, co wzmacnia znaczenie otrzymywanych wyników przybliżając je do tych które uzyskiwane są w modelach zwierzęcych (Trojan *i in.*, 2021b).

W omawianym modelu przeprowadzono ocenę patomechanizmów procesu zapalnego oraz możliwości ich modulacji przez ligandy receptora ALX/FPR2 używając

w tym celu endotoksyny bakteryjnej, zgodnie ze schematem opisanym we wcześniejszych badaniach (Trojan *i in.*, 2021a).

Ze względu na ograniczenia płynące z badań z zastosowaniem związku MR-39, w kolejnym etapie badań wykorzystano mocznikopochodne ligandy receptora ALX/FPR2 II generacji. Wprowadzenie przez grupę prof. Lacivita zmian w rusztowaniu związku MR-39, poprzez zastąpienie ugrupowania fenylocyklopropylowego ugrupowaniami o innych wymiarach, doprowadziło do identyfikacji związku AMS21. Związek ten aktywuje receptor ALX/FPR2 w stężeniu nanomolowym ($EC_{50}$ = 26 nM) i ma dobrą stabilność metaboliczną w szczurzych mikrosomach ($t_{1/2}$ = 21 min) (Mastromarino *i in.*, 2022). Dalsze modyfikacje strukturalne doprowadziły do identyfikacji kolejnego agonisty czyli związku CMC23, wykazującego najbardziej zrównoważoną kombinację siły działania na receptor ALX/FPR2 oraz stabilność metaboliczną ($EC_{50}$= 130 nM, $t_{1/2}$ = 44 min w mikrosomach wątroby szczura). Analiza biodystrybucji *in vivo* wykazała także, że ligand CMC23 gromadzi się w mózgu myszy, co oznacza, że ma zdolność do penetracji przez barierę krew-mózg (Mastromarino *i in.*, 2022). Oba związki CMC23 (Tylek *i in.*, 2023a) oraz AMS21 (Tylek *i in.*, 2023b) badano pod kątem ich potencjału przeciwzapalnego i pro-wyciszeniowego w warunkach *ex vivo*.

Wykazano, że podobnie jak w przypadku badań *in vitro* endotoksyna zwiększa poziom uwolnionego LDH oraz NO po 24-godzinnej inkubacji w hodowlach OHC. Oba ligandy normalizowały podwyższone poziomy LDH, a związek CMC23 także ilość uwalnianego NO, co wskazuje na jego potencjał antyoksydacyjny. Warto podkreślić, że działanie ochronne mocznikopochodnych ligandów II generacji obserwowano już w stężeniach nanomolarnych w obecności mikrogleju, astrocytów i komórek neuronalnych, co potwierdzono w pracy Tylek *i in.* 2023b. Badania dotyczące obecności receptora ALX/FPR2 na komórkach OUN w dalszym ciągu pozostają niejednoznaczne (Schröder *i in.*, 2020; Zhu *i in.*, 2021). Dlatego też, w pracy Tylek *i in.* 2023b wykonano barwienia fluorescencyjne, które po raz pierwszy pokazały zróżnicowaną ekspresję tego receptora w warunkach hodowli OHC. Wykazano, że eliminacja mikrogleju poprzez zastosowanie klodronatu czyli znanego bifosfonianu pierwszej generacji o potencjale hamującym proliferację tych komórek (Makkonen *i in.*, 1996; Dehghani *i in.*, 2004; Lee *i in.*, 2012), obniżyła nie tylko ilość komórek mikrogleju, ale także poziom receptora ALX/FPR2, co wskazuje, że komórki te są głównym miejscem jego lokalizacji. Jednocześnie pozostałe komórki w hodowlach OHC wykazują znacznie niższy poziom tego receptora mierzony

metodami fluorescencji. Można więc postulować, że pomimo iż efekty działania mocznikopochodnych ligandów II generacji są wynikiem ich interakcji z receptorami ALX/FPR2 zlokalizowanymi na wszystkich komórkach w warunkach hodowli OHC, to w szczególny sposób w odpowiedzi tej uczestniczą receptory na komórkach mikrogleju. W tym kontekście znaczenia nabiera fakt, że zastosowana w opisywanych badaniach endotoksyna bakteryjna aktywuje głównie komórki mikrogleju. Znajduje to swoje odzwierciedlenie w odmiennym niż w warunkach fizjologicznych panelu antygenów powierzchniowych. W przeprowadzonych badaniach wykazano wzrost ekspresji genów głównie o profilu prozapalnym w tym: *Ccl2, Cd40, Il-1β, Il-6* oraz *Il-18*. Nowe ligandy, CMC23 oraz AMS21, obniżały ekspresję *Cd40*, którego wzrost ekspresji wraz z innymi antygenami np. *MhcII, Cd86* zachodzi na wczesnym etapie aktywacji mikrogleju. Efekt działania badanych agonistów receptora ALX/FPR2 w hamowaniu odpowiedzi zapalnej widoczny jest więc już na etapie inicjacji procesu zapalnego (Ponomarev *i in.*, 2006; Salemi *i in.*, 2011). Jednocześnie wykazano, że związek CMC23 miał nieco szerszy potencjał modulacji fenotypu mikrogleju, wpływając także na ekspresję *Cd68*, a tym samym na aktywność fagocytarną tych komórek. Ligand ten obniżał także ekspresję genu chemokiny *Ccl2*. Ta ciekawa obserwacja ma istotne znaczenie, gdyż ekspresja genów enzymów zaangażowanych w syntezę SPM (5-LOX i 15-LOX) oraz potencjalizację wyciszania procesu zapalnego jest odwrotnie zależna od poziomu ekspresji genu tej chemokiny, której silne właściwości chemotaktyczne potęgują jego przebieg(Gutiérrez *i in.*, 2022).

W obu omawianych pracach przeprowadzono także ocenę przeciwzapalnego i pro-wyciszeniowego działania badanych związków zwracając szczególną uwagę na ich potencjał w modulacji syntezy cytokin prozapalnych i przeciwzapalnych, których wzrost uwalniania obserwuje się w warunkach immunoaktywacji wywołanej podaniem LPS. Pokazano, że ligandy w mediowany przez receptor ALX/FPR2 sposób obniżają poziom dwóch kluczowych w procesie zapalnym cytokin: IL-1β oraz IL-6. Mając na uwadze tą obserwację w pracy Tylek *i in.*, 2023b w której stosowano związek AMS21, podjęto próbę oceny mechanizmów działania tego liganda. W procesach uwalniania aktywnych form cytokin z rodziny IL-1 (IL-1β oraz IL-18) istotne znaczenie odgrywa aktywacja kompleksu inflammasomu NLRP3 (Heneka *i in.*, 2013; Hanslik i Ulland, 2020; Huang *i in.*, 2021). Jak wykazano, aktywacja platformy inflamasomu NLRP3 (cząsteczki sensorowej NLRP3), białka adaptorowego PYCARD oraz pro-kaspazy 1 indukowana jest procesem zapalnym i przebiega dwuetapowo (Heneka *i in.*, 2013; Huang *i in.*, 2021).

W badaniach własnych wykazano, że podniesiony w wyniku zastosowania LPS, poziom wszystkich komponentów tego kompleksu normalizowany był przez ligand AMS21. Co istotne, efekt ten mediowany był przez receptor ALX/FPR2. Ta istotna obserwacja wskazuje na kluczowy udział interakcji ligand-receptor w hamowaniu ścieżki inflamasomu NLRP3, a tym samym przeciwzapalnym i pro-wyciszeniowym efekcie obserwowanym po zastosowaniu związku AMS21 w hodowlach OHC. Warto także wspomnieć, że w naszych wcześniejszych badaniach przeprowadzonych z wykorzystaniem myszy transgenicznych (knockout FPR2$^{-/-}$) obserwowano brak przeciwzapalnego działania związku MR-39 oraz jego wpływu na ścieżkę NLRP3, pomimo że efekt ten był widoczny u zwierząt kontrolnych (Trojan *i in.,* 2021a). Wyniki przedstawione w pracy Tylek *i in.,* 2023b uzupełniły te obserwacje i pokazały, że aktywacja receptora ALX/FPR2 ma istotne znaczenie w modulacji aktywności inflammasomu NLRP3, a szczególnie w hamowaniu aktywności kaspazy-1 przez agonistów mocznikopochodnych, także II generacji.

Ciekawą obserwacją jest także wykazanie, że w hodowlach OHC w których zastosowano klodronat dodanie endotoksyny nie zwiększało poziomu IL-1β oraz komponent inflamasomu NLPR3. Co więcej, eliminacja komórek mikrogleju całkowicie zniosła przeciwzapalny i pro-wyciszeniowy efekt działania związku AMS21. Prawdopodobnie brak największej (jak wspomniano wcześniej) puli receptora ALX/FPR2 na komórkach mikrogleju ograniczył biologiczną aktywność badanego liganda, a jego interakcja z receptorem ALX/FPR2 na innych komórkach w hodowlach OHC nie była wystarczająca do mediowania jego działania. Tym niemniej, niektóre badania wskazują, że także astrocyty mogą być źródłem uwalnianych cytokin prozapalnych (John *i in.,* 2003; Gorina *i in.,* 2011; Ma *i in.,* 2013), w tym także IL-1β, dlatego też obecność tej cytokiny po stymulacji endotoksyną w hodowlach OHC, traktowanych uprzednio klodronatem prawdopodobnie może mieć pochodzenie astrocytarne. Przeprowadzona ocena przeciwzapalnego i pro-wyciszeniowego potencjału związku AMS21 mocno wskazuje na udział zależnego od receptora ALX/FPR2 hamowania ścieżki inflammasomu NLRP3 w jego korzystnych efektach w modelu neuronalno-glejowej immunoaktywacji.

W kolejnych badaniach stanowiących podstawę niniejszej rozprawy w których zastosowano związek CMC23 zwrócono szczególną uwagę na potencjał tego liganda w modulacji poziomu IL-23, która jest regulatorem późniejszych etapów procesu zapalnego (Kleinschek *i in.,* 2006; Meeks *i in.,* 2009; Town *i in.,* 2009). W budowie IL-23, wyróżnia się dwie podjednostki: konserwatywną p19 oraz współdzieloną z IL-12 podjednostkę p40

(Croxford *i in.*, 2014; Chyuan i Lai, 2020; Nitsch *i in.*, 2021). Wykazano, że indukowany podaniem LPS wzrost poziomu IL-12/23p40 oraz IL-23p19 był normalizowany przez ligand CMC23 w sposób częściowo zależny od aktywacji receptora ALX/FPR2. Efekty biologiczne IL-23 są w dużej mierze związane z fosforylacją czynnika transkrypcyjnego STAT3. Jednakże czynnik ten aktywowany może być także przez IL-6, TGF-β i IL-10 (Cua *i in.*, 2003; Cevey *i in.*, 2019), dlatego wydaje się, że wykazane normalizujące działanie CMC23 na fosforylację STAT3 ma szerszy wymiar i może stanowić kluczowy mechanizm w regulacji RoI. Uzyskane wyniki nabierają także dodatkowego znaczenia w obliczu ostatnich doniesień wskazujących, że również endogenne ligandy receptora ALX/FPR2, w tym AXA1 mają działanie pro-wyciszeniowe poprzez hamowanie fosforylacji STAT3 (Liu *i in.*, 2022). Obserwacje te pozwalają zatem na sugestię, że pokazany w pracy Tylek *i in*. (2023a) mechanizm działania CMC23 jest istotnym mechanizmem ograniczającym chroniczny (przedłużony) proces zapalny stanowiący podłoże rozwoju wielu patologii w mózgu.

Warto uzupełnić analizę potencjału RoI badanych związków o obserwacje przeprowadzone w obu pracach wskazujące, że w przeciwieństwie do silnego działania przeciwzapalnego polegającego na istotnym ograniczaniu uwalniania cytokin prozapalnych CMC23 i AMS21 nie modulowały podwyższonego przez LPS poziomu cytokin przeciwzapalnych TGF-β oraz IL-10. W istocie, wysoki poziom IL-10 zapobiega uwalnianiu pro-IL-1β. Można zatem postulować, że taki poziom ma swoje odzwierciedlenie w ograniczaniu dalszego uwalniania IL-1β przez komórki mikrogleju w warunkach immunoaktywacji zapalnej (Cevey *i in.*, 2019) i/lub możliwości normalizacji poziomu IL-1β przez badane ligandy. Sugestię tą, wzmacniają uzyskane wyniki potwierdzające efektywność związku AMS21 do zwiększania ekspresji genu *Il-1Ra*, którego transkrypt odpowiedzialny jest za hamowanie ekspresji receptora dla IL-1β i w konsekwencji regulację aktywności zapalnej mediowanej właśnie przez IL-1β (Stama *i in.*, 2017). Dodatkowo, IL-1RA działa synergistycznie z TGF-β oraz IL-10 (Saghazadeh *i in.*, 2019). W badaniach innych autorów wykazano, że IL-10 ma zdolność do indukcji syntezy SOCS3 (inhibitora aktywacji ścieżki STAT3) ograniczając w ten sposób odpowiedź prozapalną w tym uwalnianie IL-1β (Cianciulli *i in.*, 2015; Porro *i in.*, 2019). Tym samym wysoki poziom inhibitora czynnika transkrypcyjnego STAT3 pokazany w hodowlach OHC w których analizowano działanie liganda CMC23, może mieć istotne znaczenie w warunkach deficytów RoI po aktywacji zapalnej (Zheng *i in.*, 2022).

Tym niemniej, te skomplikowane interakcje sugerowane w oparciu o wyniki uzyskane w modelu hodowli OHC wskazujące na przeciwzapalny i pro-wyciszeniowy kierunek aktywacji receptora ALX/FPR2 po interakcji z ligandami II generacji, niewątpliwie wymagają dalszych badań.

***Badania in vivo***

W niniejszej wersji rozprawy zawarto także wyniki badań nieopublikowanych, które przeprowadzono u dorosłych szczurów poddanych uogólnionej reakcji zapalnej. Istotą tego modelu było jednorazowe dootrzewnowe (ang. *intraperitoneal*, i.p.) podanie endotoksyny bakteryjnej – lipopolisacharydu (Yirmiya, 1996; Gauthier *i in.*, 2022). Co ważne, procedura ta spełnia podstawowe kryteria dotyczące spójności predykcyjnej, zewnętrznej oraz konstruktu (Lasselin *i in.*, 2020). Zaobserwowano, że LPS działając przez receptor TLR4 (Ciesielska *i in.*, 2021) prowadzi do zwiększonej syntezy cytokin prozapalnych oraz pojawienia się u zwierząt zachowań określanych jako „*sickness behavior*", do których należy m.in. wzrost temperatury, spadek aktywności eksploracyjnej, lokomotorycznej oraz socjalnej. Co więcej, obserwowana jest także anhedonia i zmiany masy ciała, a objawy te w dużej mierze wydają się być tożsame z symptomami chorobowymi u pacjentów w tym także z epizodami depresyjnymi (Mullington *i in.*, 2000; Draper *i in.*, 2018). Warto zaznaczyć, że w modelach zwierzęcych w których stosowano LPS pokazano efektywne działanie leków przeciwdepresyjnych w normalizacji zmian w zachowaniu oraz hamowaniu reakcji zapalnej (O'Connor *i in.*, 2009).

Opisany model wykorzystany został do oceny behawioralnych efektów działania LXA4 oraz egzogennych agonistów receptora ALX/FPR2 związków MR-39 i CMC23 u dorosłych zwierząt z wywołaną uogólnioną reakcją zapalną. Ze względu na brak jednoznacznych dowodów wskazujących, że LXA4 jak i pozostali agoniści receptora ALX/FPR2 (w momencie prowadzenia tych badań) penetrują barierę krew-mózg związki podawano dokomorowo (Zhang *i in.*, 2022). Co więcej, biorąc pod uwagę krótkotrwałe protekcyjne/korzystne działanie LXA4 w badaniach *in vitro* oraz fakt jej szybkiej inaktywacji *in vivo* przez układ enzymów metabolicznych, analizę wpływu ligandów receptora ALX/FPR2 na parametry w teście Porsola wykonano zarówno 1 godzinę, jak i 4 godziny po dokomorowej iniekcji ligandów. Warto wspomnieć, że o wyborze pozostałych związków do badań zadecydował fakt, iż związek MR-39 jako jedyny do czasu

przeprowadzenia doświadczeń posiadał udokumentowane działanie *in vivo* (Cristiano *i in.*, 2022), natomiast CMC23 w badaniach *in vitro* wykazał przeciwzapalną oraz pro-wyciszeniową efektywność w stężeniach porównywalnych z LXA4 (nanomolarnych).

Prezentowane wyniki potwierdziły, że podanie LPS prowadzi do obecności tzw. „*sickness behavior*". U zwierząt obserwowano wydłużenie czasu bezruchu i skrócenie czasu wspinania w teście Porsolta. Wyniki te są spójne z naszymi wcześniejszymi doniesieniami (Duda *i in.*, 2017). Zastosowanie w niniejszych badaniach zmodyfikowanej wersji testu Porsolta (Detke *i in.*, 1995) umożliwiło także ocenę trzeciego parametru czyli czasu wspinania, co dało możliwość zróżnicowania u dorosłych szczurów zachowań zależnych od komponenty serotoninergicznej (pływanie) oraz noradrenergicznej (wspinanie). Zaprezentowane w rozprawie wyniki są zgodne z badaniami innych autorów, którzy obserwowali, że endotoksyna w teście wymuszonego pływania, ale także w teście zawieszenia za ogon redukuje czas wspięć (Zhang *i in.*, 2019; Yin *i in.*, 2023).

Dotychczasowe dane wskazują, że test Porsolta stosowany jest także do weryfikacji farmakologicznej związków w modelach zwierzęcych (Pollak *i in.*, 2010). Dlatego też, w prezentowanych badaniach wykonano go po dokomorowych podaniach wybranych agonistów receptora ALX/FPR2. Uzyskane wyniki wskazują, że LXA4 oraz CMC23 normalizowały wydłużony przez podanie endotoksyny czas bezruchu, oraz wydłużały czas pływania. Dodatkowo CMC23 wydłużał (normalizował do wartości kontrolnych) czas wspinania u zwierząt po podaniu LPS. Należy jednak podkreślić, że korzystne działanie LXA4 na deficyty behawioralne u szczurów obserwowano tylko 1 godzinę od jej podania, podczas, gdy w przypadku CMC23 było one widoczne nawet po 4 godzinach. Niniejsze obserwacje są pierwszymi wskazującymi na możliwość normalizacji deficytów zachowania u modelu uogólnionej aktywacji zapalnej przez agonistę CMC23, co świadczy o ich nowatorstwie. Odmienne obserwacje uzyskano w przypadku liganda MR-39, który nie modulował deficytów w modelu *„sickness behavior"*. Związek ten był dotychczas przedmiotem tylko kilku badań w tym grupy Cristiano *i in.* (2022), która w modelu autyzmu wykazała, iż dootrzewnowe podania MR-39 przez 8 dni myszom eksponowanym w okresie prenatalnym na kwas walproinowy, poprawiają zachowania socjalne w teście interakcji socjalnych. Można zatem sugerować, że specyfika zastosowanego modelu i/lub gatunek zwierząt mają wpływ na potencjał modulacji przez ligand MR-39 zachowania u zwierząt.

Kolejnym krokiem badań *in vivo* była analiza biochemiczna przeprowadzona w strukturach mózgu po podaniach agonistów receptora ALX/FPR2. W homogenatach struktur uczestniczących w pojawieniu się deficytów w modelu *„sickness behavior”* czyli w korze czołowej oraz hipokampie wykonano pomiar poziomu dwóch głównych cytokin prozapalnych TNF-α i IL-1β. Wykazano, że w hipokampie podwyższone po podaniu LPS poziomy TNF-α normalizowane były zarówno przez LXA4 jak i oba mocznikopochodne ligandy (MR-39 i CMC23), a efekt przeciwzapalny obserwowano w obu punktach czasowych: 1 godzinę oraz 4 godziny po ich podaniach. W korze czołowej natomiast, w przeciwieństwie do korzystnego działania LXA4 oraz CMC23, nie wykazano wpływu związku MR-39 na poziom tej cytokiny.

Podniesiony poziom TNF-α dotychczas obserwowano w wielu modelach immunoaktywacji po podaniach LPS (Grigoleit *i in.*, 2011; Ferguson *i in.*, 2013; Kox *i in.*, 2014). Także w badaniach klinicznych wykazano, że endotoksyna podnosi poziomy TNF-α oraz IL-1β w surowicy (Dorresteijn *i in.*, 2010; Engler *i in.*, 2017), wywołując jednocześnie zmiany w zachowaniu w tym objawy lęku czy depresji (Reichenberg *i in.*, 2001). Co więcej, zarówno obwodowe, jak i ośrodkowe infuzje IL-1β i TNF-α indukują szereg objawów towarzyszących depresji jak: zmniejszona aktywność lokomotoryczna, anhedonia, zaburzenia snu czy zmiany w funkcjach poznawczych (Goshen *i in.*, 2008; Kaster *i in.*, 2012). Jak dotąd wpływ mocznikopochodnych agonistów receptora ALX/FPR2 na poziomy cytokin prozapalnych nie był jeszcze badany w żadnym doświadczalnym modelu immunoaktywacji. Warto podkreślić, że przeprowadzona w niniejszej rozprawie ocena wpływu agonistów receptora ALX/FPR2 na poziom IL-1β w modelu „*sickness behavior*” potwierdziła ograniczony przeciwzapalny potencjał liganda endogennego LXA4. Związek ten ulega bardzo szybkiej inaktywacji metabolicznej, która w mózgu zachodzi głównie w mikrogleju i obejmuje dehydrogenację do 15-oksy-lipksyny A4 przez dehydrogenazę 15-hydroksyprostaglandynową (15-PGDH) (Romano, 2010). Niemniej jednak, w innych badaniach pokazano, że wykazuje on potencjał pro-wyciszeniowy, który związany jest z hamowaniem prozapalnej aktywacji mikrogleju oraz indukcją produkcji cytokiny przeciwzapalnej IL-10 (Medeiros *i in.*, 2013).

Tym samym, skoro przydatność endogennych ligandów o potencjale RoI pozostaje ograniczona, badania pozytywnie weryfikujące pro-wyciszeniowe właściwości nowych mocznikopochodnych ligandów receptora ALX/FPR2 w modelach immunoaktywacji, mogą

stanowić podstawy do rozwoju nowej strategii farmakoterapii wyciszania procesów zapalnych w ośrodkowym układzie nerwowym.

# 9. Podsumowanie i wnioski

1. W hodowlach pierwotnych mikrogleju stymulowanych lipopolisacharydem (LPS) wykazano protekcyjne, antyoksydacyjne oraz pro-wyciszeniowe działanie endogennych agonistów receptora ALX/FPR2 związków: LXA4 oraz AT-LXA4, a także egzogennego mocznikopochodnego liganda tego receptora – MR-39.
2. Działanie przeciwzapalne i pro-wyciszeniowe związku MR-39 pomimo, że obserwowane w stężeniach wyższych (mikromolarnych) niż ligandów endogennych było dłuższe, a mechanizmy tego działania związane z hamowaniem tych samych szlaków przekazu sygnału (ERK1/2 oraz NF-κB), co w przypadku ligandów endogennych.
3. W badaniach *ex vivo* prowadzonych w hodowlach organotypowych hipokampa stymulowanych LPS wykazano największą ekspresję receptora ALX/FPR2 na komórkach mikrogleju, a w mniejszym stopniu także na neuronach i astrocytach.
4. W hodowlach organotypowych hipokampa pokazano neuroprotekcyjne i pro-wyciszeniowe działanie mocznikopochodnego liganda receptora ALX/FPR2 – związku CMC23 w stężeniu nanomolarnym. Wskazano także na istotną rolę modulacji szlaku sygnałowego STAT3/SOCS3 w mechanizmach jego działania.
5. Związek AMS21 w stężeniu nanomolarnym wykazał działanie neuroprotekcyjne, antyoksydacyjnie oraz pro-wyciszeniowe przez wpływ na obecny na komórkach mikrogleju receptor ALX/FPR2. W mechanizmach przeciwzapalnego działania tego liganda w warunkach *ex vivo* istotną rolę odgrywa hamowanie ścieżki kanonicznej inflammasomu NLRP3.
6. W modelu uogólnionej reakcji zapalnej wywołanej jednorazowym dootrzewnowym podaniem lipopolisacahrydu u dorosłych szczurów ujawniono deficyty behawiorlane (tzw. *„sickness behaviour”*), które w teście wymuszonego pływania (test Porsolta) obserwowano jako: wydłużenie czasu bezruchu, skrócenie czasu pływania oraz wspinania. Jednocześnie w hipokampie i korze czołowej zwierząt w modelu immunoaktywacji wykazano wzrost poziomu cytokin prozapalnych IL-1β oraz TNF-α.
7. U szczurów po dokomorowym podaniu agonistów receptora ALX/FPR2 (LXA4, CMC23) wykazano normalizację deficytów behawioralnych wywołanych podaniem endotoksyny bakteryjnej, w tym skrócenie czasu bezruchu oraz wydłużenie czasu

pływania. Działanie LXA4 było krótkotrwałe w porównaniu do korzystnego efektu związku CMC23, który wydłużał także czas wspinania u szczurów oceniany w teście Porsolta.

8. W hipokampie oraz korze czołowej dorosłych szczurów wykazano przeciwzapalne działanie agonistów receptora ALX/FPR2. Podwyższony poziom cytokiny prozapalnej TNF-α w hipokampie obniżany był przez wszystkie ligandy (LXA4, MR-39 oraz CMC23), natomiast w korze czołowej zmiany dotyczyły LXA4 oraz CMC23. Jednocześnie wykazano, że podwyższony poziom cytokiny IL-1β normalizowany był przez mocznikopochodne ligandy I i II generacji receptora ALX/FPR2, natomiast LXA4 miała działanie krótkotrwałe.

**W oparciu o przedstawione w rozprawie wyniki badań uzyskane w modelach immunoaktywacji można wnioskować, że receptor ALX/FPR2 poprzez możliwość przyłączania ligandów o różnej budowie chemicznej stanowi niezwykle ważny punkt uchwytu dla nowych związków, które mogą wielokierunkowo modulować odpowiedź zapalną wspomagając proces jej wyciszenia.**

**Poszukiwanie nowych ligandów receptora ALX/FPR2 o większej aktywności oraz korzystnych parametrach metabolicznych i farmakokinetycznych wydaje się stanowić istotę nowej strategii modulacji procesów zapalnych w ośrodkowym układzie nerwowym, których niekontrolowany oraz przedłużony przebieg stanowi podłoże rozwoju chorób cywilizacyjnych w tym psychicznych i neurodegeneracyjnych.**

## 10.Bibliografia


Allen, R.G. & Tresini, M. (2000) Oxidative stress and gene regulation. *Free Radic. Biol. Med.*, **28**, 463–499.

Alpizar, Y.A., Boonen, B., Sanchez, A., Jung, C., López-Requena, A., Naert, R., Steelant, B., Luyts, K., Plata, C., De Vooght, V., Vanoirbeek, J.A.J., Meseguer, V.M., Voets, T., Alvarez, J.L., Hellings, P.W., Hoet, P.H.M., Nemery, B., Valverde, M.A., & Talavera, K. (2017) TRPV4 activation triggers protective responses to bacterial lipopolysaccharides in airway epithelial cells. *Nat. Commun.*, **8**, 1059.

Amor, S., Peferoen, L.A.N., Vogel, D.Y.S., Breur, M., van der Valk, P., Baker, D., & Van Noort, J.M. (2014) Inflammation in neurodegenerative diseases - an update. *Immunology*, **142**, 151–166.

Araújo, I.M. & Carvalho, C.M. (2005) Role of nitric oxide and calpain activation in neuronal death and survival. *Curr. Drug Targets CNS Neurol. Disord.*, **4**, 319–324.

Ariel, A., Fredman, G., Sun, Y.-P., Kantarci, A., Van Dyke, T.E., Luster, A.D., & Serhan, C.N. (2006) Apoptotic neutrophils and T cells sequester chemokines during immune response resolution through modulation of CCR5 expression. *Nat. Immunol.*, **7**, 1209–1216.

Bachstetter, A.D. & Van Eldik, L.J. (2010) The p38 map kinase family as regulators of proinflammatory cytokine production in degenerative diseases of the CNS. *Aging Dis.*, **1**, 199–211.

Bao, L., Gerard, N.P., Eddy, R.L., Shows, T.B., & Gerard, C. (1992) Mapping of genes for the human C5a receptor (C5AR), human FMLP receptor (FPR), and two FMLP receptor homologue orphan receptors (FPRH1, FPRH2) to chromosome 19. *Genomics*, **13**, 437–440.

Basta-Kaim, A., Szczesny, E., Glombik, K., Stachowicz, K., Slusarczyk, J., Nalepa, I., Zelek- Molik, A., Rafa- Zablocka, K., Budziszewska, B., Kubera, M., Leskiewicz, M., & Lason, W. (2014) Prenatal stress affects insulin-like growth factor-1 (IGF-1) level and IGF-1 receptor phosphorylation in the brain of adult rats. *Eur. Neuropsychopharmacol.*, **24**, 1546–1556.

Batista, C.R.A., Gomes, G.F., Candelario-Jalil, E., Fiebich, B.L., & de Oliveira, A.C.P. (2019) Lipopolysaccharide-Induced Neuroinflammation as a Bridge to Understand Neurodegeneration. *Int. J. Mol. Sci.*, **20**, 2293.

Beaino, W., Janssen, B., Vugts, D.J., de Vries, H.E., & Windhorst, A.D. (2021) Towards

PET imaging of the dynamic phenotypes of microglia. *Clin. Exp. Immunol.*, **206**, 282–300.

Becker, E.L., Forouhar, F.A., Grunnet, M.L., Boulay, F., Tardif, M., Bormann, B.-J., Sodja, D., Ye, R.D., Woska Jr, J.R., & Murphy, P.M. (1998) Broad immunocytochemical localization of the formylpeptide receptor in human organs, tissues, and cells. *Cell Tissue Res.*, **292**, 129–135.

Bena, S., Brancaleone, V., Wang, J.M., Perretti, M., & Flower, R.J. (2012) Annexin A1 interaction with the FPR2/ALX receptor: Identification of distinct domains and downstream associated signaling. *J. Biol. Chem.*, **287**, 24690–24697.

Bennett, M. & Gilroy, D.W. (2016) Lipid Mediators in Inflammation. *Microbiol. Spectr.*, **4**.

Bennett, T.A., Maestas, D.C., & Prossnitz, E.R. (2000) Arrestin binding to the G protein-coupled N-formyl peptide receptor is regulated by the conserved „DRY" sequence. *J. Biol. Chem.*, **275**, 24590–24594.

Block, M.L., Zecca, L., & Hong, J.S. (2007) Microglia-mediated neurotoxicity: Uncovering the molecular mechanisms. *Nat. Rev. Neurosci.*, **8**, 57–69.

Boillat, M., Carleton, A., & Rodriguez, I. (2021) From immune to olfactory expression: neofunctionalization of formyl peptide receptors. *Cell Tissue Res.*, **383**, 387–393.

Boonen, B., Alpizar, Y.A., Sanchez, A., López-Requena, A., Voets, T., & Talavera, K. (2018) Differential effects of lipopolysaccharide on mouse sensory TRP channels. *Cell Calcium*, **73**, 72–81.

Boulay, F., Tardif, M., Brouchon, L., & Vignais, P. (1990) Synthesis and use of a novel N-formyl peptide derivative to isolate a human N-formyl peptide receptor cDNA. *Biochem. Biophys. Res. Commun.*, **168**, 1103–1109.

Brandenburg, L.O., Konrad, M., Wruck, C., Koch, T., Pufe, T., & Lucius, R. (2008) Involvement of formyl-peptide-receptor-like-1 and phospholipase D in the internalization and signal transduction of amyloid beta 1-42 in glial cells. *Neuroscience*, **156**, 266–276.

Brandenburg, L.O., Konrad, M., Wruck, C.J., Koch, T., Lucius, R., & Pufe, T. (2010) Functional and physical interactions between formyl-peptide-receptors and scavenger receptor MARCO and their involvement in amyloid beta 1-42-induced signal transduction in glial cells. *J. Neurochem.*, **113**, 749–760.

Burguillos, M.A., Hajji, N., Englund, E., Persson, A., Cenci, A.M., Machado, A., Cano, J., Joseph, B., & Venero, J.L. (2011) Apoptosis-inducing factor mediates dopaminergic cell death in response to LPS-induced inflammatory stimulus. Evidence in Parkinson's

disease patients. *Neurobiol. Dis.*, **41**, 177–188.

Bylicky, M.A., Mueller, G.P., & Day, R.M. (2018) Mechanisms of Endogenous Neuroprotective Effects of Astrocytes in Brain Injury. *Oxid. Med. Cell. Longev.*, **2018**, 1–16.

Cattaneo, F., Parisi, M., & Ammendola, R. (2013) *Distinct signaling cascades elicited by different formyl peptide receptor 2 (FPR2) agonists*, International Journal of Molecular Sciences.

Cekanaviciute, E. & Buckwalter, M.S. (2016) Astrocytes: Integrative Regulators of Neuroinflammation in Stroke and Other Neurological Diseases. *Neurotherapeutics*, **13**, 685–701.

Cevey, Á.C., Penas, F.N., Alba Soto, C.D., Mirkin, G.A., & Goren, N.B. (2019) IL-10/STAT3/SOCS3 axis is involved in the anti-inflammatory effect of benznidazole. *Front. Immunol.*, **10**, 1–13.

Chen, W.W., Zhang, X., & Huang, W.J. (2016) Role of neuroinflammation in neurodegenerative diseases (Review). *Mol. Med. Rep.*, **13**, 3391–3396.

Chyuan, I.T. & Lai, J.H. (2020) New insights into the IL-12 and IL-23: From a molecular basis to clinical application in immune-mediated inflammation and cancers. *Biochem. Pharmacol.*, **175**, 113928.

Cianciulli, A., Dragone, T., Calvello, R., Porro, C., Trotta, T., Lofrumento, D.D., & Panaro, M.A. (2015) IL-10 plays a pivotal role in anti-inflammatory effects of resveratrol in activated microglia cells. *Int. Immunopharmacol.*, **24**, 369–376.

Ciesielska, A., Matyjek, M., & Kwiatkowska, K. (2021) TLR4 and CD14 trafficking and its influence on LPS-induced pro-inflammatory signaling. *Cell. Mol. Life Sci.*, **78**, 1233–1261.

Cooray, S.N., Gobbetti, T., Montero-Melendez, T., McArthur, S., Thompson, D., Clark, A.J.L., Flower, R.J., & Perretti, M. (2013) Ligand-specific conformational change of the G-protein-coupled receptor ALX/FPR2 determines proresolving functional responses. *Proc. Natl. Acad. Sci. U. S. A.*, **110**, 18232–18237.

Corminboeuf, O. & Leroy, X. (2015) FPR2/ALXR Agonists and the Resolution of Inflammation. *J. Med. Chem.*, **58**, 537–559.

Cristiano, C., Volpicelli, F., Crispino, M., Lacivita, E., Russo, R., Leopoldo, M., Calignano, A., & Perrone-Capano, C. (2022) Behavioral, Anti-Inflammatory, and Neuroprotective Effects of a Novel FPR2 Agonist in Two Mouse Models of Autism. *Pharmaceuticals*, **15**, 1–16.

Croxford, A.L., Kulig, P., & Becher, B. (2014) IL-12-and IL-23 in health and disease. *Cytokine Growth Factor Rev.*, **25**, 415–421.

Cruz, J.V.R., Batista, C., Diniz, L.P., & Mendes, F.A. (2023) The Role of Astrocytes and Blood–Brain Barrier Disruption in Alzheimer's Disease. *Neuroglia*, **4**, 209–221.

Cua, D.J., Sherlock, J., Chen, Y., Murphy, C.A., Joyce, B., Seymour, B., Lucian, L., To, W., Kwan, S., Churakova, T., Zurawski, S., Wiekowski, M., Lira, S.A., Gorman, D., Kastelein, R.A., & Sedgwick, J.D. (2003) Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature*, **421**, 744–748.

Cunha, C., Gomes, C., Vaz, A.R., & Brites, D. (2016) Exploring New Inflammatory Biomarkers and Pathways during LPS-Induced M1 Polarization. *Mediators Inflamm.*, **2016**.

Dantzer, R. (2001) Cytokine-Induced Sickness Behavior: Where Do We Stand? *Brain. Behav. Immun.*, **15**, 7–24.

Dehghani, F., Conrad, A., Kohl, A., Korf, H., & Hailer, N. (2004) Clodronate inhibits the secretion of proinflammatory cytokines and NO by isolated microglial cells and reduces the number of proliferating glial cells in excitotoxically injured organotypic hippocampal slice cultures. *Exp. Neurol.*, **189**, 241–251.

Detka, J., Kurek, A., Kucharczyk, M., Głombik, K., Basta-Kaim, A., Kubera, M., Lasoń, W., & Budziszewska, B. (2015) Brain glucose metabolism in an animal model of depression. *Neuroscience*, **295**, 198–208.

Detke, M.J., Rickels, M., & Lucki, I. (1995) Active behaviors in the rat forced swimming test differentially produced by serotonergic and noradrenergic antidepressants. *Psychopharmacology (Berl).*, **121**, 66–72.

Devanney, N.A., Stewart, A.N., & Gensel, J.C. (2020) Microglia and macrophage metabolism in CNS injury and disease: The role of immunometabolism in neurodegeneration and neurotrauma. *Exp. Neurol.*, **329**, 113310.

Devosse, T., Guillabert, A., D'Haene, N., Berton, A., De Nadai, P., Noel, S., Brait, M., Franssen, J.-D., Sozzani, S., Salmon, I., & Parmentier, M. (2009) Formyl Peptide Receptor-Like 2 Is Expressed and Functional in Plasmacytoid Dendritic Cells, Tissue-Specific Macrophage Subpopulations, and Eosinophils. *J. Immunol.*, **182**, 4974–4984.

Dorresteijn, M.J., Draisma, A., van der Hoeven, J.G., & Pickkers, P. (2010) Lipopolysaccharide-stimulated whole blood cytokine production does not predict the inflammatory response in human endotoxemia. *Innate Immun.*, **16**, 248–253.

Draper, A., Koch, R.M., van der Meer, J.W., AJ Apps, M., Pickkers, P., Husain, M., & van

der Schaaf, M.E. (2018) Effort but not Reward Sensitivity is Altered by Acute Sickness Induced by Experimental Endotoxemia in Humans. *Neuropsychopharmacology*, **43**, 1107–1118.

Du, L., Zhang, Y., Chen, Y., Zhu, J., Yang, Y., & Zhang, H.L. (2017) Role of Microglia in Neurological Disorders and Their Potentials as a Therapeutic Target. *Mol. Neurobiol.*, **54**, 7567–7584.

Duda, W., Kubera, M., Kreiner, G., Curzytek, K., Detka, J., Głombik, K., Ślusarczyk, J., Basta-Kaim, A., Budziszewska, B., Lasoń, W., Regulska, M., Leśkiewicz, M., Roman, A., Zelek-Molik, A., & Nalepa, I. (2017) Suppression of pro-inflammatory cytokine expression and lack of anti-depressant-like effect of fluoxetine in lipopolysaccharide-treated old female mice. *Int. Immunopharmacol.*, **48**, 35–42.

Dufton, N. & Perretti, M. (2010) Therapeutic anti-inflammatory potential of formyl-peptide receptor agonists. *Pharmacol. Ther.*, **127**, 175–188.

Dunn, H.C., Ager, R.R., Baglietto-Vargas, D., Cheng, D., Kitazawa, M., Cribbs, D.H., & Medeiros, R. (2014) Restoration of Lipoxin A4 Signaling Reduces Alzheimer's Disease-Like Pathology in the 3xTg-AD Mouse Model. *J. Alzheimer's Dis.*, **43**, 893–903.

Engler, H., Brendt, P., Wischermann, J., Wegner, A., Röhling, R., Schoemberg, T., Meyer, U., Gold, R., Peters, J., Benson, S., & Schedlowski, M. (2017) Selective increase of cerebrospinal fluid IL-6 during experimental systemic inflammation in humans: association with depressive symptoms. *Mol. Psychiatry*, **22**, 1448–1454.

Falcicchia, C., Tozzi, F., Arancio, O., Watterson, D.M., & Origlia, N. (2020) Involvement of p38 mapk in synaptic function and dysfunction. *Int. J. Mol. Sci.*, **21**, 1–14.

Feng, X., Valdearcos, M., Uchida, Y., Lutrin, D., Maze, M., & Koliwad, S.K. (2017) Microglia mediate postoperative hippocampal inflammation and cognitive decline in mice. *JCI Insight*, **2**.

Ferguson, J.F., Patel, P.N., Shah, R.Y., Mulvey, C.K., Gadi, R., Nijjar, P.S., Usman, H.M., Mehta, N.N., Shah, R., Master, S.R., Propert, K.J., & Reilly, M.P. (2013) Race and gender variation in response to evoked inflammation. *J. Transl. Med.*, **11**, 63.

Filep, J.G. (2013) Biasing the lipoxin A 4 /formyl peptide receptor 2 pushes inflammatory resolution. *Proc. Natl. Acad. Sci.*, **110**, 18033–18034.

Fiore, S., Maddox, J.F., Perez, H.D., & Serhan, C.N. (1994) Identification of a human cDNA encoding a functional high affinity lipoxin A4 receptor. *J. Exp. Med.*, **180**, 253–260.

Fox, S., Leitch, A.E., Duffin, R., Haslett, C., & Rossi, A.G. (2010) Neutrophil apoptosis:

Relevance to the innate immune response and inflammatory disease. *J. Innate Immun.*, **2**, 216–227.

Fredriksson, R., Lagerström, M.C., Lundin, L.G., & Schiöth, H.B. (2003) The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol. Pharmacol.*, **63**, 1256–1272.

Freire, M.O. & Van Dyke, T.E. (2013) Natural resolution of inflammation. *Periodontol. 2000*, **63**, 149–164.

Gauthier, A.E., Rotjan, R.D., & Kagan, J.C. (2022) Lipopolysaccharide detection by the innate immune system may be an uncommon defence strategy used in nature. *Open Biol.*, **12**.

Ge, Y., Zhang, S., Wang, J., Xia, F., Wan, J.B., Lu, J., & Ye, R.D. (2020) Dual modulation of formyl peptide receptor 2 by aspirin-triggered lipoxin contributes to its anti-inflammatory activity. *FASEB J.*, **34**, 6920–6933.

Gong, Q., He, L., Wang, M., Zuo, S., Gao, H., Feng, Y., Du, L., Luo, Y., & Li, J. (2019) Comparison of the TLR4/NFκB and NLRP3 signalling pathways in major organs of the mouse after intravenous injection of lipopolysaccharide. *Pharm. Biol.*, **57**, 555–563.

Gorina, R., Font-Nieves, M., Márquez-Kisinousky, L., Santalucia, T., & Planas, A.M. (2011) Astrocyte TLR4 activation induces a proinflammatory environment through the interplay between MyD88-dependent NFκB signaling, MAPK, and Jak1/Stat1 pathways. *Glia*, **59**, 242–255.

Goshen, I., Kreisel, T., Ben-Menachem-Zidon, O., Licht, T., Weidenfeld, J., Ben-Hur, T., & Yirmiya, R. (2008) Brain interleukin-1 mediates chronic stress-induced depression in mice via adrenocortical activation and hippocampal neurogenesis suppression. *Mol. Psychiatry*, **13**, 717–728.

Graeber, M.B. (2014) Neuroinflammation: No rose by any other name. *Brain Pathol.*, **24**, 620–622.

Grigoleit, J.-S., Kullmann, J.S., Wolf, O.T., Hammes, F., Wegner, A., Jablonowski, S., Engler, H., Gizewski, E., Oberbeck, R., & Schedlowski, M. (2011) Dose-Dependent Effects of Endotoxin on Neurobehavioral Functions in Humans. *PLoS One*, **6**, e28330.

Guo, S., Wang, H., & Yin, Y. (2022) Microglia Polarization From M1 to M2 in Neurodegenerative Diseases. *Front. Aging Neurosci.*, **14**.

Gutiérrez, I.L., Novellino, F., Caso, J.R., García-Bueno, B., Leza, J.C., & Madrigal, J.L.M. (2022) CCL2 Inhibition of Pro-Resolving Mediators Potentiates Neuroinflammation in Astrocytes. *Int. J. Mol. Sci.*, **23**.

Guzman-Martinez, L., Maccioni, R.B., Andrade, V., Navarrete, L.P., Pastor, M.G., & Ramos-Escobar, N. (2019) Neuroinflammation as a common feature of neurodegenerative disorders. *Front. Pharmacol.*, **10**, 1–17.

Han, P.F., Che, X. Da, Li, H.Z., Gao, Y.Y., Wei, X.C., & Li, P.C. (2020) Annexin A1 involved in the regulation of inflammation and cell signaling pathways. *Chinese J. Traumatol. - English Ed.*, **23**, 96–101.

Hanslik, K.L. & Ulland, T.K. (2020) The Role of Microglia and the Nlrp3 Inflammasome in Alzheimer's Disease. *Front. Neurol.*, **11**, 1–9.

Hanson, J., Ferreirós, N., Pirotte, B., Geisslinger, G., & Offermanns, S. (2013) Heterologously expressed formyl peptide receptor 2 (FPR2/ALX) does not respond to lipoxin A4. *Biochem. Pharmacol.*, **85**, 1795–1802.

Hawkins, K.E., DeMars, K.M., Alexander, J.C., de Leon, L.G., Pacheco, S.C., Graves, C., Yang, C., McCrea, A.O., Frankowski, J.C., Garrett, T.J., Febo, M., & Candelario-Jalil, E. (2017) Targeting resolution of neuroinflammation after ischemic stroke with a lipoxin A4 analog: Protective mechanisms and long-term effects on neurological recovery. *Brain Behav.*, **7**, 1–14.

He, H.Q. & Ye, R.D. (2017) The formyl peptide receptors: Diversity of ligands and mechanism for recognition. *Molecules*, **22**.

He, M., Cheng, N., Gao, W., Zhang, M., Zhang, Y., Ye, R.D., & Wang, M. (2011) Characterization of Quin-C1 for its anti-inflammatory property in a mouse model of bleomycin-induced lung injury. *Acta Pharmacol. Sin.*, **32**, 601–610.

He, R., Browning, D.D., & Ye, R.D. (2001) Differential Roles of the NPXXY Motif in Formyl Peptide Receptor Signaling. *J. Immunol.*, **166**, 4099–4105.

Helmut, K., Hanisch, U.K., Noda, M., & Verkhratsky, A. (2011) Physiology of microglia. *Physiol. Rev.*, **91**, 461–553.

Heneka, M.T., Kummer, M.P., Stutz, A., Delekate, A., Schwartz, S., Vieira-Saecker, A., Griep, A., Axt, D., Remus, A., Tzeng, T., Gelpi, E., Halle, A., Korte, M., Latz, E., & Golenbock, D.T. (2013) NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. *Nature*, **493**, 674–678.

Hilger, D., Masureel, M., & Kobilka, B.K. (2018) Structure and dynamics of GPCR signaling complexes. *Nat. Struct. Mol. Biol.*, **25**, 4–12.

Hu, S., Sheng, W.S., Ehrlich, L.C., Peterson, P.K., & Chao, C.C. (2000) Cytokine Effects on Glutamate Uptake by Human Astrocytes. *Neuroimmunomodulation*, **7**, 153–159.

Huang, X., Guo, M., Zhang, Y., Xie, J., Huang, R., Zuo, Z., Saw, P.E., & Cao, M. (2023)

Microglial IL-1RA ameliorates brain injury after ischemic stroke by inhibiting astrocytic CXCL1 -mediated neutrophil recruitment and microvessel occlusion. *Glia*, **71**, 1607–1625.

Huang, Y., Xu, W., & Zhou, R. (2021) NLRP3 inflammasome activation and cell death. *Cell. Mol. Immunol.*, **18**, 2114–2127.

Hyam, S.R., Lee, I.-A., Gu, W., Kim, K.-A., Jeong, J.-J., Jang, S.-E., Han, M.J., & Kim, D.-H. (2013) Arctigenin ameliorates inflammation in vitro and in vivo by inhibiting the PI3K/AKT pathway and polarizing M1 macrophages to M2-like macrophages. *Eur. J. Pharmacol.*, **708**, 21–29.

Iraz, M., Iraz, M., Eşrefoğlu, M., & Aydin, M.Ş. (2015) Protective effect of β-glucan on acute lung injury induced bylipopolysaccharide in rats. *TURKISH J. Med. Sci.*, **45**, 261–267.

Jia, Y., Jin, W., Xiao, Y., Dong, Y., Wang, T., Fan, M., Xu, J., Meng, N., Li, L., & Lv, P. (2015) Lipoxin A4 methyl ester alleviates vascular cognition impairment by regulating the expression of proteins related to autophagy and ER stress in the rat hippocampus. *Cell. Mol. Biol. Lett.*, **20**.

John, G.R., Lee, S.C., & Brosnan, C.F. (2003) Cytokines: Powerful regulators of glial cell activation. *Neuroscientist*, **9**, 10–22.

Jones, H.R., Robb, C.T., Perretti, M., & Rossi, A.G. (2016) The role of neutrophils in inflammation resolution. *Semin. Immunol.*, **28**, 137–145.

Kaster, M.P., Gadotti, V.M., Calixto, J.B., Santos, A.R.S., & Rodrigues, A.L.S. (2012) Depressive-like behavior induced by tumor necrosis factor-α in mice. *Neuropharmacology*, **62**, 419–426.

Katsumoto, A., Lu, H., Miranda, A.S., & Ransohoff, R.M. (2014) Ontogeny and Functions of Central Nervous System Macrophages. *J. Immunol.*, **193**, 2615–2621.

Kenakin, T. & Williams, M. (2014) Defining and characterizing drug/compound function. *Biochem. Pharmacol.*, **87**, 40–63.

Kleinschek, M.A., Muller, U., Brodie, S.J., Stenzel, W., Kohler, G., Blumenschein, W.M., Straubinger, R.K., McClanahan, T., Kastelein, R.A., & Alber, G. (2006) IL-23 Enhances the Inflammatory Cell Response in Cryptococcus neoformans Infection and Induces a Cytokine Pattern Distinct from IL-12. *J. Immunol.*, **176**, 1098–1106.

Kooij, G., Troletti, C.D., Leuti, A., Norris, P.C., Riley, I., Albanese, M., Ruggieri, S., Libreros, S., van der Pol, S.M.A., van het Hof, B., Schell, Y., Guerrera, G., Buttari, F., Mercuri, N.B., Centonze, D., Gasperini, C., Battistini, L., de Vries, H.E., Serhan, C.N.,

& Chiurchiù, V. (2020) Specialized pro-resolving lipid mediators are differentially altered in peripheral blood of patients with multiple sclerosis and attenuate monocyte and blood-brain barrier dysfunction. *Haematologica*, **105**, 2056–2070.

Kox, M., van Eijk, L.T., Zwaag, J., van den Wildenberg, J., Sweep, F.C.G.J., van der Hoeven, J.G., & Pickkers, P. (2014) Voluntary activation of the sympathetic nervous system and attenuation of the innate immune response in humans. *Proc. Natl. Acad. Sci.*, **111**, 7379–7384.

Kretschmer, D., Rautenberg, M., Linke, D., & Peschel, A. (2015) Peptide length and folding state govern the capacity of staphylococcal β-type phenol-soluble modulins to activate human formyl-peptide receptors 1 or 2. *J. Leukoc. Biol.*, **97**, 689–697.

Kwon, H.S. & Koh, S.H. (2020) Neuroinflammation in neurodegenerative disorders: the roles of microglia and astrocytes. *Transl. Neurodegener.*, **9**, 1–12.

Kyvelidou, C., Sotiriou, D., Zerva, I., & Athanassakis, I. (2018) Protection Against Lipopolysaccharide-Induced Immunosuppression by IgG and IgM. *Shock*, **49**, 474–482.

Lagerspetz, K.Y.. & Väätäinen, T. (1987) Bacterial endotoxin and infection cause behavioural hypothermia in infant mice. *Comp. Biochem. Physiol. Part A Physiol.*, **88**, 519–521.

Lasselin, J., Benson, S., Hebebrand, J., Boy, K., Weskamp, V., Handke, A., Hasenberg, T., Remy, M., Föcker, M., Unteroberdörster, M., Brinkhoff, A., Engler, H., & Schedlowski, M. (2020) Immunological and behavioral responses to in vivo lipopolysaccharide administration in young and healthy obese and normal-weight humans. *Brain. Behav. Immun.*, **88**, 283–293.

Lawrence, T., Willoughby, D.A., & Gilroy, D.W. (2002) Anti-inflammatory lipid mediators and insights into the resolution of inflammation. *Nat. Rev. Immunol.*, **2**, 787–795.

Le, Y., Gong, W., Tiffany, H.L., Tumanov, A., Nedospasov, S., Shen, W., Dunlop, N.M., Gao, J.L., Murphy, P.M., Oppenheim, J.J., & Wang, J.M. (2001b) Amyloid (beta)42 activates a G-protein-coupled chemoattractant receptor, FPR-like-1. *J. Neurosci.*, **21**, 2–6.

Le, Y., Oppenheim, J.J., & Wang, J.M. (2001a) Pleiotropic roles of formyl peptide receptors. *Cytokine Growth Factor Rev.*, **12**, 91–105.

Le, Y., Yazawa, H., Gong, W., Yu, Z., Ferrans, V.J., Murphy, P.M., & Wang, J.M. (2001c) Cutting Edge: The Neurotoxic Prion Peptide Fragment PrP106–126 Is a Chemotactic Agonist for the G Protein-Coupled Receptor Formyl Peptide Receptor-Like 1. *J.*

*Immunol.*, **166**, 1448–1451.

Lee, J.C., Seong, J., Kim, S.H., Lee, S.J., Cho, Y.J., An, J., Nam, D.H., Joo, K.M., & Cha, C.I. (2012) Replacement of microglial cells using Clodronate liposome and bone marrow transplantation in the central nervous system of SOD1G93A transgenic mice as an in vivo model of amyotrophic lateral sclerosis. *Biochem. Biophys. Res. Commun.*, **418**, 359–365.

Leng, F. & Edison, P. (2021) Neuroinflammation and microglial activation in Alzheimer disease: where do we go from here? *Nat. Rev. Neurol.*, **17**, 157–172.

Leszek, J., E. Barreto, G., G|siorowski, K., Koutsouraki, E., Ávila-Rodrigues, M., & Aliev, G. (2016) Inflammatory Mechanisms and Oxidative Stress as Key Factors Responsible for Progression of Neurodegeneration: Role of Brain Innate Immune System. *CNS Neurol. Disord. - Drug Targets*, **15**, 329–336.

Leuti, A., Maccarrone, M., & Chiurchiù, V. (2019) Proresolving lipid mediators: Endogenous modulators of oxidative stress. *Oxid. Med. Cell. Longev.*, **2019**.

Liang, T.S., Wang, J.M., Murphy, P.M., & Gao, J.L. (2000) Serum amyloid A is a chemotactic agonist at FPR2, a low-affinity N-formylpeptide receptor on mouse neutrophils. *Biochem. Biophys. Res. Commun.*, **270**, 331–335.

Liu, H., He, J., Wu, Y., Du, Y., Jiang, Y., Chen, C., Yu, Z., Zhong, J., Wang, Z., Cheng, C., Sun, X., & Huang, Z. (2021) Endothelial Regulation by Exogenous Annexin A1 in Inflammatory Response and BBB Integrity Following Traumatic Brain Injury. *Front. Neurosci.*, **15**, 1–13.

Liu, M., Chen, K., Yoshimura, T., Liu, Y., Gong, W., Le, Y., Gao, J.L., Zhao, J., Wang, J.M., & Wang, A. (2014) Formylpeptide receptors mediate rapid neutrophil mobilization to accelerate wound healing. *PLoS One*, **9**, 1–7.

Liu, X., Zhou, L., Xin, W., & Hua, Z. (2022) Exogenous Annexin 1 inhibits Th17 cell differentiation induced by anti-TNF treatment via activating FPR2 in DSS-induced colitis. *Int. Immunopharmacol.*, **107**, 108685.

Livne-Bar, I., Wei, J., Liu, H.-H., Alqawlaq, S., Won, G.-J., Tuccitto, A., Gronert, K., Flanagan, J.G., & Sivak, J.M. (2017) Astrocyte-derived lipoxins A4 and B4 promote neuroprotection from acute and chronic injury. *J. Clin. Invest.*, **127**, 4403–4414.

Lohse, M.J. (2010) Dimerization in GPCR mobility and signaling. *Curr. Opin. Pharmacol.*, **10**, 53–58.

Lu, Y.-C., Yeh, W.-C., & Ohashi, P.S. (2008) LPS/TLR4 signal transduction pathway. *Cytokine*, **42**, 145–151.

Lucki, I. (1997) The forced swimming test as a model for core and component behavioral effects of antidepressant drugs. *Behav. Pharmacol.*, **8**, 523–532.

Ma, D., Jin, S., Li, E., Doi, Y., Parajuli, B., Noda, M., Sonobe, Y., Mizuno, T., & Suzumura, A. (2013) The neurotoxic effect of astrocytes activated with toll-like receptor ligands. *J. Neuroimmunol.*, **254**, 10–18.

Machado, F.S., Johndrow, J.E., Esper, L., Dias, A., Bafica, A., Serhan, C.N., & Aliberti, J. (2006) Anti-inflammatory actions of lipoxin A4 and aspirin-triggered lipoxin are SOCS-2 dependent. *Nat. Med.*, **12**, 330–334.

Majumder, S., Zhou, L.Z., Chaturvedi, P., Babcock, G., Aras, S., & Ransohoff, R.M. (1998) p48/STAT-1alpha-containing complexes play a predominant role in induction of IFN-gamma-inducible protein, 10 kDa (IP-10) by IFN-gamma alone or in synergy with TNF-alpha. *J. Immunol.*, **161**, 4736–4744.

Makkonen, N., Hirvonen, M.R., Teräväinen, T., Savolainen, K., & Mönkkönen, J. (1996) Different effects of three bisphosphonates on nitric oxide production by RAW 264 macrophage-like cells in vitro. *J. Pharmacol. Exp. Ther.*, **277**, 1097–1102.

Marín-Teva, J.L., Dusart, I., Colin, C., Gervais, A., van Rooijen, N., & Mallat, M. (2004) Microglia Promote the Death of Developing Purkinje Cells. *Neuron*, **41**, 535–547.

Mastromarino, M., Favia, M., Schepetkin, I.A., Kirpotina, L.N., Trojan, E., Niso, M., Carrieri, A., Leśkiewicz, M., Regulska, M., Darida, M., Rossignolo, F., Fontana, S., Quinn, M.T., Basta-Kaim, A., Leopoldo, M., & Lacivita, E. (2022) Design, Synthesis, Biological Evaluation, and Computational Studies of Novel Ureidopropanamides as Formyl Peptide Receptor 2 (FPR2) Agonists to Target the Resolution of Inflammation in Central Nervous System Disorders. *J. Med. Chem.*, **65**, 5004–5028.

Matejuk, A. & Ransohoff, R.M. (2020) Crosstalk Between Astrocytes and Microglia: An Overview. *Front. Immunol.*, **11**.

Medeiros, R., Kitazawa, M., Passos, G.F., Baglietto-Vargas, D., Cheng, D., Cribbs, D.H., & LaFerla, F.M. (2013) Aspirin-Triggered Lipoxin A4 Stimulates Alternative Activation of Microglia and Reduces Alzheimer Disease–Like Pathology in Mice. *Am. J. Pathol.*, **182**, 1780–1789.

Medzhitov, R. (2008) Origin and physiological roles of inflammation. *Nature*, **454**, 428–435.

Meeks, K.D., Sieve, A.N., Kolls, J.K., Ghilardi, N., & Berg, R.E. (2009) IL-23 Is Required for Protection against Systemic Infection with Listeria monocytogenes . *J. Immunol.*, **183**, 8026–8034.

Meseguer, V., Alpizar, Y.A., Luis, E., Tajada, S., Denlinger, B., Fajardo, O., Manenschijn, J.-A., Fernández-Peña, C., Talavera, A., Kichko, T., Navia, B., Sánchez, A., Señarís, R., Reeh, P., Pérez-García, M.T., López-López, J.R., Voets, T., Belmonte, C., Talavera, K., & Viana, F. (2014) TRPA1 channels mediate acute neurogenic inflammation and pain produced by bacterial endotoxins. *Nat. Commun.*, **5**, 3125.

Mészáros, Á., Molnár, K., Nógrádi, B., Hernádi, Z., Nyúl-Tóth, Á., Wilhelm, I., & Krizbai, I.A. (2020) Neurovascular Inflammaging in Health and Disease. *Cells*, **9**, 1614.

Michell-Robinson, M.A., Touil, H., Healy, L.M., Owen, D.R., Durafourt, B.A., Bar-Or, A., Antel, J.P., & Moore, C.S. (2015) Roles of microglia in brain development, tissue maintenance and repair. *Brain*, **138**, 1138–1159.

Minogue, A.M., Barrett, J.P., & Lynch, M.A. (2012) LPS-induced release of IL-6 from glia modulates production of IL-1β in a JAK2-dependent manner. *J. Neuroinflammation*, **9**, 1.

Moore, R., Johnson, B., & Berry, L. (1977) Nutritional effects of salmonellosis in mice. *Am. J. Clin. Nutr.*, **30**, 1289–1293.

Mullington, J., Korth, C., Hermann, D.M., Orth, A., Galanos, C., Holsboer, F., & Pollmächer, T. (2000) Dose-dependent effects of endotoxin on human sleep. *Am. J. Physiol. Integr. Comp. Physiol.*, **278**, R947–R955.

Nelson, J.W., Leigh, N.J., Mellas, R.E., McCall, A.D., Aguirre, A., & Baker, O.J. (2014) ALX/FPR2 receptor for RvD1 is expressed and functional in salivary glands. *Am. J. Physiol. - Cell Physiol.*, **306**, 178–185.

Neurath, M.F. (2019) Resolution of inflammation: from basic concepts to clinical application. *Semin. Immunopathol.*, **41**, 627–631.

Nitsch, L., Schneider, L., Zimmermann, J., & Müller, M. (2021) Microglia-Derived Interleukin 23: A Crucial Cytokine in Alzheimer's Disease? *Front. Neurol.*, **12**, 1–8.

Norel, X. & Brink, C. (2004) The quest for new cysteinyl-leukotriene and lipoxin receptors: recent clues. *Pharmacol. Ther.*, **103**, 81–94.

O'Connor, J.C., Lawson, M.A., André, C., Moreau, M., Lestage, J., Castanon, N., Kelley, K.W., & Dantzer, R. (2009) Lipopolysaccharide-induced depressive-like behavior is mediated by indoleamine 2,3-dioxygenase activation in mice. *Mol. Psychiatry*, **14**, 511–522.

Ortega-Gómez, A., Perretti, M., & Soehnlein, O. (2013) Resolution of inflammation: An integrated view. *EMBO Mol. Med.*, **5**, 661–674.

Pacher, P., Beckman, J.S., & Liaudet, L. (2007) Nitric Oxide and Peroxynitrite in Health and

Disease. *Physiol. Rev.*, **87**, 315–424.

Pamplona, F.A., Ferreira, J., Menezes de Lima, O., Duarte, F.S., Bento, A.F., Forner, S., Villarinho, J.G., Bellocchio, L., Wotjak, C.T., Lerner, R., Monory, K., Lutz, B., Canetti, C., Matias, I., Calixto, J.B., Marsicano, G., Guimarães, M.Z.P., & Takahashi, R.N. (2012) Anti-inflammatory lipoxin A 4 is an endogenous allosteric enhancer of CB 1 cannabinoid receptor. *Proc. Natl. Acad. Sci.*, **109**, 21134–21139.

Panigrahy, D., Gilligan, M.M., Serhan, C.N., & Kashfi, K. (2021) Resolution of inflammation: An organizing principle in biology and medicine. *Pharmacol. Ther.*, **227**, 107879.

Paolicelli, R.C., Sierra, A., Stevens, B., Tremblay, M.-E., Aguzzi, A., Ajami, B., Amit, I., Audinat, E., Bechmann, I., Bennett, M., Bennett, F., Bessis, A., Biber, K., Bilbo, S., Blurton-Jones, M., Boddeke, E., Brites, D., Brône, B., Brown, G.C., Butovsky, O., Carson, M.J., Castellano, B., Colonna, M., Cowley, S.A., Cunningham, C., Davalos, D., De Jager, P.L., de Strooper, B., Denes, A., Eggen, B.J.L., Eyo, U., Galea, E., Garel, S., Ginhoux, F., Glass, C.K., Gokce, O., Gomez-Nicola, D., González, B., Gordon, S., Graeber, M.B., Greenhalgh, A.D., Gressens, P., Greter, M., Gutmann, D.H., Haass, C., Heneka, M.T., Heppner, F.L., Hong, S., Hume, D.A., Jung, S., Kettenmann, H., Kipnis, J., Koyama, R., Lemke, G., Lynch, M., Majewska, A., Malcangio, M., Malm, T., Mancuso, R., Masuda, T., Matteoli, M., McColl, B.W., Miron, V.E., Molofsky, A.V., Monje, M., Mracsko, E., Nadjar, A., Neher, J.J., Neniskyte, U., Neumann, H., Noda, M., Peng, B., Peri, F., Perry, V.H., Popovich, P.G., Pridans, C., Priller, J., Prinz, M., Ragozzino, D., Ransohoff, R.M., Salter, M.W., Schaefer, A., Schafer, D.P., Schwartz, M., Simons, M., Smith, C.J., Streit, W.J., Tay, T.L., Tsai, L.-H., Verkhratsky, A., von Bernhardi, R., Wake, H., Wittamer, V., Wolf, S.A., Wu, L.-J., & Wyss-Coray, T. (2022) Microglia states and nomenclature: A field at its crossroads. *Neuron*, **110**, 3458–3483.

Perego, C., Fumagalli, S., & De Simoni, M.-G. (2011) Temporal pattern of expression and colocalization of microglia/macrophage phenotype markers following brain ischemic injury in mice. *J. Neuroinflammation*, **8**, 174.

Perretti, M. & Godson, C. (2020) Formyl peptide receptor type 2 agonists to kick-start resolution pharmacology. *Br. J. Pharmacol.*, **177**, 4595–4600.

Perry, V.H. & Teeling, J. (2013) Microglia and macrophages of the central nervous system: The contribution of microglia priming and systemic inflammation to chronic neurodegeneration. *Semin. Immunopathol.*, **35**, 601–612.

Pollak, D.D., Rey, C.E., & Monje, F.J. (2010) Rodent models in depression research:

Classical strategies and new directions. *Ann. Med.*, **42**, 252–264.

Ponomarev, E.D., Shriver, L.P., & Dittel, B.N. (2006) CD40 Expression by Microglial Cells Is Required for Their Completion of a Two-Step Activation Process during Central Nervous System Autoimmune Inflammation. *J. Immunol.*, **176**, 1402–1410.

Porro, C., Cianciulli, A., Trotta, T., Lofrumento, D.D., & Panaro, M.A. (2019) Curcumin regulates anti-inflammatory responses by JAK/STAT/SOCS signaling pathway in BV-2 microglial cells. *Biology (Basel).*, **8**.

Porsolt, R.D., Anton, G., Blavet, N., & Jalfre, M. (1978) Behavioural despair in rats: A new model sensitive to antidepressant treatments. *Eur. J. Pharmacol.*, **47**, 379–391.

Prossnitz, E.R. & Ye, R.D. (1997) The N-formyl peptide receptor: A model for the study of chemoattractant receptor structure and function. *Pharmacol. Ther.*, **74**, 73–102.

Raabe, C.A., Gröper, J., & Rescher, U. (2019) Biased perspectives on formyl peptide receptors. *Biochim. Biophys. Acta - Mol. Cell Res.*, **1866**, 305–316.

Rabiet, M.-J., Huet, E., & Boulay, F. (2005) Human mitochondria-derivedN-formylated peptides are novel agonists equally active on FPR and FPRL1, whileListeria monocytogenes-derived peptides preferentially activate FPR. *Eur. J. Immunol.*, **35**, 2486–2495.

Ransohoff, R.M. (2016a) A polarizing question: Do M1 and M2 microglia exist. *Nat. Neurosci.*, **19**, 987–991.

Ransohoff, R.M. (2016b) How neuroinflammation contributes to neurodegeneration. *Science (80-. ).*, **353**, 777–783.

Recchiuti, A., Isopi, E., Romano, M., & Mattoscio, D. (2020) Roles of specialized pro-resolving lipid mediators in autophagy and inflammation. *Int. J. Mol. Sci.*, **21**, 1–23.

Regulska, M., Szuster-Głuszczak, M., Trojan, E., Leśkiewicz, M., & Basta-Kaim, A. (2020) The Emerging Role of the Double-Edged Impact of Arachidonic Acid- Derived Eicosanoids in the Neuroinflammatory Background of Depression. *Curr. Neuropharmacol.*, **19**, 278–293.

Reichenberg, A., Yirmiya, R., Schuld, A., Kraus, T., Haack, M., Morag, A., & Pollmächer, T. (2001) Cytokine-Associated Emotional and Cognitive Disturbances in Humans. *Arch. Gen. Psychiatry*, **58**, 445.

Rindflesch, T.C., Blake, C.L., Cairelli, M.J., Fiszman, M., Zeiss, C.J., & Kilicoglu, H. (2018) Investigating the role of interleukin-1 beta and glutamate in inflammatory bowel disease and epilepsy using discovery browsing. *J. Biomed. Semantics*, **9**, 25.

Rock, R.B., Gekker, G., Hu, S., Sheng, W.S., Cheeran, M., Lokensgard, J.R., & Peterson,

P.K. (2004) Role of Microglia in Central Nervous System Infections. *Clin. Microbiol. Rev.*, **17**, 942–964.

Romano, M. (2010) Lipoxin and aspirin-triggered lipoxins. *ScientificWorldJournal.*, **10**, 1048–1064.

Ronaldson, P.T. & Davis, T.P. (2020) Regulation of blood–brain barrier integrity by microglia in health and disease: A therapeutic opportunity. *J. Cereb. Blood Flow Metab.*, **40**, S6–S24.

Saghazadeh, A., Ataeinia, B., Keynejad, K., Abdolalizadeh, A., Hirbod-Mobarakeh, A., & Rezaei, N. (2019) A meta-analysis of pro-inflammatory cytokines in autism spectrum disorders: Effects of age, gender, and latitude. *J. Psychiatr. Res.*, **115**, 90–102.

Salemi, J., Obregon, D.F., Cobb, A., Reed, S., Sadic, E., Jin, J., Fernandez, F., Tan, J., & Giunta, B. (2011) Flipping the switches: CD40 and CD45 modulation of microglial activation states in HIV associated dementia (HAD). *Mol. Neurodegener.*, **6**, 3.

Sama, M.A., Mathis, D.M., Furman, J.L., Abdul, H.M., Artiushin, I.A., Kraner, S.D., & Norris, C.M. (2008) Interleukin-1β-dependent Signaling between Astrocytes and Neurons Depends Critically on Astrocytic Calcineurin/NFAT Activity. *J. Biol. Chem.*, **283**, 21953–21964.

Schaedler, R.W. & Dubos, R.J. (1961) THE SUSCEPTIBILITY OF MICE TO BACTERIAL ENDOTOXINS. *J. Exp. Med.*, **113**, 559–570.

Schafer, D.P. & Stevens, B. (2015) Microglia Function in Central Nervous System Development and Plasticity. *Cold Spring Harb. Perspect. Biol.*, **7**, a020545.

Schaldach, C.M., Riby, J., & Bjeldanes, L.F. (1999) Lipoxin A 4 : A New Class of Ligand for the Ah Receptor. *Biochemistry*, **38**, 7594–7600.

Schepetkin, I.A., Khlebnikov, A.I., Giovannoni, M.P., Kirpotina, L.N., Cilibrizzi, A., & Quinn, M.T. (2014) Development of Small Molecule Non-peptide Formyl Peptide Receptor (FPR) Ligands and Molecular Modeling of Their Recognition. *Curr. Med. Chem.*, **21**, 1478–1504.

Schett, G. & Neurath, M.F. (2018) Resolution of chronic inflammatory disease: universal and tissue-specific concepts. *Nat. Commun.*, **9**.

Schröder, N., Schaffrath, A., Welter, J.A., Putzka, T., Griep, A., Ziegler, P., Brandt, E., Samer, S., Heneka, M.T., Kaddatz, H., Zhan, J., Kipp, E., Pufe, T., Tauber, S.C., Kipp, M., & Brandenburg, L.O. (2020) Inhibition of formyl peptide receptors improves the outcome in a mouse model of Alzheimer disease. *J. Neuroinflammation*, **17**, 1–15.

Schwartz, M. & Baruch, K. (2014) The resolution of neuroinflammation in

neurodegeneration: Leukocyte recruitment via the choroid plexus. *EMBO J.*, **33**, 7–22.

Sensoy, O. & Weinstein, H. (2015) A mechanistic role of Helix 8 in GPCRs: Computational modeling of the dopamine D2 receptor interaction with the GIPC1–PDZ-domain. *Biochim. Biophys. Acta - Biomembr.*, **1848**, 976–983.

Serhan, C.N. (2014a) Pro-resolving lipid mediators are leads for resolution physiology. *Nature*, **510**, 92–101.

Serhan, C.N. (2014b) Novel Pro-Resolving Lipid Mediators in Inflammation Are Leads for Resolution Physiology. *Nature*, **510**, 92–101.

Serhan, C.N., Chiang, N., & Van Dyke, T.E. (2008) Resolving inflammation: Dual anti-inflammatory and pro-resolution lipid mediators. *Nat. Rev. Immunol.*, **8**, 349–361.

Serhan, C.N., Hamberg, M., & Samuelsson, B. (1984) Lipoxins: novel series of biologically active compounds formed from arachidonic acid in human leukocytes. *Proc. Natl. Acad. Sci.*, **81**, 5335–5339.

Singh, D. (2022) Astrocytic and microglial cells as the modulators of neuroinflammation in Alzheimer's disease. *J. Neuroinflammation*, **19**, 206.

Siracusa, R., Fusco, R., & Cuzzocrea, S. (2019) Astrocytes: Role and Functions in Brain Pathologies. *Front. Pharmacol.*, **10**.

Sivandzade, F., Bhalerao, A., & Cucullo, L. (2019) Analysis of the Mitochondrial Membrane Potential Using the Cationic JC-1 Dye as a Sensitive Fluorescent Probe. *Bio-Protocol*, **9**, 1–13.

Skvortsov, S.S. & Gabdoulkhakova, A.G. (2017) Formyl peptide receptor polymorphisms: 27 most possible ways for phagocyte dysfunction. *Biochem.*, **82**, 426–437.

Ślusarczyk, J., Trojan, E., Głombik, K., Piotrowska, A., Budziszewska, B., Kubera, M., Popiołek-Barczyk, K., Lasoń, W., Mika, J., & Basta-Kaim, A. (2018) Targeting the NLRP3 inflammasome-related pathways via tianeptine treatment-suppressed microglia polarization to the M1 phenotype in lipopolysaccharide-stimulated cultures. *Int. J. Mol. Sci.*, **19**, 1–23.

Sodin-Semrl, S., Spagnolo, A., Mikus, R., Barbaro, B., Varga, J., & Fiore, S. (2004) Opposing regulation of interleukin-8 and NF-kB responses by lipoxin A4 and serum amyloid a via the common lipoxin a receptor. *Int. J. Immunopathol. Pharmacol.*, **17**, 145–155.

Soehnlein, O. & Lindbom, L. (2010) Phagocyte partnership during the onset and resolution of inflammation. *Nat. Rev. Immunol.*, **10**, 427–439.

Sofroniew, M. V. (2009) Molecular dissection of reactive astrogliosis and glial scar

formation. *Trends Neurosci.*, **32**, 638–647.

Soliman, A.M. & Barreda, D.R. (2022) Acute Inflammation in Tissue Healing. *Int. J. Mol. Sci.*, **24**, 641.

Sousa, C., Biber, K., & Michelucci, A. (2017) Cellular and molecular characterization of microglia: A unique immune cell population. *Front. Immunol.*, **8**.

Stama, M.L., Ślusarczyk, J., Lacivita, E., Kirpotina, L.N., Schepetkin, I.A., Chamera, K., Riganti, C., Perrone, R., Quinn, M.T., Basta-Kaim, A., & Leopoldo, M. (2017) Novel ureidopropanamide based N-formyl peptide receptor 2 (FPR2) agonists with potential application for central nervous system disorders characterized by neuroinflammation. *Eur. J. Med. Chem.*, **141**, 703–720.

Stephenson, J., Nutma, E., van der Valk, P., & Amor, S. (2018) Inflammation in CNS neurodegenerative diseases. *Immunology*, **154**, 204–219.

Suffredini, A.F. & Noveck, R.J. (2014) Human Endotoxin Administration as an Experimental Model in Drug Development. *Clin. Pharmacol. Ther.*, **96**, 418–422.

Sugimoto, M.A., Sousa, L.P., Pinho, V., Perretti, M., & Teixeira, M.M. (2016) Resolution of inflammation: What controls its onset? *Front. Immunol.*, **7**.

Szczesny, E., Basta-Kaim, A., Slusarczyk, J., Trojan, E., Glombik, K., Regulska, M., Leskiewicz, M., Budziszewska, B., Kubera, M., & Lason, W. (2014) The impact of prenatal stress on insulin-like growth factor-1 and pro-inflammatory cytokine expression in the brains of adult male rats: The possible role of suppressors of cytokine signaling proteins. *J. Neuroimmunol.*, **276**, 37–46.

Taetzsch, T., Levesque, S., McGraw, C., Brookins, S., Luqa, R., Bonini, M.G., Mason, R.P., Oh, U., & Block, M.L. (2015) Redox regulation of <scp>NF</scp> -κB p50 and M1 polarization in microglia. *Glia*, **63**, 423–440.

Takano, T., Fiore, S., Maddox, J.F., Brady, H.R., Petasis, N.A., & Serhan, C.N. (1997) Aspirin-triggered 15-epi-lipoxin A4 (LXA4) and LXA4 stable analogues are potent inhibitors of acute inflammation: Evidence for anti-inflammatory receptors. *J. Exp. Med.*, **185**, 1693–1704.

Tansey, M.G., Wallings, R.L., Houser, M.C., Herrick, M.K., Keating, C.E., & Joers, V. (2022) Inflammation and immune dysfunction in Parkinson disease. *Nat. Rev. Immunol.*, **22**, 657–673.

Tiberi, M. & Chiurchiù, V. (2021) Specialized Pro-resolving Lipid Mediators and Glial Cells: Emerging Candidates for Brain Homeostasis and Repair. *Front. Cell. Neurosci.*, **15**, 1–11.

Tiefenthaler, M., Amberger, A., Bacher, N., Hartmann, B.L., Margreiter, R., Kofler, R., & Konwalinka, G. (2001) Increased lactate production follows loss of mitochondrial membrane potential during apoptosis of human leukaemia cells. *Br. J. Haematol.*, **114**, 574–580.

Tiffany, H.L., Lavigne, M.C., Cui, Y.H., Wang, J.M., Leto, T.L., Gao, J.L., & Murphy, P.M. (2001) Amyloid-β Induces Chemotaxis and Oxidant Stress by Acting at Formylpeptide Receptor 2, a G Protein-coupled Receptor Expressed in Phagocytes and Brain. *J. Biol. Chem.*, **276**, 23645–23652.

Town, T., Bai, F., Wang, T., Kaplan, A.T., Qian, F., Montgomery, R.R., Anderson, J.F., Flavell, R.A., & Fikrig, E. (2009) Toll-like Receptor 7 Mitigates Lethal West Nile Encephalitis via Interleukin 23-Dependent Immune Cell Infiltration and Homing. *Immunity*, **30**, 242–253.

Trojan, E., Bryniarska, N., Leśkiewicz, M., Regulska, M., Chamera, K., Szuster-Głuszczak, M., Leopoldo, M., Lacivita, E., & Basta-Kaim, A. (2019) The Contribution of Formyl Peptide Receptor Dysfunction to the Course of Neuroinflammation: A Potential Role in the Brain Pathology. *Curr. Neuropharmacol.*, **18**, 229–249.

Trojan, E., Tylek, K., Leśkiewicz, M., Lasoń, W., Brandenburg, L.O., Leopoldo, M., Lacivita, E., & Basta-Kaim, A. (2021a) The N-formyl peptide receptor 2 (Fpr2) agonist mr-39 exhibits anti-inflammatory activity in lps-stimulated organotypic hippocampal cultures. *Cells*, **10**.

Trojan, E., Tylek, K., Schröder, N., Kahl, I., Brandenburg, L.O., Mastromarino, M., Leopoldo, M., Basta-Kaim, A., & Lacivita, E. (2021b) The N-Formyl Peptide Receptor 2 (FPR2) Agonist MR-39 Improves Ex Vivo and In Vivo Amyloid Beta (1–42)-Induced Neuroinflammation in Mouse Models of Alzheimer's Disease. *Mol. Neurobiol.*, **2**.

Tucureanu, M.M., Rebleanu, D., Constantinescu, C.A., Deleanu, M., Voicu, G., Butoi, E., Calin, M., & Manduteanu, I. (2017) Lipopolysaccharide-induced inflammation in monocytes/macrophages is blocked by liposomal delivery of Gi-protein inhibitor. *Int. J. Nanomedicine*, **Volume 13**, 63–76.

Tylek, K., Trojan, E., Leśkiewicz, M., Francavilla, F., Lacivita, E., Leopoldo, M., & Basta-Kaim, A. (2023a) Stimulation of Formyl Peptide Receptor-2 by the New Agonist CMC23 Protects against Endotoxin-Induced Neuroinflammatory Response: A Study in Organotypic Hippocampal Cultures. *ACS Chem. Neurosci.*, **14**, 3869–3882.

Tylek, K., Trojan, E., Leśkiewicz, M., Ghafir El Idrissi, I., Lacivita, E., Leopoldo, M., & Basta-Kaim, A. (2023b) Microglia Depletion Attenuates the Pro-Resolving Activity of

the Formyl Peptide Receptor 2 Agonist AMS21 Related to Inhibition of Inflammasome NLRP3 Signalling Pathway: A Study of Organotypic Hippocampal Cultures. *Cells*, **12**, 1–21.

Tylek, K., Trojan, E., Leśkiewicz, M., Regulska, M., Bryniarska, N., Curzytek, K., Lacivita, E., Leopoldo, M., & Basta-Kaim, A. (2021b) Time-dependent protective and pro-resolving effects of fpr2 agonists on lipopolysaccharide-exposed microglia cells involve inhibition of nf-κb and mapks pathways. *Cells*, **10**, 1–27.

Tylek, K., Trojan, E., Regulska, M., Lacivita, E., Leopoldo, M., & Basta-Kaim, A. (2021a) Formyl peptide receptor 2, as an important target for ligands triggering the inflammatory response regulation: a link to brain pathology. *Pharmacol. Reports*,.

Urbán, N., Blomfield, I.M., & Guillemot, F. (2019) Quiescence of Adult Mammalian Neural Stem Cells: A Highly Regulated Rest. *Neuron*, **104**, 834–848.

Valente, M., Dentoni, M., Bellizzi, F., Kuris, F., & Gigli, G.L. (2022) Specialized Pro-Resolving Mediators in Neuroinflammation: Overview of Studies and Perspectives of Clinical Applications. *Molecules*, **27**, 1–25.

Vaughn, M.W., Proske, R.J., & Haviland, D.L. (2002) Identification, Cloning, and Functional Characterization of a Murine Lipoxin A4 Receptor Homologue Gene. *J. Immunol.*, **169**, 3363–3369.

Venero, J.L., Burguillos, M.A., Brundin, P., & Joseph, B. (2011) The executioners sing a new song: Killer caspases activate microglia. *Cell Death Differ.*, **18**, 1679–1691.

Venkatakrishnan, A.J., Deupi, X., Lebon, G., Tate, C.G., Schertler, G.F., & Madan Babu, M. (2013) Molecular signatures of G-protein-coupled receptors. *Nature*, **494**, 185–194.

Wang, J., He, W., & Zhang, J. (2023) A richer and more diverse future for microglia phenotypes. *Heliyon*, **9**, e14713.

Wang, Y.P., Wu, Y., Li, L.Y., Zheng, J., Liu, R.G., Zhou, J.P., Yuan, S.Y., Shang, Y., & Yao, S.L. (2011) Aspirin-triggered lipoxin A4attenuates LPS-induced pro-inflammatory responses by inhibiting activation of NF-κB and MAPKs in BV-2 microglial cells. *J. Neuroinflammation*, **8**, 95.

Weiß, E. & Kretschmer, D. (2018) Formyl-Peptide Receptors in Infection, Inflammation, and Cancer. *Trends Immunol.*, **39**, 815–829.

Wu, J., Ding, D., Wang, X., Li, Q., Sun, Y., Li, L., & Wang, Y. (2019a) Regulation of aquaporin 4 expression by lipoxin A4 in astrocytes stimulated by lipopolysaccharide. *Cell. Immunol.*, **344**, 103959.

Wu, J., Ding, D.H., Li, Q.Q., Wang, X.Y., Sun, Y.Y., & Li, L.J. (2019b) Lipoxin A4

regulates lipopolysaccharide-induced BV2 microglial activation and differentiation via the notch signaling pathway. *Front. Cell. Neurosci.*, **13**, 1–17.

Wu, Y., Wang, Y.-P., Guo, P., Ye, X.-H., Wang, J., Yuan, S.-Y., Yao, S.-L., & Shang, Y. (2012a) A Lipoxin A4 Analog Ameliorates Blood–Brain Barrier Dysfunction and Reduces MMP-9 Expression in a Rat Model of Focal Cerebral Ischemia–Reperfusion Injury. *J. Mol. Neurosci.*, **46**, 483–491.

Wu, Y., Zhai, H., Wang, Y., Li, L., Wu, J., Wang, F., Sun, S., Yao, S., & Shang, Y. (2012b) Aspirin-triggered lipoxin A 4 attenuates lipopolysaccharide- induced intracellular ROS in BV2 microglia cells by inhibiting the function of NADPH oxidase. *Neurochem. Res.*, **37**, 1690–1696.

Yao, C., Yang, D., Wan, Z., Wang, Z., Liu, R., Wu, Y., Yao, S., Yuan, S., & Shang, Y. (2014) Aspirin-triggered lipoxin A4 attenuates lipopolysaccharide induced inflammatory response in primary astrocytes. *Int. Immunopharmacol.*, **18**, 85–89.

Ye, R.D., Boulay, F., Ji, M.W., Dahlgren, C., Gerard, C., Parmentier, M., Serhan, C.N., & Murphy, P.M. (2009) International union of basic and clinical pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family. *Pharmacol. Rev.*, **61**, 119–161.

Yin, R., Zhang, K., Li, Y., Tang, Z., Zheng, R., Ma, Y., Chen, Z., Lei, N., Xiong, L., Guo, P., Li, G., & Xie, Y. (2023) Lipopolysaccharide-induced depression-like model in mice: meta-analysis and systematic evaluation. *Front. Immunol.*, **14**.

Yirmiya, R. (1996) Endotoxin produces a depressive-like episode in rats. *Brain Res.*, **711**, 163–174.

Zhang, B., Wang, P.-P., Hu, K.-L., Li, L.-N., Yu, X., Lu, Y., & Chang, H.-S. (2019) Antidepressant-Like Effect and Mechanism of Action of Honokiol on the Mouse Lipopolysaccharide (LPS) Depression Model. *Molecules*, **24**, 2035.

Zhang, D., Zhao, Q., & Wu, B. (2015) Structural studies of G protein-coupled receptors. *Mol. Cells*, **38**, 836–842.

Zhang, J., Li, Z., Fan, M., & Jin, W. (2022) Lipoxins in the Nervous System: Brighter Prospects for Neuroprotection. *Front. Pharmacol.*, **13**, 1–18.

Zhang, S., Gong, H., Ge, Y., & Ye, R.D. (2020) Biased allosteric modulation of formyl peptide receptor 2 leads to distinct receptor conformational states for pro- and anti-inflammatory signaling. *Pharmacol. Res.*, **161**, 105117.

Zhang, W., Tian, T., Gong, S.-X., Huang, W.-Q., Zhou, Q.-Y., Wang, A.-P., & Tian, Y. (2021) Microglia-associated neuroinflammation is a potential therapeutic target for

ischemic stroke. *Neural Regen. Res.*, **16**, 6.

Zhang, W., Xiao, D., Mao, Q., & Xia, H. (2023) Role of neuroinflammation in neurodegeneration development. *Signal Transduct. Target. Ther.*, **8**, 267.

Zheng, Z.V., Chen, J., Lyu, H., Lam, S.Y.E., Lu, G., Chan, W.Y., & Wong, G.K.C. (2022) Novel role of STAT3 in microglia-dependent neuroinflammation after experimental subarachnoid haemorrhage. *Stroke Vasc. Neurol.*, **7**, 62–70.

Zhu, J., Li, L., Ding, J., Huang, J., Shao, A., & Tang, B. (2021) The Role of Formyl Peptide Receptors in Neurological Diseases via Regulating Inflammation. *Front. Cell. Neurosci.*, **15**, 1–13.

Zhu, J., Yu, B., Fu, C., He, M., Zhu, J., Chen, B., Zheng, Y., Chen, S., Fu, X., Li, P., & Lin, Z. (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.

Zhu, M., Wang, X., Hjorth, E., Colas, R.A., Schroeder, L., Granholm, A.-C., Serhan, C.N., & Schultzberg, M. (2016) Pro-Resolving Lipid Mediators Improve Neuronal Survival and Increase Aβ42 Phagocytosis. *Mol. Neurobiol.*, **53**, 2733–2749.

Zorova, L.D., Popkov, V.A., Plotnikov, E.Y., Silachev, D.N., Pevzner, I.B., Jankauskas, S.S., Babenko, V.A., Zorov, S.D., Balakireva, A. V., Juhaszova, M., Sollott, S.J., & Zorov, D.B. (2018) Mitochondrial membrane potential. *Anal. Biochem.*, **552**, 50–59.

## 11.Oświadczenia

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**OŚWIADCZENIE**

Oświadczam, że mój udział w pracy:

1. Tylek K., Trojan E., Regulska M., Lacivita E., Leopoldo M., Basta-Kaim A. Formyl peptide receptor 2, as an important target for ligands triggering the inflammatory response regulation: a link to brain pathology. Pharmacol Rep. 2021 Aug;73(4):1004-1019. doi: 10.1007/s43440-021-00271-x.

   polegał na: przygotowywaniu części manuskryptu powyższej publikacji, zbieraniu i opracowaniu danych literaturowych, przygotowywaniu figur, odpowiedzi na pytania recenzentów.

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2. Tylek K., Trojan E., Leśkiewicz M., Regulska M., Bryniarska N., Curzytek K., Lacivita E., Leopoldo M., Basta-Kaim A. Time-Dependent Protective and Pro-Resolving Effects of FPR2 Agonists on Lipopolysaccharide-Exposed Microglia Cells Involve Inhibition of NF-κB and MAPKs Pathways. Cells. 2021 Sep 9;10(9):2373. doi: 10.3390/cells10092373.

   polegał na: przygotowywaniu hodowli pierwotnych mikrogleju, wykonywaniu analiz biochemicznych, przygotowaniu części manuskryptu powyższej publikacji, przygotowaniu wykresów oraz schematów, zbieraniu i opracowaniu literatury, odpowiedzi na pytania recenzentów.

Oświadczam, że mój udział w pracach:

3. Tylek K., Trojan E., Leśkiewicz M., Francavilla F., Lacivita E., Leopoldo M., Basta-Kaim A. Stimulation of Formyl Peptide Receptor-2 by the New Agonist CMC23 Protects against Endotoxin-Induced Neuroinflammatory Response: A Study in Organotypic Hippocampal Cultures. ACS Chem Neurosci. 2023 Oct 18;14(20):3869-3882. doi: 10.1021/acschemneuro.3c00525.

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4. Tylek K., Trojan E., Leśkiewicz M., Ghafir El Idrissi I., Lacivita E., Leopoldo M., Basta-Kaim A. Microglia Depletion Attenuates the Pro-Resolving Activity of the Formyl Peptide Receptor 2 Agonist AMS21 Related to Inhibition of Inflammasome NLRP3 Signalling Pathway: A Study of Organotypic Hippocampal Cultures. Cells. 2023 Nov 3;12(21):2570. doi: 10.3390/cells12212570.

polegał na: zakładaniu hodowli organotypowych hipokampa, wykonaniu analiz biochemicznych, analizie otrzymanych wyników, wykonaniu wykresów oraz schematów, zbieraniu i opracowaniu literatury, odpowiedzi na pytania recenzentów.

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   polegał na: pomocy przy wykonywaniu analiz biochemicznych, przygotowywaniu części manuskryptu powyższej publikacji, przygotowywaniu literatury, odpowiedzi na pytania recenzentów.

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polegał na: pomocy przy zakładaniu hodowli organotypowych hipokampa, analizie części otrzymanych wyników, przygotowywaniu literatury, odpowiedzi na pytania recenzentów.

Wyrażam zgodę na wykorzystanie publikacji w postepowaniu doktorskim Pani Kingi Tylek oraz oświadczam, że wyniki nie zostaną ponownie wykorzystane w innych postępowaniach o nadanie stopnia doktora lub doktora habilitowanego.

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1. Tylek K., Trojan E., Leśkiewicz M., Regulska M., Bryniarska N., Curzytek K., Lacivita E., Leopoldo M., Basta-Kaim A. Time-Dependent Protective and Pro-Resolving Effects of FPR2 Agonists on Lipopolysaccharide-Exposed Microglia Cells Involve Inhibition of NF-κB and MAPKs Pathways. Cells. 2021 Sep 9;10(9):2373. doi: 10.3390/cells10092373.

   polegał na: pomocy przy przygotowywaniu hodowli pierwotych mikrogleju, planowaniu części eksperymentów, wykonywaniu oznaczeń biochemicznych, analizie otrzymanych wyników, pisaniu części manuskryptu powyższej publikacji.

Oświadczam, że mój udział w pracach:

2. Tylek K., Trojan E., Leśkiewicz M., Francavilla F., Lacivita E., Leopoldo M., Basta-Kaim A. Stimulation of Formyl Peptide Receptor-2 by the New Agonist CMC23 Protects against Endotoxin-Induced Neuroinflammatory Response: A Study in Organotypic Hippocampal Cultures. ACS Chem Neurosci. 2023 Oct 18;14(20):3869-3882. doi: 10.1021/acschemneuro.3c00525.

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   polegał na: pomocy przy wykonywaniu oznaczeń biochemicznych, stymulacji hodowli organotypowych hipokampa agonistami receptora FPR2, analizie otrzymanych wyników.

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1. Tylek K., Trojan E., Regulska M., Lacivita E., Leopoldo M., Basta-Kaim A. Formyl peptide receptor 2, as an important target for ligands triggering the inflammatory response regulation: a link to brain pathology. Pharmacol Rep. 2021 Aug;73(4):1004-1019. doi: 10.1007/s43440-021-00271-x.

   polegał na: przygotowywaniu części manuskryptu związanej z endogennymi agonistami receptora formylowego 2 oraz na przygotowywaniu odpowiedzi na pytania recenzentów.

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polegał na: wykonaniu izolacji mRNA z hodowli pierwotnych mikrogleju, przeprowadzeniu analizy ekspresji genów za pomocą metody qRT-PCR, analizie części otrzymanych wyników w manuskrypcie powyższej publikacji oraz odpowiedzi na pytania recenzentów dotyczących wykonanej części badań.

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polegał na: barwieniu immunocytochemicznym komórek pochodzących z hodowli pierwotnej mikrogleju, przygotowaniu oraz analizie zdjęć z mikroskopu konfokalnego, napisaniu podrozdziałów 2.10 oraz 2.11 w rozdziale „Materials and Methods" oraz podrozdziału 3.2 w rozdziale „Results" w manuskrypcie powyższej publikacji, odpowiedzi na pytania od recenzentów dotyczących zagadnień związanych z barwieniem immunocytochemicznym.

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November 6th 2023

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   consisted of answering the reviewers' questions related to chemical issues.

I declare, that my participation in the following publications:

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   consisted of the designing and synthesizing of formyl peptide receptor 2 agonists (MR-39, AMS21, CMC23), writing parts of manuscripts of the above publications related to chemistry and pharmacokinetic properties of FPR2 agonists, and answering the reviewers' questions related to chemical issues.

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consisted of synthesizing compound AMS21 [(S)-1-(3-(4-cyanophenyl)-1-(in-129 dolin-1-yl)-1-oxopropan-2-yl)-3-(4-fluorophenyl)urea].

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I hereby consent to the use of the publication in the doctoral proceedings of Ms. Kinga Tylek, and declare that the results will not be reused in other proceedings to confer a doctoral or post-doctoral degree.

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1. Tylek K., Trojan E., Regulska M., Lacivita E., Leopoldo M., Basta-Kaim A. (2021) Formyl peptide receptor 2, as an important target for ligands triggering the inflammatory response regulation: a link to brain pathology. Pharmacological Reports. 73(4):1004-1019.

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   polegał na opracowaniu koncepcji badań, pozyskaniu finansowania, koordynacji wykonywanych doświadczeń oraz nadzorze merytorycznym nad ich przebiegiem, pisaniu części powyższych manuskryptów, merytorycznej korekcie prac oraz przygotowaniu odpowiedzi na pytania recenzentów.

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## 12.Artykuły naukowe stanowiące podstawę rozprawy doktorskiej

**Formyl peptide receptor 2, as an important target for ligands triggering the inflammatory response regulation: a link to brain pathology.**

Tylek K., Trojan E., Regulska M., Lacivita E., Leopoldo M., Basta-Kaim A.

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SPECIAL ISSUE: REVIEW

# Formyl peptide receptor 2, as an important target for ligands triggering the inflammatory response regulation: a link to brain pathology

**Kinga Tylek[1] · Ewa Trojan[1] · Magdalena Regulska[1] · Enza Lacivita[2] · Marcello Leopoldo[2] · Agnieszka Basta-Kaim[1]**

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## Abstract

Formyl peptide receptors (FPRs) belong to the family of seven-transmembrane G protein-coupled receptors. Among them, FPR2 is a low affinity receptor for N-formyl peptides and is considered the most promiscuous member of FPRs. FPR2 is able to recognize a broad variety of endogenous or exogenous ligands, ranging from lipid to proteins and peptides, including non-formylated peptides. Due to this property FPR2 has the ability to modulate both pro- and anti-inflammatory response, depending on the nature of the bound agonist and on the different recognition sites of the receptor. Thus, FPR2 takes part not only in the proinflammatory response but also in the resolution of inflammation (RoI) processes. Recent data have indicated that the malfunction of RoI may be the background for some central nervous system (CNS) disorders. Therefore, much interest is focused on endogenous molecules called specialized pro-resolving mediators (SPMs), as well as on new synthetic FPR2 agonists, which kick-start the resolution of inflammation (RoI) and modulate its course. Here, we shed some light on the general characteristics of the FPR family in humans and in the experimental animals. Moreover, we present a guide to understanding the "double faced" action of FPR2 activation in the context of immune-related diseases of the CNS.

**Keywords** Formyl peptide receptors · Inflammation · Lipoxins · Resolvins · SPM's (small pro-resolving mediators) · Immune-related brain disorders

## Abbreviations

| | |
|---|---|
| Aβ | Amyloid β |
| AD | Alzheimer's disease |
| Akt | Protein kinase B |
| ANXA1 | Annexin A1 |
| AP-1 | Activator protein 1 |
| ASA-COX2 | Acetylated cyclooxygenase |
| ATL | Aspirin-triggered lipoxin |
| AT-LXA4, AT-LXB4 | Aspirin-triggered lipoxins |
| cAMP | Cyclic adenosine monophosphate |
| CNS | Central nervous system |
| COX-2 | Cyclooxygenase 2 |
| DHA | Docosahexaenoic acid |
| DAMP | Damage-associated molecular patterns |
| EPA | Eicosapentaenoic acid |
| ERK 1/2 | Extracellular signal-regulated kinases |
| fMLF | N-formyl-methionyl-leucylphenylalanine |
| FPR | Formyl peptide receptor |
| GPCR | G-protein-coupled receptors |
| GPR32 | G-protein-coupled receptor 32 |
| IFN-γ | Interferon-γ |
| IL | Interleukin |
| LOX | Lipoxygenase |
| LPS | Lipopolysaccharide |
| LXA4 | Lipoxin A4 |
| MAPK | Mitogen-activated protein kinase |
| MARCO | Macrophage scavenger receptor |
| NF-κB | Nuclear factor-κB |
| Nrf2 | Nuclear factor erythroid 2-related factor 2 |

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| | |
|---|---|
| P38MAPK | P38 mitogen-activated protein kinases |
| PAMP | Pathogen-associated molecular patterns |
| PPARγ | Peroxisome proliferator-activated receptor gamma |
| PI3K | Phosphoinositide 3-kinase |
| PKC | Protein kinase C |
| PLC | Phospholipase C |
| $PrP^c$ | Cellular prion protein |
| PRR | Pathogen recognition receptors |
| PUFA | Polyunsaturated fatty acid |
| RoI | Resolution of inflammation |
| ROS | Reactive oxygen species |
| RvD1 | Resolvin D1 |
| SAA | Serum amyloid A |
| SOCS | Cytokine signaling suppressors |
| SPMs | Specialized pro-resolving mediators |
| TLR4 | Toll-like receptor 4 |
| TNF-α | Tumor necrosis factor α |

## Introduction

Formyl peptide receptors (FPRs) belong to the largest and functionally diverse family of 7 transmembrane chemoattractant G-protein-coupled receptors. FPRs are classified as Pathogen Recognition Receptors (PRRs) located on immune cells that play a key role in innate immunity due to their ability to recognize both, pathogen associated and damage-associated molecular patterns (PAMPs and DAMPs). In fact, FPRs were first identified on myeloid cell membrane, but subsequently their expression was demonstrated on neuronal, glial, endothelial and epithelial cells [1]. FPRs participate not only in host defense and regulation of inflammatory response but also in the migration, proliferation, superoxide production and in several physio-pathological processes due to their unique binding properties and interaction with structurally diverse ligands [2]. Actually, FPRs can interact with a wide range of compounds belonging to different chemical structures, from various endogenous peptides and proteins to non-peptide host-derived lipids and eicosanoids, but also covers many small-molecule ligands [3, 4].

Among FPRs, the FPR2 receptors are an attractive therapeutic target for researchers due to the functionality related to biased agonism and the diversity of bound ligands. In this review, we shed some light on the general characteristics of FPRs in humans and in experimental animals. Moreover, we present some of the crucial FPR2 ligands which may open opportunities for research in the context of immune-related diseases of the central nervous system including Alzheimer's disease, depression and ischemia.

## Formyl peptide receptor family—an overview

The nomenclature of FPRs family is diverse, due to the fact that terminology of the same receptors was associated with the different manners of classification [5]. Therefore, to unify the terminology, the International Union of Basic and Clinical Pharmacology (IUPHAR) established a new lexicon based on the interaction of the receptor with the agonist. Based on these guidelines three members of FPRs family were identified in humans, namely FPR1, FPR2 and FPR3. On the other hand, despite the ordering of these nomenclature, the FPR2 and FPR3 receptors in the literature still often appear under other names, such as FPR1L and FPRL2 (these names refer to the common homology with other family members). Furthermore, the names ALX, FPR2/ALX, LXA4R are often used for the FPR2 receptor to refer to its interaction with the endogenous ligand A4 lipoxin (LXA4) [6, 7]. It is also worth to mention that in humans, at the beginning, the naming criterion for FPR1, FPR2 and FPR3 was based on the binding of formylated bacterial product formyl-methioninyle-leucyl-phenylalanine (*f*MLF), because the formyl receptor was first discovered as a target for this PAMP [8].

In humans all genes of the formyl receptor are located on chromosome 19. Moreover, they are characterized by high homology, e.g., the hFPR1 and hFPR2 receptors share sequence identity of 69%, hFPR1 and hFPR3 of 56%, while hFPR2 and hFPR3 about 83% (Table 1). Despite this sequence similarity, hFPR2 is more ubiquitous and was created as a result of gene amplification. According to the sequence analysis, hFPR3 is evolutionarily "the youngest" member of the FPR receptor family and seems to be

**Table 1** Formyl peptide receptors (FPRs) family names (IUPHAR-recommended and used previously)

| IUPHAR-recommended FPR names | Other names (used previously) | Homology with FPR1 | Homology with FPR2 |
|---|---|---|---|
| FPR1 | FPR, NFPR, FMLPR, FMLP | – | – |
| FPR2 | FPR2/ALX, FPRH1, FPRL1, ALXR, RFP, LXA4R, FMLPX, HM63, FPR2A | 69% | – |
| FPR3 | FPRL2, FMLPY, FPRH2 | 56% | 83% |

more related to hFPR2 than to hFPR1, suggesting that it arose from gene duplication [9, 10]. They are all expressed on monocytes; in addition, hFPR1 and hFPR2 are also expressed on neutrophils [11] and hFPR1 and hFPR3 on dendritic cells (DC) [12, 13]. Formyl receptors, especially hFPR2, also maintain a relatively strong expression on cells of the nervous system including astrocytes and microglia [14].

Importantly formyl receptors also share overlapping functions. Originally, these receptors were thought to be only involved in neutrophil chemotaxis, but later discoveries have begun to highlight other functions, including: calcium efflux, clearance of infection, recruitment of immune cells, pro-resolving properties, but also a role as a background in the multiple diseases. The wide range of functions caused by the diversity of endogenous FPR ligands are not limited only to N-peptides [4]. FPR1 was for the very first time isolated from HL-60 cells that were differentiated into granulocytes [15] and prefer to bind short and flexible structures, such as *f*MLF for which they have a strong affinity [16]. The chimeric receptor approach showed that the affinity of FPR1 for *f*MLF was 400 times higher than that of FPR2 [17, 18].

Nevertheless, FPR2 is the only member of the formyl receptor family that interacts with all types of ligands, i.e., lipids, peptides, and proteins preferring mainly long, amphipathic peptides with a helix structure [16, 19]. To date, the evolutionarily youngest FPR3 is the least known member of the FPR family. Interestingly, only one peptide ligand with a high affinity for FPR3 is known [10, 11]. Furthermore, FPR3 receptor is highly phosphorylated, indicating that it rapidly internalizes after binding its ligands and thus may serve as a "decoy" receptor to restrict the binding of available ligands to other receptors [20]. Recently, some data have indicated a role of FPR3 in promoting calcium mobilization or chemotaxis [10, 11] but it certainly requires further research.

## Formyl peptide receptor family—animal species distribution

The formyl receptor family has also become the focus of animal research. The presence of FPRs was found in guinea pigs, primates, rabbits, horses, rats, and mice, among others [5]. Considering that formyl receptors are present in a wide range of species, their structure, functionality, nomenclature, and homology with the human FPR family are very diverse. Currently, the most widely known formyl receptors in animals are those found in mice. The murine formyl receptor family includes 8 described formyl receptors: *mFpr1, mFpr2, mFpr-rs1, mFpr-rs3, mFpr-rs4, mFpr-rs6, mFpr-rs7, and mFpr-rs8* located on chromosome 17A3.2 [4] (Fig. 1). Scientific research has mainly targeted two direct orthologs between mouse and human with *mFpr1* and *mFpr2* represented by hFPR1 and hFPR2, respectively [21]. Although the human FPR family has murine orthologs whose high level of expression is also similar to that of humans on phagocytic leukocytes, the binding affinity for individual ligands is different. Literature data show a 100-fold lower affinity of *mFpr1* for *f*MLF and structural differences in the ligand binding domain. The mFpr1 receptor appears to be more similar to FPR2 in terms of its human ortholog [22, 23]. The structural differences between hFPR1 and *mFpr1* do not cover all aspects of functionality. Mice with the $mFpr1^{-/-}$ phenotype revealed its strong association with host defense regulation. The targeted deletion of genes encoding *mFpr1* but also *mFpr2* seems to confirm these results. Animals with that deletion show reduced resistance to bacterial infections; however, the fertility and viability of the animals are not affected [24, 25]. The *mFpr2* and *mFpr-rs1* receptors show high homology to human FPR2 and FPR3. Moreover,

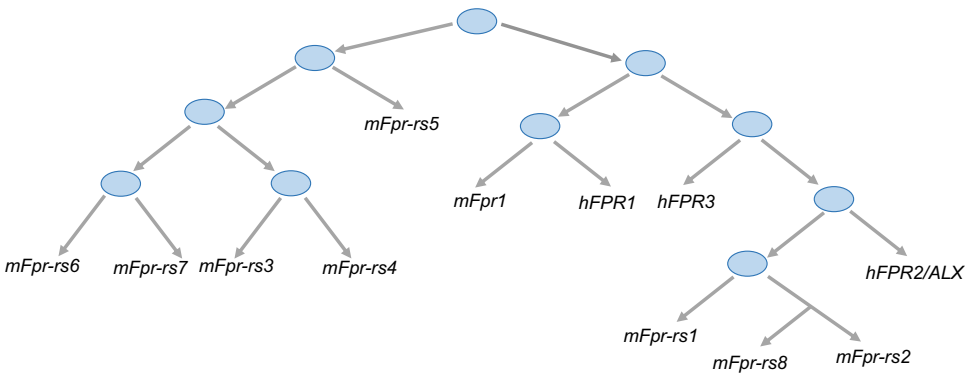


**Fig. 1** Homology between the human (h) and mouse (m) FPR family member genes. The "human group" contains three FPR proteins, the "mice group" includes eight FPR-related forms. *hFPR1* and *mFpr1* are in the same cluster, while *mFpr-rs1, mFpr-rs2* (called also *mFpr2*) and *mFpr-rs8* belong to the another cluster. They are closely related to hFPR2/ALX and hFPR3. Based on protein sequences, *mFpr-rs3, mFpr-rs4, mFpr-rs6, mFpr-rs7* and *mFpr-rs5* are closely related

the knockout study mice *mFpr2*$^{-/-}$ revealed the possibility of a functional crossover between hFPR3, hFPR2, and *mFpr2,* respectively [26, 27]. Studies based on these animals have also established that despite mFpr2 has a low affinity for *f*MLF, it binds with high affinity to several peptide agonists that activate human FPR2/ALX, including the amyloidogenic proteins serum amyloid A [28–31] and amyloid $\beta_{(1-42)}$. Mouse Fpr2 is also a receptor for F2L (which is also a strong agonist of hFPR3). These findings indicate that mouse Fpr2 share pharmacological properties with human FPR2/ALX. It is very important in the context of the result of studies conducted in FPRs-deficient mice, indicating their translational potential.

The remaining members of the formyl receptor family do not seem to exhibit as many distinctive features. It may be related to the complex evolution of genes and sequence divergence between orthologs. Among the genes encoding *mFpr,* there is the pseudogene *mFpr-rs5 (ψmFpr-rs3)* which does not encode a functional receptor, but it does not possess the features characteristic of pseudogene [4, 21]. *mFpr-rs1, mFpr-rs3, mFpr-rs4, mFpr-rs6, and mFpr-rs7* represent chemosensory vomeronasal GPCR receptors [32, 33]. The biological function of *mFpr-rs1* is still unclear. Although *mFpr-rs1* overlaps many functions and structural features with hFPR2 its ability to activate the human and mice ligands is very low. The underlined data demonstrate the commonality of many structural and pharmacological features of both human and mice members of the FPR family.

## Conformational changes and biased agonism of FPRs

According to the literature, the FPR family is a group of G-protein-coupled receptors and belongs to one of the most diverse groups of receptors, namely: 7 transmembrane receptors (7 TM) [34, 35]. In general, the FPR family receptors consists of a few conservative elements: the extracellular N-terminus, seven transmembrane domains (TM1–7) connected via three intracellular and extracellular loops (IL1–3, EL1–3) and the intracellular C-terminus. Furthermore, in some receptors, there is an extra eighth helix in the polypeptide chain that is parallel to the inner surface of the cell membrane [36, 37]. The extracellular domains (EL1–3) are responsible for the detection of ligands and their access to the structural core, while intracellular domains (IL1–3) bind to a variety of cytoplasmatic systems, such as G proteins, arrestin or receptor kinases coupled with G proteins [38]. Transmembrane TM1–7 helixes participate in binding and signal transmission into the cell through conformational changes that are essential for receptor activity [39]. Two highly conserved motifs are directly involved in the conformational changes: NPXXY in TM7, which is responsible for activating the receptor and E/DRY (combining TM3 and TM6), which acts as a specific "ion blocker" that maintains the stabilization of the receptor conformation [40, 41].

It is intriguing to observe that among formyl peptide receptors, FPR2 have properties to functional changes, which depend on this receptor conformation. Emerging data suggest that FPRs form higher order structures (e.g., FPR1/FPR2 heterodimers, FPR2 homodimers, FPR1 homodimers), which leads to altering the downstream intracellular signaling pathways by allowing colocalization of effector domains, enhancing intracellular activation, or creating new ligand specificity [42, 43]. Cooray et al. have indicated that FPR2 homodimers and FPR2–FPR1 heterodimers occur constitutively in leukocytes and alters the activation of signaling pathways in response to specific ligands [44]. Peptide ligands also play a role in dimerization: annexin A1 (ANXA1) and LXA4 promote FPR2 homodimerization, while peptide Ac2–26 stimulates FPR2–FPR1 heterodimerization. Interestingly, FPRs also form oligomers with scavenger MARCO receptors (macrophage receptor with collagenous structure). Interactions between FPR and MARCO receptors have been demonstrated by bioluminescence and co-immunoprecipitation studies and fulfill their functions in agonist-evoked changes in cyclic adenosine monophosphate (cAMP) levels and extracellular signal-regulated kinases (ERK1/2) phosphorylation, as well as signal transduction in glial cells via Aβ1–42 [45]. Importantly, the FPR2 conformational changes (ligand-dependent) determines its action [46].

On the other hand, protein and lipid ligands bind to different FPR2 binding sites (Fig. 2). Lipoxins A4 (LXA4) bind at 7TM and 3rd extracellular loop, while peptide ligands, such as ANXA1 or serum amyloid A (SAA), bind at the NH2-terminal domain or 1st two extracellular loops [47, 48].

After binding of the ligand, FPR2 is activated and triggers several agonist-dependent signal transduction pathways through the involvement of the Gαi1, Gαi2 and Gαi3 G-receptor subunits [5]. In fact, the effects observed after FPR2 activation include the activation of phospholipase A2 (PLA2), phospholipase C (PLC) isoforms, protein kinase C (PKC), phosphoinositide 3-kinase (PI3K), protein kinase B (Akt), mitogen-activated protein kinase (MAPK) pathway as well as p38MAPK, which modulate proliferation, differentiation, apoptosis, cellular communication and other intracellular functions. Furthermore, phosphorylation of cytosolic tyrosine kinases, phosphorylation and nuclear translocation of regulatory transcription factors, calcium release and oxidant production so far were demonstrated [49]. Among the post-translational modifications of FPR2, phosphorylation processes, which are determined by a balance between protein kinases and protein phosphatases, seem to be of great importance [1]. Therefore, despite the fact that protein phosphorylation is limited to specific phospho-sites, and it is not the only post-translational change (which also

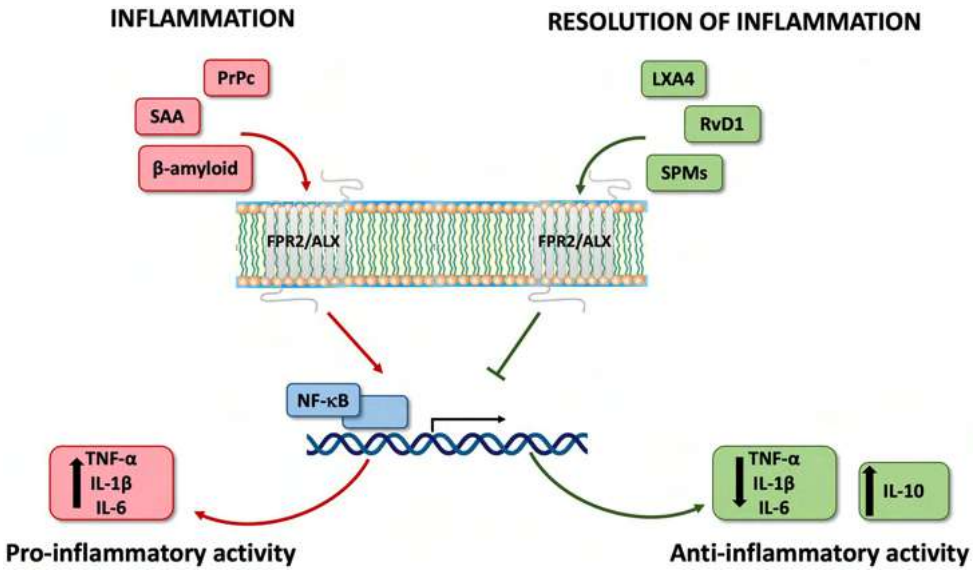


**Fig. 2** Ligand-biased signaling via FPR2 leads to dual effects: pro-inflammatory on the one hand and pro-resolving on the other. A variety of endogenous ligands exert pro-inflammatory (*SAA* Serum Amyloid A, *PrPc* Prion Protein, *ANXA1* Annexin A1) and pro-resolving (*LXA4* Lipoxin A4, *RvD1* Resolvin D1, *SPMs* Specialized Pro-resolving Mediators) effects. SAA, PrPc, amyloid-β elicit proinflammatory signals and stimulate the release of pro-inflammatory cytokines (e.g., TNF-α, IL-1β, IL-6). This FPR2 signaling is counteracted by pro-resolving agonists that suppress the expression of pro-inflammatory cytokines and increase the release of anti-inflammatory factors (e.g., IL-10). (Image generated by Biorender)

include methylation, acetylation, sumoylation or ubiquitination) occurring after FPR activation by agonists, understanding the mechanisms of their regulation may be crucial for the development of new pharmacotherapy of CNS diseases. Ligand-dependent stimulation of G-protein-coupled receptors may also lead to transactivation process, which activates signaling from receptors tyrosine kinase (RTK) [50]. Among others, TrKA receptor activation results in phosphorylation of various tyrosine residues (e.g., Y490, Y751 or Y785). Phosphorylated tyrosine residues form docking sites for other proteins and trigger the activation of Ras/MAPK, PI3K/Akt as well as PLC γ/PKC pathways [51]. Interestingly, several features of TrkA receptor transactivation are noteworthy and differ significantly from other transactivation events, first of all, because it is slower. However, given the role of the mentioned signaling cascades in the physiological and pathological processes in the brain and in the action of CNS-active drugs, the TrkA transactivation by FPRs agonists may provide an innovative strategy for the treatment schizophrenia, depression and other mental illnesses.

At the same time, it should be strongly emphasized that the FPR2 downstream signaling pathway activation, depends not only on the chemical structure of the ligand but also on the cell type involved [14, 52], which is important in understanding how FPR2 activation elicits different cellular responses leading to inflammation and its resolution. For example, SAA binding increases the expression of the NF-κB, whereas LXA4 suppresses NF-κB activity [46]. Therefore, FPR2 enables the switch of both pro-inflammatory action to pro-resolving because of the diversity of intracellular signaling cascades from GPCR activation.

In 2019, Raabe et al. discussed the biased perspectives on FPRs. According to those authors, the "classic" view about ligand/receptor interactions accounts only for agonists (which leads to activation) and antagonists (inhibit the activation) [53, 54]. FPR2–ligand interactions lead to totally different cellular responses, a finding, which completely questions this classical concept of receptor–ligand interaction. This phenomenon—biased agonism—explains how different FPR2 agonists do not lead to the same effects and why FPR2 agonists play essential roles in the control of active inflammation resolution and host defense. What is more, FPR2 is unusual, because it can switch from a pro-inflammatory to anti-inflammatory response while at the same time maintaining the former at a low but possibly life-saving level [55].

## Inflammatory response and FPR2 ligands

The inflammatory response is one of the main process in the organism. Among the processes of inflammatory origin, acute inflammation is a protective, self-limiting process that disappears after the removal of the insult in the absence of major damage to the body. Several phases of inflammation, including initiation, propagation, and resolution, have

been demonstrated. Recently it has been suggested that these phases do not develop sequentially but rather overlap [56]. The physiological outcome of the acute inflammatory response is the restoration of tissue homeostasis and functionality, culminating in tissue repair, and is followed by the resolution phase [57, 58]. However, when the mechanisms controlling this complex reaction, triggered by several factors including proteins, lipids and stimulatory signals derived from injured cells or by inflammatory mediators (e.g., chemokines, cytokines) fail, an uncontrolled inflammatory reaction can be detrimental, which indeed is a driving pathogenetic mechanism for a wide range of immune-related diseases.

There is a lot of evidence that in the course of the prolonged inflammatory response and neurodegeneration, non-formyl peptide FPRs agonists are involved. This group of ligands activates FPRs independently from the presence of an *N*-formyl group, showing a particular preference for the interaction and activation of FPR2.

Undoubtedly, the serum acute-phase protein (SAA) is of particular importance among them because of an unfavorable role in chronic inflammation and amyloidosis. The pro-inflammatory effects of SAA caused by stimulation of FPR2 in phagocytes, epithelial cells and T lymphocytes, lead to the production of inflammatory mediators [43, 59–61]. Interestingly, some data postulated that native SAA may exhibit cytokine-like properties but whether this effect is related to FPR2 activation still remains unclear and is a subject of scientific debate [62, 63].

Among the amyloidogenic agonists of FPR2 the cellular prion protein fragment ($PrP^c$), a glycoprotein highly expressed in the brain is highlighted. The role of $PrP^C$ in amyloid β (Aβ) oligomer-induced synaptic impairment is of great interest [64]. In fact, impairment of LTP by Aβ oligomers isolated from the brains of AD patients was attenuated by pretreatment with an anti-$PrP^C$ antibody [65, 66]. Moreover, some data pointed out the role of $PrP^C$ in synaptotoxicity mediated by soluble Aβ. On the other hand, in some studies the effects of $PrP^C$ in the LTP alterations and memory deficits in mouse models of AD were not seen [67, 68]. $PrP^C$ fragment, through its interaction with FPR2 in glial cells, induces calcium mobilization, enhances chemotaxis (e.g., via MCP-1) and leads to potentiation of the inflammatory response. Among the cytokines released from glia cells in response to $PrP^C$, there are: TNF-α, IL-1β, IFN-γ, or IL-6, which reportedly accelerate AD progression in both AD patients and in the animal model of AD [69, 70].

In addition to SAA and $PrP^c$, two other amyloidogenic peptides have also been described: 42-amino acid form of Aβ amyloid peptide (Aβ42) and humanin, which exert an agonistic effect on FPR2. Despite the fact that both peptides, by activating FPR2, induce migration and increase the phagocytic activity of monocytes in the brain, they have a different role in the course of Alzheimer's disease. Aβ42 is involved in the fibrillary tangle formation and deposition in the brain of AD patients [71, 72]. Moreover, via interaction of microglial cells with FPR2 Aβ42 increase the inflammatory cytokines production, including TNF-α, interleukins (IL-1β, IL-6), interferon-γ, and chemokines, such as CCL2, CXCL8, CXCL10 and CCL3 [73, 74].

In contrast, the already mentioned humanin has the opposite, i.e., neuroprotective activity [75]. In fact, humanin, by inhibiting Aβ interaction with FPR2 in phagocytes, probably reduces aggregation and generation of fibrillary formations. Perhaps also the ability of humanin to interact with other FPRs, e.g., FPR3 [75, 76] play a crucial role in these phenomena.

The "dual-faced" FPR2 agonists include annexin A1 (ANXA1) and its bioactive N-terminus domains (Ac2–26 and Ac9–25). ANXA1 is a glucocorticoid-regulated phospholipid-binding protein of 37 kDa, expressed in a variety of cell types. It seems that the dual properties manifested by ANXA1 are mediated by peptides derived from its N-terminus domain (Ac2–26 and Ac9–25), which are presumably generated at sites of inflammation. Interestingly, at high concentration the ANXA1 peptides fully activate FPR1, just as the conventional agonists and induce pro-inflammatory response. On the other hand, at low concentrations they only demonstrate a partial activity at FPR1, leading to the inhibition of adhesion and transmigration of leukocytes, reducing the intensity and duration of the inflammatory response while intensifying proliferation and invasion of epithelial cells [77]. Moreover, it is suggested that both (Ac2–26 and Ac9–25) peptides use FPR2 for their anti-inflammatory actions [78], but there are also data postulating that other receptors, including FPR3, are involved in these pro-resolving effects [79].

Moreover, the role of ANXA1 in the behavioral disturbances, such as anxiety is widely discussed. In fact, the absence of ANXA1 protein even more than the absence of its main receptor (namely FPR2/3) is indispensable to the suppressive action of glucocorticoids on the HPA axis, as well as to the hippocampal homeostasis by preventing neuronal damage in the course of depression [80]. On the other hand, in FPR2/3-deficient mice data showed a behavioral disinhibition and reduced anxiety [81], manifested by the increased climbing exploratory activity in an open-field test, as well as superior performance on a novel object recognition test, just to mention a few. These effects were accompanied by an increase in blood plasma corticosterone, which does not exclude the possibility of a compensatory effect and/or changes in ANXA1 level. This issue undoubtedly requires further detailed studies. Nonetheless, the crucial role of FPR2 receptors in mediating the behavioral deficits at the cognitive–emotional interface are clearly confirmed by the Boc-2 administration to wild-type mice, which followed

the deficits observed in the above-mentioned FPR2/3-deficient mice [81].

Recently, data have demonstrated that in the brain, ANXA1 is engaged in the regulation of the blood–brain barrier (BBB) integrity of patients with multiple sclerosis [82]. Furthermore, ANXA1 may be involved in the occurrence and progression of acute severe traumatic brain injury [83]. Moreover, Wang et al. found that the expression of ANXA1 decreased after cerebral hemorrhage, and the increase in the expression of ANXA1 could alleviate neuronal necrosis, and reduce brain edema after cerebral hemorrhage [84]. Interestingly, Luo et al. found that ANXA1 could also exert neuroprotective effects on brain damage by polarizing microglia cells into M2 phenotypes [85].

ANXA1 was reported to also be associated with the early stage of AD in patients and in animal models. By inhibiting the secretion of inflammatory mediators stimulated by Aβ, ANXA1 could stimulate microglial phagocytosis of Aβ and reduce the level of Aβ [86]. In fact, some data show that ANXA1 expression is reduced in AD patients, which may be related to an increased degree of neurodegeneration. The decreased expression of ANXA1 in patients with mild cognitive impairment and AD might contribute to the increased neuroinflammation and cognitive deficits [87].

## FPR2 agonists in the course of the resolution of inflammation

The correct flow of the resolution of inflammation (RoI), which is an active process, requires proper endogenous activation that induces a switch from the release of proinflammatory molecules to the secretion of pro-resolving mediators. In this event, the so-called specialized pro-resolving lipid mediators (SPMs) play a prominent role, because they modulate leukocyte infiltration and activities, as well as anti-inflammatory cytokine release to terminate inflammation [88]. These molecules, including lipoxin A4 (LXA4), derived from arachidonic acid (AA), and the D-series resolvins (RvD1) derived from docosahexaenoic acid (DHA) are key paracrine and autocrine biochemical signaling molecules in the CNS. They are reported to be involved not only in the RoI by triggering the processes that reduce the expression of pro-inflammatory response, but also, in the case of RoI deficits, in the progression of neurodegenerative and neuropsychiatric diseases [89, 90]. In fact, SPMs activate cascades that induce remodeling within sites damaged by inflammatory processes. Most of the effects of RoI are mediated through FPR2, which is able to promote several processes crucial for resolution of inflammation, including neutrophil extravasation blockade, promotion non-phlogistic monocyte recruitment, suppression of proinflammatory mediators while potentiating anti-inflammatory cytokines release and macrophage phagocytosis and efferocytosis, altering macrophages phenotype and instructing cells to favor repair [91, 92]. Interestingly, the anti-inflammatory effects rely mostly on suppressive action, while pro-resolving effects are mediated by the activation of specific inherent processes; however, the RoI is the final result of both [91, 93]. Recently, it has been found that SPMs elicit "mild to moderate effects", which, led to the balance between proinflammatory and anti-inflammatory reactions [91]. It should be mentioned, that in the brain the course of inflammatory response is slightly different due to the collective interaction of various brain cells (microglia, astrocytes, oligodendrocytes, and NG2 glia) and, in some cases, peripheral immune cells. Therefore, a great deal of importance is given to SPMs which can act on both glia and neurons [93] and they include lipoxins and resolvins.

### Lipoxins

Lipoxins have emerged as prominent chemical mediators whose synthesis is switched on during an inflammatory response, which allows the RoI. In classical lipoxin biosynthesis in leukocytes and epithelial cells, arachidonic acid undergoes double, transcellular oxidation catalyzed by lipoxygenases (LOX), resulting in the formation of two derivatives of lipoxin A (LXA4) and lipoxin B (LXB4) [93]. On the other hand, in the second pathway of lipoxin synthesis, aspirin-dependent lipoxin epimers: AT-LXA4 and AT-LXB4 are formed under the influence of acetylated cyclooxygenase (ASA-COX2). Lipoxin A4 (LXA4) and its AT-LXA4 epimer act primarily through the FPR2 receptor [94]. In addition, LXA4 can activate other receptors, such as an orphan G-protein-coupled receptor (GPR32), aryl hydrocarbon receptor, estrogen receptor and high affinity cysteinyl leukotriene receptor [95–97].

Binding of LXA4 to FPR2 receptor results in the activation of many intracellular signaling pathways. Simultaneously, the conformational changes following the attachment of LXA4 prevents binding of other ligands, e.g., amyloid β or SAA to the FPR2 [19]. Among signaling cascades, the cell-dependent activation of the PI3K/AKT pathway by LXA4 is of key interest [98]. LXA4-mediated modulation of the neutrophil recruitment to the site of inflammation by increasing cytosolic calcium levels is important in the resolution of inflammation [99]. Moreover, LXA4 anti-inflammatory effect is also associated with the inhibition of the NF-κB (nuclear factor-κB), which in turn, leads to a reduction in the transcription of pro-inflammatory cytokines. Simultaneously, LXA4 increases the level of mRNA for cytokine signaling suppressors (SOCS). On the other hand, LXA4, by inhibiting the activation of transcription factors including NFκB and AP-1 (Activator protein 1) [100], up-regulates the levels of nuclear factor erythroid 2-related factor 2 (Nrf2)

and peroxisome proliferator-activated receptor gamma (PPARγ), which are the factors which suppress the expression of pro-inflammatory genes [98] (Fig. 3). Resolving the inflammation and restoring LXA4 signaling has been shown to reduce the severity of Alzheimer's disease such as neuropathology including the decrease in amyloid plaques, tau phospohorylation and inflammation as well as leading to the improvement in the cognitive performance in the 3xTg-AD mouse model [101]. Moreover, the combined administration of LXA4 and resolving E1 terminated inflammation in a murine model of AD [102]. The mechanism of LXA4 and AT-LXA4 has not been defined unequivocally, nevertheless it is postulated that both agonists reduce the secretion of pro-inflammatory mediators, such as TNFα, while LXA4 has also been shown to promote the release of anti-inflammatory factors and to exhibit the ability to reduce Aβ and phosphorylated tau levels [89].

## Resolvins

Resolvins are the second important class of FPR2 agonists that play an important role in the positive regulation of inflammatory processes. They are a group of compounds, derivatives of docosahexaenoic acid (DHA)—resolvin D and eicosapentaenoic acid (EPA)—resolvin E. The formation of resolvins is the result of the process taking place at the final stage of acute inflammation as a result of the interaction of cells, i.e., neutrophils, macrophages, platelets or endothelial cells (transcellular biosynthesis). The synthesis of D-series resolvins from docosahexaenoic acid (DHA) is catalyzed by lipoxygenase (15-LOX) or acetylated aspirin cyclooxygenase-2 (COX-2). The initially formed 17R-hydroperoxydocosahexaenoic acid (17R-HDHA) is transformed by epoxidation and with the participation of 5-LOX into D resolvins 1 to 4, which differ in the stereochemical asymmetry of the carbon chain. In parallel, the transformation of DHA under the influence of ASA-COX-2 leads to the formation of AT-RvD1 to 4 [103]. The synthesis of E-series resolvins occurs by conversion of eicosapentaenoic acid (EPA) catalyzed by ASA-COX-2 and 5-LOX leads to resolvin E1 (RvE1) and resolvin E2 (RvE2) formation.

RvD1 interacts with the GPCR-32 receptor as a potent agonist to signal for pro-resolving responses but can also directly activate FPR2 with a high affinity [77]. Numerous studies have shown that resolvins inhibit the migration of inflammatory cells, stimulate macrophages to phagocytosis of apoptotic neutrophils, inhibit NF-κB activation and secretion of proinflammatory cytokines, thereby contributing to

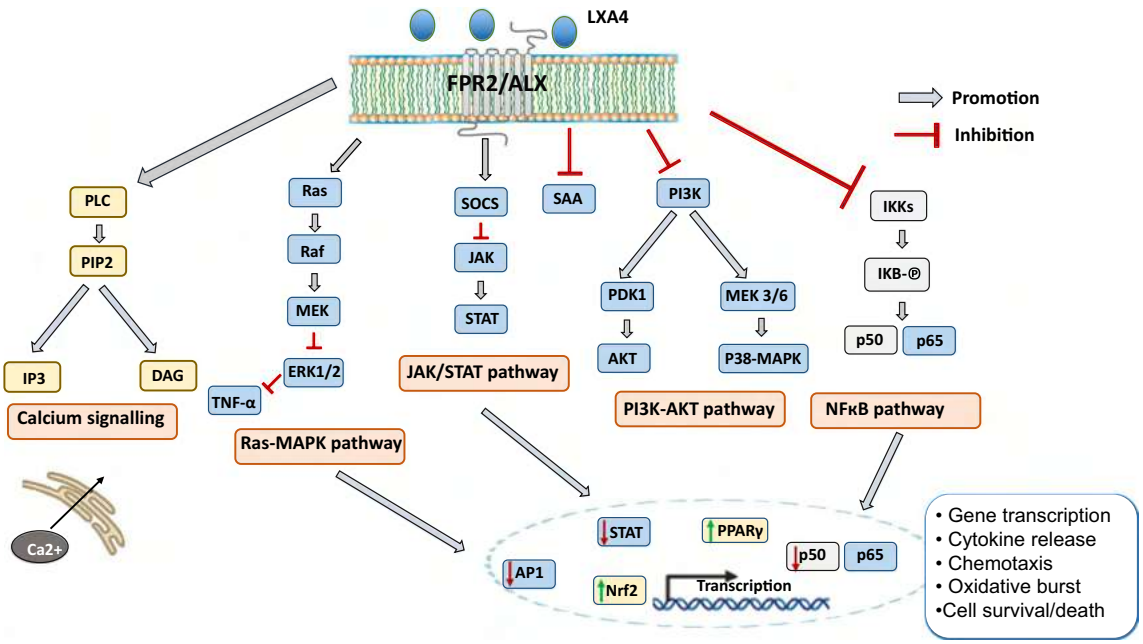


**Fig. 3** Binding of LXA4 to FPR2 receptor results in the activation of many intracellular signaling pathways. Depending on the cell type, LXA4 have different effects on PI3K/AKT signaling pathway. In macrophages, lipoxins have an anti-inflammatory effect through the activation of PI3K/AKT pathways which leads to an increase in their life span. By increasing cytosolic calcium levels, LXA4 is involved in the recruitment of neutrophils at the site of inflammation. LXA4 controls the synthesis of pro-inflammatory cytokines by inhibiting the activation of the NF-κB and by increasing the SOCS mRNA level. By inhibiting the activation of transcription factors including NFkB and AP-1, LXA4 up-regulates the levels of Nrf2 and PPARγ—factors which suppress the expression of pro-inflammatory genes. *LXA4* Lipoxin A4, *NF-κB* nuclear factor-κB, *SOCS* cytokine signaling suppressors, *AP-1* activator protein 1, *Nrf-2* nuclear factor erythroid 2-related factor 2, *PPARγ* peroxisome proliferator-activated receptor gamma

the suppression of inflammatory processes [104]. Moreover, RvD1 can promote cell survival by calcium release, Erk1/2 and PI3K/Akt signaling activation or blocking the TNF-α signaling as well as caspase-3 activity. Furthermore, RvD1 could promote bcl-xL expression, Interaction with FPR2 negatively regulates downstream IRAK1/TRAF6/NF-κB or MAPKs signaling pathways [80, 105]. All of the above data indicate that RvD1 may modulate microglial pro-inflammatory polarization and may play an important role in the resolution of inflammation (Fig. 4).

In line, in PC12 cell cultures the beneficial impact of RvD1 on the IL-4 induced expression of alternative microglia stimulation markers was observed. This anti-inflammatory and pro-resolving effects of RvD1 was related to the activation of STAT6 and PPAR-γ signaling pathways [106]. In addition, it was found that an increase in the production of D1 resolvin may be one of the mechanisms protecting the cells against ischemic injury, resulting in the protective effect on CA1 neurons of the hippocampus and cognitive functions. This action of RvD1 is probably also related to its modulatory impact on the PPAR-γ pathway [85].

Some data postulate a possible therapeutic potential of RvD1 in Parkinson's disease. It is based on the observation from the PC12 cultures, where RvD1 dose-dependently inhibited MPP + induced upregulation of cell apoptosis and cellular damage evoked by TNF-α and IL-6 production via suppression and ERK and p-38 pathways [107]. In addition, in an in vivo model of Parkinson's disease in rats induced by 30-day LPS administration, the combined treatment with RvD1 and RvD2 prevented the development of behavioral deficits and the activation of the TLR4/NF-κB pathway [108].

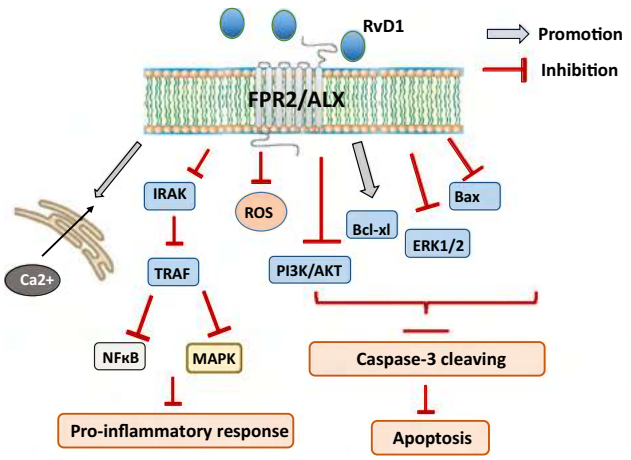


**Fig. 4** After binding to FPR2, RvD1 can promote cell survival by calcium release, Erk1/2 and PI3K/Akt signaling activation or blocking the TNF-α signaling as well as caspase-3 activity. RvD1 could also promote bcl-xL expression, leading to cell survival. Interaction with FPR2 negatively regulates downstream IRAK1/TRAF6/NF-κB or MAPKs signaling pathways. *RvD1* Resolvin D1, *NF-κB* nuclear factor-κB, *TNF-α* tumor necrosis factor α

Nevertheless, much more data points to the antidepressant potential of RvD1 in many experimental models. For instance, it was described, that in animal models of depression, some resolvins counteracted the depressive-like behavior. In fact, intraventricular administration of RvD1 or RvD2 attenuated the LPS-induced depression-like behaviors in the tail suspension test (TST) and forced swim test (FST) in murine chronic unpredictable stress (CUS) model, which may indicate an antidepressant effect of RvD1 and RvD2 [109]. Also, in studies using the murine model of depression, RvD1 has been shown to have an antidepressant effect, strongly dependent on the activation of FPR2 and in consequence, on MAP/ERK, PI3K/Akt but also AMPA signaling [110]. Importantly, in the mouse model of fibromyalgia-associated depression, intravenous RvD1 and RvD2 administration increased dopamine and glutamine cortical levels and limited the deficiencies of serotonin, suggesting the positive effect on neurotransmitter imbalance in depression [111]. Simultaneously some clinical studies suggest that RvD1 may be an attractive marker in manic, depressive and euthymic states of bipolar disorders. In fact, the levels of RvD1 were enhanced in manic and depressive states in comparison with the appropriate control groups [112]. Since RvD1 level correlated with an increase in the c-reactive protein, it is possible that RvD1 concentration should also be indicative of the presence of a subclinical inflammation, especially in the course of acute episodes to compensate for the inflammatory response. The usefulness of RvD1 as an indicator of the anti-inflammatory process has been confirmed by a positive correlation between RvD1 and neutrophil count. Thus, the assessment of RvD1 may be a new potential marker in studies of psychiatric disorders associated with inflammatory processes.

Various reports postulate that also RvD1 of the AT-RvD1 series, which was formed as a result of the action of ASA-COX2, exerts anti-inflammatory and pro-resolving effects and is many times more stable that LXA4 and RvD1. In fact, the data from in vitro and in vivo studies show that the peripheral administration of AT-RvD1 prevented astrogliosis and improved short- and long-term potentiation (LTP) enhancement of the hippocampus in mice [113]. Furthermore, improvement in the sensorimotor function and memory after traumatic brain injury (TBI) in mice leads to the conclusion that the reduction of long-term inflammation limits the decline in neurological function [114]. Simultaneously, beneficial responses were observed after intravenous administration of AT-RvD1 expressed as increased levels of cortical dopamine and glutamate and reduced depletion of serotonin in a mouse model of depression associated with fibromyalgia, which suggests that AT-RvD1 activity normalizes neurotransmitters levels in depression [111].

## Synthetic FPR2 agonists

Lipoxins and resolvins exert strong endogenous anti-inflammatory effects but their chemical and metabolic liability [115] greatly hamper their development as potential pro-resolving drugs. In fact, LXA4 is subject to metabolism by prostaglandin dehydrogenase at $C_{15}$ and ω-oxidation at $C_{20}$. Therefore, there has been and still, there is a great interest to develop lipoxin analogs less susceptible to metabolic deactivation with a longer biological half-life [116, 117].

The first generation of lipoxin analogs was designed to enhance biostability at $C_{15}$ and the ω-end. For example, compound **1** (Fig. 5) was able to inhibit the transmigration of human neutrophils at a dose range comparable to LXA4 [22]. However, the therapeutic potential of these analogs was limited due to rapid in vivo clearance after oral or intravenous administration.

The second generation of lipoxin mimetics featured a benzene ring to replace the triene system of LXA4 (the so-called benzo-LXA4), exemplified by compound **2** (Fig. 5), which demonstrated potent potential therapeutic in several models of peripheral inflammation [44, 45].

The high lipophilicity of the second generation of lipoxin mimetics led to the development of the less lipophilic third generation in which the benzene ring was replaced with heteroaromatic rings (imidazole, oxazole). These compounds, exemplified by compound **3** (Fig. 5), showed in vitro anti-inflammatory activity being able to attenuate LPS-induced NF-κB activity with a potency similar to LXA4 [46, 47] and reduced the inflammatory process in vivo in a model of zymosan-induced peritonitis. None of the lipoxin mimetics has been tested in animal models of neurodegenerative diseases and, thus, there are data about their ability to cross the blood–brain barrier and to accumulate into the brain.

Besides lipoxin mimetics, several small-molecule FPR2 agonists with promising therapeutic potential have been developed from both pharmaceutical companies and academia. The FPR2 agonist BML-111 (Fig. 5) is able to reduce inflammation and neutrophil infiltration and to potentiate the release of anti-inflammatory factors (e.g., IL-4, IL-10) in various inflammatory-based disorders [106, 116, 118–120]. A recent study demonstrated the efficacy of BML-111 in the cerebral ischemia–reperfusion injury in rats (Fig. 2) [94]. In the ischemic brain treatment LXA(4)ME suppressed neutrophils infiltration and lipid peroxidation levels; inhibited the activation of microglia and astrocytes, reduced the expression of pro-inflammatory cytokines (e.g., TNF-α and IL-1β), while up-regulated the expression of anti-inflammatory cytokines (e.g., IL-10 and TGF-β1). Interestingly, the activation of NF-κB was also inhibited by LXA(4)ME, which suggested that LXA(4)ME afforded a strong neuroprotective effect against cerebral ischemia–reperfusion injury, and that these effects might be associated with its anti-inflammatory property [121].

Among the small-molecule FPR2 agonists, the quinazolinone derivative Quin-C1 (Fig. 5) is a potent agonist as it induces FPR2-mediated intracellular $Ca^{2+}$ mobilization in the nanomolar range. Quin-C1 showed anti-inflammatory properties in a mouse model of bleomycin-induced lung injury being able to decrease the expression of IL-1β and TNF-α [122]. Another small-molecule FPR2 agonist, which is also an FPR1 agonist, is the chloropyrazolone derivative "Compound 43" (Fig. 5). This compound is able to mobilize intracellular $Ca^{2+}$ and inhibit PMN migration stimulated by IL-8 and *f*MLF [123]. In a recent study, the intracellular signalling pathways activated by Compound 43 and by the pyridazin-3(2*H*)-one FPR2 agonist known as "compound 17b" have been comparatively studied evidencing biased-agonist properties for the two compounds. In CHO cell overexpressing FPR2 and in primary cardiomyocites "compound 17b" showed a marked biased effect as it induced ERK1/2 and Akt1/2/3 phosphorylation along with 30-fold bias away from intracellular $Ca^{2+}$ mobilization relative to "compound 43". In addition, "compound 17b" reduced necrosis in isolated cardiomyocytes and inhibited the release of pro-inflammatory IL-1β after stimulation with TGF-β [124].

The pyrrolidinone FPR2 agonist BMS-986235 (Fig. 5), recently disclosed by Bristol-Meyer Squibb, shows high potency and selectivity for FPR2 and is able to inhibit neutrophil chemotaxis and stimulate macrophage phagocytosis in cellular assays. BMS-986235 is also able to improve cardiac function in a mouse model of heart failure [125].

We have contributed to the field of FPR2 agonists by developing a series of ureidopropanamide-based agonists [126, 127] that has its origin from the gastrin-releasing peptide receptor antagonist PD-175266 and the neuromedin B receptor antagonist PD-168368, both potent FPR1/FPR2 agonists. A medicinal chemistry campaign led to the identification of the selective FPR2 agonist MR39 (Fig. 5) [126] that shows favorable pharmacokinetic properties. In fact, MR39 is stable to oxidative metabolism in rat liver microsomes ($t_{1/2}$ = 48 min) and shows good passive permeability through an hCMEC/D3 cells monolayer, an in vitro model of the blood–brain barrier. MR39 demonstrated protective and anti-inflammatory properties as it lowered IL-1β and TNF-α levels in LPS-stimulated primary rat microglia cell cultures [126]. Moreover, MR39 and related analogs exerted neuroprotective effects in LPS-stimulated rat primary microglial cells at dose ranges comparable to LXA4 but lasting longer (unpublished data). MR39 provided promising results also in relation to the shift to the alternative microglia activation and the synthesis of anti-inflammatory cytokines. Thus, MR39 and its analogs are prospective tools to study the therapeutic potential of FPR2 agonists in the pharmacotherapy

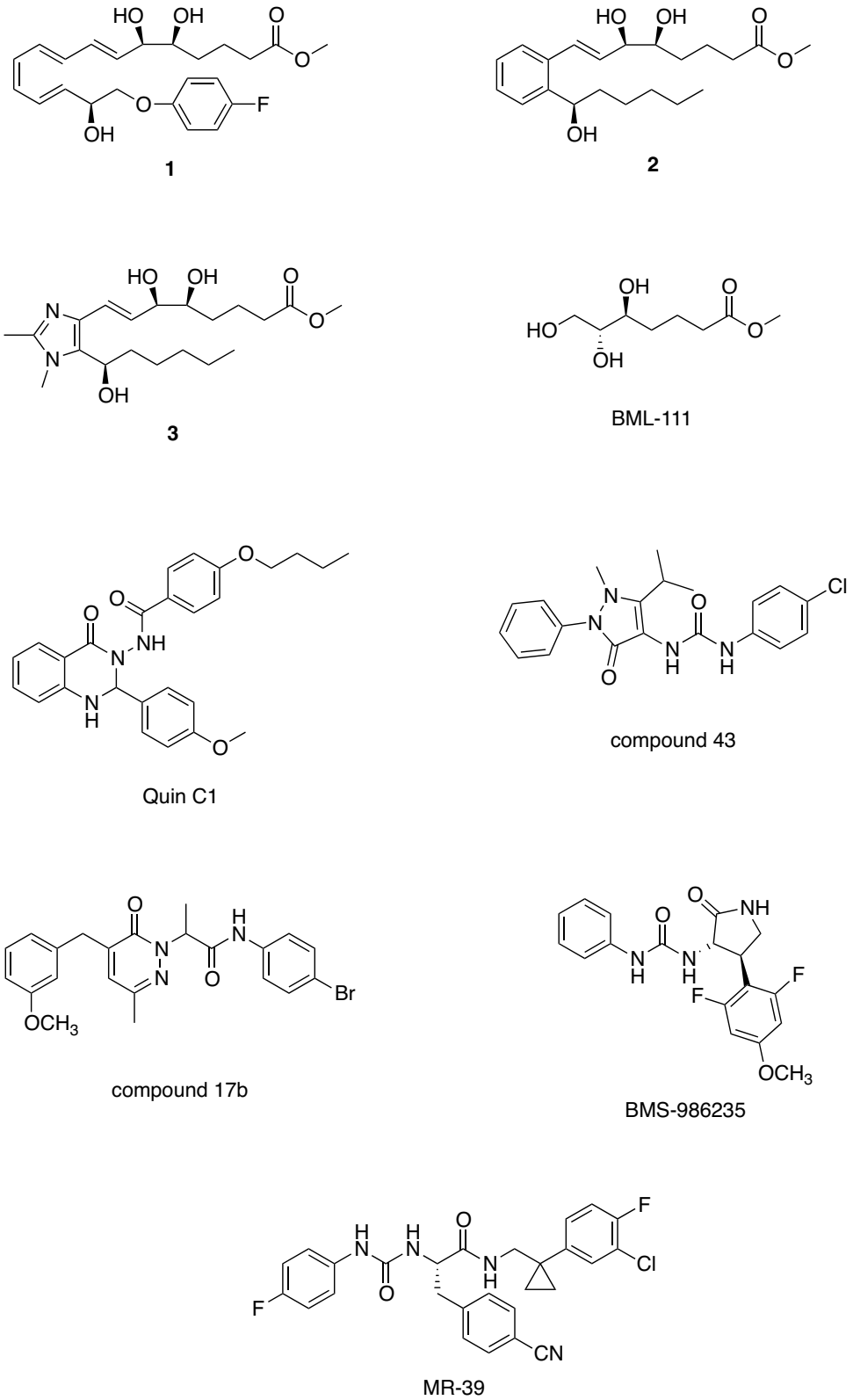


**Fig. 5** Structures of the lipoxin mimetics and small-molecule Formyl peptide receptor 2 (FPR2) agonists

of CNS diseases [127]. It worth noting that the wide chemical diversity of FPR2 agonists might imply biased FPR2 signaling. Therefore, a detailed pharmacological analysis of existing FPR2 agonists will provide valuable pieces of information in the search of FPR2 agonists effective in the resolution of inflammation.

## Conclusions

The FPR2 is a versatile transmembrane protein belonging to the class of G-protein-coupled receptor family. FPR2 recognize various ligands with significantly different structures, such as non-formyl peptides, endogenous peptides, structurally unrelated lipids as well as synthetic small pro-resolving molecules. Therefore, FPR2s is highly "promiscuous" in terms of ligand recognition, which means that it can be activated by agonists with pro-inflammatory as well as pro-resolving properties. This creates a unique opportunity for switching from pro- to anti-inflammatory profile of FPR2 activation. This is of utmost importance for the treatment of various chronic CNS inflammatory-related diseases, since traditional anti-inflammatory therapies only reduce the mounting of the inflammatory response but also impair some relevant mechanisms that trigger the resolution phase. Therefore, a novel and innovative approach to modulating the inflammatory response is needed. Opportunities are given by SPMs, which in addition to their well-recognized role as modulators of inflammation promote RoI by regulating several molecular and cellular pathways. Hence, the search for ligands characterized by an adequate pharmacological profile and bioavailability, which may become widely used to promote endogenous RoI through FPR2 activation, appears advisable and may be a promising strategy for resolution pharmacology in the future.

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## Declarations

**Conflict of interest** The author declares no conflict of interest.



## References

1. Annunziata MC, Parisi M, Esposito G, Fabbrocini G, Ammendola R, Cattaneo F. Phosphorylation sites in protein kinases and phosphatases regulated by formyl peptide receptor 2 signaling. Int J Mol Sci. 2020;21(11):3818.
2. Li L, Chen K, Xiang Y, Yoshimura T, Su S, Zhu J, et al. New development in studies of formyl-peptide receptors: critical roles in host defense. J Leukoc Biol. 2016;99(3):425–35.
3. Cattaneo F, Parisi M, Ammendola R. Distinct signaling cascades elicited by different formyl peptide receptor 2 (FPR2) agonists. Int J Mol Sci. 2013;14:7193–230.
4. He HQ, Ye RD. The formyl peptide receptors: diversity of ligands and mechanism for recognition. Molecules. 2017;22(3):455.
5. Ye RD, Boulay F, Ji MW, Dahlgren C, Gerard C, Parmentier M, et al. International union of basic and clinical pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family. Pharmacol Rev. 2009;61(2):119–61.
6. Hanson J. Heterologously expressed formyl peptide receptor 2 (FPR2/ALX) does not respond to lipoxin A4. Biochem Pharmacol. 2013;85(12):1795–802.
7. Fiore S, Maddox JF, Perez HD, Serhan CN. Identification of a human cDNA encoding a functional high affinity lipoxin A4 receptor. J Exp Med. 1994;180(1):253–60.
8. Boulay F, Tardif M, Brouchon L, Vignais P. Synthesis and use of a novel N-formyl peptide derivative to isolate a human N-formyl peptide receptor cDNA. Biochem Biophys Res Commun. 1990;168(3):1103–9.
9. Muto Y, Guindon S, Umemura T, Kőhidai L, Ueda H. Adaptive evolution of formyl peptide receptors in mammals. J Mol Evol. 2015;80(2):130–41.
10. Rabiet MJ, Macari L, Dahlgren C, Boulay F. N-formyl peptide receptor 3 (FPR3) departs from the homologous FPR2/ALX receptor with regard to the major processes governing chemoattractant receptor regulation, expression at the cell surface, and phosphorylation. J Biol Chem. 2011;286(30):26718–31.
11. Krepel SA, Wang JM. Chemotactic ligands that activate G-protein-coupled formylpeptide receptors. Int J Mol Sci. 2019;20(14):3426.
12. Becker EL, Forouhar FA, Grunnet ML, Boulay F, Tardif M, Bormann BJ, et al. Broad immunocytochemical localization of the formylpeptide receptor in human organs, tissues, and cells. Cell Tissue Res. 1998;292(1):129–35.
13. Devosse T, Guillabert A, D'Haene N, Berton A, De Nadai P, Noel S, et al. Formyl peptide receptor-like 2 is expressed and functional in plasmacytoid dendritic cells, tissue-specific macrophage subpopulations, and eosinophils. J Immunol. 2009;182(8):4974–84.
14. Cattaneo F, Guerra G, Ammendola R. Expression and signaling of formyl-peptide receptors in the brain. Neurochem Res. 2010;35(12):2018–26.
15. Boulay F, Tardif M, Brouchon L, Vignais P. The human N-formylpeptide receptor. Characterization of two cDNA isolates and evidence for a new subfamily of G-protein-coupled receptors. Biochemistry. 1990;29(50):11123–33.
16. Kretschmer D, Rautenberg M, Linke D, Peschel A. Peptide length and folding state govern the capacity of staphylococcal β-type phenol-soluble modulins to activate human formyl-peptide receptors 1 or 2. J Leukoc Biol. 2015;97(4):689–97.
17. Ye RD, Cavanagh SL, Quehenberger O, Prossnitz ER, Cochrane CG. Isolation of a cDNA that encodes a novel granulocyte N-formyl peptide receptor. Biochem Biophys Res Commun. 1992;184(2):582–9.

18. Quehenberger O, Prossnitz ER, Cavanagh SL, Cochrane CG, Ye RD. Multiple domains of the N-formyl peptide receptor are required for high- affinity ligand binding. Construction and analysis of chimeric N-formyl peptide receptors. J Biol Chem. 1993;268(24):18167–75.
19. Dufton N, Perretti M. Therapeutic anti-inflammatory potential of formyl-peptide receptor agonists. Pharmacol Ther. 2010;127(2):175–88.
20. Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, et al. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget. 2018;9(6):7204–18.
21. Gao JL, Chen H, Filie JD, Kozak CA, Murphy PM. Differential expansion of the N-formylpeptide receptor gene cluster in human and mouse. Genomics. 1998;51(2):270–6.
22. Rabiet MJ, Huet E, Boulay F. Human mitochondria-derived N-formylated peptides are novel agonists equally active on FPR and FPRL1, while Listeria monocytogenes-derived peptides preferentially activate FPR. Eur J Immunol. 2005;35(8):2486–95.
23. Southgate EL, He RL, Gao J-L, Murphy PM, Nanamori M, Ye RD. Identification of formyl peptides from listeria monocytogenes and staphylococcus aureus as potent chemoattractants for mouse neutrophils. J Immunol. 2008;181(2):1429–37.
24. Gao JL, Lee EJ, Murphy PM. Impaired antibacterial host defense in mice lacking the N-formylpeptide receptor. J Exp Med. 1999;189(4):657–62.
25. Liu M, Chen K, Yoshimura T, Liu Y, Gong W, Wang A, et al. Formylpeptide receptors are critical for rapid neutrophil mobilization in host defense against Listeria monocytogenes. Sci Rep. 2012;2:1–7.
26. Gao J-L, Guillabert A, Hu J, Le Y, Urizar E, Seligman E, et al. F2L, a peptide derived from heme-binding protein, chemoattracts mouse neutrophils by specifically activating Fpr2, the low-affinity N-formylpeptide receptor. J Immunol. 2007;178(3):1450–6.
27. Gobbetti T, Coldewey SM, Chen J, McArthur S, Le Faouder P, Cenac N, et al. Nonredundant protective properties of FPR2/ALX in polymicrobial murine sepsis. Proc Natl Acad Sci USA. 2014;111(52):18685–90.
28. Liang TS, Wang JM, Murphy PM, Gao JL. Serum amyloid A is a chemotactic agonist at FPR2, a low-affinity N-formylpeptide receptor on mouse neutrophils. Biochem Biophys Res Commun. 2000;270(2):331–5.
29. Tiffany HL, Lavigne MC, Cui Y-H, Wang J-M, Leto TL, Gao J-L, et al. Amyloid-$^{1\text{-}42}$ induces chemotaxis and oxidant stress by acting at formylpeptide receptor 2, a G protein-coupled receptor expressed in phagocytes and brain. J Biol Chem. 2001;276(26):23645–52.
30. Migeotte I, Riboldi E, Franssen J-D, Grégoire F, Loison C, Wittamer V, et al. Identification and characterization of an endogenous chemotactic ligand specific for FPRL2. J Exp Med. 2005;201(1):83–93.
31. Tiffany HL, Lavigne MC, Cui YH, Wang JM, Leto TL, Gao JL, et al. Amyloid-β induces chemotaxis and oxidant stress by acting at formylpeptide receptor 2, a G Protein-coupled receptor expressed in phagocytes and brain. J Biol Chem. 2001;276(26):23645–52.
32. Rivière S, Challet L, Fluegge D, Spehr M, Rodriguez I. Formyl peptide receptor-like proteins are a novel family of vomeronasal chemosensors. Nature. 2009;459(7246):574–7.
33. Liberles SD, Horowitz LF, Kuang D, Contos JJ, Wilson KL, Siltberg-Liberles J, et al. Formyl peptide receptors are candidate chemosensory receptors in the vomeronasal organ. Proc Natl Acad Sci. 2009;106(24):9842–7.
34. Fredriksson R, Lagerström MC, Lundin LG, Schiöth HB. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol Pharmacol. 2003;63(6):1256–72.
35. Hilger D, Masureel M, Kobilka BK, Struct N, Biol M. Structure and dynamics of GPCR signaling complexes HHS public access author manuscript. Nat Struct Mol Biol. 2018;25(1):4–12.
36. Venkatakrishnan AJ, Deupi X, Lebon G, Tate CG, Schertler GF, Madan BM. Molecular signatures of G-protein-coupled receptors. Nature. 2013;494(7436):185–94.
37. Sensoy O, Weinstein H. A mechanistic role of Helix 8 in GPCRs: Computational modeling of the dopamine D2 receptor interaction with the GIPC1–PDZ-domain. Biochim Biophys Acta BBA Biomembr. 2015;1848(4):976–83.
38. Zhang D, Zhao Q, Wu B. Structural studies of G protein-coupled receptors. Mol Cells. 2015;38(10):836–42.
39. Skvortsov SS, Gabdoulkhakova AG. Formyl peptide receptor polymorphisms: 27 most possible ways for phagocyte dysfunction. Biochem Mosc. 2017;82(4):426–37.
40. Bennett TA, Maestas DC, Prossnitz ER. Arrestin binding to the G protein-coupled N-formyl peptide receptor is regulated by the conserved 'DRY' sequence. J Biol Chem. 2000;275(32):24590–4.
41. He R, Browning DD, Ye RD. Differential roles of the NPXXY motif in formyl peptide receptor signaling. J Immunol. 2001;166(6):4099–105.
42. Lohse MJ. Dimerization in GPCR mobility and signaling. Curr Opin Pharmacol. 2010;10(1):53–8.
43. Sodin-Semrl S, Spagnolo A, Mikus R, Barbaro B, Varga J, Fiore S. Opposing regulation of interleukin-8 and NF-kB Responses by lipoxin A4 and serum amyloid a via the common lipoxin a receptor. Int J Immunopathol Pharmacol. 2004;17(2):145–55.
44. Cooray SN, Gobbetti T, Montero-Melendez T, McArthur S, Thompson D, Clark AJL, et al. Ligand-specific conformational change of the G-protein-coupled receptor ALX/FPR2 determines proresolving functional responses. Proc Natl Acad Sci U S A. 2013;110(45):18232–7.
45. Brandenburg LO, Konrad M, Wruck CJ, Koch T, Lucius R, Pufe T. Functional and physical interactions between formyl-peptide-receptors and scavenger receptor MARCO and their involvement in amyloid beta 1–42-induced signal transduction in glial cells. J Neurochem. 2010;113(3):749–60.
46. Filep JG. Biasing the lipoxin A4/formyl peptide receptor 2 pushes inflammatory resolution. Proc Natl Acad Sci. 2013;110(45):18033–4.
47. Bena S, Brancaleone V, Wang JM, Perretti M, Flower RJ. Annexin A1 interaction with the FPR2/ALX receptor. J Biol Chem. 2012;287(29):24690–7.
48. Chiang N, Fierro IM, Gronert K, Serhan CN. Activation of lipoxin a4 receptors by aspirin-triggered lipoxins and select peptides evokes ligand-specific responses in inflammation. J Exp Med. 2000;191(7):1197–208.
49. Ammendola R, Parisi M, Esposito G, Cattaneo F. Pro-resolving FPR2 agonists regulate NADPH oxidase-dependent phosphorylation of HSP27, OSR1, and MARCKS and activation of the respective upstream kinases. Antioxidants. 2021;10(1):134.
50. Cattaneo F, Guerra G, Parisi M, De Marinis M, Tafuri D, Cinelli M, et al. Cell-surface receptors transactivation mediated by G protein-coupled receptors. Int J Mol Sci. 2014;15(11):19700–28.
51. Cattaneo F, Russo R, Castaldo M, Chambery A, Zollo C, Esposito G, et al. Phosphoproteomic analysis sheds light on intracellular signaling cascades triggered by formyl-peptide receptor 2. Sci Rep. 2019;9(1):17894.
52. Krishnamoorthy S, Recchiuti A, Chiang N, Yacoubian S, Lee CH, Yang R, et al. Resolvin D1 binds human phagocytes with evidence for proresolving receptors. Proc Natl Acad Sci USA. 2010;107(4):1660–5.
53. Raabe CA, Gröper J, Rescher U. Biased perspectives on formyl peptide receptors. Biochim Biophys Acta Mol Cell Res. 2019;1866(2):305–16.

54. Kenakin T, Williams M. Defining and characterizing drug/compound function. Biochem Pharmacol. 2014;87(1):40–63.
55. Schepetkin IA, Khlebnikov AI, Giovannoni MP, Kirpotina LN, Cilibrizzi A, Quinn MT. Development of small molecule non-peptide formyl peptide receptor (FPR) Ligands and molecular modeling of their recognition. Curr Med Chem. 2014;21(13):1478–504.
56. Headland SE, Jones HR, Norling LV, Kim A, Souza PR, Corsiero E, et al. Neutrophil-derived microvesicles enter cartilage and protect the joint in inflammatory arthritis. Sci Transl Med. 2015;7(315):315ra190-315ra190.
57. Nathan C, Ding A. Nonresolving inflammation. Cell. 2010;140(6):871–82.
58. Murakami M, Hirano T. The molecular mechanisms of chronic inflammation development. Front Immunol. 2012;3(NOV):1–3.
59. He R, Sang H, Ye RD. Serum amyloid A induces IL-8 secretion through a G protein–coupled receptor, FPRL1/LXA4R. Blood. 2003;101(4):1572–81.
60. O'Hara R, Murphy EP, Whitehead AS, FitzGerald O, Bresnihan B. Local expression of the serum amyloid A and formyl peptide receptor-like 1 genes in synovial tissue is associated with matrix metalloproteinase production in patients with inflammatory arthritis. Arthritis Rheum. 2004;50(6):1788–99.
61. Bozinovski S, Uddin M, Vlahos R, Thompson M, McQualter JL, Merritt A-S, et al. Serum amyloid A opposes lipoxin A4 to mediate glucocorticoid refractory lung inflammation in chronic obstructive pulmonary disease. Proc Natl Acad Sci. 2012;109(3):935–40.
62. Sano T, Huang W, Hall JA, Yang Y, Chen A, Gavzy SJ, et al. An IL-23R/IL-22 circuit regulates epithelial serum amyloid a to promote local effector Th17 responses. Cell. 2015;163(2):381–93.
63. Atarashi K, Tanoue T, Ando M, Kamada N, Nagano Y, Narushima S, et al. Th17 cell induction by adhesion of microbes to intestinal epithelial cells. Cell. 2015;163(2):367–80.
64. Li S, Selkoe DJ. A mechanistic hypothesis for the impairment of synaptic plasticity by soluble Aβ oligomers from Alzheimer's brain. J Neurochem. 2020;154(6):583–97.
65. Barry AE, Klyubin I, Mc Donald JM, Mably AJ, Farrell MA, Scott M, et al. Alzheimer's disease brain-derived amyloid- -mediated inhibition of LTP in vivo is prevented by immunotargeting cellular prion protein. J Neurosci. 2011;31(20):7259–63.
66. Klyubin I, Nicoll AJ, Khalili-Shirazi A, Farmer M, Canning S, Mably A, et al. Peripheral administration of a humanized Anti-PrP antibody blocks Alzheimer's disease a synaptotoxicity. J Neurosci. 2014;34(18):6140–5.
67. Balducci C, Beeg M, Stravalaci M, Bastone A, Sclip A, Biasini E, et al. Synthetic amyloid-β oligomers impair long-term memory independently of cellular prion protein. Proc Natl Acad Sci. 2010;107(5):2295–300.
68. Calella AM, Farinelli M, Nuvolone M, Mirante O, Moos R, Falsig J, et al. Prion protein and Aβ-related synaptic toxicity impairment. EMBO Mol Med. 2010;2(8):306–14.
69. Heneka MT, Carson MJ, Khoury JE, Landreth GE, Brosseron F, Feinstein DL, et al. Neuroinflammation in Alzheimer's disease. Lancet Neurol. 2015;14(4):388–405.
70. Heppner FL, Ransohoff RM, Becher B. Immune attack: the role of inflammation in Alzheimer disease. Nat Rev Neurosci. 2015;16(6):358–72.
71. Le Y, Yazawa H, Gong W, Yu Z, Ferrans VJ, Murphy PM, et al. Cutting edge: the neurotoxic prion peptide fragment PrP 106–126 is a chemotactic agonist for the G protein-coupled receptor formyl peptide receptor-like 1. J Immunol. 2001;166(3):1448–51.
72. Yazawa H, Yu Z-X, Takeda K, Le Y, Gong W, Ferrans VJ, et al. β Amyloid peptide (Aβ 42) is internalized via the G-protein-coupled receptor FPRL1 and forms fibrillar aggregates in macrophages 1. FASEB J. 2001;15(13):2454–62.
73. Domingues C, da Cruz e Silva OAB, Henriques AG. Impact of cytokines and chemokines on Alzheimer's disease neuropathological hallmarks. Curr Alzheimer Res. 2017;14(8):870–882.
74. Lyons A, Griffin RJ, Costelloe CE, Clarke RM, Lynch MA. IL-4 attenuates the neuroinflammation induced by amyloid-β in vivo and in vitro: IL-4 attenuates the neuroinflammation induced by amyloid-βin vivo andin vitro. J Neurochem. 2007;101(3):771–81.
75. Ying G, Iribarren P, Zhou Y, Gong W, Zhang N, Yu Z-X, et al. Humanin, a newly identified neuroprotective factor, uses the G protein-coupled formylpeptide receptor-like-1 as a functional receptor. J Immunol. 2004;172(11):7078–85.
76. Harada M, Habata Y, Hosoya M, Nishi K, Fujii R, Kobayashi M, et al. N-Formylated humanin activates both formyl peptide receptor-like 1 and 2. Biochem Biophys Res Commun. 2004;324(1):255–61.
77. Cattaneo F, Parisi M, Ammendola R. Distinct signaling cascades elicited by different formyl peptide receptor 2 (FPR2) agonists. Int J Mol Sci. 2013;14(4):7193–230.
78. Perretti M, Chiang N, La M, Fierro IM, Marullo S, Getting SJ, et al. Endogenous lipid- and peptide-derived anti-inflammatory pathways generated with glucocorticoid and aspirin treatment activate the lipoxin A4 receptor. Nat Med. 2002;8(11):1296–302.
79. Karlsson J, Fu H, Boulay F, Dahlgren C, Hellstrand K, Movitz C. Neutrophil NADPH-oxidase activation by an annexin AI peptide is transduced by the formyl peptide receptor (FPR), whereas an inhibitory signal is generated independently of the FPR family receptors. J Leukoc Biol. 2005;78(3):762–71.
80. Liu G-J, Tao T, Wang H, Zhou Y, Gao X, Gao Y-Y, et al. Functions of resolvin D1-ALX/FPR2 receptor interaction in the hemoglobin-induced microglial inflammatory response and neuronal injury. J Neuroinflammation. 2020;17(1):239.
81. Gallo I, Rattazzi L, Piras G, Gobbetti T, Panza E, Perretti M, et al. Formyl peptide receptor as a novel therapeutic target for anxiety-related disorders. PLoS ONE. 2014;9(12):e114626 (**Rubino T, editor**).
82. Cristante E, McArthur S, Mauro C, Maggioli E, Romero IA, Wylezinska-Arridge M, et al. Identification of an essential endogenous regulator of blood-brain barrier integrity, and its pathological and therapeutic implications. Proc Natl Acad Sci. 2013;110(3):832–41.
83. Zhao J, Wang T, Lv Q, Zhou N. Expression of heat shock protein 70 and Annexin A1 in serum of patients with acutely severe traumatic brain injury. Exp Ther Med. 2019. https://doi.org/10.3892/etm.2019.8357.
84. Wang Z, Chen Z, Yang J, Yang Z, Yin J, Zuo G, et al. Identification of two phosphorylation sites essential for annexin A1 in blood–brain barrier protection after experimental intracerebral hemorrhage in rats. J Cereb Blood Flow Metab. 2017;37(7):2509–25.
85. Luo ZZ, Gao Y, Sun N, Zhao Y, Wang J, Tian B, et al. Enhancing the interaction between annexin-1 and formyl peptide receptors regulates microglial activation to protect neurons from ischemia-like injury. J Neuroimmunol. 2014;276(1–2):24–36.
86. Lu J, Yu Y, Zhu I, Cheng Y, Sun PD. Structural mechanism of serum amyloid A-mediated inflammatory amyloidosis. Proc Natl Acad Sci. 2014;111(14):5189–94.
87. Tao Y, Han Y, Yu L, Wang Q, Leng SX, Zhang H. The predicted key molecules, functions, and pathways that bridge mild cognitive impairment (MCI) and Alzheimer's disease (AD). Front Neurol. 2020;3(11):233.
88. Recchiuti A, Isopi E, Romano M, Mattoscio D. Roles of specialized pro-resolving lipid mediators in autophagy and inflammation. Int J Mol Sci. 2020;21(18):6637.
89. Biringer RG. The role of eicosanoids in Alzheimer's disease. Int J Environ Res Public Health. 2019;16(14):2560.

90. Yui K, Imataka G, Nakamura H, Ohara N, Naito Y. Eicosanoids derived from arachidonic acid and their family prostaglandins and cyclooxygenase in psychiatric disorders. Curr Neuropharmacol. 2015;13(6):776–85.
91. Perretti M, Leroy X, Bland EJ, Montero-Melendez T. Resolution pharmacology: opportunities for therapeutic innovation in inflammation. Trends Pharmacol Sci. 2015;36(11):737–55.
92. Perretti M, Godson C. Formyl peptide receptor type 2 agonists to kick-start resolution pharmacology. Br J Pharmacol. 2020;177(20):4595–600.
93. Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology. Nature. 2014;510(7503):92–101.
94. Hawkins KE, DeMars KM, Alexander JC, de Leon LG, Pacheco SC, Graves C, et al. Targeting resolution of neuroinflammation after ischemic stroke with a lipoxin $A_4$ analog: protective mechanisms and long-term effects on neurological recovery. Brain Behav. 2017;7(5):e00688.
95. Schaldach CM, Riby J, Bjeldanes LF. Lipoxin A 4: a new class of ligand for the Ah receptor. Biochemistry. 1999;38(23):7594–600.
96. Russell R, Gori I, Pellegrini C, Kumar R, Achtari C, Canny GO. Lipoxin $A_4$ is a novel estrogen receptor modulator. FASEB J. 2011;25(12):4326–37.
97. Gronert K, Martinsson-Niskanen T, Ravasi S, Chiang N, Serhan CN. Selectivity of recombinant human leukotriene D4, leukotriene B4, and Lipoxin A4 receptors with aspirin-triggered 15-epi-LXA4 and regulation of vascular and inflammatory responses. Am J Pathol. 2001;158(1):3–9.
98. Prieto P, Cuenca J, Través PG, Fernández-Velasco M, Martín-Sanz P, Boscá L. Lipoxin A4 impairment of apoptotic signaling in macrophages: implication of the PI3K/Akt and the ERK/Nrf-2 defense pathways. Cell Death Differ. 2010;17(7):1179–88.
99. Dorward DA, Lucas CD, Chapman GB, Haslett C, Dhaliwal K, Rossi AG. The role of formylated peptides and formyl peptide receptor 1 in governing neutrophil function during acute inflammation. Am J Pathol. 2015;185(5):1172–84.
100. Delerive P, De Bosscher K, Besnard S, Vanden Berghe W, Peters JM, Gonzalez FJ, et al. Peroxisome proliferator-activated receptor α negatively regulates the vascular inflammatory gene response by negative cross-talk with transcription factors NF-κB and AP-1. J Biol Chem. 1999;274(45):32048–54.
101. Dunn HC, Ager RR, Baglietto-Vargas D, Cheng D, Kitazawa M, Cribbs DH, et al. Restoration of Lipoxin A4 signaling reduces alzheimer's disease-like pathology in the 3xTg-AD mouse model. J Alzheimers Dis. 2014;43(3):893–903.
102. Kantarci A, Aytan N, Palaska I, Stephens D, Crabtree L, Benincasa C, et al. Combined administration of resolvin E1 and lipoxin A4 resolves inflammation in a murine model of Alzheimer's disease. Exp Neurol. 2018;300:111–20.
103. Sun Y-P, Oh SF, Uddin J, Yang R, Gotlinger K, Campbell E, et al. Resolvin D1 and its aspirin-triggered 17R epimer. J Biol Chem. 2007;282(13):9323–34.
104. Dona M, Fredman G, Schwab JM, Chiang N, Arita M, Goodarzi A, et al. Resolvin E1, an EPA-derived mediator in whole blood, selectively counterregulates leukocytes and platelets. Blood. 2008;112(3):848–55.
105. Nelson JW, Leigh NJ, Mellas RE, McCall AD, Aguirre A, Baker OJ. ALX/FPR2 receptor for RvD1 is expressed and functional in salivary glands. Am J Physiol-Cell Physiol. 2014;306(2):C178–85.
106. Li H, Wu Z, Feng D, Gong J, Yao C, Wang Y, et al. BML-111, a lipoxin receptor agonist, attenuates ventilator-induced lung injury in rats. Shock. 2014;41(4):311–6.
107. Xu J, Gao X, Yang C, Chen L, Chen Z. Resolvin D1 attenuates Mpp+-induced Parkinson disease via inhibiting inflammation in PC12 cells. Med Sci Monit. 2017;23:2684–2691.
108. Tian Y, Zhang Y, Zhang R, Qiao S, Fan J. Resolvin D2 recovers neural injury by suppressing inflammatory mediators expression in lipopolysaccharide-induced Parkinson's disease rat model. Biochem Biophys Res Commun. 2015;460(3):799–805.
109. Ishikawa Y, Deyama S, Shimoda K, Yoshikawa K, Ide S, Satoh M, et al. Rapid and sustained antidepressant effects of resolvin D1 and D2 in a chronic unpredictable stress model. Behav Brain Res. 2017;332:233–6.
110. Giacobbe J, Benoiton B, Zunszain P, Pariante CM, Borsini A. The anti-inflammatory role of omega-3 polyunsaturated fatty acids metabolites in pre-clinical models of psychiatric, neurodegenerative, and neurological disorders. Front Psychiatry. 2020;28(11):122.
111. Klein CP, Sperotto NDM, Maciel IS, Leite CE, Souza AH, Campos MM. Effects of D-series resolvins on behavioral and neurochemical changes in a fibromyalgia-like model in mice. Neuropharmacology. 2014;86:57–66.
112. Kok Kendirlioglu B, Unalan Ozpercin P, Yuksel Oksuz O, Sozen S, Cihnioglu R, Kalelioglu T, et al. Resolvin D1 as a novel anti-inflammatory marker in manic, depressive and euthymic states of bipolar disorder. Nord J Psychiatry. 2020;74(2):83–8.
113. Terrando N, Gómez-Galán M, Yang T, Carlström M, Gustavsson D, Harding RE, et al. Aspirin-triggered resolvin D1 prevents surgery-induced cognitive decline. FASEB J. 2013;27(9):3564–71.
114. Harrison JL, Rowe RK, O'Hara BF (2016) Resolvins AT-D1 and E1 differentially impact functional outcome, post-traumatic sleep, and microglial activation following diffuse brain injury in the mouse. Brain Behav Immun. 2015;47:131–40.
115. Parkinson JF. Lipoxin and synthetic lipoxin analogs: an overview of anti-inflammatory functions and new concepts in immunomodulation. Inflamm Allergy Drug Targets. 2006;5(2):91–106.
116. Conte FP, Menezes-De-Lima OJ, Verri WA, Cunha FQ, Penido C, Henriques MG. Lipoxin A 4 attenuates zymosan-induced arthritis by modulating endothelin-1 and its effects. Br J Pharmacol. 2010;161(4):911–24.
117. Maciuszek M, Cacace A, Brennan E, Godson C, Chapman TM. Recent advances in the design and development of formyl peptide receptor 2 (FPR2/ALX) agonists as pro-resolving agents with diverse therapeutic potential. Eur J Med Chem. 2021;213:113167.
118. Gong J, Guo S, Li HB, Yuan SY, Shang Y, Yao SL. BML-111, a lipoxin receptor agonist, protects haemorrhagic shock-induced acute lung injury in rats. Resuscitation. 2012;83(7):907–12.
119. Li YS, Wu P, Zhou XY, Chen JG, Cai L, Wang F, et al. Formyl-peptide receptor like 1: a potent mediator of the Ca2+ release-activated Ca2+ current ICRAC. Arch Biochem Biophys. 2008;478(1):110–8.
120. Wang YZ, Zhang YC, Cheng JS, Ni Q, Li PW, Han W, et al. Protective effects of BML-111 on cerulein-induced acute pancreatitis-associated lung injury via activation of Nrf2/ARE signaling pathway. Inflammation. 2014;37(4):1120–33.
121. Ye X-H, Wu Y, Guo P-P, Wang J, Yuan S-Y, Shang Y, et al. Lipoxin A4 analogue protects brain and reduces inflammation in a rat model of focal cerebral ischemia reperfusion. Brain Res. 2010;1323:174–83.
122. He M, Cheng N, Gao W, Zhang M, Zhang Y, Ye RD, et al. Characterization of Quin-C1 for its anti-inflammatory property in a mouse model of bleomycin-induced lung injury. Acta Pharmacol Sin. 2011;32(5):601–10.
123. Dufton N (2010) Therapeutic anti-inflammatory potential of formyl-peptide receptor agonists. Pharmacol Ther. 2010;127(2):175–88.
124. Qin CX, May LT, Li R, Cao N, Rosli S, Deo M, et al. Small-molecule-biased formyl peptide receptor agonist compound 17b protects against myocardial ischaemia-reperfusion injury in mice. Nat Commun. 2017;8:1–13.

125. Asahina Y, Wurtz NR, Arakawa K, Carson N, Fujii K, Fukuchi K, et al. Discovery of BMS-986235/LAR-1219: a potent formyl peptide receptor 2 (FPR2) selective agonist for the prevention of heart failure. J Med Chem. 2020;63(17):9003–19.
126. Stama ML, Ślusarczyk J, Lacivita E, Kirpotina LN, Schepetkin IA, Chamera K, et al. Novel ureidopropanamide based N-formyl peptide receptor 2 (FPR2) agonists with potential application for central nervous system disorders characterized by neuroinflammation. Eur J Med Chem. 2017;141:703–20.
127. Mastromarino M, Lacivita E, Colabufo NA, Leopoldo M. G-Protein coupled receptors involved in the resolution of inflammation: ligands and therapeutic perspectives. Mini-Rev Med Chem. 2021;20(20):2090–103.

**Time-Dependent Protective and Pro-Resolving Effects of FPR2 Agonists on Lipopolysaccharide–Exposed Microglia Cells Involve Inhibition of NF-κB and MAPKs Pathways.**

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# Time-Dependent Protective and Pro-Resolving Effects of FPR2 Agonists on Lipopolysaccharide-Exposed Microglia Cells Involve Inhibition of NF-κB and MAPKs Pathways

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**Abstract:** Prolonged or excessive microglial activation may lead to disturbances in the resolution of inflammation (RoI). The importance of specialized pro-resolving lipid mediators (SPMs) in RoI has been highlighted. Among them, lipoxins (LXA4) and aspirin-triggered lipoxin A4 (AT-LXA4) mediate beneficial responses through the activation of N-formyl peptide receptor-2 (FPR2). We aimed to shed more light on the time-dependent protective and anti-inflammatory impact of the endogenous SPMs, LXA4, and AT-LXA4, and of a new synthetic FPR2 agonist MR-39, in lipopolysaccharide (LPS)-exposed rat microglial cells. Our results showed that LXA4, AT-LXA4, and MR-39 exhibit a protective and pro-resolving potential in LPS-stimulated microglia, even if marked differences were apparent regarding the time dependency and efficacy of inhibiting particular biomarkers. The LXA4 action was found mainly after 3 h of LPS stimulation, and the AT-LXA4 effect was varied in time, while MR-39's effect was mainly observed after 24 h of stimulation by endotoxin. MR-39 was the only FPR2 ligand that attenuated LPS-evoked changes in the mitochondrial membrane potential and diminished the ROS and NO release. Moreover, the LPS-induced alterations in the microglial phenotype were modulated by LXA4, AT-LXA4, and MR-39. The anti-inflammatory effect of MR-39 on the IL-1β release was mediated through FPR2. All tested ligands inhibited TNF-α production, while AT-LXA4 and MR-39 also diminished IL-6 levels in LPS-stimulated microglia. The favorable action of LXA4 and MR-39 was mediated through the inhibition of ERK1/2 phosphorylation. AT-LXA4 and MR39 diminished the phosphorylation of the transcription factor NF-κB, while AT-LXA4 also affected p38 kinase phosphorylation. Our results suggest that new pro-resolving synthetic mediators can represent an attractive treatment option for the enhancement of RoI, and that FPR2 can provide a perspective as a target in immune-related brain disorders.

**Keywords:** microglia; lipopolysaccharide; lipoxin A4; aspirin-triggered lipoxin A4; MR-39; formyl peptide receptor 2



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## 1. Introduction

A large body of evidence has demonstrated that microglia manage innate and adaptive immune responses in various pathological and regenerative processes in the central nervous system (CNS) [1,2]. Among others, it is believed that active microglia can clear cellular debris by phagocytosis, thereby promoting tissue repair and regulating the response to pathogens. On the other hand, prolonged or excessive activation leads to the functional changes and switch of microglia from regulatory to inflammatory/neurotoxic

functions [3–5], which allows us to infer that microglia are highly sensitive indicators of the brain condition [6,7]. Recently, the microglia heterogeneity has become one of the crucial and controversial topics in neuroimmunology. Although the view that microglia heterogeneity is context-dependent [8–11] is gaining more and more followers, there are still researchers who support the classical (M1-like) and alternative (M2-like) concept of microglia polarization [12,13]. In spite of that, in response to immune stimulation, microglia upregulate a number of pro-inflammatory surface proteins (e.g., CD40 and MHC II), cytokines (IL-18, IL-1β, TNF-α, and IL-6), and neurotoxic mediators, such as nitric oxide (NO), prostaglandin (PG), and reactive oxygen species (ROS) [14]. In contrast, the anti-inflammatory response leads to the expression of various markers (e.g., Arg-1 and CD206) and mediators (e.g., insulin growth factor 1 (IGF-1) and/or IL-10) and is involved in the limitation of inflammation and the restoration of homeostasis [13,15,16].

According to current views, inflammation is a multistage self-resolving process mediated by various factors that "switch off" the inflammatory response [17]. Nevertheless, disturbances in the resolution of inflammation (RoI) [18,19] can be involved in the pathogenesis of brain-related inflammatory diseases, mainly due to the constant stimulation of the immune system, overproduction of pro-inflammatory cytokines, oxidative stress, and potential impairment in the maintenance of homeostasis [20–23]. Recently, the importance of RoI has been realized for specialized pro-resolving lipid mediators (SPMs), which limit inflammatory signals and resolve inflammation at multiple levels. Thus, in contrast to other "anti-inflammatory" treatments, SPMs not only block the production of pro-inflammatory mediators as NSAIDs and other anti-inflammatory drugs do but also stimulate physiological signals to resolve and terminate the inflammatory reaction through particular receptor–ligand interactions and specific endogenous mechanism activation [24–26].

Among the known SPMs, lipoxin (LXA4) is the most specific endogenous ligand and it is synthesized from arachidonic acid via interactions of the 5-lipoxygenase and 15-lipoxygenase pathways [27]. Moreover, it was discovered that the acetylation of cyclooxygenase-2 (COX-2) by aspirin could lead to the transcellular biosynthesis of epi-lipoxins, the so-called aspirin-triggered lipoxins (AT-LXA4), which are LXA4 analogs. Lipoxin expression was identified in neural stem cells, neurons, astrocytes, and microglia [28,29] The release of LXA4 under physiological brain conditions is limited, while its synthesis is upregulated under pathological stimulation [30–32]. The data available thus far have demonstrated that LXA4 and its analog AT-LXA4 are biologically active with mostly anti-inflammatory and pro-resolving profiles. Studies have underlined the protective role of LXA4 via its impact on neuronal survival and enhancement of microglial phagocytic and anti-inflammatory potential [32,33]. Moreover, LXA4 inhibits microglial activation and diminishes neuroinflammation after spinal cord hemisection [34].

Several studies have demonstrated that LXA4 mediates responses related to RoI through the activation of N-formyl peptide receptors (FPRs) belonging to the G-protein coupled receptor family [22,32]. FPRs form higher-order structures (e.g., FPR1/FPR2 heterodimers, FPR2 homodimers, FPR1 homodimers), which lead to altering the downstream intracellular signaling pathways by allowing the co-localization of effector domains, enhancing intracellular activation, or creating new ligand specificity [35–37]. The beneficial role in the suppression of inflammation is primarily mediated through the FPR2 receptor [32,38]. In fact, LXA4 can directly bind to FPR2 with high affinity (Kd of 1.7 nM), but also to a variant of mouse mFpr-rs-1 [22,39]. The expression of FPR2 has been reported in the brainstem, spinal cord, thalamus/hypothalamus, cerebral neocortex, hippocampus, cerebellum, and striatum [33] in selected neurons [40] and also by microglia [34], in which FPR2 is rapidly upregulated following an inflammatory insult [35]. Moreover, the FPR2 is also expressed in many other cell types including neutrophils, eosinophils, monocytes, macrophages, T cells, synovial fibroblasts, and intestinal and airway epithelial cells [41], as well as neural stem cells [42]. Interestingly, FPR2 can mediate both pro-inflammatory and pro-resolving effects, depending on the chemical structure of the agonist. Therefore, this receptor may represent a unique target for balancing the inflammatory process and,

consequently, for developing new therapeutic strategies for brain disorders characterized by persistent neuroinflammation.

However, the unfavorable pharmacokinetic properties of lipoxin A4 (LXA4) and/or aspirin-triggered lipoxin A4 (AT-LXA4) represent a limitation of further studies. Thus, we have recently proposed novel ureidopropanamide FPR2 agonists as new agents to promote the resolution of inflammation [43]. From among them we have selected MR-39 as it shows in vitro favorable pharmacokinetic properties (i.e., stability toward hepatic oxidative metabolism and good passive permeability in a model of the blood–brain barrier) and has the potential to inhibit some signs of the inflammatory response [43]. To further elucidate the engagement of FPR2 in RoI, we conducted time-dependent studies covering the influence of lipoxin A4 (LXA4), aspirin-triggered lipoxin A4 (AT-LXA4), and MR-39, a new ureidopropanamide FPR2 agonist, on lipopolysaccharide (LPS)-induced changes in microglial cells. We assessed the effect of FPR2 agonists on cell death/viability by lactate dehydrogenase release, mitochondrial membrane potential modulation, and caspase 3 activation, whereas their putative antioxidant potential was estimated by measuring the reactive oxygen species (ROS) level and nitric oxide (NO) release. The effect of the tested agonists on LPS-evoked changes in FPR2 levels in microglia was visualized by immunofluorescence methods. Consequently, we also assessed the impact of LXA4, AT-LXA4, and MR-39 on the pro-inflammatory and anti-inflammatory microglia markers as well as on the synthesis of various cytokines using a specific FPR2 antagonist (WRW4). Finally, to better characterize the molecular mechanisms underlying the effect of FPR2 agonists on RoI, we studied their impact on intracellular pathways (e.g., ERK1/2, p38 MAPK, NF-κB) activated upon FPR2 stimulation in microglial cells.

## 2. Materials and Methods

### 2.1. Animals

Sprague-Dawley rats (200–250 g) were obtained from Charles River (Sulzfeld, Germany) and kept under standard conditions at room temperature (23 °C) under a 12/12 h light/dark cycle with lights on at 8.00 with food and water available *ad libitum*. One week after arrival, vaginal smears were taken daily from the female rats to determine the phase of the estrous cycle. On the proestrus day, females were placed with males for 12 h, and afterward, the presence of sperm in vaginal smears was checked. Pregnant females were left undisturbed in their home cages. The experiments were approved by the Local Ethics Committee, Kraków, Poland (approval no. 204/2018, 28.06.2018).

### 2.2. Chemicals

FPR2 agonists LXA4 and AT-LXA4 were obtained from Cayman Chemical Company, Ann Arbor, USA). Compound MR-39 ((S)-3-(4-cyanophenyl)-N-[[1-(3-chloro-4-fluorophenyl) cyclopropyl]methyl]-2-[3-(4-fluorophenyl)ureidopropanamide) was prepared as we described previously [43,44]. The FPR2 antagonist WRW4 was purchased from Alomone Labs, Israel. The bacterial endotoxin lipopolysaccharide (LPS; *Escherichia coli* 0111:B4) was obtained from Sigma-Aldrich, St. Louis, MO, USA.

### 2.3. Cell Culture

The cultures of the microglial cells were prepared from the cortices of 1–2-day-old Sprague-Dawley rat pups according to the procedure described by Zawadzka and Kaminska (2005) [45] with our slight modifications [46,47]. Briefly, after decapitation, the brains were removed and the cerebral cortices were cut into small pieces. The minced tissue was incubated in Hanks' balanced salt solution (HBSS, Gibco, Waltham, MA, USA) containing glucose, bovine serum albumin (BSA), and HEPES with 0.025% trypsin at 37 °C for 20 min. The trypsinization process was stopped by adding the trypsin inhibitor Glycine max (soybean) (Sigma-Aldrich, St. Louis, MO, USA). A completely dissociated suspension of the tissue was prepared by mild trituration. Next, the cells were plated at a density of 3 × 105 cells/cm2 in a culture medium consisting of Dulbecco's modified Eagle's medium

(DMEM) with GlutaMax and high glucose (4.5 g/L) supplemented with heat-inactivated 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (all reagents obtained from Gibco, Waltham, MA, USA) in poly-L-lysine-coated 75-$cm^2$ culture flasks. After 3 days, the culture medium was removed and replaced with a fresh medium. On the 9th day in vitro (37 °C, 5% $CO_2$), the flasks were agitated on a horizontal shaker (1 h, 37 °C, 80 rpm). After centrifugation, the cells were resuspended in the culture medium and seeded at a final density of $1.25 \times 10^6$ cells/well in 6-well plates, $2 \times 10^5$ cells/well in 24-well plates, or $4 \times 10^4$ cells/well in 96-well plates. Two days after plating the cells were used for experiments. One hour before the cell treatment, the culture medium was changed to a medium with 1% FBS. The purity of microglial cell cultures was assessed as previously described [43,44] using the specific microglia marker anti-Iba-1 antibody (ab5076, Abcam, Cambridge, UK). Images were captured using a confocal microscope (Leica Microsystems CMS GmbH, Mannheim, Germany). We obtained a homogeneous microglia population (greater than 95% Iba-1 positivity) (representative fluorescence images of microglia cells acquired by confocal microscopy, in Supplementary Materials).

### 2.4. Cell Treatment

The cells were pretreated for 1 h with various concentrations of FPR2 agonists, i.e., LXA4, AT-LXA4, and MR-39, and then stimulated for 3, 6, and/or 24 h with LPS (0.1 μg/mL). Additionally, in some experiments, to confirm the involvement of the FPR2 receptor in the effects of the examined ligands, the FPR2 antagonist synthetic peptide WRW4 (10 μM) was added to the cell cultures 30 min before the tested agonists. Stock solutions of the examined compounds were prepared as follows: LXA4 and AT-LXA4 (1 mM ethanol), MR-39 (1 mM DMSO), WRW4 (1 mM distilled water), and LPS (1 mg/mL PBS). The final solutions of the tested compounds were prepared in distilled water. Each experimental set of the control cultures was supplemented with the appropriate vehicles, and the solvent was present in cultures at a final concentration of 0.1% ($v/v$).

### 2.5. Lactate Dehydrogenase (LDH) Release Assay

To estimate cell damage 3, 6, or 24 h after LPS treatment, the lactate dehydrogenase (LDH) release into the culture media was measured as previously described [48]. Cell culture supernatants were incubated with the reagent mixture according to the supplier's instructions (cytotoxicity detection kit, Roche, Mannheim, Germany). The intensity of the red color formed in the assay, measured at a wavelength of 490 nm using an Infinite M200PRO microplate reader (TECAN, Männedorf, Switzerland), is proportional to the LDH activity and to the number of damaged cells. The data were normalized to the activity of the LDH released from vehicle-treated cells (100%) and expressed as a percentage of the control ± SEM.

### 2.6. Mitochondrial Membrane Potential ($\Delta\psi m$) Assay

JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide, Cayman Chemical Company, Ann Arbor, MI, USA) is a positively charged cationic dye that exhibits membrane potential-dependent accumulation in mitochondria. It was used to study the change in the mitochondrial membrane potential of microglial cells as previously described [49]. Briefly, the cells were seeded into 96-well black plates and treated with MR-39, LXA4, or AT-LXA4 for 1 h before the LPS (0.1 μg/mL) was added to the cultured cells for 3 h or 24 h. Next, the cells were stained with JC-1 for 30 min at 37 °C. In healthy cells with high mitochondrial potential, JC-1 forms complexes with intense red fluorescence (535 nm excitation and 595 nm emissions); however, in apoptotic or unhealthy cells with low potential, JC-1 remains in the monomeric form, showing green fluorescence (485 nm excitation and 535 nm emissions). Fluorescence intensities were measured using an Infinite M200PRO microplate reader (TECAN, Männedorf, Switzerland), and the ratio of fluorescence intensity was used as an indicator of cell health. A decrease in the red/green

fluorescence intensity ratio was interpreted as a loss of Δψm, whereas an increase in the ratio was interpreted as a gain in Δψm.

### 2.7. Caspase-3 Activity

Caspase-3 activity was detected using a caspase-3 colorimetric assay kit (BioVision, Milipitas, CA, USA). Microglial cells were lysed 3 h or 24 h after treatment with cell lysis buffer (BioVision, Milipitas, CA, USA), incubated on ice for 10 min and centrifuged (1 min, 4 °C, 14,000 rpm). The obtained supernatant was incubated with a reaction buffer containing dithiothreitol (DTT, 10 mM) and DEVD-p-nitroaniline substrate (DEVD-pNA, 200 μM) for 2 h at 37 °C. The chromophore p-NA light emission was quantified using an Infinite M200PRO microplate reader (TECAN, Männedorf, Switzerland) at a wavelength of 405 nm. The data (expressed as the mean relative fluorescence units, RFU) were normalized to the protein level (measured by the BCA method) and then calculated as a percent of control cultures and presented as the mean ± SEM.

### 2.8. Intracellular ROS Assay

To determine the intracellular level of the reactive oxygen species (ROS), the 2′,7′-dichlorofluorescin diacetate (DCFH-DA) test was used according to the manufacturer's instructions (Cell Biolabs, San Diego, CA, USA) as previously reported [47]. After 3 h or 24 h of microglial treatment, the cells were washed with a phosphate-buffered saline buffer and then incubated with DCFH-DA (10 μM) for 30 min at 37 °C. DCFH-DA diffuses into cells and is deacetylated by cellular esterase to nonfluorescent 2′,7′-dichlorodihydrofluorescin (DCFH), which is rapidly oxidized to highly fluorescent 2′,7′- dichlorodihydrofluorescein by ROS. The fluorescence intensity is proportional to the ROS levels within the cell cytosol. The fluorescence intensity was detected using an Infinite M200PRO microplate reader (TECAN, Männedorf, Switzerland) with excitation and emission wavelengths of 485 nm and 535 nm, respectively. The data were normalized against the fluorescence intensity of the control cells (100%) and presented as a percentage of the control ± SEM.

### 2.9. NO Release Assay (Nitrite Ion in Solution)

To assess the production of nitric oxide (NO) from LPS-treated microglial cells, the extracellular release of nitrite ($NO^{2-}$) was measured using the Greiss reaction as previously described [47]. Next, 3 h and 24 h after treatment, 50 μL of cell culture medium was collected and mixed with an equal volume of Griess reagent (0.1% N-1-naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid) in a 96-well plate and incubated for 10 min at room temperature. Absorbance was measured at 540 nm in an Infinite M200PRO microplate reader (TECAN, Männedorf, Switzerland). The data were normalized to the NO released from vehicle-treated cells (100%) and expressed as a percentage of the control ± SEM.

### 2.10. Immunocytochemistry

Immunofluorescent staining and confocal imaging were performed as described previously [50]. Briefly, microglial cells were seeded on glass coverslips in 24-well plates. Cells were fixed with 4% paraformaldehyde for 20 min and permeabilized in cool (4 °C) 0.1% Triton X-100. Subsequently, the cells were blocked with 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) at 4 °C for 1 h. Cells were incubated with an FPR2 rabbit polyclonal antibody (Huabio, Greater Boston, MA, USA; 1:50) or anti-Iba1 antibody (Abcam, Cambridge, UK; 1:200) overnight at 4 °C and then incubated with a secondary goat anti-rabbit antibody conjugated with the fluorescent dye AlexaFluor 647 (Abcam, Cambridge, UK; 1:300) or donkey anti-goat antibody conjugated with the fluorescent dye AlexaFluor 555 (Abcam, Cambridge, UK; 1:300) for 4 h at room temperature (RT) in the dark. Finally, the cells were incubated with phalloidin conjugated with AlexaFluor 488 dye (Invitrogen, Waltham, MA, USA; 1:200) at RT for 1 h in the dark. Cell nuclei were stained with DAPI or Hoechst 33,342 (Invitrogen, Waltham, MA, USA; 1:5000) for 15 min

at RT in the dark. Images were acquired on 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).

### 2.11. Quantitative Analysis of Confocal Fluorescent Images of Microglia

The cell spread area was determined from actin cytoskeleton images by applying a threshold allowing us to cover the spread area of each analyzed cell. Then, the area of the thresholded object was determined in ImageJ by function analysis particles. Fluorescence intensity was derived from images showing fluorescently stained FPR2 in the microglia. The threshold for FPR2 intensity for the thresholded area was determined in ImageJ by a function analysis of the particles as previously described by Prauzner-Bechcicki et al. (2015) and Bollmann et al. (2015) [51,52].

### 2.12. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Microglial cells were lysed by adding 200 µL TRI® Reagent (Sigma-Aldrich, St. Louis, MO, USA) 24 h after LPS (0.1 µg/mL) treatment and stored at −20 °C until isolation. Total RNA was extracted from the microglial cells following the TRIzol® reagent user guide instructions (Thermo Fisher Scientific, Waltham, MA, USA). The RNA concentration was determined by a NanoDrop spectrophotometer (ND/1000 UV/Vis, Thermo Fisher NanoDrop, Waltham, MA, USA). The synthesis of complementary DNA (cDNA) was performed via reverse transcription from equal amounts of RNA (600 ng) using an NG dART RT kit (EURx, Gdansk, Poland) according to the manufacturer's instructions. cDNA was amplified with a FastStart Universal Probe Master (Rox) kit (Roche, Basel, Switzerland) and TaqMan probes (Thermo Fisher Scientific, Waltham, MA, USA) for the following genes: *Cd40* (*cluster of differentiation 40;* Rn01423590_m1), *Cd68* (*cluster of differentiation 68;* Rn01495634_g1), *Cd206* (*cluster of differentiation 206;* Rn01487342_m1), *Arg1* (*arginase 1;* Rn00691090_m1), *Igf-1* (*insulin-like growth factor 1;* Rn00710306_m1), *Il-1β* (*interleukin 1β;* Rn00580432_m1), *Il-10* (*interleukin 10;* Rn01644839_m1), and *Tnf-α* (*tumor necrosis factor α;* Rn00562055_m1) (all obtained from Thermo Fisher Scientific, Waltham, MA, USA). Next, amplification was carried out in a total volume of 20 µL containing 10 µL FastStart Universal Probe Master (Rox), 1 µL cDNA used as the PCR template, 1 µL TaqMan forward and reverse primers, and 250 nM hydrolysis probe labeled with the fluorescent reporter dye fluorescein (FAM) at the 5′-end and a quenching dye at the 3′-end and 8 µL RNase-free water. The thermal cycling conditions were 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The samples were run in 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. Furthermore, *B2m* (*beta-2 microglobulin;* Rn00560865_m1) (Thermo Fisher Scientific, Waltham, MA, USA) was used as the reference gene.

### 2.13. Enzyme-Linked Immunosorbent Assay (ELISA)

The cytokines TNF-α (tumor necrosis factor-α), IL-1β (interleukin 1-β), IL-6 (interleukin-6), and IL-10 (interleukin 10) were measured in supernatants harvested 3 h or 24 h after LPS treatment. The protein levels of the cytokines TNF-α (Rat TNF-alpha uncoated ELISA kit, Thermo Fisher, Waltham, MA, USA), IL-1β (Rat interleukin 1-beta, Bioassay Technology Laboratory, Shanghai, China), IL-6 (Rat interleukin 6 ELISA kit, Bioassay Technology Laboratory, Shanghai, China), and IL-10 (Rat interleukin 10 ELISA kit, Bioassay Technology Laboratory, Shanghai, China) were measured using commercially available enzyme-linked immunosorbent assay kits according to the manufacturers' instructions. The detection limits were as follows: TNF-α, 16 pg/mL; IL-1β, 10.27 pg/mL; IL-6, 0.052 ng/L; IL-10, and 1.51 pg/mL. The inter assay precision was as follows: TNF-α < 8.8%; IL-1β < 10%; IL-6 < 10%; IL-10 < 10%, The intra assay precision was as follows: TNF-α: < 2.1%; IL-

1β: <8%; IL-6 < 8%; and IL-10 < 8%. Positive controls for each assay were provided by the manufacturers.

### 2.14. Western Blot Analyses in Homogenates of Microglial Cells

Western blot analyses were conducted as previously described [47,53]. Briefly, 30 min (for the ERK1/2 pathway) or 24 h (for the p38 and NF-κB pathways) after LPS treatment (0.1 μg/mL), the cells were lysed with the RIPA lysis buffer containing protease inhibitors, phosphatase inhibitors, 1 mM sodium orthovanadate, and 1 mM phenylmethanesulfonyl fluoride (all reagents were from Sigma-Aldrich, St. Louis, MO, USA). The lysates (equal amounts of protein) and the buffer (4 × Laemmli buffer, Roche, Basel, Switzerland) were mixed and boiled for 6 min before they were loaded onto the gel. The proteins were separated using 4–20% CriterionTM TGXTM Precast Midi Protein Gels, with 12-well plates (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 room temperature, and incubated overnight at 4 °C with the antibodies diluted in a SignalBoost Immunoreaction Enhancer kit (Millipore, Warsaw, Poland): anti-phospho-NF-κB (1:1000, #3033, Cell Signaling, MA, USA), anti-phospho-p38 (1:500, sc-101759), anti-phospho-ERK1/2 (1:500, sc-81492) (both from Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and anti-vinculin (1:15,000, V9264, Sigma-Aldrich, St. Louis, MO, USA). After incubation, the membranes were washed with a TBS containing 0.1% Tween-20 (TBST) and incubated with horseradish peroxidase-linked secondary antibodies: horse anti-mouse immunoglobulin G (IgG, 1:10,000, PI-2000 Vector Laboratories) and goat-anti-rabbit IgG (1:10,000, PI-1000, Vector Laboratories) at room temperature for 1 h. Next, the membranes were washed, and the immune complexes were detected using Pierce® ECL Western blotting substrate (Thermo Fisher, Waltham, MA, USA) and visualized using a Fujifilm LAS-1000 system (Fuji Film, Tokyo, Japan). After phospho-NF-κB, phospho-p38, and phospho-ERK1/2 determination, the blots were stripped in a stripping buffer containing 100 μL of Tris-HCl (pH = 6.7), 2% SDS, and 700 μL of 2-mercaptoethanol (all from Sigma-Aldrich, St. Louis, MO, USA). They were then re-probed with antibodies against unphosphorylated NF-κB (1:1000, #6956), ERK1/2 (1:2000, #9102) (both from Cell Signaling, Beverly, MA, USA), and p38 (1:500, sc-7972, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) diluted in a SignalBoost Immunoreaction Enhancer kit for the normalization of all bands. The relative levels of immunoreactivity were densitometrically quantified using Fujifilm Multi Gauge software (Fuji Film, Tokyo, Japan).

### 2.15. Statistical Analysis

The results were derived from independent microglial cultures and are presented as the mean ± SEM (standard error of the mean). The results of the cell viability/death processes, mitochondrial membrane potential, caspase-3, and oxidative stress (NO, ROS) are presented as the mean ± SEM percentage of the control (vehicle-treated cells). The data obtained in the ELISA study are presented as the mean ± SEM percentage of the control (vehicle-treated cells); those for RT-PCR are presented as an average fold ± SEM, and for the Western blot analysis, the results are presented as the mean ± SEM percentage of the control (vehicle-treated cells). The data obtained from confocal imaging are presented as the mean value of the calculated parameter ± SEM, while the differences between groups were compared with Student's *t*-test. All of the other groups were compared by a one-way or two-way analysis of variance (ANOVA), followed by Duncan's post hoc test to assess the differences between the treatment groups. A *p*-value less than or equal to 0.05 was considered statistically significant. * $p < 0.05$ vs. the control group; # $p < 0.05$ vs. the LPS group; ^ $p < 0.05$ vs. the agonist + LPS group. All graphs were prepared using GraphPad Prism 5.

## 3. Results

### *3.1. The Time-Dependent Impact of LXA4, AT-LXA4, and MR-39 on Lactate Dehydrogenase Release in Microglial Cells Stimulated with Lipopolysaccharide*

In the first part of the experiments, we evaluated the time-dependent properties of lipoxin A4 (LXA4), aspirin-triggered lipoxin A4 (AT-LXA4), and MR-39 against LPS-induced lactate dehydrogenase (LDH) release, which is a marker of cell death after damage to the plasma membrane. Exposure of microglial cells to LPS (0.1 μg/mL) for 3, 6, and 24 h caused a significant increase in LDH activity. The tested compounds did not change the LDH release under basal conditions. LXA4 at concentrations of 0.01 μM ($p < 0.0001$) and 0.1 μM ($p < 0.0001$) inhibited the LDH release only after 3 h of LPS stimulation (Figure 1). The AT-LXA4-evoked effect was long-lasting because this ligand diminished LDH release after 3 h (0.001; $p = 0.000398$; 0.01; $p = 0.000102$; and 0.1 μM; $p < 0.0001$), 6 h (0.01; $p = 0.02292$ and 0.1 μM; $p = 0.037909$) and 24 h (0.1 μM; $p = 0.000211$) of LPS exposure (Figure 2). On the other hand, MR-39 inhibited cell death only after 24 h of incubation with LPS at concentrations of 1 ($p < 0.0001$) and 5 μM ($p = 0.000104$) (Figure 3). Importantly, the pretreatment of microglial cells with the FPR2 receptor antagonist WRW4 (10 μM) reversed the inhibitory effect of the examined compounds, in the case of LXA4 at 0.1 μM ($p = 0.047844$) and AT-LXA4 at 0.1 μM ($p = 0.032741$) after 3 h of stimulation with LPS while for MR-39 at a dose of 1 μM and 5 μM ($p = 0.033522$; $p = 0.015338$, respectively) after 24 h of endotoxin presence in the microglial cultures. This suggests that the observed effects are mediated through the interaction with FPR2 (Figure 4). Based on these data we selected LXA4 (at the dose of 0.1 μM), AT-LXA4 (at the dose of 0.1 μM), as well as MR-39 (at the dose of 1 μM) for the vast majority of our further research. Moreover, considering the time-dependent protective studies of the agonists on the LPS-evoked LDH release, which only in the case of AT-LXA4 showed an influence on this parameter after 6 h, we performed further studies after 3 h and 24 h of LPS stimulation.

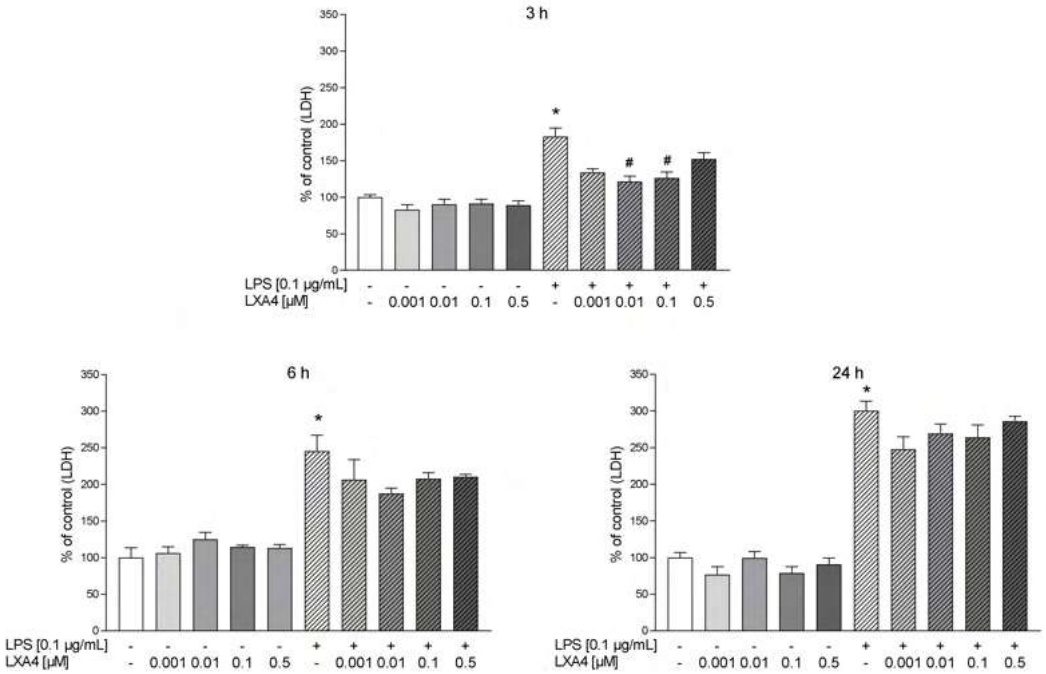


**Figure 1.** The impact of LXA4 on LPS-induced LDH release in rat microglial cultures. The cells were preincubated with LXA4 (0.001–0.5 μM) for 1 h and then treated with 0.1 μg/mL LPS for 3, 6, and 24 h. Control cultures were treated with the appropriate vehicle. The data are presented as the mean ± SEM percentage of control (vehicle-treated cells) of independent experiments, $n$ = 2–5 in each experiment. * $p < 0.05$ vs. control group, # $p < 0.05$ vs. LPS group. LXA4–lipoxin A4; LPS–lipopolysaccharide; LDH–lactate dehydrogenase.

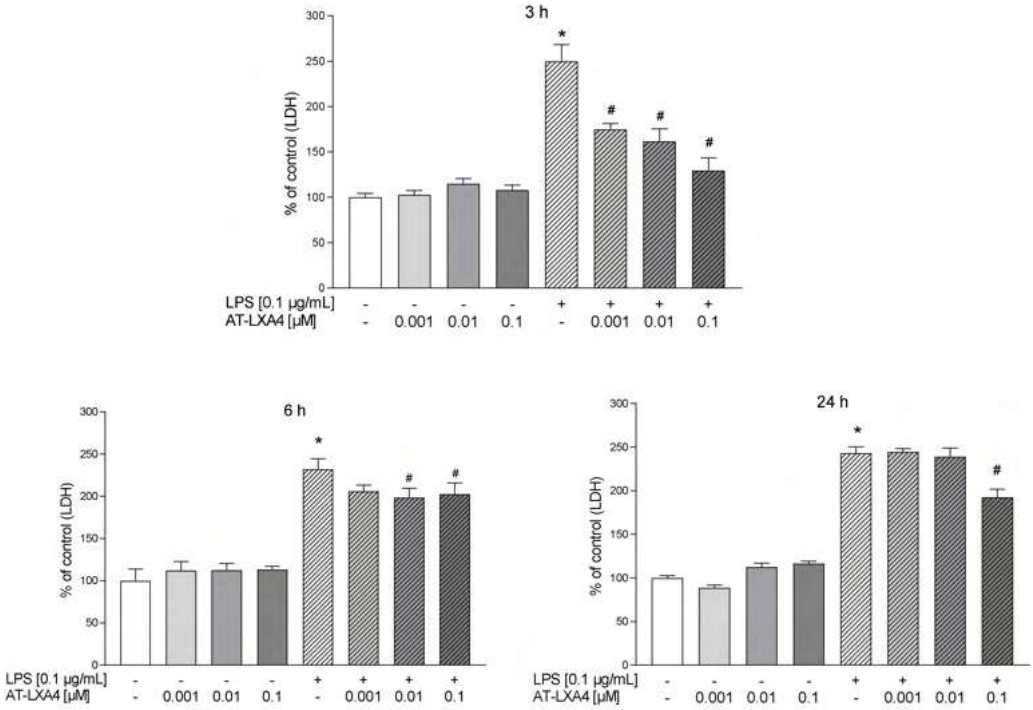


**Figure 2.** The impact of AT-LXA4 on LPS-induced LDH release in rat microglial cultures. The cells were preincubated with AT-LXA4 (0.001–0.1 µM) for 1 h and then treated with 0.1 µg/mL LPS for 3, 6, and 24 h. Control cultures were treated with the appropriate vehicle. The data are presented as the mean ± SEM percentage of control (vehicle-treated cells) of independent experiments, $n$ = 2–5 in each experiment. * $p < 0.05$ vs. control group, # $p < 0.05$ vs. LPS group. AT-LXA4–aspirin-triggered lipoxin A4; LPS–lipopolysaccharide; LDH–lactate dehydrogenase.

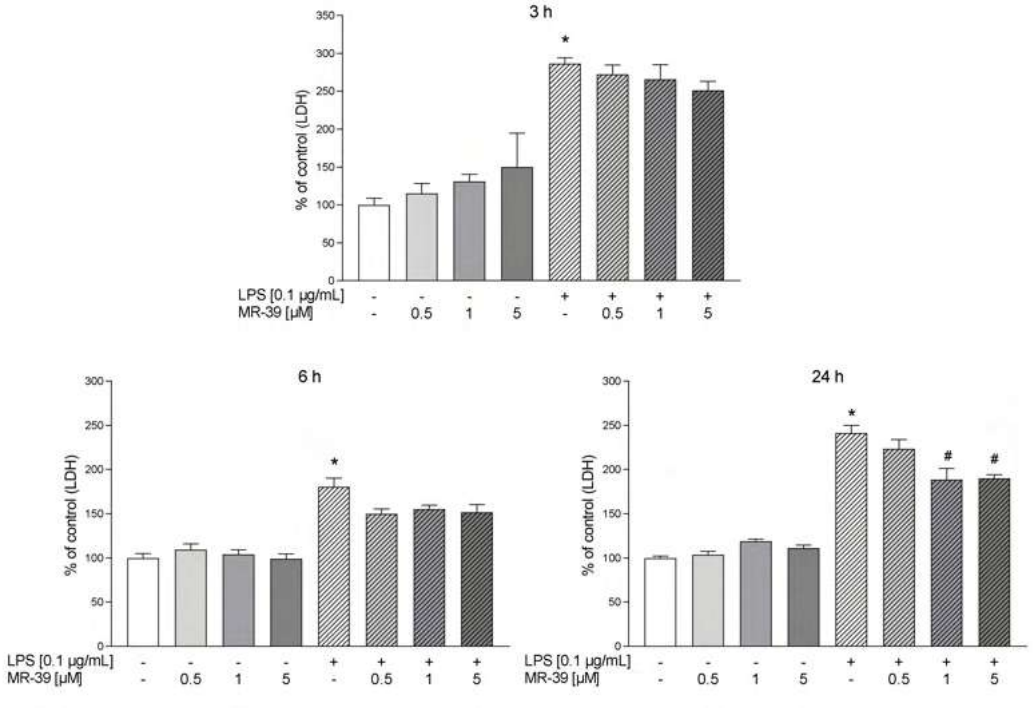


**Figure 3.** The impact of MR-39 on LPS-induced LDH release in rat microglial cultures. The cells were preincubated with MR-39 (0.5–5 µM) for 1 h and then treated with 0.1 µg/mL LPS for 3, 6, and 24 h. Control cultures were treated with the appropriate vehicle. The data are presented as the mean ± SEM percentage of the control (vehicle-treated cells) from independent experiments, $n$ = 2–5 in each experiment. * $p < 0.05$ vs. control group, # $p < 0.05$ vs. LPS group. LPS–lipopolysaccharide; LDH–lactate dehydrogenase.

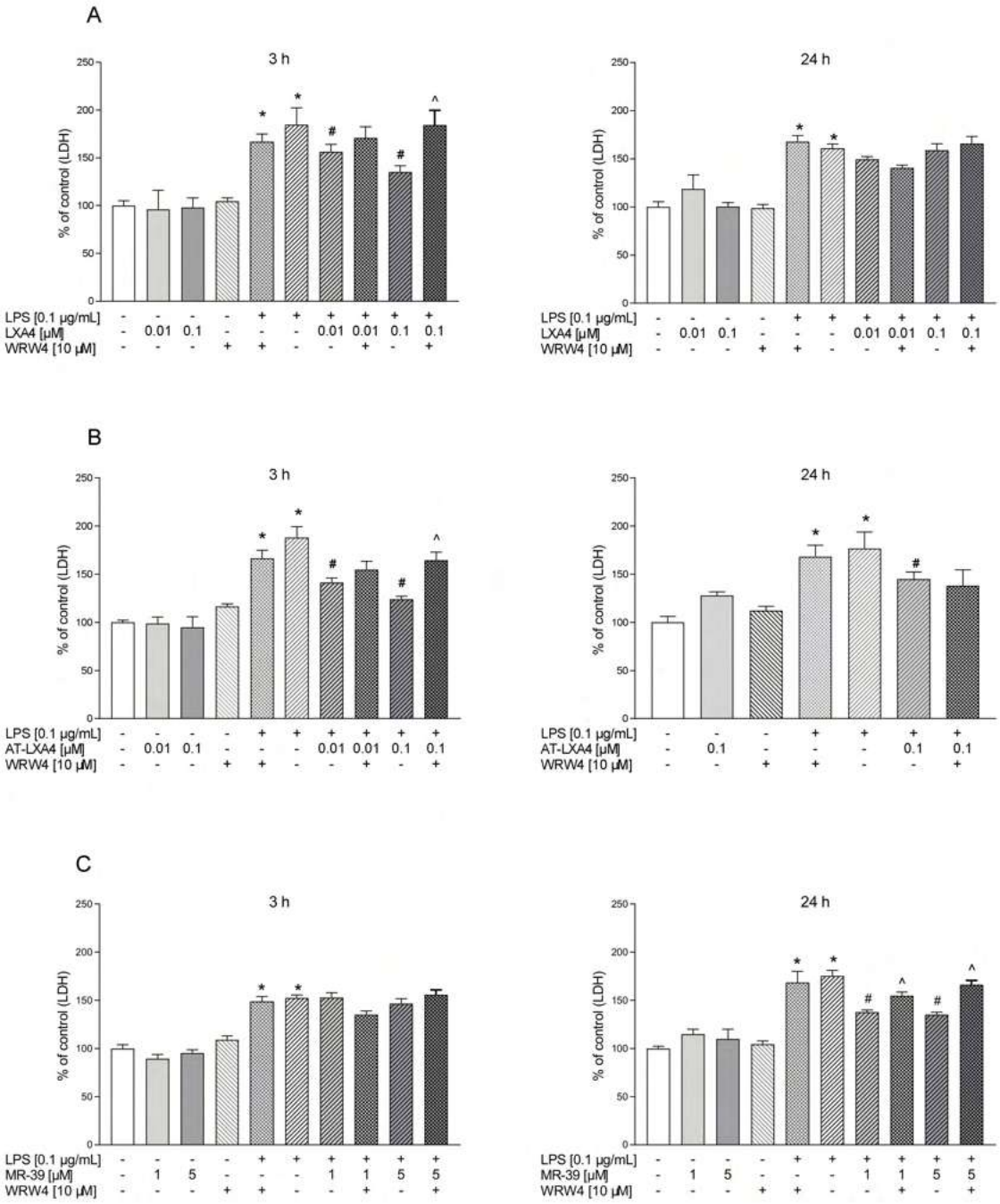


**Figure 4.** The time-dependent impact of LXA4 (**A**), AT-LXA4 (**B**), and MR-39 (**C**) on LPS-induced LDH release in rat microglial cultures. The cells were pretreated for 30 min with the FPR2 antagonist WRW4 (10 μM). After that, LXA4 (0.01 μM or 0.1 μM), AT-LXA4 (0.01 μM or 0.1 μM), or MR-39 (1 or 5 μM) was added for 1 h, and then the cells were stimulated for 3 or 24 h with lipopolysaccharide (LPS; 0.1 μg/mL). Control cultures were treated with the appropriate vehicle. The data are presented as the mean ± SEM percentage of the control (vehicle-treated cells) of independent experiments, $n$ = 2–5 in each experiment. * $p < 0.05$ vs. the control group, # $p < 0.05$ vs. the LPS group, ^ $p < 0.05$ vs. the agonist + LPS group. LXA4–lipoxin A4; AT-LXA4–aspirin-triggered lipoxin A4; LPS–lipopolysaccharide; LDH–lactate dehydrogenase.

### *3.2. Visualization of FPR2 Presence in Microglial Cells Stimulated with Lipopolysaccharide*

Although it is widely accepted that FPR2 is expressed in microglial cells [30,40,43,54], most data point to the low expression of FPR2 under basal conditions. In contrast, after stimulation with various immunogens, FPR2 expression is upregulated. In the present study, by confocal microscopy, we showed the presence of FPR2 in microglial cells under basal conditions (Figure 5A,B). Moreover, as demonstrated in Figure 5C,D, there was a significant increase in FPR2 fluorescence intensity after long-lasting (24 h) LPS stimulation (339.74 ± 24.26) in comparison to the control group. However, this effect was not observed after 3 h of LPS treatment (63.24 ± 15.91).

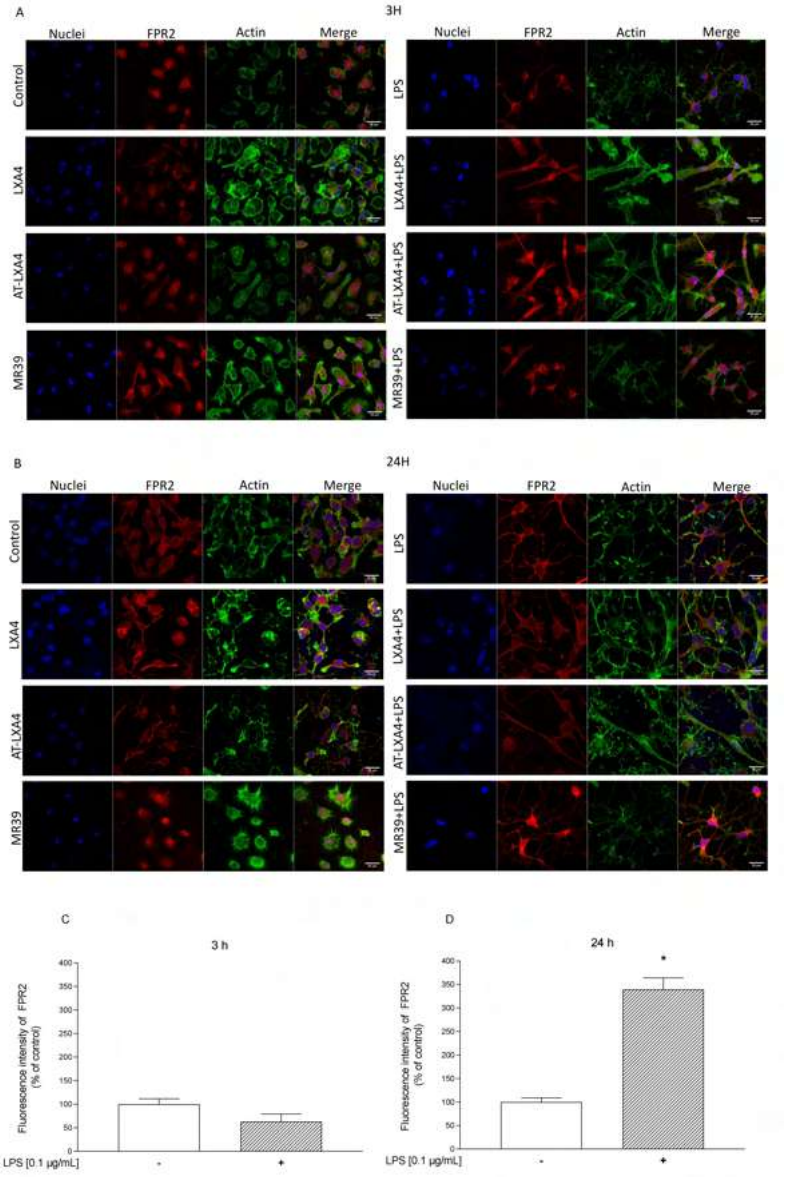


**Figure 5.** Representative fluorescence images of microglial cells acquired by confocal microscopy 3 h (**A**) and 24 h (**B**) after FPR2 agonists ((LXA4 (0.1 µM), AT-LXA4 (0.1 µM), or MR-39 (1 µM)) and/or lipopolysaccharide (LPS; 0.1 µg/mL) stimulation. Fluorescence intensity of the FPR2 receptor was calculated from images recorded with the use of a fluorescent confocal microscope. Data are derived for control microglia and microglia activated by LPS after 3 h (**C**) and 24 h (**D**) of treatment. Bars present the mean intensity value normalized to the control ± SEM. Nuclei appear in blue, FPR2 in red, and AlexaFluor 488-labeled phalloidin for F-actin in green. Scale bar: 20 µm is located in the bottom right corner of each image. * $p < 0.05$ vs. the control group.

### *3.3. The Impact of LXA4, AT-LXA4, and MR-39 on the Mitochondrial Membrane Potential in Microglial Cells Stimulated with Lipopolysaccharide*

Changes in the mitochondrial membrane potential (Δψm) have been shown to be involved in microglial activation and the production of pro-inflammatory factors [55]. In "untreated cells" with a normal Δψm, JC-1 dye enters and accumulates in energized and negatively charged mitochondria and spontaneously forms red fluorescent J-aggregates. In contrast, in affected or apoptotic cells, JC-1 dye also enters the mitochondria but to a lesser degree since the inside of the mitochondria is less negative because of increased membrane permeability and the consequent loss of electrochemical potential. Under this condition, JC-1 does not reach a sufficient concentration to trigger the formation of JC-1 aggregates, thus retaining its original green fluorescence. Therefore, the fluorescently sensitive probe JC-1 was used to check the effect of LPS alone and together with the tested FPR2 ligands on the status of the microglial mitochondrial membrane potential. As shown in Figure 6A, the microglia displayed a collapse of Δψm after 3 h ($p = 0.0006$) and 24 h ($p = 0.001781$) of exposure to LPS (0.1 µg/mL). MR-39 (1 µM) slightly attenuated the LPS-induced decrease in mitochondrial potential after 3 h of LPS stimulation ($p = 0.021667$). This effect was prolonged and also observed after 24 h, ($p = 0.024291$). In contrast, we did not observe any impact of LXA4 or AT-LXA4 on LPS-induced changes in mitochondrial membrane potential (Figure 6A).

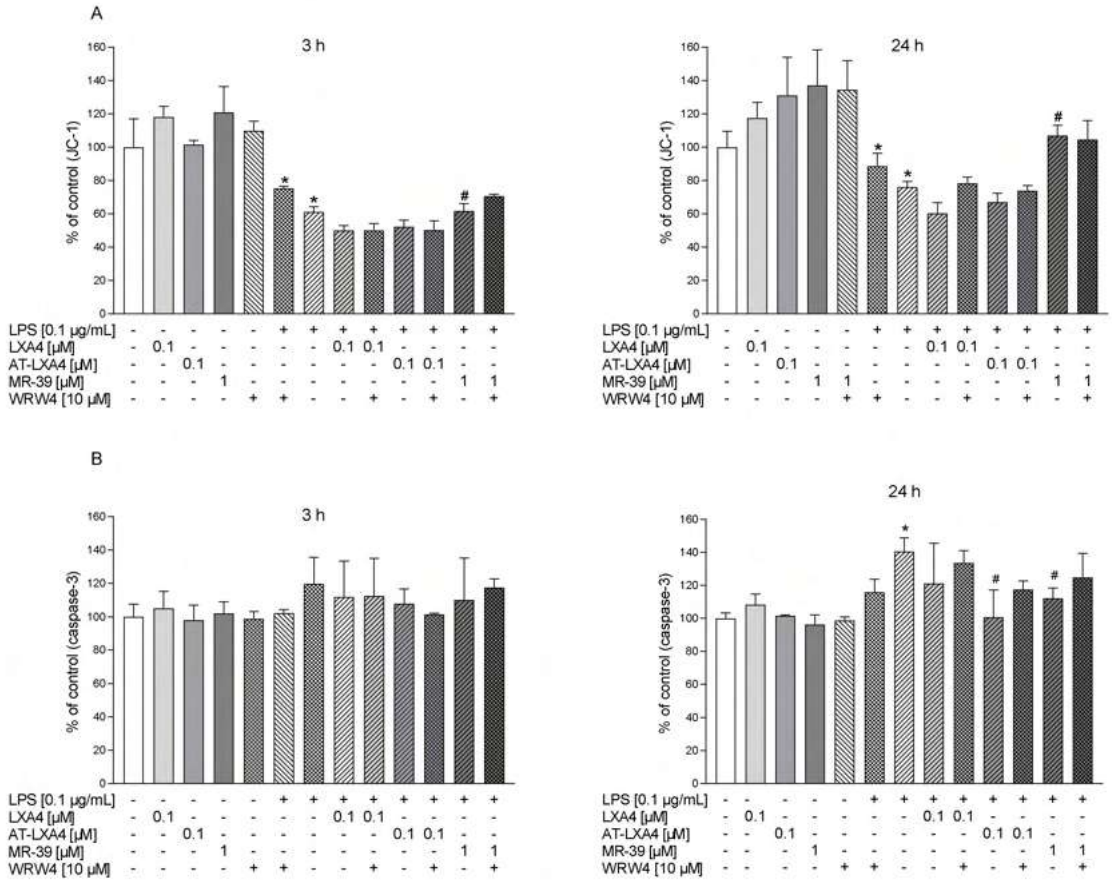


**Figure 6.** The impact of LXA4, AT-LXA4, and MR-39 on the mitochondrial membrane potential (**A**) and caspase-3 activity (**B**) in rat microglial cultures. The cells were pretreated for 30 min with the FPR2 antagonist WRW4 (10 µM). After that, LXA4 (0.1 µM), AT-LXA4 (0.1 µM), or MR-39 (1 µM) was added for 1 h, and then the cells were stimulated for 3 h or 24 h with lipopolysaccharide (LPS; 0.1 µg/mL). Control cultures were treated with the appropriate vehicle. The data are presented as the mean ± SEM percentage of control (vehicle-treated cells) of independent experiments, $n = 2–5$ in each experiment. * $p < 0.05$ vs. control group, # $p < 0.05$ vs. LPS group. LXA4: lipoxin A4; AT-LXA4: aspirin-triggered lipoxin A4; LPS: lipopolysaccharide.

### 3.4. The Impact of LXA4, AT-LXA4, and MR-39 on Caspase-3 Activity in Microglial Cells Stimulated with Lipopolysaccharide

Caspase-3 is a well-known executor of apoptotic cell death, and its activation also promotes the pro-inflammatory activation of microglial cells [56]. Therefore, in the next set of experiments, we determined the effect of LXA4, AT-LXA4, and MR-39 on the LPS-induced activity of caspase-3. As revealed in Figure 6B, after 3 h of incubation the lack of neither LPS nor tested ligands were observed. On the other hand, after 24 h of incubation LPS significantly potentiated the activation of caspase-3 ($p = 0.000333$). The FPR2 ligands alone had no effect on caspase-3 activation, but AT-LXA4 (0.1 μM; $p = 0.00034$) and MR-39 (1 μM; $p = 0.00244$) significantly reduced the LPS-induced changes (Figure 6B), while LXA4 did not affect this parameter. On the other hand, pretreatment with WRW4 (10 μM) did not change the effects of AT-LXA4 and MR-39.

### 3.5. The Impact of LXA4, AT-LXA4, and MR-39 on Reactive Oxygen Species (ROS) Production in Microglial Cells Stimulated with Lipopolysaccharide

Microglial cells subjected to various stimulators, including bacterial endotoxins, produce ROS. Excessive production of ROS by microglia is associated with neuroinflammation and can stimulate the microglial release of pro-inflammatory mediators, which can strongly prolong microglial activation. In the next set of experiments, we assessed the potential time-dependent antioxidant properties of the tested FPR2 agonists after stimulation with LPS microglial cultures. As shown in Figure 7A, we demonstrated that LPS treatment (0.1 μg/mL) enhanced ROS levels compared with untreated cells after 3 h ($p = 0.025314$) and 24 h ($p = 0.002313$) of incubation. The ROS intensity measurement in the microglial cells revealed that 0.1 μM LXA4 ($p = 0.033531$) and 1 μM MR-39 ($p = 0.009297$) reduced ROS production in the LPS-treated group after 3 h and 24 h of incubation, respectively (Figure 7A). Unfortunately, this effect was not modulated by the WRW4 pretreatment (data not shown).

### 3.6. The Impact of LXA4, AT-LXA4, and MR-39 on Nitric Oxide Release (NO) in Microglial Cells Stimulated with Lipopolysaccharide

The generation of ROS may lead to nitric oxide production from microglial cells. Moreover, NO causes the formation of peroxynitrite via a reaction with superoxide, which kills cells by disturbing mitochondrial processes and potentiates harmful pro-inflammatory responses [57]. Since we found that 3 h of LPS stimulation did not affect the NO release (data not shown), we evaluated the effects of the examined FPR2 ligands on the production of NO under basal conditions and after 24 h of LPS stimulation. LXA4, AT-LXA4, and MR-39 did not evoke any change in the NO levels under basal conditions. Treatment of microglial cells with LPS dramatically increased the nitric oxide release ($p < 0.0001$), which was significantly attenuated only by the higher dose of MR-39 (5 μM; $p = 0.015135$). Notwithstanding, the pretreatment of microglial cells with an antagonist of the FPR2 receptor WRW4 did not fully block the favorable effect of MR-39 on NO secretion (Figure 7B).

### 3.7. The Impact of LXA4, AT-LXA4, and MR-39 on Pro- and Anti-Inflammatory Factors' Expression in Microglial Cells Stimulated with Lipopolysaccharide

Microglia, as the pivotal immune reactive cells of the central nervous system, are the initial responders to pathogens or tissue damage and are responsible for the maintenance of or return to homeostasis. Recently, the presence of M1/M2 microglia phenotypes has been controversial and a subject of debate. Despite the view that microglia heterogeneity is context-dependent, and while the concept of functional polarization is gaining followers, the shift from the pro-inflammatory to anti-inflammatory activity is necessary for the proper repair of damaged tissue and the resolution of inflammation.

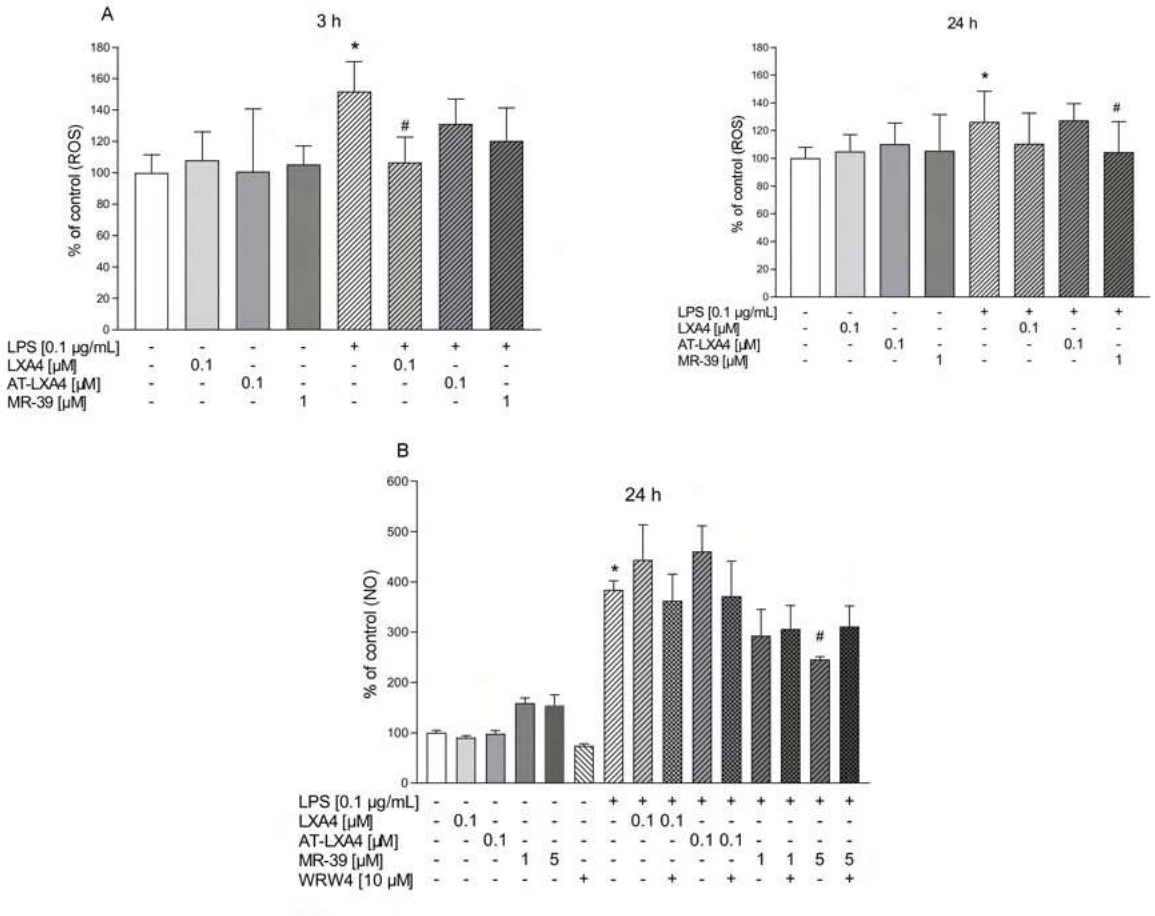


**Figure 7.** The impact of LXA4, AT-LXA4, and MR-39 on reactive oxygen species (**A**) and nitric oxide (**B**) release in rat microglial cultures. The cells were pretreated for 30 min with the FPR2 antagonist WRW4 (10 µM). After that, LXA4 (0.1 µM), AT-LXA4 (0.1 µM), or MR-39 (1 µM or 5 µM) was added for 1 h, and then the cells were stimulated for 3 h or 24 h with lipopolysaccharide (LPS; 0.1 µg/mL). Control cultures were treated with the appropriate vehicle. The data are presented as the mean ± SEM percentage of control (vehicle-treated cells) of independent experiments, $n$ = 2–5 in each experiment. * $p < 0.05$ vs. control group, # $p < 0.05$ vs. LPS group. LXA4: lipoxin A4; AT-LXA4: aspirin-triggered lipoxin A4; LPS: lipopolysaccharide; ROS: reactive oxygen species; NO: nitric oxide.

To demonstrate the impact of FPR2 stimulation through LXA4, AT-LXA4, or MR-39 on the microglial markers we evaluated the expression levels of various genes after 3 h and 24 h of LPS stimulation. As shown in Table 1, after 3 h of LPS exposure, we observed an elevated mRNA expression of *Il-1β* and *Tnf-α* ($p = 0.032$, $p < 0.0001$, respectively).

The statistical analysis revealed that MR-39 (5 µM) effectively decreased ($p = 0.028$) expression of *Il-1β*, whereas LXA4 (0.1 µM) and AT-LXA4 (0.1 µM) ($p = 0.009$, $p = 0.0002$, respectively) increased Il-1β mRNA expression. Importantly, all FPR2 agonists tested, i.e., LXA4 (0.1 µM), AT-LXA4 (0.1 µM), and MR-39 (5 µM) ($p = 0.044$, $p = 0.003$, $p < 0.0001$, respectively) significantly attenuated the LPS-induced increase in the expression of the *Tnf-α* gene. On the other hand, 3 h of LPS incubation upregulated *Arg-1* ($p = 0.002$) mRNA expression but downregulated *Igf-1* ($p = 0.006$) mRNA levels. A statistical analysis revealed that MR-39 effectively decreased ($p < 0.0001$) Arg-1. Importantly, all FPR2 agonists tested, i.e., LXA4, AT-LXA4, and MR-39 ($p = 0.014$, $p = 0.016$, $p = 0.012$) significantly increased the mRNA expression of IL-10 in LPS-stimulated microglial cells. We did not observe significant changes in the expression of *Cd206 expression* (Table 1A).

**Table 1.** The time-dependent effect of 3 h (**A**) and 24 h (**B)** of lipopolysaccharide stimulation and FPR2 ligands: MR-39, LX-A4, or AT-LXA4 treatment on the gene expression of pro-inflammatory (*Cd40*, *Il-1β*, *Tnf-α*, and *Cd68)* and anti-inflammatory (*Cd206*, *Arg1*, *Igf-1*, and *Il-10*) microglia markers. Control cultures were treated with the appropriate vehicle. The mRNA levels were measured using qRT-PCR from independent experiments; $n$ = 2–4 in respective experiments. The results are presented as the average fold change ± SEM. * $p < 0.05$ vs. control, # $p < 0.05$ vs. LPS group.

| **A** | | | | | |
|---|---|---|---|---|---|
| | **Control** | **LPS** | **MR + LPS** | **LXA4 + LPS** | **AT-LXA4 + LPS** |
| **Pro-inflammatory markers** | | | | | |
| *Cd40* | 1.04 ± 0.27 | 5.44 ± 1.67 | 1.42 ± 0.68 | 3.40 ± 1.99 | 4.16 ± 3.23 |
| *Il-1β* | 1.06 ± 0.19 | 19.57 ± 1.57 * | 13.84 ± 2.04 # | 42.34 ± 9.95 # | 35.61 ± 2.83 # |
| *Tnf-α* | 1.22 ± 0.70 | 19.13 ± 0.68 * | 4.10 ± 0.10 # | 9.17 ± 3.12 # | 9.55 ± 1.89 # |
| *Cd68* | 1.00 ± 0.00 | 0.22 ± 0.1 * | 0.10 ± 0.04 | 0.20 ± 0.10 | 0.17 ± 0.03 |
| **Anti-inflammatory markers** | | | | | |
| *Cd206* | 1.07 ± 0.38 | 0.21 ± 0.09 | 0.07 ± 0.03 | 0.16 ± 0.03 | 0.11 ± 0.01 |
| *Arg1* | 1.00 ± 0.07 | 1.81 ± 0.02 * | 0.27 ± 0.03 # | 1.02 ± 0.65 | 1.40 ± 0.88 |
| *Igf-1* | 1.02 ± 0.19 | 0.13 ± 0.05 * | 0.03 ± 0.01 | 0.13 ± 0.07 | 0.09 ± 0.01 |
| *Il-10* | 1.05 ± 0.17 | 1.69 ± 0.38 | 15.60 ± 7.95 # | 18.88 ± 9.67 | 29.58 ± 14.80 # |
| **B** | | | | | |
| **Pro-inflammatory markers** | | | | | |
| *Cd40* | 1.01 ± 0.07 | 0.46 ± 0.14 * | 0.62 ± 0.18 | 0.39 ± 0.04 | 0.68 ± 0.25 |
| *Il-1β* | 1.07 ± 0.13 | 9.06 ± 1.79 * | 13.24 ± 1.42 | 35.19 ± 7.12 # | 52.54 ± 13.15# |
| *Tnf-α* | 1.02 ± 0.10 | 0.33 ± 0.07 * | 0.88 ± 0.32 | 0.56 ± 0.12 | 0.88 ± 0.35 |
| *Cd68* | 1.01 ± 0.07 | 0.04 ± 0.01 | 0.07 ± 0.02 | 0.10 ± 0.02 | 0.03 ± 0.02 |
| **Anti-inflammatory markers** | | | | | |
| *Cd206* | 1.01 ± 0.06 | 0.01 ± 0.00 * | 0.01 ± 0.00 | 0.02 ± 0.01 | 0.03 ± 0.01 |
| *Arg1* | 1.04 ± 0.16 | 0.38 ± 0.10 * | 0.22 ± 0.06 | 0.48 ± 0.07 | 0.67 ± 0.19 |
| *Igf-1* | 1.05 ± 0.19 | 0.01 ± 0.00 * | 0.01 ± 0.00 | 0.04 ± 0.01 | 0.03 ± 0.01 |
| *Il-10* | 1.04 ± 0.09 | 4.46 ± 0.72 | 8.45 ± 1.61 # | 23.78 ± 2.58 # | 31.56 ± 5.77 # |

Simultaneously, the 24 h stimulation of microglia with LPS caused a significant decrease in *Cd40* and *Tnf-α* ($p = 0.046$ and $p = 0.005$, respectively) mRNA expression while stimulating the expression of Il-1β ($p = 0.015$) gene expression. After 24 h, we did not observe an inhibitory effect of the tested FPR2 ligands on the pro-inflammatory markers. Moreover, for LXA4 and AT-LXA4, we observed a statistically significant increase in the mRNA expression of *Il-1β* ($p = 0.038$, $p = 0.045$, respectively) compared to that in cells treated with LPS alone. Furthermore, we demonstrated that 24 h of LPS stimulation downregulated the mRNA expression of various anti-inflammatory markers, including *Cd206, Arg-1,* and *Igf*-1 ($p < 0.0001$, $p = 0.028$, $p < 0.0001$, respectively), in microglial cultures. Treatment with LXA4 ($p < 0.0001$), AT-LXA4 ($p < 0.0001$), and MR-39 ($p = 0.011$) upregulated the expression of *Il-10* after stimulation for 24 h with LPS microglial cultures (Table 1B). In both time-dependent experimental conditions, that is, after short or longer stimulation with bacterial endotoxin, we did not observe significant changes in microglial gene expression after the use of ligands alone compared to the control groups (data not shown).

### *3.8. The Impact of LXA4, AT-LXA4, and MR-39 on Pro- and Anti-Inflammatory Cytokine Production in Microglial Cells Stimulated with Lipopolysaccharide*

To determine the anti-inflammatory and pro-resolving impact of LXA4, AT-LXA4, and MR-39, we measured the production of the pro-inflammatory factors IL-1β, TNF-α, and IL-6 and the anti-inflammatory factor–IL-10 in LPS-stimulated microglial cells using ELISA. Additionally, to check whether the observed effect is mediated through the ligand interaction with FPR2, we used WRW4, i.e., a selective FPR2 antagonist.

We observed that 3 h of LPS treatment increased the TNF-α ($p = 0.00018$) and IL-6 ($p = 0.015095$) response, while after longer (24 h) LPS stimulation, all pro-inflammatory cytokines were measured, namely, TNF-α ($p < 0.0001$), IL-1β ($p = 0.000651$), and IL-6 ($p = 0.006116$).

We found that 3 h after LPS treatment only LXA4 (0.1 μM) significantly attenuated TNF-α release ($p = 0.00018$) and this effect was affected by WRW4 pretreatment ($p = 0.0343$). In contrast, after 3 h of LPS stimulation we did not found the effect of AT-LXA4 and MR-39 on TNF-α release as well as all tested FPR2 ligands on IL-1β production (Figure 8).

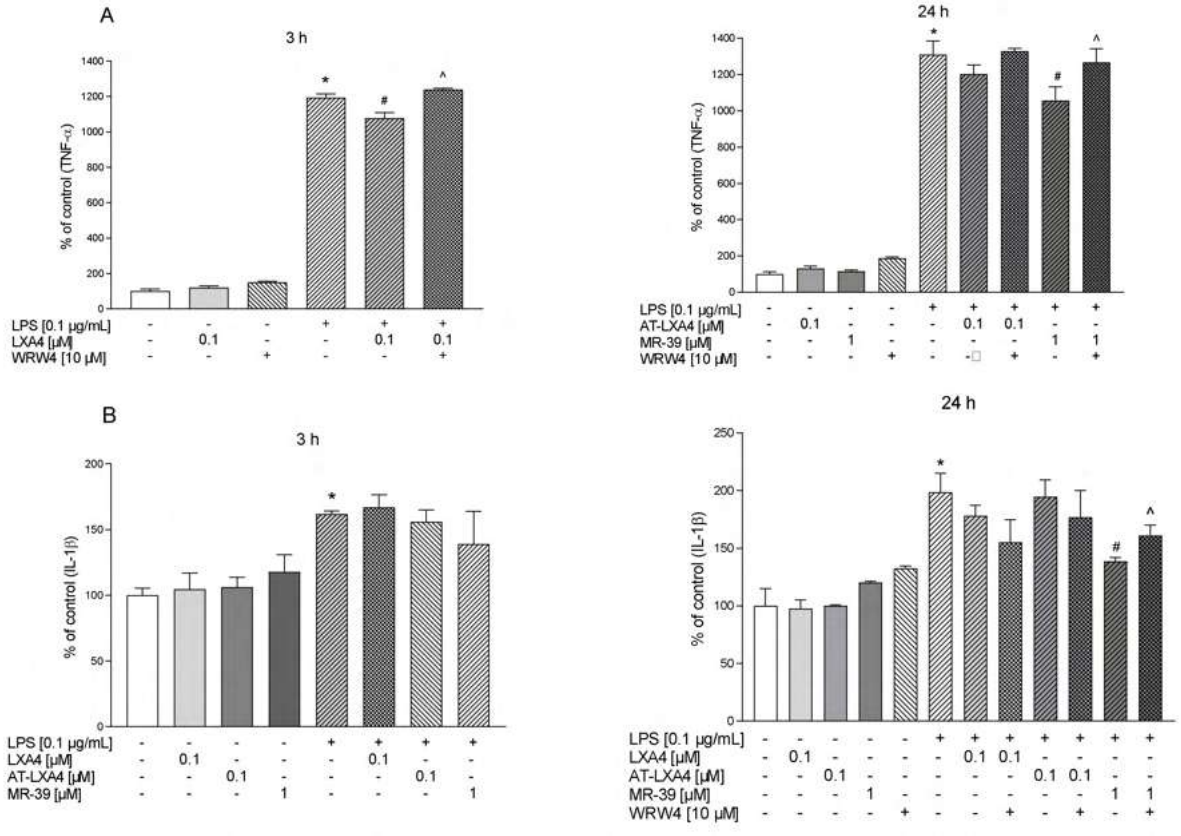


**Figure 8.** The impact of LXA4, AT-LXA4, and MR-39 on pro-inflammatory cytokines: TNF-α (**A**) and IL-1β (**B**) production in rat microglial cultures. The cells were pretreated for 30 min with the FPR2 antagonist WRW4 (10 μM). After that, LXA4 (0.1 μM), AT-LXA4 (0.1 μM), or MR-39 (1 μM) was added for 1 h, and then the cells were stimulated for 3 h or 24 h with lipopolysaccharide (LPS; 0.1 μg/mL). Control cultures were treated with the appropriate vehicle. The data are presented as the mean ± SEM percentage of the control (vehicle-treated cells) of independent experiments, $n = 2–5$ in each experiment. * $p < 0.05$ vs. the control group, # $p < 0.05$ vs. the LPS group, ^ $p < 0.05$ vs. the agonist + LPS group. LXA4: lipoxin A4; AT-LXA4: aspirin-triggered lipoxin A4; LPS: lipopolysaccharide; TNF-α: tumor necrosis factor α; IL-1β: interleukin 1β.

Importantly, after 24 h of stimulation, MR-39 (1 μM) significantly downregulated the LPS-induced enhancement of all pro-inflammatory factors examined, TNF-α ($p = 0.07855$), IL-1β ($p = 0.003631$), and IL-6 ($p = 0.004908$) (Figures 9 and 10). Pretreatment of microglial cells with WRW4 blocked the effect of MR-39 on TNF-α production ($p < 0.035578$) and IL-1β release ($p = 0.04590$) (Figure 8).

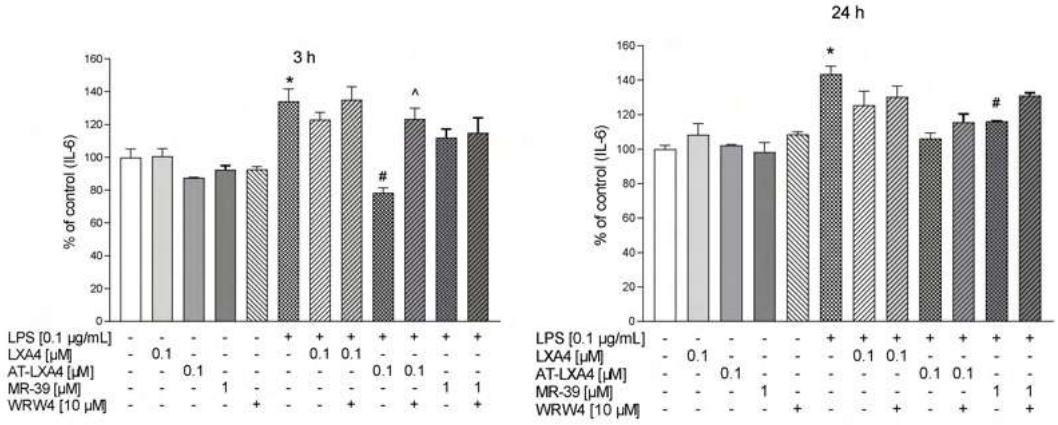


**Figure 9.** The impact of LXA4 and AT-LXA4 and MR-39 on IL-6 production in rat microglial cultures. The cells were pretreated for 30 min with the FPR2 antagonist WRW4 (10 μM). After that, LXA4 (0.1 μM), or AT-LXA4 (0.1 μM), or MR-39 (1 μM) was added for 1 h, and then the cells were stimulated for 3 h or 24 h with lipopolysaccharide (LPS; 0.1 μg/mL). Control cultures were treated with the appropriate vehicle. The data are presented as the mean ± SEM percentage of the control (vehicle-treated cells) of independent experiments, $n$ = 2–5 in each experiment. * $p < 0.05$ vs. the control group, # $p < 0.05$ vs. the LPS group, ^ $p < 0.05$ vs. the agonist + LPS group. LXA4: lipoxin A4; AT-LXA4: aspirin-triggered lipoxin A4; LPS: lipopolysaccharide; IL-6: interleukin 6.

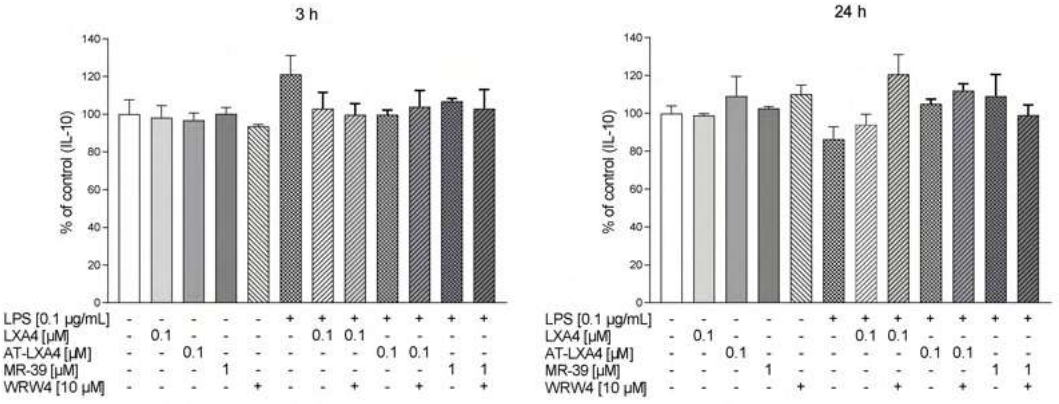


**Figure 10.** The impact of LXA4, AT-LXA4, and MR-39 on anti-inflammatory cytokines (IL-10) production in rat microglial cultures. The cells were pretreated for 30 min with the FPR2 antagonist WRW4 (10 μM). After that LXA4 (0.1 μM), AT-LXA4 (0.1 μM), or MR-39 (1 μM) was added for 1 h and then the cells were stimulated for 3 h or 24 h with lipopolysaccharide (LPS; 0.1 μg/mL). Control cultures were treated with the appropriate vehicle. The data are presented as the mean ± SEM percentage of the control (vehicle-treated cells) of independent experiments, $n$ = 2–5 in each experiment-. LXA4: lipoxin A4; AT-LXA4: aspirin-triggered lipoxin A4; LPS: lipopolysaccharide; IL-10: interleukin 10.

Moreover, AT-LXA4 (0.1 μM) inhibited LPS-induced IL-6 release after 3 h ($p = 0.027459$) of incubation. This result seems to be FPR2-dependent because WRW4 was able to block this AT-LXA4 action ($p = 0.011351$) (Figure 9).

On the other hand, no changes were observed in the IL-10 release after 3 h or 24 h of LPS stimulation as well as after ligands treatment (Figure 10). Collectively, the data indicated that the selected FPR2 agonists exerted anti-inflammatory effects in LPS-treated cells, while the biological profile of their beneficial action was slightly different.

### *3.9. The Impact of LXA4, AT-LXA4, and MR-39, FPR2 Ligands, on the ERK1/2, p38, and NF-κB Pathways in Microglial Cells Stimulated with Lipopolysaccharide*

The MAPK pathway is a major cellular signaling cascade that regulates the immune response and pro-inflammatory mediators. Moreover, MAPK phosphorylates a large number of substrates and induces the activation of transcription factors such as NF-κB, which

play a pivotal role in regulating the expression of a number of pro-inflammatory factors (including ROS and NO release) by microglial cells. To investigate the intracellular mechanism of the antioxidative and anti-inflammatory effects of FPR2 ligands in LPS-activated microglial cultures, we measured the active phosphorylated forms of ERK1/2, p38, and NF-κB proteins. We demonstrated that LPS stimulation for 30 min led to the activation of ERK1/2 kinase ($p$ = 0.000901). Pretreatment with LXA4 (0.1 μM) and MR-39 (1 μM) markedly blocked LPS-evoked ERK1/2 phosphorylation ($p$ = 0.03590 and $p$ = 0.024410, respectively) (Figure 11). Next, we measured the phosphorylation levels of the p38 and NF-κB proteins after 24 h of LPS stimulation. As shown in Figure 11, LPS stimulation enhanced the phosphorylation of p38 kinase ($p$ = 0.0001435) and NF-κB ($p$ = 0.002668). The LPS-induced increase in p38 kinase phosphorylation was only mitigated by AT-LXA4 administration ($p$ = 0.040349). On the other hand, AT-LXA4 ($p$ = 0.000431) and MR-39 ($p$ = 0.011622) pretreatment significantly suppressed NF-κB phosphorylation.

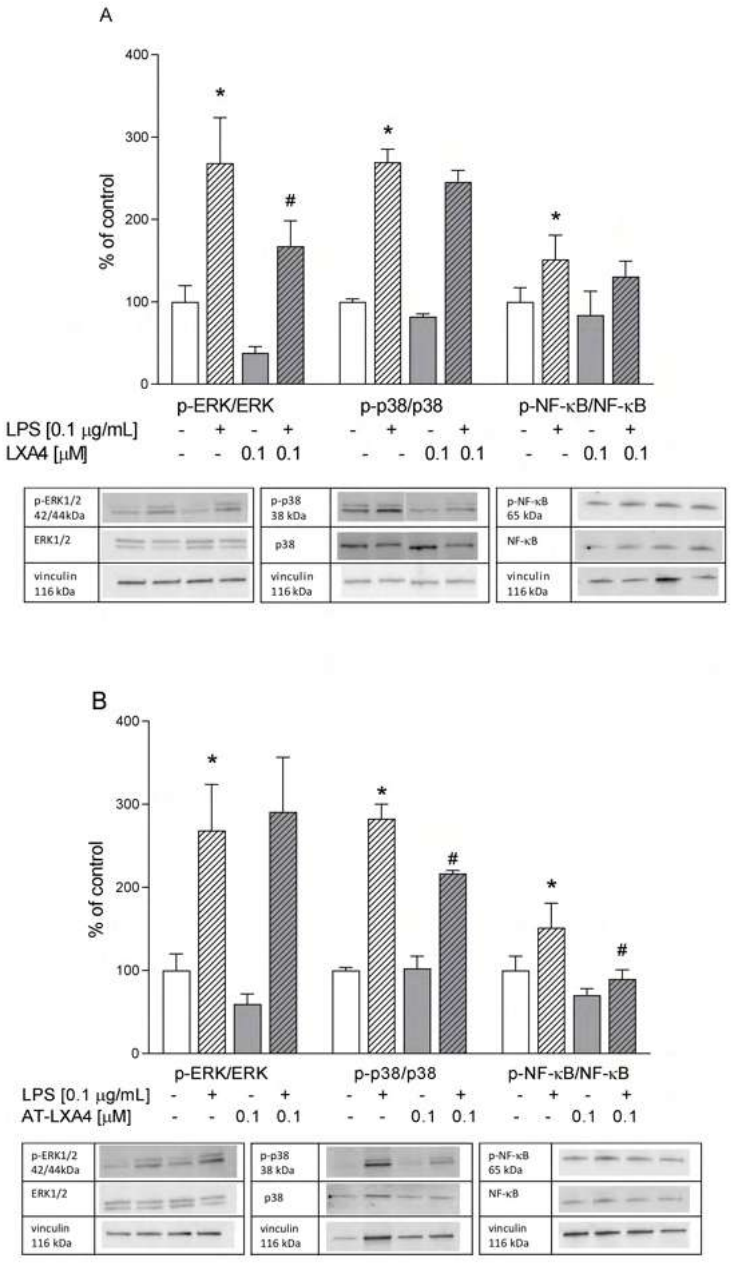


**Figure 11.** *Cont.*

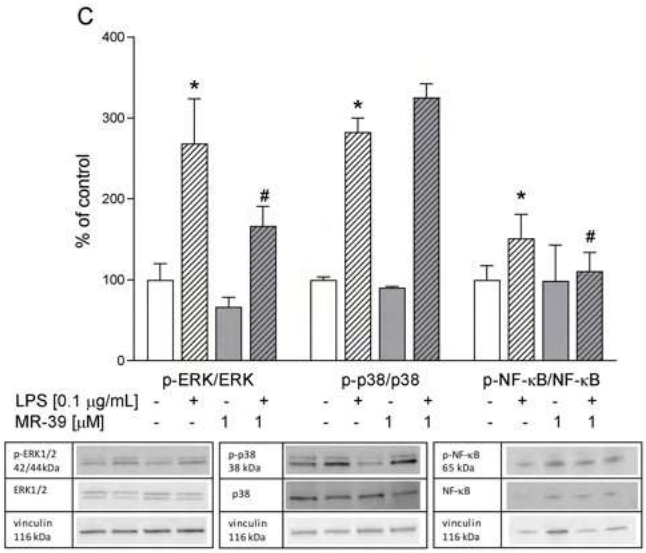


**Figure 11.** The impact of LXA4, AT-LXA4, and MR-39 on extracellular kinase 1/2 (ERK1/2), *p*-38 mitogen-activated protein kinases (*p*-38 MAPK), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways measured using Western blot analyses of microglial cells. The cells were treated with LXA4 (0.1 μM; **A**), AT-LXA4 (0.1 μM; **B**), or MR-39 (1 μM; **C**) for 1 h and then stimulated with LPS (0.1 μg/mL). Control cultures were treated with the appropriate vehicle. The data are presented as the mean ± SEM percentage of the control (vehicle-treated cells) of independent experiments, $n = 2–5$ in respective experiments. Representative immunoblots are presented under each graph. * $p < 0.05$ vs. the control group, # $p < 0.05$ vs. the LPS group.—LXA4: lipoxin A4; AT-LXA4: aspirin-triggered lipoxin A4; LPS: lipopolysaccharide.

Therefore, the ERK1/2, p38, and NF-κB proteins may be postulated to be important signaling pathways in the beneficial antioxidant and pro-resolving action of the examined ligands in microglial cells stimulated by LPS.

## 4. Discussion

Modulation of the resolution of inflammation (RoI) has been proposed as a new strategy to treat acute and chronic CNS disorders, and the FPR2 receptor is a recently discovered target for pro-resolving agents. Because endogenous FPR2s are chemically instable and poorly bioavailable, the search for new ligands of FPR2 is necessary. The present study evaluated the pro-resolving and anti-inflammatory effects of two endogenous FPR2 agonists, LXA4 and AT-LXA4, and one of the synthetic ureidopropanamide FPR2 agonists, MR-39, in microglial cells exposed to LPS, i.e., an in vitro model of neuroinflammation. Our study showed the protective impact of all tested FPR2 agonists on LPS-induced changes in microglial cells, although there were marked differences between the agonist effects regarding time dependency and efficacy in inhibiting particular biomarkers.

First, we found that LXA4 diminished LDH release only after 3 h, while the new agonist MR-39 exerted this effect 24 h after endotoxin stimulation. Moreover, the effects of LXA4 and MR-39 were inhibited by pretreatment with the FPR2 antagonist WRW4, which confirms the receptor specificity of these compounds. On the other hand, the inhibitory effect of AT-LXA4 on cell damage was long-lasting, but it was blocked by WRW4 only after 3 h of incubation with LPS. These differences may be due to dynamic changes in FPR2 expression in LPS-stimulated microglial cells. Although it is firmly accepted that FPR2 is expressed in microglial cells [30,40,43,54], some data have shown that under basal conditions, the microglial expression of FPR2 is low, and only after stimulation with various immunogens is it strongly upregulated. In the present study, immunofluorescent staining and confocal imaging visualized the presence of FPR2 in microglial cells, both after 3 h and after 24 h of incubation. However, the fluorescence intensity of FPR2 was strongly time-

dependent. Therefore, our observation is partially in line with that of other investigators, who reported that the FPR2 function in murine microglial cells was upregulated between 12 h and 24 h after LPS administration either by promoting receptor gene transcription and protein synthesis or by priming the responsiveness of the existing receptors [58].

The LDH test is accepted as an indicator of cell death processes. Furthermore, if the release of cytosolic enzymes is measured, it may be proposed as an indicator of the membrane integrity of cells. In fact, increased lactate production follows the loss of mitochondrial membrane potential [59]. Therefore, we assessed the impact of FPR2 agonists not only on lactate dehydrogenase release but also the applied JC-1, which is a novel cationic carbocyanine dye that accumulates in mitochondria and is a sensitive marker for mitochondrial membrane potential [60,61]. The loss of ΔΨM is one of the major events occurring in mitochondria and is associated with the opening of mitochondrial permeability pores and the loss of the electrochemical gradient [62]. Thus, ΔΨM is an essential parameter of mitochondrial function that can be used as a marker of cell status. We demonstrated that LPS diminished JC-1 accumulation. Interestingly, of the three FPR2 agonists tested, only MR-39 was able to significantly attenuate the LPS-induced decrease in mitochondrial potential (ΔΨM). Mitochondria are inherently involved in the apoptotic process of cells, while caspases, a family of proteases, are executors of apoptotic cell death, and their activation is considered to be a commitment to cell death [63]. Beyond their involvement in apoptosis, an important role of caspases, including caspase-3, in the control of microglial activation and neuroinflammation has been described [64,65]. Thus, the suppressive effect of ATL-LXA4 and MR-39 on LPS-induced caspase-3 activity observed in the present study may reflect not only the anti-apoptotic properties of FPR2 agonists but also the mechanism of FPR2 agonists based on the inhibition of microglial activation. However, the involvement of FPR2 in LPS-induced caspase-3 activity in our model should be further confirmed because WRW4 only tends to antagonize the effect of FPR2 agonists on this parameter. Therefore, although we have observed the protection potential of FPR2 ligands, some beneficial MR-39 effects may exert through other molecular targets, the understanding of which requires further study.

The second main finding of our study is the observation that FPR2 agonists exert antioxidative properties in microglial cultures affected by LPS. The activation of microglial cells contributes to harmful processes promoted by neurotoxic factors such as pro-inflammatory cytokines and nitric oxide (NO). Moreover, reactive oxygen species (ROS) play a role as important pro-inflammatory modulators [66]. Activated microglia potentiate the release of superoxide ion ($O^{2-}$), hydrogen peroxide ($H_2O_2$) and hydroxyl radicals (•OH) [67]. The dual role of ROS is implemented by maintaining homeostasis, while enhanced ROS levels elevate the mRNA expression of apoptotic genes and inflammatory mediators [68]. In turn, pro-inflammatory cytokines, through a feedback loop, upregulate the synthesis of ROS by activating NADPH oxidase, leading to redox disequilibrium and oxidative damage [69]. Recently, the crucial role of NADPH oxidase and mitochondria derived ROS in metabolic re-programing in functionally distinct microglia has been considered [11]. In the present study, we demonstrated that LXA4 after short-term LPS stimulation inhibited the production of ROS in microglial cells, while MR-39 diminished ROS levels after 24 h of stimulation with LPS. It may be suggested that the effect of FPR2 agonists on ROS production, although different in dynamics, contributes to restoring the redox equilibrium and/or immunometabolism changes in LPS-activated microglia.

Likewise, NO is a potentially neurotoxic factor because exaggerated production of NO results in the formation of peroxynitrite by reacting with superoxide, which leads to malfunctions in various mitochondrial processes [57]. Large amounts of NO are produced in the brain after the induction of the expression of iNOS in glial cells [70]. In the present study, MR-39 was the only FPR2 ligand that significantly attenuated the LPS-evoked increase in NO levels. The physiological significance of the MR-39 inhibitory effect on the LPS-induced NO release remains uncertain because of the NO ability to promote neuronal survival or neuronal death, depending on the NO concentration and the site of action [71].

Activated microglia cells express various markers and produce a wide array of pro-inflammatory cytokines. We observed that after short-term LPS stimulation, the expression of *Il-1β* and *TNF-α* gene expression was significantly upregulated. Conversely, prolonged endotoxin stimulation led to *Cd40* and *Tnf-α* gene expression downregulation. The anti-inflammatory properties of LXA4, AT-LXA4, and MR-39 confirmed that all FPR2 agonists inhibit *Tnf-α* mRNA expression. Simultaneously, we demonstrated that LXA4 (after 3 h) and MR-39 (after 24 h) also diminished TNF-α protein levels in a receptor-specific manner. Interestingly, only MR-39 attenuated LPS-induced *Il-1β* expression (after 3 h) and IL-1β production in microglial cultures. As in the case of TNF-α, this effect of MR-39 was also abolished by the WRW4 pretreatment, which clearly demonstrated the engagement of FPR2 in the anti-inflammatory action of MR-39.

It should also be mentioned that microglia treated with LXA4 and AT-LXA4 and stimulated by LPS had increased *Il-1β* expression. This surprising observation may suggest that the anti-inflammatory effects of LXA4 and AT-LXA4 are limited, probably due to strong LXA4 inactivation, which takes place in microglial cells and involves initial dehydrogenation to 15-oxo-lipoxin A4 [72]. In addition, the anti-inflammatory effect of MR-39 and AT-LXA4 was demonstrated as the ability of both agonists to inhibit the elevated IL-6 level after LPS stimulation. Therefore, the profile of the activity of the ligands tested in the present study on the inhibition of the LPS-induced inflammatory response is slightly different; nevertheless, MR-39 appears to have the most consistent inhibitory effects on the parameters.

The inflammatory response and the process of RoI are achieved by the shifting of the functional microglia polarization as well as the synthesis of pro- and anti-inflammatory factors. Therefore, the effect of the tested agonists on the anti-inflammatory status of activated microglia was also assessed. We found that brief LPS stimulation upregulates *Arg-1*, which competes with *iNos* for arginine substrates and may be at least in part responsible for changes in the NO release or as compensatory participation in repairing microglial damage after LPS stimulation [73]. Moreover, we found prolonged downregulation of *Igf-1* expression, followed by the suppression of *Cd206* gene expression. Next, we found that LXA4, AT-LXA4, and MR-39 upregulated *Il-10* gene expression; although the protein level of this cytokine was not elevated. Moreover, our data agree with previous findings showed that LXA4 and AT-LXA4 exert anti-inflammatory properties during acute and chronic inflammatory conditions [74]. An important role of LXA4 and AT-LXA4 has been shown in the brain, including neural stem cell proliferation and differentiation [75,76], as well as in ischemic/reperfusion models [77] after subarachnoid hemorrhage [32], and in astrocytes stimulated with LPS [30,78]. Recently, it has been shown that LXA4 exerts an anti-inflammatory effect through the upregulation of the anti-inflammatory mediator IL-10, which acts through the Notch signaling pathway [30]. Furthermore, the inhibitory effect of AT-LXA4 on the inflammatory activation of microglia has also been demonstrated [70]. Indisputably, our study adds to these reports showing the time-dependent, although variable, beneficial effects of LXA4, AT-LXA4, and MR-39 in an experimental model of inflammation in microglial cells and underlying their potent protective, anti-inflammatory, and pro-resolving properties.

It is well known that treatment with LPS causes the activation of mitogen-activated protein kinases (MAPKs) and transcriptional nuclear factor κB (NF-κB) [25,47,79]. Among the MAPK family of proteins, p-38 and ERK1/2 appear to be particularly involved in the production of pro-inflammatory mediators in microglial cells [80–82]. Indeed, LPS potentiates the phosphorylation of ERK1/2 and p38 in a dose- and time-dependent manner, leading among others to increase in the TNF-α release [83,84]. We found that the treatment of microglia with LXA4 and MR-39 significantly reduced LPS-induced ERK1/2 phosphorylation. Moreover, we observed that only AT-LXA4 attenuated LPS-stimulated p-38 phosphorylation.

These data suggest that the anti-inflammatory properties of both ligands at least in part result from inhibition of the ERK1/2 or p38 pathways. Of note, inhibition of

the ERK1/2 pathway could also suppress the caspase-3 pathway [65], which may point to the favorable impact of MR-39 on this caspase activity evoked by the LPS treatment. Interestingly, our results are in line with the research by Qin et al. (2017) [85], in which they showed that other synthetic FPR2 agonists, including compound 43 and compound 17b, also reduced ERK1/2 phosphorylation in a model of cardiovascular disorders. The present results also indicate the involvement of the NF-κB pathway in the action of FPR2 agonists in microglial cultures. NF-κB, a heterotrimeric complex present in the cytoplasm after activation, exposes nuclear localization signals on the p50/p65 complex, leading to their nuclear translocation and binding to the specific regulated sequences in the DNA, thus controlling gene transcription [86,87]. We found that AT-LXA4 and MR-39 attenuated the LPS-evoked phosphorylation of a specific serine in the p65 NF-κB subunit in microglial cells. NF-κB is considered a crucial factor in the regulation of the inflammatory response due to its ability to induce the transcription of pro-inflammatory genes and upregulate TNF-α and IL-6 release [88,89]. Therefore, it may be postulated that FPR2 ligands, through inhibition of ERK1/ERK2 and/or p-38 activation, as well as by suppression of phosphorylation of NF-κB factors, exert a protective and supporting RoI action in microglial cells stimulated by bacterial endotoxin.

We are aware that our study has some limitations. Firstly, the three studied agonists induced their effects at different concentrations with MR-39 being active in the micromolar range. These findings seem to be in agreement with the different potencies of the selected agonists in FPR2 activation. In fact, LXA4 induced $Ca^{2+}$ mobilization at nanomolar concentrations (EC50 = 1 nM) [90,91], whereas MR-39 elicited the same effect in the micromolar range (EC50 = 3.9 nM) [43]. On the other hand, we have to admit that some of the protective effects observed after the use of ligands were not unequivocally mediated by FPR2, as the use of WRW4 did not reverse them. Therefore, this requires further investigation, especially in the context of the interaction within the FPR's family and/or with regard to the structure-dependent ligand activation of FPR2. Secondly, microglial cultures exposed to LPS do not fully reflect the neuroinflammation observed in the brain in pathological conditions, where the response is complex and includes the interactions between neuronal and glial cells [92]. Notwithstanding the foregoing, based on the available data, the LPS model may be useful for a time-dependent assessment of the pro-resolving and anti-inflammatory activity of various ligands via receptors present in microglial cells [93,94].

## 5. Conclusions

Taken together, the present findings show that LXA4, AT-LXA4, and MR-39 exhibit time-dependent protective and anti-inflammatory effects in LPS-stimulated microglia. Among the tested agonists, MR-39 had the widest range of protective mechanisms, expressed by its ability to reduce the LDH release and mitochondrial membrane depolarization as well as its ability to inhibit caspase-3. Moreover, MR-39 showed antioxidative activity, thereby lowering ROS levels and inhibiting NO release. The resolution of inflammation in microglial cultures was also promoted by MR-39 via inhibiting the FPR2-dependent synthesis of pro-inflammatory cytokines, i.e., TNF-α and IL-1β. Furthermore, we demonstrated that the abovementioned effects were mediated through pathways including ERK1/2 and NF-κB inhibition (Figure 12).

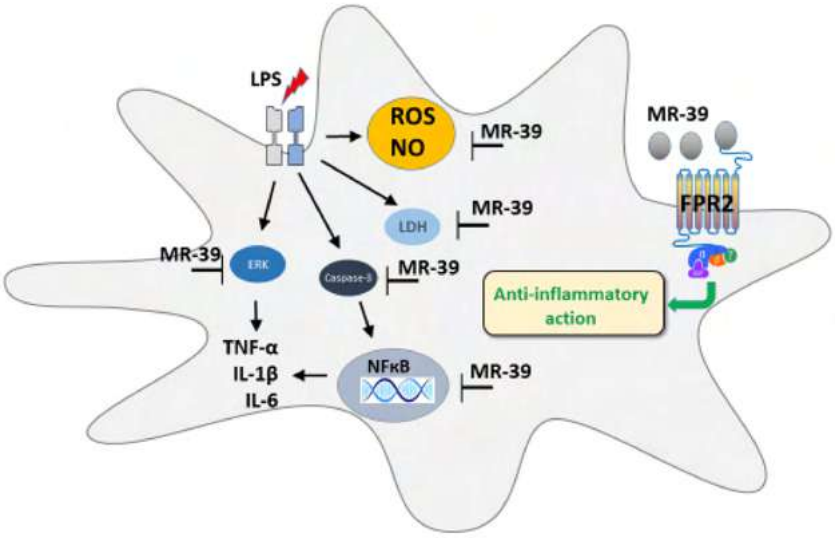


**Figure 12.** Schematic illustration of the targets of beneficial MR-39 action in LPS-stimulated microglial cells. The varied action of MR-39 in microglia cells includes reduction of the lactate dehydrogenase release, inhibition of the caspase-3 activity, and reactive oxide production as well as nitric oxide release evoked by bacterial endotoxin treatment. Moreover, MR-39 exerts an anti-inflammatory effect related to the inhibition of the synthesis of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6). This action is mediated by the reduction of ERK1/2 and the NF-κB transcription factor phosphorylation. Abbreviations: FPR2: formyl peptide receptor2; LDH: lactate dehydrogenase; LPS: lipopolysaccharide; ERK1/2: extracellular signal-regulated kinases; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; ROS: reactive oxygen species; NO: nitric oxide; TNF-α: tumor necrosis factor α; IL-1β: interleukin 1β; IL-6: interleukin 6.

In conclusion, the search for new and more potent FPR2 agonists may open a new perspective for innovative treatment of various brain disorders related to the malfunction of the endogenous resolution of inflammation processes.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/cells10092373/s1, Figure S1: Representative fluorescence images of microglia cells acquired by confocal microscopy.

**Author Contributions:** Conceptualization, E.T., K.T. and A.B.-K.; methodology, E.T., K.T., M.L. (Monika Leśkiewicz), M.R., N.B. and K.C.; formal analysis, E.T. and M.L. (Monika Leśkiewicz), M.R., K.C. and N.B.; investigation, M.L. (Monika Leśkiewicz), M.R., E.T., K.T. and K.C.; resources, A.B.-K.; data curation, M.L. (Monika Leśkiewicz), M.R., K.C., N.B. and K.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. (Monika Leśkiewicz), M.R., E.L., M.L. (Marcello Leopoldo) and A.B.-K.; supervision, A.B.-K.; project administration, A.B.-K.; 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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**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).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** All data supporting the conclusions of this manuscript are provided in the text, figures and tables.

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**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

## References

1. Graeber, M.B.; Streit, W.J. Microglia: Biology and pathology. *Acta Neuropathol.* **2010**, *119*, 89–105. [CrossRef] [PubMed]
2. Yang, G.; Meng, Y.; Li, W.; Yong, Y.; Fan, Z.; Ding, H.; Wei, Y.; Luo, J.; Ke, Z.-J. Neuronal MCP-1 Mediates Microglia Recruitment and Neurodegeneration Induced by the Mild Impairment of Oxidative Metabolism. *Brain Pathol.* **2011**, *21*, 279–297. [CrossRef] [PubMed]
3. Polazzi, E.; Monti, B. Microglia and neuroprotection: From in vitro studies to therapeutic applications. *Prog. Neurobiol.* **2010**, *92*, 293–315. [CrossRef] [PubMed]
4. Goldmann, T.; Prinz, M. Role of microglia in CNS autoimmunity. *Clin. Dev. Immunol.* **2013**, *2013*, 208093. [CrossRef] [PubMed]
5. Kohman, R.A.; Rhodes, J.S. Neurogenesis, inflammation and behavior. *Brain. Behav. Immun.* **2013**, *27*, 22–32. [CrossRef]
6. Szalay, G.; Martinecz, B.; Lénárt, N.; Környei, Z.; Orsolits, B.; Judák, L.; Császár, E.; Fekete, R.; West, B.L.; Katona, G.; et al. Microglia protect against brain injury and their selective elimination dysregulates neuronal network activity after stroke. *Nat. Commun.* **2016**, *7*, 1–13. [CrossRef]
7. Rock, R.B.; Gekker, G.; Hu, S.; Sheng, W.S.; Cheeran, M.; Lokensgard, J.R.; Peterson, P.K. Role of microglia in central nervous system infections. *Clin. Microbiol. Rev.* **2004**, *17*, 942–964. [CrossRef] [PubMed]
8. Hu, X. Microglia/macrophage polarization: Fantasy or evidence of functional diversity? *J. Cereb. Blood Flow Metab.* **2020**, *40*, S134–S136. [CrossRef]
9. Masuda, T.; Sankowski, R.; Staszewski, O.; Prinz, M. Microglia Heterogeneity in the Single-Cell Era. *Cell Rep.* **2020**, *30*, 1271–1281. [CrossRef]
10. Ransohoff, R.M. A polarizing question: Do M1 and M2 microglia exist. *Nat. Neurosci.* **2016**, *19*, 987–991. [CrossRef]
11. Bordt, E.A.; Polster, B.M. NADPH oxidase- and mitochondria-derived reactive oxygen species in proinflammatory microglial activation: A Bipartisan affair? *Free Radic. Biol. Med.* **2014**, *76*, 34–46. [CrossRef] [PubMed]
12. Orihuela, R.; McPherson, C.A.; Harry, G.J. Microglial M1/M2 polarization and metabolic states. *Br. J. Pharmacol.* **2016**, *173*, 649–665. [CrossRef] [PubMed]
13. Jurga, A.M.; Paleczna, M.; Kuter, K.Z. Overview of General and Discriminating Markers of Differential Microglia Phenotypes. *Front. Cell. Neurosci.* **2020**, *14*, 1–18. [CrossRef] [PubMed]
14. Kierdorf, K.; Prinz, M. Factors regulating microglia activation. *Front. Cell. Neurosci.* **2013**, *7*, 1–8. [CrossRef]
15. Cherry, J.D.; Olschowka, J.A.; O'Banion, M.K. Neuroinflammation and M2 microglia: The good, the bad, and the inflamed. *J. Neuroinflamm.* **2014**, *11*, 1–15. [CrossRef]
16. Kwon, H.S.; Koh, S.H. Neuroinflammation in neurodegenerative disorders: The roles of microglia and astrocytes. *Transl. Neurodegener.* **2020**, *9*, 1–12. [CrossRef] [PubMed]
17. Wickstead, E.S.; Karim, H.A.; Manuel, R.E.; Biggs, C.S.; Getting, S.J.; McArthur, S. Reversal of β-Amyloid-Induced Microglial Toxicity in Vitro by Activation of Fpr2/3. *Oxidative Med. Cell. Longev.* **2020**, *2020*, 2139192. [CrossRef] [PubMed]
18. Feghali, C.A.; Wright, T.M. Cytokines in acute and chronic inflammation. *Front. Biosci.* **1997**, *2*, A171. [CrossRef]
19. Lintermans, L.L.; Stegeman, C.A.; Heeringa, P.; Abdulahad, W.H. T cells in vascular inflammatory diseases. *Front. Immunol.* **2014**, *5*, 1–12. [CrossRef] [PubMed]
20. Medzhitov, R. Origin and physiological roles of inflammation. *Nature* **2008**, *454*, 428–435. [CrossRef] [PubMed]
21. Ashley, N.T.; Weil, Z.M.; Nelson, R.J. Inflammation: Mechanisms, costs, and natural variation. *Annu. Rev. Ecol. Evol. Syst.* **2012**, *43*, 385–406. [CrossRef]
22. Schett, G.; Neurath, M.F. Resolution of chronic inflammatory disease: Universal and tissue-specific concepts. *Nat. Commun.* **2018**, *9*, 1–8. [CrossRef]
23. Leszek, J.; Barreto, G.E.; Gsiorowski, K.; Koutsouraki, E.; Ávila-Rodrigues, M.; Aliev, G. Inflammatory Mechanisms and Oxidative Stress as Key Factors Responsible for Progression of Neurodegeneration: Role of Brain Innate Immune System. *CNS Neurol. Disord. Drug Targets* **2016**, *15*, 329–336. [CrossRef] [PubMed]
24. Serhan, C.N. Novel Pro-Resolving Lipid Mediators in Inflammation Are Leads for Resolution Physiology. *Nature* **2014**, *510*, 92–101. [CrossRef]
25. Trojan, E.; Bryniarska, N.; Leśkiewicz, M.; Regulska, M.; Chamera, K.; Szuster-Głuszczak, M.; Leopoldo, M.; Lacivita, E.; Basta-Kaim, A. The Contribution of Formyl Peptide Receptor Dysfunction to the Course of Neuroinflammation: A Potential Role in the Brain Pathology. *Curr. Neuropharmacol.* **2019**, *18*, 229–249. [CrossRef] [PubMed]

26. Kantarci, A.; Aytan, N.; Palaska, I.; Stephens, D.; Crabtree, L.; Benincasa, C.; Jenkins, B.G.; Carreras, I.; Dedeoglu, A. Combined administration of resolvin E1 and lipoxin A4 resolves inflammation in a murine model of Alzheimer's disease. *Exp. Neurol.* **2018**, *300*, 111–120. [CrossRef] [PubMed]
27. Chiang, N.; Serhan, C.N.; Dahlén, S.-E.; Drazen, J.M.; Hay, D.W.P.; Rovati, G.E.; Shimizu, T.; Yokomizo, T.; Brink, C. The Lipoxin Receptor ALX: Potent Ligand-Specific and Stereoselective Actions in Vivo. *Pharmacol. Rev.* **2006**, *58*, 463–487. [CrossRef] [PubMed]
28. Regulska, M.; Szuster-Głuszczak, M.; Trojan, E.; Leśkiewicz, M.; Basta-Kaim, A. The Emerging Role of the Double-Edged Impact of Arachidonic Acid- Derived Eicosanoids in the Neuroinflammatory Background of Depression. *Curr. Neuropharmacol.* **2020**, *19*, 278–293. [CrossRef]
29. Serhan, C.N. Lipoxins and aspirin-triggered 15-epi-lipoxins are the first lipid mediators of endogenous anti-inflammation and resolution. *Prostaglandins Leukot. Essent. Fat. Acids* **2005**, *73*, 141–162. [CrossRef] [PubMed]
30. Wu, J.; Ding, D.H.; Li, Q.Q.; Wang, X.Y.; Sun, Y.Y.; Li, L.J. Lipoxin A4 regulates lipopolysaccharide-induced BV2 microglial activation and differentiation via the notch signaling pathway. *Front. Cell. Neurosci.* **2019**, *13*, 1–17. [CrossRef]
31. Lee, T.H. Lipoxin A4: A novel anti-inflammatory molecule? *Thorax* **1995**, *50*, 111–112. [CrossRef] [PubMed]
32. Guo, Z.; Hu, Q.; Xu, L.; Guo, Z.; Ou, Y.; He, Y.; Yin, C.; Sun, X.; Tang, J.; Zhang, J.H. Lipoxin A4 Reduces Inflammation Through Formyl Peptide Receptor 2/p38 MAPK Signaling Pathway in Subarachnoid Hemorrhage Rats. *Stroke* **2016**, *47*, 490–497. [CrossRef]
33. Vital, S.A.; Becker, F.; Holloway, P.M.; Russell, J.; Perretti, M.; Granger, D.N.; Gavins, F.N.E. Formyl-peptide receptor 2/3/Lipoxin A4 receptor regulates neutrophil-platelet aggregation and attenuates cerebral inflammation: Impact for therapy in cardiovascular disease. *Circulation* **2016**, *133*, 2169–2179. [CrossRef]
34. Martini, A.C.; Berta, T.; Forner, S.; Chen, G.; Bento, A.F.; Ji, R.R.; Rae, G.A. Lipoxin A4 inhibits microglial activation and reduces neuroinflammation and neuropathic pain after spinal cord hemisection. *J. Neuroinflamm.* **2016**, *13*, 1–11. [CrossRef]
35. Tylek, K.; Trojan, E.; Regulska, M.; Lacivita, E.; Leopoldo, M.; Basta-Kaim, A. Formyl peptide receptor 2, as an important target for ligands triggering the inflammatory response regulation: A link to brain pathology. *Pharmacol. Rep.* **2021**, 1–16. [CrossRef]
36. Lohse, M.J. Dimerization in GPCR mobility and signaling. *Curr. Opin. Pharmacol.* **2010**, *10*, 53–58. [CrossRef]
37. Sodin-Semrl, S.; Spagnolo, A.; Mikus, R.; Barbaro, B.; Varga, J.; Fiore, S. Opposing regulation of interleukin-8 and NF-kB responses by lipoxin A4 and serum amyloid a via the common lipoxin a receptor. *Int. J. Immunopathol. Pharmacol.* **2004**, *17*, 145–155. [CrossRef] [PubMed]
38. Hawkins, K.E.; DeMars, K.M.; Alexander, J.C.; De Leon, L.G.; Pacheco, S.C.; Graves, C.; Yang, C.; McCrea, A.O.; Frankowski, J.C.; Garrett, T.J.; et al. Targeting resolution of neuroinflammation after ischemic stroke with a lipoxin A4 analog: Protective mechanisms and long-term effects on neurological recovery. *Brain Behav.* **2017**, *7*, 1–14. [CrossRef] [PubMed]
39. He, H.Q.; Ye, R.D. The formyl peptide receptors: Diversity of ligands and mechanism for recognition. *Molecules* **2017**, *22*, 455. [CrossRef] [PubMed]
40. Liu, G.J.; Tao, T.; Wang, H.; Zhou, Y.; Gao, X.; Gao, Y.Y.; Hang, C.H.; Li, W. Functions of resolvin D1-ALX/FPR2 receptor interaction in the hemoglobin-induced microglial inflammatory response and neuronal injury. *J. Neuroinflamm.* **2020**, *17*, 1–17. [CrossRef] [PubMed]
41. Duvall, M.G.; Levy, B.D. DHA- and EPA-derived resolvins, protectins, and maresins in airway inflammation. *Eur. J. Pharmacol.* **2016**, *785*, 144–155. [CrossRef] [PubMed]
42. Wang, G.; Zhang, L.; Chen, X.; Xue, X.; Guo, Q.; Liu, M.; Zhao, J. Formylpeptide Receptors Promote the Migration and Differentiation of Rat Neural Stem Cells. *Sci. Rep.* **2016**, *6*, 2–11. [CrossRef] [PubMed]
43. Stama, M.L.; Ślusarczyk, J.; Lacivita, E.; Kirpotina, L.N.; Schepetkin, I.A.; Chamera, K.; Riganti, C.; Perrone, R.; Quinn, M.T.; Basta-Kaim, A.; et al. Novel ureidopropanamide based N-formyl peptide receptor 2 (FPR2) agonists with potential application for central nervous system disorders characterized by neuroinflammation. *Eur. J. Med. Chem.* **2017**, *141*, 703–720. [CrossRef]
44. Mastromarino, M.; Lacivita, E.; Colabufo, N.A.; Leopoldo, M. G-protein coupled receptors involved in the resolution of inflammation: Ligands and therapeutic perspectives. *Mini Rev. Med. Chem.* **2020**, *20*, 2090–2103. [CrossRef] [PubMed]
45. Zawadzka, M.; Kaminska, B. A novel mechanism of FK506-mediated neuroprotection: Downregulation of cytokine expression in glial cells. *Glia* **2005**, *49*, 36–51. [CrossRef] [PubMed]
46. Ślusarczyk, J.; Trojan, E.; Głombik, K.; Budziszewska, B.; Kubera, M.; Lasoń, W.; Popiołek-Barczyk, K.; Mika, J.; Wędzony, K.; Basta-Kaim, A. Prenatal stress is a vulnerability factor for altered morphology and biological activity of microglia cells. *Front. Cell. Neurosci.* **2015**, *9*, 1–14. [CrossRef]
47. Ślusarczyk, J.; Trojan, E.; Głombik, K.; Piotrowska, A.; Budziszewska, B.; Kubera, M.; Popiołek-Barczyk, K.; Lasoń, W.; Mika, J.; Basta-Kaim, A. Targeting the NLRP3 inflammasome-related pathways via tianeptine treatment-suppressed microglia polarization to the M1 phenotype in lipopolysaccharide-stimulated cultures. *Int. J. Mol. Sci.* **2018**, *19*, 1965. [CrossRef]
48. Basta-Kaim, A.; Ślusarczyk, J.; Szczepanowicz, K.; Warszyński, P.; Leśkiewicz, M.; Regulska, M.; Trojan, E.; Lasoń, W. Protective effects of polydatin in free and nanocapsulated form on changes caused by lipopolysaccharide in hippocampal organotypic cultures. *Pharmacol. Rep.* **2019**, *71*, 603–613. [CrossRef]
49. Leskiewicz, M.; Regulska, M.; Budziszewska, B.; Jantas, D.; Jaworska-Feil, L.; Basta-Kaim, A.; Kubera, M.; Jagla, G.; Nowak, W.; Lason, W. Effects of neurosteroids on hydrogen peroxide- and staurosporine-induced damage of human neuroblastoma SH-SY5Y cells. *J. Neurosci. Res.* **2008**, *86*, 1361–1370. [CrossRef] [PubMed]

50. Kubiak, A.; Chighizola, M.; Schulte, C.; Bryniarska, N.; Wesołowska, J.; Pudełek, M.; Lasota, M.; Ryszawy, D.; Basta-Kaim, A.; Laidler, P.; et al. Stiffening of DU145 prostate cancer cells driven by actin filaments—Microtubule crosstalk conferring resistance to microtubule-targeting drugs. *Nanoscale* **2021**, *13*, 6212–6226. [CrossRef] [PubMed]
51. Prauzner-Bechcicki, S.; Raczkowska, J.; Madej, E.; Pabijan, J.; Lukes, J.; Sepitka, J.; Rysz, J.; Awsiuk, K.; Bernasik, A.; Budkowski, A.; et al. PDMS substrate stiffness affects the morphology and growth profiles of cancerous prostate and melanoma cells. *J. Mech. Behav. Biomed. Mater.* **2015**, *41*, 13–22. [CrossRef] [PubMed]
52. Bollmann, L.; Koser, D.E.; Shahapure, R.; Gautier, H.O.B.; Holzapfel, G.A.; Scarcelli, G.; Gather, M.C.; Ulbricht, E.; Franze, K. Microglia mechanics: Immune activation alters traction forces and durotaxis. *Front. Cell. Neurosci.* **2015**, *9*, 1–16. [CrossRef]
53. Basta-Kaim, A.; Budziszewska, B.; Leśkiewicz, M.; Fijał, K.; Regulska, M.; Kubera, M.; Wędzony, K.; Lasoń, W. Hyperactivity of the hypothalamus-pituitary-adrenal axis in lipopolysaccharide-induced neurodevelopmental model of schizophrenia in rats: Effects of antipsychotic drugs. *Eur. J. Pharmacol.* **2011**, *650*, 586–595. [CrossRef] [PubMed]
54. Liu, M.; Chen, K.; Yoshimura, T.; Liu, Y.; Gong, W.; Le, Y.; Gao, J.L.; Zhao, J.; Wang, J.M.; Wang, A. Formylpeptide receptors mediate rapid neutrophil mobilization to accelerate wound healing. *PLoS ONE* **2014**, *9*, e99541. [CrossRef] [PubMed]
55. Zhu, J.; Qu, C.; Lu, X.; Zhang, S. Activation of microglia by histamine and substance P. *Cell. Physiol. Biochem.* **2014**, *34*, 768–780. [CrossRef] [PubMed]
56. Shen, X.; Burguillos, M.A.; Joseph, B. Guilt by association, caspase-3 regulates microglia polarization. *Cell Cycle* **2017**, *16*, 306–307. [CrossRef]
57. Pacher, P.; Beckman, J.S.; Liaudet, L. Nitric Oxide and Peroxynitrite in Health and Disease. *Physiol. Rev.* **2007**, *87*, 315–424. [CrossRef] [PubMed]
58. Cui, Y.-H.; Le, Y.; Gong, W.; Proost, P.; Van Damme, J.; Murphy, W.J.; Wang, J.M. Bacterial Lipopolysaccharide Selectively Up-Regulates the Function of the Chemotactic Peptide Receptor Formyl Peptide Receptor 2 in Murine Microglial Cells. *J. Immunol.* **2002**, *168*, 434–442. [CrossRef]
59. Tiefenthaler, M.; Amberger, A.; Bacher, N.; Hartmann, B.L.; Margreiter, R.; Kofler, R.; Konwalinka, G. Increased lactate production follows loss of mitochondrial membrane potential during apoptosis of human leukaemia cells. *Br. J. Haematol.* **2001**, *114*, 574–580. [CrossRef]
60. Zorova, L.D.; Popkov, V.A.; Plotnikov, E.Y.; Silachev, D.N.; Pevzner, I.B.; Jankauskas, S.S.; Babenko, V.A.; Zorov, S.D.; Balakireva, A.V.; Juhaszova, M.; et al. Mitochondrial membrane potential. *Anal. Biochem.* **2018**, *552*, 50–59. [CrossRef] [PubMed]
61. Sivandzade, F.; Bhalerao, A.; Cucullo, L. Analysis of the Mitochondrial Membrane Potential Using the Cationic JC-1 Dye as a Sensitive Fluorescent Probe. *Bio Protocol* **2019**, *9*, 1–13. [CrossRef]
62. Green, D.R.; Reed, J.C. Mitochondria and apoptosis. *Science* **1998**, *281*, 1309. [CrossRef] [PubMed]
63. Burguillos, M.A.; Hajji, N.; Englund, E.; Persson, A.; Cenci, A.M.; Machado, A.; Cano, J.; Joseph, B.; Venero, J.L. Apoptosis-inducing factor mediates dopaminergic cell death in response to LPS-induced inflammatory stimulus. Evidence in Parkinson's disease patients. *Neurobiol. Dis.* **2011**, *41*, 177–188. [CrossRef]
64. Venero, J.L.; Burguillos, M.A.; Brundin, P.; Joseph, B. The executioners sing a new song: Killer caspases activate microglia. *Cell Death Differ.* **2011**, *18*, 1679–1691. [CrossRef] [PubMed]
65. Nelson, J.W.; Leigh, N.J.; Mellas, R.E.; McCall, A.D.; Aguirre, A.; Baker, O.J. ALX/FPR2 receptor for RvD1 is expressed and functional in salivary glands. *Am. J. Physiol. Cell Physiol.* **2014**, *306*, 178–185. [CrossRef]
66. Park, J.; Min, J.S.; Kim, B.; Chae, U.-B.; Yun, J.W.; Choi, M.S.; Kong, I.K.; Chang, K.T.; Lee, D.S. Mitochondrial ROS govern the LPS-induced pro-inflammatory response in microglia cells by regulating MAPK and NF-κB pathways. *Neurosci. Lett.* **2015**, *584*, 191–196. [CrossRef]
67. Block, M.L.; Zecca, L.; Hong, J.S. Microglia-mediated neurotoxicity: Uncovering the molecular mechanisms. *Nat. Rev. Neurosci.* **2007**, *8*, 57–69. [CrossRef] [PubMed]
68. Allen, R.G.; Tresini, M. Oxidative stress and gene regulation. *Free Radic. Biol. Med.* **2000**, *28*, 463–499. [CrossRef]
69. Brown, G.C.; Neher, J.J. Inflammatory neurodegeneration and mechanisms of microglial killing of neurons. *Mol. Neurobiol.* **2010**, *41*, 242–247. [CrossRef]
70. Wang, Y.P.; Wu, Y.; Li, L.Y.; Zheng, J.; Liu, R.G.; Zhou, J.P.; Yuan, S.Y.; Shang, Y.; Yao, S.L. Aspirin-triggered lipoxin A4attenuates LPS-induced pro-inflammatory responses by inhibiting activation of NF-κB and MAPKs in BV-2 microglial cells. *J. Neuroinflamm.* **2011**, *8*, 95. [CrossRef] [PubMed]
71. Araújo, I.M.; Carvalho, C.M. Role of nitric oxide and calpain activation in neuronal death and survival. *Curr. Drug Targets CNS Neurol. Disord.* **2005**, *4*, 319–324. [CrossRef]
72. Romano, M. Lipoxin and aspirin-triggered lipoxins. *Sci. World J.* **2010**, *10*, 1048–1064. [CrossRef] [PubMed]
73. Yang, T.; Xu, G.; Newton, P.T.; Chagin, A.S.; Mkrtchian, S.; Carlström, M.; Zhang, X.M.; Harris, R.A.; Cooter, M.; Berger, M.; et al. Maresin 1 attenuates neuroinflammation in a mouse model of perioperative neurocognitive disorders. *Br. J. Anaesth.* **2019**, *122*, 350–360. [CrossRef] [PubMed]
74. Serhan, C.N.; Yacoubian, S.; Yang, R. Anti-Inflammatory and Proresolving Lipid Mediators. *Annu. Rev. Pathol. Mech. Dis.* **2008**, *3*, 279–312. [CrossRef] [PubMed]
75. Wu, Y.; Ye, X.H.; Guo, P.P.; Xu, S.P.; Wang, J.; Yuan, S.Y.; Yao, S.L.; Shang, Y. Neuroprotective effect of lipoxin a4 methyl ester in a rat model of permanent focal cerebral ischemia. *J. Mol. Neurosci.* **2010**, *42*, 226–234. [CrossRef] [PubMed]

76. Wada, K.; Arita, M.; Nakajima, A.; Katayama, K.; Kudo, C.; Kamisaki, Y.; Serhan, C.N. Leukotriene B 4 and lipoxin A 4 are regulatory signals for neural stem cell proliferation and differentiation. *FASEB J.* **2006**, *20*, 1785–1792. [CrossRef]
77. Wu, Y.; Zhai, H.; Wang, Y.; Li, L.; Wu, J.; Wang, F.; Sun, S.; Yao, S.; Shang, Y. Aspirin-triggered lipoxin A 4 attenuates lipopolysaccharide- induced intracellular ROS in BV2 microglia cells by inhibiting the function of NADPH oxidase. *Neurochem. Res.* **2012**, *37*, 1690–1696. [CrossRef]
78. Wu, J.; Ding, D.; Wang, X.; Li, Q.; Sun, Y.; Li, L.; Wang, Y. Regulation of aquaporin 4 expression by lipoxin A4 in astrocytes stimulated by lipopolysaccharide. *Cell. Immunol.* **2019**, *344*, 103959. [CrossRef]
79. Miller, Y.I.; Viriyakosol, S.; Worrall, D.S.; Boullier, A.; Butler, S.; Witztum, J.L. Toll-like receptor 4-dependent and -independent cytokine secretion induced by minimally oxidized low-density lipoprotein in macrophages. *Arterioscler. Thromb. Vasc. Biol.* **2005**, *25*, 1213–1219. [CrossRef]
80. Koistinaho, M.; Koistinaho, J. Interactions between Alzheimer's disease and cerebral ischemia—Focus on inflammation. *Brain Res. Rev.* **2005**, *48*, 240–250. [CrossRef]
81. Bachstetter, A.D.; Van Eldik, L.J. The p38 map kinase family as regulators of proinflammatory cytokine production in degenerative diseases of the CNS. *Aging Dis.* **2010**, *1*, 199–211.
82. Falcicchia, C.; Tozzi, F.; Arancio, O.; Watterson, D.M.; Origlia, N. Involvement of p38 mapk in synaptic function and dysfunction. *Int. J. Mol. Sci.* **2020**, *21*, 5624. [CrossRef] [PubMed]
83. Lee, S.C.; Dickson, D.W.; Liu, W.; Brosnan, C.F. Induction of nitric oxide synthase activity in human astrocytes by interleukin-1β and interferon-γ. *J. Neuroimmunol.* **1993**, *46*, 19–24. [CrossRef]
84. Hwang, J.; Zheng, L.T.; Ock, J.; Lee, M.G.; Suk, K. Anti-inflammatory effects of m-chlorophenylpiperazine in brain glia cells. *Int. Immunopharmacol.* **2008**, *8*, 1686–1694. [CrossRef] [PubMed]
85. Qin, C.X.; May, L.T.; Li, R.; Cao, N.; Rosli, S.; Deo, M.; Alexander, A.E.; Horlock, D.; Bourke, J.E.; Yang, Y.H.; et al. Small-molecule-biased formyl peptide receptor agonist compound 17b protects against myocardial ischaemia-reperfusion injury in mice. *Nat. Commun.* **2017**, *8*, 1–13. [CrossRef]
86. Moynagh, P.N. The NF-κB pathway. *J. Cell Sci.* **2005**, *118*, 4589–4592. [CrossRef] [PubMed]
87. Zou, J.Y.; Crews, F.T. TNFα potentiates glutamate neurotoxicity by inhibiting glutamate uptake in organotypic brain slice cultures: Neuroprotection by NFκB inhibition. *Brain Res.* **2005**, *1034*, 11–24. [CrossRef] [PubMed]
88. Majumder, S.; Zhou, L.Z.; Chaturvedi, P.; Babcock, G.; Aras, S.; Ransohoff, R.M. p48/STAT-1alpha-containing complexes play a predominant role in induction of IFN-gamma-inducible protein, 10 kDa (IP-10) by IFN-gamma alone or in synergy with TNF-alpha. *J. Immunol.* **1998**, *161*, 4736–4744. [PubMed]
89. Hyam, S.R.; Lee, I.A.; Gu, W.; Kim, K.A.; Jeong, J.J.; Jang, S.E.; Han, M.J.; Kim, D.H. Arctigenin ameliorates inflammation in vitro and in vivo by inhibiting the PI3K/AKT pathway and polarizing M1 macrophages to M2-like macrophages. *Eur. J. Pharmacol.* **2013**, *708*, 21–29. [CrossRef]
90. Le, Y.; Murphy, P.M.; Wang, J.M. Formyl-peptide receptors revisited. *Trends Immunol.* **2002**, *23*, 541–548. [CrossRef]
91. De Gaetano, M.; Butler, E.; Gahan, K.; Zanetti, A.; Marai, M.; Chen, J.; Cacace, A.; Hams, E.; Maingot, C.; McLoughlin, A.; et al. Asymmetric synthesis and biological evaluation of imidazole- and oxazole-containing synthetic lipoxin A4 mimetics (sLXms). *Eur. J. Med. Chem.* **2019**, *162*, 80–108. [CrossRef]
92. Harry, G.J.; Kraft, A.D. Neuroinflammation and microglia: Considerations and approaches for neurotoxicity assessment. *Expert Opin. Drug Metab. Toxicol.* **2008**, *4*, 1265–1277. [CrossRef] [PubMed]
93. Li, L.; Wu, Y.; Wang, Y.; Wu, J.; Song, L.; Xian, W.; Yuan, S.; Pei, L.; Shang, Y. Resolvin D1 promotes the interleukin-4-induced alternative activation in BV-2 microglial cells. *J. Neuroinflamm.* **2014**, *11*, 1–11. [CrossRef] [PubMed]
94. Obuchowicz, E.; Bielecka, A.M.; Paul-Samojedny, M.; Pudełko, A.; Kowalski, J. Imipramine and fluoxetine inhibit LPS-induced activation and affect morphology of microglial cells in the rat glial culture. *Pharmacol. Rep.* **2014**, *66*, 34–43. [CrossRef] [PubMed]

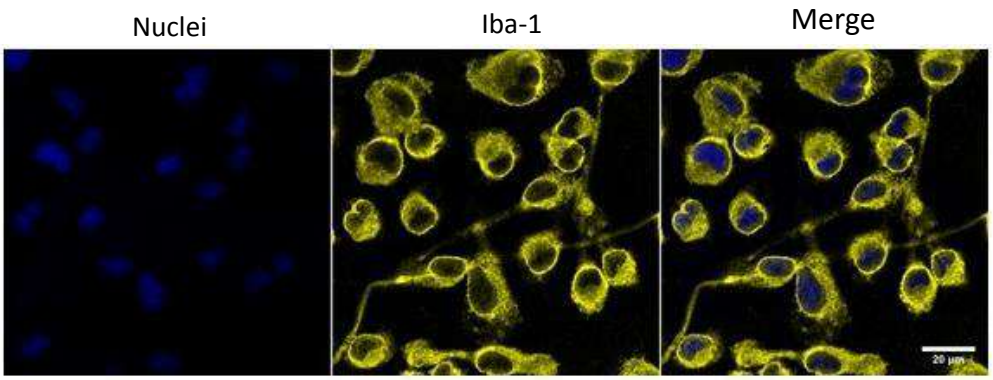


Figure S1. Representative fluorescence images of microglia cells acquired by confocal microscopy. Nuclei appear in blue and Iba-1 in yellow. Scale bar: 20 μm is located in the bottom right corner of each image.

**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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# 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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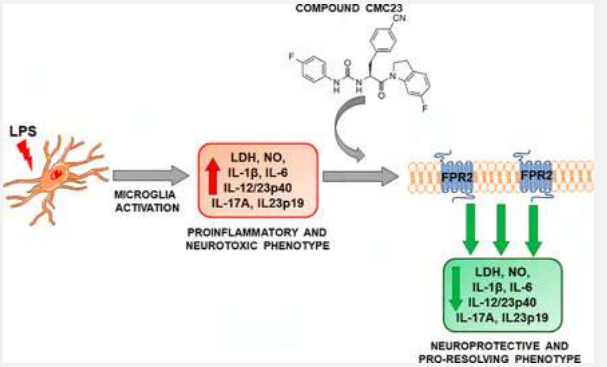
**ABSTRACT:** A substantial body of evidence demonstrates an association between a malfunction in the resolution of acute inflammation and the development of chronic inflammation. Recently, in this context, the importance of formyl peptide receptor 2 (FPR2) has been underlined. FPR2 activity is modulated 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-1$\beta$ 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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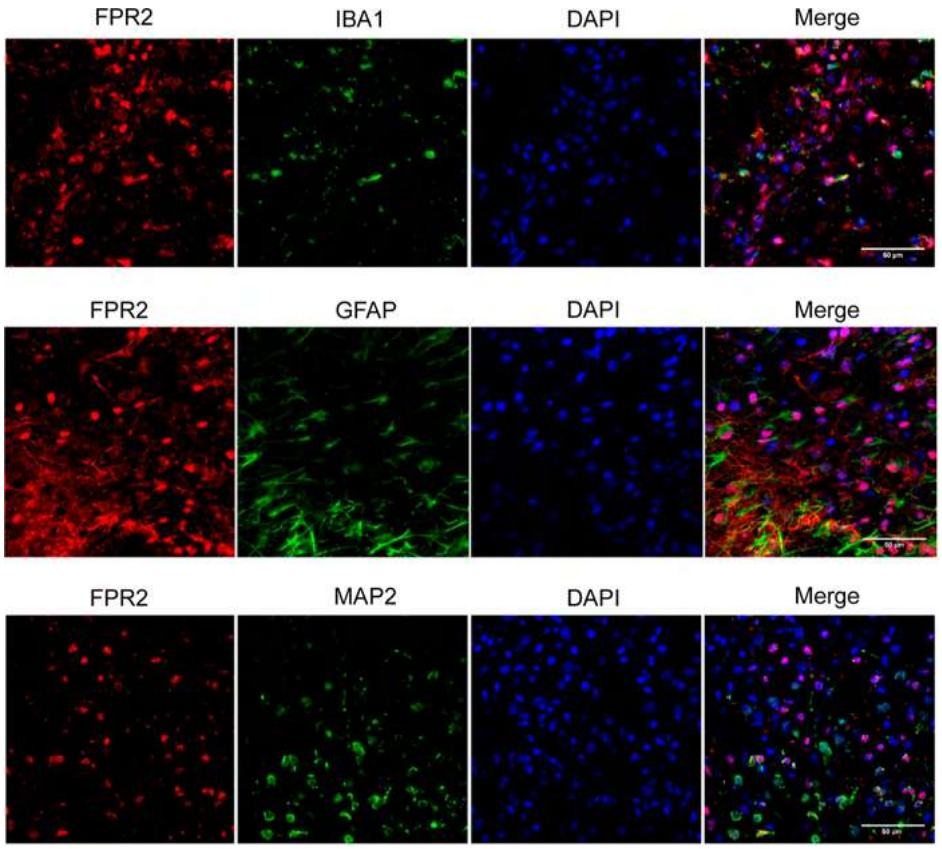


**Figure 1.** Representative fluorescence images of control (vehicle-treated) OHCs were obtained from the CA1 area of the OHCs using confocal microscopy. Nuclei appear in blue due to Hoechst 33342 staining, FPR2 was labeled in red using Alexa Fluor 647, IBA1 was labeled using Alexa Fluor 555, and GFAP and MAP2 were both labeled in green using Alexa Fluor 488. Scale bar: 50 $\mu$m 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-1$\beta$, IL-6, IL-10, IL-12/23p40, IL-17A, IL-23p19, and TGF-$\beta$) 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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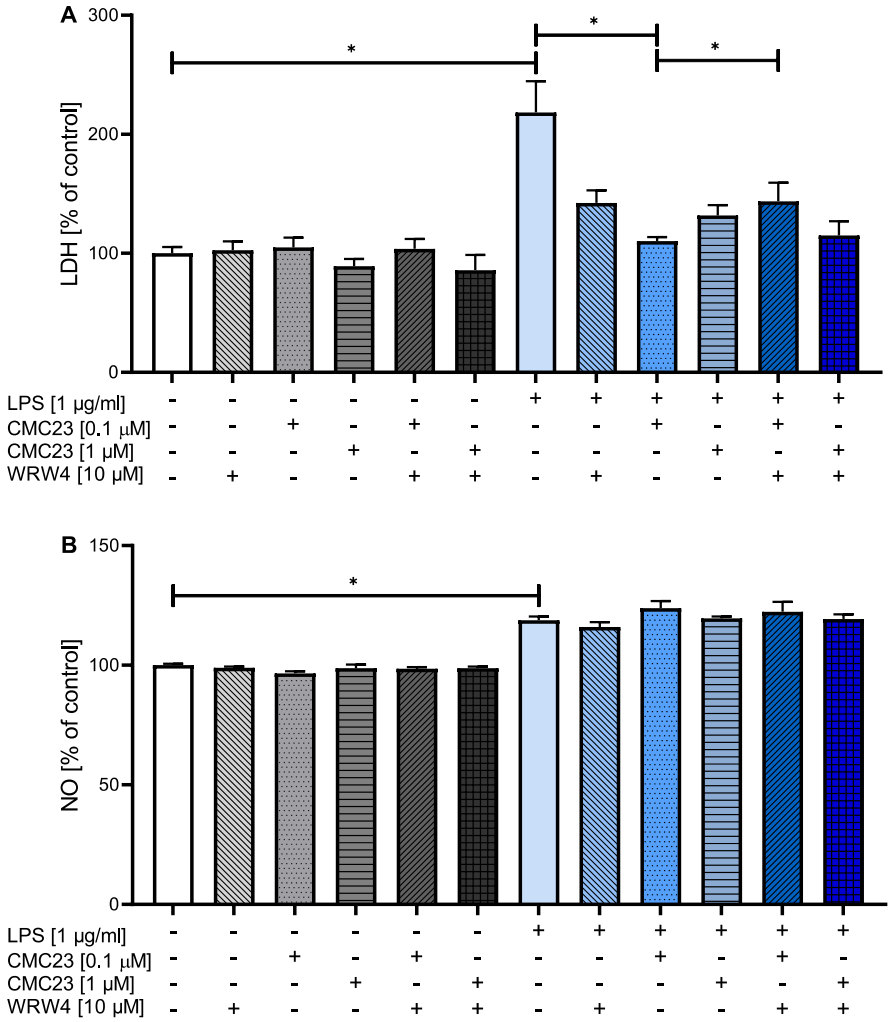


**Figure 2.** Impact of CMC23 and WRW4 treatment on the LPS-stimulated LDH (A) and NO (B) release. OHCs were pretreated with the FPR2 antagonist WRW4 (10 $\mu$M) for 30 min. Subsequently, OHCs were treated with CMC23 (0.1 or 1 $\mu$M) for 1 h and then stimulated with lipopolysaccharide (LPS; 1 $\mu$g/mL) 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 $\mu$M, 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 $\mu$M) did not significantly change the levels of NO and LDH in control OHCs. Stimulation of OHCs with LPS (1 $\mu$g/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 $\mu$M ($p = 0.000056$) (Figure 2A) but not at 1 $\mu$M (Figure 2A). Significantly, pretreatment of the OHCs with the FPR2 antagonist WRW4 attenuated the protective activity of CMC23 (0.1 $\mu$M) ($p = 0.047104$) (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 $\mu$M 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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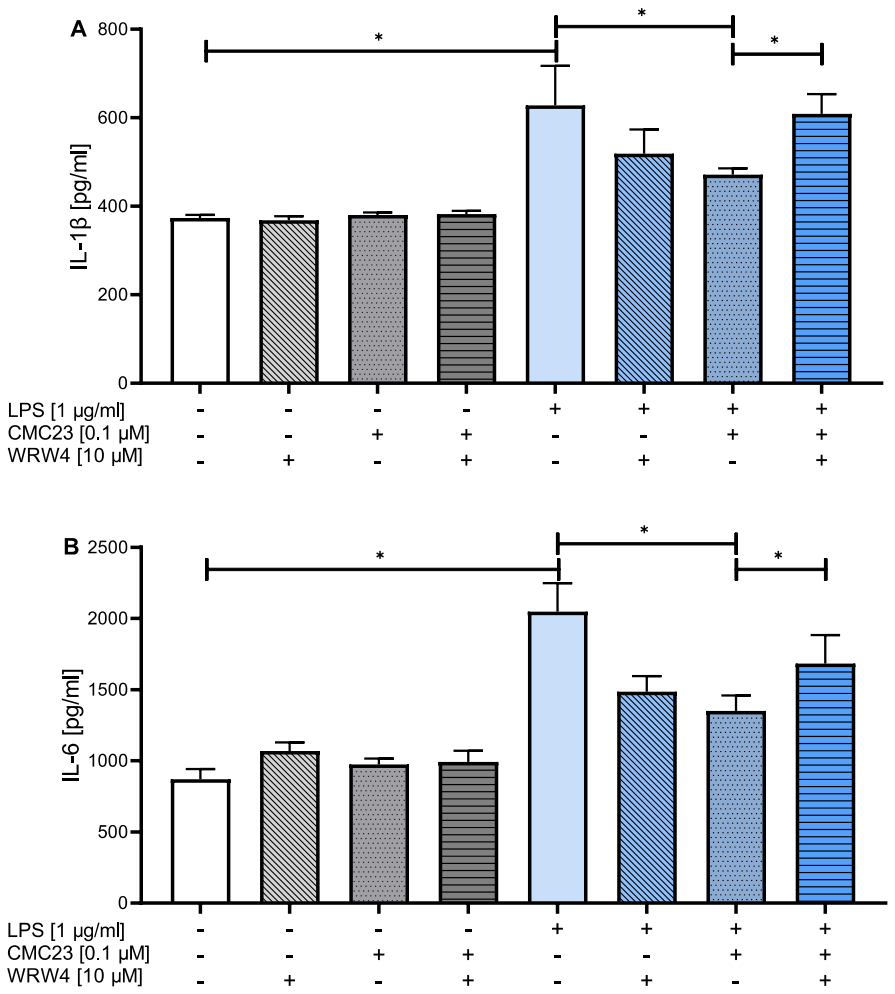


**Figure 3.** Impact of CMC23 and WRW4 on the release of proinflammatory cytokines IL-1$\beta$ (A) and IL-6 (B) in LPS-stimulated OHCs. OHCs were pretreated with the FPR2 antagonist WRW4 (10 $\mu$M) for 30 min. Subsequently, OHCs were treated with CMC23 (0.1 $\mu$M) for 1 h and then stimulated with lipopolysaccharide (LPS; 1 $\mu$g/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-1$\beta$—interleukin 1$\beta$; 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 $\mu$M), 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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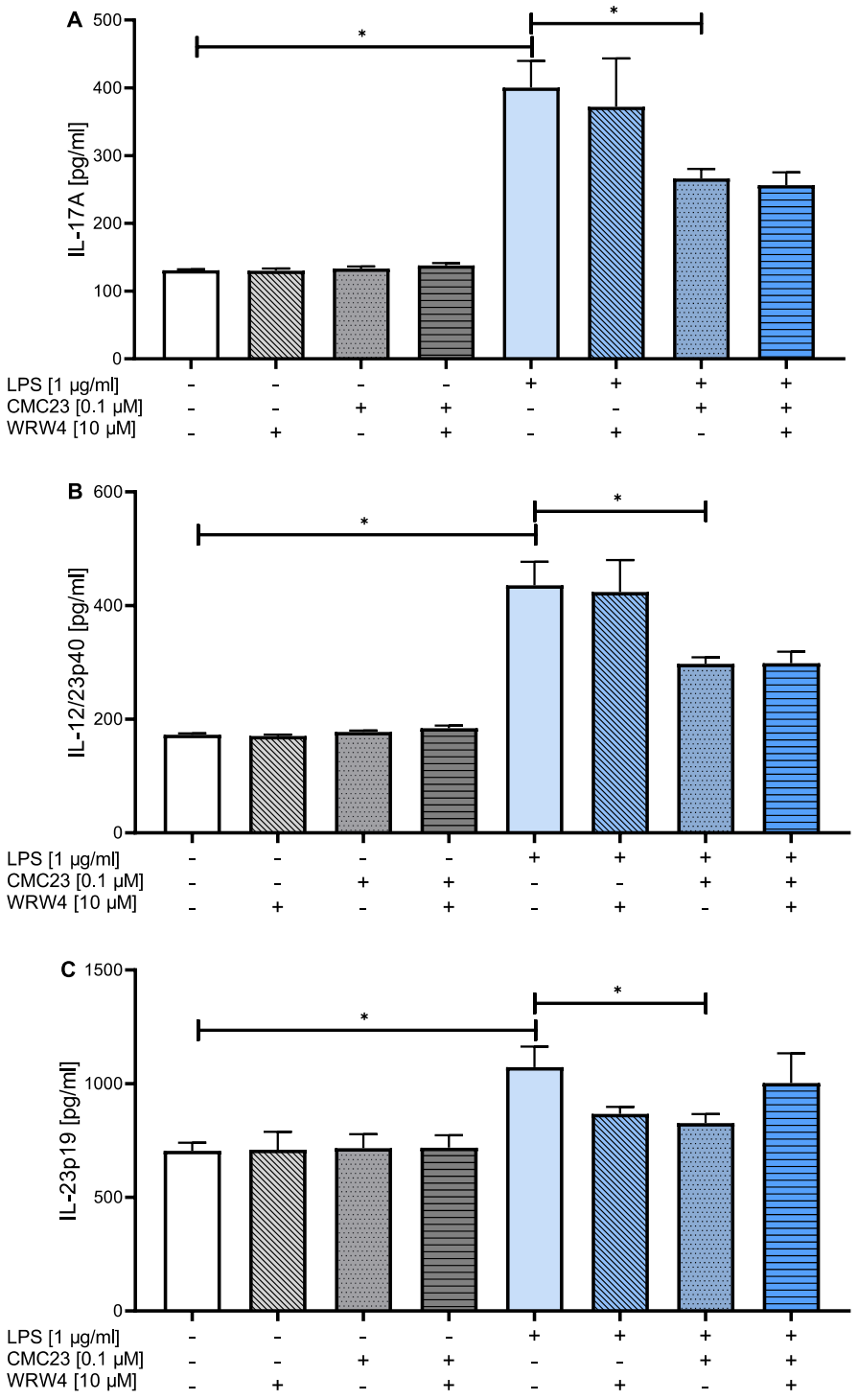


**Figure 4.** Impact of CMC23 and WRW4 on the release of IL-17A (A), IL-23 subunits IL-12/23p40 (B), and IL-23p19 (C) in LPS-stimulated OHCs. OHCs were pretreated with the FPR2 antagonist WRW4 (10 $\mu$M) for 30 min. Subsequently, OHCs were treated with CMC23 (0.1 $\mu$M) for 1 h and finally with lipopolysaccharide (LPS; 1 $\mu$g/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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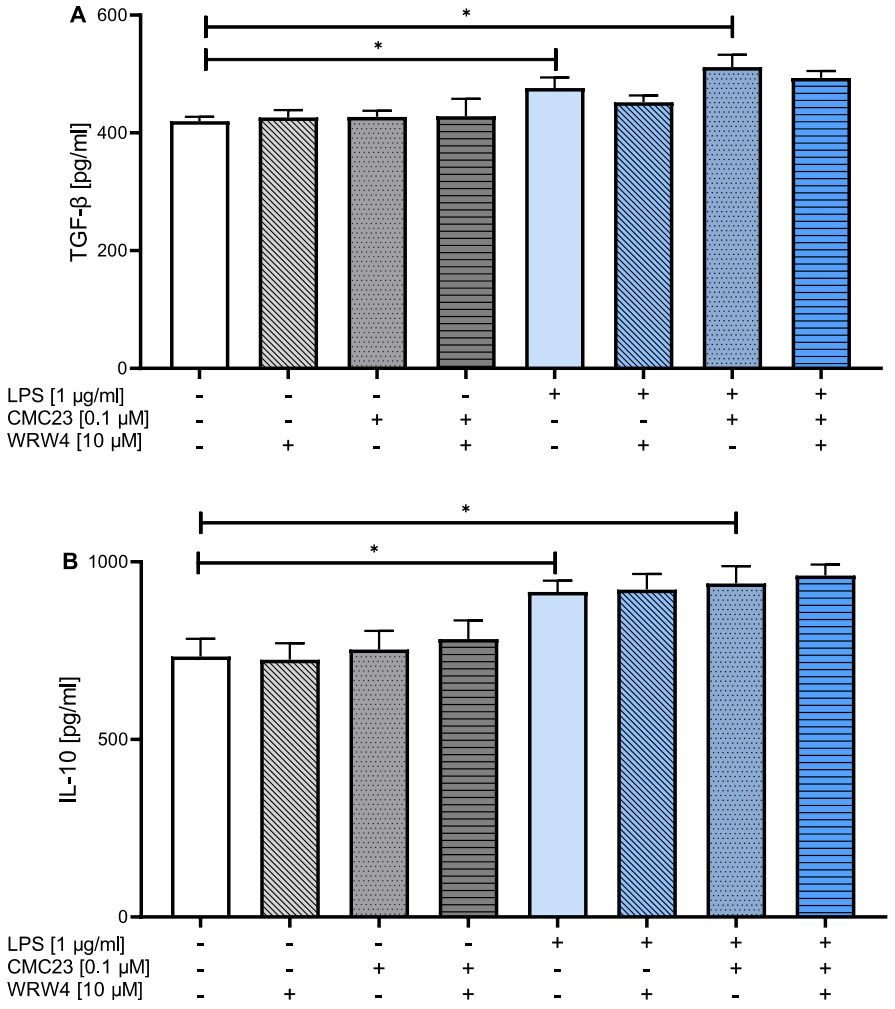


**Figure 5.** Impact of CMC23 and WRW4 on the release of anti-inflammatory cytokines TGF-$\beta$ (A) and IL-10 (B) in LPS-stimulated OHCs. OHCs were pretreated with the FPR2 antagonist WRW4 (10 $\mu$M) for 30 min. Subsequently, the OHCs were treated with CMC23 (0.1 $\mu$M) for 1 h and then with lipopolysaccharide (LPS; 1 $\mu$g/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-$\beta$ − transforming growth factor − $\beta$; 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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| 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 |

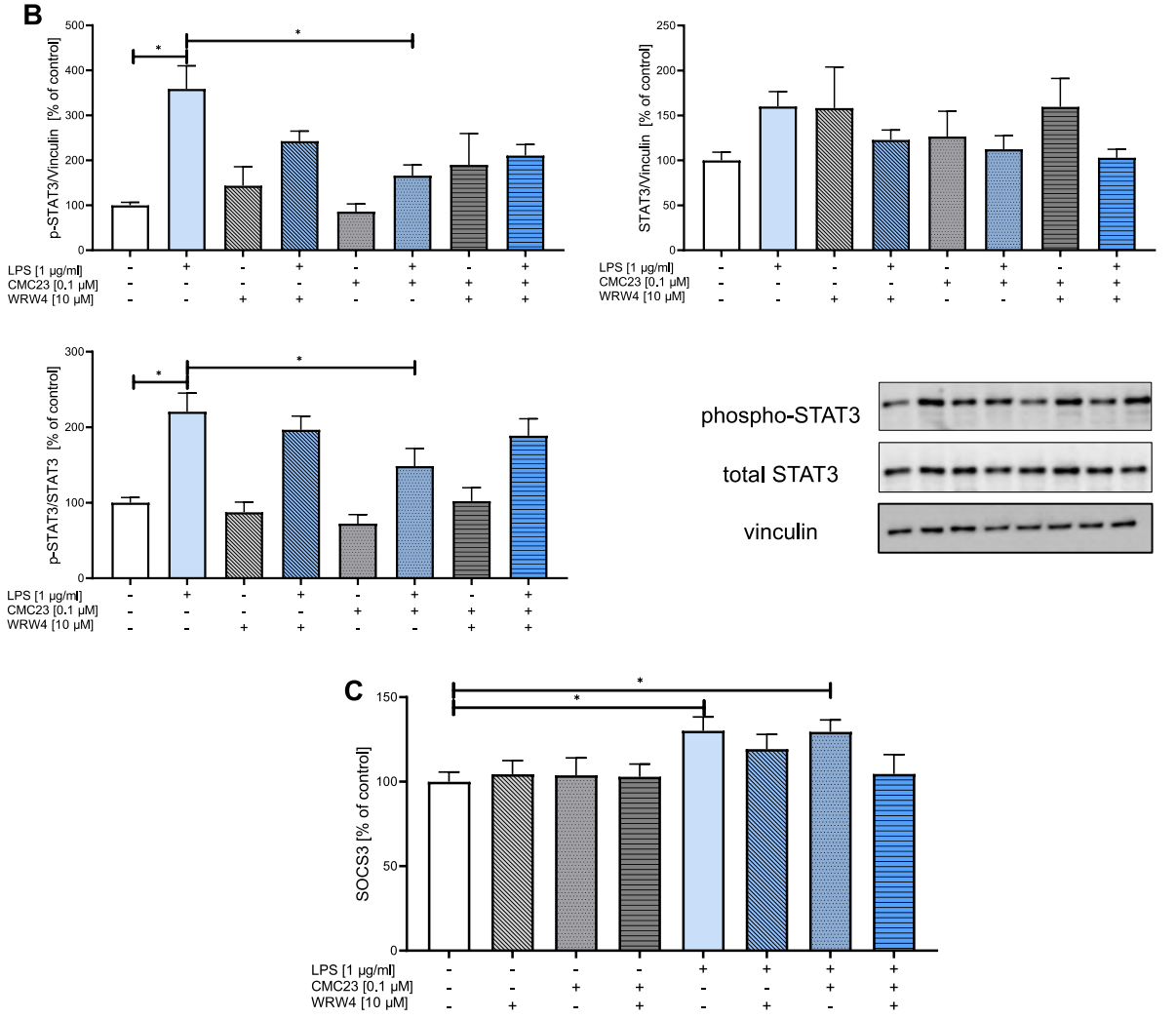


**Figure 6.** Impact of CMC23 and WRW4 on the gene expression of *Jak1*, *Jak2*, and *Stat3* (A) and protein level of phospho-STAT3, STAT3 (B), and SOCS3 (C) in LPS-stimulated OHCs. OHCs were pretreated with the FPR2 antagonist WRW4 (10 $\mu$M) for 30 min. Subsequently, OHCs were treated with CMC23 (0.1 $\mu$M) for 1 h and then stimulated with lipopolysaccharide (LPS; 1 $\mu$g/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-$\beta$ 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-$\alpha$, IL-1$\beta$, 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-1$\beta$ expression. Thus, the balance between IL-10 induction and the level of pro-IL1$\beta$ potentially determines the final level of IL-1$\beta$,[44] which in our study was reduced by CMC23 treatment. Our previous studies showed that the FPR2 agonist MR-39 suppresses LPS-evoked IL-1$\beta$ release by inhibiting the NLRP3/NF-$\kappa$B 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-1$\beta$ 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-$\beta$ 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-1$\beta$, 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-1$\beta$.[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, $\beta$2 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 1$\beta$ (IL-1$\beta$), 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-$\beta$). Furthermore, OHCs were lysed in 160 $\mu$L 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-1$\beta$, IL-12p40, IL-17A, IL-23p19, and TGF-$\beta$); 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-1$\beta$ < 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-$\beta$ < 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 $\mu$L of Tris−HCl (pH = 6.7), 2% SDS, and 700 $\mu$L 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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## ■ REFERENCES


(1) Schwartz, M.; Baruch, K. The resolution of neuroinflammation in neurodegeneration: Leukocyte recruitment via the choroid plexus. *EMBO J.* **2014**, *33* (1), 7−22.

(2) Perretti, M.; Leroy, X.; Bland, E. J.; Montero-Melendez, T. Resolution Pharmacology: Opportunities for Therapeutic Innovation in Inflammation. *Trends Pharmacol. Sci.* **2015**, *36* (11), 737−755.

(3) Scott, M. C.; Bedi, S. S.; Olson, S. D.; Sears, C. M.; Cox, C. S. Microglia as therapeutic targets after neurological injury: strategy for cell therapy. *Expert Opin. Ther. Targets* **2021**, *25* (5), 365−380.

(4) Tiberi, M.; Chiurchiù, V. Specialized Pro-resolving Lipid Mediators and Glial Cells: Emerging Candidates for Brain Homeostasis and Repair. *Front. Cell. Neurosci.* **2021**, *15*, 673549.

(5) Trojan, E.; Bryniarska, N.; Leśkiewicz, M.; Regulska, M.; Chamera, K.; Szuster-Głuszczak, M.; Leopoldo, M.; Lacivita, E.; Basta-Kaim, A. The Contribution of Formyl Peptide Receptor Dysfunction to the Course of Neuroinflammation: A Potential Role in the Brain Pathology. *Curr. Neuropharmacol.* **2020**, *18* (3), 229−249.

(6) Tylek, K.; Trojan, E.; Regulska, M.; Lacivita, E.; Leopoldo, M.; Basta-Kaim, A. Formyl peptide receptor 2, as an important target for ligands triggering the inflammatory response regulation: a link to brain pathology. *Pharmacol. Rep.* **2021**, *73*, 1004−1019.

(7) Fullerton, J. N.; Gilroy, D. W. Resolution of inflammation: A new therapeutic frontier. *Nat. Rev. Drug Discovery* **2016**, *15* (8), 551−567.

(8) Serhan, C. N.; Levy, B. D. Resolvins in inflammation: emergence of the pro-resolving superfamily of mediators. *J. Clin. Invest.* **2018**, *128* (7), 2657−2669.

(9) Maderna, P.; Cottell, D. C.; Toivonen, T.; Dufton, N.; Dalli, J.; Perretti, M.; Godson, C. FPR2/ALX receptor expression and internalization are critical for lipoxin A 4 and annexin-derived peptide-stimulated phagocytosis. *FASEB J.* **2010**, *24* (11), 4240−4249.

(10) Serhan, C. N. Pro-resolving lipid mediators are leads for resolution physiology. *Nature* **2014**, *510* (7503), 92−101.

(11) Mastromarino, M.; Favia, M.; Schepetkin, I. A.; Kirpotina, L. N.; Trojan, E.; Niso, M.; Carrieri, A.; Leśkiewicz, M.; Regulska, M.; Darida, M.; Rossignolo, F.; Fontana, S.; Quinn, M. T.; Basta-Kaim, A.; Leopoldo, M.; Lacivita, E. Design, Synthesis, Biological Evaluation, and Computational Studies of Novel Ureidopropanamides as Formyl Peptide Receptor 2 (FPR2) Agonists to Target the Resolution of Inflammation in Central Nervous System Disorders. *J. Med. Chem.* **2022**, *65* (6), 5004−5028.

(12) Romano, M. Lipoxin and aspirin-triggered lipoxins. *ScientificWorldJournal* **2010**, *10*, 1048−1064.

(13) Schebb, N. H.; Kühn, H.; Kahnt, A. S.; Rund, K. M.; O'Donnell, V. B.; Flamand, N.; Peters-Golden, M.; Jakobsson, P. J.; Weylandt, K. H.; Rohwer, N.; Murphy, R. C.; Geisslinger, G.; FitzGerald, G. A.; Hanson, J.; Dahlgren, C.; Alnouri, M. W.; Offermanns, S.; Steinhilber, D. Formation, Signaling and Occurrence of Specialized Pro-Resolving Lipid Mediators—What is the Evidence so far? *Front. Pharmacol* **2022**, *13*, 838782.

(14) Corminboeuf, O.; Leroy, X. FPR2/ALXR agonists and the resolution of inflammation. *J. Med. Chem.* **2015**, *58* (2), 537−559.

(15) Dahlgren, C.; Gabl, M.; Holdfeldt, A.; Winther, M.; Forsman, H. Basic characteristics of the neutrophil receptors that recognize formylated peptides, a danger-associated molecular pattern generated by bacteria and mitochondria. *Biochem. Pharmacol.* **2016**, *114*, 22−39.

(16) Lee, H. Y.; Jeong, Y. S.; Lee, M.; Kweon, H. S.; Huh, Y. H.; Park, J. S.; Hwang, J. E.; Kim, K.; Bae, Y. S. Intracellular formyl peptide receptor regulates naive CD4 T cell migration. *Biochem. Biophys. Res. Commun.* **2018**, *497* (1), 226−232.

(17) Devosse, T.; Guillabert, A.; D'Haene, N.; Berton, A.; De Nadai, P.; Noel, S.; Brait, M.; Franssen, J.-D.; Sozzani, S.; Salmon, I.; Parmentier, M. Formyl Peptide Receptor-Like 2 Is Expressed and Functional in Plasmacytoid Dendritic Cells, Tissue-Specific Macrophage Subpopulations, and Eosinophils. *J. Immunol.* **2009**, *182* (8), 4974−4984.

(18) Brandenburg, L. O.; Konrad, M.; Wruck, C. J.; Koch, T.; Lucius, R.; Pufe, T. Functional and physical interactions between formyl-peptide-receptors and scavenger receptor MARCO and their involvement in amyloid beta 1−42-induced signal transduction in glial cells. *J. Neurochem.* **2010**, *113* (3), 749−760.

(19) Liu, G. J.; Tao, T.; Wang, H.; Zhou, Y.; Gao, X.; Gao, Y. Y.; Hang, C. H.; Li, W. Functions of resolvin D1-ALX/FPR2 receptor interaction in the hemoglobin-induced microglial inflammatory response and neuronal injury. *J. Neuroinflammation* **2020**, *17* (1), 239.

(20) Becker, E. L.; Forouhar, F. A.; Grunnet, M. L.; Boulay, F.; Tardif, M.; Bormann, B. J.; Sodja, D.; Ye, R. D.; Woska, J. R., Jr.; Murphy, P. M. Broad immunocytochemical localization of the formylpeptide receptor in human organs, tissues, and cells. *Cell Tissue Res.* **1998**, *292* (1), 129−135.
(21) Perretti, M.; Godson, C. Formyl peptide receptor type 2 agonists to kick-start resolution pharmacology. *Br. J. Pharmacol.* **2020**, *177* (20), 4595−4600.
(22) Stama, M. L.; Lacivita, E.; Kirpotina, L. N.; Niso, M.; Perrone, R.; Schepetkin, I. A.; Quinn, M. T.; Leopoldo, M. Functional N -Formyl Peptide Receptor 2 (FPR2) Antagonists Based on the Ureidopropanamide Scaffold Have Potential To Protect Against Inflammation-Associated Oxidative Stress. *ChemMedChem* **2017**, *12* (22), 1839−1847.
(23) Tylek, K.; Trojan, E.; Leśkiewicz, M.; Regulska, M.; Bryniarska, N.; Curzytek, K.; Lacivita, E.; Leopoldo, M.; Basta-Kaim, A. Time-dependent protective and pro-resolving effects of fpr2 agonists on lipopolysaccharide-exposed microglia cells involve inhibition of nf-κb and mapks pathways. *Cells* **2021**, *10* (9), 2373.
(24) Wu, J.; Ding, D. H.; Li, Q. Q.; Wang, X. Y.; Sun, Y. Y.; Li, L. J. Lipoxin A4 regulates lipopolysaccharide-induced BV2 microglial activation and differentiation via the notch signaling pathway. *Front. Cell. Neurosci.* **2019**, *13*, 19.
(25) Liu, M.; Chen, K.; Yoshimura, T.; Liu, Y.; Gong, W.; Le, Y.; Gao, J. L.; Zhao, J.; Wang, J. M.; Wang, A. Formylpeptide receptors mediate rapid neutrophil mobilization to accelerate wound healing. *PLoS One* **2014**, *9* (3), No. e90613.
(26) Carow, B.; Rottenberg, M. E. SOCS3, a major regulator of infection and inflammation. *Front. Immunol.* **2014**, *5*, 58.
(27) Trojan, E.; Tylek, K.; Schröder, N.; Kahl, I.; Brandenburg, L. O.; Mastromarino, M.; Leopoldo, M.; Basta-Kaim, A.; Lacivita, E. The N-Formyl Peptide Receptor 2 (FPR2) Agonist MR-39 Improves Ex Vivo and In Vivo Amyloid Beta (1−42)-Induced Neuroinflammation in Mouse Models of Alzheimer's Disease. *Mol. Neurobiol.* **2021**, *58*, 6203−6221.
(28) Cristiano, C.; Volpicelli, F.; Crispino, M.; Lacivita, E.; Russo, R.; Leopoldo, M.; Calignano, A.; Perrone-Capano, C. Behavioral, Anti-Inflammatory, and Neuroprotective Effects of a Novel FPR2 Agonist in Two Mouse Models of Autism. *Pharmaceuticals* **2022**, *15* (2), 161.
(29) Trojan, E.; Tylek, K.; Leśkiewicz, M.; Lasoń, W.; Brandenburg, L. O.; Leopoldo, M.; Lacivita, E.; Basta-Kaim, A. The n-formyl peptide receptor 2 (Fpr2) agonist mr-39 exhibits anti-inflammatory activity in lps-stimulated organotypic hippocampal cultures. *Cells* **2021**, *10* (6), 1524.
(30) Possel, H.; Noack, H.; Putzke, J.; Wolf, G.; Sies, H. Selective upregulation of inducible nitric oxide synthase (iNOS) by lipopolysaccharide (LPS) and cytokines in microglia: In vitro and in vivo studies. *Glia* **2000**, *32* (1), 51−59.
(31) Wang, X.; Zhu, M.; Hjorth, E.; Cortés-Toro, V.; Eyjolfsdottir, H.; Graff, C.; Nennesmo, I.; Palmblad, J.; Eriksdotter, M.; Sambamurti, K.; Fitzgerald, J. M.; Serhan, C. N.; Granholm, A.; Schultzberg, M. Resolution of inflammation is altered in Alzheimer's disease. *Alzheimer's Dement.* **2015**, *11* (1), 40.
(32) Zhu, J.; Li, L.; Ding, J.; Huang, J.; Shao, A.; Tang, B. The Role of Formyl Peptide Receptors in Neurological Diseases via Regulating Inflammation. *Front. Cell. Neurosci.* **2021**, *15*, 753832.
(33) Schröder, N.; Schaffrath, A.; Welter, J. A.; Putzka, T.; Griep, A.; Ziegler, P.; Brandt, E.; Samer, S.; Heneka, M. T.; Kaddatz, H.; Zhan, J.; Kipp, E.; Pufe, T.; Tauber, S. C.; Kipp, M.; Brandenburg, L. O. Inhibition of formyl peptide receptors improves the outcome in a mouse model of Alzheimer disease. *J. Neuroinflammation* **2020**, *17* (1), 131.
(34) Ponomarev, E. D.; Shriver, L. P.; Dittel, B. N. CD40 Expression by Microglial Cells Is Required for Their Completion of a Two-Step Activation Process during Central Nervous System Autoimmune Inflammation. *J. Immunol.* **2006**, *176* (3), 1402−1410.
(35) Salemi, J.; Obregon, D. F.; Cobb, A.; Reed, S.; Sadic, E.; Jin, J.; Fernandez, F.; Tan, J.; Giunta, B. Flipping the switches: CD40 and CD45 modulation of microglial activation states in HIV associated dementia (HAD). *Mol. Neurodegener.* **2011**, *6* (1), 3.
(36) Gutiérrez, I. L.; Novellino, F.; Caso, J. R.; García-Bueno, B.; Leza, J. C.; Madrigal, J. L. M. CCL2 Inhibition of Pro-Resolving Mediators Potentiates Neuroinflammation in Astrocytes. *Int. J. Mol. Sci.* **2022**, *23* (6), 3307.
(37) Gutiérrez, I. L.; Dello Russo, C.; Novellino, F.; Caso, J. R.; García-Bueno, B.; Leza, J. C.; Madrigal, J. L. M. Noradrenaline in Alzheimer's Disease: A New Potential Therapeutic Target. *Int. J. Mol. Sci.* **2022**, *23* (11), 6143.
(38) Bratt, J. M.; Zeki, A. A.; Last, J. A.; Kenyon, N. J. Competitive metabolism of L -arginine: arginase as a therapeutic target in asthma. *J. Biomed. Res.* **2011**, *25* (5), 299−308.
(39) Campbell, L.; Saville, C. R.; Murray, P. J.; Cruickshank, S. M.; Hardman, M. J. Local arginase 1 activity is required for cutaneous wound healing. *J. Invest. Dermatol.* **2013**, *133* (10), 2461−2470.
(40) Doma, M. K.; Parker, R. RNA Quality Control in Eukaryotes. *Cell* **2007**, *131* (4), 660−668.
(41) Houseley, J.; Tollervey, D. The Many Pathways of RNA Degradation. *Cell* **2009**, *136* (4), 763−776.
(42) Mazille, M.; Buczak, K.; Scheiffele, P.; Mauger, O. Stimulus-specific remodeling of the neuronal transcriptome through nuclear intron-retaining transcripts. *EMBO J.* **2022**, *41* (21), No. e110192.
(43) Liu, Y.; Beyer, A.; Aebersold, R. On the Dependency of Cellular Protein Levels on mRNA Abundance. *Cell* **2016**, *165* (3), 535−550.
(44) Cevey, Á. C.; Penas, F. N.; Alba Soto, C. D.; Mirkin, G. A.; Goren, N. B. IL-10/STAT3/SOCS3 axis is involved in the anti-inflammatory effect of benznidazole. *Front. Immunol.* **2019**, *10*, 1267.
(45) Cua, D. J.; Sherlock, J.; Chen, Y.; Murphy, C. A.; Joyce, B.; Seymour, B.; Lucian, L.; To, W.; Kwan, S.; Churakova, T.; Zurawski, S.; Wiekowski, M.; Lira, S. A.; Gorman, D.; Kastelein, R. A.; Sedgwick, J. D. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* **2003**, *421* (6924), 744−748.
(46) Nitsch, L.; Petzinna, S.; Zimmermann, J.; Schneider, L.; Krauthausen, M.; Heneka, M. T.; Getts, D. R.; Becker, A.; Müller, M. Astrocyte-specific expression of interleukin 23 leads to an aggravated phenotype and enhanced inflammatory response with B cell accumulation in the EAE model. *J. Neuroinflammation* **2021**, *18* (1), 101.
(47) Town, T.; Bai, F.; Wang, T.; Kaplan, A. T.; Qian, F.; Montgomery, R. R.; Anderson, J. F.; Flavell, R. A.; Fikrig, E. Toll-like Receptor 7 Mitigates Lethal West Nile Encephalitis via Interleukin 23-Dependent Immune Cell Infiltration and Homing. *Immunity* **2009**, *30* (2), 242−253.
(48) Kleinschek, M. A.; Muller, U.; Brodie, S. J.; Stenzel, W.; Kohler, G.; Blumenschein, W. M.; Straubinger, R. K.; McClanahan, T.; Kastelein, R. A.; Alber, G. IL-23 Enhances the Inflammatory Cell Response in Cryptococcus neoformans Infection and Induces a Cytokine Pattern Distinct from IL-12. *J. Immunol.* **2006**, *176* (2), 1098−1106.
(49) Meeks, K. D.; Sieve, A. N.; Kolls, J. K.; Ghilardi, N.; Berg, R. E. IL-23 Is Required for Protection against Systemic Infection with Listeria monocytogenes. *J. Immunol.* **2009**, *183* (12), 8026−8034.
(50) Oppmann, B.; Lesley, R.; Blom, B.; Timans, J. C.; Xu, Y.; Hunte, B.; Vega, F.; Yu, N.; Wang, J.; Singh, K.; Zonin, F.; Vaisberg, E.; Churakova, T.; Liu, M.; Gorman, D.; Wagner, J.; Zurawski, S.; Liu, Y. J.; Abrams, J. S.; Moore, K. W.; Rennick, D.; De Waal-Malefyt, R.; Hannum, C.; Bazan, J. F.; Kastelein, R. A. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* **2000**, *13* (5), 715−725.
(51) Croxford, A. L.; Kulig, P.; Becher, B. IL-12-and IL-23 in health and disease. *Cytokine Growth Factor Rev.* **2014**, *25* (4), 415−421.
(52) Chyuan, I. T.; Lai, J. H. New insights into the IL-12 and IL-23: From a molecular basis to clinical application in immune-mediated inflammation and cancers. *Biochem. Pharmacol.* **2020**, *175*, 113928.
(53) Gao, Y.; Zhao, H.; Wang, P.; Wang, J.; Zou, L. The roles of SOCS3 and STAT3 in bacterial infection and inflammatory diseases. *Scand. J. Immunol.* **2018**, *88* (6), No. e12727.


https://doi.org/10.1021/acschemneuro.3c00525
ACS Chem. Neurosci. XXXX, XXX, XXX−XXX

(54) Nitsch, L.; Schneider, L.; Zimmermann, J.; Müller, M. Microglia-Derived Interleukin 23: A Crucial Cytokine in Alzheimer's Disease? *Front. Neurol.* **2021**, *12*, 639353.
(55) Woś, I.; Tabarkiewicz, J. Effect of interleukin-6, −17, −21, −22, and −23 and STAT3 on signal transduction pathways and their inhibition in autoimmune arthritis. *Immunol. Res.* **2021**, *69* (1), 26−42.
(56) Yang, X. O.; Panopoulos, A. D.; Nurieva, R.; Chang, S. H.; Wang, D.; Watowich, S. S.; Dong, C. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J. Biol. Chem.* **2007**, *282* (13), 9358−9363.
(57) Liu, X.; Zhou, L.; Xin, W.; Hua, Z. Exogenous Annexin 1 inhibits Th17 cell differentiation induced by anti-TNF treatment via activating FPR2 in DSS-induced colitis. *Int. Immunopharmacol.* **2022**, *107*, 108685.
(58) Cua, D. J.; Tato, C. M. Innate IL-17-producing cells: The sentinels of the immune system. *Nat. Rev. Immunol.* **2010**, *10* (7), 479−489.
(59) Chen, J.; Liu, X.; Zhong, Y. Interleukin-17A: The Key Cytokine in Neurodegenerative Diseases. *Front. Aging Neurosci.* **2020**, *12*, 566922.
(60) Qin, H.; Yeh, W. I.; De Sarno, P.; Holdbrooks, A. T.; Liu, Y.; Muldowney, M. T.; Reynolds, S. L.; Yanagisawa, L. L.; Fox, T. H.; Park, K.; Harrington, L. E.; Raman, C.; Benveniste, E. N. Signal transducer and activator of transcription-3/suppressor of cytokine signaling-3 (STAT3/SOCS3) axis in myeloid cells regulates neuroinflammation. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109* (13), 5004−5009.
(61) Cianciulli, A.; Dragone, T.; Calvello, R.; Porro, C.; Trotta, T.; Lofrumento, D. D.; Panaro, M. A. IL-10 plays a pivotal role in anti-inflammatory effects of resveratrol in activated microglia cells. *Int. Immunopharmacol.* **2015**, *24* (2), 369−376.
(62) Porro, C.; Cianciulli, A.; Trotta, T.; Lofrumento, D. D.; Panaro, M. A. Curcumin regulates anti-inflammatory responses by JAK/STAT/SOCS signaling pathway in BV-2 microglial cells. *Biology (Basel)* **2019**, *8* (3), 51.
(63) Zheng, Z. V.; Chen, J.; Lyu, H.; Lam, S. Y. E.; Lu, G.; Chan, W. Y.; Wong, G. K. C. Novel role of STAT3 in microglia-dependent neuroinflammation after experimental subarachnoid haemorrhage. *Stroke Vasc. Neurol.* **2022**, *7* (1), 62−70.
(64) Hillmer, E. J.; Zhang, H.; Li, H. S.; Watowich, S. S. STAT3 signaling in immunity. *Cytokine Growth Factor Rev.* **2016**, *31* (80), 1−15.
(65) Yin, Y.; Liu, W.; Dai, Y. SOCS3 and its role in associated diseases. *Hum. Immunol.* **2015**, *76* (10), 775−780.
(66) Tajiri, K.; Shimojo, N.; Sakai, S.; Machino-Ohtsuka, T.; Imanaka-Yoshida, K.; Hiroe, M.; Tsujimura, Y.; Kimura, T.; Sato, A.; Yasutomi, Y.; Aonuma, K. Pitavastatin regulates helper T-Cell differentiation and ameliorates autoimmune myocarditis in mice. *Cardiovasc. Drugs Ther.* **2013**, *27* (5), 413−424.
(67) Szczesny, E.; Basta-Kaim, A.; Slusarczyk, J.; Trojan, E.; Glombik, K.; Regulska, M.; Leskiewicz, M.; Budziszewska, B.; Kubera, M.; Lason, W. The impact of prenatal stress on insulin-like growth factor-1 and pro-inflammatory cytokine expression in the brains of adult male rats: The possible role of suppressors of cytokine signaling proteins. *J. Neuroimmunol.* **2014**, *276* (1−2), 37−46.
(68) Stoppini, L.; Buchs, P. A.; Muller, D. A simple method for organotypic cultures of nervous tissue. *J. Neurosci. Methods* **1991**, *37* (2), 173−182.
(69) Basta-Kaim, A.; Ślusarczyk, J.; Szczepanowicz, K.; Warszyński, P.; Leśkiewicz, M.; Regulska, M.; Trojan, E.; Lasoń, W. Protective effects of polydatin in free and nanocapsulated form on changes caused by lipopolysaccharide in hippocampal organotypic cultures. *Pharmacol. Rep.* **2019**, *71* (4), 603−613.
(70) Ślusarczyk, J.; Trojan, E.; Głombik, K.; Piotrowska, A.; Budziszewska, B.; Kubera, M.; Popiołek-Barczyk, K.; Lasoń, W.; Mika, J.; Basta-Kaim, A. Targeting the NLRP3 inflammasome-related pathways via tianeptine treatment-suppressed microglia polarization to the M1 phenotype in lipopolysaccharide-stimulated cultures. *Int. J. Mol. Sci.* **2018**, *19* (7), 1965.
(71) Chomczynski, P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques* **1993**, *15* (3), 532-4−536-7.
(72) Ślusarczyk, J.; Piotrowski, M.; Szczepanowicz, K.; Regulska, M.; Leśkiewicz, M.; Warszyński, P.; Budziszewska, B.; Lasoń, W.; Basta-Kaim, A. Nanocapsules with Polyelectrolyte Shell as a Platform for 1,25-dihydroxyvitamin D3 Neuroprotection: Study in Organotypic Hippocampal Slices. *Neurotoxic. Res.* **2016**, *30* (4), 581−592.
(73) Basta-Kaim, A.; Budziszewska, B.; Leśkiewicz, M.; Fijał, K.; Regulska, M.; Kubera, M.; Wędzony, K.; Lasoń, W. Hyperactivity of the hypothalamus-pituitary-adrenal axis in lipopolysaccharide-induced neurodevelopmental model of schizophrenia in rats: Effects of antipsychotic drugs. *Eur. J. Pharmacol.* **2011**, *650* (2−3), 586−595.
(74) Gogolla, N.; Galimberti, I.; DePaola, V.; Caroni, P. Staining protocol for organotypic hippocampal slice cultures. *Nat. Protoc.* **2006**, *1* (5), 2452−2456.


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# Supporting information

## Stimulation of Formyl Peptide Receptor-2 (FPR2) by the new agonist CMC23 protects against endotoxin-induced neuroinflammatory response: a study in organotypic hippocampal cultures

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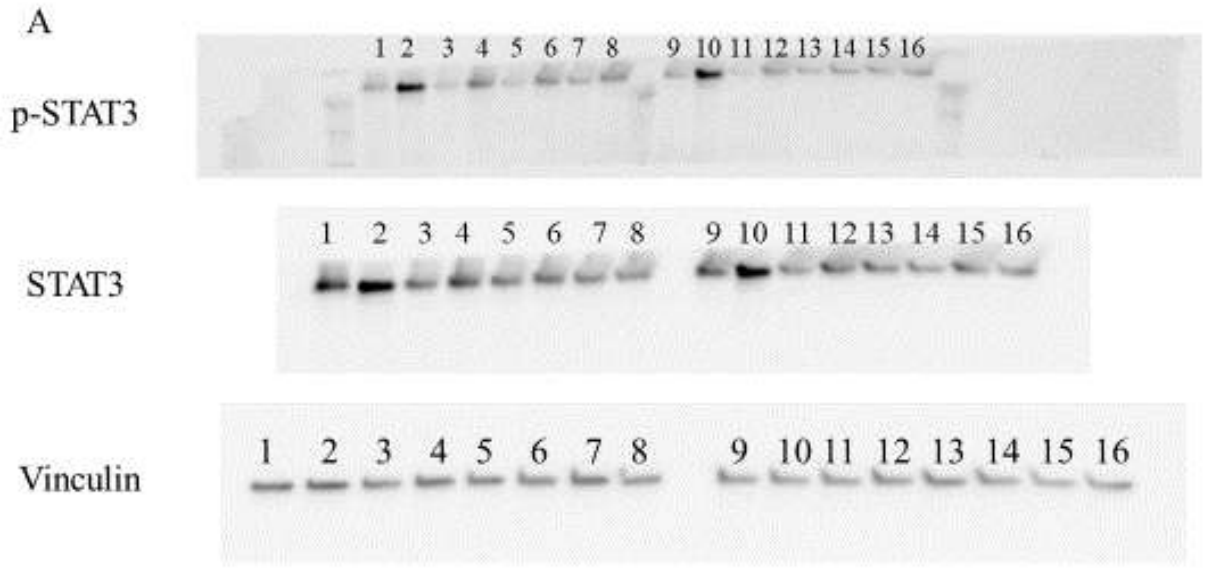
A

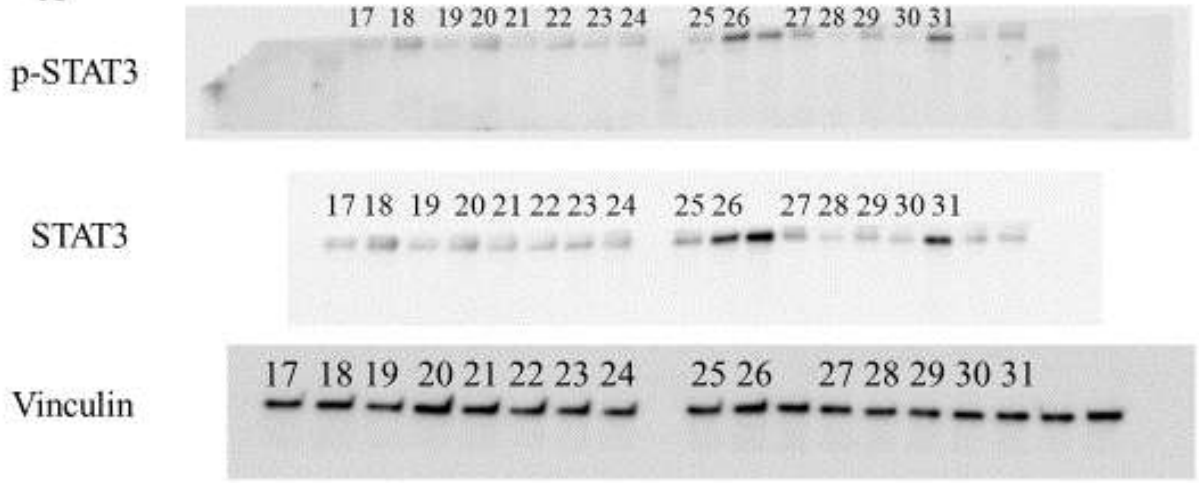



B

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| LPS 1 µg/ml | - | + | - | + | - | + | - | + |
| WRW4 10 µM | - | - | + | + | - | - | + | + |
| CMC23 0.1 µM | - | - | - | - | + | + | + | + |
| Sample ID | 1, 9 | 2, 10 | 3, 11 | 4, 1, | 5, 13 | 6, 14 | 7, 15 | 8, 16 |
| N | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |

**Supplementary Figure S1** Western blot analysis **(A)** of lysates from organotypic hippocampal cultures (OHCs) demonstrating protein level of phospho-STAT3 and total STAT3 protein. Vinculin was used as a normalizing control. Stripping and re-probing were used to investigate the levels of phosphorylated proteins before assessing the total expression of the same protein. Description of the arrangement of the samples (1-16) **(B)**.

A

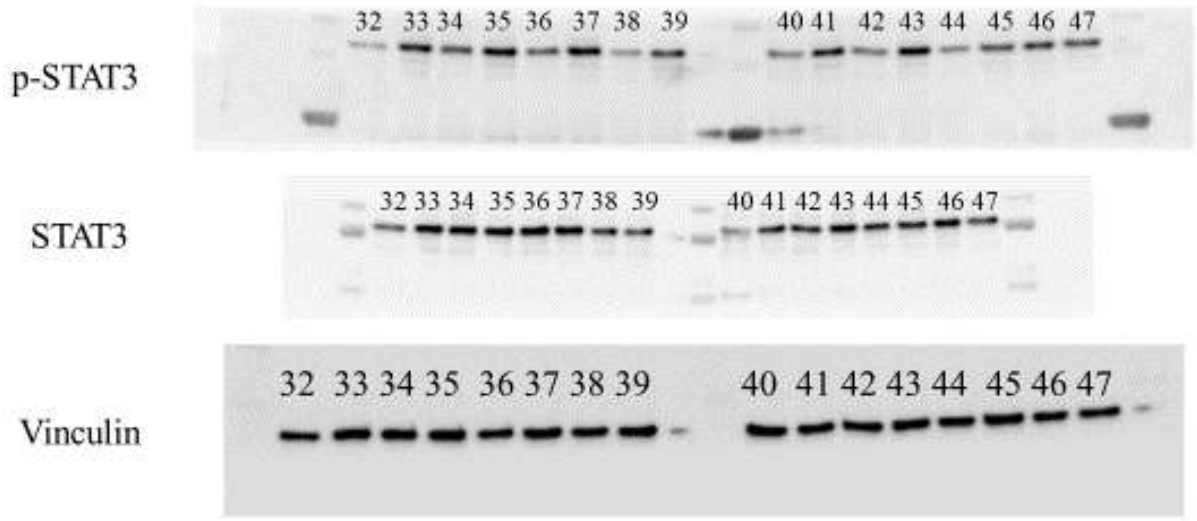



B

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| LPS 1 µg/ml | - | + | - | + | - | + | - | + |
| WRW4 10 µM | - | - | + | + | - | - | + | + |
| CMC23 0.1 µM | - | - | - | - | + | + | + | + |
| Sample ID | 17, 25 | 18, 26 | 19 | 20, 27 | 21, 28 | 22, 29 | 23, 30 | 24, 31 |
| N | 2 | 2 | 1 | 2 | 2 | 2 | 2 | 2 |

**Supplementary Figure S2** Western blot analysis **(A)** of lysates from organotypic hippocampal cultures (OHCs) demonstrating protein level of phospho-STAT3 and total STAT3 protein. Vinculin was used as a normalizing control. Stripping and re-probing were used to investigate the levels of phosphorylated proteins before assessing the total expression of the same protein. Description of the arrangement of the samples (17-31) **(B)**.

A

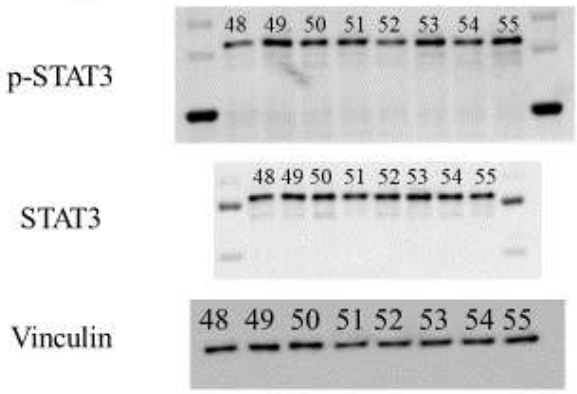



B

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| LPS 1 µg/ml | - | + | - | + | - | + | - | + |
| WRW4 10 µM | - | - | + | + | - | - | + | + |
| CMC23 0.1 µM | - | - | - | - | + | + | + | + |
| Sample ID | 32, 40 | 33, 41 | 34, 42 | 35, 43 | 36, 44 | 37, 45 | 38, 46 | 39, 47 |
| N | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |

**Supplementary Figure S3** Western blot analysis **(A)** of lysates from organotypic hippocampal cultures (OHCs) demonstrating protein level of phospho-STAT3 and total STAT3 protein. Vinculin was used as a normalizing control. Stripping and re-probing were used to investigate the levels of phosphorylated proteins before assessing the total expression of the same protein. Description of the arrangement of the samples (32-47) **(B)**.

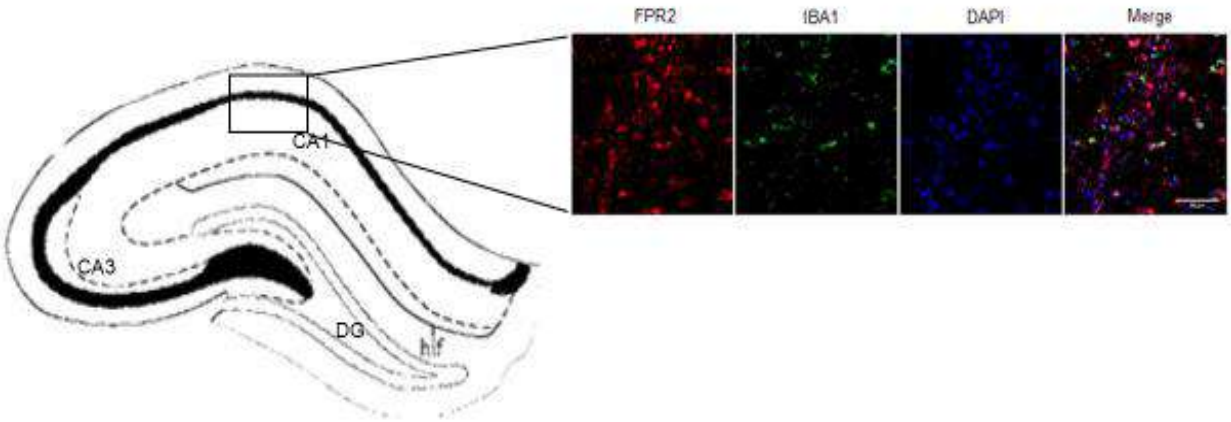
A




B

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| LPS 1 µg/ml | - | + | - | + | - | + | - | + |
| WRW4 10 µM | - | - | + | + | - | - | + | + |
| CMC23 0.1 µM | - | - | - | - | + | + | + | + |
| Sample ID | 48 | 49 | 50 | 51 | 52 | 53 | 54 | 55 |
| N | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

**Supplementary Figure S4** Western blot analysis **(A)** of lysates from organotypic hippocampal cultures (OHCs) demonstrating protein level of phospho-STAT3 and total STAT3 protein. Vinculin was used as a normalizing control. Stripping and re-probing were used to investigate the levels of phosphorylated proteins before assessing the total expression of the same protein. Description of the arrangement of the samples (48-55) **(B)**.




**Supplementary Figure S5** Schematic representation of the hippocampus. The fluorescence images were taken from the CA1 hippocampus region (labeled with a square on the scheme).

**Microglia Depletion Attenuates the Pro-Resolving Activity of the Formyl Peptide Receptor 2 Agonist AMS21 Related to Inhibition of Inflammasome NLRP3 Signalling Pathway: A Study of Organotypic Hippocampal Cultures.**

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# Microglia Depletion Attenuates the Pro-Resolving Activity of the Formyl Peptide Receptor 2 Agonist AMS21 Related to Inhibition of Inflammasome NLRP3 Signalling Pathway: A Study of Organotypic Hippocampal Cultures

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**Abstract:** Microglial cells have been demonstrated to be significant resident immune cells that maintain homeostasis under physiological conditions. However, prolonged or excessive microglial activation leads to disturbances in the resolution of inflammation (RoI). Formyl peptide receptor 2 (FPR2) is a crucial player in the RoI, interacting with various ligands to induce distinct conformational changes and, consequently, diverse biological effects. Due to the poor pharmacokinetic properties of endogenous FPR2 ligands, the aim of our study was to evaluate the pro-resolving effects of a new ureidopropanamide agonist, compound AMS21, in hippocampal organotypic cultures (OHCs) stimulated with lipopolysaccharide (LPS). Moreover, to assess whether AMS21 exerts its action via FPR2 specifically located on microglial cells, we conducted a set of experiments in OHCs depleted of microglial cells using clodronate. We demonstrated that the protective and anti-inflammatory activity of AMS21 manifested as decreased levels of lactate dehydrogenase (LDH), nitric oxide (NO), and proinflammatory cytokines IL-1β and IL-6 release evoked by LPS in OHCs. Moreover, in LPS-stimulated OHCs, AMS21 treatment downregulated NLRP3 inflammasome-related factors (CASP1, NLRP3, PYCARD) and this effect was mediated through FPR2 because it was blocked by the FPR2 antagonist WRW4 pre-treatment. Importantly this beneficial effect of AMS21 was only observed in the presence of microglial FPR2, and absent in OHCs depleted with microglial cells using clodronate. Our results strongly suggest that the compound AMS21 exerts, at nanomolar doses, protective and anti-inflammatory properties and an FPR2 receptor located specifically on microglial cells mediates the anti-inflammatory response of AMS21. Therefore, microglial FPR2 represents a promising target for the enhancement of RoI.

**Keywords:** formyl peptide receptor 2; ureidopropanamide agonist; neuroinflammation; lipopolysaccharide; inflammasome NLPR3-related pathway; hippocampus



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## 1. Introduction

Microglia, the brain's resident immune cells, play a crucial role in health and disease [1,2]. They defend the brain against infections and injury [3], contribute to neurogenesis and synaptic conditions, and interact with neurons and other brain cells to control homeostasis [4–7]. Importantly, microglial cells are susceptible to the surrounding environment [8] and possess various receptors to sense microenvironment changes that regulate their functions [9,10]. Therefore, during inflammatory stimulation, the brain environment

plays a crucial role in the proper resolution of inflammation (RoI). In fact, triggering microglial reactivity and prolonged stimulation, they alter homeostasis, leading to neuronal and other glial tissue damage [11–13]. Once activated, microglia change their morphology, proliferate and enhance the expression of various cluster of differentiation (CD) markers (e.g., CD40 and CD68). Moreover, microglia are a prominent source of proinflammatory cytokines such as IL-1β, IL-18, IL-6 and tumour necrosis factor alpha (TNF-α), as well as neurotoxic mediators including NO, ROS, prostaglandin (PG) E2, and the superoxide anion [14,15].

One of the crucial players in the development of the neuroinflammatory response is the nucleotide-binding oligomerisation domain-like (NOD-like) receptor pyrin-containing 3 inflammasome (NLRP3), which is highly expressed in microglia [16–18]. NLRP3 is a multiprotein complex consisting of NLRP3, procaspase-1, and apoptosis-associated speck-like protein containing a caspase recruitment domain (PYCARD). Various environmental and endogenous molecules activate the NLRP3 complex. Additionally, this complex is indirectly activated by a primary component of the endotoxin from Gram-negative bacterial cell walls, lipopolysaccharide (LPS) [19,20]. The two-step NLRP3 inflammasome activation facilitates the oligomerisation of inactive NLRP3, PYCARD, and procaspase-1. This complex, in turn, catalyses the conversion of procaspase-1 to caspase-1, which contributes to the production and secretion of microglial mature proinflammatory cytokines, mainly IL-1β and IL-18 [21–23].

In addition, to proinflammatory polarisation, microglial cells also have an anti-inflammatory phenotype and are involved in the suppression of inflammation and in the restoration of homeostasis inter alia by the release of transforming growth factor (TGF-β), insulin-like growth factor 1 (IGF-1), and IL-10 [24–26]. Alterations in microglial polarisation are associated with excessive microglial inflammatory activation and disturbances in the RoI [11,13]. Since microglial cells are highly heterogeneous, as identified via novel transcriptomic and microscopy studies [27,28], it has been suggested that by targeting specific receptors, microglial cells can be "reprogrammed" to adopt new functional and molecular identities in a context-dependent manner [29].

Formyl peptide receptors (FPRs) belong to the seven-transmembrane G protein-coupled receptor family. As demonstrated in previous scientific research in the brain, FPR2 is expressed mainly in microglial cells, neurons, and astroglia not only in physiological but also pathological conditions [30–35]. Among them, FPR2 is a low-affinity receptor for N-formyl peptides and is considered the most promiscuous member of the FPR family. FPR2 can recognise various endogenous and exogenous ligands, ranging from lipids to proteins and peptides, including non-formylated peptides [36,37]. FPR2 also shows diversity in receptor signalling leading to different and sometimes opposite downstream effects, which have been ascribed to conformational changes that underline biased signalling [38,39]. Biased agonism explains at least in part the role of different FPR2 agonists in modulating the inflammatory response [40]. In fact, after the binding of the ligand, FPR2 is activated and triggers several agonist-dependent signal transduction pathways [41,42]. However, it should be strongly emphasised that FPR2 downstream signalling pathway activation depends not only on the ligand but also on the cell type involved [43,44], which is crucial in understanding how FPR2 activation can modulate cellular processes, including the RoI.

Therefore, identifying new bioactive compounds that can target microglial FPR2 and inflammatory pathways to dampen the neuroinflammatory response may be a useful approach in preventing or delaying the onset of neuroinflammation-based brain diseases.

We recently identified a series of ureidopropanamide-based FPR2 agonists endowed with high agonist potency. Among the studied compounds, derivative AMS21 ((S)-1-(3-(4-cyanophenyl)-1-(indolin-1-yl)-1-oxopropan-2-yl)-3-(4-fluorophenyl)urea) was able to activate FPR2 at nanomolar concentrations (EC50 = 0.026 μM), which was was 10-fold selective over FPR1 subtype and showed acceptable resistance to oxidative metabolism, which are promising properties for prospective in vivo preclinical studies [45]. In addition, in rat primary microglial cells stimulated with LPS, the anti-inflammatory effects of AMS21 were

counterbalanced by the FPR2 antagonist WRW4, suggesting these were mainly mediated through the interaction with FPR2 [45].

In the present study, we assessed the pro-resolving effects of compound AMS21 on the neuroinflammatory response stimulated by bacterial endotoxin treatment using organotypic hippocampal cultures (OHCs). Moreover, to confirm the contribution of FPR2 activation to the observed effects of AMS21, we used the FPR2 receptor antagonist WRW4. Next, we focused on the impact of AMS21 on FPR2 localised on microglial cells, assessing the anti-inflammatory activity of this ligand in OHCs depleted of microglial cells using clodronate. The intracellular mechanisms of the effects of AMS21, with particular emphasis on the pathway associated with the NLRP3 inflammasome under standard conditions and in microglia-deficient cultures, were also evaluated.

## 2. Materials and Methods

### 2.1. Animals

Sprague Dawley rats were obtained from Charles Rivers (Sulzfel, Germany) and kept under standard conditions. The rats were maintained at room temperature (23 °C) on a 12/12 h light/dark cycle (lights on at 06:00 am) with food and water available ad libitum. After one week of acclimatisation, the presence of the proestrus cycle was identified by taking vaginal smears daily. On the proestrus day, females and males were mated overnight. Next, pregnant females were kept under standard conditions in home cages without any disruptions. 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 June 2018).

### 2.2. Chemicals

The formyl peptide receptor 2 agonist compound AMS21 ((S)-1-(3-(4-cyanophenyl)-1-(indolin-1-yl)-1-oxopropan-2-yl)-3-(4-fluorophenyl)urea) was synthesised at the Department of Pharmacy, University of Bari, as previously reported [45]. The FPR2 antagonist WRW4 was purchased from Alomone Labs, Israel. The bacterial endotoxin lipopolysaccharide (LPS; *Escherichia coli* 0111:B4) was obtained from Sigma-Aldrich, St. Louis, MO, USA. Microglial cells were depleted using clodronate (disodium salt) obtained from Millipore, Burlington, MA, USA. Stock solutions of the used compounds were prepared as follows: AMS21 in DMSO to 1 mM concentration, LPS in PBS to 1 mg/mL, WRW4, and clodronate in distilled water to 1 mM and 1 mg/mL concentration, respectively. The final concentrations of the used compounds were in distilled water.

### 2.3. Establishment of Organotypic Hippocampal Cultures (OHCs)

Organotypic hippocampal cultures were prepared according to the protocol of Stoppini et al. [46] with slight modifications. The cultures were prepared from six- to seven-day-old Sprague Dawley females. The animals were decapitated, and the isolated brains were placed directly into 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). Afterwards, the hippocampi were placed on Teflon discs and cut into 350 µM sections using a McIlwain™ Tissue Chopper (Surrey, UK). Then, selected sections were transferred into ThinCerts™ (Minneapolis, MN, USA) inserts with 0.4 µM pore size membranes (Greiner Bio-one, Kremsmunster, Austria) in 6-well plates containing 1 mL of initial medium with 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, London, UK)). OHCs were grown for 7 days in vitro (DIV) in an incubator (37 °C) with an adjustable $CO_2$ flow (5%). The culture medium was changed every 48 h, and horse serum concentration was tapered down according to the following schedule: on 1st to 3rd DIV 25% medium; on 5th DIV 10% medium (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, London, UK)); and lastly on 7th DIV serum-free medium (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).

### 2.4. Treatment

Treatment of OHCs was divided into two parts depending on clodronate presence or absence. In the groups without clodronate on the 7th DIV, pre-treatment was performed with the FPR2 antagonist WRW4 (10 μM) for 30 min. Then, the new FPR2 agonist compound AMS21 at two doses, 0.1 μM and 1 μM, was added to the culture medium for an hour, and, afterwards, OHCs were stimulated with 1 μg/mL LPS for 24 h.

In the second part of the experiments, to deplete microglia, clodronate was prepared and used according to the manufacturer's instructions. Clodronate was administered to the culture medium in the 1st DIV for 24 h. Afterwards, the culture medium was removed, 6-well plates were washed twice with warm, sterile PBS, and a new medium was added. On the 7th DIV, OHCs were treated with AMS21 at a dose of 0.1 μM (selected in the first part of the experiments), and an hour later, bacterial endotoxin at 1 μg/mL dose was added for 24 h. Control OHCs were treated with PBS as a vehicle.

### 2.5. Lactate Dehydrogenase (LDH) Assay

A lactate dehydrogenase (LDH) assay was conducted using a Cytotoxicity Detection Kit (Roche, Germany) as previously described [47,48]. Briefly, 24 h after LPS administration, the culture medium was collected, and 50 μL of each sample was placed into a 96-well plate. Then, an equal amount of reagent mixture prepared according to the manufacturer's instructions was mixed with the samples. After incubation at 37 °C, the intensity of the red colour formed in the colorimetric assay was measured at a wavelength of 490 nm (Infinite® 200 PRO plate reader, Tecan, Zurich, Switzerland) and was proportional to the number of damaged/dead cells.

### 2.6. Nitric Oxide (NO) Assay

The amount of nitric oxide (NO) was detected as we previously described [49,50] using a colorimetric Griess reaction in accordance with the protocol. An equal volume of the collected samples (50 μL), Griess A (0,1% N-1-naphthylethylenediamine dihydrochloride), and Griess B (1% sulfanilamide in 5% phosphoric acid; Sigma-Aldrich, St. Louis, MO, USA), was mixed in a 96-well plate. The intensity of the formed colour was measured at a wavelength of 540 nm (Infinite® 200 PRO plate reader, Tecan, Zurich, Switzerland).

### 2.7. RNA Extraction and cDNA Preparation

Twenty-four hours after LPS administration, slices were lysed using 200 μL TRI® Reagent (Sigma-Aldrich, St. Louis, MO, USA) and stored at −20 °C until isolation. Total RNA extraction was performed according to the User Guide (TRIzol® Reagent User Guide Instructions; Thermo Fisher Scientific, Waltham, MA, USA) based on the Chomczynski [51] method. Then, the RNA concentration was assessed using a NanoDrop spectrophotometer (ND/1000 UV/Vis, Thermo Fisher NanoDrop, Waltham, MA, USA). The synthesis of cDNA was performed using an NG dART RT Kit (EURx, Gdansk, Poland) according to the manufacturer's instructions.

### 2.8. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The 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: *Casp1* (*Caspase 1*; Rn00562724_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-1ra* (*Interleukin 1 receptor antagonist*; Rn02586400_m1), *Il-1β* (*Interleukin 1β*; Rn00580432_m1), *Il-6* (*Interleukin 6*; Rn01410330_m1), *Il-18* (*Interleukin 18*; Rn01422083_m1), *Nlrp3* (*NLR family pyrin domain containing 3*; Rn04244620_m1), *Pycard*

(*Apoptosis-associated speck-like protein containing a caspase recruitment domain*; Rn00597229_g1), *Tgf-β1* (*Transforming growth factor β1*; Rn00572010_m1) (all obtained from Thermo Fisher Scientific, Waltham, MA, USA). The *B2m* gene (*β2 microglobulin*; Rn00560865_m1) was used as a normalising control. Thermal cycling conditions were as follows: 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The threshold value (Ct) for each sample was set in the exponential phase of PCR, and the ΔΔCt method was used for data analysis.

### 2.9. Enzyme-Linked Immunosorbent Assay (ELISA)

The OHCs medium was collected 24 h after LPS administration for further experiments. Furthermore, OHCs were lysed using 160 µL of RIPA buffer with Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA, USA). Protein isolation was performed, and the total concentration of the protein was assessed using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Optical density was measured at a wavelength of 562 nm using an Infinite® 200 PRO plate reader (Tecan, Zurich, Switzerland). The levels of interleukin 1β, interleukin 6, interleukin 10 and transforming growth factor β were measured in the collected supernatants, and the levels of caspase 1, NLR family pyrin domain containing 3, and apoptosis-associated speck-like protein containing a caspase recruitment domain were assessed in the protein isolated from OHCs. All tests were performed using commercially available enzyme-linked immunosorbent assays (ELISA) obtained from Wuhan Fine Biotech Co., Ltd. Wuhan, China (IL-1β, TGF-β, CASP1, NLRP3, PYCARD), BD Biosciences, Franklin Lakes, NJ, USA (IL-6, IL-10) in accordance with the manufacturer's protocols. The detection limits were as follows: IL-1β < 18.75 pg/mL, IL-6 < 78 pg/mL, IL-10 < 15.6 pg/mL, TGF-β < 18.75 pg/mL, CASP1 < 37.5 pg/mL, NLRP3 < 0.188 ng/mL, and PYCARD < 46.875 pg/mL. The inter-assay precision of all ELISA kits was CV% < 10%. The intra-assay precision of all ELISA kits was CV% < 8%.

### 2.10. Western Blot Analyses

Western blot analyses were carried out as we described previously [4,6,52]. The samples (equal protein concentration) were mixed with Laemmli buffer (Roche, Basel, Switzerland) in a 4:1 ratio and heated in an Eppendorf Thermomixer comfort (Sigma-Aldrich, St. Louis, MO, USA) for 8 min at 95 °C. Afterwards, the samples were resolved by SDS-PAGE on 4–20% Criterion™ TGX™ Precast Gels (Bio-Rad, Hercules, CA, USA) and transferred to PVDF membranes (Sigma-Aldrich, St. Louis, MO, USA) using Trans-Blot Turbo (Bio-Rad, Hercules, CA, USA). Membranes with transferred protein were briefly washed with Tris-buffered saline (TBS) (pH 7.5), blocked in 5% bovine serum albumin (5% BSA dissolved in TBS with 0.1% Tween 20 (TBST)) for 1 h at room temperature (RT), and washed with TBST 3 times for 10 min. Then, the membranes were incubated with primary antibodies overnight at 4 °C anti-IBA1 (NBP2-19019, 1:500, Novus Biologicals, Centennial, CO, USA) and anti-vinculin (1:15,000, V9264, Sigma-Aldrich, St. Louis, MO, USA) diluted in Signal Boost Immunoreaction Enhancer Kit Buffer (Millipore, Warsaw, Poland). The next day, the membranes were washed in TBST 3 times for 10 min and incubated with horse anti-mouse IgG (1:10,000, PI-2000 Vector Laboratories, Newark, CA, USA) and goat anti-rabbit IgG (1:10,000, PI-1000, Vector Laboratories, Newark, CA, USA) secondary antibodies for an hour. Finally, the membranes were washed again with TBST, and blots were detected using Pierce™ ECL Western blotting substrate (Thermo Fisher, Waltham, MA, USA) and visualised using a Fujifilm LAS1000 system (Fuji Film, Tokyo, Japan). The relative levels of immunoreactivity were quantified using Fujifilm Multi Gauge V3.0 software (Fuji Film, Tokyo, Japan).

### 2.11. Immunofluorescence Staining of Organotypic Hippocampal Cultures

Immunofluorescence staining of OHCs was performed in accordance with Gogolla et al. [53] with slight modifications. Twenty-four hours after LPS administration, OHCs were fixed with 4% paraformaldehyde (PFA, ChemCruz®, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) 1 mL above and 1 mL underneath the insert for an hour. The slices were washed with PBS 3 times for 5 min, and the sections were kept at 4 °C until experiments. Afterwards, OHCs were carefully removed from the inserts, placed into 12-well plates, and permeabilised in 0.5% Triton X-100 in PBS for up to 18 h at 4 °C. Then, the sections were blocked with 20% BSA in PBS solution (20% bovine serum albumin, Sigma-Aldrich, St. Louis, MO, USA) overnight at 4 °C or for 4 h at RT. All blocked sections were first incubated with an anti-FPR2 rabbit polyclonal antibody (Huabio, Greater Boston, MA, USA; 1:50) and then with a goat anti-rabbit antibody conjugated with the fluorescent dye AlexaFluor® 647 (Abcam, Cambridge, UK; 1:300) overnight at 4 °C in a closed wet chamber. Secondary staining was carried out using the primary antibody anti-IBA1 goat polyclonal antibody (Abcam, Cambridge, UK; 1:50) under the same conditions. Subsequently, the slices were incubated with donkey anti-goat secondary antibody conjugated with the fluorescent dye Alexa Fluor® 555 (Abcam, Cambridge, UK; 1:300). OHCs were briefly washed with 5% BSA in PBS and stained with Hoechst 33342 (Invitrogen, Waltham, MA, USA; 1:5000) for 15 min at RT to stain the nuclei. Dyed OHCs were placed onto microscope slides and 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 63× 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).

### 2.12. Quantitative Analyses of Confocal Fluorescence Images of Organotypic Hippocampal Cultures

The fluorescence intensity of IBA1 and FPR2 was measured using ImageJ. Briefly, the intensity in single images was measured after applying the colour threshold separately for IBA1 and FPR2. Then, the thresholded area derived from the entire picture was determined, and the data are presented as the control mean ± SEM.

### 2.13. Statistical Analysis

The data were analysed using Statistica 13.3 software (Stat Soft, Tulsa, OK, USA). All biochemical experiments were conducted under the same conditions for all samples, regardless of the type of treatment. The results were obtained from independent experiments carried out under the same conditions and are presented as the mean ± SEM. Data obtained from LDH, NO, and confocal microscopy are presented as the mean percentage ± SEM of the control. The results obtained from the ELISA 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.

## 3. Results

### 3.1. The Effect of WRW4 and AMS21 Treatment on Lactate Dehydrogenase and Nitric Oxide Release in OHCs

To establish the most efficient dose of the FPR2 agonist AMS21, our initial studies focused on the assessment of the release of lactate dehydrogenase (LDH), which is a cell death marker, and nitric oxide (NO) induced by LPS stimulation. For this purpose, we treated cultures with AMS21 at two doses: 0.1 μM and 1 μM. Furthermore, OHCs were also pre-treated with the FPR2 antagonist WRW4 to determine whether the observed effect of the tested agonist was mediated through FPR2.

Treatment of OHCs with AMS21 at both doses and WRW4 did not affect LDH release in the control groups (Figure 1A). However, we observed a decrease in NO release after

combined AMS21 and WRW4 administration ($p = 0.045149$ and $p = 0.034001$ for 0.1 µM and 1 µM AMS21 doses, respectively) (Figure 1B). Stimulation of OHCs with LPS (1 µg/mL) elevated the levels of both LDH ($p = 0.008050$) (Figure 1A) and NO ($p = 0.000033$) (Figure 1B) release. LDH release was decreased after AMS21 administration in LPS-stimulated OHCs; however, this beneficial effect was observed only after a lower (0.1 µM) dose ($p = 0.003820$) (Figure 1A). Moreover, we observed a significant attenuation of NO release at 0.1 µM ($p = 0.000057$) and 1 µM ($p = 0.000057$) AMS21 doses (Figure 1B) in LPS-challenged groups. Pre-treatment with the FPR2 antagonist WRW4 did not significantly modulate favourable AMS21 properties in LPS-stimulated groups. Since AMS21 attenuated the release of LDH and NO only at 0.1 µM, this dose was chosen for further studies.

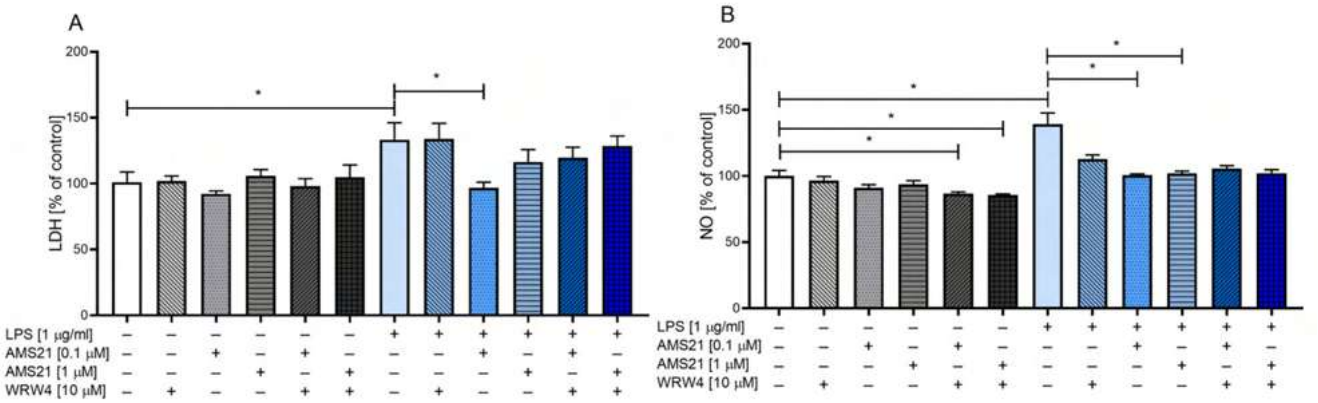



**Figure 1.** The effect of WRW4 and AMS21 treatment on lactate dehydrogenase (LDH) (**A**) and nitric oxide (NO) (**B**) release in LPS-stimulated OHCs. Cultures were pre-treated with the FPR2 antagonist WRW4 (10 µM) for 30 min. Afterwards, OHCs were treated with two doses of AMS21 (0.1 µM and 1 µM) for one hour and then stimulated with lipopolysaccharide (LPS; 1 µg/mL) for 24 h. Control groups were treated with an appropriate vehicle. The data are presented as the mean percentage ± SEM of the control of independent experiments, $n = 4$–6 in each experiment. Statistical analysis was carried out 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.

### *3.2. The Effect of WRW4 and AMS21 Treatment on the Release of the Proinflammatory Cytokines IL-1β and IL-6 in OHCs*

To examine whether AMS21 has anti-inflammatory properties, we assessed the levels of two proinflammatory cytokines, IL-1β and IL-6, using ELISA. Our findings indicate that AMS21 (0.1 µM) and WRW4 (10 µM) did not affect the levels of IL-1β (Figure 2A) and IL-6 (Figure 2B) under basal conditions. As expected, LPS (1 µg/mL) stimulation significantly elevated the levels of both the proinflammatory cytokines IL-1β ($p = 0.007455$) (Figure 2A) and IL-6 ($p = 0.000039$) (Figure 2B). Furthermore, AMS21 attenuated the inflammatory response caused by LPS since decreased levels of IL-1β ($p = 0.022065$) (Figure 2A) and IL-6 ($p = 0.029148$) (Figure 2B) were observed. In the case of IL-1β, the anti-inflammatory effect of AMS21 was mediated via FPR2, and pre-treatment with WRW4 blocked the beneficial effect of AMS21 ($p = 0.000732$) (Figure 2A).

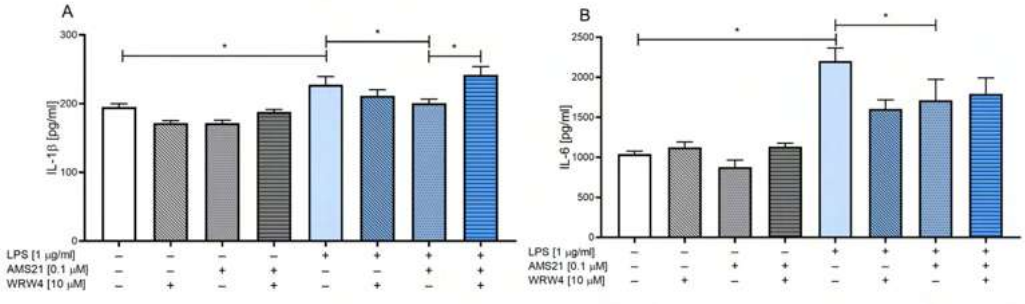



**Figure 2.** The impact of WRW4 and AMS21 on the release of proinflammatory IL-1β (**A**) and IL-6 (**B**) cytokines in LPS-stimulated OHCs. Cultures were pre-treated with the FPR2 antagonist WRW4 (10 μM) for 30 min. Afterwards, OHCs were stimulated with AMS21 (0.1 μM) for one hour and then with lipopolysaccharide (LPS; 1 μg/mL) for 24 h. Control groups were treated with the appropriate vehicle. The data are presented as the mean ± SEM of independent experiments, $n$ = 4–8 in each experiment. Statistical analysis was carried out 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.

### *3.3. The Effect of WRW4 and AMS21 Treatment on the Release of Anti-Inflammatory Cytokines TGF-β and IL-10 in OHCs*

In the next part of our research, we determined the pro-resolving properties of AMS21 by assessing the protein levels of two anti-inflammatory cytokines, TGF-β and IL-10. Treatment of OHCs with WRW4 (10 μM) and AMS21 (0.1 μM) under basal conditions reduced the protein level of TGF-β ($p$ = 0.016505) (Figure 3B). Nevertheless, an equivalent effect was not observed in the case of IL-10 (Figure 3A) under basal conditions. Our findings indicate elevated levels of both cytokines TGF-β ($p$ = 0.010386) (Figure 3A) and IL-10 ($p$ = 0.021561) (Figure 3B) in LPS-stimulated groups. Moreover, AMS21 administration slightly increased the TGF-β ($p$ = 0.000818) (Figure 3A) level and maintained an enhanced level of IL-10 ($p$ = 0.018393) (Figure 3B) in the LPS-challenged group in comparison to the control group. Importantly, the favourable effect of AMS21 was mediated via FPR2 in the case of TGF-β release ($p$ = 0.000025) (Figure 3A) since we observed a diminished level of this factor in WRW4 pre-treated groups.

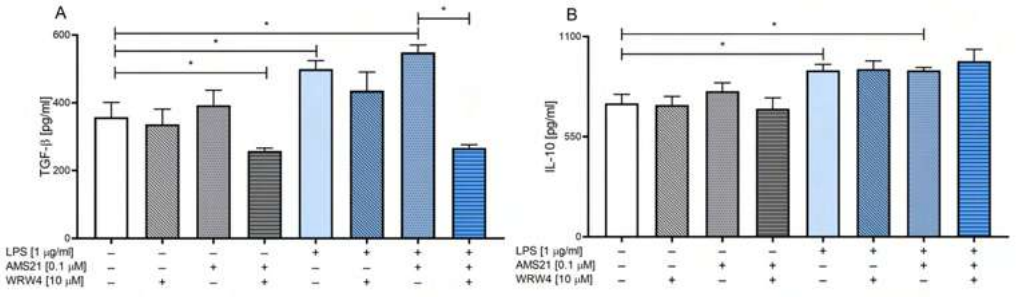



**Figure 3.** The impact of WRW4 and AMS21 on the release of anti-inflammatory TGF-β (**A**) and IL-10 (**B**) cytokines in LPS-stimulated OHCs. Cultures were pre-treated with the FPR2 antagonist WRW4 (10 μM) for 30 min. Afterwards, OHCs were stimulated with AMS21 (0.1 μM) for one hour and then with lipopolysaccharide (LPS; 1 μg/mL) for 24 h. Control groups were treated with the appropriate vehicle. The data are presented as the mean ± SEM of independent experiments, $n$ = 4–8 in each experiment. Statistical analysis was carried out 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.

### *3.4. The Effect of AMS21 on the mRNA Expression of Proinflammatory and Anti-Inflammatory Genes*

Considering the favourable role of AMS21 in modulating anti-inflammatory and pro-resolving action and the fact that microglia are crucial immune cells in the central nervous system, we assessed proinflammatory and anti-inflammatory gene profiles with particular consideration of microglial markers.

We determined the mRNA expression levels of proinflammatory (*Cd40*, *Cd68*, *Il-1β*, *Il-6*, *Il-18*) (Table 1 part A) and anti-inflammatory genes (*Igf-1*, *Il-1Ra*, *Tgf-β*) (Table 1 part B). The statistical analysis of proinflammatory genes revealed the upregulation of the *Cd40* ($p = 0.037870$), *Il-1β* ($p = 0.000159$), *Il-6* ($p = 0.036641$), and *Il-18* ($p = 0.028277$) (Table 1 part A) genes in LPS-treated (1 μg/mL) OHCs. Importantly, the impact of AMS21 (0.1 μM) was noticed as the downregulation of *Cd40* ($p = 0.042920$ and *Il-18* ($p = 0.030416$) genes after LPS stimulation. As shown in Table 1 part B, LPS administration decreased *Igf-1* ($p = 0.002364$) and increased the *Il-1Ra* ($p = 0.008516$) mRNA levels. AMS21 potentiated the elevation of *Il-Ra* ($p = 0.031202$) evoked by LPS.

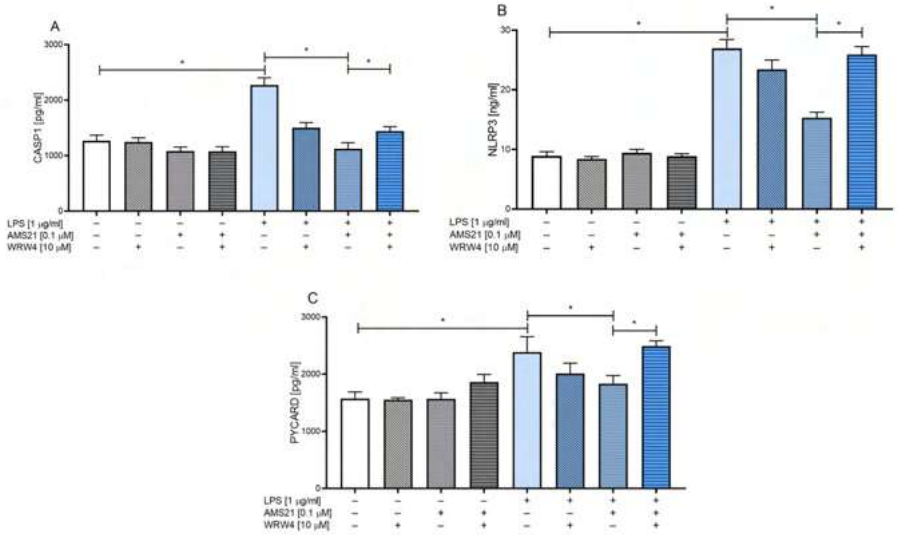
**Table 1.** The impact of AMS21 on the mRNA expression of proinflammatory (*Cd40*, *Cd68*, *Il-1β*, *Il-6*, *Il-18*) (A) and anti-inflammatory (*Igf-1*, *Il-1Ra*, *Tgf-β*) (B) genes in LPS-stimulated OHCs. OHCs were stimulated with AMS21 (0.1 μM) for an hour and subsequently with lipopolysaccharide (LPS; 1 μg/mL) for 24 h. Control groups were treated with the appropriate vehicle. The data are presented as the average fold change ± SEM of independent experiments, $n = 3$–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 the LPS group; # $p < 0.05$ LPS compared to the AMS21 + LPS group.

| | **Control** | **LPS** | **AMS21** | **AMS21 + LPS** |
|---|---|---|---|---|
| **A** | | | | |
| ***Proinflammatory factors*** | | | | |
| *Cd40* | 1.04 ± 0.18 | **2.83 ± 1.51** * | 1.02 ± 0.25 | **0.96 ± 0.14** # |
| *Cd68* | 1.06 ± 0.18 | 1.07 ± 0.39 | 0.92 ± 0.20 | 0.94 ± 0.16 |
| *Il-1β* | 1.06 ± 0.15 | **27.05 ± 3.70** * | 1.02 ± 0.18 | **27.58 ± 1.90** * |
| *Il-6* | 1.07 ± 0.27 | **3.57 ± 0.33** * | 3.23 ± 1.19 | 3.42 ± 0.24 |
| *Il-18* | 1.05 ± 0.18 | **2.52 ± 0.86** * | 1.04 ± 0.29 | **0.97 ± 0.18** # |
| **B** | | | | |
| ***Anti-inflammatory factors*** | | | | |
| *Igf-1* | 1.04 ± 0.15 | **0.33 ± 0.02** * | 1.06 ± 0.18 | 0.47 ± 0.10 |
| *Il-1Ra* | 1.06 ± 0.28 | **7.18 ± 1.63** * | 1.59 ± 0.25 | **11.74 ± 1.98** # |
| *Tgf-β* | 1.03 ± 0.16 | 0.45 ± 0.14 | 1.15 ± 0.21 | 0.84 ± 0.12 |

### *3.5. The Effect of WRW4 and AMS21 Treatment on the NLRP3-Related Pathway in OHCs*

We also determined the effect of AMS21 on the protein level and mRNA expression of all NLRP3 inflammasome subunits, which is a multiprotein complex containing sensor protein (NLRP3), adaptor protein (PYCARD) and caspase-1 protein [21,54].

As revealed in Figure 4, AMS21 (0.1 μM) and WRW4 (10 μM) did not affect the protein level of all examined subunits under basal conditions. However, LPS (1 μg/mL) stimulation led to a significant increase in CASP1 ($p = 0.000057$) (Figure 4A), NLRP3 ($p = 0.000028$) (Figure 4B) and PYCARD ($p = 0.000814$) (Figure 4C) in OHCs. Moreover, AMS21 attenuated LPS-induced elevations in CASP1 ($p = 0.000028$), NLRP3 ($p = 0.000055$) and PYCARD ($p = 0.018303$), while pre-treatment with WRW4 blocked these beneficial properties of the agonist (CASP1 ($p = 0.037574$), NLRP3 ($p = 0.000062$), PYCARD ($p = 0.006172$)).




| | Control | LPS | AMS21 | AMS21 + LPS |
|---|---|---|---|---|
| **D** | | | | |
| ***Factor*** | | | | |
| *Casp1* | 1.09 ± 0.25 | 1.83 ± 0.42 | 1.18 ± 0.35 | 0.99 ± 0.15 |
| *Nlrp3* | 1.02 ± 0.13 | **1.70 ± 0.26** * | 1.40 ± 0.13 | **0.95 ± 0.14** # |
| *Pycard* | 1.08 ± 0.30 | **0.58 ± 0.12** * | 1.41 ± 0.15 | 0.56 ± 1.06 |

**Figure 4.** The impact of WRW4 and AMS21 on the protein levels of CASP1 (**A**), NLRP3 (**B**), and PYCARD (**C**) and mRNA expression (**D**) in LPS-stimulated OHCs. Cultures were pre-treated with WRW4 (10 µM) for 30 min. Afterwards, OHCs were administered AMS21 (0.1 µM) for one hour and lipopolysaccharide (LPS; 1 µg/mL) for 24 h. Control groups were treated with the appropriate vehicle. The data are presented as the mean ± SEM of independent experiments (**A**–**C**), $n$ = 6–8 in each experiment and as the average fold change ± SEM (**D**), $n$ = 3–6 in each experiment. Statistical analysis was carried out 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 * $p < 0.05$ (**A**–**C**), and * $p < 0.05$ control compared to the LPS group; # $p < 0.05$ LPS compared to the AMS21 + LPS group (**D**). CASP1—caspase 1; NLRP3—nod-like receptor pyrins 3; PYCARD—apoptosis-associated speck-like protein containing a caspase recruitment domain; LPS—lipopolysaccharide.

Analysis of mRNA expression revealed an elevated level of *Nlrp3* ($p$ = 0.017181) and downregulation of *Pycard* ($p$ = 0.044698) genes in LPS-stimulated OHCs (Figure 4D). Furthermore, we observed restored homeostasis in the *Nlrp3* ($p$ = 0.011565) gene after AMS21 treatment in LPS-challenged groups. Although we did not observe statistical significance, *Casp1* tended to be elevated after LPS administration and restored after AMS21 treatment.

### *3.6. The Effect of Clodronate Treatment on Microglia in OHCs*

Formyl peptide receptor 2 is widespread in peripheral immune cells and in central nervous system cells, including neurons, astrocytes, and microglia [30–33]. Since our research has shown that AMS21 exerts its biological function via FPR2, we determined whether the presence of microglial FPR2 is required for the anti-inflammatory and pro-resolving action of AMS21. As a preliminary part of these experiments, we confirmed that clodronate depleted microglia using immunofluorescence staining (in Supplementary Materials Figure S2) and Western blot analysis.

As we demonstrated in Figure 5A, the fluorescence intensity of IBA1 in LPS-treated OHCs (1 µg/mL) was significantly increased ($p$ = 0.005184). Moreover, after clodronate (150 µg/mL) administration, the fluorescence intensity decreased in both the control

($p$ = 0.000443) and LPS-challenged group ($p$ = 0.000061). This finding seems to be in line with the fluorescence intensity of FPR2 (Figure 5B), as we observed a diminished level of FPR2 in control ($p$ = 0.0004026) and LPS-stimulated OHCs ($p$ = 0.012620) after clodronate treatment. Nevertheless, the fluorescence intensity of FPR2 remains not completely silenced, as in the microglial marker IBA1.

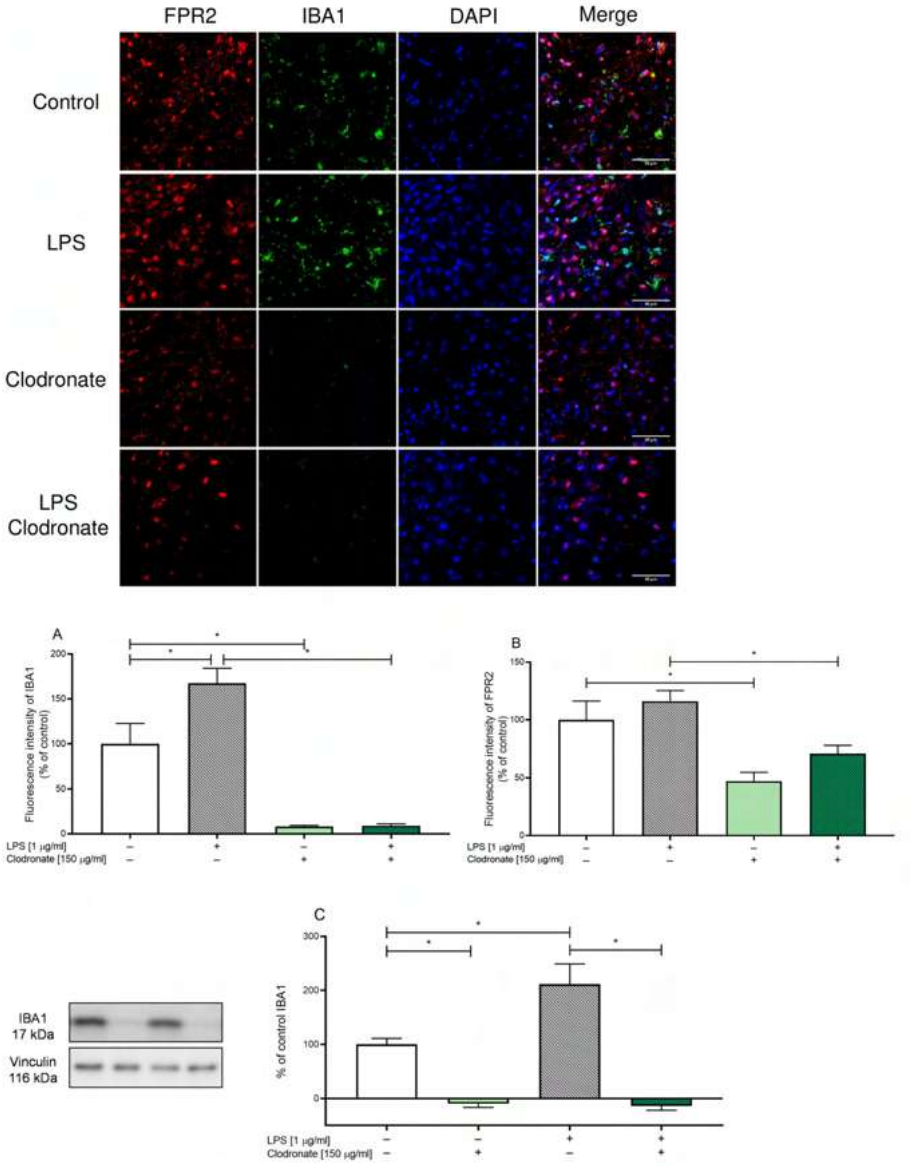



**Figure 5.** Representative fluorescence images of OHCs obtained using confocal microscopy (**A**,**B**) and the protein level of IBA1 (**C**) after LPS and clodronate administration. Microglia depletion was performed on the 1st DIV using clodronate (150 µg/mL). On the 7th DIV, cultures were treated with lipopolysaccharide (LPS; 1 µg/mL) for 24 h. Control groups were treated with the appropriate vehicle. Fluorescence intensity data are derived for IBA1 (**A**) and FPR2 (**B**). Nuclei appear in blue Hoechst 33342, FPR2 was labelled using AlexaFluor® 647 in red and IBA1 was labelled using AlexaFluor® 555. The data are presented as the mean ± SEM of independent experiments, $n$ = 6–9 for fluorescence images, and $n$ = 4 for Western blot analysis. Statistical analysis was carried out 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 * $p < 0.05$. Scale bar: 50 µM is located at the bottom right corner of each image.

In the second part of this experiment, to ensure that clodronate depleted microglia, we performed Western blot analysis (Figure 5C and Supplementary Materials Figure S1). As expected, LPS administration elevated the protein level of IBA1 ($p = 0.002265$), and clodronate stimulation diminished the microglial marker in both control ($p = 0.002506$) and LPS-treated ($p = 0.000070$) OHCs.

### *3.7. The Effect of AMS21 on the Protein Level of NLRP3 Inflammasome Pathway-Related Factors in Microglia-Depleted OHCs*

Finally, we investigated the proinflammatory and pro-resolving properties of AMS21 in microglia-depleted OHCs. Our research revealed that clodronate (150 µg/mL) and AMS21 (0.1 µM) administration did not change the protein levels of IL-1β (Figure 6A) and CASP1 (Figure 6B) under basal conditions. Nevertheless, we observed the influence of combined treatment with clodronate and AMS21 in vehicle-treated groups since the protein levels of NLRP3 ($p = 0.015504$) (Figure 6C) and PYCARD ($p = 0.032702$) (Figure 6D) were decreased compared to those in the AMS21-treated group. As expected, all examined factor levels were elevated in LPS-stimulated OHCs (IL-1β ($p = 0.001421$), CASP1 ($p = 0.000054$), NLRP3 ($p = 0.000055$), PYCARD ($p = 0.000054$)), and AMS21 exhibited favourable properties by decreasing the amount of NLRP3 inflammasome pathway-related factors (IL-1β ($p = 0.016751$), CASP1 ($p = 0.000028$), NLRP3 ($p = 0.000032$), PYCARD ($p = 0.000034$)) (Figure 6A–D).

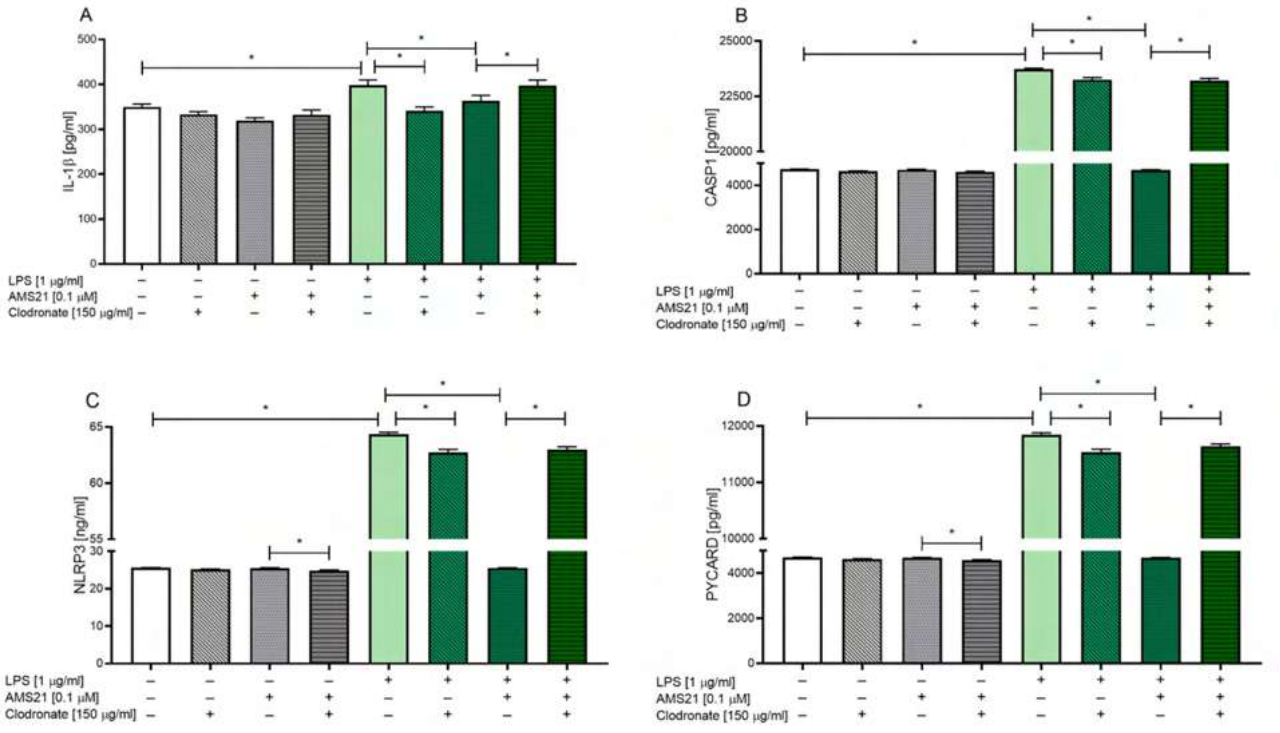



**Figure 6.** The impact of clodronate and AMS21 on the protein levels of IL-1β (**A**), CASP1 (**B**), NLRP3 (**C**), and PYCARD (**D**) in LPS-stimulated OHCs. Microglia depletion was performed on the 1st DIV using clodronate (150 µg/mL). On the 7th DIV, cultures were stimulated with AMS21 (0.1 µM) for one hour and then with lipopolysaccharide (LPS; 1 µg/mL) for 24 h. Control groups were treated with the appropriate vehicle. The data are presented as the mean ± SEM of independent experiments, $n = 7–8$ in each experiment. Statistical analysis was carried out 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 * $p < 0.05$. CASP1—caspase 1; NLRP3—nod-like receptor pyrins—3; PYCARD—apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD); LPS—lipopolysaccharide.

Importantly, the proinflammatory potential of LPS administration was attenuated in clodronate-treated OHCs (IL-1β ($p$ = 0.000280), CASP1 ($p$ = 0.000114), NLRP3 ($p$ = 0.000062), PYCARD ($p$ = 0.000061)) (Figure 6A–D). Moreover, AMS21 seems to be microglia-dependent since we noticed the beneficial potential of this compound only in the absence of clodronate administration. In fact, OHCs treatment with clodronate inhibited the beneficial impact of AMS21 on the protein level of NLRP3 inflammasome-related factors in LPS-stimulated OHCs (IL-1β ($p$ = 0.014792), CASP1 ($p$ = 0.000054), NLRP3 ($p$ = 0.000055), PYCARD ($p$ = 0.000054)) (Figure 6A–D).

## 4. Discussion

In this study, we found that the FPR2 agonist AMS21 limits lactate dehydrogenase and nitric oxide release in OHCs stimulated by LPS. Moreover, AMS21 significantly attenuated LPS-evoked microglial marker expression, including *Cd40* and *Il-18*, while upregulating *Il-1Ra* expression, a negative regulator of the inflammatory response. The FPR2-dependent pro-resolving ability of AMS21 was related to the limitation of proinflammatory cytokines: IL-1β and partial IL-6 release in stimulated OHCs. Importantly, our results are the first to firmly point to the crucial role of FPR2 expressed by microglial cells in the anti-inflammatory activity of AMS21 since the depletion of microglial cells abolished this effect. Moreover, the molecular mechanism of the pro-resolving potential of AMS21 treatment in control OHCs was identified as the suppression of the NLRP3 inflammasome complex and the decrease in IL-1β release in the neuroinflammatory response.

Considering the inconclusive data related to the expression of FPR2 in CNS cells and the fact that microglial cells perform their functions in the brain mainly through interactions with other cells that are highly sensitive to the surrounding environment, in this study, we used an ex vivo model of hippocampal organotypic cultures. This model preserves the functional interactions between brain cells, and the influence of various biological brain components, including immunological components, on the studied effects has not been affected [55]. Moreover, this experimental model allowed us to demonstrate the role of microglial cells in the studied mechanisms related to FPR2 activation through simple pharmacological modulation.

Modulation of the resolution of inflammation (RoI) has been proposed as a new strategy to treat CNS disorders based on inflammation, and the FPR2 receptor has been recently identified as a target of pro-resolving agents [9,56,57]. Since endogenous FPR2 agonists (such as LXA4 and ATL-LXA4) are characterised by high chemical lability and poor bioavailability, the identification of "drug-like" FPR2 agonists is necessary [58,59]. During the last decade, intensive work has been carried out to identify compounds that could combine the favourable profile of endogenous ligands, including RoI, with favourable pharmacokinetic properties and high bioavailability. We identified the first series of FPR2 agonists based on an ureidopropanamide scaffold able to reduce the intracellular levels of proinflammatory mediators in rat primary microglial cell cultures stimulated with lipopolysaccharide (LPS) [31,60]. 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 barrier model. However, the most promising ligand, MR-39, produces beneficial in vitro effects in the micromolar range. Thus, this limits its use for in vivo preclinical studies because a high dosage would imply the risk of unpredictable and confounding off-target effects. Therefore, in a subsequent study, the FPR2 agonist potency of the ureidopropanamide derivatives was improved and led to the identification of compound AMS21, which was able to activate FPR2 at nanomolar concentrations [45]. In the present study, we evaluated the neuroprotective and anti-inflammatory effects of AMS21 in OHCs exposed to LPS, a well-accepted neuroinflammation model.

First, we demonstrated that AMS21 at nanomolar concentrations attenuated LDH release and NO production evoked by LPS. These data are consistent with our previous observations in the model of primary microglial cultures [45] and confirm the neuroprotective effects of AMS21 in the three-dimensional cell system that maintains neuronal–glial

interactions, thus bringing us closer to preclinical studies in vivo. The endotoxin of Gram-negative bacteria is one of the most potent bacterial inducers of cytokine release, including proinflammatory cytokines such as IL-1β and IL-6 [61–63], and the gene expression of various other proinflammatory markers and factors. Consistent with these observations, in this study, LPS upregulated the expression of *Cd40*, *Il-1β*, *Il-18*, and *Il-6* in OHCs. Moreover, we observed an increased production of IL-1β and IL-6. AMS21 treatment abolished the stimulatory effect of LPS administration on the expression of proinflammatory markers, namely, *Cd40* and *Il-18*. Concurrently, the administration of this ligand diminishes the IL-1β and IL-6 levels. This effect was FPR2-dependent because it was counterbalanced by pre-treatment with the FPR2 antagonist WRW4 in LPS-stimulated OHCs. Therefore, the results reported in this study are in agreement with those obtained in other experimental models using the FPR2 agonist MR-39 [31,48,50]. Nevertheless, we showed that AMS21 in LPS-evoked cultures did not disturb the increase in the release of anti-inflammatory cytokines (TGF-β, IL-10), whose role as a "stop signal" in inflammatory processes is crucial. IL-10 exerts an anti-inflammatory response at least in part by regulating IL-1β production [50]. LPS specifically activates IL-10, triggering the induction of IL-1β secretion, whose level, together with the amount of pro-IL-1β, determines the final level of IL-1β [64]. Since we found that the antagonist WRW4 inhibits the synthesis of IL-10 and TGF-β, it can be suggested that the activation of FPR2 by AMS21 plays an indirect role in maintaining a proper balance between pro- and anti-inflammatory cytokines, thus contributing to the regulation of RoI and return to homeostasis after LPS-induced immune activation. Consistent with this hypothesis, we found that AMS21 upregulates *Il-1Ra* in OHCs. This protein inhibits the IL-1β receptor and is an essential factor in regulating IL-1-mediated inflammation [65]. Interestingly, some data also indicate the positive synergistic effects of IL-1Ra with TGF-β and IL-10 cytokines [66], pointing to the potential new mechanism for promoting RoI by AMS21, but this observation requires further verification.

As we have shown that the beneficial activity of AMS21 is mainly associated with the inhibition of the proinflammatory response related to the IL-1 family, we assessed the molecular mechanism underlying the observed anti-inflammatory and pro-resolving effects of AMS21, focusing our attention on the NLRP3 inflammasome. NLRP3 inflammasome activation leads to the release of the active cytokines IL-1β and IL-18 in a model of neuroinflammation in OHCs [50]. Indeed, IL-1β is biologically inactive and must be cleaved and transformed into its biologically active form by the enzymatic activity of caspase-1 [17,54,67]. Therefore, upon activation, NLRP3 nucleates PYCARD helical clusters through PYD-PYD interactions. The oligomerised PYCARD CARDs then form the platform for caspase-1 CARD to nucleate into filaments, which, in turn, activates caspase-1 [54,68]. Unique among inflammasomes, NLRP3 requires a priming step for canonical activation. In experimental practice, LPS treatment is able to induce NLRP3 expression. In the next step, NLRP3 inflammasome assembly leads to inflammasome activation [54]. We found that LPS stimulation upregulated *Nlrp3* and *Pycard* subunits expression in OHCs. AMS21 attenuated the impact of endotoxin on the mRNA expression of the *Nlrp3* subunit. Furthermore, we demonstrated that the LPS-evoked increase in CASP-1, NLRP3, and PYCARD levels in OHCs was inhibited by AMS21 administration. Notably, this beneficial effect of AMS21 was abolished by WRW4 pre-treatment, thus confirming that the effect is FPR2-mediated. This observation suggests a potential interaction between FPR2 activation and the suppression of the canonical NLRP3 inflammasome pathway in OHCs. Interestingly, the anti-inflammatory activity of AMS21 was observed at nanomolar concentrations, providing grounds for further research on the pro-resolving potential of this compound in vivo. Moreover, in our previous study, we observed that the levels of caspase-1 in OHCs from KO FPR2 mice were higher than those in OHCs obtained from WT mice, suggesting enhanced cleavage of pro-IL-1β into active IL-1β and a consequential increase in the level of IL-1β in OHCs from KO FPR2 mice. Additionally, MR-39 was able to diminish caspase-1 activation only in KO FPR2 mice [50].

To date, most of the data indicate the expression of FPR2 on microglial cells and the unique ability of these receptors to differentiate responses based on the structure of its ligands following the described agonist bias [69,70]. Nevertheless, other authors point to the expression of FPR2 on other brain cells, including neurons, astrocytes, and even oligodendrocytes [35,71]. To shed more light on this still controversial issue, in the following research stage, we used a method of eliminating microglial cells at the stage of OHCs establishment. To date, experimental approaches to characterise microglia's functional receptors and repertoire have relied on pharmacological and genetic methodologies. Among these approaches is the use of bisphosphonate clodronate, which deactivates cells belonging to the monocyte lineage [72,73]. Clodronate is a first-generation bisphosphonate that exerts an effect on the brain's peripheral macrophages and perivascular cells [73,74]. Simultaneously, the most spectacular direct effect of clodronate on microglial cells involves inhibiting proliferation [75]. Accordingly, we have shown, using anti-IBA1 staining, that clodronate eliminated microglial cells, while stimulation with bacterial endotoxin did not increase the IBA1 protein level. At the same time, using confocal microscopy, we showed the colocalisation of FRP2 with IBA1-positive cells, which was absent after microglia depletion in OHCs. Nevertheless, the measurement of FPR2 fluorescence demonstrated no complete decrease in FPR2 level, which suggests the presence of this receptor in OHCs after clodronate treatment, although it was significantly reduced. These observations indicate the localisation of FPR2 in other cells in OHCs, probably astrocytes and/or neurons [76].

Moreover, the various FPR2-mediated activities of microglial cells were affected by clodronate treatment. We demonstrated that clodronate diminished IL-1β release in OHCs compared to control cultures, but only after LPS treatment. Other studies have reported that TNF-β and IL-6 production is also limited after immune stimulation in microglia-depleted OHCs [75]. According to data on RAW 264 macrophages, proinflammatory cytokines and NO production decreased following clodronate treatment [77]. The mechanisms of action of bisphosphonates have not been fully clarified. In 1997, Frith et al. [78] showed that clodronate can be metabolised, and the metabolites inhibit the DNA-binding activity of NF-κB and the production of proinflammatory cytokines. In the present study, we demonstrated for the first time that NLRP3 pathway activation is attenuated, including caspase-1 level and other protein subunits, following clodronate administration in OHCs.

Additional intriguing points raised in our study are that microglia depletion completely abolished the anti-inflammatory potential of AMS21 administration observed in control OHCs. It may be hypothesised that the depletion of FPR2, which is located mainly on microglial cells, significantly hampered the possibility of the ligand to interact with its own receptor, thus limiting its beneficial effect. Interestingly, in our previous study, we observed that the beneficial impact of the structurally related FPR2 agonist MR-39 was also limited to the suppression of microgliosis but not astrogliosis in an *in vivo* model of Alzheimer's disease [48].

Although the data indicate that clodronate mainly affects microglia, the possibility that the impact of clodronate on OHCs was also induced indirectly via other glial cells on which FPR2 was preserved cannot be ruled out. Accordingly, clodronate in adult mice leads to microenvironment changes that decrease neuronal markers and blood vessel integrity [79]. In contrast, clodronate administration in vitro improves the purity of astrocytes and increases the postsynaptic current frequency in OHCs [80,81]. Moreover, in microglia-eliminated cultures, astrocytes produced IL-6, while IL-1β followed the activation of the JAK/STAT3 pathway [80]. Recent advances in genomics and multiomics have yielded novel insight into astrocyte reactivity, in which astrocytes undergo a broad spectrum of morphological, molecular and functional changes to become reactive astrocytes [82,83]. The transformation to reactive phenotypes involves a variety of molecular regulators and signalling pathways [84]. Nevertheless, despite many efforts, the mechanism of astrocyte activation, such as their response to TLR ligands, including LPS, remains highly debated [85]. Among others, CD14 was found in astrocytes, which serve as a high-affinity receptor for LPS and correspond to the interaction with TLR4 activated by this

endotoxin [85]. Moreover, rodent astrocytes have been shown to be highly sensitive to IL-1β, while the types of inflammatory genes induced by this cytokine resemble those of LPS-activated microglia, suggesting that astrocytes are capable of mounting potent immune responses [86–88]. Therefore, it can be suggested that the IL-1β level observed in OHCs treated with clodronate, at least in part, has an astrocytic origin.

The next intriguing observation from the present study is NLRP3 activation in clodronate-depleted OHCs. Although some data suggest that the NLRP3 inflammasome is limited to microglia but not astrocytes [89], increasing evidence supports the presence of the NLRP3 inflammasome in other brain cells, such as oligodendrocytes and astrocytes, in pathological conditions and some disease models [90,91]. Our research showed the activation of not only CASP1 but also of the remaining two NLRP3 subunits evoked by LPS in microglia-depleted OHCs. This finding strongly suggested the presence of the NLRP3 inflammasome in astroglia. It should be emphasised that other caspases can also activate NLRP3 by non-classical and/or alternative activation pathways. An emerging body of research has supported the role of caspase-4, caspase-5, and caspase-11 in regulating caspase-1 activation and inducing the inflammatory form of cell death called pyroptosis [92,93]. Moreover, the role of caspase-12, which is expressed by astrocytes and is important for caspase-1 and NLRP3 activation, should also be considered [94].

## 5. Conclusions

The FPR2 receptor is a versatile transmembrane receptor that recognises a wide variety of chemically diverse endogenous ligands. This creates a unique opportunity to switch from a pro- to an anti-inflammatory activation profile of this receptor in the brain. In this context, our study provides new data on the molecular mechanisms underlying the anti-inflammatory and pro-resolving properties of our second-generation FPR2 agonist AMS21 in an ex vivo model of an experimentally induced neuroinflammatory response. Our data showed that AMS21 modulates the proinflammatory response related to the IL-1 family through different mechanisms that include the modulation of inflammasome NLRP3 assembly and the upregulation of the IL-1Ra protein. These data propose a new mechanism for the pro-resolving effect of AMS21 that will be further studied in detail in future studies. Finally, our findings suggest a crucial role of microglial cells and the FPR2 receptor located on these glial cells in mediating the anti-inflammatory response of compound AMS21.

Therefore, these studies have significant implications for the translation of FPR2 activation and modulation of downstream signalling as a potential therapeutic agent for inflammatory pathologies.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cells12212570/s1. Figure S1: Western blot analysis of lysates from organotypic hippocampal cultures (OHCs) demonstrating protein level of IBA1 protein. Vinculin was used as a normalising control. Description of the arrangement of the samples (1–16).

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## References

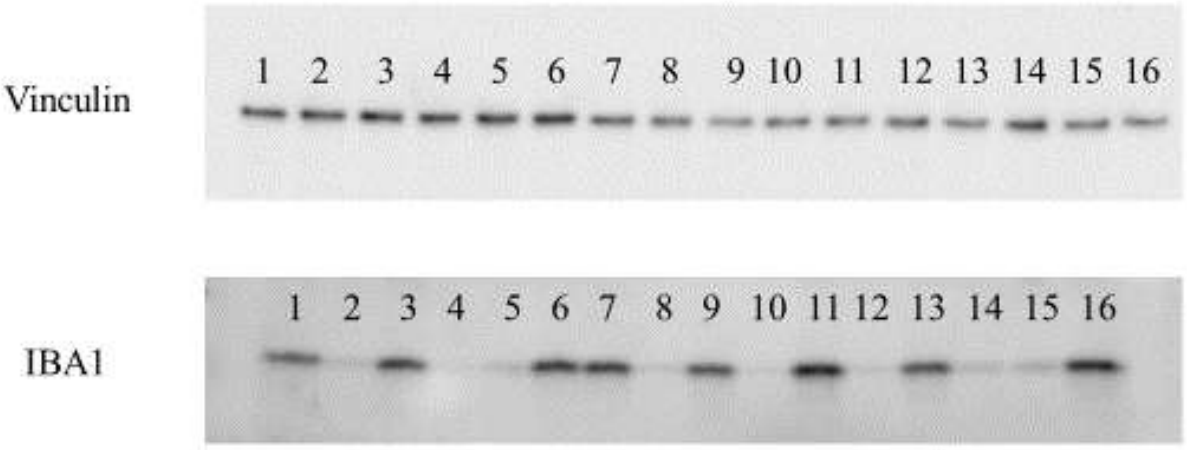
1. Woodburn, S.C.; Bollinger, J.L.; Wohleb, E.S. The Semantics of Microglia Activation: Neuroinflammation, Homeostasis, and Stress. *J. Neuroinflamm.* **2021**, *18*, 258. [CrossRef] [PubMed]
2. Borst, K.; Dumas, A.A.; Prinz, M. Microglia: Immune and Non-Immune Functions. *Immunity* **2021**, *54*, 2194–2208. [CrossRef]
3. Napoli, I.; Neumann, H. Microglial Clearance Function in Health and Disease. *Neuroscience* **2009**, *158*, 1030–1038. [CrossRef]
4. Ślusarczyk, J.; Trojan, E.; Głombik, K.; Piotrowska, A.; Budziszewska, B.; Kubera, M.; Popiołek-Barczyk, K.; Lasoń, W.; Mika, J.; Basta-Kaim, A. Targeting the NLRP3 Inflammasome-Related Pathways via Tianeptine Treatment-Suppressed Microglia Polarization to the M1 Phenotype in Lipopolysaccharide-Stimulated Cultures. *Int. J. Mol. Sci.* **2018**, *19*, 1965. [CrossRef] [PubMed]
5. Paolicelli, R.C.; Bolasco, G.; Pagani, F.; Maggi, L.; Scianni, M.; Panzanelli, P.; Giustetto, M.; Ferreira, T.A.; Guiducci, E.; Dumas, L.; et al. Synaptic Pruning by Microglia Is Necessary for Normal Brain Development. *Science* **2011**, *333*, 1456–1458. [CrossRef] [PubMed]
6. Chamera, K.; Trojan, E.; Kotarska, K.; Szuster-Głuszczak, M.; Bryniarska, N.; Tylek, K.; Basta-Kaim, A. Role of Polyinosinic:Polycytidylic Acid-Induced Maternal Immune Activation and Subsequent Immune Challenge in the Behaviour and Microglial Cell Trajectory in Adult Offspring: A Study of the Neurodevelopmental Model of Schizophrenia. *Int. J. Mol. Sci.* **2021**, *22*, 1558. [CrossRef] [PubMed]
7. McKee, C.G.; Hoffos, M.; Vecchiarelli, H.A.; Tremblay, M.-È. Microglia: A Pharmacological Target for the Treatment of Age-Related Cognitive Decline and Alzheimer's Disease. *Front. Pharmacol.* **2023**, *14*, 1125982. [CrossRef]
8. Carvalho-Paulo, D.; Neto, J.B.T.; Filho, C.S.; de Oliveira, T.C.G.; de Sousa, A.A.; dos Reis, R.R.; dos Santos, Z.A.; de Lima, C.M.; de Oliveira, M.A.; Said, N.M.; et al. Microglial Morphology Across Distantly Related Species: Phylogenetic, Environmental and Age Influences on Microglia Reactivity and Surveillance States. *Front. Immunol.* **2021**, *12*, 683026. [CrossRef] [PubMed]
9. Tylek, K.; Trojan, E.; Regulska, M.; Lacivita, E.; Leopoldo, M.; Basta-Kaim, A. Formyl Peptide Receptor 2, as an Important Target for Ligands Triggering the Inflammatory Response Regulation: A Link to Brain Pathology. *Pharmacol. Rep.* **2021**, *74*, 1004–1019. [CrossRef] [PubMed]
10. Šimončičová, E.; de Andrade, E.G.; Vecchiarelli, H.A.; Awogbindin, I.O.; Delage, C.I.; Tremblay, M.-È. Present and Future of Microglial Pharmacology. *Trends Pharmacol. Sci.* **2022**, *43*, 669–685. [CrossRef]
11. Dokalis, N.; Prinz, M. Resolution of Neuroinflammation: Mechanisms and Potential Therapeutic Option. *Semin. Immunopathol.* **2019**, *41*, 699–709. [CrossRef] [PubMed]
12. Kaur, N.; Chugh, H.; Sakharkar, M.K.; Dhawan, U.; Chidambaram, S.B.; Chandra, R. Neuroinflammation Mechanisms and Phytotherapeutic Intervention: A Systematic Review. *ACS Chem. Neurosci.* **2020**, *11*, 3707–3731. [CrossRef]
13. Moyse, E.; Krantic, S.; Djellouli, N.; Roger, S.; Angoulvant, D.; Debacq, C.; Leroy, V.; Fougere, B.; Aidoud, A. Neuroinflammation: A Possible Link between Chronic Vascular Disorders and Neurodegenerative Diseases. *Front. Aging Neurosci.* **2022**, *14*, 827263. [CrossRef] [PubMed]
14. Jana, M.; Jana, A.; Liu, X.; Ghosh, S.; Pahan, K. Involvement of Phosphatidylinositol 3-Kinase-Mediated Up-Regulation of IκBα in Anti-Inflammatory Effect of Gemfibrozil in Microglia. *J. Immunol.* **2007**, *179*, 4142–4152. [CrossRef] [PubMed]
15. Lee, Y.Y.; Park, J.S.; Jung, J.S.; Kim, D.H.; Kim, H.S. Anti-Inflammatory Effect of Ginsenoside Rg5 in Lipopolysaccharide-Stimulated BV2 Microglial Cells. *Int. J. Mol. Sci.* **2013**, *14*, 9820–9833. [CrossRef]
16. Trojan, E.; Chamera, K.; Bryniarska, N.; Kotarska, K.; Leśkiewicz, M.; Regulska, M.; Basta-Kaim, A. Role of Chronic Administration of Antidepressant Drugs in the Prenatal Stress-Evoked Inflammatory Response in the Brain of Adult Offspring Rats: Involvement of the NLRP3 Inflammasome-Related Pathway. *Mol. Neurobiol.* **2019**, *56*, 5365–5380. [CrossRef] [PubMed]
17. Hanslik, K.L.; Ulland, T.K. The Role of Microglia and the Nlrp3 Inflammasome in Alzheimer's Disease. *Front. Neurol.* **2020**, *11*, 570711. [CrossRef]
18. Kaufmann, F.N.; Costa, A.P.; Ghisleni, G.; Diaz, A.P.; Rodrigues, A.L.S.; Peluffo, H.; Kaster, M.P. NLRP3 Inflammasome-Driven Pathways in Depression: Clinical and Preclinical Findings. *Brain. Behav. Immun.* **2017**, *64*, 367–383. [CrossRef]

19. Fan, Z.; Liang, Z.; Yang, H.; Pan, Y.; Zheng, Y.; Wang, X. Tenuigenin Protects Dopaminergic Neurons from Inflammation via Suppressing NLRP3 Inflammasome Activation in Microglia. *J. Neuroinflamm.* **2017**, *14*, 256. [CrossRef] [PubMed]
20. Moretti, J.; Blander, J.M. Increasing Complexity of NLRP3 Inflammasome Regulation. *J. Leukoc. Biol.* **2021**, *109*, 561–571. [CrossRef]
21. Swanson, K.V.; Deng, M.; Ting, J.P.Y. The NLRP3 Inflammasome: Molecular Activation and Regulation to Therapeutics. *Nat. Rev. Immunol.* **2019**, *19*, 477–489. [CrossRef]
22. Shao, B.Z.; Xu, Z.Q.; Han, B.Z.; Su, D.F.; Liu, C. NLRP3 Inflammasome and Its Inhibitors: A Review. *Front. Pharmacol.* **2015**, *6*, 262. [CrossRef] [PubMed]
23. Sutterwala, F.S.; Haasken, S.; Cassel, S.L. Mechanism of NLRP3 Inflammasome Activation. *Ann. N. Y. Acad. Sci.* **2014**, *1319*, 82–95. [CrossRef]
24. Shabab, T.; Khanabdali, R.; Moghadamtousi, S.Z.; Kadir, H.A.; Mohan, G. Neuroinflammation Pathways: A General Review. *Int. J. Neurosci.* **2017**, *127*, 624–633. [CrossRef] [PubMed]
25. Kwon, H.S.; Koh, S.H. Neuroinflammation in Neurodegenerative Disorders: The Roles of Microglia and Astrocytes. *Transl. Neurodegener.* **2020**, *9*, 42. [CrossRef] [PubMed]
26. Subhramanyam, C.S.; Wang, C.; Hu, Q.; Dheen, S.T. Microglia-Mediated Neuroinflammation in Neurodegenerative Diseases. *Semin. Cell Dev. Biol.* **2019**, *94*, 112–120. [CrossRef]
27. Paolicelli, R.C.; Sierra, A.; Stevens, B.; Tremblay, M.-E.; Aguzzi, A.; Ajami, B.; Amit, I.; Audinat, E.; Bechmann, I.; Bennett, M.; et al. Microglia States and Nomenclature: A Field at Its Crossroads. *Neuron* **2022**, *110*, 3458–3483. [CrossRef] [PubMed]
28. Wang, Z.; Weaver, D.F. Microglia and Microglial-Based Receptors in the Pathogenesis and Treatment of Alzheimer's Disease. *Int. Immunopharmacol.* **2022**, *110*, 109070. [CrossRef]
29. Wright-Jin, E.C.; Gutmann, D.H. Microglia as Dynamic Cellular Mediators of Brain Function. *Trends Mol. Med.* **2019**, *25*, 967–979. [CrossRef]
30. Liu, G.J.; Tao, T.; Wang, H.; Zhou, Y.; Gao, X.; Gao, Y.Y.; Hang, C.H.; Li, W. Functions of Resolvin D1-ALX/FPR2 Receptor Interaction in the Hemoglobin-Induced Microglial Inflammatory Response and Neuronal Injury. *J. Neuroinflamm.* **2020**, *17*, 239. [CrossRef]
31. Tylek, K.; Trojan, E.; Leśkiewicz, M.; Regulska, M.; Bryniarska, N.; Curzytek, K.; Lacivita, E.; Leopoldo, M.; Basta-Kaim, A. Time-Dependent Protective and pro-Resolving Effects of Fpr2 Agonists on Lipopolysaccharide-Exposed Microglia Cells Involve Inhibition of Nf-Kb and Mapks Pathways. *Cells* **2021**, *10*, 2373. [CrossRef]
32. Liu, M.; Chen, K.; Yoshimura, T.; Liu, Y.; Gong, W.; Le, Y.; Gao, J.L.; Zhao, J.; Wang, J.M.; Wang, A. Formylpeptide Receptors Mediate Rapid Neutrophil Mobilization to Accelerate Wound Healing. *PLoS ONE* **2014**, *9*, e90613. [CrossRef] [PubMed]
33. Wu, J.; Ding, D.H.; Li, Q.Q.; Wang, X.Y.; Sun, Y.Y.; Li, L.J. Lipoxin A4 Regulates Lipopolysaccharide-Induced BV2 Microglial Activation and Differentiation via the Notch Signaling Pathway. *Front. Cell. Neurosci.* **2019**, *13*, 19. [CrossRef] [PubMed]
34. Ong, W.-Y.; Chua, J.E. Role of Formyl Peptide Receptor 2 (FPR2) in the Normal Brain and in Neurological Conditions. *Neural Regen. Res.* **2019**, *14*, 2071–2072. [CrossRef]
35. Ho, C.F.Y.; Ismail, N.B.; Koh, J.K.Z.; Gunaseelan, S.; Low, Y.H.; Ng, Y.K.; Chua, J.J.E.; Ong, W.Y. Localisation of Formyl-Peptide Receptor 2 in the Rat Central Nervous System and Its Role in Axonal and Dendritic Outgrowth. *Neurochem. Res.* **2018**, *43*, 1587–1598. [CrossRef]
36. Cattaneo, F.; Parisi, M.; Ammendola, R. Distinct Signaling Cascades Elicited by Different Formyl Peptide Receptor 2 (FPR2) Agonists. *Int. J. Mol. Sci.* **2013**, *14*, 7193–7230. [CrossRef] [PubMed]
37. Qin, C.X.; Norling, L.V.; Vecchio, E.A.; Brennan, E.P.; May, L.T.; Wootten, D.; Godson, C.; Perretti, M.; Ritchie, R.H. Formylpeptide Receptor 2: Nomenclature, Structure, Signalling and Translational Perspectives: IUPHAR Review 35. *Br. J. Pharmacol.* **2022**, *179*, 4617–4639. [CrossRef] [PubMed]
38. Maciuszek, M.; Cacace, A.; Brennan, E.; Godson, C.; Chapman, T.M. Recent Advances in the Design and Development of Formyl Peptide Receptor 2 (FPR2/ALX) Agonists as pro-Resolving Agents with Diverse Therapeutic Potential. *Eur. J. Med. Chem.* **2021**, *213*, 113167. [CrossRef] [PubMed]
39. Park, J.; Langmead, C.J.; Riddy, D.M. New Advances in Targeting the Resolution of Inflammation: Implications for Specialized Pro-Resolving Mediator GPCR Drug Discovery. *ACS Pharmacol. Transl. Sci.* **2020**, *3*, 88–106. [CrossRef] [PubMed]
40. Raabe, C.A.; Gröper, J.; Rescher, U. Biased Perspectives on Formyl Peptide Receptors. *Biochim. Biophys. Acta BBA Mol. Cell Res.* **2019**, *1866*, 305–316. [CrossRef] [PubMed]
41. Ye, R.D.; Boulay, F.; Ji, M.W.; Dahlgren, C.; Gerard, C.; Parmentier, M.; Serhan, C.N.; Murphy, P.M. International Union of Basic and Clinical Pharmacology. LXXIII. Nomenclature for the Formyl Peptide Receptor (FPR) Family. *Pharmacol. Rev.* **2009**, *61*, 119–161. [CrossRef] [PubMed]
42. Liao, Q.; Ye, R.D. Structural and Conformational Studies of Biased Agonism through Formyl Peptide Receptors. *Am. J. Physiol. Cell Physiol.* **2022**, *322*, C939–C947. [CrossRef] [PubMed]
43. Cattaneo, F.; Guerra, G.; Ammendola, R. Expression and Signaling of Formyl-Peptide Receptors in the Brain. *Neurochem. Res.* **2010**, *35*, 2018–2026. [CrossRef] [PubMed]
44. Krishnamoorthy, S.; Recchiuti, A.; Chiang, N.; Yacoubian, S.; Lee, C.H.; Yang, R.; Petasis, N.A.; Serhan, C.N. Resolvin D1 Binds Human Phagocytes with Evidence for Proresolving Receptors. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 1660–1665. [CrossRef] [PubMed]

45. Mastromarino, M.; Favia, M.; Schepetkin, I.A.; Kirpotina, L.N.; Trojan, E.; Niso, M.; Carrieri, A.; Leśkiewicz, M.; Regulska, M.; Darida, M.; et al. Design, Synthesis, Biological Evaluation, and Computational Studies of Novel Ureidopropanamides as Formyl Peptide Receptor 2 (FPR2) Agonists to Target the Resolution of Inflammation in Central Nervous System Disorders. *J. Med. Chem.* **2022**, *65*, 5004–5028. [CrossRef] [PubMed]
46. Stoppini, L.; Buchs, P.A.; Muller, D. A Simple Method for Organotypic Cultures of Nervous Tissue. *J. Neurosci. Methods* **1991**, *37*, 173–182. [CrossRef]
47. Basta-Kaim, A.; Ślusarczyk, J.; Szczepanowicz, K.; Warszyński, P.; Leśkiewicz, M.; Regulska, M.; Trojan, E.; Lasoń, W. Protective Effects of Polydatin in Free and Nanocapsulated Form on Changes Caused by Lipopolysaccharide in Hippocampal Organotypic Cultures. *Pharmacol. Rep.* **2019**, *71*, 603–613. [CrossRef]
48. Trojan, E.; Tylek, K.; Schröder, N.; Kahl, I.; Brandenburg, L.O.; Mastromarino, M.; Leopoldo, M.; Basta-Kaim, A.; Lacivita, E. The N-Formyl Peptide Receptor 2 (FPR2) Agonist MR-39 Improves Ex Vivo and In Vivo Amyloid Beta (1–42)-Induced Neuroinflammation in Mouse Models of Alzheimer's Disease. *Mol. Neurobiol.* **2021**, *2*, 6203–6221. [CrossRef]
49. Ślusarczyk, J.; Trojan, E.; Głombik, K.; Budziszewska, B.; Kubera, M.; Lasoń, W.; Popiołek-Barczyk, K.; Mika, J.; Wędzony, K.; Basta-Kaim, A. Prenatal Stress Is a Vulnerability Factor for Altered Morphology and Biological Activity of Microglia Cells. *Front. Cell. Neurosci.* **2015**, *9*, 82. [CrossRef] [PubMed]
50. Trojan, E.; Tylek, K.; Leśkiewicz, M.; Lasoń, W.; Brandenburg, L.O.; Leopoldo, M.; Lacivita, E.; Basta-Kaim, A. The N-Formyl Peptide Receptor 2 (Fpr2) Agonist Mr-39 Exhibits Anti-Inflammatory Activity in Lps-Stimulated Organotypic Hippocampal Cultures. *Cells* **2021**, *10*, 1524. [CrossRef] [PubMed]
51. Chomczynski, P. A Reagent for the Single-Step Simultaneous Isolation of RNA, DNA and Proteins from Cell and Tissue Samples. *Biotechniques* **1993**, *15*, 532–537. [PubMed]
52. Chamera, K.; Szuster-Głuszczak, M.; Trojan, E.; Basta-Kaim, A. Maternal Immune Activation Sensitizes Male Offspring Rats to Lipopolysaccharide-Induced Microglial Deficits Involving the Dysfunction of CD200-CD200R and CX3CL1-CX3CR1 Systems. *Cells* **2020**, *9*, 1676. [CrossRef] [PubMed]
53. Gogolla, N.; Galimberti, I.; DePaola, V.; Caroni, P. Staining Protocol for Organotypic Hippocampal Slice Cultures. *Nat. Protoc.* **2006**, *1*, 2452–2456. [CrossRef] [PubMed]
54. Huang, Y.; Xu, W.; Zhou, R. NLRP3 Inflammasome Activation and Cell Death. *Cell. Mol. Immunol.* **2021**, *18*, 2114–2127. [CrossRef] [PubMed]
55. Noraberg, J.; Poulsen, F.R.; Blaabjerg, M.; Kristensen, B.W.; Bonde, C.; Montero, M.; Meyer, M.; Gramsbergen, J.B.; Zimmer, J. Organotypic Hippocampal Slice Cultures for Studies of Brain Damage, Neuroprotection and Neurorepair. *Curr. Drug Targets CNS Neurol. Disord.* **2005**, *4*, 435–452. [CrossRef] [PubMed]
56. Perretti, M.; Leroy, X.; Bland, E.J.; Montero-Melendez, T. Resolution Pharmacology: Opportunities for Therapeutic Innovation in Inflammation. *Trends Pharmacol. Sci.* **2015**, *36*, 737–755. [CrossRef]
57. Trojan, E.; Leśkiewicz, M.; Lacivita, E.; Leopoldo, M.; Basta-Kaim, A. The Formyl Peptide Receptor 2 as a Target For Promotion of Resolution of Inflammation. *Curr. Neuropharmacol.* **2022**, *20*, 1482–1487. [CrossRef] [PubMed]
58. Maderna, P.; Godson, C. Lipoxins: Resolutionary Road. *Br. J. Pharmacol.* **2009**, *158*, 947–959. [CrossRef] [PubMed]
59. Chen, J.; Li, W.; Yao, H.; Xu, J. Insights into Drug Discovery from Natural Products through Structural Modification. *Fitoterapia* **2015**, *103*, 231–241. [CrossRef] [PubMed]
60. Stama, M.L.; Lacivita, E.; Kirpotina, L.N.; Niso, M.; Perrone, R.; Schepetkin, I.A.; Quinn, M.T.; Leopoldo, M. Functional N-Formyl Peptide Receptor 2 (FPR2) Antagonists Based on the Ureidopropanamide Scaffold Have Potential To Protect against Inflammation-Associated Oxidative Stress. *ChemMedChem* **2017**, *12*, 1839–1847. [CrossRef] [PubMed]
61. Batista, C.R.A.; Gomes, G.F.; Candelario-Jalil, E.; Fiebich, B.L.; de Oliveira, A.C.P. Lipopolysaccharide-Induced Neuroinflammation as a Bridge to Understand Neurodegeneration. *Int. J. Mol. Sci.* **2019**, *20*, 2293. [CrossRef] [PubMed]
62. Catorce, M.N.; Gevorkian, G. LPS-Induced Murine Neuroinflammation Model: Main Features and Suitability for Pre-Clinical Assessment of Nutraceuticals. *Curr. Neuropharmacol.* **2016**, *14*, 155–164. [CrossRef]
63. Facchin, B.M.; dos Reis, G.O.; Vieira, G.N.; Mohr, E.T.B.; da Rosa, J.S.; Kretzer, I.F.; Demarchi, I.G.; Dalmarco, E.M. Inflammatory Biomarkers on an LPS-Induced RAW 264.7 Cell Model: A Systematic Review and Meta-Analysis. *Inflamm. Res.* **2022**, *71*, 741–758. [CrossRef] [PubMed]
64. Sun, Y.; Ma, J.; Li, D.; Li, P.; Zhou, X.; Li, Y.; He, Z.; Qin, L.; Liang, L.; Luo, X. Interleukin-10 Inhibits Interleukin-1β Production and Inflammasome Activation of Microglia in Epileptic Seizures. *J. Neuroinflamm.* **2019**, *16*, 66. [CrossRef] [PubMed]
65. Li, M.; Han, L.; Xiao, J.; Zhang, S.; Liu, G.; Sun, X. IL-1ra Treatment Prevents Chronic Social Defeat Stress-Induced Depression-like Behaviors and Glutamatergic Dysfunction via the Upregulation of CREB-BDNF. *J. Affect. Disord.* **2023**, *335*, 358–370. [CrossRef] [PubMed]
66. Saghazadeh, A.; Ataeinia, B.; Keynejad, K.; Abdolalizadeh, A.; Hirbod-Mobarakeh, A.; Rezaei, N. A Meta-Analysis of pro-Inflammatory Cytokines in Autism Spectrum Disorders: Effects of Age, Gender, and Latitude. *J. Psychiatr. Res.* **2019**, *115*, 90–102. [CrossRef] [PubMed]
67. Heneka, M.T.; Kummer, M.P.; Stutz, A.; Delekate, A.; Schwartz, S.; Vieira-Saecker, A.; Griep, A.; Axt, D.; Remus, A.; Tzeng, T.; et al. NLRP3 Is Activated in Alzheimer's Disease and Contributes to Pathology in APP/PS1 Mice. *Nature* **2013**, *493*, 674–678. [CrossRef] [PubMed]

68. Piancone, F.; La Rosa, F.; Marventano, I.; Saresella, M.; Clerici, M. The Role of the Inflammasome in Neurodegenerative Diseases. *Molecules* **2021**, *26*, 953. [CrossRef]
69. Zhu, J.; Li, L.; Ding, J.; Huang, J.; Shao, A.; Tang, B. The Role of Formyl Peptide Receptors in Neurological Diseases via Regulating Inflammation. *Front. Cell. Neurosci.* **2021**, *15*, 753832. [CrossRef] [PubMed]
70. Wickstead, E.S.; Irving, M.A.; Getting, S.J.; McArthur, S. Exploiting Formyl Peptide Receptor 2 to Promote Microglial Resolution: A New Approach to Alzheimer's Disease Treatment. *FEBS J.* **2022**, *289*, 1801–1822. [CrossRef]
71. Brandenburg, L.O.; Konrad, M.; Wruck, C.J.; Koch, T.; Lucius, R.; Pufe, T. Functional and Physical Interactions between Formyl-Peptide-Receptors and Scavenger Receptor MARCO and Their Involvement in Amyloid Beta 1-42-Induced Signal Transduction in Glial Cells. *J. Neurochem.* **2010**, *113*, 749–760. [CrossRef] [PubMed]
72. Asai, H.; Ikezu, S.; Tsunoda, S.; Medalla, M.; Luebke, J.; Haydar, T.; Wolozin, B.; Butovsky, O.; Kügler, S.; Ikezu, T. Depletion of Microglia and Inhibition of Exosome Synthesis Halt Tau Propagation. *Nat. Neurosci.* **2015**, *18*, 1584–1593. [CrossRef] [PubMed]
73. Lee, J.C.; Seong, J.; Kim, S.H.; Lee, S.J.; Cho, Y.J.; An, J.; Nam, D.H.; Joo, K.M.; Cha, C.I. Replacement of Microglial Cells Using Clodronate Liposome and Bone Marrow Transplantation in the Central Nervous System of SOD1G93A Transgenic Mice as an in Vivo Model of Amyotrophic Lateral Sclerosis. *Biochem. Biophys. Res. Commun.* **2012**, *418*, 359–365. [CrossRef] [PubMed]
74. Makkonen, N.; Hirvonen, M.R.; Teräväinen, T.; Savolainen, K.; Mönkkönen, J. Different Effects of Three Bisphosphonates on Nitric Oxide Production by RAW 264 Macrophage-like Cells In Vitro. *J. Pharmacol. Exp. Ther.* **1996**, *277*, 1097–1102. [PubMed]
75. Dehghani, F.; Conrad, A.; Kohl, A.; Korf, H.; Hailer, N. Clodronate Inhibits the Secretion of Proinflammatory Cytokines and NO by Isolated Microglial Cells and Reduces the Number of Proliferating Glial Cells in Excitotoxically Injured Organotypic Hippocampal Slice Cultures. *Exp. Neurol.* **2004**, *189*, 241–251. [CrossRef]
76. Tylek, K.; Trojan, E.; Leśkiewicz, M.; Francavilla, F.; Lacivita, E.; Leopoldo, M.; Basta-Kaim, A. Stimulation of Formyl Peptide Receptor-2 by the New Agonist CMC23 Protects against Endotoxin-Induced Neuroinflammatory Response: A Study in Organotypic Hippocampal Cultures. *ACS Chem. Neurosci.* **2023**, *14*, 3869–3882. [CrossRef]
77. Makkonen, N.; Salminen, A.; Rogers, M.J.; Frith, J.C.; Urtti, A.; Azhayeva, E.; Mönkkönen, J. Contrasting Effects of Alendronate and Clodronate on RAW 264 Macrophages: The Role of a Bisphosphonate Metabolite. *Eur. J. Pharm. Sci.* **1999**, *8*, 109–118. [CrossRef] [PubMed]
78. Frith, J.C.; Mönkkönen, J.; Blackburn, G.M.; Russell, R.G.G.; Rogers, M.J. Clodronate and Liposome-Encapsulated Clodronate Are Metabolized to a Toxic ATP Analog, Adenosine 5′-(β,γ-Dichloromethylene) Triphosphate, by Mammalian Cells In Vitro. *J. Bone Miner. Res.* **1997**, *12*, 1358–1367. [CrossRef] [PubMed]
79. Han, X.; Li, Q.; Lan, X.; EL-Mufti, L.; Ren, H.; Wang, J. Microglial Depletion with Clodronate Liposomes Increases Proinflammatory Cytokine Levels, Induces Astrocyte Activation, and Damages Blood Vessel Integrity. *Mol. Neurobiol.* **2019**, *56*, 6184–6196. [CrossRef] [PubMed]
80. Kumamaru, H.; Saiwai, H.; Kobayakawa, K.; Kubota, K.; van Rooijen, N.; Inoue, K.; Iwamoto, Y.; Okada, S. Liposomal Clodronate Selectively Eliminates Microglia from Primary Astrocyte Cultures. *J. Neuroinflamm.* **2012**, *9*, 116. [CrossRef]
81. Ji, K.; Akgul, G.; Wollmuth, L.P.; Tsirka, S.E. Microglia Actively Regulate the Number of Functional Synapses. *PLoS ONE* **2013**, *8*, e56293. [CrossRef] [PubMed]
82. Guttenplan, K.A.; Weigel, M.K.; Prakash, P.; Wijewardhane, P.R.; Hasel, P.; Rufen-Blanchette, U.; Münch, A.E.; Blum, J.A.; Fine, J.; Neal, M.C.; et al. Neurotoxic Reactive Astrocytes Induce Cell Death via Saturated Lipids. *Nature* **2021**, *599*, 102–107. [CrossRef] [PubMed]
83. Guttikonda, S.R.; Sikkema, L.; Tchieu, J.; Saurat, N.; Walsh, R.M.; Harschnitz, O.; Ciceri, G.; Sneeboer, M.; Mazutis, L.; Setty, M.; et al. Fully Defined Human Pluripotent Stem Cell-Derived Microglia and Tri-Culture System Model C3 Production in Alzheimer's Disease. *Nat. Neurosci.* **2021**, *24*, 343–354. [CrossRef] [PubMed]
84. Colombo, E.; Farina, C. Astrocytes: Key Regulators of Neuroinflammation. *Trends Immunol.* **2016**, *37*, 608–620. [CrossRef]
85. Tarassishin, L.; Suh, H.-S.; Lee, S.C. LPS and IL-1 Differentially Activate Mouse and Human Astrocytes: Role of CD14. *Glia* **2014**, *62*, 999–1013. [CrossRef] [PubMed]
86. John, G.R.; Lee, S.C.; Brosnan, C.F. Cytokines: Powerful Regulators of Glial Cell Activation. *Neuroscientist* **2003**, *9*, 10–22. [CrossRef] [PubMed]
87. Ma, D.; Jin, S.; Li, E.; Doi, Y.; Parajuli, B.; Noda, M.; Sonobe, Y.; Mizuno, T.; Suzumura, A. The Neurotoxic Effect of Astrocytes Activated with Toll-like Receptor Ligands. *J. Neuroimmunol.* **2013**, *254*, 10–18. [CrossRef] [PubMed]
88. Gorina, R.; Font-Nieves, M.; Márquez-Kisinousky, L.; Santalucia, T.; Planas, A.M. Astrocyte TLR4 Activation Induces a Proinflammatory Environment through the Interplay between MyD88-Dependent NFκB Signaling, MAPK, and Jak1/Stat1 Pathways. *Glia* **2011**, *59*, 242–255. [CrossRef]
89. Gustin, A.; Kirchmeyer, M.; Koncina, E.; Felten, P.; Losciuto, S.; Heurtaux, T.; Tardivel, A.; Heuschling, P.; Dostert, C. NLRP3 Inflammasome Is Expressed and Functional in Mouse Brain Microglia but Not in Astrocytes. *PLoS ONE* **2015**, *10*, e0130624. [CrossRef] [PubMed]
90. Abulafia, D.P.; De Rivero Vaccari, J.P.; Lozano, J.D.; Lotocki, G.; Keane, R.W.; Dietrich, W.D. Inhibition of the Inflammasome Complex Reduces the Inflammatory Response after Thromboembolic Stroke in Mice. *J. Cereb. Blood Flow Metab.* **2009**, *29*, 534–544. [CrossRef]

91. Halle, A.; Hornung, V.; Petzold, G.C.; Stewart, C.R.; Monks, B.G.; Reinheckel, T.; Fitzgerald, K.A.; Latz, E.; Moore, K.J.; Golenbock, D.T. The NALP3 Inflammasome Is Involved in the Innate Immune Response to Amyloid-β. *Nat. Immunol.* **2008**, *9*, 857–865. [CrossRef] [PubMed]
92. Huang, X.; Feng, Y.; Xiong, G.; Whyte, S.; Duan, J.; Yang, Y.; Wang, K.; Yang, S.; Geng, Y.; Ou, Y.; et al. Caspase-11, a Specific Sensor for Intracellular Lipopolysaccharide Recognition, Mediates the Non-Canonical Inflammatory Pathway of Pyroptosis. *Cell Biosci.* **2019**, *9*, 31. [CrossRef] [PubMed]
93. Viganò, E.; Diamond, C.E.; Spreafico, R.; Balachander, A.; Sobota, R.M.; Mortellaro, A. Human Caspase-4 and Caspase-5 Regulate the One-Step Non-Canonical Inflammasome Activation in Monocytes. *Nat. Commun.* **2015**, *6*, 8761. [CrossRef] [PubMed]
94. Liu, L.; Chen, M.; Lin, K.; Xiang, X.; Zheng, Y.; Zhu, S. Inhibiting Caspase-12 Mediated Inflammasome Activation Protects against Oxygen-Glucose Deprivation Injury in Primary Astrocytes. *Int. J. Med. Sci.* **2020**, *17*, 1936–1945. [CrossRef] [PubMed]

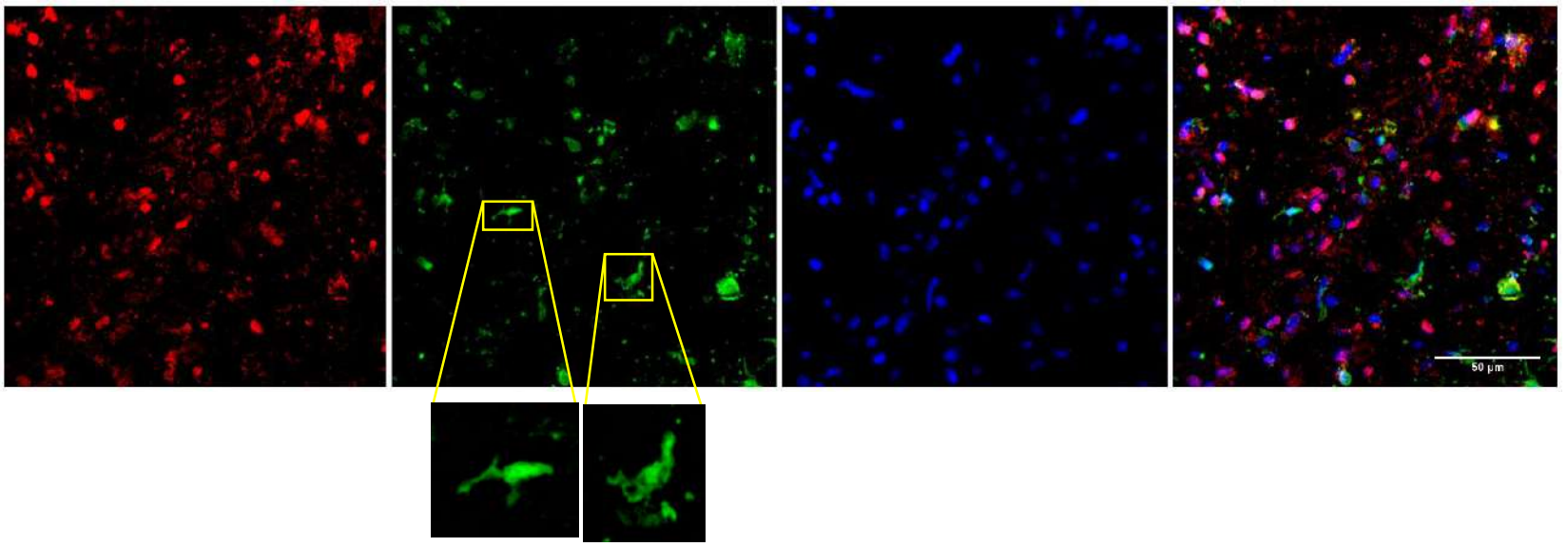



| | | | | |
|---|---|---|---|---|
| LPS 1 µg/ml | - | + | - | + |
| Clodronate 150 µg/ml | - | - | + | + |
| Sample ID | 1, 3, 6, 7 | 9, 11, 13, 16 | 2, 4, 5, 8 | 10, 12, 14, 15 |
| N | 4 | 4 | 4 | 4 |

**Supplementary Figure S1** Western blot analysis of lysates from organotypic hippocampal cultures (OHCs) demonstrating protein level of IBA1 protein. Vinculin was used as a normalizing control. Description of the arrangement of the samples (1-16).

FPR2 IBA1 DAPI Merge

Control




FPR2 IBA1 DAPI Merge

LPS