Cromolyn Inhibits the Secretion of Inflammatory Cytokines by Human Microglia (HMC3)

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Abstract

Cromolyn is a known mast cell stabilizer and is approved for treatment of asthma and for other allergic indications. Cromolyn, in a new redesigned dry powder formulation, is being tested in a pivotal clinical trial in combination with low dose ibuprofen to treat early Alzheimer's Disease (AD) subjects. To better understand the mechanistic effect cromolyn has in slowing down or halting the neuroinflammatory response associated with AD progression, we tested the effect of cromolyn to dampen the inflammatory response in the human HMC3 microglia cell line. The direct effect of cromolyn on HMC3 microglia is on cytokines and chemokines production following their activation by the inflammatory cytokine TNF-α. Cromolyn and a new fluorinated analog dramatically reduced the secretion of a wide spectrum of inflammatory mediators, which included cytokines such as IL-1β, IL-6, IL-8 and IFN-γ, and chemokines such as CXCL10, CCL2, CCL3 and CCL4. These results bolster our understanding of how our cromolyn platform modulates toxic microglia behavior as a dynamic future treatment option for neurodegenerative disorders.

Summary

Cromolyn is a known mast cell stabilizer and is approved for treatment of asthma and for other allergic indications. Cromolyn, in a new redesigned dry powder formulation, is being tested in a pivotal clinical trial in combination with low dose ibuprofen to treat early Alzheimer's Disease (AD) subjects. To better understand the mechanistic effect cromolyn has in slowing down or halting the neuroinflammatory response associated with AD progression, we tested the effect of cromolyn to dampen the inflammatory response in the human HMC3 microglia cell line. The direct effect of cromolyn on HMC3 microglia is on cytokines and chemokines production following their activation by the inflammatory cytokine TNF-α. Cromolyn and a new fluorinated analog dramatically reduced the secretion of a wide spectrum of inflammatory mediators, which included cytokines such as IL-1β, IL-6, IL-8 and IFN-γ, and chemokines such as CXCL10, CCL2, CCL3 and CCL4. These results bolster our understanding of how our cromolyn platform modulates toxic microglia behavior as a dynamic future treatment option for neurodegenerative disorders.

Introduction

Microglia are the resident macrophages of the central nervous system (CNS) and have critical roles with immune surveillance, maintenance of synapses, and neuronal health [1]. Microglia are distinct from other myeloid cells, such as monocytes and peripheral macrophages, and are extremely reactive to changes within the brain, collectively existing as heterogeneous populations determined by the homeostatic requirements of a given region of the CNS that respond to extracellular changes to protect from infection [2-4]. Microglia mediate these important and diverse functions through the secretion of cytokines, chemokines as well as growth and neurotrophic factors [5]. In addition, microglia exhibit direct anti-microbial activity through phagocytosis and the production of microbicidal Reactive Oxygen Species (ROS) and nitric oxide (NO) [6].

The prominent roles microglia have in regulating inflammation are usually designated by one of two phenotypes: the M1-type where microglia produce pro-inflammatory, neurotoxic cytokines whose chronic production contributes to dysfunction of the neural network and propagates inflammation reaction; or the M2 type where microglia secrete anti-inflammatory mediators and neurotrophic factors that are involved in restoring brain homeostasis [6]. Simplifying microglia into only two designations makes it simpler to refer to a generalized activation state of microglia, but it is more likely that microglia exist on a dynamic spectrum that activate specifically in response to chronic stressors that lead to neurodegeneration in the CNS [7].

It is generally accepted that early activation of monocytes and microglia has potential to decelerate neurodegenerative progression by modulating immune responses to increase the intrinsic phagocytic capacity of monocytes and microglia without triggering secretion of pro-inflammatory cytokines that could worsen neurodegeneration [8]. Strategies to modulate monocyte and microglial activity have been studied, especially those that can protect against microglia-mediated neurotoxicity [9-11]. We are focused on studies that would establish the utility of our cromolyn analogs as a platform to modulate the pathology of neurodegenerative diseases such as Alzheimer's Disease (AD) and Amyotrophic Lateral Sclerosis (ALS). The role of the neuro-inflammatory response in the presence of amyloid plaques and neurofibrillary tangles in the brain and its associated neuronal loss in the pathology of AD is well established and extensively studied [12-15]. Numerous studies show that microglial-mediated inflammation contributes to the progression of AD and that microglial cells are found in close association with amyloid-β (Aβ) deposits [16].

Cromolyn is a small molecule approved for the treatment of asthma [17]. Although the molecular mechanism of action is not well understood, cromolyn has been known for some time as a "mast cell stabilizer" [18] and to have other anti-inflammatory effects on polymorphonuclear neutrophil (PMN) [19] and macrophages [20]. Screening of a pharmaceutical library of compounds that have reached clinical trial stages in the US identified cromolyn as having potential to reduce Aβ42:Aβ40 burden in human iPSC-derived neurons by 20% and by more than 50% in combination with topiramate and bromocriptine [21]. This independently verifies the *in vivo* cromolyn study by Hori et al., that reduced soluble Aβ by 50% in one week and showed alterations in microglia economy [22]. More recently, cromolyn has been shown to promote the non-inflammatory phagocytosis of Aβ42 by murine BV2 microglial cells as well as improved clearance of TBS-soluble Aβ40 and Aβ42 and improved neuroprotection in Tg2576 AD model mice [10]. Furthermore, cromolyn reduced the levels of pro-inflammatory cytokines and mast cell activity in SOD1 transgenic mouse models from ALS [23].

Our group has provided pre-clinical evidence for the role of cromolyn in the treatment in APP/PS1 mice as an animal model of AD [10, 22]. On this basis, cromolyn is currently under evaluation in a late-stage clinical trials for early onset AD (NCT02547818 – A Phase III Safety and Efficacy Study of ALZT-OP1 in Subjects with Evidence of Early Alzheimer's Disease) and ALS (NCT04428775 – A Phase II Safety and Biomarker Study of ALZT-OP1a in Subjects with Mild-Moderate ALS Disease). To determine whether cromolyn inhibits secretion of pro-inflammatory mediators by microglia, we examined the effect of

cromolyn on HMC3 microglial cell line [24] after stimulation with TNF-α. Cromolyn dramatically reduced the secretion of a wide spectrum of inflammatory mediators, which included cytokines such as IL-1β, IL-6, IL-8 and IFN-γ, and chemokines such as CXCL10, CCL2, CCL3 and CCL4. Experiments in our group to modify the cromolyn structure to increase lipophilicity and blood-brain barrier uptake while maintaining the phagocytic profile of microglia resulted in substitution of the bridge hydroxyl group for fluorine to produce F-cromolyn [25]. Here we show that the core cromolyn structural motif was able to impact cytokine and chemokine release by activated human HMC3 microglia and these benefits are retained with the modified F-cromolyn. These results expand the robust cromolyn platform for promotion of non-inflammatory phagocytic activity of microglia to include inhibition of inflammatory mediators secreted by microglia as well, further bolstering cromolyn as an exciting therapeutic to combat neurodegeneration.

Materials And Methods

Chemicals and reagents

DMEM (Dulbecco's Modified Eagle Medium) (Cat#1995065), DMEM without phenol red (Cat#21063029), PBS-pH7.2 (Cat#20012050), L-glutamine (Cat#25030081), Trypsin-EDTA (Cat#25200056), and penicillin-streptomycin (Cat#15140122) were products of Gibco, Thermo Fisher Scientific. FBS (fetal bovine serum) (Cat#F4135), DMSO (dimethyl sulfoxide) (Cat#D2438), and TWEEN-20 (Cat#P1379) were purchased from Sigma-Aldrich. 3 mL Syringe/Needle Combination with Luer-Lok™ Tip (Cat#8936G82), 13 mm syringe filter (PVDF, 0.22 µm) (Cat#1159T77) were purchased from Thomas Scientific. Recombinant human TNF-α (Cat#300-01A), recombinant human IFN-γ (Cat#300-02) were purchased from PeproTech. Cromolyn and F-cromolyn were provided by AZTherapies and DMEM was used as diluent to achieve final concentrations as indicated.

Cell line and cell culture

Human microglial cell line HMC3 (CRL-3304) was purchased from ATCC (American Type Culture Collection). HMC3 cell line was cultured in DMEM medium with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. Cells were maintained in 37°C incubator at 5% CO_2.

Flow cytometry

HMC3 cells were resuspended, counted using the LUNA-II Automated Cell Counter, seeded in the 6-well plate (300 K cells/2 mL medium/well), and incubated overnight to allow cells to attach. Cells were treated with TNF-α or IFN-γ at five different concentrations (0, 10, 30, 100, 300 ng/mL) for 24 hr to induce cell activation. The supernatant medium was completely aspirated and 1 mL of fresh DMEM was added to wash the cell layer to remove detached cells and debris. The attached cells were resuspended and washed with PBS. Cells were washed with FACS buffer (2% FBS in PBS) prior to incubation with Zombie Violet Fixable Viability Dye (#423114, Biolegend, 1:500 dilution) and Human Fc Receptor Blocking Solution (#422302, Biolegend, 1:20) diluted in FACS buffer at RT for 10 min in the dark. Cells were then washed with FACS buffer and incubated at RT for 20 min with the following cell-surface antibodies: FITC

anti-MHC II (#361706, Biolegend), PerCP/Cy5.5 anti-CD11b (#301328, Biolegend), BV605 anti-CD40 (#334336, Biolegend), PE anti-CD86 (#374206, Biolegend), PE/Cy7 anti-CD163 (#326514, Biolegend), APC anti-CD206 (#321110, Biolegend), and PerCP/Cy5.5 anti-CD14 (#367110, Biolegend). All of the cell-surface antibodies were diluted 1:20 in FACS buffer. Cells were permeabilized using the Transcription Factor Fixation/Permeabilization Buffer Set (#424401, Biolegend). Then cells were incubated with the following intracellular antibodies: APC/Cy7 anti-CD68 (#333822, Biolegend), purified anti-IBA1 (#PA5-27436, Invitrogen), Goat anti-Rabbit-Alexa Fluor 647 (#A21245, Invitrogen), purified anti-GFAP (#644702, Biolegend), and Goat anti-Mouse-Alexa Fluor 488 (#11029, Invitrogen). All of the intracellular antibodies were diluted 1:100 in the Permeabilization buffer (1x). Cells were washed with Permeabilization buffer and fixed in Fixation Buffer (#420801, Biolegend). Flow cytometry was performed using the Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific). Analysis of flow cytometry data was performed via FCS Express 7 (DeNovo Software).

Cell viability assay by AO/PI stain

Cell viability was assessed with a LUNA-FL Automated fluorescence cell counter. Viable nucleated cells show green fluorescence and dying nucleated cells show red fluorescence. 18 µL of sample was mixed with 2 µL Acridine Orange/Propidium Iodide (AO/PI) Stain.

MSD U-PLEX assay platform – cytokine and chemokine secretions

HMC3 cells were resuspended, counted using the LUNA-II Automated Cell Counter, seeded in the 6-well plate (400 K cells/2 mL medium/well), and incubated overnight to allow cells to attach. The media and detached cells were removed next day. The cell layer was washed three times in PBS and once in serum- and phenolred-free DMEM (SPFM). Cells were incubated in SPFM for 4 hr prior to treatment with TNF-α (0.3 µg/mL) and/or Cromolyn (0.3 µM, 3 µM) or F-cromolyn-diacid (0.3 µM, 3 µM) for 24 hr. The conditioned medium was collected and centrifuged at 1,000 x RCF (g) for 5 min to pellet detached cells and large debris, which was subsequently passed through a 0.22 µm filter with PVDF membrane to remove smaller debris. Samples of the supernatant medium were put in a CoolRack (#07210041, Fisher Scientific) on dry ice for Snap-freezing and kept at -80ºC until use.

Meso-scale U-PLEX plates that detect a cytokine panel including IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, TNF-α, and a chemokine panel including IP-10/CXCL10, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, Eotaxin/CCL11, were used as per manufacture's protocol. 25 µL of the conditioned medium was used in each well of the MSD plates to detect the analytes. The plates were then analyzed on an MSD QuickPlex SQ120 instrument. All standard concentration curves for cytokines and chemokines tested in this study are provided in the Supplemental Figure S1.

Quantification and statistical Analysis

All the data were presented as mean ± standard error from at least three times, each done in triplicate. The statistical significance between two groups was determined by Student's t test, whereas the

comparisons of multiple groups were carried out by one-way ANOVA, followed by Bonferroni's post-test using GraphPad Prism 7 (GraphPad Software, Inc.). A probability value of * $p < 0.05$ was considered to be significant.

Results

F-cromolyn justification

A significant hurtle to overcome when treating CNS disorders is delivery of a given drug into the CNS while maintaining *in vivo* stability and subsequent drug activity. Fluorine substitutions are utilized in medicinal chemistry to increase important pharmacokinetic properties of drugs such as blood-brain barrier permeability, metabolic stability, and bioavailability [26]. C-F bonds have higher bond energies than C-H and C-O bonds and thus greater stability against proteolytic degradation and increased relative lipophilicity [26]. The log P of compound is a measure of its lipophilicity and is associated with its ability to cross the blood-brain barrier, and the substitution of hydrogen and other functional groups for fluorine is known to modulate a compound's log P [27]. As the hydroxyl group is considered a bioisostere of fluorine, with C-F and C-O bonds having similar bond length and isoelectronic properties [28], the fluorine-substituted F-cromolyn compound in our experiments is expected to have increased metabolic stability and a lipophilicity for better uptake across cell membranes and more attuned to crossing the blood-brain barrier into the CNS, making F-cromolyn potentially useful as an orally available drug [25].

HMC3 microglia exhibit inflammatory phenotype after TNF-α exposure

Mast cells are an integral component of the innate immune system and inflammatory process in the skin and mucosal surfaces of the body but are also prevalent in the human brain [29]. Once matured, their degranulation and subsequent release of cytokines and other pro-inflammatory mediators and attractants initiate complex and robust responses to help eliminate infection. In the case of an asthmatic, allergic immune response, mast cell degranulation can be dampened with the inhaled use of cromolyn to alleviate an asthmatic attack [17]. Inflammation is a predominant characteristic of aging and neurodegenerative disorders [30] and genetic mutations in microglial proteins such as TREM2 that have regulatory roles in microglial inflammation [31] and others are risk factors for developing AD [32]. We therefore sought to determine the ability of our cromolyn platform to dampen the secretion of inflammatory mediators in HMC3 human microglia that behave similarly to microglia in the CNS.

The human microglial clone 3 (HMC3) microglia cell line was developed through SV40 immortalization of human primary microglial cells and is capable of reacting to inflammatory stimuli with regulation of typical markers of microglia activation [24]. The density of microglia is not uniform in all regions of the brain and the presence of surface markers for activated microglia differ depending on their location in the CNS [33]. As such, a microglial model of neuroinflammation need not exhibit all possible surface markers for inflammation, though common surface markers for activated microglia of an M1-like inflammatory phenotype include CD40, CD86, MHC-II, IBA-1, CD11b, CD14, and CD68 [33], all of which in our experiments, except for CD11b, are increased after IFN-γ administration (**Figure 1a**). Generalized surface

markers of microglia such as CD11b and IBA-1 are found inconsistently upregulated in multiple brain regions of AD patients, but MHC-II and CD68 activation markers are more consistently increased in AD [34]. Interestingly, we observe expression of the astrocyte biomarker glial fibrillary acidic protein (GFAP) in resting, untreated HMC3 microglia and subsequent increase in GFAP after TNF-α and IFN-γ treatment, respectively (**Figure 1a**). To our knowledge GFAP has not been reported to be expressed in HMC3 cells, but others have found microglia to be morphologically dynamic and can exhibit classical astrocyte markers from a variety of injurious stimuli [35-37]. Importantly, due to the anti-inflammatory phagocytic surface markers CD163 and CD206 remaining unexpressed and unchanged prior to and after the addition of either IFN-γ or TNF-α, it is reasonable that HMC3 microglia behave as expected for a model of neuroinflammation [24].

We verified through cell viability experiments that both TNF-α and IFN-γ exert microglia necrosis in a concentration-dependent manner up to 300 ng/mL for each, with microglia death at 19.7% with IFN-γ and 15.6% with TNF-α (**Figure 1b**). Representative AO/PI fluorescence images for 300 ng/mL TNF-α are presented at 4x and 10x magnification in **Figure 1c**. Interestingly, we observed a more robust range of cytokine and chemokine secretion in response to TNF-α than with IFN-γ stimulation (data not shown). This aspect is partially due to TNF-α inducing less cell death than IFN-γ at the same concentration and thus TNF-α was subsequently used to induce cytokine and chemokine secretion by HMC3 microglia prior to addition of cromolyn and F-cromolyn.

Cromolyn and F-cromolyn inhibit secretion of inflammatory cytokines and chemokines from HMC3 human microglial cells

The cytokine TNF-α is known to be amply produced by monocytic cells, including microglia, and strongly influences many other inflammatory mediators to be upregulated by innate immune cells upon TNF-α exposure [38]. Our experiments verify that treatment of HMC3 cells with TNF-α potently induces inflammatory cytokine secretions by HMC3 cells as evidenced by the dramatic increase in cytokine concentration in cell culture supernatant after 24 hours of TNF-α treatment (**Figure 2**). Compared to untreated HMC3 cells (control), treatment with TNF-α (0.3mg/mL) resulted in >177-fold increase in IL-1β ($p < 0.001$), IL-6 ($p < 0.001$), and IL-8 ($p < 0.001$) and caused the previously quiescent cells to secrete IFN-γ. We then tested the effect of both cromolyn and F-cromolyn at a low (0.3 μM) and higher (3 μM) concentration against TNF-α induced cytokine secretion by HMC3 microglia. IFN-γ ($p = 0.0033$), IL-6 ($p = 0.0065$), IL-1β ($p = 0.0005$), and IL-8 ($p = 0.0006$) concentrations were decreased after addition of the lower 0.3 μM concentration of cromolyn and F-cromolyn. This inhibitory effect is dose-dependent for cromolyn, because the higher 3 μM concentration resulted in >40% inhibition of IFN-γ, IL-6, and IL-1β, and 25.8% inhibition of IL-8. Interestingly, F-cromolyn also decreases cytokine release in a dose-dependent manner, with the higher 3 μM concentration achieving >50% inhibition of both IFN-γ and IL-1β and 39.8% and 27.9% inhibition of IL-6 and IL-8, respectively.

We next tested the effect of cromolyn and F-cromolyn on the production of extracellular chemokines after the treatment of HMC3 cells with 0.3 μg/mL TNF-α (**Figure 3**). Treatment with cromolyn (0.3 μM) resulted

in a statistically significant reduction in the concentration of extracellular IP-10 (CXCL10) ($p < 0.001$), MCP-1 (CCL2) ($p = 0.0016$), MIP-1α (CCL3) ($p = 0.0118$), and MIP-1β (CCL4) ($p < 0.001$) compared to cells treated with TNF-α alone for 24 hours. Importantly, we observed an even greater reduction in extracellular cytokine production at a higher concentration of cromolyn (3 μM), with >40% inhibition of IP-10 (CXCL10), MCP-1 (CCL2), and MIP-1β (CCL4), and 35.6% inhibition of MIP-1α (CCL3) compared to TNF-α cytokine induction levels. Additionally, we observed that like cromolyn, F-cromolyn (0.3 μM) can inhibit the production of inflammatory chemokines IP-10 (CXCL10) ($p < 0.001$), MCP-1 (CCL2) ($p < 0.001$), MIP-1α (CCL3) ($P = 0.0081$), and MIP-1β (CCL4) ($p < 0.001$). We also observed F-cromolyn (3 μM) retained the dose-dependent inhibitory ability of HMC3 microglia chemokine secretion, including >40% inhibition of IP-10 (CXCL10), MCP-1 (CCL2), and MIP-1β (CCL4), and 36.0% inhibition of MIP-1α (CCL3). We conclude that both cromolyn and F-cromolyn can dose-dependently inhibit the release of several inflammatory chemokines by HMC3 microglia cells.

Since cromolyn and F-cromolyn were found to reduce concentrations of secreted neurotoxic cytokines, we next carried out cell necrosis experiments to verify if this property translated to enhanced cell viability. **Figure 4** shows that administration of TNF-α (0.3 μg/mL) to HMC3 cells was sufficient to increase cell death to approximately 20% of the total population, over four times that of controls. Addition of 0.3 μM cromolyn and F-cromolyn, respectively, significantly reduced cell death. Addition of higher 3 μM concentrations of cromolyn and F-cromolyn significantly reduced cell death even further, to increasing viability by approximately 40% over TNF-α induced HMC3 cells alone. We conclude that cromolyn and F-cromolyn are able to prevent HMC3 microglia cell death induced by TNF-α in a concentration-dependent manner.

Discussion

Despite the robust cytokine and chemokine release by HMC3 microglia after addition of TNF-α, we observed several mediators that exhibited modest or no significant change in our experiments (**Supplementary Figure S2**). IL-2 is modestly expressed in HMC3 microglia after TNF-α exposure in our experiments and cromolyn appears to have no significant effect on the amount of IL-2 secretion. IL-2 is primarily produced by T cells and dendritic cells in the response to bacterial lipopolysaccharide (LPS) and is involved in T and B cell proliferation and differentiation [39]. Nuclear factor of activated T cells (NFAT) signaling mediates production of many pro-inflammatory mediators in dendritic cells, including IL-2 and TNF-α [39]. Although experiments with murine microglia have been shown to express NFAT isoforms, TNF-α does not significantly induce NFAT-driven luciferase activity in microglia as LPS does, suggesting that NFAT signaling in microglia may be more specifically induced than other peripheral immune cells [40]. Despite that microglia express IL-2 receptors and potently respond to exposure to IL-2 by activating expression of other cytokines [41], TNF-α itself is not a significant inducer of IL-2 secretion by HMC3 microglia and cromolyn does not significantly change this behavior.

IL-10 is anti-inflammatory cytokine strongly produced by microglia in response to pathogen-associated molecular patterns (PAMPs) such as microbial lipopolysaccharides (LPS), against which the body is well

known to initiate strong and lasting immune responses [42]. TNF-α has been found to induce IL-10 secretion alone in a dose-dependent manner in primary human fetal microglia over a 48-hour exposure. However, our experiments with HMC3 microglia utilized 3 times the concentration of TNF-α (0.1 μg/mL vs 0.3 μg/mL) [43] and yielded approximately 6 orders of magnitude less the amount of IL-10 (**Supplementary Figure S2**). This result was surprising, but others have found that HMC3 microglia do not produce detectable amounts of IL-10 at rest [24] as we can confirm. Neither cromolyn or F-cromolyn had any significant effect on altering IL-10 expression in HMC3 microglia. Additionally, *in vivo* experiments with the $SOD1^{G93A}$ mice model of ALS found that 5 days per week intraperitoneal cromolyn treatment for approximately two months beginning 60 days postnatal until onset of major paralysis did not significantly alter IL-10 levels in spinal cord lysates despite mice exhibiting delayed onset of disease [23]. Future experiments will warrant exploring co-stimulatory factors to model anti-inflammatory IL-10 expression in this particular microglial cell line to further elucidate whether cromolyn or F-cromolyn has any effect on its secretion.

IL-4 is generally considered an anti-inflammatory cytokine when not chronically expressed that can downregulate the expression of pro-inflammatory mediators including TNF-α, IL-1β, IL-6, IL-8, and IL-12 [44]. Though microglia are particularly responsive to exogenous IL-4 and the cytokine been associated with reducing expression of MHC-II, IL-4 is primarily produced by mast cells, basophils, eosinophils, and T helper cells [45]. As we observe little expression of IL-4 after administration of TNF-α to HMC3 microglia (**Supplementary Figure S2**), it is reasonable that IL-4 acts primarily on microglia to influence actions against neuroinflammation and that microglia do not appreciably secrete IL-4. Additionally, neither cromolyn or F-cromolyn significantly affected IL-4 secretion by HMC3 microglia induced by TNF-α.

CCL11 is a chemokine eosinophil attractant involved in allergic reactions and is found to be upregulated in blood sera and in the CNS in aging individuals [46]. Although microglia express CCR3 surface receptors for the CCL11 chemokine and produce neurotoxic reactive oxygen species in response to CCL11, it is activated astrocytes that are primarily found to produce CCL11 in the presence of 10 ng/mL TNF-α and not microglia [46]. Our experiments utilizing a much higher concentration (0.3 μg/mL) of TNF-α promoted detectable amounts of CCL11 (>60 pg/mL) in HMC3 microglia but neither cromolyn nor F-cromolyn significantly altered their CCL11 secretion (**Supplementary Figure S2**).

Though the exact molecular mechanism of action for cromolyn's effect of dampening the inflammatory response in microglia is not clear from this study, experiments with mast cells pretreated with cromolyn have higher amounts of externalized annexin-A1 attached to formyl peptide receptors (FPR) on the cell surface, suppressing cell activation and further degranulation [17]. Interestingly, annexin-A1 is expressed more readily in AD microglia than in normal-aged microglia, which has roles in inflammatory microglia activation resolution and promotion of phagocytic signaling between phosphatidylserine on apoptotic neurons and FPR2 surface receptors on microglia [47]. Cerebral organoids are three-dimensional neural cell models that have been used to express familial mutations in presenilin 1 and amyloid precursor protein to illustrate that amyloid β-protein deposition and phosphorylated tau pathology can be modeled *in vitro* [48] and these cell models have been used to characterize altered microglia morphology, their

phagocytic and inflammatory activation states, and their cytokine and chemokine release [49]. Clearly, further experiments will be necessary to determine the impact cromolyn may have on other AD-related surface markers of microglia and how it may translate to ameliorating toxic, inflammatory microglia activation.

As this study focused on the ability of cromolyn and F-cromolyn to prevent cytokine and chemokine secretion in HMC3 microglia, imminent experiments with RNA sequencing, proteomic binding assays, and biomarker panels of multiple cell types are needed to fully verify whether the mechanism of action of cromolyn to prevent cytokine secretion may be due to altering upstream/downstream cytokine gene transcription or by specific inhibition of cellular export either through exocytosis or secretion through the constitutive secretory pathway.

We have verified that HMC3 microglia exhibit robust inflammatory profiles when activated with TNF-α. Cromolyn and F-cromolyn are found to reduce the release of several key cytokines and chemokines related to neuroinflammation from TNF-α activated HMC3 microglia cells in a dose-dependent manner. Further, we found that cromolyn and F-cromolyn were able to prevent cell death in a dose-dependent manner compared to TNF-α activated controls. These results further expand our promising cromolyn platform for the potential treatment of neurodegenerative disorders to dampen the inflammatory profile of activated microglia.

Declarations

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Disclosures: Y.W., A.M., M.A.D., and P.G.A.-R are employed by AZTherapies. D.R.E. is an Associate Professor at Harvard Medical School and holds a part time employee position at Massachusetts General Hospital and serves as an executive in AZTherapies.

Author Contributions: Y.W. and A.M. performed the lab work, M.A.D. worked on the manuscript and P.G.A.-R. and D.R.E. lead the project.

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