EVALUATION OF THE ANTI-INFLAMMATORY POTENTIAL OF AN AGONISTIC CD200R1 APTAMER ON ACTIVATED GLIAL CULTURES

Master in Translational Medicine

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ABSTRACT

Neuroinflammation constitutes a fundamental process involved in the progression of several neurodegenerative disorders as well as other neurological diseases. Its study has gained interest since it could help to treat different brain pathologies. The involvement of microglial cells on neuroinflammation has been known for a long time, but its function is not fully understood. CD200-CD200R1 ligand-receptor system has been described as essential for the inhibitory control of the inflammatory response as its signalig can decrease microglial activation. The present work was aimed at reducing microglial activation through the modulation of CD200-CD200R1 signaling pathway. To achieve so, primary mice glial cell cultures were treated with the aptamer molecule M52, which was expected to act as an agonist for CD200R1, and consequently, produce an anti-inflammatory effect.

ACKNOWLEDGEMENTS

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INDEX

ABBREVIATIONS

ANOVA: Analysis of Variance
BSA: Bovine Serum Albumin
CD200: Cluster of Differentiation 200
CD200R1: Cluster of Differentiation 200 Receptor 1
CNS: Central Nervous System
Cox2: Cyclooxygenase 2
Dok1/2: Downsteram of Tyrosine Kinase 1/2
DMEM: Dulbecco's Modified Eagle's Medium
DMSO: Dimethyl Sulfoxide
ERK: Extracellular Signal-Regulated Kinases
FBS: Fetal Bovine Serum
GFAP: Glial Fibrillary Acidic Protein
Iba-1: Ionized Calcium Binding Adaptor Molecule-1
IFN-γ: Interferon-γ
IL: Interleukin
iNOS: Inducible Nitric Oxide Synthase
LPS: Lypopolysaccharide
L/I: Lypopolysaccharide + Interferon-γ
MAPKs: Mitogen-Activated Protein Kinases
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NO: Nitric Oxide
PAGE: Polyacrylamide Gel Electrophoresis
PBS: Phosphate Buffer Saline
PEI: Polyethylenimine
PFA: Paraformaldehyde
PI: Propidium Iodide
PI3K: Phosphoinositidie 3-kinase
qRT-PCR: Quantitative Real Time Polymerase Chain Reaction
SELEX: Systematic Evolution of Ligands by Exponential Enrichment
SEM: Standard Error of the Mean
TLR: Toll-Like Receptor
TNF-α: Tumor Necrosis Factor α

1. INTRODUCTION

1.1 Neuroinflammation and Microglia

Neuroinflammation is described as an essential and complex biological process where the reactive central nervous system (CNS) elements respond to altered homeostasis [1]. It differs from peripheral inflammation in various ways, mainly regarding the cellular types involved which principally are microglia cells and also astrocytes [2]. Microglia, known as the resident macrophage of the CNS, is the principal effector of neuroinflammation and can become promptly activated with different stimuli, for example in the presence of pathogens, tissue damage, abnormal stimulation, neurotoxins, infection or injury [3].

Unless the neuroinflammatory situation is resolved in a certain period of time, it might develop into a chronic neuroinflammation where microglia will remain activated, releasing cytokines and other molecules [4]. It is believed that neuroinflammation may cause or accelerate long-term neurodegenerative diseases, playing a central role in its very early development [2]. Furthermore, microglial over-activation may also accelerate the process of other neurological pathologies including CNS infection, ischemic stroke and psychiatric disorders [5]. The specific mechanisms that lead to the initiation of brain inflammation in these diseases are still not well understood, neither are the mechanisms that contribute to chronic neuroinflammation [6].

In normal conditions, microglia remains in a resting/surveilling state characterized by small cell bodies with thin ramifications that are continuously controlling the microenvironment. Microglia is also implied in normal development, connectivity and plasticity [7]. Its activation is usually accompanied by a morphological shift where their ramifications are shortened and they become amoeboid with numerous vacuoles [8,9]. In this state, they increase the expression of myeloid cell markers [10]. Activated microglia can secrete pro-inflammatory and neurotoxic factors, besides, they are capable of phagocytosis [11].

Nevertheless, it is important to bear in mind that changes in morphology do not reliably reflect the function being undertaken by this cell [12]. Moreover, microglial activation involves a great variety of phenotypes which probably coexist at the same time in different microglial populations to restore brain homeostasis and diminish neuronal damage [13].

1.2 CD200 signaling pathway

Cluster of differentiation 200 (CD200) is a transmembrane glycoprotein that in the CNS is predominantly expressed by neurons and astrocytes. It binds to the receptor CD200 receptor-1 (CD200R1), which is confined in myeloid lineage cells, such as microglia [14].

When the ligand binds the receptor, the receptor is phosphorylated which causes the phosphorylation of adaptor molecules downstream of tyrosine kinase 1 (Dok1) and Dok2. This follows the recruitment and activation of Ras GTPase effector enzyme (RasGAP) that induces the inhibition of Ras and downstream MAPKs (mitogen-activated protein kinases) phosphoinositide 3-kinase (PI3K) and extracellular signal-regulated kinases (ERK) activation. Ultimately, the pathway leads to the inhibition of inflammatory cytokine production, in other words, it results in a suppression of inflammation [15].

In the normal brain, CD200-CD200R1 ligand-receptor system is one of the inhibitory mechanisms that helps to maintain microglial reactivity under control [16]. Alterations in these inhibitory signals are known to be altered in multiple sclerosis [17], Alzheimer's disease [18] as well as in the aging brain [19]. In all these situations, there is a down-regulation of CD200 expression that has been related to microglial activation [18]. For this reason, there are evidences suggesting that the restoration of CD200-CD200R1 signaling pathway could be a potential anti-inflammatory treatment for some neurological diseases [20]. One way to achieve the enhancement of this pathway is by using agonists of CD200R1.

1.3 Aptamers

Aptamers are short synthetic single-stranded nucleic acids, either DNA or RNA that can bind specific ligands with high affinity and selectivity due to their three-dimensional structure [21]. They are isolated through a process named systematic evolution of ligands by exponential enrichment (SELEX) which consists of short DNA sequences that are screened for their binding to the specific target [22].

Because of their advantageous properties, such as relatively small size, quick production, high stability and lack of immunogenicity, aptamers have been suggested to be extremely suitable as a therapeutic and diagnostic tool [23].

Several DNA aptamers were developed as agonists of CD200R1. The results showed that two of them (M52 and M49) were able to potentiate the CD200 signaling pathway by inducing the phosphorylation of CD200R1, likewise CD200 would act [24].

2. WORKING HYPOTHESIS AND OBJECTIVES

Neuroinflammation, which is mainly driven by activated microglial cells, has been closely linked to the pathophysiology of neurodegenerative diseases [25]. Previous studies of the group showed that CD200R1 mRNA expression was lower in microglial cells in response to LPS [6,16]. The central **hypothesis** of this study is that an exogenous alteration by an agonist molecule of the CD200-CD200R1 ligand-receptor system will lead to a decrease of the microglial activation produced by a pro-inflammatory stimulus.

The main **objective** was to study the effect of aptamer molecules on microglial activation by using mice primary glial cell cultures. The specific objectives were the following:

1. To select the aptamer concentration to work with based on cell viability and nitrite production in mixed glial cell cultures.

2. To determine morphological alterations of the effect of aptamer exposition in induced glial activation by a pro-inflammatory stimulus in mixed glial and microglial cell cultures.

3. To determine functional changes as a result of the aptamer exposition in induced glial activation by a pro-inflammatory stimulus in microglial cell cultures.

3. MATERIALS AND METHODS

Experiments were carried out in accordance with European Union directives (86/609/EU) and Spanish regulations (BOE 67/8509-12, 1988) on the use of laboratory animals, and were approved by the Ethics and Scientific Committees of Universitat de Barcelona and the Hospital Clínic Provincial de Barcelona.

3.1 Primary Cell Cultures

Primary mixed glial cell culture was prepared from cerebral cortices of C57BL/6 (Charles River, Lyon, France) mice between 0 and 3 days of age as described elsewhere [26]. The medium used consisted of Dulbecco's modified Eagle's medium (DMEM)/F-12 nutrient mixture (Invitrogen, Carlsbad, CA), 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), 0.1% penicillin-streptomycin (Invitrogen) and amphotericin B (Fungizone; Invitrogen). Once a single cell suspension was obtained, it was filtered through a 100 µm pore-mesh. Cells were counted using a Neubauer chamber, brought to a final density of 350,000 cells/mL and cultured at 37ºC in 5% CO_2/ 95% air. Medium was replaced every 5-7 days *in vitro*. Cells were confluent at 8-12 days *in vitro* and used at day 21 (see Supplementary Figure S1- S2).

Primary microglial cell culture was obtained from brains of postnatal 0- 3 days mouse pups as described elsewhere [27]. The medium used consisted of DMEM (Invitrogen), 10% FBS (Invitrogen) and 1% of penicillin/streptomycin (Invitrogen). Single cell suspension was obtained by mechanical isolation. Cells were plated on polyethyleneimine (PEI; Sigma-Aldrich, St. Louis, MO)-coated T75 culture flasks and incubated at 37ºC in 5% CO_2/ 95% air. At 48h, the medium was changed, no additional medium was added until total detachment of astrocytes, at 14-16 days *in vitro*. Then, cells were trypsinized and seeded using the medium DMEM, FBS 1% and penicillin/streptomycin 1%. The number of cells were counted with Neubauer chamber and brought to a final density of 200,000 cells/mL or 250,000 cells/mL in 48-well, 24-well plate, respectively (Supplementary Figure S1).

3.2 Treatments

Cultures were pre-treated with different concentrations of DNA aptamers C (Control) and M52 (putative CD200R1 agonist) at 6.25-400nM (Supp Table S1) or dexamethasone (0.5-2.5nM) 1 hour before the treatment with lipopolysaccharide (LPS, Sigma-Aldrich) or LPS plus interferon-γ (IFN-γ, Sigma-Aldrich). Two kinds of aptamers were used: ultramers and polyacrylamide gel electrophoresis (PAGE) purified aptamers (Integrated DNA Technologies, Coralville, IA). LPS was used at 100 ng/mL and interferon, at 0.1 ng/mL. Cells were treated during 6h for RNA extraction, 24h for immunocytochemistry and cell viability and 48h for nitrite assays.

3.3 MTT assay

The MTT assay of cellular metabolism was used as a first estimation of cell mortality. Cell viability was estimated by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). MTT (Sigma-Aldrich) was added and incubated for 30 minutes. After that time, the medium was removed and dimethyl

sulfoxide (DMSO) was added to dissolve the blue crystals formed. The absorbance was read at 570nm and 650nm using a microplate reader (Multiskan Spectrum; Thermo Electron Corporation, Waltham, CA). Results were expressed as a percentage of the control.

3.4 Propidium Iodide/ Hoechst staining

Propidium Iodide (PI) and Hoechst 33342 (Molecular Probes, INC., Eugene, OR) were added to label nuclei of dead and alive cells, respectively. PI stains dead cells in red while Hoechst can label all cells with blue fluorescence. Images were obtained under an Olympus IX70 microscope (Olympus, Okoya, Japan) and a digital camera (CC-12, Olympus Soft Imaging Solutions GmbH, Hamburg, Germany).

3.5 Nitric Oxide Production

Liberation of nitric oxide (NO) was monitored using the colorimetric Griess assay. Conditioned media was collected 48 hours after treatment. Standard curve was prepared using a solution of $NaNO_2$. Griess reactive was prepared by mixing 1:1 reactive A (1% sulphanilamide and 5% phosphoric acid) and reactive B (0.1% dihidroclorur of N-(1-naftil)-ethylendiamine). Absorbance was read at 540nm using a microplate reader (Multiskan Spectrum).

3.6 Immunocytochemistry

Culture cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes and permeated using a solution of 0.3% Triton X-100 in phosphate buffer saline (PBS) containing 1% bovine serum albumin (BSA) and 10% donkey serum (Vector, Peterborough, UK). The primary antibodies were incubated overnight at 4ºC. Secondary antibodies were incubated for 1 hour at room temperature (Supp Table S2). Images were obtained using an Olympus IX70 microscope (Olympus).

3.7 Quantitative Real Time PCR (qRT-PCR)

RNA was extracted from microglial cell cultures using PureLink RNA micro kit (Invitrogen) and from mixed glial cell cultures using High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany). Samples were retrotranscripted from RNA to cDNA using qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD). 3ng of cDNA were used to perform qRT-PCR. Primers used (Integral DNA Technology) are shown in the Supplementary material (Supp Table S3). Relative gene expression was calculated using the 2-ΔΔCt method with the CFX software (Bio-Rad Laboratories, Hercules, CA). Ct values were corrected with the reference genes actin and S18.

3.8 Statistical Analysis

Results are presented as the mean + standard error of the mean (SEM) values. Statistical analysis was performed with GraphPad Prism 4 Software (GraphPad Software Inc., San Diego, CA). One-way analysis of variance (ANOVA) followed by Newman-Keuls Multiple Comparison Test were used to compare groups. Values of $p<0.05$ were considered statistically significant.

4. RESULTS

Results are presented in two parts. The first corresponds to the study performed with ultramers (Ult C and Ult M52) whereas the second part corresponds to the study done with PAGE purified aptamers (Apt C and AptM52).

4.1 Ultramers

4.1.1 Viability

In a first approach, cell metabolism measured by the MTT assay was used to estimate cell viability. Regarding the selection of an adequate concentration of aptamer to work with, viability was determined in a range of concentrations. Glial cells were treated at concentrations of ultramers of 100, 200, 300 and 400nM in the absence or presence of LPS plus IFN-γ (L/I) (Figure 1). We observed a significant decrease in MTT reduction in cells treated with all the concentrations of Ult C. In the case of Ult M52, a significant decrease in MTT reduction was seen in cells treated at 300nM and 400nM plus L/I.

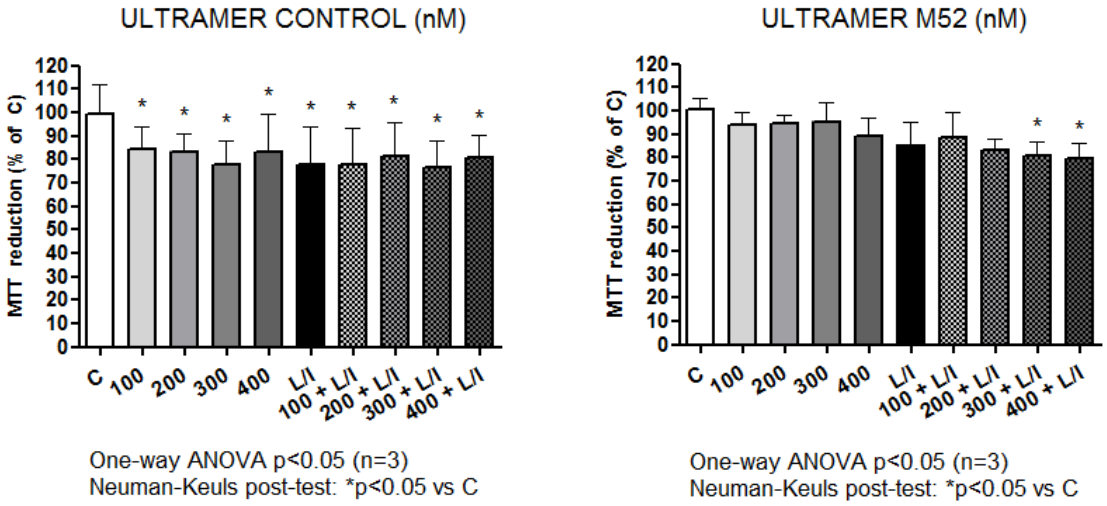

Figure 1. Effect of Ult C (left) and Ult M52 (right) on MTT reduction in mixed glial cell cultures.

To confirm whether the observed decreases were due to cell death, cell viability was also evaluated through PI/Hoechst staining (Figure 2). In all experimental situations, the number of PI positive nuclei (dead cells) was similar to the observed in the control group.

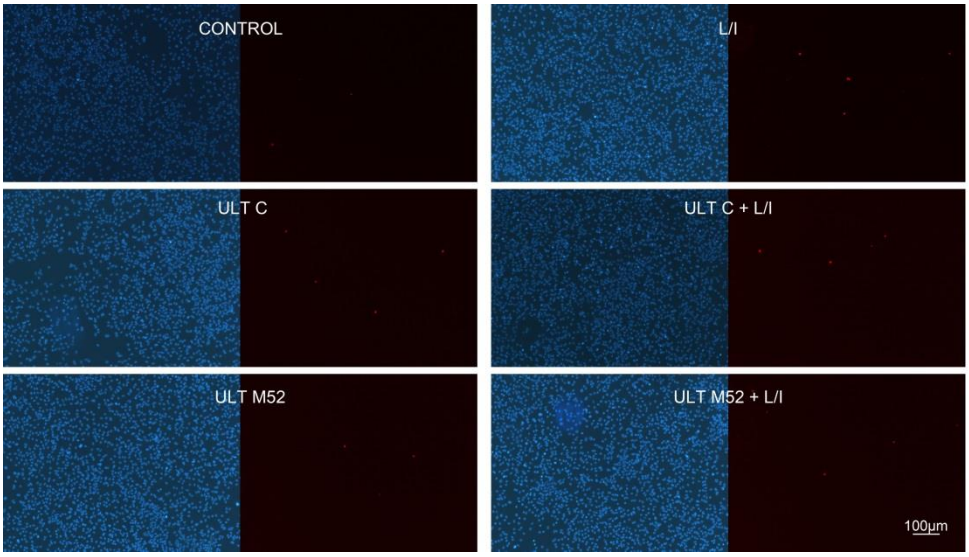

Figure 2. Microscope images of PI/Hoechst staining for Ult C and Ult M52 in mixed glial cell cultures. First and third columns correspond to the Hoechst staining (blue), whereas, second and fourth columns correspond to PI staining (red).

4.1.2 Morphological changes

Ult C and Ult M52 treatment induced morphological changes in glial cell cultures. In mixed glia (Figure 3 and 4), the treatment of aptamers produced a morphological shift analogous to the characteristic L/I alteration: superficial microglia is no longer seen bright and rounded as in the control, yet it loses its spherical form and luminosity being more attached to the well's surface. Besides, microglial cells seemed to contain more granules in response to the treatment. This change could be seen from 100nM Ult C or Ult M52, but not at lower concentrations.

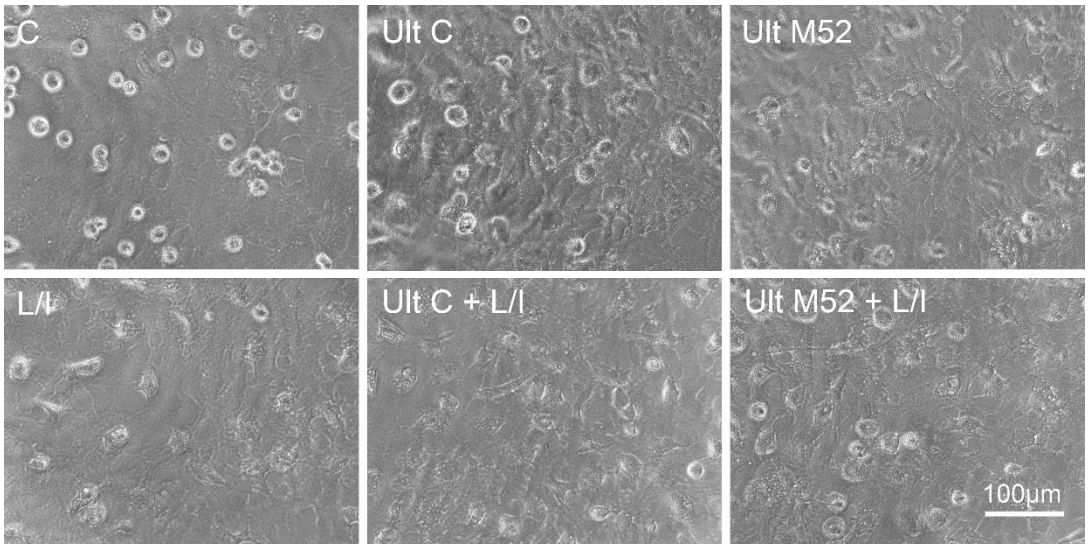

Figure 3. Morphological changes in a mixed glial cell culture treated with 400nM ultramers. Phase contrast images.

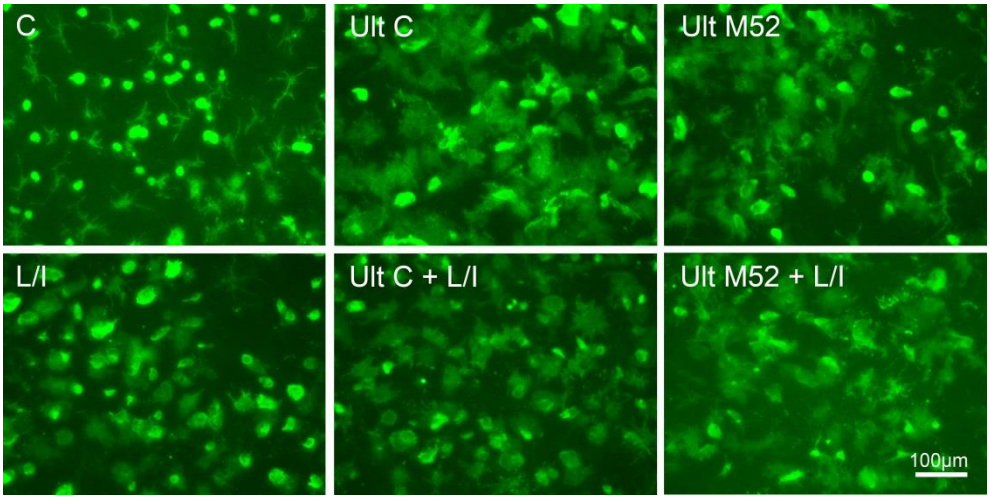

Figure 4. Iba1 and CD68 immunolabeling in mixed glial cell cultures. Ultramers were administered at 400nM.

In microglial cell cultures, morphological alterations were also apparent after the ultramer treatment (Figure 5). Control cells appeared to be more rounded with some large and thin ramifications. However, following the treatment, they turned into bigger and more ramified cells with granules. These changes were visible with both Ult C and Ult M52.

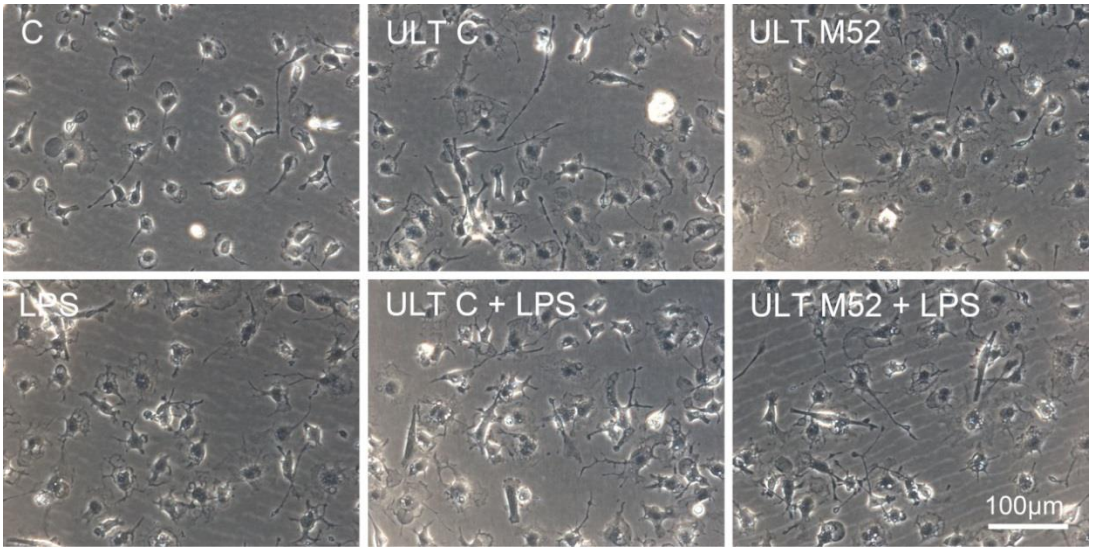

Figure 5. Morphological changes in a microglial cell culture. Phase contrast images. Cells were treated with ultramers at 400nM.

4.1.3 Nitrite production

NO production was determined as an index of glial activation after L/I treatment. We evaluated whether ultramer treatment had some effect on NO production in L/I treated cultures. As expected, L/I generated a significant increase on NO production (Figure 6). This production was not observed in glial cells treated with the different concentrations of ultramers. L/I induced NO production was not modified by Ult C and Ult M52. Surprisingly, a trend to increase NO production was seen in L/I treated

cultures pre-treated with 400nM Ult M52. Lower concentrations of ultramers (12.5, 25, 50nM) were also analyzed (results not shown), but a reduction of NO production could neither be seen.

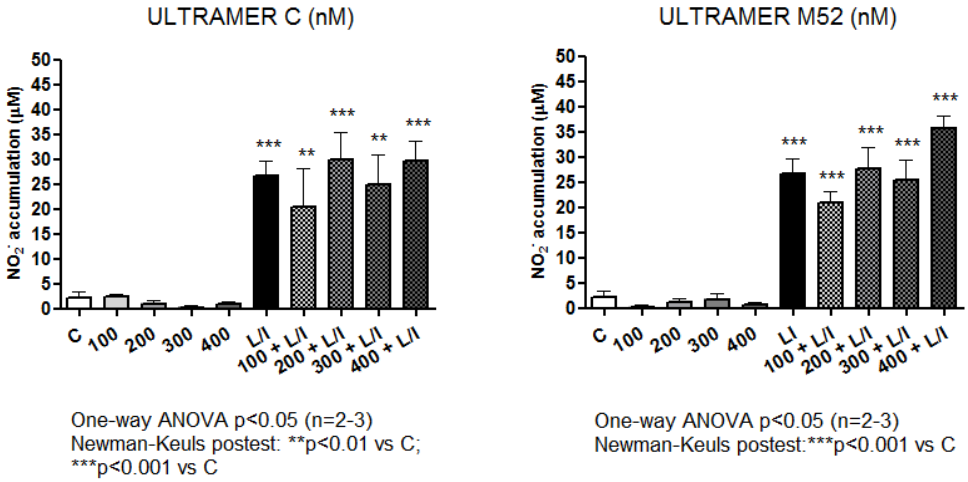

Figure 6. Effect of ultramer C (left) and M52 (right) treatment on NO production in mixed glial cell culture.

In parallel, we used an already known anti-inflammatory drug, dexamethasone, as a positive control for the inhibition of inflammation. NO production induced by L/I was significantly reduced when dexamethasone was administered (Figure 7). Previously, cell viability and morphological changes were also evaluated in mixed glial cell cultures treated with dexamethasone (Supplementary Figure S3).

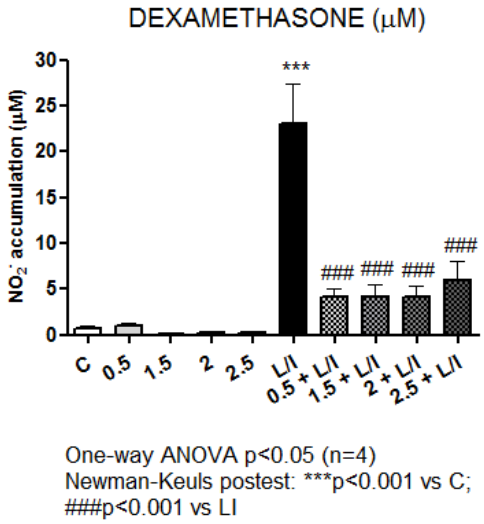

Figure 7. Effect of dexamethasone tretament on NO production in mixed glial cell culture.

4.1.4 Gene expression

Alterations in mRNA levels were determined in microglial cell cultures treated with ultramers at 400nM both in the absence and presence of LPS (Figure 8). The expression of pro-inflammatory genes, such as IL-1β, IL-6, TNF-α, Cox2 and iNOS was analyzed.

As expected, LPS induced an increase in the expression of all mRNAs tested. An increase in the expression of most of the mRNAs evaluated was also observed after Ult C and Ult M52 treatment, although to a lesser extent than with LPS. In general, L/I induced increases in mRNA expression were not modified by Ult C and Ult M52 pre-treatment. This experiment was repeated three times (not shown) with equivalent results. A representative culture is presented in Figure 8.

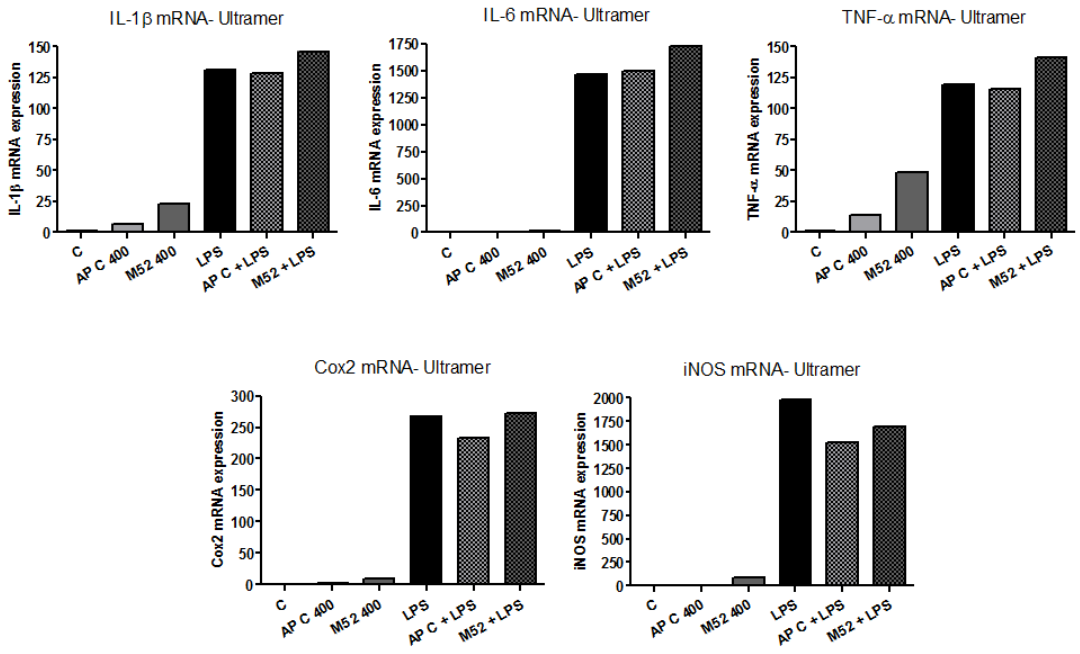

Figure 8. mRNA expression in microglial cells treated with ultramers C and M53 at 400nM. (n=1)

Again, dexamethasone was used as a positive control for the inhibition of inflammation (Figure 9). As expected, dexamethasone reduced the expression of all genes induced by L/I.

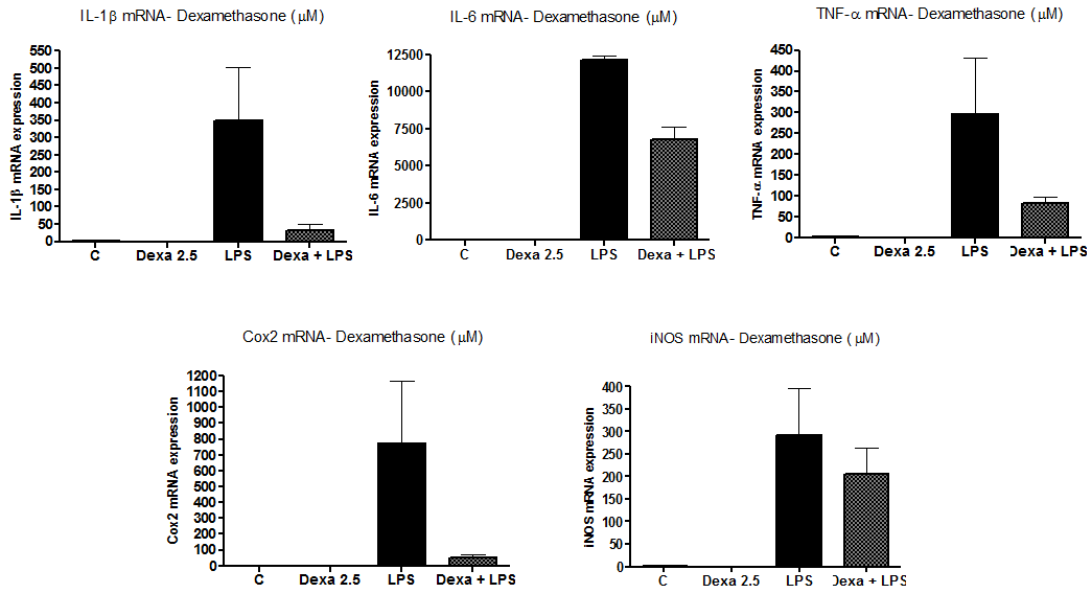

Figure 9. mRNA expression in microglial cells treated with dexamethasone (2.5μM). (n=2)

4.2 PAGE purified aptamers

DNA aptamers purified by PAGE were used in this section (Apt C and Apt M52). A lower range of concentrations (6.25, 12.5, 25, 50 and 100nM) was used than in the experiments performed with ultramers.

4.2.1 Viability

MTT assay and IP/Hoechst staining were also performed in mixed glial cell cultures treated with PAGE purified aptamers (data not shown). The results obtained did not indicate a decreased cell viability.

4.2.2 Morphological changes

Morphological changes were also distinguished in glial cultures treated with 100nM Apt C and Apt M52. Regarding mixed glial cell cultures, similar alterations as those seen using ultramers were observed: microglial cells located over the astroglial cell layer lose their refringence and their spherical shape resembling the alterations seen with L/I (Figure 10). Treatment with the Apt C produced the same effect than the observed in Apt M52 treatment (not shown). These morphological changes were not seen at 50nM.

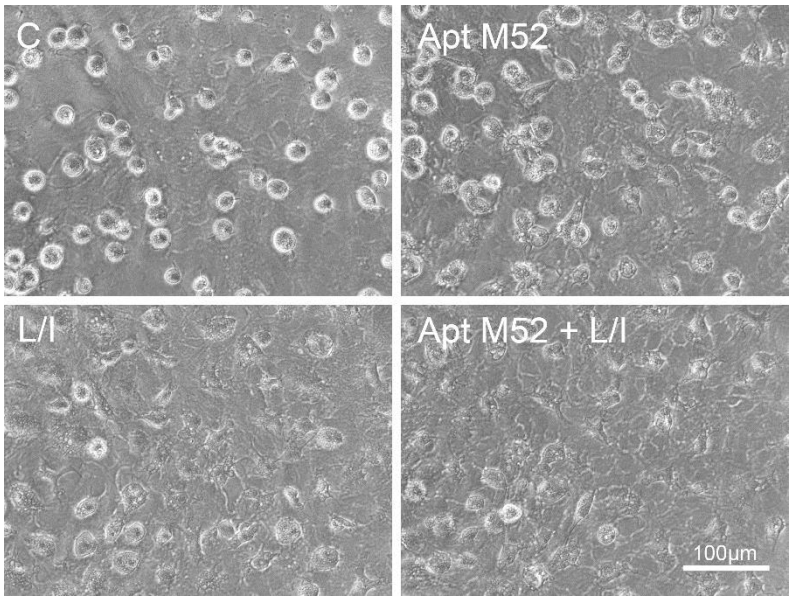

Figure 10. Morphological changes in mixed glial cell cultures. Phase contrast images. Cells were treated with 100nM Apt M52.

Microglial cell cultures treated with 100nM Apt C and Apt M52 also presented changes in morphology (Figure 11), although at 50nM these alterations could not be seen. When comparing with the control, it could be noticed that the aptamer treated cells presented a much globose morphology where it was more difficult to determine the contours of each cell. The administration of L/I also produced this morphological alteration.

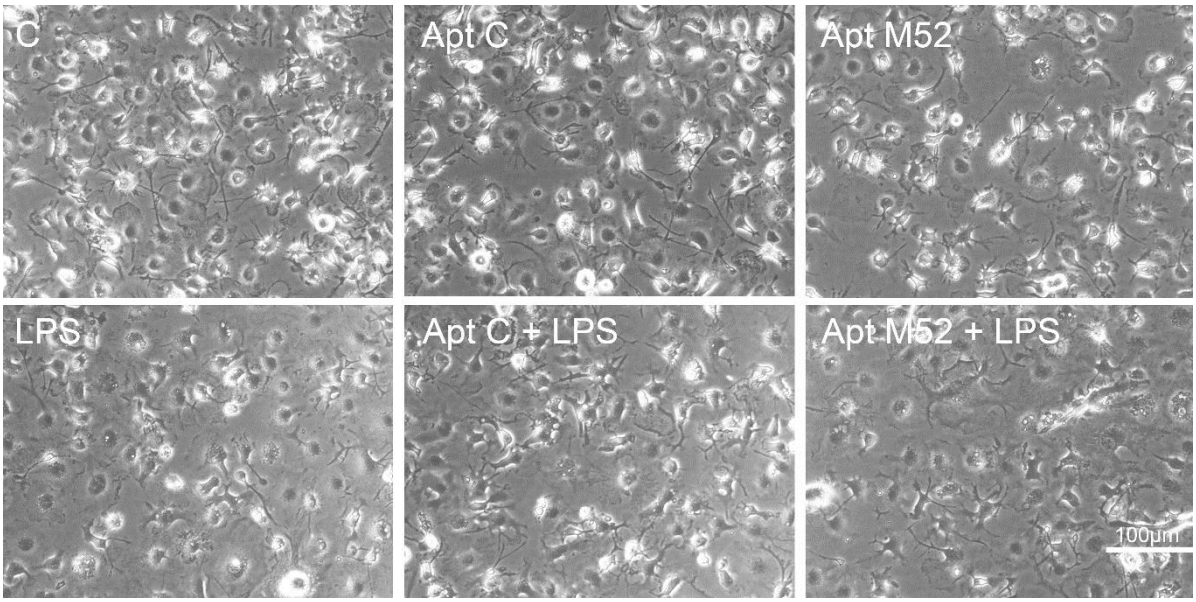

Figure 11. Morphological changes in a microglial cell culture treated with 100nM Apt C and Apt M52. Phase contrast images.

4.2.3 Nitrite production

NO production was also examined in mixed glial cultures treated with Apt C and Apt M52. (Figure 12). L/I induced an increase in NO production, as expected. NO production was not observed in glial cells treated with Apt C or Apt M52. In Apt C, L/I induced NO production was not modified. However, in Apt M52, L/I induced NO production was decreased in cultures pre-treated with 25 and 50nM Apt M52.

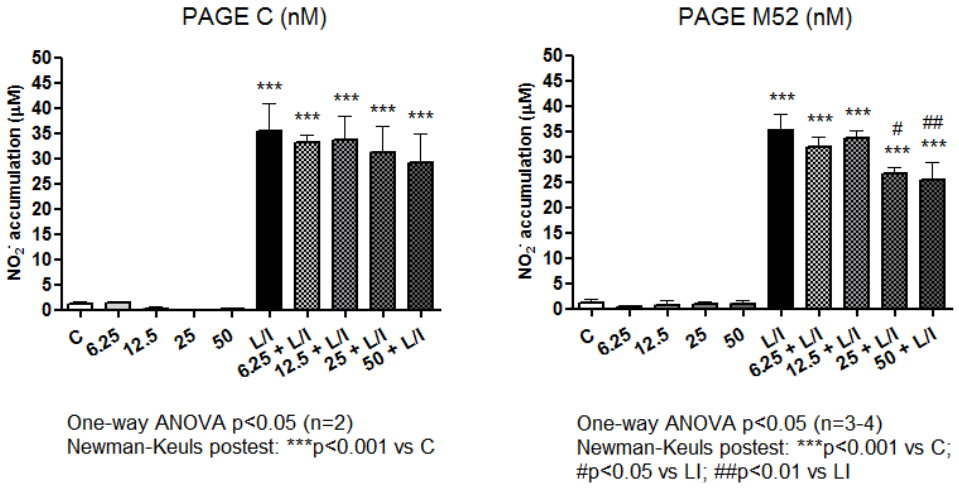

Figure 12. NO production for aptamer PAGE C and M52 in mixed glia cells.

4.2.4 Gene expression

IL-1β, IL-6, TNF-α, Cox2 and iNOS mRNA expression was determined in mixed glial cells treated with Apt C and Apt M52 (Figure 13). As expected, L/I induced an increase in the expression of all mRNAs tested. mRNA expression of the analyzed genes was not detected when cells were treated with aptamers. Both aptamers showed a trend to decrease mRNA expression of IL-1β, IL-6, TNF-α, Cox2 and iNOS induced by L/I.

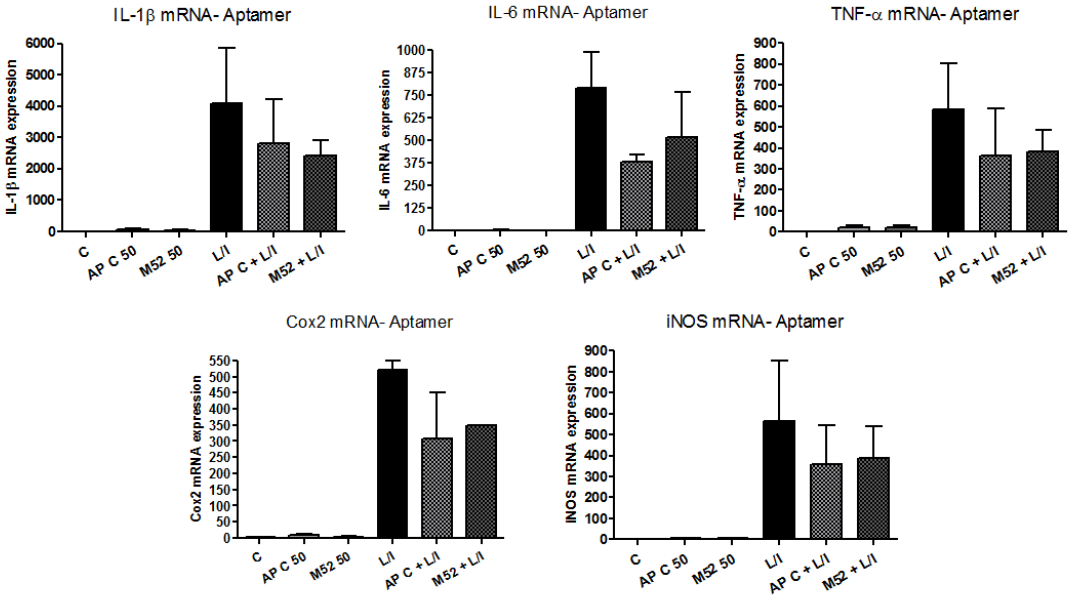

Figure 13. mRNA expression levels in mixed glial cells treated with 50nM aptamers PAGE. (n=2).

Gene expression of the same genes was also analyzed in mixed glial cultures treated with 2.5μM dexamethasone (Figure 14). A significant decrease in L/I induced IL-1β, TNF-α and Cox2 mRNA expression was observed when cells were pre-treated with dexamethasone. The same observation could be seen as a tendency in IL-6 mRNA. mRNA expression of the genes evaluated was not detected after dexamethasone treatment. In general, L/I induced increases in mRNA expression of IL-1β, TNF-α and Cox2 was significantly decreased by dexamethasone pre-treatment. Dexamethasone showed a trend to decrease IL-6 mRNA expression produced by L/I.

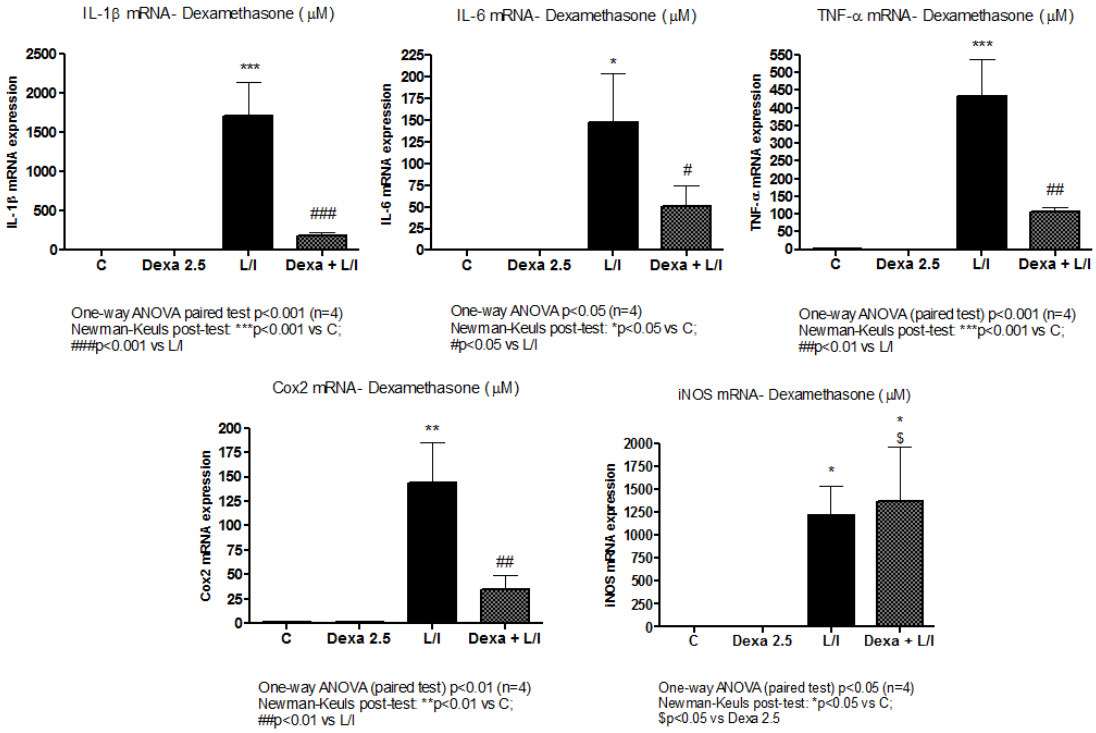

Figure 14. mRNA expression levels in mixed glial cells treated with 2.5μM dexamethasone.(n=4).

5. DISCUSSION

With this project, we studied the possible agonistic function of a DNA aptamer on CD200R1 using mouse primary mixed glial and microglial cell. To this end, we treated cells with aptamers in the absence and presence of pro-inflammatory stimulus and determined cell viability, morphological changes, nitrite production and changes in the expression of pro-inflammatory genes. As a positive control, we evaluated the anti-inflammatory properties of dexamethasone in the same cell cultures.

Prodeus et al. (2014) reported the development and characterization of DNA aptamers which bind to murine CD200R1 and act as CD200R1 agonists. Their study was not performed on glial cells, but on T lymphocytic cultures and murine skin allografts. They hypothesized that aptamers would mimic the functional (agonistic) properties of protein ligands such as CD200, in binding to CD200R1 and repressing immune cell response [24]. It is known that CD200 signaling pathway is implied in several immune-related and autoimmune disorders where it has been described that the expression of CD200 correlates with the suppression of immunity [28]. Their results indicated that several aptamers were capable of repressing immune cell responses since they suppressed cytotoxic T-lymphocyte induction at levels comparable to that of a soluble CD200Fc ligand [24]. CD200Fc, a CD200 fusion protein, is known to act as agonist of the CD200R1. An injection of CD200Fc has been established to decrease markers of microglial activation in rats [34].

When employing a new substance, one of the first aspects to bear in mind is the appropriate concentration to work with. The right dose would be the one that does not cause death or major alterations to the cell yet it produces some desired effect. In this study, the aptamer concentration was first established from the published article mentioned above where they used aptamers in a range of concentrations from 100nM to 900nM. Based on that, we decided to work with aptamers at 100, 200, 300 and 400nM.

We asked Integrated DNA Technologies to synthesize a control aptamer and an agonistic CD200R1 aptamer (M52) according to the sequences published by Prodeus et al. (2014). The synthesis of the aptamers could be obtained by different protocols. We first obtained ultramers which were purified by high performance liquid chromatography (HPLC).

First of all, cell viability was tested with the MTT assay which lies in the fact that MTT is reduced and it produces a quantifiable colorimetric solution. Even though this is one of the most widely approach used for assessing viability, it does not measure the number of alive cells, but rather the activity of some enzymes related to cell metabolism [29]. For this reason, there is the possibility of significant over or underestimation with this assay due to changes in the cell metabolism. However, we could compare the supposed death by the MTT assay with the actual cell death seen under the microscope with IP/Hoechst staining. Thanks to this, we were able to

determine that there was not an increased cell mortality following the treatment with aptamers, even if some statistically significant differences were found in the MTT assay. We believe that those differences were produced due to alterations in cell metabolism and do not correspond to a significant increment of mortality.

Despite of not seeing any variance on viability, we did observe morphological alterations in the first range of concentrations used (100-400nM). These changes resembled the ones characterized by LPS. Although morphological changes in microglia might not relate to the function undertaken by this cell, we were not expecting any variation in the Control aptamer because it consists of a random nucleotide sequence that does not bind to the receptor studied.

These morphological variations together with the trend to increase NO production seen in the ultramers, made us think that we were using excessively high concentrations. Therefore, nitrite production in ultramers was tested at a lower concentration range, from 6.25 to 100nM, but the tendency to rise was still seen. Moreover, results obtained for gene expression were inconclusive. We studied the expression of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) and pro-inflammatory enzymes (Cox2 and iNOS). Lower concentrations, 4 and 40nM of ultramer, were also tested for gene expression; a diminished expression of pro-inflammatory genes was neither seen. The stimulation of CD200R1 is known to result in the inhibition of the pro-inflammatory phenotype in microglial cells [30].

Overall, the exposure to ultramers C and M52 apparently produced an alteration on glial cells. Nonetheless, this response was probably not acting through the CD200-CD200R1 signaling pathway because the ultramer C is not supposed to bind the receptor. Since it was possible that there might be something interfering with the expected action of ultramers, we decided to use the same two aptamers sequence produced through a different synthesis process. These aptamers are PAGE purified aptamers.

The main difference between ultramers and PAGE purified aptamers lies on the processes of synthesis and purification. Ultramers are purified through HPLC which can separate according to hydrophobicity or charge difference. On the other hand, PAGE aptamers are purified with polyacrylamide gel electrophoresis (PAGE) which is a technique that uses a denaturing environment to separate oligonucleotides based on molecular weight. This last technique is subjected to a low yield. Despite increasing the purity, it substantially reduces the amount of final aptamer product. Consequently, with PAGE aptamers we could not work at the same concentrations as ultramers and 100nM was the highest concentration we used.

Firstly, cell viability after treatment with PAGE aptamers was also assessed and it was not found to be altered. Regarding nitrite production, the tendency to increase noticed in ultramers was no longer observed. Apt M52 at 25 and 50nM, but not Apt C generated a statistically significant decrease of the production of nitrites caused by L/I.

Concerning morphological changes, they were also seen with the treatment with Apt C and Apt M52 at 100nM, but not at 50nM. Therefore, 50nM was the chosen concentration to analyze gene expression. The results of mRNA levels did not clarify the activity undertaken by aptamers because it was appreciated a trend to decrease the levels of pro-inflammatory genes by aptamer C and M52.

Dexamethasone was used in this study as a positive control of the inhibition of inflammation. It is a corticosteroid with potent anti-inflammatory properties. It has been described that this substance can efficiently control LPS-induced neuroinflammation [31]. We could see that dexamethasone, at the tested concentrations, did not affect cell viability and was effective at reducing nitrite production caused by the pro-inflammatory stimulus L/I. It reduced the expression of the pro-inflammatory genes analyzed too.

It has been described that CD200-deficient mice respond more profoundly to LPS and other Toll-like receptor (TLR) agonists since microglia exhibits more activated characteristics [32]. In these animals, microglia takes a morphology typically associated with activation and generates a more rapid onset of symptoms in inflammatory disease [33]. We wanted to study the effect of M52 aptamer in CD200 knockout mice primary mixed glial cell cultures. The objective was to determine whether the aptamer could compensate the deficiency of CD200 ligand. However, due to aptamers lack of effect, this was not examined.

In the recent years, the possibility to promote microglia's neuroprotective phenotype has become a therapeutic goal for neurodegenerative diseases as it may help to retard their progression [8]. One promising way to achieve so, is by modulating the CD200-CD200R1 ligand-receptor system. As already mentioned, CD200Fc can bind CD200R1 and act as agonist. This fusion protein can activate the CD200-CD200R1 signaling pathway by phosphorylating the adaptor molecule Dok1/2 [35]. Withal, the usage of this CD200Fc agonist remains limited by reason of its big size and elevated price. Thus, the selection of aptamers, which are small and very easily produced, seemed a good alternative. We also tried to detect the phosphorylation of Dok1/2 by using Western blot so we could know whether the M52 aptamer was actually binding to the expected receptor and initiating signal transduction, but we had some problems with the antibody and work is still in progress in regards of this point.

Taking all in consideration, aptamers did not have an agonistic effect in glial cells as it was previously described in lymphocytic cultures [24]. However, aptamers have been proposed to be ideal candidates for diagnostic and therapeutic applications considering that they are cheap, non-immunogenic and easy to modify. Still, aptamers have not been commonly used thus far. In fact, there is only one approved aptamer, Macugen (or Pegaptanib) for therapeutic application [36].

6. CONCLUSIONS

The conclusions obtained from this study were the following:

1. The viability of the glial cell cultures was not affected by the treatment with aptamers.
2. The treatment with concentrations of ultramers and PAGE purified aptamers above 100nM, either the control aptamer or the putative CD200R1 agonist, produced morphological changes in microglial cells in glial cultures.
3. Mixed glial cell cultures treated with 25 and 50nM M52 PAGE purified aptamer, but not with the control aptamer, presented a significant decrease on L/I induced NO production.
4. A decrease on the L/I induction of the expression of pro-inflammatory could not be seen by the treatment with ultramers. In the case of PAGE purified aptamers, some inhibitory effect was observed by both Apt C and Apt M52.
5. The treatment with dexamethasone significantly reduced the L/I induced glial activation in primary mouse glial cell cultures.

Consequently, the immunosuppressive properties of the M52 CD200R1 agonistic aptamer reported by Prodeus et al. (2014) could not be corroborated in glial cell cultures. Further studies are needed to understand the role of aptamers in glial cells.

7. REFERENCES

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8. SUPPLEMENTARY MATERIAL

Supplementary Table S1. Nucleotide sequence for aptamer C and M52.

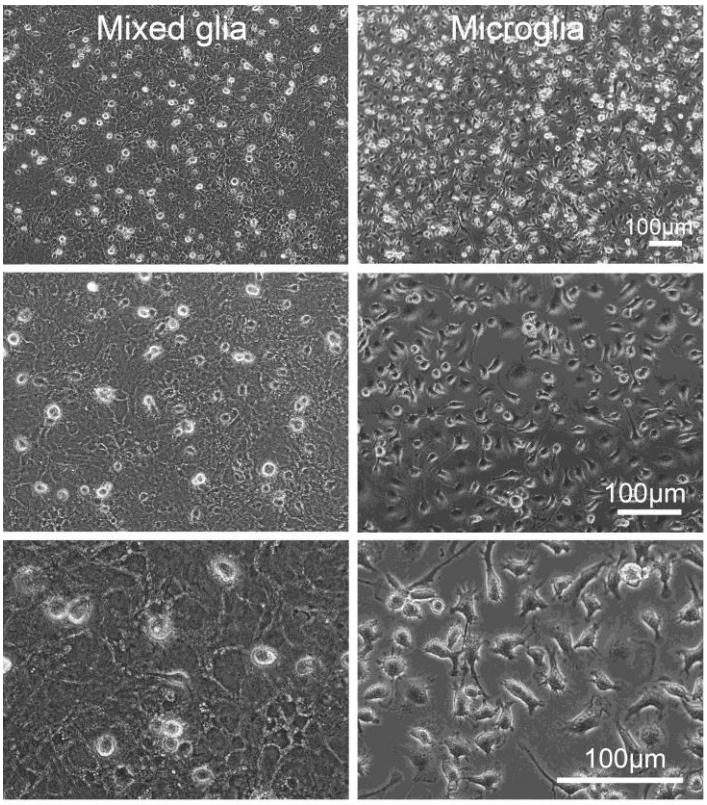
	GACGATAGCGGTGACGGCACAGACG-$N_{(25)}$-CGTATGCCGCTTCCGTCCGTCGCTC
APT C	TCCCGCATCCTCCGCCGTGCCGACC
M52	TTTATTACCATTATGCCTATGTAA

Supplementary Table S2. Antibodies used for immunofluorescency.

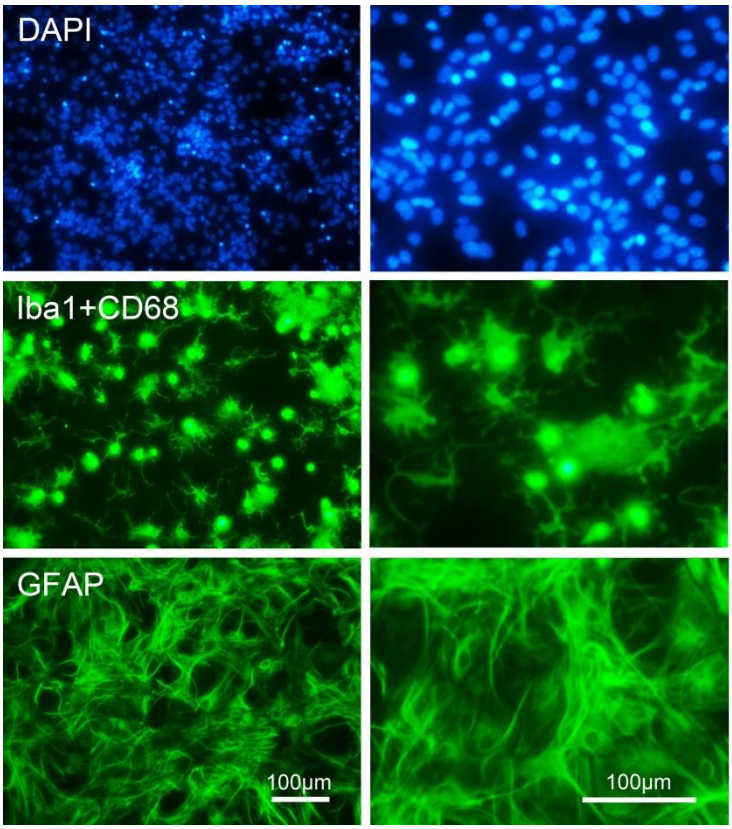
Primary antibody	Specie	Company	Dilution	Secondary antibody	Company	Dilution
GFAP	Mouse	Sigma-Aldrich	1/2000	Alexa 488 donkey anti-mouse	Invitrogen	1/1000
IBA-1	Rabbit	WAKO	1/500	Alexa 488 donkey anti-rabbit	Invitrogen	1/1000
CD68	Rat	AbD Serotec	1/1000	Alexa 488 donkey anti-rat	Invitrogen	1/1000

Supplementary Table S3. Primers of the genes analyzed by qRT-PCR.

Target mRNA	Accession Number	Forward primer (5'→3')	Reverse primer (5'→3')
COX2	NM_011198.4	TGCAGAATTGAAAGCCCTCT	CCCCAAAGATAGCATCTGGA
IL-1β	NM_008361.4	TGGTGTGTGACGTTCCCATTA	CAGCACGAGGCTTTTTTGTTG
IL-6	NM_031168.2	CCAGTTTGGTAGCATCCATC	CCGGAGAGGAGACTTCACAG
iNOS	NM_010927.3	GGCAGCCTGTGAGACCTTTG	GGCAGCCTGTGAGACCTTTG
TNF-α	NM_013693.3	TGATCCGCGACGTGGAA	ACCGCCTGGAGTTCTGGAA
β-actin	NM_007393.5	CAACGAGCGGTTCCGATG	GCCACAGGATTCCATACCCA
Rn18s	NR_003278.3_	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

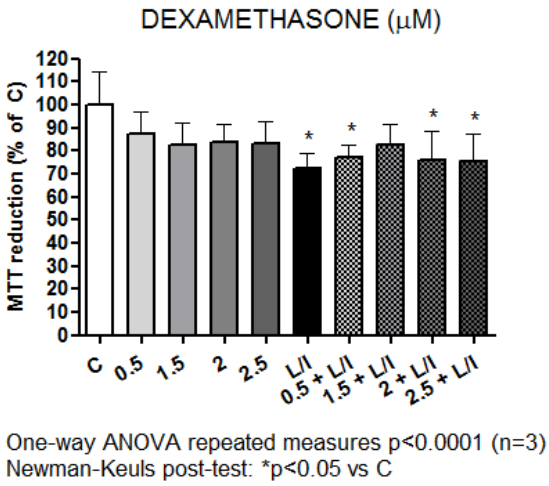


Supplementary Figure S1. Phase-contrast images of mixed glial cell culture (left) and microglial cell culture (right).



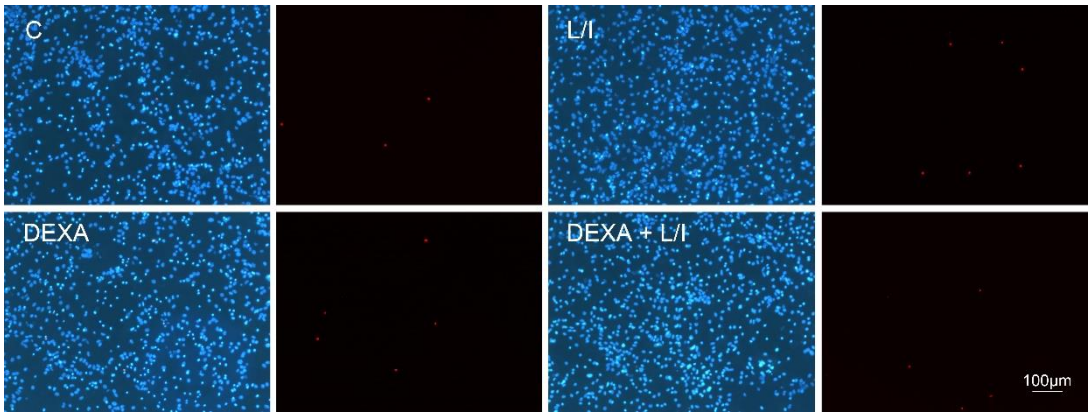
Supplementary Figure S2. DAPI (nuclei), Iba1+CD68 (microglia) and GFAP (astrocytes) immunostaining in mixed glial cell cultures.

A

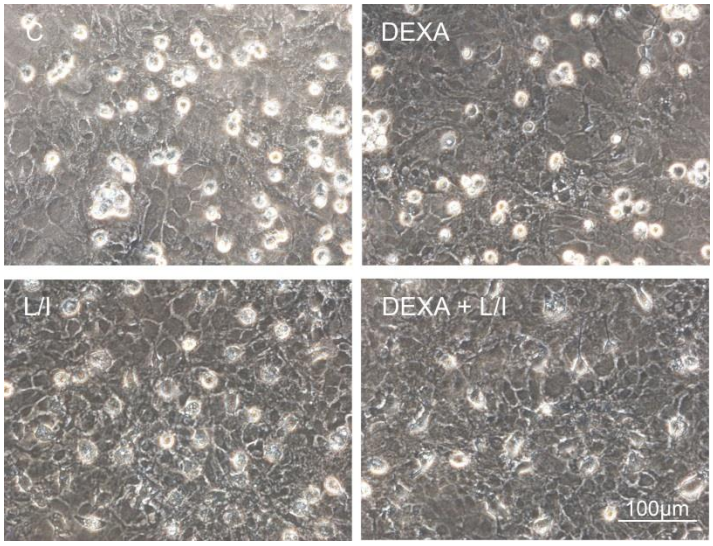


One-way ANOVA repeated measures p<0.0001 (n=3)
Newman-Keuls post-test: *p<0.05 vs C

B



C



Supplementary Figure S3. A. Effect of dexamethasone on MTT reduction in mixed glial cell cultures. **B.** Microscope images of IP/Hoechst staining of dexamethasone treated in a mixed glial cell culture. Lefts images correspond to Hoechst staining (blue) whereas right images correspond to PI staining (red). **C.** Morphological changes in a mixed glial cell culture treated with 2.5μM dexamethasone. Phase contrast images.