Investigation of immunomodulatory properties of anti-secretory factor in experimental brain tumor models

Undersökning av immunmodulerande egenskaper hos antisekretorisk faktor i experimentella hjärntumörmodeller

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Abstract

Introduction: Glioblastoma is the most aggressive form of primary brain tumor in adults. Current treatment including chemotherapy poses a limitation due to consequences of systemic toxicity. Further studies for alternative, multimodal treatments are necessary. Antisecretory factor is an endogenous protein. Studies have shown that it has anti-inflammatory and immune modulatory effects in experimental models. The active peptide of antisecretory factor, AF-16, modulates cytokine and chemokine secretion from myeloid and tumor cells in a manner that affects immune suppression, immune cell recruitment and treatment efficacy.

Aims: To investigate the immunomodulatory effect of AF-16 in *in vitro* cell cultures of glioblastoma cells and myeloid immune cells (monocytes and macrophages). To investigate AF-16 mediated treatment efficacy in *in vivo* glioma mouse models.

Methods: Cell cultures (human monocytes, macrophages and glioblastoma cells) treated with different doses of AF-16 were analyzed using different protein platforms (Olink Proteomics, Mesoscale Discovery). 3-day mini-osmotic pumps (Alzet®) were used to deliver AF-16 to tumor bearing mice and survival was assessed between treated and non-treated mice. Immune cell populations in tumors from treated and non-treated mice were analyzed by immunohistochemistry.

Results: AF-16 affected the expression of several inflammatory proteins. 2/8 mice survived in the AF-16 treated group compared to 0/8 in the non-treated group. However, the median survival time was equal between groups therefore data was not statistically significant. Immunohistochemical images indicated modest modification in macrophage concentration at the tumor site in treated mice, however no quantitative analysis was performed.

Conclusion: AF-16 affects expression of several inflammatory proteins in immune- and glioblastoma cells. AF-16 treatment has the potential to cure animals with brain tumors and modulates the distribution of macrophages at the tumor site. The exact therapeutic benefit and mechanism of action remains to be elucidated but we provide indications that it involves the myeloid branch of the immune system of the host.

Populärvetenskaplig sammanfattning

Glioblastom är den vanligaste och svåraste formen av hjärntumörer hos vuxna. De tillhör gruppen gliom som utgår från gliacellerna i centrala nervsystemet och utgör 80% av all gliom. Välkända riskfaktorer inkluderar exponering för joniserande strålning och vissa genetiska syndrom såsom Li-Fraumeni syndrom och Neurofibromatos typ 1. Medelåldern för insjuknandet är 59 år och trots behandling med kirurgi, kemoterapi och strålning är prognosen oerhört dålig. Behandling med kemoterapi är begränsat då starka doser medför systemisk toxicitet och trots detta botas få patienter. Dessutom begränsar blodhjärnbarriären upptaget av cellgifter i hjärnan. Forskning kring nya behandlingsmetoder är ständigt aktuellt. Behandlingar med mindre biverkningar är t.ex. olika strategier som utnyttjar patientens eget immunförsvar för att bekämpa tumören.

Antisekretoriskt faktor (AF) är ett kroppseget protein som tidigare studerats i olika sammanhang. AF-16, den aktiva peptidsekvensen i antisekretorisk faktor, har egenskapen att kunna reglera den osmotiska balansen i tarmarna, vilket har utnyttjats för att bota diarré hos barn. Man har även sett att AF-16 har en trycksänkande effekt i hjärnan, vilket har använts i behandling mot hjärnskador för att effektivt få ner den skadliga tryckökningen som sker. Denna trycksänkande egenskapen har varit till fördel hos patienter med glioblastom då växande tumörceller orsakar högt intratumoralt tryck som skadar omgivande vävnad och begränsar distribution av läkemedel i tumören. Med hjälp av AF-16 har man alltså kunnat sänka det intratumorala trycket och samtidigt främja upptaget av cytostatika lokalt i tumören, som i sin tur medfört mindre cellgiftsdoser med färre biverkningar.

Då glioblastom, tillsammans med olika myeloida celler såsom makrofager bidrar till en immunosuppressiv miljö ville vi undersöka om AF-16 kunde påverka proteiner som hämmar ett aktivt anti-tumoralt immunförsvar. Detta för att missgynna tumörcellerna i deras tillväxt och utbredning. Vi ville även undersöka hur AF-16 påverkar överlevnaden hos möss med hjärntumörer. I vår studie fann vi att AF-16 påverkade utsöndringen av flertalet inflammatoriska cytokiner både i immunceller och tumörceller. Vi studerade även en grupp glioblastom-bärande möss för att jämföra överlevnaden hos AF-16 behandlade och obehandlade möss. Vi fann att behandlingen främjar överlevnad till viss mån men fler studier är nödvändiga för att kunna dra en signifikant slutsats.

Abbreviations

GBM	Glioblastoma
BBB	Blood brain barrier
CED	Convection-enhanced delivery
AF	Antisecretory factor
CNS	Central nervous system
SPC	Specially Processed Cereals
NKCC	Na/K/Cl-cotransporter
IFP	Interstitial fluid pressure
AF-16	The active peptide sequence of AF
COX-2	Cyclooxygenase 2
THP-1	Human monocytic cell line
RAW	Mouse macrophages
M0	Human macrophages
NPX	Normalized Protein eXpression
LOD	Lowest limit of Detection
LLOQ	Lowest Limit of Quantification
ULOQ	Upper limit of Quantification
GL261	Mouse glioma cell line
VEGF	Vascular endothelial growth factor
CXCL10	Interferon gamma-induced protein 10 (IP-10)
MCP-1	Monocyte chemoattractant protein-1
MIP	Macrophage Inflammatory Protein
TNF	Tumor Necrosis Factor
OPG	Osteoprotegerin
PGE2	Prostaglandin E2

Introduction

Glioblastoma is the most common and aggressive form of primary brain tumor in adults. Glioblastoma is a form of glioma, a group of malignant tumors that are derived from glial cells in the central nervous system. Gliomas comprise about 30% of all brain tumors and include astrocytomas, oligodendromas and ependymomas (1). Glioblastoma is the most malignant form of astrocytoma and accounts for approximately 80% of the gliomas. Although primary glioblastoma can occur at any age, the median age for diagnosis is around 59 years and the incidence are slightly higher in males than in females (1.58:1). Despite current treatment combination of surgery, chemotherapy and radiotherapy, it has a significant mortality with a median survival of 7,5 months and 2,0% of 5-years all survival (1; 2).

Current treatment including systemic chemotherapy poses a limitation due to the consequences of systemic toxicity. Limitations of blood brain barrier (BBB) permeability require higher doses of chemotherapeutic drugs to achieve the desired distribution in the tumor. This reasons for studies for alternative, multimodal treatments. Recent studies have indicated that intratumoral administration of chemotherapeutic drugs (for example convection-enhanced delivery, CED) leads to a prolonged survival in mouse brain tumor models (3). CED-administration bypasses the BBB which allows a higher concentration of the chemotherapeutic drug within the tumor, which in turn reduces the systemic side effects (4).

Immunotherapy that take advantage of the patient's own immune system is a therapeutic intervention with less side effect, that could be utilized against glioblastomas. Antisecretory factor (AF) is a 41 kDa protein that is found in most mammals including humans. It was first discovered in the 1980's by Stefan Lange and Ivar Lönnroth when studying experimental diarrhea caused by cholera toxin in mice and rats, where AF was shown to be a potent inhibitor of intestinal fluid secretion (5). Earlier studies have shown that AF is expressed by macrophages, B-cells and dendritic cells in lymphoid organs including thymus, spleen, gut-associated lymphoid tissue and in the perivascular area in the central nervous system (CNS). This discovery has suggested that AF might have a role in regulating the immune system (6).

Earlier studies have shown that AF has an antisecretory and anti-inflammatory effect. For therapeutic studies, AF has been concentrated in the form of an egg yolk extract (Salovum) and specially processed cereals (SPC) which can be administrated orally (7). As AF is found naturally in the human body, it is not classified as a drug but as a nutrient with medical benefits. Salovum has previously been used to treat diarrhea among children in endemic areas.

Functioning as an inhibitor of NKCC-channels in the intestines it thereby regulates the osmotic balance (8). In addition, Salovum is now tested in Phase 1 Clinical Trial in the treatment of patients with GBM (personal communication, Anna Darabi).

Moreover, the NKCC-channel has been shown to be overexpressed in glioblastomas where it contributes to tumor aggressiveness. NKCC count increases with glioma grade and has a role in invasion and survival of tumor cells where it facilitates osmotic adaption (9). Earlier studies have shown that the active peptide sequence of antisecretory factor, AF-16, functions as a NKCC-blocker (10). Hence, isolated AF-16 has antitumor features in glioblastoma models in mice where it reduces the interstitial fluid pressure, IFP, as well as promotes chemotherapeutic drug uptake which prolonged the survival of the animals (9). Furthermore, AF-16 is confirmed to have an immunomodulatory effect (11).

Within the tumor microenvironment, glioblastoma cells are known to reduce co-stimulatory molecules and secrete a variety of factors that help suppress the immune system (12). Thus, intratumoral immunosuppression is a key factor that accounts for the overall poor prognosis in patients with GBM (13). It is known that AF-16 affects the secretion of soluble factors, which in turn can modify the cellular response in different tissues (14). Cytokines have a broad range of functions including functioning as chemoattractants, activating immune cells and regulating immune responses. The finding that AF-16 affect cytokine expression might contribute to a new approach towards immunotherapy in glioblastomas.

Through modulation of inflammation, it is possible to inhibit cyclooxygenase 2 (COX-2), which in turn decreases the production of inflammatory prostaglandins (15). Prostaglandins inhibit attraction of various proinflammatory cells and modulate chemokine production (16). Prostaglandins are a part of the CNS immune-suppressive network utilized by microglia and neural stem cells under physiologic and pathologic conditions such as trauma, autoimmunity and neoplasia (17). Prostaglandins are produced in the tumor tissue itself which contributes to its immunosuppressed microenvironment (18). With this knowledge it is presumable that AF-16, if able to inhibit COX-2, could induce an immune reaction that help combat the tumor. Attracting immune cells to the site, along with changes in immune modulatory factors such as COX-2, could have a positive effect in glioblastoma treatment.

Aims and scientific questions

The aim of this study is to investigate the immunomodulatory effect of AF in *in vitro* cell cultures of glioblastoma cells and immune cells and also *in vivo* glioma mouse models. Our questions at issue are:

- Which immune-related proteins are altered when myeloid and tumor cells are treated with different doses of antisecretory factor?
- Is there a difference in the composition of immune cells in glioma mouse models that have been treated with antisecretory factor?
- Does AF-16 treatment have an effect on survival in tumor-bearing mice?

Glioblastoma has a serious impact on patients' quality of life and indirectly, the society. Current treatments have significant side effects and do not primarily take advantage of the patient's own immune system. With this approach we hope to contribute to a better understanding on how AF-16 helps modify the immune system and how it affects the interaction between immune cells and tumor cells. Studies like this have not yet been performed. Further experiments will hopefully contribute to a change in treatment protocol with less side effects.

Methods

Experimental design

This study is part of a larger experimental study performed at Glioma Immunotherapy group at Lund University, Sweden. Due to the current COVID-19 situation the material of this study is composed of the research group's already collected data. All parts were analyzed, composed and approved by both authors.

To study immune modulation of AF-16 *in vitro*, we will expose monocytes/macrophages and human glioblastoma cells to different concentrations of AF-16. We will characterize the changes in inflammatory factors by analyzing supernatants from untreated and treated cells on multi-array protein platforms (Olink Proteomics, Mesoscale Discovery) covering >100 proteins.

To confirm if AF-16 also has immunomodulatory effects *in vivo*, mice with induced orthotopic brain tumors (GL261) will be treated with AF-16. AF-16 will be delivered intratumorally via mini osmotic pumps. To investigate the immunomodulatory functions of AF-16, the composition of immune cells, and released factors will be analyzed in brain tumor sections at

protein level using immunohistochemistry. We reviewed images from previously stained samples.

Cell line and cell culture medium

The human monocytic cell line (THP-1), and the mouse macrophage cell line (RAW) were a kind gift from Prof. Håkan Ericsson, Department of Biomedical Laboratory Science, Faculty of Health and Society, Malmö University, SE-205 06 Malmö, Sweden. The human glioblastoma cell line (GBM) was established as previously described (19).

The cells were incubated for growth at 37°C, 21% O_2 and 5% CO_2. The used culture medium was RPMI 1640 supplemented with 2 mM L-Glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 50 µg/ml gentamicin (Invitrogen AB, Sweden) and 10% FBS (Fetal Bovine Serum, Biochrom AB, Berlin, Germany) (R10). Cells were passaged regularly to avoid overgrowth. Adherent GBM-cells and RAW-macrophages were passaged by first being rinsed in phosphate buffered saline/PBS without Ca^{2+} and Mg^{2+} (GIBCO® Life Technologies) followed by rinse out eventual trypsin neutralizers and detached with Trypsin-EDTA (GIBCO®, Life Technologies) at incubation at 37°C for 5-10 minutes. After confirming that the cells had detached, the passaged cells were centrifuged and resuspended in 2/3 fresh medium and 1/3 conditioned medium. Non-adherent THP-1 cells were passaged every 3-4 day by resuspending 1/3 of a confluent culture in 2/3 fresh cell culture medium.

THP-1-cells (100.000 cells/ml) were seeded in 24-well plates and treated with different concentrations of AF-16 (0,02 mg/ml, 0,2 mg/ml or 2 mg/ml) for 24 h (AF-16 was a kind gift from professor Stefan Lange, Gothenburg University, Sweden). Maturation of THP-1 cells into macrophages (M0) were performed by treating 100.000 THP-1 cells with 100 µM PMA for 72 hours. PMA (phorbol 12-myristate 13-acetate) is the most common phorbol ester and is a potent activator of Protein kinase C (PKC) and therefore activates NF-kB, a transcription factor that controls the transcription of several genes related to cell survival and immune function. PMA containing medium was discarded and replaced with fresh R10 medium for 3-4 hour after which M0 cells were treated with different concentrations of AF-16 (0,02 mg/ml, 0,2 mg/ml or 2 mg/ml) for 24 h. The supernatants were collected and kept at -80°C until analyzed.

Protein profiling using the Olink assay

Human monocytes (THP-1) and glioblastoma cells (GBM) were analyzed for inflammatory factors by in situ proximity extension assay (PEA, Olink Biosciences, Uppsala, Sweden) according to the manufacturer's recommendations. Olink offers protein profiling of 92 proteins

using only 1μL of for example cell culture supernatant. By using pairs of oligonucleotide-labeled antibodies ("probes") that bind pairwise to the target protein present in the sample in a homogeneous assay, the risk of cross reactions is limited. When the two probes are in close proximity hybridization occurs and DNA-polymerization forms a new PCR sequence that is quantified. If cross reaction occurs, this will not be detected with Olink's panels since only matched probes will we amplified. This allows for scalable multiplexing without loss of specificity and sensitivity, which is a common problem in many multiplex immunoassays. NPX (Normalized Protein eXpression) is the unit used to present the collected values. NPX is in Log2 scale which reflects a doubling of the protein concentration for each unit increase.

Cytokine profiling using MesoScale V-PLEX®

Supernatants from human cells (THP-1, M0, GBM) were analyzed in duplicates with MesoScale V-PLEX® (Meso Scale Discovery, Rockville, MD, USA) Proinflammatory Panel 1 (IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-α), Cytokine Panel 1 (GM-CSF, IL-1α, IL-5, IL-7, IL-12/IL-23p40, IL-15, IL-16, IL-17A, TNF-β, VEGFA) and Chemokine Panel 1 (Eotaxin, Eotaxin-3, IL-8, IL-8 (HA), IP-10, MCP-1, MCP-4, MDC, MIP-1α, MIP-1β, TARC) according to the manufacturer's instructions. Supernatants from mouse cells (RAW) were analyzed in duplicates with MesoScale V-PLEX® Proinflammatory panel 1 (IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70, and TNF-α) according to the manufacturer's instructions. Due to low number of replicates, no statistical calculations were made. When analyzing data LOD and LLOQ were used as detection limits. LOD (limit of detection) refers to the lowest quantity of an analyte that can be distinguished from a blank sample. LLOQ (lowest limit of quantification) describes the lowest value of quantification that is possible to use in statistic calculations. Values between LLOQ and ULOQ (upper limit of quantification) provide analytes that can be accurately quantified.

MesoScale offers high sensitivity detection of cytokines using a small volume sample. The technique involves a plate integrated with underlying electrodes that is pre-coated with captured antibodies for specific cytokines. Thereafter, the sample is added followed by secondary detection antibodies that are conjugated with electro-chemiluminescent labels (MSD SULFO-TAG™). Voltage is applied to the electrodes which causes the captured labels on the secondary antibodies to emit light proportional to the amount of analyte present in the sample.

Brain tumor model

Mice were housed at the BMC Conventional Animal Facility at BMC, Lund University, Sweden. The GL261 mouse glioma cell line, used for brain tumor inoculation, was a kind gift from Dr. G Safrany ”Frédéric Joliot-Curie”, NHHR, Hungary. In brief brain tumors were induced in mice on day 0 by inoculation of 5x103/5µl GL261 tumor cells into the right frontal lobe. Mice were anaesthetized with 2.5% Isoflurane-Forene (Abbot Scandinavia AB) and then fixed and immobilized in stereotactic frame (Kopf Instrument) for the procedure. A medial sagittal skin incision was performed and a small hole was drilled into the skull approximately 1.5mm to the right and 1.0mm anterior of the bregma. A Hamilton syringe was used to inject the suspension of GL261 cells slowly over the course of 5 minutes. The skin was later sealed with a metal clip. In total of 16 mice were utilized, whereof 8 mice were treated with AF-16 (300µg/72µl) and 8 mice were kept as a control group without any treatment.

Preparation of mini-osmotic pumps

On day 7 after tumor inoculation, the previous skin incision was reopened and 3-day mini-osmotic pumps Alzet® model 1003D were used for treatment with AF-16. The pump assembly filled with AF-16 was implanted into a subcutaneous pocket in the midscapular area and was later removed within 4-5 days after implantation, according to manufacturer’s instruction. The mice were monitored and sacrificed when showing neurological symptoms of tumor growth and the brains were harvested for cryopreservation. The Kaplan-Meier curve was plotted to compare survival of AF-16 treated mice (n=8) and non-treated mice (n=8) followed by a log rank test.

Cryopreserving and Immunohistochemistry

Brains were collected and snap-frozen in isopentane (VWR International AB), cooled to -55°C using dry ice and stored at -80°C until sectioning. Brains were sectioned into 6 µm-thick sections using a cryostat (Leica, Germany) and mounted on Super frost glass slides. After this procedure they were stored at –80°C. The sections were thawed and fixed in 4% paraformaldehyde. PBS (GIBCO-Life technologies) were used to re-hydrate and wash the sections and 5% goat-serum were applied as blockage for 20 minutes to prevent off-target secondary binding.

Sections were incubated with rabbit anti-COX-2 (rabbit anti-mouse 5µg/ml, Abcam, Cambridge, UK) for 60 minutes, followed by incubation with the secondary antibody

conjugated to Alexa 594 (goat anti-rabbit 5µg/ml, Molecular Probes, Life Technologies, Stockholm, Sweden) for 30 minutes. Double labeling was performed by adding F4/80^{+} (rat anti-mouse 5µg/ml, AbD Serotec, Düsseldorf, Germany), followed by a secondary antibody conjugated to Alexa 488 (goat anti-rat 5µg/ml, Molecular probes). Sections were washed from unbound antibodies with PBS between steps to prevent them from unspecific binding and false-positive staining.

Pro-long Gold anti-fading reagent containing DAPI was added as nuclear stain (Molecular probes). Images were taken using a fluorescent microscope (BX-53, Olympus LRI instrument AB). To cover a larger area of the section, several images were merged using Multi image alignment (cellSens Dimension software, Olympus LRI instrument AB, Lund, Sweden).

Ethical considerations

Animal experiments have been approved by the regional ethics committee for animal research (Ethical permit M151/15). Experiments using patient tissue for cell culturing were approved by the Local Ethical Board of the University of Lund, Sweden (LU307-98). For the purpose of reducing the number of experimental animals to a minimum, sample size and power calculations were performed in advance to assure that valid results could be obtained. To prevent further suffering, animals were sacrificed when showing symptoms based on predetermined protocols. We analyzed survival data post-hoc, we did not handle living animals.

Results

Protein profiling using the Olink assay

To study the immunomodulatory role of AF-16 *in vitro*, human THP-1 and GBM-cells were exposed to different concentrations of AF-16. Cell supernatants were analyzed using the Olink platform for changes in the secretion of immune system related proteins. Olink Proteomics provide data from over 92 proteins. However, we chose to focus on only some of these, as shown in Figure 1 and 2.

In THP-1-cells, levels of selected proteins (VEGF, IL-8, MCP-1 and MIP-1α) increased after treatment with the lowest and middle dose of AF-16 (0,02 mg/ml and 0,2 mg/ml) (Fig. 1). When increasing the dose further (2 mg/ml) expression of VEGF, IL-8, MCP-1 and MIP-1α decreased. Expression of MIP-1β and CXCL10 increased with every increasing dose. Values are expressed in NPX where each increasing unit reflects a doubling of the protein

concentration, as earlier described. Expression of IL-8 showed the most pronounced difference between untreated THP-1-cells and the intermediate dose with an eight-fold increase compared to untreated levels. On the contrary, VEGF expression showed the least change after treatment, with approximately a one-fold increase.

The same factors as described for THP-1-cells were selected in GBM-cells with the addition of OPG (Fig. 2). In brief, protein expression showed an increase in VEGF, IL-8, MIP-1α and MIP-1β when comparing untreated cells with the highest dose (2 mg/ml). In contrary, the expression of OPG and MCP-1 decreased two-fold and four-fold overall, respectively when GBM-cells were treated with AF-16 compared with untreated values. CXCL10 secretion decreased when GBM-cells were treated with the lowest dose (0,02 mg/ml) but later increased when treated with the intermediate and highest dose of AF-16.

Protein profiling using Mesoscale V-PLEX®

Changes in expression of immune system related factors in human THP-1, human M0 and RAW-macrophages were further investigated using Mesoscale Discovery assay. Figure 3 shows differences in the expression of IL-8, VEGF and MCP-1 between untreated and treated (0,2 mg/ml AF-16) THP-1 cells. Fig. 4 shows difference in the expression of VEGF, MCP-1, MIP-1α, MIP-1β, IL-8HA, CXCL10 and TNF-α between untreated and treated (0,2 mg/ml AF-16) M0-cells. AF-16 induced a distinct increase of IL-8 concentration in both THP-1 and M0-cells. Expression of MIP-1α, MIP-1β and TNF-α also increased extensively in M0-cells when treated with AF-16. The MIP-1α value when M0 were treated with 0,2 mg/ml AF-16 (921,04 pg/ml) was modified to the value of ULOQ (743 pg/ml) as it exceeded the limit. Only expression of CXCL10 decreased when treated with AF-16.

In RAW-macrophages, only TNF-α showed a remarkable difference in expression when cells were treated with different concentrations of AF-16 (Fig. 5). The most distinct increase was detected when cells were treated with the highest dose (2 mg/ml AF-16) where expression reached 4075,71 pg/ml. This is a 24-fold increase in concentration relative to the untreated RAW-macrophages which expressed 168,11 pg/ml of TNF-α.

Immunohistochemistry

Frozen brain sections from AF-16 treated and untreated mice harboring GL261 gliomas were stained for $F4/80^+$ monocytes/macrophages (green) and COX-2 producing cells (red). $F4/80^+$/COX-2 double labeled cells (yellow), indicative of immune suppressive macrophages,

were present despite treatment. No significant change in macrophage count could be detected between the AF-16 treated and untreated brain tumors. However, an altered distribution of macrophages in the respective tumors were noted (Fig. 6). Comparing immunohistochemical images (Fig. 6A and 6C), there is a moderate indication of higher macrophage-concentration in the tumor site in AF-16 treated mice than in the untreated mice. No change in COX-2 producing cell-count could either be observed. No statistical quantitative analysis was performed.

Treatment with AF-16 in the GL261 glioma mouse model

Survival between AF-16 treated mice and non-treated mice was compared. The last mouse from the non-treated group was sacrificed due to tumor symptoms at day 52. Two mice in the AF-16 treated group were still alive at the time of monitoring closure (day 160). Median survival in both the AF-16 treated group and the non-treated group was 40,5 days. Mean survival in the non-treated group was 38,3 days, considerably shorter compared to the 64,5 days in the AF-16 treated group. Log rank test showed no significance in median survival time (*p=0,5401*).

Discussion

Today's treatment protocols for glioblastoma require improvement and modulation in order to achieve cure of glioblastoma patients. In this study, we hereby propose an area of interest in further research for an alternative multimodal treatment. The effect of AF-16 in glioblastoma has not yet been completely established. AF is a protein that has been used to influence the osmotic balance in the intestines through NKCC-channels (8). It has also been shown to decrease IFP in the brain, operating through the same channel, and enhancing chemotherapeutic drug uptake (9). Since brain tumors thrive in an immunosuppressive environment with high pressure (12), we asked whether AF-16 has a role in regulating immunological inflammatory factors expressed by various cell types at the tumor site. Further investigation of how immunomodulation with AF-16 influences the tumor microenvironment, as well as survival of glioblastoma-exposed individuals, is important in order to understand the pre-requisites of clinical implementation.

Data analysis using Olink and Mesoscale indicates notable changes in cytokine and chemokine expression in supernatants from human monocytes (THP-1), macrophages (M0) and glioblastoma cells (GBM) after treatment with AF-16. We chose to focus on only a few of the most outstanding values. In THP-1-cells we saw a notable change in expression of IL-8 from Mesoscale and Olink data. IL-8 is a strong neutrophil chemoattractant and an increase causes a

greater inflammatory response in the site surrounding the tumor, mainly by attracting neutrophils (20). Further experiments will reveal whether AF-16 induce an enhanced neutrophil infiltration in the tumor of treated animals. Expression of VEGF in THP-1-cells was not affected by AF-16 to the same extent as IL-8. While Olink data suggests a small increase (Fig. 1), Mesoscale analysis suggested the contrary; a decrease when treated with AF-16 (Fig. 3). Taking into consideration that these are two different protein platforms that analyze and measure protein with their individual methods, different results could be obtained. Hence, no absolute conclusions can be done regarding the role of VEGF in glioblastoma after AF-16 treatment. It would have more beneficial if VEGF decreased when treated with AF-16 considering that VEGF induces angiogenesis and therefore facilitates tumor survival and growth. Furthermore, angiogenesis in cancer development often results in weak leaky vessels that increase the intratumoral pressure and causes central necrosis.

CXCL10 was the only analyzed chemokine that decreased when M0-cells were treated with AF-16. On the contrary, production of CXCL10 increased in THP-1 and GBM-cells. CXCL10, also known as IP-10, is known to have antitumor actions by attracting immune cells and inhibiting angiogenesis in a macrophage-, T- or NK-cells independent manner. Earlier studies have shown that an increased concentration of CXCL10 was correlated with reduced tumor growth in several cancers including lymphoma and lung adenocarcinoma (21). Simultaneously, upregulation of CXCL10 in glioblastomas seemed to contribute to tumor growth (22), despite its aforesaid anti-tumorigenic properties. The exact role of CXCL10 in glioblastomas has not been completely described and further studies are needed in order to elucidate its function.

Both Olink and Mesoscale data imply that concentrations of MIP-α, MIP-β and MCP-1 were elevated in each respective human cell type (THP-1, M0, GBM) when treated with AF-16. The MIP-family consists of proinflammatory cytokines expressed mainly by macrophages and monocytes and help recruit other proinflammatory cells and activate them (23). MCP is a chemokine family produced by several cell types and is known to stimulate monocyte and macrophage recruitment (24). This suggests that AF-16 help activate the immune system both through immune cells (THP-1, M0) and the tumor cells (GBM) and promotes inflammation at the tumor site.

Furthermore, increased expression of TNF-α was noted both in human and mouse macrophages. This protein, expressed by both monocytes and macrophages, is known for regulating proliferation, survival, differentiation and apoptosis (25). Macrophages are both the major producer of TNF-α and are highly responsive to the same cytokine. TNF-α has a necrotizing

activity against tumor cell lines, which could be a critical characteristic in immunological treatment against glioblastoma. On the contrary, expression of OPG in glioblastoma cells decreased when treated with AF-16. OPG, Osteoprotegerin, is mainly involved in bone biology but is also a soluble receptor for TNF-related apoptosis inducing ligand (TRAIL). It has been found to inhibit apoptosis in OPG-expressing tumors and to promote angiogenesis (26). This suggest that AF-16 affects the tumor cells in an unfavorable manner.

As seen in figure 1 and figure 2, expression of most cytokines showed a pronounced increase when treated with intermediate dose of AF-16 (0,2 mg/ml), which later on diminished when increasing the dose further (2 mg/ml). This has also been recognized by Lantmännen AB, an agricultural cooperative that fabricates Salovum and SPC-flakes, who have not been able to explain the phenomenon (personal communication, Jan Kopecky). Possibly, this suggest that AF-16 works in a dose-dependent manner where the middle dose gives an outlier value. This has been discussed in earlier studies by Ewa Johansson et.al where AF-16 was shown to decrease intracranial hypertension in dose-dependent manner (27). Alternatively, some sort of negative feedback could be involved in which cytokines inhibit their own production when reaching a too high concentration.

Proteomic data analysis via Mesoscale and Olink show that several proinflammatory and antitumoral cytokines tend to increase when THP-1, M0, GBM and RAW-macrophages are treated with AF-16. However, this was not the case for all factors. Since glioblastoma thrives in an immunosuppressive microenvironment it is possible to assume that AF-16 treatment helps combat the tumor *in vivo* by utilizing the hosts own immune system. Whether increased inflammation in the tumor site is simply favorable for the host is uncertain. Inflammation promotes infiltration of immune cells that help oppose the tumor cells and scavenge cellular debris. At the same time, inflammation may induce necrosis and edema that theoretically could influence other brain functions. Optimally, a balance is required which demands greater understanding of the mechanism of action behind AF-16. Further investigation on this topic is necessary.

Immunohistochemical analysis on frozen brain sections was performed to examine AF-16 immunomodulatory effect *in vivo*, with main focus on the effect on the composition of macrophage population ($F4/80^{+}$) and expression of cyclooxygease-2 (COX-2). It has previously been found that COX-2 has a role in glioblastoma progression (18). COX-2 is an enzyme involved in the production of several prostaglandins including prostaglandin E_2 (PGE_2), the most abundant prostaglandin *in vivo*. Specific COX-2 inhibitors, such as some nonsteroidal

anti-inflammatory drugs (NSAIDs), have shown a reduced risk for several cancer forms such as cancer in the esophagus and stomach. This proposed that PGE2 had pro-oncogenic activity by primarily stimulating angiogenesis and immunosuppression (18). Indeed, further studies have shown that inhibiting COX-2 in combination with immunotherapy prolonged survival in established rat and mouse brain tumors. Furthermore, increased infiltration of immune cells, primarily T-lymphocytes, was noted (28). This finding evokes interest for further investigations asking if AF-16 is able to inhibit COX-2, which in turn could induce an immune reaction that help combat the tumor.

On gross inspection, no major difference in macrophage count nor change in COX-2 expression between AF-16 treated and non-treated brain tumor mouse models could be distinguished. Nevertheless, an adjusted distribution of the macrophages was noted with a higher concentration in the brain tumor area. However, no quantitative analysis of the percentage of intratumoral $F4/80^{+}$ stained area was performed and therefore no confident conclusion can be made. Even if results *in vitro* implied affected expression of immunomodulatory factors induced by AF-16, this does not ensure that it reflects on the results *in vivo*. Additional immunohistochemical stainings of brain tumors would be required to be able to make a more specific conclusion.

To examine the effect by intratumoral administration of AF-16 on mice with brain tumors, GL261 tumor-bearing mice were treated with AF-16 according to the setup described earlier. In summary, 2 out of 8 mice treated with AF-16 survived until the end of monitoring on day 160. However, the majority of the treated animals died early in the course of the experiment, rendering the median survival time equal to that of the control group (40,5 days). Therefore, we postulate that AF-16 has the potential to cure brain tumors in mice, however the overall survival remains unaffected ($p=0,5401$). More studies with larger sample size and repeated measurements are required to prove treatment efficacy. All studies performed on animals have inherent limitations as findings cannot directly be applied to humans due to apparent differences in physiology. Moreover, mouse glioma models are presumably more homogenous than primary human gliomas and produce more consistent results.

Limitations

Before concluding, a few limitations in this study merit further discussion. Firstly, a larger study population would be required in order to receive more significant results in the *in vivo* experiments. However, due to ethical considerations, number of experimental animals should be kept to a minimum. In this study, small sample size allowed us to obtain fairly valid results while fulfilling the 3Rs principle (reduction, replacement, refinement). In addition, mice in the control group were not treated with mini osmotic pumps containing placebo-treatment due to limited resources. Whether a placebo treatment with e.g. saline solution would have given different results was not examined in this study. However, unpublished data on similar experiments from Glioma immunotherapy group, Lund University, showed no difference in survival days in mice receiving saline in pumps versus no pumps (personal communication, Jan Kopecky).

Furthermore, only one brain from each group was harvested for immunohistochemical analysis, thus limiting the power of conclusions. Due to few tumor sections, no quantitative analysis of the percentage of intratumoral $F4/80^+$ stained area nor COX-2 producing cells could be performed, and no quantitative data was retrieved.

To draw more specific conclusions regarding the effect of AF-16 on glioma cells, cell death should have been measured through flow cytometry. As earlier mentioned, this study was part of a larger experimental study where we received data for analysis and interpretation due to the current pandemic. Initially, flow cytometry was scheduled to be performed but data had not yet been produced during the time writing this essay. If given other circumstances, we would have attended laboratory sessions and obtained our own data. However, considering current conditions as well as our moderate experience in the field of research, this study is well composed.

Conclusions

In this study, we found that AF-16 affects expression of inflammatory factors in human monocytes (THP-1), macrophages (M0) and glioblastoma-cells (GBM) as well as mouse macrophages (RAW). The *in vivo* effect of AF-16 is promising, however further studies are required to elucidate its full potential as a treatment against brain tumors. We could see that AF-16 treatment possibly cured 2 out of 8 tumor bearing mice as well as modulated the distribution of macrophages at the tumor site. Our study provides an insight into AF-16 immunomodulatory role and pose as useful framework for future studies.

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Figures and tables

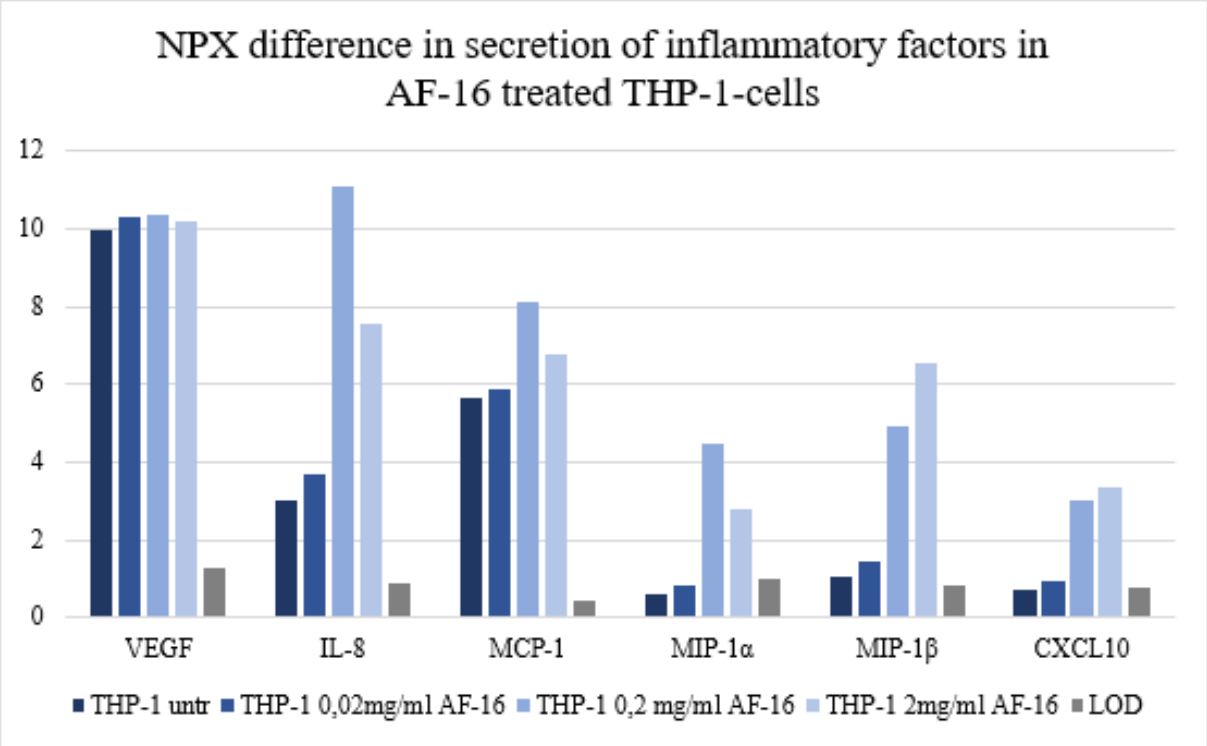

Figure 1: **Change of inflammatory factors in AF-16 treated human monocytes (THP-1)**. Diagram represents inflammatory factors VEGF, IL-8, MCP-1, MIP-1α, MIP-1β, CXCL10 and their respective change of expression in THP-1-cells treated with different doses of AF-16 (untreated, 0,02 mg/ml, 0,2 mg/ml and 2 mg/ml). Data is based on protein profiling using the Olink assay. Values are expressed in NPX. LOD is shown.

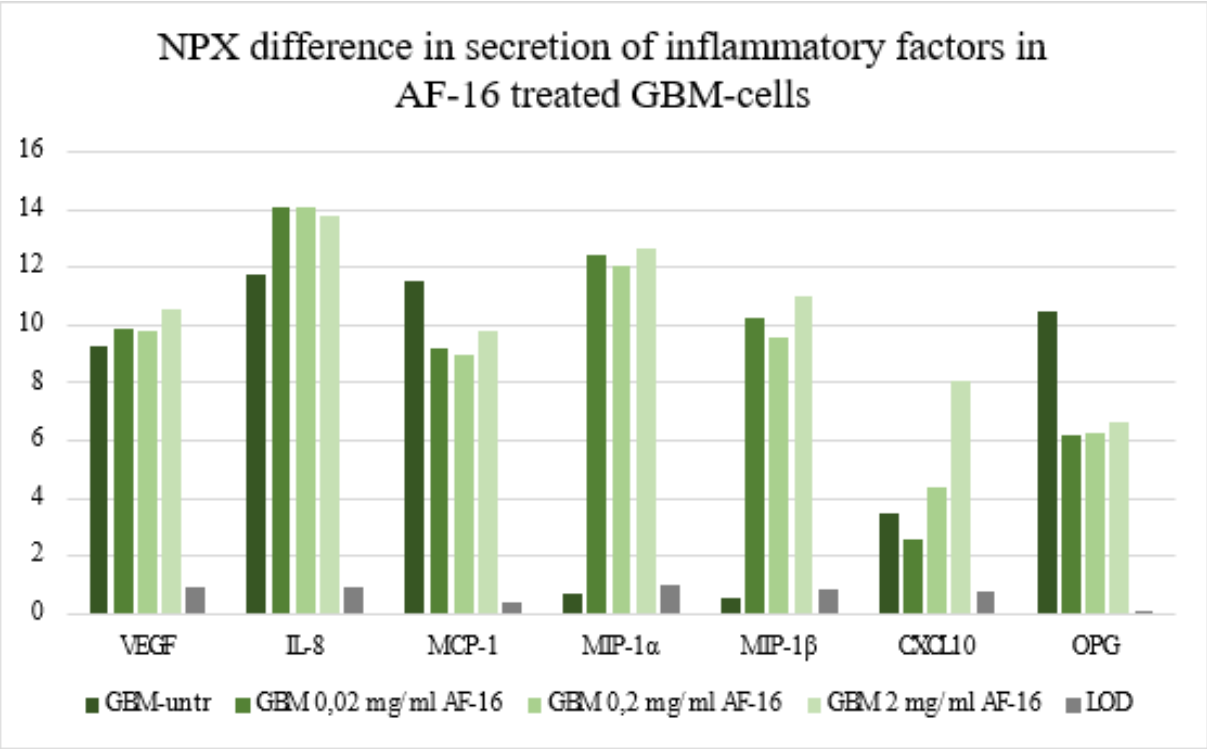

Figure 2: **Change of inflammatory factors in AF-16 treated GBM-cells**. Diagram represents inflammatory factors VEGF, IL-8, MCP-1, MIP-1α, MIP-1β, CXCL10, OPG and their respective change of expression in GBM-cells treated with different doses of AF-16 (untreated, 0,02 mg/ml, 0,2 mg/ml and 2 mg/ml). Data is based on protein profiling using the Olink assay. Values are expressed in NPX. LOD is shown.

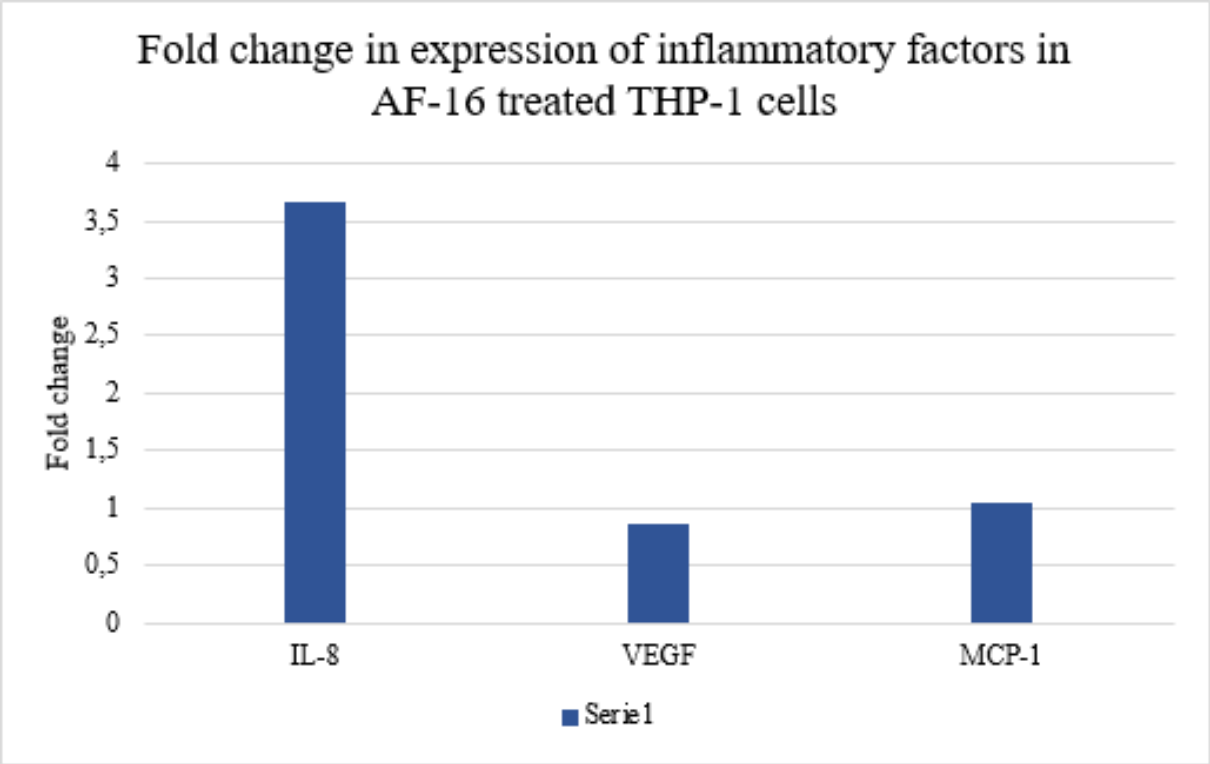

Figure 3: **Fold change in expression of inflammatory factors in untreated compared to treated THP-1 cells**. Diagram represents inflammatory factors IL-8, VEGF, MCP-1 and their respective percental change of expression in THP-1 cells treated with AF-16 (0,2 mg/ml). Data is based on analysis of supernatants with MesoScale V-PLEX®.

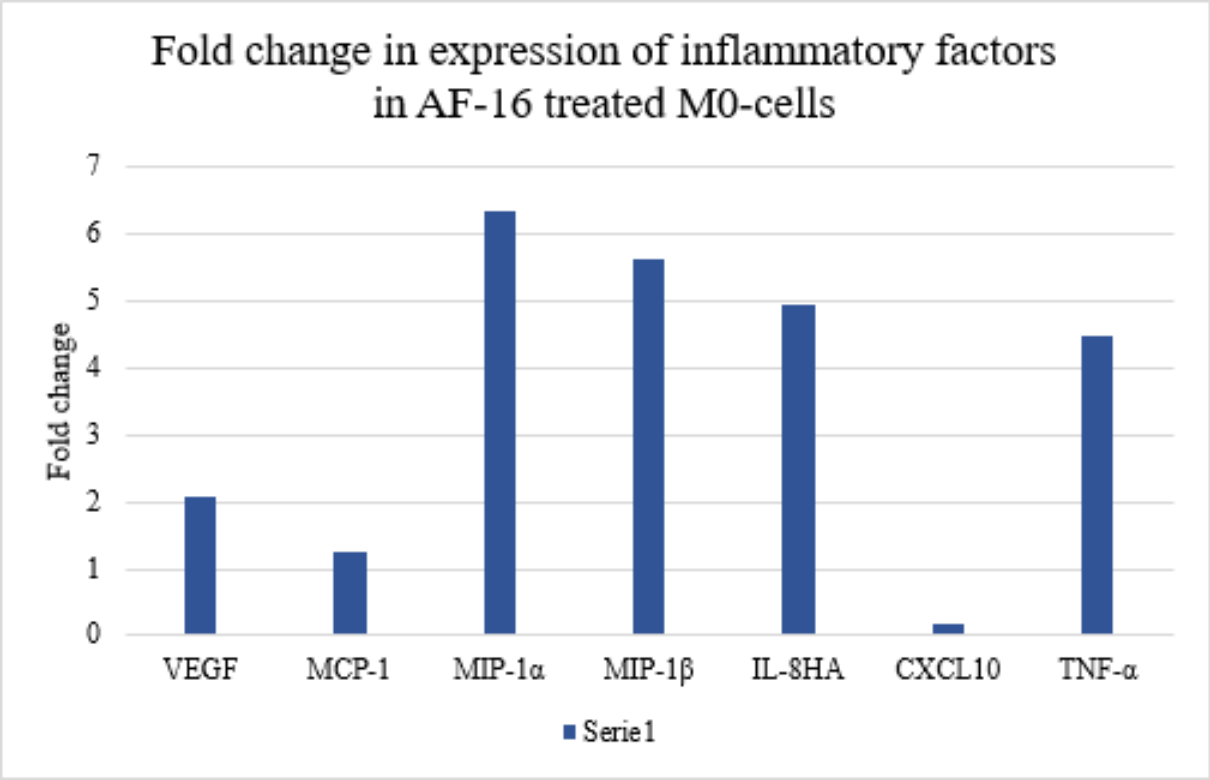

Figure 4: **Fold change of cytokine expression in untreated compared to treated M0**. Diagram represents inflammatory factors VEGF, MCP-1, MIP-1α, MIP-1β, IL-8(HA), CXCL10, TNF-α and their respective percental change of expression in M0-cells treated with AF-16 (0,2 mg/ml). Data is based on analysis of supernatants with MesoScale V-PLEX®.

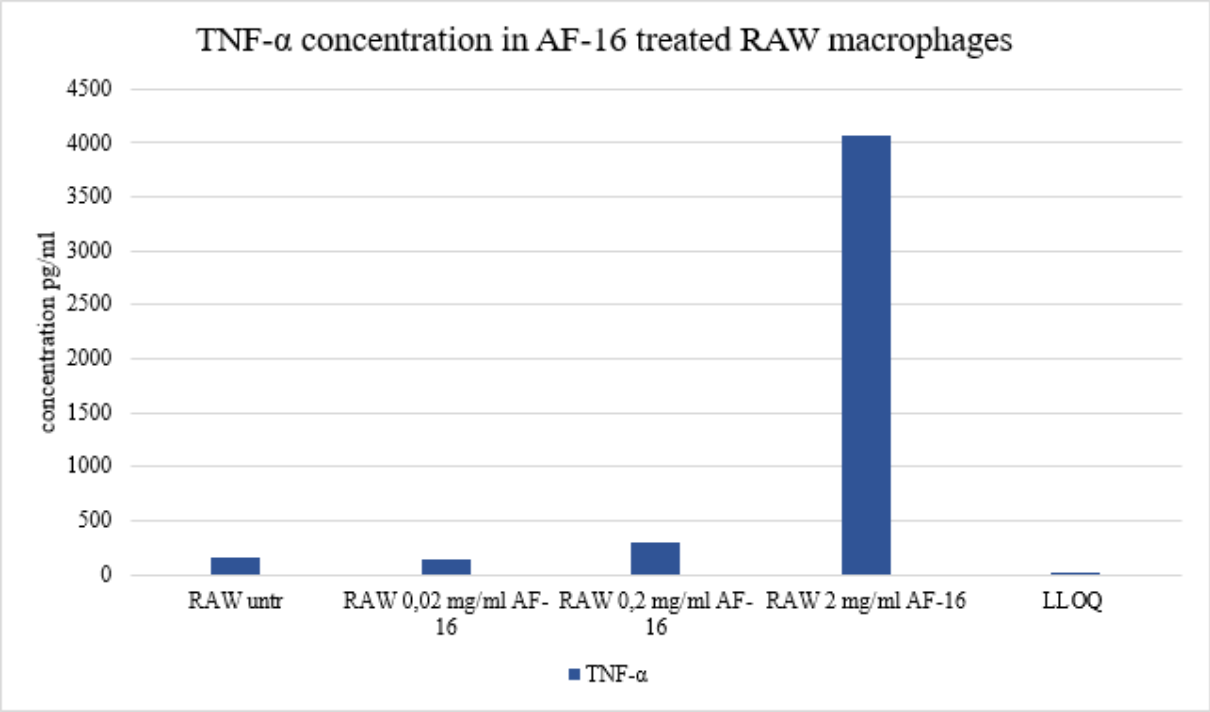

Figure 5: **TNF-α concentration change in AF-16 treated RAW mouse macrophages** after treatment with different doses of AF-16 (untreated, 0,02 mg/ml, 0,2 mg/ml, 2 mg/ml) for 24 hours. Data is based on analysis of supernatants using MesoScale V-PLEX®. LLOQ is shown.

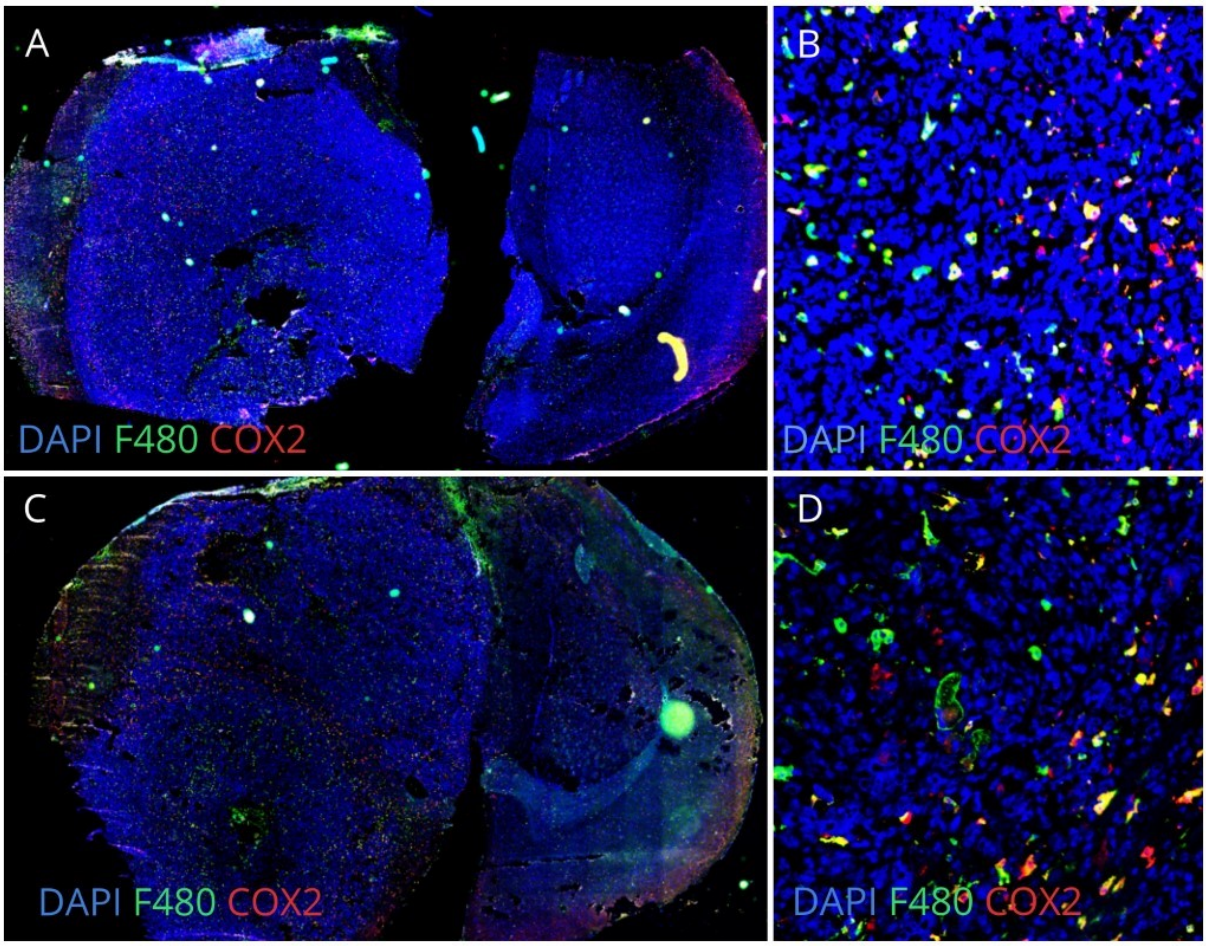

Figure 6: **Immunohistochemistry on frozen brain sections harboring GL261 glioma**, groups included were mice treated with AF-16 (n=8) and non-treated control group (n=8). Sections were stained for F4/80^{+} macrophages in green and COX-2 producing cells in red. Double labeled cells (F4/80^{+}/COX-2) are seen in yellow. DAPI was used as a nuclear staining. Images show representative staining from one animal from each treatment group. Image A present brain section from untreated mice and image C brain section from mice treated with AF-16. Images were taken at 10x magnification. Image B and D respectively present zoomed-in images from a representative area in brain sections from untreated mice (B) and mice treated with AF-16 (D).

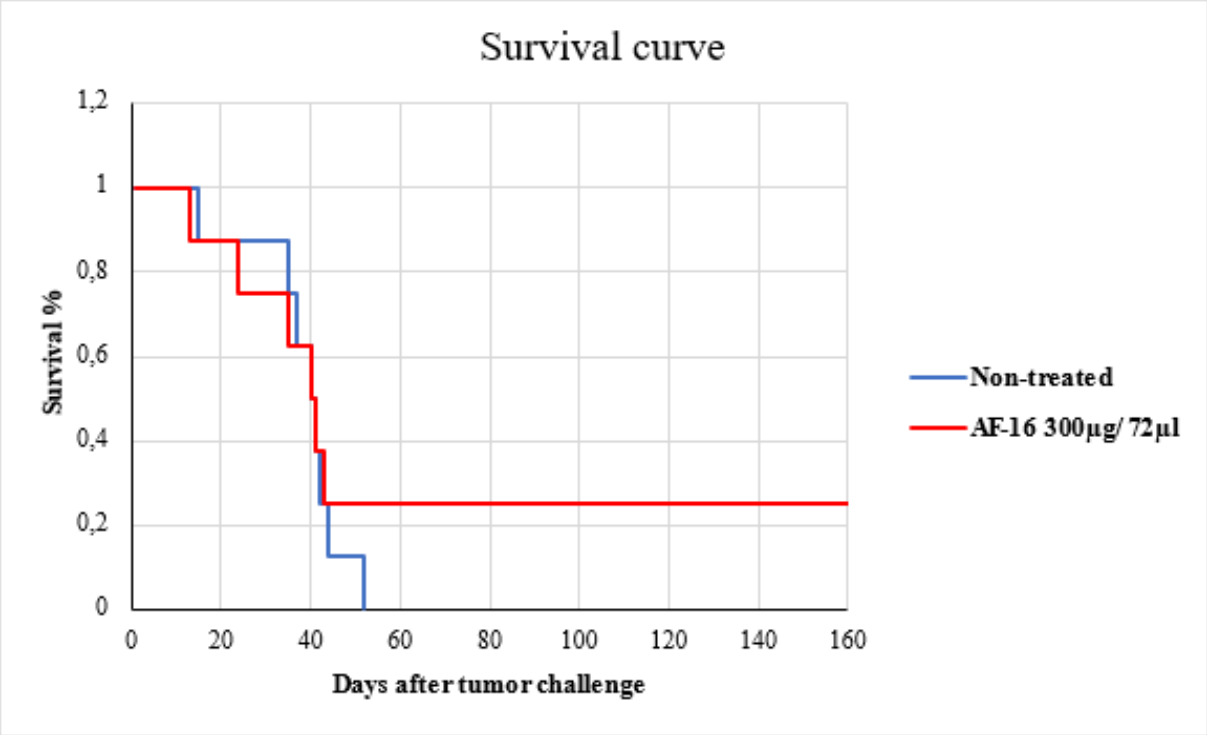

Figure 7: **Kaplan-Meier survival curve** shows effect on survival after AF-16 treatment in GL216 tumor-bearing mice. GL261-bearing mice receiving CED of AF-16 for 3 days (day 7-10 after tumor inoculation) using a mini-osmotic pump (n=8) shown in red, and mice receiving no treatment (n=8) shown in blue. Survival was monitored for 160 days.