The Effect of of SARS-CoV-2 Nucleocapsid Protein on IL-6 Cytokine Release from Glial Cells

Bachelor's Thesis
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I. Abstract

Since 2019 the world has experienced global pandemics of COVID-19 caused by SARS-CoV-2, resulting in the transmission of the disease to hundreds of millions of people all around the globe. To this day the majority of mechanisms by which virus disseminates and proliferates inside the cells, leading to severe cases of disease are not known. Despite the fact that COVID-19 is a respiratory disease, 30% of the patients in acute stages of disease show numerous neurological symptoms, including headache, anosmia, and ageusia implementing that viral cells have a serious effect on the CNS probably by inducing cytokine release syndrome and leading to cytokine storm as in other tissues. Glial cells are of a particular interest as they are the major source of inflammatory cytokines in the nervous system. It was found in the previous research that a structural protein of SARS-CoV-2, particularly nucleocapsid (N) protein, induces the release of numerous cytokines from alveolar cells, macrophages, and monocytes. IL-6 is one of the major inflammatory particles released from types of cells studied before, during the cytokine storm, and it was shown that it might lead to the severe neurological response. Previous studies also showed that alveolar cells and macrophages release IL-6 when transfected with or cultured in the presence of N protein. The main goal of this research is to identify the effect of the SARS-CoV-2 N protein on the release of IL-6 from U-87 MG glial cells by culturing the cells in its presence. The results are then interpreted by using supernatants of treated cells for dot-blot assay, ELISA, and qRT-PCR.

II. Introduction

SARS-CoV-2 and neurological infection dissemination

The outbreak of COVID-19 in 2019 brought one of the most massive global pandemics and resulted in transmission of the virus to around 257 million people around the world in the last 3 years [1]. Although patients with the mild course of disease often experience cough, fever, and muscle fatigue, a significant number of patients showed signs of pneumonia and even organ failure [2].

Despite the fact that Severe acute respiratory syndrome 2 (SARS-CoV-2) is primarily a respiratory disease, the analysis of cerebrospinal fluid of infected people often indicates the presence of the virus in the central nervous system (CNS) [3]. The information on the virus dissemination mechanism within CNS is still limited, but it was found that 36.4% of patients with severe cases

had numerous neurological symptoms such as headache, anosmia and ageusia [3]. Particularly, it is proposed that SARS-CoV-2 infects neuronal and glial cells, which gives access to numerous pathways for the spread of the infection to the entire CNS [4]. It was proposed that astrocytes might as well be affected by SARS-CoV-2. They have an effect on the production of neurotransmitters and trophic factors for the differentiation of neurons, they are also located along the vasculature, affecting the blood-brain barrier (BBB) formation and activity, which might be the reason for the neurological symptoms associated with virus [3]. One of the most common consequences of cases with the development of neurological symptoms is the development of cytokine release syndrome (CRS) which leads to the neurotoxic response that further stimulates brain damage in severe cases [5]. Upon entry virus induces glial cells to uncontrollably release inflammatory particles (interferons, Tumor necrosis factor α (TNFα), interleukins (IL-1β, IL-6 and IL-10)) causing cytokine storm. It leads to an increase in permeability of the BBB and damage to neurons [6]. In all inflammatory reactions, the cytokines that are created and released by immune cells play a role in both the initiation and effect stages. For example, the acute-response cytokines such as TNF and IL-1β can be found in the blood of an infected person within minutes after infection, followed by a gradual increase in IL-6 [6]. In the later stages of the infections initially released cytokines induce the production of other inflammatory particles leading to the cytokine storm [6]. Later with the increase in their concentration of this. However, the crucial step is activating the transcription mechanism of cytokines to then stimulate their secretion [7]. Taken as a whole, these findings imply that SARS-CoV-2 could have the ability to infect multiple neural cells, such as astrocytes, and thus potentially cause neurological symptoms in individuals with COVID-19. This conclusion is based on an analysis of the various cytokines and their direct effects on the inflammation initiation. Therefore, studying the effect of the virus on the release of cytokines from cells of CNS is highly crucial for the further understanding of the course of the disease.

The role of IL-6 in inflammation

Interleukin 6 (IL-6) is an important cytokine that is responsible for the acute inflammation in response to the viral infection [8]. It is released primarily by the cells of immune system (T lymphocytes, macrophages, monocytes), endothelial cells and fibroblasts, and has major influence

on differentiation and production of antibodies released by B lymphocytes, activation of cytotoxic T lymphocytes (CTL), differentiation of hematopoietic stem cells and many more [9]. But one of its major functions is its effect on the activation of inflammatory particles and growth particles when it interacts with viral particles. Its activation is controlled by several transcription factors, such as NF-κB, which can be transcribed in response to the bacterial, viral, or mitagen infection, where it interacts with a particular DNA binding site and activates IL-6 transcription activation [10]. A further signal transduction involves the binding of IL-6 to its receptor IL-6R, which creates a complex that subsequently binds to a transmembrane glycoprotein known as gp130 [9]. This binding activates intracellular signal transduction which include. Both a transmembrane IL-6R and a soluble form (sIL-6R) exist, and IL-6 can bind to both forms [9]. When glial cells such as astrocytes were tested for the ability to produce IL-6 cytokines in response to LPS treatment, they showed that the cell supernatants contained an increased concentration of IL-6 (through ELISA) after 2 hours of treatment [11]. It further induces the various inflammatory reactions in the CNS.

SARS-CoV-2 virion and genome structure, role of N protein in production of virus

Four main structural proteins (S, M, N, E) of SARS-CoV-2 are responsible for the entry and replication of the virus within the cells [12]. Previously conducted research suggests that among four proteins the one that is responsible for the induction of cytokine release in cells is nucleocapsid (N) protein [3].

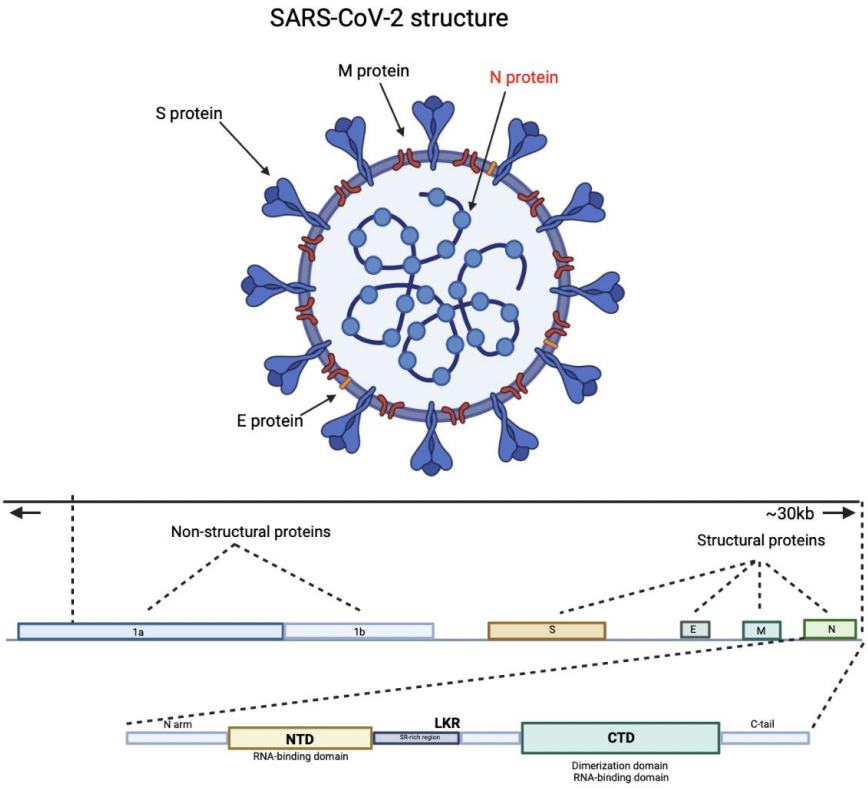
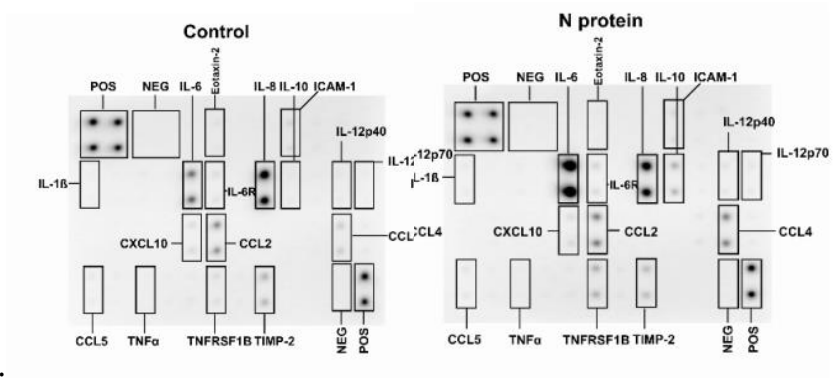


Figure 1. The structure of SARS-CoV-2 virion and its genome.

The N protein is a RNA-binding protein with two folded domains (NTD and CTD) connected by a disordered region (LKR) containing potential phosphorylation sites. There are also two disordered regions on both sides of the NTD and CTD (N-arm and C-tail) that help regulate RNA binding and oligomerization [13]. It participates in numerous processes during the infection, including the entry of the SARS-CoV-2 into the cells as well as the release and assembly of new viral particles [14]. Therefore, the use of an NF-κB inhibitor called pyrrolidine dithiocarbonate can help alleviate lung injury induced by SARS-CoV-2 N-protein [15]. Previous research observed if macrophages and monocytes that were cultured in the presence of N protein express a comparatively higher level of the inflammatory cytokines such as IL-6, IL1B, IL10, and many more in comparison with untreated cells [10].

The expression of IL-6 in response to N protein in macrophages and monocytes

A.



B.

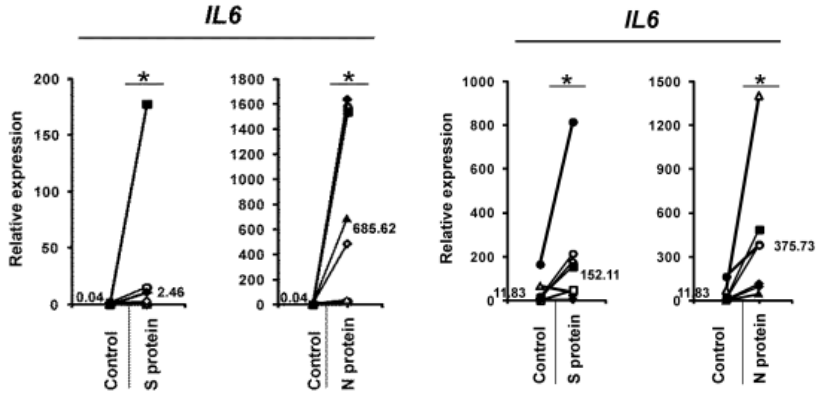


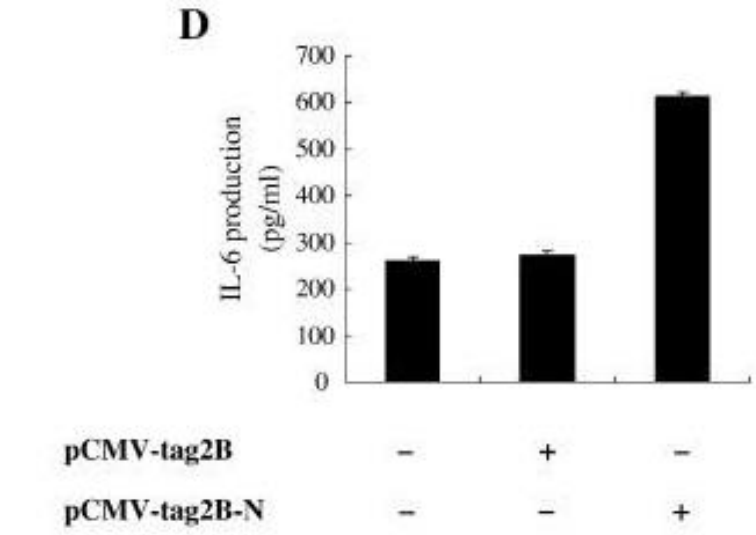
Figure 2. The change in the expression of IL-6 in macrophages and monocytes. (**A**)The supernatants of cells incubated in the presence of N protein were analyzed using Human Inflammatory Array C3 (**B**) The increase in the expression of cytokines in response to N protein measured with real time PCR. From Karwaciak, I., 2021.

The expression of IL-6 in macrophages and monocytes increased 30-fold in response to 1ug/ml of N protein as it can be seen in Figure 1 [10]. It was established as well that the upregulated release of cytokines from this type of cells not only leads to cytokine storm, but also reduces the response from CD8+ T lymphocytes by inhibiting the activity of T helper 1 (Th1) within the organism [10]. The segment of the N protein that goes from amino acids 341 to 422, also known as the C-terminus, is necessary for the viral protein to trigger the expression of the IL-6 gene. If this region is removed, the ability to activate IL-6 is lost [10].

N protein of SARS-CoV is also shown to induce the release of cytokines (IL-6) from alveolar cells by interacting with NF-κB promoter that is responsible for the expression and induction of IL-6 release from cells [16].The degree to which IL6 mRNA was triggered varied greatly, ranging from

30 to several thousand times (as shown in Figure 2A and DataSet S1). This supports the idea that differences in proteins responsible for identifying viral proteins, like pattern recognition receptors and signaling molecules, caused by genetic diversity such as polymorphisms and mutations, can result in varying degrees of risk for severe and imbalanced inflammatory responses, which can lead to cytokine storm syndrome [16].

The expression of IL-6 in response to N protein in alveolar cells (A549)



A.

B.

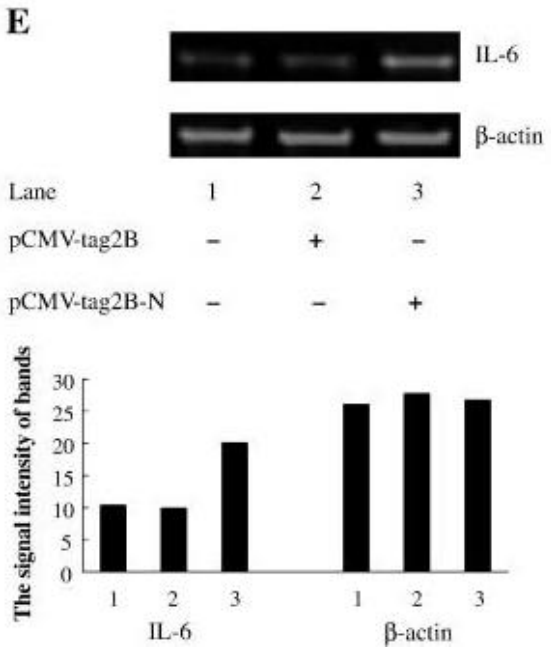


Figure 3. The change in the expression of IL-6 cytokine in alveolar cells. (**A**) The expression of IL-6 of the cells transfected with pCMV plasmid containing a sequence for N protein gene was analyzed with ELISA (**B**) Semi-quantitative PCR was used in order to find the change in the expression of IL-6. From Zhang, X., 2007.

The release of IL-6 into the bloodstream of the CNS can pose a great danger, resulting in the demyelination of the axons and damage to neuronal cells [6]. Glial cells, particularly astrocytes, are important to study, as these are the major cell types releasing the cytokines in the CNS [9]. The effect of N protein on the release of cytokines in glial cells is important to be investigated, as it might be a key to the future treatment of the disease. The particular type of astrocytes chosen for the experimental procedure is U-87 MG cells obtained from a malignant glioblastoma. The main reason for U-87 MG cells' application is their high transfection efficiency, around 91.83% [17].

III. Materials and methods

U-87 MG cells culturing and plating.

U-87 MG is a type of adherent astrocyte cell with epithelial-like morphology that was chosen for the research. Cell line was obtained from a female patient with malignant glioma. During the procedure cells are cultured according to the protocol on ATCC website in Eagle's Minimum Essential Medium (EMEM) (under 37C, 95% air, 5% CO2) in T25 or T75 flasks (40000 cells/cm2

and 27000 cells/cm2). Cells are grown, until the confluence reaches 80%, then 2 ml of trypsin is added to the flasks in order to detach the cells from the surface. Next, 4 ml of EMEM is added to T25 to neutralize trypsin, then cell suspension is mixed thoroughly and transferred to a 15 tube. Next the suspension is centrifuged at 900 rpm for 5 minutes, supernatant (SN) is then aspirated . 1 ml of EMEM is added to the centrifuged cells and mixed thoroughly, so that no precipitate is left on the bottom of the tube. 10 ul of cell suspension is transferred to 1 ml tube with 90 ul of PBS mixed, then 10 ul of cell suspension diluted in PBS is transferred to a different 1 ml tube with 10 ul of Trypan Blue 0.4% and mixed thoroughly. 10 ul of the stained cells is put onto a hemocytometer to observe the cells under the microscope (30X) and count the number of cells in the original suspension. Cells are seeded on a 6-well plate (500K/well) for further stimulation with N protein.

Reagents	**Source**	**Catalog number**
U-87 MG cells	ATCC	HTB-14
Trypsin (100X)	Sigma-Aldrich	T2600000
Eagle's Minimum Essential Medium (EMEM)	Thomas Scientific	M0894
PBS	Thermofisher	18912014
Trypan Blue	Sigma-Aldrich	T8154

Table 1. Reagents used for cell culturing.

Stimulation of U87-MG cells with SARS-CoV-2 N protein

SARS-CoV-2 N protein used for the stimulation is obtained from Cusabio (cat# CSB-EP3325GMY) from *E.coli* (P0DTC9). Cells are plated according to the scheme below.

1	2	3
500K	500K	500K
500K	500K	500K
4	5	6

Table 2. Cell seeding scheme.

1st and 4th well contain cells in CM, without any stimulatory particles. PBS (10 ul) is added to the 2nd and 5th as a control. 3rd and 6th wells contain cells that were stimulated with N protein (10 ul of 200 ug/mL). For the final step of stimulation, the cells are incubated for 48 hours at 37C and 5% CO2.

Human Inflammation Antibody Array

Human Inflammation Antibody Array was purchased from Abcam (cat# ab134003). After stimulating U87-MG cells with 2 ug of N protein for 48 hours, supernatants are then collected for the dot-blot array. Chemiluminescence was measured using Bio-Rad Chemidoc Chemiluminescence Gel Imaging System (12003153), and ImageJ is used for analysis of the results.

RNA isolation

RNA isolation is performed using RNeasy Mini Kit, for a subsequent synthesis of cDNA. The U-87 MG cells seeded in a 6-well plate are used for the isolation of RNA. For that cells are removed from the flasks, and cell suspension is then centrifuged at 900 rpm for 5 minutes. The supernatant is removed and 350 ul of RLT buffer from the kit is added to the cells and mixture is pipetted 10 times. Then 350 ul of 70% ethanol is added to the tube and pipetted with a syringe 10 times, this mixture is then transferred to the elution column, that is centrifuged for 15 seconds at 10000 rpm, supernatant is removed. Next, 700 ul of RW1 is added, the column is centrifuged for 15s at 10000 rpm, and the supernatant is removed again. Following this, 500 ul of RPE is added, the column is centrifuged for 15s at the same speed, and the supernatant is removed. Another 500 ul of RPE is added, the column is centrifuged for 2 minutes at 10000 rpm, and the supernatant is removed. In the following stage 50 ul of RNase-free water is added for the 1st elution, the column is centrifuged for 1 minute at 10000 rpm, the obtained RNA is transferred to the tube. RNA should be kept in the -80C freezer for longer storage. In order to identify the concentration and purity of the final products the isolated RNA is analyzed with the help of NanoDrop.

cDNA synthesis and qRT-PCR

In order to identify the concentration of the produced mRNA levels of IL-6 protein, complementary DNA (cDNA) is synthesized from the isolated RNA. Reverse transcriptase

included in the Maxima Enzyme mix produces the complementary strand DNA from the initial sample. DNA synthesis starts with obtaining 2 ug of RNA that is used to synthesize cDNA. First, 1 ul of 10xDNase and 1ul of ds DNase are added to the tube with 2 ug of RNA, mixed, and centrifuged for 30 seconds at 5000 rpm. The mix is incubated for 2 minutes at 37 C in a thermocycler, then cooled on ice for 2 minutes. The tube is then placed in a centrifuge for 30 seconds at 5000 rpm. 4 ul of 5X reaction mix is added to the tube, and 2ul of Maxima Enzyme is added to the tube. Control NRT sample does not need Maxima Enzyme to be added to it, so the same amount of water is added instead. Finally 4 ul of RNase-free water is added to result in the total volume of 20 ul, mixed and centrifuged for 30 seconds at 5000 rpm. The mixture is then placed in thermocycler (10 minutes at -25C, 15 minutes at -50C, and 5 minutes at -85C).cDNA should be kept in -30 freezer.

For qRT-PCR 6 ul of the obtained cDNA (1:100) is treated with 2 ul of forward (10 uM) and 2 ul of reverse (10 uM) primers (Karw-IL6 and SDHA), and 10 ul of Sso Advanced Universal SYBR green Supermix is added to the tube. The sample is put in the thermocycler to the following parameters: initial denaturation -95C for 30 seconds, 40 cycles of denaturation at -95C for 30 seconds, annealing at -53C for 30 seconds, extension at -72C for 10 seconds, and 53-95C (0.5C step) for 4 seconds. No template control (NTC) that contains only reagents for PCR is used as a clarity and quality test for reagents used.

Primer	**Sequence**
Karw-IL-6 forward	5′-CCTGAACCTTCCAAAGATGG-3′
Karw-IL-6 reverse	5′-GGTCAGGGGTGGTTATTGC-3′
SDHA forward	5′-GAGATGTGGTGTCTCGGTCCAT-3′
SDHA reverse	5′-GCTGTCTCTGAAATGCCAGGCA-3′
b_actin forward	5′-CACCATTGGCAATGAGCGGTTC-3′
b_actin reverse	5′-AGGTCTTTGCGGATGTCCACGT-3′
GAPD forward	5′-GTCTCCTCTGACTTCAACAGCG-3′

GAPD reverse	5′-ACCACCCTGTTGCTGTAGCCAA-3′

Table 3. The primers used for qRT-PCR.

Statistical analysis

For the statistical analysis of the results obtained from the experimental procedures t-test p value is used to see what concentration of recombinant N protein showed significant difference in expression of IL-6 from glial cells. The normalized signal density values are also analyzed to find what cytokines expression was affected significantly compared to the control sample.

IV. Results

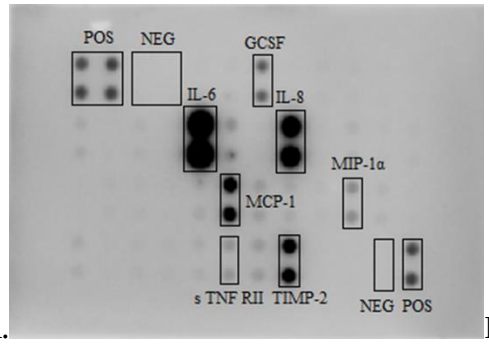
The production of IL-6 and other cytokines increases after stimulation with N protein.

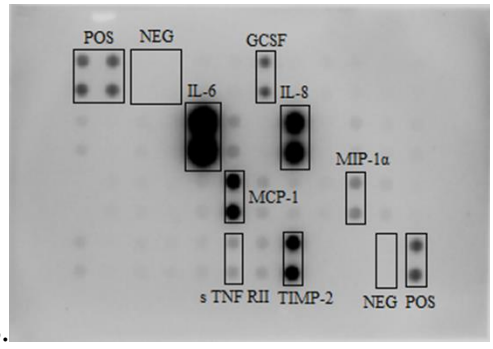
After stimulating U87-MG cells with 1ug/mL recombinant N protein and incubating them for 2 days the supernatants of the cells were collected to conduct dot-blot assay. Increase in the stimulation is indicated by a higher intensity signal in the experimental sample compared to the control which was incubated with PBS. Figure 4 shows the results of the 2 experiments conducted one after another, different cytokines that were expressed by U87-MG cells in the presence (Figure 3B and 3D) and absence (Figure 3A and 3C) of the N protein. The goal of the study was to determine the effect of SARS-CoV-2 on IL-6 production, so the intensity of the signal for the control sample should be lower, the confirmation to it can be seen in Figure 3. Based on the results of the first experiment (Figure 3A and 3B) it was established that IL-6 produced a comparatively higher signal intensity after it was stimulated with nucleocapsid protein. ImageJ was used to find the intensity of bands of the sample for cells incubated with viral protein compared to the control. The results are recorded in Table 3. The quantitative measurements were obtained for further analysis and normalization of values to positive and PBS controls. It allowed us to compare the results not only for the cytokine of interest but for all particles that were expressed by U87-MG cells.

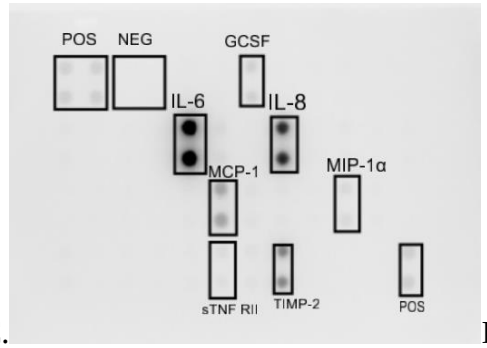
According to Figure 4A and 4B the expression of IL-6 increases when the cells are stimulated with N protein, even though for the 1st experiment the increase was considerably more drastic than for the 2nd trial. IL-6 shows a significant difference for both trials, indicating that N protein has an effect on its expression measured by signal intensity. IL-8, MCP-1, and TIMP-2 show significant differences for the 1st, and MIP-1α was upregulated for the 2nd stimulation with N protein. On the other hand, average normalized expression values (Figure 4C) do not show any significant

difference (in terms of p values), but the rise in the IL-6 and other cytokines (IL-8,MCP-1, TIMP-2) can still be observed. The results are also consistent with the values for the expression relative to PBS sample (Figure 4D, Figure 4E, and Figure 4F). However, the change in the expression of cytokine of interest for the 2nd stimulation is minor. The average relative expression showed a significant increase in the expression of IL-8 and sTNF-RII.

Besides IL-6, the change in the release of other cytokines as a result of stimulation with N protein can be observed from Figure 5 (IL-8, TIMP-2, MCP-1, sTNF-RII, GCSF, MIP-1α). Among the highest expressed cytokines are IL-8 and TIMP-2, IL-8 plays a crucial role in the glial tumor progression and neovascularity, so its expression might be explained by the fact that U87-MG cells were obtained from a malignant glioma [18]. It also acts as an inflammatory chemoattractant, gathering immune cells to the site of infections [18]. TIMP-2 is also a important factor in the immune defense mechanisms, as it is responsible for the cell proliferation, and it is expressed in response to lipopolysaccharide (LPS) contained in the cell walls of bacteria, which possibly shows that it is released in response to the external stimuli [19]. MCP-1 and GCSF expression can also be observed in the U87-MG cells; the difference in their expression was also found to be the most significant. If this observation will be repeated in the further experiments, it might be necessary to test the effect of N protein on these two cytokines particularly. The release of MCP-1 is connected with the differentiation of glial cells and also acts as a transcription factor for a protein responsible for cell apoptosis [20]. GCSF has a primary role in neutrophil production and regulation of T cell response, showing a great importance in the immune response [21]. According to normalized expression, MIP-1α and sTNF-RII were affected the least among all the cytokines expressed during the stimulation with recombinant N protein. Nevertheless, average relative expression indicated a significant difference in the levels of sTNF-RII, which is a receptor for another pro-inflammatory cytokine TNFα, expressed in high concentrations upon the host cell interaction with pathogens [22].

A. 

B. 

C. 

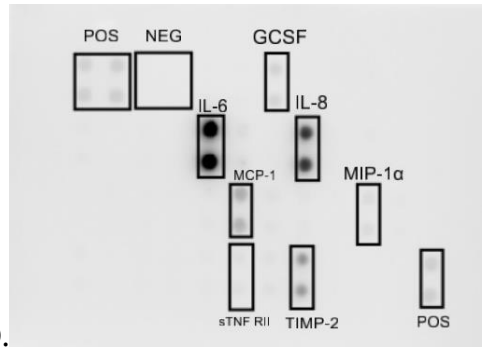
D. 

Figure 3. The results of dot-blot analysis. **A.** Sample treated with PBS (1st experiment) **B.** Sample stimulated with N protein (1st experiment) **C.** Sample treated with PBS (2nd experiment) **D.** Sample stimulated with N protein (2nd experiment)

	1	2	3	4	5	6	7	8	9	10	11	12
1	267.9	331.3					78.7					
2	320.6	372.3					100.1					
3					6558.6			5516.8				
4					7090.9			5880.8				
5						967.5				55.9		
6						972.8				85.3		
7						46.9		1919.9				473.9
8						66.9		1688.5				432.6

Table 4. Raw signal intensity for the control sample stimulated with PBS measured on ImageJ for the 1st experiment.

	1	2	3	4	5	6	7	8	9	10	11	12
1	475.5	464.0					153.7					
2	549.6	576.3					162.4					
3					7128.8			5647.8				
4					6889.9			4863.0				
5						747.4				107.4		
6						733.5				110.7		
7						45.5		1649.1				457.2
8						68.9		1973.6				514.7

Table 5. Raw signal intensity for the sample stimulated with N protein measured on ImageJ for the 1st experiment.

	1	2	3	4	5	6	7	8	9	10	11	12
1	439.9	348.2					400.5					

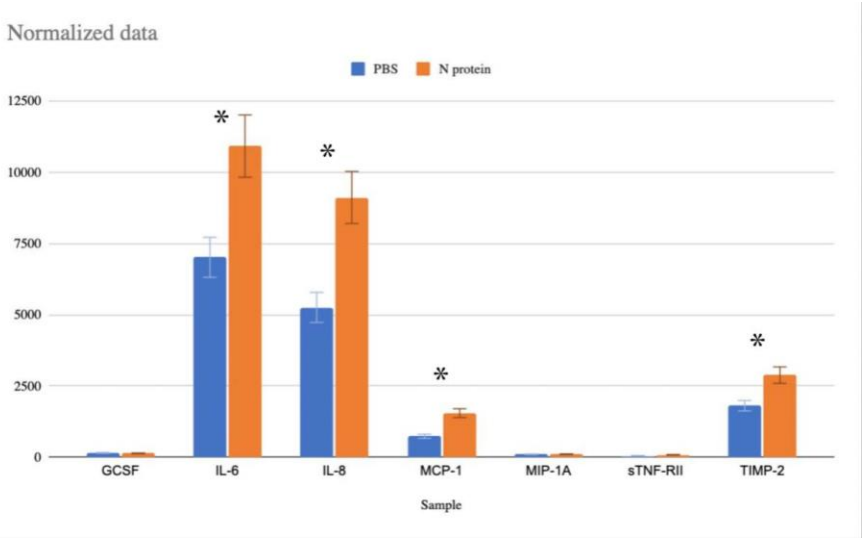
2	463.6	193.2					296.4					
3					9353.0			4306.3		44.5		
4					9581.9			4525.9		40.5	40.5	
5						2193.8				186.6	47.5	47.5
6						1807.9				166.4	8.0	24.1
7						408.2		5998.6				5708.5
8						308.7		5596.6				2068.4

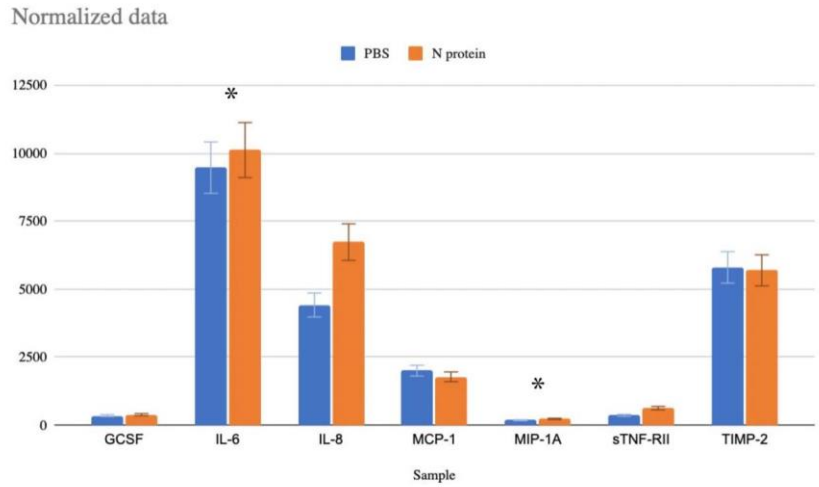
Table 6. Raw signal intensity for the control sample stimulated with PBS measured on ImageJ for the 2nd experiment.

	1	2	3	4	5	6	7	8	9	10	11	12
1	313.1	313.1					377.2					
2	367.4	362.5					341.7					
3					9486.5			8244.1		0.0		
4					9496.2			4386.3		25.5		
5						1868.0				220.4	192.8	
6						1463.	39.0	80.8		205.8	48.5	
7	124.3					693.7	222.1	5301.3				1466.5
8	155.8					473.2	186.0	5379.4				1208.0

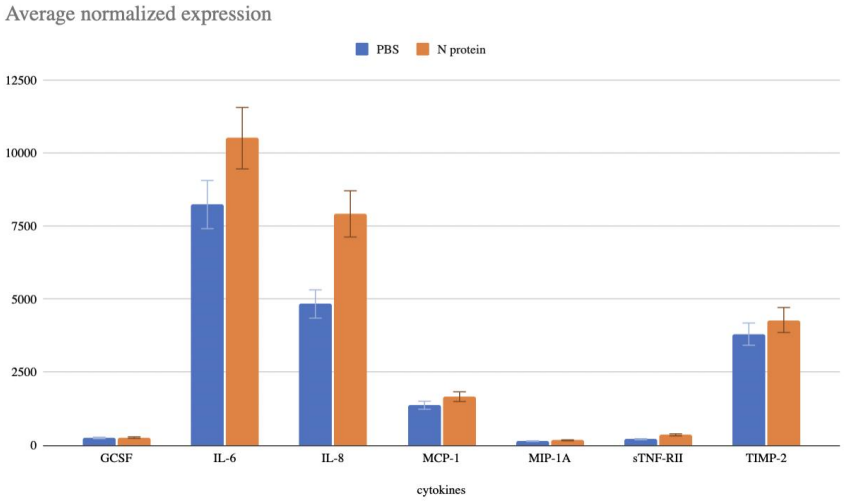
Table 7. Raw signal intensity for the sample stimulated with N protein measured on ImageJ for the 2nd experiment.

A.

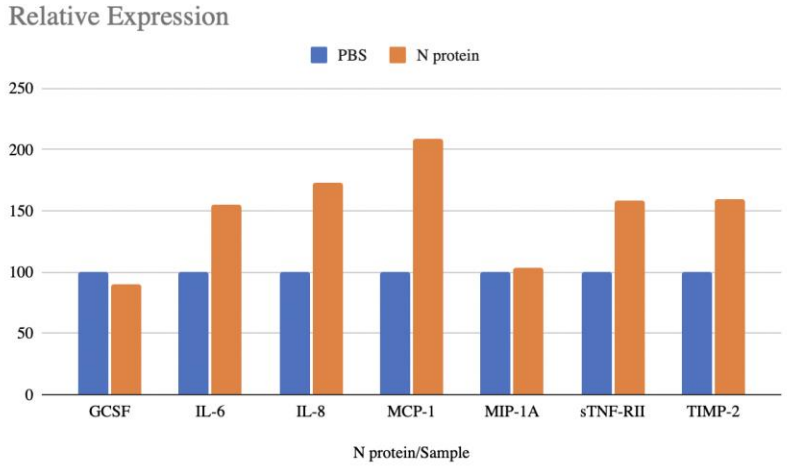



Normalized data
PBS
N protein
12500
10000
7500
5000
2500
0
*
*
GCSF
IL-6
IL-8
MCP-1
MIP-1A
sTNF-RII
TIMP-2
Sample

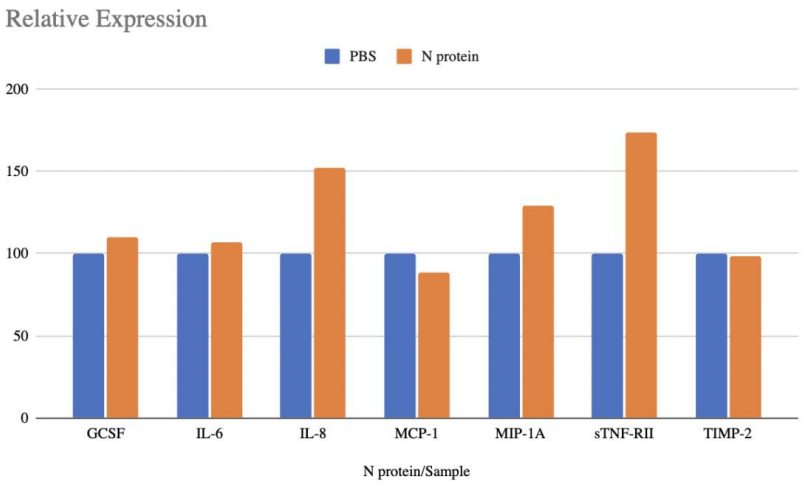
B.


Average normalized expression
PBS
N protein
12500
10000
7500
5000
2500
0
GCSF
IL-6
IL-8
MCP-1
MIP-1A
sTNF-RII
TIMP-2
cytokines

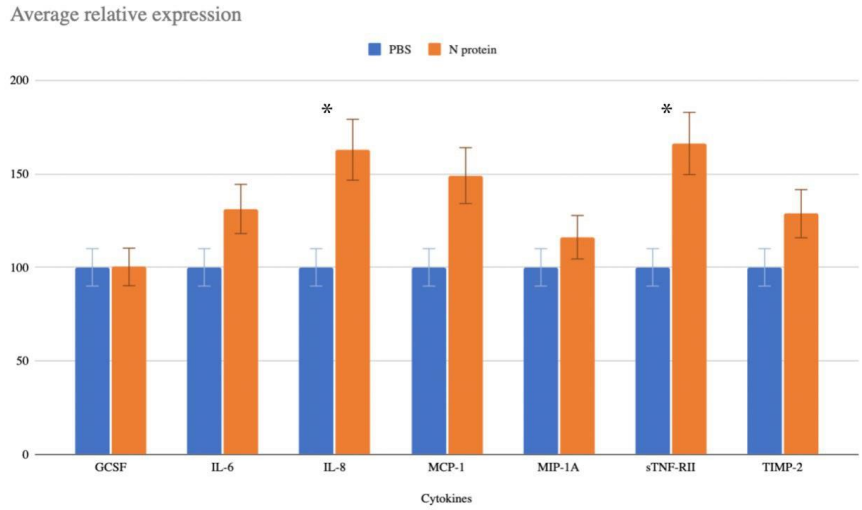
C.


Relative Expression
PBS
N protein
250
200
150
100
50
0
GCSF
IL-6
IL-8
MCP-1
MIP-1A
sTNF-RII
TIMP-2
N protein/Sample

D.



E.



F.

Figure 5. Normalized expression of the cytokines in U87-MG cells in response to N protein stimulation. **A.** Normalized expression relative to positive control (1st experiment) **B.** Normalized expression relative to positive control (2nd experiment) **C.** Average normalized expression relative to positive control for 2 experiments **D.** Expression of cytokines relative to PBS control (1st experiment) **E.** Expression of cytokines relative to PBS control (2nd experiment).**F.** Average expression of cytokines relative to PBS control for 2 experiments. An asterisk indicates a statistically significant difference at $p<0.05$.

The next step of analysis involved ELISA on the U87-MG cells at different concentrations and incubation time. Experimental setup for stimulation with recombinant N protein is the same, except for the fact that 5 different concentrations of N proteins were used (0ng/mL, 300ng/mL, 1ug/mL, 3ug/mL, 10ug/mL) at different incubation times

(12h, 24h, 48h). The measurements for the samples that were used for the standard curve were inconsistent, so it was hard to build the curve to analyze the results. Therefore, we used only raw values for the IL-6 concentration measured to see what samples showed the greatest difference. Figure 6 shows that the most significant difference in the concentration was for the sample of U87-MG cells that were stimulated with 10ug/mL for 24 hours, as p values for this sample was around 0.04.

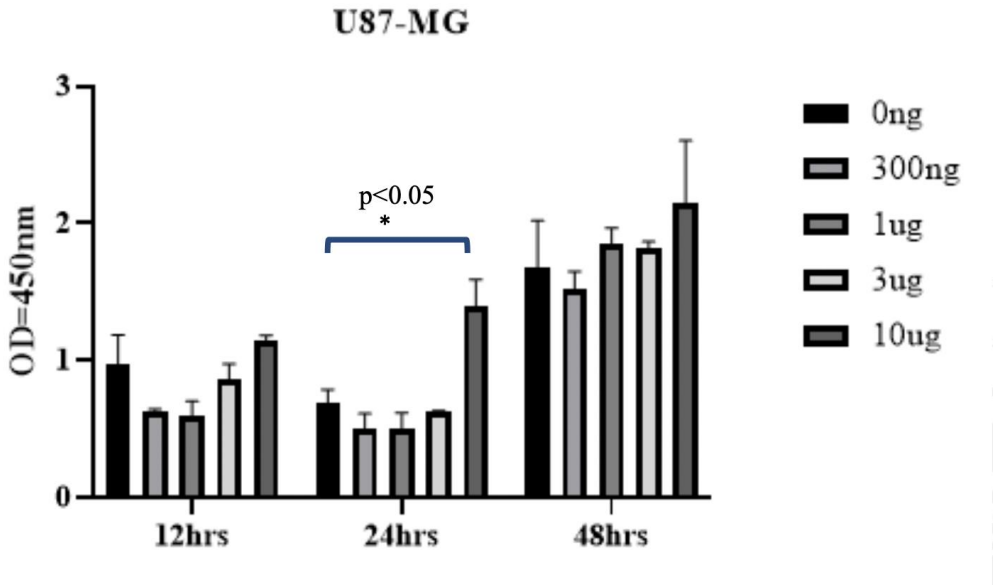


Figure 6. The concentration of IL-6 expressed in U87-MG cells after treatment with 0ng/mL, 300ng/mL, 1ug/mL, 3ug/mL, and 10ug/mL recombinant N protein. An asterisk indicates a statistically significant difference at $p<0.05$.

mRNA level of IL-6 increases in response to N protein.

To see if the levels of IL-6 on mRNA level changed when stimulated with N protein qRT-PCR was conducted. After 48 hours of incubation RNAs of U87-MG cells from experimental and control groups were isolated using RNeasy Mini Kit. The obtained samples of RNA were then checked on quality and purity using NanoDrop (Table 6). We used the first two elutions (1st and 2nd tubes) due to a higher quality and purity of these samples.

#	Name	C, ng/µL	260/280	260/230	V, µL	m, µg
1st elution						

1st tube	N protein	360.2	2.10	2.05	40	14.41
2nd tube	PBS	413.8	2.08	2.07	40	16.55
2nd elution						
3rd tube	N protein	8.84	1.95	1.10	40	0.35
4th tube	PBS	32.40	2.08	1.46	40	1.30

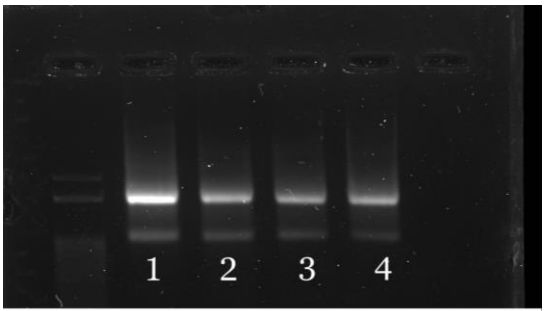
Table 8. Concentration and purity of RNA samples isolated from U87-MG cells for the 1st experiment.

#	Name	C, ng/µL	260/280	260/230	V, µL	m, µg
1st elution						
1st tube	PBS	847.5	2.07	1.73	40	33.90
2nd tube	N protein	556.6	2.06	1.60	40	22.26
2nd elution						
3rd tube	PBS	530.5	2.06	1.62	40	21.22
4th tube	N protein	372.0	2.05	2.13	40	14.88

Table 9. Concentration and purity of RNA samples isolated from U87-MG cells for the 2nd experiment.

#	Name	C, ng/µL	260/280	260/230	V, µL	m, µg
1st elution						
1st tube	PBS	885.2	2.13	2.09	40	35.40
2nd tube	N protein	808.9	2.14	2.18	40	32.35
2nd elution						
3rd tube	PBS	42.51	2.05	0.91	40	1.7
4th tube	N protein	29.11	2.01	0.29	40	1.1

Table 10. Concentration and purity of RNA samples isolated from U87-MG cells for the 3rd experiment.



#	Name	C, ng/µL	260/280	260/230	V, µL	m, µg
1st elution						
1st tube	PBS	847.5	2.07	1.73	40	33.90
2nd tube	N protein	556.6	2.06	1.60	40	22.26
2nd elution						
3rd tube	PBS	530.5	2.06	1.62	40	21.22
4th tube	N protein	372.0	2.05	2.13	40	14.88

Figure 7. The results of RNA electrophoresis for the 2nd experiment.

Obtained RNA samples were then used for synthesis of cDNA, which in turn is used for qRT-PCR. For the qRT-PCR we used one prime designed for IL-6 cytokine, to see the change in its concentration (primer sequence for IL-6 is used from previous paper [10]) and a primer for a housekeeping gene that is consistently expressed in the cells.

The raw values obtained from the first experiment (Table 10) show that the average Ct measurements were lower compared to the Ct value of the control sample, indicating that the levels IL-6 have increased after the cells were treated with recombinant N protein (1ug/mL). Based on the raw data it is hard to analyze the difference in Ct values, so the delta delta-Ct method was applied to compare the expression of IL-6 in an experimental sample with the control [23]. The expression fold change was found to be 137.38%, which indicates that the IL-6 levels increased by 37.38% in the stimulated cells compared to the control in the first experiment.

There are noticeable differences in the outcomes of the second and third procedures as well in comparison to the first one. Several additional primers for housekeeping genes were used (b_actin, GAPD) to see the difference in expression of IL-6 compared to the control, but SDHA is used for the comparison of the IL-6 expression between the experiments. The raw data of the two other experiments both show a lower Ct value for the experimental sample, but the difference is lower than in the previous experiment. After finding the expression fold change with the help of delta delta-Ct method only values for the SDHA primer showed very insignificant change in the expression (60.29% and 106.66% for 1st and 2nd trials respectively) of IL-6, or a change by -39.71 and 6.66%. Other 2 primers (b-actin, GAPD) show a great decrease in the concentration of the cytokine (6.38%, 44.13%, and 101.4%, 102.89% respectively).

Sample	**Karw_IL6**						
	cDNA			**NRT**	**NTC**	Karw_IL6	AVE Ct
N protein	24.51	24.28	24.42	0.00	0	N protein	24.40
PBS	25.43	25.51	25.42	0.00	0.00	PBS	25.45
Sample	**SDHA**						
	cDNA			**NRT**	**NTC**	SDHA	AVE Ct
N protein	24.68	24.03	24.13	0.00	39.54	N	24.28

					[redacted]	protein	
PBS	24.90	25.04	24.78	39.61	39.54	PBS	24.90

Table 11. Raw data for the Ct values of experimental and control samples for the 1st experiment

23/02/22	Average Experimental Ct value (**Karw_IL6**)	Average Experimental Ct value (**SDHA**)	Average Control Ct value (**Karw_IL6**)	Average Control Ct value (SDHA)	ΔCt value (Experimental) (SDHA)	ΔCt value (Control) (SDHA)	ΔΔCt value (SDHA)	Expression fold change (SDHA)
N protein	24.40	24.28	25.45	24.90	0.12	0.55	-0.43	134.38%

Table 12. Expression fold change calculated with delta delta-Ct method for the 1st experiment.

Sample	**Karw_IL6**						
	cDNA			**NRT**	**NTC**	Karw_IL6	AVE Ct
N protein	21.44	22.11	19.58	N/A	[redacted]	N protein	21.04
PBS	21.26	21.70	21.28	N/A	N/A	PBS	21.41
Sample	**b_actin**						
	cDNA			**NRT**	**NTC**	b_actin	AVE Ct
N protein	22.57	24.24	25.35	N/A	[redacted]	N protein	24.05
PBS	26.81	29.13	29.24	N/A	N/A	PBS	28.39
Sample	**GAPD**					GAPD	
N protein	20.24	20.82	20.41	35.95	[redacted]	N protein	20.49
PBS	21.32	20.60	20.60	35.63	35.24	PBS	20.84
Sample	**SDHA**						
	cDNA			**NRT**	**NTC**	SDHA	AVE Ct
N protein	27.26	28.82	28.41	N/A	[redacted]	N protein	27.84
PBS	29.38	28.49	29.01	40.56	N/A	PBS	28.94

Table 13. Raw data for the Ct values of experimental and control samples for the 2nd experiment

23/04/10	Average Experimental Ct value (**Karw_IL6**)	Average Experimental Ct value (b_actin)	Average Control Ct value (**Karw_IL6**)	Average Control Ct value (b_actin)	ΔCt value (Experimental) (b_actin)	ΔCt value (Control) (b_actin)	ΔΔCt value (b_actin)	Expression fold change (b_actin)
N protein	21.04	24.05	21.41	28.39	-3.01	-6.98	3.97	6.38%

23/04/10	Average Experimental Ct value (**Karw_IL6**)	Average Experimental Ct value (GAPD)	Average Control Ct value (**Karw_IL6**)	Average Control Ct value (GAPD)	ΔCt value (Experimental) (GAPD)	ΔCt value (Control) (GAPD)	ΔΔCt value (GAPD)	Expression fold change (GAPD)
N protein	21.04	20.49	21.41	20.84	0.55	0.57	-0.02	101.40%
23/04/10	Average Experimental Ct value (**Karw_IL6**)	Average Experimental Ct value (**SDHA**)	Average Control Ct value (**Karw_IL6**)	Average Control Ct value (SDHA)	ΔCt value (Experimental) (SDHA)	ΔCt value (Control) (SDHA)	ΔΔCt value (SDHA)	Expression fold change (SDHA)
N protein	21.04	27.84	21.41	28.94	-6.79	-7.52	0.73	60.29%

Table 14. Expression fold change calculated with delta delta-Ct method for the 2nd experiment.

Sample	**Karw_IL6**						
	cDNA			**NRT**	**NTC**	Karw_IL6	AVE Ct
N protein	24.14	24.07	24.08	36.90		N protein	24.10
PBS	24.77	24.85	24.72	38.18	N/A	PBS	24.78
Sample	**b_actin**						
	cDNA			**NRT**	**NTC**	b_actin	AVE Ct
N protein	21.75	21.71	21.55	N/A		N protein	21.67
PBS	23.52	24.17	22.92	36.72	38.51	PBS	23.54
Sample	**GAPD**						
	cDNA			**NRT**	**NTC**	GAPD	AVE Ct
N protein	17.95	17.79	17.85	32.72		N protein	17.86
PBS	18.49	18.58	18.45	33.25	33.00	PBS	18.51
Sample	**SDHA**						
	cDNA			**NRT**	**NTC**	SDHA	AVE Ct
N protein	24.17	23.61	23.55	37.43		N protein	23.78
PBS	24.46	24.41	24.24	39.50	38.33	PBS	24.37

Table 15. Raw data for the Ct values of experimental and control samples for the 3rd experiment.

23/04/17	Average Experimental Ct value (**Karw_IL6**)	Average Experimental Ct value (b_actin)	Average Control Ct value (**Karw_IL6**)	Average Control Ct value (b_actin)	ΔCt value (Experimental) (b_actin)	ΔCt value (Control) (b_actin)	ΔΔCt value (b_actin)	Expression fold change (b_actin)
N protein	24.10	21.67	24.78	23.54	2.42	1.24	1.18	44.13%

23/04/17	Average Experimental Ct value (**Karw_IL6**)	Average Experimental Ct value (GAPD)	Average Control Ct value (**Karw_IL6**)	Average Control Ct value (GAPD)	ΔCt value (Experimental) (GAPD)	ΔCt value (Control) (GAPD)	ΔΔCt value (GAPD)	Expression fold change (GAPD)
N protein	24.10	17.86	24.78	18.51	6.23	6.27	-0.04	102.89%
23/04/17	Average Experimental Ct value (**Karw_IL6**)	Average Experimental Ct value (**SDHA**)	Average Control Ct value (**Karw_IL6**)	Average Control Ct value (SDHA)	ΔCt value (Experimental) (SDHA)	ΔCt value (Control) (SDHA)	ΔΔCt value (SDHA)	Expression fold change (SDHA)
N protein	24.10	23.78	24.78	24.37	0.32	0.41	-0.09	106.66%

Table 16. Expression fold change calculated with delta delta-Ct method for the 3rd experiment.

V. Discussion

The effect of SARS-CoV-2 on the nervous system and its cells is still being thoroughly studied, and there is limited understanding on how it infects and disseminates within it. However, glial cells that are particularly responsible for the inflammatory response and consequent release of cytokines for the recruitment of immune cells to the site of infection [22].

a. Dot-blot assay

We can see from the outcomes of the results of dot-blot assay that in both experiments there was a consistent set of the cytokines (IL-8, TIMP-2, MCP-1, sTNF-RII, GCSF, MIP-1α) that were expressed and release of which might have been affected by the stimulation with N protein. Cytokines that were expressed by U87-MG cells cultured in the presence of N protein show different set of cytokines compared to stimulated macrophages and monocytes in the past papers, indicating that glial cells express different particles in normal conditions as well as when affected by external stimuli. We can see that the same cytokines are released by both experimental and control groups. The raw signal density values that were then normalized, show that IL-6 and IL-8 are affected by N protein the most, as both experiments show their higher level in these samples than in control cells. However, the difference is more noticeable in the 1st experiment than in the 2nd one, so in order to see if the degree of upregulation of expression is repeatable. In addition, t-test showed that the difference in the levels of IL-6 and IL-8 is not significant, as p values for both are >0.05. Concentration of TIMP-2 and MCP-1 also rose in response to N protein, but only for the 1st experiment, p value for MCP-1 was <0.05, showing a significant difference in the release of this cytokine. The second trial did not show a great difference, but in turn indicated a slight

decrease in their expression, so the results are not consistent. Lastly MIP-1α, sTNF-RII, and GSCF were as well released by U87-MG, and particularly the release of GCSF has been affected the most according to the p value for the first experiment. As we can see besides IL-6, IL-8 and TIMP-1 are affected by the stimulation with N protein, so it seems to be reasonable to test the difference in their expression further with the use of qRT-PCR, for which specific primers will be designed to measure the change in their concentration as a result of interaction with nucleocapsid protein of SARS-CoV-2. It is also necessary to repeat dot-blot assay several more times to see if the results are consistent with each other and the observations in the expression of cytokines are repeatable within the trials.

b. ELISA

The problem with the previous part of the procedure could have been caused by the unsuitable conditions for the concentration of recombinant N protein and incubation time, as these were established based on the previous paper [10]. So, we conducted the ELISA test after 3 different incubation times (12h, 24, and 48h) and various concentrations (0ng/mL, 300ng/mL, 1ug/mL, 3ug/mL, 10ug/mL). Outcomes indicate that the most significant difference can be observed in a sample incubated for 24 hours at concentration of 10ug/mL. Therefore, for the next attempts of dot-blot and ELISA the change in these variables will be applied to get more accurate results with a greater difference in the expression of some cytokines. However, during the ELISA procedure the building of the standard curve was impossible as the concentration of the samples used for the standard were incorrect, so before using these conditions for the next experiment it is important to conduct at least two more ELISA to make sure these parameters will actually show a significant difference in the concentration of IL-6.

c. qRT-PCR

Next, we measured the concentration of mRNA IL-6 in experimental and control samples with the help of qRT-PCR and specifically designed primer (Karw_IL-6) that was used in the previous study on macrophages and monocytes [10]. Two qRT-PCR were performed and in both cases the decrease in Ct value or increase in concentration of IL-6 in the experimental sample can be observed. The results of the delta delta-Ct method using the raw Ct values for the first experiment show that there was a noticeable upregulation of expression (34.38%) in the levels of the cytokine of interest. Nevertheless, for the second and third procedures despite the fact that Ct value was lower for the sample with N protein, the delta delta-Ct method did not show any significant

difference in the concentration of IL-6 with different primers. The average change in the expression for SDHA primer is 100.4%, which indicates that there is no significant difference in the concentration of IL-6 mRNA in the experimental samples. Main reason for that is the fact that the second experiment showed negative results, lowering the average expression change. It shows that it is necessary to conduct qRT-PCR several more times to see if the upregulation of the expression for different trials follows the trend and is consistent. Here, as well as for dot-blot assay, it might be necessary to change the conditions for the further experiments, as the concentration of recombinant N protein used for these experiments might not be sufficient to significantly affect the release of IL-6.

VI. Conclusion

SARS-CoV-2 N protein has an effect on the release of various inflammatory cytokines from the infected cells, such as alveolar cells, macrophages and monocytes, leading to the rise of cytokine storm. In CNS glial cells are the major producers of cytokines, might also contribute to the acute inflammation and severe symptoms might also be affected by N protein. Our findings show that the expression IL-6 that is particularly responsible for the inflammatory signal transduction during the infection is affected when stimulated with the nucleocapsid protein of SARS-CoV-2. In addition, in response to this viral particle other inflammatory proteins are released by U87-MG cells, showing the importance in studying how far the concentration of IL-8 and TIMP-2 can change in response to it. Despite the fact that it is hard to judge, based on our findings, how significantly the upregulation of IL-6 and other cytokines happen when cultured in presence of N protein, we can see that with a modified approach we will be able to obtain the desired outcomes.

VII. Acknowledgments

I would like to thank my PI Dr. Olena Filchakova for offering me this opportunity to work and conduct experiments in her laboratory, for always giving directions and trying to help me anytime I struggled with procedure or theory part of my thesis.

In addition, I want to thank my RAs Zhanar Kunushpayeva and Bakhytgul Gadilgereyeva for teaching me all the necessary skills and assisting me during experiments.

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IX. Appendix

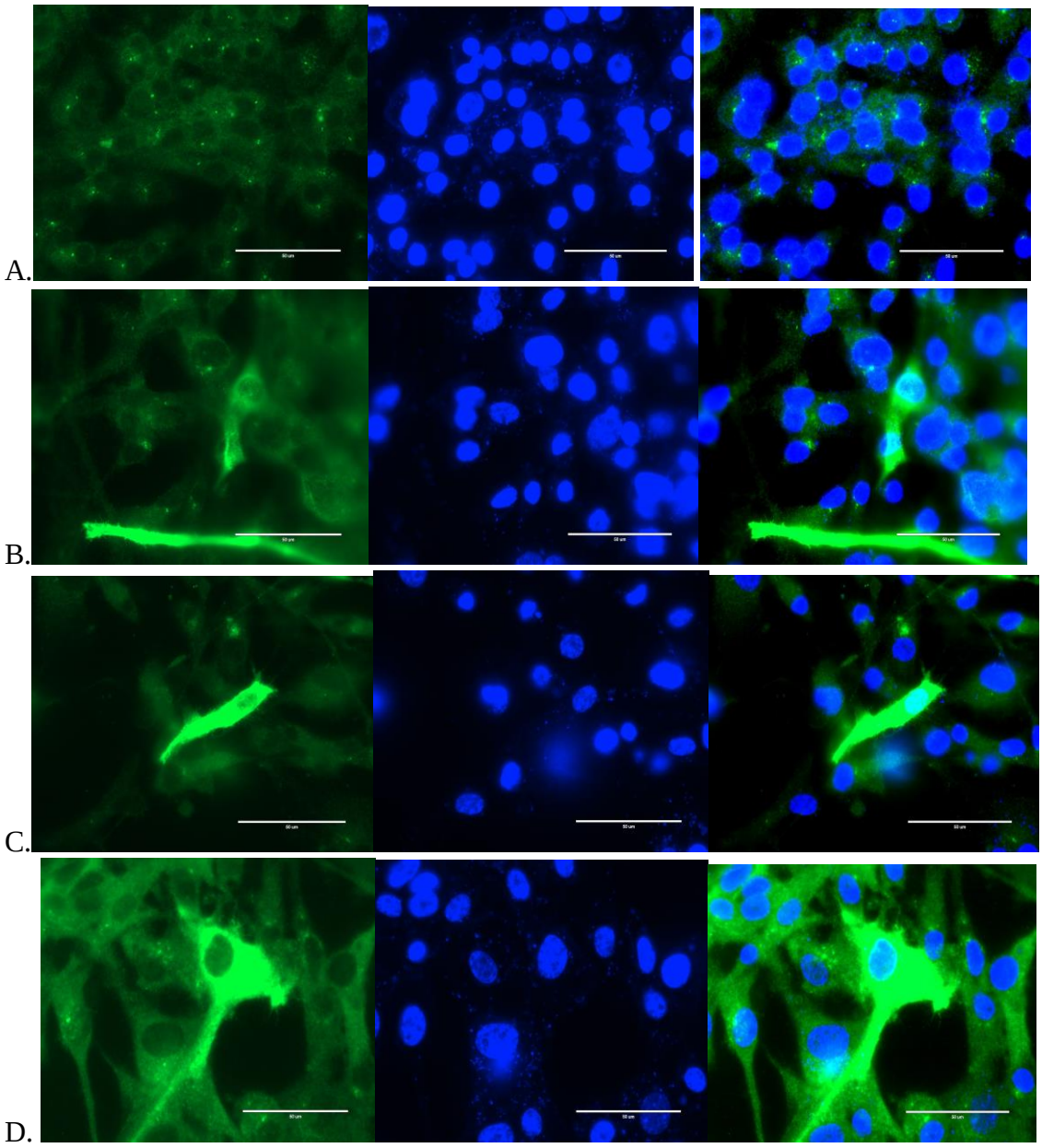
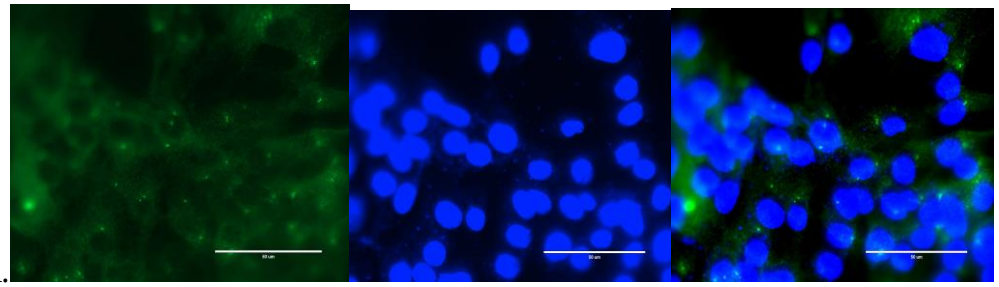


Figure 8. U-87 MG cells trasnfected with pCMV plasmid containing myc-DDK vector for N protein gene observed on EVOS (60X). (**A**) The results for cells in the first well, first trial. (**B**) The results for cells in the second well, first trial. (**C**) The results for cells in the first well, second trial. (**D**) The results for cells in the second well, second trial

A.


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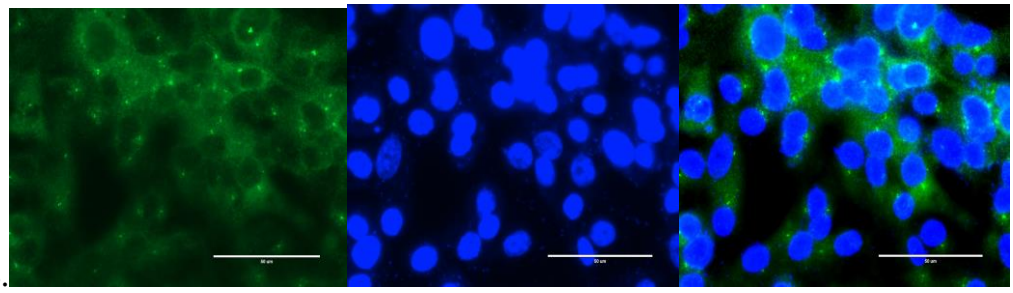
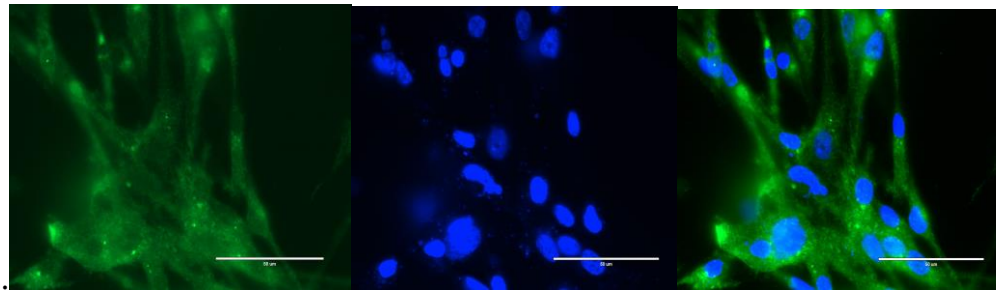


C.


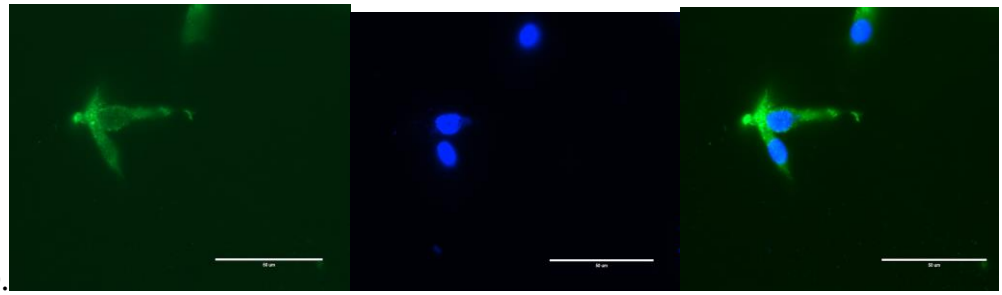
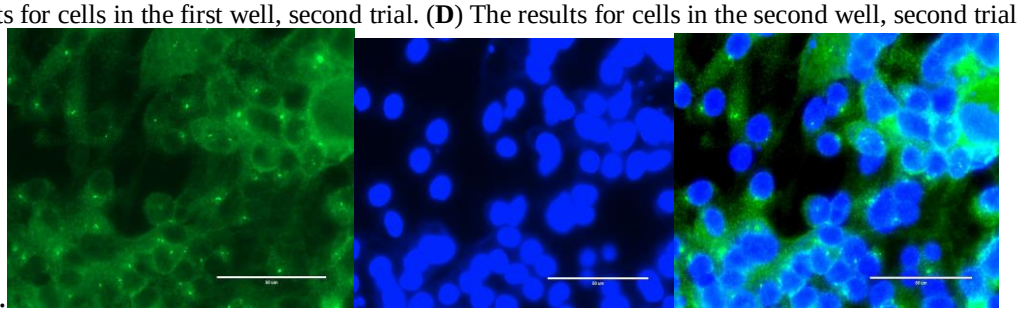
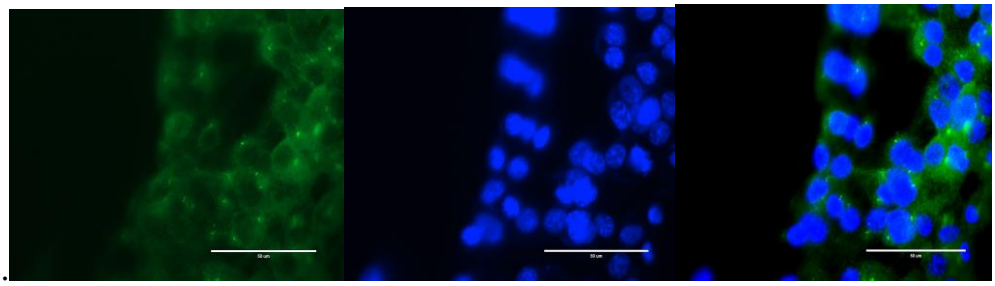
D.


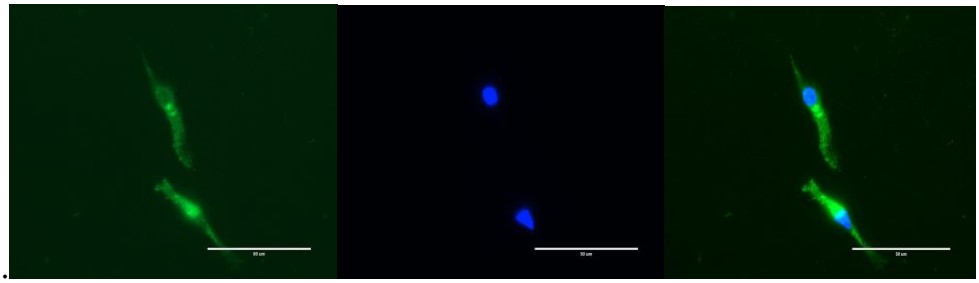
Figure 9. U-87 MG cells trasnfected with an empty pCMV plasmid observed on EVOS (60X). (**A**) The results for cells in the first well, first trial. (**B**) The results for cells in the second well, first trial. (**C**) The results for cells in the first well, second trial. (**D**) The results for cells in the second well, second trial

A.


B.



C.



D.

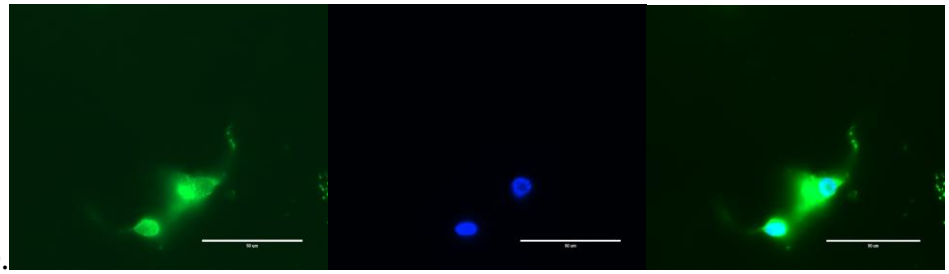
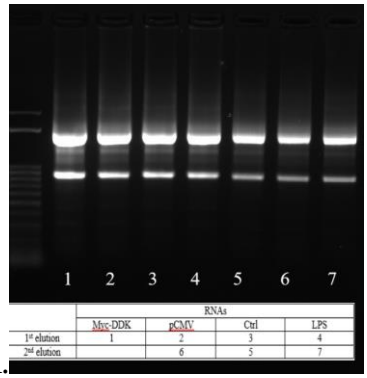
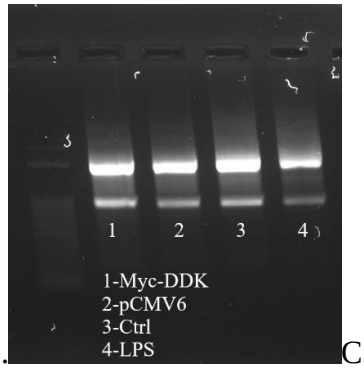


Figure 10. U-87 MG control cells. (**A**) The results for cells in the first well, first trial observed on EVOS (60X). (**B**) The results for cells in the second well, first trial. (**C**) The results for cells in the first well, second trial. (**D**) The results for cells in the second well, second trial

A.



B.



C.

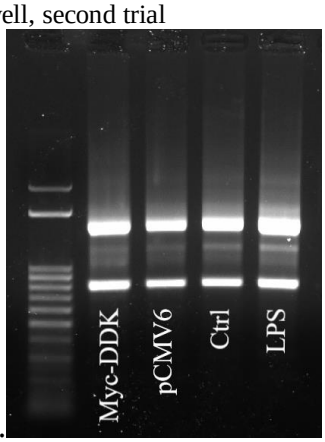


Figure 11. The results of RNA electrophoresis. (**A**)First trial of RNA electrophoresis, procedure is performed on 22/10/10. (**B**) Second trial of RNA electrophoresis. Procedure is performed on 22/10/21 (**C**) Second trial of RNA electrophoresis. Procedure is performed on 22/11/03

#	Name	C, ng/µL	260/280	260/230	V, µL	m, µg
1st elution						
1st tube	Myc-DDK	802.5	2.10	2.20	45	36.11
2nd tube	pCMV6	1639	2.14	1.94	45	73.75
3rd tube	Ctrl	839.2	2.12	1.59	45	37.78
4th tube	LPS	691.6	2.13	1.32	45	31.12
2nd elution						
5th tube	Myc-DDK	327.3	2.08	2.17	45	14.73
6th tube	pCMV6	511.4	2.01	2.03	45	23.01
7th tube	Ctrl	412.9	2.07	1.87	45	18.58
8th tube	LPS	521.5	2.02	1.79	45	23.47

Table 17. The quality of RNA samples for the first experiment. Procedure is performed on 22/10/07

#	Name	C, ng/µL	260/280	260/230	V, µL	m, µg
1st elution						
1st tube	Myc-DDK	349.4	2.07	2.22	45	15.72
2nd tube	pCMV6	359.9	2.08	1.56	45	16.19
3rd tube	Ctrl	277.3	2.10	1.61	45	12.48
4th tube	LPS	322.3	2.09	1.30	45	14.50
2nd elution						
5th tube	Myc-DDK	116.4	2.09	2.27	45	5.24
6th tube	pCMV6	71.01	2.12	1.00	45	3.19
7th tube	Ctrl	115.8	2.15	0.56	45	5.21
8th tube	LPS	177.7	2.10	1.78	45	8.00

Table 18. The quality of RNA samples for the second experiment. Procedure performed on 22/10/20

#	Name	C, ng/µL	260/280	260/230	V, µL	m, µg
1st elution						
1st tube	Myc-DDK	316.5	2.08	2.09	45	14.24
2nd tube	pCMV6	195.5	2.08	1.99	45	8.8
3rd tube	Ctrl	365.4	2.05	2.12	45	16.4
4th tube	LPS	355.8	2.05	1.92	45	16.01
2nd elution						
5th tube	Myc-DDK	182.2	2.05	2.18	45	8.2
6th tube	pCMV6	112.3	2.01	2.06	45	5.05
7th tube	Ctrl	241.8	2.05	2.11	45	10.88
8th tube	LPS	146.8	2.02	2.12	45	6.61

Table 19. The quality of RNA samples for the first experiment. Procedure performed on 22/11/03

Conc	LOG conc	Ct1	Ct2	AVE Ct			
1.00E-05	-5.00	0.00	0.00	0.00	y =		
1.00E-04	-4.00		38.04	38.04	-3.3385x		
1.00E-03	-3.00	33.76	35.58	34.67	+ 24.55		$R^2 = 0.9902$
1.00E-02	-2.00	30.51	30.71	30.61	Slope	Intercept	Efficiency
1.00E-01	-1.00	28.22	28.31	28.27	-3.3385	24.55	99.3%

Table 20. Efficiency of Zhang primer. Procedure was performed on 22/07/07

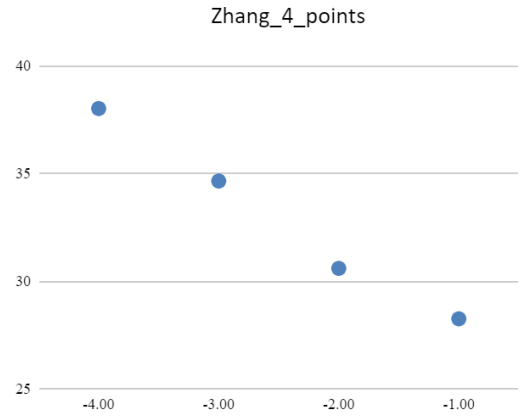


Figure 12. Graph built to calculate the efficiency of the primer

Conc	LOG conc	Ct1	Ct2	AVE Ct
1.00E-05	-5.00	28.87	28.79	28.83
1.00E-04	-4.00	25.14	25.03	25.09
1.00E-03	-3.00	21.18	21.26	21.22
1.00E-02	-2.00	17.42	17.66	17.54
1.00E-01	-1.00	14.59	14.52	14.56

y = -3.527x + 10.783		R^2=0.9967
Slope	Intercept	Efficiency
-3.527	10.783	92.1%

Table 21. Efficiency of GAPD primer. Procedure was performed on 22/10/03

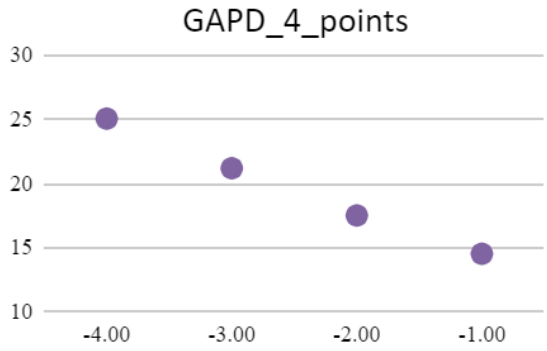
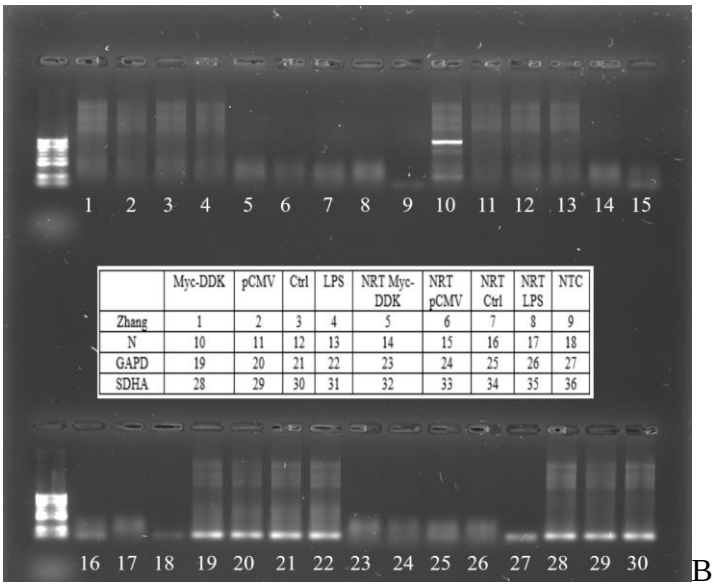
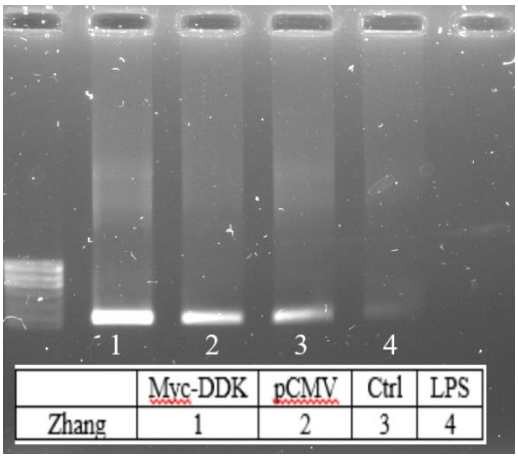


Figure 13. Graph built to calculate the efficiency of the primer



	Myc-DDK	pCMV	Ctrl	LPS	NRT Myc-DDK	NRT pCMV	NRT Ctrl	NRT LPS	NTC
Zhang	1	2	3	4	5	6	7	8	9
N	10	11	12	13	14	15	16	17	18
GAPD	19	20	21	22	23	24	25	26	27
SDHA	28	29	30	31	32	33	34	35	36

B.



	Myc-DDK	pCMV	Ctrl	LPS
Zhang	1	2	3	4

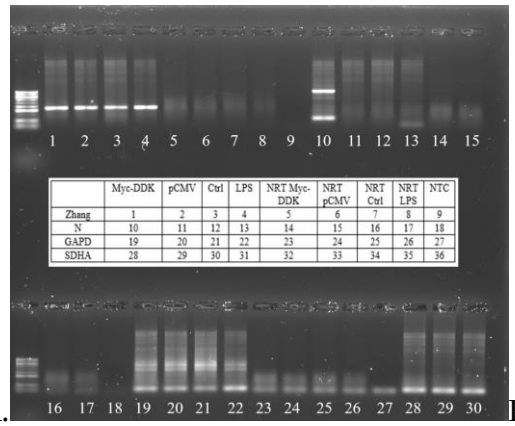
Figure 14. Electrophoresis of synthesized DNA samples for the first trial.(**A**) Procedure was performed on 22/10/07. (**B**) Procedure was performed on 22/10/11

Exp	Myc-DDK	pCMV6	Ctrl	LPS
Zhang				
N	2032			
GAPD	379	355	355	355
SDHA	379	379	379	

Linear	Myc-DDK	pCMV6	Ctrl	LPS
Zhang				
N	2035			
GAPD	380	356	356	356
SDHA	380	380	380	

Table 21. Bands of obtained DNA samples for the first trial

A.



B.

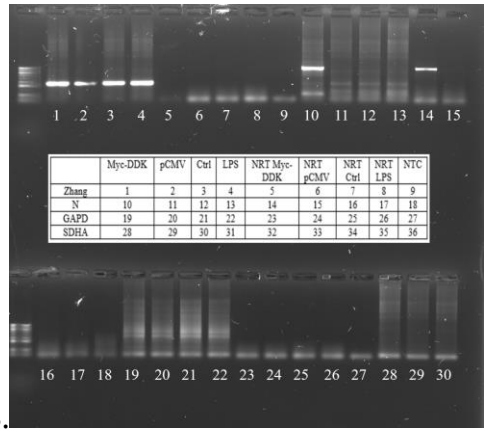


Figure15. Gel electrophoresis of synthesized DNA samples for the second trial.(**A**) Gel electrophoresis performed on 22/10/21 (**B**) Gel electrophoresis performed on 22/11/07

A.

Exp	Myc-DDK		pCMV6	Ctrl	LPS	Linear	Myc-DDK		pCMV6	Ctrl	LPS
Zhang	407		442	442	442	Zhang	407		442	442	442
N	1394	270				N	1393	270			
GAPD	240		240	240	240	GAPD	241		241	241	241
SDHA	240		240	240	132	SDHA	241		241	241	132

B.

Exp	Myc-DDK		pCMV6	Ctrl	LPS
Zhang	495		495	495	507
N	1413	304			
GAPD	239		239	239	239
SDHA	239		239	239	127

Table 22. Bands of obtained DNA samples for the second trial. (**A**) Procedure performed on 22/10/21 (**B**) Procedure performed on 22/11/07

A.

Sample	Zhang				
	cDNA			NRT	NTC
Myc-DDK	24.07	24.11	24.01	0	0
pCMV6	25.31	25.30	25.47	0	
Ctrl	23.51	23.59	23.70	0	
LPS	25.13	25.09	25.19	0	

Zhang	AVE Ct
Myc-DDK	24.06
pCMV6	25.36
Ctrl	23.60
LPS	25.14

B.

Sample	GAPD				
	cDNA			NRT	NTC
Myc-DDK	18.47	18.23	18.70	35.86	35.24
pCMV6	18.98	18.94	19.01	36.18	
Ctrl	18.57	18.41	19.01	35.53	
LPS	18.40	18.62	18.54	36.57	

GAPD	AVE Ct
Myc-DDK	18.47
pCMV6	18.98
Ctrl	18.66
LPS	18.52

C.

22/10/12	Average Experimental Ct value (**Zhang**)	Average Experimental Ct value (**GAPD**)	Average Control Ct value (**Zhang**)	Average Control Ct value (GAPD)	ΔCt value (Experimental) (GAPD)	ΔCt value (Control) (GAPD)	ΔΔCt value (GAPD)	Expression fold change (GAPD)
Myc-DDK	24.06	18.47	23.60	18.66	5.60	4.94	0.66	63.29%
pCMV6	25.36	18.98	23.60	18.66	6.38	4.94	1.45	36.69%

Table 23. CT values for U-87 MG after real time PCR. (**A**) Ct values for Zhang primer for the experiment performed on 22/10/12. (**B**) Ct values for GAPD primer for the experiment performed on 22/10/12 (**C**) The analysis of obtained results using delta delta method

A.

Sample	Zhang				
	cDNA			NRT	NTC
Myc-DDK	25.18	25.18	24.90	N/A	40.97
pCMV6	25.42	26.16	25.61	N/A	
Ctrl	25.79	26.01	26.75	40.49	
LPS	26.67	26.28	26.39	N/A	

Zhang	AVE Ct
Myc-DDK	25.09
pCMV6	25.73
Ctrl	26.18
LPS	26.45

B.

Sample	GAPD				
	cDNA			NRT	NTC
Myc-DDK	17.51	17.65	17.45	34.20	33.13
pCMV6	18.16	18.03	18.08	33.76	
Ctrl	18.74	18.64	18.35	34.37	
LPS	19.02	18.94	18.86	34.93	

GAPD	AVE Ct
Myc-DDK	17.54
pCMV6	18.09
Ctrl	18.58
LPS	18.94

C.

22/11/10, cDNA of 3rd experiment	Average Experimental Ct value (**Zhang**)	Average Experimental Ct value (**GAPD**)	Average Control Ct value (**Zhang**)	Average Control Ct value (GAPD)	ΔCt value (Experimental) (GAPD)	ΔCt value (Control) (GAPD)	ΔΔCt value (GAPD)	Expression fold change (GAPD)
Myc-DDK	23.45	18.49	24.19	18.84	4.95	5.35	-0.39	131.34%
pCMV6	23.57	18.99	24.19	18.84	4.59	5.35	-0.76	169.35%

D.

Sample	Zhang				
	cDNA			NRT	NTC
Myc-DDK	23.53	23.29	23.52	40.45	N/A
pCMV6	23.59	23.60	23.53	N/A	
Ctrl	24.26	24.08	24.22	N/A	
LPS	23.27	25.16	25.19	N/A	

Zhang	AVE Ct
Myc-DDK	23.45
pCMV6	23.57
Ctrl	24.19
LPS	24.54

E.

Sample	GAPD				
	cDNA			NRT	NTC
Myc-DDK	18.59	18.38	18.51	33.66	32.89
pCMV6	18.98	19.04	18.94	33.70	
Ctrl	18.86	18.78	18.88	33.57	
LPS	18.71	18.9	18.95	36.1	

GAPD	AVE Ct
Myc-DDK	18.49
pCMV6	18.99
Ctrl	18.84
LPS	18.85

F.

22/11/10, cDNA of 2nd experiment	Average Experimental Ct value (**Zhang**)	Average Experimental Ct value (**GAPD**)	Average Control Ct value (**Zhang**)	Average Control Ct value (GAPD)	ΔCt value (Experimental) (GAPD)	ΔCt value (Control) (GAPD)	ΔΔCt value (GAPD)	Expression fold change (GAPD)
Myc-DDK	25.09	17.54	26.18	18.58	7.55	7.61	-0.06	104.01%
pCMV6	25.73	18.09	26.18	18.58	7.64	7.61	0.03	97.72%

Table 24. CT values for U-87 MG cells after real time PCR. (**A**)Ct values for Zhang primer for the experiment performed on 22/11/10. (**B**) Ct values for GAPD primer for the experiment performed on 22/11/10. (**C**) The analysis of the obtained data using the delta delta method. (**D**) Ct values for Zhang primer for the experiment performed on 22/11/10. (**E**) Ct values for GAPD primer for the experiment performed on 22/11/10. (**F**) The analysis of the obtained data using the delta delta method