University of South Bohemia in České Budějovice

Faculty of Science

Antiviral activity of selected IFN-β-stimulated genes

Bachelor thesis

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Annotation

The main aim of this thesis is to determine the possible antiviral activity of IFI6 and IFI27 genes in case of TBEV infection in DAOY cells. Both genes belong to the large family of interferon-stimulated genes and were previously shown to be highly up-regulated in DAOY cells treated with IFN-β, which lead to the significant decrease of TBEV titres. To investigate the possible role of IFI6 and IFI27 in this effect, they were both cloned separately into mammalian expression vectors and their contribution to an anti-viral state in DAOY cells was elucidated, using an *in vitro* transient over-expression.

Declaration

I hereby declare that I have worked on my bachelor's thesis independently and used only the sources listed in the bibliography.

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Armig Kabrelian

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I would also like to express my gratefulness to my loving family for their profound support and love throughout my hard times.

And lastly, I would like to dedicate this thesis to the memory of my father, Dr. Vasken Kabrelian, whom I miss every day, but I am glad that he could see my progresses throughout my life, and who always offered me support, as well as plenty of encouragement.

Abstract

This project consists of two parts: Cloning of IFI6 and IFI27 genes into mammalian expression vectors, and *in vitro* analyses of IFI6 and IFI27 antiviral effect in case of tick-borne encephalitis virus infection in human medulloblastoma cell line (DAOY). The results of the first part showed that the genes of interest were successfully cloned into the mammalian expression vector. For the second part, in case of DNA transfection of IFI6/IFI27 constructs, the viability assay showed significant inhibition of virus-induced cytopathic effect, but the plaque assay couldn't show any significant decrease in viral titres. Both, IFI6 and IFI27 couldn't be detected on mRNA and protein levels using real-time PCR and western blot (using anti-IFI6/27 as well as anti-V5 tag antibodies). The procedure of transfection was repeated with mRNA instead of plasmid DNA, and still no difference was found in plaque titration, indicating that probably the overexpression didn't work again. Further experiments are in progress to optimize the proper transfection conditions.

List of used abbreviations

AP = Alkaline phosphatase

BBB = Blood-brain barrier

BCIP = 5-bromo-4-chloro-3-indoxylphophate

cDNA = Complementary Deoxyribonucleic acid

CDP-star = Disodium 2-chloro-5-(4-methoxyspiro[1,2-dioxetane-3,2′-(5-chlorotricyclo[3.3.1.1$^{3.7}$]decan])-4-yl]-1-phenyl phosphate

CMC = Carboxymethyl cellulose

CMV = Cytomegalovirus

CNS = Central nervous system

CPE = Cytopathic effect

DAOY = Desmoplastic cerebellar human medulloblastoma cells

DEPC = Diethyl pyrocarbonate

D-MEM = Dulbecco's modified eagle medium

DMSO = Dimethyl sulfoxide

DNA = Deoxyribonucleic acid

dNTPs = Deoxyribose nucleoside triphosphate

FBS = Foetal bovine serum

HPRT = Hypoxanthine guanine phosphoribosyl transferase

HBSS = Hank's balanced salt solution

HCMV = Human cytomegalovirus

HIV = Human immunodeficiency virus

IFITM = Interferon-induced transmembrane

IFN = Interferon

IRF9 = Interferon regulatory factor 9

ISGF3 = Interferon-stimulated gene factor-3 complex

ISGs = Interferon-stimulated genes

JAK = Receptor-associated Janus kinase

MDB = Membrane desalting buffer

MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Mx1 = Murine myxovirus resistance 1

NBT = Nitro blue tetrazolium chloride

NS = Non-structural proteins

OAS = Oligoadenylate synthetase

ORF = Open reading frame

PAMPs = Pathogen-associated molecular patterns

PBS = Phosphate-buffered saline

PBS-T = Phosphate-buffered saline with Tween-20

PCR = Polymerase chain reaction

PRRs = Pattern recognition receptors

PTS = Precolostral calf serum

PVDF = Polyvinylidene fluoride

RIPA buffer = Radioimmunoprecipitation assay buffer

RNA = Ribonucleic acid

RNaseL = Endoribonuclease L

SARS-CoV = Severe acute respiratory syndrome coronavirus

STAT = Signal transducers and activators of transcription

TAE buffer = Tris-acetate-EDTA buffer

TBEV = Tick-borne encephalitis virus

TE buffer = Tris-EDTA buffer

ß-ME = ß-mercaptoethanol

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1 Introduction

Central European tick-borne encephalitis virus (TBEV) is a causative agent of one of the most dangerous viral meningoencephalitides. It enters the central nervous system (CNS) through the blood-brain barrier (BBB) and causes severe encephalitis. Around 3,000 patients annually are reported to be affected by TBEV worldwide (Gelpi et al., 2005). TBEV belongs to the tick-borne flavivirus group, family *Flaviviridae*, genus Flavivirus (Lindquist, Vapalahti, 2008). Although the disease can be prevented by vaccination, no treatment is available up to date. Therefore, the possibility of triggering any antiviral activity within the CNS would be noteworthy.

1.1 Tick-Borne Encephalitis Virus

As mentioned above, tick-born encephalitis belongs to the *Flaviviridae* family. It is known to be one of the most dangerous agents that causes lethal central nervous system diseases, featuring symptoms of influenza-like syndrome, severe meningitis, neurological sequelae, and characteristic neuropathologic changes that feature a multinodular to patchy polioencephalomyelitis accentuated in spinal cord, brainstem, and cerebellum (Gelpi et al., 2005). TBEV has three different subtypes: European, Far Eastern, and Siberian.

All the flaviviruses share common structural features: virions have the form of 50 nm sphere that contains viral RNA of about 11 kb encoding for one open reading frame (ORF) translated into one single polyprotein of 3414 amino acids, which is co- and post-translationally cleaved by viral and cellular proteases to three structural proteins (membrane protein (M), envelope glycoprotein (E) and a capsid protein (C) as represented in Figure 1) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Lindquist, Vapalahti, 2008). The capsid protein C has the role of encapsulating the viral RNA, whereas the glycoprotein E plays an important role during the reproductive stages of the virus and also facilitates the interaction between the viral surface and the host cells. On the other hand, the E protein acts as the most important antigen and functions both as a ligand to the cell receptor and as a fusion protein. Virions use clathrin-mediated endocytosis to enter the cells. The low pH in the endosomes triggers fusion between the virus and host cell resulting in the release of infectious viral genomes to the host cytoplasm. Further, translation of the viral genome results in the replication and production of new viruses (Lindquist, Vapalahti, 2008).

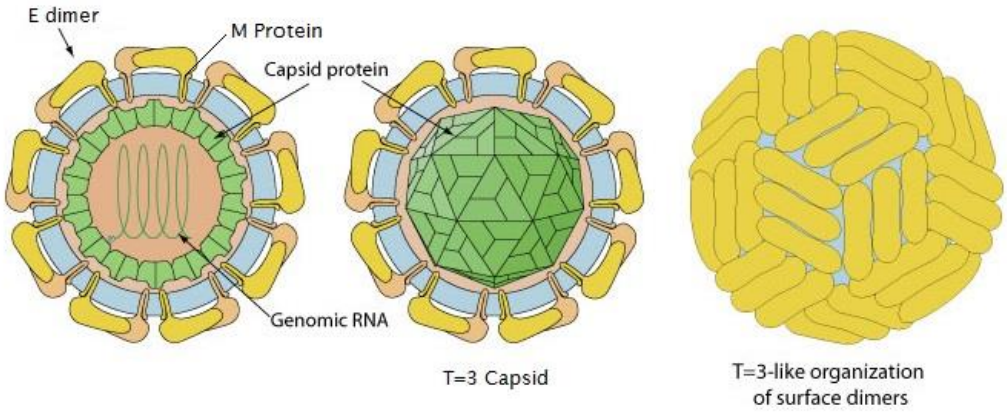


Figure 1: Structure of the tick-born encephalitis virus. Source: https://laboratoryinfo.com/zika-virus-structure-epidemiology-pathogenesis-symptoms-laboratory-diagnosis-and-prevention/

1.2 Vectors

The tick-borne encephalitis virus is an arbovirus that uses vectors like *Ixodes ricinus* tick in order to circulate in nature. The TBEV circulates within a 'parasitic triangle' of interactions between virus, vector ticks, and tick hosts (Süss, 2011). Tick vectors have their natural habitats in the forests of Europe and Asia, where ticks can find high humidity. Once ticks are infected, they remain infected throughout their life cycle and transmit virus to vertebrate hosts when feeding on them, since they provide a possible environment for the virus to multiply and circulate in the nature.

Ticks are globally distributed, and around 850 species have been classified (Süss, 2011). One of the most important tick species is *I. ricinus* that except transmitting TBEV in Europe and Asia transmits also many different disease agents like *Borrelia burgdorferi* s.l. in Eurasia and North America, human babesiosis, which is caused by *Babesia microti* in North America and *Ba. divergens* in Europe (Süss, 2011).

Most frequent endemic areas of *I. ricinus* ticks are found in European countries and Asia. Humans get infected by the virus through the bite of an infected tick vector, but they are just a dead-end infection host for the virus (Mandl, 2005). The following Figure 2 shows the distribution of different tick vectors worldwide.

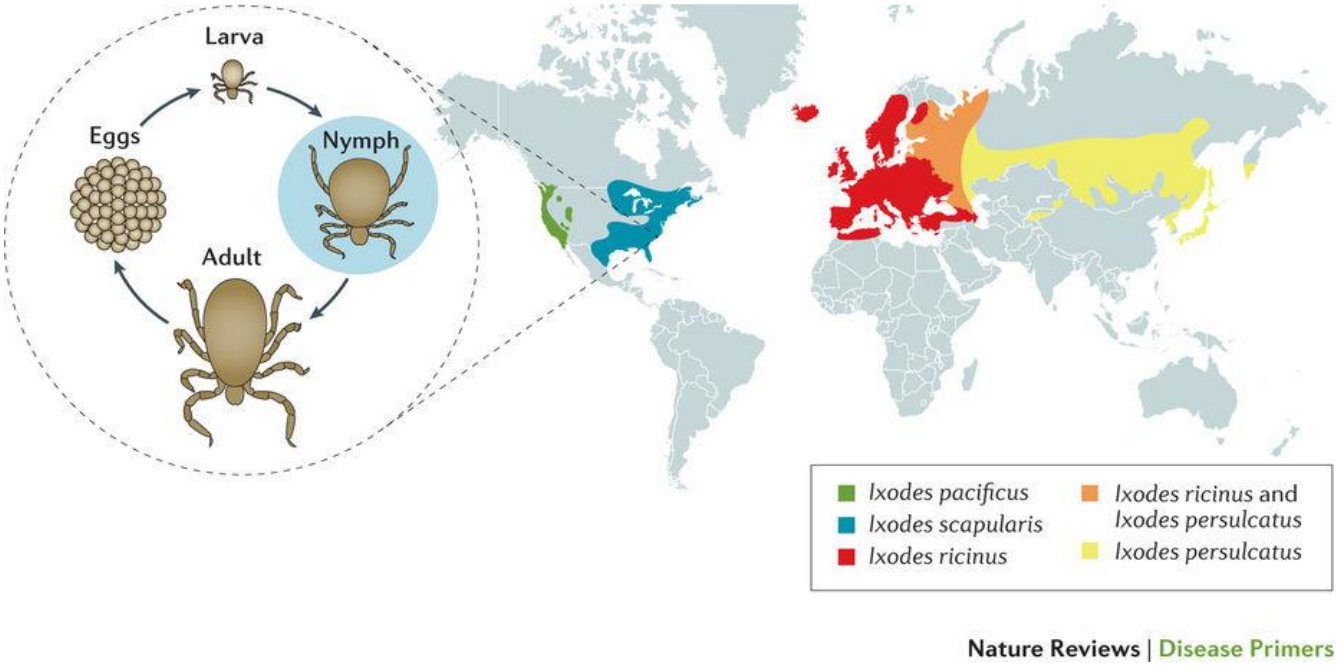


Figure 2: Global distribution of *Ixodes* tick vectors. Source: http://www.nature.com/articles/nrdp201690/figures/2

1.3 Immune system

The immune system is a complex network of specialized cells and molecules, which represents the defence system of the body against external pathogens, such as bacteria, parasites, and viruses. The activity of immune system is based on its ability to find and distinguish pathogens and to deactivate them. Once the immune system is exposed to certain antigens, it gains a memory, that leads to more efficient immune response upon re-exposure to the same antigen. The immune system is classified into two major responses: Innate response and Adaptive response.

1.4 Innate response

Innate immunity, or also known as natural non-specific immunity, is the first line of defence of the body, which also carries a significant burden of defence against viral pathogens. It consists of humoral immunity and a cellular immunity.

The components of humoral immunity are complement, coagulation, and plasma and serum proteins. The cellular immunity consists of phagocytes, like natural killers and macrophages which are specialized in degrading and eliminating pathogens, mast cells that are inflammatory regulators, and dendritic cells. The innate response is the primary defence line of the host body, which eliminates the pathogens (Alberts, 2002). Furthermore, the innate response has specific receptors, known as pattern recognition receptors (PRRs), that

recognize the viral genome or proteins and get activated, which triggers the activation of signalling cascade that produces interferons (Crosse et al., 2018).

1.5 Adaptive response

Adaptive immunity is a specialized defence that gets activated after an exposure to an antigen, at its first encounter to the antigen it takes longer time establish its component but later it works even quicker than innate response. The activation of adaptive response depends on the innate response, which provides information for the adaptive response to mobilize. The main parts that make up the adaptive response are cell-mediated immune response (T cells) and humoral immune response (B cells). T cells form in the bone marrow, then migrate to thymus to mature and become differentiated T cells. B cells, which also form in bone marrow, mature there then move to lymphatic system and circulate until they encounter an antigen which causes them to mature and produce antibodies. Both T cells and B cells are essential for antiviral defence. Adaptive immunity is also known to have an immunological memory by which it ensures long-term protection on re-exposure to the same antigen (Alberts, 2002).

1.6 Interferons

Interferons (IFNs) were firstly discovered in 1950, and later described by Isaacs & Lindenmann (Isaacs et al., 1957, Sorgeloos et al., 2013) as small soluble proteins that are secreted by the cells after the PRR detects the pathogen-associated molecular patterns (PAMPs). It was demonstrated that upon IFN production when influenza virus was present, it interferes with the viral infection (Schneider et al., 2014). IFNs have variety of functions like inhibition of virus growth, antitumoral, antiviral, immunomodulatory, and antiparasitic actions.

Up to date, 3 types of IFNs have been identified:

- Type I, contains IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω, are the largest subgroup of interferon proteins that help to regulate the activity of the immune system (Rosebeck, Leaman, 2008).
- Type II, contains IFN-γ, is produced in activated T-cells and natural killer cells and has some anti-viral and anti-tumour effects.
- Type III, contains IFN-λ.

IFNs are produced in many cell types in case of viral infections (Sorgeloos et al., 2013). But only type I IFNs have been shown to have a particular importance in restricting viral infections, which was documented for a wide spectrum of neurotropic viruses, like Theiler's encephalomyelitis virus, La Crosse encephalitis virus, and Sindbis virus in mice lacking the IFNAR-I subunit of the type I IFN receptor (Schneider et al., 2014, Selinger et al., 2017, Sorgeloos et al., 2013).

Interferons can act on two different levels, intracellularly and extracellularly. In case of intracellular signalling they cause the activation of immune system in infected cells by binding of IFNs onto the cell receptors and activating the receptor-associated Janus kinases. On extracellular level they signal to nearby cells to increase their anti-viral defences. Thus, the IFN-mediated innate immune response provides a robust first line of defence against invading pathogens. Upon IFN binding to cell surface receptors, a signal is transmitted through JAK-STAT signalling pathway, leading to dramatic changes in cellular properties that involve the activation of specific signalling receptors and the transcription of several antiviral genes.

1.7 JAK-STAT

JAK-STAT is a signalling pathway, which is triggered upon IFN binding to the receptor. The IFN specific receptor is called type-I IFN receptor complex that is on the cell surface and composed of two subunits (IFNAR-1 and IFNAR-2) (Booy et al., 2014). When INF-α or -β bind to the specific type-I IFN receptor, this causes the receptor to dimerize, which brings the receptor-associated Janus kinases (JAKs) into close proximity, where the JAKs phosphorylate each other. Once JAKs are activated they phosphorylate the signal transducers and activators of transcription ($STAT_1$ and $STAT_2$), and those form a dimer that in turn associates with interferon regulatory factor 9 (IRF9) to form interferon-stimulated gene factor-3 complex (ISGF3). This complex is translocated to the cell nucleus and bounds to specific gene sequences that regulate the expression of interferon-stimulated genes (ISGs). Furthermore, there are different numbers of STAT proteins in various cells, and activation of different receptors will lead to the formation of different STAT dimers, and in turn this leads to the transcription of different genes. Figure 3 gives a brief overlook into the whole JAK-STAT signalling cascade.

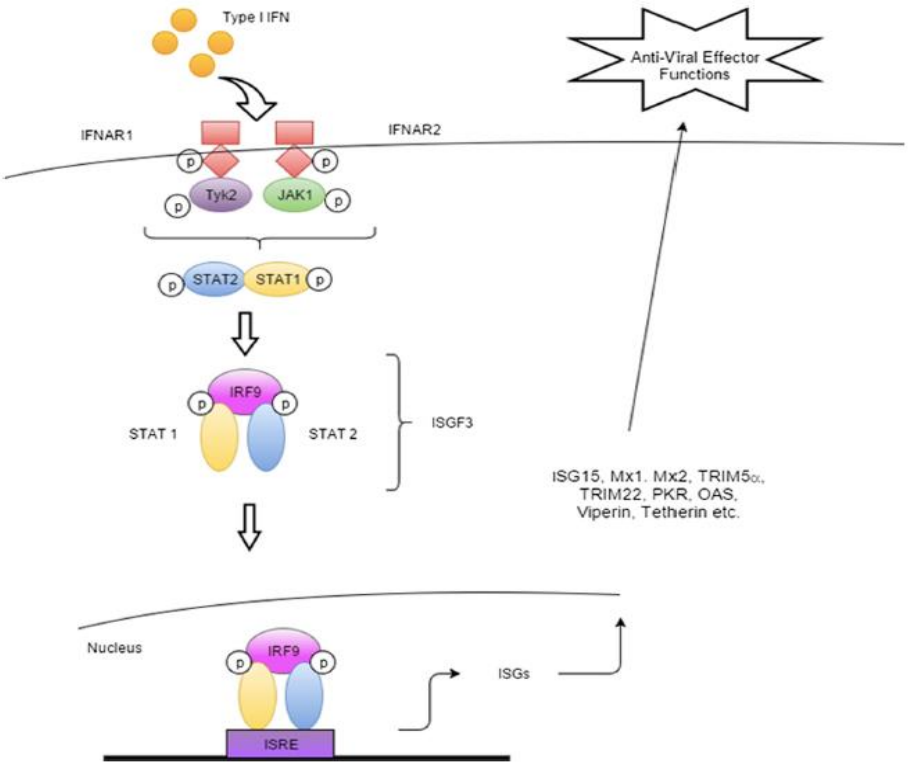


Figure 3: Representation of JAK-STAT signalling pathway, where upon binding of the cytokine, JAK2 molecules get activated, which results in the phosphorylation of STAT molecules, STAT molecules form a heterodimers that translocate to nucleus and bind the DNA (Raftery, Stevenson, 2017).

1.8 Interferon-stimulated genes

Interferon-stimulated genes are a large set of genes, which are expressed upon IFN binding to specific cell receptors, and a signal being transduced through JAK-STAT signaling pathway towards the cell nucleus. ISGs are activated upon encounter of viral pathogen (Hayes, Chayama, 2017), and they have several functions starting from surveillance (PRRs), cytoskeleton remodelling, to apoptosis that leads the cells to death (Sadler, Williams, 2008). ISGs have a significant effect in controlling the viral infection by upregulating chemokines which in turn enhances cell-to-cell communication. This results in a remarkable antiviral state, which is effective against positive-, negative-, double-stranded RNA viruses, and DNA viruses. Some of the well-known ISGs with identified functions are Oligoadenylate synthetase (OAS), Murine myxovirus resistance 1 (Mx1), interferon-induced transmembrane (IFITM) and viperin (Schneider et al., 2014).

OAS: Oligoadenylate synthetase is a type of ISG complex, that detects viral RNA and triggers the synthesis of 2′-5′-oligoadenylates, which then act as intracellular second messengers to activate the RNaseL. This leads to indiscriminate cleavage of both host and viral RNA (Schneider et al., 2014).

Mx1: Murine myxovirus resistance 1 gene is one of the first described inhibitors of virus entry. Mx1 is broadly inhibitory, acts prior to genome replication at the early post-entry step of HIV-1 virus life cycle (Schneider et al., 2014).

IFITM: IFITM protein is the only ISG shown to have a credible role in blocking virus entry and it is enriched in late endosomes and lysosomes. These proteins recently showed to be potent inhibitors of influenza A virus, whereas IFITM1 type showed to be an inhibitor of SARS-coronavirus (CoV), the filoviruses, and Ebola (Schneider et al., 2014).

Viperin: Also known as RSAD2, its expression is stimulated by human cytomegalovirus (HCMV) glycoprotein B and is one of the most highly induced antiviral effectors (Schneider et al., 2014).

Furthermore, a recent study has shown that pretreatment of DAOY human-medulloblastoma cells with IFN-β has resulted in a decreased TBEV production. Transcriptome analysis revealed that there are two ISGs – IFI6 and IFI27 – that are up-regulated in the brain cells

when treated with IFN-β and they could have a direct involvement in the antiviral defence in case of TBEV infection (Selinger et al., 2017).

1.9 IFI6 and IFI27

IFI6 and IFI27 are proteins belonging to the FAM14 family of interferon-stimulated genes (Parker, Porter, 2004). IFI6 is believed to be involved in apoptosis regulation. Whereas, IFI27 is involved in sensitizing the infected cells to apoptotic stimuli. Further studies showed that over-expression of IFI6 leads to the restriction of hepatitis C virus replication in the cells (Itsui et al., 2006) and IFI27 over-expression leads to reducing the production of West Nile virus and Saint Louis encephalitis virus (Cho et al., 2013), thus suggesting possible roles of IFI6 and IFI27 in inhibiting the TBEV infection in DAOY cells.

2 Aims

The aim of this work is:

- to clone genes for IFI6 and IFI27 into mammalian expression vector *via* Gateway cloning
- subsequent *in vitro* analyses of their antiviral effect in the case of tick-borne encephalitis virus infection in human neural cell line DAOY.

3 Materials and methods

3.1 Viruses

The virus used was TBEV Neudoerfl strain (Western European subtype). In more detail, fourth brain passage from suckling mice was used with a concentration of 5 MOI (multiplicity of infection).

Entire work with infectious material was conducted in a BSL2 laboratory using biosafety cabinet. Working aliquot of TBEV Neudoerfl had the concentration of $7.5*10^7$ PFU/ml and its volume needed for the infection was calculated by the following formula:

$$Needed\ viral\ volume\ per\ well\ = \frac{MOI * cell\ number}{\text{titre}\ [\frac{\text{PFU}}{\text{ml}}]}$$

In case of 96-well plate, 100 µl of viral suspension was added to each well; for 24-well plates 200 µl were added per well. The virus was let to attach to the cells for 1-2 hours and then the cells were washed with PBS and fresh cultivation medium was added.

3.2 Cells, media, and passaging

As mentioned earlier, TBEV infects special parts of the brain such as spinal cord or brainstem, and the cerebellum as the most affected brain part.
The DAOY HTB-186 human medulloblastoma cell line, which were derived from desmoplastic cerebellar medulloblastoma (Jacobsen et al., 1985), was used in our study.
The DAOY cultivation medium was low glucose D-MEM + 10% foetal bovine serum (FBS) + antibiotics/antimycotics (Amphotericin B 0.25 µg/ml, Penicillin G 100 units/ml, Streptomycin 100 µg/ml) + 1% L-alanyl-L-glutamine, with 5% CO_2 atmosphere.
In case of plaque titrations (chapter 3.19), PS (Porcine kidney) cell line was used. The PS cultivation medium was L15 + 3% precolostral calf serum (PTS) + 1% ATB + 1% L-alanyl-L-glutamine, without 5% CO_2 atmosphere.

DAOY cells were passaged by detaching the cells from the flask surface *via* trypsinization (0,025% trypsin in Hank's balanced salt solution (HBSS)) and incubation at 37°C for ca 5 minutes. The cells were count using Bürker chamber and classical light microscope. For the count only 25 squares were counted, and the following formula was used:

$$Cell\ concentration\ \left[\frac{cells}{ml}\right] = average\ cell\ count\ x\ 2\ x\ 10{,}000$$

Cells were prevented from reattaching by storing the solution in the fridge while the counting of the cells took place. Secondly, the cells were seeded in particular tissue culture plate by calculating the dilution ratio for seeding the cells depending on required values following the formula:

Dilution ratio = (current cell concentration) / (desired cell concentration)

Then the total amount of needed cell suspension was calculated and the amount of media which needed to dilute the cell suspension. The cells were let to attach for 12 h by incubating them at 37°C in 5% CO2 atmosphere.

3.3 RNA isolation

3.3.1 Total RNA isolation using NucleoSpin® RNA II kit

Total RNA has been isolated from cells which were treated with IFN-β (10 ng/ ml) 12 hours before the harvesting following the NucleoSpin® RNA II manual by MACHEREY-NAGEL. Briefly, 350 µl RA1 Buffer and 3.5 µl ß-mercaptoethanol (ß-ME) were added to the cell pellet and vortexed. Then a NucleoSpin® Filter was placed into a collection tube, to which the mixture was applied and centrifuged for 1 min at 11,000 x *g*. 350 µl ethanol (70 %) was added to the filtered lysate and mixed by pipetting in order to adjust RNA binding conditions. Then the lysate was loaded on a NucleoSpin® RNA II Column, which was fixed to a collection tube and this was centrifuged for 30 s at 11,000 x *g*. Then the NucleoSpin® RNA II Column was placed in a new collection tube to which 350 µl MDB (Membrane Desalting Buffer) was added and centrifuged at 11,000 x *g* for 1 min to dry the membrane. Further, DNA digestion took place by preparing DNase reaction mixture where 10 µl reconstituted rDNase was mixed with 90 µl Reaction Buffer for rDNase and mixed. 95 µl of the DNase reaction mixture was added onto the centre of the silica membrane of the column and incubated at room temperature for 15 min. The silica membrane was washed 3 times with 200 µl RA2 Buffer, 600 µl RA3 Buffer, and 250 µl Buffer RA3, and each wash was centrifuged for 30 s at 11,000 x *g*, whereas the third wash was centrifuged for 2 mins to dry the membrane completely. The isolated RNA was eluted with 60 µl RNase-free H_2O, and centrifuged at 11,000 x *g* for 1 min.

3.3.2 Total RNA isolation using RNAblue

Phase separation:

To the cell homogenate a 200 µl of RNA Blue reagent (Top-Bio) was added, then 40 µl of chloroform was added, vortexed for 15 sec and centrifuged at 12,000 x *g* for 15 min at 4 °C. The upper phase contained the RNA, which was transferred to a new tube.

RNA precipitation:

100 µl of isopropanol was added to the obtained aqueous solution and incubated at room temperature for 10 min, then centrifuged at 12,000 x *g* for 8 min at 4 °C, the formed precipitate contained the RNA.

RNA wash step:

The supernatant was removed from the precipitate. The pellet was washed with 75% ethanol, vortexed and centrifuged at 7500 x *g* for 5 min at 4 °C.

RNA solubilization:

After the ethanol was removed, the RNA pellet was air dried for 5 min. Then it was dissolved in DEPC-treated water and incubated for 10 min at 55-60 °C.

3.4 cDNA synthesis

Complementary DNA (cDNA) has been synthesized from the isolated RNA using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) as follows:

All the reagents were centrifuged and kept on ice. 1 µg of total RNA was mixed with 1 µl oligo $(dT)_{18}$ primer and the mixture were filled up with nuclease-free water to 11 µl. Reaction was mixed gently, centrifuged briefly, and incubated at 65 °C for 5 min. Then it was chilled on ice, spun down, and placed back on ice. Then, 4 µl of 5x Reaction Buffer, 1 µl of RiboLock RNase Inhibitor (20 U/µl), 2 µl of 10 mM dNTP Mix, and 2 µl of M-MuLV Reverse Transcriptase (20 U/µl) were added. The whole mixture was then mixed gently, centrifuged, and incubated for 60 min at 37°C. The cDNA synthesis reaction was terminated by heating it at 70 °C for 5 min. Reaction was used for subsequent PCR amplification of IFI6 and IFI27 genes.

3.5 PCR amplification of IFI6 and IFI27 genes

For the amplification of IFI6 and IFI27 coding sequences, we used Q5 High-Fidelity DNA Polymerase (New England Biolabs, NEB). Primers were designed to amplify the whole coding sequences beginning with ATG start codon but lacking the stop codon (Table 1).

Reactions were prepared according to the manufacturer's protocol (Table 2). The expected size of IFI6 gene was 393 bp, and IFI27 was 369 bp.

Table. 1: Cloning primer sequences.

Primer	Sequence (5' → 3')
IFI6-F	ATGCGGCAGAAGGCGGTATCG
IFI6-R	CTCCTCATCCTCCTCACTATCG
IFI27-F	ATGGAGGCCTCTGCTCTCACC
IFI27-R	GTAGAACCTCGCAATGACAGCCG

Table 2: Q5 High-Fidelity PCR reaction composition.

Component	for 50 µl Reaction	Final concentration
5X Q5 Reaction Buffer	10 µl	1X
2 mM dNTPs	5 µl	200 µM
10 µM Forward Primer	2.5µl	0.5 µM
10 µM Reverse Primer	2.5 µl	0.5 µM
cDNA	2 µl	
Q5 High-Fidelity DNA Polymerase	0.5 µl	0.02 U/µl
5X Q5 High GC Enhancer	10 µl	(1X)
Nuclease-Free Water	to 50 µl	

The reaction mixture was mixed gently, and transferred to a PCR machine, where the thermocycling took place following the conditions in Table 3.

Table 3: Thermocycling Conditions for a PCR.

STEP	**TEMP**	**TIME**
Initial Denaturation	98 °C	30 seconds
40 Cycles		
	98 °C	10 seconds
	68 °C (IFI6) 71 °C1 °C (IFI27)	20 seconds
	72 °C	30 seconds
Final Extension	72 °C	2 minutes
Hold	4 °C	

3.6 Isolation of DNA fragment from agarose gel

For the purification of DNA fragments from agarose gel, the High Pure PCR Product Purification Kit by Roche was used, where the isolated DNA band of interest was loaded on a 1.2 % agarose gel, for preparation of which 1x TAE running buffer was used. Electrophoresis took place until DNA band of interest was isolated from adjacent contaminating fragments. The bands were visualized by the SYBR Green dye, which was present in the loading buffer. The desired DNA band was cut from the gel using an ethanol-cleaned scalpel. Afterwards, the excised agarose gel slice was placed in a sterile 1.5 ml microcentrifuge tube. To every 100 mg agarose gel slice 300 µl Binding Buffer was added inside the microcentrifuge tube. In order to release the DNA from the agarose gel, the suspension was incubated for 10 min at 56 °C, vortexed briefly every 2 – 3 min during incubation. After the agarose gel slice was completely dissolved, 150 µl isopropanol was added to every 100 mg agarose gel slice inside the tube and vortexed thoroughly. Then, one High Pure Filter Tube was inserted into one Collection Tube, into which the entire content of the microcentrifuge tube was pipetted, and centrifuged for 60 s at maximum speed at 25 °C. The flow-through was discarded. Then, the High Pure Filter Tube was reconnected to a Collection Tube, and to the Filter Tube 500 µl Wash Buffer was added, centrifuged 1 min at maximum speed, and the flow-through was discarded. The Filter Tube was recombined with the same Collection Tube and 200 µl Wash Buffer was added, the tube was centrifuged for 1 min at maximum speed. The flow-through and Collection Tube were discarded. The Filter Tube was reconnected to a clean 1.5 ml microcentrifuge tube, to which 50 µl Elution Buffer was added and was centrifuged 1 min at maximum speed. The microcentrifuge tube at this step contained the purified DNA.

3.7 Gateway cloning system

Gateway cloning technology is a rapid and efficient universal cloning method where DNA fragments can be cloned into plasmids. The Gateway method takes advantage of the site-specific recombination properties of bacteriophage lambda, which is used to move fragments between different plasmids that contain recombination sites suitable for lambda recombinase machinery. The scheme of the Gateway system is represented in Figure 4.

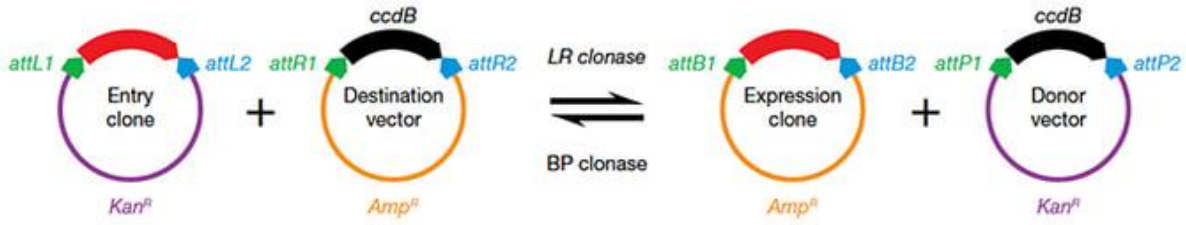


Figure 4: Gateway cloning system. Source: http://smolkelab.weebly.com/gateway-cloning.html

In this project we used both, Gateway BP clonase and LR clonase to clone human cDNA containing the IFI6 and IFI27 genes, into pDONR207 vector, and subsequently into pDEST40 expression vector. Figures 5 and 6 illustrate the construction of donor and destination vectors.

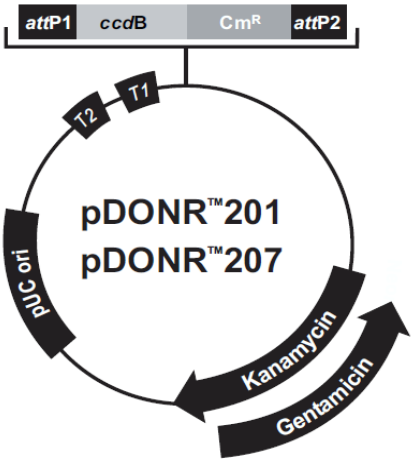


Figure 5: Donor vector representation.

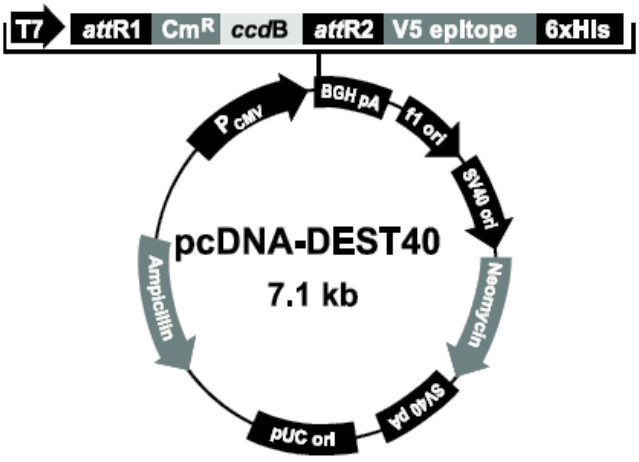


Figure 6: Destination vector representation

3.7.1 Addition of attB sites to IFI6 and IFI coding sequences

A necessary condition of Gateway cloning is the presence of the attB sequence on both ends of DNA insert. In this work, a PCR-based addition of attB sites was performed using primers listed in Table 4; the reaction composition is described in Table 5.

Table. 4: Gateway cloning primer sequences (attB sites in lowercase letters).

Primer	Sequence (5' → 3')
attB-IFI6-F	ggggacaagtttgtacaaaaaagcaggcttcATGCGGCAGAAGGCGGTATCG
attB-IFI6-R	ggggaccactttgtacaagaaagctgggttCTCCTCATCCTCCTCACTATCG
attB-IFI27-F	ggggacaagtttgtacaaaaaagcaggcttcATGGAGGCCTCTGCTCTCACC
attB-IFI27-R	ggggaccactttgtacaagaaagctgggttGTAGAACCTCGCAATGACAGCCG

Table 5: Q5 High-Fidelity PCR reaction composition.

Component	for 50 µl Reaction	Final concentration
5X Q5 Reaction Buffer	10 µl	1X
2 mM dNTPs	5 µl	200 µM
10 µM Forward Primer	2.5µl	0.5 µM
10 µM Reverse Primer	2.5 µl	0.5 µM
PCR fragment	2 µl	
Q5 High-Fidelity DNA Polymerase	0.5 µl	0.02 U/µl
5X Q5 High GC Enhancer	10 µl	(1X)
Nuclease-Free Water	to 50 µl	

The reaction mixture was mixed gently, and transferred to a PCR machine, where the thermocycling took place following the conditions in Table 6.

Table 6: Thermocycling conditions for a PCR.

STEP	**TEMP**	**TIME**
Initial Denaturation	98 °C	30 seconds
40 Cycles		
	98 °C	10 seconds
	72 °C	20 seconds
	72 °C	30 seconds
Final Extension	72 °C	2 minutes
Hold	4 °C	

3.7.2 Gateway BP Clonase

The *att*B × *att*P reaction is mediated by Gateway® BP Clonase™ II enzyme mix from Invitrogen/Thermo Fisher Scientific which catalyses *in vitro* recombination between an *att*B-PCR product and an *att*P-containing donor vector to generate an *att*L-containing entry clone. This method was achieved following the Thermo Fisher Scientific manual for BP Clonase. A volume between 1-7 µl of the previously obtained cDNA (150 ng) with *att*B sites was mixed with a 1 µl donor vector (150 ng/ µl) and the mixture was filled up with TE buffer of pH 8.0 up to 8 µl. Afterwards, BP Clonase™ II enzyme mix was thawed on ice for about 2 minutes, vortexed and mixed and 2 µl out of this was added to the previous mixture mixed and

vortexed once more and centrifuged. The whole reaction was then incubated at 25 °C for 1 hour. Then the reaction was inhibited by 1 µl of Proteinase K solution, vortexed, and incubated at 37 °C for 10 min.

Transformation:

After the Gateway BP Clonase reaction, transformation into bacterial cells took place. 1 µl of BP Clonase reaction mixture was mixed with 50 µl of competent *E. coli* strain NovaBlue Singles (Merck). The cell mixture was incubated on ice for 30 min, then cells underwent heat-shock by incubation at 42 °C for 30 sec. This was followed by addition of 250 µl of S.O.C. medium and incubated at 37 °C for 1 hour with shaking. 20 µl and 100 µl of each transformation reaction were plated onto selective LB agar plates which contained 10 µg/ml gentamycin.

Colony PCR:

To verify the presence of IFI6 or IFI27 coding sequences in gentamycin-selected *E. coli* clones, we performed PCR reaction with gene-specific primers using FastStart PCR Master Kit (Roche). For this purpose, a PCR mix was prepared as mentioned in Table 7.

Table 7: PCR reaction composition.

Component	Volume	Final concentration
Master Mix, 2x	12.5 µl	1x
Forward primer (10 µM)	0.75 µl	300 nM
Reverse primer (10 µM)	0.75 µl	300 nM
Water	11 µl	
Total Volume	25 µl	

The bacteria were directly added to the Mix solution (using a sterile tip) then put into PCR machine, where the thermocycling took place following the set-up in Table 8.

Table 8: Thermocycling conditions for the PCR.

STEP	Cycles	TEMP	TIME
Initial denaturation	1	95 °C	4 min
Denaturation	35	95 °C	30 s
Annealing		60 °C	30 s
Elongation		72 °C	60 s
Final Extension	1	72 °C	7 min

Plasmid miniprep isolation:

Small-scale (mini) preparation took place to purify the plasmid DNA for subsequent sequencing verification. The procedure was achieved using High Pure Plasmid Isolation Kit by Roche.

Firstly, the binding buffer was put on ice, then the materials were prepared by pelleting the bacterial cells from *E. coli* culture that were inoculated the day before. The bacterial pellet was resuspended in 250 µl of suspension buffer with RNase A, 250 µl of lysis buffer was added, and mixed gently by inverting the tube several times. To the lysed solution 350 µl chilled binding buffer was added and mixed gently, then incubated on ice for 5 min. The cloudy solution was centrifuged for 10 min at 13,000 x *g*. After the centrifugation, the supernatant was transferred into High Pure Filter Tube which is assembled to a collection tube, and this assembly was centrifuged for 1 min at full speed. The flow-through was discarded. The Filter Tube was washed with 500 µl of wash buffer I, then centrifuged for 1 min. The flow-through was discarded. Then, 700 µl of wash buffer II was added to the Filter Tube and centrifuged for 1 min. The flow-through was discarded. The Filter Tube assembly was centrifuged for additional 1 min, then the collection tube was discarded. The Filter Tube that contains the bound DNA, was put into microcentrifuge tube, the DNA was eluted by adding 100 µl Elution Buffer and centrifuged for 1 min. The eluted plasmid DNA was collected in the microcentrifuge tube and final concentration was measured using NanoPhotometer, Implen.

3.7.3 Gateway LR Clonase

The *att*L × *att*R reaction is mediated by Gateway® LR Clonase™ II enzyme mix from Invitrogen manufacturer, which catalyzes *in vitro* recombination between an entry clone *att*L-flanked "gene" and an *att*R-containing destination vector to generate an *att*B-containing

expression clone. This method was achieved following Thermo Fisher Scientifi manual for LR Clonase. For this cloning step the following components were added: 1-7 µl entry clone (150 ng), 1 µl destination vector (150 ng/ µl), TE buffer of pH 8.0 up to 8 µl. The LR Clonase™ II enzyme mix was thawed on ice for about 2 minutes, vortexed, and 2 µL of it was added to the previous mixture, vortexed, and then centrifuged. The reaction mixture was incubated at 25 °C for 1 hour. Afterwards, the reaction was inhibited by adding 1 µl of the Proteinase K solution to the reaction mixture, vortexed, and incubated at 37°C for 10 minutes.

Transformation:

1 µl of the LR reaction was transformed into 50 µl of competent *E. coli* strain NovaBlue Singles (Merck). The cells were incubated on ice for 30 minutes; then underwent heat-hock by incubating them at 42 °C for 30 sec. 250 µl of S.O.C. medium was added to the cells and incubated at 37 °C for 1 hour with shaking. 20 µl and 100 µl of each transformation reaction were plated onto selective LB agar plates containing 100 µg/ml carbenicilin.

To verify the presence of IFI6 or IFI27 coding sequences in carbenicilin-selected *E. coli* clones, we performed colony PCR reaction with gene-specific primers using FastStart PCR Master Kit (Roche) as described in chapter 3.4.1. Afterwards, plasmid miniprep isolation (see chapter 3.4.1.) was performed to validate ORF sequences of IFI6 and IFI27 incorporated in pDEST40 vector *via* sequencing.

Maxiprep:

To get a sufficient amount of plasmid DNA for further transfection experiments, HiSpeed Plasmid Maxi Kit (Qiagen) for rapid purification of transfection-grade plasmid DNA was used.

A selected clone was picked from a freshly streaked selective plate and a culture of 6 ml LB medium containing the appropriate selective antibiotic was inoculated and incubated for approx. 8 h at 37 °C with vigorous shaking. The cultures were diluted in 1/1000 ratio (v/v) into 250 ml of selective LB medium. Bacteria were incubated at 37 °C for 12–16 h with vigorous shaking. The bacterial cells were harvested by centrifugation at 4,000 x g for 10 min at 4 °C. The supernatant was discarded, whereas the bacterial pellet was resuspended in 10 ml of Buffer P1. Later, 10 ml of Buffer P2 was added, mixed, and incubated at room temperature for 5 min. During the incubation, the QIAfilter Cartridge was prepared by screwing the cap

onto the outlet nozzle of the QIAfilter Maxi Cartridge and the QIAfilter Cartridge was placed into a convenient tube. 10 ml chilled Buffer P3 was added to the lysate and mixed. The lysate was poured into the barrel of the QIAfilter Cartridge and incubated at room temperature for 10 min. The HiSpeed Maxi Tip was equilibrated by applying 10 ml of Buffer QBT. The cap was removed from the QIAfilter outlet nozzle. The plunger was inserted into the QIAfilter Maxi Cartridge and the cell lysate was filtered into the previously equilibrated HiSpeed Tip. HiSpeed Maxi Tip was washed with 60 ml Buffer QC. The isolated DNA was eluted with 15 ml of Buffer QF. The DNA was then precipitated by adding 10.5 ml isopropanol to the eluted DNA, mixed, and incubated at room temperature for 5 min. During the incubation, the plunger was removed from a 30 ml syringe and the QIAprecipitator Maxi Module was attached onto the outlet nozzle. The QIAprecipitator was placed over a waste bottle, the elute/isopropanol mixture was transferred into 30 ml syringe, and the plunger was inserted. The elute/isopropanol mixture was filtered through QIAprecipitator using constant pressure. Afterwards, the QIAprecipitator was removed from the 30 ml syringe and plunger was pulled. The QIAprecipitator was re-attached and 2 ml 70% ethanol was added to the syringe. The DNA was washed by inserting the plunger and pressing the ethanol through the QIAprecipitator using constant pressure. Then the QIAprecipitator was removed from the 30 ml syringe and plunger was pulled out. The QIAprecipitator was then attached to the 30 ml syringe again, the plunger was inserted, and the membrane was dried by pressing air through the QIAprecipitator quickly and forcefully. This step was repeated.
The outlet nozzle of the QIAprecipitator was dried with absorbent paper to prevent ethanol carryover. The plunger was removed from a new 5 ml syringe and the QIAprecipitator was attached onto the outlet nozzle. The outlet of the QIAprecipitator was held over a 1.5 ml collection tube. 1 ml of Buffer TE was added to the 5 ml syringe. The plunger was inserted and the DNA was eluted into the collection tube using constant pressure. The QIAprecipitator was removed from the 5 ml syringe, the plunger was pulled out, and the QIAprecipitator was reattached to the 5 ml syringe. The eluate from the previous step was transferred to the 5 ml syringe and eluted for a second time into the same 1.5 ml tube.

3.8 Preparation of IFI6 and IFI27 mRNA *in vitro*

The *in vitro* preparation of capped RNA coding sequence of IFI6/IFI27 with poly(A) tail was done *via* following steps.

3.8.1 Q5 PCR amplification of T7 promoter-IFI6/IFI27 DNA fragment

First step in the preparation of capped mRNA was to amplify IFI6/IFI27 coding sequence with linked T7 promoter, which is necessary for subsequent *in vitro* RNA transcription based on T7 RNA polymerase. The list of used primers is depicted in Table 9; PCR reaction composition as well as the cycling conditions are described in Tables 10 and 11, respectively.

Table. 9: T7 promoter primer sequences.

Primer	Sequence (5' → 3')
T7-F	TAATACGACTCACTATAG
bGH-R	TAGAAGGCACAGTCGAGG

Table 10: PCR mix for T7-IFI6 or T7-IFI27 fragment amplification.

Component	for 50 µl Reaction	Final concentration
5X Q5 Reaction Buffer	10 µl	1X
2 mM dNTPs	5 µl	200 µM
10 µM Forward Primer	1.5 µl	0.5 µM
10 µM Reverse Primer	1.5 µl	0.5 µM
Template DNA	variable	100 ng
Q5 High-Fidelity DNA Polymerase	0.5 µl	0.02 U/µl
5X Q5 High GC Enhancer (optional)	5 µl	(1X)
Nuclease-Free Water	to 50 µl	

Table 11: Thermocycling conditions for the PCR.

STEP	**TEMP**	**TIME**
Initial Denaturation	98 °C	30 seconds
40 Cycles		
	98 °C	10 seconds
	54 °C	15 seconds
	72 °C	20 seconds
Final Extension	72 °C	2 minutes
Hold	4 °C	

3.8.2 Q5 PCR addition of poly(T) tail

The addition of poly(T)$_{80}$ tail was performed using Q5 PCR with primers listed below (Table 12); reaction composition as well as cycling conditions are described in Tables 13 and 14, respectively.

Table 12: Primer sequences for addition of poly(T)$_{80}$ tail.

Primer	Sequence (5' → 3')
T7-F	TAATACGACTCACTATAG
bGH-poly(T)-R	TAGAAGGCACAGTCGAGG-(T)$_{80}$

Table 13: PCR mix for T7-IFI6-poly(T) or T7-IFI27-poly(T) fragment amplification.

Component	for 50 µl Reaction	Final concentration
5X Q5 Reaction Buffer	10 µl	1X
2 mM dNTPs	5 µl	200 µM
10 µM Forward Primer	1.5 µl	0.5 µM
10 µM Reverse Primer	1.5 µl	0.5 µM
Template DNA	variable	100 ng
Q5 High-Fidelity DNA Polymerase	0.5 µl	0.02 U/µl
5X Q5 High GC Enhancer (optional)	5 µl	(1X)
Nuclease-Free Water	to 50 µl	

Table 14: Thermocycling conditions for the PCR.

STEP	**TEMP**	**TIME**
Initial Denaturation	98 °C	30 seconds
40 Cycles		
	98 °C	10 seconds
	54 °C	15 seconds
	72 °C	25 seconds
Final Extension	72 °C	2 minutes
Hold	4 °C	

3.8.3 T7 RNA *in vitro* transcription with cap analog

For *in vitro* RNA transcription the MEGAscript kit (Ambion/Thermo Fisher Scientific) was used according to the manufacturer's instructions. To synthesize capped transcripts, a cap

analog (m7G(5')ppp(5')G; NEB) was added to the reaction (resulting concentration ratio of GTP:cap analogue was 1:1). The composition of the reaction is described below in the Table 15.

Table 15: PCR reaction composition.

Component	for 20 µl Reaction
ATP 75 mM	2 µl
GTP 75 mM	1 µl
GTP - cap analog (NEB) - 50 mM	1.5 µl
UTP 75 mM	2 µl
CTP 75 mM	2 µl
10x reaction buffer	2 µl
T7 Enzyme Mix	2 µl
Rnase-free water	6.5 µl
DNA (PCR fragment) - 160 ng	1 µl

The reaction was incubated at 37 °C for 4 hours. Then, 1 µl of TURBO DNase was added, and the reaction was further incubated for 15 min at 37 °C. This was followed by RNA precipitation with LiCl according to the manufacturer's protocol. The resulting pellet was resuspended in 50 µl water and the concentration was measured using NanoPhotometer, Implen.

3.9 *In vitro* transient transfection

For *in vitro* transfections of both, DNA and RNA, PolyJet transfection reagent (SignaGen) was used. The procedure was performed according to the manufacturer's instructions with minor modifications. DAOY cells were seeded one day prior the transfection at cell counts depending on the well format (see Table 16). At the day of the transfection, fresh medium was added to the cells and transfection mixtures were prepared and added to the cells as described in Table 16.

Table 16: Preparation of cell/media solution for seeding on 24-well plate for *in vitro* transient transfection.

					Mixing ratio for the suspension	
Average of counted cells	Actual concentration [cells/mL]	Total volume of suspension needed [mL]	Final conc. to be reached [cells/mL]	Cells/well	Cells [mL]	Medium [mL]
39	780000	40	300,000	300,000	15.4	24.6

3.9.1 DNA transfection

In the case of DNA transfections, mammalian expression vector pDEST40 carrying IFI6/IFI27 coding sequence was used. As a negative control, empty pDEST40 was used.

96-well panel:
For each sample a plasmid DNA (100 or 200 ng/ well) was diluted with basic low glucose D-MEM medium up to 12,5 µl. Simultaneously, 0.15 or 0.3 µl of PolyJet were mixed with basic low glucose D-MEM medium as well. The solutions were mixed together and incubated for 10 minutes at room temperature. Then, the whole mixture was added to the respective well. In case of higher amount of identical reactions, the mastermix was prepared, and 25 µl of the resulting mixture were added to each well.
24-well panel:
For each sample a plasmid DNA (500 or 1000 ng/ well) was diluted with basic low glucose D-MEM medium up to 25 µl. Simultaneously, 0.75 or 1.5 µl of PolyJet were mixed with basic low glucose D-MEM medium as well. The solutions were mixed together and incubated for 10 minutes at room temperature. Then, the whole mixture was added to the respective well. In case of higher amount of identical reactions, the mastermix was prepared, and 50 µl of the resulting mixture were added to each well.

3.9.2 RNA transfection

In case of RNA transfections, *in vitro* transcribed ssRNA with G(5‘)ppp(5‘)G RNA Cap, coding sequence of IFI6/IFI27/MBP with poly(A) tail was used. MBP (Maltose-binding protein) gene was used as a negative control.

24-well panel:

For each sample 60 ng of particular ssRNA was diluted with basic low glucose D-MEM medium up to 25 µl. Simultaneously, 1 µl of PolyJet was mixed with basic low glucose D-MEM medium as well. The solutions were mixed together and incubated for 10 minutes at room temperature. Then, the whole mixture was added to the respective well. In case of higher amount of identical reactions, the mastermix was prepared, and 50 µl of the resulting mixture were added to each well.

3.10 Real-time PCR

The qPCR master mix was prepared following the KAPA SYBR® FAST Universal One-Step qRT-PCR Kit instructions. The experiment included an RT- control that would enable detection of DNA contamination. Table 17 shows the qPCR component mixture.

Table 17: PCR reaction composition.

Component	For 15 µl reaction	Final concentration
PCR-grade water	5.3 µl	N/A
2X KAPA SYBR FAST qPCR Master Mix2	7.5 µl	1x
10 µM Forward Primer	0.45 µl	300 nM
10 µM Reverse Primer	0.45 µl	300 nM
50X KAPA RT Mix	0.3 µl	1x
Template RNA	4 µl	80 ng

Set up of qRT-PCR:

Table 18: Represents the program of the PCR cycling protocol.

Table 18: Cycling protocol of qRT-PCR.

Step	Temp	Duration	Cycles
Reverse Transcription	42 °C	10 min	Hold
Enzyme inactivation	95 °C	5 min	Hold
Denaturation	95 °C	5 sec	40
Annealing/ extension/data acquisition	60 °C	30 sec	

3.11 Viability assay

To measure the cell viability, we used the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to analyse the cytopathic effect (CPE) inhibition in DAOY cells expressing IFI6 and IFI27. The cells were seeded on 96 well plate using two different concentrations – 10,000 cells/ml and 20,000 cells/ml. Afterwards, the cells were transfected with two different concentrations of IFI6/IFI27/pDEST40 – 100 ng and 200 ng. Then, the infection took place using TBEV Neudoerfl strain with 5 MOI on half of the plate side to compare the CPE effect in infected and non-infected cells which were expressing IFI6/IFI27, the analysis of the cell viability was performed 5 days post infection: MTT (5 mg/ml in PBS) was added to fresh cultivation medium (final concentration 0,5 mg/ml), which was subsequently added to PBS-washed DAOY cells (100 µl/well). Cells were incubated under normal cultivation conditions for 4 hours. Then, medium was discarded and formazan crystals (product of MTT reduction by cellular enzymes) were dissolved in 200 µl of DMSO. The amount of produced formazan was quantified *via* absorbance measurement at 570 nm.

3.12 Plaque titration assays

Firstly, sufficient amount of the PS cells suspension was prepared with the concentration of 300,000 cells/ml. Secondly, 24-well plate was used, where each well was filled with 180 µl of L15 cultivation media. Then 20 µl of the respective viral media was added to each first well, mixed well, then 10-fold dilution in a row took place. Afterwards, 300 µl of the prepared PS cell suspension was added into each well. The plate was put inside a plastic box

filled with 150 ml of CO_2 gas and incubated for 5 hours at 37 °C. This was followed by preparing a CMC + 2x concentrated L15 medium mixture (1:1; v:v) and the cells in each well were covered with 400 µl by this mixture and avoided shaking. Plates were put back to the plastic box and filled again with 150 ml CO_2 gas and incubated for 5 days at 37 °C.

Staining:

After 5 days, staining of the plate took place, where the wells were washed with physiological solution was prepared (9 g of sodium chloride/1 L deionized water), and then the cells were stained by naphthalene black solution (0.1% naphthol blue-black, 6% glacial acetic acid and 1.36% sodium acetate) for 45 min.

Once the plates were dry, the plaque numbers could be counted and using the following formula, the concentration of virus titre was calculated in PFU/ml:

$$C[PFU/ml]\frac{Dilution\ factor\ x\ Number\ of\ Plaques}{Volume\ of\ the\ virus\ media}$$

3.13 Immunodetection of IFI6/IFI27 using western blot

After removal of the medium, the cells from a 24-well plate have been resuspended in the RIPA lysis buffer (150 mM NaCl, 1% Octylphenoxy poly(ethyleneoxy)ethanol, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfat, 50 mM Tris, pH 8.0), containing protease and phosphatase inhibitors then the cells with RIPA buffer were sonicated to disrupt the cells. Afterwards, the obtained proteins were blotted. Firstly, 15 % acrylamide gels have been prepared. The samples prior to loading onto the gels were prepared in the following way: 15 µl of each sample was mixed with 5 µl LB/dithiothreitol dye. 20 µl of each prepared sample was loaded onto the gel in separate wells, and on the far end well a 5 µl of protein marker VI (10-245) prestained from PanReac AppliChem manufacturer was loaded. The Mini-PROTEAN Tetra cell was used for the electrophoresis and run for 1.5 h at 120 V using electrophoresis cell from Bio-Rad. Then the gels were blotted semi-dry in a sandwich way: paper immersed in 1xblotting buffer, PVDF membrane immersed in methanol, gel, paper. The sandwich was blotted using Trans-Blot® Turbo™ Transfer system for 30 min at 25 V at a maximum current of 1 A. Afterwards, the gel was stained with PageBlue™ protein staining solution by Thermo Scientific.

In the meantime, the membrane was kept in water for 2 min, then blocked in 10 ml 5% skimmed milk in PBS-T for 1h. Then, new 10 ml of 5% milk in PBS-T solution was added this time containing 20 µl primary antibodies of anti-IFI6 (Bioss; bs-15552R), anti-IFI27

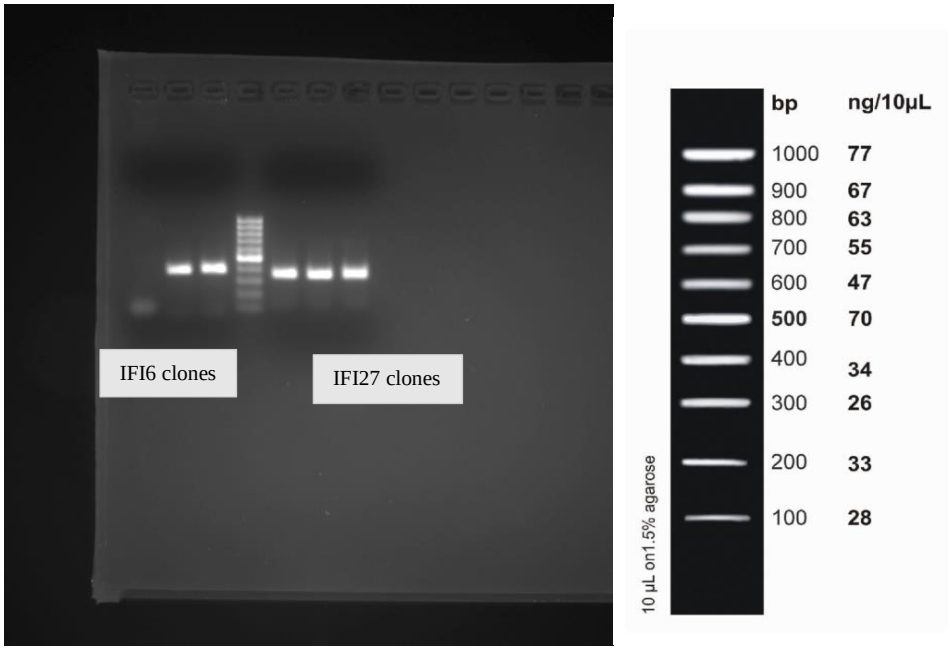
(Bioss; 15549R), or anti-V5 tag (Abcam; ab27671), which were incubated shaking overnight at 4 °C h (all antibodies at 1:500 dilution ratio). The same procedure was repeated for AP-conjugated secondary anti-goat (1:1000 dilution ratio) and AP-conjugated tertiary anti-horse (1:2000 dilution ration) antibodies, but with 3 x wash steps in between using PBS-T. Novex Chemiluminescent CDP-Star substrate was used for the detection of chemiluminescent signal which was documented using the GBox CHemi XX6 (Syngene).

4 Results

4.1 Cloning of human IFI6 and IFI27 coding sequences

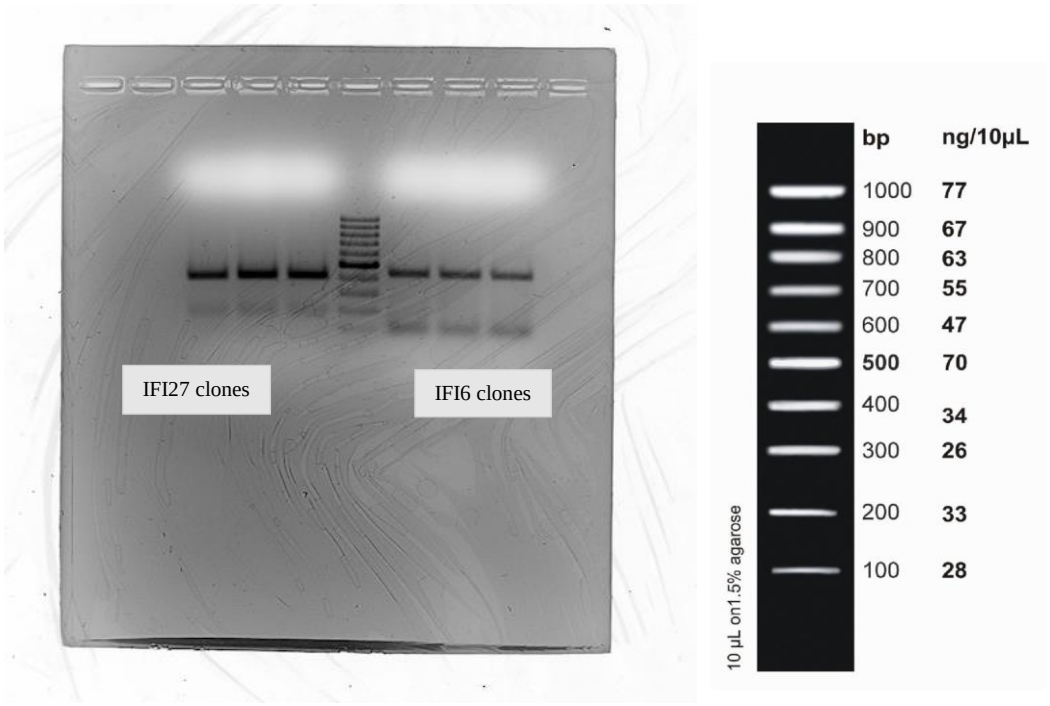
To express the genes of interest in human DAOY cells, cloning had to take place using Gateway cloning system, where the IFI6 and IFI27 genes were firstly cloned into pDONR207 vector, and secondly into pDEST40 expression vector.

For this purpose, the total RNA was isolated from IFN- β pre-treated cells that were prepared by the supervisors. Afterwards, the synthesis of cDNA took place. Then Q5 PCR amplification of IFI6 and IFI27 was performed. Figure 7 shows the gel electrophoresis outcome after Q5 PCR, the larger bands on the left are the IFI6 with the size of 393 bp, the smaller bands on the right are the IFI27 with the size of 369 bp. Then the addition of attB sites to DNA fragments of IFI6 and IFI27 took place. Figure 8 shows the gel electrophoresis outcome after the addition of attB sites, the IFI6 bands on the right have bigger sizes than the IFI27 bands on the left.



(A) (B)

Figure 7: (A) Q5-PCR amplification of IFI6 and IFI27 after cDNA synthesis. The IFI6 gene has the size of 393 bp, and IFI27 has the size of 369 bp. (B) The used DNA marker in the middle of the gel.

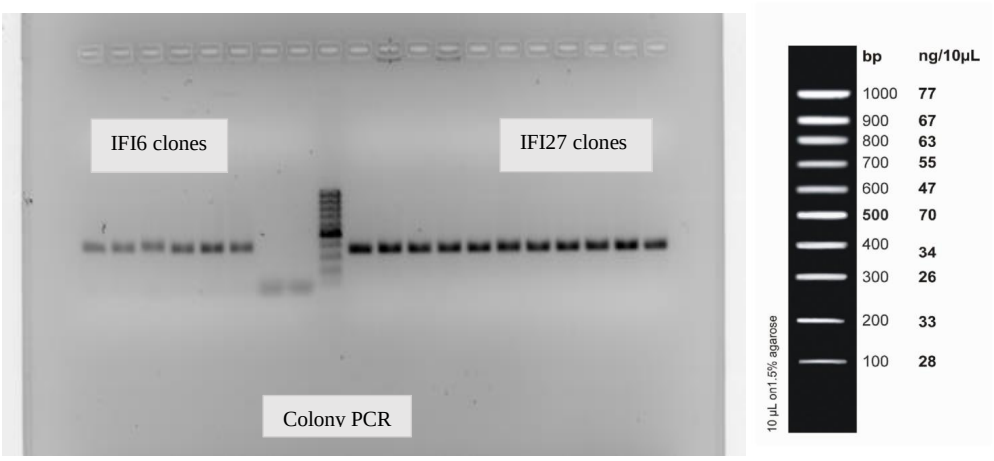


(A) (B)

Figure 8: (A) Addition of attB sites. The IFI6 gene has the size of 393 bp, and IFI27 has the size of 369 bp. (B) The used DNA marker in the middle of the gel.

Then the IFI6 and IFI27 were cloned into pDONR207 vector, *via* BP clonase reaction. *E. coli* (NovaBlue Singles strain; Merck) were used for the transformation and the efficiency of the

transfection was analysed by colony PCR. The Figure 9 shows the gel electrophoresis outcome after colony PCR.



(A) (B)

Figure 9: (A) Gel electrophoresis results after a colony PCR of IFI6 gene on the left with the size of 393 bp and IFI27 on the right side with the size of 369 bp. (B) The used DNA marker in the middle of the gel.

A miniprep isolation took place of the clones 1 to 3 for IFI6 and 9 to 11 for IFI27 and these positive clones were sent for sequencing to GATC Biotech centre to verify the ORF sequence. Table 19 represents the used primer sequences. Figures 10, 11 are the sequence alignments of IFI6/27 using Geneious. The results from the alignments show that the ORF of practically obtained IFI6/27 genes match the literature database ORF of IFI6/27, meaning that the right targeted genes have been amplified.

Table 19: Primer sequences.

Primer	Sequence (5' → 3')
pDONR-F	TCGCGTTAACGCTAGCATGGATCTC
pDONR-R	GTAACATCAGAGATTTTGAGACAC

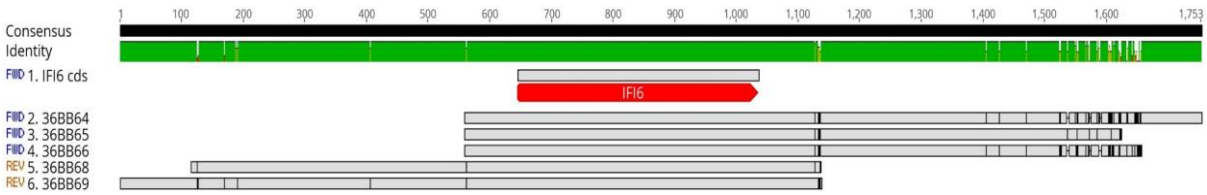


Figure 10: Aligned sequence of IFI6.

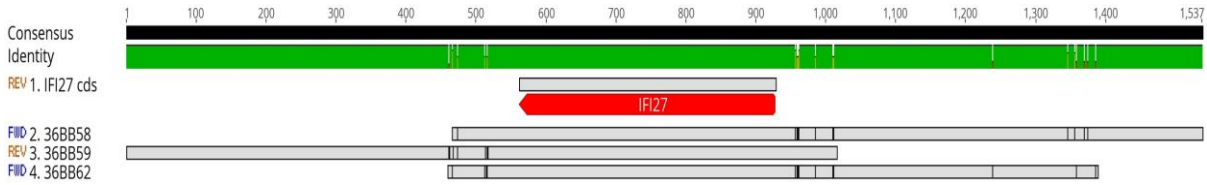
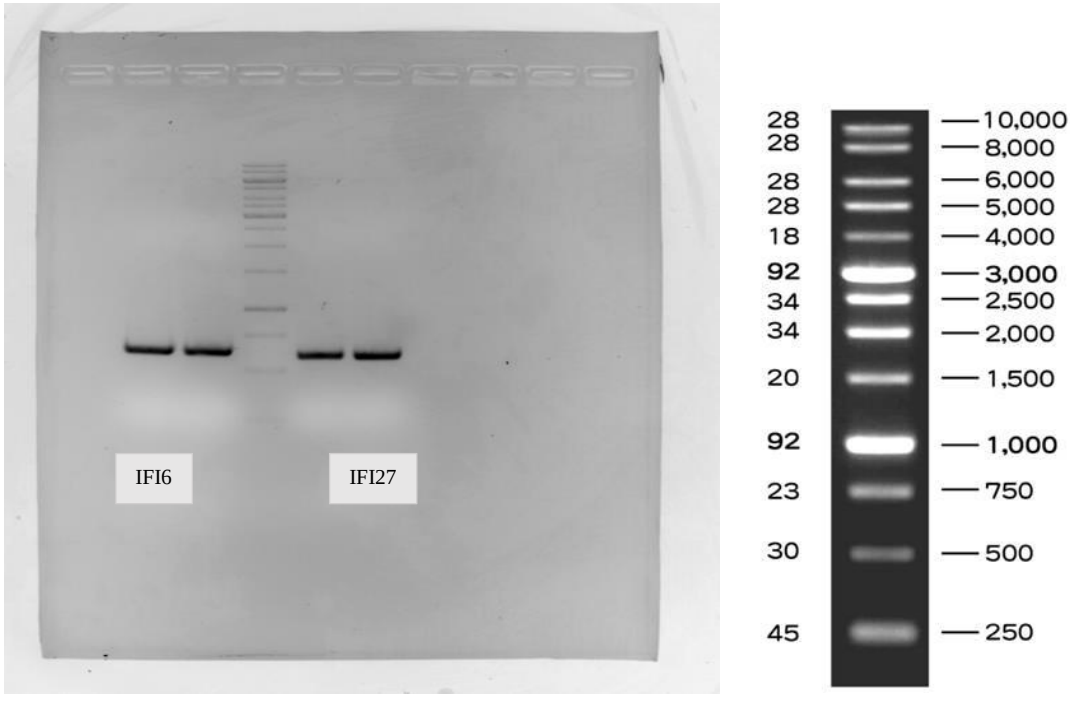


Figure 11: Aligned sequence of IFI27.

The positive selected clones were used for cloning into pDEST40 vector, *via* LR clonase reaction. This particular vector was chosen for the fact that it contains CMV promoter suitable for over-expression in mammalian cells, and the inserted ORF is tagged with the V5 epitope as well as 6xHis tag; the vector also contains a T7 promoter that is used for *in vitro* RNA transcription. *E. coli* (NovaBlue Singles strain; Merck) were used for the transformation and the efficiency of the clones was proved by colony PCR. Figure 12 shows the gel electrophoresis outcome after colony PCR.



(A) (B)

Figure 12: (A) PCR product of IFI6 and IFI27 were run in gel electrophoresis. IFI6 on the left has the size of 393 bp, IFI27 on the right has the size of 369 bp. (B) The used DNA marker in the middle of the gel.

Miniprep isolation took place of the four selected positive clones shown on Figure 12 and the clones were sent for sequencing to verify the ORF sequence. Used primers are represented in Table 20, and Figures 13, 14 are the sequence alignments of IFI6/27 using Geneious. The results from the alignments show that the practical obtained IFI6/27 genes after being cloned

into donor vector, match the literature database, meaning that the right target genes have been amplified.

Table 20: Primer sequences.

Primer	Sequence (5' → 3')
T7-F	TAATACGACTCACTATAG
bGH-R	TAGAAGGCACAGTCGAGG

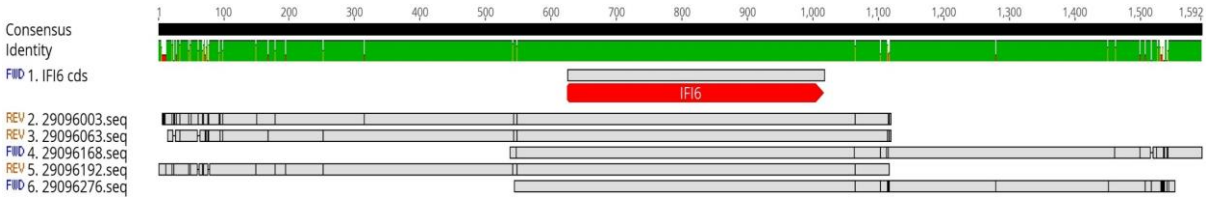


Figure 13: Aligned sequence of IFI6.

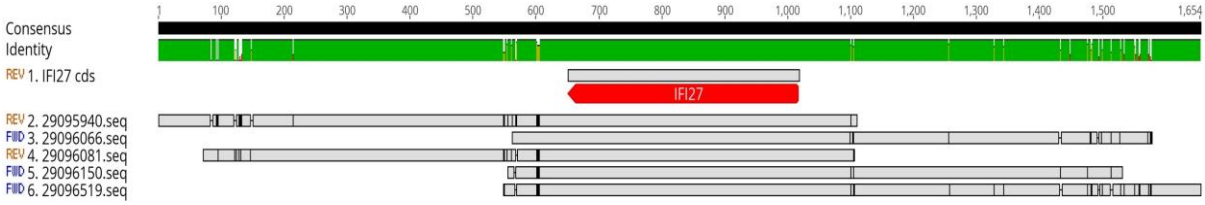


Figure 14: Aligned sequence of IFI27.

After the sequencing, the positive selected clones of IFI6 and IFI27 were used for maxiprep isolation for the following transfection experiments. Table 21 shows the concentrations of isolated plasmids.

Table 21: Concentration of the isolated genes and their purity.

clone	**c [ng/µl]**	**A260/A280**	**A260/A230**
IFI6	522	1,881	2,047
IFI27	1203	1,885	2,477

4.2 Analysis of *in vitro* antiviral characteristics of IFI6 and IFI27

The second part of this work was mainly based on *in vitro* analysis and characterization of IFI6 and IFI27 antiviral effect. This was carried out to test whether these genes of interest would have any antiviral effect in the case of TBEV infection. For this, the isolated pDEST40 constructs with IFI6 and IFI27 coding sequences were used for transient transfection. To analyse the CPE inhibition effect, the MTT viability assay was used. Moreover, viral titres

were determined in IFI6 and IFI27 transfected cells. For this analysis, two different transfection approaches were used – transfection of plasmid DNA and transfection of *in vitro* synthesized mRNA of IFI6/27.

4.2.1 Analysis of IFI6/IFI27 antiviral characteristics based on plasmid DNA transfection

For this step, the cells were seeded, then transfected with the plasmid DNA of IFI6/pDEST40 or IFI27/pDEST40, then infection was performed (at 24 hours post transfection), and cell viability analysis was performed5 days post infection.

This analysis was used with DAOY cells that were transfected by 100 ng/200 ng DNA plasmid of IFI6 and IFI27 that corresponds to $12{,}96x10^9/25{,}92x10^9$ copies of plasmid, the used virus was TBEV Neudoerfl strain, with a concentration of 5 MOI, following 5 days post infection. The Figure 15 represents the results of this experiment, that was statistically tested using Student's t-test, N = 6, where the p-values were:

P-value (100 ng IFI6) = 0,0124

P-value (100 ng IFI27) = 0,0060

P-value (200 ng IFI6) = 0,2350

P-value (200 ng IFI27) = 0,1966

When p-value < 0.05 (it is significant).

The obtained percentages were compared and showed higher cell viability in the case that contained over-expressed IFI6/27. Therefore, indicating the possible antiviral effect of IFI6 and IFI27.

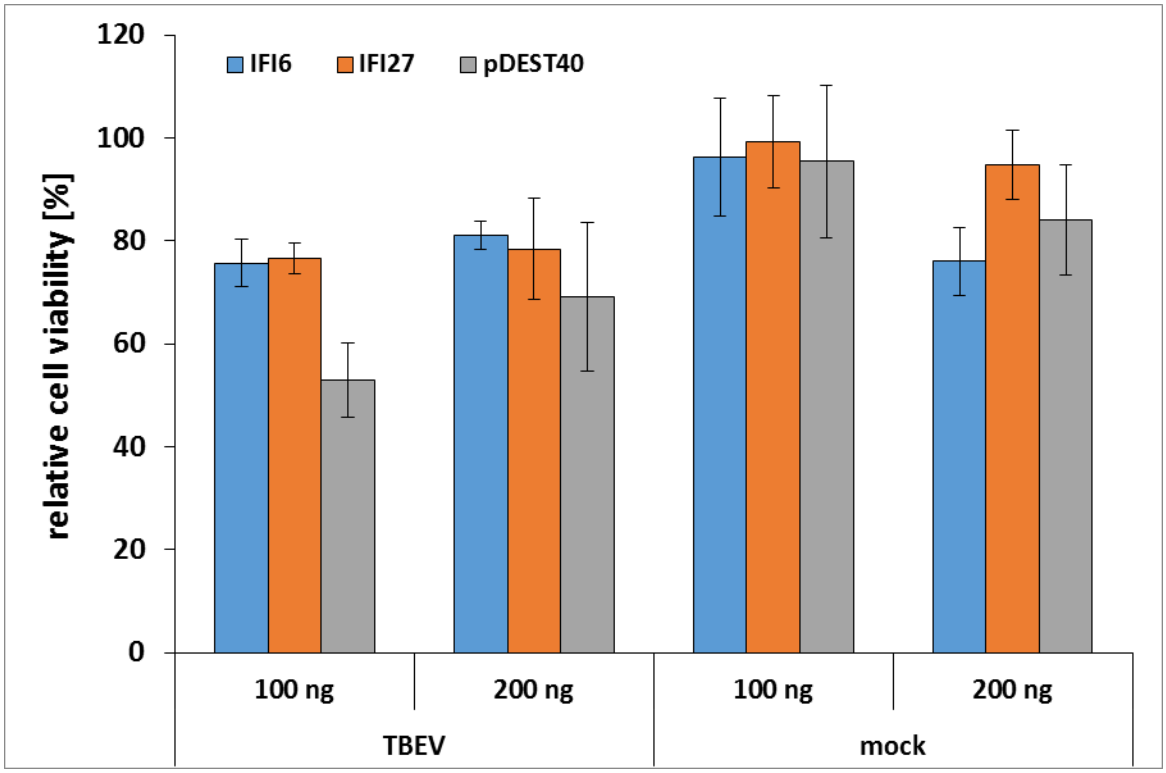


Figure 15: This graph shows the relative cell viability between different cases of infection. On the left we find the TBEV-infected DAOY cells with over-expressed IFI6 and IFI27 genes and the empty pDEST vector, and on the right, no TBEV-infection, only mock cells with over-expressed IFI6 and IFI27 and the empty pDEST vector. While both being compared with different amounts of IFI6 and IFI27 DNA genes (100ng and 200ng). Blue bar means that the cells were transfected by IFI6 DNA, orange bar means that the cells were transfected by IFI27 DNA, gray bar means that the cells were transfected by pDEST40 empty vector DNA.

In order to further characterize the possible antiviral effect of both genes, the cultivation media from 24 hours post infection were collected and tested by plaque assay method for virus production. Virus titres in Figure 16 shows that no difference was detected when IFI6 and IFI27 expressing samples were compared with negative control (empty pDEST). IFN-β pre-treated DAOY cells were used as a positive control.

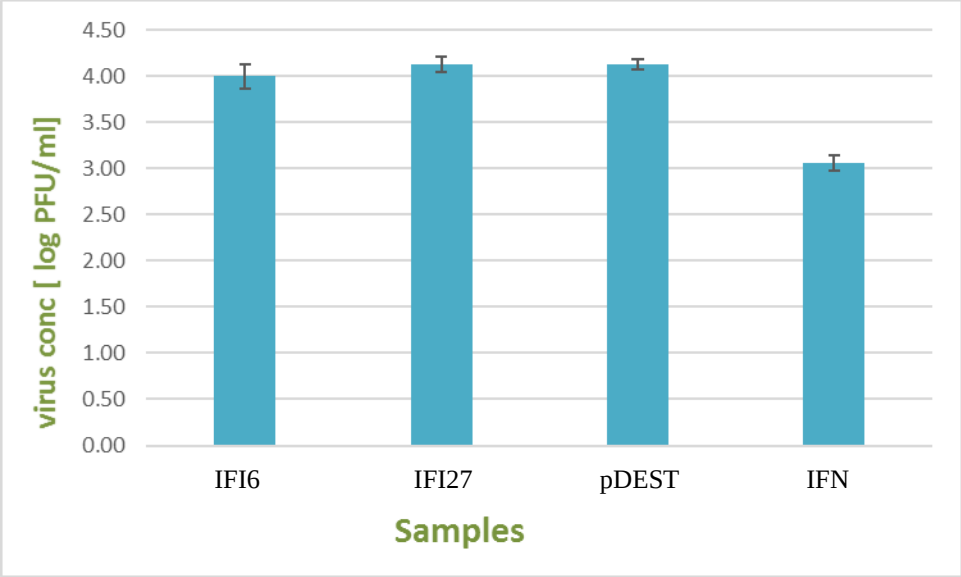


Figure 16: Viral production was compared between IFI6 and IFI27 gene expressing cells and an empty pDEST vector.

Despite the statistically significant decrease in CPE in IFI6 and IFI27 transfected cells as seen in Figure 15, we did not document any decrease in viral titres as seen in Figure 16. Therefore, we decided to verify the levels of over-expressed IFI6 and IFI27 in the transfected cells by detecting the mRNA levels using qPCR and protein levels using western blot and immunofluorescence microscopy.

Therefore, the work was followed by extraction of total RNA from of IFI6 and IFI27 transfected cells and testing for the presence of respective mRNAs. Relative quantification using ΔΔct method with HPRT as a housekeeping gene was performed. Moreover, the melting curves (Figures 17-19) illustrate that in case of IFI6 amplification, the reaction was not fully specific when compared to IFI27 and HPRT case.

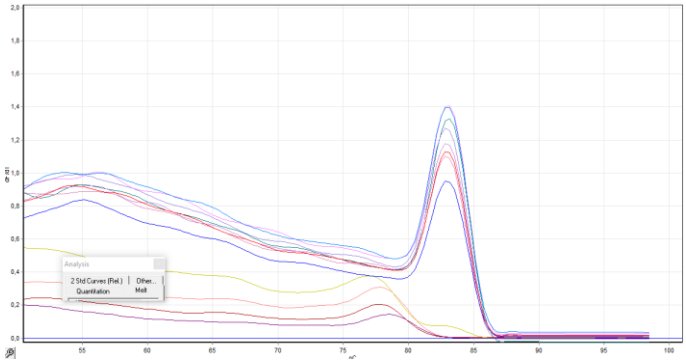


Figure 17: Melting curves from qRT-PCR of IFI27 gene.

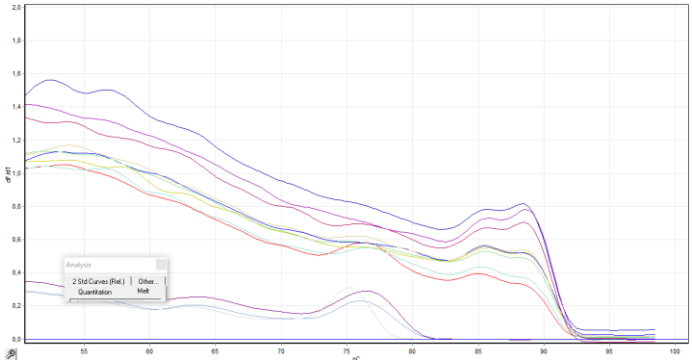


Figure 18: Melting curves from qRT-PCR of IFI6 gene.

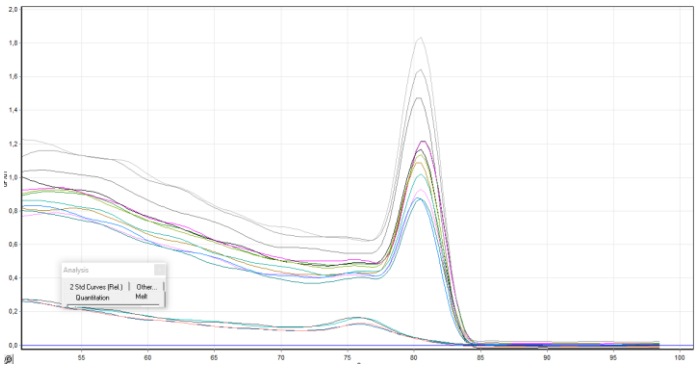


Figure 19: Melting curves from qRT-PCR of HPRT gene.

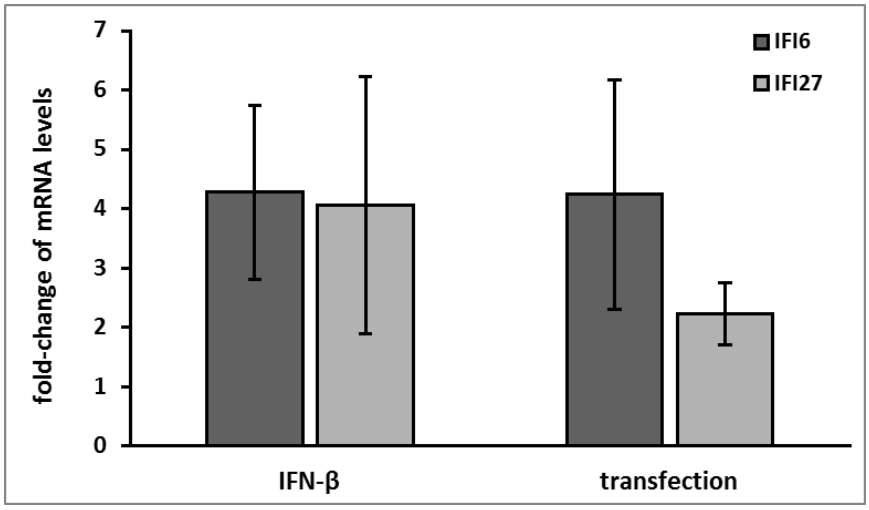
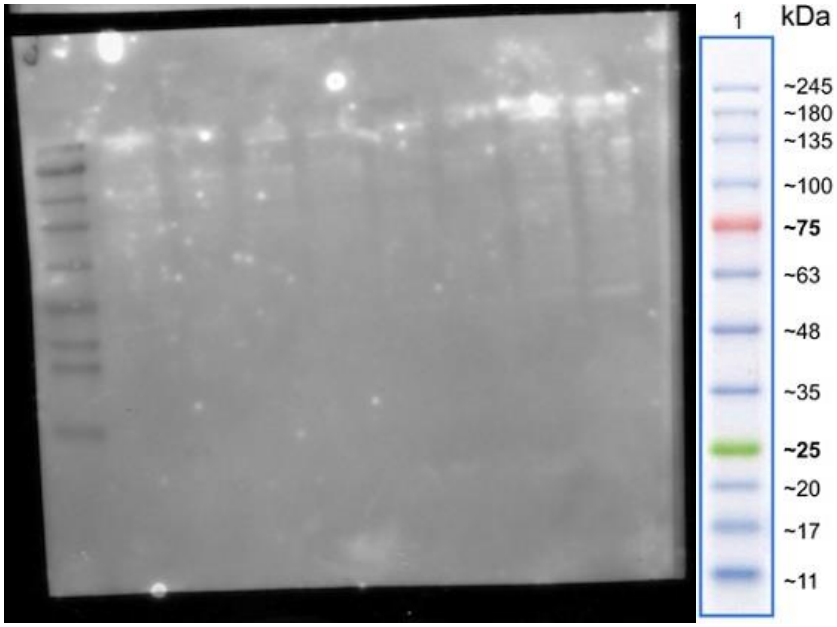


Figure 20: Expressed mRNA levels in case of DAOY cells over-expressing IFI6 and IFI27 on the right, and in case of IFN-β pre-treated DAOY cells on the left.

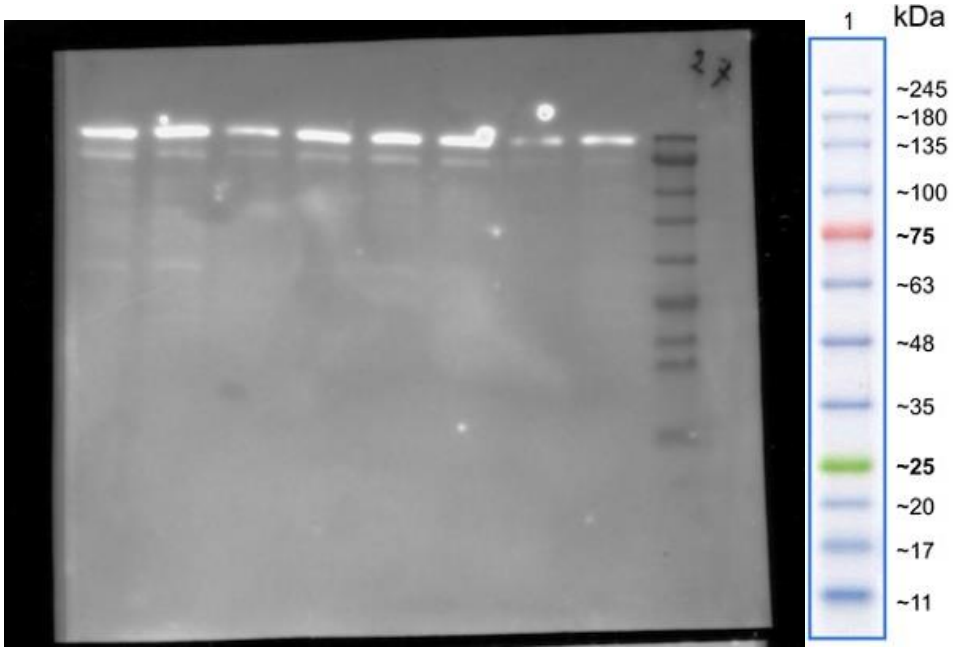
Next, the proteins from the samples used for plaque assays (Figure 16) were extracted from the cells and tested on the presence of IFI6/IFI27 using western blot. Firstly, the detection took place using anti-IFI6 and anti-IFI27 antibodies, which wasn't successful in detecting the

IFI6 and IFI27 proteins as represented in Figures 21 and 22. The IFI6 protein had the size of 12,9 kDa and IFI27 the size of 11,5 kDa and a protein marker of size 10-245 kDa.



(A) (B)

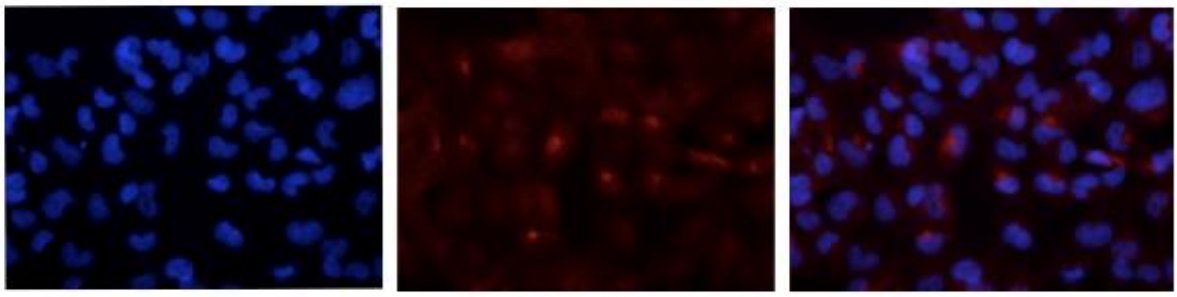
Figure 21: (A) PVDF membrane containing the blotted IFI6 protein using anti-IFI6 and Novex Chemiluminescent CDP-Star substrate to develop the chemiluminescent signal and documented using the GBox CHemi XX6 Syngene gel device.
(B) The used DNA marker in the middle of the gel.



(A) (B)

Figure 22: (A) PVDF membrane containing the blotted IFI27 protein using anti-IFI27 and Novex Chemiluminescent CDP-Star substrate to develop the chemiluminescent signal and documented using the GBox CHemi XX6 Syngene gel device.
(B) The used DNA marker in the middle of the gel.

The following Figure 23 represents the images of DAOY cells treated with IFN-β (10 ng/ml) 12 hours prior fixation captured with the immunofluorescence microscopy. IFN-β increases the expression of IFI6 and IFI27 according to previous study [Selinger et al. 2017]. According to the Figures we see that very low signals were detected in case of IFI6.



(A) **(B)** **(C)**

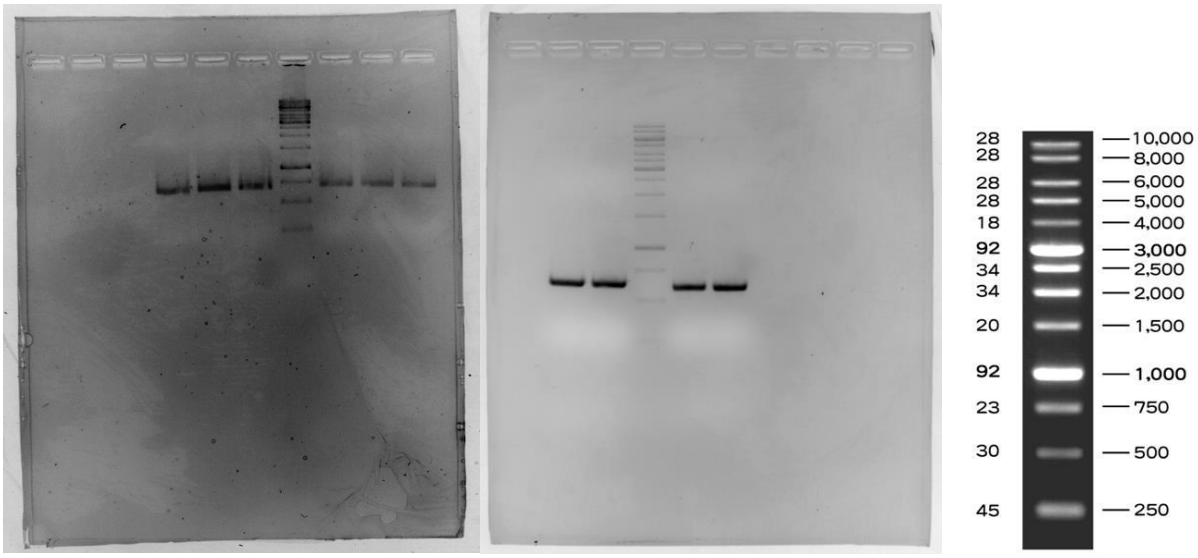
Figure 23: (A) DAPI-stained nuclei of IFN-treated DAOY cells (magnification: 400x). (B) Signal for IFI6 in IFN-treated DAOY cells (magnification: 400x). (C) Merged Figure of (A) and (B).

After all these negative results, this led to the conclusion that probably there is something wrong with the transfection step. Therefore, it was decided to try transfection of IFI6/IFI27 mRNA instead of the DNA construct.

4.2.2 Analysis of IFI6/IFI27 antiviral characteristics based on *in vitro* synthesized RNA transfection

Hence, T7 RNA *in vitro* transcription was used to create capped RNA of IFI6/IFI27 transcript tailed with poly(A). Firstly, the pDEST40 vector with IFI6/IFI27 coding sequence was used and amplified the T7-IFI6 or T7-IFI27 fragment using Q5-PCR (Figure 24 (B)). Afterwards, the poly(T) tail was linked to the 3‘end using Q5-PCR. The resulting DNA fragments T7-IFI6-poly(T) or T7-IFI27-poly(T) were used for *in vitro* RNA synthesis with cap analogue (NEB; G(5‘)ppp(5‘)G RNA Cap).

The successful amplification of T7-IFI6-poly(T)$_{80}$ and T7-IFI27-poly(T)$_{80}$ fragments for T7 RNA *in vitro* transcription is documented in Figure 24 (A). The resulting bands from the samples – T7-IFI6-poly(T) ~ 900 bp and T7-IFI27-poly(T) ~ 876 bp – were cut out of gel and isolated; the isolated IFI6 were pooled together also the IFI27 the same way and used for *in vitro* RNA transcription.



(A) (B) (C)

Figure 24: (A) Gel electrophoresis of amplified T7-IFI6-poly(T)$_{80}$ on the right and T7-IFI27-poly(T)$_{80}$ on th left. (B) Gel electrophoresis of amplified T7-IFI6 on the left and T7-IFI27 on the right using Q5-PCR. (C) Is the used DNA DNA marker in the middle of the gel.

The resulting RNA was afterwards purified by DNase treatment and precipitated according to MEGAscript kit manual using LiCl. Afterwards, the IFI6, IFI27, IFI6 and IFI27 mRNA mixture were used for the analogous experiment as in the previous part, where the DAOY cells were transfected this time by 60 ng of RNA, which corresponds to 148,2x10^9 of copies. Then infection with TBEV took place the same way. The harvested media were used in plaque assays and the cell lysates were used for western blot. The following Figure 25 represents the viral titres from this experiment that showed no significant difference when the viral titres were compared between IFI6 and IFI27 expressing cells and an empty pDEST vector.

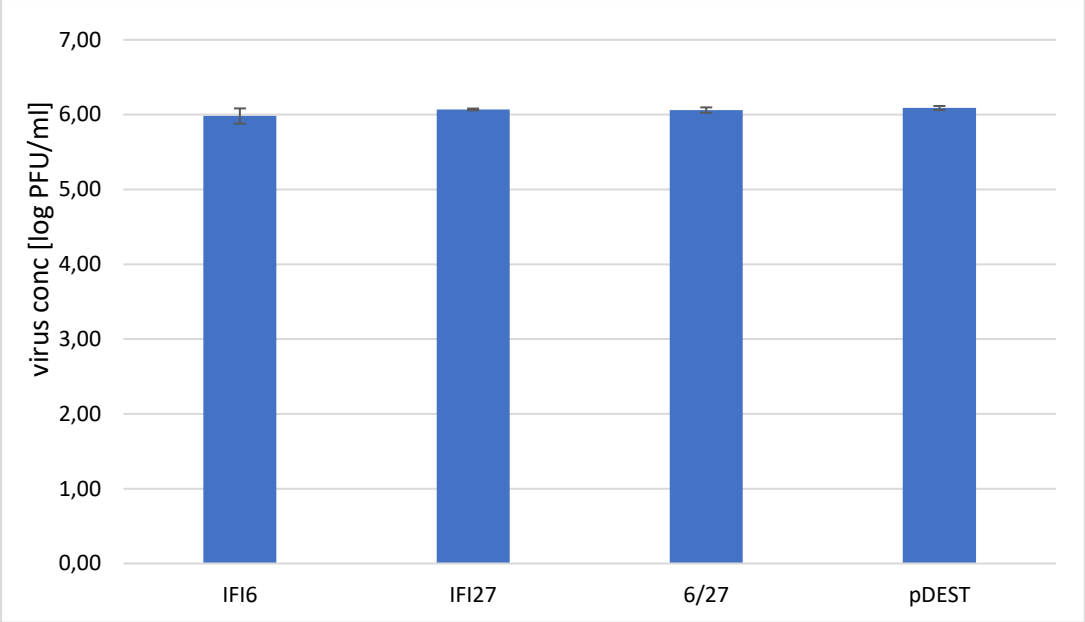
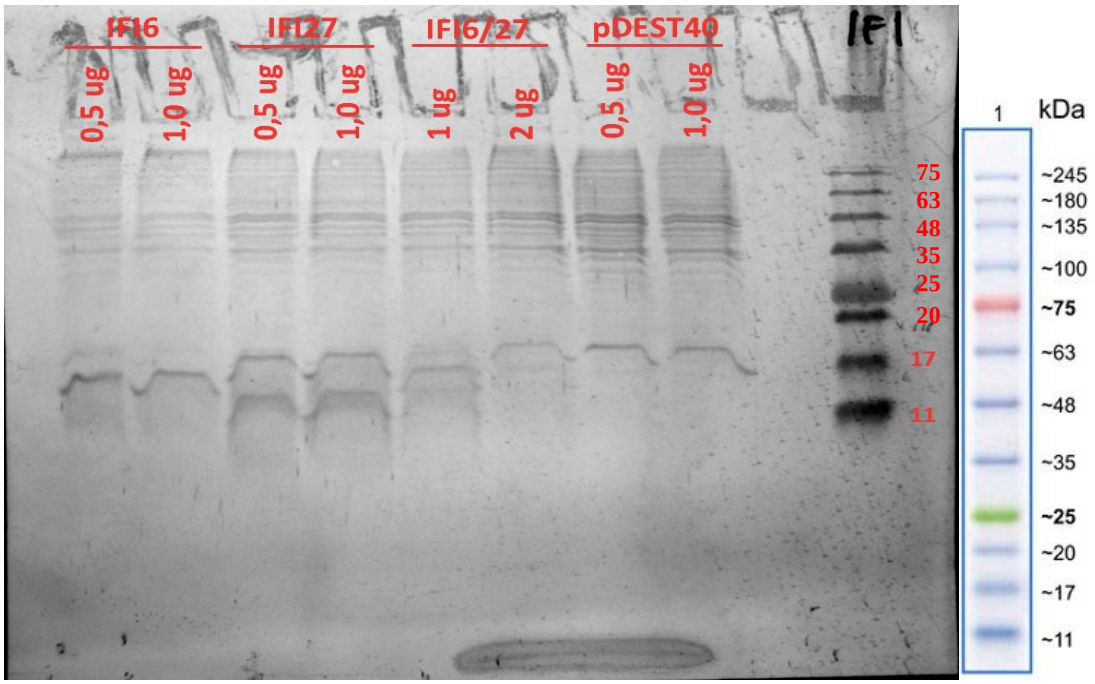


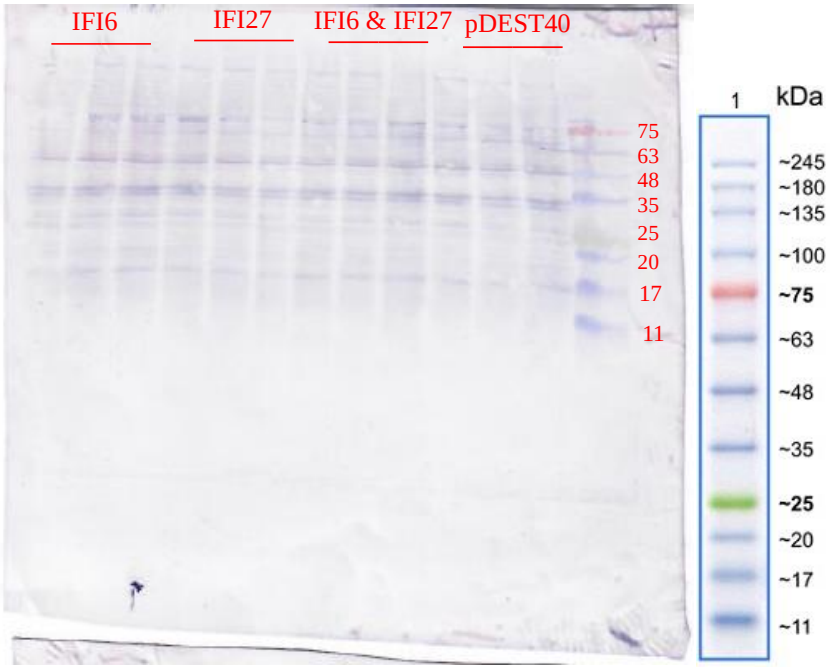
Figure 25: Viral production was compared between IFI6 and IFI27 expressing cells to an empty pDEST vector.

The western blot results are presented in Figures 26-29 that are membranes developed using the NBT/BCIP substrates (nitro-blue tetrazolium chloride with 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) for alkaline phosphatase. As seen from the following Figures the protein detection signals for IFI6 and IFI27 using anti-V5 tag antibodies were too low.



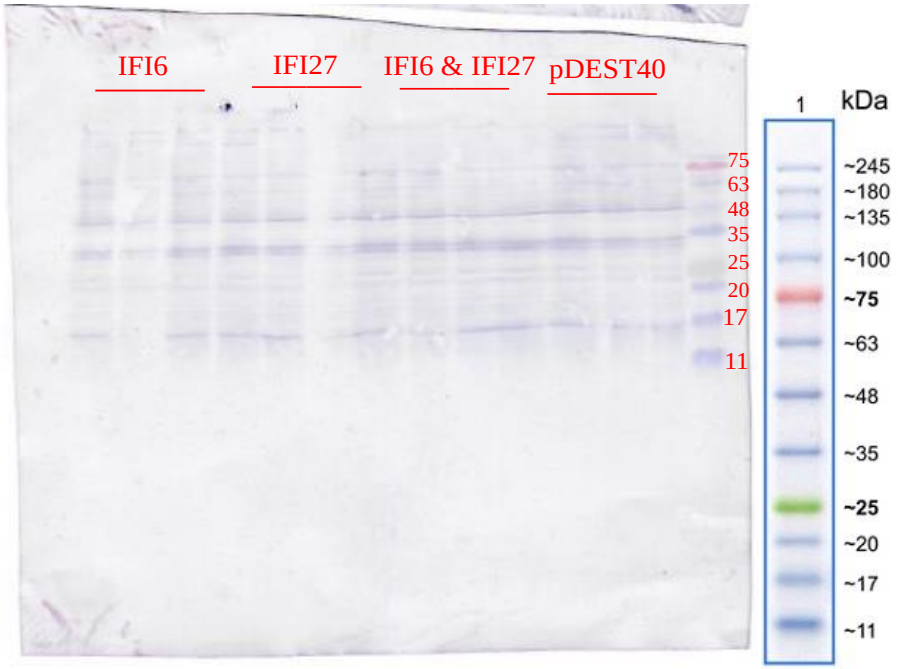
(A) (B)

Figure 26: (A) Western blot using anti-V5 tag. Two different amounts of each sample have been blotted. The IFI6 protein had the size of 12,9 kDa and IFI27 the size of 11,5 kDa. (B) The used DNA marker in the middle of the gel.



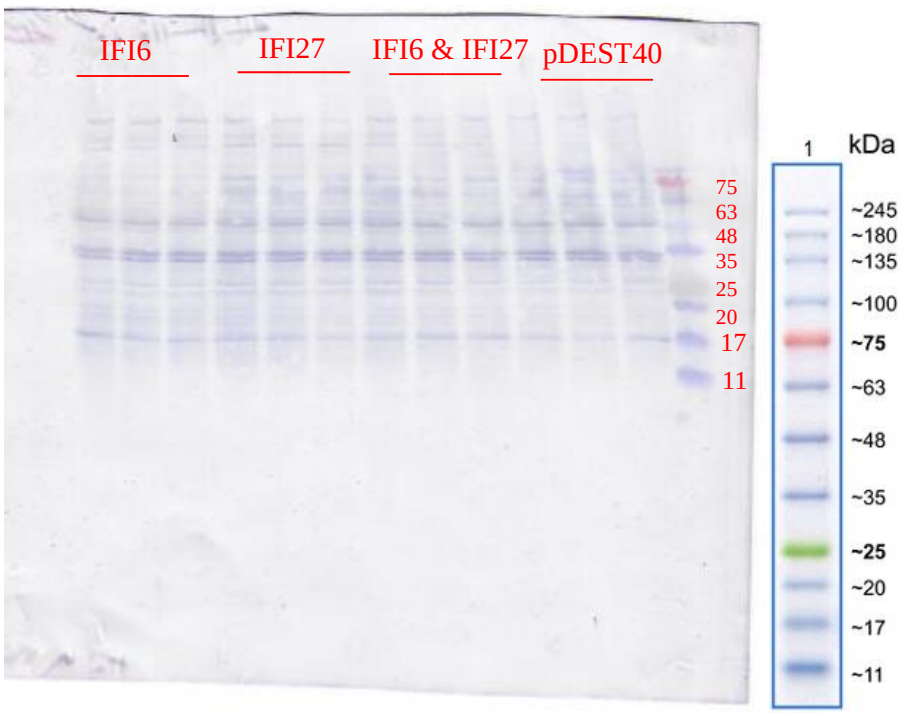
(A) (B)

Figure 27: (A) Western blot of the proteins from 0 hours post infection using anti-V5 tag. The IFI6 protein had the size of 12,9 kDa and IFI27 the size of 11,5 kDa and the protein marker is on the far right. (B) The used DNA marker in the middle of the gel.



(A) (B)

Figure 28: (A) Western blot of the proteins from 24 hours post infection using anti-V5 tag. The IFI6 protein had the size of 12,9 kDa and IFI27 the size of 11,5 kDa and the protein marker is on the far right. (B) The used DNA marker in the middle of the gel.



(A) (B)

Figure 29: (A) Western blot of the proteins from 48 hours post infection using anti-V5 tag. The IFI6 protein had the size of 12,9 kDa and IFI27 the size of 11,5 kDa and the protein marker is on the far right. (B) The used DNA marker in the middle of the gel.

5 Discussion

When DAOY cells were pre-treated with IFN-β prior to TBEV infection, the viral titres turned out to be 10^4 times lower in comparison to non-IFN treated cells, and according to the transcriptome analysis conducted in a previous research, IFI6 and IFI27 were found to be mostly up-regulated after IFN-β treatment [Selinger et al. 2017]. Other previous studies also showed that over-expressed IFI6 leads to the restriction of hepatitis C virus replication in the Huh7 cells (Itsui et al., 2006), and over-expressed IFI27 leads to reducing the production of West Nile virus and Saint Louis encephalitis virus in granule cell neurons of the cerebellum and cortical neurons from the cerebral cortex (Cho et al., 2013). This gave us the motivation to further investigate the antiviral activity of IFI6/27 in the case of TBEV. Therefore, the aim of this study was to clone the genes of interest, IFI6 and IFI27, into mammalian expression vector, and to investigate the possible antiviral activity of both IFI6 and IFI27 in human DAOY cells when infected by TBEV.

Using the Gateway cloning technology, the IFI6/27 were first cloned into a pDONR207 donor vector, then into a pDEST40 destination vector to be expressed in the mammalian

cells. The target genes were successfully cloned into the specific vectors, and the clones were validated by sequencing.

The second part of the project was focused on investigating the antiviral effect of the IFI6/27 *in vitro*. For the DNA transfection part, the isolated pDEST40 constructs with IFI6 and IFI27 coding sequences were used to transient transfection, where cell viability analysis took place, and gave us a promising antiviral effect as seen in Figure 15, which showed statistically significant antiviral effect of IFI6/27. When the plaque assay took place, Figure 16 indicates that there was not any decrease in viral titres. Therefore, I decided to verify the levels of over-expressed IFI6 and IFI27 in transfected cells, by detecting the mRNA levels using real-time PCR and protein levels using western blot and immunofluorescent microscopy. The results from qPCR showed that the melting curves (Figures 17-19) illustrate that in case of IFI6 amplification, the reaction was not fully specific, whereas in case of IFI27 it shows a nice melting curve, the western blot with anti-IFI6 and anti-IFI27 antibodies couldn't detect the proteins of interest. The reason for this could be that the target genes weren't over-expressed successfully or that the antibodies weren't able to detect the proteins. The immunofluorescence microscopy method showed a weak signal as seen in Figure 23 using anti-IFI6 antibodies (results shown only for positive control IFN-β-treated cells) indicated that the signal was too low, and the reason could be again that the either anti-IFI6 did not work or the DNA transfection method did not work.

All those results led us to try optimization of the method, Therefore, the transfection procedure was repeated with mRNA instead DNA and the western blot was repeated using anti-V5 tag antibodies instead anti-IFI6/27. For mRNA transfection, capped RNA of IFI6/IFI27 transcript was created and tailed with poly(A). The plaque assay for mRNA transfection didn't show any decrease in viral titre as seen in Figure 25, as well as no protein levels were detected in transfected cells when using western blot with anti-V5 tag antibody. The problem could be that the transfection of the corresponding genes wasn't successful either even with mRNA case leading to errors in overexpression. Based on previous studies [Selinger et al. 2017] and (Gjermandsen et al., 2000), both IFI6 and IFI27 were able to be overexpressed when stimulated with IFN-β in case of infections. Therefore, it is believed that IFI6 and IFI27 proteins are involved in apoptosis signalling pathway, and their up-regulation could lead to an apoptosis of infected cell. However, the overexpression levels of ISGs is influenced by the used cell lines as well (Gjermandsen et al., 2000), and since in our case the over-expression didn't work, this led to difficulties in detection because of the low protein

levels, and in case of qRT-PCR there had been pipetting error that could have lowered the detection limit. Therefore, optimization of the transfection method is needed to be further optimized.

6 Conclusion

- Both IFI6 and IFI27 were successfully cloned into a pDONR207 donor vector, then into a pDEST40 destination vector and validated via sequencing.
- The antiviral effect of IFI6/27 couldn’t be fully proven in case of DNA transfection, the plaque assay could not show any significant antiviral effect of IFI6/IFI27, the used antibodies of western blot could not detect the IFI6/27 proteins.
- The immunofluorescence microscopy did not show high signal for IFI6, therefore it wasn’t repeated in case of IFI27.
- The transfection step was repeated, using capped RNA of IFI6/IFI27 transcript and tailed with poly(A). And the results of plaque assay and western blot did not prove the antiviral effect of IFI6/27 either.
- We still believe that IFI6 and IFI27 have an antiviral role, but different approach should be used to prove this point.

7 References

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