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„Cell Based Assays: Cell Viability Profiling and TNFα Quantification“

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Abstract

Tumor necrosis factor alpha (TNFα) not only plays a key role in the human immune system, but also has a cytotoxic effect on tumor cells. Owing to this function, TNFα can become a considerable candidate for chemotherapy but needs to be delivered locally. One starting point for the solution of this problem is the transgene expression of TNFα by local cells by methods of gene therapy. Towards this goal, the present work includes the in vitro transfection of plasmids coding for TNFα into cells by LPEI (linear polyethylenimine) based nanoparticles (NPs) as well as the optimization of L929-MTT cytotoxicity bioassay for estimation of the bioactivity and the amount of secreted TNFα. The L929-MTT cytotoxicity bioassay is based on TNFα induced cytotoxicity on L929 cells, which was measured by MTT assay (a colorimetric cell viability assay). First, MTT assay was optimized by adapting from an original protocol based on the insights of Supino R. and Chiba K. et al., by testing different intermediate steps and solvents like DMSO, isopropanol, acidified isopropanol and SDS/HCl, and comparing the R squared values of the different variations, showing the correlation between the seeded cell number and the measured absorbance. This study showed that acceptable and reliable results (based on R squared values) can be obtained with the MTT assay without an additional intermediate step of removing the whole medium after first incubation, adding MTT working solution in PBS up to a final concentration of 0.45 mg/ml in each well and using DMSO as a solvent. Second, the optimized MTT protocol was used for establishing the L929-MTT assay for quantification of bioactive TNFα produced as a result of nanoparticle mediated TNFα gene delivery. It was possible to validate the transfection efficiency of LPEI based nanoparticles (10 kDA, N/P = 6) and to compare the transfection efficiency with commercially available lipofectamine (LF). After transfection of A549 cells with TNFα plasmid based LPEI nanoparticles, TNFα is secreted by the A549 cells. Bioactivity of the secreted TNFα was measured by transferring different dilutions of the A549-supernatant containing the TNFα to L929 cells and afterwards investigating the cell viability with the MTT assay and by implication the cytotoxic effect of TNFα on L929 cells. For transfection pCpG-hCMV-EF1α-TNFα (pEF1α-TNFα) and pCpG-hCMV-SCEP-TNFα (pSCEP-TNFα) were used, two plasmids carrying the same cDNA for murine TNFα but under two different promoters

namely EF1α and SCEP respectively. The in vitro TNFα transgene expression kinetic was also analyzed in this assay for both plasmids over duration of 4 days. Afterwards the transgene expression of the two promoters was compared. For quantification of the secreted TNFα, the cytotoxic effect of the unknown amount of TNFα measured was compared with the cytotoxic effect achieved with known amounts of recombinant murine TNFα. It was also shown that the cytotoxic effect of the secreted TNFα on L929 cells reached 91.5% for transfection with 200 ng pEF1α-TNFα using LPEI, 91.3% for 200 ng pSCEP-TNFα using LPEI and 91.4% for the transfection with 200 ng pEF1α-TNFα using LF. This indicates high bioactivity of TNFα, which is secreted by the A549 cells as a result of nanoparticle mediated transfection. Also, there is no significant difference between transgene expression of TNFα when compared between the two plasmids having different promoters. Another conclusion is that the transfection efficiency of LPEI based nanoparticles is comparable to LF. After performing the L929-MTT assay with rTNFα the amount of secreted TNFα was calculated. After transfection with 200 ng pEF1α-TNFα using LPEI, 0.039 ng TNFα is estimated to be present in 10 µl of the A549-supernatant. Furthermore, analyzing the in vitro TNFα transgene expression kinetics showed that both plasmids have an expression peak on the second day after the transfection, with an amount of 0.023 ng TNFα secreted for pEF1α-TNFα and 0.0226 ng for pSCEP-TNFα. The transgene expression of pEF1α-TNFα was higher on all 4 days. The comparison of fresh and frozen A549-supernatant with secreted TNFα revealed that a freezing period can reduce the bioactivity of TNFα.

Zusammenfassung

Tumornekrosefaktor alpha (TNFα) spielt nicht nur eine Schlüsselrolle im menschlichen Immunsystem, sondern hat auch einen zytotoxischen Effekt auf Tumorzellen. Dank dieser Funktion kann TNFα zu einem bedeutenden Hilfsmittel während der Chemotherapie werden, allerdings muss es dafür lokal verabreicht werden. Ein Ansatzpunkt für die Lösung des Problems ist die transgene Expression von TNFα durch lokale Zellen mit Hilfe der Gentherapie. Deshalb beinhaltet diese Arbeit die in vitro Transfektion von Zellen mit Plasmiden die für TNFα kodieren, durch Nanopartikel die mit LPEI (Lineares Polyethylenimin) gebildet wurden sowie auch die Optimierung des L929-MTT Zytotoxizitätbioassays für die Beurteilung der Bioaktivität bzw. der Menge des sezernierten TNFα. Der L929-MTT Zytotoxizitätsbioassay basiert auf der Zytotoxizität von TNFα auf L929 Zellen, gemessen mit dem MTT Assay, einem kolorimetrischen Zellviabilitätsassay. Zunächst, wurde der MTT Assay optimiert, indem ein Originalprotokoll basierend auf den Erkenntnissen von Supino R. und Chiba K. et al., durch das Testen von unterschiedlichen Arbeitsschritten sowie Lösungsmitteln wie DMSO, Isopropanol, angesäuertes Isopropanol und SDS/HCl angepasst wurde, und anschließenden die R^2 Werte der unterschiedlichen Variationen verglichen wurden, die die Korrelation zwischen der gesäten Zellzahl und der gemessenen Absorption beschreiben. Diese Studie verdeutlichte, dass brauchbare und verlässliche Ergebnisse (basierend auf den R^2 Werten) mit dem MTT Assay erzielt werden können, ohne den zusätzlichen Arbeitsschritt nach der ersten Inkubationszeit, bei dem das gesamte Zellmedium entfernt wurde, durch das Hinzufügen der MTT Arbeitslösung in PBS bis zu einer Konzentration von 0.45 mg/ml in jedem Well, und durch die Verwendung von DMSO als Lösungsmittel. Anschließend wurde das optimierte Protokoll für den L929-MTT Assay verwendet, für die Quantifizierung des bioaktiven TNFα, dass infolge der Genübertragung durch Nanopartikel produziert wurde. Ebenfalls war es möglich die Transfektionseffizienz der LPEI basierten Nanopartikel (10 kDa, N/P = 6) zu bewerten und mit der des handelsüblichen Lipofectamine (LF) zu vergleichen. Nach der Transfektion der A549 Zellen mit den auf LPEI basierenden Nanopartikeln aus für TNFα kodierenden Plasmiden, wird TNFα von diesen sezerniert. Die Bioaktivität des sezernierten TNFα wurde gemessen, indem unterschiedliche Verdünnungen des

Überstandes der A549 Zellen, der das sezernierte TNFα enthält, auf die L929 Zellen aufgebracht wurden um anschließend die Zellviabilität und somit im Umkehrschluss auch den zytotoxischen Effekt des TNFα auf die L929 Zellen mit dem MTT Assay zu bestimmen. Für die Transfektion wurden pCpG-hCMV-EF1α-TNFα (pEF1α-TNFα) und pCpG-hCMV-SCEP-TNFα (pSCEP-TNFα) verwendet, zwei Plasmide, die dieselbe cDNA für murines TNFα tragen, allerdings unterschiedliche Promoter besitzen, und zwar EF1α und SCEP. Die Kinetik der transgenen Expression von TNFα wurde in vitro über einen Zeitraum von 4 Tagen ebenfalls mit diesem Assay analysiert. Die Ergebnisse der beiden Promoter wurden anschließend miteinander verglichen. Für die Quantifizierung der Menge an sezerniertem TNFα, wurde der zytotoxische Effekt der unbekannten Mengen an TNFα mit dem zytotoxischen Effekt verglichen, der durch bekannte Mengen an rekombinanten murinen TNFα verursacht wurde. Der zytotoxische Effekt des sezernierten TNFα auf die L929 Zellen erzielte einen Wert von 91,5% nach der Transfektion mit 200 ng pEF1α-TNFα mit LPEI, 91,3% wurde nach der Transfektion mit 200 ng pSCEP-TNFα mit LPEI erreicht und 91,4% nach der Transfektion mit 200 ng pEF1α-TNFα mit LF. Das weist auf eine hohe Bioaktivität des TNFα hin, das von den A549 Zellen nach der Transfektion mit den Nanopartikeln sezerniert wird. Des Weiteren, gibt es keinen signifikanten Unterschied zwischen den transgenen Expressionen von TNFα, beim Vergleich der beiden Plasmide bezogen auf die unterschiedlichen Promoter. Ein weiteres Ergebnis ist, dass die Transfektionseffizienz von LPEI basierten Nanopartikeln vergleichbar mit der von LF ist. Nachdem der L929-MTT Assay auch mit dem rTNFα durchgeführt wurde, wurde die Menge des sezernierten TNFα berechnet. Es wurde berechnet, dass nach der Transfektion mit 200 ng pEF1α-TNFα unter Verwendung von LPEI, 0,039 ng TNFα in 10 µl des Überstandes der A549 Zellen vorhanden sind. Außerdem hat die in vitro Untersuchung der Kinetik der transgenen Expression gezeigt, dass beide Plasmide ein Expressionsmaximum am zweiten Tag nach der Transfektion haben, wobei durch pEF1-TNFα 0,023 ng TNFα sezerniert wurden und durch pSCEP-TNFα 0,0226 ng. Die transgene Expression war an allen 4 Tagen höher für pEF1α-TNFα. Abschließend, hat der Vergleich zwischen frischem und bereits gefrorenem A549-Überstand mit dem sezernierten TNFα veranschaulicht, dass das Einfrieren die Bioaktivität des TNFα reduzieren kann.

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ABBREVIATIONS

AAF-R110	Bis-Ala-Ala-Phe-rhodamine 110
AFC	Aminofluorocoumerin
AP-1	Activator protein 1
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
Calcein AM	Calcein acetoxymethyl
cDNA	Complementary Deoxyribonucleic acid
cfu	Colony forming unit
cIAP	Cytoplasmic inhibitor of apoptosis
CMV	Cytomegalovirus
CMV IEP	Cytomegalovirus major immediate early promoter
CV	Coefficient of variation
DAPI	4',6-diamidino-2′-phenylindole
DD	Death domain
dH_2O	Distilled H_2O
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
DTP	Developmental Therapeutics Program
E. coli	Escherichia coli
ED_{50}	Median effective dose
EF1α	Elongation factor 1 alpha

ELAM	Endothelial leukocyte adhesion molecules
ELISA	Enzyme-linked Immunosorbent Assay
EP	Endogenous pyrogen
ER	Endoplasmatic reticulum
Expt	Experiment
FBS	Fetal bovine serum
GF-AFC	Glycyl-phenylalanyl-aminofluorocoumerin
G3-HD-OEI	Generation3-1,6-hexandioldiacrylate-oligoethylenimine
H	Hour
HBS	HEPES-buffered saline
HCl	Hydrochloric acid
hCMV	Human Cytomegalovirus
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
IBP	Inflammatory bowel disease
IL-1	Interleukin 1
JRA	Juvenile rheumatoid arthritis
kDa	Kilodalton
La	DNA-ladder
LB	Luria-Bertani
LDH	Lactate Dehydrogenase
LF	Lipofectamine® 2000
LPEI	Linear Polyethylenimine
LPS	Lipopolysaccharide
MALT	Mucosa Associated Lymphoid Tissue
min	Minute

MQ	Milli-Q, ultrapure water
MTS	5-(3-carboxymethoxyphenyl)-2-(4, 5-dimethyl-thia- zoly)-3-(4-sulfophenyl) tetrazolium
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NaOH	Sodium hydroxide
NAD/NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCE	New chemical entity
NCI	National Cancer Institute
NCCD	Nomenclature Committee on Cell Death
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	Natural Killer cell
NP	Nanoparticle
N/P ratio	Nitrogen/Phosphate ratio
OD_{600}	Optical density measured at 600 nm
OMEM	Opti-MEM® I Reduced-Serum Medium
PBS	Phosphate-buffered saline
pDNA	Plasmid DNA
PI	Propidium iodide
PKCζ	Protein Kinase C zeta
QSAR	Quantitative structure-activity relationship
R^2	R squared value
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
rpm	Rotations per minute

RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
rTNFα	Recombinant tumor necrosis factor alpha
SB	Sodium borate
SCEP	Shuffled CMV EF-1 promoter
SD	Standard deviation
SDS	Sodium dodecyl sulfate
Sec	Second
SRB	Sulphorhodamin b assay
Supern.	Supernatant
TACE	TNFα-converting-enzyme
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TNF-R1	Type I TNF receptor
TNF-R2	Type II TNF receptor
TRADD	TNF-R1-associated death domain
TRAF 2	TNF receptor-associated factor 2
WST-1	4-[3-4-iodophenyl]-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulfonate
XTT	2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-carboxanilide-2H-tetrazolium
ZO-1	Zonula occludens-1

1. INTRODUCTION

1.1. CELL VIABILITY ASSAYS

Cell viability assays are frequently used instruments for the determination of cell proliferation and cytotoxicity of new chemical entities (NCE) in the field of drug discovery. Especially the possibility of testing new compounds in an economic, repeatable, straightforward and reliable way aroused great interest of the pharmaceutical industry in the past with the intention of detecting new chemotherapeutic substances in pre-clinical in vitro screenings, such as the NCI 60 human tumor cell lines screen. This method was originated by the Developmental Therapeutics Program (DTP) of the National Cancer Institute (NCI) in the early 1990s and makes use of 60 human tumor cell lines that are treated with different concentrations of the compound to be tested. After a defined incubation time, the cytotoxicity is ascertained by staining with sulphorhodamin B (Holbeck et al., 2010), a colorimetric cell viability assay (Voigt, 2005). In 2010 already 100.000 compounds have been examined by the NCI and an additional 50.000 products extracted from natural sources (Holbeck et al., 2010).

According to the definition by the Nomenclature Committee on Cell Death (NCCD), a cell is considered dead as soon as the plasma membrane is non-reversibly permeable to the extent that the selectivity is lost or the cell is entirely fractionalized (Galluzzi et al., 2015), which is accompanied by the decay of metabolic activity. These are three of various conditions cell viability assays make use of, either by visualizing the number of viable or the number of dead cells. The different types of experiments are described in the following points.

1.1.1. COMMON METHODS IN THE DETERMINATION OF CELL VIABILITY

Existing cell viability assays are utilizing different working points of the cells, like the penetrability of the cytoplasmic membrane, the ability of proliferation, the specific amount of DNA, the ATP production or the metabolic activity of the cells. To find the appropriate assay for planed experiments, it is necessary to consider different factors like the type of cells used for the experiment, the experimental design and the possible

interactions caused by the compound and components of the medium or the cell viability assay. Furthermore, the effect of the compound on the cell, meaning whether it leads to apoptosis or necrosis or has a cytostatic impact, should be considered.

Counting the cells directly using a hemocytometer and an optical microscope, is one possibility for determining the cell number and viability. For a hemocytometer, a cell suspension in an appropriate concentration is required. A predefined volume is pipetted into the counting chamber, a space between the object slide and the cover slip and the cells that should be evenly spread over the counting grid are counted. In order to visualize the dead cells and thus make them more distinguishable from the viable cells, trypan blue an exclusion dye that only penetrates into dead cells can be added to the suspension (Ng and Schantz, 2010). Advantages of this method are the simplicity and affordability, whereas it is not suitable for high-throughput screening, because it is time-consuming. In addition, there exists a high error, explained by the Poisson distribution that reveals, that the standard deviation (SD) of *n* counted cells is $\sqrt{n}$ and the coefficient of variation (CV) in percentage terms is calculated by the formula $CV = 100/n^{0.5}$. That implies that the CV for 100 counted cells amounts to 10% calculated for an optimal counting procedure (Shapiro, 2005). Supplementary there are other sources of error, like for instance insufficient mixing of the cell suspension, not adding the correct volume of suspension to the counting chamber, due to imprecise procedure or not calibrated pipettes, using the wrong cover glass or deficient counting. To exclude the abovementioned human errors, automated cell counters are also used.

The colony forming assay is used mainly for measuring the effect of ionizing radiation but also for the cytotoxic effect of NCE. After the specific treatment, the cells are counted and afterwards plated in a very low density. Colonies of at least 50 cells formed by single cells, still viable and proliferative after a defined duration, are fixed, stained and counted. This method is very time-consuming and therefore not convenient for a high-throughput screening (Rafehi et al., 2011). Another disadvantage is the fact, that this assay is not suitable for all cell lines, because not all cell lines arrange in colonies (Langdon, 2004).

Another a possible approach for the determination of the cell viability is the ATP assay. The amount of ATP released on lysis of viable cells is proportional to the cell number. After inactivation of the endogenous ATPase and adding luciferase and luciferin, whereby luciferin reacts to oxiluciferin, a luminescent signal appears that can be measured. This assay is very fast due to the missing incubation step, is suitable for high-throughput screening and has the highest sensitivity among all assays that can be applied for microplates. Up to 10 cells per well can be determined, because of the minor interfering signal of the background. Drawbacks of this assay are the severe temperature-dependency of the enzyme-catalyzed reaction and that some compounds could act as luciferase inhibitors (Chen, 2009).

Another method used for the determination of the cell number is the quantification of DNA using fluorescent stains. DAPI and PI are not able to penetrate the cell membrane of living cells and therefore are used for staining dead cells. PI can be combined with calcein AM that permeates living cells and is then split by cellular esterases, whereupon the cell membrane impermeable fluorescent dye calcein is formed (Fakhrullin and Choi, 2014). The fluorescent dye Hoechst 33342 penetrates the membrane quickly and intercalates with the DNA. It is frequently applied for staining living cells. Cyanine dyes like CyQuant and PicoGreen are also often used because their fluorescent increases strongly after binding the DNA. PicoGreen quantifies double-stranded DNA (dsDNA) so that there is no problem with single-stranded DNA, RNA or free nucleotides. CyQuant binds to DNA and RNA and therefore the application of an RNAse could be necessary in order to get a precise result. These fluorescent assays are convenient for high-throughput screenings performed in 96-well plates and more sensitive than colorimetric assays (Quent et al., 2010a).

1.1.2. Colorimetric Cell Viability Assays

Colorimetric assays make use of the Beer-Lambert Law that describes a linear relation between the concentration of a chromophore molecule in a solution and its absorbance in the visible spectrum, measured with a spectrophotometer, for a constant molar extinction coefficient and path length (Housecroft and Constable, 2006). The chromogen develops during metabolic activities, that only occur in viable cells, accordingly the concentration of the chromogen compound is proportional to the

number of viable cells and hence to the measured absorbance. Colorimetric assays are of advantage because they can be applied to cell suspension as well as adherent cells and they are suitable for high-throughput screenings. Furthermore, these assays are sensitive and reproducible as long as they are optimized for the required experimental setup (Vega-Avila and Pugsley, 2011).

The Alamar Blue® assay is an often-applied colorimetric cell viability assay that uses resazurin, a dye and redox indicator. It is based on the reduction of resazurin to resorufin in viable cells which leads to an alteration of the medium color and the emergence of fluorescence, thus the sample can be analyzed by colorimetric and fluorometric measurements (Al-Nasiry et al., 2007; Wu, 2010). A relevant benefit of this assay is the fact that it is atoxic and therefore convenient for the observation of cell proliferation over a longer period (Ahmed et al., 1994). It is sensitive enough to measure 200 cells/well in a 96-well plate (Page et al., 1993).

Apart from the assays using the metabolic activity of cells, the sulphorhodamin b assay (SRB) determines the cell number by the pH dependent linking to cellular proteins. The amount of bound dye can also be quantified by colorimetric and fluorometric measurements and is proportional to the cell number calculated afterwards. Due to the necessity of fixing the cells, it is an endpoint assay and it can be used for a high-throughput screening (Vichai and Kirtikara, 2006). It is possible to observe down to 500 cells per well in a 96-well plate with this assay (van Tonder et al., 2015).

The detection of protease activity in viable cells that decreases rapidly in dead cells is also a method for the determination of the cell number and cell viability. GF-AFC is a substrate that passes the cell membrane and through the enzymatic cleavage by cellular aminopeptidases, AFC is released. The amount of AFC is proportional to the number of living cells and its fluorescence signal can be measured. To detect the dead cells AAF-R110 can be used, a compound that is not able to penetrate the intact membrane of viable cells and is split by a special protease, released through the permeable membrane of dead cells. The originated rhodamine 110 creates a fluorescent that is measured and from which the number of dead cells can be

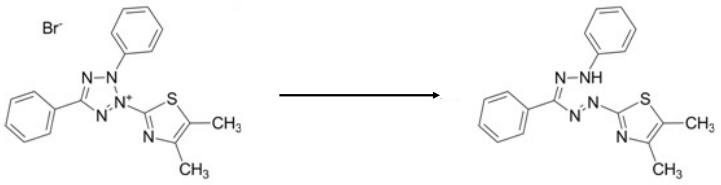
concluded. These two assays can be meld with each other and also with other assays (Chen, 2009; Niles et al., 2009; Stoddart, 2011).

Another option for determining the number of dead cells consists in measuring the amount of the cytosolic enzyme LDH that is released into the medium by necrotic cells with a ruptured membrane. Lactate and NAD^+ are added to the culture medium and contained LDH oxidizes lactate to pyruvate whereby NAD^+ is reduced to NADH. After adding a tetrazolium salt, the recently synthesized NADH reduces it to formazan which can be measured colorimetrically (Chan et al., 2013; Decker and Lohmann-Matthes, 1988).

One of the most frequently used colorimetric assays for the determination of cell proliferation compared to MTS, WST-1, AlamarBlue and also in comparison with the cyanine dyes PicoGreen and CyQuant is the MTT assay, based on the tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Quent et al., 2010b), presented in 1.1.3..

1.1.3. MTT Assay

The MTT assay offers the possibility to quantify the number of viable cells and hence the toxic effect of compounds on mammalian cells, using a high-throughput screening, with a sensitivity down to 200 cells/well (Mosmann, 1983). Typical applications for this cell viability assay is the evaluation of the impact of specific drugs on the viability of cells in vitro and hence the resistance of cells (Hubeek et al., 2006). Also the interplay between different drugs can be analyzed with the MTT assay (Kaspers et al., 1995). The tetrazolium salt MTT converts from the yellowish compound to a purple formazan crystal, triggered by metabolic active cells that split the tetrazole ring via reduction (Berridge et al., 2005) (Fig. 1).



MTT
(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

Formazan
(E,Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan

Figure 1: Reduction of water-soluble, yellowish MTT to water-insoluble purple formazan. Modified figure from Riss et al. (Riss et al., 2015).

The exact procedure of this conversion is not yet completely clear. Several studies concluded that the water soluble tetrazolium salt is not able to permeate the cell membrane and therefore infiltrates the cell via endocytosis, where afterwards the reduction to the non-water-soluble colored formazan crystals takes place (Liu et al., 1997)(Molinari et al., 2005). Having said this, there exist also studies that assume that due to the positive charge of the tetrazolium salt the molecule passes the cell membrane readily (Berridge et al., 2005; Diaz et al., 2007). This consideration gets reinforced by the quantitative structure-activity relationship (QSAR) that states, that tetrazolium salt is a lipophilic positively charged molecule with both, an apolar and a polar side and should cumulate as per computation in the mitochondria and the endoplasmic reticulum (ER) (Stockert et al., 2012). Also, the concrete location of the MTT reduction is not resolved yet. Former studies showed that it was reduced by succinate dehydrogenase, an enzyme complex located in the inner membrane of the mitochondria and a part of the citrate cycle and the respiratory chain (Koolman and Röhm, 2003; Slater et al., 1963). A later study nevertheless illustrated that the main part of the reduction is associated with processes depending on NADH and NADPH that are not located in the inner membrane of the mitochondria (Berridge and Tan, 1993). Furthermore it was shown that in viable cells 25-45% of the formazan could be detected at the mitochondria after an incubation time of 25 min, the rest was located in other parts of the cell, like subcellular compartments, the cytoplasm or at the cytoplasmic membrane (Bernas and Dobrucki, 2002). It is also not yet clarified where

the originated formazan accumulates in the cell and whether, respectively how it gets removed by the cell. It is supposed that the formazan that is unloaded and according to the QSAR lipophilic, amasses in lipid bodies in the cytosol (Diaz et al., 2007; Stockert et al., 2012). The reduced tetrazolium salt also gets removed by the cell and forms spicular crystals on the exterior. This process is as well not clarified yet. One assumption is that it works via exocytosis (Liu and Schubert, 1997; Liu et al., 1997) whereas a more recent study excludes this process (Diaz et al., 2007). The water-insoluble formazan finally has to be dissolved in an organic solvent before it can be measured spectrophotometrically, with an absorbance peak at approximately 570 nm, what makes the MTT cell viability assay to an endpoint assay due to the cell lysing effect of the solvents. In consequence of this, other assays were developed using tetrazolium salts, like XTT, MTS and WST-1, producing water soluble formazan (Berridge et al., 2005), what simplifies the execution of the experiments. Like already mentioned, the MTT assay still is frequently used, even though there are some potential error sources that necessitate an optimization for the specific experimental setup.

1.2. TUMOR NECROSIS FACTOR ALPHA - TNFα

1.2.1. ROLE OF TNFα IN THE HUMAN IMMUNE SYSTEM

TNFα is a pro-inflammatory cytokine of the innate immune system, together with IL-1 heavily involved in inflammatory processes of the acute phase and plays an important role during infections caused by bacteria, viruses or parasites. It is mainly liberated by macrophages activated through TLR, as well as T- and B-cells, neutrophils and natural killer (NK) cells. TNFα is a transmembrane protein that can be split by the TNF-α-converting enzyme (TACE) whereby the soluble and the extracellular form emerges, for which two receptors exist: the type I TNF receptor (TNF-R1; Fig. 2) localized on all cells and the type II TNF receptor (TNF-R2) mainly expressed on cells of the immune system (Bradley, 2008). TNF-R1 plays a more important role and is responsible for most of the biological effects of TNFα, like death or survival of the cells, cell proliferation and increasing of inflammatory activities (Horssen et al., 2006)(Fig. 3). The main difference between the two receptors is the death domain (DD) on the cytoplasmic side of the type I receptor that does not exist in the type II receptors. Soluble and transmembrane

TNFα can activate TNF-R1 whereupon the TNF-R1-associated death domain protein (TRADD) on the cytosolic side of the receptor binds to the death domain and forms complexes with different signaling pathways (Kalliolias and Ivashkiv, 2016). On the one hand apoptosis, can be initiated through the activation of caspase 8 whereby the signaling pathway of the caspases starts and also the signaling pathway of the mitochondria, both leading to apoptosis. On the other hand the apoptosis can be prevented via the TNF receptor-associated factor 2 (TRAF2) that activates the cytoplasmic inhibitor of apoptosis protein (cIAP) and the transcription factors AP-1 as well as NF-κB (Horssen et al., 2006; Micheau and Tschopp, 2003). AP-1 regulates genes important for the proliferation and differentiation of the cells but also for the apoptosis (Ameyar et al., 2003). NF-κB prevents apoptosis through the synthesis of proteins that interfere either in the caspase activity or the release of cytochrome c, moreover it increases the transcription of chemokines, cytokines, enzymes and adhesion molecules and also leads to cell proliferation (Marks et al., 2007). To TNF-R2 mainly the transmembrane form binds and ensures the viability of the cells and the renewal of the tissue, therefore it accomplishes an homeostatic effect (Kalliolias and Ivashkiv, 2016). TNFα has a great impact on the cells of the vascular endothelium and leads to an upregulation of the endothelial leukocyte adhesion molecules (ELAMs) and therefore to the formation of a leukocyte extravasation into inflamed tissue (Pober, 2002). Furthermore TNFα increases the permeability of endothelial cells by decreasing important proteins of the tight junctions like ZO-1 and Claudin-5 via the PKCζ signaling pathway that leads to the activation of the NF-κB transcription factor also involved in the rising permeability (Aveleira et al., 2010). It engenders an increased synthesis of chemotactics as well as proinflammatory cytokines and activates B-, T-cells and macrophages (Hochberg et al., 2014).

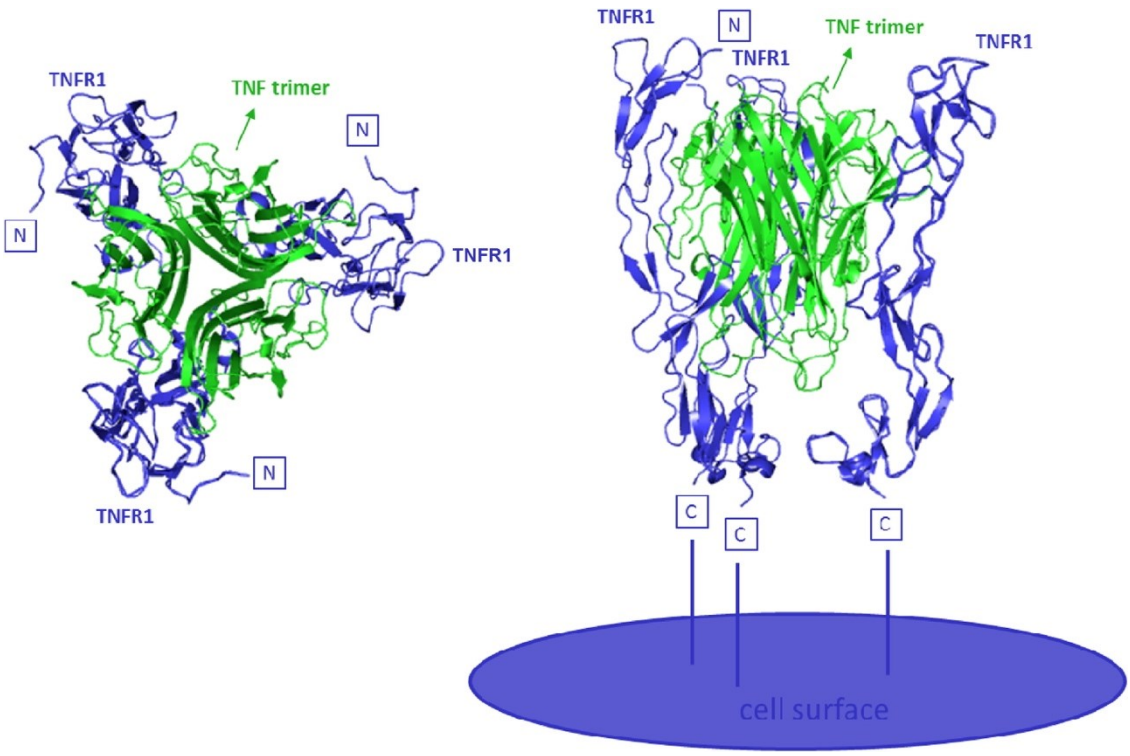


Figure 2: Crystallographic description of trimeric structure of TNFα (green) binding to TNF-R1 (blue); plan view on the left, lateral view shown on the right. Figure from Puimège et al. (Puimège et al., 2014)

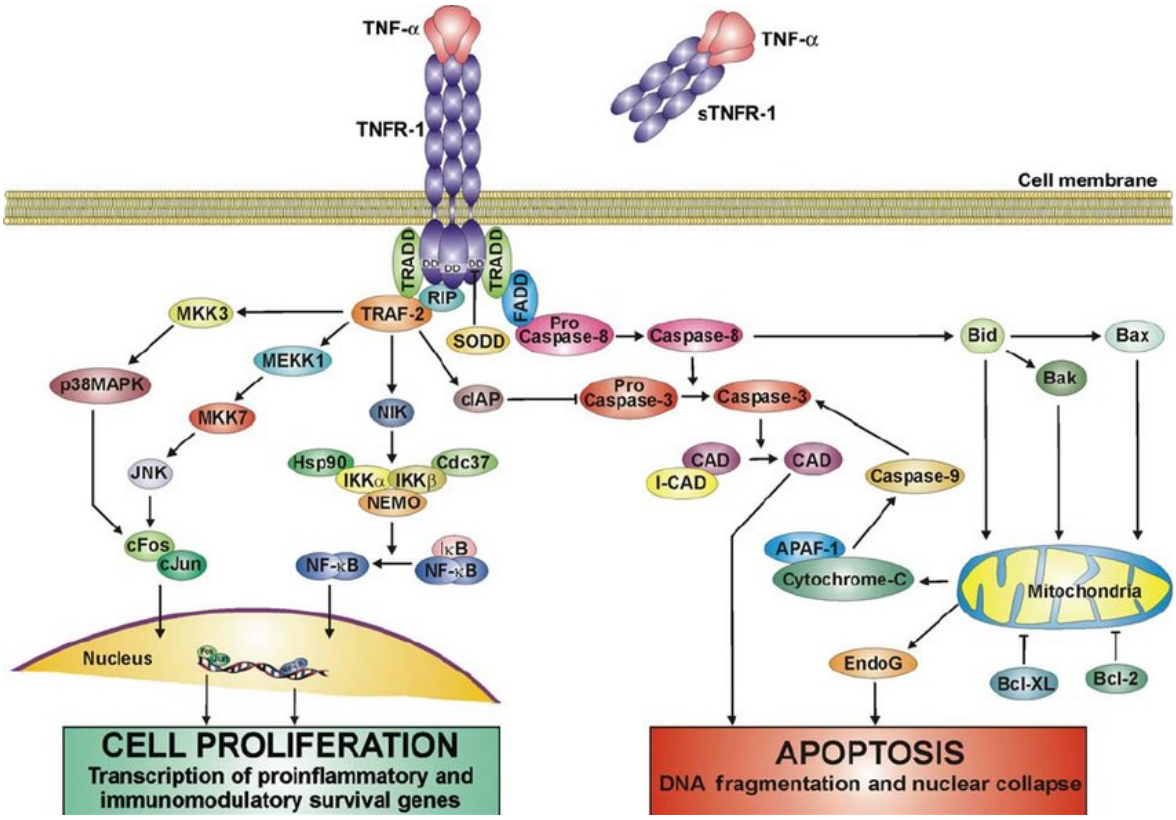


Figure 3: Signaling cascade after binding of TNFα to the TNF-R1. Figure from Horssen et al. (Horssen et al., 2006)

1.2.2. Role of TNFα in Disease Pathogenesis

TNFα acts in an autocrine and paracrine way, thus the effect is mainly limited to the local area of inflammation. It is also an endogenous pyrogen (EP) and causes systemic effects like fever provoked by exogenous pyrogens like bacterial toxins or LPS (Netea et al., 2000). Another systemic effect is the septic shock that often has a lethal outcome. It occurs during a sepsis because of the heavy release of TNFα in organs like the liver and endothelial cells (Murphy et al., 2012). Due to its proinflammatory effects an excess production of TNFα is associated with a lot of inflammatory and autoimmune diseases like rheumatoid arthritis (RA), juvenile rheumatoid arthritis (JRA), inflammatory bowel disease (IBD), Bekhterev syndrome or psoriasis (Kalliolias and Ivashkiv, 2016). This is the reason for the development of TNFα antagonists like the monoclonal antibodies infliximab, adalimumab and golimumab as well as the fusion protein etanercept and the antigen-binding fragment certolizumab for the

therapy of this diseases (Mitoma et al., 2016). One serious adverse drug effect of the therapy with TNFα antagonists is the possible activation of a silent tuberculosis despite minding the avoidance instructions (Guinard et al., 2016). It also exists a linkage between inflammatory diseases and tumorigenesis, like for example the correlation between a gastritis caused by Helicobacter pylori and stomach cancer or MALT lymphoma and the correlation between hepatitis B or C and malignant hepatoma (Karin, 2006). The tumor necrosis factor was initially described by Carswell et al. in 1975 as a compound liberated by cells after the treatment with endotoxin and initiating the specific necrosis of malign tumor cells. This impact was analyzed for L929 cells that responded the most, with a cytotoxic effect after 48 hours, for sarcoma cells, that showed a cytostatic effect and for mouse embryonic fibroblasts, where no ramification could be demonstrated (Carswell et al., 1975). Furthermore, the cytotoxic effect only occurs with highly dosed TNFα. Little amounts released in the cellular environment of the tumor can even function as a tumor booster by activating the transcription factors NF-κB and AP-1 resulting in cell proliferation and production of chemokines, cytokines and angiogenic factors (Balkwill, 2006). Nevertheless, TNFα is used in the therapy of soft-tissue sarcoma together with melphalan. Because of the required high dose of TNFα it is necessary to briefly cut off the systemic bloodstream from the extremity where the application takes place in order to avoid adverse drug effects like the systemic inflammatory response syndrome. Melphalan primarily amasses in the tumor due to the fact that TNFα mainly increases the permeability of tumor endothelial cells (Podleska et al., 2015). These two described impacts of TNFα, namely the cytotoxicity towards tumor cells and the increasing vascular permeability of tumor endothelial cells make this cytokine very attractive for the field of gene therapy. One way to circumvent the problem of undesirable systemic effects of TNFα in gene therapy is the direct injection of adenoviral vectors like TNFerade (Ad_{GV}EGR.TNF.11D) into tumor tissue, containing the cDNA coding for TNFα. TNFerade showed promising successes in preclinical researches but in randomized phase III tests with study participants suffering from pancreatic cancer, it did not have a life-prolonging effect combined with the medication of the treatment guidelines (Herman et al., 2013). Another possibility of gene therapy is using bacterial plasmids encoding

for TNFα. Good results were achieved with pCpG-hCMV-EF1α-TNFα for both, in vitro and animal tests. Cytotoxicity and increasing permeability of the endothelium could be ascertained in vitro. The animal tests with mice proved, that the transgenic expression of TNFα after a parental administration of the plasmid complexed with G3-HD-OEI, mainly took place in the tumor tissue and that the permeability of the endothelial cells increased. In combination with doxorubicin also higher amounts of the anthracycline in the tumor were achieved (Su et al., 2013).

1.2.3. TNFα and Gene Therapy

As already mentioned one potential method for gene delivery is the usage of bacterial plasmids. The plasmids pCpG-hCMV-EF1α-TNFα and pCpG-hCMV-SCEP-TNFα both have a CpG-free backbone with a human Cytomegalovirus (hCMV) enhancer and cDNA encoding for the murine TNFα. The plasmid backbone is free of the unmethylated dinucleotides CpG, that mainly occur in bacterial DNA and therefore can provoke an immune response (Yew, 2000). Another advantage is the elongation of the expression of the transgenes (Hodges, 2004). The two plasmids only differ in their promoters, the human elongation factor 1 alpha (EF1α) and the shuffled CMV EF-1 promoter (SCEP). It was shown that the combination of the EF1α promoter and the human CMV enhancer leads to a secular high transgene expression (Navarro et al., 2010). SCEP is a combination of the two promoters CMV-IEP and EF-1, and was created to obtain an optimized promoter with a higher and more enduring expression in vivo (Magnusson et al., 2011).

1.3. Aims of the Study

The goal of the study was to establish a cell based assay for profiling TNFα bioactivity and for quantification of TNFα transgene expression. For this reason, the L929-MTT assay was established, consisting of the L929 cell cytotoxicity based bioassay and the MTT assay, a cell viability assay. It was also intended to use the L929-MTT assay for evaluating the transfection efficiency of LPEI and for analyzing the in vitro TNFα transgene expression kinetics as a function of promoter type over duration of 4 days. First step of the L929-MTT assay was the transfection of A549 cells with TNFα plasmid based LPEI nanoparticles. During the second step, the L929 bioassay, the supernatant

of A549 cells containing the secreted TNFα was transferred to L929 cells and afterwards the cytotoxic effect of TNFα was measured using the MTT assay. Towards this, the MTT assay was optimized first, for reliable and reproducible measurements of the decrease of cell viability during the L929 cell cytotoxicity based bioassay in presence of TNFα. For optimizing the MTT assay, initially a protocol based on the insights of a report by Supino R. and Chiba K. et al. was tested and subsequently different variations were performed. Considerations during planning the varying parameters concerned on one hand the avoidance of mechanical disturbance of seeded cells and formed formazan crystals during the aspiration process. On the other hand, the avoidance of disturbing factors during the absorbance measurement like FBS or phenol red. FBS is contained in full cell culture medium and able to precipitate with certain solvents like isopropanol, and phenol red, a pH indicator contained in full cell culture medium as well as basal medium, with an absorbance maximum in the same wavelength range as solved formazan. The different tested variations were validated by reference to the correlation between the seeded number of A549 cells and the measured absorbance, described as R squared values. The transfection success of A549 cells with pCpG-hCMV-EF1α-TNFα and pCpG-hCMV-SCEP-TNFα based LPEI nanoparticle mediated gene delivery was also validated during the performance of the L929-MTT assay and compared with the transfection efficiency of Lipofectamine. The transfection reagent LPEI was used, because it yields a high transfection efficiency compared to other polymers (Brissault et al., 2003). Transfected A549 cells secreted TNFα into the supernatant and the cytotoxic effect was investigated by applying different dilutions of A549-supernatant on L929 cells, murine fibroblasts, sensitized with Actinomycin D, and measuring the cytotoxic effect, thus the bioactivity of TNFα, using the optimized MTT assay (Fig. 4). For quantification of secreted TNFα, known amounts of recombinant murine TNFα (rTNFα) were tested with the L929-MTT assay and the results of the cytotoxicity measurement were used for calculating the unknown amount of secreted TNFα by using the correlation between the known amount of the tested rTNFα and the cytotoxic effect attained with it. Furthermore, the impact of a freezing period on the bioactivity of TNFα was investigated by comparing the cytotoxic effect of TNFα in fresh A549-supernatant of the L929-MTT assay to

results of the same experiment performed 6-12 days later, with the same A549-supernatant that was frozen during this duration.

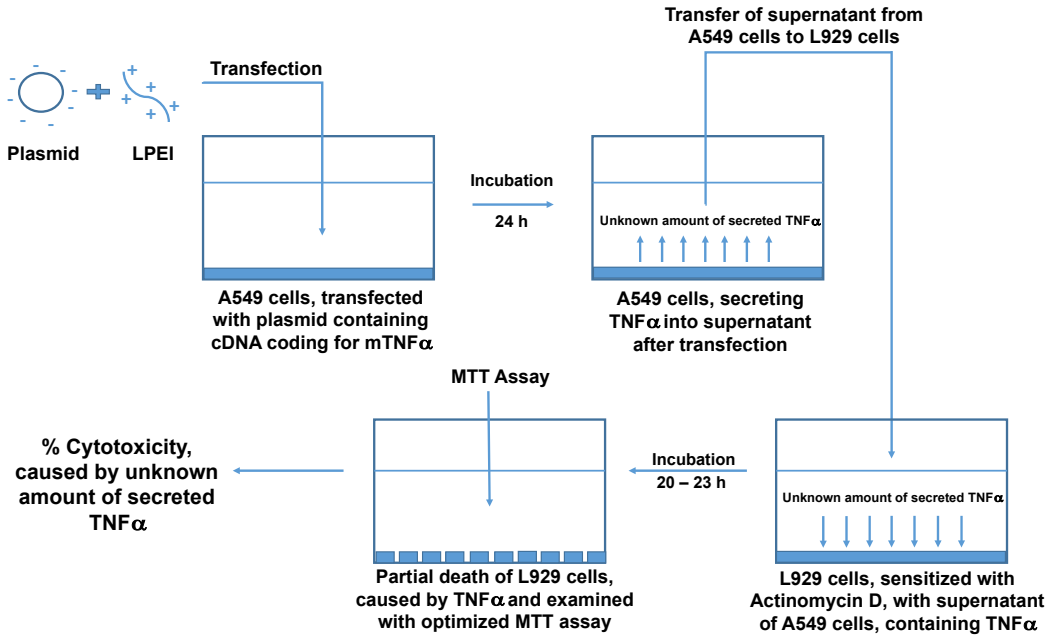


Figure 4: Overview of working steps performed during nanoparticle mediated in vitro transfection with TNFα plasmid and L929-MTT assay for the measurement of cytotoxicity caused by secreted TNFα. A549 cells were transfected with pEF1α-TNFα and pSCEP-TNFα using LPEI based NPs. After incubation, supernatant of the transfected cells, containing secreted TNFα, was transferred to L929 cells (sensitized with Actinomycin D). Following this, after expiration of the incubation time, previously optimized MTT assay was used to determine the cytotoxic effect of TNFα on L929 cells. This effect was later compared with cytotoxicity data collected for the same assay, performed with known amounts of recombinant murine TNFα .

2. MATERIALS AND METHODS

Ubiquitous used materials are listed in table 1, the specific used materials are listed in the following, respective subitems.

Table 1: Materials generally used in the experiments.

Consumable Material	Supplier, Catalogue Number
Centrifuge tube, 15 ml	Starlab, E1415-0200
Centrifuge tube, 50 ml	Starlab, E1450-0200
Cuvette	Brand, 759150
Microcentrifuge tube, 0.5 ml	Nerbe Plus, 04-322-9200
Microcentrifuge tube, 1.5 ml	Nerbe Plus, 04-212-9200
Microcentrifuge tube, 2 ml	Nerbe Plus, 04-232-9200
Pipette tip, 10 µl	Nerbe Plus, 07-372-2015
Pipette tip, 200 µl	Nerbe Plus, 07-376-2015
Pipette tip, 1250 µl	Nerbe Plus, 07-379-2015
Serological pipette, 5 ml	Sarstedt, 86.1253.001
Serological pipette, 10 ml	Sarstedt, 86.1254.001
Serological pipette, 25 ml	Sarstedt, 86.1685.001

2.1. EXPANSION OF TNFα BASED PLASMIDS

2.1.1. LB AGAR PLATES AND LB MEDIUM

Preparation of LB Agar Plates

Materials

- LB Agar (Lennox) (Carl Roth, X965.3)
- dH_2O
- Zeocin™ (*Phleomycin D1)* (InvivoGen, ant-zn-1)
- Ampicillin sodium salt (Sigma-Aldrich, A0166) (stock solution: 50 mg/ml in water)
- Petri dishes, sterilized (Kord-Valmark, 800 452 9070, “Superior”)

The LB agar was prepared as described by the manufacturer, in brief 600 ml of dH_2O were added to 21 g of the LB agar powder and mixed thoroughly. The solution was autoclaved and after cooling down to 55 °C it was separated into three parts of 200 ml each. The antibiotics were added according to table 2.

Table 2: Antibiotics used for the preparation of the LB Agar plates and the LB medium.

	Stock Solution [mg/ml]	Working Concentration [μg/ml]	Purpose of Use
Ampicillin	50	100	Negative Control
Zeocin™	100	25	Selection Plate

The solutions were mixed and poured in the petri dishes to the indicated line. After 1 h the culture dishes were closed, sealed with Parafilm® and stored at 4 °C for at least 12 h.

Preparation of LB Medium

- LB Medium (Lennox) (Roth, X964.3)
- dH_2O

The LB medium was mixed as described by the manufacturer, therefore 20 g of the LB medium powder were dissolved in 1 L of dH_2O and autoclaved. The needed amount of antibiotics was calculated according to table 2.

2.1.2. Bacterial Transformation

Materials

- Chemically competent E.coli DB3.1
- pCpG-hCMV-SCEP-TNFα (Magnusson et al., 2011)

Chemically competent E.coli DB3.1 were taken out from the freezer at –80 °C and slowly defrosted on ice for 20–30 min. 50 μl of the cell suspension were pipetted in precooled 1.5 ml tubes and 100 ng of the DNA were added, mixed gently by flicking

the bottom of the tube a few times and put on ice for 20–30 min. After that the competent cell/DNA mixture was heat shocked for 60 seconds at 42 °C in a preheated water bath (Ultrasonic Cleaner USC-TH, VWR) and subsequently put back on ice for 2 min. In order to afford the cells, the time to synthesize the antibiotic resistance proteins encoded on the plasmid backbone, 400 µl of LB-medium without antibiotics (see 2.1.1) were added and the tube was then placed on a shaking incubator (MaxQ 4450 Benchtop Incubating Orbital Shaker, Thermo Scientific™) at 37 °C for 45 min. The plasmid pCpG-hCMV-SCEP-TNFα incorporates the resistance gene for Phleomycin D1 (Zeocin™). In the meantime, LB agar plates were transferred from 4 °C to 37 °C to an incubator for bacterial cultures (WTB Binder, 1811530000202). After the incubation time was finished, 50 µl of the transformation were plated onto one LB agar plate containing Zeocin™, and 300 µl onto a second one with a view to improve the growth of single bacterial colonies. As a negative control 50 µl were plated onto a plate with Ampicillin, and as a positive control the same volume was plated onto a plate containing no antibiotic. All plates were incubated at 37 °C overnight.

2.1.3. Streaking the Transformed Bacteria from a Glycerol Stock

Materials

- Glycerol stock with pCpG-hCMV-EF1α-TNFα
- LB agar plates (2.1.1)

The glycerol stock with the E.coli DB3.1 already transformed with pCpG-hCMV-EF1α-TNFα was taken out from the freezer at –80 °C and directly stored on ice that was precooled at –80 °C. Using inoculation loops (Sarstedt, 86.1567.050) the bacteria from the glycerol stock were gently spread over the LB agar dish containing Zeocin™ as a selection plate, because pCpG-hCMV-EF1α-TNFα carries the antibiotic resistance gene for Phleomycin D1. As a negative control the bacteria were streaked over a plate with Ampicillin as component, and as a positive control a LB-agar plate without an antibiotic was used. The plates were stored at 37 °C overnight.

2.1.4. Transformation Efficiency

The efficiency of the bacterial transformation was calculated for the transformation of the E.coli DB3.1 competent cells with the pCpG-hCMV-SCEP-TNFα. Therefor the grown colonies on the selection plate containing Zeocin™ were counted, the amount of pDNA used for the transformation was calculated and the following formula was applied:

$$\boldsymbol{Transformants\ per\ \mu g\ DNA} = \frac{\boldsymbol{Number\ of\ Colonies\ on\ the\ Plate}}{\boldsymbol{\mu g\ of\ Plated\ DNA}}$$

2.1.5. Liquid Culture

- LB-Medium (Lennox) (Roth, X964.3)
- 50% glycerol solution: 50% sterile filtered glycerol (Sigma-Aldrich®, 49781) in sterile water

After the overnight incubation, the bacterial growth of the different plates was examined by visual assessment. The plates with Zeocin™ should show bacterial growth with single colonies whereas the plates with Ampicillin should not show any bacterial growth as a negative control and the plates for the positive control not containing any antibiotic should also show a distinct bacterial growth. As soon as the mentioned criteria were verified the selection plate containing Zeocin™ was checked for single colonies with the same morphologic features concerning the color and the diameter. In order to calculate the transformation efficiency, the single colonies from the selection plate were counted and this number of colony forming units (cfu) was divided by the DNA amount used for the transformation in μg like described in 2.1.4. of materials and methods. Afterwards a colony was selected, picked with a sterile pipette tip and transferred to a 50 ml tube (Starlab, E1450-0200) containing 5 ml of LB medium and the adequate amount of Zeocin™ (Tab. 1) for the preparation of a bacterial preculture. From the same plate a second single colony was picked and transferred to a tube with 5 ml LB medium and the appropriate volume of Ampicillin (Tab. 1) as a negative control. As a positive control a third colony was picked and added to LB medium without an antibiotic. The tubes were put in the shaking incubator (MaxQ 4450 Benchtop Incubating Orbital Shakers, Thermo Scientific™) at 37

°C and 200 rpm with slightly opened lids, fixed with sticky tape for 7–8 h. After the expiration of this term the tubes were screened for the required bacterial growth identifiable as a cloudy haze in the medium containing Zeocin™. For the overnight culture 250 ml of the LB medium filled into a baffled-bottom flask with a membrane screw cap (Duran®, 2I 283 54 59) were preheated in the incubator at 37 °C, the appropriate volume of Zeocin™ (Tab. 1) and 25 µl of the preculture were added in order to obtain a 1:10 000 dilution. The flask was placed in the orbital shaker at 37 °C and 200 rpm overnight. The next morning the optical density of the overnight culture was measured at 600 nanometers (OD_{600}) using a spectrophotometer (GeneQuant, GE Healthcare). To make a part of the transformed bacteria storable at –80 °C, 500 µl of the bacteria suspension were mixed with 500 µl 50% glycerol (see above) in a cryovial (Thermo Scientific™, 374502).

2.1.6. Isolation of the Plasmids

The plasmids were isolated with the GeneJET Plasmid Maxi Prep Kit (Thermo Scientific™, K0491) following Protocol A of the manufacturer information for the plasmid purification using a low speed centrifuge (Heraeus Megafuge 16R Centrifuge, Thermo Scientific™) aside from two variations: in step 1, the centrifugation was performed for 15 min at 4800 rpm and in step 15, the elution of the pDNA was executed in two steps by first adding 0.7 ml of the elution buffer provided in the kit and after the centrifugation, adding the remnant 0.3 ml.

2.1.7. Isopropanol Precipitation

Materials

- Isopropanol (VWR®, A3928,1000GL)
- 80% Ethanol (VWR®, 1.11727.2500)

The isopropanol precipitation was performed in order to concentrate and desalt the pDNA from the elution buffer applied in the last step of the GeneJET Plasmid Maxi Prep Kit as described in 2.1.5, using isopropanol as an anti-solvent. In the first step 500 µl of the pDNA solution were transferred to a sterile 1.5 ml tube and 1000 µl isopropanol were added. The tubes were stored at –20 °C overnight. The next day the

pDNA isopropanol solution was centrifuged at 15000 x g at 4 °C for 15 min and afterwards the supernatant was cautiously discarded, trying not to empty out the remaining pDNA pellet. The tubes were placed opened in a tilted position in the laminar flow cabinet so that the remnant isopropanol could evaporate. In the next step the pellet was washed, therefor 750 µl of ice cold 80% ethanol were added and the sample was vortexed for 20–30 sec until the pellet started to float. The resulting mixture was centrifuged at 15000 x g for 5 min, the supernatant was carefully discarded and the washing procedure was repeated one time in the same way. After the second wash round the tubes were centrifuged again at 1000 x g for 5 sec to collect the residual ethanol which was then removed with a sterile pipette. The pellet was air dried in the opened tube in the laminar flow cabinet for approximately 0.5 h and finally the pellet was resuspended in 200 µl of nuclease free water and stored at 4 °C for 1 to 5 days for a proper resolving of the pellet before measuring the concentration of the pDNA.

2.1.8. DNA Quantification

Materials

- Nuclease free water

The pDNA was quantified after the isopropanol precipitation using the NanoVue Plus™ spectrophotometer (4282 V2.0.4, VWR®). For more precise results, the pDNA resolved in nuclease free water was diluted 1:5 and 1:10 also with nuclease free water, which was also used as a blank. 2 µl of each dilution were quantified. The absorbance of the pDNA at 260 nm was applied for the calculation of the undiluted pDNA concentration. The values of this measurement should be greater than 0.1 for precise results. The measured and calculated concentrations were averaged out. The absorbance ratio 260/280 nm was used to check the purity of the pDNA solution and should be in a range of 1.7–1.9. Afterwards the pDNA was either stored at -80 °C for later use or at 4 °C for maximal 5 days.

2.1.9. DIAGNOSTIC RESTRICTION DIGEST

Materials

- SB buffer *(sodium borate buffer)* 20x stock solution
    - 47 g boric acid (Sigma-Aldrich®, B0394-1KG-D)
    - 8 g NaOH (PanReac AppliChem, A6829,1000)
    - dissolved in 800 ml dH_2O
    - pH adjusted to 8.0 - 8.2 with HCl/NaOH
    - filled up to a final volume of 1000 ml with dH_2O
    - autoclaved
- Agarose (SERVA, 11404.04)
- Ethidium bromide (Sigma-Aldrich®, E1510)
- FastDigest™ HindIII restriction enzyme (Thermo Scientific™, FD0504)
- FastDigest™ EcoRI restriction enzyme (Thermo Scientific™, FD0274)
- FastDigest™ NdeI restriction enzyme (Thermo Scientific™, FD0583)
- FastDigest™ NheI restriction enzyme (Thermo Scientific™, FD0973)
- FastDigest™ XbaI restriction enzyme (Thermo Scientific™, FD0684)
- 10x FastDigest™ Green Buffer (Thermo Scientific™, B72)
- FastRuler Middle Range DNA Ladder (Thermo Scientific™, SM1113)

The diagnostic restriction digest was performed to validate the plasmids by cutting them with the restriction enzymes into fragments, with known lengths. The restriction maps for the plasmids were produced with the ApE A Plasmid Editor (M. Wayne Davis, version 1.13 for Windows). By reference to these maps, the appropriate restriction enzymes were chosen and the base pair numbers of the originated pDNA sequences were calculated, for the evaluation of the gel electrophoresis at the end of this procedure. For the diagnostic restriction digest, samples with a total volume of 20 µl were prepared by mixing approximate 250 ng of the pDNA to be analyzed with 2 µl 10x FastDigest™Green Buffer and 1 µl of each required restriction enzyme in PCR tubes (Nerbe Plus, 04-032-0200). The volume was filled up to 20 µl with nuclease free water. The samples were mixed and spun down in a mini-centrifuge (Biosan, FVL-2400N) for 10 sec. For every diagnostic restriction digest, always one uncut sample without any

restriction enzymes, and one single cut sample of the plasmids were prepared, so that in the end the band pattern of the supercoiled and the linear version could be examined. The specimens containing pDNA and restriction enzymes were digested with the thermocycler (Eppendorf, Mastercycler ep gradient S) starting with 37 °C for 40 min, followed by 80 °C for 10 min and in the end 4 °C for 2 min. The sample without the restriction enzyme was stored at 4 °C during the digest.

For the gel electrophoresis, a 0.8% agarose gel was prepared. Therefor a SB buffer working solution was made by diluting 50 ml of the 20x SB buffer stock solution with 950 ml of distilled water. Afterwards 0.48 g of the agarose was mixed with 60 ml of the SB buffer working solution in a wide-neck Erlenmeyer flask. The mixture was heated in a microwave (Sharp, R-207) until the agarose was completely dissolved and there were no particles visible anymore. As soon as the mixture reached a temperature of approximately 60 °C, 3 µl ethidium bromide stock solution with a concentration of 10 mg/ml in methanol were added and mixed well. Afterwards the gel was poured into the gel trough, bubbles were removed and it was left there for 40 to 60 min to solidify. As soon as the gel was congealed, it was transferred to the migration chamber (Wide Mini-Sub® Cell GT, Bio-Rad), covered with SB buffer working solution and loaded with 20 µl of each sample and 8 µl of the FastRuler middle range DNA ladder. The gel electrophoresis was started at 100 V for 1 h (Power Pac 200, Bio-Rad). After this term the gel was photographed with the Molecular Imager® Gel Doc™ XR (Bio-Rad, 170-8170) and the pictures were processed using the Quantity One 4.6.3 software for windows.

2.2. CELL CULTURE

The cell culture work was performed under aseptic conditions using a laminar flow cabinet (Herasafe™ KS, Class II Biological Safety Cabinet 1.2m, Thermo Scientific™). The incubation of the cells always took place in CO_2 incubator for cell culture (Heracell™ 150i CO_2 Incubator, Thermo Scientific™) at constant temperature (37 °C) and 5% CO_2.

2.2.1. CELL CULTIVATION

Cell Culture Medium for A549 Cells

- RPMI 1640 Medium (Sigma-Aldrich®, R0883)
- FBS *(fetal bovine serum)* (Sigma-Aldrich®, F7524)
- L-Glutamine 200 mM (Sigma-Aldrich®, 59202C)

The full RPMI 1640 medium, used as cell culture medium for the A549 cells, was prepared by adding FBS up to 10% and 10.25 ml L-Glutamine 200 mM per 500 ml of RPMI 1640 medium.

Cell Culture Medium for L929 Cells

- RPMI 1640 Medium (Sigma-Aldrich®, R0883)
- FBS (Sigma-Aldrich®, F7524)
- L-Glutamine 200 mM (Sigma-Aldrich®, 59202C)
- Penicillin - Streptomycin (Sigma-Aldrich®, P4333)

The full RPMI 1640 medium, used as culture medium for the L929 cells, was prepared by adding FBS up to 10%, 10.25 ml L-Glutamine 200 mM per 500 ml of the medium and 1% Penicillin-Streptomycin.

Cell Cultivation

Materials

- PBS *(Dulbecco`s Phosphat Buffered Saline)* (Sigma-Aldrich®, D8537)
- TrypLE™ Express (1X) *(Stable Trypsin Replacement Enzyme)* (Gibco®, 12605-010)

As soon as the adherent cells reached certain confluency the spent medium was removed using serological pipettes (Sarstedt, 86.1253.001, 86.1254.001, 86.1685.001). The flask was rinsed two times with 10 ml PBS, which was preheated in the water bath (Ultrasonic Cleaner, VWR®) at 37 °C. After removing the PBS, the cells were covered with 1 ml Trypsin and stored in the incubator (Heracell™ 150i CO_2 Incubator, Thermo Scientific™) for 5 min. Following this, the flask was tapped with the heel of the hand to

detach the cells from the bottom and after checking the detachment of the cells, 10 ml of the appropriate preheated medium were added. The cell suspension was flushed a few times and transferred to tubes (Starlab, E1415-0200) for the centrifugation (Heraeus Megafuge 16R Centrifuge, Thermo Scientific™) at 200 x g for 5 min at RT. The supernatant medium was removed and the cell pellet was resuspended in 1 ml of the appropriate medium. The received cell suspension was then used for the cell counting with the Neubauer Chamber (Marienfeld-Superior, 0640010, Neubauer-improved). To retain the cell line, a partition of the cells was filled into a new 75 cm^2 cell culture flask (Sarstedt, REF 83.3911.002, tissue culture treated) with 15 ml of preheated medium and stored in the incubator.

2.2.2. Cell Counting with Hemocytometer

Material

- Neubauer Chamber (Marienfeld-Superior, 0640010, Neubauer-improved)

The Neubauer Chamber was prepared as described in the manual. The cells were split like it is described in 2.2.1. Depending on the confluency of the cells, the 1 ml cell suspension had to be diluted with the corresponding medium so that in the end the counted cell number in one square with the size of 1 mm^2 amounted to 25–50 cells. 10 µl of the diluted cell suspension were mixed well and pipetted between the chamber base and the cover glass. The counting was performed like it is described in the manual and for the calculation of the total cell number in 1 ml, the following formula was applied:

$$\boldsymbol{Cell\ Number\ in\ 1\ ml} = \frac{\boldsymbol{Number\ of\ Counted\ Cells}}{\boldsymbol{Counted\ Area}\ [\boldsymbol{mm^2}]} \times \boldsymbol{Dilution\ Factor} \times \mathbf{10000}$$

2.3. MTT Assay Optimization

2.3.1. MTT Stock and Working Solution

Preparation of MTT 2 mg/ml Stock Solution:

Materials

- MTT *(Thiazolyl Blue Tetrazolium Bromide)* (Sigma-Aldrich®, M2128)
- PBS *(Dulbecco`s Phosphat Buffered Saline)* (Sigma-Aldrich®, D8537)

For the preparation of a stock solution with the concentration of 2 mg/ml MTT in PBS, 115 mg MTT were dissolved in 57.5 ml PBS and merged by repeatedly pipetting. The working steps took place under the laboratory hood with switched off lights. Remaining clots of the MTT powder were dissolved by sonicating (Ultrasonic Cleaner USC-TH, VWR®) the solution for 5 seconds a few times in sequence. The solute MTT stock solution was drawn up into a syringe (syringe: BD Discardit™, 300296; needle: 100 Sterican® Braun, 466 5791, PZN 2057984), and sterile filtered through a filter with a pore size of 0.22 μm. Afterwards aliquots of the sterile stock solution were filled in 15 ml tubes (Starlab, E1415-0200) wrapped in aluminum foil and stored at –20 °C.

Preparation of MTT 0.4 mg/ml Working Solution:

Materials

- MTT 2 mg/ml stock solution
- RPMI 1640 medium (Sigma-Aldrich®, R0883)

For the preparation of a MTT 0.4 mg/ml working solution an already prepared MTT 2 mg/ml stock solution in PBS was thawed under light protection and 1 ml of this stock solution was freshly diluted with 4 ml of pure RPMI 1640 medium in order to get a MTT 0.4 mg/ml working solution. Both solutions were wrapped in aluminum foil and stored at 4 °C after usage.

2.3.2. MTT ASSAY

Materials

- MTT 2 mg/ml stock solution in PBS (see 2.3.1.)
- PBS *(Dulbecco`s Phosphate Buffered Saline)* (Sigma-Aldrich®, D8537)
- Pure RPMI 1640 Medium (Sigma-Aldrich®, R0883)
- Full RPMI 1640 (RPMI 1640, 10% FBS, 1% L-Glutamine)
- 2-Propanol (PanReac AppliChem, A3928,1000GL)

This assay was performed following the original protocol based on the insights of Supino R. et al. and Chiba K. et al., as a starting point for the following alterations of parameters during the process of MTT assay optimization.

Day 1 – Seeding A549 cells

On the first day, a confluent culture flask with A549 cells was split as specified in 2.2.1., the cells were counted with the Neubauer Chamber as described in 2.2.2. in order to calculate the needed volume of cell suspension for seeding the cells in different numbers from 5 x 10^3 to 3 x 10^4 cells per well as a triplet in a 96-well plate (Greiner Bio-One, Cellstar®, 655 180). Before plating the cells, the correlating volume of full RPMI medium was pipetted in the wells so that in the end a final volume of 200 µl was reached in every well. Afterwards the required number of cells was added. The plate was lidded and stored in the incubator for 19–20 h overnight.

Day 2 – MTT Assay

On the second day, the medium was carefully aspirated, 200 µl of the MTT 0.4 mg/ml working solution (see 2.3.1.) were pipetted in the wells and the 96-well plate was again stored in the incubator for 4 h. After the expiration of this term the total MTT working solution in the wells was cautiously removed by using a single channel pipet trying not to detach the developed formazan crystals or the cells attached to the bottom of the wells. Following this, 200 µl 2-Propanol were added to every well as a

solvent and additionally to 3-6 empty wells on the same plate, later used as a blank for the calculation of the corrected absorbance. Afterwards the plate was placed on an orbital shaker (Eppendorf, ThermoMixer® C, 5382000015) for 30 min at RT. Afterwards the resolving of the formazan crystals was checked under the microscope, before the absorbance of the samples was determined with a microplate reader (Infinite® 200Pro, Tecan) at 540 nm. For the calculation of the corrected absorbance the absorbance of 200 µl pure isopropanol was subtracted from the measured values. The three corrected values from each cell number were averaged out for the evaluation of the R squared values (R^2).

The working steps of the MTT assay are shown in figure 5 and table 3.

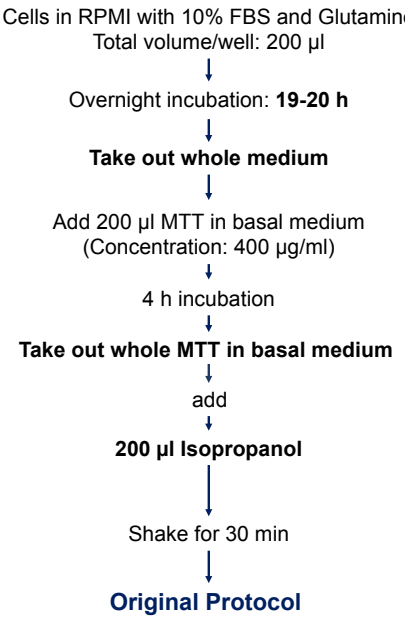


Figure 5: Working steps of the original MTT assay protocol based on the insights of Supino R. et al. and Chiba K. et al.

Table 3: Overview of the working steps during the performance of the MTT assay following the original protocol based on the insights Supino R. et al. and Chiba K. et al.

	WORKING STEPS	REMARK
ORIGINAL PROTOCOL	1. Cells seeded in total volume of 200 µl full cell culture medium 2. After an overnight incubation, whole medium was removed 3. 200 µl MTT in basal medium working solution with a concentration of 400 µg/ml were added 4. After 4 h incubation whole supernatant was removed 5. 200 µl pure isopropanol were added as solvent 6. 96-well plate was stored on a shaker for 30 min 7. Direct absorbance measurement after 30 min	• Seeding cells in 200 µl full cell culture medium made it necessary to remove medium after overnight incubation because of the restricted volume per well • The whole medium was removed after the overnight incubation to eliminate FBS, because it could precipitate with the solvent and be a disturbance factor of absorbance measurement; aspiration of the medium was an additional working step and potential disturbance for cells • MTT in basal medium was added to avoid the presence of FBS • Whole medium was removed after 4 h incubation, so that phenol red could not disturb absorbance measurement, because it has an absorbance maximum in the same wavelength range like formazan • For better lysis of cells as well as formazan crystals and mixing of the solution, plate was stored on shaker

2.3.3. MTT VARIATIONS

Materials

- 5 mg/ml MTT in PBS working solution
    - 2.5 g MTT *(Thiazolyl Blue Tetrazolium Bromide)* (Sigma-Aldrich®, M 2128)
    - solved in 500 ml PBS *(Dulbecco`s Phosphat Buffered Saline)* (Sigma-Aldrich®, D8537)
    - sterile filtered using a filter with a pore size of 0.2 μm
    - aliquoted, wrapped in aluminum foil and stored at –80 °C for max. 12 months or at 4 °C for max. 6 months
- DMSO *(dimethylsulfoxid)* (Sigma-Aldriche®, D5879-500ML)
- SDS *(sodium dodecyl sulfate)* (Amresco®, 0837-500ML)
- HCl *(hydrochloric acid)* (Sigma-Aldrich®, 30721-2.5L-GL)
- Isopropanol (AppliChem, A3928,1000GL)

With the intention of optimizing the protocol based on the insights of Supino R. and Chiba K. et al. for the MTT assay (Fig. 5), different steps of the protocol were changed and examined. The executed variations are shown in Figure 6.

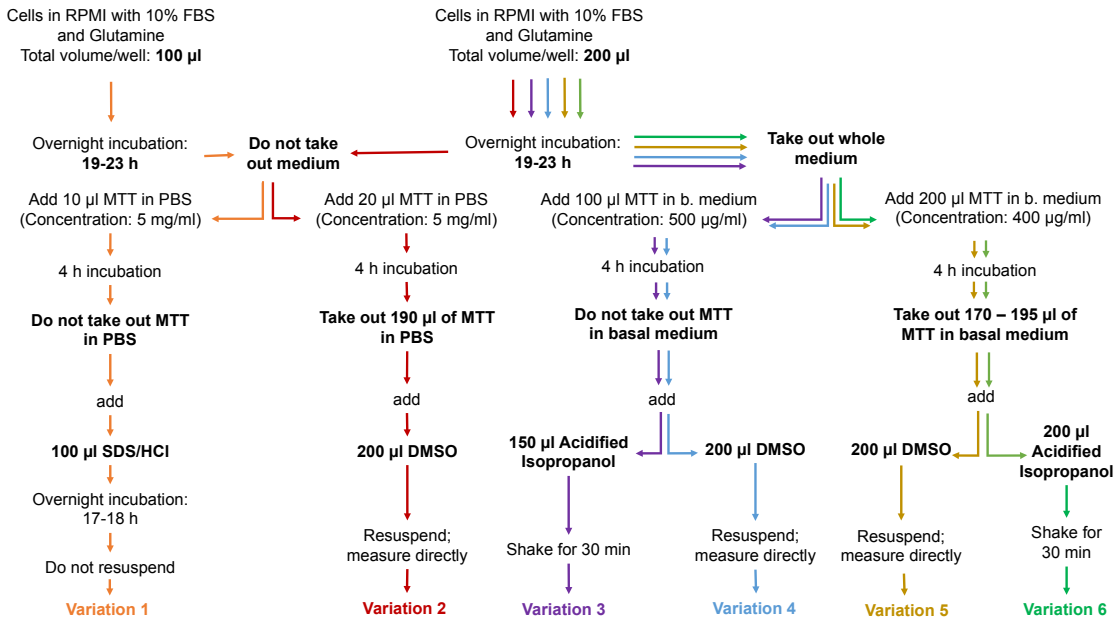


Figure 6: Different variations tested for optimization of MTT assay using the A549 cells.

Table 4: Overview of different variations tested during optimization of MTT assay (Fig. 6). Variations 1 to 6 are listed, describing varying parameters from original protocol based on the insights of Supino R. et al. and Chiba K. et al. shown in 2.3.2. and remarks referring to changed parameters.

VARIATIONS (FIG. 6)	VARYING PARAMETERS FROM THE ORIGINAL PROTOCOL	REMARK
VARIATION 1	1. Cells seeded in a total volume of 100 µl full cell culture medium 2. Medium was not removed after overnight incubation 3. 10 µl of MTT in PBS with a concentration of 5 mg/ml were added 4. Supernatant was not removed after 4 h incubation 5. 100 µl of SDS/HCl were added as solvent 6. Additional overnight incubation was performed 7. Absorbance was measured without resuspending the supernatant before	• Cells were seeded in smaller volume → remaining components were added without removing supernatant → no disturbance of cells or formazan crystals and less working steps • MTT in PBS was added undiluted → no additional working step for dilution and volume of each well stayed small → no supernatant had to be removed • HCl as addition to solvent → shifting the absorbance maximum of phenol red; SDS prevented precipitation of present FBS • Due to additional overnight incubation after adding solvent, formazan crystals and cells were completely lysed the next day, but experiment has longer duration • Resuspending the solution right before absorbance measurement was not

		possible, because of strong appearance of air bubbles in the wells, disturbing the measurement
VARIATION 2	1. Medium was not removed after overnight incubation 2. 20 µl of MTT in PBS with a concentration of 5 mg/ml were added 3. After 4 h incubation, 190 µl of supernatant were removed 4. 200 µl DMSO were added as solvent 5. Absorbance measurement directly after adding the solvent and resuspending adequately	• Medium was not removed after overnight incubation → less disturbance of cells and less working steps • No dilution of MTT stock solution necessary → less working steps • 30 µl of supernatant were left in the wells after 4 h incubation → less disturbance of the formazan crystals • DMSO caused a complete solution of formazan crystals directly after adding; but single cell fragments remained visible • Resuspension after adding the solvent was possible → better mixing before absorbance measurement
VARIATION 3	1. After overnight incubation and removing whole medium, 100 µl MTT in basal medium working solution with a concentration of 500 µg/ml were added 2. After 4 h incubation MTT in basal medium was not removed 3. 150 µl acidified isopropanol were added	• Only 100 µl of MTT in basal medium were added → the solvent was added after 4 h incubation without additional removing of supernatant → no disturbance of formazan crystals • No FBS in the supernatant before adding the solvent → no risk of precipitation • Absorbance maximum of phenol red in the supernatant was shifted with HCl in solvent
VARIATION 4	1. After overnight incubation and removing whole medium, 100 µl MTT in basal medium working solution with a concentration of 500 µg/ml were added 2. After 4 h incubation, MTT in basal medium was not removed 3. 200 µl DMSO were added as solvent 4. After resuspending the absorbance was directly measured	• Only 100 µl of MTT in basal medium were added → the solvent was added after 4 h incubation without additional removing supernatant → no disturbance of formazan crystals • No FBS in the supernatant before adding the solvent → no risk of precipitation • Absorbance maximum of existing phenol red in supernatant was not changed → potential disturbance factor • DMSO lysed formazan crystals completely, but cell fragments

		remained after short resuspension
VARIATION 5	1. After 4 h incubation 170-195 µl of the supernatant were removed 2. 200 µl of DMSO were added as solvent 3. After resuspending, the absorbance was directly measured	• Not whole supernatant was removed after the 4 h incubation time → less disturbance of formazan crystals • No FBS in the supernatant before adding the solvent → no risk of precipitation • Less phenol red was left in each well before adding the solvent → small disturbing potential • DMSO lysed formazan crystals completely, but cell fragments remained after short resuspension
VARIATION 6	1. After 4 h incubation 170-195 µl of the supernatant were removed 2. 200 µl of acidified isopropanol were added as solvent	• Not whole supernatant was removed after 4 h incubation time → less disturbance of formazan crystals • No FBS in the supernatant before adding the solvent → no risk of precipitation • Less phenol red was left in each well before adding the solvent and HCl shifted absorbance maximum → no disturbance of absorbance measurement

In every performed assay the A549 cells were counted with the Neubauer Chamber and seeded in numbers from 2.5 x 10^3 to 4 x 10^4 cells like described in 2.3.2.. Another constant was the 4 h incubation time at 37 °C after adding the MTT solution. The solvent for the formazan lysis was variable and the tested solvents were DMSO, acidified isopropanol and 10% SDS in 0.01 N HCl. For the SDS/HCl solvent 75 ml of a 20% SDS solution were added to 75 ml of a 0.02 N HCl to obtain 150 ml 10% SDS in 0.01 N HCl, which was stored in a glass bottle at room temperature. After adding SDS/HCl, an additional overnight incubation took place and the absorbance was measured at the next day, without resuspending the wells to avoid the formation of air bubbles that could disturb the absorbance measurement. DMSO, the second solvent tested, was sterile filtered just before pipetting it to the cells using a filter with a pore size of 0.22 µm. After a thoroughly resuspension the absorbance was measured

directly. Acidified isopropanol was also prepared just before using it, therefor 9900 µl of isopropanol were mixed with 100 µl of 1 N HCl to receive 0,01 N HCl in isopropanol. Every cell number was seeded in triplicates and averaged for the evaluation of R^2. As a blank for all the variations full medium was used, added to extra wells on the same plate and treated the same way like the cells. After checking the resolving of the formazan crystals under the microscope, the absorbance was measured with the microplate reader (Infinite® 200Pro, Tecan) at 570 nm with a shaking duration of 3 s and a shaking amplitude of 1.5 mm.

2.4. Cell Based Assay for TNFα Expression and Quantification

2.4.1. In Vitro Transfection with TNFα Plasmids and L929 Cytotoxicity Based TNFα Assay

Materials

- HBS *(HEPES buffered saline)* (kind gift from J. Sperhansl)
    - 2.3831 g HEPES (20 mM) (PanReac AppliChem, A3724,0500)
    - 4.383 g sodium chloride (150 mM)
    - solved in 400 ml dH_2O
    - pH adjusted to 7.4 with HCl and NaOH
    - filled up to a final volume of 500 ml with dH_2O and sterile filtered with a pore size of 0.22 µm
    - stored at room temperature
- LPEI *(linear polyethylenimine)* 10 kDa, stock solution 3.071 mg/ml (kind gift from A. Taschauer and J. Sperhansl, MMCT, University of Vienna, Austria)
- OMEM (Opti-MEM® I Reduced-Serum Medium, Gibco®, 11058-021)
- Lipofectamine® 2000 (Invitrogen®, 11668-019)
- Actinomycin D (PanReac AplliChem, A1489,0005)

Day 1 – Seeding A549 Cells

On the first day of the TNFα assay the A549 cells were seeded. Therefor a confluent T75 flask was split and the cells were counted with the Neubauer Chamber (see 2.2.1.

and 2.2.2.). The cells were seeded in a number of 10^4 cells/well in 200 µl full RPMI 1640 cell culture medium per well, in a sterile 96-well cell culture plate (Greiner Bio-One, Cellstar®, 655 180). Afterwards the plate was stored in the incubator for 18–23 h overnight.

Day 2 – Transfection of A549 Cells and Seeding L929 Cells

On the second day, the A549 cells from day 1 were transfected with the needed plasmids, listed in table 5.

Table 5: Plasmids used for the transfection of the A549 cells for the TNFα assay.

Plasmid	Purpose	Origin
pCpG-hCMV-EF1α-TNFα	TNFα assay	(Magnusson et al., 2011)
pCpG-hCMV-SCEP-TNFα	TNFα assay	(Magnusson et al., 2011)
pCpG-hCMV-EF1α-LucSH	Negative control for TNFα assay	Kind gift from K. Völckers
pCpG free promoter Lucia	Negative control for TNFα assay	Kind gift from K. Völckers

For the transfection, the plasmids stored at −80 °C were slowly thawed on ice and the concentration was verified. Therefor the pDNA solution was diluted 1:5 and 1:10 with nuclease free water and the concentration was measured with the NanoVue Plus™ spectrophotometer (4282 V2.0.4, VWR®). Nuclease free water was used as a blank and the concentrations were averaged out. In one half of the plate the cells were transfected with 100 ng of the plasmids and in the other half 200 ng were used for the transfection. The required amount of LPEI for the nitrogen/phosphate (N/P) charge ratio of 6 was calculated by using the following formula (Taschauer et al., 2016):

$$\mu g\ LPEI = \frac{\mu g\ DNA \times 43 \times (N/P\ ratio)}{330}$$

Dilutions were made of the pDNA and the LPEI with HBS so that equal volumes could be used for making the polyplexes. The LPEI was added to the DNA and immediately

after the transfer the mixture was flash pipetted up and down 20 times to avoid the development of aggregates. Afterwards the LPEI-DNA mixture was incubated for 20 min at RT. Meanwhile the positive control with Lipofectamine® 2000 was prepared. Following the manufacturers instruction, the pDNA and the Lipofectamine were diluted with OMEM, again to get equal volumes. In this case the DNA dilution was added to the Lipofectamine dilution. The mixture was rapidly pipetted 5 times up and down and incubated for 5 min at RT. For the transfection the medium in the wells of the 96-well plate with the seeded A549 cells from day 1 was aspirated completely and 100 µl of fresh, preheated (37 °C) basal RPMI 1640 medium without FBS and L-Glutamine were added into each well. After the incubation times the DNA-LPEI mixture and the DNA-Lipofectamine mixture were pipetted into the wells. Some of the wells were left untreated. The plate was stored in the incubator for 4 h. After this time the volume was filled up to 200 µl, with full RPMI 1640 medium containing 10% FBS and L-Glutamine and the plate was again stored in the incubator for 20–22.5 h. In later experiments the supernatant with leftover polyplexes was removed completely after the 4 h incubation time, and 200 µl of full medium were added in order to exclude cytotoxic effects of the remaining polyplexes in the supernatant. Also on day 2 the L929 cells were seeded in a number of 1.25 x 10^4 cells/well in 200 µl full RPMI 1640 medium with 10% FBS, L-Glutamine and 1 % P/S into a 96-well plate (Greiner Bio-One, Cellstar®, 655 180). The plate with the seeded L929 cells was stored in the incubator overnight for 20–24 h.

Day 3 – Collection of Supernatant form A549 Cells and Addition to L929 Cells

On the third day 5 mg of Actinomycin D were dissolved in 5 ml DMSO. 2.5 ml of this 1 mg/ml solution were sterile filtered through a pore size of 0.22 µm and subsequently aliquoted in parts of 100 µl, wrapped up in aluminum foil and together with the not sterile filtered Actinomycin D - DMSO solution stored at –80 °C. For the experiments, only the sterile filtered stock solution was used. From the aliquots of the filtered stock solution a working solution was freshly produced by diluting the 1 mg/ml solution 1:100 with full RPMI medium for the L929 cells, to obtain a concentration of 0.01 µg/µl. After this preparation, the plates with the transfected A549 cells and the seeded

L929 cells from day 2 were taken out of the incubator and the supernatant from the L929 cells was completely aspirated. It was substituted with 100 µl of the A549 supernatant containing the secreted TNFα. Some of the wells were filled with 100 µl fresh, preheated (37 °C) full L929 medium and later used as untreated cells for the calculation of the cytotoxicity. There were also made tenfold serial dilutions of the supernatant from the transfected A549 cells with full RPMI 1640 medium in a range from 100 µl, therefore undiluted, to 0.001 µl, in order to have more data for the later comparison with the cytotoxicity of the recombinant TNFα. In the end 11 µl of the Actinomycin D working solution were pipetted into each well so that in total the Actinomycin D concentration in each well was 0.1 µg/100 µl. Afterwards the plate with the L929 cells was stored in the incubator and left there overnight for 20-24 h. The remnant supernatant from the A549 cells was transferred to a fresh 96-well plate and stored at −80 °C for further experiments.

Day 4 – MTT Assay

On the fourth day, the MTT assay was performed to measure the cell viability and furthermore to calculate the cytotoxicity of the secreted TNFα. After the incubation duration, 11 µl of a 5 mg/ml MTT in PBS solution were added to each well, to obtain a final concentration of 0.45 mg/ml. Subsequently the plate was incubated for 4 h at 37 °C and 5% CO_2. After this duration 92 µl of the supernatant were removed, 200 µl of sterile filtered DMSO were added and resuspended nicely before the absorbance was measured within 20 min, using the microplate reader (Infinite® 200Pro, Tecan) at the wavelengths 570 nm and 630 nm. The correct absorbance was calculated by subtracting the absorbance measured at 630 nm from the absorbance measured at 570 nm. The cytotoxic effect of TNFα secreted by the transfected A549 cells, on the L929 cells, sensitized with Actinomycin D, was calculated with the following formula:

$$\boldsymbol{Cytotoxicity}\ \% = \frac{\boldsymbol{Absorbance\ of\ 100\%\ Viable\ Cell\ Control\ Well - Absorbance\ of\ Test\ Well}}{\boldsymbol{Absorbance\ of\ 100\%\ Viable\ Cell\ Control\ Well}} \times \mathbf{100}$$

The absorbance of the L929 cells that were treated the same way like the test cells but without adding the supernatant from the A549 cells containing the cytotoxic TNFα, was used as the absorbance of 100% viable cells for the calculation. Instead of the supernatant, 100 µl of full RPMI 1640 medium were added.

Additionally, the difference between the cytotoxic impact of the fresh supernatant, like described above, and the cytotoxicity of the same supernatant after freezing at −80 °C for duration of 6 to 12 days was investigated. Therefore, after performing the TNFα assay with a serial dilution from 10 to 0,001 µl, the remaining supernatant of the A549 cells transfected with 200 ng of LPEI pCpG-hCMV-EF1α-TNFα was transferred to a sterile 96-well plate and stored at −80 °C. 6 to 12 days later the supernatant was thawed and the same TNFα assay, with the same serial dilution was executed. The results were averaged and compared.

Table 6: Overview of the working steps during 4 days of in vitro transfection with TNFα plasmids and L929 cytotoxicity based TNFα assay.

	In Vitro Transfection	L929 Bioassay
Day 1	Seeding A549 cells	-
Day 2	Transfection with TNFα-plasmids	Seeding L929 cells
Day 3	Collection of supernatant	Addition of supernatant from A549 cells
Day 4	-	MTT assay

2.4.2. L929 Cytotoxicity based TNFα Assay with Recombinant Murine TNFα

Materials

- Recombinant Murine TNFα (PeproTech®, 315-01A)
- Actinomycin D (PanReac AplliChem, A1489,0005) working solution 0,01 µg/µL
- Dilution buffer 0.2% BSA in PBS
    - BSA *(bovine serum albumine)* (Sigma®, A9647-50G)
    - Sterile PBS *(Dulbecco`s Phosphat Buffered Saline)* (Sigma-Aldrich®, D8537)
- MTT working solution 5 mg/ml in PBS (see above)

Day 1 – Seeding L929 Cells

For the experiments utilizing the recombinant murine TNFα, L929 cells were seeded in a number of 1.25 x 10^4 cells per well into a sterile 96-well cell culture plate (Greiner Bio-One, Cellstar®, 655 180) with a total volume of 200 µl full RPMI 1640 per well. The plate was stored in the incubator at 37 °C and 5% CO_2 for 20–23 h overnight.

Day 2 – Preparation of rTNFα and Addition to L929 Cells

The recombinant murine TNFα needed to be reconstitute, therefore the vial with the recombinant TNFα powder was initially centrifuged for 30 secs in a micro centrifuge and afterwards 20 µl of autoclaved Millipore (MQ) water (Arium®pro VF, Sartorius) were added to the 20 µg protein, to receive a concentration of 1000 ng/µl (Stock A). Stock A was diluted 1:10 with the dilution buffer to obtain Stock B with a concentration of 100 ng/µl. Stock B also was diluted 1:10 to get Stock C with a concentration of 10 ng/µl, which then was aliquoted as single use aliquots of 10 µl and stored at –80 °C. Directly before every recombinant TNFα assay one aliquot of Stock C was thawed on ice and a tenfold serial dilution was prepared using a dilution buffer, like shown in table 7.

Table 7: Serial dilution of recombinant murine TNFα with dilution buffer 0.2% BSA in PBS.

Dilution Number	Volume taken	Volume Dilution Buffer [µl]	Final Volume [µl]	C [ng/µl]	Sample Volume [µl]	Amount of Protein per Sample [ng]
1	5 µl from Stock C	45	50	1	10	10
2	5 µl from 1	45	50	10^{-1}	10	1
3	5 µl from 2	45	50	10^{-2}	10	10^{-1}
4	5 µl from 3	45	50	10^{-3}	10	10^{-2}
5	5 µl from 4	45	50	10^{-4}	10	10^{-3}
6	5 µl from 5	45	50	10^{-5}	10	10^{-4}
7	5 µl from 6	45	50	10^{-6}	10	10^{-5}
8	5 µl from 7	45	50	10^{-7}	10	10^{-6}
9	-	50	50	-	10	-

Dilutions shown in table 8 were mixed with full RPMI 1640 medium.

Table 8: Serial dilution of recombinant murine TNFα with full cell culture medium RPMI 1640.

Dilution Number	Total Volume [µl]	C [ng/µl]	Added Volume of Full RPMI 1640 Medium [µl]	Final Volume [µl]	Sample Volume Added per Well [µl]	Amount of Protein per Sample [ng]
1	40	1	360	400	100	10
2	40	10^{-1}	360	400	100	1
3	40	10^{-2}	360	400	100	10^{-1}
4	40	10^{-3}	360	400	100	10^{-2}
5	40	10^{-4}	360	400	100	10^{-3}
6	40	10^{-5}	360	400	100	10^{-4}
7	40	10^{-6}	360	400	100	10^{-5}
8	40	10^{-7}	360	400	100	10^{-6}
9	40	-	360	400	100	-

The medium of the seeded L929 cells from day 1 was removed completely and the dilutions were added in triplets, with a total volume of 100 µl per well. The dilution number 9 (Tab. 4) without recombinant TNFα was added to 3–6 wells with L929 cells and used as 100% viable control wells for the calculation of the cytotoxicity on day 3. After adding the serial dilution 11 µl of the freshly produced Actinomycin D working solution with a concentration of 0.01 µg/µl were pipetted into every well, also the control wells. The plate was incubated at 37 °C and 5% CO_2 for 20–21 h overnight.

Day 3 – MTT Assay

On the third day 11 µl of the 5 mg/ml MTT in PBS solution were added to every well. The plate was incubated for 4 h and after this duration 92 µl of the supernatant were removed. The wells were filled up with 200 µl sterile filtered DMSO and resuspended. Within 20 min after adding the solvent the absorbance was measured with the plate reader at 570 nm and 630 nm. The cytotoxicity was calculated with the formula shown in 2.4.1..

2.4.3. TNFα Transgene Expression Kinetics in Vitro

Day 1 – Seeding A549 Cells

On the first day of the assay profiling the kinetics of TNFα transgene expression the A549 cells were seeded in a number of 7 x 10^4 cells per well in a clear 24 well plate, tissue culture treated with lid (Sigma-Aldrich®, Greiner CELLSTAR® multiwall culture plate, M8812 SIGMA) in a total volume of 500 µl full RPMI 1640 medium per well. For this purpose, a confluent cell culture flask with A549 cells was split and counted like described in 2.2.1. and 2.2.2. The plate was stored in the incubator at 37 °C and 5% CO_2 overnight for 20.5 h.

Day 2 – Transfection of A549 Cells

On the second day, the transfection of the A549 cells from day 1 took place like described in 2.4.1. but with 1000 ng of each plasmid, shown in table 9.

Table 9: Plasmids used for transfection of A549 cells for the assay, used for profiling TNFα transgene expression kinetics.

Plasmid	Purpose	Origin
pCpG-hCMV-EF1α-TNFα	Kinetic assay	(Magnusson et al., 2011)
pCpG-hCMV-SCEP-TNFα	Kinetic assay	(Magnusson et al., 2011)
pCpG-hCMV-EF1α-LucSH	Negative control for TNFα assay	Kind gift from K. Völckers

4 wells of the seeded A549 cells were left untreated without a transfection. After the 4 h incubation duration of the transfected cells in basal medium, the whole medium was removed and replaced with 500 µl of full RPMI 1640 medium. The plate was stored in the incubator overnight.

Day 3, 4, 5 and 6 – Collection and Freezing of Supernatant

On the following 4 days, the supernatant of every well was individually collected in 1.5 ml tubes after an overnight incubation of 23 h. The cells were washed with 300 µl PBS, which was carefully aspirated and 500 µl of fresh, preheated (37 °C) full RPMI 1640 medium were added. The plate was stored in the incubator. The tubes with the collected supernatant were centrifuged with a micro centrifuge (VWR®, Micro Star

17R) at 500 x g at 4 °C for 5 min. 450 µl of the supernatant were transferred from every tube to a new one, without disturbing the not visible pellet at the bottom of the tube. For later analysis, the tubes were stored at –80 °C.

Day 6 – Seeding L929 Cells

On the sixth day, the L929 cells were seeded in a number of 1.25 x 10^4 cells per well in 200 µl full RPMI 1640 into a 96-well cell culture plate (Greiner Bio-One, Cellstar®, 655 180). The plate was stored in the incubator overnight.

Day 7 – Addition of Collected Supernatant to L929 Cells

On the seventh day, the frozen supernatant samples collected the days before were thawed on ice and serial dilutions were prepared shown in table 10.

Table 10: Serial dilutions of collected supernatants of transfected A549 cells from the assay used for profiling TNFα transgene expression kinetics.

Dilution Number	Volume Taken [µl]	Added Volume of Full RPMI 1640 Medium [µl]	Added Volume per Well [µl]	Amount of Original Supernatant per Well [µl]
1	90 from collected supernatant, undiluted	810	100	10
2	90 from 1	810	100	1
3	400 from 2	400	100	0.5
4	400 from 3	400	100	0.25
5	90 from 2	810	100	0.1
6	400 from 5	400	100	0.05

The first three dilutions of the collected supernatant (Tab. 6) were not used, because a previous test run showed the appropriate range. These serial dilutions were prepared for all three plasmids shown in Table 9 and for all four days. Each dilution was applied in triplets.

Also recombinant TNFα was thawed on ice and serial dilutions were prepared shown in table 11. They were also applied in triplets.

Table 11: Serial dilution of recombinant murine TNFα.

Dilution Number	Volume taken [µl]	Added Volume of Dilution Buffer [µl]	C [ng/µl]	Volume taken from Dilution [µl]	Added Volume of Full RPMI 1640 Medium [µl]	C [ng/µl]	Added Volume per Well [µl]	Amount of rTNFα per well [ng]
1	10 from Stock C (10 ng/µl)	90	1	80	720	0.1	100	10
2	10 from 1	90	0.1	80	720	0.01	100	1
3	10 from2	90	0.01	80	720	0.001	100	0.1
4				400 from 3	400	0.0005	100	0.05
5				400 from 4	400	0.00025	100	0.025
6	10 from 3	90	0.001	80	720	0.0001	100	0.01
7				400 from 6	400		100	0.0025
8	10 from 6	90	0.0001	80	720	0.00001	100	0.001

Because of the amount of the wells needed for this assay, more than one 96-well plate was needed. On every plate, there were always left 3–6 wells with seeded L929 cells untreated, used for the later calculation of the cytotoxicity.

After adding the serial dilution 11 µl Actinomycin D working solution with the concentration of 0.01 µg/µl were added to every well and the plates were stored in the incubator at overnight for approximately 21 h.

Day 8 – MTT Assay

On the last day of this assay 11 µl of the MTT working solution in PBS with a concentration of 5 mg/ml were added. The plates were stored in the incubator for 4 h and after this duration, 92 µl of the supernatant were removed and 200 µl of sterile filtered DMSO were added. After resuspending, the absorbance was measured at 570 nm and 630 nm with the plate reader and the cytotoxicity was calculated just as described in 2.4.1..

3. RESULTS

3.1. OPTIMIZATION OF THE MTT ASSAY

3.1.1. MTT ASSAY

The protocol based on the results of Supino R. and Chiba K. et al. was tested initially to determine the correlation between seeded cell number and measured absorbance. Cells were seeded in different cells numbers (in triplicates) and after overnight incubation, complete cell culture medium was removed and replaced by 200 µl MTT working solution with a concentration of 0.4 mg/ml. After 4 h of incubation, the MTT solution was aspirated completely and 200 µl isopropanol were added. After 30 min on the shaker, absorbance was measured at 540 nm. For calculation of corrected absorbance, the absorbance of pure isopropanol was used as a blank. The R^2 value for the first assay was with 0.77 lower than the second with R^2 = 0.97 and the third with R^2 = 0.95 (Fig. 7). Because of these variations different parameters (as described in Table4 and Figure6) like the solvents of the formazan crystals and the intermediate steps were changed and tested during the optimization. Other factors like seeded number of cells and incubation time of 4 h after adding the MTT solution stayed constant.

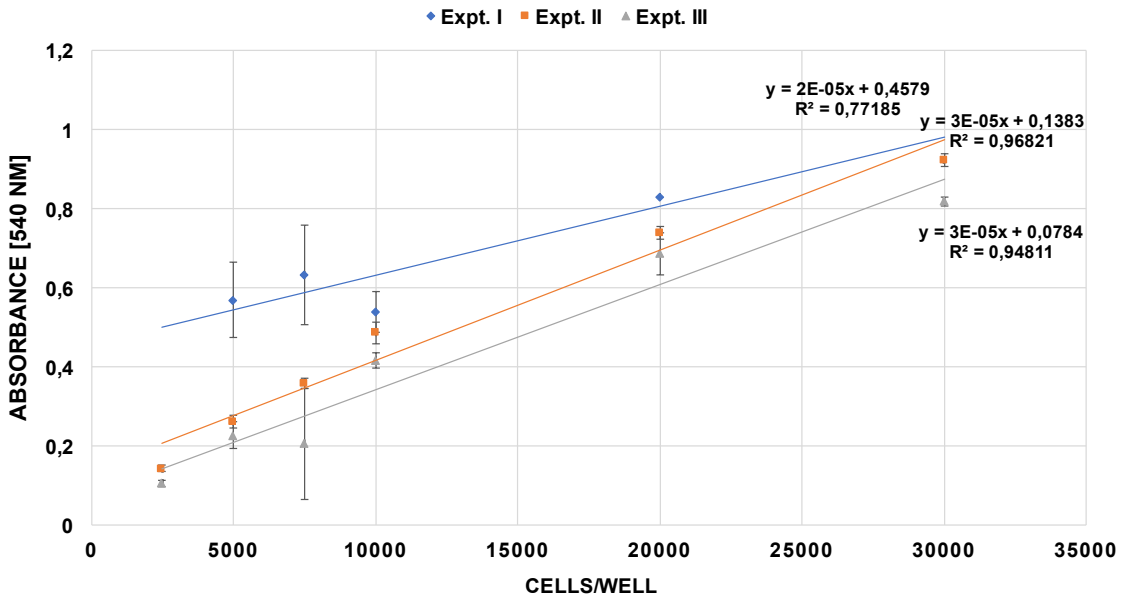


Figure 7: Performance of the original protocol of MTT assay: Correlation between the measured absorbance and the number of seeded A549 cells by MTT assay as per the original protocol (Supino R. and Chiba K. et al. as shown in section 2.3.2. and figure 5), illustrated as R^2 value. Data of 3 independent experiments, each performed in triplicates; mean of the triplicates is shown, ± SD. $R^2_{(Expt.\ I)}$ = 0.77185, $R^2_{(Expt.\ II)}$ = 0.96821, $R^2_{(Expt.\ III)}$ = 0.94811.

3.1.2. Investigation of Parameters Affecting MTT Assay

Main considerations during testing the different variations were the mechanical disturbance of attached cells and formazan crystals during the process of aspirating the medium. One solution was the variation where we tried not removing supernatant after first incubation and not removing the whole supernatant after 4 h incubation so that 30 µl of supernatant was left in each well. Another consideration was the possibility of precipitation of FBS, a component of full cell culture medium and solvents like isopropanol, that could disturb the absorbance measurement. Phenol red, also a component of full cell culture medium as well as of basal medium, has an absorbance maximum in the same wavelength range like formazan and thus is also a disturbance factor for the absorbance measurement. These problems were tried to solve with the following variations of different working steps and solvents.

Variation 1:

Selected parameters of the MTT assay were changed like described in 2.3.3. and results were validated by comparing R^2 values of each assay. As a blank for the calculation of corrected absorbance without background absorbance of components of the medium or MTT solution, mean absorbance of 3-7 wells filled with full cell culture medium but without cells and treated like the other wells, was used. Following the protocol for variation 1, the cells were seeded in 100 µl full cell culture medium, after overnight incubation medium was not removed and 10 µl of MTT in PBS stock solution with a concentration of 5 mg/ml were added so that the concentration of MTT in each well amounted 0.45 mg/ml. As soon as the 4 h incubation time were over, 100 µl 10 % SDS in 0.01 N HCl were added as a solvent. The 96 well plate was incubated for an additional night and absorbance was measured the next day without resuspending before. HCl had the function to reduce the pH to avoid the interference of pH indicator phenol red during measuring the absorbance. Furthermore, the risk of disturbing the attached cells in the wells during aspiration of the medium was eliminated by seeding the cells in 100 µl of full medium so that there was enough space in each well for adding the MTT working solution and the solvent without the necessity of removing medium. The additional overnight incubation with SDS/HCl was necessary for a complete lysis of cells and formazan crystals. Before measuring the absorbance, the solution was not resuspended to avoid the formation of air bubbles in the wells that could disturb the measurement. This variation was performed in three independent experiments and the results are shown in Figure 8.

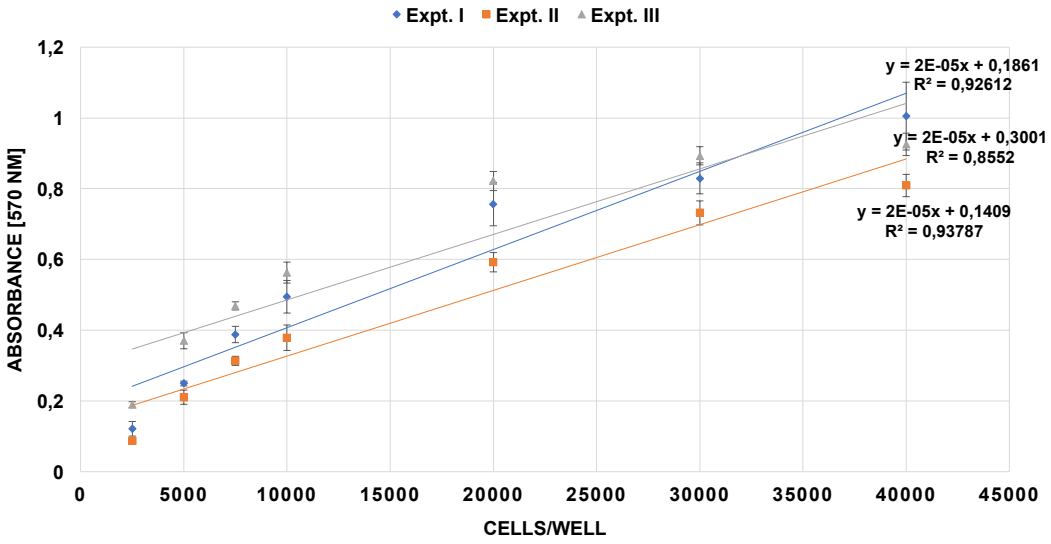


Figure 8: Performance of Variation 1 of MTT assay: Correlation between the measured absorbance and the number of seeded A549 cells by MTT assay as per the protocol of variation 1 (as shown in section 2.3.3. and figure 6), illustrated as R^2 value. Data of 3 independent experiments, each performed in triplicates; mean of the triplicates is shown, ± SD. $R^2_{(Expt.\ I)}$ = 0.92612, $R^2_{(Expt.\ II)}$ = 0.8552 and $R^2_{(Expt.\ III)}$ = 0.93787. As a blank the averaged absorbance of 7 wells on the same plate, without cells but with the same volume of full RPMI 1640 medium, treated the same way like described in 2.3.3. was used.

Variation 2:

For the second variation shown in 2.3.3. A549 cells were seeded in 200 µl of full RPMI medium and after overnight incubation 20 µl MTT in PBS with the concentration of 5 mg/ml was added up to a concentration of 0.45 mg/ml in each well, without removing the medium before. After 4 h incubation, the medium was aspirated, leaving 30 µl in each well. 200 µl of DMSO served as solvent and the absorbance was measured at 570 nm directly after resuspending each well. R^2 values of this variation showed a high correlation (Fig. 9).

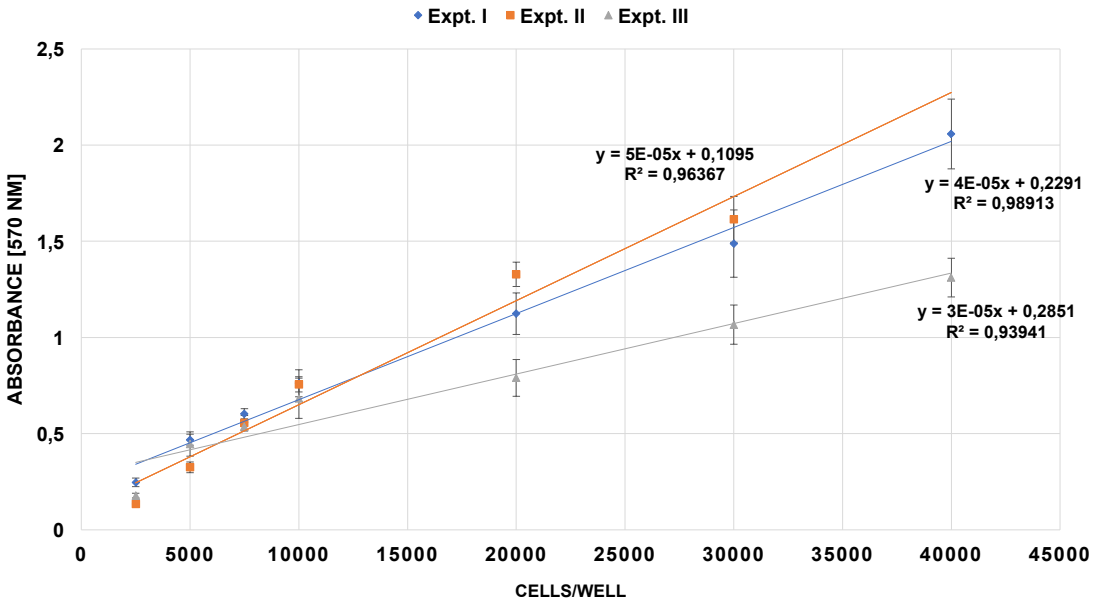


Figure 9: Performance of Variation 2 of MTT assay: Correlation between the measured absorbance and the number of seeded A549 cells by MTT assay as per the protocol of variation 2 (as shown in section 2.3.3. and figure 6), illustrated as R^2 value. Data of 3 independent experiments, each performed in triplicates; mean of the triplicates is shown, ± SD. $R^2_{(Expt.\ I)}$ = 0.98913, $R^2_{(Expt.\ II)}$ = 0.96367, $R^2_{(Expt.\ II)}$ = 0.93941.

During variation 1 and 2, the medium used for seeding the cells in 96-well plates, containing FBS, Glutamine and phenol red was not removed after overnight incubation. Consequently, the attached cells were not disturbed because of an aspiration process.

Variations 3 and 6:

Variation 3 and 6 comprised acidified isopropanol as solvent. Cells were seeded in 200 µl full cell culture medium, after overnight incubation whole medium was removed. Following the protocol for variation 3, 100 µl of MTT in basal medium working solution with a concentration of 500 µg/ml were added. After 4 h incubation the supernatant was not removed and 150 µl of acidified isopropanol were added. The absorbance was measured after the plate was stored on a shaker for 30 min. Following the protocol for variation 6, 200 µl of MTT working solution in basal medium with a concentration of 400 µg/ml were added and 170-195 µl of supernatant were removed after 4 h incubation. 200 µl acidified isopropanol were added afterwards and the absorbance was measured after the plate was stored on a shaker for 30 min. HCl shifted the

absorbance maximum of phenol red, so that it could not interfere with the absorption maximum of formazan. It was possible to use isopropanol as solvent, because the full medium containing FBS for seeding cells was removed almost completely after overnight incubation, and MTT was added in basal medium. Consequently, there was no FBS in the wells that could precipitate with the solvent and interfere the absorbance measurement. MTT concentration in each well amounted 0.5 mg/ml for variation 3, and 0.4 mg/ml for variation 6. Main difference was the missing intermediate step in variation 3 of removing the supernatant after 4 h incubation time, containing the MTT working solution. The correlation between the cell number and the absorbance is shown in Figure 10.

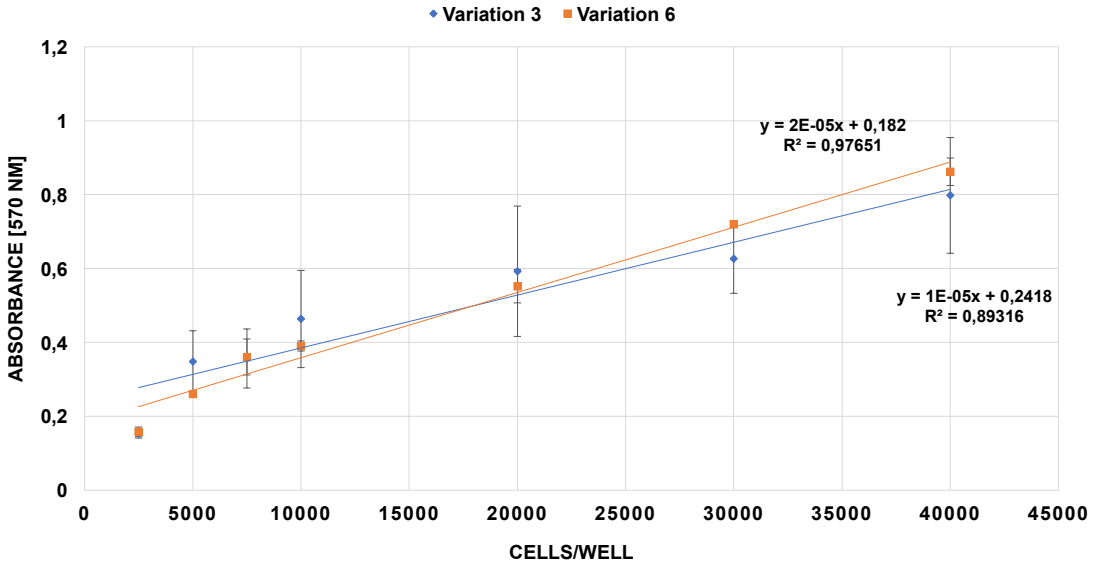


Figure 10: Performance of variation 3 and 6 of MTT assay: Correlation between the measured absorbance and the number of seeded A549 cells by MTT assay as per the protocol of variation 3 and 6 (as shown in section 2.3.3. and figure 6), illustrated as R^2 value. Data of 2 independent experiments, each performed in triplicates; mean of the triplicates is shown, ± SD. The solvent for both assays was acidified isopropanol. $R^2_{(Variation\ 3)}$ = 0.89316, $R^2_{(Variation\ 6)}$ = 0.97651.

Variations 4 and 5:

DMSO was used as a solvent for the remaining variations 4 and 5. In variation 4 the MTT concentration in each well was 0.5 mg/ml and only one aspiration step was performed. After overnight incubation, the full medium was removed and 100 µl of MTT working solution in basal medium, with the concentration of 500 µg/ml were

added. After 4 h incubation, the MTT solution was not aspirated and 200 µl DMSO were added. The R^2 values are shown in Figure 11.

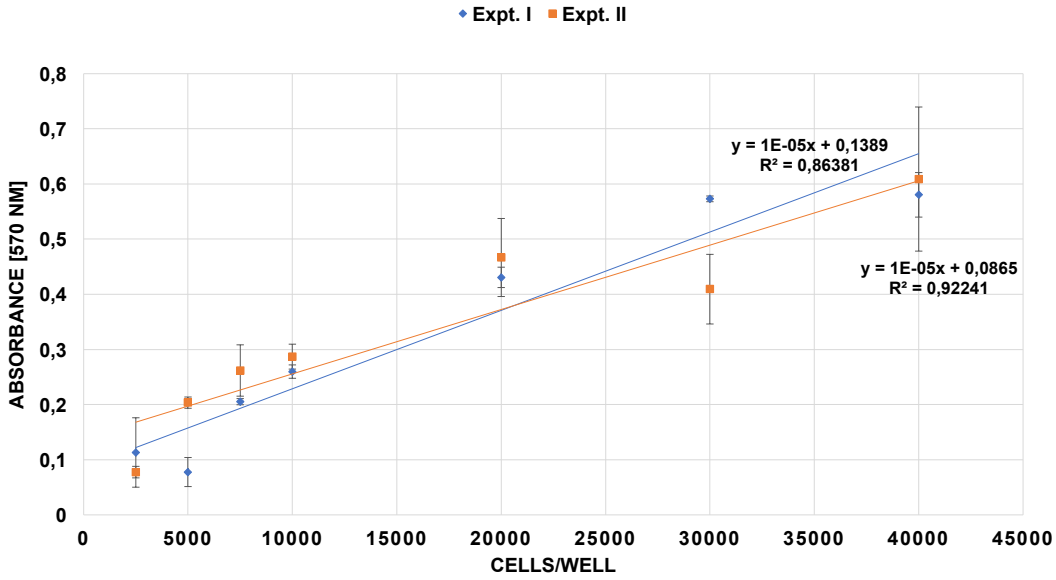


Figure 11: Performance of Variation 4 of MTT assay: Correlation between the measured absorbance and the number of seeded A549 cells by MTT assay as per the protocol of variation 4 (as shown in section 2.3.3. and figure 6), illustrated as R^2 value. Data of 2 independent experiments, each performed in triplicates; mean of the triplicates is shown, ± SD. $R^2_{(Expt.\ I)}$ = 0.92241, $R^2_{(Expt.\ II)}$ = 0.86381.

During variation 5 the full cell culture medium was removed after overnight incubation and 200 µl MTT working solution in basal medium, with a concentration of 400 µg/ml were added so that the MTT concentration per well was 0.4 mg/ml. The assay was performed with two intermediate steps. Primarily medium was removed after overnight incubation and after 4 h incubation time with the MTT solution, 170 - 195 µl of the supernatant were aspirated. In order, not to disturb either the formed formazan crystals after the incubation time, nor the cells, not the whole MTT working solution was removed. The R^2 values are shown in Figure 12.

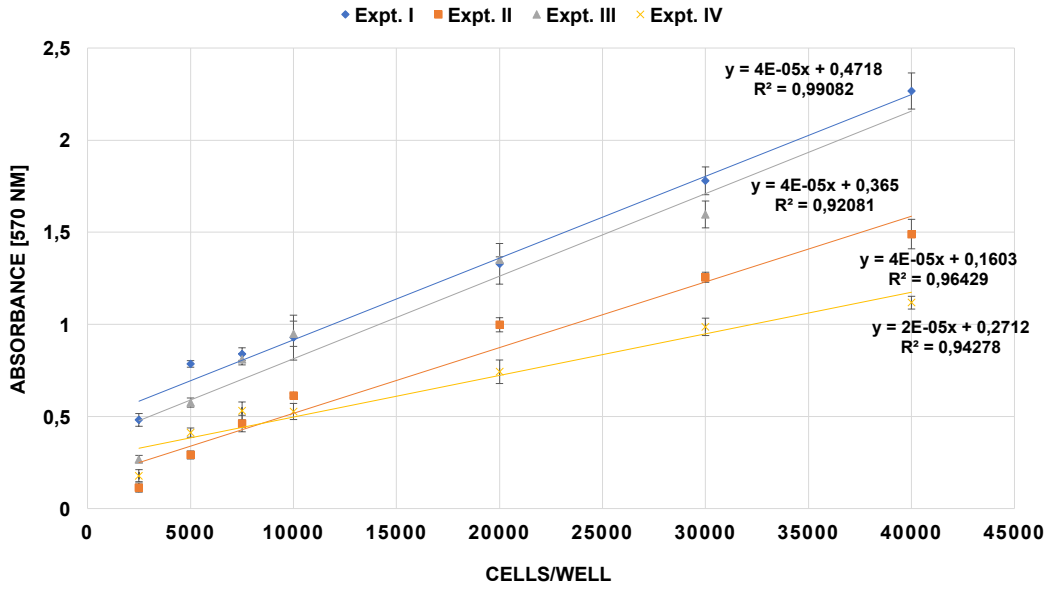


Figure 12: Performance of Variation 5 of MTT assay: Correlation between the measured absorbance and the number of seeded A549 cells by MTT assay as per the protocol of variation 5 (as shown in section 2.3.3. and figure 6), illustrated as R^2 value. Data of 4 independent experiments, each performed in triplicates; mean of the triplicates is shown, ± SD. In Expt. I 195 µl of the MTT supernatant were removed before adding DMSO, in Expt. III 175 µl and in Expt. IV 170 µl were removed. Expt. II was performed divergently: only 100 µl instead of 200 µl of the MTT working solution with a concentration of 0.5 mg/ml instead of 0.4 mg/ml were added and after the 4 h incubation time 60 µl of the supernatant were aspirated. $R^2_{(Expt.\ I)}$ = 0.99082, $R^2_{(Expt.\ II)}$ = 0.96429, $R^2_{(Expt.\ III)}$ = 0.92081, $R^2_{(Expt.\ IV)}$ = 0.94278.

To evaluate the different variations of the MTT assay, the R^2 values of the different variations were averaged out and compared (Fig. 13). Variation 3 and 6 only were performed once and therefore have no statistical pertinence.

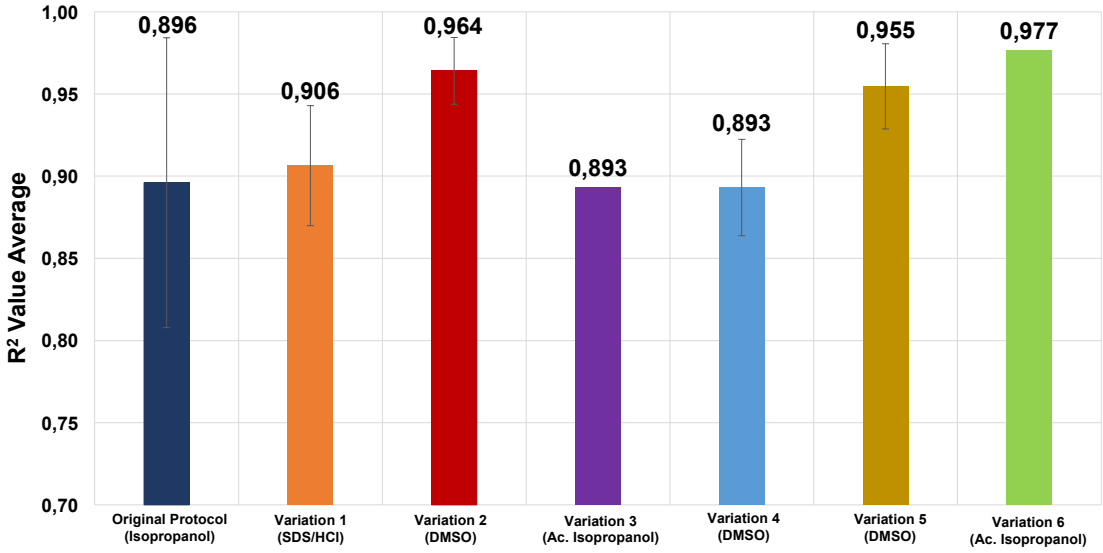


Figure 13: Performance of different variants of MTT assay in terms of averaged R^2 values of the 17 independent experiments for the original protocol based on the insights of Supino R. and Chiba K. et al. and for the different variations described in 2.3.3. tested for the optimization of the MTT assay. Each experiment was performed in triplicates, for the optimization of the MTT assay. The original protocol and variations are described in section 2.3.3. and figure 5-6. Mean of the triplicates was used for the calculation of the R^2 values. Used solvents are mentioned in brackets. Variation 3 and 6 were only performed once and not repeated. The mean of R^2 values is shown, ± SD. Number of experiments (n): $n_{(original\ protocol)} = 3$, $n_{(variation\ 1)} = 3$, $n_{(variation\ 2)} = 3$, $n_{(variation\ 3)} = 1$, $n_{(variation\ 4)} = 2$, $n_{(variation\ 5)} = 4$, $n_{(variation\ 6)} = 1$.

Comparing the averaged R^2 values shown in Figure 13 variation 2 and 5 have the highest correlation between the number of seeded cells and the measured absorbance. Variation 6 has admittedly the highest R^2 value with 0.977, but was performed only once, because this variation just like variation 3, was with the preparation time of the fresh solvent and 30 min on the shaker too time-consuming, and thereby has no statistical validity. Because of that, variation 2 represents the optimized protocol for the MTT assay. In summary, it can be stated, that the most reliable and reproducible results can be achieved, if cells are not disturbed with an aspiration step after the first incubation and if 30 µl of the supernatant are left in each well after the second 4 h incubation. This seems to be enough volume not to disturb cells and formazan crystals during aspiration, but not too much so that FBS or phenol red interfere the absorbance measurement. MTT concentration of 0.45 mg/ml showed good results for the forming of formazan crystals and DMSO worked good as a solvent.

3.2. Propagation of TNFα Plasmids (pCpG-hCMV-EF1α-TNFα and pCpG-hCMV-SCEP-TNFα)

3.2.1. Bacterial Growth and Selection

The agar plates, streaked with the transformed E.coli DB3.1 bacteria, were visually examined and showed the expected growth after overnight incubation at 37°C (Fig. 14).

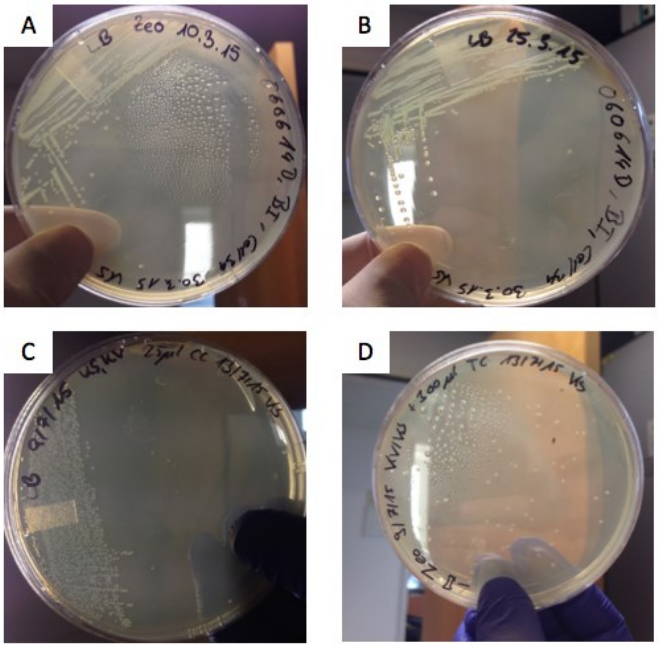


Figure 14: Bacterial growth after the incubation at 37 °C. (A) Bacteria transformed with pCpG-hCMV-EF1α-TNFα from a glycerol stock, plated on the selection plate containing Zeocin. Bacterial growth is identifiable. (B) Bacteria transformed with pCpG-hCMV-EF1α-TNFα from a glycerol stock, plated on an agar plate without antibiotics, as a positive control. Bacterial growth is identifiable (C) 25 µl of competent cells, later used for the transformation with pCpG-hCMV-SCEP-TNFα, plated on an agar plate not containing antibiotics, as a positive control. Bacterial growth is identifiable. (D) 300 µl of the newly transformed competent cells with pCpG-hCMV-SCEP-TNFα, plated on the selection agar plate containing Zeocin. Bacterial growth is identifiable.

3.2.2. Transformation Efficiency

The transformation efficiency could not be calculated for the transformation with pCpG-hCMV-EF1α-TNFα, because a glycerol stock with already transformed bacteria was used for streaking the agar plates, but for the transformation with pCpG-hCMV-SCEP-TNFα the calculation was possible, as shown in table 12.

Table 12: Calculated transformation efficiency for competent E.coli DB3.1 transformed with pCpG-hCMV-SCEP-TNFα.

Amount of DNA plated [μg]	Number of colonies	Calculated Transformation Efficiency
0.245	63	256.51 transformants per μg DNA

3.2.3. Isopropanol Precipitation

The isopropanol precipitation was performed for pCpG-hCMV-EF1α-TNFα and pCpG-hCMV-SCEP-TNFα. During this procedure, a certain proportion of DNA was lost, the remaining yield is shown in table 13.

Table 13: DNA yield after isopropanol precipitation of pCpG-hCMV-EF1α-TNFα and pCpG-hCMV-SCEP-TNFα.

	Number of Maxiprep	Amount of DNA before Isopropanol Precipitation [μg/ml]	Amount of DNA after Isopropanol Precipitation [μg/ml]	DNA yield [%]
pCpG-hCMV-EF1α-TNFα	1	522	392.5	75.2
	2	807	652.5	80.9
	3	844.6	672.5	79.6
pCpG-hCMV-SCEP-TNFα	1	1680	742	44

3.2.4. Diagnostic Restriction Digest

The restriction enzymes for the diagnostic restriction digest were selected following the restriction map shown in Figure 15, that was prepared using SnapGene®. The restriction map depicts the pCpG-hCMV-EF1α-TNFα with a total length of 3666 bp and the restriction enzymes able to cleave the plasmid.

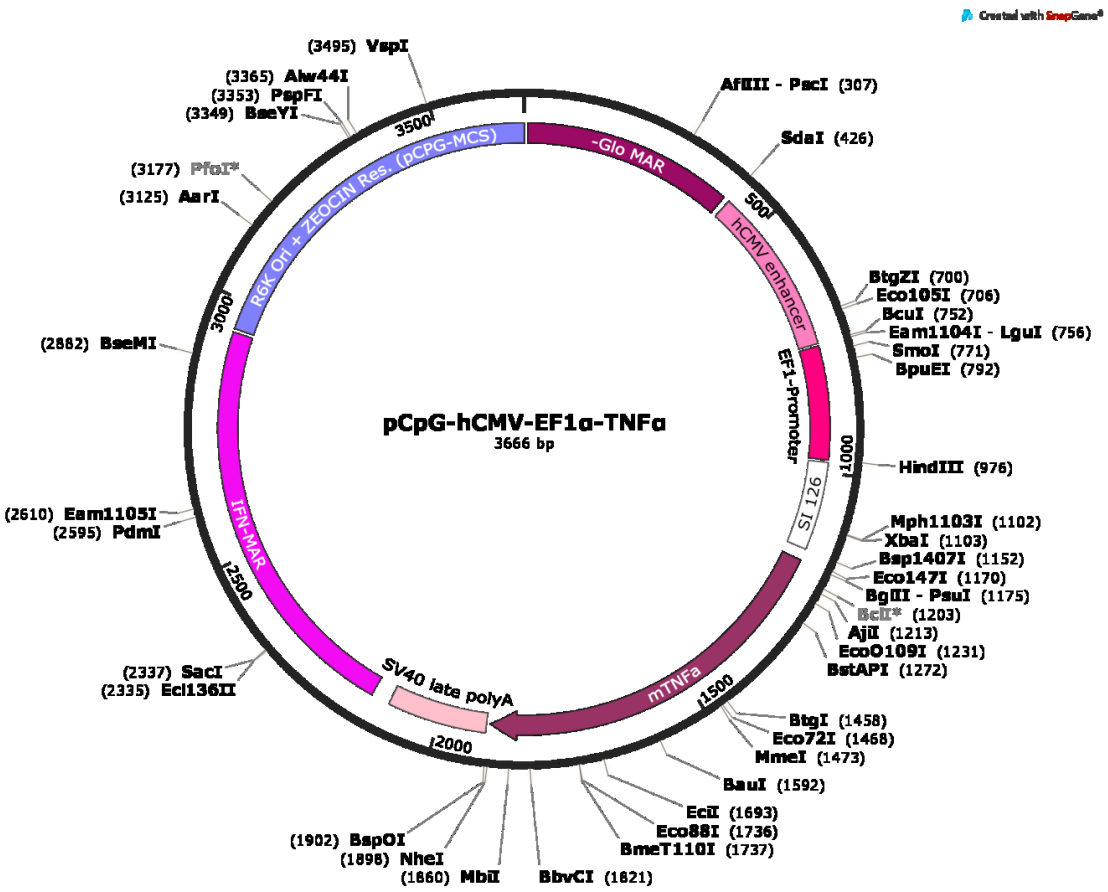


Figure 15: Restriction map for pCpG-hCMV-EF1α-TNFα. Different restriction enzymes able to cleave the plasmid are shown, as well as number and location of the recognition sequences. (Created with SnapGene®)

For both plasmids, the same combination of restriction enzymes was used shown in table 14.

Table 14: Applied restriction enzymes for validation of pCpG-hCMV-EF1α-TNFα and pCpG-hCMV-SCEP-TNFα via gel electrophoresis.

Well	Restriction Enzyme	Expected Number of Bands	Expected Number of Base Pairs
A	non	1	3666
B	HindIII	1	3666
C	HindIII, EcoRI	3	1950 1156 560
D	NdeI	4	1872 1305 473 16
E	NheI, XbaI	2	2871 795

The uncut, supercoiled form of the plasmids (position A) always migrated faster than the single cut, linear form (position B), like expected. There was an additional band in the slot of the uncut plasmid in every DRD, representing the nicked form of the plasmids, that migrates slower than the supercoiled form. For every gel electrophoresis, on one gel the expanded and purified plasmid was always let run parallel to the source plasmid, that was used for the transformation. Both plasmids were treated with the same restriction enzymes. In this way, a direct comparison was possible and proved the same identity for the plasmids in all DRDs. The other bands of the gel electrophoresis presented the calculated and expected patterns compared to the DNA ladder, and therefore confirmed the identity of the plasmids (Fig. 16 and 17).

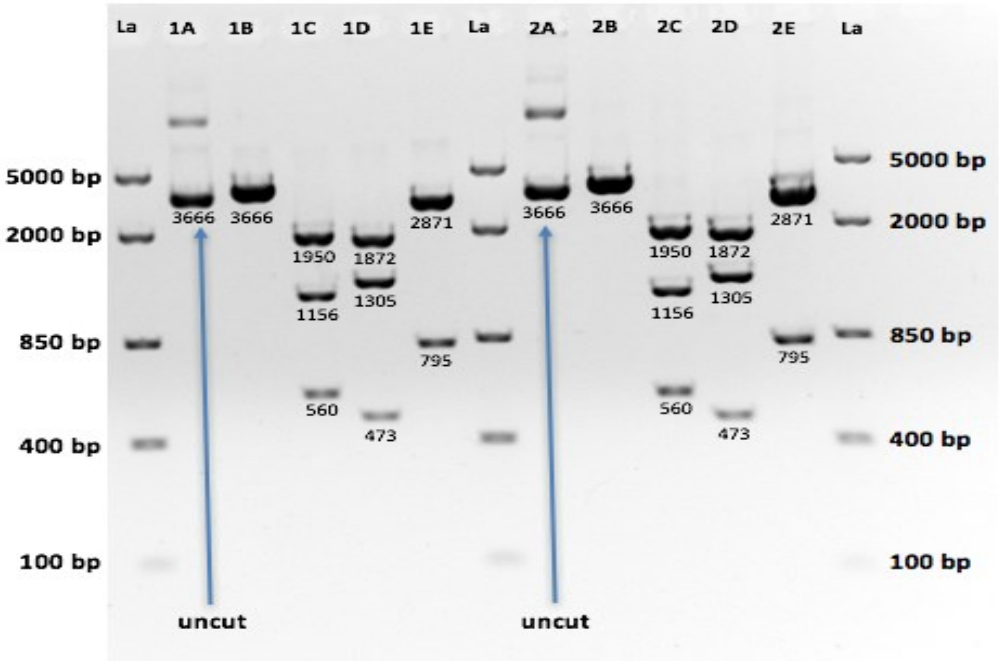


Figure 16: Image of the gel from gel electrophoresis of pCpG-hCMV-EF1α-TNFα after DRD. 1A – 1E represent digested samples from expanded and purified pCpG-hCMV-EF1α-TNFα. 2A – 2E show digested samples from pCpG-hCMV-EF1α-TNFα source plasmid that was used for transformation. 1A and 2A: uncut plasmid migrated faster than linearized plasmid in position 1B and 2B, because of its supercoiled form; faint band in position 1A and 2A that migrated slower than supercoiled form represents nicked plasmid. 1D and 2D: fourth band consisting of 16 base pairs (bp) could not be shown, because size was too small. The digits represent the calculated number of bp, expected after correct digest. La = DNA-ladder.

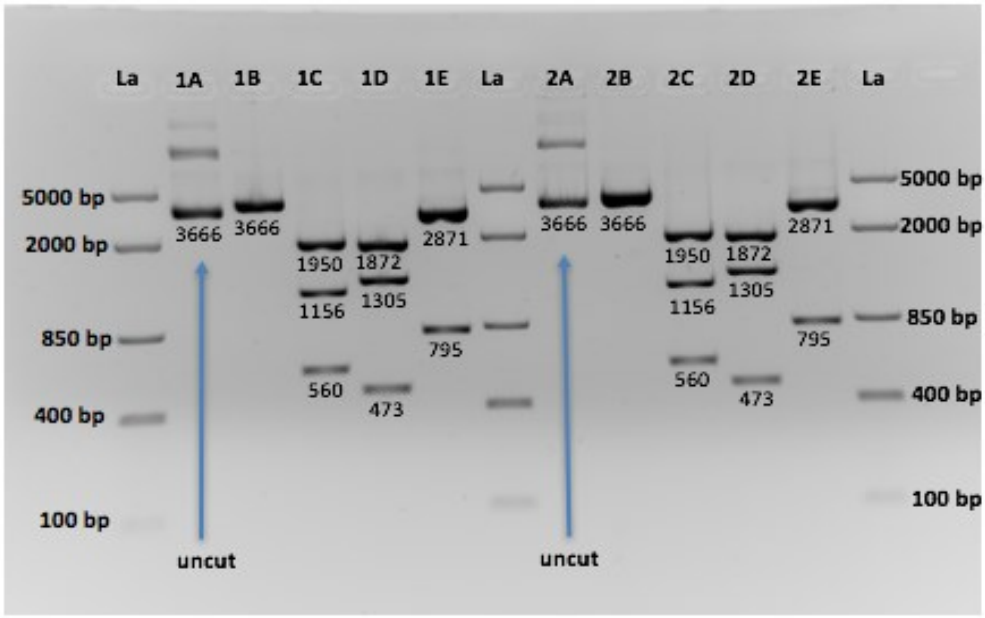


Figure 17: Image of the gel from gel electrophoresis of pCpG-hCMV-SCEP-TNFα after DRD. 1A – 1E show expanded and purified plasmid. 2A – 2E represent fragments of source plasmid that was used for transformation. 1A and 2A: uncut plasmid migrated faster than linearized in position 1B and 2B, because it is the supercoiled form; faint band in 1A and 2A that migrated slower than supercoiled form represents nicked DNA. 1D and 2D: fourth band consisting of 16 bp could not be shown, because size was too small. The digits represent the calculated number of bp, expected after correct digest. La = DNA-ladder.

The gel electrophoreses showed the expected patterns of bands, thereby validating the identity of the two plasmids.

3.3. Optimization of Cell Based Assays for TNFα Expression and Quantification

3.3.1. In Vitro Transfection mediated Expression of Bioactive TNFα and L929 Cytotoxicity Based TNFα Assay

Cytotoxicity and thus the bioactivity of secreted TNFα were measured to validate the transfection efficiency of TNFα plasmid based LPEI nanoparticles in comparison to Lipofectamine (LF). A549 cells were transfected with pCpG-hCMV-EF1α-TNFα (pEF1α-TNFα) and pCpG-hCMV-SCEP-TNFα (pSCEP-TNFα) after complexation with LPEI (10 kDa, N/P = 6) in HBS, for 4 h in basal medium. After this duration, the basal medium was removed and replaced by 200 µl full cell culture medium and cells were left undisturbed for 20h. After this, 100 µl of the total 200 µl supernatant was transferred

to the already attached L929 cells for the performance of the L929 bioassay to investigate the cytotoxic effect of TNFα, secreted by cells transfected with 100 ng of the plasmid (Fig. 18) as well as with 200 ng (Fig. 19). The measured cytotoxic effects were almost the same for the transfection with 100 ng and 200 ng plasmid (Fig. 18, Fig. 19). Results for the transfection with pEF1α-TNFα and pSCEP-TNFα always achieved a cytotoxic effect in the range of minimum 88,2% (except for one measurement with LPEI SCEP-TNFα, 100 ng, Fig. 18) and maximum 93.5%, for both LPEI and LF. This illustrates the high transfection efficiency of LPEI, that is in the same range like LF, and the high bioactivity of secreted TNFα (Fig. 18, Fig. 19). The negative controls pEF1α-LucSH and Lucia showed a much lower cytotoxic effect compared to the TNFα-plasmids, like expected (Fig. 18, Fig. 19). For the transfection with LPEI pEF1α-TNFα an averaged cytotoxic effect of 91.9% for 100 ng and 91.5% for 200 ng was measured. For LPEI pSCEP-TNFα the averaged cytotoxic effect of 74.6 % for 100 ng and 91.3% for 200 ng plasmid was achieved (Fig. 20). The cytotoxicity reached with LPEI pEF1α-TNFα was 91.9% and was only marginally higher than the 91.0% cytotoxic effect of the pEF1α-TNFα complexed with LF. The negative control with pCpG-hCMV-EF1α-LucSH (pEF1α-LucSH) was chosen because of the same enhancer and promoter like pEF1α-TNFα. pEF1α-LucSH showed a minor cytotoxic effect, whereby the cytotoxicity of the plasmid complexed with LPEI was lower for 100 and 200 ng plasmid in comparison to the same plasmid complexed with LF. The pCpG free promoter Lucia was also utilized as a negative control, because in contrast to pEF1α-LucSH, Luciferase is secreted by transfected cells. LPEI Lucia also showed a slight cytotoxic effect, that was lower than the effect measured for LF Lucia. The cytotoxic effect of pEF1α-TNFα and pSCEP-TNFα was nevertheless clearly distinguishable from the effect of the negative controls (Fig. 18 and 19).

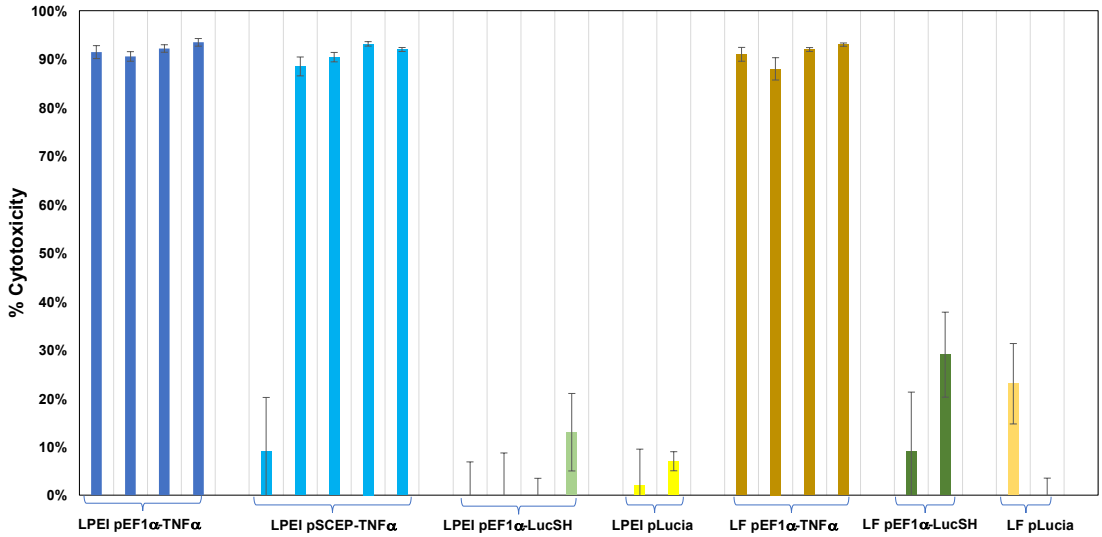


Figure 18: In vitro transfection (100 ng plasmid) mediated expression of secreted TNFα and estimation of its bioactivity in terms of TNFα induced L929 cytotoxicity: Percentage cytotoxic effect of 100 µl supernatant from the A549 cells transfected with following NP formulations (loaded with 100 ng plasmid): LPEI pEF1α-TNFα, LPEI pSCEP-TNFα, LPEI pEF1α-LucSH, LPEI pLucia, LF pEF1α-TNFα, LF pEF1α-LucSH, LF pLucia. 100 µl supernatant (containing the secreted TNFα) were added to L929 cells and cytotoxicity measured via MTT assay. LPEI and LF stand for linear polyethylenimine (N/P=6) and lipofectamine based NPs respectively, with LF used as positive control. As negative controls, pEF1α-LucSH and pLucia were complexed with LPEI and LF. pEF1α-TNFα and pSCEP-TNFα both have the cDNA coding for TNFα, leading to a secretion of TNFα by transfected A549 cells. pEF1α-LucSH is the negative control, carrying the same enhancer and promoter like pEF1α-TNFα but without the cDNA for TNFα. Data from 6 independent experiments with different compositions of tested plasmids; each MTT assay was performed in triplicates, mean is shown, ± SD.

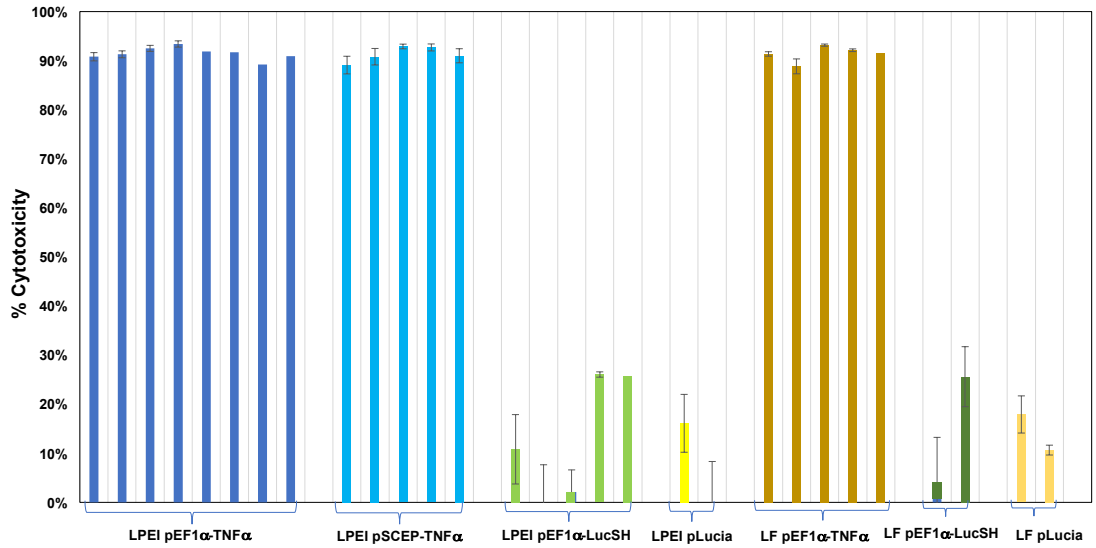


Figure 19: In vitro transfection (200 ng plasmid) mediated expression of secreted TNFα and estimation of its bioactivity in terms of TNFα induced L929 cytotoxcity: Percentage cytotoxic effect of 100 µl supernatant from the A549 cells transfected with following NP formulations (loaded with 200 ng plasmid): LPEI pEF1α-TNFα, LPEI pSCEP-TNFα, LPEI pEF1α-LucSH, LPEI pLucia, LF pEF1α-TNFα, LF pEF1α-LucSH, LF pLucia. 100ul supernatant (containing the secreted TNFα) were added to L929 cells and cytotoxicity measured via MTT assay. LPEI and LF stand for linear polyethylenimine (N/P=6) and lipofectamine based NPs respectively, with LF used as positive control. As negative controls, pEF1α-LucSH and pLucia were complexed with LPEI and LF. pEF1α-TNFα and pSCEP-TNFα both have the cDNA coding for TNFα, leading to a secretion of TNFα by transfected A549 cells. pEF1α-LucSH is the negative control, carrying the same enhancer and promoter like pEF1α-TNFα but without the cDNA for TNFα. Data from 6 independent experiments with different compositions of tested plasmids; each MTT assay was performed in triplicates, except of 4 MTT assays of LPEI pEF1α-TNFα with the results of 91.9 %, 91.7 %, 89.2 % and 90.9 % and one result of LF pEF1α-TNFα of 91.6%, each of them was performed in a single well; mean is shown, ± SD.

The averaged values of the cytotoxic effect shown in Figure 20 illustrate that the positive control LF pEF1α-TNFα reached an average of 91.0 % and 91.4 % for the transfection with 100 and 200 ng, and therefore had almost the same effect like the transfection agent LPEI with 91.9 % for 100 ng pEF1α-TNFα and 91.5 % for 200 ng pEF1α-TNFα. For LPEI as well as for LF there were almost no differences in the measured cytotoxicity referred to the transfection with 100 or 200 ng pEF1α-TNFα. For LPEI pSCEP-TNFα the results showed a higher effect for the transfection with 200 ng plasmid compared to the results using 100 ng (Fig. 20). The cytotoxic effect of the negative control with pEF1α-LucSH was in general much lower than the effect of pEF1α-TNFα and pSCEP-TNFα, but nevertheless there was a measurable cytotoxicity,

that was higher for transfection with LF compared to LPEI (Fig. 20). For the second negative control Lucia, a plasmid that causes secretion of luciferase, the cytotoxic effect was much lower than the effect of pEF1α-TNFα and pSCEP-TNFα. The measured cytotoxicity of 2.3 – 6.7% for 100 ng LPEI Lucia and 0 – 16.1% for 200 ng LPEI Lucia was lower than the effect of LF with 0 – 23.3% for 100 ng LF-Lucia and 10.6 – 17.9% for 200 ng LF-Lucia (Fig. 18 and 19).

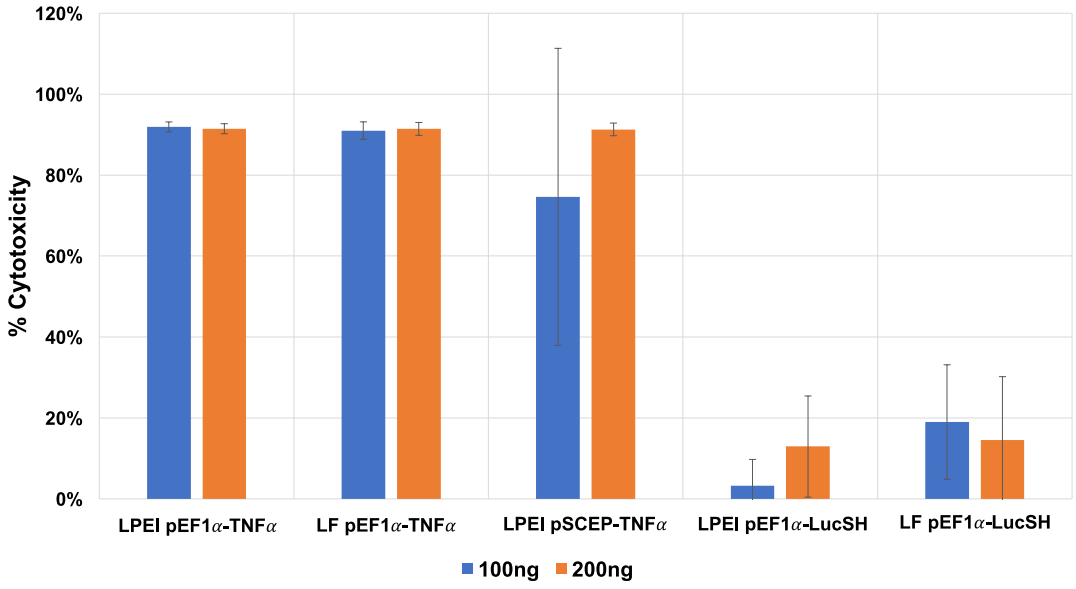


Figure 20: Comparison of TNFα induced L929 cytotoxcity between NP formulations loaded with 100 ng and 200 ng plasmid: Averaged percentage cytotoxic effect of 100 µl supernatant from the A549 cells transfected with following NP formulations (loaded with 100 ng/200 ng plasmid): LPEI pEF1α-TNFα, LPEI pSCEP-TNFα, LPEI pEF1α-LucSH, LF pEF1α-TNFα, and LF pEF1α-LucSH. 100 µl supernatant (containing the secreted TNFα) were added to L929 cells and cytotoxicity measured via MTT assay. LPEI and LF stand for linear polyethylenimine (N/P=6) and lipofectamine based NPs respectively, with LF used as positive control. As negative controls, pEF1α-LucSH and pLucia were complexed with LPEI and LF. pEF1α-TNFα and pSCEP-TNFα both have the cDNA coding for TNFα, leading to a secretion of TNFα by transfected A549 cells. pEF1α-LucSH is the negative control, carrying the same enhancer and promoter like pEF1α-TNFα but without the cDNA for TNFα. Data from independent experiments; each MTT assay was performed in triplicates, mean is shown, ± SD. Number of experiments (n): $n_{(LPEI\ pEF1\alpha\text{-}TNF\alpha,\ 100\ ng)} = 4$, $n_{(LPEI\ pEF1\alpha\text{-}TNF\alpha,\ 200\ ng)} = 8$, $n_{(LF\ pEF1\alpha\text{-}TNF\alpha,\ 100\ ng)} = 4$, $n_{(LF\ pEF1\alpha\text{-}TNF\alpha,\ 200\ ng)} = 5$, $n_{(LPEI\ pSCEP\text{-}TNF\alpha,\ 100\ ng)} = 5$, $n_{(LPEI\ pSCEP\text{-}TNF\alpha,\ 200\ ng)} = 5$, $n_{(LPEI\ pEF1\alpha\text{-}LucSH,\ 100\ ng)} = 4$, $n_{(LPEI\ pEF1\alpha\text{-}LucSH,\ 200\ ng)} = 5$, $n_{(LF\ pEF1\alpha\text{-}LucSH,\ 100\ ng)} = 2$, $n_{(LF\ pEF1\alpha\text{-}LucSH,\ 200\ ng)} = 2$.

For investigating the dose response relationship between different amounts of secretd TNFα and percentage cytotoxicity, 100 µl supernatant of transfected A549 cells was diluted in different amounts to investigate the profile of cytotoxicity as a function of decrease in TNFα amounts (Fig. 21). Like shown in Figure 21, cytotoxic effect declines significantly from 91.3% for 10 µl to 77.1% for 1 µl of supernatant from A549 cells transfected with 200 ng of LPEI pEF1α-TNFα, and declines to a cytotoxic effect of 11.2% for 0.001 µl supernatant. Data demonstrate that the toxic impact on L929 cells is almost not varying within the range of 100-10 µl supernatant. That indicates, that the MTT assay is not sensitive enough in this range (Fig. 21). However, there is a dose response relationship between different volumes of supernatant (corresponding to different amounts of TNFα) used for L929 assay and percentage cytotoxicity.

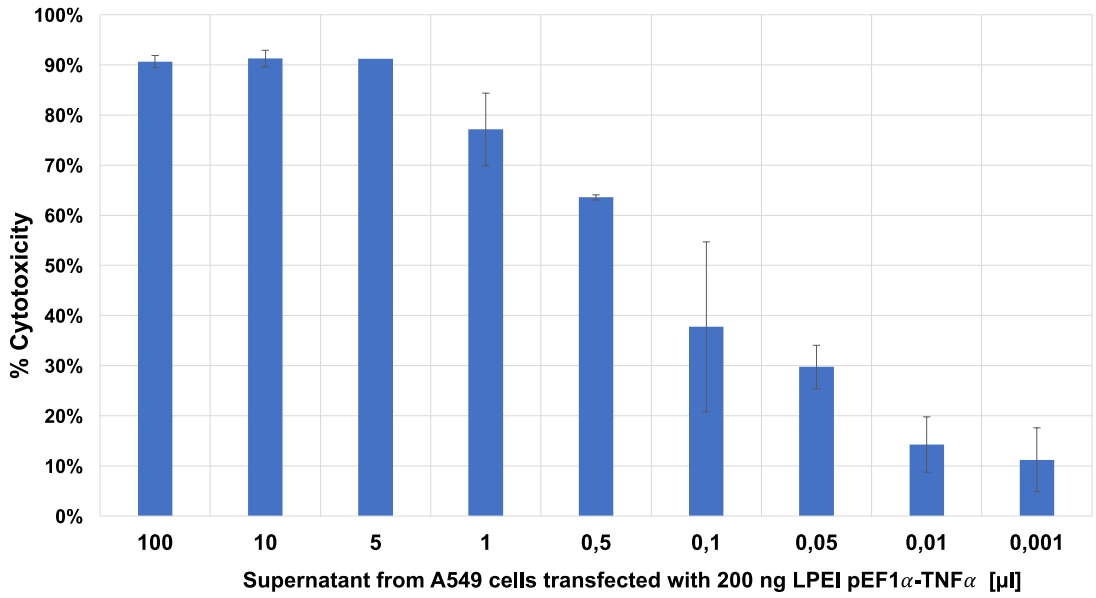


Figure 21: Dose response relationship between different amounts of secreted TNFα and percentage cytotoxicity. Cytotoxic effect of different amounts of TNFα on L929 cells measured with MTT assay. Different volumes of supernatant from A549 cells (transfected with 200 ng LPEI pCpG-hCMV-EF1α-TNFα) containing different amounts of TNFα were transferred to L929 cells and the cytotoxic effect was tested with MTT assay. The supernatant was diluted and transferred to the L929 cells. Number of experiments (n): $n_{(100\ µl)} = 5$, $n_{(10\ µl)} = 9$, $n_{(5\ µl)} = 1$, $n_{(1\ µl)} = 8$, $n_{(0.5\ µl)} = 2$, $n_{(0.1\ µl)} = 8$, $n_{(0.05\ µl)} = 2$, $n_{(0.01\ µl)} = 8$, $n_{(0.001\ µl)} = 8$. Data of independent experiments; MTT assay was performed in triplicates, mean cytotoxic effect is shown, ± SD.

Further, effect of freezing of supernatant (having the TNFα) from A549 cell transfections on L929 cytotoxicity was investigated. Figure 22 demonstrates the results of the comparison between the cytotoxicity of fresh supernatant directly taken from A549 cells and the same supernatant measured after an additional freezing period of 6-12 days at –80 °C (Fig. 22). 10 µl frozen supernatant show the same effect like 10 µl of fresh supernatant. The cytotoxicity of 1 µl frozen supernatant decreases faster, and for 0.01 µl and 0.001 µl there is no quantifiable impact of frozen supernatant anymore. This indicates that freezing might have a negative effect on the bioactivity of TNFα (Fig. 22).

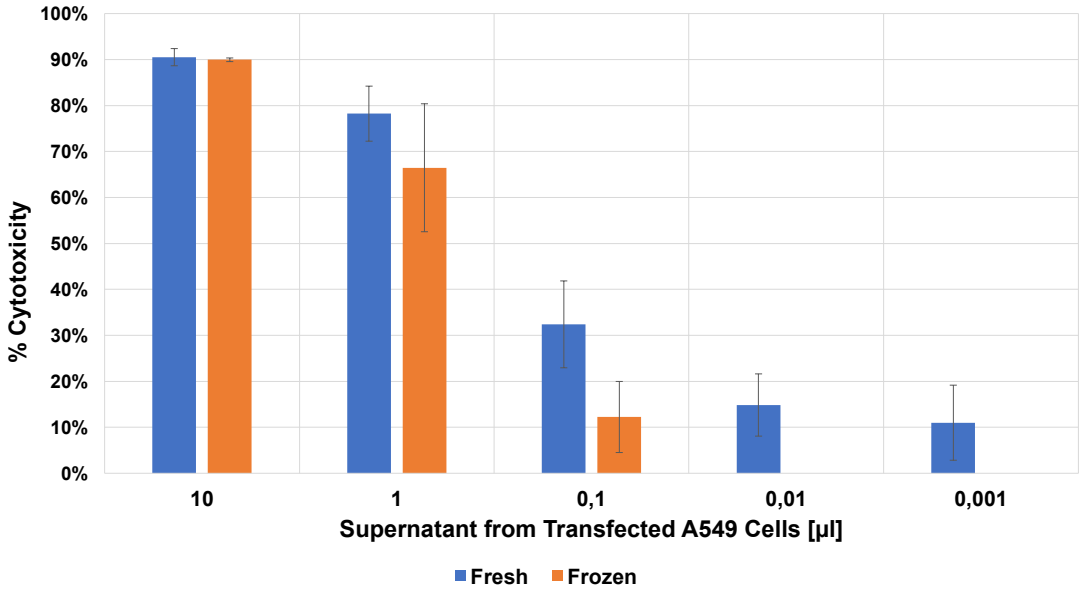


Figure 22: Effect of freezing the supernatant (having the TNFα from A549 cell transfections) on L929 cytotoxicity as measured with MTT assay. Comparison between cytotoxic effect of dilutions of supernatant from A549 cells transfected with 200 ng LPEI pCpG-hCMV-EF1α-TNFα (N/P = 6), containing the secreted TNFα, before and after freezing at -80 °C for 6 to 12 days. The assays firstly were processed with the fresh supernatant and after the mentioned duration, repeated in the same way with the frozen supernatant. Number of experiments (n): n = 4. The MTT assay was performed in triplicates, mean cytotoxic effect is shown, ± SD.

3.3.2. Quantification of TNFα Transgene Expression: L929 Cytotoxicity based TNFα Assay with Recombinant Murine TNFα

Serial dilutions of recombinant murine TNFα illustrate the correlation between the amount of rTNFα and the cytotoxic effect on L929 cells treated with Actinomycin D

(Fig. 23). For 10 ng and 1 ng rTNFα the cytotoxic effect is almost the same (Fig. 23). Cytotoxicity starts to decrease for 0.1 ng rTNFα with a measured value of 90.72% down to 1.87% for 0.00025 ng rTNFα. Median effective dose (ED_{50}) of rTNFα amounts to 0.0036 ng (Fig. 23). The linear range from 0.05-0.00025 ng rTNFα (Fig. 23) was used to create a regression line with R^2 = 0.98807, defined by the formula y = 0.1716ln(x) + 1.4679 like shown in Figure 24.

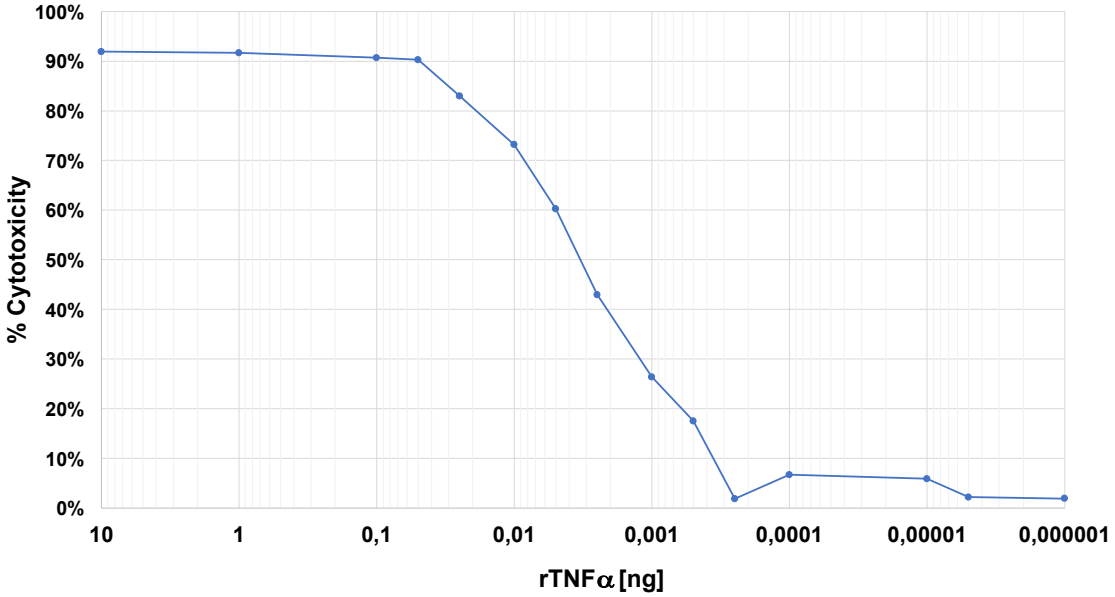


Figure 23: Dose response relationship between different amounts of recombinant TNFα and percentage cytotoxicity. Cytotoxic effect of rTNFα ascertained with serial dilutions added to L929 cells (pre-treated with Actinomycin D) and measured by MTT assay. rTNFα was diluted with full cell culture medium and added to the cells in a total of 100 µl. ED_{50} = 0.0036 ng. MTT assay was performed in triplicates and the mean cytotoxic effect is shown.

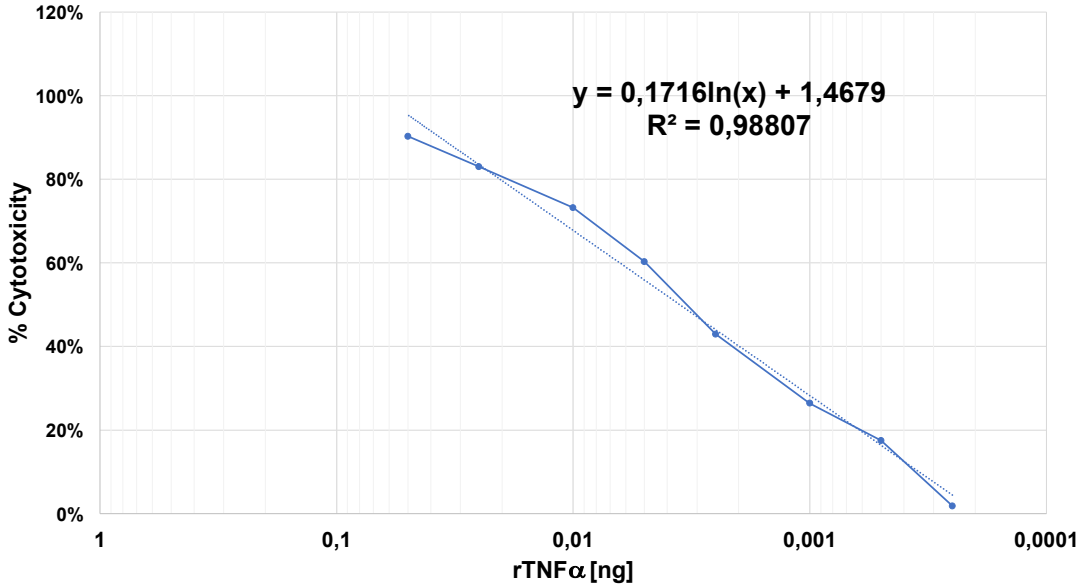


Figure 24: Dose response relationship between different amounts of recombinant TNFα and percentage cytotoxicity: Linear range of the cytotoxic effect of rTNFα from Figure 23. Cytotoxic effect of rTNFα ascertained with serial dilutions added to L929 cells (pre-treated with Actinomycin D) and measured by MTT assay. The regression line is described by the formula y = 0.1716ln(x) + 1.4679. R^2 = 0.98807. ED_{50} = 0,00355 ng. Number of experiments (n): $n_{(0.05\ ng)}$ = 6, $n_{(0.025\ ng)}$ = 4, $n_{(0.01\ ng)}$ = 8, $n_{(0.005\ ng)}$ = 8, $n_{(0.0025\ ng)}$ = 4, $n_{(0.001\ ng)}$ = 10, $n_{(0.0005\ ng)}$ = 5, $n_{(0.00025\ ng)}$ = 2. MTT assay was performed in triplicates, the mean cytotoxic effect is shown.

The regression line (Fig. 24) was later used for the calculation of unknown amount of secreted TNFα by A549 cells transfected with 200 ng LPEI pEF1α-TNFα, via the cytotoxicity and the formula: y = 0.1716ln(x) + 1.4679 (Fig. 25).

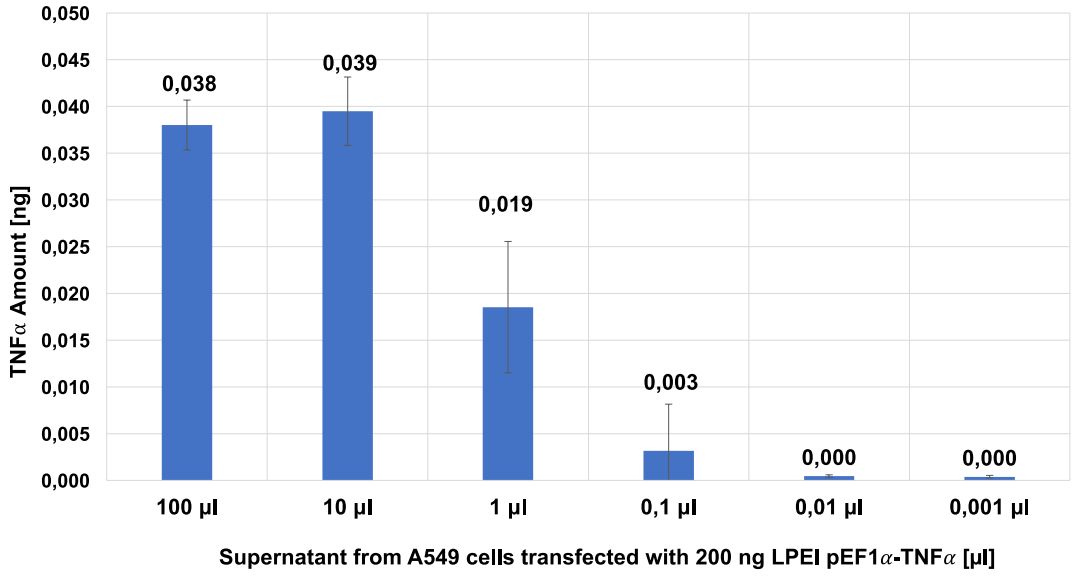


Figure 25: Calculated amount of secreted TNFα in ng for different volumes of supernatant from A549 cells (that were transfected with 200 ng LPEI pCpG-hCMV-EF1α-TNFα (N/P = 6)). The cytotoxicity measured with MTT assay for different amounts of supernatant from the transfected A549 cells was used for the calculation using the regression line of the linear range of the rTNFα (Fig. 24). The known cytotoxicity values were used for the calculation of the unknown amount of secreted TNFα. The cytotoxic effect on L929 cells (pre-treated with Actinomycin D) was measured by MTT assay. The Calculated TNFα amounts are: $TNF\alpha_{(100\ \mu l)}$ = 0.038 ng, $TNF\alpha_{(10\ \mu l)}$ = 0.039 ng, $TNF\alpha_{(1\ \mu l)}$ = 0,019 ng, $TNF\alpha_{(0,1\ \mu l)}$ = 0,003 ng. Number of experiments (n): $n_{(100\ \mu l)}$ = 5, $n_{(10\ \mu l)}$ = 9, $n_{(1\ \mu l)}$ = 8, $n_{(0.1\ \mu l)}$ = 8, $n_{(0,01\ \mu l)}$ = 8, $n_{(0.001\ \mu l)}$ = 8. The mean of the independent experiments is shown, ± SD.

3.3.3. Comparison of TNFα Transgene Expression Kinetics In Vitro as a Function of Promoter Type

The L929-MTT assay was as well used for profiling TNFα transgene expression kinetics in vitro as a function of two different promoters during a duration of 4 days after transfection with pCpG-hCMV-EF1α-TNFα and pCpG-hCMV-SCEP-TNFα complexed with LPEI (N/P = 6). A549 cells were transfected with 1000 ng of each plasmid in a total volume of 500 µl full RPMI medium in each well. Supernatant was collected for four days and stored at –80 °C after a short centrifugation, to separate the supernatant from dead cells and cell debris. After these four days, collected supernatants were thawed, diluted like described in 2.4.3. and added to L929 cells treated with Actinomycin D. Cytotoxic effect was measured with the MTT assay. The amount of TNFα contained in the volume of supernatant from A549 cells was calculated with the

formula shown in Figure 24, for the regression line of the cytotoxic effect, measured for the known amount of rTNFα. pEF1α-TNFα had a secretion peak on day 2 after the transfection (Fig.26). This was also observed for the assay used for profiling the TNFα transgene expression kinetics performed with pSCEP-TNFα (Fig. 27). The secreted amount of TNFα on day 2 was higher for transfection with pEF1α-TNFα with 0.0232 ng TNFα calculated for 0.25 µl of supernatant, compared to the secretion of A549 cells transfected with pSCEP-TNFα with 0.0226 ng TNFα in 0.25 µl supernatant (Fig. 28).

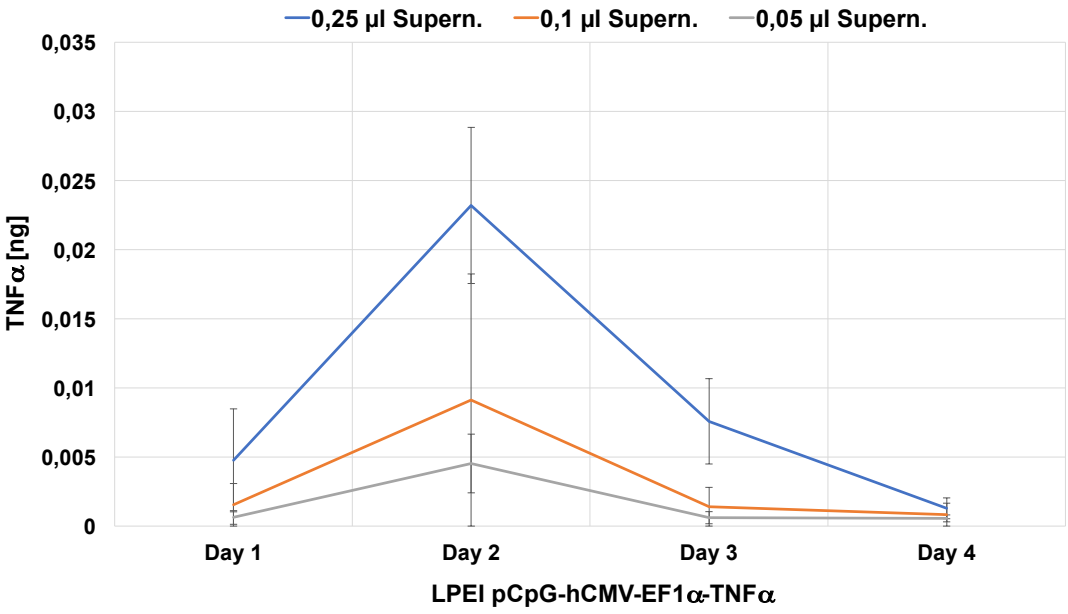


Figure 26: In vitro TNFα transgene expression kinetics after transfection with pCpG-hCMV-EF1α-TNFα. A549 cells were transfected with 1000 ng pEF1α-TNFα complexed with LPEI (N/P = 6) and supernatant collected daily over a duration of 4 days. Different volumes of the supernatant (0.25 µl, 0.1 µl, 0.05 µl) of the transfected A549 cells were transferred to the L929 cells and the cytotoxic effect was measured with MTT assay. TNFα amount was calculated with the formula describing the regression line for the recombinant TNFα (Fig. 24). The assay used for profiling the TNFα transgene expression kinetics took place over a duration of four days; the supernatant was removed every single day, stored at –80 °C and replaced by fresh full RPMI medium. The secretion has a peak on day 2. Number of experiments (n): $n_{(Day1-4,\ 0.25\ µl)}$ = 4, $n_{(Day\ 1-4,\ 0.1\ µl)}$ = 5, $n_{(Day\ 1-4,\ 0.05\ µl)}$ = 5. The mean amount of TNFα is shown, ± SD. Supern. = Supernatant.

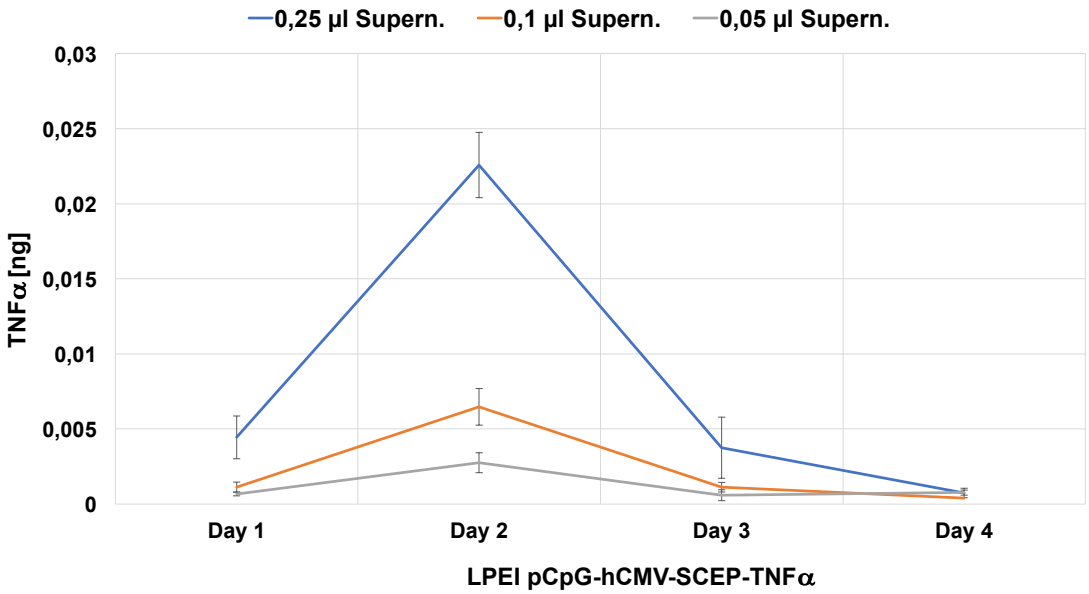


Figure 27: In vitro TNFα transgene expression kinetics after transfection with pCpG-hCMV-SCEP-TNFα. A549 cells were transfected with 1000 ng pSCEP-TNFα complexed with LPEI (N/P = 6) and supernatant collected daily over a duration of 4 days. Different volumes supernatant (0.25 µl, 0.1 µl, 0.05 µl) of transfected A549 cells were transferred to the L929 cells and the cytotoxic effect was measured with MTT assay. TNFα amount was calculated with the formula describing the regression line for the recombinant TNFα (Fig. 24). The assay used for profiling the TNFα transgene expression kinetic took place over a duration of four days; the supernatant was removed every single day, stored at –80 °C and replaced by fresh full RPMI medium. The secretion has a peak on day 2. Number of experiments (n): $n_{(Day\ 1\text{-}4,\ 0,25\ µl)}$ = 4, $n_{(Day\ 1,\ 0,1\ and\ 0.05\ µl)}$ = 5, $n_{(Day\ 2\ and\ 3,\ 0,1\ and\ 0.05\ µl)}$ = 4, $n_{(Day\ 4,\ 0,1\ and\ 0.05\ µl)}$ = 2. The mean amount of TNFα is shown, ± SD. Supern. =Supernatant.

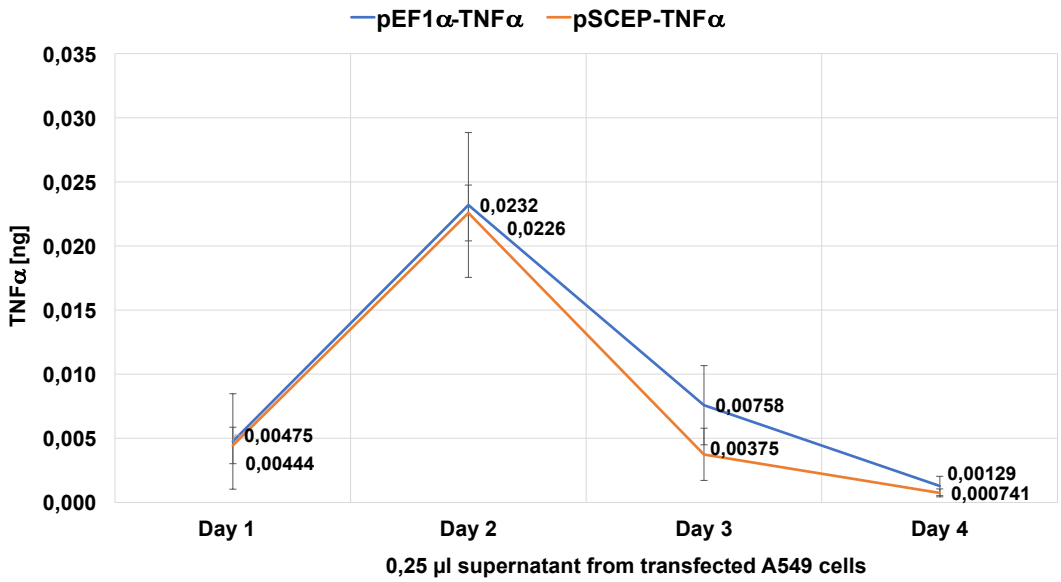


Figure 28: Comparison of TNFα transgene expression kinetics as a function of promoter type. Comparison between the amount of TNFα, contained in 0.25 µl supernatant, secreted by A549 cells transfected with 1000 ng pCpG-hCMV-EF1α-TNFα and pCpG-hCMV-SCEP-TNFα complexed with LPEI (N/P = 6) over a duration of 4 days. The A549 cells were transfected, the supernatant transferred to the L929 cells and the cytotoxic effect was measured with MTT assay. 0.25 µl collected supernatant of each day were transferred to the L929 cells treated with Actinomycin D. The amount was calculated with the formula describing the regression line for rTNFα (Fig. 24). The mean amount of TNFα is shown, ± SD.

4. DISCUSSION

4.1. MTT ASSAY OPTIMIZATION

The MTT assay is an often-used colorimetric assay for determination of cell viability or by implication cytotoxic effect caused by tested compounds. Main problem is that different factors affect the results, what makes it necessary to optimize the MTT assay for given conditions of specific experimental setups. One factor is the tested compound and the mechanism of its cytotoxic effect. Cytotoxic agents that change the metabolic activity of the cells can lead to false results, like for example sodium trinitride or chloroquine, that lead to a higher cell viability tested with the MTT assay compared to results of the ATP assay or neutral red (Weyermann et al., 2005). Another example is doxorubicin that leads to an increase of the amount of mitochondria in MTLn3 cells, what leads to an increased measured cell viability using the MTT assay (Kluza et al., 2004). Also the usage of liposomes can cause a higher cell viability measured with the MTT assay compared to the cell count combined with a Trypan Blue dye (Angius and Floris, 2015). But besides that, this study was about optimizing the basic protocol of the MTT assay firstly without including a test compound, and therefore other factors were initially more important, like the cell line, the ingredients of the cell medium like pH indicator phenol red and proteins of FBS. Phenol red in normal pH of a cell culture can interfere with the absorbance measurement of formazan, what can be avoided by shifting the pH with HCl for example (Mosmann, 1983). FBS contains proteins, that can precipitate with solvents like isopropanol and disturb the measurement with the occurring turbidity. This can be evaded by removing as much of the cell culture medium as possible, without disturbing the attached cells and the formazan crystals, before adding the solvent. Accordingly, also the incubation time for the treatment with the MTT working solution and the remaining cell culture medium in the wells before adding the solvent are important coefficients, as well as the solvent itself (Twentyman and Luscombe, 1987).

The focus of this optimization was on analyzing the change in linearity of the correlation between a known range of cell numbers and the measured absorbance using different solvents. Other factors were also analyzed, like the impact of different

volumes of remaining medium in the wells containing phenol red and FBS. First, a protocol based on the insights of Supino R. and Chiba K. et al. was tested for the A549 cells (Chiba et al., 1998; Supino, 1995). Afterwards different variations were executed and evaluated. The best linearity was achieved with variation 2 (Section 2.3.3.), in which the seeding medium of the A549 cells containing phenol red and FBS was not removed before adding the MTT working solution in PBS with a concentration of 5 mg/ml, in order not to disturb the attached cells. The concentration of MTT was 0.45 mg/ml in each well and after 4 h incubation, whole supernatant was removed, except for 30 µl, so that cells and formazan crystals were not disturbed. DMSO was applied as a solvent and the absorbance was measured at 570 nm directly after adding, resuspending and checking the resolving of the formazan crystals under the microscope. The modification with the second-best linearity was variation 5: the seeding medium was removed after overnight incubation and the MTT working solution in basal medium was added up to a concentration of 0.4 mg/ml in each well. After 4 h incubation time the supernatant was likewise removed except for 5 to 30 µl and afterwards DMSO was applied. Following these results, the most effective solvent tested was DMSO in combination with a MTT concentration of 0.4-0.45 mg/ml in each well and a remaining volume of cell medium before adding the solvent of 30 µl. As a blank for the calculation of the corrected absorbance without the background absorbance, full cell medium treated the same way like cells, was used for both variations. This conclusions match to other results of studies in this issue-area (Twentyman and Luscombe, 1987). Nevertheless, variation 2 has a higher R^2 value, thus is more precise and because of the missing aspiration step after the overnight incubation of the cells, it is also more feasible and less time-consuming. Another factor saving time during the assay is, that there is no need to dilute the MTT working solution with a concentration of 5 mg/ml in PBS. On the other hand, variation 5 could be more suitable for experiments, testing compounds that interact with the MTT solution, like strong reducing ones, and therefore need to be removed before adding the MTT working solution. This should be investigated before starting the specific experiment.

One limitation of this study design is that the optimization was only tested for A549 cells in the range of 2.5 x 10^3 – 4 x 10^4 cells and is consequently restricted in its validity.

A possible solution for the disturbance of phenol red could also be the usage of phenol red free medium (Denizot and Lang, 1986) or measuring an additional reference wavelength to calculate the correct absorbance (Mueller, 2004).

4.2. L929 CYTOTOXICITY BASED TNFα ASSAY – L929-MTT ASSAY

The aim of this part of the study was firstly the expansion of plasmids containing the cDNA coding for the cytokine TNFα, and following the transfection of A549 cells with these plasmids via LPEI nanoparticles for establishing the L929-MTT assay. Success of the transfection was confirmed with the cytotoxicity assays performed on the L929 cells, sensitized with Actinomycin D. The amount of secreted TNFα was determined via the comparison of the cytotoxic effect of an unknown amount secreted by the transfected A549 cells, with the same cytotoxic effect of a known amount of recombinant murine TNFα. Finally, an assay used for profiling the TNFα transgene expression kinetic was performed to analyze the TNFα secretion behavior over a duration of 4 days of transfected A549 cells.

The cytotoxic effect and therefore the performance of the transfection of the A549 cells using the transfection reagent LPEI was analyzed with the L929 bioassay based on the protocol of Su et al. (2012). The MTT assay was performed based on the optimization executed before and described as variation 2 in the subitem 2.3.3. in materials and methods with the difference that it was performed on L929 cells and not on the A549 cells and that the absorbance was not only measured at 570 nm but also at 630 nm, as a reference absorbance for the calculation of the cytotoxic effect. In this study, firstly the cytotoxicity of different dilutions of the supernatant from the transfected A549 cells, containing the unknown amount of secreted TNFα of a duration of 20-22.5 h was analyzed. Therefore, the transfection protocol is different to the one described by Su et al. (2012). The A549 cells were seeded in a cell number of 10^4 cells per well in a 96-well plate and incubated overnight. The following day the

pDNA was complexed with LPEI in HBS (N/P ratio = 6) and the transfection took place with 100 and 200 ng of the plasmids EF1α-TNFα, SCEP-TNFα as well as EF1α-LucSH as a negative control. The transfection was performed in basal medium for 4 h, afterwards the complete supernatant was removed and replaced by 200 µl of full medium containing 10% FBS and L-Glutamine. The transfected A549 cells were incubated for 20-22.5 h. For the L929 bioassay 1.25 x 10^4 cells per well were seeded and after an overnight incubation, different dilutions of the supernatant from the transfected A549 cells were added, as well as the Actinomycin D up to a concentration of 0.1 µg/100 µl. After 20-24 h the supernatant of the L929 cells was removed and the MTT assay was performed. MTT in PBS stock solution was added up to a concentration of 0.45 mg/ml without removing supernatant. After 4 h incubation time the supernatant was removed, except for 30 µl, and as a solvent 200 µl DMSO were added. The absorbance was measured at 570 nm with a reference absorbance at 630 nm.

Additionally, the behavior of the TNFα secretion of the transfected A549 cells was analyzed over the duration of 4 days. For the transfection 7 x 10^4 A549 cells per well were seeded in a 24 well plate, in a total of 500 µl. 1000 ng of the plasmids pEF1α-TNFα, pSCEP-TNFα and as a negative control pEF1α-LucSH were complexed for each well with LPEI with the N/P ratio of 6. The transfection was performed in basal medium for 4 h, afterwards the medium was removed and replaced by 500 µl full medium. After an overnight incubation, the supernatant was collected and replaced by fresh medium. The collected supernatant of 4 days was stored at −80°C for the following L929 bioassay, that was also performed like described by Su et al. (2012).

There exist also some variations between this study and the protocol of Su et al. concerning the transfection. In this study LPEI 10 kDa was used for the condensation of the pDNA for the transfection in basal medium, whereas in the study of Su et al. G3-HD-OEI was applied for the transfection in full medium containing FBS. The main gap between this study and the one performed by Su et al. is however the application of the MTT assay in this study, because it was used for the calculation of TNFα amount secreted by transfected A549 cells through the comparison between the cytotoxic

effect of the recombinant murine TNFα and the secreted TNFα. In the other study, MTT assay was merely used for validation of cytotoxic effect in general and thus transfection efficiency. For the determination of the exact amount of secreted TNFα an ELISA assay was applied. This represents a drawback of this study, because there was no second experiment performed for the determination of the TNFα amount, like an ELISA. Also, no additional cell viability assay was performed, to see whether the results of the MTT assay showed an overestimation or underestimation of the cytotoxic effect. One possibility could have been the validation using propidium iodide (Trost and Lemasters, 1994). In the study of Trost et al. PI was added to the cells and the fluorescent was measured in the beginning, every 2-6 h after adding the recombinant human TNFα and in the end, after the cells were totally lysed. With this results the percental shift of cell viability during a duration of 72h after adding the TNFα was calculated. Trost et al. also described the increase of sensitivity of the L929 cells towards the cytotoxic effect of TNFα, after the treatment with Actinomycin D.

During the profiling of the TNFα transgene expression kinetic in this study it was ascertained, that the peak of the TNFα secretion appears on the second day after the transfection like it is shown in the study of Su et al.. One point to be considered in this assay is that the supernatant containing the TNFα was frozen at −80 °C and like already described, this freezing procedure might have a negative impact on the bioactivity of TNFα.

Future studies aim at verification of the calculated amounts of TNFα with the help of a more precise method that measures the amount of TNFα directly, like e.g. an ELISA, or at least the comparison with another cell viability assay, investigating a different factor than the metabolic activity of the tested cells.

4.3. CONCLUSION

In conclusion, the MTT assay was successfully optimized and it was shown that with this assay reproducible results can be achieved. Nevertheless, an adaption to specific experimental setups needs to be performed before. The TNFα transfection using LPEI based nanoparticles showed good results for gene delivery of pCpG-hCMV-EF1α-TNFα

and pCpG-hCMV-SCEP-TNFα. With the optimized L929-MTT assay it was possible to investigate the cytotoxic effect of the secreted TNFα on L929 cells, sensitized with Actinomycin D. By measuring the cytotoxicity of known amounts of recombinant murine TNFα, it was feasible to calculate the amount of the secreted TNFα from the transfected A549 cells. Furthermore, the TNFα transgene expression kinetics by transfected A549 cells over a duration of 4 days could be determined using the L929-MTT assay. It was demonstrated, that both promoters had an expression peak of TNFα on the second day after transfection and showed similar expression kinetics.

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