Identification of Fundamental Gene Regulatory Networks and Signaling Pathways in Cytokine-Induced Senescence

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"Not everything that is faced can be changed,
but nothing can be changed until it is faced."

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James A. Baldwin (1924 – 1987)

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%	Percent
°C	Degree Celsius
[^{3}H]	Hydrogen-3 (*Tritium*)
A	Ampere
AGO2	Argonaute-2 protein
AKT or PKB	Protein kinase B
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ANT2	Adenine nucleotide translocase 2
APC	Antigen-presenting cell
APS	Ammonium persulfate
AP-1	Activator protein 1
ATM	Ataxia telangiectasia mutated
AUF1	AU-rich binding factor 1
BCA	Bicinchoninic acid
Bcl-2	B cell lymphoma 2
BCR	B cell receptor
BRAF	V-raf murine sarcoma viral oncogene homolog B
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
cGAS	Cyclic GMP-AMP synthase
c-IAP	Cellular inhibitor of apoptosis protein
Cip1	CDK-interacting protein 1
CIS	Cytokine-induced senescence
CSNK1A1	Casein kinase 1 alpha 1
CO_2	Carbon dioxide
cpm	Counts per minute
CTLA4	Cytotoxic T-lymphocyte-associated protein 4

Ctrl	Control
CXCL	C-X-C motif chemokine ligand
ddH_2O	Double distilled water
DDR	DNA damage response
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-SCARS	DNA segments with chromatin alterations reinforcing senescence
DNase	Deoxyribonuclease
DNMT	DNA methyltransferase
DTX	Docetaxel
E2F	E2F transcription factor
EDTA	Ethylenediaminetetraacetic acid
e.g.	For example (*exempli gratia*)
EGFR	Epidermal growth factor receptor
et al.	And others (*et alii*)
EtOH	Ethanol
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FDA	Food and Drug Administration
g	Gram
GAS	Gamma-activated sequences
GMP	Guanosine monophosphate
GO	Gene ontology
h	Hours
H_2O_2	Hydrogen peroxide
HAEC	Human aortic endothelial cells
HCl	Hydrochloric acid
HDAC	Histone deacetylase
HEPES	Hydroxyethyl piperazine ethane sulfonic acid
HER2	Human epidermal growth factor receptor 2
HIF-1α	Hypoxia-inducible factor 1 alpha

HP1	Heterochromatin protein 1
HPV	Human papillomavirus
HSP	Heat shock protein
HUVEC	Human umbilical vein endothelial cells
ICB	Immune checkpoint blockade
i.e.	That is (*id est*)
IFN	Interferon
IFNGR	Interferon-gamma receptor
IKK	Inhibitor of κB kinase
IL	Interleukin
INK4A	Inhibitors of CDK4
IP-10	Interferon-gamma-induced protein 10 kDa
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
IT	IFN-γ + TNF
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
Ki-67	Marker of proliferation Ki-67
Kip1	Kinase-inhibitory protein 1
L	Liter
LAG-3	Lymphocyte activation gene 3
lncRNA	Long non-coding RNA
M	Molar
MAPK	Mitogen-activated protein kinase
MC	Medium control
mCi	Millicurie
MEM	Minimum essential medium
MeOH	Methanol
mg	Milligram
MHC	Major histocompatibility complex

MIG	Monokine induced by gamma interferon
miRNA	MicroRNA
mL	Milliliter
mM	Millimolar
mm	Millimeter
µCi	Microcurie
µg	Microgram
µL	Microliter
µM	Micromolar
min	Minutes
mol	Mole
mRNA	Messenger RNA
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NF-κB	Nuclear factor “kappa-light-chain-enhancer” of activated B cells
ng	Nanogram
Nox4	NADPH oxidase 4
NP-40	Nonidet P-40 substitute
o/n	Overnight
OIS	Oncogene-induced senescence
p	Phospho
PAGE	Polyacrylamide gel electrophoresis
P-bodies	Processing bodies
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCNA	Proliferating cell nuclear antigen
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
Pen/Strep	Penicillin/Streptomycin
pg	Picogram
PIAS	Protein inhibitor of activated STAT

PICS	PTEN-loss-induced cellular senescence
PI3K	Phosphatidylinositol 3-kinase
PoMiCS	Post-mitotic cellular senescence
PTEN	Phosphatase and tensin homolog
PTP1B	Protein tyrosine phosphatase 1B
PVDF	Polyvinylidene fluoride
Rb	Retinoblastoma protein
RIN	RNA integrity number
RIP	Rat insulin promoter
RIPA	Radioimmunoprecipitation assay
RIPK	Receptor-interacting protein kinase
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SA-β-gal	Senescence-associated beta-galactosidase
SAHF	Senescence-associated heterochromatin foci
SASP	Senescence-associated secretory phenotype
SCAP	Senescent cell anti-apoptotic pathways
SD	Standard deviation
SDS	Sodium dodecyl sulfate
sec	Seconds
Ser	Serine
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SMS	Senescence-messaging secretome
SOCS	Suppressors of cytokine signaling
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes

TAA	Tumor-associated antigen
TACE	TNF-α converting enzyme
Tag	Simian virus 40 large T antigen
T-bet	T-box expressed in T cells
TBS	TRIS-buffered saline
TBST	TBS + Tween 20
TCEP	Tris-(2-carboxyethyl)-phosphine
TCR	T cell receptor
TEMED	Tetramethyl ethylenediamine
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TGF	Transforming growth factor
Th1	T-helper 1 cells
Thr	Threonine
TIL	Tumor-infiltrating lymphocyte
TIS	Therapy-induced senescence
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRADD	TNF receptor 1-associated death domain
TRAF	TNF receptor-associated factor
Treg	Regulatory T cells
TRIS	Tris-(hydroxymethyl)-aminomethane
Tyr	Tyrosine
UV	Ultraviolet
V	Volt
VIP	Variable importance in projection
v/v	Volume per volume
w/v	Weight per volume
WAF1	Wild type p53-activated fragment 1
WHO	World Health Organization
Wig1	Wild type p53-induced gene 1

Summary

Cancer is a global health threat with multifaceted disease manifestations and distinct characteristics dependent on the respective context. These circumstances make its treatment challenging and finding the right cure for the individual patient is not always possible. Besides the classical interventions with surgery, radio- and chemotherapy a variety of other quite advanced treatment strategies is available today. Especially the emerging field of immunotherapy, as for example the use of immune checkpoint blockade therapies, makes cancer treatment more specific by now. Since most of the treatment options are mainly designed to destroy and eradicate the tumor, a novel therapeutic option could also be the induction of senescence, which causes a permanent growth arrest of the malignant cells. This may help to control disease progression of not-easy-to-treat tumors and extend the therapeutic window for following treatment decisions.

In this context, we established a therapy with non-cytotoxic T-helper 1 (Th1) cells in a mouse model of multistage carcinogenesis that mediates cytokine-induced senescence (CIS) in the endogenous cancer cells *in vivo*. The main effector cytokines of Th1 cells are interferon-gamma (IFN-γ) and tumor necrosis factor (TNF), and the direct administration of both cytokines also induced CIS *in vitro* in the absence of Th1 cells. Despite the already known dependence of CIS on the presence of TNF receptor 1 (TNFR1) and the signal transducer and activator of transcription 1 (STAT1) protein, detailed data regarding the general senescence induction process and the involved signaling pathways were still missing. In this thesis, another validated model for CIS was used to investigate these questions on a simplified, cell-based level *in vitro*. By transcriptome analyses the underlying signal transduction pathways were examined. In addition, Western blot analyses of samples taken at different time points during senescence induction revealed the kinetics of the regulation of important targets on the protein level.

The major drivers of CIS are the IFN-γ-regulated Janus kinase (JAK)/STAT axis that utilizes the transcription factor STAT1, and the simultaneous stimulation of the TNF signaling pathway, which triggers activation of the mitogen-activated protein kinase (MAPK) p38 and the nuclear factor “kappa-light-chain-enhancer” of activated B cells (NF-κB) transcription factor. Single treatments with IFN-γ or TNF are not sufficient to suppress the cellular proliferation and to induce senescence. Each cytokine alone mediated transient effects that were not persistent over time and mainly lasted for hours only. The sole exception of this rule was STAT1: while TNF alone only led to activated p38 MAPK and increased NF-κB signaling during the first 24 h, treatment with IFN-γ alone induced sustained STAT1 activation for up to 96 h, thereby covering the entire treatment phase. In contrast to the short-lived effects of IFN-γ and TNF alone, the combined action of both cytokines enforced a strong and long-lasting signaling response that was characterized by 1.) hyperactivation of STAT1 and 2.) hyper-activation of NF-κB.

While hyperactivation of STAT1 was also observed for single treatment with IFN-γ alone, activated p38 MAPK signaling and hyperactivation of NF-κB were only present in the combined treatment with both cytokines and not achieved by single treatment with TNF alone. This long-lasting effect created a state of opposing cell fates where on one hand the treatment provoked stress- and death-inducing signals, while at the same time counteracting pro-survival signals were stimulated and reinforced. Therefore, CIS can be considered as a condition which resembles the phenotype of an early apoptosis that has been induced, but not executed. This was also shown by accumulation of the inactive pro-caspase-3 and elevated levels of anti-apoptotic factors like c-IAP2. Taken together, these events halted the cellular proliferation and established the senescent phenotype in form of a permanent growth arrest that was mediated and maintained by the cell cycle regulators p21 and p27. Interestingly, CIS in human A204 cancer cells was independent of p16 since no detectable levels of this senescence-associated protein were found.

In summary, only combined application of IFN-γ and TNF enables cancer control, as each cytokine alone failed to permanently activate the two essential signaling pathways that establish CIS.

Zusammenfassung

Krebs stellt weltweit eine der häufigsten Erkrankungen dar, die mit einer vielfältigen Krankheitsmanifestation und unterschiedlichen, vom jeweiligen Kontext abhängigen Merkmalen einhergeht. Auf Grund dieser Umstände wird die Behandlung häufig zu einer Herausforderung und es ist nicht immer möglich für jeden einzelnen Patienten die optimale Therapie zu finden. Neben den klassischen Behandlungsformen zu denen die chirurgische Entfernung des Tumors, sowie die Radio- und Chemotherapie zählen, steht heutzutage eine Vielzahl an weiteren und recht fortschrittlichen Therapieoptionen zur Verfügung. Vor allem das wachsende Gebiet der Immuntherapien, wie zum Beispiel der Einsatz von Immun-Checkpoint Inhibitoren, sorgt mittlerweile für eine deutlich zielgerichtetere Krebstherapie. Obwohl die meisten Behandlungen nach wie vor auf eine Zerstörung des Tumors setzen, kann ein neuartiges therapeutisches Konzept auch die Induktion der Seneszenz sein, die zu einem dauerhaften Wachstumsstopp der malignen Zellen führt. Diese Option könnte dazu beitragen, den Krankheitsverlauf bei schwer zu behandelnden Tumoren zu kontrollieren und das therapeutische Fenster für nachfolgende Behandlungsentscheidungen zu erweitern.

Vor diesem Hintergrund wurde von unserer Forschungsgruppe eine Therapie mit nicht-zytotoxischen T-Helfer 1 (Th1) Zellen in einem Mausmodell mit mehrstufiger Krebsentstehung etabliert, durch die eine Zytokin-induzierte Seneszenz (ZIS) in den endogenen Krebszellen der Mäuse vermittelt wird. Dabei spielen Interferon-gamma (IFN-γ) und Tumornekrosefaktor (TNF) als wichtige Effektorzytokine der Th1 Zellen eine bedeutende Rolle, da sich auch durch die direkte Verabreichung von beiden Zytokinen in Abwesenheit von Th1 Zellen eine Seneszenz *in vitro* induzieren ließ. Trotz der bereits nachgewiesenen Abhängigkeit der ZIS von der Expression des TNF-Rezeptors 1 (TNFR1) und des Signaltransduktor und Aktivator der Transkription 1 (STAT1) Proteins fehlten bislang allerdings noch detaillierte Daten über den Prozess der Seneszenzinduktion und die daran beteiligten Signalwege. Im Rahmen dieser Arbeit wurde nun ein weiteres validiertes Modell der ZIS verwendet, um diese Fragen auf einer vereinfachten, zellbasierten Ebene *in vitro* näher zu untersuchen. Anhand von Transkriptomanalysen wurden die zugrunde liegenden Signalwege aufgedeckt und analysiert. Darüber hinaus konnte mit Western blot Analysen eine Kinetik der Regulation von wichtigen Molekülen auf Proteinebene erstellt werden.

Als Haupttreiber der ZIS wurde dadurch zum einen die über IFN-γ regulierte Januskinase (JAK)/STAT-Achse identifiziert, welche den Transkriptionsfaktor STAT1 nutzt, und zum anderen die gleichzeitige Stimulation des TNF-Signalweges, der wiederum zur Aktivierung der Mitogen-aktivierten Proteinkinase (MAPK) p38 und des nukleärer Faktors κB (NF-κB) führt. Einzelbehandlungen mit IFN-γ oder TNF reichten allerdings nicht aus, um die Proliferation der Zellen nachhaltig zu unterdrücken und eine Seneszenz zu induzieren. Jedes Zytokin alleine vermittelte flüchtige Effekte, die meist nur für wenige Stunden anhielten.

Die einzige Ausnahme von dieser Regel war STAT1: Während TNF allein nur innerhalb der ersten 24 Stunden zu einer Aktivierung der p38 MAPK und von NF-κB führte, induzierte die Behandlung mit IFN-γ allein eine anhaltende STAT1-Aktivierung von bis zu 96 Stunden, wodurch die gesamte Behandlungsphase abgedeckt wurde. Im Gegensatz zu den kurzlebigen Effekten der Einzelbehandlungen mit IFN-γ und TNF bewirkten beide Zytokine in Kombination eine starke und langanhaltende Signalantwort, die durch 1.) Hyperaktivierung von STAT1 und 2.) Hyperaktivierung von NF-κB gekennzeichnet war.

Obwohl die Hyperaktivierung von STAT1 auch bei der Einzelbehandlung mit IFN-γ beobachtet wurde, kam es nur durch die kombinierte Behandlung mit beiden Zytokinen zur dauerhaften Aktivität der p38 MAPK und zur Hyperaktivierung von NF-κB, was durch die Einzelbehandlung mit TNF nicht erreicht werden konnte. Diese langanhaltenden Effekte erzeugten einen Zustand von an sich gegensätzlichen Zellschicksalen, in dem die Behandlung einerseits zur Induktion von mit Stress- und Zelltod-assoziierten Signalen führte, während andererseits überlebensfördernde Signale stimuliert und verstärkt wurden. Daher kann die ZIS als ein Zustand betrachtet werden, der dem Phänotyp einer frühen Apoptose ähnelt, die zwar induziert aber nicht ausgeführt wurde. Dies wurde auch durch die Anreicherung der inaktiven Pro-Caspase-3 und anhand des erhöhten Protein-Spiegels von anti-apoptotischen Faktoren wie c-IAP2 gezeigt. Zusammen stoppten diese Ereignisse die zelluläre Proliferation und etablierten den seneszenten Phänotyp in Form eines permanenten Wachstumsarrests, der durch die Zellzyklus-Regulatoren p21 und p27 vermittelt und auch aufrechterhalten wurde. Interessanterweise war die ZIS in den menschlichen A204 Krebszellen unabhängig von p16, da keine nachweisbaren Spuren dieses Seneszenz-assoziierten Proteins gefunden wurden.

Somit lässt sich schlussfolgern, dass nur die kombinierte Anwendung von IFN-γ und TNF eine effektive Kontrolle des Tumorwachstums ermöglicht, da jedes Zytokin für sich alleine nicht in der Lage war die beiden wesentlichen Signalwege der ZIS dauerhaft zu aktivieren und dadurch eine Seneszenz der Krebszellen zu induzieren.

1. Introduction

1.1. Cancer: an overview

Cancer is one of the leading causes of death worldwide and the World Health Organization (WHO) estimated for 2018 that globally 9.6 million deaths were caused by and associated with cancer, while approximately 18 million new patients were diagnosed with cancer [1]. Regarding Germany, epidemiological evaluations list about 510,000 newly registered cases and approximately 230,000 deaths for the year 2016 [2]. In general, cancer comprises a group of different diseases that develop from the body's own cells by their uncontrolled growth. Carcinogenesis, i.e., the process of cancer development, involves multiple factors and stages. It occurs as a result of different elements affecting each other, including internal causes (i.e., the patient's age and own genetic predisposition that may harbor inherited defects or mutations) and external factors (i.e., exposure to and interaction with mutagens and/or carcinogens). Such factors comprise chemical substances (e.g., tobacco smoke in case of lung cancer and others [3]) or physical causes (e.g., ultraviolet (UV) radiation in case of melanoma [4]). Moreover, certain parasites, bacteria, or viruses can cause chronic infections that lead to the malignant transformation of cells and finally to cancer (e.g., cervical cancer due to infection with human papillomavirus (HPV)) [5-7]. Depending on the localization and origin of the tumor surgical removal is the means of choice in most cases. Other common therapeutic options include: (i) chemotherapy with cytotoxic and cytostatic drugs, (ii) radiotherapy via irradiation of the tumor, (iii) (anti-) hormone therapy to influence and interfere with the endocrine system, (iv) targeted therapies based on the use of small molecules (e.g., inhibitors) as therapeutic agents, and (vi) immunotherapy, which exploits immune responses as therapeutic modality against cancer [8].

1.2. Cancer development: causes and consequences

Different models have been described that explain occurrence of carcinogenesis on a molecular level. The classical two-hit model originally proposed by Carl O. Nordling in 1953 and later refined by Alfred G. Knudson in 1971 to what is now generally known as the "Knudson hypothesis" describes how sequential events cause genetic alterations and lead to tumor formation [9, 10]. On basis of observations of retinoblastoma patients Knudson found that the hereditary form manifests mostly in both eyes and quite early in life (especially in children), while sporadic tumors occur with later age [10]. This prompted him to suggest that two events (hits) altering the DNA were needed for cancer formation. Regarding the hereditary form of retinoblastoma, the first hit is inherited while the second hit is acquired during lifetime, causing early onset of the disease [10]. In contrast, patients with sporadic tumors have no genetic predisposition and the tumor only develops after both hits occurred, which results in the late form of retinoblastoma [10]. Later on, the gene responsible for the disease was discovered and called *RB1*, which is now known as an important tumor suppressor gene [11-14].

Since each gene consists of two alleles, Knudson's observation could be explained on a genetic basis by the loss of heterozygosity: the loss or inactivation of one allele is not sufficient to cause cancer as the function of the intact allele is preventive, but when both alleles of a tumor suppressor gene are dysfunctional cancer development can take place [15, 16]. Besides tumor suppressor genes which have a recessive nature, there are also oncogenes that act in a dominant way. Normally, proto-oncogenes are involved in important cellular processes like cell division, proliferation and growth, while their oncogenic (hyper-) activity abrogates the normal regulation of these processes and contributes to cellular transformation and carcinogenesis [17, 18]. In addition to the "Knudson hypothesis" other causative models for cancer development could also demonstrate that a single hit is not sufficient to cause cancer. An example is the "initiation-promotion model", where a genotoxic compound (i.e., a mutagen) initiates carcinogenesis by mutating the DNA [19, 20]. As a consequence, the affected cells are irreversibly altered (as are the arising daughter cells), making them susceptible for a second hit delivered by another, primarily non-genotoxic substance that promotes cellular proliferation and tumor growth [19, 20]. Application of a promoter alone generally fails to induce tumor development without an initiating event, while a complete carcinogen is instead sufficient to induce tumor development on its own, as it is both able to initiate and to promote carcinogenesis [19, 20]. Other more recent investigations besides these classical DNA damage- and mutation-based models focus also on the role of epigenetics, the surrounding tissue and microenvironment, or even the microbiome that can impact the development of cancer as well as its progression and therapy in different ways [21-23]. Finally, the "cancer stem cell hypothesis" relates carcinogenesis to a subset of malignant cells that possess stem cell-like characteristics. Malignant cells with such properties are thought to be either derived from transformed stem cells, or from differentiated cells that acquired these features. Cancer stem cells may not only initiate and maintain tumor growth, but they may also be a cause for disease relapse [24-27].

1.3. Common characteristics: hallmarks of cancer

Although cancers often greatly differ in their biological properties and the etiology of the tumor, there are nevertheless shared features that define and characterize cancer cells that were collectively compiled and described by Douglas Hanahan and Robert A. Weinberg as the "hallmarks of cancer" [28, 29]. First of all, these cells are abnormal and they left the regulated and controlled path of cell division and cell death by accumulating a variety of distinct alterations. While normal cells need growth promoting factors to divide (i.e., growth factors like hormones), cancer cells are self-sufficient in these proliferative signals and different strategies to accomplish this include (i) autocrine signaling by producing the necessary growth factors themselves, (ii) inhibition of antagonistic signaling, and (iii) constitutive activation of the underlying signaling pathways. As normal cells stop proliferation due to contact inhibition with neighboring cells, this preventive mechanism is not present in tumor cells. Another way to evade growth suppression and deregulate normal proliferation is the loss of tumor suppressor gene function.

Especially alterations of the p53/Rb pathway as well as other events enable an indefinite proliferative capacity and grant the tumor cells replicative immortality. Naturally, the number of cell division cycles is limited, but upregulation of telomerase activity prevents telomere shortening and helps cancer cells to escape irreversible growth arrest (i.e., senescence induction; see chapter 1.7). The uncontrolled proliferation leads also to DNA damage, mutations, and chromosomal aberrations that cause genomic instability. The accumulation of such alterations and damages would normally trigger apoptosis, the process of programmed cell death that clears abnormal cells. However, cancer cells are quite resistant towards cell death-inducing events and they circumvent apoptosis either by influencing the molecules and pathways involved in the damage sensing (e.g., impaired function of p53), or via alterations of the factors necessary for proper apoptosis execution (e.g., overexpression of anti-apoptotic molecules). Together with the uncontrolled proliferation, disruption of apoptosis and the decrease in cell death can promote tumor formation and cancer development. Since cancer cells are programmed to grow and divide, they have an increased energy consumption as well as a high demand for biosynthetic components. Hence, they display a dysregulated metabolism by using unusual metabolic pathways for their energetic supply and biosynthetic pathways.

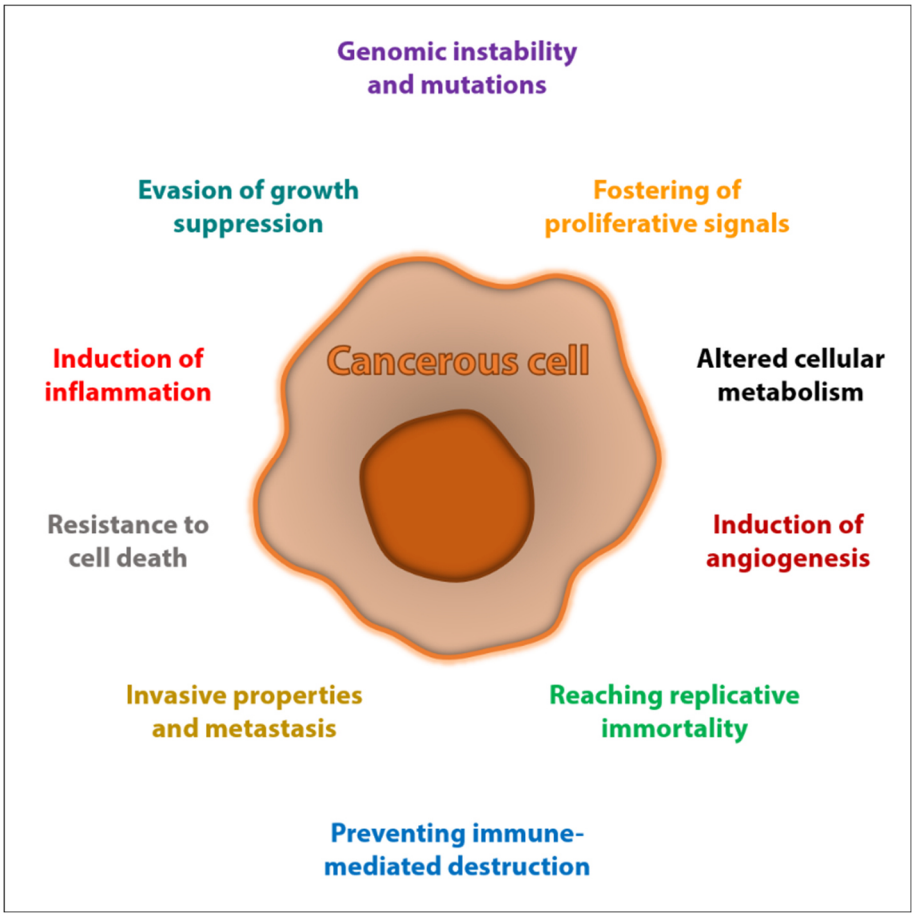


Figure 01: Schematic overview of the different hallmarks of cancer. The process of malignant transformation is critical to cancer development that is further accompanied by distinct cellular changes. In contrast to normal cells, these characteristic alterations are typically acquired by and found in cancerous cells, and are therefore commonly referred to as the hallmarks of cancer. Figure modified from Hanahan and Weinberg, 2011 [29].

In contrast to normal cells, the tumor cells prefer the inefficient process of glycolysis over the oxidative phosphorylation, an observation called the "Warburg effect". As a consequence of continued growth, cancer cells enable an increased formation of new blood vessels to supply the tumor sufficiently with nutrients and oxygen. Angiogenesis is also induced by chronic inflammation, which in turn can be tumor promoting too. While inflammation is caused by immune responses, tumor cells are also able to efficiently avoid recognition and destruction through the immune system, or to modulate the immune response in order to survive. Other events occurring during disease progression are invasion and metastasis: the cancer cells gain the ability to invade surrounding tissues, and by reaching blood and/or lymph vessels disseminate from their original (primary) location throughout the body to form secondary tumors (metastases) at distant sites.

1.4. The immune system: the body's intrinsic defense

The immune system is composed of the innate and the adaptive immunity. The innate branch is a first line of defense against pathogens and infections that consists of different barriers, soluble factors, and innate immune cells, such as natural killer cells, macrophages or granulocytes [30]. Although innate immunity is rapidly activated and directly reacts to pathogenic threats, the response is quite unspecific and no lasting immunity is induced. In contrast, the adaptive immune system is more refined and needs time to react, but offers long-lasting protection in form of specific immunity [30]. Components of the adaptive branch are lymphocytes, such as T cells and B cells, antibodies, and the formation of immunological memory [30]. The specificity of the adaptive immune system is achieved through rearrangements of the genes that encode immune cell receptors (i.e., T cell receptors (TCRs) and B cell receptors (BCRs)) via somatic recombination, thereby creating and providing an almost infinite potential of adaption to foreign antigens during the whole lifetime [30]. B cells as well as T cells are derived from a common lymphoid progenitor and go through different selection steps during their development and differentiation. Maturation of B cells takes place in the bone marrow and in secondary lymphoid organs, such as lymph nodes and spleen, whereas T cells originate from lymphoid progenitors that migrated to the thymus [30]. Since various subtypes exist, T cells are mainly divided into the following groups that express different factors, such as cluster of differentiation (CD) molecules on their surface, which are used as markers to characterize and discern the cells. Cytotoxic T lymphocytes are $CD8^+$ and mediate killing of cellular targets after activation through TCR-dependent antigen recognition on major histocompatibility complex (MHC) class I molecules and other co-stimulatory signals [30]. T-helper (Th) cells are $CD4^+$, generally non-cytotoxic and interact with other immune cells through direct contact and the secretion of effector cytokines that are released after their activation through co-stimulatory factors and antigens presented on MHC class II molecules expressed by professional antigen-presenting cells (APCs) [30]. Depending on the cytokines that are present when activation takes place, naïve $CD4^+$ cells can differentiate into several effector cell types that have varying function and produce different cytokines.

Differentiation of activated $CD4^+$ cells into Th1 cells is induced through interleukin 12 (IL-12) and interferon-gamma (IFN-γ) that are secreted by activated APCs [30]. Downstream transcription factors that are critical for Th1 cell differentiation include the signal transducer and activator of transcription (STAT) family members 1 and 4, as well as T-bet (i.e., T-box expressed in T cells) and others [30]. On a functional basis, Th1 cells are relevant for the elimination of intracellular pathogens by promoting cell-mediated immune reactions, and they primarily produce IL-2, IFN-γ, and tumor necrosis factor (TNF) as effector cytokines [30]. As Th1 cells and their effector cytokines IFN-γ and TNF are of importance for the work presented in this thesis, the following paragraph will focus on the related cytokine signaling.

1.5. IFN-γ and TNF signaling: a close-up on the underlying signal transduction

Interferons are important mediators of the immune system mainly involved in the anti-viral defense, and their family consists of 3 different groups: type I, type II, and type III interferons [31]. IFN-γ is a homodimeric cytokine and the sole member of type II interferons that exerts its action via binding to the respective IFN-γ receptor (IFNGR) on the cell surface. The receptor is a multimeric complex made up of different subunits, two ligand-binding α-chains (IFNGR1) and two non-ligand-binding β-chains (IFNGR2), that utilizes the Janus kinase (JAK)/STAT pathway for intracellular signal transduction [32-35]. Upon receptor activation JAK1 and JAK2 are phosphorylated and in turn phosphorylate the α subunits of the IFNGR complex creating a binding site for STAT1. The STAT1 molecules are then phosphorylated by the JAKs, form homodimers, translocate into the nucleus, and finally bind to so-called GAS elements (gamma-activated sequences) in the promoter regions of interferon-stimulated genes (ISGs) to induce transcription of these targets [36-39]. Two well-known downstream targets of IFN-γ are the C-X-C motif chemokine ligand 9 (CXCL9), which is also known as monokine induced by gamma interferon (MIG) [40], or the C-X-C motif chemokine ligand 10 (CXCL10) that is also referred to as interferon-gamma-induced protein 10 kDa (IP-10) [41]. Both CXCL9 and CXCL10 are strongly upregulated after IFN-γ stimulation [42, 43].

Like IFN-γ, TNF (formerly referred to as TNF-α) is another cytokine of the immune system that plays a critical role as an inflammatory mediator. It belongs to the TNF superfamily of transmembrane proteins that consists of 19 different members, and acts as homotrimer either membrane-bound, or in its soluble form after cleavage through the TNF-α converting enzyme (TACE) [44]. TNF can bind to two distinct cellular receptors: TNF receptor 1 (TNFR1), which binds soluble as well as membrane-bound TNF, and TNF receptor 2 (TNFR2) that is mainly activated by the transmembrane form of TNF [45]. On a structural basis the receptor molecules differ in their composition and mediate different effects. TNFR1 contains a death domain in its intracellular region and is therefore a death receptor [46], whereas TNFR2 is considered as an activating non-death receptor due to the lack of a death domain [47]. Upon binding of TNF to the extracellular domain of TNFR1 trimerization of receptor

molecules occurs, and TNF receptor 1-associated death domain (TRADD) proteins are recruited that bind to the respective intracellular domain of the receptor [48]. This is followed by recruitment and binding of other proteins, such as TNF receptor-associated factors (TRAFs) or receptor-interacting protein kinases (RIPKs), which leads to the subsequent formation of different signaling complexes that mediate varying cellular responses [49]. Dependent on the context, cells can be subject to different forms of regulated cell death, i.e., apoptosis or necroptosis. TNF-induced apoptosis through TNFR1 signaling can be mediated by two different complexes. Although formation and composition of complex IIa and IIb is different, both are relevant for the downstream activation of caspases (an acronym for cysteine-dependent aspartate-directed proteases) [50]. The whole process starts with cleavage of pro-caspase-8, which initiates a cascade of molecular interactions that results in the activation of executioner caspases, such as caspase-3, and finally in apoptotic cell death [51]. Under certain conditions also necroptosis may take place after the activation of caspase-8, which involves the formation of the necrosome [52]. In addition to its death-inducing function, receptor binding of TNF can also trigger the activation of downstream transcription factors, such as nuclear factor "kappa-light-chain-enhancer" of activated B cells (NF-κB), which regulates the expression of various genes and mediates cellular survival [53, 54]. This process involves the initial signaling complex I that activates different kinases, such as the inhibitor of κB kinases (IKKs), or members of the mitogen-activated protein kinase (MAPK) family [55]. Examples for MAPKs activated in response to TNF are p38, the c-Jun N-terminal kinases (JNKs), and the extracellular signal-regulated kinases (ERKs) [56].

1.6. Immunotherapy: utilizing immune responses to fight cancer

Treatment approaches based on and involving the patient's immune system consist of cell-based therapies, the use of immunomodulatory agents (e.g., cytokines), and therapeutic antibodies. Tumor-infiltrating lymphocytes (TILs) are a representation of the immune system attacking the malignant cells, a circumstance that is generally associated with a better prognosis and treatment outcome in cancer patients [57, 58]. A therapeutic approach using adoptive transfer of *ex vivo* expanded TILs was initially developed by Steven Rosenberg et al. as a treatment for metastatic melanoma in the late 1980s and is nowadays also available as a therapy for other cancers [59-61]. Besides the expansion of naturally occurring immune cells that have already infiltrated the tumor, there are also strategies to use genetically engineered cells if the patient's immune system failed to recognize and attack the tumor. One strategy is to equip autologous T cells with an engineered TCR that is specific for the respective tumor antigen expressed, while T cells with a chimeric antigen receptor (CAR) can also target cancer cells that do not express tumor antigens via MHC class I molecules [62]. To date, no TCR therapy received green light for clinical use, but the first CAR T cell therapies were approved by the U.S. Food and Drug Administration (FDA) in 2017 for hematological malignancies, one for the treatment of diffuse large B cell lymphoma (axicabtagene ciloleucel) and the other one also for B cell precursor acute lymphoblastic leukemia (tisagenlecleucel) [63, 64].

Instead of transferring immune cells as a treatment, the direct administration of recombinant cytokines is another possible application of immune-based therapy. Cytokine therapies have been described for different factors, including interferons (e.g., IFN-α), interleukins (e.g., IL-2), and granulocyte-macrophage colony-stimulating factor (GM-CSF). They are generally used after or along other conventional therapies to stimulate the immune system with the aim to achieve an anti-tumor immune response [65, 66]. During the last years the approval and clinical use of blocking antibodies against negative immune regulatory checkpoints to overcome cancer-induced dampening of the immune system has revolutionized the therapy of many patients [67]. This so-called immune checkpoint blockade (ICB) therapy utilizes monoclonal antibodies directed against the cytotoxic T lymphocyte-associated protein 4 (CTLA-4) (e.g., ipilimumab) and the programmed cell death 1 (PD-1) receptor on immune cells (e.g., nivolumab and pembrolizumab), or the programmed cell death 1 ligand 1 (PD-L1) on tumor cells (e.g., atezolizumab) to antagonize and disrupt the inhibitory function of those surface molecules that mediated immunological tolerance for the cancer cells [67-75]. As a result, ICB reactivates the previously downregulated immune responses and turns it against the tumor. The importance of understanding fundamental functions of the immune system that pave the way for novel immunotherapies like ICB was also highlighted by awarding the Nobel Prize 2018 in Physiology or Medicine to James P. Allison and Tasuku Honjo "for their discovery of cancer therapy by inhibition of negative immune regulation" [76].

1.7. Senescence: an endogenous barrier against malignant transformation

Senescence, a term derived from *senex* the Latin word for "old", is a form of cellular aging that was first described by Leonard Hayflick and Paul S. Moorhead in 1961: they discovered that primary human fibroblasts cultured *in vitro* at some point reach a state that allows no further cell division, and are thus not immortalized – a finding that was quite the opposite of the common belief that *in vitro* cultured cells are dividing indefinitely [77]. This phenomenon was due to the induction of replicative senescence in the cultured cells that aged over time and defined the so-called "Hayflick limit", which determines the number of potential cell division cycles that normal human cells are able to pass through before their proliferation ultimately stops [78-80]. Originally defined as a state of irreversible growth arrest in normal human diploid cells *in vitro* (i.e., induction of replicative senescence due to a finite number of replication cycles), senescence was also found *in vivo* where it plays an important role during embryogenesis, organ development, and later on in tissue homeostasis, remodeling and repair (e.g., during wound healing) [81-86]. In this context, senescence-like phenotypes were also found in terminally differentiated cells and tissues that rarely divide or ceased to proliferate (e.g., cardiomyocytes or neurons), and thus described as "post-mitotic cell senescence" (PoMiCS) [87-89]. Accumulation of senescent cells also contributes to organismal aging and age-related diseases [90-92]. In addition, senescence acts as a tumor-suppressive mechanism that limits tumorigenesis by restricting the proliferation of damaged or pre-malignant cells, thus blocking cancer development [93-99].

If cells overcome this barrier and re-enter the cell cycle, they likely give rise to tumors or initiate cancer relapse. As studies already indicated potential senescence escape mechanisms and the evasion of an "irreversible" growth arrest (e.g., by loss, suppression or mutation of p53), senescence should rather be seen as a permanent state that can be induced by various stresses and stimuli [100-112].

1.8. Senescence induction: triggers and their mode of action

Different modes of senescence induction have been described, including intrinsic and extrinsic triggers. Since senescence is commonly associated with its original description (i.e., related to aging), the term "premature senescence" or other more precise naming is often used instead to define stimuli and stresses that induce a senescent state independent of the cells replicative age, as briefly summarized in the following section. As already mentioned before, replicative senescence is associated with the aging of the cells by each cell division, and it is induced through telomere shortening: the eroding telomeres trigger a DNA damage response (DDR) that is quite similar to those found for DNA double-strand breaks and leads to senescence induction, while telomerase activity was shown to reverse this kind of senescence through elongation of the telomeres [78, 113-116]. Other triggers for senescence are activated oncogenes (e.g., *RAS* or *RAF*) and the related signaling of the stress-associated p38 MAPK pathway and others, as well as the induction of DNA damage checkpoints and the upregulation of tumor-suppressive factors like p53, $p21^{Waf1/Cip1}$ (hereinafter referred to as p21), $p16^{INK4A}$ (hereinafter referred to as p16), and Rb [94, 99, 117-121].

The oncogene-induced senescence (OIS) stops the malignant transformation and further tumor development through a stable growth arrest, as for example found in melanocytic nevi of the skin: these benign lesions harbor aberrant activity of the v-raf murine sarcoma viral oncogene homolog B (BRAF) caused by the common V600E mutation (i.e., a substitution of the amino acid valine by glutamic acid) and express high levels of p16 that in turn primarily mediates the growth arrest [96, 122]. In this context, not only the (re-) activation of oncogenes, but also the inactivation or the loss of tumor suppressor genes can trigger the induction of senescence: in case of the "PTEN-loss-induced cellular senescence" (PICS) the tumor-suppressive phosphatase and tensin homolog (PTEN) is inactivated, which induces a p53-dependent growth arrest in the absence of DNA damage that restricts cancer development [98, 123, 124]. Therapy-induced senescence (TIS) is a general term which refers to the effect of common cancer treatments, such as radio- or chemotherapeutic approaches, that can also lead to a cytostatic state instead of killing the tumor cells [125-131]. While most of these treatments include genotoxic substances (e.g., doxorubicin) that induce a DDR and lead to the formation of reactive oxygen species (ROS) that mediate oxidative stress, other therapeutic drugs induce growth arrest and a senescent phenotype by interfering with the cell cycle, such as the FDA approved cyclin-dependent kinase (CDK) 4/6 inhibitor palbociclib that mimics the function of natural cell cycle regulators [132-134].

Moreover, therapeutic agents like histone deacetylase (HDAC) inhibitors (e.g., trichostatin A or suberanilohydroxamic acid) or DNA methyltransferase (DNMT) inhibitors (e.g., 5-aza-2'-deoxy-cytidine) that are also used in cancer treatment were shown to induce epigenetic changes and senescence [135-142]. Besides these different triggers of senescence also other stress-induced forms of senescence exist (e.g., through cytokines, as outlined in chapter 1.10). All forms of senescence are typically accompanied by distinct cellular changes as illustrated in the next paragraph.

1.9. Characteristics of senescent cells: associated markers and phenotypes

As with cancer cells there are pronounced differences between normal proliferating cells and senescent cells. The cellular characteristics of senescence include various changes with the most prominent features being the common change in morphology and shape (i.e., cellular enlargement, flattening, and/or multinucleation), and the increase in senescence-associated β-galactosidase (SA-β-gal) activity at pH 6.0 [143-146]. Another emerging biomarker besides the classical blue staining for SA-β-gal activity is lipofuscin, a pigment that mainly consists of protein and lipid aggregates, which can be detected by Sudan Black B staining (or alternatively by the autofluorescence of the cells) [147-150]. Other associated markers are the typical growth arrest, the upregulation of CDK inhibitors and cell cycle regulators like p16 or p21, the downregulation of proliferation markers like Ki-67, as well as the resistance to apoptosis [151-160]. The senescent state is also characterized by changes of the nucleus that affect its structure and the DNA. Nuclear remodeling is frequently found in senescent cells and involves loss or downregulation of lamin B1, which is a component of the nuclear lamina that determines the nuclear integrity and interacts with the chromatin [161].

The reorganization of the chromatin structure through epigenetic modifications, such as trimethylation of histone 3 at lysine 9 (H3K9me3) and phosphorylation of the heterochromatin protein 1 gamma (HP1γ) are linked to the formation of senescence-associated heterochromatin foci (SAHF) that are associated with the silencing of E2F target genes [162, 163]. Senescent cells may also exhibit phosphorylation of the DNA damage marker histone H2AX (γH2AX) that is not only found in transient and repairable foci but also in persistent DNA lesions (i.e., DNA-SCARS: "DNA segments with chromatin alterations reinforcing senescence") [116, 164, 165]. Furthermore, senescent cells present an altered gene expression profile and recently certain types of RNA (i.e., small non-coding microRNAs (miRNAs) as well as long non-coding RNAs (lncRNAs)) have also been associated with the regulation of senescence [166-168]. Moreover, senescent cells are metabolically (hyper-) active and usually establish a distinct secretome generally referred to as the senescence-associated secretory phenotype (SASP) or alternatively as the senescence-messaging secretome (SMS) [169, 170]. The SASP is quite heterogeneous and consists of a large number of factors, including pro-inflammatory cytokines, chemokines, growth factors, proteases and others [169, 171, 172]. These secreted factors can not only reinforce the senescent state of the cells, but they can also lead to (chronic) inflammation

and even promote tumor formation, cancer progression, and relapse by influencing the cells in the surrounding tissue microenvironment [169-173]. Nevertheless, the SASP also contains factors that direct certain immune cells to the senescent cells, which are then cleared [174-178].

As the secretome of senescent cells can further contain extracellular vesicles that are loaded with functional biomolecules like lipids, proteins, or nucleic acids (DNA as well as mRNAs and miRNAs) as cargo, these structures also contribute to the intercellular communication [179-183]. It is noteworthy that the senescent state as well as its distinct phenotype may vary in different cell types dependent on the mode of induction and the underlying trigger. Hence, a single feature of the aforementioned characteristics is not sufficient to specifically define senescence, especially as not all cell types might express the respective markers. This circumstance highlights the necessity to analyze multiple parameters in order to accurately detect and classify senescence.

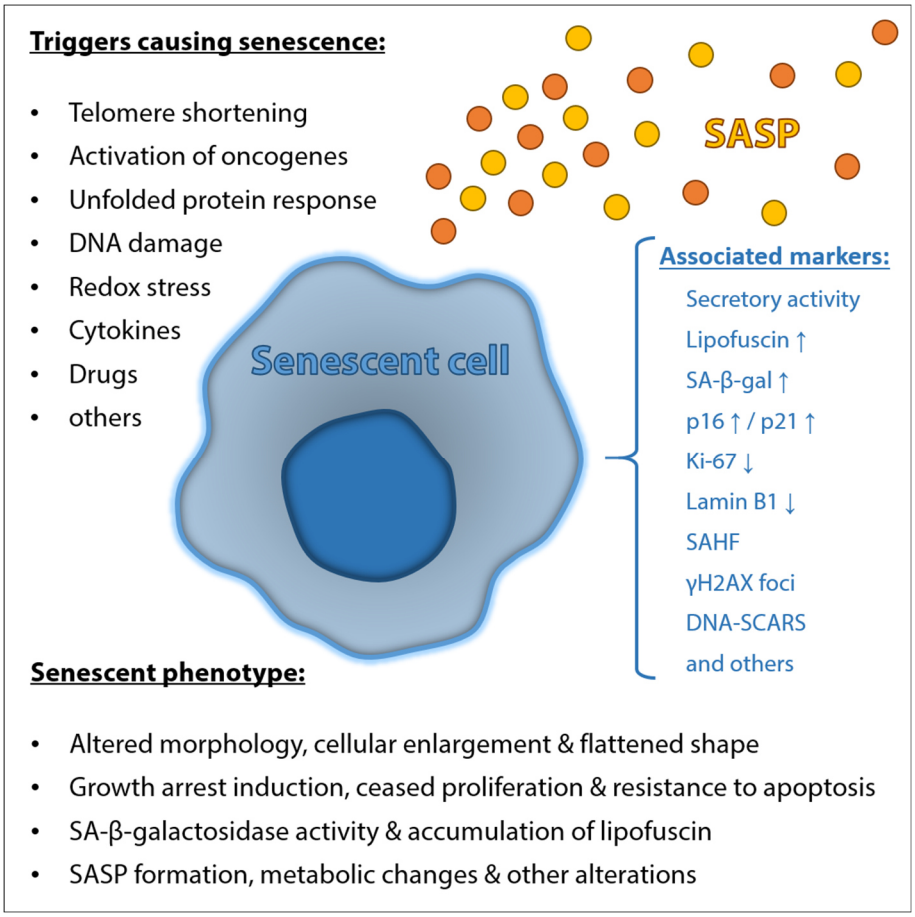


Figure 02: Schematic overview of the different triggers and characteristics of senescence. A variety of different triggers leads to the induction of senescence, a cellular response to stress that permanently stops the proliferation. This growth arrest is also considered as an endogenous barrier against the malignant transformation of cells that would otherwise cause cancer. Senescent cells show a characteristic phenotype with typical changes and alterations that are distinct from normal cells. They further express associated markers, such as increased SA-β-galactosidase activity or the upregulation of p16 and/or p21, while markers of proliferation like Ki-67 are downregulated. In addition, senescent cells establish the so-called SASP, a secretory program that includes many pro-inflammatory factors that act on surrounding tissue and neighboring cells as well as on the secreting cells itself. Figure modified from Sharpless and Sherr, 2015 [184].

1.10. Cytokine-induced senescence: a novel type of immune-mediated tumor control

In addition to the aforementioned forms of senescence, we recently identified a novel type of induction that uses a combination of IFN-γ and TNF to exert its effect on cancer cells. It was therefore termed cytokine-induced senescence (CIS) [185, 186]. This concept was based on initial *in vivo* data from a multistage carcinogenesis model where the adoptive transfer of tumor-associated antigen (TAA)-specific Th1 cells into the tumor-bearing RIP-Tag mice induced tumor dormancy without signs of elevated apoptosis [187]. The transgenic RIP-Tag mice express the oncogenic simian virus 40 large T antigen (Tag) under control of the rat insulin promoter (RIP) [188]. Within 12 weeks the mice develop endogenous tumors of the insulin-producing β-cells of the pancreas: these hyperplastic islets undergo an angiogenic switch and become vascularized carcinomas with invasive capacity that finally metastasize [188, 189]. The novel therapeutic approach to target the endocrine β-cell tumors in the cancer-prone RIP-Tag mice with TAA-specific (Tag-directed), non-cytotoxic Th1 cells showed homing and localization of these mediators of adaptive immunity in the surrounding micro-environment of the pancreatic tumors [187]. Tumor dormancy was then achieved by the Th1 cell-mediated immune response through secretion of the effector cytokines IFN-γ and TNF that act locally by inhibiting angiogenesis and further tumor growth without destroying the cancer cells [187]. Since therapy failed and transition of hyperplastic islets into carcinomas occurred in RIP-Tag mice injected with neutralizing anti-IFN-γ antibodies, or in knockout mice deficient for TNF receptor 1 (Tnfr1), direct evidence was provided that the permanent growth arrest of the cancer cells found in the respective Tnfr1 and IFN-γ proficient RIP-Tag mice was omitted in the absence of cytokine activity [187]. Thus, the therapeutic response depends on intact cytokine signaling mediated by IFN-γ and TNF [187].

The follow-up study uncovered in detail that the previously described tumor dormancy is actually a new type of senescence [185]. Again, the RIP-Tag model was used since one important feature in this model system is the constant inactivation of p53 that enables cancer development and tumor growth [190]. This circumstance allowed differential analysis of the tumor-directed and immune-mediated senescence induction process from OIS [185]. After adoptive transfer, the Tag-reactive Th1 cells induced a permanent cytokine-mediated growth arrest in the Tag-expressing β-cell tumors *in vivo*, thereby prolonging the survival of the tumor-bearing RIP-Tag mice and reducing the tumor volume [185]. This protective effect was dependent on the presence of Tnfr1 since the Th1 cell-based immunotherapy failed in experiments with RIP-Tag mice that have dysfunctional cytokine signaling [185]. *Ex vivo* analyses of isolated islets and β-cancer cells showed a decreased expression of the proliferation marker Ki-67 and upregulation of senescence-associated markers like phosphorylated HP1γ in the nucleus, trimethylation of histone 3 at lysine 9 (H3K9me3), and high levels of p16 (encoded by the *Cdkn2a* gene) as well as growth arrest induction after treatment with Th1 cells [185].

Additional *in vitro* studies using the Th1 cell cytokines IFN-γ and TNF confirmed these observations and further revealed increased SA-β-gal activity, hypophosphorylation of the Rb protein, and induction of a G0/G1 cell cycle arrest [185]. Knockdown experiments with short hairpin RNAs (shRNAs) targeting p16 verified the role of this cell cycle regulator in the senescence induction process that was also shown to depend on the combined action of both cytokines, since β-cancer cells derived from knockout mice deficient for Tnfr1 or Stat1 were resistant to senescence induction [185]. The direct application of both cytokines together was also tested *in vitro* on various human and murine cancers (established cell lines as well as primary tumors and isolated tumor cells) that also responded with induction of senescence, whereas single treatments with each cytokine alone failed to induce a permanent growth arrest, and to upregulate senescence-associated markers at the same level as the combination of IFN-γ and TNF. This pivotal characterization finally coined the term cytokine-induced senescence (CIS) [185]. Most recently, another study from our group demonstrated that immunotherapy with immune checkpoint inhibitors (i.e., anti-PD-L1 or anti-LAG-3) achieves cancer control by the induction of tumor cell senescence in two different mouse models (in case of RIP-Tag mice also in combination with TAA-specific Th1 cells) [191]. The underlying mechanisms which induce a stable arrest in the tumor cells are important cell cycle regulators that exert their function in an interferon-dependent manner: either the loss of p16 (encoded by the *Cdkn2a* gene) or p21 (encoded by the *Cdkn1a* gene) caused failure of the immunotherapy [191]. Moreover, the immune-mediated senescence induction was abrogated in *Stat1*-deficient mice highlighting the role of interferon-signaling as a necessity in this process [191].

During the last years, CIS or CIS-like phenotypes were also described by others as outlined below. A study analyzing the senescence-inducing capacities of different DNA damaging substances and genotoxic drugs, such as hydroxyurea, distamycin A, aphidicolin, or bromodeoxyuridine, and the related signaling events in different human cancer cell lines discovered a long-lasting activation of the JAK/STAT pathway leading to downstream gene expression (especially of ISGs) and cytokine production, including members of the transforming growth factor (TGF) family, IL-1, and TNF [192]. Moreover, the senescent cells maintained the active JAK/STAT signaling in an auto- and/or paracrine manner through the expression and secretion of activating ligands like interleukins (IL-6, IL-10, IL-20, and IL-24) and interferons (IFN-β and IFN-γ) [192]. The senescence-associated secretome of the senescent cells is also able to influence surrounding cells through paracrine signaling, a phenomenon called “bystander” senescence [193-195]. These “bystander” effects were further assessed by the work of Sapega et al. who analyzed and compared the senescent phenotypes of different cell types after senescence induction with docetaxel (DTX), a chemotherapeutic drug, or a cytokine cocktail of IFN-γ and TNF [196]. By using the murine cancer cell lines TC-1 (a lung tumor) and B16F10 (a melanoma entity) it was shown that the resulting outcome of the treatments varied heavily: in both cell lines DTX induced the expected outcome in terms of senescence markers (SA-β-gal positive cells and a stable

growth arrest mediated by increased expression of p21) and secreted SASP factors that could in turn induce "bystander" senescence, while the application of IFN-γ and TNF failed to achieve similar results [196]. Features of senescence were only observed in B16F10 cells, but the growth arrest was not permanent as the cells continued to proliferate after withdrawal of the cytokines [196]. Moreover, the cytokine-treated B16F10 cells led to tumor formation after injection into C57BL/6 mice and they could not mediate "bystander" senescence as seen for the treatment with DTX [196]. Collectively, these findings highlight distinct differences between varying triggers used to induce senescence and the cell lines analyzed [196]. The results further indicate that immune-mediated processes like the induction of CIS might not lead to the classical state of senescence that is described as "irreversible" and hint to the potential risk of cells that start to regrow after the end of the cytokine treatment [196].

In 2014, Wang et al. found senescence induction through IFN-γ in normal melanocytes of the human skin [197]. The constant treatment with IFN-γ mediated various effects on the melanocytes, including increased levels of intracellular ROS. Furthermore, the melanocytes showed decreased viability accompanied by the induction of apoptosis and senescence that was characterized by a p21-dependent cell cycle arrest, increased SA-β-gal activity, secretion of heat shock protein 70 (HSP70) and IL-6, as well as alterations in the morphology and pigmentation of the cells [197]. Since the aforementioned effects were reversed by the use of a ROS scavenger (i.e., N-acetyl cysteine) or through small interfering RNAs (siRNAs) targeting components within the interferon signaling cascade (e.g., JAK2 and STAT1), the study illustrates the impact of IFN-γ on melanocytes and their function (i.e., melanin production) that contributes to the pathogenesis of skin diseases, such as vitiligo [197].

Schilbach et al. demonstrated in an *in vivo* model using humanized mice with sarcoma engraftment the potent anti-cancer activity of an antibody-coupled IL-12 construct (NHS-IL12) that can bind to the histones of necrotic cells [198]. When combined with engineered IL-2 or IL-7 constructs, the NHS-IL12 therapy activated both innate and adaptive immunity leading to extended survival of the mice. Induction of senescence in the tumor cells was demonstrated by a stop of proliferation, upregulation of p16, and accumulation of p-HP1γ in the nucleus [198]. Since IL-12 is a driver of immune responses involving IFN-γ, the established cocktail of IFN-γ and TNF was used as another validated senescence model for the treatment of patient-derived rhabdomyosarcoma cell lines as well as for the human A204 rhabdomyosarcoma cell line *in vitro* [198]. As rhabdomyosarcomas resistant to senescence induction via IFN-γ and TNF showed no expression of p16, this observation confirmed its role in CIS [198]. Furthermore, the cytokines provoked expression of muscle-specific genes leading to myogenic differentiation of A204 cells, while the growth arrest was accompanied by a strong induction of p21 and increased activity of SA-β-gal [198]. This circumstance adds to the growing evidence that effective anti-tumor immunity is not primarily achieved by killing and cytotoxicity, but also includes further processes, such as induction of senescence and differentiation to promote cancer control [198].

The senescence-inducing capacities of IFN-γ were also emphasized by Hubackova et al. who identified NADPH oxidase 4 (Nox4) and adenine nucleotide translocase 2 (ANT2) as important factors in the downstream signaling cascade [199]. Only the combination of IFN-γ and TNF led to expression of NADPH oxidases like Nox4, induction of oxidative stress, activation of the DDR, and senescence in tumor cells of mice, whereas application of INF-γ alone was able to provoke a similar response in human cells [199]. As IFN-γ induced NADPH oxidase expression through JAK/STAT signaling and the secretion of TGFβ that activated the SMAD pathway, it also suppressed the expression of ANT2. These contrary events enhance the cellular ROS levels and the resulting DNA damage finally implements CIS [199].

In another study focusing on TNF-mediated effects on human umbilical vein endothelial cells (HUVECs), Kandhaya-Pillai et al. showed that this pro-inflammatory cytokine induces senescence via an autocrine STAT-dependent feedback loop that leads to ROS production and DNA damage, promotes secretion of cytokines, and reinforces expression of an underlying interferon-responsive gene network [200]. This senescent state of the HUVECs was characterized by increased levels of SA-β-gal, p16 and p21, and critically involves activation of STAT1 and STAT3 [200]. Interestingly, inhibition of these factors was not able to prevent growth arrest induction, but rather altered and modulated the TNF-induced senescence: TNF treatment not only activated the JAK/STAT pathway, but also p38 MAPK signaling and triggered ROS production that together led to senescence induction via a positive feedback loop as described [200]. Although the use of a JAK inhibitor blocked activation of STAT1 and STAT3 and led indeed to decreased expression of interferon-responsive genes, diminished ROS levels and a reduced cytokine production, it also increased the expression of senescence-associated markers like p21 or the number of SA-β-gal positive cells, and further suppressed cell cycle genes and inhibited S-phase entry of the cells instead of reversing the growth arrest [200]. Therefore, these findings indicate a dual role of the JAK/STAT pathway for TNF-induced senescence that works in a reciprocal fashion [200].

Rosemblit et al. reported a STAT1-mediated induction of apoptosis and senescence through application of Th1 cell cytokines and inhibition of the human epidermal growth factor receptor 2 (HER2) in a breast cancer model [201]. This dose-dependent effect was achieved in HER2-expressing cells by either administration of $CD4^+$ Th1 cells or direct treatment with the cytokines IFN-γ and TNF. After loss of HER2 activity by an induced knockdown or specific blockade with therapeutic antibodies (e.g., trastuzumab) increased levels of senescence and apoptosis induction were found in trastuzumab-sensitive as well as -resistant breast cancer cells upon treatment with Th1 cells or the respective cytokines [201]. In the case of triple negative breast cancers, additional inhibition of EGFR (i.e., HER1) was necessary to sensitize the cells to IFN-γ and TNF, since otherwise only minimal effects were achieved. Mechanistically, IFN-γ and TNF induced the activation of STAT1 by its increased

phosphorylation at different sites, while the activity of STAT3 was reduced [201]. By this, blockade of an oncodriver in combination with IFN-γ and TNF mediates senescence induction via activation of the transcription factor STAT1 that facilitates expression of senescence-associated genes [201].

Besides the senescence-inducing properties of Th1 cells and its effector cytokines IFN-γ and TNF on tumors and cancer cells in humans and mice, Liu et al. recently found senescence induction also in immune cells, i.e., in human $CD4^+$ effector T cells after interaction with regulatory T cells (Tregs) [202]. During their cross-talk a metabolic competition between both cell types occurs, which leads to DNA damage and the induction of a senescence program in the effector T cells that involves important signaling pathways comprising ATM, STAT1, STAT3, ERK1/2, and p38 MAPK activation [202]. Thus, senescence induction in effector T cells is another cell suppressive mechanism of Tregs that allows therapeutic intervention during Treg-mediated disease or cancer immunotherapy [202]. Taken together, CIS is now an established biological concept that plays an important role in maintaining tissue homeostasis under physiological as well as pathological conditions.

1.11. The Argonaute-2 protein: involvement in senescence induction processes and CIS

Argonaute proteins are molecules involved in RNA interference (RNAi). They mediate post-transcriptional gene silencing through binding of different types of small RNAs (especially miRNAs and siRNAs) that are complementary to the respective mRNA targets [203]. These proteins consist of different functional domains and their assembly with other factors forms the RNA-induced silencing complex (RISC) that regulates the gene expression in different ways, e.g., through mRNA degradation [204]. In this context, the *EIF2C2*-encoded Argonaute-2 protein (AGO2) plays a critical role in the gene silencing through RNAi, since it exerts endonuclease activity which is needed for targeted RNA cleavage [205-207]. The cellular distribution of AGO2 is variable: it is mainly localized in the cytoplasm where it is found in stress granules or processing bodies (P-bodies) that are relevant for RNA decay [208, 209] but it can also shuttle into the nucleus, a process that is mediated by importin-8 [210, 211]. The nuclear functions of Argonaute proteins are still not fully understood, but involvement of AGO2 in transcriptional regulation and alternative splicing [212-214] as well as DNA repair [215-217] has been described and discussed. Furthermore, different roles of AGO2 expression were associated with various processes, including embryogenesis and early development [205, 218, 219], differentiation [220-222], angiogenesis [223-225], proliferation and quiescence [226-228], as well as cellular senescence as outlined below.

The link between AGO2 and senescence was first described by Benhamed et al. in 2012 who found accumulation of AGO2 in the nucleus of cells undergoing senescence that was induced by different stimuli (e.g., expression of oncogenic RAS, treatment with doxorubicin, or re-expression of Rb) [229].

The authors further showed an interaction of this protein with Rb and miRNAs (e.g., let-7 family members) that led to repression of E2F target genes involved in cell cycle regulation and associated with proliferation [229]. This so-called senescence-associated transcriptional gene silencing was achieved by binding of AGO2/miRNA complexes to target gene promotors, thus modifying the chromatin state to repress expression [229]. An important factor for the post-transcriptional regulation of gene expression that is necessary for the loading of miRNAs like let-7 on AGO2 was identified to be the AU-rich binding factor 1 (AUF1) [230]. AUF1 is a RNA-binding protein and was earlier described to counteract premature senescence and aging by preserving the integrity of the DNA through increased expression of genomic maintenance proteins [231].

In 2014, Yang et al. reported that protein tyrosine phosphatase 1B (PTP1B) is inactivated by ROS produced in response to oncogenic RAS activity [232]. In this study, phosphorylation of AGO2 at tyrosine residue 393 was found to inhibit not only the loading of miRNAs on AGO2 but also the transcriptional gene repression, while active PTP1B would normally reverse these effects by dephosphorylating the Tyr393 residue [232]. Therefore, the proposed mode of action in their model of OIS followed the inactivation of PTP1B by RAS-induced ROS that led to enhanced phospho-Tyr393 levels of AGO2, which in turn inhibited the miRNA loading and the post-transcriptional repression of important genes like p21 that subsequently implemented the senescent phenotype [232]. The emphasis of phosphorylation events that regulate AGO2 function and the associated gene silencing by miRNA was also described by Golden et al. who identified casein kinase 1 alpha 1 (CSNK1A1) and a functional serine/threonine protein phosphatase 6 complex as the enzymes responsible for the underlying phosphorylation/dephosphorylation cycle [233]. In another study performed with the MCF-7 cell line, Kim et al. demonstrated that the wild type p53-induced gene 1 (Wig1) is important for the mRNA stability of p21 by controlling its association with AGO2: Wig1 was shown to be essential for the cellular proliferation, as its expression facilitated the miRNA-mediated degradation of p21 mRNA while depletion of Wig1 induced premature senescence [234].

Furthermore, Laudadio et al. uncovered that AGO2 influences telomere maintenance by regulating telomerase activity: normally, telomere shortening leads to senescence induction over time while activity of telomerases counteracts this process [235]. AGO2 was shown to control the telomere length via its interaction with telomerase RNA component (TERC), and by stimulating the association between telomerase reverse transcriptase (TERT) and TERC [235]. Recently, AGO2 was found to be relevant for OIS in a KRAS-driven model of pancreatic cancer development [236]. Shankar et al. demonstrated that loss of AGO2 promoted formation of premalignant lesions, but progression to pancreatic ductal adenocarcinoma was prevented by the induction of senescence (OIS) through alterations in the miRNA profile and changes in the cellular signaling activating oncogenic KRAS [236]. They previously showed a direct interaction between AGO2 and KRAS that is involved in

cellular transformation, and currently found that pancreatic cancer progression was accompanied by an increased AGO2/KRAS co-localization at the plasma membrane of the cells [236, 237]. Moreover, EGFR-mediated Tyr393 phosphorylation of AGO2 disrupted this interaction and as a consequence regulated oncogenic KRAS activity, which enabled cancer progression by overcoming OIS [236].

Involvement of AGO2 was also described in CIS. First of all, we established the methods to study AGO2 in cellular senescence: MCF-7 breast cancer cells were used and treated with doxorubicin to induce senescence, since a role of AGO2 in this system was already described [229]. During drug-induced senescence, accumulation of AGO2 was found in the nucleus of non-proliferating cells as shown by immunofluorescence staining for AGO2 and the proliferation marker Ki-67, and verified by Western blot analyses of nuclear and cytoplasmic cell extracts [238]. In the transition to CIS, it was shown that the MCF-7 cells responded with induction of growth arrest and SA-β-gal activity upon treatment with the cytokines, and this was again accompanied by nuclear translocation of AGO2 [238]. To further assess the role of AGO2 in CIS, the A204 rhabdomyosarcoma cell line was used: the cytokine-sensitive cancer cells also showed the typical growth arrest accompanied by SA-β-gal activity after stimulation with IFN-γ and TNF [185, 198, 238]. For A204 cells, it was also shown that a quite fast translocation of AGO2 into the nucleus of non-proliferation cells occurred as early as 24 h after cytokine treatment [238]. After withdrawal of the cytokines, the effect was slowly reversed and AGO2 shuttled back into the cytoplasm of the cells [238].

A functional consequence of the cytokine-induced nuclear accumulation of AGO2 was the suppression of AGO2-regulated genes involved in cell cycle control: the expression of cyclin A2 (*CCNA2*), cyclin E2 (*CCNE2*), cell division cycle associated 8 (*CDCA8*), cell division control protein 2 (*CDC2*; also known as cyclin-dependent kinase 1 (*CDK1*)), and proliferating cell nuclear antigen (*PCNA*) was markedly decreased after 24 h of treatment with IFN-γ and TNF [238]. All those cell cycle control genes were repressed during the whole treatment phase with the exception of *PCNA* that returned to its basal level of expression after 96 h of treatment [238]. Lastly, by siRNA-mediated knockdown of AGO2 its influence on the cytokine-mediated growth arrest was investigated: downregulation of AGO2 during the first 48 h of cytokine treatment interfered with the induction of growth arrest and improved the growth properties of the cytokine-treated AGO2 knockdown cells in comparison with cytokine-treated cells that received a non-targeting siRNA instead [238]. This observation was also supported by the fact that no difference in the activity of SA-β-gal was found in both groups, i.e., the application of IFN-γ and TNF failed to increase the number of SA-β-gal positive cells upon knockdown of AGO2 [238].

In the context of cytokine function, Polioudakis et al. discovered by AGO2 immunoprecipitation and other assays a connection between the Myc transcription factor and miRNA-22 that regulates the

expression of cell cycle control genes as well as the response to IFN-β stimulation allowing cellular transition from the quiescent G0 phase to proliferation [239]. Taken together, AGO2 contributes to or prevents senescence induction in different ways as shown by its association with a variety of different stimuli and cell types.

2. Aim of the Thesis

In the treatment of cancer, most therapeutic approaches focus primarily on the eradication of the tumor either by surgical removal or, if possible, by targeted destruction. Among other treatment options, various strategies have also been pursued in the field of immunotherapy: on one hand it is possible to influence the patient's own immune system in order to trigger a desired anti-tumor immune response. Especially therapies with antibodies directed against important immune checkpoints such as CTLA4 or PD-1/PD-L1 (re-) activate the immune system and trigger an anti-tumor response. On the other hand, cell-based therapies are available with the oldest one being the transplantation of bone marrow. Nowadays, it is also possible to expand patient-derived immune cells *ex vivo* and re-implant them as therapeutic agents. In addition, *ex vivo* modification of patient-derived immune cells is also an option in order to specifically fight the cancer (e.g., CAR T cells). While these strategies are mainly based on the primary effector function of the immune cells used, and therefore focus on the direct killing of the targets (i.e., the malignant cells), a quite novel approach is the use of other immune cells for cancer control like the adoptive transfer of Th1 cells. Besides some cytotoxic subsets, these $CD4^+$ cells are not able to directly attack and kill the malignant cells, but they exert an anti-tumor effect via the secretion of soluble factors that act locally on the tumor. This secondary mechanism of tumor control is mediated by the combined action of their main effector cytokines IFN-γ and TNF that drive cancer cells into a state of permanent growth arrest known as cytokine-induced senescence. Therefore immune-mediated senescence induction can be regarded as a novel therapeutic option in cancer treatment.

Since cell-based therapies involve a lot of effort in regard to their approval and clinical application, the approach of using recombinant cytokines instead of transferring immune cells provides an easy way for a potential anti-tumor therapy. In this study, the treatment was based on the direct application of these Th1 cell cytokines to cancer cells. As previous work has already demonstrated a dependence on STAT1 and TNFR1, and it has also been shown that both cytokines are required for senescence induction, the aim was now to decipher the complex nature of the *in vivo* setting in a simplified cell-based model. To understand the combined and synergistic effects of IFN-γ and TNF, human cancer cells were treated for up to 96 h with a cocktail of both cytokines to induce senescence *in vitro*. During the whole treatment phase, time course samples were generated to investigate the kinetic and dynamic alterations of the cells in response to the cytokine treatment. In the first step, we therefore performed a transcriptome analysis to provide an overview on the underlying regulation of genes and signaling networks involved in the senescence induction process. Targets identified by this screening were then analyzed in detail at the protein level to further characterize their activation status and turnover under treatment with both cytokines, either alone or in combination. In addition, other senescence-associated characteristics like growth arrest and cellular proliferation were analyzed, measured, and evaluated.

3. Materials

3.1. Laboratory equipment

Table 01: List of laboratory equipment

Name	Company
Airflow Controller AC2	Waldner Laboreinrichtungen GmbH & Co. KG
Biofuge Fresco	Thermo Fisher Scientific, Inc.
Biofuge Pico	Thermo Fisher Scientific, Inc.
BioPhotometer 6131	Eppendorf AG
Cryogenic Storage System CryoCE 24K	Taylor Wharton Germany GmbH
Eppendorf Research Multichannel Pipette 300 µL	Eppendorf AG
Eppendorf Research Plus Multichannel Pipette 100 µL	Eppendorf AG
Eppendorf Research Plus Multichannel Pipette 300 µL	Eppendorf AG
Eppendorf Reference Pipette 1000 µL	Eppendorf AG
Eppendorf Reference Pipette 100 µL	Eppendorf AG
Eppendorf Reference Pipette 10 µL	Eppendorf AG
Eppendorf Research Pipette 1000 µL	Eppendorf AG
Eppendorf Research Pipette 100 µL	Eppendorf AG
Eppendorf Research Pipette 10 µL	Eppendorf AG
Eppendorf Research Plus Pipette 1000 µL	Eppendorf AG
Eppendorf Research Plus Pipette 100 µL	Eppendorf AG
Eppendorf Research Plus Pipette 10 µL	Eppendorf AG
FilterMate Universal Harvester	PerkinElmer, Inc.
Heating Block HB-LS2	VLM GmbH
HeraCell 240 CO_2 Incubator	Thermo Fisher Scientific, Inc.
HeraFreeze Ultra-low Temperature Freezer	Thermo Fisher Scientific, Inc.
HeraSafe KS 18 Class II Biological Safety Cabinet	Thermo Fisher Scientific, Inc.
IKA Magnetic Stirrer RCT Basic	IKA-Werke GmbH & Co. KG
IKA Minishaker MS 2	IKA-Werke GmbH & Co. KG
Impulse Bar Sealer HPL 450 AS	Hawo GmbH

Continuation of Table 01

Name	Company
Laboratory Balance EW 1500-2M	Kern & Sohn GmbH
Laboratory Balance Kern 770	Kern & Sohn GmbH
Laboratory pH Meter Type CG842	SI Analytics GmbH
Leitz IL DM microscope	Leica Microsystems GmbH
Liebherr Comfort Refrigerator	Liebherr-International S.A.
Liebherr Premium NoFrost Fridge-freezer	Liebherr-International S.A.
Liquid Nitrogen Storage Cylinder XL-240	Taylor Wharton Germany GmbH
Mini-PROTEAN Gel Combs (1.5 mm, 10-well and 15-well)	Bio-Rad Laboratories, Inc.
Mini-PROTEAN Short Plates	Bio-Rad Laboratories, Inc.
Mini-PROTEAN Spacer Plates (with 1.5 mm integrated spacer)	Bio-Rad Laboratories, Inc.
Mini-PROTEAN Tetra Cell Casting Stands & Casting Frames	Bio-Rad Laboratories, Inc.
Mini-PROTEAN Tetra Cell Vertical Electrophoresis System	Bio-Rad Laboratories, Inc.
Multifuge 3 S-R	Thermo Fisher Scientific, Inc.
Multiskan EX Microplate Reader	Thermo Fisher Scientific, Inc.
Nalgene Mr. Frosty Freezing Container	Thermo Fisher Scientific, Inc.
Neubauer Improved Counting Chamber	Glaswarenfabrik Karl Hecht GmbH & Co. KG
Odyssey Sa Infrared Imaging System	LI-COR Biosciences, Inc.
Pipette Controller Pipetboy Acu	Integra Biosciences AG
Platform Rocker STR8	Stuart Scientific Co. Ltd.
Power Supply PowerPac 300	Bio-Rad Laboratories, Inc.
Precision Balance CP224S-0CE	Sartorius AG
REAX Top Shaker	Heidolph Instruments GmbH & Co. KG
Scotsman Flake Ice Maker AF 156	Scotsman Ice S.r.l.
SkyLine Digital Rocking Shaker DRS-12	ELMI Ltd.
Sprout Mini Centrifuge	Heathrow Scientific, LLC.
Transfer System Trans-Blot Turbo	Bio-Rad Laboratories, Inc.
Vacuum Impulse Sealer CASO VC 10	CASO Design Braukmann GmbH
Wallac 1450 MicroBeta TriLux LSC & Luminescence Counter	PerkinElmer, Inc.
Waterbath Type 1004	GFL Gesellschaft für Labortechnik mbH

3.2. Laboratory consumables

Table 02: List of laboratory consumables

Name	Company	Catalog number
Biosphere Filter Tips 0.1-20 µL	Sarstedt AG & Co. KG	70.1114.210
Biosphere Filter Tips 2-100 µL	Sarstedt AG & Co. KG	70.760.212
Biosphere Filter Tips 1250 µL	Sarstedt AG & Co. KG	70.1186.210
Cellstar Cell Culture Flask 250 mL, 75 cm²	Greiner Bio-One GmbH	658175
Cellstar Cell Culture Flask 550 mL, 175 cm²	Greiner Bio-One GmbH	660175
Cellstar centrifuge tube 15 mL	Greiner Bio-One GmbH	188271
Cellstar centrifuge tube 50 mL	Greiner Bio-One GmbH	227 261
Cell scraper	Corning Incorporated	3010
Cryo.s freezing tube	Greiner Bio-One GmbH	126 263
Immobilon-FL PVDF Transfer Membrane	Merck KGaA	IPFL00010
Printed Filtermat A glass fiber filter 90 x 120 mm	PerkinElmer, Inc.	1450-421
Sample Bag for MicroBeta	PerkinElmer, Inc.	1450-432
Safe-lock reaction tube 0.5 mL	Eppendorf AG	0030 121.023
Safe-lock reaction tube 1.5 mL	Eppendorf AG	0030 120.086
Safe-lock reaction tube 2.0 mL	Eppendorf AG	0030 120.094
Stripette serological pipette 5 mL	Corning Incorporated	4051
Stripette serological pipette 10 mL	Corning Incorporated	4101
Stripette serological pipette 25 mL	Corning Incorporated	4251
TipOne pipette tips 10 µL	Starlab International GmbH	S1111-3000
TipOne pipette tips 200 µL	Starlab International GmbH	S1111-1006
TipOne pipette tips 1000 µL	Starlab International GmbH	S1111-6001
Tissue Culture Plate 6-well	Corning Incorporated	353224
Tissue Culture Plate 96-well	TPP Techno Plastic Products AG	92096
UV transparent disposable cuvettes	Sarstedt AG & Co. KG	67.759
Whatman 17 Chr cellulose chromatography paper	GE Healthcare Life Science	3017-915

3.3. Chemicals and reagents

Table 03: List of chemicals and reagents

Name	Company	Catalog number
[Methyl-^{3}H]-thymidine, 1 mCi	PerkinElmer, Inc.	NET027001MC
2-mercaptoethanol	Sigma-Aldrich Chemie GmbH	M3148-500ML
Albumin fraction V, from bovine serum	Carl Roth GmbH + Co. KG	8076.4
Ammonium persulfate (APS)	Merck KGaA	A3678-100G
Betaplate scint	PerkinElmer, Inc.	1205-440
Bromophenol blue, sodium salt	Carl Roth GmbH + Co. KG	A512.1
Caspase-3 Control Cell Extracts	Cell Signaling Technology, Inc.	9663
cOmplete EDTA-free Protease Inhibitor Cocktail Tablets, EASYpack	Roche Diagnostics GmbH	04693132001
Descosept AF	Dr. Schumacher GmbH	00-311-005
Dimethyl sulfoxide (DMSO)	Carl Roth GmbH + Co. KG	A994.1
Ethanol absolute (EtOH)	VWR International S.A.S.	20821.330
Glycerol	Carl Roth GmbH + Co. KG	3783.1
Glycine	Carl Roth GmbH + Co. KG	3908.2
Hydrochloric acid (HCl), fuming 37 %	Carl Roth GmbH + Co. KG	4625.1
Isopropyl alcohol	Honeywell International, Inc.	33539-2.5L
Methanol (MeOH)	Honeywell International, Inc.	32213-2.5L
Nonidet P-40 substitute (NP-40)	Sigma-Aldrich Chemie GmbH	74385
PhosSTOP Phosphatase Inhibitor Cocktail Tablets, EASYpack	Roche Diagnostics GmbH	04906837001
PageRuler Plus pre-stained protein ladder (10 to 250 kDa)	Thermo Fisher Scientific, Inc.	26619
Rotiphorese Gel 30 ready-to-use acrylamide solution 30 %	Carl Roth GmbH + Co. KG	3029.1
Sodium chloride (NaCl)	Carl Roth GmbH + Co. KG	3957.1
Sodium deoxycholate	Sigma-Aldrich Chemie GmbH	D6750-10G
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH + Co. KG	5136.1
Sodium hydroxide (NaOH)	Merck KGaA	31.05.14
Tetramethylethylenediamine (TEMED)	Merck KGaA	T9281
Tris-(hydroxymethyl)-aminomethane (TRIS), blotting grade	Carl Roth GmbH + Co. KG	0188.2
Tween 20	Carl Roth GmbH + Co. KG	9127.1

3.4. Buffers and solutions

Table 04: List of commercially available buffers and solutions

Name	Company	Catalog number
Dulbecco's Phosphate-buffered saline (PBS)	Sigma-Aldrich Chemie GmbH	D8537-500ML
Ampuwa Aqua ad iniectabilia	Fresenius Kabi AG	B23067A
Odyssey Blocking buffer (PBS)	LI-COR Biosciences, Inc.	927-40000
Intercept Blocking buffer (PBS)	LI-COR Biosciences, Inc.	927-70001
Fetal Bovine Serum (FBS)	Sigma-Aldrich Chemie GmbH	F7524
HEPES buffer solution, 1 M (50x)	Biochrom GmbH	L1613
Sodium pyruvate, 100 mM	Biochrom GmbH	L0473
MEM amino acids solution (50x)	Biochrom GmbH	K0363
Trypan Blue solution, 0.4 %	Thermo Fisher Scientific, Inc.	15250061
Penicillin/Streptomycin (Pen/Strep) (10,000 U/mL Pen & 10,000 µg/mL Strep)	Biochrom GmbH	A2213
Roswell Park Memorial Institute (RPMI) 1640 medium	Biochrom GmbH	FG1215
0.05 % Trypsin-EDTA (1x) solution	Life Technologies Limited	25300-054

Table 05: List of buffers and solutions prepared in the laboratory

Name	Composition	Concentration or Volume
Cell culture medium	RPMI 1640 medium	500 mL
	FBS	10 % (v/v)
	Pen/Strep	1 % (v/v)
	Sodium pyruvate	1 % (v/v)
	HEPES buffer	1 % (v/v)
	MEM amino acids	1 % (v/v)
Laemmli buffer (2x)	Glycerol	20 % (v/v)
	2-mercaptoethanol	10 % (v/v)
	SDS	4 % (w/v)
	Bromophenol blue	0.004 % (w/v)
	TRIS-HCl, pH 8.0	125 mM
	Adjust to pH 6.8	

Continuation of Table 05

Name	Composition	Concentration or Volume
RIPA lysis buffer	TRIS-HCl, pH 8.0	50 mM
	NaCl	150 mM
	NP-40	1.0 % (v/v)
	Sodium deoxycholate	0.5 % (w/v)
	SDS	0.1 % (w/v)
Running buffer (10x)	TRIS	248 mM
	Glycine	1.9 M
	SDS	1 % (w/v)
	For 1x Running buffer: dilute stock 1:10 in ddH_2O	
Separating gel (10 %)	ddH_2O	4.0 mL
	Acrylamide solution 30 %	3.3 mL
	TRIS buffer 1.5 M, pH 8.8	2.5 mL
	SDS solution 10 % (w/v)	100 µL
	APS solution 10 % (w/v)	100 µL
	TEMED	10 µL
Separating gel (12 %)	ddH_2O	3.3 mL
	Acrylamide solution 30 %	4.0 mL
	TRIS buffer 1.5 M, pH 8.8	2.5 mL
	SDS solution 10 % (w/v)	100 µL
	APS solution 10 % (w/v)	100 µL
	TEMED	10 µL
Stacking gel (5 %)	ddH_2O	2.8 mL
	Acrylamide solution 30 %	833 µL
	TRIS buffer 0.5 M, pH 6.8	1.25 mL
	SDS solution 10 % (w/v)	50 µL
	APS solution 10 % (w/v)	50 µL
	TEMED	5 µL
Stripping solution	NaOH	0.5 M

Continuation of Table 05

Name	Composition	Concentration or Volume
TRIS-buffered saline (TBS) (10x)	TRIS	0.2 M
	NaCl	1.37 M
	Adjust to pH 7.6	
TBS + Tween 20 (TBST) (1x)	Dilute TBS stock 1:10 in ddH_2O and add 0.05 % (v/v) Tween 20	
Transfer buffer (semi-dry)	TRIS	48 mM
	Glycine	29 mM
	SDS	0.038 % (w/v)
	MeOH	20 % (v/v)
	Adjust to pH 8.5	
TRIS buffer for separating gel	TRIS	1.5 M
	SDS	0.4 % (w/v)
	Adjust to pH 8.8	
TRIS buffer for stacking gel	TRIS	0.5 M
	SDS	0.4 % (w/v)
	Adjust to pH 6.8	

3.5. Kits and recombinant proteins

Table 06: List of kits

Name	Company	Catalog number
NucleoSpin RNA Kit	Macherey-Nagel GmbH & Co. KG	740955
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific, Inc.	23225

Table 07: List of recombinant proteins

Name	Company	Catalog number
recombinant human IFN-gamma protein 100 µg	R&D Systems, Inc.	285-IF-100
recombinant human TNF-alpha protein 20 µg	R&D Systems, Inc.	210-TA-020

3.6. Antibodies

Table 08: List of primary antibodies

Target	Reactivity	Working dilution	Company	Catalog number
β-actin	rabbit anti-human	1:1000	Cell Signaling Technology, Inc.	4970
β-actin	mouse anti-human	1:5000	Merck KGaA	MAB1501R
phospho-AKT (Ser473)	rabbit anti-human	1:2000	Cell Signaling Technology, Inc.	4060
AKT (pan)	rabbit anti-human	1:1000	Cell Signaling Technology, Inc.	4691
Caspase-3 (active + pro)	mouse anti-human	1 µg/mL	OriGene Technologies, Inc.	AM00162PU-N
c-IAP2	rabbit anti-human	1:1000	Cell Signaling Technology, Inc.	3130
p16 INK4A	rabbit anti-human	1:1000	Cell Signaling Technology, Inc.	80772
p21 Waf1/Cip1	rabbit anti-human	1:1000	Cell Signaling Technology, Inc.	2947
p27 Kip1	rabbit anti-human	1:1000	Cell Signaling Technology, Inc.	3686
phospho-p38 (Thr180/Tyr182)	rabbit anti-human	1:1000	Cell Signaling Technology, Inc.	4511
p38	rabbit anti-human	1:1000	Cell Signaling Technology, Inc.	9212
phospho-p44/42 (Thr202/Tyr204)	rabbit anti-human	1:1000	Cell Signaling Technology, Inc.	9101
p44/42	rabbit anti-human	1:1000	Cell Signaling Technology, Inc.	9102
phospho-p65 (Ser536)	rabbit anti-human	1:1000	Cell Signaling Technology, Inc.	3033
p65	rabbit anti-human	1:1000	Cell Signaling Technology, Inc.	4764
phospho-p105 (Ser933)	rabbit anti-human	1:1000	Cell Signaling Technology, Inc.	4806

Continuation of Table 08

Target	Reactivity	Working dilution	Company	Catalog number
p105/p50	rabbit anti-human	1:1000	Cell Signaling Technology, Inc.	3035
PTEN	rabbit anti-human	1:1000	Cell Signaling Technology, Inc.	9188
phospho-STAT1 (Tyr701)	rabbit anti-human	1:1000	Cell Signaling Technology, Inc.	9167
STAT1	rabbit anti-human	1:1000	Cell Signaling Technology, Inc.	9172
phospho-STAT3 (Tyr705)	rabbit anti-human	1:1000	Cell Signaling Technology, Inc.	9145
STAT3	rabbit anti-human	1:1000	Cell Signaling Technology, Inc.	4904

Table 09: List of secondary antibodies

Name	Working dilution	Company	Catalog number
IRDye 680RD goat anti-mouse IgG	1:15,000	LI-COR Biosciences, Inc.	926-68070
IRDye 680RD goat anti-rabbit IgG	1:20,000	LI-COR Biosciences, Inc.	926-68071
IRDye 800CW goat anti-mouse IgG	1:15,000	LI-COR Biosciences, Inc.	926-32210
IRDye 800CW goat anti-rabbit IgG	1:15,000	LI-COR Biosciences, Inc.	926-32211

3.7. Software programs and applications

Table 10: List of software programs and applications

Name	Version	Company
Adobe Illustrator CS6	16.0.3 (64-bit)	Adobe, Inc.
Adobe Reader DC	2019.021.20056	Adobe, Inc.
Ascent Software for Multiskan	2.6	Thermo Fisher Scientific, Inc.
EndNote X9	9.3.3	Clarivate Analytics PLC

Continuation of Table 10

Name	Version	Company
GraphPad Prism 8	8.1.0 (325)	GraphPad Software, Inc.
GNU Image Manipulator Program 2.10	2.10.18	The GIMP Team
Image Studio Lite	3.1.4	LI-COR Biosciences, Inc.
Microsoft Office Professional Plus 2010	14.0.7237.5000	Microsoft Corp.
Odyssey Sa Application Software	1.1.7	LI-COR Biosciences, Inc.
Wallac 1450 MicroBeta Windows Workstation	4.01.014	PerkinElmer, Inc.

4. Methods

4.1. Cell line

The human cancer cell line A204 (synonymously A-204) was used as a model to investigate the effects of cytokine treatment during senescence induction at the cellular level *in vitro*. Generally classified as rhabdomyosarcoma, a malignant tumor arising from undifferentiated skeletal muscle precursors, the cell line was first established in 1973 by Donald J. Giard et al. and originates from the tumor of a 13-months-old female child [240]. The cells used in this study were kindly provided by Prof. Dr. Karin Schilbach from the Department of General Pediatrics, Oncology/Hematology at the University Children's Hospital Tübingen, and were originally obtained from the American Type Culture Collection (ATCC HTB-82).

4.2. Cell culture and passaging

Human cancer cells were cultivated in RPMI 1640 medium supplemented with additives at 37 °C in a humidified incubator containing 5 % CO_2. For passaging of cells, the culture medium was taken off and discarded. Then, the cell layer was rinsed with PBS to remove residues of the serum-containing medium completely. After taking off the PBS, trypsin-EDTA solution was added to the flask to detach the adherent cells. The flask was incubated for 5 min at 37 °C in the incubator before culture medium was added to stop the effect of the trypsin-EDTA solution. The cell suspension was then transferred to a tube and centrifuged at 400 x g for 5 min. After discarding the supernatant, the cell pellet was resuspended in fresh culture medium, the number of living cells was determined by cell counting, and the desired cell density was seeded to new cell culture vessels.

4.3. Cell counting

The number of living cells was determined under a Leitz IL DM microscope from Leica Microsystems by Trypan blue exclusion using a Neubauer improved counting chamber, 10 µL of the diluted cell suspension, and the following equation:

$$\text{cells/mL} = \left(\frac{(\text{number of cells counted x dilution factor})}{\text{number of large squares counted}} \right) \text{x } 10{,}000$$

4.4. Freezing and thawing of cells

For the cryopreservation, cells were resuspended in FBS supplemented with 10 % DMSO and filled in freezing tubes, which were then placed in a pre-cooled Mr. Frosty freezing container and stored at -80 °C until the next day. The frozen cells were later transferred to a cryogenic storage system cooled by liquid nitrogen for long-term preservation.

For experimental purposes, the cells were thawed by putting the frozen tubes for approximately 90 sec in a water bath at 37 °C. The cells were subsequently pipetted into a 15 mL tube containing warm medium, then centrifuged and, after resuspension in fresh medium, seeded onto suitable culture flasks.

4.5. *Mycoplasma* testing

Cell culture supernatants were collected in 1.5 mL tubes, shortly heat-inactivated at 95 °C, centrifuged and 100 µL of the cell-free solution of each sample were then used for a *mycoplasma* test via polymerase chain reaction that was routinely performed on a monthly basis at the Department of Dermatology, University Hospital Tübingen. Only *mycoplasma*-free batches of cells were used for experiments.

4.6. Cytokine treatment

Human cancer cells were treated *in vitro* with recombinant human cytokines IFN-γ [100 ng/mL] and TNF [400 pg/mL] either in combination or as single treatments for the indicated period of time [185, 238]. The cytokines were dissolved in sterile PBS. Adequate amounts of PBS were added to the culture medium and served as solvent control for the mock treatment.

4.7. RNA isolation

To isolate RNA, the cells were harvested from 6-well plates as follows: first, the supernatant was removed and discarded. The wells were then filled with PBS to wash the cells. After removing the PBS, lysis buffer RL1 (including tris-(2-carboxyethyl)-phosphine (TCEP) as a reducing agent) was directly added into the wells to harvest the cells with a cell scraper. The samples were transferred to 1.5 mL tubes and stored at -80 °C until RNA isolation. RNA isolation was performed with the NucleoSpin RNA kit from Macherey-Nagel according to the manufacturer's instructions (Protocol 5.1 "RNA purification from cultured cells and tissue" 02/2013, Rev. 15). In brief: the cell lysates were purified using different columns and buffers to bind nucleic acids. The genomic DNA was then digested on-column using DNase and, after subsequent washing and drying steps, the RNA was finally eluted in nuclease-free water. Lastly, the concentration of the isolated RNA samples was determined using an Eppendorf BioPhotometer 6131.

4.8. Microarray measurement

For the examination of the cellular transcriptome, the RNA samples were sent overnight on dry ice to the Pasteur Institute in Paris, France. The quality control of the RNA was performed with the Agilent 2100 Bioanalyzer system. For the subsequent analyses, only samples with a RNA integrity number (RIN) greater than 8 were included. 100 ng RNA per sample were used and measured with the GeneChip Scanner 3000 system from Affymetrix according to the manufacturer's instructions.

The preparation of cRNA as well as the hybridization and scanning of the Affymetrix Human Transcriptome Arrays 2.0 was performed by Grégory Doré, a technician in the research group of Dr. Oliver Bischof at the Pasteur Institute in Paris, France. After measurement, the raw array intensity data were provided as CEL files for the following bioinformatic processing.

4.9. Bioinformatic processing and data mining

The complete raw data sets were analyzed by Dr. Mohamed Ali Jarboui from the Core Facility for Medical Bioanalytics at the Institute for Ophthalmic Research, University Hospital Tübingen. All of the generated results were discussed, and the figures were then kindly provided and approved for use in the context of this thesis.

4.10. Protein extraction

Cell lysates were prepared on 6-well plates as follows: after the supernatant was taken off and discarded, the wells were rinsed with PBS. Once the PBS was removed, the plates were put on ice and lysis buffer was pipetted into each well. For cell lysis RIPA buffer supplemented with Roche's protease inhibitor (cOmplete tablets) and phosphatase inhibitor (PhosSTOP tablets) cocktails was used. The cells were then detached using a cell scraper and the lysates were collected in 1.5 mL tubes. In order to solubilize the cells completely, the samples were incubated on ice for 45 min and vortexed every 10 min. Subsequently, the lysates were centrifuged for 5 min at maximum rotational speed to pellet the remaining cellular debris before the cleared supernatants were transferred to new 1.5 mL tubes. The protein concentration of each lysate was either determined directly after preparation or the samples were stored at -80 °C until measurement.

4.11. Quantitation of protein concentration and sample preparation

The protein concentration of the cell lysates was determined using the colorimetric Pierce bicinchoninic acid (BCA) protein assay kit from Thermo Fisher Scientific. The assay was performed according to the manufacturer's protocol. In brief: first, diluted albumin standards were prepared in lysis buffer (i.e., RIPA) covering a concentration range starting from 50 ng/mL up to 1500 ng/mL of protein. Then, 10 µL of each of the diluted albumin standards and the samples were pipetted into a 96-well plate in duplicates, while RIPA buffer served as blank. Subsequently, 200 µL of BCA working reagent were added to each well and the solution was mixed by gently shaking the plate. After incubating the plate 30 min at 37 °C followed by cooling to RT, the absorbance was measured at 540 nm with the Multiskan EX photometric microplate reader (Thermo Fisher Scientific) and the protein content of each sample was calculated. For the following analyses, 10 µg of each lysate were transferred to 1.5 mL tubes and filled up with RIPA buffer to 12.5 µL if necessary. The samples were then mixed with 12.5 µL Laemmli buffer (2x) and incubated at 95 °C for 5 min to denature the proteins. When the samples had cooled down they were ready for further processing.

4.12. SDS-PAGE

To separate proteins according to their molecular weight, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Laemmli method [241]. First of all, the necessary gels were cast using the respective equipment of the Mini-PROTEAN Tetra cell system from Bio-Rad: casting stand, casting frames, glass plates (short and spacer plates), and the respective gel mixtures. Each gel consists of a lower (bigger) part, the so-called separating gel, and an upper (smaller) part named stacking gel. The separating gel was poured first between two glass plates and covered with a layer of isopropanol to allow polymerization of the acrylamide. After polymerization of the separating gel the isopropanol was taken off and the stacking gel was poured on top of it. A comb was then inserted into the liquid gel to form the loading wells for the samples.

When the polymerization of the stacking gel was completed, the comb was removed and the gel was ready for use. The glass plates containing the gels were then placed in the running module, which in turn was placed inside the tank. After filling the tank with running buffer (1x), the wells of the gels were loaded with the prepared samples and a pre-stained protein ladder before the lid was put on and an electric field was applied (voltage and duration of the individual runs were dependent on the percentage of the used gels, e.g., 90 V and 120 min for a 12 % gel). Once electrophoresis was completed, denatured proteins (i.e., unfolded polypeptide chains) were separated from each other by their size and prepared for the following transfer onto hydrophobic polyvinylidene fluoride (PVDF) membranes.

4.13. Western blotting

After separation of the proteins, the gels were carefully peeled off the glass plates and placed on pieces of Whatman filter paper that were previously soaked in semi-dry transfer buffer. Next, the blotting sandwiches were assembled by placing methanol-activated and transfer buffer-rinsed Immobilon FL PVDF membranes on top of the gels, followed by another piece of buffer-soaked filter paper. The prepared blotting sandwiches were then placed upside down on the bottom of the transfer cassette (anode) and a roller was used to remove air bubbles from within the different layers. When the lid (cathode) was put on, the whole cassette was inserted into the Trans-Blot Turbo transferring system from Bio-Rad and semi-dry transfer was performed using the pre-installed protocol for 1.5 mm gels (2.5 A, 25 V, 10 min). Once the transfer was completed, the membranes carrying the proteins were blocked at RT for at least 1 h with Odyssey Blocking buffer (or later with its successor, the Intercept Blocking buffer) from LI-COR Biosciences. After blocking, the membranes were incubated overnight at 4 °C on a rocking platform with the primary antibodies for the respective target of interest and a reference protein (β-actin). The next day, the antibody solution was removed and the membranes were washed three times for 15 min with TBST (1x) before being incubated at RT for 1 h with the secondary antibodies. Following incubation, the membranes were washed four times with TBST (1x) and once with TBS (1x) for 10 min each and subsequently measured with LI-COR's Odyssey Sa

infrared imaging system: images were taken using the Odyssey Sa Application software, while they were later on adjusted and analyzed with the Image Studio Lite software provided by LI-COR Biosciences. If additional targets were to be analyzed on the same blot, the membrane was stripped after imaging with 0.5 M NaOH for 10 min to remove the bound antibodies. Afterwards, the membrane was washed twice with TBST (1x) for 10 min and blocked again. All subsequent steps were then performed as previously described above.

4.14. Semi-quantitative densitometric analysis

On basis of the measured intensity values for the respective protein bands, a target/reference ratio was calculated. In case of negative intensity measures obtained for the target protein, the values were set as 1 to allow calculation of the target/reference ratio.

4.15. Proliferation assay using [^{3}H]-thymidine incorporation

Cellular proliferation during the senescence induction process was assessed by the incorporation of [^{3}H]-thymidine into the cells' DNA [185, 238]. In detail, the cancer cells were seeded onto 96-well plates and treated with the recombinant cytokines (IFN-γ and/or TNF) for the indicated period of time. For short time samples (i.e., 6 h and 12 h) 10 µL of diluted [^{3}H]-thymidine [0.25 µCi] were added in parallel to each well until the end of incubation. In all other cases (i.e., 24 h, 48 h, 72 h, and 96 h), the [^{3}H]-thymidine solution was applied 24 h before the incubation ended. The incubation was stopped by freezing the samples at -20 °C, and the plates were stored in the freezer until all probes had been collected. After thawing, the 96-well plates were harvested using the equipment of a FilterMate Harvester (PerkinElmer) to collect the cellular DNA on glass fiber filter mats (PerkinElmer's Printed Filtermat A). Once the filter mats had dried, they were placed in sample bags which were sealed after addition of the scintillation cocktail (PerkinElmer's Betaplate scint). The samples were then mounted into filter cassettes and placed inside a MicroBeta TriLux 1450 LSC & luminescence counter (PerkinElmer), and the amount of incorporated [^{3}H]-thymidine was quantified.

4.16. Growth arrest assay

The growth arrest assay was performed *in vitro* as essentially described before [185]. Human cancer cells were seeded with a density of 0.2 x 10^6 cells/well in 2 mL culture medium on 6-well plates (passage -1). The next day, the cells were treated for 96 h with the recombinant cytokines or PBS as previously mentioned (see chapter 4.5). At the end of the 96 h incubation, the treatment was stopped by removing the supernatant and washing the cells once with PBS. The cells were then trypsinized and the number of living cells was determined by Trypan blue exclusion. Subsequently, the cells were reseeded on 6-well plates (passage 0) with the initial density of 0.2 x 10^6 cells/well in 2 mL culture medium and grown until confluency of the mock-treated control cells. After determination of the cell numbers at this stage (passage 1) the cells were reseeded for an additional passage and again

trypsinized and counted when the control cells reached confluency. At this point (passage 2) the growth assay was stopped and evaluated. The following equation was used to calculate the proliferation index of the cells:

$$\text{Proliferation index} = \left(\frac{\text{number of counted cells}}{\text{number of seeded cells}}\right) \text{ x proliferation factor of previous passage}$$

4.17. Statistical analysis

Statistical analysis was performed and graphs were built with GraphPad Prism version 8 (GraphPad Software, Inc.). Values were represented as mean ± standard deviation (SD). Nonparametric one-way analysis of variance (ANOVA) was performed by the Kruskal-Wallis test and Dunn's multiple comparisons test was used as post hoc test. P-values < 0.05 were considered as statistically significant.

5. Results

Previous work already established the concept of CIS and demonstrated a dependency on the simultaneous presence of both Th1 cell cytokines IFN-γ and TNF [185, 187]. Furthermore, the human A204 cancer cell line used in this thesis has already been described as a useful and reliable model system for the *in vitro* study of CIS [185, 198, 238]. To further investigate the cytokine-mediated effects on cancer cells in more detail, different *in vitro* assays and analyses were performed to enable a comparison between the single treatments and the dual application of IFN-γ and TNF.

5.1. The combined action of IFN-γ and TNF is needed for senescence induction of human cancer cells *in vitro*

One of the most characteristic features of cellular senescence is the permanent stop of proliferation, known as growth arrest. Therefore, *in vitro* growth arrest assays based on the previously published procedure were performed. This was done to assess the senescence-inducing effect of the double treatment with IFN-γ and TNF as compared to the outcome of single treatments with these cytokines [185, 198, 238]. While single treatment with TNF for 96 h had virtually no effect on the cell growth during the subsequent passages, IFN-γ alone mediated an initial growth decline, which was not sufficient to stably arrest the cells during further passaging. In contrast, only the combined action of IFN-γ and TNF was able to induce a stable growth arrest that persisted over two passages after the end of the treatment (Figure 03).

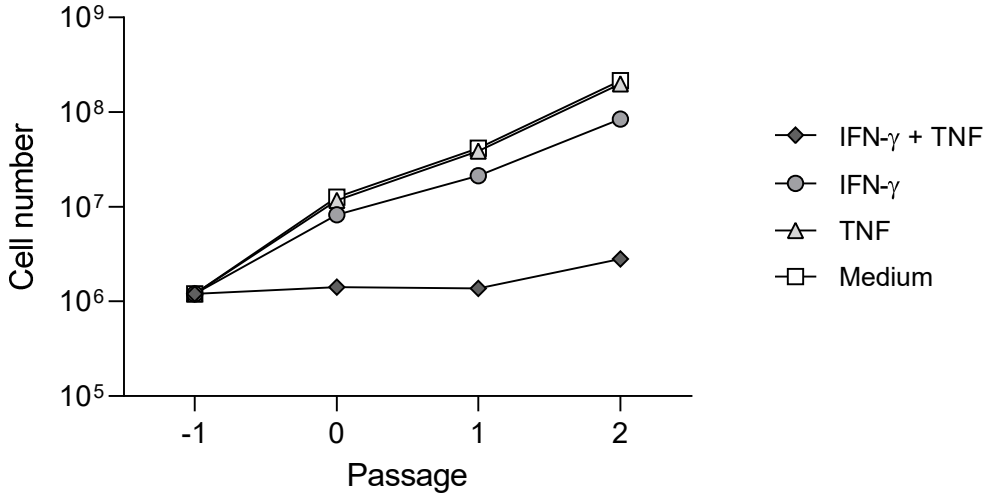


Figure 03: IFN-γ and TNF induce a stable and long-lasting growth arrest only in combination, but not as single treatments. Human A204 cancer cells were treated for 96 h with IFN-γ [100 ng/mL] and TNF [400 pg/mL] either alone or in combination. Medium containing PBS served as control. After the incubation the cells were washed, counted, and cultivated for two additional passages in the absence of the cytokines (passage 1 and 2). A representative growth arrest assay is shown and the results were independently reproduced three times with similar outcome.

Since the *in vitro* growth arrest assay clearly demonstrated the cytokine-mediated senescence induction in the cancer cells after the continuous 96 h treatment, the next objective was to investigate how the growth behavior of the tumor cells changes during the treatment period. For this purpose, an *in vitro* proliferation assay was used to measure the cellular proliferation by uptake and incorporation of radioactively labeled [^{3}H]-thymidine into the DNA of the cells [185, 238].

As for the *in vitro* growth arrest assay, the proliferation was analyzed in the presence of both cytokines alone or in combination. Although measurable uptake of [^{3}H]-thymidine already occurred after 6 h of incubation in all groups, distinct differences between the groups were first observed after 24 h of treatment. These differences were even more pronounced during the next 72 h of treatment. In comparison with the results of the *in vitro* growth arrest assay (Figure 03), the outcome of the *in vitro* proliferation assay was similar: while the medium control showed normal and enhanced proliferation of the cells over time, administration of TNF alone was not able to reduce the cellular proliferation as seen for the treatment with IFN-γ, where a marked decrease in the counts per minute was observed after 96 h. However, only the combined treatment with both cytokines led to a significant reduction of incorporated [^{3}H]-thymidine that prevailed over time, indicating the stop of proliferation and induction of growth arrest in these cells (Figure 04).

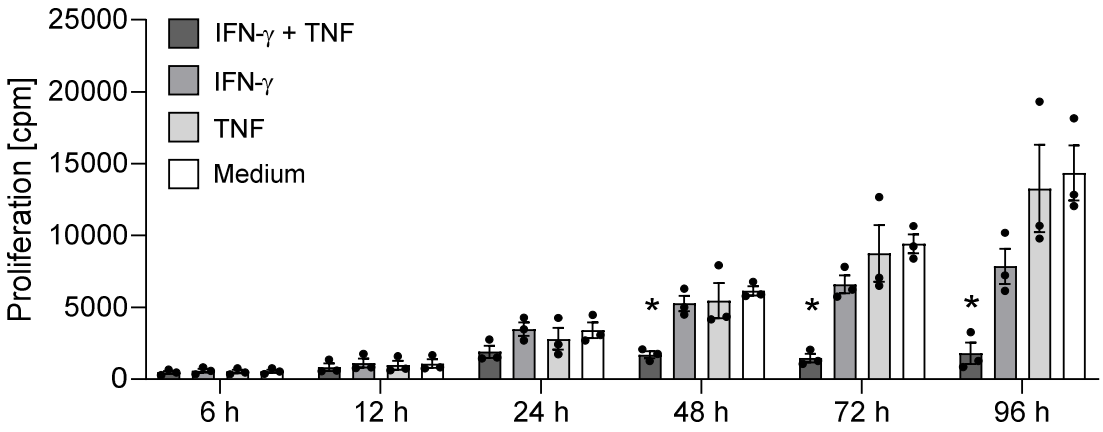


Figure 04: IFN-γ and TNF inhibit the cellular proliferation only in combination, but not as single treatments. Human A204 cancer cells were treated for up to 96 h with IFN-γ [100 ng/mL] and TNF [400 pg/mL] either alone or in combination. Medium containing PBS served as control. [^{3}H]-thymidine was added to the respective samples 24 h before the end of incubation. Only for the short-time incubation, the samples received [^{3}H]-thymidine in parallel to the cytokine treatment for 6 h and 12 h, respectively. Afterwards, the incorporation of [^{3}H]-thymidine into the DNA of a given cell population was measured in counts per minute (cpm). Data are given as mean ± SD (n=3), asterisks indicate significant differences (p-value < 0.05) between treatment (IFN-γ + TNF) and control (medium).

The next step was to elucidate changes in the cellular gene expression during the senescence induction process, since the growth-inhibitory effect of the double treatment with IFN-γ and TNF already occurred as early as 24 h and clearly lasted over the whole treatment phase of 96 h as shown by the *in vitro* proliferation assay.

5.2. Time-dependent changes in the cellular transcriptome during the cytokine-mediated senescence induction process

A transcriptome analysis was used to examine alterations in the global gene expression pattern upon stimulation with IFN-γ and TNF over the course of time as illustrated by the heatmap (Figure 05). In general, the following pattern was observed: genes found to be strongly expressed in proliferating cells were downregulated by the cytokine treatment over time, whereas initially repressed genes were instead induced. However, some of these genes were indeed upregulated in response to the cytokine stimulation, but their expression was not long-lasting and declined during further incubation.

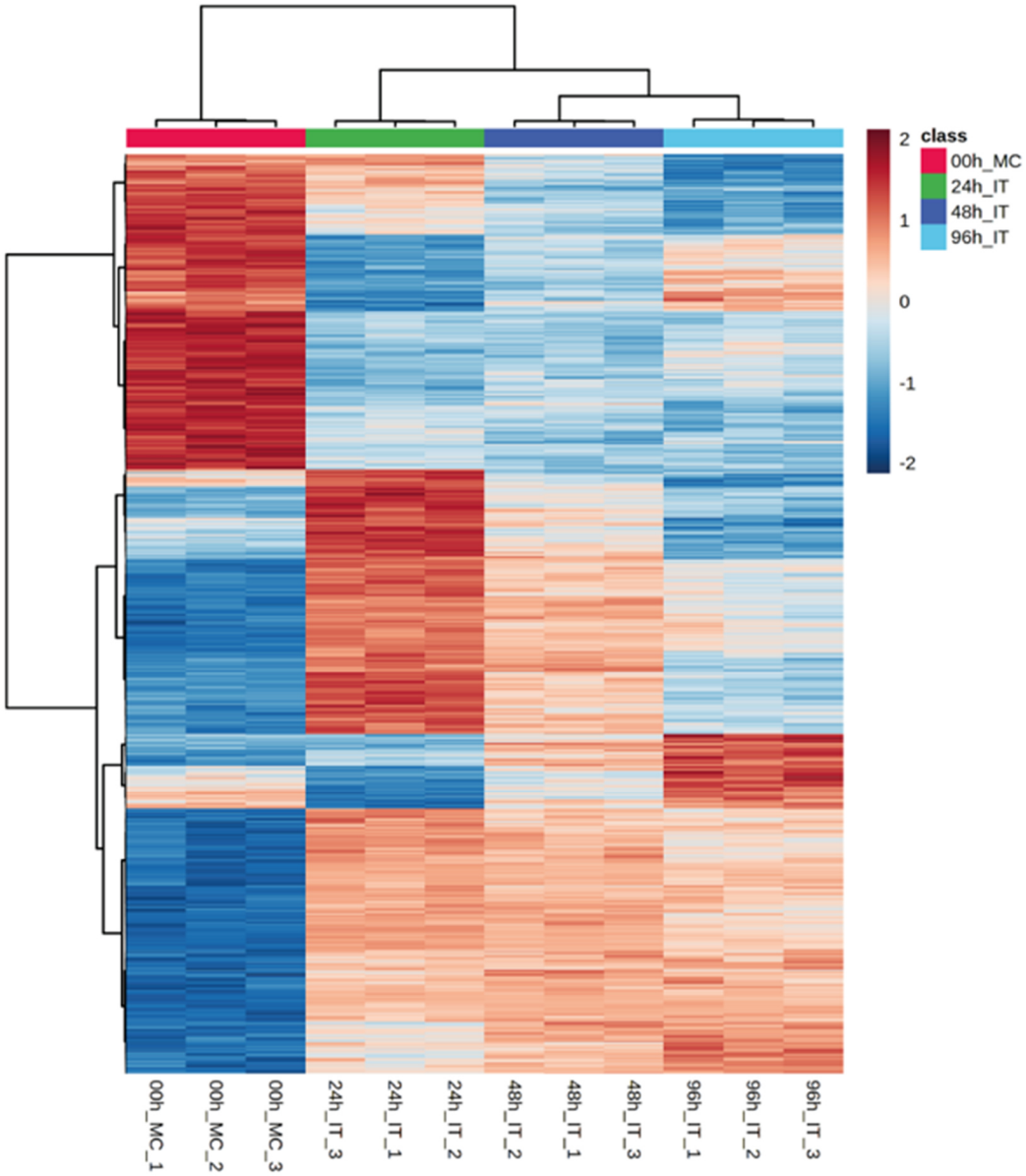


Figure 05: IFN-γ and TNF alter the transcriptomic landscape of human cancer cells during senescence induction. RNA samples isolated from human A204 cancer cells after 24 h, 48 h, and 96 h of treatment with IFN-γ [100 ng/mL] and TNF [400 pg/mL] were analyzed and compared to the respective 0 h medium control using RNA microarray technology. The heatmap depicts the general change in the expression of protein-coding genes identified in all replicates from all groups over time. Each column represents an individual sample from batches that were generated in independent experiments, respectively (n=3).

The basis for this heatmap representation was the hierarchical clustering of the individual samples, which is shown in the appendix (Supplementary Figure 01). In this context, also the principal component analysis (PCA) of the different time points showed a distinct cluster formation and grouping of the samples from each time point analyzed (Supplementary Figure 02). The time-dependent change in the gene expression of the cytokine-treated and thereby senescence-prone cells is also reflected in the following chart. The Venn diagrams below show the number of up- and downregulated genes that are unique to each condition, as well as shared by the different conditions. Regarding the upregulated genes, a core consisting of 276 common genes was found to be highly induced by the cytokine treatment with IFN-γ and TNF across all time points (Figure 06 A), whereas a total of 174 genes were generally repressed and downregulated in all settings (Figure 06 B).

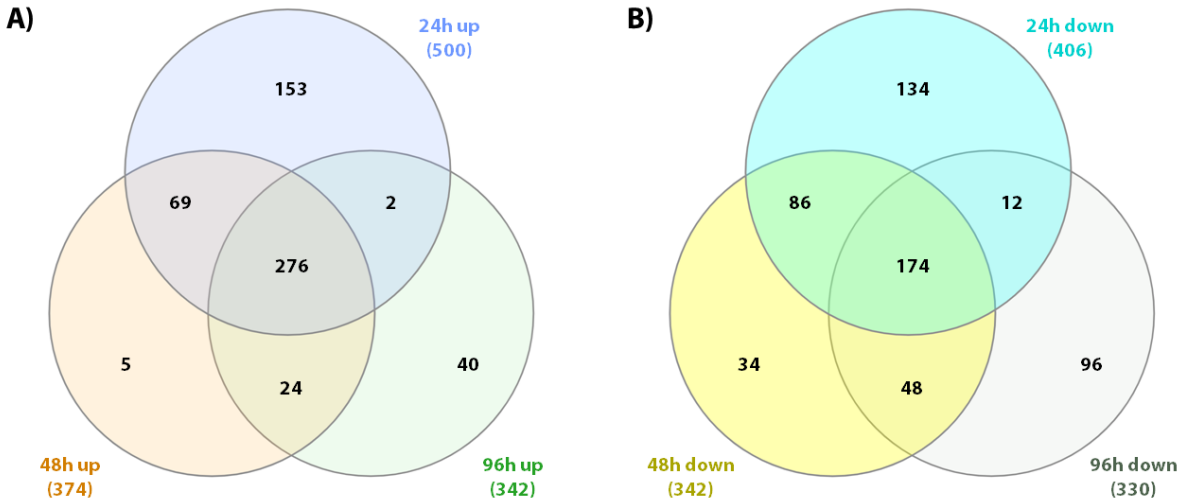


Figure 06: IFN-γ and TNF influence the gene expression in a time-dependent manner during senescence induction. To better visualize the time-dependent changes in the gene expression between the different time points (24 h, 48 h, and 96 h) triple intersections were created for induction and repression. A) Venn diagram comparing significantly upregulated genes in human A204 cancer cells after 24 h, 48 h, and 96 h of treatment with IFN-γ [100 ng/mL] and TNF [400 pg/mL] in relation to the basal expression of the respective 0 h medium control. B) Venn diagram comparing the significantly downregulated genes in human A204 cancer cells after 24 h, 48 h, and 96 h of treatment with IFN-γ [100 ng/mL] and TNF [400 pg/mL] in relation to the basal expression of the respective 0 h medium control. Numbers in parentheses indicate the total amount of genes associated with each condition.

This differential gene regulation was further assessed by a gene ontology (GO) enrichment analysis. The main purpose of such an analysis is to see which GO terms are either over- or underrepresented within a given set of genes found to be regulated. The annotations assigned to each GO term are based on curated biological processes and molecular functions that were already identified for each gene. The gene set analyzed here includes the upregulated genes after 96 h of treatment with IFN-γ and TNF (Figure 07). Based on the enriched GO terms and categories different color-coded clusters (C1 – C9) were formed to group processes and functions associated with one another. All other GO terms were instead regarded as unassigned and therefore not grouped with a distinct cluster (Figure 07).

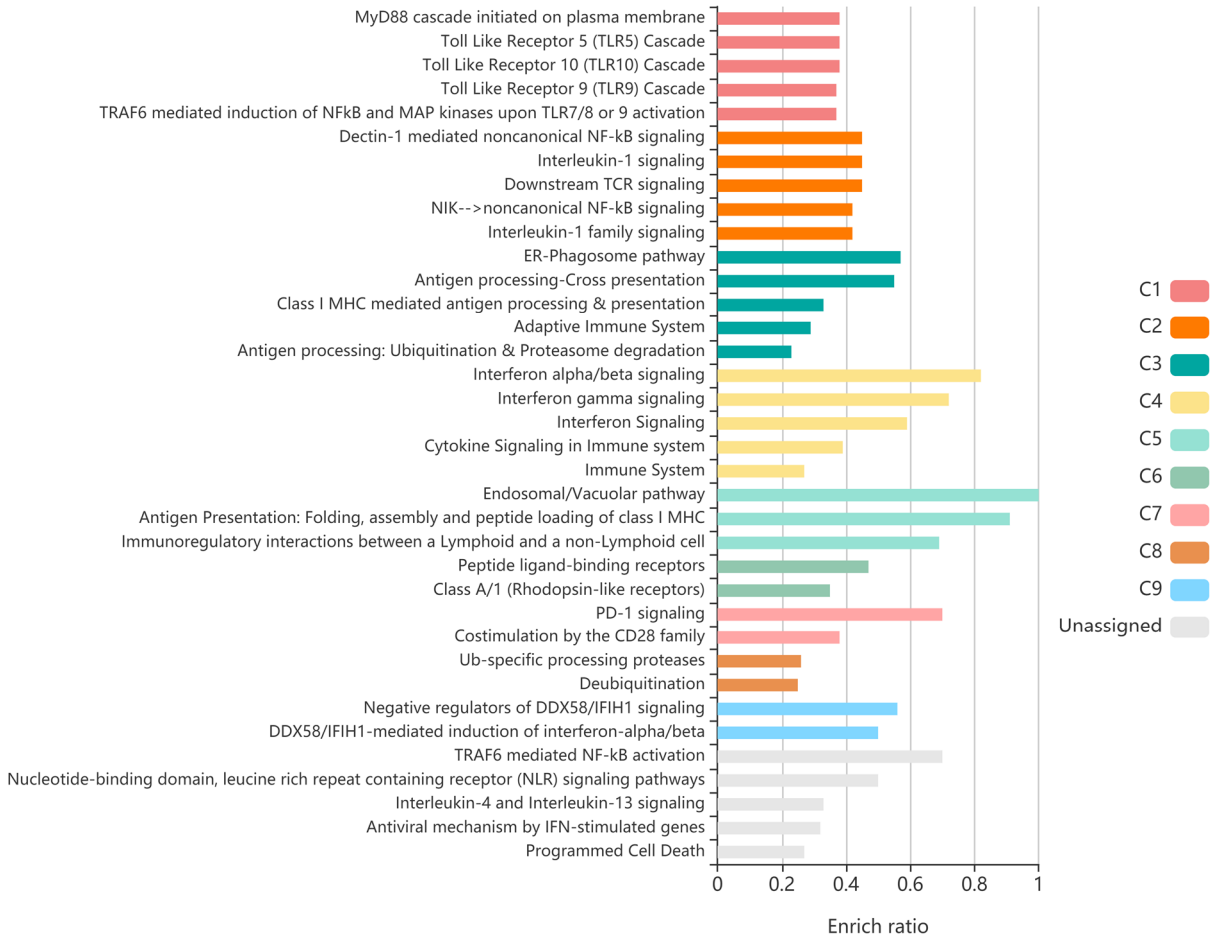


Figure 07: GO enrichment analysis of differentially expressed genes after 96 h of treatment with IFN-γ and TNF. The bar chart depicts the enrichment ratios of GO terms that are assigned to the significantly upregulated genes after 96 h of treatment with IFN-γ [100 ng/mL] and TNF [400 pg/mL]. Coding genes with a t-test-based p-value < 0.05 were clustered in functionally similar groups gathered from the KEGG pathway database.

In general, the GO enrichment analysis revealed many immune-related processes and functions that were upregulated in response to the cytokine treatment. Especially the interferon-associated GO terms of cluster C4 as well as the antigen-related processes of cluster C5 and C3 were highly enriched. Moreover, many processes involved in NF-κB activation were found among the GO terms of different clusters such as C1 and C2, or within the unassigned GO terms (Figure 07).

Since most of the biological processes and molecular functions identified by the GO enrichment analysis are related to effects mediated by IFN-γ and TNF, a pathway and network analysis was performed. One of the important pathways found to be highly regulated on basis of the differentially expressed genes was the JAK/STAT signaling axis (Figure 08). Upregulation of important factors within this IFN-γ stimulated signaling pathways was observed for cytokines and their receptors, the JAKs and STATs, as well as interferon regulatory factors (IRFs) such as IRF9, whereas negative regulators of this pathway like suppressors of cytokine signaling (SOCS) or protein inhibitor of activated STATs (PIAS) members were found to be downregulated after 96 h of cytokine treatment.

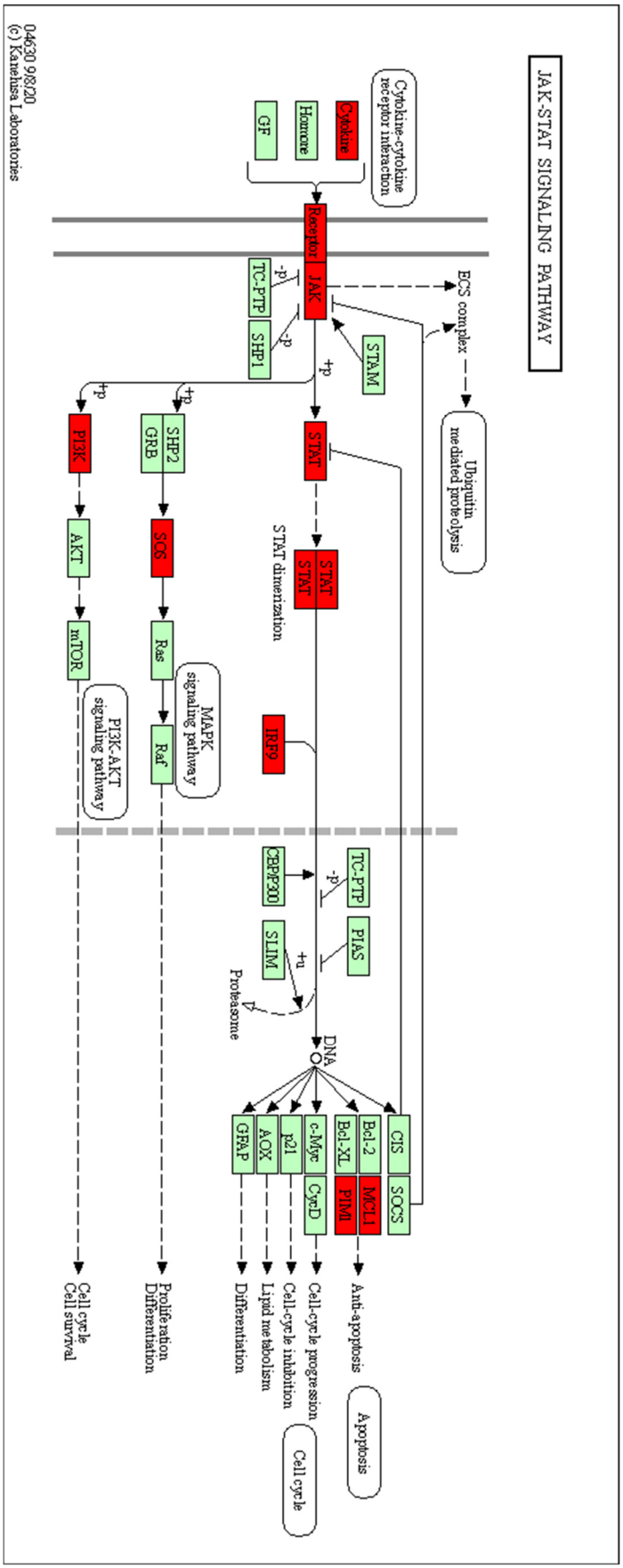


Figure 08: The JAK/STAT signaling pathway is a major driver of CIS. Pathway analysis after 96 h of treatment with IFN-γ [100 ng/mL] and TNF [400 pg/mL] identified the JAK/STAT signaling pathway as one of the essential signaling networks leading to CIS. Red indicates upregulated genes, green indicates non- or downregulated genes.

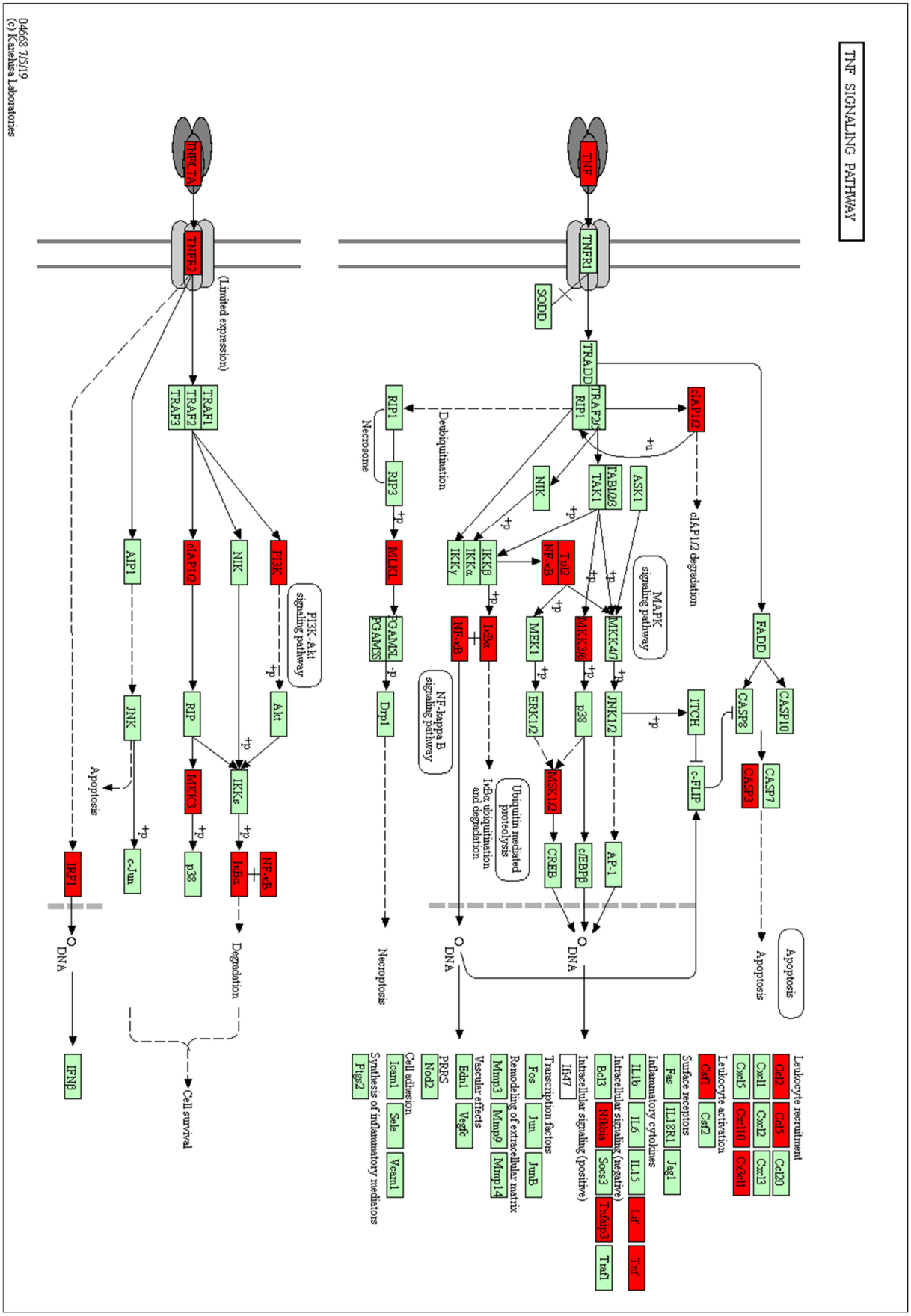


Figure 09: The TNF signaling pathway is another major driver of CIS. Pathway analysis after 96 h of treatment with IFN-γ [100 ng/mL] and TNF [400 pg/mL] identified the TNF signaling pathway as another essential signaling network leading to CIS. Red indicates upregulated genes, green indicates non- or downregulated genes.

While factors associated with cell cycle progression (e.g., cyclin D) or its inhibition (e.g., p21) were also downregulated, upregulation was found for the anti-apoptotic factors MCL1 and PIM1 that are also connected to the cell cycle and its regulation. Moreover, the active JAK/STAT signaling had also an influence on other cellular pathways, namely the MAPK signaling and the phosphatidylinositol 3-kinase (PI3K)/AKT axis. These findings collectively highlight the importance of interferon-driven signaling during the whole senescence induction process of 96 h that finally establishes CIS.

The pathway and network analysis further revealed that the TNF signaling pathway plays also a crucial role and has to be considered as the second important signaling cascade of CIS (Figure 09). As seen for the JAK/STAT signaling, several factors within the TNF pathway were still upregulated after 96 h of treatment with the cytokines. These factors include TNF itself as well as its receptor TNFR2. Most striking was the induction of NF-κB and related components (e.g., IκBα or TNFAIP3), since this transcription factor plays a pivotal role in the regulation of many different processes, including cell death and survival. Surprisingly, genes associated with apoptosis were also upregulated in response to the cytokines. However, not only pro-apoptotic factors (e.g., caspase-3) were strongly upregulated, but also anti-apoptotic factors (e.g., c-IAP1/2) that counteract apoptosis and secure cellular survival. In contrast to NF-κB, other transcription factors were downregulated after 96 h. These include the activator protein 1 (AP-1) and its components (i.e., Fos, c-Jun, or JunB), as well as related factors like JNK1/2. A downregulation was also observed for other kinases of the MAPK signaling, such as ERK1/2 or the p38 family. The signaling associated with TNFR2 showed a similar regulation of such factors and also indicated a general downregulation of AKT, which was already observed in the analysis of the JAK/STAT signaling. Lastly, also members of the cytokine/chemokine family (e.g., CCL2, CCL5, and others) were strongly induced, while other secreted factors that are typically regarded as SASP components, such as IL-1b, IL-6, or IL-15 were instead downregulated. Together, these findings demonstrate that the long-lasting TNF signaling during the 96 h treatment period is a core component of CIS.

In addition, a variable importance in projection (VIP) analysis was performed. This final approach was used to identify key genes that contribute to the segregation between the biological groups of the time course data sets. A high VIP score correlates with a greater importance of the respective factor within the underlying network. By ranking the VIP scores from top to bottom, the top 35 contributing factors were identified as listed below (Figure 10). Generally spoken, most of these factors represent interferon-regulated genes, which further reflects the predominant role of the interferon signature in CIS. While the vast majority of these genes (including *STAT1*) were induced and upregulated in response to the cytokine treatment, some cytokine-mediated effects pointed in the opposite direction for others. These genes (i.e., *NID1*, *PEG10*, and *SPARC*) showed a downregulated expression over time.

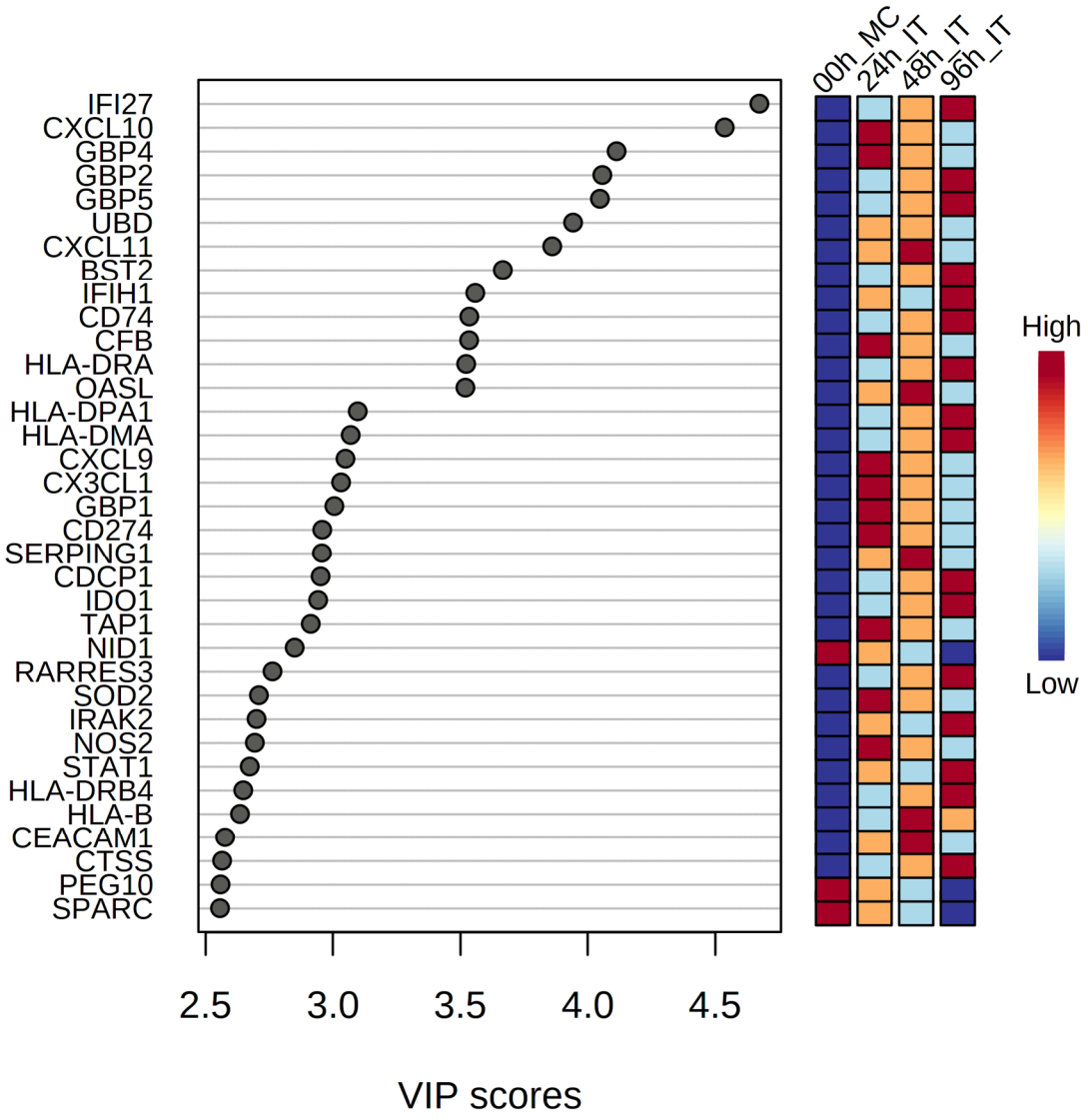


Figure 10: Representation of the most relevant genes that distinguish CIS from proliferating cancer cells. The graph shows in descending order the VIP score ranking of the top 35 genes that are most relevant for the variance between the cytokine-treated cells (IT) and the respective medium control (MC). The colored boxes on the right side indicate either an increase (red) or a decrease (blue) in the expression of the respective gene through the cytokine treatment at the given time point.

5.3. Impact and kinetics of continuous cytokine treatment on the activation status and the total protein turnover of selected targets

In order to verify the RNA data and the associated gene regulation within the underlying signaling networks, the activation state as well as the total protein level of selected targets was investigated. Western blot analyses covering the whole senescence induction phase for up to 96 h were performed for the double treatment and the single treatments with IFN-γ and TNF to allow a direct comparison of the respective effects over time.

5.3.1. Fundamental IFN-γ- and TNF-regulated signaling networks

STAT1 is the main STAT protein transducing the IFN-γ signal in the cells via the JAK/STAT pathway. The transcriptome analysis revealed a strong induction and upregulation of genes relevant for this signaling cascade, including the *STAT* family (Figure 08). Since the screenings provided a mere overview on the underlying gene regulation over time, Western blot analyses were used to determine the protein expression of STAT1 in response to the cytokine stimulation.

As expected, treatment with IFN-γ and TNF led to a fast and considerable activation of STAT1 by reversible phosphorylation after 6 h. Surprisingly, this initially high level of phosphorylated STAT1 did not decline during the following hours and was persistent over time and detectable for up to 96 h (Figure 11 A & D). Besides the long-lasting phosphorylation of STAT1, the protein expression was also induced upon treatment with both cytokines and the total protein accumulated over time leading to the highest STAT1 levels after 96 h (Figure 11 A & E).

Regarding the application of IFN-γ alone, a similar effect on the STAT1 activation and expression was found as for the double treatment: phosphorylation of STAT1 was detected as early as 6 h and activated STAT1 levels were abundant until 96 h of treatment (Figure 11 B & F). Moreover, total STAT1 levels were rising during the whole treatment phase and accumulation of the unphosphorylated protein peaked after 96 h (Figure 11 B & G).

As opposed to the observations made for the treatments with IFN-γ alone or in combination with TNF, the single treatment with TNF alone failed to induce both activation and expression of STAT1 during the complete course of time that was analyzed (Figure 11 C, H & I).

In addition to STAT1 the regulation of STAT3 was also analyzed, as the transcriptome analysis highlighted a long-lasting induction and upregulation of the *STAT3* gene (data not shown). The Western blot data for activation and expression of the STAT3 protein are shown in the appendix (Supplementary Figure 03 A – I).

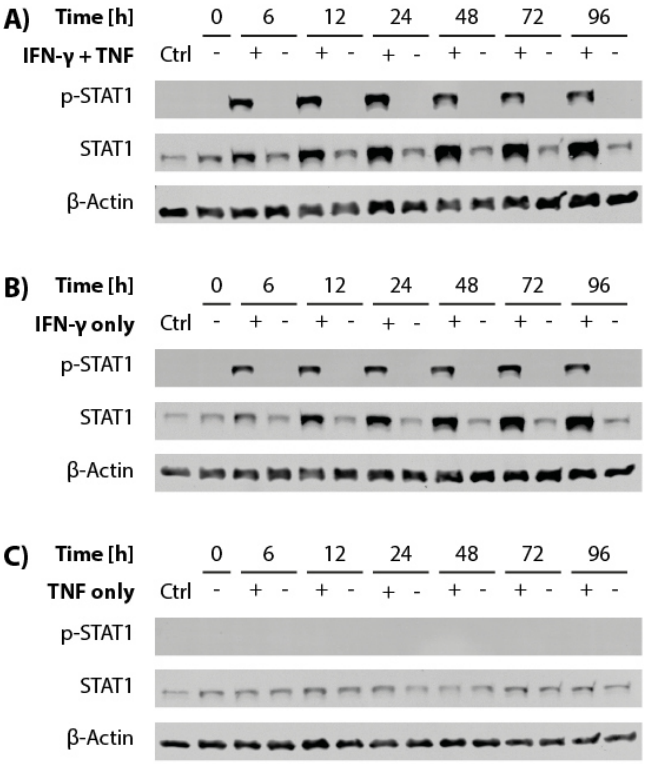
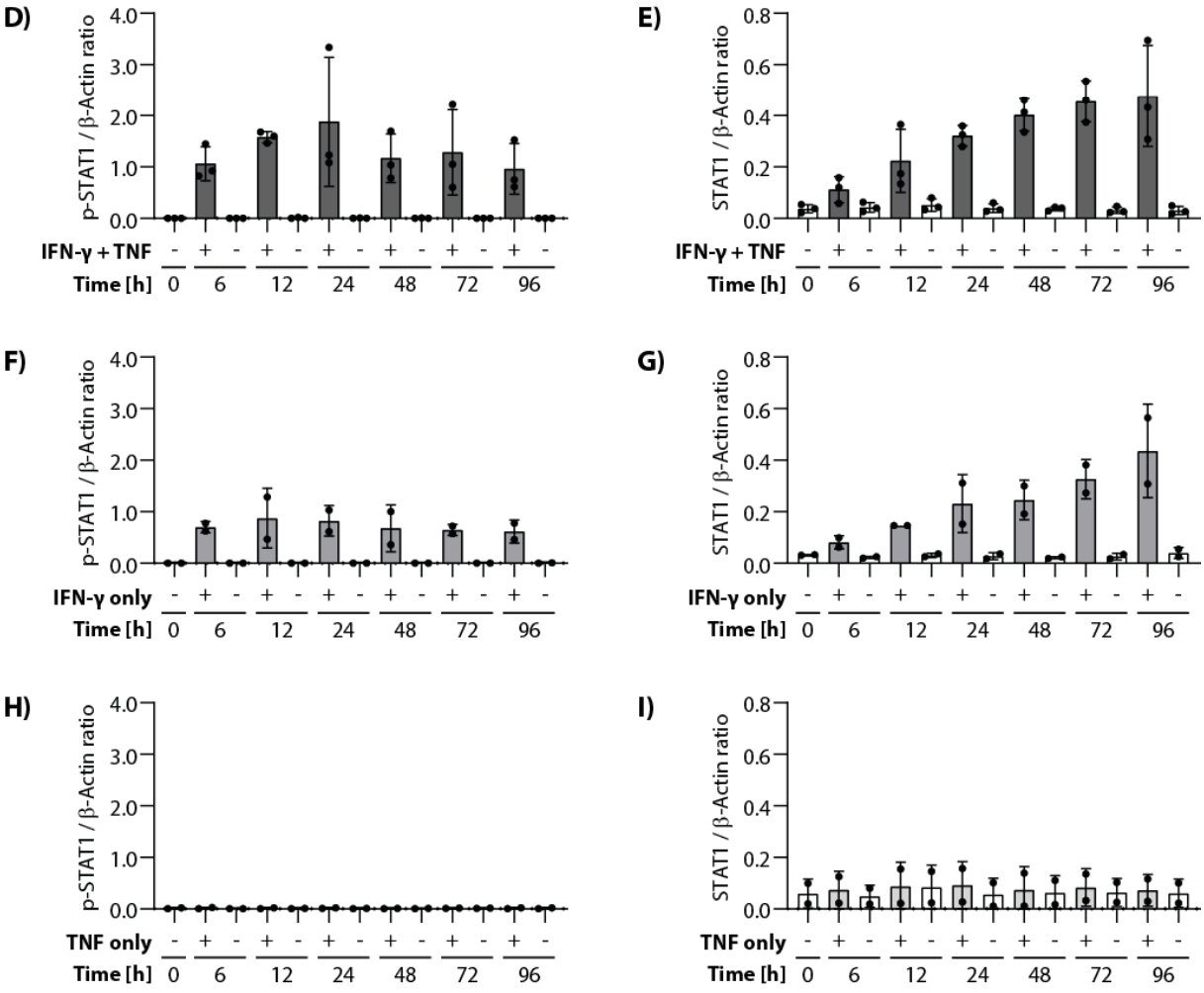


Figure 11: Regulation of STAT1 protein levels upon cytokine treatment. Human A204 cancer cells were treated for up to 96 h with IFN-γ [100 ng/mL] and TNF [400 pg/mL] either alone or in combination. Medium containing PBS served as control. During the indicated treatment, protein extracts were prepared and analyzed by Western blot. HeLa cell lysates were used as a control (Ctrl). Protein levels of phospho-STAT1 (p-STAT1) and STAT1 were determined for the cytokines either in combination with IFN-γ + TNF (A), or as single treatments with IFN-γ only (B) and TNF only (C). Semi-quantitative densitometric analyses of the respective Western blots were used to evaluate the activation and expression levels of STAT1 for treatment with IFN-γ + TNF (D & E), or IFN-γ only (F & G) and TNF only (H & I). Representative Western blots and quantifications are shown (mean ± SD; n=3 for IFN-γ + TNF; n=2 for IFN-γ only and TNF only, respectively).



The p38 MAPK family comprises 4 protein kinases named p38-α (encoded by *MAPK14*), p38-β (encoded by *MAPK11*), p38-γ (encoded by *MAPK12*), and p38-δ (encoded by *MAPK13*) that are activated by different stimuli, such as cytokines, and thereby promote a cellular stress response. Therefore, protein analyses were performed to identify the expression and activation status of p38 MAPK.

Cytokine treatment with IFN-γ and TNF in combination led to a fast and long-lasting activation of p38 MAPK: phosphorylation of p38 was detected as early as 6 h after treatment, also representing the peak in its activation. Afterwards, the phosphorylation levels began to decline steadily, and starting from 48 h onwards stayed quite constant until the end (Figure 12 A & D). The antibody used for the analysis of general p38 MAPK expression detects total levels of p38-α, p38-β, and p38-γ, but not of p38-δ. Other than for the activation state of p38 MAPK, no regulation of the unphosphorylated protein was observed over time (Figure 12 A & E).

The single treatment with IFN-γ alone implemented no activation of p38 MAPK, since the slightly elevated phosphorylation levels after 6 h and 12 h of treatment were also detected in the respective medium controls (Figure 12 B & F). As already seen for the double treatment with both cytokines, the protein level of unphosphorylated p38 MAPK was generally unchanged during treatment with IFN-γ alone (Figure 12 B & G).

Contrary to the lack of p38 MAPK activation by IFN-γ alone, administration of TNF alone had a similar impact on the initial protein phosphorylation as in combination with IFN-γ: the p38 MAPK phosphorylation was already observed after 6 h of incubation and was subsequently reduced during the first 24 h. Afterwards, it was reverted to an undetectable level until the end of the treatment (Figure 12 C & H). However, the protein level of p38 MAPK was not regulated by the single treatment with TNF alone for up to 96 h (Figure 12 C & I).

Other signaling relevant MAPKs besides the p38 family that were additionally analyzed are ERK1 (p44 MAPK encoded by *MAPK3*) and ERK2 (p42 MAPK encoded by *MAPK1*), and the respective data are shown in the appendix (Supplementary Figure 04 A – I).

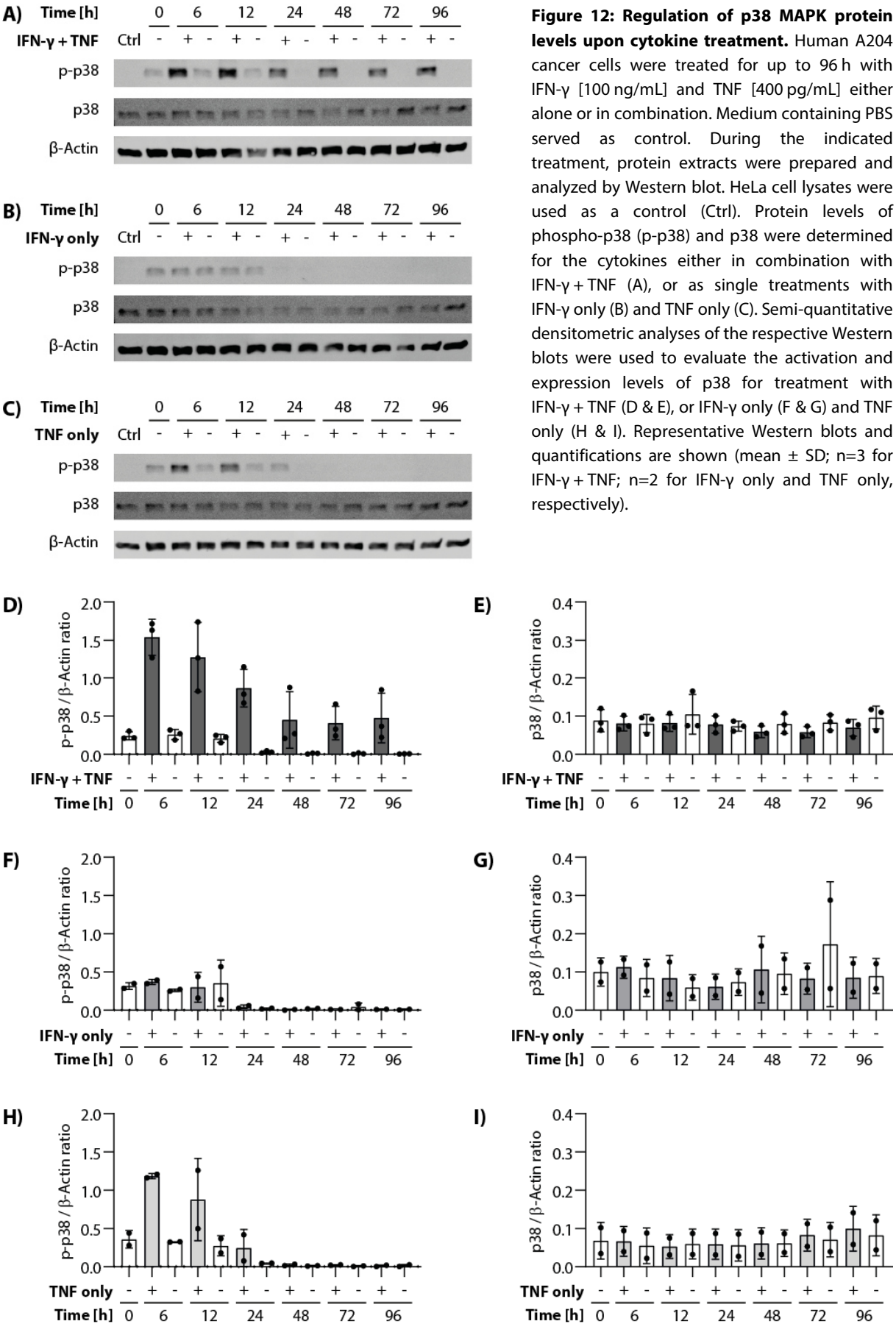


Figure 12: Regulation of p38 MAPK protein levels upon cytokine treatment. Human A204 cancer cells were treated for up to 96 h with IFN-γ [100 ng/mL] and TNF [400 pg/mL] either alone or in combination. Medium containing PBS served as control. During the indicated treatment, protein extracts were prepared and analyzed by Western blot. HeLa cell lysates were used as a control (Ctrl). Protein levels of phospho-p38 (p-p38) and p38 were determined for the cytokines either in combination with IFN-γ + TNF (A), or as single treatments with IFN-γ only (B) and TNF only (C). Semi-quantitative densitometric analyses of the respective Western blots were used to evaluate the activation and expression levels of p38 for treatment with IFN-γ + TNF (D & E), or IFN-γ only (F & G) and TNF only (H & I). Representative Western blots and quantifications are shown (mean ± SD; n=3 for IFN-γ + TNF; n=2 for IFN-γ only and TNF only, respectively).

Activation and induction of the transcription factor NF-κB is a cellular response to different stressors and various stimuli, including cytokines. NF-κB acts as a dimer and is comprised of different subunits, e.g., p65. Since a strong upregulation of the p65 encoding *RELA* gene was found on the transcription level for up to 96 h of treatment (data not shown), the activity and protein expression of this NF-κB subunit was analyzed by Western blot.

For the double treatment with IFN-γ and TNF, a strong activation in terms of phosphorylated p65 levels was observed that ultimately lasted for up to 96 h (Figure 13 A & D). In accordance with the RNA data, the protein expression of p65 was also enhanced by the combination of IFN-γ and TNF, and the total protein accumulated over the course of time with the highest level been found after 96 h of treatment (Figure 13 A & E).

Unlike the cytokine cocktail, the single treatment with IFN-γ alone failed to activate p65 by phosphorylation, and also to induce its protein expression during the whole treatment phase of 96 h (Figure 13 B, F & G).

Most interestingly, p65 was rapidly activated upon stimulation with TNF alone reaching a peak already after 12 h. Afterwards, the phosphorylation of p65 started to decline and was only present during the first 24 h of treatment (Figure 13 C & H). In addition, elevated levels of the un-phosphorylated p65 protein were found for up to 48 h with the TNF single treatment, which did not exceed the prominent effect of the combined action of IFN-γ and TNF (Figure 13 C & I).

Taken together, only the combination of IFN-γ and TNF led to a permanent activation of the cytoprotective NF-κB pathway over the whole treatment phase of 96 h.

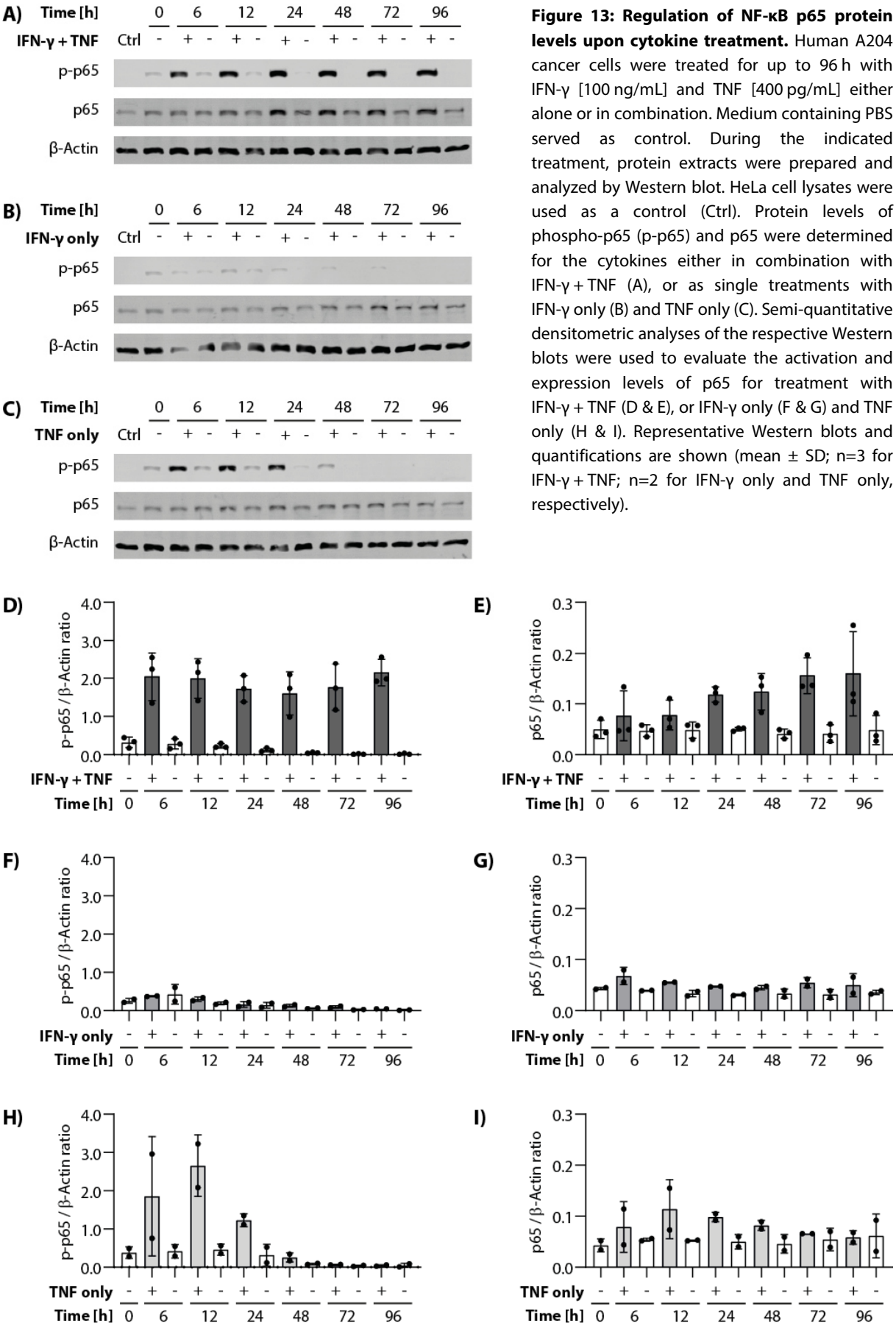


Figure 13: Regulation of NF-κB p65 protein levels upon cytokine treatment. Human A204 cancer cells were treated for up to 96 h with IFN-γ [100 ng/mL] and TNF [400 pg/mL] either alone or in combination. Medium containing PBS served as control. During the indicated treatment, protein extracts were prepared and analyzed by Western blot. HeLa cell lysates were used as a control (Ctrl). Protein levels of phospho-p65 (p-p65) and p65 were determined for the cytokines either in combination with IFN-γ + TNF (A), or as single treatments with IFN-γ only (B) and TNF only (C). Semi-quantitative densitometric analyses of the respective Western blots were used to evaluate the activation and expression levels of p65 for treatment with IFN-γ + TNF (D & E), or IFN-γ only (F & G) and TNF only (H & I). Representative Western blots and quantifications are shown (mean ± SD; n=3 for IFN-γ + TNF; n=2 for IFN-γ only and TNF only, respectively).

Besides p65, the expression of another NF-κB subunit was assessed: p105 is a protein encoded by the *NFKB1* gene that was also found in the transcriptome analysis to be highly upregulated under treatment with IFN-γ and TNF (data not shown). In addition, proteasomal processing during translation creates a smaller variant of the protein known as p50 that is a functional subunit of NF-κB.

Western blot analyses focusing on the activation of p105 clearly showed a strong induction of the protein phosphorylation with IFN-γ and TNF after 6 h of treatment that rapidly declined during the first 24 h, but was nevertheless persistent over time for up to 96 h (Figure 14 A & D). Contiguous to the activation state of p105, an increased expression of the unphosphorylated protein was observed that was kept at a high level during the whole treatment phase (Figure 14 A & E). Since the antibody used to analyze p105 also detects p50, a similar expression pattern as for total p105 was found, but with a general higher protein level (Figure 14 A; quantification of p50 not shown).

As already described for p65, IFN-γ alone was not sufficient to induce activation of p105, although slightly elevated levels of the total protein were observed during the 96 h treatment (Figure 14 B, F & G). For the p50 subunit only a modest increase was observed after 48 h of treatment with IFN-γ that was kept on a constant level until the end of the 96 h incubation (Figure 14 B; quantification of p50 not shown).

Stimulation with TNF alone led to a fast and strong phosphorylation of p105 as early as 6 h of treatment. After this time point, the levels of phosphorylated p105 started to decline and were only detectable for up to 24 h of treatment (Figure 14 C & H). While an increase in the total protein level of p105 was also found over time with the strongest effect seen during the first 24 h, overall induction and expression of p50 upon TNF treatment was even stronger and lasted for up to 96 h (Figure 14 C & G; quantification of p50 not shown).

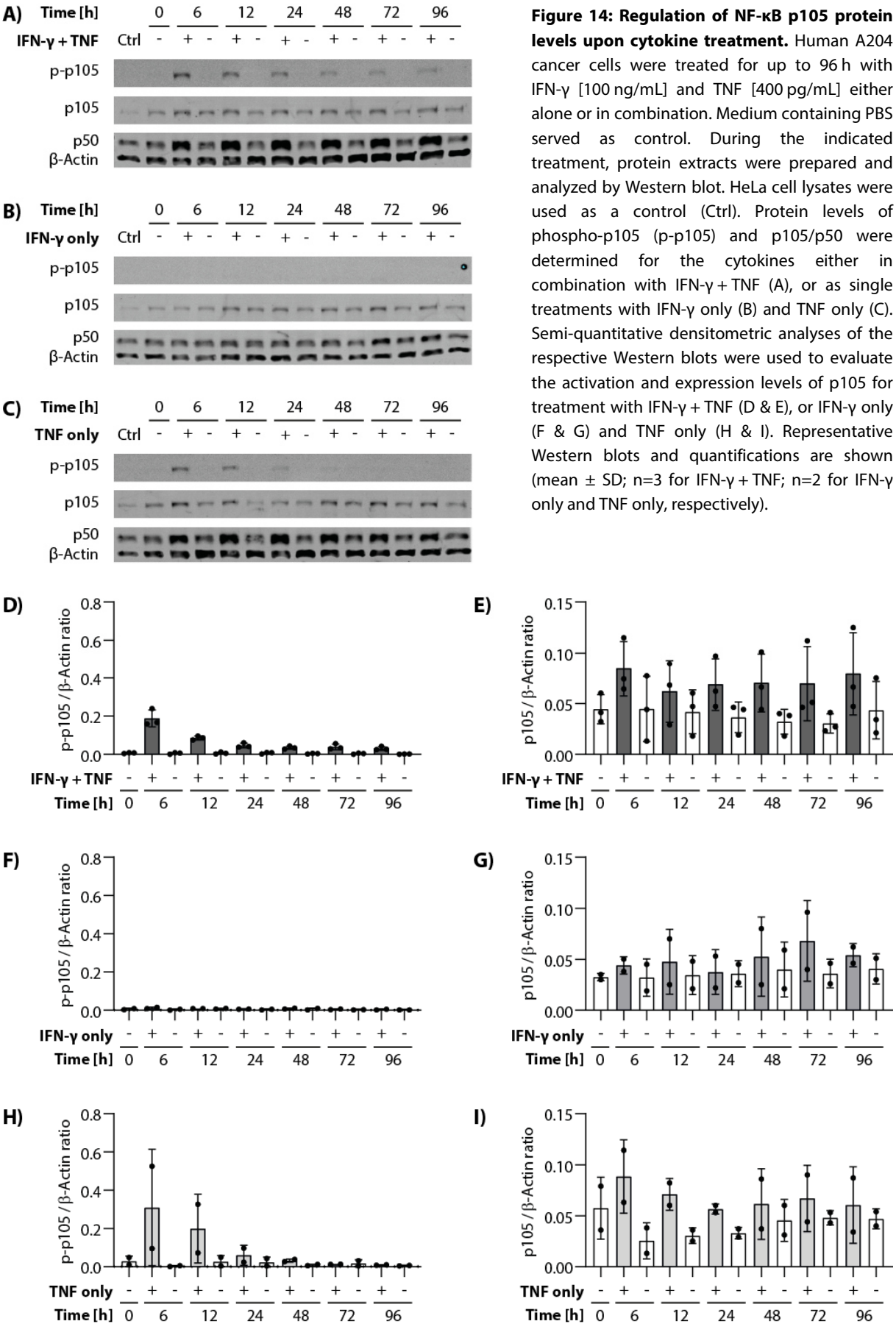


Figure 14: Regulation of NF-κB p105 protein levels upon cytokine treatment. Human A204 cancer cells were treated for up to 96 h with IFN-γ [100 ng/mL] and TNF [400 pg/mL] either alone or in combination. Medium containing PBS served as control. During the indicated treatment, protein extracts were prepared and analyzed by Western blot. HeLa cell lysates were used as a control (Ctrl). Protein levels of phospho-p105 (p-p105) and p105/p50 were determined for the cytokines either in combination with IFN-γ + TNF (A), or as single treatments with IFN-γ only (B) and TNF only (C). Semi-quantitative densitometric analyses of the respective Western blots were used to evaluate the activation and expression levels of p105 for treatment with IFN-γ + TNF (D & E), or IFN-γ only (F & G) and TNF only (H & I). Representative Western blots and quantifications are shown (mean ± SD; n=3 for IFN-γ + TNF; n=2 for IFN-γ only and TNF only, respectively).

5.3.2. Senescence-associated markers and cell cycle regulators

Expression of the cell cycle regulator p16 is a known senescence-associated marker in many cell types and was analyzed for the A204 cancer cells upon cytokine treatment. The respective Western blots for the combination of the cytokines as well as for the single treatments with IFN-γ and TNF showed no protein bands, and p16 expression was only detected in the positive control (i.e., untreated HeLa cell lysate) (Figure 15 A – C).

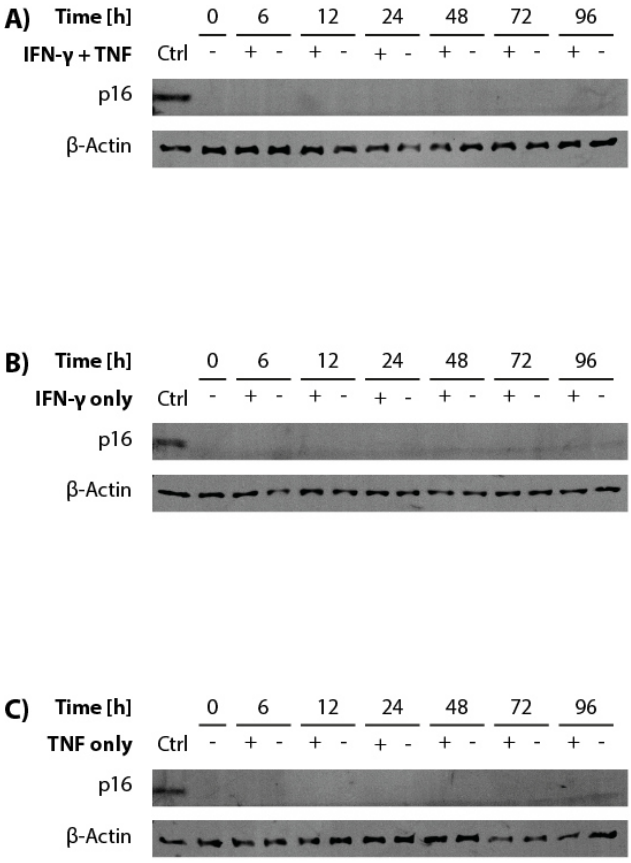


Figure 15: Regulation of p16 protein levels upon cytokine treatment. Human A204 cancer cells were treated for up to 96 h with IFN-γ [100 ng/mL] and TNF [400 pg/mL] either alone or in combination. Medium containing PBS served as control. During the indicated treatment, protein extracts were prepared and analyzed by Western blot. HeLa cell lysates were used as a control (Ctrl). Protein levels of p16 were determined for the cytokines either in combination with IFN-γ + TNF (A), or as single treatments with IFN-γ only (B) and TNF only (C). Representative Western blots are shown (n=3 for IFN-γ + TNF; n=2 for IFN-γ only and TNF only, respectively).

Since p16 expression was not detectable in the A204 cell line during senescence induction, another important regulator of the cell cycle was investigated. p21 is a p53-regulated target encoded by the *CDKN1A* gene that likewise acts as a CDK inhibitor and is also associated with senescence. Upon treatment with the cytokine mix of IFN-γ and TNF, a fast induction of p21 was observed after 6 h that slightly declined until 48 h of treatment. Later on, the upregulation was restored until the end of treatment after 96 h (Figure 16 A & D). In contrast to the effect of the double treatment, no induction and upregulation of p21 was found for stimulation with IFN-γ alone (Figure 16 B & E), while single treatment with TNF induced p21 expression during the first 24 h of incubation (Figure 16 C & F).

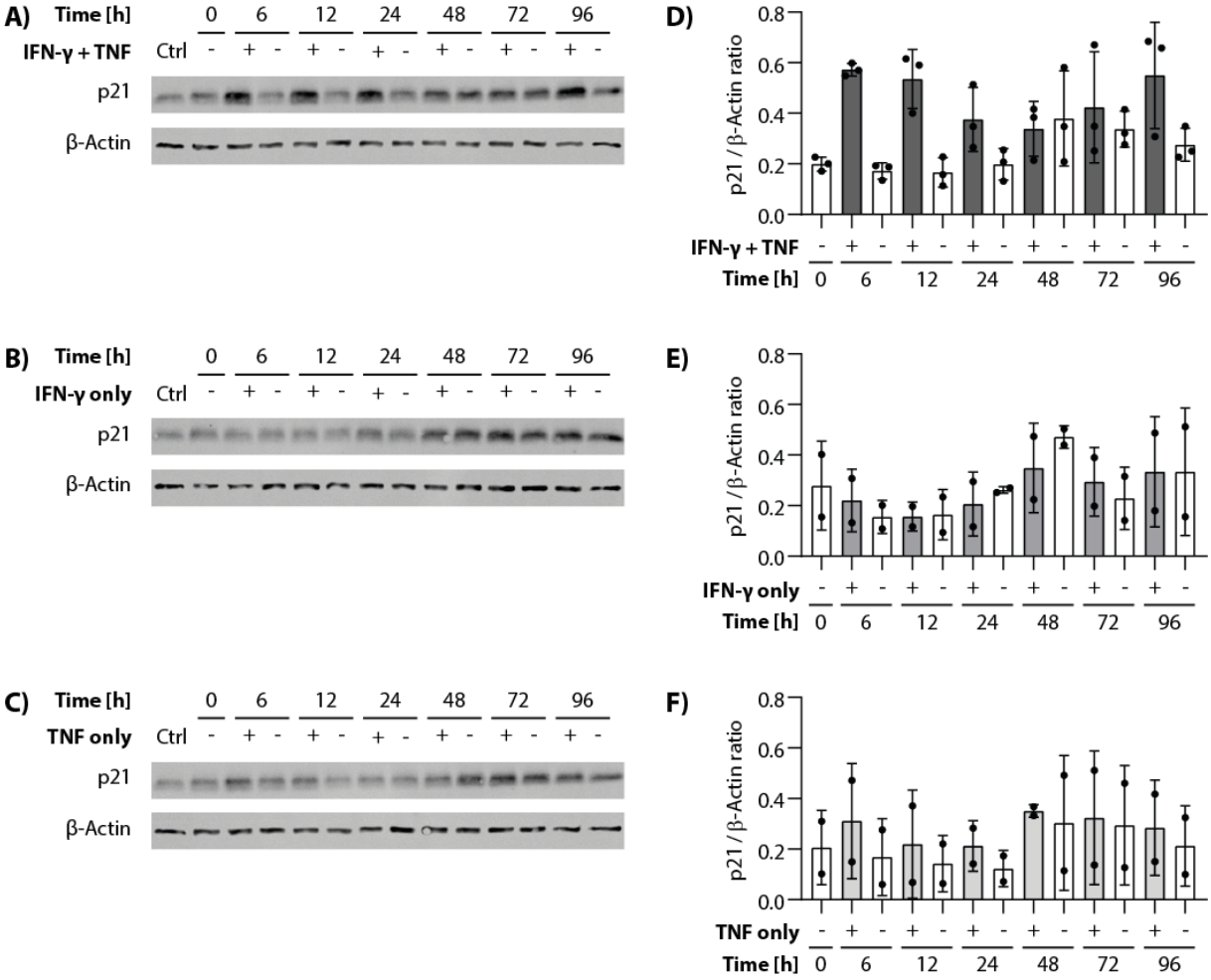


Figure 16: Regulation of p21 protein levels upon cytokine treatment. Human A204 cancer cells were treated for up to 96 h with IFN-γ [100 ng/mL] and TNF [400 pg/mL] either alone or in combination. Medium containing PBS served as control. During the indicated treatment, protein extracts were prepared and analyzed by Western blot. HeLa cell lysates were used as a control (Ctrl). Protein levels of p21 were determined for the cytokines either in combination with IFN-γ + TNF (A), or as single treatments with IFN-γ only (B) and TNF only (C). Semi-quantitative densitometric analyses of the respective Western blots were used to evaluate the expression levels of p21 for treatment with IFN-γ + TNF (D), or IFN-γ only (E) and TNF only (F). Representative Western blots and quantifications are shown (mean ± SD; n=3 for IFN-γ + TNF; n=2 for IFN-γ only and TNF only, respectively).

In addition to the CDK inhibitor p21, the protein turnover of $p27^{Kip1}$ (hereinafter referred to as p27) was assessed. As a member of the CDK-interacting protein (Cip)/kinase-inhibitory protein (Kip) family, p27 acts as a regulator of the cell cycle in controlling G1 progression. Application of both cytokines led to a fast induction of p27 after 6 h of treatment that was maintained for up to 24 h. After this initial peak in the expression of p27, the protein levels returned to the basal expression and remained unchanged for up to 96 h (Figure 17 A & D). For single treatments with IFN-γ and TNF alone neither induction nor regulation of the p27 protein level was observed over time (Figure 17 B & E, C & F).

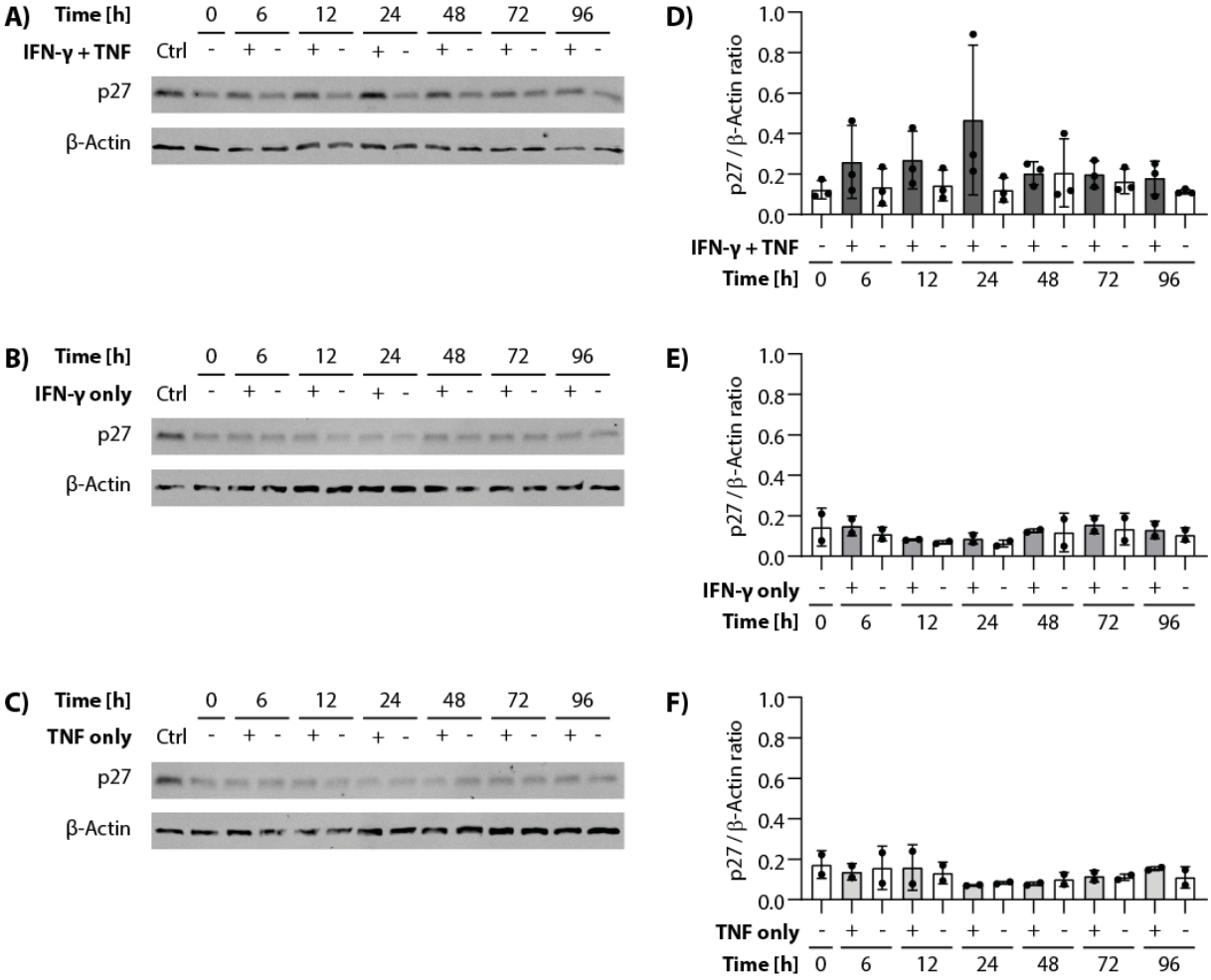


Figure 17: Regulation of p27 protein levels upon cytokine treatment. Human A204 cancer cells were treated for up to 96 h with IFN-γ [100 ng/mL] and TNF [400 pg/mL] either alone or in combination. Medium containing PBS served as control. During the indicated treatment, protein extracts were prepared and analyzed by Western blot. HeLa cell lysates were used as a control (Ctrl). Protein levels of p27 were determined for the cytokines either in combination with IFN-γ + TNF (A), or as single treatments with IFN-γ only (B) and TNF only (C). Semi-quantitative densitometric analyses of the respective Western blots were used to evaluate the expression levels of p27 for treatment with IFN-γ + TNF (D), or IFN-γ only (E) and TNF only (F). Representative Western blots and quantifications are shown (mean ± SD; n=3 for IFN-γ + TNF; n=2 for IFN-γ only and TNF only, respectively).

5.3.3. Pro- and anti-apoptotic factors relevant for cellular survival

Treatment with IFN-γ and TNF induced a strong upregulation of caspase-3 already after 6 h that remained over time and led to an accumulation of the protein (Figure 18 A & D). These results fit well with the transcriptome analysis that also showed upregulation of the respective gene after 96 h (Figure 09). In contrast, single treatments with each cytokine alone had only moderate effects: IFN-γ treatment as well as TNF administration showed slightly elevated protein levels of caspase-3 (Figure 18 B & E, C & F). Most importantly, the cleaved form of caspase-3 was not detected in the different treatment groups over time, while the positive control (lysate of Jurkat cells treated with cytochrome c) clearly showed activation and processing of caspase-3 as seen by a decrease of the inactive pro-form and an increase of the smaller and active form of the protein (Figure 18 A – C).

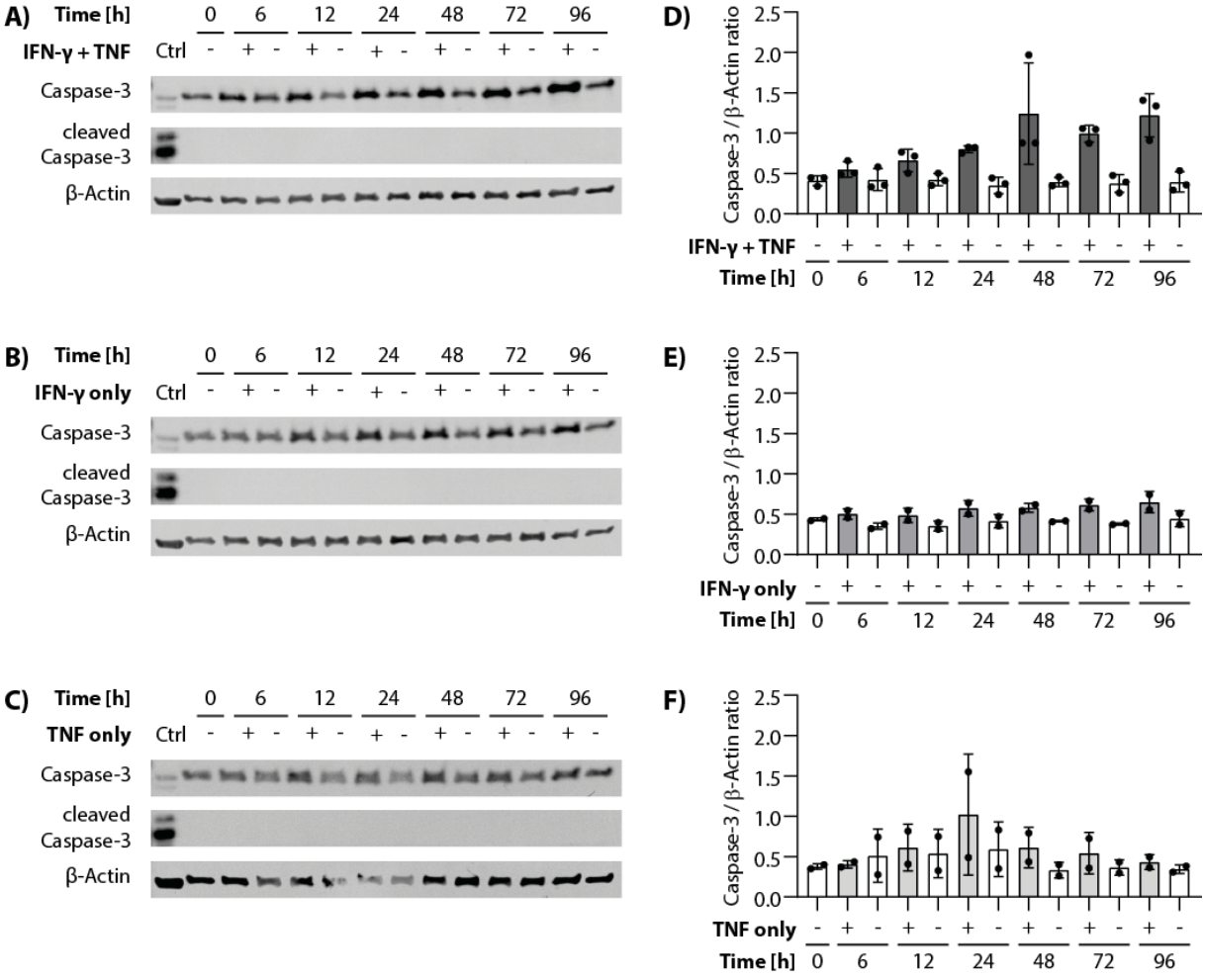


Figure 18: Regulation of caspase-3 protein levels upon cytokine treatment. Human A204 cancer cells were treated for up to 96 h with IFN-γ [100 ng/mL] and TNF [400 pg/mL] either alone or in combination. Medium containing PBS served as control. During the indicated treatment, protein extracts were prepared and analyzed by Western blot. Cytochrome c treated Jurkat cell extracts were used as a control (Ctrl). Protein levels of caspase-3 were determined for the cytokines either in combination with IFN-γ + TNF (A), or as single treatments with IFN-γ only (B) and TNF only (C). Semi-quantitative densitometric analyses of the respective Western blots were used to evaluate the expression levels of caspase-3 for treatment with IFN-γ + TNF (D), or IFN-γ only (E) and TNF only (F). Representative Western blots and quantifications are shown (mean ± SD; n=3 for IFN-γ + TNF; n=2 for IFN-γ only and TNF only, respectively).

Analysis of the anti-apoptotic protein c-IAP2 revealed a strong and long-lasting induction, which already occurred after 6 h of treatment with the combination of both cytokines. The intensity of the protein bands was also increasing over time indicating an accumulation of c-IAP2 with its maximum reached after 96 h (Figure 19 A & D). A corresponding induction of the c-IAP2 encoding *BIRC3* gene was also seen with the microarrays that were used to analyze the RNA expression (Figure 09). For single treatment with IFN-γ, a similar time pattern for c-IAP2 was observed. However, the overall expression level and protein accumulation was much weaker as seen for the double treatment (Figure 19 B & E). Application of TNF alone had initially comparable effects regarding the expression of c-IAP2 as compared to the cytokine cocktail. After the peak at 24 h, the protein level started to decline steadily during the last 2 days of treatment (Figure 19 C & F).

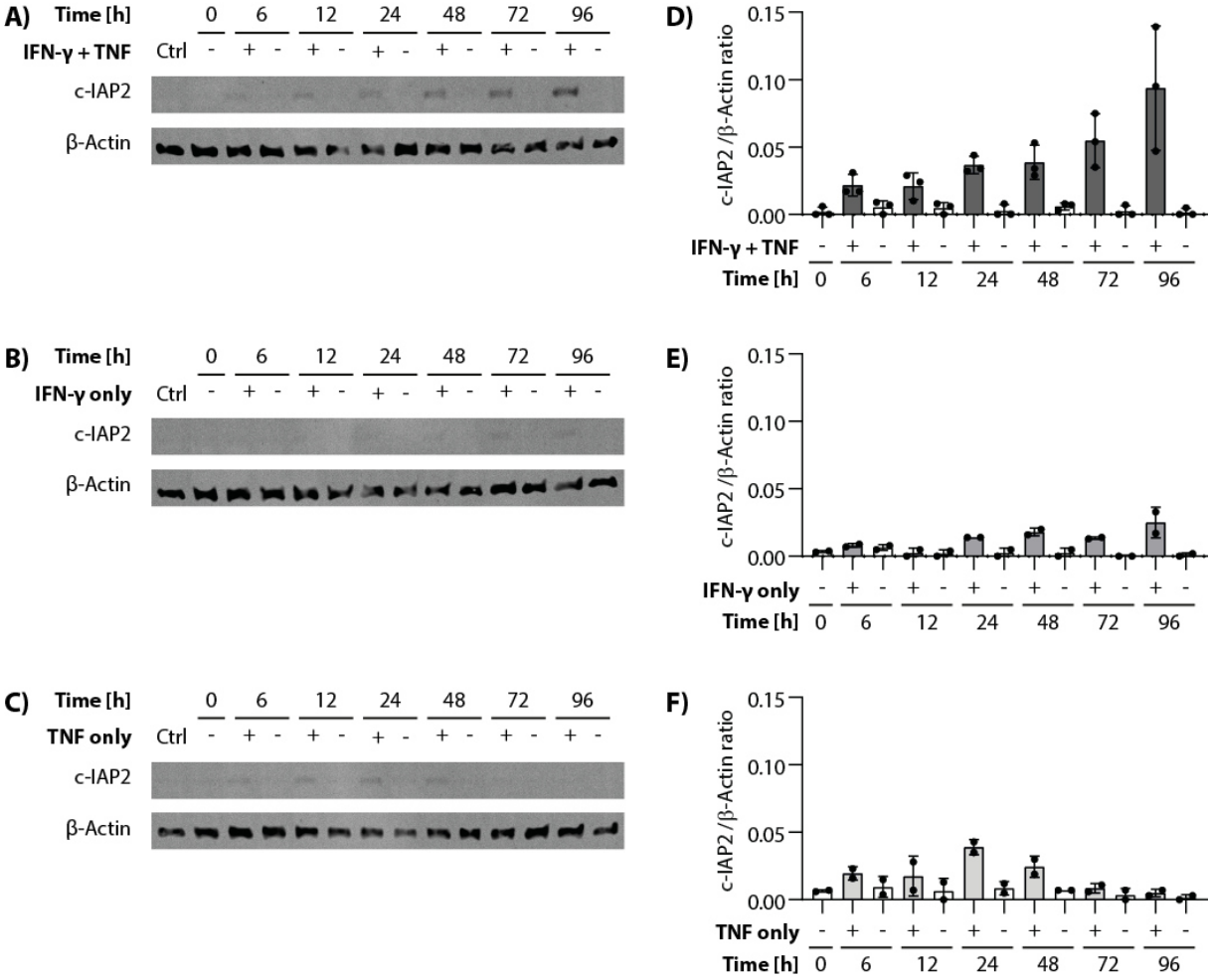


Figure 19: Regulation of c-IAP2 protein levels upon cytokine treatment. Human A204 cancer cells were treated for up to 96 h with IFN-γ [100 ng/mL] and TNF [400 pg/mL] either alone or in combination. Medium containing PBS served as control. During the indicated treatment, protein extracts were prepared and analyzed by Western blot. HeLa cell lysates were used as a control (Ctrl). Protein levels of c-IAP2 were determined for the cytokines either in combination with IFN-γ + TNF (A), or as single treatments with IFN-γ only (B) and TNF only (C). Semi-quantitative densitometric analyses of the respective Western blots were used to evaluate the expression levels of c-IAP2 for treatment with IFN-γ + TNF (D), or IFN-γ only (E) and TNF only (F). Representative Western blots and quantifications are shown (mean ± SD; n=3 for IFN-γ + TNF; n=2 for IFN-γ only and TNF only, respectively).

5.3.4. Other regulated factors involved in CIS

The tumor suppressor gene *PTEN* was another relevant factor found to be downregulated by the cytokine stimulation in the transcriptome analysis (data not shown). PTEN is part of the PI3K/AKT axis and acts as a negative regulator of this pathway by dephosphorylating phosphatidyl-inositol (3,4,5)-triphosphate to phosphatidylinositol (4,5)-bisphosphate, thereby interfering with the downstream activation of AKT. Upon treatment with IFN-γ and TNF, PTEN was continuously downregulated over the course of time reaching the lowest level of expression after 72 h (Figure 20 A & D). A similar effect on the total protein level of PTEN was neither observed for the single treatment with IFN-γ alone (Figure 20 B & E) nor for the administration of TNF alone (Figure 20 C & F).

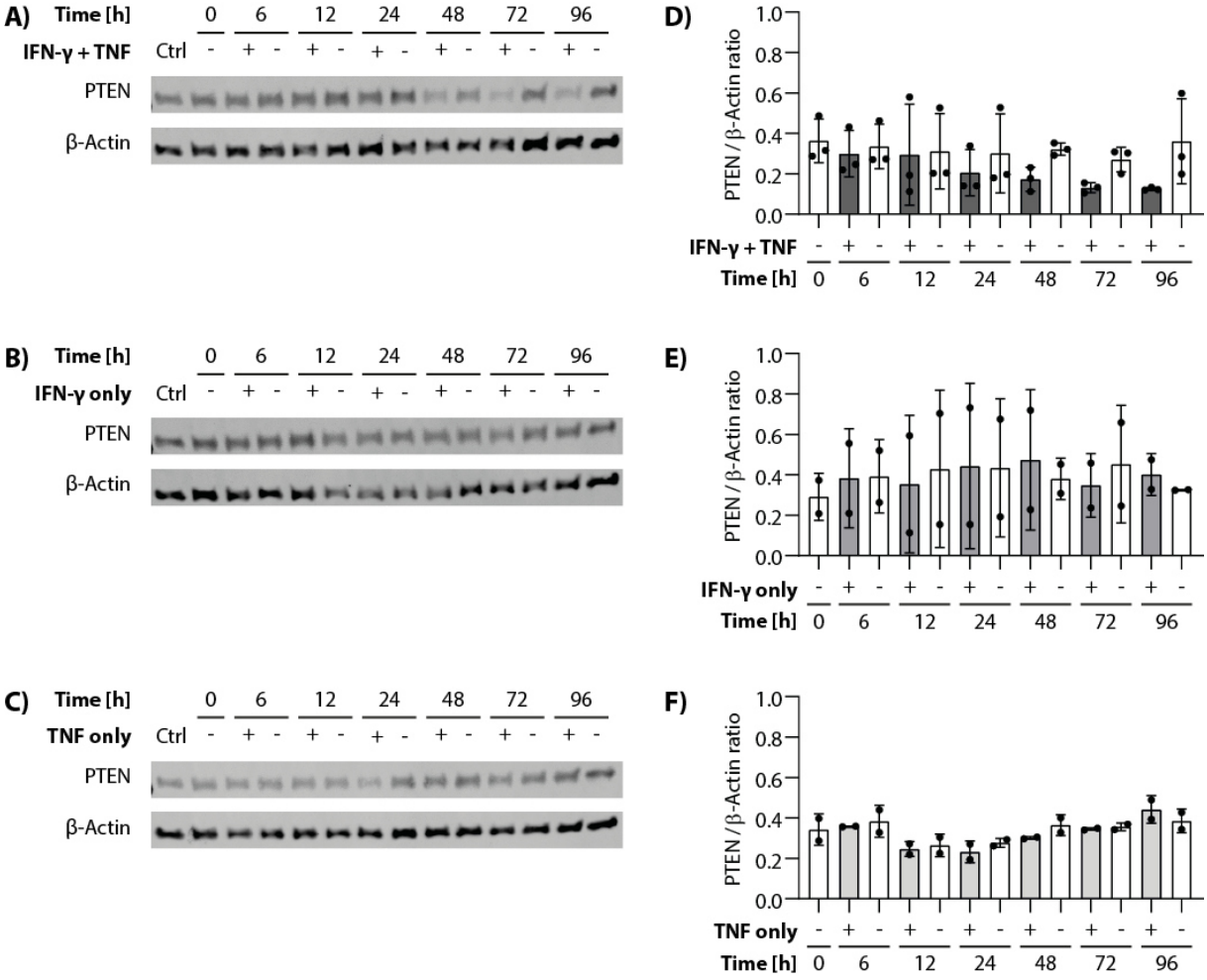


Figure 20: Regulation of PTEN protein levels upon cytokine treatment. Human A204 cancer cells were treated for up to 96 h with IFN-γ [100 ng/mL] and TNF [400 pg/mL] either alone or in combination. Medium containing PBS served as control. During the indicated treatment, protein extracts were prepared and analyzed by Western blot. HeLa cell lysates were used as a control (Ctrl). Protein levels of PTEN were determined for the cytokines either in combination with IFN-γ + TNF (A), or as single treatments with IFN-γ only (B) and TNF only (C). Semi-quantitative densitometric analyses of the respective Western blots were used to evaluate the expression levels of PTEN for treatment with IFN-γ + TNF (D), or IFN-γ only (E) and TNF only (F). Representative Western blots and quantifications are shown (mean ± SD; n=3 for IFN-γ + TNF; n=2 for IFN-γ only and TNF only, respectively).

In conjunction with the observed downregulation of PTEN, the phosphorylation and expression of AKT (also referred to as protein kinase B (PKB)) was analyzed by Western blot. The double treatment with IFN-γ and TNF mediated suppression of AKT activation over time for up to 96 h, and clearly reduced the phosphorylation level compared to the respective medium controls (Figure 21 A & D). Both cytokines also led to diminished protein levels of AKT after 24 h of treatment that were persistent until the end of the 96 h incubation (Figure 21 A & E).

In contrast to the effects of the double treatment with both cytokines neither a clear reduction of phosphorylated AKT (Figure 21 B & F) nor a general downregulation of the total AKT protein (Figure 21 B & G) was observed for the single treatment with IFN-γ alone over the course of time.

However, the situation was different for the application of TNF alone. TNF mediated a slight reduction of phosphorylated AKT during the first 48 h of treatment (Figure 21 C & H), whereas no substantial decrease of the total AKT level was observed over time, except for a moderate decline after 48 h (Figure 21 C & I).

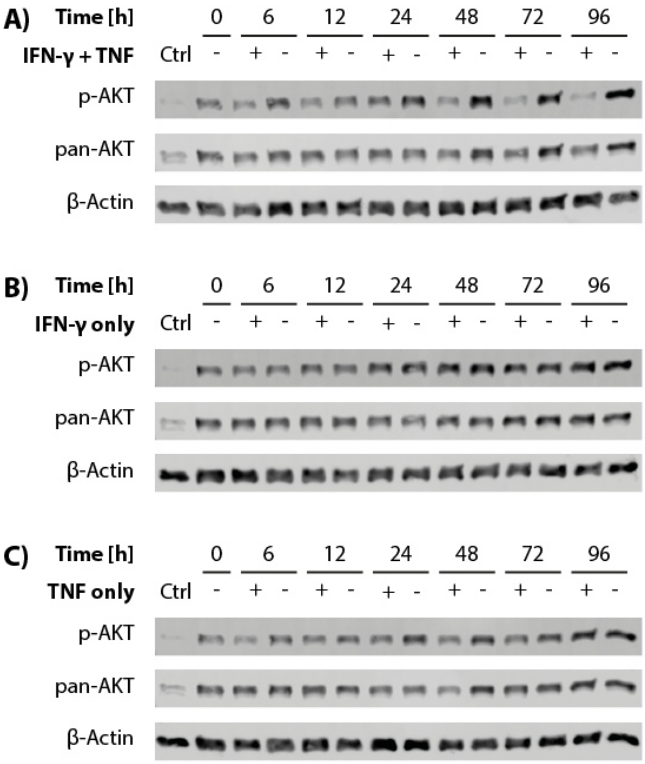
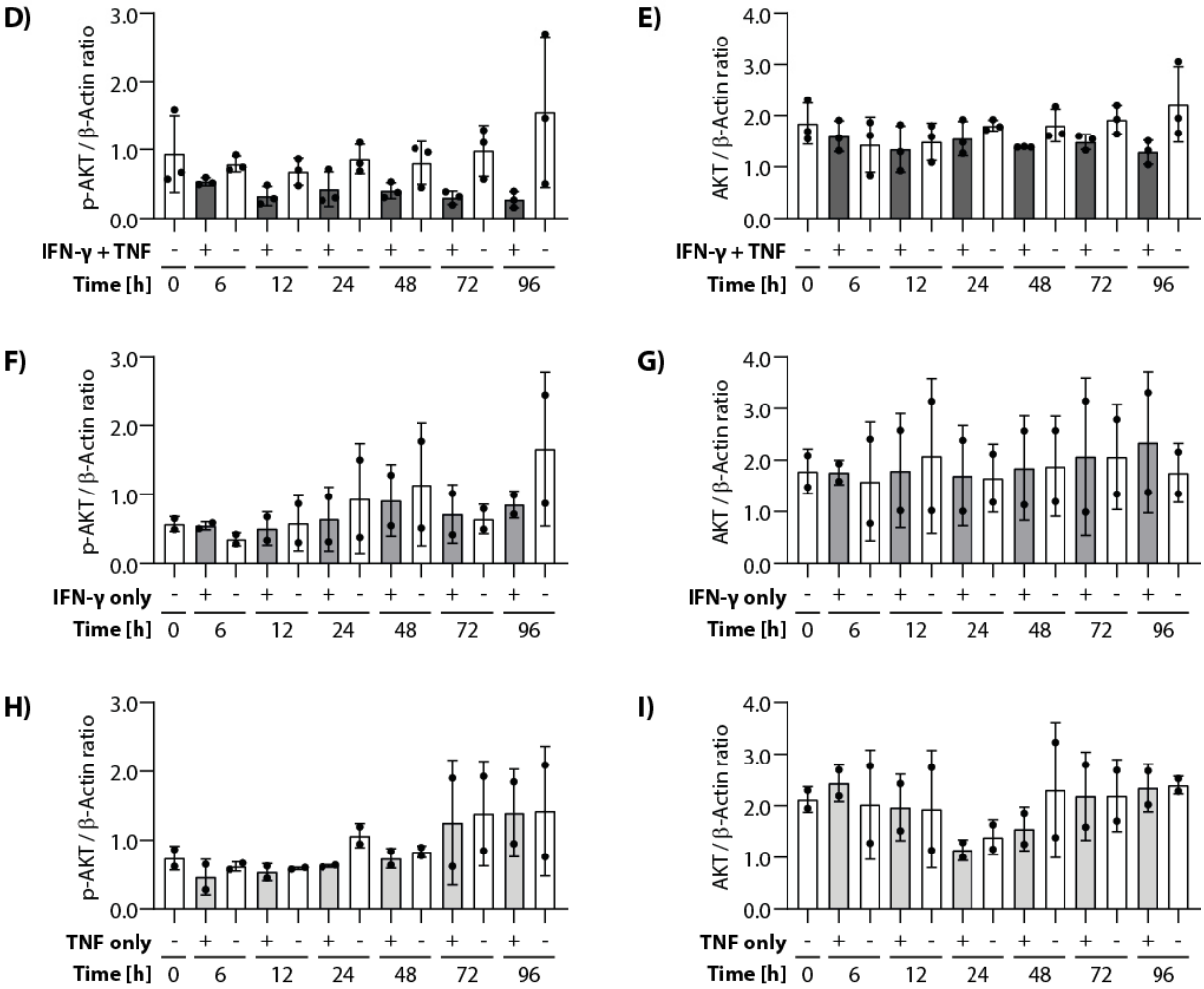


Figure 21: Regulation of AKT protein levels upon cytokine treatment. Human A204 cancer cells were treated for up to 96 h with IFN-γ [100 ng/mL] and TNF [400 pg/mL] either alone or in combination. Medium containing PBS served as control. During the indicated treatment, protein extracts were prepared and analyzed by Western blot. HeLa cell lysates were used as a control (Ctrl). Protein levels of phospho-AKT (p-AKT) and AKT (pan-AKT) were determined for the cytokines either in combination with IFN-γ + TNF (A), or as single treatments with IFN-γ only (B) and TNF only (C). Semi-quantitative densitometric analyses of the respective Western blots were used to evaluate the activation and expression levels of AKT for treatment with IFN-γ + TNF (D & E), or IFN-γ only (F & G) and TNF only (H & I). Representative Western blots and quantifications are shown (mean ± SD; n=3 for IFN-γ + TNF; n=2 for IFN-γ only and TNF only, respectively).



6. Discussion

Senescence in general acts as potent anti-tumor mechanism that prevents uncontrolled proliferation through induction of a permanent growth arrest. Moreover, senescence induction can also be the consequence of cellular stress and damaging events leading to ceased proliferation [184, 242]. Since senescent cells are viable, persistent, metabolically (hyper-) active, and secrete different factors commonly referred to as the SASP, they are relevant for organismal aging, the formation of age-related diseases, and also potentially harmful in the context of cancer development, as components of their secretome can mediate pro-tumorigenic effects [173, 243-245]. Although studied for many years, the underlying mechanisms and processes leading to senescence are still not fully understood and have yet to be characterized in more detail.

With reference to the recently discovered CIS that utilizes mediators of the immune system for senescence induction (i.e., the combined action of IFN-γ and TNF) a systematic analysis was performed. Starting with a transcriptome-based screening to uncover the underlying gene regulatory networks of CIS over time in the cytokine-sensitive A204 rhabdomyosarcoma cell line [185, 198, 238], several important signaling pathways and biological processes have been identified to be highly regulated. These include the interferon-triggered JAK/STAT signaling axis, the TNF-triggered p38 MAPK and NF-κB pathway, genes and factors involved in cell cycle regulation, SASP-related factors such as cytokines and chemokines, as well as regulation of the apoptosis cascade on all levels. This concomitant induction of multiple signaling pathways and senescence-associated factors indicates an interrelated and complex molecular regulation that is involved in the establishment and maintenance of CIS. Considering the long treatment phase of 96 h that is needed to induce CIS, it is remarkable that the quite fast regulation of gene expression, which is usually seen as a short-lived response, persists over time. This circumstance highlights the robust long-term effect triggered by the combination of IFN-γ and TNF, as the cytokines were initially applied once without further replenishment during the 96 h treatment phase. Given the massive alterations of the underlying gene expression profiles this observation could explain the outcome of the growth arrest assays, which clearly demonstrated that only application of both cytokines creates a synergistic effect and leads to senescence induction. Similar results demonstrating the dependence of CIS on the presence of both cytokines were already described [185, 187, 191, 198]. Furthermore, the proliferation assays conducted during the 96 h treatment phase also showed the necessity of a concomitant presence of IFN-γ and TNF, as each cytokine alone failed to stably suppress the cellular proliferation. This also fits with previous reports that described anti-proliferative effects of the pro-inflammatory cytokines in combination when they are either used directly *in vitro* or released through Th1 cells *in vivo* [185, 187, 238, 246-249]. On basis of this comprehensive screen, certain members of the most regulated pathways were selected to validate the implications of CIS on the protein level.

This in-depth analysis covers the entire senescence induction phase of 96 h from start to end by a tight time frame, and reveals clear differences in the outcome of single treatments with either IFN-γ or TNF alone as compared to the dual application of both cytokines. Furthermore, additional studies focusing on the activation state of different target proteins were performed to complete the analysis of the general expression level. Since IFN-γ signaling is mediated via the JAK/STAT pathway and *STAT1* together with other interferon-induced targets was among the upregulated genes induced by the cytokine treatment, the protein analysis started with this factor. Besides accumulation of the total protein over time, STAT1 was also activated through phosphorylation at tyrosine residue 701, a site known to be phosphorylated by different kinases, such as JAK1 and JAK2, that further mediates STAT1 dimerization [37, 250-254]. This hyperactivation of STAT1, which was also observed in the single treatment with IFN-γ alone, might explain the predominant role of the interferon signature in the process of CIS induction. Also, *CXCL10* being on the second place in the VIP score ranking accounts for the long-lasting and interferon-dominated effects found in the transcriptome after 96 h.

The importance of undisrupted IFN-γ signaling for the induction of CIS was previously described for murine β-cancer cells of the RIP-Tag model *in vitro* and *in vivo* [185, 187, 191]. Furthermore, other reports about CIS identified STAT1 as an important factor. Novakova et al. showed activated JAK/STAT signaling in senescent cells of different origin that were treated with genotoxic drugs [192]. The interferon pathway activation was persistent, as the senescent cells expressed stimulating ligands as part of their secretory phenotype that reinforced the JAK/STAT signaling in an autocrine and paracrine manner [192]. Although IFN-γ was among the expressed factors, it was IFN-β that was highly induced and mediated the activation of STAT1, as did IL-6 [192]. This was shown, as phosphorylation of STAT1 at tyrosine residue 701 and at serine residue 727 was observed in the senescent cells [192]. Nevertheless, functional experiments revealed a less dominant and more subordinate role of the JAK/STAT signaling axis for the cellular phenotypes of drug-induced senescence [192]. As the results described by Novakova et al. represent a secondary phenomenon that is induced as a consequence of effects of the primary triggers of senescence (i.e., genotoxic substances), Wang et al. demonstrated the direct senescence-inducing properties of IFN-γ [197]. Analyzing normal human melanocytes instead of cancer cell lines, a functional role of JAK2 and STAT1 for the senescence induction was reported that was further characterized by ROS formation and the upregulation of p21 [197]. Moreover, Hubackova et al. demonstrated similar results when they analyzed CIS in different human and murine cell lines to unravel the mechanistic links between the cytokine signaling, oxidative stress, and DNA damage [199]. Senescence induction in murine cancer cells required the concomitant presence of IFN-γ and TNF, while for human cancer cell lines TNF was omittable and IFN-γ alone mediated the senescence-promoting effects [199]. They observed phosphorylation of STAT1 at tyrosine 701 and also found activation of STAT2, STAT3, and STAT5 as early as 15 min after stimulation with IFN-γ, and further demonstrated that even a short-term

treatment for 24 h with IFN-γ alone was able to induce a robust and long-lasting cellular response that ultimately leads to senescence [199]. Rosemblit et al. also noted that STAT1 phosphorylation on tyrosine 701 and other sites was present in breast cancer cells after treatment with IFN-γ alone, or in combination with TNF and other substances [201]. While phospho-STAT1 levels were increased, STAT3 activity was decreased, leading to a STAT1-mediated induction of apoptosis and senescence when the underlying oncogene was inhibited [201]. The authors further demonstrated that early changes induced through an initial treatment were maintained over time, even after the end of treatment and subsequent passaging of the breast cancer cells [201]. In contrast to these evidential results shown for IFN-γ and senescence, Kandhaya-Pillai et al. uncovered a STAT-dependent feedback loop for TNF-induced senescence in HUVECs that also shows an interferon signature and further involves oxidative stress, DNA damage, as well as the release of different cytokines [200]. The TNF treatment further led to activation of the JAK/STAT pathway as seen by the phosphorylation of STAT1 and STAT3, and the expression of interferon response genes [200]. The authors showed that activity of STAT1 and STAT3 is needed for ROS formation and the production of cytokines, such as IFN-γ or IL-6, that act in an autocrine manner and reinforce the senescent state through sustained inflammation [200]. Conversely, inhibition of STAT activation was not sufficient to stop the senescence induction process, as the use of a JAK2 inhibitor only changed the cellular response to TNF by modifying the senescent phenotype [200].

Besides these mostly cytokine-related studies, a role of STAT1 in the induction of senescence in response to angiotensin II and H_2O_2 treatment was reported for human glomerular mesangial cells of the kidney, and the authors further demonstrated that application of losartan (an angiotensin II receptor blocker) inhibits STAT1 activation and protects the cells from premature senescence [255-257]. Another recent report deciphering the transcriptomic basis of senescent fibroblasts, human aortic endothelial cells (HAECs), and HUVECs detected upregulated mRNA levels of the *STAT1* transcript in all senescence models analyzed (i.e., replicative senescence as well as oncogene-, irradiation-, and drug-induced senescence) [258]. This ubiquitous induction prompts the assumption of a fundamental role of STAT1 and the related signaling cascade for cellular senescence. Another group reported at the same time the basic gene expression signature of premature senescence in human cancer cells [259]. Comparing the transcriptome of senescent MCF-7 cells after irradiation with the effects of an induced overexpression of p21, Wu et al. discovered a core network of shared up- and downregulated genes in both conditions [259]. They also found differences between the two groups and distinguished p21-induced senescence from radiation-induced senescence by a pathway analysis [259]. The identified pathways include for instance the interferon signaling and genes related to this process. However, *IFNGR1*, *STAT1*, *IRF1*, or *IRF9* were only upregulated in the irradiated MCF-7 cells. Those cells also exhibited DNA damage, whereas no DDR was present in the cells overexpressing p21 [259].

Although not analyzed in this thesis and for the A204 cell line, DNA damage that is itself a potent trigger for senescence was reported by others in the context of cytokine treatment [199, 200, 202, 260, 261]. To exclude such p53-mediated effects, Braumüller et al. established the concept of CIS in the RIP-Tag mouse model [185]. This was succeeded by the recently published follow-up study that further expanded the senescence analysis, and also addressed DNA damage in this p53-deficient system and in other models [191]. As staining for the DNA damage-associated markers was mainly negative, it was shown that CIS occurs in the absence of excessive DNA damage, which could activate the cyclic guanosine monophosphate (GMP) adenosine monophosphate (AMP) synthase (cGAS) and the stimulator of interferon genes (STING) pathway that can also mediate senescence and involves the induction of a type I interferon response [191, 262-270]. Since the JAK/STAT axis is an important signaling pathway that is activated in response to different cytokines, including type I interferons such as IFN-α and IFN-β, the signal transduction involves different STAT molecules and is not limited to STAT1 [271-273]. Previous reports already discovered that STAT family members such as STAT3 and STAT5 contribute to senescence, and their involvement was shown in different systems [274-279]. As *STAT2* and *STAT3* were also found to be upregulated after challenge with IFN-γ and TNF in our transcriptome analysis (data not shown), this combination of cytokines probably leads to convergent signaling events and/or the secretion of other factors that might explain their transcription. In addition, an increased protein level of STAT3 was observed over time that resembled the kinetics of STAT1, as the effect was strongest in response to both cytokines. The single treatment with IFN-γ was similar to these results, whereas even application of TNF alone induced STAT3 expression but to a lesser extent. In contrast to this, the weak but long-lasting phosphorylation of STAT3 was limited to the treatment with IFN-γ, either alone or in combination with TNF (Supplementary Figure 3 A – I). In line with this, Hubackova et al. also detected activation of STAT3 and other members (i.e., STAT1, STAT2, and STAT5) after treatment with IFN-γ alone [199], while the results presented by Kandhaya-Pillai et al. showed phosphorylation of STAT1 and STAT3 even in response to TNF [200]. Since senescence induction through Th1 cells and their effector cytokines already demonstrated a potent mechanism of tumor control that failed in mice lacking Stat1 function, or by the use of neutralizing antibodies against IFN-γ, the impact of an intact interferon response became apparent [185, 191].

Moreover, a general role of the interferon signaling could be proposed for cellular senescence. Several studied published by the research group of Michael Tainsky since the early 2000s identified the interferon signaling pathway and related components to be repressed during cellular immortalization, a process that is closely related to cancer development and naturally antagonized through the induction of senescence. First of all, it was shown by comparative gene expression profiling of proliferating Li-Fraumeni fibroblasts and their senescent counterparts that (spontaneous) cellular immortalization was accompanied by the epigenetic silencing of different factors, including various genes within the interferon signaling cascade [280]. Another report from the same group revealed that a total of three

cellular pathways were essentially altered when comparing senescence and immortalization. The epigenetically silenced pathways found in immortalized cells contained genes that were related to (i) the cytoskeleton, (ii) the cell cycle, and (iii) the interferon signaling [281]. Based on the repeated finding of a disturbed interferon pathway in immortalized cells, this issue was further investigated. The following study observed the downregulation of *STAT1* in response to immortalization, but its overexpression in the immortal cells was neither able to inhibit their proliferation nor to induce senescence [282]. The conclusion was that there are also other factors besides STAT1, which act differently in senescence and immortalization. It was later shown that overexpression of IRF5 and IRF7 either alone or in combination led to the activation of interferon-regulated genes, a repressed growth rate, and the induction of senescence in the immortalized cells [283]. Since the interferon pathway is a conserved defense mechanism against viral infections that is activated in response to double-stranded RNAs and others, it was again found in the immortalized Li-Fraumeni fibroblasts that these cells overexpressed Dicer and also showed a better tolerability to the higher content of created miRNAs [284]. In contrast to this, the cells with intact interferon signaling were more sensitive to the abundance of miRNAs, which triggered the expression of ISGs that not only resulted in cell death induction but also in growth inhibition and senescence [284].

These collective findings that uncovered alterations of the interferon pathway in immortalized cells were further confirmed by a follow-up study that compared the gene expression profiles of four different types of senescence induction to one another [285]. The analysis of genes that were associated with all forms of senescence tested consistently revealed involvement of pathways related to (innate) immune processes and interferon responses. While upregulation of IFR5 was present in all four settings, IRF7 was only upregulated in replicative senescence and after treatment with 5-aza-2'-deoxycytidine, indicating variations in the activation of the interferon pathway in senescent cells [285]. Also, the discovery that cGAS is an essential factor for senescence can be related to the aforementioned observations and the interferon pathway in general, as cGAS can mediate a type I IFN response [265, 266]. Furthermore, it was shown that DNA sensing and STING activation not only involve cGAS but also IFI16, a member of the interferon-inducible protein family [286-288]. The latter has already been associated with the induction of senescence in human prostate epithelial cells and fibroblasts, counteracting the effects of cellular immortalization in both systems [289, 290]. Another study of the same group demonstrated that the increased expression of IFI16 led to the downregulation of TERT, an enzyme that normally prevents telomere shortening and was already shown to circumvent replicative senescence [114, 291]. Taken together, these reports provide evidence that inactivating or suppressive alterations in the interferon pathway can be linked to cellular immortalization, as they contribute to the bypass of senescence induction. Although interferon signaling is not the only pathway that leads to senescence, it is nevertheless an important one, especially in the context of CIS that relies on the combined action of IFN-γ and TNF.

In this regard, the TNFR1-related signaling is the second major driver for CIS whose relevance has also been demonstrated before [185, 187]. The TNF pathway engages various factors, including the p38 MAPK, which is also involved in the non-canonical signaling of different interferons, such as IFN-γ [292-296]. Furthermore, it was previously shown that activation of STAT1 is not only limited to JAK family members, as phosphorylation at different sites was also described for other kinases, including MAPKs like ERK1/2 and p38 [297-301]. The activity of p38 MAPKs has already been associated with stress-induced senescence and the SASP, and was now analyzed in the context of CIS [120, 302-306]. Although p38 MAPK expression was not regulated on the total protein level, the phosphorylation pattern was similar to that observed for STAT1: the treatment with both cytokines induced a persistent activation for up to 96 h as seen by the phosphorylation of threonine 180 and tyrosine 182. In clear contrast, the single treatment with TNF alone mediated only transient effects during the first 24 h. This finding highlights a robust induction of TNF-related signaling through the synergism of both cytokines that suggests the existence of a self-reinforcing mechanism, whereby IFN-γ drives the expression of TNF. This notion fits well with reports that IFN-γ can induce TNF as well as TNF receptor expression [307-315], since our results derived from the transcriptome analysis also showed upregulation of TNF and TNFR2 during the 96 h treatment. Moreover, activation of p38 MAPK through TNF was shown to be dependent on TNFR1 that further utilizes JAK2, an interaction that can also activate the transcription factor STAT3 [316].

Since activated p38 MAPK signaling is of importance for the regulation of senescence, Kandhaya-Pillai et al. investigated its role in TNF-induced senescence [200]. In this setting, pathway analysis initially highlighted the involvement of p38 MAPK, and the use of a chemical inhibitor demonstrated its contribution during the senescence induction process [200]. Inhibition of p38 MAPK activity not only prevented induction of senescence in general, but led further to a decreased STAT1 phosphorylation, the reduced expression of IL-6, and diminished ROS levels in response to the TNF treatment [200]. Similar to these observations and comparable to the findings presented in this thesis, phosphorylation of p38 MAPK at the residues threonine 180 and tyrosine 182 was also reported by Rosemblit et al. for treatment with IFN-γ and TNF [201]. They considered this effect as a secondary and TNF-mediated signaling event that occurred in parallel to the IFN-γ-driven activation of STAT1 [201]. If the Th1 cell cytokines were further combined with an oncodriver inhibition that also activated p38, this MAPK provided the additional phosphorylation of STAT1 at serine 727 [201]. A similar interaction of both MAPK signaling and STAT transcription factor activity in response to DNA damage was reported by Liu et al. who analyzed Treg-mediated senescence induction in effector T cells [202]. An earlier report published by the same group illustrated the functional cooperation between the MAPKs p38 and ERK1/2, and the activation of STAT1 and STAT3 that jointly regulate and control the senescence program on a molecular basis [317]. In addition to p38 MAPK, also ERK1/2 expression was analyzed in this thesis. While activated MAPK signaling via ERK1/2 was

present in the A204 cell line under medium control conditions, it was further enhanced through the cytokine treatment with IFN-γ and TNF as seen by the phosphorylation of ERK1 and ERK2, with the latter being more intense. In comparison, only the single treatment with TNF induced an initial increase in the phosphorylation of ERK1/2 during the first 24 h that adapted to the basal level afterwards (Supplementary Figure 4 A – I). Since the phosphorylation of ERK2 was stronger as compared to the level found for ERK1, this could indicate a predominant role of the p42 MAPK during CIS. In case of OIS, a study published by Shin et al. unveiled that it was indeed the loss of ERK2, which abrogated the senescent state and led to continuous proliferation [318]. Although activity of ERK1/2 is generally related to the cellular response to mitogens and proliferation, its role in senescence was instead associated with the induction of growth arrest [319, 320]. This is achieved, since MAPKs like ERK1/2 and p38 can influence the expression of important cell cycle regulators such as p16 or p21 in different ways [117, 120, 318, 321-324].

Besides the aforementioned observations that so far linked CIS to the JAK/STAT axis and the activation of p38 MAPK signaling, another important factor that contributed to the process of senescence induction was NF-κB. While the transcriptome analysis showed upregulation of relevant genes associated with NF-κB signaling, the expression of the subunits p65 (encoded by the *RELA* gene) and p105 (encoded by the *NFKB1* gene) was further analyzed on the protein level. Similar to STAT1 and the p38 MAPK, a strong phosphorylation of NF-κB p65 was present during the whole treatment phase with IFN-γ and TNF that was accompanied by the accumulation of the total p65 protein. In sharp contrast to that, the initial phosphorylation induced by TNF was rapidly abolished after 24 h, whereas treatment with IFN-γ alone completely failed to phosphorylate the p65 subunit of NF-κB. In case of NF-κB p105 a similar phosphorylation pattern was present that again only prevailed in the treatment schedule with both cytokines and quickly faded after stimulation with TNF alone. Phosphorylation of the serine residue 933 and at other sites of the precursor molecule is mediated by IKKs and causes the subsequent processing of p105 via the proteasome system [325-331]. While the total protein level of p105 was slightly elevated in response to IFN-γ and TNF, a much stronger enrichment of the processed and mature p50 subunit was detected for the double treatment, as well as the single treatment with TNF alone. Moreover, the observed phosphorylation of p65 at serine 536 is facilitated by different kinases including the IKKs, and has been generally associated with the activation of NF-κB after stimulation with different factors, such as TNF in the majority of studies [332-342], albeit some reports either found no correlation or indicated an opposing role [343, 344]. A study based on a phosphomimetic p65 mutant linked phosphorylated Ser536 to the induction of apoptosis and senescence [345], while a novel biphasic pattern of NF-κB activation was currently identified in senescent cells [346]. Regarding cytokines such as interferons or TNF, protective effects of NF-κB and its activity have already been described [347-355]. Thapa et al. identified the manganese-dependent superoxide dismutase MnSOD (encoded by *sod2*) as an important factor that

counteracts accumulation of ROS and prevents cell death induction through IFN-γ [356]. This finding might also translate to CIS, as upregulation of the antioxidant gene *SOD2* was observed in A204 cells after cytokine treatment with IFN-γ and TNF, therefore suggesting that induction of the senescent state presumably occurs independent of ROS.

Although the dependence of CIS on the expression of TNFR1 was demonstrated as another determinant besides STAT1 [185, 187, 191], most of the other cytokine-related reports about senescence lack detailed downstream analyses of the TNF signaling. This is partly due to the fact that senescence induction following single cytokine treatment with IFN-γ was also observed in other systems that thus did not rely on the application of TNF [197, 199]. Wieder et al. proposed a possible mode-of-action model, which involves synergistic effects of IFN-γ and TNF that together induce a cell cycle arrest in G0/G1 and prevent S-phase transition via STAT1-mediated gene expression and the activation of NF-κB signaling [357]. In this context, Hubackova et al. found for the NF-κB subunit p65 that nuclear translocation occurred after treatment with both cytokines [199]. Despite missing functional analyses, they assumed a greater impact of IFN-γ over TNF that could even activate NF-κB in the absence of TNF, as they also demonstrated senescence induction with IFN-γ alone [199]. In the opposite case, Kandhaya-Pillai et al. induced senescence with TNF alone and they surprisingly uncovered involvement of STAT1 in this process [200]. Even though a pathway analysis identified NF-κB signaling and a gene expression analysis confirmed upregulation of *NFKB2*, *NFKBIA*, and *NFKBIZ* no further functional analyses were conducted to determine its role for the TNF-induced senescence, as it might be of subordinate relevance compared to the identified interferon signature [200]. Nevertheless, activation of NF-κB signaling has also been described in other settings of senescence, and its activity was closely linked to the expression of pro-inflammatory genes and the SASP [341, 358-361]. Besides its role in the SASP formation, NF-κB also contributes to senescence and growth arrest induction by interfering with the cell cycle and its regulation [362-366]. For example, upregulation of p21 was shown to be mediated through NF-κB, even in settings with mutated and inactive p53 [367-371].

The initially identified cell cycle regulators for CIS are p16 and Rb, as the RIP-Tag mouse model has inactivated and non-functional p53 that prevents downstream activation of p21 [185, 190]. Data presented by others also demonstrated induction of p16 and/or p21 for CIS in different cell-based systems ranging from normal cells to cancer cells, and from mice to men [191, 196, 197, 199-202]. In case of the human A204 cell line that expresses wild type p53 activity and was already characterized in earlier studies which analyzed CIS, upregulation of *CDKN1A* (the gene encoding p21) was described [198, 372]. Although *CDKN1A* was not ranked among the most upregulated genes, the expression of p21 was analyzed on the protein level. While no expression of p16 was detected in the A204 cells at all, the cytokine treatment with IFN-γ and TNF increased p21 levels as early as 6 h after

stimulation. In addition, expression of p27 was also increased in response to IFN-γ and TNF, but the effect was quite short-lived and not persistent over time. The role of p21 as the leading cell cycle inhibitor for CIS in the A204 system also fits with previous studies that reported its induction in response to TNF as well as interferons, such as IFN-α, IFN-β, and IFN-γ [368, 370, 373-384]. It is also noteworthy that p21 is essential for the viability and survival of senescent cells in response to persistent DNA damage [385]. By fostering senescence, p21 prevents activation of the ATM kinase and NF-κB, which would normally activate JNKs and different caspases (including caspase-3) resulting in cell death [385]. Furthermore, evidence for binding of p21 to pro-caspase-3 was shown, which prevents its activation and the execution of apoptosis [386].

Speaking of cell death and caspases, another observation revealed by our transcriptome-based pathway analysis was the strong induction of players of the apoptosis cascade on all levels during the 96 h cytokine treatment. Although IFN-γ and TNF are both well-known inducers of apoptosis [387, 388], this effect was mainly attributed to the TNF signaling, and covers the induction of ligands (e.g., TNF itself) and receptors (e.g., TNFR2) as well as different downstream effectors, such as caspase-3. Astonishingly, the protein analysis revealed accumulation of the inactive proenzyme over time in the absence of detectable levels of active cleaved caspase-3. Consistent with these results was the observation that not only pro-apoptotic genes were highly upregulated upon treatment but also anti-apoptotic factors, which counteract the cell death fate and mediate cellular survival. These factors include for instance members of the cellular inhibitor of apoptosis protein (c-IAP) family that are able to prevent the activation of different caspases and thereby apoptotic cell death [389-392]. As an example, c-IAP2 (encoded by the *BIRC3* gene) was analyzed by Western blot, which revealed its expression in response to the cytokine treatment. This effect was strongest when both cytokines were present, as it led to an accumulation of the protein over time, whereas only a minimal induction was observed for the single treatments with either TNF or IFN-γ alone.

In addition to the aforementioned TNF-related effects, a possible contribution of IFN-γ to the process of apoptotic cell death and its modulation was for instance observed by the induction of the interferon alpha-inducible protein 27 (*IFI27*, also known as *ISG12a*) [393-396] and the interferon-induced helicase C domain-containing protein 1 (*IFIH1*, also known as melanoma differentiation-associated protein 5, *MDA5*, or Helicard) [397-401]. An upregulation of these factors together with other interferon response genes was also found within the interferon signature identified by Kandhaya-Pillai et al. in the model of TNF-driven senescence [200]. Another interesting interconnection of the interferon and TNF signaling pathways in the context of cell death and apoptosis is the reciprocal relationship between STAT1 and caspases. Some studies reported upregulation as well as activation of different caspases, such as caspase-3 through stimulation with IFN-γ or other substances, and demonstrated involvement of STAT1 in these processes [402-408].

Vice versa, caspase-mediated cleavage of STAT1 was identified as a post-translational modification of the protein that was further associated with its inactivation and the regulation of cell death pathways, including apoptosis [409-412]. Furthermore, Wang et al. identified a TNF-dependent protein-protein interaction of STAT1 with TNFR1 and TRADD that suppresses downstream NF-κB activation and promotes apoptosis induction [413]. This matter was further analyzed by Wesemann et al. who reasoned that stimulation with IFN-γ could interfere with this association by reducing the ability of STAT1 to bind to TNFR1, and thus leading to sustained activity of NF-κB [414]. They demonstrated recruitment and binding of STAT1 to TNFR1 in response to TNF, while additional treatment with IFN-γ was able to abrogate their interaction through the activation and nuclear translocation of STAT1, which in turn permits augmented NF-κB activity [414]. Another report published by the same group identified the existence of a TRADD-STAT1 complex found in the cytoplasm and nucleus after IFN-γ stimulation [415]. This observation indicates a role of TRADD in the downstream regulation of IFN-γ signaling that is independent of the TNF pathway [415]. It was recently shown that STAT1 is involved in the apoptotic process induced through transmembrane TNF, which is a precursor form of the soluble molecule [416]. Eventually, it was also discovered that even unphosphorylated STAT1 can influence the expression of different genes (e.g., *IFI27* or *IFIH1*) [417], and mediate resistance to apoptosis [418, 419].

The original description of CIS by Braumüller et al. revealed that the Th1 cell-mediated senescence induction in the tumor cells occurs in the absence of enhanced apoptosis as shown by various assays, including staining for caspase-3 [185]. This confirmed the initial results obtained from *in vivo* experiments in the RIP-Tag mouse model that indicated formation of tumor dormancy through cellular growth arrest instead of tumor cell destruction, or apoptosis induction [187]. While only few other reports about senescence induction through cytokines exist and not all of them even addressed apoptosis, some studies demonstrated induction of both, senescence and apoptosis, in response to the cytokines IFN-γ and/or TNF [197, 201]. A criterion of senescent cells is their resistance to apoptotic cell death and senescent cell anti-apoptotic pathways (SCAPs) have been identified and highlighted in the last years. Important factors associated with SCAPs include anti-apoptotic members of the Bcl-2 family, HSP90, serpins (an acronym for serine protease inhibitors), hypoxia-inducible factor 1 alpha (HIF-1α), p53 and p21, as well as the PI3K/AKT signaling axis that is generally associated with cellular survival [420-422]. While PTEN normally acts as a potent antagonist that negatively regulates the PI3K/AKT pathway [423-426], we found a notable downregulation of this tumor suppressor in the transcriptome analysis, which was also confirmed for the protein itself. This effect was only observed in response to the treatment with IFN-γ and TNF, and gradually occurred over the course of time. Although senescence induction through the loss of PTEN has already been described in the literature, it is most likely that the expression of PTEN is repressed by TNF and NF-κB, which leads to its incremental decline during CIS [98, 123, 124, 427, 428].

Downregulation of *PTEN* might further explain the decrease of the initially upregulated p27 protein level, since this cell cycle regulator is a known downstream mediator of PTEN [429-434]. Contrary to our initial expectations, the protein analysis for AKT revealed a reduced phosphorylation at the serine residue 473 that was further linked to a moderate downregulation of pan-AKT during treatment with both cytokines. In this context, the functional consequences of the reduced AKT level and its activity remain enigmatic, as phosphorylation at threonine 308 plays also a role in the activation of the protein [435-437]. Since AKT is not only involved in processes regulating cell death and survival but also associated with cell cycle progression and proliferation [438], its downregulation might be linked to the induction of the cell cycle regulators p21 and p27. This is likely, as AKT can phosphorylate both proteins and thereby prevent their nuclear translocation, which is necessary for the p21- as well as p27-mediated cell cycle arrest [439-445]. Altogether, the molecular crosstalk of the different signaling pathways as outlined above mediates growth arrest and CIS through the suppression of proliferation while maintaining cellular survival.

In conclusion, the data presented in this thesis broaden the understanding of the cellular events and the underlying signaling networks that finally lead to the senescent state of CIS. By combining computational modeling to analyze complex biological data sets (i.e., the transcriptome of the cytokine-treated cells and its modulation over time) and functional analyses to determine the cellular outcome (i.e., protein analyses of highly regulated targets in terms of general expression and their activation), we were able to identify the fundamental networks of gene regulation as well as the cellular signaling cascades driving CIS. As a result of the continuous treatment with pro-inflammatory cytokines, a hyperactivation of the respective signaling pathways was triggered that together mediated the cytostatic effects. Through the accumulation of non-lethal stress during the 96 h of treatment, CIS phenotypically resembles a pro-apoptotic state where apoptosis is induced, but not executed. This finding is in line with the original observation that CIS occurred in cells escaping from apoptosis mediated by IFN-γ and TNF, and also fits with the fact that senescent cells are resistant to apoptosis [155-160, 185]. Further studies comparing the direct effects of IFN-γ and TNF in different cell types and settings (i.e., *in vitro* vs. *in vivo*) over time, or even with other triggers of senescence will reveal the distinct nature of CIS and the senescent state in general. The careful evaluation of available data indicated that senescence induction through cytokines as well as the targets involved may vary in different model systems [185, 191, 192, 197-202]. Moreover, Sapega et al. even showed reversal of the growth arrest after withdrawal of the cytokines [196]. As CIS might not be irreversible and rather represents a long-lasting growth arrest that is induced as a consequence of the continuous treatment, its impact on the epigenetic landscape of the cells should also be addressed in the future. Current investigations also showed that senescence is not a static cell fate but a quite dynamic state that relies on the constant action of important molecules, such as transcription factors and epigenetic modifications for its maintenance [111, 446, 447].

Hence, it depends on further examinations of the senescent cells whether or not induction of an epigenetic memory exists in the context of CIS. This will elucidate if these cells remember an initial stress beyond the treatment phase, or if a continuing stimulation of the underlying signaling pathways is mandatory to keep the cells in this fragile state.

Regarding a potential therapy based on antigen-specific $CD4^+$ Th1 cells and CIS, different studies provide growing evidence that Th1 cell-mediated anti-tumor immunity leads to a beneficial treatment outcome and prolonged survival in patients with different cancers [357, 448-454]. This is further supported by various observations that a therapeutic response correlates with the successful activation of IFN-γ signaling [191, 455-458]. In addition, another recent study reported senescence-inducing properties of another T cell subset, namely γδ T cells, that when stimulated with IL-12 and IL-18 also secrete the pro-inflammatory cytokines IFN-γ and TNF, and mediate anti-tumor immunity [459]. Since cell-based immunotherapies are effective but quite complex, the direct use of the effector cytokines could be applied instead. Currently no (systemic) cytokine therapy is approved for IFN-γ and TNF. Nevertheless, clinical investigations using these cytokines for isolated limb perfusions (with or without additional chemotherapeutic drugs) demonstrated therapeutic responses in the past [460-466]. Aside from that, IFN-α as a single agent or in combination with other substances is already in clinical use for the treatment of chronic hepatitis B and C, and patients with different cancers, such as malignant melanoma could also benefit from this therapy [467-471]. This cytokine might be a suitable alternative for IFN-γ, since downstream signaling of IFN-α also involves STAT1 and senescence induction through type I interferons has been described [270, 472-476]. However, additional application of TNF might still be a limiting factor for the therapeutic use of CIS as a cytokine therapy. Other limiting factors in this regard are the dependencies on undisrupted cytokine signaling and on intact cell cycle regulators [185, 187, 191].

As a novel immune-mediated type of tumor control, CIS stably limits the growth of different cancer cells, but it still harbors the potential risk of relapse. In addition, the expected formation of a SASP that could reinforce the senescent state might also exert pro-tumorigenic effects on the surrounding tissue. Therefore, clearance of the potentially harmful senescent cells seems to be a reasonable approach to prevent such adverse effects and cancer regrowth [91, 92, 110, 477]. Since CIS exhibits traits of an early apoptosis and involves SCAPs that mediate cellular survival, the subsequent application of senolytic drugs (e.g., ABT-263, also known as navitoclax, which is an inhibitor of anti-apoptotic Bcl-2 family members) to selectively remove the senescent tumor cells could also be a promising strategy in the adjuvant treatment of cancer [478-480].

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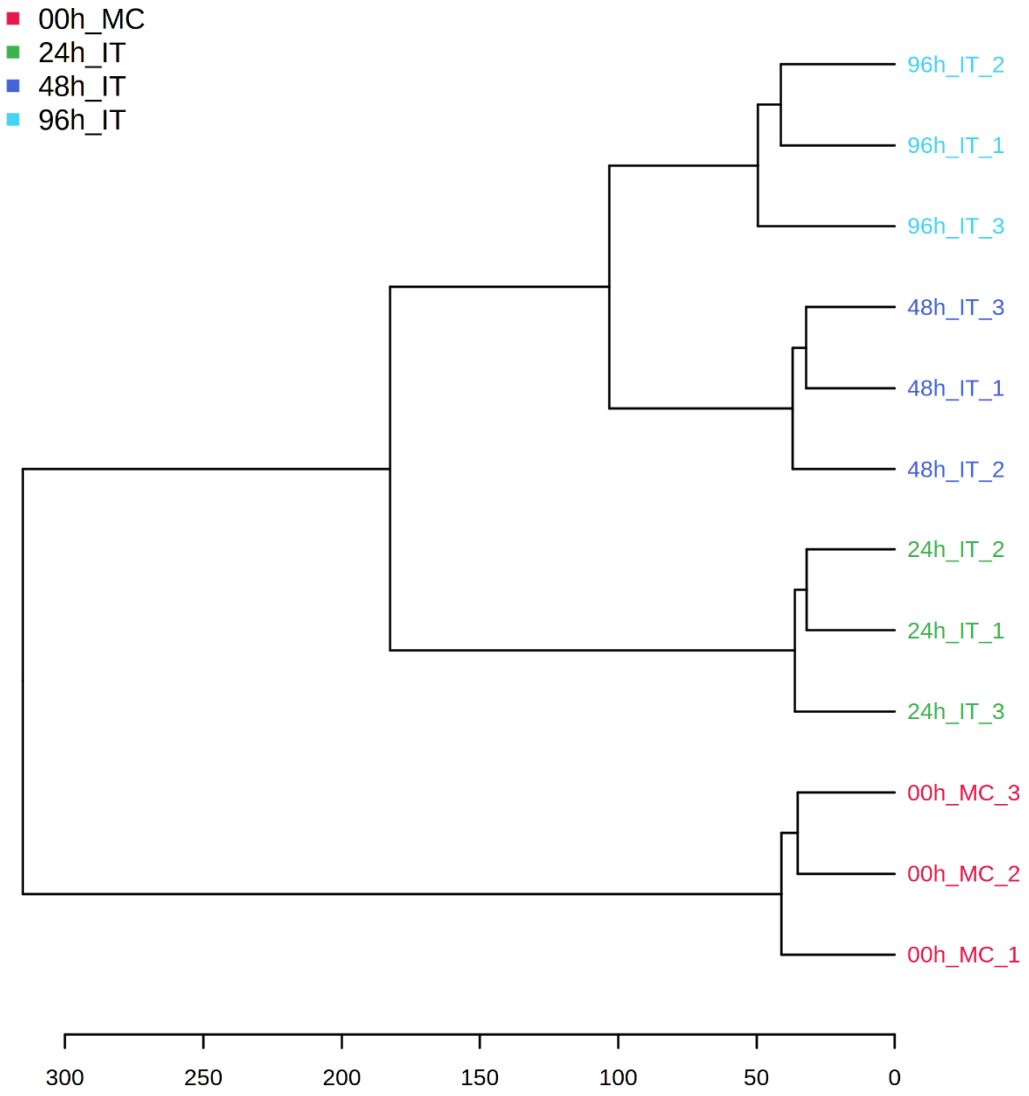
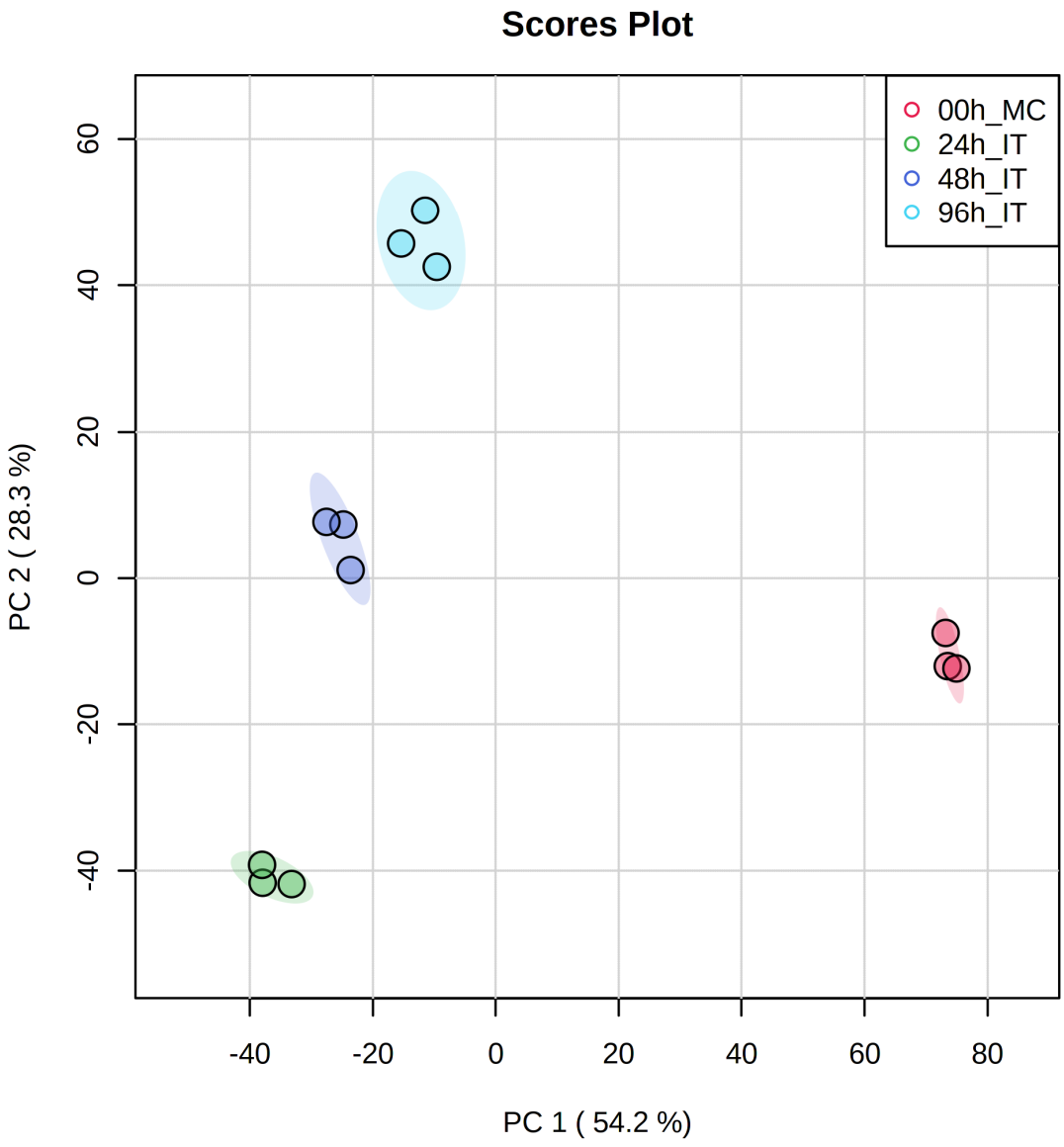
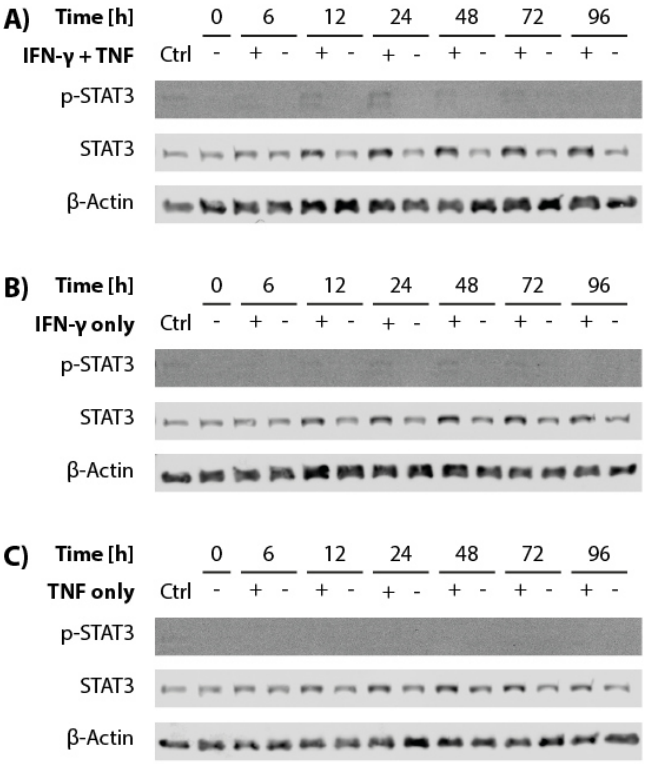
8. Appendix



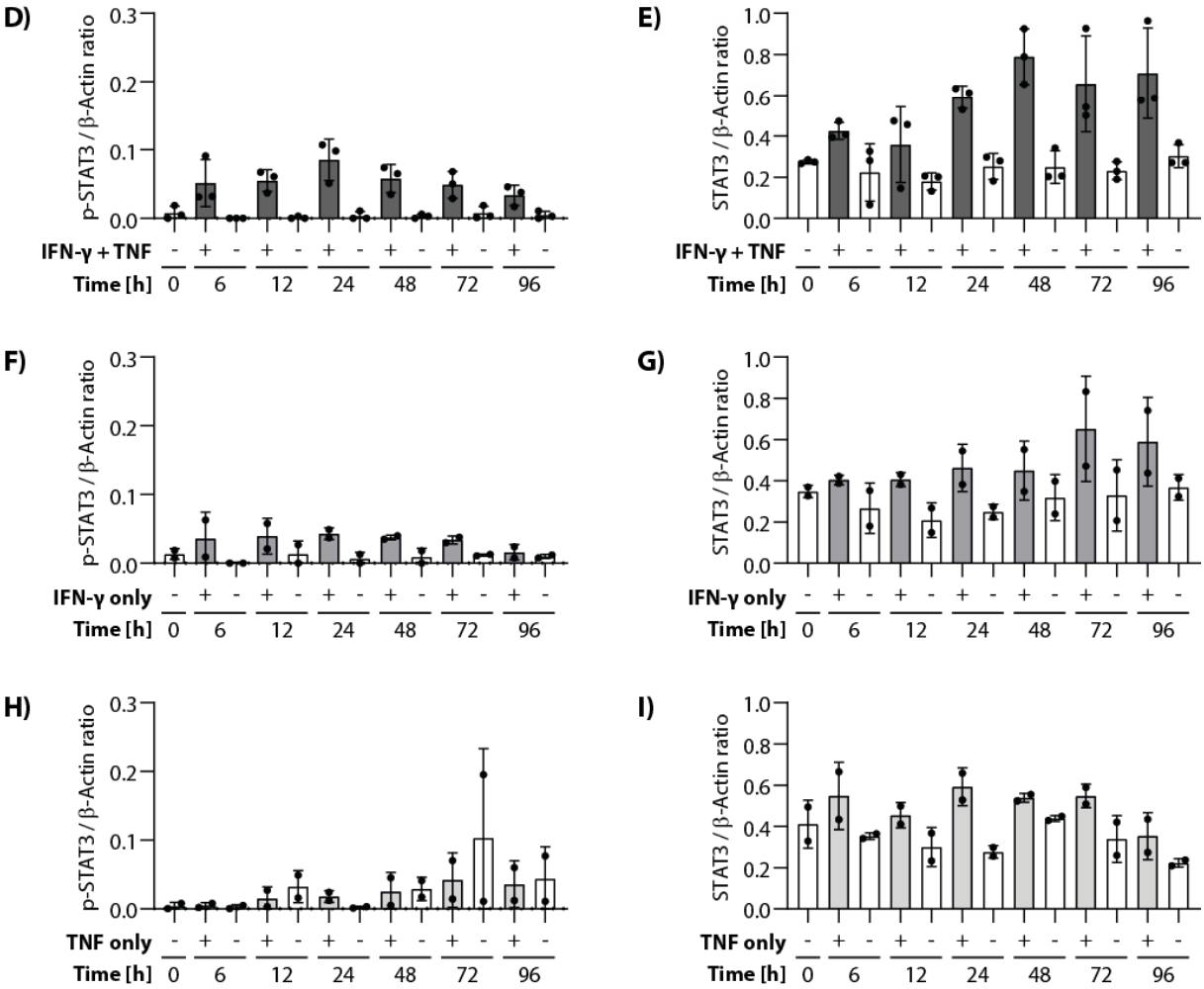
Supplementary Figure 01: Graphical representation of the hierarchical cluster analysis used to group the samples. On basis of a hierarchical cluster analysis encompassing all samples a tree-like dendrogram was generated. The scale bar indicates a virtual distance that is used to measure the differences of the individual data sets to one another. By this, similar samples are grouped and joined together as seen above.

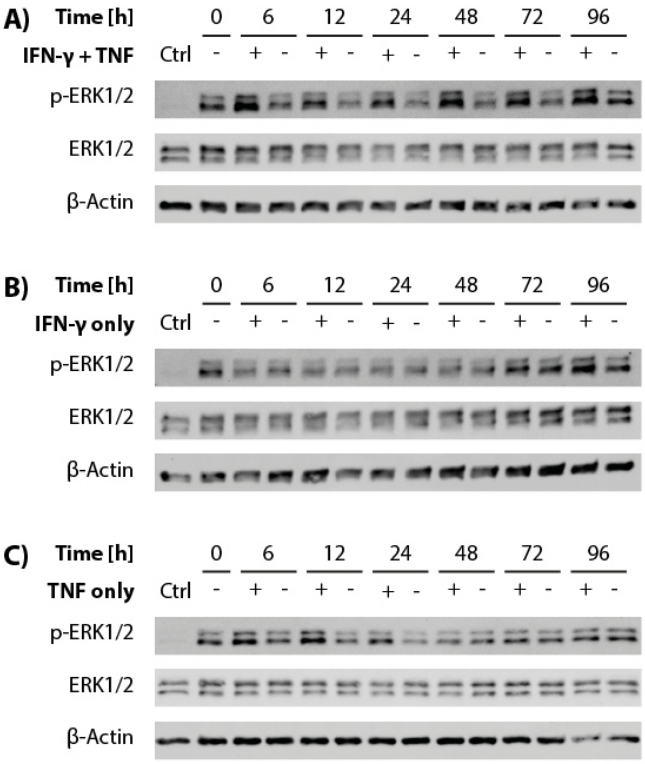


Supplementary Figure 02: PCA scores plot of the microarray expression data from all biological replicates of each group. 95 % confidence areas (colored ellipses) were plotted around the respective biological replicates (colored circles) from each group (n=3 for 0 h MC, 24 h IT, 48 h IT, and 96 h IT). The analysis clearly shows that biological groups cluster separately, indicating a differential response to the treatment with IFN-γ [100 ng/mL] and TNF [400 pg/mL].

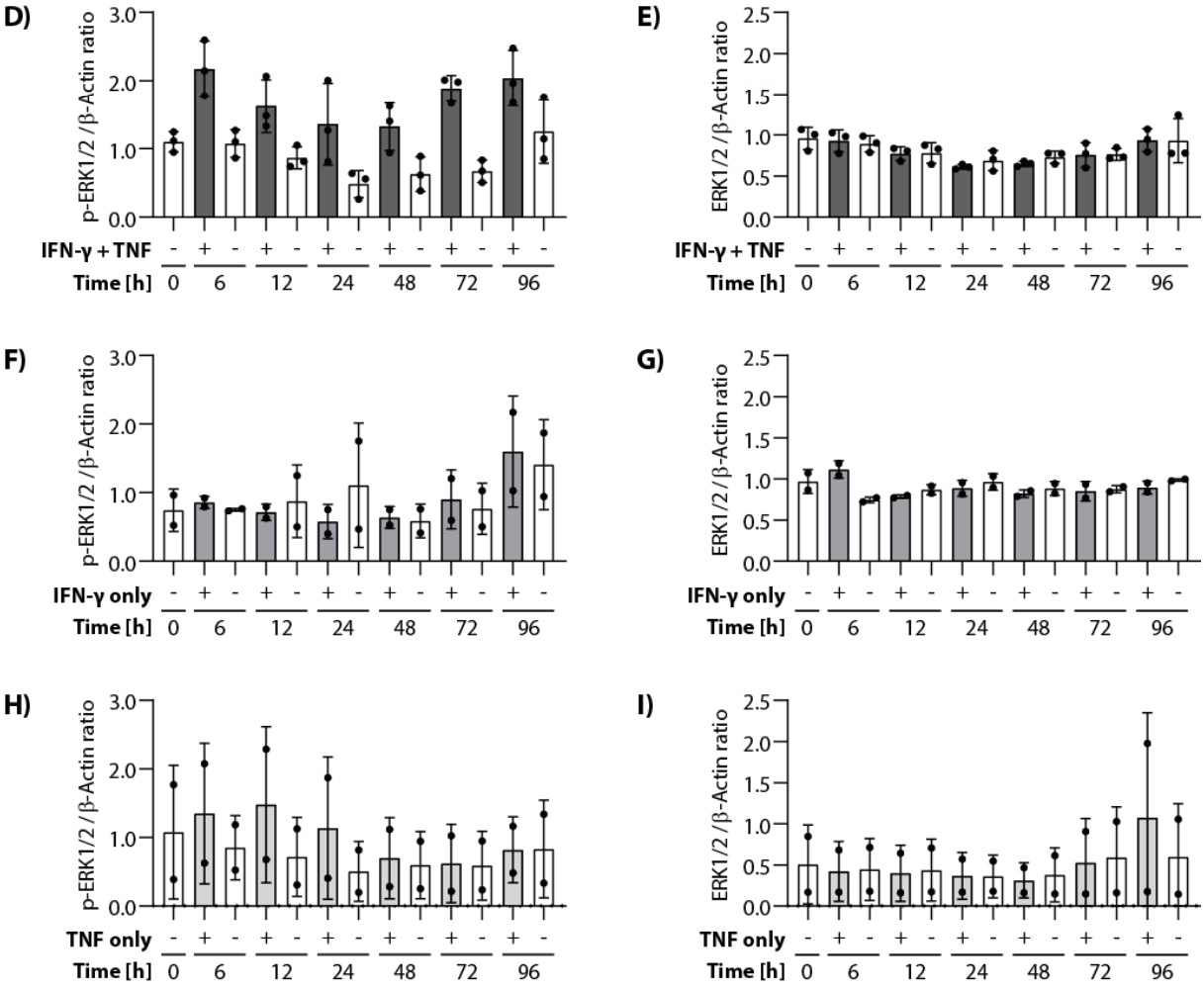


Supplementary Figure 03: Regulation of STAT3 protein levels upon cytokine treatment. Human A204 cancer cells were treated for up to 96 h with IFN-γ [100 ng/mL] and TNF [400 pg/mL] either alone or in combination. Medium containing PBS served as control. During the indicated treatment, protein extracts were prepared and analyzed by Western blot. HeLa cell lysates were used as a control (Ctrl). Protein levels of phospho-STAT3 (p-STAT3) and STAT3 were determined for the cytokines either in combination with IFN-γ + TNF (A), or as single treatments with IFN-γ only (B) and TNF only (C). Semi-quantitative densitometric analyses of the respective Western blots were used to evaluate the activation and expression levels of STAT3 for treatment with IFN-γ + TNF (D & E), or IFN-γ only (F & G) and TNF only (H & I). Representative Western blots and quantifications are shown (mean ± SD; n=3 for IFN-γ + TNF; n=2 for IFN-γ only and TNF only, respectively).





Supplementary Figure 04: Regulation of ERK1/2 protein levels upon cytokine treatment. Human A204 cancer cells were treated for up to 96 h with IFN-γ [100 ng/mL] and TNF [400 pg/mL] either alone or in combination. Medium containing PBS served as control. During the indicated treatment, protein extracts were prepared and analyzed by Western blot. HeLa cell lysates were used as a control (Ctrl). Protein levels of phospho-ERK1/2 (p-ERK1/2) and ERK1/2 were determined for the cytokines either in combination with IFN-γ + TNF (A), or as single treatments with IFN-γ only (B) and TNF only (C). Semi-quantitative densitometric analyses of the respective Western blots were used to evaluate the activation and expression levels of ERK1/2 for treatment with IFN-γ + TNF (D & E), or IFN-γ only (F & G) and TNF only (H & I). Representative Western blots and quantifications are shown (mean ± SD; n=3 for IFN-γ + TNF; n=2 for IFN-γ only and TNF only, respectively).



9. List of Publications

I. Rentschler M, Chen Y, Pahl J, Soria-Martinez L, Braumüller H, Brenner E, Bischof O, Röcken M and Wieder T. ***Nuclear Translocation of Argonaute 2 in Cytokine-Induced Senescence.*** Cell Physiol Biochem. 2018; 51(3):1103-1118. doi: 10.1159/000495490.

II. Gassenmaier M*, Rentschler M*, Fehrenbacher B, Eigentler TK, Ikenberg K, Kosnopfel C, Sinnberg T, Niessner H, Bösmüller H, Wagner NB, Schaller M, Garbe C and Röcken M. ***Expression of DNA Methyltransferase 1 Is a Hallmark of Melanoma, Correlating with Proliferation and Response to B-Raf and Mitogen-Activated Protein Kinase Inhibition in Melanocytic Tumors.*** Am J Pathol. 2020 Oct; 190(10):2155-2164. doi: 10.1016/j.ajpath.2020.07.002.

* These authors contributed equally to this work.

III. Rentschler M, Braumüller H, Briquez PS and Wieder T. ***Cytokine-Induced Senescence in the Tumor Microenvironment and Its Effects on Anti-Tumor Immune Responses.*** Cancers (Basel). 2022 Mar 8; 14(6):1364. doi: 10.3390/cancers14061364.

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