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The Effects of Conventional Chemotherapeutics on the Activation of Type I Interferons (IFN-α/β) in Hepatic Cancer Cells *in vitro*: The Significance of Endogenous Type III Interferons (IFN-λ)

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Hiermit erkläre ich, die Dissertation mit dem Titel "The Effects of Conventional Chemotherapeutics on the Activation of Type I Interferons (IFN-α/β) in Hepatic Cancer Cells *in vitro*: The Significance of Endogenous Type III Interferons (IFN-λ)" eigenständig angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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List of Abbreviations

-/-	Homozygous knockout
AcD	Actinomycin D
AMP	Adenosine monophosphate
AP-1	Activator protein 1
ATF2	Activating transcription factor 2
ATP	Adenosine 5'-triphosphate
BCG	Bacillus Calmette-Guérin
BCLC	Barcelona Clinic Liver Cancer staging system
bp	Basepair
Cas/*cas*	CRISPR-associated protein/gene
CD	Cluster of differentiation (protein)
cGAS	Cyclic GMP-AMP synthase
cGAMP	Cyclic GMP-AMP
CHX	Cycloheximide
CRISPR	Clustered regularly interspaced short palindromic repeats
CT	Threshold cycle
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
DAMP	Danger/Damage-associated molecular pattern
DEAE-dextran	Diethylaminoethyl-dextran
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
- cDNA	Complementary DNA
- gDNA	Genomic DNA
dNTPs	Deoxynucleoside triphosphates
DOX	Doxorubicin
DPBS	Dulbecco's Phosphate Buffered Saline
ds	Double-strand
EASL	European Association for the Study of the Liver
ECOG	Eastern Cooperative Oncology Group Score

EGFR	Epidermal growth factor receptor
EORTC	European Organisation for Research and Treatment of Cancer
FBS	Fetal Bovine Serum
GMP	Guanosine monophosphate
GEM	Gemcitabine
GFP	Green fluorescence protein
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HGNC	HUGO Gene Nomenclature Committee
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HMGB1	Non-histone chromatin-binding nuclear protein high-mobility group protein B1
HPRT/*Hprt*	Hypoxanthine phosphoribosyltransferase gene (human/murine genome)
HR	Homologous repair
IC_{50}	Half maximal inhibitory concentration
ICD	Immunogenic cell death
ICGHN	International Consensus Group for Hepatocellular Neoplasia
IFN	Interferon (protein)
- IFN-α	Interferon-alpha (protein)
- IFN-β	Interferon-beta (protein)
- IFN-γ	Interferon-gamma (protein)
- IFN-λ	Interferon-lambda (protein)
IFN/*Ifn*	Interferon gene (human/murine genome)
- *IFNA*/*Ifna*	Interferon-alpha (gene)
- *IFNB*/*Ifnb*	Interferon-beta (gene)
- *IFNL*/*Ifnl*	Interferon-lambda (gene)
- *IFNG*/*Ifny*	Interferon-gamma (gene)
IFNAR	IFN-α/β-receptor
IFNGR	IFN-γ receptor complex
IFNLR1	IFN-λ receptor subunit 1

IL28A	Interleukin-28A (equivalent to IFN-λ2)
IL28B	Interleukin-28B (equivalent to IFN-λ3)
IL29	Interleukin-29 (equivalent to IFN-λ1)
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene (human/murine genome)
- *CXCL10*/*Cxcl10*	C-X-C motif chemokine ligand 10 (equivalent to *IP10*)
- *IP10*/*Ip10*	C-X-C motif chemokine ligand 10 (equivalent to *CXCL10*)
- *MX1*/*Mx1*	Myxovirus resistance dynamin like GTPase 1
- *USP18*/*Usp18*	Ubiquitin specific peptidase 18
IP10	C-X-C motif chemokine ligand 10 (equivalent to CXCL10)
ISRE	Interferon-stimulated regulatory element
JAK	Janus kinase
LGP2	Laboratory of genetics and physiology 2
MAVS	Mitochondrial antiviral-signaling protein
MDA5	Melanoma differentiation associated antigen 5
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MMHC	Mouse Models of Human Cancer
mt	Mitochondrial
MyD88	Myeloid differentiation primary response 88
MX1	Myxovirus resistance dynamin like GTPase 1
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor κ-light-chain enhancer of activated B-cells
NHEJ	Nonhomologous end joining
NK	Natural killer (cell)
NTCP	Sodium taurocholate co-transporting peptide
OXA	Oxaliplatin
PAM	Protospacer adjacent motif
PAMP	Pathogen-associated molecular pattern

PCR	Polymerase chain reaction
- qPCR	Quantitative/real-time PCR
- qRT-PCR	Quantitative/real-time reverse transcriptase PCR
- RT-PCR	Reverse transcriptase PCR
PD-1/PD-L1	Programmed cell death protein 1/programmed cell death ligand 1
PEI	Percutaneous ethanol injection
Poly(I:C)	Polyriboinosinic acid and polyribocytidylic acid complexes
PRR	Pattern-recognition receptor
RFA	Radiofrequency ablation
RIG-I	Retinoic-acid inducible gene-I
RNA	Ribonucleic acid
- crRNA	CRISPR RNA
- pre-crRNA	Precursor CRISPR RNA
- sgRNA	Single guide RNA
- siRNA	Short interfering RNA
- tracrRNA	Trans-encoded/trans-activating CRISPR RNA
RNase	Ribonuclease
SNP	Single nucleotide polymorphism
ss	Single-strand
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
TACE	Trans-arterial chemoembolization
TCGA	The Cancer Genome Atlas
TICAM	TIR domain-containing adaptor molecule
TLR	Toll-like receptor
TNM	Tumor-node-metastasis classification
TRIF	TIR domain-containing adaptor inducing IFN-β
USP18	Ubiquitin specific peptidase 18
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
wt	Wildtype

1. Introduction

1.1 Hepatocellular Carcinoma

Cancer has been firmly set within the top spots of leading global causes of death for years. Currently responsible for 4.5 million or roughly 30% of premature deaths, cancer is second only to cardiovascular diseases (Cao et al. 2020). Of all cancer cases worldwide, liver malignancies account for around 4.7% or 841.000 new cases annually, according to the current global cancer statistic GLOBOCAN 2018 and the corresponding work published by Freddie Bray et al. (2018). Unfortunately, liver cancer is characterized by high fatality, causing 8.2% or 782.000 of all cancer deaths worldwide (Bray et al. 2018). In total, liver cancer is currently the sixth most commonly diagnosed cancer as well as the fourth leading cause of cancer death worldwide, and incidence and mortality rates are expected to rise even further in the upcoming years (Balogh et al. 2016; Bray et al. 2018).

Of the primary liver cancer entities, hepatocellular carcinoma (HCC) makes up 75% to 85%, while cholangiocarcinoma and an array of rare tumors account for the remaining part (Saxena et al. 2010; Theise et al. 2010; Kew 2017; Bray et al. 2018). Additionally, the liver is prone to be the target of metastases of other cancer entities, with metastatic disease accounting for the highest number of liver tumors in total (Iacobuzio-Donahue and Ferrell 2010; Saxena et al. 2010).

If assessing gender-separated populations, liver cancer incidence tends to be two to three times higher in males than in females, thus making liver cancer the second most common cause of cancer death in men (Nordenstedt et al. 2010; Theise et al. 2010; McGlynn and London 2011; Bray et al. 2018). This is generally attributed to a higher prevalence of risk factors in men, however, hormonal influences have also been suggested (Hou J et al. 2013; Theise et al. 2014). Additionally, the epidemiology of liver cancer exhibits regional differences, as 72.5% of all new liver cancer cases occur in Asia, while Europe and North America together account for only 14.8% (Bray et al. 2018). The highest incidence rates are found in regions of Eastern Asia, South-Eastern Asia and Africa, where liver cancer is the most commonly diagnosed cancer in men in various countries (Bray et al. 2018). While this can once again be at least partly credited to higher rates of predisposing conditions and risk factor exposure, studies assessing different descents within populations have also pointed towards the involvement of genetic and epigenetic factors (Calvisi et al. 2007; Theise et al. 2010;

McGlynn and London 2011). This implies the existence of subpopulations with an increased risk for developing liver cancer and therefore HCC.

Etiological factors for the development of HCC are manifold. The occurrence of liver malignancies is generally associated with chronic liver disease facilitating the malignant transformation of precancerous lesions (ICGHN 2009; Lata 2010; Theise et al. 2010; McGlynn and London 2011; Baffy et al. 2012). While cirrhosis is often mentioned in this context, it is neither a typical premalignant lesion nor an obligatory requirement for liver cancer (Theise 1996; Chen et al. 2006; Theise et al. 2010). Neil Theise (1996) describes cirrhosis and HCC development as two independent processes, that do, however, often go side by side. Cirrhosis should thus be regarded as an indicator of an underlying chronic liver disease, which poses the actual risk factor. Nonetheless, patients with liver cirrhosis are at a high risk for developing HCC (Balogh et al. 2016).

In their evaluation of the Global Burden of Disease Study 2015 Tomi Akinyemiju et al. (2017) investigated all deaths from liver cancer between 1990 and 2015 and reported viral hepatitis to be the major cause, accounting for 33% of deaths from liver cancer through hepatitis B and 21% of deaths from liver cancer through hepatitis C, respectively. Both the hepatitis B virus (HBV) and hepatitis C virus (HCV) have been identified as hepatocarcinogenic, however, only up to 25% of chronically infected patients develop HCC (Gurtsevitch 2008; Bouvard et al. 2009; Yang et al. 2010; Crissien and Frenette 2014). In the current World Cancer Report Chien-Jen Chen (2020) describes the process of hepatocarcinogenesis on the basis of hepatitis as being multistage, of multifactorial etiology, and influenced by manifold circumstances, including various mechanisms of immune tolerance and clearance.

In adults, less than 5% of HBV infections lead to chronic hepatitis B (Tassopoulos et al. 1987). However, children are at a much higher risk, as around 90% of perinatal infections and 20% to 50% of infections before the age of five advance to chronic disease (Stevens et al. 1975; Beasley et al. 1982; Coursaget et al. 1987). The annual HCC incidence in HBV carriers has been shown to vary between 0.4% and 2.5%, but incidence rates are greatly influenced by factors such as descend, age, age at point of infection, and occurrence of cirrhosis (Beasley et al. 1981; Sakuma et al. 1988; Koike et al. 2002; Bruix and Sherman 2005). Risk factors for the progression from chronic hepatitis B to HCC include antigen-serostatus, viral load, viral genotype and specific mutations as well as co-infection with HCV or human immunodeficiency virus (HIV) (Chen et al. 2006; Yang et al. 2010; Huang et al. 2011; Tseng et al. 2012; Crissien and Frenette 2014; Chen 2020). The Risk Evaluation of Viral Load

Elevation and Associated Liver Disease/Cancer (REVEAL) study identified genotype variants of the sodium taurocholate co-transporting peptide (NTCP), an HBV receptor, to be influencing both the antigen serostatus and the rate of advancement of chronic HBV infection to HCC (Hu et al. 2016). However, hepatitis B is beginning to decline in importance, as the HBV immunization programs have significantly reduced the burden of disease, especially in children, therefore greatly decreasing HCC incidence among vaccinated populations (Chang et al. 1997; Chiang et al. 2013; Chen 2018).

In contrast to HBV, HCV causes chronic infection in the majority of cases, with approximately 75% of patients developing chronic hepatitis C (Grebely et al. 2014). Symptoms tend to be mild or non-specific, which is why chronic HCV infections are typically not diagnosed until cirrhosis has occurred and the liver function has been greatly impaired (Tong et al. 1995; Theise et al. 2014; Balogh et al. 2016; Evon et al. 2018). Chronic hepatitis C leads to liver fibrosis and subsequently cirrhosis, based on which HCC develops with an incidence of around 2% to 8% annually or a five year rate of 7% to 13.4% (Fattovich et al. 1997; Yoshida et al. 1999; Degos et al. 2000). While HBV has been reported to lead to HCC without cirrhosis especially in patients of Asian descent, HCV rarely causes HCC independently from cirrhosis (Niederau et al. 1998; Yoshida et al. 1999; Evans et al. 2002; Crissien and Frenette 2014). Risk factors for the progression of chronic hepatitis C infection towards cirrhosis and HCC are, inter alia, co-infection with HIV or HBV as well as alcohol consumption (Roudot-Thoraval et al. 1997; Cacciola et al. 1999; Giordano et al. 2004; Hutchinson et al. 2005; Squadrito et al. 2013). Additionally, the HCV genotype is also an important determinant, as genotype 1b has been shown to greatly increase the risk of HCC occurrence in patients with cirrhosis (Bruno et al. 2007).

The mechanism of how HCV perseveres and leads to chronic infection is not completely understood. One viral factor, which could facilitate escaping host immunity, may be HCV's tendency to mutate rapidly, resulting in extremely heterogenous virus populations and thus obstructing immune clearance (Farci et al. 2000). HCV-specific cluster of differentiation protein 4 positive ($CD4^+$) T-cells have been shown to play a vital role in acquiring immunity against HCV (Grakoui et al. 2003). Several favorable predictors that come with an increased rate of spontaneous HCV clearance and thus decreased rate of chronic hepatitis C have been identified so far. Among those are female sex, the HCV genotype 1 as well as host genetic factors such as certain major histocompatibility complex (MHC) class II genotypes, specifically human leukocyte antigen (HLA)-DRB1 and HLA-DQB1 alleles, and a polymorphism

near interleukin-28B (*IL28B*), which has since been renamed to interferon-lambda-3 (*IFNL3*) (Thursz et al. 1999; Grebely et al. 2014). The role of type III interferons (IFNs), i.e. IFN-λ, in the context of hepatitis C and more importantly HCC is at the very center of thesis and will be further explored in the upcoming sections.

Next to chronic hepatitis, major causes of HCC are alcohol consumption and aflatoxin exposure. Alcohol consumption leads to liver injury, fibrosis, cirrhosis, and subsequently HCC and has been shown to be responsible for around 30% of all liver cancer deaths (Baan et al. 2007; Gelband et al. 2015; Akinyemiju et al. 2017). While alcohol is an important factor globally, the hepatotoxic aflatoxins are mainly prevalent in Africa, Asia and Latin America due to high local exposure rates, causing HCC independently as well as synergistically with chronic hepatitis or alcohol consumption (Pitt et al. 2012; Chu et al. 2017; Chu et al. 2018).

Especially in countries with high human development indices the distribution of the etiological factors of liver cancer has been shown to shift, as profiles of predisposing conditions are changing and the survival of patients with chronic liver disease has improved (Theise et al. 2010; McGlynn and London 2011; Baffy et al. 2012; Balogh et al. 2016). As the global development of obesity, diabetes and non-alcoholic fatty liver disease (NAFLD) is reaching epidemic levels, these known metabolic risk factors are gaining in importance (Calle et al. 2003; Reddy and Sambasiva Rao 2006; Larsson and Wolk 2007; Nordenstedt et al. 2010; Baffy et al. 2012; Wang et al. 2012; Gao et al. 2013). Patients affected by obesity have been shown to be at an up to four times higher risk of developing HCC, which might be mediated through NAFLD or non-alcoholic steatohepatitis (NASH), as these conditions are a common occurrence in obese patients and have been shown to increase the risk of liver cancer (Larsson and Wolk 2007; Michelotti et al. 2013). Overall, the causes of HCC tend to interact synergistically, ergo, the accumulation of risk factors further increases HCC incidence.

While the etiological factors of HCC presented up to this point are either acquirable or habitual, rare hereditary conditions may also cause liver cancer. Genetic dispositions with an increased risk for liver cancer include haemochromatosis, α1-antitrypsin deficiency, Wilson's disease, and certain types of porphyria (Theise et al. 2010; McGlynn and London 2011; Balogh et al. 2016). But even outside of hereditary diseases, HCC has shown familial accumulation tendencies independently from common environmental factors (Chen 2020). This once again suggests genetic and epigenetic factors altering the susceptibility towards risk factors. Genetic factors that have been identified so far include the already mentioned polymorphisms of the HBV receptor NTCP, HLA alleles, and genes for type III IFNs (Thursz et

al. 1999; Grebely et al. 2014; Hu et al. 2016). An array of other metabolism enzymes, oncogenes, tumor suppressor genes, and the androgen receptor have also been shown to be associated with an increased risk of HCC (Chen 2018). Further investigating the genetic factors influencing the vulnerability of individuals affected by HCC may be the key to a deeper understanding of this disease, as current HCC management remains unsatisfactory.

Unfortunately, hepatocellular carcinoma is characterized by high mortality and limited therapy options, resulting in a median survival rate of only 11 months (Greten et al. 2005). Due to this, both the primary prevention and early detection of HCC are currently of the highest priority. Universal newborn vaccination against HBV, the routine screening of blood products for HCV, antiviral therapies, and the reduction of aflatoxins in food have already been shown to lead to a decrease of HCC incidence within populations exposed to these risk factors (Chang et al. 1997; McGlynn and London 2011; Wogan et al. 2012; Chiang et al. 2013; Hosaka et al. 2013; Theise et al. 2014). The global targets drafted by the World Health Organization for 2030 in their agenda against HCC include extensive HBV vaccination coverage, the prevention of mother-to-child HBV transmission, safety in blood transfusions and injection practices, early and thorough diagnosis of hepatitis B and C as well as the treatment of hepatitis in all eligible patients (World Health Organization 2017). Further measures have been suggested to reduce alcohol consumption, exposure to aflatoxins, liver fluke infections, and obesity in regions with high prevalence of these risk factors (Gelband et al. 2015).

Next to preventing the development of liver malignancies by removing predisposing factors, the early and reliable diagnosis of HCC is also of great importance. As liver malignancies generally remain clinically silent until they have reached an advanced stadium and have led to impairment of the liver function, patients are often diagnosed too late for curative therapy options (Theise et al. 2014; Balogh et al. 2016; Kew 2017). Early detection of HCC can therefore be regarded as one of the most important factors in increasing overall patient survival in the current setting, and the establishment of screening methods has been shown to greatly reduce HCC mortality (Zhang et al. 2004). Routine HCC screening is recommended for all individuals with chronic liver disease or at otherwise increased risk (Bruix and Sherman 2005; Bruix and Sherman 2011; Theise et al. 2014; Balogh et al. 2016; EASL 2018).

In order to offer the best possible treatment to each patient, HCC staging is performed to evaluate the tumor burden, the gravity of co-existing cirrhosis, the liver function impairment as well as the overall health condition of the patient (Kew 2017; EASL 2018; Chen 2020). Recommended by the European Association for the Study of the Liver (EASL) guidelines

for clinical decision making, the Barcelona Clinic Liver Cancer (BCLC) staging system offers a validated tool to separate patients into five categories, from very early stage to terminal stage, with subsequent prognostic prediction and treatment recommendations (Forner et al. 2010; EASL 2018). According to the current guidelines, the stage dependent prognosis is defined by tumor status, i.e. tumor size, number of tumors, invasion of vessels or lymph nodes as well as extrahepatic manifestation, the liver impairment as defined by bilirubin, portal hypertension and preserved liver function, and the health status of the patient according to the Eastern Cooperative Oncology Group (ECOG) (Forner et al. 2010; EASL 2018).

Only few patients are diagnosed early enough to be sorted into BCLC-0, the very early stage of HCC, characterized by a single small tumor below 2 cm in diameter without invasion or dissemination, good health status (ECOG-0) and preserved liver function, evaluated for example via the Child-Pugh score (Forner et al. 2010; EASL 2018). With the current screening programs 5% to 10% of patients in Western countries are diagnosed at this stage, but diagnosis rates in countries with less screening established can be assumed to be well below 5% (EASL 2018). Treatment of choice for this HCC stage is surgical resection, or if the tumor is located unfavorably for resection, locoregional therapy via radiofrequency ablation (RFA) (Forner et al. 2010; EASL 2018). After hepatic resection, the median survival has been shown to reach 80% to 90% at five years and similar results have been reported for RFA (Takayama et al. 1998; Livraghi et al. 2008; Hasegawa et al. 2013; Roayaie et al. 2013).

Early stage of HCC, BCLC-A, is defined by either a single tumor exceeding 2 cm in diameter or up to three nodules below 3 cm in diameter in patients in good health (ECOG-0) and without liver function impairment (Child-Pugh A or B) (Forner et al. 2010; EASL 2018). Survival without treatment at this stage is estimated to be around 36 months, while median survival after treatment has been shown to be around 50% to 70% at five years (Bruix et al. 1996; Llovet et al. 1999; Forner et al. 2010). Treatment options for BCLC-A stage HCC include surgical resection of solitary tumors if possible, alternatively liver transplantation in suitable candidates as defined by the Milan criteria or, if neither is an option, locoregional disease control via ablation techniques (Mazzaferro et al. 1996; EASL 2018). However, of the therapy options available, only surgical resection and liver transplantation are potentially curative. As only about 15% of all HCC patients qualify for these options, treatment with the intention to cure remains available to only a small subset of patients (O'Grady and Lawless 2015; Balogh et al. 2016; Kew 2017). Locoregional therapy options include the above mentioned RFA as well as microwave ablation, laser ablation and cryoablation in the

category of thermal ablation, or chemical tumor ablation via percutaneous ethanol injection (PEI) (Lencioni and Crocetti 2012). RFA is currently favored over PEI due to superior rates of overall survival, disease-free survival and recurrence, to an extent where RFA has also proven to be an alternative to surgical resection in well-selected patients (Cho et al. 2009; Orlando et al. 2009; Cho et al. 2010; Germani et al. 2010; Cucchetti et al. 2013; EASL 2018).

If multiple malign nodules are present but the liver function is still adequate with a Child-Pugh score of A or B, patients are categorized into the BCLC-B or intermediate stage (Forner et al. 2010; EASL 2018). Median survival for untreated BCLC-B stage HCC has been reported to be 16 months or 49% at two years (Cabibbo et al. 2010). As these tumors are mostly unresectable, locoregional disease control via trans-arterial chemoembolization (TACE) is considered to be the first-line therapy option, increasing median survival to 19.4 months in a systematic review of 10.108 cases and to up to 40 months in well-selected patients, respectively (Burrel et al. 2012; Malagari et al. 2012; Takayasu et al. 2012; Lencioni et al. 2016). TACE is performed by the intra-arterial infusion of a cytotoxic agent either emulsified with Lipiodol® or carried by drug-eluting beads, and subsequent embolization of the tumor-feeding arterial vessel to exert both cytotoxic and ischemic pressure onto the tumor, while sparing surrounding liver tissue supplied through the portal venous system (EASL 2018). The most common cytotoxic agents used in this technique are doxorubicin, epirubicin, cisplatin and miriplatin, while idarubicin is under evaluation with promising results in the IDASPHERE II phase II trial (Boulin et al. 2016; Lencioni et al. 2016; Guiu et al. 2019). TACE is a well-established treatment for unresectable HCC, but can also be utilized as a bridging or downsizing technique in patients with non-resectable early-stage HCC in order to meet the Milan criteria to allow for liver transplantation (Raoul et al. 2011; Park et al. 2015; EASL 2018).

However, there is significant heterogeneity within the intermediate HCC stage, and subgroups of patients may also benefit from surgical resection or liver transplantation, especially after bridging or tumor downsizing via locoregional techniques (D'Amico et al. 2006; Bruix and Sherman 2011; EASL and EORTC 2012; Tsochatzis et al. 2013; Tsochatzis et al. 2014; Yao et al. 2015; EASL 2018). Similarly, certain subgroups are better served with systemic therapy or best supportive care in line with more advanced disease stages (EASL 2018). Further strategies to define and identify subclasses within BCLC-B are thus needed (Bolondi et al. 2012; Hucke et al. 2014; EASL 2018).

Once HCC has become symptomatic, patients are suffering from at least advanced disease or BCLC-C (Forner et al. 2010). This stage is characterized by increased tumor burden with

invasive growth into vessels or extrahepatic metastases and an impact onto the patient's overall health, represented by an increase in the ECOG score (EASL 2018). Survival is severely limited to a median survival of six to eight months or 25% at one year (Llovet and Bruix 2003; Cabibbo et al. 2010). These patients are offered systemic therapy in the means of targeted therapy; however, all substances currently available have shown limited success with overall survival extended by only a few months (Forner et al. 2010; EASL 2018). It took 30 years of research for the development of the multi-tyrosine kinase inhibitor sorafenib, which has been the first-line therapy for BCLC-C stage HCC since its approval in 2007 and has been shown to increase median overall survival to around 10 months (Llovet et al. 2008; Cheng et al. 2009; Iavarone et al. 2011; Reig et al. 2013; Lencioni et al. 2014; Ganten et al. 2017). Sorafenib may also be offered to patients with BCLC-B stage HCC with contraindications for TACE or progression under the same (Llovet et al. 2008; Raoul et al. 2011; Forner et al. 2014). If the disease progresses further, regorafenib has been shown to have effect as a second-line treatment (Bruix et al. 2017). Additionally, lenvatinib has been found non-inferior as first-line therapy, cabozantinib may be utilized as second- or third-line therapy, and the immunotherapeutic checkpoint inhibitor nivolumab is under evaluation as a second-line alternative (El-Khoueiry et al. 2017; Abou-Alfa et al. 2018; Kudo et al. 2018).

The prognosis for patients with BLCL-D stage HCC, terminal disease, is very poor with a median survival of only three to four months or 11% at one year (Cabibbo et al. 2010). This stage is characterized by severe tumor-related disability, reflected for example by a Child-Pugh C status, and very poor performance status (ECOG-3 to ECOG-4) (Forner et al. 2010; EASL 2018). If liver transplantation is not an option for these patients, they should receive palliative therapy in the means of best supportive care (EASL 2018).

Overall, therapy options for HCC remain scarce and of limited effect despite decades of extensive research. Recurrence and progression rates are high, contributing to low overall survival rates. HCC recurrence after curative hepatic resection occurs with a rate of around 20% to 30% at one year and 70% to 80% at five years post-surgery (Kumada et al. 1997; Llovet et al. 1999; Iizuka et al. 2003; Imamura et al. 2003). Even though significantly lower recurrence rates have been reported for orthotopic liver transplantation with 8% to 21% overall or around 16% for patients receiving downstaging measures prior to transplantation, tumor recurrence is nevertheless the most common cause of death in HCC patients after liver transplantation, ranging from 15% overall to 60% in patients with HCC and cirrhosis (Mazzaferro et al. 1996; Bismuth et al. 1999; Jonas et al. 2001; Parikh et al. 2015). HCC

recurrence is caused by either intrahepatic metastases or de novo tumors (Llovet et al. 2005). Differentiations of reoccurred tumors have shown that around ⅔ are true recurrences caused by priorly undetected intrahepatic metastases, while ⅓ are de novo HCCs (Ng et al. 2003).

HCC is recognized to be one of the most chemo-resistant malignancies and besides the already mentioned targeted therapy options, no systemic drug has proven to be effective against this malignant disease – a remarkable, albeit fateful, individual characteristic within the field of oncology (Brito et al. 2016; Dutta and Mahato 2017). Across the different chemotherapeutic agents, combinatory regimes and application techniques available, response rates have consistently been unsatisfactory in various trials (Kew 2017). Even in the search for further targeted therapeutics and immunotherapeutic agents to follow in the footsteps of sorafenib many phase III trials have been unsuccessful due to high toxicity and low efficacy, but possibly also due to insufficient study design (Llovet and Hernandez-Gea 2014).

Multiple factors may influence the chemoresistance of liver cancer. The majority of HCC patients also suffers from liver cirrhosis, which may alter the metabolism necessary for many chemotherapeutics to take effect as well as increase toxicity of these substances, aggravated by the immunosuppression present in patients with cirrhosis (EASL 2018). Chemotherapy for non-cirrhotic HCC patients has however not been thoroughly investigated, as the overall evidence was not sufficient enough to recommend further trials. Nonetheless, works like the study presented by Julien Edeline et al. (2009), which demonstrated response rates of 22%, tumor control rates of 52%, and good tolerance of chemotherapy in non-cirrhotic patients, have called for a differentiation between cirrhotic and non-cirrhotic patients. The EASL has thus acknowledged the need for further investigations especially for non-cirrhotic HCC patients, calling for focused patient selection and trial design (EASL 2018).

A second factor in HCC's poor response rates towards chemotherapy may be its intertumoral heterogeneity resulting from genetic instability leading to the accumulation of numerous mutations (Guichard et al. 2012; Kan et al. 2013; Lee 2015; Schulze et al. 2015). Various somatic genetic alterations have been observed (Alexandrov et al. 2013; Totoki et al. 2014; Castelli et al. 2017). However, the broad mutational landscape of HCC may also offer an opportunity to identify prognostic or therapeutic factors. Thus the EASL has proposed investigating of the role of biomarkers and molecular subclasses of HCC with regards to therapy response and outcome (EASL 2018). Insight into both the genetic background of the cancer cells as well as the tumor microenvironment might offer new or improved markers for prevention, diagnosis and treatment of HCC.

An interesting study by Victor Ho et al. (2015) drew attention to another aspect by investigating the treatment of HCC with a combination of sorafenib and lysine-stabilized polyriboinosinic acid and polyribocytidylic acid complexes (poly(I:C)), a toll-like-receptor (TLR) 3 agonist, in an attempt to recruit the patient's immune system and thus control HCC progression via immune activation. Their data suggested significant tumor control via direct growth reduction both *in vitro* and *in vivo*, as well as the potent activation of host immune responses within the tumor microenvironment (Ho et al. 2015).

The rationale of their investigation was to attempt to regulate or negate the known immunemodulatory characteristics of sorafenib and thus increase the efficacy of its anti-proliferative and anti-angiogenetic effects (Wilhelm et al. 2008; Ho et al. 2015). Sorafenib has been suggested to influence the tumor microenvironment by reducing natural killer (NK) cell and dendritic cell activation as well as inhibit regulatory T cells, thus facilitating tumor progression (Hipp et al. 2008; Zhang et al. 2013; Chen ML et al. 2014). In contrast, the activation of TLR3 has been shown to reverse immunosuppression of the tumor microenvironment via proinflammatory effects, which has been demonstrated to relate to improved patient survival in HCC as well as induce tumor cell death in multiple cancer cell lines (Salaun et al. 2006; Morikawa et al. 2007; Salaun et al. 2007; Chew et al. 2010; Chew et al. 2012a).

By combining the two substances, Victor Ho et al. (2015) hoped to disrupt immunosuppressive pathways and shift host immune activity towards a beneficiary state. They first demonstrated an increase in apoptosis and a decrease in tumor cell proliferation in HCC cell lines *in vitro*, and subsequently investigated the effect of combinational therapy in two mouse models, one with transplanted Hepa 1-6 tumors and one with tumors induced via the *Sleeping Beauty* transposon (Ho et al. 2015). *In vivo*, tumor cell apoptosis was also increased and an induction of host immune responses within the tumor microenvironment was observed, namely the activation of NK cells, T cells, macrophages, and dendritic cells, leading to improved tumor growth control (Ho et al. 2015).

With their results, Victor Ho et al. (2015) have set the basis for additional research to evaluate the impact of immune activation in the context of hepatocellular carcinoma. To further set the foundation for this thesis' investigation, the following section unravels the concept of immunogenic cell death (ICD) and the increasingly recognized role of the immune system in the fight against cancer.

1.2 The Concept of Immunogenic Cell Death

The chemoresistance of HCC and its high reoccurrence rates, suggesting inefficiency of the available therapy regimens, are a prime example of the limitations that current anticancer therapies are facing. Long-term success of cancer treatment is generally dictated by the efficacy of tumor cell eradication to prevent reoccurrence; however, a different approach has been investigated within the field of immunotherapy.

Based on the idea that an efficient long-term treatment can only be achieved by enabling the patient's immune system to recognize and thus eradicate the cancerous cells by itself, efforts have been directed at recruiting the immune system to target tumor cells as it would infectious pathogens following a vaccination. In their review, Laurence Zitvogel et al. (2008) stressed the importance of including immunotherapy in cancer therapies to achieve anticancer immune responses, which they argued to be essential to eliminate residual cancer cells or at least control metastases after conventional therapies. In line with this, immunotherapy, if applied successfully, has been shown to feature more long-term efficacy than conventional therapies or targeted therapies, which – even though they are highly effective – often only achieve temporary responses (Trinchieri and Abastado 2014). The combination of established anticancer therapies with immunotherapeutic approaches may offer the ability to mediate cancer resistance and thus take a significant step from treating advanced cancer towards curing it (Zitvogel et al. 2008; Restifo et al. 2012).

Tumors have been known to elicit immune responses similar to infectious diseases under certain circumstances. This is best demonstrated by paraneoplastic diseases, where effective antitumor immune responses utilizing antibodies aimed at neuronal antigens expressed on the targeted cancer cells also lead to neurological degeneration (Albert and Darnell 2004). Many attempts have been made to utilize the immune system for cancer therapy. Successful examples range from the use of bacillus Calmette-Guérin (BCG) to treat cancer of the bladder, to pro-inflammatory cytokines like type I IFNs as well as various antibodies targeted for example against tumor antigens or components of the tumor microenvironment, which can recruit effector cells, mediate cytotoxicity or directly inhibit tumor-favoring receptors and factors (Weiner et al. 2010; Mellman et al. 2011; Trinchieri and Abastado 2014; Morales et al. 2017). Nonetheless, the success of cancer vaccines to stimulate resistance has been modest at most (Trinchieri and Abastado 2014).

The relationship of tumors and the host immune system is a complicated one. There are mechanisms in place to recognize and eliminate transformed cells with natural killer (NK) cells and activated T lymphocytes reacting to deoxyribonucleic acid (DNA) damage posing an important defense line (Kepp et al. 2009; Chen and Mellman 2017). NK cells are part of the innate immune system and have been demonstrated to play an important role in the mediation of resistance to carcinogenesis and metastasis for example in the liver and the lung (Molgora et al. 2017; Mantovani et al. 2020). Tumor infiltration by NK or T cells has been associated with improved prognosis in multiple human neoplastic diseases such as melanoma, ovarian cancer and colorectal cancer (Zhang et al. 2003; Pagès et al. 2005; Haanen et al. 2006). In contrast, impairment of the immune system as found in immunosuppressed patients comes with increased cancer incidence (Trinchieri and Abastado 2014).

Similarly to the response to infectious agents, B lymphocytes are also able to cause adaptive anti-tumor immune reactions leading to the production of specific antibodies and subsequent T lymphocyte-dependent anti-tumor immunity (Mantovani et al. 2020). Characteristics functioning as antigens can be either tumor-specific or tumor-associated and are usually caused by mutations, genetic instability or overexpression (Trinchieri and Abastado 2014; Schumacher and Schreiber 2015; Chen and Mellman 2017; Fridman et al. 2017; Wei et al. 2018; Mantovani et al. 2020). Even nascent tumor cells have been shown to be eliminated due to either the co-expression of ligands for activating receptors on innate immune cells or tumor antigens that are recognized by immune receptors of the adaptive immune system (Schreiber et al. 2011). Generally, the presence of T lymphocytes, type 1 immune responses and IFN signatures is associated with improved prognosis in human tumors (Fridman et al. 2017; Pagès et al. 2018). This has led to the development of several immunoscores such as the assessment of T-cell infiltration in colorectal cancer, which has proved to be an independent prognostic addition to the tumor-node-metastasis (TNM) classification (Pagès et al. 2018).

However, tumors are also keen on staying invisible to the immune system and exert strategies to both escape host immunity as well as to recruit immune cells in their favor, leading to the suppression of anti-tumoral immune responses and tumor progression (Dunn et al. 2002; Zitvogel et al. 2006; Mantovani et al. 2008; Mantovani et al. 2017). They have been shown to produce cytokines and growth factors such as the vascular endothelial growth factor (VEGF) to promote growth and progression in an autocrine as well as paracrine manner (Langley et al. 2014). The crosstalk between cancer cells and their so called tumor microenvironment, which includes soluble factors like cytokines and the complement system,

components of the extracellular matrix as well as stromal, endothelial and immune cells, has been identified as playing a central role in the neoplastic process (Galluzzi et al. 2012b; Trinchieri and Abastado 2014; Mantovani et al. 2020). The process of interaction between the tumor, its microenvironment and the host immune system, also termed immunoediting, includes mechanisms such as the loss or mutation of tumor antigens and the decrease or loss of MHC, the suppression of T cell-mediated responses, the depression of antigen-presenting cell, T and B lymphocyte activity as well as the stimulation of regulatory T cells and myeloid-derived suppressor cells (MDSC) (Schreiber et al. 2011; Trinchieri and Abastado 2014; Bronte 2018). Constant adaptation leads to progression from a stage in which the tumor is eliminated by the immune system to a stage of equilibrium and ultimately to the tumor avoiding and escaping host immunosurveillance, which has been proposed as a potential seventh hallmark of cancer (Dunn et al. 2002; Zitvogel et al. 2008; Hanahan and Weinberg 2011; Schreiber et al. 2011; Trinchieri and Abastado 2014). The discovery of the tumor-immune system interaction has allowed for the development of checkpoint inhibitors targeting for example the programmed cell death protein 1 (PD-1)/programmed cell death ligand 1 (PD-L1) axis to restore anti-tumoral T cell cytotoxicity (Mantovani et al. 2020).

Most conventional chemotherapeutics are innately immunosuppressive, as they act on immune cells, lymphatic tissue and bone marrow just as much as on the growing tumor and have been chosen for their efficacy in killing proliferating cells (Galluzzi et al. 2012b). However, several chemotherapeutic agents as well as targeted therapies have been shown to trigger initially unintended immunomodulatory effects that affect their therapeutic efficacy and the development of antitumor immunity (Galluzzi et al. 2012b). Gemcitabine, for example, has been shown to increase the expression of class I HLA on malignant cells, enhance the cross-presentation of tumor antigens to CD8+ T cells and selectively kill MDSC, both *in vitro* and *in vivo*, thus facilitating T cell-dependent anticancer immunity (Nowak et al. 2003a; Nowak et al. 2003b; Liu et al. 2010; Vincent et al. 2010; Mundy-Bosse et al. 2011). The stimulation of HLA expression by oxaliplatin, cyclophosphamide or the epidermal growth factor receptor (EGFR) inhibitors erlotinib and cetuximab, and the reduction of immunosuppressive mechanisms via inhibition of signal transducer and activator of transcription 6 (STAT6)-regulated PD-L2 expression by platinum-derived compounds are only two further examples of many (Liu et al. 2010; Lesterhuis et al. 2011; Pollack et al. 2011).

In addition to these off-target immunostimulatory and immunomodulatory effects, which are considered mainly side effects, specific substances and stimuli have been demonstrated to

lead to a targeted antitumor immune response as well as immunological memory following tumor cell death (Galluzzi et al. 2012b; Kroemer et al. 2013; Zitvogel et al. 2013; Ghiringhelli and Apetoh 2014). This specific instance of tumor cell death triggering an immune reaction has been named immunogenic cell death (ICD) and was first described in vaccination experiments performed by Noelia Casares et al. (2005), in which immunocompetent murine models were employed to demonstrate an effective antitumor immune response against colorectal carcinoma and melanoma cells following treatment with doxorubicin.

ICD may be triggered by various lethal stimuli. Chemotherapeutic triggers that have been identified so far include the anthracyclines doxorubicin and epirubicin as well as oxaliplatin, cyclophosphamide, etoposide and bleomycin to only name a few (Apetoh et al. 2007b; Obeid et al. 2007a; Guerriero et al. 2008; Tesniere et al. 2010; Tongu et al. 2010; Fucikova et al. 2011; Martins et al. 2011; Schiavoni et al. 2011; Sistigu et al. 2011; Bénéteau et al. 2012; Bugaut et al. 2013; Galluzzi et al. 2013b; Chen X et al. 2014; Gou et al. 2014). But not only chemotherapy can mediate ICD, other effective stimuli have been identified such as for example ionizing irradiation, certain oncolytic viruses or capsaicin (Obeid et al. 2007a; Obeid et al. 2007b; Perez et al. 2009; Schildkopf et al. 2011; Formenti and Demaria 2012; D'Eliseo et al. 2013; Galluzzi et al. 2013a; Liikanen et al. 2013; Vacchelli et al. 2013a; Gameiro et al. 2014; Gorin et al. 2014; Pol et al. 2014; Gilardini Montani et al. 2015). Additionally, substances may also act as adjuvants to increase immunogenicity. For instance, studies by Oliver Kepp and Laurie Menger have demonstrated the capacity of cardiac glycosides to trigger ICD in situations where cell death would usually remain non-immunogenic (Kepp et al. 2012; Menger et al. 2012; Menger et al. 2013). Similarly, CD40 co-stimulation has been shown to increase immunogenicity of treatment with 5-fluorouracil, which, as a standalone intervention, has not been found to trigger ICD (Liljenfeldt et al. 2014).

Taking a closer look, ICD has been described to feature characteristics of apoptosis and is therefore a form of regulated cell death (Kroemer et al. 2009; Galluzzi et al. 2012a; Kroemer et al. 2013). However, apoptosis as a physiological process has previously been considered non-immunogenic and has thus been expected to stay either unrecognized by the immune system or even induce tolerance to inhibit possible auto-immune responses (Gallucci et al. 1999; Abud 2004; Henson and Hume 2006; Kepp et al. 2014). Similarly, apoptosis of tumor cells caused by conventional chemotherapeutics has been assumed to not be recognized by the immune system, as only necrosis has been expected to cause the activation of the immune system leading to inflammatory responses (Albert et al. 1998; Gallucci et al. 1999). The

description of ICD as well as multiple new insights into regulated cell death and the mechanisms and regulation of necrosis forced a reevaluation of the traditional separation of cell death into the two distinct categories of apoptosis and necrosis and lead to a much more heterogenous view on the mechanisms of cell death than previously (Candi et al. 2005; Casares et al. 2005; Kepp et al. 2009; Vandenabeele et al. 2010; Cirone et al. 2012; Krysko et al. 2012; Kroemer et al. 2013; Berghe et al. 2014; Galluzzi et al. 2014; Kepp et al. 2014).

In order for apoptotic tumor cells to trigger ICD, a series of immunostimulatory signals is required, rendering the occurring cell death detectable to the host immune system (Garg et al. 2010; Zitvogel et al. 2010; Krysko et al. 2012; Garg et al. 2013; Honeychurch et al. 2013; Hou W et al. 2013; Krysko et al. 2013; Melis et al. 2013; Garg et al. 2014; Inoue and Tani 2014; Kepp et al. 2014). The basic concept is similar to that of pathogen-associated molecular patterns (PAMPs), which signal an infection and trigger targeted immune responses by binding to their respective pattern-recognition receptors (PRRs) (Matzinger 2002b; Zitvogel et al. 2010). Based on the danger model introduced by Polly Matzinger in 1994, immunostimulatory signals in the context of ICD are called danger- or damage-associated molecular patterns (DAMPs), which can trigger signaling cascades and enable the host immune system to differentiate between the apoptotic demise of a tumor cell versus the physiological turnover of a host cell (Matzinger 1994; Matzinger 2002b; Seong and Matzinger 2004; Krysko et al. 2011; Krysko et al. 2013).

A multitude of signals from the cell's nucleus and cytoplasm can act as a DAMP, including nuclear acids, nucleotides, heat shock proteins and hyaluronan fragments (Taylor et al. 2004; Korbelik et al. 2005; Scheibner et al. 2006; Spisek et al. 2007; Vega et al. 2008; Brusa et al. 2009; Aguilera et al. 2011; Fredly et al. 2011; Krysko et al. 2011; Garg et al. 2012b; Lv et al. 2012; D'Eliseo et al. 2013; Krysko et al. 2013). Their common characteristic is their displacement, either through release or through display on the outer cell membrane, allowing for contact to PRRs and thus to the immune system (Matzinger 2002b; Matzinger 2002a; Seong and Matzinger 2004; Lotze et al. 2007; Pouwels et al. 2014; Minn 2015).

However, only few members of the heterogenous groups of DAMPs have been identified in detail. Among them are three that have been described as central components of ICD (Kepp et al. 2011b). These are in detail the secretion of the nucleotide adenosine 5'-triphosphate (ATP), the release of the non-histone chromatin-binding nuclear protein high-mobility group protein B1 (HMGB1), and the presentation of the endoplasmic reticulum chaperone calreticulin on the outer cell membrane (Rovere-Querini et al. 2004; Apetoh et al. 2007b; Obeid

et al. 2007a; Obeid et al. 2007c; Brusa et al. 2009; Candolfi et al. 2009; Martins et al. 2009; Garg et al. 2010; Kepp et al. 2011b; Michaud et al. 2011; Garg et al. 2012b; Kohles et al. 2012; Martins et al. 2012; Stoetzer et al. 2012; Arnold et al. 2013; Hou W et al. 2013; Kepp et al. 2013; Ma et al. 2013b; Stoetzer et al. 2013; Wang et al. 2013; Lavieri et al. 2014; Martins et al. 2014).

If released during the demise of a tumor cell, ATP has been shown to attract immune cells and promote differentiation of the same, as well as activating antigen-presenting cells and leading to an increase in pro-inflammatory cytokines (Steer et al. 2005; Virgilio 2007; Elliott et al. 2009; Ghiringhelli et al. 2009; Iyer et al. 2009; Aymeric et al. 2010; McDonald et al. 2010; Riteau et al. 2010; Riteau et al. 2012; Couillin et al. 2013; Ma et al. 2013b; Ma et al. 2013a; Xiang et al. 2013; Cauwels et al. 2014; England et al. 2014).

HMGB1 has been found to increase the processing and presentation of antigens to T cells, act on several immune cell receptors and possess strong chemotactic abilities in a complex with C-X-C motif chemokine ligand 12 (CXCL12) (Scaffidi et al. 2002; Kokkola et al. 2005; Lotze and Tracey 2005; Park et al. 2006; Yu et al. 2006; Apetoh et al. 2007b; Bianchi and Manfredi 2007; Dong et al. 2007; Curtin et al. 2009; Pathak et al. 2012; Schiraldi et al. 2012). Especially the interaction of HMGB1 with toll-like-receptor 4 (TLR4) has been demonstrated to be of great importance, as both deficiency of HMGB1 and TLR4 have been shown to diminish host immune responses to ICD (Apetoh et al. 2007b; Apetoh et al. 2007a; Vacchelli et al. 2012; Vacchelli et al. 2013b; Aranda et al. 2014; Yamazaki et al. 2014).

Similarly, calreticulin repositioned to the outer membranes of dying tumor cells has been described to provide a strong signal to dendritic cells, triggering and increasing phagocytosis of tumor cell debris and thus providing potential antigens, which is an essential and presumably obligatory mechanism in the development of an adaptive immune response (Basu et al. 2001; Casares et al. 2005; Krysko et al. 2006; Obeid et al. 2007a; Obeid et al. 2007c; Krysko et al. 2008; Krysko and Vandenabeele 2008; Panaretakis et al. 2009; Chao et al. 2010; Krysko and Vandenabeele 2010; Weiss et al. 2010; Garg et al. 2012a; Lauber et al. 2012; Gilardini Montani et al. 2015). The occurrence of autophagy is central in the context of ICD, as it sets the basis for the activation, differentiation and survival of immune cells and allows for effective cross-presentation and subsequently the establishment of protective anticancer immunity (Li et al. 2011; Zitvogel et al. 2011; Ma et al. 2013c; Senovilla et al. 2014).

Additionally, there is evidence pointing towards endogenous DNA and ribonucleic acid (RNA) serving as DAMPs in what has been described by authors like Antonella Sistigu, Andy J. Minn and David Roulois et al. as “viral mimicry” (Sistigu et al. 2014; Minn 2015; Roulois et al. 2015), since these damaged, unusual, or misplaced nuclear acids resulting from genomic disintegration caused by genotoxic agents have been shown to trigger immune reactions in a way similar to viral DNA or RNA following an infection (Zhang et al. 2010; Sistigu et al. 2014; Minn 2015). The ability of RNAs to induce immune responses outside of the context of viral infection was discovered as a side effect during the evaluation of short interfering RNAs (siRNAs), which had been introduced into cells to degrade specific targets and reduce specific gene expression as a possible therapeutic intervention but unexpectedly also triggered type I IFN responses in some cases (Bridge et al. 2003; Sledz et al. 2003; Karikó et al. 2004; Hornung et al. 2005). However, the further identification and characterization of endogenous DNA or RNA DAMPs has proved to be difficult, and even though there is evidence for the contribution of mtDNA, dsRNA and ssRNA to ICD, especially via the induction of IFN and the respective effector genes, further investigations are needed to establish detailed knowledge of which and how nucleic acids may act as DAMPs (Collins et al. 2004; Wang and Carmichael 2004; Hornung et al. 2005; Zhang et al. 2010; Sistigu et al. 2014; White et al. 2014; Chiappinelli et al. 2015; Härtlova et al. 2015; Roulois et al. 2015).

To initiate an appropriate immune reaction, DAMPs caused by genotoxic stress and tumor cell death are bound and recognized by specific pattern-recognition receptors (PRRs). In the case of ectopic or altered nucleic acids, three major pathways of recognition have been identified: Toll-like receptors (TLRs) for endosomal sensing of RNA or DNA, retinoic-acid inducible gene-I (RIG-I)-like receptors for cytosolic RNA and the stimulator of interferon genes (STING) pathway for cytosolic ssDNA or dsDNA (Takeda and Akira 2005; Matsumoto and Seya 2008; Yoneyama and Fujita 2009; Slater et al. 2010; Yoneyama and Fujita 2010; Minn 2015; Gaston et al. 2016). Other pathways dependent on the transport of extracellular dsRNA into the cell as well as additional mechanisms of DNA sensing within the cytoplasm are suspected but subject to further investigations (Alexopoulou et al. 2001; Matsumoto and Seya 2008; Yoneyama and Fujita 2010).

Toll-like receptors (TLRs) are a large family of PRRs expressed on many cell types and able to recognize a broad array of signals generally associated to infectious pathogens such as lipopolysaccharides, dsRNA or bacterial DNA (Hoshino et al. 1999; Hemmi et al. 2000; Akira 2001; Janeway and Medzhitov 2002; Takeda and Akira 2005). Of the numerous TLRs,

TLR3 has been identified as playing a major role in viral infections such as hepatitis C and activation of the same has been demonstrated to increase tumor cell death in several cancer cell lines *in vitro* including HCC and the also highly chemo-resistant renal cell carcinoma, as well as correlate with improved survival in HCC patients (Salaun et al. 2006; Morikawa et al. 2007; Salaun et al. 2007; Askar et al. 2009; Wang et al. 2009; Chew et al. 2012b).

TLR3, which is found for example in the endosomal compartment of epithelial cells, was first described for its ability to sense viral infection via dsRNA released by dying cells in the vicinity (Akira 2001; Alexopoulou et al. 2001; Janeway and Medzhitov 2002; Matsumoto and Seya 2008). The immune-recognition receptor is activated by various types of dsRNA, including viral and synthetic RNAs such as the double-stranded polynucleotide poly(I:C) (Field et al. 1967; Dianzani et al. 1968; Alexopoulou et al. 2001; Matsumoto and Seya 2008). TLR3 has been classified as part of the innate immune system and its activation has been demonstrated to lead to the induction of a potent immune response via multiple signaling pathways and the activation of innate antiviral cytokines such as the interferons IFN-α, IFN-β and IFN-λ (Alexopoulou et al. 2001; Ank et al. 2006; Takeuchi and Akira 2009). However, TLR3 has also been shown to mediate mechanisms of adaptive immunity by inducing the maturation of dendritic cells and activating NK cells as well as cytotoxic T cells via cross-presentation, thus pathing the way for establishing cellular immunity (Matsumoto and Seya 2008; Chew et al. 2012b).

All TLRs depend on specific adaptors to induce downstream signaling cascades. TLR3 has been shown to utilize both TIR domain-containing adaptor inducing IFN-β (TRIF), also known as TIR domain-containing adaptor molecule 1 (TICAM1), as well as myeloid differentiation primary response 88 (MyD88) as adaptor molecules (Yamamoto et al. 2002; Oshiumi et al. 2003; Yamamoto et al. 2003; Akira et al. 2006; Matsumoto and Seya 2008; Yoneyama and Fujita 2010). TRIF or TICAM1 activation leads to the induction of the transcription factors nuclear factor κ-light-chain enhancer of activated B-cells (NF-κB), interferon regulatory factor (IRF)-3, IRF-7 and activator protein 1 (AP-1) (Oshiumi et al. 2003; Yamamoto et al. 2003). These transcription factors are essential for the production of type I IFNs, type III IFNs and several chemokines as well as dendritic cell maturation (Alexopoulou et al. 2001; Oshiumi et al. 2003; Yamamoto et al. 2003; Onoguchi et al. 2007; Österlund et al. 2007; Matsumoto and Seya 2008; Li et al. 2012).

While TLR3 is responsible for the endosomal sensing of RNA, the ubiquitously expressed RIG-I-like receptors recognize cytosolic RNAs (Yoneyama et al. 2008; Yoneyama and

Fujita 2009; Slater et al. 2010; Yoneyama and Fujita 2010; Yoneyama et al. 2015). Also called RNA helicases for their DExD/H-box-containing helicase domains, the family of RIG-I-like receptors consists of three distinct members: RIG-I, melanoma differentiation associated antigen 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) (Yoneyama et al. 2008; Yoneyama and Fujita 2009; Slater et al. 2010). The exact function of LGP2 has not been fully elucidated, however, RIG-I and MDA5 have been shown to play important roles in the recognition of cytosolic dsRNA, originally demonstrated for their ability to bind viral RNA in the context of infectious insults (Yoneyama and Fujita 2009; Yoneyama and Fujita 2010). Upon contact to dsRNA, RIG-I and MDA5 activate the transcription factors IRF-3, IRF-7, NF-κB and activating transcription factor 2 (ATF2)/c-jun via the adaptor mitochondrial antiviral-signaling protein (MAVS) in conjunction with multiple signaling molecules (Yoneyama and Fujita 2009; Yoneyama and Fujita 2010). As mentioned above, IRF-3, IRF-7 and NF-κB activation leads to the expression of several cytokines including type I IFNs and type III IFNs, of which RIG-I and MDA5 are thus important inducers (Yoneyama et al. 2008; Yoneyama and Fujita 2009; Yoneyama and Fujita 2010; Yoneyama et al. 2015).

When it comes to the sensing of cytosolic dsDNA and ssDNA, the STING pathway has been identified as an important recognition mechanism (Sun et al. 2013; Wu et al. 2013; Gentili et al. 2015). This pathway consists of the cyclic GMP-AMP synthase (cGAS), which is the actual cytosolic DNA sensor, cyclic GMP-AMP (cGAMP) as a signaling molecule and finally STING, an transmembranous endoplasmic reticulum adaptor protein that is activated by cGAMP (Ishikawa and Barber 2008; Sun et al. 2009; Sun et al. 2013; Wu et al. 2013; Gentili et al. 2015; Wang et al. 2015). STING activation ultimately leads to the nuclear translocation of IRF-3, IRF-7 and NF-κB, once again culminating in the induction of type I IFNs, type III IFNs and interferon-stimulated genes (ISGs) (Ishikawa et al. 2009; Abe et al. 2013; Barker et al. 2013; Schneider et al. 2014). While STING activation was originally described as a response to an infectious insult, it also occurs as an answer to genotoxic stress, presumably via the recognition of DNA DAMPs (Gaston et al. 2016). Substances acting as STING agonists have been demonstrated to induce anti-tumoral immune reactions via activating IFN production in tumor infiltrating lymphocytes (Corrales et al. 2015).

Successful activation of TLR3, RIG-I-like receptors or the STING pathway by nuclear acids acting as DAMPs thus culminates in the activation of several downstream effectors, including type I and type III IFNs and ISGs. While a detailed insight into the history of IFNs and

especially type III IFNs is given in section 1.3, the role of type I IFNs in the context of ICD will be displayed in the upcoming paragraphs.

The type I IFNs IFN-α and IFN-β were first described in the context of viral infections and for their anti-viral properties, which are mediated through the stimulation of certain ISGs and are an important pillar in the immune response, connecting the innate and adaptive immune system (Muller et al. 1994; MacMicking 2012). Additionally, IFN induction was also demonstrated in response to DNA damage caused by chemotherapeutic substances like etoposide and anthracyclines, which are known inducers of ICD (Brzostek-Racine et al. 2011; Sistigu et al. 2014).

Type I IFNs belong to the large group of cytokines essential for the activation and differentiation of immune cells and is typically produced and secreted by the same (Le Page et al. 2000; Woo et al. 2015). However, more recent investigations of the role of type I IFNs in the context of chemotherapy and anti-tumoral immune responses have shown that cancer cells themselves can also express type I IFNs autonomously in response to certain chemotherapeutic stimuli and the resulting DAMPs, which may be central for the development of targeted anti-tumor immune responses and success of chemotherapy (Sistigu et al. 2014; Legrier et al. 2016). In their work published in 2014, Antonella Sistigu et al. utilized a syngeneic tumor mouse model to demonstrate the ability of anthracyclines to lead to type I IFN production in cancer cells stimulated by an unidentified RNA DAMP (Sistigu et al. 2014). This IFN induction was dependent on TLR3 signaling and involved autocrine as well as paracrine stimulation of tumor cells (Sistigu et al. 2014). Additionally, they investigated the chemosensitizing effects of type I IFNs and were able to demonstrate the significance of IFN signaling in neoplastic cells over that of host cells, as an intact downstream IFN signaling cascade within the tumor cells proved to be essential for the chemotherapeutic response in *in vivo* tumor models (Sistigu et al. 2014). ICD can thus be assumed to not be as one directional as immune cells reacting to DAMPs generated by damaged tumor cells, but rather an intricate interaction between tumor cells, DAMPs and the immune system, resulting in immunoediting to form an anticancer immune response (Minn 2015).

Once induced, type I IFNs act on the IFN-α/β-receptor (IFNAR), which consists of the two subunits IFNAR1 and IFNAR2 and is expressed on the cell surface of most cells including immune cells such as dendritic cells and NK cells (Novick et al. 1994; Matsumoto and Seya 2008; Yoneyama and Fujita 2009; Schneider et al. 2014; Sistigu et al. 2014). Since IFNs are secreted by stimulated cells, they may act in an autocrine manner on the secreting cell as

well as in a paracrine manner on surrounding tumor or host cells and recruited immune cells (Yoneyama and Fujita 2009; Sistigu et al. 2014).

As all IFN receptors, IFNAR signals via the intracellular Janus kinase signal transducer and activator of transcription (JAK-STAT) pathway to enhance the transcription of hundreds of ISGs and thus lead to the typical IFN signature in gene expression (Yoneyama and Fujita 2009; Schneider et al. 2014). Signaling through JAK-STAT pathway has been shown to be extremely rapid due to a baseline expression of all required components, due to which no time-consuming *de novo* synthesis is necessary during the initial cascade (Larner et al. 1984; Larner et al. 1986). The activation of the IFNAR leads to the activation of a bound Janus kinase (JAK) via transphosphorylation, which in turn activates signal transducer and activator of transcription (STAT) proteins (Heim et al. 1995; Schneider et al. 2014). Activated STAT proteins form the transcription activator ISG factor 3 (ISGF3) complex with IRF-9 and translocate into the nucleus to induce ISG expression via IFN-stimulated regulatory elements (ISREs) within the DNA upstream of ISGs (Levy et al. 1986; Reich et al. 1987; Levy et al. 1988; Levy et al. 1989; Fu et al. 1990; Schindler et al. 1992; Melén et al. 2001; Fagerlund et al. 2002; McBride et al. 2002; Yoneyama and Fujita 2009; Schneider et al. 2014).

As hundreds of genes are induced by IFNs, ISGs are a large and heterogenous group coding not only for direct antiviral proteins such as the Myxovirus resistance 1 (MX1) protein, but also for PRRs like the already mentioned RIG-I and MDA5 receptors as well as TLRs and cGAS, for signaling molecules like JAK2, MyD88, MAVS, for a multitude of transcription factors such as IRFs and STATs, and last but not least for various regulatory proteins such as the negative regulator ubiquitin specific peptidase 18 (USP18) (Schneider et al. 2014; Hubel et al. 2019). The upregulation of chemokines and chemokine receptors facilitates cell-to-cell communication and allows for the recruitment and activation of immune cells as well as tumor infiltration by T lymphocytes (Matsumoto and Seya 2008; Harlin et al. 2009; Zhu et al. 2010; Schneider et al. 2014). Under certain conditions, interferon-stimulated proapoptotic proteins may even induce cell death (Schneider et al. 2014). ISGs thus include not only antiviral or antitumoral effectors, but also many components of the IFN signaling cascade itself, multiplying IFN induction and exerted effects via positive feedback and enhancement loops. They additionally provide negative regulation to allow for the restrain and termination of IFN induced alarm states to regain homeostasis within the cell (Schneider et al. 2014).

Two ISGs will be introduced within this section, as they served as indicators for type I and type III IFN signatures during the work of this thesis.

In the context of ICD, IFN triggered chemokines seem especially relevant due to their ability to attract and activate immune cells. One of the chemokine encoding genes induced by type I IFNs is the C-X-C motif chemokine ligand 10 (*CXCL10*), also called *IP-10*, and its corresponding ortholog within the murine genome *Cxcl10/Ip-10* (Crawford et al. 2010; Cheon et al. 2014; Braschi et al. 2019; Bult et al. 2019; HGNC Database; MMHC Database). The encoded protein CXCL10/Cxcl10 (human/murine) has been identified as a chemokine of the CXC subfamily and an important ligand of the receptor C-X-C motif chemokine receptor 3 (CXCR3), which is found mainly on the cell surface of T lymphocytes and NK cells (Karin and Razon 2018; Karin 2020).

Via CXCR3, CXCL10 attracts T lymphocytes and facilitates their differentiation to effector cells, which may play a vital role in the development of anti-tumor immunity and thus may qualify CXCL10 as a potential target for cancer immunotherapy (Karin and Razon 2018; Karin 2020). This notion is supported by various studies associating low expression of CXCL10 with poor prognosis in cancer patients as well as observations that connect CXCL10 production in the microenvironment of human or murine tumors and administration of CXCL10 with T cell infiltration and tumor suppression (Arenberg et al. 2001; Jiang et al. 2010; Zhu et al. 2010; Hong et al. 2011; Barash et al. 2014; Li C et al. 2014, S. 10; Rainczuk et al. 2014; Zumwalt et al. 2014; da Silva et al. 2015; Peng et al. 2015; Flores et al. 2017; Karin 2020). This includes HCC, as Jing Zhang et al. (2019) demonstrated an association of high CXCL10 levels with improved survival. This is in line with findings of Valerie Chew et al., who demonstrated a correlation between inflammatory HCC tumor microenvironments with T cell infiltration and improved survival in humans as well as the predictive value of CXCL10 including gene signatures for survival of HCC patients (Chew et al. 2010; Chew et al. 2012a). However, CXCL10 may not always be beneficial, as these effects could not be confirmed in other cancer entities such as renal and breast cancer or multiple myeloma (Mulligan et al. 2013; Lunardi et al. 2014; Bolomsky et al. 2016; Karin 2020). As for ICD, CXCL10 has been shown to be induced by anthracyclines and, more importantly, be obligatory in order for anthracyclines to trigger ICD (Sistigu et al. 2014; Minn 2015). Additionally, CXCL10 was demonstrated to be able to restore tumor sensitivity to anthracyclines in IFNAR-deficient tumor models and sensitize tumors to more ineffective ICD inducers such as cisplatin (Minn 2015).

While CXCL10's primary effect is that of chemoattraction, the genes Myxovirus resistance dynamin like GTPase 1 (*MX1*) and the murine ortholog *Mx1* are an example for an ISG with

direct effector functions (Schneider et al. 2014; Braschi et al. 2019; Bult et al. 2019; HGNC Database; MMHC Database). The encoded dynamin-like large guanosine triphosphatase MX1/Mx1 (human/murine) is an important inhibitor of viral infections as it directly interferes with the entry of the virus into the cell (Hubel et al. 2019). Supposedly not for its ability to trap intruding viral components but as an indicator for an IFN gene signature, MX1 has been demonstrated to be induced by chemotherapy, predict response to chemotherapy and correlate to survival in human breast cancer (Weichselbaum et al. 2008; Popovici et al. 2010; Tabchy et al. 2010; Desmedt et al. 2011; Hatzis et al. 2011; Iwamoto et al. 2011; Horak et al. 2013; Kim et al. 2016; Legrier et al. 2016). Thus, MX1 may serve as an indicator for more effective or more easily stimulated IFN responses to DAMP generation, possibly predicting the ability of a patient to react to ICD and form an anti-tumor immune response (Sistigu et al. 2014).

The induction of ISGs has been shown to play an essential part for ICD and the success of chemotherapy. However, due to their heterogenous nature, ISGs may exert many different functions including tumor-favorable effects in the context of ICD and more detailed insight into the exact mechanisms of ICD, possible ICD variants and the distinct roles of ISGs is subject to further investigations (Kepp et al. 2014; Gaston et al. 2016). Additionally, malfunction of any element within the signaling cascade leading from DAMP recognition to IFN secretion and ISG induction could prevent ICD and thus effective immunotherapy, but also pose possible targets for personalized immunotherapy, i.e. the adjuvant use of type I IFNs or CXCL10 (Sistigu et al. 2014; Yang et al. 2014).

To summarize, recent investigations within the field of immunotherapy have shown that the immune system is able to form an adaptive immune response against cancer cells and can thus play a major part especially in the long-term success of anti-tumoral therapy, setting the basis for immunochemotherapeutic approaches (Olsson and Ebbesen 1978; Matzinger 2002b; Casares et al. 2005; Zitvogel et al. 2008; Palombo et al. 2014; Trinchieri and Abastado 2014). Under the right circumstances, tumor cell death caused by certain agents can be immunogenic, thus triggering immune responses (Kepp et al. 2014). ICD leads to the release of DAMPs and induces chemokines promoting the crosstalk between tumor cells and the immune system as well as between the tumor microenvironment and the immune system (Galluzzi et al. 2012b). Especially type I IFNs have been identified to play a major role in mounting an immune response to ICD and may originate from both host and tumor cells to contribute to the therapeutic effect of chemotherapy (Sistigu et al. 2014).

Novel evaluations of chemotherapeutic substances have therefore shifted from the search for the most cytotoxic agent to the most immunostimulatory one, trying to identify new ICD inducers and form effective therapy and vaccination protocols based on tumor-specific effector memory T cells (Galluzzi et al. 2012b). As Oliver Kepp et al. put it in their work, the goal of utilizing ICD is to "facilitate the development of next-generation anticancer regimens, which kill malignant cells and simultaneously convert them into a cancer-specific therapeutic vaccine" (Kepp et al. 2014). Immunotherapy could offer new perspectives on cancer therapy and thus open up opportunities to target cancers with high reoccurrence rates and resistance to traditional chemotherapeutic regimes, for which HCC is only one example. There are promising results for the use of adjuvant immunotherapy, i.e. the use of activated cytokine induced T lymphocytes and NK cells in HCC patients, which was shown to increase recurrence-free and overall survival (Lee et al. 2015).

To allow for the development of successful immunochemotherapeutic strategies, many variables have to be investigated more in depth. For one, collected evidence so far points to a distinct kinetic of DAMP emission and downstream signaling to be obligatory for effective ICD (Garg et al. 2010; Zitvogel et al. 2010; Krysko et al. 2012; Garg et al. 2013; Honeychurch et al. 2013; Hou W et al. 2013; Krysko et al. 2013; Melis et al. 2013; Garg et al. 2014; Inoue and Tani 2014; Kepp et al. 2014). The admission of ICD inducers and possibly adjuvant substances thus has to be precisely coordinated in detailed regimens (Zitvogel et al. 2008; Galluzzi et al. 2012b).

Secondly, as the success of ICD is largely dependent on the ability of the host organism to hoist an anti-tumor immune response, the efficacy of immunochemotherapy also relies on the careful selection of patients predicted to benefit from the administration of the same to spare other patients the toxicity of the utilized substances (Galluzzi et al. 2012b; Mantovani et al. 2020). To do so, and at the same time ensure financial sustainability, these so-called responders have to be identified, which calls for the establishment of predictive biomarkers (Galluzzi et al. 2012b; Kepp et al. 2014; Mantovani et al. 2020). Biomarkers may include for example tumor antigen-specific circulating autoantibodies, single nucleotide polymorphisms (SNPs) in genes that mediate and modulate immune responses, NK cell characteristics, immune-related gene signatures as well as local indicators such as infiltration of immune cells (Schilsky 2010; Zitvogel et al. 2011; Galluzzi et al. 2012b).

Considering the parallels between ICD triggered anti-tumoral immunity and anti-viral immunity, biomarkers for the efficacy of anti-viral immune responses in the liver may pose as

a starting point for investigations into ICD biomarkers for HCC. For example, variants of the gene for the type III IFN IFN-λ4 detected by Ludmila Prokunina-Olsson et al. (2013) were demonstrated to be of predictive value for viral clearance in hepatitis C. As this thesis concentrates on the role of type III IFNs in the context of ICD, they are introduced in depth in the next segment.

1.3 Type III Interferons: Interferon-λ

Multiple mediators are involved in the process of ICD and hoisting anti-tumoral immune responses, including the broad family of interferons (IFNs). While largely investigated for their role in the context of viral infections such as hepatitis C, multiple findings have hinted towards the complex role and impact of IFNs also within the field of oncology.

The term "interferon" was established by Alick Isaacs and Jean Lindenmann in 1957 when they discovered a cytokine with the ability to interfere with the influenza A virus in chicken cells (Isaacs et al. 1957a; Isaacs et al. 1957b). Concurrently, Yasuichi Nagano et al. investigated viral interference in rabbits and described a virus interference factor, today's IFN (Nagano et al. 1954). This virus-interfering cytokine was subsequently identified to be a small protein and produced by cells as a reaction to PAMPs stimulating PRRs via pathways described in detail in the previous section (Lengyel 1982; Schneider et al. 2014; Wu and Chen 2014). Up until today, three distinct classes within the family of IFNs have been identified.

IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω make up the heterogenous group of type I IFNs in humans, which signal through the IFN-α receptor (IFNAR), a heterodimeric receptor complex made up of IFNAR1 and IFNAR2 subunits (Pestka et al. 2004; de Weerd et al. 2007; Uzé et al. 2007). The respective genes for all type I IFNs are located on chromosome 9 and may be expressed by just about every cell, however plasmacytoid dendritic cells have been identified as responsible for the majority of type I IFNs produced (Siegal et al. 1999; Pestka et al. 2004; Liu 2005; Schneider et al. 2014). Further details concerning type I IFNs especially in the context of ICD can be found in section 1.2.

Only a single IFN belongs to the class of type II IFNs, IFN-γ. IFN-γ signals via its own receptor complex, the IFN-γ receptor complex (IFNGR), is mostly produced by immune cells but sensed by a broad array of cells, and functions as a regulator of immune functions as well as a bridge between innate and adaptive responses (Valente et al. 1992; Walter et al. 1995; Schroder et al. 2004; Schneider et al. 2014). IFN-γ has been shown to prime the type I IFN response and vice versa (Decker et al. 1989; Lew et al. 1989; Levy et al. 1990; Fenner et al. 2006; Fujimoto and Naka 2010).

The newest addition to the family of IFNs are type III IFNs, also called IFN-λ. Even though IFNs had been discovered for nearly 50 years at that time, at the turn of the year 2002/2003 two independent groups around Sergei Kotenko and Paul Sheppard discovered a new group of cytokines featuring characteristics of IFNs on one hand and IL-10 cytokines on the other

hand (Kotenko et al. 2003; Sheppard et al. 2003). The three new proteins were named IFN-λ1, IFN-λ2 and IFN-λ3 or IL-29, IL-28A and IL-28B, respectively, and are collectively referred to as type III IFNs (Kotenko et al. 2003; Sheppard et al. 2003; Donnelly and Kotenko 2010). The respective genes *IFNL1*, *IFNL2* and *IFNL3* are situated on chromosome 19 in humans (Kotenko et al. 2003; Sheppard et al. 2003). When investigating the three genes for example with the Genome Data Viewer provided by the National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine, it can be observed that *IFNL1* and *IFNL2* are aligned in the same direction, while *IFNL3* is oriented in the opposite direction (NCBI). Furthermore, the *IFNL* genes feature high sequence homology and are thus examples for paralogous genes (Sheppard et al. 2003; Lasfar et al. 2011). As for exon-intron structure, *IFNL1* contains five exons, while *IFNL2* and *IFNL3* each contain six exons (Sheppard et al. 2003).

A fourth member of the class of type III IFNs, IFN-λ4, was discovered even later than 2003, when a polymorphism in close proximity to *IFNL3* turned out to be a new gene that had so far escaped all genome mapping (Prokunina-Olsson et al. 2013). Single-nucleotide polymorphisms in the area of *IFNL3* had drawn attention as they were offering an explanation to observed differences in the success of treatment between patients of different ethnic backgrounds as well as a solution to the mystery of spontaneous virus clearance (Marcello et al. 2006; Ge et al. 2009; Thomas et al. 2009; Montes-Cano et al. 2010; Tillmann et al. 2010; Jiménez-Sousa et al. 2013). Ludmila Prokunina-Olsson et al. (2013) described the induction of a sequence upstream of *IFNL3* in human hepatocytes after stimulation with the synthetic dsRNA poly(I:C) to mimic HCV infection. They furthermore identified a frameshift variant within this gene sequence, denoted ss469415590 (TT > ΔG), with the ΔG variant enabling the expression of a novel gene, subsequently designated *IFNL4* and featuring strong though negative correlation to HCV clearance and treatment response (Prokunina-Olsson et al. 2013). The open reading frame due to the ΔG variant in ss469415590 was shown to correlate with poor spontaneous clearance of viral infection with HCV and worse outcome after IFN-α treatment (Bibert et al. 2013; Hamming et al. 2013b; Prokunina-Olsson et al. 2013; Aka et al. 2014). Inactivation of *IFNL4* via the loss of the open reading frame was thus demonstrated to be beneficial, resulting in lower rates of chronic HCV infection and better response to treatment of the same (Bibert et al. 2013; Prokunina-Olsson et al. 2013). However, when recombinant IFN-λ4 is administered it exerts strong antiviral effects, presenting the paradox of the disadvantageous expression of an otherwise antiviral effector (Hamming et al. 2013b). Interestingly, *IFNL4* expression is associated with higher hepatic expression of ISGs,

however up-regulation of ISGs has been correlated to poor treatment response in chronic hepatitis C (Honda et al. 2010; Amanzada et al. 2013; Hamming et al. 2013a; Huschka and Mihm 2021).

The murine *IFNL* orthologs, designated *Ifnl*, are located on chromosome 7 but encode only two functional proteins, IFN-λ2 and IFN-λ3, while the gene for IFN-λ1 has lost exon 2 and gained a stop codon in exon 1, rendering it inoperative (Lasfar et al. 2006; Lasfar et al. 2011). As no corresponding region could be identified for a murine *Ifnl4*, neither *IFNL1* nor *IFNL4* have appropriate murine orthologs (Paquin et al. 2015). Similar to their human counterparts, *Ifnl2* and *Ifnl3* are paralogous genes due to their nearly identical sequences, located in close proximity and orientated opposite of each other (Lasfar et al. 2006; NCBI).

Type III IFNs are induced in response to DNA damage, e.g. after treatment with etoposide, a known ICD inducer (Brzostek-Racine et al. 2011). Activators involved in this process are IRFs and NF-κB, as the *IFNL* gene promoters possess binding sites for both (Onoguchi et al. 2007; Österlund et al. 2007; Brzostek-Racine et al. 2011). In comparison, type I IFNs are regulated directly by IRFs but not NF-κB, however NF-κB has been shown to be required the induction of type I IFNs, even though the gene loci do not include NF-κB binding sites (Génin et al. 2009). The activation of both type I and type III IFNs adheres to specific time-dependent kinetics, as demonstrated by Sabrina Brzostek-Racine et al. (2011), with type III IFNs peaking earlier than type I IFNs, possibly due to the direct induction of IFN-λ via NF-κB released by DNA damage, while the IRFs rely on induction via NF-κB themselves, resulting in a slight delay in the expression of IFN-α.

Type III IFNs signal through a receptor complex composed by the IFN-λ receptor subunit 1 (IFNLR1) and a subunit of the main receptor of the IL-10 cytokine family, IL-10R2 (Kotenko et al. 2003; Sheppard et al. 2003; Hamming et al. 2013b). While IL-10R2 can be found on a variety of cells, IFNLR1 is unique to epithelial cells including hepatocytes (Sommereyns et al. 2008; Dickensheets et al. 2013; Schneider et al. 2014). Similar to the type I IFN signaling cascade elaborated on in section 1.2, downstream signaling of type III IFNs involves the activation of JAKs leading to the induction of STAT1 and STAT2 phosphorylation, subsequently the activation of ISRE via IRFs, and ultimately the expression of ISGs (Levy et al. 1986; Reich et al. 1987; Levy et al. 1988; Levy et al. 1989; Fu et al. 1990; Schindler et al. 1992; Heim et al. 1995; Melén et al. 2001; Fagerlund et al. 2002; McBride et al. 2002; Yoneyama and Fujita 2009; Prokunina-Olsson et al. 2013; Schneider et al. 2014).

Even though the signaling cascades are very similar and many of the same ISGs are induced, differences between the effects of type I and type III IFNs can be observed nonetheless.

As the type III IFN receptor is limited to certain tissues and organs, the effects of type III IFNs are expected to be more targeted than those of type I IFNs, possibly offering a more efficient therapeutic approach with less side-effects (Marcello et al. 2006; Zhou et al. 2007; Sommereyns et al. 2008; Dickensheets et al. 2013; Bolen et al. 2014; Schneider et al. 2014). Additionally, Tobias Marcello et al. investigated the kinetics of signal transduction as well as gene regulation for type I and type III IFNs when inhibiting HCV and observed distinct differences (Marcello et al. 2006). While type I IFNs led to an early peak and a rapid decline in ISGs levels, ISGs induction by type III IFNs was slower but more steady (Marcello et al. 2006).

However, type I and type III IFNs do not simply coexist, but rather interact. Thus, IFN-λ was demonstrated to play a role in the activation of IFN-α and may modulate IFN-α-induced signaling (Syedbasha and Egli 2017). Nonetheless, the exact effects of type III IFNs and the specifics of interaction with type I IFNs is the subject of ongoing research. IFN-λ4 has been demonstrated to feature comparable antiviral activity to IFN-λ3 when administered, acting against viruses such as HCV, human coronavirus strain 229E and MERS-CoV (Hamming et al. 2013a; Hamming et al. 2013b). It has been demonstrated that IFN-λ4 may exert negative effects for example by preactivating IFN signaling and thus preventing further activation by type I and type III IFNs (Prokunina-Olsson et al. 2013). Other possible explanations for the previously described disadvantageous effects of IFN-λ4 in the context of HCV clearance are undiscovered receptor complexes or IFN-λ4 acting as a competitive antagonist for the IFNLR1, reducing effective IFN-λ signaling (Prokunina-Olsson et al. 2013).

To specifically investigate the effect of type III IFNs in murine HCC cells, this thesis intended to generate *Ifnl2/3*-deficient clones of the murine Hepa 1-6 cell line by performing a gene knockout for *Ifnl2* and *Ifnl3*. In order to achieve this, the new state of the art CRISPR/Cas genetic engineering technique was employed, providing a reliant, fast and reproducible tool for altering the corresponding gene loci.

1.4 Revolutionizing Genetic Engineering: CRISPR/Cas Systems

The prospect of altering genetic sequences in order to study the function of specific genes has always been of interest to scientists in a multitude of research fields, with oncology and immunology only serving as examples. The replacement of a chromosome segment in *Saccharomyces cerevisiae* by Stewart Scherer and Ronald W. Davis in 1979 was the first major key event that set the basis for the development of genetic engineering techniques (Scherer and Davis 1979; Doudna and Charpentier 2014). Though many different tools were discovered throughout the years such as meganucleases, zinc-finger nucleases or transcription activator-like effector nucleases, so far none of these have been able to reach the success of the CRISPR/Cas technology, which surpassed all other methods within a few years of its first application and was awarded the Nobel Prize in chemistry in 2020 (Doudna and Charpentier 2014; Nobel Media AB 2022).

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) is the name of an adaptive immune system found in prokaryotes, providing archaea and bacteria with a specific defense mechanism against viral and plasmid challenge (Jansen et al. 2002; Makarova et al. 2006; Barrangou et al. 2007; Marraffini and Sontheimer 2010a; Sapranauskas et al. 2011). It can be found in up to 90% of archaeal and around 50% of bacterial species and is characterized by its unique but heterogenous architecture, up until today subjected to rapid evolution (Grissa et al. 2007; Makarova et al. 2015). The diverse CRISPR/Cas systems effectively target and silence foreign nucleic acids relying on information obtained from prior contact and preserved in what can be described as a genetic library (Makarova et al. 2006; Barrangou et al. 2007; Marraffini and Sontheimer 2010a; Deltcheva et al. 2011). Three major elements are found on the CRISPR/Cas gene loci, being firstly the eponymous short palindromic repeats, then a group of associated genes encoding for the Cas proteins, and lastly spacer segments interspersed between the repeats and harboring the target information (Jansen et al. 2002; Bolotin et al. 2005; Haft et al. 2005; Mojica et al. 2005; Pourcel et al. 2005; Makarova et al. 2015). CRISPR/Cas genetic engineering technology has revolutionized the field of genetic research due to its relative simplicity, specificity, and broad availability and has so far come closer to *in vivo* application as gene therapy than everything before it (Jinek et al. 2012; Doudna and Charpentier 2014; Charpentier et al. 2019).

In 1987 Ishino et al., while investigating the nucleotide sequence of the gene for alkaline phosphatase in the bacterial genome of *Escherichia coli*, incidentally discovered short and

repetitive nucleotide sequences, spaced by distinct segments. This was a novel finding at that point as these segments had not been described in prokaryotes prior to that incident (Ishino et al. 1987). However, the authors were not able to identify the function of the sequence they had discovered, and its potential was not recognized until decades later.

As uneventful as its initial discovery had been, the developments around CRISPR/Cas sped up rapidly beginning in the early 2000s. The terms CRISPR for the repetitive sequences and *cas* for genes found in close proximity to the repeats were coined by Jansen et al. in 2002, who also proposed a functional relationship between the two elements. Nevertheless, they were not able to identify the origin or function of the spacer sequences, only noticed that they were usually present as only single copies and that each organism had their own set of unique spacers (Jansen et al. 2002).

It took another three years until three independent studies were able to demystify the spacer sequences that were so unique within the prokaryotes' genomes. As the comparison of the spacers between different prokaryotes had not yielded conclusive results, they were subsequently compared with known genes of viral and plasmid origin, surprisingly showing homology to these extrachromosomal elements (Bolotin et al. 2005; Mojica et al. 2005; Pourcel et al. 2005). The existence of viral and plasmid sequences within the prokaryotic genome without evidence for an infection gave way to the proposition of the existence of a targeted immune system in prokaryotes (Mojica et al. 2005; Makarova et al. 2006).

This hypothesis was confirmed by Rodolphe Barrangou et al. in 2007 by the example of a phage-sensitive strain of the lactic acid bacterium *Streptococcus thermophilus*, in which exposure to a bacteriophage led to the integration of new spacers from the viral genome and resulted in the acquirement of resistance against the respective virus (Barrangou et al. 2007). Thus, the CRISPR arrays of repeats and spacers in combination with the *cas* genes were finally recognized as a specific and adaptive immune system, providing bacteria and archaea with a protective mechanism against viral and plasmid challenge (Barrangou et al. 2007). Anyhow, the exact function of each of the Cas proteins remained to be discovered.

The next key point in the development of CRISPR/Cas into a technique suited for genetic engineering was the description of a new type of CRISPR/Cas systems in bacteria like *Streptococcus thermophilus* and *Streptococcus pyogenes* by the works of Elitza Deltcheva et al. and Rimantas Sapranauskas et al. in 2011. In these systems, referred to as type II CRISPR/Cas systems, a specific Cas protein, Csn1, was identified by the authors as being

essential to CRISPR/Cas acquired immunity as it proved to be the sole protein responsible and necessary for executing the silencing of foreign nucleic acids (Deltcheva et al. 2011; Sapranauskas et al. 2011). Csn1 would later be renamed to Cas9. It was further demonstrated that the CRISPR arrays were transcribed into CRISPR RNAs (crRNAs) complementary to viral or plasmid sequences (Brouns et al. 2008; Marraffini and Sontheimer 2010a; Deltcheva et al. 2011). Besides that, type II systems were discovered to employ a second type of crRNA, the trans-encoded or trans-activating CRISPR RNA (tracrRNA) (Deltcheva et al. 2011). The tracrRNA proved to direct maturation of crRNAs by the non-Cas protein RNase III as well as aid Cas9 in target binding (Deltcheva et al. 2011; Jinek et al. 2012). Thus, CRISPR/Cas9 systems were demonstrated to rely on only a few elements, mainly the Cas9, a set of crRNAs and the ubiquitous RNase III. In contrast, the other CRISPR/Cas systems described up until that point included multiple *cas* genes and only a few of the encoded Cas proteins had been successfully characterized, e.g. the endoribonuclease Cas6, which had been identified as responsible for crRNA maturation in these systems (Carte et al. 2008).

The proposed simplicity of the CRISPR/Cas9 system, and Rimantas Sapranauskas et al. demonstrating in 2011 that it could be successfully cloned and transferred from one bacterium to another, made it the perfect candidate for further investigation.

In 2012, research groups around the French microbiologist, geneticist and biochemist Emmanuelle Charpentier and the US-American biochemist and molecular biologist Jennifer Doudna majorly contributed to the success of CRISPR/Cas9 technology by identifying the Cas9 protein as a DNA endonuclease with two endonuclease domains, guided by a set of specific RNAs to cleave target DNA via double-strand breaks (Jinek et al. 2012). By the incorporation of both crRNA and tracrRNA traits into a chimeric single guide RNA (sgRNA) and designing sgRNAs for specific target sequences, Martin Jinek et al. were the first to utilize the CRISPR/Cas9 system for fast, targeted and precise cleaving of determined dsDNA sequences and thus recognized its potential as a tool for "RNA-programmable genome editing" (Jinek et al. 2012).

The demonstration of Martin Jinek et al. that they had not only understood the CRISPR/Cas9 system but were able to use it in a targeted manner resulted in what Elizabeth Pennisi dubbed "the CRISPR craze" (Pennisi 2013), and it took only a few months until CRISPR/Cas9 was shown to enable genetic engineering not only in bacteria but in a multitude of organisms as well as animal and human cells and even *in vivo* in zebrafish (Cho et al. 2013; Cong et al. 2013; Hwang et al. 2013; Jiang et al. 2013; Jinek et al. 2013; Mali et al. 2013b). Thus, the

CRISPR/Cas systems and especially the CRISPR/Cas9 gene technology became one of the great discoveries of this century.

In their updated classification of CRISPR/Cas systems in 2015, Kira S. Makarova et al. differentiated between two classes of CRISPR/Cas systems (Makarova et al. 2015). According to their categorization, class 1 systems, all characterized by relying on a multisubunit complex of crRNA and effector proteins as the key agent, include the type I systems with their signature Cas3 protein, the heterogeneous group of type III systems around the Cas10 protein, and the so far not fully characterized type IV systems, which are minimalist variants of Cas protein systems often found without adjacent CRISPR arrays. In contrast, the class 2 systems in this classification do not require the formation of a multisubunit complex, for they utilize a single protein capable of carrying out all tasks. Conforming to the classification of Kira S. Makarova et al., the CRISPR/Cas9 technology used in this thesis is classified as a type II-A system and, like all type II systems, belongs to class 2 along with the rare type V system. Kira S. Makarova et al. split up the five types of CRISPR/Cas systems into 16 subtypes in total, due to different makeup of genes included, gene arrangement, and intended function. As CRISPR/Cas is still evolving rapidly and it is for certain that not all variants of its dynamic gene loci have been discovered yet, not all systems fit into the current classification and thus may be rated as rare or unclassifiable (Makarova et al. 2015).

As described above, most CRISPR/Cas systems are characterized by the *cas* gene loci they feature. The Cas proteins encoded are a large and heterogeneous group of protein families and greatly differ in their function (Haft et al. 2005). By the year 2005, 45 different Cas protein families had been identified (Haft et al. 2005). These protein families can be separated into a total of four key roles, with three of them corresponding to the phases of adaptive immunity via CRISPR/Cas (Makarova et al. 2015). A fourth functional group, the ancillary module, presumably carries out regulatory and auxiliary tasks, which have not yet been completely identified (Makarova et al. 2015).

Taking a closer look at the exact mechanism, the acquirement of adaptive immunity via CRISPR/Cas can be separated into three distinctive phases: adaption, expression, and interference (van der Oost et al. 2009; Terns and Terns 2011).

The phase of adaption results in the acquirement of new spacers into the CRISPR array, which provides the prokaryote with the ability to recognize foreign nucleic acids during reinfection. Upon primary contact to foreign nucleic acids during viral or plasmid exposure,

sequences of the same are integrated as spacers into the CRISPR array to form a memory bank, thus adapting the prokaryote's genome to its challenges (Marraffini and Sontheimer 2010a; Bhaya et al. 2011). The respective complementary sequence on the foreign genetic element is termed protospacer (Bhaya et al. 2011; Makarova et al. 2011). Spacer acquisition was first demonstrated by Rodolphe Barrangou et al. in 2007 (Barrangou et al. 2007). Each spacer is accompanied by a repeat duplicated during the integration process (Bhaya et al. 2011; Nuñez et al. 2014). The Cas proteins usually responsible for the incorporation of new sequences into the CRISPR array have been identified as Cas1 and Cas2, due to which it is no surprise that they are present in the majority of CRISPR/Cas systems and all type II systems (Makarova et al. 2006; Datsenko et al. 2012; Yosef et al. 2012; Nuñez et al. 2014; Makarova et al. 2015). New repeat-spacer segments are incorporated in a directed manner at the leader end of the CRISPR locus (Pourcel et al. 2005; Barrangou et al. 2007). Therefore, newer spacers as evidence for recent contact are found further towards the leader end of the array (Pourcel et al. 2005).

The protospacers are not chosen randomly but marked by a short motif in close proximity, the so-called protospacer adjacent motif (PAM), which provides a recognition site to the Cas nucleases (Deveau et al. 2008; Mojica et al. 2009). Each CRISPR/Cas system has its own unique PAM dictating which protospacers are selected from an invading virus or plasmid and subsequently which sequence of the invader will be targeted during the interference phase (Deveau et al. 2008; Horvath et al. 2008; Mojica et al. 2009). Consequently, mutations in the PAM region have shown to allow viruses to escape recognition by the CRISPR/Cas system (Deveau et al. 2008; Mojica et al. 2009).

Once a new spacer has been incorporated into the CRISPR array, the spacer and the adjacent repeat are transcribed during the phase of expression, often also referred to as biogenesis (Marraffini and Sontheimer 2010a; Bhaya et al. 2011). The transcript resulting from the expression of the repeat-spacer segment is a long precursor CRISPR RNA (pre-crRNA) which is consecutively processed into a shorter mature crRNA (Brouns et al. 2008; Marraffini and Sontheimer 2010a; Deltcheva et al. 2011; Makarova et al. 2011). Each crRNA contains a sequence complementary to the targeted protospacer to enable recognition, as well as a sequence derived from the repeat segment (Brouns et al. 2008). The sequence homologous to the repeat provides on the one hand a binding site to Cas proteins and is on the other hand elemental for the distinction between self and non-self during the interference phase (Brouns et al. 2008; Marraffini and Sontheimer 2010b; Marraffini and Sontheimer 2010a). A

prokaryote's library of crRNAs may therefore be compared to a human's assortment of antibodies, reflecting past contact to pathogens and providing defense against future infections (Marraffini and Sontheimer 2010a; Deltcheva et al. 2011).

While the adaption via Cas1 and Cas2 is standard in most CRISPR/Cas systems, the phases of expression and interference differ with regard to the Cas proteins and auxiliary elements involved (Makarova et al. 2015). Some systems rely on multisubunit complexes made up of various Cas proteins, crRNA, and additional protein subunits, depending on the CRISPR/Cas system class (Brouns et al. 2008; Carte et al. 2008; Makarova et al. 2015). The so-called Cas complex then performs crRNA processing and subsequent interference (Brouns et al. 2008; Carte et al. 2008). Other CRISPR/Cas systems, however, utilize only single Cas proteins for analogous tasks, such as the Cas9 in type II systems (Deltcheva et al. 2011; Sapranauskas et al. 2011; Jinek et al. 2012). In addition, CRISPR/Cas9 systems depend on the transcription of tracrRNA in combination with crRNA, as already mentioned, in order to recruit the Cas9 for crRNA maturation as well as target binding (Deltcheva et al. 2011; Jinek et al. 2012).

The crRNAs resulting from protospacer integration during the adaption phase and subsequent array transcription as well as processing during the expression phase are combined with either multiple Cas proteins or a single Cas protein plus tracrRNA to ribonucleoprotein complexes (Brouns et al. 2008; Fineran and Charpentier 2012). These crRNA-Cas complexes pose the actual effectors of the CRISPR/Cas mediated immunity, surveying the nucleic acid sequences within the prokaryote to detect a possible infection and, if necessary, direct the destruction of invasive elements during the phase of interference (Brouns et al. 2008; Fineran and Charpentier 2012).

If the virus or plasmid enters the prokaryote again, the crRNA complementary to the respective protospacer binds to the foreign nucleic acid sequence, if applicable with the help of tracrRNA, and a Cas protein or a complex of Cas proteins cleaves the invading nucleic acid to disable it (Brouns et al. 2008; Deltcheva et al. 2011; Jinek et al. 2012). By this manner, invasive DNA as well as RNA is cleaved at specific sites within the protospacer sequence and is thus rendered inoperable (Hale et al. 2009; Garneau et al. 2010).

A mechanism to discriminate between self and non-self DNA is imperative for the prokaryote to avoid targeting the own CRISPR locus and was investigated by the works of Luciano Marraffini and Erik Sontheimer (2010a; 2010b). The authors described crRNAs to include a part complementary to the repeat sequence, which is only bound if paired with the CRISPR

locus and effectively prohibits interference by Cas proteins. On foreign DNA, however, the repeat sequence is not present and the homologous part of the crRNA remains unbound, identifying it as non-self and resulting in interference (Marraffini and Sontheimer 2010a; Marraffini and Sontheimer 2010b).

The CRISPR/Cas9 system is one of the first and one of the few in which the interference phase was fully understood. Martin Jinek et al. described the existence of two domains within Cas9. One of them, the HNH nuclease domain, cleaves the complementary strand, while the other, the RuvC-like domain, cleaves the noncomplementary strand, resulting in a double-strand break and providing an explanation for the mutagenesis caused by CRISPR/Cas9 technology in eukaryotic cells (Jinek et al. 2012; Jinek et al. 2013).

If applied to eukaryotic cells, the mechanism of CRISPR/Cas9 remains similar to the phases described above, however, the crRNA and tracrRNA are replaced by a sgRNA targeting a gene on the cells genome instead of a protospacer sequence on a viral or plasmid genome (Jinek et al. 2012; Jinek et al. 2013). Nevertheless, the result is still a double-strand break in the target sequence, in this case, the chosen gene (Jinek et al. 2012; Jinek et al. 2013). The cells can react to a double-strand break via two repair mechanisms to avoid being compromised (Burma et al. 2006). The highly accurate mechanism of homologous repair (HR) can only be performed during cell cycle phases in which a sister chromatid is present (Burma et al. 2006). The other mechanism, nonhomologous end joining (NHEJ), is much more frequent as it can be carried out quickly during all phases of the cell cycle (Burma et al. 2006). However, it is highly error-prone and leads to insertions and deletions, so-called indel mutations (Kruskal 1983; Burma et al. 2006). The high frequency of NHEJ and its infidelity can thus be utilized to cause indel mutations at the cleavage site on the targeted gene locus with a certain efficiency, which leads to frameshifts causing missense or nonsense mutations (Cho et al. 2013; Cong et al. 2013; Jinek et al. 2013; Mali et al. 2013b; Mali et al. 2013a; Xiao et al. 2013). Thus, genetic engineering via CRISPR/Cas9 technology results in a non-functional version of the targeted gene, effectively knocking out specific gene loci.

1.5 Workflow

Hepatocellular carcinoma features poor response to systemic chemotherapy, high reoccurrence rates and high lethality. Escaping host immune surveillance may be one of the mechanisms involved. The induction of ICD and subsequent activation of targeted immune reactions has been demonstrated to be able to control tumor growth and increase the efficacy of chemotherapy (Olsson and Ebbesen 1978; Matzinger 2002b; Casares et al. 2005; Zitvogel et al. 2008; Galluzzi et al. 2012b; Palombo et al. 2014; Trinchieri and Abastado 2014). In preclinical models, the success of chemotherapeutic agents has been found to rely on a molecular pattern-driven activation of tumor-derived type I IFN boosting an antitumor response (Sistigu et al. 2014). Additionally, there is emerging evidence not only from viral, e.g. hepatitis C virus (HCV), but also from bacterial infections, that type III IFNs may modulate type I IFN-induced signaling (Syedbasha and Egli 2017).

This investigation aims at learning about the capacity of hepatoma cells to activate type I and type III IFNs, i.e. the two paralogous genes *Ifnl2* and *Ifnl3*, as well as typical ISGs upon treatment with various chemotherapeutic drugs in a first instance. Besides that, and intending to establish preclinical models enabling integrative mechanistic studies, murine transplantable *Ifnl2/3*-deficient Hepa 1-6 cells were generated via CRISPR/Cas9 engineering technology, validated, and compared to their parental counterparts with regard to chemosensitivity and their capability to activate type I and type III IFNs and IFN effectors.

Hepa 1-6 is one of the few spontaneous hepatoma cell lines that can be utilized for syngeneic tumor models in *in vivo* experiments, as it can be transplanted into immunocompetent mice (Heindryckx et al. 2009; Reiberger et al. 2015; Li et al. 2019; Ou et al. 2019). Since this thesis aims at setting the basis for possible subsequent *in vivo* studies, it was important to choose a cell line that could be used for further research after having been genetically modified. Syngeneic tumor models allow for the investigation of immune mechanisms in an immunocompetent tumor-bearing system, while the transplanted tumor cells themselves are non-immunogenic, thus ruling out possible confounding antitumor immune responses as demonstrated for example by Da-Liang Ou et al. in their investigations of the implications of PD-L1 expression in an orthotopic liver cancer model (Olson et al. 2018; Li et al. 2019; Ou et al. 2019). Derived from C57BL/6J mice, Hepa 1-6 can be utilized to generate either subcutaneous or orthotopic mouse models, with the latter ensuring a more accurate imitation of the tumor microenvironment (Brown et al. 2018; Li et al. 2019). The inoculation of immunocompetent mice with malignant cells, which have been exposed to a lethal stimulus *in*

vitro beforehand, to subsequently observe the development of intact tumor cells of the same entity serves as a vaccination assay, the current gold-standard to detect ICD as stated in the Consensus guidelines for the detection of immunogenic cell death published by Oliver Kepp et al. in 2014 (Casares et al. 2005; Kroemer et al. 2013; Kepp et al. 2014; Vacchelli et al. 2014). This is then to be followed by confirmatory assays, i.e. the assessment of the therapeutic effect of ICD inducers against established neoplastic lesions, best performed in syngeneic tumor models such as Hepa 1-6 (Apetoh et al. 2007b; Obeid et al. 2007a; Ghiringhelli et al. 2009; Vesely et al. 2011; Menger et al. 2012; Kroemer et al. 2013; Kepp et al. 2014).

However, the investigation of type I and type III IFNs in the context of ICD in a syngeneic mouse model was not the goal of this thesis, it was to establish a suitable genetically modified *Ifnl2/3*-deficient cell clone of the hepatoma cell line Hepa 1-6, and subsequently perform a comparative analysis of the *Ifnl2/3*-deficient clones and the parental cell line. In order to achieve this, the workflow of this thesis was separated into several steps of investigation, with the evaluation of the ability of Hepa 1-6 to express type I and type III IFNs as well as ISGs as the first step.

In preparation for the comparative analysis of Hepa 1-6 wildtype cells and *Ifnl2/3*-deficient clones, the ability of Hepa 1-6 cells to express type I and type III IFNs as well as ISGs had to be investigated. IFN expression in the undisturbed cell is usually below detection levels, as it is a reactive signaling substance (Sheppard et al. 2003). Thus, stimulatory protocols had to be established to induce both type I IFNs, type III IFNs and ISGs. The ISGs investigated were *Mx1* as well as *Cxcl10*.

The synthetic double-stranded polynucleotide poly(I:C) acts as a dsRNA analog and has been shown to induce IFN production in cells upon exposure (Field et al. 1967; Dianzani et al. 1968). The induction is achieved by the activation of several signaling pathways. The most important of these acts via TLR3, a pathogen-associated molecular pattern receptor specifically recognizing viral infection via dsRNA and initiating an immune reaction as mentioned in the previous section (Alexopoulou et al. 2001; Matsumoto and Seya 2008). Nonetheless, IFN expression caused by poly(I:C) alone is small in quantity and detection requires identification of the optimal exposure time as well as assessment time (Dianzani et al. 1968). Matching descriptions of improved TLR3 activation by larger dsRNA molecules, high molecular weight poly(I:C) has shown to induce higher levels of type I as well as type III IFNs rather than low molecular weight poly(I:C) (Zhou et al. 2013).

TLR3 is mostly situated inside of endosomes to recognize dsRNA or poly(I:C) that has entered the cell by the means of endocytosis (Alexopoulou et al. 2001; Matsumoto and Seya 2008; Yoneyama and Fujita 2010). However, this seems to be only one of the possible pathways. Extracellular dsRNA is also recognized by intracellular receptors, but the transport pathways have not been identified yet (Alexopoulou et al. 2001; Matsumoto and Seya 2008). Once inside the cell, dsRNA can bind to the cytosolic RIG-1-like receptors or be transported to endosomes activate TLR3 anew (Matsumoto and Seya 2008; Yoneyama and Fujita 2010).

As recognition via TLR3 inside of endosomes has been identified as the major pathway resulting in IFN production, poly(I:C) treatment was combined with the transfector DEAE-dextran to increase its effect. The cationic DEAE-dextran facilitates the uptake of poly(I:C) into the cell by binding the negatively charged polynucleotide, resulting in a complex with positive net charge (Schenborn and Goiffon 2000). The complex accumulates to the cell membrane and is taken up via endocytosis, raising the amount of TLR3 activated (Schenborn and Goiffon 2000). Since the discovery of its potential in transferring both RNA by Antti Vaheri and Joseph Pagano in 1965 and DNA by James McCutchan and Joseph Pagano in 1968, DEAE-dextran has been utilized as a standard method for transient transfections *in vitro* (Vaheri and Pagano 1965; McCutchan and Pagano 1968). Dianzani et al. demonstrated that the combination of poly(I:C) and DEAE-dextran treatment resulted in IFN yields up to 100 times higher than poly(I:C) exposure alone (Dianzani et al. 1968).

In addition, cell stimulation was also performed by combining poly(I:C) priming with consecutive protein and RNA synthesis inhibition, adhering to an established superinduction protocol for type I IFNs by Edward A. Havell and Jan Vilček (Havell and Vilček 1972).

Further details on the stimulation protocols are displayed in section 2.2.4.

The second step of investigation was to assess the effect of chemotherapeutic agents on the induction of type I and type III IFNs as well as ISGs in Hepa 1-6 in order to ensure Hepa 1-6 to be a fitting model for chemotherapy-induced ICD and immunostimulation in hepatoma.

However, chemotherapy has currently nearly no place in the therapy of HCC as it lacks efficacy, hence there was no representative set of chemotherapeutic drugs to be chosen for assessment (EASL 2018). The investigated drugs were thus chosen because of their roles in pancreatic, gastric or colorectal cancer therapies, and to reflect different substance groups as well as different mechanisms of action.

Gemcitabine as 2',2'-difluoro 2'-deoxycytidine functions as an antimetabolite and unfolds its antitumoral effects by various ways of inhibiting DNA synthesis (Mini et al. 2006). After influx into the cell the cytidine analog is converted to active metabolites (Mini et al. 2006). These metabolites then impact multiple aspects of DNA synthesis, e.g. through the inhibition of the DNA polymerase and the ribonuclease reductase or through masked chain termination (Mini et al. 2006). Having been initially tested for possible antiviral properties, gemcitabine has become an important cytotoxic agent and is applied alone or in combinations in chemotherapy for carcinomas of the pancreas, bladder, lung, ovaries, and breasts (Mini et al. 2006).

Oxaliplatin is a third-generation platinum compound that, in contrast to its related substances cisplatin and carboplatin, contains diaminocyclohexane ligands, resulting in a wide antitumor activity while surpassing resistance mechanisms negating the effect of other platinums (Di Francesco et al. 2002). Therefore, it has found its place in chemotherapy regimes as an addition to 5-fluorouracil for colorectal cancer, which had until that point been resistant to platinums (Raymond et al. 2002; Simpson et al. 2003).

Similar to gemcitabine, oxaliplatin is taken up into the cell where it is biotransformed to its active metabolites (Raymond et al. 2002). As a cytotoxic agent, oxaliplatin forms mainly intrastrand cross-links, thus disrupting DNA replication and transcription (Raymond et al. 1998; Di Francesco et al. 2002). The resulting cell cycle arrest leads to apoptosis (Raymond et al. 1998; Di Francesco et al. 2002).

In contrast to the other substances, doxorubicin is not a classic chemotherapeutic for it is an early member of the group of anthracycline antibiotics (Arcamone et al. 2000; Thorn et al. 2011). Discovered by Federico Arcamone et al. in 1969, doxorubicin was found to have potent antineoplastic effects in addition to its antibiotic properties, and thus classifies as an antitumor antibiotic (Arcamone et al. 2000; Thorn et al. 2011).

Doxorubicin intercalates between adjacent DNA base pairs, which leads to malfunctioning of the topoisomerase II, causing DNA lesions through double-strand breaks (Hortobágyi 1997). Besides that, doxorubicin generates free radicals (Hortobágyi 1997). In combination, this causes a strong cytotoxic effect due to which doxorubicin has found its place in various cancer treatments, e.g. gastric, lung, breast and ovarian cancer (Hortobágyi 1997; Thorn et al. 2011). However, clinical application is limited due to resistance as wells as strong side effects such as cardiotoxicity and myelosuppression, mainly due to a heightened sensitivity to damage caused by free radicals (Hortobágyi 1997; Thorn et al. 2011).

Of the chosen chemotherapeutics, doxorubicin and oxaliplatin have been established as ICD inducers as demonstrated by Antonella Sistigu et al. (2014) and Antoine Tesniere et al. (2010). In their work, Antonella Sistigu et al. demonstrated the induction of type I IFNs as well as a type I IFN fingerprint in tumors through autocrine and paracrine mechanisms in response to treatment with doxorubicin (Sistigu et al. 2014). They identified self RNA from dying cells to act as a mediator and TLR3 as a vital receptor in this mechanism, and linked the success of chemotherapy to the presence of type I IFN or the ISG CXCL10 in the neoplastic cells rather than the host cells (Sistigu et al. 2014). A similar type I IFN response was demonstrated for neoplastic cells exposed to oxaliplatin (Sistigu et al. 2014).

Gemcitabine has not been identified as an ICD inducer, however several immunostimulatory effects have been described including an increase in the expression of class I HLA on malignant cells, the enhancement of cross-presentation of tumor antigens and a decrease in MDSC to boost T cell-dependent anticancer immunity (Nowak et al. 2003a; Nowak et al. 2003b; Liu et al. 2010; Vincent et al. 2010; Mundy-Bosse et al. 2011). Similarly, treatment with oxaliplatin was also linked to a shift in the tumor-immune microenvironment, increased levels of CD8+ T cells and reduced levels of MDSC (Gonzalez-Aparicio et al. 2011).

Appropriate dosage had to be evaluated for each substance via cell viability tests, as ICD induction relies on a certain level of cytotoxicity to create a sufficient stimulus (Kepp et al. 2014). Sub-therapeutic dosages of cytotoxic drugs that affect but do not kill cells have been shown to fail at inducing protective immunity (Obeid et al. 2007a; Menger et al. 2012).

For further details on cell culture stimulation with the mentioned chemotherapeutic agents see section 2.2.4.

The next step within the work for this thesis aimed at evaluating the role and nature of possible mediators in the observed effects.

As outlined in section 1.2 a multitude of signals may act as a DAMP, which are essential mediators in ICD. Co-culturing experiments were performed in order to investigate the mediating DAMPs involved in the process of ICD and IFN signaling in the forementioned setup. To identify possible mediators of stimulation between cell cultures, unstimulated cell cultures were incubated with medium from previously stimulated cell cultures. Recipient cell cultures were then investigated for the expression of IFNs and ISGs.

Additionally, the transferred culture medium was combined with either ribonuclease A to specifically degrade RNAs, DNase I to target DNAs or Benzonase® nuclease to inhibit both RNAs and DNAs. This approach was utilized to further the characterization of the involved DAMPs.

Detailed protocols of the co-culturing experiments can be found in section 2.2.4.

The fourth and final step of the outlined workflow was to assess the effect of type III IFN induction on tumor viability, leading to a comparative analysis of Hepa 1-6 wt and *Ifnl2/3*-deficient Hepa 1-6 clones.

Ifnl2/3-deficient clones of Hepa 1-6 cells were generated utilizing the CRISPR/Cas9 gene engineering technology with an IL-28 double nickase transfection. To ensure the successful knockout of both *Ifnl2* and *Ifnl3*, and thus the lack of both functioning *IFNL* orthologs, each cell clone was assessed on both gDNA and mRNA level. For details on the CRISPR/Cas9 double nickase transfection and *Ifnl2/3* knockout design see section 2.2.5. Further information on the knockout validation can be found in section 2.2.6.

The established stimulation protocols were employed to investigate the ability of *Ifnl2/3*-deficient clones to hoist a type I IFN response, analyzing for both *Ifna* and the ISGs *Mx1* and *Cxcl10*. Additionally, clones were assessed for differences in their morphology as well as base cell viability to investigate whether the *Ifnl2/3* deficiency would influence tumor growth or viability. Cell viability was assessed via cell counting with dye exclusion, as end-stage plasma membrane permeabilization is one of the most reliable indicators of cell death (Galluzzi et al. 2009; Kepp et al. 2011a). To evaluate the cell viability and proliferation after exposure to chemotherapy the metabolic activity of cell cultures was recorded utilizing an MTS cell viability and proliferation assay, of which detailed information can be found in section 2.2.1. The data obtained allowed for a comparative analysis of the *Ifnl2/3*-deficient Hepa 1-6 clones and their parental wild type Hepa 1-6 cells.

Regarding the context of Prof. Mihm's research focus, this thesis aims at establishing a firm basis to further investigate the extent of IFN-λ participation or modulation in the process of preclinical *in vivo* immunoediting and thus the anticancer immune response. This includes knocking out IFN-λ genes, which was intended to be achieved by CRISPR/Cas 9 technology, a quite novel tool at that time.

2. Material and Methods

2.1 Material

2.1.1 Cell Line and Culturing Conditions

The murine hepatoma cell line Hepa 1-6, derived from the BW7756 tumor in a C57L *Mus musculus*, was supplied as a frozen culture by the Leibniz Institute's DMSZ-German Collection of Microorganisms and Cell Cultures GmbH and is registered under ACC 175.

Following a standard protocol and adhering to the supplier's recommended seeding density of 1-2 x 10^6 cells/25 cm^2, the frozen cells were thawed and seeded into cell culture flasks with 90% of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 4.5 g/L glucose (Thermo Fisher Scientific Inc.), 10% of Fetal Bovine Serum (FBS) (Biowest Europe) and 100 U/ml Penicillin G/Streptomycin sulfate (Thermo Fisher Scientific Inc.). Cell cultures were incubated at standard conditions of 37 °C and 5% CO_2. Culture splitting was performed in 1:5 to 1:15 ratios every second to third day by washing the cells with Dulbecco's Phosphate Buffered Saline (DPBS) (Thermo Fisher Scientific Inc.) and detaching the cells utilizing Trypsin-EDTA (Thermo Fisher Scientific Inc.). Stocks of the supplied cells as well as of acquired Hepa 1-6 clones were stored in a nitrogen tank in 70% DMEM, 20% FBS and 10% dimethyl sulfoxide (CAS 67-68-5, Sigma-Aldrich Chemie GmbH).

In accordance with the DMSZ cell culture data sheet concerning the morphology of Hepa 1-6, the hepatoma cells showed characteristics of epithelial cells and grew as adherent monolayers, as depicted in figure 1.

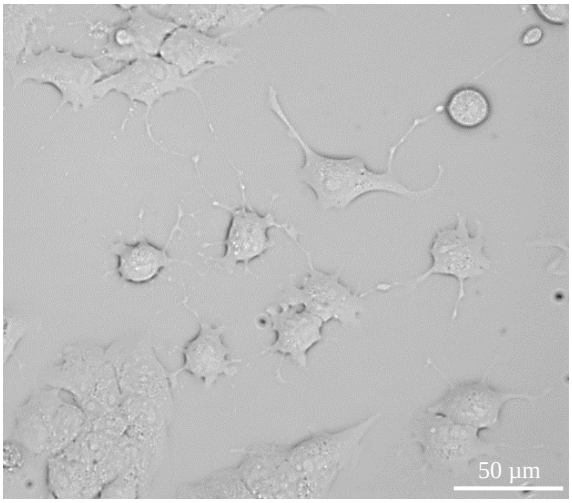


Figure 1: Phenotype of Hepa 1-6 Wildtype

Hepa 1-6 wildtype cells were seeded into 6-well plates at a density of 2 x 10^5 cells/well. After 24 h of incubation at 37 °C and 5% CO_2, cell cultures were photographed using bright light exposure and the Leica DMi8 Advanced Fluorescence Imaging System (Leica Mikrosysteme Vertrieb GmbH). As expected, cells had grown as an adherent epithelial monolayer.

2.1.2 Substances

Table 1: Substances Utilized Organized According to Function

Cell Culture Supplies	Supplied by
0.5% Trypsin-EDTA (10X)	Gibco™ by Thermo Fisher Scientific Inc. MA, USA
DMEM (1X) Dulbecco's Modified Eagle Medium [+] 4.5 g/L D-Glucose, L-Glutamin [-] Pyruvate	Gibco™ by Thermo Fisher Scientific Inc. MA, USA
DPBS (1X) Dulbecco's Phosphate Buffered Saline [-] $CaCl_2$ [-] $MgCl_2$	Gibco™ by Thermo Fisher Scientific Inc. MA, USA
Fetal Bovine Serum Premium	Biowest Europe Nuaillé, France
Chemotherapeutics & Antibiotics	**Supplied by**
Actinomycin D ≥ 95% (HPLC), CAS 50-76-0	Sigma-Aldrich Chemie GmbH Munich, Germany
Cycloheximide CAS 66-81-9	Sigma-Aldrich Chemie GmbH Munich, Germany
Doxorubicin hydrochloride 98.0 – 102.0% (HPLC), CAS 25316-40-9	Sigma-Aldrich Chemie GmbH Munich, Germany
Gemcitabine hydrochloride ≥ 98% (HPLC), CAS 122111-03-9	Sigma-Aldrich Chemie GmbH Munich, Germany
Oxaliplatin CAS 61825-94-3	Sigma-Aldrich Chemie GmbH Munich, Germany
Pen Strep 10.000 U/ml Penicillin G, Streptomycin sulfate CAS 61-33-6; CAS 3810-74-0	Gibco™ by Thermo Fisher Scientific Inc. MA, USA
Puromycin dihydrochloride CAS 58-58-2	Santa Cruz Biotechnology, Inc. TX, USA
Enzymes	**Supplied by**
Benzonase® Nuclease CAS 9025-65-4	Sigma-Aldrich Chemie GmbH Munich, Germany
DNase I RNase-free EC 3.1.21.1	Invitrogen™ by Thermo Fisher Scientific Inc. MA, USA
Ribonuclease A	Sigma-Aldrich Chemie GmbH

CAS 9001-99-4; EC 3.1.27.5	Munich, Germany
Taq DNA Polymerase Recombinant	Invitrogen™ by Thermo Fisher Scientific Inc. MA, USA

General Chemicals	Supplied by
2-Mercaptoethanol CAS 60-24-2	Merck KGaA Darmstadt, Germany
50 mM $MgCl_2$ CAS 7791-18-6 (in water)	Invitrogen™ by Thermo Fisher Scientific Inc. MA, USA
Agarose Standard Roti®garose CAS 9012-36-6	Carl Roth GmbH + Co. KG Karlsruhe, Germany
Ampuwa® water for injections	Fresenius Kabi Deutschland GmbH Bad Homburg, Germany
Bidistilled water	In-house production of Universitaetsmedizin Goettingen, Germany
Dimethyl sulfoxide CAS 67-68-5	Sigma-Aldrich Chemie GmbH Munich, Germany
Ethanol absolute p.a. CAS 64-17-5	CHEMSOLUTE® by Th. Geyer GmbH & Co. KG Renningen, Germany
TBE 5X Tris/Borate/EDTA	In-house production of Universitaetsmedizin Goettingen, Germany

Other Reagents and Chemicals	Supplied by
100 bp DNA Ladder	Invitrogen™ by Thermo Fisher Scientific Inc. MA, USA
10X PCR Rxn Buffer [-] $MgCl_2$	Invitrogen™ by Thermo Fisher Scientific Inc. MA, USA
DNA Gel Loading Dye (6X)	Thermo Scientific™ by Thermo Fisher Scientific Inc. MA, USA
DEAE-Dextran hydrochloride CAS 9064-91-9	Sigma-Aldrich Chemie GmbH Munich, Germany
dNTP Mix 10 Mm (2.5 mM *ea*)	Applied Biosystems® by Thermo Fisher Scientific Inc.

	MA, USA
GeneAmp® dNTP Mix with dTTP (2.5mM)	Applied Biosystems® by Thermo Fisher Scientific Inc. MA, USA
Midori Green Advance DNA Stain CAS 7732-18-5	Nippon Genetics Europe GmbH Dueren, Germany
Plasmid Transfection Medium CAS 31852-29-6	Santa Cruz Biotechnology, Inc. TX, USA
Poly(I:C) (HMW)	InvivoGen Toulouse, France
RNeasy RLT Plus Lysis Buffer (Part of AllPrep DNA/RNA Mini Kit)	QIAGEN GmbH Hilden, Germany
TaqMan® Universal PCR Master Mix, No AmpErase® UNG	Applied Biosystems® by Thermo Fisher Scientific Inc. MA, USA
Trypan Blue Solution 0.4% CAS 72-57-1	Sigma-Aldrich Chemie GmbH Munich, Germany
UltraCruz® Transfection Reagent	Santa Cruz Biotechnology, Inc. TX, USA

2.1.3 Assays, Kits, Plasmids and Primers

Table 2: Detailed Listing of Utilized Assays, Kits, Plasmids and Primers Including Gene Sequences

Assays	Supplied by
CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay	Promega Corporation WI, USA
TaqMan™ Gene Expression Assays	Applied Biosystems® by Thermo Fisher Scientific Inc. MA, USA
HPRT	ID: Mm00446968_m1
IFNL2/3	ID: Mm04204155_gH
IFNa4	ID: Mm00833969_s1
Mx1	ID: Mm00487796_m1
CXCL10/IP10	ID: Mm00445235_m1

Kits	Supplied by
AllPrep DNA/RNA Mini Kit (50)	QIAGEN GmbH Hilden, Germany
NucleoSpin® Gel and PCR Clean-up Kit	MACHEREY-NAGEL GmbH & Co. KG Düren, Germany
QuantiTect® Reverse Transcription Kit	QIAGEN GmbH Hilden, Germany
RNeasy Mini Kit (250)	QIAGEN GmbH Hilden, Germany

Plasmids	
Control Double Nickase Plasmid	Santa Cruz Biotechnology, Inc. TX, USA
IL-28a Double Nickase Plasmid (m2) (sc-437298-NIC2)	Santa Cruz Biotechnology, Inc. TX, USA

Primers	
Primer random $p(dN)_6$	Roche Diagnostics International AG Rotkreuz, Switzerland
Custom DNA Oligo Primers	Invitrogen™ by Thermo Fisher Scientific Inc. MA, USA
IFNL2-3 ko for	5’-AAGAACCCAAGCTGACCCTG-3’
IFNL2-3 ko rev	5’-GCAGCTCTTTTGGGGACAGA-3’
IFNL2-3 ko2 for	5’-TGTCCCAAAGAGCTGC-3’
IFNL2-3 ko2 rev	5’-TGCACAAAGTGTGGAGACCA-3’
IFNL2 span ex1 for	5’-CTGCCACAAAACCGAACCAAAG-3’
IFNL2 span ex1 rev	5’-TCCCAGTTAGCATAAGGGATGA-3’
IFNL3 span ex1 for	5’-AAGTCAGCCCACTGCACAAA-3’
IFNL3 span ex1 rev	5’-CCAAGCTTCTTGTGGGTAGC-3’

2.1.4 General Supplies

Table 3: General Laboratory Supplies

Flasks	Supplied by
CELLSTAR® Cell Culture Flask, 50 ml, 25 cm², Red Filter Screw Cap, Sterile	Greiner Bio-One International GmbH Kremsmuenster, Austria
CELLSTAR® Cell Culture Flask, 550 ml, 175 cm², Red Filter Screw Cap, Sterile	Greiner Bio-One International GmbH Kremsmuenster, Austria
TC-Flask T75, Ventilation Cap	Sarstedt AG & Co. Nuembrecht, Germany
TC-Flask T175, Ventilation Cap	Sarstedt AG & Co. Nuembrecht, Germany
Pipette Tips	**Supplied by**
Combitips advanced® 0.1 ml Sizes 0.1 ml, 0.5 ml, 1.0 ml, 2.5 ml	Eppendorf AG Hamburg, Germany
SafeSeal-Tips® premium Ultra Micro Pipette Tips Sizes 0.1 – 10 µl, 100 µl	Biozym Scientific GmbH Hessisch Oldendorf, Germany
TipOne® 10 µl Graduated Tip	STARLAB GmbH Hamburg, Germany
TipOne® 10/20 µl Graduated, Filter Tip, RPT	STARLAB GmbH Hamburg, Germany
TipOne® 100 µl Bevelled, Filter Tip, RPT	STARLAB GmbH Hamburg, Germany
TipOne® 200 µl Yellow, Ultra Point Graduated Tip	STARLAB GmbH Hamburg, Germany
TipOne® 1000 µl Blue, Graduated Tip	STARLAB GmbH Hamburg, Germany
Tip One® 1000 µl Filter Tip	STARLAB GmbH Hamburg, Germany
Pipettes (single use)	**Supplied by**
Aspiration Pipette Size 2ml	Sarstedt AG & Co. Nuembrecht, Germany
Serological Pipette Sizes 2 ml, 25 ml, 50 ml	Sarstedt AG & Co. Nuembrecht, Germany
Serological Pipette Graduated Sizes 5 ml, 10 ml	Greiner Bio-One International GmbH Kremsmuenster, Austria

Plates and Dishes	Supplied by
CELLSTAR® Cell Culture Microplate, 96 Well, F-Bottom	Greiner Bio-One International GmbH Kremsmuenster, Austria
CELLSTAR® 24 Well Cell Culture Plate, Sterile	Greiner Bio-One International GmbH Kremsmuenster, Austria
MicroAmp® Fast 96-Well Reaction Plate (0.1 ml)	Applied Biosystems® by Thermo Fisher Scientific Inc. MA, USA
TC-Plate 6 Well, Standard, F	Sarstedt AG & Co. Nuembrecht, Germany
TC-Plate 12 Well, Sterile	Greiner Bio-One International GmbH Kremsmuenster, Austria
TC Dish 100, Standard	Sarstedt AG & Co. Nuembrecht, Germany
Thincerts with PET-Membrane 0.4 µm pores	Greiner Bio-One International GmbH Kremsmuenster, Austria

Reaction Tubes	Supplied by
Eppendorf Tubes® Safe-Lock Sizes 1.5 ml, 2.0 ml	Eppendorf AG Hamburg, Germany
Injekt® Syringe Luer Lock Solo Sizes 5 ml, 10 ml	B. Braun Melsungen AG Melsungen, Germany
Micro Tubes Sizes 1.5 ml, 2.0 ml	Sarstedt AG & Co. Nuembrecht, Germany
Nunc™ CryoTube™ Vials 1.0 ml	Nalgene Labware by Thermo Fisher Scientific Inc. MA, USA
PCR Tube 0.5 ml Thin-walled	Peqlab by VWR International PA, USA
Tubes Sizes 5 ml, 15 ml, 50 ml	Sarstedt AG & Co. Nuembrecht, Germany

Various Items	Supplied by
Desco Wipes DT	Dr. Schumacher GmbH Malsfeld, Germany
DESOMED® RAPID AF	Desomed - Dr. Trippen GmbH Freiburg, Germany

DURAN® Erlenmeyer Flasks	DURAN Group GmbH Wertheim/Main, Germany
Kimtech Science Professional Precision Wipes	Kimberly-Clark Corporation TX, USA
Kimtech Science Professional Delicate Task Wipes	Kimberly-Clark Corporation TX, USA
LABSOLUTE® Syringe Filters 0.22 µm, Sterile	Th. Geyer GmbH & Co. KG Renningen, Germany
MicroAmp® Optical Adhesive Film	Applied Biosystems® by Thermo Fisher Scientific Inc. MA, USA
Nitrile gloves/gants standard Cat. III	LLG Labware by Lab Logistics Group GmbH Meckenheim, Germany
RNase Zap®	Ambion® by Thermo Fisher Scientific Inc. MA, USA
Semperguard® Nitrile Comfort powder-free gloves	Semperit Technische Produkte GmbH Wien, Austria
Simax® Measuring Cylinders	Kavalierglass, a.s. Prague, Czech Republic
Sterillium® classic pure	BODE Chemie GmbH Hamburg, Germany
UVette®	Eppendorf AG Hamburg, Germany

2.1.5 Devices and Software

Table 4: Detailed List of Utilized Devices and Applied Software

Cell Culture Devices	Supplied by
BINDER INC CB 220 (E6) CO_2-Incubator	BINDER GmbH Tuttlingen, Germany
Fluid aspiration system BVC control with VacuuHandControl VHC^{pro}	VACUUBRAND GMBH + CO KG Wertheim, Germany
Safe 2020 Class II Biological Safety Cabinet	Thermo Scientific™ by Thermo Fisher Scientific Inc. MA, USA

Waterbath WNB 14	Memmert GmbH + Co. KG Schwabach, Germany

Centrifuges	Supplied by
Centrifuge 5415 R	Eppendorf AG Hamburg, Germany
Heraeus™ Megafuge™ 16 Centrifuge	Thermo Scientific™ by Thermo Fisher Scientific Inc. MA, USA
Plate centrifuge PerfectSpin P	Peqlab by VWR International PA, USA

Fridges and Nitrogen Tanks	Supplied by
CS 35 BA Nitrogen Storage Vessel	CRYO Anlagenbau GmbH Wilnsdorf, Germany
HERAfreeze™ -80°C	Thermo Scientific™ by Thermo Fisher Scientific Inc. MA, USA
LIEBHERR Refrigerator Comfort -20°C LIEBHERR Refrigerator ProfLine 4°C	Liebherr-International Deutschland GmbH Biberach an der Riß, Germany

Microscopes	Supplied by
Invertoskop ID03	Carl Zeiss Microscopy GmbH Göttingen, Germany
Leica DMi8 Advanced Fluorescence Imaging System	Leica Mikrosysteme Vertrieb GmbH Wetzlar, Germany

Pipettes	Supplied by
Eppendorf Multipette® plus	Eppendorf AG Hamburg, Germany
Eppendorf Reference® Sizes 2.5 µl, 10 µl, 20 µl, 100 µl, 200 µl, 1000 µl	Eppendorf AG Hamburg, Germany
PIPETBOY acu 2	INTEGRA Biosciences GmbH Biebertal, Germany
pipetus®	Hirschmann Laborgeraete GmbH & Co. KG Eberstadt, Germany
Transferpette® -12 20 – 200 µl	BRAND GMBH + CO KG Wertheim, Germany

Software	Supplied by
AUTOsoft 2.6.4 PHOmo Software	Autobio Diagnostics Co., Ltd Zhengzhou, China
BLAST® Align Sequences Nucleotide	National Center for Biotechnology Information, U.S. National Library of Medicine Bethesda MD, USA
Chromas 2.6.2 DNA Sequencing Software	Technelysium Pty Ltd South Brisbane, Australia
DataAssist™ Software v3.01	Applied Biosystems® by Thermo Fisher Scientific Inc. MA, USA
Genome Data Viewer Genome Browser Version 4.8.2	National Center for Biotechnology Information, U.S. National Library of Medicine Bethesda MD, USA
GraphPad Prism 7.03	GraphPad Software, Inc. CA, USA
Image Lab™ Software	Bio-Rad Laboratories, Inc. CA, USA
Leica Application Suite X	Leica Mikrosysteme Vertrieb GmbH Wetzlar, Germany
Primer-BLAST®	National Center for Biotechnology Information, U.S. National Library of Medicine Bethesda MD, USA
STATISTICA 13 (campus license)	StatSoft (Europe) GmbH Hamburg, Germany
StepOne™ Software v2.3	Applied Biosystems® by Thermo Fisher Scientific Inc. MA, USA

Various Devices	Supplied by
DPU414 Desktop Thermal Serial Printer	Seiko Instruments GmbH Neu Isenburg, Germany
Eppendorf BioPhotometer®	Eppendorf AG Hamburg, Germany
FlexCycler	Analytik Jena AG Jena, Germany

lab dancer	IKA®-Werke GmbH & CO. KG Staufen, Germany
LABOKLAV 55-195	SHP Steriltechnik AG Detzel Schloss/Satuelle, Germany
MS1 Minishaker	IKA®-Werke GmbH & CO. KG Staufen, Germany
Multiple Tally Denominator	The Denominator Company CT, USA
Neubauer counting chamber (0.1 mm)	Hecht Glaswarenfabrik GmbH & Co KG Sondheim vor der Rhoen, Germany
PerfectBlue™ Gel System Mini M	Peqlab by VWR International PA, USA
PHILIPS Whirpool M611 Space Cube High Power Line Microwave	Philips GmbH Market DACH Hamburg, Germany
PHOmo Elisa Reader	Autobio Diagnostics Co., Ltd Zhengzhou, China
PowerPac™ HC High-Current Power Supply	Bio-Rad Laboratories, Inc. CA, USA
Sartorius Analytical Balance AC 210 P	Sartorius AG Goettingen, Germany
Short period timer WB 388	Lab Unlimited Dublin, Ireland
SPROUT® Mini Centrifuge	Heathrow Scientific IL, USA
StepOnePlus™ Real-Time PCR System	Applied Biosystems® by Thermo Fisher Scientific Inc. MA, USA
ThermoMixer® compact	Eppendorf AG Hamburg, Germany
Universal Hood II	Bio-Rad Laboratories, Inc. CA, USA

2.2 Methods

2.2.1 Cell Viability and Proliferation Tests

Cell viability as well as metabolic activity were assessed through cell counting with dye exclusion via Trypan Blue Solution (Sigma-Aldrich Chemie GmbH) and by employing the MTS cell viability and proliferation assay (CellTiter 96 AQ One Solution Cell Proliferation Assay, Promega). In order to estimate viable cell numbers for seeding, cell density was evaluated in Neubauer counting chambers (Hecht Glaswarenfabrik GmbH & Co KG).

Hemocytometers such as the Neubauer counting chamber allow for easy assessment of cell density by taking up a calibrated volume of cell suspension onto a microscope slide divided into standardized squares (Absher 1973; Strober 1997a). The number of cells within each square is counted under a light microscope at 10x magnification. Cell density of the sample tested is then calculated using the following equation (Strober 1997a):

(Total of cells counted/squares counted) x 10^4 = cells/ml

Cells to be counted were washed, detached by trypsinization and dispersed by gentle pipetting before being resuspended in DMEM culturing medium. Based loosely on protocols by Warren Strober (Strober 1997a; Strober 1997b; Strober 2015), dye exclusion was performed by adding 5 µl of 0.4% trypan blue dye to 100 µl of cells suspended in DMEM. Subsequently, cells were counted in a Neubauer counting chamber, counting non-stained and stained cells separately. Figure 2 depicts an excerpt of such a Neubauer counting chamber under a light microscope (Invertoskop ID03, Carl Zeiss Microscopy GmbH). Cell numbers were then calculated as described above. As the dilution performed by adding the dye is negligibly small, adjusting the equation was not necessary. Resulting numbers were compared to receive percentages of stained and non-stained cells, respectively.

To extend cell culture assessment and improve evaluation of cell viability and proliferation after drug exposure, the metabolic activity of cell cultures was recorded by MTS reduction assays (CellTiter 96 AQ One Solution Cell Proliferation Assay, Promega).

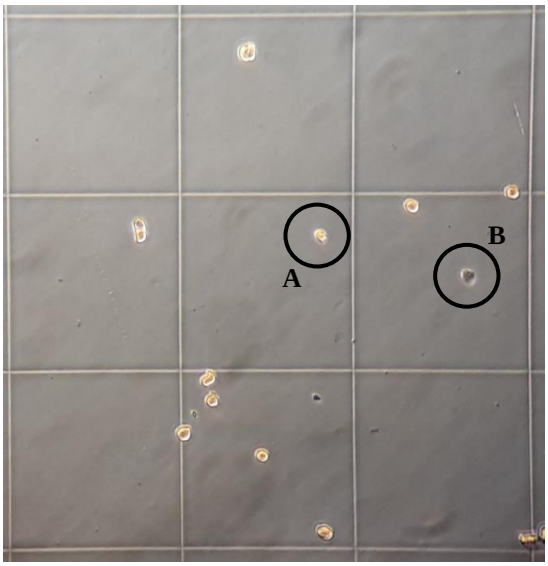


Figure 2:
Cell Counting with Trypan Blue Dye Exclusion

Cells were detached by trypsinization, dispersed by gentle pipetting and resuspended in DMEM. 5 µl of 0.4% trypan blue dye was added to 100 µl of cell suspension. Cells were then counted under a light microscope at 10x magnification using a Neubauer counting chamber to calculate the amount of intact as well as compromised cells per ml. Circle A marks an intact cell; circle B marks a compromised and thus presumably dead cell.

According to the supplier's instructions, the MTS assay was prepared by seeding cells into flat-bottom 96-well-plates (CELLSTAR® Cell Culture Microplate, 96 Well, F-Bottom, Greiner Bio-One International GmbH) with 2 x 10^3 cells/100 µl density. To reduce evaporation of culture medium, the plates were incubated in a humidified atmosphere. An exemplary schematic of an MTS assay plate is depicted in figure 3. Once the cells had grown sufficiently, the drug to be examined was applied in quadruplicates of different concentrations. 24 hours later culturing medium was replaced as suggested by a work of Manish I. Patel et al. to improve accuracy (Patel et al. 2005), before adding 20 µl of MTS reagent to each well. Following one hour to four hours of incubation at 37 °C and 5% CO2, the plates were assessed via photometry at $\lambda = 492$ nm in a PHOmo Elisa Reader (Autobio Diagnostics Co., Ltd) with extinction referring to metabolic activity.

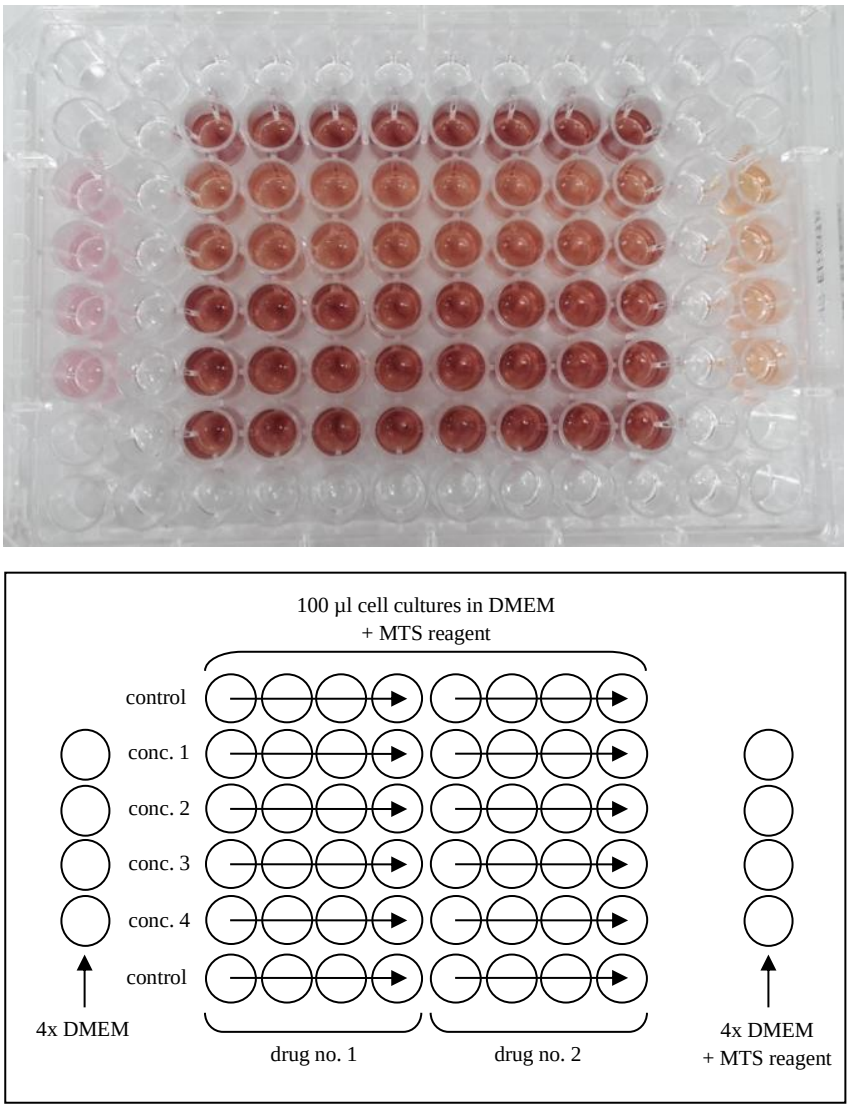


Figure 3: MTS Cell Metabolic Activity Assay Schematic and Exemplary Plate

The set of wells on the sides were filled with 100 µl of DMEM culturing medium each. The left quadruplicate functioned as a blank for medium only to assess potential interference by the culturing medium. The quadruplicate on the right side provided blanks for the actual MTS assay. The sets of quadruplicates in the middle consisted of 100 µl cell cultures in DMEM, having been seeded 24 h to 48 h prior. Two different drugs were administered to the cells in four different concentrations with the lowest concentration in the last row. The top row of cell cultures functioned as a control. 24 h after drug exposure, cells were washed and the culture medium was exchanged. 20 µl of MTS reagent was added. Photometry at $\lambda = 492$ nm was performed after 1.5 h and 3 h of incubation. The photographed plate depicts an MTS assay after 3 h of incubation, demonstrating differences in metabolic activity measurable not only in photometry, but also visible to the eye, as the second and third row of cell cultures, having been subjected to high drug concentrations, were remarkably lighter in color than the control above or the lower concentrations below.

2.2.2 Purification of Nucleic Acids

2.2.2.1 Extraction of Nucleic Acids

In order to determine genotypes as well as to quantify mRNA, gDNA, and total cellular RNA, respectively, nucleic acids had to be extracted from cell cultures.

Nucleic acid extraction from mammalian cells requires a series of steps to isolate either genomic DNA (gDNA) or RNA. The following procedure is based on protocols by Cheryl Koh, Ximeng Liu and Shuko Harada (Koh 2013; Liu and Harada 2013a; Liu and Harada 2013b). After harvesting and lysing the respective cells, contaminants such as proteins have to be eliminated. Usually by the means of organic extraction or silica spin-column absorption, nucleic acids are separated from other cell components and then retrieved. When aiming at extracting RNA, all supplies need to be free of ribonucleases (RNases), while when separating for gDNA treatment with RNases can be used to eliminate residual RNA.

To ensure successful and comparable extractions, commercially available extraction kits were utilized in this thesis. RNAs were extracted and purified using the Allprep DNA/RNA Mini Kit (Qiagen GmbH) or the RNeasy Mini Kit (Qiagen GmbH) according to the supplier's protocol as well as the appended protocol for purification of total RNA containing small RNAs. DNAs were extracted using the Allprep DNA/RNA Mini Kit (Qiagen GmbH) or the Invisorb® Genomic DNA Kit II (Stratec Molecular GmbH) adhering once again to the supplied protocols. Cells were harvested by either direct lysis using lysis buffer included in the extraction kits combined with 2-Mercaptoethanol or by trypsinizing to detach and then lysing the harvested cells subsequently. All kits utilized employ the silica spin-column absorption method.

Thus, samples containing RNA or gDNA from harvested cell cultures were obtained, with the exact amount of nucleic acid per sample to be determined in the next step.

2.2.2.2 Photometric Quantification of Nucleic Acids

Upon extraction, the amount and purity of the nucleic acids in each sample was evaluated via spectrophotometry.

Assessing nucleic acids by measuring absorbance at a wavelength of λ = 260 nm has proved to be easy to use and replicate without the need for additional markers such as ethidium bromide (Gallagher 2011). Detection range spans from around 1 µg/ml of nucleic acid to about 50 µg/ml for DNA and 40 µg/ml for RNA (Gallagher 2011).

The knowledge of the absorbance behavior of different substance groups allows for their differentiation. As summarized by the works of Sean Gallagher and Katrin Kaeppler-Hanno et al., pure nucleic acids show peak absorbance at λ = 260 nm, while proteins absorb at λ = 280 nm (Gallagher 2011; Kaeppler-Hanno et al. 2015). An increase of absorbance at λ = 280 nm in comparison to λ = 260 nm, resulting in a lower absorbance ratio A260/A280, indicates sample contamination with proteins (Gallagher 2011; Kaeppler-Hanno et al. 2015). Kaeppler-Hanno et al. recommend aiming at A260/A280 ratios of 1.8 to 2.0 for pure nucleic acids. Interference caused by floating particles in the sample or stains on the cuvettes can be evaluated at λ = 320-325 nm, while organic solvents, aromatic substrates as well as salts absorb at λ = 230 nm (Gallagher 2011; Kaeppler-Hanno et al. 2015). To rule out contamination with organic compounds, the absorbance ratio A260/A230 should be above 2.0 (Kaeppler-Hanno et al. 2015).

Besides the assessment of purity, absorbance measured at λ = 260 nm can be utilized to calculate nucleic acid concentrations with the following equations (Gallagher 2011):

Concentration of dsDNA [µg/ml] = absorbance at λ = 260 nm / 0.02

Concentration of ssRNA [µg/ml] = absorbance at λ = 260 nm / 0.025

Samples were analyzed in distilled water using UVette® cuvettes (Eppendorf AG) and the Eppendorf BioPhotometer® (Eppendorf AG). The calculations for nucleic acid concentrations were automatically performed by the spectrophotometer with preprogrammed equations for ssRNA or dsDNA respectively.

As customary, absorbance was measured at λ = 260 nm for nucleic acid concentration as well as at λ = 280 nm for possible protein residue, at λ = 230 nm for organic/aromatic contamination and at λ = 320 nm for interference caused by stains on cuvettes or large particles

in the solution. Absorbance ratios were determined to facilitate interpretation of purity. An exemplary reading obtained by this method is illustrated in table 5.

Table 5: Photometric Quantification of Hepa 1-6 Clone DNA Extracts

	Absorbance at Different Wavelengths				Absorbance Ratios		
Sam-ple	230 nm	260 nm	280 nm	320 nm	A260/A280	A260/A230	Concentra-tion [µg/µl]
Blank	0.000	0.000	0.000	0.000	-	-	0.000
7A6	0.019	0.037	0.020	0.001	1.82	1.94	0.074
7E6	0.015	0.018	0.009	0.001	1.91	1.19	0.035
9A4	0.021	0.040	0.021	0.001	1.90	1.87	0.080
9H2	0.018	0.041	0.022	0.001	1.81	2.22	0.081

2 µl samples of DNA extracted from Hepa 1-6 clone cell colonies were analyzed in 98 µl of distilled water. Blank value was set at the base absorbance of 100 µl distilled water. Absorbance values are given for λ = 230 nm, 260 nm, 280 nm, and 320 nm. Absorbance ratios are given for A260/A280 and A260/A230. Concentrations are given as µg/µl. Goal ratios are A260/A280 between 1.8 and 2.0 and A260/A230 below 2.0. As the A260/A230 ratio of sample 9H2 is above 2.0, this sample might be contaminated with organic or aromatic compounds, i.e. residues of extraction buffers, and thus the measurement and, if necessary, the DNA extraction should be repeated to ensure a pure sample and minimize interference in further analysis.

Results were then utilized to dilute samples to aliquots containing specific amounts of DNA or RNA per volume for further analysis, e.g. 4 ng/µl for gene expression assays or 20 ng/µl for agarose gel electrophoresis.

2.2.2.3 Integrity of Nucleic Acids: Agarose Gel Electrophoresis

To ascertain the successful extraction of intact nucleic acids and to rule out possible degradation, nucleic acids were assessed via agarose gel electrophoresis. In addition, products of gene assays were assessed for fragment size.

Gel electrophoresis as a method of separating proteins and nucleic acids according to size and charge has been long since established in a wide range of laboratory applications. The horizontal electrophoresis chamber system generally utilized was first established by Michael W. McDonell et al. in 1976. In it, negatively charged substances such as DNA and RNA migrate in an electric field towards the positive pole and when placed into a porous agarose gel move depending on their size as well as charge (McDonell et al. 1977; Voytas 2000). For nucleic acids, the phosphate in each basepair (bp) adds an equal negative charge to the structure, resulting in a fixed charge to mass ratio (Voytas 2000). Nucleic acids of the same length can therefore not differ in charge and if examined via gel electrophoresis will be found within close proximity on the gel, resulting in fragment separation according to length (McDonell et al. 1977; Southern 1979b; Southern 1979a). Agarose gel electrophoresis is thus a reliable tool in determining sizes of DNAs.

Fragment separation depends on fragment size as well as the gel's agarose concentration (Southern 1979a). To ensure linear resolution, concentrations should be adapted to the expected fragment length (Koontz 2013). For optimal separation, agarose concentrations ranging from 0.6% to 2% were utilized for this thesis, i.e. 0.6% gels for the assessment of RNA, 1.5% gels for estimated 800 bp amplicons, and 2% gels for assays with amplification products around 200 bp. The gels were prepared with TRIS-Borate-EDTA-buffer (TBE), which has been shown to improve resolution for nucleic acids smaller than 3,000 bp (Koontz 2013).

In order to visualize the result of gel electrophoresis, nuclear acids have to be stained or made susceptible to stimulation by for example ultraviolet (UV) light. Therefore nucleic acid intercalators such as ethidium bromide have been implemented into basic gel electrophoresis protocols (Voytas 2000; Koontz 2013). For safety reasons the mutagen and possible carcinogen ethidium bromide has today been largely replaced by other DNA stains. Midori Green Advance (Nippon Genetics Europe GmbH), which was utilized for this thesis is non-toxic, non-carcinogenic and less mutagenic than ethidium bromide while delivering similar results according to the provider's data sheet. Adding this nucleic acid stain allowed for visualizing DNA or RNA bands by UV light with wavelengths between 270 nm and 290 nm.

To evaluate DNA fragment size a 100 bp DNA ladder (Thermo Fisher Scientific Inc.) was added alongside the samples, marking specific nucleic acid lengths. In lack of an RNA ladder, the DNA ladder was also utilized as a relative standard to evaluate RNA integrity.

Applied voltage gradients ranged between 125 V and 200 V for 20 min to 40 min or until sufficient migration was indicated by a DNA gel loading dye (Thermo Fisher Scientific Inc.). Figure 4 provides a representative result of an agarose gel electrophoresis assessing the integrity of extracted RNA and gDNA. Pictures were taken using a UV lightbox with a camera and evaluated utilizing the Image Lab™ Software (Bio-Rad Laboratories, Inc).

As described in detail later on, agarose gel electrophoresis was also used to visualize the results of gene assays generating multiple gene sequence replicates via conventional polymerase chain reaction (PCR). This allowed for the discrimination between successful and unsuccessful amplification of target sequences, and consecutively drawing conclusions concerning the existence of certain gene sequences. Agarose gel electrophoresis therefore proved invaluable during the phase of gene knockout validation.

A detailed protocol of this thesis' standard gel electrophoresis based on protocols designed by Daniel Voytas (2000) and Laura Koontz (2003) is provided on the next page.

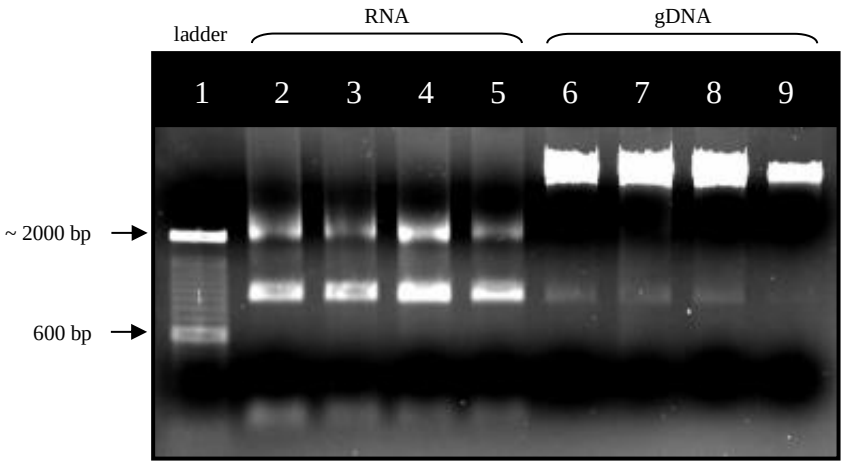


Figure 4: Agarose Gel Electrophoresis of RNA and gDNA from Hepa 1-6 Wildtype and Clones

Electrophoresis was performed on a 1% agarose gel including 4 µl Midori Green. Slot 1 contains a 100 bp DNA ladder, while slots 2 to 9 contain 10 µl equaling 200 ng of RNA or DNA with 2 µl DNA Gel Loading Dye each. 125 V was applied for 20 minutes. The gel was then photographed under a UV camera and evaluated via Image Lab™ Software. Samples in slots 2 to 5 show intact RNA. Slots 6 to 9 contain samples of intact gDNA.

Protocol: Agarose Gel Electrophoresis

Material:

Agarose Standard Roti®garose, CAS 9012-36-6 (Carl Roth GmbH + Co. KG)
TBE 5X, Tris/Borate/EDTA (in-house production of Universitaetsmedizin Goettingen)
DNA Gel Loading Dye (6X) (Thermo Scientific™ by Thermo Fisher Scientific Inc.)
100 bp DNA Ladder (Invitrogen™ by Thermo Fisher Scientific Inc.)
Midori Green Advance DNA Stain, CAS 7732-18-5 (Nippon Genetics Europe GmbH)
RNA or DNA samples (20 ng/µl)

Procedure:

Weigh the appropriate amount of agarose depending on gel size as well as desired concentration using a precision scale, i.e. 0.75 g of agarose for a 75 ml 1% gel, adapting concentrations to the expected fragment sizes.

Transfer agarose to an Erlenmeyer flask and carefully add the appropriate amount of TBE, i.e. 75 ml for a small gel. Swirl gently to mix. Heat the mixture in a microwave until boiling, take out and swirl. Repeat heating and swirling until homogenous. Once achieved, cool the gel mixture by gently swirling while holding the Erlenmeyer flask into cold water. Be careful not to cause air bubbles in the gel. Wearing gloves to avoid contact, add Midori Green, i.e. 4 µl to a small gel, and swirl to mix. Then fill the gel mixture into a gel tray, make sure there are no air bubbles and add a comb with the desired number of teeth to create slots. The gel then needs to cool out and harden for about 30 minutes.

When the gel has fully hardened, remove the comb and place the tray holding the gel into an electrophoresis chamber filled with TBE buffer. The first slot is generally loaded with a DNA ladder, i.e. a 100 bp DNA ladder, to mark sample sizes. In lack of an RNA ladder, the DNA ladder can also be utilized to estimate RNA sizes. Add samples to the remaining slots in volumes of 10 µl equaling to 200 ng of nucleic acid plus 2 µl of loading dye. Depending on the gel's size apply voltage gradients of 125 V to 200 V and assess gels after 20 min and 40 min via UV light.

2.2.3 Quantification of Gene Expression via real-time RT-PCR

To quantify the expression of selected IFNs and IFN effectors, samples extracted from Hepa 1-6 cells were analyzed via quantitative reverse transcriptase PCR (qRT-PCR).

The method of PCR has come a long way from its early beginnings when first patented by Kary B. Mullis in 1987 (Mullis 1987). His invention of this rapid, simple and reproducible *in vitro* DNA amplification technique was honored with the Nobel Prize for chemistry in 1993 and paved the way for a new era of molecular and genetic research (Mullis et al. 1987; Mullis and Faloona 1987; Mullis 1990; Nobel Media AB 2019).

As simple a technique as it is, the introduction of PCR was revolutionary. Mullis' discovery gave access to analyzing nucleic acids in an unprecedented dimension. Even minuscule amounts of DNA samples from a multitude of sources could now be specifically amplified in order for them to be detected and analyzed sufficiently (Erlich 1989; Mullis 1990; Canene-Adams 2013; Garibyan and Avashia 2013). This led PCR to a broad field of application and made numerous impactful works like the Human Genome Project possible (Erlich 1989; Garibyan and Avashia 2013).

Basic PCR is performed using a template DNA and a target-specific pair of oligonucleotides, so-called primers (Mullis 1987; Mullis et al. 1987). These primers mark the sequence to be amplified by binding to their complementary sites on the DNA and furthermore provide a starting point for the amplifying enzyme, a DNA polymerase (Mullis and Faloona 1987; Erlich 1989; Mullis 1990; Garibyan and Avashia 2013). The DNA polymerase then synthesizes DNA sequences complementary to the presented target sequence using deoxynucleoside triphosphates (dNTPs) of the four bases adenosine, cytidine, guanosine and thymidine (Erlich 1989; Garibyan and Avashia 2013). Target DNA, primers, dNTPs and DNA polymerase are mixed in one reaction tube with a reaction buffer including, inter alia, magnesium cations as an enzyme cofactor (Erlich 1989; Dorado et al. 2019b).

PCR is performed as a single-tube reaction in a thermal cycler with three specific phases (Erlich 1989; Garibyan and Avashia 2013; Dorado et al. 2019b):

1) Denaturation at 94 – 96 °C: separation of dsDNA to ssDNA
2) Annealing at 45 – 60 °C: binding of primers to target sequences
3) Extension at 70 – 72 °C: synthesis of target copies by the DNA polymerase

Heating above the dsDNA melting point leads to separation of the DNA strands, allowing for primers to hybridize to their specific targets during the next step, with annealing temperature depending on the composition of each primer pair (Erlich 1989; Garibyan and Avashia 2013). This is followed by raising the temperature once more to the DNA polymerase optimum to allow the enzyme to synthesize new DNA strands complementary to the sequence marked by primers (Erlich 1989; Garibyan and Avashia 2013). If the primer annealing temperature is high enough, the steps of annealing and extension may be combined to increase simplicity (Dorado et al. 2019b). If at maximum efficiency, each PCR cycle results in the duplication of the target sequence (Erlich 1989; Garibyan and Avashia 2013; Dorado et al. 2019b). Even as few as twenty cycles at 100% efficiency produce about a million copies of a single starting template by exponential accumulation, hence twenty to forty cycles usually yield enough copies fur further analysis (Erlich 1989; Dorado et al. 2019b).

In the early beginnings, thermolability of the utilized DNA polymerase posed a problem as the enzyme was destroyed during denaturation and therefore had to be added during every cycle, while lower extension temperatures also resulted in lower specificity due to increased mispriming (Erlich 1989; Dorado et al. 2019b). The discovery of thermostable DNA polymerases such as the *Taq* polymerase from the bacteria *Thermus aquaticus* enabled the automation of the PCR process in closed-tube systems at lower expenses, increased specificity, and better yields (Saiki et al. 1988; Erlich 1989; Dorado et al. 2019b).

The basic PCR technique has been frequently modified and a multitude of variations is routinely utilized, however, the conventional technique of PCR has not lost its application in the means of qualitative PCR.

Qualitative or conventional PCR is a fast, reproducible and reliable method to assess the presence or absence of a DNA sequence by the means of endpoint analysis (Garibyan and Avashia 2013). Conventional PCR is commonly combined with agarose gel electrophoresis, sequencing or blotting to analyze the resulting amplicons (Erlich 1989; Bustin 2000; Canene-Adams 2013; Garibyan and Avashia 2013). While conventional PCR cannot be used to quantify gene expression, its ability to amplify targeted gDNA sequences proved indispensable during the knockout validation process as described in detail in section 2.2.6.

If planning to not only detect the existence of certain sequences but to assess gene copy numbers or gene expression, which equals to messenger RNA (mRNA) production, endpoint analysis proved to be unsatisfactory (Dymond 2013). During amplification three plot phases

can be identified (Dymond 2013; Dorado et al. 2019b): In the early phase of amplification, the reaction is performed close to maximum efficiency, resulting in near duplication of the target sequence per cycle. The amplification plot is therefore exponential. With time, however, reaction substrates are decreasing and the enzyme reaches saturation. Additionally, increasing numbers of amplicons begin to inhibit the reaction. This causes the amplification to slow down, entering a linear phase. Once the substrates are consumed, no more amplicons are produced, equating to a plateau phase of the amplification plot. Progression through the phases is unique to each reaction, depending mainly on the starting concentration of the template (Dymond 2013). Once in the plateau phase, differences in starting concentration are however indistinguishable (Dymond 2013). Endpoint analysis, meaning evaluation of amplicons after the amplification series and thus in the plateau phase, is therefore not fit to compare different samples with regard to their quantities but can only provide information about presence or absence of sequences (Dymond 2013).

In order to assess cells' responses to stimuli with regard to gene expression via PCR, two problems had to be solved. There first of them is the fact that quantification of amplicons is only possible during the exponential phase of the amplification plot and therefore requires amplicon analysis simultaneously to the PCR reaction (Klein 2002; Dymond 2013). This led to the introduction of quantitative or real-time PCR (qPCR) and the integration of visualization methods into the PCR process.

To reliably monitor the accumulation of sequence copies, labeling techniques had to be developed. While there are different approaches to visualization, all methods rely on fluorescence, with fluorescent emission being proportional to the copy numbers present in the reaction (Gibson et al. 1996; Bustin 2000). Next to fluorescent intercalating dyes, molecular beacons and hybridization probe pairs, hydrolysis probes of the TaqMan™ type are frequently used, having been introduced through works by Christian A. Heid and Ursula E. M. Gibson in 1996 (Gibson et al. 1996; Heid et al. 1996; Bustin 2000). Besides enabling the quantification of gene expression, the fact that with TaqMan™ fluorogenic probes no post-PCR handling of samples is required and analysis can be performed in an automated closed-tube setting, allows for less contamination, less expenditure of time and higher performance (Gibson et al. 1996; Heid et al. 1996; Klein 2002; Dymond 2013).

TaqMan™ probes as designed by Christian Heid and Ursula Gibson in 1996 consist of the usual target-specific primer pair, but with an additional dual-labeled internal probe. This probe carries two dyes with overlapping emission spectra, a fluorescent reporter dye on its

5’ end and a quencher dye or black hole quencher on its 3’ end (Gibson et al. 1996; Heid et al. 1996; Dymond 2013). As long as the probe is intact and these two dyes are in close proximity, the fluorescent emission of the reporter dye is canceled out due to a phenomenon called fluorescence resonance energy transfer (Heid et al. 1996; Dymond 2013). If copies of the target sequence are present, the probe hybridizes to the target sequence during the annealing phase, resulting in a short double-strand region within the target sequence (Heid et al. 1996; Dymond 2013). During extension, the DNA polymerase starts copying the template beginning at the 3’-OH end of the normal primer. In addition to its polymerization activity, the *Taq* polymerase also acts as a double-strand-specific 5’-exonuclease to free the template strand during synthesis if necessary (Heid et al. 1996). Due to this, the polymerase nicks the probe upon contact, separating the two dyes. The reporter dye is no longer quenched and its fluorescent emission can be measured (Heid et al. 1996; Dymond 2013). The more amplicons are synthesized, the more fluorescence can be detected (Gibson et al. 1996; Klein 2002). If no amplification is performed, the probe stays intact and no signal is emitted (Bustin 2000; Dymond 2013). The TaqMan™ technique offers added specificity over simple DNA intercalators, as only target-specific synthesis is detected as opposed to detection of all double-strand regions including primer dimers or unspecific PCR products (Dymond 2013).

Fluorescent emission is measured in real-time via a sequence detector every 8.5 seconds (Gibson et al. 1996; Heid et al. 1996; Dymond 2013). To make this possible, the PCR machines expanded from being a simple thermocycler to containing an excitation source, a camera for detection as well as a computer with fitting software for data processing (Dymond 2013). To account for differences in reaction composition, the delta of emission intensity is calculated using normalization of reporter emission to quencher emission, as the quencher dye emission stays relatively constant (Gibson et al. 1996). The TaqMan™ assay thus comes with a built-in normalization method. During the early cycles fluorescence is below detection levels, providing a baseline (Gibson et al. 1996; Heid et al. 1996). Based on the medium baseline emission through cycles one to fifteen, an arbitrary threshold is established at ten standard deviations above the baseline (Heid et al. 1996; Dymond 2013). With rising copy numbers, more and more reporter dye is liberated. The amplification cycle at which the normalized fluorescent emission surpasses this threshold is called the threshold cycle or in short CT (Heid et al. 1996; Dymond 2013). The CT value correlates with the amount of starting template and is per definition set within the exponential phase of the amplification plot, thus enabling quantification (Heid et al. 1996; Dymond 2013). Visualization via TaqMan™

assays does not require any post-PCR handling whatsoever as it combines amplification, detection, and quantification into one reaction (Gibson et al. 1996; Heid et al. 1996).

The second problem to be solved was the fact that PCR can only be performed on DNA, as the polymerase is DNA-specific (Erlich 1989; Bustin 2000). Aiming at the assessment of gene expression, mRNA had to be made accessible to PCR. Thus, reverse transcription was used to synthesize cDNA from mRNA to provide a template to the DNA polymerase, leading to the development of reverse transcriptase PCR (RT-PCR) (Tan and Weis 1992; Bustin 2000). If combined with real-time analysis, this PCR is called real-time or quantitative reverse transcriptase PCR (qRT-PCR), which is up until today the best method for mRNA analysis with regard to sensitivity and flexibility (Bustin 2000; Dorado et al. 2019b).

After eliminating any gDNA residue, which could otherwise lead to confounding of the amplification, mRNA is reverse transcribed by a reverse transcriptase (Bustin 2000; Maddocks and Jenkins 2017). This retroviral enzyme, in detail an RNA-dependent DNA polymerase, produces DNA complementary to the template mRNA using random oligonucleotide primers and dNTPs (Bustin 2000; Maddocks and Jenkins 2017). On the one hand, this allows for the otherwise unstable mRNA to be stored indefinitely as cDNA, on the other hand this opened up the field of gene expression to analysis, enabling PCR to become one of the most substantial techniques in a multitude of experimental as well as clinical applications in fields such as microbiology, oncology and the diagnostics of genetic diseases (Bustin 2000; Garibyan and Avashia 2013).

With mRNA being accessible to PCR via cDNA synthesis and established visualization methods available, the last step in the preparation of qRT-PCR is the design of a primer pair.

The assessment of gene expression is a targeted method and therefore depends upon careful selection of primers for specific sequences to be investigated. Designing primers requires consideration of multiple factors such as primer length, nucleotide composition, amplicon size, position within the gene and relatively to exon-exon borders to only name a few (Dymond 2013; Maddocks and Jenkins 2017; Dorado et al. 2019b). Each primer pair comes with a different set of optimal conditions with regard to buffer magnesium concentration and annealing temperature (Erlich 1989; Dymond 2013; Dorado et al. 2019b). As primer design, evaluation and optimization can be a very laborious and time-consuming task, use of commercially available primers is oftentimes recommended (Dymond 2013; Maddocks and Jenkins 2017; Dorado et al. 2019b). This also allows for the easy incorporation of the TaqMan™

visualization method via a fitted internal primer. However, if investigating, for example, gene knockouts, a suitable and well-designed primer pair is essential and has to be specifically designed for the individual setting as described in section 2.2.6.

At the end of a qPCR or qRT-PCR reaction, the quantity of amplicons is represented by CT values, with samples including higher starting amounts of target molecules exceeding the threshold earlier than those with lower starting amounts. Further quantification may be performed as either absolute or relative quantification.

To specify the absolute copy number of a template and thus calculate starting template concentration, the amplification plot is compared to a standard curve, having been established using reference DNA of known quantities (Heid et al. 1996; Dymond 2013). Absolute quantification is an important analytical tool in settings that require exact information about sequence numbers, e.g. when investigating the viral load in an HIV-infected patient (Piatak et al. 1993; Heid et al. 1996).

In comparison, relative quantification as a description of fold-change in gene expression is usually sufficient when assessing the increase or decrease of gene expression under various stimuli (Dymond 2013). The ΔΔCT method of relative quantification is based on the detection of fluorescence emission resulting in CT values in a manner predictive of quantity (Gibson et al. 1996; Heid et al. 1996; Klein 2002). However, raw CT values are not fit for direct comparison. Smallest differences in the composition of the reaction or amount of sample added are magnified during the extremely sensitive qPCR reaction, confounding the resulting CT values (Heid et al. 1996; Dymond 2013). To allow comparison of different samples, these possible differences in reaction composition have to be accounted for. For this eventuality, a so-called housekeeping gene is assessed simultaneously to act as an internal control to normalize CT values (Heid et al. 1996; Klein 2002; Dymond 2013).

Representative normalization of CT values relies on careful selection of the internal control, i.e. the housekeeping gene (Klein 2002). To function as a reliable and accurate control, the housekeeping gene should show stable expression independent from stimuli and be expressed in all cells (Dymond 2013). In addition to allowing for normalization, the housekeeping gene can also be utilized as an internal control of the quality of nucleic acid quantification and subsequent dilution prior to the PCR. Stable housekeeping gene expression over all samples indicates high accuracy of nucleic acid extraction resulting in equal starting template amounts per sample (Heid et al. 1996). However, amplification does not only depend

on starting template concentration but is also influenced by factors such as primer efficiency, which is in turn determined by primer design. To ensure that CT differences are due to variation in starting template concentrations, amplification efficiencies need to be matched as closely as possible (Heid et al. 1996; Dymond 2013). Only then the normalization via a housekeeping gene is representative.

Normalization is performed versus the housekeeping gene, resulting in ΔCT values (Livak and Schmittgen 2001; Dymond 2013; Dorado et al. 2019b). Consecutively, ΔCT values are compared between investigated sample and control sample or knockout and reference strand, calculating for ΔΔCT values and fold induction, with the latter presenting fold change in gene expression in comparison to the untreated control or reference strand (Livak and Schmittgen 2001; Dymond 2013). As already mentioned, in order for this method to be precise, the target and housekeeping assay reaction efficiencies should be as similar as possible to minimize variation caused by differences in amplification efficiency (Livak and Schmittgen 2001; Dymond 2013).

Calculations for relative quantification were performed as follows (Livak and Schmittgen 2001; Dymond 2013; Dorado et al. 2019b):

ΔCT = CT target gene – CT housekeeping gene

$\Delta\Delta CT = \Delta CT$ treated sample – ΔCT control sample

Fold induction = $2^{-\Delta\Delta CT}$

Sole CT values should only be given in situations that do not allow for relative quantification, as raw non-normalized CT values can be confounded for various reasons and thus graphics derived of those CT values may be misleading (Livak and Schmittgen 2001). However, if assessing genes without basal expression in the control samples, such as IFNs, analyzing CT or ΔCT values is unavoidable, as ΔΔCT and fold induction cannot be calculated.

For this thesis, RNA was extracted and assessed for quantity and integrity as described in sections 2.2.2.1 to 2.2.2.3. Synthesis of cDNA was performed using the QuantiTect® Reverse Transcription Kit (Qiagen GmbH) with 120 pmol of p(dN)6 random primers (150 pmol/2 µl, Roche Diagnostics International AG) in a volume of 1.6 µl according to the supplier's protocol, resulting in cDNA samples each corresponding to 6.4 ng of RNA.

All primer pairs utilized were commercially available and validated TaqMan™ Gene Expression Assays supplied by Applied Biosystems® by Thermo Fisher Scientific Inc. The gene for hypoxanthine phosphoribosyltransferase (*Hprt*) was chosen as the housekeeping gene and investigated via HPRT assay (ID: Mm00446968_m1). As for IFNs, expression of *Ifnl2/3* and *Ifna4* was assessed via IFNL2/3 assay (ID: Mm04204155_gH) and IFNa4 assay (ID: Mm00833969_s1), respectively. ISGs as downstream effectors were represented by the gene for interferon-induced GTP-binding protein Mx1 (*Mx1*) and the gene for C-X-C motif chemokine 10 (*Cxcl10* or *Ip10*) and evaluated using an Mx1 assay (ID: Mm00487796_m1) and a CXCL10/IP10 assay (ID: Mm00445235_m1).

QRT-PCR was performed utilizing the referenced TaqMan™ assays with the TaqMan Universal Master Mix (Thermo Fisher Scientific Inc.) and carried out in 10 µl reactions in a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific Inc.). Gene expression was given as CT values by StepOne™ Software v2.3 and calculated for ΔCT and ΔΔCT values by DataAssist™ Software v3.01, both supplied by Thermo Fisher Scientific Inc. Fold induction was calculated as elaborated above. All samples were tested in duplicates at first, followed by triple testing in case of inconclusive results or high variation. Controls were included either as untreated samples or Hepa 1-6 wt depending on the experiment.

2.2.4 Stimulation of Cell Cultures

This thesis aims at assessing Hepa 1-6 cells' response to drug exposure with regard to IFN expression as well as viability. Expression of IFNs and ISGs as downstream effectors requires stimulation of the cell culture, which was conducted via various protocols.

Cell cultures were subjected to either high molecular weight complexes of polyriboinosinic acid and polyribocytidylic acid (poly(I:C)) (2 mg/ml, InvivoGen) alone or a 1:1 mixture of poly(I:C) and diethylaminoethyl (DEAE)-dextran (20 mg/ml, CAS 9064-91-9, Sigma-Aldrich Chemie GmbH) for 1 h or 16 h at 36 °C, respectively. Acting as a dsRNA analog, the synthetic double-stranded polynucleotide poly(I:C) has been demonstrated to induce IFN production, especially in its high molecular weight variant (Field et al. 1967; Dianzani et al. 1968; Zhou et al. 2013). The addition of DEAE-dextran to transfect the poly(I:C) into the cell has been shown to significantly increase IFN levels (Dianzani et al. 1968).

In addition, cell stimulation was also performed by combining poly(I:C) priming with consecutive protein and RNA synthesis inhibition, adhering to an established superinduction protocol for type I IFNs (Havell and Vilček 1972).

Following this protocol introduced by Edward Havell and Jan Vilček in 1972, the cells were exposed to poly(I:C) or poly(I:C) combined with DEAE-dextran for one hour, after which the culture medium was exchanged and 50 µg/ml of cycloheximide (CHX) (CAS 66-81-9, Sigma-Aldrich Chemie GmbH) was added for five hours. During the last 30 minutes, 1 µg/ml of actinomycin D (AcD) (CAS 50-76-0, Sigma-Aldrich Chemie GmbH) was added to the cell culture as well. Afterwards, cells were immediately harvested and gene expression was assessed.

By the means of consecutive exposure to the two antibiotics CHX, inhibiting the protein synthesis, and AcD, which effectively subdues RNA production, substantially higher IFN yields can be achieved compared to stimulation with poly(I:C) or poly(I:C) and DEAE-dextran (Reich et al. 1961; Ennis and Lubin 1964; Havell and Vilček 1972). CHX was found to cause an increase in IFN expression while AcD delayed the usually rapid decline in production (Havell and Vilček 1972). Thus, superinduction results in increased and prolonged IFN production (Havell and Vilček 1972).

To assess the effect of different drugs on the activation of IFNs in Hepa 1-6 wt and clones, the stimulation protocols were combined with exposure to gemcitabine (CAS 122111-03-9),

doxorubicin (CAS 25316-40-9) or oxaliplatin (CAS 61825-94-3), all supplied by Sigma-Aldrich Chemie GmbH. For cell culture stimulation, each agent was added to the cell medium in a concentration of 10 µM. As for chemosensitivity assays, concentrations utilized ranged between 0,8 µM and 50 µM for gemcitabine and oxaliplatin, while doxorubicin was tested in a range of 0,3 µg/ml to 20 µg/ml. Further details on the chemotherapeutic substances chosen can be found in section 1.5.

To investigate possible mediators of stimulation in between cell cultures, co-culturing experiments were performed with the addition of ribonuclease A (CAS 9001-99-4, EC 3.1.27.5, Sigma-Aldrich Chemie GmbH), Benzonase® nuclease (CAS 9025-65-4, Sigma-Aldrich Chemie GmbH) or DNase I (EC 3.1.21.1, Thermo Fisher Scientific Inc.). This allowed for the detection of nucleic acid mediators as well as the distinction between mediators of RNA and DNA origin. The concentrations utilized were 30 U/ml of ribonuclease A (RNase A), 60 U/ml of Benzonase® nuclease and 60 U/ml of DNase I as recommended by the suppliers.

2.2.5 CRISPR/Cas9 Technology: IL-28 Double Nickase Transfection

The CRISPR/Cas9 technology utilized in this thesis in order to generate *Ifnl2/3*-deficient Hepa 1-6 clones is categorized as a class 1 type II-A system and was originally derived from *Streptococcus pyogenes* (Charpentier et al. 2019). Type II systems are the rarest of the CRISPR/Cas systems and can only be found in bacteria, albeit at the low rate of 5% (Chylinski et al. 2014). CRISPR/Cas9 technology has been frequently modified to not only perform gene knockouts but to enable for example the modification or insertion of genetic information as well as gene regulation or flagging certain sequences without altering them (Charpentier and Doudna 2013; Mali et al. 2013a; Doudna and Charpentier 2014).

Some of the most frequent problems encountered when working with CRISPR/Cas9 technology are varying efficiency and unexpected off-target effects, which limit the specificity (Doudna and Charpentier 2014). For reliable gene editing and especially when aiming at *ex vivo* and *in vivo* gene therapy, however, obtaining a high specificity must be of top priority.

Target binding for Cas9-dependent gene alteration relies on the 20 nucleotides of the sgRNA complementary to the targeted sequence (Jinek et al. 2012; Cong et al. 2013; Mali et al. 2013b). Nevertheless, Cas9 tolerates a variable number of mismatches within the base-paired sequence, especially in the PAM-distal region (Cong et al. 2013; Jiang et al. 2013; Mali et al. 2013a; Kuscu et al. 2014). Thus, mutagenesis can be caused not only on-target but also off-target due to non-specific sgRNA binding and subsequent cleavage by the sgRNA-Cas9 complex (Cho et al. 2013; Cradick et al. 2013; Mali et al. 2013a). The first to describe off-target effects caused by CRISPR/Cas9 were the three research groups around Patrick Hsu, Vikram Pattanayak and Yangang Fu in 2013. Their investigations showed that the frequency of off-target effects depends not only on mismatches but also on the concentration of sgRNA and Cas9, nonetheless the sequence of the sgRNA was confirmed to be the major factor (Fu et al. 2013; Hsu et al. 2013; Pattanayak et al. 2013). In 2014 Wu et al. described that in their experimental setting 70% of off-target sites were associated with genes, demonstrating the impact off-target mutations could potentially have and highlighting the need for specificity-increasing alterations to the CRISPR/Cas9 genetic engineering technology (Wu et al. 2014).

Different approaches to optimize the CRISPR/Cas9 specificity were tested, such as choosing sgRNAs with sequences as original as possible, titrating sgRNAs and Cas9 to optimal concentrations or increasing the sgRNA length, but all were limited in their success (Cho et al. 2013; Hsu et al. 2013; Ran et al. 2013a). Choosing original sgRNA sequences greatly

complicates targeting various genes, and optimal concentrations could not reduce off-target activity to a satisfying level (Hsu et al. 2013; Pattanayak et al. 2013). The introduction of longer sgRNAs, presumably increasing sequence binding specificity, was shown to result in the sgRNAs being processed to the usual 20 nucleotide length and thus having no effect on the specificity whatsoever (Ran et al. 2013a).

The most promising development with regard to specificity proved to be a mutated D10A Cas9 designed by Cong et al., who substituted an aspartate to alanine within the RuvC-like domain to silence it (Cong et al. 2013). Thus, this Cas9 mutant exhibited only one nuclease domain, the HNH domain, and could only nick instead of cleave the dsDNA to cause single-strand breaks. Cong et al. therefore termed this protein Cas9 nickase or Cas9n. On the basis of the Cas9n design and the proposition by Prashant Mali et al. to reduce off-target effects by the use of cooperative nicking, Fei Ann Ran et al. developed a Cas9n double-nicking technique using a pair of Cas9n and two sgRNAs targeting sequences opposite and with up to 20 bp distance of each other (Mali et al. 2013a; Ran et al. 2013a; Ran et al. 2013b). This alteration to the CRISPR/Cas9 technology has been shown to result in 50- to 1,000-fold fewer off-target effects for Cas9n double-nicking while maintaining similar on-target efficiency as the wildtype Cas9 (Ran et al. 2013a; Cho et al. 2014).

In D10A Cas9n double-nicking each sgRNA-Cas9n complex nicks the strand complementary to its sgRNA, resulting in two single-strand breaks within a small offset and thus mimicking a double-strand break (Cong et al. 2013; Ran et al. 2013b; Ran et al. 2013a; Cho et al. 2014). As per usual, the double-strand break is primarily repaired by the error-prone NHEJ, causing the desired mutations in a fashion identical to the wildtype CRISPR/Cas9 system (Ran et al. 2013b; Ran et al. 2013a; Cho et al. 2014). On-target efficiency of the two techniques is therefore comparable, while on-target specificity is considerably higher in Cas9n double-nicking due to the doubled amount of correct base-pairing needed to result in a double-strand break (Ran et al. 2013a; Cho et al. 2014). Off-target cleavage is however greatly decreased by the utilization of the two sgRNAs, as the chances of both sgRNAs binding similar off-target sequences are quite low or close to non-existing in a proper sgRNA design (Ran et al. 2013a). As long as only one sgRNA binds to an off-target sequence merely a single-strand break is caused, which is repaired via the high-fidelity base excision repair mechanism and does not result in unwanted genetic editing (Caldecott 2001; Dianov and Hübscher 2013). To minimize off-target mutagenesis, a CRISPR/Cas9 double-nicking approach with a D10A Cas9 nickase was chosen to perform the intended *Ifnl2/3* knockout.

In order to design a set of sgRNAs for CRISPR/Cas9 double-nicking, detailed knowledge of the targeted gene locus is imperative. The *IFNL* gene locus in man is composed of four genes, *IFNL1* to *IFNL4* (Kotenko et al. 2003; Sheppard et al. 2003; Prokunina-Olsson et al. 2013). The murine genome, however, harbors only *Ifnl2* and *Ifnl3* on the seventh chromosome (Lasfar et al. 2006; Lasfar et al. 2011). Their gene sequence is nearly identical, suggesting them being paralogous genes as a result of gene duplication (Lasfar et al. 2006; NCBI). Therefore, in order to create a truly *Ifnl*-deficient clone, it was necessary to knock out both genes, preferably during one transfection. This required a set of sgRNAs with the ability to mark both genes for editing.

Next to detailed knowledge about the targeted genes and their sequences, designing fitting sgRNAs requires careful consideration of multiple factors (Cradick et al. 2013; Cho et al. 2014). Similar to the principles established for primer design, the sequence chosen should be as unique as possible, set within in an exon and ideally not in regions with single nucleotide polymorphisms to increase sgRNA specificity (Cho et al. 2014). A 20 nucleotide long sequence complementary to a segment of the gene locus is required to determine the target (Jinek et al. 2012; Cong et al. 2013; Mali et al. 2013b). Additionally, the targeted sequence has to be in the direct neighborhood of a PAM determined by the employed Cas9 and similar sequences should be found on as few off-targets as possible (Jinek et al. 2012; Mali et al. 2013b; Mali et al. 2013a; Cho et al. 2014). In case of Cas9n double nicking, the two sgRNAs need to bind target sequences with an offset of 0 to 20 bp and orientated in a matter resulting in 5’ overhangs (Ran et al. 2013a).

The Hepa 1-6 *Ifnl2/3* knockout in this thesis was performed with an *Ifnl2/3* double nickase plasmid pair assay (sc-437298-NIC-2) developed on demand by Santa Cruz Biotechnology, Inc., adhering to the manufacturer’s provided transfection protocol and recommendations.

In brief, cells were seeded at a density of 1.5 x 10^5 cells/3 ml in 6-well plates before being transfected with 1.5 µg of the plasmid pair on the following day for a total of 48 hours. The plasmids each encoded the D10A mutated Cas9 nuclease as well as sgRNAs with a length of 20 nucleotides, offset by approximately 20 bp and targeting both exons 1 of *Ifnl2* and *Ifnl3*. Additionally, one plasmid of the pair contained the gene for green fluorescence protein (GFP). The transient expression of the GRP gene allowed for confirmation of successful transfection via fluorescence as depicted in figure 5. The other plasmid of the pair carried a puromycin resistance gene, which enabled selection of successfully transfected cells by exposing them to culturing medium containing puromycin. A concentration of 4 µg/ml of

puromycin had been determined to kill 100% of Hepa 1-6 wildtype cells within 48 hours to 72 hours previous to the transfection.

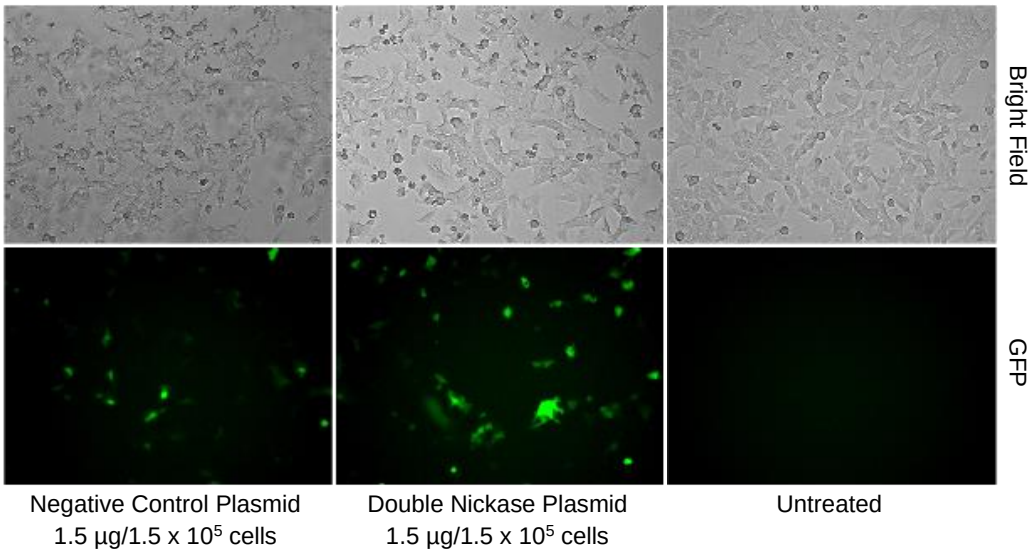


Figure 5: Hepa 1-6 GFP Expression 48 h Post-transfection

Success of the transfection was assessed via fluorescence after 48 h. Pictures in bright field exposure versus fluorescent light are depicted for cell cultures treated with the negative control plasmid, the double nickase plasmid or no treatment, respectively. Plasmids were applied in concentrations of 1.5 µg/1.5 x 10^5 cells. Successful transfection was confirmed by the expression of GFP, visible under fluorescent light, while non-transfected or untreated cells showed no fluorescence.

Transfection was visually confirmed after 48 hours. Afterward, cells were subjected to puromycin selection for another 48 hours before being serially diluted to a final density of 0.5 cells/100 µl and seeded into 96-well-plates to obtain single-cell colonies of transfected clones. Single clones were then expanded for stock generation and gDNA extraction while keeping replica plates to maintain single clones in culture. Cell culturing conditions remained similar to the Hepa 1-6 wildtype conditions described in section 2.1.1.

Thus, isogenic Hepa 1-6 clones were generated via CRISPR/Cas9 genetic engineering and could subsequently be assessed to validate a possible *Ifnl2/3* deficiency.

2.2.6 Knockout Validation

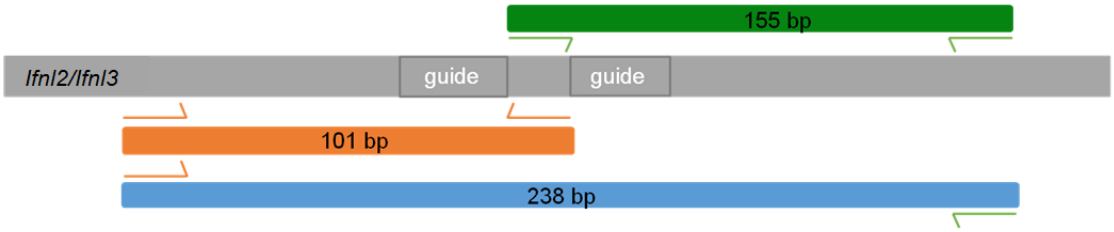
While the GFP expression allowed for confirmation of a successful transfection of the plasmid into the cell and successfully transfected cells could be selected by applying puromycin, the CRISPR/Cas9 technology does not include mechanisms to confirm the actual knockout of the targeted gene. Validation of the biallelic knockout in the selected cell clones was therefore performed subsequently on gDNA level via conventional PCR and Sanger sequencing as well as on mRNA level per cDNA via conventional PCR.

Designing a reliable knockout validation proved more challenging than anticipated. Due to the nature of its mechanism, CRISPR/Cas9 genetic engineering results in random mutations, affecting each allele of the targeted gene separately. As the *Ifnl* knockout performed included modifying two paralogous genes, this resulted in four alleles to be assessed for a successful knockout. Only Hepa 1-6 clones with effective mutations in all four alleles, thus with biallelic knockouts for both *Ifnl2* and *Ifnl3*, could be expected to lack expression of any *Ifnl* whatsoever.

As the mutations caused by the double-strand break within the target sequence are random, designing a primer to directly prove the knockout was impossible. Therefore, rather than verifying the presence of a mutated sequence, the absence of the wildtype sequence was assessed via conventional PCR with two independent gene assays, one primer each set directly within the targeted region. The assays were designed to amplify only wildtype sequences, thus the absence of an amplicon due to inability of primers to bind could be interpreted as absence of wildtype, indirectly confirming an alteration of the wildtype sequence.

In order to design the knockout validation assay, the position of each sgRNA supplied by Santa Cruz Biotechnology Inc. was identified on the exons 1 of *Ifnl2/3* to design primers that could bind the region between the two guide sequences in a wildtype Hepa 1-6. Around the targeted locus, two assays were designed as depicted in figure 6 and utilizing the Genome Data Viewer provided by the NCBI, U.S. National Library of Medicine. The first assay, referred to as IFNL2-3 ko, yielded a 101 bp amplicon in Hepa 1-6 wt, while the second assay, IFNL2-3 ko 2, resulted in a 155 bp amplicon in Hepa 1-6 wt. To provide a positive control, the outer primers of each assay were combined to provide a third assay flanking the target sequence and yielding a 238 bp amplicon in Hepa 1-6 wt, confirming the existence of the overall sequence targeted. Hepa 1-6 *Ifnl2/3* knockout clones were expected to lack both the 101 bp and the 155 bp amplicon while yielding an amplicon resulting from the combined

assay which was expected to differ in size, due to indel mutations within the sequence (figure 6).



	101 bp	155 bp	238 bp	Interpretation
Hepa 1-6 wt	+	+	+	wildtype
Hepa 1-6 knockout clone	-	-	different size	no wildtype

Figure 6: *Ifnl2/3* Knockout Validation Assay Design

Two *Ifnl2/3* knockout validation PCR assays were designed to amplify the respective locus in Hepa 1-6 wt cells, the first yielding a 101 bp amplicon in Hepa 1-6 wt (orange), the second resulting in a 155 bp amplicon in Hepa 1-6 wt (green). As a positive control the outer primers of each assay were combined into a third assay spanning the target sequence and yielding a 238 bp amplicon in Hepa 1-6 wt. While amplification of Hepa 1-6 wt gDNA resulted in three amplicons of appropriate sizes, Hepa 1-6 *Ifnl2/3* knockout clones were expected to lack both the 101 bp and the 155 bp amplicon while the amplicon resulting from the span assay was expected to differ in size, due to indel mutations within the sequence. Thus, each Hepa 1-6 clone was characterized according to presence or absence of the wildtype Hepa 1-6 *Ifnl2/3* genotype, with the latter corresponding to a presumably successful Hepa 1-6 *Ifnl2/3* knockout.

As already described in section 2.2.3, successful and reliable amplification via PCR relies on careful primer design. To ensure primer quality, primer pairs should exhibit high levels of specificity with minuscule tendencies for primer dimerization to avoid confounding (Álvarez-Fernández 2013; Dymond 2013). Primers aiming at assessing mRNA expression are best to be set on an exon-exon border to avoid amplifying residual gDNA in the sample, while primers set within an intron can only target gDNA (Dymond 2013). Additional factors in primer design are annealing temperature, primer length as well as nucleotide composition

and amplicon size (Álvarez-Fernández 2013; Dymond 2013; Maddocks and Jenkins 2017; Dorado et al. 2019b). To ensure high quality of the primers as well as maximum specificity, primers were designed with the help of the Primer-BLAST® software provided by the NCBI, U.S. National Library of Medicine.

The gene assays were performed with custom-made Invitrogen™ DNA oligonucleotide primer pairs supplied by Thermo Fisher Scientific Inc. The respective primer sequences were chosen as follows:

IFNL23 ko for 5'-AAGAACCCAAGCTGACCCTG-3'

IFNL23 ko rev 5'-GCAGCTCTTTTGGGGACAGA-3'

IFNL23 ko2 for 5'-TGTCCCCAAAAGAGCTGC-3'

IFNL23 ko2 rev 5'-TGCACAAAGTGTGGAGACCA-3'

To allow for gene-specific sequencing later on, another two assays were designed to specifically amplify a larger region of exons 1 of *Ifnl2* or *Ifnl3* on the basis of sequences upstream of the genes. These assays were referred to as IFNL2 span ex1 and IFNL3 span ex1 and yielded 810 bp and 795 bp products, respectively. Also supplied by Thermo Fisher Scientific Inc., forward and reverse Invitrogen™ DNA oligonucleotide primer sequences targeting each gene individually were chosen as follows:

IFNL2 span ex1 for 5'-CTGCCACAAAACCGAACCAAAG-3'

IFNL2 span ex1 rev 5'-TCCCAGTTAGCATAAGGGATGA-3'

IFNL3 span ex1 for 5'-AAGTCAGCCCACTGCACAAA-3'

IFNL3 span ex1 rev 5'-CCAAGCTTCTTGTGGGTAGC-3'

In addition to confirming the knockout via absence of wildtype sequences on gDNA level and due to the location of its complementary sequences within the exonic gene region, the primer pair of the IFNL23 ko assay could be utilized to amplify cDNA to assess the absence of *Ifnl2/3* mRNA, providing further independent evidence. As the outer primer of the IFNL23 ko2 assay could not be placed within an exon due to the location of the guide sequence in relation to exon-intron boundaries, this approach could not be transferred to the second primer pair.

Nucleic acid extraction and preparation for selected Hepa 1-6 clones as well as Hepa 1-6 wildtype for reference was performed as described in section 2.2.2.1 to 2.2.2.3. If investigating mRNA, cell colonies were subjected to stimulation prior to mRNA extraction and cDNA synthesis was performed as described in section 2.2.3 in order to provide a template. Subsequently, gDNA or cDNA templates were assessed via conventional PCR utilizing the five assays with the respective primer pairs as described above. A detailed protocol for the conventional PCR as performed in this thesis is provided at the end of this section.

Following amplification, samples were then assessed via agarose gel electrophoresis as described in chapter 2.2.2.3 on 2% agarose gels for the assays yielding 101 bp and 238 bp amplicons and on 1.5% agarose gels for the amplicons ranged around 800 bp. Amplicons of Hepa 1-6 clones were compared to those of Hepa 1-6 wildtype with regard to presence and size to identify clones lacking wildtype *Ifnl2/3* gene sequences.

In order to investigate the exact alterations caused within the *Ifnl2* and *Ifnl3* gene sequence of each non-wildtype clone, the amplicons resulting from the combined assay as well as the gene-specific span assays were isolated via agarose gel electrophoresis and extracted utilizing the NucleoSpin® Gel and PCR Clean-up Kit supplied by Macherey-Nagel GmbH Co. KG. Having confirmed successful extraction of nucleic acids via photometry, Sanger sequencing was performed by Microsynth Seqlab, Goettingen, Germany. Results were interpreted utilizing Chromas 2.6.2 DNA Sequencing Software (Technelysium Pty Ltd), while comparisons between investigated sequences and wildtype sequences were performed with the help of the BLAST® Align Sequences Nucleotide software provided by the NCBI, U.S. National Library of Medicine.

In 1977 Sanger et al. introduced Sanger sequencing as one of the main techniques utilized in first-generation sequencing (Sanger et al. 1977). In brief, the DNA sequence to be investigated is amplified by a variation of PCR using a mix of deoxynucleotides and fluorescence-marked dideoxynucleotides (Dorado et al. 2019a). Built-in dideoxynucleotides terminate further polymerization, while deoxynucleotides extend amplicons as usual (Sanger et al. 1977; Dorado et al. 2019a). This results in a mixture of ssDNA amplicons of different sizes, every single one with a dideoxynucleotide in the terminal position according to the template sequence (Sanger et al. 1977; Dorado et al. 2019a). Amplicons are then separated by size via electrophoresis and the terminal dideoxynucleotide is identified via fluorescence (Dorado et al. 2019a). Thus, the base in every nucleotide position can be identified by the means of dideoxy-termination and represented in a virtual electrofluorogram in which each color

represents a certain base and the color sequence allows for identification of the DNA sequence (Dorado et al. 2019a).

As described above, even if separating the two paralogous genes by gene-specific PCR assay, the resulting amplicons are still a mix of two alleles. As each allele is subject to CRISPR/Cas9 genetic alteration separately, chances are high that two different, hence heterozygous, mutations are caused. Sequencing, however, can only result in a clear signal if only a single sequence is present, as two templates due to heterozygous mutations cause sections with double signals. It was therefore expected that sequencing would show mixed signals and thus not be successful for the majority of the amplicons tested. However, if homozygous mutations were present on the two alleles, Sanger sequencing provided valuable insight into the nature of the mutations caused as well as possible offset mutations within the range of the span assay. In order to specifically investigate heterozygous mutations, it would have been necessary to clone the alleles onto plasmids and then analyze each allele in sequencing (Li K et al. 2014). Due to scheduling, this was not performed in this thesis.

Protocol: Sequence-specific Amplification via Conventional PCR

Material:

TaqMan® Universal PCR Master Mix, No AmpErase® UNG

GeneAmp® dNTP Mix with dTTP (2,5mM)

Taq-Polymerase Mix: 1 part *Taq* DNA Polymerase Recombinant, 4 parts distilled water

Custom DNA Oligo Primers forward and reverse

DNA sample (10 ng/2,5 µl)

Eppendorf Tubes® Safe-Lock

FlexCycler

Procedure:

Choose primers according to the gene sequence to be amplified. Preheat Flexcycler. In small Eppendorf tubes, mix the following substances:

2.5 µl	DNA sample (= 10 ng)
+ 7.75 µl	Distilled water
+ 3.25 µl	TaqMan® Universal PCR Master Mix
+ 5 µl	Primer forward (3 µM)
+ 5 µl	Primer reverse (3 µM)
+ 0.5 µl	dNTPs (10 mM)

Store on ice at all times. When the FlexCycler is ready, add the *Taq*-Polymerase:

+ 1 µl *Taq*-Polymerase Mix = ∑ 25 µl per sample in small PCR tube

Immediately transfer to preheated FlexCycler and run the following program:

1) 94°C 5 min
2) 94°C 1 min
3) 60°C 50 sec
4) 72°C 50 sec
5) 72°C 9 min

Repeat steps 2 to 4 for 40 cycles

6) 4°C hold

Continue cooling the samples. Amplicons may now be evaluated via agarose gel electrophoresis or sequencing.

2.2.7 Data Evaluation and Statistics

Gene expressions obtained in qRT-PCR experiments were given as CT values by StepOne™ Software and evaluated for ΔCT and ΔΔCT values by DataAssist™ Software, both supplied by Thermo Fisher Scientific Incorporation. To allow for comparison, gene expressions as percentages of the respective control were assessed graphically for normal distribution using a quantile-quantile plot with the software STATISTICA 13 by StatSoft GmbH. Significance was determined by one-way ANOVA with Bonferroni correction. The adjusted level of significance was set at $p < 0.017$.

With regard to viability and proliferation assessment via MTS reduction assay, mean metabolic activities of quadruplicate sets of microcultures were referenced to the respective untreated control. Standard deviations, 95%-confidence-intervals, and IC_{50} values were analyzed in a variable slope four-parameter dose-response curve by using GraphPad Prism 7 by GraphPad Software Inc.

Assistance in statistical analyses was provided by the consulting services of the Department of Medical Statistics, University Medical Center Goettingen.

3. Results

3.1 Expression of Type I and Type III Interferons in Hepa 1-6

3.1.1 Inducibility of IFN-α, IFN-λ and ISG Expression by Poly(I:C):DEAE-dextran

In order to investigate the significance of endogenous type III IFN expression in the context of chemotherapy and the process of immunoediting in Hepa 1-6, the cell line's overall ability to express both type I and type III IFNs was initially investigated.

As depicted in figure 7, the murine hepatoma cell line Hepa 1-6 was shown to react to stimulation with IFN-α and IFN-λ gene expression, represented by the genes *Ifna4* and *Ifnl2/3*. Various stimulatory regimes were utilized as described in detail in section 2.2.4, relying mainly on the RNA analog and pattern recognition receptor agonist poly(I:C). While sole exposure to poly(I:C) did not reliably result in measurable IFN induction, the combination with the transfection agent DEAE-dextran led to reproducible IFN-α and IFN-λ gene expression depending on length of exposure and harvest time.

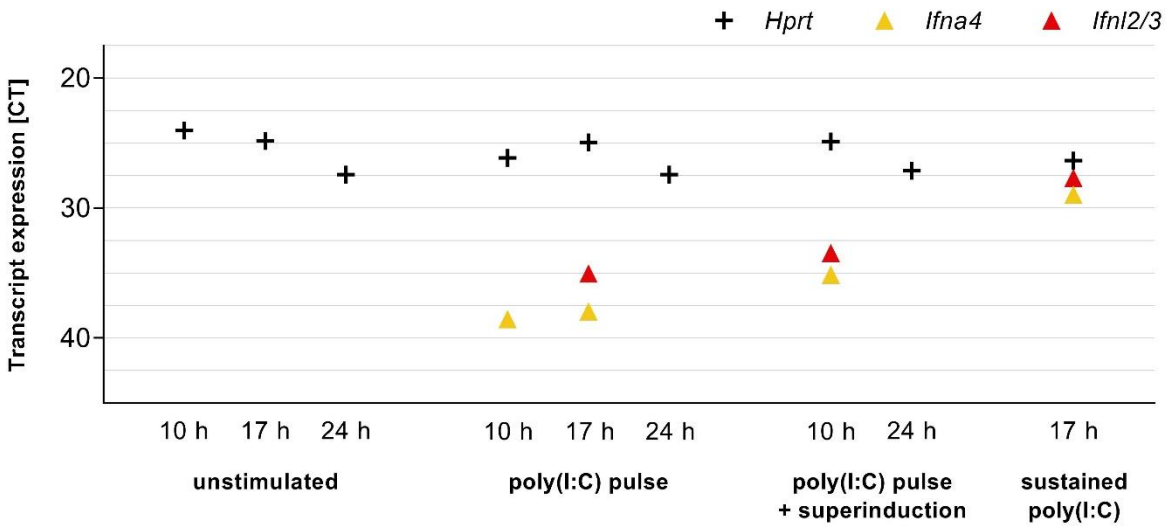


Figure 7: Poly(I:C)-based Stimulation of Type I and Type III IFN Expression in Hepa 1-6

$3x10^5$ Hepa 1-6 cells were seeded in 6-well plates. On the following day, cells were treated with 10:100 µg/ml poly(I:C):DEAE-dextran for either a 1 h pulse, a 1 h pulse in combination with the superinduction protocol, or 16 h of sustained exposure. Cultures were harvested at the indicated time points and analyzed for gene expression. Data on *Hprt*, *Ifna4* and *Ifnl2/3* are given as mean values of up to six independent data sets and depicted as raw CT values to illustrate the absence or presence of transcription as unbiased as possible.

Investigating *Ifna4* as a surrogate for type I IFNs, a transient expression could be detected after a one-hour 10:100 µg/ml poly(I:C):DEAE-dextran pulse and cell harvest at t = 10 h or t = 17 h compared to non-stimulated controls (figure 7). *Ifna4* expression levels at t = 10 h could be slightly increased by combining pulse treatment with the superinduction regimen consisting of timed inhibition of protein and RNA synthesis by exposure to CHX and AcD as specified in 2.2.4 (figure 7). However, *Ifna4* expression was shown to be of short duration, as the transcription activity was below detection limits at t = 24 h (figure 7).

Both the poly(I:C):DEAE-dextran pulse treatment as well as the poly(I:C):DEAE-dextran plus superinduction regimen were found to be sufficient to additionally induce *Ifnl2/3* expression, however with a slightly different kinetic as for *Ifna4*. The one-hour pulse exposure resulted in measurable *Ifnl2/3* transcription levels at t = 17 h, while levels at t = 10 h remained subliminal (figure 7). The combination with the superinduction protocol increased *Ifnl2/3* expression sufficiently for it to be detected at t = 10 h (figure 7). Nonetheless, *Ifnl2/3* expression was back below detection limits at t = 24 h in concordance with the *Ifna4* expression pattern (figure 7). While the comparison of harvest times at t = 10, 17 and 24 h was not sufficient to establish a detailed account of IFN-α and IFN-λ gene expression kinetics, it can, however, be assumed that there is a relevant time dynamic and that the peak of *Ifna4* expression upon stimulation precedes that of *Ifnl2/3*.

The highest numbers of type I and type III IFN transcripts were yielded after 16 hours of sustained exposure to poly(I:C):DEAE-dextran, exceeding the numbers obtained by the superinduction regimen by more than 50-fold on average (figure 7). However, cells exposed to this regimen showed distinctive signs of distress when assessed via microscopy due to the increased toxicity of long-time DEAE-dextran exposure.

Having established the ability of Hepa 1-6 to express type I as well as type III IFNs, the effect of stimulation and IFN gene transcription on the expression of ISGs as downstream effectors was investigated subsequently. As the ISGs are a large and heterogeneous group, the two genes *Mx1* and *Cxcl10*, also referred to as *Ip10*, were chosen as representatives.

As depicted in figure 8, cell cultures were subjected to the stimulation regimes that had proven to induce IFN-α and IFN-λ gene expression and were harvested at either t = 10 h or t = 17 h, the harvest times at which IFNs were detectable simultaneously. *Mx1* expression levels reacted only minimally to stimulation with a maximum induction of 6.23-fold in cultures exposed to sustained poly(I:C):DEAE-dextran in comparison to the untreated control.

In contrast and as expected with a downstream effector, *Cxcl10* transcription levels were found to correlate to the induction of IFN-α and IFN-λ. At t = 10 h, the chemokine *Cxcl10* was induced 10.41-fold by a poly(I:C):DEAE-dextran pulse and 17.75-fold by the pulse treatment with superinduction. At t = 17 h, *Cxcl10* activation was found to be increased 3.46-fold by pulse treatment, while sustained stimulation resulted in a maximum induction of 92.41-fold.

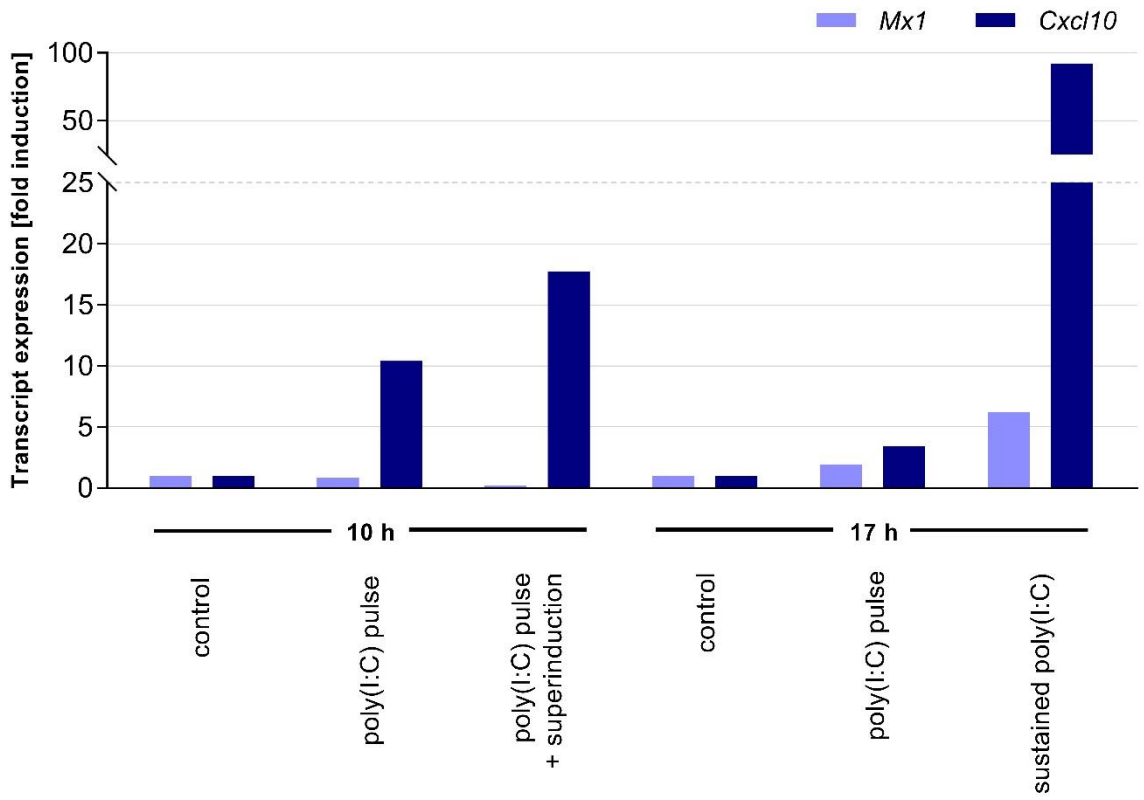


Figure 8: Effect of Poly(I:C)-based Stimulation on Selected ISGs in Hepa 1-6

$3x10^5$ Hepa 1-6 cells were seeded in 6-well plates. On the following day, cells were treated with 10:100 µg/ml poly(I:C):DEAE-dextran for either a 1 h pulse, a 1 h pulse in combination with a superinduction protocol or 16 h of sustained exposure. Cultures were harvested at the indicated time points and analyzed for gene expression of the selected ISGs *Mx1* and *Cxcl10*. Data on *Mx1* and *Cxcl10* are given as mean values of up to five independent data sets and displayed as fold induction in comparison to the respective control.

Accordingly, Hepa 1-6 was shown to be capable of both type I IFN and type III IFN expression after exposure to the synthetic nucleic acid poly(I:C) if combined with the transfector DEAE-dextran to allow for cytoplasmatic exposure to the RNA analog (figure 7). Additionally, a superinduction protocol was demonstrated to result in higher transcript numbers or

earlier detection due to increased transcription (figure 7). Both induction regimens provided reliable priming methods for further experiments with exposure to chemotherapeutics.

Furthermore, IFN-α and IFN-λ induction was related to the expression of two ISGs, *Mx1* and *Cxcl10*. While *Mx1* expression was not greatly impacted by stimulation, *Cxcl10* transcription levels increased concordantly to *Ifna4* and *Ifnl2/3* expression (figure 8). In contrast to the short term IFN-α and IFN-λ gene induction, increase of *Mx1* and *Cxcl10* transcription was also demonstrated to be detectable at t = 24 h, suggesting slower activation or longer sustain of activation.

Moreover, sustained poly(I:C):DEAE-dextran exposure was shown to provide an additional technique to assess the overall capacity of Hepa 1-6 clones to express type III IFNs, since a lack of expression in clones in contrast to high expression levels in Hepa 1-6 wt could serve as a confirmation of *Ifnl2/3* deficiency, as the results could be expected to not be masked by subliminal expression levels.

3.1.2 Inducibility of IFN-α, IFN-λ and ISG Expression by Chemotherapeutics

The capability of conventional chemotherapeutics to induce type I and type III IFNs in Hepa 1-6 with possible immunomodulatory effects was assessed for the nucleoside analog gemcitabine, the DNA intercalator doxorubicin and the DNA cross-linker oxaliplatin, combined with the established poly(I:C) stimulation regimes to achieve priming (figure 9).

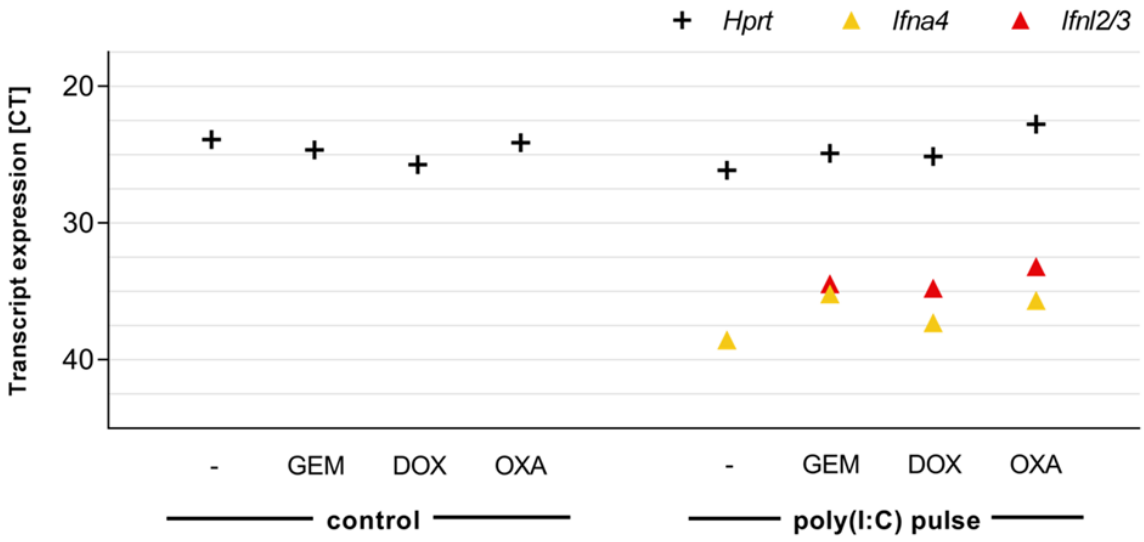


Figure 9: Induction of Type I and Type III IFNs by Chemotherapeutic Drugs in Hepa 1-6

$3x10^5$ Hepa 1-6 cells were seeded in 6-well plates. On the following day, cultures were treated with 10 µM of gemcitabine (GEM), doxorubicin (DOX) or oxaliplatin (OXA) as indicated or left untreated. One hour later, the indicated cultures were pulsed with 10:100 µg/ml poly(I:C):DEAE-dextran. After another hour, the culture medium was replaced before cells were harvested at t = 10 h. Transcript expression of *Hprt*, *Ifna4* and *Ifnl2/3* are given as either mean values of two independent data sets (GEM) or single representative results (DOX, OXA) and are displayed as raw CT values to illustrate the absence or presence of transcription as unbiased as possible.

As depicted in figure 9, Hepa 1-6 cells were treated with the chosen chemotherapeutics for two hours with or without one hour of poly(I:C):DEAE-dextran pulsing and harvested at t = 10 h. Neither chemotherapeutic substance tested was shown to induce *Ifna4* or *Ifnl2/3* in detectable amounts by itself, while priming alone, as expected, did induce *Ifna4* but not *Ifnl2/3* transcription above the detection limit. Combined treatment of chemotherapeutics and poly(I:C):DEAE-dextran priming, however, resulted in *Ifnl2/3* expression in all cell cultures, demonstrating the ability of these genotoxic drugs to induce type III IFNs in Hepa 1-6. With regard to type I IFNs and evaluating *Ifna4* CT values normalized to the corresponding *Hprt* levels to compensate for sample size differences, only gemcitabine was shown to

also increase IFN-α expression in Hepa 1-6, while treatment with doxorubicin and oxaliplatin did not raise *Ifna4* transcription above priming levels.

As overall *Ifna4* and *Ifnl2/3* transcription levels were low and their increase of short duration, the effect of chemotherapeutic treatment with and without priming on the expression of the ISGs *Mx1* and *Cxcl10* as possible effectors was also investigated as depicted in figure 10.

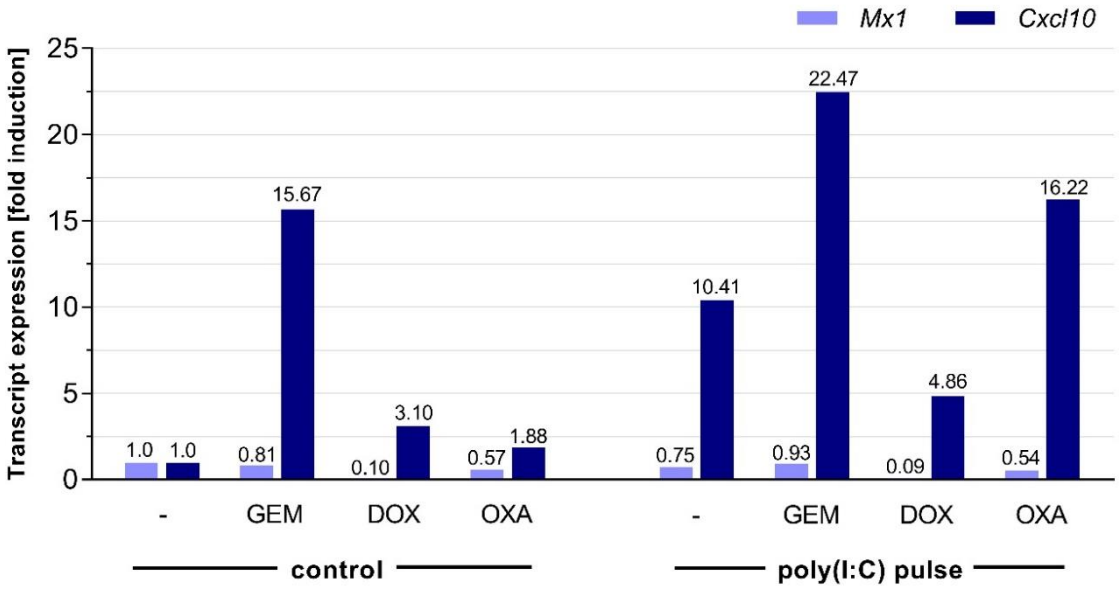


Figure 10: Induction of Selected IFN Effectors by Chemotherapeutic Drugs in Hepa 1-6

$3x10^5$ Hepa 1-6 cells were seeded in 6-well plates. On the following day, cultures were treated with 10 µM of gemcitabine (GEM), doxorubicin (DOX) or oxaliplatin (OXA) as indicated or left untreated. One hour later, the indicated cultures were pulsed with 10:100 µg/ml poly(I:C):DEAE-dextran. After another hour, the culture medium was replaced before cells were harvested at t = 24 h. Data on *Cxcl10* and *Mx1* expression are given as either mean values of two independent data sets (GEM) or single representative results (DOX, OXA) and depicted as fold induction compared to the untreated control.

Comparing expression levels in treated cell cultures to the basal expression of *Mx1* and *Cxcl10* in an unstimulated control, the effects of chemotherapeutics on the expression of the selected chemokines were not as uniform as their previously demonstrated *Ifnl2/3* induction had suggested. Nonetheless, *Mx1* levels were again shown to be either largely unaffected or even slightly suppressed by stimulation with or without priming (figure 10). Especially exposure to doxorubicin both with and without poly(I:C):DEAE-dextran priming led to the diminution of *Mx1* expression down to 11.11 times and 10 times fewer transcripts than in the unstimulated control, respectively (figure 10).

With regard to *Cxcl10* expression, stimulation by poly(I:C):DEAE-dextran alone resulted in a 10.41-fold induction of transcription compared to the unstimulated control, representing the downstream effect of type I and type III IFN induction (figure 10). The exposure to doxorubicin and oxaliplatin resulted in modest *Cxcl10* induction up to 3.1-fold and 1.88-fold, respectively (figure 10). The induction was increased by poly(I:C):DEAE-dextran priming to 4.86-fold for doxorubicin and 16.22-fold for oxaliplatin (figure 10). Thus, *Cxcl10* levels in cultures stimulated with doxorubicin and poly(I:C):DEAE-dextran priming remained below the *Cxcl10* expression in cultures subjected to poly(I:C):DEAE-dextran priming alone, while primed oxaliplatin treatment increased *Cxcl10* expression in a synergistic manner.

Surprisingly, treatment with a two-hour pulse of gemcitabine without priming already induced high levels of *Cxcl10* at 15.67-fold induction compared to the unstimulated control (figure 10). Therefore, gemcitabine was found to be a potent inducer of *Cxcl10* in the absence of any measurable type I or type III IFN induction, suggesting this to be a direct effect of gemcitabine exposure. Combined treatment with gemcitabine and poly(I:C):DEAE-dextran priming further increased *Cxcl10* induction to 22.47-fold, overall resulting in the highest *Cxcl10* induction of all tested substances (figure 10). However, the increase of expression by the addition of priming remained below the effect of priming alone (figure 10).

Taken together, Hepa 1-6 cells were found to be responsive to the selected chemotherapeutics with regard to *Ifnl2/3* expression if combined with a poly(I:C):DEAE-dextran priming (figure 9). The potentially synergistic IFN-λ induction achieved by gemcitabine or oxaliplatin treatment and poly(I:C):DEAE-dextran exposure may result from a complementary stimulation of cytoplasmatic inner receptors via poly(I:C) transfection and membranous outer receptors via DAMPs released by dying cells. Hepa 1-6 was therefore shown to pose a suitable cell line to investigate the impact of drug-driven *Ifnl2/3* expression on immunoediting in hepatocellular carcinoma *in vitro* and, due to its transplantability, *in vivo*.

Furthermore, activation of type I and type III IFNs is also mirrored by the downstream effector *Cxcl10* (figure 10). Additionally, a two-hour exposure to gemcitabine was shown to independently induce *Cxcl10* with high effectivity and independent of priming as well as IFN induction (figure 10). This characteristic of gemcitabine had not been described prior to this thesis.

3.1.3 Mediating Factors in Drug-induced *Ifnl2/3* Expression

The proposed model of immunoediting credits immunomodulatory effects to mediating factors such as DAMPs released by dying cells as well as autocrine effects of IFN produced by stimulated cells. In order to investigate possible mediating factors in the drug-induced *Ifnl2/3* expression in Hepa 1-6, a set of experiments with washing steps and nuclease addition was performed.

Initially, the overall existence of factors released into the culture medium after the exposure to gemcitabine was investigated by comparing the established protocol of primed chemotherapy exposure to a protocol containing an additional washing step. As depicted in figure 11 A, the treatment of a Hepa 1-6 cell culture with gemcitabine and a priming pulse resulted in measurable *Ifnl2/3* transcription levels at t = 24 h. This induction was negated by a timed culture medium exchange at t = 7 h, supporting the involvement of extracellular compounds released in the early hours of drug-mediated cytotoxicity (figure 11A).

To further specify the mediating compound, stimulated Hepa 1-6 cell cultures were subsequently treated with either Benzonase® nuclease, ribonuclease A (RNase A) or DNase I and the cultures' *Ifnl2/3* and *Ifna4* expression were compared to that of a stimulated control without nuclease treatment, as depicted in figure 11B. *Ifna4* induction did not show significant differences in cultures subjected to Benzonase® nuclease (98.5% ± 94.045, $p = 0.98$) or RNase A (69.5% ± 24.749, $p = 0.58$). *Ifnl2/3* expression, however, was distinctively lower in cultures treated with Benzonase® nuclease (40% ± 31.113, $p = 0.04$), degrading both RNA and DNA, and RNase A (53% ± 9.899, $p = 0.07$), degrading only RNA, suggesting the mediating factor in drug-induced *Ifnl2/3* activation to be of RNA origin. Albeit the data did not reach the adjusted level of significance of $p < 0.017$, the visible differences suggest a contribution of extracellular ribonucleic acids to the IFN-λ gene expression resulting from gemcitabine exposure and cytoplasmatic pattern recognition receptor stimulation via poly(I:C). Thus, adding RNA-targeting nucleases negates the stimulation of membranous outer receptors and attenuates the activation of *Ifnl2/3*.

Additionally, exposure to DNase I did not result in suppression of IFN induction but increased both *Ifna4* (240 % ± 28.284, $p = 0.05$) and *Ifnl2/3* (169% ± 21.213, $p = 0.02$) expression levels, presumably by degradation of an unspecified inhibitory factor (figure 11B).

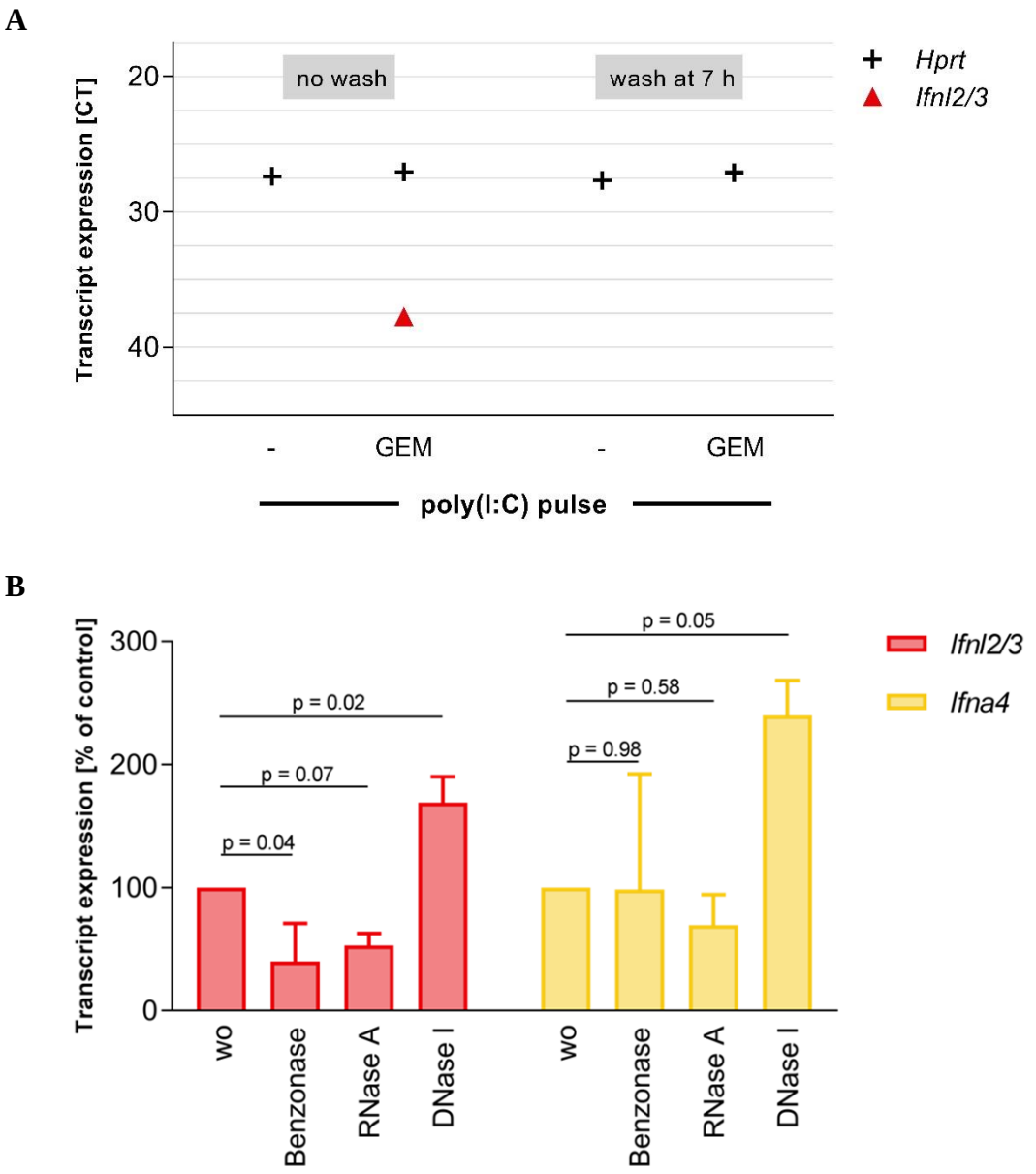


Figure 11: Mediating Factors in IFN-α and IFN-λ Induction by Gemcitabine in Hepa 1-6

$3x10^5$ Hepa 1-6 cells were seeded in 6-well plates. The next day, indicated cultures (A) or all cultures (B) were treated with 10 µM gemcitabine. After 1 h, the cultures were pulsed with 10:100 µg/ml poly(I:C):DEAE-dextran. Another hour later, culture medium was replaced before cells were either washed or left unwashed at t = 7 h and harvested at t = 24 h (A), or were either left untreated (wo) or treated with nucleases (Benzonase® nuclease 60 U/ml, ribonuclease A (RNase A) 30 U/ml, DNase I 60 U/ml) and harvested at t = 24 h (B). Data are given as CT values of single representative results (A) or as mean values and standard deviations with p-values (ANOVA) for two independent experiments as percentages of the control (B). Gene expressions as percentages of the respective control were assessed graphically for normal distribution using a quantile-quantile plot with the software STATISTICA 13 by StatSoft GmbH and significance was determined by one-way ANOVA with Bonferroni correction. The adjusted level of significance was set at $p < 0.017$.

To further investigate the role and impact of the proposed mediating compound a set of co-culturing as well as supernatant experiments was performed as depicted in figure 12.

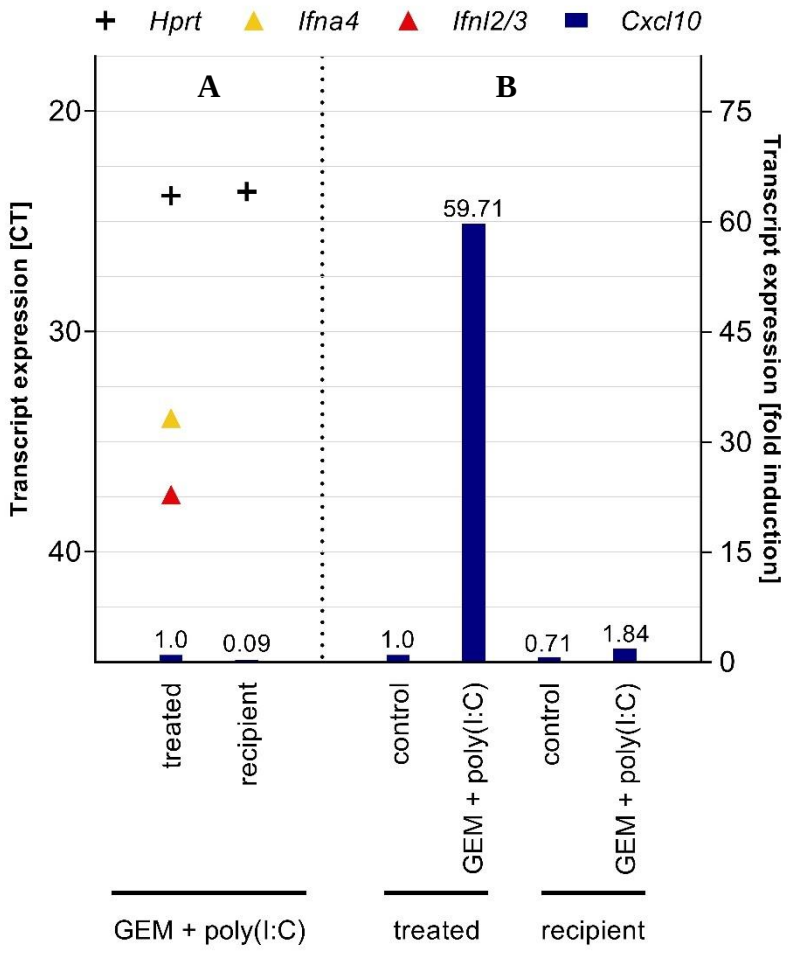


Figure 12: Transmissibility of IFN-α, IFN-λ and *Cxcl10* Induction

A) 3x10^5 Hepa 1-6 cells were seeded into 6-well-plates as well as into inlets (Thincerts with PET-Membrane 0.4 µm pores, Greiner Bio-One International GmbH). On the following day, cells in the 6-well-plate were treated with 10 µM gemcitabine. At t = 1 h these cells were pulsed with 10:100 µg/ml poly(I:C):DEAE-dextran, before being washed at t = 2 h. 6-well-plate cultures were then either left untreated (control) or treated with nucleases (Benzonase® nuclease 60 U/ml, ribonuclease A (RNase A) 30 U/ml, DNase I 60 U/ml) and inlets with cells were added to each well. Treated cells in the 6-well-plate along with recipient cells in the inlets were harvested separately at t = 17 h. Data are given as a single representative result and transcript expression is displayed as either raw CT values (*Hprt*, *Ifna4*, *Ifnl2/3*) or fold induction (*Cxcl10*).

B) 3x10^5 Hepa 1-6 cells were seeded into 6-well-plates. On the following day, cells in the 6-well-plate were treated with 10 µM gemcitabine. At t = 1 h these cells were pulsed with 10:100 µg/ml poly(I:C):DEAE-dextran, before being washed at t = 2 h. Simultaneously, a second plate was seeded with 3x10^5 Hepa 1-6 cells. After 24 h of incubation, the supernatant of the first plate was collected

and the treated cells harvested. The culturing medium of the second plate of cells was replaced with the collected supernatant. Recipient cells were incubated with the supernatant for another 22 h before being harvested. Data are given as a single representative result and *Cxcl10* transcript expression is displayed fold induction.

Figure 12A displays the results of a co-culturing experiment. Hepa 1-6 cells were treated with two hours of gemcitabine and a one-hour poly(I:C):DEAE-dextran pulse. After the treatment, the culturing medium was exchanged and inlets with Hepa 1-6 cells were added to each well, so that a previously treated Hepa 1-6 cell colony shared culturing medium with an untreated Hepa 1-6 cell colony. However, neither *Ifna4* nor *Ifnl2/3* gene expression was mediated from the treated culture to the recipient culture via the shared culturing medium (figure 12A). Consistently, *Cxlc10* activation levels in the recipient culture were 10 times lower than in the treated culture (figure 12A). In addition, a second set of Hepa 1-6 cultures was treated with conditioned medium derived from the supernatant of Hepa 1-6 cell cultures treated with gemcitabine and poly(I:C):DEAE-dextran pulsing as depicted in figure 12B. The comparison of *Cxcl10* gene expression of treated and recipient cells to the respective control confirmed the lack of transmissibility of *Cxcl10* induction via culturing medium, as *Cxcl10* transcript expression remained at 1.84-fold induction in cells exposed to supernatant from treated cultures, while treated cells expressed *Cxcl10* at 59.71-fold the level of the control (figure 12B).

Thus, *Ifnl2/3* induction by stimulation with gemcitabine was shown to be suppressible by medium exchange (figure 11A) or RNA targeting nucleases (figure 11B), proposing the existence of a mediating factor impacting *Ifnl2/3* expression. However, the direct impact of the treatment onto the cell colony was shown to be imperative for IFN and ISG induction (figure 12), suggesting the IFN-inducing effect to be the result of an initial direct effect onto the cell and subsequent amplification via a mediating RNA factor.

Additionally, possible mediating factors influencing the induction of *Cxcl10* by gemcitabine were assessed as displayed in figure 13. *Cxcl10* activation was neither transmissible by conditioned medium (figure 13A, figure 12), nor impaired by medium replacement (figure 13C), nor abated by the addition of nucleases (figure 13B). These observations support the thesis of *Cxcl10* induction being a direct effect of gemcitabine exposure and not mediated by factors released into the culturing medium.

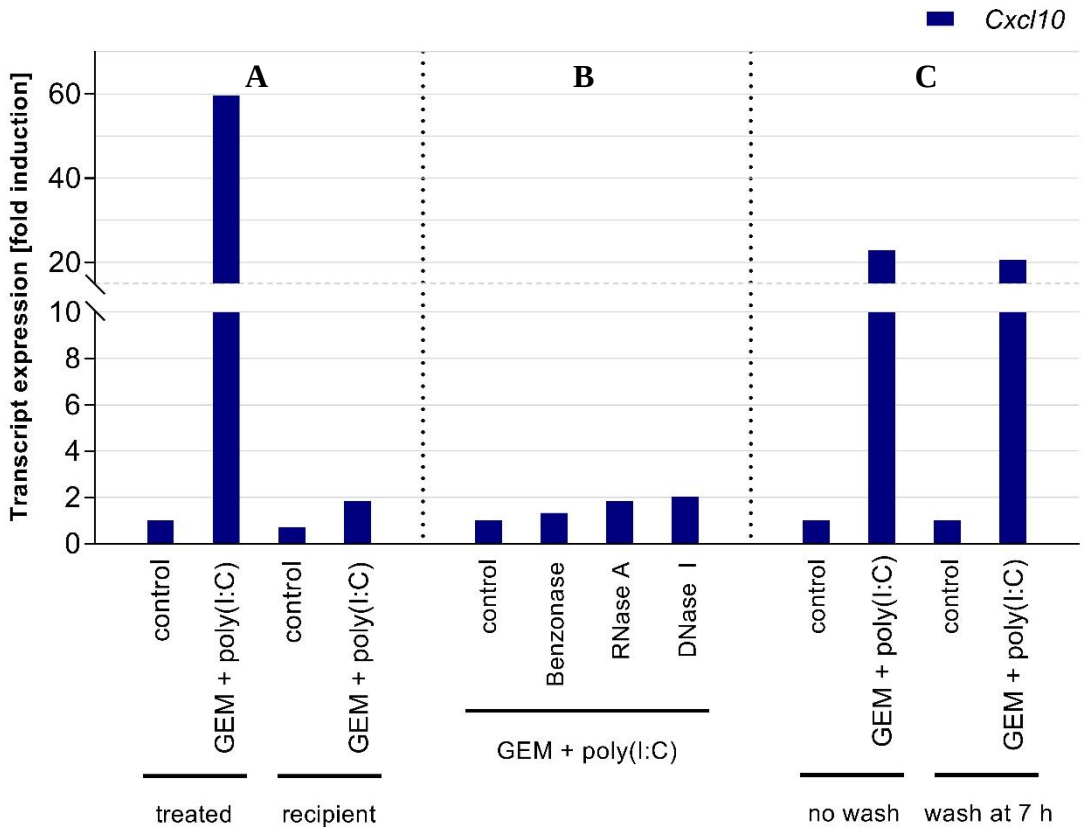


Figure 13: *Cxcl10* Induction by Gemcitabine Exposure

$3x10^5$ Hepa 1-6 cells were seeded into 6-well-plates (A, B, C) as well as into inlets (A) (Thincerts with PET-Membrane 0.4 µm pores, Greiner Bio-One International GmbH). On the following day, indicated cultures in the 6-well-plates were treated with 2 h of 10 µM gemcitabine and a 1 h pulse of 10:100 µg/ml poly(I:C):DEAE-dextran, before being washed at t = 2 h.

A) After 24 h of incubation, the supernatant of the treated 6-well-plate was collected and the treated cells were harvested. The culturing medium of a second plate of cells was replaced with the collected supernatant. Recipient cells were incubated with the supernatant for 22 h before being harvested.

B) After washing, 6-well-plate cultures were either left untreated (control) or treated with nucleases (Benzonase® nuclease 60 U/ml, ribonuclease A (RNase A) 30 U/ml, DNase I 60 U/ml). Inlets with cells were added to each well. Treated cells in the 6-well-plate and recipient cells in the inlets were harvested separately at t = 17 h.

C) Cell cultures were either washed or left unwashed at t = 7 h and harvested at t = 24 h.

Data are given as single representative results and displayed as fold induction in comparison to the respective control.

3.2 Characterization of *Ifnl2/3*-deficient Hepa 1-6 Clones

3.2.1 *Ifnl2/3* Knockout Validation

In order to evaluate the impact of IFN-λ on the inducibility of type I IFNs and effector chemokines as well as chemosensitivity, CRISPR/Cas9 genetic engineering technology was utilized as described in detail in section 2.2.5 to create isogenic *Ifnl2/3*-deficient Hepa 1-6 clones to compare to the parental Hepa 1-6 cell line.

During the process of CRISPR/Cas9-mediated *Ifnl2/3* knockout, the success of the transfection with the DNA altering double nickase plasmid pair was confirmed via fluorescence microscopy, as the one of the double nickase plasmids employed carried the gene for GFP, resulting in a green fluorescent signal (figure 14). The cells of the successfully transfected cell colony were selected via exposure to puromycin based on a transfected transient puromycin resistance and consecutively serially diluted to receive single cell colonies. Ten Hepa 1-6 clone cell lines were chosen for the knockout validation process.

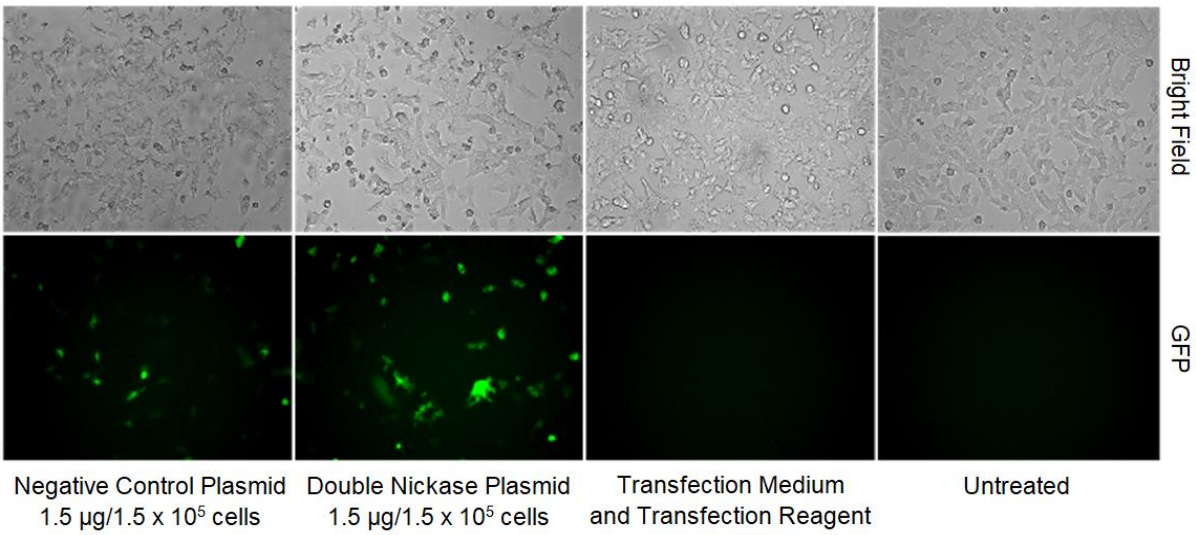


Figure 14: Hepa 1-6 GFP Expression 48 h Post-transfection Extended

Successful transfection was confirmed by the expression of GFP, visible under fluorescent light after 48 h. Pictures in bright field exposure versus fluorescent light are depicted for cell cultures treated with the negative control plasmid, the double nickase plasmid, transfection medium and reagent without plasmid, or no treatment, respectively. Plasmids were applied in concentrations of 1.5 µg/1.5 x 10^5 cells. As expected, only cells exposed to plasmids were expressing GFP, thus confirming successful transfection, while cells treated without the addition of plasmid or untreated cells showed no fluorescence. However, the exposure to transfection medium and reagent with or without plasmids

resulted in increased cell stress, as cultures showed more vesicles, detached and dead cells in bright field as opposed to untreated cell cultures.

Ifnl2/3 knockout validation was performed on gDNA level via conventional PCR and Sanger sequencing as well as on cDNA level via conventional PCR. The knockout validation PCR assays utilized in this thesis are described in detail in section 2.2.6. In brief, primers were designed so that one of each pair bound directly within the guide region on the *Ifnl2/3* locus in Hepa 1-6 wt, expecting impairment of binding in Hepa 1-6 clones with altered sequences in this region. The assays were thus utilized to demonstrate absence of original sequences within the targeted *Ifnl2/3* region on gDNA and subsequently also on mRNA via cDNA. As a positive control, *Ifnl2/3*-specific sequences of the region outside of the guide sequences were also amplified. These were expected to be detectable on gDNA level of both Hepa 1-6 wt as well as Hepa 1-6 *Ifnl2/3*-deficient clones. Additionally, assays were designed to generate gene-specific amplicons on the basis of sequences upstream of each of the paralogous *Ifnl* genes to validate specific changes within *Ifnl2* and *Ifnl3*, respectively, via sequencing.

In order to select Hepa 1-6 clones with a successful knockout from the possible candidates, cultures of the ten Hepa 1-6 clones were harvested and the extracted clone gDNA was assessed via *Ifnl2/3* knockout validation PCR assays. Designed to amplify the targeted *Ifnl2/3* gene sequence in Hepa 1-6 wt cells, the first assay yielded a 101 bp amplicon in Hepa 1-6 wt and the second resulted in a 155 bp amplicon in Hepa 1-6 wt. As a positive control the outer primers of each assay were combined into a third assay spanning the target sequence and yielding a 238 bp amplicon in Hepa 1-6 wt. Resulting amplicons were subsequently examined utilizing agarose gel electrophoresis, photographed under a UV camera and evaluated via Image Lab™ Software as depicted in figure 15. While amplification of Hepa 1-6 wt gDNA resulted in three amplicons of appropriate sizes, *Ifnl2/3*-deficient clones were expected to lack both the 101 bp as well as the 155 bp amplicon, and the amplicon resulting from the span assay was expected to be present but differ in size due to indel mutations within the sequence.

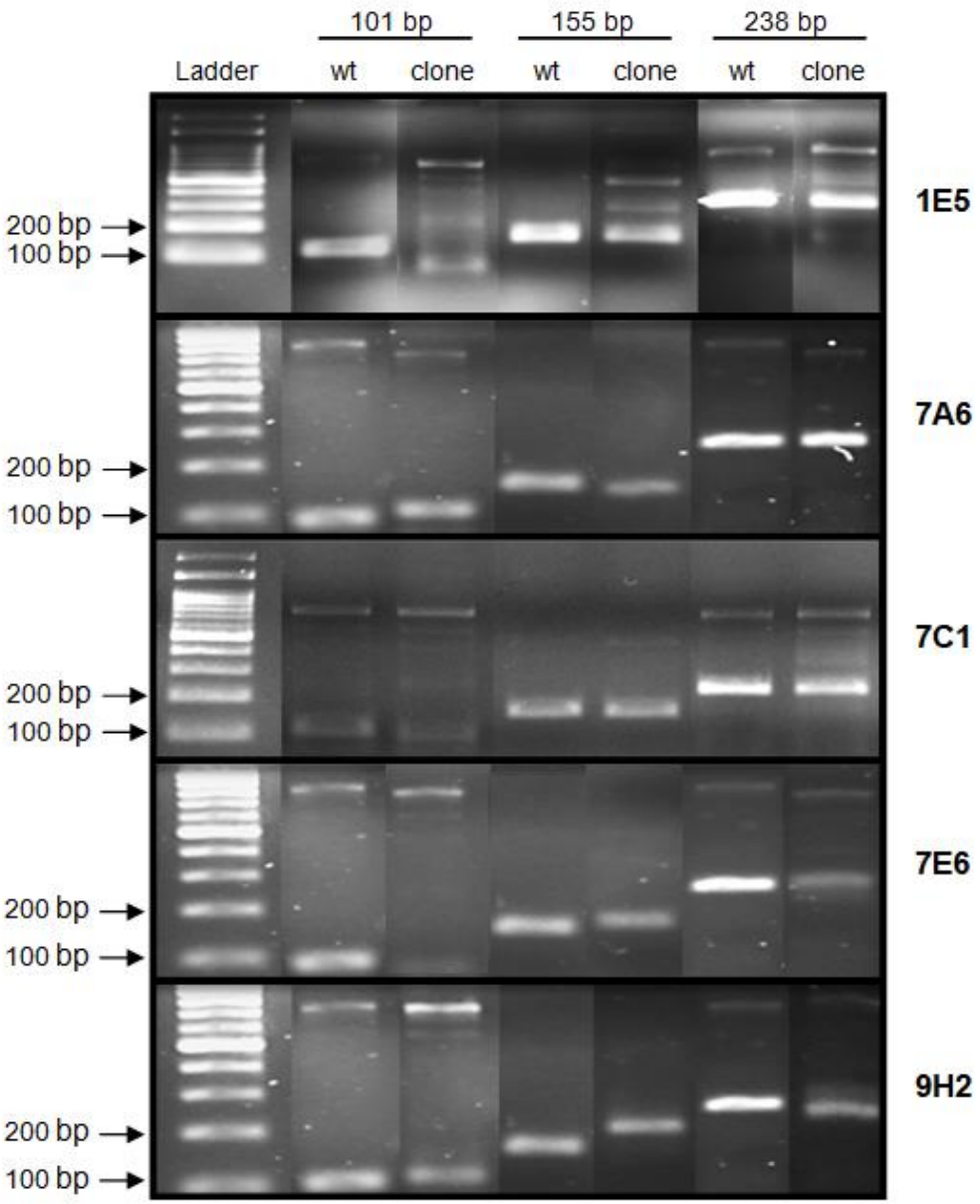


Figure 15: *Ifnl2/3* Knockout Validation via PCR Assay on gDNA Level

Cultures of Hepa 1-6 wt and the selected Hepa 1-6 clones were harvested for gDNA and assessed via *Ifnl2/3* knockout validation PCR assays. The first assay yielded a 101 bp amplicon in Hepa 1-6 wt, the second resulted in a 155 bp amplicon in Hepa 1-6 wt. As a positive control the outer primers of each assay were combined into a third assay spanning the target sequence and yielding a 238 bp amplicon in Hepa 1-6 wt. Resulting amplicons were subsequently examined utilizing agarose gel electrophoresis, photographed under a UV camera and evaluated via Image Lab™ Software. In comparison to Hepa 1-6 wt, each Hepa 1-6 clone was characterized according to presence, absence or size of amplicons.

However, assay results were not as clear as their design suggested. Figure 15 displays representative results of the knockout validation PCR assay for five Hepa 1-6 clones. Of those,

only two clones showed clear absence of an amplicon (1E5 and 7E6), one showed distinctive difference in size in one amplicon (9H2), but none showed absence of both, which would have been the expected result in non-wt clones. Two clones (7A6 and 7C1) showed similar amplicon patterns as the parental Hepa 1-6 wt, however amplicons seemed to differ slightly in size. Thus, assays were combined with sequencing of either the *Ifnl2/3* amplicons resulting from the 238 bp yielding assay or of gene-specific amplicons for *Ifnl2* and *Ifnl3* individually to further investigate the *Ifnl2/3* gene status. Sanger sequencing was performed by Microsynth Seqlab, Goettingen, Germany, and results were interpreted utilizing Chromas 2.6.2 DNA Sequencing Software (Technelysium Pty Ltd) as well as the BLAST® Align Sequences Nucleotide software provided by the NCBI, U.S. National Library of Medicine. The result for Hepa 1-6 1E5 *Ifnl2* is displayed in figure 16, while the sequencing results of the other clones can be found in the addenda.

As expected, sequencing was hindered by heterogeneity in alleles resulting in mixed signals. Since the investigated clones could be heterozygous for either two knockout alleles or for a knockout and a wildtype allele, sequencing results had to be cross-referenced to the validation PCR assay results to specify the *Ifnl2/3* knockout status as depicted in table 6.

Table 6: Hepa 1-6 Clone Characterization and Knockout Validation

Hepa 1-6 Clone	Conventional PCR		Sanger Sequencing	
	Wt Sequence on gDNA Level	Wt Sequence on mRNA Level	*Ifnl2*	*Ifnl3*
1E5	-	-	16 bp Deletion	No Amplicon
7A6	+	+	*Ifnl2/3* Exon 1 Wildtype Sequence	
7C1	+	nd	3 bp Deletion*	153 bp Insertion
7E6	-	-	Mixed Signals	Mixed Signals
9H2	-	nd	Mixed Signals	Mixed Signals

Hepa 1-6 clones were assessed via conventional PCR on gDNA and mRNA level for the presence of wildtype *Ifnl2/3* sequences as well as Sanger sequencing. Sequencing approaches were carried out on amplicons generated either by primers recognizing exon 1 sequences of both *Ifnl2* and *Ifnl3* genes or by primers complementary to intronic sequences allowing discrimination of *Ifnl2* or *Ifnl3* gene specific sequences. nd = not done, mixed signal = heterozygous alleles, * = no frameshift

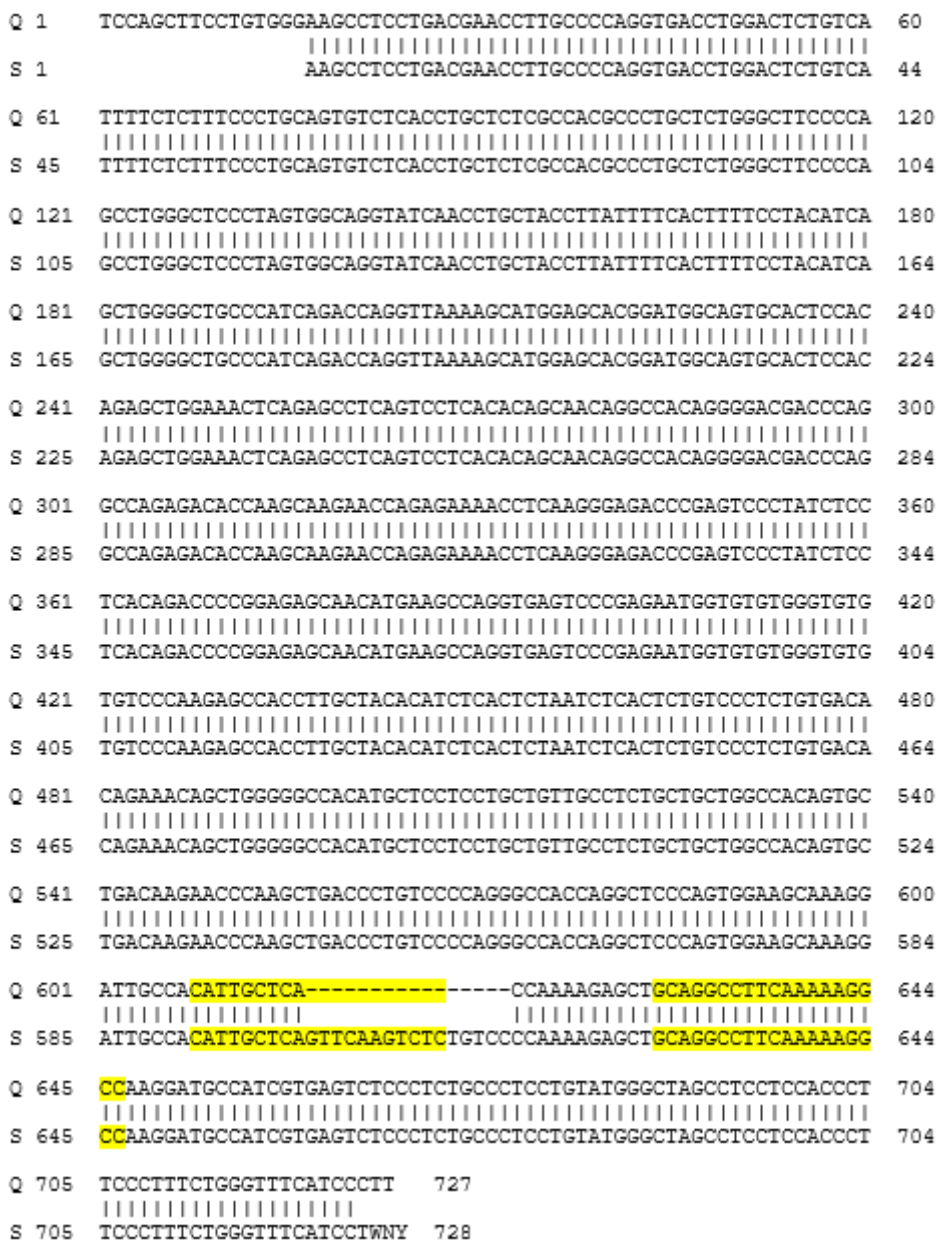


Figure 16: *Ifnl2* Sequencing Results for Hepa 1-6 1E5

Gene sequences were amplified using the IFNL2 span ex1 gene assay with Invitrogen™ DNA oligonucleotide primer sequences provided by Thermo Fisher Scientific Inc. and amplicon integrity was ensured via agarose gel electrophoresis. Sanger sequencing was performed by Microsynth Seqlab, Goettingen, Germany. Sequence alignment was performed using the NCBI´s nucleotide alignment tool BLAST® provided by the NCBI, U.S. National Library of Medicine. The Hepa 1-6 wildtype *Ifnl2* sequence is displayed as subject sequence (S), while the Hepa 1-6 1E5 *Ifnl2* sequence is marked as query sequence (Q). Highlighted areas mark the primer target sequences utilized in the *Ifnl2/3* gene knockout.

As depicted in figure 15 and table 6, Hepa 1-6 clone 7E6 showed absence of the 100 bp amplicon in the conventional PCR, suggesting absence of wildtype *Ifnl2/3* sequences. Gene-specific sequencing of this clone, however, resulted in mixed signals for each IFN-λ gene (table 6). By combining the absence of wt sequences as suggested by the PCR and the presence of heterozygous alleles resulting from sequencing, Hepa 1-6 clone 7E6 was thus confirmed to be *Ifnl2/3*-deficient and was consecutively referred to as Hepa 1-6 *Ifnl2/3* -/- 7E6.

Similarly, Hepa 1-6 clone 1E5 also showed absence of Hepa 1-6 wt *Ifnl2/3* by lacking the 100 bp amplicon in the knockout validation assay (figure 15, table 6). Sequencing of 1E5 amplicons revealed a 16 bp deletion in both the *Ifnl2/3* amplicon and the gene-specific amplicon for *Ifnl2* (table 6, figure 16). However, *Ifnl3* could not be sequenced due to lack of amplification by the gene-specific assay, which suggested a larger deletion on this gene impairing amplification of the same (table 6). So, Hepa 1-6 clone 1E5 was also confirmed to be *Ifnl2/3*-deficient and renamed to Hepa 1-6 *Ifnl2/3* -/- 1E5.

Even though their amplicons seemed to be similar to those of Hepa 1-6 wt, the two clones 7A6 and 7C1 were nonetheless assessed via sequencing. As expected, Hepa 1-6 7A6 was confirmed to harbor a wildtype *Ifnl2/3* gene locus, thus subsequently referred to as Hepa 1-6 7A6 wt (table 6). Still, differences between Hepa 1-6 7A6 wt and the native Hepa 1-6 wt cell line due to off-target effects not revealed by PCR or sequencing could not be excluded.

Sequencing of the other clone characterized as wildtype by the conventional PCR, Hepa 1-6 7C1, however, revealed a 3 bp deletion for *Ifnl2* and a 153 bp insertion for *Ifnl3* (table 6). It can be assumed that the 3 bp deletion on *Ifnl2* was not sufficient to disrupt primer binding for the knockout validation PCR assay, thus confounding the result of the PCR assay to mimic that of a wildtype Hepa 1-6. With the clear sequencing result, Hepa 1-6 7C1 qualified as an *Ifnl2/3*-deficient clone. Due to the lack of the one amino acid encoded by the deleted nucleic acids, the resulting protein can be expected to not fold properly and should thus be detected as irregular and eliminated by the cell, rendering Hepa 1-6 7C1 a functionally *Ifnl2/3*-deficient clone.

The last Hepa 1-6 clone assessed via sequencing was Hepa 1-6 9H2. The 9H2 amplicon resulting from the second knockout validation PCR assay was distinctively larger in size than the expected 155 bp, indicating the presence of an alteration within the targeted *Ifnl2/3* sequence that did not disrupt primer binding but resulted in a larger amplicon (figure 15).

Consecutive sequencing, however, was not able to elucidate the caused mutation further as it showed only mixed signals indicating heterozygous alleles (table 6).

Of the five Hepa 1-6 clones investigated via conventional PCR as well as Sanger sequencing on gDNA level, Hepa 1-6 *Ifnl2/3* -/- 1E5 and Hepa 1-6 *Ifnl2/3* -/- 7E6 were chosen as *Ifnl2/3*-deficient cell lines to compare to the parental Hepa 1-6 wt, while Hepa 1-6 7A6 wt was selected to serve as a control.

To improve validation security, the clones were additionally subjected to stimulation and investigated for *Ifnl2/3*-mRNA transcription. As expected, the *Ifnl2/3*-deficient clones Hepa 1-6 *Ifnl2/3* -/- 1E5 and Hepa 1-6 *Ifnl2/3* -/- 7E6 lacked *Ifnl2/3* transcript expression (figure 17A). However, due to scheduling and to avoid possible further mutagenesis caused by selection within the Hepa 1-6 clone cell culture, the second part of this thesis was performed utilizing a second stock of cells for each clone, that had been established after the initial knockout validation and stored in nitrogen tanks. As per usual and for security reasons, the new stocks were subjected to the same assessment via stimulation experiment, expecting similar results. Surprisingly, these cells expressed detectable amounts of *Ifnl2/3*-mRNA in the control experiment (figure 17A, Hepa 1-6 clone 7E6). This phenomenon was even more obvious in sustained exposure to poly(I:C):DEAE-dextran (figure 17A, Hepa 1-6 clone 1E5 and Hepa 1-6 clone 7E6).

Nonetheless, the knockout validation PCR assays repeated on gDNA level for these stocks showed the same results as for the first stock of clones, confirming absence of wildtype *Ifnl2/3* for clones 1E5 and 7E6 on gDNA level. The validation PCR assays were thus repeated on mRNA level by stimulating the clones with the potent IFN-inducing regimen of 16 h of sustained poly(I:C):DEAE-dextran exposure, harvesting the cell cultures at t = 17 h and assessing extracted mRNA via cDNA utilizing the 101 bp yielding knockout assay IFNL23 ko, for this assay could also be used on cDNA. The assay confirmed the absence of the wildtype *Ifnl2/3* sequence within the guide sequence region on mRNA level for both clone 1E5 and 7E6 (figure 17B, table 6).

Taken together, even though original sequences in the targeted region of the clones were absent on gDNA and mRNA level (figure 15, figure 17, table 6), *Ifnl2/3*-specific sequences of the region outside of the guide sequences could unexpectedly be found on mRNA level. With simultaneous absence of wildtype sequences this result was interpreted as being caused by fragments of non-functional transcripts.

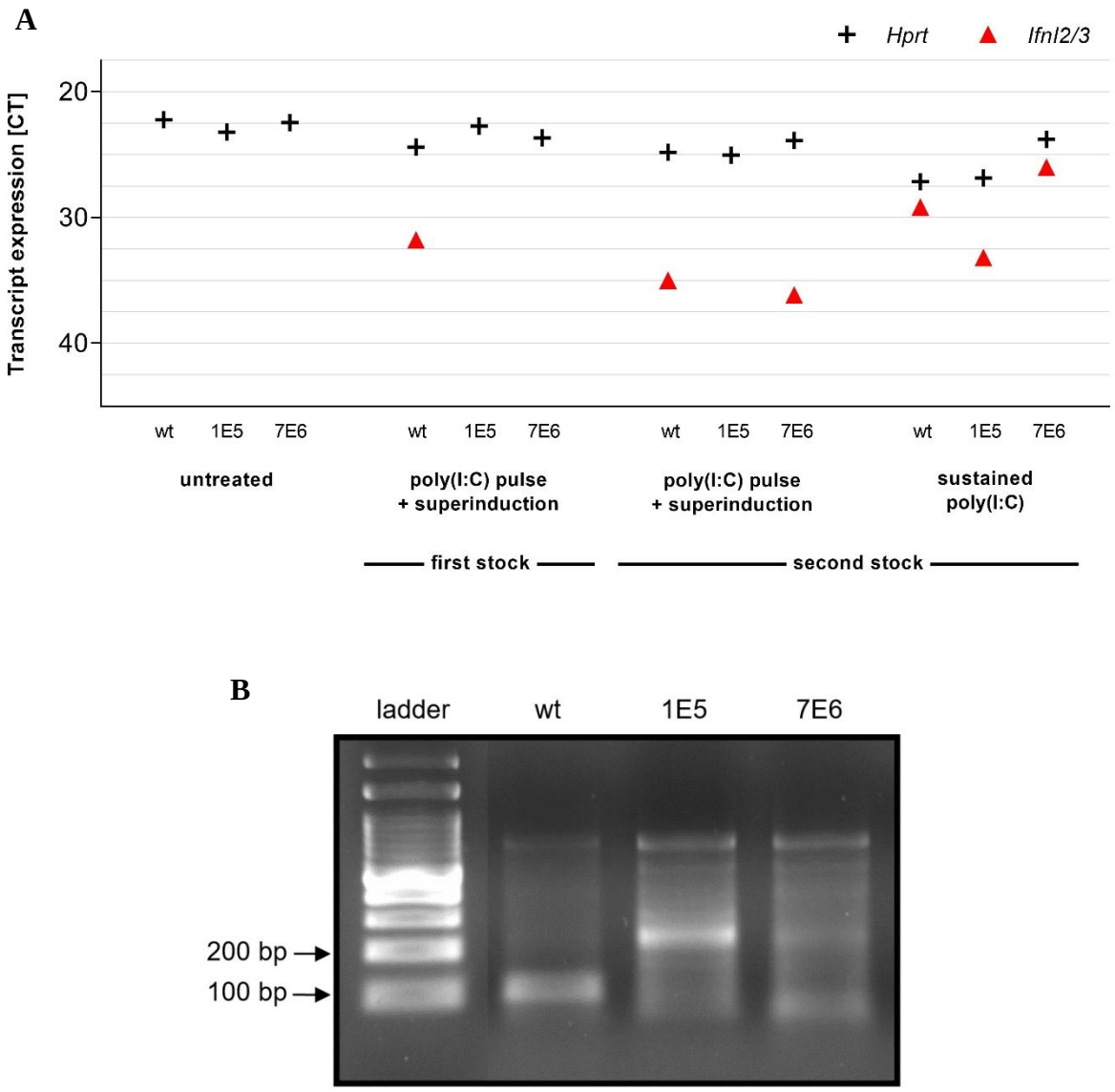


Figure 17: *Ifnl2/3* Expression in Hepa 1-6 Clones and Knockout Validation on mRNA Level

A) $3x10^5$ Hepa 1-6 wt, Hepa 1-6 clone 1E5 and Hepa 1-6 clone 7E6 were seeded into 6-well-plates. On the following day, cells were subjected to a 2 h 10:100 µg/ml poly(I:C):DEAE-dextran pulse along with the established superinduction protocol. Cells were then harvested at t = 10 h and assessed for *Hprt* and *Ifnl2/3* gene expression. Data are given as CT values of single representative data sets.

B) The knockout validation PCR assays were repeated on mRNA level. Cell cultures of Hepa 1-6 wt, Hepa 1-6 clone 1E5 and Hepa 1-6 clone 7E6 were stimulated with 16 h of sustained 10:100 µg/ml poly(I:C):DEAE-dextran exposure and harvested at t = 17 h. Extracted mRNA was utilized to synthesize cDNA and assessed using the knockout assay IFNL23 ko, known to yield a 101 bp in Hepa 1-6 wt while failing to amplify in early stocks of Hepa 1-6 clones 1E5 and 7E6.

3.2.2 Hepa 1-6 Clone Morphology

In order to assess the morphology of the selected Hepa 1-6 *Ifnl2/3* -/- clones as well as Hepa 1-6 7A6 wt, Hepa 1-6 7C1 and Hepa 1-6 9H2, colonies of each Hepa 1-6 clone were assessed under a microscope and compared to the native Hepa 1-6 wt cell line (figure 18).

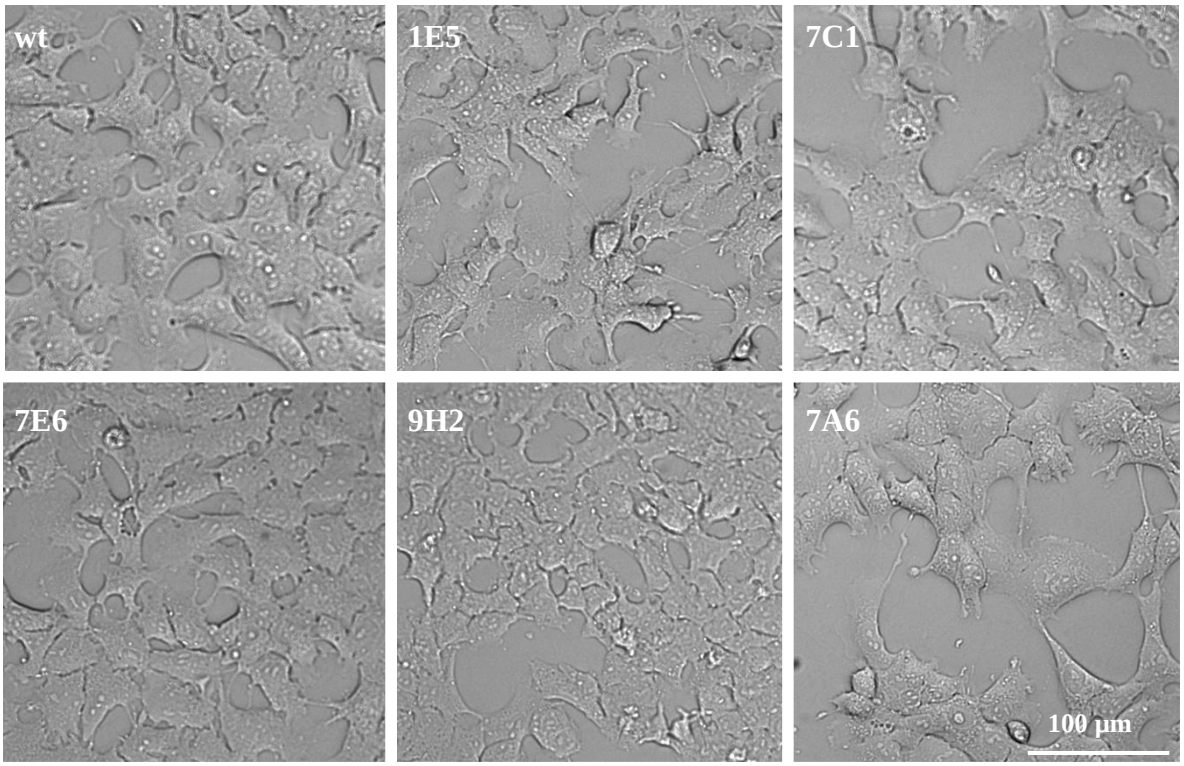


Figure 18: Morphology of Hepa 1-6 Wildtype and Isogenic Clones

Hepa 1-6 wt and Hepa 1-6 clones were seeded into 6-well-plates. The respective cell cultures were subsequently assessed via bright field microscopy at 60 – 80% confluence. Similar picture sections were chosen for each cell culture to allow for comparison. All cell cultures photographed displayed typical features of Hepa 1-6 with adherent monolayer cultures of epithelial cells. Status of *Ifnl2/3* did not influence viability, proliferation or colony formation as assessable via microscopy.

As expected in isogenic cell lines differing only in their *Ifnl2/3* locus, no effect of *Ifnl2/3* gene status on the morphology was observed. Independently of their *Ifnl2/3* status, Hepa 1-6 cells were growing in adherent monolayer colonies of similar patterns, displaying typical features of epithelial cells without signs for increased stress, cell death or disruption of colony formation (figure 18).

3.3 Effect of *Ifnl2/3*-Deficiency

3.3.1 Effect of *Ifnl2/3*-Deficiency on Type I IFN and ISG Expression

After the knockout validation process, the selected Hepa 1-6 *Ifnl2/3* -/- clones were assessed with regards toward their ability to express type I IFNs as well as ISGs to evaluate possible effects of the CRISPR/Cas9 engineered *Ifnl2/3*-deficiency.

As depicted in figure 19A, Hepa 1-6 wt and the isogenic clones were subjected to the established stimulation regime of 10:100 µg/ml poly(I:C):DEAE-dextran pulsing combined with the superinduction protocol. Subsequently, cells were harvested at t = 10 h and assessed with regard to mean values of *Ifna4*, *Mx1* and *Cxcl10* expression for two (for clones) or three (for wildtype) independent data sets. Type I IFN expression in stimulated Hepa 1-6 clones 1E5 and 7A6 was comparable in extent to expression levels in Hepa 1-6 wt (figure 19A). However, mean *Ifna4* transcript expression in Hepa 1-6 *Ifnl2/3* -/- 7E6 was distinctively increased (figure 19A).

The expression of ISGs in Hepa 1-6 isogenic clones is depicted in figure 19A as well as in figure 19B, with the latter utilizing a logarithmic scale for better visibility. Both *Ifnl2/3*-deficient clones showed lowered basal expression of *Mx1* with 16.67 times fewer transcripts in 1E5 and 9.09 times fewer transcripts in 7E6 compared to unstimulated Hepa 1-6 wt (figure 19A, 19B). Of note, Hepa 1-6 clone 7A6 wt also expressed *Mx1* 16.67 times fewer than the native Hepa 1-6 wt (figure 19A, 19B). All three clones' *Mx1* expression was suppressed down to a level where gene transcription was subliminal in one of the displayed data sets (figure 19A, 19B). Stimulation via poly(I:C):DEAE-dextran and superinduction did not increase *Mx1* expression in the isogenic clones (figure 19A, 19B). Hepa 1-6 *Ifnl2/3* -/- 7E6 showed stable suppression of *Mx1* expression of 0.09-fold induction compared to 0.11-fold induction in the unstimulated cell culture (figure 19A, 19B). Similarly, Hepa 1-6 7A6 wt showed a 0.06-fold induction of Mx1 in both the stimulated and unstimulated control (figure 19A, 19B). Only Hepa 1-6 *Ifnl2/3* -/- 1E5 showed further suppression of *Mx1* by stimulation with a 0.01-fold induction in the stimulated culture compared to the 0.06-fold basal induction, equaling to 100 times fewer *Mx1* transcripts in the stimulated culture compared to unstimulated Hepa 1-6 wt (figure 19A, 19B).

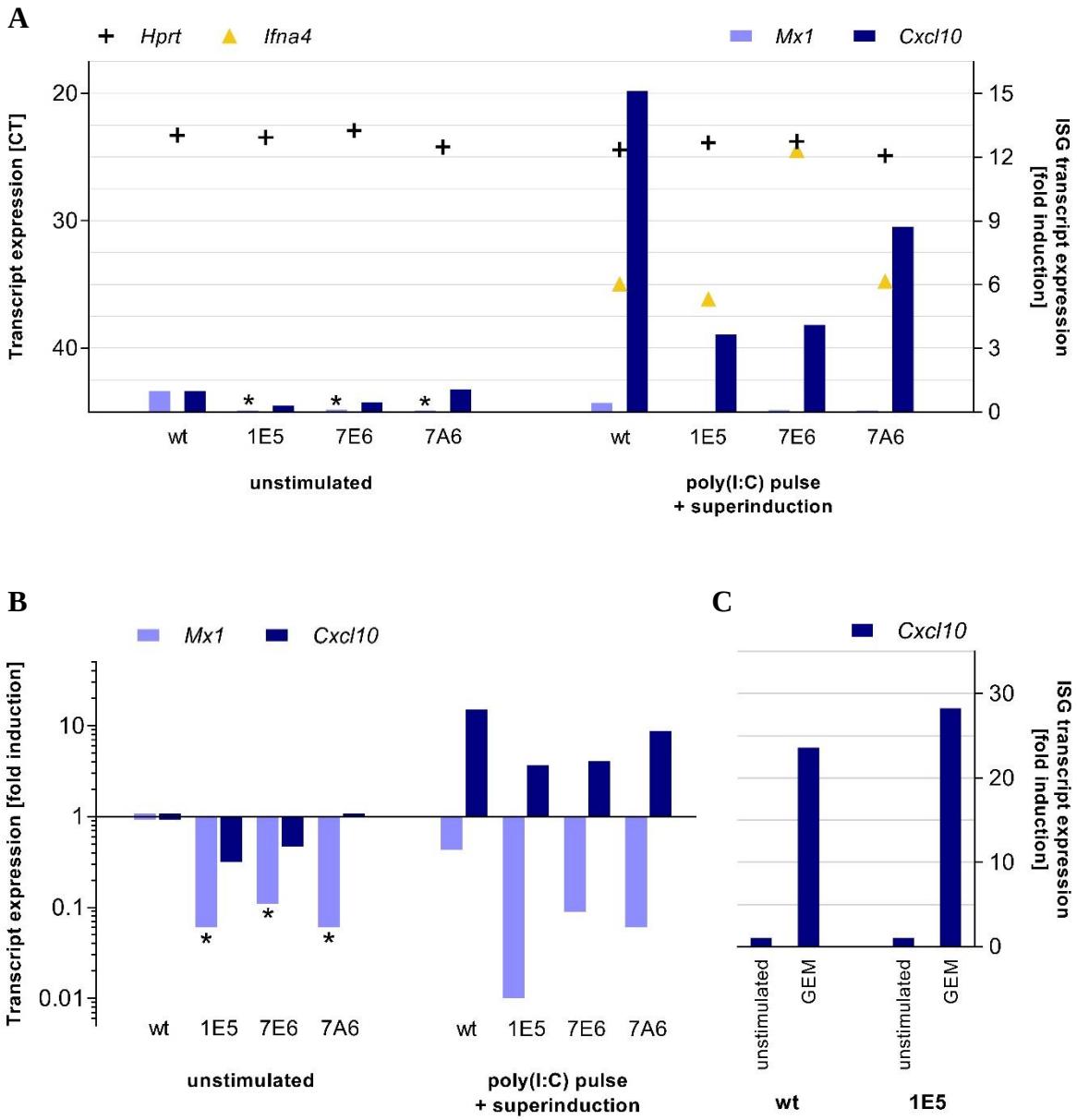


Figure 19: Type I IFN and ISG Expression in Hepa 1-6 Isogenic Clones

$3x10^5$ cells of Hepa 1-6 wt, Hepa 1-6 Ifnl2/3 -/- 1E5, Hepa 1-6 *Ifnl2/3* -/- 7E6 and Hepa 1-6 7A6 wt were seeded into 6-well-plates. On the following day, cells were either treated with a 2 h 10:100 µg/ml poly(I:C):DEAE-dextran pulse plus superinduction protocol and harvested at t = 10 h (A, B) or treated with 10 µM gemcitabine for 2 h and harvested at t = 10 h (C). Data are given as medium values of two (clones) and three (wildtype) independent data sets (A, B) or as a single representative result (C). Gene expression of *Hprt* and *Ifna4* are given as CT values, while *Mx1* and *Cxcl10* transcript expression is depicted as fold induction compared to the unstimulated Hepa 1-6 wt culture (A, B) or the unstimulated control (C). Transcription values marked by * were above the detection limit in only one of the two sets of experiments, while the other set was undetectable.

The expression of the second selected ISG *Cxcl10* was investigated simultaneously. In this case, basal expression in Hepa 1-6 7A6 wt did not differ to that of Hepa 1-6 wt with a fold induction of 1.07 (figure 19A, 19B). Nonetheless, the *Ifnl2/3*-deficient clones 1E5 and 7E6 showed decreased basal expression for the second ISG as well with 3.23 and 2.17 times fewer *Cxcl10* transcripts than Hepa 1-6 wt, respectively (figure 19A, 19B). Additionally, *Cxcl10* responsiveness seemed to be linked to *Ifna4* and *Ifnl2/3* expression, as transcription was higher in all stimulated cultures compared to the unstimulated control, but lower in extent in Hepa 1-6 *Ifnl2/3*-deficient clones (figure 19A, 19B). Stimulation increased *Cxcl10* gene activation to a 15.10-fold induction in Hepa 1-6 wt, while Hepa 1-6 *Ifnl2/3* -/- clones showed a 3.64-fold induction for 1E5 and 4.09-fold induction in 7E6, *Cxcl10* expression levels thus staying well below those of Hepa 1-6 wt with 4.15 times fewer transcripts in 1E5 and 3.69 times fewer transcripts in 7E6 (figure 19A, 19B). In comparison, Hepa 1-6 7A6 wt also showed less induction of Cxcl10 gene activation with an 8.73-fold induction, equaling 1.73 times fewer transcription (figure 19A, 19B). Expressed in percentages, *Cxcl10* expression was set at 24.22% of stimulated Hepa 1-6 wt in stimulated Hepa 1-6 *Ifnl2/3* -/- 1E5, at 27.09% in stimulated Hepa 1-6 *Ifnl2/3* -/- 7E6 and at 57.81% in Hepa 1-6 7A6 wt.

As Hepa 1-6 wt had been shown react to gemcitabine exposure by expressing *Cxcl10* independently of IFN activation, this effect was also investigated in Hepa 1-6 *Ifnl2/3* -/- 1E5, depicted in figure 19C. Consistent with the previous results, Hepa 1-6 wt showed a 23.57-fold induction of *Cxcl10* transcript expression upon stimulation with a two-hour gemcitabine treatment and harvest at t = 10 h compared to the untreated control (figure 19C). Similarly, *Cxcl10* activation was also found in gemcitabine exposed Hepa 1-6 *Ifnl2/3* -/- 1E5 with a 28.25-fold induction compared to unstimulated Hepa 1-6 *Ifnl2/3* -/- 1E5 (figure 19C). This biological response induced by gemcitabine was thus comparable in extent in the isogenic *Ifnl2/3*-deficient clone.

Taken together, *Ifnl2/*3-deficiency was demonstrated to lower basal expression of *Mx1* and *Cxcl10*, diminishing *Mx1* induction by stimulation and attenuating *Cxcl10* induction by stimulation (figure 19A, 19B). Hepa 1-6 7A6 wt was found to differ in its expression of ISGs to the parental Hepa 1-6 wt cell line, which could suggest for example undetected off-target effects in this clone caused by the CRISPR/Cas9 technology (figure 19A, 19B).

Moreover, in addition to activating *Cxcl10* independently of stimulation, medium replacement or addition of nucleases as described in section 3.1.3, gemcitabine was shown to induce *Cxcl10* in the isogenic *Ifnl2/3*-deficient Hepa 1-6 clone 1E5 (figure 19C). *Cxcl10* induction

in Hepa 1-6 by gemcitabine was hence demonstrated to be truly independent from *Ifnl* activation as it was not only observable in the absence of any measurably type I or type III IFN activation but also found in CRISPR-Cas9 engineered *Ifnl2/3*-deficient clones.

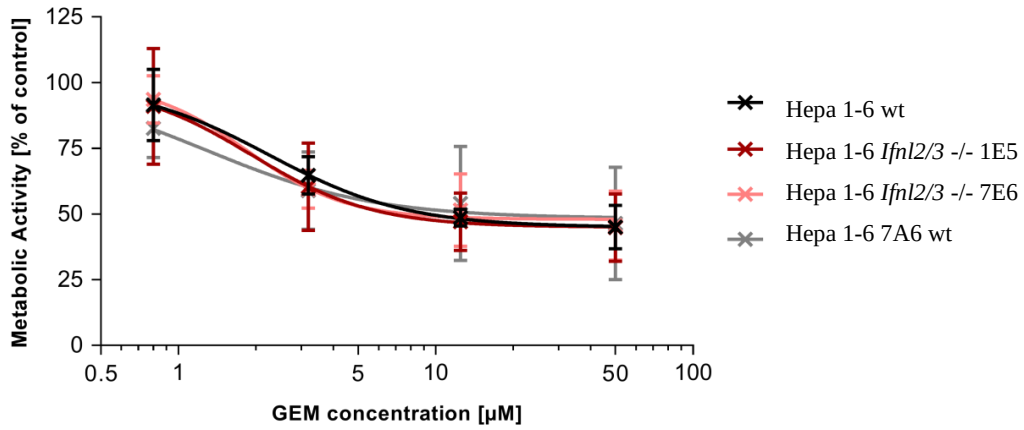
3.3.2 Effect of *Ifnl2/3*-Deficiency on Viability and Chemosensitivity

In order to evaluate the importance of IFN-λ for the susceptibility of hepatic cancer cells towards conventional chemotherapy *in vitro*, Hepa 1-6 *Ifnl2/3*-deficient clones were assessed with regards to viability as well as chemosensitivity.

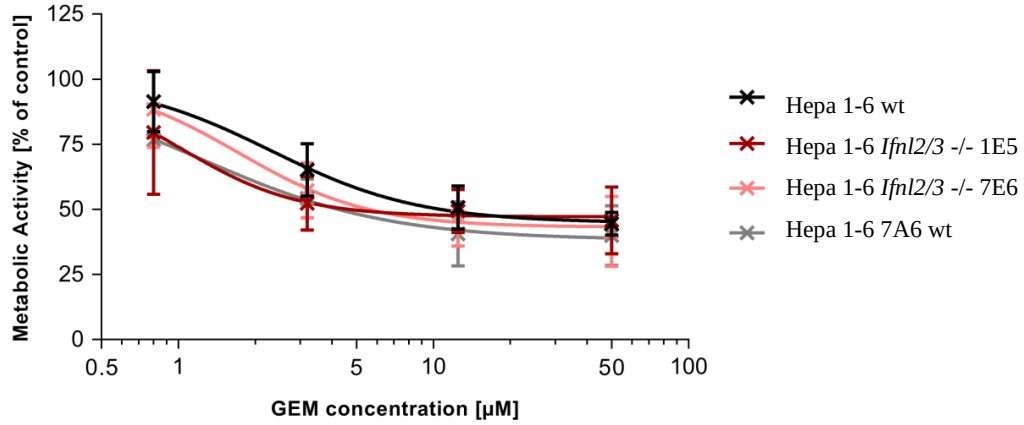
As already described in section 3.2.2, Hepa 1-6 isogenic clones did not distinctively differ to the parental Hepa 1-6 cell line with regards to basal viability, showing no signs for increased stress or cell death. This impression from the microscopic assessment of the clones' morphology was supported by comparable numbers of intact and compromised cells during cell counting with Trypan Blue exclusion. Hepa 1-6 clone viability was subsequently assessed in detail via MTS reduction assay (CellTiter 96 AQ One Solution Cell Proliferation Assay, Promega) as described in section 2.2.1. The Hepa 1-6 clones were compared to the parental Hepa 1-6 wt cell line with regard to chemosensitivity towards gemcitabine, doxorubicin and oxaliplatin to evaluate the impact of *Ifnl2/3* on the *in vitro* effects of these genotoxic drugs.

As depicted in figure 20, the selected Hepa 1-6 clones as well as Hepa 1-6 wt were subjected to four different concentrations of gemcitabine (figure 20.I), doxorubicin (figure 20.II) and oxaliplatin (figure 20.III) for either a short two-hour treatment (A), a short treatment with a one-hour poly(I:C):DEAE-dextran pulsing (B) or a sustained treatment of 17 h (C). Cell cultures were then assessed for metabolic activity utilizing the MTS reduction assay at either t = 17 h (C) or t = 24 h (A, B) and compared to the respective controls. Resulting percentages of metabolic activities were displayed as mean values of three to six independent data sets in dependency of the drugs' concentrations and analyzed in a variable slope four-parameter dose-response-curve using GraphPad Prism 7 (GraphPad Software Inc.). The comparison of the resulting curves showed no distinctive differences in chemosensitivity between Hepa 1-6 wt, Hepa 1-6 *Ifnl2/3* -/- 1E5, Hepa 1-6 *Ifnl2/3* -/- 7E6, and Hepa 1-6 7A6 wt (figure 20). Status of *Ifnl2/3* gene activation was thus demonstrated to not contribute to *in vitro* chemosensitivity towards gemcitabine, doxorubicin or oxaliplatin, as metabolic activities were found to be independent of *Ifnl2/3* expression.

A: Short Exposure



B: Short Exposure with Poly(I:C) Pulse



C: Sustained Exposure

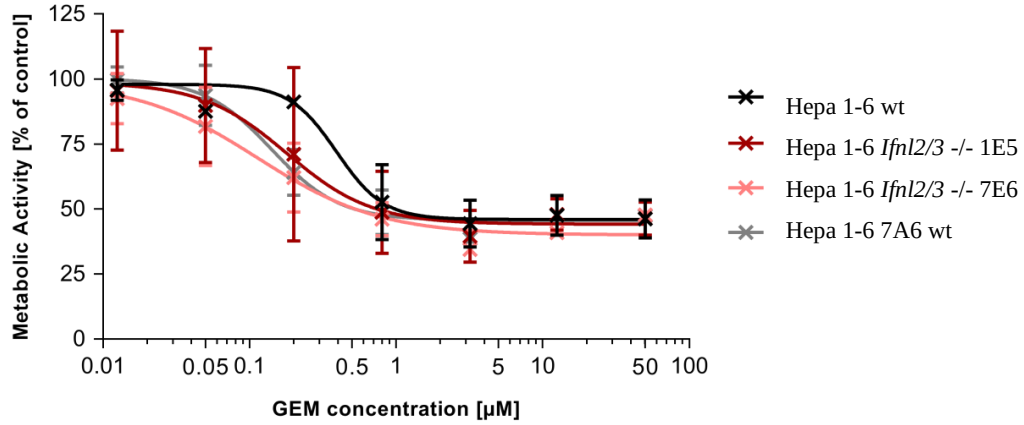
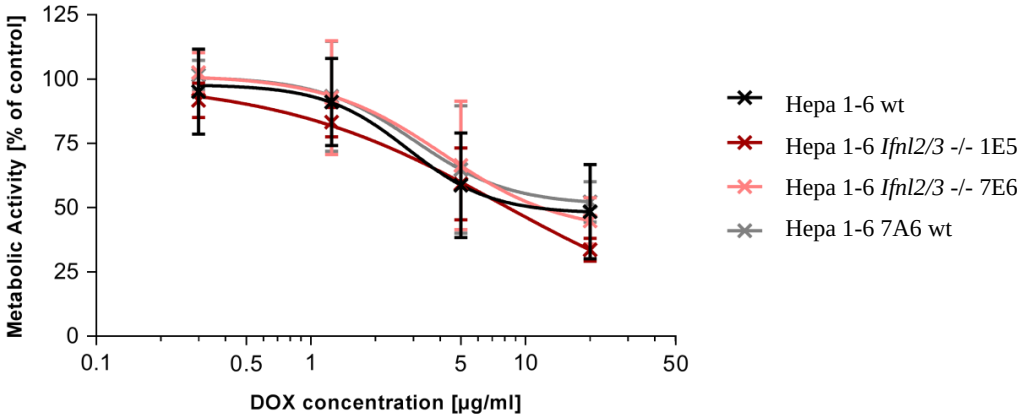
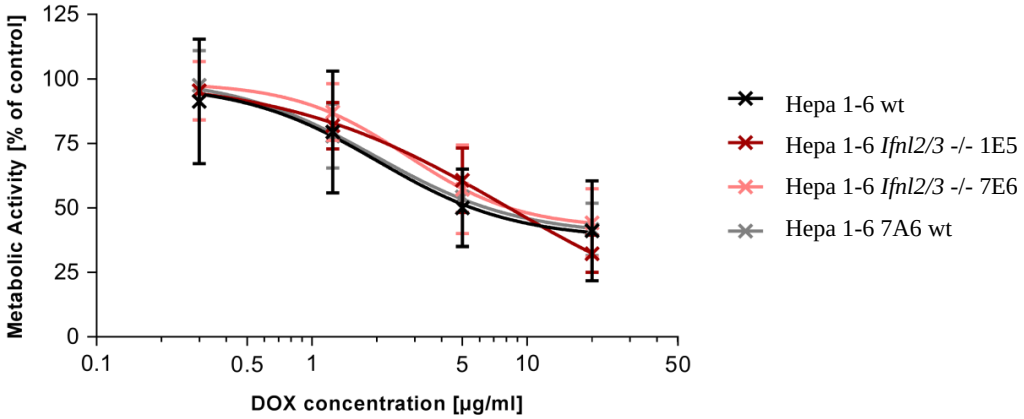


Figure 20.I: Chemosensitivity of Hepa 1-6 Wildtype and Isogenic Clones to Gemcitabine

A: Short Exposure



B: Short Exposure with Poly(I:C) Pulse



C: Sustained Exposure

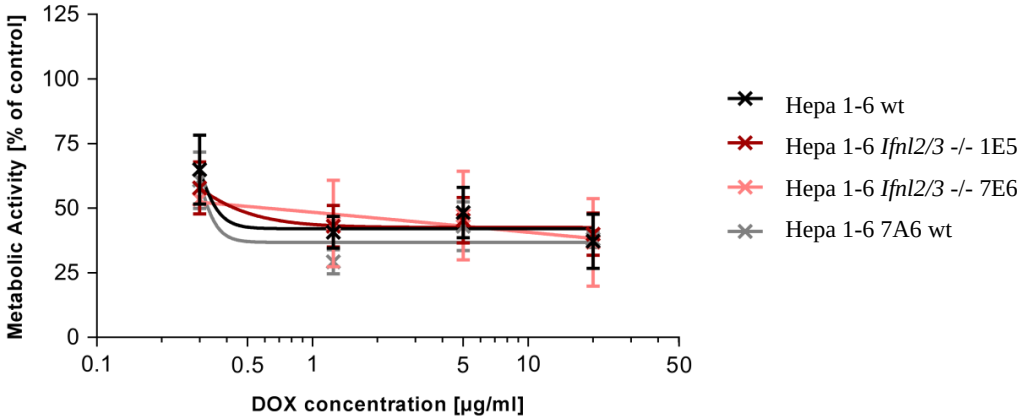
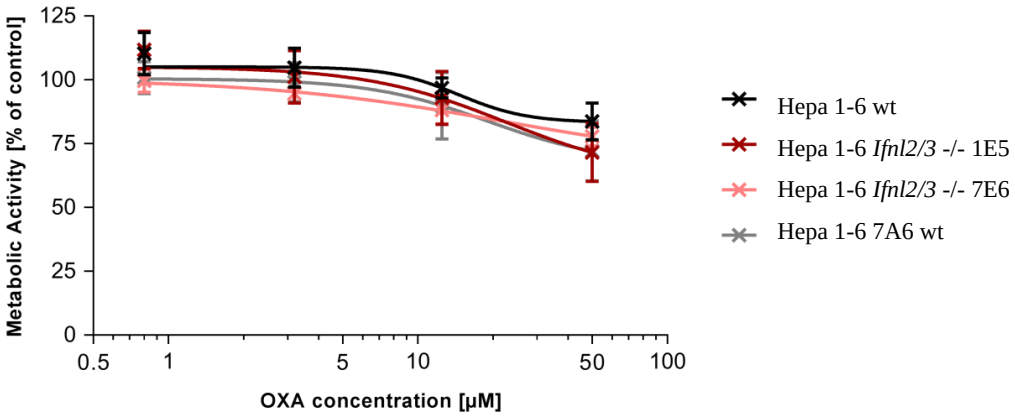
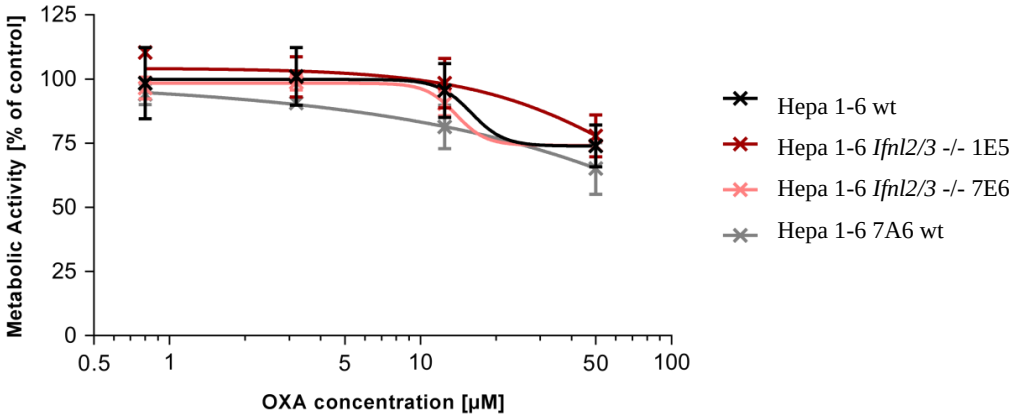


Figure 20.II: Chemosensitivity of Hepa 1-6 Wildtype and Isogenic Clones to Doxorubicin

A: Short Exposure



B: Short Exposure with Poly(I:C) Pulse



C: Sustained Exposure

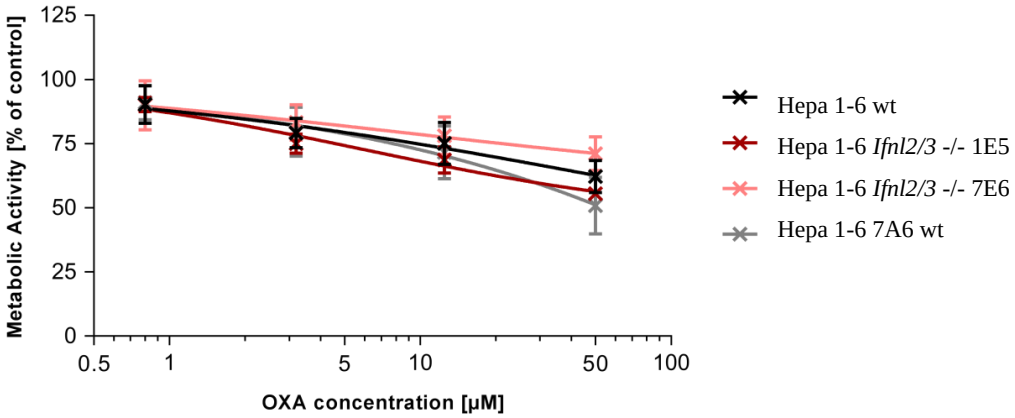


Figure 20.III: Chemosensitivity of Hepa 1-6 Wildtype and Isogenic Clones to Oxaliplatin

Figure 20.I-III: Chemosensitivity of Hepa 1-6 Wildtype and Isogenic Clones

Figure 20 depicts the chemosensitivity of Hepa 1-6 wt, *Ifnl2/3*-deficient clones and Hepa 1-6 7A6 wt towards gemcitabine (GEM) (figure 19.IA-IC), doxorubicin (DOX) (figure 19.IIA-IIC) and oxaliplatin (OXA) (19.IIIA-IIIC), respectively. $8x10^3$ cells of Hepa 1-6 wt and indicated clones were seeded as quadruplicate sets of 100 µl microcultures into 96-well-plates as depicted in detail in section 2.2.1. On the following day, cell cultures were treated with the indicated concentrations and chemotherapeutic drugs. Cultures were then either washed at t = 2 h (A), pulsed with poly(I:C):DEAE at t = 1 h and washed at t = 2 h (B), or left unwashed and thus continuously exposed to the chemotherapeutic (C). Cell cultures were assessed for metabolic activity via MTS reduction assay (CellTiter 96 AQ One Solution Cell Proliferation Assay, Promega) at t = 24 h (A, B) or t = 17 h (C). Medium extinctions of the quadruplicates were referenced to the respective untreated controls to receive percentages of metabolic activity. Percentages of metabolic activities were displayed in dependency of the applied drug concentration and are given as mean values ± standard deviation of three to six independent experiments. Percentages were analyzed in a variable slope four-parameter dose-response-curve for standard deviations and 95%-confidence intervals using GraphPad Prism 7 (GraphPad Software Inc.).

The analysis of the metabolic activity in a variable slope four-parameter dose-response-curve additionally allowed for calculating the half-maximal inhibitory concentrations (IC_{50}s) of the tested chemotherapeutics for each clone in comparison to Hepa 1-6 wt as displayed in table 7. However, the tested four concentrations per drug allowed for only a rough fit in the variable slope model, decreasing accuracy of the calculations or, in cases marked in table 7 by * or ~, resulting in very wide standard deviations indicating impractical (*) or greatly imprecise (~) analysis.

Nonetheless, the viability of non-primed Hepa 1-6 wt cells was shown to be inhibited dose-dependently by a two-hour exposure to gemcitabine (figure 20.IA) and doxorubicin (figure 20.IIA) with calculated IC_{50}s of 2.25 µM ± 0.44 and 2.83 µg/ml ± 1.20 (table 7), respectively, while being nearly unaffected by exposure to oxaliplatin (figure 20.IIIA). *Ifnl2/*3-deficient clones were not found to differ significantly from parental Hepa 1-6 cells or from Hepa 1-6 7A6 wt in their response to short chemotherapeutic treatment, neither in the absence of a poly(I:C) stimulus (figure 20.IA, 20.IIA, 20.IIIA) nor in its presence (figure 20.IB, 20.IIB, 20.IIIB), suggesting that *Ifnl2/3* activation is unlikely to contribute to antiproliferative effects.

Table 7: IC_{50}s of Hepa 1-6 Wildtype and Isogenic Clones for Selected Chemotherapeutics

	IC_{50}			
Cell Line	Gemcitabine [µM]	Doxorubicin [µg/ml]	Oxaliplatin [µM]	Exposure
Hepa 1-6 wt	2.25 ± 0.44	2.83 ± 1.20	~ 14.59 ± 16.43	Short
	0.39 ± 0.11	~ 0.28 ± 18.81	*	Sustained
Hepa 1-6 *Ifnl2/3* -/- 1E5	1.92 ± 0.66	7.75 ± 9.25	~ 22.62 ± 55.83	Short
	0.18 ± 0.07	0.19 ± 0.15	5.913 ± 4.11	Sustained
Hepa 1-6 *Ifnl2/3* -/- 7E6	1.85 ± 0.51	4.13 ± 1.90	~ 22.46 ± 33.70	Short
	0.11 ± 0.04	*	~ 9.32 ± 51.14	Sustained
Hepa 1-6 7A6 wt	1.29 ± 0.71	3.04 ± 1.16	~ 19.00 ± 34.93	Short
	0.14 ± 0.03	~ 0.28 ± 98.27	*	Sustained

$8x10^3$ cells of Hepa 1-6 wt and indicated clones were seeded as quadruplicate sets of 100 µl micro-cultures into 96-well-plates as depicted in detail in section 2.2.1. On the following day, cell cultures were treated with different concentrations of the indicated chemotherapeutic drugs for 2 h before the culturing medium was replaced. Cell cultures were then incubated and assessed for metabolic activity via MTS reduction assay (CellTiter 96 AQ One Solution Cell Proliferation Assay, Promega) at t = 24 h. Metabolic activities as medium extinction of the quadruplicates were referenced to the respective untreated controls and resulting percentages were displayed in dependency of the applied drug concentration. Mean percentages of three to six independent experiments were analyzed in a variable slope four-parameter dose-response-curve to receive IC_{50} values and standard deviations using GraphPad Prism 7 (GraphPad Software Inc.).

* analysis impossible (slope fit ambiguous), ~ analysis inaccurate (standard deviation very wide)

Chemosensitivity assessment and IC_{50} calculation were repeated for sustained exposure to the chemotherapeutic drugs as displayed in parts C of figure 20 as well as table 7. Similar to the short treatment regimens, long exposure to gemcitabine showed dose-dependent viability inhibition for Hepa 1-6 wt and isogenic clones (figure 20.IC) with IC_{50}s of 0.39 ± 0.11 for Hepa 1-6 wt, 0.18 ± 0.07 for Hepa 1-6 *Ifnl2/3* -/- 1E5, 0.11 ± 0.04 for Hepa 1-6 *Ifnl2/3* -/- 7E6, and 0.14 ± 0.03 for Hepa 1-6 7A6 wt (table 7). With regard to doxorubicin, long exposure showed high impact for all concentrations in Hepa 1-6 wt and the assessed clones, confounding the variable slope dose-response-curves and prohibiting accurate IC_{50} analysis

(figure 20.IIC, table 7). Just as the short treatment, sustained exposure to oxaliplatin did not greatly inhibit cell viability either, suggesting the tested concentrations to be ineffective in this cell line (figure 20.IIIC). For both oxaliplatin treatment regimens calculations of IC_{50} values were thus highly inaccurate (table 7).

For both the short and the long treatment regimens, calculation of significance was not performed as results were expected to likely be imprecise due to the small number of concentrations assessed and the therefore lowered quality of the variable slope model fit. Nonetheless, a lack of distinctive differences in viability and chemosensitivity of the parental Hepa 1-6 cell line and the CRISPR/Cas9 engineered isogenic clones can be assumed from visually comparing the assessed metabolic activities (figure 20). It was thus demonstrated that *Ifnl2/3*-deficiency does not have a distinctive impact on *in vitro* cell viability as well as the *in vitro* growth inhibitory capacity of gemcitabine, doxorubicin or oxaliplatin in the hepatic cancer cell line Hepa 1-6, setting the basis for future investigations of the effect of drug-driven tumor cell-intrinsic *Ifnl2/3* gene activation on immunoediting in hepatocellular carcinoma *in vivo*.

4. Discussion

The importance of ICD and resulting antitumoral immune responses in the search for more efficient cancer therapy regimes has been increasingly brought to attention by recent investigations. For instance, the activation of TLR3, simulated with the RNA mimetic poly(I:C), has been shown to exert both direct and indirect antitumoral effects in murine and human hepatoma cell lines, decreasing proliferation upon *in vitro* treatment and enhancing tumor control and local immune responses *in vivo* (Chew et al. 2012b; Ho et al. 2015). The infiltration of the tumorous tissue by immune cells and the evidence of inflammatory responses like TLR3 expression in the tumor microenvironment has been associated with increased survival in HCC, linking the observed local reactions to overall outcomes (Chew et al. 2010; Chew et al. 2012a; Lee et al. 2015). NK cells and T cells have been identified to be a central component of antitumor immunity as they are the principal effector cells in tumor-targeted immune responses, and activation of and infiltration by the same was correlated to increased TLR3 expression in patient samples by Valerie Chew et al. (Matsumoto and Seya 2008; Chew et al. 2012b). These findings were substantiated by investigations done by Victor Ho et al., who demonstrated improved tumor control by combining the established HCC therapeutic Sorafenib with a TLR3 agonist, indicating how further investigations and a deeper understanding of immunological mechanisms may offer new or improved therapeutical approaches to HCC and other highly chemoresistant cancers (Ho et al. 2015).

Several chemotherapeutic drugs have been shown to lead to cancer cell-intrinsic activation of type I IFNs provoking an antitumoral immune response (Sistigu et al. 2014; Minn 2015; Garg and Agostinis 2017). Additionally, drug efficacy of ICD inducing substances has been linked to the induction of IFNs and downstream effectors, as inhibition of the same impaired the antitumoral effects (Sistigu et al. 2014). Type I IFN activation *in vivo* has been shown to result from the emission of DAMPs such as ectopic aberrant nucleic acids resulting from DNA damage in stressed or dying cancer cells (Sistigu et al. 2014; Minn 2015; Garg and Agostinis 2017). The contribution of IFN-λ to immunoediting has not yet been elucidated.

The importance of IFN activation for the efficacy of chemotherapy and tumor control is not unique to HCC, however, it has been demonstrated to not always be favorable as investigations by Julie Gaston et al. (2016) revealed cell-intrinsic type I IFN signaling and subsequent ISG upregulation to contribute to resistance to treatment in breast cancer. Nevertheless, an IFN signature was shown to be favorable and predictive for a pathological complete response in several cohorts of breast cancer patients (Sistigu et al. 2014; Kim et al. 2016; Legrier et

al. 2016). This demonstrates the importance of investigating immunomodulatory effects individually for different tissues, species, and settings alike.

In a first step to investigate the contribution of type III IFNs to immunoediting during this thesis, the transplantable murine hepatoma cell line Hepa 1-6 was demonstrated to be capable of expressing type I and type III IFNs when properly stimulated. To achieve stimulation, cytoplasmatic exposure to the RNA analog poly(I:C) was obligatory. The transfection of poly(I:C) via DEAE was utilized to mimic the stimulation by an RNA DAMP or PAMP, that has been taken up into the cell. The exposure to poly(I:C) reliably led to the induction of *Ifna4* and *Ifnl2/3*, representatives for type I and type III IFNs, as well as increased levels in *Cxcl10* transcription and decreased expression of *Mx1*, a negative regulator of the IFN/ISG signaling cascade. The demonstrated type I IFN induction by poly(I:C) is consistent with data on other murine and human hepatoma cell lines showing decreased proliferative activity upon in vitro treatment as a direct antitumoral effect (Chew et al. 2012b; Ho et al. 2015). Additionally, it is also in line with preclinical data showing heterotopic Hepa 1-6 tumors to respond to poly(I:C) in terms of enhancing tumor control in combination with a boosted local immune response as an indirect antitumoral effect by immunoediting (Chew et al. 2012b; Ho et al. 2015). The demonstration of type III IFN gene activation was also an essential precondition to utilize Hepa 1-6 cells for *in vivo* studies on the contribution of type III IFNs in immunoediting.

Consecutively, the ability of Hepa 1-6 to induce IFNs in response to chemotherapy was investigated. While sole exposure to the chosen chemotherapeutic drugs doxorubicin, oxaliplatin and gemcitabine did not lead to measurable IFN induction, the combination with priming by the established stimulatory protocol showed a modulation of the poly(I:C)-primed *Ifnl2/3* activation. This effect was mirrored by an increase in *Cxcl10* levels as a downstream effector of *Ifnl2/3*. The lack of response to a sole chemotherapeutic stimulus in terms of IFN activation under the conditions tested might appear to be opposing to findings by Sabrina Brzostek-Racine et al. (2011), who described type III IFN (IFN-λ1) induction in primary human monocytes and human non-liver cancer cell lines by etoposide and other genotoxic agents *in vitro*. However, for murine cells, they found a rather modest activation of both type I and type III IFNs (Brzostek-Racine et al. 2011). IFN levels after exposure to chemotherapeutics alone in the setting of this thesis may thus have been below detection levels. These findings stress the need to elucidate antitumor mechanisms in the integrated preclinical settings that transplantable tumor cells within immunocompetent mice do provide.

With regard to type III IFN activation an additive, possibly synergistic, action of poly(I:C) priming and treatment with gemcitabine was demonstrated. This observation is compatible with the idea of synergistic or complementary interactions of two different kinds of DAMPs, namely the stimulant poly(I:C) acting upon transfection on cytoplasmic RNA sensors on one hand and an RNase-sensitive compound released from gemcitabine-treated dying tumor cells to the extracellular compartment on the other. This observation conceptually reflects current trials on TLR agonists as pharmacological adjuvants for the development of anti-cancer therapies: TLR agonists have been recognized as promising components if combined with conventional chemotherapeutics or with radiotherapy (Iribarren et al. 2016; Mikulandra et al. 2017). Recombinant IFN-λ itself is being tested in preclinical models as an anticancer agent for various tumor entities (Stiff and Carson 2015; Eslam and George 2016; Yan et al. 2017).

The observed IFN induction was diminished by timed culture medium exchange or the addition of RNA targeting nucleases, suggesting the release of a mediating compound. Albeit not reaching the adjusted level of significance, the data does suggest a contribution of a release and an extracellular supply of ribonucleic acids to cytoplasmic poly(I:C)-mediated PRR stimulation, yielding *Ifnl2/3* gene expression. This finding was supported by co-culturing experiments, in which IFN induction by gemcitabine was transferrable via culture medium. IFN induction was therefore demonstrated to be mediated, at least to some extent, by soluble factors. This is in line with findings by Antonella Sistigu et al., who utilized syngeneic tumor mouse models to demonstrate the induction of type I IFNs and the release of *Cxcl10* to rely on the release of self-RNA, which they identified to be a major mediator (Sistigu et al. 2014). Moreover, Antonella Sistigu et al. connected the rise of type I IFNs one to four days after chemotherapy to the occurrence of cell death within the tumor and the beginning of infiltration of the tumor by immune cells, suggesting the antitumor immune response caused by chemotherapeutics to stem from tumor-derived type I IFNs affecting both the tumor cells in an autocrine manner and the tumor microenvironment and immune cells in a paracrine manner (Michaud et al. 2011; Ma et al. 2013a; Sistigu et al. 2014).

In the setting of this thesis, the addition of RNase supposedly prohibited external stimulation of the cell by either RNA-derived DAMPs or other mediating factors. Nonetheless, IFN induction was not fully inhibited as the cytoplasmatic stimulation by poly(I:C):DEAE was not disrupted, accounting for the residual IFN levels measured. This suggests combined cytoplasmatic and extracellular exposure to be necessary for the generation of a measurable response in the experimental setting of this thesis. In regimes with only chemotherapy

induction as performed by Antonella Sistigu et al. (2014), complete extinction of the reaction could be achieved by the addition of RNase, however, the low levels of IFN induction in Hepa 1-6 in this thesis' setting did not allow for regimens with chemotherapy induction only to reproduce this result.

Hepa 1-6 cells were thus found to be responsive to conventional genotoxic drugs and suitable to address the impact of drug-driven tumor cell-intrinsic *Ifnl2/3* activation on immunoediting in HCC *in vitro* and *in vivo*. In order to investigate the significance of type III IFNs on the activation of type I IFNs by conventional chemotherapeutics, *Ifnl2/3*-deficient isogenic clones to the parental Hepa 1-6 cell line were generated via CRISPR/Cas9 gene engineering technology with an IL-28 double nickase transfection. The resulting clones were validated with regard to induced mutations and lack of *Ifnl2/3* expression. Of the five Hepa 1-6 clones investigated, Hepa 1-6 *Ifnl2/3* -/- 1E5 and Hepa 1-6 *Ifnl2/3* -/- 7E6 were chosen as *Ifnl2/3*-deficient cell lines to compare to the parental Hepa 1-6 wt, while Hepa 1-6 7A6 wt was selected to serve as a control.

Both Hepa 1-6 *Ifnl2/3* -/- 1E5 and Hepa 1-6 *Ifnl2/3* -/- 7E6 repeatedly showed absence of the targeted wildtype *Ifnl2/3* sequence on gDNA and mRNA level in independent assays. Additionally, no *Ifnl2/3* mRNA was detected when subjecting early stocks of these clones to the established stimulatory protocols. Interestingly, stocks of the same clones, having been stored in nitrogen tanks, were able to express low but measurable amounts of *Ifnl2/3* mRNA, while still lacking the wildtype *Ifnl2/3* sequence. It can thus be safely assumed, that the detected *Ifnl2/3* mRNA consisted of fragments and thus non-functional transcripts, and that all stocks utilized as *Ifnl2/3* -/- clones were representative for *Ifnl2/3*-deficient Hepa 1-6 cells.

To allow for comparative analysis, all selected isogenic Hepa 1-6 clones, the *Ifnl2/3*-deficient clones as well as Hepa 1-6 7A6, were subjected to the same experimental settings as their parental wildtype Hepa 1-6 cells and investigated for type I IFN and ISG expression.

While *Ifna4* expression levels in Hepa 1-6 *Ifnl2/3* -/- 1E5 and Hepa 1-6 7A6 did not differ from those in Hepa 1-6 wt, Hepa 1-6 *Ifnl2/3* -/- 7E6 showed remarkably higher levels of type I IFN expression. Functional *Ifnl2/3* transcripts were, as already investigated during the process of gene knockout validation, not detected for both Hepa 1-6 *Ifnl2/3* -/- 1E5 and Hepa 1-6 *Ifnl2/3* -/- 7E6. Hepa 1-6 7A6 was able to express *Ifnl2/3* as expected.

Additionally, lack of *Ifnl2/3* expression and functionality was indirectly confirmed by lower basal expressions of the associated ISGs *Mx1* and *Cxcl10* in *Ifnl2/3*-deficient clones. Basal expression of *Mx1* was decreased with 16.67 times fewer transcripts in Hepa 1-6 *Ifnl2/3* -/- 1E5 and 9.09 times fewer transcripts in Hepa 1-6 *Ifnl2/3* -/- 7E6 compared to unstimulated Hepa 1-6 wt. Hepa 1-6 *Ifnl2/3* -/- 1E5 and Hepa 1-6 *Ifnl2/3* -/- 7E6 showed decreased basal expression for the second ISG investigated as well with 3.23 and 2.17 times fewer *Cxcl10* transcripts than Hepa 1-6 wt, respectively.

When subjected to stimulation via poly(I:C):DEAE-dextran and superinduction, Hepa 1-6 *Ifnl2/3* -/- 7E6 showed only minimal suppression of *Mx1* expression from 0.11-fold basal expression in the unstimulated cell culture to 0.09-fold induction after stimulation. In Hepa 1-6 *Ifnl2/3* -/- 1E5 however, *Mx1* expression was suppressed by stimulation from the 0.06-fold basal expression to a 0.01-fold induction compared to unstimulated Hepa 1-6 wt. As for *Cxcl10*, results after stimulation were more notable. While all stimulated cultures showed an increase in *Cxcl10* expression, *Cxcl10* levels were remarkably lower in Hepa 1-6 *Ifnl2/3*-deficient clones, with a 15.20-fold induction in Hepa 1-6 wt compared to 3.64-fold induction in Hepa 1-6 *Ifnl2/3* -/- 1E5 and 4.09-fold induction in Hepa 1-6 *Ifnl2/3* -/- 7E6, which equals to 4.15 and 3.69 times fewer transcripts in the *Ifnl2/3*-deficient clones or 24.22% and 27.09% of *Cxcl10* expression compared to Hepa 1-6 wt, respectively.

As for Hepa 1-6 7A6, *Cxcl10* basal expression did not differ from that of Hepa 1-6 wt. However, Hepa 1-6 7A6 also expressed *Mx1* 16.67 times less than the native Hepa 1-6 wt. Stimulation did not impact *Mx1* expression in Hepa 1-6 7A6, while stimulated *Cxcl10* expression measured at 57.81% of that of stimulated Hepa 1-6 wt. This may be the result of off-target effects or modifications of the Ifn-λ gene locus that went undetected in the knockout validation. This stresses the importance of utilizing positive controls and investigating specifically for off-target effects.

Nonetheless, these results link *Cxcl10* responsiveness to *Ifna4* and *Ifnl2/3* expression, as *Ifnl2/3* deficiency leads to lower *Cxcl10* expression levels. This may be caused either by the lack of direct stimulation by *Ifnl2/3* or indirectly via the loss of the effects that type III IFNs exert on type I IFNs.

Valerie Chew et al. (2010; 2012a) demonstrated inflammatory tumor microenvironments, resulting T cell infiltration, and gene signatures including *CXCL10* to predict survival in patients with HCC. This was substantiated by findings of Jing Zhang et al. (2019) linking

high *CXCL10* expression in human HCC tumor tissue with improved overall survival. Baseline *CXCL10* expression may thus offer a way to sort HCC patients into high and low risk groups to improve therapy recommendations for example in the broad group of patients classified as BCLC-B. Similarly, baseline *MX1* expression may indicate an immune system's responsiveness to DAMPs as it has been linked to predictive IFN gene signatures for example in breast cancer (Sistigu et al. 2014). ISGs such as *CXCL10* could possibly be central biomarkers for the efficacy of ICD in HCC, as they have been shown to be induced by anthracyclines, which are important ICD triggers (Sistigu et al. 2014; Minn 2015). Therapies including stimulants of *CXCL10* expression could thus perhaps achieve improved survival.

The effect of type III IFNs on antitumor immunity may be mediated via CXCL10 or via impacting immunogenicity and inflammation in ways that have not been identified yet.

IFNL expression in humans varies due to polymorphisms as described by Ludmila Prokunina-Olsson et al. (2013). In HCV infections, *IFNL4* expression in humans has been shown to be associated with high expression of ISGs, which is however connected to poor treatment response for chronic hepatitis C, resulting in IFNL4 being non-favorable in the context of HCV infection (Honda et al. 2010; Hamming et al. 2013a; Prokunina-Olsson et al. 2013). If *IFNL4* status does impact *CXCL10* expression levels in the context of HCC and subsequently tumor infiltration by T cells and NK cells, evaluation of the same may thus be a useful tool to identify HCC patients who respond to and therefore profit from immunochemotherapy. However, a recent study by Henriette Huschka and Sabine Mihm (2020) analyzing The Cancer Genome Atlas (TCGA) data did not find a correlation of *IFNL* gene status and disease outcome for HCC as opposed to pancreatic ductal adenocarcinoma, where presence of *IFNL4* was shown to be an independent and favorable predictor. Nonetheless, *IFNL* gene status may be a predictor on the immune system's ability to react to ICD and hoist a targeted immune response, but of no consequence in the current therapy regime. If immunochemotherapy was offered to selected patients, an influence on disease outcome might become apparent.

When investigating ICD and antitumor immune responses, *in vivo* experiments are pivotal as only these can approximate the immune mechanisms of the living organism. However, *in vitro* experiments serve to exclude any direct effects of the gene knockout on the cells' viability and chemosensitivity that could confound the result of *in vivo* tumor models. Accordingly, the Hepa 1-6 clones as well as the parental Hepa 1-6 cell line were subjected to

gemcitabine, doxorubicin and oxaliplatin, and evaluated for metabolic activity as a marker for viability to compare the chemosensitivity of *Ifnl2/3*-deficient clones to that of Hepa 1-6 wt.

Neither the *Ifnl2/*3-deficient clones nor Hepa 1-6 7A6 diverged from Hepa 1-6 wt in their response to exposure to the selected chemotherapeutics, both with and without poly(I:C) stimulus. *Ifnl2/3*-deficiency can thus be assumed to not affect chemosensitivity to gemcitabine, doxorubicin or oxaliplatin *in vitro*. However, these results still require confirmation, as the number of both concentrations and observations for the chemosensitivity experiment in this thesis is not sufficient to allow for a reliable evaluation of significance or the calculation of IC_{50}s. However, both from theoretical considerations and the visual assessment of the experiments performed, *Ifnl2/3* activation is unlikely to greatly contribute to direct antiproliferative effects of the assessed chemotherapeutics. This is important groundwork for the future application of the generated *Ifnl2/*3-deficient Hepa 1-6 clones in syngeneic mouse models to assess the importance of type III IFNs in HCC *in vivo*.

While not an intended target of assessment, gemcitabine was additionally and unexpectedly found to be a potent inducer of the ISG *Cxcl10*, independent of priming and IFN induction, both in parental and *Ifnl2/3*-deficient Hepa 1-6 cells. While investigating the correlation of *Cxcl10* expression to the induction of type I and type III IFN mRNA, short exposure to gemcitabine was also observed to induce *Cxcl10* in the absence of any measurable type I or type III IFN induction. The strength of activation was substantially higher than that was achieved by poly(I:C) stimulation alone. This was demonstrated to be an effect of gemcitabine specifically, as no enhancement of chemokine expression was found for both oxaliplatin and doxorubicin, neither in unprimed nor in poly(I:C) primed cultures. Gemcitabine appeared to activate *Cxcl10* transcription directly, as its induction was neither impaired by medium replacement, nor transmissible by conditioned medium, nor abated by the addition of nucleases. Moreover, induction of *Cxcl10* by gemcitabine was observed in isogenic *Ifnl2/3*-deficient clones as well and in the absence of any measurable type I or type III IFN activation both in Hepa 1-6 wt cells and in the isogenic *Ifnl2/3*-deficient clones, suggesting an *Ifnl2/3*-independent activation of *Cxcl10*.

This finding is consistent with the demonstration of the existence of IFN-independent pathways to CXCL10 activation in primary human hepatocytes, suggesting *Cxcl10* expression to be less strictly indicative for IFN activation than other ISGs like *Mx1* (Brownell et al. 2013). The fact that *Cxcl10* inducibility is attainable by gemcitabine but not by doxorubicin

or oxaliplatin might be related to their modes of action. The three compounds all hamper duplication of genomic DNA, however by different mechanisms. The exact mode of action remains to be investigated, as well as the possible implications for the utilization, resulting advantages or also possibly disadvantages of gemcitabine as a chemotherapeutic in HCC.

The utilization of CRISPR/Cas9 gene technology to generate *Ifnl2/3*-deficient Hepa 1-6 clones for this thesis was the first use of this technique in this working group. Extensive protocols provided by Santa Cruz Biotechnology, Inc. allowed for quick and easy understanding of the experimental setup and process. While the initial genome editing was a quick and relatively easy process, the knockout validation and clone evaluation was everything but, taking up a large portion of the work and time invested in this thesis.

Even though specificity and efficiency of the CRISPR/Cas genome editing technique has been greatly enhanced during the years since its first application in 2012, off-target mutagenesis remains a major weak point (Jinek et al. 2012; Fu et al. 2013; Hsu et al. 2013; Pattanayak et al. 2013; Doudna and Charpentier 2014; Wu et al. 2014). Albeit improvements such as the double nickase approach and a multitude of sgRNA designing tools as a result of a deeper understanding of the mechanisms at work, off-target effects cannot be completely ruled out (Cong et al. 2013; Ran et al. 2013a; Cho et al. 2014).

Additionally, incomplete gene knockout had to be faced due to the approach of this thesis, as not one but two gene loci were targeted, resulting in a total of four target sequences in each cell. This greatly increased the potential for ineffective or incomplete gene knockout, as demonstrated by Hepa 1-6 clones with residual wildtype alleles not effectively leading to silencing of the targeted gene.

As demonstrated by the Hepa 1-6 clone sequences provided in the addenda, the extent of the mutation caused by the use of CRISPR/Cas9 gene technology cannot be predicted and ranged from a small 3 bp deletion to large indel mutations in this setting. This testifies to the impossibility of precisely estimating the results of genome editing. While this may not be as much of a problem in *in vitro* settings, it is certainly a point of limitation when planning to utilize this technique *in vivo*. This thesis' setup allowed for the extensive evaluation of the clones before assessing them in experimental setups to identify optimal candidates, but that may not be true for other settings. This stresses the need for thorough and critical evaluations of any genome editing performed to ensure accurate results and rule out unwanted off-target effects or accidental survival advantages or disadvantages. This process requires

determination as it is time consuming and often tedious, however it is imperative for conclusive and reliable research results.

5. Summary

HCC is characterized by limited therapy options, poor response rates to systemic chemotherapy, and high lethality. Preclinical cancer models have revealed the efficacy of genotoxic drugs to rely on the release of type I IFNs by neoplastic cells boosting an antitumor response. This thesis elucidates the capacity of the transplantable hepatoma cancer cell line Hepa 1-6 to activate type I IFNs (IFN-α/β), type III IFNs (IFN-λ) and IFN effectors, and compares the parental Hepa 1-6 cell line to CRISPR/Cas9-engineered *Ifnl2/3*-deficient isogenic clones in view of preclinical models.

The murine hepatoma cell line Hepa 1-6 was demonstrated to be able to express both type I and type III IFNs if sufficiently stimulated via transfection with the PRR agonist poly(I:C), which mimics exposure to mRNA PAMPs or DAMPs. Additionally, the chemotherapeutic substances gemcitabine, oxaliplatin and doxorubicin were shown to induce type I and type III IFNs in Hepa 1-6 when combined with priming by a PRR agonist. This may be due complementary stimulation of cytoplasmic inner receptors (via poly(I:C) transfection) and membranous outer receptors (via DAMPs induced by the chemotherapeutic). Chemotherapy was thus demonstrated to be able to modulate poly(I:C)-primed *Ifnl2/3* activation. IFN induction was also reflected in Cxcl10 induction rather than Mx1 levels. Both IFN and ISG induction were mediated by soluble factors, as they were diminished by culture medium exchange or the addition of RNA targeting nucleases.

Furthermore, gemcitabine was revealed to incite *Cxcl10* expression in Hepa 1-6 independently from IFN-λ, as this effect was reproducible in both Hepa 1-6 wildtype and *Ifnl2/3*-deficient cells. The *Cxcl10* induction appeared to be a direct effect, as it was neither impaired by medium replacement, nor transmissible by conditioned medium, nor abated by the addition of nucleases. These novel findings can be expected to impact interactive immunoediting in HCC *in vivo*.

The secretion of type I IFNs by tumor cells has been identified as central to antitumoral immune responses, but the role of type III IFNs has not yet been thoroughly investigated. The effect of type I IFNs is mainly mediated through autocrine effects on the tumor itself as well as paracrine effects on the tumor microenvironment including immune cells. The excretion of chemokines adds to the process of immunoediting by complementing activation via DAMPs. This effect, leading to ICD, is by mechanism expected to be mainly observable *in vivo*, as it requires the delicate interaction of tumor and host immune system. To elucidate

the impact of type III IFNs on type I IFN activation, tumor growth and eventually tumor immunogenicity, transplantable isogenic *Ifnl2/3*-deficient Hepa 1-6 cell clones were established via CRISPR/Cas9 gene technology utilizing an IL-28 double nickase transfection. While this thesis concentrates on *in vitro* effects of *Ifnl2/3*-deficiency, the working group's goal of establishing an *Ifnl2/3*-deficient Hepa 1-6 clone to utilize in a future *in vivo* mouse model, the gold standard for ICD research, was also kept in mind.

The selected clones were confirmed to lack *Ifnl2/3* and showed distinctive differences in ISG expression. To ensure that *Ifnl2/3*-deficiency itself did not influence viability or chemosensitivity, the *Ifnl2/3*-deficient Hepa 1-6 clones were subjected to the same experiments as the parental wildtype cell line, demonstrating comparable *in vitro* chemosensitivity to gemcitabine, oxaliplatin and doxorubicin. The direct effect of these chemotherapeutics on tumorous cells did thus not rely on expression of *Ifnl2/3*, excluding this possible confounding factor from all future experiments. Therefore, the established Hepa 1-6 cell clones are suitable to assess the role of type III IFNs in HCC viability, chemosensitivity and immunogenicity *in vivo*. It can be expected that lack of type III IFNs affects the impact of drug-driven tumor cell-intrinsic IFN-α/β gene expression and subsequently the tumor growth and the establishment of an antitumoral immune response in immunocompetent mice *in vivo*. The impact of gemcitabine-induced IFN-λ and Cxcl10 on immunoediting and ICD in HCC poses an additional and interesting question for further research.

6. Addenda - Hepa 1-6 Clone Sequences:

Sequences are provided for the clones Hepa 1-6 1E5, Hepa 1-6 7A6, Hepa 1-6 7C1, Hepa 1-6 7E6 and Hepa 1-6 9H2, and aligned against Hepa 1-6 wt.

Sequence alignment was performed using the NCBI´s nucleotide alignment tool BLAST® provided by the NCBI, U.S. National Library of Medicine.

Q = Query, S = Sbjct (Subject)

Primer target sequences

6.1 Hepa 1-6 1E5

Alignment Hepa 1-6 wt and Hepa 1-6 clone 1E5 for IFNL23

```
Q 1     GYNCATACAGGAGGGCAGAGGGAGACTCACGATGGCATCCTTGGCCTTTTTGAAGGCCTG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 1     NN---TACAGGAGGGCAGAGGGAGACTCACGATGGCATCCTTGGCCTTTTTGAAGGCCTG  57

Q 61    CAGCTCTTTTGGGGACAGAGACTTGAACTGAGCAATGTGGCAATCCTTTGCTTCCACTGG  120
        ||||||||||||                ||||||||||||||||||||||||||||||||
S 58    CAGCTCTTTTGG----------------TGAGCAATGTGGCAATCCTTTGCTTCCACTGG  101

Q 121   GAGCCTGGTGGCCCTGGGGACAGGGTCAGCTTGGGTTCT   159
        |||||||||||||||||||||||||||||||||||||||
S 102   GAGCCTGGTGGCCCTGGGGACAGGGTCAGCTTGGGTTCTT
```

Alignment Hepa 1-6 wt and Hepa 1-6 clone 1E5 for IFNL2

```
Q 1    TCCAGCTTCCTGTGGGAAGCCTCCTGACGAACCTTGCCCCAGGTGACCTGGACTCTGTCA  60
                     ||||||||||||||||||||||||||||||||||||||||||||||
S 1                  AAGCCTCCTGACGAACCTTGCCCCAGGTGACCTGGACTCTGTCA  44

Q 61   TTTTCTCTTTCCCTGCAGTGTCTCACCTGCTCTCGCCACGCCCTGCTCTGGGCTTCCCCA  120
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 45   TTTTCTCTTTCCCTGCAGTGTCTCACCTGCTCTCGCCACGCCCTGCTCTGGGCTTCCCCA  104

Q 121  GCCTGGGCTCCCTAGTGGCAGGTATCAACCTGCTACCTTATTTTCACTTTTCCTACATCA  180
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 105  GCCTGGGCTCCCTAGTGGCAGGTATCAACCTGCTACCTTATTTTCACTTTTCCTACATCA  164

Q 181  GCTGGGGCTGCCCATCAGACCAGGTTAAAAGCATGGAGCACGGATGGCAGTGCACTCCAC  240
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 165  GCTGGGGCTGCCCATCAGACCAGGTTAAAAGCATGGAGCACGGATGGCAGTGCACTCCAC  224

Q 241  AGAGCTGGAAACTCAGAGCCTCAGTCCTCACACAGCAACAGGCCACAGGGGACGACCCAG  300
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 225  AGAGCTGGAAACTCAGAGCCTCAGTCCTCACACAGCAACAGGCCACAGGGGACGACCCAG  284

Q 301  GCCAGAGACACCAAGCAAGAACCAGAGAAAACCTCAAGGGAGACCCGAGTCCCTATCTCC  360
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 285  GCCAGAGACACCAAGCAAGAACCAGAGAAAACCTCAAGGGAGACCCGAGTCCCTATCTCC  344

Q 361  TCACAGACCCCGGAGAGCAACATGAAGCCAGGTGAGTCCCGAGAATGGTGTGTGGGTGTG  420
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 345  TCACAGACCCCGGAGAGCAACATGAAGCCAGGTGAGTCCCGAGAATGGTGTGTGGGTGTG  404

Q 421  TGTCCCAAGAGCCACCTTGCTACACATCTCACTCTAATCTCACTCTGTCCCTCTGTGACA  480
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 405  TGTCCCAAGAGCCACCTTGCTACACATCTCACTCTAATCTCACTCTGTCCCTCTGTGACA  464

Q 481  CAGAAACAGCTGGGGGCCACATGCTCCTCCTGCTGTTGCCTCTGCTGCTGGCCACAGTGC  540
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 465  CAGAAACAGCTGGGGGCCACATGCTCCTCCTGCTGTTGCCTCTGCTGCTGGCCACAGTGC  524

Q 541  TGACAAGAACCCAAGCTGACCCTGTCCCCAGGGCCACCAGGCTCCCAGTGGAAGCAAAGG  600
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 525  TGACAAGAACCCAAGCTGACCCTGTCCCCAGGGCCACCAGGCTCCCAGTGGAAGCAAAGG  584

Q 601  ATTGCCACATTGCTCA-----------CCAAAAGAGCTGCAGGCCTTCAAAAAGG  644
       ||||||||||||||||           ||||||||||||||||||||||||||||
S 585  ATTGCCACATTGCTCAGTTCAAGTCTCTGTCCCCAAAAGAGCTGCAGGCCTTCAAAAAGG  644

Q 645  CCAAGGATGCCATCGTGAGTCTCCCTCTGCCCTCCTGTATGGGCTAGCCTCCTCCACCCT  704
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 645  CCAAGGATGCCATCGTGAGTCTCCCTCTGCCCTCCTGTATGGGCTAGCCTCCTCCACCCT  704

Q 705  TCCCTTTCTGGGTTTCATCCCTT   727
       |||||||||||||||||||
S 705  TCCCTTTCTGGGTTTCATCCTWNY  728
```

6.2 Hepa 1-6 7A6

Alignment Hepa 1-6 wt and Hepa 1-6 clone 7A6 for IFNL23

- Alignment against Hepa 1-6 WT for

```
Q 1     GTNTCTGTCCCCAAAAGAGCTGCAGGCCTTCAAAAAGGCCAAGGATGCCATCGTGAGTCT  60
        || ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 1     GTCTCTGTCCCCAAAAGAGCTGCAGGCCTTCAAAAAGGCCAAGGATGCCATCGTGAGTCT  60

Q 61    CCCTCTGCCCTCCTGTATGGGCTAGCCTCCTCCACCCTTCCCTTTCTGGGTTTCATCCCT  120
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 61    CCCTCTGCCCTCCTGTATGGGCTAGCCTCCTCCACCCTTCCCTTTCTGGGTTTCATCCCT  120

Q 121   TATGCTAACTGGRATGAAAATGGTCTCC                  148
        |||||||||||| ||||||||||||||
S 121   TATGCTAACTGGNATGAAAATGGTCTCCACACTTTGTGCAAAA  163
```

- Alignment against Hepa 1-6 WT rev

```
Q 1     GYNCATACAGGAGGGCAGAGGGAGACTCACGATGGCATCCTTGGCCTTTTTGAAGGCCTG  60
          | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 1     NNN-ATACAGGAGGGCAGAGGGAGACTCACGATGGCATCCTTGGCCTTTTTGAAGGCCTG  59

Q 61    CAGCTCTTTTGGGGACAGAGACTTGAACTGAGCAATGTGGCAATCCTTTGCTTCCACTGG  120
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 60    CAGCTCTTTTGGGGACAGAGACTTGAACTGAGCAATGTGGCAATCCTTTGCTTCCACTGG  119

Q 121   GAGCCTGGTGGCCCTGGGGACAGGGTCAGCTTGGGTTCT    159
        |||||||||||||||||||||||||||||||||||||
S 120   GAGCCTGGTGGCCCTGGGGACAGGGTCAGCTTGGGTTCTTN  160
```

6.3 Hepa 1-6 7C1

Alignment Hepa 1-6 wt and Hepa 1-6 clone 7C1 for IFNL2

```
Q 1                        AAGCCTCCTGACGAACCTTGCCCCAGGTGACCTGGACTCTG  41
                           |||||||||||||||||||||||||||||||||||||||||
S 1    GCCTCCAGCTTCCTGTGGGAAGCCTCCTGACGAACCTTGCCCCAGGTGACCTGGACTCTG  60

Q 42   TCATTTTCTCTTTCCCTGCAGTGTCTCACCTGCTCTCGCCACGCCCTGCTCTGGGCTTCC  101
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 61   TCATTTTCTCTTTCCCTGCAGTGTCTCACCTGCTCTCGCCACGCCCTGCTCTGGGCTTCC  120

Q 102  CCAGCCTGGGCTCCCTAGTGGCAGGTATCAACCTGCTACCTTATTTTCACTTTTCCTACA  161
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 121  CCAGCCTGGGCTCCCTAGTGGCAGGTATCAACCTGCTACCTTATTTTCACTTTTCCTACA  180

Q 162  TCAGCTGGGGCTGCCCATCAGACCAGGTTAAAAGCATGGAGCACGGATGGCAGTGCACTC  221
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 181  TCAGCTGGGGCTGCCCATCAGACCAGGTTAAAAGCATGGAGCACGGATGGCAGTGCACTC  240

Q 222  CACAGAGCTGGAAACTCAGAGCCTCAGTCCTCACACAGCAACAGGCCACAGGGGACGACC  281
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 241  CACAGAGCTGGAAACTCAGAGCCTCAGTCCTCACACAGCAACAGGCCACAGGGGACGACC  300

Q 282  CAGGCCAGAGACACCAAGCAAGAACCAGAGAAAACCTCAAGGGAGACCCGAGTCCCTATC  341
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 301  CAGGCCAGAGACACCAAGCAAGAACCAGAGAAAACCTCAAGGGAGACCCGAGTCCCTATC  360

Q 342  TCCTCACAGACCCCGGAGAGCAACATGAAGCCAGGTGAGTCCCGAGAATGGTGTGTGGGT  401
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 361  TCCTCACAGACCCCGGAGAGCAACATGAAGCCAGGTGAGTCCCGAGAATGGTGTGTGGGT  420

Q 402  GTGTGTCCCAAGAGCCACCTTGCTACACATCTCACTCTAATCTCACTCTGTCCCTCTGTG  461
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 421  GTGTGTCCCAAGAGCCACCTTGCTACACATCTCACTCTAATCTCACTCTGTCCCTCTGTG  480

Q 462  ACACAGAAACAGCTGGGGGCCACATGCTCCTCCTGCTGTTGCCTCTGCTGCTGGCCACAG  521
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 481  ACACAGAAACAGCTGGGGGCCACATGCTCCTCCTGCTGTTGCCTCTGCTGCTGGCCACAG  540

Q 522  TGCTGACAAGAACCCAAGCTGACCCTGTCCCCAGGGCCACCAGGCTCCCAGTGGAAGCAA  581
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 541  TGCTGACAAGAACCCAAGCTGACCCTGTCCCCAGGGCCACCAGGCTCCCAGTGGAAGCAA  600

Q 582  AGGATTGCCACATTGCTCAGTTCAAGTCTCTGTCCCCAAAAGAGCTGCAGGCCTTCAAAA  641
       |||||||||||||   |||||||||||||||||||||||||||||||||||||||||||
S 601  AGGATTGCCACATT---CAGTTCAAGTCTCTGTCCCCAAAAGAGCTGCAGGCCTTCAAAA  657

Q 642  AGGCCAAGGATGCCATCGTGAGTCTCCCTCTGCCCTCCTGTATGGGCTAGCCTCCTCCAC  701
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 658  AGGCCAAGGATGCCATCGTGAGTCTCCCTCTGCCCTCCTGTATGGGCTAGCCTCCTCCAC  717

Q 702  CCTTCCCTTTCTGGGTTTCATCCTWNY  728
        ||||||||||||||||||||||||
S 718  CCTTCCCTTTCTGGGTTTCATCCCTT   743
```

Alignment Hepa 1-6 wt and Hepa 1-6 clone 7C1 for IFNL3

```
S 1     TAAGTCAGCCCCACTGCACAAAGTGTGGAGACCATTTTCATTCCAGTTAGCATAAGGGAT  60

Q 1                     AGGNTGGAGGAGGCTAGCCCATACAGGAGGGCAGAGGGAGACTC  44
                        ||| ||||||||||||||||||||||||||||||||||||||||
S 61    GAAACCCAGAAAGGGAAGGGTGGAGGAGGCTAGCCCATACAGGAGGGCAGAGGGAGACTC  120

Q 45    ACGATGGCATCCTTGGCCTTTTTGAAGGCCTGCAGCTCTTTTGGGGACAGAGACTTGAAC  104
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 121   ACGATGGCATCCTTGGCCTTTTTGAAGGCCTGCAGCTCTTTTGGGGACAGAGACTTGAAC  180

Q       ------------------------------------------------------------

S 181   ATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGAGAATA  240

Q       ------------------------------------------------------------

S 241   GCAGGCATGCTGGGGAGCGGCCGCAGGAACCCCTAGTGATGGAGTTGGCCACTCCCTCTC  300

Q 105   ----------------------------------TGAGCAATGTGGCAATCCTTTGCTTCC  131
                                           ||||||||||||||||||||||||||
S 301   TGCGCGCTCGCTCGCTCACTGAGGCCGCCGAACTGAGCAATGTGGCAATCCTTTGCTTCC  360

Q 132   ACTGGGAGCCTGGTGGCCCTGGGGACAGGGTCAGCTTGGGTTCTTGTCAGCACTGCGGCC  191
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 361   ACTGGGAGCCTGGTGGCCCTGGGGACAGGGTCAGCTTGGGTTCTTGTCAGCACTGCGGCC  420

Q 192   AGCAGCAGAGGCAACAGCAGGAGGAGCATGTGGCCCCCAGCTGTTTCTGTGTCACAGAGG  251
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 421   AGCAGCAGAGGCAACAGCAGGAGGAGCATGTGGCCCCCAGCTGTTTCTGTGTCACAGAGG  480

Q 252   GACAGAGTGAGATTAGAGTGAGATGTGTAGCAAGGTGGCTCTTGGGACACACACCCACAC  311
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 481   GACAGAGTGAGATTAGAGTGAGATGTGTAGCAAGGTGGCTCTTGGGACACACACCCACAC  540

Q 312   ACCATTCTCGGGACTCACCTGGCTTCATGTTGCTCTCCGGGGTCTGTGAGGAGATAGGGA  371
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 541   ACCATTCTCGGGACTCACCTGGCTTCATGTTGCTCTCCGGGGTCTGTGAGGAGATAGGGA  600

Q 372   CTCGGGTCTCCCTTGAGGTTTTCTCTGGTTCTTGCTTGGTGTCTCTGGCCTGGGTCGTCC  431
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 601   CTCGGGTCTCCCTTGAGGTTTTCTCTGGTTCTTGCTTGGTGTCTCTGGCCTGGGTCGTCC  660

Q 432   CCTGTGGCCTGTTGCTGTGTGAGGACTGAGGCTCTGAGTTTCCAGCTCTGTGGAGTGCAC  491
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 661   CCTGTGGCCTGTTGCTGTGTGAGGACTGAGGCTCTGAGTTTCCAGCTCTGTGGAGTGCAC  720

Q 492   TGCCATCCGTGCTCCATGCTTTTAACCTGGTCTGATGGGCAGCCCCAGCTGATGTAGGAA  551
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 721   TGCCATCCGTGCTCCATGCTTTTAACCTGGTCTGATGGGCAGCCCCAGCTGATGTAGGAA  780

Q 552   AAGTGAAAATAAGGTAGCAGGTTGACACCAGCCACTAGGAGAGCCCAGGCTGGGGAAGCC  611
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 781   AAGTGAAAATAAGGTAGCAGGTTGACACCAGCCACTAGGAGAGCCCAGGCTGGGGAAGCC  840

Q 612   CAGAGCAGGGTGTGGCGAGAGCAGGTGAGACACTGCAGGGAAAGAGAAAATGACAGAGTC  671
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 841   CAGAGCAGGGTGTGGCGAGAGCAGGTGAGACACTGCAGGGAAAGAGAAAATGACAGAGTC  900
```

6.4 Hepa 1-6 7E6

Alignment Hepa 1-6 wt and Hepa 1-6 clone 7E6 for IFNL2

```
Q 1                AAGCCTCCTGACGAACCTTGCCCCAGGTGACCTGGACTCTGTCATTT  47
                   |||||||||||||||||||||||||||||||||||||||||||||||
S 1    NGCTTCCTGTGGGAAGCCTCCTGACGAACCTTGCCCCAGGTGACCTGGACTCTGTCATTT  60

Q 48   TCTCTTTCCCTGCAGTGTCTCACCTGCTCTCGCCACGCCCTGCTCTGGGCTTCCCCAGCC  107
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 61   TCTCTTTCCCTGCAGTGTCTCACCTGCTCTCGCCACGCCCTGCTCTGGGCTTCCCCAGCC  120

Q 108  TGGGCTCCCTAGTGGCAGGTATCAACCTGCTACCTTATTTTCACTTTTCCTACATCAGCT  167
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 121  TGGGCTCCCTAGTGGCAGGTATCAACCTGCTACCTTATTTTCACTTTTCCTACATCAGCT  180

Q 168  GGGGCTGCCCATCAGACCAGGTTAAAAGCATGGAGCACGGATGGCAGTGCACTCCACAGA  227
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 181  GGGGCTGCCCATCAGACCAGGTTAAAAGCATGGAGCACGGATGGCAGTGCACTCCACAGA  240

Q 228  GCTGGAAACTCAGAGCCTCAGTCCTCACACAGCAACAGGCCACAGGGGACGACCCAGGCC  287
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 241  GCTGGAAACTCAGAGCCTCAGTCCTCACACAGCAACAGGCCACAGGGGACGACCCAGGCC  300

Q 288  AGAGACACCAAGCAAGAACCAGAGAAAACCTCAAGGGAGACCCGAGTCCCTATCTCCTCA  347
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 301  AGAGACACCAAGCAAGAACCAGAGAAAACCTCAAGGGAGACCCGAGTCCCTATCTCCTCA  360

Q 348  CAGACCCCGGAGAGCAACATGAAGCCAGGTGAGTCCCGAGAATGGTGTGTGGGTGTGTGT  407
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 361  CAGACCCCGGAGAGCAACATGAAGCCAGGTGAGTCCCGAGAATGGTGTGTGGGTGTGTGT  420

Q 408  CCCAAGAGCCACCTTGCTACACATCTCACTCTAATCTCACTCTGTCCCTCTGTGACACAG  467
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 421  CCCAAGAGCCACCTTGCTACACATCTCACTCTAATCTCACTCTGTCCCTCTGTGACACAG  480

Q 468  AAACAGCTGGGGGCCACATGCTCCTCCTGCTGTTGCCTCTGCTGCTGGCCACAGTGCTGA  527
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 481  AAACAGCTGGGGGCCACATGCTCCTCCTGCTGTTGCCTCTGCTGCTGGCCACAGTGCTGA  540

Q 528  CAAGAACCCAAGCTGACCCTGTCCCCAGG-GCCACCAGGC-TCCCAGTGGAAGCAAAGGA  585
       |||||||||||||| |    | ||   |  |||     |  || |  |     |    |
S 541  CAAGAACCCAAGCTSAMMMWGGCCMMGGATGCCMTMGKGWGTCTCYSTMTGCMCWCSWGT  600

Q 586  TTGCCACAT--TGCT---CAGTTCAAGTCTCTGTCCCCAAAAGAGCTGCAGGCCTTCAA-  639
        ||    |   | ||   |  |||   | ||||               ||  |||
S 601  RTGRSMTAGCYTMCTYCWCCCTTCMM-TWTCTGSRTKT----------CATCCCTNANSN  649

Q 640  AAAGGCCAAGGATGCCATCGTGAGTCTCCCTCTGCCCTCCTGTATGGGCTAGCCTCCTCC  699
       ||| |    |||     |||||||||||||||||||||||||||||||||||||||||||
S 650  AAARGKG--GGAANT--TCGTGAGTCTCCCTCTGCCCTCCTGTATGGGCTAGCCTCCTCC  705

Q 700  ACCCTTCCCTTTCTGGGTTTCATCCTWNY        728
       |||||||||||||||||||||||||
S 706  ACCCTTCCCTTTCTGGGTTTCATCCCTTANTWAAM  740
```

Alignment Hepa 1-6 wt and Hepa 1-6 clone 7E6 for IFNL3

```
Q 1    NTTTTTTTGTAGCCTCCTGACGAACCTTGCCCCAGGTGACCTGGACTCTGTCATTTTCTC  60
                                                        ||||||||
S 1                                                     ATTTTCTC  8

Q 61   TTTCCCTGCAGTGTCTCACCTGCTCTCGCCACACCCTGCTCTGGGCTTCCCCAGCCTGGG  120
       |||||||||| ||||||| |||||||| ||  ||||||||| ||||||||||||||||||
S 9    TTTCCCTGCANTGTCTCANCTGCTCTCNCCNMACCCTGCTCNGGGCTTCCCCAGCCTGGG  68

Q 121  CTCTCCTAGTGGCTGGTGTCAACCTGCTACCTTATTTTCACTTTTCCTACATCAGCTGGG  180
       |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
S 69   CTCTCCTAGTGGCTGGTRTCAACCTGCTACCTTATTTTCACTTTTCCTACATCAGCTGGG  128

Q 181  GCTGCCCATCAGACCAGGTTAAAAGCATGGAGCACGGATGGCAGTGCACTCCACAGAGCT  240
       |||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
S 129  GCTGCCCATCAGACCAGGTTAAAAGCATGGAGCACGGATGGMAGTGCACTCCACAGAGCT  188

Q 241  GGAAACTCAGAGCCTCAGTCCTCACACAGCAACAGGCCACAGGGGACGACCCAGGCCAGA  300
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 189  GGAAACTCAGAGCCTCAGTCCTCACACAGCAACAGGCCACAGGGGACGACCCAGGCCAGA  248

Q 301  GACACCAAGCAAGAACCAGAGAAAACCTCAAGGGAGACCCGAGTCCCTATCTCCTCACAG  360
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 249  GACACCAAGCAAGAACCAGAGAAAACCTCAAGGGAGACCCGAGTCCCTATCTCCTCACAG  308

Q 361  ACCCCGGAGAGCAACATGAAGCCAGGTGAGTCCCGAGAATGGTGTGTGGGTGTGTGTCCC  420
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 309  ACCCCGGAGAGCAACATGAAGCCAGGTGAGTCCCGAGAATGGTGTGTGGGTGTGTGTCCC  368

Q 421  AAGAGCCACCTTGCTACACATCTCACTCTAATCTCACTCTGTCCCTCTGTGACACAGAAA  480
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 369  AAGAGCCACCTTGCTACACATCTCACTCTAATCTCACTCTGTCCCTCTGTGACACAGAAA  428

Q 481  CAGCTGGGGGCCACATGCTCCTCCTGCTGTTGCCTCTGCTGCTGGCCGCAGTGCTGACAA  540
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 429  CAGCTGGGGGCCACATGCTCCTCCTGCTGTTGCCTCTGCTGCTGGCCGCAGTGCTGACAA  488

Q 541  GAACCCAAGCTGACCCTGTCCCCAGGGCCACCAGGCTCCCAGTGGAAGCAAAGGATTGCC  600
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 489  GAACCCAAGCTGACCCTGTCCCCAGGGCCACCAGGCTCCCAGTGGAAGCAAAGGATTGCC  548

Q 601  ACATTGCTCAGTTCAAGTCTCTGTCCCCAAAAGAG----CTGCAGGCCTTCAAAAAG-GC  655
       |||||||||||  | ||  |     ||     |||    |  | | |||  |  | | ||
S 549  ACATTGCTCAGGYCMAGGATR----CCWKCRTGAGTYTCCMTCTGCCCTCGAGTATGKGC  604

Q 656  CAAGGA----------TGCCATCGTGAGTCTC---CCTCTGCCCTCCTGT--ATGGGC-T  699
         ||            | || |  || ||  |   |||    ||  ||||  |||    |
S 605  TRAGCTCCTCCMYSCYTYCCTTTCTGGGTTACAYYCCTYMTSCCAWCTGTTAATGAARWT  664

Q 700  AGCCTCC-----------------------------TCCANC-CT                714
          ||||                               |||  | ||
S 665  KSTCTCCAYACTTTGWGTNGGGGGRAAAWAWKTTAATCCCACACTTTTGTGCATGGGGSY  724

S 725  K  725
```

6.5 Hepa 1-6 9H2

Alignment Hepa 1-6 wt and Hepa 1-6 clone 9H2 for IFNL2

```
Q 1                    AAGCCTCCTGACGAACCTTGCCCCAGGTGACCTGGACTCTGTCAT  45
                       |||||||||||||||||||||||||||||||||||||||||||||
S 1    CNAGCTTCCTGTGGGAAGCCTCCTGACGAACCTTGCCCCAGGTGACCTGGACTCTGTCAT  60

Q 46   TTTCTCTTTCCCTGCAGTGTCTCACCTGCTCTCGCCACGCCCTGCTCTGGGCTTCCCCAG  105
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 61   TTTCTCTTTCCCTGCAGTGTCTCACCTGCTCTCGCCACGCCCTGCTCTGGGCTTCCCCAG  120

Q 106  CCTGGGCTCCCTAGTGGCAGGTATCAACCTGCTACCTTATTTTCACTTTTCCTACATCAG  165
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 121  CCTGGGCTCCCTAGTGGCAGGTATCAACCTGCTACCTTATTTTCACTTTTCCTACATCAG  180

Q 166  CTGGGGCTGCCCATCAGACCAGGTTAAAAGCATGGAGCACGGATGGCAGTGCACTCCACA  225
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 181  CTGGGGCTGCCCATCAGACCAGGTTAAAAGCATGGAGCACGGATGGCAGTGCACTCCACA  240

Q 226  GAGCTGGAAACTCAGAGCCTCAGTCCTCACACAGCAACAGGCCACAGGGGACGACCCAGG  285
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 241  GAGCTGGAAACTCAGAGCCTCAGTCCTCACACAGCAACAGGCCACAGGGGACGACCCAGG  300

Q 286  CCAGAGACACCAAGCAAGAACCAGAGAAAACCTCAAGGGAGACCCGAGTCCCTATCTCCT  345
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 301  CCAGAGACACCAAGCAAGAACCAGAGAAAACCTCAAGGGAGACCCGAGTCCCTATCTCCT  360

Q 346  CACAGACCCCGGAGAGCAACATGAAGCCAGGTGAGTCCCGAGAATGGTGTGTGGGTGTGT  405
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 361  CACAGACCCCGGAGAGCAACATGAAGCCAGGTGAGTCCCGAGAATGGTGTGTGGGTGTGT  420

Q 406  GTCCCAAGAGCCACCTTGCTACACATCTCACTCTAATCTCACTCTGTCCCTCTGTGACAC  465
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 421  GTCCCAAGAGCCACCTTGCTACACATCTCACTCTAATCTCACTCTGTCCCTCTGTGACAC  480

Q 466  AGAAACAGCTGGGGGCCACATGCTCCTCCTGCTGTTGCCTCTGCTGCTGGCCACAGTGCT  525
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 481  AGAAACAGCTGGGGGCCACATGCTCCTCCTGCTGTTGCCTCTGCTGCTGGCCACAGTGCT  540

Q 526  GACAAGAACCCAAGCTGACCCTGTCCCCAGGGCCACCAGGCTCCCAGTGGAAGCAAAGGA  585
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 541  GACAAGAACCCAAGCTGACCCTGTCCCCAGGGCCACCAGGCTCCCAGTGGAAGCAAAGGA  600

Q 586  TTGCCACATTGCTCAGTTCAAGTCTCTGTCCCCAAAAGAGCTGCAGGCCTTCAAAAAGGC  645
        ||
S 601  NTG                                                          603

Q 646  CAAGGATGCCATCGTGAGTCTCCCTCTGCCCTCCTGTATGGGCTAGCCTCCTCCACCCTT  705


Q 706  CCCTTTCTGGGTTTCATCCTWNY  728
```

Alignment Hepa 1-6 wt and Hepa 1-6 clone 9H2 for IFNL3

```
Q 1     NTTTTTTTGTAGCCTCCTGACGAACCTTGCCCCAGGTGACCTGGACTCTGTCATTTTCTC  60

S       M-------------------------------------------------------------

Q 61    TTTCCCTGCAGTGTCTCACCTGCTCTCGCCACACCCTGCTCTGGGCTTCCCCAGCCTGGG  120
                                          ||||||||||||||||||||||||||||
S 2     --------------------------------ACCCTGCTCTGGGCTTCCCCAGCCTGGG  29

Q 121   CTCTCCTAGTGGCTGGTGTCAACCTGCTACCTTATTTTCACTTTTCCTACATCAGCTGGG  180
        |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
S 30    CTCTCCTAGTGGCTGGTRTCAACCTGCTACCTTATTTTCACTTTTCCTACATCAGCTGGG  89

Q 181   GCTGCCCATCAGACCAGGTTAAAAGCATGGAGCACGGATGGCAGTGCACTCCACAGAGCT  240
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 90    GCTGCCCATCAGACCAGGTTAAAAGCATGGAGCACGGATGGCAGTGCACTCCACAGAGCT  149

Q 241   GGAAACTCAGAGCCTCAGTCCTCACACAGCAACAGGCCACAGGGGACGACCCAGGCCAGA  300
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 150   GGAAACTCAGAGCCTCAGTCCTCACACAGCAACAGGCCACAGGGGACGACCCAGGCCAGA  209

Q 301   GACACCAAGCAAGAACCAGAGAAAACCTCAAGGGAGACCCGAGTCCCTATCTCCTCACAG  360
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 210   GACACCAAGCAAGAACCAGAGAAAACCTCAAGGGAGACCCGAGTCCCTATCTCCTCACAG  269

Q 361   ACCCCGGAGAGCAACATGAAGCCAGGTGAGTCCCGAGAATGGTGTGTGGGTGTGTGTCCC  420
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 270   ACCCCGGAGAGCAACATGAAGCCAGGTGAGTCCCGAGAATGGTGTGTGGGTGTGTGTCCC  329

Q 421   AAGAGCCACCTTGCTACACATCTCACTCTAATCTCACTCTGTCCCTCTGTGACACAGAAA  480
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 330   AAGAGCCACCTTGCTACACATCTCACTCTAATCTCACTCTGTCCCTCTGTGACACAGAAA  389

Q 481   CAGCTGGGGGGCCACATGCTCCTCCTGCTGTTGCCTCTGCTGCTGGCCGCAGTGCTGACAA  540
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S 390   CAGCTGGGGGGCCACATGCTCCTCCTGCTGTTGCCTCTGCTGCTGGCCGCAGTGCTGACAA  449

Q 541   GAACCCAAGCTGACCCTGTCCCCAGGGCCACCAGGCTCCCAGTGGAAGCAAAGGATTGCC  600
        ||||||||||||||||||||||||||||||||||||||||||||||||||  |||  |   |
S 450   GAACCCAAGCTGACCCTGTCCCCAGGGCCACCAGGCTCCCAGTGGAAGCWMAGGMTKSAC  509

Q 601   ACATTGCTCA-GTTCAA-----GTCTCTGT------CC-----CCAAAAGAG--------  635
         |  |   || |  |||     | ||| ||      ||     ||    |
S 510   RCCWTSTKCAAGGKCAAWGRAKGCCTCYGTRRKYTTCCTWYKKCCTYCYGKWYKGGSYWR  569

Q 636   ----CTGCAGGCCTTCAAAAA--GG--------CCAAGGATGCC----ATCGTGA--GTC  675
            || ||  | | |        ||        ||   |    |    ||    |  |
S 570   CCYYCTMCAMCCTTYCCWTTCTKGGTTTSCWCCCCTRWGSYKACTWGRATYRAAAWKGKT  629

Q 676   TCC----CTCTGCCCT---CCTGTA--------TGGGC--------TAGCCTCCTC----  708
        | |    ||  |   |   ||  |         ||||         | | |||| |
S 630   TYCMWTWCTTGGTSNTNNSCCYNTWAMYTWAACTGGGNATGAAAATTGGTCTCCACACTT  689

Q 709   ----CANC---CT   714
            ||     ||
S 690   TGTGCATGGGGCTG  703
```

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