INAUGURAL-DISSertation

Submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg
for the degree of
Doctor of Natural Sciences

Presented by
Diplom-Biologe Holger Wilden
born in Simmerath

Date of oral examination:
18.07.2007
Tumour selective replication of Newcastle Disease Virus as the result of defects in the basal and inducible expression of interferon-related proteins

Referees:       Prof. Dr. Volker Schirrmacher
                Prof. Dr. Michael Wink
Meinen Eltern und
meinem Bruder
Das Staunen ist eine Sehnsucht nach Wissen.

(Matthias Claudius, 1740 – 1815)
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Acknowledgements

I am indebted to many people without whom I could not have finished this thesis and to whom I would like to express my gratitude.

First, I would like to thank Professor Dr. Volker Schirrmacher for giving me the opportunity to do my PhD thesis at his department and for all the stimulating discussions and his ideas that were a great help in carrying out this thesis.

I am also grateful for the willingness of Professor Dr. Michael Wink to be a referee for this thesis.

Professor Dr. Ulrich Hilgenfeldt and Professor Dr. Werner Buselmaier I would like to thank for their readiness to be examiners for my oral defence.

I want to thank Dr. Philippe Fournier for the supervision and for the helpful feedback and discussions concerning my experiments.

The meetings with Professor Dr. Rainer Zawatzky helped me a lot to understand the intricacies of the interferon system and in addition I would like to thank him for the interferon bioassays and for supplying me with crucial cell lines and mouse strains for my work.

Dr. Anne Krug provided me with the knock out mice that were used to carry out important analyses for this thesis and I would like to express my gratitude to her. I am grateful to PD Dr. Ulrich Massing for the help in doing the liposome encapsulation experiments.

Thanks also to all the members of my research group for their help and for the nice working atmosphere that I always enjoyed in the three years I have worked at the DKFZ. A special thanks to Dr. Hujie Bian for introducing me to working with mice and to Annette Arnold for helping me with a thousand details in the lab work and for her support with the Western blot analyses.

Finally, I want to express my gratitude to my parents for their continual support and encouragement during the making of this thesis as well as during all the time of my studies. Without you I would never have come this far!
Abstract

Newcastle Disease Virus (NDV) is an avian Paramyxovirus that preferentially replicates in cancer cells and that has already been used successfully in the treatment of cancer. It has been suggested that defects in the interferon response present in many neoplastic cells make them prone to NDV infection. However, the exact mechanisms that underlie the tumour selective replication of NDV are not known. In this thesis the relation between the NDV susceptibility of cells and the basal and inducible expression of different type I interferon-related proteins was analysed. It was assessed what the differences in the interferon response were between tumour and normal cells and if these differences could explain the tumour selectivity of NDV.

It could be shown that tumour cells have defects in the basal and inducible expression of several antiviral, interferon-related genes. These genes include cytosolic viral RNA receptors such as RIG-I, transcription factors such as IRF3 and IRF7, type I interferons and antiviral effector molecules such as PKR. In addition tumour cells displayed defects in the responsiveness to interferon pretreatment with regard to the induction of an antiviral state by the upregulation of antiviral genes. A statistically significant, negative correlation was found between the expression of many of the tested interferon-related genes and the susceptibility to NDV infection. Furthermore the functional significance of some of the genes for the establishment of an antiviral response could be evaluated by the analysis of gene knock out macrophages. These functional analyses revealed the crucial importance of the type I interferon receptor and to a lesser degree also of IRF3/7 for NDV resistance and interferon-related gene expression. Some tumour cells were found to be unusually resistant to NDV infection, which could be connected to an exceptionally strong expression of antiviral genes.

All in all the results strongly indicate multiple defects in the interferon response of tumour cells as the cause for the tumour selective replication of NDV. There seems to be a general downregulation of the antiviral gene expression rather than defects in the expression of only a few interferon-related genes. These findings shed new light on the processes that take place in normal and tumour cells after NDV infection. In the future they might be used to improve the efficacy of cancer treatment with NDV by the prediction of the virus susceptibility of a tumour with the help of gene expression analysis before virus treatment.
Zusammenfassung


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Ø</td>
<td>Diameter</td>
</tr>
<tr>
<td>+/-</td>
<td>Gene knock out</td>
</tr>
<tr>
<td>$A_{260}$</td>
<td>Absorbance at the wavelength 260 nm</td>
</tr>
<tr>
<td>APC</td>
<td>Allo-phycocyanin</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>ATF-2/c-Jun</td>
<td>Activating transcription factor-2/c-Jun</td>
</tr>
<tr>
<td>ATV-NDV</td>
<td>NDV-modified autologous tumour cell vaccine</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase recruitment domain</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>Copy DNA</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukaemia</td>
</tr>
<tr>
<td>$c_T$</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
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<tr>
<td>CXCL10</td>
<td>CXC-chemokine ligand 10</td>
</tr>
<tr>
<td>d</td>
<td>Days</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>Doubly distilled H$_2$O</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double-stranded RNA</td>
</tr>
<tr>
<td>DKFZ</td>
<td>Deutsches Krebsforschungszentrum (German Cancer Research Centre)</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>Deoxynucleoside triphosphate</td>
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<tr>
<td>$\varepsilon$</td>
<td>Extinction coefficient</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
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<tr>
<td>EGFP</td>
<td>Enhanced green fluorescence protein</td>
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<tr>
<td>eIF-2$\alpha$</td>
<td>Eukaryotic initiation factor 2$\alpha$</td>
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<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>EMCV</td>
<td>Encephalomyocarditis virus</td>
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<tr>
<td>F</td>
<td>Fusion protein</td>
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<td>FACS</td>
<td>Fluorescence activated cell sorter / sorting</td>
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<tr>
<td>FAM</td>
<td>6-Carboxy-fluorescein</td>
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<tr>
<td>FCS</td>
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<td>FITC</td>
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<td>5-Fluorouracil</td>
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<td>G</td>
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<td>Goat anti-mouse</td>
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<td>HBV</td>
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<td>HEPES</td>
<td>N-2-Hydroxy-ethylpiperazine-N′-2-ethanesulfonic acid</td>
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<tr>
<td>HLA</td>
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<tr>
<td>HN</td>
<td>Hemagglutinin-neuraminidase</td>
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<td>HPV</td>
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<td>HSV</td>
<td>Herpes simplex virus</td>
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<tr>
<td>HU</td>
<td>Hemagglutination unit</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>IFNAR</td>
<td>IFN-α/β receptor</td>
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<td>IκB kinase-i</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
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<td>ISG</td>
<td>Interferon-stimulated gene</td>
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<tr>
<td>ISGF3</td>
<td>Interferon-stimulated gene factor 3</td>
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<tr>
<td>ISRE</td>
<td>Interferon-stimulated response element</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
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<tr>
<td>i.v.</td>
<td>Intravenously</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kaposi's sarcoma-associated herpesvirus</td>
</tr>
<tr>
<td>LGP2</td>
<td>Likely ortholog of mouse D11lgp2</td>
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<td>LRR</td>
<td>Leucine rich repeat</td>
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<tr>
<td>M</td>
<td>Matrix protein</td>
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<td>MAVS</td>
<td>Mitochondrial anti-viral signalling protein</td>
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<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MDA5</td>
<td>Melanoma differentiation-associated gene-5</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>MMTV</td>
<td>Mouse mammary tumour virus</td>
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<td>MRD</td>
<td>Minimal residual disease</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<td>Mx</td>
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<td>Myeloid differentiation primary-response protein 88</td>
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<td>N&lt;sub&gt;A&lt;/sub&gt;</td>
<td>Avogadro constant (= 6.022045*10&lt;sup&gt;23&lt;/sup&gt;)</td>
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<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
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<td>NDV</td>
<td>Newcastle disease virus</td>
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<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
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<td>NK cells</td>
<td>Natural killer cells</td>
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<tr>
<td>NS1</td>
<td>Nonstructural protein 1</td>
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<td>OAS1a</td>
<td>2'-5' Oligoadenylate synthetase 1A</td>
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<td>Oligonucleotide</td>
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<td>Pro analysis</td>
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<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
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<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
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<td>PE</td>
<td>Phyco-erythrin</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein kinase PKR</td>
</tr>
<tr>
<td>PO</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>poly (I:C)</td>
<td>Polyriboinosinic polyribocytidylic acid</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>R</td>
<td>Correlation coefficient</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>REU</td>
<td>Relative expression units</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic acid-inducible gene I; Ddx58</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse-transcriptase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>SARS</td>
<td>Severe acute respiratory syndrome</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneously</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SFV</td>
<td>Semliki forest virus</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single-stranded RNA</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>SV</td>
<td>Simian virus</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumour-associated antigen</td>
</tr>
<tr>
<td>TAMRA</td>
<td>6-Carboxy-tetramethyl-rhodamine</td>
</tr>
<tr>
<td>TBK-1</td>
<td>TANK-binding kinase-1</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TIF</td>
<td>Tagged image file</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1 receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>TRAF3</td>
<td>TNF receptor-associating factor 3</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adaptor protein inducing IFN-β</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris-hydroxymethyl-aminomethane</td>
</tr>
<tr>
<td>TYK2</td>
<td>Tyrosine kinase 2</td>
</tr>
<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
<td>---------------------------------------------</td>
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<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>vs.</td>
<td>Versus</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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</tbody>
</table>
1 Introduction

1.1 General introduction to cancer
Cancer is a leading cause of death worldwide. According to the World Health Organization (WHO; fact sheet no. 297, February 2006) cancer accounted for 7.6 million (or 13%) of all deaths in the year 2005. In Germany 224,000 deaths (or 26.6%) were attributable to cancer, making it the second leading cause of death after cardiovascular diseases (47.4%). The main types of cancer leading to overall cancer mortality are in terms of deaths per year: lung (1.3 million), stomach (1.0 million), liver (0.7 million), colon (0.7 million) and breast cancer (0.5 million). 40% of all cancer cases could be prevented by a healthy diet, physical activity and not using tobacco. Tobacco use is the single largest preventable cause of cancer in the world and causes a large variety of cancer types. In addition one-fifth of cancers worldwide are due to chronic infections, mainly from HBV (liver), HPV (cervix), Helicobacter pylori (stomach) schistosomes (bladder) and HIV (Kaposi sarcoma and lymphomas).

Cancer occurs because of changes of the genes responsible for cell growth and repair. These changes are the result of the interaction between genetic host factors and external agents such as physical carcinogens (e.g. UV or ionizing radiation), chemical agents (e.g. asbestos or tobacco smoke) and biological carcinogens (e.g. viruses, bacteria or contamination of food by mycotoxins like aflatoxins).

1.1.1 The development of cancer
Cancer arises from a single cell. The transformation from a normal cell into a tumour cell is a multi-stage process, typically a progression from a pre-cancerous lesion to malignant tumours. The development of cancer may be initiated by external agents and inherited genetic factors. Ageing is another fundamental factor for the development of cancer. The incidence of cancer rises dramatically with age, most likely due to risk accumulation over the life course combined with the tendency for cellular repair mechanisms to be less effective as a person grows older.

Cancer cells have acquired defects in regulatory circuits that govern normal cell proliferation and homeostasis. According to Hanahan et al. (2000) there are six essential alterations in cell
physiology that collectively dictate malignant growth (figure 1-1): self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis.

The order in which these capabilities are acquired is likely to vary across the spectrum of cancer types, but they will ultimately be shared by most kinds of tumours.

1.1.2 Cancer therapy

1.1.2.1 Classical therapy approaches

Treatment of cancer is aimed at curing, prolonging life and improving the quality of life of patients with cancer. Some of the most common cancer types such as breast cancer, cervical cancer and colorectal cancer have a high cure rate when detected and treated early. Classical treatment methods are surgery, radiation therapy and chemotherapy.

Surgery can cure cancer if the tumour can be completely removed. This is the case if the tumour has not spread throughout the body and if its removal does not damage vital organs such as the brain or the liver. However, if the tumour cannot be completely removed, other
treatment methods have to be applied as well. One of these methods is radiation therapy, the use of ionising radiation to kill cancer cells. Radiation therapy works by damaging the DNA of cells and since cancer cells generally proliferate more and have acquired defects in the DNA damage repair, they are more susceptible to radiation-induced DNA damage than normal, non-malignant cells. Another important way to treat cancer is chemotherapy, the use of drugs that interfere with cell division in different ways. Most forms of chemotherapy target all rapidly dividing cells and are not specific for cancer cells. There are several subgroups of chemotherapeutic substances, for example, alkylating agents such as cisplatin that work directly on the DNA and prevent the cell division process by cross-linking and breaking the DNA strands and causing abnormal base pairing. There are several other subgroups such as antimetabolites (e.g. 5-fluorouracil (5FU)), anthracyclines, monoclonal antibodies and so on. Since most chemotherapeutic drugs target all proliferating cells, normal replicating cells of the body such as bone marrow cells, intestinal cells or cells of hair follicles are also killed. This can lead to side-effects such as immunosuppression, diarrhoea or hair loss.

In addition to these classical treatment methods there are many other therapeutic approaches for cancer treatment. In general the best successes in cancer therapy can be achieved if different treatments are combined, for example, by using chemotherapy after surgical removal of the primary tumour to target residual tumour cells in the body.

1.1.2.2 The use of viruses in cancer treatment

Viruses can be used to treat cancer in different ways, because many tumour cells have acquired defects in their antiviral interferon response that might give them a growth and survival advantage but that makes them also more susceptible to virus infections (Stojdl et al., 2000). The two principal approaches in virus therapy are the stimulation of the immune system with virus-modified tumour cell vaccines and the use of live virus to target and kill tumour cells in the body. Tumour cell vaccines can be generated by infecting autologous tumour cells ex vivo with virus and by gamma-irradiating them with a radiation dose that prevents further replication but that does not kill the cells immediately. The virus-modified tumour cell vaccine is then injected back into the patient. The idea behind the treatment is that in this vaccine multiple tumour-associated antigens from the patient-derived tumour cells are linked in the same cell with multiple danger signals from the virus infection such as dsRNA,
Introduction

viral surface proteins or interferons. This linkage is thought to specifically activate innate or adaptive immune responses against the tumour cells. The vaccine can be further improved by the genetic engineering of viruses that express immune stimulatory molecules such as GM-CSF. Examples of a successful application of virus-modified tumour cell vaccines can be found for the Newcastle disease virus (Ockert et al., 1996; Schirrmacher et al., 2002). As the tumour cell vaccines require an intact immune system, this approach is more likely to be used in early disease stages when the body is not yet markedly weakened by tumour- or therapy-related effects.

The second principal approach in the treatment of cancer with viruses is the use of oncolytic viruses in vivo. Oncolytic viruses selectively infect cancer cells, but not normal cells of the body. Some viruses are naturally attenuated (such as some strains of Newcastle disease virus (NDV), reovirus or vesiculostomatitis virus), while other viruses are genetically modified to mediate oncolytic effects (such as herpes simplex virus type 1 or adenovirus). The viruses are given intratumourally or systemically into the blood stream and then they infect and kill tumour cells. One advantage of oncolytic viruses compared to other anticancer drugs is the possibility to add genes by recombinant DNA technology that code for toxic or immune-stimulatory products or that improve the tumour targeting of the virus. In addition oncolytic viruses are self-replicating in cancer cells and thereby self-amplify their anti-tumour effect and they can also target metastases if given systemically. Problems that can occur especially with regard to systemic application are the immune clearance of the virus, insufficient tumour targeting and limited intratumoural dissemination.

An example of an oncolytic virus that has been used in clinical trials is the adenovirus H101, which is the only oncolytic virus to be tested in a phase III study to date (Xia et al., 2004) and which has been approved for cancer treatment in China in 2006 (Garber, 2006). NDV has also been used successfully as an oncolytic virus in a phase I study with the NDV strain PV701 (Pecora et al., 2002) and in a phase I/II study with the NDV strain HUJ (Freeman et al., 2005). Before the use of NDV in cancer therapy will be discussed in detail, it is necessary to take a closer look at its molecular biology.


1.2 Newcastle Disease Virus (NDV)

The following information was mainly taken from Nagai et al. (1989) and Modrow et al. (2002) if not mentioned otherwise. Newcastle disease virus (NDV) belongs to the family of the Paramyxoviridae which are widespread and can cause severe diseases in animals and humans. The measles and the mumps virus are examples of members of the Paramyxoviridae that are pathogenic for humans. NDV causes Newcastle disease which is a highly contagious bird disease affecting many domestic and wild avian species. NDV is not infectious for humans and can cause only mild conjunctivitis and influenza-like symptoms, for example, when workers in the poultry industry are exposed to high virus doses during an outbreak of Newcastle disease.

1.2.1 The biology of NDV

NDV is an enveloped virus of 150 to 300 nm size containing a non-segmented, single-stranded negative RNA genome of 15 kb size. The NDV genome codes for the following six genes given in order from the 3’-end (figure 1-2): nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and large protein (L).

![Figure 1-2: The structure of the NDV virion.](https://www.microbiologybytes.com/virology/Paramyxoviruses.html)
The surface protein HN is anchored in the viral envelope and it is indispensable for the attachment of the virus to sialic acid-containing cell surface receptors (Iorio et al., 1992). These receptors can be found on most mammalian cells and hence NDV binds to most mammalian cell types. HN can also cause hemagglutination, i.e. the agglutination of red blood cells, a characteristic that is used to quantify NDV dilutions. The F protein, which is also anchored in the viral envelope, mediates the fusion of the viral envelope with the host cell membrane and thereby enables the entry of the viral capsid. It is synthesised as an F\textsubscript{0} precursor protein that is cleaved into the two parts F\textsubscript{1} and F\textsubscript{2} which remain connected via a disulfide bridge. The cleavage exposes a strongly hydrophobic area in the F\textsubscript{1} part that is thought to be important for the membrane fusion. The sequence of the the F protein cleavage site contributes to NDV pathogenicity (Romer-Oberdorfer et al., 2003). The M protein is located between the viral capsid and the envelope and is important for the generation and packaging of viral RNA as well as for the assembly of new virus particles.

The nucleocapsid consists of three viral proteins that form a complex with the RNA genome. About 2,200 – 2,600 subunits of the NP protein form the nucleocapsid. A complex of the NP, the L and the P protein are involved in the transcription of the RNA genome. The L protein is an RNA-dependent RNA polymerase that is active only in a complex with the NP and the P protein.

The replication of NDV (figure 1-3) takes place solely in the cytosol with no participation of the nucleus. After HN-mediated binding the F protein induces the fusion of the viral envelope with the host cell membrane and the nucleocapsid enters the cytosol. Then the RNA genome is uncoated and transcribed into mRNAs by the L-NP-P protein complex and the viral proteins are transcribed. Later on when enough NP protein has been produced a continuous positive RNA strand (antigenome) is synthesised that serves as matrix for the generation of new viral genomes. Finally all the viral components accumulate at the plasma membrane and are assembled into progeny virus. The virus is released from cells by budding.
In the process of RNA-dependent RNA synthesis viral double-stranded RNA (dsRNA) is generated in the cytosol. Viral dsRNA is thought to be a general pathogen-associated molecular pattern (PAMP) that appears during the replication of many viruses and that can induce antiviral responses mediated by pattern recognition receptors (PRRs) in cells. In mammalian cells dsRNA is thought to be an important signal limiting NDV infection (Kato et al., 2005; Yoneyama et al., 2004), as will be discussed later.

1.2.2 The classification of NDV strains

NDV strains can be classified as highly virulent (velogenic), intermediate (mesogenic) or non-virulent (lentogenic), based on the results of the mean death time in chicken (Beard and Hanson, 1984). The virulence of Newcastle disease virus is determined by the cleavage site of the fusion protein and by both the stem region and globular head of the hemagglutinin-neuraminidase protein (de Leeuw et al., 2005). In addition NDV can be classified into monocylically replicating strains according to the ability to generate infective progeny virus in mammalian cells. Besides NDV strains can be categorised as lytic or non-lytic. Viruses of both strain types can kill cancer cells, but lytic strains have the potential to do this more quickly because they damage the plasma membrane of infected cells. Non-lytic strains appear to kill by interfering with cell metabolism.

In this thesis mostly the lentogenic, non-lytic, monocylically replicating NDV strain Ulster and the velogenic, lytic, multicyclically replicating NDV strain Italien were used. In addition the two recombinant, EGFP-expressing NDV variants NDFL-EGFP and NDFLtag-EGFP were used in some experiments in order to quickly follow NDV infection by flow cytometry.
Both EGFP-expressing NDV variants were based on the lentogenic NDV strain La Sota, but the NDFL-tagEGFP was mesogenic due to a modified cleavage site of the F protein (Al-Garib et al., 2003; Peeters et al., 1999).

1.2.3 The use of NDV in cancer therapy
Since Newcastle disease virus is not pathogenic in humans and because it replicates only in the cytosol of host cells, which makes a virus-induced malignant transformation of infected cells unlikely, NDV is well suited for cancer therapy trials. NDV can replicate up to 10,000 times better in cancer cells than in most normal cells (Bar-Eli et al., 1996; Reichard et al., 1992; Schirrmacher et al., 1999a; Tzadok-David et al., 1995). The ability of NDV to replicate efficiently in human cancer cells has been demonstrated in both laboratory studies and animal studies. The lytic NDV strain 73-T has been shown to kill a variety of human cancer cells, while it did not harm most normal human cells (Lorence et al., 1988; Reichard et al., 1992; Zorn et al., 1994). Other NDV strains that have been demonstrated to replicate well in tumour cell lines are the lytic NDV Italien in human melanoma cell lines (Ahlert and Schirrmacher, 1990), the lytic strain MTH-68/H in a wide range of transformed cells (Fabian et al., 2007) and the non-lytic NDV Ulster in a variety of human tumour cell lines (Schirrmacher et al., 1999a). The ability of NDV strains to kill human cancer cells in vivo has been examined in xenograft studies. Intratumoural injection of the NDV strain 73-T caused the regression of xenografts in athymic mice from different human tumours such as neuroblastoma or fibrosarcoma (Lorence et al., 1994a/b; Phuangsab et al., 2001).

Other laboratory and animal studies have shown that NDV and NDV-infected cancer cells can stimulate a variety of immune system responses that are essential for the successful immunotherapy of cancer. It could be shown that the infection of human immune system cells with NDV in vitro causes the cells to produce the cytokines interferon-α and tumour necrosis factor-α (Lorence et al., 1988; Zorn et al., 1994). Additional studies have shown that NDV-infected human cancer cells are better at activating human lymphocytes than uninfected cancer cells (Haas et al., 1998b). Bispecific antibodies that bind to the viral HN and to CD3 or CD28 on T cells could further increase the T cell stimulatory capacity of NDV-infected human cancer cells (Haas et al., 1998a, 1999, 2005, 2006). There are indications from in vitro (Schirrmacher et al., 1997, 1999b) and in vivo experiments (Heicappell et al., 1986) that virus
proteins inserted into the plasma membrane of NDV-infected cancer cells may help the immune system to recognise tumour-specific antigens better, thereby leading to an enhanced killing of cancer cells.

The clinical anticancer potential of NDV has been evaluated for oncolysates, whole cell vaccines or infection of patients with lytic NDV. Oncolysates and whole cell vaccines improve the recognition of tumour-associated antigens by the immune system by their association with viral antigens. It has been found that whole cell vaccines can stimulate the immune system more efficiently than oncolysates (Kobayashi et al., 1969), probably due to the importance of tumour cell membrane integrity for CTL activation (Schirrmacher and von Hoegen, 1993). NDV oncolysates have been mostly applied in the treatment of melanoma in phase I and phase II trials, in which an improved overall survival could be observed (Cassel and Murray, 1992; Murray et al., 1977). Improved disease-free survival could be found in a phase II trial with NDV oncolysates in the treatment of advanced renal cancer (Anton et al., 1996).

Whole cell vaccines in the form of autologous tumour cell vaccines (ATV-NDV) increased the 2-year survival rate in glioblastoma multiforme (Steiner et al., 2004) and disease-free (Schlag et al., 1992) or overall survival (Ockert et al., 1996) in phase II trials for colorectal and for renal cell cancer (Pomer et al., 1995). In addition an improved median survival in ovarian cancer could be observed after treatment with ATV-NDV (Möbus et al., 1993).

The infection of patients with NDV has the advantage that the cytotoxic effect is amplified by the generation of virus offspring and that NDV can spread to every cancer cell in the body. The drawbacks of this approach are virus clearance of the immune system by virus-neutralising antibodies. In case studies with the lytic NDV strain MTH-68/H in the treatment of glioblastoma multiforme a dramatical increase in survival was reported for the 4 treated patients when NDV was given on a daily basis (Csatary et al., 2004). Three phase I clinical trials with the oncolytic NDV strain PV701 demonstrated partial tumour regression in some patients (Hotte et al., 2007; Laurie et al., 2006; Pecora et al., 2002). In addition it was shown that a desensitisation with a low initial NDV dose and a low infusion rate can increase the maximum tolerable dose and can reduce flu-like side effects. A phase I/II trial with the oncolytic NDV strain HUJ found good tolerability of NDV and there was one complete response (Freeman et al., 2006).
1.3 The antiviral interferon response

1.3.1 Interferons

Upon virus infection the expression of a class of cytokines called interferons (IFNs) is induced. The name interferon is derived from their ability to interfere with viral replication in previously uninfected tissue culture cells (Isaacs and Lindenmann, 1957) and there are at least three distinct types of interferons: types I, II and III (Pestka at al., 2004). Type I IFNs are composed of various genes including IFN-α, -β (Taniguchi et al., 1980) and others such as IFN-ω, -ε and -κ (Pestka et al., 2004) and are the major antiviral IFNs. Type II IFN is referred to as IFN-γ that is represented by a single gene and that is structurally unrelated to type I IFNs. IFN-γ is not directly induced by most viruses but is produced later by cells of the immune system such as T cells or NK cells and plays a role in the adaptive immune response against intracellular pathogens (Farrar and Schreiber, 1993). The recently discovered type III IFNs IFN-λ1, -λ2 and -λ3 (also known as interleukin-29 (IL-29), IL-28A and IL28B, respectively) are produced in virally infected cells and these IFN genes may also be induced by mechanisms similar to those of IFN-α and -β (Honda et al., 2006a). Since the type I IFNs IFN-α and -β are thought to be the key cytokines defining antiviral responses, the induction and effects of these two IFNs will be discussed in the following.

In humans and mice there are 13 and 14 IFN-α genes, respectively, while there is only a single IFN-β gene (Weissmann and Weber, 1986). All IFN-α/β genes lack introns. Although virtually all cells can produce IFN-α/β in response to viral pathogens, plasmacytoid dendritic cells (pDCs) are the strongest IFN-α/β producers in the body (Colonna et al., 2004). The transcription of IFN-α/β is induced by viral-associated molecular patterns such as viral nucleic acids that are detected by pattern recognition receptors (PRRs) of the innate immune system. IFN-α/β signal both through a common cell surface receptor, the IFNAR, leading to the transcription of hundreds of genes. Some of the induced proteins increase the resistance of cells to infection by directly inhibiting viral replication. In addition IFNs upregulate the expression of MHC class I and activate NK cells. NK cells preferentially kill cells with a low MHC class I level. Some viruses selectively prevent the export of MHC class I molecules to the cell surface. Therefore, IFNs protect uninfected cells from NK cells by increasing the expression of MHC class I molecules, while infected cells are killed either by the better antigen presentation to T cells or by the activated NK cells.
1.3.2 The regulation of type I IFN gene transcription

The induction of IFN-α/β is regulated primarily at the transcriptional level, wherein IFN regulatory factors (IRFs) play central roles (Taniguchi et al., 2001). The transcription of IFN-α/β in a cell is induced upon detection of invariant molecular structures shared by pathogens of various origin, so called pathogen-associated molecular patterns (Medzhitov and Janeway, 2002). Toll-like receptors (TLRs) 3, 7, 8 and 9 are the major pattern recognition receptors (PRRs) that recognise distinct types of virally-derived nucleic acids and activate signalling cascades that induce the production of IFN-α/β (Akira and Takeda, 2004). Recently, cytosolic receptors for viral RNA such as the retinoic acid inducible gene I (RIG-I) or the melanoma differentiation associated gene 5 (MDA5) have been identified and could be shown to induce IFN-α/β in a TLR-independent manner (Yoneyama et al., 2004, 2005). The TLRs and the cytosolic receptors constitute two different receptor systems that are located in different cellular compartments (figure 1-4).

TLRs detect viral nucleic acids mainly in endosomes and are expressed mainly by specialised cell types such as macrophages or dendritic cells. TLRs sample material entering cells from the outside and thus do not detect the presence of infection from within. The material often consists of viral particles that become degraded upon endosomal acidification, thereby exposing viral nucleic acids for recognition. After ligand binding through the leucine rich repeats (LRR) the TLRs dimerise and undergo the conformational change required for the recruitment of downstream signalling molecules such as MyD88 or TRIF via the TIR domain (Akira and Takeda, 2004). The further signalling leads ultimately to the activation of transcription factors such as NF-κB or IRF3/7. At that point the TLR signalling converges with the signalling of the cytosolic viral receptors.

The second receptor system that is TLR-independent is represented by cytosolic viral RNA receptors such as RIG-I or MDA5. These receptors, which belong to the DExD/H box RNA helicases (Yoneyama et al., 2005; Zhang et al., 2000), are expressed ubiquitously and detect viral RNA in the cytosol of an infected cell (Stetson and Medzhitov, 2006). They bind viral RNA with their helicase domain leading to the activation of a downstream signalling cascade via the interaction of the CARD domain of the helicases with the CARD domains of a MAVS adaptor protein (also known as IPS-1, VISA or CARDIF) that is located in the membrane of mitochondria (Kawai et al., 2005; Seth et al., 2005). Ultimately this signalling cascade
activates transcription factors such as NF-κB or IRF3/7 mainly through phosphorylation by activated kinases.

The activated transcription factors induce the transcription of IFN genes in the nucleus which are secreted and act in an auto- and paracrine manner by signalling through the IFNAR. However, the IFN-α/β genes are not expressed at the same level or with the same kinetics after virus infection. The expression of IFN-β and IFN-α4 is induced early through the action of the constitutively expressed IRF3 (Juang et al., 1998), while the expression of the other IFN-α genes depends on IRF7. The transcription factor IRF7 is expressed at a low level in most cells in the absence of virus infection and is induced through a positive feedback loop by the early interferons IFN-α4/β (Levy et al., 2002; Yeow et al., 2000). Only after induction of IRF7 by the early IFNs are the other IFN-α genes transcribed, resulting in a full interferon response.

Figure 1-4: Regulation of type I IFN gene transcription.

There are two different receptor systems that signal upon the detection of virus-associated molecular patterns: Toll-like receptors (TLRs) and cytosolic receptors such as RIG-I. Both systems converge in the activation of transcription factors such as IRF3/7 that in turn induce the transcription of IFNs. There is a positive feedback loop that upregulates the expression of IRF7 by the early interferons IFN-β and IFN-α4, which is indispensable for the expression of other IFN-α genes and for a full interferon response. Adapted from Seth et al., 2006.

The binding of IFN-α and IFN-β to the IFNAR activates janus kinase family members which in turn phosphorylate and activate the signal transducer and activator of transcription 1
(STAT1) and STAT2 proteins. These transcription factors associate with IRF9 to form a heterotrimeric complex, the IFN-stimulated gene factor 3 (ISGF3). ISGF3 initiates the transcription of several interferon stimulated genes (ISGs) by binding to the IFN-stimulated response elements (ISRE) in their promoter regions. Examples for ISGs with ISREs are the transcription factor IRF7 or genes with direct antiviral effector functions such as PKR, OAS1a or Mx1. These proteins together with other important molecules of the interferon response will be discussed in the next chapter.

1.3.3 Key molecules of the interferon response

1.3.3.1 The DExD/H-box RNA helicases RIG-I, MDA5 and LGP2

The cytosolic proteins RIG-I and MDA5 sense intracellular viral RNA and trigger a signal for innate antiviral responses including the production of type I interferons. RIG-I and MDA5 are members of the DExD/H-box helicase family and have a C-terminal RNA helicase domain as well as two N-terminal CARD domains (Yoneyama et al., 2004, 2005). In addition the C-terminal domain of RIG-I contains a repressor domain that is responsible for auto-repression by interacting with both CARD and helicase domains (Saito et al., 2007). RIG-I selectively binds with the dsRNA analogue poly (I:C) as well as with untranslated regions of HCV genomic RNA, but not with double-stranded DNA (Sumpter et al., 2005; Yoneyama et al., 2004).

These results suggest that RIG-I is a specific sensor for dsRNA, but in the case of influenza A virus IFN gene activation occurs without detectable dsRNA accumulation (Pichlmair et al., 2006). A recent hypothesis views 5´-triphosphate RNA as the ligand for RIG-I and as the means for the discrimination of self and non-self in a cell (Hornung et al., 2006). 5´-triphosphate RNA is abrogated in cellular RNAs by posttranscriptional RNA processing in eukaryotes, but it is present in many RNA viruses.

The activation of RIG-I is ATP-dependent since a single mutation in the ATP binding motif of the helicase domain renders RIG-I into a dominant inhibitor (Yoneyama et al., 2004). The binding of viral dsRNA or 5´-triphosphate ssRNA together with ATP binding changes the conformation of RIG-I and releases CARD for relaying signalling to the downstream molecule MAVS (Yoneyama and Fujita, 2007). MAVS is localised in the mitochondria and this localisation is crucial for its function (Li et al., 2005; Lin et al., 2006; Loo et al., 2006),
Introduction

although the precise mechanism is not known. RIG-I and MDA5 both appear to transmit signals via the MAVS protein (Kawai et al., 2005; Kumar et al., 2006). The signal is branched at MAVS, resulting in the activation of NF-κB by the protein kinases IKK-α/β/γ and of IRF3 and IRF7 via TRAF3 (Saha et al., 2006) and the protein kinases TBK-1 and IKK-i(ε) (Hemmi et al., 2004; Perry et al., 2004). Recently it has been suggested that the functions of RIG-I, MDA5 and MAVS are negatively regulated by proteasomal degradation after conjugation to ubiquitin mediated by the ubiquitin ligase RNF125 (Arimoto et al., 2007).

A homologous protein to RIG-I and MDA5 is LGP2 (Miyoshi et al., 2001), which in contrast to the former two lacks the CARD domain and is incapable of transmitting a positive signal. LGP2 has been suggested as a negative regulator, but its role in vivo has yet to be established (Komuro and Horvath, 2006; Rothenfusser et al., 2005; Yoneyama et al., 2005). So far the data indicate a disparate regulatory role for LGP2 in the triggering of innate immune signalling pathways following RNA virus infection (Venkataraman et al., 2007).

The importance of RIG-I seems to be cell type specific. RIG-I is essential in NDV-induced IFN production, but it is dispensable for virus-induced IFN production by pDCs (Kato et al., 2005). pDCs use a distinct signalling cascade to produce high levels of IFN-α and sense viral infection by TLR7/8 and TLR9 (Honda and Taniguchi, 2006b; Takeda and Akira, 2005). RIG-I and MDA5 have differential roles in the recognition of RNA viruses. RIG-I is essential for the production of interferons in response to viruses such as NDV, Sendai virus, VSV and influenza virus, while MDA5 is critical for picornavirus detection (Kato et al., 2006).

Several viruses express proteins that counteract the functions of RIG-I and MDA5. HCV expresses the NS3/4A protein that cleaves MAVS from the mitochondrial membrane, thereby preventing RIG-I or MDA5 downstream signalling (Li et al., 2005; Lin et al., 2006; Loo et al., 2006). The NS1 protein from the influenza A virus inhibits RIG-I signalling by sequestering dsRNA and by binding to RIG-I (Mibayashi et al., 2007; Pichlmair et al., 2006). The V protein of paramyxoviruses binds to MDA5 and inhibits its downstream signalling (Andrejeva et al., 2004).

1.3.3.2 The transcription factors IRF3 and IRF7

The interferon-regulatory factor (IRF) family of transcription factors was initially found to be involved in the induction of genes that encode type I interferons. IRFs have now been shown
Introduction

to have functionally diverse roles in the regulation of the immune system. The mammalian IRF family comprises the nine members IRF1-9 that all contain a conserved DNA-binding domain (Lohoff and Mak, 2005; Taniguchi et al., 2001). This region recognises an ISRE consensus DNA sequence that is found in the promoters of the genes that encode the type I IFNs themselves as well as in the promoters of many other genes that are involved in immunity and oncogenesis (Honda et al., 2006). IRFs are essential in the signalling of PRRs that recognise PAMPs and that link innate and adaptive immune responses. There are two classes of PRRs: transmembrane PRRs such as the TLRs and cytosolic PRRs like RIG-I and MDA5. In the following the role of IRFs in cytosolic PRR signalling will be discussed.

The four IRFs IRF1/3/5/7 have been implicated as positive regulators of the transcription of type I IFN genes (Mamane et al., 1999; Taniguchi et al., 2001). However, IRF1 and IRF5 do not seem to be essential for type I gene expression since the induction of IFN-α/β by NDV in IRF1−/− and IRF5−/− MEFs is not changed (Matsuyama et al., 1993; Takaoka et al., 2005).

IRF3 and IRF7 are the two crucial transcription factors in the signalling of cytosolic PRRs. IRF3 is constitutively expressed, while IRF7 is present only at low levels in most cells. Upon the recognition of viral nucleic acids by cytosolic PRRs they are both activated by phosphorylation and form homo- or heterodimers. After translocation to the nucleus they induce the dimer-specific transcription of type I interferons and chemokines (Lin et al., 2000). The binding of type I interferons to the IFNAR results in the activation of the heterotrimeric transcriptional ISGF3 that is responsible for the induction of IRF7 (Marie et al., 1998; Sato et al., 1998). IRF3 is a potent activator of the IFN-β gene but not the IFN-α genes, except for the IFN-α4 gene, whereas IRF7 efficiently activates both IFN-α and IFN-β genes (Marie et al., 1998; Sato et al., 1998, 2000). In addition it was demonstrated that the induction of type I interferons was severely impaired in IRF7−/− MEF (Honda et al., 2005). Therefore a two-step induction of type I interferons has been suggested in which IRF3 and low initial amounts of IRF7 are important for the early secretion of IFN-β and some IFN-α subtypes. These early interferons induce the expression of IRF7 in a positive-feedback loop through the IFNAR. In the late phase the newly synthesised IRF7 enables the expression of high amounts of most IFN-β and IFN-α genes (figure 1-5). As mentioned before IRF3 and IRF7 are phosphorylated by the protein kinases TBK1 and IKK-i that are activated after viral nucleic acid detection by RIG-I and MDA5 and subsequent downstream signalling via MAVS and TRAF3.
Introduction

1.3.3.3 The IFNAR

The IFNAR is the only known receptor for type I interferon signalling. It is a cell-surface receptor with an extracellular ligand binding domain and an intracellular kinase domain that is activated after ligand-induced dimerisation (Darnell et al., 1994). The IFNAR has two chains, IFNAR1 and IFNAR2c, which are both necessary for most of its functions (van Boxel-Dezaire et al., 2006). Interferon binding induces the phosphorylation of the JAK1 or TYK2 Janus kinases and of certain IFN-α subtypes such as IFN-α4. a) In the later phase secreted IFNs bind and activate the type I IFN receptor (IFNAR) in an auto- or paracrine manner, which leads to the activation of ISGF3, a heterotrimer formed by STAT1+2 and IRF9. ISGF3 translocates to the nucleus and induces the transcription of IRF7. Newly synthesised IRF7 is then activated following the recognition of viral nucleic acids by cytosolic PRRs, leading to the expression of large amounts of IFN-β and many of the IFN-α proteins. Expression of the cytosolic PRRs RIG-I and MDA5 is also induced by type I IFNs (not shown). Adapted from Honda et al., 2006.

Figure 1-5: Interferon-regulatory factors in positive-feedback regulation of type I interferon genes.

1) In the early phase of a virus infection, IRF3 and IRF7 are phosphorylated, form homo- or heterodimers and translocate to the nucleus. There they induce the expression of chemokines such as CXCL10 and of small amounts of IFN-β and of certain IFN-α subtypes such as IFN-α4. b) In the later phase secreted IFNs bind and activate the type I IFN receptor (IFNAR) in an auto- or paracrine manner, which leads to the activation of ISGF3, a heterotrimer formed by STAT1+2 and IRF9. ISGF3 translocates to the nucleus and induces the transcription of IRF7. Newly synthesised IRF7 is then activated following the recognition of viral nucleic acids by cytosolic PRRs, leading to the expression of large amounts of IFN-β and many of the IFN-α proteins. Expression of the cytosolic PRRs RIG-I and MDA5 is also induced by type I IFNs (not shown). Adapted from Honda et al., 2006.
Because different subtypes of type I IFN activate the same cell-surface receptor complex to mediate variable responses, it seems likely that different ligand-receptor interactions cause diverging downstream responses, probably by differences in critical amino acid residues among the different type I IFN subtypes (Deonarain et al., 2002). There is, for example, a distinct binding site on IFNAR1 for IFN-β and some hybrid IFN-α subtypes (Platis and Foster, 2003) and IFN-β also forms complexes with the extracellular domains of IFNAR2c and IFNAR1 with higher affinity than IFN-α2 (Lamken et al., 2004).

The IFNAR is crucial in the defence against viral infection. Mice lacking the IFNAR are highly susceptible to a wide range of viral infections (van den Broek et al., 1995; Hwang et al., 1995; Luker et al., 2003). Measles virus suppresses type I interferon responses by the inhibition of IFNAR signalling through the association of the MeV-accessory proteins C and V with the IFNAR1, which blocks the phosphorylation of JAK1 (Yokota et al., 2003). In addition IFNAR−/− MEF and peritoneal macrophages show severe defects in NDV-induced IFN-α/β gene expression (Harada et al., 1996).

1.3.3.4 The antiviral effector molecules PKR, OAS1a and Mx1
The signalling through the IFNAR activates the inducible expression of hundreds of genes that together establish an antiviral state, i.e. an increased ability to resist viral infection (van Boxel-Dezaire et al., 2006; Stark et al., 1998). The exact function of many of these genes has not yet been established, but for some molecules the antiviral mechanisms have been demonstrated in detail. Three of these will be discussed in the following.

A well-studied IFN-induced antiviral effector that is induced by IFNs is the protein kinase PKR (Clemens and Elia, 1997; Meurs et al., 1990). Activation of PKR by dsRNA leads to the phosphorylation of the translation initiation factor eIF-2α, causing the blockade of the translation of most cellular and viral mRNAs. PKR has profound effects on cell growth and apoptosis (Garcia et al., 2006; Tan and Katze, 1999). Many viruses have developed strategies to downregulate the activity of PKR so that virus replication is not compromised by this molecule (Gale and Katze, 1998; Langland et al., 2006).

2′-5′ oligoadenylate synthetase (OAS) family proteins are induced by IFNs (Kumar et al., 2000; Rebuillat and Hovanessian, 1999) and also have broad antiviral effects. They are
activated by dsRNA and produce 2′-5′ oligoadenylates that in turn activate the latent ribonuclease RNase L by inducing its dimerisation. RNase L activation results in the degradation of host and viral mRNAs (Samuel, 2001). In addition RNase L has been implicated in specific apoptotic actions (Zhou et al., 1997) which could also contribute to its antiviral effects.

A third group of IFN induced proteins with antiviral activities are the myxovirus resistance (Mx) proteins. These proteins are GTPases that were first discovered as anti-influenza virus effector molecules (Staeheli, 1990). Mx proteins inhibit myxovirus multiplication by blocking either viral nucleocapsid transport or viral RNA synthesis (Haller et al., 1998). Mx proteins increase the resistance of cells against viruses of the Orthomyxoviridae (e.g. influenza A + C virus), Rhabdoviridae (e.g. VSV) or Paramyxoviridae (e.g. measles virus) among others (Samuel, 2001), although the mechanism of the antiviral effect has not yet been established.

1.3.4 The antiviral response upon NDV infection

Many viruses have developed strategies that limit the antiviral responses in the host cell by the interaction of viral proteins with key molecules for the establishment of an interferon response (Haller et al., 2006). Paramyxoviruses can inhibit the interferon response by the interaction of their V proteins with the cytosolic viral RNA receptor MDA5 (Andrejeva et al., 2004; Yoneyama et al., 2005). In addition the V protein can interact with and inhibit STAT-mediated signalling from the IFNAR (Horvath, 2004). The IFN antagonist activity of the the NDV V protein, which is derived from the P protein via RNA editing (Steward et al., 1993), is species specific and only inhibits the interferon response in avian and not in mammalian cells (Park et al., 2003).

NDV is a potent inducer of type I interferons in mammalian cells. In mouse cells NDV stimulates IFN secretion in vitro in murine spleen cells and fibroblasts (Ito et al., 1982; Yeow et al., 1997) and in vivo NDV rapidly induces the transcription of IFN-β and IFN-α4 mRNA (Guha-Thakurta and Majde, 1997). In human tumour cells the induction of interferons as well as of HLA, cell adhesion molecules and chemokines could be observed after NDV infection (Washburn and Schirrmacher, 2002). In addition NDV induces interferon-stimulated antiviral proteins such as MxA and PKR in human PBMC and tumour cells (Fiola et al., 2006). Antiviral responses seem to be stimulated by dsRNA and by the NDV HN protein via a
hemagglutinin lectin-cell interaction (Fournier et al., 2003; Zeng et al., 2002a/b).

1.3.5 The interferon response in tumour cells
As discussed before (chapter 1.2.3) NDV replicates selectively in cancer cells. However, the mechanism by which NDV kills human tumour cells and the role of IFN in the spread of the virus in mammalian cells is not entirely known. Many tumour cells have acquired defects in the interferon response that might give them a growth and survival advantage (Pansky et al., 2000; Paucker et al., 1962; Pitha, 2000; Stojdl et al., 2000; Sun et al., 1998; Wagner et al., 2004). Since type I interferons are key components in the antiviral response of cells against NDV it has been suggested that defects in the interferon response make tumour cells much more susceptible to NDV infection than normal cells. For some viruses such as VSV it is known that defects in the interferon response of tumour cells determines tumour selective replication and oncolytic effects (Barber, 2005; Wollmann et al., 2007). The tumour selectivity of NDV has also been related to a restricted interferon response in human fibrosarcoma cells (Krishnamurthy et al., 2006) or in human MCF-7, BT-20 and Jurkat tumour cells (Fiola et al., 2006).

1.4 Aims of the project
The exact mechanisms that underlie the tumour selective replication of NDV are not clear. There are indications that differences in the interferon response in normal and tumour cells play an important role for the differences in NDV susceptibility. The working hypothesis to explain the variations in NDV susceptibility is shown in figure 1-6. Normal mammalian cells have an intact interferon response that is activated upon NDV infection and that induces the expression of antiviral genes. These genes establish an antiviral state in the cell that limits NDV replication. In contrast the interferon response in tumour cells is limited, causing only a low level of antiviral gene expression that is not sufficient to establish an effective antiviral state.
Introduction

In this thesis the NDV susceptibility and the expression of interferon-related antiviral genes should be investigated in different normal and tumour cell types. It should be found out if there were correlations between the degree of NDV susceptibility and parameters of the interferon response such as the basal and inducible expression of cytosolic viral nucleic acid receptors, type I IFNs, essential type I interferon transcription factors or antiviral effector molecules. A possible correlation should be analysed first in a cell-type homogeneous system consisting of murine macrophages and macrophage-like tumour cells. In order to determine general differences in antiviral gene expression between normal and tumour cells and to calculate the degree of correlation between viral and antiviral gene expression, several normal and tumour cells should be analysed before and after NDV infection. The functional significance of certain key components of the interferon response for NDV susceptibility and antiviral gene expression should be tested in murine knock out macrophages. In addition the expression of the human RIG-I molecule was related to the expression of NDV genes during infection of normal and malignant human cells.

Figure 1-6: Working hypothesis to explain differences in NDV susceptibility between normal and tumour cells.

NDV replicates better in tumour than in normal cells. In the above model this is implicated by a recombinant EGFP-expressing NDV that leads to an EGFP signal in tumour but not in normal cells. The hypothesis is that normal cells induce a strong interferon response when danger signals such as viral dsRNA are detected. In the course of the antiviral response IFNs are secreted that induce an antiviral state in the cell due to the expression of interferon-stimulated antiviral genes. In tumour cells the interferon response is defective, preventing the establishment of an antiviral state and enabling NDV to replicate and express EGFP.

In this thesis the NDV susceptibility and the expression of interferon-related antiviral genes should be investigated in different normal and tumour cells types. It should be found out if there were correlations between the degree of NDV susceptibility and parameters of the interferon response such as the basal and inducible expression of cytosolic viral nucleic acid receptors, type I IFNs, essential type I interferon transcription factors or antiviral effector molecules. A possible correlation should be analysed first in a cell-type homogeneous system consisting of murine macrophages and macrophage-like tumour cells. In order to determine general differences in antiviral gene expression between normal and tumour cells and to calculate the degree of correlation between viral and antiviral gene expression, several normal and tumour cells should be analysed before and after NDV infection. The functional significance of certain key components of the interferon response for NDV susceptibility and antiviral gene expression should be tested in murine knock out macrophages. In addition the expression of the human RIG-I molecule was related to the expression of NDV genes during infection of normal and malignant human cells.
2 Materials

2.1 Equipment

Binocular microscope Zeiss, Jena
Biological safety cabinet Baker, Sanford (USA)
Cell culture incubator Labotec, Göttingen
Cell homogeniser Ultra-Turrax® T25 basic IKA®-Werke, Staufen
Centrifuge Biofuge fresco Heraeus, Hanau
Centrifuge Megafuge 2.0R Heraeus, Hanau
ELISA reader Perkin-Elmer, Überlingen
Film developing machine Agfa, Cologne
Flow cytometer FACSCalibur Becton Dickinson, Heidelberg
Flow cytometer FACSScan Becton Dickinson, Heidelberg
Freezer -20 °C Liebherr, Biberach an der Riss
Freezer -80 °C Thermo Fisher Scientific, Karlsruhe
Gel dryer B. Braun Scientific Instruments, San Francisco (USA)
GeneAmp® 5700 Sequence Detection System Perkin-Elmer, Überlingen
System
Glass pipettes Hirschmann, Eberstadt
Glassware Schott, Mainz
Heatable magnetic stirrer Heidolph Instruments, Schwabach
Heat block Grant Instruments, Cambridgeshire (England)
Milli-Q water purification system Millipore, Eschborn
Mikrowave oven Bosch, Heidelberg
Neubauer cell counting chamber B. Braun, Melsungen
pH meter Wissenschaftliche Technische Werkstätten, Weilheim
Pipettes (2 – 1000 µL) Eppendorf, Hamburg
Pipetting aid Pipetboy acu INTEGRA Biosciences, Fernwald
Photometer GeneQuant pro Amersham Biosciences, Freiburg
Power supply for electrophoresis Pharmacia, Freiburg
Materials

Quartz cuvette
Scales
SDS-PAGE electrophoresis equipment
Shaker Mixer 5432
Shaker Reax 2000
Table centrifuge
Thermocycler PTC-100
UV transilluminator (254 nm)
Water baths
Western blot equipment

2.2 Software

Basic Local Alignment Search Tool (BLAST), Web Interface
Cellquest Pro 4
GeneAmp® 5700 SDS 1.3
GraphPad Prism 3
ImageJ 1.37
Microsoft Excel 2000
OpenOffice 2.0
Primer3, Web Interface
WinMDI 2.8

2.3 Consumables

Aluminium foil
Cell scrapers
Cell culture flasks (25, 75 and 125 cm²)
Cell culture dishes
Cell culture plates (6, 12, 24 and 48 well)
Cell strainer (40 µm)
Materials

Centrifuge tubes (15 and 50 mL)  Greiner, Frickenhausen
Cover slips  R. Langenbrinck, Teningen
Cryo vials  Costar, Cambridge
Disposable scalpels  PfM AG, Cologne
FACS tubes  Greiner, Frickenhausen
Injection needles (Ø 0.3 – 0.7 mm)  Becton Dickinson, Heidelberg
Laboratory gloves  Kimberly-Clark, Zaventem (Belgium)
LeucoSep centrifuge tubes  Greiner, Frickenhausen
Micro test tubes (1.5 – 2.0 mL)  Eppendorf, Hamburg
Nitrocellulose membrane  Schleicher & Schüll, Dassel
Parafilm  American National Can, Greenwich (USA)
Pasteur pipettes  Carl Roth, Karlsruhe
Petri dishes  Renner, Dannstadt
Pipette tips (1 – 1000 µL)  Greiner, Frickenhausen
Pipette filter tips (1 – 1000 µL)  Starlab, Ahrensburg
Real-time PCR plates, 96 well  Biozym, Hessisch-Oldendorf
Slides  Renner, Dannstadt
Syringes  (1 – 2 mL)  Becton Dickinson, Heidelberg
  (5 – 50 mL)  Terumo, Leuven (Belgium)
Syringe-driven filter units, pore size 22 µm  Millipore, Eschborn
Whatman 3MM paper  Schleicher & Schüll, Dassel
X-ray film  Amersham Biosciences, Freiburg

2.4 Chemicals

Acrylamide  Carl Roth, Karlsruhe
Agarose  Gibco BRL, Eggenstein
ε-Aminocaproic acid  Sigma-Aldrich, Schnelldorf
APS  Serva, Heidelberg
Bromine phenol blue  Serva, Heidelberg
DMSO  Merck, Darmstadt
ECL  Amersham Biosciences, Freiburg
EDTA  Merck, Darmstadt
## Materials

- **Ethanol, p.a.** Merck, Darmstadt
- **Ethidium bromide solution (10 mg/mL)** Merck, Darmstadt
- **Ficoll Paque Plus** Pharmacia, Freiburg
- **GelCode® Blue Stain Reagent** Pierce, Bonn
- **Glacial acetic acid** Merck, Darmstadt
- **Glycerol** Merck, Darmstadt
- **Glycine** Carl Roth, Karlsruhe
- **HEPES** Merck, Darmstadt
- **High-Range Rainbow™ protein marker** Amersham Biosciences, Freiburg
- **Isopropanol, p.a.** Merck, Darmstadt
- **β-Mercaptoethanol** Gibco BRL, Eggenstein
- **Methanol, p.a.** Merck, Darmstadt
- **Molecular weight marker for Western blot** (High-Range Rainbow™) Amersham Biosciences, Freiburg
- **Milk powder** Carl Roth, Karlsruhe
- **PBS powder** Biochrom, Berlin
- **Potassium chloride** Merck, Darmstadt
- **di-Potassium hydrogen phosphate** Merck, Darmstadt
- **Propidium iodide** Sigma-Aldrich, Schnelldorf
- **SDS, 10 %** Merck, Darmstadt
- **Sodium azide** Merck, Darmstadt
- **Sodium chloride** Merck, Darmstadt
- **di-Sodium hydrogen phosphate** Merck, Darmstadt
- **Sodium hydroxide pellets** Carl Roth, Karlsruhe
- **TEMED** Sigma-Aldrich, Schnelldorf
- **Tris-Base** Sigma-Aldrich, Schnelldorf
- **Tris HCl** Sigma-Aldrich, Schnelldorf
- **Trypan blue** Serva, Heidelberg
- **Trypsin-EDTA solution, sterile** Life Technologies, Karlsruhe
- **Tween20** Merck, Darmstadt
## 2.5 Commercial kits

<table>
<thead>
<tr>
<th>Name</th>
<th>Used for</th>
<th>Provider</th>
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<tr>
<td>Plasmid Maxi Kit</td>
<td>Preparation of plasmids from bacteria</td>
<td>Qiagen, Hilden</td>
</tr>
<tr>
<td>QIAprep Spin Miniprep Kit</td>
<td>Preparation of plasmids from bacteria</td>
<td>Qiagen, Hilden</td>
</tr>
<tr>
<td>QIAquick Gel Extraction Kit</td>
<td>Preparation of DNA from agarose gels</td>
<td>Qiagen, Hilden</td>
</tr>
<tr>
<td>RNeasy Mini Kit</td>
<td>Preparation of RNA from cells or tissues</td>
<td>Qiagen, Hilden</td>
</tr>
<tr>
<td>qRT-PCR Core Kit</td>
<td>Real-time PCR amplification and detection of DNA</td>
<td>Eurogentec, Seraing (Belgium)</td>
</tr>
<tr>
<td>qRT-PCR Core Kit for SYBR® green</td>
<td>Real-time PCR amplification and detection of DNA</td>
<td>Eurogentec, Seraing (Belgium)</td>
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</tbody>
</table>

## 2.6 Enzymes / Reagents

- CellLytic™-M: Sigma-Aldrich, Schnelldorf
- dNTP Mix: Invitrogen, Karlsruhe
- jetPEI™ transfection reagent: Biomol, Hamburg
- Lipofectamine™ 2000 transfection reagent: Invitrogen, Karlsruhe
- Oligo(dT)₁₂₋₁₈ Primer: Invitrogen, Karlsruhe
- Protease Inhibitor Cocktail for use with mammalian cell and tissue extracts: Sigma-Aldrich, Schnelldorf
- RNAlater: Ambion, Austin, Rexas (US)
- RNaseOUT Recombinant Ribonuclease Inhibitor: Invitrogen, Karlsruhe
- SuperScript™ II Reverse Transcriptase: Invitrogen, Karlsruhe

## 2.7 Oligonucleotides

### 2.7.1 Primer pairs used for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Gene</th>
<th>Primer [nM]</th>
<th>MgCl₂ [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arbp_1/2</td>
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<td>Acidic ribosomal phosphoprotein PO</td>
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<tr>
<td>β-2-microg_1/2</td>
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<td>β-2-Microglobulin</td>
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<td>β-Actin</td>
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<tr>
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<td>Human</td>
<td>β-Actin</td>
<td>300</td>
<td>3.5</td>
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## Materials

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Gene</th>
<th>Primer [nM]</th>
<th>MgCl₂ [mM]</th>
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<tbody>
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<tr>
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<td>Retinoic acid-inducible gene I</td>
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<td>3.5</td>
</tr>
</tbody>
</table>

The primers were either designed with the Primer3 software (optimised for a melting temperature of 60 °C) based on the respective mRNA sequence (as in the NCBI nucleotide database) or they were taken from publications. The primers were ordered from MWG Biotech, Ebersberg bei München. The sequences and a detailed description of the primers and their origin can be found in the appendix (see table 7.2.1).

### 2.7.2 Labelled oligonucleotides used for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Detection chemistry</th>
<th>Oligo [nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>Mouse</td>
<td>FAM-TAMRA</td>
<td>100</td>
</tr>
<tr>
<td>Matrix protein</td>
<td>NDV</td>
<td>FAM-TAMRA</td>
<td>100</td>
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</tbody>
</table>

The sequences and a detailed description of the primers can be found in the appendix (table 7.2.2).

### 2.8 Plasmids

Human RIG-I wildtype and human RIG-I IKA-mutated plasmids were kindly provided by Dr. Takashi Fujita, Tokyo Metropolitan Organization for Medical Research, Tokyo (Japan)
Materials

EGFP expression plasmid 'psuperEGFP' kindly provided by Prof. Peter Altevogt, DKFZ Heidelberg

Plasmid 241 (coding for an anti-CD3-IL2 construct) own production

2.9 Antibodies

Primary antibodies

Mouse anti-NDV HN, IgG2a, clone HN.B Ioro (USA), own production
Rat anti-mouse F4/80 Cedarlane Laboratories, Burlington (Canada)

Secondary antibodies

Goat F(ab')2 anti-mouse IgM, IgG, IgA (H+L), PE-conjugated Southern Biotech, Birmingham (USA)
Goat anti-rabbit IgG, PO-conjugated Dianova, Hamburg

Polyclonal antibodies

Rabbit anti-human RIG-I AXXORA, Lörrach

Antibodies for media supplementation

Anti-mouse IFN-α, hybridoma clone 4EA1 kindly provided by Prof. Rainer Zawatzky, DKFZ Heidelberg
Anti-mouse IFN-β, hybridoma clone 7FD3 kindly provided by Prof. Rainer Zawatzky, DKFZ Heidelberg
2.10  Cell culture media and supplements

2.10.1  Media

αMEM  Cambrex, Verviers (Belgium)
DMEM  Gibco Invitrogen, Karlsruhe
RPMI 1640  Gibco Invitrogen, Karlsruhe

2.10.2  Media supplements

FCS  Gibco Invitrogen, Karlsruhe
HEPES  Gibco Invitrogen, Karlsruhe
Murine interferon-α4  Provided by Prof. Dr. Rainer Zawatzky (Division of Viral Transformation Mechanisms, German Cancer Research Center Heidelberg)
M-CSF-containing supernatant  Prepared from M-CSF-producing L929 cells
β-Mercaptoethanol  Sigma-Aldrich, Schnelldorf
Penicillin / streptomycin  Gibco Invitrogen, Karlsruhe

2.11  Cells

2.11.1  Primary cells

Human PBMC  Prepared from centrifuged human blood (buffy coat); blood was acquired from the blood bank in Heidelberg
Murine macrophages  Generated from murine bone marrow cells
Murine bone marrow cells  Prepared from murine bones
Murine spleen cells  Prepared from murine spleen

2.11.2  Murine cell lines

B16  Skin melanoma; ATCC, Rockville (USA)
CT26 (p/wt)  Colon carcinoma; ATCC, CT26wt from Rockville (USA); CT26p were kindly provided Dr. O. van Tellingen, The Netherlands Cancer Research Institute, Amsterdam (The Netherlands)
DA3  Breast adenocarcinoma (strain DBA/2); ATCC, Rockville (USA)
Eb  Non-metastasising T cell lymphoma (Schirrmacher et al., 1979)
Esb 289  Highly metastasising T cell lymphoma (Larizza et al., 1984)
L929    Fibroblast; ATCC, Rockville (USA)
J774A.1  Macrophage-derived reticulum cell sarcoma (strain BALB/c); ATCC, Rockville (USA)
MEF     Embryonic fibroblast (strain C57BL/6); ATCC, Rockville (USA)
NIH/3T3  Embryonic fibroblast; ATCC, Rockville (USA)
RAW 264.7  Macrophage-derived Abelson murine leukemia virus-induced tumour (strain BALB/c); ATCC, Rockville (USA)

2.11.3 Human cell lines
Hela     Cervical adenocarcinoma; ATCC, Rockville (USA)
Jurkat  Lymphoblastoid T cell lymphoma; ATCC, Rockville (USA)
MCF-7    Breast adenocarcinoma; ATCC, Rockville (USA)
U937     Histiocytic lymphoma; ATCC, Rockville (USA)

2.12 Newcastle Disease Virus strains
NDV strains can be differentiated on the basis of their pathogenicity for chickens into strains of high (velogenic), intermediate (mesogenic) or low (lentogenic) virulence. The following strains of NDV were used in this thesis:

NDFL-EGFP  Lentogenic recombinant strain expressing the jellyfish EGFP protein, based on the NDV strain LaSota, provided by Dr. Ben Peeters (Institute for Animal Science and Health, Lelystad (The Netherlands)), see also Al-Garib et al., 2003
NDFLtag-EGFP Mesogenic recombinant strain expressing the jellyfish EGFP protein, based on the NDV strain LaSota, provided by Dr. Ben Peeters (Institute for Animal Science and Health, Lelystad (The Netherlands)), see also Al-Garib et al., 2003
NDV Italien Velogenic strain, provided by H.D. Klenk (University of Giessen)
NDV Ulster Lentogenic strain, provided by Dr. P. Russell (Department of Microbiology and Parasitology, Royal Veterinary College, University of London (England))

2.13 Mouse strains
BALB/c wildtype    Fa. Charles River, Sulzfeld
B6/129 mixed background IFNAR−/− Provided by Prof. Dr. Rainer Zawatzky (Division of
<table>
<thead>
<tr>
<th>Animal Type</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 knock out mice</td>
<td>Provided by Dr. Anne Krug (Institute for Medical Microbiology, Immunology and Hygiene, Technical University Munich)</td>
</tr>
<tr>
<td>(IRF3⁻/⁻, IRF7⁻/⁻, TLR3⁻/⁻, TLR7⁻/⁻)</td>
<td></td>
</tr>
<tr>
<td>C57BL/6 wildtype</td>
<td>Fa. Charles River, Sulzfeld</td>
</tr>
<tr>
<td>DBA/2 wildtype</td>
<td>Fa. Charles River, Sulzfeld</td>
</tr>
</tbody>
</table>

Viral Transformation Mechanisms, German Cancer Research Center Heidelberg)
3 Methods

3.1 Molecular biological methods

3.1.1 Buffers and solutions

TAE, 50x

- 242 g/L Tris base
- 37.2 g/L Na₂EDTA·2H₂O
- 57.1 mL/L glacial acetic acid
- in ddH₂O, pH 8.5, storage at room temperature

3.1.2 Preparation of agarose gels

For a 1 % (w/v) gel 1 g agarose was dissolved in 100 mL 1x TAE buffer and was brought to boil in a microwave oven to melt the agarose. After cooling down to about 50 °C 1 µL ethidium bromide stock solution (10 mg/mL) was added and it was mixed thoroughly but gently to avoid introducing bubbles. The agarose solution was poured into a clean, dry gel tray and allowed to set for about 30 minutes.

3.1.3 Preparation of DNA from agarose gels

For the extraction of DNA from agarose gels the QIAquick Gel Extraction Kit from Qiagen was used according to the manufacturers instructions (Qiagen, www.qiagen.com, QIAquick® Spin Handbook as at July 2002).

3.1.4 Preparation of total RNA from animal cells or tissues

Cell pellets were either frozen at -80 °C after removal of the medium or used immediately for RNA preparation. Animal tissue samples were used immediately for RNA preparation or stored in RNAlater. For archival storage the tissue samples in RNAlater were kept first at 4 °C for 1 – 2 days to enable the RNAlater to penetrate the tissue and then they were stored at -20 °C.

For the extraction of total RNA from animal cells or tissues the RNeasy Mini Kit from Qiagen was used according to the manufacturers instructions (Qiagen, www.qiagen.com, RNeasy® Mini Handbook as at April 2006). For cells the 'Purification of Total RNA from Animal Cells...
Methods

Using Spin Technology protocol (pp. 25-30) was used and for animal tissues the 'Purification of Total RNA from Animal Tissues' protocol (pp. 39-44). The samples were homogenised using a rotor-stator homogeniser (21,500 rpm, step 3b in the protocol). The optional step 9 of the protocol was left out. In step 10 the RNA was eluted with 40 µL RNase-free water and before using the extracted RNA in further experiments the RNA concentration was determined photometrically (see chapter 3.1.7).

3.1.5 Reverse transcription of total RNA
Total RNA was reversely transcribed using the SuperScript™ II Reverse Transcriptase from Invitrogen according to the manufacturers instructions (Invitrogen, www.invitrogen.com, SuperScript™ II Reverse Transcriptase protocol as at 11 November 2003). In general about 500 ng total RNA per sample were reversely transcribed using oligo(dT)12-18 primers and the generated cDNA was used in subsequent PCR reactions.

3.1.6 Quantitative real-time PCR
Quantitative real-time PCR is a technique to quantify a specific part of a given DNA molecule. In contrast to traditional PCR it is not only possible to determine whether a sequence is present in a sample, but it is also possible to determine the amount of this sequence. The PCR is termed 'real-time' since the DNA amount is measured after each cycle during the PCR and not only after the last cycle as in traditional PCR. When combined with reverse transcription quantitative real-time PCR can be used to determine gene expression by determining the mRNA amount of a gene in a given sample.

3.1.6.1 Optimisation of the primer pairs
Only primers that bound just once in the genome when tested in a database search (BLAST, NCBI) were chosen for PCR. The primers were optimised to show a melting curve with one peak only (as in figure 3-1a) and to have approximately the same amplification efficiency as the gene used for normalisation (as in figure 3-1b, described in: User Bulletin #2, ABI PRISM 7700 Sequence Detection System. Applied Biosystems, December 11, 1997 (updated 10/2001), p. 11-15; www.appliedbiosystems.com). The master mix was prepared as recommended by the manufacturer (Eurogentec, Seraing (Belgium)) and the concentrations for the primers and for MgCl2 were adjusted as described in the above table (2.7.1).
3.1.6.2 Relative quantification of mRNA content

For the quantification of the analysed genes the comparative \( c_T \) method was used as described in the User Bulletin #2 for the ABI Prism 7700 Sequence Detection System (www.appliedbiosystems.com, as at December 11, 1997 (updated 10/2001), p. 11-15). The amount of the target genes analysed throughout this thesis was mostly expressed as follows: relative gene expression = \( 2^{\Delta c_T \cdot 1000} \), with \( \Delta c_T = c_T \) (target gene) – \( c_T \) (reference gene). The ratio of the relative gene expressions from different experiments can be used to compare the gene expression in these experiments. For example, if the gene expression is 40 REU (relative expression units) in experiment 1 and 80 REU in experiment 2, the gene expression is twice as high in the latter experiment.

In some cases the target gene expression was given relative to its expression in a certain reference sample. For instance, in the kinetics of gene expression the time point 0 \( (t_0) \) was often used as reference and the relative gene expression was calculated as \( 2^{\Delta \Delta c_T} \), with \( \Delta \Delta c_T = \Delta c_T (t_0) – \Delta c_T \) (sample).

The \( c_T \) for the target and the reference gene in a sample was determined in triplicates and the \( c_T \) was set as the mean of these measurements \pm the standard deviation. The standard deviation of the \( \Delta c_T \) was calculated as follows: \( SD_{\Delta c_T} = \sqrt{SD_{cT1}^2 + SD_{cT2}^2} \).

Figure 3-1: Typical real-time PCR melting curve and amplification efficiency plot.

(A) Melting curve for the acidic ribosomal phosphoprotein PO (Arbp) amplicon generated during a real-time PCR. \( T = \) temperature (B) Correlation between the \( c_T \) and the cDNA dilution for the NDV matrix (■) and the murine β-actin gene (▲), and relative efficiency plot for the two genes (♦).
3.1.7 Determination of nucleic acid concentration

The DNA/RNA concentration in a sample was determined photometrically via the absorbance at 260 nm \( A_{260} \) using the following formula: concentration of RNA sample [µg/mL] = \( \varepsilon \times A_{260} \times \text{dilution factor} \) with \( \varepsilon_{\text{DNA}} = 50 \) and \( \varepsilon_{\text{RNA}} = 40 \). The ratio \( A_{260}/A_{280} \) was taken as a measure of the purity of an RNA sample.

3.2 Cell biological methods

3.2.1 Buffers and solutions

**PBS**
- 8 g/L NaCl
- 0.2 g/L KCl
- 0.2 g/L KH\(_2\)PO\(_4\)
- 2.85 g/L Na\(_2\)HPO\(_4\) *12 H\(_2\)O
- in ddH\(_2\)O, pH 7.2, storage at 4 °C

**Freezing medium**
- 20 % (v/v) DMSO
- 80 % (v/v) FCS
- prepared freshly before use

**Trypsin / EDTA**
- 0.5 g/L trypsin
- 0.2 g/L EDTA
- in sterile PBS, storage at 4 °C

**Trypan blue solution**
- 0.16 % (w/v) trypan blue
- 0.9 % (w/v) NaCl
- 0.1 % (w/v) NaN\(_3\)
- in ddH\(_2\)O, filtered (0.45 µm), storage at 4 °C

**RBC lysis solution**
- 8.3 g/L NH\(_4\)Cl
- 1 g/L KHCO\(_3\)
- 0.037 g/L EDTA
- in ddH\(_2\)O, pH 7.2 – 7.4, autoclave, storage at 4 °C
3.2.2 Cell culture methods

3.2.2.1 Culture of cells
All cells were grown at 37 °C in a cell incubator in a 5 % carbon dioxide / 100 % humidity atmosphere. If not mentioned otherwise all cell culture media were supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin. The FCS added to the media was heat inactivated at 56 °C for 1 h before use. All media supplements were filtered (pore size: 22 µm) before addition to the media.

Most adherent cells were detached from the surface of the cell culture flasks with the help of a trypsin / EDTA solution. Before the detachment the growth medium was removed and the cells were washed by the careful addition and subsequent removal of 10 – 20 mL PBS. Then 40 µL trypsin / EDTA solution per square centimetre surface was added and the cells were kept for 5 – 10 minutes at 37 °C in a cell incubator. When the cells were detached they were washed in 10 – 20 mL growth medium to inactivate the trypsin / EDTA and used for further experiments.

Murine macrophages
The murine macrophages used in the experiments were always generated from bone marrow cells. They were generated in DMEM supplemented with 10 % FCS and 20 % M-CSF-containing L929 cell supernatant. The macrophages grew adherently and showed the typical spindle-like morphology. For a detailed description of the generation and culture of macrophages see chapter 3.2.3.

Murine spleen and bone marrow cells
The murine spleen and bone marrow cells were cultured in RPMI 1640 supplemented with 10 % FCS. Both cell populations consisted of round cells of varying size that did not adhere to the surface of the cell culture dishes. Since the two cell populations did not proliferate and were mostly cultured for two days at the most, no splitting was necessary.
Methods

J774A.1, RAW 264.7 cells

The macrophage-like J774A.1 and RAW 264.7 cells grew adherently and were cultured in DMEM supplemented with 10 % FCS. Originally the J774A.1 were derived from a reticulum cell sarcoma and the RAW 264.7 cells were induced by Abelson murine leukemia virus. They showed a heterogeneous morphology ranging from round to spindle-like forms. In order to culture the cells they were harvested when the cell layer in the cell culture flask was 80 – 90 % confluent. To harvest the cells from the flask the growth medium was removed and the cell layer was washed once with PBS. Then PBS was added again (e.g. 10 mL PBS for a flask with 75 cm² surface area) and the cells were carefully detached from the surface with a cell scraper. After washing once with 10 – 20 mL growth medium (250 g, 5 min.) 1/5ᵗʰ to 1/20ᵗʰ of the cells were transferred into a new cell culture flask.

MEF, NIH/3T3 and L929 cells

These three mouse fibroblast cell lines grew adherently and were harvested using trypsin / EDTA solution when there was a confluent monolayer. They were cultured in DMEM supplemented with 10 % FCS and usually split 1:10 twice every week.

CT26wt/p, DA3, Eb and Esb cells

The CT26wt/p and DA3 mouse tumour cell lines grew adherently and were harvested using trypsin / EDTA solution. The two murine colon carcinoma cell lines CT26wt and CT26p differed in their origin (see 2.11.2). They were cultured in DMEM with 10 % FCS and split 1:10 twice every week. The mouse breast carcinoma cell line DA3 was grown in RPMI 1640 with 10 % FCS supplemented with β-mercaptoethanol in a final concentration of 0.05 mM and split twice every week 1:15. The murine non-metastasising T cell lymphoma cell line Eb and the highly metastasising Esb 289 cell line grew in suspension in RPMI 1640 supplemented with 10 % FCS. For the Esb cells the growth medium was also supplemented with 0.05 mM β-mercaptoethanol. Both cell lines were split 1:10 twice a week.
Methods

Jurkat, U937, MCF-7 and Hela cells

The human T cell lymphoma cell line Jurkat and the human histiocytic lymphoma cell line U937 grew in suspension and were cultured in RPMI 1640 with 10 % FCS. They were split 1:10 twice a week. The human breast carcinoma cell line MCF-7 and the human cervical adenocarcinoma cell line Hela grew adherently and were harvested using trypsin / EDTA solution. They were cultured in RPMI 1640 with 10 % FCS and were split twice a week 1:8.

3.2.2.2 Freezing and thawing of cells

In order to freeze mammalian cells, 1*10^6 to 1*10^7 cells were suspended in 500 µL of the respective growth medium. To the cell suspension 500 µL freezing medium (see 3.2.1) was added and the samples were transferred immediately to -80 °C. After one week at -80 °C the cells were transferred to liquid nitrogen containers and stored at -196 °C.

In order to thaw cells, 37 °C warm growth medium was added to the frozen cells with a Pasteur pipette. One washing step was performed in 10 mL growth medium (250 g, 5 min.) to remove the DMSO. After that the cells were seeded in fresh medium in a cell culture flask.

3.2.2.3 Determination of cell number and viability

Cells were counted with the help of a hemocytometer (Neubauer cell counting chamber, depth 0.1 µL) and an optical microscope. To distinguish live and dead cells, trypan blue was added to the cell suspension in different dilutions ranging from 1:2 up to 1:10. Trypan blue stains only dead cells. The suspension was diluted enough so that the cells did not overlap each other on the counting grid. Cells that overlapped the top or left ruling of a large square were counted, whereas cells overlapping the bottom or right ruling were not counted. At least 100 living cells were counted for each sample in order to produce a statistically significant count. The cell titre was calculated using the following formula:

\[
\text{Cells} [\text{mL}^{-1}] = \frac{\text{Total cell count}}{\text{Number of counted large squares}} \times \text{Dilution factor} \times 10^4.
\]

The viability of a cell population could be determined by calculating the percentage of living cells.
3.2.3 **Generation of macrophages from murine bone marrow cells**

The macrophages used for this thesis were always generated from murine bone marrow cells. 4*10^6 freshly harvested bone marrow cells were seeded into 10 cm diameter cell culture dishes and were grown for 8 – 12 days in RPMI 1640 with 10 % FCS and 20 % M-CSF containing supernatant from L929 cells. After removal of the medium the macrophages were harvested in PBS with a cell scraper and washed once in growth medium (250 g, 5 min.). Before the use in further experiments the phenotype was tested with an anti-F4/80 antibody.

To generate the L929 supernatant, a dense L929 cell monolayer was split 1:10 and then grown for one week in DMEM with 10 % FCS. Then the supernatant was harvested, centrifuged (250 g, 5 min.) and used in further experiments.

3.2.4 **Generation of supernatant containing anti-IFN-α and anti-IFN-β antibodies**

To generate supernatant containing anti-IFN-α and anti-IFN-β antibodies for blocking assays, 7FD3 and 4EA1 hybridoma cells were grown in RPMI 1640 with 10 % FCS until there was a dense monolayer. Then the cells were harvested using trypsin / EDTA solution and the cells were seeded again in a vessel of the same size in RPMI 1640 without FCS to avoid the presence of foreign proteins in the supernatant. After four days the supernatants was harvested and the cells were removed by centrifugation (250 g, 5 min.). Finally the neutralisation titre of the supernatants against mouse interferon was determined.

3.2.5 **Preparation of human PBMC**

The human PBMC used in the experiments were prepared from buffy coats, a fraction of a centrifuged blood sample that contains most of the leukocytes. LeucoSep centrifuge tubes were filled with 15 mL Ficoll solution and centrifuged shortly (250 g, 1 min.). Theuffy coat was diluted 1:4 in serum-free RPMI 1640 and loaded onto the prepared LeucoSep tubes with 35 mL volume per tube. The tubes were centrifuged (800 g, 20 min., no brake), leading to an interphase enriched in PBMC between the Ficoll solution and the plasma. This interphase was collected with a Pasteur pipette and washed with serum-free RPMI 1640 (800 g, 10 min.). Two more washing steps with serum-free RPMI 1640 followed (250 g, 5 min. and 100 g, 5 min.). The cell pellet was resuspended in PBS and filtered with a cell strainer (40 µm).
3.2.6 Transfection of mammalian cells with jetPEI
To transfect mammalian cells with plasmid DNA, the cationic polymer transfection reagent jetPEITM was used according to the manufacturers instructions (PolyPlus, www.polyplus-transfection.com, In vitro Transfection Protocol, Ref: CPT 101, Version H). The transfections were usually carried out on a 12 to 24 well scale.

3.2.7 Determination of interferon in supernatants
The determination of biologically active interferon was done in the laboratory of Prof. Dr. Rainer Zawatzky, Division of Viral Transformation Mechanisms, German Cancer Research Center Heidelberg. The assay was based on the induction of an antiviral state in murine L929 cells protecting against subsequent VSV infection.

The supernatants to be tested were diluted in steps of two and these dilutions were given to L929 cells over night. In parallel a dilution series of a solution with known IFN-α/β concentration was used to later generate a standard curve. The next day VSV was added to all pretreated L929 cells and cell lysis was measured 2 days later. The dilution of a supernatant that protected 50 % of the cells against lysis contained by definition 1 IU/mL interferon. The standard curve generated with the known amounts of IFN-α/β was used to correct for variations in interferon responsiveness of the L929 cells in different assays.

3.3 Virological methods

3.3.1 Production of NDV stocks
The different NDV strains used in this thesis were propagated in embryonated chicken eggs. Fertilised eggs were first incubated for 10 days at 37 °C and 62 % humidity. During this period they were turned over every 4 hours. At day 10 the eggs were candled to sort out dead embryos and to mark the air cell on the eggshell. Through the air cell 100 µL of an NDV inoculation stock was injected into the egg and the puncture in the shell was sealed with paraffin. After further incubation for 3 days the eggs were incubated for 4 hours at 4 °C to kill the embryos. The eggshell above the air cell was removed and the allantoic fluid was harvested with a Pasteur pipette. Debris was removed from the allantoic fluid by centrifugation (1800 g, 30 min., 4 °C) and the NDV was sedimented by ultra-centrifugation.
(50000 g, 1 h, 4 °C). The NDV pellet was resuspended in 0.5 mL PBS and purified by ultra-
centrifugation (97000 g, 1 h, 4 °C) on a saccharose cushion. The band containing the NDV
particles was harvested and the NDV was sedimented again by ultra-centrifugation (50000 g,
1 h, 4 °C). The NDV pellet was resuspended in a PBS / 0.1 % EDTA solution and the NDV
amount was quantified by a hemagglutination assay (see chapter 3.3.2). Thereafter, the NDV
solution was diluted and divided up into appropriate aliquots if desired and the virus solutions
were kept at -70 °C or in liquid nitrogen for archival storage.

3.3.2 NDV quantification with the hemagglutination assay
After NDV production the virus amount was determined in a hemagglutination assay that uses
the ability of NDV to adsorb to and agglutinate sheep erythrocytes. Sheep erythrocytes were
washed twice in PBS and adjusted to a concentration of 1 % (w/v) in PBS. In 96 well round
bottom plates 1:2 PBS dilution series of the virus samples to be tested were seeded in
duplicates. Then the 1 % sheep erythrocyte solution was added to the wells and after an
incubation period of 60 – 90 minutes at room temperature the virus titre was read out. 1
hemagglutination unit (HU) was defined as the smallest virus concentration leading to
visible sheep erythrocyte agglutination.

3.3.3 Infection of cells with NDV
Cells were washed with serum-free growth medium (250 g, 5 min.) and counted. The cells
were then mixed with the respective amount of NDV in 100 µL serum-free medium per
10⁶ cells. The mixture was kept at 37 °C for 1 h and every 15 minutes during that time the
samples were homogenised with a cell shaker. The cells were washed twice in their respective
growth medium and for NDV binding assays the infected cells were now used in flow
cytometric analysis. For the study of viral replication the infected cells were seeded into cell
culture flasks or plates to be harvested at a later time point for analysis.

3.3.4 UV inactivation of NDV
NDV was UV inactivated for some experiments by exposure for 5 min. to UV-light (254 nm,
2 mW/cm², 7 cm distance). After UV inactivation the virus particles were thought to be intact
but the NDV was no longer able to replicate due to irreparable damage to its RNA genome.
3.4 Protein chemical methods

3.4.1 Buffers and solutions

**SDS poly-acrylamide gel electrophoresis**

- **Acrylamide solution**
  - 37.5 % acrylamide
  - 1 % bis-acrylamide

- **Sample buffer, 2x**
  - 0.5 M Tris HCl (pH 6.8)
  - 10 % (w/v) glycerol
  - 2 % SDS
  - 5 % β-mercaptoethanol
  - 0.1 % (v/v) bromophenol blue
  - storage at 4 °C

- **Separating gel (12.5 %)**
  - 4.525 mL ddH₂O
  - 4.15 mL acrylamide/bis-acrylamide
  - 1.325 mL Tris HCl (3 M, pH 8.8)
  - 0.1 mL SDS (10 %)
  - 40 µL APS
  - 5 µL TEMED

- **Stacking gel**
  - 3 mL ddH₂O
  - 0.675 mL acrylamide/bis-acrylamide
  - 1.25 mL Tris HCl (0.5 M, pH 6.8)
  - 50 µL SDS (10 %)
  - 50 µL APS
  - 5 µL TEMED

- **Electrophoresis buffer**
  - 25 mM Tris-HCl (pH 8.3)
  - 0.19 M glycine
  - 0.1 % (w/v) SDS
  - storage at room temperature

**Western blot**

- **Buffer 1**
  - 0.04 M ε-aminocaproic acid
  - 0.025 M Tris HCl
  - pH 9.4, storage at room temperature
## Methods

| Buffer 2 | 0.3 M Tris HCl  
pH 10.4, storage at room temperature |
|----------|------------------------------------------------|
| Buffer 3 | 0.025 M Tris HCl  
pH 10.4, storage at room temperature |
| TBS solution | 10 mM Tris HCl  
150 mM NaCl  
pH 8.0, storage at room temperature |
| T-TBS solution (0.1 %) | 0.1 % (v/v) Tween 20  
in TBS |
| Blocking buffer (5 %) | 5 % (w/v) powdered milk  
in T-TBS (0.05 %) |

### 3.4.2 Generation of cell lysates

Cells were harvested, washed once in PBS (250 g, 3 min.) and counted. The PBS was removed again by centrifugation (250 g, 3 min.) and 125 µL CellLytic™-M (mixed with 2 % (v/v) Protease Inhibitor Cocktail) was added per 1*10^7 cells. The cells were homogenised in the buffer by pipetting up and down and then they were incubated for 15 minutes on a shaker at room temperature. After centrifugation in a table centrifuge (16.000 g, 15 min.) the protein-containing supernatant was transferred to a chilled test tube and stored at -80 °C.

### 3.4.3 Bradford protein assay

The Bradford protein assay is a method commonly used to determine the total protein concentration of a sample. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilise the anionic form of the dye, causing a visible colour change. Within the linear range of the assay it can be assumed that the more protein is present, the more Coomassie dye binds to proteins. Standardised samples with known FCS concentration (0.1 – 1.4 mg/mL) were used to
generate a standard curve. 100 µL of the standardised and the test samples were transferred onto a 96 well flat bottom plate in duplicates. Then 100 µL Coomassie Brilliant Blue was added to each well and after 5 minutes the absorbance at 595 nm was read out in an ELISA reader.

3.4.4 SDS poly-acrylamide gel electrophoresis
SDS poly-acrylamide gel electrophoresis (SDS-PAGE) is used to determine the molecular weight and the purity of proteins. SDS is used to denature the proteins to the same linear shape and to coat them with many negative charges. In poly-acrylamide the proteins migrate towards the positive pole when an electric field is applied and the migration speed depends only on the size of the protein. The separation starts in an ion gradient to enhance the sharpness of the protein bands within the stacking gel, which represents a rather wide-meshed polymer. The actual separation takes place in the fine-meshed separating gel.

The separating gel was prepared as described earlier (3.4.1) and poured into the gel cassette until the gel level reached a position of about one centimetre from the bottom of the comb that would form the loading wells. After solidification the stacking gel was poured on top and also allowed to solidify. The cassette with the complete gel was fixed in the electrophoresis chamber which was then filled with electrophoresis buffer. The samples were mixed 1:1 with sample buffer and heated for 5 minutes at 95 °C. 20 µL of these samples were loaded into the wells and a molecular weight marker was loaded in parallel in a separate well. The electrophoresis was run with a constant current of 75 mA until the bromophenol blue of the sample buffer had run out of the gel. At that point the electrophoresis was stopped and the separating gel was taken out of the cassette.

3.4.5 Staining of poly-acrylamide gels with Coomassie
The gels were washed three times for 20 minutes in distilled water on a shaker (50 rpm) and then they were incubated for 45 minutes in 20 mL of the GelCode® Blue Stain Reagent. Then the gels were washed three times for 30 minutes in distilled water.

3.4.6 Drying of gels
To preserve the gels they were placed on a piece of Whatman paper and covered with cling film. The gels were then dried at 80 °C for 30 minutes in a vacuum gel drier.
3.4.7 Western blot

3.4.7.1 Transfer of proteins onto anitrocellulose membranes
After gel electrophoresis (3.4.4) the separating gel was transferred onto a nitrocellulose membrane using a semi-dry apparatus. 6 sheets of Whatman paper were soaked in buffer 2 and three sheets were soaked in buffer 3. The pre-wetted paper sheets were then placed on the graphite plate constituting the anode without the inclusion of air bubbles. The next layer was the nitrocellulose membrane that had been washed in buffer 3 and pre-wetted in methanol. On the membrane the separating gel was placed after pre-wetting in buffer 3. The last layer consisted of 9 sheets of Whatman paper that had been soaked in buffer 1. On top the cathode plate was placed and the blot was run for 90 minutes at 0.8 mA/cm². After the blot was finished the bands of the molecular weight marker were marked on the membrane.

3.4.7.2 Staining of nitrocellulose membranes
After the protein transfer the nitrocellulose membranes were blocked for one hour in 5 % blocking buffer on a shaker (50 rpm). Then the primary antibody binding to the protein of interest was diluted in 10 mL of 5 % blocking buffer and added to the membranes. After incubating for 2 hours at room temperature or over night at 4 °C the membranes were washed once for 15 minutes in 0.1 % T-TBS solution and twice for 5 minutes in 0.05 % T-TBS solution. A peroxidase-conjugated secondary antibody was diluted in 10 mL of 2.5 % blocking buffer (0.08 µg antibody / mL) and added to the membranes for one hour at room temperature. The membranes were washed again once in 0.1 % T-TBS solution and twice in 0.05 % T-TBS solution.

Detection was carried out with the ECL system. The detection solutions were mixed 1:1 and given to the membranes for one minute. Excess solution was removed with paper towels and the membranes were placed in cling film. The chemiluminescence on the membranes was detected with x-ray film and the time for development varied depending on the intensity of the chemiluminescence.

3.4.7.3 Densitometric analysis of the Western blot films
The Western blot films were analysed with the ImageJ software (Wayne Rasband, National Institutes of Health). The films were scanned and then the digital image was inverted and
stored in TIF format. The mean signal intensity of the background was measured and automatically subtracted from all subsequent measurements. Then an area was drawn around the largest band and the mean of the bands was determined be moving this area over the band to be analysed before measurement of the mean.

### 3.5 Immunobiological methods

#### 3.5.1 Buffers and solutions

FACS buffer

- 5 % (v/v) FCS
- 0.1 % (w/v) NaN₃
- in sterile PBS, storage at 4 °C

PI solution

- 1 ng/µL propidium iodide
- in PBS, lightproof storage at 4 °C

#### 3.5.2 Flow cytometry

**3.5.2.1 Determination of cell surface antigens with flow cytometry**

Flow cytometry or FACS can be used to distinguish cells according to their size, structure, cell surface properties and internal composition. Often cells are labelled with antibodies that bind to certain cellular structures. These antibodies are either conjugated themselves with a fluorescing dye or are detected with a secondary antibody that is connected to a fluorescing dye. In addition cells can be labelled with fluorescing dyes that stain, for example, only dead cells. In a flow cytometer the cells are moving in a laminar flow and pass a laser beam. On the one hand the light excites the dyes that subsequently emit a fluorescence of a characteristic wave length that is detected by sensors. On the other hand the light is refracted by the cell depending on its size and granularity and the refraction can be used to determine the latter two properties of a cell.

In order to measure the fluorescence of cells expressing EGFP or cells stained with dye-labelled antibodies 1*10⁵ to 1*10⁶ cells per sample were resuspended in FACS buffer and loaded into FACS tubes. The cells were washed once in FACS buffer (250 g, 3 min., 4 °C)
and EGFP expressing cells were taken up in 200 µL FACS buffer and used immediately in flow cytometric analysis. To stain surface antigens the cells were resuspended in 40 µL FACS buffer containing an appropriate amount of the antibody (usually around 10 µg/mL) binding to the antigen in question. After incubation for 20 minutes on ice in the dark the cells were washed twice in FACS buffer (250 g, 3 min., 4 °C) and if the antibody was directly labelled with a fluorescing dye the cells were taken up in FACS buffer and analysed by FACS. If the primary antibody was not labelled, the cells were now resuspended in 40 µL FACS buffer containing a dye-labelled secondary antibody. After incubation for 20 minutes on ice in the dark the cells were washed twice in FACS buffer (250 g, 3 min., 4 °C) and used in flow cytometry.

When the cells were analysed in a flow cytometer the settings were adjusted with unstained control cells. The forward and the side scatter were adjusted to distinguish the main cell populations and the autofluorescence of the cells was set in the first decade of the fluorescence channels. If the cells had been stained with more than one antibody and/or dye with overlapping fluorescence spectra, the signals in a channel derived from dyes other than the one to be measured in this channel were neutralised by compensation. For this purpose, samples that generated only a single fluorescence signal were used, for example cells that were only stained with one type of a dye-labelled antibody.

After the settings and the compensation were completed, 10,000 to 100,000 cells of each sample were measured. The analysis was carried out later on with the WinMDI 2.8 flow cytometry data analysis software.

3.5.2.2 Determination of dead cells with propidium iodide

Propidium iodide can be used to distinguish dead and living cells in flow cytometry. Dead cells have disruptions in their cell and nuclear membrane and propidium iodide (PI) can enter the cells and intercalate in the DNA. Dead cells show therefore a fluorescence signal in the FACS channel three induced by the PI, whereas living cells have intact membranes and show no PI induced fluorescence signal in this channel.

Just before flow cytometry PI was added to the cell samples in FACS buffer to a final concentration of 1 µg/mL.
3.6 **In vivo experiments**

3.6.1 **Preparation of murine spleen cells**
Mice were sacrificed by CO₂ inhalation and the fur was sterilised with 70 % ethanol. All the following steps were carried out under sterile conditions in a tissue culture hood. The mice were placed on a preparation pad and fastened with metal pins. They were then cut open on the ventral side through the skin and the peritoneum, and the spleen was taken out and placed in a 60 mm dish filled halfway with PBS. With a cell scraper the cells were carefully pushed out of the spleen by repeated strokes from the centre of the spleen towards its ends. This procedure was continued until all of the cells were in suspension in the PBS and only the outer skin of the spleen remained. The cell suspension was filtered with a 40 µm cell strainer to remove tissue residues. The cells were washed once in PBS (250 g, 5 min.) and to the cell pellet 1 mL RBC lysis buffer was added. After 1 minute the cells were washed in PBS (250 g, 5 min.) and in growth medium (250 g, 5 min.) and then they were counted and used in the subsequent experiments.

3.6.2 **Preparation of murine bone marrow cells**
To prepare bone marrow cells, mice were sacrificed by CO₂ inhalation and the fur was sterilised with 70 % ethanol. All the following steps were carried out under sterile conditions in a tissue culture hood. The mice were cut open and the femurs were taken out and placed in a 60 mm dish filled halfway with PBS. For sterilisation the femurs were first transferred in a 60 mm dish filled with 70 % ethanol and after one minute they were again transferred into a new dish filled with PBS. In yet another dish filled with PBS the femurs were carefully cut at the ends and the bone marrow cells were flushed out with a syringe with a 0.4 mm Ø injection needle. The femurs were flushed three times from every end with 2 mL PBS. After flushing the femurs the cell suspension in the dish was filtered with a 40 µm cell strainer to remove tissue residues. The cells were washed once in PBS (250 g, 5 min.) and to the cell pellet 1 mL RBC lysis buffer was added. After 1 minute the cells were washed in PBS (250 g, 5 min.) and in growth medium (250 g, 5 min.) and then they were counted.

3.6.3 **Organ preparation for RNA extraction**
For organ preparation the mouse was sacrificed by CO₂ inhalation and the fur was sterilised
Methods

with 70 % ethanol. The mouse was placed on a preparation pad and fastened with metal pins. It was then cut open on the ventral side through the skin and the peritoneum which were fixed with metal pins. The organs such as liver, kidney or spleen were removed carefully by severing their main blood vessels and their main tissue connections. To remove the lung the rib cage was cut open along the sternum and fixed on the side. The organs were placed on 6 cm diameter petri dishes and 3 squares with an edge length of about 3 mm were cut from different regions of the organ with a disposable scalpel. This was done to get an average of the whole organ, for example, by taking one square of each the outer, middle and inner part of the organ. The organ samples were immersed in 500 µL RNAlater and kept at 4 °C for 1 – 2 days before they were transferred to -20 °C for archival storage.

3.7 Statistical methods

To test the statistical significance of the results, the p-value was calculated using the Student's t-test. The unpaired t-test was employed and experimental results were regarded as statistically significant if the two-tail p-value was below 0.05. The groups that were compared were labelled in the figures and the exact p-values were given in the figures or in the legends. All error bars indicated the standard deviation if not mentioned otherwise.

Correlation analyses were done calculating the Pearson product-moment correlation coefficient using the Microsoft Excel spreadsheet software. Correlations were regarded as strong if the absolute value of the correlation coefficient was higher than 0.5.
4 Results

4.1 NDV infection of spleen and DA3 tumour cells

NDV can replicate substantially better in cancer cells than in most normal cells (Reichard et al., 1992; Schirrmacher et al., 1999a). The most important protection mechanism against NDV infection is the interferon response, which is mediated by the type I interferons IFN-α and IFN-β. Many tumour cells have acquired defects in the interferon response that might give them a growth and survival advantage (Fiola et al., 2006, Stojdl et al., 2000). To investigate a connection between NDV susceptibility and the antiviral interferon response in normal and tumour cells, primary mouse spleen cells and the murine mammary carcinoma cell line DA3 were compared with regard to NDV infection and the expression of genes involved in the antiviral response. Before the results of these analyses are shown, the sensitivity of the real-time PCR detection system will be examined, since most of the gene expression results were obtained with this technique.

4.1.1 The sensitivity of the real-time PCR detection system

The gene expression in many different cell types and tissues was analysed in this thesis with the help of quantitative real-time PCR after reverse transcription of mRNA into cDNA. The PCR is a sensitive molecular biological method since the target DNA is amplified manifold and in theory one copy of target DNA is sufficient to yield a signal. In order to find out what the practical limit of detection for the real-time PCR system used for this thesis was, a dilution series of samples with known copy number was analysed for the expression of the NDV matrix and the murine β-actin gene. In addition, the copy number per cell for the murine β-actin gene was calculated to determine the lowest number of cells necessary for gene expression analysis.

The real-time PCR detection limit for the NDV matrix gene was determined with a plasmid containing the M gene sequence of the NDV strain La Sota (kindly provided by Ben Peeters, Institute for Animal Science and Health (ID-Lelystad), The Netherlands). A 10-fold dilution series of this plasmid was tested in real-time PCR and the detection limit was found to be at 4.98*10^{-11} µg plasmid. The plasmid was 6551 bp long and contained 1 copy of the M gene.
Results

With an average base pair weight of 660 g/mol the detection limit for the M gene was calculated as follows: \(4.98 \times 10^{-17} \text{ g} / (6651 \text{ bp} \times 660 \text{ g/mol}) \times N_A = 6.83\). That is, about 7 copies of the M gene per sample could still be detected with the real-time PCR system used in this thesis.

The detection limit for the murine β-actin gene that was used to normalise the M gene expression was determined with a PCR product amplified from murine cDNA containing a part of the β-actin gene. The PCR product had a length of 677 bp and was amplified using primers designed with the Primer3 software (forward primer: 5´-AGC CAT GTA CGT AGC CAT CC-3´; reverse primer: 5´-ACA TCT GCT GGA AGG TGG AC-3´). This PCR product was cut out from an agarose gel and after photometrically determining the DNA concentration a 10-fold dilution series of the amplicon was tested in real-time PCR. The detection limit was found to be at \(2.4 \times 10^{-17} \text{ g} \) PCR product, corresponding to a copy number of \(2.4 \times 10^{-17} \text{ g} / (677 \text{ bp} \times 660 \text{ g/mol}) \times N_A = 32.34\). Hence, about 32 copies of the murine β-actin gene per sample could still be detected.

To estimate the number of mRNA copies in a tumour cell total RNA was extracted from one million DA3 mouse mammary carcinoma tumour cells. After reverse transcription of 240 ng total RNA, 3 out of 40 µL of a 1-500 dilution of the cDNA was used for the detection of β-actin in real-time PCR. The ensuing \(c_T\) (24.13) was used in the following formula derived from a standard curve generated with a dilution series of the above mentioned β-actin PCR product: copy number = \(10^{-0.3072 \times c_T + 11.876}\). Therefore, the β-actin mRNA copy number per DA3 cell was \(29,058 \times 500 \times (40/3) / 1 \times 10^6 = 194 \text{ copies per DA3 cell}\). Translated into cell numbers the detection limit for β-actin was \(32/194 = 0.165\) DA3 cells.

In conclusion it can be said that the real-time RT-PCR technology applied in this thesis was very sensitive and could detect down to a few copies of the NDV M gene and the murine β-actin gene. It was also possible to analyse the gene expression of a single cell since the β-actin detection limit was at 1/6 cell. The exact number of cells that can still be detected per sample might vary depending on the cell type since cells with a larger volume such as certain tumour cells can be expected to contain more β-actin mRNA copies than normal cells and will therefore have a lower cell detection limit.
4.1.2 NDV susceptibility

In general NDV can successfully infect only tumour cells, whereas primary, non-malignant cells are almost completely resistant to infection. In order to compare the susceptibility to NDV infection between primary murine spleen cells and murine mammary adenocarcinoma DA3 cells these two cell types were infected with the monocyclically replicating NDV strain Ulster. An infection kinetics was established over a period of two days by extracting RNA at different time points and determining the amount of the viral M gene in the cDNA as a measure for viral replication with the help of real-time RT-PCR. In addition, spleen cells were infected with different amounts of NDV Ulster to analyse the correlation between the virus infection level and the ensuing M gene expression level and to determine the maximum M gene expression level in these cells.

Figure 4-1a shows that there was a large difference in M gene expression after NDV Ulster infection between spleen and DA3 cells. Spleen cells reached a plateau of expression at 8 hours after infection which was only slightly higher than the initial level at 4 hours. After 8 hours the expression decreased slightly. DA3 cells showed a strong increase in M gene expression between 4 and 8 hours after infection and reached the maximum at 24 hours. The maximum M gene expression in DA3 cells was approximately 5000 times higher than that in spleen cells. In figure 4-2b it can be seen that the plot follows a saturation curve culminating at about 500 REU (relative expression units), which was about 20 times lower than the maximum M gene expression obtained in DA3 cells.
Results

The spleen cells were highly resistant to NDV infection compared to DA3 cells even when infected with high doses of NDV. Antiviral mechanisms in spleen cells seem to have become active early after infection and to have stopped viral replication. Since there were pronounced differences in NDV replication between spleen and DA3 cells, there must have been differences in the antiviral response of these two cell types.

4.1.3 Interferon-related gene expression

In order to find out more about the differences in the antiviral response that could cause the strong differences in NDV susceptibility between murine spleen and DA3 tumour cells, the expression of genes related to an antiviral interferon response in these two cell types was analysed. The RNA samples taken in the NDV Ulster infection kinetics as shown before (4.1.2) were used to analyse the expression of seven genes known to be important for the establishment and effectiveness of an antiviral response (see for example Sen, 2001; Seth et al., 2006). The change in gene expression relative to the basal level was determined with real-time RT-PCR, first within the murine spleen cells.

Figure 4-2 shows an increase in the expression of all of the seven genes analysed after

![Figure 4-2](image-url)
infection with NDV Ulster. The expression increased until it peaked at 8 hours and decreased afterwards. The expression level at 48 hours was still higher than the basal level before NDV infection. The highest increase in expression could be observed for the antiviral effector molecule Mx1. The RIG-I gene expression showed only a slight increase. For the other genes the maximum expression level was about 30 – 50 times higher than the basal expression.

The spleen cells showed a clear increase in the expression of all analysed genes after NDV infection. The gene expression kinetics correlated with the NDV M gene expression observed before in that it peaked at 8 hours and declined thereafter.

Next the RNA samples taken in the NDV Ulster infection kinetics for the DA3 cells as shown before (4.1.2) were used to analyse the expression kinetics of the interferon-related genes.

Figure 4-2: Inducible expression of genes related to the interferon response in murine spleen cells infected with NDV Ulster.

Murine spleen cells were infected with 10 HU NDV Ulster per 10^6 cells and total RNA was prepared 0, 4, 8, 24 and 48 hours after infection (see Figure 4-1). After reverse transcription the respective gene was quantified by real-time PCR with the comparative C\text{t} method using \(\beta\)-2-microglobulin for normalisation. The results are represented as the mean of three measurements. The expression level of each gene is shown relative to the basic level at 0 hours and the results are represented as the mean of three measurements. Bars indicate standard deviation.
Results

In figure 4-3 it can be seen that the expression of the analysed genes increased during infection with NDV Ulster except for the IRF7. The increase peaked at 24 hours except for the RIG-I gene, which displayed a maximum at 8 hours. The increase compared to the basal expression was highest for the antiviral effector molecule OAS1a being about 45-fold, followed by Mx1 with a 30-fold increase. For the other genes the expression increased 2 – 10-fold.

![Figure 4-3: Inducible expression of genes related to the interferon response in murine DA3 tumour cells infected with NDV Ulster.](image)

Murine DA3 mammary tumour cells were infected with 10 HU NDV Ulster per 10^6 cells and total RNA was prepared 0, 4, 8, 24 and 48 hours after infection (see Figure 4-1). After reverse transcription the respective gene was quantified by real-time PCR with the comparative C_t method using β-2-microglobulin for normalisation. The expression level of each gene is shown relative to the basic level at 0 hours and the results are represented as the mean of three measurements. Bars indicate standard deviation.

The DA3 cells showed a heightened gene expression that peaked at a later time point then in the spleen cells. In general, the increase relative to the basal expression level was lower than in spleen cells.

In order to compare the absolute gene expression in DA3 and spleen cells during NDV
Results

infection, the expression levels were plotted relative to the basal level of the DA3 cells.

Figure 4 shows that the expression level for the IRF7 transcription factor, for interferon-β and for Mx1 were clearly higher in spleen cells during NDV infection. For OAS1a the same maximum expression was reached at 8 hours for the spleen cells and at 24 hours for the DA3 cells. The basal expression level of all analysed genes was comparable except for MDA5 and PKR, which were lower in spleen cells (10-fold and 100-fold respectively).

Figure 4: Comparison of the interferon-related gene expression in spleen cells and DA3 tumour cells after infection with NDV Ulster.

Murine spleen cells and DA3 mammary tumour cells were infected with 10 HU NDV Ulster per 10^6 cells and total RNA was prepared 0, 4, 8, 24 and 48 hours after infection (see Figure 4). After reverse transcription the respective gene was quantified with by real-time PCR the comparative C_T method using β-2-microglobulin for normalisation. The expression level of each gene is shown relative to the basic level at 0 hours in DA3 cells and the results are represented as the mean of three measurements. Bars indicate standard deviation.

The main difference in the antiviral interferon response between spleen and DA3 cells seemed to be a much faster and stronger induction of the expression of some antiviral genes in spleen cells. These differences could explain the high susceptibility of DA3 tumour cells and the resistance of spleen cells to NDV infection. The basal gene expression levels were similar and
hence could not be connected with the differences in NDV susceptibility.

However, since spleen cells represent a heterogeneous mixture of different normal cell types such as T and B cells, dendritic cells, macrophages and the like, it would be more informative to compare primary and tumour cells of the same cell type to compensate for expression differences related to cell type. This approach was followed in the next but one chapter. Before the question if NDV could also induce gene expression \textit{in vivo} was addressed.

\subsection*{4.1.4 Interferon-related gene expression \textit{in vivo} after systemic NDV application}

NDV can be used systemically in tumour therapy for the generation of anti-tumour vaccines or as a direct oncolytic agent in virotherapy. To find out whether and to what extent NDV also induces the expression of interferon-related genes \textit{in vivo}, NDV was injected into the bloodstream of mice, and spleen and bone marrow cells were extracted after 12 hours. After RNA extraction real-time RT-PCR was used to determine the expression level of the NDV M gene and of seven genes related to an antiviral interferon response.

NDV M gene expression could only be detected in the cDNA from spleen cells at a low level (0.27 REU). No M gene could be detected in the bone marrow samples (data not shown).

Figure 4 -5 shows an increase in the gene expression in spleen cells 12 hours after the systemic application of NDV Ulster for all genes. In bone marrow cells an increase in gene expression could only be observed for the IRF7 transcription factor and for the two antiviral effector molecules PKR and Mx1. In general the gene expression level in the PBS control mice was several times higher in bone marrow than in spleen cells.
Results

The fact that spleen cells displayed a higher induction of gene expression than the bone marrow cells could be explained by a better accessibility of the spleen for NDV. Firstly, M gene expression can only be detected in the spleen samples and secondly, the spleen has an exceptionally good connection to blood vessels since its main purposes are the collection of antigens from the blood and the disposal of senescent red blood cells. The generally higher basal expression of interferon-related genes in bone marrow cells especially in the PBS control mice points to a higher responsiveness of these cells to virus infection. This could be due to a higher number of plasmacytoid dendritic cells, which are known to produce large amounts of type I interferons in response to virus infection (Siegal et al., 1999) and which have been shown to be several times more frequent in the mouse bone marrow compared to spleen (Asselin-Paturel et al., 2003).

Figure 4-5: Changes in interferon-related gene expression in vivo after systemic application of NDV Ulster.

750 HU NDV Ulster or PBS were injected with a volume of 300 µL into the tail vein of DBA/2 mice. Spleen and bone marrow cells were isolated 12 hours later and after total RNA extraction and reverse transcription a real-time PCR was carried out to determine the expression levels of the listed genes. The expression level of the genes was determined with the comparative C_T method using β-2-microglobulin for normalisation. The expression level of each gene is shown relative to the level in spleen cells from mice treated with PBS. The results are represented as the mean of three measurements. Bars indicate standard deviation.
4.2 NDV infection of macrophages and the macrophage-derived tumour cell lines J774A.1 and RAW 264.7

In order to examine the properties that make tumour cells susceptible to NDV infection it is best to compare tumour and normal cells that belong to the same cell type. By this approach one can rule out that the differences that are observed between tumour and normal cells are due to a difference in cell type and not due to variation in malignancy. For this purpose, murine bone marrow-generated macrophages and the two murine macrophage-like tumour cell lines J774A.1 and RAW 264.7 were used. These three cell types were compared with regard to NDV susceptibility, growth characteristics, basal and NDV-induced interferon-related gene expression and interferon secretion upon infection. Moreover the influence of the pretreatment with anti-interferon-α+-β antibodies and with interferon-α4 on the aforementioned cell properties was analysed. In the end it should be determined what cellular characteristics correlate with NDV susceptibility and whether these characteristics could be found predominantly in tumour cells.

4.2.1 Expression of the F4/80 macrophage marker

It was important to determine if the macrophage-like phenotype is comparable in the generated macrophages and in the two cell lines. An equal macrophage-like phenotype was an important prerequisite for the following experiments to exclude that observed variations were due to cell type differences and not due to differences in malignancy. To determine the macrophage-like phenotype, the expression of the pan-macrophage marker F4/80, a cell surface glycoprotein that is expressed on a wide range of mature tissue macrophages (Leenen et al., 1994), was tested.

Figure 4-5 shows that the F4/80 antigen was expressed similarly on the three cell types.
Results

After it was ensured that the macrophage-like phenotype was comparable for the three cell types, their further characterisation was continued.

4.2.2 NDV infection after interferon or anti-interferon antibody pretreatment

The susceptibility for NDV infection of the bone marrow-derived macrophages and the two macrophage-like tumour cell lines J774A.1 and RAW 264.7 was assessed to correlate it with other cell characteristics measured later on. In addition the influence of externally added interferon or of interferon secreted by the cells themselves before NDV infection was tested. It is known, for example, that a low secretion of interferon can also be found in the absence of virus infection and that this secretion is important for the ability of cells to defend against virus infection (Prof. Rainer Zawatzky, oral communication).

4.2.2.1 NDV infection without pretreatment

It was necessary to determine the infectability of the three cell types with NDV since according to the working hypothesis an increased NDV susceptibility of a cell type points to defects in the antiviral response mechanisms which will be analysed later in this chapter. To this end the cells were infected with the lentogenic NDV Ulster and the velogenic NDV Italien and then cell samples were taken at different time points after infection for RNA extraction. The RNA was used for M gene expression analysis by real-time RT-PCR and also for antiviral gene expression analysis (4.2.5 and 4.2.6). In addition the supernatants were

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Figure 4 -6: The expression of the F4/80 macrophage marker on bone marrow-derived macrophages, J774A.1 and RAW 264.7 cells.

Bone marrow-derived macrophages, J774A.1 and RAW 264.7 cells were stained with a FITC-conjugated anti-F4/80 antibody and the surface expression of this marker was determined in flow cytometry.
Results

collected and analysed for the interferon content (4.2.4).

Figure 4-7 shows distinct differences in M gene expression for the three cell types analysed. After 2 hours M gene expression was similar for all cells with the J774A.1 cells having a slightly lower level for both viruses. For the macrophages the M gene level remained at this low starting level of about 10 REU (NDV Ulster) or even decreased at later time points (NDV Italien). For the other two cell types the M gene level increased with a similar kinetics to reach a plateau at 12 hours after infection. The M gene expression of the RAW 264.7 cells was generally about 10-fold higher than that of the J774A.1 cells. At the plateau the ratio of J774A.1 versus RAW 264.7 M gene was about 1:18 (240 versus 4200 REU) for NDV Ulster and about 1:6 (1225 versus 7160 REU) for NDV Italien. When the peak in M gene expression for the macrophages (11 REU for NDV Ulster and 23 REU for NDV Italien) is compared to the highest expression of the two cell lines, it can be said that the J774A.1 cells were 20 – 50 times better infectable than the macrophages and the RAW 264.7 300 – 400 times better. Overall the M gene expression was 2 – 5 times higher when the three cell types were infected with NDV Italien compared to the NDV Ulster infections.

A  NDV Ulster  

B  NDV Italien

Figure 4-7: M gene expression in macrophages, J774A.1 and RAW 264.7 cells after infection with NDV Ulster or NDV Italien, respectively.

Bone marrow-derived macrophages from BALB/c mice, J774A.1 and RAW 264.7 tumour cells were infected with 10 HU NDV Ulster (A) or NDV Italien (B) per 10^6 cells. At 0, 2, 4, 8, 12 and 24 hours the cells were harvested and total RNA was extracted. After reverse transcription the expression level of the NDV M gene was determined by real-time PCR with the comparative C_T method using β-actin for normalisation. At 0 hours no M gene could be detected (data not shown). The results are represented as the mean of three measurements. Bars indicate standard deviation.
The NDV infection kinetics revealed pronounced differences in the M gene expression levels for the three cell types. The macrophages proved to be NDV resistant with a low and even decreasing M gene level during infection. The RAW 264.7 cells were highly susceptible to NDV infection with an M gene level several hundred times that of the macrophages. The J774A.1 cells showed an intermediate M gene expression.

To confirm these results for NDV susceptibility of the three cell types, another parameter for NDV replication was used. The cells were infected with recombinant NDV variants that expressed EGFP during the viral replication cycle and the EGFP signal was taken as indicator of viral replication. The cells were infected with NDFL-EGFP and NDFLtag-EGFP and one day later the EGFP signal of the cells was measured in flow cytometry.

Figure 4 -8 demonstrates that the RAW 264.7 cells had the highest EGFP signal, followed by the J774A.1 and then by the macrophages, which again showed to have the lowest signal. There was no clear difference in the EGFP signal between the lentogenic NDFL-EGFP and mesogenic NDFLtag-EGFP.
Results

The analysis of the EGFP signal derived from the infection with recombinant EGFP confirmed the results of the M gene expression after infection. The RAW 264.7 had the strongest signal, the macrophages displayed almost no signal and the strength of the EGFP signal of the J774A.1 cells was in between that of the two other cell lines.

4.2.2.2 Binding of NDV to J774A.1 and RAW 264.7 tumour cells

Since the differences observed in the M gene or EGFP signal after NDV infection could be due to differences in the ability of NDV to bind to the cells, the cell binding of NDV was analysed. J774A.1 and RAW 264.7 cells were infected with NDV Ulster and one hour later the NDV HN surface molecule was stained on the cells and measured by flow cytometry. At this time point the majority of the HN molecules on the cell surface could only come from bound virus particles, since the virus replication and expression of newly synthesised HN takes more than one hour.

Figure 4-8: EGFP expression after infection of macrophages, J774A.1 and RAW 264.7 tumour cells with NDFL-EGFP or NDFLtag-EGFP, respectively.

Bone marrow-derived macrophages from BALB/c mice, J774A.1 and RAW 264.7 tumour cells were infected with 10 HU per 10^6 cells NDFL-EGFP (A) or NDFLtag-EGFP (B, J774A.1 and RAW 264.7 only). After 24 h EGFP expression was determined in flow cytometry.
Figure 4-9 shows that NDV Ulster bound similarly to the J774A.1 than to the RAW 264.7 cells.

The RAW 264.7 cells displayed a higher M and EGFP signal after virus infection than the J774A.1 cells, but the virus binding was equal. Hence the virus binding could not explain the higher NDV susceptibility of the RAW 264.7 cells. It was likely that there were differences in the antiviral interferon response of the three macrophage-like cells. Important factors in this response are interferon secretion and responsiveness to external interferon, which were analysed in the next chapter.

4.2.2.3 Influence of interferon-α4 or interferon-α and -β antibody pretreatment on NDV infection

To control viral infection it is important for cells to secrete type I interferons and to react to type I interferon in an auto- or paracrine fashion, both at a low level before and also after infection. In order to analyse these parameters in the three macrophage cell types, one part of the cells was pretreated with anti-interferon-α and -β antibodies before infection to prevent the effect of a low basal interferon secretion that could be important for a constitutively increased defensiveness against virus infection. Without the effect of this basal interferon secretion the cells should be rendered more susceptible to NDV infection. Another part of the cells was

10⁶ J774A.1 or RAW 264.7 tumour cells, respectively, were incubated for one hour with 10 HU NDV Ulster. The cells were then stained with a mouse antibody against the NDV surface protein HN and with a PE-labelled GaM secondary antibody.
pretreated with interferon-α4, a crucial player in the first phase of the interferon response (Marié et al., 1998), in order to start an antiviral response before contact of the virus with the cells. So the main points that should be compared between the cells were the secretion of interferon and the responsiveness to external interferon. To that end the cells were pretreated as described before and then the percentage of EGFP-expressing cells was measured in flow cytometry one day after infection with NDFL-EGFP. Additionally the M gene expression was determined by real-time RT-PCR one day after infection with NDV Ulster.

Figure 4-10 demonstrates that the EGFP and the M gene expression after NDV infection could almost be completely blocked in the macrophages pretreated with interferon-α4. Also in the J774A.1 cells a strong blocking could be observed after the pretreatment with interferon-α4. In the RAW 264.7 cells the EGFP and M gene expression after infection could also be blocked, but only by 63%. With IFN-α4 pretreatment the RAW 264.7 cells displayed still an EGFP and M gene expression level that was about twice as high as that of J774A.1 cells without pretreatment.

The pretreatment with anti-interferon-α and -β antibodies slightly increased the EGFP and M gene expression except for the EGFP in macrophages and for the M gene in J774A.1 cells. For macrophages the M gene expression was increased 2-fold, for J774A.1 the EGFP expression 2.5-fold and for RAW 264.7 cells the increase was 1.1-fold for the EGFP and 1.4-fold for the M gene.
Results

Interferon-α4 pretreatment could block NDV infection almost completely in macrophages and the J774A.1, whereas the blocking was incomplete in RAW 264.7 cells. This pointed to a lower IFN-α4 responsiveness in the latter cells. The anti-interferon-α and -β antibody pretreatment did slightly increase the EGFP and M gene expression. The increase was higher in macrophages and J774A.1 cells than in RAW 264.7 cells. In order to analyse the kinetics of NDV infection after IFN-α4 pretreatment, the M gene expression was determined at several time points after infection with NDV Ulster with the help of real-time RT-PCR.

Figure 4-11 shows that in J774A.1 cells after IFN-α4 pretreatment no increase in the M gene expression could be observed at any time point. In RAW 264.7 cells between 4 and 8 hours the M gene expression increased much more in the control cells than in the pretreated cells. Between 8 and 20 hours the rate of increase is similar for the pretreated and for the control RAW 264.7 cells. Again the IFN-α4 pretreated RAW 264.7 cells displayed an M gene expression level that was about twice as high as that of J774A.1 cells without pretreatment.

Bone marrow-derived macrophages, J774A.1 and RAW 264.7 tumour cells were grown for 7 days either in normal growth medium (■ control group) or in medium supplemented with 1500 U/mL anti-IFN-α and 1900 U/mL anti-IFN-β antibody (■ anti-IFN-α + -β group and ■ IFN-α4 group). For 16 hours before NDV infection one group of cells (■ IFN-α4 group) was grown in medium supplemented with 120 IU/mL IFN-α4. The percentage of EGFP expression blocking in comparison to the control is indicated. (A) 24 hours after infection with 10 HU NDFL-EGFP per 10^6 cells EGFP expression of the cells was determined in flow cytometry. (B) 24 hours after infection with 10 HU NDV Ulster per 10^6 cells the expression level of the NDV M gene was determined by real-time RT-PCR with the comparative C_T method using β-actin for normalisation. The results are represented as the mean of three measurements. The percentage of M gene expression blocking in comparison to the control is indicated. Bars indicate standard deviation.
Results

When pretreated with IFN-α4 gene expression after NDV infection was suppressed in J774A.1 cells at every time point analysed. In RAW 264.7 cells the rate of increase in M gene expression at early time points was clearly lower in the IFN-α4 pretreated cell population compared to the control. At later time points the rate of increase became similar. In summary the results indicated a much lower IFN-α4 responsiveness in RAW 264.7 cells compared to macrophages and J774A.1 cells.

4.2.3 Growth characteristics of J774A.1 and RAW 264.7 cells

In order to further characterise the two macrophage-like tumour cell lines J774A.1 and RAW 264.7, their growth was followed in the presence or absence of IFN-α4. Macrophages were not included in the experiments, because as most differentiated cells they were not expected to show any growth.

The working hypothesis was that the more malignant the phenotype of a cell, the faster its growth and the lower the antiproliferative effect of IFN. The generation time G, the time it takes the cells to double, was calculated using the following formula:

\[ G = t \frac{\log N - \log N_0}{\log 2} \]

where \( N \) = final cell number, \( N_0 \) = initial cell number and \( t \) = elapsed time.

Figure 4 -11: The influence of IFN-α4 pretreatment on the NDV Ulster infection kinetics in J774A.1 and RAW 264.7 cells.

J774A.1 and RAW 264.7 tumour cells were grown in normal growth medium ( ■ control group) or in medium supplemented with IFN-α4 in a final concentration of 120 IU/mL ( ■ IFN-α4 group) 1 day before infection with NDV Ulster. For the infection 10 HU per 10⁶ cells NDV Ulster was used and total RNA was extracted at 0, 4, 8 and 20 hours after infection. After a reverse transcription the expression level of the NDV M gene was determined by real-time PCR with the comparative C_T method using β-actin for normalisation. The results are represented as the mean of three measurements. Bars indicate standard deviation.
Results

Figure 4-12 shows that without IFN-α4 pretreatment the J774A.1 cells grew slower than the RAW 264.7 cells. The generation time $G$ between day 0 and day 5 was 1.87 days for the J774A.1 cells and 1.32 days for the RAW 264.7 cells, i.e. the RAW 264.7 grew about 40% faster than the J774A.1 cells. With IFN-α4 pretreatment the generation time was increased for the J774A.1 cells to 4.4 days, less than half the normal generation time. For the RAW 264.7 the generation time was with 1.25 days slightly lower than that of the control.

![Graph showing cell growth](image)

Figure 4-12: The influence of IFN-α4 on the growth of J774A.1 and RAW 264.7 cells.

$3 \times 10^6$ J774A.1 and RAW 264.7 cells per well were seeded in 6 well plates in normal growth medium (control) or in medium supplemented with 120 IU/mL IFN-α4. Afterwards at each time point up to 6 days the number of living cells per well was determined with a hemocytometer and an optical microscope. $G = \text{generation time}$

Without pretreatment the RAW 264.7 cells grew distinctly faster than the J774A.1 cells. IFN-α4 pretreatment could more than halve the generation time of the J774A.1 cells, whereas the the RAW 264.7 cells did not respond to IFN-α4 with a significant change in the generation time.

Next the growth of the two macrophage-like cell lines in the presence of NDV was analysed to find out if and how long they could resist a single virus infection. J774A.1 and RAW 264.7 cells were infected once with NDV Ulster or NDV Italien and the cell growth was followed for several days.

As can be seen in figure 4-13 the J774A.1 cells stopped to grow after infection with NDV Ulster or NDV Italien. The total cell number fell after 1 day and than it stabilised at about...
Results

330,000 per well for NDV Ulster and at about 150,000 for NDV Italien. When infected with NDV Italien almost no living RAW 264.7 cells could be found any more from the first day after infection on. After infection with NDV Ulster the number of RAW 264.7 cells decreased at day 1, but then it started to increase again and the growth rate was as high as in the control between day 3 and day 4. The generation time for the control from day 0 to day 4 was 2.04 for the J774A.1 cells and 1.19 for the RAW 264.7 cells.

The most striking difference between the growth of the two cell lines was that the RAW 264.7 cells started to grow again after infection with the monocyclically replicating NDV Ulster, whereas the J774A.1 cell number remained at a constant level. Thus a fraction of both cell lines survived the infection, but the J774A.1 cells did not start to grow again, perhaps due to their ability to respond to danger signals such as interferon (as in figure 4-12). When infected with the multicyclically replicating NDV Italien, a part of the J774A.1 cells survives and the cell number remains constant. In contrast, almost all RAW 264.7 cells die, probably due to a higher susceptibility to NDV Italien. The few RAW 264.7 cells that did survive did not start to regrow, perhaps as a result of the viable viral offspring that was generated during infection and that could reinfect the cells.

Figure 4-13: Growth of J774A.1 and RAW 264.7 cells after NDV infection.

7.5*10^6 J774A.1 (A) and RAW 264.7 (B) cells were infected with 10 HU NDV Ulster or Italien and seeded in 6 well plates. Subsequently at each time point up to 4 days the number of living cells in one well was counted with a hemocytometer and an optical microscope. In order to stain dead cells trypan blue was used.
4.2.4 Interferon secretion after NDV infection

An important part in an antiviral response of cells is the synthesis and release of interferons into the supernatant as part of a positive feedback loop to induce an effective interferon response, for example, by stimulating the expression of crucial transcription factors such as IRF7 (Sato et al., 1998). In order to analyse the interferon secreted upon NDV infection in the three macrophage-like cell types, samples of the supernatants were taken at different time points after infection. The amount of biologically active interferon in these samples was determined in a VSV protection assay (3.2.7), in which L929 cells were pretreated with the supernatants and then infected with VSV. The protection against VSV-induced cell death due to the stimulation of an antiviral state conferred by the interferons in the supernatant was then used to determine the amount of interferons.

Figure 4-14 shows that the macrophages already started to secrete interferons 4 hours after infection, whereas the two tumour cell lines J774A.1 and RAW 264.7 began only after 12 hours to secrete detectable amounts of interferon. The amount of interferon in the supernatant of macrophages peaked 12 hours after infection at 700 IU (international units) and then it slightly decreased. Total interferon in the supernatants of the two tumour cell lines peaked at 24 hours and was 1200 IU for the J774A.1 cells and 120 IU for the RAW 264.7 cells.

![Figure 4-14: Interferon secretion of macrophages, J774A.1 and RAW 264.7 cells after infection with NDV Ulster.](image)

Bone marrow-derived macrophages from BALB/c mice, J774A.1 and RAW 264.7 tumour cells were infected with 10 HU per 10^6 cells NDV Ulster and 2*10^6 cells per well were seeded in 12 well plates with 2 mL growth medium (see Figure 4-7). After 2, 4, 8, 12 and 24 hours supernatant was collected and the interferon-α/β level was determined in a VSV based interferon bioassay (3.2.7).
Results

The most striking difference between the primary macrophages and the two tumour cell lines was the early beginning of interferon secretion in macrophages. The total amount of interferon secreted was higher in the J774A.1 tumour cells than in the primary cells. Since the J774A.1 cells were more susceptible to NDV than macrophages, the rapid secretion of interferons seemed to be of greater importance for NDV resistance than the total amount of secreted interferons at later time points.

4.2.5 Basal expression of interferon-related genes

In order to determine the responsiveness to viral infection in macrophages and J774A.1 and RAW 264.7 tumour cells before virus infection, the basal expression level of important genes of the interferon response was determined by real-time RT-PCR. The genes analysed were the two cytosolic double-stranded RNA receptors RIG-I and MDA5, the two transcription factors IRF3 and IRF7, interferon-β and the three antiviral effector molecules PKR, OAS1a and Mx1.

Figure 4 -15 demonstrates that the basal expression level of all the analysed genes was higher in macrophages and than in the two tumour cell lines. The lowest expression showed the RAW 264.7 cells and J774A.1 always had an intermediate expression level. The biggest differences in the gene expression level could be found for IRF7 and MDA5, in which the expression in macrophages was 12 to 15 times higher than in RAW 264.7 cells. On average the gene expression in macrophages was about 8.0 times and in J774A.1 2.3 times higher than in RAW 264.7 cells.
Results

The basal gene expression level of the analysed interferon-related genes correlated inversely with the NDV susceptibility, with the RAW 264.7 cells showing a low, the J774A.1 an intermediate and the macrophages a high expression level.

4.2.6 Inducible interferon-related gene expression

An important characteristic of the interferon response are positive feedback mechanisms that are necessary for an effective antiviral response. By these feedback mechanisms the expression of key molecules such as IRF7, which in the absence of viral infection is expressed only at low level in most cells, is stimulated. Only in the presence of a sufficient amount of IRF7 and other key molecules can there be an effective interferon response. To find out if there were differences in the inducibility of interferon-related genes in the course of viral infection, the expression of these genes was followed after NDV infection. In some cases

![Graph showing basal expression level of interferon-related proteins in macrophages, J774A.1 and RAW 264.7 cells.](image)

The basal gene expression level of the analysed interferon-related genes correlated inversely with the NDV susceptibility, with the RAW 264.7 cells showing a low, the J774A.1 an intermediate and the macrophages a high expression level.
Results

NDV was UV-inactivated before infection and a successful inactivation of the virus was confirmed by the absence of M gene expression in the respective samples (data not shown).

4.2.6.1 The cytosolic double-stranded RNA receptors RIG-I, MDA5 and LGP2

The retinoic acid-inducible gene I (RIG-I) and the melanoma differentiation associated gene 5 (MDA5) are cytosolic double-stranded RNA receptors that signal the presence of viral dsRNA in cells (Yoneyama et al., 2005). They have overlapping functions, but it seems that RIG-I is of more importance for the signalling after NDV infection then MDA5 (Kato et al., 2006). The D11lgp2 gene (LGP2) belongs to the same class of molecules but acts as a negative regulator interfering with RIG-I and MDA5. LGP2 lacks that signalling domain necessary for the signal transmission after binding of double-stranded RNA.

In order to assess the inducibility of these three genes in macrophages and the two macrophage-like tumour cell lines J774A.1 and RAW 264.7, the gene expression level was measured at different time points after infection with real-time RT-PCR. The infections were done with NDV Ulster and/or NDV Italien and in some cases also UV-inactivated NDV Ulster was used.

Figure 4 -16 shows the inducible expression of RIG-I. When infected with live NDV Ulster (part A), RIG-I expression was induced in all three cell types. The induction of expression started at 2 hours after infection in macrophages and J774A.1 cells, and at 8 hours in RAW 264.7 cells. The maximal expression level compared to the basal level in RAW 264.7 cells was highest for macrophages with 67-fold, lower for J774A.1 cells with 42-fold and lowest for RAW 264.7 cells with 15-fold. After infection with UV-inactivated NDV Ulster (part B), the induction of RIG-I expression was highest for macrophages 8 hours after infection with 69-fold. In J774A.1 cells the maximum induction of expression was 15-fold and in RAW 264.7 cells it was 4-fold. In contrast to infection with live NDV Ulster, the RIG-I gene expression dropped sharply after 8 hours. After infection with NDV Italien (part C), the general picture was similar to that after infection with NDV Italien. In macrophages induction was highest with 41-fold, in J774A.1 cells it was lower with 23-fold and RAW 264.7 cells it was lowest with 10-fold.
Results

RIG-I expression was inducible after NDV infection with the highest expression levels reached in macrophages, lower levels attained in J774A.1 cells and the lowest levels found in RAW 264.7 cells. NDV Ulster infection led to slightly higher RIG-I expression levels than infection with NDV Italien. UV-inactivated NDV Ulster induced a high RIG-I expression in macrophages that subsided early 8 hours after infection, pointing to a mechanism of induction that was independent of NDV replication. Next, the inducible expression of the cytosolic dsRNA receptor MDA5 after NDV infection was tested.

Figure 4-16 shows that after infection with NDV Ulster the expression of MDA5 was induced in all three cell types. The maximum of induction compared to the basal level in

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Figure 4-16: Changes in retinoic acid-inducible gene I (RIG-I) gene expression after NDV infection.

Bone marrow-derived macrophages from DBA/2 mice, J774A.1 and RAW 264.7 tumour cells were infected with 10 HU per 10⁶ cells live NDV Ulster (A), UV-inactivated NDV Ulster (B) or live NDV Italien (C). At different time points after the infection total RNA was extracted and after reverse transcription the level of retinoic acid-inducible gene I (RIG-I) expression was determined by real-time PCR with the comparative Cₜ method using the acidic ribosomal phosphoprotein PO for normalisation. The gene expression level is shown relative to the level in RAW 264.7 cells. The results are represented as the mean of three measurements. Bars indicate standard deviation.

RIG-I expression was inducible after NDV infection with the highest expression levels reached in macrophages, lower levels attained in J774A.1 cells and the lowest levels found in RAW 264.7 cells. NDV Ulster infection led to slightly higher RIG-I expression levels than infection with NDV Italien. UV-inactivated NDV Ulster induced a high RIG-I expression in macrophages that subsided early 8 hours after infection, pointing to a mechanism of induction that was independent of NDV replication. Next, the inducible expression of the cytosolic dsRNA receptor MDA5 after NDV infection was tested.

Figure 4-17 shows that after infection with NDV Ulster the expression of MDA5 was induced in all three cell types. The maximum of induction compared to the basal level in
Results

RAW 264.7 cells was reached earliest in macrophages at 12 hours with 312-fold and at 24 hours for J774A.1 and RAW 264.7 cells with 84-fold or 12-fold, respectively. When infected with UV-inactivated NDV Ulster (part B) there was a strongly increased MDA5 expression after 8 hours for macrophages with 91-fold. The expression dropped again sharply after this time point. In J774A.1 cells the expression increased up to 16-fold and in RAW 264.7 cells there was no induction of MDA5 expression by UV-inactivated NDV Ulster. Infection with NDV Italien (part C) induced MDA5 gene expression in macrophages to 203-fold at 12 hours and after 12 hours the gene expression had dropped to 1/4th of this value. For J774A.1 and RAW 264.7 cells the induced expression level of 102-fold or 27-fold respectively fell only slightly after the maximum had been reached.

![Graph A: Live NDV Ulster](image)

![Graph B: UV-inactivated NDV Ulster](image)

![Graph C: Live NDV Italien](image)

Figure 4-17: Changes in melanoma differentiation associated protein 5 (MDA5) gene expression after NDV infection.

Bone marrow-derived macrophages from DBA/2 mice, J774A.1 and RAW 264.7 tumour cells were infected with 10 HU per 10^6 cells live NDV Ulster (A), UV-inactivated NDV Ulster (B) or live NDV Italien (C). At different time points after the infection total RNA was extracted and after reverse transcription the level of melanoma differentiation associated protein 5 (MDA5) expression was determined by real-time PCR with the comparative C_T method using the acidic ribosomal phosphoprotein PO for normalisation. The gene expression level is shown relative to the level in RAW 264.7 cells. The results are represented as the mean of three measurements. Bars indicate standard deviation.
MDA5 expression was inducible by all types of NDV except for UV-inactivated NDV Ulster in RAW 264.7 cells. The induction was always strongest in macrophages at 8 hours after which it markedly declined. The stimulation of MDA5 expression was always lower in J774A.1 and lowest in RAW 264.7 cells. In contrast to the macrophages the induced gene expression fell only slightly in the two tumour cell lines in the course of the experiments. The general induction pattern of MDA5 gene expression was similar to that of RIG-I. One minor difference was that for macrophages the MDA5 expression fell markedly after attaining the maximum 8 hours after infection, whereas the RIG-I expression usually was more stable.

Since the LGP2 molecule is thought to act as a negative regulator of the RIG-I and MDA5 signalling, an analysis of the LGP2 expression kinetics could indicate its role after NDV infection. One possible hypothesis could be, for example, that LGP2 is expressed more strongly in tumour cells and that it makes them therefore more susceptible to NDV infection by inhibiting the signalling of cytosolic dsRNA receptors.

Figure 4-18 demonstrates that LGP2 was highly inducible after NDV Ulster infection in the three cell types. The increase compared to the basal level in RAW 264.7 cells was strongest in macrophages with 925-fold, lower in J774A.1 cells with 395-fold and lowest for RAW 264.7 cells with 194-fold. The expression maximum was reached earlier in macrophages (12 hours) than in the two tumour cell lines (24 hours).

![Figure 4-18](image-url)

**Figure 4-18: Changes in likely ortholog of mouse D11lgp2 (LGP2) gene expression after NDV infection.**

Bone marrow-derived macrophages from DBA/2 mice, J774A.1 and RAW 264.7 tumour cells were infected with 10 HU per 10^6 cells live NDV Ulster. At different time points after the infection total RNA was extracted and after reverse transcription the level of likely ortholog of mouse D11lgp2 (LGP2) expression was determined by real-time PCR with the comparative C_T method using the acidic ribosomal phosphoprotein PO for normalisation. The gene expression level is shown relative to the level in RAW 264.7 cells. The results are represented as the mean of three measurements. Bars indicate standard deviation.
Results

The inducible LGP2 expression pattern was similar to that of RIG-I and MDA5 with the macrophages having the highest expression level, followed by the J774A.1 and then by the RAW 264.7 cells. The relative values of expression were high because the basal expression level in RAW 264.7 cells, to which they were related, was very low. Since the LGP2 expression pattern was analogous to that of the two cytosolic dsRNA receptors and since the LGP2 expression level corresponded inversely with the NDV susceptibility, the expression of the LGP2 protein could not explain the differences in sensitivity to NDV infection.

4.2.6.2 The transcription factors IRF3 and IRF7

The transcription factors IRF3 and IRF7 are activated upon virus infection by signalling cascades beginning with pattern recognition receptors such as RIG-I, which recognise virus associated molecular patterns such as double-stranded RNA. After activation IRF3 and IRF7 induce the transcription of interferons and are therefore crucial in the establishment of an antiviral response. IRF3 is constitutively expressed in most cell types (Au et al., 1995) whereas IRF7 is induced in most cells by interferons (Sato et al., 1998). The expression of these two transcription factors was followed after NDV infection by real-time RT-PCR in macrophages, J774A.1 and RAW 264.7 cells.

Figure 4-19 shows that the IRF3 expression was increased after NDV Ulster infection. The macrophages had already a 6-fold higher basal expression level than the RAW 264.7 cells and they attained the highest level with 17-fold. The second highest expression level was found for the J774A.1 cells with 5-fold, followed by the RAW 264.7 cells with 2-fold.
IRF3 also proved to be inducible upon virus infection, but the increase in expression in relation to the basal level was low compared to the other genes analysed so far, as could be expected from the literature. The situation was expected to be different for IRF7, which was known to be inducible by interferons.

Figure 4-20 demonstrates that IRF7 was induced upon infection with NDV Ulster (part A) with the highest expression in macrophages with 760-fold, followed by the J774A.1 and the RAW 264.7 cells with 263- and 37-fold respectively. A marked increase in IRF7 expression started earlier in macrophages (4 hours after infection) than in the two tumour cell lines (12 hours after infection). UV-inactivated NDV Ulster (part B) stimulated a peak of IRF7 expression at 8 hours with 604-fold that levelled out at about 250-fold. The maximum expression level reached for J774A.1 cells was 39-fold, whereas no IRF7 expression was induced in RAW 264.7 cells. Upon infection with NDV Italien (part C) the maximum IRF7 expression level was slightly higher in macrophages (378-fold) than in J774A.1 cells (291-fold). At 24 hours after infection the expression level for macrophages fell below that of the J774A.1 cells. In RAW 264.7 cells the IRF7 level attained a maximum at 38-fold.
Results

The expression of IRF7 turned out to be highly inducible in the tested cells, with macrophages showing the highest induction followed by the J774A.1 and finally by the RAW 264.7 cells. In macrophages NDV Ulster induced a higher IRF7 expression than NDV Italien, a fact that had also been observed for other genes such as RIG-I or MDA5. Interestingly, UV-inactivated NDV Ulster markedly increased the IRF7 expression which remained at a high level up to the end of the experiment. This pointed to a mechanism of induction that was independent of NDV replication and that could nevertheless sustain IRF7 expression at a high level in macrophages. Compared to IRF3 the IRF7 gene was highly inducible by NDV infection.

Bone marrow-derived macrophages from DBA/2 mice, J774A.1 and RAW 264.7 tumour cells were infected with 10 HU per 10⁶ cells live NDV Ulster (A), UV-inactivated NDV Ulster (B) or live NDV Italien (C). At different time points after the infection total RNA was extracted and after reverse transcription the level of interferon regulatory factor 7 (IRF7) expression was determined by real-time PCR with the comparative C_T method using the acidic ribosomal phosphoprotein PO for normalisation. The gene expression level is shown relative to the level in RAW 264.7 cells. The results are represented as the mean of three measurements. Bars indicate standard deviation.

The expression of IRF7 turned out to be highly inducible in the tested cells, with macrophages showing the highest induction followed by the J774A.1 and finally by the RAW 264.7 cells.
4.2.6.3 Interferon-β

Interferon-β is synthesised early in a virally induced interferon response and is an important factor in a positive feedback loop by which other key components such as IRF7 are induced (Juang et al., 1998). To find out whether this function in an early interferon response is reflected in its expression pattern, the interferon-β expression was followed after NDV infection by real-time RT-PCR.

Figure 4-21 shows that upon infection with live NDV Ulster (part A) only the macrophages had a strong induction of interferon-β expression up to 459-fold of that of the basal RAW 264.7 expression. This marked induction quickly fell soon afterwards. For macrophages infected with UV-inactivated NDV Ulster and for J774A.1 and RAW 264.7 cells there was only a low induction up to 20-fold. After infection with NDV Italien (part B) all three cell types displayed an increased interferon-β expression which was earliest and highest in macrophages (161-fold) and later and lower for J774A.1 and RAW 264.7 cells (95- and 32-fold respectively). The interferon-β fell markedly for all three cell types 12 hours after infection.

Figure 4-21: Changes in interferon-β (IFN-β) gene expression after NDV infection.

Bone marrow-derived macrophages from DBA/2 mice, J774A.1 and RAW 264.7 tumour cells were infected with 10 HU per 10^6 cells live or UV-inactivated NDV Ulster (A) or live NDV Italien (B). At different time points after the infection total RNA was extracted and after reverse transcription the level of interferon-β (IFN-β) expression was determined by real-time PCR with the comparative C_T method using the acidic ribosomal phosphoprotein PO for normalisation. The gene expression level is shown relative to the level in RAW 264.7 cells. The results are represented as the mean of three measurements. Bars indicate standard deviation.

The induction of interferon-β gene expression was strongest at early time points after
Results

infection and dropped substantially thereafter in all three cell types. This was different to the other genes analysed so far and pointed to a special importance of this gene in the early stages of viral infection. Except for macrophages NDV Italien induced a stronger expression than NDV Ulster.

4.2.6.4 The antiviral effector molecules PKR, OAS1a and Mx1

The three proteins protein kinase PKR (PKR), 2'-5' oligoadenylate synthetase 1A (OAS1a) and Myxovirus (influenza virus) resistance 1 (Mx1) are induced by interferons and are known to have antiviral activities. The PKR inhibits protein translation, OAS1a leads to the degradation of viral RNA and Mx1 inhibits viral nucleocapsid transport or viral RNA synthesis. To analyse if the expression pattern of these molecules in macrophages, J774A.1 and RAW 264.7 tumour cells corresponded to the NDV susceptibility, their expression was followed after NDV infection by real-time RT-PCR, starting with the PKR.

Figure 4-22 demonstrates that the PKR expression was increased the most in J774A.1 cells when infected with live NDV Ulster (part A) or NDV Italien (part C, 40-fold or 54-fold respectively). The induction was lower in macrophages (25-fold and 43-fold respectively) and lowest in RAW 264.7 cells (13-fold or 23-fold respectively). After infection with UV-inactivated NDV Ulster (part B) the induction of PKR expression was highest for macrophages (22-fold), lower for J774A.1 cells (16-fold) and for RAW 264.7 cells no induction could be observed. In macrophages the expression declined again markedly after the peak had been reached, whereas it remained stable in the J774A.1 cells.
Results

In contrast to the genes analysed so far the J774A.1 and not the macrophages displayed the strongest induction of expression for the PKR gene. This was true for the infection with NDV Ulster as well as for that with NDV Italien. Only after the infection with UV-inactivated NDV Ulster was the PKR level highest in macrophages and declined it at later time points after infection. Next, the expression kinetics of the OAS1a was analysed.

Figure 4-23 demonstrates that OAS1a expression was induced earlier in macrophages than in the two tumour cell lines (8 hours versus 24 hours) and that macrophages attained a slightly higher maximal expression level than the J774A.1 tumour cells. The OAS1a induction was clearly lower than that of the other two cell types. The expression kinetics after infection with NDV Ulster (part A) or NDV Italien (part C) was similar, the only distinct difference being that the maximum for J774A.1 cells was reached at a later time point. After infection with

Figure 4-22: Changes in protein kinase PKR (PKR) gene expression after NDV infection.

Bone marrow-derived macrophages from DBA/2 mice, J774A.1 and RAW 264.7 tumour cells were infected with 10 HU per 10^6 cells live NDV Ulster (A), UV-inactivated NDV Ulster (B) or live NDV Italien (C). At different time points after the infection total RNA was extracted and after reverse transcription the level of protein kinase PKR (PKR) expression was determined by real-time PCR with the comparative C_T method using the acidic ribosomal phosphoprotein PO for normalisation. The gene expression level is shown relative to the level in RAW 264.7 cells. The results are represented as the mean of three measurements. Bars indicate standard deviation.

In contrast to the genes analysed so far the J774A.1 and not the macrophages displayed the strongest induction of expression for the PKR gene. This was true for the infection with NDV Ulster as well as for that with NDV Italien. Only after the infection with UV-inactivated NDV Ulster was the PKR level highest in macrophages and declined it at later time points after infection. Next, the expression kinetics of the OAS1a was analysed. 
Results

UV-inactivated NDV Ulster (part B) macrophages showed a peak of expression after 8 hours which was comparable to that reached after infection with live NDV, but which fell rapidly thereafter. In J774A.1 cells there was only a low but persistent increase in OAS1a expression after infection with UV-inactivated NDV Ulster, while in RAW 264.7 cells the expression was not increased.

Figure 4 -23: Changes in 2'-5' oligoadenylate synthetase 1A (OAS1a) gene expression after NDV infection.

Bone marrow-derived macrophages from DBA/2 mice, J774A.1 and RAW 264.7 tumour cells were infected with 10 HU per 10^6 cells live NDV Ulster (A), UV-inactivated NDV Ulster (B) or live NDV Italien (C). At different time points after the infection total RNA was extracted and after reverse transcription the level of 2'-5' oligoadenylate synthetase 1A (OAS1a) expression was determined by real-time PCR with the comparative C_T method using the acidic ribosomal phosphoprotein PO for normalisation. The gene expression level is shown relative to the level in RAW 264.7 cells. The results are represented as the mean of three measurements. Bars indicate standard deviation.

For the OAS1a gene the induction of expression after NDV infection was slightly higher for macrophages than for J774A.1 cells, while RAW 264.7 cells displayed merely a low increase in OAS1a expression. A clear increase in gene expression could be observed earlier in
Results

macrophages than in the two tumour cells lines. Last, the expression kinetics of the Mx1 gene was determined after NDV infection.

Figure 4 -24 shows that the Mx1 expression after infection with NDV Ulster (part A) was noticeably increased early on, reaching a maximum of 581-fold. The induction was clearly lower and later for the J774A.1 cells reaching a maximum of 65-fold, while the RAW 264.7 cells displayed no clear increase in Mx1 expression. After infection with UV-inactivated NDV Ulster (part B) a slight increase in gene expression could be observed for J774A.1 cells, whereas the Mx1 expression level of RAW 264.7 cells declined during the infection.

For the Mx1 expression kinetics there was a pronounced difference between the three cell types. The macrophages showed a strong and early induction of expression, the J774A.1 displayed a lower and delayed induction and in RAW 264.7 cells gene expression remained the same or even decreased after infection.

In summary it can be said that the inducible expression of the genes analysed in this chapter correlates with NDV resistance. An early and high induction could usually be found in macrophages, which were most resistant to NDV infection. The J774A.1 cells, which had an
intermediate NDV resistance, generally displayed a later induction of gene expression with a lower maximum. The RAW 264.7 cells mostly showed an increase in expression at similar time points as the J774A.1 cells, but the maximum level that was reached in the course of the infection was clearly lower.

The velogenic NDV Italien induced in general a lower gene expression than the lentogenic NDV Ulster, which might be connected to the increased cytotoxicity of the former. The hypothesis in this case would be that if a virus stimulates a weaker antiviral response, it can replicate better and is for that reason more cytotoxic.

Viral replication seemed to be necessary to induce a strong and sustainable interferon response, since UV-inactivated NDV, which could not replicate any more, induced only a low increase in gene expression. One interesting phenomenon was that macrophages when infected with UV-inactivated NDV Ulster displayed a strong peak of expression at 8 hours after infection which could not be found in the tumour cell lines and which declined sharply after this time point. This indicates a mechanism of inducing interferon-related gene expression in the absence of viral replication which was only active in the very early phase of an infection and only in macrophages. This mechanism could be related to the HN molecule of NDV, which is known to be able to induce interferons in the absence of viral replication (Fournier et al., 2003).

4.2.7 The influence of IFN-α4 on the interferon-related gene expression
Since it had been demonstrated that the pretreatment with IFN-α4 could block NDV infection in macrophages and two macrophage-like tumour cells, it was speculated that this pretreatment induced the expression of genes with antiviral properties to an extent that correlated with the degree of the blocking. According to this hypothesis it was expected, for example, that in macrophages the induction of interferon-related gene expression was strongest since they displayed the most complete blocking. In order to test this hypothesis the expression of RIG-I, IRF3, IRF7 and IFN-β was analysed with and without IFN-α4 pretreatment before and after NDV infection with real-time RT-PCR.

Figure 4 -25 shows that the IFN-α4 pretreatment induced RIG-I expression in all three cell types. The induction was about 5-fold in macrophages, 2-fold in J774A.1 cells and 3-fold in RAW 264.7 cells. In all three cell types NDV Ulster infection induced a stronger RIG-I
expression than the pretreatment with IFN-α4. When pretreated with IFN-α4 macrophages displayed a higher RIG-I expression level after NDV infection, while in the two tumour cell lines the level was reduced compared to the control. In addition the pretreated macrophages had a basal RIG-I expression level that was higher than that in the tumour cells after NDV infection.

RIG-I could be induced by IFN-α4 in all three cell types and the extent of induction correlated with NDV resistance and the blocking of NDV infection as determined before. The induction was strongest in macrophages, lower in J774A.1 cells and lowest in RAW 264.7 cells. A difference between the macrophages and the two tumour cell lines with regard to IFN-α4 responsiveness was that after NDV infection the RIG-I expression level was higher in pretreated macrophages than in pretreated tumour cells. Following the analysis of the cytosolic dsRNA receptor RIG-I, the IRF3 transcription factor expression was determined with and without IFN-α4 pretreatment.

Bone marrow-derived macrophages from BALB/c mice, J774A.1 and RAW 264.7 tumour cells were grown for 7 days either in normal growth medium (control group) or in medium supplemented with 1500 U/mL anti-IFN-α and 1900 U/mL anti-IFN-β antibody (IFN-α4 group). To the latter group IFN-α4 was added with a final concentration of 120 IU/mL 16 hours before NDV infection. The cells were then infected with 10 HU NDV Ulster per 10⁶ cells (see Figure 4 -10) and total RNA was extracted directly before infection (0 h) or 20 hours after infection. The level of RIG-I expression was determined by real-time RT-PCR with the comparative C_T method using the acidic ribosomal phosphoprotein PO for normalisation. The results are represented as the mean of three measurements. Bars indicate standard deviation.

Figure 4 -25: The influence of IFN-α4 pretreatment on the RIG-I expression in macrophages, J774A.1 and RAW 264.7 tumour cells after NDV infection.

RIG-I could be induced by IFN-α4 in all three cell types and the extent of induction correlated with NDV resistance and the blocking of NDV infection as determined before. The induction was strongest in macrophages, lower in J774A.1 cells and lowest in RAW 264.7 cells. A difference between the macrophages and the two tumour cell lines with regard to IFN-α4 responsiveness was that after NDV infection the RIG-I expression level was higher in pretreated macrophages than in pretreated tumour cells. Following the analysis of the cytosolic dsRNA receptor RIG-I, the IRF3 transcription factor expression was determined with and without IFN-α4 pretreatment.
Figure 4-26 demonstrates that IRF3 was not inducible by IFN-α4. The IRF3 expression level was almost the same in the pretreated as in the control cells or it even decreased slightly as in the RAW 264.7 cells. NDV Ulster infection only stimulated IRF3 expression in macrophages and not in the two tumour cell lines. There was no clear difference in the IRF3 expression level between the IFN-α4 pretreated and the control group for all three cell types.

In contrast to RIG-I, IRF3 expression did not seem to be IFN-α4-dependent. In the interferon signalling cascade IRF3 stimulates the transcription of IFN-α4 after activation, but there did not appear to be a feedback loop that in turn influenced the IRF3 expression via IFN-α4. Next, the induction of IRF7 by IFN-α4 was investigated.

Figure 4-27 shows that IRF7 was inducible by IFN-α4 in all three cell types, with the induction being strongest in macrophages with about 30-fold, lower in J774A.1 cells with about 8-fold and lowest in RAW 264.7 cells with about 4-fold. Compared to the IFN-α4 pretreatment infection with NDV Ulster stimulated the IRF7 expression much more.

Bone marrow-derived macrophages from BALB/c mice, J774A.1 and RAW 264.7 tumour cells were grown for 7 days either in normal growth medium (control group) or in medium supplemented with 1500 U/mL anti-IFN-α and 1900 U/mL anti-IFN-β antibody (IFN-α4 group). To the latter group IFN-α4 was added with a final concentration of 120 IU/mL 16 hours before NDV infection. The cells were then infected with 10 HU NDV Ulster per 10⁶ cells (see Figure 4-10) and total RNA was extracted directly before infection (0 h) or 20 hours after infection. The level of IRF3 expression was determined by real-time RT-PCR with the comparative C_T method using the acidic ribosomal phosphoprotein PO for normalisation. The results are represented as the mean of three measurements. Bars indicate standard deviation.
Results

pronouncedly in all three cell types. IFN-α4 pretreatment reduced the IRF7 expression after NDV infection compared to the control.

![Graph showing relative IRF7 expression in macrophages, J774A.1 and RAW 264.7 tumour cells after NDV infection.]

Figure 4-27: The influence of IFN-α4 pretreatment on the IRF7 expression in macrophages, J774A.1 and RAW 264.7 tumour cells after NDV infection.

Bone marrow-derived macrophages from BALB/c mice, J774A.1 and RAW 264.7 tumour cells were grown for 7 days either in normal growth medium (control group) or in medium supplemented with 1500 U/mL anti-IFN-α and 1900 U/mL anti-IFN-β antibody (IFN-α4 group). To the latter group IFN-α4 was added with a final concentration of 120 IU/mL 16 hours before NDV infection. The cells were then infected with 10 HU NDV Ulster per 10^6 cells (see Figure 4-10) and total RNA was extracted directly before infection (0 h) or 20 hours after infection. The level of IRF7 expression was determined by real-time RT-PCR with the comparative C_T method using the acidic ribosomal phosphoprotein PO for normalisation. The results are represented as the mean of three measurements. Bars indicate standard deviation.

In contrast to IRF3, IRF7 turned out to be highly inducible by IFN-α4. As for RIG-I the extent of IRF7 induction correlated with NDV resistance and the blocking of NDV infection as determined before. The induction was strongest in macrophages, lower in J774A.1 cells and lowest in RAW 264.7 cells. Compared to the basal expression level the IFN-α4 pretreatment induced IRF7 expression markedly stronger than the expression of RIG-I. Last, the expression of IFN-β after IFN-α4 pretreatment was analysed.

Figure 4-28 demonstrates that IFN-β expression was induced after IFN-α4 pretreatment only in macrophages, while it remained stable in the two tumour cell lines. Again, NDV Ulster infection induced gene expression in almost all cells and except for macrophages the IFN-β expression after NDV infection was lower than in the control cells.
Results

The IFN-β expression appeared to depend little on the presence of IFN-α, since only a slight increase in expression could be observed only in macrophages. Solely NDV infection produced a marked increase in IFN-β expression.

In summary it could be said that IFN-α induced the expression of IRF7 and RIG-I and also to a lower degree that of IFN-β, whereas the expression of IRF3 proved to be independent of IFN-α in the three cell types. The responsiveness to IFN-α with regard to interferon-related gene expression correlated with NDV resistance and blocking of NDV infection as determined before (4.2.2.3). In macrophages the increase in gene expression was noticeably higher than in J774A.1 and RAW 264.7 tumour cells. NDV infection induced gene expression more strongly than the IFN-α pretreatment, perhaps because upon NDV infection the secretion of IFN-α4 is stronger and additional danger signals such as other interferons are secreted. In macrophages often the IFN-α4 pretreatment did not reduce gene expression after NDV infection, although the infection was completely blocked by the pretreatment and no replication could be expected to take place. This could have been due to the ability of macrophages to increase antiviral gene expression even in the absence of NDV replication, as

Figure 4-28: The influence of IFN-α pretreatment on the IFN-β expression in macrophages, J774A.1 and RAW 264.7 tumour cells after NDV infection.

Bone marrow-derived macrophages from BALB/c mice, J774A.1 and RAW 264.7 tumour cells were grown for 7 days either in normal growth medium (control group) or in medium supplemented with 1500 U/mL anti-IFN-α and 1900 U/mL anti-IFN-β antibody (IFN-α4 group). To the latter group IFN-α4 was added with a final concentration of 120 IU/mL 16 hours before NDV infection. The cells were then infected with 10 HU NDV Ulster per 10⁶ cells (see Figure 4-10) and total RNA was extracted directly before infection (0 h) or 20 hours after infection. The level of IFN-β expression was determined by real-time RT-PCR with the comparative C_t method using the acidic ribosomal phosphoprotein PO for normalisation. The results are represented as the mean of three measurements. Bars indicate standard deviation.
Results

had been observed before in infections with UV-inactivated NDV (4.2.6).

4.3 Analysis of knock outs in genes involved in the interferon response

The experiments carried out so far in macrophages and macrophage-like tumour cells pointed to the importance of key molecules of the interferon response being connected to an increased susceptibility to NDV infection. In order to analyse the functional significance of some of the proteins involved in an antiviral response, macrophages were generated from mice with gene knock outs of these antiviral molecules. Five single gene knock outs could be obtained and investigated with regard to NDV infection and to an antiviral response. They were knock outs for the transcription factors IRF3 and IRF7, the two Toll-like receptors TLR3 and TLR7, recognizing viral double-stranded or single-stranded RNA respectively, and for the type I interferon receptor IFNAR. The parameters analysed were NDV infection with IFN or anti-IFN pretreatment, virally induced interferon secretion and interferon-related gene expression.

4.3.1 NDV replication in knock out macrophages

The first characteristic measured in the knock out macrophages was the infection with NDV. Since the proteins missing in the knock outs were important factors in the establishment of an interferon response, it was hypothesised that compared to the wildtype there might be an increase in NDV susceptibility at least in some of the knock outs. The macrophages were pretreated with anti-IFN-β or IFN-α4 and then infected with NDFL-EGFP. The EGFP signal was measured by flow cytometry one day later.

Figure 4 -29 shows that IFN-α4 pretreatment completely blocked an EGFP signal upon NDFL-EGFP infection in all knock out macrophages except for the IFNAR knock out. The latter knock out displayed the highest EGFP signal of all cells for the control and for the two pretreatment groups. In addition the EGFP expression in IFNAR knock out macrophages that were pretreated with anti-IFN-β or IFN-α4 was lower than in the control. The EGFP signal of the two Toll-like receptors was very similar to that of the wildtype. In the IRF3 and IRF7 knock outs the EGFP signal was slightly increased in comparison with the wildtype, with the signal in IRF3 knock outs being higher than in the IRF7 knock outs. In general the anti-IFN-β pretreatment either did not change the EGFP expression (wildtype, TLR3−/−, TLR7−/−) or it
slightly decreased it (IRF3\(^{-/-}\), IRF7\(^{-/-}\)).

In comparison to the wildtype the strongest increase in the EGFP expression was found in IFNAR knock out macrophages, pointing to the outstanding importance of this molecule in an antiviral response. It was also evident that IFN-α\(^4\) signalling took place only via the IFNAR, since compared to the control and to the anti-IFN-β pretreated cells there was no clear blocking of infection in the IFNAR knock outs in contrast to all other macrophages tested. IFN-α\(^4\) could block NDV infection when compared to the control, indicating that for example the induction of IRF7 by IFN-α\(^4\), which had been shown before, did not appear to be necessary for an effective antiviral response in this experimental setting. The IRF3 knock outs exhibited a higher EGFP signal than the wildtype and to a lesser degree this was also true for the IRF7 knock outs. This indicated that the early phase in the interferon response, which depends on IRF3, was more important in impeding NDV infection than the late phase. TLR3 and TLR7 did not seem to be important for an anti-NDV response in the cells as well as interferon-β secreted before NDV infection, since the anti-IFN-β pretreatment did not clearly

![Diagram](image-url)

Figure 4 -29: EGFP expression in macrophages with different knock-outs in interferon-related genes infected with NDFL-EGFP after pretreatment with anti-IFN-β antibody or IFN-α\(^4\).

Wildtype C57BL/6 macrophages and macrophages with knock-outs in the genes IRF3, IRF7, TLR3, TLR7 and IFNAR were grown for 7 days either in normal growth medium (control group) or in medium supplemented with 1900 U/mL anti-IFN-β antibody (anti-IFN-β group and IFN-α\(^4\) group). For 4 hours before NDV infection one group of cells (IFN-α\(^4\) group) was grown in medium supplemented with 120 IU/mL IFN-α\(^4\). 24 hours after infection with 10 HU NDFL-EGFP per 10\(^6\) cells EGFP expression of the cells was determined in flow cytometry.
influence the EGFP signal. Another parameter that was determined next to analyse NDV infection was the viral M gene, which was measured in the knock outs after NDV Ulster infection with real-time RT-PCR.

Figure 4 demonstrates that also the M gene expression was highest in IFNAR knock outs and that IFN-α4 pretreatment had no influence on the M gene expression after NDV Ulster infection. In contrast IFN-α4 pretreatment strongly reduced the M gene expression in the wildtype and the IRF3 and IRF7 knock outs. On average the M gene expression in IRF3 knock outs was about 10 times lower than that in IFNAR knock outs and about 40 times lower in IRF7 knock outs. Related to the wildtype M gene expression in IRF3 and IRF7 knock outs was markedly higher with about 100-fold or around 25-fold respectively.

Figure 4: NDV Ulster M gene expression in macrophages with different knock-outs in interferon-related genes after pretreatment with anti-IFN-β antibody or IFN-α4.

Wildtype C57BL/6 macrophages and macrophages with knock-outs in the genes IRF3, IRF7 and IFNAR were grown for 7 days either in normal growth medium (● control group) or in medium supplemented with 1900 U/mL anti-IFN-β antibody (● anti-IFN-β group and □ IFN-α4 group). For 4 hours before NDV infection one group of cells (● IFN-α4 group) was grown in medium supplemented with 120 IU/mL IFN-α4. 20 hours after infection with 10 HU NDV Ulster per 10⁶ cells the expression level of the NDV M gene was determined by real-time RT-PCR with the comparative C_T method using β-actin for normalisation. The results are represented as the mean of three measurements. Part B of the figure shows the same data as part A without the IFNAR-/- samples to better visualise the M gene expression differences between the IRF3-/- and the IRF7-/- cells. Bars indicate standard deviation.

The analysis of the M gene expression in the knock out macrophages after NDV infection underlined the crucial importance of the IFNAR for the generation of an antiviral state and for the induction of NDV resistance by IFN-α4. It also proved the role of IRF3 and to a lesser extent that of IRF7 in limiting M gene expression after NDV infection. In addition it seemed
Results

again that interferon-β that might be secreted constitutively without viral infection did not influence the M gene expression after NDV infection. To gain further insight in the importance of the genes that were knocked out especially with regard to their influence at certain time points after infection, a kinetics of the M gene expression after NDV Ulster infection was generated.

Figure 4 -31 shows that none of the knock outs had a higher M gene expression than the wildtype 4 hours after NDV Ulster infection. There was also no clear difference in M gene expression between the wildtype and the TLR3 and TLR7 knock outs at any other time point. The M gene level compared to the wildtype was increased 8 and 20 hours after NDV infection in the IRF3 and IRF7 knock outs. 8 hours after infection the M gene expression was similar between the IRF3 and IRF7 knock out, but 20 hours after infection the M gene level in IRF3 knock out macrophages had increased, whereas it had decreased in IRF7 knock outs.

Figure 4 -31: NDV Ulster M gene expression kinetics in macrophages with different knock-outs in interferon-related genes.

Wildtype C57BL/6 macrophages and macrophages with knock-outs in the genes IRF3, IRF7, TLR3 and TLR7 were infected with 10 HU NDV Ulster per 10^6 cells and total RNA was extracted 4, 8 and 20 hours later. After reverse transcription the expression level of the NDV M gene was determined by real-time PCR with the comparative C_T method using β-actin for normalisation. The results are represented as the mean of three measurements. Bars indicate standard deviation.

The kinetics of the M gene expression after NDV infection demonstrated again that TLR3 and TLR7 did not have an influence on the M gene level. At earlier time points IRF3 and IRF7 appeared to have the same influence on the M gene expression, but at later time points the M
Results

gene level could be reduced in IRF7 knock outs, whereas it increased in IRF3 knock outs. This pointed to a higher importance of the IRF3 gene in the containment of the M gene expression at later time points after NDV infection. Next, the influence of anti-IFN-β antibodies and IFN-α4 on the M gene expression kinetics after NDV infection was determined.

Figure 4-32 shows that when macrophages were pretreated with anti-IFN-β antibodies (part A) the M gene expression compared to the wildtype was increased in IRF3 and IRF7 knock outs at 8 and 20 hours after infection with NDV Ulster. At 8 hours the two knock outs showed around the same level, while at 20 hours the level had increased in the IRF3 knock out macrophages and decreased in IRF7 knock out macrophages. When pretreated with IFN-α4 (part B) M gene expression was very low compared to the anti-IFN-β pretreated cells. The M gene expression level was slightly increased at 4 and 8 hours for the IRF7 knock outs, but a 20 hours all three cell types had about the same M gene level.

Wildtype C57BL/6 macrophages and macrophages with knock-outs in the genes IRF3 and IRF7 were grown for 7 days in medium supplemented with 1900 U/mL anti-IFN-β antibody (A + B). For 4 hours before NDV infection one group of cells was grown in medium supplemented with 120 IU/mL IFN-α4 (B). 4, 8 and 20 hours after infection with 10 HU NDV Ulster per 10⁶ cells total RNA was extracted and the expression level of the NDV M gene was determined by real-time RT-PCR with the comparative C₇ method using β-actin for normalisation. The results are represented as the mean of three measurements. Bars indicate standard deviation.

The M gene expression kinetics of cells pretreated with anti-IFN-β antibodies was very
Results

similar to that of control cells without pretreatment analysed before, thereby indicating that IFN-β secretion ahead of NDV infection did not play an important role in acting upon NDV replication. The results demonstrated again that IRF3 was important to lower M gene expression at later time points, whereas IRF7 seemed to have less influence on M gene expression at later time points after infection. Finally, the role of the IFNAR in the M gene expression kinetics after NDV infection was tested.

Figure 4 -33 demonstrates that there was a strong increase in M gene expression between 4 and 8 hours after NDV Ulster infection. Between 8 and 20 hours there was again an increase in the M gene expression level of about 50 % in all three groups. The macrophages pretreated with anti-IFN-β antibodies had a somewhat higher M gene level, while in macrophages pretreated with IFN-α4 the expression level was slightly lower.

Figure 4 -33: NDV Ulster M gene expression kinetics in IFNAR-/- macrophages after NDV Ulster infection.

Wildtype C57BL/6 macrophages and macrophages with knock-outs in the IFNAR gene were grown for 7 days either in normal growth medium (control group) or in medium supplemented with 1900 U/mL anti-IFN-β antibody (anti-IFN-β group and IFN-α4 group). For 4 hours before NDV infection one group of cells (IFN-α4) was grown in medium supplemented with 120 IU/mL IFN-α4. 4, 8 and 20 hours after infection with 10 HU NDV Ulster per 10⁶ cells the expression level of the NDV M gene was determined by real-time RT-PCR with the comparative C₅₇ method using β-actin for normalisation. The results are represented as the mean of three measurements. Bars indicate standard deviation.

The results demonstrated that the IFNAR was essential in containing the M gene expression already at early time points after NDV infection. The differences between the pretreated and the control macrophages were unlikely to be genuine and probably due to chance variations since no influence of type I interferons could be conceived in the absence of the only known receptor for these interferons.
To summarise the results of the NDV infections in the analysed knock out macrophages it can be stated that the IFNAR clearly plays the most important role in the defence against NDV since its absence caused the strongest EGFP or M gene signals. It also became clear that type I interferon signalling in macrophages took place only via the IFNAR, for IFN-α4 produced a strong blocking of NDV infection in all except the IFNAR knock out macrophages.

In addition the IRF3 and IRF7 transcription factors also appeared to be involved in an anti-NDV response, with the IRF3 having a somewhat stronger influence than the IRF7 protein with regard to the M gene expression at later time points. One explanation would be that in the IRF3 knock out macrophages there was no early phase of the interferon response that is usually mediated by IRF3. Without this early phase there was probably also no second phase, since this second phase has to be induced by the first phase. In the IRF7 knock out macrophages on the other hand there was at least a functioning first phase in the interferon response that could in part block NDV replication.

The TLR3 and TLR7 seemed to have no measurable influence on the outcome of an NDV infection. After determining the influence of the knock out genes on NDV infection, their role in shaping other parameters of an interferon response such as interferon secretion or the expression of interferon-related genes was tested.

4.3.2 Interferon secretion after NDV infection in knock out macrophages

The secretion of type I interferons upon viral infection is considered to be an important factor in the establishment of an antiviral state in cells. An effective interferon response depends strongly on positive feedback loops for the induction of key components such as IRF7. These feedback signals are transmitted by type I interferons that signal in an auto- or paracrine manner exclusively via the IFNAR. In order to investigate the influence of genes related to an interferon response on the secretion of type I interferons after NDV infection, macrophages from knock out mice were generated, infected with NDV and then the interferon content in the supernatants was determined in a bioassay detecting only biologically active interferons (3.2.7). The macrophages were also pretreated with anti-IFN-β antibodies and with IFN-α4 before infection to examine the influence of the knocked out genes on the effect of these pretreatments.
Figure 4-34 shows that in the wildtype the secretion of interferons was equal in the control and in the anti-IFN-β antibody pretreated macrophages, while it was reduced to about 1/3rd in the IFN-α4 pretreated cells. In the absence of the IFNAR no interferon was secreted after NDV Ulster infection. The IRF7 knock out macrophages displayed a low interferon secretion and secreted 7 times less interferon then the controls for all three conditions tested. In the IRF3 knock out macrophages the interferon secretion was slightly lower than in the wildtype for the control and for the IFN-α4 pretreated cells. In the anti-IFN-β pretreated cells the IFN secretion was twice that in the control. Compared to the wildtype the macrophages with knock outs in the TLR3 and TLR7 genes showed a lower interferon secretion in the control and anti-IFN-β groups and a higher interferon level in the IFN-α4 groups.

The IFNAR appeared to be a crucial molecule for the secretion of interferons in response to viral infections, indicating also the importance of positive feedback loops in the induction of interferon secretion mediated by this receptor. IRF7 proved to be of importance in the induction of type I interferon secretion since its absence strongly reduced the interferon secretion.
Results

levels. IRF3 seemed to have only a minor function in interferon secretion except in the presence of anti-IFN-β antibodies. The strong increase in the interferon level in the presence of these antibodies could indicate a negative regulatory effect of IFN-β in IRF3 knock out macrophages. The two Toll-like receptor knock outs only had a minor effect by slightly reducing interferon secretion. Regarding the pretreatments in general IFN-α4 led to a slight increase in interferon, perhaps by limiting viral replication and viral danger signals that could induce interferons, whereas anti-IFN-β did not show a clear effect. Next an interferon secretion kinetics was used to test for the importance of the gene knock outs at specific times after infection.

Figure 4 -35 shows that in the control medium (part A) at 4 hours after infection interferon could only be detected in the supernatants of wildtype macrophages. There was interferon secretion in IFNAR knock out macrophages that led to a detectable interferon concentration from 8 hours on, but the interferon level remained at a low level that was about 100-fold lower than that of wildtype macrophages 20 hours after infection. In IRF3 knock out macrophages interferon was not detectable until 20 hours after infection, at which point it attained almost the same level as the wildtype. In IRF7 knock out macrophages interferon secretion could be observed from 8 hours on, but the final level was about 10 times lower than that in the wildtype. When pretreated with anti-IFN-β antibodies there was a similar picture for the interferon secretion in IRF3 and IRF7 knock out macrophages (part B). In the IRF3 knock out cells interferon could only be observed 20 hours after infection, but at that point it had reached the same level as the wildtype. In the IRF7 knock out macrophages interferon could already be detected after 4 hours, but the final level was about 8 times lower than that of the wildtype.
**Results**

The results of the interferon secretion kinetics suggested different roles for the IRF3 and the IRF7 transcription factors. IRF3 seemed to be important for the secretion of interferons at early time points after infection, but its absence did not diminish the final interferon level. In contrast IRF7 did not appear to be important for the interferon secretion at early time points, but was necessary for reaching normal interferon levels at later time points. Since the IRF3 knock out macrophages were more susceptible to NDV infection than the IRF7 knock outs, it could be hypothesised that in the tested setting the early secretion of low type I interferon amounts was more important for NDV resistance than the later secretion of high interferon amounts. A low interferon secretion upon NDV infection as seen in the IFNAR knock out macrophages could explain the high NDV susceptibility of these cells. As the last parameter to be tested remained the interferon-related gene expression with or without IFN-α4 before and after NDV infection.

**4.3.3 Expression of interferon-related genes in knock out macrophages**

In order to ascertain the influence of the five gene knock outs on antiviral gene expression, the expression level of RIG-I, IRF3, IRF7 and IFN-β was determined by real-time RT-PCR. The basal as well as the NDV-inducible expression levels were determined and in addition the
knock out macrophages were pretreated with IFN-α4 to determine how the IFN-α4-induced gene expression was influenced by the knocked out genes.

Figure 4-36 demonstrates that the basal expression level of RIG-I was lowered in the absence of the IFNAR, whereas it was unaltered in the IRF3 and IRF7 knock outs. When grown in normal medium (part A), the induction of RIG-I by NDV Ulster infection was delayed in all knock outs compared to the wildtype. The final RIG-I expression level in IRF3 knock out macrophages was the same as in the wildtype, while it was slightly reduced in the IRF7 and IFNAR knock outs. After IFN-α4 pretreatment (part B) the final RIG-I level after NDV infection was lower in all knock out macrophages compared to the wildtype. In the IFNAR knock out the basal level and the expression at 8 hours after infection was several times lower than that of all other macrophages.

![Figure 4-36: RIG-I expression in IRF3, IRF7 and IFNAR knock-out macrophages after infection with NDV Ulster.](image)

Wildtype C57BL/6 macrophages and macrophages with knock-outs in the genes IRF3, IRF7 and IFNAR were grown for 7 days either in normal growth medium (A) or in medium supplemented with 1900 U/mL anti-IFN-β antibody (B). For 4 hours before NDV infection one group of cells (B) was grown in medium supplemented with 120 IU/mL IFN-α4. At 0, 4, 8 and 20 hours after infection with 10 HU NDV Ulster per 10⁶ cells the expression level of the RIG-I gene was determined by real-time RT-PCR with the comparative Cₜ method using the acidic ribosomal phosphoprotein PO for normalisation. The results are represented as the mean of three measurements. Bars indicate standard deviation.

The expression of RIG-I was not strongly regulated by IRF3 and IRF7, since their absence did just slightly lower and delay the inducible RIG-I expression. IFNAR appeared to be necessary for both the basal and the inducible RIG-I expression, but at later time points RIG-I
Results

expression was also increased in the IFNAR knock outs, pointing to IFNAR-independent ways of RIG-I induction. Next, the IRF3 expression in the knock out macrophages was analysed.

Figure 4 - 37 shows that the basal and NDV-inducible IRF3 expression in IRF7 and IFNAR knock out macrophages was slightly reduced compared to the wildtype (part A). This reduction in expression was most pronounced 20 hours after NDV Ulster infection. The pretreatment with IFN-α4 did not stimulate the IRF3 expression before or in the course of NDV infection.

Figure 4 - 38 demonstrates that the basal and inducible IRF7 expression depended markedly on the IFNAR and to a lesser degree on IRF3. In the IFNAR knock out macrophages almost

Wildtype C57BL/6 macrophages and macrophages with knock-outs in the genes IRF7 and IFNAR were grown for 7 days either in normal growth medium (A) or in medium supplemented with 1900 U/mL anti-IFN-β antibody (B, wildtype only). For 4 hours before NDV infection one group of cells (B) was grown in medium supplemented with 120 IU/mL IFN-α4. At 0, 4, 8 and 20 hours after infection with 10 HU NDV Ulster per 10⁶ cells the expression level of the IRF3 gene was determined by real-time RT-PCR with the comparative Cₜ method using the acidic ribosomal phosphoprotein PO for normalisation. The results are represented as the mean of three measurements. Bars indicate standard deviation.

The results confirmed that IRF3 is not strongly inducible neither by IFN-α4 nor by NDV infection. IRF7 and the IFNAR seemed to play only a minor role in IRF3 expression, because their absence decreased IRF3 expression only little. A transcription factor that had been shown to be strongly inducible was IRF7, which was analysed next.

Figure 4 - 38 demonstrates that the basal and inducible IRF7 expression depended markedly on the IFNAR and to a lesser degree on IRF3. In the IFNAR knock out macrophages almost
no IRF7 expression could be detected before or during infection with NDV Ulster. When grown in control medium (part A) the IRF7 expression remained nearly undetectable in IRF3 knock out macrophages except for the 20 hour time point, at which it reached about half the expression level of the wildtype. In both the wildtype and the IRF3 knock out macrophages IFN-α4 strongly increased the basal IRF7 expression level (part B). Compared to the wildtype the IRF7 expression level was slightly reduced in IRF3 knock out macrophages and reached about half the level of the wildtype at 0 and 20 hours in IFN-α4 pretreated cells.

Figure 4.38: IRF7 expression in IRF3, IRF7 and IFNAR knock-out macrophages after infection with NDV Ulster.

Wildtype C57BL/6 macrophages and macrophages with knock-outs in the genes IRF3, IRF7 and IFNAR were grown for 7 days either in normal growth medium (A) or in medium supplemented with 1900 U/mL anti-IFN-β antibody (B). For 4 hours before NDV infection one group of cells (B) was grown in medium supplemented with 120 IU/mL IFN-α4. At 0, 4, 8 and 20 hours after infection with 10 HU NDV Ulster per 10^6 cells the expression level of the IRF7 gene was determined by real-time RT-PCR with the comparative C_T method using the acidic ribosomal phosphoprotein PO for normalisation. The results are represented as the mean of three measurements. Bars indicate standard deviation.

The IFNAR seemed to be indispensable for the IRF7 expression, since nearly no IRF7 signal could be detected in any of the IFNAR knock out macrophage groups. IRF3 appeared to be important in the early phase of IRF7 induction, because the IRF7 level was strongly reduced at early time points after NDV infection. This strong reduction could not be found when the IRF3 knock out macrophages had been pretreated with IFN-α4. An interpretation would be that IRF3 was necessary for the induction of a positive feedback loop via IFN-α4 in the early phase of an interferon response, as had been shown in the literature (Sato et al., 1998). When IFN-α4 was added this early-phase feedback was short-circuited and IRF7 could be induced
Results

without IRF3. As a last interferon-related gene the IFN-β expression was determined in the knock out macrophages.

Figure 4-39 demonstrates that when infected with NDV Ulster in control medium (part A) almost no IFN-β expression could be found in the IRF7 and in the IFNAR macrophages. Compared to the wildtype the expression of IFN-β was delayed in IRF3 knock out macrophages, but 20 hours after infection the IFN-β was about 4 times of that in the wildtype. When pretreated with IFN-α4 a low IFN-β expression in IFNAR knock out macrophages could only be observed 20 hours after infection. In the other three macrophages types IFN-β expression peaked 8 hours after infection and was markedly lower at 20 hours. The peak in expression was highest in the wildtype, about 2.5 times lower in IRF3 knock outs and around 8 times lower in IRF7 knock out macrophages.

Figure 4-39: IFN-β expression in IRF3, IRF7 and IFNAR knock-out macrophages after infection with NDV Ulster.

Wildtype C57BL/6 macrophages and macrophages with knock-outs in the genes IRF3, IRF7 and IFNAR were grown for 7 days either in normal growth medium (A) or in medium supplemented with 1900 U/mL anti-IFN-β antibody (B). For 4 hours before NDV infection one group of cells (B) was grown in medium supplemented with 120 IU/mL IFN-α4. At 0, 4, 8 and 20 hours after infection with 10 HU NDV Ulster per 10^6 cells the expression level of the IFN-β gene was determined by real-time RT-PCR with the comparative C_{T} method using the acidic ribosomal phosphoprotein PO for normalisation. The results are represented as the mean of three measurements. Bars indicate standard deviation.

IRF7 and the IFNAR seemed to be crucial for a strong IFN-β expression and in their absence almost no IFN-β could be detected in the course of an NDV infection. IRF3 appeared to be important for an early expression of IFN-β, since in contrast to the wildtype no IFN-β could be detected in IRF3 knock outs 4 and 8 hours after infection. An important factor in the
induction of IFN-β expression could be the secretion of IFN-α4 induced by IRF3, because its addition led to almost normal IFN-β expression.

In summary it could be said that the expression of RIG-I and IRF3 was relatively independent from any of the genes that were knocked out in the macrophages. However, the basal and inducible expression of IRF7 was completely dependent on the IFNAR. The expression of IRF7 also depended to a lower degree on IRF3, especially with regard to early time points after NDV infection. The IFN-β expression depended substantially on both the IRF7 and the IFNAR. In the absence of IRF3 the IFN-β expression was delayed in the course of infection.

4.3.4 NDV infection and gene expression analysis of IFNAR<sup>−/−</sup> MEF

Another cell type in which the role of the IFNAR in NDV infection and in the NDV-induced interferon response was analysed were mouse embryonic fibroblast (MEF) cells. The MEF cell lines had been grown for a long time in vitro and could be expected to respond less to growth inhibitory signals as an adaptation to the in vitro culture. The IFN responsiveness of the MEF was therefore expected to be lower than that of bone marrow-derived macrophages. Wildtype and IFNAR knock out MEF were compared with regard to NDV replication, basal and inducible gene expression and IFN responsiveness.

4.3.4.1 NDV replication

The first parameter that was tested in the MEF was EGFP or M gene expression after infection with NDVF-EGFP or NDV Ulster respectively. Before NDV infection some of the MEF were pretreated with anti-IFN-α/β antibody to determine the influence of the basal interferon secretion of the MEF. Another group of cells was pretreated with IFN-α4 to look for IFN-induced changes in the resistance to NDV infection. The EGFP expression was measured in flow cytometry and the M gene level was determined with the help of real-time RT-PCR.

Figure 4-40 demonstrates that the MEF wildtype had a much lower EGFP (part A+B) and M gene expression (part C) after NDV infection than the IFNAR knock out. The EGFP and M gene signal in the wildtype were not influenced by the anti-IFN-α/β antibody pretreatment. Nevertheless, the MEF pretreated with IFN-α4 caused a marked reduction especially with regard to M gene expression. In the IFNAR knock out MEF no pretreatment changed the
Results

EGFP or M gene signal compared with the control medium. Concerning the EGFP signal upon NDFL-EGFP infection in the IFNAR knock out cells (part B) there were apparently two populations, one with a low and one with an about 100 times stronger signal (10 versus 1000 relative fluorescence units). The M gene expression kinetics (part D) revealed that the main increase in expression took place between 8 and 20 hours after infection.

Wildtype and IFNAR−/− mouse embryonic fibroblasts were grown for 7 days either in normal growth medium (control group) or in medium supplemented with 1900 U/mL anti-IFN-β antibody (anti-IFN-α/β group and IFN-α4 group). For 4 hours before NDV infection one group of cells (IFN-α4 group) was grown in medium supplemented with 120 IU/mL IFN-α4. Afterwards the cells were infected with 10 HU NDFL-EGFP per 10⁶ cells. (A + B) The EGFP expression was determined by flow cytometry 20 hours later. (C) 20 hours after infection total RNA was extracted and the expression level of the NDV M gene was determined by real-time RT-PCR with the comparative CT method using β-actin for normalisation. The results are represented as the mean of three measurements. The percentage of M gene expression blocking in comparison to the control is indicated. (D) 0, 4, 8 and 20 hours after infection total RNA was extracted from the control and the IFN-α4 group and the expression level of the NDV M gene was determined by real-time RT-PCR with the comparative CT method using β-actin for normalisation. The results are represented as the mean of three measurements. Bars indicate standard deviation.
The IFNAR seemed to be a crucial factor in NDV resistance, since the IFNAR knock out MEF turned out to be much more susceptible to NDV infection than the wildtype MEF. The EGFP signal in the IFNAR knock out MEF pointed to two populations with markedly different susceptibilities to NDV, possibly representing two different stages in the cell cycle. According to this hypothesis the highly NDV susceptible cells were proliferating and provided the virus with a high amount of the necessary cellular components for replication. The less susceptible cells could be non-proliferating and would therefore be less suitable for NDV replication.

Interestingly, the M gene level after NDV infection in the non-tumourigenic wildtype MEF was higher than that in tumour cells such as the macrophage-like RAW 264.7 cells analysed before (4.2.2.3), perhaps attributable to phenotypic changes in the MEF as an adaptation to prolonged in vitro passaging. On the other hand unlike the RAW 264.7 tumour cells the MEF responded strongly to interferons and the M gene expression could almost be completely blocked by IFN-α4 pretreatment. As expected IFN-α4 did not influence the NDV infection in IFNAR knock out MEF, since the IFNAR is the only known receptor for IFN-α4.

4.3.4.2 Interferon-related gene expression

One explanation for the clear differences in NDV susceptibility between wildtype and IFNAR knock out MEF could be differences in the expression of genes related to the antiviral interferon response. The expression of three genes important in the establishment of an interferon response was followed after NDV infection by real-time RT-PCR.

Figure 4-41 shows that the basal expression of RIG-I, IRF7 and IFN-β was moderately higher in the IFNAR than in the wildtype MEF. The IFN-α4 pretreatment increased the basal gene expression only in the wildtype MEF and solely in these cells it reduced the gene expression after NDV infection. The increase in basal gene expression by IFN-α4 pretreatment was highest for IRF7 (7-fold) and lower for RIG-I (3-fold) and IFN-β (2-fold). After infection with NDV Ulster the IFN-β expression in the IFNAR knock out MEF was 10 – 15 times higher and the RIG-I expression 3 times higher than in the wildtype. The IRF7 expression after infection was comparable in both MEF cell types.
Results

Overall the relative gene expression in MEF was substantially lower than in macrophages. IFN-α4 pretreatment increased basal gene expression and led to a reduced expression level after NDV infection only in wildtype and not in IFNAR knock out MEF, corresponding to the assumption that IFN-α4 signals exclusively through the IFNAR.

In contrast to the macrophages analysed earlier (4.3.3) the NDV-inducible expression of IFN-β and RIG-I was higher in the IFNAR knock out than in the wildtype MEF and the IRF7 expression was comparable. The stronger gene expression in the IFNAR knock out MEF of IFN-β and RIG-I after NDV infection could be explained by more viral danger signals due to the better NDV replication in the IFNAR knock outs. IFN-β and RIG-I expression did not
Results

appear to be strongly dependent on the IFNAR signalling in macrophages (4.3.3) and therefore the IFNAR knock out MEF were not expected to be markedly inhibited in their ability to synthesise these molecules. Surprisingly, the IRF7 gene expression was also not inhibited although in macrophages it could be shown that IRF7 expression strongly depends on IFNAR signalling. Evidently the long in vitro culture had strongly reduced the ability of wildtype MEF to express IRF7 since compared to macrophages the basal and inducible IRF7 expression was decidedly lower than in macrophages.

4.4 Connection between antiviral gene expression and NDV susceptibility in tumour and normal cells

In order to investigate whether the lower basal and inducible expression of interferon-related genes that could be observed in some tumour cells (4.1, 4.2) represented a general feature of many tumour cells and could therefore explain the generally high NDV susceptibility of tumour cells, several normal and tumour cells were tested and compared regarding NDV replication and antiviral gene expression. The aim was to find out if genes involved in the interferon response were differently expressed in normal and in tumour cells. In addition it should be examined to what degree NDV resistance could be correlated with the expression levels of the analysed genes.

4.4.1 Analysis of RIG-I, IRF3, IRF7, IFN-β and M gene expression in normal and tumour cells

The first parameter that was compared between normal and tumour cells was the M gene expression after NDV infection. To this end several normal and tumour cell types were infected with NDV Ulster and the M gene expression was measured with real-time RT-PCR one day after infection.

Figure 4 -42 shows that the M gene expression one day after infection with NDV was higher in tumour than in normal cells. The difference was not statistically significant. In contrast to the normal cells there was a high variation in M gene expression in the tumour cells and three groups could be distinguished. One group with relatively low M gene expression comprised the CT26p (23 relative expression units (REU)), J774A.1 (64 REU) and RAW 264.7 cells (3749 REU). A medium expression was found in DA3 (20821 REU), CT26wt (21063 REU)
and B16 cells (19973 REU), and a high expression displayed the Esb cells (44529 REU). The M gene level in normal cells was always lower than 31 REU.

The results demonstrated that the M gene expression after NDV infection was markedly higher in tumour than in normal cells. The lack of statistical significance could be explained by the high variation of the M gene expression in the tumour cells. Next the expression of four interferon-related genes was determined in normal and tumour cells before and after NDV infection with the help of real-time RT-PCR. In addition the degree of correlation between the M and the antiviral gene expression was calculated.

Figure 4-42 demonstrates that the basal and inducible RIG-I expression was significantly higher in normal than in tumour cells. The average basal RIG-I level (part A) was 47 REU in normal cells and 7 REU in tumour cells. The NDV-inducible RIG-I level (part C) was on average 237 REU in normal and 49 REU in tumour cells. There was a negative correlation between the RIG-I expression with the M gene expression one day after infection (part B+D) which was stronger for the basal RIG-I level ($R = -0.8598$) than for the inducible level ($R = -0.6271$). The normal and the tumour cells clearly grouped together with the IFNAR knock out macrophages and the CT26p cells being the only exceptions.
Results

It could be shown that RIG-I is significantly more expressed in normal than in tumour cells before as well as after NDV infection. In addition both the basal and the inducible RIG-I expression levels correlate negatively with the M gene expression, i.e. the higher the RIG-I

Figure 4-43: Analysis of the basal and inducible RIG-I expression in primary and tumour cells before and after NDV infection.

The RIG-I expression level was measured in several primary and tumour cell types before and 24 hours after infection with 10 HU NDV Ulster or NDFL-EGFP by real-time RT-PCR. The gene expression was determined with the comparative Ct method using the acidic ribosomal phosphoprotein PO for normalisation. (A) The basal RIG-I expression of several primary (macrophages from BALB/c, C57BL/6 and DBA/2 mice, lung tissue, liver tissue) and tumour cell types (CT26p, CT26wt, J774A.1, RAW 264.7, DA3, Esb and B16) is shown. (B) The basal RIG-I level of several primary (♦ macrophages from BALB/c, C57BL/6 and DBA/2 mice; IFNAR-/- macrophages) and tumour cell types (♦ CT26wt, J774A.1, RAW 264.7, DA3, Esb and B16; ♦ CT26p) is plotted against the NDV M gene expression level after 24 hours (see Figure 4-42). (C) The inducible RIG-I expression level is indicated for several primary (macrophages from BALB/c, C57BL/6 and DBA/2 mice) and tumour cell types (CT26p, CT26wt, J774A.1, RAW 264.7, DA3, Esb and B16). (D) The inducible RIG-I level of several primary (♦ macrophages from BALB/c, C57BL/6 and DBA/2 mice; IFNAR-/- macrophages) and tumour cell types (♦ CT26wt, J774A.1, RAW 264.7, DA3, Esb and B16; ♦ CT26p) is plotted against the NDV M gene expression level after 24 hours (see Figure 4-42). The p value was calculated using an unpaired student's t-test. Bars indicate standard deviation. The correlation coefficient was calculated with the mean expression values.
gene expression, the lower the M gene expression. The next interferon-related gene analysed was the IRF3 gene.

Figure 4 shows that IRF3 was expressed significantly higher in normal than in tumour cells before as well as after infection with NDV. The average basal IRF3 expression (part A) was 54 REU for normal and 7 REU for tumour cells. On average the NDV-inducible IRF3 expression (part B) was 32 REU for normal and 4 REU for tumour cells. The negative correlation of the IRF3 expression with the M gene expression one day after infection (part B+D) was stronger for the basal IRF3 level (R = -0.8308) than for the inducible level (R = -0.5725). Again the normal and the tumour cells formed two distinguishable groups with the exception of the IFNAR knock out macrophages and the CT26p and J774A.1 tumour cells.
Results

The basal and NDV-inducible IRF3 expression turned out to be significantly higher in normal than in tumour cells. Additionally there was a negative correlation between the basal and inducible IRF3 expression level and the M gene level. Subsequently the IRF7 gene expression was investigated.

Figure 4-44: Analysis of the basal and inducible IRF3 expression in primary and tumour cells before and after NDV infection.

The IRF3 expression level was measured in several primary and tumour cell types before and 24 hours after infection with 10 HU NDV Ulster or NDFL-EGFP. The gene expression was determined by real-time RT-PCR with the comparative C\textsubscript{T} method using the acidic ribosomal phosphoprotein PO for normalisation. (A) The basal IRF3 expression of several primary (macrophages from BALB/c, C57BL/6 and DBA/2 mice, lung tissue, liver tissue) and tumour cell types (CT26p, CT26wt, J774A.1, RAW 264.7, DA3, Esb and B16) is shown. (B) The basal IRF3 level of several primary (♦ spleen cells, macrophages from BALB/c and C57BL/6 mice; ♦ IFNAR\textsuperscript{−}/\textsuperscript{−} macrophages) and tumour cell types (♦ CT26wt, J774A.1, RAW 264.7, DA3, Esb and B16; ♦ CT26p) is plotted against the NDV M gene expression level after 24 hours (see Figure 4-42). (C) The inducible IRF3 expression level is indicated for several primary (spleen cells, macrophages from BALB/c and C57BL/6 mice) and tumour cell types (CT26p, CT26wt, J774A.1, RAW 264.7, DA3, Esb and B16). (D) The inducible IRF3 level of several primary (♦ spleen cells, macrophages from BALB/c and C57BL/6 mice; ♦ IFNAR\textsuperscript{−}/\textsuperscript{−} macrophages) and tumour cell types (♦ CT26wt, J774A.1, RAW 264.7, DA3, Esb and B16; ♦ CT26p) is plotted against the NDV M gene expression level after 24 hours (see Figure 4-42). The p value was calculated using an unpaired student's t-test. Bars indicate standard deviation. The correlation coefficient was calculated with the mean expression values.
Results

Figure 4-45 demonstrates that the basal and NDV-induced IRF7 expression was significantly higher in normal than in tumour cells. The average basal IRF7 expression (part A) was 83 REU in normal and 11 REU in tumour cells, and the average inducible IRF7 expression (part C) 5841 REU for normal and 240 REU for tumour cells. The negative correlation between the basal IRF7 level and the M gene expression (part B) was stronger (R = -0.9116) than that between the inducible IRF7 and M gene level (part D, R = -0.8244). The normal and the tumour cells formed two distinct groups with regard to IRF7 and M gene expression with the exception of the IFNAR knock out macrophages and the CT26p and J774A.1 tumour cells.
Results

The basal and NDV-inducible IRF7 expression turned out to be significantly higher in normal than in tumour cells. Additionally there was a negative correlation between the basal and inducible IRF7 expression level and the M gene expression. The last antiviral gene analysed

The IRF7 expression level was measured in several primary and tumour cell types before and 24 hours after infection with 10 HU NDV Ulster or NDFL-EGFP. The gene expression was determined by real-time RT-PCR with the comparative C\textsubscript{T} method using the acidic ribosomal phosphoprotein PO for normalisation. (A) The basal IRF7 expression of several primary (spleen cells, bone marrow cells, macrophages from BALB/c, C57BL/6 and DBA/2 mice, lung tissue, liver tissue) and tumour cell types (CT26p, CT26wt, J774A.1, RAW 264.7, DA3, Esb and B16) is shown. (B) The basal IRF7 level of several primary (♦ spleen cells, macrophages from BALB/c, C57BL/6 and DBA/2 mice; † IFNAR\textsuperscript{−/−} macrophages) and tumour cell types (♦ CT26wt, J774A.1, RAW 264.7, DA3, Esb and B16; ♦ CT26p) is plotted against the NDV M gene expression level after 24 hours (see Figure 4-42). (C) The inducible IRF7 expression level is indicated for several primary (♦ spleen cells, macrophages from BALB/c, C57BL/6 and DBA/2 mice) and tumour cell types (CT26p, CT26wt, J774A.1, RAW 264.7, DA3, Esb and B16). (D) The inducible IRF7 level of several primary (♦ spleen cells, macrophages from BALB/c, C57BL/6 and DNA/2 mice; † IFNAR\textsuperscript{−} macrophages) and tumour cell types (♦ CT26wt, J774A.1, RAW 264.7, DA3, Esb and B16; ♦ CT26p) is plotted against the NDV M gene expression level after 24 hours (see Figure 4-42). The p value was calculated using an unpaired student's t-test. Bars indicate standard deviation. The correlation coefficient was calculated with the mean expression values.

The basal and NDV-inducible IRF7 expression turned out to be significantly higher in normal than in tumour cells. Additionally there was a negative correlation between the basal and inducible IRF7 expression level and the M gene expression. The last antiviral gene analysed
Results

in this context was the IFN-β.

Figure 4 demonstrates that the basal and the NDV-inducible IFN-β expression were higher in normal than in tumour cells. The difference was in both cases not significant. The average basal IFN-β expression level (part A) was 26 REU for normal and 1 REU for tumour cells. For the inducible IFN-β level (part C) the average was 96 REU for normal and 55 REU for tumour cells. There was a negative correlation between the basal IFN-β and the M gene expression (part B, R = -0.7166). However, there was only a low correlation between the inducible IFN-β and the M gene expression (R = -0.0995).
Results

It could be demonstrated that IFN-β was expressed more strongly in normal than in tumour cells before as well as after NDV infection. However, this difference was not statistically significant. There was a negative correlation between the basal IFN-β expression and the M gene expression, but for the inducible IFN-β level no clear correlation could be found.

Figure 4-46: Analysis of the basal and inducible IFN-β expression in primary and tumour cells before and after NDV infection.

The IFN-β expression level was measured in several primary and tumour cell types before and 24 hours after infection with 10 HU NDV Ulster or NDFL-EGFP. The gene expression was determined by real-time RT-PCR with the comparative C_T method using the acidic ribosomal phosphoprotein PO for normalisation. (A) The basal IFN-β expression of several primary (spleen cells, bone marrow cells, macrophages from BALB/c, C57BL/6 and DBA/2 mice, lung tissue, liver tissue) and tumour cell types (CT26p, CT26wt, J774A.1, RAW 264.7, DA3, Esb and B16) is shown. (B) The basal IFN-β level of several primary (♦ spleen cells, macrophages from BALB/c, C57BL/6 and DBA/2 mice; IFNAR−/− macrophages) and tumour cell types (♦ CT26wt, J774A.1, RAW 264.7, DA3, Esb and B16; ♦ CT26p) is plotted against the NDV M gene expression level after 24 hours (see Figure 4-42). (C) The inducible IFN-β expression level is indicated for several primary (♦ spleen cells, macrophages from BALB/c, C57BL/6 and DBA/2 mice; IFNAR−/− macrophages) and tumour cell types (♦ CT26p, CT26wt, J774A.1, RAW 264.7, DA3, Esb and B16). (D) The inducible IFN-β level of several primary (♦ spleen cells, macrophages from BALB/c, C57BL/6 and DNA/2 mice; IFNAR−/− macrophages) and tumour cell types (♦ CT26wt, J774A.1, RAW 264.7, DA3, Esb and B16; ♦ CT26p) is plotted against the NDV M gene expression level after 24 hours (see Figure 4-42). The p value was calculated using an unpaired student's t-test. Bars indicate standard deviation. The correlation coefficient was calculated with the mean expression values.

It could be demonstrated that IFN-β was expressed more strongly in normal than in tumour cells before as well as after NDV infection. However, this difference was not statistically significant. There was a negative correlation between the basal IFN-β expression and the M gene expression, but for the inducible IFN-β level no clear correlation could be found.
Results

In conclusion it could be stated that the basal and inducible expression levels of RIG-I, IRF3, IRF7 and IFN-β were higher in normal than in tumour cells. These differences were statistically significant except for the IFN-β gene. The expression of these four genes correlated inversely with NDV susceptibility determined by the M gene expression. For IFN-β the correlation was weaker for the basal expression level compared to the other three genes. In contrast to the other three genes there was almost no correlation between the inducible IFN-β gene and the M gene expression. The results confirmed the hypothesis that tumour cells in general have a lower expression of antiviral genes and that this lower gene expression makes them more susceptible to NDV infection than normal, non-malignant cells.

4.4.2 Differences in NDV infection in CT26 cell lines with different origin

Experiments done by Leonidas Apostolidis (Department of Cellular Immunology, DKFZ Heidelberg) had shown that the CT26p tumour cell line was much more resistant to NDV infection than the CT26wt cell line. The sole difference between the two CT26 cell lines was their origin (see 2.11.2). The differences in NDV susceptibility could be confirmed in the correlation analysis (4.4.1) and in addition it was found out that they had a markedly diverging basal and inducible expression of interferon-related genes. In order to investigate the differences in NDV susceptibility and antiviral gene expression further, CT26p and CT26wt cells were infected with different NDV strains and the infection was followed with regard to viral and interferon-related gene expression. Furthermore the binding of NDV to the cells was tested to ascertain whether the differences in infection could only be explained by physiologic differences between the cells.

4.4.2.1 NDV susceptibility

In order to further analyse the NDV infection CT26p and CT26wt cells were infected with NDFL-EGFP and the EGFP signal was measured after one day by flow cytometry. Additionally, the two cell lines were infected with NDV Ulster and the M gene expression was determined by real-time RT-PCR. The binding of NDFL-EGFP after one hour was ascertained by measuring NDV HN protein on the cell surface in flow cytometry. It was assumed that after this time no virus replication had taken place and that all HN molecules on the cell surface must come from bound or fused NDV particles.
Figure 4-47 shows that there were pronounced differences in NDV infectability between the CT26p and the CT26wt cells. After infection with NDFL-EGFP (part A) EGFP expression after one day was clearly detectable in CT26wt cells, whereas there was almost no signal in the CT26p cells. The differences in M gene expression after infection with NDV Ulster (part B) was even stronger and the relative M gene expression in CT26wt cells was around 900 times stronger than in the CT26p cells. The HN signal one hour after incubation with NDFL-EGFP was similar for the two cell lines.

Figure 4-47: Differences in NDV infectability between CT26 cells with different origin.

(A) Two types of CT26 colon carcinoma cell lines with different origins, denominated CT26wt and CT26p (see 2.11.2), were infected with 10 HU NDFL-EGFP per 10^6 cells and expression of EGFP was determined by flow cytometry 24 hours later. (B) Total RNA was extracted 24 hours after NDV infection and the expression level of the NDV M gene was determined by real-time RT-PCR with the comparative C_T method using β-actin for normalisation. The results are represented as the mean of three measurements. (C) The binding of NDV to the two CT26 cell lines was determined after one hour incubation with 10 HU NDFL-EGFP by flow cytometry. The cells were stained with a murine anti-HN antibody and a PE-conjugated GaM antibody.

The differences in NDV infectability between the CT26p and the CT26wt cells did not appear to be attributable to differences in NDV binding, which was very similar in both cell lines.
Results

The substantial differences in the EGFP and M gene signal after NDV infection could therefore be best explained by cell physiologic differences. To analyse this aspect, the antiviral gene expression before and after NDV infection was characterised.

4.4.2.2 Antiviral gene expression

Since the differing NDV susceptibilities appeared to be due to intrinsic differences between the two CT26 cell lines, the expression of the interferon-related genes RIG-I, IRF3, IRF7 and IFN-β was analysed by real-time RT-PCR.

Figure 4-48 demonstrates that the basal expression level of all genes analysed was higher in the CT26p than in the CT26wt cells, while for the gene expression level after infection with NDFL-EGFP there was no such trend. The differences in basal gene expression was highest for IRF7 (41 times), followed by RIG-I (13 times), IFN-β (6 times) and IRF3 (2 times). The NDV-inducible gene expression was approximately equal for RIG-I and IRF3. The inducible IRF7 expression was three times higher in CT26p cells and the IFN-β expression was 9 times lower.
When the CT26p and CT26wt cells were compared with regard to the interferon-related gene expression, the most striking differences could be found in the basal expression level, which was substantially higher in the NDV resistant CT26p cells. If the expression of the four genes tested was used to explain the differences in NDV susceptibility between the two cell lines, it would seem that an increased basal gene expression was much more important than an enhanced ability to induce gene expression after NDV infection. A high basal antiviral gene expression could enable the cells to react more rapidly to viral infections without the need of preceding gene expression.

Figure 4 - 48: Expression of interferon-related genes in different CT26 cell lines.

The two cell lines CT26wt and CT26p were infected with 10 HU NDFL-EGFP per 10^6 cells. Total RNA was extracted before and 24 hours after infection and the expression level of the RIG-I (A), the IRF3 (B), the IRF7 (C) and the IFN-β (D) gene was determined by real-time RT-PCR with the comparative C_T method using the acidic ribosomal phosphoprotein PO for normalisation. The results are represented as the mean of three measurements. Bars indicate standard deviation.

When the CT26p and CT26wt cells were compared with regard to the interferon-related gene expression, the most striking differences could be found in the basal expression level, which was substantially higher in the NDV resistant CT26p cells. If the expression of the four genes tested was used to explain the differences in NDV susceptibility between the two cell lines, it would seem that an increased basal gene expression was much more important than an enhanced ability to induce gene expression after NDV infection. A high basal antiviral gene expression could enable the cells to react more rapidly to viral infections without the need of preceding gene expression.
4.5 The functional significance of RIG-I in NDV infection

In order to investigate the functional significance of RIG-I in NDV infection, an expression plasmid coding for the human RIG-I was obtained and different cell types were transfected with this plasmid before NDV infection. The working hypothesis was that if RIG-I was an important factor in the detection of NDV infection, an increased RIG-I level could enhance the ability of cells to defend against virus infection. This enhanced virus resistance should be detectable as a reduction of the expression of viral genes such as the EGFP or the M gene after NDV infection. In addition more information about the function of RIG-I was deduced from an analysis of its expression in human cells by Western blot and real-time RT-PCR because up to that point only mouse cells had been used to analyse RIG-I.

4.5.1 NDV infection after transfection of cells with human RIG-I expression plasmids

Different human and murine cells were transfected with a plasmid coding for the human RIG-I protein to elucidate its role in NDV infection. As control an irrelevant plasmid and a plasmid coding for a mutated, non-functional RIG-I (RIG-I IKA) were transfected in parallel. It was thought that the RIG-I IKA, which was still able to bind dsRNA, might be able to reduce the NDV-induced signalling by sequestering viral dsRNA from receptors such as wildtype RIG-I or MDA5. One or two days after transfection the cells were infected with NDFL-EGFP and the EGFP expression was measured with flow cytometry. In addition the M gene expression was determined by real-time RT-PCR to obtain an additional parameter of NDV infection. The transfections were also done with the two macrophages-like tumour cell lines J774A.1 and RAW 264.7, but the transfection efficiencies were too low (data not shown) and therefore other cell lines were used subsequently.

Figure 4-49 shows that in MCF-7 cells there was no clear difference in the EGFP signal one day after infection with NDFL-EGFP (part A) between the cells transfected with the functional, wildtype RIG-I (RIG-I wt) and the two control transfections (control and RIG-I IKA). The transfection efficiency was about 40%. Transfection alone reduced the EGFP signal from 48% to 7.5% in the control transfected with an irrelevant plasmid. The EGFP signals in cells transfected with RIG-I wt or RIG-I IKA were 4.2 and 4.6%, respectively. The M gene signal (part B) was also strongly reduced by the transfection with an irrelevant plasmid alone (from 2033 to 235 REU). The M gene expression for RIG-I wt and RIG-I IKA
Results

were both lower than for the irrelevant plasmid with 29 and 56 REU, respectively.

![Graph A](image)

**Figure 4-50:** NDV infection of MCF-7 tumour cells after transfection with human RIG-I expression plasmids.

MCF-7 human breast cancer cells were transfected with expression plasmids coding for the wildtype human RIG-I (pRIG-I wt), a mutated, non-functional RIG-I IKA (pRIG-I IKA) or an irrelevant plasmid (pcontrol, plasmid no 241, see chapter 2.8). jetPEI™ was used as transfection reagent and as transfection efficiency control a plasmid coding for EGFP was used. After 48 hours the cells were infected with NDFL-EGFP and 24 hours after infection the EGFP expression was determined by flow cytometry (A). Also, total RNA was extracted 24 hours after infection and the expression level of the NDV M gene was determined by real-time RT-PCR with the comparative C\textsubscript{T} method using \(\beta\)-actin for normalisation. The results are represented as the mean of three measurements. Bars indicate standard deviation.

There was no marked difference in the NDV-induced signals in MCF-7 cells that had been transfected with RIG-I wt or RIG-I IKA before NDV infection. Only the M gene signal was slightly higher in cells that had been transfected with RIG-I IKA. The strong reduction of the EGFP or M gene expression by transfection alone could prevent differences in the NDV-induced signals from becoming visible. Therefore other cells were used in the following experiments that did not show this signal reduction after transfection. First, CT26wt cells were employed, but the transfection efficiency was too low (~ 15 %). L929 cells turned out to be well transfectable and did not display a marked reduction of NDV-induced signals by transfection.

**Figure 4-50** demonstrates that there was a clear difference in the expression of NDV genes between the L929 cells transfected with the different RIG-I plasmids. The cells transfected with RIG-I wt showed a lower EGFP signal (28 % positive cells, part A) than the control cells transfected with an irrelevant plasmid (39 %). The RIG-I IKA transfected cells displayed a
Results

high EGFP signal with 62%. There was only a slight reduction in the EGFP signal by the transfection alone when the transfected cells were compared with the not transfected cells (78%). The transfection efficiency was 56%. The M gene expression (part B) was also clearly lower in RIG-I wt than in RIG-I IKA transfected cells (222 and 16679 REU, respectively). Transfection reduced the M gene expression from 30910 to 725 REU in the control transfected with an irrelevant plasmid.

Figure 4 -50: NDV infection of L929 fibroblast cells after transfection with human RIG-I expression plasmids.

L929 murine fibroblast cells were transfected with expression plasmids coding for the wildtype human RIG-I (pRIG-I wt), a mutated, non-functional RIG-I IKA (pRIG-I IKA) or an irrelevant plasmid (pcontrol, plasmid no 241, see chapter 2.8). jetPEI™ was used as transfection reagent and as transfection efficiency control a plasmid coding for EGFP was used. After 48 hours the cells were infected with NDFL-EGFP and 24 hours after infection the EGFP expression was determined by flow cytometry (A). In addition, total RNA was extracted 24 hours after infection and the expression level of the NDV M gene was determined by real-time RT-PCR with the comparative C_T method using β-actin for normalisation. The results are represented as the mean of three measurements (B). Bars indicate standard deviation.

In L929 cells the expression of NDV-related proteins could be influenced by the induced expression of different types of RIG-I proteins. Wildtype RIG-I could reduce the EGFP and M gene expression, whereas RIG-I IKA could increase it. As an additional indicator that externally induced RIG-I could change the expression of NDV genes, the two plasmids RIG-I wt and RIG-I IKA were mixed in different ratios and were then transfected in L929 cells. This was done in order to determine dose-dependent effects of RIG-I on NDV infection.

Figure 4 -51 shows that in this experiment the EGFP expression upon NDFL-EGFP infection was clearly lower in the transfected than in the non-transfected cells. Without transfection
98% of the cells showed EGFP expression, while in the transfected cells the proportion of EGFP-expressing cells ranged from 13 to 45%. The percentage of EGFP expression was around 13% except if the amount of RIG-I wt in the plasmid mixture was lower than 0.33 out of 1. At 0.17 parts out of 1 RIG-I wt the percentage of EGFP expressing cells increased to 31 and if only RIG-I IKA was transfected EGFP expression was highest with 45%. The transfection efficiency was 51%.

![Graph](image-url)

**Figure 4-51:** NDV infection of L929 fibroblast cells after transfection with different ratios of human RIG-I wt versus RIG-I IKA expression plasmids.

L929 murine fibroblast cells were transfected with different ratios of expression plasmids coding for the wildtype human RIG-I (pRIG-I wt) and the mutated, non-functional RIG-I IKA (pRIG-I IKA). jetPEI® was used as transfection reagent and as transfection efficiency control a plasmid coding for EGFP was used. After 48 hours the cells were infected with NDFL-EGFP and one day after infection the EGFP expression was determined by flow cytometry.

There were no differences in the percentage of EGFP expressing cells for most of the RIG-I wt / IKA ratios tested. A dose-dependent increase in the EGFP signal with a decreasing amount of the RIG-I wt plasmid could only be observed at a very low RIG-I wt / IKA ratio.

In general there were only slight changes in the expression of viral proteins after the transfection of plasmids coding for RIG-I wt or RIG-I IKA. In some cells there were no clear changes at all in the EGFP or M gene expression, as for the MCF-7 or CT26 cells. In other cell types such as the L929 fibroblasts there was an increase or decrease in the viral signals for RIG-I IKA or RIG-I wt, respectively. In conclusion it could be said that the expression of additional RIG-I or of a mutated, non-functional RIG-I did only have a slight influence on the
outcome of an NDV infection depending on the cell type.

4.5.2 The basal and inducible expression of RIG-I in human cell lines
Since so far mostly murine cells had been analysed with regard to the influence of RIG-I on NDV infection, human cells were analysed next to obtain information on the importance of RIG-I in the human system. After establishing the NDV susceptibility in human PBMC and several human tumour cell lines the RIG-I expression in these cells was determined by Western blot analysis and by real-time RT-PCR to find out if there were correlations between NDV resistance and the basal and inducible RIG-I expression.

4.5.2.1 NDV replication
Human PBMC and the human tumour cells MCF-7, Jurkat, U937 and Hela were infected with NDFL-EGFP to test their susceptibility to NDV infection. The infection was followed by measuring the EGFP expression and the M gene expression level in infected and uninfected cells with flow cytometry and real-time RT-PCR, respectively.

Figure -52 demonstrates that there was almost no detectable EGFP signal in human PBMC, while the tumour cells all displayed EGFP expression (part A). The strongest EGFP expression could be found in the Hela cells, followed by the Jurkat, the MCF-7 and the U937 cells. There was also nearly no M gene signal in the PBMC in contrast to the four tumour cell types (part B). Here again the Hela cells had the strongest signal, followed by the U937, the Jurkat and the MCF-7 cells. The EGFP and the M gene signals correlated in all tumour cells except for the U937, which had the lowest EGFP signal, but the second highest M gene expression level.
Results

As in the mouse the primary human PBMC proved to be very resistant to NDV infection, while the tumour cells showed different degrees of NDV susceptibility. The MCF-7 cells seemed to be most resistant to NDV, while the Hela were most susceptible in this experimental setting.

4.5.2.2 RIG-I expression

After having established the NDV susceptibility the expression of RIG-I with and without NDV infection was determined by Western blot analysis and by real-time RT-PCR.

Figure 4-52: NDV replication in human PBMC and human tumour cell lines.

Human PBMC and the human tumour cell lines MCF-7, Jurkat, U937 and Hela were infected with 10 HU per 10⁶ cells NDFL-EGFP and EGFP expression was determined 48 hours after infection (A). In addition, total RNA was extracted 24 hours after infection and the expression level of the NDV M gene was determined by real-time RT-PCR with the comparative C₉ method using human β-actin for normalisation. Uninfected control cells for each cell type were negative for M gene expression (data not shown). The results are represented as the mean of three measurements (B). Bars indicate standard deviation.

As in the mouse the primary human PBMC proved to be very resistant to NDV infection, while the tumour cells showed different degrees of NDV susceptibility. The MCF-7 cells seemed to be most resistant to NDV, while the Hela were most susceptible in this experimental setting.

4.5.2.2 RIG-I expression

After having established the NDV susceptibility the expression of RIG-I with and without NDV infection was determined by Western blot analysis and by real-time RT-PCR.

Figure 4-53 shows that in the Western blot (part A+B) the basal RIG-I expression was highest in the MCF-7 cells, followed by the Hela and the Jurkat cells. The PBMC had the lowest RIG-I expression together with the U937 cells. The highest NDV-inducible RIG-I level was reached in MCF-7 and Hela cells. PBMC showed an intermediate inducible RIG-I expression level, while the expression was low in U937 and almost absent in Jurkat cells. In the real-time RT-PCR analysis the basal RIG-I gene expression was by a wide margin the highest in PBMC, while it was similarly low in the tumour cell lines (part C). NDV infection strongly induced RIG-I expression in all cell types except for the Jurkat cells. The inducible RIG-I expression level was higher in U937 and Hela cells than in PBMC and MCF-7 cells.
Results

The Western blot and the real-time RT-PCR analysis of RIG-I yielded divergent results. While the basal RIG-I expression measured by real-time RT-PCR was high in PBMC and low in the tumour cell lines corresponding to the NDV resistance, the PBMC showed the lowest RIG-I expression of all cells when measured by Western blot. The diverging results obtained with the two methods could be due to differences in the regulation of RIG-I mRNA.

Figure 4-53: RIG-I expression in human PBMC and human tumour cell lines before and after NDV infection.

Human PBMC and the human tumour cell lines MCF-7, Jurkat, U937 and Hela were infected with 10 HU per 10^6 cells NDFL-EGFP. (A) 48 hours after infection the cells were lysed and the lysates were used for Western blot analysis of the RIG-I expression. As loading control human β-actin was used. (B) The intensity of the bands in the Western blot was determined with a software for densitometric analysis. The intensities of the RIG-I bands were normalised with the intensities of the β-actin band intensities. (C) Total RNA was extracted 48 hours after NDV infection and the expression level of the RIG-I gene was determined by real-time RT-PCR with the comparative C_\text{\textit{T}} method using β-actin for normalisation. The results are represented as the mean of three measurements. Bars indicate standard deviation.
processing that could lead to different RIG-I mRNA translation rates. Another possible reason could be unspecific binding of the polyclonal anti-RIG-I serum, which generated a high background on the Western blot x-ray film (data not shown).

In summary it could be stated that the basal expression of RIG-I measured by real-time RT-PCR corresponded to the NDV resistance. However, since the Western blot analysis yielded different results it remained unclear whether this correlation of the basal RIG-I expression and the NDV resistance was true.
5 Discussion

In this thesis the basal and NDV-inducible interferon response was analysed in normal and tumour cells. The working hypothesis was that a defective interferon response in tumour cells could explain the tumour selective replication of NDV (figure 1-6). It could be shown that the expression of antiviral genes was higher in normal than in tumour cells. Antiviral gene expression was examined in macrophages, macrophage-like tumour cells and other normal or tumour cell types. Normal cells displayed a high basal expression of molecules important during an interferon response such as cytosolic viral RNA receptors, transcription factors for interferons, interferons and molecules with direct antiviral properties. It could be demonstrated that upon NDV infection normal cells reacted with a much stronger induction of these genes than tumour cells. The IFN responsiveness was also much higher in normal cells after IFN treatment with regard to antiviral gene expression and inhibition of NDV replication. A strong, negative correlation between NDV susceptibility and the expression of genes important in the interferon response was discovered. This could explain the ability of some tumour cells to resist NDV infection much better than most other tumour cells.

Furthermore the role of some interferon-induced antiviral proteins in NDV infection could be investigated in knock out macrophages. It could be demonstrated that the two most important transcription factors for the induction of type I interferons, IRF3 and IRF7, are important at distinct time points during the interferon response. The absence of IRF3 limited antiviral gene expression in the early but not in the late phase of the interferon response, while IRF7 appeared to be crucial for the strong expression of antiviral genes after virus infection. In addition, the IFNAR proved to be a bottleneck in the interferon signalling and its absence dramatically increased the NDV susceptibility of cells.

In summary the results support the hypothesis that defects in the interferon response in tumour cells can explain the tumour selective replication of NDV. Tumour cells showed a diminished expression of pivotal antiviral genes and a low expression of these genes was strongly correlated with NDV susceptibility.
5.1 Quantification of gene expression by real-time RT-PCR

Quantitative real-time RT-PCR was used throughout this thesis to determine the expression of viral or interferon-related genes. The employment of the real-time RT-PCR technique for gene detection and quantification offers the advantages of high sensitivity and reproducibility, combined with an extremely broad dynamic detection range. Since the cDNA to be analysed is amplified in the real-time PCR, a low amount of tissue or a low number of cells is sufficient to carry out an analysis. It could be shown that the sensitivity of some of the primer systems applied in this thesis still detected down to a few copies of the target sequences (chapter 4.1.1). Hence the RNA that is extracted from a sample of one million cells, a typical sample size that was used in the experiments of this thesis, is sufficient for multiple determination of the expression of a high number of genes and to repeat experiments several times. Furthermore the analysis of gene expression does not depend on living cells as, for example, flow cytometric analysis, and because RNA or cDNA samples can be stored almost indefinitely at low temperatures, the samples can be used again to determine the expression of additional genes when new questions arise in the course of a series of experiments. Compared to conventional RT-PCR, real-time RT-PCR is significantly less variable (Heid *et al.*, 1996).

Quantification of gene expression by real-time RT-PCR can either be done by using an external standard curve or by employing one or more co-amplified internal control mRNAs for normalisation (Bustin, 2000). In this thesis the samples were normalised relative to internal housekeeping genes, i.e. genes that are thought to be expressed at a constant level irrespective of the conditions in which a cell is growing. This method of quantification has the advantage of compensating for all the steps that are required to obtain the final PCR measurement such as different input RNA amounts used in the reverse transcription step due to varying amounts of biological material available for RNA extraction. A possible disadvantage of the quantification using endogenous control genes is that the expression of housekeeping genes is sometimes not absolutely constant.

In general the mRNA expression levels of a gene can be expected to correlate strongly with the protein level. Since there are further steps and regulatory mechanisms that influence the gene expression on the protein level it is nevertheless possible that the mRNA and the protein level for a gene vary. Yet, when the NDV M gene expression was analysed with real-time
RT-PCR and with flow cytometry, the results were very similar (figure 4-10, 4-40 or 4-52), pointing to a strong connection between the mRNA and the protein level.

The ability of real-time RT-PCR to generate accurate quantitative data has had a huge impact on the study of viral agents of infectious disease. It is helping to clarify disputed infectious disease processes and demonstrates links between specific viral sequences and patient clinical symptoms (Mackay et al., 2002). There are, for example, various viruses targeting the respiratory tract such as influenza virus types A and B or RSV, which are important causes of severe lower respiratory tract disease in elderly and immunocompromised patients with significant morbidity and mortality (Bustin and Mueller, 2005). Since these respiratory viral pathogens produce very similar clinical symptoms, differential diagnosis of the pathogens is required. Real-time RT-PCR assays have been developed to distinguish these viruses and their subtypes and to adjust the therapy accordingly (Kuypers et al., 2004; Stone et al., 2004). During the outbreak of SARS in China real-time RT-PCR proved to be more sensitive and faster than other methods such as conventional RT-PCR and more suitable for the necessary high throughput analysis of samples. Real-time RT-PCR was invaluable for the rapid, early diagnosis of SARS (Poon et al., 2003), the elucidation of viral pathogenesis (Peiris et al., 2003) and the provision of prognostic information for clinical management (Cheng et al., 2004). Other examples of the widespread use of real-time RT-PCR in clinical virus detection are HBV, HCV and HIV-1 (Candotti et al., 2004).

The real-time RT-PCR assay that was applied in this thesis to detect the NDV M gene has been developed to measure NDV RNA in clinical samples (Wise et al., 2004). It could be shown that most NDV isolates can be detected with this assay, presumably because the assay amplifies a relatively conserved region near the 5´ end of the M gene (Seal et al., 2000). It is also possible to differentiate NDV strains by a melting-curve analysis after a real-time RT-PCR amplification of a region of the F gene (Pham et al., 2005).

In cancer treatment real-time RT-PCR can be employed to monitor disease-specific markers and to evaluate MRD (minimal residual disease). For example, the detection of typical cytogenetic abnormalities in CML (chronic myeloid leukaemia) by real-time RT-PCR is an important prognostic parameter for the assessment of complete remission and long-term survival. Real-time RT-PCR has been found to be more sensitive and reliable than other methods for molecular follow-up of CML (Kim et al., 2002; Raanani et al., 2004). Since the
monitoring of the dynamics of residual disease in CML patients can predict clinical relapse, the real-time RT-PCR data are useful in guiding clinical therapeutic decisions (Goldman et al., 1999; Moravcova et al., 1999).

Apart from determining the expression of viral genes or MRD, real-time RT-PCR has also been used in this thesis to measure the relative expression of cellular genes. The rationale was that if a gene played a role in special cellular conditions such as a virus infection, its expression level was likely to change compared to control conditions without virus. If a gene product is indicated to play a role in cellular processes under certain physiological, pathophysiological or developmental conditions, for example, due to indications from its known function or because of previous microarray data, it is a common approach to corroborate this indication by real-time RT-PCR expression analysis (Valasek and Repa, 2005). All in all it can be said that real-time RT-PCR has become a reliable and widely used method for the rapid and accurate assessment of changes in gene expression to enhance the understanding of biological processes. For clinical molecular diagnostics real-time RT-PCR has become indispensable to measure viral loads or to evaluate cancer status.

5.2 NDV infection in normal and tumour cells

It is known that in general NDV can replicate substantially better in cancer cells than in most normal cells (Reichard et al., 1992; Schirrmacher et al., 1999a). Tumour cells are in general more susceptible to virus infection as a result of their unrestricted growth, their deficiency in controlling nucleic acid synthesis and defects in their antiviral interferon response (Fiola et al., 2006, Stojdl et al., 2000). In addition it has been suggested that for efficient NDV replication an activated ras-pathway is necessary (Lorence et al., 1994b). In contrast to many other viruses the tumour selectivity of NDV is inherent and it does not have to be genetically modified to grow specifically in tumour cells. This makes NDV an attractive candidate for cancer therapy (Csatary et al., 2004; Freeman et al., 2006; Lorence et al., 2007; Schirrmacher, 2005; Sinkovics and Horvath, 2000). Inherent tumour selectivity is a characteristic of many RNA viruses such as echovirus type 1 (Shafren et al., 2005), poliovirus (Ochiai et al., 2006), mumps virus (Myers et al., 2005), VSV (Barber, 2004) or reovirus (Figova et al., 2006). Naturally occurring infections with these viruses cause either asymptomatic or relatively mild disease in humans and therefore these viruses have a good intrinsic safety profile. In contrast
there are oncolytic viruses that are derived from viruses that naturally target also non-malignant cells. These viruses can be made tumour selective by modifying the cellular tropism at the level of replication in a way that they become dependent on specific characteristics of tumour cells for viral replication. This can be achieved by deleting viral genes that are critical for viral replication in healthy cells but are dispensable upon infection of neoplastic cells. Other approaches to make viruses tumour selective are the use of tumour specific promoters upstream of viral genes or the modification of the viral coat to enable tumour selective binding and uptake. Examples of viruses that have been modified to replicate only in tumour cells are adenovirus (Relph et al., 2005), HSV (Shen and Nemunaitis, 2006) and vaccinia virus (Thorne et al., 2005).

Here it could be demonstrated that several strains of NDV replicated substantially better in tumour than in normal cells. Even when high doses of NDV were used for infection, normal cells proved to be strongly resistant to NDV replication (figure 4-1). In macrophage-like tumour cells there were pronounced differences in NDV susceptibility. Viral replication was much stronger in the RAW 264.7 than in the J774A.1 cells. When compared to the J774A.1 cells, the RAW 264.7 cells displayed a 50% higher growth rate and a lower IFN responsiveness with regard to the blocking of NDV infection and to the reduction of proliferation after IFN pretreatment. In addition the RAW 264.7 cells showed a lower basal and inducible expression of important interferon-related antiviral genes. So the RAW 264.7 cells appeared to have a more malignant phenotype with faster proliferation and insensitivity to anti-growth signals. The RAW 264.7 cells were derived from a virus-induced tumour (chapter 2.11.2) and could therefore be expected to be more susceptible to virus infection. These data could explain the higher NDV susceptibility of the RAW 264.7 cells. They point to an increased ability of NDV to replicate in tumour cell subtypes that display a more malignant phenotype.

Even within the same tumour cell line there can be substantial differences in NDV susceptibility as was observed for CT26 colon carcinoma cells (figure 4-47). In general CT26 cells are well infectable by NDV (Schirrmacher et al., 2001) or other oncolytic viruses (Malhotra et al., 2007; Smyth et al., 2005). The two CT26 cell lines here were derived from different sources and displayed strong variations in the basal expression of antiviral genes. These variations could explain the differences in NDV replication. The differences in gene
expression had probably been acquired because of varying ways of cultivation at the laboratories from which the cells were obtained. Perhaps the more NDV susceptible CT26 cells were cultivated for a longer time and had therefore an increased probability to acquire mutations that gave them a selective advantage. This could have been an increase in the growth rate by the downregulation of interferon-related genes which led to an increased responsiveness to anti-growth effects of IFN but at the same time made the cells more susceptible to virus infection.

A high variation in NDV susceptibility of tumour cells could also be found in the comparison of other normal and tumour cells (figure 4-42). The variation seemed to be connected to the extent to which the tumour cells had acquired defects in the antiviral interferon response (figure 4-43 – 4-46). The importance for an intact interferon response for NDV resistance could be clearly seen in cells that had gene knock outs for crucial antiviral genes such as the IFNAR. However, since IFNAR^{-} macrophages are still more NDV resistant than most tumour cells there must be other factors influencing NDV susceptibility.

Low cell proliferation rates could explain the NDV resistance of normal cells even when they show a strongly reduced interferon response due to the knock out of crucial antiviral genes such as the IFNAR. In highly replicating tumour cells the cellular machinery that is necessary for the replication of the cell as well as that of the virus is expressed much stronger than in resting normal cells. That makes it possible for a virus to replicate better in tumour cells than in normal cells. This hypothesis could also explain that NDV infects quickly proliferating, wildtype MEF cells better than resting IFNAR^{-} macrophages. A detailed discussion of the interferon response and other properties different between normal and tumour cells and their influence on NDV infection can be found in the next chapter.

When different strains of NDV were used for the infection of normal and tumour cells, different levels of viral gene expression could be observed. Infection of macrophages with the velogenic NDV strain Italien and to a lesser degree also with the mesogenic strain NDFLtag-EGFP generated a higher expression of viral genes than the infection with the lentogenic strains Ulster and NDFL-EGFP (figure 4-7). As might have been expected it seems that the more virulent an NDV strain is, the better it can replicate in a cell. One explanation could be an increased ability of velogenic strains to limit the induction of antiviral responses. This hypothesis is substantiated by the fact that in macrophages the induction of antiviral gene
expression was generally lower for NDV Italien than for NDV Ulster.

5.3 The interferon response in normal and tumour cells in the context of NDV infection

Defects in the interferon response are thought to make many tumour cells susceptible to viruses that do not infect normal, non-neoplastic cells. This has been suggested for NDV (Fiola et al., 2006), VSV (Stojdl et al., 2000, 2003) and influenza virus (Muster et al., 2004) and others. A defective interferon response is thought to give tumour cells a selective advantage by making them less sensitive to growth-inhibitory effects of interferons and allowing them to grow faster in the presence of low basal interferon levels that can be found in the absence of viral infection (chapter 1.3.5). The exact nature of the defects and the molecules that are affected in the signalling chains necessary for the establishment of an antiviral response are not known. An important question is, for example, if only a limited number of key molecules of the interferon response are downregulated or defective, or if many components of the antiviral response are involved. It is also not known which points in the signalling cascade that leads to a cellular response are affected: are the relevant differences more at the level of the basal or the inducible antiviral gene expression, the secretion of interferons or the ability to respond to external interferons? These questions could be answered in the course of the experiments of this thesis and will be discussed in the following.

5.3.1 Cytosolic pattern recognition receptors

Cytosolic pattern recognition receptors are indispensable for the detection of virus infection inside a cell. They are one of two different receptor systems for virus infection which are located in different cellular compartments. The other receptor system is constituted by TLRs that are located mainly on endosomal membranes (chapter 1.3.2). Cytosolic pattern recognition receptors belong to the family of DExD/H-box helicases and recognise viral RNAs. One important factor for the differentiation of self and non-self RNA that has recently been discovered is 5´-triphosphate RNA (Hornung et al., 2006; Pichlmair et al., 2006). In addition, the 5´-triphosphate ended viral leader transcript of measles virus, which is a member of the paramyxoviridae like NDV, was found to be an activator of the RIG I-mediated
interferon response (Plumet et al., 2007).

Since RNA helicases are crucial molecules to start an antiviral response after virus infection, a different expression of RIG-I or MDA5 could be related to differences in NDV susceptibility between normal and tumour cells. Indeed it could be observed in macrophages that the basal and inducible expression level of these genes correlated negatively with NDV susceptibility. The correlation was underlined by the fact that the J774A.1 cells with an intermediate NDV susceptibility also showed intermediate basal and inducible gene expression levels for the two helicases. In addition there was also a strong, short-lived induction of RIG-I and MDA5 expression by UV-inactivated NDV, pointing to replication-independent ways of induction. NDV Ulster induced a stronger gene expression than NDV Italien, which could explain the higher virulence of the latter strain by its ability to limit the expression of antiviral genes.

The importance of RIG-I and MDA5 for virus recognition has shown to depend on the virus. For NDV, RIG-I seems to be more important than MDA5 (Kato et al., 2006). This might be due to the fact that most paramyxoviruses target MDA5, but not RIG-I with their V proteins (Childs et al., 2007). The functional significance of RIG-I in NDV infection could be confirmed here in transfection experiments with plasmids encoding wildtype or mutated, non-functional RIG-I. MDA5 was induced stronger in macrophages upon NDV infection than RIG-I but that does not necessarily indicate a higher importance of MDA5. The basal expression level of these viral detection molecules is probably more important than the inducible level to limit NDV infection. A substantial induction after NDV infection of RIG-I and MDA5 mRNA took 8 to 12 hours, so that on the protein level at least half a day passes until there is a significant increase in danger signalling. After 12 hours NDV has already reached its maximum of gene expression (see for example figure 4-7), so the induction of viral detection molecules would be too late to significantly restrict NDV replication. The importance of an early induction of an antiviral response is underlined by the higher NDV susceptibility of IRF3 than of IRF7 knock out macrophages, since IRF3 is important in the early phase of an interferon response. The special importance of RIG-I for the early induction of antiviral responses has also been shown in the infection with other viruses such as RSV (Liu et al., 2007).

When several normal and tumour cell types were compared (figure 4-43) it could be demonstrated that RIG-I was significantly higher expressed in normal cells than in tumour
cells. Furthermore there was a strong negative correlation between the RIG-I expression and NDV susceptibility. Exceptionally NDV resistant CT26 tumour cells or uncommonly NDV susceptible normal cells such as IFNAR−/− macrophages had respectively an unusually high or low RIG-I level. These findings suggest that differences in RIG-I expression in normal and tumour cells can account for differences in NDV susceptibility.

In human cells a high basal rather than inducible RIG-I expression correlated with NDV resistance at the mRNA level (figure 4-53). At the protein level the tendency was similar, although some tumour cells showed a higher RIG-I level than PBMC. In contrast to the tested mouse cell types the NDV-inducible RIG-I level in human cells was not correlated to NDV susceptibility. This might point to differences in the regulation of RIG-I expression in murine and human cells. However, this suggestion needs to be confirmed by testing more human normal and tumour cells before general conclusions can be drawn.

It is not yet clear what the regulatory mechanisms are that could lead to a downregulation of RIG-I in tumour cells. RIG-I is inducible by IFN pretreatment in macrophages (figure 4-25 and 4-36) and dendritic cells (Yount et al., 2007). In contrast, the expression of MDA5 seems to be independent of type I interferons but it appears to be strongly linked to IRF3 (Yount et al., 2007). RIG-I expression must also be regulated by IFN-independent mechanisms since the basal and NDV-inducible RIG-I expression in IFNAR or IRF7 knock out macrophages was not much lower than in the wildtype. These mechanisms are probably similar to the general regulation of DExD/H box RNA helicases. These can be expected to be widely expressed because they are active in all processes involving RNA molecules including transcription, editing, splicing, ribosome biogenesis, RNA export, translation, RNA turnover, and organelle gene expression (Tanner and Linder, 2001). In human endothelial cells RIG-I expression was upregulated by IFN-γ (Imaizumi et al., 2004) and in human keratinocytes by IFN-γ or TNF-α (Kitamura et al., 2007), pointing to a role of RIG-I in inflammatory processes.

The RNA helicase LGP2 has been suggested as a negative regulator of RIG-I and MDA5 signalling since it lacks the CARD domain necessary for signal transmission after the binding of viral RNA (Rothenfusser et al., 2005; Yoneyama et al., 2005). Results indicate that LGP2 can inhibit antiviral signaling independently of dsRNA or virus infection intermediates by engaging in a protein complex with MAVS (Komuro and Horvath, 2006). The expression of LGP2 was therefore analysed in normal and tumour cells to investigate whether differences in
expression can be related to differences in NDV susceptibility. However, the results did not suggest LGP2 as a factor involved in the varying NDV replication in normal and tumour cells. Normal macrophages showed the highest basal and inducible LGP2 level (figure 4-18). It is of course possible that there are differences in protein modification after the translation process that cannot be seen at the mRNA level. There is, for example, a repressor domain in RIG-I and LGP2 (Saito et al., 2007) that might function differently in normal and tumour cells.

5.3.2 Membrane-bound pattern recognition receptors

Apart from the cytosolic pattern recognition receptors, membrane-bound pattern recognition receptors constitute the second major detection system for viral infection and the induction of type I interferons. Most members of these receptors belong to the TLRs and each member recognises a different repertoire of pathogen-associated molecular patterns. The main TLRs for the detection of virally-derived nucleic acids are TLR 3, 7, 8 and 9, and they are mostly located on the membranes of endosomes (chapter 1.3.2). TLRs are important danger signal receptors in tissues because they detect viral nucleic acids that are released from other infected and lysed cells and can thereby induce an antiviral state in so far uninfected cells. By this mechanism the spread of viruses in tissues can be effectively limited. TLRs are expressed mainly by specialised cell types such as macrophages and dendritic cells and are crucial for the secretion of large amounts of type I IFNs by pDCs (Colonna et al., 2004). In macrophages TLRs play central roles in the induction of proinflammatory cytokine expression during viral infection (Mogensen and Paludan, 2005).

The results obtained with the TLR3 and TLR7 knock out macrophages with regard to NDV replication and secretion of type I interferons were generally very similar to those obtained with the wildtype macrophages. The secretion of IFN-α/β appeared to be slightly lower in the two knock outs but since the experiment could only be carried out once it could not be said if this slight difference was significant. All in all the results indicated that the two TLRs were not involved in the signalling of NDV infection in the tested experimental setting. TLR3 recognises dsRNA (Alexopoulou et al., 2001) and TLR7 viral ssRNA (Diebold et al., 2004; Heil et al., 2004) and since the two receptors face the outside of a cell the nucleic acids have to be located in the extracellular fluid to be recognised. In the infection experiments carried out here there could not be any viral nucleic acids in the extracellular space since the
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Macrophages were rarely killed by the NDV infection and because the experiment lasted only about one day. This period of time was too short for the release of large amounts of viral RNA from NDV-infected cells that might have died following NDV infection. The TLR3/7 knockout mice can be assumed to have a much stronger effect on virus resistance in vivo. A comparison between fibroblasts and macrophages revealed the importance of TLR3 for the latter cell type in response to viral PAMPs. The ability of bone marrow derived murine macrophages to respond to dsRNA was largely dependent on TLR3 (Alexopoulou et al., 2001), while in fibroblasts the response was TLR3-independent (Hemmi et al., 2004). TLR7 could also be shown to be critical for antiviral responses in vivo since TLR7 knockout mice were defective in their response to VSV and influenza virus (Lund et al., 2004).

5.3.3 Interferon regulatory factors

IRF3 and IRF7 are the two most important transcription factors for the induction of type I interferons. They are activated by cytosolic RNA helicases as well as TLRs upon viral infection. On the gene expression level the major difference between IRF3 and IRF7 is that the former is expressed constitutively whereas the expression of IRF7 is not and depends on induction. During the two-step induction of type I interferons, IRF3 is more important in the early phase while IRF7 is crucial in the later phase (chapter 1.3.3.2).

In macrophages IRF3 expression increased slightly after NDV infection while the expression remained almost constant in macrophage-like tumour cells. When the induction of IRF3 is compared with all other interferon-related genes analysed in this thesis it can be said to be very moderate. Therefore there is no contradiction to the general assumption that IRF3 action is mostly constitutive, as shown for example by Juang et al. (1998). In the macrophage cell types there was a correlation between NDV resistance and IRF3 expression, suggesting a role of this molecule for NDV resistance of cells. A low expression of IRF3 in tumour cells could lead to a diminished signal transduction after the detection of viral danger signals and would lead to a weaker antiviral response. This hypothesis was corroborated by the analysis of several normal and tumour cells types. IRF3 was significantly higher expressed in normal cells at the basal as well as at the inducible level and there was a strong negative correlation between IRF3 expression and NDV susceptibility (figure 4-44).

IRF7 was found to be highly inducible by NDV infection in spleen cells (figure 4-4) and in
macrophages and macrophage-like tumour cells (figure 4-20), confirming its strong inducibility described in the literature (Marie et al., 1998; Sato et al., 1998). In the macrophage cell types there was a negative correlation between NDV susceptibility and IRF7 expression level after NDV infection, indicating IRF7 as a further factor involved in NDV resistance. This conclusion was corroborated by the analysis of several normal and tumour cell types (figure 4-45). IRF7 was significantly more expressed in normal cells and there was a strong negative correlation between IRF7 expression and NDV susceptibility. In addition NDV Ulster induced IRF7 expression more strongly than NDV Italien, pointing to an increased ability of NDV Italien to limit antiviral gene expression, which could explain its higher virulence.

IRF7 is a crucial molecule for the induction of antiviral responses. Its importance for the enhancement and diversification of IFN induction has been confirmed in earlier studies (Levy et al., 2002). It has even been proposed that IRF7 is the master regulator of type I interferon-dependent immune responses and that all elements of IFN responses, whether the systemic production of IFN in innate immunity or the local action of IFN from pDCs in adaptive immunity, are under the control of IRF7 (Honda et al., 2005). IRF7 is often targeted by viruses to limit antiviral responses and to increase virus survival and replication. For example, a Kaposi's sarcoma-associated herpesviral protein inhibits virus-mediated induction of type I interferon by blocking IRF7 phosphorylation and nuclear accumulation (Zhu et al., 2002). Also the herpes simplex virus ICP0 RING finger domain inhibits IRF3- and IRF7-mediated activation of interferon-stimulated genes (Lin et al., 2004). Often viral proteins increase the degradation of IRF7 such as the rotavirus NSP1 protein (Barro and Patton, 2007) or the KSHV immediate-early transcription factor RTA that encodes a ubiquitin E3 ligase activity that targets IRF7 for proteasome-mediated degradation (Yu et al., 2005).

When macrophages were treated with IFN-α4 there was a strong induction of IRF7 expression, whereas the IRF3 expression did not change (figure 4-26 and 4-27). This is in accordance with the view that IRF3 is constitutively expressed while IRF7 is highly inducible. For IRF7 the increase in expression brought about by IFN-α4 correlated also with NDV susceptibility and was much higher in normal macrophages than in the macrophage-like tumour cells lines. These results showed that tumour cells can have defects not only in the ability to secrete IFN upon virus infection, but also in the ability to respond to external IFNs.
The expression of IRF7 is thought to be induced by early IFNs and depends on the ISRE present in the IRF7 promoter (Lu et al., 2000). In addition IFN-independent mechanisms for the induction of IRF7 by virus infection have been suggested (Ning et al., 2005). It is likely that both mechanisms play a role for the differential expression of IRF7 in normal and tumour cells because on the one hand tumour cells displayed limits in their ability to secrete interferons, but on the other hand the J774A.1 cells secreted high amounts of type I interferons at later time points after NDV infection and still showed only a low IRF7 expression (figure 4-14). Another explanation for the low expression of IRF7 in spite of the considerable IFN secretion after NDV infection would be defects in the signalling of the IFNAR or a downregulation of the IFNAR.

The functional importance of IRF3 and IRF7 for NDV resistance and the interferon response became apparent in the analysis of knock out macrophages. The absence of both transcription factors increased the susceptibility of macrophages for NDV infection. The increase was higher in the IRF3 than in the IRF7 knock out cells. The kinetics of NDV infection showed that virus replication was comparable at early time points. Thereafter it increased in the IRF3 knock outs while it decreased in the IRF7 knock outs (figure 4-31). This suggests that the secretion of early IFNs, which depends on IRF3, is more important for limiting NDV infection than the secretion of high amounts of all IFN types. Furthermore the lack of IRF3 clearly delays and limits the expression of IRF7 (figure 4-38) so that until later time points the IRF3 knock out macrophages display almost a IRF3/7 double knock out phenotype and are therefore especially vulnerable to NDV infection. On the other hand IRF7 knock out macrophages still express almost the same amount of IRF3 than the wildtype (figure 4-37) and are therefore not inhibited in their ability to establish the early phase of the interferon response.

The IFN-α4 pretreatment was sufficient to limit NDV replication in the IRF3 and IRF7 knock out macrophages almost as strongly as in the wildtype. It seems that in the tested experimental setting no additional secretion of IFN-α4 or of other type I IFNs mediated by IRF3/7 was necessary to strongly inhibit NDV replication. However, it cannot be concluded that IFN-α4 is the only IFN that plays a role in the interferon response to NDV infection. It is known that NDV infection stimulates the expression of several IFN-α subtypes (Marie et al., 1998). Unfortunately a real-time RT-PCR assay could not be established to elucidate the expression
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of different IFN-α genes at different time points after NDV infection. There are more than a
dozen IFN-α genes in the mouse and it proved to be very difficult to find primer systems that
detect only one member of this gene family.

The IFN secretion upon NDV infection was clearly reduced in IRF7 knock out macrophages,
while IRF3 knock out macrophages showed an IFN secretion similar to the wildtype. This
underlines the role of IRF7 as the master regulator of the IFN response but confirms also that
for NDV resistance \textit{in vitro} an early interferon response is more important than the ability of a
cell to secrete large amounts of type I IFNs. The IFN-α4 pretreatment reduced the type I
interferon secretion in the IRF3/7 knock outs as well as in the wildtype probably because of
the reduction of NDV replication and hence the decreased concentration of IFN-stimulating
viral danger signals.

IRF3 and IRF7 did not seem to be required for the induction of RIG-I by NDV infection or by
IFN-α4 since the RIG-I expression in the knock outs was comparable with the wildtype.
Nevertheless, the expression of IFN-β was strongly compromised in the IRF3/7 knock outs
(figure 4-39). In the absence of IRF3 the IFN-β expression was clearly delayed, while almost
no IFN-β was expressed at any time point after NDV infection when IRF7 was lacking. This
indicates that although IFN-β is regarded as an early IFN it requires not only the presence of
IRF3 but also that of IRF7 for its expression. An initial model suggested that only IRF3 is
involved in the early phase of IFN-β induction by forming a multi-protein complex called an
enhanceosome (Maniatis \textit{et al.}, 1998). Meanwhile it could be shown in knock out mice that
the presence of IRF7 is also crucial for the IFN-β induction after HSV, VSV and EMCV
infection (Honda \textit{et al.}, 2005). So the low amounts of IRF7 that are expected to be present in
the absence of virus infection seem to be of importance for IFN-β induction. The high IFN-β
expression in the IRF3 knock outs at the latest time point occurred probably because of the
higher NDV replication in these cells that could be translated into a stronger response when
finally enough IRF7 had been synthesised.

It can thus be concluded that IRF3 and IRF7 play a role in the generation of differences in
NDV susceptibility between normal and tumour cells. Firstly, it could be shown that the
expression of both transcription factors is significantly downregulated in tumour cells and
secondly, their importance for NDV resistance and the establishment of an interferon response
could be demonstrated in knock out macrophages. However, they cannot be the only antiviral
factors for which differences exist between normal and tumour cells, because the IRF3/7 knock out macrophages display still a low viral replication compared to tumour cells.

**5.3.4 Type I interferons and the IFNAR**

In the experiments for this thesis the expression of the type I IFN-β could be followed in normal and tumour cells upon NDV infection by real-time RT-PCR. IFN-β expression is induced early in an antiviral interferon response by IRF3 and it is important for positive feedback mechanisms and the induction of key molecules such as IRF7. IFN-β signals through the IFNAR which is the only known receptor for type I interferon signalling. Upon binding of type I IFNs the signal is transmitted via the activation of STAT transcription factors which form the protein complex ISGF3. This protein complex translocates into the nucleus and induces the expression of hundreds of genes containing an ISRE motif in their promoter.

The basal expression of IFN-β was found to be substantially lower in tumour than in normal cells. Its NDV-inducible expression was generally also lower in tumour cells, although in both cases the difference was not statistically significant. Hence a downregulation of IFN-β in tumour cells before virus infection seemed to better explain the higher NDV susceptibility of tumour cells than a limited inducible IFN-β level. One possible reason for the lower basal IFN-β level in tumour cells in accordance with the data gathered in this thesis would be the lower IRF3 level in tumour cells. IRF3 is important for the induction of IFN-β (Sato *et al.*, 2000) and if there is a lower level of IRF3 in tumour cells in the absence of virus infection, the induction of IFN-β stimulated by a low constitutive IFN secretion can also expected to be limited. The kinetics of IFN-β expression during NDV infection demonstrated that after induction it was more strongly downregulated than the other analysed genes, pointing to strong negative regulatory mechanisms. There was no positive feedback regulation of IFN-β by IFN-α4 since no change in the IFN-β expression could be found after IFN-α4 treatment.

The IFN-β gene induction is a highly ordered process and it is regulated by multiple transcription factors (Honda *et al.*, 2006a). Its importance for NDV resistance could be shown in IRF3 knock out macrophages, in which the lack of the early induction of IFN-β made them even more susceptible to NDV infection than the IRF7 knock out macrophages. The importance of IFN-β for virus resistance can also be deduced from the fact that the IFN-β-
inducing IRF3 is commonly targeted by viruses to limit the induction of antiviral responses. In the case of influenza A virus, the viral nonstructural protein 1 prevents the induction of the IFN-β promoter by inhibiting the activation of transcription factors including IRF3 (Mibayashi et al., 2007). The HCV NS3/4A serine protease blocks the phosphorylation and effector action of IRF3 (Foy et al., 2003) and the induction of IFNs by HSV was shown to be counteracted by ICP27 targeting NF-κB and IRF3 (Melchjorsen et al., 2006). Paramyxoviruses also target IRF3 and downregulate IFN-β to inhibit antiviral responses, usually via their V proteins. Sendai virus C and V proteins target signalling pathways leading to IRF3 activation for the negative regulation of interferon-β production (Komatsu et al., 2004) and in another study the V proteins of simian virus 5 and other paramyxoviruses inhibited the induction of IFN-β (Poole et al., 2002). In the same study it was found that NDV is a potent inducer of IFN-β in mammalian cells. Furthermore a strong induction of type I IFNs by NDV has been shown in several other studies (Fournier et al., 2003; Washburn et al., 2002; Zeng et al., 2002a; Zorn et al., 1994). These findings underline the importance of IFN-β in antiviral responses and suggest that the limited IFN-β expression in tumour cells is related to their high NDV susceptibility. Moreover, the homozygous deletion of IFN-α/β genes in human leukemia and derived cell lines have been associated with tumour development (Diaz et al., 1988) and recombinant type I interferons are used in the treatment of different kinds of cancers (Vannucchi et al., 2007; Yoshida et al., 2004).

The importance of the IFNAR during NDV infection could be assessed with the help of IFNAR knock out macrophages and MEF. It was shown that the IFNAR is crucial for NDV resistance. In its absence the cells were much more susceptible to NDV infection than the wildtype and also than the IRF3/7 knock out cells. Considering the M gene expression, the IFNAR knock out macrophages were almost as susceptible to NDV infection as tumour cells. In addition the cells became completely irresponsive to IFN-α4 pretreatment with regard to inhibition of NDV replication or to induction of interferon-related gene expression.

The wildtype MEF cells were susceptible to NDV infection although they are non-malignant cells. The MEF constitute an immortalised cell line that grows permanently in contrast to the macrophages. They therefore provide a better environment for NDV replication since much of the cellular machinery that is required for cell proliferation is also necessary for virus replication. Resting cells such as macrophages are already more resistant to NDV infection.
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than MEF cells since there are only low concentrations of proteins involved in cell proliferation. Another factor for NDV susceptibility in MEF was apparently the downregulation of important interferon-related genes such as RIG-I, IRF7 or IFN-β. The expression of these molecules might have decreased because cells with a low expression of interferon-related proteins are less sensitive to anti-growth effects of IFN and have therefore a higher proliferation rate and a selective advantage in *in vitro* cell culture. Nevertheless, when the IFNAR knock out MEF cells were compared with the wildtype there was a strong increase in NDV replication and a complete abrogation of the responsiveness to IFN-α4. This indicated that the defects acquired in the IFN response in wildtype MEF were probably not directly concerning the IFNAR but were rather related to other components of the IFN system. Interestingly, the IFNAR knock out had almost no influence on the expression of interferon-related genes (figure 4-41), possibly because these genes were already expressed at a relatively low level in the wildtype MEF.

The data suggest a crucial role of the IFNAR in the defence against NDV infection. The importance of the IFNAR in antiviral responses has also been shown for other viruses. IFNAR knock out mice proved to be highly susceptible to infection by SFV and EMCV (Hwang *et al.*, 1995) and to infection by VSV or vaccinia virus (van den Broek *et al.*, 1995). However, the IFNAR seems not to be essential in the defence against MMTV infections (Maillard *et al.*, 1998). The IFNAR represents a bottleneck in the type I interferon signalling required to induce antiviral responses because it is the only receptor for type I interferons. It is therefore an attractive target for virus interference. Vaccinia virus, for example, expresses a soluble IFN-α/β receptor that binds to the cell surface and protects cells from the antiviral effects of IFN (Alcami *et al.*, 2000). Measles virus suppresses IFN-α signalling by the suppression of Jak1 phosphorylation and association of viral accessory proteins C and V with the IFNAR complex (Yokota *et al.*, 2003) and the V protein of mumps virus induces the dissociation of STAT-1 from the IFNAR complex (Kubota *et al.*, 2002). The NDV V protein can also inhibit the IFN response (Huang *et al.*, 2003), but its IFN antagonist activity is species specific and in contrast to avian cells it cannot prevent the induction of a strong interferon response in mammalian cells (Park *et al.*, 2003).

The mechanism that leads to the dramatic increase in NDV susceptibility in the absence of the IFNAR is likely the abrogation of type I interferon signalling, the main road via which
antiviral responses are induced. There is also no more secretion of IFN-α/β without the IFNAR (figure 4-34), demonstrating the crucial importance of positive feedback signalling for the induction of IFNs. In the IFNAR knock outs it could also nicely be seen that type I IFNs are essential for the induction of IRF7 and IFN-β but not for RIG-I or IRF3.

There are indications that defects in the IFNAR signalling are involved in tumourigenesis and present a selective advantage for tumour cells. In mice lacking a functional IFNAR tumour development and ensuing mortality were enhanced, indicating that endogenous IFN-alpha/beta production is a mediator of natural immunity against tumour development (Picaud et al., 2002). The IFNAR was found to be expressed on epithelial tumours but was missing in lymphomas, sarcomas and endocrine tumours (Navarro et al., 1996), suggesting an involvement of this receptor in tumourigenesis of the latter cancer types. In addition the transfection of tumour cells with a type I IFN receptor chain increased the rate of apoptosis and sensitivity to the antiproliferative effects of IFNs (Wagner et al., 2004). In melanoma cells defects in the IFNAR JAK-STAT pathway could have led to cancer progression through loss of growth-restraining functions of IFN (Pansky et al., 2000).

5.3.5 Antiviral effector molecules

Among the hundreds of genes that are induced by IFNs there are genes that directly interfere with viral infection. These antiviral effector molecules usually inhibit crucial steps of the viral replication inside a cell such as RNA translation or the assembly of viral particles. In this thesis the expression of the three antiviral effector molecules PKR, OAS1a and Mx1 has been analysed in tumour and normal cells to find out whether there are indications that these molecules are involved in the generation of differences in NDV susceptibility.

The basal expression of all three antiviral effector molecules was found to be higher in normal than in macrophage-like tumour cells and correlated negatively with NDV susceptibility. This was also true for the NDV-inducible expression of OAS1a and Mx1, but for PKR one tumour cell line showed a higher inducible expression than the primary macrophages. These results suggest that the differences in NDV infection in normal and tumour cells could be due to the different expression of the three analysed molecules. The highest expression of the PKR in a more resistant macrophage-like tumour cell line could be one reason for the relatively high NDV resistance of these cells compared to other tumour cells. Mx1 was induced about 10-
fold more than PKR and OAS1a, pointing to a higher sensitivity of this gene to IFN-induced transcription factors. This could be explained, for example, by a different promoter organisation. However, it is difficult to generalise that high Mx1 expression is of special significance for NDV resistance.

The importance of Mx proteins in the defence against viral infections was first established when the increased susceptibility of inbred mice against influenza and influenza-like viruses could be related to defects in the Mx1 gene (Staeheli et al., 1988). Mx proteins could be shown to be crucial for the defence against orthomyxoviruses or bunyaviruses (Haller et al., 1998), but they can also inhibit paramyxovirus infections (Schneider-Schaulies et al., 1994).

The antiviral effects of PKR have been well-studied. PKR inhibits infection by many viruses. It is therefore a common target for interference by many different viruses. HCV, for example, inhibits PKR via its NS5A and its E2 protein (Gale et al., 1998; Taylor et al., 1999) and HSV suppresses PKR activation (Peters et al., 2002) or reverses the phosphorylation of the eIF-2α by PKR, thereby precluding the shut-off of protein synthesis (He et al., 1997). Furthermore, the influenza A virus protein NS1 binds to PKR and prevents its activation (Dauber et al., 2006; Li et al., 2006) and the reovirus sigma3 protein blocks the activation of PKR (Yue and Shatkin, 1997). The role of PKR for paramyxovirus infection is less clear, but it could be shown that PKR expression upon NDV infection correlates negatively with NDV susceptibility of normal and tumour cells (Fiola et al., 2006).

The OAS family proteins have also been demonstrated to possess broad antiviral activities and some viruses target these proteins for inhibition. The influenza A virus, for example, binds viral RNA by its NS1 protein to inhibit the OAS/RNase L pathway (Min and Krug, 2006) and cellular OAS is inhibited by the herpes simplex virus type 1 Us11 protein (Sanchez and Mohr, 2007). During NDV infection the expression of OAS did not correlate with NDV susceptibility in malignant and non-malignant cells in contrast to PKR (Fiola et al., 2006).

The above data suggest an important role of PKR, OAS1a and Mx1 in viral defences. Therefore their different expression in tumour and normal cells could in part explain differences in NDV susceptibility. The possible involvement of the three analysed antiviral effector molecules in tumorigenesis has not been addressed thoroughly. An involvement can be expected, for example, because of pro-apoptotic actions which are known for PKR (Gil
and Esteban, 2000; Lee and Esteban, 1994) or OAS1a (Castelli et al., 1998; Zhou et al., 1998). Some studies have implicated the involvement of PKR in the development of chronic lymphocytic leukemia (Hii et al., 2004) and other cancer types (Jagus et al., 1999). PKR is switched off in tumour cells due to ras transformation (Mundschau and Faller, 1995) and defects in translational regulation renders tumour cells insensitive to PKR action (Balachandran and Barber, 2004). In addition several tumour suppressors such as MDA7 regulate PKR to induce apoptosis in tumour cells (Pataer et al., 2002) and oncogenes repress PKR action such as the NPM oncogene in Karpas 299 tumour cells (Friedrich et al., 2005). RNase L, which is activated by OAS, has been implicated in the pathology of prostate and colorectal cancer (Bisbal and Silverman, 2007).

There could be other factors involved in the generation of differences in NDV susceptibility between normal and tumour cells that were not analysed in this study. The existence of such factors can be deduced from the finding that cells from so-called triple knock-out mice lacking PKR, RNaseL and Mx still exhibit a limited IFN-induced antiviral state (Zhou et al., 1999).

### 5.3.6 The replication-independent induction of antiviral responses by NDV

A main mechanism for the induction of antiviral responses in cells infected by NDV starts with recognition of viral RNAs in the cytosol by receptors such as RIG-I. After the recognition of the RNAs a signal is transmitted downstream and leads ultimately to the induction of IFNs and IFN-related genes. However, in the analysis of NDV-induced gene expression in macrophages and macrophage-like tumour cells in this thesis there was a characteristic peak in expression after 8 hours when UV-inactivated NDV was used. This inactivated NDV did not replicate any more and therefore no viral RNA was generated in the cytosol. The peak in expression could only be observed in primary macrophages and had the same magnitude as the gene expression induced by live virus. After reaching the peak the expression usually decreased quickly for cells treated with UV-inactivated NDV and levelled out somewhat higher than the basal level. In contrast the infection with live NDV usually led to a sustained increase in gene expression.

These data strongly suggest another mechanism for the induction of IFN by NDV independent
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of viral RNA. The only viral RNA in the treatments with inactivated NDV could come from the viral capsid. It is hard to imagine that this alone could induce gene expression almost as well as the large amounts of viral RNA that are generated during infection with live NDV. It is conceivable that some viral proteins such as the membrane proteins F or HN interact with pattern recognition receptors on the cell surface such as TLRs to induce a response. This hypothesis is supported by the fact that the NDV HN protein could induce the production of IFN-α in human PBMCs while the F protein did not induce IFNs (Fournier et al., 2003; Zeng et al., 2002a). It has been suggested that this induction of IFN-α is based on the interaction of the viral HN molecule with cellular sialic acid expressing receptors (Zeng et al., 2002b). These could possibly be coupled to TLRs.

Nucleic acid-independent ways of virus recognition are also known for other viruses. In most of these cases viral proteins are recognised by TLR2 and TLR4. For example, the hemagglutinin protein of wild-type measles virus activates TLR2 signalling (Bieback et al., 2002) and human cytomegalovirus activates inflammatory cytokine responses via CD14 and TLR2 (Boehme et al., 2006; Compton et al., 2003). In addition TLR4 and again CD14 respond to the fusion protein of RSV to mediate antiviral responses (Kurt-Jones et al., 2000) and MMTV activates B cells via interaction with TLR4 (Rassa et al., 2002).

Tumour cells did not respond to inactivated NDV in contrast to normal cells. Limitations of malignant cells to use replication-independent ways of NDV recognition have been observed before (Fiola et al., 2006; Washburn and Schirrmacher, 2002). This could be another reason why tumour cells are more susceptible to NDV infection than normal cells.

5.4 The correlation between interferon-related gene expression and NDV resistance

In most instances NDV replicates much better in tumour than in normal cells. However, there are deviations from this rule. On the one hand some tumour cells are almost resistant to NDV infection such as the CT26p colon carcinoma cell line (figure 4-48). On the other hand some normal cells are exceptionally susceptible to NDV infection such as the IFNAR knock out macrophages. CT26p cells showed a basal expression of some interferon-related genes that was unusually high for tumour cells and the IFNAR knock outs only weakly expressed these genes compared to other normal cells. Thus, the expression of interferon-related antiviral
genes in cells can strongly influence susceptibility to infection by NDV.

The analysis of about a dozen normal and tumour cells revealed a strong negative correlation between the expression of RIG-I, IRF3, IRF7 and in part of IFN-β and the susceptibility to NDV (figure 4-43 – 4-46). When NDV replication was plotted against the basal and inducible gene expression level of normal and tumour cells two clusters could be distinguished. The correlation coefficient R was between -0.6 and -0.9. In general a correlation coefficient that is above 0.5 or below -0.5 is regarded as a strong indication of a connection between two variables (oral communication, Axel Benner, Central Unit Biostatistics, DKFZ Heidelberg). A correlation of two variables is not necessarily a proof of a causal relation, but because the antiviral effects of the analysed molecules are known the correlation strongly indicates a functional role of the molecules for NDV resistance. In addition the correlation can also explain the exceptions mentioned above.

In summary it can be concluded that defects in the basal or inducible expression of interferon-related genes makes cells susceptible to infection by NDV. In principal such defects can be acquired by any cell type but because they confer survival and growth advantages they usually develop in tumour cells. This is a main reason for the high susceptibility of most tumour cells to NDV infection. The above conclusion also predicts differences in NDV susceptibility in tumour cells depending on the severity of the acquired defects in the interferon system. This is a new finding that has implications for the use of viruses in cancer treatment that will be discussed in the next chapter.

5.5 Implications for the use of NDV in tumour therapy

NDV is not pathogenic in humans and replicates substantially better in tumour than in normal cells. These two properties make NDV a promising candidate for the application in cancer therapy. There are two major therapeutical approaches: active-specific immunotherapy (ASI), i.e. the anti-tumour stimulation of the immune system with oncolysates or whole cell vaccines, and oncolytic virus therapy with lytic NDV strains that directly kill tumour cells. For both approaches clinical studies have been undertaken that demonstrate the good tolerability and apparent anti-tumour efficacy.

In this thesis it was confirmed that NDV replicates selectively in tumour cells. In addition a
strong induction of IFNs and interferon-related genes could be observed in normal cells upon NDV infection. This underlines the role of NDV in the stimulation of the immune system that is crucial for ASI involving NDV but that can also be a beneficial side-effect for oncolytic virus therapy.

The results of this thesis have additional implications for the use of NDV in tumour therapy. It could be shown that there can be a great variability in NDV susceptibility of tumour cells and that some tumour cells are almost as resistant to NDV infection as normal cells. Even with tumour cell lines of the same origin, but maintained in different laboratories, there were tremendous differences in NDV susceptibility as observed with the colon carcinoma cell line CT26. Furthermore it could be shown that the expression of interferon-related genes strongly correlates with NDV susceptibility. This correlation could also explain the exceptional resistance to NDV infection of certain tumour cell lines.

An important finding with implications for the use of NDV in cancer treatment was the strong correlation of the basal expression of certain antiviral genes with NDV resistance. If this correlation could be reproduced in human cells, this knowledge would enable a clinician to pretest the tumour of the patient to evaluate its suitability for NDV virotherapy. A small tumour sample would be sufficient for RNA extraction and the subsequent expression analysis of interferon-related genes such as RIG-I or IRF7. By this proceeding oncolytic NDV therapy would be used preferentially in those cases for which a high susceptibility of the tumour would be predicted. For the tumours that show a relatively high expression of the analysed genes and that would therefore likely be resistant to NDV other therapeutical approaches had to be chosen.

The analysis of basal gene expression to predict the susceptibility of a tumour for NDV infection has important advantages over other methods. An alternative would only be the preparation of living tumour material and its subsequent infection with NDV. This proceeding would have several disadvantages. The isolation, culture and infection of tumour material would be more laborious and time consuming than the immediate RNA extraction and PCR analysis. In addition, more tumour material of a better quality would be needed for the latter approach. A possible confounding factor for the analysis of the basal gene expression of tumour tissue could be non-malignant cells such as stromal cells that can also be found in a tumour. This point could be tested, for example, in a mouse model in which the tumour cells
would be injected into mice to form tumours before the RNA of these tumours would be prepared and used for a gene expression analysis. By a comparison of *in vitro* and *in vivo* results one could evaluate if the confounding of the results by normal cells is strong. If the latter is the case the problem could be circumvented by analysing areas from the tumour tissue that contain only tumour cells. These areas could be selected, for example, by a pathologist in a tissue slice. Only little tissue material would be necessary for a PCR analysis.

The general approach of analysing a cancer patient and its special tumour type before the selection of a treatment protocol is becoming more and more widespread to improve treatment efficacy (Gasparini *et al.*, 2006). It is likely that the expression of certain antiviral genes strongly correlates with the susceptibility to the infection with other viruses than NDV. Hence the analysis of gene expression to select tumours that are especially suitable for virotherapy might also be extended to other viruses and could improve therapy efficacy.

The use of oncolytic viruses in cancer therapy is a rapidly developing field and for many viruses clinical trials have been carried out. Table 5-1 gives an overview over the oncolytic viruses that have been tested in clinical studies:

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mechanism of tumour targeting</th>
<th>Phase of development</th>
<th>Results</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>Targets to tumour antigens; conditionally replicating</td>
<td>Phase III conducted; approval for cancer treatment in China</td>
<td>Recombinant H101 in combination with cisplatin and 5-FU showed efficacy in patients with squamous cell cancer of head and neck or oesophagus after intratumoural injection</td>
<td>1, 2</td>
</tr>
<tr>
<td>Reovirus</td>
<td>Selectively infects ras-transformed cells</td>
<td>Phase I conducted</td>
<td>Systemic treatment well tolerated in patients with metastatic disease, observation of virus-induced tumour necrosis associated with intratumoural viral replication</td>
<td>3</td>
</tr>
<tr>
<td>Herpes simplex virus 1</td>
<td>Only replicates in tumour cells</td>
<td>Phase I conducted</td>
<td>G207 and HSV1716 were found to be well tolerated when given intratumourally in patients with glioma</td>
<td>4, 5</td>
</tr>
<tr>
<td>Newcastle disease virus</td>
<td>Selectively replicates in interferon-defective cells</td>
<td>Phase I/II conducted</td>
<td>Systemic administration of PV701 and NDV-HUJ was found to be well tolerated and anti-tumour responses was found in some patients</td>
<td>6, 7</td>
</tr>
</tbody>
</table>
Discussion

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mechanism of tumour targeting</th>
<th>Phase of development</th>
<th>Results</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinia virus</td>
<td>Gains access to tumour by vascular leakiness</td>
<td>Phase I conducted</td>
<td>Recombinant vaccinia was found to be well tolerated in phase I clinical trials when given intratumourally into melanomas or systemically for pancreatic cancer or various advanced tumours</td>
<td>8, 9, 10</td>
</tr>
<tr>
<td>Coxsackievirus</td>
<td>Selectively infects tumour cells that overexpress DAF</td>
<td>Phase I conducted</td>
<td>Coxsackievirus A21 was found to be well tolerated when administered by intratumoural injection in patients with melanoma</td>
<td>11</td>
</tr>
<tr>
<td>Measles virus</td>
<td>Virus re-targeting to tumour antigens; overexpression of virus receptor (CD46) on some tumour cells</td>
<td>Phase I ongoing</td>
<td>Intraperitoneal treatment was well tolerated in patients with ovarian cancer</td>
<td>11</td>
</tr>
</tbody>
</table>

Table 5-1: Clinical development of oncolytic viral vectors.


Oncolytic viruses are promising tools in the treatment of cancer. They have the potential to amplify their anti-tumour effect by the generation of virus progeny and they can reach disseminated tumour cells in the body when given systemically. In addition they can be genetically modified to improve tumour selectivity, tumour killing or the stimulation of immune cells. However, there are problems in the clinical application of viruses that have to be addressed such as tumour targeting, intratumoural dissemination, immune clearance or safety issues. Regarding the latter point NDV is an ideal therapeutic because it is naturally tumour selective and does not cause disease in humans. Furthermore NDV replication takes place in the cytosol of the host cell and there is no danger of malignant transformation by the integration of parts of the virus into the host genome. NDV has also a high immunostimulatory potential and induces a strong interferon response as could be shown in this and other studies (Washburn and Schirrmacher, 2002). The stimulation of the immune system by NDV is important for immunotherapy but can also be helpful for the oncolytic approach to achieve the establishment of post-oncolytic protective anti-tumour immunity.

It remains to be seen if in future studies the good safety profile of NDV can be complemented with an improved oncolytic efficacy. If so, cancer therapy with NDV may become a widely
available therapeutic tool that can be used in a multi-modal approach together with standard therapies such as radio- or chemotherapy to achieve the highest possible anti-cancer effect.
References


References


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References


References


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189. Sato, M., Suemori, H., Hata, N., Asagiri, M., Ogasawara, K., Nakao, K., Nakaya, T.,


243. Yeow, W.S., Au, W.C., Juang, Y.T., Fields, C.D., Dent, C.L., Gewert, D.R. and


7 Appendix

7.1 Systemic in vivo application of NDV

7.1.1 Biodistribution of NDV after systemic application

The data of this chapter has already been published in Bian et al. (2006).

NDV can be applied systemically as an oncolytic virus therapeutic to target cancer cells (see for example Laurie et al., 2006). In order to investigate the biodistribution kinetics of native NDV, the M gene expression was followed in different mouse organs after systemic application of NDV Italien. This velogenic NDV strain was used because it is more virulent and has therefore a higher oncolytic potential than the lentogenic NDV strains employed so far in this thesis. In addition NDV Italien replicates multicyclically and generates viable offspring that can reinfect tumour cells in the body.

Figure 7-1 shows that M gene expression could be detected in all the analysed organs after systemic application of NDV Italien. The M gene level declined in all organs in the course of the treatment. A high M gene expression could be found in lung, blood, liver and spleen. In the lung and in the spleen M gene expression could still be detected after 14 days. In the liver M gene expression lasted four days and in the blood no M gene expression could be detected later than 12 hours. In the kidney the M gene expression was low and lasted until day 2, while in the thymus a low M gene signal could only be detected at 12 hours.
The biodistribution kinetics demonstrated that most of the M gene expression could be found in the lung. The lung is well supplied with blood and it was the second organ after the blood itself which was reached after NDV had entered the body via the tail vein and had then passed the heart. The blood showed high M gene levels because this is the organ into which NDV had been injected. However, NDV appeared to be cleaned quickly from the blood, probably by binding to endothelial cells in the network of blood vessels that supplied other organs and tissues in the body or by active removal by the immune system. Besides the lung, the liver and the spleen are also well supplied by blood vessels, which could explain the relatively high amounts of virus found there.

### 7.1.2 Biodistribution after systemic application of encapsulated NDV

NDV binds to sialic acid containing receptors that are present on most mammalian cells. It can be expected that a large fraction of NDV does not reach tumour cells when applied systemically due to unspecific binding to all kinds of cells in the body. This unspecific binding could be demonstrated in the preceding chapter. One approach to improve the targeting of NDV to tumour cells is to encapsulate the virus in liposomes. The rationale is that encapsulated NDV will be prevented from unspecific binding to, for example, erythrocytes and could therefore circulate for a longer time in the blood system. The targeting could be

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**Figure 7-1: Biodistribution of NDV Italien in mice.**

1500 HU NDV Italien was injected i.v. into DBA/2 mice. Different organs (lung, liver, spleen, kidney, thymus and blood) were collected at different time points (2 mice for each analysis) after virus administration. Total RNA was extracted and the expression level of the NDV M gene was determined by real-time RT-PCR with the comparative C\textsubscript{T} method using β-actin for normalisation. The results are represented as the mean of three measurements. Bars indicate standard deviation.
further improved because the liposomes might be expected to leave the blood vessels of tumour tissues more readily than that of healthy, normal tissues, since the endothelium of blood vessels in tumours is thought to be more leaky. In addition, the circulation time and thereby the chance of an encounter of NDV with a tumour cell could be improved by an increased protection against immunological clearance of encapsulated NDV.

The encapsulation of NDV Ulster was carried out with the help and in the laboratory of PD Dr. Ulrich Massing, Tumor Biology Center, Department of Clinical Research/Phospholipids, Freiburg, Germany. The virus particles were encapsulated in a mixture of egg phosphatidylcholine 3 (EPC3) / cholesterol (55/45 mol%, manufactured by Lipoid Germany). The virus was mixed with the lipoid and also with 100 % (w/w) glass beads and then the vials were placed into a mixer. The vials were mixed several times and in between they were cooled on ice.

As a first step Eb-M7 tumour cells that were used later on to generate tumours in mice were infected with native and encapsulated virus to ascertain if there were differences in the ability to infect these cells. As a measure for NDV Ulster infection the HN expression on the cell surface was determined by flow cytometry.

Figure 7-2 shows that after the infection of Eb-M7 cells with different amounts of native and encapsulated NDV Ulster the HN expression on the cell surface was comparable. Only when infected with 100 HU per 10^6 cells the HN signal was slightly lower for the encapsulated NDV.
After it had been confirmed that the encapsulation did not significantly reduce the ability of NDV to infect Eb-M7 cells, the biodistribution of encapsulated NDV Ulster after systemic application in mice was tested. In the mice Eb-M7 cells had been injected subcutaneously about one week before the virus was given.

Figure 7-3 demonstrates that the M gene expression at a similar level could be detected in the tumours for the native and for the encapsulated NDV 1, 6 and 36 hours after systemic application. At 24 hours M gene expression could only be detected for the native virus and at all other time points no M gene expression could be observed. The M gene level was highest at 6 and 24 hours and lower at 1 and 36 hours after virus application.

Encapsulated and native NDV Ulster was used to infect Eb-M7 cells with 10 HU per 10^6 cells. After 24 hours the HN expression of the cells was determined by flow cytometry after staining with a murine primary antibody against the viral surface protein HN and a secondary GaM PE-labelled antibody.

Figure 7-2: HN surface expression on Eb-M7 cells after infection with encapsulated or native NDV Ulster.
The results showed that the M gene expression was comparable for the native and the encapsulated virus. The M gene level rose between 1 and 6 hours because of the progressing virus replication probably related to more virus reaching the tumour. Surprisingly there was no detectable M gene expression at 12 hours and at 24 hours only in the mice treated with native NDV there was an NDV signal. This could either mean that NDV infection stopped after 12 hours and restarted again at 24 hours or was more likely related to variations because of the small sample size, because only one mouse was sacrificed per time point and only a part of the tumours was taken for RNA extraction.

At time points later than 36 hours no M gene could be observed in tumours any more, indicating that virus replication and reinfection of tumour cells could not be sustained. Possible reasons for this could have been the clearance of the virus by the immune system or limited viral spread in the tumour mass due to NDV resistant stromal cells or necrotic areas. Next lung and liver tissue was tested for viral replication to ascertain whether liposome encapsulation changed the amount of virus that reached these tissues after systemic application.

Figure 7-4 demonstrates that the encapsulation reduced the M gene signal after systemic application.
application of NDV Ulster compared to the native virus at all measured time points (part A). For both virus batches the M gene expression levels increased up to 12 hours after the virus application and then they declined again until 120 hours. The average M gene expression for the whole time course was 77 REU for the native NDV and 20 REU for the encapsulated NDV. In the liver samples (part B) the M gene expression levels were similar at most time points. Only at 12 hours the M gene level was higher in the mice treated with encapsulated NDV.

Figure 7-4: M gene expression in lung and liver tissue after i.v. application of encapsulated or native NDV Ulster.

DBA/2 mice were irradiated with 4.5 Gray at day -8 and 5*10⁶ Eb-M7 cells in 300 µL volume were injected subcutaneously at day -7. At day 0 2000 HU native or liposome-encapsulated NDV Ulster were injected into the tail vein of the mice. At the given time points after virus application one animal was sacrificed for each group and total RNA was extracted from the lung or liver, respectively. The expression level of the NDV M gene was determined by real-time RT-PCR with the comparative Cₜ method using β-actin for normalisation. The results are represented as the mean of three measurements. Bars indicate standard deviation.

Liposome encapsulation strongly reduced the amount of virus that reached the lung after systemic application, whereas it did not diminish the M gene expression in liver tissue. There seemed to be some factor that specifically limited the binding of encapsulated NDV to lung tissue.

In conclusion it could be said that liposome encapsulation of NDV proved to feasible without reducing the ability of the virus to infect cells. However, in the setting tested no improvement of the targeting of NDV to tumours could be observed.
Appendix

7.1.3 Tumour targeting of NDV with bispecific molecules

The data of this chapter has already been published in Bian et al. (2006).

Another approach to improve tumour targeting that had already been successfully employed by our research group was the use of bispecific adaptor proteins. These proteins block the native cell binding site of NDV and introduce a new binding site for tumour-associated targets (Bian et al., 2005a-c). In the following experiments the influence of the modification of NDV particles with bispecific molecules was analysed regarding side effects induced by systemic application. The bispecific protein introduced a binding site for the IL-2 receptor (IL-2R). The Eb-M7 tumour cells displayed a high expression of the IL-2R and were obtained by successive sorting. In addition to the side effects the targeting of modified NDV to tumour cells was also determined. In contrast to earlier studies with lentogenic NDV strains the velogenic NDV Italien was used for the analysis.

As a parameter for NDV-induced side effects the body weight was followed in the course of NDV treatment. The anti-tumour effects were determined by measuring the tumour volumes at different time points after NDV application.

Figure 7-5 shows that there was a significant difference in body weight variation on day 4 and 6 between the medium dose native NDV (NAT MED) and the medium dose modified NDV (MOD MED) group (part A). All groups treated with NDV displayed a reduction in the body weight in contrast to the PBS treated group. The reduction increased up to day 4 when it reached -4 – -9 %. On day 6 the body weight of the mice in the NDV groups had partly recovered and reached from -5 – 0 %. The tumour volume in the NDV treatment groups was always lower than in the PBS control group (part B). This difference was statistically significant on day 9. The tumour volumes in the NDV treatment groups were comparable at all time points.
The results demonstrated that the modification of NDV with bispecific molecules reduced the cytopathic effects in systemic therapy. At the same time it could be shown that NDV could be systemically applied to reduce the growth of a locally growing tumour and that the modification did not substantially diminish the anti-tumour effects. Therefore the modification protocol might be used to reduce side effects in future NDV tumour therapy approaches.

Figure 7-5: Anti-tumour effects and side effects after systemic NDV application.

Irradiated DBA/2 mice were s.c. inoculated with 5*10⁶ Eb-M7 (IL-2R⁺) cells. One day later, they received 500 HU of NDV Ulster i.v. as a means of desensitization. For therapy, the animals received three i.v. injections of different NDV Italien preparations at days 2, 3 and 4. PBS was added to the virus preparation to a final volume of 300 µL. Modified NDV was obtained by incubation of 2000 HU of the virus with 250 µg αHN-IL-2 in a final volume of 300 µL for 1 hour on ice. (A) Mouse body weights were measured every other day. The mean body weight change was calculated as the percentage of the body weight ± standard error of the mean (SEM) among a cohort of 10 mice in each group. NAT MED, native NDV Italien 1000 HU per injection; MOD MED, modified NDV Italien/αHN-IL-2 1000 HU per injection; MOD HIGH, modified NDV Italien/αHN-IL-2 2000 HU per injection. The P-values were calculated: *P<0.0006, **P=0.03. (B) The tumor volumes were determined in 10 mice at the time-points indicated. The P-values were corrected for multiple testing. *P<0.05.
## Appendix

### 7.2 Description of the oligonucleotides used for quantitative real-time PCR

#### 7.2.1 Oligonucleotides

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Gene</th>
<th>Sequence 5´ - 3´</th>
<th>Oligo (bp)</th>
<th>Amplicon (bp)</th>
<th>Origin</th>
</tr>
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<tbody>
<tr>
<td>bactin_1</td>
<td>Mouse</td>
<td>β-Actin</td>
<td>ACGGCCAGGTGATCATGTA</td>
<td>21</td>
<td>84</td>
<td>Bian et al., Int J Oncol. 2006 Dec;29(6):1359-69.</td>
</tr>
<tr>
<td>IFNb_1</td>
<td>Mouse</td>
<td>Interferon β</td>
<td>AGCTCCAAGAAAGGACCAAT</td>
<td>22</td>
<td>82</td>
<td>Doyle et al., Immunity. 2002 Sep;17(3):251-63.</td>
</tr>
<tr>
<td>IFNb_2</td>
<td>Mouse</td>
<td>Interferon β</td>
<td>GCCCTGTAGGTGAGGGTGATCT</td>
<td>22</td>
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<td>Doyle et al., Immunity. 2002 Sep;17(3):251-63.</td>
</tr>
<tr>
<td>IRF3_7</td>
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<td>Interferon regulatory factor 3</td>
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<td>IRF3_8</td>
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<td>Interferon regulatory factor 3</td>
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<td>IRF7_1</td>
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<td>20</td>
<td>148</td>
<td>Prakash et al., J Biol Chem.</td>
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<tr>
<td>Name</td>
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<td>Gene</td>
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<tr>
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<td>Melanoma differentiation-associated gene-5</td>
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<td>20</td>
<td></td>
<td>NM_027835</td>
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<tr>
<td>Mx_1</td>
<td>Mouse</td>
<td>Myxovirus (influenza virus) resistance 1</td>
<td>AAACCTGATCCGACCTCC</td>
<td>23</td>
<td>83</td>
<td>Doyle et al., Immunity. 2002 Sep;17(3):251-63.</td>
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<tr>
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<td>24</td>
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<td>Doyle et al., Immunity. 2002 Sep;17(3):251-63.</td>
</tr>
<tr>
<td>OAS1a_5</td>
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<td>OAS1a_6</td>
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<td>2'-5' Oligoadenylate synthetase 1A</td>
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### Oligonucleotide probes

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<th>Oligo (bp)</th>
<th>Modification</th>
<th>Origin</th>
</tr>
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<td>b-actin</td>
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<td>β-Actin</td>
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<td>21</td>
<td>5´ 6-FAM 3´ TAMRA</td>
<td>Jens Derbinski, DKFZ Heidelberg; oral communication</td>
</tr>
</tbody>
</table>

The primer pairs were either designed with the Primer3 software (Rozen and Skaletsky, 2000; web software provided by the Whitehead Institute for Biomedical Research, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) based on the sequence of the mentioned mRNA locus or were taken from the quoted publications.

### 7.2.2 Oligonucleotide probes
7 Appendix