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IMMUNOLOGICAL BOOSTING AND PERSONALIZATION OF ONCOLYTIC VIROTHERAPIES FOR CANCER TREATMENT

Mari Hirvinen

ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Pharmacy of the University of Helsinki, in Biocentrum 2, Auditorium 1041, on 1st of April 2016, at 12 noon.

Helsinki 2016

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Dissertationes Scholae Doctoralis Ad Sanitatem Investigandam Universitatis Helsinkiensis

No. 15/2016
ISBN 978-951-51-1935-3 (paperback)
ISBN 978-951-51-1936-0 (PDF)

http://ethesis.helsinki.fi
ISSN 2342-3161 (print)
ISSN 2342-317X (online)

Unigrafia
Helsinki 2016

“It’s far more important to know what person the disease has

than what disease the person has”

– Hippocrates

(c. 470 – 370 BC)

ACKNOWLEDGEMENTS

This thesis work was carried out at the University of Helsinki, mainly in the ImmunoViroTherapy Lab (Centre for Drug Research, Division of Pharmaceutical Biosciences, Faculty of Pharmacy in Viikki) and partly in the Cancer Gene Therapy Group (formerly part of the Molecular Cancer Biology Program in Biomedicum, currently affiliated with the Faculty of Medicine, University of Helsinki and Helsinki University Hospital Comprehensive Cancer Center). My deepest gratitude goes out to all current and former members of both of these groups in which I felt like I was part of a large family.

I wish to thank the following persons for providing excellent research facilities and educational opportunities: Professor Jouni Hirvonen, the dean of the Faculty of Pharmacy, Professor Heikki Vuorela, the head of Centre for Drug Research and Professor Arto Urtti, the former head of CDR and Professor Marjo Yliperttula, the head of the Division of Pharmaceutical Biosciences. I also would like to thank Professor Hannu Sariola and Docent Eeva Sievi, the director and the coordinator of the DSHealth graduate school, respectively, and Professor Heikki Ruskoaho and Doctor Ilkka Reenilä, the director and the coordinator of the Doctoral Programme in Drug Research (DPDR).

I would like to express my sincere appreciation to esteemed Professor Maria Croyle for accepting the role of the thesis opponent and to Professor Arto Urtti, the custos of the defence for making my dissertation possible. I wish to thank Professor Ari Hinkkanen and Docent Veijo Hukkanen for the professional review of my thesis. I would also like to thank my thesis committee members Prof. Pia Vuorela, Doc. Pia Siljander and Doc. Susanna Fagerholm for valuable comments and guidance regarding my thesis studies.

I am most grateful to my supervisor Assistant Professor Vincenzo Cerullo. Vince, you have always supported me in everything and offered me opportunities to grow as a researcher and, more importantly, as a person, I really appreciate that. You have been very flexible and you have trusted me and let me work independently. I admire your “scientific mind”, the source of endless ideas, and especially your optimism! Thanks also for the fun group days at Strömsö. I am also really grateful to my co-supervisor Professor Akseli Hemminki, the leader of Cancer Gene Therapy Group. Aksu, first of all thank you for accepting me to join your group as a master student, without that opportunity I would most probably have never had chance to explore this interesting world of oncolytic viruses and cancer immunotherapy! You gave me the rare and priceless opportunity to learn and closely follow the development of cancer drugs from bench to bedside. Working in CGTG taught me such a wide repertoire of techniques, methods, manners as well as other valuable tips and tricks for my future for which I am truly thankful. For both of you Vince and Aksu: I truly appreciate your desire to speed up the development of novel cancer therapeutics and the willingness to push forward in a field that has so many obstacles.

I want to express many thanks to all former and present members of the IVT lab, especially to Cris, Lukasz and Mariangela. It has been such a fun time working with you and I really appreciate all your help during the years. I also want to thank all the people in CDR for the help and friendship. I also want to thank all the past and present members of CGTG. I

learned so much and had such a wonderful time while working in the group. Special thanks go to Siri, Suvi, Simona and Mikko who made me feel like I would have still been part of the CGTG group even years after moving to Viikki! Special thanks go also to all the precious technicians! And to Minna.

I also want to thank all the co-authors and collaborators who helped obtain the results of this thesis work.

Last but not least I wish to thank my family and friends for all the support and encouragement throughout the years. Thanks for always believing in me! Especially I would like to thank my beloved Ate for withstanding all the ups and downs during the years, and for all your help to push me forward and keep me going.

My PhD work and conference trips were financially supported by the University of Helsinki, Finnish Cultural Foundation, Emil Aaltonen Foundation, Biomedicum Helsinki Foundation, Finnish Cancer Organizations, Waldemar von Frenckels Foundation, Päivikki and Sakari Sohlberg Foundation, Maud Kuistila Memorial Foundation, Foundation for Research on Viral Diseases and the American Society of Gene and Cell Therapy.

Helsinki, February 2016

Mari Hirvinen

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications that are referred to in the text by Roman numerals.

I. HIRVINEN M, Rajecki M, Kapanen M, Parviainen S, Rouvinen-Lagerström N, Diaconu I, Nokisalmi P, Tenhunen M, Hemminki A and Cerullo V. *Immunological effects of a TNF-alpha armed oncolytic adenovirus.* Human Gene Therapy, 26, 134-44, 2015.

II. HIRVINEN M, Capasso C, Guse K, Garofalo M, Vitale A, Ahonen M, Kuryk L, Vähä-Koskela M, Hemminki A, Fortino V, Greco D, Cerullo V. *Expression of DAI by an oncolytic vaccinia virus boosts the immunogenicity of the virus and enhances anti-tumor immunity.* Molecular Therapy – Oncolytics, accepted for publication, 2016.

III. HIRVINEN M, Heiskanen R, Oksanen M, Pesonen S, Liikanen I, Joensuu T, Kanerva A, Cerullo V and Hemminki A. *Fc-gamma receptor polymorphisms as predictive and prognostic factors in patients receiving oncolytic adenovirus treatment*. Journal of Translational Medicine, 21;11:193, 2013.

IV. Capasso C, HIRVINEN M, Garofalo M, Romaniuk D, Kuryk L, Sarvela T, Vitale A, Antopolsky M, Magarkar A, Viitala T, Suutari T, Bunker A, Yliperttula M, Urtti A, Cerullo V. *Oncolytic adenoviruses coated with MHC-I tumor epitopes increase the anti-tumor immunity and efficacy against melanoma.* OncoImmunology, published online October 29, 2015. http://tandfonline.com/doi/full/10.1080/2162402X.2015.1105429

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PERSONAL CONTRIBUTION

Study I: I carried out the design of the studies, acquisition, analysis and interpretation of data, statistical analyses and drafted the manuscript with the help of supervisors and other co-authors. The virus was cloned by Dr. Rajecki.

Study II: The DAI-VV interaction studies were performed by Dr. Guse. The viruses were designed and cloned by Dr. Guse. Analyses of the gene expression data were performed by Drs. Cerullo and Greco. I carried out the design of the rest of the studies, acquisition, analysis and interpretation of data, statistical analyses and drafted the manuscript with the help of supervisors and other co-authors.

Study III: I carried out the design of the studies, acquisition, analysis and interpretation of data, statistical analyses and drafted the manuscript with the help of supervisors and other co-authors.

Study IV: I carried out the design and acquisition of animal studies and helped draft the manuscript. I am also one of the inventors of the PeptiCRAd platform.

ABBREVIATIONS

Ab	Antibody
Ad	Adenovirus
Ad3	Adenovirus serotype 3
Ad5	Adenovirus serotype 5
ADCC	Antibody-dependent cellular cytotoxicity
ADCVI	Antibody-dependent cell-mediated virus inhibition
AIM2	Absent in melanoma 2 –protein
ALP	Alkaline phosphatase
APC	Antigen presenting cell / Allophycocyanin
ATAP	Advanced Therapy Access Program
ATP	Adenosine triphosphate
bp	base pair
CAR	Coxsackie-adenovirus receptor
CAR T cell	Chimeric antigen receptor T cells
CBA	Cytometric bead array
CD	Cluster of differentiation
CD40L	CD40-ligand
CEV	Cell-associated enveloped virus
cGAS	Cyclic guanosine monophosphate-adenosine monophosphate synthase
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CP	Cyclophosphamide
CPE	Cytopathic effect
CR	Complete response
CT	Computed tomography
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
DAI	DNA-dependent activator of interferon-regulatory factors
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DMEM	Dulbecco´s modified eagle medium
dsDNA	Double-stranded DNA
DSG2	Desmoglein 2 protein
E1	Early region 1
EEV	Extracellular enveloped virus
EGFR	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked ImmunoSpot assay

EMA	European Medicines Agency
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FcγR	Fc gamma receptor
FDA	Food and Drug Administration
FIMEA	Finnish Medicines Agency
FITC	Flurescein Isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GM	Growth medium
GM-CSF	Granulocyte macrophage colony-stimulating factor
Gy	Gray, SI unit of absorbed radiation (J/kg)
HLA	Human leukocyte antigen
HMGB-1	High-mobility group box 1 protein
HSV	Herpes Simplex virus
HUCH	Helsinki University Central Hospital
ICC	Immunocytochemistry
ICD	Immunogenic cell death
IFI16	Gamma-interferon-inducible protein 16
IFN	Interferon
IL	Interleukin
IMV	Intracellular mature virus
i.p.	Intraperitoneal
IRF	Interferon regulatory factor
ISRCTN	International Standard Registered Clinical/soCial sTudy Number
i.t.	Intratumoral
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
ITR	Inverted terminal repeat
KKTK	Lysine-Lysine-Threonine-Lysine motif
i.v.	Intravenous
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
Luc	Luciferase
MAPK	Mitogen-activated protein kinase
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MRI	Magnetic resonance imaging
MTD	Maximum tolerated dose
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
MyD88	Myeloid differentiation primary response gene 88

Nab	Neutralizing antibody
NF-κB	Nuclear factor kappa-B
NK cell	Natural killer cell
OAd	Oncolytic adenovirus
OS	Overall survival
OV	Oncolytic virus
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death 1 protein
PE	Phycoerythrin
PEG	Polyethylene glycol
PET	Positron emission tomography
PFS	Progression free survival
pfu	Plaque-forming unit
PR	Partial response
PSA	Prostate-specific antigen
Rb	Retinoblastoma
RECIST	Response Evaluation Criteria in Solid Tumors
RGD	Arginine-Glycine-Aspartic acid motif
s.c.	Subcutaneous
SD	Stable disease
STING	Stimulator of interferon genes -protein
TAA	Tumor-associated antigen
$TCID_{50}$	50% tissue culture infective dose
TCR	T cell receptor
TERT	Telomerase reverse transcriptase
TGF-β	Transforming growth factor beta
TIL	Tumor infiltrating lymphocyte
TK	Thymidine kinase
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TRAF	TNF receptor associated factors
Treg	Regulatory T cell
TRIF	TIR-domain-containing adapter-inducing interferon-β
VGF	Vaccinia growth factor
VP	Virus particle
VV	Vaccinia virus
WHO	World Health Organization
wt	Wild type
XRT	Irradiation (X-ray therapy)

ABSTRACT

Cancer is the leading cause of death worldwide. It is estimated that the incidence rate is rising despite that in recent decades, the range of treatment options and drugs have expanded considerably. Often conventional cancer drugs are also associated with significant adverse event profiles from both on- and off-target effects. Thus, there is an urgent need for novel treatment options that are more efficient but at the same time safer and more specific. Oncolytic viruses (i.e. viruses that specifically kill cancerous cells) have shown a solid safety profile with only mild adverse events in several clinical trials, but still these treatments are often non-curative. Hence, the efficacy of the oncolytic virotherapies needs to be enhanced. Currently researchers are trying to improve the immunogenicity of the viruses to boost the activation of anti-tumor immune responses by, for example, arming the viruses with immunostimulatory molecules. Actually, oncolytic viruses are nowadays considered immunotherapies because of their ability to stimulate many arms of the host immune system to fight against cancer. These immunomodulatory viruses have shown promising efficacy in some trials, one oncolytic virus (talimogene laherparepvec (T-Vec), trade name Imlygic), was even approved for clinical use by the FDA (Food and Drug Administration) just recently in October 2015; also EMA (European Medicines Agency) recommended its approval and it will most probably be accepted for treatment soon in the EU as well. However, oncolytic viruses, like most other cancer drugs as well, have only been effective in a small group of patients. This is due to genetic and/or other variability between the treated patients and their tumors. Understanding the effects of individual differences in treatment responses has spurred the era of personalized medicine. Choosing only patients who most probably would respond to the treatments, choosing the correct dosing for each patient, or tailoring individually designed drugs for each case would enable safer, more cost-effective and more efficacious treatments.

The purpose of this thesis was to improve the efficacy of oncolytic virotherapies by A) boosting the immunogenicity of the viruses and B) by finding means to optimize the therapies by selecting a favorable patient population and by developing a method to tailor the drug individually for each patient.

We first considered whether the efficacy of oncolytic viruses could be enhanced by boosting their immunogenicity. An oncolytic adenovirus was modified to express human tumor necrosis factor alpha (hTNFα), which is a potent multifunctional cytokine. TNFα expression was hypothesized to improve the cytotoxic potency of the virus and its ability to activate and attract immune cells to the tumor site. We showed effective tumor cell killing by the Ad5/3-Δ24-hTNFα virus both *in vitro* and *in vivo*, which was associated with signs of immunogenic cell death. The virus also enhanced recruitment of adaptive immune cells to the infection site. We also studied the hTNFα-expressing virus in combination with radiotherapy and saw some enhanced tumor-growth control and hence potential for combining the Ad5/3-Δ24-hTNFα therapy with radiation. In another study we enhanced

the immunogenicity of an oncolytic vaccinia virus by modifying it to express a pattern recognition receptor, DNA-dependent activator of interferon-regulatory factors (DAI), which was shown to sense vaccinia virus DNA and to induce innate immune responses during virus infection. The DAI-producing oncolytic virus was tested *in vitro* and *in vivo* for anti-tumor efficacy and immunogenic potential. We showed that the virus induces expression of several genes involved in immune responses, and treatments with the DAI-virus showed improved cancer-killing efficacy and immunogenicity in murine and human melanoma models. This study suggests that expression of a self-recognizing receptor by vaccinia virus could be a novel way to improve the adjuvant potency of viral drugs and could have potential applications in vaccine design.

There is a great deal of variation in the response rates after oncolytic virotherapies between the patients, and there is a lack of markers that would help predict the patient cohorts who would benefit from the therapy. We screened over 200 cancer patients who had received oncolytic adenovirus therapies for two Fc gamma receptor (FcγR) polymorphisms to determine if these polymorphisms could serve as biomarkers for selection of suitable patients for clinical trials. We hypothesized that FcγRs, as important links between cellular and humoral immune responses, could have a role in defining the effectiveness of oncolytic virotherapy, which is nowadays known to greatly depend on the activity of the immune system. We therefore determined if polymorphic variation in these receptors would affect the responsiveness to the treatments. We observed a certain FcγR genotype combination (FcγRIIIa-VV + FcγRIIa-HR) to be predictive of poor overall survival after oncolytic adenovirus treatments. Additionally, we found some genotype combinations to be beneficial for treatments with viruses armed with immunostimulatory molecules: FcγRIIIa-FF + FcγRIIa-HR and FcγRIIIa-FF + FcγRIIa-RR combinations for treatments with GM-CSF-armed viruses and FcγRIIIa-FF + FcγRIIa-HH combination for CD40L-virus treatments. If confirmed in a larger and more controlled study, these genotype combinations could serve as exclusion or inclusion markers for the oncolytic adenovirus therapy.

Because each patient and each tumor is unique, the same drug is not effective for all cases. To tailor the oncolytic virotherapy for enhanced specificity for different tumors, we developed a novel platform to quickly and easily coat a virus with tumor-specific antigens (peptides) to improve the induction of cancer-specific immune responses. This peptide-coated conditionally replicating (i.e. oncolytic) adenovirus is called PeptiCRAd. Efficacy and immunogenic potency of the PeptiCRAd coated with known model peptides were shown in several *in vivo* models, both mouse and human melanomas. Our results suggest that administration of tumor-specific peptides on the surface of oncolytic viruses increases the anti-tumor efficacy compared to treatments with viruses or peptides alone. We think the PeptiCRAd platform could in the future be used as a carrier and adjuvant for patient-specific peptides to trigger anti-tumor immunity in a personalized manner.

REVIEW OF LITERATURE

1. Introduction

Cancer is a devastating disease and a major cause of death, causing over 8 million deaths a year worldwide (IARC-International Agency for Research on Cancer (WHO)). As the percentage of aging population is growing, the cancer incidence rates are rising regardless of the development of tools for earlier diagnosis. The treatment methods for cancer have also developed a great deal during the past decades - still there are some major problems that need to be addressed, like side effects, unspecificity, and resistance developed against the treatments. New, better targeted, more specific, safer and more efficient treatments are needed.

Oncolytic viruses, i.e. viruses that specifically kill tumor cells, are one potential treatment option for cancers, even advanced ones. There are several clinical trials to test oncolytic virotherapies for treating cancer (Sheridan 2015). Although this is a highly promising field, most trials still have failed to show sufficient efficacy. However, the first oncolytic virus, H101, was approved over ten years ago in China for treating head and neck cancer (Yu & Fang 2007). And just recently in October 2015, the first oncolytic virus, a herpes simplex virus encoding for GM-CSF (talimogene laherparepvec (T-Vec), trade name Imlygic, Amgen) was approved by the FDA for the treatment of recurrent melanoma (Andtbacka et al. 2015); this is a very encouraging step for the field.

Some of the most commonly used viruses in oncolytic virotherapies are adenoviruses and vaccinia viruses, which will be the main focus of this thesis. Both viruses are effective in infecting cells and fast in cell lysis. They can be easily modified to be even more efficient and immunogenic in order to enhance their tumor killing potency.

The role of the immune system in the progression and eradication of cancer is widely acknowledged nowadays and immunotherapy is a fast evolving and growing form of therapy for cancer. Also in the field of oncolytic virotherapies, in recent years, the common trend has turned towards "immunovirotherapies", in which viruses are used as adjuvants to boost the host immune responses against cancer (Coffin 2015, Lichty et al. 2014, Tong et al. 2011).

Even though the virus could be engineered to be as efficient as possible, the treatment most probably would not work for every patient. Thus, the concept of "personalized medicine" is spreading, and selection of the right patients for the treatments is generally agreed to be a way to reduce unnecessary use of drugs, to minimize side effects and save money (Rodriguez-Antona & Taron 2015, Fisher 2011, Heuckmann & Thomas 2015, Gasparini & Longo 2012). The recent innovations also aim to design drugs so that they can easily be customized for each patient and even for each tumor.

In the future, patient-tailored drugs, selecting the right patients who most probably respond to the treatments and combining different therapies will possibly offer better solutions to defeat cancer.

2. Challenges of cancer treatment

Cancers are a group of heterogeneous diseases in which cells begin to proliferate independently causing a mass of malformed cells – a tumor. Carcinogenesis, the formation of a tumor, is a multistep process involving various changes in the genetic material and the effect of the environment. Tumors are considered malignant when cells have gained an ability to evade the primary tumor to travel to other parts of the body and give rise to new secondary tumors called metastases (Leber & Efferth 2009).

Cancer is caused by multiple gain-of-function and loss-of-function mutations in a single cell, which becomes insensitive to growth controlling signals. Mutations can originate from any cell type of the body. Usually the mutations leading to the development of cancer take place in genes that normally code for proteins that regulate cell growth and proliferation (tumor suppressor genes and oncogenes) or DNA repair mechanisms (Osborne, Wilson & Tripathy 2004). In 2000, Hanahan and Weinberg proposed the six "hallmarks of cancer" (Hanahan & Weinberg 2000) that differentiate tumor cells from normal cells: i) Cancer cells stimulate their own growth, ii) they resist anti-growth signals, iii) they can evade apoptotic cell death, iv) they can multiply infinitely, v) they stimulate angiogenesis and vi) they become invasive and can spread to distant sites (Hanahan & Weinberg 2000). A few years ago, in 2011, they added four more traits characteristic to cancer cells, two of which recognize the importance of the immune system in the tumor progression and eradication: inflammation and immune evasion (Hanahan & Weinberg 2011). The immune system both prevents malformed cells from growing and erases them, but if some tumor cells have a chance to evade the immune system, they start evolving towards less immunogenic forms and eventually hide from the immune system. This development process of the tumor towards lower immunogenicity is called "immunoediting" (Dunn et al. 2002). In 2004 Dunn et al. divided the main events of immunoediting into "three Es": in the *E*limination phase, the immune system is still able to recognize and eliminate cancerous cells from the body, but in the *E*quilibrium phase the surviving cancer cells remain in a dynamic equilibrium with the immune system and the "strongest" tumor cells that can hide from the immune system are selected by Darwinian law. These strongest cancer cells can eventually grow in an immunologically intact environment; this last phase is called the *E*scape phase. At this stage it becomes very challenging to treat the cancer, and today there are many attempts to provoke the immunogenicity of the cancer and to wake up the host immune system to the "elimination phase" when the tumor cells will again be recognized and destroyed (Dunn, Old & Schreiber 2004).

These characteristics of cancer form the tumor microenvironment, a constantly evolving environment where new mutations arise and selections are made. It is a constant battle

between the body and a tumor. Immunotherapies have emerged as potential strategies to treat certain tumors that tend to overexpress some proteins important for tumor survival or that present tumor-specific antigens. Since tumors always originate from the body´s own cells, the immune system needs to find epitopes that are foreign for the tumor cell to be recognized as malfunctioning and to be killed. Sometimes mistakes in this identification process cause a problem, known as autoimmunity, in which the immune system starts to attack the body´s own healthy cells (Amos et al. 2011). Thus, it is very important that drugs designed to wake up the host immune system against cancer, e.g. peptide vaccines, contain only tumor-specific antigens, not self-antigens. Although immunotherapies have shown success in some cases, they are often quite limited, targeting only one or two pathways, which often is not enough to block the growth of the tumor. Therefore combining different therapeutic strategies of therapies would be more efficient.

Foreign antigens are loaded into MHC I molecules that are recognized by cytotoxic T cells (CTLs). Tumor cells, however, have often acquired a way to reduce the presentation of tumor antigens by down-regulating MHC I expression and function. Also the activity of T cells and antigen presenting cells in the tumors is usually low due to several mechanisms of the immunosuppressive microenvironment. Tumor cells secrete immunosuppressive cytokines, like transforming growth factor beta (TGF-β), which can recruit regulatory T cells (Tregs) and directly inhibit the activity of T cells. Tumors can also overexpress surface proteins, like CTLA-4 and PD-1 ligand that also inhibit T cell activation. Myeloid-derived suppressor cells (MDSCs) also constitute part of the immunosuppressive environment by inducing all the above-mentioned mechanisms. Tumor-associated macrophages with an M2 phenotype can as well promote immune evasion by secreting anti-inflammatory molecules, such as IL-10 and TGF- β. (Schlosser et al. 2014)

Heterogeneity is the major challenge for cancer therapies; each patient, each tumor and even each cell within the tumor is unique. Finding a cure that works in all cancer cases is not possible; thus finding a way to easily customize a drug for each case is the future challenge in the field.

3. Cancer therapies

3.1. Conventional therapies

Traditionally cancer is treated first with surgery, if possible, and thereafter by radiation and/or chemotherapy to remove the remaining cancerous cells from the body and to reduce the re-growth of the tumor or growth of metastases to other parts of the body. Often a combination of different therapies provides the best treatment response.

Surgery is a standard treatment for cancer after the diagnosis, but is sometimes limited by the anatomical location or extent of the tumor. Radiation therapy is normally used to eliminate the remaining cancerous cells after surgery, but also as a palliative treatment and for tumors that are inoperable. Radiation therapy can be divided into three main

categories: i) teletherapy, in which an external source of radiation is used (Pisansky 2006, McGovern & Mahajan 2012), ii) brachytherapy, in which the source of radiation is directly implanted into the tumor (Pisansky et al. 2008) and iii) systemic radioisotope therapy, in which radioisotopes are delivered orally or systemically into tumor sites by specific transporters (Porter, Ben-Josef & Davis 1994). The effects of radiotherapy are mediated through ionizing radiation that creates single- and double-strand breaks in the cellular DNA and it also creates free hydroxyl radicals (Russi, Raber-Durlacher & Sonis 2014, Kavanagh et al. 2013). Cells have several mechanisms to repair DNA breaks; nevertheless if the amount of radiation is strong enough, damages cannot be compensated and they eventually lead to cell cycle arrest and finally induction of cell death in the irradiated area. Thus, cancer eradication is dependent on the dose of irradiation (Kavanagh et al. 2013, Valicenti et al. 2000), although the risks for severe side effects increase with higher doses (Russi, Raber-Durlacher & Sonis 2014).

Chemotherapy is a widely used and often effective therapy that uses chemical substances to kill cancer cells. Chemotherapeutics can be divided into alkylating agents, antimetabolites, cytotoxic antibiotics, plant alkaloids, topoisomerase inhibitors and other anti-tumor agents. These drugs are cytotoxic agents that have an effect on cell division or DNA synthesis. Chemotherapeutic drugs act dose-dependently, but the effect can be directed mostly to fast dividing cells with a controlled dosage. In low doses some chemotherapeutics can mediate immunomodulating functions and immunogenic cell death (Shevchenko et al. 2013, Tesniere et al. 2010).

Fortunately conventional therapy methods have developed a great deal over the past decades, but some aggressive or advanced cancers typically develop resistance against radiation and chemotherapeutics and become refractory to these therapies. These therapies are also unspecific, thus similar damage is done to both tumor and normal healthy cells, especially rapidly dividing cells.

3.2. Biological cancer therapies

In addition to the conventional therapies, several other treatment modalities for cancer have been developed.

In the late 1800s, Dr. William Coley noticed that induction of a fever and immune reactions following use of a killed bacterial mixture (called Coley's toxin) were followed by tumor regression in some cancer patients (Coley 1891, Nauts, Swift & Coley 1946). Later, following Coley's observation, a weakened form of a live tuberculosis bacterium originally used for vaccination against tuberculosis showed also to have anti-cancer properties, and this Bacillus Calmette Guérin (BCG) vaccine became the first biological therapy approved by the FDA for treatment of cancer. BCG is still used as an immunomodulatory therapy for bladder cancer due to its adjuvant potential (Morales, Eidinger & Bruce 2002).

In some cancer types, the tumor can use the body's own hormones as a growth signal, or they can even acquire the ability to overexpress hormones. For this kind of hormone-

sensitive or hormone-dependent cancers, hormonal therapies, in which the excess production of hormones is blocked, can be useful. Hormonal therapies have been used for cancer treatment since mid-1900s when Drs. Huggins and Hodges showed that patients with metastatic prostate cancer benefited from testosterone suppression (Huggins & Hodges 2002). Still today it is a widely used therapy option for cancer types like prostate, breast, ovarian and thyroidal cancers. However, hormone therapy usually is only palliative rather than curative.

Often when high doses of chemo- and/or radiotherapy are used to treat a patient, it also destroys a patient´s stem cell repertoire; thus stem cell transplantation (Weiden et al. 1979) is a means to replace these cells that have been destroyed and reconstitute the body´s ability to produce blood cells. There are two types of stem cell transplants: autologous, where the cells are taken from the patient’s own bone marrow and returned after treatments, and allogeneic transplants, where the cells are from a donor with a matched HLA type. The latter has some advantages over autologous transplant; donated transplants are free of cancer cells (that might be present in autologous samples) and donor stem cells can produce immune cells that can better recognize tumor cells and destroy any remaining cancerous cells; this phenomenon is called the graft-versus-cancer effect. Therefore bone marrow transplants have been used to treat leukemia and even some solid tumors (Dougan & Dranoff 2009). However, allogeneic transplants also carry certain risks. The graft might be rejected, i.e. the donor cells could die or be destroyed by the patient’s body before engraftment in the bone marrow. The immune cells from the donor might in some cases also attack the patient´s healthy cells in addition to the cancer cells, which is referred to as graft-versus-host disease. There is also a small risk of infection from the donor cells, even though donors are extensively tested before donation.

Gene therapy is a relatively novel approach where genetic material, DNA or RNA, is introduced into patient’s cells to fight disease (Mulligan 1993). The genetic material is delivered into cells inside a vector. Viral vectors are the most used mainly because of their efficient gene delivery rates compared to non-viral vectors, like for example liposomes (Robbins & Ghivizzani 1998). Previously, most gene therapy approaches concentrated on introducing a functional version of a gene mutated in a monogenic disease, but nowadays most (64%) of all gene therapy trials focus on cancer (Gene Therapy Trials Worldwide). Cancer gene therapy can be used for instance to replace a deficient tumor suppressor gene or to silence an activated oncogene. Also various enzyme-activated prodrug systems have been constructed, e.g. HSV-TK/GCV combination treatment, where the cells are introduced with a herpes simplex virus thymidine kinase (HSV-TK) gene, which encodes an enzyme that can convert a separately administered non-toxic prodrug Ganciclovir (GCV) into a toxic metabolite GCV triphosphate (GCVTP) (Dachs et al. 2009). Also introducing genes that block or silence genes that are essential for tumor growth (like genes related to angiogenesis) has been widely investigated (Llovet et al. 2008, Yoo et al. 2007, Guse et al. 2010).

Nowadays it is also known that there are cells in tumors responsible for cancer initiation, progression, metastasis, recurrence and drug resistance: cancer stem cells (CSCs). There are a growing number of studies on the development of CSC-related therapies and the identification of key molecules involved in controlling the properties of CSCs (Chen, Huang & Chen 2013). In addition, different immunotherapy methods are rapidly being developed to harness a patient´s own immune system to fight the disease.

3.2.1. Immunotherapies

Cancer immunotherapy aims at waking up the patient’s own immune system to target and kill tumor cells (Mellman, Coukos & Dranoff 2011, Melief 2008). Together with Coley’s toxin, recombinant cytokines are one of the oldest applications of using immunologically active biological substances for treating cancer. For example interferon-α, TNFα, GM-CSF and IL-2 have been used for cancer treatment because of their direct and indirect cytotoxic effects as well as immunostimulatory properties. Interferons (IFNs) are cytokines that the body normally produces to fight pathogens. IFNα increases MHC-I expression and thus presentation of tumor-associated antigens (Anderson et al. 1994) and is therefore useful for treating hematological malignancies, melanomas, hepatocellular carcinomas and bladder cancer (Floros & Tarhini 2015). TNFα has several ways to stimulate the immune system; it can, e.g., induce production of other inflammatory cytokines and maturation of dendritic cells (Mocellin et al. 2005). It also induces necrotic and apoptotic cell death when present at high local concentrations (Ashkenazi & Dixit 1998, Salako et al. 2011, van Horssen, Ten Hagen & Eggermont 2006, Rath & Aggarwal 1999, Balkwill et al. 1986). However, its systemic administration is toxic, so it has only been approved for treatment of soft tissue sarcoma and melanoma patients by isolated limb perfusion (Mocellin et al. 2005, Lienard et al. 1992). GM-CSF, granulocyte-macrophage colony-stimulating factor, also promotes DC maturation and antigen presentation and it can activate lymphocytes making it a powerful cytokine for immunotherapy. Currently it is used as a combination therapy with other therapies, including cancer vaccines and oncolytic virotherapies (Pol et al. 2015, Andtbacka et al. 2015, Ranki et al. 2014). IL-2, a T cell growth factor, has shown best promise in the treatment of metastatic melanoma (Atkins et al. 1999). Recombinant cytokines have shown to be effective in treating some cancers, but their systemic administration is often toxic and mediates serious side effects that hinder their use. However their local expression by gene therapy approaches is a way to reduce these side effects.

Currently the most predominant forms of immunotherapy are monoclonal antibodies (mAbs). These drugs are designed to specifically target cancer-associated proteins that block pathways relevant to cancer survival. MAbs act in several ways to kill tumor cells: they can directly kill tumor cells by blocking tumor cell signaling or by delivery of cytotoxic molecules (antibody-drug conjugates), they can induce tumor eradication by specific effects on tumor vasculature or stroma or they can induce immune-mediated cell killing by complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity

(ADCC) or by regulation of T cell functions. So far 19 mAbs have been approved by the FDA (The Antibody Society 2015) as cancer therapeutics of which maybe the best known example is trastuzumab (Herceptin, Roche) for treatment of HER2 positive breast cancer (Ewer & Ewer 2015). In recent years, the patents on the first approved mAbs have expired; thus a rapid development period of biosimilars has recently started (Rak Tkaczuk & Jacobs 2014).

Immunomodulatory antibodies have recently achieved clinical success. In many cancers, the immune checkpoint pathways that control the immune system´s ability to maintain self-tolerance and duration of immune responses are often disrupted; tumors and tumor-associated cells often produce molecules, like for instance PD-L1, to suppress immunity. Therefore there are mAbs designed to target these molecules. For example, ipilimumab (Yervoy, Bristol-Myers Squibb) is an antibody against CTLA-4 (cytotoxic T lymphocyte-associated protein 4) that blocks the interaction of this inhibitory T cell receptor and the B7 costimulatory molecules on APCs and thus prevents the negative feedback loop of CTL activation. Ipilimumab has been approved for treating advanced melanomas by the FDA (Hodi et al. 2010). Also antibodies against programmed cell death protein 1 (PD-1) and its ligand (PD-L1) are of great interest. There are two mAbs, pembrolizumab (Keytruda, Merck) (Khoja et al. 2015) and nivolumab (Opdivo, Bristol-Myers Squibb) (Johnson, Peng & Sosman 2015) approved by the FDA and EMA to block the PD-1 pathway, also preventing the inhibition of CTL activation.

The first drug of the novel class of bispecific monoclonal antibodies, blinatumomab (Blincyto, Amgen) was just approved in 2014 for treatment of B cell acute lymphoblastic leukemia. These bispecific T cell engagers (BiTEs) consist of two monoclonal antibodies joined together; one end of the BiTE binds to T cells and the other end binds to a molecule on cancer cells (Buie et al. 2015).

Therapeutic cancer vaccines are also an emerging type of cancer immunotherapy in which immunogenic tumor-associated antigens or cell-based strategies are used to induce anti-tumor immune responses. The greatest success in the field of therapeutic cancer vaccines so far is a vaccine against advanced prostate cancer, called sipuleucel-T (Provenge, Dendreon). It is a mixture of a patient´s own PBMCs, GM-CSF and a prostate cancer associated antigen, prostatic acid phosphatase (Kantoff et al. 2010, Mellman, Coukos & Dranoff 2011). The therapeutic cancer vaccine field is intriguing and full of potential, but so far it has failed to show decent efficacy (except in the case of sipuleucel-T). There are also prophylactic cancer vaccines to protect from oncogenic viruses and formation of cancer, e.g. against papilloma virus or hepatitis B virus induced cancers (Dougan & Dranoff 2009).

There is also a way to engineer a patient´s own immune cells to attack tumor cells. This is called adoptive cell therapy. Cells are collected from the patient and modified and amplified in laboratory conditions prior to reinfusing them back to the patient (Dudley & Rosenberg 2003). For example use of tumor-infiltrated lymphocytes (TILs) showed good

anti-tumor responses in metastatic melanoma patients (Rosenberg et al. 2011). Alternatively circulating T cells can be adopted from a patient and they can be genetically modified to express artificial T cell receptors (TCRs) against tumor antigens. These T cells are then able to recognize the tumor antigen bound to MHC-I molecules on tumor cell surfaces. This approach is, however, limited, since MHC-I expression is often down-regulated in many cancer types and it may also cause autoimmune reactions due to off-target toxicity (Hinrichs & Rosenberg 2014). To circumvent the need of proper MHC-I binding, another MHC-independent strategy has been developed. By using artificial chimeric antigen receptors (CARs) the T cells can be modified to be more flexible in terms of antigen binding. The CAR molecule normally consists of an antigen-binding variable fragment of a monoclonal antibody fused to intracellular T cell signaling domains (Bridgeman et al. 2010). These CAR T cells have shown promise at least in treating B cell leukemia by targeting CD19 antigen on the tumor cell surface (Maude, Shpall & Grupp 2014).

It is common that the therapeutic efficacy of immunotherapies is difficult to observe reliably, since these therapies often lead to swelling and fibrosis in the tumor site. Therefore the Response Evaluation Criteria in Solid Tumors (RECIST), originally developed to measure responses of chemotherapy, may not provide decent evaluations for immunotherapies.

4. Oncolytic viruses

In the mid-1800s and the beginning of 1900s, it was discovered that some cancer patients with concurrent infections of e.g. influenza (Kelly & Russell 2007) or chicken pox (Bierman et al. 1953) had tumor regression. In the 1950s and 1960s, some of the first clinical trials were conducted to study viruses for treating cancer but after this, the field of virotherapy was nearly abandoned for almost 50 years due to the lack of technology required to modify viruses and the early success stories of chemo- and radiotherapies. However, now these virotherapies have gained widespread attention and have shown promise as cancer therapeutics.

Oncolytic viruses (OVs) are viruses that selectively replicate in cancer cells leading to tumor cell lysis while leaving normal cells unharmed (Post 2002) (**Figure 1**). They can be either naturally tumor-selective viruses, like reovirus and Seneca Valley virus, or genetically modified so that their replication is dependent on cancer-related pathways, like in the case of adeno- vaccinia, herpes simplex, measles and Newcastle disease viruses. Ideally, when the tumor cells are lysed, the released new virions can also infect surrounding tumor cells as a bystander effect and even spread through circulation into distant metastases.

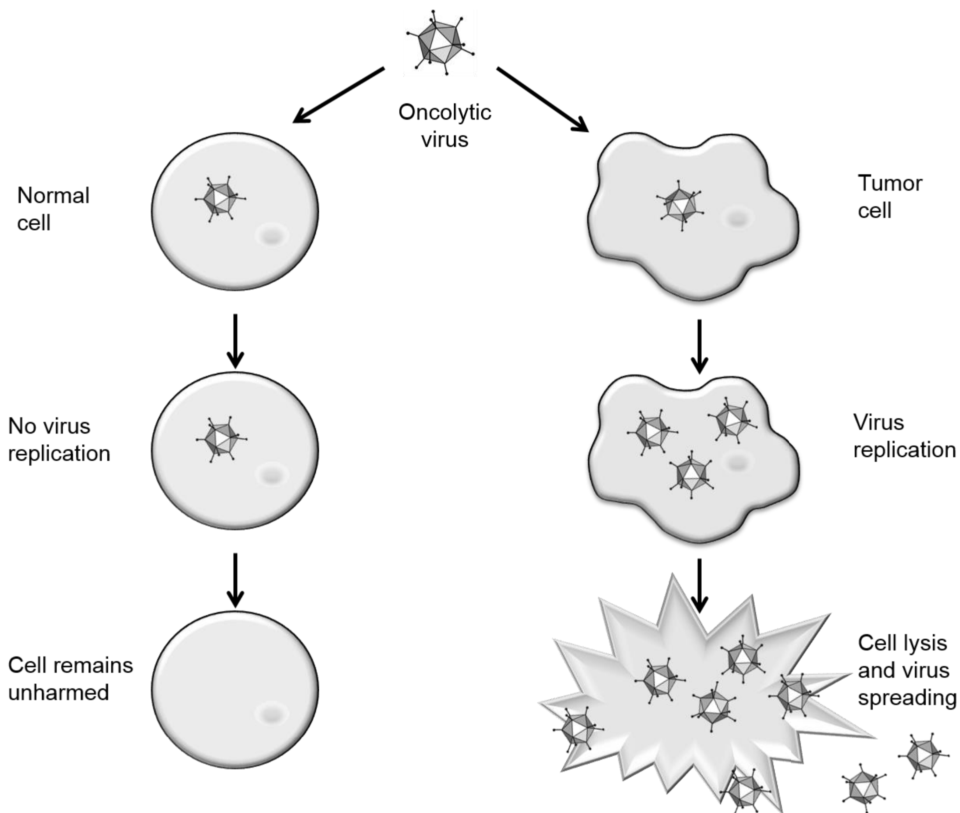


Figure 1. Oncolytic virus action. *Oncolytic viruses are viruses that can only kill cancer cells. They can infect both normal and cancerous cells, but in normal cells the replication is inhibited through mutations in the viral genome, therefore leading to virus clearance without cell damage. Since most cancer cells bear mutations allowing continuous cell proliferation, the modified viruses are able to replicate in them which eventually leads to cell lysis and release of a new virus progeny.*

In addition to the lysing effect, oncolytic viruses can act as adjuvants and stimulate host immune responses against tumors (Lichty et al. 2014). When the tumor cell is lysed, pieces of the cell, i.e. cell-associated antigens are released to the tumor microenvironment (Miller & Fraser 2003, Nakamori et al. 2004). Some of these antigens are tumor-specific that can be picked up by antigen-presenting cells, like dendritic cells (DCs), which can then process and present the antigens to naïve T cells in lymph nodes. The activated anti-tumor T cells can then travel to the tumor and recognize and kill cancer cells. This can ideally lead to long-term anti-tumor memory. Since OVs can release unique tumor epitopes from each patient's own tumor they could thus be considered *in situ* cancer vaccines (Tuve et al. 2009). Viruses also induce native immune responses when infecting the tumor cells. The viruses are recognized by several pathogen associated molecular pattern (PAMP) receptors, which once activated induce production of several chemokines and other cytokines that can recruit immune cells into the tumor site. So, oncolytic viruses have the

ability to break the immunotolerance of tumors and divert the immunosuppressive tumor environment, which can lead to beneficial anti-tumor effects. In fact, the immunological mechanisms-of-action of oncolytic viruses are currently the most studied ways to improve oncolytic virotherapies and the field has widely shifted towards the concept of "immunovirotherapy" and oncolytic viruses are even considered to be part of cancer immunotherapeutics (Coffin 2015).

The clinical and pre-clinical studies on oncolytic viruses have revealed the tested viruses to be remarkably safe and there are many candidates that can offer potential for treatment of many cancers that currently lack curative therapy (Zamarin & Pesonen 2015).

4.1. Adenoviruses

Adenovirus (Ad) was discovered in 1953 by Rowe et al. from adenoids, lymphatic tissues behind the nasal cavity (Rowe et al. 1953). Since then the culturing and propagation methods and the knowledge of the structure of the adenoviruses have developed rapidly, which has made it a well-known and easy-to-use vector for several purposes, in fact it is the most frequently selected vector for cancer gene therapy applications (Choi et al. 2012).

Adenoviruses belong to the Adenoviridae family consisting of five genera, of which the human adenoviruses constitute the Mastadenovirus genus. So far, over 60 serotypes of human Ads have been identified and they are divided into seven species A-G (Liu et al. 2012, Wadell & Norrby 1969). This thesis focuses on serotype 5 and 3 Ads, which belong to species C and B, respectively. These serotypes are commonly found in human populations and cause usually mild respiratory diseases or conjunctivitis in healthy people (Kunz & Ottolini 2010, Lynch, Fishbein & Echavarria 2011).

Adenoviruses are attractive cancer gene therapy vectors because of their lytic life cycle, ability to infect both dividing and non-dividing cells and due to the fact that their genome does not integrate into the host genome, thus the risk for insertional mutagenesis is low. Adenoviruses can also be propagated efficiently in high titers (up to 10^{13} pfu/ml) and are easily genetically engineered for various purposes; they can accommodate foreign DNA up to 105% of the size of their normal genome (36 kb) (Choi et al. 2012). Adenoviruses have the ability to infect a wide range of species but their replication is species-specific, thus the viral replication is quickly arrested in foreign host cells (Jogler et al. 2006).

4.1.1. Adenovirus structure and life cycle

Adenoviruses are the largest known non-enveloped DNA viruses (~90-100 nm) and they have a unique spiked, icosahedral shape (**Figure 2**). The adenoviral double-stranded DNA genome is located in a core inside a protein capsid. In the core, with the linear ~36 kbp genome, there are also proteins that provide structure and link the genome to the capsid. These core proteins are also needed for packaging of new virions when the virus replicates. The terminal core proteins attached to the ITRs (inverted terminal repeats) in both 5´ ends

of the genome, are important for the initiation of viral DNA replication (Rekosh et al. 1977).

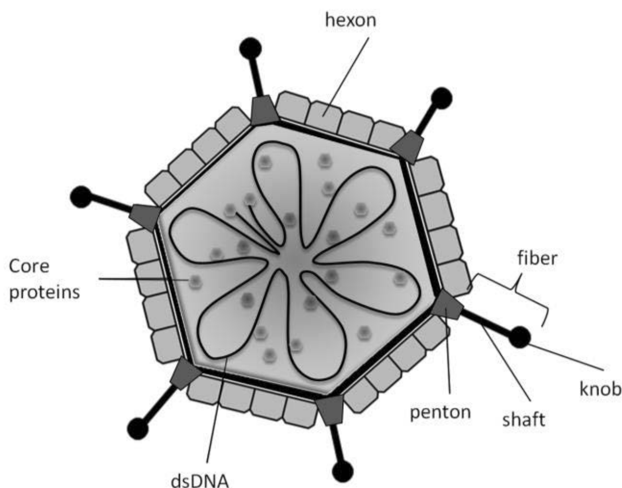


Figure 2. Adenovirus structure. *Adenoviruses are ~100 nm sized non-enveloped viruses with double- stranded DNA genome (36 kbp). The icosahedral protein capsid is mainly composed of hexons and penton bases with protruding fibers.*

The adenoviral nucleocapsid consists mainly of hexon proteins and of penton bases on the corners of the capsid where the characteristic ´spikes´, called fibers, protrude. The fibers act as moieties that mediate attachment of the viruses to cell surface receptors. Adenoviruses have 12 fibers linked to penton bases connected to their shaft, and on the C-terminus there is a knob structure that is mainly responsible for the receptor interactions. Most adenoviruses, including serotype 5 Ads are known to interact primarily with the coxsackie-adenovirus receptor (CAR) located in tight junctions between cells. CAR has high affinity to all other Ad subgroups except species B. CD46 has been identified to be the main cell entry receptor for these species B viruses (Gaggar, Shayakhmetov & Lieber 2003), but at least serotype 3 Ads require interaction also with a recently identified high affinity receptor called desmoglein-2 (DSG2) (Wang et al. 2011). This interaction has interestingly been shown to open tight junctions and this advantage can therefore be used in e.g. Ad5 therapies to enhance interactions between viruses and CARs.

After binding to a primary entry receptor, the fiber bends allowing the binding of RGD (Arg-Gly-Asp) penton base motifs to αβ-integrins. This, with other interactions like the shaft binding to heparin sulfate glycosaminoglycans, initiates the internalization of the virus into clathrin-coated pits by endocytosis (Roelvink et al. 1999, Wiethoff & Nemerow 2015). The change of pH from neutral to acidic inside the endosome induces disruption of the viral capsid and release of the viral DNA, which is thereafter transferred to the host cell nucleus where the viral replication takes place (**Figure 3**).

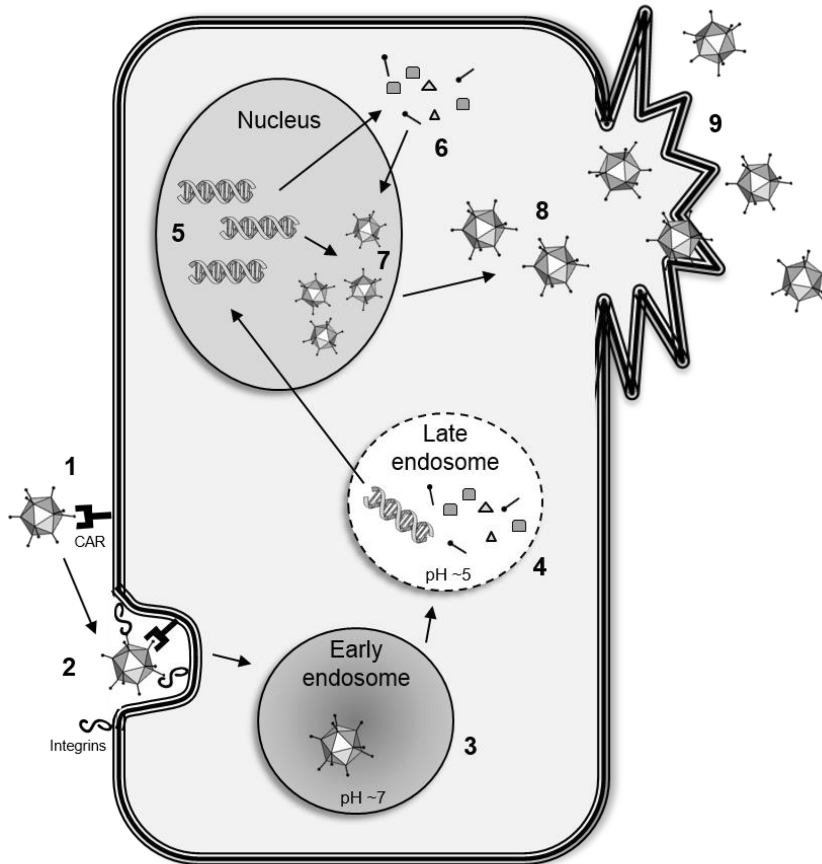


Figure 3. Adenovirus life cycle. *Serotype 5 adenovirus attaches to the cell membrane by binding through CAR **(1)**. The initial virus binding is followed by integrin binding and formation of a clathrin-coated vesicle **(2)** and the virus is then endocytozed **(3)**. Change in pH in the late endosome **(4)** disrupts the viral capsid and releases the viral DNA, which can then be transferred into nucleus. Thereafter, viral DNA is replicated in the nucleus **(5),** while the viral capsid proteins are produced in the cytosol **(6)**. Immature viral particles are assembled in the nucleus **(7)** and released into the cytosol for maturation **(8)**. The mature viral particles then induce lysis of the cell and virions are released to the cell surroundings **(9)**.*

The adenoviral genome can be divided into early (E) and late (L) genes based on their time-of-function before and after the DNA replication, respectively. The early genes (E1A, E1B, E2A, E2B, E3 and E4) encode proteins needed for regulation of DNA synthesis and initiation of replication. The late genes (L1-L5) are transcribed after the initiation of DNA replication and they encode structural proteins (Russell 2000). E1A is the first gene expressed after the viral genome reaches the host cell nucleus (Volpers & Kochanek 2004). It has an important role in the initiation of viral replication; it activates the transcription of other early genes. E1A binds to retinoblastoma protein (pRb), thus releasing the cellular E2F transcription factor that in turn activates transcription of the E2 viral gene and production of cellular S-

phase proteins. E1B-55K inhibits p53 tumor suppressor thus allowing DNA replication and preventing the cell from undergoing apoptosis together with E1B-19K. E2 proteins are needed for viral DNA replication, whereas E3 proteins are important in initiation of mechanisms against anti-adenoviral immune responses (Russell 2000). For instance the E3-gp19K protein prevents translocation of major histocompatibilty complex I (MHC I) proteins to cell surface, thus reducing the recognition of infected cells by the host immune system. The E3-14.5K/10.4K complex and E3-14.7K inhibit TNFα and Fas mediated cytolysis and apoptosis (Chen et al. 1998). E3 also encodes for the adenoviral death protein (ADP) needed for lysis of the host cell. E4 proteins have many roles for example in viral replication and transcription of the late genes. The entire adenovirus life cycle usually takes about 24-36 hours (Russell 2000).

4.1.2. Transduction, transcription and biodistribution: targeting of adenoviruses to tumors

In cancer gene therapy the tumor-restricted replication and tumor-targeted delivery are keys to safe and responsive therapy. Many types of adenovirus targeting have been investigated, most of which include genetic modifications of viral surface structures or viral genes. Transductional targeting goals for increased transduction i.e. vector intake, whereas transcriptional targeting methods aim at restricting the viral replication into tumor cells.

As previously discussed, the expression of CAR on the surface of tumor cells is a limiting factor for efficient adenoviral infection, at least for serotype 5 Ads. Because many tumor types have down-regulated CAR expression (Okegawa et al. 2004), a great deal of Ad research has focused on circumventing the CAR-dependence (Kanerva & Hemminki 2004). Fiber pseudotyping is one way to circumvent the lack of CAR expression. For example changing the knob structure of serotype 5 adenovirus to a serotype 3 virus knob retargets the virus to other entry receptors, CD46 (Gaggar, Shayakhmetov & Lieber 2003) and desmoglein 2 (Wang et al. 2011) that have a wider distribution in cancer cells than CAR (Tuve et al. 2006). This modification has shown to enhance the transduction of the virus *in vitro* and *in vivo* (Krasnykh et al. 1996, Kanerva et al. 2002b, Kanerva et al. 2003). These pseudotype viruses are called 5/3 chimeras. Also chimeras with other species B Ad knobs that use same entry receptors as Ad3 have been developed, like Ad5/35 and Ad5/11 (Yu et al. 2007, Suominen et al. 2006).

Some other attempts to enhance the transduction of adenoviruses include addition of tumor-homing peptides on the surface of the virus, e.g. modification of the fibers by adding a RGD-4C motif to the HI-loop has been shown to target αv-integrins, which are selectively expressed on various cells in tumors (Dmitriev et al. 1998). Also attaching so-called bridging molecules or adapters (e.g. antibody) that target the virus to some specific receptor or epitope on a target cell have been studied (Douglas et al. 1996).

It is known that adenoviruses are, at least in *in vivo* settings, rapidly cleared from the circulation by specific liver macrophages called Kupffer cells (Alemany, Suzuki & Curiel

2000). The entry of adenoviruses into the liver can result in decreased vector delivery into the target cells, and might cause some liver toxocity (Ranki et al. 2007). To increase target cell transduction and reduce toxicity, liver de-targeted adenoviruses have been constructed. For example deleting the KKTK-motif from the fiber shaft makes the virus unable to enter the Kupffer cells. Unfortunately KKTK-mutated viruses have shown lower transduction efficacy to target tissues (Bayo-Puxan et al. 2006, Koski et al. 2009), this may be because the KKTK motif of Ad5 fiber was shown to be important for the fiber interaction with heparin sulphate proteoglycans (HSPGs), one of the cell entry receptors of Ad5 viruses (Smith et al. 2003, Wu et al. 2003). Some other ways to reduce liver tropism and liver toxicity of the systemically administered adenovirus have been suggested too. Chemical modifications of the virus capsid or coating it with polymers or usage of delivery vehicles or matrix may reduce the toxicity and enable improved delivery and exposure to the target tissue. For instance pegylation, attachment of polyethylene glycol (PEG) polymers on the surface of adenovirus was shown to reduce the clearance of the virus from blood, but in addition it also reduced the infectivity of the virus (Alemany, Suzuki & Curiel 2000). Also other polymers have been exploited to prevent Ad interactions with the host immune system, like cationic poly-L-lysine polymers (Ahn et al. 2004), N[2-hydroxypropyl] methacrylamide (HPMA) copolymers (Fisher, Seymour 2010), arginine-grafted bio-reducible polymers (ABPs) (Kim et al. 2011) and cationic polyamidoamine (PAMAM) cascade dendrimers (Grunwald et al. 2013). Polymers can also include various targeting molecules, e.g. tumor-homing peptides (Yao et al. 2012). Silica gels have also been successfully used to deliver adenoviruses to the target site without hindering the activity of the viruses (Quintanar-Guerrero et al. 2009).

Adenoviruses can bind to different coagulation factors, such as coagulation factors X (FX) and IX (FIX) (Jonsson et al. 2009) and the complement factor C4BP (Shayakhmetov et al. 2005). The factor X is positively charged, thus the negatively charged Ads easily bind to it in the circulation (Waddington et al. 2008). Administration of an anticoagulant drug warfarin prior to Ad vector injection has been shown to prevent the formation of functional FX leading to decreased binding of the Ad vector (Parker et al. 2006).

To avoid unwanted damage in normal healthy tissues the oncolytic viruses are usually genetically modified so that they can replicate only in tumor cells and are therefore also called conditionally replicating viruses. Usually the design of conditionally replicating adenoviruses (CRAds) takes advantage of commonly known mutations present in cancerous cells. Type I CRAds bear a deletion in the viral genome important for viral transcription and replication, which is compensated by a gain-of-function mutation in cancer cells. For example a deletion of 24 base pairs in the viral E1A gene makes the expressed mutated E1A protein unable to bind retinoblastoma protein (pRb) (**Figure 4**). This interaction is needed in normal cells to activate the E2F transcription factor that leads to induction of S-phase of the cell cycle (Berk 1986). Almost all human tumor cells are defective in the pRb/p16-pathway and they constantly express the E2F transcription factor, thus making them independent of pRb binding (i.e. they can overcome the G1-S

checkpoint) (Imperiale et al. 1984, Sherr & McCormick 2002). The deletion-bearing virus is also able to infect normal cells but its replication in these cells is restricted because of the dysfunctional E1A protein (Pesonen, Kangasniemi & Hemminki 2011). The viruses bearing the 24bp deletion in their E1A gene are commonly referred to as Δ24-viruses. The first and most common oncolytic adenovirus in clinical trials, ONYX-015, has a deletion in another viral gene, E1B-55K, which makes the virus replication dependent on a deficient p53 pathway related to most cancers (Bischoff et al. 1996).

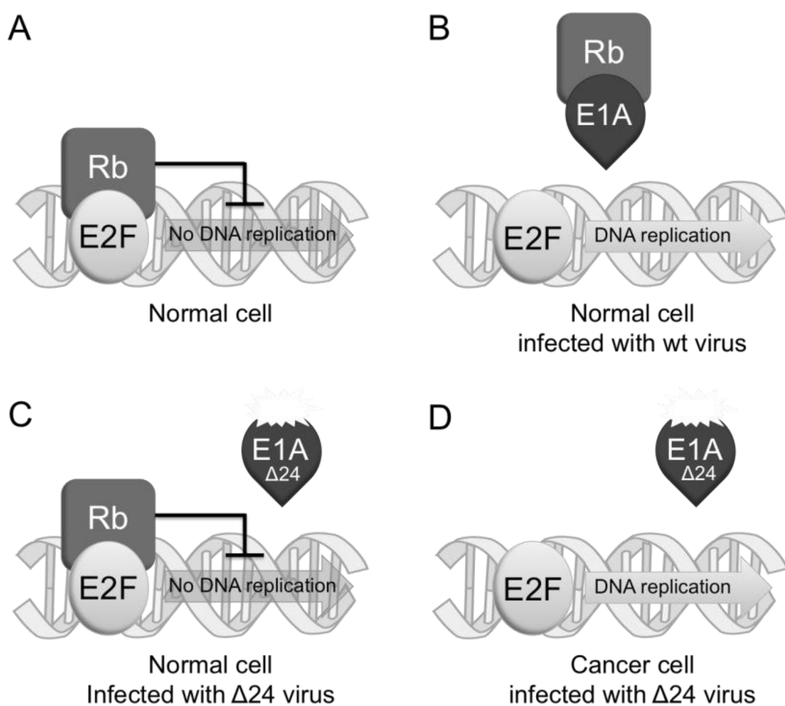


Figure 4. Illustration of the mechanism-of-action of Δ24 adenoviruses. A) *In normal cells, retinoblastoma (Rb) protein binds to transcription factor E2F arresting DNA replication.* ***B)*** *If wild-type adenovirus infects normal cells, E1A adenoviral protein binds to and inactivates pRb. This results in the release of E2F, which then promotes DNA replication also allowing replication of the viral DNA.* ***C)*** *A 24-base-pair deletion in the E1A of a modified adenovirus makes the viral E1A unable to bind pRb. The Rb protein remains then bound to the E2F halting the replication of the Δ24 adenovirus in normal cells.* ***D)*** *However, if a Δ24 adenovirus infects a cancer cell, the modified adenovirus will be able to replicate, due to loss-of-function of the Rb protein and thus constant E2F activity in most tumor cells. (Figure inspired by Gomez-Manzano et al., Neurology, 2004)*

Type II CRAds are restricted to replicate only in cancer cells by regulation of viral early genes by a tumor specific promoter that enables the expression of the virus and the possible transgenes only in cells where transcription factors for this promoter exist (Nettelbeck 2008, Glasgow et al. 2004). For example hTERT (human telomerase reverse transcriptase) promoter targets the vector to cells that have high division rates and thus

active telomerase production, i.e. preferentially cancer cells (Kim et al. 1994). In addition to tissue-specific promoters, treatment-responsive promoters can be used. Transcription can in this case be regulated for example by irradiation (Mauceri et al. 2009) or certain chemicals (Harrington, Linardakis & Vile 2000), if a radiation- or chemically inducible promoter is used to activate gene expression, respectively.

Even though these above-mentioned strategies have shown enhanced Ad targeting, there is still limited efficacy seen in pre-clinical and clinical trials mostly because of insufficient transduction and replication rates in target cells (Yamamoto & Curiel 2010). Therefore, viruses targeted by multiple strategies simultaneously are an option to circumvent this problem. For example, a double-targeted CRAd, Ad5/3-Δ24 has shown increased transduction and anti-tumor efficacy both *in vitro* and *in vivo* compared to Ad5 virus (Kangasniemi et al. 2006, Guse et al. 2007, Raki et al. 2008).

4.1.3. Immune responses to adenoviruses

An unfortunate case in a clinical trial of adenoviral gene therapy for ornithine transcarbamylase deficiency (OTD), was the death of a patient, Jesse Gelsinger, in 1999, after a massive immune response triggered by the viral vector, revealed the immunogenic characteristics of the adenovirus (Liu & Muruve 2003). Ads are potent inducers of both early innate and later adaptive immune responses.

4.1.3.1. Innate immune responses

When adenoviruses infect cells, the structural components of the virus are immediately recognized by cellular pattern recognition receptors (PRRs) (Russell 2009, Cerullo et al. 2007, Hartman et al. 2007). Toll-like receptors (TLRs) are a group of cellular receptors on the cell surface or in the cytosol that can sense pathogen-associated molecular patterns (PAMPs) (Cerullo et al. 2007, Hartman, Black & Amalfitano 2007, Rhee et al. 2011). The most important TLRs reported for adenoviral recognition are TLR-2 and TLR-9, on the cell surface and in the endosomes, respectively (Appledorn et al. 2008, Cerullo et al. 2007). TLR-9 recognizes the unmethylated CpG-rich sequences of the viral DNA (Hemmi et al. 2000). The adenoviral DNA can also be sensed by other endosolmal sensors, e.g. DNA-dependent activator of IFRs (DAI) (Schulte et al. 2013), nucleotide oligomerization domain (NOD)-like receptors (Thaci et al. 2011), absent in melanoma 2 (AIM2), gamma-interferon-inducible protein 16 (IFI16) and cyclic GMP-AMP synthase (cGAS) (Lam, Stein & Falck-Pedersen 2014). Ad sensing by the PRRs leads to activation of adaptor proteins like MyD88, TRIF, TRAFs and MAPKs (Akira 2006) and further to activation of the effector molecules such as NF-κB or interferon-regulatory factors (IRFs) depending on the signaling pathway (Kawai & Akira 2006, Zhu, Huang & Yang 2007). These signaling cascades finally lead to secretion of several inflammatory cytokines like IL-5, IL-6, IL-8, IL-12, TNFα, RANTES, MIP-2 and type I interferons (Girardin, Sansonetti & Philpott 2002, Inohara & Nunez 2003). Adenoviruses can also be opsonized and recognized by the complement system (Appledorn et al. 2008, Jiang et al. 2004, Kiang et al. 2006).

The adenovirus infection-mediated secretion of interferons and other cytokines recruits macrophages and other phagocytic cells to destroy the invader (Muruve 2004, Kiang et al. 2006). These early inflammatory responses to Ad occur within 24 hours after infection (Muruve et al. 1999). NK cells are activated in response to the interferon and macrophage-derived cytokine release. NK cells are part of the fast-acting innate immune system that can recognize infected cells. Their name “natural killer cells” derives from the notion that they are able to kill cells that are missing the MHC-I “self”-antigen presentation and they do not require activation signals from other cells. NK cells can cause apoptosis of the infected cell by releasing cytolytic granzymes into the target cell after binding to a Fas ligand, which indicates infection of a cell. They can also kill infected cells by a mechanism called antibody-dependent cellular cytotoxicity (ADCC) if the opsonization antibodies are recognized by the Fc gamma III (CD16) receptors expressed on NK cells. NK cells also secrete cytokines, TNFα and IFNγ, to activate other immune cells (Reefman et al. 2010).

4.1.3.2. Adaptive immune responses

The local inflammation leads to recruitment of various effector leukocytes to the infection site and the amplification of immune responses finally activate the immune cells belonging to the adaptive immune system. Dendritic cells (DCs) are in a key role between the innate and to the adaptive immune system, DCs phagocytose viral and tumor antigens and act as the principal cross-presenting antigen presenting cells (APCs) (Schulz et al. 2005). When matured after antigen intake, they can migrate to secondary lymphoid organs where they present the antigens to T cells (Mercier et al. 2004). DCs stimulate the T cells via costimulatory molecules CD40, CD80 (B7-1) and CD86 (B7-2) and present the antigens via MHC class I or II molecules. CD4+ T cells, the helper T cells, are activated via MHC II presentation, whereas CD8+ cytotoxic T cells via MHC I presentation (Perreau & Kremer 2006). The activated T cells can then migrate to the site of infection and kill the infected cells. Recently, oncolytic adenoviruses have been shown to induce immunogenic cell death in cancer cells. Virus-treated cells show elevated ATP and High-mobility group box 1 protein (HMGB-1) secretion and increased exposure of calreticulin on their cell surface (Diaconu et al. 2012). All these three marker molecules are known to interact with dendritic cells thus leading to activation of T cell immunity (Tesniere et al. 2010, Kepp et al. 2011).

This activation of the adaptive cellular responses typically occurs from four to seven days after the adenoviral infection, after viral gene expression (Muruve 2004, Liu et al. 2000). In addition to the T cell responses, antibodies play an important role in the anti-adenoviral immunity. When a B cell encounters a specific antigen via its B cell receptor and receives an additional activating signal from a helper T cell, it matures, expands clonally and becomes a short-lived plasma B cell able to produce antibodies specific to the encountered antigen. Some of the expanded B cells may also become memory cells, which upon reencountering the same antigen can expand and start rapidly producing antibodies (McHeyzer-Williams & McHeyzer-Williams 2005, Yang, Greenough & Wilson 1996). Also

part of the T cells can differentiate into memory subsets, and together these long-living memory B and T cells can induce long-term immunity against the virus (Russell 2009).

Many adenovirus serotypes, including Ad5, are endemic pathogens, thus basically everyone has acquired immunological memory and produce neutralizing antibodies (Nabs) against Ads (Zaiss, Machado & Herschman 2009, Wadell 1969). Therefore repeated administration of an adenoviral vector induces a strong serotype-specific immune reaction against the virus, which usually leads to quick neutralization of the systemically administered virus by the anti-viral antibodies. Treatments combining different serotypes may prevent the neutralizing effect (Hemminki et al. 2011).

4.1.4. Translation to clinics (clinical studies with oncolytic adenoviruses)

The first type I CRAd published was ONYX-015, a virus bearing two deletions in its E1B-55K protein making it dependent on the inefficient p53/p14-pathway (Bischoff et al. 1996). Although safety of this virus has been demonstrated in various clinical studies since 1996 with MTD up to 2 x 10^{12} VP (Nemunaitis et al. 2001), its efficacy remains poor (Crompton, Kirn 2007). Because of the limited results, ONYX-015 was also studied in combination with chemotherapy, which showed substantial objective responses, including several complete responses; by 6 months, none of the responding tumors had progressed, whereas all non-injected tumors treated with chemotherapy alone had progressed (Khuri et al. 2000). H101 (Oncorine, Shanghai Sunway Biotech), also an E1B-55K deleted adenovirus with additional E3 gene deletion, is the first and thus far the only oncolytic adenovirus approved for clinical use as a drug. In China it has been available for cancer patients suffering from head and neck carcinoma since 2005 (Yu & Fang 2007); however its survival data was inconclusive restricting its importation to European or US markets (Guo & Xin 2006, Sheridan 2011, Alemany 2007). The first type II CRAd tested in clinical trials was CV706 that contains a PSA promoter and enhancer to drive E1A expression. It was well tolerated and showed some clinical activity, as reflected by changes in serum PSA, encouraging further clinical investigation (DeWeese et al. 2001). There are several ongoing trials with OAds and nowadays most of the tested viruses are type II CRAds expressing some immunostimulatory molecules to provoke the activation of the host immune system. For example CG0070 (Cold Genesys Inc.) and ONCOS-102 (Oncos Therapeutics Ltd.) viruses are both serotype 5 Ads targeted to replicate in Rb-mutated cancer cells and they both express GM-CSF. CG0070 is already in phase III testing (NCT01438112) for treating non-muscle invasive bladder cancer after showing good safety profiles in phase I and II trials (Burke et al. 2012, Zamarin & Pesonen 2015). Treatment of refractory solid tumors with ONCOS-102 in the phase I study were also well tolerated. Intriguingly, it also showed clear evidence of anti-tumor immunity established in the treated patients; infiltration of lymphocytes (TILs) into treated as well as into untreated lesions were correlated with better OS, suggesting involvement of systemic immunity in the treatment efficacy (Ranki et al. 2014, Vassilev et al. 2015, Zamarin & Pesonen 2015).

Combinations with conventional therapies, like radio- or chemotherapies, could result in additional or even synergistic effects and therefore OV therapies are often tested together with other therapies (Ottolino-Perry et al. 2010). At the moment there are over 10 open clinical trials testing oncolytic adenoviruses for treating cancer (ClinicalTrials.gov).

4.1.5. Challenges of adenoviral therapies

Although adenoviruses are very appealing as cancer gene therapy vectors, they also have certain limitations that hinder development of Ad therapies. In addition to the already mentioned limitations, like rapid clearance of systemic adenovirus by liver cells (at least in mice) and Nabs or the limited transduction efficiency to tumor cells due to low CAR expression, the host-specificity of Ads creates an additional problem for Ad-based drug development (Jogler et al. 2006). Nevertheless preclinical studies in animal models with replicating adenoviruses have shown promising efficacy against cancer, these results cannot usually be repeated in human trials. This is at least partly due to the many differences between human disease and animal models used. Due to the fact that adenovirus replication is very species-specific, oncolytic adenovirus therapies lack proper animal models with which the real effects of the oncolysis could be shown. However, a Syrian hamster was recently found to be semi-permissive to human Ad5 replication, so its use in preclinical studies has expanded (Dhar, Toth & Wold 2012).

4.1.6. Safety of adenoviruses

Adenoviruses are common pathogens and normally the adenoviral infection appears asymptomatic or causes mild respiratory or conjunctival infections. Because of their low pathogenicity in immunocompetent individuals, little effort has been put into development of anti-adenoviral drugs. Yet an adenovirus is an immunogenic virus and can potentially cause liver toxicity or serious inflammatory symptoms in some immunocompromised patients or if used with too large systemic doses as in the unfortunate case of Jesse Gelsinger (Raper et al. 2003). However, the modified oncolytic adenoviruses are less pathogenic due to the deletions and insertions in their genome, in fact no mortality reported related to oncolytic adenovirus treatments has been reported. If uncontrolled infection in an adenovirally treated patient occurs and causes health-threatening side effects, anti-viral drugs can be used to inhibit viral replication. For example Cidofovir can be used to target the viral DNA polymerase and stop the viral replication cycle (Naesens et al. 2005). Ribavirin is another drug to inhibit adenovirus infections but it has shown to be less effective than Cidofovir (Lenaerts & Naesens 2006).

4.2. Vaccinia viruses

Vaccinia viruses belong to the Poxviruses of the Poxviridae family. They are large, enveloped DNA-viruses that are perhaps best known of their use as a vaccine against small pox, the first human disease completely eradicated through purposeful global vaccination. Actually, the word ´vaccine´ derives from this historical use of vaccinia virus as the first vaccine, an idea described by Edward Jenner in 1798. Moreover, the vaccinia virus derived its name from the organism it was originally discovered from, namely the cow, ´vacca´ in

Latin. Vaccinia virus is closely related to the cowpox and variola viruses, but it has been attenuated due to repeated cultivation and passaging in the laboratories for several decades (Sanchez-Sampedro et al. 2015). Nowadays vaccinia viruses are actively studied for the development of next generation vaccines but also commonly for their use as vectors in various gene delivery applications, including oncolytic virotherapy (Verheust et al. 2012, Gomez et al. 2011).

Vaccinia viruses have many advantages as vectors for cancer gene therapy. Their wide host tropism (various species and cell types) (Kroon et al. 2011), lack of mutagenetic risk due to their cytoplasmic life cycle, easily modifiable and large genome, which can take large amounts of foreign DNA, and fast replication cycle, makes them desirable study targets (Guse, Cerullo & Hemminki 2011).

4.2.1. Vaccinia virus structure and life cycle

Vaccinia viruses (VV) are large (360 x 270 x 250 nm) (Cyrklaff et al. 2005) DNA-viruses with a linear double-stranded genome of about 190 kb that encodes approximately 250 genes (**Figure 5**). VVs belong to Poxviruses, which are unique among DNA viruses because their entire replication cycle takes place in the cytosol, in discrete structures called viral factories outside the nucleus. Hence a large number of genes are needed to enable the production of new virions. These ~250 viral genes code for different enzymes and other proteins needed to initiate viral replication, including DNA polymerase and transcription factors. (Chan & McFadden 2014)

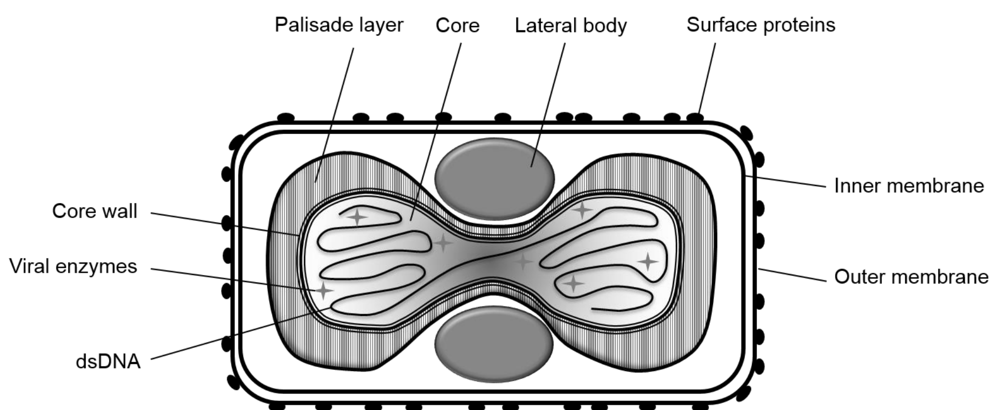


Figure 5. Vaccinia virus structure. *Vaccinia viruses are relatively large (~360 X 260 nm), brick-shaped enveloped viruses. The viral core includes a large dsDNA genome of ~190 kbp and viral enzymes for replication. Inside the membrane layers there are two lateral bodies composed of proteins that counteract host antiviral defense mechanisms and modulate the host cell to facilitate virus replication.*

Vaccinia virus is a brick-shaped virus with one or more lipoprotein envelopes (**Figure 5**) depending on its route of maturation during the replication cycle. VVs can produce four infectious forms: intracellular mature virions (IMV), intracellular enveloped virions (IEV),

cell-associated enveloped virions (CEV) and extracellular enveloped virions (EEV), IMVs being the most abundant form. According to the current knowledge, IMVs have only a single membrane, whereas CEVs and EEVs are surrounded by two membranes. The IEV particles have three envelopes. The IMVs are thought to be the form responsible for spreading between hosts. The CEVs are believed to spread from the infected cell to neighboring cells and the EEVs are instead thought to be important for spreading to distant sites inside the host. (Ward & Moss 2001)

Vaccinia viruses have a wide tropism to different mammalian cell types. The specific cellular receptors for VV entry are still unknown but it is thought that the virus-cell interaction must be through some ubiquitously expressed receptors considering the wide tropism of the virus. It is at least known that IMVs use heparan sulfate and/or laminin in their attachment to cells (Chan & McFadden 2014). After attachment to the host cell surface VV is internalized by fusion of the viral and cellular membranes, thus releasing the viral core into the cytosol (**Figure 6**). Vaccinia virus entry is then followed by the release of proteins from the two lateral bodies, which suppress the host anti-viral immune defense mechanisms and prepare the host cell for viral replication (Schmidt et al. 2013). The viral replication takes place in viral factories where the viral DNA is multiplied, viral proteins are transcribed and new virions are assembled. The new virions, intracellular mature virions (IMVs) can then be released from the host cell by lysis. The IMV particles can also take another route inside the cell and be processed by the Golgi complex receiving an additional double membrane. These particles are thereafter called intracellular enveloped virions (IEVs), which can bud through the cell membrane. When fusing to the cell membrane they lose their outer third membrane leaving only two membranes left. As long as the particle is connected to the cell membrane it is called a cell-associated enveloped virion (CEV). CEVs can infect adjacent cells by active penetration assisted by actin tails (**Figure 6 (9)**) or they can be budded out from the cell and become EEVs (extracellular enveloped virions) (**Figure 6 (10)**). EEVs can also be formed by budding of IMVs through the cell membrane. The vaccinia virus infection cycle is fast; the whole replication cycle is completed within 24 hours after infection (Salzman 1960).

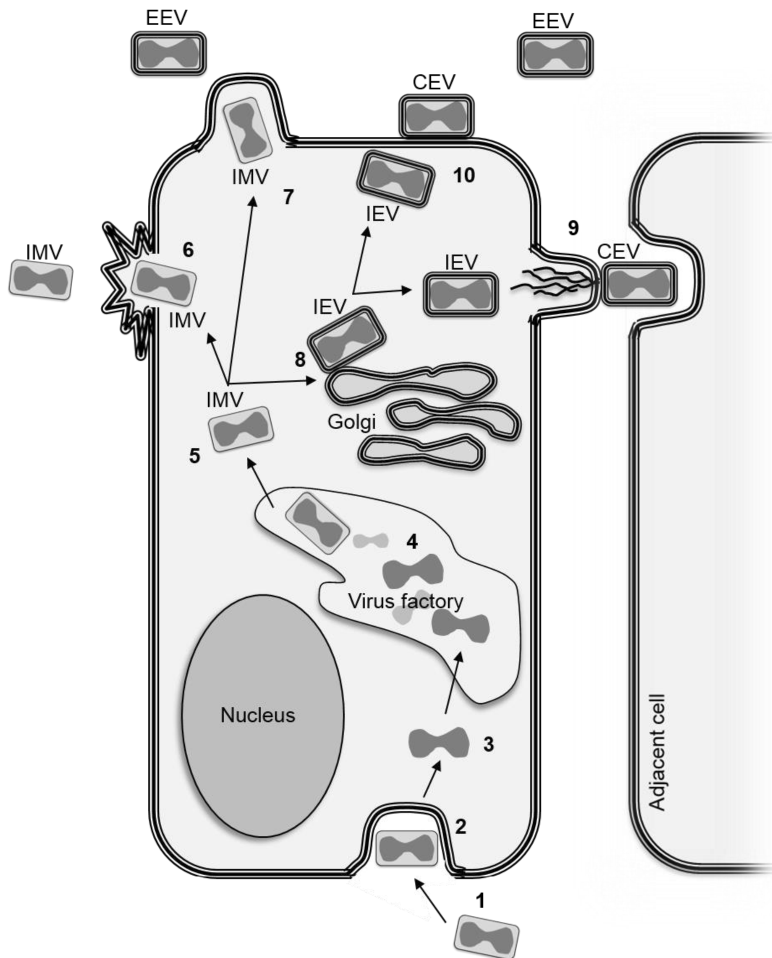


Figure 6. Vaccinia virus life cycle. *Vaccinia virus attaches to the cell membrane by interaction via membrane receptors* ***(1)*** *and is internalized by fusion of viral and cellular membranes* ***(2)****. The viral core is released* ***(3)*** *and viral replication and assembly of new virions occur in the cytosol in a so-called virus factory* ***(4)****. Intracellular mature viruses (IMVs) are formed* ***(5)****, which can then be released via cell lysis* ***(6)****. IMVs can also form extracellular enveloped viruses (EEVs) by budding through the cell membrane* ***(7)****. Some IMV particles can gain an extra membrane by traversing the Golgi complex and are then called IEVs (intracellular enveloped viruses)* ***(8)****. IEVs can be actively transported via actin tails into neighboring cells* ***(9)*** *or fused through the cell membrane* ***(10)*** *and become EEVs. As long as the viral particles are attached to the cell membrane, they are called cell-associated enveloped viruses (CEVs).*

4.2.2. Targeting vaccinia viruses to cancer

It has been observed that VVs already possess natural tropism towards cancer cells, possibly because cancer cells are generally living in a more immunosuppressed environment that does not hamper the virus proliferation (Chan & McFadden 2014). A higher proliferation rate of tumor cells also enables better conditions for viral replication,

and the leaky vasculature of tumors enables a virus to enter cancer cells more readily than normal cells (Thorne et al. 2007). However, mechanisms for a more selective replication in tumor cells have been investigated for a safer use of VVs as therapeutic agents.

Due to a scarcity of information on the cell receptor interactions of vaccinia viruses, the strategies to target VVs to tumor cells are currently focusing on viral transcriptional mechanisms in order to derive methods that take advantage of cancer specific pathways differing from those in normal cells. Tumor-targeting of VVs has been achieved by genetic mutations in the viral genome. Genes that are needed for viral replication in normal but not in tumor cells can be deleted restricting the replication only to cancer cells.

The vaccinia growth factor (VGF) gene has been deleted to improve the safety of the virus. VGF is expressed early during the virus infection and it acts as a virulence factor. It is a homologue of the endothelial growth factor (EGF), which is able to bind to EGF receptors (EGFR) on the cell surface and activates the Ras-pathway leading to activation of the cell cycle and production of factors needed for viral replication. The Ras-pathway is constantly activated in most cancers (Hanahan & Weinberg 2000) and therefore the production of VGF is not needed in cancer cells to enable viral replication (Chan & McFadden 2014).

Also deleting the viral thymidine kinase (TK) gene has shown good results in terms of selectivity. TK is involved in the synthesis of deoxyribonucleotides needed for DNA synthesis. The cellular thymidine kinase is expressed only during the S-phase of the cell cycle, i.e. during cellular division; so in normal cells TK is not constantly present. Therefore VV needs its own TK production to be able to replicate in normal cells. However, in cancer cells the common oncogenic mutations lead to constantly active cell proliferation and TK is present in the cells, thus the production of viral TK is not needed in tumor cells. The TK deletion therefore restricts the replication to cells that overexpress E2F transcription factor (i.e. most tumor cells) that regulates the expression of cellular TK (Chan & McFadden 2014).

The deletion of both of the above-mentioned genes, VGF and TK, has been shown to reduce the pathogenicity of the virus in normal tissues and to increase the selectivity to tumors compared to the single-deleted controls (McCart et al. 2001) (**Figure 7**). These double-deleted VVs are referred to as vvdd.

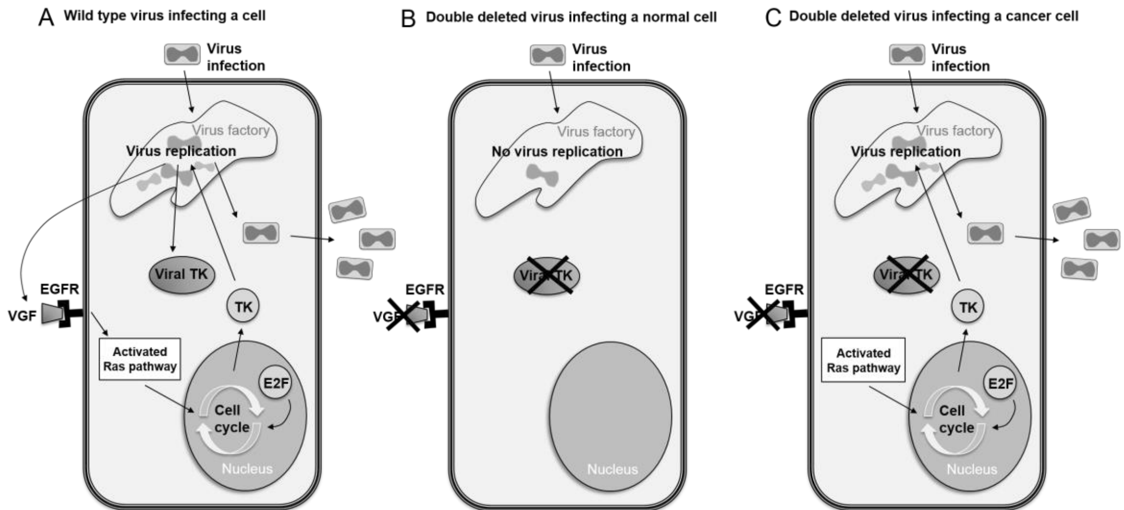


Figure 7. Illustration of the controlled replication of double-deleted vaccinia viruses. A) *In normal cells, the wild-type virus produces viral proteins that adapt the cell for viral replication: viral thymidine kinase (TK) and vaccinia growth factor (VGF), which activates the epidermal growth factor receptor (EGFR) and thereafter the Ras pathway.* ***B)*** *Oncolytic vaccinia viruses that carry deletions in the viral VGF and TK genes are unable to adapt the normal cell for viral replication and production of the virus is blocked.* ***C)*** *In cancer cells, common oncogenic mutations compensate for viral gene deletions allowing viral replication.*

4.2.3. Vaccinia virus induced immune responses

Vaccinia viruses have been considered immunogenic viruses based on their successful use as a small pox vaccine. It is true, the infection sets alarm signals to the host immune system to destroy the invader and many antiviral responses are activated. However, vaccinia viruses are well prepared to hide from the host immune attack and they actually encode several immunosuppressive genes and they also carry immunosuppressive proteins in their lateral bodies, which are released immediately after the viral core is released following the internalization of the virus into the host cell. These proteins can for instance bind and neutralize host cytokines (Smith, Bryant & Alcami 2000, Smith et al. 2013, Schmidt et al. 2013).

4.2.3.1. Innate immune responses

Immediately after VV infection the host innate immune responses are evoked by secretion of proinflammatory cytokines, like type I interferons (IFNα and IFNβ) (Samuel 1991). The IFN response is initiated upon sensing of viral PAMPs by pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and cytosolic DNA sensors. At least TLR-2 (Zhu, Huang & Yang 2007, Quigley et al. 2009), TLR-8 (Martinez, Huang & Yang 2010) and AIM2 (Rathinam et al. 2010) have been reported to sense VV infections. Also recently, a newly discovered DNA sensor cGAS (cyclic GMP-AMP synthase) was reported to induce IFN responses upon vaccinia virus infection through STING (stimulator of IFN genes) -mediated

pathway (Dai et al. 2014). PRR activation induces activation of transcription factors, such as IFN-regulatory factor (IRF) 3 and IRF7, and NF-κB, whose translocation into the nucleus induces production of type I IFNs, cytokines and chemokines (Dai et al. 2014).

Also the complement system is quickly activated and the enveloped viral particles and the infected cells can be directly destroyed by lysis (by the membrane attack complex) or the viruses can be opsonized and phagocytized by macrophages and neutrophils. VVs have developed a method to evade the complement though, they encode a vaccinia virus complement control protein (VCP) that can bind complement proteins C3b and C4b and thus blocks the activation of the complement cascade (Girgis et al. 2011, Kotwal & Moss 1988). Nevertheless, neutralizing antiviral antibodies can hamper the efficacy of VV therapies by clearance of viral particles before they have even infected the target cell. EEV particles are more resistant than IMVs to the neutralizing antibodies, because they have the outer membrane originating from the host cell membrane, and can thus evade the inactivation by the complement system (Smith, Vanderplasschen & Law 2002). Vaccinia virus, however, is not an endemic virus and the last worldwide vaccinations for small pox were given in the 1970s; therefore only elderly people could have pre-existing antiviral antibodies in their circulation.

The interferons and macrophage-derived inflammatory cytokines attract natural killer (NK) cells to the site of infection. They can directly kill VV infected cells by lysis or apoptosis by releasing granzymes. Antibody-opsonized infected cells can also be recognized by NK cell Fc gamma III (CD16) receptors, which induced antibody-dependent cellular cytotoxicity (ADCC). NK cells can also secrete cytokines (IFNγ and TNFα) and thus work to control viral infections. IFNγ activates phagocytosis by macrophages and TNFα promotes direct NK-dependent cell killing. Upon infection, vaccinia viruses, as many other viruses, down-regulate the surface exposure of MHC-I molecules, thus minimizing the presentation of viral antigens to immune cells. NK cells are able to recognize infected cells from healthy cells by a so-called "missing-self recognition": it has been shown that NK cells can recognize cells that express low levels of MHC I on their surface indicating viral infection (or cancer). Indeed, depletion of NK cells has been shown to enhance the virulence of vaccinia virus (Bukowski et al. 1983).

As the infection leads to elevated immunoreactivity in the infection site, the increased amounts of pro-inflammatory cytokines, chemokines and activated innate immune cells can not only induce immune responses against the virus but also against the infected cells, e.g. tumor cells in the case of cancer therapy (Chan & McFadden 2014). This advantage can be exploited e.g. in the concept of oncolytic cancer vaccines where the virus is used as an adjuvant.

4.2.3.2. Adaptive immune responses

Advanced, cellular responses are also developed later following the VV infection. Antigen presenting cells, such as dendritic cells (DCs) orchestrate the development of the adaptive

immune responses by presenting antigens to other immune cells. DCs can engulf free VV particles and process them for presentation on their cell surface receptors. These exogenously derived antigens are loaded on MHC II molecules and presented to CD4+ helper T cells (Th) (Guidotti & Chisari 2001). The activated Th cells can then stimulate B cells to produce antibodies against the viral antigen. When a tumor cell is lysed after VV multiplication, antigens of both the endogenously produced virions and the lysed tumor cell (self and cancer-specific antigens) will spread to the cell surroundings and the antigens can be phagocytized by DCs. Before lysis the infected cell can present viral antigens and tumor antigens on their MHC I molecules. Immature dendritic cells are also able to phagocytize pieces of cell membrane, a process called nibbling. DCs migrate to lymph nodes where they mature and present the endogenously derived antigens on MHC I to CD8+ cytotoxic T cells (CTLs). When activated, these CTLs can migrate to the infection site (tumor) and kill the target cell. The antigen-specific T and B cells may further become memory cells that retain long-lasting immunity against the virus or the tumor.

VVs have developed mechanisms to suppress development of Th responses, i.e. VVs are naturally not so efficient in cross-priming antigen-specific immune responses (Xu et al. 2004, Smith et al. 2013). However, some oncolytic VVs with enhanced immunogenic potential have shown to enhance activation of adaptive immune responses, and even an increase of anti-tumor CTLs has been observed in infections with CD40L expressing VV (Parviainen et al. 2014).

4.2.4. Translation to clinics (clinical studies with oncolytic vaccinia viruses)

The wide tropism to various tissues including most cancer types and the naturally lytic life cycle exhibited by VV make it appealing to use as an oncolytic agent. The different strains of VV, Western Reserve, Wyeth, Lister and Copenhagen are currently being developed as oncolytic virotherapeutics for several types of cancer, including melanoma, hepatocellular carcinoma and lung cancer (Chan & McFadden 2014). The highly attenuated strains Modified Vaccinia Ankara (MVA) and New York Vaccinia (NYVAC) do not replicate in mammalian cells, thus lacking utility in oncolytic applications. The Western Reserve strain seems to bear the strongest efficacy *in vitro* and *in vivo* (Naik et al. 2006) and has been safely used in humans (Zeh et al. 2015).

The first clinical trial using vaccinia virus to treat cancer was in 1964, when wild-type VV was repeatedly injected into melanoma lesions. The results were encouraging, almost 50% of the 44 treated patients had objective tumor response and even 25% of the cases had complete regression (Burdick & Hawk 1964, Hunter-Craig et al. 1970, Thorne, Bartlett & Kirn 2005). Afterwards, several trials for testing the safety and efficacy of oncolytic vaccinia viruses were conducted. At the moment the most studied drug candidate is a TK-deleted Wyeth strain VV that expresses human GM-CSF, called Pexa-Vec (Pexastimogene devacirepvec, also known as JX-594, Jennerex Inc. & co.). Pexa-Vec treatments have been well tolerated up to MTD of 1 x 10^9 pfu (Park et al. 2008, Merrick, Ilett & Melcher 2009) and the virus has been tested so far for treating melanoma (Mastrangelo et al. 1999) and

hepatocellular carcinoma (HCC) (Park et al. 2008, Merrick, Ilett & Melcher 2009) in phase I and II trials. Pexa-Vec has shown immunological responses, like inflammation and infiltrated eosinophils and lymphocytes in the injected tumors as well as perivascular lymphocyte infiltration (Mastrangelo et al. 1999). Intriguingly, responses were seen also in non-injected tumor lesions (Park et al. 2008, Merrick, Ilett & Melcher 2009). Pexa-Vec has been also tested in combination with a multikinase inhibitor drug sorafenib (Nexavar, Bayer) for the treatment of HCC (Llovet et al. 2008). The results indicated that Pexa-Vec could sensitize the tumor cells for sorafenib treatment leading to fast necrosis of treated tumors (Heo et al. 2011).

There has also been a completed phase I trial with a double-deleted (TK- and VGF-deleted) Western Reserve strain oncolytic vaccinia virus that expresses cytosine deaminase (CD) and somatostatin receptor (SR). This vvdd-CDSR (also known as JX-929) virus did not show dose-limiting toxicity with doses up to 3×10^9 pfu and some evidence of anti-tumor immunity has been reported (Zeh et al. 2015). The side effects seen in the clinical trials are usually mild flu-like symptoms including fever, fatigue, musculoskeletal pain, nausea, and vomiting. Currently the oncolytic VVs in ongoing clinical trials include the above-mentioned Jennerex viruses and another virus developed by Genelux, GL-ONC1. GL-ONC1 is an attenuated Lister strain VV. It was well tolerated with minimal toxicity in the phase I trial where it also showed preliminary evidence of anti-tumor activity (NCT01584284).

4.2.5. Safety concerns

Normally, vaccinia virus infections are mild in otherwise healthy people causing only mild rash and fever. But in immunocompromised individuals wild-type vaccinia viruses may cause even life-threatening complications: encephalitis, vaccinia necrosum, eczema vaccinatum, or roseola vaccinia (Isaacs 2012). It has to be taken into consideration with VV therapies and vaccinations that the treated person may disseminate the virus through the sites of infection and through secretions. VV is quite stable in the environment. Infectious VV has been recovered from a scab collected from the site of small pox vaccination even after storing it at room temperature for 13 years (Wolff & Croon 1968). VV can also remain infectious in +4°C water for at least four months (Essbauer et al. 2007) and infectious VV has been found in feces, urine, and intestines of infected mice (Ferreira et al. 2008). Normally wild-type VV spreads through contact with eyes or wounds in the skin, so safety precautions must be followed if working with VVs. However, the genetically modified oncolytic vaccinia viruses are much less pathogenic than the wild-type VV, since they are able to replicate only in cancer cells. Moreover, the double deleted VV (-VGF, -TK) has a low risk of recovering wild-type functions by homologous recombination with the wt virus. The double-deleted (vvdd) virus backbone has been used already in clinical trials and has been reported to be safe (Breitbach et al. 2011, Zeh et al. 2015). The tumor-selectivity of this viral backbone has already been proven decades ago (Buller et al. 1985, Gnant et al. 1999, Thorne et al. 2007).

Fortunately, there are several antiviral agents available, like vaccinia immunoglobulin and Cidofovir that are effective against undesirable replication in patients. In case adverse events or excessive viral replication occurs after VV therapy, these drugs can be used to stop the virus from spreading.

4.3. Armed oncolytic viruses and cancer immunovirotherapy

To further improve the efficacy of an oncolytic virus, an anti-tumor transgene can be integrated into the viral genome (Alemany 2007). The transgene can code for example for a directly cytotoxic protein, an anti-angiogenic (Yoo et al. 2007, Buckel et al. 2013) or a pro-apoptotic protein (Sathaiah et al. 2012). Also suicide genes, which convert non-toxic prodrugs to cytotoxic compounds, have been introduced in oncolytic viruses (Dias et al. 2010, Price et al. 2010).

Formerly it was thought that the host immune response against the oncolytic virus treatment only hinders the efficacy of the therapy and was considered an unwanted side effect. It was even proposed to use immunosuppressive drugs in combination with the OV therapy to minimize the anti-viral responses. For example cyclophosphamide, an alkylating agent that can inhibit innate immunity and down-regulate regulatory T-cells, has been used to enhance virotherapy (Di Paolo et al. 2006, Fulci et al. 2006, Ghiringhelli et al. 2007). However, the current approach considers that the immune system is “a friend” and OVs are even considered immunotherapy agents (Alemany & Cascallo 2009).

It has been recently observed that oncolysis by oncolytic viruses can trigger immunogenic cell death that leads to release of danger-signals (DAMPs) (Liikanen et al. 2013, Parviainen et al. 2014, Diaconu et al. 2012, Workenhe & Mossman 2014). Oncolysis also releases tumor-associated antigens to the tumor microenvironment. These, together with sufficient cytokine stimuli after the virus infection can outbalance the immunotolerance of the advanced tumors. This can eventually promote the mounting of antitumor immune responses (Melcher et al. 2011, Steele et al. 2011) (**Figure 8**). Furthermore, the anti-viral immune responses can actually nowadays be seen as beneficial. It has been shown that anti-viral T cells are able to attack the infected tumor cells thus enhancing the anti-tumor responses (Tuve et al. 2009). It has indeed been demonstrated in several preclinical experiments with various tumor models and with different oncolytic viruses that OVs are able to promote the activation of anti-tumor immune responses (Tuve et al. 2009, Diaz et al. 2007, Prestwich et al. 2009, Fukuhara & Todo 2007, Choi et al. 2006, Parviainen et al. 2014, Diaconu et al. 2012). Immune activation at the tumor site has been shown to correspond to treatment benefits in humans as well (Zamarin & Pesonen 2015, Lichty et al. 2014).

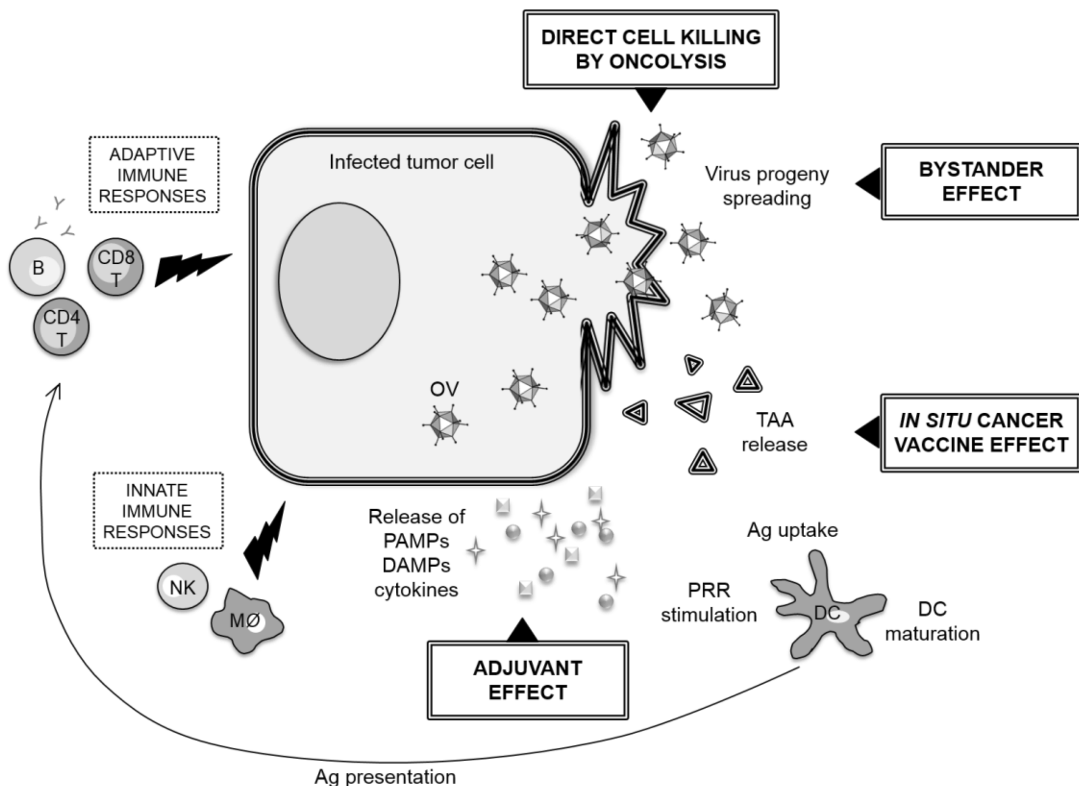


Figure 8*. *Oncolytic immunovirotherapy. *Infection of a tumor cell by an oncolytic virus triggers several anti-tumor actions. First of all, oncolytic viruses induce direct cell killing by oncolysis. The new virions released after lysis of the cell can trigger a so-called bystander effect by spreading to, replicating in, and killing the surrounding tumor cells. Oncolytic viruses can also act as adjuvants, as infection induces production and release of different cytokines and danger signals that can activate and attract immune cells to the tumor site. Tumor cell lysis also releases tumor-associated antigens ("in situ cancer vaccination"), which can be picked up by dendritic cells. The matured DCs can then cross-present the antigens to T and B cells; this can lead to activation of long-term adaptive anti-tumor immune responses. Abbreviations: Ag = antigen; B = B cell; CD4 T = CD4+ helper T cell; CD8 T = CD8+ cytotoxic T cell; DC = dendritic cell; MØ = macrophage; NK = natural killer cell; OV = oncolytic virus; PAMP/DAMP = pathogen/damage associated molecular pattern; TAA = tumor-associated antigen.*

4.3.1. Arming with cytokines

Nowadays oncolytic viruses are increasingly modified to become even more immunogenic and they are used to promote the activation of anti-tumor immune responses (Lichty et al. 2014). Immunostimulatory molecules have been inserted into the viral genome to further enhance the immune cell attack against infected cancer tissue (Chang et al. 2009, Cerullo et al. 2010). For instance, oncolytic viruses expressing interleukins, such as IL-12, IL-4 or IL-24 have shown to enhance anti-tumor immune responses in murine models (Lee et al. 2006, Zhang et al. 2009, Post et al. 2007). Also in studies using an oncolytic adenovirus

expressing RANTES the researchers observed recruitment of dendritic cells, NK cells, macrophages, and CTLs into the tumor (Lapteva et al. 2009).

Maybe the most frequently applied immunostimulatory molecule is the granulocyte-macrophage colony-stimulating factor (GM-CSF), which has been used to arm many different oncolytic viruses and actually the leading OV candidates harnessed in recent clinical trials, like Pexa-Vec (Breitbach et al. 2015b), T-VEC (Andtbacka et al. 2015, Senzer et al. 2009), ONCOS-102 (Vassilev et al. 2015) and CG0070 (Burke et al. 2012) express GM-CSF. GM-CSF can mediate antitumor effects by recruiting natural killer cells and by activation of tumor-specific cytotoxic T-lymphocytes as it stimulates proliferation and maturation of dendritic cells (Cerullo et al. 2010). However, its high systemic concentrations can activate immunosuppressive myeloid-derived suppressor cells (MDSCs) (Serafini et al. 2004), which may hinder the therapeutic efficacy of oncolytic viruses.

Also CD40-ligand (CD40L) has been used as an immunomodulatory transgene to arm oncolytic viruses (Gomes et al. 2009, Parviainen et al. 2014, Diaconu et al. 2012). CD40L acts as a costimulatory ligand mostly on the CD4+ T cell surface, whose binding to the CD40 receptor on APCs and B cells enables antigen presentation (Grewal & Flavell 1998, van Kooten 2000). CD40L expression by an OV can also induce apoptosis (Gomes et al. 2009, Diaconu et al. 2012, Loskog, Fransson & Totterman 2005) as well as immunogenic cell death in preclinical models (Parviainen et al. 2014, Autio et al. 2014). An oncolytic Ad expressing CD40L was already shown to be safe and to induce T cell immune responses in patients with solid tumors (Malmstrom et al. 2010, Pesonen et al. 2012b).

Arming an oncolytic virus with a cytokine has advantages over the traditional method of systemic cytokine therapy. Local replication of the virus restricts the expression of the cytokine to the target site thus reducing systemic toxicity often related to cytokine therapies (Arellano & Lonial 2008).

4.3.1.1. Tumor necrosis factor alpha (TNFα)

Tumor necrosis factor alpha (TNFα) is a multifunctional cytotoxic molecule that has multiple properties of a powerful anti-cancer agent. In fact, already in 1975, TNFα was noticed to have anti-cancer properties (Carswell et al. 1975, Old 1985). It has also been proposed that TNFα is the “active ingredient” in Coley’s toxin (Old 1985, Balkwill 2009). In the first clinical studies performed with recombinant TNFα in the 1980s, its antitumor properties were confirmed but it also led to severe systemic toxicity, such as septic shock or even death (Mueller 1998, Schiller et al. 1991). Thus, systemic use was soon abandoned and its only current clinical use is in isolated limb perfusion (Lienard et al. 1992, Eggermont, de Wilt & ten Hagen 2003).

TNFα can induce tumor cell death directly by apoptosis and necrosis (Kobelt et al. 2014, Rath, Aggarwal 1999); it induces apoptosis via TNF receptor 1 (TNF-RI) (Ashkenazi, Dixit 1998, Salako et al. 2011) and it can induce secretion of blood clotting and adhesion

proteins in vascular endothelium resulting in vascular thrombosis and necrosis of the tumor tissue (van Horssen, Ten Hagen & Eggermont 2006). It can also stimulate release of other cytokines (such as GM-CSF, IFN-γ, IL-6, IL-1 and IL-8) and activate and recruit immune cells, including macrophages, neutrophils, DCs, T cells and NK cells (van Horssen, Ten Hagen & Eggermont 2006, Fiers 1991). TNFα is also able to synergize with radiation and chemotherapy by increasing the apoptotic and necrotic cell death and vascular damage (Seki et al. 2011, Balkwill 2009, Hallahan et al. 1990, Mauceri et al. 2009). These multiple cytotoxic mechanisms make TNFα an appealing molecule to be used to enhance oncolytic virotherapies. Introducing TNFα as a transgene expressed by a viral vector enables its delivery and production locally and therefore reduces systemic toxicity. For example TNFerade (GenVec, Inc.), a replication deficient adenovirus with human TNFα expression and a radiation-inducible promoter, has already been studied in phase I, II, and III trials. It was well tolerated and showed good response rates in preclinical studies and in phase I and II trials, but unfortunately failed to show a statistically significant efficiency in the phase III trial of advanced pancreatic cancer (Mauceri et al. 2009, Fisher 2011).

Interestingly, TNFα has been shown to increase viral entry into tumors by extravasation through a Rho A/Rho kinase pathway (Seki et al. 2011) and actually many cell types are known to become highly susceptible to the cytotoxic effects of TNFα after viral infection (Fiers 1991). However, at least adenoviruses have evolved a mechanism to protect the cell from apoptosis, they encode two inhibitor proteins, E3-14.5K/10.4K complex and E3-14.7K, which can inhibit TNFα and Fas mediated cytolysis and apoptosis (Li et al. 1999, Chen et al. 1998).

4.3.2. Improving the viral immunogenicity by stimulating pattern recognition pathways

In addition to arming the virus with a single cytokine, another way to improve the immunogenicity of the viruses is to arm them with molecules that can orchestrate the regulation of a set of different cytokines. This strategy has potential to generate a more robust immunological response (Lladser et al. 2011, Smith et al. 2013). For example a non-replicating adenovirus was armed with myeloid differentiation primary response gene 88 (MyD88) to amplify the TLR-mediated adenovirus immune response (Hartman et al. 2010). MyD88 acts as a universal adapter protein for almost all TLRs (except TLR-3) to activate NF-κB. Overexpression of MyD88 by an adenoviral vector was shown to enhance adaptive immune responses and to inhibit local tumor immunosuppression, resulting in significantly inhibited local and systemic growth of multiple tumor types (Hartman et al. 2010). MyD88 was also used as a genetic adjuvant to enhance intrinsic immunogenic properties of a DNA vaccine (Ullas, Desai & Madhusudana 2014). Also another approach to stimulate TLR-9 signaling has been explored. Cerullo et al. introduced repeated CpG sequences into the oncolytic adenovirus backbone, which was shown to increase anti-tumor T cells and to decrease the activation of MDSCs (Cerullo et al. 2012). Similarly oncolytic parvovirus

carrying CpG islands showed enhanced immunogenicity, induced markers of cellular immunity and dendritic cell activation (Raykov et al. 2008).

Arming the virus with a molecule that induces production of anti-viral cytokines may sound controversial; however, it has been shown that arming viruses with e.g. some anti-viral cytokines like IFN-γ or type I interferons have potential to enhance also anti-tumor responses (Li, Huang & Kung 2005, Chiocca et al. 2008, Sterman et al. 2010, Odaka et al. 2002), perhaps by sending alarm signals to immune cells in the tumor site and by stimulating expression of MHC molecules on cancer cells (Propper et al. 2003).

4.3.2.1. DNA-dependent activator of interferon-regulatory responses (DAI)

DAI, DNA-dependent activator of IFN-regulatory responses (also known as Z-binding protein, ZBP1 or DLM-1) is a sensor of double-stranded DNA. It is a pattern recognition receptor (PRR) located in the cytoplasm of cells and acts as a potent activator of innate immune responses (Takaoka et al. 2007, Wang et al. 2008). DNA sensing by DAI consequently activates NF-κB and interferon regulatory factor 3 (IRF3) signaling pathways (Zhang et al. 2013), which first leads to production of IFN-β. IFN-β promotes gene expression of IFN-inducible genes and eventually leads to production of IFN-α, chemokines and pro-inflammatory cytokines (Lladser et al. 2011, Wang et al. 2008). The secretion of pro-inflammatory molecules and type I IFNs may ultimately promote adaptive T cell responses. Lladser et al. actually showed that a DAI-expressing plasmid could efficiently be used as a genetic vaccine to generate a pro-inflammatory microenvironment essential for activation of antigen-specific T cell responses and long-lasting antitumor immunity in tumor-bearing mice (Lladser et al. 2011). DAI has been reported to sense DNA of different viruses, at least adenoviruses (Schulte et al. 2013) and HSVs (Triantafilou, Eryilmazlar & Triantafilou 2014, Pham et al. 2013, Furr et al. 2011).

5. Personalized cancer therapies

Today it is known that an individual´s genetic background can influence responsiveness to treatments and drugs, as well as susceptibility to certain diseases. The Human Genome Project, a large-scale automated sequencing project begun in 1984 and completed in 2003, enabled assessment of the entire genetic code of a man. Together with the fast development of techniques, such as genome-wide association studies (GWAS) and genotyping, the Human Genome Project has enabled fast identification of genetic variations between individuals for diagnostic and therapeutic purposes (Low et al. 2014). The concept of “personalized medicine” is emerging as a new way to ensure safer and more efficient treatments. It may also provide earlier diagnoses of diseases if predictive biomarkers are found to correlate with a disease (Rodriguez-Antona & Taron 2015).

The genomic information of cancer patients and especially their tumors is essential as many novel cancer therapies rely on certain mutations. Thus, tumor genotyping aimed at

matching the patient with the right drug has been foreseen to become a clinical routine (Heuckmann & Thomas 2015).

As the regulatory authorities, such as the Food and Drug Administration and the European Medicines Agency are responsible for ensuring that drugs are safe and effective, the agencies have become increasingly interested in personalized medicine and are trying to integrate genetic and biomarker information with drug development for clinical use (FDA, Paving the Way for Personalized Medicine 2013). The FDA has suggested that patient-specific genetic background variation should be taken into account when dosing the drugs, and the FDA already requires certain genetic biomarker data on patients before the use of targeted drugs in order to ensure safety and correct dosing.

There are some ethical issues that have been raised since the rapid accumulation of personal genetic information. Patient privacy needs to be respected, for instance the questions about the owner rights to the results and data from the gene tests. For example, is it good for a person to know about a possible predisposition to disease? Or, should other family members also have the right to know about genetic predispositions or risks? It should also be ensured that the test results are well explained to the patients by educated professionals and that the information will not eventually be misused by employers or insurers.

The efficacy of oncolytic virotherapies also seems to vary between patients, some benefitting more than the others. The reason for this remains unknown, but it can be presumed that patients' genetic background and for example differences in the immune system functions may play a role.

5.1. Finding the right patients for a given therapy

Researches are with increasing energy trying to find biomarkers that would help predict treatment responses to enable better targeted use of drugs. This is especially important in the field of cancer therapies, since these drugs often cause serious side effects. Thus the use of drugs with predicted inefficiency or toxicity in given patients should be avoided.

Assessing cancer biomarkers for each patient would markedly increase diagnostics costs though, and drugs that would benefit only a small cohort of patients would of course become more expensive. Personalized medication could, however, also have clear economic benefits. The ability to identify patients who benefit most from a treatment would reduce unnecessary costs caused by ineffective or toxic drug use. Use of genetic information would also enable more refined selection of patients for clinical tests and trials resulting in smaller cohorts, and faster throughput and would increase the probability of drug approval.

5.1.1. Biomarkers

Biomarkers are measurable indicators used for diagnostic, prognostic or therapeutic purposes (Strimbu, Tavel 2010, Biomarkers Definitions Working Group. 2001). Commonly,

biomarkers are changes in the expression levels of certain proteins known to correlate with susceptibility to or progression of a disease, or with the treatment responsiveness. However, genetic variations (polymorphisms, copy number variations, chromosomal abnormalities and epigenetic variations) or anatomical and physiological phenotypes can be considered biomarkers as well.

A commonly used diagnostic and/or prognostic biomarker in cancer medicine is the prostate-specific antigen (PSA), which is overexpressed in most prostate cancers and some other cancers too (Duffy 2014). PSA levels in blood correlate with progression to prostate cancer. Other well-known diagnostic biomarkers are mutations in the tumor suppressor genes BRCA 1 and 2. These mutations indicate risk to at least breast and ovarian cancers (Struewing et al. 1997).

Cancer tissues are highly heterogeneous and bear often multiple unique, but also some common mutations found among different cancers. The realization that these mutations cause a differentiation of cancer cells from normal cells has led to the development of cancer-specific drugs that use the mutated phenotype of the cancer. These drugs are designed to work only if the mutation occurs, thus there are several biomarkers defined for various cancer therapeutics to predict the effectiveness of a certain drug in the patient. There are already several cancer drugs that have been approved for the use in patients whose tumors display specific genetic characteristics. Maybe the most familiar example of targeted cancer drugs is trastuzumab (trade names Herceptin and Herclon), which only works in cancers that overexpress HER2 (human epidermal growth factor receptor 2) protein. Slamon et al. discovered in 1987 that mutation in the HER2 gene could cause overexpression of HER2 on the surface of breast cancer cells (Slamon et al. 1987). This overexpression has been observed to occur in about 20-30% of breast cancers and it has been associated with an aggressive form of the disease (Slamon et al. 1987). These observations made HER2 an appealing biomarker and target for breast cancer treatment. Eleven years after (1998) Dr. Slamon’s discovery, the FDA approved trastuzumab for treating HER2-positive breast cancers. Trastuzumab is only useful for cancer patients with the HER2 mutation and can even cause harm to patients who do not overexpress the protein, thus underscoring the need for genetic screening prior to starting the trastuzumab therapy. Also several other cancer biomarkers have been discovered and are being used to assist cancer diagnosis and to develop targeted therapies as well as predict treatment responsiveness (Kelloff & Sigman 2012). For instance EGFR and KRAS are used as biomarkers for cetuximab therapy, BRAF for dabrafenib therapy (Rodriguez-Antona & Taron 2015), and mutations in the EGFR1 gene indicate patients' risk for non-small cell lung cancer (NSCLC) and the probability to respond to gefitinib or erlotinib therapies (Herbst et al. 2005, Lynch et al. 2004). Chronic myelogenous leukemia (CML) patients are known to feature a reciprocal translocation (“Philadelphia” translocation) between chromosome 9 and 22, which gives rise to an oncogenic fusion gene called BCR-ABL. BCR-ABL is used as a biomarker for CML, and a specific drug, a tyrosinase kinase inhibitor called imatinib (trade

names Gleevec and Glivec) has been developed to specifically target and inhibit proliferation of BCR-ABL-expressing hematopoietic cells (Moen et al. 2007).

5.1.2. Polymorphisms

Polymorphisms are genetic variations between individuals. Polymorphisms are often related to the susceptibility to many diseases and severity of the illness. They can also be the reason why some patients respond differently to drugs, chemicals, vaccines or pathogens for instance. Some, for example, metabolize certain drugs faster than others. Usually polymorphisms are variations of single nucleotides (SNPs) in the genome. Nowadays the role of polymorphisms (SNPs) in therapy responsiveness is widely recognized and they are studied for personalized medicine purposes (Low et al. 2014).

5.1.2.1. Fc gamma receptor polymorphisms

Fc gamma receptors (FcγR) are cell surface receptors expressed on leukocytes. They play an important role in host defense and immune regulation mechanisms; they serve as a link between humoral and cell-mediated immune systems (Li et al. 2009, Forthal & Moog 2009). FcγRs bind to the Fc part of immunoglobulin G (IgG).

FcγRs are divided into three classes, FcγRI, FcγRII and FcγRIII, which differ in structure, cell distribution and affinity to different IgG subtypes. The classes can be split into subclasses, indicated with letters (a, b or c). FcγRI exhibits high affinity to monomeric IgG, while FcγRII and FcγRIII bind effectively only aggregated, multimeric IgG, which is bound to an antigen (van Sorge, van der Pol & van de Winkel 2003).

Depending on whether the FcγRs stimulate or inhibit the effector leukocyte functions, they can be defined as activatory or inhibitory receptors depending on their signaling subunit type. FcγRIIb is the only inhibitory receptor subtype having an ITIM (immunoreceptor tyrosine-based inhibition motif) signaling unit in its cytoplasmic domain. All other FcγRs are activatory receptors and they have an ITAM subunit (immunoreceptor tyrosine-based activation motif). The function of the effector cell is determined by the ratio of inhibitory and activatory receptors activated (Nimmerjahn & Ravetch 2008). When the FcγR binds to an antigen-bound IgG, the ITAM/ITIM signaling unit becomes phosphorylated by tyrosine kinases, which then leads to changes in the effector cell functions. The effector cells (i.e. leukocytes) can destroy the antigen-antibody complex by multiple mechanisms: phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), degranulation, cytokine and chemokine expression or activation of B cells to produce antibodies (Cohen-Solal et al. 2010, Nimmerjahn & Ravetch 2008, van Sorge, van der Pol & van de Winkel 2003).

Altogether eleven polymorphic regions have been identified in the Fc gamma receptor genes (Bournazos et al. 2009). The polymorphisms have an effect on the binding affinity to IgG and thus on the efficacy of immunological defense mechanisms (van Sorge, van der Pol & van de Winkel 2003). As Fc gamma receptors serve as a crucial link between the innate and adaptive branches of the immune system, their polymorphisms are commonly accepted to have potential in predicting the outcome of cancer immunotherapies (Concetti

& Napolioni 2010). FcγRIIa and FcγRIIIa subclasses are the most common receptor types and play important roles in the immune regulation. Polymorphisms found in these genes are often associated with diseases and disease severity in previous studies.

FcγRII is the most widely distributed Fc gamma receptor among different cell types. FcγRIIa (CD32a), one of the FcγRII subtypes, has a wide cellular distribution. It is mainly expressed on phagocytic cells such as macrophages and neutrophils (Huang et al. 2011), but is also found on platelets (Li et al. 2009). A SNP in the amino acid position 131 in the FcγRIIa receptor´s extracellular ligand-binding domain, FcγRIIa-H131R (rs1801274), alters the binding affinity of the receptor to IgG between the two possible receptor allotypes. This missense mutation (adenine (A) to guanine (G)) results in an amino acid change (histidine (H) to arginine (R)). The FcγRIIa-R allotype binds weaker to IgG than the ancestral H-allotype, thus, individuals homozygous for FcγRIIa-H are more effective in clearing Ag-Ab immunocomplexes from the body than FcγRIIa-R homozygous individuals. Heterozygous individuals (FcγRIIa-HR) have effector cells bearing both allotypes of the receptor and are referred to as "intermediate immune responders" (van Sorge, van der Pol & van de Winkel 2003).

FcγRIIIa receptors instead, are found mostly on NK cells, but also on tissue-specific macrophages (e.g. Kupffer cells in the liver) and on small subsets of monocytes, γδ T cells and DCs. Another polymorphism in FcγRIIIa, V158F (rs396991), also leads to different IgG binding affinity between the two different isoforms of the receptor. A thymine (T) to guanine (G) codon missense mutation in the amino acid position 158 changes the valine (V) to phenylalanine (F) in the ligand-binding domain of the receptor. Of these two allotypes, the V allotype binds stronger to IgG than the F form (van Sorge, van der Pol & van de Winkel 2003).

SNPs in FcγRIIa and FcγRIIIa have been associated with susceptibility to various diseases, such as autoimmune diseases (Ravetch & Bolland 2001, Dijstelbloem, van de Winkel & Kallenberg 2001), infectious diseases (Pleass & Woof 2001) and coagulation disorders (Forthal & Moog 2009). They have also been linked to responsiveness to immunotherapies, such as mAb therapies (Clynes et al. 2000, Musolino et al. 2008) and cancer vaccines (Weng, Czerwinski & Levy 2007, Wang et al. 2010). Usually individuals carrying the allotype that binds weaker to IgG (FcγRIIIa-F or FcγRIIa-R allotypes) are more susceptible to certain immunologic diseases or more severe disease because of insufficient clearance of immunocomplexes (Li et al. 2009). Surprisingly though, the strong-binding FcγRIIIa-V allotype was associated with the severity and progression of systemic lupus erythematosus (SLE) an autoimmune disease affecting intracellular structures. This was supposed to be due to more fulminant local inflammatory responses in the V allotype carrying patients (Alarcon et al. 2006). However, on the contrary, the weakly binding R allotype of FcγRIIa was related to higher risk and disease severity of SLE (Duits et al. 1995, Karassa et al. 2002, Haseley et al. 1997). Furthermore, the FcγRIIa-RR genotype was associated with progression of HIV infection (Forthal et al. 2007), whereas it was shown to be beneficial for

anti-TNFα therapy (infliximab) for rheumatoid arthritis because of slower clearance of Ab-Ag complexes (Canete et al. 2009). It has actually been shown in multiple studies that FcγR-mediated ADCC plays a clear role in the efficacy of mAb therapies (Gianni 2008, Musolino et al. 2008). For example, Musolino et al. reported that the "strong-binding" genotypes FcγRIIIa-VV and FcγRIIa-HH both correlate with an objective response rate and progression-free survival of trastuzumab treated breast cancer patients; also the combination of these genotypes, VVHH was found beneficial. They actually showed that the PBMCs of these patients induce higher trastuzumab-mediated ADCC (Musolino et al. 2008). Usually the results logically indicate that the patients homozygous for the strong-binding allotypes benefit more from the mAb therapies, however, there are conflicting data for the genotype providing the best outcome (Bibeau et al. 2009, Zhang et al. 2007, Pander et al. 2010).

NK cells are considered the most important mediators of innate immune responses against malignancies. Triggering of FcγRIIIa on NK cells induces ADCC and lymphokine production to defend the organism against viral infections and malignancies (Iwasaki et al. 2011, van Sorge, van der Pol & van de Winkel 2003). FcγRIIIa on macrophages (e.g. Kupffer cells in the liver) are thought to play a role in the clearance of viruses (Li et al. 2009, van Sorge, van der Pol & van de Winkel 2003). Furthermore, FcγR-bearing immune cells can prevent viral infections by neutralization and degradation of cell-free viral particles in the circulation (Cotter, Zaiss & Muruve 2005, Leopold et al. 2006, Triulzi et al. 2010).

5.2. Tailoring the therapy for the patient

In addition to the selection of the right patients for defined therapies, personalized medicine may also be thought of as tailoring the treatment according to the specific characteristics and needs of the patient.

Formerly the ideology of medication relied more on a concept of "one-dose-fits-all" but nowadays it is generally thought that it would be safer and much more efficient to tailor the treatments individually for each patient. It is known that genetic variations can influence the pharmacokinetic (PK) profile of a drug. Knowing the PK variability in patients would enable more optimal dosing of drugs thus minimizing toxic side effects as well as too low, ineffective doses (Rodriguez-Antona & Taron 2015). The study of an individual's genome to provide a more tailored drug prescription is commonly referred to as pharmacogenomics.

The metabolic rate of a patient is known to highly influence the drug therapy efficacy and toxicity. Cytochrome P450 proteins (CYPs) are enzymes responsible of regulating the metabolism of most drugs. For example, the efficacy of tamoxifen cancer therapy has been shown to depend on the CYP2D6 phenotype. Patients with the CYP2D6*1 allele are slower metabolizers, thus the drug is less effective and can cause more serious side effects in these patients (Jin et al. 2005, de Leon, Armstrong & Cozza 2006).

In addition to more optimal dosing, cancer therapies can be designed to fit certain patients who e.g. express specific tumor-epitopes or antigens. There are several tumor-associated

antigens (TAAs) that are known to be present in various cancers. These TAAs are used to vaccinate cancer patients in several platforms (Melief & van der Burg 2008, Okada et al. 2015). There are currently several Phase III trials ongoing to test tumor-peptide vaccines (Aranda et al. 2013) for example to treat melanoma by a melanoma-associated antigen-A3 (MAGE-A3) vaccine (Kruit et al. 2013, Ulloa-Montoya et al. 2013) and to treat metastatic pancreatic cancer with a telomerase reverse transcriptase peptide $TERT_{611–626}$ (GV1001) vaccine (Kyte 2009). Also for gliblastoma an EGFRvIII peptide is going through phase III testing. The EGRFvIII vaccination has shown promising responses in newly diagnosed glioblastoma patients prolonging both PFS and OS compared to historically matched controls (Batich & Sampson 2014). Even though the tumor peptide vaccines have shown promising results in some patients, they still suffer from low efficacy in most cases, especially in cases of advanced diseases (Middleton et al. 2014). This may be due to the highly immunosuppressed tumor microenvironment, and thus the peptide vaccines need a powerful adjuvant that could break the immunosuppression (Rhee et al. 2011). Several adjuvants to enhance peptide vaccinations have been tried, such as CpG oligodeoxynucleotides (Murad et al. 2007, Ashkar & Rosenthal 2002), poly-ICLC (Ming Lim et al. 2013), and IFN-α (Kameshima et al. 2013, Zeestraten et al. 2013). Also the low antigen presentation by tumor cells can hinder the effect of peptide vaccines (Aranda et al. 2013).

Because of the tumor heterogeneity and the constantly changing chemical, physical and cellular environment in the tumors, there will never be one cancer drug that would work in all cancer cases. Finding a way to design the drug individually for each case taking into account the patient- and tumor-specific characteristics would perhaps offer even safer and more efficient cancer therapies. Researchers have already started to establish methods to identify specific antigens from each individual tumor that can be used as cancer immunotherapeutics (Admon & Bassani-Sternberg 2011). Tumor cells produce disease-associated proteins, which are presented as foreign antigens on the tumor cell surface by MHC molecules, which can then be recognized by specific CTLs. This presented peptide repertoire (immunopeptidome) is therefore a very valuable source for antitumor vaccine development (Purcell, McCluskey & Rossjohn 2007). There are already some protocols established to study the immunopeptidomes from tumors by extracting the peptides from MHC molecules and analyzing the yielded peptide pools by LC-MS/MS (Antwi et al. 2009, Fortier et al. 2008). Still, it is challenging to distinguish peptides that are truly tumor-specific to avoid possible auto-immune and inflammatory responses due to undesirable use of self-peptides (Marcilla & Lopez de Castro 2008) and to find the peptides that are most immunogenic.

6. AIMS OF THE STUDY

The aim of the thesis studies was to *improve the efficacy of oncolytic virotherapies* by **A)** boosting the immunogenicity of the viruses in order to enhance the activation of anti-tumor immune responses and by **B)** finding means to personalize the therapies to provide the right treatment for the right patients.

The more specific aims for each study are:

Study I: To boost immunogenicity and cytotoxic potential of an oncolytic adenovirus by arming it with TNFα.

Study II: To improve the vaccine and adjuvant potency of an oncolytic vaccinia virus by arming it with a self-recognizing receptor DAI.

Study III: To study the role of FcγR polymorphisms in the efficacy of oncolytic virotherapy to find the patient populations that would benefit the most from the oncolytic adenovirus treatments.

Study IV: To develop a novel oncolytic vaccine platform (PeptiCRAd) that carries tumor-associated antigens on the adenoviral surface to induce tumor-specific immune responses.

7. MATERIALS AND METHODS

The studies in which the following methods have been used are marked with the corresponding Roman numerals in parentheses.

7.1. Cell lines

All cells were cultured in the recommended conditions and maintained in humidified 37°C incubators with 5% CO_2. 293, 911, A549, PC-3MM2, UT-SCC8, CACO-2, A2058 and HS294T cells were cultured in DMEM supplemented with 10% FBS, 1% L-glutamine and 1% penicillin-streptomycin antibiotics. RPMI-1640 with 10% FBS and 1% L-glutamine and 1% penicillin-streptomycin was used for B16-F10, B16-OVA, EJ, THP-1, Jurkat, MDA-MB-435 and WEHI-13VAR cells. EMEM with 1% L-glut and 1% Pen-Strep was used for SK-MEL-2 and CV-1 cells. Some cell lines needed additional supplements. The growth medium of PC-3MM2 cells was supplemented with 1% sodium pyruvate, UT-SCC8 with 1% non-essential amino acids and B16-OVA with 5mg/ml G-418 (Gentamicin).

Characteristics of the cell lines used are described in **Table 1**.

Table 1. Cell lines used in studies.

Cell line (name)	Species	Description	Source	Used in study
293 (HEK-293)	human	embryonic kidney cells	ATCC[1]	I
911	human	embryonic retinoblasts	ATCC	I
A2058	human	melanoma	ATCC	II
A549	human	lung adenocarcinoma	ATCC	I, II, IV
B16-F10	mouse	melanoma	ATCC	II, IV
B16-OVA	mouse	melanoma, expresses chicken ovalbumine	Dr. Vile[2]	I, II, IV
CACO-2	human	colorectal adenocarcinoma	ATCC	IV
CV-1	monkey	kidney epithelial cells	ATCC	II
EJ	human	bladder carcinoma	Dr. Eliopoulos[3]	I
HS294T	human	melanoma	ATCC	II, IV
Jurkat	human	immortalized T lymphocytes	Dr. Tienari[4]	II
MDA-MB-435	human	breast cancer	ATCC	I
PC-3MM2	human	prostate adenocarcinoma	Dr. Fidler[5]	I, II
SK-MEL-2	human	melanoma	ATCC	II, IV
THP-1	human	monocytes	ATCC	II
UT-SCC8	human	head and neck squamous cell carcinoma	PromoCell[6]	I
WEHI-13VAR	mouse	fibroblasts, sensitive to TNFα	ATCC	I

[1]American Type Culture Collection, Manasas, VA, USA
[2]Provided by Professor R. Vile, Mayo Clinic, Rochester, MN, USA
[3]Provided by Dr. A.G. Eliopoulos, University of Crete Medical School and Laboratory of Cancer Biology, Heraklion, Crete, Greece
[4]Provided by Dr. P. Tienari, Program of Molecular Neurology, University of Helsinki, Helsinki, Finland
[5]Provided by Dr. I. J. Fidler, MD Anderson Cancer Center, Houston, TX, USA
[6]PromoCell GmbH, Heidelberg, Germany

7.2. Viruses

7.2.1. Adenoviruses

Adenoviruses used in the studies of this thesis were serotype 5 viruses and genetically modified (except the wild-type Ad300Wt control) to be cancer specific by a 24 bp deletion (Δ24) in the constant region CR2 of the E1A gene. This deletion averts binding of E1A to Rb protein, which then disrupts the viral replication in normal cells, whereas in cancer cells the replication is still possible despite the deletion since in most of the cancers (Sherr 1996) the Rb-p16 pathway is continuously active because of the Rb mutation (see **Figure 4**).

Some viruses were also modified for increased transductional targeting to tumor cells by changing the knob structure of the fiber of serotype 5 to a serotype 3 knob. These viruses are thus called 5/3 chimeric viruses.

The replication-deficient adenovirus, Ad5/3-Luc1, used as control, is an E1 and E3 deleted first-generation adenovirus. The luciferase transgene is located in the deleted E1 under the control of a cytomegalovirus (CMV) promoter.

The replication-competent i.e. oncolytic adenoviruses were produced in A549 cells and the replication-deficient adenovirus, Ad5/3-Luc1, was amplified in 293 cells. Viruses were purified following a standard protocol on double cesium chloride gradients (Luo et al. 2007). The titers of the produced viruses were measured by OD_{260} spectroscopy to determine the amount of viral particle concentration (VP/ml) and by $TCID_{50}$ assay on 293 cells to determine the amount of infectious viral particles (pfu/ml). In Study IV the protein concentrations of the virus preparations were determined by Bradford assay (Bradford 1976) using the Biorad Protein Assay Dye Reagent Concentrate (Biorad Laboratories; Hercules, CA, US). Viruses were also characterized by PCR and restriction enzyme analysis for the presence of gene deletions and transgenes and for absence of wild-type virus contamination.

The viruses used to treat patients included in Study III were produced according to Good Manufacturing Practices (GMP) by Oncos Therapeutics, Inc. (Helsinki, Finland), which was regulated by the Gene Technology Board.

In Study I, we generated a new oncolytic adenovirus expressing human tumor necrosis factor alpha (hTNFα). To create the oncolytic Ad5/3-Δ24-hTNFα (see Figure 10), we constructed a pTHSN-hTNFα plasmid containing the human TNFα transgene in the E3 region of the adenoviral genome deleted for 6.7K/gp19K. To construct the pTHSN-hTNFα, pORF-hTNFα (InvivoGen, San Diego, CA, US) was digested with *SgrAI* and *MheI* restriction enzymes and then ligated with *BsiWI* and *MfeI*-linearized pTHSN. pAdEasy-1.5/3-Δ24-hTNFα was generated by homologous recombination in *Escherichia coli* BJ5183 cells (Qbiogene Inc., Irvine, CA, USA) between *FsbI*-linearized pTHSN-hTNFα and *SfrI*-linearized pAdEasy-1.5/3-Δ24 (Kanerva et al. 2005), a rescue plasmid containing the serotype 3 knob

and a 24-bp deletion in E1A. The genome of Ad5/3-Δ24-hTNFα was released by *Pac*I digestion and subsequently transfected into 911 cells.

All the adenoviruses used in the thesis studies are listed in **Table 2**.

Table 2. Adenoviruses used in the studies.

	Virus (name)	Transductional targeting (Fiber modification)	Transcriptional targeting (E1 gene modification)	Transgene (E3 gene modification)	Reference	Used in study
Replication deficient	Ad5/3-Luc1	Ad3 knob	E1 deleted	luciferase	(Kanerva et al. 2002b)	I
Oncolytic	Ad300Wt (wt Ad5)	Wt Ad5	wt	-	ATCC	I
	Ad5/3-Δ24	Ad3 knob	24bp deletion in E1A	-	(Kanerva et al. 2003)	I
	Ad5/3-Δ24-hTNFα	Ad3 knob	24bp deletion in E1A	human TNF alpha	Study I	I
	Ad5-Δ24	Wt Ad5	24bp deletion in E1A	-	(Heise et al. 2000)	IV
	Ad5-Δ24-CpG	Wt Ad5	24bp deletion in E1A	CpG	(Cerullo et al. 2012)	IV
	Ad5-Δ24-GM-CSF	Wt Ad5	24bp deletion in E1A	GM-CSF	(Cerullo et al. 2010)	IV

7.2.1.1. PeptiCRAd – Peptide-coated adenovirus

In Study IV we generated a novel system where tumor-specific peptides can be attached onto the surface of an oncolytic adenovirus to boost the anti-peptide, i.e. the anti-tumor immunity. We call this technology PeptiCRAd (**Pepti**de-coated **C**onditionally **R**eplicating **Ad**enovirus). To allow electrostatic interactions between peptides and the negative viral surface, we added a positively charged poly-lysine (polyK) chain to the peptide sequence.

PeptiCRAd complex formation

PeptiCRAd complexes were prepared by mixing oncolytic adenoviruses and polyK-peptides using a 1:500 ratio: i) for each μL of virus used, the corresponding ug of protein was calculated; ii) then, for each μg of viral protein 500 μg of peptides was added; iii) after vortexing, the mix was incubated at room temperature for 15 minutes; iv) the solution was again vortexed before use (**Figure 9**). New PeptiCRAds were prepared before each experiment by using fresh reagents every time. All the dilutions of virus and peptides required before the incubation were made in sterile MilliQ water adjusted to pH 7.4. Then, the PeptiCRAds were diluted with the buffer required by the assay.

The peptides used for preparation of different PeptiCRAds were polyK-SIINFEKL ($OVA_{257-264}$), SIINFEKL-polyK, polyK-AHX-SIINFEKL, polyK-SVYDFFVWL ($TRP\text{-}2_{180-188}$), polyK-

KVPRNQDWL ($hgp100_{25-33}$) and polyK-SLFRAVITK ($MAGE\text{-}A1_{96-104}$). The peptides were purchased from Zhejiang Ontores Biotechnologies Co. (Zhejiang; CN). The purity of all the peptides was estimated to be >80% and they were analyzed by mass spectrometry.

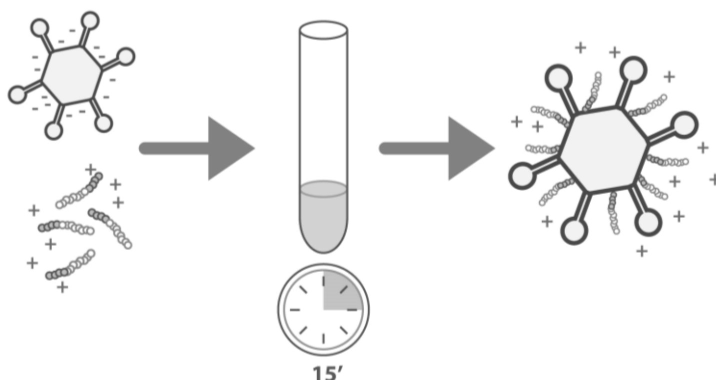


Figure 9. Conceptual scheme of the PeptiCRAd cancer vaccine platform. *(**A**) MHC-I restricted tumor epitopes are modified in order to display a strong positive charge by adding a poly lysine mini-chain at their N-terminus. (**B**) The positive tumor epitopes interact with the negative capsid of the adenovirus by electrostatic interaction. A saturating amount of peptide in the reaction ensures the efficient coating of the viral surface, and prevents the aggregation of viral particles, thus stabilizing the complex. (**C**) The complex is allowed to stabilize for 15 minutes at room temperature.*

7.2.2. Vaccinia viruses

Vaccinia viruses used in this thesis work belong to Western Reserve (WR) strain and they have been made cancer specific by deletions in two viral genes: the vaccinia growth factor (VGF) and thymidine kinase (TK). These deletions make the vaccinia virus selective for epidermal growth factor receptor pathway mutations and hypermetabolism, respectively, which are common defects in tumor cells. All viruses have also been modified to contain a marker gene (tdTomato, GFP or luciferase) for imaging and biosafety purposes.

Viruses were produced in CV-1 cells and amplified in A549 cells. Virus purification was done with sucrose gradient centrifugation, a general method described in (Earl et al. 2001, McCart et al. 2001). Virus concentrations (pfu/ml) were determined by a standard crystal violet staining assay (Hemminki et al. 2003) on A549 cells.

In Study II we cloned to new oncolytic vaccinia viruses, one expressing human DAI and one expressing mouse DAI. For generation of the vvdd-tdTomato-mDAI and vvdd-tdTomato-hDAI (see Figure 28), the mDAI and hDAI cDNA was inserted under the control of the pE/L promoter of pSC65-tdTomato plasmid (Parviainen et al. 2015, Chakrabarti, Sisler & Moss 1997) to create pSC65-tdTomato-mDAI and pSC65-tdTomato-hDAI. These shuttle plasmids were co-transfected with vvdd-luc in CV-1 cells, and the produced viruses were amplified and purified as described above.

The oncolytic vaccinia viruses used are listed in **Table 3**.

Table 3. Vaccinia viruses used in the studies.

Virus (name)	Marker gene	Transgene	Reference	Used in study
vvdd-GFP	green fluorescent protein	-	(McCart et al. 2001)	II
vvdd-luc	luciferase	-	(Guse et al. 2010)	II
vvdd-tdTomato	tdTomato	-	(Parviainen et al. 2014)	II
vvdd-tdTomato-hDAI	tdTomato	human DAI	Study II	II
vvdd-tdTomato-mDAI	tdTomato	mouse DAI	Study II	II

7.3. Human specimens

7.3.1. Patients included in the genotyping of FcγR polymorphisms (III)

For Study III, 235 cancer patients were analyzed for two Fc gamma receptor polymorphisms, FcγRIIa-H131R and FcγRIIIa-V158F. These patients (98 males and 137 females; median age 58 years) had advanced solid tumors refractory to conventional treatment modalities. The patients were treated with oncolytic adenoviruses in an Advanced Therapy Access Program (ATAP) (see more below). 169 (71.9 %) of patients included in Study III had been treated with GM-CSF-armed viruses and 39 (15.7 %) with CD40L-armed viruses (all viruses used are listed in **Table 4**). 17 (7.2 %) of these patients had received both GM-CSF- and CD40L-armed viruses and 42 (17.9 %) patients received viruses not armed with either GM-CSF or CD40L. Other relevant patient data can be found in **Table 5**.

7.3.1.1. Advanced Therapy Access Program (ATAP)

ATAP (ISRCTN ID 10141600) was a personalized therapy program (not a clinical trial) where cancer patients with advanced solid tumors refractory to available treatment modalities received oncolytic adenovirus therapies between years 2007 and 2012. Altogether 290 patients were treated during these years at Docrates Hospital, Helsinki, Finland. ATAP was regulated by the Finnish Medicines Agency (FIMEA) as determined by European Committee regulation No 1394/2007 on advanced therapy medicinal products, amending directive 2001/83/EC and regulation No 726/2004. ATAP was also evaluated by the Gene Technology Board and the Medicolegal Department of the Finnish Ministry of Social Affairs and Health.

All patients treated in ATAP voluntarily contacted the clinic and provided written informed consent for experimental therapy. Treatments were performed according to Good Clinical Practice (GCP) and based on Section 35 of the Helsinki Declaration of World Medical

Association. Treatment decisions were based on individual characteristics of the patients and their tumors.

Patients included in ATAP treatments had advanced solid tumors refractory to other treatments and a WHO performance score ≤3 at baseline. Patients who had major organ dysfunctions, organ transplants, known brain metastasis, HIV or other major immunosuppression, elevated bilirubin, alanine transaminase (ALT) or aspartate transaminase (AST) levels ≥ 3x normal limits, severe thrombocytopenia or other severe diseases were excluded from the ATAP program.

Table 4. Oncolytic adenoviruses used to treat ATAP patients included in Study III.

Virus (name)	Transductional targeting (Fiber modification)	Transcriptional targeting (E1 gene modification)	Transgene (E3 gene modification)	Reference
ICOVIR-7	RGD	E2F promoter	-	(Rojas et al. 2009)
Ad5/3-Cox2L-Δ24	Ad3 knob	Cyclooxygenase 2 (*COX-2*) promoter and 24bp deletion in E1A	-	(Bauerschmitz et al. 2006)
Ad5-Δ24-GMCSF (=CGTG-101)	Wt Ad5	24bp deletion in E1A	GM-CSF	(Cerullo et al. 2010)
Ad5/3-Δ24-GMCSF (=CGTG-102 /ONCOS-102)	Ad3 knob	24bp deletion in E1A	GM-CSF	(Koski et al. 2010)
Ad5-RGD-Δ24-GMCSF (=CGTG-103)	RGD	24bp deletion in E1A	GM-CSF	(Pesonen et al. 2012a)
Ad3-hTERT-E1A (=CGTG-201)	Wt Ad3	hTERT promoter	-	(Hemminki et al. 2012)
Ad5/3-hTERT-CD40L (=CGTG-401)	Ad3 knob	hTERT promoter	CD40L	(Diaconu et al. 2012)
Ad5/3-E2F-Δ24-GMCSF (=CGTG-602)	Ad3 knob	E2F1 promoter and 24bp deletion in E1A	GM-CSF	(Cerullo et al. 2010)

Table 5. Characteristics of patients included in Study III.

No. of patients	235
Age (age when treatments started)	
Range	3 - 82
Median	58
Sex (n (%))	
Female	137 (58.3%)
Male	98 (41.7%)
Cancer type (n (%))	
Colorectal, intestinal and anal	42 (17.9%)
Ovarian (also tubal)	35 (14.9%)
Breast	29 (12.3%)
Sarcomas	21 (8.9%)
Pancreatic and papilla vater	20 (8.5%)
Lung	18 (7.7%)
Neuroblastoma, neuroendocrine or head and neck	14 (6.0%)
Prostate	12 (5.1%)
Skin and melanomas	11 (4.7%)
Liver and mesothelioma	9 (3.8%)
Gastric	6 (2.6%)
Biliary tract or cholangio	5 (2.1%)
Urinary tract or bladder	4 (1.7%)
Cervix and endometrial	3 (1.3%)
Renal	3 (1.3%)
Thyroid, thymus or parathydoid	2 (0.9%)
Esophageal	1 (0.4%)

Survival and response evaluation

For evaluation of the effectiveness of the oncolytic adenovirus treatments on the ATAP patients, survival and treatment response criteria were applied.

Overall survival (OS) was calculated from the date of initiation of the adenovirus therapy until death or to the date of last follow-up when data were censored.

To evaluate the treatment response tumors were monitored before and after treatments. Tumor sizes were assessed by contrast-enhanced computed tomography (CT), positron emission tomography-computed tomography (PET-CT) or magnetic resonance imaging (MRI). Response evaluations were performed by professional radiologists. Maximum tumor diameters were determined according to RECIST v1.1 (Eisenhauer et al. 2009). Evaluations applied to overall disease status including injected and non-injected lesions. The criteria were: CR = complete response (complete disappearance of all tumors), PR = partial response (≥ 30% reduction in the sum of tumor diameters), SD = stable disease (no

response and no progression), PD = progressive disease (≥ 20% increase in the sum of tumor diameters or appearance of a new lesion). MR = minor response (10-29% reduction in the sum of tumor diameters) was also added to the criteria to better determine small changes in response. Also tumor markers were evaluated before and after treatments, the same percentages were used to determine the tumor marker responses.

In Study III, all the outcome (i.e. response) data were combined, averaged and divided into categories, disease control (DC) = stable disease or better, or to progressive disease (PD) to make the analyses clearer.

7.3.2. Blood samples for genotyping of FcγR polymorphisms (III)

Peripheral blood samples that had originally been collected between years 2007 and 2011 from patients for assessing presence of virus (biosafety, efficacy and safety implications) were used in this study for genotyping the FcγR polymorphisms with permission by the HUCH Operative Ethics committee (HUS 62/13/03/02/2013).

7.3.3. Buffy coats for PBMC extraction (II, IV)

Buffy coats (i.e. blood from which most of the plasma and erythrocytes have been removed by gradient centrifugation) from healthy donors were obtained from Finnish Red Cross Blood service.

7.4. *In vitro* studies

7.4.1. Cytokine expression/production analyses (I, II)

In Study I, the expression levels of the hTNFα transgene were examined from the supernatants of Ad5/3-Δ24-hTNFα infected (10 VP/cell) human cell lines and from tumor lysates and serum samples of virus treated mice. In Study II, IL-6 and TNFα concentrations in the supernatants of vaccinia virus infected cells were determined. Analyses were done by FACSarray (BD Biosciences, San Jose, CA) using Cytometric Bead Array (CBA) Human Soluble Protein Flex Sets and Human Soluble Protein Master Buffer Kit (BD Biosciences) according to manufacturer's instructions. BD FACS Array System software and FCAP Array v1.0.2 software (BD Biosciences) were used for data analysis.

7.4.2. Biofunctionality assay for TNFα (I)

hTNFalpha expressed by the Ad5/3-Δ24-hTNFα virus was tested using WEHI-13VAR mouse fibrolast cells, which are highly sensitive to TNFα when the assay is performed in the presence of 500 ng/ml actinomycin D. Supernatants from Ad5/3-Δ24-hTNFα and Ad5/3-Δ24 infected A549 cells 48 h after infection were added onto WEHI-13VAR cells and cultured in actinomycin D containing medium. After 24 hours, MTS cell viability assay was performed.

7.4.3. Cell viability assays (I, II, IV)

Tumor cells were seeded at 1×10^4 cells per well on 96-well plates. On the next day, viruses were diluted in growth media with 2% fetal calf serum, cells were infected for 1 h at 37 °C and then incubated in 5% FCS containing media at 37 °C for 3 to 5 days. Cell viability was determined by MTS assay according to the manufacturer's protocol (Cell Titer 96 AQueous One Solution Cell Proliferation Assay; Promega, Nacka, Sweden), measuring optical density with spectrophotometer at 490 nm.

7.4.4. Infectivity assay (immunocytochemistry (ICC) assay) (IV)

Tumor cells were seeded at 2×10^5 cells per well on 24-well plates in 3 or 5 replicates. On the following day, the cells were infected with 100 μl of viral dilutions. The plates were centrifuged for 90 minutes at 1000 rcf at +37°C and then transferred to the incubators for 48 hours. After the incubation period, the culture medium was removed and cells were fixed with 250 μl of ice-cold methanol for 15 minutes. Once methanol was disposed, cells were washed 3 times with 300 μl of PBS supplemented with 1% Bovine Serum Albumin (BSA). Afterwards, cells were stained with 250 μl of 1:2000 diluted mouse monoclonal anti-hexon antibody (Novus Biologicals, Littleton, CO, US) for 1 hour at RT in the dark. Cells were washed and stained with 250 μl of 1:500 diluted Biotin-Streptiavidin-conjugated goat anti-mouse antibody with PBS/1% BSA for 1 hour at RT in the dark. Cells were then incubated for 30 minutes at RT with 250 μl of 1:200 diluted extravidin-peroxidase (Sigma-Aldrich, St. Louis, MO, US). Cells were washed extensively and DAB staining solution (Sigma-Aldrich, St. Louis, MO, US) was prepared according to manufacturer´s instruction. A total of 250 μl of DAB staining solution was applied to each well. Cells were monitored under microscope for the appearance of dark spots. When optimal signal to noise ratio was reached the reaction was quenched by addition of PBS/1% BSA (500 μl per well). For each replicate (i.e. well) 5 images of non-overlapping fields were acquired using an AMG EVO XL microscope. For determinig the infectious titer, the following formula was used:

$$Infectious\,titer = x * \frac{well\ area}{field\ area} * \frac{1}{dilution\ factor} * \frac{1\ ml}{Volume\ of\ dilution\ applied}$$

7.4.5. Jurkat cell silencing for DAI (II)

Jurkat cells (kindly provided by Dr. Pentti Tienari, Program of Molecular Neurology, University of Helsinki, Finland), which are immortalized human T lymphocytes, were transfected with psiRNA-DAI or the control plasmid psiRNA-luc (Invivogen, San Diego, CA), both of which carry the zeocin resistance gene, using GenCarrier Cell Transfection Reagent (Epoch Biolabs, Sugar Land, TX). Stably transfected clones were selected after culturing in growth medium supplemented with 0.25 mg/ml zeocin (Invitrogen, Carlsbad, CA) for 6 weeks.

7.4.6. ELISA assays (I, II)

In Study II, hTNFα levels after Ad5/3-Δ24-hTNFα infection (100 VP/cell) were determined from the growth media of murine cells (B16-OVA) using a human TNFα ELISA kit (KHC3011, Invitrogen, Frederick, MD, US).

To study the IFN-β production from Jurkat cells infected with vvdd-GFP (in Study II), a high sensitive human interferon beta ELISA (PBL interferon source, Piscataway, NJ) was performed on growth media from infected and non-infected cells according to manufacturer's instructions.

7.4.7. IFN-beta qPCR (II)

Total cellular RNA was isolated with the Qiagen RNeasy kit. Isolated RNA (2 µg) was reverse-transcribed into cDNA in TaqMan RT buffer with 5.5 mM MgCl2, 500 µM dNTPs, 2.5 µM oligo d(T)16, 0.4 U/µL RNase inhibitor, and 1.25 U/µL MultiScribe RT (Applied Biosystems, Foster City, CA, USA). cDNA samples were then amplified in TaqMan universal PCR master mix buffer (Applied Biosystems) with TaqMan Pre-Developed Assay-on-demand Gene Expression Reagent kits (Applied Biosystems) to analyze mRNA levels for IFN-β1 (Hs00277188_s1). GAPDH was used as endogenous control for normalization. Each sample was amplified in duplicate or triplicate with a Roche Lightcycler sequence detector (Roche, Basel, Switzerland).

7.4.8. Whole genome gene expression profiling (II)

Human HS294T and THP-1 cells were treated with vvdd-tdTomato-hDAI, vvdd-tdTomato or PBS as a non-infected control. Total RNA was purified from tumor cells after infection with 0.1 pfu/cell of virus for 16 hours. RNeasy Plus Mini kit (Qiagen, Venlo, NL) was used according to manufacturer´s instructions.

Independent pools of two RNA samples each (total of 600 ng) were labeled using a T7 RNA polymerase amplification method (Low Input Quick Amp Labeling Kit, Agilent Technologies, Inc., Santa Clara, CA, USA), according to the instructions of the manufacturer. cRNAs were then labeled with Cy3 and Cy5 dyes (Agilent Technologies) and hybridized to the Agilent 2-color 60-mer oligo arrays (Agilent SurePrint G3 Human GE 8x60K). The slides were washed and scanned with a G2505C Agilent Microarray Scanner (Agilent Technologies) and the raw intensity values were obtained with the Feature Extraction software, version 11.0.1.1 (Agilent Technologies). Raw data was quality checked according to Agilent standard procedures. The microarray data were deposited in NCBI Gene Expression Omnibus (GEO) database and are accessible through GEO Series accession number GSE76208.

Data pre-processing and differential expression analysis of the gene expression data were done in R. First, the probe profile expression data were normalized using quantile normalization and corrected for batch processing effects using ComBat function. Next, after mapping from probsets to Ensemble gene IDs, the differentially expressed genes (DE genes) between pre- and post-treatment samples were identified using a limma package.

For the analysis using the limma package, genes were defined as being differentially expressed after satisfying a minimum fold-change of ±1.5 and a maximum Benjamini-Hochberg adjusted p-value of 0.01. Finally, functional categorization of DE genes was performed using a novel R-based package namely BACA. It queries the DAVID knowledge base and builds a chart showing multiple enrichment analysis results across different conditions/treatments. Each annotation in the BACA chart is represented as a bubble with a size indicating how many genes in a list of DE genes are associated with it, and a color indicating whether the genes are down-regulated (green) or up-regulated (red).

7.4.9. Analysis of apoptotic and necrotic cells (I)

Cells were plated onto 6-well plates, 2 x 10^5 cells/well. Cells were infected with 10 VP/cell of adenovirus (or PBS for mock). The amounts of apoptotic and necrotic cells were measured 48 hours post infection with a TACS Annexin V-FITC kit (Trevigen Inc., Gaitherburg, MD, US) and BD Accuri C6 flow cytometer (BD Biosciences) according to manufacturer´s instructions.

7.4.10. Immunogenic cell death (ICD) (I)

Calreticulin exposure:

Cells in triplicate were infected for 2 hours with 100 VP/cell of adenovirus. Twelve hours (human cells) or 48 hours (mouse cells) later, cells were harvested and stained with 1:1000 diluted rabbit polyclonal Anti-Calreticulin antibody (ab2907, Abcam, Cambridge, UK) for 40 min at 4 °C and then with 1:100 diluted Alexa-Fluor 488 IgG as secondary antibody (A21206, Invitrogen, Carlsbad, CA) for flow cytometric analysis.

ATP release:

Cells in triplicate were infected for 2 hours with 100 VP/cell of adenovirus. Supernatants were collected after 12 hours (human cells) or 48 hours (mouse cells) and analyzed with ATP Determination Kit (A22066, Molecular Probes; Invitrogen).

HMGB-1 release:

Cells in triplicate were infected with 100 VP/cell. 24 hours later, supernatant was collected and HMGB-1 was measured with an ELISA kit (ST51011; IBL International GmbH, Hamburg, DE).

7.4.11. Zeta potential and Dynamic Light Scattering (DLS) analysis (IV)

Virus-peptide complexes were vortexed and diluted to a final volume of 700 ul with sterile MilliQ water adjusted to pH 7.4. Then the samples were transferred to a polystyrene disposable cuvette, to determine the size of the complex (DLS). Afterwards, the samples were recovered from the cuvettes and transferred to the disposable capillary cell DTS1070 (Malvern; Worcestershire, UK) for zeta potential measurements. All the measurements were taken at 25°C with the Nanosizer ZS (Malvern).

7.4.12. Surface Plasmon Resonance (SPR) (IV)

Virus-peptide interaction was measured by Surface Plasmon Resonance. A multi-parametric SPR Navi™ 220A instrument (Bionavis Ltd, Tampere, Finland) was used for measurements. Milli-Q water (pH 7.4) was used as a running solution. A constant flow rate of 30 µl/min, a temperature of +20°C and a 670-nm laser light were used for all experiments. A sensor slide with a silicon dioxide surface was activated by treatment with plasma for 3 minutes followed by coating with APTES ((3-aminopropyl)triethoxysilane) by incubating the sensor in 50 mM APTES in toluene solution for 1 h. The sensor was then placed into the SPR device, and the viruses were immobilized *in situ* on the sensor surface by injecting 50 µg/ml viruses in MilliQ water for approximately 12 min, followed by a 3 min wash with 20 mM CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate). The second flow channel was used as a reference and was injected with MilliQ water only, followed by washing with CHAPS. The baseline was observed for at least 10 min before sample injections. PolyK-SIINFEKL or SIINFEKL peptides were then injected into both flow channels of the flow cell in parallel, with increasing concentrations.

7.4.13. Irradiation of cells (I)

To test the combination of oncolytic adenoviral treatments and radiotherapy *in vitro,* cells were irradiated 24 hours after virus infection with 4, 8 or 10 Gy. The cells in 96-well plates were placed in a 1-cm thick plastic phantom bottom filled with 1 cm of water. Irradiation was performed at the HUCH Department of Oncology with a medical linear accelerator (Clinac 600 C/D, Varian Medical Systems, Palo Alto, CA) in a 6 MV photon beam using a dose rate of about 4 Gy/min (400 MU/min).

7.5. *In vivo* studies

All animal experiments were performed with permission from the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland. Mice were obtained at 3-6 weeks of age and quarantined at least 1-2 weeks before experiments were started. Mice were kept in standard cages with constant food and water supply. The health status of the mice was monitored daily and as soon as signs of pain or distress were evident, they were euthanized. Mice were, at the latest, euthanized when a single tumor reached an average diameter of 17 mm, or 15mm in experiments where the mice had two tumors. Euthanasia was done by using CO_2 and neck dislocation. During the experiments mice were anesthetized either with isoflurane inhalation or with i.p. injection of 0.5 mg/kg medetomidine and 50 mg/kg ketamine diluted in 0.9% sodium chloride. In all the experiments, the formula (length × $(\text{width})^2$ × 0.5) was used to calculate the tumor volumes.

7.5.1. Syngeneic melanoma models (I, II, IV)

The *in vivo* efficacy and immunogenicity of the viruses were tested in immunocompetent C57BL/6 mice bearing syngeneic melanoma tumors. Mice were obtained from Harlan Laboratories Inc. (Indianapolis, IN, USA) or from Scanbur (Karlslunde, DK) for Study IV.

In Study I, 5-week-old C57BL/6 female mice (N = 7 per group) received 1x10^5 B16-OVA cells subcutaneously on a shaved flank (one tumor per mouse). In the “mild” regimen experiment, the virus was injected intratumorally at 10^8 VP/tumor on days 0, 1, 4 and 5 and in the “high” regimen experiment the same amount of virus was given every other day (a total of 7 times) starting when tumors reached the size of ~4x4 mm.

In Study II, B16-OVA melanoma tumors were established by injecting 1 × 10^5 B16-OVA cells subcutaneously into one flank of 10-week-old female C57BL/6J mice (N = 12 per group). Tumors (one tumor per mouse, ~4×4 mm in diameter) were injected i.t. with vaccinia viruses in a volume of 50 µl on day 0 with 3 × 10^6 pfu/tumor and on day 2 with 1 × 10^6 pfu/tumor. Mock tumors were injected with PBS only. In the re-challenging experiment where the mice received a second tumor contralaterally, either B16-OVA cells or B16-F10 cells 3 x 10^5 cells, were injected 10 days after the first virus treatment.

In Study IV, melanoma tumors were injected into both flanks of C57BL/6 mice. The number of tumor cells injected into each flank was: 3 x 10^5 for B16-OVA, 1 x 10^5 for B16-F10 and 2 x 10^6 for SK-MEL-2. The treatment of tumors started when tumors reached an average diameter of 4 mm. In all the experiments the mice were administered the virus three times per day, on days 0, 2 and 4 with 1 x 10^9 VP/tumor.

7.5.2. Human prostate cancer xenograft model (I)

To study the efficacy of the Ad5/3-Δ24-hTNFα virus in Study I, human xenografts were established by injecting 5 × 10^6 PC-3MM2 cells subcutaneously into the flanks of 6-week-old male NMRI nu/nu mice (N = 6 per group) obtained from Scanbur (Karlsunde, Denmark). Tumors (two tumors per mouse, ~5x5 mm in size) were treated i.t. with a volume of 50 µl for four times on days 0, 1, 4 and 5 with 10^8 VP/tumor and control tumors were injected with PBS only.

7.5.3. Humanized mice (II, IV)

To gain better understanding of the immunogenicity and efficacy of the viruses in a more translational system, we used mice with human peripheral blood mononuclear cells (PBMCs) and human melanoma tumors. For these studies highly immunodeficient NSG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice were obtained from The Jackson Laboratory (Bar Harbor, Maine, CA, US). Human melanoma cells (HS294T in Study II or SK-MEL-2 in Study IV) were injected subcutaneously into both flanks of the NSG mice, 5 × 10^6 of HS294T cells or 2x10^6 of SK-MEL-2 cells per flank. When tumors reached the average size of 4×4 mm in size, human PBMCs were injected intravenously into the tail vein in 200 µl of PBS, 2 × 10^7 cells/mouse. Two days later virus treatments were started. In Study II, tumors were treated on days 0 and 6 with 1 × 10^6 pfu/tumor in volume of 50 µl, mock mice were treated with PBS only. In Study IV, tumors were treated on days 0, 2 and 4 with 2x10^8 VP/tumor in 50 µl of NaCl, mock mice received NaCl only. Part of the mice were left without PBMCs as a study control lacking the immune system.

7.5.4. Irradiation of mice (I)

In Study I, mice received two times 2-Gy whole-body irradiation 24 hours after virus treatments. Mice were kept awake in standard plastic cages during the irradiation. Irradiation was performed with a medical linear accelerator (Clinac 600 C/D, Varian Medical Systems, Palo Alto, CA, US) in a 6 MV photon beam using a dose rate of about 4 Gy/min (400 MU/min).

7.6. *Ex vivo* studies

7.6.1. Analysis of immune cells from organs (I, II, IV)

After euthanizing the mice with CO_2 and neck dislocation, tumors, spleens and lymph nodes of treated mice were collected into tubes containing 10% RPMI-1640 medium, and the tubes were kept on ice until further processing. Organs were smashed through 70-µm cell strainers and single cell suspensions were stained with fluorochrome-conjugated monoclonal antibodies and analyzed using a FACSAria (BD Biosciences) or Gallios (Beckmann Coulter) flow cytometer and FlowJo software (TreeStar, Ashland, OR, US). To study tumor-specific T cells from B16-OVA tumor bearing mice, APC-conjugated SIINFEKL MHC-I pentamer (PROIMMUNE, Oxford, UK) was used together with a FITC-conjugated anti-mouse CD8 and a PE-conjugated anti-mouse CD19 antibodies respectively to gate the CD8+ cell population and to subtract unspecific binding of the pentamer to B cells. Other antibodies used in the studies are described in detail in the corresponding publications.

7.6.2. ELISpot assay (II)

The number of IFNγ producing T cells were studied from spleens of vaccinia virus treated C57BL/6 mice bearing B16 tumors. A mouse IFN-γ ELISpot^{PLUS} (ALP) kit (Mabtech, 3321-4APT) was used according to manufacturer´s instructions. Splenocytes were stimulated for 40 hours with PepMix™ VACV (vaccinia virus) peptide pool (MVA093L, JPT), ovalbumin peptide SIINFEKL (P93, Proimmune), gp100 peptide KVPRNQDWL (P1333, Proimmune) or TRP-2 peptide SVYDFFVWL (P185, Proimmune) in a concentration of 50 ng/well. Concanavalin A (Sigma-Aldrich) was used as positive control (500 ng/well). ELISpot plates were read and evaluated by ZellNet Consulting, Inc. (Fort Lee, NJ, US).

7.6.3. Analysis of apoptotic and necrotic cells in tumors (I)

Tumors were smashed through a 70-µm cell strainer to obtain single cell suspension and the cells were cultured in 10% RPMI-1640 medium overnight. Cells were then stained and analyzed with a TACS Annexin V-FITC kit (Trevigen Inc., Gaitherburg, MD, US) and BD Accuri C6 flowcytometer (BD Biosciences) according to manufacturer´s instructions.

7.6.4. DNA extraction (III)

Genomic DNA was extracted from patient blood clot samples by using a QiAmp Blood Mini Kit (Qiagen, Venlo, NL). First the samples were thawed in a 37°C water bath and then the

clots were transferred into clot spin basket filters (Qiagen, Germany). Samples were spun through the filter by centrifugation (2000 rcf, 5 min., RT). Qiagen QiAmp Blood Mini Kit DNA extraction protocol was used for the DNA extraction. DNA was eluted in nuclease free water (Amresco LLC, Solon, OH, USA) and stored in -20°C freezer until genotype analysis. DNA concentrations were measured by using NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). For genotype analyses DNA samples were diluted in nuclease free water (Amresco) to obtain a final concentration of 1 ng/µl.

7.6.5. Genotyping of FcγR polymorphisms (III)

In Study III, all the patients for whom there were available blood samples were included in the genotyping. Patients were genotyped for two different Fc gamma receptor polymorphisms, FcγRIIa-H131R (rs1801274) and FcγRIIIa-V158F (rs396991) using TaqMan technology on Applied Biosystems' (AB) 7500 Fast Real-Time PCR system (Applied Biosystems Inc., CA, USA). Probes and primers (TaqMan SNP Assays for rs1801274 and rs396991) were ordered from Applied Biosystems. Genotyping was performed by manufacturer´s instructions. Briefly, polymerase chain reactions were prepared in MicroAmp Fast Optical 96-well Reaction Plates (AB) in final volume of 25 µl. The reaction mixes consisted of 2X TaqMan Genotyping Master Mix (AB) and 20X TaqMan SNP Assay Mix (AB) for either FcγRIIa or FcγRIIIa polymorphism. A total of 10 ng of genomic template DNA sample was added per well. Each sample was set up in triplicate. Nuclease free water (Amresco) was used as No Template Control (NTC). The following PCR program was used: initiation at 60°C for 2 minutes and AmpliTaq Gold Enzyme activation at 95°C for 10 minutes followed by 45 cycles of denaturation at 92°C for 15 seconds and annealing and extension at 60°C for 1 minute, allelic discrimination plate read was performed at 60°C for 1 minute. The FcγR genotypes were determined using Allelic Discrimination protocol in the Sequence Detection System (SDS) software provided by Applied Biosystems.

7.6.6. PBMC extraction (II, IV)

Human peripheral blood mononuclear cells (PBMCs) were isolated from a buffy coat. Buffy coat blood was diluted 1:1 with PBS, and PBMCs were isolated by Ficoll-Paque PLUS (GE Healthcare, Little Chalfont, UK) gradient centrifugation according to manufacturer´s instructions.

7.6.7. HLA typing of PBMCs and tumor cell lines (II, IV)

First genomic DNA was extracted from the HS294T tumor cell line and human PBMCs for HLA typing. Qiagen DNeasy blood and tissue kit (Qiagen, Venlo, NL) was used to extract DNA by manufacturer´s instructions and the HLA genotyping and analysis was carried out in an EFI (European Federation for Immunogenetics) accredited HLA Laboratory in Haartman Institute, University of Helsinki. The genotyping of *HLA-A, -B* and *-C* genes were performed using sequence specific primers (Olerup SSP HLA-A-B-C Combi Tray, Applied Biosciences, Stockholm, SE). The reactions were performed according to manufacturers'

instructions providing at least four-digit resolution (for example, *HLA-A*01:01*). PCR reactions from agarose-gel were evaluated manually and the alleles were called with SCORE software. The HLA alleles were assessed using HLA nomenclature release 3.5.0 (IMGT/HLA database) and carefully interpreted by two persons.

7.6.8. Cross-presentation experiment (IV)

Fresh spleens were collected from naïve C57BL/6 mice and smashed through a 70 µm cell strainer. Red blood cells were lysed by incubating samples with 5 ml of ACK lysing buffer (Life Technologies) for 5 minutes at room temperature. Then, splenocytes were washed and prepared for the assay ($2x10^6$ cells in 800 ul of 10% RPMI-1640 culture media for each condition). A total of 200 ul of SIINFEKL, polyK-SIINFEKL, SIINFEKL-polyK or SIINFEKL-AHX-polyK peptide dilutions (0.19 µg/µl) was added to splenocytes. The PeptiCRAd complex was prepared as described above. After 2 hrs of incubation, cells were washed with PBS and stained with either APC anti-mouse $H\text{-}2K^b$ bound to SIINFEKL -antibody or APC Mouse IgG1, κ Isotype Ctrl (Biolegend, San Diego, CA, US). After 30 minutes of incubation on ice, samples were washed and analyzed by flow cytometry.

7.7. Statistics

In Studies I, II and IV GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, US) was used to determine the statistical differences between study groups. Usually two-tailed Student's t-test was used to determine differences in the *in vitro* and *ex vivo* experiments, but two-way ANOVA was used in some cases in Study IV (statistical analyses used in study IV are explained in detail in the figure legends of the article). A nonparametric Mann–Whitney U-test was used to determine statistical differences between *in vivo* study groups. The area under the curve (AUC) was used in Study IV to determine the size of the tumors of humanized mice. Kaplan-Meier survival curves and log rank statistics were used to compare survival of the treated mice. In Study III the Pearson´s Chi-square (χ^2) test was used to assess Hardy-Weinberg equilibrium of genotype and allele frequencies by using the Online Encyclopedia for Genetic Epidemiology studies (OEGE) Hardy-Weinberg equilibrium calculator (Rodriguez, Gaunt & Day 2009). CubeX (Cubic Exact Solution) software (OEGE - Online Encyclopedia for Genetic Epidemiology studies) and EM (expectation-maximization) algorithms (Excoffier & Slatkin 1995) were used to analyze linkage disequilibrium between the two polymorphic loci. The χ^2 test was used to compare outcomes of the patients according to FcγR polymorphisms. Kaplan-Meier survival estimations (Kaplan & Meier 1958) and log-rank statistics (Mantel 1966) were used to determine the differences in the overall survival (OS) and median time to death. The statistical data were obtained by using IBM SPSS Statistics 20 software for Windows (SPSS Inc., Chicago, IL, US).

Results are presented as mean ±SD, or as mean ±SEM for tumor growth. All P values were two-sided and considered statistically significant when ≤ 0.05. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001.

8. RESULTS AND DISCUSSION

8.1. Boosting immunogenicity and cytotoxic potential of an oncolytic adenovirus by arming with TNFα (I)

In Study I we developed a new oncolytic adenovirus that expresses human tumor necrosis factor alpha aiming at enhancing the cancer-killing potency and the immunogenicity of the virus.

8.1.1. Characterization of the TNFα expressing Ad5/3-Δ24-hTNFα *in vitro*

We successfully generated a new chimeric oncolytic adenovirus that expresses human TNFα (Ad5/3-Δ24-hTNFα) (**Figure 10**). This virus was designed to have adenovirus serotype 3 knobs on the Ad5 backbone, since switching the Ad5 knob into Ad3 knob has previously been shown to enhance the infection rate of cancer cells compared to wt Ad5 virus (Tuve et al. 2006, Krasnykh et al. 1996, Kanerva et al. 2002a, Kanerva et al. 2003). To make the virus selectively replicative in cancer cells, 24 base-pairs were deleted from its E1A region. It has already been shown several years ago that this Δ24 deletion reduces killing of normal cells drastically and thus makes the virus relatively safe to use (Pesonen, Kangasniemi & Hemminki 2011).

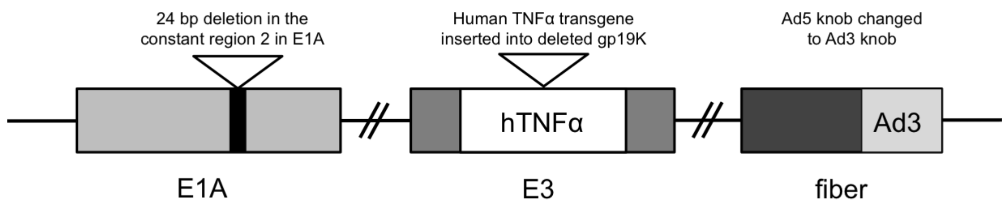


Figure 10. *A schematic view of the genome of the double-targeted human TNF-alpha expressing oncolytic adenovirus Ad5/3-Δ24-hTNFα. (Hirvinen et al. 2015, Human Gene Therapy [Study I])*

TNFα has been used in cancer treatment for decades, but most attempts have failed due to systemic toxicity of TNFα (Mocellin et al. 2005, McLoughlin et al. 2005, Corti 2004). Recent studies have therefore focused on local delivery of TNFα. A replication deficient adenovirus expressing hTNFα (TNFerade, GenVec Inc.) has been studied in clinical trials, but it failed to show good enough efficacy in the phase III trial (Mauceri et al. 2009, Fisher 2011). We believe it may have been because of the non-replicative system used. Delivering genes by an oncolytic vector that can both lyse the infected cells and also multiply the transgene expression is a huge benefit compared to a non-replicating vector that can produce only one gene product per vector. Thus, we wanted to generate a replication-competent oncolytic adenovirus that expresses TNFα from its E3 gp19K region. Intratumoral administration of this Ad5/3-Δ24-hTNFα virus was hypothesized to result in high local

concentrations of TNFα in the tumor site; however, without systemic toxicity due to tumor-selective replication of the virus.

To study the functionality of the newly generated virus we first measured the expression of human TNFα by the virus in two different human cell lines, the A549 lung adenocarcinoma cell line and the 293 embryonic kidney cells that feature transgenic E1A expression. The amount of hTNFα secreted was determined from the supernatants at several time points after virus infection by a Cytometric Bead Array (**Figure 11**). The hTNFα levels were increased with time even up to 659 ng/ml in A549 cells and up to 279 ng/ml in 293 cells 72 hours after infection with Ad5/3-Δ24-hTNFα. The almost three-fold difference between the two cell lines also shows the selectivity of the viral replication, since the TNFα production was more prominent in a cancer cell line (A549) than in a transformed "normal" cell line (293). We also measured TNFα production in a murine melanoma cell line B16-OVA to define whether the virus can be tested later in syngeneic *in vivo* tumor models. In the murine cells the expression levels of hTNFα were much lower, rising only up to 83 pg/ml 72 hours after infection most probably due to the fact that adenoviruses are quite species-specific and do not produce infective virions in foreign species (Jogler et al. 2006). The levels of hTNFα were detectable at around 24 to 36 hours after the infection in human cells and 48 hours p.i. in mouse cells.

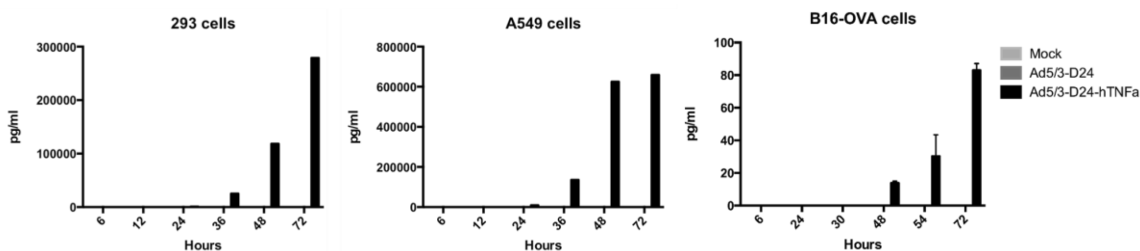


Figure 11. Expression of human TNFα in 293, A549 and B16-OVA cells. *Cells were infected with 10 VP/cell of either Ad5/3-Δ24-hTNFα virus or the control virus Ad5/3-Δ24. Supernatants were collected at indicated time points and FACSArray analysis was performed to quantify human TNFα. (Hirvinen et al. 2015, Human Gene Therapy [Study I])*

After we confirmed that the virus is able to express TNFα, we wanted to determine if the produced cytokine is also biologically functional. The functionality was tested in a TNFα-sensitive cell line, a mouse fibroblast cell line WEHI-13VAR, which die in the presence of TNFα and actinomycin. We infected the cells with different doses of Ad5/3-Δ24-hTNFα virus and a control virus TNFα (Ad5/3-Δ24). The viability of Ad5/3-Δ24-hTNFα infected cells decreased with increasing viral dose while controls survived which proves that hTNFα produced by the virus is biologically active (**Figure 12**).

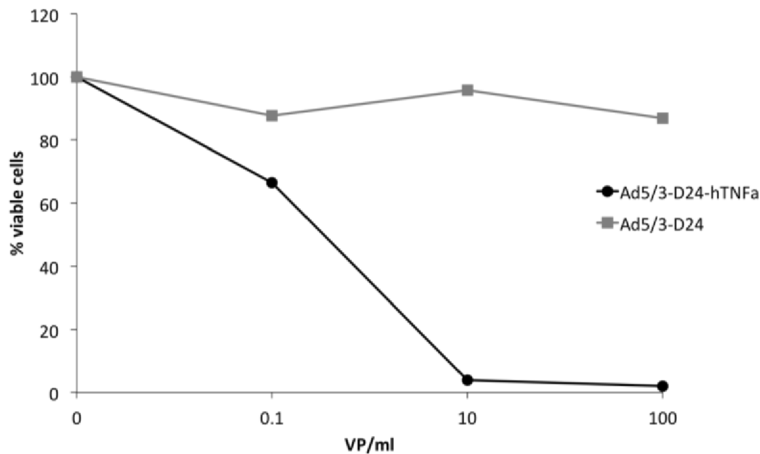


Figure 12. Biological functionality of the virus *was checked by adding supernatant from Ad5/3-Δ24-hTNFα or Ad5/3-Δ24 infected A549 cells onto WEHI-13VAR cells. WEHI-13VAR cells are sensitive to TNFα when cultured in actinomycin D containing medium. Cell viability was measured by MTS assay. (Hirvinen et al. 2015, Human Gene Therapy [Study I])*

Next we wanted to determine if the insertion and function of the hTNFα transgene in the viral genome have an impact on the cell killing efficiency of the virus. We tested the oncolytic potency of the virus in the human cancer cell lines PC-3MM2, A549, UT-SCC8 and MDA-MB-435 and the mouse cell line B16-OVA by infecting them with different amounts of Ad5/3-Δ24-hTNFα or the Ad5/3-Δ24 control and measured the cell viability by MTS assay. Interestingly, Ad5/3-Δ24-hTNFα killed the tumor cells even faster than the control virus did, so at least the insertion of the gene did not hinder oncolysis by the virus. Also, as stated above, the virus produced high concentrations of TNFα in cancer cells, which may be the mechanism behind the observed enhanced cytotoxicity compared to the control virus. It has indeed been known for a long that at high local concentrations TNFα displays anti-tumor properties (Fiers 1991, Mocellin et al. 2005).

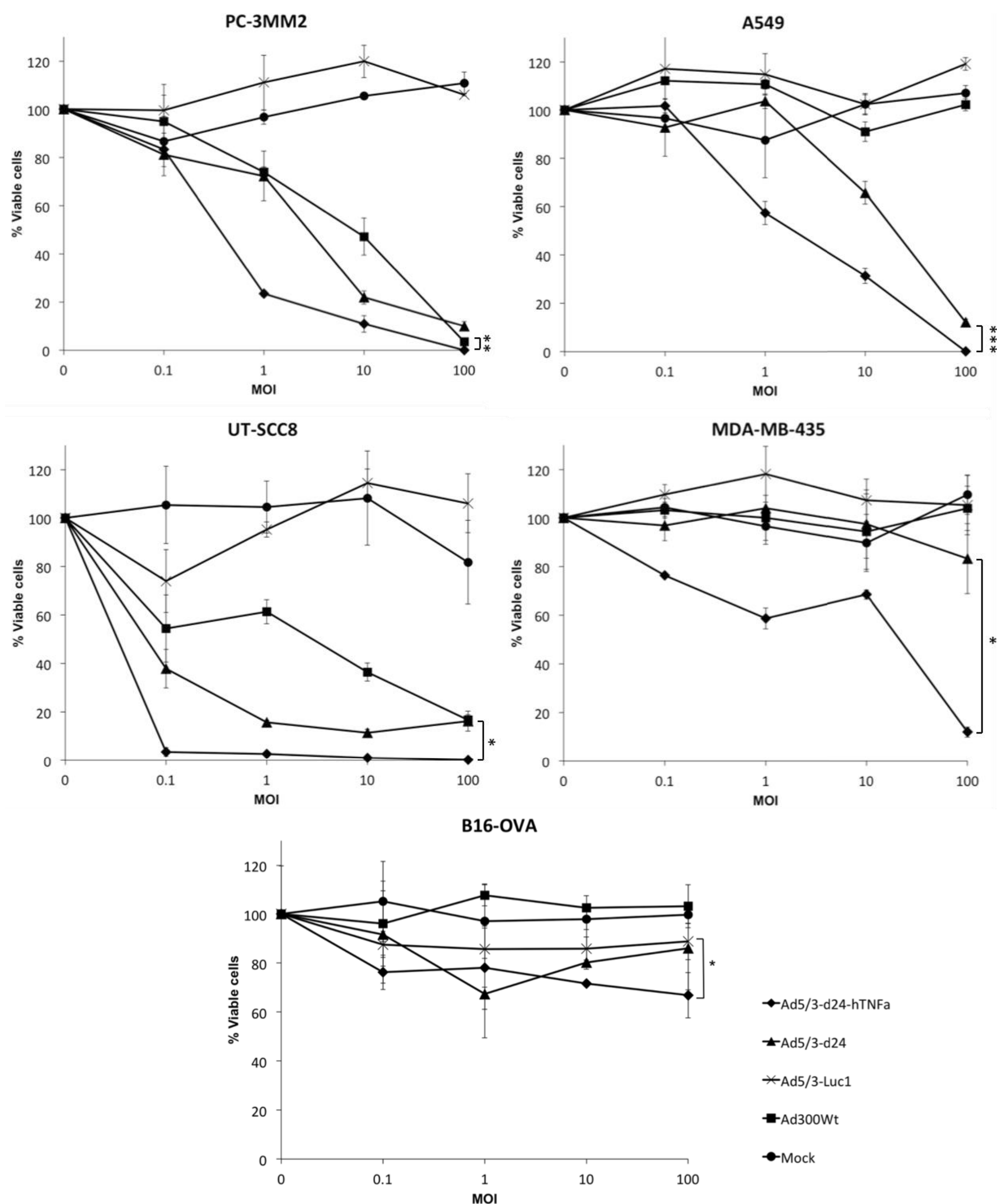


Figure 13. In vitro tumor-killing efficacy of Ad5/3-Δ24-hTNFα. *Oncolytic efficacy of Ad5/3-Δ24-hTNFα measured by MTS cell viability assay in different tumor cell lines. (Hirvinen et al. 2015, Human Gene Therapy [Study I])*

TNFα has the ability to induce direct tumor cell death by inducing apoptosis (Ashkenazi & Dixit 1998, Salako et al. 2011) and necrosis (van Horssen, Ten Hagen & Eggermont 2006, Laster, Wood & Gooding 1988). Thus, we measured the amount of early apoptotic cells (Annexin-V$^+$) and late apoptotic/necrotic cells (PI$^+$) after virus infection in different cancer cell lines (A549, PC-3MM2 and B16-OVA). The levels of both early and late

apoptotic/necrotic cells were elevated in all tested cell lines infected with the Ad5/3-Δ24-hTNFα virus compared to controls but no significant difference was found between the TNFα-expressing and non-expressing virus (**Figure 14**).

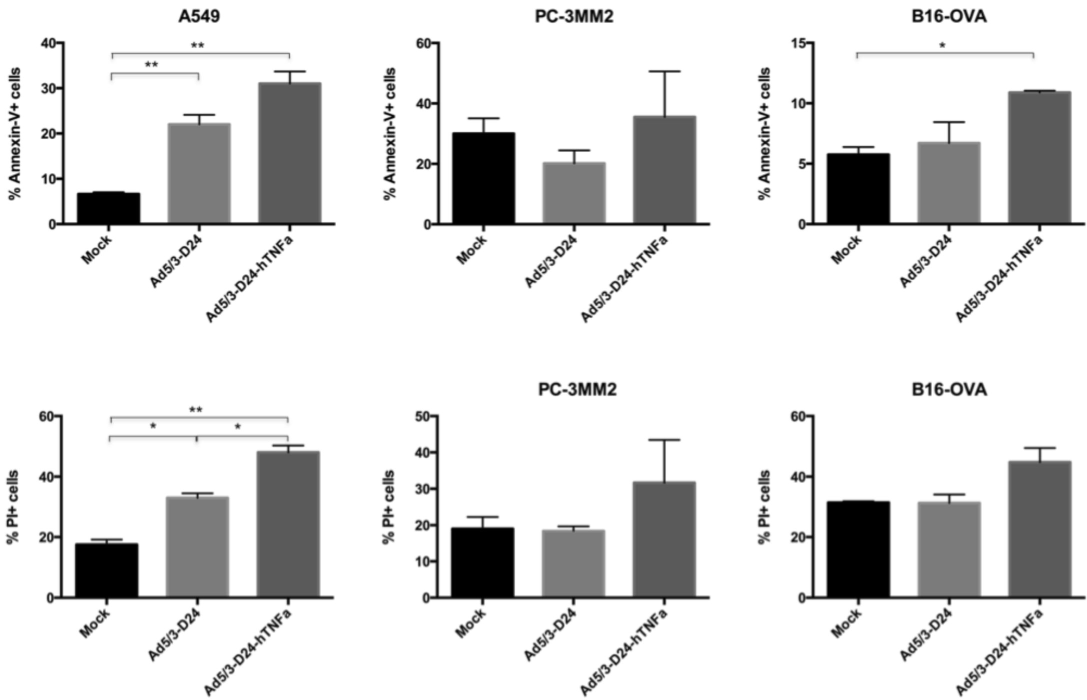


Figure 14. *The amounts of early apoptotic cells (**above**) and late apoptotic or necrotic cells (**below**) 48 hours after virus infection were analyzed by flow cytometry from PC-3MM2, A549 and B16-OVA cell lines. FITC-labeled Annexin-V was used to indicate the early apoptotic cells and PI (propidium iodide) the necrotic or late apoptotic cells. (Hirvinen et al. 2015, Human Gene Therapy [Study I])*

We and others have recently shown that immunogenic cell death is one important mechanism for tumor eradication by oncolytic viruses (Kanerva et al. 2013, Parviainen et al. 2014, Diaconu et al. 2012, Workenhe & Mossman 2014). When we measured immunogenic cell death markers, i.e. the exposure of calreticulin (CRT) on the cell membrane, and the release of adenosine triphosphate (ATP) and the nuclear protein high-mobility group box 1 (HMGB1) in virus-treated tumor cells, we observed that ATP release was significantly increased in all human cell lines (PC-3MM2, A549, EJ) treated with Ad5/3-Δ24-hTNFα compared to Ad5/3-Δ24. Also CRT exposure on the surface of Ad5/3-Δ24-hTNFα treated cells and the HMGB1 release were elevated but not significantly (**Figure 15**).

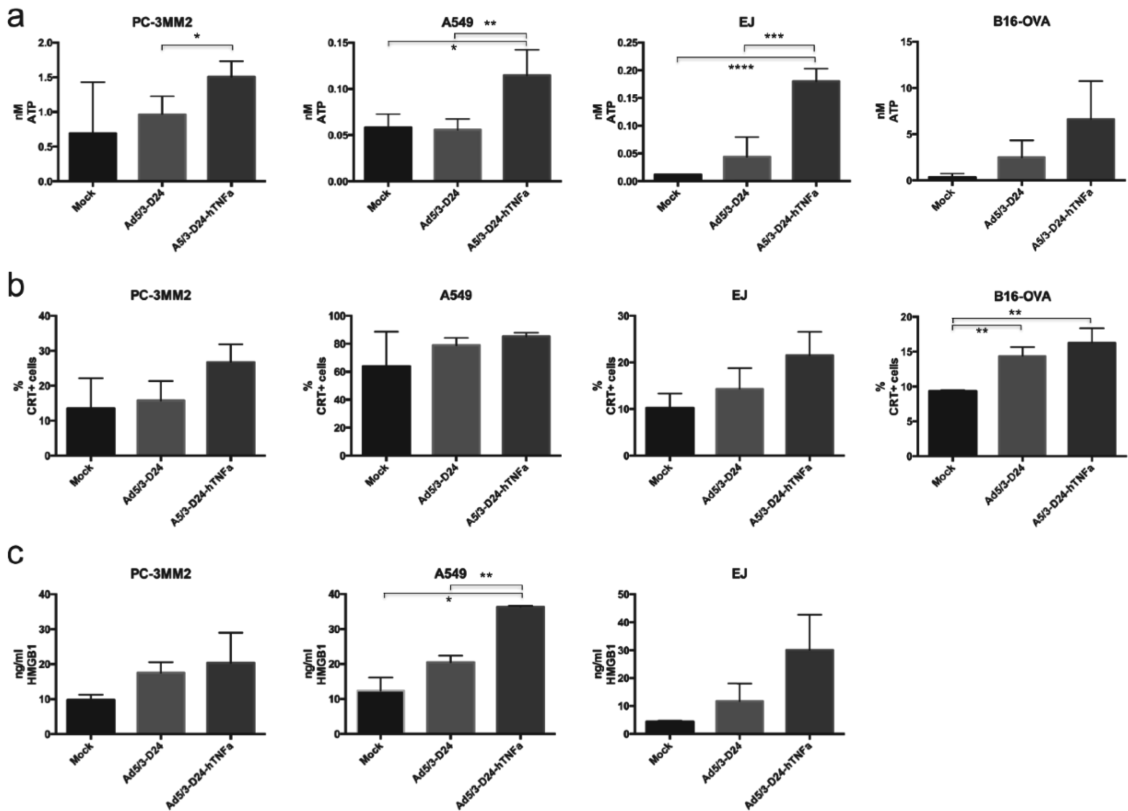


Figure 15. Expression of immunogenic cell death markers upon Ad5/3-Δ24-hTNFα infection. a) *ATP was measured from supernatants of cells infected with 100 VP/cell, 12 hours post infection with an ATP luciferase kit.* ***b)*** *Calreticulin exposure on cell surface was measured from cells infected with 100 VP/cell, 12 hours post infection (or 48 hrs p.i. for B16-OVA) with flow cytometry.* ***c)*** *HMGB1 (High-mobility group protein B1) excretion was measured from supernatants of cell cultures infected with 100 VP/cell, 24 hours post infection with ELISA. (Hirvinen et al. 2015, Human Gene Therapy [Study I])*

To summarize, the *in vitro* results of the studies with Ad5/3-Δ24-hTNFα, we can conclude that the TNFα expressed from Ad5/3-Δ24-hTNFα virus is biologically functional and capable of inducing efficient tumor cell killing. It also increases release of markers indicating immunogenic cell death.

8.1.2. *In vivo* efficacy and immunogenic potential of the Ad5/3-Δ24-hTNFα

After showing the efficacy of the virus *in vitro*, we started *in vivo* studies. The *in vivo* oncolytic activity of Ad5/3-Δ24-hTNFα was tested in a prostate cancer xenograft model. Groups of nude mice (NMRI nu/nu) were injected with PC-3MM2 human prostate cancer cells subcutaneously. These prostate cancer cells were chosen for the xenograft experiment based on the good results seen in the *in vitro* cell killing assays, and because of the aggressive nature of this cell line, to be able to show the power of the virus in a

challenging model. When the tumors reached an average diameter of 4-5mm the mice were injected intratumorally $1x10^8$ VP/tumor of virus (or PBS) on days 0, 1, 4 and 5. Results showed that the tumors were significantly smaller ($P < 0.001$ at day 40) in the Ad5/3-Δ24-hTNFα-treated mice compared to the control virus, and most of the Ad5/3-Δ24-hTNFα-treated tumors were fully cured within 40 days after the first virus injection (**Figure 16**). However, soon thereafter regrowth of some of the treated tumors was observed. This may be because the treatments were only given during the first five days of the experiment and actually the amount of virus administered per tumor during the treatments was quite low, only 4×10^8 VP/tumor in total. Also the lack of adaptive immune cells in nude mice most probably explains the regrowth of the tumors: nude mice do not develop memory cells against the tumor, thus some tumor cells that are still left can resume growing after the effect of the virus disappears. To maintain the tumor-eradicating potency of the virus in immunocompromised animals, repeated administration of the virus would be needed. However, in immunocompetent mice and in humans neutralizing antibodies against the virus may hinder the repeated use of the same virus (Zaiss, Machado & Herschman 2009), thus use of different Ad serotypes or totally different viruses could maintain the treatment efficacy. In addition, the group of mice treated with Ad5/3-Δ24-hTNFα had significantly longer survival compared to other groups ($P = 0.0002$).

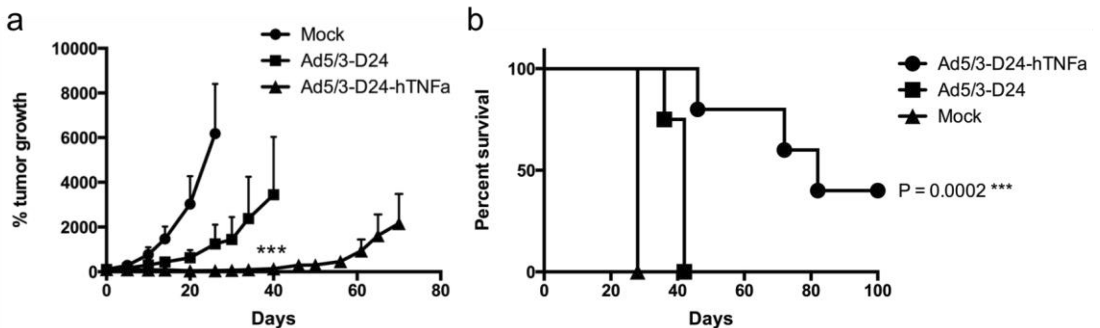


Figure 16. In vivo efficacy of Ad5/3-Δ24-hTNFα in a xenograft model. a) *Nude mice bearing PC-3MM2 tumors were treated intratumorally with PBS (Mock) or with $1x10^8$ VP/tumor of Ad5/3-Δ24 or Ad5/3-Δ24-hTNFα virus on days 0,1,4 and 5. Tumor growth was measured over time.* ***b)*** *Survival of nude mice bearing PC-3MM2 tumors was followed over time. Mice were treated with PBS or viruses as in a). Mice were euthanized when tumors exceeded 1.5 cm in diameter. (Hirvinen et al. 2015, Human Gene Therapy [Study I])*

We also determined the amount of apoptotic and necrotic cells in the treated PC-3MM2 tumors of the nude mice, but no statistical difference was found between the Ad5/3-Δ24-hTNFα and Ad5/3-Δ24-treated groups (**Figure 17**). Perhaps the viral replication itself is already such a powerful inducer of tumor cell death by these mechanisms (Bartlett et al. 2013) that TNFα does not bring much additional effect and benefit. The efficacy of the hTNFα-producing virus therefore more probably has an impact via a general inflammation reaction induced by TNFα in the Ad5/3-Δ24-hTNFα-treated mice followed by activation and

recruitment of NK cells into the treated tumors (Fiers 1991). NK cells have been shown to play a central role in tumor suppression after oncolytic adenovirus treatments (Cerullo et al. 2012). It would be interesting to further determine whether the efficacy of Ad5/3-Δ24-hTNFα was in fact because of NK cell infiltration to tumors.

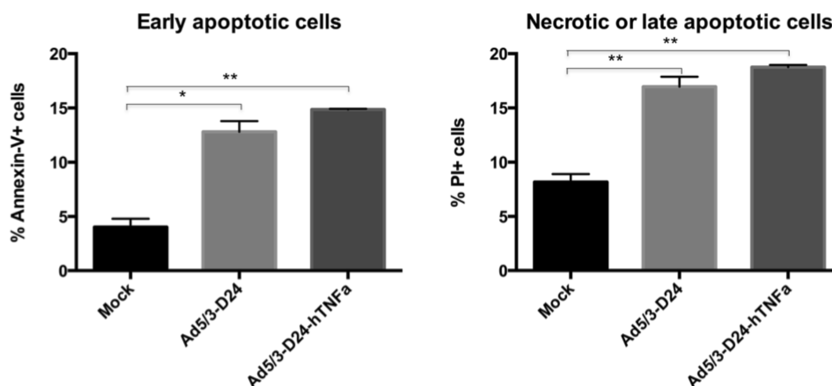


Figure 17. *The amounts of apoptotic and necrotic cells in the treated tumors were analyzed by flow cytometry. FITC-labeled Annexin-V was used to indicate the early apoptotic cells, and PI (propidium iodide) the necrotic or late apoptotic cells. (Hirvinen et al. 2015, Human Gene Therapy [Study I])*

As the very crucial role of the adaptive immune system is now well recognized in determining the long-term efficacy of OV therapies (Sobol et al. 2011), we continued to study the efficacy of our TNFα-virus in an immunocompetent mouse model. We performed studies with C57BL/6 mice with syngeneic melanoma tumors (B16-OVA) to study the immunological responses induced by the Ad5/3-Δ24-hTNFα virus *in vivo*. It has been shown that mouse TNFα receptor TNF-RI binds both human and mouse TNFα (Fiers 1991, Cauwels, Fiers & Brouckaert 1996), so human TNFα is active in mice, but with less activity than in humans (Han et al. 2007, Asher et al. 1987, Talmadge et al. 1988). We tested two different regimens of virus, “mild” and “high”. In the “mild” regimen, the mice were treated intratumorally with $1x10^8$ VP/tumor on early days (days 0, 1, 4 and 5), whereas in the “high” regimen the mice received the virus every other day (days 0, 2, 4, 6 and 8). The tumor growth was significantly slower with both regimens in the Ad5/3-Δ24-hTNFα-treated group of mice compared to the Ad5/3-Δ24 treated group (“mild” P <0.0001 and “high” <0.01) (**Figure 18**); however the tumors were not completely eradicated. Because human adenovirus does not replicate in murine cells, the tumor-suppressing effect was likely due to the inserted transgene, hTNFα. The B16-OVA cell line has actually been reported to be quite insensitive to TNFα (Fransen et al. 1986) and it is also a very aggressively growing cell line, which may explain the relatively low efficacy seen in these experiments.

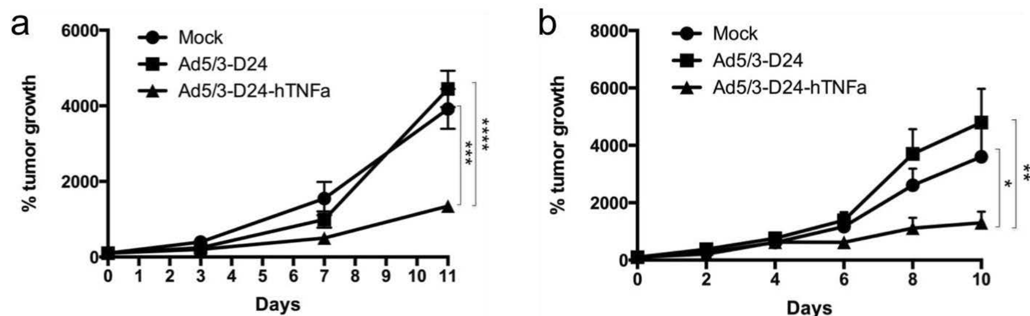


Figure 18. Effects of the expressed hTNFα in immunocompetent mice. *C57BL/6 mice bearing syngeneic B16-OVA tumors were treated intratumorally with PBS (Mock) or with 1x10*^{8} *VP/tumor of Ad5/3-Δ24 or Ad5/3-Δ24-hTNFα virus* ***a)*** *on days 0, 1, 4 and 5 or* ***b)*** *every other day on days 0, 2, 4, 6 and 8. Tumor growth was measured over time. (Hirvinen et al. 2015, Human Gene Therapy [Study I])*

The B16-OVA cell line expresses chicken ovalbumin (OVA), a foreign protein to the mouse system, which can be detected by an OVA-peptide specific MHC-I pentamer antibody in flow cytometric assays. Because TNFα has been reported to activate and recruit lymphocytes (Fiers 1991, Calzascia et al. 2007), we were interested in studying whether our TNFα-virus can increase T cell infiltration into the tumors of treated mice. Usage of the B16-OVA model enabled us to determine the degree of tumor-specific immunity developed in the treated mice by analyzing the OVA-specific (anti-tumor) cytotoxic T cells. We characterized the immune responses in tumors, spleens and lymph nodes collected from the mice 9 days after the first virus treatment (**Figure 19**). To our delight, we observed that the amount of CD8+ cytotoxic T cells was increased in the TNFα-virus treated tumors compared to mock and Ad5/3-Δ24 treated tumors ($P < 0.05$). Moreover, the amount of CD19+ cells (B cells) was significantly elevated in the Ad5/3-Δ24-hTNFα treated tumors compared to mock ($P < 0.05$). However, although increased amounts of OVA-specific (tumor-specific) T cells in the Ad5/3-Δ24-hTNFα treated tumors could be observed, the differences were not statistically significant compared to controls.

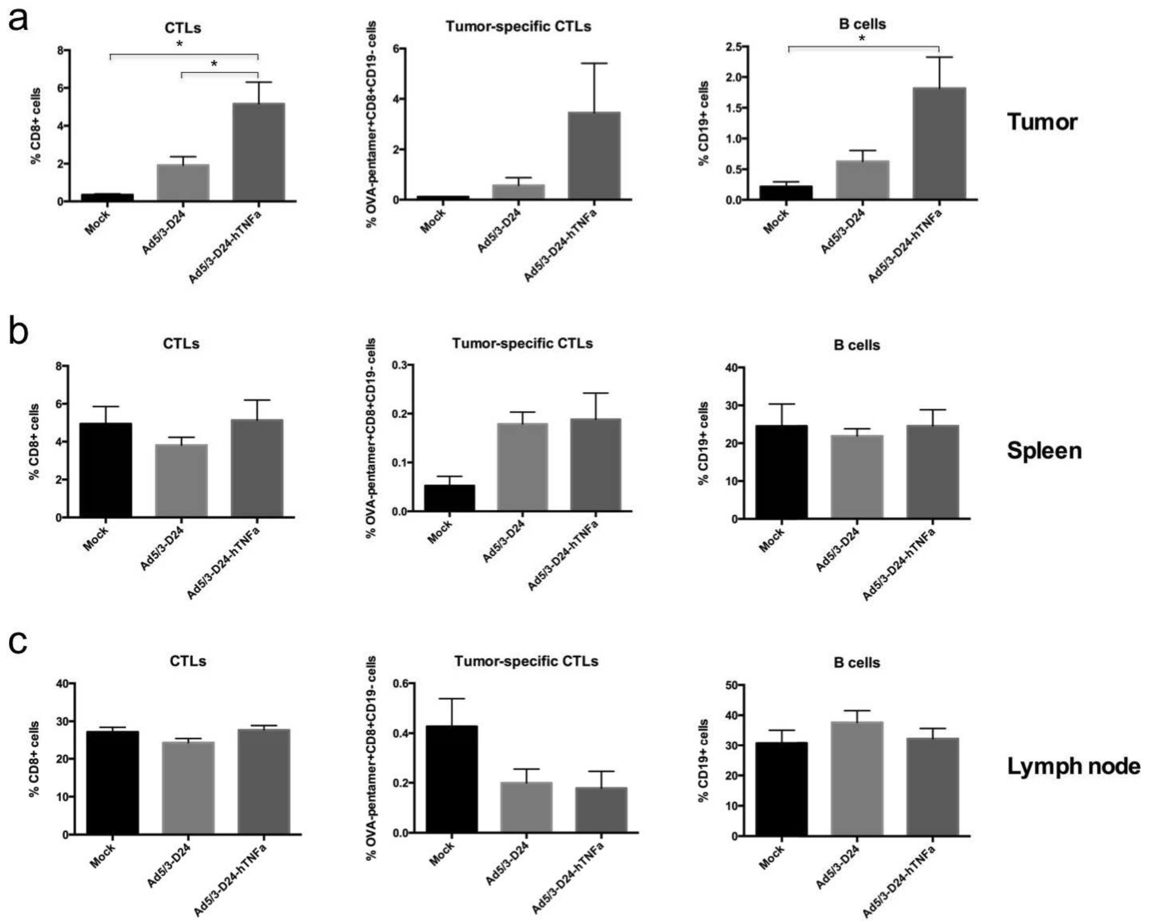


Figure 19. Immune cells in tumors, spleens and lymph nodes of C57BL/6 mice bearing B16-OVA tumors. *General CD8+ cytotoxic T lymphocytes (CTLs), tumor-specific (OVA-specific) CTLs and CD19+ B cells were measured from a) tumors, b) spleens and c) inguinal lymph nodes of mice treated every other day with PBS (mock) or with $1x10^8$ VP/tumor of Ad5/3-Δ24 or Ad5/3-Δ24-hTNFα virus. The organs were collected 9 days after the first virus injection and then smashed through a filter, cultured overnight, and analyzed by flow cytometry. (Hirvinen et al. 2015, Human Gene Therapy [Study I])*

In spleens and lymph nodes of the treated mice we did not find any differences in the amounts of infiltrated immune cells; however, the tumor-draining lymph nodes were visibly bigger in the Ad5/3-Δ24-hTNFα treated mice (**Figure 20**) compared to control virus suggesting higher activation of immune responses.

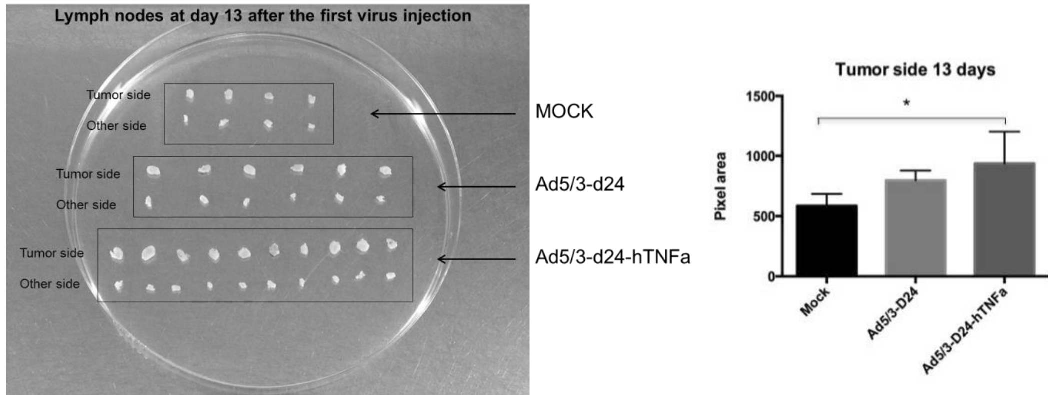


Figure 20. *a) Picture of the tumor draining and non-tumor side inguinal lymph nodes of the treated mice on day 13 after the first virus injection. b) The sizes of the lymph nodes were measured from the images using Image J software.*

We measured the production of hTNFα in the tumors collected 10 days after the first virus treatment; the mean amount of human TNFα in the Ad5/3-Δ24-hTNFα-treated tumors was 878 pg/ml (SD ±665). As leakage of TNFα in high concentrations into circulation has been shown to lead to severe toxic consequences, such as septic shock and even death (Mueller 1998, Schiller et al. 1991, Fiers 1991), we measured the amount of hTNFα also in the serum of the treated mice at several time points (on days 4, 8 and 10 after the first virus injection). No significant amounts of hTNFα in blood circulation were observed after the Ad5/3-Δ24-hTNFα treatments (**Figure 21**), even though hTNFα was quite high in the tumors. The fact that hTNFα produced by the virus seems to stay mostly in the tumors and not leak into circulation indicates that the treatment is safe when injected intratumorally and should not lead to systemic toxicity even with higher doses.

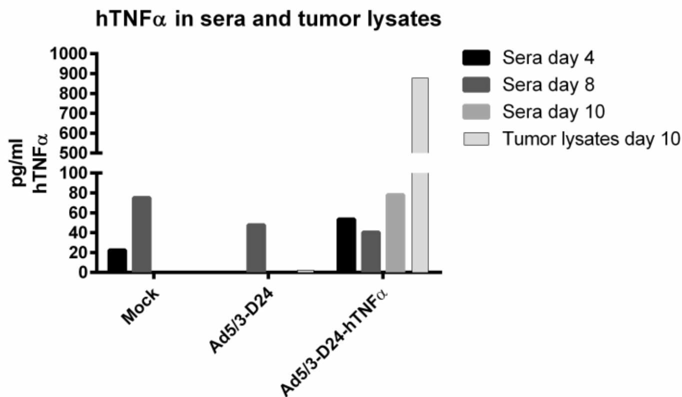


Figure 21. Human TNFα levels in tumor lysates and sera. *C57BL/6 mice bearing B16-OVA tumors treated i.t. with PBS or viruses (on days 0, 1, 4 and 5) were analyzed for hTNFα levels by cytokine bead array. (Hirvinen et al. 2015, Human Gene Therapy [Study I])*

TNFα has been reported to induce secretion of other cytokines too (at least GM-CSF, IFN-gamma, IL-6, IL-1 and IL-8) (Fiers 1991, Balkwill 2009), which together with the production of endothelial adhesion factors can activate and recruit various immune cells (neutrophils, macrophages, eosinophils, NK-cells and lymphocytes) to attack the TNF production site (tumor in our case). To determine if hTNFα produced by the virus would induce secretion of other cytokines, serum was collected from the treated mice at different time points (9 and 13 days after the first treatment) and analyzed for the cytokine levels (mGM-CSF, mIL-6, mIFN-γ and mIL-1α) by Cytometric Bead Array (**Figure 22**). We also measured the cytokines in the tumors 13 days after the first treatment. All in all, the results showed that there were no differences in the cytokine amounts between the treatment groups on the selected days. Maybe these sampling points were timely too far from the first treatment and perhaps elevated amounts could be observed at earlier time points. However, these results indicate that there is no systemic toxicity related to the Ad5/3-Δ24-hTNFα therapy, in contrast to earlier reports, where elevated, long-lasting concentrations of these pro-inflammatory cytokines in blood correlated with treatment-related toxicity (Tarrant 2010).

The results of the *in vivo* studies presented here show that arming the oncolytic adenovirus with hTNFα results in enhanced anti-tumor efficacy in mice. The results suggested that the enhanced tumor-killing potency at least partly was due to an increased infiltration of immune cells into the treated tumors, but more thorough studies (e.g. histological studies) of the mechanisms-of-action of the virus would be needed to fully understand its efficacy. TNFα is known to increase extravasation of viruses to tumors through activation of a Rho A/Rho kinase pathway (Seki et al. 2011), thus it should be investigated whether the TNFα production by the TNF-virus increases viral uptake in the treated tumors. Moreover, many cell types are known to become highly susceptible to the cytotoxic effects of TNFα after viral infection (Fiers 1991), which may also explain the effect of our viral construct.

8.1.3 Combining Ad5/3-Δ24-hTNFα with radiotherapy

TNFα has been reported to also have an ability to sensitize cells to radiation (Balkwill 2009). Therefore, we wanted to test whether combining the Ad5/3-Δ24-hTNFα treatment with radiation would result in enhanced tumor cell killing. First, we tested the combination treatment *in vitro* with three cell lines (A549, PC-3MM2 and B16-OVA) (**Figure 23**). The cells were infected with low dose of viruses (0.1 VP/cell) or with PBS (mock) and then irradiated with different doses (0, 4, 8 and 10 Gy) 24 hours later. Cell viability was measured by MTS assay. For unknown reason the irradiation itself had no effect on cell viability regardless of dose. There was also no significant difference between Ad5/3-Δ24-hTNFα and Ad5/3-Δ24 infections with any of the cell lines. These results are fairly surprising since it was shown by Rajecki et al. that with 15 Gy irradiation and with the same dose of virus (0.1 VP/cell) the cell viability dropped to around 30 percent (Rajecki et al. 2009). It could, however, be that 10 Gy, the highest dose we used, was not enough to kill the cells and perhaps we should have used higher radiation doses.

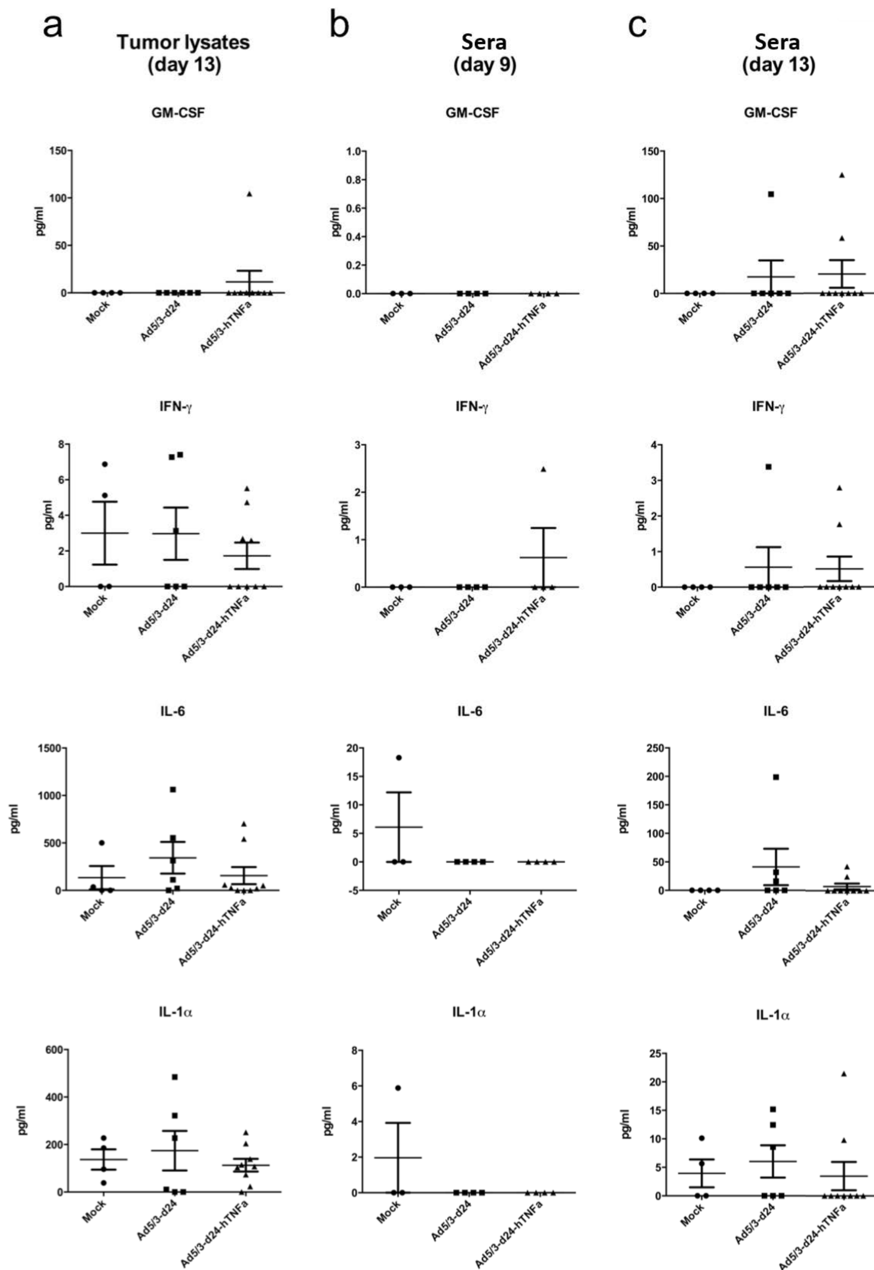


***Figure 22. Cytokine concentrations in sera and tumor lysates**. Levels of different cytokines (mouse GM-CSF, mouse IFN-gamma, mouse IL-6 and mouse IL-1α) were measured from **a)** tumor lysates (13 days after the first virus injection) and from serum samples **b)** 9 and **c)** 13 days after the first virus injection) with a cytokine bead array. The samples were from C57BL/6 mice bearing B16-OVA tumors treated every other day on days 0, 2, 4, 6 and 8 with PBS, Ad5/3-Δ24 or Ad5/3-Δ24-hTNFα. (Hirvinen et al. 2015, Human Gene Therapy [Study I])*

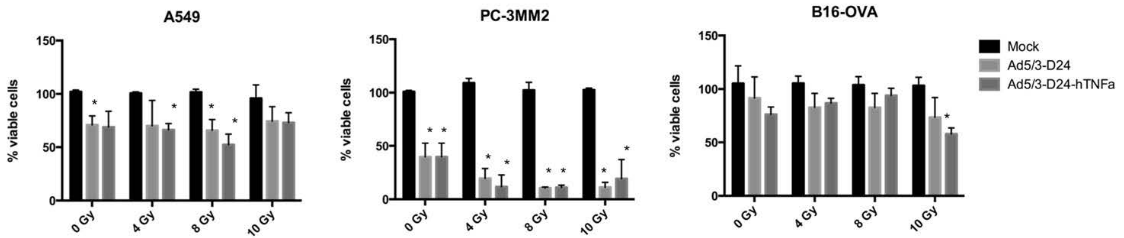


Figure 23. In vitro tumor cell killing in combination with radiation. *Cell killing was measured with MTS assay in A549, PC-3MM2, and B16-OVA cells after treatment with different doses of irradiation (0, 4, 8 and 10 Gy). Cells were treated with growth medium only (mock) or with Ad5/3-Δ24-hTNFα or Ad5/3-Δ24 (0.1 VP/cell) 24 hours before irradiation. Statistical significances are against mock. (Hirvinen et al. 2015, Human Gene Therapy [Study I])*

Despite the disappointing results *in vitro*, we however continued to study the combination therapy *in vivo.* To this end, we first performed an experiment with a prostate cancer xenograft model (nude mice bearing PC-3MM2 tumors) (**Figure 24**). The mice were first treated intratumorally with viruses (or PBS for mock) two days in a row (days 0 and 1) and then they received the first round of irradiation (2-Gy whole-body irradiation). Thereafter mice were allowed to recover for one day, and the same treatment scheme was repeated (virus treatment on days 4 and 5 and irradiation on day 6). The virus doses used per injection were $1x10^8$ VP/tumor. In this prostate cancer xenograft model, we saw a clear reduction in tumor growth in the Ad5/3-Δ24-hTNFα -treated group compared to the Ad5/3-Δ24 group ($P < 0.01$ at day 34). Combining the Ad5/3-Δ24-hTNFα therapy with radiation really boosted the effect of irradiation alone (mock) or the empty control virus. Maybe this is, indeed, as mentioned above, due to TNFα -mediated sensitization of the cells to the cytotoxic effects of irradiation. To investigate this, we analyzed the amount of apoptotic and necrotic cells in the tumors by flow cytometry (**Figure 25**). We compared the results to those from a similar experiment done without irradiation (**see Figure 17**). We noticed that indeed, the combination therapy using radiation and the Ad5/3-Δ24-hTNFα virus increases the amount of necrotic/late apoptotic cells ($P < 0.01$), which may partly explain the drastic tumor growth control seen in this group of mice (**Figure 24**).

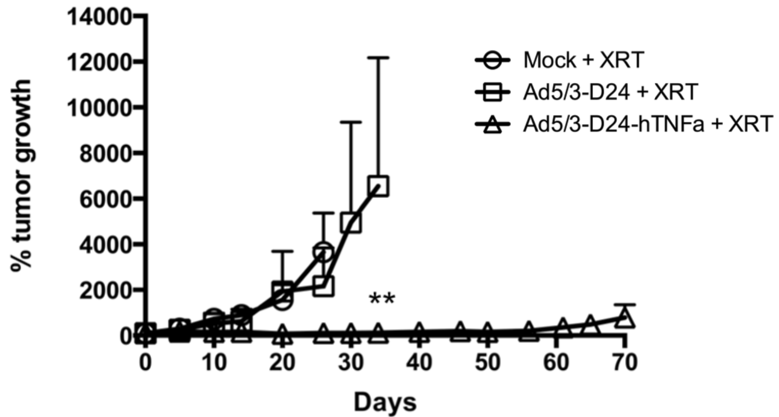


Figure 24. Tumor growth after combination treatment with virus and radiation in the prostate cancer xenograft model. *Nude mice bearing PC-3MM2 tumors were treated intratumorally with PBS (mock) or 1x10^{8} VP/tumor of Ad5/3-Δ24 or Ad5/3-Δ24-hTNFα virus on days 0, 1, 4 and 5. On days 2 and 6, the mice received 2-Gy whole-body irradiation. Tumor growth was measured over time. (XRT=irradiation). (Hirvinen et al. 2015, Human Gene Therapy [Study I])*

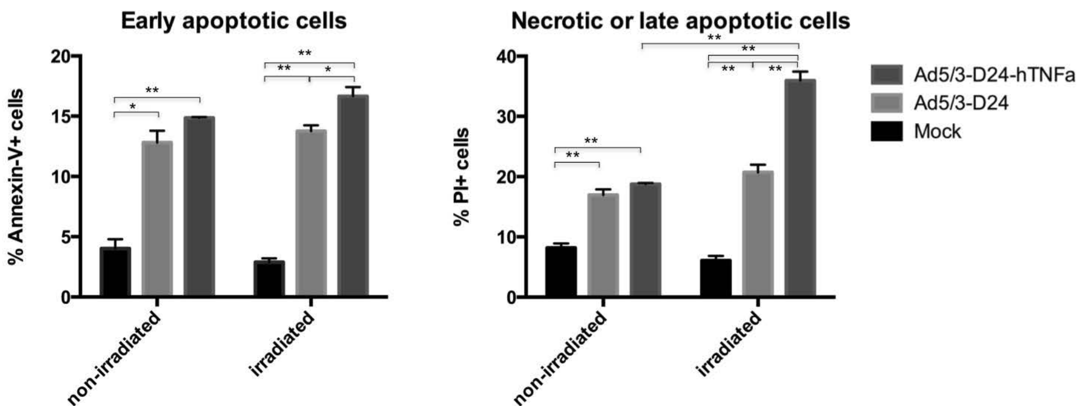


Figure 25. Apoptotic and necrotic PC-3MM2 tumor cells in irradiated and non-irradiated tumors. *The amount of early apoptotic and late apoptotic or necrotic cells at day 8 after the first virus infection with and without 2 times 2Gy irradiation were analyzed by flow cytometry from PC-3MM2 tumors of the nude mice. FITC-labeled Annexin-V was used to indicate early apoptotic cells and PI (propidium iodide) necrotic or late apoptotic cells. (Hirvinen et al. 2015, Human Gene Therapy [Study I])*

We also studied the combination therapy in a syngeneic melanoma model in immunocompetent mice (C57BL/6 mice bearing B16-OVA tumors) with a treatment scheme identical to that used in the prostate cancer xenograft model. In this immunocompetent model, tumor growth was significantly reduced ($P < 0.05$) in the Ad5/3-Δ24-hTNFα treated group compared to mock-treated group, but there was no significant difference between Ad5/3-Δ24-hTNFα treated and Ad5/3-Δ24 treated groups (**Figure 26**).

The lack of efficiency of Ad5/3-Δ24-hTNFα may be because of the known radioresistant nature of melanomas (Fertil & Malaise 1985); however, the duration of the follow-up period in this experiment was so short due to the aggressive nature of the B16-OVA cell line that no definitive conclusions can be drawn.

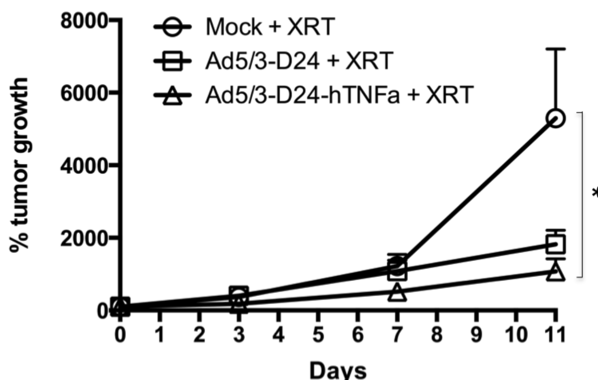


Figure 26. Tumor growth after combination treatment with radiation in a syngeneic melanoma model. *C57BL/6 mice bearing B16-OVA tumors were treated intratumorally with PBS (mock) or with 1x10*^{8} *VP/tumor of Ad5/3-Δ24 or Ad5/3-Δ24-hTNFα virus on days 0, 1, 4 and 5. On days 2 and 6 the mice received 2-Gy whole-body irradiation. Tumor growth was measured over time. (Hirvinen et al. 2015, Human Gene Therapy [Study I])*

In summary, in Study I we showed effective tumor cell killing by the Ad5/3-Δ24-hTNFα virus both *in vitro* and *in vivo*, which was associated with signs of immunogenic cell death. The virus also enhanced recruitment of adaptive immune cells to the infection site. We saw some potential for combining the Ad5/3-Δ24-hTNFα therapy with radiation: it was shown to result in enhanced tumor growth control and increased necrotic tumor cell death. Further studies to discover the mechanisms-of-action of the virus and deeper insight into its effects on the immune system should be conducted. Because TNFα is known to cause selective permeabilization of tumor vasculature and thereby increase the uptake of therapeutic agents into tissues (Seki et al. 2011, Balkwill 2009), a combination therapy with chemotherapeutics would also be worth considering.

8.2. Improving the vaccine and adjuvant potency of oncolytic vaccinia virus by arming with self-recognizing receptor DAI (II)

Vaccinia viruses (VVs), like adenoviruses, are one of the most extensively used viruses in immunotherapy studies and have a long track record of safety as well as a fast replication cycle making them intriguing vectors for oncolytic virotherapy. However, VVs encode several immunosuppressive genes and are thus well prepared to hide from the host immune system, which hampers their applicability as immunovirotherapeutics. Therefore, in Study II we developed a new oncolytic vaccinia virus that expresses a pattern recognition receptor DAI (DNA-dependent activator of interferon-regulatory factors) to improve the

immunostimulatory potency of vaccinia virus for enhanced activation of anti-tumor immune responses. We hypothesized that arming a cancer therapy virus with a receptor that regulates and induces the production of a number of different immunostimulatory molecules would generate a more robust immunological response than just arming the virus with a single cytokine (Lladser et al. 2011, Smith et al. 2013). This fierce immune response could eventually break the immunosuppressed conditions often found in advanced tumors (Schreiber, Old & Smyth 2011, Devaud et al. 2013, Igney & Krammer 2002) and in VV infected tissues (Smith et al. 2013).

8.2.1. Is DAI able to sense vaccinia virus infection?

PRRs, such as toll-like receptor 2 (TLR2) (Zhu, Huang & Yang 2007, Quigley et al. 2009) and TLR8 (Martinez, Huang & Yang 2010), as well as AIM2 (Rathinam et al. 2010) have been identified as mediators of innate immune responses against vaccinia virus. However, none of these mediators is known to trigger type I interferon expression, which however is known to be elicited upon VV infection. Zhu et al. showed that an additional, as yet unidentified TLR-independent pathway also contributes to the response (Zhu, Huang & Yang 2007). A little later, Takaoka et al. demonstrated that a cytosolic DNA sensor called DAI (also known as DLM-1 or ZBP1) can induce type I interferons upon binding to double-stranded DNA (Takaoka et al. 2007). Since DNA is normally contained in the nucleus of a cell, it uses cytosolic PRRs, such as DAI as indicators of a viral infection. Indeed, DAI has recently been reported to sense at least adenoviral (Schulte et al. 2013) and HSV DNA (Triantafilou, Eryilmazlar & Triantafilou 2014, Pham et al. 2013). Because the whole life cycle of VV is retained in the cytosol of the host cell, we hypothesized that DAI could be a sensor also for VV infection and play a role in the induction of innate immune responses.

In Study II, we first examined the role of DAI in controlling infection with live vaccinia virus. We used Jurkat cells silenced for DAI by stable transfection of a plasmid encoding siRNA against DAI. As a control we used Jurkat cells expressing siRNA against luciferase. We observed that when the cells were infected with VV (vvdd-GFP), IFNβ production, as analyzed by IFNβ gene expression (qPCR) and protein expression (ELISA) after VV infection, was significantly reduced in the DAI-silenced cells ($P = 0.005$ and $P = 0.002$, respectively) (**Figure 27a and b**). On the contrary, in control cells, IFNβ production increased 2.7-fold compared to uninfected cells.

Type I interferons activate interferon regulatory factors (IRFs). This promotes synthesis and secretion of pro-inflammatory cytokines through the NF-κB pathway. Thus, we investigated whether DAI would have an effect on the secretion of cytokines also after VV infection. We measured interleukin 6 (IL-6) and tumor necrosis factor alpha (TNFα) secretion in the supernatants of infected and uninfected modified Jurkat cells 24 hours after infection (**Figure 27c**). Prior to infection, the cytokine levels were very low or undetectable in both cell lines. After VV infection Jurkat-psiRNA-luc cells showed significantly higher Il-6 ($P = 0.04$) and TNFα ($P = 0.01$) concentrations compared to DAI-silenced cells. These results suggest that DAI might be an important mediator of innate immunity during vaccinia virus

infection, which moreover suggests utility of this PRR as an adjuvant for VV vectors to boost innate immune responses followed by possible activation of adaptive immunity. Actually, recently Dai et al. reported that a newly discovered cytosolic DNA sensor called cGAS can also trigger type I IFN responses after modified vaccinia virus Ankara (MVA) infection through STING-mediated pathway in conventional murine dendritic cells (Dai et al. 2014). This just shows that little is still known about vaccinia virus immunity and that our finding of DAI – vaccinia virus interaction is only one of the many ways vaccinia viruses interact with their hosts.

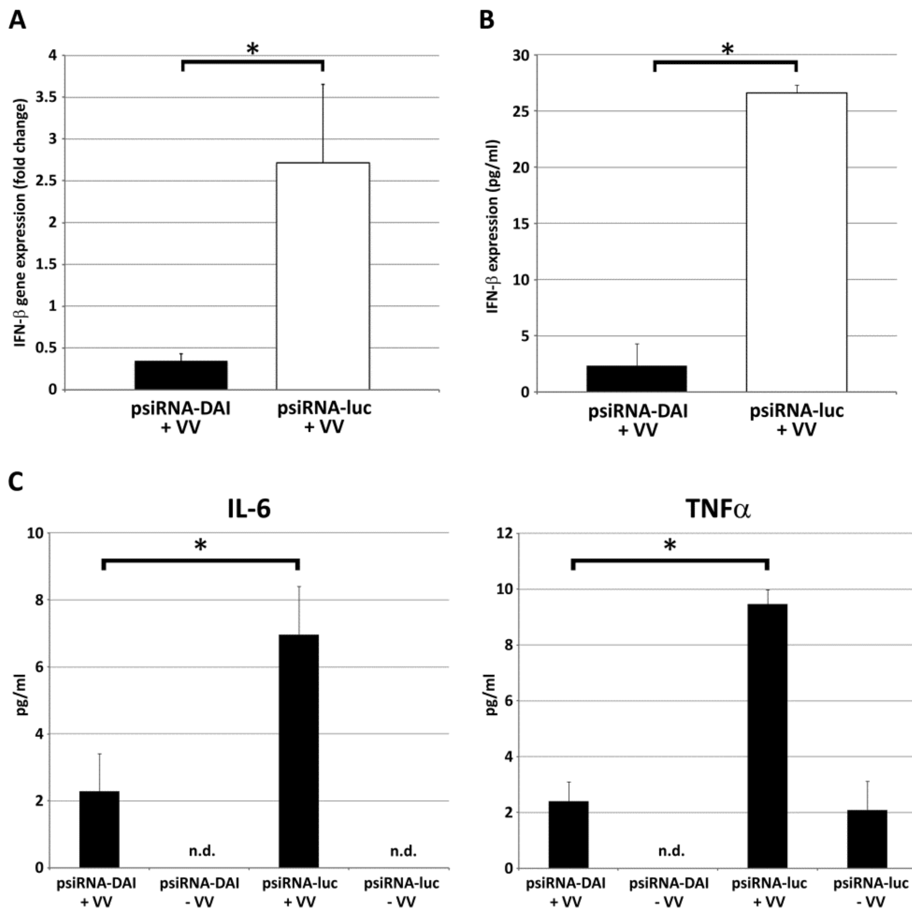


Figure 27. DAI mediates induction of type I interferon and cytokine release during VV infection. *Jurkat cells silenced for DAI with psiRNA-DAI or control cells transfected with psiRNA-luc were infected with vvdd-GFP and IFN-β was quantified by* ***a)*** *qPCR and* ***b)*** *ELISA.* ***c)*** *Il-6 and TNFα was measured by FACSarray from the supernatant before (-VV) and 24 hours after vvdd-GFP infection (+VV).*

8.2.2. *In vitro* oncolytic efficacy of DAI-armed vaccinia virus

The finding that DAI can induce activation of innate immune responses during VV infection encouraged us to generate a novel oncolytic vaccine for cancer immunovirotherapy; our approach uses an oncolytic vaccinia virus construct that rapidly replicates specifically in tumor cells and expresses DAI at the infection site. Since the virus amplifies the expression of a receptor that senses viral DNA and triggers cytokine expression, the generated virus is a self-amplifying system that can boost viral immunogenicity and cellular immune responses at the tumor site regardless of cellular expression levels of DAI.

We cloned two oncolytic VVs, one coding for human (vvdd-tdTomato-hDAI) and the other coding for mouse DAI (vvdd-tdTomato-mDAI) (**Figure 28**). The viruses are of the Western Reserve strain and double-deleted for the viral thymidine kinase (TK) gene and for the vaccinia growth factor (VGF) gene (Parviainen et al. 2014, McCart et al. 2001, Guse et al. 2010). These deletions make the virus able to replicate only in cancer cells where the corresponding pathways are mutated and constantly activated (Thorne et al. 2007, Buller et al. 1988) (see **Figure 7**). The viruses also express tdTomato, a red fluorescent protein for imaging purposes and biosafety concerns (Parviainen et al. 2014, Shaner et al. 2008).

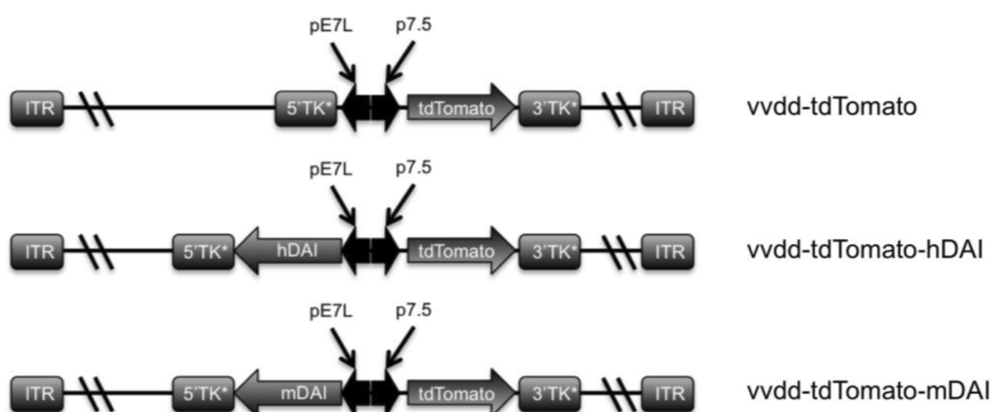


Figure 28. Schematics of the generated double-deleted oncolytic vaccinia viruses expressing DAI and tdTomato. *(Hirvinen et al. 2016, Molecular Therapy – Oncolytics [Study II])*

The functionality of the viruses, vvdd-tdTomato-hDAI and vvdd-tdTomato-mDAI, was tested in tumor cell killing assays (MTS viability assays) with several cancer cell lines (human PC-3MM2, A549, HS294T, SK-MEL-2, A2058 and murine B16-OVA cells). The cancer cell killing potency of the DAI-viruses was compared to a control virus without DAI expression (vvdd-tdTomato). We found no difference in the cell-killing efficacy between the DAI-virus and the control; hence we can conclude that the inserted transgene at least does not hinder oncolytic activity of the virus (**Figure 29**).

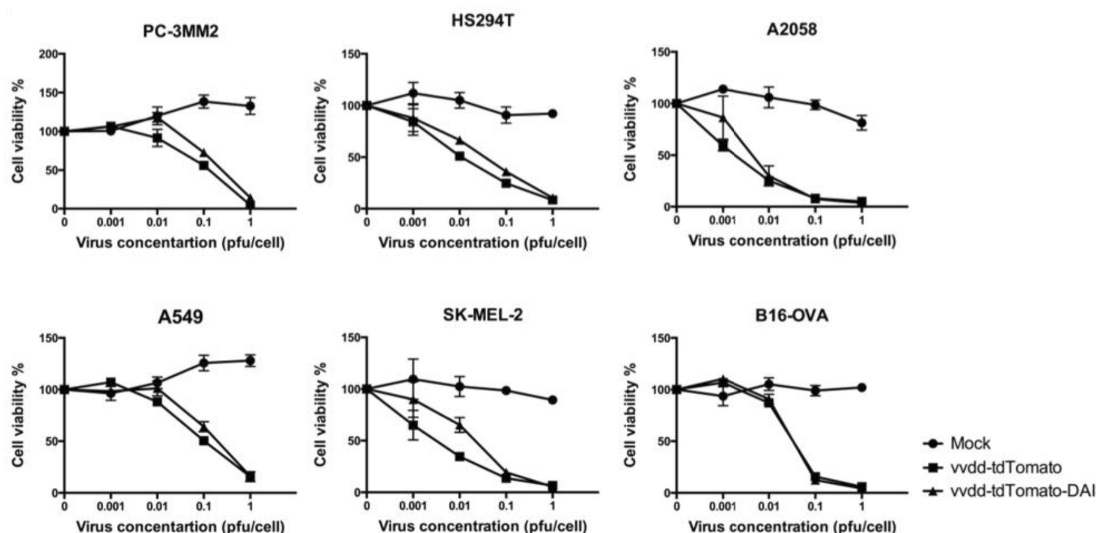


Figure 29. Cancer cell-killing efficacy in vitro. *MTS cell viability assays were performed in several human and mouse cancer cell lines 5 days after infection with vvdd-DAI or control virus. (Hirvinen et al. 2016, Molecular Therapy – Oncolytics [Study II])*

8.2.3. Effects of vvdd-tdTomato-DAI infection on cellular gene expression

Next we studied the effect of DAI expression by the virus on the gene expression profile of infected cells. We infected human melanoma cells (HS294T) and human monocyte cells (THP-1) with vvdd-tdTomato-hDAI or with the control virus vvdd-tdTomato (0.1 pfu/cell). Sixteen hours after infection, whole genomic mRNA was extracted and a wide-genome gene expression profile was analyzed and the gene expression levels were compared to non-infected cells (mock). In the DAI-virus infected cells, there was approximately a 7-fold increase in the expression levels of DAI in both cell lines (7.4 and 7.2 in THP1 and HS294T, respectively) compared to control cells infected with unarmed virus, as expected. Differentially expressed genes were plotted by BACA representing tools (Fortino, Alenius & Greco 2015) according to different biological processes (Figure 2 of Study II). The BACA plot analysis revealed highly differing expression patterns in the THP-1 monocyte cell line infected with DAI-expressing or the non-expressing viruses. An ingenuity pathway analysis (IPA) showed that the most up-regulated genes in the DAI-virus infected cells belong to important immunological pathways, including maturation of dendritic cells, communication between the innate and adaptive immune systems and virus sensoring PRRs (Supplementary Figure S2 of Study II). These findings are very intriguing because they show that DAI has the ability to alter the immunogenicity of an oncolytic vaccinia virus and it can induce activation of genetic pathways related to innate and adaptive immune systems. For instance the notion that DAI has potential to activate pathways belonging to DC activation is intriguing because we know that oncolysis can release tumor-associated antigens to the microenvironment, to be further presented to T cells by DCs followed by

possible activation of adaptive, tumor-specific immunity (Miller & Fraser 2003, Nakamori et al. 2004).

8.2.4. *In vivo* efficacy and immunogenicity of vvdd-tdTomato-mDAI in syngeneic mouse melanoma model

We then studied the *in vivo* efficacy of the DAI-virus in an immunocompetent murine melanoma model. The immunogenic potency of the DAI-virus was tested *in vivo* using an aggressive syngeneic melanoma model. C57BL/6 mice bearing subcutaneous B16-OVA tumors (12 mice/ group, one tumor/ mouse) were treated intratumorally with vvdd-tdTomato-mDAI or the control vvdd-tdTomato or PBS (mock) two times on day 0 and 2. Tumor growth in mice treated with mDAI-expressing virus was significantly ($P < 0,001$) reduced compared to mice treated with the control virus (**Figure 30**); in fact the majority of tumors treated with mDAI-virus were completely cured, which is a very promising result in such an aggressive tumor model.

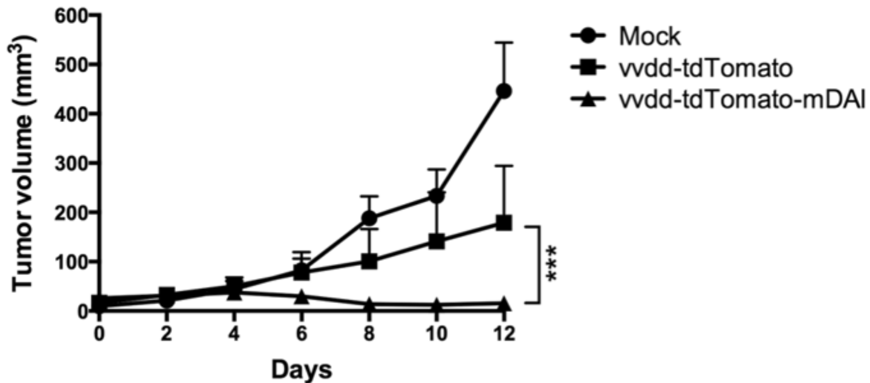


Figure 30. In vivo efficacy of i.t. vvdd-tdTomato-mDAI in treatment of syngeneic B16-OVA melanomas. *Immunocompetent C57BL/6 mice bearing B16-OVA tumors (1 tumor/mouse, n=12 mice/group) were treated intratumorally with PBS (mock) or with viruses on days 0 and 2. (Hirvinen et al. 2016, Molecular Therapy – Oncolytics [Study II])*

To study the induction of immune responses after vvdd-DAI treatments, we collected tumors of some of the mice (4/group) 12 days after the first virus treatment. Infiltration of T cells (CD8+, CD4+ and tumor-specific (OVA-specific) cytotoxic T cells) were analyzed by flow cytometry. We did not observe significant differences in the amounts of T cells between the groups, probably due to the low number of tumors analyzed. But there was a positive trend towards an increased amount of T cells in mDAI-virus treated tumors (Figures 3b and c of Study II).

Anti-tumor immunity induced by the vvdd-tdTomato-mDAI was also determined in untreated distant tumors. This was tested by re-challenging the treated mice by injection of a second tumor of same origin. We did not treat the mice anymore; we only monitored whether the second tumor would grow or not, i.e. if systemic immunity was generated

against the tumor that would prevent the growth of new tumors. We actually re-challenged part of the mice with exactly the same tumor cell line (B16-OVA) and the rest of the mice with B16-F10 cells, which is a parental line of B16-OVA and without expression of the immunogenic chicken ovalbumin protein. We gladly saw that the mice, whose first primary tumor had been treated with vvdd-tdTomato-mDAI, completely rejected the engrafted second B16-OVA tumor, whereas in mice not treated with virus (mock) or treated with the control virus secondary tumors developed (**Figure 31**).

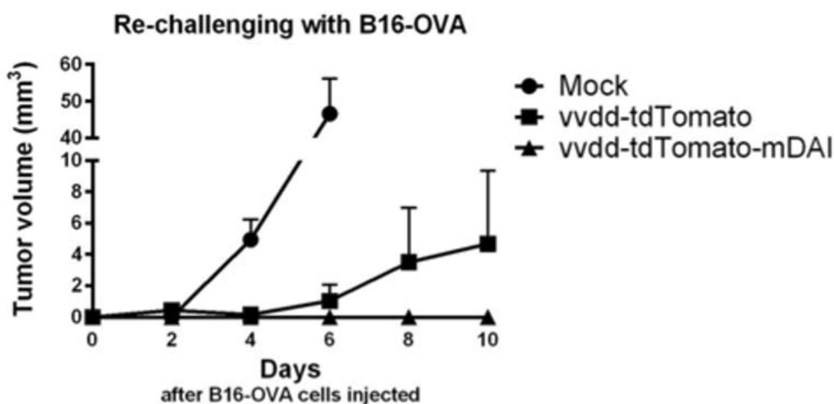


Figure 31. Growth of untreated distant tumors after vvdd-mDAI therapy. *C57BL/6 mice first received one B16-OVA tumor, which was treated on days 0 and 2. Ten days after the first treatment, the mice were re-challenged with another tumor. The second tumor was not treated; only the tumor growth was monitored. (Hirvinen et al. 2016, Molecular Therapy – Oncolytics [Study II])*

The treatment with the mDAI-virus was also able to control, at least to some extent, the growth of a secondary B16-F10 tumor as only half of the mice developed a second tumor, whereas all the mice treated with the control virus or PBS developed a second tumor (Figure 4C of Study II). These results suggest that the DAI-virus treated mice had developed systemic anti-tumor immunity. This immune response was mostly directed towards the immunogenic foreign OVA protein, as deduced from the tumor growth results; however immunity against other tumor-specific antigens would probably develop as well. To investigate this, we collected the spleens of the treated, re-challenged mice and analyzed the amount of IFNγ producing (i.e. activated) T cells in each animal group by ELISpot assay. We pulsed the splenocytes with SIINFEKL peptide (OVA-specific peptide) and with peptides derived from the natural melanoma-associated antigens TRP-2 (SVYDFFVWL) and gp100 (KVPRNQDWL) to analyze anti-tumor T cell responses. To also analyze the anti-viral responses, we challenged the cells with vaccinia peptides.

The anti-tumor T cell responses were increased in the DAI-virus treated mice, especially when the mice were re-challenged with the B16-OVA tumor cell line (**Figure 32**), and intriguingly, we also saw responses against the clinically relevant melanoma antigens TRP-2

and gp100. Interestingly, the anti-viral responses were relatively similar in both vvdd-tdTomato and vvdd-tdTomato-mDAI treated mice (**Figure 32**), even though the mechanism-of-action of the virus was hypothesized to rely on enhanced recognition of the virus DNA and thereby lead to induction of enhanced immune responses in the infection site (i.e. tumor).

We showed that the DAI-armed vaccinia virus can significantly reduce tumor growth and has potential to induce T cell infiltration and to activate tumor-specific immunity in immunocompetent mice with B16 melanoma tumors. We also showed that systemic anti-tumor response induced by the DAI-virus may even control growth of distant non-treated tumors. However, to be able to draw any definitive conclusions about anti-tumor immunity in the DAI-virus treated mice, these experiments should be repeated with larger groups to increase the statistical power.

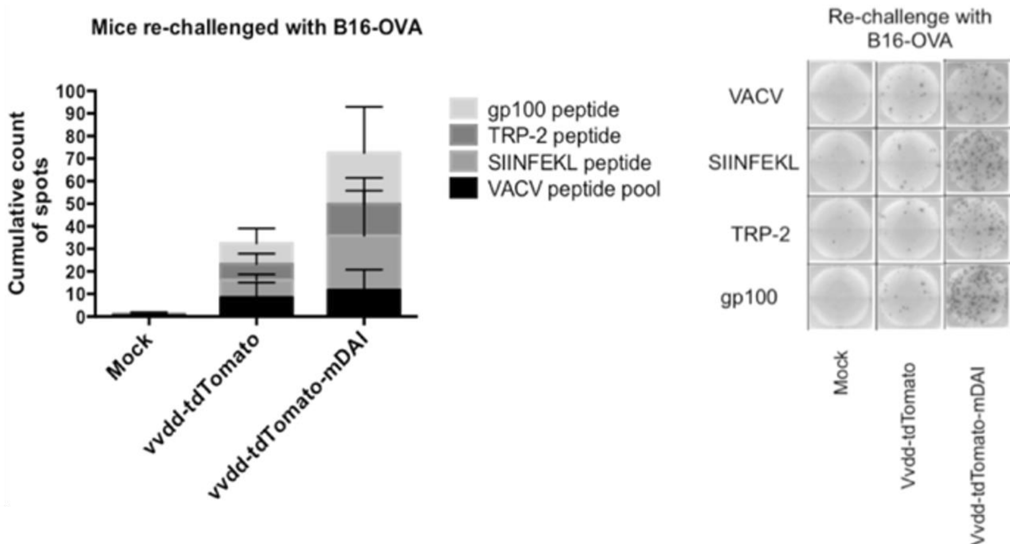


Figure 32. IFNγ producing T cells after vvdd-mDAI therapy. *Splenocytes from mice 22 days after virus treatments were cultured with tumor-associated or virus-derived peptides for 36 hours and the amount of activated T cells was assessed by ELISpot. Cumulative spot count and representative pictures of the ELISpot wells of B16-OVA re-challenged mice are shown. (Hirvinen et al. 2016, Molecular Therapy – Oncolytics [Study II])*

8.2.5. *In vivo* efficacy of vvdd-tdTomato-hDAI in humanized mice

To illustrate the translational potency of the DAI-virus in treatment of human cancers we performed a study engrafting mice with human melanoma tumors (HS294T) and some elements of the human immune system. We injected HS294T cells into the flanks of highly immunosuppressed NSG mice and allowed the tumors to grow until injectable. We then injected human peripheral blood mononuclear cells (PBMCs) obtained from a healthy donor into the tail vein of the mice to partially reconstruct the human immune system.

PBMCs (HLA-A*03*32, -B*07*27, -C*06*07) and the tumor cells (HLA-A*01*25, -B*07*08, -C*07*07) were partly HLA-matched. We then treated the tumors two times with vvdd-hDAI or controls. vvdd-hDAI treatments resulted in significant inhibition of tumor growth ($P < 0.001$) (**Figure 33a**). Indeed, the treatment efficacy was absent in mice that had not received human PBMCs (**Figure 33b**), i.e. lacked matching cellular immunity, suggesting an important role for T-cell/HLA interactions in tumor eradication by the DAI-virus.

Because the immune cells and tumor cells were not completely HLA-matched, there was undoubtedly risk of alloreactivity, i.e. graft-versus-host responses, which also could have affected tumor growth (observed as slow growth of untreated tumors). However, the mock group controls the effect of viruses and therefore, regardless of the possible alloreactivity the effect of DAI-virus could be determined. To be able to rule out the graft-versus-host reactions, completely matched tumors and immune cells should be used in these humanized mouse models. This, however, would require samples from actual cancer patients, which at this point could not be obtained.

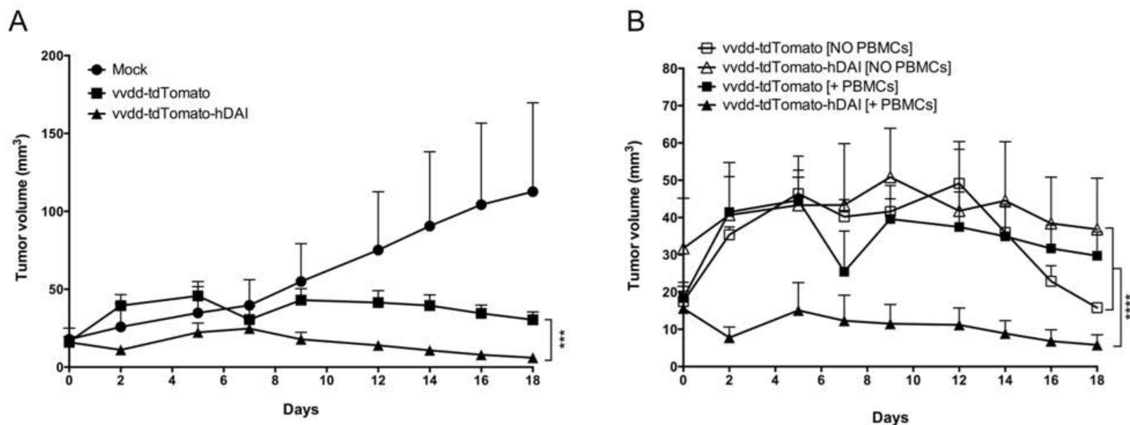


Figure 33. In vivo efficacy of vvdd-hDAI in a humanized mouse melanoma model. a) *Growth of human melanoma HS294T tumors in human PBMC engrafted NSG mice after virus treatments on days 0 and 6.* ***b)*** *Comparison of the tumor growth in hPBMC-engrafted mice and mice that did not receive hPBMCs. (Hirvinen et al. 2016, Molecular Therapy – Oncolytics [Study II])*

To further elucidate the possible mechanism behind the enhanced anti-cancer efficacy of the hDAI-virus, we analyzed the relative amounts of human CD8+ and CD4+ T cells by flow cytometry in tumors, spleens and lymph nodes of treated mice 18 days after the first treatment. There was increased infiltration of human cytotoxic cells in tumors of vvdd-hDAI treated mice compared to control virus treated mice ($P < 0.01$) (**Figure 34**).

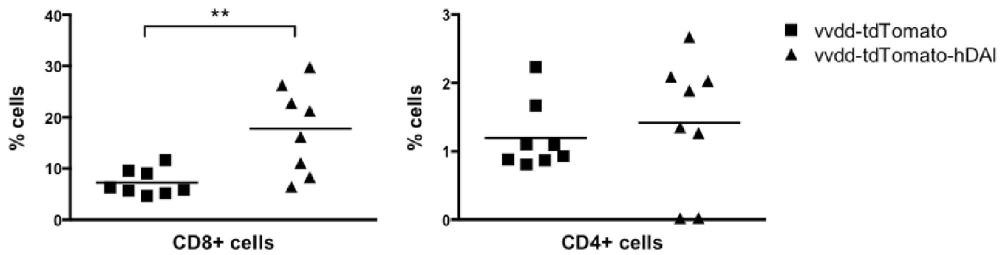


Figure 34. Presence of human T cells in the tumors of vvdd-hDAI treated humanized mice. *Human CD8+ and CD4+ T cells were analyzed by flow cytometry from the HS294T human melanoma tumors 18 days after the first virus treatment. (Hirvinen et al. 2016, Molecular Therapy – Oncolytics [Study II])*

There was also a trend (not significant) towards an increased presence of T lymphocytes in the spleens and blood of the DAI-virus treated mice (Figure 6B and 6C of Study II). Also Lladser et al. have shown that DAI is an efficient genetic adjuvant for DNA vaccines; they showed enhanced induction of antigen-specific T cell responses after DAI incorporation (Lladser et al. 2011).

In summary, in Study II we generated a novel oncolytic virotherapy platform for cancer immunotherapy. The engineered oncolytic vaccinia virus expresses a DAI protein, a molecular pattern sensor able to bind VV DNA produced in the cytoplasm of infected cells and effectively boost innate immune responses during infection. Insertion of the DAI transgene was well tolerated and did not hinder the oncolytic potency of the virus. Most importantly, the DAI-expressing virus showed improved efficacy and immunogenicity *in vivo* both in immunocompetent murine and human melanoma models. Expression of a receptor that recognizes viral DNA seems to be a potent and novel way to improve the adjuvant potency of viral drugs. This platform has potential applications in vaccine design where improved immunogenicity and efficacy are beneficial. We hypothesized that arming the virus with a receptor that induces multiple immunostimulatory molecules would generate a more robust immunological response than just arming the virus with a single cytokine; therefore, it would be interesting in the future to compare the efficacy of this DAI expressing virus with some other oncolytic VVs that express a single cytokine or other immunostimulatory molecules.

8.3. FcγR polymorphisms favorable to cancer therapy: Identification of patients with increased responsiveness to oncolytic adenovirus treatments (III)

With the increasing experience accumulating from studies with oncolytic viruses and different cancer patient populations, it is becoming clear that there is wide variance in treatment responsiveness between patients. A similar phenomenon has also been

observed with other immunotherapeutics including monoclonal antibodies (Rodriguez et al. 2012) and cancer vaccines (Weng, Czerwinski & Levy 2007, Wang et al. 2010). Oncolytic viruses are nowadays considered immunotherapeutics as well, and the immune system is increasingly being reported as an important entity governing the efficacy of OV therapies (Kanerva et al. 2013, Cerullo et al. 2010, Diaconu et al. 2012, Alemany & Cascallo 2009). Thus, genetic and phenotypic differences in the immune system between patients might at least in part explain the variance seen in the response to oncolytic virotherapy. Fc gamma receptors (FcγRs) are key players in the immune defense against infections and malignancies (van Sorge, van der Pol & van de Winkel 2003), and polymorphisms occurring in these receptors have been associated with both the disease severity (van Sorge, van der Pol & van de Winkel 2003, Li et al. 2009) and the responsiveness to immunological cancer therapies (Clynes et al. 2000, Musolino et al. 2008). As oncolysis by viral infection of cancer cells also seems dependent on cooperation with a patient's immune system, we wanted to analyze in Study III whether patient FcγR genotypes would dictate and correlate with responsiveness to oncolytic adenovirus treatments, and if these polymorphisms could perhaps serve as prognostic and/or predictive biomarkers in oncolytic virotherapy to better enable stratification of patients for the selected therapy. To implement this we genotyped more than 200 cancer patients, who had undergone adenovirus therapy, for two prominent Fc gamma receptor (FcγR) polymorphisms.

8.3.1. Genotypic frequencies of polymorphisms

We identified Fc gamma receptor genotypes of 235 patients treated with oncolytic adenoviruses in the Advanced Therapy Access Program (ATAP) conducted in Docrates Cancer Center in Helsinki from 2007 to 2011. The patients had various advanced cancers refractory to conventional therapies (see **Table 5**). DNA was extracted from blood clot sample leftovers from the samples previously collected for monitoring efficacy and safety implications from the serum. We genotyped the patients for two FcγR single nucleotide polymorphisms (SNPs), FcγRIIa-H131R and FcγRIIIa-V158F, by TaqMan-based qPCR. Samples were run in triplicate. Genotyping was successful in 233 patients; the quality of the extracted DNA was too poor in two samples and we were unable to obtain reliable genotyping results; therefore these two patients were excluded from the study.

The obtained frequencies of FcγRIIa and FcγRIIIa polymorphisms did not differ significantly from the expected ratios of the Hardy-Weinberg equilibrium with $\chi^2=0.72$ ($P<0.5$) and $\chi^2=0.01$ ($P<0.9$), respectively. The distribution of FcγRIIa and FcγRIIIa genotypes obtained in our study population was also relatively similar to those previously reported for Caucasian (Zhang et al. 2007, van der Pol et al. 2003, Leppers-van de Straat et al. 2000, Van Den Berg et al. 2001, Applied Biosystems a, Applied Biosystems b) and Finnish (Sarvas, Vesterinen & Makela 2002) populations (**Table 6**). This confirms the reliability of the genotyping performed in this study.

We observed a weak linkage disequilibrium (LD), i.e. non-random distribution of alleles, between these two polymorphic receptor loci by using a 2-locus linkage disequilibrium analysis (D’=0.28; *P*<0.01) (**Table 6**). This is in line with previously reported studies in Dutch (van der Pol et al. 2003) and Spanish (Morgan et al. 2006) populations.

Table 6. Observed FcγR allotype frequencies and linkage disequilibrium statistics

Allele frequencies				Genotype frequencies						Haplotype frequencies				LD statistics			
FcgRIIa		FcgRIIIa		FcgRIIa			FcgRIIIa										
H	R	V	F	HH	HR	RR	VV	VF	FF	HV	HF	RV	RF	D´	r^2	χ^2	*P*
0,500	0,500	0,274	0,726	0,264	0,472	0,264	0,077	0,396	0,528	0,175	0,325	0,099	0,401	0,278	0,0293	6,89	< 0,01

Abbreviations: FcgR, fragment c gamma receptor; H, FcgRIIa histidine allele; R, FcgRIIa arginine allele; V, FcgRIIIa valine allele; F, FcgRIIIa phenylalanine allele; LD, linkage disequilibrium; D´, LD/LDmax (LD is complete when D´=1); r^2, correlation coefficient (LD is perfect when $r^2 = 1$); χ^2 , chi square.

(Hirvinen et al. 2013, J. Trans Med [Study III])

8.3.2. Correlations of FcγR genotypes with OV treatment efficacy and patient survival

The role of FcγR polymorphisms in determining the efficacy of immunotherapies is increasingly recognized. Therefore, we assessed the association of FcγRIIa and FcγRIIIa with clinical response to, and survival post oncolytic adenovirus therapy.

We first correlated the different FcγR genotypes (FcγRIIa-HH, HR or RR, and FcγRIIIa-VV, VF or FF) with the overall survival (OS) of the patients (i.e. time from the first treatment with oncolytic adenoviruses until the end of the follow-up period or death). The survival estimations were performed for each genotype using Kaplan-Meier analysis. The median OS in the study cohort was 130 days. We did not observe statistically significant differences in OS between any of the FcγRIIa/IIIa genotypes (FcγRIIa HH vs. HR vs. RR, *P*=0.335; FcγRIIIa VV vs. VF vs. FF, *P*=0.193) (**Figure 35**).

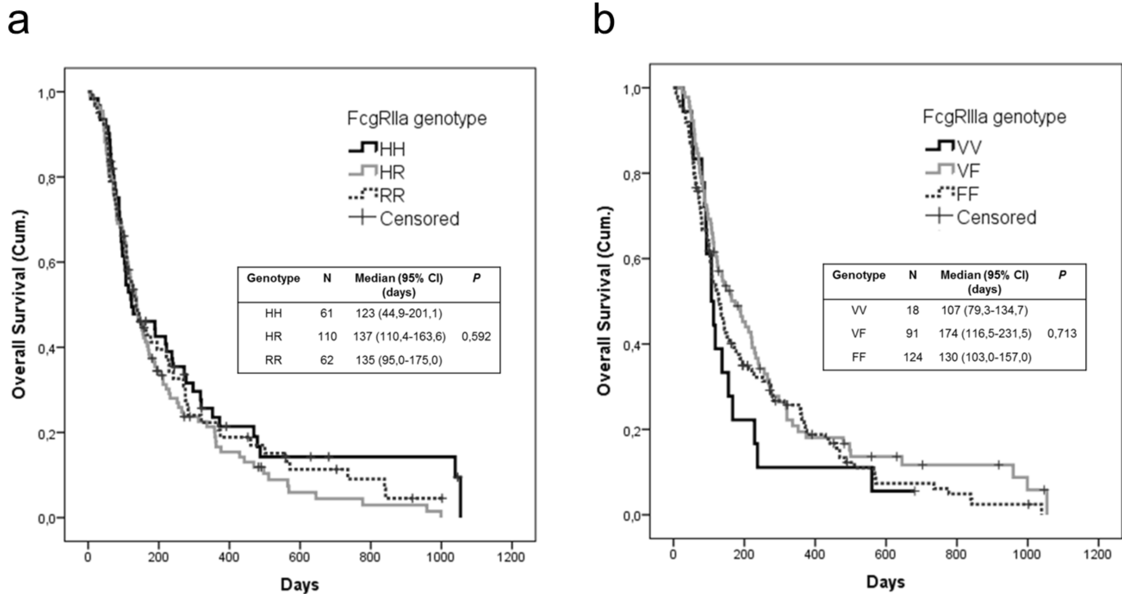


Figure 35. FcγRIIa or FcγRIIIa genotype correlations with patient survival. *Kaplan-Meier estimates of overall survival by **(a)** FcγRIIa-H131R and **(b)** FcγRIIIa-V158F genotypes. Survival time is presented in days after the first treatment with oncolytic adenovirus. Total N of patients included in the analyses is 233. Censored refers to patients still alive at the time of performing the analysis. CI = confidence interval. (Hirvinen et al. 2013, J. Trans Med [Study III])*

Moreover, we wanted to determine if the FcγR polymorphisms would correlate with clinical response to the treatments. In order to do this, we categorized the patients into two groups based on their tumor imaging and tumor marker data: i) Disease control (DC) group, i.e patients with stable disease or better and ii) patients with Progressive disease (PD). As with survival correlations, we did not find any differences in the distribution of the clinical responses between patients with different genotypes (**Figure 36**).

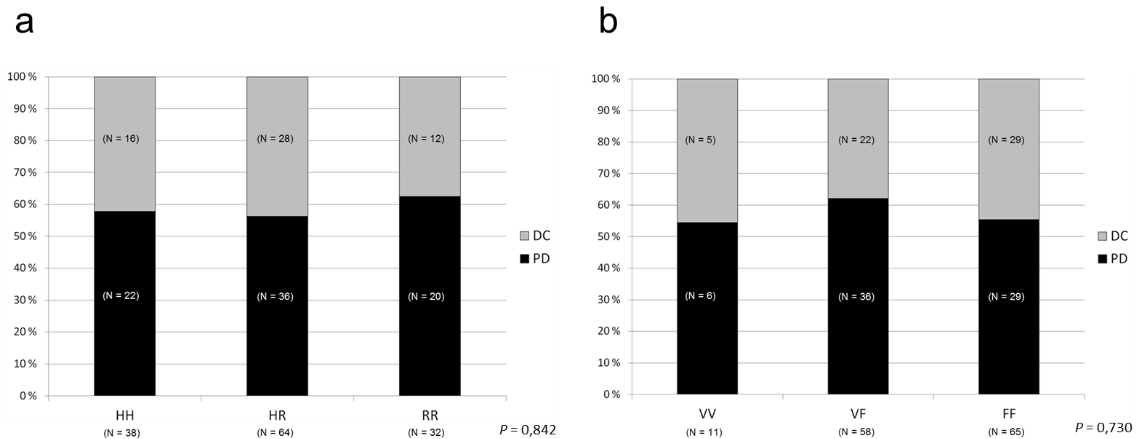


Figure 36. Clinical outcome of patients treated with oncolytic adenoviruses by (a) FcγRIIa-H131R and (b) FcγRIIIa-V158F genotypes. *Objective clinical outcome could be determined for 134 patients. DC= disease control (i.e. stable disease or better); PD= progressive disease. Correlations were analyzed by* χ^2 *test. (Hirvinen et al. 2013, J. Trans Med [Study III])*

Thus, in summary, no correlation was found between the individual FcγR genotypes to survival or clinical response. Before conducting the correlation studies we were expecting "strong-binding" genotypes (FcγRIIa-HH and FcγRIIIa-VV) to predict response to therapy, because we thought that more efficient effector cells would clear the Ab-coated tumor cells faster. Many advanced tumors have however gained an ability to escape from the host immune system by lowering the expression of tumor-associated antigens on their surface. Therefore, these tumors are no longer recognized as foreign and thus not bound by IgG, which makes the tumors "invisible" for the FcγR-bearing effector cells, thus explaining why the "strong-binding" genotypes showed no association with the outcome parameters or better survival.

8.3.3. Correlation of FcγR genotype combinations with survival and outcome after OV therapy

Different classes of FcγRs mediate Ag-Ab complex binding in a cell-specific manner, for example, FcγRII is mostly found on antigen presenting cells, while FcγRIII is more relevant for this activity of NK cells (Li et al. 2009). FcγRs function in concert with each other and their interplay defines the function of the effector cell (Nimmerjahn & Ravetch 2008). Also, co-expression of different FcγRs is supposed to result in a synergistic activation of the effector cells leading to enhanced efficacy for IC clearance (Li et al. 2009). Hence, we aimed to study the effects of multiple different genotype combinations of the allelic FcγRIIa-H131R and FcγRIIIa-V158F polymorphisms on patient survival and treatment outcome. There are nine possible genotype combinations of FcγRIIa and FcγRIIIa (**Table 7**).

Table 7. Observed frequencies of genotype combinations in the study cohort.

FcgRIIa-H131R \ FcgRIIIa-V158F			
	VVHH 7	VFHH 31	FFHH 24
	VVHR 10	VFHR 40	FFHR 61
	VVRR 1	VFRR 22	FFRR 39

(Hirvinen et al. 2013, J. Trans Med [Study III])

We first assessed the survival estimations for all nine possible genotype combinations using Kaplan-Meier analysis (**Figure 37**).

Interestingly, there was one genotype combination, FcγRIIIa-VV plus FcγRIIa-HR (VVHR), that stood out as a prognostic marker for *poor survival* after oncolytic adenovirus treatments (**Figure 37h**). The survival estimate after oncolytic virotherapy for VVHR patients was significantly (P = 0.032) shorter than for patients with any other genotype combinations with a median of 113 days (95% CI: 54.1-171.9) versus 138 days (95% CI: 112.6-163.4), respectively (**Figure 38**).

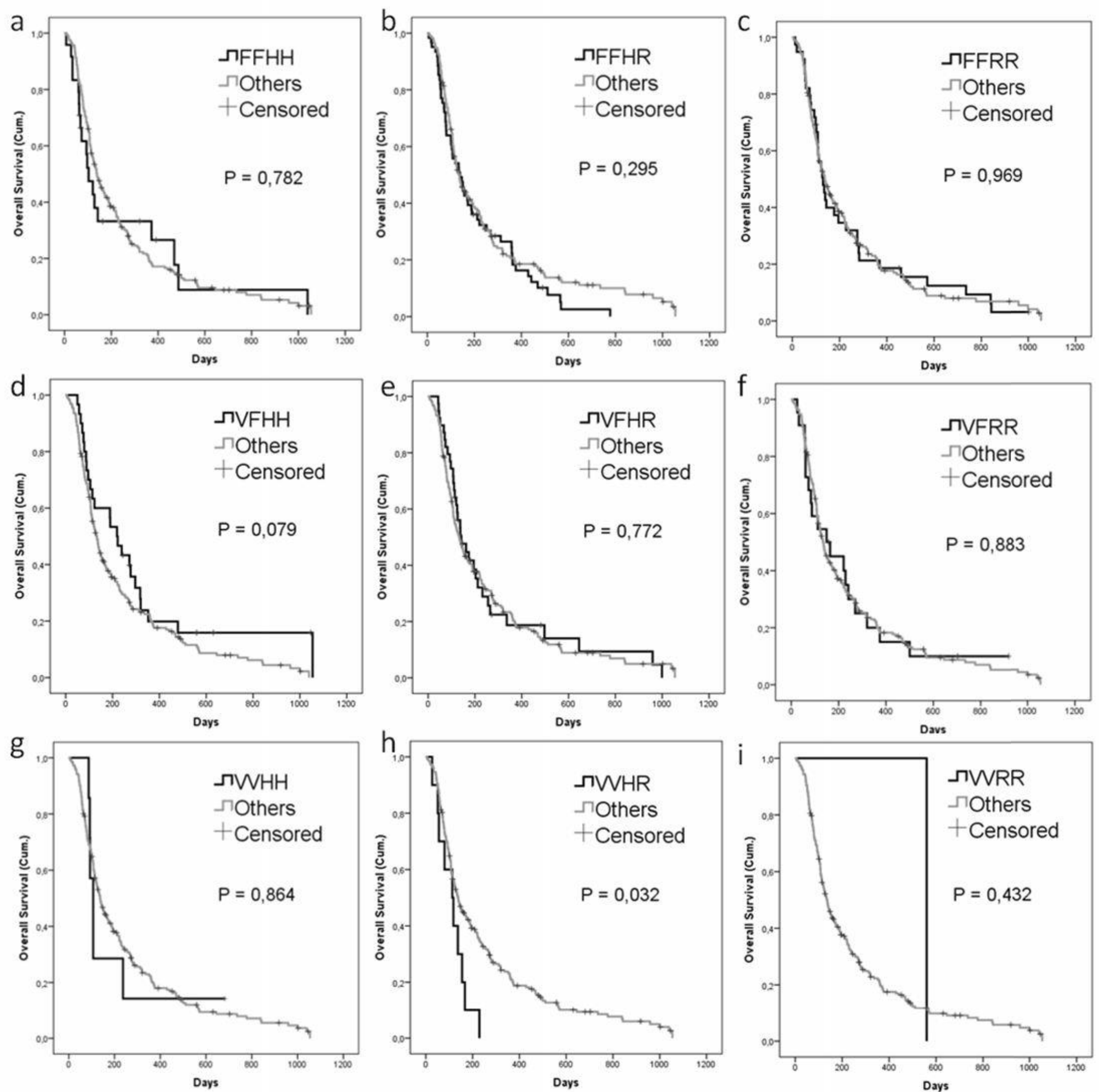
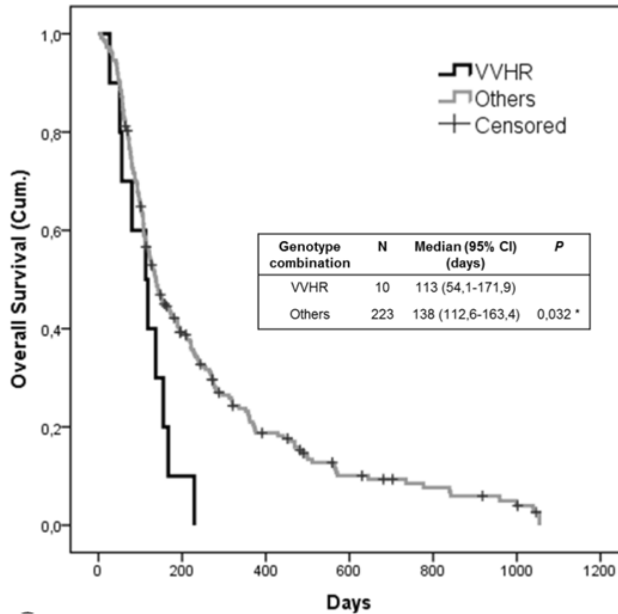


Figure 37. Survival estimates of each FcγR genotype combination. *Overall survival of cancer patients plotted for each FcγR genotype combination versus others (Kaplan-Meier estimate).* ***(a)*** *FFHH (n=24),* ***(b)*** *FFHR (n=61),* ***(c)*** *FFRR (n=39),* ***(d)*** *VFHH (n=30),* ***(e)*** *VFHR (n=39),* ***(f)*** *VFRR (n=22),* ***(g)*** *VVHH (n=7),* ***(h)*** *VVHR (n=10),* ***(i)*** *VVRR (n=1). Censored refers to patients still alive at the time of performing the analysis. (Hirvinen et al. 2013, J. Trans Med [Study III])*

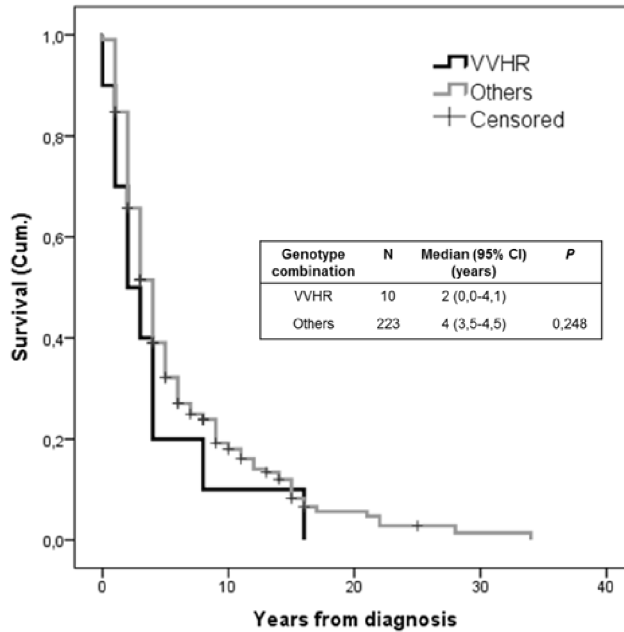


Genotype combination	N	Median (95% CI) (days)	P
VVHR	10	113 (54,1-171,9)	
Others	223	138 (112,6-163,4)	0,032 *

***Figure 38**. **Survival estimate of VVHR genotype combination in oncolytic adenovirus treatments.** Kaplan-Meier estimate of OS after the first treatment with oncolytic adenovirus until death or end of follow-up as plotted by VVHR genotype combination versus all others. The OS estimation for patients with VVHR genotype combination is significantly (P = 0.032) worse than with any other genotype combination, implying application in oncolytic adenovirus therapy. (Hirvinen et al. 2013, J. Trans Med [Study III])*

To clarify if the VVHR genotype combination was predicting survival only in oncolytic virotherapy or was a useful measure for predicting OS of cancer patients in general, we compared the *survival after cancer diagnosis* between VVHR patients and all other genotypes. No significant correlation was found between the analyzed groups (P = 0.248) indicating that the VVHR genotype is not prognostic for cancer patients *per se*, but only in the context of oncolytic virotherapy (**Figure 39**).

To investigate whether the VVHR genotype combination is also predictive for patient outcome after the oncolytic adenovirus treatments, we performed correlations of the clinical data (i.e. imaging and tumor marker data) with the genotype combinations versus all other patients (**Figure 40**). Unfortunately, outcome evaluation data was available only for five patients with the VVHR genotype combination, thus lowering the statistical power of the analysis. However, there was a trend towards a poor outcome estimate for the VVHR patients supporting the similar data of poor OS estimate in the survival analysis.



Genotype combination	N	Median (95% CI) (years)	P
VVHR	10	2 (0,0-4,1)	
Others	223	4 (3,5-4,5)	0,248

Figure 39. Survival estimate of VVHR genotype combination after cancer diagnosis. *Kaplan-Meier estimate of patient survival from the year of cancer diagnosis until the year of death or last follow-up plotted as VVHR genotype combination against all other combinations. Results reveal that the VVHR genotype is not prognostic per se. (Hirvinen et al. 2013, J. Trans Med [Study III])*

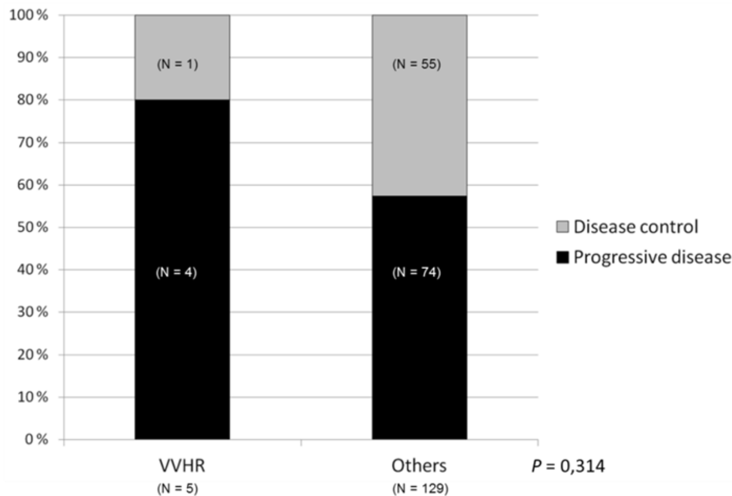


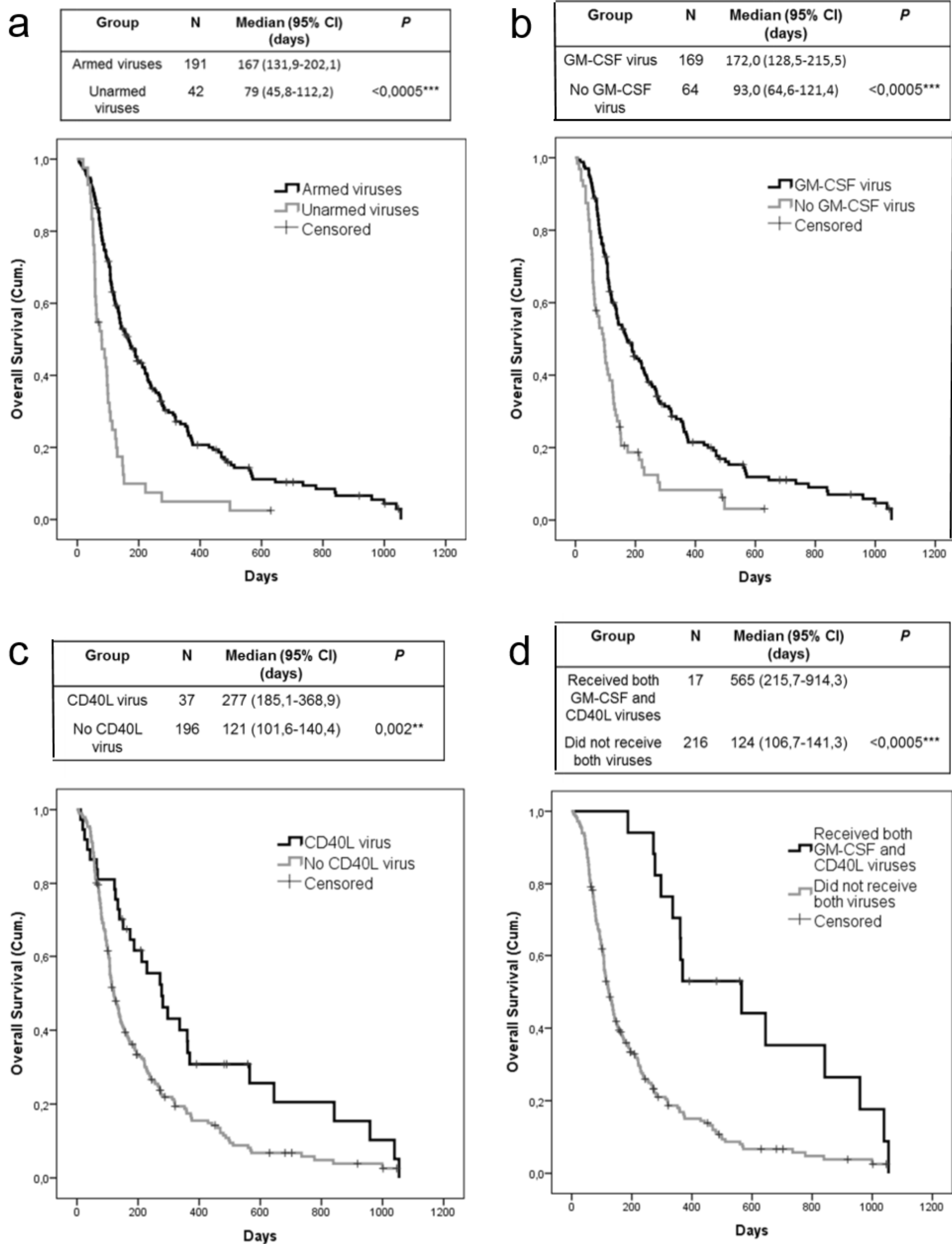
Figure 40. Clinical outcome of patients with VVHR genotype combination versus all others. *Objective clinical outcome could be determined for 134 patients. Outcome data was available only for 5 patients with the VVHR genotype combination. Correlations were analyzed by χ^2-test. (Hirvinen et al. 2013, J. Trans Med [Study III])*

FcγRIIIa receptors are mainly expressed on natural killer cells (Ravetch & Bolland 2001). Together with CTLs, NK cells have a crucial role in determining the efficacy of oncolytic viruses (Alvarez-Breckenridge et al. 2012). NK cells can mediate tumor cell killing in an antibody-dependent manner by ADCC (van Sorge, van der Pol & van de Winkel 2003). Instead, FcγRIIa receptors are widely distributed on many immune cell types, but are considered to be prominent on phagocytic cells, including antigen presenting cells (APC) (Jefferis & Lund 2002). Decent presentation of tumor-associated antigens by APCs is a key for induction of long-term T cell memory against tumors.

We hypothesized that perhaps the strong binding to tumor cells of FcγRIIIa-VV -expressing NK cells causes effective ADCC and the tumor cell is eliminated fast by NK cells also resulting in fierce anti-viral ADCVI (antibody-dependent cell-mediated virus inhibition) (Forthal & Moog 2009) before the virus has had time to replicate. Hence, such highly efficient NK cell response and only an intermediate antigen-presentation by FcγRIIa-HR expressing APCs of VVHR individuals may reduce the efficacy of OV therapy, and therefore this genotype combination is predictive of poor survival post OV therapy. We could speculate that individuals with VVRR would have an even worse survival estimate due to the even "weaker-binding" FcγRIIa-RR genotype. Unfortunately there was only one patient with such genotype combination in the study cohort, so we had no opportunity to verify this result.

8.3.4. Studies on the effect of different arming molecules on survival of patients with different FcγR genotypes

Most of the patients included in this study had been treated with viruses armed with immunostimulatory transgenes (GM-CSF-armed viruses: 71.9 % and CD40L-armed viruses: 15.7 %). As shown in several clinical studies already (Ranki et al. 2014, Andtbacka et al. 2015, Burke et al. 2012), arming oncolytic viruses with effectors like GM-CSF or CD40L can be beneficial for patients due to the ability of these molecules to recruit immune cells to the tumor site. Armed viruses were also beneficial ($P < 0.0005$) for the patients of this study cohort (**Figure 41**), with both insert molecules. The treatment benefit was even clearer if the patient received both the GM-CSF and CD40L-bearing virus during the treatment (**Figure 41d**).



a

Group	N	Median (95% CI) (days)	P
Armed viruses	191	167 (131,9-202,1)	
Unarmed viruses	42	79 (45,8-112,2)	<0,0005***

b

Group	N	Median (95% CI) (days)	P
GM-CSF virus	169	172,0 (128,5-215,5)	
No GM-CSF virus	64	93,0 (64,6-121,4)	<0,0005***

c

Group	N	Median (95% CI) (days)	P
CD40L virus	37	277 (185,1-368,9)	
No CD40L virus	196	121 (101,6-140,4)	0,002**

d

Group	N	Median (95% CI) (days)	P
Received both GM-CSF and CD40L viruses	17	565 (215,7-914,3)	
Did not receive both viruses	216	124 (106,7-141,3)	<0,0005***

Figure 41. Overall survival of patients irrespective of their FcγR genotypes as correlated with the type of virus used. *OS estimates of patients treated with **(a)** armed viruses vs. unarmed viruses, **(b)** GM-CSF-armed viruses vs. other viruses, **(c)** CD40L-armed viruses vs. other viruses **and (d)** patients who received both GM-CSF and CD40L-armed viruses during their treatment.*

It has been shown that many inflammatory and costimulatory molecules, including GM-CSF and CD40L can alter FcγR expression and FcγR-mediated immune responses. GM-CSF and IFN-γ have been shown to increase the expression of FcγRIIa and FcγRIIIa (Hartnell, Kay & Wardlaw 1992, Capsoni et al. 1991, Rossman et al. 1993) thus activating biological activity of effector cells. In contrast, some studies have shown that immunostimulatory molecules may distract and downregulate FcγR-mediated immune functions (Kruger et al. 1996, Buckle & Hogg 1989, Huang et al. 2011). Therefore we wanted to determine whether the inserted effector molecule also has an impact on the survival of patients who have different FcγR genotypes and genotype combinations (see all results from Study III article, Table 2 and Supplementary Table S3). Our results revealed two genotype combinations, FcγRIIIa-FF + FcγRIIa-HR (FFHR) and FcγRIIIa-FF + FcγRIIa-RR (FFRR), which were beneficial if the patient also received GM-CSF-armed viruses ($P < 0.004$ and $P < 0.006$, respectively) (**Figure 42a and b**). In contrast, treatment of these patients with unarmed viruses correlated with significantly shorter survival after OV therapy ($P < 0.0005$ and $P = 0.016$, respectively). Additionally, treating FcγRIIIa-FF + FcγRIIa-HH (FFHH) individuals with a CD40L-armed virus resulted in more prolonged survival compared to treatment with other viruses ($P = 0.047$) (**Figure 42c**). As, however, this genotype group treated with the CD40L-armed virus only included four patients the reliability of the results remains limited.

Interestingly, all those genotype combinations (FFHR, FFRR, and FFHH) whose treatment with the recombinant viruses was found to be beneficial contained the FcγRIIIa-FF genotype. Due to these "weak-binding" receptors on their NK cells, their tumors may exert less innate resistance to NK-cell -mediated killing. When a potent NK recruitment signal is eventually provided, i.e. by virally produced GM-CSF or CD40L, NK anti-tumor efficacy may increase. So, it can be speculated that a hypothetical lower baseline activity of the NK cells is compensated by the immunostimulatory features of GM-CSF and CD40L resulting in immunological activation that eventually results in longer survival in individuals with FFHR, FFRR, and FFHH genotype combinations.

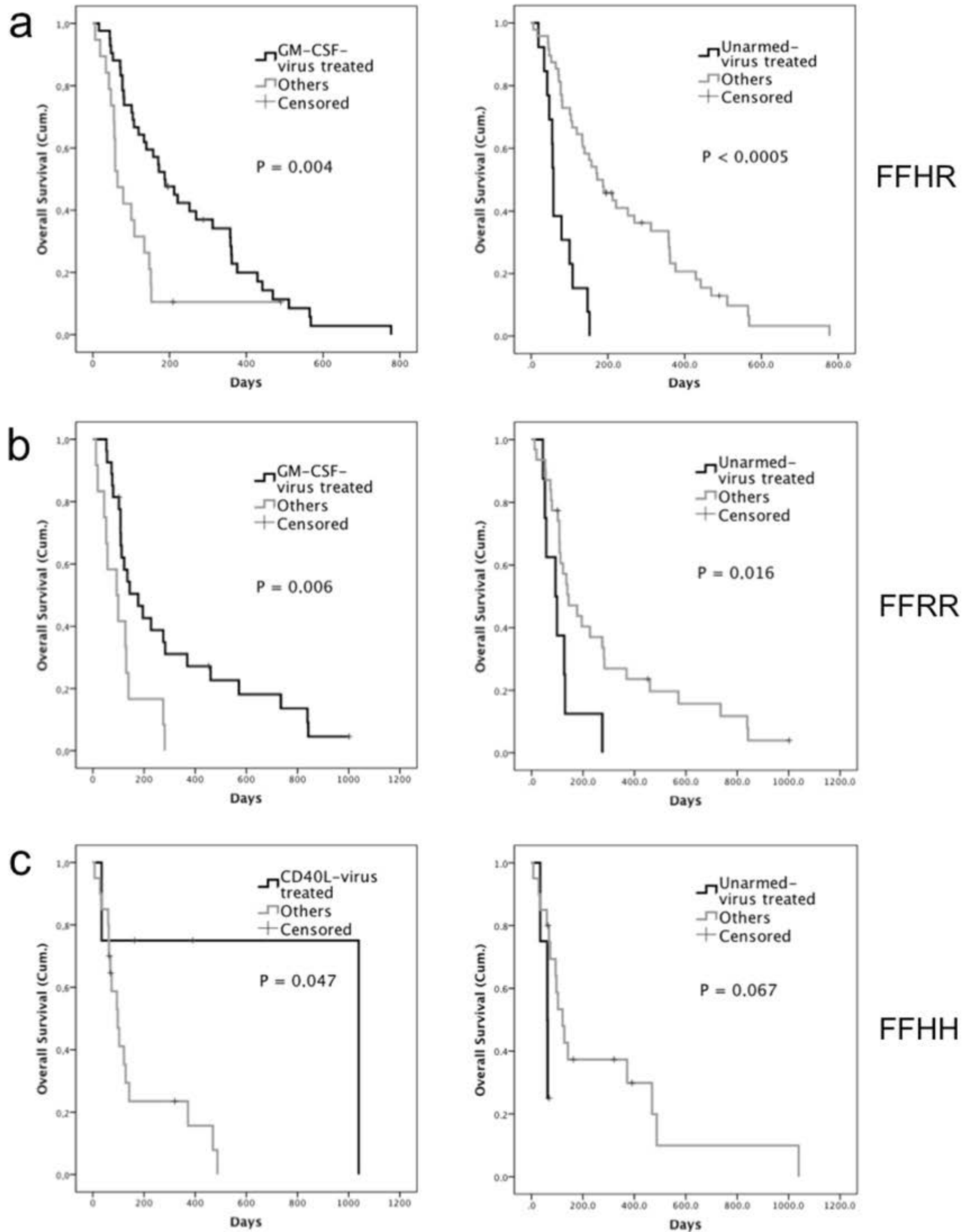


Figure 42. Correlation of patient survival with different FcγR polymorphisms and the differently armed viruses revealed three genotype combinations beneficial to treatment outcome. *Kaplan-Meier analyses show the effect of insert (GM-CSF, CD40L, or unarmed) on genotype combinations. Calculations were made by first restricting the study population to individual genotype combinations and then comparing the OS between treatment virus types vs. others. (Hirvinen et al. 2013, J. Trans Med [Study III])*

In summary, in Study III we found one genotype combination (FcγRIIIa-VV + FcγRIIa-HR) predictive of poor overall survival after oncolytic adenovirus therapy. Additionally some genotype combinations were found to be beneficial for treatments with cytokine-armed oncolytic adenoviruses: FcγRIIIa-FF + FcγRIIa-HR (FFHR) and FcγRIIIa-FF + FcγRIIa-RR (FFRR) for GM-CSF-armed viruses and FcγRIIIa-FF + FcγRIIa-HH (FFHH) for CD40L-viruses. These results suggest the possibility that FcγRs may play a role in the efficacy of oncolytic adenovirus therapies; however the study cohort was quite small and very heterogeneous, thus, to draw any definitive conclusions on the influence of FcγR polymorphisms on OV therapy responsiveness, the correlations should be repeated in a larger and more controlled study population. Nevertheless, our study is one of the first studies designed to find predictive biomarkers for oncolytic virotherapies to enable better selection of patients for clinical studies, which I think is the best way to optimize the treatment efficacy and safety.

8.4. Development of a novel oncolytic vaccine platform (PeptiCRAd) which carries tumor-associated antigens on the adenoviral surface to induce tumor-specific immune responses (IV)

In Study IV we developed a novel strategy to customize oncolytic adenovirus therapies to adapt the therapy to individual characteristics of different tumors. We engineered a viral platform where tumor-specific peptides can be attached onto the viral capsid (PeptiCRAd) so as to allow development of cancer eradicating tumor-specific immunity (European patent (EU PCT/EP2015/060903) “Modified adenovirus for cancer vaccines development” was filed).

8.4.1. *In vitro* characterization of the modified peptides and the PeptiCRAd complex

Development of a tumor-specific CTL response is a key to achieve efficient and long-lasting eradication of cancer. In order to acquire such a response, tumor-associated antigens (TAAs) need to be available for antigen presenting cells to be cross-presented to T cells. To enhance the availability and presentation of tumor antigens, we developed a method to deliver peptides efficiently into tumors using an adenovirus as a vector. The adenovirus serves as an adjuvant e.g. by inducing release of cytokines. Moreover, lysis of the tumor cells caused by replication of the virus may improve the tumor killing efficacy directly as well as by enabling spreading of other TAAs to the tumor surroundings.

Our first aim in Study IV was to develop a method to attach tumor-associated peptides onto the adenoviral surface. The protein capsid of the adenovirus is known to be negatively charged (Fasbender et al. 1997) (Supplementary Figure 1 of Study IV). We thus modified the peptides to become positively charged, so that they would adhere to the

negatively charged capsid by electrostatic interactions. To make the peptides positively charged, a poly-lysine (polyK) chain was covalently added to the peptide sequence.

To verify the concept of our novel platform, we used a well-known model peptide, SIINFEKL, an MHC-I epitope of chicken ovalbumin (OVA). We first analyzed whether the positively charged polyK-modified SIINFEKL peptide (Figure 2a of Study IV) is able to bind to the virus surface. The surface of plasmon resonance (SPR) analysis plate was then coated with oncolytic adenovirus (OAd) (Supplementary Figure 2 of study IV) and increasing amounts of the lysine-modified or unmodified SIINFEKL peptide were added onto the plate and the net charge of the formed complex was measured (**Figure 43 left panel**). The SPR analysis revealed that the unmodified peptide was unable to bind onto the virus (**Figure 43 left panel, dashed line**), whereas a concentration-dependent interaction was observed with the modified peptide (**Figure 43 left panel, solid line**) demonstrating that addition of the polyK chain to a peptide sequence is a feasible method to attach peptides onto adenovirus capsid.

Next, we studied how the amount of polyK-peptide attached to the virus affects the size and charge of the Ad-peptide complex (**Figure 43, right panel**). The size (hydrodynamic diameter) of the complex was measured by dynamic light scattering (DLS) (**Figure 43 right panel, solid line**). Also the charge (zeta-potential) of the complex was determined with different peptide-virus µg ratios (**Figure 43 right panel, dashed line**) (OAd alone, 1:5 OAd:peptide, 1:50, 1:100 and 1:500). The size of naked adenovirus is ~100 nm, and the surface charge of Ad is negative (-29.7±0.5 mV). The charge of the complex was observed to reach saturation with 1:50 virus-peptide ratio at approx. +18 mV. With low peptide concentration (1:5) the size of the complex increased to 800±13.5 nm, because of the heavy aggregation of the complexes close to neutral charge. Although the charge of the complex was seen to plateau beyond 1:50 Ad-peptide ratio, the size of the complex was close to a normal-sized adenovirus only at the highest peptide concentration (1:500 ratio). Hence, 1:500 was estimated to be the optimal mass ratio and was chosen to coat the adenovirus.

These results showed that the oncolytic adenovirus capsid could be coated with positively charged peptides to form a complex that we call PeptiCRAd (**Pepti**de-coated **C**onditionally **R**eplicating **Ad**enovirus).

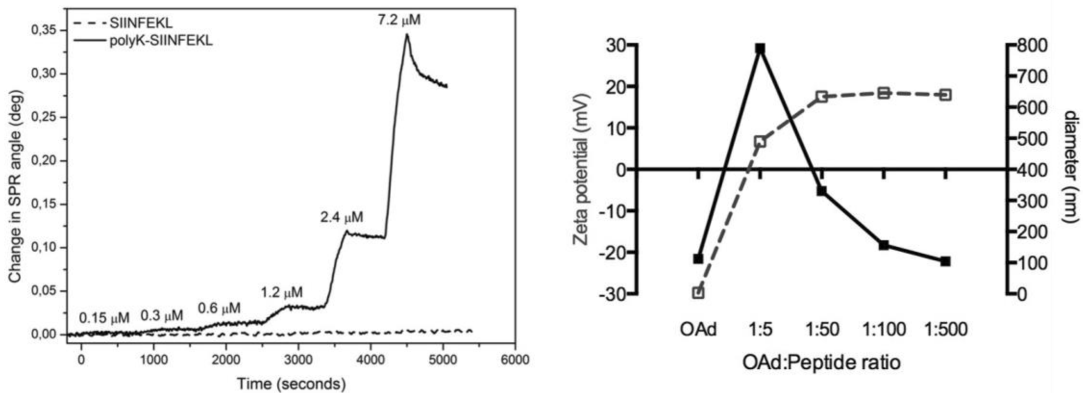


Figure 43. Physical characterization of the interaction between the modified SIINFEKL peptide and oncolytic adenovirus. *Virus-peptide interaction was measured by Surface Plasmon Resonance. An APTES Silica SiO_2 sensor was coated with OAd (Ad5-Δ24) and increasing concentrations (0.15, 0.3, 0.6, 1.2, 2.4 and 7.2 µM) of either SIINFEKL (dashed line) or polyK-SIINFEKL (solid line) were injected into the flowing system (**left panel**). OAd was incubated with polyK-SIINFEKL using different OAd-peptide (µg) ratios. Zeta potential, i.e. the charge of the complex (dashed gray line) and hydrodynamic diameter, i.e. size of the complex (solid black line) were determined. The averages of three consecutive measurements are shown in the **right panel**. (Capasso et al. 2015, OncoImmunology [Study IV])*

To trigger an effective immune response, peptides need to be taken up, processed and efficiently cross-presented by APCs to T cells on the MHC molecules. Cross-presentation is a process that allows presentation of exogenous antigens (e.g. tumor antigens) on MHC-I and thus priming of cytotoxic (CD8+) T cells. Normally exogenous antigens would be presented on MHC-II and the process would require infection of dendritic cells (Rock & Shen 2005). Cross-presentation is agreed to be an important mechanism by which tumor-specific CTL immunity is developed after many immunotherapies, such as OV therapy and peptide vaccinations (Fehres et al. 2014).

Therefore, we investigated whether modification of the peptide affects its cross-presentation and if the position of the polyK chain (i.e. on the N- or C-terminus of the peptide sequence) affects the efficiency of the cross-presentation. To this end, we performed a cross-presentation assay where we pulsed splenocytes (from C57BL/6 mice) with either the natural unmodified SIINFEKL (positive control) or its two different lysine-extended versions: polyK-SIINFEKL (N-terminus extended) and SIINFEKL-polyK (C-terminus extended). Also an extended SIINFEKL containing an N-terminal amino caproic residue (AHX) was included in the assay as a negative control. AHX is a well-known lysine analogue, an unnatural D-amino acid that cannot be processed by the proteasome. To assess the cross-presentation of SIINFEKL we used an antibody that specifically recognizes MHC-I loaded with SIINFEKL (Deng et al. 1998) and analyzed the amount of SIINFEKL-presenting cells by flow cytometry. A total of 98.5% of the splenocytes pulsed with the unmodified

SIINFEKL cross-presented the peptide. Intriguingly, also the N-terminal poly-lysine-containing peptide was effectively presented (94.5%). In contrast, when the splenocytes were pulsed with the C-terminal SIINFEKL-polyK, the cross-presenting only 27.1% of the splenocytes were able to present the peptide (**Figure 44a**). We speculate that the low cross-presentation of the C-terminally modified peptide might result from a slower proteasomal cleavage pathway, which requires that the antigen first escapes from the phagosome, then undergoes proteasomal degradation in the cytosol to be processed by endoplasmic reticulum (ER) aminopeptidases, before the mature peptide finally can be loaded on MHC-I molecules and exposed onto the cell membrane (Heath & Carbone 2001). On the other hand, as the N-terminally-modified peptide is perhaps processed faster, directly by the ER aminopeptidases (van Endert 2011), it could therefore be directly processed inside the phagosomes leading to more rapid and efficient presentation. These results made us choose the N-terminally-extended version (polyK-SIINFEKL) for further studies.

Next we investigated whether the polyK-SIINFEKL peptide could be efficiently cross-presented when complexed onto the surface of the adenovirus. Results showed that the peptide is cross-presented as efficiently as the free peptide (**Figure 44b**).

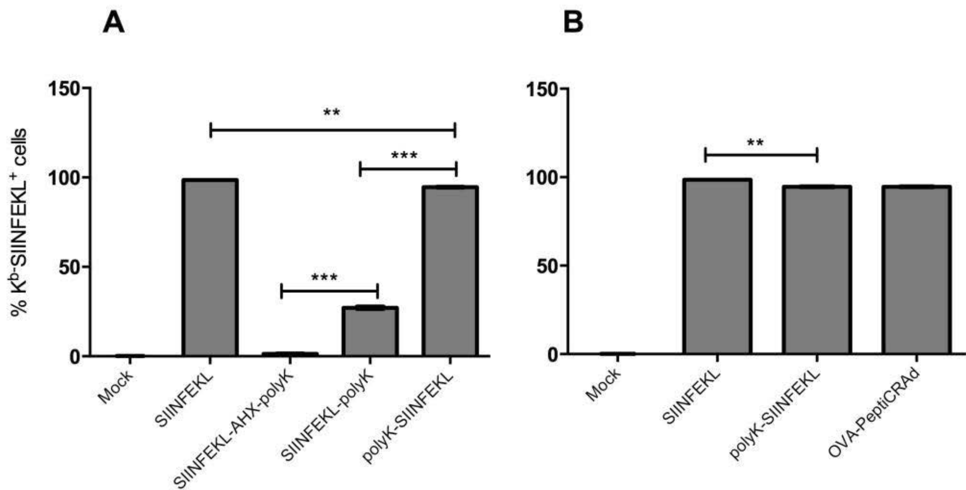


Figure 44. Cross-presentation of modified SIINFEKL analogues. *Spleens were collected from C57BL/6 mice (H2-K^b).* ***a)*** *Splenocytes were pulsed with unmodified SIINFEKL (positive control), aminocaproic acid-containing SIINFEKL-AHX-polyK (negative control), C-terminus extended SIINFEKL-polyK or with N-terminus extended polyK-SIINFEKL. Cells were stained with an APC-conjugated anti-H2-K^b SIINFEKL-binding antibody.* ***b)*** *Splenocytes were infected with of OVA-PeptiCRAd (100 vp/cell + 37.5 µg of peptide) or pulsed with 37.5 ug of SIINFEKL (positive control) or polyK-SIINFEKL. Samples were extensively washed and analyzed by flow cytometry using the same antibody as in panel a). (Capasso et al. 2015, OncoImmunology [Study IV])*

Then, in order to test if coating of the oncolytic adenovirus would hinder the oncolytic, i.e. tumor cell killing potency, we infected different cancer cells with PeptiCRAd or with uncoated oncolytic adenovirus (Ad5-Δ24) and measured the cell viability by MTS assay (**Figure 45a**). We saw no difference in the cell killing efficacy between the coated or uncoated viruses, thus the peptide coating does not affect the oncolytic efficacy of the oncolytic adenovirus.

We also wanted to determine if PeptiCRAd was capable of infecting cancer cells efficiently. We performed an ICC immunocytochemistry assay to measure the amount of infected cells after PeptiCRAd or AD5-Δ24 infection (**Figure 45b**). Interestingly, we observed increased infectivity ($P < 0.01$) of two (CACO-2 and A2058) of the three tested cell lines by PeptiCRAd compared to the naked virus. This is most probably because the cell membrane is negatively charged, hence the positive charge of the PeptiCRAd complex helps internalization into the cells. It has also been shown by others that transduction of the adenovirus is increased when complexed with natural positively charged cell penetrating peptides (Nigatu et al. 2013) or if coated with polycationic polymers and/or cationic lipids (Fasbender et al. 1997).

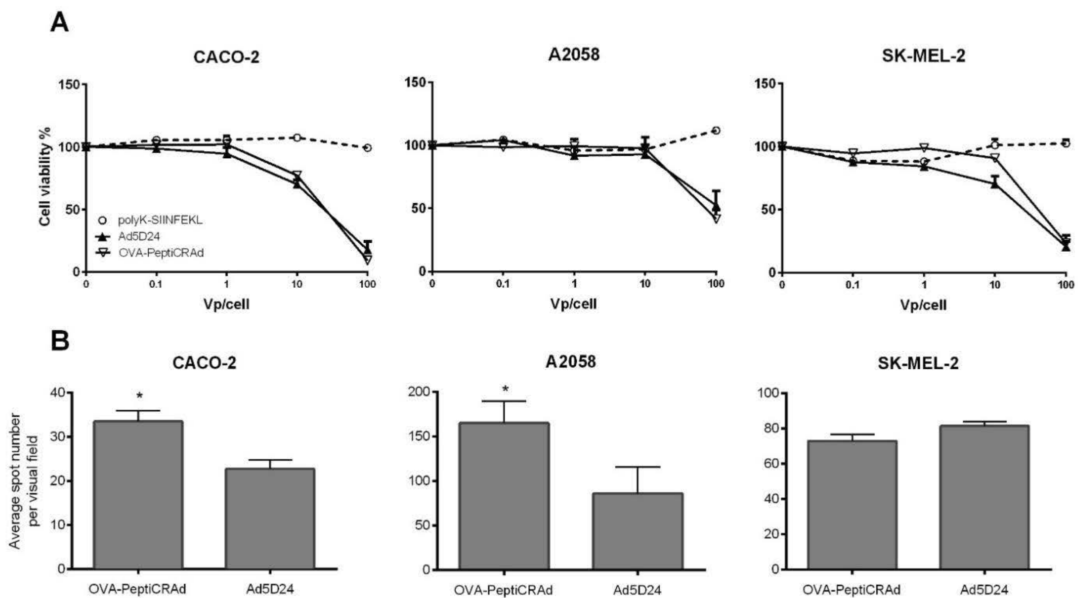


Figure 45. Oncolytic potency and infectivity of PeptiCRAd. a) *Cell viability assay in different malignant cell lines.* ***b)*** *Infectivity assay by immunocytochemistry. The average number of spots per visual field is presented. (Capasso et al. 2015, OncoImmunology [Study IV])*

In summary, the *in vitro* studies on PeptiCRAd show that we are able to coat oncolytic adenoviruses with polyK-modified peptides and the coating does not hinder the oncolytic potency nor the cross-presentation of the peptide and that the coating even increases the infectivity of the virus.

8.4.2. *In vivo* immunity and efficacy of PeptiCRAd in mouse melanoma models

To study the anti-tumor efficacy and immunity promoted by PeptiCRAd, we first used a murine model of melanoma over-expressing chicken ovalbumin (B16-OVA). B16-OVA cells were injected s.c. in the flanks of C57BL/6 mice and the established tumors were treated i.t. either with OVA-PeptiCRAd (i.e. PolyK-SIINFEKL-coated OAd), a non-complexed OAd in combination with SIINFEKL, OAd alone, peptide alone (SIINFEKL) or saline (mock). The experiment was first performed with a regular oncolytic adenovirus bearing the 24 bp deletion in E1 (Ad5-Δ24) (Supplementary Figure 3 of Study IV) and then repeated with a CpG-riched adenovirus (Ad5-Δ24-CpG) to boost the immunity even further (**Figure 46**). PeptiCRAd significantly reduced the growth of the syngeneic tumors compared to mock treatment or the mixture of OAd and SIINFEKL peptide (P < 0.01). The average volume of the tumors in the OVA-PeptiCRAd-treated mice was lower than in all other groups on day 16 (120.4±31.6 mm^3 versus 697.7±350 mm^3 in mock; 255±61.5 mm^3 in SIINFEKL; 713.7±292.6 mm^3 in Ad5-Δ24-CpG; 489.7±73.2 mm^3 in Ad5-Δ24-CpG+SIINFEKL).

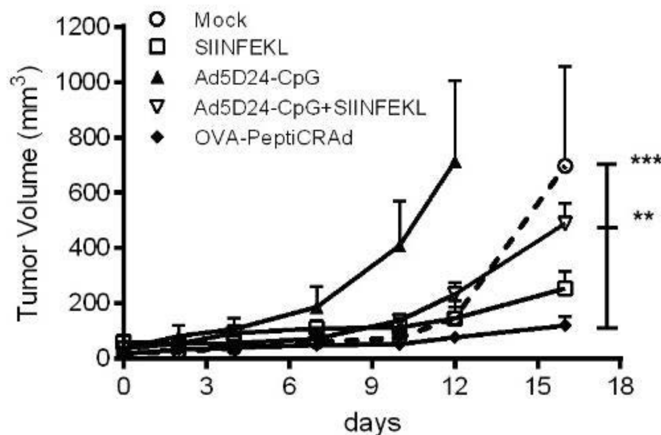


Figure 46. Anti-tumor efficacy of OVA-PeptiCRAd in vivo. *C57BL/6 mice (n= 6-8) with 2x B16-OVA tumors were treated i.t. with: saline solution (mock), peptide alone (SIINFEKL), virus alone (Ad5-Δ24-CpG), virus and peptide mix (Ad5-Δ24-CpG+SIINFEKL) or PeptiCRAd complex (OVA-PeptiCRAd). Treatment of mice was three times total (days 0, 2 and 7). Tumor volume is presented. (Capasso et al. 2015, OncoImmunology [Study IV])*

To assess the immunological effects of PeptiCRAd we collected the spleens, draining lymph nodes and tumors of the treated mice. We measured the levels of SIINFEKL-specific (i.e. OVA-specific, tumor-specific) $CD8^+$ T cells in the organs by flow cytometry. We observed increased amounts of tumor-specific T cells in the samples from the organs of PeptiCRAd treated mice, although the differences were not statistically significant. (**Figure 47, upper panel**).

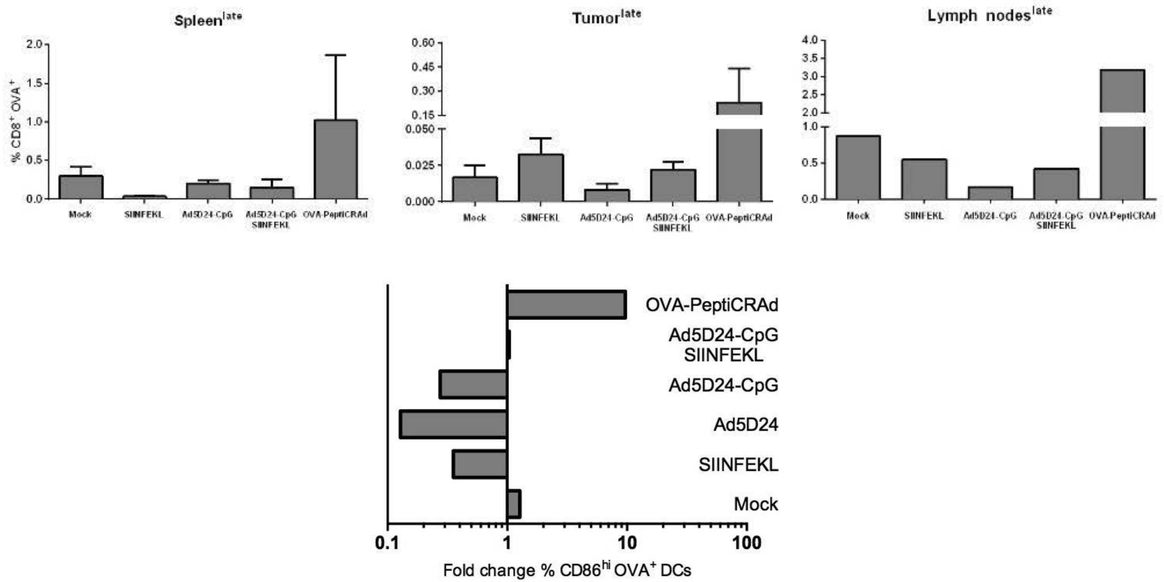


***Figure 47. Immunological analysis of antigen-specific CD8⁺ T-cells and dendritic cells after OVA-PeptiCRAd treatment. Above**: Tumors, spleens and the inguinal lymph nodes from mice were collected 16 days after virus treatments and SIINFEKL-specific CD8⁺ T-cells were determined by flow cytometry. **Below**: Fold change of dendritic cells with mature phenotype ($CD19^{-}CD3^{-}CD11c^{+}CD86^{high}$) cross-presenting SIINFEKL on their MHC-I molecules. (Capasso et al. 2015, OncoImmunology [Study IV])*

To study the potential of PeptiCRAd to trigger antigen-presentation *in vivo* we evaluated by flow cytometry the amount of mature dendritic cells ($CD19^{-}$ $CD3^{-}$ $CD11c^{+}$ $CD86^{high}$ cells) presenting the SIINFEKL peptide on MHC-I in the spleens of the mice. Sixteen days after the first virus treatment, the mice treated with PeptiCRAd showed significantly increased amounts of mature SIINFEKL-presenting DCs ($P < 0.05$) compared to mice treated with the non-complexed Ad5-Δ24-CpG+SIINFEKL (Supplementary Figure 4b of study IV). The amount of mature DCs was also evaluated at an early time point, 7 days after the first treatment. When compared between the early (day 7) and the late (day 16) time points, the biggest increase in the DC levels were in the amounts of mature, cross-presenting DCs in the PeptiCRAd group (9.67-fold change) (**Figure 47, panel below**).

Targeting multiple tumor antigens is important in order to limit the escape of malignant cells from the immune surveillance (Matsushita et al. 2012) and to combat the variability of tumor cells (Andersen & Ohlfest 2012). Indeed, it could be shown quite recently that patients who responded to multiple tumor epitopes had a significantly better treatment outcome estimate (Walter et al. 2012). In addition, a multivalent approach would be especially useful in the case of tumors where one HLA allele is down-regulated (Carretero et al. 2008). Hence, we designed experiments to address whether a double-coated PeptiCrad is more effective than a PeptiCRAd coated with only a single peptide. We also wanted to know if we are able to induce anti-tumor responses with clinically relevant

melanoma antigens (gp100 and TRP-2) in analogy to the SIINFEKL model peptide used in the proof-of-concept studies. We chose an SVYDFFVWL peptide (TRP-$2_{180\text{-}188}$; restricted to the murine MHC-I molecule H-$2K^b$) and KVPRNQDWL peptide (human $gp100_{25\text{-}33}$; restricted to the murine MHC-I molecule H-$2D^b$) (Joffre et al. 2012), both expressed by B16-F10 cells (Sijts & Kloetzel 2011). We injected B16-F10 mouse melanoma cells into the other flank of C57BL/6 mice and treated the established tumors with i) saline solution (mock), ii) naked oncolytic virus (Ad5-Δ24-CpG), iii) single-coated hgp100-PeptiCRAd, iv) single-coated TRP-2-PeptiCRAd, and v) double-coated TRP-2-hgp100-PeptiCRAd. The double-coated PeptiCRAd was shown to significantly reduce ($P < 0.001$) the growth of the treated tumors compared to both the mock and the hgp100-PeptiCRAd groups (**Figure 48, left panel**).

We also tested whether systemic immunity was triggered by PeptiCRAd whether it would be able to also control growth of distant, untreated tumors. To demonstrate this, we injected B16-F10 cells in one flank of the treated mice (described above) and followed the development of the second tumor (Figure 6A of Study IV). We saw that the multivalent PeptiCRAd bearing two autologous tumor antigens was able to significantly inhibit the growth of the second tumor compared to the treatments with naked virus or saline ($P < 0.01$) (**Figure 48, right panel**). A clear reduction of the primary and secondary tumor growth was observed in mice treated with TRP-2-hgp100-PeptiCRAd compared to TRP-2-PeptiCRAd and hgp100-PeptiCRAd, however, the differences were not statistically significant.

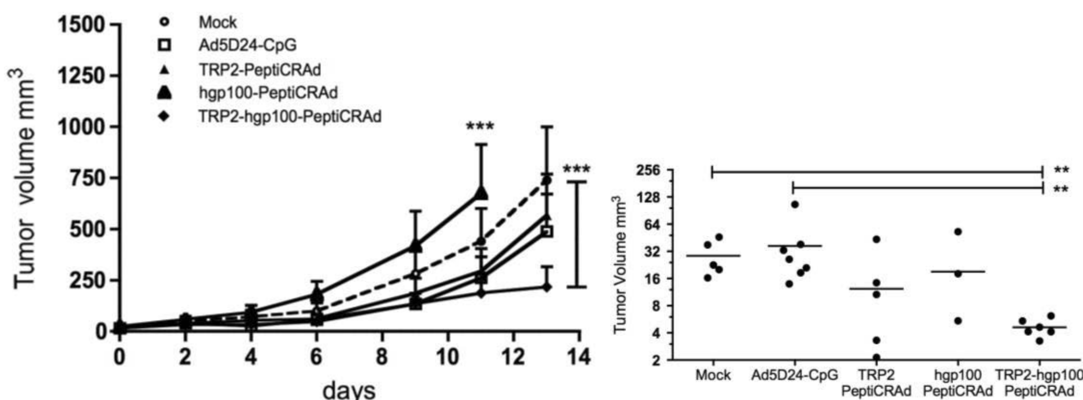


***Figure 48. PeptiCRAd loaded with natural tumor antigens inhibits regrowth of previously treated tumors but also engraftment of new distant melanoma tumors**. C57BL/6 mice bearing B16-F10 melanoma tumors were treated with viruses coated with natural melanoma-associated peptides. The growth of the treated tumors (**left**) and the size of the untreated secondary tumors (**right**) are shown. (Capasso et al. 2015, OncoImmunology [Study IV])*

We also analyzed tumor-specific T cell responses against both epitopes by pentamer staining and flow cytometry analysis of spleens and lymph nodes of the treated mice. The

highest cumulative amount of epitope-specific CD8+ T cells was found in the mice treated with TRP-2-hgp100 PeptiCRAd (**Figure 49**).

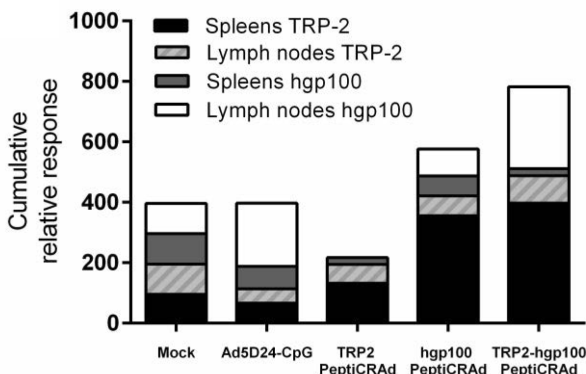


Figure 49. Tumor-specific T cell responses after multivalent PeptiCRAd therapy. *Spleens and inguinal lymph nodes were collected and the levels of TRP-2 and hgp100 specific CD8+ T-cells were determined by Class-I pentamer staining. The amount of epitope-specific CD8+ T-cells found in each organ was normalized against the mock and is presented as cumulative relative response for each experimental group. (Capasso et al. 2015, OncoImmunology [Study IV])*

These results from the syngeneic murine melanoma model indicate that PeptiCRAd has potential to increase tumor-antigen presentation and thus activation of adaptive immune responses against cancer. Our data shows that targeting two antigens simultaneously (TRP-2 and gp100) increases the anti-tumor efficacy of the PeptiCRAd platform.

8.4.3. Efficacy and immunogenicity of PeptiCRAd in humanized mice

Finally, we wanted to test the efficacy of PeptiCrad in a model closer to a clinical scenario: the humanized mouse model in which components of the human immune system were egrafted into immunodeficient mice. As in the Study II, we first injected human melanoma cells (SK-MEL-2) s.c. into the flanks of highly immunodeficient NSG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice, which lack mature T cells, B cells and functional NK cells, and are deficient in cytokine signaling, leading to efficient engraftment of human blood cells. When the tumors reached a palpable size, HLA-A3 matched human PBMCs from a healthy donor were engrafted into the mice. Subsequently, the mice were treated i.t. with PeptiCRAd, uncoated virus or saline solution (mock). For coating the PeptiCRAd in this experiment, we chose a polyK-modified epitope derived from melanoma-associated antigen A1 (MAGE-A1$_{96\text{-}104}$; SLFRAVITK), which is a peptide already used in peptide vaccine clinical trials (Toungouz et al. 2001). We also chose a GM-CSF-expressing adenovirus (Ad5-Δ24-GM-CSF), because GM-CSF viruses are the most frequently used OVs in clinical trials at the moment (Breitbach et al. 2015a, Andtbacka et al. 2015, Senzer et al. 2009, Vassilev et al. 2015, Burke et al. 2012). GM-CSF also enables better stimulation of the immune system.

Treatments with the MAGE-A1 PeptiCRAd resulted in reduced growth of the human melanoma tumors in immunodeficient mice engrafted with human PBMCs (**Figure 50**) and a complete cure was obtained in all the mice, while in control mice without human immune cells, no difference was observed between PeptiCRAd and the uncoated virus (Figure 7C of Study IV) underscoring the important role of the immune system in defining the efficacy of PeptiCRAd.

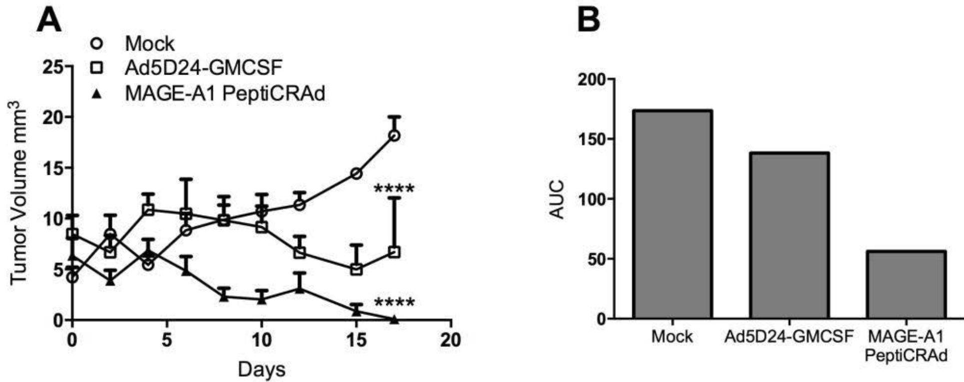


Figure 50. Efficacy of PeptiCRAd in humanized mice bearing human melanomas. *NGS mice with human melanoma tumors (SK-MEL-2) and engrafted with human PBMCs (N=6 tumors/group) were treated intratumorally on days 0, 2 and 4 with: i) saline solution (mock); ii) Ad5-Δ24-GMCSF; iii) MAGE-A1 PeptiCRAd.* ***a)*** *The tumor volume is presented.* ***b)*** *For each group of mice, the area under the curve (AUC) relative to the size of the tumors is presented. (Capasso et al. 2015, OncoImmunology [Study IV])*

To analyze whether anti-tumor immunity developed in the treated mice, we collected the spleens at the end of the experiment (17 days after the first treatment), stained the cells with a MAGE-A1 pentamer and ran the flow cytometric analysis. We found more human MAGE-A1-specific CD8+ T cells in the spleens of PeptiCRAd treated mice (**Figure 51**) (n.s.). Unfortunately, we could not analyze the T cells in the tumors (which would be more relevant when assessing anti-tumor immune responses), since the tumors were completely cured in the PeptiCRAd group in this experiment.

In summary, in Study IV, we were able to develop a method to coat an oncolytic adenovirus with tumor-associated, MHC-I restricted peptides by electrostatic interaction and showed the efficacy and immunogenic potency of the PeptiCRAd platform in several *in vivo* models. The PeptiCRAd platform is a very quick and easy way to generate tumor-antigen carrying viruses and it overcomes the difficulties of genetic or chemical modifications previously used for the same purposes. It is also easy to customize and it allows targeting of multiple antigenic entities simultaneously, which enables to compete with the heterogeneous and continuously evolving tumor environment. In addition, we have shown that the PeptiCRAd platform can be used also for efficient tumor delivery of complex of L-carnosine peptide drug, a natural anti-tumor peptide drug. This peptide-

adenovirus complex showed enhanced anti-tumor efficacy compared to OAd or L-carnosine treatment separately (Garofalo et al. 2016).

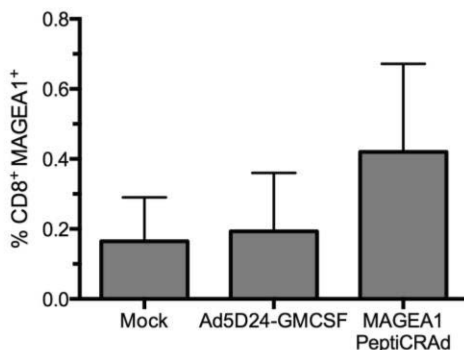


Figure 51. Tumor-specific T cells in the spleens. *At day 17, spleens of the treated NSG mice were collected and the amount of MAGE-A1 specific CD8+ T cells was analyzed by flow cytometry. (Capasso et al. 2015, OncoImmunology [Study IV])*

Our results suggest that administration of tumor-specific peptides in combination with an oncolytic virus serving as an adjuvant may increase the efficacy of cancer treatment, compared to treating with virus or peptide alone. Our results are consistent with the recent study by Li et al. where they show that adjuvant-antigen complexes are more effective than the single components because of a superior targeting of APCs (Li et al. 2013). It is increasingly understood that personalized approaches are needed to increase the efficacy and, most importantly, to reduce the side effects associated with use of unspecific cancer therapies. We think our PeptiCRAd platform could be used in the future as a carrier and adjuvant for patient- and tumor-specific peptides to trigger fierce anti-tumor immunity in a personalized manner.

9. CONCLUSIONS AND FUTURE PROSPECTS

The strategies introduced in this thesis for the improvement oncolytic virotherapies fit in the prevailing trends of a new era of cancer therapies: Immunotherapy and personalized medicine.

The thesis studies showed how the enhancement of the immunogenic potential of oncolytic viruses can improve the immune reactivity against cancer and hence potentiate the efficacy of virotherapies. This is in line with the results of other studies using viruses that have been armed with immunostimulatory molecules (Breitbach et al. 2015a, Bartlett et al. 2013, Ranki et al. 2014, Pesonen et al. 2012b, Cerullo et al. 2010). It has been realized that oncolysis alone is not powerful enough to trigger tumor responses, the immunosuppressive tumor environment needs to be counteracted and reshaped in order to be able to achieve antitumor responses critical for achieving long-term control against cancer (Lichty et al. 2014).

At the moment it seems that we are living in the golden times of immunotherapies. New strategies to enhance anti-tumor immune responses are currently being avidly investigated. Checkpoint inhibitors, which block the inhibitory signals to immune cells enabling activation of anti-tumor immune responses, are now generally accepted as critical immunotherapeutic agents studied extensively, both as single agents and in combination with other therapies. They are also being tested in a combination with oncolytic viruses (Rojas et al. 2015). It was just recently shown that combination of oncolytic Newcastle Disease Virus with a CTLA-4 blocker led to rejection of pre-established distant B16 tumors and the therapeutic effect was associated with tumor infiltration with activated $CD8^+$ and $CD4^+$ cells (Zamarin & Pesonen 2015). Also we have previously shown that anti-CTLA-4 antibody expressed by an oncolytic adenovirus enhances the cancer killing potency of the virus (Dias et al. 2011). Ongoing clinical trials are testing one of the leading cancer virotherapy agents, T-Vec (trade name Imlygic, Amgen), a herpes simplex virus expressing GM-CSF, with the novel immune checkpoint inhibitors ipilimumab (anti-CTLA-4) (NCT01740297) and pembrolizumab (anti-PD-1) (NCT02263508) (Zamarin & Pesonen 2015, Ascierto, Marincola & Atkins 2015).

Taken together, it is fairly easy to realize that combination therapies, involving both conventional and novel therapies, will be the future of cancer treatment. Also in this thesis (in Study I), I came to better understand the synergism that is possibly gained by combining two different strategies, oncolytic adenovirus treatment and radiotherapy. Oncolytic viruses have the potential to exert additive or even synergistic effects with various other treatments, like chemotherapy (Nguyen, Ho & Wan 2014, Liikanen et al. 2013) or different cell-based immunotherapies (Song 2013, Tahtinen et al. 2015), since they can act as adjuvants and they have the ability to release tumor antigens by cell lysis (*in-situ* vaccines).

The common ideology of cancer medication is shifting from a "one-dose-fits-all" concept to personalized medicine, and in the future drugs and treatments will most probably be increasingly patient-tailored, and the dosing and choice of a drug will take patient's genomics and the tumor microenvironment into account (Gasparini & Longo 2012, de Wit et al. 2015, Park et al. 2015). As for most cancer therapies, as well as for oncolytic virotherapies, it is common that the treatments are not equally efficient for all patients. Currently there is a lack of useful markers that would help in selecting the patient population that most probably benefits from the OV treatments, thus, Study III of this thesis focused on finding new biomarkers to predict the treatment efficacy. However, the steps towards optimization of tailored therapies still require discovery of new biomarkers, insight and detailed knowledge of tumor biology and immunology, further development of faster pharmacogenetic analyses, and wider collaboration and communication between experts in different fields: biologists, medical doctors, bioinformaticians, pharmacists, IT specialists, in essence, the actors between the bench and bedside, etc. These developments would most probably lead to generating highly patient-tailored drugs and approaches that could even be individually customized to each tumor of the patient. If (and when) the methods to screen tumor-specific antigens (peptides) (Admon & Bassani-Sternberg 2011) from individual cancer patients grows quicker and more efficient to use, it would be possible to also quickly generate efficient, highly specific therapeutic cancer vaccines. Our novel platform developed in Study IV to carry tumor-associated peptides on a surface of a virus (PeptiCRAd) already provides an easily customizable and highly-effective immunity-triggering vector for this purpose.

10.REFERENCES

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